Contact-Dependent Transmission of Langat and Tick-Borne Encephalitis Virus in Type I Interferon Receptor 1-Deficient Mice

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ABSTRACT Tick-borne encephalitis virus (TBEV) is transmitted to humans primarily through tick bites or oral consumption of accordingly contaminated unpasteurized milk or milk products. The detection of TBEV RNA in various body fluids in immunosuppressed human patients is documented. However, the risk of direct contact exposure remains unclear. Interferon alpha receptor 1-deficient (*Ifnar1*^{-/-}) mice, which lack the interferon alpha/beta responses, develop neurologic manifestations after infection with TBEV and Langat virus (LGTV). We showed that subcutaneous, intranasal, and peroral infections of LGTV lead to disease, whereas mice with intragastric application of LGTV showed no disease signs. With LGTV infection, mice exhibit seroconversion, and significant viral RNA levels were detected in saliva, eye smear, feces, and urine. As a result, TBEV and LGTV are transmitted between mice from infected to naive cocaged sentinel animals. Although intranasal inoculation of LGTV is entirely sufficient to establish the disease in mice, the virus is not transmitted by aerosols. These pooled results from animal models highlight the risks of exposure to TBEV contaminants and the possibility for close contact transmission of TBEV in interferon alpha receptor 1-deficient laboratory mice.

IMPORTANCE Tick-borne encephalitis is a severe disease of the central nervous system caused by the tick-borne encephalitis virus (TBEV). Every year, between 10,000 and 12,000 people become infected with this flavivirus. TBEV is usually transmitted to humans via the bite of a tick, but infections due to consumption of infectious milk products are being increasingly reported. Since there is no therapy for TBEV infection and the mechanisms of virus persistence in reservoir animals are unclear, it is important to highlight the risk of exposure to TBEV contaminants and know the possible routes of transmission of this virus. The significance of our research is in identifying other infection routes of TBEV and LGTV and the possibility of close contact transmission.

KEYWORDS Langat virus, mouse model, tick-borne encephalitis virus, virus transmission

The tick-borne encephalitis virus (TBEV) occurs in large areas of Europe and Asia and causes a severe zoonotic vector-borne disease. TBEV belongs to the family *Flaviviridae*, which includes West Nile virus (WNV), Japanese encephalitis virus (JEV), dengue virus (DENV), and Zika virus (ZIKV). The annual incidence of human cases is in the range of 10,000 to 12,000 people (1, 2). The disease has a typical biphasic course in about 72 to 84% of patients infected with TBEV-Eu (2). In the first phase, which occurs during the early 2 to 28 days after infection, one-third of infected people show flu-like symptoms, such as fever, headache, body aches, and, less often, diarrhea (3, 4). Thirty percent of the patients with clinical symptoms move into a second phase, which is associated with neurological syndromes such as meningitis and encephalitis. In rare cases, patients develop a postencephalitic syndrome that includes neuropsychiatric symptoms, severe headaches, and a general decrease in quality of life (5–7). The overall mortality rate of 2% in Russia is comparable

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to that of subtypes in Europe (1, 8, 9). Viral and host factors, such as infectious dose, subtype, age, genotype, and health status, can influence the severity of the disease in humans (3, 10–12). A vaccination against TBEV is available in some countries, but so far, there is no drug therapy (13).

Among ticks, virus transmission through sexual contact has been described in *Ixodes persulcatus* and *Hyalomma antalicum* ticks or during cofeeding under laboratory conditions (14, 15). In humans, TBEV is usually transmitted by the bite of hard ticks, in Europe mainly by ticks of the genus *Ixodes* (16), and rarely by the consumption of contaminated dairy products (17–19). In addition, rare cases of accidental laboratory infection by highly infectious aerosol inhalation are known (20, 21). Other natural transmission routes, such as inhalation of aerosolized rodent excretions in the case of hantavirus infections (22) or sexual transmission between humans as for ZIKV (23, 24), have not been described for TBEV so far. Excretions and the persistence of contagious body fluids in infected humans are documented only in immunocompromised patients for TBEV (25, 26). The risk of contact exposure to infectious body fluids remains unclear. To investigate the impact of different infection routes and horizontal vector-free transmission of TBEV or Langat virus (LGTV) in an immunodeficient host, we used alternative infection routes and cohousing experiments with mice. Here, we describe the transfer of TBEV and LGTV in mice and show that this occurs exclusively by close contact and not by the spread aerosols.

