Endothelial JAM-A Promotes Reovirus Viremia and Bloodstream Dissemination

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Viruses that cause systemic disease often spread through the bloodstream to infect target tissues. Although viremia is an important step in the pathogenesis of many viruses, how viremia is established is not well understood. Reovirus has been used to dissect mechanisms of viral pathogenesis and is being evaluated in clinical trials as an oncolytic agent. After peroral entry into mice, reovirus replicates within the gastrointestinal tract and disseminates systemically via hematogenous or neural routes. Junctional adhesion molecule-A (JAM-A) is a tight junction protein that serves as a receptor for reovirus. JAM-A is required for establishment of viremia and viral spread to sites of secondary replication. JAM-A also is expressed on the surface of circulating hematopoietic cells. To determine contributions of endothelial and hematopoietic JAM-A to reovirus dissemination and pathogenesis, we generated strains of mice with altered JAM-A expression in these cell types and assessed bloodstream spread of reovirus strain type 1 Lang (T1L), which disseminates solely by hematogenous routes. We found that endothelial JAM-A but not hematopoietic JAM-A facilitates reovirus T1L bloodstream entry and egress. Understanding how viruses establish viremia may aid in development of inhibitors of this critical step in viral pathogenesis and foster engineering of improved oncolytic viral vectors.

Keywords. reovirus; viremia; junctional adhesion molecule-A; endothelial cells; viral receptors; viral dissemination; viral pathogenesis.

Clinical manifestations of viral infections are often dictated by tropism of the virus for a particular cell type or organ. Many viruses spread systemically by bloodstream routes to reach sites at which disease is produced. Knowledge gained from studies to determine precisely how viruses disseminate can be used to block this key step in viral pathogenesis and improve vector targeting for clinical applications.

Mammalian orthoreoviruses (reoviruses) are nonenveloped, double-stranded RNA viruses that are being tested in phase 1–3 clinical trials as anticancer therapeutics [1]. Although reoviruses infect virtually all mammals, disease is limited to the very young [1]. Following peroral inoculation of newborn mice, reovirus replicates in the small intestine and systemically disseminates hematogenously or neurally, reaching high titers in most visceral organs [2–5]. Viremia is a well-established, essential step in reovirus pathogenesis [2]; however, how the virus gains entry into and amplifies to high titers within the bloodstream is unknown.

Reovirus engages 2 types of cellular receptors, sialylated glycans and junctional adhesion molecule-A (JAM-A), using attachment protein σ1 [6–11]. Reovirus binding to sialylated glycans enhances the neurovirulence of serotype 3 reovirus strains [12], whereas binding to JAM-A is required for establishment of viremia and subsequent bloodstream spread to sites of secondary replication of all reovirus serotypes [2]. However, JAM-A is not required for reovirus replication at sites of secondary replication [2]. JAM-A is a tight junction protein that serves to maintain barrier function of endothelium and epithelium [13–15]. JAM-A also is expressed on

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hematopoietic cells and platelets, promoting leukocyte extravasation from the bloodstream and platelet activation, respectively [13, 16–18]. Reovirus infection of polarized endothelial cells is dependent on engagement of both sialic acid and JAM-A [19]. Although JAM-A is required for establishment of reovirus viremia in mice [2], it is not known whether expression of JAM-A on endothelial cells, hematopoietic cells, or both promotes reovirus seeding of or exit from the vasculature.

To determine whether endothelial or hematopoietic JAM-A facilitates reovirus bloodstream dissemination, mice with diminished expression of endothelial cell-specific JAM-A (EndoJAM-AKD; knock-down [KD]) were established and infected by peroral and intravascular routes. Because EndoJAM-AKD mice also lack JAM-A expression in hematopoietic cells, mice lacking or expressing JAM-A solely in the hematopoietic cell compartment (HematoJAM-AKO [knock-out (KO)] and HematoJAM-A, respectively) also were established to determine whether hematopoietic JAM-A contributes to reovirus bloodstream spread. We found that EndoJAM-AKD mice had lower levels of viremia and lower viral titers at sites of secondary replication following either peroral or intravascular reovirus inoculation. In addition, HematoJAM-AKO mice had viral titers in blood and at sites of secondary replication similar to those observed in wild-type mice following either inoculation route. In contrast, HematoJAM-A mice had low levels of viremia and diminished titers at sites of secondary replication following peroral or intravascular reovirus inoculation. Together, these data suggest that endothelial JAM-A but not hematopoietic JAM-A is required for bloodstream dissemination of reovirus.

METHODS

Viruses and Cells

Murine L929 fibroblast cells were grown in suspension or monolayer cultures as described previously [3, 20]. Reovirus strain T1L is a laboratory stock. Strain rsT3D/T1LS1 was recovered by reverse genetics [3]. Virus was purified from infected L929 cells as described elsewhere [21]. Viral titers were determined by plaque assay using L929 cells [22].

Generation of Mouse Strains

EndoJAM-AKD mice, which have decreased expression of endothelial JAM-A and lack hematopoietic JAM-A, or HematoJAM-AKO mice, which lack JAM-A only within the hematopoietic cell compartment, were established using Cre recombinase (Cre). Female JAM-A flox/flox mice [23] were bred to male Tek-cre transgenic (tg) mice [15] to establish EndoJAM-AKD mice or male Vav-cre tg mice [24] to establish HematoJAM-AKO mice (Figure 1*A*). HematoJAM-A mice, which lack native JAM-A but express JAM-A on hematopoietic cells, were obtained by first establishing mice in which JAM-A expression is driven by the hematopoietic-specific Vav1 promoter (Figure 1*B*). These mice were then bred to JAM-AKO mice to obtain animals expressing JAM-A solely in hematopoietic cells (Figure 1*B*). Despite 3 attempts, we were unable to establish mice that express JAM-A under control of the Tek promoter (data not shown). All strains were maintained on a C57BL/6 background. No growth or breeding defects were noted in any of the mouse strains. Cell-surface expression of JAM-A in endothelial and hematopoietic cells was assessed in the different mouse strains using flow cytometry. Mouse genotypes were confirmed using polymerase chain reaction (PCR); primer sequences are shown in Table 1.

