Porcine epidemic diarrhea virus (PEDV) has catastrophic impacts on the global pig industry. Although the fecal–oral route is generally accepted, an increased number of reports indicate that airborne transmission may contribute to PEDV outbreak. Here, we show that PEDV could cause typical diarrhea in piglets through a nasal spray. Firstly, PEDV can develop a transient nasal epithelium infection. Subsequently, PEDV-carrying dendritic cells (DCs) allow the virus to be transferred to CD3+ T cells via the virological synapse. Finally, virus-loaded CD3+ T cells reach the intestine through the blood circulation, leading to intestinal infection via cell-to-cell contact. Our study provides evidence for airborne transmission of a gastrointestinal infected coronavirus and illustrates the mechanism of its transport from the entry site to the pathogenic site.
A large-scale outbreak of porcine epidemic diarrhea (PED), characterized by watery diarrhea, dehydration, and vomiting, with up to 90% mortality in suckling piglets, occurred in swine farms in Asia, Europe, and America\textsuperscript{1,2,3}. As the causative agent of PED, porcine epidemic diarrhea virus (PEDV) has spread rapidly between pig farms even over long distances, demonstrating greater transmission potential than other seasonal diarrhea viruses\textsuperscript{4,5}. Fecal–oral transmission is believed to be the main mode of PEDV transmission\textsuperscript{6}. As many large-scale pig farms in developed countries are equipped with improved disinfection and management systems (sanitation of the pigsty and feed safety are strictly controlled)\textsuperscript{7,8}, the pathogen is less likely to be spread via the digestive tract (feed or feces). These observations suggest that PEDV may have other routes of infection. Recently, many researchers have highlighted the possible role of airborne transmission of PEDV. Increasing evidence for airborne transmission of gastrointestinal infectious diseases has been reported (e.g., Norwalk viruses and rotavirus)\textsuperscript{9,10}, but the underlying mechanisms have not been confirmed. Additionally, if PED outbreaks occur in one farm, then neighboring farms can acquire an increased risk of PEDV infection, and the orientation of PEDV cavity of PEDV-negative piglets in the same room but without contact with piglets inoculated with PEDV\textsuperscript{11}. The infected DCs then migrate to the submucosal layer\textsuperscript{12}. The infected DCs then migrate to the submucosal layer\textsuperscript{13}. In addition, PEDV can survive for a long time (up to 9 months) in the environment and be transmitted over long distances in the field (even 10 miles from pig farms with a PEDV outbreak)\textsuperscript{14,15}. Therefore, the cross-talk between PEDV and the respiratory tract merits further attention.

In a recent study, preserved PEDV was detected in the nasal cavity of PEDV-negative piglets in the same room but without contact with piglets inoculated with PEDV\textsuperscript{16}. The nasal mucosa is considered a vital gateway for many pathogens (including respiratory and nonrespiratory pathogens). As indicated in previous studies, many dendritic cells (DCs) are widely distributed beneath the nasal mucosa of pigs\textsuperscript{17,18} and show a certain degree of susceptibility to PEDV\textsuperscript{19}.

Submucosal DCs are professional antigen-presenting cells with a potent capacity to capture luminal antigens by forming trans epithelial dendrites (TEDs). Such antigen-bearing DCs migrate to the nearby lymph nodes, presenting foreign antigens to T cells and further initiating an effective adaptive immune response\textsuperscript{20–22}. Paradoxically, submucosal DCs may sometimes be harnessed by viruses to help them overcome the epithelial barrier, serving as a “Trojan Horse” to evade antiviral immune responses and disseminate into the submucosal layer\textsuperscript{23}. The infected DCs then migrate to the nearby lymph nodes and transmit the virus to T lymphocytes (productive or recessive infection)\textsuperscript{24,25}. Typically, HIV is a DC-hijacking virus, and DCs might be conducive to its pathogenesis, a mechanism that promotes HIV transmission and infection of CD4\textsuperscript{+} T cells and further dissemination into the body via the migration of T cells\textsuperscript{26–29}. Additionally, DCs are a crucial target of Middle East respiratory syndrome coronavirus (MERS-CoV) replication and a driver of dissemination\textsuperscript{30}. Taken together, these data suggest that submucosal DCs in nasal cavity are likely to uptake PEDV and serve as a virus carrier to help PEDV enter and disseminate beyond the nasal mucosa. When DCs are hijacked by the virus, their endocytosis and antigen-processing abilities are limited, and the virus is maintained on dendrites for efficient transfer to T lymphocytes\textsuperscript{31}. The virus can then modulate the migratory ability of T cells; sometimes the increased motility of infected T cells facilitates the use of motile cells as drivers to disseminate within and between tissues\textsuperscript{32}. Virus-carrying lymphocytes can reach the small intestine and other structures via the blood circulation, penetrate the intestinal mucosa, and transmit the virus to target cells, becoming a vital source of infection\textsuperscript{32,33}. The virus-carrying lymphocytes transmit the virus to host cells, which is called transfer infection. For instance, researchers have suggested that varicella-zoster virus (VZV)-infected T cells can enter the gut and establish latency in enteric neurons in vivo\textsuperscript{34}. Moreover, PEDV can enter the blood and induce viremia in suckling piglets, which implies that PEDV possesses the ability to colonize some cell types in the peripheral blood\textsuperscript{35}.

