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Storage Primes Erythrocytes for Necroptosis and Clearance

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Abstract

Background/Aims: Like nucleated cells, erythrocytes (red blood cells, RBCs) are capable of executing programmed cell death pathways. RBCs undergo necroptosis in response to CD59-specific pore-forming toxins (PFTs). The relationship between blood bank storage and RBC necroptosis was explored in this study.

Methods: Human RBCs were stored in standard blood bank additive solutions (AS-1, AS-3, or AS-5) for 1 week and hemolysis was evaluated in the context of necroptosis inhibitors and reactive oxygen species (ROS) scavengers. Activation of key factors including RIP1, RIP3, and MLKL was determined using immunoprecipitations and western blot. RBC vesiculation and formation of echinocytes was determined using phase-contrast microscopy. The effect of necroptosis and storage on RBC clearance was determined using a murine transfusion model.

Results: Necroptosis is associated with increased RBC clearance post-transfusion. Moreover, storage in AS-1, AS-3, or AS-5 sensitizes RBCs for necroptosis. Importantly, storage-sensitized RBCs undergo necroptosis in response to multiple PFTs, regardless of specificity for CD59. Storage-sensitized RBCs undergo necroptosis via NADPH oxidase-generated ROS. RBC storage led to RIP1 phosphorylation and necrosome formation in an NADPH oxidase-dependent manner suggesting the basis for this sensitization. In addition, storage led to increased RBC clearance post-transfusion. Clearance of these RBCs was due to Syk-dependent echinocyte formation.

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Disclosure Statement

The authors have no conflicts of interest to declare.

Conclusion: Storage-induced sensitization to RBC necroptosis and clearance is important as it may be relevant to hemolytic transfusion reactions.

Keywords

Necroptosis; Erythrocyte; Red blood cell; Storage; Blood bank; Transfusion; Syk; Clearance; Cell death; Eryptosis; Apoptosis

Introduction

Erythrocytes (red blood cells, RBCs) undergo necroptosis in response to certain bacterial pore-forming toxins (PFTs) [1, 2]. This is significant as eryptosis, a programmed cell death (PCD) pathway specific to RBCs, was previously considered to be the only mechanism through which these cells die [3–5]. It has been recommended that the term eryptosis not be used due to the opinion that RBCs exist in a questionable state between life and death [6]. This, however, is an outdated view as RBCs have more recently been appreciated as functional, terminally-differentiated cells [7]. The existence of PCD pathways like necroptosis and eryptosis in RBCs is particularly striking as these cells lack traditional cellular machinery, such as nuclei and mitochondria. The two requirements for RBC necroptosis are membrane pore formation and necrosome formation, which requires phosphorylation of RIP1 and RIP3 [1]. Bacterial PFTs capable of RBC necroptosis engage the CD59 receptor which induces signaling leading to the phosphorylation of RIP1, RIP3 and necrosome formation [1, 2]. Accordingly, PFTs that do not engage CD59 induce RBC death, but not via necroptosis. However, when combined with artificial stimulation of CD59 these PFTs indeed induce necroptosis of RBCs [1].

Following engagement of CD59 by PFTs, Src family kinases lead to the phosphorylation of Syk kinase [1]. Activated Syk kinase then phosphorylates Band 3 leading to RBC vesiculation and the formation of echinocytes, a vesiculating phase of RBCs [1]. This is consistent with the mechanism of echinocyte formation in other systems [8, 9]. Erythrocyte shape changes, such as echinocyte formation, are associated with increased RBC clearance via extravascular hemolysis [10–12]. This is driven by large groups of macrophages and phagocytic cells present in reticular connective tissue of the liver and spleen which remove the altered RBCs [10]. Within the spleen, RBCs must pass through the Cords of Billroth to enter into the sinusoids. Changes to RBC morphology, such as echinocyte formation, hinders the passage into the sinusoids leading to the phagocytic removal of such cells [10].

Necroptosis involves the stimulation of glycolysis, the electron transport chain, and NADPH oxidases to generate reactive oxygen species (ROS) [13–17]. The ROS are key players in necroptosis, serving both signaling and damaging roles [15, 18]. Recently, we showed that increased levels of glucose primes necroptosis in several cell types including RBCs [13]. This is relevant as RBCs are routinely stored in FDA-approved solutions which contain ≥ 45 mM glucose, in contrast to the 5 mM glucose present in normal serum [19].

Since we have previously shown that RBC necroptosis is associated with echinocyte formation [1], in the present study we aim to determine if RBC necroptosis leads to increased clearance post-transfusion. In addition, we analyze the effect of RBC storage on

necroptosis, echinocyte formation, and post-transfusion clearance. Moreover, we determine that NADPH oxidase-dependent ROS promote the sensitization of stored RBCs to necroptosis.

Materials and Methods

Pharmacologic inhibitors

Inhibition of RIP1, RIP3, and MLKL was achieved with 50 μM necrostatin-1 (EMD Millipore), 2 μM GSK'872 (EMD Millipore), or 0.5 μM necrosulfonamide (NSA, EMD Millipore), respectively. Inhibition of AGE formation was achieved with 1 mM pyridoxamine (Acros Organics). For inhibition of Syk kinase, Syk kinase inhibitor IV, Bay 61–3606 (EMD Millipore) was used at 2 μM. Inhibition of NADPH oxidases was achieved with 10 μM VAS2870 (EMD Millipore).

Human erythrocytes and storage solutions

Fresh human RBCs were purchased from Zen-Bio. Upon arrival, RBCs were leukoreduced using Purecell Neo leukocyte reduction filters (Haemonetics) according to the manufacturer's instructions. At the start of all experiments, leukoreduced RBCs were 2 days old. Storage solutions used were AS-1, AS-3, and AS-5. The composition of AS-1 was 154 mM NaCl, 2 mM adenine, 111 mM glucose, 41 mM mannitol. The composition of AS-3 was 70 mM NaCl, 23 mM NaH_2PO_4, 2 mM citric acid, 23 mM sodium citrate, 2 mM adenine, 55 mM glucose. The composition of AS-5 was 150 mM NaCl, 2.2 mM adenine, 45 mM glucose, 45.5 mM mannitol. Leukoreduced RBCs were stored with adherence to current FDA standards in the indicated solutions for the indicated time periods.

