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Title	A polymorphism of the TIM-1 IgV domain: Implications for the susceptibility to filovirus infection
Author(s)	Kuroda, Makoto; Fujikura, Daisuke; Noyori, Osamu et al.
Citation	Biochemical and biophysical research communications, 455(3-4), 223-228 https://doi.org/10.1016/j.bbrc.2014.10.144
Issue Date	2014-12-12
Doc URL	https://hdl.handle.net/2115/57926
Type	journal article
File Information	BBRCv.455p.223.pdf



**A polymorphism of the TIM-1 IgV domain: Implications for the susceptibility to
filovirus infection**

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Abstract

Filoviruses, including Ebola and Marburg viruses, cause severe hemorrhagic fever in humans and nonhuman primates with mortality rates of up to 90%. Human T-cell immunoglobulin and mucin domain 1 (TIM-1) is one of the host proteins that have been shown to promote filovirus entry into cells. In this study, we cloned TIM-1 genes from three different African green monkey kidney cell lines (Vero E6, COS-1, and BSC-1) and found that TIM-1 of Vero E6 had a 23-amino acid deletion and 6 amino acid substitutions compared with those of COS-1 and BSC-1. Interestingly, Vero E6 TIM-1 had a greater ability to promote the infectivity of vesicular stomatitis viruses pseudotyped with filovirus glycoproteins than COS-1-derived TIM-1. We further found that the increased ability of Vero E6 TIM-1 to promote virus infectivity was most likely due to a single amino acid difference between these TIM-1s. These results suggest that a polymorphism of the TIM-1 molecules is one of the factors that influence cell susceptibility to filovirus infection, providing a new insight into the molecular basis for the filovirus host range.

1. Introduction

Filoviruses (viruses in the family *Filoviridae*) are filamentous, enveloped, and nonsegmented negative-stranded RNA viruses. The genus *Marburgvirus* consists of one species, *Marburg marburgvirus*, which has two distinct viruses, Marburg virus (MARV) and Ravn virus (RAVV). In contrast, the genus *Ebolavirus* consists of five distinct species, *Zaire ebolavirus*, *Sudan ebolavirus*, *Tai forest ebolavirus*, *Bundibugyo ebolavirus*, and *Reston ebolavirus*, represented by Ebola virus (EBOV), Sudan virus, Tai forest virus, Bundibugyo virus, and Reston virus (RESTV), respectively [1]. A novel filovirus named Lloviu virus (LLOV), which belongs to the genus *Cuevavirus*, was recently detected from dead bats in Spain [2,3]. Ebola and Marburg hemorrhagic fevers have occurred mainly in Africa [4,5]. Infection causes severe hemorrhagic fever in humans and nonhuman primates with high mortality rates of up to 90%. It has been shown that other animals such as pigs and fruit bats are also susceptible to these viruses [6,7,8], suggesting a broader host range than thus far assumed.

The envelope glycoprotein (GP) is the only viral surface protein responsible for both receptor binding (i.e., attachment) and membrane fusion during entry of filoviruses into cells. The expression pattern of receptors/coreceptors is believed to be one of the major factors determining the host range and tissue tropism of filoviruses [9]. While several

cellular molecules have been proposed to be filovirus receptors or coreceptors (i.e., attachment or fusion factors), the molecular mechanisms underlying filovirus entry into cells are not fully understood. Human T-cell immunoglobulin and mucin domain 1 (TIM-1) is known to contribute to filovirus entry through the recognition of phosphatidylserine (PtdSer) exposed on the virus envelope and facilitates viral attachment and virus uptake independently of GP [10,11,12]. The IgV domain, which forms a PtdSer-binding pocket, is thought to recognize PtdSer on viral envelopes and facilitate the binding of virus particles to cell surfaces [10,12,13], suggesting that the IgV domain is essential for the TIM-1-mediated enhancement of viral infection.

