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Bat Red Blood Cells Express Nucleic Acid–Sensing Receptors and Bind RNA and DNA

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ABSTRACT

RBCs demonstrate immunomodulatory capabilities through the expression of nucleic acid sensors. However, little is known about bat RBCs, and no studies have examined the immune function of bat erythrocytes. In this study, we show that bat RBCs express the nucleic acid–sensing TLRs TLR7 and TLR9 and bind the nucleic acid ligands, ssRNA, and CpG DNA. Collectively, these data suggest that, like human RBCs, bat erythrocytes possess immune function and may be reservoirs for nucleic acids. These findings provide unique insight into bat immunity and may uncover potential mechanisms by which virulent pathogens of humans are concealed in bats. ImmunoHorizons, 2022, 6: 299–306.

INTRODUCTION

A fundamental role of the immune system is nucleic acid sensing, which is essential for pathogen detection and coordination of host defense (1, 2). TLRs are critical for nucleic acid recognition and include TLR9, which detects DNA containing unmethylated CpG motifs present in bacterial, viral, and mitochondrial DNA, and TLR7, which recognizes ssRNA present in viruses and host RNA. Most studies on TLR function have focused on their roles in classical immune cells, although some enucleated cells, such as platelets, also express TLRs (3, 4). Because mammalian RBCs are devoid of organelles, the prevailing assumption has been that they lack immune function. However, contrary to this dogma, a DNA-sensing role for RBCs through the expression of TLR9 has recently been discovered (5). Whether bat RBCs bind nucleic acids or express TLRs is unknown.

Members of the order Chiroptera (bats) are exceptional in their ability to harbor deadly viruses without developing disease (6). Although substantial evidence supports a bat origin for numerous viruses, how bats tolerate such viruses and other pathogens remains unknown (7). Bats are hosts to diverse intraerythrocytic hemosporidian parasites and coevolved with these parasites (8). In addition, as an adaptation to flight, the number of erythrocytes per microliter of blood is 2- to 5-fold higher than in humans, increasing the total red cell surface area (9). Despite these observations, little is known regarding bat erythrocytes and their interaction with the immune system. Because we identified TLRs on mammalian RBCs, we asked whether bat RBCs similarly express TLRs and bind nucleic acids.
**MATERIALS AND METHODS**

**Animals and ethic statements**
Animal studies were conducted using terminal blood samples obtained from bats in rehabilitation and provided by the Pennsylvania Game Commission. All studies were done in accordance with the Institutional Animal Care and Use Committee. Studies using human specimens were approved by the Institutional Review Board. Healthy volunteers provided informed consent. Healthy volunteers provided informed consent before study participation.

**Bat blood collection**
Bats were anesthetized with isoflurane and then euthanized via cervical dislocation. Blood was collected either via cardiac puncture or from major vessels after decapitation and placed in EDTA microtainers. Samples were centrifuged for 15 min at 3500 rpm (maximum relative centrifugal force = 1534 × g) and then transferred to the University of Pennsylvania for further analysis.

**Human RBC isolation**
Healthy volunteers provided informed consent. Whole blood was collected in EDTA tubes before centrifugation at 3000 × g for 10 min. The plasma and buffy coat were aspirated. RBCs were purified from the remaining packed red cell fraction using MACS as previously described (5).

**Blood processing and RBC isolation**
Whole blood was centrifuged at room temperature for 5 min at 800 × g. Plasma and buffy coat were isolated and saved. The remaining packed RBCs were resuspended in 1 ml PBS for washing. Then, the cells were centrifuged, and the supernatant was aspirated along with a small amount of the packed RBCs on the top. To determine the yield of RBCs, we counted serially diluted packed RBCs on a hemacytometer. Immediately after this, the RBCs were either used for functional assays, fixed, or frozen in aliquots. To freeze RBCs, we added 10^7 RBCs to DNA diluted packed RBCs on a hemacytometer. Immediately after

The cells were resuspended in 300 μl PBS and analyzed by flow cytometry (FC; BD Fortessa). Immediately before data acquisition, the cells were resuspended by pipetting up and down repeatedly.