Hemolysis assays

For these studies, 1 hemolytic unit (HU) is defined as the unit of toxin needed to achieve 50% hemolysis of fresh RBCs. Fresh human RBCs (purchased from Zen-Bio) at a concentration of 0.5% (v/v) or those stored in AS-1, AS-3, or AS-5 for 1 week were treated with 0.2 HU of the indicated toxins for 30 min at 37°C. Stored RBCs were washed in PBS prior to toxin treatment. In cases where inhibitors were used they were added to RBCs 30 min prior to toxin treatment. Following toxin treatment, RBCs were centrifuged and hemoglobin release into the supernatant was used as a measure of hemolysis. Hemoglobin in RBC supernatants was measured in an Eppendorf 2200 plate reader at an absorbance of 415 nm.

Immunoprecipitations and immunoblots

Fresh human RBCs (Zen-Bio) at a concentration of 20% (v/v) or those stored for 1 week in AS-1 were treated with 0.2 HU of the indicated toxins for 30 min at 37°C. Stored RBCs were washed in PBS prior to toxin treatment. Following toxin treatment, RBCs were sonicated. Anti-human RIP1 mAb (BD) was added to pre-cleared sonicates and allowed to incubate with gentle mixing overnight at 4°C. Protein A/G beads (ThermoFisher) were added to sonicates for 2 h at room temperature with gentle mixing. Precipitates were washed several times in PBS followed by SDS-PAGE and western blot. The mAbs used for western blots were purchased from Cell Signaling Technology and were all used at a 1:1000 dilution.

Secondary HRP-conjugated mAbs and chemiluminescence were used to develop blots. For detection of high MW RIP1 oligomers, non-reducing SDS-PAGE and western blot was performed.

Phase-contrast microscopy

Fresh human RBCs (Zen-Bio) at a concentration of 0.5% (v/v) or those stored for 1 week in AS-1 were treated with 0.2 HU of the indicated toxins for 10 min at room temperature. Unstained RBCs were viewed under bright-field using an Axio Observer.Z1 microscope (Zeiss, Thornwood, NY, USA) and an AxioCam MRm digital camera (Zeiss).

In vivo transfusion model

All animal procedures were performed under a protocol approved by the IACUC of Columbia University. Human CD59 transgenic mice on C57BL/6 background [2] were used as donor mice. Following sacrifice of donor mice, RBCs were obtained via cardiac puncture and washed in PBS. Donor RBCs were treated with sub-lytic doses of vaginolysin (VLY) or pneumolysin (PLY) for 30 min at 37°C. In some experiments, donor RBCs were stored in AS-1 at 4°C for 72 h prior to treatment with VLY or PLY. In cases where inhibitors were used, they were added to RBCs 30 min prior to toxin treatment. Following treatment, unbound toxins were removed via centrifugation. RBCs were then labeled with lipophilic tracer dyes, diO, diI, diD using the Vybrant Multicolor Cell-Labeling Kit (ThermoFisher). Labeled RBCs were transfused at 50% hematocrit retro-orbitally into healthy, recipient C57BL/6 mice (Jackson Laboratory). At the designated time points, blood was obtained via tail bleed and labeled RBCs were quantified using a FACSverse flow cytometer (BD). Each recipient mouse received an equal quantity of labeled toxin-treated RBCs and untreated control RBCs. The ratio of toxin-treated RBCs:untreated control RBCs was measured to quantify RBC clearance post-transfusion.

Statement of Ethics

All animal procedures were in accordance with the American Veterinary Medical Association (AVMA) and performed under a protocol approved by the IACUC of Columbia University.

Results

Necroptosis is associated with increased RBC clearance post-transfusion

Previously, we demonstrated that vaginolysin (VLY), a PFT produced by *Gardnerella vaginalis*, induced RBC necroptosis while pneumolysin (PLY), a PFT produced by *Streptococcus pneumoniae*, did not [1, 2]. To determine if RBC necroptosis is associated with increased clearance post-transfusion, we treated human CD59 transgenic murine RBCs with sub-hemolytic quantities of VLY or PLY for 30 min, followed by labeling with lipophilic tracer dyes (diO, diI, diD). Labeled toxin-treated transgenic RBCs were mixed with labeled untreated transgenic RBCs and then transfused into healthy recipient WT mice. The ratio of toxin-treated: untreated RBCs was quantified over a 24 h period via flow cytometry. Treatment with VLY led to clearance of greater than 50% of transfused RBCs in as little as 30 min, while treatment with PLY did not (Fig. 1A). The RIP1 inhibitor

nec-1s did not prevent the clearance of VLY-treated RBCs (Fig. 1B). However, inhibition of Syk kinase, which is associated with echinocyte formation during RBC necroptosis [1], prevented the clearance of VLY-treated RBCs significantly (Fig. 1C). These results indicate that necroptosis leads to RBC clearance in a manner that depends on Syk-induced echinocytes.

Storage in AS-1, AS-3, and AS-5 primes RBCs for necroptosis

We examined three FDA-approved RBC storage solutions to determine the effect of RBC storage on necroptosis. We chose additive solution 1 (AS-1), additive solution 3 (AS-3), and additive solution 5 (AS-5) which contain glucose concentrations of 111, 55, and 45 mM, respectively [19]. Human RBCs were stored in AS-1, AS-3, or AS-5 at 4°C for 1 week, followed by treatment with VLY or intermedilysin (ILY) (which induce RBC necroptosis), or PLY and listeriolysin O (LLO) (which do not induce RBC necroptosis) for 30 min at 37°C and measurement of hemolysis. Storage in AS-1 resulted in increased hemolysis upon treatment with VLY or ILY (Fig. 2A–B). We confirmed that this increased hemolysis was due to necroptosis, as it was prevented by nec-1s (RIP1 inhibitor), GSK'872 (RIP3 inhibitor), and necrosulfonamide (NSA, MLKL inhibitor) (Fig. 2A–B). Interestingly, following storage in AS-1, hemolysis induced by PLY and LLO also increased and was prevented by nec-1s, GSK'872, and NSA (Fig. 1C–D). Similar results for all four PFTs were obtained following storage in AS-3 (Fig. 3) and AS-5 (Fig. 4). These results indicate that storage in these solutions primes RBCs for necroptosis in response to PFTs, regardless of their specificity for CD59. Importantly, 1 week of RBC storage in the absence of toxin treatment causes <10% hemolysis (Fig. S1 – for all supplementary material see www.cellphysiolbiochem.com).