Mouse Infection Studies

Two- to 3-day-old mice were inoculated perorally or intravenously with purified reovirus strain T1L. Peroral inoculations were performed as described previously [2, 3]. Intravenous inoculations were performed following anesthesia by hypothermia [25, 26]. Anesthetized mice were positioned on a transilluminator (Phillips) to visualize the superficial temporal vein. A dose of 50 μL of virus inoculum was administered via a 33-gauge 0.25-inch needle (Cadence). At defined intervals post-inoculation, mice were euthanized and organs were excised, submerged into phosphate-buffered saline (PBS), subjected to 2 freeze–thaw cycles, and sonicated until homogenized. Viral titers in organ homogenates were determined by plaque assay [22]. Blood was collected into an equal volume of Alsever's solution (Sigma), subjected to 2 freeze–thaw cycles, sonicated, and processed for viral titer determination by plaque assay [22].

Animal husbandry and experimental procedures were performed in accordance with United States Public Health Service policy on Humane Care and Use of Laboratory Animals and approved by the Vanderbilt University School of Medicine Institutional Animal Care and Use Committee.

Assessment of Hematopoietic JAM-A Expression by Flow Cytometry

Hematopoietic cells were harvested from peripheral blood or spleens of 6- to 8-week-old mice. Erythrocytes were lysed using ammonium–chloride–potassium lysis buffer (Invitrogen) at room temperature for 5 minutes. Leukocytes were collected by centrifugation at $1000 \times g$ for 5 minutes, and hematopoietic cell subsets were identified using antibodies specific for granulocytes (Gr-1), B cells (B220), T cells (TCRβ), macrophages (CD11b), and dendritic cells (CD11c). Expression of cell-surface JAM-A was assessed using antibody AF1077 (R&D Systems). Reovirus binding to or infectivity of hematopoietic cells was assessed using Alexa Fluor–conjugated reovirus-specific antiserum [27]. For infectivity studies, cells were fixed in 1% paraformaldehyde either prior to or after permeabilization using Cytofix/Cytoperm (BD Biosciences) prior to addition of Alexa Fluor–conjugated reovirus-specific antiserum as described elsewhere [28, 29]. The threshold level of detection for infected cells using this assay is ~3% [19].

