



Novel Functions of Therapeutic Platelets as “Immune Cells”

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Abstract

Platelet components are commonly transfused to bleeding in patients in the absence of a suitable alternative. If one exempts granulocyte concentrates, this labile blood component is associated with the most common infectious and immune-related hazards. Though infection can be prevented, immune-related reactions are more difficult to combat because the physiopathology is not fully understood. Recent research has placed platelets in the immune continuum as cells fully licensed for innate immunity and inflammation, possibly bridging innate and adaptive immunity. This novel paradigm provides clues for understanding how platelets can mediate immune-related side effects when transfused to a patient in need, and to address prevention strategies. The present paper discusses novel functions associated with platelets and their potential response upon encounters with a recipient's cells during transfusion.

Keywords: Platelets; Transfusion; Innate immunity; Immune cells; Transfusion adverse effects; Cytokines

Introduction

Platelet component (PC) transfusion is indispensable in the treatment of hematological diseases when a defect exists in the production of platelets in the bone marrow or excessive peripheral destruction because of hemorrhage or cytolytic or neutralizing anti-platelet antibodies. Two strategies are used for PC transfusion, prophylactic or therapeutic, but the threshold for prescribing transfusion varies based on the patient's age, primary pathology, treatment schedule (i.e. invasive exploration or surgery) and recommendations dependent on “schools of hematology” [1]. PCs are usually found as two types of products, with variants for each. PCs may be obtained from a single donor by apheresis, or been pooled from several whole blood (WB) donations with ABO and RH:1 (RhD antigen) matching. Despite several processes being available, the vast majority of blood establishments use manual or automated pooling of several buffy-coats. The most recent variants that can be applied to both types of PCs are: i) reduction of the plasma by roughly 65% and substitution by platelet conservation adapted medium termed Platelet Additive Solution or PAS; ii) deferral (for this type of donation) of females having been pregnant to avoid plasma anti-HLA antibodies [2]; and iii) implementation of pathogen reduction/inactivation (PI/PR) procedures, three of which have been described thus far, though one is used on a relatively broad basis in Europe and the Middle East [3].

In general, platelets are delivered “fresh”, optimally 1 to 5 days after collection in most countries except Japan, where delivery is limited to day 3, and in certain blood transfusion systems when PCs are tested for bacterial safety or secured by PI/PR technology (PRT), in which case delivery can be extended to day 7. Under certain circumstances, platelets can be delivered thawed from a frozen inventory, such as in the case of a rare donor/recipient platelet antigen group. Dried platelets can be a substitute for emergencies in military field operations [4]. The paradigm is that the fresher the better, especially when ABO RH:1 groups match both ways (no cell mismatch, no plasma antibody (Ab) mismatch). However, no consensus exists on the most appropriate dose per kilogram or body surface [5,6] or the most appropriate read out for efficacy. Common sense dictates that efficacy can be judged by bleeding cessation or the non-occurrence of bleeding (including micro-hemorrhage), and on a “reasonable” time schedule between two prescriptions. Measurable read-outs have been set up, however, that are principally the corrected count increment (CCI), but some clinicians

cast doubt on the strict usefulness of such a measure because platelets are not specifically given by transfusion to re-circulate in a patient, especially in a patient who is prone to bleeding. Several reviews on this topic have been published recently [7-10].

The Earlier Paradigm in Primary Haemostasis

If one exempts granulocyte transfusions, PC transfusions lead to the highest level of adverse (unwanted or deleterious) effects in patients. Most effects are minor, but some are major or occasionally lethal [11-14]. The most feared adverse effect is acute bacterial infection, which has a poor prognosis [13]. In some parts of the world acute Chagas disease infections are seen, and all reported cases thus far have been the result of PC transfusions [12]. A complete safety is expected from PI/PR technology (PRT) implementation; bacterial testing has not shown full efficacy but must be re-tested after the technology moves forward [15]. Another feared severe complication is transfusion acute lung injury (TRALI), though PCs do not recapitulate all cases; a dramatic decrease has been observed with the reduction of plasma and the avoidance of anti-HLA Abs by donor selection and lab testing [16-19]. Obviously, PC donors are tested for HIV, HCV, HBV, HTLV, and syphilis, as well as malaria and Chagas pathogens depending on epidemiology. CMV testing is optional, but providing CMV-free PCs is sometimes mandatory for certain patients. PRT avoid CMV replication and, thus, the need for testing and making special inventory [4]. PRT also avoids irradiation, which is frequently required for PC transfusions given the primary pathology or treatment of patients in need [15]. Last, platelet antigen matching is sometimes needed in cases of rare groups or allo-immunization [20].

