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Red blood cells store and release interleukin-33

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Abstract

Interleukin-33 (IL-33) is a member of the IL-1 cytokine superfamily that potently drives production of a variety of cytokines and contributes to the pathogenesis of inflammatory diseases. IL-33 is a nuclear protein and is released from apoptotic or necrotic cells. Serum IL-33 levels are increased in various diseases, such as atopic dermatitis, chronic hepatitis C infection, and asthma. Here, we show that red blood cells (RBCs) are one of the major sources of plasma IL-33. IL-33 levels are significantly increased in supernatants from lysed RBCs. Plasma IL-33 levels are increased in patients during hemolysis and plasma IL-33 levels show a positive correlation with degree of hemolysis. IL-33 protein and mRNA levels were detected in the late stages of differentiation in *ex vivo* primary human erythroid progenitor cell cultures, suggesting that IL-33 is expressed during maturation of RBCs. Furthermore, hemoglobin depleted red cell lysates induced IL-8 expression in human epithelial cells. This effect was attenuated in IL-33 decoy receptor expressing cells and was enhanced in IL-33 receptor expressing cells. These results suggest that erythroid progenitor cells produce IL-33 and circulating RBCs represent a major source of IL-33 that is released upon hemolysis.

Introduction

Interluekin-33 (IL-33), a relatively new member of the IL-1 cytokine superfamily has been reported to play a pathogenic role in inflammatory diseases including acute lung injury (ALI) (1, 2), asthma (3, 4), pulmonary fibrosis (5, 6), and rheumatoid arthritis (RA) (7–9). IL-33 binds to ST2, a member of the IL-1 receptor/Toll-like receptor superfamily. ST2 protein consists of two types: a soluble (sST2, a decoy receptor) and a membrane-bound (ST2L) isoform (10–12). IL-33 activates the MAPK signal transduction cascade and increases chemokine release through ligating to ST2L (2, 7, 10, 13). We and others have

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shown that down-regulation of ST2L attenuated IL-33-induced IL-8 release in human lung epithelial cells (2, 13). ST2 protein expression is regulated at the transcriptional (14, 15) and post-translational level (2). Post-translational ST2L regulatory mechanisms include phosphorylation and ubiquitination (2). The mechanisms that regulate IL-33 expression are incompletely understood. IL-33 is localized to nuclei of fibroblasts (16), endothelial cells (11, 17, 18), and epithelial cells (17, 19, 20). It is released from apoptotic and necrotic cells and is considered to be a “danger signaling” molecule (21–23). Increased serum IL-33 levels have been detected in patients with atopic dermatitis (24), RA (25, 26), asthma (27), and scleroderma (28); however, the source of increased IL-33 in these conditions has not been well studied.

Hemolysis is a general term for excessive breakdown of red blood cells (RBCs). Hemolysis can occur within the circulatory system (intravascular hemolysis) or in the reticuloendothelial system (extravascular hemolysis) (29). Hemolysis also occurs during storage of RBCs and the amount of storage hemolysis increases with the length of time in storage (30). In mammals, circulating mature RBCs maintain a very specialized flexible biconcave discoid shape. Mature RBCs are enucleate and lack organelles providing maximum space for their primary cargo hemoglobin. Circulating RBCs have a limited lifespan (120 days in humans) in the circulation and old RBCs are removed from the circulation by macrophages in the spleen; thus extravascular hemolysis is part of the natural life cycle of a circulating RBC. Intravascular hemolysis can be caused by bacterial infections, toxins, drugs, medications, autoimmune responses and alloimmune responses (31–33). Intravascular hemolysis results in the release of RBC contents into the circulation, which when excessive can cause more hemolysis and vascular dysfunction. Storage hemolysis also causes the release of potentially harmful, vasoactive, RBC-derived components into the RBC unit prior to transfusion into patients (30). These storage related changes may harm patients when older RBC units are transfused into patients and contribute to the RBC “storage lesion” (30). Accumulating evidence indicates that stored RBCs have increased cytokine content. Levels of IL-1 and IL-8 are significantly higher in RBC units that have been stored for 40 days compared to the levels of these cytokines observed in freshly collected RBC units (34). Darbonne, WC et al. demonstrated that ^{125}I-labeled IL-8 rapidly and efficiently bound to RBCs (35). In addition to IL-8, RBCs also bind monocyte chemotactic peptide-1 (MCP-1) (35). A recent study from Lee JS, et. al., showed that longtime storage of RBCs increases the production of IL-8-bound RBC-derived microparticles (36). RBCs also bind insulin and insulin-like growth factors (37, 38). These results support the role of circulating RBCs as carriers of bioactive peptides including cytokines. Diffuse alveolar hemorrhage plays a critical role in the pathogenesis of ALI (39, 40). Recent studies have demonstrated that hemolysis induces inflammatory responses (34, 35, 37, 38, 41, 42); however, the mechanisms have not been well characterized.