In the present study, the hypothesis for airborne PEDV caused intestine infection is proposed. A PEDV intranasal challenge experiment was performed to verify our hypothesis. A series of coculture models were established in vitro using nasal epithelial cells (NECs), DCs, T lymphocytes, and Vero cells to gain more insights into the mechanism by which PEDV enters the intestinal epithelium via the nasal cavity. This study is the first to demonstrate evidence of the airborne transmission of a gastrointestinal infected coronavirus. Moreover, pigs are closely related to humans in terms of anatomy, genetics, and physiology\textsuperscript{36}. Thus, the use of pigs as an animal model may help to reveal the pathogenicity of human viruses that possess similar pathogenic characteristics to PEDV.

Results

PEDV intranasal inoculation causes typical PED symptoms. To detect whether the PEDV can infect piglets through intranasal inoculation, we carried out animal challenge experiments. Piglets were assigned to 3 groups (I: Control, II: PEDV intranasal inoculation, and III: PEDV oral inoculation). Clinical signs included severe watery diarrhea with vomiting that were first detected in oral PEDV-inoculated piglets at 46 h post-infection (hpi). At 60 hpi, the intranasal inoculated piglets from group II began to exhibit classical PEDV symptoms, including acute, severe watery diarrhea, depression, and lethargy. Abundant yellow, foul-smelling watery stools were also observed around the perianal region of the piglets (Fig. 1a). Then, the piglets were anesthetized with pentobarbital sodium (100 mg kg\textsuperscript{-1}) and sacrificed for macroscopic examination at 66 hpi. After intranasal inoculation, all piglets exhibited moderately thin and transparent intestinal walls in the small intestine, with an accumulation of large amounts of fluid in the intestinal lumen (Fig. 1a). Pathological examination of the intestine of piglets with diarrhea after intranasal inoculation revealed multifocal to diffuse villous atrophy (Fig. 1b). Immunofluorescence analysis (IFA) showed a large amount of PEDV-positive cells in the small intestine of piglets with diarrhea, and PEDV antigens were mainly observed in the cytoplasm of villus epithelial cells (Fig. 1b). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) showed viral RNA expression in different tissues of the piglets after intranasal inoculation. PEDV mainly colonized the jejunum and ileum, and peak viral RNA titers reached up to 5.19 log\textsubscript{10} in the jejunum, this titer was significantly higher than that in the other tissues (Fig. 1c). PEDV also showed a broad tissue tropism, and low-level RNA expression was detected in the epithelium of nasal and lung tissues (Fig. 1c). Western blot results further validated the PEDV level in different tissues, and a significant quantity of PEDV N protein was detected in the jejunum and ileum. However, no immunoreactivity was observed with proteins of the nasal epithelium or lung despite the low level of RNA expression (Fig. 1d).

Replication of PEDV in the nasal cavity of piglets. After nasal inoculation, the virus was first detected in the nasal mucosa, trachea, and lung, while no viral RNA was detected in the gastrointestinal tissue (Fig. 2a). The virus showed a certain degree of
replication in the nasal epithelium within 24 h, with RNA titers reaching a peak of 3.14 log10 within 12 h and declining at 24 hpi. At 24 h, many PEDV gene copies were detected in the jejunum and ileum at higher levels than those in other tissues. The nasal mucosa of piglets was isolated at different time points after intranasal infection to further investigate the replication features of PEDV in NECs. Similar to the PEDV RNA expression in the nasal epithelium, fluorescence-activated cell sorting (FACS) analyses showed that PEDV colonized NECs, with 0.79% positive cells observed at 3 h, and maintained the infection between 12 and 24 h (PEDV-positive cells ranging from 1.81 to 2.02%). In contrast, the level of virus in NECs declined at 66 hpi (Fig. 2b, c). Moreover, after infection, the nasal cavity was fixed after infection for 12 h and subjected to IHC analysis. Virus-positive cells were unevenly distributed throughout the cytoplasm of NECs in regions I–IV (Fig. 2d, Supplementary Fig. 1c). In contrast, section IV in which most DCs and other lymphocytes accumulated, showed an increased proportion of PEDV-positive cells (Fig. 2d, e).

