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Accepted Manuscript

Thrombosis and Haemostasis

Ferroptosis of Endothelial Cells Triggered by Erythrophagocytosis Contribute to Thrombogenesis in Uremia

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DOI: 10.1055/a-2117-7890

Please cite this article as: Li Z, Yan M, Wang Z et al. Ferroptosis of Endothelial Cells Triggered by Erythrophagocytosis Contribute to Thrombogenesis in Uremia. Thromb Haemost 2023. doi: 10.1055/a-2117-7890

Conflict of Interest: The authors declare that they have no conflict of interest.

This study was supported by PhD Fund of Harbin Medical University-Daqing, QXBSQDJ201902, Heilongjiang Provincial Postdoctoral Science Foundation (http://dx.doi.org/10.13039/5011000010009), LHQ19127, Marshal Initiative Funding, HMUMIF-22005, National Natural Science Foundation of China (http://dx.doi.org/10.13039/501100001809), S2070140.82270134, Yu Weihan Foundation of Harbin Medical University, 31021180167, DQYWH201801, Center of diagnosis and treatment of disease in cold place, Harbin Medical University, CXZX-ZKXT01, Natural Science Foundation of Heilongjiang Province (http://dx.doi.org/10.13039/5011000005046), LH2020H0299

Abstract:

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Ferroptosis of Endothelial Cells Triggered by Erythrophagocytosis Contribute to Thrombogenesis in Uremia

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Abstract

Although thrombosis event is the leading complication of uremia, its mechanism is largely unknown. The interaction between endothelial cells (ECs) and red blood cells (RBCs) in uremic solutes and its prothrombotic role need to be investigated. Here, we established an in vitro co-incubation model of uremic RBC and EC as well as a uremic rat model by adenine. Using flow cytometry, confocal microscopy, and electron microscopy, we found increased erythrophagocytosis by EC accompanied with increased reactive oxygen species (ROS), lipid peroxidation and impairment of mitochondria, indicating that EC undergo ferroptosis. Further investigations showed increased proteins expression of heme oxygenase-1 (HO-1) and ferritin (FTN) and labile iron pool (LIP) accumulation in EC, which could be suppressed by deferoxamine (DFO). The ferroptosis negative regulators GPX4 and SLC7A11 were decreased in our erythrophagocytosis model and could be enhanced by ferrostatin-1 (Fer-1) or DFO. In vivo, we observed that vascular EC phagocytosed RBC and underwent ferroptosis in the kidney of the uremic rat, which could be inhibited by blocking the phagocytic pathway or inhibiting of ferroptosis. Next, we found the high tendency of thrombus formation was accompanied by erythrophagocytosis-induced ferroptosis in vitro and in vivo. Importantly,
we further revealed that upregulated TMEM16F expression mediated phosphatidylserine externalization on ferroptotic EC, which contributed to a uremia-associated hypercoagulable state. Our results indicate that erythrophagocytosis triggered ferroptosis and followed phosphatidylserine exposure of EC may play a key role in uremic thrombotic complication, which may be a promising target to prevent thrombogenesis of uremia.

**Keywords:** Erythrophagocytosis, Ferroptosis, Endothelial cell, Thrombosis, Uremia

**Introduction**

Uremic patients are at high risk of venous thromboembolism (VTE), stroke and acute coronary syndrome\(^1\)-\(^3\), which may represent the predominant cause of mortality.\(^4\) A better understanding of the pathogenesis of thrombotic tendencies in uremia could help to find novel therapeutic targets to prevent thrombosis in uremia.

Phosphatidylserine, an anionic phospholipid, is usually confined to the inner leaflet of the cell membrane.\(^5\),\(^6\) It is externalized to the outer membrane when cells apoptosis or are activated. Exposed phosphatidylserine on cells as a critical “eat me” signal for phagocytes provides a catalytic surface for the assembly of tenase and prothrombinase complexes.\(^7\)-\(^9\) Our previous studies had shown that uremic solutes indoxyl sulfate and indole-3-acetic acid (IAA) induced eryptosis and enhanced the procoagulant activity of RBCs through phosphatidylserine exposure and microparticle release, which contributed to the hypercoagulable state in uremia.\(^10\),\(^11\) However, the fate of these phosphatidylserine exposed RBCs was not investigated. It is well known that macrophages play critical roles in the clearance of senescent RBC and recycling iron in physiological processes, which are called erythrophagocytosis.\(^12\) But, in certain clinical situations such as malaria, immunoglobulin G (IgG)-mediated hemolytic transfusion reactions, transfusion of storage-damaged RBCs and uremia, shortened RBC lifespan and increased or pathologic erythrophagocytosis may make professional macrophages overwhelmed, dysfunction and loss.\(^13\),\(^14\) Endothelial cells (ECs) are amateur phagocytes which exist in a large amount on the walls of blood vessels and have the potential phagocytic ability. Recent studies have reported that ECs have phagocytic properties for lactadherin-opsonized RBC.\(^15\),\(^16\) In this study, we found that injured or eryptotic RBCs by uremic toxins were engulfed by vascular endothelial cells (vECs), and then, we investigated the effects of erythrophagocytosis on ECs.

Ferroptosis is a recently identified iron-dependent cell death characterized by increased iron-dependent reactive oxygen species (ROS) and lipid peroxides, which is morphologically, biochemically and genetically distinct from apoptosis, necroptosis and autophagy.\(^17\),\(^18\) Ferroptosis plays an important regulatory role in
many diseases such as tumors, cardiomyopathy, acute kidney injury and ischemia/reperfusion. However, the role of ferroptosis in the occurrence and development of thrombosis is currently unknown. In this study, we first demonstrated pathologic erythrophagocytosis disrupting the homeostasis of iron metabolism and resulted in iron overload, subsequently, the increased “free” iron induced ferroptosis of EC through the Fenton reaction producing multiple ROS and lipid peroxidation products. Following ferroptosis, phosphatidylserine was exposed on EC, which enhanced the procoagulant activity of EC and contributed to the thrombophilic state in the uremic condition. Our data showed the relationship between erythrophagocytosis, ferroptosis and thrombogenesis, which further explained coagulation disorders, renal anemia, and inflammation complications in uremic patients.

Materials and Methods

Reagents

Detailed description of all reagents can be found in the supplemental methods.

Cells and animal model

Peripheral blood was drawn from healthy volunteers and approval from the Ethics Committee of Harbin Medical University according to the Helsinki Declaration. All animal studies were approved by the Animal Care Committee of Harbin medical university and conformed to the international guidelines on the ethical use of animals. Detailed information about co-incubation model of uremic RBC and EC in vitro, a uremic rat model induced by adenine and flow restriction in the IVC is available in the supplemental methods. The following were performed as previously described: phagocytosis assay, flow cytometry, electron microscopy, confocal microscopy, HE and MSB staining, western blot, FXa and prothrombinase assays, thromboelastography testing, MDA, GSH, CAT, H2O2, SOD and iron quantification assays. Detailed information about these methods is available in the supplemental methods.

Statistical analysis

All values were presented as the mean ± standard deviation (SD). Groups were compared using the 2-tailed Student's test or one-way analysis of variance with Tukey's post hoc test. Differences with a P value < .05 were statistically significant. Statistical analysis was analyzed by GraphPad Prism 8.0.

