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REVIEW

The immune functions of phosphatidylserine in membranes of dying cells and microvesicles

Benjamin Frey · Udo S. Gaipl

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Abstract Lipids in the cytoplasm membrane fulfill numerous functions. We focus on how lipid asymmetry is generated and its physiological and pathophysiological mission. The role of phosphatidylserine (PS), a prominent phospholipid that gets exposed during cell death, in health and disease as well as in the clearance process will be outlined in detail. Attraction signals, bridging molecules, and danger signals being involved in the PS-dependent clearance of apoptotic and necrotic cells and in subsequent immune modulation are presented. Furthermore, modulations of immune responses by PS-exposing cells, organisms, microparticles, and by the PS-binding protein annexin A5 are discussed. Interference with PS-dependent clearance of apoptotic tumor cells by macrophages fosters uptake and presentation of cancer antigens by dendritic cells and thereby induces specific anti-tumor immunity. The lipid composition of microvesicles is also depicted. Tumor microvesicles are often rich in PS and thereby contribute to tumor escape mechanisms. Understanding the role of PS in membranes of dying cells and microvesicles will help to develop novel drugs and treatment options for controlling immune-mediated diseases like chronic autoimmunity and cancer.

Keywords Phosphatidylserine · Annexin A5 · Apoptosis · Microvesicles · Clearance · Cancer

B. Frey · U. S. Gaipl (✉)
Department of Radiation Oncology, Radiation Immunobiology,
University Hospital Erlangen, Friedrich-Alexander
University of Erlangen-Nürnberg,
Universitätsstr. 27,
91054 Erlangen, Germany
e-mail: udo.gaipl@uk-erlangen.de

Introduction

The structure and the functions of cellular membranes are linked to each other. They determine cell's live and physiology. The plasma membrane of the cells is in the opinion of many scientists the most important cellular membrane because its solidity is a matter of life or death for the cell. It is at first the barrier between the internal space (cytoplasm) and the environment. Either the release or the uptake of substances to/from the environment may be toxic for the environment or the cell itself. The understanding of the membranes of the cells was growing since the early nineteenth century, when Groter and Grendel proposed a bilayer matrix of lipids, which surrounds each single cell [21, 31]. During the years, it became evident that the plasma membrane of the cells is not a crude bilayer of lipids that blocks the transfer of water and solubles between out- and inside of the cell; moreover, the membrane acts as a cellular organelle which has different duties and responsibilities for the cell itself, for the environment, and the whole organism which harbors the single cell [79]. The present understanding of cellular membranes was first established by Singer and Nicolson. They proposed in 1972 a fluid mosaic model of the plasma membrane [151]. Based on this model, many investigators advanced this model still based on the proposed lipid bilayer as backbone [37].

The initial function of mostly every biological membrane in eukaryotic cells is to serve as a barrier between the cytoplasmic and the extracellular (plasma membrane) or intra-organelle space (internal membrane), respectively. This function is performed by the lipid bilayers, which display a barrier with a high hydrophobic core and

a high hydrophilic outer face. The bilayer is able to divide mostly aqueous compartments [79]. However, the membranes of eukaryotic cells are not only a motionless barrier between compartments built by a simple bilayer of several lipids. Nowadays, it has become clear that eukaryotic cells use about 5% of their genes to synthesize thousands of different lipids. The specific function of numerous lipids is well characterized, but a fully description of the whole lipid repertoire of eukaryotes (and that of other species) is still elusive. Generally, lipids are known to fulfill three general functions: (a) lipids are used for the storage of energy, (b) lipids can act as first or second messenger, and (c) polar lipids are able to form cellular membranes [159]. The last two functions of the lipids will be further highlighted in this review. In addition, we will focus on a distinct phospholipid of cellular membranes, namely phosphatidylserine (PS), and its role in diseases, clearance, and modulation of the immune system. The role of PS in membranes of microvesicles will also be outlined.

General composition of the cytoplasm membrane of eukaryotic cells

For the formation of the cytoplasm membrane, the eukaryotic cell uses different phospholipids (PL). These PL are derived either from glycerol, a three carbon alcohol, or sphingosine, a long-chain unsaturated amino alcohol [27, 36]. However, the main structural PL in biological membranes are the glycerophospholipids [159]. Glycerophospholipids consist of a glycerol backbone with two fatty acid chains, the diacylglycerol "tail". The latter is esterified with various phosphorylated alcohol "heads". A common scheme of the PL, which can be found in nearly any eukaryotic cytoplasm membrane, is depicted in Fig. 1a. The composition of the PL depends on the membrane type. The plasma membrane of an eukaryotic cell consists mainly of the aminophospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SPM; a phospholipid with ceramide as backbone instead of glycerol) in different amounts, as shown in Fig. 1b. Furthermore, the plasma membrane is enriched in cholesterol, which gives the bilayer of about 5 nm thickness a mechanical stability [79]. The membranes of the cellular organelles are mostly built with PC, and the other PL also show a different distribution compared to the cytoplasm membrane. Furthermore, the lipids of the membranes of organelles contain a different ration between cholesterol (CHOL) and PL (CHOL/PL<1) than the cytoplasm membrane (CHOL/PL=1) [79, 159].

Generation of lipid asymmetry

The composition of the membranes can be further characterized by analyzing the orientation of the lipids to the cytosol. Whereas the membrane of the endoplasmic reticulum (ER) displays a symmetric distribution of the PL between the inner and outer layer of the lipid bilayer, the membranes of Golgi apparatus and cytoplasmic endosomes as well as the cytoplasm membrane display strong asymmetric distribution of their membrane lipids [33, 159, 178]. The loss of the asymmetry, especially the appearance of PS on the outer membrane leaflet, will lead to many physiologic and pathologic phenomena (as discussed later in this article). Figure 2 shows schematically the asymmetric distribution of the lipids in eukaryotic cytoplasm membranes. Predominantly PE, PI, and PS are retained in the cytoplasmic leaflet, while PC and SPM are exposed to the extracellular space [91, 165, 180]. This asymmetry in PL has attracted the interest of many investigators. One could expect that the transport leading to asymmetry of the membrane lipids is a passive process, which is true for neutral lipids like cholesterol and charged lipids in a protonated form [33, 121]. However, all findings up to now point out that the cells invest a distinct amount of energy to induce and preserve their lipid asymmetry. The cells have developed concerted proteinaceous machinery, which controls the asymmetric distribution of the PL within the membranes. The passive process of lateral diffusion of PL within one leaflet of the bilayer is very fast (exchange rates of about 10^7 s^{-1}), whereas the transverse diffusion (also called flip-flop) is very slow and has a half-time for exchange of hours to days, depending on the phospholipid [33]. However, several studies have shown that the transversal transport to the inner leaflet of the plasma membrane is achieved within minutes for labeled PS and within hours for labeled PE. Interestingly, PC and SPM stay nearly completely in the outer leaflet of the plasma membrane [33]. These observations lead to the hypothesis that different proteinaceous transporters (Fig. 3) will handle the transverse transport of the different lipid classes. At least three distinct activities will handle the asymmetry: two of them are energy requiring and the third is Ca^{2+}-ion dependent.

The *aminophospholipid translocase* (also known as *flippase*) processes the inward movement of aminophospholipids from the exoplasmic to the cytosolic leaflet of the membrane. The *flippase* may belong to the P-type ATPases family, a large family of transmembrane proteins. Furthermore, the *flippase* has been shown to be responsible for the selective movement of PS and PE ($t_{1/2}$=5–10 min for PS). The transport conducted by the *flippase* is strongly dependent upon ATP. Up to now, the *aminophospholipid translocase* has not been definitely identified, but it became evident that the *flippase* is a Mg^{2+} ATPase with a molecular mass of