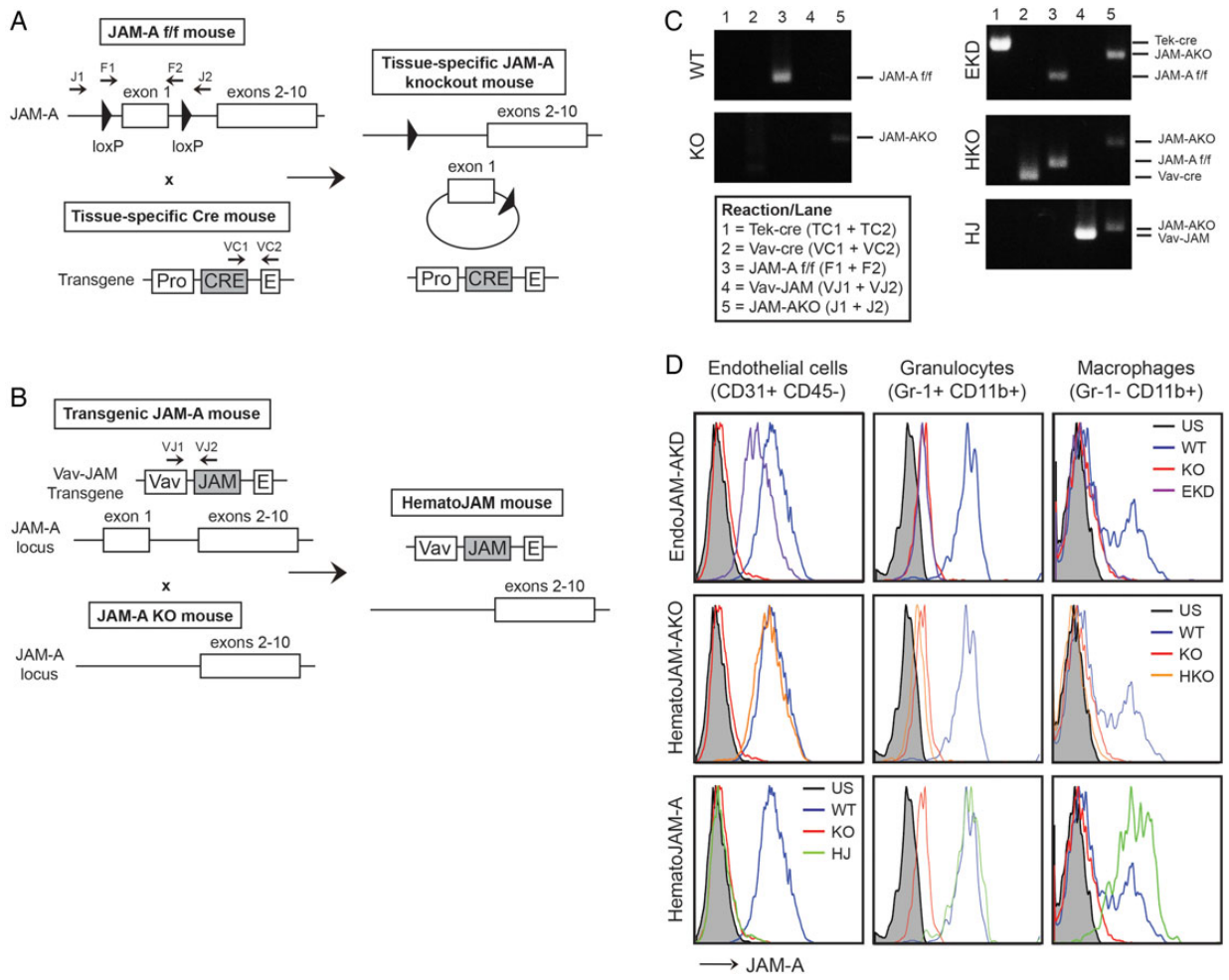

Figure 1. Generation and characterization of mouse strains. *A*, A schematic depicting the establishment of EndoJAM-AKD and HematoJAM-AKO mice used in infection experiments. Exon 1 of junctional adhesion molecule-A (JAM-A) is flanked by loxP sites in JAM-A f/f mice. Cre recombinase expression in tissue-specific Cre tg mice is driven by Tek (endothelial) or Vav1 promoter and enhancer sequences from an inserted transgene. Crosses between JAM-A f/f and tissue-specific Cre mice generate mice in which exon 1 of JAM-A is excised in tissues where Cre is expressed. Arrows indicate primer-binding sites for JAM-A primers (J1, J2), JAM-A f/f primers (F1, F2), Tek-Cre primers (TC1, TC2), and Vav-Cre primers (VC1, VC2). *B*, Mice that express JAM-A only within the hematopoietic cell compartment (HematoJAM-A mice) were obtained by establishing tg mice expressing a transgene in which JAM-A expression is driven by Vav1 promoter and enhancer sequences. JAM-A expression in other tissues was abolished by breeding the tg mice with JAM-AKO mice. Arrows indicate primer-binding sites for Vav-JAM primers (VJ1, VJ2). *C* and *D*, JAM-A expression was assessed in 6- to 8-week-old mice by genomic DNA polymerase chain reaction (PCR) and flow cytometry. *C*, Agarose gels displaying bands corresponding to regions of genomic DNA amplified in genotyping PCR reactions. Bands from the following reactions are shown for each mouse strain: JAM-A f/f, Tek-Cre, Vav-Cre, Vav-JAM, and JAM-AKO. *D*, Hematopoietic cells were collected from blood and spleens, and endothelial cells were cultured from lungs. JAM-A expression was quantified using flow cytometry. Flow cytometric profiles of endothelial and hematopoietic cells (peripheral blood granulocytes and macrophages) from each mouse strain. Abbreviations: CRE, Cre recombinase; E, enhancer; EKD, EndoJAM-AKD; HJ, HematoJAM-A; HKO, HematoJAM-AKO; JAM, JAM-A coding sequence; KO, JAM-AKO; PRO, promoter; US, unstained; Vav, Vav promoter; WT, wild-type (JAM-A f/f).

Assessment of Endothelial JAM-A Expression by Flow Cytometry

Primary lung endothelial cells [30] were cultured at 37°C for 5–7 days in EBM-2 medium supplemented to contain EGM-2 MV SingleQuots (human epidermal growth factor, hydrocortisone, gentamicin, amphotericin B, vascular endothelial growth factor, human fibroblast growth factor-B, insulin-like growth factor-1, ascorbic acid, and heparin; Lonza), washed twice with PBS on day 3, and supplemented with fresh EBM-2 medium. Expression of cell-surface JAM-A was assessed using flow cytometry following staining of cells with antibodies specific for hematopoietic cells (CD45), endothelial cells (CD31), and JAM-A. All cell staining was quantified using FlowJo software (Tree Star).

Brain Vascular Permeability Studies

Two- to 3-day-old mice were inoculated perorally with 5×10^6 plaque-forming units (PFU) of reovirus T1L or PBS as a

Table 1. Primer Sequences Used to Genotype Mouse Strains With Altered Junctional Adhesion Molecule-A Expression[a]

Mouse Strain	Region Amplified	Forward Primer	Reverse Primer
JAM-A f/f (WT)	JAM-A Exon 1	TCT TTT CAC CAA TCG GAA CG	AAA AAC TCT AGG AAC TCA CCC AGG A
JAM-AKO (KO)	JAM-A Exon 1	TCT TCT TCA GAC GCC GAA CCT	CCT CTC TTT TCA CCA ATC GGA
Tek-Cre (EKD)	Tek P to Cre	CCC TGT GCT CAG ACA GAA ATG AGA	CGC ATA ACC AGT GAA ACA GCA TTG C
Vav-Cre (HKO)	Vav P to Cre	GAA GGA ACG AGG GTG CAC	TGC CTG TCC CTG AAC ATG TC
Vav-Cre (HKO)	Cre to Vav E	ATG CAG GCT GGT GGC TGG	GGC TCG CGA GGT TTT ACT TGC
Vav-JAM (HJ)	Vav P to JAM-A	GAA GGA ACG AGG GTG CAC	GTG CAG GTC AAT TTG ATG GAC TCG
Vav-JAM (HJ)	JAM-A to Vav E	CAG CTG TCC TGG TAA CAC TGA TTC	GGC TCG CGA GGT TTT ACT TGC

[a] In cases in which 2 sets of primers for a particular mouse strain are shown, only 1 was used at any time to determine the genotype.

negative control. Four or 8 days post-inoculation, mice were inoculated intraperitoneally with 100 μL of 10% sodium fluorescein (Sigma). Ten minutes later, mice were euthanized using isoflurane and perfused. Blood was collected, and brains were excised and flash frozen. To quantify sodium fluorescein in the tissue, brains were thawed and homogenized, and an aliquot of brain homogenate was mixed with 7.5% trichloroacetic acid (Sigma). Sodium fluorescein fluorescence in the sample was quantified using a Synergy HT multi-mode microplate reader (BioTek) with 485 nm excitation and 528 nm emission filters. Fluorescein concentration was determined from the fluorescein isothiocyanate fluorescence intensity using a standard curve generated with known concentrations of sodium fluorescein. Mice were inoculated with 1 mg/kg lipopolysaccharide (LPS; Sigma) 3 hours prior to euthanasia as a positive control.

Ex Vivo Infection of Lymphocytes

Splenocytes were isolated from spleens of 5-day-old mice, stained with antibodies specific for dendritic cells (CD11c), macrophages (CD11b), T cells (CD3), and B cells (B220), and separated using fluorescence-activated cell sorting (FACS).

Cells were adsorbed with rsT3D/T1LS1 at a multiplicity of infection of 100 PFU/cell on ice for 1 hour. Cells were washed once with FACS buffer, resuspended in lymphocyte medium (Roswell Park Memorial Institute 1640 medium supplemented to contain 10% fetal bovine serum, 1% penicillin/streptomycin, 1% HEPES (pH 7.4), 1% sodium pyruvate, 1% L-glutamine, and 0.1% β-mercaptoethanol) and incubated at 37°C. Cells were collected at 0 and 24 hours post-adsorption and subjected to 2 freeze–thaw cycles; viral titers in cell lysates were determined by plaque assay.

