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Abstract

Transfusion-related hemolysis is classically the result of an interaction between antibodies produced by the recipient and blood group antigens carried by the donor red blood cells. This reaction may be life threatening, especially in sickle cell patients when they develop hyper hemolysis with concomitant accelerated clearance of their own red blood cells. The complement system is a key participant in the pathophysiology of post-transfusion hemolysis. Complement can trigger the hemolytic reaction, amplify the inflammatory response and increase tissue damage. Complement is activated by the classical pathway but may also be activated by the alternative pathway in sickle cell disease. The hemolysis-derived products permanently released by sickle cell patients with chronic hemolytic anemia may affect the potency of complement activation. All the observations in sickle cell patients as well as in vitro experiments and in vivo data in animal models support the conclusion that complement is key disease driver and a promising therapeutic target in the context of transfusion-related hemolysis and hyper hemolysis.

Key Words: complement, sickle cell disease, post-transfusion hemolysis.

Introduction

Hyper hemolysis is the most severe form of post-transfusion hemolytic transfusion reaction. It is mainly encountered in patients with sickle cell disease. The innate immune complement system is a crucial parameter of post-transfusion hemolysis reaction, contributing to red blood cells (RBC) destruction.

The complement system consists of plasma and cell membrane proteins, which interact with each other as a cascade of binding steps, conformational changes and enzymatic reactions [1]. Its biological functions are to protect the body from infections and to maintain the host homeostasis. The complement cascade can be activated by a classical, lectin or alternative pathways, depending on the context. The classical pathway is initiated after recognition of IgG- or IgM-containing immune complexes by the recognition complex C1. The lectin pathway is activated after recognition of particular sugar motifs on bacterial surfaces. The alternative pathway is permanently active due to continuous spontaneous activation of the central complement component C3, allowing a rapid attack on any invading pathogen. This is achieved by opsonization with C3 activation fragments, like C3b, which favors phagocytosis, by inflammation, provoked by the anaphylatoxins C3a and C5a and by the pathogen killing by the membrane attack complex (MAC) of the terminal complement pathway. This cascade is tightly controlled spatially and temporarily to allow destruction of pathogens but protection of host tissue. In pathological conditions, though, complement can be mistargeted to the host cells, causing damage and injury, as is the case of the RBC in hyper hemolysis.

Complement is involved at different steps in hyper hemolysis: (i) trigger of the reaction, through the classical pathway, but likely also through the alternative pathway as suggested by many cases without detectable antibodies in sickle cell disease (SCD); (ii) amplification of the inflammatory response through release of pro-inflammatory components, and finally (iii) enhancer of tissue damage (Fig.1). Moreover, in SCD, the permanent chronic anemia with release of free heme and free hemoglobin may affect the extent of complement activation.

The post-transfusion hemolysis

Transfusion-related hemolysis is typically the result of interaction between a red blood cell antigen expressed by the donor cells and anti-erythrocyte antibodies produced by the recipient. Two situations occur in a transfusion context: immediate post-transfusion hemolysis, and delayed post-transfusion hemolysis.

In the first case, the antibodies are pre-existing, and have not been taken into account. This is seen in ABO hemolysis, resulting from a human error in which the correct red blood cell (RBC) unit was not delivered to the right patient. The hemolysis is immediate; the antibodies of the ABO system are IgM or IgG that fix complement. Beside antibodies characteristics, the high density of ABO antigens at the RBC surface (1 million sites/RBC) favor complement cascade activation to completion, forming the membrane attack complex. ABO incompatibility most frequently results in intravascular hemolysis. Immediate post-transfusion intravascular hemolysis may also result from complement-fixing non-ABO antibodies which were not detected during pre-transfusion testing. [2]

