Peer Review File

Manuscript Title: LDLRAD3 is a receptor for Venezuelan equine encephalitis virus

Editorial Notes:

Redactions – unpublished data

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

In the article, the authors have identified low-density lipoprotein (LDL) receptor class A domain containing 3 (LDLRAD3) as a receptor for Venezuelan equine encephalitis virus (VEEV) by CRISPR-Cas9 based screening of sgRNA library in N2a neuroblastoma cells. Reduction of VEEV infection by knock-down of Ldlrad3 in mouse and human cells could be restored by transfection of Ldlrad3 gene into gene-edited cells. Expression of Ldlrad3 in Ldlrad3 KO cells enhances the viral binding and internalisation. Assessments of different Ldlrad3 domains (D1, D2 and D3) of Ldlrad3 in viral infections indicate that D1 domain is necessary for viral binding and infection. Animal studies using Ldlrad3 knock-out mice and administration of Ldlrad3-Fc fusions in immunocompetent mice demonstrates the importance of Ldlrad3 in VEEV infections in mice.

The authors have presented experimental evidences to demonstrate that Ldlrad3 is a receptor for VEEV. The research is original; data are comprehensive and demonstrates for the first time that Lalrad3 is a receptor for VEEV. The results presented are of considerable interest to researchers investigating the mechanisms of alphavirus infections and could be helpful in designing appropriate therapeutic strategies for the control of VEEV infections.

Although there are no major concerns with this study, some clarifications and explanations are required. In addition, as humans can be occupationally (laboratory workers, veterinarians or field workers) exposed to VEEV via aerosol routes, it is important to assess the effectiveness of Ldlard3 in aerosol VEEV infections in mice.

Major Comments

1. Fig 1a - It is interesting that the best candidate binding to SINV-VEEV-TrD(IAB) was Ldlrad3 while laminin-binding protein (LBP, a putative receptor) was not found in the list of the candidates. Does GeCKO v2 sgRNA library has sgRNAs for laminin-binding protein? In the context of the reports of LBP as a receptor for VEEV, the authors should examined the importance of LMB protein as a receptor in VEEV infections.

2. Fig 1b - Complementation of Ldlrad3 does not restore the replication of SINV-VEEV (IC) and SINV-VEEV (ID) in KO+ Ldlrad3-N2a cells to the same extent as SINV-VEEV-TrD (IAB) strain. Discuss the structural differences of E2 protein between VEEV(IAB) strains and IC and ID strains.

Fig 1c - In BV2 microglial cells - does complementation of ΔB4galt7ΔLdlrad3 with Ldlrad3 restore the infection to the same levels seen in N2 cells?

3. Fig 1d shows multi-step growth curve of SINV-VEEV infection in ΔB4galt7 ΔLdlrad3 N2a cells. As comparison suggest including multi-step growth curve of VEEV-TrD infection in complemented ΔB4galt7 ΔLdlrad3N2a cells and N2a cells with intact GAG pathway. Infection with VEEV-TrD viruses was not restored by complementation (Fig 1f) to the same extent as SINV-VEEV virus infection (Fig 1b).

4. Line 111 - no effects were observed with KO of Ldlrda3 in N2a cells for replication of SINV strains. As shown in Fig 1g and 1h, KO of Ldlrad3 increased virus infection more than in WT cells and complementation with Ldlrda3 did not reduce the infection to the levels seen with WT cells. Also VEEV (TrD) replication was not restored in KO+ Ldlrad3 N2a cells to the same extent as observed for SINV-VEEV (IAB) strain (Fig 1a and Fig 1f).

5. Fig 2d - PLC treatment of ΔB4galt7ΔLdlrad3 cells complemented with GPI anchored Ldlrad3 reduced the SINV-VEEV-TrD infection. To demonstrate the importance of GAG pathways in VEEV infection, include results of PLC treatment of ΔLdlrad3 N2a cells with intact GAG pathway complemented with Ldlrad3.

6. Extended Fig 4b - Expression of D1 domain (Flag) was very poor and also complementation of cells with D1 domain (Flag) in ΔB4galt7ΔLdlrad3 cells resulted in small increase in SINV-VEEV-Trd-GFP positive cells (Left panel). For better comparison of effects of different domains, the authors should include data showing the complementation of ΔB4galt7ΔLdlrad3 cells with D1, D1+D2, D2+D3 and Ldlrad3 on the relative infection as shown by SINV-VEEV-GFP positive cells (Extended Fig 4a). Also it is better to include the results of Domain 1 and VEEV infection in Fig 1e rather than in Extended Fig 4b for comparison of effects of Domain 1 to Domain 1+2 and Domain 2+3 constructs.