African green monkey kidney Vero E6 cells are known to be highly susceptible to filovirus infection and are commonly used for filovirus studies. Though a TIM-1 knockdown experiment has demonstrated that Vero cell-derived TIM-1 largely contributes to efficient filovirus entry into cells [11], information on other African green monkey cell lines such as COS-1 and BSC-1, which are also used for analysis of filovirus infection, is limited [14,15,16]. In this study, we found that Vero E6 TIM-1 had different primary structures and a greater ability to promote infectivity of vesicular stomatitis viruses (VSVs) pseudotyped with filovirus GPs than TIM-1s derived from the other cell lines tested. Interestingly, the increased ability of Vero E6 TIM-1 is most

likely due to a single amino acid difference at amino acid position 48. Our results suggest that a polymorphism of the TIM-1 molecules is one of the factors that influence the cell susceptibility to filovirus infection.

2. Materials and methods

2.1. Viruses and cell lines

Replication-incompetent VSVs containing the green fluorescent protein (GFP) and luciferase genes instead of the VSV G protein gene (VSV Δ G-G) and VSVs pseudotyped with filovirus GPs were generated as described previously [16]. A neutralizing monoclonal antibody against the VSV G protein, VSV-G(N)1-9 [17], was used to abolish the background infectivity of parental VSV Δ G-G. Viral infectious units used to calculate the multiplicity of infection (MOI) were determined by counting the number of cells expressing GFP. For quantitative comparison of the infectivity in human embryonic kidney 293T cells and those expressing wild-type (WT) and mutant TIM-1s, the luciferase activity in infected cell lysates was measured using a Luciferase assay kit (Promega). The relative luciferase activity was determined by setting the value of infected control (i.e., empty vector-transduced) cells to 1.0. 293T, 293T-derived Platinum-GP (Plat-GP) (Cell Biolabs) [18], and African green monkey kidney cell lines (Vero E6, COS-1, and BSC-1) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin-streptomycin.

2.2. Cloning of TIM-1 genes

The coding region of the Vero E6 TIM-1 gene was PCR-amplified from a full-length cDNA library prepared from Vero E6 cells (In-Fusion SMARTer Directional cDNA Library Construction Kit; Clontech Laboratories) using primers EcoRI-TIM-1 (5'-CGGAATTCTCAGATACCATCTGGTAGGGTGT-3'), containing an *EcoRI* restriction site, and TIM-1-XhoI (5'-CCCTCGAGACTGACATGTTGGAAGGCCA-3'), containing an *XhoI* restriction site. Coding regions of the COS-1 and BSC-1 TIM-1 genes were PCR-amplified from cDNA prepared from total RNA extracted from these cells using the same primer pairs. After sequence confirmation (DDBJ accession number AB969733, AB969734, and AB969735 for Vero E6, COS-1, and BSC-1, respectively), these PCR products were cloned into a murine leukemia virus-based retroviral vector, pMXs-IRES-GFP (pMXs-IG) [18].

2.3. Generation of TIM-1-expressing cells

To generate the retrovirus, Plat-GP cells were cotransfected with pMXs-IG encoding Vero E6, COS-1, or BSC-1 TIM-1 cDNAs and the expression plasmid pCAGGS encoding VSV G cDNA, using Lipofectamine 2000 (Invitrogen) [18]. Two days later, the culture supernatants containing retroviruses were collected, clarified through

0.45- μ m filters, and then used to infect 293T cells. Transduced GFP-positive cells were collected using a MoFlo Astrios cell sorter (Beckman Coulter). The percentages of GFP-positive cells in sorted samples were verified as >95% by using flow cytometry.

2.4. Flow cytometry

293T cells were detached using 0.25% trypsin, washed with cold PBS with 2% FCS, and incubated with a goat anti-TIM-1 polyclonal antibody (R&D Systems). Primary antibody binding was detected with Alexa Fluor 647-conjugated anti-goat IgG (Jackson ImmunoResearch Laboratories). After several washes, the mean fluorescent intensities (MFIs) of the cells were analyzed using a FACS Canto flow cytometer (BD Biosciences) and FlowJo software (Tree Star).

2.5. TIM-1 mutagenesis

Mutant TIM-1 genes were generated using a PrimeSTAR Mutagenesis Basal kit (Takara) with primers containing the desired nucleotide substitutions. All mutations were confirmed by DNA sequencing.

3. Results

3.1. Expression of TIM-1s in 293T cells and comparison of amino acid sequences among African green monkey kidney cell lines.