RESULTS

Adult mice support efficient LGTV transmission. The manifestation of encephalitis was not observed in LGTV-infected wild-type C57BL/6 mice. In contrast, we previously showed that intraperitoneal (i.p.) infection with LGTV leads to viremia and the development of encephalitis in *Ifnar1*^{−/−} mice (27). We validate *Rag2* γ ^{−/−} and *Ifnar1*^{−/−} mice as a potential model to study LGTV transmission. Highly susceptible *Rag2* γ ^{−/−} or *Ifnar1*^{−/−} mice were infected intraperitoneally with 10² focus-forming units (FFU) (index) (Fig. 1A). One index mouse was cohoused with several naive *Rag2* γ ^{−/−} or *Ifnar1*^{−/−} mice (contact). Mouse-to-mouse transmission was assessed in contact mice by determination of disease signs. *Rag2* γ ^{−/−} index mice showed body weight loss as the earliest disease sign, beginning from day 10 postinfection, followed by hunchback posture (Fig. 1B). All *Rag2* γ ^{−/−} index mice died 13 days postinfection. All contact mice developed the same symptoms and had to be killed from day 17 to 22. *Ifnar1*^{−/−} index mice developed the same symptoms as *Rag2* γ ^{−/−} mice but died already at 4 to 5 days postinfection (Fig. 1C and D). All *Ifnar1*^{−/−} contact mice developed symptoms and had to be killed 2 to 3 days after the first signs of illness appeared on days 9, 10, 11, 12, 14, 16, 17, 18, 19, and 20. Analysis of viral titers in the spleen, serum, and olfactory bulb (OB) of *Ifnar1*^{−/−} index mice revealed productive infection and viral spread to the brain (Fig. 1E).

Since all animals died with the same disease progression over a period of up to 11 days, we assume that mouse-to-mouse transmission of LGTV is possible in immunodeficient *Rag2* γ ^{−/−} and *Ifnar1*^{−/−} mice. But, contact mice were infected not only by the index mice but also by infected contact mice.

High titers are necessary for LGTV transmission. In contrast to the infection of *Rag2* γ ^{−/−} and *Ifnar1*^{−/−} mice, LGTV infection in wild-type C57BL/6 mice led to low viral replication (28). To test whether high viral titers in the index mice are necessary to enable transmission, wild-type mice were infected with 10² FFU LGTV (index) and cohoused with naive *Ifnar1*^{−/−} mice (contact) (Fig. 2A). Neither the index mice nor the susceptible contact mice developed any signs of disease or died (Fig. 2B). To investigate whether contact mice get in contact with the virus and can control the infection, we determined whether the mice developed neutralizing antibodies against LGTV (Fig. 2C). Neutralizing antibodies were detectable in infected wild-type index mice but not in cohoused contact mice. These data indicate that wild-type mice are not able to transmit the virus and that high viral titers in both the index mice and susceptible contact mice promote mouse-to-mouse transmission.

FIG 1 Mouse-to-mouse transmission of LGTV. (A) Schematic depiction of the experimental design. Mice were infected intraperitoneally with 10^2 FFU of LGTV (index, red) and cohoused with naive mice (contact, black) in the same cage. (B) Survival of *Rag2yc*^{-/-} mice plotted as Kaplan-Meier curves (index mice, $n=1$; contact mice, $n=4$). Data are representative of one experiment. Survival differences were tested for statistical significance by the log rank (Mantel-Cox) test ($P=0.0455$). (C) Survival of *Ifnar1*^{-/-} mice plotted as Kaplan-Meier curves (index mice, $n=5$; contact mice, $n=16$). Data are representative of five independent experiments. Survival differences were tested for statistical significance by the log rank (Mantel-Cox) test ($P<0.0001$). (D) Body weight analysis of individual mice over 20 days. Data are representative of one experiment (index mice, $n=1$; contact mice, $n=5$). (E) Viral titers in organs from noninfected (black) and LGTV-infected (red) *Ifnar1*^{-/-} mice determined by focus formation assay on day 4 postinfection. Results are representative of two independent experiments ($n=6$). Statistical analysis was done by a nonparametric t test and the Mann-Whitney test (**, $P=0.0022$).

Aerosols do not transmit LGTV during cohousing. To distinguish between direct contact and indirect airborne contact routes of transmission, infected *Ifnar1*^{-/-} index and contact mice were placed in housing boxes. Direct contact was prevented by the presence of a 2-mm mesh, providing a 4-cm separation distance, for 21 days. In this housing scenario, the airflow between the two compartments was unrestricted, but index and contact mice did not have direct contact with each other and did not share food and water sources (Fig. 3A). Although susceptible *Ifnar1*^{-/-} index mice developed disease signs and died on day 4 postinfection, cohoused contact mice developed no disease signs and survived (Fig. 3B). These data indicate that aerosol transmission is not involved in the mouse-to-mouse transmission of LGTV.