**Viral RNA binding assays**
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and Zika virus (ZIKV) RNA were provided by Dr. K. Jurado (University of Pennsylvania). A total of 10^7 RBCs in 100 μl sterile PBS were transferred to 2 ml DNA/RNA lo-bind tube (86-924; Eppendorf) and mixed with the indicated amount of viral RNA diluted in sterile PBS. The mix was incubated at 37°C for 2 h on a nutator. At 1 h after incubation, tubes were rotated to ensure cells remain resuspended. After the incubation, cells were washed with 1 ml sterile PBS three times and frozen at −80°C before RNA extraction.

**Surface Ab staining**
The cells were resuspended in FACS buffer (PBS + 2% FBS) to 10^7 cells/ml. For each staining reaction, 10^6 RBCs were pelleted in round-bottom polystyrene tubes and stained in 100 μl of an Ab mix diluted in FACS buffer. The cells were rocked at room temperature for 1 h in the dark. The cells were washed with 1 ml PBS twice as described earlier and analyzed by FC.

**RBC fixation**
RBCs were resuspended to 10^7 cells/ml in PBS, and an equal volume of 0.1% glutaraldehyde diluted in PBS was added to the cells for a final concentration of 0.05%. The cells were fixed at room temperature in the dark for 10 min. Fixation was stopped by adding equal volume of FACS buffer to the cells. The cells were pelleted at 800 × g for 5 min and washed a total of three times. For long-term storage, fixed cells were resuspended in FACS supplemented with 0.05% sodium azide and stored at 4°C.

**Intracellular staining with FC**
A total of 10^6 RBCs were used in each staining reaction. Fixed RBCs were permeabilized in 0.1% Triton X-100 diluted in PBS.
for 15 min at room temperature. The cells were washed and pelleted in FACS buffer, blocked in PBST (PBS + 0.05% Tween 20) supplemented with 1% BSA for 1 h, and then pelleted and resuspended in Ab diluted in PBST. Information for the Abs used is listed later. For FC staining, cells were incubated with either Ab or isotype controls diluted in FACS buffer for 1 h in the dark at room temperature under gentle shaking conditions. Subsequently, the cells were washed with FACS buffer twice and stained with a secondary Ab diluted in PBST for 45 min at room temperature. The cells were then washed in FACS buffer three times before analysis.

Immunofluorescent staining
RBCs were fixed, permeabilized, and blocked as described earlier. The primary Ab was diluted in PBST with 1% BSA, and incubation was performed overnight at 4°C. Subsequently, cells were washed in PBST three times and resuspended in a secondary Ab diluted in PBST. After 1 h of staining at room temperature, the cells were washed in PBST three times. After the final wash, the buffer was aspirated, and the residual volume of the RBCs was further reduced by pipetting. The remaining cells were resuspended in no more than 10 μl PBS. To mount cells on a slide, no more than 5 μl of Fluoromount G was added to a microscope slide, and 5 μl RBCs was mixed in it, and samples were sealed with a coverslip.

Abs used and their applications
For all Abs, the target, clone, dilution, and vendor are listed: TLR3, 40C1285.6, FC: 5 μg (Novus Biological); TLR7-4G6, immunofluorescence (IF): 1:200, FC: 1:200 (Novus Biological); TLR7-726606, IF: 1:200, FC: 5 μg (R&D Systems); TLR9-5G5, FC: 5 μg, TLR9-ab37154, IF: 1:200 (Abcam); TLR9-26C593.2, FC: 5 μg (Novus Biological/Abcam).

Microscopy and quantification
All micrographs were taken on Nikon A2 microscope. Manual counting quantification was performed by at least two personnel, one of which is blinded.

Imaging FC
RBCs were washed in PBS three times. A total of $7 \times 10^5$ RBCs were then incubated with the specified oligonucleotides at 100 or 1000 nM at a final volume of 200 μl for 2 h at 37°C. Cells were washed in PBS twice and fixed in 0.05% glutaraldehyde for 10 min. Before ImageStream (Amnis) analysis, the cells were washed in FACS buffer twice. Analysis was performed using IDEAS software. The automated feature finder was used to discriminate between RBC populations of different morphology. The aspect ratio (the length-to-width ratio or elongation of the cell) and DNA binding differentiated populations that displayed distinct RBC morphology (10).

DNA extraction
A total of $10^7$ frozen RBCs were thawed on ice. DNA was extracted using DNeasy blood/tissue kit (Qiagen) according to the manufacturer's protocol. DNA was eluted in 152 μl elution buffer at the final step.