TIM-1 genes were cloned from three different African green monkey kidney cell lines (Vero E6, COS-1, and BSC-1) and introduced into 293T cells, which naturally lack cell surface expression of TIM-1 [11]. The expression of these TIM-1 proteins on the 293T cell surface was verified by flow cytometry (Fig. 1A). Amino acid sequence analyses revealed that Vero E6 TIM-1 had deletions (1 and 23 amino acids) and 6 amino acid substitutions compared with those of COS-1 and BSC-1 cells whose sequences were completely identical (Fig. 1B). Thus, we used TIM-1s of Vero E6 and COS-1 in the following experiments to analyze their ability to promote infectivity of VSVs pseudotyped with filovirus GPs.

3.2. Difference in the ability to promote entry of VSV pseudotyped with EBOV GP between Vero E6- and COS-1-derived TIM-1s.

To compare the potential to promote filovirus infection between Vero E6 and COS-1 TIM-1s, we prepared 293T cells stably expressing these TIM-1s and examined the infectivity of VSV pseudotyped with EBOV GP (VSV Δ G-EBOV GP) (Fig. 1C). We

found that both Vero E6 and COS-1 TIM-1s enhanced the infectivity of VSVΔG-EBOV GP, and that the virus infected cells expressing Vero E6 TIM-1 more efficiently than those expressing COS-1 TIM-1. The enhancement of the VSVΔG-G infectivity was minimal and no significant difference was found between Vero E6 and COS-1 TIM-1s. Expression levels of TIM-1s on the cell surface were quantified with MFI values obtained by flow cytometry and found to be similar in these cells (Data not shown. See Fig. 3C). These results suggested that Vero E6 TIM-1 had greater potential to promote EBOV entry into cells than COS-1 TIM-1.

3.3. Importance of the IgV domain for the increased ability of Vero E6 TIM-1 to promote entry of VSV pseudotyped with EBOV GP.

The IgV domain of TIM-1 is thought to be essential for the TIM-1-mediated enhancement of viral infection [10,12,13] and the cytoplasmic tail of TIM-1 is assumed to be involved in intracellular signaling [19,20,21]. To clarify whether the IgV domain and/or cytoplasmic tail of Vero E6 TIM-1 were involved in the ability to promote infectivity of VSVΔG-EBOV GP, we constructed TIM-1-deletion mutants lacking the IgV domain (ΔIgV) or cytoplasmic tail (ΔCT) and examined the viral infectivity in 293T cells expressing these TIM-1 mutants (Fig. 2A,B). As expected, with deletion of

the IgV domain, TIM-1 completely lost its ability to promote the infectivity of VSVΔG-EBOV GP. Interestingly, ΔCT enhanced the viral infectivity but its efficiency was reduced compared to WT TIM-1. Expression levels of these TIM-1s on the cell surface were quantified with MFI values obtained by flow cytometry and found to be similar (Fig. 2C,D). These results indicated that the IgV domain played an essential role in promoting infectivity of VSVΔG-EBOV GP, and that the cytoplasmic tail was also involved in the enhanced infection but not indispensable.

3.4. Importance of an amino acid at position 48 in the IgV domain for the increased ability of Vero E6 TIM-1 to promote entry of VSV pseudotyped with EBOV and other filovirus GPs.

Comparison of amino acid sequences of the TIM-1 IgV domain revealed that there was only one amino acid difference, at position 48, between Vero E6 and COS-1 TIM-1s (Fig. 1B). To confirm that the difference of this amino acid residue affected the potential of TIM-1 to promote efficient filovirus entry, we constructed TIM-1 mutants with single amino acid substitutions in the TIM-1 IgV domain (Fig. 3A) and examined the efficacies of these mutants in promoting VSVΔG-EBOV GP infection. Expression levels of these TIM-1s on the cell surface were quantified with MFI values obtained by

flow cytometry and found to be similar (Fig. 3B,C). We found that N48H (asparagine to histidine) substitution in the Vero E6 TIM-1 IgV domain significantly decreased the virus infectivity (Fig. 3D). In contrast, H48N (histidine to asparagine) substitution in the COS-1 TIM-1 IgV domain significantly increased the virus infectivity. These results suggested that the amino acid at position 48 was responsible for cell susceptibility to the increased potential of Vero E6 TIM-1 to promote EBOV entry.

