Red Blood Cells Homeostatically Bind Mitochondrial DNA through TLR9 to Maintain Quiescence and to Prevent Lung Injury

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Abstract

Rationale: Potentially hazardous CpG-containing cell-free mitochondrial DNA (cf-mtDNA) is routinely released into the circulation and is associated with morbidity and mortality in critically ill patients. How the body avoids inappropriate innate immune activation by cf-mtDNA remains unknown. Because red blood cells (RBCs) modulate innate immune responses by scavenging chemokines, we hypothesized that RBCs may attenuate CpG-induced lung inflammation through direct scavenging of CpG-containing DNA.

Objectives: To determine the mechanisms of CpG-DNA binding to RBCs and the effects of RBC-mediated DNA scavenging on lung inflammation.

Methods: mtDNA on murine RBCs was measured under basal conditions and after systemic inflammation. mtDNA content on human RBCs from healthy control subjects and trauma patients was measured. Toll-like receptor 9 (TLR9) expression on RBCs and TLR9-dependent binding of CpG-DNA to RBCs were determined.

A murine model of RBC transfusion after CpG-DNA-induced lung injury was used to investigate the role of RBC-mediated DNA scavenging in mitigating lung injury *in vivo*.

Measurements and Main Results: Under basal conditions, RBCs bind CpG-DNA. The plasma-to-RBC mtDNA ratio is low in naive mice and in healthy volunteers but increases after systemic inflammation, demonstrating that the majority of cf-mtDNA is RBC-bound under homeostatic conditions and that the unbound fraction increases during inflammation. RBCs express TLR9 and bind CpG-DNA through TLR9. Loss of TLR9-dependent RBC-mediated CpG-DNA scavenging increased lung injury *in vivo*.

Conclusions: RBCs homeostatically bind mtDNA, and RBC-mediated DNA scavenging is essential in mitigating lung injury after CpG-DNA. Our data suggest a role for RBCs in regulating lung inflammation during disease states where cf-mtDNA is elevated, such as sepsis and trauma.

Keywords: Toll-like receptor 9; mitochondrial DNA; CpG-DNA; RBC

Cell-free mitochondrial DNA (cf-mtDNA) is released into the blood and other compartments during normal cellular turnover and is found in increased amounts during pathologic states, including periods

of excessive cell death and immune cell activation (1, 2). Elevated cf-mtDNA is associated with malignancy, autoimmune disease, trauma, and sepsis and is associated with cellular injury and organ dysfunction

(3–6). Similar to bacterial DNA, mitochondrial DNA contains CpG motifs that incite proinflammatory signaling through the endosomal, nucleotide-sensing Toll-like receptor, TLR9 (7–9). These

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At a Glance Commentary

Scientific Knowledge on the

Subject: CpG-containing cell-free mitochondrial DNA (cf-mtDNA) is elevated in critically ill patients and is associated with increased morbidity and mortality. How the body prevents excessive innate immune activation and eliminates cf-mtDNA from the circulation under homeostatic conditions and during inflammatory states remains unknown.

What This Study Adds to the

Field: Here we demonstrate that a novel function of red blood cells (RBCs) is mtDNA scavenging. cf-mtDNA partitions with RBCs under homeostatic conditions in mice and healthy human subjects, and the plasma-to-RBC mtDNA ratio is low. However, after systemic inflammation in mice and trauma in humans, plasma mtDNA is elevated and the majority of the cf-mtDNA is detected in the plasma. DNA binding to RBCs was mediated by Toll-like receptor 9 (TLR9), which is expressed on RBCs. *In vivo*, RBC TLR9-mediated mtDNA scavenging was necessary to mitigate lung injury. Collectively, these studies uncover a critical role for RBCs in regulating systemic inflammation by scavenging cf-mtDNA and demonstrate the importance of RBC-mediated DNA scavenging in limiting lung injury. Because elevated cf-mtDNA is implicated in organ injury during inflammatory states including sepsis and trauma, our findings suggest further research into RBC-mediated DNA scavenging in the development of organ injury during these states is warranted.

findings and clinical observations that suggest an association between cf-mtDNA and disease severity in trauma and mortality in sepsis underscore the importance of cell-free DNA in tissue injury during acute inflammation (3, 4).