NADPH oxidases and ROS drive storage-primed RBC necroptosis

Increased cellular glucose leads to the formation of reactive oxygen species (ROS) and advanced glycation end products (AGEs) [20]. Therefore, we examined the role of ROS and AGEs in storage-primed RBC necroptosis using inhibitors of AGEs (pyridoxamine) and NADPH oxidases (VAS2870), as well as an antioxidant (N-aceytlcysteine). Treatment with VAS2870 or N-acetylcysteine prevented the increased hemolysis seen after 1 week of AS-1 storage, while pyridoxamine did not (Fig. 5). The dependence of RBC necroptosis on NADPH oxidases is consistent with our previous observations [2]. NADPH oxidases are also considered a significant source of ROS in RBCs [21]. N-acetylcysteine prevented hemolysis of fresh RBCs by VLY, ILY, PLY, and LLO, suggesting that some level of ROS induction is involved in PFT-induced hemolysis of fresh RBCs.

Production of ROS is critical for necroptosis, as it induces oligomerization and autophosphorylation of RIP1 [18]. We examined if these events occurred during storage-primed RBC necroptosis. As determined by non-reducing PAGE and western blot, high MW RIP1 oligomers form following treatment of fresh RBCs with the necroptosis-inducing toxin, VLY, but not the non-necroptotic PLY (Fig. 6A). Following 1 week of storage in AS-1 we observed an increase in RIP1 oligomers in response to treatment with either VLY or PLY, as well as an increase in the phosphorylation levels of RIP1 (Fig. 6A). We performed immunoprecipitation (IP) on AS-1 stored RBCs treated with VLY or PLY and

determined that RIP3 and MLKL co-precipitated with RIP1 (Fig. 6A). Co-precipitation of RIP3 and MLKL with RIP1 indicates formation of the necrosome. RIP1 oligomerization and phosphorylation, as well as co-precipitation of RIP3 and MLKL with RIP1 decreased following inhibition of NADPH oxidases in all cases of AS-1 stored RBCs (Fig. 6A). Indeed, storage in AS-1 for 1 week without toxin treatment led to phosphorylation of RIP1 and co-precipitation of RIP3 and MLKL with RIP1, which was prevented by inhibition of NADPH oxidases (Fig. 6B). These results indicate that NADPH oxidases and ROS play a role in the sensitization of stored RBCs for necroptosis.

Syk-dependent echinocyte formation increases during storage-primed RBC necroptosis

Previously, we characterized Syk kinase-dependent echinocyte formation as a feature of RBC necroptosis [1]. Therefore, we examined Syk-dependent echinocyte formation in the context of storage-primed RBC necroptosis. As determined previously [1], the RBC necroptosis-inducing toxin VLY caused echinocyte formation, while the non-necroptotic PLY did not (Fig. 7). Storage of RBCs for 1 week in AS-1 resulted in increased echinocyte formation following treatment with either VLY or PLY (Fig. 7). Inhibition of Syk kinase prevented echinocyte formation in both cases. The morphology of PLY-treated RBCs still indicated damage following inhibition of Syk kinase, however, RBC morphology returned to normal following inhibition of PLY with a neutralizing mAb (Fig. 7). These results demonstrate that storage in AS-1 sensitizes RBCs for Syk-dependent echinocyte formation following contact with PFTs.

Storage-primed necroptosis leads to increased RBC clearance post-transfusion

To determine the role of storage-primed necroptosis on RBC clearance post-transfusion, we treated fresh human CD59 transgenic murine RBCs or those stored in AS-1 for 72 h with sub-lytic quantities of VLY or PLY. Following labeling with lipophilic tracer dyes (diO, diI, diD), toxin-treated transgenic RBCs were mixed with untreated transgenic RBCs and then transfused into healthy recipient WT mice. The ratio of toxin-treated:untreated RBCs was quantified over a 24 h period via flow cytometry. As in Fig. 1, treatment of fresh RBCs with VLY led to clearance of ~50% transfused RBCs after 30 min (Fig. 8A). Storage in AS-1 prior to VLY treatment led to clearance of significantly more transfused RBCs (~75%, Fig. 8A). As before, treatment of fresh RBCs with sub-lytic quantities of PLY did not lead to clearance post-transfusion. However, storage in AS-1 prior to PLY treatment led to clearance of ~50% transfused RBCs (Fig. 8B). Inhibition of Syk kinase prevented the increased clearance seen following storage of RBCs in AS-1 and treatment with VLY or PLY (Fig. 8B). These results indicate that storage-primed necroptosis leads to increased Syk-dependent RBC clearance following membrane pore formation.

