Summer 2014

The role of TIM-1 in enveloped virus entry

Sven Henrik Moller-Tank

*University of Iowa*

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THE ROLE OF TIM-1 IN ENVELOPED VIRUS ENTRY

by

Sven Henrik Moller-Tank

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology in the Graduate College of The University of Iowa

August 2014

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This is to certify that the Ph.D. thesis of

Sven Henrik Moller-Tank

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ACKNOWLEDGMENTS

I would like to thank Dr. Wendy Maury for her incredible guidance and mentorship. She has always made herself available for discussion and I am grateful for her patience during all the times I hassled her with requests or for advice. I have developed as a scientist due to the freedom she gave me to explore new avenues of my research and her willingness to fund those ventures. Most importantly, she has always been approachable, outgoing, and receptive to my humor. I could not have asked for more from a mentor. I would also like to thank my committee members, Dr. Richard Roller, Dr. Stanley Perlman, Dr. Aloysius Klingelhutz, and Dr. Patrick Sinn, for providing valuable input into my projects and their assistance with various questions I have had over the years.

My graduate career would not have been as enjoyable without the lab members I had to work with: Andrew Kondratowicz, Nick Lennemann, Bethany Rhein, and Rachel Brouillette. They have provided great comradery in the lab and have been fantastic sources of information and laughs. My research would not have had a starting point without the work of Andrew Kondratowicz paving the way and would not have been completed without the training and aid provided by everyone in the lab.

My studies benefitted from the assistance of great collaborators. Paul Rennert provided us with TIM-1-specific monoclonal antibodies that were essential for many of my studies. Our studies with infectious Ebola virus would not have been possible without the work done by Robert Davey in the BSL-4 laboratory. In addition, Lorraine Albritton played a significant role in the design of some of our mucin-like domain exchange studies.

Finally, I would like to thank my parents, sister, girlfriend, and other loved-ones for giving me the opportunities to pursue a career that I love. Because of their support, I have the pleasure of working in a lab doing science and, because of them, a reason to take a break once in a while.
ABSTRACT

Ebola viruses, and other members of the family Filoviridae, are enveloped, negative sense, RNA viruses that can cause hemorrhagic fever. Currently, there are no antivirals or approved vaccines available that target or protect from Ebola virus infection. However, recently, T-cell immunoglobulin and mucin domain-1 (TIM-1) has been identified as an epithelial-cell receptor for filoviruses and could be a potential target for antivirals. Little is known about how TIM-1 enhances virus entry and the role of TIM-1 during infection.

In order to determine the key residues of TIM-1 involved in interaction with virus, we generated a panel of point-mutations in the immunoglobulin-like variable (IgV) domain of TIM-1. We determined that several residues within the IgV domain that are involved in binding of phosphatidylserine (PtdSer) are also critical for Ebola virus entry. Further, we found that TIM-1 interacts with Ebola virus through binding of PtdSer on the viral envelope. PtdSer liposomes, but not phosphatidylcholine liposomes, competed with TIM-1 for EBOV pseudovirion binding and transduction. In addition, annexin V (AnxV) was able to substitute for the IgV domain of TIM-1, supporting a PtdSer-Binding dependent mechanism of virus entry. Our findings suggest that TIM-1-dependent uptake of EBOV occurs by apoptotic mimicry. We also determined that TIM-1 expression can enhance infection of a wide range of enveloped viruses, including alphaviruses and a baculovirus. As further evidence of the critical role of enveloped virion associated PtdSer in TIM-1-mediated uptake, TIM-1 enhanced internalization of pseudovirions and virus-like particles (VLPs) lacking a glycoprotein, providing evidence that TIM-1 and PtdSer-binding receptors can mediate virus uptake independent of a glycoprotein. These results provide evidence for a broad role of TIM-1 as a PtdSer-binding receptor that mediates enveloped virus uptake.

The PtdSer-binding activity of the IgV domain is essential for both virus binding and internalization by TIM-1. However, another member of the TIM family, TIM-3, whose IgV domain also binds PtdSer, does not effectively enhance virus entry. These data indicate that
other domains of TIM proteins are functionally important. We investigated the domains of the TIM family members that play a role in the enhancement of enveloped virus entry, thereby defining the features necessary for a functional PtdSer-mediated virus entry enhancing receptor (PVEER). Using a variety of chimeras and deletion mutants, we found that in addition to a functional PtdSer binding domain PVEERs require a stalk domain of sufficient length, containing sequences that promote an extended structure. Neither the cytoplasmic nor the transmembrane domain of TIM-1 is essential for enhancing virus entry, provided the protein is still plasma membrane bound. Based on these defined characteristics, we generated a mimic lacking TIM sequences and composed of annexin V, the mucin-like domain of α-dystroglycan, and a glycophosphatidylinositol anchor that functioned as a PVEER to enhance transduction of virions displaying Ebola, Chikungunya, Ross River, or Sindbis virus glycoproteins. This identification of the key features necessary for PtdSer-mediated enhancement of virus entry provides a basis for more effective recognition of unknown PVEERs.

Provided that expression of TIM-1 in cells enhances virus entry through binding of PtdSer on the viral membrane, we wanted to determine whether virus entry would still be enhanced if this interaction was reversed with TIM-1 present on the viral membrane. Further, we reasoned that this might allow for targeting of virus to cells with greater amounts of PtdSer exposed on their outer leaflet, such as cancer cells. In order to test this hypothesis, we generated virions in cells coexpressing a glycoprotein and one of the TIM family members. We found that expression of TIMs in virus-producing cells resulted in TIM proteins being released into the virus-containing medium and enhanced Ebola virus GP pseudovirion titers. Further, this enhancement was dependent on the amount of PtdSer exposed on the target-cell membrane. However, we also determined that TIMs were not being incorporated into virions and that coexpression of TIMs with non-ebolavirus glycoproteins in virus-producing cells resulted in virus stocks with both reduced titers and the quantity of virions.
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LIST OF ABBREVIATIONS

aa  amino acids
AcMNPV  Autographa californica multicapsid nucleopolyhedrovirus
ADAM  a disintegrin and metalloprotease
AMPK  adenosine monophosphate activating kinase
Ano6 mut  anoctamin 6 D408G mutant
Ano6  anoctamin 6
APC  antigen-presenting cell
BAI1  brain-specific angiogenesis inhibitor 1
BCA  bicinchoninic acid
BDBV  Bundibugyo virus
Bmax  maximum percent infection
BSA  bovine serum albumin
BSL  biosafety level
CDC  Centers for Disease Control
CHIKV  Chikungunya virus
CMV  Cytomegalovirus
CRISPRs  clustered regularly interspaced short palindromic repeats
DC-SIGN  dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMEM  dulbecco modified eagle medium
DMSO  dimethyl sulfoxide
DV  Dengue virus
EBOV  Ebola virus
EDTA  ethylenediaminetetraacetic acid
EGF  epidermal growth factor
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EIPA</td>
<td>5-(N-Ethyl-N-isopropyl) amiloride</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FIV</td>
<td>feline immunodeficiency virus</td>
</tr>
<tr>
<td>Gas6</td>
<td>growth-arrest-specific 6</td>
</tr>
<tr>
<td>GP</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>GP64</td>
<td>glycoprotein of AcMNPV</td>
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<tr>
<td>GPC</td>
<td>glycoprotein precursor</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HAV</td>
<td>Hepatitis A virus</td>
</tr>
<tr>
<td>HAVCR-1</td>
<td>Hepatitis A virus cellular receptor 1</td>
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<tr>
<td>HBSS</td>
<td>Hanks' balanced salt solution</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
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<tr>
<td>hMGL</td>
<td>human macrophage galactose lectin</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpes simplex virus 1</td>
</tr>
<tr>
<td>ID50</td>
<td>infective doses 50%</td>
</tr>
<tr>
<td>IFNAR</td>
<td>interferon alpha receptor</td>
</tr>
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<td>IFN-α/β</td>
<td>alpha/beta interferon</td>
</tr>
<tr>
<td>IgC</td>
<td>immunoglobulin constant</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
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<td>IgV</td>
<td>immunoglobulin variable</td>
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<td>JUNV</td>
<td>Junin virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>KIM-1</td>
<td>kidney injury molecule 1</td>
</tr>
<tr>
<td>LASV</td>
<td>Lassa virus</td>
</tr>
<tr>
<td>LSECTtin</td>
<td>liver/lymph node sinusoidal endothelial cell C-type lectin</td>
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<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MACH</td>
<td>Machupo virus</td>
</tr>
<tr>
<td>MARV</td>
<td>Marburg virus</td>
</tr>
<tr>
<td>MFG-E8</td>
<td>milk fat globule-EGF factor 8 protein</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>mSAP</td>
<td>murine serum amyloid protein</td>
</tr>
<tr>
<td>mTIM-1</td>
<td>murine TIM-1</td>
</tr>
<tr>
<td>MuLV</td>
<td>murine leukemia virus</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cutoff</td>
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<tr>
<td>NGS</td>
<td>normal goat serum</td>
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<tr>
<td>NP</td>
<td>nucleoprotein</td>
</tr>
<tr>
<td>NPC1</td>
<td>Niemann-Pick 1</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotides</td>
</tr>
<tr>
<td>P/S</td>
<td>penicillin/streptomycin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethylenimine</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>PRR</td>
<td>proline rich region</td>
</tr>
<tr>
<td>PSS-1/-2</td>
<td>PtdSer synthetase-1/-2</td>
</tr>
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<td>PtdEth</td>
<td>phosphoethanolamine</td>
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<tr>
<td>PtdSer</td>
<td>phosphatidylserine</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>PTK</td>
<td>protein tyrosine kinase</td>
</tr>
<tr>
<td>PVEER</td>
<td>PtdSer-mediated virus entry enhancing receptors</td>
</tr>
<tr>
<td>RAGE</td>
<td>receptor for advance glycation end products</td>
</tr>
<tr>
<td>RBD</td>
<td>receptor-binding domain</td>
</tr>
<tr>
<td>RGD</td>
<td>Arg-Gly-Asp</td>
</tr>
<tr>
<td>RRV</td>
<td>Ross river virus</td>
</tr>
<tr>
<td>rVSV</td>
<td>recombinant vesicular stomatitis virus</td>
</tr>
<tr>
<td>SARs</td>
<td>severe acute respiratory syndrome</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SINV</td>
<td>Sindbis virus</td>
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<tr>
<td>SOCS</td>
<td>suppressor of cytokine signaling</td>
</tr>
<tr>
<td>STAT1</td>
<td>signal transducers and activators of transcription 1</td>
</tr>
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<td>SUDV</td>
<td>Sudan virus</td>
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<tr>
<td>TAM</td>
<td>Tyro3, Axl, and Mer</td>
</tr>
<tr>
<td>TCID50</td>
<td>tissue culture infectious dose 50%</td>
</tr>
<tr>
<td>Th</td>
<td>helper T-cell</td>
</tr>
<tr>
<td>TIM-1</td>
<td>T-cell immunoglobulin and mucin domain protein 1</td>
</tr>
<tr>
<td>TIMD1</td>
<td>T-cell immunoglobulin and mucin domain 1</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>TSRs</td>
<td>thrombospondin repeats</td>
</tr>
<tr>
<td>VLPs</td>
<td>virus-like particles</td>
</tr>
<tr>
<td>VP</td>
<td>viral protein</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>VSVΔG</td>
<td>VSV in which the glycoprotein, G, gene has been replaced with EGFP</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>Xkr8</td>
<td>Xk-Related Protein 8</td>
</tr>
<tr>
<td>αDG</td>
<td>alpha-dystroglycan</td>
</tr>
<tr>
<td>β-Gal</td>
<td>β-galactosidase</td>
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CHAPTER I
INTRODUCTION

Filoviruses

Overview

The family of Filoviridae is composed of three genera: Ebolavirus, Marburgvirus, and "Cuevavirus". These viruses are enveloped, non-segmented, negative-stranded RNA viruses with genomes of roughly 19 kb in length. The seven genes encoded in the viral genome, beginning from the 3’ end, include: the nucleoprotein (NP), viral protein (VP) 35, VP 40, the glycoprotein (GP), VP30, VP24, and the RNA-dependent RNA polymerase (L). The viral RNA genome is encased in NP, which along with VP35, VP30, and L, form the replication complex (1, 2). VP40 and VP24 are major and minor matrix proteins, respectively, of which VP40 is sufficient for budding of virus-like particles (VLPs) (3).

The genus Ebolavirus contains five species: Ebola (formerly Zaire) (EBOV), Reston, Bundibugyo (BDBV), Sudan (SUDV), and Taï Forest (4). Ebolavirus infection can cause hemorrhagic fever, with mortality rates as high as 90% (5). Most recently in December 2013, an outbreak of EBOV occurred in Guinea that has spread to the neighboring countries of Liberia and Sierra Leone. As of July 6th, 2014, there have been 844 suspected and confirmed cases and 518 deaths (CDC; http://www.cdc.gov/vhf/ebola/outbreaks/guinea/index.html). Due to their propensity for severe pathogenicity and high mortality rates and the absence of any approved vaccines or antivirals, ebolaviruses have been classified as Category A potential bioterrorist agents.

Filovirus tropism

Initial infection with filoviruses occurs through direct transmission from the host reservoir, which is believed to be bats (6). Outbreaks among humans can also be initiated by consumption of infected intermediary animals such as primates (7). Subsequent spread from
human to human can occur through oral, blood, and aerosol routes, with an incubation period of 5 to 9 days after exposure (8). Once inside the body, the virus targets a broad range of cells, infecting monocytes, macrophages, and dendritic cells early during infection, and spreads to a variety of cell types, including epithelial cells, in the visceral organs (9, 10). Transport of infected immune cells and free virus through the blood stream is believed to contribute to virus spread from the site of initial infection (9). In vitro, ebolaviruses have a very broad tropism and infect most cell types, except lymphocytes (11-13). However, in vivo, infection of other cell types results in induction of apoptosis in uninfected lymphocytes (14), leading to profound lymphopenia during infection.

Filovirus entry

Filoviruses encode for a class I envelope GP that is sufficient for entry and fusion. The GP is encoded as a single polyprotein (~160 kDa) and cleaved in the Golgi by furin into two subunits, GP1 (~140 kDa) and GP2 (~26 kDa) (15). These two subunits heterodimerize through intersubunit formation of disulfide bonds and then associate with two other heterodimers to form the trimer that is the mature GP. GP1 contains the receptor-binding domain (RBD) believed to interact with one or more cellular receptors, while GP2 mediates virus/host membrane fusion events (16-19). The crystal structure of the EBOV GP reveals the formation of a chalice-like shape in which the RBD is located within the chalice bowl (20). In addition to the RBD, GP1 contains a heavily N- and O-glycosylated mucin-like domain (MLD) and a N-glycan containing cap. While this MLD is not essential for entry or fusion (21), it is believed to play an important role in vivo in shielding the GP from binding of neutralizing antibodies (22). However, this protection is not perfect as several neutralizing antibodies have been shown to target the MLD itself (23). Nonetheless, deletion of this glycan-rich region enhances titers in vitro (21, 24), suggesting a selective pressure to maintain this domain in vivo.
The complex steps of filovirus entry are not fully understood. Ebolaviruses bind to the cell surface via attachment proteins and are internalized into endosomes through unknown signaling events. Several studies have implicated triggering of macropinocytosis as critical for internalization, but no virus/receptor interactions have been associated with this activity (25-29). Details of filovirus/cell surface receptor interactions are highlighted in the next section. Within endosomes, low pH-dependent cathepsins L and B process the heavily glycosylated GP1 into a 17-19 kDa protein that retains the RBD (30, 31). Exposure of the RBD permits GP1 interaction with the late endosomal/lysosomal protein Neimann-Pick C1 (NPC1) (32-35). This interaction with NPC1 is known to occur before GP2-dependent fusion and to be essential for entry of filoviruses, yet the role of this interaction and effect binding has on virion entry are unknown. Low pH is necessary but not sufficient for this fusion step (30). After fusion, the nucleocapsid is released into the cytoplasm where the virus replicates.

**Cell surface receptors**

At the start of this project, several cell surface receptors had been identified to mediate filovirus uptake into endosomes including: TAM receptors (Tyro 3, Axl, and Mer) (36-38), lectins (LSECTin, DC-SIGN, L-SIGN, mannose-binding lectin, and hMGL) (39-43), and beta 1 integrins (44). While C-type lectins and beta 1 integrins may mediate this process in some cell types, these proteins are not present on all permissive cells. These proteins also interact with virus through binding of glycans on the viral GP and thus are not considered specific receptors. TAM receptors enhance virus entry but do not interact with EBOV GP directly and thus are not themselves considered receptors (36). These results suggest additional entry factors remain to be described.

In order to identify new factors involved in EBOV entry, our group used a comparative genetics analysis approach that was previously successful in identification of adeno-associated virus receptors (45, 46). Vesicular stomatitis virus (VSV) pseudotyped with
either the EBOV GP or that of VSV, G, were used to transduce a panel of human tumor cell lines for which expression of a broad spectrum of known genes was previously characterized. Genes important in EBOV entry were then identified by determining which genes were expressed in the cells most permissive for EBOV GP, but not VSV G, pseudovirions. This correlation led to the identification of T-cell immunoglobulin and mucin-1 (TIM-1; also called HAVCR-1, TIMD1, or KIM-1) as a receptor for filoviruses (47).

The following chapters within this thesis discuss in detail several studies performed to characterize TIM-1-mediated enhancement of EBOV entry. However, after we began our initial work on this topic, it came to light that not only does TIM-1 enhance entry of EBOV, but also entry of several other enveloped viruses. This occurs through interaction with phosphatidylserine (PtdSer) on the viral envelope. During the course of our studies, work from several other groups led to the identification of additional receptors that function through this shared mechanism of PtdSer binding. In order to provide a more complete understanding of these receptors for context, we have included the findings of these other studies below while our studies are described in the subsequent chapters.

**Apoptotic Mimicry**

**Overview of Apoptotic Mimicry**

A variety of both RNA and DNA viruses envelop their capsids in a lipid bilayer. This outer membrane is obtained during virus budding from either plasma or organelle membranes. While reliance on an envelope sensitizes viruses to desiccation, detergents, and heat, these envelopes provide a number of benefits for the virus, including protection of viral structural proteins from immune recognition and neutralizing antibodies by reducing the number of necessary extracellular proteins and epitopes, a platform for displaying viral proteins, a barrier to enclose viral and cellular proteins necessary for early steps during infection, and a mechanism for virus egress without lysing infected cells. Further, unlike the surface-exposed structural proteins of nonenveloped viruses that must maintain intimate
protein-protein interactions with each other, envelope proteins can tolerate extensive glycosylation and variation in their surface exposed regions to escape antibody responses. In addition, a more recently appreciated benefit of viral envelopes is the incorporation of phospholipids. Presentation of phosphatidylserine (PtdSer) on the outer leaflet of these membranes disguises viruses as apoptotic bodies, thereby tricking cells into engulfing virions through cell clearance mechanisms. This mechanism of enhanced virus entry is termed apoptotic mimicry.