The injurious potential of extracellular or cell-free mtDNA is highlighted in mice by expression of a transmembrane mutant TLR9 that localizes to the cell surface rather than the endosome. Under these

circumstances, extracellular CpG-containing DNA (hereafter, CpG-DNA) led to lethal inflammation and anemia, suggesting that mechanisms, such as scavenging or clearance from the circulation, must exist to prevent access of mtDNA to the endosomal compartment, preventing unnecessary inflammation (10). Importantly, the lack of elevated cf-mtDNA in healthy hosts also suggests the presence of an efficient scavenging mechanism for extracellular nucleic acids. Binding and clearance by inflammatory cells that express TLR9, however, would be expected to lead to immune activation, perhaps incompatible with normal homeostasis. Thus, although alternative mechanisms for cf-mtDNA clearance from the circulation must exist, the mechanisms by which this occurs remain unknown.

Erythrocytes are the most abundant cell type in the circulation, and mounting evidence has demonstrated that red blood cells (RBCs) possess many functions beyond oxygen and carbon dioxide transport. RBCs bind pathogens through glycoprotein A and modulate nitric oxide bioavailability (11, 12). RBCs also bind chemokines through the transmembrane receptor Duffy antigen receptor for chemokines and regulate plasma chemokine concentrations (13, 14). Given the role of RBCs in the binding and transport of numerous substances, including gaseous molecules and chemokines, we hypothesized that circulating RBCs homeostatically scavenge cf-mtDNA and prevent lung inflammation. Some of the results of these studies have been previously reported in the form of an abstract (15).

Methods

Please see the online supplement.

Results

mtDNA Is Present on Human and Murine RBCs under Basal Conditions, and RBCs Bind CpG-DNA

To determine the distribution of cell-free mtDNA present during homeostasis, qPCR of mitochondrially encoded gene *MT-COI* (mitochondrially encoded cytochrome *c* oxidase subunit I) was used to quantify mtDNA in the RBC and plasma fractions of whole blood from healthy

human subjects. Surprisingly, most of the mtDNA was found in the RBC fraction rather than in plasma, suggesting the possibility of mtDNA binding to RBCs (Figures 1A and 1B). Because RBCs do not contain an intrinsic source of nucleic acids such as mitochondria or nuclei, we asked whether RBCs bind exogenous mtDNA. RBCs bound CpG-DNA in a concentration-dependent fashion (Figure 1C).

To ensure that mtDNA associated with RBCs obtained from whole blood was not due to leukocyte contamination of the RBCs, we next determined the presence of mtDNA on erythrocytes obtained from freshly isolated leukoreduced whole blood from healthy human donors. qPCR of the mitochondrially encoded genes *MT-COI* and *MT-ND1* (mitochondrially encoded NADH: ubiquinone oxidoreductase core subunit 1) was used to quantify mtDNA and qPCR for cytochrome *c* oxidase subunit 4 (*COX4*) was used to quantify nuclear DNA on human RBCs. As shown in Figure E1 in the online supplement, a number of donors exhibited substantial amounts of mtDNA on RBCs, consistent with normal turnover of cells *in vivo*. Notably, no nuclear DNA was detected on freshly isolated RBCs (data not shown).

We next determined the distribution of cf-mtDNA in mice under basal conditions. Similar to human subjects, mtDNA partitioned with RBCs in naive mice (Figure 1D). Murine RBCs bind CpG-DNA in a concentration-dependent fashion and bind authentic mtDNA (Figures 1E and 1F). Collectively, these studies demonstrate that under basal conditions mtDNA is present on human and murine RBCs and RBCs bind CpG-containing DNA.

mtDNA Accumulates on Human and Murine RBCs after Cell Death

Cell-free mtDNA is elevated during inflammatory states and after cell death. To determine whether RBCs bind mtDNA generated from dying cells, we took advantage of the observation that mitochondria and mtDNA are released from cells after a form of regulated cell death called “necroptosis” (16). We have previously observed lung necrosome formation and danger signal release after RBC transfusion, suggesting necroptosis *in vivo*. We therefore asked whether