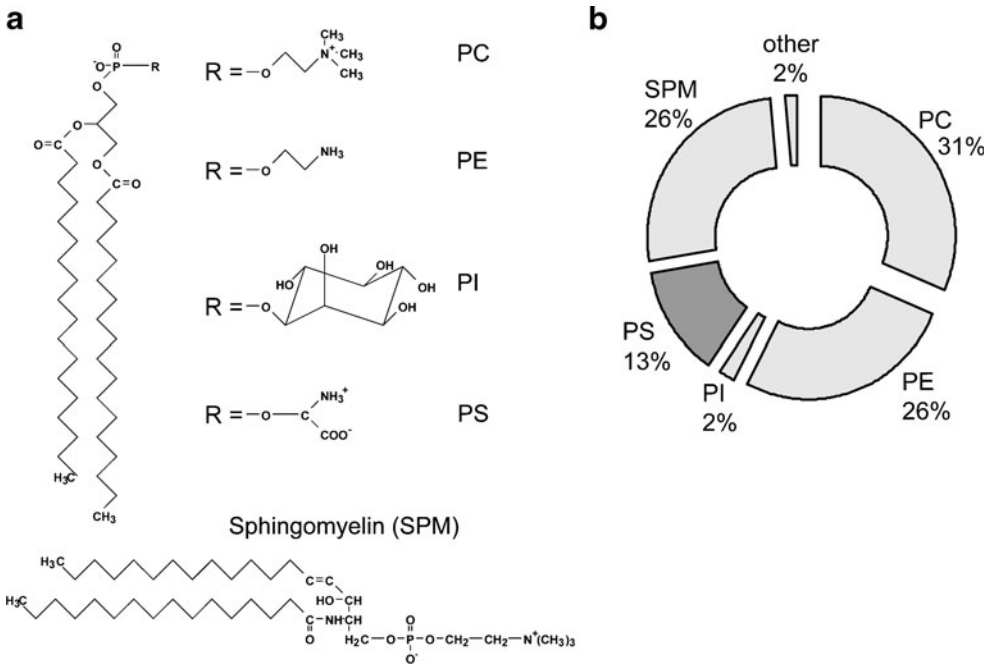

Fig. 1 Membrane lipids of eukaryotic cells. **a** The structure of glycerophosphate-based lipids and sphingomyelin, the main lipids of the cytoplasm membrane of eukaryotic cells, is displayed. **b** Phospholipid composition of the cytoplasm membrane of a eukaryotic cell. The data are expressed as weight percent of total PL. The data are the mean values of PL composition analyses published by [36, 130, 131, 159]. *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PS* phosphatidylserine, *SPM* sphingomyelin

115–120 kDa possibly associated with a 31-kDa protein at the endofacial membrane side. The *flippase* showed sensitivity to vanadate and to sulfhydryl oxidation. In red blood cells, it was evidenced that per molecule of transported aminophospholipid one molecule of ATP is hydrolyzed. Beyond the plasma membrane, the *flippase* activity can be detected in various intracellular membranes, to keep PS to the cytosolic leaflet [121, 180, 181].

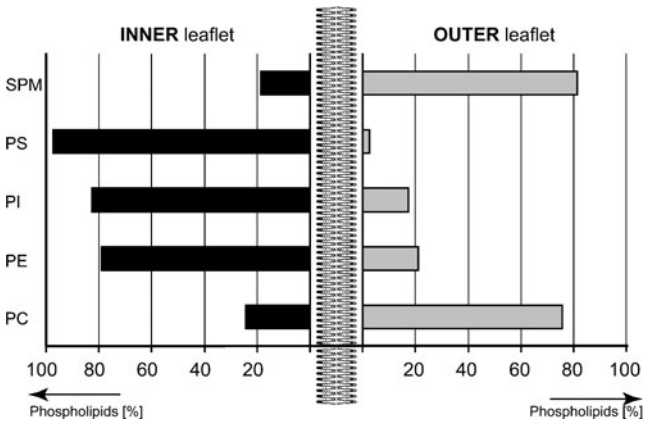

Fig. 2 Lipid asymmetry in the bilayer of the cytoplasm membrane of eukaryotic cells. The amount (percent) of each lipid found in the inner and outer leaflet of the plasma membrane of a viable cell is depicted. The amounts are the mean values of already described analyses of asymmetric distributions. The data were collected from [31, 33, 47, 131, 165, 178]. *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PS* phosphatidylserine, *SPM* sphingomyelin

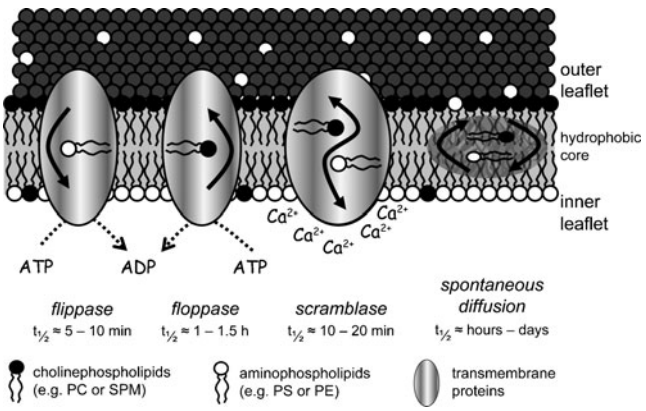

Fig. 3 Schematic overview of the known phospholipid transporters in the cytoplasm membrane of eukaryotic cells. A schematic drawing of the transporter-controlled phospholipid exchange between the both leaflets of eukaryotic cytoplasm membrane is depicted. The unidirectional phospholipid transport is maintained by the *flippase* (*inward transport* of PS and PE) and *floppase* (*outward transport* of PC and SPM). Both transporters are ATP dependent and move phospholipids against their respective concentration gradient. Furthermore, the bidirectional *scramblase* is shown, which scrambles phospholipids between the two leaflets in an ATP independent manner. However, this transporter is highly Ca^{2+} dependent and does not work under normal physiologic concentrations of Ca^{2+}. In addition, the mechanism of spontaneous diffusion of phospholipids which is neither ATP nor Ca^{2+} dependent and takes hours to days is displayed. The corresponding half-life ($t_{1/2}$) of the transportation time for phospholipids needed by each transporter is also shown. The drawing is a composition of figures of the following articles [121, 180, 181]. For detailed explanations, please refer to the main text

Besides the location of PS and PE on the membrane inner leaflet, SPM and PC are mostly found on the outer leaflet of the cytoplasm membrane (Fig. 2). The so-called *floppase*, an ATP-dependent transporter, is responsible to transport cholin- and aminophospholipids from the inner to the outer leaflet of the bilayer. It works in a similar manner to the *flippase*. However, the transport is ten times slower than the transport realized with the *flippase* and has a $t_{1/2}$= 1.5 h for PC [121, 180, 181]. The *floppase* belongs to the ATP-binding cassette (ABC) transporter which is encoded by the ABCC1 gene. This protein was formerly known as the multidrug resistance protein MRP1 and belongs to the superfamily of ABC transporters. The ABCC1 is a large integral membrane protein (M_W ~180 kDa). Further experiments have indicated that the inhibition of the *floppase* results in a slow redistribution and furthermore a more random distribution of PC. However, the asymmetric distribution of PS and PE cannot be disturbed by *floppase* inhibition, pointing out that this transporter is highly substrate specific [27, 121, 181].

One could mention that the inhibition of both *flippase* and *floppase*, especially via ATP depletion, will simply raise the random distribution of the PL between the leaflets. This experiment was often conducted, resulting in a not rapidly distribution of PS on the outer membrane leaflet. However, as seen in activated platelets and apoptotic cells, the exposure of PS appears more rapidly [181]. This allows the conclusion that cells also have an ATP-independent mechanism to move PL via the membrane leaflets. The so-called *scramblase* is an ATP-independent but Ca^{2+}-dependent mechanism to scramble PL bidirectional. The scrambling involves all major phospholipid classes with comparable scramble rates ($t_{1/2}$=10–20 min), expect SPM that is moved significantly slower [121, 180, 181]. The *scramblase* has been proposed to be a 37-kDa membrane protein, called scramblase 1 being responsible for the Ca^{2+}-dependent lipid scrambling. However, this protein may serve only as one good candidate. Nevertheless, it was shown that scramblase 1 disturbs the asymmetry of membranes with PS exposure on apoptotic cells [121, 150, 181].

Disturbance of lipid asymmetry—exposure of phosphatidylserine

The disintegration of the phospholipid asymmetry, especially the exposure of PS on the outer leaflet of the membrane, has complex physiological impact on not only the cell itself but also on the whole tissue and the whole organism. Therefore, the necessity of the development of an efficient mechanism by the cells restricting PS to the inner leaflet of the plasma membrane bilayer becomes evident. Inappropriate exposure of PS on the cell surface may lead to the "destruction" of the cell but is also important in the case of apoptotic cell removal [169]. Apoptotic cells are not able to maintain the lipid asymmetry of their membranes for longer times. They expose PS and to a lower extend also PE on the outer surface of the membranes [44, 48]. The mechanism leading to the disturbance of the lipid asymmetry and consecutive to the exposure of PS on the outer surface appears to be elementary but displays a challenging and also highly regulated mechanism. Different theories exist how PS gets exposed on the cytoplasm membrane, but it is as sure as fate that apoptotic cells possess a complete loss of the asymmetric distribution of the plasma membrane PL [27, 44, 59, 69, 122, 150, 156]. Much knowledge about the transmembrane transporters was obtained by analyzing platelets, erythrocytes, or model membranes. However, the PS exposure on apoptotic cells seems to be different from the activation-induced PS exposure on platelets or other model systems [69].

