# Phosphatidylserine-expressing cell by-products in transfusion: A pro-inflammatory or an anti-inflammatory effect?

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# Phosphatidylserine-expressing cell by-products in transfusion: a pro-inflammatory or an anti-inflammatory effect?

## Rôle des débris cellulaires exprimant la phosphatidylserine en transfusion : un effet pro- ou anti-inflammatoire ?

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**Summary:**

Labile blood products contain phosphatidylserine (PtdSer)-expressing cell dusts, including apoptotic cells and microparticles. These cell byproducts are produced during blood product process or storage and derived from the cells of interest that exert a therapeutic effect (red blood cells or platelets). Alternatively, PtdSer-expressing cell dusts may also derived from contaminating cells, such as leukocytes, or may be already present in plasma, such as platelet-derived microparticles. These cell byproducts present in labile blood products can be responsible for transfusion-induced immunomodulation leading to either TRALI or increased occurrence of post-transfusion infections or cancer relapse. In this review, we report data from the literature and our laboratory dealing with interactions between antigen-presenting cells and PtdSer-expressing cell dusts, including apoptotic leukocytes and blood cell-derived microparticles. Then, we discuss how these PtdSer-expressing cell byproducts may influence transfusion.

Key words: Apoptotic cells; Microparticles;Macrophages; Dendritic cells; TRALI; Tolerance; Inflammation; Transfusion; Treg; labile blood products

**Résumé :**

Les produits sanguins labiles contiennent des débris cellulaires qui expriment à leur surface la phosphatidylsérine, un phospholipide exprimé à la face interne de la membrane plasmique des cellules viables. Ces débris, générés durant la conservation ou la filtration des produits sanguins labiles, correspondent à des cellules en apoptose ou des microparticules. Ces débris peuvent donc influencer la transfusion et conduire à une activation du système immunitaire du patient transfusé, à l'origine du TRALI par exemple. Au contraire,ces débris cellulaires peuvent concourir à une inhibition du système immunitaire après transfusion et conduire àla survenue d'infections ou encore favoriser les récidivesde cancer. Dans cette revue, nous rapportons les données de la littérature et de notre laboratoire concernant les interactions entre les débris exprimant la phosphatidylsérine (cellules apoptotiques et microparticules) et les cellules présentatrices d'antigènes et nous discutons des éléments permettant d'expliquer le rôle pro- ou anti-inflammatoire de ces débris cellulaires lors de la transfusion.

Mots clés : Cellules apoptotiques ;Microparticules ;Macrophages ;Cellules dendritiques ; TRALI ; Tolérance ; Inflammation ;Transfusion ;Lymphocytes T régulateurs ; produits sanguins labiles

**1. Introduction**

Labile blood products contain the cells of interest (that are red blood cells [RBC], platelets) or plasma that exert their therapeutic effects,but also cell byproducts or cell dusts. These cell byproducts may play a critical role in transfusion. Here, we will discuss the influence of phosphatidylserine (PtdSer)-expressing cell dusts on immune responses and their consequences in transfusion setting. These cell byproducts may activate innate immune cells (such as antigen-presenting cells [APC]) and deliver a pro-inflammatory response observed, for instance, in antibody-independent transfusion-related acute lung injury (TRALI). Today, TRALI is on the scrutiny of the transfusion community.Indeed, TRALI is the leading cause of transfusion-related morbidity and mortality. Recent publications have reported the frequency of TRALI according to the number of transfused blood products indifferent countries [1, 2]. However, the risk of TRALI is not equal for all blood products. Single donor plasma transfusion carries the highest risk of TRALI (mainly immune-mediated TRALI) [3], while RBC transfusion exhibits the lowest risk of all blood products for antibody-associated TRALI [4]. Among the blood products implicated in TRALI, half are fresh-frozen plasma (FFP)[2]. Moreover, the risk is higher in intensive care units where 40% of critically ill patients receive at least one unit of RBC with a mean of 5 units per patient [5]. A “two hit” model is used to explain the occurrence of TRALI[2, 4, 5]. The first hit is related to the transfused patients and several recipient-related risk factors have been identified[2, 4, 5]. The analysis of experimental models of TRALI suggests that factors may protect or attenuate TRALI, such as T cells[4]. The second hit is linked to the transfused blood product[2, 4, 5]. Transfusion-related risk factors have been identified or suspected for plasma, RBC and platelets[4]. Some of these risks have been taken into account in transfusion practice and have significantly reduced TRALI occurrence, such as the prevention of anti-HLA antibody infusion by using male donor plasma[2, 3, 6]. Depending on factors present in blood products and triggering TRALI, TRALI can be divided up into immune (antibody-mediated) TRALI and non immune (antibody-independent) TRALI[2, 4, 5]. It has been suggested that substances released during blood product storage may cause non immune TRALI, such as free hemoglobin, iron, nitric oxide or microparticles[5].On the