Figure 1. Mitochondrial DNA (mtDNA) is present on human and murine red blood cells (RBCs) under basal conditions and partitions with RBCs rather than the soluble fraction. (A) Cell-free mtDNA partitions with RBCs rather than plasma in healthy human subjects: $*P = 0.029$, $n = 4$. (B) Percentage of total mtDNA is greater in RBCs than in plasma for each healthy subject tested (98.67 vs. 1.30% for RBCs vs. plasma; $P < 0.001$). (C) Human RBCs bind CpG-DNA under basal conditions. (D) Cell-free mtDNA partitions with RBCs rather than plasma in naive mice: $*P = 0.008$, $n = 5$ mice, two independent studies. (E) Murine RBCs bind CpG-DNA. (F) Murine RBCs were incubated with 200 pg of purified murine Mt-Co1 (gene encoding cytochrome *c* oxidase subunit 1) amplicon for 1 hour at 37°C . The cells were then washed, lysates were prepared, and PCR for Mt-Co1 was performed. Murine RBCs acquired mtDNA: $*P < 0.01$. Bar graph shows mean $\pm$ SEM. The Mann-Whitney rank sum test was used where data were not normally distributed. FITC = fluorescein isothiocyanate.

mtDNA would accumulate on RBCs after transfusion-induced necroptosis *in vivo*. Wild-type (WT) mice and mice lacking the essential mediator of necroptosis, receptor-interacting serine/threonine protein kinase

3 knockout (RIP3-KO), were transfused with a high dose of RBCs to induce necroptosis as previously described; the same murine packed RBC unit was used to transfuse both WT and RIP3-KO mice (17).

We observed increased copies of mtDNA on the purified RBCs from WT RBC-transfused mice when compared with RBCs from WT phosphate-buffered saline (PBS)-treated mice (Figure 2A). In contrast

Figure 2. Mitochondrial DNA (mtDNA) accumulates on human and murine red blood cells (RBCs) after necroptosis, and the plasma-to-RBC mtDNA ratio is elevated after systemic inflammation. (A) Murine RBCs bind mtDNA after necroptosis: mtDNA on RBCs obtained from phosphate-buffered saline (PBS)-treated versus RBC-transfused mice. mtDNA is increased on RBCs obtained from transfused mice. mtDNA is higher on RBCs obtained from wild-type (WT) transfused mice when compared with receptor-interacting serine/threonine protein kinase 3 knockout (RIP3 KO) transfused mice. Each symbol represents three mice and each shade represents an individual study: $n = 9$ mice per group, three independent studies, $^*P = 0.05$. (B) The plasma-to-RBC mtDNA ratio is increased 2 hours after systemic inflammation. mtDNA in naive and TNF α -zVAD-treated mice: Mann-Whitney rank sum test, $^*P = 0.032$. (C) There is increased mtDNA in the RBC fraction when compared with the plasma of naive mice ($^*P = 0.008$) but not after systemic inflammation: $n = 5$ mice, two independent studies for B and C. (D) Human RBCs bind mtDNA released after lung endothelial cell necroptosis. Preincubation with the necroptosis inhibitor necrosulfonamide (NSA) attenuates mtDNA acquisition by RBCs: $^*P < 0.01$. PCR results for MT-CO1 are shown. Three units were tested, and two representative units are shown (Unit 1 and Unit 2). (E) RBC and plasma mtDNA in healthy subjects and trauma patients: $n = 4$ healthy subjects, $n = 6$ trauma patients, $^*P = 0.0381$. (F) The plasma-to-RBC mtDNA ratio is increased after trauma or systemic inflammation: $^*P = 0.019$, Mann-Whitney rank sum test. Bar graphs show mean $\pm$ SEM. Box-and-whisker plots show median, interquartile range, and maximum and minimum values. EC = endothelial cells; MT-CO1 = mitochondrially encoded cytochrome c oxidase 1; TNF = tumor necrosis factor; zVAD = {benzyloxycarbonyl-L-valyl-L-alanyl-[(2S)-2-amino-3-(methoxycarbonyl)propionyl]}fluoromethane.