Just a couple of decades ago, PC transfusion commonly resulted

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Received November 13, 2011; **Accepted** December 21, 2011; **Published** January 05, 2011

Citation: Garraud O, Chavarin P, Fabrigli P, Hamzeh-Cognasse H, Cognasse F (2011) Novel Functions of Therapeutic Platelets as “Immune Cells”. J Blood Disord Transfus S1:005. doi:10.4172/2155-9864.S1-005

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in chills, fever, skin rashes, and inflammatory symptoms, such that pre-medication, sometimes using corticoids, was almost the rule. Reduction of the plasma volume has achieved a reduction of such symptoms, but the major contribution to reducing these side effects was most likely leukoreduction (by filtration) of PCs, especially when carried out "in process" rather than at the patient's bedside [21]. This process is thought to have resulted in an almost complete prevention of infusion of strongly pro-inflammatory leukocyte-originating cytokines and chemokines, such as IL-1 β , TNF- α , IL-6, and IL-8 [22].

To summarize the common paradigm, platelets are essential for onco-hematology patients. Careful preparation and observance of delivery rules avoid the major complications, and leukoreduction prevents discomfort and a large number of non-hemolytic febrile transfusion reactions (NHFRs). However, some reactions may still occur, and in a much larger proportion than transfusions of packed red blood cells (RBCs), which are also leukoreduced in process (but refrigerated and generally delivered later, by day 10 or after).

The Current Paradigm in Immunology: Innate Immunity and the Danger Theory

Platelets have been acknowledged for a long time as being more than "sticky material" that initiates clotting; platelets interfere with injured vessels through precise molecular interactions, and alteration of certain crucial molecules on the surface of platelets, inside platelet granules, or in an open canalicular system (OCS) sometimes leads to severe pathology and is frequently inherited (genetically controlled). Platelet functions in haemostasis are based on the non-random release of the critical factors that are needed, such as Ca²⁺ ions, ADP, ATP, and haemostasis molecules such as GpIIb/IIIa. Platelets indeed select within their secretion capacity which is the most appropriate to fit the situation; because platelets are anucleate cells there is no gene rearrangement that, in other cell types, define a program; however, the selection of the best fit proteins to couple with opposite (endothelium and plasma) proteins/glycoproteins resemble a "program". However, research on platelet functioning thought, de facto, to be "licensed" for de novo secretion of proteins.

Fifteen years ago, immunity was completely revisited after the work of Polly Matzinger and rediscovery of the non-self infectious danger theory, almost a century after the grand pioneer Elie Metchnikoff [23-25]. Pathogen-associated molecule patterns (PAMPs) and corresponding ligands, pathogen recognition receptors (PRRs), were discovered on "dangerous" pathogen immune cells and innate immune cells, respectively, in the most primitive systems, such as flies, as well as in mammals and humans [26,27]. These discoveries prompted immunologists to revisit "old" cells and describe "new" functions.

The most famous cell family is certainly the dendritic cells but also encompasses the B-lymphocytes and macrophages [28,29]. These cells express pathogen sensors, FcRs for Abs and complement (C)-Rcs, and secrete polarized panels of cytokines, chemokines, and likely other immunomodulators. They usually bridge innate and adaptive immunity, especially when expressing MHC molecules capable of presenting antigen to TcR-selected T lymphocytes [28,29]. Platelets are known to share some of these properties (i.e. Fc γ R, Fc γ R, Fc α R, and CR2 expression) [30,31] and to use them to intervene, generally with some efficacy, in innate immunity against infectious pathogens (e.g., helminth parasites) and allergens [32] by participating in the (hyper)-inflammatory reaction deployed by the injured body [33]. This function has been known for decades, but because platelets are devoid of nuclei, scientists have paid little attention to the significance of innate immunity

tools on cells aiming to stick to injured vessels. However, much interest is expressed in studying allo-immunization in individuals transfused with donors' platelets in order to better program transfusions, but no serious question has been addressed regarding the presence of HLA class I molecules on platelets (the most frequent immunogens before HPA molecular variants of haemostasis glycoproteins) [34,35].