In the second situation, post-transfusion hemolysis results from the re-stimulation of an allo-antibody [3]. The patient has already been exposed to the antigen via previous transfusion or pregnancy. Antibodies from primary immunization, often evanescent, are no longer detectable. A rise in red blood cell antibody titers can occur as early as 24-48 hours after the transfusion but usually takes 5-15 days before it is noticed, accompanied by either a fall or failure of hemoglobin increment, rise in indirect bilirubin, and/or a positive direct antiglobulin test indicating sensitization of transfused red blood cells by the antibodies. Depending on the characteristics of the antibodies (class, sub-class, titers), but also of the target antigen on RBCs (number of antigen sites), activation of the complement goes either to C3b or to completion forming membrane attack complex. However, most allo-antibodies (anti-RH, anti-KEL, anti-FY, anti-MNS, anti-JK) destroy RBCs by extra vascular mechanisms, either because they do not fix complement or they do not activate complement to MAC [2]. Sensitized RBCs are mainly destroyed by macrophages within the reticulo-endothelial system. Macrophages have Fc receptors that specifically recognize certain classes of immunoglobulins in an immune complex and certain complement components such as C3b, iC3b are recognized by the complement receptors CR1, CR3 and CR4. Macrophage interaction with RBCs coated with immunoglobulin and/or complement may result in the destruction of RBCs through phagocytosis, but also, cytotoxicity. Natural killer cells can also

be involved through their cytotoxic activity. However, the distinction between extravascular and intravascular hemolysis in post transfusion reaction is not always precise, mechanisms are frequently mixed. If detected, hemoglobinuria and hemoglobinemia are often signs of intra-vascular destruction of RBCs.

The immuno-hemolytic accidents have extremely variable clinical-biological characteristics, the signs range from poor post-transfusion increment without associated symptomatology, to very severe symptomatology, including shock, disseminated intravascular coagulation with acute organ failure leading in some cases to the death of the patient. Symptoms such as fever, chills, chest pain, hypotension, nausea are frequently encountered. The biological characteristics are those of hemolytic anemia, with increase of LDH, haptoglobin depletion, and an increase in bilirubin. Intra-vascular hemolysis is usually more serious clinically.

Beside antibodies and target antigens, a large number of other parameters determine the severity of the event, such as the presence of co-morbidity in the patient, but also, most likely, parameters related to the transfused product, such as its age or the storage conditions. In SCD patients, some parameters, such as inflammation or chronic heme release that certainly modify the response to transfusion are likely a crucial issue.

Post-transfusion hemolysis of the sickle cell patient

During sickle cell disease, post-transfusion hemolysis has particular characteristics. It is most often delayed, from 3 to 4 days after the transfusion. The main clinical signs are the recurrence of a vaso-occlusive crisis for which the patient had been transfused, or the appearance of it. Non-regenerative anemia with a lower hemoglobin level than was present pre-transfusion is observed in the most severe cases. It is in these cases that we speak of hyper hemolysis, especially since we not only observe a destruction of the RBCs transfused but also a destruction of autologous RBC [4]. Finally, the urine is dark and the levels of LDH are increased at levels higher than those observed during a vaso-occlusive crisis. Because of the frequency of vaso-occlusive symptoms, these events are underestimated. On the immuno-hematological level, serological studies might not provide an explanation for the hemolytic transfusion reaction. A certain number of these cases seem to be related to the restimulation of allo-antibodies, classically involved in delayed post-transfusion hemolysis (anti-Jkb, anti-S, anti-Fya). However, among the patients developing detectable antibodies

demonstrated post-transfusion, 30% develop antibodies which are not conventionally considered to be clinically significant: autoantibodies, antibodies without characterized specificity [5,6].

Interestingly, it was suggested that heme, released during a hemolytic process, could modulate the reactivity of IgG, broadening the spectrum of recognized antigens [7-9]. This so called induced polyreactivity is an unconventional strategy for antibody diversification [10], which in the context of SCD, could be misdirected to RBCs, triggering their lysis without prior immunization. Further studies are needed to elucidate the potential contribution of the heme-induced polyreactivity in DHTR.