to WT mice, RBC transfusion did not increase RBC-bound mtDNA in necroptosis-deficient RIP3-KO mice (Figure 2A). To ensure that our observations of decreased mtDNA on circulating RBCs from RIP3-KO mice were due to mtDNA release after necroptosis *in vivo* and not to an inability of RIP3-KO RBCs to acquire mtDNA, we tested the ability of RIP3-KO RBCs to bind mtDNA *in vitro*. As shown in Figure E2, RIP3-KO mice have intact mtDNA acquisition. These data demonstrate that mtDNA binds to circulating RBCs after transfusion-induced necroptosis.

Given our observations of mtDNA partitioning with RBCs rather than the supernatant or plasma of human and murine RBC preparations, we asked whether the localization of cf-mtDNA changed after acute inflammation. Mice subjected to systemic inflammation with tumor necrosis factor- α -zVAD demonstrated increased plasma/RBC mtDNA content when compared with naive mice (Figure 2B). Consistent with our earlier observations, significantly more mtDNA was detected on RBCs than in the plasma under basal conditions, but after systemic inflammation plasma and RBC mtDNA concentrations were not significantly different (Figure 2C).

We next asked whether human RBCs bind mtDNA generated from dying cells. Because we have previously observed lung endothelial necroptosis after exposure of lung endothelial cells (ECs) to RBCs, we asked whether extracellular mtDNA is increased after treatment of lung ECs with RBCs (17). Although only one of the RBC units tested increased supernatant mtDNA, all led to a significant increase in mtDNA associated with the RBCs (Figure E3). RBCs acquired mtDNA from necroptotic rather than intact ECs as inhibition of the critical necroptotic protein MLKL (mixed lineage kinase domain-like protein) attenuated mtDNA accumulation on RBCs after endothelial exposure (Figure 2D). Elevated plasma mtDNA has been associated with increased morbidity in trauma patients, and cellular necrosis is common after trauma (18). We next measured RBC and plasma mtDNA content in healthy volunteers and trauma patients to determine the distribution of cf-mtDNA after systemic inflammation in humans. Consistent with previous reports, plasma mtDNA was elevated in trauma patients (Figure 2E)

(18). After traumatic injury, the plasma-to-RBC mtDNA ratio was increased when compared with healthy subjects (Figure 2F), suggesting that cf-mtDNA is RBC-bound under basal conditions but detectable in the plasma during inflammatory states; this may be due to increased generation of cell-free mtDNA or abnormal scavenging of mtDNA by RBCs after trauma.

TLR9 Is Expressed on Human and Murine Erythrocytes, and CpG Binding to RBCs Is Dependent on TLR9

To determine the mechanism by which mtDNA binds to RBCs, we questioned whether the canonical TLR that binds CpG-DNA is present on RBCs. TLR9 is a nucleotide-sensing TLR that recognizes DNA containing CpG motifs, present in both bacterial-derived and mitochondrial DNA. Although not previously described on mature red blood cells, reports have described the presence of functional TLR9 on nonnucleated cells (19, 20). We measured intracellular TLR9 expression on RBCs obtained from leukoreduced RBC units by flow cytometry and found that more than one-half of the units demonstrated more than 5% positive cells (Figure 3A). TLR9 expression on RBCs was predominantly intracellular with little detectable TLR9 on the surface (data not shown). To confirm that TLR9 expression on RBCs was not due to platelet-derived microvesicles or secondary to reticulocytes present in the preparation, we stained the RBCs for CD235 (a mature red cell marker) and CD41 (a platelet marker). As shown in Figure 3B, the TLR9-positive cells were CD235 positive and CD41 negative. TLR9 expression on RBCs was confirmed by confocal imaging (Figures 3C and 3D), indicating TLR9 in vesicular structures in the submembranous region of the RBCs. Furthermore, immunoprecipitation of TLR9 from normal human RBCs revealed association with band 3, an essential red cell membrane protein (Figure E4a). Proximity ligation studies confirmed the interaction between band 3 and TLR9 in the submembranous region (Figure E4b). These results unequivocally indicate the expression of TLR9 on RBCs.