Two main theories exist up to now, both very valid, but lacking the ultimate proof, how PS gets exposed on apoptotic cells. The first theory, which combines various findings of many researches, states as follows: The simple inhibition of *aminophospholipid translocase* (*flippase*) does not alone results in collapse of plasma membrane PL asymmetry, as long as normally low Ca^{2+} concentration is maintained. In addition, passive and spontaneous translocation of PL is very slow [150]. The increase in Ca^{2+} concentration in the cytosol yet can induce a rapid movement of PL between the plasma membrane leaflets [150, 169]. These experiments could be proven by the induction of apoptosis with thapsigargin. The latter is a non-competitive inhibitor of endoplasmic reticulum Ca^{2+} ATPases and raises cytosolic calcium concentration by blocking the pumping of calcium into the sarcoplasmic and endoplasmic reticula and causes depletion of these storages. At least prolonged exposure to thapsigargin can induce apoptosis. Furthermore, the PS exposure could be abolished by addition of zVAD-fmk, a cell-permeant pan caspase inhibitor which irreversibly binds to the catalytic site of nearly all caspases and therefore inhibits induction of apoptosis [69, 169]. It was further described that lymphocytes of patients with Scott syndrome, a rare bleeding disorder that reflects impaired expression of PS by activated platelets because of a mutation in the scramblase proteins, do not have any defects in apoptotic cell clearance; even the latter is triggered by PS exposure (further details are discussed later in this review) [45, 69, 150, 169]. Over and above experiments showed dependency as well as independency of caspases, BH3-only proteins (small apoptosis regulating proteins), and Ca^{2+} concentration on the exposure of PS on the surface of apoptotic cells [45, 169], indicating that multiple exposure pathways have to exist. Alterations in *flippase* and

scramblase activity are not solely responsible for PS exposure on apoptotic cells [169].

Another main model for the exposure of PS by dying cells has been proposed by Tyurina and colleagues [156]. They observed that oxidized PS (oxPS) is crucial for uptake of apoptotic cells and therefore hypothesized that oxPS, formed during apoptosis, stimulates exposure of PS via increased rates of PS and/or oxPS transmembrane diffusion [83]. Examination of the mechanisms by which the loss of membrane asymmetry is regulated and induced is a fertile future research field. Interestingly, the mechanisms of PS exposure on apoptotic cells are similar to the effects exerted by distinct forms of dying cells (inflammation or anti-inflammation), namely it depends on the death stimulus [95, 169].

Information on the mechanisms how cells dying not exclusively via apoptosis expose PS are very scarce. Cells dying by autophagic cell death (autophagy fails and induces apoptosis) seem to behave similar to normal apoptotic cells [94]. Necrotic cells should be divided into secondary and primary necrotic cells (for extensive review, see [63]). Necrotic cells are characterized by disturbed membrane integrity. Because secondary necrotic cells are proposed to have passed an apoptotic program, the exposed PS on those kinds of necrotic cells may have had a similar fate like that in apoptotic cells. Primary necrotic cells display a high binding of annexin A5 (AnxA5), a protein that binds with high specificity and affinity in a Ca^{2+}-dependent manner to PS. Due to the disrupted plasma membrane of necrotic cells, PS gets accessible to AnxA5 binding on the inner and on the outer leaflet of the membrane, but until now no investigations have been undertaken to characterize the exposure of PS on the outer leaflet of necrotic cells. Nevertheless, the clearance of necrotic cells is also mediated by PS and various other surface molecules that play roles in apoptotic cell clearance [19]. The procedure of necrotic cell removal has to be further specified in the future and to be adapted to cell death forms similar to necrosis, like necroptosis [13].

The exposure of PS on the outer leaflet of the plasma membrane is mostly an early event of apoptosis and may under certain circumstances also be reversible. PS exposure also occurs under physiological circumstances that are not connected to cell death [94]. The initial function of the PS exposure on viable cells is manifold and somehow open-ended. Table 1 gives examples for situations where PS is exposed on membranes of non-dying cells. The PS exposure of cells that do not show any signs of apoptosis (e.g., caspase activation) is even thought to avoid clearance. Detailed explanations about PS-dependent clearance mechanisms are outlined later in this review.

Phosphatidylserine and microparticles

The description of microparticles (MP) can be found in the literature years before apoptotic bodies were mentioned. The first known report of MP, also called microvesicles, emerged from 1967, when Wolf described the existence of fragments derived from platelets in human plasma [171]. Nowadays, it has become obvious that a variety of microparticles derived from the plasma membrane of platelets, leukocytes, endothelial cells, and several other cell types are present in human blood [35, 148]. A precise definition of the term MP is still missing and will be discussed in much more detail in other articles of *in this issue*. We define here MP as a heterogeneous population of small phospholipid bilayer coated vesicles with a diameter of 0.1–2.0 μm. The MP are derived from cell's plasma membrane during activation or apoptosis of the cell. The type of stimulation can be correlated with the spectrum of released MP phenotypes. Stimulation causes the MP formation by membrane budding of the cell [35, 75, 125, 148]. First descriptions of MP suggested them to be dormant cell debris, released after distinct stimuli. Nowadays, it is well established that MP are able to serve as physiologically active effectors, playing important roles in inflammation, hemostasis, thrombosis, angiogenesis, cancer, and vascular reactivity [6]. The small size of the MP allows them to circulate easily in the vasculature. The distribution of the MP via the circulation allows not only a local but also a long-range signaling. MP bind to cells via surface ligands, enabling a long distance cell–cell interaction for cells typically dislodged from each other [6, 35, 125, 148].

MP are derived from the membrane of the ejecting cell. Furthermore, the release of the MP occurs by budding of the membrane in concerted action with the cytoskeleton. The detailed process of the budding and the interaction with the cytoskeleton are reviewed *in this issue* and elsewhere [6, 35, 75, 148]. We want to point out the following: During the budding process, the normal membrane lipid asymmetry of the cytoplasma membrane gets already lost [35, 75].

Characterizations of the surface of MP have been mostly performed by analyzing membrane surface proteins, which cause lot of the signaling function of the MP. In contrast, little is known about the lipid composition of the MP. When the MP were discovered, they were described as platelet dust because platelets shed a huge number of MP upon activation. These MP express PS on the outer leaflet, detected by staining of the MP with AnxA5 [75, 125, 148]. In Fig. 4a, the PL formations of platelet-derived MP are exemplarily displayed according to the experiments of Weerheim et al. [167]. Their analyses revealed that the main PL in MP ejected from platelets are PC and SPM [167]. These MP also show a prominent exposure of PS

Table 1 Exposure of PS in the outer leaflet of the membranes of various non-dying cells

PS exposure in the outer leaflet of "normal" cells	
Myoblasts	Large fraction of PS and PE in outer leaflet while differentiating to myotubes
Megakaryocytes	After release of platelets from progenitor cells
B Lymphocytes	Bone marrow and resting B cells; plays a role in antigen receptor-mediated signaling
Murine CD 4+ T Lymphocytes	A suggested role in T cell migration
Mast cells	Reversible PS exposure upon IgE stimulation in the absence of other features of apoptosis
Macrophages	Cell requires PS expression for bridging molecules and uptake of apoptotic cells
Various cell types	Upon *Chlamydia trachomatis* and *Chlamydia pneumoniae* infection, without caspase activation
Tumor cells	Projected anti-inflammatory clearance of single tumor cells during escape of the tumor from the immune system

The table was modified from [12, 46, 47]

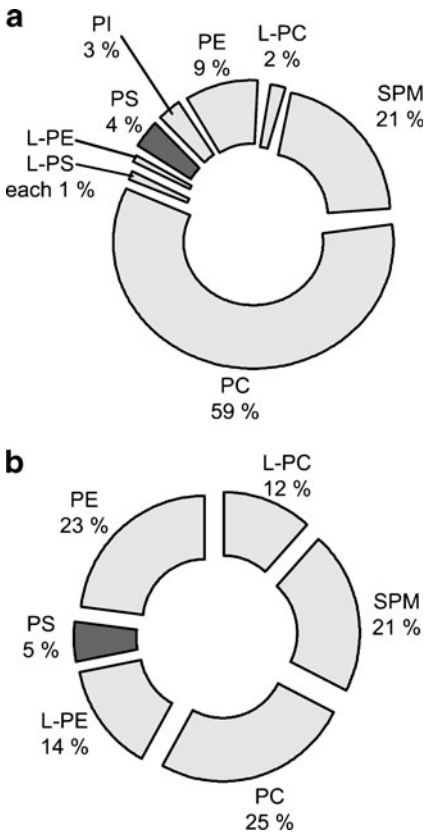

Fig. 4 Lipid composition of microparticle membrane bilayer. The composition of the microparticle membrane bilayer of eukaryotic cells is depicted. **a** The composition of the lipids which can be found in the bilayer of microparticles shed by platelets after activation. **b** In comparison to microparticles of activated platelets, the microparticles found in the synovial fluid of an inflamed joint have a different composition of membrane lipids. Note: The data are expressed as weight percent of total PL. The amounts are the mean values of already described analyses of asymmetric distributions. The data were collected from [53, 167]. *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PS* phosphatidylserine, *SPM* sphingomyelin, *L* lyso