Another aspect, by which heme could modulate the immune response in SCD is by modulation of the phenotype of monocytes and T cells. Peripheral regulatory T cell and B cell suppressive function and altered T-helper cell responses with higher circulating IFN-γ, but lower IL-10 levels have been reported in alloimmunized as compared with non-alloimmunized SCD patients [11,12]. Heme-loaded monocytes/macrophages change their phenotype towards immunosuppressive functions [13]. In the setting of adequate heme oxygenase (HO-1) upregulation in CD16+ monocytes, T-reg/T-helper polarization will be switched toward a regulatory response (higher T-reg/lower Th1) that is less conducive to alloimmunization [14]. However, when IL-12 levels in CD16+ monocytes are not optimally inhibited by hemin as a result of insufficient HO-1 activity/level, T-regs will not expand and Th1 proliferation will dominate, thereby increasing the risk of alloimmunization. These data suggest that unlike alloimmunized patients, non-alloimmunized SCD CD16+ monocytes in response to transfused RBC breakdown products promote an anti-inflammatory state that is less conducive to alloimmunization [14].

Finally, in 30 to 40% of cases, no new detectable antibody is evidenced, which raises the question of the "trigger" of the hemolytic reaction. Finally, even RBCs phenotypically matched with multiple patient antigens and with all identified antibodies might be hemolyzed.

The severity of the reaction does not correlate with the type of antibody apparently triggering the reaction, and more enigmatically still, does not depend on the presence or

absence of detectable antibodies. In all cases of hyper hemolysis, complement activation up to MAC is strongly suspected. First, because principal features of intravascular hemolysis are found (hemoglobinuria and hemoglobinemia), and secondly, because of the demonstrated efficiency of anti-C5 blocking antibody on the hemolytic process, in a series of published cases (Table 1). In view of the antibodies demonstrated, and not classically leading in vivo intravascular hemolysis, and also in view of the absence of antibodies in other cases, the question of the mechanism of intravascular hemolysis arises.

One important feature in SCD post-transfusion hemolysis is the development of a more severe anemia than was present prior to transfusion. As previously indicated, it is likely that the patient's own red blood cells are destroyed, but it is also due to reticulocytopenia with a significant decrease in the absolute reticulocyte level compared with the patient's usual value.[4,5] This could be due to destruction of reticulocytes in the blood vessel as a part of the intravascular hemolytic process, but also due to suppressed erythropoiesis with dramatic effects in patients who already display short RBCs survival. Then, severe and life-threatening anemia can develop rapidly (aplastic crisis). In the most severe cases, a multi-organ failure can set in quickly, leading to the death of the patient. Post-transfusion hyper hemolysis accounts for approximately 5% of the causes of death in the course of the disease, and death occurs in 5 to 6% of the patients experiencing a DHTR [5].

Besides being profound, the consequences of this hemolysis during SCD may be rapidly dramatic, as the release of heme and free hemoglobin is not inhibited by hemopexin and haptoglobin, which are already diminished in these chronic hemolytic patients. These molecules activate a vascular endothelium already altered during SCD, with an increase in the adhesion phenomenon of red blood cells, but also leucocytes, and an increase in thromboses.

Activation of complement by hemolysis-derived products

A series of studies provide a link between intravascular hemolysis and complement overactivation [15]. These phenomena could contribute to the RBCs destruction in DHTR [16].

Evidence for activation of the alternative complement pathway in SCD emerged during the 1970s when authors described decreased levels of C3 and Factor B in sera from SCD patients

[17]. Few years later, elevated C3b-Properdin complexes were detected in 89% of SCD patient sera [18]. Further, activation of the alternative pathway on RBCs of SCD patients has been described, due to phosphatidylserine/phosphatidylethanolamine exposure on erythrocytes promoting anchoring of C3b [19,20]. SCD RBC microvesicles also activated the alternative pathway in vitro [21]. In addition to C3 deposition on SS erythrocytes, Bb, C4d and C3a fragments were detected in SCD patients' plasma, as well as C3 deposition on RBC [20,22]. Interestingly, detection level of this activated fragments depended on patient status. For instance, elevated C3a and C4d fragments were correlated with continuous pain, while Bb and C3a fragments were highly increased during painful crisis, suggesting different activation profiles of the complement system.