Here, we report that RBCs contain IL-33. This is the first study to demonstrate IL-33 expression in differentiating erythroid progenitor cells, and IL-33 is released during hemolysis. IL-33 release during hemolysis may contribute to hemolysis-induced inflammatory responses.

Materials and methods

Cell culture and reagents

Purified human CD34+ progenitor cells were derived from GCSF–treated peripheral blood cells of healthy donors. These cells were grown at 37°C with 5% CO2 in serum-free medium consisting of Iscove's modified Dulbecco's medium (IMDM) with 1-thioglycerol, BIT9500 supplement (BITS) (Stem Cell Technologies), BSA (Sigma-Aldrich), and the indicated cytokines (PeproTech). The cells initially underwent 72 h of expansion with 100 ng/ml SCF (PeproTech), 100 ng/ml FMS-like tyrosine kinase 3 ligand (FLT3 ligand) (PeproTech), 100 ng/ml thrombopoietin (TPO) (PeproTech), and 50 ng/ml IL-3 (PeproTech). After expansion cells were then seeded in erythroid differentiation medium, which contains recombinant human erythropoietin at 4.5 U/ml (Procrit; Amgen), 10 ng/ml SCF and BIT9500 supplement. Human bronchial epithelial cells (Beas2B) were grown at 37°C with 5% CO2 in DMEM medium with 10% FBS. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies, reverse transcriptase, and real-time PCR SosoFast reagents were purchased from Bio-Rad Laboratories (Hercules, CA). Amicon Ultra centrifuge filters were purchased from EMD Millipore (Billerica, MA). Anti-IL-33 antibody was from R&D Systems (Minneapolis, MN). Anti-LarminA/C antibody was from Santa Cruz (Dallas, TX). Anti-GAPDH and β-actin antibodies and RBC lysis buffer were from Sigma (St. Louis, MO).

RBC lysates preparation

Human or mouse whole blood was collected in EDTA-treated blood collection tubes, followed by centrifugation at 500 g for 10 min. The RBC fraction was transferred to new tubes and split into two groups: the non-lysed control group was incubated in isoosmotic 0.9% NaCl, the hemolyzed group was incubated with RBC lysis buffer. Both groups were incubated at room temperature for 10 minutes. After a brief centrifugation at 500 g for 10 min, the supernatants of the two groups were subjected to IL-33 immunoblotting.

Hemoglobin free RBC lysate preparation

To remove hemoglobin (64 kDa) from RBC lysates, Amicon ultra (50 kDa) cutoff filter was used. Briefly, RBC lysates were subjected to Amicon ultra (50 kDa) cutoff filter, followed by a centrifugation at 5,000 g for 20 min.

Immunoblotting

Cells were washed with cold PBS and collected in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 5 mM β-glycerophosphate, 1 mM MgCl2, 1% Triton X-100, 1 mM sodium orthovanadate, 10 μg/ml protease inhibitors, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. Equal amounts of cell lysates (20 μg) or RBC supernatants were subjected to SDS-PAGE, electrotransferred to nitrocellulose membranes and immunoblotted with indicated antibodies.