**Nasal epithelium culture and susceptibility to PEDV.** We used a nasal epithelial cell model (NECM) (Fig. 3a) to verify the consequences of PEDV infection in vitro. Morphological examination of NECs cultures at 6 days revealed successful establishment of the NECM model (Fig. 3b). The stripped nasal mucosa was shown in Supplementary Fig. 2a, and the cilia structure in some of the isolated nasal epithelium was preserved (Fig. 3b). The NECM was shown in Supplementary Fig. 2d. The apical side of the NECM/DCs coculture system was affected by DCs or PEDV. Barrier disruption was performed by the addition of EDTA to the apical medium (Supplementary Fig. 3b). Of note, DCs coculture or PEDV challenge did not modify the morphological characteristics of tight junctions (Supplementary Fig. 3c). Moreover, the TEER of the NECM/DCs coculture system was not influenced by DCs or PEDV. Barrier disruption was performed by the addition of EDTA to the apical medium (Supplementary Fig. 3d). The apical side of the NECM/DCs coculture system was inoculated with fluorescence-labeled PEDV (Fig. 4c). In the coculture system, inoculation with live PEDV resulted in a greater number of PEDV-loaded submucosal DCs than did inoculation with inactivated virus (Fig. 4d, e). The number of virus-loaded submucosal DCs markedly increased with the increased PEDV inoculation dose (Fig. 4f). To explain how PEDV entered the DCs in the coculture system, we used an antibody against histo-compatibility complex class II (MHCII) to identify DCs in z-orthogonal views using confocal laser scanning microscopy (CLSM) (Fig. 4g). DCs were clearly visualized as enlarged TEDs.

![Image](https://example.com/image1.png)

**Fig. 1** PEDV causes typical PED symptoms after intranasal inoculation. a) Acute watery diarrhea and gross lesions of the intestine in piglets after PEDV intranasal inoculation at 66 hpi. b) Hematoxylin and eosin (H&E) staining and IFA of the intestine of an intranasal inoculated pig at 66 hpi. PEDV antigen was located in the atrophied intestinal villus (white arrowhead). In: intranasal inoculation. The scale bar represents 50 μm. Blue, DAPI; green, PEDV. c) Viral RNA expression in different tissues of the diarrheic piglets after intranasal inoculation, n = 6 from 3 piglets per group. Data are the mean ± SD. Statistical significance was using one-way ANOVA. **P < 0.01. d) Protein expression of PEDV in different tissues of diarrheic piglets as determined by Western blotting with a mouse mAb against N protein. At least three independent experiments were performed.
was detected by PEDV N protein staining, individual cells isolated from the nasal mucosa (both left and right nasal cavity) were gated based on CK18 after intranasal PEDV challenge, 4 epithelial cells (Fig. 4h). Transmission electron microscopy (TEM) observations of the NECM/DCs coculture system further confirmed that DCs extended dendrites into the filter and captured PEDV (Fig. 4i). Integral PEDV virions could be found in the extended dendrites and cytoplasm of DCs on the basolateral side (Fig. 4i).

CCL25 and NF-κB pathway are involved in DCs recruitment. DCs maturation is a key step in the subsequent antigen-specific immune response. In the coculture system, although the levels of MHCII stimulated by PEDV were not different from those of the control, the increases in swine workshop cluster 3a (SWC3a) and cluster of differentiation 1a (CD1a) after PEDV inoculation were virus dose-dependent, demonstrating the maturation of submucosal DCs (Fig. 5a, b). To better understand the molecular cues required for DCs recruitment and TED formation in the nasal passage, we assessed the expression of many chemokines associated with DCs migration. Chemokine C–C motif ligand 25 (CCL25) was upregulated at both RNA and protein levels after PEDV inoculation (Fig. 5c, d). Additionally, after PEDV inoculation, an increase in P65 in the nucleus and p-P65 in the cytoplasm confirmed the activation of nuclear factor kappa B (NF-κB) in NECs (Fig. 5e). However, pretreatment of the NECM with an inhibitor of NF-κB (BAY 11-7082), but not with the DMSO control significantly reduced the number of PEDV-containing DCs and TEDs on the apical side in the coculture system (Fig. 5f–h).

T cells acquire PEDV from DCs and enter the peripheral blood. PEDV was detected in CD3+ T cells in peripheral blood mononuclear cells (PBMCs) of piglets within 24 h after intranasal inoculation. The proportion of virus-loaded CD3+ T cells increased at an early stage and peaked with 4.15% at 12 h (Fig. 6a, b). Virus in CD3+ cells may be acquired from DCs, which take up virus into the lumen across the nasal mucosa, migrate, and engage with T cells. Coculture of DCs and T cells was employed to study
PEDV transmission from DCs to T cells. FACS analysis showed that PEDV could be transferred from DCs to CD3+ T cells, with 2.64 and 5.33% PEDV+ CD3+ T cells detected at 2 and 12 h of coculture, respectively (Fig. 6c, d). In contrast, the PEDV RNA expression level in T cells remained stable within 48 h, inconsistent with the FACS results (Fig. 6e).