Detailed studies of the complement-activation pathway of DHTR are lacking. Frequently offending allo-antibodies are not detected in some patients. This alone cannot firmly exclude the activation of the classical pathway, Nevertheless, in our prospective studies, when a diagnosis of DHTR without detectable antibodies was made, all tests were performed, including evaluation of red cell eluates. Therefore, the negative results may indirectly favor alternative pathway activation[6,23].

In experimental animals hyper hemolysis could be modeled by hemorrhagic shock and resuscitation with injection of prolonged storage red blood cells. In such a transfusion model of stored blood in guinea pigs, intravascular hemolysis occurred, associated with acute hypertension, vascular injury and kidney dysfunction [24]. These effects were driven by hemoglobin (Hb), since they were prevented by injection of the Hb scavenger protein haptoglobin. Re-analysis of the data from the proteomic assessment of the kidneys in this model revealed significant increase of the components of the alternative complement pathway C3 convertase: C3, Factor B (FB) and Factor D (FD), suggesting complement activation. The levels of C3b, FB and FD were decreased after treatment with haptoglobin, pointing directly to the casual role of hemolysis-derived products in complement activation (Fig. 2). Moreover, in patients with SCD as well as in SCD mouse models deposits of C3 activation fragments and C5b-9 were found in different organs [21,25,26]. Biomarkers of complement activation are also detected in plasma of patients with SCD [18,19,22,25,27-29]. Nevertheless, it remains possible that complement activation could be a mere bystander phenomenon, related to hyper hemolysis without importance for tissue injury.

The pathological relevance of complement in models of hyper hemolysis was addressed by triggering RBC destruction in mice by phenylhydrazine [30]. In this setting the C3-/- animals were protected from the kidney and liver injury, contrary to the wild type mice [21,31,32]. Blockade of C5 also partially prevented the liver injury [31]. Moreover, in a mouse model of SCD, blockade of C5 or C5aR1 prevented vaso-occlusion [26]. Taken together, these results demonstrate a key role of complement in the disease process of SCD, which is further exacerbated by hyper hemolysis.

The triggering factor for complement activation may be the oxygen-carrying prosthetic group heme. Indeed, in vivo complement activation, measured as kidney deposits of C3 activation fragments, was reproduced by injection of hemoglobin and prevented by haptoglobin, but also reproduced by injection of heme and prevented by pre-treatment with hemopexin [21,31,33]. Importantly, the hemoglobin-mediated complement deposits were prevented by hemopexin, suggesting that heme has to be released from hemoglobin to exert this effect [21]. In vitro data showed that alternative pathways can be activated by heme and heme-injured endothelium [31,34,35]. Heme, though, is highly hydrophobic and does not remain free in circulation. RBC microvesicles are heme carriers, especially in the context of SCD [36,37]. Indeed, SCD RBC microvesicles activated complement by a mixed mechanism, dependent on exposed phosphatidylserine [20] and on heme [21].

The activation of complement in SCD, especially during DHTRs, is a multifactorial process, dependent on the classical pathway activation by anti-RBC antibodies, on the endothelial alteration after hypoxia and on the intravascular hemolysis, ultimately triggering primarily the alternative pathway [16]. Therefore, complement and hemolysis could synergize to promote endothelial activation organ injury [15,16,38,39]. A better understanding the link between activation of complement system and hemolysis could help treat many complications of SCD and hyper hemolysis.