RNA extraction and Real time RT-PCR

Total RNA were extracted from cells by TRIzol (Sigma) according manufacturer's instructions. Reverse transcription was performed using a cDNA synthesis kit (Bio-Rad) and Real-time PCR was performed to assess cytokine expression using primers specific to human IL-33 and IL-8 mRNA transcripts. Amplicon expression in each sample was normalized to its 18S mRNA transcript level. The relative abundance of target mRNA in each sample was calculated using the relative crossing time ratio. As 2 raised to the negative of its threshold cycle value times 10^6 after being normalized to the abundance of its corresponding 18S [e.g., $2^{-(\text{IL-33R2 Threshold Cycle})}/2^{-(\text{18S Threshold Cycle})} \times 10^6$].

Statistics analysis

All results were subjected to statistical analysis using two-way analysis of variance, and, wherever appropriate, analyzed by Student–Newman-Keuls test. Data are expressed as mean ± S.D.. $p<0.05$ were considered statistically significant.

Results and Discussion

IL-33 exists in RBC lysates

Circulating IL-33 levels are increased in patients with allergic, inflammatory, rheumatologic, and infectious diseases (5, 23, 24, 26–28). However, the source of IL-33 has not been well characterized. RBCs have been shown to bind plasma cytokines, growth factors, and chemokines (35, 37, 38). To investigate whether RBCs are one of the sources of plasma IL-33, we collected mouse whole blood, and compared IL-33 levels in supernatants from lysed RBCs and non-lysed RBC controls by immunoblotting as described in the Materials and Methods. Figure 1 shows that the supernatants from hemolyzed samples contain much higher levels of IL-33 compared to non-lysed controls. These results suggest that IL-33 exists in RBC lysates. This is a novel finding and has important implications in hemolytic diseases and RBC storage in blood banking. RBCs are known to bind IL-8, MCP-1, insulin, and insulin-like growth factors (35, 37, 38), but have not been shown to bind IL-33 until now. IL-8 and MCP-1 bind to the Duffy antigen on circulating RBCs (43). Insulin binds to the insulin receptor on the surface of RBCs (44). Lee JS et al. has also shown that microparticles from RBCs contain IL-8 (36), suggesting that IL-8 is one of internal components in RBC-derived microparticles. The localization of IL-33 in the RBCs remains unclear.

IL-33 levels are increased in sickle cell patients with higher levels of hemolysis

To further investigate the role of the RBC as a storage pool for circulating IL-33, we compared the IL-33 levels in plasma from sickle cell patients known to have undergone low levels or high levels of hemolysis (Table 1). Sickle cell disease patients from our previous Walk-PHASST study were randomly selected within the highest and lowest quartiles of hemolytic component. The hemolytic component is a composed index for hemolysis based on a principle component analysis of factors associated with hemolysis, including lactate dehydrogenase, aspartate, aminotransferase, reticulocyte percentage and total bilirubin in 415 hemoglobin SS patients had a mean of 0 (SD=1.50) (45). IL-33 levels are significantly higher in plasma samples from high hemolyzers compared to the plasma samples from low

hemolyzers (Fig. 2). These results support the idea that RBCs are a major source of IL-33 in the circulation. These results also suggest that plasma IL-33 levels may be used as a biomarker for hemolytic disorders, such as sickle cell disease. This finding connects RBC damage during hemolysis directly to a cytokine release.