RESULTS

Characterization of Mice With Targeted Disruption of JAM-A Expression

To determine the role of endothelial JAM-A in reovirus infection, we established mice with diminished JAM-A expression in the endothelial cell compartment (Figure 1). We used flow cytometry to assess expression of JAM-A on pulmonary vascular endothelial cells and hematopoietic cells collected from EndoJAM-AKD mice. We found that expression of cell-surface JAM-A was decreased on endothelial cells in these mice and absent on hematopoietic cells (Figure 1*D* and Supplementary Figure 1). Compared with that seen in wild-type mice, endothelial JAM-A expression in EndoJAM-AKD mice was diminished approximately 2-fold (Figure 1*D*). It is possible that JAM-A expression on other types of endothelial cells is diminished to a greater degree.

To determine the role of hematopoietic JAM-A in reovirus infection, we established mice that either lack or express JAM-A solely in the hematopoietic compartment (Figure 1*A* and 1*B*). We assessed endothelial and hematopoietic JAM-A expression in HematoJAM-AKO and HematoJAM-A mice using flow cytometry. As expected, endothelial JAM-A expression in HematoJAM-AKO mice mirrored that seen in wild-type endothelial cells (Figure 1*D*), whereas endothelial JAM-A expression in HematoJAM-A mice was similar to that observed in JAM-AKO endothelial cells (Table 1, Figure 1*D*). JAM-A expression on hematopoietic cells collected from HematoJAM-AKO mice was undetectable, but hematopoietic JAM-A expression in HematoJAM-A mice was identical to that seen in wild-type mice (Figure 1*D* and Supplementary Figure 1). The JAM-A expression phenotypes of the mouse strains used in this study are summarized in Table 2.

Table 2. Junctional Adhesion Molecule-A Expression in Mouse Strains Used in Studies of Reovirus Bloodstream Spread

	Junctional Adhesion Molecule-A Expression	
Mouse Strain	Endothelial Cells	Hematopoietic Cells
JAM-A f/f (WT)	++[a]	++
JAM-A KO (KO)	–[b]	–
EndoJAM-A KD (EKD)	+[c]	–
HematoJAM-A KO (HKO)	++	–
Hemato-JAM-A (HJ)	–	++

[a] Wild-type expression.

[b] Absent expression.

[c] Expression decreased approximately 50%.

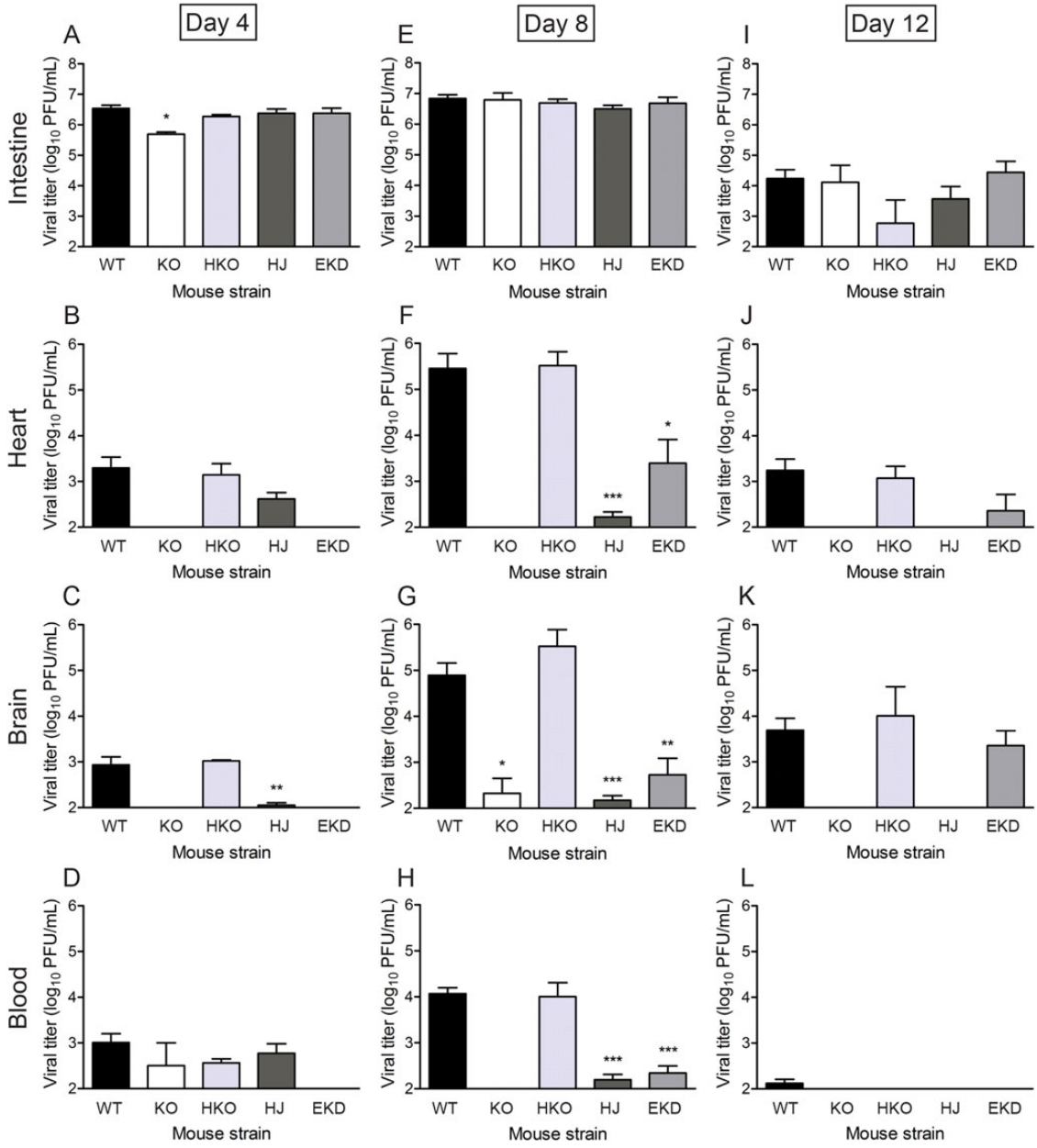

Figure 2. Endothelial junctional adhesion molecule-A is required for reovirus bloodstream spread. Newborn (2–3 days) mice were inoculated perorally with reovirus strain T1L at 1000 PFU per mouse. At 4, 8, and 12 days post-inoculation, intestine, heart, and brain were excised, and blood was collected into an equal volume of Alsever's solution for determination of viral titer by plaque assay. Results are presented as mean viral titer. Error bars indicate standard deviation. For each time point and mouse strain, 3 to 13 mice were used. Abbreviations: EKD, EndoJAM-AKD; HJ, HematoJAM-A; HKO, HematoJAM-AKO; KO, JAM-AKO; WT, wild-type (JAM-A f/f). $*P < .05$, $**P < .005$, $***P < .001$ by Student t test.

