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The evolving erythrocyte: RBCs as modulators of innate immunity

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Abstract

The field of red cell biology is undergoing a quiet revolution. Long assumed to be inert oxygen carriers, red blood cells (RBCs) are emerging as important modulators of the innate immune response. Erythrocytes bind and scavenge chemokines, nucleic acids, and pathogens in circulation. Depending on the conditions of the microenvironment, erythrocytes may either promote immune activation or maintain immune quiescence. We examine erythrocyte immune function through a comparative and evolutionary lens, as this framework may offer perspective into newly recognized roles of human RBCs. Next, we review the known immune roles of human RBCs and discuss their activity in the context of sepsis, where erythrocyte function may prove important to disease pathogenesis. Given the limited success of immunomodulatory therapies in treating inflammatory diseases, we propose that the immunologic function of RBC provides an understudied and potentially rich area of research that may yield novel insights into mechanisms of immune regulation.

Introduction

Erythrocytes are the most abundant cell type in the human body, numbering between 20 and 30 trillion and accounting for nearly 70 percent of the total cell count in the average adult(1). Erythropoiesis begins with the differentiation of multipotent hematopoietic stem cells in the bone marrow, which then give rise to erythroid-committed precursors. In the final stages of the process, known as terminal erythropoiesis, the nucleus and other organelles are extruded. These enucleated reticulocytes are then released into the bloodstream to complete their

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maturation process(2). After a lifespan of approximately 120 days, RBCs are cleared by macrophages in the spleen and liver(3).

Importantly, mammals are the only vertebrates whose erythrocytes are enucleated in their mature state. The extrusion of the nucleus and other organelles occurs through a series of complex stages, including chromatin condensation, budding, and mitophagy(2, 4). Loss of the erythroid nucleus is thought to have facilitated the evolution of mammalian endothermy, as it allowed erythrocytes to further specialize for efficient gas exchange(4).

In the classical literature, human erythrocytes have long been typecast as mere oxygen transporters. However, mounting evidence suggests that these cells also play an important role in the innate immune system(5–7). From an evolutionary perspective, the involvement of human erythrocytes in innate immunity should not be surprising, as the nucleated erythrocytes of birds, amphibians, and fishes actively participate in the immune response via production of cytokine-like factors, upregulation of viral response genes, and sequestration of pathogens through surface binding or phagocytosis(8–20), Despite their deletion of organelles during erythropoiesis and subsequent inability to carry out transcription and translation, mammalian erythrocytes retain the ability to interact with endogenous and exogenous inflammatory molecules in the blood. Research over the past several decades demonstrates that, like their evolutionary counterparts, these cells retain the ability to bind and interact with a variety of inflammatory molecules — including chemokines, nucleic acids, and pathogens — thereby regulating and modulating immune responses. Internal components of erythrocytes such as hemoglobin and heme are also formidable facets of innate immunity, capable of generating antimicrobial reactive oxygen species (ROS) to defend against invading hemolytic microbes, as well as promoting pathologic inflammatory and auto-immune responses. Lastly, the involvement of erythrocytes in host-pathogen interactions may render them crucially important in the context of sepsis and septic shock, a state of dysregulated host response to infection where the body is overwhelmed with endotoxins and other inflammatory mediators. A deeper understanding of erythrocyte function will likely be vital to elucidating the ways in which these cells contribute to the pathogenesis of a variety of acute and chronic diseases.

Evolutionary Perspectives

Erythrocyte origins

Throughout the course of evolution, the cellular components of the blood have undergone functional specialization. The earliest blood cells are thought to have been relative generalists, involved in both phagocytosis and nutrient delivery. However, they did not serve a function in oxygen transport(21, 22). Prior to the emergence of circulatory systems, small invertebrates accomplished tissue oxygenation by simple diffusion alone; this mechanism is still observed today in primitive animals such as the sponges, cnidarians, and flatworms(21, 23). Later, the emergence of larger body plans necessitated more efficient oxygen delivery mechanisms, leading to the evolution of increasingly elaborate circulatory systems(23) and a variety of oxygen-carrying respiratory pigments(24, 25). Hemoglobin appears to have arisen first among them, followed by the hemerythrins, the molluscan hemocyanins, and eventually the arthropod hemocyanins(25). Although hemoglobin likely began as a free-floating

protein, the emergence of cells to encapsulate the pigment (i.e., erythrocytes) appears to have occurred soon after(24, 25). All modern vertebrates — with the notable exception of white-blooded ice fish from the family *Channichthyidae*(26) — accomplish oxygen delivery by means of circulating, hemoglobin-rich erythrocytes(24). Similar cells are sporadically observed in numerous invertebrate groups as well, including the echinoderms, phoronids, annelids, nemerteans, and mollusks(25, 27). Erythrocytes may have arisen separately in multiple animal lineages or once prior to the divergence of the animal phyla, although the precise evolutionary history remains unresolved(21, 22, 27).