Apoptotic mimicry was first hypothesized to be used by hepatitis B virus (48), but was experimentally confirmed with vaccinia virus (49). Inactivation of vaccinia virus by NP-40-mediated lipid depletion was shown to be rescued by incubation with PtdSer liposomes (50). However, the contribution of PtdSer to infection was not understood. Mercer et al. furthered these studies and determined that not only is PtdSer present on the surface of some vaccinia infectious particles, but annexin V (AnxV), a PtdSer-binding protein, can bind to PtdSer on viral envelopes and inhibit vaccinia infection (49). These results were confirmed by another study that found substitution with a non-biologically relevant isomer of PtdSer restored infectivity (51). In addition, a role for viral envelope PtdSer during infection was demonstrated for Pichinde virus and HIV-1 (52, 53).

A protein complex composed of growth-arrest-specific 6 (Gas6) and the tyrosine kinase receptor, Axl, was the first set of cellular proteins to be implicated in PtdSer-binding enhancement of viral entry (54). The soluble protein Gas6 binds to PtdSer on the virion surface and bridges virus to the cell surface via interaction with Axl and formation of this complex is necessary for enhancement of virus entry. This study found in addition to vaccinia virus, the Gas6/Axl complex enhances binding and entry of lentiviruses pseudotyped with Ross River GP, baculovirus GP64, or Sindbis env, demonstrating for the first time that PtdSer binding can enhance entry of viral particles bearing a variety of different viral glycoproteins. More recently, a variety of additional PtdSer-binding virus receptors were identified, including T-cell immunoglobulin and mucin domain 1 and 4 (TIM-1 and 4)
proteins and MFG-E8/integrin αvβ3 or αvβ5 complexes (55-58). We have termed this group of viral receptors PtdSer-mediated virus entry enhancing receptors or PVEERs.

**TIM family members**

Human TIM-1 and TIM-4, along with TIM-3, belong to the TIM family of type I, cell-surface glycoproteins. These proteins and their murine counterparts share a common structure with an amino terminal immunoglobulin variable (IgV)-like domain that extends from the plasma membrane by a heavily O-linked-glycosylated MLD, which is attached to the cell surface by a transmembrane domain followed by a cytoplasmic tail (Figure 1.1) (59). TIM family members bind PtdSer via a pocket in their IgV domains (60-63). The crystal structure of the murine TIM-4 IgV domain reveals that the PtdSer binding pocket is located between two loops (Figure 1.2). Conserved aspartate and asparagine residues within the upper loop are involved in coordination of a cation that in conjunction with residues of the lower loop hydrogen bond with the phosphate and serine groups of PtdSer (61). Mutation of asparate and/or asparagine or chelating free cations with EGTA results in significant loss in PtdSer binding (60-62). Recent studies of TIM-4 have also implicated additional residues outside of the pocket in the binding of PtdSer and suggest TIM-4 is more sensitive to differences in the amount of PtdSer on a membrane compared to TIM-1 and TIM-3 (64). While TIM-1 and TIM-3 both signal through phosphorylation of cytoplasmic tail tyrosines (65-68), TIM-4 does not (69).

The expression patterns differ between the TIM family members (see Table 1.1). TIM-1 and TIM-3 are expressed on distinct populations of activated CD4+ T cells. TIM-1 is expressed on Th2 cells (70) and TIM-3 on Th1 and Th17 cells (71-73). TIM-1 expression has also been detected on mucosal and kidney epithelial cells as well as hepatocytes (47, 74). TIM-3, meanwhile, is expressed on a variety of immune cells such as dendritic cells and macrophages (75-77) and bronchial epithelial cells (62). Unlike TIM-1 and TIM-3, TIM-4 is absent on T cells and primarily expressed on antigen-presenting cells (60, 78-80) and
peritoneal B-1 B cells (79). In addition, expression of TIM-4 may be associated with activation of macrophages and dendritic cells (81).

Given their disparate expression profiles, the TIM family members are believed to have unique functions. TIM-1 is involved in activation and trafficking of T cells (70, 82). In addition, TIM-1 has been shown to be important for natural killer T cell activation after binding to apoptotic bodies (83, 84) and clearance of apoptotic cells in the kidney after injury (85). Depending on the cell type on which it is expressed, TIM-3 functions to promote either pro-inflammatory or anti-inflammatory responses. In naïve cells, expression of TIM-3 on dendritic cells enhances phagocytosis for antigen presentation (86) and promote TNFα expression and inflammation (76, 77). However, after activation, TIM-3 is also expressed on Th1 cells where it binds galectin-9 and stimulates anti-inflammatory signaling (87). TIM-4 is involved in regulating immune tolerance (63, 79), adaptive immunity (88), and naïve T cell proliferation (80) through phagocytosis of apoptotic bodies and T cells. TIM-4 is also necessary to maintain homeostasis of resident peritoneal macrophages (89).

**Protein S and Gas6/Tyro 3, Axl, and Mer**

Tyro3, Axl, and Mer (TAM) are members of the TAM family of receptor tyrosine kinases. These highly related proteins contain two N-terminal immunoglobulin like domains, followed by two fibronectin type III domains, a single transmembrane domain, and a cytoplasmic protein tyrosine kinase (PTK) domain (Figure 1.1). TAM N-terminal interaction with Gas6 leads to activation of the TAM receptors and autophosphorylation of tyrosines within the PTK domain (90). Tyro3 and Mer, but not Axl, are similarly activated by binding Protein S (90, 91). Both Gas6 and Protein S consist of an N-terminal domain rich in γ-carboxyglutamic acid residues (Gla) that binds to PtdSer (Figure 1.2) (92-94), a loop region, four epidermal growth factor-like repeats, and two C-terminal laminin G-like domains forming a sex hormone-binding globulin-like structure that binds to the Ig-like
domains of the TAM receptors (95, 96). Dimerization of TAM receptors occurs after binding of their ligands, resulting in a 2:2 complex, and is necessary for signaling (95).

The TAM receptors are expressed on dendritic cells, macrophages, NK cells, and platelets (97-100). The receptors and their ligands have also been detected in various cells of the reproductive and nervous systems (90, 101-103). While many cell types express two or all three receptors, some cell types express a single TAM receptor (104). The TAM receptors in conjunction with their ligands play a critical role in inhibiting activation of DCs and inflammation (105, 106). This occurs through interaction with IFNAR and activation of STAT1, which subsequently induces expression of suppressor of cytokine signaling (SOCS) (105). TAM receptors also regulate NK cell activation by inducing their expression of activating and inhibitory receptors (98). Another primary function of the TAM receptors is the maintenance of homeostasis through the phagocytosis and clearance of apoptotic cells (101, 107-109).

All TAM receptors in combination with their Gas6 or Protein S ligand have been shown to serve as a PVEER, although studies suggest that the relative effectiveness of the three TAM members as PVEERs varies (54-56, 110). This may be due to differences in binding affinities between the TAM receptors and their ligands (111) or relative expression. As the PtdSer-binding activity of PVEERs is essential for efficacy, in the case of the TAM receptors, this occurs through the Gla domain of Gas6/Protein S and removal of this domain eliminates PVEER function of the TAMs (54, 55). Similarly mutation of the Gas6 binding residues of Axl inhibits virus uptake by the complex (55). Thus, the PVEER efficacy of the TAM ligand/receptor complexes requires both efficient binding of PtdSer by ligand and binding of ligand by receptor.

Signaling through the PTK domain of TAM receptors is essential for enhancement of viral infection (55, 110). While the Gas6/Axl complex is still able to enhance WNV binding and internalization without kinase activity, subsequent infection is significantly impaired compared to infection of cells expressing WT Axl. This kinase activity is important
in vivo for TAM-receptor-inhibition of DC activation and inflammation (105, 106). Viruses appear to utilize this anti-inflammatory signaling pathway to dampen the immune response and promote replication (110). While TAM signaling is triggered by interaction with ligand alone, signaling is significantly enhanced by the presence of virus which helps to facilitate the interaction of TAM ligands with their receptors (110). Thus, the TAM receptors enhance virus infection through PVEER functions by enhancing virus binding and internalization and non-PVEER functions by inhibiting innate immunity; both of which require binding to PtdSer on the virion surface and likely contribute to enhancing in vivo virus loads.

**MFG-E8/ integrin αvβ3 and 5**

Milk fat globule-EGF factor 8 (MFG-E8), the most recently identified PVEER, is a secreted protein that contains two N-terminal EGF repeats followed by a C1 and C2 domain, of which the latter binds to PtdSer (Figure 1.1) (112, 113). Several key positively charged residues within the C2 domain are responsible for interacting with the phosphate and serine groups, while hydrophobic residues of the domain stabilize the positioning of PtdSer (Figure 1.2) (113). Like the Gas6, MFG-E8 bridges PtdSer containing membranes to cells through interaction with a membrane bound protein, either integrin αvβ3 or αvβ5 (112, 114). This occurs through an Arg-Gly-Asp (RGD) motif present in the second EGF repeat of MFG-E8 and mutation of this motif inhibits PVEER efficacy (58).

Several aspects of MFG-E8 biology may contribute to infection. Similar to the Gas6/Axl complex, MFG-E8/integrin complexes have been shown to induce production of anti-inflammatory cytokines such as IL-10 (115, 116), some of which are initiated by activation of STAT-3 (117, 118). Interestingly, STAT-3 has also been identified as an upstream regulator of TIM-1 expression (119). However, currently MFG-E8 induced signaling activity has not yet been associated with enhancement of virus entry.

MFG-E8 or its receptors, αvβ3 and αvβ5, are expressed from a variety of cell types including mammary epithelial cells, stimulated macrophages and endothelial cells, immature
dendritic cells, brain tissue, germinal centers of spleen and lymph nodes, the vasculature system, and mammary glands (114, 120-131). In macrophages, MFG-E8 is involved with recognition of apoptotic bodies for phagocytosis (114). In mammary glands MFG-E8 stabilizes milk fat globules (120) and has been suggested to be involved in the clearance of apoptotic cells during milk gland remodeling (114). MFG-E8 has also been implicated to play an essential role in neovascularization (122) and clearance of B-cells from germinal centers (121). Interestingly, upregulation of MFG-E8 and its receptors is associated with obesity (132) and may contribute to severity of infection (133).

**Uptake of apoptotic bodies**

Assuming PVEER enhancement of virus entry occurs through misrecognition of viruses as apoptotic bodies, it stands to reason that the pathways of PVEER-mediated virus internalization overlap with those involved in apoptotic body uptake. Initially it was believed that apoptotic bodies were internalized through macropinocytosis (134, 135) which involves the ruffling of membrane to form large cups that engulf fluid (136). Macropinocytosis can be stimulated by receptor signaling induced by a variety of ligands including growth factors (137) or PMA (136). While spontaneous ruffling occurs (138), only signaling-induced macropinocytosis leads to complete closure of the macropinosomes (139). Contrary to what was previously thought, studies using SEM revealed that while necrotic cells are taken up through macropinocytosis, apoptotic bodies are phagocytosed (140). Unlike macropinocytosis, which does not specifically engulf cargo, phagocytosis involves direct interaction between ligands and receptors. During phagocytosis, binding of a receptor to its ligand facilitates the binding of adjacent receptors and, because the target to be engulfed is entirely covered in ligand, results in complete enclosure of the target (141). This mechanism is appropriately compared to a zipper (142).
Phosphatidylserine

PtdSer is an anionic phospholipid that makes up 2-10% of the total cellular phospholipid content (143). In healthy cells, PtdSer is present within the inner leaflet of the plasma membrane, faced towards the cytosol (144). This asymmetry is maintained by the activity of flippases and floppases that transfer phospholipids unidirectionally from either the extracellular side to the cytosolic side or the reverse, respectively (145). Meanwhile, scramblases disrupt asymmetry by mediating random bidirectional transfer of phospholipids.

Exposure of PtdSer can be induced by several mechanisms, including apoptosis and elevated levels of intracellular calcium (145). When cells undergo apoptosis, Xk-Related Protein 8 (Xkr8) is activated by caspase cleavage and is hypothesized to mediate the flipping of PtdSer to the outer leaflet, although studies have yet to show this directly (146). The exposure of PtdSer on apoptotic cells then acts as a marker for uptake by phagocytic cells (147, 148). The density of PtdSer on the surface must also be sufficiently high enough (149). The presence of PtdSer alone on the outer leaflet, however, is not sufficient for recognition and phagocytosis by macrophages (150). Although, the required factors have yet to be identified, but could include exposure of calreticulin or modification of anti-phagocytosis receptors such as CD31 (151). For non-apoptotic cells, exposure of PtdSer after elevation of intracellular calcium levels can be important for biological functions such as platelet coagulation (152). This is mediated by the calcium-sensitive scramblase, anoctamin 6 (Ano6; also known as TMEM16F) (153).

PtdSer is synthesized in mammalian cells by serine-exchange reactions using either phosphatidylcholine (PtdChl) or phosphoethanolamine (PtdEtn) as initial substrates. These reactions are catalyzed by PtdSer synthetase-1 (PSS-1) and PSS-2 respectively (154, 155) and occurs at mitochondria-associated membranes, where these two enzymes are localized (156, 157). The production of PtdSer is negatively regulated by direct interaction between the enzymes and their substrate, PtdSer (158, 159).
Annexin V

Annexin V (AnxV) is a cellular protein belonging to the annexin family of calcium-dependent membrane binding proteins. Like MFG-E8 and Gas6, AnxV is a soluble PtdSer binding protein and, unlike them, is not considered to be a PVEER. A study did suggest a potential role for AnxV in influenza A virus entry (160), but these data have not been confirmed or shown to be true for any other viruses. However, AnxV does play an important role in vivo in coagulation as AnxV is expressed in cells of the vasculature and skeletal systems (161), where, during coagulation, it links platelets to the cytoskeleton (162). This likely occurs through interaction with PtdSer present on the platelet envelope. More recently, AnxV has also been proposed to be involved in cell membrane repair (163).

The annexin family members share a variable N-terminal domain linked to a conserved structure composed of four homologous domains that are arranged around a hydrophilic hole (164). The individual domains contain four α-helices, A-D, that are bundled together and capped by a fifth, E (Figure 1.2) (165). Each domain of AnxV can coordinate up to three Ca\(^{2+}\) ions (166). These calcium ions are critical for the three PtdSer binding sites of AnxV. The first site, termed that "apical" binding site, was identified with the crystal structure of rat AnxV with glycerophosphoserine (167). Two Ca\(^{2+}\) ions positioned by several Glu and Asp residues present within the A and B helices are critical for this interaction. Several residues from AnxV also directly interact with PtdSer at this site, including a Thr that hydrogen bonds to the serine head group of PtdSer. A second binding site, termed the "equatorial" binding site was determined by molecular modeling (168). This site involves a single Ca\(^{2+}\) ion shared with the "apical" site and several negatively charged residues present within the D helix. Finally, a third site is likely present near the third Ca\(^{2+}\) binding site between the D and E helices as determined by molecular dynamics simulation (169). Interestingly, while all four domains of AnxV share these binding sites, domain 1 is most critical for interaction with PtdSer (170, 171).
Oncolytic Viruses

Overview of Oncolytic Viruses

Cancer cells often have disrupted metabolism and cell cycle regulation that permits continuous and unchecked growth (172). Frequently the changes required for transformation disrupt the innate immune response and production or response to cytokines (173, 174). These cellular conditions are ideal for some viruses to grow, allowing for aberrant virus replication without the checks and balances imposed by a healthy cell. Thus, tumor cells are more sensitive to viral replication. Ultimately these viruses lead to cell death and lysis and so they are termed oncolytic viruses (175).

The potential of using these viruses for therapeutic use has been appreciated since the turn of the 20th century when it was observed that some cancer patients went into remission after infection with influenza (176). Since then, interest in the field of oncolytic viruses has fluctuated, with a significant crash in the 1970s and 80s that has been attributed to the regulatory barriers associated with using non-attenuated viruses (177). In the past few decades however there has been resurgence in the field due to advances in genome engineering that permits direct manipulation of viruses to obtain ideal traits while previous methods relied on directed evolution. Thus, with these new technologies, oncolytic viruses can be developed to maintain or enhance their oncolytic potential while attenuating their pathogenicity and side effects.

Delivery

There are several methods for delivery of oncolytic viruses to patients. The virus can be injected into the blood stream, allowing for transport throughout the body or directly injected into the tumor itself. An alternative delivery method that has been developed is using patient cells infected with virus (178). The motivation behind this method being that by using cells that traffic to the site of metastatic tumors, such as immune cells, virus can be delivered to the appropriate sites. For example, infected mesenchymal stem cells have been
used to successfully transport oncolytic measles virus to tumors in mice (179). This “Trojan horse” delivery mechanism was more effective than injection of virus alone. In addition to providing transport, as the virus is contained intracellularly, this Trojan horse delivery provides the added benefit of protection viral epitopes from neutralizing antibodies (180).

**Mechanism of Killing**

Oncolytic viruses function to kill cells through several mechanisms either innate or designed (181). First, these viruses can directly kill cells through replication and subsequent induction of apoptosis, necrosis, pyroptosis, and autophagic cell death. Second, infection triggers production of signaling molecules in uninfected, inflammatory cells that results in disruption of tumor vasculature and subsequent hypoxia and necrosis (182-184). Third, these viruses can be used as gene therapy vectors for delivery of cytotoxic or cancer killing genes such as toxins (i.e. diphtheria toxin or pseudomonas exotoxin A) or cellular genes such as caspases (185). Alternatively, genes encoding signaling molecules like granulocyte-macrophage colony-stimulating factor can be delivered to enhance the immune response and consequently the final mechanism of oncolytic virus efficacy and perhaps most important, antitumor immunity (181, 186-188). Infection by oncolytic viruses can tip the balance of immune suppression that occurs within cancer cells and result in induction of an anticancer immune response (181, 189). Once developed, this response can then target cancer cells throughout the body without the prerequisite oncolytic virus infection.

**Types of oncolytic viruses**

Cancer cells originate from various different tissues and consequently there exists great variety amongst tumors. This demands that oncolytic viruses have diverse tropisms, something that is naturally present in some groups of viruses. Currently, there are viruses from nine different families that are in clinical trials (190). Some of these viruses have an innate preference for replication in cancer cells (reoviruses and autonomously replicating parvoviruses) (189). However, many of these viruses infect a broad array of healthy tissues
(measles virus, poliovirus, vaccinia virus, adenovirus, herpes simplex virus, vesicular stomatitis virus). Consequently, modifications of these viruses have been introduced to promote preferential or restricted replication in tumor cells. Frequently these modifications involve deletion of viral genes required for growth in healthy cells but unnecessary in cancer cells. For example, deletion of immune suppressive viral genes leads to infection triggering innate immune responses, requiring the immune suppressed environment of cancer cells for virus replication (191, 192).