Hematopoitic progenitor cells express IL-33

IL-33 is a nuclear protein. It is unclear how IL-33 gets inside of anucleate RBCs. A speculative mechanism is that IL-33 is left behind in the reticulocyte after enucleation of erythroid progenitor cells during the late stages of maturation. To investigate IL-33 synthesis during human erythropoiesis, IL-33 protein expression was examined during different stages of erythroid progenitor cell differentiation using an *ex vivo* human CD34+ hematopoietic progenitor cell culture system as described in the Materials and Methods. We collected erythroid progenitor cells on days 1, 5, and 9 of erythroid differentiation. As shown in Figure 3, human erythroid progenitor cells show a progressive decrease in expression of the nuclear protein, LaminA/C, while IL-33 expression progressively increases, peaking at day 9. Cytoplasm proteins (IQGAP1 and β-actin) remain no changes. Reduction of LaminA/C indicates the erythroid progenitor cells were differentiated to mature RBC-like cells. IL-33 mRNA, but not IL-8 mRNA levels progressively increase as erythropoiesis proceeds in the human erythroid progenitor cell cultures (Fig. 4). These results suggest that IL-33 is synthesized during RBC differentiation and maturation, and that IL-33 is not stuck on the membrane of RBCs, but is soluble and inside the RBCs. CD34+ progenitors from chronic myeloid leukemia patients have been shown to express ST2L, proliferate and produce cytokine in response to IL-33 (46), while this study is the first to demonstrate CD34+ progenitors also express its ligand, IL-33. Watari K et al. reported that human hematopoietic progenitor cells produce IL-1β (47). These results also support the idea that IL-33 is inside circulating erythrocytes.

Hemoglobin depleted RBCs lysates increase IL-8 mRNA expression in human lung epithelial cells

We and others have shown that IL-33 treatment increases IL-8 expression in human lung epithelial and endothelial cells (2, 13, 21). To investigate if the IL-33 contained in RBC lysates can increase IL-8 expression, independent of hemoglobin, we removed hemoglobin from lysed RBCs using size exclusion filters, since hemoglobin can affect cytokine release. Beas2B cells were treated with hemoglobin depleted RBC lysates for 3 h and IL-8 mRNA expression was determined by real-time PCR. As shown in Fig. 5, hemoglobin depleted RBC lysates induced IL-8 mRNA expression ~23 fold, this effect was attenuated in IL-33 decoy receptor, sST2-overexpressing cells. Further, hemoglobin depleted RBC lysates-induced IL-8 mRNA expression was enhanced in IL-33 receptor, ST2L over-expressing cells. These results suggest that hemolysis via the release of intra-crythrocyte IL-33 may contribute to IL-8 expression (Fig. 6). Hemolysis can be caused by infection, inflammation, oxidative damage, auto-antibodies, allo-antibodies, toxins, drugs, medications, and inherited genetic lesions (29–32). Hemolysis also contributes to inflammatory responses, endothelial barrier dysfunction, and platelet activation, the effects are similar to IL-33 treatment. This study provides new evidence that IL-33 is synthesized in erythroid progenitor cells and can

be released from RBCs undergoing hemolysis. IL-33 may be involved in hemolysis-induced inflammatory responses.

Acknowledgments

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Abbreviations

RBC	red blood cell
IL	interleukin
RA	rheumatoid arthritis
IMDM	Iscove's modified Dulbecco's medium
ALI	acute lung injury

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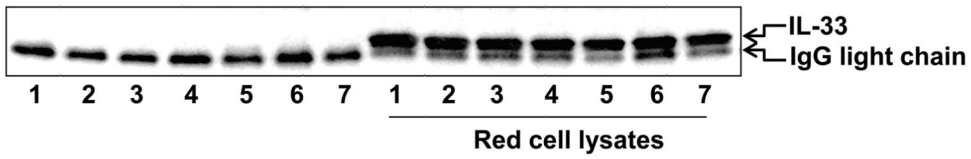

Figure 1. IL-33 is released during hemolysis *in vivo*

Isolated mouse RBCs (7 samples/group) were incubated with or without RBC lysis buffer for 10 min. After a brief centrifugation, supernatants were subjected to SDS-PAGE and immunoblotting analysis with an anti-IL-33 antibody.

