Antithrombin protects against Plasmodium falciparum histidine-rich protein II-mediated inflammation and coagulation

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Abstract:
Plasmodium falciparum (Pf)-derived histidine-rich protein II (HRPII) has been shown to inhibit heparin-dependent anticoagulant activity of antithrombin (AT) and induce inflammation in vitro and in vivo. In a recent study, we showed that HRPII interacts with the AT-binding vascular glycosaminoglycans (GAGs) to not only disrupt the barrier-permeability function of endothelial cells but also inhibit the anti-inflammatory signaling function of AT. Here we investigated the mechanisms of the pro-inflammatory function of HRPII and the protective activity of AT in cellular and animal models. We found that AT competitively inhibits the GAG-dependent HRPII-mediated activation of NF-κB and expression of intercellular cell adhesion molecule 1 (ICAM1) in endothelial cells. Furthermore, AT inhibits HRPII-mediated histone H3 citrullination and neutrophil extracellular trap (NET) formation in HL60 cells and freshly isolated human neutrophils. In vivo, HRPII induced Mac1 expression on blood neutrophils, MPO release in plasma, neutrophil infiltration and histone H3 citrullination in the lung tissues. HRPII also induced endothelial cell activation as measured by increased ICAM1 expression and elevated vascular permeability in the lungs. AT effectively inhibited HRPII-mediated neutrophil infiltration, NET formation and endothelial cell activation in vivo. AT also inhibited HRPII-mediated deposition of platelets and fibrin(ogen) in the lungs and circulating level of von Willebrand factor in the plasma. We conclude that AT exerts protective effects against pathogenic effects of Pf-derived HRPII in both cellular and animal models.

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Running title: Protective effect of AT against HRPII

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Key Points
1. HRPII binds vascular GAGs and activates coagulation and inflammation
2. AT exerts protective effects against HRPII in cellular and animal models

Abstract

Plasmodium falciparum (Pf)-derived histidine-rich protein II (HRPII) has been shown to inhibit heparin-dependent anticoagulant activity of antithrombin (AT) and induce inflammation in vitro and in vivo. In a recent study, we showed that HRPII interacts with the AT-binding vascular glycosaminoglycans (GAGs) to not only disrupt the barrier-permeability function of endothelial cells but also inhibit the anti-inflammatory signaling function of AT. Here we investigated the mechanisms of the pro-inflammatory function of HRPII and the protective activity of AT in cellular and animal models. We found that AT competitively inhibits the GAG-dependent HRPII-mediated activation of NF-κB and expression of intercellular cell adhesion molecule 1 (ICAM1) in endothelial cells. Furthermore, AT inhibits HRPII-mediated histone H3 citrullination and neutrophil extracellular trap (NET) formation in HL60 cells and freshly isolated human neutrophils. In vivo, HRPII induced Mac1 expression on blood neutrophils, MPO release in plasma, neutrophil infiltration and histone H3 citrullination in the lung tissues. HRPII also induced endothelial cell activation as measured by increased ICAM1 expression and elevated vascular permeability in the lungs. AT effectively inhibited HRPII-mediated neutrophil infiltration, NET formation and endothelial cell activation in vivo. AT also inhibited HRPII-mediated deposition of platelets and fibrin(ogen) in the lungs and circulating level of von Willebrand factor in the plasma. We conclude that AT exerts protective effects against pathogenic effects of Pf-derived HRPII in both cellular and animal models.

Keywords: Plasmodium falciparum, HRPII, antithrombin, glycosaminoglycans,
neutrophil extracellular trap
**Introduction**

Malaria is a serious infectious disease, afflicting nearly 250 million people annually with ~1 million fatalities (1-3). Among five different types of plasmodium species causing malaria, infection by Plasmodium falciparum (Pf) is the deadliest, leading to severe malaria that is responsible for most of malaria related fatalities. Infection by Pf can lead to numerous serious complications including thrombocytopenia (4), coagulopathy (5), cerebrovascular stroke (6), severe anemia (7), acute renal failure (8), and acute respiratory distress syndrome (9). Pathophysiology of severe malaria is multifactorial and remains obscure; however, increasing evidence suggests Pf-mediated endothelial cell-leukocyte interaction induces vascular dysfunction leading to activation of coagulation and inflammation (1,2,8). The course of infection begins by the parasite entering the circulation, invading host red blood cells and undergoing an asexual growth cycle during which the parasite expresses numerous membrane and secretory proteins as an essential process for maintaining viability and virulence (1). A well-studied Pf-derived membrane protein is called Pf Erythrocyte Membrane Protein 1 (PfEMP1), which has been demonstrated to downregulate the protein C anticoagulant pathway by binding to endothelial protein C receptor (EPCR), thereby downregulating activation of protein C and its anticoagulant and anti-inflammatory functions (10,11). A Pf-derived secretory protein that has been shown to also promote activation of pro-inflammatory and procoagulant pathways is histidine-rich protein II (HRPII) (1,12,13). It has been demonstrated HRPII binds anticoagulant glycosaminoglycans (GAGs), thereby promoting coagulation pathway by inhibiting the heparan sulfate-dependent anticoagulant function of antithrombin (AT) (14). Moreover, it has been found HRPII through activation of inflammasome compromises integrity of tight junctions and
endothelial cell permeability function, suggesting it may play a key role in pathogenesis of cerebral malaria by disrupting the blood-brain barrier (13).