We next asked whether human RBCs expressing TLR9 bind CpG-DNA more compared with RBCs that do not express TLR9, taking advantage of the observation

that not all RBCs express TLR9. RBC preparations from leukoreduced RBC units and healthy donors were examined. DNA-binding capability of TLR9-expressing cells versus TLR9-negative cells within each RBC preparation was compared by flow cytometry (Figures 3E and 3F). Although we observed heterogeneity in DNA binding in the RBC preparations, we found that TLR9-positive cells acquired more CpG-DNA than did TLR9-negative cells. Imaging flow cytometry confirmed these findings and revealed extensive membrane damage and RBC crenation in TLR9-positive cells that acquired CpG (Figures E4c–E4g). Collectively, these data demonstrate that human RBCs express TLR9 and bind CpG-DNA, which alters the RBC membrane.

We next asked whether murine RBC-CpG binding was dependent on TLR9. As there are no suitable antibodies against murine TLR9 for flow cytometry, we used a functional approach to ask whether RBCs obtained from mice lacking *Tlr9* would bind CpG-DNA with the same efficacy as WT RBCs. We first measured mtDNA on RBCs from naive WT and *Tlr9*-KO mice. RBCs from *Tlr9*-KO mice contained less mtDNA than did RBCs obtained from WT mice (Figure 3G). We next used flow cytometry to measure binding of CpG-DNA to WT and KO RBCs. RBCs were obtained from WT and *Tlr9*-KO mice and labeled with the membrane dyes PKH26 and PKH67, respectively. The RBCs were then combined and mixed with fluorescently labeled (allophycocyanin) CpG-DNA for 2 hours. Apparent binding of CpG-DNA to WT RBCs was greater than to *Tlr9*-KO RBCs both in terms of the intensity of binding per cell (Figure 3H) as well as the percentage of cells that bound DNA (Figure 3I). These data indicate the presence of TLR9 on murine erythrocytes and demonstrate that CpG-DNA binding at this concentration is dependent on TLR9.

RBCs Scavenge CpG-DNA from the Circulation, and Defective RBC DNA Scavenging Enhances CpG-DNA-mediated Lung Injury

To determine whether RBC binding to CpG-DNA *in vivo* is dependent on TLR9, we administered CpG to WT mice immediately after a low-dose infusion of

Figure 3. Human and murine red blood cells (RBCs) bind CpG-DNA through Toll-like receptor 9 (TLR9). (A) TLR9 expression on RBCs obtained from healthy human donors and leukoreduced RBC units: $n = 39$. (B) TLR9-positive cells obtained from a healthy donor are CD235 positive and CD41 negative. (C) Confocal imaging confirms the presence of TLR9 on human RBCs: green, TLR9; red, membrane stain (PKH); secondaries alone are shown on the left. (D) Arrows depict areas of colocalization of TLR9 with RBC membrane. (E and F) TLR9-positive RBCs bind more CpG-DNA than TLR9-negative RBCs:

PKH-labeled WT or *Tlr9*-KO cells. We did not observe any CpG-DNA–positive RBCs in the circulation 1 hour after administration of DNA, indicating rapid clearance (data not shown). RBCs were isolated from the spleen 60 minutes after transfusion, using positive selection for the murine erythrocyte marker Ter-119 (Figure 4A). Erythrocytes obtained from the spleen revealed that CpG-DNA binding was increased in WT RBCs when compared with *Tlr9*-KO RBCs (Figure 4B). Collectively, these data show that RBCs scavenge cell-free DNA *in vivo* through TLR9.

