Red blood cells serve as a vehicle for Coronavirus PEDV transmission

Jianda Li[1], Chen Yuan[1], Peng Liu[1], Yuchen Li[1], En Zhang[1], Penghao Zhang[1], and Qian Yang[1]

[1]Nanjing Agricultural University College of Veterinary Medicine

August 11, 2020

Abstract

Red blood cells (RBCs) are the most abundant cell type in the bloodstream, serving for oxygen transport. Although RBCs have been considered as possible vehicles of virus transmission to target cells, the mechanism is much less well understood. Here, we showed that porcine epidemic diarrhea virus (PEDV), a coronavirus that caused acute and devastating intestinal disease in suckling piglets, could cause typical diarrhea in newborn piglets through hijacking RBCs. Firstly, PEDV could bind and internalize into neonatal RBCs through CD71 and clathrin-mediated endocytosis, and maintain its viability for 12 h. Subsequently, after autotransfusion with PEDV-loaded RBCs, PEDV could infect and colonize intestinal epithelial cells, causing typical diarrhea symptoms in newborn piglets. Moreover, PEDV-loaded RBCs could transfer the virus to CD3+ T cells by formation conjugation structure. PEDV could continue to hitchhike CD3+ T cells to reach intestine mucosa and cause infection. Finally, PEDV-loaded RBCs were found in nasal capillary after intranasal infection with PEDV. Further, higher oxygen concentration was determined as a promoter of PEDV binding RBCs. Therefore, nasal capillary was speculated to be the entry for PEDV binding RBCs. Collectively, our studies illustrated the mechanism that PEDV could cause intestine infection through hijacking RBCs, further providing a novel insight into the role of RBCs in coronavirus pathogenesis as potential cells for viral transmission.

Introduce

Red blood cells (RBCs) are the most abundant cell type in the bloodstream and serve for transporting oxygen and carbon dioxide via blood circulatory system. RBCs are differentiated from multipotent hematopoietic stem cells in the bone marrow and circulated in the body for about 100-120 days, and then are recycled by macrophages in spleen and liver (de Back, Kostova, van Kraaij, van den Berg, & van Bruggen, 2014). Although RBCs lack of nucleus and organelles, RBCs still have numerous cell surface receptors for binding chemokines (Darbonne et al., 1991; Fukuma et al., 2003) and nucleic acids (Hotz et al., 2018). Moreover, the receptors could be employed by pathogens to adhere or invade in RBCs. For instance, *Plasmodium falciparum* invaded RBCs by adhering to glycophorin A (GYPA) (Cowman, Berry, & Baum, 2012) and *Plasmodium vivax* attached RBCs via Duffy Ag receptor for chemokines (DARC) (Boddey & Cowman, 2013; Horuk et al., 1993). Additionally, HIV-1 persisted on RBCs through binding to DARC and presented virion to $CD4^+$ T cells (He et al., 2008). Although several reports have indicated that certain viruses could bind to RBCs (Beck et al., 2009; Hess et al., 2002; Sutherland et al., 2016), the underlying mechanisms have not been confirmed.

The recent outbreak of porcine epidemic diarrhea (PED) is characterized as vomiting, severe watery diarrhea, dehydration and high mortality in suckling piglets, resulting in a huge loss in swine industry worldwide. As the causative agent of PED porcine epidemic diarrhea virus (PEDV) is an enveloped, single-stranded, and positive-sense RNA virus, belonging to the Coronaviridae family (C. Lee, 2015). Due to immature immune system, suckling piglets are more susceptible to PEDV infection, especially 1-2 days old neonatal piglets. PEDV infection causes severe destruction of intestinal villous enterocytes and villous atrophy in the jejunum

and ileum (Jung & Saif, 2015). Moreover, PEDV can enter the blood and induce viremia in suckling piglets (Jung et al., 2014; Wang, Hayes, Byrum, & Zhang, 2016), though the mechanism has been much less well understood.

Due to lack of ribosomes or genomes, mature RBSs are inability of gene transcription and protein synthesis, meaning that RBCs are invulnerable to viral infection. However, several researches have claimed that RBCs might be possible vehicles for virus transmission. For instance, RBCs could bind to HIV-1 virions and enrich virus infectious (Beck et al., 2009). In addition, a recent study has reported that complete genome sequence of the DNA of African swine fever virus (ASFV) was determined from porcine RBCs, suggesting that RBCs might serve as a carrier for virus transmission (Olesen et al., 2018). Our previous study demonstrated that PEDV could be captured by dendritic cells (DCs) from nasal epithelium, transferred to $CD3^+$ T cell and entered the peripheral blood, causing intestine infection (Li et al., 2018). Although PEDV could be carried into the blood by T cells, is it possible that RBCs, as the most abundant cell type, serve as another potential vehicle for PEDV transmission in the blood?

In the present study, the hypothesis for PEDV transmission in the blood by hijacking RBCs was proposed. To verify our hypothesis, neonatal RBCs were infected with PEDV. Subsequently, an animal challenge through autotransfusion with PEDV-loaded RBCs was performed to validate whether PEDV could cause intestine infection through hijacking RBCs in vivo. A co-culture model of PEDV-loaded RBCs and $CD3^+$ T cells was established to determine the mechanism of which PEDV entering the intestinal mucosa from the blood. Moreover, through intranasal challenge with PEDV in newborn piglets, the PEDV-loaded RBCs were found in the nasal capillary, consequently, nasal capillary was speculated to be the possible entry for PEDV binding RBCs. This study was first time to demonstrate that the coronavirus PEDV could spread throughout the body and cause intestinal infection by hijacking RBCs in newborn piglets. Therefore, our study provides a novel insight into the role of RBCs in coronavirus pathogenesis as potential cells for virus transmission.

Result

PEDV could hijack neonatal RBCs

To detect whether PEDV binds to neonatal red blood cells (RBCs), neonatal RBCs were isolated from newborn piglets and infected with PEDV strain ZJ (MOI=0.1). After washing to remove unbound virus, cells were repeatedly frozen and thawed and then detected by plaque assays. We found that the virus titer was continuously decreased, until disappeared at 24 hpi (Fig 1A), indicating that PEDV could bind to neonatal RBCs, but couldn't survive for long. Following, the levels of PEDV-N protein were gradually decreased after infection (Fig 1B). Although the low level of PEDV-N protein was still detected at 24 hpi, the virus had almost lost its viability and infectivity, speculating that despite virus loss of activity, the residual viral structural protein could continue to adhere on RBCs. Transmission electron microscopy (TEM) observation visualized that single or clustered PEDV virion could be internalized in neonatal RBCs (Fig 1C). These data suggested that PEDV virion could hijack neonatal RBCs and maintain its viability and infectivity for up to 12 h. In general, a blood circulation takes about 45 s for blood to circulate from the heart, all around the body, and back to the heart again. Thus, once PEDV hijacks neonatal RBCs, it is enough to spread all over the body.