Limitations of oncolytic viruses

One limitation to the use of oncolytic viruses for cancer therapy is the presence of pre-existing immunity and antibodies to many of the therapeutic viruses being developed (190). For enveloped viruses this can be avoided readily through use of surface proteins from different serotypes and generation of chimeric viruses (190). This becomes more difficult for viruses with only one serotype, such as measles virus, in which case glycoproteins from other members of the same family can substitute (193). This is provided, however, that these substitutions are functional. Designing non-enveloped viruses that evade an antibody response provides additional challenges as there are multiple different proteins with exposed epitopes that must be exchanged or modified (194). This can be addressed with chemical modifications to provide protection of epitopes (195-197). However, these modifications do have some drawbacks as they can only be done on input virus and can reduce viral titers. Thus, while pre-existing immunity limits efficacy of some viruses, adjustment of viral proteins through genome engineering or chemical modification.

Using viruses, there is always a concern for uncontrolled replication and pathogenesis. Continued advancement in the targeting of oncolytic viruses to tumor cells rather than healthy cells will reduce off-target effects and enhance efficacy. Tropism of viruses for cancer cells can be modulated or narrowed through modification of attachment and/or fusion proteins. One method to achieve this is passage of virus or screening virus
libraries in the presence of selective pressures to obtain ideal tropism (198, 199). More

designed approaches involve the introduction of new binding domains such as designed
ankyrin repeat proteins (200, 201) or antibodies, either directly (202) or indirectly through
introduction of an antibody binding sequence (203). Alternatively, introduction of ligands
such as IL-13 or epidermal growth factor allows for targeting to specific receptors (204, 205).

The tissue specificity of viruses can also be regulated through gene expression. This
can occur through introduction of tissue specific promoters (185, 206). This regulation,
however, cannot be used by RNA viruses that do not transcribe from DNA. Alternatively,
microRNA target sites for tissue specific microRNAs can be inserted into the untranslated
regions of viral transcripts for both DNA and RNA viruses (207-210). However, it is
difficult to identify tissue-specific promoters or microRNA target sites for cancer cells as
these cells share many of these promoters or target sites with the healthy cells from which
they originate. Nonetheless, several tumor-associated promoters and microRNA target sites
have been identified (190) and these molecular control elements can be combined to further
specify viral replication in cancer cells.

**PtdSer as a marker of tumors**

The presence of PtdSer on the outer leaflet has been implicated as a marker for
tumors. Anti-PtdSer antibodies and AnxV have been used intravenously to successfully label
a variety of tumor models in mice and rats (211-213). In these model tumors, the exposure
of PtdSer occurs within the tumor blood vessels, but not in healthy tissues, and is attributed
to exposure of these cells to hypoxia/reoxygenation, inflammatory cytokines, thrombin, and
acidity (211). In addition, studies have shown that some tumor cell lines have endogenously
elevated levels of exposed PtdSer without environmental stresses or signals (214, 215).
Interestingly, this exposure of PtdSer by tumors may enhance the binding of PtdSer binding
receptors such as CD300a and inhibit their killing by natural killer cells, thereby promoting
tumor growth (214).
As a potential marker for tumors, PtdSer-binding has been used as a mechanism for targeting therapeutics. PtdSer binding antibodies alone are sufficient to inhibit tumor growth as they enhance recruitment of monocytes and macrophage infiltration (216). Anti-PtdSer antibodies have also been shown to enhance the efficacy of other treatments as evidenced with the chemotherapeutic docetaxel or radiation therapy (217, 218). Use of these antibodies is currently in phase I and II clinical trials (219). However, using antibodies has several drawbacks as they are readily cleared from the blood (213, 220) and could cause anti-phospholipid antibody syndrome (219).

Given that there are several cellular proteins with PtdSer-binding domains, there have been efforts to use these domains as alternative to antibodies. There has been some success killing cancer cells *in vitro* by fusing AnxV to cytotoxic enzymes (221-223). Interestingly, there is also evidence that several cytotoxic peptides selected to specifically kill cancer cells do so by targeting PtdSer (224, 225). However, these treatments, like PtdSer binding antibodies, have significant downsides. For example, the accumulation of AnxV in the heart can lead to detrimental heart problems (226). Nonetheless, these studies suggest PtdSer is a viable target for specific delivery of chemotherapeutics to cancer cells.

**Rationale and objectives for current study**

TIM-1 was identified as a receptor for entry of filoviruses. Previous studies revealed that TIM-1 bound to EBOV pseudovirions, however little was known about the specificities of this interaction. We initially hypothesized this interaction was occurring due to binding between the IgV domain of TIM-1 and the EBOV GP. However, the key residues of TIM-1 and the EBOV GP that are important for this interaction were unknown. In addition, nothing was known about how this interaction triggers internalization of virus into cells and the domains of TIM-1 that might be responsible for that activity. The following studies, Chapters 2 and 3, were designed to address this lack of knowledge.
After discovering the ability of TIMs to enhance virus entry through the binding of PtdSer, we wanted to explore the potential use of TIMs to target virus to tumors. We hypothesized that TIMs could be incorporated into virus envelopes, thereby enhancing their binding to cancer cells with exposed PtdSer. To our knowledge, this had not been attempted before our studies and consequently we wanted to explore the viability of this approach to enhance the specificity of enveloped oncolytic viruses. Chapter 4 summarizes the progress of our studies into this venture.

List of Specific Aims

1. Determine the residues of TIM-1 that are critical for interaction with EBOV.
2. Determine mechanism of TIM-1/EBOV interaction.
3. Determine the contribution of the TIM-1 domains to enhancement of virus entry.
4. Characterize the binding sites of various TIM-1 specific monoclonal antibodies.
5. Determine the impact of TIM-1 when expressed in virus-producing cell lines.

Knowledge gained by current study

1. The key residues of the TIM-1 IgV domain that interact with EBOV are located within and around the PtdSer binding pocket.
   a. The most critical residues include Asn 114 and Asp 115.
   b. Mutation of these residues inhibits the ability of TIM-1 to enhance entry of EBOV GP pseudovirions or recombinant infectious VSV expressing EBOV GP.
   c. These residues are also important for entry of Marburg virus.
2. PtdSer liposomes inhibit entry of EBOV into TIM-1 expressing cells.
   a. Greater than 90% inhibition of entry occurs at 25 µM of PtdSer liposomes.
   b. Liposomes do not need to be added prior to addition of virus for effective inhibition.
3. Both murine TIM-1 and human TIM-4 also enhance EBOV entry.
4. EBOV pseudovirions contain PtdSer within the viral envelope.

5. TIM-1 binds to PtdSer on the viral envelope and PtdSer liposomes.
   a. Mutation of residues within the PtdSer binding pocket inhibits the ability of TIM-1 to bind pseudovirions.
   b. TIM-4 also effectively binds to pseudovirions.
   c. The presence of mAbs ARD5 or A6G2, EGTA, or PtdSer liposomes inhibits TIM-1 binding to pseudovirions.

6. Expression of the PtdSer-binding protein, RAGE, does not enhance EBOV entry.

7. TIM-1 enhances entry of VSV virions pseudotyped with Ross River GP, Baculovirus GP64, Chikungunya virus env, or Sindbis virus env and mutation of the PtdSer binding pocket or the presence of inhibitors (mAbs ARD5, A6G2, or A8E5, or PtdSer liposomes) inhibits this enhancement.

8. TIM-1 enhancement of entry is not limited to VSV pseudovirions as entry of FIV pseudovirions, WT RRV, and recombinant Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) are also enhanced by TIM-1 expression.

9. AnxV can substitute for the IgV domain of TIM-1 to enhance virus entry.

10. Binding and internalization of VSV pseudovirions occurs independently of the presence of a viral glycoprotein.
    a. Binding and internalization are enhanced by the expression of TIM-1.
    b. Internalization of these naked pseudovirions is as efficiently inhibited by PtdSer liposomes as EBOV GP pseudotyped VSV.

11. Virus harvested from infected mice effectively enters cells in a TIM-1 dependent manner.

12. TIM-3 does not effectively enhance EBOV entry.

13. The TIM-3 IgV domain can functionally substitute for that of TIM-1.

14. Cytoplasmic and transmembrane domains of TIM family members are not necessary for enhancement of virus entry.
15. Deletion of the cytoplasmic tails from murine and human TIM-1 reduces their expression.

16. Deletions within the TIM-1 MLD reduce enhancement of EBOV entry.
   a. Efficacy correlates with length of the MLD and plateaus at roughly 120 amino acids.
   b. Defect in entry associated with reduced MLD length is due to defect in ability to bind virus.
   c. Deletions within MLD of soluble TIM-1 do not affect ability to bind to PtdSer.

17. TIM-1 MLD mutants with longer lengths do not synergize with shorter length MLD mutants.

18. MLD of TIM-1 can be entirely substituted by that of α-dystroglycan without loss of PVEER function.
   a. Addition of α-dystroglycan MLD to that of TIM-1 does not further enhance PVEER efficacy beyond that of WT TIM-1.

19. MLD of TIM-1 cannot be effectively substituted by structured sequences such as the IgC2 domains of RAGE
   a. Insertion of TIM-1 MLD downstream of structured sequences rescues PVEER function.

20. A TIM-1 mimic generated by replacement of TIM-1 domains with functional equivalents acts as an artificial PVEER.

21. Expression of human TIM-1, TIM-3, and TIM-4, their GPI-anchored equivalents, and murine TIM-1 in virus-producing cells results in release of TIMs into medium.

22. Coexpression of TIMs in virus-producing cells with EBOV GP enhances titer of pseudovirions.
   a. Extent of titer enhancement is dependent on extent of PtdSer exposure on target cells.
b. Optimal enhancement is obtained after transfecting GP and TIM expression plasmids at a mass-ratio of 1 to 2, respectively.

c. Expression of TIM-4 and TIM-4 GPI in virus-producing cells most effectively enhanced virus titers.

d. Expression of TIM-4 GPI also enhances virus titers of SUDV, BDBV, and full-length EBOV GP pseudovirions.

23. EBOV GP pseudovirions produced in cells expressing both TIM-4 GPI and EBOV GP enter several cancer cell lines more effectively than those produced in cells expressing EBOV GP alone.

   a. Entry was most enhanced into SF-539 and SK-MEL-5 cells and minorly enhanced into Hop62, MCF-7, and HT-1080 cells.

   b. Entry was slightly reduced into Hec50, Hec1A, and A549 cells, which is attributed to their expression of TIMs.

24. TIM-4 GPI does not directly incorporate into pseudovirions.

   a. Purification of viruses results in a loss of TIM-4 GPI from virus stocks and loss of enhanced entry.

   b. Concentration of virus stocks using a MWCO filter allows for retention of TIM-4 GPI and enhanced entry.

25. Expression of TIM-4 GPI and TIM-1 GPI in virus-producing cells inhibits release and reduces titers of Ross River, Lassa, Chikunguyna, Machupo, Junín, and AcMNPV GP pseudovirions.

   a. The presence of ARD5 does not rescue the reduced titers due to expression of TIM-1 GPI in virus-producing cells.

   b. Titers of cell-associated virus are also reduced.

   c. Incorporation of JUNV and EBOV GPs are reduced when virus-producing cells coexpress TIM-4 GPI.
PtdSer-binding receptors that function as PVEERs. Cartoon representations of Gas6/Axl (representatives of TAM ligand and receptor kinases), TIM-1, TIM-4, and MFG-E8/αvβ3 integrin are displayed. Estimations of N-linked glycan sites are represented as blue tridents and O-link glycoslyations (TIM-1 and TIM-4 only) are shown as green lines. Domains for which calcium-binding is necessary for interaction with PtdSer are indicated with calcium ions (orange spheres). Gas6 and Axl interact through their respective laminin G-like and IgC2 domains. MFG-E8 contains an Arg-Gly-Asp (RGD) motif in the second EGF domain that likely binds at the interface of the αvβ3/5 integrin complex (227).
Figure 1.2 Structures of PtdSer-binding domains. Representative renders of the PtdSer-binding domains of TIM-1/-3/-4, CD300a, RAGE, BAI-1, Gas6/Protein S, Annexin V, MFG-E8, and Stabilin-1/2 are shown. PtdSer-like ligands (red) and calcium ions (orange) are also displayed for structures with which they were solved. For domains that do not have solved structures, equivalent domains from other proteins are shown. The IgV domain of TIM-1/-3/-4 is represented by the mTIM-4 IgV domain (2OR8) (61). The human CD300a IgV domain crystal structure (228) is shown with residues hypothesized for interaction (229). The region of the RAGE IgV domain (3O3U) that corresponds to the binding pockets of CD300a and TIM-1 IgV domains is shown for comparison (230); however, this domain is only hypothesized to interact with PtdSer and no residues have been implicated (231). The thrombospondin type-1 repeats (TSRs) of BAI1 are represented by TSR domain 3 of human thrombospondin-1 (3R6B) (232). Gas6 binds PtdSer via a Gla domain, represented by that of bovine prothrombin (1NL2) with residues identified that interact with PtdSer (red) (92). For AnxV, the structure of one annexin repeat domain from rat Anx V is shown (1A8A) with residues identified that interact with PtdSer (red) (167). The C2 domain of bovine MFG-E8 binds to PtdSer (3BN6) (233) and while the structure was not crystallized with PtdSer, key residues involved in interaction have been determined experimentally (113). An EGF-like domain of stabilin-1 or -2 is represented by that from human heregulin alpha (1HRE) (234).
## Table 1.1  PtdSer receptors: distribution and efficacy as a viral receptor

<table>
<thead>
<tr>
<th>Protein Name (s)</th>
<th>PtdSer Binding Domain</th>
<th>Tissue/Cell Type Expression</th>
<th>Pseudoviruses for which entry is enhanced (&gt;2 fold)</th>
<th>Pseudoviruses for which entry is unaffected (&lt;2 fold)</th>
</tr>
</thead>
</table>
| TIM-1 IgV(60)    |                       | **Immune cells:** B cells (235), mast cells (76), T<sub>1</sub>2 CD<sub>4</sub><sup>+</sup> T cells (70, 75, 78), and NKT cells (83, 84)  
**Epithelial cells:** Kidney (47, 74) and airway and eye mucosa (47) | **Filovirus:** Ebola<sup>a</sup> and Marburg (47, 56, 57)  
**Alphavirus:** Ross River<sup>a</sup>, Chikungunya<sup>a</sup>, Sindbis<sup>a</sup>, Eastern Equine Encephalitis (56-58)  
**Baculovirus:** Autographa californica multicapsid nucleopolyhedrovirus<sup>a</sup> (57)  
**Rhabdovirus:** Vesicular stomatitis virus (58)  
**New World Arenavirus:** Amapari, Tacaribe<sup>a</sup>, Junín, and Machupo virus (56)  
**Flavivirus:** West Nile Virus<sup>a</sup>, Dengue<sup>a</sup>, Yellow Fever virus<sup>a</sup> (55, 56) | **Old World Arenavirus:** Lassa Virus, LCMV (56, 57)  
**New World Arenavirus:** Oliveros (56)  
**Influenza A virus:** H7N1, H1N1<sup>a</sup> (56)  
**Coronavirus:** SARS (56)  
**Rhabdovirus:** Vesicular stomatitis virus (56)  
**Herpes simplex virus:** HSV-1<sup>a</sup> (55) |
| TIM-3 IgV(62)    |                       | **Immune cells:** T<sub>1</sub>1 and T<sub>1</sub>7 CD<sub>4</sub><sup>+</sup> T cells (71-73, 75), mast cells (76), DCs (62, 77), and monocytes (77)  
**Epithelial cells:** Bronchial (62) | **Filovirus:** West Nile Virus, Dengue Virus<sup>a</sup> (55, 56)  
**New World Arenavirus:** Tacaribe (56) | **Alphavirus:** Sindbis (58)  
**Filovirus:** Ebola, Marburg (56)  
**Old World Arenavirus:** LASV, LCMV (56)  
**New World Arenavirus:** Tacaribe, Junín, Machupo, Oliveros (56)  
**Influenza A virus:** H7N1 (56)  
**Rhabdovirus:** Vesicular stomatitis virus (56) |
| TIM-4 IgV(60, 61, 63) |                       | **Immune cells:** Macrophages and mature DCs (60, 62, 63, 78, 80), and B-1 cells (79)  
**Tissues:** Spleen, lymph node, and peritoneum (63, 78) | **Filovirus:** Ebola, Marburg (56, 57)  
**Alphavirus:** Sindbis, Ross River (58), Eastern Equine Encephalitis (56)  
**New World Arenavirus:** Tacaribe, Junín, Machupo (56)  
**Baculovirus:** Autographa californica multicapsid nucleopolyhedrovirus (58)  
**Rhabdovirus:** Vesicular stomatitis virus (56)  
**Flavivirus:** West Nile Virus<sup>a</sup>, Dengue<sup>a</sup>, Yellow Fever virus<sup>a</sup> (55, 56) | **New World Arenavirus:** Oliveros (56)  
**Old World Arenavirus:** LASV, LCMV (56)  
**Influenza A virus:** H7N1 (56)  
**Herpes simplex virus:** HSV-1<sup>a</sup> (55) |
Table 1.1 Continued

<table>
<thead>
<tr>
<th>TAM ligand (Protein S or Gas6) / TAM receptor kinases (Tyro3, Mer, or Axl)</th>
<th>Gla (92, 94) domain of TAM ligand</th>
<th><strong>Blood cells:</strong> Platelets (100)</th>
<th>Filovirus: Ebola, Marburg (38, 56)</th>
<th><strong>Old World Arenavirus:</strong> LASV, LCMV (56)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Immune cells: Macrophages, NK cells, NKT cells, and DCs (97-99)</td>
<td>Rhabdovirus: Vesicular stomatitis virus (58)</td>
<td><strong>New World Arenavirus:</strong> Machupo (56), Oliveros (56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Connective tissue: Bone marrow stromal cells (98)</td>
<td>Baculovirus: Autographa californica multicapsid nucleopolyhedrovirus (54, 58)</td>
<td>Rhabdovirus: Vesicular stomatitis virus (56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissues: Testes (101, 102), CNS (90, 103), retina (91), and foreskin fibroblasts (36)</td>
<td>Alphavirus: Sindbis, Ross River (54, 58)</td>
<td>Influenza A virus: H7N1 (56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pox virus: Vaccinia Virus&lt;sup&gt;a&lt;/sup&gt; (54)</td>
<td>Herpes simplex virus: HSV-1&lt;sup&gt;a&lt;/sup&gt; (55)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>New World Arenavirus: Amapari, Tacaribe, Junín (56)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Alphavirus: Chikungunya, Eastern Equine Encephalitis (56)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Flavivirus: Dengue&lt;sup&gt;a&lt;/sup&gt; (55)</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>MFG-E8/αvβ3-5 integrin</th>
<th>C2 domain (112, 113)</th>
<th><strong>Immune cells:</strong> Macrophages (114, 121, 126, 127), Immature DCs (123, 128)</th>
<th>Alphavirus: Ross River and Sindbis (58)</th>
<th>Rhabdovirus: Vesicular stomatitis virus (58)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tissues: Mammary glands (124, 125), spleen, lymph node, brain (121, 129, 130), and vascular system (122, 131)</td>
<td>Baculovirus: Autographa californica multicapsid nucleopolyhedrovirus (58)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Confirmed using infectious virus
CHAPTER II
ROLE OF THE PHOSPHATIDYL SERINE RECEPTOR TIM-1 IN ENVELOPED VIRUS ENTRY

Introduction

Ebolavirus, along with Marburgvirus, is a member of the Filoviridae family of enveloped, nonsegmented, negative-stranded RNA viruses. Infection can cause hemorrhagic fever, with mortality rates as high as 90% (5). The virus targets a broad range of cells, infecting monocytes, macrophages, and dendritic cells early during infection and spreads to a variety of cell types, including epithelial cells, in the visceral organs (9, 10). Ebolaviruses encode a viral class I glycoprotein (GP) that in the mature form is composed of a trimer of disulfide-bonded GP1/GP2 heterodimers. GP1 contains the receptor-binding domain (RBD) believed to interact with one or more cellular receptors, while GP2 mediates virus/host membrane fusion events (16-19).