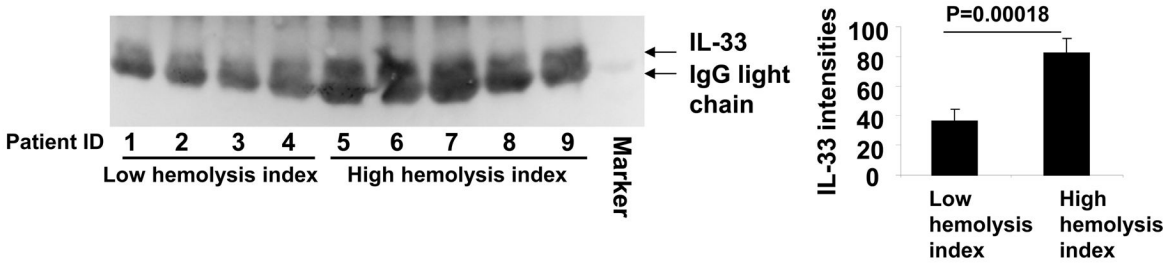

Figure 2. Plasma IL-33 levels are increased in sickle cell patients with higher hemolysis

A. IL-33 levels in plasma from patients with low hemolytic rates and high hemolytic rates were analyzed by immunoblotting with an anti-IL-33 antibody. **B.** Analysis of immunoblot densitometry was performed using Image J software.

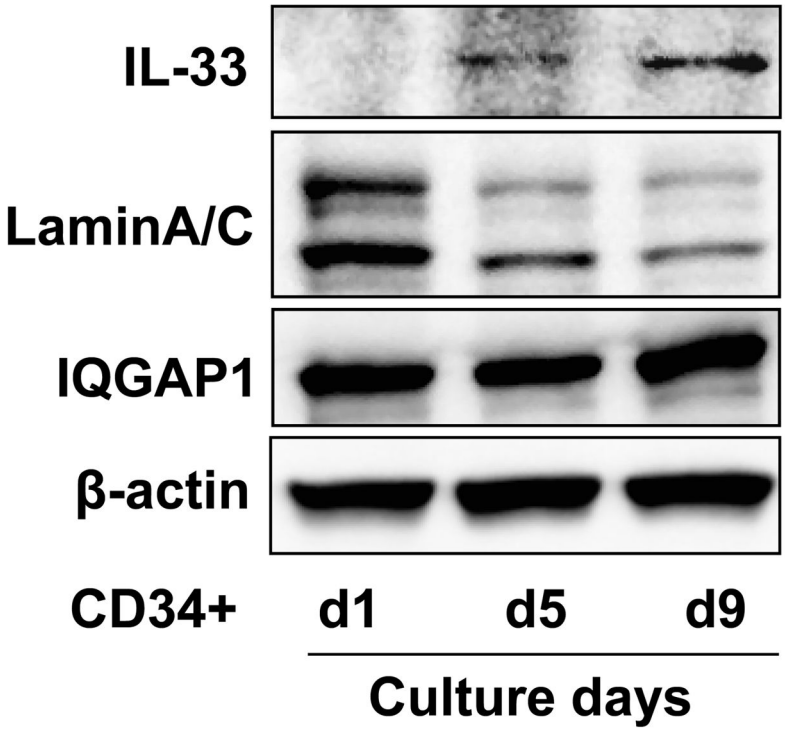

Figure 3. IL-33 protein express in erythroid progenitor cells

Primary human CD34+ hematopoitic progenitor cells were expanded and subjected to unilineage erythroid differentiation culture conditions. Cells were harvested on days 1, 5, and 9 of erythroid differentiation. IL-33, LaminA/C, GAPDH, and β-actin expression were analyzed by immunoblotting.