Revisiting the Current Paradigm in Innate Immunity to Include Platelets

Nearly a decade ago, platelets were found to convey roughly 95% of CD40L found in plasma, CD40L otherwise known as CD154 being the ligand for CD40 and exerting important immunoregulatory properties [36,37]. This finding was important because it was one of the first pieces of evidence that platelets secrete factors in vivo that are not involved in haemostasis or direct anti-disease actions. Both CD40 and CD40L belong to the super-tumor necrosis factor-Rc family for antigen-presenting cells (APCs; i.e. dendritic cells, macrophages, memory B-lymphocytes): both are also major immunoregulatory molecules controlling major biological pathways encompassing activation, differentiation, apoptosis, gene selection (with a direct action on the chromatin) etc. In B-lymphocytes, CD40 and CD40L are seminal to terminal differentiation and Ab class switching [38-40]. Such a molecule set was not really expected in association with platelets.

We have been interested in deciphering the various biological tools that best stimulate the CD40/CD40L tandems on B-lymphocytes and sought to study the platelet-produced sCD40L [41]. Our interest in platelets prompted us to examine their different stages of activation in regards to immunological markers, and we made an attempt to examine whether human platelets express PRRs and Toll-like receptors (TLRs). We were among the first to demonstrate the presence of three TLR types on platelet membranes and in platelet cytoplasm: TLR2, TLR4, and TLR9 [42-44]; we were not able to find TLR1 or TLR6 (in contrast to Shiraki et al. [45]), which are usually companions of TLR2. These five TLR types are likely present on resting, non-activated human platelets [42]. We were then able to further study the biology of TLR expression on platelets when activated by thrombin or ligands of TLR4 or TLR2, either gross bacterial components or synthesized peptide analogs. Several groups, including ours, have demonstrated simultaneously that platelets comprise a functional signalosome. We also provided evidence that, depending on the nature of the ligand coupled to membrane TLR4, platelets secrete different varieties of cytokines, chemokines, and likely molecules [45-49]. This data provide important information, that platelets can have differential secretion products depending on the danger sensed by their membrane PRRs [50-52]. Work on platelet biology has moved very fast during the past couple of years and evidence has come from a team in Salt Lake City (Utah, USA); platelets are not "dead end cells" once in the circulation and can even transform and evolve [53]. Platelets can secrete certain de novo proteins, despite not having a nucleus and DNA apart from the mitochondrial DNA, by using a spliceosome [54]. Platelets and platelet molecules have been found to play roles in diverse pathologies, taking part in the inflammatory part of, for example, cardiovascular disease, HIV disease progression, rheumatoid arthritis, diabetes, sepsis, skin diseases, allergy, and liver disease [55]. Accumulated evidence indicates that platelets are innate immune cells that play a greater role than initially thought in general inflammatory processes in the body, in great part through their capacity to secrete an enormous (>300 have been recorded) number of molecules, some of them exerting prominent inflammatory activity [31,56]. Notably, platelet molecules are considered to have three origins: i) inherited from the megakaryocyte, the mother cell, as platelets are

fragmented parts of its cytoplasm; ii) absorbed from plasma and the environment; and iii) possibly produced de novo.