To investigate the potential of TIM-1 to promote infectivities of other filoviruses and the importance of the amino acid at position 48, we prepared VSV pseudotyped with GPs of other filoviruses (RESTV, MARV, RAVV, and LLOV) and examined their infectivities in 293T cells expressing WT and mutant TIM-1s (Fig. 3E). Consistent with the result for VSV Δ G-EBOV GP, infectivities of VSVs pseudotyped with RESTV, MARV, and RAVV, and LLOV GPs (VSV Δ G-RESTV, -MARV, -RAVV, and -LLOV GPs, respectively) were significantly enhanced in 293T cells expressing TIM-1s and the distinct ability due to the single amino acid substitution was commonly observed between WT and mutant TIM-1s. It was noted that the extent of enhancement by TIM-1 expression was much lower for VSV Δ G-MARV and -RAVV GPs than for the other viruses tested.

4. Discussion

Several polymorphisms are found on amino acid sequences of the TIM-1 extracellular region consisting of the IgV and mucin domain in humans, mice, and monkeys [22,23,24,25]. The polymorphisms based on deletions or insertions in the mucin domain are known to be associated with severity in several diseases, including asthma, allergic diseases and hepatitis A virus-induced liver disease [24,25]. Since it was recently reported that the length of the mucin domain of TIM proteins regulates their ability to promote virus entry [26], the polymorphism in the mucin domain might regulate the function of TIM via a mechanism dependent on the length of the mucin domain. Other polymorphisms at N48 and K108 in the IgV region of TIM-1 were also found (Fig. 1B) [23]. K108 has been shown to be responsible for the binding of anti-TIM-1 monoclonal antibody 190/4, which blocks hepatitis A virus infection, suggesting that K108 might be associated with the virus-recognition of TIM-1 [23]. Here we focused on the other amino acid substitution at position 48 in the IgV domain and found that this mutation might regulate the activity of TIM-1s promoting filovirus cellular entry.

Although we found that the single amino acid residue at position 48 was responsible for the difference in the potential to promote infectivity of filoviruses between Vero E6

and COS-1 TIM-1s, the role of this particular amino acid in TIM-1-mediated viral entry remains unclear. Alanine scanning mutagenesis of the human TIM-1 IgV gene identified some important amino acid residues for TIM-1-mediated viral infection [10]: F55, R106, G111, N114, and D115 located on the CC' loop (44-64 aa) and FG loop (101-125 aa), which form the PtdSer-binding pocket. The crystal structure of the human TIM-1 IgV domain, predicted based on that of mouse TIM-1 using PHYRE2 (protein homology/analogy recognition engine 2) [10], revealed that the structure of the PtdSer-binding pocket was conserved between human and mouse TIM-1s. We also predicted the crystal structures of the TIM-1 IgV domains of Vero E6 and COS-1 using the same approach and found that amino acids N48 and H48 were positioned on the edge of the CC' loop in the PtdSer-binding pocket (data not shown), suggesting that the amino acid substitution focused on in the present study might regulate the function of TIM-1 by affecting the formation of the PtdSer-binding pocket. Interestingly, however, we found that virus-like particles (VLPs) attached to 293T cells expressing Vero E6 TIM-1 less efficiently than cells expressing COS-1 TIM-1 (data not shown), suggesting that the IgV domain including the amino acid at position 48 might be involved in not only viral attachment but also subsequent entry steps (e.g., viral internalization and membrane fusion).

Previous studies demonstrated that the human TIM-1 cytoplasmic tail was not required or nonessential to promote cellular entry of enveloped viruses, including EBOV [26,27]. In this study, decreased ability to promote virus infectivity was observed in TIM-1 lacking its cytoplasmic tail, suggesting the involvement of the TIM-1 cytoplasmic tail in promoting virus entry, most likely through intracellular signaling pathways. The TIM-1 cytoplasmic tail has at least one tyrosine phosphorylation site that could potentiate the intracellular signaling regulating immune responses [19,20,21]. In addition, it has previously been reported that the TIM-1 tyrosine phosphorylation in its cytoplasmic tail activates the phosphatidylinositol 3-kinase (PI3K) pathway [28] that is responsible for macropinocytosis [29,30]. Considering these findings together, we hypothesize that TIM-1-induced signaling may partially contribute to enhanced viral uptake by macropinocytosis.