The complex steps of filovirus entry are currently being elucidated. Ebolaviruses bind to the cell surface and are internalized into endosomes where low-pH-dependent cathepsins L and B process the heavily glycosylated GP1 into a 17- to 19- kDa protein that retains the RBD (30, 31). Processed GP1 interacts with the late endosomal/lysosomal protein Neimann-Pick C1 (32-35). Subsequent poorly defined events lead to GP2-dependent fusion of endosomal and viral membranes, resulting in release of the viral core into the cytoplasm.

We and others sought to identify cell surface receptors that mediate filovirus uptake into endosomes (38-42). C-type lectins may mediate this process in some cell types; however, these proteins are not present on all permissive cells, indicating additional filovirus surface receptors also exist. Recently, we demonstrated that T-cell immunoglobulin and mucin-1 (TIM-1) (also called HAVCR-1, TIMD1, or KIM-1), expressed on many epithelial cells, mast cells, B cells, and activated CD4+ T cells (47, 70, 74, 236), serves as a receptor for filoviruses (47).
The TIM family members (TIM-1, TIM-3, and TIM-4 in humans) share a type I, cell-surface glycoprotein structure. The ectodomains of these proteins contain an amino terminal immunoglobulin variable (IgV)-like domain that is extended from the plasma membrane by a heavily O-linked-glycosylated mucin-like domain (MLD) (59). TIM proteins also contain a transmembrane spanning domain followed by a short cytoplasmic tail. The CC’ and FG loops of the TIM IgV domains form a pocket that binds phosphatidylserine (PtdSer) (Figure 2.1) (60-63). PtdSer on the surface of apoptotic cells interacts with TIM-4 on macrophages, TIM-3 on several types of innate immune cells, and TIM-1 on kidney tubule cells, leading to engulfment of apoptotic bodies (60, 85, 237). In addition to serving as a receptor for filoviruses, human TIM-1 is reported to serve as a receptor for hepatitis A virus (HAV) (238, 239) and, most recently, for a variety of flaviviruses, alphaviruses, and arenaviruses (55, 56).

Here, we define the molecular interactions between TIM-1 and Ebola virus (EBOV), formally Zaire ebolavirus. Residues located in the PtdSer binding pocket of the TIM-1 IgV domain are critical for infection. Further, contrary to our initial findings that TIM-1 and EBOV GP directly interact (47), we now show that PtdSer on the virus surface directly interacts with TIM-1, resulting in virion internalization. Additionally, TIM-1 enhances infection of members of both the alphavirus and baculovirus families, indicating the breadth of the importance of PtdSer binding receptors for enveloped virus entry.

**Materials and Methods**

**Cell lines**

HEK 293T cells, a human embryonic kidney cell line, H3 cells, 293T cells, and Vero cells, an African green monkey kidney epithelial cell line, were maintained in Dulbecco’s modified Eagle medium (DMEM) (Gibco BRL) with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). H3 cells are a clonal population of HEK 293T cells that stably express TIM-1 due to integration of a transfected TIM-1 expression plasmid (47).
Production and titer determination of pseudovirions and infectious viruses

Feline immunodeficiency virus (FIV) and vesicular stomatitis virus (VSV) pseudovirions were produced as previously described (11, 16, 47, 240). To produce VSVΔG-EGFP pseudovirions, VSV (strain Indiana) virions with genomes in which the G glycoprotein gene is replaced with enhanced green fluorescent protein (EGFP), HEK 293T cells were transfected with plasmids expressing either EBOV GP lacking the mucin domain of GP1, full-length EBOV GP (EBOV FL), Marburg Virus (MARV) GP, Sindbis virus (SINV) 2.2 1L1L env, Ross River virus (RRV) GP, GP64, Chikungunya virus (CHIKV) env (OPY1), or Lassa virus (LASV) precursor glycoprotein (GPC) and transduced 24 h later with VSVΔG-EGFP pseudovirions. After 4 h of virus uptake, the plates were washed and repleted with medium. Pseudotyped virions were collected in supernatant 48 and 72 h following transduction, pooled, and filtered through 0.45 µm filter. MARV and EBOV FL pseudotyped virions were concentrated by centrifuging supernatants at 5,380 x g overnight at 4°C and resuspending pellet in fresh medium to achieve higher-titer stocks. Virus aliquots were stored at -80°C.

Fluorescein isothiocyanate (FITC) labeled "No GP" and EBOV GP pseudovirions were generated as described above, with No GP pseudovirions generated by adding starter virus to cells not expressing a glycoprotein. Pseudovirions were concentrated by centrifuging supernatants overnight at 5,380 x g at 4°C. The pellet was resuspended in 500 mM carbonate buffer, pH 9.5 (-100-fold concentration of virus). Ten milligrams of FITC "Isomer 1" (Invitrogen) was resuspended in 1mL dimethyl sulfoxide (DMSO). One microliter of FITC solution was added per 100µL of reaction buffer. Pseudovirions were incubated at 4°C for 1 h in the dark. The reaction solution was dialyzed using 10,000-molecular-weight-cutoff (MWCO) Slide-A-Lyzer dialysis cassettes (Thermo Scientific) into 1x phosphate-buffered saline (PBS), 3 times for 2 h each at 4°C, and protected from light. Pseudovirions were purified through a 20% sucrose cushion by centrifugation at 80,000 x g
for 2 h at 4°C. Pellets were resuspended in 1x PBS, filtered through a 0.45 µm syringe filter, aliquoted, and stored at -80°C.

No GP VP40-GFP VLPs were generated by transfecting HEK 293T cells with a plasmid expressing EBOV VP40 fused to green fluorescent protein (GFP) (241). Meanwhile, EBOV GP pseudotyped VLPs were generated using EBOV GP plasmid at a 1:1 ratio with VP40-GFP plasmid. Supernatants were collected 48 and 72 h after transfection. VLPs were concentrated by centrifuging supernatants overnight at 5,380 x g at 4°C. Pellets were resuspended in 1x PBS and purified through a sucrose cushion as described above. Pellets were resuspended in 1x PBS, filtered through a 0.45 µm syringe filter, aliquoted, and stored at -80°C.

To produce FIV pseudovirions, HEK 293T cells were transfected with three plasmids as previously described (36, 47). One plasmid expressed an FIV reporter construct that contains a psi sequence and expressed β-galactosidase (β-Gal). A second plasmid expressed the FIV gag and pol genes and a third plasmid expressed a viral glycoprotein gene of choice (EBOV or LASV). Supernatants were collected 24, 48, and 72 h following transfection, filtered, and either aliquoted or concentrated ~200-fold by 16-h centrifugation at 5,380 x g at 4°C in a Sorval GSA rotor, and resuspended in 1x PBS. Virus aliquots were stored at 80°C until use.

Recombinant, replication-competent VSV expressing the EBOV GP with the mucin domain deleted and EGFP in place of the G glycoprotein (EBOV GP-rVSV-EGFP) was produced as previously described (47). Briefly, the EBOV GP gene was inserted upstream of EGFP gene that had replaced the G gene in the genome of a recombinant VSV (Indiana) and infectious virus was produced using a multiplasmid transfection protocol as described previously (242). EBOV GP-rVSV-EGFP stocks were produced in Vero cells using a low multiplicity of infection (MOI) (~0.001) of input virus and maintaining the infection for 3 days prior to supernatant collection. Supernatants were filtered through a 0.45 µm filter and stored as aliquots at -80°C until use. Ross River virus (RRV) was kindly provided by Dr.
David Sanders (Purdue University) and stocks were generated in Vero cells. Supernatants were collected and filtered through a 0.45 µm filter. Aliquots were stored at 80°C. Titers of EBOV GP-rVSV-EGFP stocks were determined by endpoint dilution on Vero cells after a 2-day infection with visualization of EGFP. RRV titers were determined by endpoint dilution on Vero cells after a 7-day infection with visualization of cell death. The 50% tissue culture infective dose (TCID\textsubscript{50})/mL was calculated by the Reed-Meunch method (www.med.yale.edu/micropath/pdf/Infectivity%20calculator.xls).

Recombinant baculovirus expressing β-Gal under the cytomegalovirus (CMV) immediate early promoter was provided by Frederick Boyce (Harvard University). Titers of virus were determined by endpoint dilution on HEK 293T cells, and assayed for β-Gal activity above the background level was assayed 2 days following infection using a Galacto-Light (Applied Biosystems) detection kit as per the manufacturer's instructions.

**Protein structures**

The predicted TIM-1 IgV structures were generated by threading the TIM-1 amino acid sequence on the murine TIM-1 (mTIM-1) (2OR8) (243), mTIM-3 (2OYP) (244), or mTIM-4 (3B19) (61) crystal structure using the protein homology/analogy recognition engine 2 (PHYRE2) service (245). Crystal structures were manipulated and rendered using the PyMOL software program (246).

**TIM-1 mutagenesis**

TIM-1 point mutations were introduced by amplifying a CMV immediate early promoter-driven TIM-1 expression plasmid (Origene) with primers containing targeted nucleotide changes flanked by base pairs of identical sequence. Thermal cycling was performed with PFU Turbo polymerase (Stratagene) using an S1000 thermal cycler (Bio Rad) for 17 cycles (95°C for 30 s, 55°C for 1 min, and 68°C for 14 min). PCR products were digested with *DpnI* (New England Biolabs) to remove parental plasmid. Bacteria were transformed and single colony isolates were grown for plasmid purification. All mutations
were confirmed by DNA sequencing. Mutants that were generated during the studies are shown in Table 2.1.

**AnxB∆IgV-TIM-1**

The IgV domain of TIM-1 was replaced with annexin V (AnxV) by first introducing an NheI site between the hydrophobic signal sequence and first residues of the TIM-1 IgV domain. The IgV domain was excised between this NheI cut site and the naturally occurring MfeI site located downstream of the IgV domain. The IgV domain was replaced with a PCR product containing the AnxV coding sequence and 14 nucleotides (nt) of the TIM-1 mucin domain lost with excision of the IgV domain.

**Analysis of monoclonal antibody binding and TIM expression**

HEK 293T cells were transfected with TIM-1, mutant TIM-1, or empty vector expression plasmids using a polyethyleneimine (PEI) transfection protocol. Cells were detached 48 h later with 5 mM EDTA in PBS and washed with PBS containing 5% FBS. Cells were incubated with 0.5 µg of ARD5, A6G2, AKG7, or A8E5, previously characterized mouse anti-human TIM-1 IgG2a monoclonal antibodies (mAbs) (47, 66, 247) or 0.5 µg of mouse IgG1 or IgG2a controls in 100µL of PBS with 5% FBS for 1 h on ice. Cells were washed and incubated with an α-mouse Cy5 (Invitrogen), DyLight 649 (Jackson ImmunoResearch), or FITC (Jackson ImmunoResearch)-conjugated secondary antiserum for 20 min on ice. AnxB∆IgV-TIM-1 was also incubated with anti-AnxV polyclonal antisera (Abcam). Cells were washed, and expression was assessed by measuring the percentage of positive cells in the FL-4 or FL-1 channel using a FACSCalibur flow cytometer (BD Biosciences).
Transductions

Using trypsin, transfected HEK 293T cells were detached 24 h following transfection and a portion of the population was reseeded in a 48 well plate format while the remaining cells were reseeded in the 6 well format. Forty-eight hours after transfection, the cells in the 6 well plate were evaluated for cell surface TIM expression by flow cytometry as described above. For transduction comparisons between HEK 293T cells and H3 cells, equal numbers of cells were directly seeded onto 48 well plates. Medium was removed from the cells and replaced with 300 μL fresh medium containing VSV or FIV pseudovirions. If needed for TIM-1 mutant studies, ARD5 was added concurrently with virus at a concentration of 1.7 μg/mL.

For transductions of TIM-1 mutants, EBOV pseudovirions were used at an MOI of 0.005 as determined from titers on HEK 293T cells (MOI = 0.8 as determined from titers on Vero cells). EBOV FL pseudovirions were used at an MOI of 0.04 as determined from titers on HEK 293T cells and MARV pseudovirions were used at an MOI of 0.002 as determined from titers on HEK 293T cells. LASV pseudovirions were used at an MOI of 0.15 as determined from titers on HEK 293T cells (MOI = 0.30 as determined from titers on Vero cells). For comparison between transduction efficiency into H3 cells or HEK 293T cells, pseudovirions were added at an MOI of 0.01 or 0.03 as determined from titers on HEK 293T cells. Transduction of VSV pseudovirions was assessed by lifting cells with Accumax solution (Fisher) 24 h following transduction and detecting FL-1 intensity using a FACSCalibur flow cytometer (BD Biosciences). Flow cytometry data were analyzed using FlowJo cytometry analysis software.

A mammalian expression plasmid for receptor for advanced glycation endproducts (RAGE) (Origene) was transfected into HEK 293T cells and cells were transduced as described above. Transductions were normalized to transduction into TIM-1-transfected cells. Protein expression was determined by immunoblotting with goat anti-TIM-1
antiserum (R&D Systems) and RAGE antiserum (Abcam) and developed using the SuperSignal West Dura extended-duration substrate (Thermo/Fisher).

For transductions in the presence of ethylene glycol tetraacetic acid (EGTA), Vero cells were seeded into 48 well plates. Medium on cells was replaced with 1x PBS, 10% FBS supplemented with either 0, 0.25, 0.5, 1.0, or 2.0 mM EGTA and incubated for 1 h. EBOV and LASV VSVΔG pseudovirions, diluted in respective EGTA solutions, were added to cells for 4 h before being refreshing medium. Twenty-four hours after transduction, EGFP expression was assessed by flow cytometry. Sufficient quantities of pseudovirions were added to yield ~25% EGFP positive cells in the absence of EGTA.

For transductions in the presence of mAbs, H3 cells were seeded in a 48 well format and transduced 24 h later with VSVΔG pseudovirions as described above in the presence of 0.5 µg/mL ARD5, A6G2, or A8E5 or no antibody. Transduction was assessed 24 h later by flow cytometry.

Transductions with liposomes

L-α-Phosphatidylcholine (PtdChl) and l-α-phosphatidylyl-serine (PtdSer) (Sigma) liposomes were generated as previously described (60). Briefly, lipids were dissolved in chloroform, dried, resuspended in PBS, and sonicated for 5 min on ice. Liposomes were aliquoted and stored at -80°C until use. H3 and HEK 293T cells were seeded into a 48 well plate and transduced with pseudovirions in the presence or absence of PtdChl or PtdSer liposomes at various concentrations.

Infections

HEK 293T cells were transfected with (wild-type) WT TIM-1, mutant TIM-1, or empty vector and reseeded into 48 well and 6 well plates as described above. Forty-eight hours after transfection, expression of TIM-1 was assessed by surface staining as described above. Cells in the 48 well format were infected with an MOI of 1 as determined from titers on Vero cells with infectious EBOV GP-rVSV-EGFP. Forty-eight hours after infection, cells
were detached and fixed in 3.7% formaldehyde, washed with 1x PBS, and evaluated for EGFP expression by flow cytometry. For RRV infections, WT TIM-1 and empty vector transfected cells were reseeded into 6 well cluster plates pretreated with poly-l-lysine (Invitrogen). Cells were infected 48 h after transfection with an MOI of 0.001, 0.01, or 0.1 for 2 h at 37°C. After infection, cells were washed 3x with 1x PBS and refreshed with medium. Forty-eight hours after virus was added, supernatant aliquots were collected and stored at -80°C. Infections with Vero cells were performed similarly in the presence or absence of ARD5 (1µg/mL). ARD5 was also added to fresh medium for wells infected in presence of ARD5. Titers of supernatants were determined by endpoint dilution on Vero cells as described above.

**Infection with WT EBOV**

The studies with infectious WT EBOV were done by Robert A. Davey at the Texas Biomedical Research Institute. Vero E6 cells were grown to 70% confluence in 96-well plates. Cells were preincubated for 1 h with medium alone or PtdChl or PtdSer liposomes suspended in DMEM with 2% FBS. Cells were then transferred to biosafety level 4 (BSL4) and challenged with replication competent Ebola virus encoding GFP (248) at an MOI of 0.05 in the presence of the lipids. After 24 h, which is sufficient time for the GFP to be expressed in infected cells, the cells were fixed in formalin and cell nuclei were stained with Hoescht 33342. Cells were then imaged with a Nikon Ti Eclipse microscope using the high-content-analysis software package. Images were processed to detect total cells by counting nuclei and infected cells by GFP expression using the Cell Profiler program (Broad Institute, Massachusetts) using customized pipelines that are available from R. Davey upon request. Typically, more than 10,000 cells were analyzed per image. Data are expressed as the percentage of infected cells in the total cell population compare to that for untreated cells.
Infection with *in vivo* derived virus

IFNAR1-/- BALB/c mice lacking a functional alph/beta interferon (IFN-α/β) receptor were obtained from Joan Durbin (New York University, New York, NY). A mouse was infected intranasally with 2x10^7 infectious units of EBOV GP-rVSV-EGFP in 1% methylcellulose. Four days after infection, the mouse was sacrificed and lung, spleen, and kidney were harvested. Organs were homogenized in 1x PBS using a tissue tearer. Debris was pelleted and supernatants were filtered through a 0.45 µm syringe filter, aliquoted, and stored at -80°C. Supernatants were serially diluted and incubated with or without ARD5 (2µg/mL) on Vero cells. Forty-eight hours after infection, cells were detached and fixed in 3.7% formaldehyde, washed with 1x PBS, and evaluated for EGFP expression by flow cytometry. Mice were maintained in the animal care facility at the University of Iowa. This protocol was approved by the University of Iowa Animal Care and Use Committee and was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH).

Baculovirus transductions

HEK 293T cells or H3 cells were seeded in equal numbers into 24 well plates pretreated with poly-l-lysine. Virus was added at an MOI of 0.003 and 48 h later cells were lysed and CMV-driven β-Gal activity was assessed using Galacto-Light (Applied Biosystems) detection kit as per the manufacturer’s instructions.