Results
Phosphatidylserine exposed RBCs were engulfed, degraded and eliminated by ECs in vitro

To investigate the increased erythrophagocytosis by ECs in uremic conditions, we set up an in vitro co-incubation model of uremic RBC and cultured EC. Uremic toxin IAA was used to induce phosphatidylserine exposure on RBCs, and the ethanol was used as the vehicle because IAA was dissolved by ethanol. Phosphatidylserine exposure was detected through Annexin V-FITC binding by confocal microscopy and flow cytometry, respectively. After 24 hours of incubation, compared with RBCs cultured in Ringer solution (R-RBCs), 20μM IAA treated RBCs (I-RBCs) had a significantly higher percentage of Annexin V binding (11.08 ± 0.44 % vs 0.15 ± 0.02 %, P < 0.001), and a higher percentage (14.03 ± 1.5 %, P < 0.01) in 50μM IAA (supplemental Figure 1A), which consistent with our previous studies. Confocal microscopy furtherly showed stronger rings of green fluorescence on the outer membrane of I-RBCs compared to R-RBCs (supplemental Figure 1B-C). Therefore, in the following co-incubation experiment, we used the RBCs treated with the maximal level of IAA (50μM) found in uremic patients. Next, we determined the interaction between uremic RBC and EC in the co-incubation model. After co-incubation for 6 hours, images of representative confocal microscopy showed that significantly increased I-RBCs (red) were taken up by ECs (green) compared with R-RBCs (Figure 1A-B). By flow cytometry, we furtherly quantified the internalization of RBCs by ECs. The percentage of ECs containing RBCs was 21.47 ± 1.18 % in I-RBC group, which was significantly higher than that in R-RBC group (1.9 ± 0.6 %, P < 0.001). In addition, I-RBC showed a massive enhanced endocytosed by EC after lactadherin opsonization (31.78 ± 1.27 %, P < 0.001), while the extent of phagocytosis was dramatically suppressed when I-RBCs were pretreated with annexin V to block the exposed phosphatidylserine (6.83 ± 1.47 %, P < 0.001) (Figure 1C). We furtherly depicted the dynamic phagocytosis process by SEM and TEM. Representative images of SEM showed that four I-RBCs were trapped, anchored, and endocytosed by EC (Figure 1E), whereas none of the R-RBC was in contact with the EC during 6 hours of co-culture (Figure 1D). As shown in Figure 1F, TEM displayed that the EC was extending the pseudopodia and adhering I-RBC, and then I-RBC was captured and internalized by EC (Figure 1G). After 12 hours of co-incubation, erythrocytes were digested and degraded into fragments in EC (Figure 1H). Within 24 hours, nearly all phagocytic intracellular RBCs had disappeared, leaving only their membranes ruffles or empty vesicles (Figure 1I-J), suggesting that degradation was virtually complete. Taken together, these results indicated that uremic RBCs were indeed endocytosed by ECs and in a phosphatidylserine-dependent manner.
ECs underwent ferroptosis following enhanced erythrophagocytosis

The distinction between ferroptosis and other forms of cell death is the dramatic morphological changes of mitochondria in ferroptosis. When observing the ultrastructure of ECs through TEM, compared with the normal mitochondria in ECs without erythrophagocytosis (Figure 2A), we found detailed impairments of mitochondria, including content lost, cristae reduced or disappeared (Figure 2B-C), increased membrane density, condensed cristae (Figure 2D), and enhanced the Fiameng score (a semiquantitative evaluation method of the mitochondrial ultrastructure) in I-RBC treated ECs (Figure 2E). To further verify that increased erythrophagocytosis induced ferroptosis in ECs, cell viability, ROS and lipid peroxidation were detected respectively. We found that erythrophagocytosis led to significantly decreased viability of ECs, which could be rescued by Fer-1 and DFO, respectively (Figure 2F). In addition, compared to R-RBC treated EC, there were significantly increased DCF and C11-Bodipy fluorescence in I-RBCs treated EC after 12 hours of incubation, which were effectively reduced by treatment with Fer-1 or DFO (Figure 2G and H). These results furtherly indicated that increased erythrophagocytosis led to EC ferroptosis.

Erythrophagocytosis induced ferroptosis of ECs via iron overload and amino acid metabolism disorder

Although increasing evidence suggests that EC experience ferroptosis following phagocytosis of uremic RBCs, the underlying mechanism is poorly understood. In human, approximately 80% of heme is present in erythrocytes, we speculated that iron overload may play an important role in erythrophagocytosis induced EC ferroptosis. Therefore, the proteins that regulated intracellular iron homeostasis were detected. As we expected, there was significantly higher iron content in ECs that co-incubated with I-RBCs than that with R-RBCs, whereas the intracellular active iron was decreased in I-RBCs treated ECs with the addition of DFO to chelate iron, but it was similar between the two groups when pretreated with Fer-1 (Figure 3 A). The level of HO-1 protein expression was significantly increased in I-RBCs treated ECs, moreover, neither Fer-1 nor DFO could inhibit the upregulated HO-1 (Figure 3B). Consistent with the increased HO-1 levels, we also found increased FTN protein levels in the I-RBCs treated ECs, while the expression of FPN responsible for iron efflux did not change. Furthermore, when I-RBCs treated ECs were incubated with DFO, FTN significantly decreased, but there was no difference when pretreated with Fer-1 (Figure 3C-D), suggesting iron overload played a critical role in the ferroptosis of EC triggered by erythrophagocytosis. By western blot assay, we found that in addition to iron overload, I-RBCs treated ECs had decreased levels of GPX4 and SLC7A11 compared with R-RBCs treated ECs, moreover, both of the two proteins rescued significantly
following the administration of Fer-1 and DFO (Figure 3E-F), indicating amino acid metabolism disorder also occurred in erythrophagocytosis triggered ferroptosis by suppressing the expression of system Xc− and led to a reduced generation of GPX4.

Ferroptosis followed by erythrophagocytosis enhanced the procoagulant activity of ECs

Since ECs experienced ferroptosis after the uptake of large numbers of RBCs, we considered that this could disrupt the clotting balance of ECs. To explore whether ferroptosis enhance the procoagulant activity of ECs, we examined the procoagulant activity of ECs by one-stage recalcification time assay. As shown in Figure 4A, after 12 hours of incubation, the coagulation time of ECs cultured with I-RBCs was 151.22±10.29 s, which significantly reduced when compared with ECs cultured with R-RBCs (197.07±8.36 s, P < 0.001). To understand the role of phosphatidylserine exposure in ferroptosis induced procoagulant activity of EC, coagulation inhibition assays was furtherly performed on EC. The procoagulant activity of ECs was inhibited over 90% by 128nM lactadherin. It is well known that one of the important roles of phosphatidylserine is a catalytic surface for the assembly FXa and prothrombinase complexes. We further investigated the capacity of EC to support intrinsic, extrinsic FXa and thrombin that contribute to the procoagulant activity. As shown in Figure 4B, I-RBCs treated ECs significantly increased the production of the three procoagulant enzyme complexes compared with R-RBCs treated ECs, which were inhibited by 128nM lactadherin up to 90%. Results from inhibition assays furtherly confirmed that phosphatidylserine played a crucial role in the procoagulant activity of ECs undergoing ferroptosis. More recent studies have shown that TMEM16F served as phospholipid scramblase and was upregulated in ferroptosis. Therefore, we also evaluated the expression and role of TMEM16F in the erythrophagocytosis-induced ferroptosis. We found that I-RBCs treated ECs exhibited significantly higher TMEM16F expression and phosphatidylserine exposure compared to ECs with or without cocultured R-RBCs by western blotting and flow cytometry, respectively (Figure 4C-D). In addition, confocal microscopy visually demonstrated that TMEM16F expression (green) and phosphatidylserine exposure (green) increased significantly in the I-RBCs treated ECs compared with the R-RBCs treated ECs (Figure 4E-J).

Erythrophagocytosis and ferroptosis occurred in the kidney of uremic rats

To better understand the mechanisms of thromboembolic disorders in uremia, we established adenine-induced animal model of chronic kidney disease. Consistent with previous reports, 27 0.75 % adenine treatment for four weeks was effective in causing chronic kidney disease in rats. As shown in supplemental
Table 1, adenine-treated rats significantly decreased the body weight, RBC and hemoglobin, and significantly increased plasma urea, creatinine, uric acid, and phosphorus compared with control rats, which in line with the characteristics of uremia. Likewise, compared with control rats, representative images of HE staining from kidney of the adenine-treated rats showed the characteristic structural changes including glomerular injury, tubulointerstitial fibrosis, interstitial inflammatory infiltrates, tubular distend and vacuolar degeneration of epithelial cells (supplemental Figure 2A-B). Histological analysis of kidney sections showed that the number, diameter, and surface area of the glomerulus were markedly reduced in the adenine-treated group compared to the control group. Compared with the control group, the renal coefficient (the ratio of the left kidney’s weight to its body weight) was significantly higher in the adenine-treated group (supplemental Figure 2C-F). These results indicated adenine administration effectively induced uremia in rats. Then, we furtherly explored the association and underlying mechanisms of erythrophagocytosis, ferroptosis and thrombogenesis in our uremic rat model. Representative light microscopy image of a blood vessel in the kidney showed multiple erythrocytes were adhered to the vascular wall and internalized in vEC (Figure 5A) and accompanied by microthrombosis in the microvasculature of the uremic kidney by MSB staining (Figure 5B). More importantly, we also examined the interaction between RBC and EC inside the blood vessel by TEM. Representative image showed that the erythrocyte was trapped and anchored by the EC in the vessel of kidney (Figure 5C), and several erythrocytes were endocytosed and degraded to some fragments by the EC (Figure 5D). Similarly as observed in vitro, where we found morphological changes of the mitochondria in the EC such as cristae reduced and content lost (Figure 5E).