RNA extraction and reverse transcription
A total of $1 \times 10^7$ frozen RBCs were thawed on ice. RNA was extracted using RNaeasy Plus Kit (Qiagen) according to the manufacturer’s protocol. During the initial step, RBCs were lysed in 600 μl lysis buffer, and during the final step, RNA was eluted in 30 μl water. A total of 8 μl of freshly isolated RNA was reverse transcribed to cDNA via the Superscript First Strand Synthesis system (Thermo Fisher) following the manufacturer's protocol.

qPCR
Nucleic acids were quantified via the QuantStudio7 Flex System. For 18S rRNA and ZIKV, cDNAs were amplified in PowerUp SYBR Green master mix with the primers (18S rRNA F: 5'-AACCCGTGAAACCCATT-3' and 18S rRNA-R: 5'-CCA-TCAATCGGATAGGCG-3'; ZIKV-E: forward: 5’-TTGTGCATGATCTGCTGATTGCC-3' and reverse: 5’-CCTTTCAACAAGTCCCTATTGCC-3'; ZIKV-nonstructural protein 5 forward: 5’-GGCCACGAGTCTGATACCAAA-3' and reverse: 5’-AGCTTTCACTGGCAGTCTTC-3'). For bacterial 16S rDNA, DNA was amplified in TaqMan Fast Universal Mastermix with the primers (BSF8: 5’-AGA GTT GAC TGG TGC GCA-3' and BSR357: 5’-CTG CCT CCT YCC GTA-3') and probe (/56-FAM/TA A+CA +CAT G+CA +AGT +GGA/3BHQ_1/, where + denotes locked nucleic acid). SARS-CoV-2 was quantified using 2019-nCoV RUO Kit from IDT and TaqMan Fast Universal Mastermix.

Statistical analysis
Statistical analyses were performed with GraphPad Prism 9. Data normality was determined by Wilk–Shapiro test. Differences among groups were determined with t test or one-way ANOVA wherever appropriate.

RESULTS

Bat RBCs display diverse morphologies
Big brown bats (Eptesicus fuscus) are a hibernating North American microbat species that commonly use anthropogenic structures for hibernation and maternal colonies, even to some degree in winter, and are frequently submitted to wildlife rehabilitation facilities throughout the year, often presenting with injuries. We obtained terminal blood samples from wild-caught big brown bats admitted to Pennsylvania wildlife rehabilitators that were determined to be nonreleasable because of their conditions (Table I). All bats were consistently in a euthermic state during their time at the facility. Samples were collected post-euthanasia following transfer to the Pennsylvania Game Commission. We first prepared peripheral blood smears to
Table I. Big brown bat (Eptesicus fuscus) subjects

<table>
<thead>
<tr>
<th>Bat</th>
<th>Sex</th>
<th>Weight (g)</th>
<th>Days in Rehabilitation</th>
<th>Clinical Presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>M</td>
<td>9.8</td>
<td>91</td>
<td>Injured wing</td>
</tr>
<tr>
<td>B</td>
<td>F</td>
<td>11.2</td>
<td>70</td>
<td>Grounded because of unknown cause</td>
</tr>
<tr>
<td>C</td>
<td>F</td>
<td>16.5</td>
<td>27</td>
<td>Grounded and captured at remodeling work site</td>
</tr>
<tr>
<td>D</td>
<td>M</td>
<td>11.7</td>
<td>33</td>
<td>Multiple membrane holes and fracture to right wing, small distal fracture to left wing, possible frost bite</td>
</tr>
<tr>
<td>E</td>
<td>F</td>
<td>13.5</td>
<td>98</td>
<td>No visible injuries</td>
</tr>
<tr>
<td>F</td>
<td>M</td>
<td>12.7</td>
<td>6</td>
<td>No visible injuries</td>
</tr>
<tr>
<td>G</td>
<td>M</td>
<td>9.4</td>
<td>4</td>
<td>Shredded membrane and open fracture of left wing</td>
</tr>
<tr>
<td>H</td>
<td>F</td>
<td>12.0</td>
<td>8</td>
<td>Shredded membrane and exposed bone of right wing</td>
</tr>
<tr>
<td>I</td>
<td>M</td>
<td>11.3</td>
<td>9</td>
<td>Alopecia along left shoulder, unspecified injury to left wing, degloving injury to one toe of left foot</td>
</tr>
<tr>
<td>J</td>
<td>M</td>
<td>20.9</td>
<td>193</td>
<td>Injury and bruising to right leg; cuts along left leg, hip, and back</td>
</tr>
<tr>
<td>K</td>
<td>M</td>
<td>16.4</td>
<td>&gt;240</td>
<td>No unexplained significant weight loss despite healthy appetite</td>
</tr>
</tbody>
</table>