7. Show histopathological analysis of brain tissues of animals infected with VEEV and treated with Ldlrad3-Fc proteins.

8. Compared to subcutaneous route aerosol infection results in rapid appearance of VEEV in CNS because of infection of olfactory neuroepithelium by aerosolized VEEV particles. It is important to demonstrate VEEV pathogenesis by aerosol infections in Ldlrad3 KO mice and in mice treated with Ldlrad3-D1-Fc.

Minor Comments

1. Fig 2 or in Extended Fig 4a - show a schematic of the construct with the Flag epitope.

2. Most of the experiments have been carried out with viruses derived from SINV-VEEV-GFP constructs or VLP particles except for one experiment shown in Fig 1f. Also restoration of infection by VEEV-TrD-GFP (Fig 1f) by complementation was not as efficient as restoration by SINV-VEEV-Trd viruses (Fig 1b). Clarify whether this due to the influence of nsps on the replication of viruses.

Referee #2 (Remarks to the Author):

Using crispr-cas9 based genome screening in neuronal cells, the authors identified the cell-surface expressed membrane glycoprotein Ldlrad3 as an essential host gene candidate for infection of the encephalitic alphavirus VEEV. Targeted knockout of Ldlrad3 in neuronal cells abrogated cell binding and infection by VEEV, which was restored upon Ldlrad3 complementation. Using genetic and biochemical approaches the authors demonstrate that virus binding occurs through the N-terminal domain (D1) of Ldlrad3. Using a mouse model, the authors showed that prophylactic administration of Ldlrad3-D1-Fc protected animals from VEEV pathogenesis. Moreover, the authors demonstrate that engineered Ldlrad3 ko mice were protected from lethality and morbidity. The work described is original, highly relevant and convincingly demonstrates the essential role of Ldlrad3 as a receptor for VEEV infection of (neuronal and microglial) cells, and its relevance for VEEV infection of mice. The relevance of Ldlrad3 as an entry mediator does not extend to mosquitoes (natural hosts for VEEV), as they lack an apparent Ldlrad3 ortholog. In addition, ko of Ldlrad3 in cells had no effect on infection of two other encephalitic alphaviruses EEEV and WEEV, suggesting that alternative entry receptors exist for these related viruses.

Major:
A limitation of the study is that the authors only used two cell types (neuronal and microglial cells) to study the infection dependency on Ldlrad3. Hence it remains unknown whether Ldlrad3 cell surface expression is a more general requirement for infection of other types of mammalian cells. Authors are encouraged to study Ldlrad3 expression and its dependency for infection in other mammalian cells that are susceptible to VEEV (e.g. human leukocytes) through e.g. receptor binding competition studies using soluble Ldlrad3-D1-Fc.
Somewhat surprisingly, information is also lacking on cells that might be refractory to VEEV infection, its correlation with a lack of Ldlrad3 expression and the ability to confer virus susceptibility by ectopic expression of Ldlrad3.

Little is known on the cell and tissue distribution of Ldlrad3 in vivo. The authors refer to a transcriptome study that indicates that the Ldlrad3 is primarily expressed in neuronal cells of the brain. The study would gain strength if the in vivo tissue distribution of Ldlrad3 in the mouse is addressed (e.g. using commercially available anti-Ldlrad3 antibodies) in correlation with the cell and tissue tropism of VEEV in vivo.

Referee #3 (Remarks to the Author):

This excellent paper provides a variety of convincing evidence supporting the idea that LDLRAD3 is a receptor involved in infection of a variety of VEEV strains in mammalian cells and in mice. The experiments are very well done, the data are very nicely presented, and, overall, the paper was a pleasure to read. Assuming that my reservations can be addressed, I think the paper provides an important insight into VEEV biology, and - because VEEV is an interesting alphavirus and biodefense threat - the observations are likely to be of broad interest.