Discussion

Mechanism of storage-primed RBC necroptosis

Here we have shown that stored RBCs are primed for necroptosis resulting in increased clearance post-transfusion. Storage for 1 week in AS-1, AS-3, or AS-5 led to increased necroptosis by VLY and ILY, two toxins previously shown to induce necroptosis [1, 2]. Perhaps more notable was the finding that storage in these solutions led to necroptosis

by PLY and LLO, two toxins that cannot induce necroptosis of fresh RBCs [1, 2]. This increases the relevancy of storage-primed RBC necroptosis beyond CD59-specific toxins (VLY and ILY) but also sheds light on its potential mechanism. We have previously reported that the minimal requirements for RBC necroptosis are formation of the necrosome and membrane pores [1], which distinguishes it from necroptosis in nucleated cells [15]. At that time, we determined that necrosome formation could be induced by CD59 or Fas receptor signaling [1]. In this work we identified an additional stimulus of necrosome formation in mature RBCs, NADPH oxidase-induced ROS formation as a result of storage in high glucose solutions. Storage in AS-1 resulted in phosphorylation of RIP1 and necrosome formation in an NADPH oxidase-dependent manner, while inhibition of NADPH oxidases or ROS prevented storage-primed RBC necroptosis. This type of ROS-induced activation of RIP1 and the necrosome is in accordance with the mechanism of necroptosis in nucleated cell types [18]. As previously reported, it was necessary for necrosome formation to be paired with membrane pore formation in order for RBC demise by necroptosis [1]. The activation of NADPH oxidases by high glucose has been observed in several other cell types [22–24] providing precedent for our observations during RBC storage in high glucose solutions. In conclusion, we believe that storage sensitizes RBCs to necroptosis due to ROS-induced necrosome formation as a result of NADPH oxidase activity. Once these sensitized RBCs encounter a pore-forming protein, such as the toxins used in this study or the complement membrane attack complex, they will be primed to undergo necroptosis.

Considerations for storage solution composition

Since high glucose primes RBC necroptosis, this may represent a flaw in the current standards of RBC storage. Therefore, moving forward it may be necessary to determine the efficacy of alternate solutions for survival of stored RBCs. As levels of high glucose enhance RBC necroptosis due to glycolysis [2, 13], it is possible that 2-deoxyglucose (a non-metabolizable form of glucose) can serve as a glucose substitute in these solutions. However, since glycolysis is needed to maintain ATP in RBCs due to phosphate leakage during storage [25], this approach may not be ideal. Lower levels of glucose in the storage solutions may be a viable option, however, lower amounts of glucose may still prime RBC necroptosis [13]. A possible solution may be the replacement of glucose with intermediates of glycoysis. NADPH, which induces ROS, forms upstream of diphosphoglycerate in glycolysis but diphosphoglycerate precedes steps that produce all ATP molecules [26, 27]. Morevover, phosphoglycerate and phoshoenolpyruvate precede production of the last 2 ATP molecules [26, 27]. Since these intermediates would still result in ATP production, they may be viable substitutes for glucose during storage. Inclusion of ATP in storage solutions may be an efficient substitute for glucose as well.

Relevance to transfusions

We have shown here that storage primes RBCs for clearance post-transfusion following attack by VLY or PLY. The toxin PLY does not induce necroptosis in fresh RBCs [1, 2] and did not induce clearance post-transfusion of these cells. However, PLY and LLO (which are non-necroptotic against fresh RBCs) were capable of inducing necroptosis of stored RBCs. The clearance of storage-primed RBCs following contact with non-specific pore-forming toxins suggests that this phenomenon may be relevant to complement-dependent hemolytic

transfusion reactions [28]. In fact, we previously showed that the complement membrane attack complex induces RBC necroptosis when combined with artificial stimulation of the necrosome [1]. This suggests that storage-primed necroptosis may be a component of hemolytic transfusion reactions. It is possible that this phenomenon may also be related to the RBC "storage lesion" [29, 30]. This refers to multiple physiologic changes that RBCs undergo during storage and refrigeration [29, 30]. As these physiologic changes include the formation of echinocytes and spheroechinocytes [29, 30], there may be a connection to our observations that storage sensitizes RBCs for necroptosis and clearance via Syk-dependent echinocyte formation. Further work and evaluation of longer periods of RBC storage is necessary to firmly establish this connection, however.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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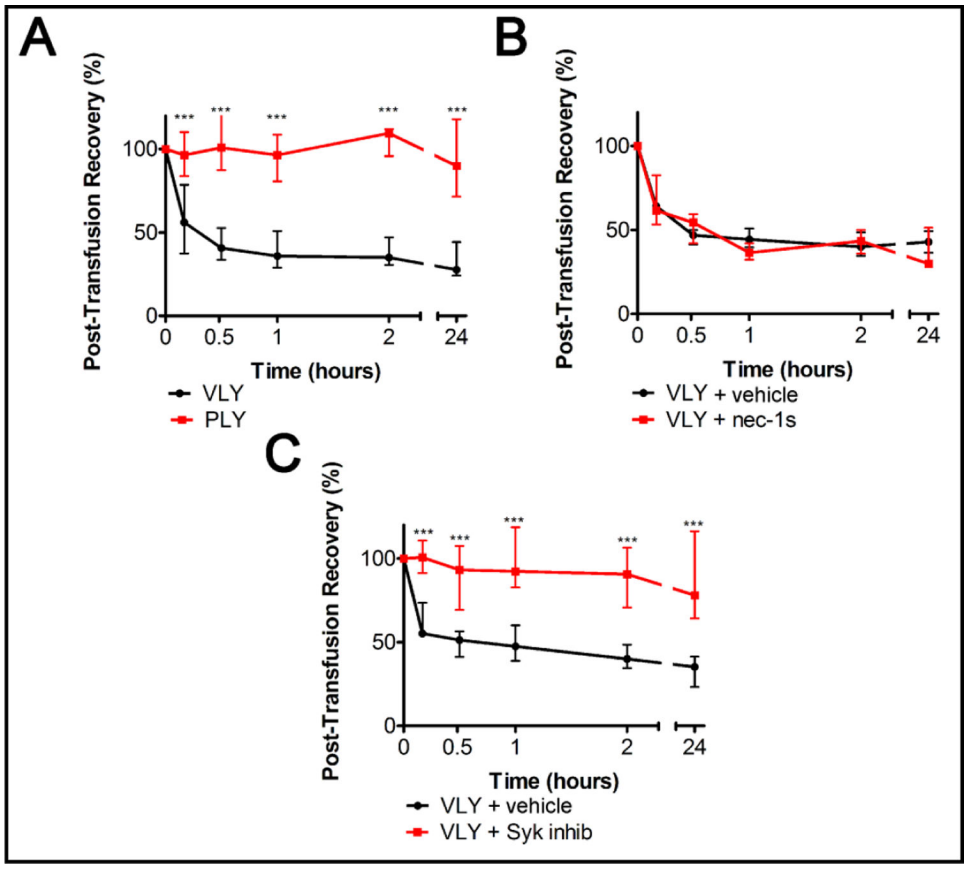

Fig. 1.