TIM-1 was shown to be highly expressed on injured kidney epithelial cells, activated CD4⁺ T cells, B cells, and mast cells [11,20,24,31,32]. Among these cells, kidney epithelial cells such as Vero E6 cells are highly susceptible to filovirus infection [11], but lymphocytes are resistant to filovirus infection although they express TIM-1 [15], indicating that TIM-1 expression alone is not sufficient to confer susceptibility to filovirus infection. Furthermore, although ectopic expression of TIM-1 dramatically

promoted filovirus GP-mediated entry into cells (Fig. 3D,E) [10,11,12], filoviruses infect macrophages and dendritic cells, which have low or undetectable expression of TIM-1 [33], suggesting that TIM-1 is not essential for filovirus infection. However, since filovirus antigens are detected in many organs, including the liver, spleen, kidneys, lymph nodes, and lungs at the late stage of infection [34], it is conceivable that TIM-1 polymorphism might affect overall disease progression in filovirus-infected individuals.

It has been demonstrated that TIM-1 directly binds to PtdSer on the viral envelope and TIM-1-mediated enhancement of viral infection has been reported in infection with several different enveloped viruses in a manner independent of specific receptor recognition by their envelope glycoproteins [10,12,13]. For example, TIM-1 enhances filovirus, alphavirus, flavivirus, and arenavirus infections but not Lassa virus, herpes simplex virus 1, influenza A virus (H7N1), and SARS coronavirus infections [10,12,27]. Interestingly, we found that the infectivities of VSVΔG-MARV GP and -RAVV GP were enhanced less efficiently by TIM-1 expression compared to VSVΔG-EBOV GP. These findings suggest that there might be viral glycoprotein-dependent mechanisms underlying TIM-1-mediated viral entry. Thus, further studies are needed to fully understand the contribution of TIM-1 to the tissue tropism and pathogenicity of enveloped viruses.

Acknowledgments

We thank Mari Ishijima for technical assistance and Kim Barrymore for editing the manuscript. This work was supported by KAKENHI, a Grant-in-Aid for Scientific Research on Innovative Areas (25115501) and for from the Ministry of Education, Culture, Sports, Science and Technology (MEXT). Funding was also provided by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID).

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Figure legends

Fig. 1.

Characterization of TIM-1s derived from three different African green monkey

kidney cell lines. (A) 293T cells stably expressing TIM-1s derived from three African green monkey kidney cell lines (Vero E6, COS-1, and BSC-1 cells) were stained with an anti-TIM-1 polyclonal antibody and analyzed by flow cytometry. Open and shaded histograms indicate the fluorescent intensities of the indicated transfectant and vector-transduced control cells, respectively. (B) The deduced amino acid sequences were aligned using GENETYX (version 10). Numbers of residues starting with the respective initiating methionine codons are shown [35]. The signal peptide and transmembrane region are indicated with single and double lines above the Vero E6 TIM-1 sequence, respectively. Black and white arrows indicate the beginning of the IgV domain and mucin domain, respectively. Gaps introduced in the sequences for the alignment are indicated by dashes. Black shading indicates non-identical amino acid residues among TIM-1s.

(C) 293T cells expressing TIM-1s and control cells were infected with VSVΔG-EBOV GP or VSVΔG-G at a MOI of 0.02-0.04. Luciferase activities were measured 24 hours postinfection. The means of three independent experiments are shown. Error bars

represent standard deviations (SDs). Significance was calculated using student's *t*-test (***P* < 0.01).

Fig. 2.

Involvement of the IgV domain and cytoplasmic tail in TIM-1-mediated

enhancement of viral infection. (A) Schematic representation of WT and deletion

mutant TIM-1s. TIM-1 is a type I transmembrane protein consisting of an N-terminal

IgV domain, a highly glycosylated mucin domain, a transmembrane region, and a

cytoplasmic tail. SP: signal peptide. TM: transmembrane region. CT: cytoplasmic tail.