ELISAs

Nunc MaxiSorp enzyme-linked immunosorbent assay (ELISA) plates were precoated overnight with ~5.2x10^6 transducing units (as determined from titers on Vero cells) of concentrated EBOV pseudovirions or PtdSer or PtdChl liposomes (50 µM) diluted in 1x TBS+ (150 mM NaCl, 25 mM Tris, and 10mM CaCl2, pH 7.2). Plates were blocked for 2 h at 4°C with 1xTBS+ with 2% bovine serum albumin (BSA), incubated with supernatants containing hemagglutinin (HA)-tagged proteins or recombinant HA-tagged AnxV for 2 h,
probed with rabbit polyclonal anti-HA antiserum (Sigma) for 1 h and horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antisera for 1 h, and developed using 3,3',5,5'-tetramethylbenzidine (TMB) substrate (1-Step Ultra TMB-ELISA; (Thermo). Plates were washed thoroughly in between steps with 1x TBS+. Absorbance was read at 450 nm.

HA- and HIS-tagged recombinant AnxV was purified from DH5α Escherichia coli cells transformed with a modified version of the expression vector pProEx.Htb.annexin V obtained from Seamus Martin (Trinity College) and using the protocol outlined by Logue et al. (249). We modified the vector pProEx.Htb.annexin V by inserting a 3x GS linker, HA tag, and stop codon directly after the last aspartate of AnxV.

HA-tagged soluble proteins used in the ELISAs were generated by transfecting expression plasmids into HEK 293T cells and collecting secreted proteins in Opti-MEM with 1% P/S medium without FBS. Supernatants were collected 48 h after transfection, filtered through 0.2 µm filters, aliquoted, and stored at -80°C. Expression of proteins in supernatants was assessed by probing Western blots with rabbit polyclonal anti-HA antiserum and imaged using Li-Core Odyssey CLx system. Soluble HA-tagged TIM-1, TIM-4, and Axl were generated by replacing the transmembrane and cytoplasmic domains with an HA tag sequence and stop codon. The HA-Axl expression plasmid was created by Nicholas Lennemann (University of Iowa). Soluble TIM-1 mutants were generated by site directed mutagenesis as described above. A murine serum amyloid protein (mSAP) expression plasmid was kindly provided by Thomas Rutkowski (University of Iowa).

ELISAs using inhibitors of TIM-1 binding were performed as described above using 1 µL of concentrated TIM-1 HA supernatants per well. Supernatants were concentrated using Amicon Ultra 30 kDa MWCO columns (Millipore). TIM-1 was diluted in 1x TBS+ and incubated with PtdSer or PtdChl liposomes (100 or 10 µM), mAb ARD5 (2 µg/mL), or IgG2a (2 µg/mL) for 30 min before adding to blocked ELISA plates. For binding in presence of EGTA, TIM-1 was diluted in 1x PBS with 2 mM EGTA.
The presence of VSV matrix on ELISA plates prebound with EBOV pseudovirions was determined by first binding virus to ELISA plates and blocking as described above. After blocking, plates were incubated with supernatants containing anti-VSV mAb 23H12 (kindly provided by Douglas Lyles; Wake Forest) and binding was assessed after incubation with HRP-conjugated secondary anti-mouse antisera and TMB substrate.

**FITC-pseudovirion and VLP binding assay**

Forty-eight hours after transfection with TIM-1, empty vector, mutant TIM-1 ND115DN, or AnxVΔIgV-TIM-1, HEK 293T cells were incubated with FITC labeled EBOV GP- or No GP-VSV pseudovirions for 1 h on ice. EBOV GP pseudovirions were added at an MOI of 2.25, as determined from titers on Vero cells, and enough No GP pseudovirions were added to achieve similar fluorescence relatively 2.5-fold more matrix on dot blots). Cells were washed 3 times with 1x PBS plus 5% FBS and cellular fluorescence was determined by flow cytometry.

**Internalization assay**

Vero cells were incubated on ice for 1 h with 1x Hanks' balanced salt solution (HBSS) (+ CaCl₂, + MgCl₂) (Gibco) with 30 mM HEPES. ARD5 (2 μg/mL) or PtdSer or PtdChl liposomes (25 μM) were also added as needed. VLPs or FITC-labeled VSV pseudovirions were bound for 1 h on ice. EBOV GP pseudovirions were added at an MOI of ~6 and No GP pseudovirions were added to achieve approximate binding mean fluorescence intensity (MFI) (relatively twice as much matrix on dot blots). A roughly 0.36 μg total protein amount of EBOV GP-VLPs and 0.48 μg total protein No GP-VLPs were added per 10,000 cells. VLP protein concentrations were determined by bicinchoninic acid (BCA) assay (Thermo). The total protein concentrations do not accurately correlate to total matrix amounts due to GP proteins present in the EBOV GP-VLPs contributing to the total protein amounts. Some cells were shifted to 37°C for 30 min to allow internalization while remaining cells were maintained on ice. After internalization, all cells were treated with 1x
trypsin-EDTA (Gibco) for 10 min at room temperature and an additional 10 min at 37°C. Cells were washed twice with 1x PBS and once with 1x PBS plus 5% FBS. Fluorescence was determined by flow cytometry.

Statistics

Transductions for mutant TIM-1 construct-expressing HEK 293T cells were normalized to WT transduction in the absence of ARD5 by dividing the sample mean obtained from replicates by the WT mean from the same experiment. EBOV, full-length EBOV, and MARV GP pseudovirion transductions into mutant TIM-1 and WT TIM-1 expressing HEK 293T cells in the absence of ARD5 were normalized to TIM-1 expression by dividing by a ratio of mutant TIM-1 expression to WT TIM-1 expression. Expression was determined by flow cytometry as described above and measured as a percentage of antibody positive cells. For data that have been normalized to a control, one-sample t-tests were used to compare transductions and infections to 100, which represents normalized WT transduction. Fold enhancement by TIM-1 or AnxVΔIgV-TIM-1 was compared to 1, which represents background transduction of the empty vector. Comparisons between experimental samples were done using two-sample t test with two tails and equal variance. Nonlinear curves were fitted to data from serial dilutions of infectious EBOV GP-rVSV-EGFP onto transfected HEK 293T cells and WT EBOV infection of Vero cells in the presence of liposomes using the GraphPad Prism software program using one-site, specific binding for EBOV GP-rVSV and dissociation, one phase exponential decay for WT EBOV. The EBOV GP-rVSV curves had R² values of 0.9708 (WT), 0.9577 (empty vector), 0.9889 (N114A construct), and 0.9737 (D115A construct). The 50% infective doses (ID₅₀) (relative to the maximum percent infection of each curve [Bmax]) for the WT, empty vector, and N114A and D115A constructs were calculated from the fitted curve to be 7.57 x 10⁻⁷, 8.10 x 10⁻⁴, 3.42 x 10⁻⁴, and 4.25 x 10⁻⁴, respectively. The PtdSer inhibition curve of WT EBOV had an R² value of 0.9834.
Results

Modeling a human TIM-1 structure.

Our previous study demonstrated that in a poorly permissive cell line, HEK 293T cells, ectopic TIM-1 expression enhances EBOV transduction, which is inhibited by the anti-TIM-1 IgV mAbs ARD5, A6G2, and A8E5 (47). This focused our initial studies on identifying TIM-1 IgV residues important for EBOV entry. Since the inhibitory mAb A6G2 has been shown to also block TIM-1 binding of PtdSer (247), we focused our attention on the PtdSer binding pocket. We aligned the amino acid sequences of the human TIM IgV domains with murine TIM-1 (mTIM-1) IgV, revealing significant conservation of residues (Figure 2.1A). Since the crystal structures of mTIM-1,-3, and -4 IgV domains are similar (61, 62, 243, 244), we threaded human TIM-1 onto these structures using PHYRE2 (protein homology/analogy recognition engine 2) (245). The human TIM-1 IgV sequence threaded on the mTIM-1 structure strongly resembled mTIM-1 with the PtdSer binding pockets being comparable (Figure 2.1B) and key residues involved in the binding of PtdSer conserved and positioned similarly (Figure 2.1C). Alanine scanning mutagenesis of human TIM-1 residues predicted to be surface exposed was performed to identify residues important for EBOV entry. Adjacent residues and non-alanine residue substitutions were also introduced after the initial screen. In total, 45 individual residues of the IgV domain of TIM-1 were mutated (Table 2.1 and Figure 2.1D).

PtdSer binding pocket residues are necessary for EBOV entry.

HEK 293T cells were transfected with TIM-1 mutants in parallel with wild-type (WT) TIM-1 and an empty vector control, evaluated for TIM-1 surface expression by flow cytometry, and transduced or infected 48 h after transfection. HEK 293T cells were selected for these studies since these cells are readily transfected, do not endogenously express any TIM family members, and in the absence of ectopic TIM-1 expression poorly support
EBOV infection (47). All mutants but one had >70% of WT surface expression as assessed by surface staining of transfected cells with four different anti-TIM-1 mAbs: ARD5, A6G2, AKG7, and A8E5 (47, 66, 247). Surface expression of the D115A construct was ~55% of WT expression, but Glu or Asn substitution rather than Ala at this position yielded expression equivalent to that of the WT. The WT surface expression of our mutants and ability of multiple IgV-specific mAbs to bind to mutants argues against an impact of the mutations on the stability and/or expression of TIM-1.

Transfected cells were transduced with vesicular stomatitis virus (VSV) pseudovirions that express EGFP as a reporter molecule in place of native G (VSVΔG). VSV was pseudotyped with either EBOV GP that has a deleted mucin domain or, as a control, Lassa virus (LASV) GPC. We used EBOV GP lacking the GP1 mucin domain since this construct confers the same tropism as the full-length Ebola virus GP and produces higher pseudovirus titers (21, 240, 250). Ebola GP-VSVΔG and LASV GPC-VSVΔG transductions were performed in parallel and analyzed for EGFP expression by flow cytometry at 24 h following transduction.

A summary of the effects of TIM-1 IgV domain mutations on EBOV transduction is shown in Table 2.1. Most TIM-1 mutations had no deleterious effect on transduction and none significantly enhanced entry. Mutation of eight residues within and adjacent to the PtdSer binding pocket inhibited EBOV transduction (Figure 2.2A). G111A, N114A, or D115A resulted in the most profound effects. EBOV transduction into cells expressing these TIM-1 mutants remained sensitive to ARD5 inhibition, suggesting that the inhibitory effect of ARD5 is not due to direct binding competition. In contrast, transduction of LASV pseudovirions was largely unaffected by expression of WT or mutant TIM-1s or the presence of ARD5 (Figure 2.2B). However, LASV pseudovirion transduction into empty vector and F113A construct-transfected cells was slightly reduced, and this difference was statistically significant. Nonetheless, the reduction was minor and no significant changes were observed
in the presence of ARD5. Mutation of the TIM-1 PtdSer binding pocket also reduced transduction of EBOV FL GP or MARV GP pseudovirions (Figure 2.2C).

These same TIM-1 mutants were evaluated for their ability to support a recombinant, infectious VSV/GFP (rVSV) that encodes and expresses EBOV GP (EBOV GP-rVSV-EGFP) (47). Similar to our transduction studies, expression of the TIM-1 mutants, N114A and D115A, resulted in little to no enhancement of EBOV GP-rVSV-EGFP infection compared to cells transfected with an empty vector (Figure 2.2D). Further, dose-response curves with EBOV GP-rVSV-EGFP demonstrated that infection in the presence of the N114A and D115A mutants was indistinguishable from that with the empty vector control (Figure 2.2E). Since these mutants were expressed at WT levels, but did not support EBOV transduction, we propose that these PtdSer binding pocket residues are influencing the ability of the mutants to serve as an EBOV receptor.

Interestingly, mutagenesis of two additional residues, Y38 and D99 not located near the PtdSer binding cleft also decreased EBOV transduction (Figure 2.3A) and infection (Figure 2.3B), but not surface expression of TIM-1 (Table 2.1). Our structural model predicts that Y38 and D99 are distal to the PtdSer binding pocket and are not surface exposed (Figure 2.3C), suggesting that mutagenesis of these residues may alter the IgV domain structure without altering the overall stability of the protein. Consistent with this possibility, mutations of surface-exposed residues adjacent to Y38 had no effect on transduction (Table 2.1).

**TIM-1-mediated uptake requires virion-associated PtdSer binding.**

Residues critical for EBOV infection are located within the PtdSer binding pocket, suggesting that virion-associated PtdSer interactions with TIM-1 contribute to transduction. Others have reported that PtdSer is present on the surface of enveloped viruses (54, 55). To assess the role of PtdSer in TIM-1-dependent entry of EBOV, competition studies were
performed in H3 cells, a clonal population of HEK 293T cells stably expressing TIM-1 (47), in the presence of increasing concentrations of PtdSer or phosphatidylcholine (PtdChl) liposomes. Similar liposomes have been used previously to inhibit HIV-1 infection (53) and uptake of apoptotic cells and virus mediated by TIM-1 (56, 60). Transduction was inhibited in a dose-dependent manner by the presence of PtdSer liposomes, with a 2.5 μM concentration abolishing transduction (Figure 2.4A). PtdChl liposomes had a minimal effect. Since binding of PtdSer by TIM-1 requires divalent cations and the presence of EGTA inhibits this interaction (85), we assessed the effect of increasing concentrations of EGTA on EBOV entry in highly permissive, TIM-1 expressing Vero cells. Transduction of EBOV was inhibited in dose-dependent manner by EGTA (Figure 2.4B). Conservation of PtdSer binding among the human TIM family members (Figure 2.1A) suggests that other family members such as TIM-4 would also function as an EBOV receptor. Indeed, upon transfection into 293T cells, TIM-4 enhanced EBOV entry equivalently to that with TIM-1 (Figure 2.4C).

We previously showed that TIM-1 binds to EBOV pseudovirions (47). In order to determine if TIM-1 was binding to PtdSer on virions, we initially determined if PtdSer was present in pseudovirion membranes by ELISA studies using purified recombinant HA-tagged AnxV. AnxV is a cellular protein that binds to PtdSer (251) and detects PtdSer on the surface of apoptotic cells (252). AnxV bound to EBOV pseudovirion-coated ELISA plates in a dose-dependent manner but not to plates lacking pseudovirions (Figure 2.5A). We also found AnxV bound to LASV pseudovirions (Figure 2.5B). Similarly, HA-tagged soluble WT TIM-1 in HEK 293T cell supernatants bound to virus and PtdSer liposomes, but N114D and D115N TIM-1 PtdSer binding pocket mutants did not (Figure 2.5C and D). Soluble, HA-tagged Axl and murine serum amyloid protein (mSAP) served as negative controls in the study and did not bind virus. While Axl has been shown to enhance pseudovirus uptake, Axl cannot bind PtdSer without bridging by Gas6 (54). Consistent with our transduction studies, HA-tagged soluble TIM-4 also bound to pseudovirions. It should
be noted that our virus preparations likely contain broken particles in addition to intact virions, since a VSV matrix specific antibody was able to bind to pseudovirions (Figure 2.5E) and we cannot rule out that AnxV is also binding to the inner membrane.

The specificity of the TIM-1/EBOV pseudovirion interaction was further examined by assessing the ability of inhibitors to interfere with TIM-1 binding (Figure 2.5F). These included the anti-TIM-1 mAbs ARD5 and A6G2, PtdSer liposomes, and EGTA, all of which dramatically inhibited TIM-1 binding. PtdChl liposomes did not affect TIM-1 binding to pseudovirions. These findings lend support for a model of PtdSer-dependent TIM-1-mediated uptake of filoviruses.

Not all PtdSer receptors mediate filovirus entry.

The ability for TIM-1 and TIM-4 to bind pseudovirions and enhance EBOV transduction might suggest that all PtdSer binding receptors may enhance EBOV entry. Receptor for advance glycation endproducts (RAGE) is a PtdSer receptor unrelated to the TIM family that also enhances apoptotic body uptake upon transfection into HEK 293 cells (231, 253). However, we found that expression of RAGE did not enhance EBOV transduction into HEK 293T cells (Figure 2.6A) although expression was detected in lysates (Figure 2.6B).

TIM-1 enhances entry of VSV pseudotyped with a wide range of viral GPs.

We hypothesized that if TIM-1 enhancement was dependent on PtdSer binding and not on interaction with EBOV GP, TIM-1 should enhance entry of VSVΔG pseudotyped with other viral GPs. VSVΔG virions were pseudotyped with the alphavirus Ross River virus (RRV) GP or Chikungunya virus (CHIKV) env or baculovirus Autographa californica nucleopolyhedrovirus (AcMNPV) GP64. Transduction of these pseudoviruses into 293T cells and TIM-1+ H3 cells was evaluated in parallel with that of EBOV GP and LASV GPC. In a manner similar to that of EBOV, transduction of RRV or GP64 pseudovirions was
enhanced by TIM-1 expression (Figure 2.7A). CHIKV entry was more modestly enhanced by about 3- to 5-fold. LASV GPC-dependent entry was unaffected by TIM-1 expression. PtdSer binding pocket mutant ND115DN (N114D and D115N double mutant) did not increase pseudovirion transduction, demonstrating that enhancement was due to the PtdSer binding pocket of TIM-1 (Figure 2.7B). Further, TIM-1 IgV domain mAbs ARD5, A6G2, and A8E5 inhibited CHIKV, RRV, or GP64 pseudovirion transduction as effectively as EBOV pseudovirion transduction (Figure 2.7C). Consistent with the possibility that virion associated PtdSer enhances alphavirus and baculovirus entry, TIM-1-enhanced transduction of RRV, GP64, and CHIKV pseudovirions was inhibited by PtdSer liposomes, although inhibition of EBOV transduction was most profound (Figure 2.8A). Interestingly, LASV was partially inhibited in both H3 and HEK 293T cells by PtdSer liposomes. Since transduction of LASV is unaffected by TIM-1 expression in HEK 293T cells and the reduction is equal in the two cell types, this inhibition is likely an off-target effect for LASV. The correlation between enhancement of transduction by TIM-1 expression and subsequent inhibition by PtdSer liposomes was evident when these two data sets were plotted, suggesting that TIM-1 binding of PtdSer is important for TIM-1-dependent enhancement of transduction (Figure 2.8B). To exclude the possibility that our findings were specific to the VSV pseudotyping system, we confirmed our results with FIV pseudovirions, although EBOV GP FIV was more modestly enhanced by TIM-1 than EBOV GP VSV (Figure 2.8C).

**AnxV substitutes for the TIM-1 IgV domain.**

Our results implicate the PtdSer binding activity of the TIM-1 IgV domain in mediating entry of a diverse group of enveloped viruses. In order to rule out other functions of the IgV domain and verify the importance of virus-associated PtdSer for the effect, we replaced the IgV domain of TIM-1 with AnxV. The chimeric construct (AnxVΔIgV-TIM-1) was expressed in transfected cells at levels equivalent to that of WT TIM-1 as detected by a
TIM-1 polyclonal antiserum and AnxVΔIgV-TIM-1 was detected on the surface of cells by both the TIM-1 MLD specific mAb AKG7 and an AnxV-specific mAb (Figure 2.9A). However, the IgV domain specific mAb ARD5 does not bind to AnxVΔIgV-TIM-1. AnxVΔIgV-TIM-1 significantly increased transduction for all of the pseudoviruses tested except LASV (Figure 2.9B), albeit with reduced efficiency compared to that of TIM-1.