Then, iron metabolism and lipid peroxidation signaling were detected in kidney tissue. Representative images of Perl’s blue plus diaminobenzidine (DAB) staining revealed increased reactive iron deposits on the microvasculature EC in the uremic kidney compared to control, which furtherly proved iron overload was the most important mechanism in erythrophagocytosis triggered EC ferroptosis (Figure 5F-I). Moreover, compared with the control group, the uremic rats had significantly higher content of iron, MDA and H$_2$O$_2$, as well as decreased GSH content, SOD and CAT activity, all of which are also characteristic markers of ferroptosis and indicate enhanced oxidative stress in adenine-induced uremic rat model (Figure 5J-O). To furtherly confirm the occurrence of ferroptosis following increased erythrophagocytosis in vivo, adenine-treated rats were simultaneously treated with annexin V to block the phagocytosis pathway, Fer-1 to inhibit ferroptosis and DFO to chelate iron, respectively. Next, we found that compared with the control group, the protein expression of HO-1, responsible for heme breakdown, was significantly increased in uremic kidney tissue. Consistent with increased HO-1 and iron, FTN responsible for storing excess iron also increased in uremic group. Both two proteins were inhibited by Fer-1, DFO and annexin V, respectively. However, FPN, the only iron efflux channel located on the surface of the cell membrane, was no different between the two
groups. Additionally, the expression levels of ferroptosis characteristic protein GPX4 and SLC7A11 in uremic renal were significantly lower than those in the corresponding control group and could be reversed by Fer-1, DFO and annexin V, which revealed that in addition to iron metabolism, amino acid metabolism also involved in erythrophagocytosis triggered ferroptosis in our uremic model. We also detected the protein expression of TMEM16F that serves as a phospholipid scramblase, consistent with the results in vitro, TMEM16F increased in the renal tissue of the adenine-treated rats but reduced in uremic rats treated with Fer-1, DFO and annexin V compared to the control group (ie, nontreated) (Figure 5P). These series of results confirmed that erythrophagocytosis induced ferroptosis indeed occurred in vivo, both iron overload and lipid peroxidation contributed to the pathological process.

**Erythrophagocytosis induced ferroptosis of ECs promoted hypercoagulability in uremic rats**

To confirm whether erythrophagocytosis induced ferroptosis plays a role in uremia-associated thrombogenesis, a flow restriction model was used. In response to the restriction of venous blood flow, all (4 of 4) uremic rats developed a thrombus within 6 hours after flow stasis in the inferior vena cava (IVC). And four control rats also formed a visible thrombus after 6 hours of stasis (Figure 6A). Moreover, weight, length, and the index of weight (mg)/length (mm) of the thrombus in uremic group were all significantly higher than in the control group, suggesting the thrombus was tighter and denser in uremic rats. Whereas treating adenine-fed rats with Fer-1, DFO or Annexin V significantly reduced the parameters of thrombus (Figure 6B-D). Next, the phosphatidylserine exposure of erythrocytes was analyzed by flow cytometry, as expected, the percentage of Annexin V positive erythrocytes significantly increased in uremic rats (5.97 ± 0.49 %) compared to healthy controls (1.22 ± 0.08 %, P < 0.001) (Figure 6E). Thromboelastography was used in our study to further monitor dynamic real-time pictures of coagulation in rats. There was a significant difference between uremic rats and control rats, with uremic rats having a shorter R time, and larger α angle, MA, and CI. Not surprisingly, with the addition of Fer-1, DFO and annexin V in uremic rats, the R time was markedly prolonged, while α angle, MA and CI were reduced compared to rats only treated with adenine (Figure 6F-J), which further proved the contribution of ferroptosis in blood hypercoagulability of uremic rats. Taken together, these results confirmed that erythrophagocytosis induced EC ferroptosis accompanying phosphatidylserine exposure led to a prothrombotic state and local thrombi formation.

**Discussion**
Uremia might be at high risk of cardiovascular and thrombotic events that require special attention. A better understanding of the prothrombotic mechanisms of uremic toxins could help to find novel therapeutic targets to prevent thrombosis in uremia. In this study, to investigate the role of uremic toxin exposure on RBC and its effect on EC function, we set up an in vitro co-incubation model of uremic RBC and EC that may mimic uremic condition and an adenine-induced chronic kidney disease rat model. We first found that uremic solute IAA-treated phosphatidylserine-exposed erythrocytes were sequestered, endocytosed, and degraded by EC in vitro. Then, increased erythrophagocytosis triggered EC ferroptosis and induced a procoagulant phenotype on EC through increased phosphatidylserine exposure that mediated by TMEM16F. Furthermore, thromboelastography and IVC ligation induced flow stasis model confirmed uremic rats exhibited the hypercoagulable state and were more prone to develop VTE, which was related to erythrophagocytosis induced ferroptosis. More importantly, we demonstrated that iron overload and perturbations of amino acid metabolism were the most important mechanism of erythrophagocytosis triggered EC ferroptosis.

It is well known that the ageing RBCs are mainly cleared by macrophage, named erythrophagocytosis. Professional phagocytes such as macrophages and dendritic cells are the main phagocyte, which have the capacity to remove dead cells and other foreign materials in the body. However, professional macrophages may become overwhelmed by the excessive amounts of eryptotic or damaged RBCs in uremia, the amateur phagocytes which can also participate in efferocytosis when needed. Amateur phagocytes are non-myeloid progenitor/nonimmune cells that include differentiated cells (e.g., epithelial cells, fibroblasts, and endothelial cells [ECs]) and stem cells. ECs as an amateur phagocytes which exist in a large amount on the walls of blood vessels, represent the first barrier in contact with erythrocytes and have the potential phagocytic ability. Ageing or damaged RBC express phosphatidylserine on their surface which can directly bind to Stabilin-2 or Tim-4 on the macrophage or via opsonins such as Gas-6, lactadherin or thrombospondin-1. The ability of uremic plasma to promote erythrophagocytosis may be associated with the proinflammatory state, enhanced oxidative stress, and the accumulation of toxins. Moreover, increased erythrophagocytosis could accompany by decreased levels of CD47, a don’t eat me signal. Our previous studies demonstrated that ECs could endocytose activated platelets, apoptotic neutrophils, and acute promyelocytic leukemia (APL) cells. Catan et al. reported that vECs were able to bind and internalize aged/glycated RBC in diabetic conditions and may promote vulnerable atherothrombotic plaques to rupture. However, the effects on ECs biology of progressively ingesting large numbers of RBCs are not completely understood, moreover, the relationship between erythrophagocytosis and procoagulant activity of ECs in uremia has not been explored. In our RBC treatment model, we found that ECs engulfed large amounts of phosphatidylserine exposed IAA-induced erythrocytes even no bridge molecules, and exhibited...
morphologic abnormal mitochondria, increased ROS and lipid peroxidation, all of which were all reduced by treatment with Fer-1 or DFO, indicating EC undergo ferroptosis following enhanced erythrophagocytosis in the uremic environment. Our previous studies have reported uremic toxins such as uric acid, IAA and indoxyl sulfate (IS) could significantly increase phosphatidylserine exposure of RBCs. In the present study, we chose one of the uremic toxins IAA, we suggested that other uremic toxins including urea, creatinine, oxalic acid, uric acid and IS also play a similar role in erythrophagocytosis triggered ferroptosis of EC and followed procoagulant activity. The effect of other toxins on this process will be detected in the future.