Major points

1. It is difficult to assess how essential LDLRAD3 expression is to mediate VEEV infection. Knockout of essential receptors such as the poliovirus receptor leads to a complete loss of infection with cells completely protected against the cytolytic effect of infection. Knockout of attachment factors or co-receptors lead to more modest decreases in viral infection due to redundant modes of entry. From the data it is unclear where LDLRAD3 lies on this scale. Although a clear reduction in infectivity is shown in LDLRAD3 knockout, a significant fraction of the cells is still being infected (ranging from ~5% to 40%) For example in SH-SY5Ycells the remaining infection for one of the clonal knockout line is ~5% (Fig. 1m), whereas another independent knockout clone shows a much higher percentage of ~40% (Extended data 2h). The authors rely heavily on one FACS-based assay to assess viral infection. To better assess and quantify the effects of LDLRAD3 expression VEEV infection, the authors should use additional assays. The multicycle experiment is only performed in one mouse cancer cell line and should be repeated in additional cell lines preferably ones that have intact HS biosynthesis like SH-SY5Y.

2. One would expect that in the context of HS biosynthesis knockout, overexpression of LDLRAD3 would strongly enhance infection, binding and internalization. Although there is a hint that LDLRAD3 can do that, the effect is very modest and not always seen with different VEEV strains (Fig 1b). Are there cellular context where LDLRAD3 is rate limiting for infection? Can one make a normally lowly LDLRAD3 expressing cell line more susceptible for VEEV infection by overexpression of LDLRAD3?

3. LDLRAD3 knockout in HS negative cells shows only a marginal reduction in binding and internalization. This suggests that there are redundant attachment and entry factors. For the minor group of rhinoviruses such redundancy is described as they can use LDLR, VLDLR, and the LDLR related protein (LRP) as receptors. Can the authors discuss or experimentally address whether other proteins containing Low Density Lipoprotein Receptor Class A domains can act as receptors for VEEV.

Minor points.

1. In several figures, statistical significance is not indicated. For example LDLRAD3 knockout seems to enhance SINV infection in fig. 1g and 1h but it is unclear if this is statistically significant or not. Consistently indicating significance (or n.s.) of the WT versus the tested conditions would make this clearer.

2. The authors conclude this from Fig. 2: "Thus, interaction with the ectodomain of Ldlrad3 may facilitate VEEV glycoprotein conformational changes required for internalization or potentiate interactions with other host factors that bridge membrane penetration and entry." This seems quite speculative. Given that the effect of knockout on VEEV binding is equal to the effect on internalization an equally likely function of Ldlrad3 can be solely mediating attachment.

3. Correlation of cell surface staining of endogenous LDLRAD3 (or RNA expression) with VEEV infectivity would more firmly establish whether LDLRAD3 can be a tropism determining entry factor.

Author Rebuttals to First Revision:

Response to Reviewers

We greatly appreciated the constructive criticisms of the reviewers and have addressed all of the major concerns in the detailed response below and in the revised paper. To summarize the major changes and new data added to the manuscript during the revision phase:

1. Multi-step growth kinetics of VEEV infection in SH-SY5Y cells (Figure 1n).

2. Assessment of <u>21 different cell lines</u>, including four primary cell lines, for correlations between Ldlrad3 surface expression and VEEV infection (Extended Data Figures 3, 4, and 5).

3. VEEV infection experiments in three additional Ldlrad3-complemented cell lines (Fig 1c, and Extended Data Figures 2, 3, and 5).

4. Additional blocking of VEEV infection experiments in cell culture using Ldlrad3-D1-Fc and newly generated anti-Ldlrad3 polyclonal antisera (Figure 2e-f and Figure 3h).

5. *In vivo* protection studies using the LDLRAD3-D1-Fc decoy receptor in mice challenged via <u>intracranial route</u> with highly pathogenic VEEV-TrD (Figure 4h and Extended Data Figures 8b and 8d).

6. Viral RNA *in situ* hybridization and hematoxylin and eosin staining of brain and spinal cord tissues from Ldlrad3-D1-Fc treated and VEEV challenged mice (Figure 4g and Extended Data Figure 9).

7. Analysis of *Ldlrad3* mRNA expression in various mouse tissues with qRT-PCR and RNA *in situ* hybridization (Extended Data Figure 10).

In summary, these experiments provide overwhelming support for our data establishing LDLRAD3 as a key receptor for VEEV infection and pathogenesis.