To detect whether PEDV could induce the conjugate structure between DCs and CD3+ T cells, we performed a conjugate formation experiment. FACS analysis of conjugate formation revealed that 42.8% of PEDV-treated DCs formed conjugates with T cells, while DCs in the control group and PEDV (inactivated)-treated group showed reduced conjugate formation compared with those in the live virus treatment group (9–15% of PBS or PEDV (inactivated)-treated DCs formed conjugates with T cells) (Fig. 6f). Furthermore, DC/T cell conjugates induced by PEDV were observed by CLSM, which revealed virus in the conjugate structures formed by the two cells (Fig. 6g). TEM imaging also showed that PEDV was taken up by DCs located within large compartments (Fig. 6h, arrowheads) at the synapse, a cell–cell contact that involves extensive interdigitation (black frame) of the respective cell membranes (Fig. 6h). The combination of the membrane encasement, deep virion channels and interdigitation between the donor and target cell membranes served to ensure that PEDV transfer to T cells occurs in a highly secluded environment. The panel II of Fig. 6h shows that PEDV was successfully transferred from DCs to T cells, and integrated coronavirus-like particles (arrowheads) were present in the cytoplasm of T cells.

**Virus-loaded T cells can cause intestine infection.** Next, to evaluate whether PEDV-loaded T cells could reach the small intestine, we performed a competitive T cell autotransfusion assay in piglets. After 24 h, more CFSE-labeled T cells (coculture with PEDV-treated DCs) entered the blood circulation and jejunum than did CM-Dil-labeled T cells (coculture with mock infection DCs) (Fig. 7a, b). Observations of frozen sections of the jejunum further confirmed the FACS results (Fig. 7c). We further assessed whether the transferred PEDV-loaded T cells could transmit the virus to host cells. T cells (donor cells) acquired the virus from DCs and were sorted and cocultured with PEDV-susceptible Vero cells (recipient cells) in two forms, contact or noncontact (Fig. 7d). Among CD3+ T cells, 3% were PEDV-positive in both the contact and noncontact groups at the original stage. Surprisingly, PEDV-positive T cells again appeared in both groups after 96 h of coculture. Particularly in the contact group, as much as 51.9% of PEDV+ CD3+ T cells were detected, which was much higher than those in the direct contact group (Fig. 7e). As shown in Fig. 7f, the absence of virus in the supernatant at 2 h eliminated the possibility of residual PEDV outside the T cells in the medium. In contrast to Vero cells in the noncontact group, Vero cells in the contact group with T cells exhibited higher PEDV RNA expression levels and...
virus titers in the medium (Fig. 7f, g). An apparent CPE appeared in Vero cells in the contact group at 96 h, while a mild lesion was observed in Vero cells in the noncontact group (Fig. 7h). Moreover, in the contact group, we observed many Vero cells displaying dramatic changes in cellular morphology, with significant increases in membrane extensions in which the filopodia appeared to be very thick, essentially forming a large branch emanating from the central body of the cell and contacting T cells (Fig. 7i).

The above donor T cells and target Vero cells were further analyzed by TEM (Fig. 7j). The variation in morphological characteristics, such as an elongated cell and cellular pseudopodium, were observed in T cells compared with mock-infected T cells (coculture with PBS-treated DCs). Moreover, the accumulation of a larger number of PEDV particles in the cytoplasm of target Vero cells (white asterisk) after 96 h by contact culture validated the transfer of infection between donor and target cells.
Discussion

Due to its devastating impact on the global pig industry in the US, PEDV has aroused vast concern worldwide. Most published research regarding PEDV has focused on its epidemic characteristics, structural features, and vaccine development; fewer studies have been conducted to delineate its mechanisms of pathogenic pathways, which are generally thought to occur via the digestive tract. However, based on its previously reported airborne characteristics, we propose that another pathogenic pathway may be involved in PEDV infection in piglets. In this study, we demonstrate an alternative pathway of PEDV infection, in which PEDV can disseminate from the entry site (nasal epithelium) to the pathogenic site (intestinal mucosa) by utilizing immune cells.

Through intranasal inoculation, we found that PEDV could cause typical PED symptoms in piglets, and the fecal titers achieved through intranasal inoculation were the same as those achieved through oral inoculation. The longer incubation period observed in the intranasal inoculation group may be attributed to the unique dissemination pathway of the virus, which spreads from the nasal epithelium to the intestine. The nasal mucosa is the first site to be directly exposed to inhaled pathogens, representing an important portal for pathogen entry. In our study, PEDV developed a transient nasal epithelium infection, and more virus was detected at the rear of the nasal cavity. Transient patterns of virus shedding were observed, with viral loads in tissues and host RNA expression peaking early in infection and decreasing over time. We speculate that PEDV primarily affects the nasal epithelium in a transient manner, by which the airborne virus can resume its activity and produce progeny virions in the nasal cavity. Moreover, according to previous research, DCs and many other lymphocytes mainly accumulate at the rear of the nasal cavity.