We then sought to explore the mechanisms of erythrophagocytosis induced ferroptosis in EC. As a complex form of cell death, ferroptosis mainly involves three biochemical metabolisms, including iron, amino acid and lipid metabolism. We hypothesized iron disorder may be the most important reason because plenty of hemoglobin was degraded in the EC after phagocytosis of RBCs. Usually, macrophages play important physiological roles in iron metabolism and recycling by increasing the expression of HO-1 and FTN to handle the increased heme and iron load, thus keeping homeostatically and protecting it from severe oxidative stress. However, the biological role of iron metabolism in erythrophagocytosis induced EC ferroptosis remains poorly understood. In the present study, we systematically studied the protein expression in the pathway of iron metabolism of ECs following clearance of RBCs. We found an increased accumulation of free iron and FTN in EC incubated with I-RBCs, while the FPN responsible for iron efflux was no different. Moreover, treatment of ECs with Fer-1 did not decrease the labile iron pool (LIP) and the content of FTN, whereas they were both reduced after treatment with the iron chelator DFO. Therefore, we speculated that iron overload in ECs eventually leads to ferroptosis. We furtherly investigated several molecules in iron and redox metabolism that have been implicated in ferroptosis. Although HO-1 was thought to be a protective antioxidant enzyme, growing data suggests it plays a positive role in ferroptosis. Recent studies have demonstrated HO-1 drives ferroptosis by promoting iron overload in cardiomyopathy, hemochromatosis and β-thalassemia. Consist with most other’s reports, HO-1 protein expression was enhanced in our model, which catalyzes heme degradation and facilitates the release of free iron. We speculate that the upregulated HO-1 in our erythrophagocytosis induced EC model was mainly attributed to the regulation of Nrf2 signaling pathway. Whether HO-1 is expressed in mature erythrocytes remains unclear, which need to be investigated in our future study. Beside the iron metabolism, we also explored the key protein in amino acid metabolism pathway. SLC7A11 is a cystine/glutamate antiporter that mediates the efflux of cellular glutamate and the influx of cystine, which plays a role in ferroptosis due to regulate the downstream synthesis of glutathione peroxidase 4 (GPX4) and then catalyzed the reaction between GSH and lipid peroxides and prevent ferroptosis. In our present cell model, erythrophagocytosis reduced the
protein expression of SLC7A11 and GPX4, which may furtherly facilitate the accumulation of lipid peroxide and contribute ferroptosis of EC. In an adenine-induced uremic rat model, we observed that RBCs showed specific binding and internalization by vECs in the kidney, we considered that this could evoke serious damage to the vEC, and similarly as observed in vitro, where we noted erythrophagocytosis induced mitochondrial damage. At the same time, we found that the content of Fe$^{3+}$, the lipid peroxide MDA and H$_2$O$_2$ were increased in the kidney, while GSH, CAT and SOD activity were decreased, all of which are characteristic indicators of ferroptosis. Furthermore, the protein expression changes of FTN, FPN, HO-1, GPX4, SLC7A11 and TMEM16F in uremic rats were consistent with our in vitro experiments and could be reversed by blocking phagocytic pathway or inhibition of ferroptosis. However, there is still a trend of increased thrombus size even with Fer-1 or DFO treatment in uremia condition, we think that phosphatidylserine-exposed RBC/platelets/myeloid cells may involve in thrombogenesis in uremia, which has been demonstrated in our previous studies. Combined, in the erythrophagocytosis model that mimics uremic environment and uremic rat model, we revealed that increased heme upregulated the expression of HO-1, which promoted the release of free iron, thus increasing a variety of ROS and lipid peroxidation products through Fenton reaction and driving ferroptosis. Several ferroptosis associated proteins including SLC7A11 and GPX4 also involved in this process. Further studies may need to elucidate the relationship of regulating mechanism between iron, glutathione and lipid metabolism in our model.

The relationship between EC dysfunction and subsequent thrombotic events is already well known in cardiovascular diseases, diabetes, and uremia. Here, we are especially concerned about the significant involvement of the EC ferroptosis in the thrombotic complication of uremia. We found that the erythrophagocytosis led to the increase of phosphatidylserine externalization on the surface of ECs, which provided binding sites for clotting factors FXa and prothrombinase complexes, thus promoting the coagulation cascade reaction and subsequently leading to a dramatic increase in thrombin generation. By lactadherin inhibition assay, we further demonstrated the increase in phosphatidylserine externalization after ferroptosis was the main reason for the enhancement of procoagulant activity in EC. TMEM16F is a Ca$^{2+}$-activated phospholipid scramblase located on the cell membrane, which moves phospholipids from the inner to the outer leaflet of the plasma membrane. Recently, evidences have been provided that TMEM16F participates in cell apoptosis, ferroptosis and pyroptosis. We found increased TMEM16F expression in renal of uremic rats and EC undergoing ferroptosis, which may explain the reason why phosphatidylserine externalization increased following ferroptosis in our model. In line with earlier reports describing that adenine-treated rats induce an increased thrombogenicity, we found an adenine-induced uremic rat model exhibited enhanced thrombotic tendency by thromboelastography and IVC model. Additional pre-treatment with Fer-1 to inhibit ferroptosis or annexin V to block the pathway of phagocytosis significantly decreased
the hypercoagulability and ameliorated the flow restriction-induced venous thrombi in uremic rat, which demonstrated erythrophagocytosis induced ferroptosis played a critical role in the high tendency of uremic thrombus formation, confirming our in vitro findings. Taken together, our present study not only indicated erythrophagocytosis induced ferroptosis in EC, but also confirmed TMEM16F mediated phosphatidylserine exposure during the process of ferroptosis plays a major role in uremia associated thrombosis. Additional studies are required to establish an in vivo experiment to inhibit the phagocytosis of macrophages, furtherly determine the phagocytosis of RBCs and other peripheral blood cells or microparticles by ECs.

In conclusion, our findings suggest uremic RBC could trigger EC phagocytosis, ferroptosis and thrombogenesis, which may be clinically relevant to the complication of uremia, such as anemia, infection, and thrombosis. Alternatively, inhibition erythrophagocytosis or ferroptosis could be a novel therapeutic strategy to alleviate the thromboembolic complication of uremia.

Acknowledgments
This work was supported by the National Natural Science Foundation of China (82070140 and 82270134), Marshal Initiative Funding (HMUMIF-22005), PhD Fund of Harbin Medical University-Daqing (XQBSQDJ201902), Natural Science Foundation of Heilongjiang Province (LH2020H0299), Heilongjiang Postdoctoral Science Foundation (LBHQ19127), Yu Weihan Foundation of Harbin Medical University (31021180167 and DQYWH201801), Center of diagnosis and treatment of disease in cold place, Harbin Medical University (CXZX-ZXKT01).

Authorship
Contribution: Z. L. performed the experiments, analyzed the data, and edited the manuscript; Z. W., M. Y., Y. A., and L. W., performed the experiments and analyzed the data; M. X., Y. X., X.W. and T. L. contributed to the study design and commented on the manuscript; C. G. designed and conducted the research, analyzed, and interpreted the data, and wrote the manuscript.
Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Figure 1 Phosphatidylserine exposed RBCs were engulfed, degraded, and eliminated by ECs in vitro. ECs were incubated with R-RBCs and I-RBCs for 6 hours, respectively. After washing and removing the non-adherent or non-phagocytosed RBCs, immunofluorescent staining was applied to definitely determine the phagocytosis of RBCs by ECs. RBCs were stained with CD235a (red). The cytoskeleton and nuclei of ECs were labelled with tubulin (green) and Hoechst 33342 (blue), respectively. R-RBCs (A) and I-RBCs (B) were taken up or engulfed by ECs. (C) RBCs were stained with 1μM CMFDA (green) for 30 minutes at room temperature, and then cocultured with ECs for 6 hours in the presence or absence of 2nM Lact or 2nM AnV. The phagocytic percentage was counted by flow cytometry. All data were representative of at least three independent experiments and values were expressed as mean ± SD. Scanning electron microscopic examination of adhered RBCs to the surface of ECs after 6 hours of incubation, several I-RBCs (arrow) were trapped, anchored and internalizing by EC (E), whereas none of the R-RBC adhered to EC (D). Transmission electron microscopic images displayed a kinetic process that I-RBCs were engulfed and degraded in ECs for 24 hours. I-RBC (arrow) was extending the pseudopodia and adhering to EC (F), and then RBCs (stars) were internalized in EC after 6 hours of coculture (G). (H) After 12 hours of co-incubation, erythrocytes were digested and degraded into fragments in EC (dotted box). (I) After 24 hours, nearly all phagocytosed intracellular RBCs had disappeared, leaving only their membranes. (J) Local magnification of RBCs membranes ruffles (star) and empty vesicles (triangle) in EC. IAA, indole-3-acetic acid; R-RBC, RBCs cultured in Ringer solution; I-RBC, RBCs cultured in Ringer solution with 50μM IAA; Lact, lactadherin; AnV, annexin V.