PEDV internalizes into RBCs via binding CD71 and clathrin-mediated endocytosis

To investigate whether the binding of PEDV to RBCs depends on RBCs from different ages pigs, non-neonatal RBCs were isolated from 40-day-old pigs and 120-day-old pigs and then infected with PEDV strain ZJ (MOI=0.1). We observed that PEDV was more affinitive to neonatal RBCs than non-neonatal RBCs (Fig 2A). In the blood circulation of newborn, there were not only mature RBCs, but also a small amount of immature $CD71^+$ RBCs (Elahi, 2014). In agreement with previous reports, we found that CD71 only expressed in neonatal RBCs, but none in non-neonatal RBCs (Fig 2B). Moreover, our study has demonstrated that CD71 was considered as a co-receptor for PEDV infection (unpublished). To investigate whether CD71 mediates PEDV binding neonatal RBCs, the expression of CD71 was detected after 1 h to 24 h of infection. The protein levels of CD71 was continuously decreased during 1 h to 24 h of PEDV infection (Fig 2C), due

to PEDV invasion exhausting CD71 expression in neonatal RBCs. Moreover, a blockade experiment was performed by using an anti-CD71 antibody. After blocking CD71, the level of PEDV virions binding to neonatal RBCs was decreased significantly (Fig 2D and 2E), suggesting that CD71 acts as a receptor for PEDV infection and mediates special binding of PEDV to neonatal RBCs. CD71 is employed to transport iron through clathrin-mediated endocytosis (Gammella, Buratti, Cairo, & Recalcati, 2017). To verify whether PEDV internalization into RBCs is mediated by clathrin-mediated endocytosis, an inhibitor chlorpromazine (CPZ) for preventing clathrin coated pit formation (Martinez, Cordo, & Candurra, 2007) were pre-treated with RBCs. After inhibition with CPZ, the virus titer and the level of PEDV-N protein in neonatal was decreased significantly (Fig 2F and 2G), implying that PEDV might be internalized in to RBCs through clathrin-mediated endocytosis. These data demonstrated that the presence of CD71 and clathrin-mediated endocytosis promoted PEDV internalizing in neonatal RBCs.

PEDV-loaded RBCs transfusion induces typical PED symptoms

To determine whether PEDV could infect newborn piglets through hijacking RBCs, an autotransfusion of PEDV-loaded RBCs were performed in newborn piglets (Fig 3A). Newborn piglets were randomly assigned to 2 groups: control group (transfusion with Dil-labeled RBCs); infection group (transfusion with PEDV-loaded and Dil-labeled RBCs). The virus titer of transfused RBCs was counted at 3.2×10^6 PFU/ 10^9 cells (Fig 3B). At 1 hpt (hour post-transfusion), the percent of Dil-labeled RBCs in infection group was the same as that in control groups, but a small amount of PEDV-loaded RBCs was detected in infection group (Fig 3C and 3E). However, at 48 hpt, the transfused PEDV-loaded RBCs in infection group were vanished in the blood circulation (Fig 3C and 3E). The newborn piglets in infection group appeared typical PED symptoms at 48 hpt, including severe watery diarrhea, dehydration and lethargy (Fig 3F). Compared with piglets in control group, the piglets in infection group revealed multifocal to diffuse villous atrophy and destruction of intestinal villous enterocytes through pathological observation (Fig 3G). Furthermore, a large amount of PEDV-positive cells was found in the intestinal villi of the piglets transfused with PEDV-loaded RBCs through immunofluorescence observation (Fig 3G). Subsequently, viral RNA levels in different tissues were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). After 1 hpt, low levels of viral RNA were found in all tissues, and the viral RNA content in the spleen was higher relatively (Fig 3H), suggesting that the transfused RBCs could carry the virus and spread throughout the body. Moreover, at 48 hpt, PEDV could already infect and colonize in small intestine, and the peak vital RNA reached up 4.8 log10 in the jejunum. Further, the PEDV-N protein was verified by Western blot and only a slight immunoreactivity with PEDV-N protein was observed in spleen at 1 hpt, while at 48 hpt, stronger levels were detected in jejunum and ileum (Fig 3I). Consequently, PEDV could colonize and infect small intestine of neonatal piglets through transfusion with PEDV-loaded RBCs.

$CD3^+$T cells acquire virus from RBCs by forming conjugation

Although RBCs could carry PEDV to the whole body, the RBCs could not directly contact the intestinal mucosa. Therefore, we speculated whether some types of immune cells could recognize and capture the virus-loaded RBCs. As 'sentry' cells in the blood, peripheral blood mononuclear cells (PBMCs) play an important role in immune reactivity. To examine whether PBMCs possess the ability to recognize and capture virus-loaded RBCs, a co-culture model of RBCs and PBMCs was established (Fig 4A). After co-culture with virus-loaded RBCs and PBMCs, the RBCs were removed by ACK Lysis Buffer and the PBMCs were detected. The virus was detected in PBMCs in only 1 h of co-cultrue by flow cytometry and Western blot (Fig 4B and 4C), suggesting that PEDV could transmit from RBCs to PBMCs. Our previous study had indicated that $CD3^+$ T cells in PBMCs could capture PEDV from DCs and transfer the virus to intestinal epithelial cells (IECs) (Li et al., 2018). We further investigated whether $CD3^+$ T cells could capture PEDV from RBCs (Fig 4D). Compared to co-culture with normal RBCs and PBMCs, higher frequency of $CD3^+$ $PEDV^+$ cells were present in co-culture of virus-loaded RBCs and PBMCs (Fig 4E). As co-culture time was prolonged, more $CD3^+$ Dil^+ cells were increased, suggesting $CD3^+$ T cell might form conjugation with RBCs to capture PEDV (Fig 4F). Moreover, the conjugate structure was visualized by TEM observation between the PEDV-loaded RBCs and $CD3^+$ T cells (Fig 5G), even though the virus transfer was not visualized.

Nasal capillary might be the entry of PEDV binding RBCs

Although we have verified that transfusion with PEDV-loaded RBCs could cause intestinal infection, the place where PEDV enter the blood of newborn piglets to hijack RBCs is poorly defined. Our previous study verified that PEDV could cause typical diarrhea through nasal spray and develop a transient NECs infection (Li et al., 2018). Moreover, we found that numerous capillaries were distributed under the nasal epithelium, and even many capillaries are immediately adjacent to the nasal epithelium (Fig 5A). To further verify whether these nasal capillaries might be the entry of PEDV binding RBCs, a nasal spray challenge was performed in newborn piglets. After 12 h of intranasal incubation with PEDV, PEDV-positive RBCs were found in the capillaries adjacent to the nasal epithelial cells (NECs) by immumohistochemical (IHC) observation (Fig 5B).