An interaction between the virus and immune cells is frequently observed, and may benefit or impede the virus. DCs are widely distributed in the mucosal lamina propria of various tissues, such as the respiratory, gastrointestinal, and genital tracts. DCs have the ability to take up antigens at these sites, predisposing them to become primary targets for viral infection. Submucosal DCs are characterized by the extension of cellular processes into the lumen for continuous surveillance. However, as a double-edged sword, the function of DCs is sometimes exploited by the virus to overcome the mucosal barrier. For instance, HIV-infected DCs are mainly responsible for establishing a foothold of infection. DCs are exploited by VZV invading the mucosa and are known as the immune system’s Achilles heel during VZV infection. Additionally, many other viruses, including Dengue virus, measles virus, and cytomegalovirus, can utilize DCs to enter the body, disseminate in humans, evade antiviral immune responses, and cause disease.

In our study, DCs formed TEDs to capture luminal PEDV virions in vitro and in piglets, and then helped PEDV overcome the nasal epithelial barrier. Furthermore, integral viral particles may be excluded from degradation by acid proteolysis for more efficient vesicle-mediated transfer to other cells. Moreover, PEDV has evolved strategies to exploit DCs maturation to promote its dissemination, while unchanged levels of MHCII expression may induce interference with MHCII-associated antigen presentation. The family of NKx3.1 transcription factors plays a central role in coordinating the expression of a wide variety of chemokines, a family of small molecules that induce chemotaxis of neutrophils, mononuclear cells, or lymphocytes. In our study, activation of NKx3.1 in NECs by PEDV was associated with the release of the chemokine CCL25 and mobilized DCs to accumulate in the submucosal regions. Taken together, these results imply that, although the virus can be released only from the apical side of the NECM, submucosal DCs may function as an important portal for PEDV entry into the nasal mucosa.

DCs can transfer HIV to CD4+ T cells in which it replicates explosively. VZV exploits DCs as “Trojan Horses”, allowing it to shuttle to the tonsils and regional lymph nodes, where VZV-infected DCs establish T cell infection. In our study, DCs could transfer PEDV to CD3+ T cells by inducing functional cross-talk within the conjugated structure between DCs and CD3+ T cells, and the virus was detected in these structures. Considering the structural similarity, the large membranous puncta from PEDV-containing DCs to T cells are potentially similar to the previously described virological synapses responsible for the HIV-1 trans-sinfection process during HIV pathogenesis. However, the detailed molecular mechanism by which PEDV is transferred from DCs to T cells has not been defined.

In addition, PEDV-loaded CD3+ T cells can enter the peripheral blood and reach the intestinal mucosa through the blood circulation. Such adventures are replete with difficulties and challenges for free PEDV, such as limited diffusion, anatomical barriers, and soluble immune factors (complement and natural antibody). Allowing infected T cells to retain robust motility and serve as migratory vehicles might therefore be a viral strategy for efficient local and systemic dissemination. Numerous high endothelial venules (HEV) are distributed in Peyer’s Patches and lymph nodes of the intestine, through which virus-carrying T cells can enter the intestinal mucosa after reaching the small intestine through the blood circulation.

Subsequently, T cells can become an important source of infection. VZV-infected T cells can potentially transfer VZV to...
the intestinal tract and establish latency in enteric neurons in vivo.

The virus-carrying lymphocyte induction of infection in susceptible cells is known as transfer infection. The specific mechanism of transfer-infection has been more extensively studied in HIV, which can spread from T cells to astrocytes and uninfected T cells via cell-to-cell contact, indicating a unique interpretation of rapid HIV transmission during the early process of infection. PEDV infection of Vero cells occurred efficiently by cell-to-cell contact with PEDV-infected CD3+ T cells. CPE of Vero cells in the contact group was apparent at 96 h, while the minor lesion was concomitantly observed in the noncontact group. PEDV could be transferred within 2 h through T cell contact coculture; in turn, large amounts of virus were transferred from infected Vero cells to T cells. We also detected large extensive membranous structures, either as membrane sheets or lopodial bridges, between Vero and CD3+ T cells, that might be