opposite to immune system activation, PtdSer-expressing cell byproducts present in blood products may inhibit innate immune cells and deliver an anti-inflammatory signal or may induce donor-specific tolerance. Such mechanismscan be involved in the immunosuppressive or tolerogenic effects of blood transfusion observed in patients. This may lead to an increased occurrence of post-transfusion infections [7]or cancer relapse[7, 8]. In addition, this may explain the effect of donor-specific transfusion (DST) in kidney transplanted patients[9-11].Thereafter, we will discuss how PtdSer-expressing cell byproducts may participate in both sides of transfusion-related immunomodulation [8]: immune activation or inhibition(Figure 1). Before that, we will review publications reporting the presence of PtdSer-expressing cell byproducts with a special emphasis in the processes that influence their generation.

**2. Phosphatidylserine-expressing cell byproducts in labile blood products**

Several publications report the presence of PtdSer-expressing cell byproducts in blood products[12-34](Table 1).These cell byproducts may be generated during the blood product process, such as filtration[19] or during storage(either cold storage for RBC [12-15, 19, 24-26, 28, 34, 35]or between 20-24°C for platelets[16, 17, 20-22, 27, 29]). Alternatively, they may be limitedby filtration [23, 31, 33, 36]. Phosphatidylserine-expressing cell byproducts can be apoptotic cells[13-17, 19-22, 27, 29, 34]. Apoptotic cells have been found in different blood products: RBC units[13-15, 19, 34], platelet concentrates[16, 17, 20-22, 27, 29]. These apoptotic cells correspond to dying cells of interest: RBC or platelets[16, 20-22, 27, 29], both enucleated cells that can undergo apoptosis [37]. Residual cells contaminating the blood products −such as leucocytes[13-15, 19, 34], including: lymphocytes[14], neutrophils [14, 34] or monocytes[34]− may also become apoptotic. Leukoreductionby filtration reduces the absolute number of contaminating leukocytes in blood products and thus may limit the number of apoptotic leukocytes[38, 39].However, filtration seems also to affect leukocyte viability favoring apoptotic leukocyte production [19]. Otherwise, PtdSer-expressing cell byproducts can also be microparticles[12, 23-26, 28, 30-33, 35, 36], also known as ectosomes[40]. This corresponds to

particles with a diameter of less than one micrometer produced from plasma membrane by a mechanism called vesiculation[40]. These microparticles are generated during apoptotic process or after cell activation [26]and may originate from the cells of interest that are transfused(i.e., RBC[12, 24-26, 28]or platelets[32])and be produced during storage [12, 18, 24-26, 28, 29, 35],or alternatively, they can already be present in the plasma as microparticles can be detected in FFP[23, 33, 36].These latter correspond to circulating microparticles. The major circulating microparticles found in healthy donors are platelet-derived microparticles (PMP)followed by red blood cell-derived microparticles (RMP)[41]. Overall, the different steps of blood product process,and especially storage at 20-24°C for platelet concentrates or at 1-6°C for RBC units, generate PtdSer-expressing cell byproducts that may have consequences in transfused patients.

## 3. Immune functions of phosphatidylserine-expressing cell byproducts and potential consequences in transfusion

### 3.1. Immune functions of apoptotic blood cells and potential consequences in transfusion

The anti-inflammatory propertiesof early stage apoptotic leukocytes are well documentedin the literature(for review [42-45])(Figure 2). An early event occurring after apoptosis is the expression of “eat-me signals”, such as the expression of PtdSer at the external leaflet of plasma membrane[45-47]. Phosphatidylserine expression by apoptotic cells favors their uptake by professional phagocytes, such as macrophages or conventional dendritic cells (cDC)(for review [42, 45]). Uptake of apoptotic cells induces the secretion of anti-inflammatory cytokines, such as IL-10 or TGF-β, as well as the inhibition of inflammatory cytokine secretion such as IL-12 or IL-1β, IL-6 and TNF (for review [42, 43]). Moreover, APC become resistant to Toll-like receptor (TLR) ligand activation[48, 49]. These APC do not express high levels of costimulatory molecules and if they interact with naive T cells they favor T cell anergy (for review [42]).By the release of TGF-β after apoptotic cell internalization,APC induce the generation of regulatory T cells (Treg)[50, 51]. In this setting, generation of Treg leads to tolerance against apoptotic cell-derived antigens.