Complement blockade as a therapeutic strategy in DHTR

Observations in patients, results of in vitro experiments, and available in vivo data in different animal models together strongly support the conclusion that complement is a key disease driver and a promising therapeutic target in the context of hyper hemolysis. Blocking

the effects of both IgG-mediated classical pathway activation and the alternative pathway activation via anti-complement therapies might allow control of the downstream reactions which cause RBC destruction and tissue injury. As shown in Table 1, anti-complement therapies, using the C5-blocking antibody Eculizumab, have already shown promising results in a series of case reports. [40-43]

Multiple novel complement inhibitors, acting at different level of the cascade, are in development, and some of them will surely reach clinic in the upcoming years [44]. Clinical trials are necessary in order to define the capacity of complement blockers to control hyper hemolysis and DHTRs. Blockade at the level of the C3 convertase versus C5 should be investigated in order to select the most appropriate strategy for patients. The capacity of therapeutics or drug candidates acting outside of the complement cascade, to block complement should be also evaluated, since animal models hint that hemopexin administration or P-selectin blockade prevent deposition of C3 fragment in vivo [16,21].

Eculizumab has been available in the clinic for a decade. It binds to C5 and stops the cleavage of C5 to C5a and C5b, and hence the generation of the anaphylatoxin C5a and the membrane attack complex C5b-9 [45]. It is used for patients with paroxysmal nocturnal hemoglobinuria [46] and atypical hemolytic uremic syndrome [47] and multiple clinical trials are ongoing for other indications [44]. Eculizumab is proposed for patients encountering post-transfusion hyper hemolysis and who continue to experience clinical deterioration despite first line agents [48]. The promising results of the case reports (Table 1) urges a clinical trial in a larger number of patients.

Appropriate biomarkers, allowing selection of those patients who might benefit most from anti-complement therapy should be defined. Soluble C5b-9 in plasma was increased in the few reported cases which benefited from Eculizumab [41-43] (Table 1). A dedicated study is necessary to determine what are the soluble C5b-9 levels in patients with DHTR complicated by hyper hemolysis to determine if measurement of soluble C5b-9 levels could predict the success of complement blocking therapy. Currently, normal levels of soluble C5b-9 do not preclude the use of complement inhibitors. Indeed, in patients with aHUS the activation of complement is local, on glomerular endothelium and does not necessarily induce increase in soluble C5b-9 in the circulation [38].

In conclusion, complement activation drives the disease process in hyper hemolysis and its inhibition holds promise to be an efficient therapeutic strategy. The available in vitro, in vivo data and case reports are a solid basis for evaluation of complement inhibition in patients with hyper hemolysis and DHTR.

Conflict of interest

None.

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Legends of the figures

Figure 1: Complement in RBCs destruction in DHTR. Complement fixing IgG against alloantigens on transfused red blood cells (RBC) can mediate DHTR through recruitment of the C1 complex (C1qC1r2C1s2) of the classical pathway and further C3b deposits on RBC membranes. The incomplete complement activation results in opsonization with C3 activation fragments and extravascular hemolysis, i.e. phagocytosis (a). However, if complement activation is complete, it induces membrane attack complex formation and direct intravascular hemolysis, leading to release of cell-free Hb and heme into the circulation (b). Non-complement binding IgG mediate RBC destruction through the extravascular reticuloendothelial system. When haptoglobin and hemopexin are overwhelmed by massive hemolysis, this leads to release of cell-free heme in the circulation. We hypothesize that heme induces alternative complement pathway activation by promoting hydrolysis of C3 into C3(H_2O), formation of a fluid phase C3 convertase directly in serum and hence a complement deposits on RBCs. Further activation of the complement system would be increased by the "amplification loop, leading to C5b-9 membrane attack complex formation.