Cell-free mtDNA is elevated in multiple disease states including autoimmune disease, trauma, and sepsis, and studies demonstrate a role for cell-free mtDNA in the pathogenesis of nonalcoholic steatohepatitis (3, 4, 18, 21, 22). Preclinical studies have demonstrated increased lung injury after systemic mtDNA administration, and mitochondrial damage-associated molecular patterns (mtDAMPs) have been reported to induce lung injury (18, 23). Given our observations of mtDNA on RBCs from naive mice, CpG-DNA binding to RBCs, and decreased systemic inflammation in transfused CpG-treated mice (Figure E5), we hypothesized that RBC-mediated scavenging of CpG-DNA plays an important role in counteracting tissue injury and that loss of RBC-mediated DNA scavenging from the lung microvasculature would augment lung injury. We therefore used a model in which transfusion of scavenging-competent (WT) or scavenging-deficient (*Tlr9*-KO) RBCs was tested for its effect on lung injury after CpG administration (24). CpG-DNA was administered intravenously to WT mice simultaneously with WT or *Tlr9*-KO RBCs. Mice were killed 6 hours after CpG administration and the lungs were examined for neutrophil infiltration (Ly6G staining) and tissue injury, which was quantified according to the American Thoracic Society lung injury scoring system (25). WT and *Tlr9*-KO RBCs alone did not

lead to lung injury when compared with control (PBS alone; data not shown). CpG administration led to enhanced neutrophil infiltration and lung injury when compared with PBS alone (Figures 4C–4E). The administration of *Tlr9*-KO RBCs augmented CpG-induced lung injury, suggesting that intact RBC nucleic acid–scavenging activity is required for the prevention of tissue injury (Figures 4C–4E). Collectively, these data demonstrate that loss of RBC-mediated CpG-DNA scavenging augments lung injury.

Human RBCs Scavenge CpG-DNA from Lung ECs and Attenuate TLR9 Activation *In Vitro*

Because RBCs are in continuous contact with vascular endothelium and CpG-DNA and mtDAMPs are known activators of lung endothelium, we asked whether human lung microvascular endothelial cell (HMVEC-L) CpG-DNA binding would be reduced in the presence of RBCs (26, 27). HMVEC-L bind CpG-DNA and RBCs scavenged CpG-DNA away from HMVEC-L (Figures 5A and 5B).

We next asked whether DNA binding by human RBCs would inhibit CpG-DNA–induced inflammation. CpG-stimulated activity of TLR9 reporter cells was attenuated in the presence of RBCs (Figure 5C).

We tested the scavenging ability of RBCs obtained from healthy donors and leukoreduced units and from trauma patients. Although we observed heterogeneity in the ability of RBCs from different individuals to scavenge CpG-DNA, RBCs from healthy donors significantly attenuated CpG-DNA–induced TLR9 activation, whereas RBCs from trauma patients did not (Figure 5D). Collectively, these data demonstrate that human RBCs from healthy donors scavenge CpG-DNA as evidenced by decreasing CpG-DNA binding to lung ECs and attenuating CpG-mediated TLR9 activation *in vitro*.

Discussion

Increasing evidence shows that circulating cell-free mtDNA is elevated during acute and chronic inflammatory states and causes tissue injury and organ dysfunction (3, 28–32). However, the mechanisms of mtDNA clearance have remained elusive. In this article, we demonstrate that RBCs homeostatically bind mtDNA and prevent CpG-DNA–induced inflammation, as defective RBC-DNA sequestration led to increased lung injury after CpG-DNA administration. Collectively, these data demonstrate a role for circulating RBCs in limiting tissue injury through the binding of potentially injurious nucleic acids.

We detected mtDNA on RBCs from healthy donors and naive mice and provide evidence for RBC-DNA binding under basal conditions. Under homeostatic conditions, the majority of the cell-free mtDNA partitioned with RBCs rather than with plasma in both humans and mice, suggesting that RBCs serve to bind cf-mtDNA under normal conditions. We also observed increased mtDNA on RBCs after necroptosis, suggesting that RBCs can bind mtDNA released during cell death. Mounting evidence over the past two decades has demonstrated that RBCs possess many functions beyond oxygen and hemoglobin transport. As examples, RBCs and their by-products regulate nitric oxide bioavailability, activate pattern recognition receptors including TLR4 and the receptor for advanced glycation end products, and alter acute lung inflammatory responses through Duffy antigen receptor for chemokines (13, 24, 33–38). Our data indicate that RBCs may possess yet another unconventional role in maintaining organismal homeostasis through the binding and sequestration of extracellular mtDNA. Given that approximately 30 trillion RBCs are in circulation in humans, the scavenging potential of RBCs is vast. We observe increased plasma-to-RBC mtDNA ratios after systemic injury in mice