RBC necroptosis leads to Syk-dependent post-transfusion clearance. A.) Human CD59 transgenic murine RBCs were treated with sub-lytic quantities of VLY or PLY ex vivo for 30 min followed by transfusion into healthy, recipient WT mice. VLY, which induces RBC necroptosis, induces RBC clearance while PLY does not. B.) VLY-induced clearance is not affected by nec-1s. Shown is the ratio of vehicle-treated to nec-1s-treated RBCs. C.) VLY-induced clearance is prevented by inhibition of Syk kinase. Shown is the ratio of toxin-treated:untreated RBCs. Results shown are from 10 transfused mice in each experiment. Mann-Whitney, ***$p<0.001$.

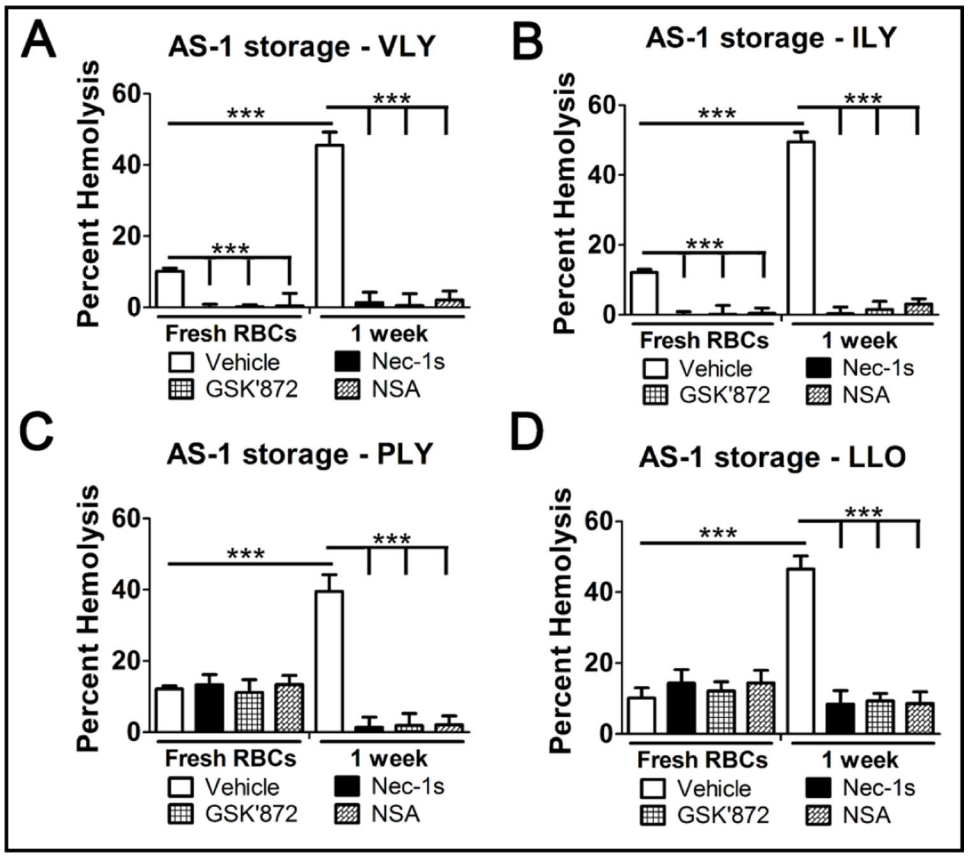

Fig. 2.

Storage in AS-1 leads to increased RBC necroptosis. Fresh human RBCs or those stored in AS-1 for 1 week were treated with the necroptosis-inducing A.) VLY, B.) ILY and the non-necroptotic C.) PLY and D.) LLO. The necroptosis-inducing toxins VLY and ILY (A-B) produce RBC death that can be inhibited by nec-1s (RIP1 inhibitor), GSK’872 (RIP3 inhibitor), or necrosulfonamide (NSA, MLKL inhibitor). This RBC necroptosis increases following 1 week of storage in AS-1 relative to fresh RBCs. The non-necroptotic toxins PLY and LLO (C-D) produced death of fresh RBCs that was not inhibited by nec-1s, GSK’872, or NSA. However, following 1 week of storage in AS-1 RBC death by PLY and LLO was prevented by nec-1s, GSK’872, and NSA. Hemolysis values were normalized to basal RBC death in the absence of toxin treatment. Results shown are from 3 independent experiments. Two-way ANOVA, ***$p<0.001$.