(B) 293T cells expressing Vero E6 WT, ΔIgV, or ΔCT TIM-1s and control cells were

infected with VSVΔG-EBOV GP at a MOI of 0.02-0.04. Luciferase activity was

measured 24 hours postinfection. The means and SDs of three independent experiments

are shown. Significance was calculated using student's *t*-test (***P* < 0.01). (C,D) 293T

cells expressing Vero E6 WT, ΔIgV, and ΔCT TIM-1s were stained with the anti-TIM-1

polyclonal antibody and analyzed by flow cytometry (C) and the TIM-1 expression was

quantified using MFI (D). Open and shaded histograms (C) indicate the fluorescent

intensities of TIM-1-expressing cells and vector-transduced control cells, respectively.

Fig. 3.

A single amino acid in the TIM-1 IgV domain responsible for the increased ability of Vero E6 TIM-1 to promote entry of VSV pseudotyped with filovirus GPs. (A)

Schematic representation of WT and mutant TIM-1s of Vero E6 and COS-1. A Vero E6 mutant TIM-1 containing an asparagine-to-histidine substitution (N48H) and a COS-1 mutant containing a histidine-to-asparagine substitution (H48N) were constructed.

(B,C) 293T cells expressing WT and mutant TIM-1s were stained with the anti-TIM-1 polyclonal antibody and analyzed by flow cytometry (B) and the TIM-1 expression was quantified using MFI (C). Open and shaded histograms (B) indicate fluorescent intensity of TIM-1-expressing cells and vector-transduced control cells, respectively.

(D,E) 293T cells expressing TIM-1s and control cells were infected with VSVΔG-EBOV, -RESTV, -MARV, -RAVV, and -LLOV GPs at a MOI of 0.02-0.04. The luciferase activity was measured 24 hours postinfection. The means and SDs of three independent experiments are shown. Significance was calculated using student's *t*-test (**P* < 0.05, ***P* < 0.01).

A



C

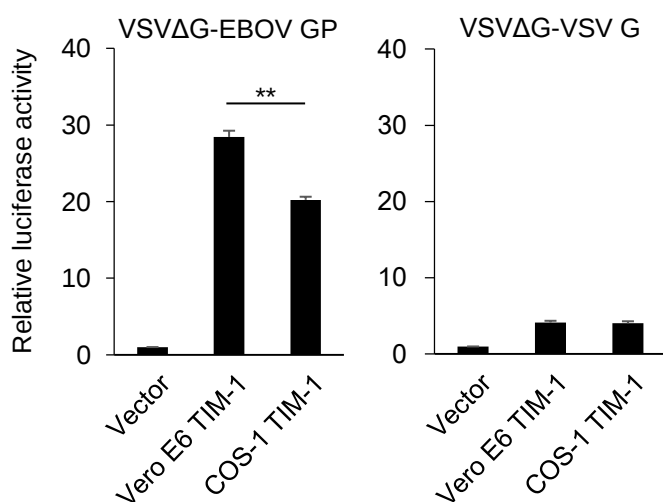
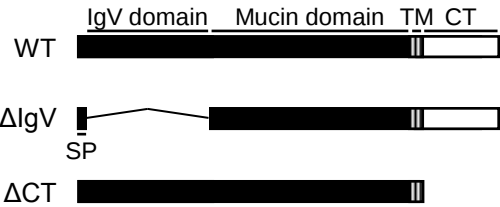
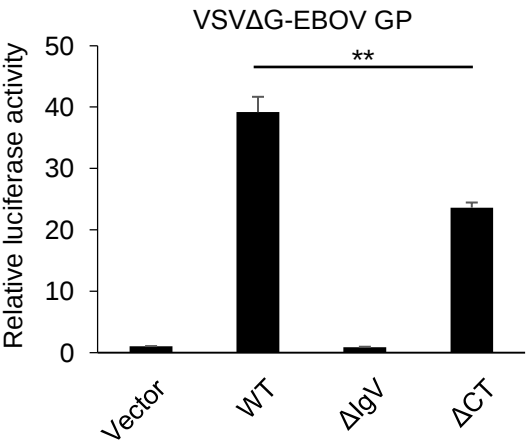