Referee #1:

In the article, the authors have identified low-density lipoprotein (LDL) receptor class A domain containing 3 (LDLRAD3) as a receptor for Venezuelan equine encephalitis virus (VEEV) by CRISPR-Cas9 based screening of sgRNA library in N2a neuroblastoma cells. Reduction of VEEV infection by knock-down of Ldlrad3 in mouse and human cells could be restored by transfection of Ldlrad3 gene into gene-edited cells. Expression of Ldlrad3 in Ldlrad3 KO cells enhances the viral

binding and internalisation. Assessments of different Ldlrad3 domains (D1, D2 and D3) of Ldlrad3 in viral infections indicate that D1 domain is necessary for viral binding and infection. Animal studies using Ldlrad3 knock-out mice and administration of Ldlrad3-Fc fusions in immunocompetent mice demonstrates the importance of Ldlrad3 in VEEV infections in mice.

The authors have presented experimental evidences to demonstrate that Ldlrad3 is a receptor for VEEV. The research is original; data are comprehensive and demonstrates for the first time that Lalrad3 is a receptor for VEEV. The results presented are of considerable interest to researchers investigating the mechanisms of alphavirus infections and could be helpful in designing appropriate therapeutic strategies for the control of VEEV infections.

Although there are no major concerns with this study, some clarifications and explanations are required. In addition, as humans can be occupationally (laboratory workers, veterinarians or field

workers) exposed to VEEV via aerosol routes, it is important to assess the effectiveness of Ldlard3 in aerosol VEEV infections in mice.

We greatly appreciate the positive comments and the lack of major concerns with the study.

Major Comments

1. Fig 1a - It is interesting that the best candidate binding to SINV-VEEV-TrD(IAB) was Ldlrad3 while laminin-binding protein (LBP, a putative receptor) was not found in the list of the candidates. Does GeCKO v2 sgRNA library has sgRNAs for laminin-binding protein? In the context of the reports of LBP as a receptor for VEEV, the authors should examine the importance of LMB protein as a receptor in VEEV infections.

The laminin-binding protein, which as the Reviewer points out, is a putative receptor for VEEV (PMID: 14664162 and 19961413). It was present in our library (gene symbol Rpsa) and ranked 2975 (see **Table S1**). *Our paper focuses on identifying and validating LDLRAD3 as a receptor for VEEV. The possible reasons we did not identify it may include its level of expression of N2a cells, its subordinate nature as a receptor, or that it is not a bona fide receptor. Respectfully, we feel it is beyond the scope of this paper to test and validate other putative receptors that were not identified in our screen.*

2. Fig 1b - Complementation of Ldlrad3 does not restore the replication of SINV-VEEV (IC) and SINV-VEEV (ID) in KO+ Ldlrad3-N2a cells to the same extent as SINV-VEEV-TrD (IAB) strain. Discuss the structural differences of E2 protein between VEEV(IAB) strains and IC and ID strains.

The differences after LDLRAD3 complementation in N2a cells that the reviewer points out are relatively small (~150% versus 200% infection compared to the wild-type control cell). While this point is interesting, it would require substantial corroboration perhaps at different levels of transgene expression to be sure the differences are meaningful and real. Moreover, we have not yet mapped the exact LDLRAD3-VEEV binding site at the atomic level (by X-ray crystallography or cryo-electron microscopy). Thus, comments about the structural and sequence differences in the E2 protein among the VEEV subtypes to us seem too speculative to put in this paper.

Fig 1c - In BV2 microglial cells - does complementation of ΔB4galt7ΔLdlrad3 with Ldlrad3 restore the infection to the same levels seen in N2 cells?

*We agree this is an important point. We have now added complementation data in the BV2 cells (***Figure 1c***).*

3. Fig 1d shows multi-step growth curve of SINV-VEEV infection in ΔB4galt7 ΔLdlrad3 N2a cells. As comparison suggest including multi-step growth curve of VEEV-TrD infection in complemented ΔB4galt7 ΔLdlrad3N2a cells and N2a cells with intact GAG pathway. Infection with VEEV-TrD viruses was not restored by complementation (Fig 1f) to the same extent as SINV-VEEV virus infection (Fig 1b).

The experiments in the original **Figure 1b and 1f** *were performed in different laboratories under slightly different infection conditions and timing. One note is that VEEV-TrD is more cytopathic than SINV-VEEV, and that highly infected cells undergo cell death more rapidly. It is clear from the data in the original* **Figure 1f** *that VEEV-TrD infection is (a) reduced markedly in LDLRAD3 KO cells and (b) restored in complemented cells. We have now included data showing that the restoration of infection in Ldlrad3 complemented cells is statistically significant (P < 0.0001) (***Figure 1f***). Experiments with highly pathogenic VEEV-TrD (a Select Agent) are currently limited due to COVID-19 work in the Regional Biocontainment Laboratory. Respectfully, given our compelling in vivo data (***Figure 4***) with VEEV-TrD and limited access to containment facilities, we*

feel that this in vitro experiment with highly pathogenic VEEV-TrD will not substantially add to the main findings of this paper. As a note, prior studies from the Klimstra laboratory have implicated a role for HS in VEEV infectivity in some cell types (PMID: 21896745).