In a recent study, we demonstrated HRPII inhibits anti-inflammatory function of AT by competitively binding to specific AT-binding 3-O-sulfate (3-OS) containing GAGs that are involved in transmitting D-helix-dependent protective signaling function of the serpin (15). Through interaction with vascular GAGs, AT initiates anti-inflammatory signaling responses by inducing prostacyclin synthesis (16), and HRPII effectively blocks this protective function of AT in endothelial cells. HRPII also inhibited barrier protective function of AT by inducing Src-dependent phosphorylation of VE-cadherin, thereby leading to disruption of adherens junction in endothelial cells (15). Here, we investigated molecular mechanisms through which HRPII promotes inflammation and coagulation in cellular and animal models and evaluated the mechanism of protective effects of AT in these systems. Results indicate the GAG-dependent binding of HRPII to endothelial cells and leukocytes induces NF-κB activation, culminating in cell surface expression of cell adhesion molecules. Furthermore, HRPII induces neutrophil extracellular trap (NET) formation in vitro that is inhibited by AT. In an in vivo system, HRPII increased Mac1 expression on blood neutrophils, enhanced their infiltration to lung tissues and promoted NET formation. HRPII also promoted activation of coagulation and increased platelet and fibrin(ogen) deposition in lungs and von Willebrand factor (VWF) secretion in the plasma. AT and an AT mutant with higher affinity for GAGs (17), but not a signaling-defective AT mutant (18), downregulated activation of inflammation and coagulation by HRPII. These results suggest AT may have therapeutic value against pathogenic effects of HRPII in patients infected with Pf.
Materials and methods

Reagents

Human plasma-derived antithrombin (AT) was purchased from Enzyme Research Laboratories (South Bend, IN, USA). Recombinant AT derivatives AT-N135Q and AT-4Mut were prepared as described (17-19). Recombinant HRPII was expressed and purified to homogeneity as described (15). The complete list of reagents is presented as Supplementary Materials.

Neutrophil isolation and extracellular trap analysis

Blood was collected from healthy adult volunteers (following an approved institutional review board protocol) in acid-citrate-dextrose buffer. Blood neutrophils were isolated according to the Polymorphprep™ (Cosmo Bio USA Cat. # AXS-1114683) protocol. Isolated neutrophils were stimulated with HRPII (100nM) in the absence or presence of AT (2.5µM) or its variants. After 4h of incubation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% TritonX-100/PBS and processed for staining. Percent of NET forming cells were calculated based on total number of cells per field as described (20).

Flow cytometry

Endothelial cells were treated with HRPII in the presence and absence of AT. Surface expression of ICAM1 was detected by flow cytometry using FITC-conjugated anti-ICAM1 antibody as described (21). Surface expression of Mac1 on LY6G-positive mouse blood neutrophils was determined as described (22).

Histological examination and immunofluorescence
C57BL/6J mice (8-12-weeks old) were injected intraperitoneally (i.p.) with either saline or HRPII (10µg/g bodyweight) with or without prior injection of AT (500µg/mouse, 20µg/gm bodyweight). Mice were sacrificed and perfused with PBS, and organs collected for histopathological analysis.

**Vascular permeability in the lung and kidney**

Mice were injected with saline or HRPII in the presence or absence of AT. After 3h, 150 µL of 1% Evans blue dye in normal saline was injected intravenously (i.v.). After 30min, mice were sacrificed, perfused with PBS and organs were collected and vascular permeability in the lung and kidney determined as described (23).

Details of all methods including Western-blotting for monitoring expression of signaling molecules and ELISA assays for determining MPO, thrombin-AT (TAT) complex and VWF levels are described in Supplementary Materials.

**Statistical analysis**

Data are presented as mean ± standard error of mean (SEM) from ≥3 independent experiments. Data were analyzed by the Student t-test, and group data were analyzed using ANOVA followed by Bonferroni post hoc test using Graph Pad Prism7 (Graph Pad Prism, CA). A p value of <0.05 was considered statistically significant.
Results

**AT inhibits GAG-dependent HRPII-mediated NF-κB activation in endothelial cells**

HRPII has been shown to inhibit the anti-inflammatory function of AT by competitive inhibition of AT binding to endothelial cell GAGs (15). In this study, we explored mechanisms of the pro-inflammatory signaling function of HRPII and the protective effect of AT using endothelial cells (hTERT-HUVECs). Western-blot analysis of lysates of endothelial cells, treated simultaneously with both HRPII and AT, showed HRPII increases phosphorylation of NF-κB p65 and this effect was counteracted by AT (Fig. 1A). Immunofluorescence data supported these findings and showed AT inhibits HRPII-mediated nuclear translocation of NF-κB p65 (Fig. 1B). Inhibitory effect of AT was mediated through D-helix-dependent interaction of AT with cellular GAGs since the AT D-helix mutant (AT-4Mut), which has normal protease inhibitory function but is incapable of binding GAGs (18,19), exhibited no protective activity against HRPII (Fig. 1B). In agreement with these results, flow cytometry data, employing FITC-labeled ICAM1, indicated AT, but not AT-4Mut, inhibits HRPII-mediated upregulation of ICAM1 expression on endothelial cells (Fig. 1C). The pro-inflammatory function of HRPII was mediated through interaction with cellular GAGs as evidenced by the GAG-antagonist, surfen, blocking binding of FITC-labeled-HRPII to endothelial cells (Fig. 1D). Immunofluorescence data also showed surfen blocks HRPII-mediated nuclear translocation of NF-κB p65 (Fig. 1E). We previously demonstrated HRPII has a potent barrier-disruptive effect in endothelial cells (15). Surfen also inhibited barrier-disruptive effect of HRPII (Fig. 1F). We have demonstrated that surfen can also inhibit the cytoprotective activity of AT in an in vivo model. (17). In agreement with HRPII and AT
interacting with overlapping binding sites on GAGs, AT-N135Q (17), which similar to β-isoform of AT (AT-β) binds GAGs with 5-10-fold higher affinity (24,25), exhibited significantly better competitive effect in blocking pro-inflammatory function of HRPII in cell permeability assay. This conclusion is based on the analysis of protective effects of increasing concentrations of AT-WT and AT-N135Q on barrier-disruptive function of HRPII in endothelial cells (Fig. 1G). Similar results were obtained in human pulmonary microvascular endothelial cells (HPMECs). Results showed both AT and surfen inhibit HRPII-mediated NF-κB p65 nuclear translocation and permeability in HPMECs (Suppl. Fig. S1A,S1B).