Nasal cavity is exposed in higher oxygen concentration condition than in intestinal tissues (Carreau, El Hafny-Rahbi, Matejuk, Grillon, & Kieda, 2011; Elad, Wolf, & Keck, 2008). Moreover, oxygen concentration exerts a significant effect on viral propagation and replication (Morinet, Parent, Bergeron, Pillet, & Capron, 2015). To further speculate whether the differences of oxygen concentration between nasal cavity and other tissues exert an effect on PEDV binding to RBCs, an experiment of viral infection in normoxic (20 % oxygen partial pressure, pO_2) or hypoxic (3 % pO_2) condition was performed. Compared in normoxic condition, RBCs exhibited lower affinity to PEDV in hypoxic condition (Fig 5C and 5E). Considering that the nasal cavity is directly connected to the environment air, the temperature is slightly lower than other tissues (Elad et al., 2008). To further validate whether temperature could affect PEDV binding to RBCs, RBCs were placed under normoxic condition at 37 °C or 33 °C (the temperature of nasal cavity) to culture and infected with PEDV, respectively. However, alteration temperature exerted no effect on PEDV binding to RBCs (Fig 5d and 5E). Therefore, the relatively high oxygen condition in nasal cavity exerted a promoting effect on PEDV binding to RBCs, rather than temperature. Although the mechanism by which PEDV enter the blood and bind to RBC has been unknown, the capillary adjacent to the NECs might allow the virus pass through to bind to RBCs.

Discussion

As it causes devastating losses to the swine industry, coronavirus PEDV has aroused wide mounting concern worldwide (Choudhury, Dastjerdi, Doyle, Frossard, & Steinbach, 2016; C. Lee, 2016). PEDV infection mainly cause diarrhea, dehydration and even death in suckling piglets. Although several reports has indicated that piglets developed viremia after PEDV infection (Chen et al., 2016; Jung et al., 2014), the mechanism of how PEDV spreads in the blood remains unclear. Our previous study has indicated that PEDV could be carried by $CD3^+$T cells to enter the blood (Li et al., 2018). In addition to $CD3^+$ T cells, could PEDV bind to other cell types to facilitate transmission? In the present study, we demonstrated that the coronavirus PEDV could hijack RBCs and spread in the blood circulation, causing intestine infection.

Red blood cells are the most abundant cell in the bloodstream, serving for oxygen transport. Despite of loss of nuclei and organelles, RBCs still express numerous cell surface receptors to contact with the exogenous agents in the blood, performing several additional immunological functions (Baum, Ward, & Conway, 2002). However, several receptors are employed by pathogens for binding RBCs to spread in the blood. The most notorious pathogen is the malaria parasite, which infects RBCs by binding to receptors on the cell surface, such as*Plasmodium vivax* and *Plasmodium knowlesi* attaching Duffy Ag receptor for chemokines (DARC) (Boddey & Cowman, 2013; Horuk et al., 1993)· and *Plasmodium falciparum* adhering glycophorin A (GYPA) (Farrow et al., 2011). Moreover, RBCs are considered as possible vehicles for virus transmission, such as HIV-1 (Beck et al., 2009), Dengue virus (Sutherland et al., 2016) and West Nile virus (WNV) (Rios, Daniel, Chancey, Hewlett, & Stramer, 2007). In this study, we verified that PEDV could bind to RBCs and maintain its viability and infectivity for 12 h.

Mounting researches have indicated that there is a small amount of immature RBCs in the circulation of fetuses and newborns, containing a nucleus and cell surface transferrin receptor (CD71) (Elahi, 2014, 2019). The $CD71^+$ RBCs are enriched during pregnancy or newborns, and have distinctive effect on suppressing

innate immune responses (Delyea et al., 2018; Dunsmore et al., 2017; Elahi, 2014; Elahi et al., 2013). Similar to previous researches, CD71 was only present in the RBCs of newborn piglets, but not of 40-120 days old piglets. As a transmembrane receptor for guaranteeing iron (Gammella et al., 2017), CD71 is also considered as a preferred entry for human pathogenic arenaviruses (Abraham, Corbett, Farzan, Choe, & Harrison, 2010; Radoshitzky et al., 2011), hepatitis C virus (HCV) (Martin & Uprichard, 2013) and malaria parasite (Gruszczyk, Huang, et al., 2018; Gruszczyk, Kanjee, et al., 2018). Moreover, our previous study has proved that CD71 was considered as a co-receptor for Transmissible gastroenteritis virus (TGEV) (Zhang, Hu, Yuan, & Yang, 2018) and PEDV infection (unpublished), which could be caught by spike protein of PEDV. In our study, the presence of CD71 exerted a significant effect on promoting PEDV adhering neonatal RBCs. CD71 is employed to internalize iron through clathrin-mediated endocytosis. Meanwhile, clathrin-mediated endocytosis also played an important role in facilitating PEDV internalizing into RBCs, just as HIV-1, Influenza and Ebola virus (EBOV) invade target cells (Aleksandrowicz et al., 2011; Mercer & Helenius, 2009). Therefore, PEDV could exploit CD71 and clathrin-mediated endocytosis to internalize in neonatal RBCs.

Through autotransfusion with PEDV-loaded RBCs, we found that PEDV could cause intestinal infection in newborn piglets. Until now, blood transfusions are still the route of transmission of many virus, as is the case for HIV-1 (D. Lee, 2006), Hepatitis E Virus (Izopet, 2018), DENV (Pozzetto, Memmi, & Garraud, 2015), HCV (Schuch, Thimme, & Hofmann, 2015) and WNV (David & Abraham, 2016). The essential characteristics of transmission through blood transfusion are that the virus could survive or maintain viability in the blood or blood components and cause infection through the blood vessels route (Glynn et al., 2013). Although coronavirus PEDV persists in RBCs and maintains its viability and infectivity for a relatively short time (about 12 h), but it is enough for PEDV to hijack RBCs to the target tissues. After 1 h of transfusion, PEDV appeared in the spleen, which is the only lymphoid organ that filters the blood. Macrophage in the spleen could phagocytose RBCs that bind to pathogens (Minasyan, 2016), such as RBCs infected by *Plasmodium* (de Back et al., 2014). However, after 48 h, transfusion with PEDV-loaded RBCs caused intestine infection and PEDV could colonize in intestinal epithelial cells. PEDV could hitchhike RBCs to reach the small intestine and colonize in IECs (Li et al., 2018). Since RBCs are thought to be not able to penetrate the vascular endothelium under normal physiological condition, we speculated that the RBCs could be recognized and transferred the virus to certain types of immune cells in the blood.