FIG 2 High viral titers in the index mice promote mouse-to-mouse transmission of LGTV. (A) Schematic representation of the experimental design. Wild-type C57BL/6 mice were infected intraperitoneally with 10^2 FFU of LGTV (index; red) and cohoused with naive *Ifnar1*^{-/-} mice (contact; black) in the same cage (index mice, $n=2$; contact mice, $n=7$). Data are representative of two independent experiments. (B) Survival of mice plotted as Kaplan-Meier curves. Survival differences were tested for statistical significance by the log rank (Mantel-Cox) test ($P>0.9999$). (C) Serum samples were collected 21 days postinfection, and a virus neutralization assay quantified LGTV-specific antibodies. Data are representative of two independent experiments.

Transmission of LGTV without direct contact. To investigate whether direct contact of index and contact mice is necessary for mouse-to-mouse transmission, transfer experiments were performed (Fig. 4A). Infected *Ifnar1*^{-/-} index mice were kept in a cage for 24 h before they were transferred to a new cage. Naive *Ifnar1*^{-/-} contact mice were transferred directly to the cage previously inhabited by the infected index mice. Contact mice had contact to the litter, feces, urine, food, and water of the index mice. The procedure was continued for 5 days. The index mice died 4 to 5 days postinfection, showing weight loss and hunchback posture (Fig. 4B). Five of nine contact mice died, developing the same symptoms as the index mice. These data indicate that no direct contact of index and contact mice is necessary for mouse-to-mouse transmission of LGTV.

Contact with urine and feces is not sufficient for mouse-to-mouse transmission of LGTV. To restrict the source of infectious agents, we modified the cage transfer experiments. Index mice were housed for 12 h in cages without litter to increase contact with excrement. The index mice were then allowed to recover in cages with litter for 12 h before this housing regime was repeated. Naive *Ifnar1*^{-/-} contact mice were transferred to the cage previously inhabited by the infected index mice. The water bot-

FIG 3 No airborne transmission of LGTV. *Ifnar1*^{-/-} mice were infected intraperitoneally with 10^2 FFU of LGTV (index; red). Naive *Ifnar1*^{-/-} mice (contact; black) were added to each transmission cage adjacent to index mice (index mice, $n=6$; contact mice, $n=7$). Data are representative of two independent experiments. (A) Schematic representation of the experimental design. (B) Survival of mice plotted as Kaplan-Meier curves. Survival differences were tested for statistical significance by the log rank (Mantel-Cox) test ($P=0.0005$).

FIG 4 Contactless transmission of LGTV. Cage transfer experiments were performed. *Ifnar1*^{-/-} mice were infected intraperitoneally with 10^2 FFU of LGTV. (A) Schematic representation of the experimental design. Every 24 h, index mice were transferred into a new cage. Naive *Ifnar1*^{-/-} contact mice were housed in cages that had been previously inhabited by the infected index mice. (B) Survival of mice plotted as Kaplan-Meier curves (index mice, $n = 8$ [red]; contact mice, $n = 9$ [black]). Data are representative of two independent experiments. Survival differences were tested for statistical significance by the log rank (Mantel-Cox) test ($P < 0.0001$). (C) To increase contact with urine and feces, index mice were kept in new cages with or without litter every 12 h. Naive *Ifnar1*^{-/-} contact mice were housed in the cages that had been previously inhabited by the infected index mice directly after the food and water sources were renewed. Survival of mice is plotted as Kaplan-Meier curves (index mice, $n = 3$; contact mice, $n = 3$). Data are representative of one experiment. Survival differences were tested for statistical significance by the log rank (Mantel-Cox) test ($P > 0.9999$).

tle and food were exchanged before each transfer. The procedure was continued for 5 days. The index mice died 5 days postinfection, showing weight loss and hunchback posture, whereas all contact mice remained without any disease symptoms (Fig. 4C). Thus, direct contact with urine and feces is not sufficient for mouse-to-mouse transmission of LGTV.

Viral RNA is detectable in various body fluids. The virus can be secreted and excreted by multiple mechanisms. To assess the source of the virus for transmission, urine, feces, saliva, eye, and nose swabs were taken from LGTV-infected diseased *Ifnar1*^{-/-} mice. Viral RNA was detectable in all samples, with the highest content of viral RNA in feces (Fig. 5). These data suggest that diseased mice could secrete and excrete the virus by different routes.