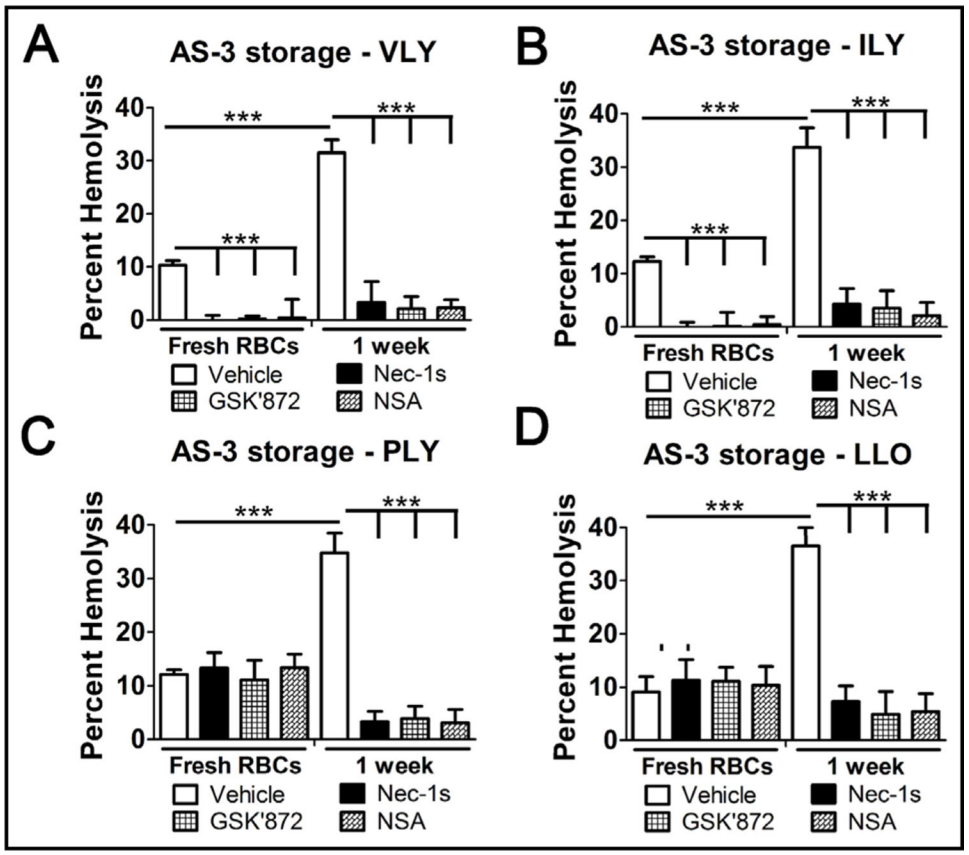

Fig. 3.

Storage in AS-3 leads to increased RBC necroptosis. Fresh human RBCs or those stored in AS-3 for 1 week were treated with A.) VLY, B.) ILY, C.) PLY, and D.) LLO. Storage in AS-3 for 1 week leads to increased RBC necroptosis by all toxins relative to death of fresh RBCs which is inhibited by nec-1s, GSK'872, and NSA. Hemolysis values were normalized to basal RBC death in the absence of toxin treatment. Results shown are from 3 independent experiments. Two-way ANOVA, ***p<0.001.

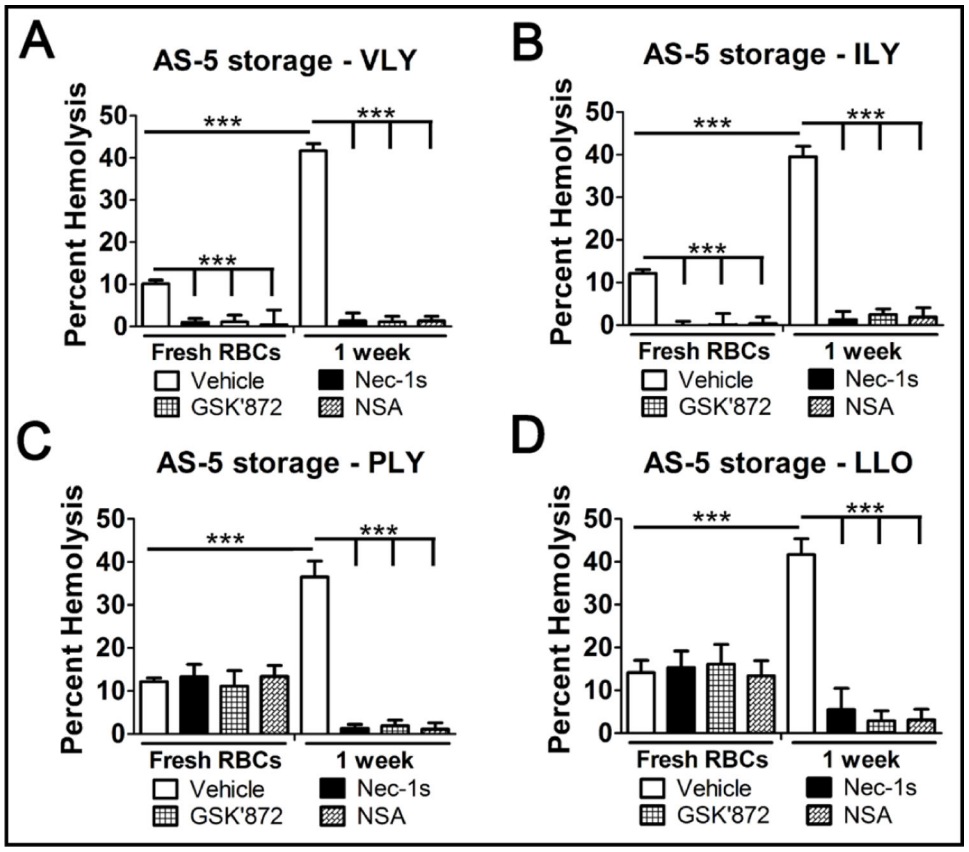

Fig. 4.

Storage in AS-5 leads to increased RBC necroptosis. Fresh human RBCs or those stored in AS-5 for 1 week were treated with A.) VLY, B.) ILY, C.) PLY, and D.) LLO. Storage in AS-5 for 1 week leads to increased RBC necroptosis by all toxins relative to fresh RBCs. RBC necroptosis after 1 week of storage is inhibited by nec-1s, GSK’872, and NSA. Hemolysis values were normalized to basal RBC death in the absence of toxin treatment. Results shown are from 3 independent experiments. Two-way ANOVA, ***$p<0.001$.