Endothelial JAM-A Promotes Bloodstream Dissemination of Reovirus

To determine whether endothelial or hematopoietic JAM-A is required for bloodstream spread of reovirus, wild-type, JAM-AKO, EndoJAM-AKD, HematoJAM-AKO, and HematoJAM-A mice were inoculated perorally with reovirus. Following peroral inoculation of newborn mice, strain T1L infects the intestine, disseminates hematogenously, and reaches high titers in most visceral organs [2, 3]. Reovirus dissemination was assessed by determining viral titers in organ homogenates and blood of infected mice 4, 8, and 12 days post-inoculation by plaque assay. As anticipated, wild-type, JAM-AKO, and the tissue-specific JAM-A–expressing mice had equivalent viral titers in the intestine (Figure 2), as replication at that site does not require JAM-A [2]. Viral titers were minimally detectable in the heart, brain, and blood of JAM-AKO mice and significantly lower in the

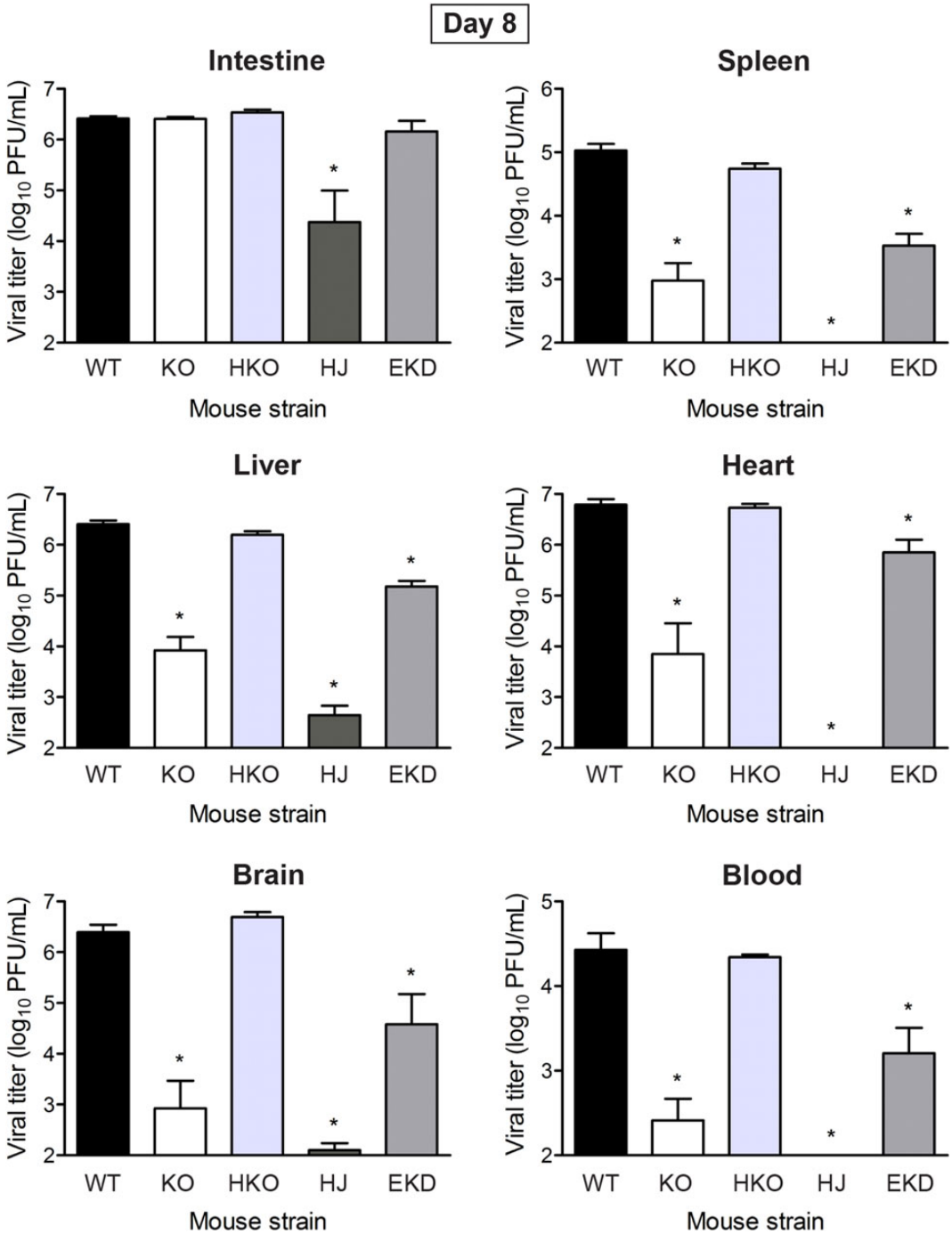

Figure 3. Endothelial junctional adhesion molecule-A is required for reovirus egress from the circulation. Newborn (2–3 days) mice were inoculated intravenously with reovirus strain T1L at 1000 PFU per mouse. At 8 days post-inoculation, intestine, spleen, liver, heart, and brain were excised, and blood was collected into an equal volume of Alsever's solution for determination of viral titer by plaque assay. Results are presented as mean viral titer. Error bars indicate standard deviation. For each time point and mouse strain, 5 to 18 mice were used. Abbreviations: EKD, EndoJAM-AKD; HJ, HematoJAM-A; HKO, HematoJAM-AKO; KO, JAM-AKO; WT, wild-type (JAM-A f/f). *$P < .05$ by Student t test.