Erythrocytes of non-mammalian vertebrates

The erythrocytes of non-mammalian vertebrates are nucleated, save rare exceptions in the fishes(28) and amphibians(29, 30). Furthermore, like many other hematopoietic cells, non-mammalian erythrocytes directly participate in the immune response. In fishes(8, 16), amphibians(19), and birds(9, 13), erythrocytes actively undergo transcription and protein production in response to environmental cues. Specifically, Atlantic salmon (*Salmo salar* L.) erythrocytes increase transcription of antigen-presenting and viral-response genes during piscine orthoreovirus (PRV) infection and upregulate interferon alpha (IFN-α) in response to infectious salmon anemia virus (ISAV), contributing to host resistance(10, 11). Erythrocytes in striped beakfish (*Oplegnathus fasciatus*) appear to coordinate immune function with leukocytes, upregulating an additional 338 genes when incubated with leukocytes and lipopolysaccharide (LPS) compared to LPS alone(18). Furthermore, both chicken and trout erythrocytes release cytokine-like factors following exposure to fungal antigens(8, 9), and express functional pattern recognition receptors (including toll-like receptors and peptidoglycan recognition proteins) on their surfaces(31, 32). Remarkably, trout erythrocytes have even been observed engaging in phagocytosis of pathogenic yeast (*Candida albicans*) (12).

Lastly, the erythrocytes of trout and chickens form cellular aggregations known as rosettes, which serve to expedite pathogen destruction by macrophages(12, 17). The rosetting behavior of piscine and avian erythrocytes stands in stark contrast to that of human erythrocytes, which is notoriously observed in severe cases of *Plasmodium falciparum* malaria. Rosetting in this context is characterized not as a defense mechanism of the human host, but rather as a virulence mechanism of the malarial parasite(20). Infected erythrocytes adhering to uninfected erythrocytes and endothelial cells results in a sequestration of blood cells in affected organs, which, particularly when localized in cerebral tissue, is associated with mortality(20). It is possible that this rosetting behavior was co-opted by the malarial parasite, or that it is the maladaptive consequence of an evolutionarily conserved mechanism of pathogen sequestration.

Mammalian erythrocytes

Mammals are the only vertebrates with enucleated erythrocytes under homeostatic conditions. Intriguingly, however, nucleated erythrocytes are sometimes observed in humans during pathologic states, constituting a striking exception and suggesting that enucleated erythrocytes are not an obligate feature of mammalian physiology(33–35). Mammalian erythrocytes also lack endoplasmic reticula, ribosomes, and mitochondria, as early

developmental processes expunge these typically vital organelles(2). It has been hypothesized that the deletion of nuclei and organelles in mature erythrocytes may have allowed for increased surface-area-to-volume ratio, facilitating increased gas exchange efficiency(36–38). Moreover, the feature allowed for elevated cellular hemoglobin capacity(4), increased pliability, and an improved ability to traverse small capillaries(38, 39).

It is possible that erythrocyte enucleation evolved in response to a reduction in Earth's atmospheric oxygen concentrations(36) or due to the increased metabolic demands of endothermy(38). In line with the latter hypothesis, erythrocytes are comparatively larger in ectothermic, phylogenetically lower organisms like fishes and herptiles(37, 40). The extrusion of mitochondria in mammalian erythrocytes may also have reduced production of damaging reactive oxygen species (ROS)(41). This is an attractive hypothesis, given that human erythrocytes generate extensive free radicals even without mitochondria, necessitating their ample arsenal of protective antioxidant molecules and enzymes(42). However, avian erythrocytes exhibit lower levels of blood oxidative stress and similar cellular hemoglobin content in comparison to mammals, despite their retaining both functional mitochondria and nuclei(4, 43), indicating that the presence of mitochondria and nuclei in erythrocytes is not necessarily a barrier to endothermy. Because endothermy arose separately in the avian and mammalian lineages, it is perhaps to be expected that alternative suites of physiologic adaptations accompanied it.

Regardless of the specific pressures driving the loss of organelles in mammalian erythrocytes, the change appears to have been an evolutionary compromise: opting for greater respiratory specialization, the forfeiture of key cellular components rendered mammalian erythrocytes more passive actors than those of other vertebrates. Without ribosomes or a genome, mammalian erythrocytes are incapable of signal transduction, gene transcription, or new protein synthesis, and are therefore unable to contribute to the immune response via *de novo* production of immunologically active signaling molecules. However, there is significant evidence that mammalian erythrocytes remain key contributors to innate immunity. Despite their loss of internal organelles, mammalian erythrocytes express a large number of cell surface receptors that mediate their interactions with both endogenous and exogenous agents in the blood(44), providing them with an extensive capacity for scavenging or sequestration of circulating molecules that impact immune function.

Hemoglobin and heme

Among the respiratory pigments, hemoglobin is the most ancient(25). Circulating hemoglobin appears to have arisen from the tissue-localized hemoglobins of primitive animals, such as the turbellarian platyhelminths(25). While oxygen carriage is their primary function, hemoglobin and free heme also participate in the innate immune response.