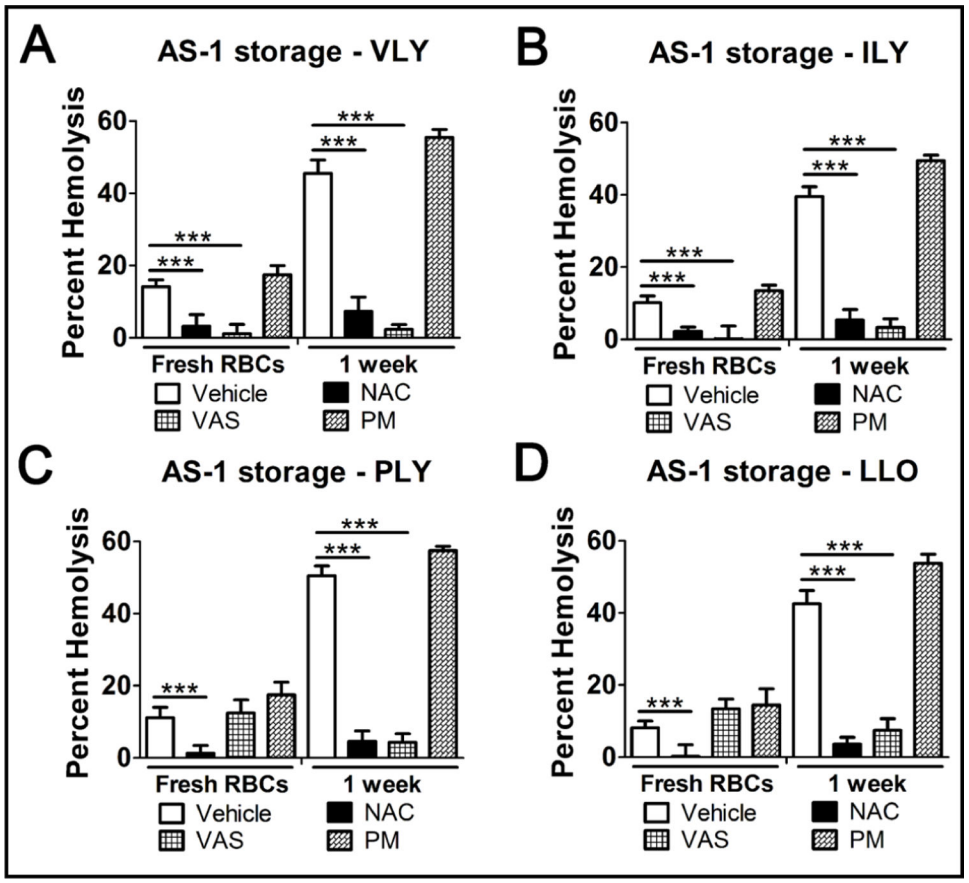

Fig. 5.

Storage-enhanced RBC necroptosis depends on ROS and NADPH oxidases. Fresh human RBCs or those stored for 1 week in AS-1 were treated with A.) VLY, B.) ILY, C.) PLY, and D.) LLO. Increased RBC necroptosis seen following storage is prevented by inhibition of ROS with N-acetylcysteine (NAC) or NADPH oxidases with VAS2870 but not by inhibition of AGEs with pyridoxamine (PM). Death of fresh RBCs by all toxins tested was prevented by inhibition of ROS with NAC. Hemolysis values were normalized to basal RBC death in the absence of toxin treatment. Results shown are from 3 independent experiments. Two-way ANOVA, ***$p<0.001$.

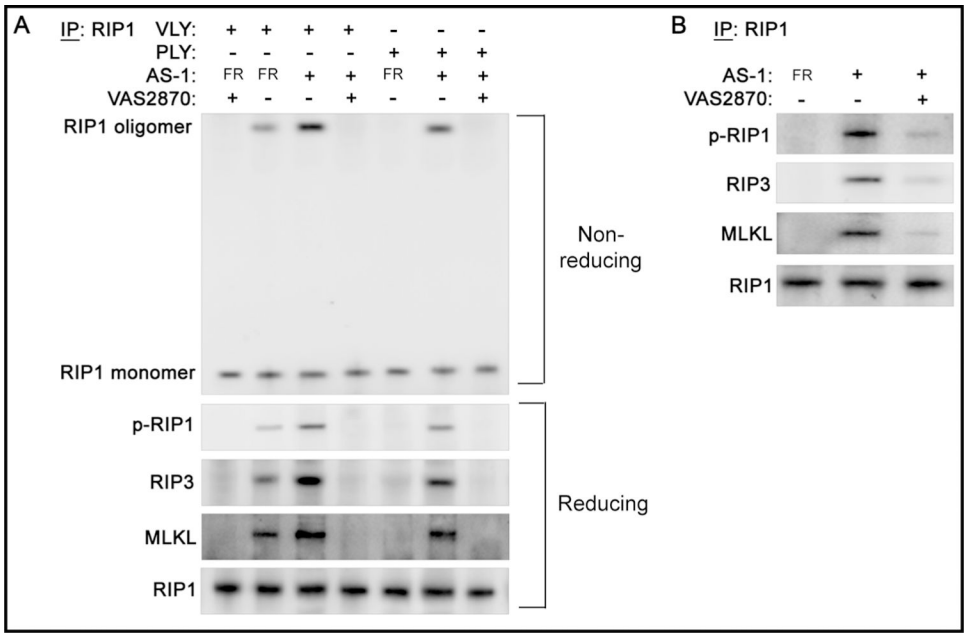

Fig. 6.

RBC storage leads to increased activation of necroptosis factors in an NADPH oxidase-dependent manner. Fresh human RBCs (FR) or those stored for 1 week in AS-1 were treated with the necroptosis-inducing toxin, VLY, or the non-necroptotic PLY followed by immunoprecipitation (IP) of RIP1. A.) VLY leads to oligomerization and phosphorylation of RIP1 in fresh RBCs and leads to necrosome formation as indicated by co-precipitation of RIP3 and MLKL. RIP1 oligomerization and phosphorylation as well as necrosome formation increase following RBC storage in response to either VLY or PLY. Increased RIP1 oligomerization, RIP1 phosphorylation, and necrosome formation were prevented by inhibition of NADPH oxidases with VAS2870 (VAS). B.) Storage of RBCs in AS-1 in the absence of toxin treatment leads to phosphorylation of RIP1 and necrosome formation which is prevented by VAS2870. Representative blots from 3 independent experiments are shown.