Cytokines and Inflammatory Proteins in ex vivo Pcs

A number of groups have extended the findings on platelet-secreted sCD40L. In particular, we showed that sCD40L is secreted by platelets prepared for transfusion PCs and stored in blood bank inventories. Not only sCD40L increased during storage (with significance by day 3), but many other factors, including PDGF-AA and sCD62P, increased [57]. We were also interested in ascertaining whether the mode of PC preparation affects platelet secretion during storage. Both mixed pools obtained from WBCs and aphaeresis had elevated levels of pro-inflammatory and immunomodulatory molecules during storage, though with small variations in the individual kinetics of production for any given product (personal correspondence). We also obtained evidence of small, but significant, differences in the production of pro-inflammatory factors secreted by stored platelets obtained with different aphaeresis separators. The differences were observed at the beginning of the process, but by day 3 and later, the inflammatory molecule profile was similar in the PCs, independent of the mode of production (submitted to publication). Taken together, the data indicate that activation markers are consistently expressed on platelets from day 3 onward. Platelets are usually collected and prepared 3 days before delivery for inventory management, and the expression of these markers must be taken into consideration when interpreting experimental and clinical data related to PC transfusion [58]. Our work has been confirmed by other groups, and consensus now exists that stored platelets release a number of factors within the supernatant (plasma and/or PAS) over time, meaning that fresher PCs deliver lower levels of external inflammatory factors to patients.

Another question we addressed concerned the propensity of platelet-released factors during PC storage to affect cells encountered in the recipient's vessels when transfused, including endothelial cells lining the catheterized vein and circulating immune cells. Evidence has been provided that inflammatory cytokines are secreted in amounts sufficient for activating, at least in vitro models, peripheral blood mononuclear cells (PBMCs), specifically T-lymphocytes, B-lymphocytes, monocytes, dendritic cells, and polymorphonuclear cells [58-60]. For example, platelet-originating sCD40L can affect circulating B lymphocytes by interfering with their Ab-production differentiation program in terms of Ab subclasses [59]. Thus, platelet-secreted products are, at least on experimental and theoretical grounds, not ineffective and possibly noxious. An as-yet unanswered question is whether some situations exist in which these pro-inflammatory products can be beneficial to the patient, particularly situations in which a limited inflammatory situation initiates an adapted immune response or faster replenishment of transplanted bone marrow.

Cytokines and Inflammatory Proteins in Transfused Pcs

Previously reported findings strongly suggest that PC transfusion provides more than therapeutic platelets aimed at preventing or stopping bleeding. Transfusion also provides plasma, and within plasma are many factors, including antibodies, active lipids from insulted platelet membranes, which are thought to be partly responsible for acute lung injury [61], and inflammatory cytokines and immunomodulators. Current practice is to limit native plasma, as it is generally considered to be unneeded, to one-third of the platelet suspension medium with replacement by additive solutions [4,62], a method that usually proves its efficacy by limiting adverse effects in recipients [63,64]. However,

the best hemostatic quality for platelets in additive solutions vs. native plasma is being reconsidered in ongoing investigations.

We recently provided evidence that sCD40L in PC products have a direct role in acute transfusion reactions (ATRs) in patients undergoing transfusion. In certain patients presenting with ATR, compared to matched control patients, the transfused platelets secreted excessive amounts of sCD40L and "squeezed" all of the cytoplasm-stored sCD40L from the cells during storage or at the time of transfusion. This sCD40L was secreted in large enough amounts to mediate biological activities on target cells, such as patients' B lymphocytes [59,65]. However, whether certain donors' platelets are prone to hyperactivation (and against which stimulus) or whether certain recipients are hyper-reactive to sCD40L remains to be determined.

More recently, we conducted another survey of 40 ATR cases, investigating 65 cytokines in each involved PC supernatant. In all cases, we found a significant increase (over controls) of only two cytokines, IL-27 and Ox40L; in all but one case they were elevated in association with sCD40L. These cytokines were present in large enough amounts to mediate in vitro biological activities on T cells (expressing Ox40 and CD69) or B cells (expressing IL-27R and CD86, and secretion of IL-6); neither IL-27 nor Ox40L was described before this work as being associated with platelets, but we received confirmation that they are already present within the megakaryocyte and not absorbed from "pathologic" plasma (submitted for publication). This observation confirmed that at least three cytokines can be significantly elevated in PC products, leading to ATRs. Whether all elevated levels or just these three cytokines are pathogenic remains to be determined. Notably, we observed a number of other cytokines that increased during storage in PC bags; however, in the present study, only sCD40L, IL-27, and Ox40L seemed to be involved in pathology according to the criteria under investigation and current hemovigilance reports. We are about to start a larger investigation to screen for the presence and amounts of cytokines in consecutive series of PCs to be delivered to patients, and to analyze data with an epidemiological perspective.