spleen and liver compared with those in wild-type animals (Figure 2 and Supplementary Figure 2), as described previously [2]. After peroral reovirus inoculation, HematoJAM-AKO mice, which lack JAM-A only in hematopoietic cells, phenocopied wild-type mice. Viral titers in the heart, brain, spleen, liver, and blood of infected HematoJAM-AKO mice were comparable to those in infected wild-type mice (Figure 2 and Supplementary Figure 2). In contrast, viral titers in the heart, brain, spleen, liver,

and blood of HematoJAM-A mice, which express JAM-A solely in hematopoietic cells, were similar to those in JAM-AKO mice (Figure 2 and Supplementary Figure 2). These data suggest that hematopoietic JAM-A is dispensable for reovirus hematogenous dissemination. Viral titers in the heart, brain, spleen, liver, and blood of EndoJAM-AKD mice were significantly lower than those observed in wild-type mice (Figure 2 and Supplementary Figure 2). Titers of virus in these animals were similar to those observed in JAM-AKO and HematoJAM-A mice, suggesting that reovirus hematogenous dissemination is dependent on endothelial JAM-A but not hematopoietic JAM-A.

Endothelial JAM-A is Required for Reovirus Egress From the Bloodstream

Because the requirement for bloodstream entry may differ from that for bloodstream egress, we used an intravenous inoculation protocol to directly assess the role of JAM-A in reovirus exit from the bloodstream. Wild-type and JAM-AKO mice were inoculated intravenously with reovirus, and viral titers in organ homogenates were determined by plaque assay 4 and 8 days post-inoculation (Figure 3). Viral titers in wild-type mice inoculated intravenously were similar to those in perorally inoculated wild-type animals (Figures 2 and 3 and Supplementary Figure 2). However, viral titers in JAM-AKO mice were significantly lower in all organs tested compared with those in wild-type mice, suggesting that reovirus egress from the bloodstream is dependent on JAM-A expression (Figure 3).

To determine whether endothelial or hematopoietic JAM-A facilitates reovirus egress from the bloodstream, EndoJAM-AKD, HematoJAM-AKO, and HematoJAM-A mice were inoculated intravenously, and viral titers were determined in organ homogenates 8 days post-inoculation. Reovirus titers in HematoJAM-AKO mice were comparable to those in wild-type mice (Figure 3), suggesting that reovirus egress from the bloodstream does not require hematopoietic JAM-A. Viral titers in the intestine, spleen, liver, heart, and brain of HematoJAM-A mice were similar to those in JAM-AKO mice (Figure 3), suggesting that hematopoietic JAM-A is not sufficient for reovirus exit from the circulation and infection of those organs. Viral titers in EndoJAM-AKD mice were significantly lower in the spleen, liver, heart, brain, and blood compared with those in wild-type mice (Figure 3). Taken together, these data suggest that reovirus egress from the bloodstream requires endothelial JAM-A but not hematopoietic JAM-A.

Reovirus Infection Does Not Increase Vascular Permeability in the Brain

Since JAM-A functions in the regulation of endothelial permeability [13], we sought to determine whether reovirus infection enhances vascular permeability as a mechanism of bloodstream egress. We quantified diffusion of sodium fluorescein into brains of reovirus-infected mice 4 and 8 days post-inoculation as a measure of vascular permeability. In contrast to mice treated with LPS, which enhances blood–brain barrier permeability [31], we found that fluorescein levels in brains of reovirus-infected mice were equivalent to those in PBS-treated mice 4 and 8 days post-inoculation (Figure 4). These data suggest that reovirus infection of newborn mice does not result in enhanced vascular permeability in the brain.

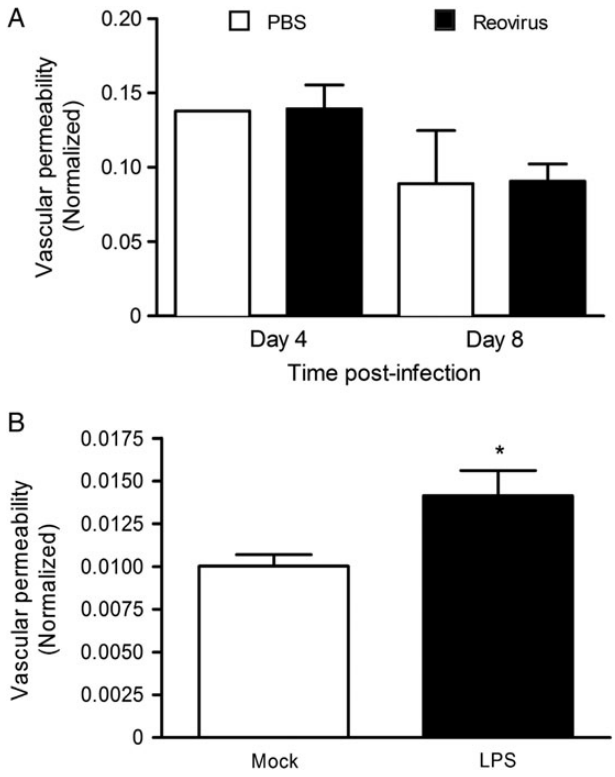

Figure 4. Reovirus infection does not increase vascular permeability in vivo. *A*, Newborn (2–3 days) wild-type mice were inoculated perorally with either phosphate-buffered saline (PBS) or reovirus strain T1L at 5×10^6 PFU per mouse. At 4 and 8 days post-inoculation, mice were injected intraperitoneally with 0.1 mL 10% sodium fluorescein. *B*, Mice (7–8 days) were inoculated with 1 mg/kg lipopolysaccharide (LPS) and 3 hours later injected intraperitoneally with 0.1 mL 10% sodium fluorescein. Ten minutes later, all mice were euthanized and perfused. Brains were homogenized, and fluorescein content was determined using a microplate reader with 485 nm excitation and 528 nm emission filters. Fluorescein content in blood was used to normalize the fluorescein content in the brain. In the PBS- and LPS-treated groups, 2 to 3 mice were used; in the reovirus-infected group, 5 mice were used at both time points. $^*P < .05$ by Student *t* test.