Impact of infection routes on the infectibility by LGTV. Since LGTV is secreted and excreted by different routes, we determined whether the uptake of the virus by different infection routes could lead to disease. LGTV was administered to *Ifnar1*^{-/-} mice by various infection routes. As with intraperitoneal infection, subcutaneous infection of mice with 10^2 FFU led to the same infection kinetics and disease symptoms, such as body weight loss and hunchback posture (Fig. 6A). Mice were also highly susceptible to intranasal infection (Fig. 6A). Intranasal infection with 10^2 FFU led to death in 89% of the mice. However, the onset of illness was delayed, and mice showed an average survival of 8 days. Higher viral titers were necessary to induce disease symptoms by peroral infections (Fig. 6B); whereas all mice survived infection with 10^2 FFU, 86% of the mice died upon infection with 10^4 FFU. Disease symptoms were not detectable in mice infected by use of feeding needles (gavage), although high viral doses up to 10^6 FFU were used (Fig. 6C). Moreover, administration of virus in milk does not lead to disease symptoms or death in the mice (Fig. 6D).

FIG 5 LGTV RNA detection in secretions and excrements. *Ifnar1*^{-/-} mice were noninfected (black) or infected (red) intraperitoneally with 10^2 FFU of LGTV. Urine, feces, saliva, nose, and eye swabs were collected 4 days postinfection. The viral RNA was detected by qRT-PCR. Results are representative of two independent experiments (noninfected, $n=4$; nose, $n=10$; eye, $n=10$; urine, $n=7$; saliva, $n=10$; feces, $n=8$). Statistical analysis was done by a nonparametric t test and the Mann-Whitney test (***, $P=0.0001$; ****, $P<0.0001$).

These data suggest that mice could get infected by different infection routes. However, for some infection routes, higher viral doses or more extended periods are needed to induce disease symptoms.

Mouse-to-mouse transmission of TBEV. To determine if TBEV could also be transmitted from mouse to mouse, we performed cohousing experiments with *Ifnar1*^{-/-} index mice infected with the TBEV strain Torö-2003 (Fig. 7A). Index mice were highly

FIG 6 The infection route influences establishment of severe infection. *Ifnar1*^{-/-} mice were infected by different injection routes with the indicated LGTV amount. Survival of mice is plotted as Kaplan-Meier curves. Survival differences were tested for statistical significance by the log rank (Mantel-Cox) test. (A) Subcutaneous (10^2 FFU, $n=11$, black) and intranasal (10^2 FFU, $n=9$, blue) infection. Data are representative of two independent experiments ($P<0.0001$). (B) Peroral infection (10^2 FFU, $n=3$, black; 10^4 FFU, $n=7$, blue). Data are representative of one (10^2 FFU) or two (10^4 FFU) independent experiments ($P=0.0277$). (C) Oral gavage (feeding needle) infection (10^2 FFU, $n=14$, black; 10^6 FFU, $n=8$, blue). Data are representative of two independent experiments ($P>0.9999$). (D) Infection by oral gavage (feeding needle) with milk (10^2 FFU, $n=9$, black; 10^6 FFU, $n=9$, blue). Data are representative of two independent experiments ($P>0.9999$).

FIG 7 TBEV transmission from mouse to mouse. (A) Schematic representation of the experimental design. (B and C) *Ifnar1*^{-/-} mice were infected intraperitoneally with 10² FFU of TBEV Torö-2003 (index; red) and cohoused with naive *Ifnar1*^{-/-} mice (contact; black) in the same cage. (B) Survival of mice plotted as Kaplan-Meier curves (index mice, *n* = 2; contact mice, *n* = 8). Data are representative of two independent experiments. Survival differences were tested for statistical significance by the log rank (Mantel-Cox) test (*P* = 0.0027). (C) Body weight analysis of individual mice over 14 days. Data are representative for one experiment (index mice, *n* = 1; contact mice, *n* = 4). (D) *Rag2*^{-/-} mice were infected intraperitoneally with 10⁴ FFU of TBEV Torö-2003 (index, red) and cohoused with naive *Rag2*^{-/-} mice (contact, black) in the same cage. Survival of mice is plotted as Kaplan-Meier curves (index mice, *n* = 3; contact mice, *n* = 4). Data are representative of three independent experiments. Survival differences were tested for statistical significance by the log rank (Mantel-Cox) test (*P* = 0.0101). (E) Wild-type C57BL/6 mice were infected intraperitoneally with 10⁴ FFU of TBEV (index; red) and cohoused with naive wild-type C57BL/6 mice (contact; black) in the same cage. Survival of mice is plotted as Kaplan-Meier curves (index mice, *n* = 1; contact mice, *n* = 4). Data are representative of one experiment. Survival differences were tested for statistical significance by the log rank (Mantel-Cox) test (*P* = 0.0455).

susceptible to infection with Torö-2003, developed disease symptoms, and succumbed to infection by day 6 (Fig. 7B and C). All contact mice developed disease symptoms and died with the same disease progression from day 9 to 15. Infection of immunodeficient *Rag2*^{-/-} mice with Torö-2003 led to prolonged survival of mice, compared to wild-type mice. However, the transmission of the virus was possible, since 25% of contact mice died upon development of symptoms during a cohousing experiment (Fig. 7D). In contrast, whereas wild-type index mice died by infection with Torö-2003, all contact mice survived a cohousing without the development of disease symptoms (Fig. 7E). These data indicate that TBEV can also be transmitted from mouse to mouse in immunodeficient mice in the absence of a tick vector but not in immunocompetent mice.