4. Line 111 - no effects were observed with KO of Ldlrda3 in N2a cells for replication of SINV strains. As shown in Fig 1g and 1h, KO of Ldlrad3 increased virus infection more than in WT cells and complementation with Ldlrda3 did not reduce the infection to the levels seen with WT cells. Also VEEV (TrD) replication was not restored in KO+ Ldlrad3 N2a cells to the same extent as observed for SINV-VEEV (IAB) strain (Fig 1a and Fig 1f).

*We are not entirely clear what the Reviewer is trying to convey here. In **Figure 1g and 1h**, we show clearly that SINV, SINV-WEEV, and SINV-EEEV __do not show a loss of infection__ when LDLRAD3 is gene edited. The fact that some of these viruses show slight increases in the KO and complemented cells could be a clonal effect or due to expression of other factors that enhance infection of these viruses. The important point is that infection is **NOT** reduced in the LDLRAD3 KO cells, suggesting that Ldlrad3 is not essential for entry of these viruses. We have clarified this in the text (p.5).*

5. Fig 2d - PLC treatment of ΔB4galt7ΔLdlrad3 cells complemented with GPI anchored Ldlrad3 reduced the SINV-VEEV-TrD infection. To demonstrate the importance of GAG pathways in VEEV infection, include results of PLC treatment of ΔLdlrad3 N2a cells with intact GAG pathway complemented with Ldlrad3.

*The Reviewer is interested in the role of GAG interactions in VEEV infection. However, our paper is focused on identification and characterization of LDLRAD3 as a key receptor for VEEV. In Figure 1, we showed the importance of LDLRAD3 in cells that lack (**Figure 1b and 1d**) or express HS (**Figure 1c and 1e**). Other published studies have already established that HS interactions can modulate VEEV pathogenesis (PMID: 11021998). The purpose of **Figure 2d** was to show the importance of the ectodomain of LDLRAD3 on VEEV infection using a GPI-anchored trans-complementing form.*

6. Extended Fig 4b - Expression of D1 domain (Flag) was very poor and also complementation of cells with D1 domain (Flag) in ΔB4galt7ΔLdlrad3 cells resulted in small increase in SINV-VEEV-Trd-GFP positive cells (Left panel). For better comparison of effects of different domains, the authors should include data showing the complementation of ΔB4galt7ΔLdlrad3 cells with D1, D1+D2, D2+D3 and Ldlrad3 on the relative infection as shown by SINV-VEEV-GFP positive cells (Extended Fig 4a). Also it is better to include the results of Domain 1 and VEEV infection in Fig 1e rather than in Extended Fig 4b for comparison of effects of Domain 1 to Domain 1+2 and Domain 2+3 constructs.

*We are not entirely sure what the Reviewer is asking. (a) The new **Extended Data Fig 7a** shows expression of the LDLRAD3 domain mutants; (b) The new **Extended Data Figure 7b-c** shows the absolute infection including the low-level expression of LDLRAD3 Domain 1 (which is likely due to a loss of chaperone binding). In **Figure 3e** we have shown the relative binding of the domain mutant constructs that express well. We assume the Reviewer is requesting the absolute infection of the other domain constructs. This has now been added to **Extended Data Figure 7b**. We prefer not to put the LDLRAD3 Domain 1 data in the main Figure since it is poorly expressed (see **Extended Data Figure 7c**). We did confirm the importance of Domain 1 in other experiments using the Ldlrad3-D1-Fc protein and antibodies against Ldlrad3 Domain 1, both of which inhibit VEEV infection.*

7. Show histopathological analysis of brain tissues of animals infected with VEEV and treated with Ldlrad3-Fc proteins.

*We have added viral RNA in situ hybridization and hematoxylin and eosin staining of brain and spinal cord from isotype control and LDLRAD3-D1-Fc treated mice (**Figure 4g and Extended Data Figure 9**). As expected, little pathology and no viral RNA is seen in the brains from LDLRAD3-D1-Fc compared to control IgG-treated mice, which have extensive infection and injury.*

8. Compared to subcutaneous route aerosol infection results in rapid appearance of VEEV in CNS because of infection of olfactory neuroepithelium by aerosolized VEEV particles. It is important to demonstrate VEEV pathogenesis by aerosol infections in Ldlrad3 KO mice and in mice treated with Ldlrad3-D1-Fc.