Because hemoglobin provides a rich source of iron for bacterial metabolism(45–47), microbial invaders have evolved both hemolytic toxins and high affinity scavenging systems in order to access it(48–50). In response, host hemoglobin provides a counterattack: activated by the extracellular proteases of virulent bacteria, the peptide emits bactericidal reactive oxygen species (ROS) in the microbial vicinity(51). Studies show that both the alpha and beta subunits of hemoglobin contain high-affinity binding sites for

lipopolysaccharide (LPS), the immune-stimulating endotoxin present in the membranes of Gram-negative bacteria. When LPS binds to hemoglobin, it stimulates the redox activity of the peptide and leads to the production of anti-microbial free radicals(52). Additionally, the presence of hemoglobin has been shown to amplify macrophage cytokine production in the presence of LPS, as well as enhance macrophage binding of LPS(53). Lastly, micromolar concentrations of hemoglobin fragments are sufficient to inhibit the growth of yeast and bacteria (both Gram-positive and Gram-negative) via ROS production, while demonstrating no toxic effects on human blood cells(54).

The immunologic functions of respiratory globins are widespread across the animal kingdom. Invertebrate horseshoe crabs (*Tachypleus tridentatus*) contain blue-tinted blood rich in hemocyanin, a cell-free protein that carries oxygen throughout the blood of many mollusks and arthropods. In these animals, hemocyanin similarly generates ROS in response to microbial challenge(51, 55). The antimicrobial properties of globins are so universal that the cattle tick (*Boophilus microplus*) can appropriate fragments of its host's hemoglobin for use in its own gut(56). Beyond their function in gas exchange, the utility of respiratory pigments as innate immune elements has been realized at multiple points throughout evolution(12, 25, 55).

While hemoglobin may confer antimicrobial protection under homeostatic conditions, research indicates that it has the opposite effect during pathologic states. Particularly when the system is overwhelmed with endotoxin, as is the case in severe sepsis, elevated levels of cell-free hemoglobin are associated with increased mortality(57, 58). Interestingly, intraperitoneal injection of globin had protective effects on endotoxemic mice(57), while intravenous injection of hemoglobin into endotoxemic mice dramatically increased rates of mortality(58). And while globin alone reduces macrophage TNF production in the presence of LPS, thereby dampening immune response to endotoxin, hemoglobin actually stimulates macrophage TNF production and triggers the release of pro-inflammatory cytokines under the same conditions(57). The conflicting nature of the hemoglobin literature appears to be due to the dualistic properties of the hemoglobin molecule itself, in which — broadly speaking — globins attenuate immune response(57, 59) while heme exacerbates it(60, 61). These effects are likely attributable to the ability of globin to scavenge excess free heme during endotoxemia, resulting in attenuated immune activation. In contrast, if heme is inflammatory, injecting hemoglobin could serve to exacerbate this effect.

Heme has also been characterized as a potent danger signal *in vitro*, capable of activating NF-κB-mediated expression of pro-inflammatory proteins (including IL-1 and TNF-α), generation of intracellular ROS, 100-fold increase in transcription of pro-inflammatory genes, and macrophage release of TNF-α and interleukin-1(62). It has been suggested that free heme is unable to cause damage on its own, but rather produces deleterious effects only in the presence of other immune modulators(63). However, others have demonstrated that free heme is an independent activator of TLR4(64), stimulating secretion of TNF-α, ROS, and leukotriene B4 from macrophages(61, 64) and release of high mobility group box 1 (HMGB1) from hepatocyte nuclei(60).

Human Erythrocytes and the Innate Immune System

Chemokine binding

A number of studies have revealed that red blood cells serve a variety of functions beyond oxygen transport(6, 7, 65, 66). One important immunomodulatory property of human erythrocytes is their propensity to bind a wide variety of chemokines. One major locus for binding is the Duffy antigen receptor for chemokines (DARC), which was first recognized by Darbonne and colleagues in 1991(7). This work demonstrated that erythrocytes act as a sink for CXCL8, thereby inactivating the CXCL8-dependent chemokine gradient and preventing neutrophil recruitment(7). Duffy binds other immunomodulatory proteins at high affinity as well, including additional CXC and CC chemokines besides CXCL8(6, 65). *In vitro* experiments by Lee et al. demonstrated that blood lacking erythroid Duffy exhibited higher plasma chemokine levels following LPS exposure, further implicating Duffy in chemokine scavenging(65). This is an evolutionarily conserved feature of DARC, as Duffy knockout mice exhibit higher chemokine (i.e., MIP-2 and KC) concentrations in the lung vascular space(65). Some have proposed that the sequestration of inflammatory molecules by red cell surface receptors functions to dampen immune response via attenuation of neutrophil signaling(7). Such an inhibitory system could confer a selective advantage, as overstimulation of the immune system can generate excessive inflammation and lead to tissue damage.