As a sentry in the peripheral blood, peripheral blood mononuclear cells (PBMCs) play a very important role in immune response against both infectious invaders. After co-culture, we verified that PEDV could transmit from RBCs to PBMCs within a short time. Further, $CD3^+$T cells were determined as the main cells types in PBMCs for capturing virus, and could form conjugation to acquire PEDV from RBCs within 1 h. Moreover, PEDV could continue to hitchhike $CD3^+$ T cells and migrate to the intestine, causing typical diarrhea (Li et al., 2018). Several reports have hypothesized that RBCs might be exploited by pathogen as a "Trojan horse" to carry them to the target cells (Baum et al., 2002; Beck et al., 2009). This would be similar with HIV-1 binding to DCs, which could transfer the virus to the susceptible cells in the lymph nodes (Harman, Kim, Nasr, Sandgren, & Cameron, 2013; Manches, Frleta, & Bhardwaj, 2014). However, the detailed mechanism of PEDV transfer from RBCs to T cells needs to be validated.

In general, RBCs circulate in the blood vessels, and are difficult to directly contact with pathogens from outside. The previous study in our group have indicated that PEDV could infect piglets through airborne transmission and develop a transient nasal epithelium infection (Li et al., 2018). Moreover, numerous capillaries are distributed under the nasal epithelial cells (NECs), and some are even adjacent to the NECs. In addition, the permeability of the capillaries in the lamina propria of the nasal mucosa was very high than that of other tissues (Watanabe, Saito, Watanabe, & Mizuhira, 1980). Through intranasal incubation with PEDV, PEDV-loaded RBCs were found in the capillary adjacent to the NECs. Although the mechanism by which PEDV could enter the nasal capillary after infection with NECs has poorly been clarified, nasal capillary might be the possible entry for PEDV binding RBCs. Further, the nasal cavity is a contacting room between respiratory system and environment air. Cells in nasal cavity are exposed in relatively high oxygen condition with 19-21 % oxygen partial pressure (pO_2), but are exposed in lower oxygen condition with 6-8

% pO2 (Carreau et al., 2011). And increasing evidences have indicated that oxygen concentration exerted significant effect on virus infection (Ebbesen & Zachar, 1998; Mazzon et al., 2013; Rastelli et al., 2016). In the present study, the relatively high oxygen concentration promoted the affinity of PEDV to RBCs. Besides, the temperature is in nasal cavity is cooler than in other tissues, with an average temperature of 33 °C (Bailey, Casey, Pawar, & Garcia, 2017; Forero et al., 2017), but reducing temperature had no effect on PEDV binding RBCs. Therefore, the relatively higher oxygen concentration in nasal cavity might be a promoting factor for PEDV binding RBCs.

In summary, our study has demonstrated for the first time that the coronavirus PEDV could hijack RBCs and spread throughout the body, causing intestine infection. PEDV could bind and be endocytosed into neonatal RBCs through CD71 and clathrin-mediated endocytosis (Fig 6). Subsequently, PEDV-loaded RBCs could be recognized and transferred the virus to $CD3^+$ T cells in PBMCs by form conjugation. Moreover, the capillary adjacent to the nasal epithelium was speculated as the entry for PEDV binding RBCs. Our studies put forward a new insight into the role of RBCs in coronavirus PEDV as potential cells for virus transmission, which might be helpful to reveal the transmission and pathogenesis of viruses with the same characteristics, even though the detailed mechanisms remain to be clarified.

Materials and methods

Virus

The wild-type PEDV strain ZJ was isolated from intestinal contents of a 2-day-old diarrheic piglet in Jiangsu Province in 2012, and this strain clustered with the emerging virulent strain according to phylogenetic analysis (Li et al., 2018).

Cells isolation

Whole blood was collected from newborn piglets, 40-day-old pigs or 120-day-old pigs. Peripheral blood mononuclear cells (PBMCs) were isolated by a density centrifugation using a porcine peripheral blood lymphocyte separation kit (Solarbio) and cultured in RPMI 1640 medium with 10 % FBS (Gibco) and 1% penicillin/streptomycin (20 ng/mL) at 37°C with 5 % CO_2. Red blood cells (RBCs) were separated as a centrifuged pellet after purification of PBMCs were washed three times in Dulbecco's phosphate saline (lacking $Ca2^+$and $Mg2^+$, DPBS) and stored in Red Cell Storaging Solution (Solarbio) at 4 °C. To isolate T cells, PBMCs were labeled with APC-CD3 antibody (BD Biosciences), incubated with anti-APC microbeads and sorted using MiniMACS Starting kits. Vero E6 cells were kindly provided by the Veterinary Medicine Research Center of the Da Bei Nong Group.

Binding of PEDV to RBCs

RBCs were infected with PEDV (MOI=0.1) for 1 h at 37 °C and then wash repeatedly to remove unbound virus. For survival assays, RBCs were cultured from 1 h to 24 h after infection with PEDV and analyzed by plaque assay and Western blot. For Transmission electron microscopy (TEM) observation, RBCs were centrifuged at 300×g for 5 min and fixed in glutaraldehyde/cacodylate buffer.

For observation the effect of oxygen concentration on PEDV binding RBCs, RBCs were pre-cultured for 2 h and then infected with PEDV (MOI=0.1) for 1 h in a fully humidified incubator supplied with pure nitrogen gas to reduce oxygen as well as with 5% CO_2 at 37 °C. After incubation with PEDV, cells were still cultured in the incubator as described above for 1 h and 6 h. For observation the effect of temperature on PEDV binding RBCs, RBCs were pre-cultured for 2 h and then infected with PEDV for 1 h in normoxic condition at 33 °C. After infection with PEDV, cells were still cultured in normoxic condition at 33 °C for 1 h and 6 h.

Anti-CD71 Ab and Drug treatment

For blocking CD71, RBCs were pre-treated with Anti-CD71 Ab (abcam) or Rabbit IgG Ab (Beyotime) for 1 h, and then infected with PEDV for 1 h at 37 °C. For inhibition of clathrin-mediated endocytosis, RBCs were pre-treated with the respective doses of chlorpromazine (CPZ) (Selleck) for 2 h and then infected with

PEDV in RPMI 1640 medium containing CPZ for 1 h at 37 °C. After infection, RBCs were collected and counted to detect viral titer.

Animals challenge

All animal procedures and experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) and followed the National Institutes of Health guidelines.

A total of 30 newborn piglets were purchased successively from a high health status herd, which were confirmed seronegative to PEDV, TGEV, Porcine Reproductive and Respiratory Syndrome virus (PRRSV), Classical Swine Fever virus (CSFV), Porcine circovirus type 2 (PCV2) and Porcine Respiratory Coronavirus (PRCV). The animals were fed with milk every 3 h throughout the experiment to meet or exceed the National Research Council (NRC) requirements for nutrients and energy for newborn piglets.