*Weight was measured at time of euthanasia. F, female; M, male.

examine RBC morphology under basal conditions (Fig. 1A). Consistent with previous reports, we noted crenated and poikilocytic cells (Fig. 1B) (9). Unlike human RBCs where crenated RBCs were primarily observed after DNA binding and during acute inflammatory states, the number of nonbiconcave/RBCs was equivalent to biconcave/discoid-appearing RBCs in bats that spent sufficient time in rehabilitation (Fig. 1C) (5). We also noted a substantial amount of heterogeneity in the appearance of crenated cells. Two of the bats (J and K) demonstrated significant hemolysis and the presence of intrerythrocytic inclusions, marked by erythrocyte ghosts and Howell-Jolly bodies, respectively (Fig. 1D).

We next performed imaging FC to examine RBC morphology at the single-cell level. Concurring with earlier observations, naive untreated bat RBCs also displayed a range of morphologies (Fig. 1E). Because we observed dramatic alterations in human RBC morphology after CpG binding, we asked whether CpG would alter bat RBCs. Consistent with our observations of human RBCs, CpG acquisition by RBCs led to marked alteration of the bat RBCs (Fig. 1F) (5).

**Nucleic acids bind to bat RBCs**

Next, we measured the ability of bat RBCs to bind RNA and DNA. Fluorescently labeled GU-rich ssRNA (RNA-40) and CpG oligodeoxynucleotides (ODNs) were incubated with bat RBCs before washing and analyzing by FC. Consistent with human RBCs, bat RBCs bound both RNA and CpG ODNs in a dose-dependent fashion (Fig. 1G, 1H). We next performed competitive binding experiments by assessing the acquisition of fluorescently labeled CpG by bat RBCs in the presence of excess unlabeled CpG. Despite heterogeneity in RBC CpG acquisition, we observed attenuation in CpG binding in the presence of high doses of unlabeled CpG, indicative of specific binding to the oligonucleotide by RBCs (Fig. 1I, 1J).

Because microbial DNA binds to human RBCs, we assayed purified bat RBCs for the presence of RNA or DNA by performing qPCR for 18S rRNA and the 16S rDNA gene (5). When compared with RBCs from healthy human donors, bat RBCs contained significantly less bacterial DNA but similar amounts of 18S rRNA (Fig. 2A, 2B). Together, these data would suggest that bat RBCs exhibit differential nucleic acid-binding capability when compared with human RBCs. Because ZIKV infection has recently been described in free-ranging bats, and persistent association of ZIKV to RBCs has been reported in the transfusion literature, we next asked whether RBCs bind RNA derived from ZIKV (11, 12). We observed a dose-dependent binding of ZIKV RNA to RBCs (Fig. 2C). Because a bat origin of SARS-CoV-2 has been proposed, we also asked whether bat RBCs would bind RNA from SARS-CoV-2. Although only one animal was available for studies, we observed robust binding of SARS-CoV-2 RNA to bat RBCs (Fig. 2D). Collectively, these data demonstrate that bat RBCs bind RNA from pathogenic viruses.

**Bat RBCs express TLR7 and TLR9**

Nucleic acid sensing in bats is a topic of intense investigation, and the TLRs have been extensively characterized in bats (13, 14). We therefore asked whether bat RBCs expressed TLRs. FC of intact, unpermeablized bat RBCs did not identify TLRs on the RBC surface (data not shown). However, staining of permeablized cells did reveal the presence of TLR9 and TLR7, although TLR3 was not detected (Fig. 3A, 3B). We confirmed these findings with IF (Fig. 3C). However, only four of the five bats tested consistently demonstrated positive TLR7 and TLR9 staining, suggesting heterogeneity in erythroid TLR expression among mammals. Notably, the bats with the highest TLR7 staining were also the bats nearest to their injury before arrival to the rehabilitation facility (Table I).