*Respectfully, we feel that aerosol challenge studies are beyond the scope of a paper that identifies a receptor through a genome-wide CRISPR screen, validates its activity, maps its key domain of interaction, develops a decoy receptor, and shows an absence of pathogenicity in KO mice as well as decoy-treated mice. As the Reviewer likely is aware, experiments with highly pathogenic VEEV-TrD (a Select Agent) and aerosol challenge are currently very limited due to the extensive COVID-19 work in the Regional Biocontainment Laboratory. We feel this is best performed in the context of a subsequent more detailed therapeutics paper. Instead, we have performed direct intracranial challenge experiments. Remarkably, peripheral administration of Ldlrad3-D1-Fc protected mice after intracranial challenge with VEEV-TrD (**see Figure 4h and Extended Data Figure 8b and d**)*

Minor Comments

1. Fig 2 or in Extended Fig 4a - show a schematic of the construct with the Flag epitope.

*We have added a scheme of the location of the N-terminal FLAG epitope to **Extended Data Figure 2c**.*

2. Most of the experiments have been carried out with viruses derived from SINV-VEEV-GFP constructs or VLP particles except for one experiment shown in Fig 1f. Also restoration of infection by VEEV-TrD-GFP (Fig 1f) by complementation was not as efficient as restoration by SINV-VEEV-Trd viruses (Fig 1b). Clarify whether this due to the influence of nsps on the replication of viruses.

*Although VEEV-TrD infection was restored with LDLRAD3 complementation it was not quite as efficiently as with SINV-VEEV-GFP. We feel this is because experiments were performed in different laboratories (Diamond, SINV-VEEV-GFP; Klimstra, VEEV-TrD) under slightly different conditions and timing. Other alternative explanations include that (a) cells that are highly infected with VEEV-TrD are more prone to cytopathic effects and thus, would be eliminated from our flow cytometric-based analysis; or (b) the non-structural proteins influence the replication of the viruses as the Reviewer alludes to. Regardless, it is clear that loss of LDLRAD3 impairs VEEV-TrD entry and infection, and this is restored by complementation. These data are supported by the in vivo experiments in **Figure 4** which were performed using SINV-VEEV (TrD), VEEV ZPC738, and VEEV TrD .*

Reviewer #2

Using crispr-cas9 based genome screening in neuronal cells, the authors identified the cell-surface expressed membrane glycoprotein Ldlrad3 as an essential host gene candidate for infection of the encephalitic alphavirus VEEV. Targeted knockout of Ldlrad3 in neuronal cells abrogated cell binding and infection by VEEV, which was restored upon Ldlrad3 complementation. Using genetic and biochemical approaches the authors demonstrate that virus binding occurs through the N-terminal domain (D1) of Ldlrad3. Using a mouse model, the authors

showed that prophylactic administration of Ldlrad3-D1-Fc protected animals from VEEV pathogenesis. Moreover, the authors demonstrate that engineered Ldlrad3 ko mice were protected from lethality and morbidity. The work described is original, highly relevant and convincingly demonstrates the essential role of Ldlrad3 as a receptor for VEEV infection of (neuronal and microglial) cells, and its relevance for VEEV infection of mice. The relevance of Ldlrad3 as an entry mediator does not extend to mosquitoes (natural hosts for VEEV), as they lack an apparent Ldlrad3 ortholog. In addition, ko of Ldlrad3 in cells had no effect on infection of two other encephalitic alphaviruses EEEV and WEEV, suggesting that alternative entry receptors exist for these related viruses.

We greatly appreciate the favorable comments made by this reviewer.

Major Comments:

A limitation of the study is that the authors only used two cell types (neuronal and microglial cells) to study the infection dependency on Ldlrad3. Hence it remains unknown whether Ldlrad3 cell surface expression is a more general requirement for infection of other types of mammalian cells. Authors are encouraged to study Ldlrad3 expression and its dependency for infection in other mammalian cells that are susceptible to VEEV (e.g. human leukocytes) through e.g. receptor binding competition studies using soluble Ldlrad3-D1-Fc.