Reovirus Does Not Bind to or Infect Hematopoietic Cells

Although reovirus does not require hematopoietic JAM-A to spread by the bloodstream, we thought it possible that reovirus transits the blood by engaging a molecule distinct from JAM-A on hematopoietic cells. To determine whether reovirus binds to or infects hematopoietic cells in vivo, wild-type mice were inoculated perorally with reovirus. Blood was collected and spleens

were excised from PBS-treated and reovirus-inoculated mice 8 days post-inoculation. Hematopoietic cell preparations were stained for reovirus antigen in addition to hematopoietic cell type-specific markers. Staining for reovirus antigen under non-permeabilizing conditions was used to quantify the amount of virus bound to the cell surface, whereas staining for reovirus under permeabilizing conditions was used to quantify intracellular viral antigen, indicative of active infection. Under both conditions, we were unable to detect appreciable reovirus staining in hematopoietic cells collected from reovirus-infected or PBS-treated mice (Figure 5*A* and Supplementary Figure 3).

Next, we examined reovirus replication in distinct leukocyte populations ex vivo (Figure 5*B*). Dendritic cells, macrophages, T cells, and B cells were isolated from the spleens of 5-day-old mice using FACS, and individual cell populations were infected with reovirus strain rsT3D/T1LS1, which disseminates hematogenously [3]. Viral titers in cell lysates were determined by plaque assay at 0 and 48 hours post-infection. Viral titers were roughly equivalent at both time points in each of these cell populations (Figure 5*B*), indicating that reovirus does not replicate in explanted hematopoietic cells. Together, these data demonstrate that reovirus does not bind to or infect hematopoietic cells in vivo and suggest that reovirus disseminates following amplification in endothelial cells by a cell-free viremia.

DISCUSSION

Reoviruses are useful experimental models to dissect mechanisms by which viruses gain access to systemic transport pathways in the host. In this study, we assessed hematogenous reovirus spread in mice with tissue-specific alterations in JAM-A expression. We hypothesized that systemic bloodstream spread of reovirus depends on JAM-A expression in the endothelium and on hematopoietic cells. The key finding in this study is that endothelial JAM-A but not hematopoietic JAM-A is required for reovirus bloodstream transmission.

To establish mice with altered JAM-A expression in the endothelial compartment, we used animals that express Cre recombinase driven by the Tek promoter. Tek is a tyrosine kinase expressed in endothelial cells, but this protein also is expressed in hematopoietic stem cells [32]. Tek-dependent Cre recombinase expression in hematopoietic stem cells likely accounts for the absence of JAM-A expression in all hematopoietic cell types in EndoJAM-AKD mice (Figure 1*D* and Supplementary Figure 2). To exclude the possibility that the absence of hematopoietic JAM-A might confound the results obtained using EndoJAM-AKD mice, we established HematoJAM-AKO and HematoJAM-A mice, which respectively lack or express JAM-A exclusively in hematopoietic cells.

Compared with wild-type mice, EndoJAM-AKD mice, which display diminished JAM-A expression on endothelial cells, have significantly lower levels of viremia after peroral or intravenous inoculation of reovirus (Figures 2 and 3 and Supplementary Figure 2). This finding suggests that endothelial JAM-A expression is required for reovirus entry into and exit from the bloodstream. Subsequent spread to sites of secondary replication (eg, brain and heart) after peroral inoculation is significantly diminished in EndoJAM-AKD mice (Figure 2), suggesting that efficient spread to those sites requires a threshold level of viremia. Although viral titers in organ homogenates prepared from EndoJAM-AKD mice are significantly lower than those observed in wild-type mice, there is detectable virus at these sites. This finding may be attributable to residual JAM-A expression in the endothelium of EndoJAM-AKD mice or the presence of another host component that facilitates systemic trafficking of reovirus. Nonetheless, our findings provide strong evidence that endothelial JAM-A promotes bloodstream entry during systemic reovirus infection.

We envision 3 possible mechanisms by which reovirus could traverse an endothelial cell layer to invade the underlying tissue. First, reovirus may infect endothelial cells from the luminal surface and be released abluminally. Endothelial cells are readily infected by reovirus in culture [19], and reovirus antigen can be detected in endothelial cells within the brains of mice infected perorally with reovirus (data not shown). Second, reovirus infection of endothelial cells may induce barrier dysfunction and vascular leak, allowing reovirus access to tissue surrounding blood vessels. However, we found that reovirus infection does not induce vascular leak into the brain at 4 and 8 days post-inoculation, which coincide with times at which infectious virus can be detected at that site (Figure 4). Third, reovirus may use hematopoietic cells to traffic hematogenously. Surprisingly, we found that viral titers in the blood and at sites of secondary replication of HematoJAM-AKO mice, which lack all hematopoietic JAM-A, are equivalent to those seen in wild-type mice after peroral or intravenous inoculation (Figures 2 and 3 and Supplementary Figure 2). Additionally, viral titers in HematoJAM-A mice, which express JAM-A only in hematopoietic cells, mirrored those seen in JAM-AKO mice (Figures 2 and 3 and Supplementary Figure 2). These data suggest that hematopoietic JAM-A is dispensable for reovirus bloodstream spread. However, it is possible that cell type-specific differences in posttranslational modification of JAM-A allows reovirus to infect and replicate in endothelial cells and not hematopoietic cells. Since reovirus associates with human dendritic cells in vivo [33], we thought that the virus might bind to or infect leukocytes using a receptor distinct from JAM-A. However, we found that reovirus does not attach to or productively infect murine hematopoietic cells in vivo (Figure 5A and Supplementary Figure 3) or ex vivo (Figure 5*B*), suggesting that reovirus strains differ in the capacity to infect leukocytes in vivo or that such interactions are species specific.