In line with these data, we have reported that intravenous apoptotic leukocyte infusion favors bone marrow engraftment when apoptotic leukocytes were infused simultaneously with an allogeneic bone marrow allograft in irradiated mice[52]. This effect is observed whatever the origin of apoptotic cells: donor, recipient and third party[52]and is dependent on TGF-β[50, 53, 54]. Apoptotic cell-induced engraftment implicates recipient macrophages, since prior depletion of host macrophages by clodronate liposome infusion inhibits the graft facilitating effect of apoptotic cells[50].This confirms previous resultson the critical role of macrophages to limit immune responses against dying cells [55, 56]. We also showed that plasmacytoid dendritic cells (PDC) are mandatory in the apoptotic cell-induced Treg both in transplantation settings, as well as in naive mice [53]. However, whereas PDC are influenced by factors (including TGF-β) released by macrophages uptaking apoptotic cells,PDC are not directly influenced by apoptotic cells [53].

Implication of PtdSer in the inhibition of both inflammation and specific immune responses has been further demonstrated using PtdSer-expressing liposomes [47, 57]and is sustained by the following observations:i) PtdSer-dependent ingestion of apoptotic cells induces TGF-β secretion and resolution of lung inflammation [57];ii) inhibition of PtdSer recognition through Annexin-V enhances the immunogenicity of irradiated tumor cells *in vivo*[58];iii) masking of PtdSer inhibits apoptotic cell engulfment and induces autoantibody production in mice [59].

Based on data from our group [52] and Peter Henson's group [57],some authors have speculated that apoptotic leukocytes present in blood products may be responsible for transfusion-related immunosuppression[38, 60]. Thus, the first consequences of PtdSer-expressing apoptotic cells in blood products may be a transient immunosuppression [38, 60]−responsible for an increase in infection rate and of cancer relapse [8]−,or tolerance induction −as observed after DST[9-11]−when Treg have been generated.However, as discussed below (see Paragraph 4), apoptotic leukocytes become secondarily necrotic in the absence of phagocytes[45, 46]. This may certainly occur in blood productbags. Necrotic cells, through the release of damage-associated molecular patterns (DAMP) [61], may become immunogeneic [42, 61]. The same process may occur for platelets [62]. Necrotic

platelets may represent the procoagulant form of platelets [62]. Thus, hemostatic activation of platelets or their byproductsmay link thrombosis and inflammation to amplify lung microvascular damage during non immune TRALI [5].

### 3.2. Immune functions of microparticles and potential consequences in transfusion

Cell surface PtdSer expression is a common feature of microparticles whatever their cell origin[40, 45, 47]. Phosphatidylserineexposure at cell surface is involved in the procoagulant activity of circulating microparticles[41]: including PMP [63], but also RMP [64]. However, their function on innate immune response is less clear. We and others have addressed the effect of PtdSer-microparticles on APC[65-72].Incubation of neutrophil-derived microparticles (NMP) inhibits secretion of inflammatory cytokines (IL-8, TNF) by macrophages in response to TLR4 ligand, LPS and TLR2 ligand, zymosan [65]. The same effect is observed using macrophages [68, 70-72] or other APC (i.e., cDC[67, 69, 71, 72], monocytes [66] or B cells [66]) incubated with other microparticles derived from different circulating cells: platelets [69, 72], neutrophils [65, 67, 71], RBC[68], T cells [69], endothelial cells [69] as well as tumors cells[66, 70]. Thus, PtdSer-expressing microparticles exert an anti-inflammatory response through PtdSer expression[65, 70] and TGF-β secretion[65, 70-72].Moreover, the Mer receptor tyrosine kinase implicated in the binding of PtdSer ligands (GAS-6 or protein S) is required for alteration of APC functions by NMP[71]. Thus, PtdSer-expressing microparticles participate in transfusion-related immunosuppression as apoptotic cells do. However, data report the increase of microparticle number in stored blood products (Table 1) and the impact of blood product storage on TRALI occurrence [5].In line with these observations, we reported recently that endothelial-derived microparticles (EMP) (found in FFPand representing around 0.5% of microparticles [33]) induce the activation of a particular subset of dendritic cells, PDC[69]. PDC play a major role in immune responses against viruses by the secretion of interferon-alpha and are implicated in autoimmune diseases, such as psoriasis or lupus[73]. Incubation of EMP induces the increase of costimulatory molecules on PDC and therefore polarization of naive $CD4^+$ T