However, an alternative model for widespread binding of chemokines by Duffy receptors may actually result in the maintenance of blood chemokine levels, as chemokines would remain in circulation rather than being cleared. In support of this view, Duffy wild type mice contain significantly higher blood chemokine concentrations than Duffy knockout mice(66). Darbonne's aforementioned experiments — which concluded that chemokine binding is superficial and easily reversible — may also hint at the potential for sustained immune activation, as erythrocyte receptors could alternately bind and release their substrates in the tissue microenvironment.

These two models for erythrocyte regulation of blood chemokine levels can be termed the "sink" hypothesis (i.e., erythrocyte chemokine binding serves to diminish immune-activating signals) and the "reservoir" hypothesis (i.e., erythrocyte binding prevents chemokine clearance, thereby prolonging chemokine half-life in the blood). While these models appear contradictory at first, they may not be mutually exclusive: Fukuma et al. suggested that erythrocytes do scavenge chemokines from sites of inflammation, but eventually release them in response to decreases in plasma chemokine concentration, effectively maintaining homeostasis(66).

Nucleic acid binding

In addition to regulating chemokines, recent research demonstrates that erythrocytes are also capable of binding another class of molecules with the potential to mediate inflammatory responses: nucleic acids. Specifically, our recent work has focused on the ability of erythrocytes to bind DNA derived from mitochondria (mtDNA)(5). Mitochondria comprise a sizable endogenous reservoir of potentially inflammatory nucleic acids, which are released

into circulation during normal cell turnover, by activated immune cells, and during cell death. Similar to the DNA of their bacterial ancestors, mtDNA is rich in immunostimulatory CpG motifs and initiates pro-inflammatory signaling through direct binding of CpG-containing DNA regions to toll-like receptor 9 (TLR9). Furthermore, mtDNA is known to activate the inflammasome(67, 68), and cytosolic mtDNA is a potent inducer of STING (stimulator of interferon genes), an ER protein that senses a cellular second messenger produced in response to cytosolic double-stranded DNA(69–72) and bacterially derived cyclic dinucleotides(69, 73). A growing body of literature implicates these mitochondrial DAMPs (damage-associated molecular patterns) in the pathogenesis of a variety of diseases, including autoimmunity, cancer, cardiovascular disease, trauma, and critical illness(74–78).

We recently reported that human erythrocytes express TLR9, potentially providing human erythrocytes with the capacity to scavenge or buffer potentially inflammatory nucleic acids in the circulation(5). Whereas in non-erythroid cells, TLR9 is trafficked to endolysosomal compartments and cleaved by lysosomal cathepsins in order to initiate innate immune signaling(79–81), it remains unknown whether erythroid surface TLR9 undergoes cleavage and signaling after binding mtDNA. We found that TLR9-positive erythrocytes bind and sequester circulating cell-free mtDNA, and *in vivo* mouse studies demonstrated that loss of this function results in enhanced lung injury during states of inflammation(5). Additionally, RBCs scavenged CpG-DNA from human microvascular endothelial cells (HMVECs) *in vitro*, which may serve to attenuate endothelial TLR9 activation(5).

Notably, there exists significant heterogeneity in erythroid TLR9 expression both between and within individuals(5). Some individuals express relatively high percentages of erythroid TLR9, while others express virtually none. Even in those who do express erythroid TLR9, the receptor is only present in a fraction of the total RBC population. Nevertheless, due to the vast number of erythrocytes in circulation — between 20 and 30 trillion in the average adult(1) — even a minority of TLR9-positive cells would have incredible potential to influence systemic function or modulate organ injury.

We are just beginning to understand the ways in which erythrocyte binding of nucleic acids influences systemic function. As in the case of RBC chemokine binding, nucleic acid scavenging may have discrepant effects on inflammation. In some instances, RBCs may attenuate inflammation in the lung compartment by scavenging mtDNA from endothelial cells; however, given the close mechanical contact between erythrocytes and the microvascular endothelium, RBCs binding mtDNA could also exacerbate inflammation by incidentally presenting the inflammatory molecules to immune or endothelial cells. Furthermore, mtDNA binding causes crenation of the erythrocyte membrane, which could result in accelerated clearance of abnormal RBCs or entrapment of the RBCs in the microvasculature.

While the recent results are consistent with a model in which RBCs scavenge mitochondrial DAMPs from the lung compartment, the dynamics of mtDNA scavenging may be more nuanced. It is possible that, similarly to findings with Duffy antigen and the chemokines that it binds, mtDNA binding could act either as a protective or pathologic mechanism, with the precise impact being largely dependent on the conditions of the local microenvironment.

It is not yet known whether RBCs are also capable of scavenging other types of nucleic acids. However, recent data suggests that Zika RNA may persist on red cells during storage(82). The recent discovery of numerous TLRs (including TLR3) in the transcriptome of mammalian reticulocytes offers a potential mechanism by which this binding could occur, and intimates that still other TLRs and nucleic acid scavengers may yet be found on the surface of mature human erythrocytes(83). Furthermore, RBC binding of viral material may be important for disease pathogenesis, and may also have implications regarding safe blood storage and transfusion practices.