(detected by AnxA5 staining). MP are a potential trigger of the clotting cascade in combination with activated platelets. The latter also expose PS on their membrane surface [125]. However, if MP are extracted from the synovial fluid of inflamed joints, the composition of the membrane has a more equal composition, as shown in Fig. 4b. The source of the MP in the inflamed joint is not clearly worked out until now because most of inflammatory immune cells (e.g., neutrophils, macrophages, and lymphocytes) as well as endothelial/synovial cells can shed MP upon inflammatory activation. Information about the allocation of the PL between the two single layers of the MP membrane are elusive; however, PS exposure seems to be ubiquitous [6, 35, 80, 92, 125, 153]. Moreover, it was shown that the membrane composition of the MP correlate with the membrane composition of the releasing cell. The PL composition of the MP correlates strongly with the cell type and the stimulus leading to the budding and the release of the MP [35]. It has to be mentioned that MP are distinct from apoptotic bodies. The apoptotic program leads to chromatin condensation and cellular rearrangement resulting in formation of blebs filled with cytoplasmic material. These MP are released very early during the apoptotic process and are swiftly cleared by macrophages. If clearance of apoptotic cells is delayed or inhibited (for review, see [55, 62, 63]), the apoptotic cell collapses and fragments. The vesicles that are passively released in later phases of apoptosis are called apoptotic bodies. They differ from MP in size and composition, since the cytoplasm membrane has been substituted by intracellular membranes [35, 56]. Again, these apoptotic bodies are in contrast to MP (blebs). The latter are released early during the apoptotic process [35, 56]. MP are also different from exosomes, which are derived from late endosomes and released by the cells upon activation. Compared to MP, exosomes form vesicles and are stored intracellularly in multivesicular bodies. The latter fuse with the cell membrane and secrete the exosomes [6, 35]. Because the membrane composition of endosomes differs from the membrane

composition of the plasma membrane, the exosome's membrane composition may differ from MP membrane composition [154, 159].

Diseases linked to loss of lipid asymmetry

Scott syndrome

The Scott syndrome is a rare, moderately severe, bleeding disorder, which is characterized by impaired blood coagulation of the patient's platelets. It is known that the patients have an ineffective scramblase resulting in an impaired scrambling of the PL after activation, leading to disturbances in the clotting cascade. Apart from that, the platelets show normal behavior after activation. However, it was shown that these cells are also not able to shed PS-expressing MP. Up to now, the molecular basis of the Scott syndrome is still elusive. Most of the studies claim that a deletion or mutation in multiple hematological lineages may lead either to an ineffective lipid scramblase activity or avoids a correct Ca^{2+}-induced activation pathway [159, 180, 181].

Antiphospholipid syndrome

Patients with the antiphospholipid syndrome (APL) demonstrate circulating "antiphospholipid" antibodies accompanied with severe arterial and venous thrombosis, recurrent abortions, and thrombocytopenia. The antiphospholipid syndrome is mostly seen in patients who suffer from systemic lupus erythematosus (SLE). While this chronic autoimmune disease can trigger an antiphospholipid syndrome, antiphospholipid antibodies can be detected in a variety of diseases that are accompanied by loss of membrane asymmetry and cell surface exposure of PS (e.g., sickle cell anemia, thalassemia, malaria, uremia, diabetes, pre-eclampsia, cancer, and diseases/conditions with elevated levels of circulating microvesicles). First it was thought that the antiphospholipid antibodies directly interact with PL. However, it has become evident that those antibodies are directed against plasma proteins like β-2-glycoprotein-1 that interact with anionic PL. While the APL is mostly accompanied by autoimmune disorders, the patients do not show a bleeding tendency but have an increased risk for thrombosis [159, 180, 181].

Sickle cell anemia

The sickle cell disease results from a point mutation in the β-chain of hemoglobin, leading to hemolytic anemia and vaso-occlusive episodes. The mutation is responsible for hemoglobin polymerization and sickling of erythrocytes when deoxygenized conditions exist. Furthermore, it leads to loss of the lipid asymmetry and exposure of PS on the surface of sickled cells and sickle cell-derived microvesicles [159, 180, 181].

Kidney stone disease

It was shown that an abnormal exposure of PS on membranes of renal epithelial cells has an emerging role in the formation of kidney stones. If cultured renal epithelial cells are treated with oxalate, they switch to apoptotic cell like phenotype and expose PS on the outer membrane. The exposed PS promotes the binding of calcium oxalate crystals and furthermore the growth of the renal stones. The mechanism of the oxalate-induced PS exposure remains unclear. However, a direct physical interaction between the oxalate and the membrane lipids of the epithelial cells is discussed [159, 180, 181].

Phosphatidylserine and clearance

"Find-me" signals of apoptotic cells

As outlined above, the exposure of PS is a key event in the apoptotic program. This anionic phospholipid acts as major "eat-me" signal that ensures efficient recognition and phagocytosis of dying cells and nuclei by phagocytes [177]. However, a prerequisite for recognition of apoptotic cells is the attraction of the professional or semi-professional phagocytes by their "prey". Since phagocytes are mostly not located in the immediate neighborhood of apoptotic cells, the secretion of chemotactic factors attracting monocytes and macrophages is very likely. Even our knowledge about the soluble factors released from apoptotic cells is rather limited, current research identified several attraction factors for phagocytes (summarized in [119]). Proteins as well as lipids have been identified to contribute to the attraction process. More than a decade ago, Horino and colleagues identified the dimer of the ribosomal protein S19 as attraction signals for apoptotic HL-60 cells [74]. Recently, CX3CL1/fractalkine, being a chemokine and intercellular adhesion molecule, has been identified to be released by apoptotic cells leading to the stimulation of macrophage chemotaxis [155]. A new class of dying cell-derived attraction signals was identified by Elliot and colleagues [42]. The selective attraction of macrophages has been shown to be mediated by the nucleotides ATP and UTP. In addition, an apoptotic cell-derived lipid acting as "find-me" signal was identified by Lauber and co-workers [97]. The phospholipid lysophosphatidylcholine, but not its metabolic derivates or related lysophospholipids, stimulates chemotaxis of macrophages [118]. Importantly, not only

single molecules but also apoptotic cell-derived microparticles are capable to attract phagocytes [144]. PS and membrane-bound TGF-beta exposed by blebs or microparticles may further contribute to anti-inflammatory processes exerted by a timely clearance of apoptotic cells by macrophages [175].

Danger signals of necrotic cells

A delayed or improper recognition and uptake of apoptotic cells lead to further changes in the membrane composition of the dying cells and finally to the disruption of the cellular membrane integrity. The cells turn from apoptosis to secondary necrosis [147]. The latter form of cell death mainly results, like primary necrosis, in immune activation and inflammatory reactions [63]. Uric acid has been identified as a danger signal released from dying cells [146]. The amount of released uric acid crystals seems to be higher in the case of secondary necrosis compared to primary necrosis [119]. The monosodium salt of uric acid, namely monosodium urate (MSU), forms birefringent crystals inducing reactive oxygen species production and release of pro-inflammatory cytokines in monocytes, in contrast to monopotassium urate, indicating that the microenvironment at the site of crystal formation is important for the immunogenic and inflammatory potential of uric acid [141]. The high mobility group box 1 (HMGB1) protein is a prominent example for a protein being also released by necrotic cells. Inside the cell nucleus, HMGB1 stabilizes chromatin and contributes to DNA bending. Outside the cell, it is a potent mediator of inflammation [136]. Tissue damage induced "Damage associate molecular patterns (DAMP)" activate the innate and adaptive immune system. Secondary necrotic cells release HMGB1 in complexed forms, since the danger signal remains bound to nucleosomes of late apoptotic cells [157]. Danger signals released from primary to secondary necrotic cells overcome the anti-inflammatory mode of action of PS. The latter gets accessible to the immune system on the inner leaflet of necrotic cells. DAMP molecules, including HMGB-1, S100 proteins, hepatoma-derived growth factor (HDGF), uric acid, ATP, altered matrix proteins, and heat shock proteins (HSP), represent important danger signals that mediate inflammatory responses [52]. Even HSP, playing key roles in the recovery from stress to acting as chaperons mainly inside the cell, exert various extracellular functions leading to immune activation. Hsp70 has been shown to function as danger signal and is released within membranous structures from the cells [163]. The release of Hsp70 has been controversial discussed because this protein does not present a secretory signal. However, a co-expression of Hsp70 and PS on the cell surface after stress conditions has recently been described [139]. Furthermore, lipid profiling experiments demonstrated that Hsp70 membrane-positive cells differ from their membrane-negative counterparts by containing significantly higher amounts of the lipid globotriaosylceramide (Gb3) [66]. Taken together, Gb3 and PS enable anchorage of Hsp70 in the plasma membrane of cells. PS interacting with proteins like HSP therefore contributes to inflammatory reactions and, as outlined above, in its pure form or complexed with bridging molecules (see below) to anti-inflammation. This discrepancy is also observed for the nucleotide ATP that functions as attraction signal for macrophages in non- or even anti-inflammatory apoptotic cell removal [42] but in the context of tissue damage in higher concentrations as immune activating danger signal [9, 77].