Figure 2. ECs undergone ferroptosis following enhanced erythrophagocytosis. The ultrastructure of mitochondria in ECs was analyzed by transmission electron microscopic in the erythrophagocytosis model. (A) Normal mitochondria in EC without erythrophagocytosis. In our erythrophagocytosis model, ECs cocultured with I-RBCs exhibit morphological changes of mitochondria around the internalized RBC fragments including mitochondria integrity impaired, content lost, cristae reduced (dotted box) (B) and disappeared (C), membrane density increased and cristae condensed (D). ECs were incubated with each group of RBCs for 12 hours with or without 5μM Fer-1 or 200μM DFO. (E) The Fiameng scores were used to evaluate the mitochondrial ultrastructure. (F) The viability of ECs was assessed by the CCK-8 test. Each group of cells was incubated with DCFH-DA or C11-BODIPY for 30 minutes at 37℃, after washing, the levels of ROS (G) and lipid peroxidation (H) in ECs were determined by flow cytometry. All data were representative of at least three independent experiments and values were expressed as mean ± SD. IAA,
indole-3-acetic acid; R-RBC, RBCs cultured in Ringer solution; I-RBC, RBCs cultured in Ringer solution with 50μM IAA; Fer-1, ferrostatin-1; DFO, deferoxamine; ROS, reactive oxygen species.

**Figure 3. Iron overload and amino acid metabolism contributed to erythrophagocytosis induced ferroptosis of ECs.** ECs were incubated with R-RBC and I-RBC with or without 5μM Fer-1 (I-RBC + Fer-1 group) or 200μM DFO (I-RBC + DFO group) for 12 hours, respectively. (A) Calcein fluorescence assays. ECs were stained with calcein-AM (1μM) and fluorescence intensity was measured by flow cytometry. The results were expressed as the mean fold changes versus untreated ECs (mean ± SD, n=3 experiments). (B-F) Protein levels of HO-1, FTN, FPN, GPX4 and SLC7A11 in ECs were detected by western blotting. The relative density of the protein bands was quantified and normalized to β-actin (mean ± SD, n=3 experiments). IAA, indole-3-acetic acid; R-RBC, RBCs cultured in Ringer solution; I-RBC, RBCs cultured in Ringer solution with 50μM IAA; LIP, labile iron pool; Fer-1, ferrostatin-1; DFO, deferoxamine; HO-1, heme oxygenase-1; FTN, ferritin; FPN, ferroportin; GPX4, glutathione peroxidase 4.

**Figure 4. Ferroptosis followed by erythrophagocytosis enhanced the procoagulant activity of ECs.** ECs were cocultured with R-RBC and I-RBC for 12 hours, respectively. After washing and removing the non-adherent or non-phagocytosed RBCs, ECs were collected for the one-stage recalcification time assay. (A) In the absence or presence of 128nM Lact, coagulation times of 100μL ECs (10^3) in each group were shown. Results were displayed as mean ± SD for at least three independent experiments. (B) Formation and inhibition assays of procoagulant enzyme complexes. FXa and thrombin production of ECs (10^3) were shown. Intrinsic FXa formation was measured in the presence of FIXa, FVIII and thrombin. Extrinsic FXa production was assessed in the presence of FVIIa. Thrombin generation was investigated in the presence of FXa and FVa. The capacity of 128nM Lact was evaluated to block procoagulant enzyme complexes on ECs. Data are mean ± SD. (C) ECs were collected and incubated with Annexin V- FITC for 30 minutes in the dark, and then flow cytometry analyzed the phosphatidylserine exposure of ECs. Results were expressed as mean ± SD. (D) The expression level of TMEM16F in ECs was detected by western blotting. The relative density of the protein bands was quantified and normalized to β-actin (mean ± SD, n=3). Expression of TMEM16F (green) in ECs was determined by immunofluorescence staining. The cytoskeleton and nuclei of ECs were labeled with tubulin (red) and Hoechst 33342 (blue), respectively. (E) No TMEM16F staining was found in the R-RBCs treated ECs. (F) Much green fluorescence was visible on ECs after coculturing with I-RBC. (G) The relative fluorescence intensity of TMEM16F. Confocal microscopy images of phosphatidylserine (green) externalized to the outer membrane of ECs coincubated with I-RBC (I) or R-RBC (H). (J) The relative fluorescence intensity of phosphatidylserine. IAA, indole-3-acetic acid; R-RBC, RBCs cultured in Ringer solution; I-RBC, RBCs cultured in Ringer solution with 50μM IAA; Lact, lactadherin.
Figure 5. Erythrophagocytosis triggered ferroptosis of kidney in the uremic rats. Histological analyses of the kidney tissue section in three different stainings (HE, MSB and DAB-enhanced Prussian blue). (A) Light microscopy image of a blood vessel shown internalization of multiple RBCs by ECs (indicated by arrow). (B) Representative image of MSB staining showed RBCs aggregates and fibrin on the microvasculature EC surface in the uremic kidney. (C) Transmission electron microscopy image of an erythrocyte trapped and anchored by EC in the vessel of the uremic kidney. (D) Several erythrocytes were endocytosed (star) and degraded to some fragments (arrow) by ECs. (E) The mitochondrial ultrastructure of renal vascular ECs from uremic rats showed cristae reduced and content lost (arrow). (F-I) Kidney sections were stained with DAB-enhanced Prussian blue to detect iron deposits on the microvasculature EC. Representative images of kidney sections from the uremia group (F and G) and control (H and I) were shown. G and I were the magnified images of selected areas from F and H, respectively. (J-O) Adenine-induced ferroptosis in the kidney of rats. (J) The iron content of the kidney tissue was measured in each group. (K) The level of lipid peroxide MDA in each group. (L) The level of H$_2$O$_2$ in each group. (M) The GSH level in each group. (N) The activity of SOD in each group. (O) The activity of CAT in each group. All data are mean ± SD of at least three independent experiments (n=5 rats/group). (P) Western blotting of HO-1, FTN, FPN, GPX4, SLC7A11 and TMEM16F protein expression of kidney tissue in control and uremic rats treated with or without 1% DMSO, 1 mg/kg Fer-1, 25 mg/kg DFO and 0.2 mg/kg AnV, respectively (n=5 rats/group). Data are expressed as the mean ± SD of at least three independent experiments. The relative density of protein bands was quantified and normalized to β-actin. MSB, Martius Scarlet Blue; HE, hematoxylin-eosin; MDA, malondialdehyde; GSH, glutathione; H$_2$O$_2$, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; Fer-1, Ferrostatin-1; DFO, deferoxamine; AnV, annexin V; HO-1, heme oxygenase-1; FTN, ferritin; FPN, ferroportin; GPX4, glutathione peroxidase 4.

Figure 6. Erythrophagocytosis induced ferroptosis contributed to hypercoagulability in uremic rats. IVC was ligated to obtain complete blood stasis in rats. After 6 hours of flow restriction, rats (n=4 per group) were euthanized and the IVC containing thrombus were harvested. (A) Representative images of thrombus from control and uremic rats treated with or without Fer-1, DFO and AnV. The weight (B), length (C) and weight/length ratio (D) of the thrombus in each group of rats were shown. (E) RBCs from control and uremic rats were stained with Annexin V-FITC and analyzed by flow cytometry. Data were expressed as the mean ± SD of four independent experiments. The entire hemostatic system was monitored by thromboelastography. An amount of 1mL of citrated whole blood was drawn [blood: citrate (sodium citrate 3.8%); 8:2] from each group of rats (n=4) via cardiac puncture and mixed with 40μL kaolin. 340μL citrated blood was mixed with 20μL CaCl$_2$ (0.2 M) for thromboelastography analysis. (F) Various representative thromboelastography traces shown a real-time and dynamic picture of coagulation in each group. Major
coagulation parameters were analyzed including R time (G), α angle (H), MA (I) and CI (J). Results were mean ± SD (n=4 rats/group). IVC, inferior vena cava; Fer-1, ferrostatin-1; DFO, deferoxamine; AnV, annexin V; R time, reaction time; α angle, alpha angle; MA, maximum amplitude; CI, clotting index.