Pathogen binding

In addition to their affinity for endogenous molecules like mitochondrial DNA and chemokines, human erythrocytes are also known to bind pathogens. Undoubtedly the most infamous and evolutionarily consequential erythrocyte pathogen belongs to the genus *Plasmodium,* a group of protists responsible for causing malaria. These pathogens have exerted the strongest evolutionary pressures on the human genome in recent millennia(84).

Each *Plasmodium* species has evolved distinct mechanisms for adhering to human erythrocytes. *Plasmodium vivax* and *P. knowlesi* attach via the aforementioned Duffy antigen receptor for chemokines(85, 86), rendering the presence of Duffy so maladaptive in high-malaria regions that nearly all West Africans and 68 percent of African Americans exhibit a Duffy-negative phenotype(87). Clearly, the benefits conferred by Duffy's ability to scavenge chemokines were dwarfed by its deleterious effects in the presence of malaria, resulting in the spread of the allele conferring Duffy negativity.

The most virulent of the human malarial parasites, *P. falciparum,* invades erythrocytes after adhering to glycophorin A, B, or C(88, 89). Although individuals deficient in any of the glycophorin types exhibit increased resistance to *P. falciparum*, glycophorin A (GYPA) paradoxically remains the most common erythrocyte surface protein in humans(12, 44, 90). Complete deficiency of erythrocyte GYPA is a viable but extremely rare phenotype, suggesting that the absence of GYPA must come at a cost(44), or that GYPA's presence must provide sufficient benefit to override the selective pressure exerted by *P. falciparum.* Otherwise, *P. falciparum* would presumably have driven the widespread disappearance of GYPA in malarial regions.

GYPA may act as a "decoy receptor," chaperoning pathogens away from important tissues and into the spleen to facilitate their destruction by macrophages(44). In support of this hypothesis, many of the pathogens that bind human glycophorins (e.g., reovirus, influenza C, Sendai, *Mycoplasma pneumoniae*, *Escherichia coli*, and *Ureaplasma urealyticum*) do not infect erythrocytes themselves(44). This could also help explain why GYPA persists on the erythrocyte surface despite selective pressure from *P. falciparum*. Engineering decoy viral receptors on erythrocytes to facilitate adsorption and prevent invasion of nucleated cells therefore provides a potentially promising approach to clear pathogens from circulation. Indeed, such an approach has been shown to attenuate coxsackievirus B infection in murine models(91).

Unfortunately, not all decoy adsorption has desirable effects. Notably, human erythrocytes bind HIV-1 virions, and this binding results in the 100-fold enrichment of viral infectivity(92). Additionally, HIV-1 binding to DARC on erythrocytes maintains virus viability, and presents viral particles to CD4-positive T cells(93). In some cases, erythrocyte binding activity may therefore facilitate trans infections of other viruses as well. As in the case of chemokine and mtDNA binding, local conditions in the bloodstream and the specific interactions between viral ligands and their host receptors may ultimately determine whether erythrocyte pathogen binding proves advantageous or detrimental to the host.

RBCs in the Context of Modern Medicine

Critical illness

Given their capacity for interaction with both exogenous and endogenous inflammatory molecules, erythrocytes likely play a major role in the context of critical illness. During sepsis, the circulation is inundated with excess cell-free nucleic acids, pathogens, and chemokines, all of which have been shown to bind to erythrocytes. Thus, any loss of erythrocyte function has the potential to dramatically alter host response and contribute to the pathogenesis of these conditions.

Research indicates that physical and chemical changes do occur to erythrocytes during periods of sepsis and endotoxemia(94). Septic shock decreases erythrocyte 2,3-bisphosphoglycerate levels, renders them less deformable, and reduces their ability to traverse microvessels(94–96).

Crenation of the RBC membrane is another potentially important component of sepsis pathology. This cellular deformation appears to be a consequence of mtDNA binding(21), and elevated levels of cell-free mtDNA during sepsis likely increase the incidence of RBC crenation. We speculate that crenation may expedite RBC clearance and turnover, potentially leading to reduced RBC counts and anemia. Lastly, crenated RBCs may also be involved in inflammation, organ failure, tissue ischemia, and vascular occlusion, all of which are central to the sepsis phenotype.

Furthermore, erythrocytes of critically ill trauma patients demonstrate diminished mtDNA scavenging ability(5). This may be highly relevant to the disease pathogenesis of severe sepsis, as recent studies have concluded that elevated levels of plasma mtDNA during sepsis predict mortality(97, 98). The loss of scavenging ability could be attributable to saturation of erythroid TLR9 receptors due to the sheer amount of excess nucleic acids in circulation or to cellular damage incurred by erythrocytes during inflammatory states. Whether defective nucleic acid sequestration by RBCs contributes to the development of lung or other organ injury during sepsis remains unknown. Additionally, future research should aim to determine whether erythroid TLR9 has the ability to bind bacterial CpG-DNA in addition to mtDNA, as this could potentially illuminate components of disease pathogenesis.