PS-dependent phagocytosis of dying cells

After attraction of the phagocytes, recognition and engulfment of the dying cells has to be managed. PS on the outer leaflet of the plasma membrane represents the key signal for triggering phagocytosis of both apoptotic as well as necrotic cells [19, 173]. How PS arrives at the plasma membrane outer leaflet during apoptosis is still fragmentarily understood [20], and the current knowledge is summarized in the top of this article. Shortly, the translocation of PS from the outer to the inner leaflet may fail due to inhibition of an aminophospholipid translocase, which is essential for the ATP-dependent maintenance of the asymmetric distribution of PL across the membrane bilayer. Alternatively or in addition, an activation of a not yet identified phospholipid scramblase, which rapidly flips PL from either side of the bilayer to the other, may contribute to PS exposure during apoptosis [169]. Though clear is that PS is recognized either directly by receptors or in combination with soluble adaptor proteins. The latter bear unique domains that bind to specialized receptors on the phagocyte. Integrins and receptor tyrosine kinases like Mer are examples that enable the transduction of the PS stimulus into various signaling outcomes [143]. Milk fat globule protein MFG-E8 [70], growth arrest-specific gene product GAS-6 (ligand for the receptor tyrosine kinase MerTK) [78], β-2-glycoprotein-1 [11], C-reactive protein (CRP) [111], serum-derived protein S [2], and annexin A1 [7] bind to PS and join apoptotic cells with the phagocyte. Changes of the glycoprotein composition of apoptotic cells often occur later than the exposure of PS [54]. They may therefore act as back-up mechanism for apoptotic cell clearance like the binding of complement components. Complement binding is an early event in necrosis and a rather late event in apoptosis/secondary necrosis [58]. Various molecules like complement, serum DNase I [60], and CRP act as back-up molecules in the clearance process

(summarized in [63]). Complement activation in the innate immune response is besides due to mannan-binding lectin and deposition of C3 by the alternative pathway also due to natural IgM antibodies and CRP [137]. Notably, all those molecules are also involved in the clearance of dying and dead cells (Fig. 5).

There exist examples in which viable cells transiently expose PS but are ignored by phagocytes [34, 40]. Exposed PS may also play a role in endocytic pathways for the internalization of annexins [84]. One big question remains: How can a phagocyte distinguish between PS on viable and on apoptotic cells? Binding studies with AnxA5, being a natural occurring ligand for PS, to apoptotic, necrotic, and viable cells, revealed that the restricted lateral mobility of PS may be one crucial point to distinguish life from death. We suggested that the PS density on membranes of viable cells is too low to induce the "eat-me" stimulus for phagocytes [5]. Kagan and co-workers suggested that oxidation of PS during apoptosis is mandatory for both the exposure of PS to the cell surface and for the "activation" of PS being an "eat-me" signal for phagocytes [82]. Another hint for this hypothesis is that oxidized PS binds adaptor proteins like MFG-E8 with higher affinity in comparison to non-oxidized PS [17]. Furthermore, a spontaneous but only ex vivo observable exposure of PS on viable B cells may be the cause of the proper function of PS as unique "eat-me" signal for dying cells [41]. Viable cells further expose a combination of "don't-eat-me" signals such as CD47, CD31, and specific carbohydrate structures to prevent their uptake by phagocytes [124].

Several macrophage scavenger receptors may also interact directly with PS on the surface of the apoptotic cells [128]. Regardless of the receptors engaged on the phagocyte, ingestion does not occur in the absence of PS. Receptors exist which are involved in "tethering" (recognition and binding) of apoptotic cells versus those inducing their uptake and signal transduction, namely their "tickling" [72]. Monoclonal antibodies (mAb) against stimulated phagocytes were produced to identify receptors for PS (PSR). The antigenic target of the mAb 217 had hallmarks of a receptor for PS, namely it recognized PS and blocked the engulfment of apoptotic cells [49]. Phenotypes of PSR-

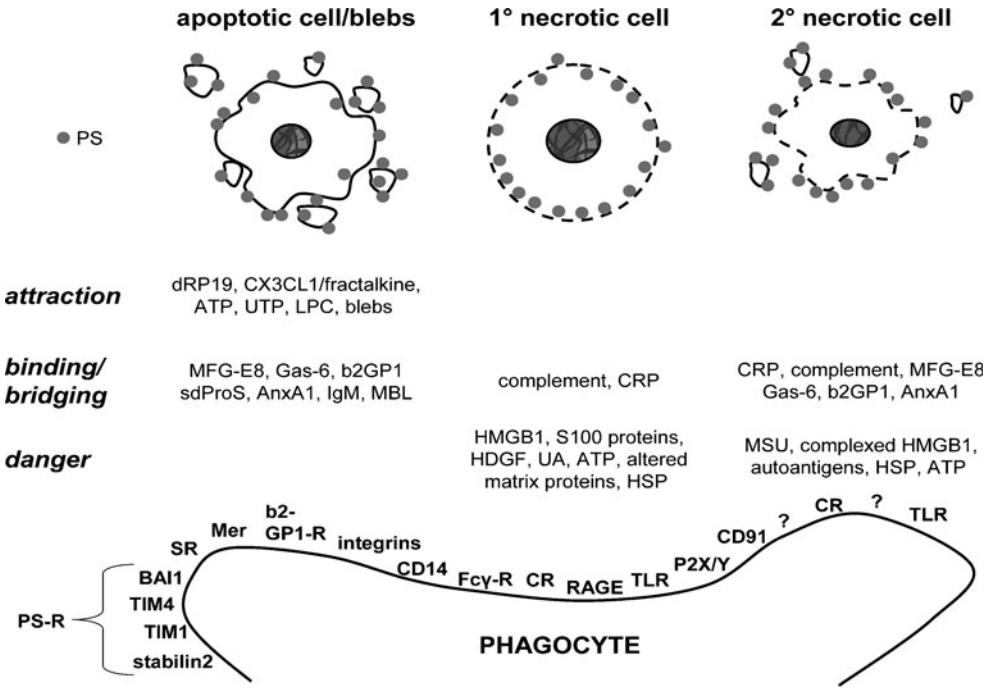

Fig. 5 Attraction signals, bridging molecules, and danger signals are involved in the clearance of apoptotic and necrotic cells by phagocytes and in consecutive immune modulation. Phosphatidylserine (*filled gray circles*) is the main recognition structure for apoptotic and necrotic cells. Apoptotic cells attract phagocytes, bind directly or via bridging molecules to receptors on phagocytes, and elicit no or anti-inflammatory immune responses. Necrotic cells release danger signals contributing to immune activation. For detailed explanation, please refer to the part of the main text dealing with "phosphatidylserine and clearance". *1°* primary, *2°* secondary, *AnxA1* annexin A1, *ATP* adenosine-5′-triphosphate, *b2GP1* beta2-glycoprotein 1, *BAI1* brain-specific angiogenesis inhibitor 1, *CR* complement receptor, *CRP* C-reactive protein, *dRP19* dimer of the ribosomal protein S19, *Gas-6* growth arrest-specific protein 6, *HDGF* hepatoma-derived growth factor, *HMGB1* high mobility group 1 protein, *HSP* heat shock protein, *LPC* lysophosphatidylcholine, *Mer* receptor tyrosine kinase, *MFG-E8* milk fat globule protein E8, *MSU* monosodium urate, *P2* purinergic receptors, *PS* phosphatidylserine, *R* receptor, *RAGE* receptor for advanced glycation end-products, *sdProS* serum-derived protein S, *SR* scavenger receptors (e.g., CD68, CD36, SR-A), *TIM* T cell immunoglobulin and mucin-domain-containing molecule, *TLR* Toll-like receptor, *UA* uric acid, *UTP* uridine-5′-triphosphate, *?* receptors to be identified

deficient mice were described consistent with a role of the PSR in the removal of apoptotic cells [96, 100]. In another line of PSR-deficient mice, where the mAb 217 still stained positive, the PSR displayed essential functions during embryogenesis but not in apoptotic cell removal [18]. Also PSR-deficient cells were fully competent to recognize, engulf, and respond to apoptotic cells [108]. It became evident that this protein is not at the cell surface but in the nucleus [140]. But there is no doubt of PS itself and its putative receptor(s) playing an important role in the uptake process of apoptotic and necrotic cells. Using library of hamster monoclonal antibodies against mouse peritoneal macrophages Tim4 (T cell immunoglobulin and mucin-domain-containing molecule) has recently be identified as one receptor for PS [109]. Tim4 was found to be expressed on human and mouse macrophages and on dendritic cells and recognized PS via its immunoglobulin domain. Interestingly, among other Tim family members, only Tim1, but neither Tim2 nor Tim3, was found to bind PS. A metal-ion-dependent ligand binding site seems to be crucial for PS binding [135]. Just recently it was demonstrated that Tim3 is also a receptor for PS and binds in a pocket on the N-terminal IgV domain in coordination with a calcium ion. However, in contrast to fibroblastic cells, T or B cells expressing Tim3 formed conjugates with but failed to engulf apoptotic cells [32]. Another PS-specific membrane receptor that binds directly to the exposed PS and provides a tickling signal has been identified by Park and colleagues. Their experiments led to the conclusion that stabilin-2 is the first of the membrane PS receptors to provide tethering and tickling signals [117]. Stabilin-2, the hepatic hyaluronan receptor, has been shown to be essential for the clearance of hyaluronan from the lymph or the blood circulation [50]. It acts like a scavenger receptor, a number of which are already known to bind native and/or oxidized PS. The brain-specific angiogenesis inhibitor 1 (BAI1) has also been shown to be a receptor that can bind PS on apoptotic cells. The thrombospondin type 1 repeats within the extracellular region of BAI1 mediate direct binding to PS [116]. BAI1 functions as engulfment receptor for both recognition and subsequent internalization of dying cells.