**AT inhibits HRPII-mediated inflammatory responses in neutrophils**

Similar to endothelial cells, HRPII induced activation of NF-κB in DMSO-differentiated HL60 (dHL60) cells, which are known to exhibit neutrophil-like properties (26,27). Time-course analysis by Western-blotting of cell lysates indicated HRPII induces phosphorylation of NF-κB p65 in dHL60 cells after 5min (the first time-point analyzed) (Fig. 2A). Simultaneous treatment with both HRPII and AT led to inhibition of NF-κB p65 phosphorylation in dHL60 cells (Fig. 2B). Immunofluorescence analysis indicated HRPII induces extracellular trap formation and histone H3 citrullination (Cit-H3) in dHL60 cells, and AT-WT and AT-N135Q effectively inhibited these pro-inflammatory responses (Fig. 2C). As expected, AT-4Mut exhibited no protective effect in this assay (Fig. 2C). Western-blot analysis of dHL60 cell lysates confirmed immunofluorescence data, showing the Cit-H3 level is significantly elevated in HRPII-treated cells (Fig. 2D). Pro-inflammatory cytokines PMA and TNFα, which are known to induce Cit-H3, were used as positive controls in these experiments (Fig. 2D). AT
effectively inhibited HRPII-mediated elevation of Cit-H3 in dHL60 cells (Fig. 2E). AT-N135Q exhibited a higher inhibitory effect, but AT-4Mut failed to block histone H3 citrullination by HRPII in dHL60 cells (Fig. 2E).

Neutrophil activation and NET formation have been shown to be involved in pathogenesis of inflammation in Pf-infected patients (28,29). However, the underlying cause of NET formation in Pf infection is not known. We investigated the possible role of HRPII in NET formation using freshly isolated human neutrophils. Results indicated HRPII induces degranulation of neutrophils, release and colocalization of myeloperoxidase (MPO) with extracellular DNA as determined by co-staining for both MPO and Sytox Green (Suppl. Fig. S2A). Formation of long extracellular mesh-like structures of DNA associated with Cit-H3, suggested HRPII induces NET formation and AT effectively inhibits this response (Suppl. Fig. S2B). It has been demonstrated Cit-H3 plays a key role in mediating NET formation (30), a process in which activated neutrophils release their nuclear contents as de-condensed chromatin fibers comprised of DNA, histones, and granular contents to neutralize invading microorganisms (31). Immunofluorescence analysis provided further support for the hypothesis that HRPII induces NET formation by appearance of fiber-like structures co-stained for both neutrophil elastase and Cit-H3 (Fig. 3A). Histone citrullination is catalyzed by nuclear peptidyl arginine deiminase 4 (PAD4), which facilitates de-condensation of chromatin and formation of NETs by innate immune cells (32,33). Confocal immunofluorescence analysis of neutrophils, co-stained for PAD4/H3/DAPI, indicated HRPII induces PAD4 nuclear translocation, thereby enhancing citrullination of histones in neutrophils (Fig. 3B), providing further support for the hypothesis that HRPII activates neutrophils to
induce NETs. HRPII-mediated PAD4 nuclear translocation was further confirmed by Western-blot analysis of nuclear extract of neutrophils (Fig. 3C). In addition to NET release, HRPII also induced secretion of TNFα, which was also inhibited by AT (Fig. 3D). Taken together, these results suggest AT effectively inhibits HRPII-mediated NET formation and blocks the release of pro-inflammatory markers in activated neutrophils.