Figure 2: Analysis of all complement proteins detected in renal tissue proteomic analysis (independent analysis of the dataset published by Baek et al. in their Supplementary Table 1[24]). Results are the ratio of proteins expression levels after transfusion on protein expression levels before transfusion (basal level). Folds are expressed in Log_2, where only a fold > 1 is considered positive. Clusterin is a regulator that inactivates the membrane attack compex (C5b-9), while CD55 (DAF) is a regulator that inhibits the C3 convertase formation. Here HO-1 is depicted as a positive control. A) Among the 2634 proteins detected in kidneys, only 6 complement proteins were present, belonging to the alternative and the terminal complement pathways. Taken individually, their presence was increased after transfusion of old blood and attenuated (except for C9) in presence of Hp. B) Statistical analysis of the behavior of the group of complement proteins in the three experimental conditions. The pattern is similar to what was observed for the proteins related to heme metabolism, to which belongs HO-1, taken as a positive control here. The data analysis is made in the same way as the results presented at Figure 5 of the paper of Baek et al. [24].

Table 1. Cases of hyper-hemolytic transfusion reactions treated with Eculizumab.

SCD	C3 level (mg/L)	C4 level (mg/L)	C5b9 level (ng/ml)	Associated treatments	Delay[a]	Outcome[b] after Eculizumab therapy	References
No	-	-	-	None	17 days	**No improvement 24 hours later. Improvement 10 days later with prednisone and EPO.**	[49]
No[c]	-	-	-	CS & Heparin infusion	1 hour	**Improvement from day 2**	[50]
Yes	-	-	-	Rituximab	3 days	**Improvment from day 7**	[40]
Patient 1 : Yes	1080	266	856	None	7 days	**Improvement from day 3**	[41]
Patient 2 : Yes	-	-	-	PLEX	7 days	**Improvement from day 21**	[41]
Patient 3 : Yes	313	104	1527	IS & CS	9 days	**Improvement from day 1**	[41]
Yes	1350	214	270	CS, IvIg & Rituximab	14 days	**Improvement from day 2**	[42]
Yes	-	-	325	CS, IvIg & Rituximab	10 days	**Improvement from day 3**	[43]
Yes	Normal range	-	-	HBOC-201, CS, IvIg, EPO, IV iron, vitamin B12	11 days	**Improvement from day 9**	[51]

C3: C3 complement component (normal range, 660-1500 mg/L); C4: C4 complement component (normal range, 93-380 mg/L); sC5B9: soluble terminal complement complex (normal range <244 ng/mL); SCD: sickle cell disease; HTR: Hemolytic Transfusion Reaction; PLEX: Plasma Exchange; CS: corticosteroids; IS: immunosuppressive regimen; IvIg: intravenous immunoglobulins.

a) Delay from RBC transfusion to Eculizumab therapy;

b) Delay correspond to the time between Eculizumab injection and evaluation of the outcome. Eculizumab therapy was considered successful since it allowed hemoglobin stabilization and a decrease in hemolysis markers (LDH and/or free bilirubin);
c) Acute hemolytic reaction was due to ABO-incompatible red blood cell transfusion