**Figure 7. Schematic diagram showing that erythrophagocytosis triggered ferroptosis contributed to hypercoagulable state in uremia.** Phosphatidylserine externalized RBCs that were induced with uremic toxin IAA were recognized and engulfed by receptors or opsonins of EC. Following erythrophagocytosis, the ingested erythrocytes were digested in the phagolysosome, leading to the release of hemoglobin and eventually heme into the cytosol by HRG1, where it can be cleaved by HMOX-1 into amounts of iron. Subsequently, iron either was stored within FTN or was exported from the cell by FPN and transported through the circulation by transferrin. In response to enhanced erythrophagocytosis in uremic solution, ECs could respond homeostatically by increasing the expression of HMOX-1 and FTN, which allowed the cell to handle the increased heme and iron load. However, given the large number of RBCs that were rapidly ingested, HMOX-1 production was insufficient to prevent oxidative stress, thus iron overload triggered ferroptosis. In addition, SLC7A11 and GPX4 protein expression decreased in an unknown mechanism, leading to the accumulation of lipid peroxides, and inducing ferroptosis in ECs. Following ferroptosis, increased TMEM16F mediated phosphatidylserine externalized on the outer membrane of ECs, then initiated the coagulation cascade through providing binding sites for FXa and prothrombinase complexes, promoting thrombin and fibrin production. Together, erythrophagocytosis triggered ferroptosis representing a novel mechanism in uremia-associated thrombophilia. IAA, indole-3-acetic acid; HMOX-1, heme oxygenase-1; LIP, labile iron pool; FTN, ferritin; FPN, ferroportin; Glu, glutamate; Cys, cysteine; GSH, glutathione; GSSG, oxidized glutathione; GPX4, glutathione peroxidase 4; L-OOH, lipid peroxides; L-OH, lipid alcohols.
What is known about this topic?

- Uremia might be at high risk of cardiovascular and thrombotic events, however, its mechanism is largely unknown.
- ECs as amateur phagocytes have phagocytic properties for lactadherin-opsonized RBC.
- The role of ferroptosis in the occurrence and development of thrombosis remains largely unclear.

What does this paper add?

- Uremic RBC could trigger EC phagocytosis, ferroptosis and thrombogenesis, TMEM16F regulated PS exposure play a key role in this process.
- Iron and amino acid metabolism were involved in EP triggered ferroptosis, which may be promising targets to prevent thrombosis of uremia.
Supplemental Materials and Methods

Reagents

Human umbilical vein endothelial cells (HUVECs) and ECs medium were from ScienCell (SanDiego, CA, USA). Indole-3-acetic acid (IAA), deferoxamine (DFO), ferrostatin-1 (Fer-1), erastin and adenine were obtained from Sigma (Saint Quentin Fallavier, France). Cell Counting Kit-8 was purchased from GLPBIO (Montclair, CA, USA). Purified annexin V was purchased from BD Pharmingen (New Jersey, USA). Cell Tracker Green CMFDA was from Molecular Probes (Invitrogen, Eugene, OR, USA). Trypsin-EDTA, Annexin V-FITC, Hoechst 33342, a-tubulin mouse anti-human monoclonal antibodies, Alexa fluor 488-labeled goat anti mouse IgG and goat anti rabbit IgG, Cy3-labeled goat anti mouse IgG and goat anti rabbit IgG, BCA kit and RIPA lysis buffer were from Beyotime (Jiangsu, China). Malondialdehyde (MDA), glutathione (GSH), hydrogen peroxide (H₂O₂), catalase (CAT), superoxide dismutase (SOD), tissue iron content assay kit, Martius Scarlet Blue (MSB) stain kit and Ringer's solution were from Solarbio (Beijing, China). DCFH-DA, test kits of malondialdehyde (MDA), glutathione (GSH), hydrogen peroxide (H₂O₂), catalase (CAT), superoxide dismutase (SOD), tissue iron content assay kit, Martius Scarlet Blue (MSB) stain kit and Ringer's solution were from Invitrogen (Carlsbad, CA, USA). ECL detection reagent was from HaiGene (Harbin, Heilongjiang, China). Primary antibodies anti-ferritin (FTN), anti-ferroportin (FPN) and anti-heme oxygenase-1 (HO-1) were from Proteintech (Wuhan, Hubei, China). Anti-TMEM16F was from Abnova (Taipei, Taiwan, China). Secondary antibodies goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were from ZSGB-BIO (Beijing, China). Recombinant human MFG-E8 protein was obtained from R&D Systems (Minneapolis, MN). Human factors Va, VIIa, IXa, X, Xa, prothrombin and thrombin were all from Haematologic Technologies (Burlington, VT, USA). Recombinant human factor VIII was from American Diagnostica Inc (Stamford, CT, USA). Chromogenic substrates S-2765 and S-2238 were from DiaPharma Group (West Chester, Ohio, USA).

Animal experiments

All animal studies were approved by the Animal Care Committee of Harbin medical university and conformed to the international guidelines on the ethical use of animals. 60 male Sprague Dawley (SD) rats weighted 200 ± 20 g were from Changchun Yisi Biotechnology (Changchun, Jilin, China) and housed in a specific pathogen free room with a 12 hours light/dark cycle under the temperature of 25 ± 1°C with free access to food and water. After one-week adaptation, the rats were subjected for uremic model construction.
Chow was purchased from TrophicDiet (Nantong, China). Briefly, 8-week-old SD rats received regular chow (control group, n=10) or chow supplemented with 0.75% adenine (n=50). After 4-week uremic modeling, the rats were divided into five groups: rats only treated with adenine (uremia group, n=10), uremic rats treated with DMSO (uremia + DMSO, n=10), uremic rats treated with Fer-1 (uremia + Fer-1, n=10), uremic rats treated with DFO (uremia + DFO, n=10) and uremic rats treated with annexin V (uremia + AnV, n=10). 1 mg/kg of Fer-1 was injected intraperitoneally every day to inhibit ferroptosis. 25 mg/kg of DFO were given once every three days to chelating iron. To inhibit phagocytosis in uremia rats, rats were injected with 0.2 mg/kg of AnV into tail vein every day. The adenine-fed rats treated daily with 1% DMSO were considered as vehicle control. After 4-week treatment, rats were sacrificed, blood samples were collected to detect renal function, blood routine indexes and thromboelastography (TEG), respectively. The kidney tissues were harvested for further analysis.

**Cell preparation**

HUVECs were cultured in EGM2 medium under standard cell culture conditions (humidified atmosphere, 5% CO₂, 37°C) in 6-well culture plates. Peripheral venous blood was drawn from healthy volunteers by antecubital venipuncture and 3.2% sodium citrate was used as anticoagulant. They did not take any drugs affecting the coagulation system for at least two weeks and had no history of thrombosis. RBCs were prepared within 30 minutes after blood collection by centrifugation for 13 minutes at 200 g at room temperature. Then, isolated RBCs were cultured in Ringer solution with or without mean and maximal uremic concentration of IAA (20 and 50 μM) for 24 hours. After washing and removing of the residual toxin on RBC, the co-incubation experiments were performed. For the phagocytosis assay, on reaching 60% to 70% confluence, the ECs were overlaid and cocultured with RBCs (at an EC: RBC ratio of 1:40). At various time points (0, 6, 12 and 24 hours), the coculture cells were washed to remove non-adherent and non-phagocytosed RBCs, and then the ECs were detached by 800μL of 0.25% trypsin-EDTA solution for 2 minutes at 37°C and harvested for further analysis.

**Flow Cytometry**

To determine PS exposure on EC and RBC, each group of cells (1×10⁶/mL) was suspended in binding buffer and stained with 5μL of Annexin V- FITC for 30 minutes at room temperature in the dark. For the phagocytosis assay, the amount of phagocytosis was evaluated by measuring the percentage of RBCs containing ECs as previously reported. In brief, each group of RBCs was labeled with 1μM CMFDA at room temperature for 30 minutes in dark, and then co-incubated with ECs in presence or absence of lactadherin (Lact) or annexin V. At determined time points, the mixed cells were washed, detached, and any
surface-bound RBCs were removed. Then samples were detected by Cytek DxP Athena flow cytometry (Cytek, CA, USA). Ten thousand events per sample were acquired and analyzed with CYTEK FJCE Software. For detection of reactive oxygen species (ROS) generation and lipid peroxidation, each group of ECs was measured by staining with (10µM) DCFH-DA or C11-BODIPY (5µM) for 30 minutes at 37°C. To measure intracellular labile iron pool (LIP), each group of ECs was washed in PBS and then ECs was incubated with 0.05µM Calcein-AM in the dark for 20 minutes at room temperature. Subsequently, ECs were washed twice and then resuspended in 400µL PBS and detected by Cytek DxP Athena flow cytometry.