For autotransfusion with virus-loaded RBCs, 12 newborn piglets with similar weight were randomly assigned to 2 groups: 6 control group (I); 6 PEDV infection group (II). Whole bloods were collected from precaval vein to isolate RBCs, respectively. Newborn piglets in control groups were infused autologous RBCs labeled with Dil via ear vein. For PEDV infection group, RBCs were infected with PEDV (MOI=0.1) for 1 h at 37 °C, then labeled with CM-Dil Dye (Invitrogen) and wash extensively to remove unbound virus and dye. The supernatants were collected and detected viral titer by plaque assays to ensure washing cleanly. In Both of I and II group, 2×10^9 RBCs were suspended in 1 mL DPBS and infused via ear vein. After 1 h of transfusion, 3 piglets from control group and 3 piglets from PEDV infection group were euthanized and sacrificed to collect blood and organ samples, individually. The remained piglets were observed daily for diarrhea symptoms. All piglets were euthanized before dying or necropsy.

For PEDV intranasal infection, 18 newborn piglets with similar weight were randomly assigned to 2 groups: 9, control group (I); 9, PEDV intranasal infection (II). Newborn piglets in group II were infected 1 mL PEDV (10^7 PFU/mL) through nasal incubation. In group I, the same volume of PBS was incubated intranasally. Subsequently, three piglets from each group were euthanatized at 3 h, 12 h, and 24 h after virus inoculation to determine the dynamic changes of PEDV in nasal cavity.

PEDV transmission assays and conjugate formation

PEDV-loaded RBCs were labeled with CM-Dil Dye, washed extensively to remove unbound virus and dye, and then co-cultured with PBMCs (RBCs: PBMCs = 1:1) for 1 h or 6 h. For detection of PEDV transmission, after co-culture, RBCs were removed by ACK Lysis Buffer and PBMCs were collected to detect by flow cytometry and Western blot. For observation the conjugated formation, CD3+ T cells were purified from PBMCs as described (Li et al., 2018). $CD3^+$ T cells were then co-cultured with virus-loaded RBCs and then centrifuged at 200×g for 5 min. The mixtures of RBCs and PBMCs were detected by flow cytometry and TEM observation.

Flow cytometry analysis

After PEDV infection, RBCs were pre-labeled with CM-Dil Dye and stained with a monoclonal antibody against PEDV-N protein. PBMCs were stained with an antibody specific for porcine CD3 (BD Biosciences). To detect PEDV, cells were resuspended in fixation/permeabilization solution (BD Cytofix/Cytoperm kit, BD Pharmingen) and stained with antibody against PEDV-N protein. BD FACS Calibur were used for flow cytometry and FlowJo X.10.0.7 was used to analyze the data.

Plaque assay

Collecting RBCs were suspended in 1 mL PBS, then repeatedly frozen and thawed to release the virus. Vero cells were seeded in 12-well plates and grown to confluent monolayers. Then cells were infected with 500 μL serial ten-fold dilutions of the frozen and thawed samples for 1 h at 37 °C. After infection, cells were overlaid with 0.7% low melting point agarose (Solarbio) in Dulbecco's Modified Eagle's Medium (DMEM) containing 2% FBS and incubated at 37 °C for 72 h. Plaques were visualized by staining with crystal violet.

Western blot

To ensure the number of cells consistent, RBCs were counted and centrifuged before collecting. Collected RBCs were lysed by RIPA lysis buffer. Tissues were grinded and lysed by RIPA lysis buffer. Protein concentrations were determined using BCA assay. All samples were detected by Western blot with specific antibodies.

Quantitative RT-PCR (qRT-PCR)

Total RNAs from tissues were extracted with RNAiso Plus (Takara) according to the manufacturer's instructions. Reverse transcriptions were completed using a PrimeScript^{TM} RT reagent Kit (Takara). qRT-PCR was performed by using a SYBR Green qPCR Kit (Takara, CN) in the Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies). PEDV load was determined by detecting the viral nucleocapsid (N) gene (Fwd: 5'-CACCTCCTGCTTCACGTACA-3' and Rev:5'-AGCTCCACGACCCTGGTTAT-3'). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Fwd: 5'-TCATCATCTCTGCCCCTTCT-3' and Rev: 5'-GTCATGAGTCCCTCCACGAT-3') was used as the reference gene. The relative levels of viral RNA were calculated by using the $2^{-\Delta\Delta^{\wedge}T}$ method.

Immunohistochemistry (IHC) and Immunofluorescence assay (IFA)

All piglets were euthanized and sacrificed to collect tissues. After fixation, tissues were dehydrated, embedded and sectioned. Subsequently, tissues were stained with primary antibody against PEDV-N protein for observing the distribution of PEDV by IHC staining. For IFA, after staining with antibody against PEDV-N protein, tissues were incubated with secondary antibodies Alex-Flour 594 goat anti-Mouse IgG (Invitrogen), and the nuclei was counterstained with DAPI (Beyotime) for 5 min.

Statistical analysis

Results was performed as the mean ± SD by using SPSS 17.0 software (SPSS Inc, Chicago, IL, USA). Data was employed one-way analysis of variance (ANOVA) among multiple groups, and employed a t-tests between the two groups to determine the difference. For all analysis, $p< 0.05$ (*) was considered statistically significant and$p < 0.01$ (**) was considered highly statistically significant.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Jianda Li performed the experiments and wrote the relative manuscript; Chen Yuan and Peng Liu prepared the reagents and helped isolating RBCs and PBMCs; Yuchen Li, En Zhang and Penghao Zhang were responsible for animals challenge experiments; Qian Yang initiated the study, designed the experiments and supplied the manuscript. All authors reviewed the manuscript.

Acknowledgements

This study was supported by 31930109 and 31772777 from the National Science Grant of China and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Data availability The data that support the findings of this study are available from the corresponding author upon request.

Reference

Abraham, J., Corbett, K. D., Farzan, M., Choe, H., & Harrison, S. C. (2010). Structural basis for receptor recognition by New World hemorrhagic fever arenaviruses. *Nat Struct Mol Biol, 17* (4), 438-444. doi:10.1038/nsmb.1772

Aleksandrowicz, P., Marzi, A., Biedenkopf, N., Beimforde, N., Becker, S., Hoenen, T., . . . Schnittler, H. J. (2011). Ebola virus enters host cells by macropinocytosis and clathrin-mediated endocytosis. *J Infect Dis, 204 Suppl 3* , S957-967. doi:10.1093/infdis/jir326

Bailey, R. S., Casey, K. P., Pawar, S. S., & Garcia, G. J. (2017). Correlation of Nasal Mucosal Temperature With Subjective Nasal Patency in Healthy Individuals. *JAMA Facial Plast Surg, 19* (1), 46-52. doi:10.1001/jamafacial.2016.1445

Baum, J., Ward, R. H., & Conway, D. J. (2002). Natural selection on the erythrocyte surface. *Mol Biol Evol, 19* (3), 223-229. doi:10.1093/oxfordjournals.molbev.a004075

Beck, Z., Brown, B. K., Wieczorek, L., Peachman, K. K., Matyas, G. R., Polonis, V. R., . . . Alving, C. R. (2009). Human erythrocytes selectively bind and enrich infectious HIV-1 virions. *PLoS One, 4* (12), e8297. doi:10.1371/journal.pone.0008297