*We agree with this comment. In the revision, we have tested a large panel of mouse and human cell lines for infectivity by SINV-VEEV and Ldlrad3 surface expression as judged by a polyclonal antibody that recently was generated in our laboratory (**Extended Data Figure 3, 4, and 5**). From this analysis, we divided cells into two groups: permissive and non-permissive. A panel of non-permissive cells (e.g., Jurkat and Raji) were transduced with Ldlrad3, which resulted in gain-of-infection (**Extended Data Figure 3b and 3d**). For selected permissive cells (e.g., 293T, 3T3, HeLa, hCMEC/D3, CADMEC, and HDF cells), which expressed Ldlrad3, SINV-VEEV infection was inhibited by gene-editing, anti-Ldlrad3 sera, and/or Ldlrad3-D1-Fc (**Figure 2f, 3h and Extended Data Figure 5**). Collectively, these studies establish the dependency of Ldlrad3 on VEEV infectivity in a wider range of cell types.*

Somewhat surprisingly, information is also lacking on cells that might be refractory to VEEV infection, its correlation with a lack of Ldlrad3 expression and the ability to confer virus susceptibility by ectopic expression of Ldlrad3.

*As described in the comment above, we now provide additional data showing that selected non-permissive cells (e.g., Jurkat and Raji) transduced with Ldlrad3 show gain-of-infection phenotypes (**Extended Data Figure 3b and 3d**).*

Little is known on the cell and tissue distribution of Ldlrad3 in vivo. The authors refer to a transcriptome study that indicates that the Ldlrad3 is primarily expressed in neuronal cells of the brain. The study would gain strength if the in vivo tissue distribution of Ldlrad3 in the mouse is addressed (e.g. using commercially available anti-Ldlrad3 antibodies) in correlation with the cell and tissue tropism of VEEV in vivo.

We agree with this comment that more information on Ldlrad3 expression in vivo would provide greater context for our studies. First, we would like to point out that commercially available anti-Ldlrad3 mAbs are neither authenticated nor specific, Indeed, they bound equally well to our WT and KO cells, and thus we feel are not reliable. Our own polyclonal anti-Ldlrad3 antisera, while specific with respect to binding Ldlrad3 on the surface of cells, does not stain tissue sections well, possibly due to low expression levels of Ldlrad3 (data not shown). To overcome these limitations and address the issue of tissue expression, we performed the following studies: (a) We generated TaqMan probes targeting D1 of Ldlrad3 and used these to screen for mRNA expression in

*different organs including brain, spinal cord, lung, heart, kidney, spleen, lymph nodes, gut, liver, pancreas, muscle, testis, ovary (**Extended Data Figure 10a-b**); (b) We performed RNA in situ hybridization using probes for Ldlrad3 and VEEV (strain ZPC-738) on brain and spinal cord tissues. These data show Ldlrad3 mRNA expression corresponds with the cells that are infected by VEEV (**Figure 4g and Extended Data Figure 10c**). We acknowledge the Ldlrads3 mRNA expression studies have limitations and thus are planning to generate a reporter gene mouse in the near future for more definitive analysis.*

Referee #3

This excellent paper provides a variety of convincing evidence supporting the idea that LDLRAD3 is a receptor involved in infection of a variety of VEEV strains in mammalian cells and in mice. The experiments are very well done, the data are very nicely presented, and, overall, the paper was a pleasure to read. Assuming that my reservations can be addressed, I think the paper provides an important insight into VEEV biology, and - because VEEV is an interesting alphavirus and biodefense threat - the observations are likely to be of broad interest.

We greatly appreciate the supportive comments on the paper.

Major points.

1. It is difficult to assess how essential LDLRAD3 expression is to mediate VEEV infection. Knockout of essential receptors such as the poliovirus receptor leads to a complete loss of infection with cells completely protected against the cytolytic effect of infection. Knockout of attachment factors or co- receptors lead to more modest decreases in viral infection due to redundant modes of entry. From the data, it is unclear where LDLRAD3 lies on this scale. Although a clear reduction in infectivity is shown in LDLRAD3 knockout, a significant fraction of the cells is still being infected (ranging from ~5% to 40%) For example in SH-SY5Y cells the remaining infection for one of the clonal knockout line is ~5% (Fig. 1m), whereas another independent knockout clone shows a much higher percentage of ~40% (Extended data 2h). The authors rely heavily on one FACS-based assay to assess viral infection. To better assess and quantify the effects of LDLRAD3 expression VEEV infection, the authors should use additional assays. The multicycle experiment is only performed in one mouse cancer cell line and should be repeated in additional cell lines preferably ones that have intact HS biosynthesis like SH-SY5Y.