**DISCUSSION**

Collectively, our data demonstrate the presence of nucleic acid-sensing TLRs on bat RBCs and nucleic acid-binding capabilities of bat RBCs. Furthermore, bat RBCs contain RNA, suggesting that these RBCs may serve as reservoirs for RNA. As a result of the preponderance of bat-borne viral pathogens with pandemic potential, substantial interest in bat immunity has emerged. Recently, an immune function of RBCs through nucleic acid sensing and delivery to classical immune cells has been identified (9).
FIGURE 1. Bat RBCs bind nucleic acids.

(A–D) Morphological characteristics of bat RBCs. (A) Peripheral smears of RBCs from six bats. (B) Percentage of normal and abnormal RBCs in individual bats. (C) Similar numbers of normally shaped and abnormally shaped RBCs are observed in bats. (D) Percentage of abnormal RBCs in bats. Arrows denote erythrocyte ghosts. (E and F) Imaging FC of CpG-treated bat RBCs. PBS- (E) and ODN2006-treated (F) RBCs (Continued)
Given the large number of circulating RBCs present in Chiropteran species and the uniquely rich lipid composition of bat RBCs, we speculate that lipid-rich bat RBCs function to shelter RNA from the immune system, permitting host tolerance; however, further studies of bat RBCs, including detailed analysis of surface proteins and lipid composition, will be needed to further investigate this hypothesis (15). Interestingly, in rehabilitated bats, RBCs exhibited a substantial number of abnormal cells and features, including excessive hemolysis, poikilocytes, Howell-Jolly bodies, and crenated cells, reminiscent of those found in hemolytic anemias, acute infection, and RBC membrane disorders, including pyropoikilocytosis and elliptocytosis. In addition, bat TLRs display mutations in ligand binding sites that may alter their ability to bind nucleic acids, yet whether these adaptations affect erythrocyte TLRs and whether the bat RBC TLRs directly bind nucleic acids similar to human and mouse RBCs remain unknown as further characterization of the RBC membrane, including the TLRs we report in this article, is needed (5, 13, 14, 16). Because bats undergo dramatic temperature changes from hypothermia during torpor to elevated temperature during flight, it is possible that the unique RBC features are not just a consequence of acclimatization to pathogens but also an adaption to rapid dynamic temperature changes, as has been observed in some congenital human red cell disorders, such as pyropoikilocytosis (17). The extreme metabolic stress of flight has also driven red cell adaptations, including increased hematocrit and smaller RBCs, allowing for greater oxygen transport, and thus may also have influenced other RBC changes (18). Although the driver of these unique RBC alterations in Chiroptera remain unclear, our findings of nucleic acid binding by bat RBCs suggest an immune function for these RBCs.

reveal four distinct populations of cells and acquisition of DNA by RBCs. (G and H) Dose-dependent binding of ssRNA (RNA40), CpG ODN1826, or ODN2006; contour plots and histograms for one representative bat are shown in (G), and summary statistics for percent positive cells in (H). n = 8 bats. (I and J) Competitive inhibition of CpG binding to bat RBCs; contour plots (I) and percent CpG-positive cells and mean fluorescence intensity (MFI) (J) are shown for two bats. **p < 0.01. ****p < 0.0001. FSC, forward scatter; SSC, side scatter.
FIGURE 3. TLR expression on bat RBCs.
(A) Intracellular staining for TLR9, TLR7, and TLR3 from three representative bats. (B) Summary statistics, percent positive cells, and mean fluorescence intensity (MFI) for TLRs. n = 5. *p < 0.05, **p < 0.01. (C) Immunofluorescent staining for TLR7 and TLR9; magenta is autofluorescence, and green is TLR staining. FSC, forward scatter; SSC, side scatter.
The discovery of an immune role for enucleate, mature RBCs prompts the question of what evolutionary pressure led to such adaptations. Mounting evidence suggests that human non–gas-exchanging RBC adaptations arose in response to the dominant selective pressure of malaria-causing parasites of the genus *Plasmodium*, with chemokine binding through Duffy Ag receptor for chemokines and hemoglobin S (sickle cell) being the two most prominent examples. Mammals in the order Chiroptera coexist with *Plasmodium* and other hemoparasitids, and some have postulated a Chiropteran origin to primate malaria (8). Future studies of mammalian erythrocyte immune function, including nucleic acid binding and complement regulation in bats and other mammalian hosts, will be necessary to elucidate the complete picture of red cell immune function, potentially providing insight into mechanisms by which pathogens lethal to humans are tolerated in other mammals.

**DISCLOSURES**

The authors have no financial conflicts of interest.

**ACKNOWLEDGMENTS**

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