cells into pro-inflammatory Th1 T cells by EMP-stimulated PDC. Stimulation of PDC byEMP induces the production of inflammatory cytokines IL-6 and IL-8 [69]. Overall, depending on the considered APC, microparticles favor inflammation.This suggests thatPtdSer-expressing microparticles present in blood products may favor and/or sustain inflammation.

**4. Conclusion and perspectives**

What are the next steps to answer the question on the role of PtdSer-expressing cell dusts in the modulation of immune responses after transfusion?The next steps are to characterize or identify factors involved in the triggering of inflammation or its inhibition and produced during blood product storage or process.Several factors influence the immune responses against dying cells [42]. We can speculate on some factors,including: i) the number of PtdSer-expressing cell byproducts contained per blood product, as the immunogenicity of apoptotic cells may be proportional to the number of cells[42]; ii) the occurrence of secondary necrosis [61](see above) and sothe passive release of intracellular DAMP that overpasses the inhibitory signals delivered byPtdSer.One of these DAMP can be the hemereleased from stored RBC which signals *via* TLR4[74]; iii)the size of cell byproducts and especially microparticles, since these latter exert different functions according to their size[30, 75]. Moreover, APC, such as PDC, respond only to lower sized synthetic particles[76]. This may explain the different responses observed between “amateur” phagocytes (PDC) versus professional phagocytes (cDC/macrophages) after incubation with microparticles [69]. The size of cell byproducts diminishes during plasma filtration as assessed by dynamic light scattering from 101 to 464 nm in unfiltered FFP*versus* 21 to 182 nm after 0.2µm filtration process [36];iv) expression of the recently described PtdSer receptors [46]on different APC subsets may also explain the different responses between PDC *versus*cDC/macrophages[69] and may impact on the overall immune response.Finally, one has to evoke that microparticles are linked with apoptotic cells and that apoptotic cells and microparticles represent a same continuum. Indeed, PMP are generated by a loss of membrane integrity [18] and PMP foundin platelet concentrates are linked to platelet apoptosis rather than platelet activation

[16]. Apoptotic blebs released early during apoptotic process are due to microvesiculation of cell membrane and thus correspond to microparticles/ectosomes[47].In contrast, late apoptotic bodies −produced at the end ofthe apoptotic process whenapoptotic cell clearance is delayed or altered− differ from microparticules in size and composition, since plasma membrane has been substituted by membranes from internal compartments[47, 77]. The function of late apoptotic bodies is different from early stage apoptotic cells. While APC incubated with early apoptotic cells generate Treg [50, 51], cDC maturated with late apoptotic blebs rather induce pro-inflammatory Th17 cells [78]. Concomitant analysis of cell dusts in blood products and collection of clinical data from transfused patients is the only way to determine the role of PtdSer-expressing cell byproduct in transfusion setting.

**Acknowledgements:**We are grateful to Sarah Odrion for her help in manuscript editing, and the members of our laboratory for their work. Our studies in the fields of this review are supported by grants from the Association pour la Recherche sur le Cancer (ARC) (#5084 to SP), the Etablissement Français du Sang (#2011-05 to SP, #2011-11 to PS), the Association Recherche etTransfusion (2010 to FGO), the APICHU CHU Besancon (“IMIB” project API 3A CHU 2011 to FGO), and the Fondation Transplantation (# to FA).