Figure 3. (Continued). * $P < 0.001$, Mann-Whitney rank sum test. RBCs not treated with DNA are also depicted in E; nine individual RBC preparations from healthy donors or RBC units were tested. (G) RBCs obtained from naive wild-type (WT) mice contain more mitochondrial DNA than do RBCs obtained from naive TLR9 knockout (KO) mice: * $P = 0.053$, $n = 5$ or 6 mice per group, one study of three shown. (H and I) WT and TLR9 KO RBCs were labeled with PKH26 and PKH67 and mixed with CpG-DNA. TLR9 KO RBCs (solid line and solid circles) bind CpG-DNA less efficiently than WT RBCs (dashed line and open circles). (H) MFI versus CpG: 0–25 nM CpG doses shown. (I) Percent CpG-DNA–positive cells versus CpG concentration for WT and TLR9 KO RBCs. Bar graph shows mean $\pm$ SEM. Box-and-whisker plot shows median, interquartile range, and maximum and minimum values. APC = allophycocyanin; FITC = fluorescein isothiocyanate; MFI = mean fluorescence intensity; MT-CO1 = mitochondrially encoded cytochrome c oxidase I; PE = phycoerythrin; PKH = membrane dye.

Figure 4. Red blood cells (RBCs) scavenge CpG-DNA *in vivo*, and loss of RBC Toll-like receptor 9 (TLR9)-mediated CpG-DNA scavenging augments lung injury. (A) Left: Spleen RBCs obtained from mice treated with phosphate-buffered saline (PBS) and CpG-DNA. Right: PKH-positive cells are detected in the RBC-CpG-DNA-treated animals. (B) Wild-type (WT) transfused RBCs have increased CpG positivity when compared with native RBCs in the spleen ($*P = 0.043$); knock-out (KO) RBCs are less CpG positive when compared with native and transfused WT RBCs ($*P < 0.001$, $n = 6$ mice per group, two independent studies). (C–E) Loss of RBC-mediated CpG-DNA scavenging augments lung injury. Lungs obtained from mice treated with PBS, CpG-DNA, CpG-DNA + WT

Figure 5. Red blood cells (RBCs) attenuate CpG-induced Toll-like receptor 9 (TLR9) activation *in vitro*. (A) RBCs scavenge CpG-DNA (100 nM) from human lung microvascular endothelial cells. FITC-CpG binding to endothelial cells (ECs) or to ECs treated with CpG-DNA in the presence or absence of RBCs is shown. EC, EC + CpG-DNA, EC + CpG-DNA + RBCs (Hct, 30 or 40% is shown). (B) MFI of CpG-DNA in the treatments in A; $n = 3$ independent studies, two of the three RBC units scavenged CpG-DNA, and one representative study is shown. (C) CpG-DNA-induced activation of TLR9 was assayed with 293 cells transfected with a TLR9 reporter. CpG-DNA (10 $\mu\text{g}/\text{ml}$) induced activation of TLR9, which was attenuated with the addition of RBCs; $*P < 0.001$. (D) RBCs from RBC units attenuated CpG-DNA-induced TLR9 activation, whereas RBCs obtained from trauma patients do not attenuate TLR9 activation: 11 units tested, $*P = 0.048$; $n = 5$ trauma patients, $P = 0.160$. Bar graph shows mean $\pm$ SEM. Box-and-whisker plot shows median, interquartile range, and maximum and minimum values. FITC = fluorescein isothiocyanate; Hct = hematocrit; MFI = mean fluorescence intensity.

and trauma in humans. It is plausible that anemia after systemic injury and during trauma may account for the shift in the plasma-to-RBC mtDNA ratio due to less red cell availability; however, we did not observe elevated plasma mtDNA in the patients with decreased red cell counts. Whether alterations in the plasma-to-RBC mtDNA

ratio are due solely to excess mtDNA release that overwhelms RBC scavenging capacity during systemic inflammation and trauma versus impaired mtDNA sequestration by RBCs during inflammatory states is currently under investigation.