Figure 2

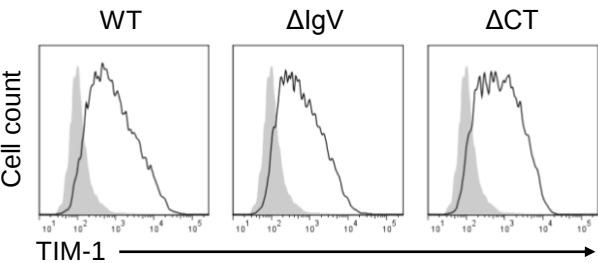
A



B



C



D

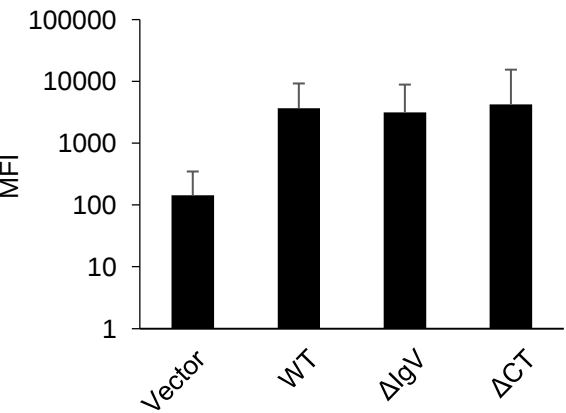
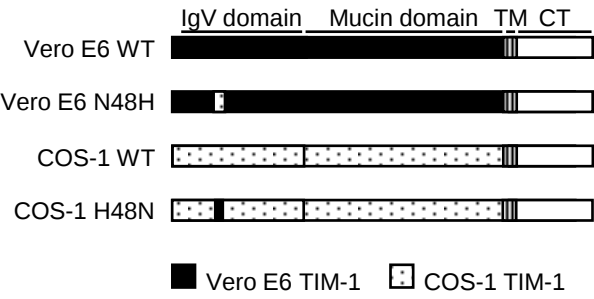
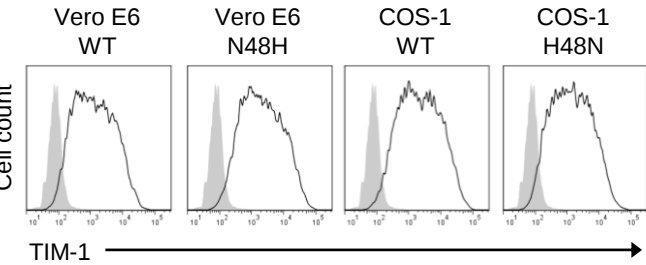


Figure 3

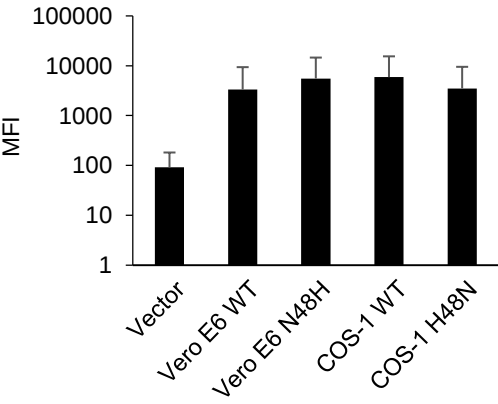
A



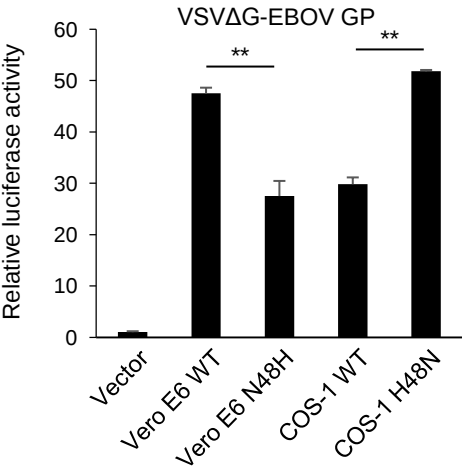
B



C



D



E