**Conflict of interest:**the authors have not declared any conflict of interest.

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**Table 1. Phosphatidylserine-expressing cell by products in labile blood products**

| Phosphatidylserine-expressing cell byproducts | Blood products in which cell byproducts have been identified | Process generating cell byproducts* | References |
|---|---|---|---|
| RMP | RBC | cold storage (day 10 to day15) | Rumsby et al., 1977[12] |
| Apoptotic leukocytes (neutrophils, lymphocytes) | RBC | cold storage (48 hours) | Frabetti et al., 1998[14] |
| Apoptotic leukocytes | RBC | cold storage | Mincheff et al., 1998[13] |
| microparticles** | platelets | cold storage (+4°C) | Reid et al., 1999[18] |
| Apoptotic leukocytes | Platelets | storage and γ-irradiation (20 Gy) | Frabetti et al., 2000[17] |
| Apoptotic platelets | Platelets | storage | Li et al., 2000[16] |
| Apoptotic leukocytes | RBC | cold storage | Martelli et al., 2000[15] |
| Apoptotic leukocytes | RBC | filtration (around 50% at day 0), storage (day 10 ≈40% in non leukoreduced vs. ≈ 90%) | Bontadini et al., 2002[19] |
| Apoptotic platelets | Platelets | storage (37°C is also tested, 3 hours) | Bertino et al., 2003[20] |
| Apoptotic platelets | Platelets | storage | Perrotta et al., 2003[21] |
| RMP | RBC | cold storage | Kriebardis et al., 2008[24] |
| PMP and RMP | Plasma | filtration (0.2µm, RMP decrease but not PMP decrease) | Lawrie et al., 2008[23] |
| Apoptotic platelets | Platelets | storage | Leytin et al., 2008[22] |
| RMP | RBC | cold storage (4°C, a 20-fold increase) | Rubin et al., 2008[25] |
| RMP | RBC | cold storage | Salzer et al., 2008[26] |
| Apoptotic platelets | Platelets | storage | Albanyan et al., 2009[27] |
| RMP | RBC | cold storage, modulation by conservation solution | Antonelou et al., 2010[28] |
| Apoptotic platelets | Platelets | storage | Cookson et al., 2010[29] |
| RMP | RBC | | Jy et al., 2010[30] |
| microparticles | FFP | filtration (“high sized” MP removal) | Lawrie et al., 2009[36] |
| PMP | RBC | filtration (PMP decrease) | Sugawara et al., 2010[31] |
| RMP | RBC | cold storage | Jy et al., 2011[35] |
| Apoptotic leukocytes (neutrophils, monocytes) | RBC | cold storage (4°C – day 10-15) | Keating et al., 2011[34] |
| PMP (> 85%) | FFP | storage (PMP decrease) | Matijevic et al., 2011[33] |
| PMP | Platelets | | Xu et al., 2011[32] |

Abbreviations used:FFP, fresh frozen plasma; PMP, platelet-derived microparticles; RBC, red blood cells; RMP, red blood cell-derived microparticles; RT, room temperature. *Conditions of storage for RBC and platelets are 42 days at 1 - 6°C (mentioned as “cold storage” in the Table unless if specified in brackets) and 5 -7 days at 20 - 24°C (unless specified in brackets), respectively. Some information was also added in brackets such as the time where cell dusts were identified or the percentage of dying/dead cells, etc. ** microparticles were not formally identified but platelets were labeled with a fluorescent marker and small fluorescent fragments were identified in supernatant.

**Figure legends**

**Figure 1. Potential consequences of phosphatidylserine-expressing cell byproducts in transfusion.**Interactions of PtdSer-expressing cell dusts (apoptotic cells or microparticles) may lead to antigen-presenting cell (APC) activation or inhibition. APC activation may trigger inflammation and be involved in TRALI while APC inhibition may exert transient immunosuppression or tolerance. Blood product process or storage may influence the generation of PtdSer-expressing cell dusts (see table 1).

**Figure 2.The immunomodulatory effects of apoptotic leukocytes.**Early during the apoptotic program, PtdSer-exposure occurs leading to apoptotic cell removal by macrophages (MΦ) or conventional dendritic cells (cDC). This uptake by APC induces the production of anti-inflammatory factors and concomitantly inhibits the synthesis of inflammatory cytokines [43]. These APC are refractory to TLR activation. This leads to a transient immunosuppressive microenvironment. If APC from this microenvironment migrate to secondary lymphoid organs, naive T cells are converted into inducible regulatory T cells. This leads to tolerance against apoptotic cell-derived antigens.Abbreviations used: HGF, hepatocyte growth factor; IL-, interleukin; NO, nitrite oxide; PGE-2, prostaglandin-E2; TGF, transforming growth factor; TNF, tumor necrosis factor.