Fig. 5 Role of CCL25 and NF-κB pathway in DCs recruitment and TED formation. a, b After incubation with medium, PEDV or PEDV plus antiserum on the apical side of the NECs for 20 h, basolateral DCs were collected. The expression of phenotypic marker including CD1a, SWC3a, and MHCII on DCs was analyzed by FACS. n = 3 per group. c After 1 h of PEDV incubation, qRT-PCR was used to determine the relative mRNA expression of a series of chemokines related to DCs in NECs, n = 3 per group. d The protein expression of CCL25, CCL20, and CXCL2 in the medium of the basolateral side were detected using ELISA kits, n = 3 per group. e After PEDV inoculation, the phosphorylation of p65 and nuclear p65 in the NECs of the coculture system was detected at the indicated time by Western blotting. f The coculture system was pretreated with an NF-κB inhibitor for 2 h (DMSO as a negative control) by upper compartment inoculation, followed by inoculation with DyLight 633-labeled PEDV for 1 h. Basolateral DCs were collected and analyzed by FACS. g IFA of filters from the coculture system and a three-dimensional (3D) rendering of representative fields showing that basolateral DCs sent dendrites (MHCII, green) to creep through ECs in response to PEDV and/or inhibitor. h Quantitative analysis of TEDs was performed. The number of TEDs was counted from five random fields of view at a unit area (0.078 mm²) for each of three individual filters and depicted as a dot plot with each dot representing a field. All data are the mean ± SD, comparisons performed with one-way ANOVA. *P < 0.05, **P < 0.01. The results are from at least three different experiments.
involved in PEDV transmission between CD3⁺ T and Vero cells. Whether these structures are present during PEDV cell-to-cell transfer in piglets or the role they play in PEDV cell-to-cell transmission will require further exploration. Cell-to-cell spread of PEDV in trans or in cis may permit efficient ongoing replication and spreading despite a variety of defense strategies in the intestinal mucosa.

Our study demonstrates an alternative pathogenic pathway of PEDV, which can cause typical pathogenic symptoms in piglets through nasal cavity inoculation. The exact mechanism by which PEDV enters the intestinal epithelium through the respiratory route has been investigated (Fig. 8). The tropism of PEDV for NECs is responsible for virus propagation and accumulation at the rear of the nasal cavity. However, DCs may play an important role in virus entry into the nasal mucosa and transfer of the virus to T cells. Furthermore, the virus-carrying T cells could reach the intestine via the blood and lymph circulation and transfer the virus to the intestinal epithelium. These findings are helpful in facilitating strategies for
PEDV prevention and providing insights into the pathogenicity of viruses with the same characteristics, although the specific molecular mechanisms remain to be elucidated.

Methods

Virus. The wild-type PEDV strain ZJ was obtained from intestinal contents of a 2-day-old diarrheic piglet on a farm in Jiangsu in 2012, and this strain clustered with the emerging virulent strain based on phylogenetic analysis. Viruses were purified using discontinuous sucrose density gradient centrifugation. Heat-inactivated viruses were prepared at 56 °C for 0.5 h and tested for complete loss of infectivity by inoculation into Vero cells multiplicity of infection (MOI) = 1 for CPE observations. Fluorescence labeling of the virus was performed with the fluorescent probe DyLight 633 NHS Ester (Thermo Fisher Scientific), according to the instructions provided by the manufacturer. Unincorporated dye was removed using commercial fluorescent dye removal columns (Thermo Fisher Scientific). Labeled viruses were stored at 4 °C and used within 2 days.

Animals. Caesarian-derived and colostrum-deprived xin huai piglets were obtained from a swine herd at the Jiangsu Academy of Agricultural Science (JAAS) and were artificially fed with milk. The swine herd was seronegative for antibodies against PEDV, PRRSV, PRCV, TGEV, and porcine circovirus type 2. Each experimental group of pigs was housed in a separate room in a high-security isolation facility. All animal procedures and experiments were performed according to protocols.
Fig. 7 PEDV-loaded T cells reach the intestine and mediate transfer infection. a Through an autotransfusion competition experiment, the percentage of PEDV-treated (CFSE) and PBS-treated (CM-DiI) cells among PBMCs and in the jejunum were analyzed by FACS. b The numbers of cells shown in (a) were quantified, n = 3 piglets per group. c After the autotransfusion competition experiment, frozen sections of the jejunum from mock-injected and fluorescence-labeled cells injected groups were stained with DAPI (blue) and observed by fluorescence microscopy. PEDV-treated (CFSE) CD3+ T cells were marked with white arrowheads. Bars, 50 μm. d Schematic of the model to study PEDV-carrying CD3+ T cell transmission of the virus to Vero cells. PEDV-pulsed DCs were cocultured with CD3+ T cells for 2 h and donor DCs were removed by MACS. Then, the remaining CD3+ T cells were cocultured with Vero cells by two methods (contact and noncontact coculture). e PEDV+CD3+ T cells in the contact and noncontact groups were detected by FACS at the indicated times. f In addition, viral titers in the supernatant of the coculture system were measured by a plaque assay, n = 3. g PEDV RNA expression in Vero cells was evaluated by qRT-PCR at 96 h, n = 3. h At 48 hpi, Vero cells in the noncontact and contact groups were observed. LM light microscopy. i Vero cells (contact group) displayed thin cell bodies with long, branched membrane protrusions toward the T cells at 24 and 96 h. Bars, 20 μm. j TEM image showing the morphological features of sorted T cells cocultured with medium or PEDV-treated DCs. Bars, 1 μm. When cocultured for 96 h, Vero cells from the contact group were processed for TEM, which revealed an accumulation of virus particles in the cytoplasm. Some virus particles are indicated by a white asterisk. Bars, 1 μm. All data represent the mean ± SD, comparisons performed with t-tests (two groups) or analysis of variance (ANOVA) (multiple groups). *P < 0.05, **P < 0.01. Data were combined from at least three independent experiments unless otherwise stated.

approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) and followed the National Institutes of Health guidelines.

Reagents and cell lines. To study DCs maturation in coculture systems, fluorescently labeled anti-pig PE-SW/C3a (1:200, ab25684), FITC-SW/C3a (1:200, ab24885), PE-CD1a (1:200, ab25599) and the respective isotype and fluorochrome-matched control antibodies mouse IgG1 (SW/C3a) and mouse IgG2α (CD1a) were purchased from Abcam. Anti-pig FITC-MHC-I (1:400, MCA2314F) and its isotype control mouse and PE-IgG2b antibodies were purchased from Bio-Rad. For analyzing the cells in complete medium Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 20 ng·mL−1 porcine GM-CSF, and 10 ng·mL−1 porcine IL-4 and plated at 106 cells·mL−1 in 6-well plates. Pig adherent granulocytes were removed by discarding the culture medium after 60 h. On day 6, cultures, the clusters were harvested and subcultured overnight for removing adherent cells. Non-adherent cells were collected after 7 days, washed, and used as DCs for the subsequent studies.

Establishment of the NECM and NECM/DCs coculture system. The NECs culture protocol was based on a modified method used for human airway epithelial cultures46. The acquired NECs were collected, adjusted to 106 cells·mL−1 and seeded at a cell concentration of 2 × 106 cells·cm−2 onto Transwell inserts (12 μm pore size; 6.5 mm membrane diameter; Corning) coated with collagen from human placenta Type IV (6 μg·mL−1) (Sigma). After the cells reached confluence, the medium was replaced with a differentiating medium, and the cells were brought to the air–liquid interface. When the NECM formed an air–liquid interface and stable TEER, the filters were turned upside down, and the DCs (5 × 105) were cultured for 4 h on the filter facing the basolateral membrane of NECs to allow the cells to attach to the filter. Medium, DyLight 633-labeled PEDV, and WIV PEDV were incubated on the apical side of the NECs and cultured at 37 °C for 1 h. Then, DCs were collected for FACS analyses. Moreover, DyLight 633-labeled PEDV were incubated on the apical side of the NECs and cultured at 37 °C for 6 days. Vero cell monolayer was collected for CLSM analyses. As a control group, DyLight 633-labeled PEDV were incubated at 4 °C with a MOI of 0.1.

PEDV infection and transmission. DCs were pulsed with PEDV (MOI = 0.1) for 1 h at 37 °C and washed extensively to remove unbound virus. PEDV-pulsed DCs were cocultured with autologous positively selected CD3+ T cells that had been maintained at 37 °C in RPMI 1640 medium supplemented with IL-2 (10 IU·mL−1) for the subsequent study. After 2 h, donor DCs were removed from CD3+ T cells using MiniMACS Starting kits (anti-Sw3a-PE and anti-PE beads). PEDV-carrying CD3+ T cells were cultured in maintenance medium for viral RNA titers determination. For TEM studies, the mixtures were centrifuged at 200×g for 1 min to facilitate conjugate formation prior to co-culturing. After 1 h in culture, the mixtures were collected by centrifugation at 300×g for 5 min and fixed in glutaraldehyde/cadecylate buffer.

Conjugate formation. The DCs obtained after 7 days of culture, untreated or treated with PEDV (MOI 0.1), were labeled with PKH26 according to the manufacturer’s instructions. T cells were labeled with 10 nM CFSE for 10 min at 37 °C. PEDV-pulsed DCs and T cells mixtures were centrifuged at 200×g for 5 min. The pellet was incubated at 37 °C for 30 min, gently resuspended in 200 μL of PBS and analyzed immediately for double-labeled cell conjugates by flow cytometry (BD FACS Calibur).