Boddey, J. A., & Cowman, A. F. (2013). Plasmodium nesting: remaking the erythrocyte from the inside out. *Annu Rev Microbiol, 67* , 243-269. doi:10.1146/annurev-micro-092412-155730

Carreau, A., El Hafny-Rahbi, B., Matejuk, A., Grillon, C., & Kieda, C. (2011). Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *J Cell Mol Med, 15* (6), 1239-1253. doi:10.1111/j.1582-4934.2011.01258.x

Chen, Q., Gauger, P. C., Stafne, M. R., Thomas, J. T., Madson, D. M., Huang, H., . . . Zhang, J. (2016). Pathogenesis comparison between the United States porcine epidemic diarrhoea virus prototype and S-INDEL-variant strains in conventional neonatal piglets. *J Gen Virol, 97* (5), 1107-1121. doi:10.1099/jgv.0.000419

Choudhury, B., Dastjerdi, A., Doyle, N., Frossard, J. P., & Steinbach, F. (2016). From the field to the lab - An European view on the global spread of PEDV. *Virus Res, 226* , 40-49. doi:10.1016/j.virusres.2016.09.003

Cowman, A. F., Berry, D., & Baum, J. (2012). The cellular and molecular basis for malaria parasite invasion of the human red blood cell. *J Cell Biol, 198* (6), 961-971. doi:10.1083/jcb.201206112

Darbonne, W. C., Rice, G. C., Mohler, M. A., Apple, T., Hebert, C. A., Valente, A. J., & Baker, J. B. (1991). Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin. *J Clin Invest, 88* (4), 1362-1369. doi:10.1172/jci115442

David, S., & Abraham, A. M. (2016). Epidemiological and clinical aspects on West Nile virus, a globally emerging pathogen. *Infect Dis (Lond), 48* (8), 571-586. doi:10.3109/23744235.2016.1164890

de Back, D. Z., Kostova, E. B., van Kraaij, M., van den Berg, T. K., & van Bruggen, R. (2014). Of macrophages and red blood cells; a complex love story. *Front Physiol, 5* , 9. doi:10.3389/fphys.2014.00009

Delyea, C., Bozorgmehr, N., Koleva, P., Dunsmore, G., Shahbaz, S., Huang, V., & Elahi, S. (2018). CD71(+) Erythroid Suppressor Cells Promote Fetomaternal Tolerance through Arginase-2 and PDL-1. *J Immunol, 200* (12), 4044-4058. doi:10.4049/jimmunol.1800113

Dunsmore, G., Bozorgmehr, N., Delyea, C., Koleva, P., Namdar, A., & Elahi, S. (2017). Erythroid Suppressor Cells Compromise Neonatal Immune Response against Bordetella pertussis. *J Immunol, 199* (6), 2081-2095. doi:10.4049/jimmunol.1700742

Ebbesen, P., & Zachar, V. (1998). Oxygen tension and virus replication.*Acta Virol, 42* (6), 417-421.

Elad, D., Wolf, M., & Keck, T. (2008). Air-conditioning in the human nasal cavity. *Respir Physiol Neurobiol, 163* (1-3), 121-127. doi:10.1016/j.resp.2008.05.002

Elahi, S. (2014). New insight into an old concept: role of immature erythroid cells in immune pathogenesis of neonatal infection.*Front Immunol, 5* , 376. doi:10.3389/fimmu.2014.00376

Elahi, S. (2019). Neglected Cells: Immunomodulatory Roles of CD71(+) Erythroid Cells. *Trends Immunol, 40* (3), 181-185. doi:10.1016/j.it.2019.01.003

Elahi, S., Ertelt, J. M., Kinder, J. M., Jiang, T. T., Zhang, X., Xin, L., . . . Way, S. S. (2013). Immunosuppressive CD71+ erythroid cells compromise neonatal host defence against infection. *Nature, 504* (7478), 158-162. doi:10.1038/nature12675

Farrow, R. E., Green, J., Katsimitsoulia, Z., Taylor, W. R., Holder, A. A., & Molloy, J. E. (2011). The mechanism of erythrocyte invasion by the malarial parasite, Plasmodium falciparum. *Semin Cell Dev Biol, 22* (9), 953-960. doi:10.1016/j.semcdb.2011.09.022

Forero, A., Fenstermacher, K., Wohlgemuth, N., Nishida, A., Carter, V., Smith, E. A., . . . Pekosz, A. (2017). Evaluation of the innate immune responses to influenza and live-attenuated influenza vaccine infection in primary differentiated human nasal epithelial cells. *Vaccine, 35* (45), 6112-6121. doi:10.1016/j.vaccine.2017.09.058

Fukuma, N., Akimitsu, N., Hamamoto, H., Kusuhara, H., Sugiyama, Y., & Sekimizu, K. (2003). A role of the Duffy antigen for the maintenance of plasma chemokine concentrations. *Biochemical and Biophysical Research Communications, 303* (1), 137-139. doi:10.1016/s0006-291x(03)00293-6

Gammella, E., Buratti, P., Cairo, G., & Recalcati, S. (2017). The transferrin receptor: the cellular iron gate. *Metallomics, 9* (10), 1367-1375. doi:10.1039/c7mt00143f

Glynn, S. A., Busch, M. P., Dodd, R. Y., Katz, L. M., Stramer, S. L., Klein, H. G., . . . November, N. E. I. D. T. F. c. (2013). Emerging infectious agents and the nation's blood supply: responding to potential threats in the 21st century. *Transfusion, 53* (2), 438-454. doi:10.1111/j.1537-2995.2012.03742.x

Gruszczyk, J., Huang, R. K., Chan, L. J., Menant, S., Hong, C., Murphy, J. M., . . . Tham, W. H. (2018). Cryo-EM structure of an essential Plasmodium vivax invasion complex. *Nature, 559* (7712), 135-139. doi:10.1038/s41586-018-0249-1

Gruszczyk, J., Kanjee, U., Chan, L. J., Menant, S., Malleret, B., Lim, N. T. Y., . . . Tham, W. H. (2018). Transferrin receptor 1 is a reticulocyte-specific receptor for Plasmodium vivax. *Science, 359* (6371), 48-55. doi:10.1126/science.aan1078

Harman, A. N., Kim, M., Nasr, N., Sandgren, K. J., & Cameron, P. U. (2013). Tissue dendritic cells as portals for HIV entry. *Rev Med Virol, 23* (5), 319-333. doi:10.1002/rmv.1753

He, W., Neil, S., Kulkarni, H., Wright, E., Agan, B. K., Marconi, V. C., . . . Ahuja, S. K. (2008). Duffy antigen receptor for chemokines mediates trans-infection of HIV-1 from red blood cells to target cells and affects HIV-AIDS susceptibility. *Cell Host Microbe, 4* (1), 52-62. doi:10.1016/j.chom.2008.06.002