**AT attenuates HRPII-mediated inflammation in vivo**

The pro-inflammatory effect of HRPII was evaluated in an in vivo model by intraperitoneal administration of HRPII in mice. HRPII increased infiltration of MPO positive neutrophils to lungs, which was significantly attenuated by AT (Fig. 4A). Immunofluorescence analysis of lung tissues suggested number of resident macrophages in lungs (F4/80-positive) does not change (Suppl. Fig. S3A), suggesting HRPII-mediated infiltrated leukocytes are primarily neutrophils. Ly6G immunostaining provided further support for this hypothesis (Suppl. Fig. S3B). Analysis of kidney tissues by Ly6G immunostaining yielded similar results, suggesting HRPII also induces neutrophil infiltration to kidneys (Suppl. Fig. S3C). AT effectively attenuated HRPII-mediated neutrophil infiltration to both lungs and kidneys (Suppl. Fig. S3B,S3C). Flow cytometry analysis showed HRPII increases surface expression of Mac1 on blood neutrophils (Fig. 4B). HRPII also increased mouse plasma MPO as determined by an antibody dependent MPO activity assay, and AT significantly suppressed this effect (Fig. 4C). Immunofluorescence analysis for Cit-H3 in lung tissues of HRPII-treated mice revealed infiltrating neutrophils were forming NETs, indicating HRPII is inducing NETs in vivo, and this effect was inhibited by AT (Fig. 4D). Western-blot analysis of lung tissue lysates showed AT significantly reduces HRPII-mediated elevation of Cit-H3 and MPO
These results suggest HRPII activates neutrophils in vivo to promote inflammation, which is attenuated by AT. Western blot analysis further indicated AT attenuates HRPII-mediated endothelial activation as measured by expression of CAMs. Expression of ICAM1 and VCAM1 was upregulated in lung tissues of mice treated with HRPII, but it was downregulated in mice treated with both HRPII and AT as determined by Western blot (Fig. 5A) and immunofluorescence analyses (Suppl. Fig. S4A, S4B), suggesting HRPII activates lung endothelial cells, thereby promoting infiltration of activated neutrophils in lung tissues as demonstrated in Fig. 4A. In agreement with this observation, HRPII also disrupted barrier-permeability function of lung endothelial cells and AT inhibited this response (Fig. 5B).

**AT attenuates HRPII-mediated activation of coagulation**

Analysis of mice blood samples indicated HRPII increases circulating plasma levels of VWF and AT effectively inhibits this response (Fig. 5C). HRPII also induced thrombocytopenia as evidenced by lower number of platelets in circulation, which was inhibited by AT (Fig. 5D). Immunofluorescence analysis showed markedly high levels of platelets (CD41-positive cells) in both lung (Fig. 5E) and kidney (Suppl. S5A) indicating increased platelet deposition in both tissues of HRPII-treated mice. These results suggest HRPII induced VWF release, thrombocytopenia and platelet deposition, which were all effectively inhibited by AT.

The coagulation activation marker, thrombin-antithrombin (TAT) complex, was also markedly increased in the plasma of HRPII-treated mice (Fig. 6A), suggesting HRPII promotes activation of coagulation and AT inhibits this procoagulant state (Fig. 6A). This conclusion was further confirmed by the analysis of fibrin(ogen) deposition in
lung tissues. Western-blot and immunofluorescence studies showed a markedly elevated level of fibrin(ogen) in lung tissues which was significantly inhibited by AT (Fig. 6B-D). H&E staining supported this conclusion as evidenced by appearance of thrombus in lung tissues of HRPII-treated mice, which was again effectively inhibited by AT (Fig. 6E). In addition to lungs, HRPII also increased fibrin(ogen) deposition and vascular leakage in the kidney, which were attenuated by AT (Suppl. Fig. S5B-D). Taken together, increased VWF and TAT levels in blood samples as well as increased platelet and fibrin(ogen) deposition in lung and kidney suggest HRPII can promote activation of coagulation and thrombosis which are attenuated by AT.

Role of neutrophils on HRPII-mediated activation of inflammation and coagulation

To delineate the role of neutrophils in HRPII-mediated activation of inflammation and coagulation, mice were treated with anti-Ly6G antibody to deplete circulating neutrophils. Pro-inflammatory effects of HRPII were measured in neutrophil-depleted mice (Suppl. Fig. S6). Western-blot analysis showed HRPII-mediated upregulation of ICAM1 and VCAM1 were reduced in neutrophil-depleted mice (Fig. 7A-C). These results indicate HRPII-mediated neutrophil activation and release of pro-inflammatory cytokines play an important role in inducing endothelial dysfunction. The MPO level in the lung was also reduced in anti-Ly6G antibody-treated mice (Fig. 7A,D); however, fibrin(ogen) deposition was not changed, suggesting circulating neutrophils may not play a significant role in HRPII-mediated coagulation activation (Fig. 7A,E). In support of this hypothesis, plasma levels of TAT-complex were also unchanged following neutrophil depletion (Fig. 7F). The VWF level in plasma, which was significantly upregulated following HRPII treatment, was reduced following neutrophil depletion (Fig.
Circulating platelets are known to bind VWF strings attached to activated endothelial cells. Neutrophil depletion rescued HRPII-mediated thrombocytopenia, supporting a role for neutrophils in activation of endothelial cells and their interaction with platelets (Fig. 7H). HRPII-mediated vascular permeability was also significantly decreased in neutrophil-depleted mice (Fig. 7I). Taken together, these results suggest neutrophils play an important role in promoting HRPII-mediated pro-inflammatory signaling but may have minimal direct role in activation of coagulation.
Discussion