We further determined the impact of infection routes by Torö-2003. Wild-type and

FIG 8 The infection route influences establishment of severe TBEV infection. Wild-type C57BL/6 and *Ifnar1*^{-/-} mice were infected by different injection routes with the indicated TBEV Torö-2003 amount. Survival of mice is plotted as Kaplan-Meier curves. Survival differences were tested for statistical significance by the log rank (Mantel-Cox) test. (A) Peroral infection of wild-type (10⁴ FFU, *n* = 5, black; 10⁶ FFU, *n* = 10, blue) and *Ifnar1*^{-/-} (10⁶ FFU, *n* = 7, blue dashed line) mice. Data are representative of one (wild-type mice, 10⁴ FFU) or two (wild-type and *Ifnar1*^{-/-} mice, 10⁶ FFU) independent experiments. *P* = 0.0199 (wild type at 10⁴ FFU versus wild type at 10⁶ FFU), *P* < 0.0001 (10⁶ FFU, wild type versus *Ifnar1*^{-/-}). (B) Peroral infection with milk of wild-type (10⁴ FFU, *n* = 9, black; 10⁶ FFU, *n* = 10, blue) and *Ifnar1*^{-/-} (10⁴ FFU, *n* = 5, black dashed line; 10⁶ FFU, *n* = 7, blue dashed line) mice. Data are representative of two independent experiments. *, *P* = 0.0383 (wild type at 10⁴ FFU versus wild type at 10⁶ FFU); *P* = 0.0192 (*Ifnar1*^{-/-} at 10⁴ FFU versus *Ifnar1*^{-/-} at 10⁶ FFU); *P* > 0.9999 (10⁴ FFU, wild type versus *Ifnar1*^{-/-}), and ns, *P* = 0.0600 (10⁶ FFU, wild type versus *Ifnar1*^{-/-}). (C) Oral gavage (feeding needle) of wild-type (10⁶ FFU, *n* = 27, blue) and *Ifnar1*^{-/-} (10⁶ FFU, *n* = 21, blue dashed line) mice. Data are representative of six independent experiments (*P* = 0.0057).

Ifnar1^{-/-} mice were administered 10⁴ or 10⁶ FFU of Torö-2003 perorally. Induction of disease symptoms was dependent on the virus concentrations: 70% of the wild-type mice died upon infection with 10⁶ FFU, whereas no disease signs were detectable in mice infected with 10⁴ FFU. All susceptible *Ifnar1*^{-/-} mice died within 5 to 6 days after peroral administration of 10⁶ FFU (Fig. 8A). Since it has been discussed that milk has a protective effect on TBEV infectivity, we infected mice perorally with Torö-2003 in milk (Fig. 8B). No protective impact of milk on infectivity and replication was detectable *in vitro* (data not shown). *In vivo*, induction of disease symptoms was dependent on the virus amount. All wild-type mice survived after infection with 10⁴ FFU, whereas 40% of wild-type mice died upon infection with 10⁶ FFU. No significant changes were determined by virus application with or without milk in wild-type mice (*P* = 0.2215). The survival of susceptible *Ifnar1*^{-/-} mice was significantly prolonged, and some mice survived the infection through administration of the virus in milk (Fig. 8B) (*P* = 0.0198).

Administration of the virus by feeding needle led to the death of 33% of *Ifnar1*^{-/-} mice and 4% of wild-type mice (Fig. 8C). These data indicate that mice could be infected through different infection routes by TBEV strain Torö-2003 and that milk does not improve the infectivity of the virus upon peroral infection.

DISCUSSION

The role of horizontal virus transmission for TBEV in human patients or adult mice has not been described so far. Here, we showed that mouse-to-mouse transmission is

possible in immunodeficient laboratory mice. Our cohousing experiments showed transmission of TBEV and LGTV from infected index mice to naive contact mice. However, certain requirements need to be fulfilled: for low-pathogenicity LGTV, the virus must be able to replicate up to high titers in the periphery of the index mice and contact mice must be highly susceptible to viral infections, whereas the transmission of TBEV in laboratory mice is possible without increased virus replication in index mice or in susceptible contact mice.