To elucidate the mechanism of mtDNA acquisition by RBCs, we examined

RBCs for the presence of TLR9. We detected TLR9 on RBCs by several methods and demonstrated enhanced CpG-DNA binding by both TLR9-positive human and murine erythrocytes. It has been demonstrated that nucleated erythrocytes in nonmammals express nucleic acid-binding TLRs including TLR3 and TLR9, and

Figure 4. (Continued). RBCs, and CpG-DNA + TLR9-KO RBCs were compared. WT or KO RBC transfusions (10 $\mu\text{L}/\text{g}$; hematocrit, 65%) were given simultaneously with CpG-DNA. (C) Ly6G; (D) hematoxylin and eosin; and (E) composite lung injury scores demonstrate increased tissue injury in CpG TLR9-KO RBC-treated mice when compared with all other groups: $*P < 0.001$, $n = 7$ –12 mice per group, three independent studies. Box-and-whisker plots show median, interquartile range, and maximum and minimum values. MFI = mean fluorescence intensity; PKH = membrane dye; Tx = transfused.

it has been further postulated that these erythrocytes actively participate in the innate immune response (39). Our observations of mtDNA scavenging by erythrocytes suggest a similar phenomenon is evident in murine and human RBCs. As TLR9 binds DNA derived from *Plasmodium* species and is vital in innate immune sensing during malaria, it is tempting to speculate that evolutionary pressures in malaria-endemic regions led to the retention of erythrocyte TLR9 (40, 41). TLR9 is present on other nonnucleated cells including platelets and functions in signal transduction and thrombus formation (20). However, whether erythrocyte TLR9 functions in signaling in addition to acting as a sink for mtDNA is still unknown and the subject of future studies.

Cell-free mtDNA is elevated in septic patients with acute respiratory distress syndrome, and multiple studies have examined the association of cf-mtDNA with acute respiratory distress syndrome in trauma patients (3, 42). *In vivo*, DAMPs derived from mitochondria-induced lung injury in rats and studies using a model of bacterial pneumonia demonstrate that extracellular mtDNA is increased in the vascular compartment and augments

permeability and lung injury (31). Collectively, these observations implicate mtDNA in the pathogenesis of lung injury during both sterile inflammation and infection. We have previously demonstrated that loss of RBC chemokine scavenging augments lung injury during endotoxemia; here we demonstrate the importance of RBC scavenging of mtDNA in the prevention of lung injury (24). *In vitro*, RBCs attenuated CpG-mediated TLR9 activation and scavenged CpG-DNA from lung ECs. In a mouse model CpG-DNA increased lung neutrophil infiltrate and tissue injury that worsened with the administration of TLR9-deficient RBCs. These studies suggest that RBC-mediated DNA scavenging plays a vital role in the prevention of tissue injury, as a reduction in RBC-mediated DNA-scavenging capacity with TLR9-KO RBC transfusion augmented injury. Alterations of RBC scavenging ability in addition to excess cell-free mtDNA generation during inflammatory states may be important in the development of organ failure during acute sterile and pathogen-induced inflammation. In support of this hypothesis are our findings that RBCs from traumatically injured patients do not scavenge CpG-DNA consistently. Our findings of altered DNA

scavenging by RBCs during trauma are limited given the small sample size. Larger studies to determine whether RBC scavenging ability is altered during trauma and other inflammatory disease states such as sepsis remain an active area of investigation.

In summary, we provide evidence for an *in vivo* scavenging mechanism for cf-mtDNA. Our findings reveal a novel, nontraditional role of RBCs in binding cf-mtDNA. We further demonstrate that alterations in RBC-mediated mtDNA scavenging during inflammatory states contribute to organ injury. Red cells may thus function to maintain quiescence and modulate innate immune responses through the binding and sequestration of mtDNA released under normal conditions, during inflammatory states, and after massive cell death. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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