Cell-free heme is also an important factor in the context of critical illness. In states of infection or inflammation, free heme can act as a damage associated molecular pattern (DAMP), contributing to excess inflammation, tissue damage, and even death in susceptible

hosts(60, 61). Various scavengers of free heme have been identified, and reduced levels of these heme scavengers are also associated with increased mortality(60). One such heme scavenger, heme oxygenase-1 (HO-1), catabolizes free heme and is upregulated following LPS exposure(99). While the carbon monoxide produced by HO-1 catabolism of heme can be a source of ROS signaling and oxidative stress, the overall effects of the HO-1/CO system are anti-inflammatory due to its reduction of TNF-α, IL-8, and MIP-1β levels and upregulation of IL-12(99). HO-1 has been shown to be protective in individuals with severe sepsis, suggesting that elevated levels of circulating free heme are central to the pathogenesis of the condition(60). Additionally, low levels of the heme sequestering protein hemopexin (HPX) are associated with increased mortality during sepsis, and administration of HPX during septic shock reduced probability of mortality(60).

Critically ill patients are prone to developing acute respiratory distress syndrome (ARDS), a life-threatening syndrome characterized by disruption of the alveolar endothelial-epithelial barrier resulting in leakage of proteinaceous fluids into the airspaces, loss of lung compliance, hypoxia, and respiratory failure(100). In pre-clinical models of lung injury, loss of erythrocyte DARC increased lung inflammation and airspace neutrophil recruitment.(101, 102) Consistent with these findings, Kangelaris et al. reported that African American patients possessing a gene polymorphism that abrogates erythroid DARC expression was associated with a 17 percent increased risk of mortality from ARDS compared to patients of European ancestry(103). African American patients with erythroid DARC, however, fared similarly to European American patients(103). Thus, it is possible that reduced erythrocyte chemokine scavenging function during critical illness may compound inflammation in susceptible hosts.

Malaria

In the context of malaria, free heme is generated following parasite digestion of hemoglobin. To protect themselves from the cytotoxic effects of heme, *P. falciparum* parasites convert the compound into an insoluble crystal known as hemozoin. Hemozoin itself is immunologically inert; however, it becomes coated with malarial DNA and subsequently presents the DNA to host TLR9, thereby magnifying the innate immune response(104). Whether erythroid TLR9 is important in the pathogenesis of malaria remains to be investigated.

Appearance of nucleated erythrocytes in disease states

Lastly, it is worth noting that there have been numerous documented cases of nucleated red blood cells (NRBCs) circulating in adult humans with severe diseases(33, 34). This is a highly abnormal phenotype, as NRBCs are typically only observed in the peripheral bloodstream of fetuses and neonates(33), yet it is observed in 10 to 30 percent of critically ill patients(35). Patient prognosis is poor in these cases, with over 50 percent of individuals dying in the hospital. The presence of NRBCs in the blood has been characterized as an independent predictor of mortality — typically portending death within one to three weeks(34).

Given what is known about the immunologic functions of nucleated erythrocytes in non-mammalian vertebrates, it is tempting to speculate that the reappearance of this

evolutionarily ancient trait in humans may serve a functional purpose. For instance, NRBCs could represent a final effort to extrude as many immunologically active cells into circulation as possible in an attempt to overcome a life-threatening insult. While this is highly speculative, further investigation may uncover previously unrecognized immune activities of human NRBCs.

Conclusions

Altogether, these findings provide mounting evidence that erythrocytes play a direct and significant role in innate immunity and inflammation. Given that erythrocytes are the most abundant cell in the human body(1), they have the capacity to exert substantial effects on immune function. Although this review outlines several immunomodulatory functions of RBCs, the full scope of erythrocyte involvement in the human immune response remains to be defined.

Numerous unanswered questions persist, including whether other pathogen recognition receptors exist on mature RBCs. Furthermore, while the metabolic functions of RBCs are well established, it is unknown whether changes in RBC metabolism may contribute to alterations in immune function. In addition, further investigation into the dualistic nature of erythrocyte immune modulation is required. Research thus far suggests that the utility of RBC binding activities is context-dependent, sometimes exacerbating the immune response and sometimes maintaining immune quiescence, but the precise mechanisms and environmental factors moderating this dualism remain enigmatic. Lastly, it remains unresolved whether RBC alterations during disease states are the product or the cause of disease pathogenesis.

Further research into the immune functions of erythrocytes will be crucial to understanding not only diseases that affect red blood cells directly (e.g., malaria and sickle cell disease), but also chronic and acute inflammatory states where regulation of DAMPs and other mediators is essential for disease control (e.g., sepsis, trauma, autoimmunity). Given the immune functions of erythrocytes in other vertebrate orders, comparative evolutionary approaches may yield insights into undiscovered functions of erythrocytes in humans. In light of recent findings demonstrating that Zika RNA persists on stored RBCs(82) and the presence of toll-like receptors on erythrocytes(5), we stress the importance and urgency of continued research into the immunologic functions of human red blood cells.