Hess, C., Klimkait, T., Schlapbach, L., Del Zenero, V., Sadallah, S., Horakova, E., . . . Schifferli, J. A. (2002). Association of a pool of HIV-1 with erythrocytes in vivo: a cohort study. *Lancet, 359* (9325), 2230-2234. doi:10.1016/s0140-6736(02)09291-7

Horuk, R., Chitnis, C. E., Darbonne, W. C., Colby, T. J., Rybicki, A., Hadley, T. J., & Miller, L. H. (1993). A receptor for the malarial parasite Plasmodium vivax: the erythrocyte chemokine receptor. *Science, 261* (5125), 1182-1184. doi:10.1126/science.7689250

Hotz, M. J., Qing, D., Shashaty, M. G. S., Zhang, P., Faust, H., Sondheimer, N., . . . Mangalmurti, N. S. (2018). Red Blood Cells Homeostatically Bind Mitochondrial DNA through TLR9 to Maintain Quiescence and to Prevent Lung Injury. *Am J Respir Crit Care Med, 197* (4), 470-480. doi:10.1164/rccm.201706-1161OC

Izopet, J. (2018). [HEV and transfusion-recipient risk]. *Ann Pharm Fr, 76* (2), 89-96. doi:10.1016/j.pharma.2017.12.007

Jung, K., & Saif, L. J. (2015). Porcine epidemic diarrhea virus infection: Etiology, epidemiology, pathogenesis and immunoprophylaxis. *Vet J, 204* (2), 134-143. doi:10.1016/j.tvjl.2015.02.017

Jung, K., Wang, Q., Scheuer, K. A., Lu, Z., Zhang, Y., & Saif, L. J. (2014). Pathology of US porcine epidemic diarrhea virus strain PC21A in gnotobiotic pigs. *Emerg Infect Dis, 20* (4), 662-665.

doi:10.3201/eid2004.131685

Lee, C. (2015). Porcine epidemic diarrhea virus: An emerging and re-emerging epizootic swine virus. *Virol J, 12* , 193. doi:10.1186/s12985-015-0421-2

Lee, C. (2016). Erratum to: Porcine epidemic diarrhea virus: An emerging and re-emerging epizootic swine virus. *Virol J, 13* , 19. doi:10.1186/s12985-016-0465-y

Lee, D. (2006). Perception of blood transfusion risk. *Transfus Med Rev, 20* (2), 141-148. doi:10.1016/j.tmrv.2005.11.006

Li, Y., Wu, Q., Huang, L., Yuan, C., Wang, J., & Yang, Q. (2018). An alternative pathway of enteric PEDV dissemination from nasal cavity to intestinal mucosa in swine. *Nature Communications, 9* (1). doi:10.1038/s41467-018-06056-w

Manches, O., Frleta, D., & Bhardwaj, N. (2014). Dendritic cells in progression and pathology of HIV infection. *Trends Immunol, 35* (3), 114-122. doi:10.1016/j.it.2013.10.003

Martin, D. N., & Uprichard, S. L. (2013). Identification of transferrin receptor 1 as a hepatitis C virus entry factor. *Proc Natl Acad Sci U S A, 110* (26), 10777-10782. doi:10.1073/pnas.1301764110

Martinez, M. G., Cordo, S. M., & Candurra, N. A. (2007). Characterization of Junin arenavirus cell entry. *J Gen Virol, 88* (Pt 6), 1776-1784. doi:10.1099/vir.0.82808-0

Mazzon, M., Peters, N. E., Loenarz, C., Krysztofinska, E. M., Ember, S. W., Ferguson, B. J., & Smith, G. L. (2013). A mechanism for induction of a hypoxic response by vaccinia virus. *Proc Natl Acad Sci U S A, 110* (30), 12444-12449. doi:10.1073/pnas.1302140110

Mercer, J., & Helenius, A. (2009). Virus entry by macropinocytosis.*Nat Cell Biol, 11* (5), 510-520. doi:10.1038/ncb0509-510

Minasyan, H. (2016). Mechanisms and pathways for the clearance of bacteria from blood circulation in health and disease.*Pathophysiology, 23* (2), 61-66. doi:10.1016/j.pathophys.2016.03.001

Morinet, F., Parent, M., Bergeron, C., Pillet, S., & Capron, C. (2015). Oxygen and viruses: a breathing story. *J Gen Virol, 96* (8), 1979-1982. doi:10.1099/vir.0.000172

Olesen, A. S., Lohse, L., Dalgaard, M. D., Wozniakowski, G., Belsham, G. J., Botner, A., & Rasmussen, T. B. (2018). Complete genome sequence of an African swine fever virus (ASFV POL/2015/Podlaskie) determined directly from pig erythrocyte-associated nucleic acid. *J Virol Methods, 261* , 14-16. doi:10.1016/j.jviromet.2018.07.015

Pozzetto, B., Memmi, M., & Garraud, O. (2015). Is transfusion-transmitted dengue fever a potential public health threat?*World J Virol, 4* (2), 113-123. doi:10.5501/wjv.v4.i2.113

Radoshitzky, S. R., Longobardi, L. E., Kuhn, J. H., Retterer, C., Dong, L., Clester, J. C., . . . Bavari, S. (2011). Machupo virus glycoprotein determinants for human transferrin receptor 1 binding and cell entry.*PLoS One, 6* (7), e21398. doi:10.1371/journal.pone.0021398

Rastelli, E., Corinaldesi, C., Petani, B., Dell'Anno, A., Ciglenecki, I., & Danovaro, R. (2016). Enhanced viral activity and dark CO2 fixation rates under oxygen depletion: the case study of the marine Lake Rogoznica. *Environ Microbiol, 18* (12), 4511-4522. doi:10.1111/1462-2920.13484

Rios, M., Daniel, S., Chancey, C., Hewlett, I. K., & Stramer, S. L. (2007). West Nile virus adheres to human red blood cells in whole blood.*Clin Infect Dis, 45* (2), 181-186. doi:10.1086/518850

Schuch, A., Thimme, R., & Hofmann, M. (2015). Priming persistence of HCV. *Oncotarget, 6* (31), 30427-30428. doi:10.18632/oncotarget.5445

Sutherland, M. R., Simon, A. Y., Serrano, K., Schubert, P., Acker, J. P., & Pryzdial, E. L. G. (2016). Dengue virus persists and replicates during storage of platelet and red blood cell units. *Transfusion, 56* (5), 1129-1137. doi:10.1111/trf.13454

Wang, L., Hayes, J., Byrum, B., & Zhang, Y. (2016). US variant porcine epidemic diarrhea virus: histological lesions and genetic characterization. *Virus Genes, 52* (4), 578-581. doi:10.1007/s11262-016-1334-x

Watanabe, K., Saito, Y., Watanabe, I., & Mizuhira, V. (1980). Characteristics of capillary permeability in nasal mucosa. *Ann Otol Rhinol Laryngol, 89* (4 Pt 1), 377-382. doi:10.1177/000348948008900415

Zhang, S., Hu, W., Yuan, L., & Yang, Q. (2018). Transferrin receptor 1 is a supplementary receptor that assists transmissible gastroenteritis virus entry into porcine intestinal epithelium. *Cell Commun Signal, 16* (1), 69. doi:10.1186/s12964-018-0283-5

Fig. 1 PEDV bind neonatal RBCs . **A.** The viral titer in neonatal RBCs was determined by plaque assay after PEDV infection (MOI=0.1). **B.** The levels of PEDV-N protein were detected at different time after PEDV infection with neonatal RBCs by Western blot.**C.** Transmission electron microscopy (TEM) observation of PEDV infection with neonatal RBCs. The fine ultrastructure of the virus particles (white arrowheads) was observed in neonatal RBCs. Bars = 200 nm. The data shown were the mean results ± SD from three independent experiments.