Reovirus replication in endothelial cells appears to promote the development of viremia and facilitates systemic spread to sites of secondary replication. Viral titers in the blood of

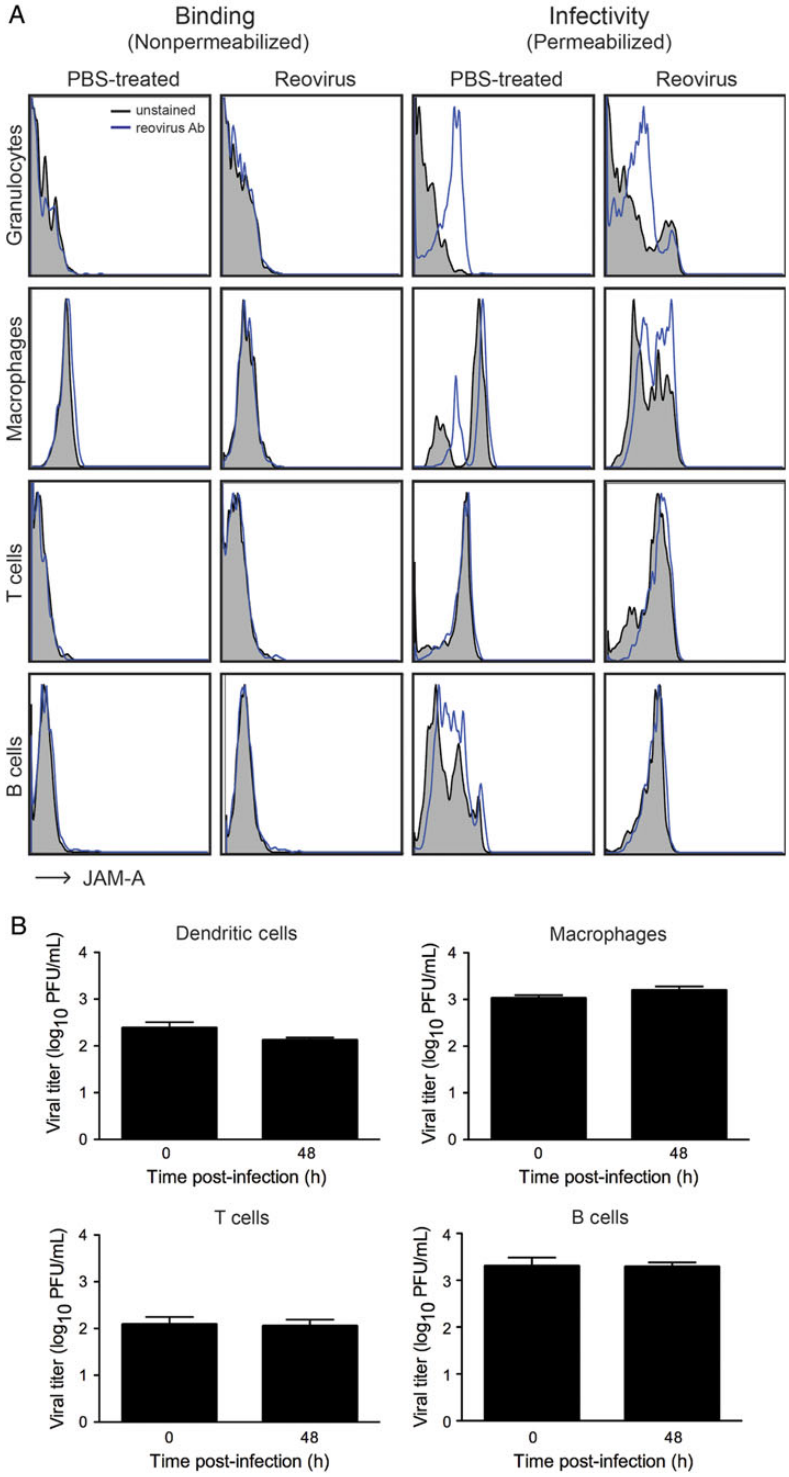

Figure 5. Reovirus does not infect hematopoietic cells in vivo or ex vivo. *A*, Newborn (2–3 days) wild-type mice were inoculated perorally with either phosphate-buffered saline or reovirus strain T1L at 5×10^6 PFU per mouse. At 8 days post-inoculation, hematopoietic cells from the blood were isolated and stained with antibodies specific for hematopoietic cell subsets and reovirus-specific polyclonal antiserum using either nonpermeabilizing (to detect binding) or permeabilizing (to detect infection) conditions. The percentage of infected cells within each cell population was determined using flow cytometry. *B*, Dendritic cells, macrophages, T cells, and B cells were isolated from the spleens of 5-day-old C57BL/6 mice by fluorescence-activated cell sorting using antibodies specific for CD11c, CD11b, CD3, and B220, respectively. Isolated cells were infected with reovirus strain rsT3D/T1LS1 at a multiplicity of infection of 100 PFU/cell, and viral titers were determined by plaque assay at 0 and 48 hours post-infection. Results are presented as mean viral titer at each time point. Error bars indicate standard deviation.

JAM-AKO mice are significantly lower than those observed in wild-type mice after peroral or intravenous inoculation (Figures 2 and 3), suggesting that JAM-A promotes establishment of viremia. Interferon-β is induced in equal amounts in the intestines of wild-type and JAM-AKO mice 24 hours post-inoculation (data not shown), suggesting that differences in viral titer in animals varying in JAM-A expression are unlikely to be attributable to differences in reovirus-induced innate immune activation in the presence and absence of JAM-A. Infection of polarized endothelial cells with reovirus requires JAM-A [19] and may serve as a means to amplify reovirus within the bloodstream. Lower viral titers in the blood of EndoJAM-AKD mice in comparison to those in HematoJAM-AKO mice after peroral or intravenous inoculation (Figures 2 and 3) suggest that endothelial JAM-A and not hematopoietic JAM-A is required for both development of viremia and viral egress from the bloodstream.

Reoviruses are superb candidates for oncolytic therapeutics due to their preferential replication in transformed cells and capacity to induce apoptosis [1]. Spread of reovirus by the bloodstream enables the virus to target even the smallest foci of tumor cells [33]. By understanding how reovirus interacts with JAM-A on the endothelium, it may be possible to design new reovirus vectors that reach target tumors more efficiently. Oncolytic serotype 3 reoviruses, which disseminate both neurally and hematogenously in newborn mice [26], likely require expression of JAM-A on endothelial cells to spread through the bloodstream in a manner similar to that observed in our study for serotype 1 reoviruses. Thus, understanding mechanisms that govern the spread of reovirus by the bloodstream sheds light on how pathogens disseminate systemically and may enhance vector targeting for therapeutic applications.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Authorship contribution. C. M. L., K. W. B., and A. J. P. designed and performed the experiments, analyzed the results, and wrote the manuscript. V. V. P. analyzed the results. C. A. P. and L. V. K. provided critical reagents and experimental advice. T. S. D. designed the experiments, analyzed the results, and wrote the manuscript.

Potential conflicts of interest. All authors: No potential conflicts of interest.

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