TBEV is a zoonosis and is transmitted across different levels. Hard ticks, such as *Ixodes ricinus* and *Ixodes persulcatus*, are the vector and central point of the transmission cycle of TBEV. They spread the virus among a variety of animal species. Ticks can retain the virus during their different life stages through transstadial and, with low efficiency, transovarial transmission (29). Furthermore, transmission between ticks might be mediated either by cofeeding or by feeding on an infected reservoir host (15). Transmission among ticks by feeding on reservoir hosts does not require viremia in these animals (30). However, infection of bank voles with TBEV-Eu strains does not support this theory by showing long-term viremia in these animals (31–33).

Despite a high prevalence of TBEV among warm-blooded reservoir hosts, little is currently known about intraspecies transmission routes. *Myodes voles*, for example, *Myodes rutilus*, are small wild rodents and known as competent reservoir hosts for TBEV, capable of maintaining the virus as persistent infections for a long time. Vertical transmission of TBEV has been shown prenatally by detection of the virus in the placenta and the embryo (34, 35), whereas no studies of vertical transmission of TBEV have been reported in humans and mice. Related flaviviruses of the same family as WNV (36), JEV, ZIKV (37, 38), and DENV (39) could be vertically transmitted with variable frequencies of 2 to 76%. Sexual transmission of TBEV in humans has not been reported, whereas sexual transmission of other flaviviruses (WNV and ZIKV) has been suspected (24, 40, 41). Interestingly, sexual transmission of TBEV between infected males and uninfected females has been previously described for laboratory mice, which are not adapted reservoir hosts for the virus (42).

Horizontal transmission of TBEV-Eu strains was excluded by cohousing experiments with bank voles as a natural reservoir host, although they showed high long-term viremia (31). However, we showed that laboratory mice, which are not adapted for the virus, showed disease signs and eventually succumbed to infection after cohousing with LGTV- or Torö-2003-infected mice. Still, some prerequisites must be fulfilled. Mice must be immunodeficient (*Ifnar1*^{−/−}, *Rag2*^{−/−}, or *Rag2*^{γc}^{−/−}) and therefore highly susceptible. Infection with as little as 1 FFU of LGTV was sufficient to induce disease in *Ifnar1*^{−/−} mice and led to the death of the mice (27). Additionally, the virus has to replicate to a great degree in the index mice, since LGTV-infected wild-type mice, which show low viral titers (27, 28), are not able to transmit the virus to highly susceptible contact mice. Thus, high titers in index mice and highly susceptible contact mice are necessary for successful horizontal transmission of the virus.

The mechanism of mouse-to-mouse transmission is not entirely understood. Transmission of the virus by aerosols should be possible. An aerosol transmission was postulated in the case of an accidental laboratory infection in humans, from a broken cell culture flask containing high amounts of the virus or by homogenization of infected mouse brains (20, 21). How intranasal infection led to the disease is unclear. Direct infection of olfactory receptor neurons and transport to the olfactory bulb were shown for vesicular stomatitis virus (VSV) in mice (43). The early detection of LGTV in the olfactory bulb of mice might support this hypothesis (28, 44). However, the disease onset in intranasally infected mice is prolonged, which suggests that the virus is not transmitted directly to the brain but reaches the brain via peripheral viremia by other mechanisms. This was in line with the observation in laboratory infection of humans, where a biphasic progression of the disease was monitored (20). Our cohousing experiments with a mesh, where direct contact of the mice was inhibited, indicated that aerosols in this experimental setting were not able to transmit the virus. This could be

due to a low concentration of virus in aerosols or the possibility that droplets are necessary for the transmission.

The secretion of TBEV in body fluids might be an important finding that could be of further interest. For example, it has been shown that the urine of immunosuppressed patients contains TBEV RNA (25). In mice, contact with secretions or excretions appears to be sufficient to infect susceptible mice. Viral RNA was detectable in eye smear, saliva, feces, and urine. Our experiments did not discriminate between feces and urine. No transmission was detectable when contact mice were housed in close contact to urine and feces of infected index mice, although these excrements showed the highest viral load. The mice were placed in a new cage every 12 h. To what extent the experimental setup changed the behavior of the mice, and thus the uptake of feces, is unknown. Besides, we could detect viral RNA only by quantitative real-time PCR (qRT-PCR). Whether feces or urine contains a replication-competent virus and how long viruses are active in feces or urine remain unclear.