Fig. 2 CD71 and clathrin-mediated endocytosis promoted PEDV internalize into neonatal RBCs. A. For FACs analyses, RBCs from different age pigs were infected with PEDV (MOI=0.1) and detected through an antibody against PEDV-N protein staining. **B.** CD71 expression was detected in different age pig's RBCs by Western blot.**C.** CD71 expression in neonatal RBCs was determined at different time after PEDV infection by Western blot. **D** and **E.**Neonatal RBCs were pre-treated with a blocking antibody against CD71, and then infected with PEDV. After blocking CD71 expression, the viral titer (**D**) and the level of PEDV-N protein (**E**) was determined by plaque assays and Western blot. **F** and **G.**Neonatal RBCs were pre-treated with the respective doses of chlorpromazine (CPZ) for 2 h and then infected with PEDV in RPMI 1640 medium containing CPZ. After infection, the viral titer (**F**) and the level of PEDV-N protein (**G**) were determined by plaque assays and Western blot. The data shown are the mean results ± SD from three independent experiments. The comparisons were performed with t-tests (two groups) or analysis of variance (ANOVA) (multiple groups). $^{*}p < 0.05$, $^{**}p < 0.01$.

Fig. 3 Transfusion with PEDV-loaded RBCs caused typical PED symptoms. A. Schematic representation of experimental design. RBCs were isolated from newborn piglets, and then infected with PEDV in vitro. After infection, RBCs were labeled with Dil and transfused via ear vein. **B.** The viral titer of transfused RBCs was determined by a plaque assays. **C**and **D.** After 1 h or 48 h of transfusion, the percent of Dil^{+} RBCs (**C**) and $PEDV^{+}$RBCs (**D**) were detected from the blood by FACS analysis.**E.** Quantification of the FACS results as shown in (**C**and **D**). **F.** Acute watery diarrhea and gross lesions of the small intestine in piglets after transfusion with $PEDV^{+}$ RBC at 48 hpt. **G.** IHC results showed the distribution of PEDV (white arrowheads), and severe destruction of intestinal villous enterocytes and villous atrophy in jejunum. Bars = 100 μm. **H.** Sections of jejunum from different group were stained with DAPI (blue) and an antibody against PEDV-N protein (red), and were visualized by confocal microscopy. Bars = 100 μm. **I.**Viral RNA expression in different tissues after 1 h and 48 h of transfusion with RBCs was determined by qRT-PCR. **K.** The levels of PEDV-N protein in different tissues after 1h and 48 h of transfusion with RBCs was detected by Western blot. The data shown are the mean results ± SD from three independent experiments. The comparisons were performed with t-tests (two groups) or analysis of variance (ANOVA) (multiple groups). $^{*}p < 0.05$, $^{**}p < 0.01$.

Fig. 4 $CD3^{+}$ T cells acquired PEDV from RBCs by forming conjugation. A. Schematic representation of experimental design. After infection with PEDV, RBCs were co-cultured with PBMCs. After co-culture, the co-cultured cells were collected and remove RBCs by ACK Lysis Buffer. **B.** The percent of $PEDV^{+}$PBMCs were determined by FACS analysis. **C.** The levels of PEDV-N protein were detected in PBMCs after co-culture. **D.** Schematic representation of experimental design. After infection with PEDV

and labeling with Dil, RBCs were co-cultured with PBMCs. The co-cultured cells were stained with $CD3^+$ antibody and PEDV-N-FITC antibody to detected PEDV transmission. **E.** The PEDV transmission was quantified by analyzing the $CD3^+PEDV^+$ population. **F.** The conjugate formation between $CD3^+$ T cells and RBCs was determined by analyzing the $CD3^+$ Dil^+ population.**G.** Quantification of the FACS results as shown in (**E**and **F**). **H** . TEM observation showed $CD3^+$ T cells form conjugation with RBCs (white arrowheads). The fine ultrastructure of the virus particles (white arrowheads) was observed in the T cells adjacent to the conjugate structure (white arrowheads). The data shown are the mean results ± SD from three independent experiments. The comparisons were performed with t-tests (two groups) or analysis of variance (ANOVA) (multiple groups). $*p < 0.05$, $**p < 0.01$.

Fig. 5 Nasal capillary might be the entry of PEDV binding RBCs.A. Capillaries were immediately adjacent to nasal epithelium cells (NECs) in nasal cavity of newborn piglets by Hematoxylin and eosin (H&E) staining. **B.** PEDV-loaded RBCs (white arrowheads) were found in the capillary adjacent (white ellipse) to NECs after 12 h of intranasal incubation with PEDV by IHC observation. **C.** No PEDV-loaded RBCs was found in in the capillary (white ellipse) of PEDV-infected intestine by IHC observation. **D.** RBCs were cultured and infected with PEDV in normoxic condition (20 % oxygen partial pressure, pO2) or hypoxic condition (3 % pO2), and then the levels of PEDV-N protein were determined by Western blot. **E.**RBCs were cultured and infected with PEDV in 37 °C or 33 °C condition, and then the levels of PEDV-N protein were determined by Western blot.**F.** For FACS analyses, RBCs were cultured and infected with PEDV in normal, hypoxic condition or 33 °C condition, and then detected by PEDV-N protein staining. **G** . Quantification of the FACS results as shown in (**F**). The data shown are the mean results ± SD from three independent experiments. The comparisons were performed with t-tests (two groups) or analysis of variance (ANOVA) (multiple groups). $*p < 0.05$, $**p < 0.01$.

Fig 6. Schematic of the proposed mechanism for PEDV transportation in newborn piglets.PEDV could bind and internalize into neonatal RBCs through CD71 and clathrin-mediated endocytosis. Moreover, the relatively high oxygen concentration in nasal cavity promoted PEDV bind to RBCs. $CD3^+$ T cells could recognize and acquire the virus from PEDV-loaded RBCs. PEDV-loaded $CD3^+$ T cells could transfer the virus to intestinal epithelial cells (IECs), causing typical PED symptoms.