We recently demonstrated HRPII binds vascular GAGs to disrupt barrier-permeability function of endothelial cells through Src-dependent phosphorylation and destabilization of VE-cadherin (15). Results here indicate HRPII by interacting with GAGs induces phosphorylation and nuclear translocation of NF-κB p65 and upregulation of ICAM1 in endothelial cells. The essential role of GAGs in pro-inflammatory function of HRPII can be gleaned from observations that, surfen 1) inhibited binding of HRPII to endothelial cells as analyzed by flow cytometry, 2) blocked nuclear translocation of NF-κB p65 as determined by immunofluorescence, and 3) inhibited barrier-disruptive function of HRPII as analyzed by a permeability assay. HRPII and AT bind overlapping sites on endothelial GAGs since simultaneous treatment of cells with both HRPII and AT-WT, but not a signaling-defective D-helix mutant (AT-4Mut), effectively blocked both binding and pro-inflammatory function of HRPII. The higher affinity GAG-variant (AT-N135Q) exhibited significantly higher competitive effect in blocking barrier-disruptive function of HRPII, further supporting the hypothesis that both molecules interact with overlapping binding sites on GAGs to transmit their signaling effects. The molecular mechanism through which HRPII and AT elicit paradoxical signaling effects through interaction with same GAGs is not fully understood. We previously demonstrated the protective signaling function of AT requires D-helix-dependent interaction of AT with 3-O-sulfate (3-OS) containing GAGs (18,34,35), a modification primarily mediated by heparan sulfate 3-O-sulfotransferase-1 (3-OST-1) in endothelial cells (35). The siRNA knockdown of 3-OST-1 abrogated both barrier-protective function of AT and barrier-disruptive function of HRPII (15), further supporting the hypothesis that both molecules competitively bind similar and
overlapping GAG sequences to exert their signaling functions. We demonstrated interaction of AT with 3-OS containing GAGs on syndecan 4 (Synd-4) culminates in recruitment of PKC-δ to cytoplasmic membrane of endothelial cells, thereby leading to phosphorylation of Synd-4 cytoplasmic domain, induction of prostacyclin synthesis and inhibition of activation of NF-κB (21). However, unlike AT, interaction of HRPII with same type of GAGs resulted in activation of NF-κB by an unknown mechanism. We hypothesize upon interaction with GAGs, as a pathogen-associated molecular pattern (PAMP), HRPII is likely directed to molecular pattern recognition receptors (PRRs). A recent study demonstrated HRPII activates inflammasome to exerts pro-inflammatory effects by a mechanism not dependent on Toll-like receptors (TLR1, TLR2, TLR5, TLR6, and TLR9) (13). However, the possible role of Receptor for Advanced Glycation End-Products (RAGE) has not been investigated. Previous results have indicated GAGs function as co-receptors for RAGE (36), thus the hypothesis that GAG-bound HRPII is presented to RAGE is a possible mechanism to account for pro-inflammatory function of HRPII warrants further investigation. However, a direct pro-inflammatory signaling by HRPII through interaction with GAGs cannot be excluded at this time.

Similar to activation of endothelial cells, HRPII also activated NF-κB in leukocytes, induced histone citrullination and NET formation in both dHL60 cells and human neutrophils. The nuclear enzyme PAD4 catalyzes histone citrullination that results in de-condensation of chromatin structure and release of nuclear contents as NETs by neutrophils during infection (30-33). Our results suggest HRPII upregulates expression of PAD4 and its nuclear localization in neutrophils as demonstrated by confocal imaging. AT inhibited PAD4 expression and NET formation in HRPII-treated
neutrophils, possibly by competitively blocking interaction of HRPII with GAGs on neutrophils. In support of this hypothesis, unlike AT-WT, AT-4Mut did not inhibit citrullination of histones and NET formation in dHL60 cells. By contrast, AT-N135Q, which exhibits a higher affinity for GAGs (17,24,25), was more effective in inhibiting these inflammatory processes in HRPII-treated dHL60 cells. Nevertheless, a recent study identified three transmembrane receptors; CD13, CD300f and LRP-1 on monocytes that bind to AT to initiate protective cell signaling (37). Thus, further studies will be required to determine whether these receptors are expressed on neutrophils and if interaction of AT with any one of these receptors contributes to inhibition of HRPII-mediated citrullination and NET formation by neutrophils. Further studies will also be required to understand the mechanism by which HRPII activates neutrophils and whether similar to interaction with endothelial cells it interacts with GAGs on neutrophils or its direct interaction with any one of PRRs is responsible for transmitting pro-inflammatory signaling of HRPII. Based on presented results and our experimental approach in this study (simultaneous incubation of cells with HRPII and AT) we hypothesize, similar to endothelial cells, both molecules competitively bind to neutrophil GAGs to exert their specific signaling effects. The observations that AT-4Mut had no effect in protecting neutrophils and AT-N135Q exhibited higher protective activity are consistent with this hypothesis.