Unwanted Complications and Transfusion Reactions

Unwanted effects of PC transfusion can be classified as acute or chronic, and they grossly comprise three families of complications: infectious, immune, and metabolic. It is not the purpose of this review to cover these general topics that have been reviewed previously [66,67]. Rather, the present review stresses the possibility that some of these complications are totally or partially linked to inflammatory factors secreted by platelets. However, the case of allergy will not be covered here because it is largely unknown in terms of physiopathology [68]. ATRs, the major presentation of NHFTRs, have already been addressed with at least three products being clearly identified: sCD40L, IL-27, and Ox40L. We could not succeed at completely neutralizing the mediated effects in vitro, possibly because of technical difficulties linked to neutralizing monoclonal Abs, or the involvement of as of yet unidentified factors or co-factors.

The case of TRALI is complex because it occurs with all labile blood products, not only platelets; however, the "double hit" theory postulates that the "first partner in crime" is an Ab, particularly in the donor's plasma or exceptionally in the patient's plasma in cases of inverted TRALI, and the second partner is either inflammatory cytokines from granulocytes or oxidized residues of platelet membranes/vesicles, or both [68,69]. A role of platelet-derived cytokines is suspected but not yet proven. Because TRALIs are rare and becoming even rarer, it will

take some time to study the latter hypothesis or extrapolate from a model animal [70].

The case of allo-immunization is even more complex. Two families of molecules harboring strong polymorphisms lead to allo-Ab formation in pregnant women and transfused patients. One series reported that 1.7% of females that never got pregnant presented with detectable anti-HLA antibody levels; this percentage reached 11.2, 22.3, 27.3, and 32.5 after 1, 2, 3, and 4 (and more) pregnancies, respectively (unpublished data; [71]). Post-transfusion allo-immunization is less frequent than it used to be before the systematic in process leukoreduction of labile blood products, but it is barely observed after RBC components, and even less often after fresh frozen plasma transfusion, but it is not uncommon after PC transfusion. Anti-HPA immunization is rare but often associated with serious consequences when it induces platelet destruction in fetuses and newborns. Active immunization against protein antigens requires a number of factors: antigens themselves; antigen presentation to T cells, with some HLA haplotypes involved in a greater capacity to present HPA1a antigens to T cells; CD4+ T-cell reactivation of B lymphocytes, with cognate interactions and cytokine-induced second signals [72-75]. In the case of platelets, cognate interactions may be helped by copious amounts of CD40/CD40L and differentiating cytokines. To what extent this allo-antibody development differs from what occurs after allogeneic RBC antigens or infectious agent antigens is not clear and would be a path to explore.

A Novel Paradigm Regarding Platelets: Consequences for Transfusion Therapy

Platelets are not only cellular tools that initiate primary haemostasis, but they may be considered major partners in the repair of injured vessels and, potentially, impaired non-vascular tissues. This function is largely due to their capacity to secrete regulatory cytokines and other factors and possibly by activating NF- κ B [76,77]. Platelets also participate in innate immunity, in surveillance against pathogenic invaders, especially those that are infectious in nature. Platelets engage in cognate interactions with other innate and adaptive immune cells via receptor-ligand interactions and secreted molecules. None of these properties are lost when platelets obtained/given by blood donors are transfused to a patient in need, and the only desired action currently is haemostatic. In the majority of cases, the pro-inflammatory actions of infused platelets are harmful; to what extent the other properties of platelets can be beneficial to the patient is unknown (immunomodulation, vascular arborescence, non-vascular tissue repair, etc). None of the labile blood products has ever been considered "passive" products, but PCs are particularly "active" components, which may renew consideration in the near future.

Acknowledgements

The authors would like to express their gratitude to Dr. Georges Andreu (INTS, Paris) for fruitful comments and advice, and Prof. Jean-Yves Muller (EFS, Saint-Denis) for sharing unpublished data.

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This article was originally published in a special issue, **Hematologic Oncology: Diagnosis & Therapeutics** handled by Editor(s). Dr. Ulrich Mahlknecht, Saarland University, Germany