TBEV could be transmitted via the digestive route. Several outbreaks upon consumption of TBEV-contaminated dairy products have been reported (17–19). The virus is stable in milk (45) and under a wide range of pH conditions (46), from which it was concluded that it could pass through the gastrointestinal tract. Virus infection by the dietary route often results in virus replication in epithelial cells of the intestinal wall. TBEV can infect human intestinal Caco-2 cells (47), and virus replication has been detected in the intestine upon infection of BALB/c mice with TBEV Sofjin strain (48). Some strains of TBEV, such as HB171/11, are associated with gastrointestinal symptoms rather than brain diseases in humans. Surprisingly, no viral replication was found in the intestines of mice infected with these strains (49). Here, we showed that direct administration of the virus into the stomach does not lead to an infection in mice. However, inoculation occurred in the absence of milk, which is suggested to be protective against inactivation of the virus.

In contrast, oral feeding of the virus led to disease symptoms in mice, which indicates an infection superior to the gastrointestinal tract. In rhesus monkeys, direct exposure of tonsils to ZIKV led to a symptomatic course of the disease with virus detection in the urine (50). Instead of tonsils, mice have nasal-associated lymphoid tissue (NALT). The NALT could serve as an entry point for TBEV or LGTV with systemic infection via the lymphatic system, which might explain the virus detection in the gastrointestinal tract as well as the virus shedding in the feces and urine of the mice. In peroral infection settings, administration of TBEV in milk led to prolonged survival (Fig. 8B), indicating no protective effect of milk for the infectivity of the virus.

Finally, it remains unknown if similar direct contact transmission modes observed in mice might occur in human populations.

MATERIALS AND METHODS

Mice and viral infections. C57BL/6OlaHsd (wild-type), B6.129S2-*Ifnar1*^{tm1Agt} (*Ifnar1*^{−/−}), B6(Cg)-*Rag2*^{tm1.1Cgn} (*Rag2*^{−/−}), and *Rag2*^{tm1.1Flv}*Il2rg*^{tm1.1Flv} (*Rag2γc*^{−/−}) mice were bred and housed under specific-pathogen-free (SPF) conditions at the Otto-von-Guericke University or the Helmholtz Centre for Infection Research. Animal experiments with TBEV infections were performed in a biosafety level 3 facility at the Helmholtz Centre. All experiments were performed with male and female mice to exclude gender effects. Six- to 10-week-old mice were infected intraperitoneally (i.p.) with 10² FFU, subcutaneously (s.c.) with 10² FFU, intranasally (i.n.) with 10² FFU, perorally with 10², 10⁴, and 10⁶ FFU, or by a feeding needle (gavage) with 10² and 10⁶ FFU of LGTV or Torö-2003 in phosphate-buffered saline (PBS) or milk. To anesthetize mice for a more extended time (intranasal and subcutaneous infections), mice were injected with 100 μl/10 g (body weight) of a sterile solution of 10% ketamine (Bela-Pharm GmbH & Co. KG), 5% xylazine (Ceva Tiergesundheit GmbH) in 0.9% NaCl. This procedure allows mice to stay unconscious for approximately 30 min. Mice that lost more than 20% of their body weight or showed pain-associated behavior like hunchback were sacrificed immediately.

Viruses and cells. LGTV strain TP21 (a kind gift of Gerhard Dobler) and Torö-2003 (51) were replicated and titrated on VeroB4 cells. LGTV and TBEV titers were determined by a focus formation assay, as previously described (51, 52).

Special housing conditions. (i) **Composition of the experiment: transmission via physical contact.** Wild-type, *Ifnar1*^{−/−}, *Rag2γc*^{−/−}, or *Rag2*^{−/−} mice were infected i.p. with 10² or 10⁴ FFU LGTV or TBEV (Torö-2003) (index). Index mice were cohoused with naive wild-type, *Ifnar1*^{−/−}, *Rag2γc*^{−/−}, or *Rag2*^{−/−} mice (contact).

(ii) Composition of the experiment: transmission via air. Infected *Ifnar1*^{-/-} (10² FFU LGTV) index mice and contact mice were placed in housing boxes with the prevention of physical contact by the presence of a 2-mm mesh, providing a 4-cm separation distance, for 21 days. In this housing scenario, the airflow between the two compartments was unrestricted, but index and contact mice did not have direct contact with each other and did not share food and water sources.

(iii) Composition of the experiment: transmission via nonphysical contact. Infected *Ifnar1*^{-/-} (10² FFU LGTV) index mice were kept in a cage for 24 h before they were transferred to a new cage. Naive *Ifnar1*^{-/-} contact mice were transferred into the cage previously inhabited by the infected index mice. Contact mice had contact with litter, feces, urine, food, and water of the index mice. The procedure was continued for 5 days.

(iv) Composition of the experiment: direct contact with feces/urine. Infected *Ifnar1*^{-/-} (10² FFU LGTV) index mice were housed alternately for 12 h in cages without litter and with litter. Naive *Ifnar1*^{-/-} contact mice were transferred to the cage previously inhabited by the infected index mice. The water bottle and food were exchanged before each transfer. The procedure was continued for 5 days.