Transfer infection. Vero cells were seeded in 24-well plates and grown to monolayers before transfer infection. PEDV-carrying CD3+ T cells were cocultured with Vero cells by two methods (contact and noncontact). In the contact method, T cells were directly cultured with Vero cells. In the noncontact method, T cells were grown in Transwell® assemblies with a pore diameter of 0.4 μm over Vero cell monolayers. Negative controls, including uninfected T cells, and
positive controls, which consisted of an identical number of PEDV viruses placed over the 0.4 μm filters, were used to verify that infectious cell-free PEDV was able to cross the filters in the Transwell assemblies. T cells and the medium of the Vero coculture were collected at the indicated times. Vero cells were washed five times with PBS to eliminate the nonabsorbed virus and T cells, followed by culture in maintenance medium for 96 h.

In vivo competition experiments. For the in vivo competition assay, CD3+ T cells were isolated from three littermate piglets and cocultured with DCs (treated or not with PEDV). Then, the CD3+ T cells were purified and labeled with CFSE (green) or CM-DiI (red, Molecular Probes), respectively, for long-term cell labeling. Equal numbers of cells (1 × 10⁷) from the two populations were then mixed and adoptively transferred into piglets (autologous) via front cavity vein injection. The recipient piglets were sacrificed 24 h after the injection. For the flow cytometry analyses, cells were isolated from PBMCs, duodenum, jejunum, and ileum. For histological analyses, the duodenum, jejunum, and ileum were washed with 0.1 M PBS (pH 7.4) and embedded in OCT compound (Sakura Finetechical). The frozen tissues were cut into 10-μm sections and mounted onto poly-L-lysine-coated glass slides. The CM-DiI or CFSE-labeled cells were observed with a fluorescence microscope (Carl Zeiss). Investigators were not blinded to experimental groups. Pathologists interpreting slides demonstrated equal protein sample loading. Uncropped Western blot images of data shown in Figs. 1 and 3 can be found in Supplementary Figures 4.

Flow cytometric analysis. In vivo, after PEDV inoculation, epithelial cells were acquired from the mucosa of the nasal cavity and stained with an antibody against CK18. After surface staining, the cells were resuspended, fixed, and analyzed by flow cytometry. CD107 + T cells were detected by FACS for 24 h. The harvested CD107 + T cells were washed twice with cold PBS and stained with fluorescent mAbs specific for porcine CD3 and PEDV N protein. The collected DCs were detected by FACS for fluorescent PEDV uptake. In addition, after NECM/DCs coculture, the cells were stimulated with PEDV for 24 h. The harvested DCs were washed twice with cold PBS and stained with fluorescent mAbs specific for porcine MHCII, SWC3a and CD1a or the respective isotype controls at 4 °C for 0.5 h, according to the manufacturer’s guidelines. After three washes with PBS, the cells were phenotypically analyzed by FACS (BD FACS Calibur).

Cytokine assays by enzyme-linked immunosorbent assay (ELISA). Cytokines were analyzed by ELISA. The production of cytokines (CCL2, CCL20, and CXCL2) was measured using ELISA kits (ebioscience), which was performed according to the manufacturer’s instructions.

Immunohistochemistry and IFA assay. PEDV-infected piglets were anesthetized and sacrificed via an intravenous injection of pentobarbital sodium (100 mg kg⁻¹) 12 h post intranasal inoculation. After fixation, histological sections of four blocks were selected according to the fractions 1/4, 2/5, 3/5, and 4/5, using the landmarks provided in the diagram presented in Supplementary Fig. 1a. Four cross-sections (I, II, III, and IV) taken at the fractions (Supplementary Fig. 1b) were subsequently selected for assessing the distribution of PEDV by immunohistochemistry (IHC) using primary antibodies directed against PEDV N protein. DCs located in nasal mucosa were immunolabeled with rat anti-Swc3a mAb and mouse anti-MHCII mAb, followed by Alexa Fluor 594-conjugated goat anti-rat IgG and Alexa Fluor 488-conjugated goat anti-mouse IgG. The colonization of PEDV in cross-sectional sections IV was detected by IFA with PEDV polyclonal antibody. Fixed filters were permeabilized in 0.2% Triton X-100 in PBS for 5 min. After blocking with 5% bovine serum albumin in PBS for 1 h, the filters were incubated with primary antibodies overnight at 4 °C.
followed by fluorescent secondary antibodies at room temperature for 1 h. Pathologists interpreting slides were blinded to PEDV-treated and control animal identifiers.

Statistical analysis. Results are expressed as the means ± SD and analyzed with SPSS 17.0. One-way analysis of variance (ANOVA) was employed to determine significant differences among multiple groups, and a t-test was employed to determine the differences between the two groups. *P < 0.05, **P < 0.01. Data were combined from at least three independent experiments unless otherwise stated.

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

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Author contributions
Yuchen Li was responsible for performing the experiments, data analysis, and writing the manuscript. Qinxing Wu and Jialu Wang were responsible for animal experiments. Lulu Huang and Chen Yuan were responsible for a series of coculture models establishment. Qian Yang was responsible for the conception and design of the study, data collection, drafting the article, and final approval of the version submitted.

Additional information
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