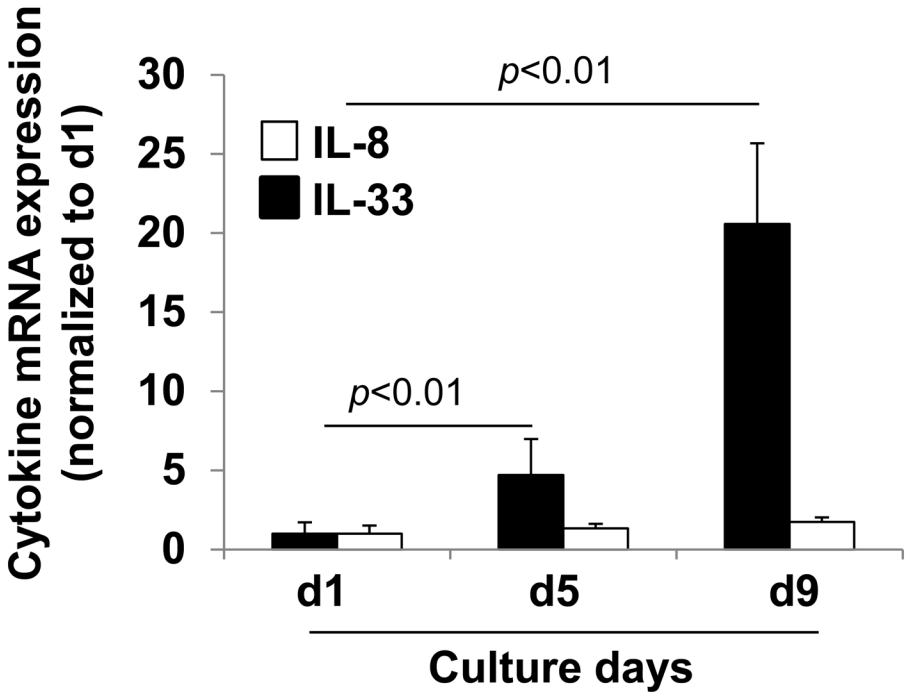

Figure 4. IL-33 mRNA expression in erythroid progenitor cells

Primary human CD34+ hematopoitic progenitor cells were expanded and differentiated as described for Figure 3. Erythroid progenitor cells were harvested on days 1, 5, and 9 of erythroid differentiation. Total RNA was extracted and IL-33 and IL-8 mRNA expression were analyzed by real-time PCR.

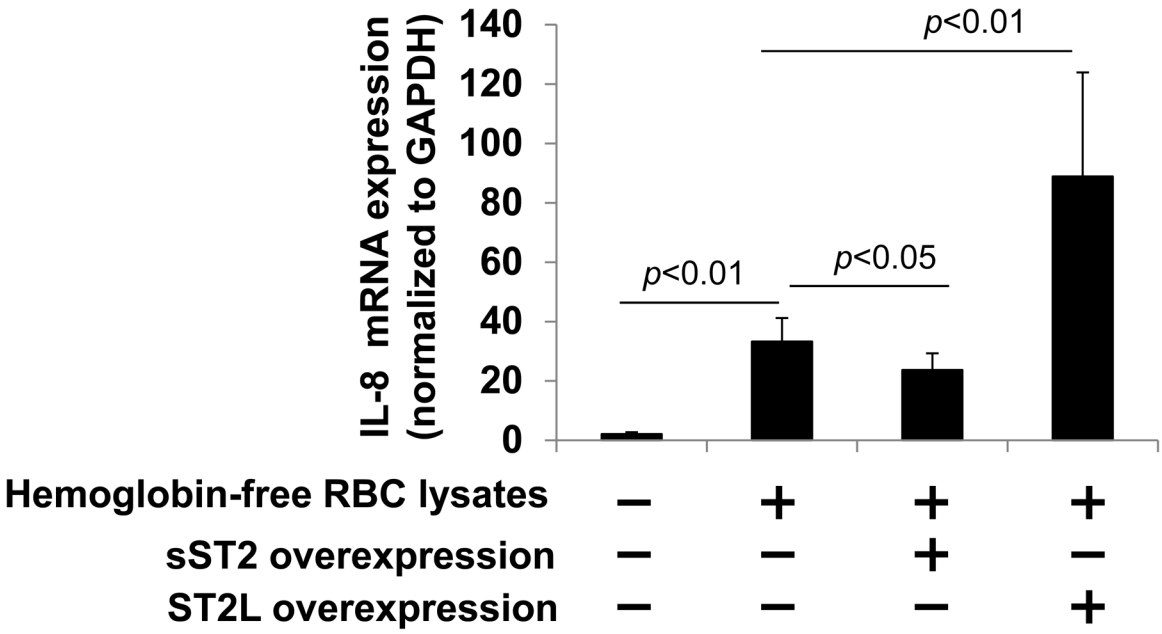

Figure 5. Hemoglobin depleted RBC lysates-induced IL-8 mRNA expression is regulated by IL-33 receptor expression

RBC lysates were filtered through a 50 kDa cutoff Amicon Ultra column to remove hemoglobin. Beas2B cells were transfected with sST2 or ST2L plasmids for 48 h prior to treatment with the hemoglobin depleted filtrates from hemolyzed RBCs for additional 3 h and total RNA was extracted. IL-8 mRNA levels were determined by real-time PCR.