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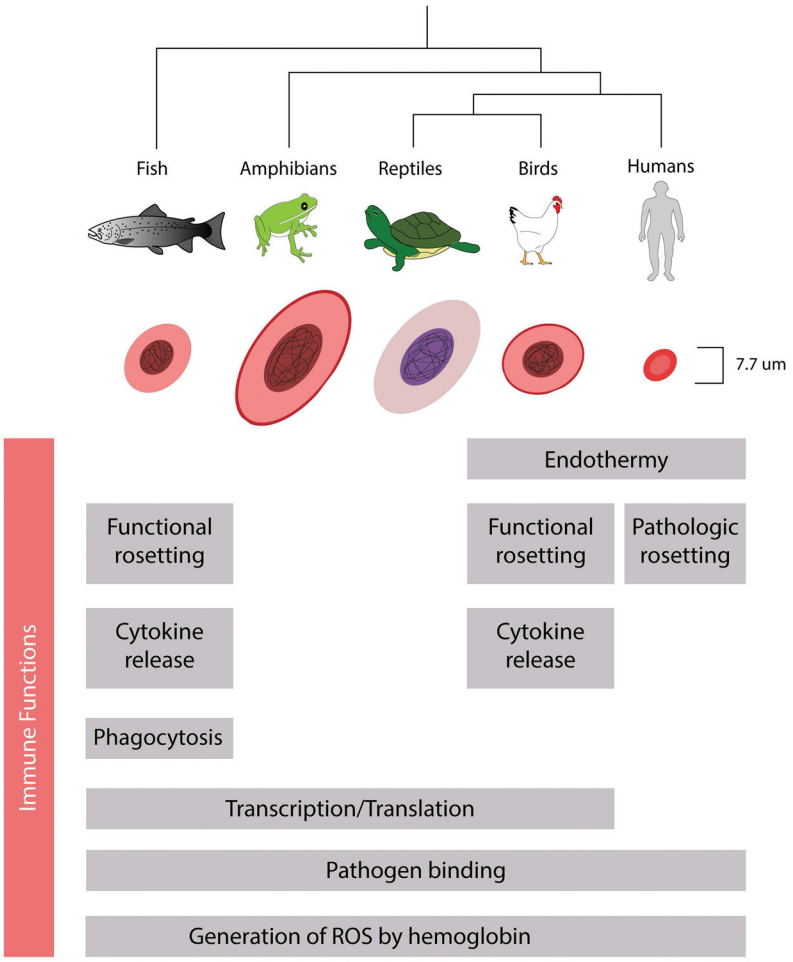

Figure 1. Comparing the immunologic properties of erythrocytes across vertebrate orders

The erythrocytes of lower vertebrates (i.e., fishes, amphibians, reptiles, and birds) are nucleated in their mature state. Erythrocytes of fish and birds are known to actively undergo transcription and translation, produce and release cytokines, form rosettes to sequester pathogens, and, in the case of trout, phagocytose pathogens. While the activities of erythrocytes in reptiles and amphibians are less well studied, it is likely that they retain many of these characteristics given that they have been maintained across the phylogenetic distance from fish to birds. Despite lacking nuclei and organelles, mammalian (and human) erythrocytes retain the ability to influence innate immunity. All erythrocytes contain hemoglobin, which participates in host defense by generating antimicrobial reactive oxygen species (ROS).