Figure 1

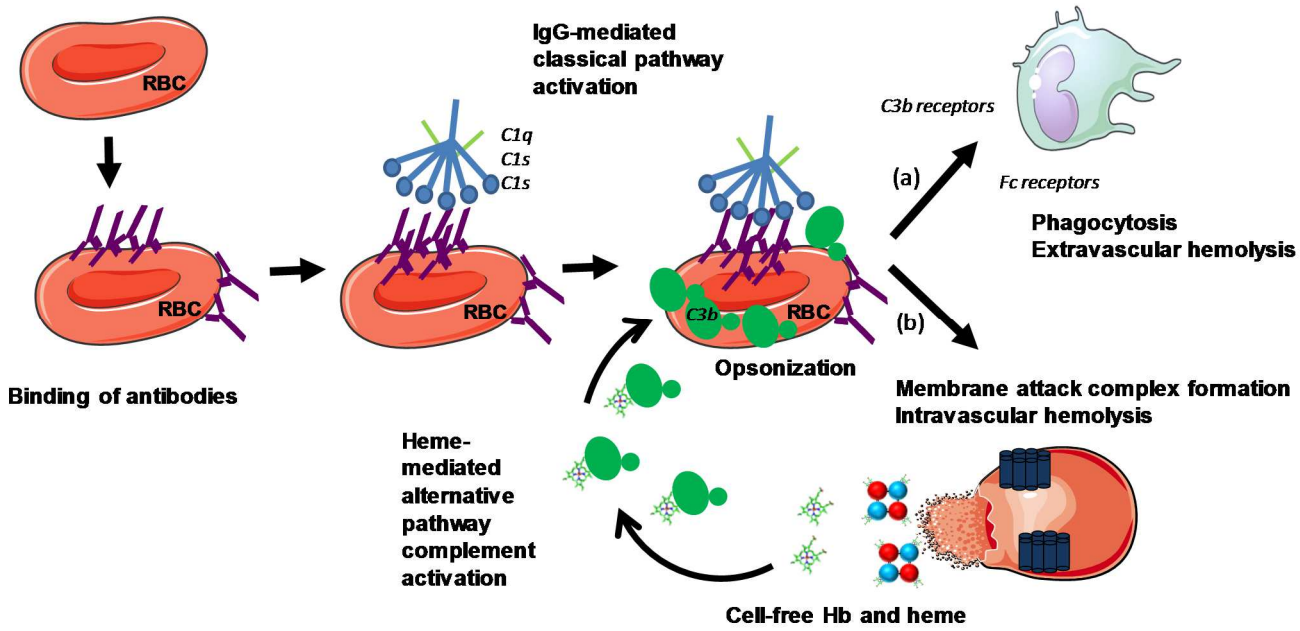
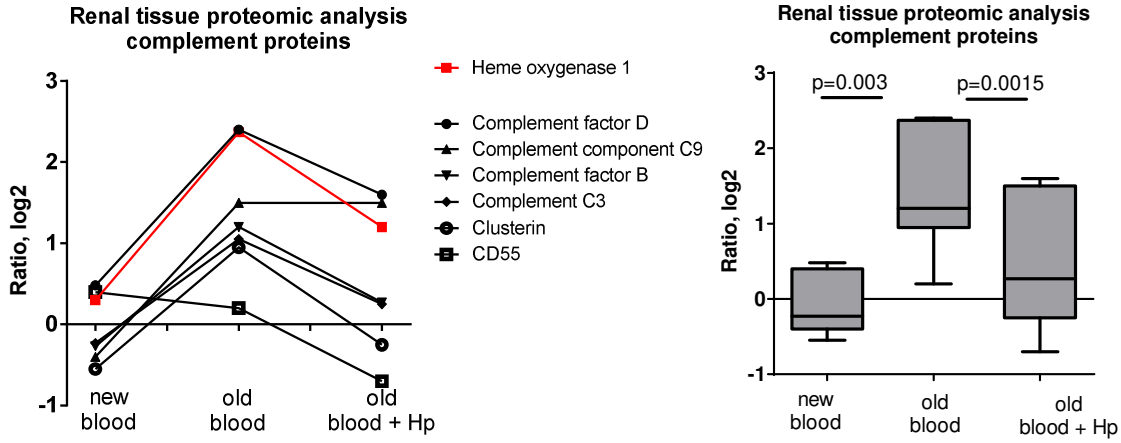

RBC
IgG-mediated
classical pathway
activation
C1q
C1s
C1s
C3b receptors
(a)
Fc receptors
Phagocytosis
Extravascular hemolysis
RBC
RBC
C3b
RBC
(b)
Binding of antibodies
Opsonization
Membrane attack complex formation
Intravascular hemolysis
Heme-
mediated
alternative
pathway
complement
activation
Cell-free Hb and heme

Figure 2


Renal tissue proteomic analysis
complement proteins
Ratio, log2
3
2
1
0
-1
new blood
old blood
old blood + Hp
Heme oxygenase 1
Complement factor D
Complement component C9
Complement factor B
Complement C3
Clusterin
CD55
Renal tissue proteomic analysis
complement proteins
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Ratio, log2
new blood
old blood
old blood + Hp