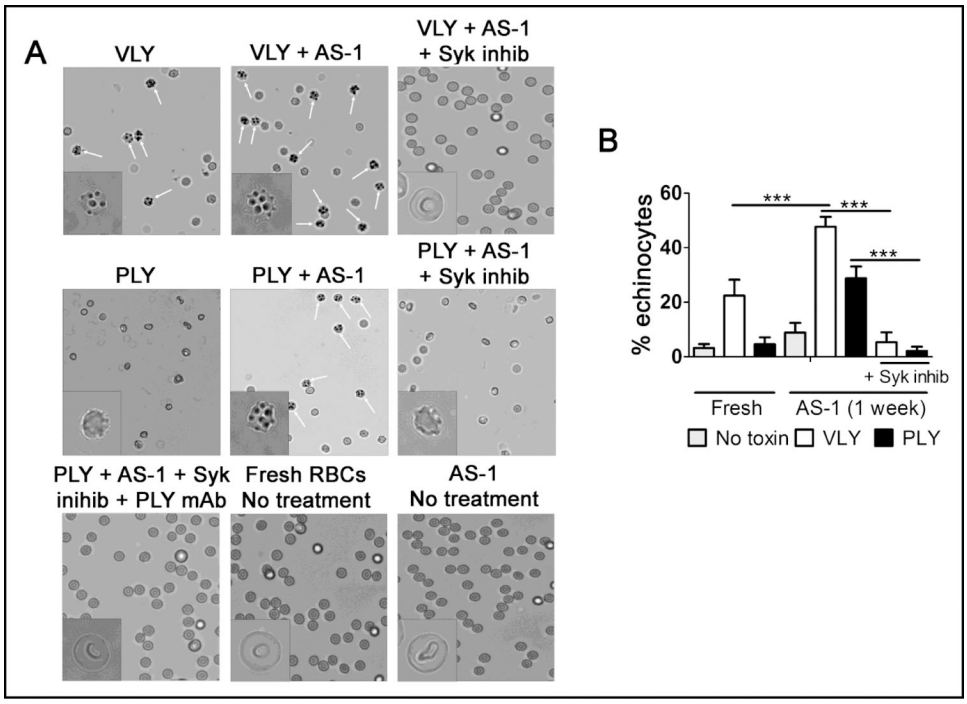

Fig. 7.

RBC storage leads to Syk-dependent echinocyte formation. A.) Fresh human RBCs or those stored for 1 week in AS-1 were treated with the necroptosis-inducing toxin, VLY, or the non-necroptotic PLY. VLY induces echinocyte formation in fresh RBCs (arrows) while PLY does not. Echinocytes increase following 1 week of storage in response to either toxin. This increased echinocyte formation following storage was prevented by inhibition of Syk kinase (Syk inhib). RBCs were still affected by PLY following Syk inhibition but no echinocytes were observed. Treatment of these cells with a neutralizing mAb against PLY returned RBC morphology to normal. Representative images from 3 independent experiments are shown. B.) Quantification of echinocyte formation. Results shown are from 3 independent experiments. 10 fields were counted in each experiment. Two-way ANOVA, ***$p<0.001$.

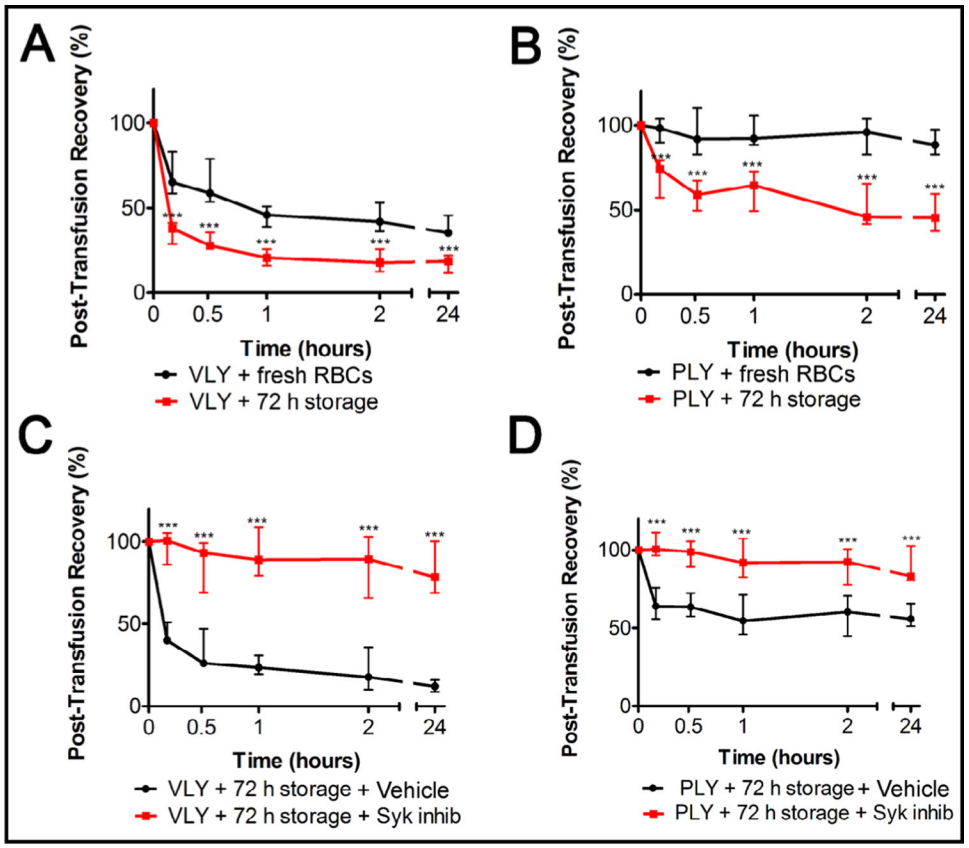

Fig. 8.

Storage leads to increased Syk-dependent RBC clearance post-transfusion. Fresh human CD59 transgenic murine RBCs or those stored in AS-1 for 72 h were treated with sub-lytic quantities of A.) VLY or B.) PLY ex vivo for 30 min followed by transfusion into healthy recipient WT mice. RBC clearance increases in in response to both toxins following storage. C-D.) Increased storage-induced RBC clearance was prevented by inhibition of Syk kinase. Shown is the ratio of toxi-treated:untreated RBCs. Results shown are from 10 transfused mice in each experiment. Mann-Whitney, ***$p<0.001$.