**Electron microscopy**

In scanning electron microscope (SEM) assays, ECs from above coincubation experiments were collected and fixed by immersion in 2.5% glutaraldehyde-phosphate fixative and stored at 4°C until processed. After several rinses in 0.1M Na-cacodylate HCl buffer, co-cultivations were postfixed in 1% OsO₄ and dehydrated in a graded series of ethanol (30%, 50%, 70%, 90%, and 100%; twice in 5 minutes). After critical drying, a layer of platinum, 10nm thick, was sprayed on the samples. All images were viewed with a S-3400N Scanning Electron Microscope (Hitachi Ltd, Yokohama, Japan) with an ultra-high- resolution mode.

In transmission electron microscope (TEM) experiments, the ECs were collected and double fixed in 2.5% glutaraldehyde and 1% OsO₄. After dehydration and embedding, ultrathin sections were prepared with Reichert Jung Ultracut Ultramicrotome (Leica, Wetzlar, Germany). Images were observed under a H7650 transmission electron microscope (Hitachi Ltd, Yokohama, Japan).

**Confocal microscopy**

To observe the PS exposure, RBCs were stained with Annexin V-FITC as previously described. To definitively determine the phagocytosis of RBCs by ECs, ECs grown on coverslips were washed in phosphate buffered saline (PBS) and fixed with prepared 4 % paraformaldehyde for 15 minutes, and then were permeabilized with 0.1% Triton X-100 in PBS for 30 minutes. After three washes, ECs were blocked in the 1% bovine serum albumin (BSA) phosphate buffered saline for 1 hours, incubated with the primary antibodies (tubulin mouse anti-human monoclonal antibody and CD235a rabbit anti-human monoclonal antibody) for 12 hours at 4°C, then secondary IgG (Alexa fluor 488-labeled goat anti-mouse IgG, Cy3-labeled goat anti-rabbit IgG) for 2 hours at room temperature, and Hoechst for 30 minutes. Immunofluorescent staining was also applied to visually identify the TMEM16F expression. Briefly, ECs were incubated with primary antibodies (a-tubulin mouse anti-human monoclonal antibody and rabbit polyclonal anti-TMEM16F) for 12 hours at 4 °C, and then incubated with secondary antibodies (Cy3-labeled goat anti mouse IgG, Alexa fluor 488-labeled goat anti rabbit IgG) for 2 hours at room temperature. The
nucleus was stained with Hoechst 33342. Antibody dilutions were as follows: tubulin, 1:100; goat anti-
mouse or anti-rabbit Alexa 488 or Cy3, 1:200; CD235a and TMEM16F antibody, 1:500. Hoechst was used at 1:3000.

**Cell viability assay**

4000 ECs/well were seeded in 96-well plates and incubated for 24 hours. Then the ECs were overlaid and
cocultured with RBCs for 12 hours in the presence or absence of Fer-1 or DFO. After washing and removing of the floating and any surface-bound RBCs, 100μL fresh medium containing 10μL Cell Counting Kit-8 (CCK-8) reagent was added to each well and incubated for 4 hours at 37°C. The absorbance of each well was detected at 450 nm by using SpectraMax 190 plate reader (Molecular Devices, CA, USA).

**HE, MSB and DAB-enhanced Prussian blue staining staining**

Hematoxylin and eosin (HE) staining was conducted as follows. Briefly, after deparaffinization and
rehydration, 5μm thin sections of kidney were stained with hematoxylin solution for 5 minutes followed by
5 dips in 1% acid ethanol (1% HCl in 70% ethanol) and then rinsed in distilled water. Then the kidney
sections were stained with eosin solution for 3 minutes and followed by dehydration with graded alcohol and
clearing in xylene. Photomicrographs were obtained using LEICA Versa (LEICA, Wetzlar, Germany). The
diameter and area of glomerulus were analyzed by ImageJ software.

In order to identify the microthrombus formation of kidney, paraffin sections of kidney tissue were
stained in accordance with the manufacturer’s recommended procedure of Martius Scarlet Blue (MSB) stain
kit and observed in LEICA Versa. For iron histochemistry, kidney sections were stained with DAB-
enhanced Prussian blue according to the manufacturer’s recommended procedure and observed in LEICA
Versa.

**Western blot analysis**

Cell or tissue lysates extracted from fresh kidney tissue were collected at the indicated times in RIPA lysis
buffer with protease inhibitor. Protein levels were quantified with a BCA kit according to the manufacturer’s
instructions. Samples were run on 8%, 10% or 12% polyacrylamide gels and transferred onto nitrocellulose
membranes. The membranes were blocked with 5% skim milk powder at room temperature for 2 hours and
then were incubated overnight at 4°C with primary antibodies. Subsequently, membranes were incubated
with secondary goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP at room temperature for 1 hour.
Protein bands were visualized with ECL detection reagent and images finally were recorded by UVP Gel
StudioTM PLUS Imager (Analytik Jena AG, Jena, Germany) and quantified using ImageJ software.
Procoagulant activity assay

Procoagulant activity (PCA) was determined by a one-stage recalcification time assay in a Start 4 coagulometer (Diagnostica stago S.A.S, France) as described previously. 100μL of the ECs (10^3) suspension was mixed 1:1 with citrated platelet-poor plasma from healthy volunteers. After 180 seconds, 100μL of warmed 25mM CaCl₂ was added to start the reaction and clotting time was recorded. For the inhibition assay of coagulation time, 100μL of ECs (10^3) were preincubated with 50μL lactadherin (final concentration 128nM) for 10 minutes at 37°C. Clotting time was then recorded as above after addition of 100μL platelet-poor plasma and 50μL of warmed 50mM CaCl₂.

FXa and prothrombinase assays

The activation of intrinsic FXa in the presence of cells was performed as follows. ECs were incubated with 1nM factor IXa, 5nM factor VIII, 0.2nM thrombin, 130nM factor X, and 5mM CaCl₂ in FXa buffer (200μL of 10% BSA, 1mL 10×TBS, and 8.8mL of ddH₂O). The reaction was stopped by EDTA at a final concentration of 7mM. FXa generation was determined immediately at 405 nm in kinetic mode on a SpectraMax 190 plate reader after incubation with 10μL of S-2765 (0.8 mM). Measurement of extrinsic FXa formation was similar to that for intrinsic FXa except that cells were mixed with 1nM factor VIIa, 130nM factor X, and 5mM CaCl₂. For the prothrombinase assay, the samples were incubated with 1nM factor Va, 0.05nM FXa, 1µM prothrombin, and 5mM CaCl₂ in prothrombinase buffer (1mL 10×TBS, 50µL 10% BSA, 8.95mL ddH₂O) for 5 minutes at room temperature. Thrombin production was evaluated immediately at 405 nm in the kinetic microplate reader with S-2238 (0.8 mM) after the addition of EDTA. For the inhibition assay of FXa and prothrombinase production, ECs were preincubated with 50μL lactadherin (final concentration 128nM) for 10 minutes at 37°C. FXa and prothrombinase production was then performed as above.

MDA, GSH, CAT, H₂O₂, SOD and iron quantification assays

To determine the activity and level of various oxidative stress markers, including MDA, GSH, CAT, H₂O₂ and SOD, we used detection kits from Solarbio according to the manufacturer’s instructions. 10% kidney tissue homogenates in rats from control and uremic groups were centrifuged (4°C) for 10 minutes at 8000 g and the supernatants were collected. Iron content in kidney tissues was detected following the manufacturer’s protocols of tissue iron content assay kit and determined at 405 nm. The content of MDA was evaluated by using a thiobarbituric acid reaction method and measured at 450, 532 and 600 nm, respectively. The levels of MDA calculated as: the content of MDA[μmol/g]=5×(ΔA532-ΔA600-2.58×ΔA450)/0.1. The contents of H₂O₂, GSH and the activity of SOD were determined at 415 nm, 412 nm
and 560 nm respectively. For CAT, the absorbance was measured at 240 nm and the activity of CAT were calculated by the following formula: the activity of CAT (U/g) = \(459 \times \Delta A_{240} \div 0.1\). The absorbance was measured by using a SpectraMax 190 plate reader.