A plethora of molecules are involved in PS recognition and binding (Fig. 5). A typical receptor that triggers internalization of apoptotic cells is Mer, originally named for its distribution in myeloid cells, epithelial cells, and reproductive tissue and responsible for the downregulation of TNF-alpha in macrophages [25]. Ligands for Mer are GAS-6 and protein S that bridge PS to Mer. Activation of Mer induces Src-dependent phosphorylation of FAK on Tyr861 finally leading to Rac1 activation and internalization of bound apoptotic cells (summarized in [173]). The activation of the small GTPase Rac leads to cytoskeletal reorganization of the phagocyte membrane allowing internalization of the PS-exposing corpse. In general, TAM receptor protein tyrosine kinases, TYRO3, AXL, and Mer, have been shown, besides fostering the phagocytosis of apoptotic cells and membranous organelles, to inhibit inflammation in both macrophages and dendritic cells most likely by suppression of Toll-like receptor (TLR) signaling [99].

The receptor for advanced glycation end-products and TLR mediate the signals of HMGB1 to the immune system [149]. The danger signal ATP activates purinergic P2RX7 receptors on DC leading to activation of the inflammasome. The latter drives the secretion of IL-1beta resulting in full differentiation of IFNgamma polarized CD8+ T cells during the priming of anti-tumor immune responses [9]. However, very recently, Bles and colleagues demonstrated that ATP released from tumor cells might also exert a tumorigenic action by stimulating the secretion of amphiregulin from DC [14]. An engagement of the P2 receptors also influences the ability of macrophages to bind apoptotic bodies. After binding of nucleotides to P2 receptor, the ability of macrophages to internalize and present antigens from the apoptotic and necrotic cells was enhanced [105]. Triggering of P2Y receptors induces the maturation of DC and leads to increased intracellular levels of cAMP [168]. Scavenger receptors and CD91 have been implicated in binding of the danger signal Hsp70 and may direct Hsp70–peptide complexes into the MHC class II presentation pathway [51]. The receptors for HDGF and MSU are not known by now. MSU seems to modulate the release of other danger signals since eosinophils exposed to MSU crystals within minutes of exposure released ATP into the extracellular milieu [90]. The identification of receptors specifically mediating the uptake of secondary necrotic cells has to be performed in future research. Uptake via complement receptors of complement opsonized secondary necrotic cells certainly contributes as back-up clearance mechanism. The molecular mechanisms of late apoptotic/secondary necrotic cell clearance were recently summarized by Poon and co-workers [124]. The attraction signals, bridging molecules, and danger signals for and of apoptotic and necrotic cells and the respective receptors on phagocytes outlined in the part "phosphatidylserine and clearance" are summarized in Fig. 5.

The PS-binding protein annexin A5

Switching from the physiological role of PS to its diagnostic application, it has to be stressed that PS has become a promising target to visualize dying and dead cells in vivo. AnxA5 is the most prominent molecular imaging agent to detect PS-bearing membranes in the body [86]. Being a natural occurring ligand for PS, AnxA5 belongs to a huge family of evolutionary related annexin proteins detected in most eukaryotic phyla. The core domain of

annexins consists of four similar repeats approximately 70 amino acids long with the exception of AnxA6, which has eight repeats. The core domain is responsible for the binding of Ca^{2+} ions and phospholipid. AnxA5 is present in both the intracellular and extracellular milieu. The concentration in the circulation is low and about 1.5 nM. However, annexins are considered to be only cytosolic proteins since they lack a 5′-leader sequence in their messenger RNA. Shedding of plasma membrane-derived microparticles might be one possibility to shuffle AnxA5 in the extracellular space like it has been described for interleukin-1beta [126]. Complex formation with other proteins and binding to PS on the inner membrane leaflet may also lead to externalization of AnxA5 [158]. Once present in the extracellular space, AnxA5 is capable to inhibit the release of microparticles by apoptotic cells but can also foster endocytosis [67]. Proteins present in the extracellular environment are endocytosed via receptor-mediated internalization or macropinocytosis. Ligands can be taken up by multiple lipid raft-mediated pathways. Just recently, it has been demonstrated that magnetic nanoparticles can be endocytosed efficiently by conjugation to a specific anti-polysulfated heparan sulfate antibody. The studies from Wittrup and colleagues revealed the chaperone glucose-regulated protein 75 as a functional constituent of a defined endocytic pathway [170]. Nevertheless, Kenis and colleagues demonstrated that AnxA5 internalization by apoptotic and viable PS-expressing tumor cells is independent from the described endocytic pathways [84]. The major prerequisite for internalization seems to be the binding of AnxA5 to PS. This pathway opens novel aspects to exploit AnxA5 as a targeting agent for delivery compounds into PS-expressing cells [142]. It was further suggested that AnxA5 blocks via this internalization mechanism the phagocytosis of dying cells by macrophages by delivering PS back to the inner leaflet of the cell membrane [85].

Modulation of inflammation by PS-exposing cells and organisms

Under healthy conditions, apoptotic cells are poorly immunogenic [123] and their clearance is non- or even anti-inflammatory. In contrast to the uptake of pathogens or FcR-mediated phagocytosis [134], the engulfment of apoptotic cells does not induce inflammatory cytokine production. Voll and colleagues demonstrated more than a decade ago that activated macrophages secrete more IL-10 and TGF-beta and less inflammatory cytokines like TNF-alpha, IL-1beta, and IL-12 after phagocytosis of apoptotic cells [166]. TGF-beta released by stimulated macrophages that ingest apoptotic cells was shown to be a central player in mediating those anti-inflammatory responses [103]. The production of TGF-beta is regulated at both transcriptional and translational steps. Transcription required p38 MAPK, ERK, and JNK whereas translation was dependent on Rho GTPase, PI3K, and Akt [174]. Cells like human monomyelocytes that do not express PS during apoptosis failed to induce TGF-beta. However, PS liposomes transferred onto the cell surface membranes were capable to restore the induction of this anti-inflammatory cytokine suggesting an important role for PS as immune suppressor [76].

PS in exosomes further contributes to an anti-inflammatory environment. Exosomes are vesicles of endocytic origin, and dendritic cells as well as macrophages are capable to spontaneously secret them. MFG-E8 being an adaptor protein for PS is one of the major exosomal proteins. It targets exosomes to other immune cells [164] or possibly to apoptotic cells. The binding of both exosomes and apoptotic cells is feasible, since MFG-E8, also called lactadherin, has two lipid binding domains [1]. The uptake of the dying cells together with exosomes might strongly contribute to the anti-inflammatory responses exerted by PS-exposing particles like apoptotic cells [173]. Ectosomes derived from polymorphonuclear neutrophils expose PS and induce immune suppression by inhibiting the maturation of DC and by inducing an increased release of TGF-beta1 [39]. The clearance of apoptotic cells is involved in the resolution of inflammation, and the regulation of pro- and anti-inflammatory cytokine production during the ingestion of dying and PS-exposing cells has been identified as one key regulatory mechanism. Kim and colleagues suggested that the pH value of the microenvironment where phagocytosis events take place further contributes to this regulation. The PS-binding activity of stabilin-2, being one receptor for PS, is enhanced in acidic areas, suggesting that low pH might act as a warning signal to foster stabilin-2-mediated phagocytosis to resolve inflammation [89].