Sampling of feces, urine, saliva, nose, and eye swabs. Feces and urine were collected by placing mice in a beaker and waiting until they voluntarily eliminated the feces and urine. To collect saliva, eye, and nose secretions from the mice, the animals were killed by CO₂ inhalation at 4 days postinfection (dpi). The smear was collected with a swab soaked in PBS (Thermo Fisher Scientific, Inc.).

Sampling of serum, spleen, and olfactory bulb. To collect serum, spleen, and olfactory bulb from the mice, the animals were sacrificed by CO₂ inhalation at 4 days postinfection (dpi). Heart blood was collected, and serum was isolated. Organs were isolated after PBS perfusion.

RNA extraction and quantitative real-time PCR (qRT-PCR). For RNA extraction, the spleen, olfactory bulb, and feces were homogenized in peqGOLD TriFast (PqLab) using a Fast Prep 24 homogenizer (MP Biomedicals). RNA from serum, urine, eye secretion, nose, and saliva was extracted using a QIAmp viral RNA minikit (Qiagen). RNA was isolated according to the manufacturer's instruction. cDNA synthesis was done with a Moloney murine leukemia virus (M-MLV) reverse transcriptase kit (Invitrogen/Life Technologies). LGTV RNA was determined by using a KAPA Probe fast qPCR kit using LGTV NS3 (forward primer, 5'-AACGGAGCCATAGCCAGTGA-3'; reverse primer, 5'-AACCCGTCGCCCACTC-3'; probe, FAM-AGAGACAGATCCCTGATGG-MGB). Samples were measured with a Light Cycler 480 II (Roche). The copy number of LGTV was analyzed by the Light Cycler software 480 II (Roche).

Focus formation assay. Virus titers in serum, spleen, and olfactory bulb were quantified by a focus formation assay as described previously (52). Briefly, serial dilutions of LGTV samples were added to VeroB4 cells for 2 h. The inoculum was removed, and cells were overlaid with 1% Avicel RC/CL in 1× Dulbecco modified Eagle medium (DMEM) supplemented with fetal calf serum (FCS) (Merck Millipore), 100 U/ml penicillin (Life Technologies), 100 µg/ml streptomycin (Life Technologies), and glutamine (Life Technologies). After 72 h, the cells were fixed with 6% paraformaldehyde (Roth) and permeabilized with 1× PBS (Thermo Fisher Scientific, Inc.), 0.5% Triton X-100 (Sigma-Aldrich), 20 mM glycine. LGTV foci were stained with mouse anti-TBEV E monoclonal antibody 19/1786 (53) and secondary rabbit anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Jackson; no. 315-035) in 1× PBS supplemented with 10% FCS. LGTV-positive foci were visualized by TrueBlue staining (KPL, Gaithersburg).

Neutralization assay. The neutralization assay was performed as previously described (54). VeroB4 cells were plated on 96-well plates in RPMI 1640 (Gibco, Life Technologies), 10% fetal bovine serum (FBS) (Merck Millipore), 1% sodium pyruvate (Gibco), 25 mM HEPES (Life Technologies), 100 U/ml penicillin (Life Technologies), and 100 µg/ml streptomycin (Life Technologies). For each serum dilution, 1.4 × 10³ FFU of LGTV virus particles were added at a ratio of 1:1, mixed, and incubated for 1 h at 37°C, 5% CO₂. The mixture was added to VeroB4 cells, incubated for 6 to 7 days at 37°C, 5% CO₂, washed with 1× PBS (Thermo Fisher Scientific, Inc.), fixed for 1 h at room temperature with 6% formaldehyde (Roth), and stained with crystal violet (Sigma-Aldrich) for 30 min.

Ethical approval. All animal experiments were performed in compliance with the animal welfare law (EU-Directive 2010/63/EU). The mice were housed and handled under good animal practices as defined by FELASA. All animal experiments were approved by the Lower Saxony State Office of Consumer Protection and Food Safety under permit number AZ 33.19-42502-04-15/1895 or by the Landesverwaltungsamt Sachsen-Anhalt AZ 42502-2-1568, University Magdeburg.

Statistical analysis. Data were analyzed and plotted by GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Survival differences were tested for statistical significance by the log rank (Mantel-Cox) test. Group differences were analyzed by a nonparametric *t* test and the Mann-Whitney test.

Data availability. All data generated or analyzed in this study are included in this published article.

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S.S. and A.K. conceived the experiments, designed the work, and wrote the paper. S. S. and K.C. performed the analyses. S.S. and A.K. interpreted the data. All authors approved the submitted version.

We declare that we have no conflicts of interest.

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