The in vivo relevance of HRPII-mediated inflammatory responses was investigated by challenging mice with HRPII in both the presence and absence of AT. Results indicated HRPII promotes both inflammation and coagulation, and AT exhibits a significant protective effect in downregulating both pathways. This conclusion is derived
from observations that HRPII increased cell surface expression of Mac1 on mouse blood neutrophils (Ly6G-positive cells) and promoted their infiltration into lung and kidney tissues. HRPII also upregulated expression of CAMs and vascular permeability in lungs and kidneys. HRPII also induced NET formation in vivo and markedly increased histone H3 citrullination in lung tissues and MPO activity in the plasma. Recent results have indicated there is a direct association between neutrophil activation and NETosis in malaria severity in Pf infected patients (28,29,38). Thus, it is tempting to speculate HRPII may contribute to NETosis in Pf-infected patients. In support of this possibility, circulating NET counts in Pf infected patients have been found to correlate with the plasma level of HRPII (28). However, the exact mechanism of NET formation in severe malaria needs further investigation since neutrophil activation and NET formation have also been observed in infection with different species of Plasmodium, which do not synthesize HRPII (29). In this context, neutrophil activation and NET formation has been shown to be associated with development of acute respiratory distress syndrome (ARDS) in Plasmodium berghei-infected mice, where neutrophil depletion protects mice from malaria associated ARDS (39). We also found neutrophil depletion rescues HRPII-mediated endothelial activation, pulmonary vascular leakage and thrombocytopenia. In addition to promotion of inflammation, HRPII also promoted procoagulant responses as evidenced by increased platelet deposition in lungs, thrombocytopenia, elevated VWF and TAT-complex in the plasma and fibrin(ogen) in lung tissues. Neutrophil depletion did not have effects on fibrin(ogen) and TAT-complex levels, possibly suggesting a minimal direct role for neutrophils in promoting activation of coagulation and thrombin generation. However, neutrophil depletion rescued HRPII-mediated thrombocytopenia
and elevated VWF release both of which are known to play critical roles in pathogenesis of Pf malaria (40). The mechanism by which HRPII induces coagulation activation requires further investigation. A prothrombotic role for NETs has been reported by their ability to support platelet adhesion, activation and aggregation (41). Moreover, NET-associated histones are known to stimulate exocytosis of VWF in endothelial cells and platelets, thereby promoting coagulation and inflammation (immuno-thrombosis) (42). The observation that neutrophil depletion rescued HRPII-mediated thrombocytopenia and elevated VWF release confirms results of these previous studies on the important role of NETs in immuno-thrombosis, however, the lack of an effect on fibrin(ogen) deposition and TAT-complex suggests HRPII also enhances thrombin generation independent of NETs. In this context, in addition to NETs, HRPII also induced TNFα expression, which may induce tissue factor expression on monocytes and endothelial cells, thereby promoting activation of coagulation. Finally, it is known activated platelets store significant amounts of polyphosphates, which not only can bind HRPII and promote its pro-inflammatory function (15), but also bind histones and promote thrombin generation by a platelet dependent mechanism (43). Thus, the possibility that activated platelet-derived polyphosphates are involved in augmenting procoagulant function of HRPII also needs further investigation. This is a clinically important question since Pf is known to store large amounts of short- and long-chain polyphosphate in different blood stages of infection (44). We have demonstrated platelet-size polyphosphates can bind to nuclear proteins and induce VWF release and platelet string formation on endothelial cells (45). Thus, further studies will be required to understand the exact mechanism through which HRPII promotes coagulation activation in our model system. Further
studies will also be required to determine whether HRPII-mediated endothelial cell or platelet activation or both contributes to increased plasma level of VWF in Pf malaria patients.

In summary our results demonstrate HRPII promotes inflammation and coagulation in both cellular and animal models, thus it may play a role in the disease pathogenesis of the Pf malaria. HRPII exerts its pro-inflammatory effect through interaction with GAGs. AT exhibits a potent protective effect by eliciting GAG-dependent anti-inflammatory responses against HRPII in both systems. Moreover, AT competitively inhibits binding of HRPII to GAGs, thereby downregulating its pro-inflammatory signaling function. Currently, there is no effective therapy for treating severe malaria. Based on our results, we propose the therapeutic potential of AT, particularly AT-N135Q, which exhibits significantly higher GAG-binding properties, in protecting against pathogenesis of the Pf parasite infection may warrant further investigation.
Data Sharing Statement

For data sharing, please contact the corresponding author at Ray-Rezaie@omrf.org.

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Authorship

I.B. designed, performed and analyzed experiments in cellular and animal models; S.R.P. designed, performed and analyzed all animal and flow cytometry experiments; X.S.C. designed and performed histological analysis and immunofluorescence experiments; H.G. measured the VWF level in the plasma; A.R.R. designed experiments, analyzed data, wrote the manuscript and supervised the project. All authors approved the final version of the manuscript.

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Disclosure of Conflict of Interests

The authors declare no conflict of interests.
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Figure 1. AT inhibits GAG-dependent HRPII-mediated inflammatory responses in endothelial cells. (A) Confluent endothelial cells (hTERT-HUVECs) were simultaneously treated with HRPII (100 nM) and AT (2.5 µM) for 15 min followed by analysis of phosphorylation of NF-κB p65 by Western blotting. Quantitative analysis presented. (B) Cells were treated similarly with HRPII (100 nM) and AT or AT-4Mut (2.5 µM) for 1 h and then fixed, permeabilized and stained with anti-NF-κB p65 antibody (rabbit) followed by Alexa Fluor 488-conjugated anti-rabbit IgG. DAPI was used to stain the nucleus. (C) Cells were treated with HRPII and AT derivatives as described above for 4 h followed by analysis of cell surface expression of ICAM-1 by flow cytometry. Quantitative analysis presented. (D) Fluorescein isothiocyanate (FITC)-conjugated HRPII (HRPII-FITC, 1 µM) binding to endothelial cells were analyzed by flow cytometry in the absence and presence of the GAG antagonist, surfen (10µM). Quantitative analysis presented. (E) Cells were treated with HRPII (100 nM) for 1 h in the absence and presence of Surfen (10µM). Cells were then fixed, permeabilized and stained with anti-NF-κB p65 (rabbit) antibody and Alexa Fluor 488-conjugated anti-rabbit IgG. DAPI was used to stain the nucleus. (F) Confluent cells were treated with HRPII in absence or presence of surfen (10µM) followed by monitoring HRPII-mediated barrier-disruptive function through measuring the influx of albumin-bound Evans blue across the cell monolayer as described in Materials and methods. (G) The same as (F) except that the competitive effect of increasing concentration of wild-type AT and AT-N135Q were monitored in presence of fixed concentration of HRPII (40 nM).