Malignant cells and some virus and parasites are taking advantage of this ubiquitous PS regulated mechanism. PS is expressed on monocytes as part of their differentiation program to macrophages [23]. Since HIV and many other viruses cause extensive apoptosis, the infected cells express elevated levels of PS which, consecutively, can also be found in the outer membrane of the enveloped retrovirus [24]. The latter therefore enter macrophages “silently”, like apoptotic cells possibly contributing to the impaired immune response accompanying these infections. We also analyzed the influence of AnxA5 on HIV-I replication in infected phagocytes and found that the virus replication in macrophages was significantly reduced after treatment with a single dose of AnxA5 [112]. An opsonin for dying cells, namely complement component C1q, was further identified to mediate the binding of HIV to erythrocytes, playing a

crucial role in the progression of the primary infection [73]. In addition, ectosomes derived from erythrocytes bear immune suppressive properties [133]. Recent research revealed that cell death in protozoan parasites also occurs in a programmed fashion [162]. The obligate intracellular pathogen *Leishmania major* survives and multiplies in professional phagocytes. The survival of the parasites inside macrophages was shown to be dependent on the presence of apoptotic parasites exposing PS. Apoptotic promastigotes from *Leishmania* parasites induced release of TGF-beta by neutrophils, suggesting that the presence of apoptotic parasites provides survival advantage for the viable parasites fostering disease development [161]. Apoptotic neutrophils with *Leishmania* parasites inside further act as "Trojan horse" to deliver the parasites into macrophages by inducing even anti-inflammation [160]. *Vaccinia virus* also uses apoptotic mimicry to infect host cells. It enters the cells via macropinocytosis after inducing the extrusion of blebs. All entry events were strictly dependent on the presence of PS in the viral membrane [106]. To block the PS-dependent "silent" uptake of tumor cells, virus, and parasites is a promising target to induce immunity against those "invaders". Figure 6 schematically displays how interference with PS-dependent clearance processes leads to immune modulation.

Modulation of PS-dependent clearance—implications in disease

An efficient clearance of PS-exposing apoptotic cells is strongly linked to homeostasis in healthy tissues. One of the most intensively examined relationship between disturbed dying cell clearance and disease pathogenesis represents chronic autoimmunity. The clearance failure of apoptotic cells leads to the accumulation of secondarily necrotic cells. The latter have lost their membrane integrity resulting in the release of intracellular antigens, danger signals, DNA, and noxious contents. Under those circumstances, dendritic cells get the chance to take up dead cell-derived antigens, and they receive further co-stimulation by danger signals released from the secondarily necrotic cells. The presentation of antigens, which is also fostered by danger signals, leads to activation of T cells, which provide survival signals for B cells leading to specific immune reactions against dead cell-derived antigens (Fig. 6). SLE is a multifactorial systemic autoimmune disease characterized by a chronic and antigen-driven autoimmune response against antigens that are cleaved, phosphorylated, and/or originated from dying or dead cells. A clustering and concentration of these molecules in the surface blebs of apoptotic cells is often observed [132]. In about half of the patients with SLE, deficiencies in the recognition and engulfment of apoptotic cells have been reported to be one main alteration [64]. Early mechanistic studies revealed that an excess of apoptotic cells leads to autoantibody production [102, 107]. When lower amounts of apoptotic cells are present but the PS on the dying cell surface is masked, autoantibody production is also induced, most likely due to impaired clearance of the apoptotic cells by macrophages [8]. Mice mutant for TAM receptor protein tyrosine kinases also bear signs of chronic autoimmunity [99]. Various mouse models displaying defects in the PS mediated clearance have confirmed that impairment in apoptotic cell removal can lead to chronic and systemic autoimmunity (summarized in [61]). Examples for deficiencies of the following molecules in mice with clearance failures are C1q, DNase I, Mer, MFG-E8, secreted IgM, serum amyloid P component, and surfactant protein-D. All of those adaptor or chromatin processing molecules are involved in the clearance process of apoptotic cells [98]. Just recently, it has been described that in Tim4-deficient mice peritoneal macrophages and B-1 cells do not efficiently clear apoptotic thymocytes in vivo [129], emphasizing that defects in receptors for PS also contribute to chronic autoimmunity.

As already mentioned, Rac inside the phagocyte is mandatory for cytoskeletal rearrangement during internalization of apoptotic cells. Rac can be antagonized by the small GTPase RhoA resulting in impaired engulfment [113]. Those mechanisms may play pivotal roles in the diseased lung. Increased levels of apoptotic cells have been observed in lung tissue of various respiratory diseases. The impact of an impaired clearance on the pathogenesis of those diseases as well as on neurological diseases and on atherosclerosis was just recently reviewed very detailed by Elliott and Ravichandran [43]. Mice deficient in typical apoptotic cell adaptor and bridging molecules develop atheriosclerotic plaques. MFG-E8 seems to be the prominent adaptor protein for clearance of apoptotic cells by microglia in the brain. Decreased levels of MFG-E8 have been shown to be associated with Alzheimer's disease [15].

The MFG-E8-dependent clearance may be beneficial in the case of removal of tumor cells. The induction of anti-inflammatory mediators could neutralize the inflammatory environment often fostering tumorigenesis [127]. However, the immune suppressive effects resulting from clearance processes could also suppress specific anti-tumor immune responses. Increased MFG-E8 levels in the tumor microenvironment have been found to foster tumor cell survival, invasion, and angiogenesis by contributing to local immune suppression. The expression of other molecules also involved in engulfment processes is upregulated in certain tumor entities [22]. Blockade of MFG-E8 cooperated with cytotoxic chemotherapy, molecularly targeted therapy, and radiation therapy to induce destruction of already established mouse tumors [81]. It has to be stressed that the

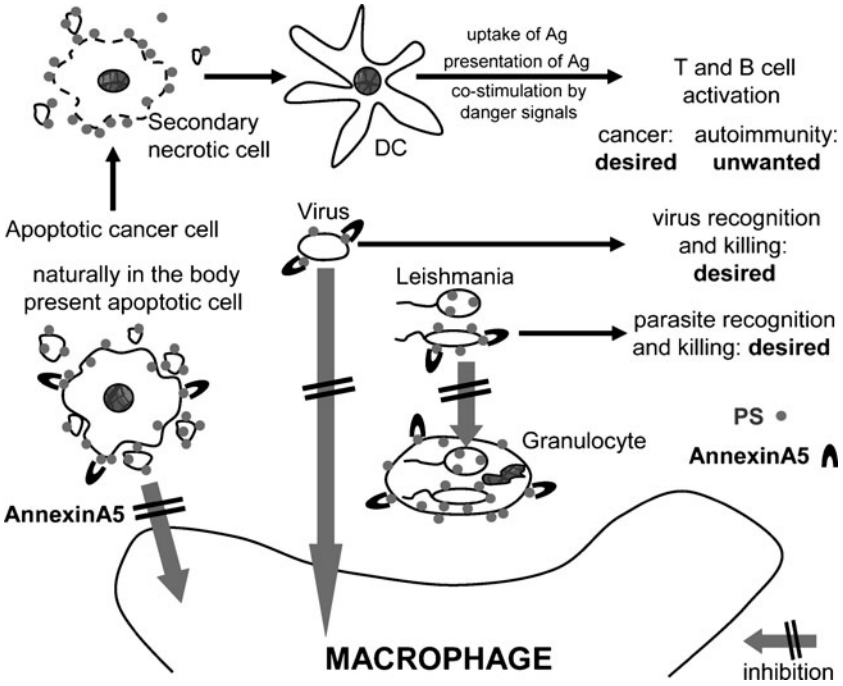

Fig. 6 PS-exposing particles enter silently in macrophages while blocking their clearance induces specific immunity. Apoptotic cells and microparticles expose PS leading to an anti-inflammatory clearance by macrophages. Clearance defects for naturally in the body occurring cells lead to the accumulation of apoptotic cells which finally proceed to secondary necrosis. Secondarily necrotic cell-derived antigens are taken up and presented by DC assumed that co-stimulation is provided by danger signals released from the dead cells. The presented antigens lead to activation of T cells, which provide survival signals for B cells leading to chronic autoimmunity. In the case of cancer cells, a conscious blocking of their clearance by, e.g., the PS-binding protein AnxA5 can lead by the same mechanism to specific anti-tumor immunity. This blocking strategy is also instrumental to induce immune reactions against PS-exposing virus or *Leishmania* parasites. Both membrane wrapped virus and *Leishmania* parasites use the PS exposure as a tool to assure their survival by fooling the immune system. *Ag* antigen, *DC* dendritic cell, *PS* phosphatidylserine