Direct role of TIM-1 for internalization of virus.

Since TIM-1 could enhance transduction by binding virus and either directly mediating entry or acting as an attachment factor to concentrate virus on the cell surface for internalization via an alternative receptor, we evaluated transduction of VSV pseudotyped with a well-characterized Sindbis virus (SINV) envelope receptor binding domain mutant 2.2 1L1L (254-256). This GP contains deletions and mutations within E2 and E1 that diminish native receptor binding activity, reducing interaction with a specific cell surface receptor, but still allowing fusion. TIM-1 enhanced SINV pseudovirion transduction through a PtdSer-dependent mechanism (Figure 2.7A to C, 2.8A and B, and 2.9B). The ability of TIM-1 to enhance transduction of pseudovirions lacking an intact RBD suggests that TIM-1-mediated enhancement is independent of receptor binding by the glycoprotein RBD.

TIM-1 expression enhances virus binding and internalization independent of glycoprotein.

As a second approach to assessing if TIM-1 is acting as an attachment factor or directly internalizing virus, we fluorescein isothiocyanate (FITC) labeled both EBOV GP-VSV pseudovirions and VSV pseudovirions without an envelope GP (No GP). We found that binding of both EBOV GP and No GP pseudovirions to HEK 293T cells was enhanced by exogenous expression of TIM-1 and AnxVΔIgV-TIM-1, but binding of pseudovirions to a PtdSer-binding mutant TIM-1, ND115DN, was minimal (Figure 2.10A). Because not all cells express the transgene after transfection, only a portion of the population shifts.
Additionally, ARD5 inhibited EBOV GP-VSV binding to TIM-1+ cells but not AnxVΔIgV-TIM-1-expressing cells.

We then used a trypsin protection assay to assess virion internalization in the presence of endogenous TIM-1 in Vero cells. Pseudovirions were bound to Vero cells on ice to prevent internalization. Subsequently, some cell populations were shifted to 37°C to allow internalization of virus. Virion internalization would be predicted to protect virus from trypsin cleavage and removal. Using these conditions, the fluorescence of cells prebound with either EBOV GP-VSV or No GP pseudovirions was assessed with or without virion internalization. In the absence of the temperature shift to 37°C, bound virus was removed by trypsin (Figure 2.10B). However, after internalizing virus at 37°C and treating cells with trypsin, FITC-labeled EBOV GP and No GP pseudovirions were readily detected, indicating that they were effectively internalized (Figure 2.10B). Internalization as detected by mean fluorescence intensity (MFI) is quantified in Figure 2.10C, demonstrating similar internalization of both EBOV GP and No Env pseudovirions. Additionally, ARD5 and PtdSer liposomes inhibited internalization of both viruses but PtdChl liposomes did not. These results were confirmed using EBOV VP40-GFP virus-like-particles (VLPs)(241) (Figure 2.10D and E) and demonstrate that enveloped virus is internalized into TIM-1-expressing cells in the absence of a viral glycoprotein.

**TIM-1 enhances Ross River virus and AcMNPV infection.**

Despite previous observations by our group and others that TIM-1 enhances entry of replication competent WT EBOV, EBOV GP-rVSV-EGFP, and dengue virus (DV) (47, 55), we were concerned that high levels of GP expression during pseudovirus production could lead to PtdSer flipping to the outer leaflet, altering the pseudovirion envelope composition compared to virus produced during an infection. Thus, we assessed if TIM-1 expression enhances infection by the alphavirus RRV or the baculovirus AcMNPV. For RRV
experiments, a range of MOIs, as titered in Vero cells, was added to HEK 293T cells transfected with an empty or TIM-1-expressing vector, and virus was collected and titered on Vero cells by endpoint dilution at 48 hours. RRV titers were enhanced by TIM-1 at low MOIs, and this effect was reduced with increasing MOIs (Figure 2.11A). Consistent with a role for TIM-1, RRV infection of TIM-1-positive Vero cells for 24 h was inhibited by ARD5 (Figure 2.11B). In a similar manner, entry of AcMNPV into mammalian cells was enhanced by the presence of TIM-1. While AcMNPV does not replicate in mammalian cells, use of an infectious AcMNPV that expresses β-Gal under a CMV promoter allowed assessment of virus entry, uncoating, and transgene expression in either HEK 293T or H3 cells. TIM-1 expression increased entry of this baculovirus by ~6 fold (Figure 2.11C). While we have already shown that WT EBOV infection of Vero cells is inhibited by ARD5 (47), we wanted to confirm that WT EBOV infection is mediated through PtdSer binding. Vero cells were infected with WT EBOV in the presence or absence of PtdSer or PtdChl liposomes (Figure 2.11D). EBOV infection was sensitive to inhibition by PtdSer liposomes in a dose dependent manner, but not to that by PtdChl liposomes. The ability of TIM-1 to mediate entry of a variety of viruses suggests a potential role as a broad receptor for enveloped virus uptake.

**Production of PtdSer-containing virions in viva**

While we have demonstrated that PtdSer-mediated entry occurs with both transducing and infectious virus generated in tissue culture, we also sought to determine if virus generated in vivo enters cells in a TIM-1-dependent manner. Replication competent EBOV GP-rVSV-EGFP was obtained from lung, spleen, and kidney homogenates of an interferon-α/β receptor (IFNAR) knockout BALB/c mouse 4 days after intranasal infection. Virus collected from homogenates was serially diluted onto Vero cells in the presence or absence of ARD5 (2 μg/mL). ARD5 inhibited virus infection at all dilutions, regardless of
the organ of origin (Figure 2.12). These results suggest that the presence of PtdSer on virus grown in tissue culture is representative of virus produced during in vivo infection.

Discussion

This study demonstrates that TIM-1 enhances infection of a variety of unrelated enveloped viruses by binding to virion-associated PtdSer, leading to virus internalization. The virus families described here include filoviruses, alphaviruses, and a baculovirus. Other recent studies have also confirmed that TIM-1 enhances filoviruses, alphaviruses, flaviviruses, and some arenaviruses to this extensive group (55, 56). Here, we have provided a number of lines of evidence to support this conclusion. First, PtdSer liposomes inhibit TIM-1 binding to virions and effectively eliminate TIM-1 dependent transduction/infection. Second, mutations in the TIM-1 PtdSer-binding pocket abrogate the enhanced transduction. Third, TIM-1 mAbs that block PtdSer binding decrease TIM-1 binding to pseudovirions and TIM-1-dependent transduction and infection. Fourth, the TIM family member TIM-4, which also has a PtdSer binding pocket, functions to enhance virus transduction. Fifth, pseudovirions or VLPs that lack a viral envelope glycoprotein are efficiently internalized into TIM-1-expressing cells. Finally, the PtdSer-binding protein AnxV effectively substitutes for the TIM-1 IgV domain on a chimeric TIM-1 receptor. These studies extend the number of virus families that use apoptotic mimicry as a mechanism of virion uptake and support the idea that enveloped viruses can commandeer the use of cell surface PtdSer-binding receptors to mediate uptake.

Our mutagenesis studies initially focused on identifying TIM-1 residues that impact filovirus infection. We identified eight residues critical for EBOV virus infection that line and surround the PtdSer binding pocket of the TIM IgV domain. These residues are generally conserved across the TIM family and a mutagenesis study of the mTIM-4 IgV domain identified a subset of these residues as critical for PtdSer binding (61). The PtdSer pocket Asn and Asp residues (N114 and D115 in TIM-1) are involved in intra- and
intermolecular interactions important for retaining a metal cation required for PtdSer binding. The mTIM-4 crystal structure also suggests that the backbone of the PtdSer pocket Gly (G111 in TIM-1) also contributes to cation binding (61). Other pocket residues contribute to PtdSer binding through hydrogen bonding to the phosphate and serine moieties of PtdSer and maintaining the spacing between the CC' and FG loops. Consistent with the important role of this binding pocket in mediating virus entry, W112, F113, N114, and D115 were recently identified as critical for PtdSer dependent uptake of several enveloped viruses by TIM-1 (55, 56).

Since many of the TIM-1 residues found to be important for EBOV infection are also important in PtdSer binding, we tested if EBOV entry was mediated through the presence of PtdSer on the surface of virions. We detected PtdSer on the surface of EBOV GP-pseudovirions with AnxV. Further, transduction of VSV pseudotyped with a variety of other viral GPs and infection of both RRV and AcMNPV at low, biologically relevant MOIs was also increased by TIM-1 surface expression. Additionally Jemielity et al. found that SINV, Tacaribe virus, and RRV infection is enhanced by TIM-1 expression (56). Thus, TIM-1 mediates infection of a broader range of viruses than previously appreciated through a PtdSer-dependent mechanism. Enhancement of AcMNPV, flavivirus, and alphavirus entry also suggests a potential role for TIM-1 in transmission of arthropod-borne viruses between mosquitoes and mammals. However, it should be noted that not all pseudovirion data, such as those for CHIKV, have been confirmed with WT virus.

The phenomenon of apoptotic mimicry, reviewed in reference (257), was first described for vaccinia virus, although no cellular PtdSer receptor was implicated (49). Their study found that depleting PtdSer from the surface of mature virions inhibited infectivity, which was restored by PtdSer reconstitution. Recently, the cell surface complex of Gas6 and Axl was identified to mediate PtdSer-dependent virus entry (54). The Gas6/Axl complex mediates uptake of both vaccinia extracellular enveloped virus and DV in HEK 293T and A549 cells (54, 55). Further, Axl enhances EBOV entry in certain cell types, which may in
part be due to Gas6/Axl complexes (25, 36, 37). However, it should be noted that mediation of EBOV entry by Axl appears to be cell type dependent and endogenous Axl expression in several cell lines does not enhance EBOV uptake. Here, our findings, along with the TIM-1 studies by Meertens et al. (55) and Jemielity et al. (56), provide strong evidence that TIM-1 mediates virus uptake through binding of virion-associated PtdSer, thereby identifying it as a second cellular receptor that mediates virus entry in a PtdSer-dependent manner. This new class of viral receptors we term phosphatidylserine-mediated virus entry enhancing receptors or PVEERs.

The mechanism of PtdSer incorporation into viral envelopes remains unclear. Apoptosis may contribute to the accumulation of PtdSer on the surface of virions, since many viruses induce this type of cell killing. For instance, CHIKV can induce apoptosis during infection (258), contributing to spread of virus through uptake of apoptotic blebs. Additionally, the natural surface-exposure of PtdSer by certain cell types or species may also contribute to PtdSer presentation as seen with macrophages (259) and cell lines (260). One possible association that we explored was between viral GP expression on the cell surface and PtdSer flipping. However, enhancement of binding and internalization of No GP pseudovirions and VLPs by TIM-1 suggests that PtdSer incorporation is independent of GP expression.

Not all enveloped virions transduced more effectively with TIM-1 expression. We found that LASV pseudovirion entry into HEK 293T cells was not enhanced by TIM-1 expression, as others also found with LASV and other viruses including herpes simplex virus 1, influenza A virus (H7N1), Oliveros virus, and severe acute respiratory syndrome coronavirus (55, 56). It is possible that the affinity between the GPs of these viruses and their native receptors may be sufficiently high that this interaction outcompetes TIM-1 for virus interactions. Additionally, these receptors may be more accessible for interaction due to size, for example the LASV receptor, α-dystroglycan (αDG), is considerably larger than TIM-1 (261). An alternative possibility, which is not mutually exclusive, is that receptor expression
on the cells under investigation is abundant, reducing the likelihood of virus/TIM-1 binding. Recently, it has been shown that LASV entry can be enhanced by expression of Axl, DC-SIGN, Tyro3, and LSECtin on cells that lack αDG (262). We are able to detect PtdSer in LASV GPC pseudovirions, and ectopic expression of TIM-1 on these αDG−/− cells may reveal that TIM-1 is able to enhance LASV entry. However, contrary to these possible explanations, expression of TIM-1 did not enhance transduction of SARS coronavirus isolates with reduced binding affinity for ACE2 (56). The mechanism of PtdSer-dependent internalization may be compatible with some glycoproteins but not with others. Additionally, mechanisms or route(s) of endocytosis may differ between PtdSer receptors, and this may explain why some PtdSer receptors, such as RAGE and BAI1 (55), do not effectively enhance enveloped-virus entry.

The identification and appreciation of the broad use by enveloped viruses of PVEERs may explain the wide tropism seen with many of these viruses, such as the filoviruses and alphaviruses (this study and that of Jemielity et al. (56)) and the flaviviruses (55). In addition to expanding the host range, cell receptor interactions with virion-associated PtdSer would provide effective protection against antibody neutralization, since exposure of GP RBD residues on the surface of extracellular virions would not be necessary. Consistent with this possibility, the EBOV GP RBD has been shown to be protected by a glycan cloud that is only removed once the virus has internalized into endosomes (30, 31). Virus use of PVEERs for internalization may relegate the role of the receptor binding domain of the viral GP to endosomal functions. For filoviruses, these functions would include intracellular binding of NPC1 and subsequent fusion events (Figure 2.13). For other enveloped viruses these functions may involve low-pH-dependent conformational changes of GP leading directly to fusion events.

TIM-1 has been postulated to function as an attachment factor (55). Alternatively, TIM-1/virion PtdSer interactions may directly mediate virus internalization. Our studies demonstrate that TIM-1 is able to enhance transduction of Sindbis env 2.2 1L1L
pseudovirions with mutated RBDs, and we and others found that TIM-1 enhances binding and internalization of No GP VLPs and pseudovirions (56), supporting the idea that TIM-1 interaction with virion-associated PtdSer mediates virus internalization. Additionally, a recent study using VSV G found that proteinaceous receptors are not necessary for fusion (263), suggesting for some viruses that once virions are internalized, endosomal conditions are sufficient to drive viral GP/cell membrane association and fusion. However, in these studies, we cannot completely rule out that TIM-1 acts as an attachment factor and virus internalization is mediated by another as yet uncharacterized receptor(s). Nonetheless, internalization of enveloped viruses independent of a GP would suggest that this second receptor is also a PVEER.

With the elucidation that EBOV entry is mediated by conserved residues of the TIM-1 PtdSer binding pocket, it is not surprising that family member TIM-4 was found to be capable of mediating transduction of EBOV. Since TIM-1 and TIM-4 are found on differing populations of cells (47, 60, 62, 63, 74, 76, 78), EBOV targeting of both receptors provides an expanded range of permissive cells. Low-level expression of these receptors may contribute to background transduction seen in otherwise mostly nonpermissive cells for many other viruses.

Future studies are needed to elucidate the *in vivo* relevance of apoptotic mimicry for viral pathogenesis. While we found that *in vivo*-derived virus is sensitive to ARD5, the relative contribution of PVEERs *in vivo* remains unclear. Interestingly, nonhuman primates do not serve as reservoirs for DV in South America as they do in Africa (264), which may be related to TIM-1 mutations and deletions that lead to TIM-1 loss-of-function in many New World primate lineages (265). The inhibition of TIM-1-mediated uptake of DV in New World primates may be sufficient to reduce virus entry, preventing robust infection and their establishment as a reservoir. It is interesting to speculate that loss of TIM-1 function in these species may be due to selective pressures resulting from virus utilization of TIM-1 as a PVEER. However, targeting PVEERs in humans may be an effective antiviral approach,
since a study found that bavituximab, a chimera of mouse mAb 3G4 that cross-links a phosphatidylserine binding protein, \( \beta_2 \text{GP1} \), to the cell surface, partially protected against lethal infection of guinea pigs with Pichinde virus and reduced the viral load (52).