Figure 2 AT inhibits HRPII-mediated inflammatory responses in HL60 cells. (A) DMSO-differentiated HL60 (dHL60) cells were treated with HRPII (100 nM) for 5-15 min
followed by the analysis of phosphorylation of NF-κB p65 by Western blotting. Densitometric analysis presented. (B) The same as (A) except that cells were treated simultaneously with HRPII and AT (2.5 µM) followed by the analysis of phosphorylation of NF-κB p65 by Western blotting. Densitometric analysis presented. (C) dHL60 cells on coverslips were treated simultaneously with HRPII (100 nM) and different AT derivatives (AT-WT, AT-N135Q and AT-4Mut (2.5 µM) for 4 h and then fixed in 4% paraformaldehyde, permeabilized with 0.2% TritonX-100/PBS and stained with anti-citrullinated H3 (rabbit polyclonal) antibody followed by Alexa Fluor 555-conjugated anti-rabbit antibody. DNA was stained with Sytox Green. (D) dHL60 cells were treated with HRPII (100 nM), TNFα (10 ng/mL) or PMA (100 nM) for 4 h followed by analysis of histone (H3) citrullination by Western blotting. Densitometric analysis presented. (E) The same as (D) except that cells were treated with HRPII and different AT derivative (AT-WT, AT-N135Q and AT-4Mut (2.5 µM) and histone (H3) citrullination were analyzed by Western blotting. Densitometric analysis presented.

Figure 3. AT inhibits HRPII activation of neutrophils and NET formation. (A) Human blood neutrophils were isolated and treated with HRPII (100 nM) in absence or presence of AT for 4 h. Cells were then fixed in 4% paraformaldehyde, permeabilized with 0.2% TritonX-100/PBS and stained with anti-neutrophil elastase (goat polyclonal) antibody, anti-citrullinated H3 (rabbit polyclonal) followed by Alexa Fluor-488 conjugated anti-goat and Alexa Fluor 555-conjugated anti-rabbit antibody. DNA was stained with DAPI. Quantification of NET forming cells and citrullinated H3-positive cells per field represented as the percentage of the total cells. (B) The same as panels above except that human blood neutrophils were fixed and stained with anti-PAD4 (rabbit polyclonal)
and anti-histone (H3) (mouse monoclonal) antibody followed by Alexa Fluor 555-conjugated anti-rabbit antibody and Alexa Fluor 488-conjugated anti-mouse antibody. DAPI was used to stain the nucleus. Quantification of PAD4 nuclear translocation is represented next to the panel. (C) Human blood neutrophils were treated with HRPII in absence and presence of AT. PAD4 level in nuclear extract were analyzed through western blot. PCNA was used as loading control. Densitometric analysis presented. (D) Human blood neutrophils were treated with HRPII in absence and presence of AT. TNFα level in supernatant of neutrophils were measured using commercial ELISA.

**Figure 4.** AT inhibits HRPII-mediated activation of neutrophils in vivo. Mice were injected intraperitoneally (i.p.) with saline or HRPII (10 µg/gm) and AT (500 µg/mouse ~20 µg/gm) followed by collection of organs after 3.5 h for analysis. (A) Cryosections of the lung tissue were fixed and permeabilized followed by analysis of infiltration of neutrophils to the lung with anti-MPO (rabbit) antibody and Alexa Fluor 555-conjugated anti-rabbit antibody. DAPI was used to stain the nucleus. Relative intensity of the MPO stain is presented. (B) Mac1 surface expression in Ly6G-positive mouse neutrophils was analyzed by flow cytometry as described in Materials and methods. (C) The MPO level in plasma (marker of neutrophil activation) was analyzed by the MPO activity assay as described in Materials and methods. (D) The same as (A) except that the lung cryosections were fixed, permeabilized and incubated with anti-citrullinated histone H3 (rabbit) and anti-Ly6G (rat) antibodies followed by Alexa Fluor 555-conjugated anti-rabbit and Alexa Fluor 488-conjugated anti-rat antibodies. DAPI was used to stain the nucleus. The arrows indicate citrullinated histone H3. (E) The PBS perfused lower right lobe of lung was dissected and dissolved in the tissue lysis buffer followed by Western-
blotting of the lysate for detection of MPO, citrullinated histone H3, histone H3 and beta actin using appropriate antibodies. Densitometric analysis of expression of these proteins are presented.

**Figure 5.** AT inhibits HRPII-mediated inflammation and coagulation. Mice were injected intraperitoneally (i.p.) with saline or HRPII (10 µg/gm) and AT (20 µg/gm) followed by sacrificing mice after 3.5 h, perfusing the lungs with PBS, dissecting the lower right lobe of the lung and dissolving it in the tissue lysis buffer. (A) Lung tissue lysates were immunoblotted for VCAM1 and ICAM1 and β-actin. Densitometric analysis of expression of these proteins are presented. (B) The barrier-protective effect of AT in mice injected with both AT and HRPII was analyzed after 3 h by the intravenous injection (i.v.) of 1% Evans blue dye. After 30 min, animals were sacrificed, perfused with PBS, lung tissue samples were collected, and vascular permeability was measured from the amount of Evans blue dye leaked into the lung as described in Materials and methods. (C) Plasma level of VWF was measured by an ELISA using a commercial kit. (D) Blood platelet counts were determined using a veterinary hematology analyzer. (E) Lung cryosections were fixed, permeabilized and incubated with anti-CD41 (rat) and anti-CD31 (rabbit) antibodies followed by Alexa Fluor 555-conjugated anti-rat and Alexa Fluor 488-conjugated anti-rabbit antibodies. DAPI was used to stain the nucleus. The arrows indicate platelet rich thrombus. Relative intensity of CD41 is presented.