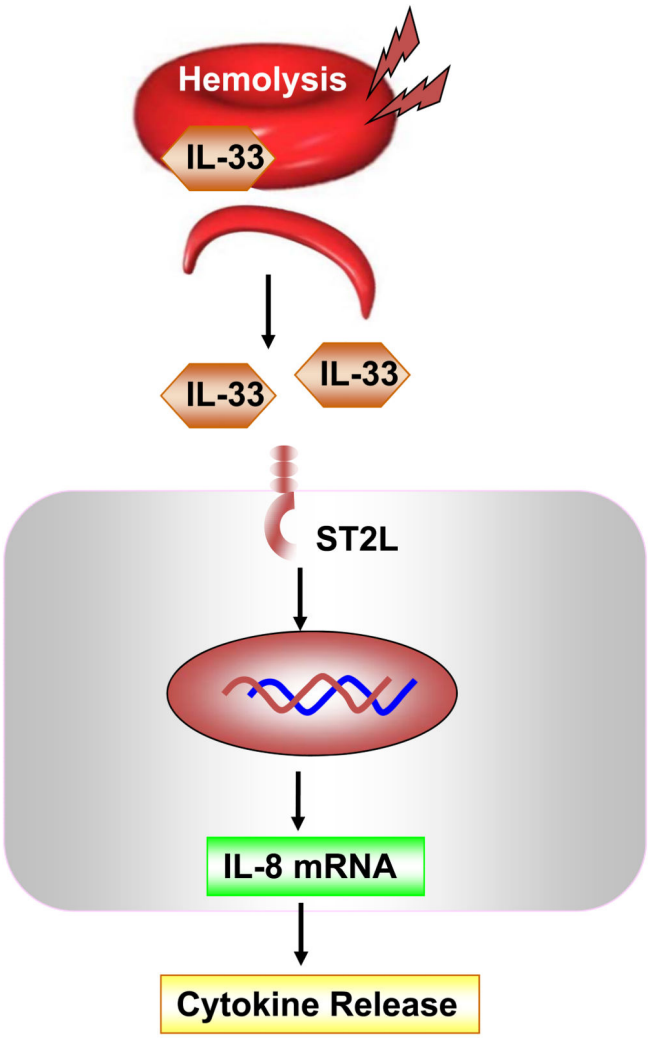

Figure 6. IL-33 releases during hemolysis

Hemolysis induces release of IL-33 from disrupted RBCs. IL-33 modulates cytokine release through ligation to its receptor, ST2L on surrounding cells.

Table 1

Plasma samples from subjects with low and high hemolytic component.

Sample ID	Hemolytic component (relative unit)	Hemolytic group	age
1	−4.0508	Low	47.4
2	−2.529	Low	22.3
3	−2.4477	Low	46.9
4	−2.4456	Low	20.7
5	2.76036	High	42.9
6	2.82356	High	48.6
7	2.9443	High	47.8
8	3.23965	High	37.0
9	3.58382	High	41.7

Plasma samples of sickle cell disease patient from the Walk-PHASST study were used for Western blot analysis (Fig. 2). Patients were randomly selected within the highest and lowest quartiles of hemolytic component of the Walk-PHASST cohort. The hemolytic component is a composed index for hemolysis based on a principle component analysis of factors associated with hemolysis, including lactate dehydrogenase, aspartate, aminotransferase, reticulocyte percentage and total bilirubin in 415 hemoglobin SS patients had a mean of 0 (SD=1.50) (45).