Additionally, hepatitis A virus was recently shown to cloak itself in host-derived membranes (266). The presence of PtdSer on these membranes might explain the use of TIM-1 as a receptor for hepatitis A virus. While these results implicate virion-associated PtdSer in enveloped virus pathogenesis, more research must be done to determine the role PVEERs play and subsequently the effectiveness of inhibitors of this process.
Figure 2.1 TIM-1 threaded on mTIM-1 crystal structure. (A) Amino acid sequences of TIM-1, mTIM-1, TIM-3, and TIM-4 IgV domains aligned using the Clustal W software program. Residues in the TIM-1 PtdSer binding pocket that were mutated are shown in bold. β sheets are marked above the alignment with lines and corresponding letters. Residue numbering is based on the TIM-1 sequence. (B) Structures of mTIM-1 IgV (20R8) (left) and a threaded model of TIM-1 IgV (right). β sheets are labeled with their corresponding letters assigned by Santiago et al. (243). (C) PtdSer binding residues of mTIM-1 (above) and TIM-1 (below) are shown on each structure. (D) All residues in the TIM-1 IgV domain mutated in this study are highlighted in black.
Figure 2.2 Identification of TIM-1 IgV residues that impact EBOV GP-dependent entry. (A to C) Relative virus transduction into HEK 293T cells mediated by mutant TIM-1 constructs compared to that with WT TIM-1. At 48 h, transfected cells were transduced with EBOV GP (A), LASV GPC (B), MARV GP (C) or full-length EBOV (C) pseudovirions. (A and B) Transductions were also done in the presence or absence of anti-human TIM-1 mAb ARD5 (1.7 µg/mL). EGFP expression was assessed 24 h later. (D) EBOV GP-rVSV-EGFP infection mediated by TIM-1 constructs in HEK 293T cells (MOI of 1 as titered in Vero cells) relative to WT TIM-1. (E) Serial dilution of EBOV GP-rVSV-EGFP onto TIM-1-transfected HEK 293T cells. EGFP expression was assessed 24 (A to C) or 48 (D and E) h later by flow cytometry. Data are shown as means ± SEM for at least three replicates. (A to D) Significance was calculated using one-sample t-test comparison to 100 (**= p<0.001, *=p<0.01). Significance for all ARD5 transductions for EBOV, p<0.001.
Figure 2.3  Two TIM-1 mutations outside of the PtdSer binding cleft that reduce transduction and infection. Relative virus transduction of EBOV GP or LASV GPC pseudovirions (A) or infection of EBOV GP-rVSV-EGFP (B) into transfected HEK 293Ts mediated by TIM-1 mutants, Y38A or D99A, compared to that of WT TIM-1. EGFP expression was assessed 24 h after transduction (A) or 48 h after infection (B). (C) Y38 and D99 are shown on hTIM-1 threaded cartoon model in dark grey. Data are shown as means ± SEM for at least three replicates. (A and B) Significance was calculated using one-sample t-test comparison to 100 (** = p<0.001, * = p<0.01).
Figure 2.4  TIM-1-mediated transduction is inhibited by liposomes and EGTA. (A) Transduction of TIM-1+ H3 cells with EBOV pseudovirions in the presence of increasing concentrations of PtdSer or PtdChl liposomes. (B) Transduction of EBOV and LASV pseudovirions into Vero cells in the presence of increasing EGTA concentrations. EGTA in PBS plus 10%FBS was incubated on Vero cells for 1 h before pseudovirions were added for 4 h. Medium was replaced, and transduction assessed after 24 h. (C) Transduction of EBOV pseudovirions into empty vector or TIM-4-transfected HEK 293T cells relative to TIM-1 transduction. Data are shown as means ± SEM for at least three replicates. Significance was calculated using one-sample t-test comparison to 100 (** = p<0.001, *=p<0.01).
Figure 2.5  TIM-1 binds to PtdSer on virions. (A and B) AnxV binds to EBOV and LASV pseudovirions. Increasing concentrations (A) or 0.5 μg/mL (B) of HA-tagged AnxV were incubated with ELISA plates prebound with pseudovirions or untreated (No Virus). (C) HEK 293T supernatants containing HA-tagged soluble Axl, mSAP, TIM-1, TIM-4, or mutants of TIM-1, N114D or D115N, were incubated with ELISA plates prebound with EBOV pseudovirions or not treated. Relative protein amounts present in supernatants are shown below in a representative Western blot using anti-HA antisera. (D) Binding of TIM-1 and N114D from supernatants to ELISA plates prebound with PtdSer (filled) or PtdChl (open) liposomes (50 μM). (E) Binding of unpurified anti-VSV-matrix mAb to ELISA plates prebound with EBOV pseudovirions. (F) Binding of HA-tagged TIM-1 in the presence of IgG2a, mAb ARD5, mAb A6G2, PtdSer or PtdChl liposomes, or 2 mM EGTA to ELISA plates prebound with EBOV pseudovirions. Approximate background absorbance is shown with dashed line. Data are shown as means ± SEM for at least three replicates. Significance was calculated using a two-sample t test for panel I (**= p<0.001, *=p<0.01).
Figure 2.6  Expression of RAGE does not increase transduction. (A) Transduction of EBOV or LASV pseudovirions into HEK 293T cells transfected with an empty vector (Empty) or a RAGE-expressing vector (RAGE) relative to WT TIM-1. Cells were transduced at 48 h following transfection and EGFP expression was assessed 24 h following transduction by flow cytometry. Data are shown as means ± SEM for at least three replicates. Significance compared to WT was calculated using one-sample $t$-test comparison to 1 (** = $p<0.001$, *= $p<0.01$). Significance between Empty and RAGE was calculated using two-sample $t$-test. (B) TIM-1 and RAGE expression for three experiments, labeled 1 to 3, are shown by immunoblot analysis.
Figure 2.7  TIM-1-mediated transduction is not specific to EBOV. (A) Transduction of HEK 293T cells (grey bars) and TIM-1+ H3 cells (black bars) with VSVΔG pseudotyped with EBOV GP, RRV GP, GP64, CHIKV env, LASV GPC, or SINV 2.2.1L1L env. Cells were seeded in equal number, transduced with a MOI of ~0.01 and 0.03, and assayed for EGFP by flow cytometry at 24 h following transduction. (B) Transduction of pseudovirions at MOI ~0.01 into HEK 293T cells transfected with empty vector, ND115DN mutant of TIM-1, or WT TIM-1. MOIs were determined in untransfected HEK 293T cells. (C) Percent inhibition of pseudovirion transduction into TIM-1+ H3 cells by mAb ARD5, A6G2, or A8E5 (0.5 μg/mL). Percent inhibition is calculated relative to no-antibody control. Data are shown as means ± SEM for at least three replicates. For panels A and B, significance was calculated using a two-sample student t-test with equal variance and for panel C significance was calculated using one-sample t-test comparison to 0 (C) (**=p<0.001, *=p<0.01).
Figure 2.8  TIM-1 enhances entry in PtdSer binding-dependent mechanism. (A) Transduction of pseudovirions into TIM-1+ H3 cells (filled) or HEK 293T cells (open) in presence of 0, 5, or 25 μM PtdSer liposomes. (B) Correlation between enhancement of transduction by TIM-1 expression and percent inhibition by PtdSer liposomes. Pseudovirions were plotted on x axis based on fold enhancement of transduction in H3 cells over HEK 293T cells and on the y axis based on percent inhibition by 25 μM PtdSer compared to that by 0 μM PtdSer liposomes. Linear regression was fitted and significance calculated using GraphPad Prism software program. (C) FIV pseudovirions expressing β-gal were used to transduce HEK 293T cells and TIM-1+ H3 cells. β-gal expression in lysates was assessed using the Galacto-Light system. Shown is the fold increase in β-gal signal in H3 cells over 293T cells. Data are shown as means ± SEM for at least three replicates.
Figure 2.9  Expression of AnxVΔIgV-TIM-1. (A) Representative histograms of TIM-1 and AnxVΔIgV-TIM-1 expression 48 h after transfection. Transfected HEK293T cells were surface stained using mAb ARD5 or AKG7, polyclonal anti-AnxV antisera, or an IgG2a control antibody (black line). Background binding of antibodies to empty vector cells are shown for comparison (filled grey). (B) Fold enhancement of VSVΔG pseudovirions transduction into HEK 293T cells transfected with TIM-1 or AnxVΔIgV-TIM-1 relative to empty vector control. Cells were transduced with an MOI of 0.01 as determined from titers in empty vector HEK 293T cells. Data are shown as means ± SEM for at least three replicates. Significance was calculated using one-sample t-test comparison to 1 (**) p<0.001, (*)=p<0.01).
Figure 2.10  Virus binding and internalization occurs independent of glycoprotein. (A) Binding of FITC-labeled EBOV and No GP VSV pseudovirions to HEK 293T cells transfected with TIM-1, mutant TIM-1 ND115DN, or AnxVΔIgV-TIM-1 in the presence or absence of ARD5 (2µg/mL). (B to E) Internalization of FITC-labeled EBOV and No GP pseudovirions (B and C) or VP40-GFP VLPs (D and E) into Vero cells. Virus was added at 4°C to Vero cells that were prebound with or without ARD5 (2µg/mL) or PtdChl or PtdSer liposomes (25µM). As noted, some cells were shifted to 37°C for 30 minutes, whereas others remained on ice. Identified cell populations were treated with trypsin and washed to remove excess virus. Fluorescence was determined by flow cytometry. Representative histograms are shown in panels A, B, and D with filled grey line representing background cellular fluorescence and black line representing virus fluorescence. Histograms are quantified in panels C and E. Data are shown as means ± SD for at least three replicates.
Figure 2.11  RRV and AcMNPV infection is enhanced by TIM-1 expression. (A) Replication of RRV in TIM-1 or empty vector transfected cells at MOIs ranging from 0.001 to 0.1. Supernatants were collected 48 h following infection and titers were determined by endpoint dilution on Vero cells. (B) Infection of TIM-1+ Vero cells with RRV in presence or absence of mAb ARD5 (1µg/mL). Supernatants were collected 48 h after infection and assessed for viral titer by end-point dilution on Vero cells. (C) Transduction of HEK 293T cells or TIM-1+ H3 cells with recombinant baculovirus expressing β-gal at an MOI of 0.003 as determined from titers on HEK 293T cells. After 48 h incubation with virus, cells were lysed and β-gal activity was assessed using Galacto-light. (D) Impact of PtdSer on WT EBOV infection. Vero cells were preincubated for 1 h with the indicated concentrations of PtdSer or PtdChl liposomes or medium alone. Cells were then challenged in the presence of the liposomes with replication competent EBOV encoding GFP. After 24 h, at which time the GFP from one round of infection can be detected, cells were fixed and the proportion of infected cells in the total cell population was determined (see Materials and Methods). The experiment was repeated 3 times with similar outcome (50% inhibitory concentration [IC50], 5 ± 1.5 µM) and the means ± SD of 4 replicates are shown for one experiment. Data for RRV infections shown as means ± SD and AcMNPV as means ± SEM for at least three replicates. Significance was calculated using two-sample t-test comparison with equal variance (**= p<0.001, *=p<0.01).
**Figure 2.12** Organ-derived virus enters cells in PtdSer-binding dependent manner. Infection with mouse organ-derived EBOV GP-rVSV of Vero cells in absence (black) or presence (grey) of ARD5. Virus obtained from lung, spleen, or kidney homogenates was serially diluted onto Vero cells and after 48 h infection was assessed by EGFP expression. Data are shown as means ± SEM for at least three replicates.
Figure 2.13  Model for Phosphatidylserine-Mediated Virus Entry Enhancing Receptors (or PVEERs) uptake of virus. (A) Virus buds from cell surface after infection, incorporating GP and PtdSer onto the viral envelope. The glycan cloud formed by extensive GP glycosylation events provides both stability of the GP in its pre-fusion state and potential steric protection against immune responses to GP residues. (B) Uptake of virus by a neighboring cell. PtdSer on the viral envelope interacts with PVEERs such as the Gas6/Axl complex and TIM family, which mediate virus internalization. (C) Conditions within the endosome promote GP-dependent fusion events. Within the endosome, low pH events can lead directly to GP conformational changes or, alternatively, protease processing of the GP, thereby reducing energy barriers required for fusion. Additional factors, such as binding NPC1 by filovirus GPs, may also be necessary.
Table 2.1 TIM-1 IgV domain mutants

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A Entry is assessed by transduction into cells expressing mutant TIM-1 relative to transduction into cells expressing WT TIM-1. ▼ indicate a decrease in transduction.

B Expression was determined by surface staining with mucin-like domain specific mAb AKG7 and grouped into 10% increments.
CHAPTER III
CHARACTERIZING FUNCTIONAL DOMAINS FOR TIM-MEDIATED ENVELOPED VIRUS ENTRY

Introduction

T-cell immunoglobulin and mucin domain 1 (TIM-1; also called HAVCR-1, TIMD1, or KIM-1) is a member of the TIM family of type 1 cell surface glycoproteins. TIM-1 enhances entry of a broad range of viruses including members of the picornavirus, filovirus, flavivirus, alphavirus, arenavirus, and baculovirus families (47, 55-57, 238, 239). Although TIM-4 is less extensively studied, all evidence suggests it enhances virus entry equivalently to TIM-1. For enveloped viruses, this enhancement is believed to occur through TIM binding to phosphatidylserine (PtdSer) on the viral envelope. By commandeering the cellular machinery involved in the uptake of apoptotic bodies, virus internalization occurs independently of virion glycoprotein interactions with cell surface receptors (56, 57). This mechanism of apoptotic mimicry was first described for vaccinia virus (49). Subsequently, the Gas6/Axl complex, TIM-1, and TIM-4 were identified as cellular proteins involved in this process (54). We have termed this class of viral receptors as PtdSer-mediated virus entry enhancing receptors (PVEERs).

The TIM family of proteins, TIM-1, -3, and -4 in humans, share a structure. Their ectodomains include an amino-terminal immunoglobulin-like variable (IgV)-like domain that extends from the plasma membrane by a heavily O-linked-glycosylated mucin-like domain (MLD) (59). The CC’ and FG loops of all human TIM IgV domains form a pocket that binds PtdSer (60-63). This pocket is highly conserved between the human TIM family members and their murine orthologs. In addition, the integrity of and the key residues within this pocket are necessary for PtdSer binding and subsequently TIM-1-mediated enhancement of virus entry (55-57). However, despite this conservation, TIM-3 much less effectively enhances virus entry (55, 56).
The mechanism by which TIM family members enhance virus entry remains unclear. They have been hypothesized to enhance entry by functioning as attachment factors and concentrating virus on the cell surface by binding PtdSer (55-57). However, this does not account for the ability of TIM-1 to enhance internalization of enveloped viruses that do not have glycoproteins (56, 57). There are two likely mechanisms of entry enhancement that are not mutually exclusive; either TIMs directly internalize virions into endosomes or TIM/virion complexes interact with additional PtdSer binding proteins that facilitate internalization.

In this study, we define the necessary features of the TIM family of proteins for the enhancement of Ebola virus (EBOV) entry. In addition to conserved PtdSer-binding elements of the IgV domain, we found that the length and the domain structure of the MLD are critical for virus binding and transduction. However, the presence of the transmembrane domain and cytoplasmic tail is nonessential for virus entry as a glycosylphosphatidylinositol (GPI)-anchor can replace these elements, demonstrating that TIM cytoplasmic tail signaling is not critical for enhancing EBOV transduction. With knowledge gained from the identification of domains that are required for virus entry, we built a chimeric molecule composed of a GPI anchor, the stalk of \(\alpha\)-dystroglycan, and annexin V that enhanced EBOV entry as efficiently as wild-type (WT) TIM-1.

**Materials and Methods**

**Cell lines and viruses**

HEK 293T cells, a human embryonic kidney cell line were maintained in Dulbecco modified Eagle medium (DMEM) (Gibco BRL) with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Vesicular stomatitis virus (VSV) pseudovirions were produced as previously described (11, 16, 47, 240). We used VSV (strain Indiana) pseudovirions whose genomes had the G glycoprotein gene replaced with enhanced green fluorescent protein (VSV\(\Delta\)G-EGFP).
produce VSV pseudovirions, HEK 293T cells were transfected with plasmids expressing either EBOV GP lacking the mucin domain of GP1, Lassa virus (LASV) glycoprotein precursor (GPC), Sindbis virus (SINV) 2.2 1L1L envelope protein (env), Ross River virus (RRV) GP, GP64, or Chikungunya virus (CHIKV) env (strain OPY1) and transduced 24 h later with VSVΔG-EGFP pseudovirions. After 4 h of virus uptake, the plates were washed and refreshed with media. Pseudotyped virions were collected in supernatant 48 and 72 h after transduction, pooled, and filtered through a 0.45 µm pore-size filter. Virus aliquots were stored at -80°C until use.

Fluorescein isothiocyanate (FITC)-labeled EBOV GP pseudovirions were generated as previously described (57). Briefly, pseudovirions were concentrated by centrifuging supernatants overnight at 4°C at 5,380 × g, resuspended in 500 mM carbonate buffer (pH 9.5) and reacted with FITC-Isomer 1 (Invitrogen) at 4°C for 1 h in the dark. Pseudovirions were then dialyzed into 1x phosphate-buffered saline (PBS) using a 10,000-molecular-weight-cutoff Slide-A-Lyzer dialysis cassettes (Thermo Scientific) and purified by ultracentrifugation through a 20% sucrose cushion at 80,000 x g at 4°C. Pellets were resuspended in 1x PBS, filtered through 0.45 µm pore-size syringe filter, divided into aliquots, and stored at -80°C until use.

**Transductions**

HEK 293T cells were transfected with plasmids as indicated in figures using polyethylenimine (PEI) according to standard protocol (267). Twenty-four hours following transfection, one portion of the cell population was transferred to a six well tissue culture plate and stained for surface expression 24 h later as described below. A second portion of the transfected cells was transferred to a 48 well tissue culture plate and transduced 24 h later with pseudovirions at the multiplicities of infection (MOIs) noted in the figure legends. At 24 h after transduction, the cells were detached with Accutase solution (Millipore), and
EGFP expression was assessed by flow cytometry as the percent positive cells in the FL-1 channel. Transduction data were normalized as indicated in the figure legends.

For the transduction into cells expressing mixtures of MLD mutants, the DNA for MLD mutant expression vectors were mixed together in equal ratios of DNA in the following combinations: (C66 alone, C66+C84, C66+C84+C101, C84 alone, C84+C101, C84+C101+C118, C101 alone, C101+C118, and C66+C84+C101+C118). All cell populations were transfected with the same total mass of DNA; thus, the mass of DNA used for one mutant expression vector in a mixture of three mutants would be one-third the mass of DNA used in the transfection of that mutant alone.

**Surface expression**

Transfected HEK 293T cells were detached with 1x PBS plus 5 mM EDTA 48 h after transfection, washed with 1x PBS plus 5% FBS, and incubated for 1 h with goat polyclonal antisera specific to mTIM-1, TIM-1, TIM-3, or TIM-4 as indicated (R&D Systems) at a concentration of 4 μg/mL. Alternatively, cells were incubated for 1 h with human TIM-1 specific mouse monoclonal antibodies (mAbs) ARD5 or AKG7 (47, 66, 247) as indicated at a concentration of 5 μg/mL. Normal goat serum or purified mouse IgG2a (R&D Systems) were used as negative controls. RAGE and AnxV chimeras were also detected using anti-RAGE and anti-AnxV rabbit, polyclonal antisera (Abcam). Cells were washed and incubated for 20 min with anti-mouse, anti-rabbit, or anti-goat secondary antisera conjugated to either Cy5 (Invitrogen) or DyLight 649 (Jackson ImmunoResearch). After incubation, cells were washed and protein expression was assessed by measuring the percentage of positive cells in the FL-4 channel using a FACSCalibur flow cytometer (BD Biosciences). All flow cytometry data were analyzed using FlowJo software (TreeStar, Inc).

**Generation of TIM mutants and chimeras**

TIM-1 (NM_012206.2), TIM-3 (NM_032782.4), TIM-4 (NM_138379.2), and mTIM-1 (NM_134248.2) cDNAs were cloned downstream and expressed from the human
cytomegalovirus (CMV) immediate-early promoter in pCMV6-XL5. All wild-type and chimeric constructs were sequenced in their entirety to verify the correct composition.

**TIM IgV chimeras.** Chimeras were generated by PCR amplification of the IgV domains with overhanging primers containing restriction enzyme sites. Subsequently, both vector and PCR product were digested with respective enzyme pairs, gel purified, and ligated together. In all cases the restriction sites excised the IgV domain from the parent vector. The sequences encoding the IgV domains of mTIM-1 or TIM-3 were cloned into a TIM-1 expression plasmid using 5’ *Kpn*I and 3’ *Mfe*I restriction sites to create chimeras m1h1 or h3h1 and cloned into a TIM-4 expression plasmid using 5’ *Eco*RI and 3’ *Bam*HI restriction sites to create m1h4 or h3h4, respectively. Initially a *Bam*HI restriction enzyme site was inserted downstream of the TIM-4 IgV domain, introducing an A134G missense mutation that had no effect on EBOV transduction (Figure 3.1A). The sequences encoding the IgV domain of TIM-1 were cloned into a TIM-3 expression plasmid using 5’ *Nhe*I and 3’ *Bst*EII restriction sites to create h1h3.

**TIM-1 MLD length mutants.** Primers pairs containing overhanging 5’ *Mfe*I and 3’ *Pst*I restriction enzyme sites were designed to amplify various regions between the *Mfe*I and *Pst*I restriction sites present within the TIM-1 MLD coding region of the expression plasmid. PCR products and TIM-1 expression vector were digested with *Mfe*I and *Pst*I, separated on an agarose gel, purified, and ligated. Mutations were confirmed by sequencing. The sequences are aligned in Figure 3.3A.

**TIM-1 cytoplasmic tail mutants.** The TIM-1 construct lacking the cytoplasmic tail domain (h1Δcyto) has been previously described (66). The TIM-3, TIM-4, and mTIM-1 cytoplasmic tail deletions (h3Δcyto, h4Δcyto, and m1Δcyto) were created by mutating Y227, L340, or S268, respectively, to stop codons, resulting in early termination after the transmembrane domain, while still retaining a few cytoplasmic domain, charged residues.