**Rat model of flow restriction in the IVC**

IVC ligation model were performed to induce the formation of deep vein thrombus (DVT) as described previously.\(^{24}\) In brief, rats were anesthetized with 10% pentobarbital sodium and a midline laparotomy was performed. Intestines were exteriorized and sterile saline was used to prevent drying after laparotomy during the whole procedure. Then, gentle separation from aorta, the infrarenal IVC and all side branches were immediately ligated with a 4.0 polypropylene suture. For the sham experiments, rats underwent the same surgical procedures except for IVC ligation (sham ligation). After surgery, peritoneum and skin were closed by monofilament absorbable suture and 4.0 silk, respectively. Rats were euthanized after 6 hours, and the vessel was excised just below the renal veins and proximal to the confluence of the common iliac veins to analyze the thrombus weight and length.

**Thromboelastography (TEG) testing**

1mL of blood was drawn into 3.8% sodium citrate tube from rats by cardiac puncture and mixed with 40μL kaolin. TEG analysis was performed according to Harmanpreet et al\(^{25}\), in brief, 340μL blood samples were placed into the prepared TEG cups with 20μL CaCl\(_2\) (0.2M), and TEG analysis using a Haemoscope TEG 5000 Thrombelastograph Hemostasis Analyzer (Haemonetics Corp., Braintree, Massachusetts, USA) started immediately according to the manufacturer’s instructions. The TEG run was stopped after all relevant parameters were assessed. Major coagulation parameters were analyzed including reaction time (R time), alpha angle (α angle), maximum amplitude (MA) and the clotting index (CI).

**Supplemental Figure Legends**

**Figure S1. Detection of PS exposure on IAA treated RBCs.** RBCs from healthy volunteers were cultured with median (20μM) and maximal (50μM) uremic concentration of IAA for 24 hours. RBCs cultured in Ringer solution with ethanol was utilized as control. (A) RBCs were collected and stained with Annexin V-FITC for 30 minutes in the dark before flow cytometric analysis. Results represent the mean ± SD of at least three independent experiments. Confocal microscopy images showing several RBCs cultured with 50μM IAA displayed strong green fluorescence (C), while only few Annexin V-FITC staining was observed on RBCs cultured in Ringer solution with ethanol (B). IAA, indole-3-acetic acid.

**Figure S2. Successful construction of uremic rat model.** Histopathological changes of control rats and adenine-fed rats for four weeks were evaluated by HE staining. Representative images of kidney sections
from normal rats (A) and adenine-fed rats accompanying with glomerular atrophy, tubulointerstitial fibrosis (star), interstitial inflammatory infiltrates (triangle), dilation of the renal tubules (circular) and vacuolation of renal tubule epithelial cells (arrow) (B). The number (C), diameter (D) and surface area (E) of glomeruli were determined from histology sections (mean ± SD, n=6 rats/group) and analyzed by Image J. And the ratio of kidney weight to body weight (F) in rats was measured (mean±SD, n=6 rats/group).
Table 1. Baseline characteristics of control and adenine-treated rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Adenine</th>
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<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>356.4±20.57</td>
<td>195.73±30.52***</td>
</tr>
<tr>
<td><strong>Urea (mmol/L)</strong></td>
<td>7.31±1.54</td>
<td>45.55±12.78***</td>
</tr>
<tr>
<td><strong>Creatinine (μmol/L)</strong></td>
<td>51.6±9.1</td>
<td>269.2±70.01***</td>
</tr>
<tr>
<td><strong>UA (μmol/L)</strong></td>
<td>112.6±38.27</td>
<td>229.8±120.13**</td>
</tr>
<tr>
<td><strong>Phosphorus (mmol/L)</strong></td>
<td>1.83±0.46</td>
<td>5.87±1.17***</td>
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<tr>
<td><strong>Magnesium (mmol/L)</strong></td>
<td>1.05±0.21</td>
<td>1.24±0.33</td>
</tr>
<tr>
<td><strong>Cholesterol (mmol/L)</strong></td>
<td>1.33±0.38</td>
<td>2.15±0.56*</td>
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<tr>
<td><strong>Homocysteine (μmol/L)</strong></td>
<td>13.36±1.86</td>
<td>5.77±1.92***</td>
</tr>
<tr>
<td><strong>RBC (10^{12}/L)</strong></td>
<td>7.97±0.33</td>
<td>6.05±0.49***</td>
</tr>
<tr>
<td><strong>Haemoglobin (g/L)</strong></td>
<td>139.2±5.45</td>
<td>102.8±6.5***</td>
</tr>
<tr>
<td><strong>Haematocrit (%)</strong></td>
<td>37.36±1.59</td>
<td>27.54±1.64***</td>
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<tr>
<td><strong>MCV (fl)</strong></td>
<td>46.9±1.82</td>
<td>45.6±1.42</td>
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<tr>
<td><strong>MCH (pg)</strong></td>
<td>17.46±0.53</td>
<td>17.06±0.75</td>
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<tr>
<td><strong>MCHC (g/L)</strong></td>
<td>372.8±6.38</td>
<td>374±8.06</td>
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<tr>
<td><strong>MPV (fl)</strong></td>
<td>6.48±0.4</td>
<td>5.54±0.25</td>
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<tr>
<td><strong>RDW-CV (%)</strong></td>
<td>13.78±0.54</td>
<td>16.18±1.4**</td>
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<td><strong>RDW-SD (fl)</strong></td>
<td>27.04±0.25</td>
<td>31.54±2.98**</td>
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<td><strong>WBC (10^{9}/L)</strong></td>
<td>4.35±1.75</td>
<td>4.15±3.33</td>
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<td><strong>Neutrophils (10^{9}/L)</strong></td>
<td>0.82±0.29</td>
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<td><strong>Lymphocytes (10^{9}/L)</strong></td>
<td>3.53±1.94</td>
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<td><strong>Basophiles (10^{9}/L)</strong></td>
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<td><strong>Platelets (10^{9}/L)</strong></td>
<td>587.4±114.76</td>
<td>473.4±68.44</td>
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<td><strong>PDW (%)</strong></td>
<td>15.34±0.11</td>
<td>14.86±0.15</td>
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<td><strong>Plateletcrit (%)</strong></td>
<td>0.38±0.08</td>
<td>0.26±0.04</td>
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Abbreviations: UA, uric acid; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MPV, mean platelet volume; RDW, red blood cell distribution width; RDW-CV, red blood cell distribution width-coefficient of variation; RDW-SD, red blood cell distribution width-standard deviation; WBC, white blood cell; PDW, platelets distribution width. Data are presented as mean ± SD. N=5 rats in each group. *P<0.05; **P<0.01; ***P<0.001.
Figure 1

Figure 2

F  G  H

E  Relative Flammng scores

Cell viability

DCF

C1 Bodipy

Table:

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<td>Viability</td>
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<td>C1 Bodipy</td>
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Statistical significance:

- P < 0.01
- P < 0.001
- NS
**Visual summary.** PS externalized RBCs that induced with uremic toxin IAA were recognized and engulfed by receptors or opsonins of EC. Following EP, the ingested erythrocytes were digested in the phagolysosome, leading to the release of hemoglobin and eventually heme into the cytosol by HRG1, where it can be cleaved by HMOX-1 into amounts of iron. Subsequently, iron either was stored within FTN or was exported from the cell by FPN and transported through the circulation by transferrin. In response to enhanced EP in uremic solution, ECs could respond homeostatically by increasing the expression of HMOX-1 and FTN, which allowed the cell to handle the increased heme and iron load. However, given the large number of RBCs that were rapidly ingested, HMOX-1 production was insufficient to prevent oxidative stress, thus iron overload triggered ferroptosis. In addition, SLC7A11 and GPX4 protein expression decreased in an unknown mechanism, leading to the accumulation of lipid peroxides and inducing ferroptosis in ECs. Following ferroptosis, increased TMEM16F mediated PS externalized on the outer membrane of ECs, then initiated the coagulation cascade through providing binding sites for FXa and prothrombinase complexes, promoted thrombin and fibrin production. Together, EP triggered ferroptosis representing a novel mechanism in uremia associated thrombophilia. IAA, indole-3-acetic acid; EP, erythropagocytosis; HMOX-1, heme oxygenase-1; LIP, labile iron pool; FTN, ferritin; FPN, ferroportin; Glu, glutamate; Cys, cysteine; GSH, glutathione; GSSG, oxidized glutathione; GPX4, glutathione peroxidase 4; L-OOH, lipid peroxides; L-OH, lipid alcohols.