*We agree with this comment and have added additional multi-step growth curves with SH-SY5Y cells using a focus-forming assay (**Figure 1n**). VEEV infection levels were not completely abolished in B4GALT7$^{+/+}$ ΔLDLRAD3 SH-SY5Y cells, suggesting possible alternative entry pathways.*

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[Redacted]

One would expect that in the context of HS biosynthesis knockout, overexpression of LDLRAD3 would strongly enhance infection, binding and internalization. Although there is a hint that LDLRAD3 can do that, the effect is very modest and not always seen with different VEEV strains (Fig 1b). Are there cellular context where LDLRAD3 is rate limiting for infection? Can one make a normally lowly LDLRAD3 expressing cell line more susceptible for VEEV infection by overexpression of LDLRAD3?

*We agree with this comment as well. Based on a comment by Reviewer #1, we screened several lines for LDLRAD3 expression (See **Extended Data Figures 3, 4, and 5**) and identified Jurkat and Raji cells with virtually no cell surface expression by flow cytometry (**Extended Data Figures 3a and 3c**). We subsequently transduced these cells with LDLRAD3 and now show enhanced infection, as predicted by the Reviewer. This data is now added in (**Extended Data Figures 3b and d**).*

2. LDLRAD3 knockout in HS negative cells shows only a marginal reduction in binding and internalization. This suggests that there are redundant attachment and entry factors. For the minor group of rhinoviruses such redundancy is described as they can use LDLR, VLDLR, and the LDLR related protein (LRP) as receptors. Can the authors discuss or experimentally address whether other proteins containing Low Density Lipoprotein Receptor Class A domains can act as receptors for VEEV?

We agree this is an interesting point. Although we did not identify other LDLRAD3-like proteins in our CRISPR-Cas9 screen, there may be a second, subordinate receptor for VEEV. It is also possible that other LDL receptor-like family members serve as primary receptors for WEEV and EEEV, which do not use LDLRAD3 for entry and infectivity. We feel that a complete evaluation of the family requires a large set of studies that is beyond the scope of this paper, which identifies LDLRAD3 as a dominant receptor in vitro and in vivo for VEEV. Notwithstanding this point, we now raise this possibility in the Discussion (p.11) as a future area of investigation.

Minor points.

1. In several figures, statistical significance is not indicated. For example, LDLRAD3 knockout seems to enhance SINV infection in fig. 1g and 1h but it is unclear if this is statistically significant or not. Consistently indicating significance (or n.s.) of the WT versus the tested conditions would make this clearer.

We have gone through the Figures and added statistical significance where appropriate. We did not include statistics on figures containing less than three independent experiments.

2. The authors conclude this from Fig. 2: "Thus, interaction with the ectodomain of Ldlrad3 may facilitate VEEV glycoprotein conformational changes required for internalization or potentiate interactions with other host factors that bridge membrane penetration and entry." This seems quite speculative. Given that the effect of knockout on VEEV binding is equal to the effect on internalization an equally likely function of Ldlrad3 can be solely mediating attachment.

We agree on the speculative nature of this comment, and have deleted it from the revision.

3. Correlation of cell surface staining of endogenous LDLRAD3 (or RNA expression) with VEEV infectivity would more firmly establish whether LDLRAD3 can be a tropism determining entry factor.

This point was also raised by Reviewer #1. We have provided infectivity data with a more comprehensive group of cells and compared LDLRAD3 expression with infectivity at the same MOI and time point. These results are now provided in **Extended Data Figures 3, 4, and 5**.

Reviewer Reports on the Second Revision:

Referee #1 (Remarks to the Author):

Most of the author's responses to the reviewer comments are satisfactory. The authors have performed additional experiments to corroborate their findings. Appropriate explanations and descriptions have been added in the text and the figure legends. The authors have carried out intracranial infections to show the importance of Ldlrad3 in VEEV infection. As aerosol infections present an important route of VEEV infections, experiments using aerosol infections will provide support for the importance of Ldlrad3 in CNS infection by VEEV.

Referee #2 (Remarks to the Author):

The authors have sufficiently addressed the comments in my previous report and improved their manuscript. I recommend it for publication.

Referee #3 (Remarks to the Author):

The authors have included a significant amount of new data further supporting their conclusions. They have satisfactorily addressed all my points and I fully support publication.