**Figure 6.** AT inhibits HRPII-mediated procoagulant responses in mice. Mice were injected i.p. with saline or HRPII (10µg/gm) and AT (20 µg/gm) followed by taking blood samples and collecting organs after 3.5 h for analysis. (A) Plasma level of the TAT-complex was measured with an established ELISA assay. (B) Perfused lower right lobe
of the lung was harvested in the tissue lysis buffer and immunoblotted for the fibrin(ogen). (C) Densitometric analysis of the fibrin(ogen) deposition in the lung tissue sample is presented. (D) Lung cryosections were fixed, permeabilized and incubated with anti-CD31 (rat) and anti-fibrin(ogen) (rabbit) antibodies followed by Alexa Fluor 488-conjugated anti-rat and Alexa Fluor 562-conjugated anti-rabbit antibodies. DAPI was used to stain the nucleus. The arrows indicate intravascular thrombosis. (E) Perfused upper left lobe of the lung was collected and processed for the histological analysis. Paraffin-embedded sections of the lung tissue were stained with H&E. Inset boxes from each group are magnified. The blue arrows indicate thrombosis and yellow arrows indicate inflammatory foci.

**Figure 7.** Depletion of neutrophils in mice decreases HRPII-mediated inflammation but not the procoagulant response. Mice were administered either control antibody or anti-Ly6G antibody (20µg/gm) 48 hours before HRPII treatment (10µg/gm). After 3.5 hours of HRPII treatment, mice were sacrificed, perfused with PBS and blood and organ collected. The lower right lobe of the lung was lysed in tissue lysis buffer. (A) Lung tissue lysates were immunoblotted for VCAM1, ICAM1, MPO, fibrin(ogen) and β-actin. Densitometric analysis of expression of these proteins are presented in (B), (C), (D), (E). β-actin were used as loading control. (F) Plasma level of the TAT-complex was measured with an established ELISA assay. (G) Plasma level of VWF was measured by an ELISA using a commercial kit. (H) Blood platelet counts were determined using a veterinary hematology analyzer. (I) To analyze the effect of neutrophil depletion on lung vascular permeability, 1% Evans blue dye were injected 3 h after HRPII treatment. After 30 min, animals were sacrificed, perfused with PBS, lung tissue samples were
collected, and vascular permeability was measured from the amount of Evans blue dye leaked into the lung as described in Materials and methods.
Figure 2

A

B

C

D

E

Fig. 2
**Fig. 3**

A. **Neutrophil Elastase**

B. **PAD4, Histone H3**

C. **PAD4 in nucleus (fold change)**

D. **TNFα (pg/ml)**
**Figure 4**

**A**

|            | Saline | HRPII | AT+HRPII |
|------------|--------|-------|----------|
| MPO, DAPI | ![Image](image1) | ![Image](image2) | ![Image](image3) |

**B**

Relative Cell Number

![Graph](image4)

**C**

![Graph](image5)

**D**

|            | Saline | HRPII | AT+HRPII |
|------------|--------|-------|----------|
| Cit-H3     | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| Ly6G       | ![Image](image9) | ![Image](image10) | ![Image](image11) |
| Ly6G, Cit-H3, DAPI | ![Image](image12) | ![Image](image13) | ![Image](image14) |

**E**

|            | Saline | HRPII | AT+ HRPII |
|------------|--------|-------|-----------|
| MPO        | ![Image](image15) | ![Image](image16) | ![Image](image17) |
| Cit-H3     | ![Image](image18) | ![Image](image19) | ![Image](image20) |
| H3         | ![Image](image21) | ![Image](image22) | ![Image](image23) |
| β-actin    | ![Image](image24) | ![Image](image25) | ![Image](image26) |
**Figure 5**

**A**

| Saline | HRPII | AT+HRPII |
|--------|-------|----------|
| 1 2 3 4 | 1 2 3 4 5 | 1 2 3 |

ICAM1, VCAM1, β-actin

**B**

Evans Blue (µg/gm tissue)

HRPII AT - - + +

*** *** **

**C**

VWF (ng/ml)

HRPII AT - - + +

*** ** *

**D**

Platelet count (10^6/ml)

HRPII AT - - + +

*** ** *

**E**

CD41, CD31, CD41, DAPI

Saline, HRPII, AT+HRPII

Relative intensity

HRPII AT - - + +

*** **
Figure 6
Figure 7

- **A**: Immunoblot analysis showing the expression of ICAM1, VCAM1, MPO, Fibrinogen, and β-actin in different groups (Control, IgG+HRPII, Ly6G IgG+HRPII).

- **B**: Graph showing the fold change in ICAM1 expression.

- **C**: Graph showing the fold change in VCAM1 expression.

- **D**: Graph showing the fold change in MPO expression.

- **E**: Graph showing the fold change in Fibrinogen expression.

- **F**: Graph showing the concentration of TAT-Complex.

- **G**: Graph showing the concentration of VWF.

- **H**: Graph showing the platelet count.

- **I**: Graph showing the concentration of Evans Blue.

Fig. 7