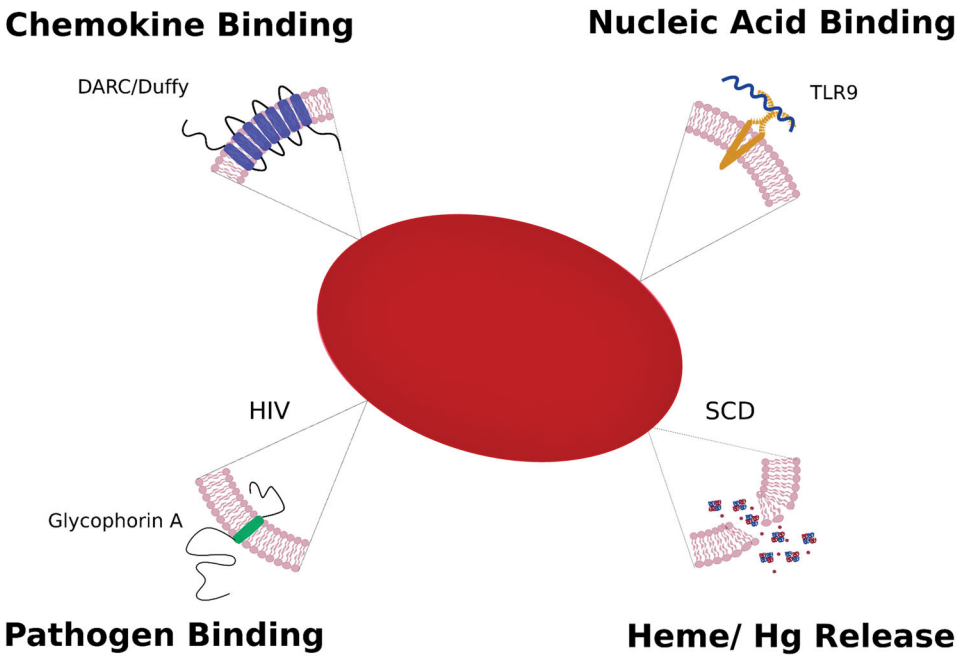

Figure 2. An overview of the binding activities of human erythrocytes

Clockwise from top left. (a) Erythrocytes bind chemokines through the Duffy antigen receptor for chemokines (DARC), thereby modulating neutrophil recruitment and immune activation. (b) Recent studies have shown that erythrocytes are capable of binding and scavenging mitochondrial DNA (mtDNA) via toll-like receptor 9 (TLR9). It is not yet known whether erythroid TLR9 can also scavenge other CpG-rich DNA fragments (e.g., bacterial DNA). (c) Lysed erythrocytes can release heme and hemoglobin into the extracellular environment. In the vicinity of invading microbes, hemoglobin can bind LPS and generate toxic reactive oxygen species (ROS) that function in host defense. However, the release of free heme during states of inflammation can result in excessive immune activation, leading to cellular injury or death. (d) Glycophorin A (GYPA) has been identified as a potential “decoy receptor” on the surface of human erythrocytes, functioning to sequester pathogens and facilitate their removal from circulation.

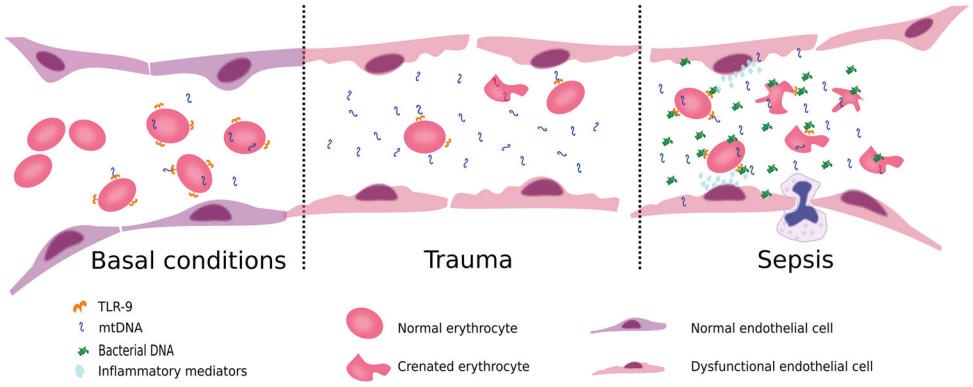

Figure 3. A systemic model of erythrocyte involvement in critical illness

(a) Under basal conditions, erythrocytes contribute to immune homeostasis by binding and scavenging mitochondrial DNA (mtDNA) via toll-like receptor 9 (TLR9). (b) During states of trauma, extensive cell death leads to increased cell-free mtDNA levels. TLR9-positive erythrocytes scavenge these nucleic acids. Erythrocytes binding mtDNA become crenated (i.e., deformed), which could either expedite their clearance from the circulation or trigger the activation of endothelial cells. Unbound mtDNA may engage immune cells leading to increased inflammation. (c) In sepsis, the circulation is overwhelmed with both bacterial and mitochondrial DNA, as well as free heme (not shown). It remains unknown whether mtDNA-binding capacity of erythrocytes is reduced during sepsis.

Table 1

The dualistic nature of RBCs in innate immune function and its relevance to human disease states

Depending on the conditions of the local and systemic microenvironment, immunomodulatory components of human RBCs may function to either repress or promote inflammatory responses.

RBC Component	Innate Immune Function	Promotes Immune Response	Maintains Immune Quiescence	Relevant Disease States
Duffy antigen receptor for chemokines (DARC)	Binds chemokines[6,7,65] Binds pathogens[85,93]	• Maintains chemokine gradient[66] • Serves as entry point for *P. vivax*[85] • Binds HIV-1[93]	• Scavenges and sequesters chemokines[7]	• HIV[93] • Malaria[85–87] • Critical Illness[103]
Toll-like receptor 9 (TLR9)	Binds nucleic acids[5]	• ? Presents mtDNA to immune or endothelial cells[5] • Results in RBC crenation[5]	• Scavenges and sequesters inflammatory nucleic acids[5]	• Trauma[5]
Glycophorin A (GYPA)	Binds pathogens[44,88,89]	• ? Facilitates *trans* infections • Serves as attachment site for *P. falciparum*[88,89]	• Facilitates pathogen destruction via decoy receptor binding[44]	• Malaria[88,89]
Heme/Hemoglobin	Generates reactive oxygen species (ROS)[51,52]	• Activates toll-like receptor 4 (TLR4)[64] • Amplifies cytokine production in the presence of LPS[53,57]	• Prevents the growth of yeast and bacteria[54]	• Malaria[103] • Sepsis[60] • *Staphylococcus* infection[48]