concentration of MFG-E8 strongly determines the immunological outcome. An excess of MFG-E8 has an inverse effect on the engulfment of apoptotic cells, meaning that apoptotic cell removal is blocked instead of fostered [176]. High MFG-E8 concentrations may therefore have similar immune effects like AnxA5 (Fig. 6). Besides concentration, the timely regulated release of immune modulating molecules is essential. The temporarily release of immune activating danger signals like HMGB1 by necrotic cells, resulting among others from impaired clearance, provide co-stimulatory signals for dendritic cells. Such danger signals act as early warning system to activate innate and adaptive immune responses. Recently, it was demonstrated that activation of a tumor antigen-specific T cell immunity involves secretion of HMGB1 protein by dying and dead tumor cells [4]. Besides HMGB1, ATP, uric acid, or extracellular HSP can act as danger signals and interact with several receptors leading to the induction of the secretion of pro-inflammatory cytokines by immune cells. Combinations of ionizing irradiation with hyperthermia resulted in increased amounts of necrotic tumor cells and extracellular HMGB1 in comparison to single treatments [104, 138]. The release of HMGB1 by tumor cells leads to cross-presentation of tumor cell-derived antigens by DC while on the tumor cell surface exposed and normally intracellular located proteins may foster the uptake of tumor cells by DC. Anthracyclines have been discovered by Dr. Obeid and co-workers to elicit immunogenic tumor cell death. The latter correlates with the exposure of the ER-resident protein calreticulin (CRT) on the tumor cell surface in very early phases of the apoptotic program [114]. Cell death-related molecules like CRT and HMGB1 have to compile a spatiotemporal code to translate cell death into a specific anti-tumor immune response [179]. It has to be stressed that the general view that necrosis always does induce inflammation and apoptosis anti-inflammation is an oversimplification. One main important step in the induction of anti-tumor immunity is to block the uptake of dying tumor cells by macrophages and to shift it toward DC [57]. We apply the PS-binding protein AnxA5 to block the uptake of apoptotic tumor cells by macrophages (Fig. 6). AnxA5 decreased apoptotic cell uptake by macrophages and concomitantly increased their uptake by DC in vitro and in vivo. Furthermore, activated macrophages secreted higher amounts of TNF-alpha and IL-1beta and lower amounts of TGF-beta after contact with dying tumor cells in the presence of AnxA5 in comparison to solely dying tumor cells [16, 57]. Vaccination and cure assays of tumor-

bearing mice proceeded significantly better when AnxA5 was present to mask PS exposed by irradiated apoptotic tumor cells [16]. PS also becomes exposed on the luminal surface of vascular endothelium in tumors. Targeting of PS with antibodies in addition to irradiation led to damage of tumor blood vessels in rats and to the induction of tumor immunity, even in glioblastoma [71]. Intervention with molecules acting as "find-me" signals may also be a promising target in anti-cancer therapies [43]. IL-10 activated macrophages showing an enhanced apoptotic cell clearance capacity sustain an anti-inflammatory environment thereby contributing to the suppression of anti-tumor immunity [115]. The "find-me" signal fractalkine (CX3CL1) seems to be involved in the recruitment of such immune suppressive macrophages [155]. Small amounts of ATP could also foster anti-inflammatory clearance processes [42], while present in high local concentrations, it acts as inflammatory danger signal.

Microvesicles as modulators of the tumor microenvironment

The cell biology of microvesicles and their influence on inflammatory processes was recently summarized in detail by Cocucci and colleagues [30]. Microvesicles often consist of mixed vesicle populations containing shedding vesicles and exosomes. An anti-inflammatory microenvironment is sustained by tumor-derived microvesicles by multiple mechanisms (Fig. 7). Macrophages secreted high amounts of TGF-beta after engulfment of tumor microvesicles being rich in PS. Pre-clinical models revealed that the metastatic potential of melanoma was enhanced in the presence of such PS-exposing microvesicles. Notably, the effects were reversed by adding AnxA5 [101]. An accumulation of cytotoxic drug and their discharge in shed vesicles is another mechanism by which tumor cells escape from therapy [145]. Inhibition of drug export in microvesicles may therefore offer new potential for reverting multidrug resistance of cancer cells [38]. The transfer of microparticles is often dependent on bridging molecules like already outlined for the uptake of apoptotic cells by macrophages. After binding of complement, microparticles have been shown to be taken up by immune cells. They thereby reduce the activation of B cells and skip the cytokine secretion profile of monocytes toward anti-inflammation [93]. Tumor-derived microvesicles significantly modulate the biological activity of monocytes resembling very closely the effects of tumor cells by themselves on immune cells [10]. Growing tumor cells shed microvesicles. The rate of shedded vesicles is often increased in malignant tumors. Analyses of the specific proteome of such microvesicles identified annexins to be involved in their biogenesis [29]. The binding of annexins to PL is strictly dependent on Ca^{2+} ions, like it was identified to be for the release of ectosomes [120]. Besides microvesicles, cancer cells also shed soluble PS [88] contributing to active immune suppression that is also sustained by secretion of TGF-beta. TGF-beta1 secretion and the exposure of PS on the surface of ectosomes both down-modulate cellular activation in macrophages [65].

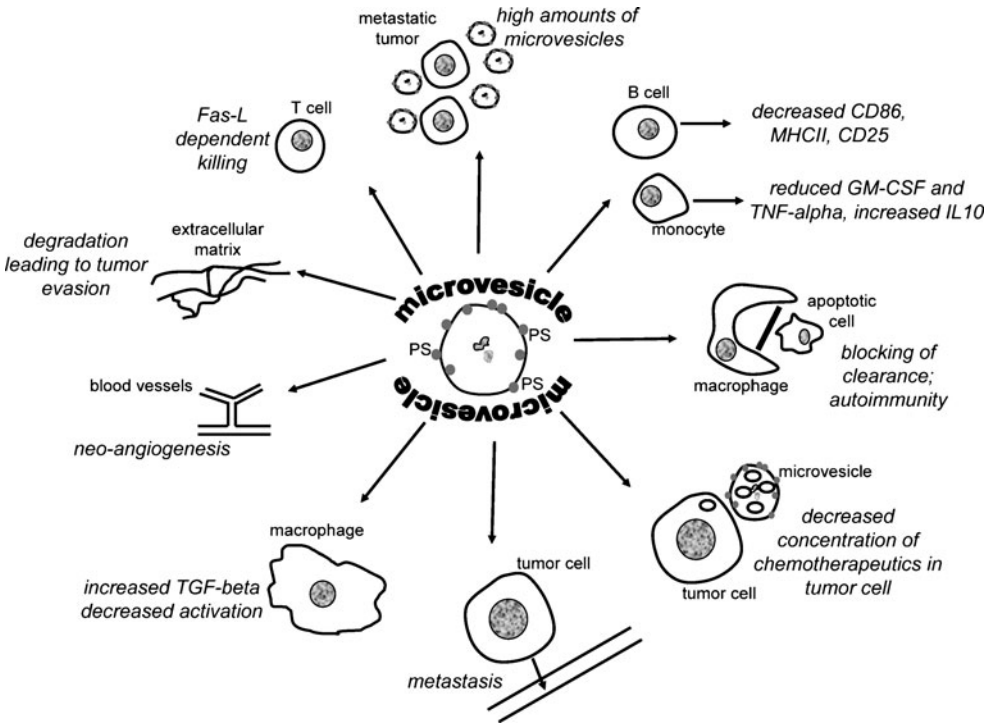

Fig. 7 The role of microvesicles in tumor escape. PS-exposing microvesicles interact with various immune cells, vessels, tumor cells, and the extracellular matrix leading to an anti-inflammatory microenvironment fostering tumor escape. For detailed explanation, please refer to the part of the main text dealing with "microvesicles as modulators of the tumor microenvironment". *PS* phosphatidylserine

The extracellular matrix is also modulated by microvesicles. Metalloproteinases and cathepsin B are main modulators of extracellular matrix and are transferred in the tumor microenvironment via microvesicles [68]. A disintegrin and metalloproteinases are expressed in various human tumors and promote cell growth as well as tumor spreading [110]. Microvesicles contain mRNA, microRNA, and angiogenic proteins and are thereby capable to deliver genetic information and proteins to recipient cells in the tumor microenvironment [152]. Another possibility for tumor progression and cancer cell immune escape exerted by microvesicles is to induce killing of immune cells. Microvesicles containing FasL induced loss of mitochondrial membrane potential, cytochrome c release, and caspase-3 cleavage leading to T cell apoptosis [87]. Taken together, the release of extracellular microvesicles is a highly important process in tumorigenesis [172].

Microvesicles further compete for macrophages with apoptotic cells. The production of PS-exposing plasma membrane-derived vesicles is increased during apoptosis, the so-called blebbing of the dying cell. The blebs may lead to workload of macrophages resulting in an accumulation of apoptotic cells. The latter can sustain the plasma membrane integrity only for a certain time before loosing it. The resulting secondarily necrotic cells foster inflammatory reactions and may contribute to the etiopathogenesis of chronic autoimmune diseases like SLE [3]. Blebs are further known for long time to be, besides their anti-inflammatory properties, responsible for spread of antigen in SLE [26]. Figure 7 summarizes how microvesicles contribute to the escape of tumors. The isolation and analysis of microvesicles from blood samples of autoimmune or tumor patients have the potential to provide information about state and progression of diseases and could ascent as innovative biomarkers [28].

Concluding remark

Resolving further pathophysiological roles of PS will be prerequisites for the development of novel drugs and in exploiting immunogenic signals of apoptotic and necrotic cells and microvesicles for controlling diseases like chronic autoimmunity and cancer.

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