The TIM-1 cytoplasmic tail exchange mutants were created by first introducing a *Bam*HI site downstream of the transmembrane domain, resulting in a YF320GS mutation.
that had no effect on expression or EBOV transduction (Figure 3.1B). The sequences encoding the cytoplasmic tails of TIM-3, TIM-4, and mTIM-1 were amplified by PCR with primers encoding overhanging BamHI (TIM-3 and TIM-4) or BglII (mTIM-1) and XbaI restriction sites. After excision of the TIM-1 cytoplasmic tail using BamHI and XbaI restriction sites, these PCR products were ligated into the modified TIM-1 plasmid to construct TIM-1 chimeras containing the cytoplasmic tail of either mTIM-1 (h1m1cyto), TIM-3 (h1h3cyto), or TIM-4 (h1h4cyto).

**TIM-1 GPI-anchored mutants.** A plasmid expressing GPI-anchored murine TIM-4 (mTIM-4) in which the transmembrane and cytoplasmic tail domains of mTIM-4 are replaced with the carboxy-terminal 37 amino acids (aa) of decay-accelerating factor (DAF) was kindly provided by Kodi Ravichandran (University of Virginia) (69). The sequences encoding the IgV and MLD domains of TIM-1, TIM-3, TIM-4, and mTIM-1 were amplified by PCR using primers encoding overhanging 5' BglII and 3' SbfI restriction enzyme sites and ligated into the GPI-anchored mTIM-4 plasmid after digestion of the vector at these same sites. This resulted in GPI-anchored mTIM-1 (m1-GPI), TIM-3 (h3-GPI), and TIM-4 (h4-GPI) expression plasmids.

A protocol used by Park et al. (69) was modified to confirm GPI-linkage of our mutants. Briefly, HEK 293T cells transfected with wild-type (WT) TIMs or GPI-anchored TIM expression constructs were plated in 24 well plates and incubated 48 h later with or without 0.4 U of phosphatidylinositol-specific phospholipase C (PI-PLC; Invitrogen) at 37°C for 1 h in 100 μL of PBS. PI-PLC cleaves the phosphoglycerol bond within GPI, releasing GPI-anchored proteins from the membrane. Flow cytometry analysis of surface stained cells confirmed that the expression of GPI-anchored TIMs was reduced from cell surface after PI-PLC treatment, whereas there was no detectable loss of WT TIMs (see Figure 3.2H).
\textit{αDG chimeras.} The MLD of TIM-1 was replaced with that of murine alpha-dystroglycan (αDG) to generate αDG-MLD. The cDNA of murine dystroglycan (DAG1) was kindly provided by Kevin Campbell (University of Iowa). The sequences encoding the MLD of murine αDG (aa 315 to 463) and the transmembrane and cytoplasmic domains of human TIM-1 (aa 295 to 364) were amplified by PCR, fused by an additional PCR amplification using an overlapping primer set, and cloned into an expression vector containing the signal peptide and IgV domain sequences of TIM-1. The percent identity between MLD of αDG (aa 314 to 483) and TIM-1 (aa 128 to 294) was determined by CLUSTAL W alignment of amino acid sequences using MegAlign software (DNASTAR).

A construct containing both the MLDs of TIM-1 and αDG, called 2xMLD, was generated by inserting the TIM-1 IgV and αDG MLD sequences from αDG-MLD upstream of the MLD and cytoplasmic domains of TIM-1. Thereby, the MLD of αDG is between the IgV and MLD of TIM-1.

\textit{AnxV- αDG-GPI chimera.} A plasmid encoding human annexin V (AnxV) was kindly provided by Seamus Martin (Trinity College, Dublin, Ireland). The sequences encoding AnxV and the MLD of murine αDG (aa 314 to 485) were amplified by PCR, joined by a second PCR amplification using primers with overhanging BglII and SbfI restriction sites, and ligated into the mTIM-4 GPI vector between the immunoglobulin κ-chain leader sequence and the DAF sequence, replacing the mTIM-4 coding sequence.

\textit{PRR chimera.} h1PRR was generated using In-Fusion HD (Clontech) following the manufacturers protocol. Two fragments encoding aa 242 to 300 and 239 to 321 of the proline rich region (PRR) from the amphotropic 4070A murine leukemia virus (MuLV) envelope gene, kindly provided by Dr. Paul McCray (University of Iowa), were amplified by PCR. These fragments were inserted in tandem between sequences encoding amino acids 129 and 295 of WT TIM-1 to create a chimera in which the MLD of TIM-1 is replaced with two copies of the PRR from MuLV env.
**RAGE chimeras.** h1RAGE was generated by first PCR amplifying the IgV domain of TIM-1 (aa 1 to 125) and sequences downstream of the human receptor for advanced glycation endproducts (RAGE) IgV (aa 119 to 404) (Origene). These amino acid residues of RAGE compose the two IgC2, transmembrane, and cytoplasmic domains of the protein. These TIM-1 and RAGE sequences were joined by a second PCR amplification and ligated into a vector, downstream of the CMV promoter.

h1RAG£h1cyto was generated using In-Fusion HD (Clontech). The h1RAGE vector and ectodomain of RAGE were amplified by PCR and joined according to the manufacturer’s protocol. The resulting construct contained the IgV domain of TIM-1 (aa 1 to 124), the extracellular domains of RAGE without the RAGE IgV domain (aa 119 to 338), and the transmembrane and cytoplasmic domains of TIM-1 (aa 295 to 364).

h1RAG£mucin was constructed similarly to h1RAGE, resulting in a portion of the TIM-1 MLD and entire transmembrane and cytoplasmic domains of TIM-1 (aa 209 to 364) being inserted downstream of the RAGE stalk sequence (aa 119 to 335) of h1RAGE.

RAGE-IgV was generated by amplifying the RAGE IgV domain (aa 1 to 123) with primers encoding 5' KpnI and 3' MfeI restriction sites. Sequences encoding aa 126 to 133 of TIM-1 were also inserted in the 3’ reverse primer, between the homologous RAGE sequence and MfeI cut site. The TIM-1 expression vector was digested with KpnI and MfeI, resulting in excision of the TIM-1 IgV domain, and the digested PCR product was ligated into the cut site.

**FITC-pseudovirion VLP binding assay**

At 48 h after transfection with TIM-1, empty vector, or MLD TIM-1 mutants, HEK 293T cells were incubated with FITC-labeled EBOV GP pseudovirions (MOI of 2.25 as determined from titers on Vero cells) for 1 h on ice. The cells were washed three times with 1x PBS plus 5% FBS and the cellular fluorescence was either determined immediately
by flow cytometry or the cells were additionally stained with AKG7 as described above prior to flow cytometry for analysis of TIM-1 cell surface expression.

**Enzyme-linked immunosorbent assays (ELISAs)**

Soluble TIM-1 was produced in HEK 293T cells by transfecting in an expression vector, previously described (57), in which the transmembrane and cytoplasmic domains of TIM-1 are replaced with a hemagglutinin (HA) tag. Supernatant was collected 48 h after transfection. TIM-1 MLD mutants were similarly generated after subcloning of the mutants described above into the soluble WT TIM-1 expression vector. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Western blot) or serial dilutions of proteins were applied directly to membranes and pulled through via vacuum by using a dot blot apparatus (dot blot). Nitrocellulose was blocked for 2 h in 1x PBS with 10% nonfat dry milk and subsequently incubated for 2 h with anti-HA antisera (1:4000) in 1x PBS with 10% milk and 0.15% Tween 20. Protein expression was imaged using a LI-CORE Odyssey CLx and quantified by measuring the total fluorescent signal and subtracting background signal using Image Studio (LI-CORE). The protein levels were normalized by first plotting fluorescent data from dot blots against the dilution factor for each mutant TIM-1 and WT TIM-1. A line was fitted to the scatter plot for each construct and data points outside the linear range due to saturation of the nitrocellulose were eliminated. The slope of these lines was then used to normalize relative amounts of supernatant to use for each mutant and WT TIM-1. Appropriate normalization of dilutions was confirmed by Western blot.

Nunc MaxiSorp ELISA plates were precoated overnight with ~5.2x10⁶ transducing units (as determined from titers on Vero cells) of concentrated EBOV pseudovirions or PtdSer or PtdChl liposomes (50 μM) diluted in 1x TBS+ (150 mM NaCl, 25 mM Tris, 10 mM CaCl₂, pH 7.2). PtdSer liposomes were generated as previously described (57). Supernatants were diluted in 1x TBS+ to normalize TIM-1 protein amounts as described above. Plates were blocked for 2 h at 4°C with 1xTBS+ with 2% BSA, incubated with serial
dilutions of supernatants for 2 h, probed with rabbit polyclonal anti-HA antisera (Sigma) for 1 h and horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody for 1 h, and developed using 1-Step Ultra TMB-ELISA (Thermo). Plates were washed thoroughly between steps with 1x TBS+. The absorbance was read at 450 nm.

**Results**

**TIM family members enhance EBOV entry.**

We and others recently identified TIM-1 and TIM-4 as PVEERs (47, 55-57). The IgV domains of TIM family members share a conserved PtdSer binding pocket responsible for interacting with PtdSer on the virion surface and essential for TIM-1-mediated enhancement of virus entry. However, despite containing a PtdSer binding pocket, TIM-3 does not effectively function as a PVEER (47, 55, 56). In these studies we sought to determine the structural basis for the high PVEER efficiency of TIM-1 compared to TIM-3. In addition, we wanted to test whether the murine homolog of human TIM-1 (mTIM-1) that shares many of the same structural features also mediates enveloped virus transduction.

PVEER efficiency was quantified by measuring transduction of pseudotyped vesicular stomatitis virus (VSV) that expressed EGFP as reporter molecule in place of native G. Although TIM-1 enhances the entry of a number of enveloped viruses, EBOV GP pseudotyped VSV (EBOV pseudovirions) entry is most strongly enhanced by TIM-1, whereas the entry of LASV GPC pseudotyped VSV (LASV pseudovirions) entry is unaffected by TIM-1 expression (47, 56, 57). Thus, stocks of these two pseudovirions were used to identify TIM sequences required for virus transduction. EBOV GP lacking the GP1 mucin domain was used since this protein confers the same tropism as the full-length Ebola virus GP and produces higher pseudovirus titers (21, 240, 250). However, entry mediated by the full-length Ebola virus GP is enhanced in an equivalent manner to the mucin domain deleted GP by the expression of TIM-1 (47, 56, 57).
HEK 293T cells transiently expressing TIM-1, mTIM-1, TIM-3, or TIM-4 at comparable and robust levels on the cell surface (Figure 3.2A) were transduced with EBOV and LASV pseudovirions. For comparison, cells transfected with empty vector were also transduced. Similar to TIM-1 and TIM-4, mTIM-1 increased EBOV transduction; but TIM-3 only slightly enhanced transduction (<2 fold) (Figure 3.2B). Consistent with previous findings (47, 56, 57), TIM proteins did not enhance LASV entry. Thus, LASV pseudovirion entry was used throughout these studies as a control.

The IgV domains from all tested TIMs mediate entry.

The ability of other TIMs to enhance EBOV entry more effectively than TIM-3 might be due to unique aspects of their IgV domains or higher affinity for PtdSer (62). This was tested by replacing the IgV domain of TIM-1 or TIM-4 with that of mTIM-1 (m1h1 and m1h4) or TIM-3 (h3h1 and h3h4). In addition, the IgV domain of TIM-3 was replaced with that of TIM-1 (h1h3). All chimeric TIM proteins were expressed abundantly on the surfaces of transfected HEK 293T cells (Figure 3.2C). The chimeras containing the IgV domain of either mTIM-1 or TIM-3 increased transduction of EBOV pseudovirions similar to WT TIM-1 (Figure 3.2D). However, the h1h3 chimera containing the TIM-1 IgV domain and the remaining domains from TIM-3 was as deficient as WT TIM-3. These results indicated the IgV domain of TIM-3 is not responsible for its reduced efficacy to enhance EBOV transduction, but rather the other domains are.

Cytoplasmic and transmembrane domains of TIM family are not essential for EBOV transduction.

Due to retention of the MLD, transmembrane domain, and cytoplasmic tail of TIM-4 or TIM-1 in our functional TIM-3 chimeras, we evaluated the contribution of these domains to transduction efficiency. TIM-1 (66), TIM-3 (65), and mTIM-1 (67, 68) have cytoplasmic tails that after tyrosine phosphorylation can potentiate cell signaling, which could contribute to transduction. However, the cytoplasmic tail of TIM-4 has no known
signaling function (69). To determine the potential role of cytoplasmic domain signaling during EBOV transduction, we tested chimeras in which the cytoplasmic tail of TIM-1 was exchanged with that of mTIM-1 (h1m1cyto), TIM-3 (h1h3cyto), or TIM-4 (h1h4cyto). Transfection of chimeric TIM proteins resulted in equivalent transduction efficiencies (Figure 3.2E), suggesting either no role for the cytoplasmic tail or a mechanism of signaling is shared between the cytoplasmic tails of TIM proteins.

To further investigate the importance of the cytoplasmic tail domains in EBOV transduction, we tested TIM mutants whose cytoplasmic tails were deleted. The TIM-3 and TIM-4 mutants (h3Δcyto and h4Δcyto) supported transduction in a manner similar to the WT proteins (Figure 3.2F), while deletion of the TIM-1 or mTIM-1 cytoplasmic tails (h1Δcyto or m1Δcyto, respectively) resulted in an approximately 60 or 70% decrease. The decrease in transduction might be explained by reduced expression and, indeed, h1Δcyto and m1Δcyto were expressed on fewer cells compared to WT (Figure 3.2G). In addition, the cytoplasmic or transmembrane domains of TIM-1 and mTIM-1 might contribute to appropriate localization of these proteins to plasma membrane. In order to examine this possibility, TIM family mutants were generated in which the ectodomains of each TIM composed of the IgV and MLD were anchored to the plasma membrane via a GPI anchor, as confirmed by PI-PLC treatment (Figure 3.2H). Unlike the cytoplasmic deletion mutants, there were no significant differences in expression (Figure 3.2H) or transduction (Figure 3.2I) of any of the GPI anchored TIMs compared to their WT counterparts. These results rule out a role for the transmembrane domain in transduction.

**Deletions in the TIM-1 MLD decrease virus binding and transduction.**

Based on the results presented above, the differences in PVEER efficacy observed between TIM-3 and the other family members cannot be accounted for by the IgV, the transmembrane, or cytoplasmic domains. Thus, focus was directed to the MLD. The MLDs
of the TIM family members differ in several ways: amino acid sequence, extent of O- and N-linked glycosylation, and overall length. Noting that TIM-3 has a considerably shorter MLD (~66 aa) than TIM-1, mTIM-1, and TIM-4 (~167, 107, and 174 aa, respectively) and that significant deletion within the TIM-1 inhibits virus uptake (56), we hypothesized that the length of the MLD might be important for PVEER function. For example, a minimal length of the MLD may be needed to project the IgV domain sufficiently far from the surface of the cell for interaction with virus. To test the role of the MLD length, a series of TIM-1 MLD mutants were generated by deleting various lengths within a highly repetitive region of the MLD. These mutants retained either amino-terminal (N), middle (M), or carboxy-terminal (C) sequences of this region and the length of the MLD is noted in the name of each MLD mutant. The amino acid sequences of the TIM-1 MLD mutants are aligned with TIM-1 in Figure 3.3A. In addition to deletions, some mutants had point mutations generated by introduction of the restriction sites.

Initial studies with this panel of mutants were performed to determine the effect that reductions of MLD length had on virus binding. We quantified FITC-labeled EBOV pseudovirion binding to cells transfected with WT TIM-1, a selection of MLD deletion mutants, or an empty vector. Mutants containing large MLD deletions such as C66 did not show appreciable increases in virion binding compared to cells expressing empty vector (Figure 3.3B). However, as the MLD lengthened, virion binding increased, reaching levels comparable to the WT, suggesting that the length of the MLD is important for TIM-1 to bind EBOV.

To determine whether the cells expressing the largest amounts of these TIM constructs bound the greatest amounts of virus, we bound virus to MLD mutant-transfected HEK 293T cells and assessed the cells for both TIM-1 expression using the MLD-specific mAb AKG7 and binding of FITC-labeled virus. Except for C66 and N66, the MLD length mutants were detected at or near wild-type levels (Figure 3.3C). An IgV domain mAb, ARD5, detected levels of surface expressed C66, N66, and other MLD mutants equivalent to
those of the WT (Figure 3.4), suggesting AKG7 detection of C66 and N66 mutants was suboptimal, perhaps due to steric hindrance by the IgV domain which is much closer to the AKG7 epitope in this mutant (268). As evidenced in the contour plots shown in Figure 3.3C, virus binding was more robust on cells expressing higher levels of TIM-1. Quantification of binding from Figure 3.3B illustrates a strong correlation between virus binding and MLD length (Figure 3.3D). To determine whether TIM-1 MLD length is only important for binding virion-associated PtdSer in the context of the cell surface or if soluble TIM-1 constructs with shortened MLDs also bound with reduced efficiency, we assessed the ability of various length soluble TIM-1 mutants to bind PtdSer liposomes in an ELISA. Serial dilutions of normalized quantities of soluble HA-tagged MLD length mutant TIM proteins, as demonstrated by an immunoblot (Figure 3.3E), were incubated with ELISA plates precoated with PtdSer liposome. All MLD length mutants bound to PtdSer liposomes similarly to the WT (Figure 3.3F). We confirmed these studies with EBOV pseudovirions and found binding to be similar for soluble MLD mutants containing short or long MLDs (Figure 3.5A). Neither WT TIM-1 nor MLD mutants bound to plates alone nor plates coated with phosphatidylcholine (Figure 3.5B). Thus, while reduced virion binding of short MLD mutants was observed when the TIM was membrane associated, this was not observed with soluble mutant proteins.

To determine whether the reduction in virus binding correlated with reduced ability to enhance virus entry, we evaluated the transduction mediated by all 18 of the MLD deletion mutants. The enhancement of EBOV transduction correlated with increasing MLD length, the effect plateauing at WT TIM-1 levels of transduction (Figure 3.6A to C). A similar plateau did not occur in the binding studies, likely due to our ability to detect binding of multiple virions to the same cell by flow cytometry, but not transduction of multiple virions into a single target cell by an increase in EGFP expression. Notably, wild-type levels of PVEER function were seen regardless of if amino-, carboxy-terminal, or combinations of both deletions were present, provided the overall length of the MLD was
-120 residues or longer. A scatter plot of the binding data from Figure 3.3D and the transduction data from Figure 3.6A showed a positive correlation between ability of the TIM-1 mutants to bind to EBOV pseudovirions and enhance transduction (Figure 3.6D).

We hypothesized that expressing MLD mutants of different lengths on the same cell might have a synergistic effect due to mutants with longer MLDs passing virus off to those with shorter MLDs, thereby enhancing the binding capacity of the shorter mutants. To test this possibility, HEK 293T cells were transfected with equivalent total amounts of DNA containing various groupings of the C66, C84, C101, and C118 MLD mutants. Transduction efficiency into mixed or single MLD mutants, WT, or empty vector populations was plotted against the average MLD length (Figure 3.6E). If synergy was occurring, we would expect that transduction into mixed MLD populations to be equivalent to transduction into cells expressing only the longest MLD mutant from that group. However, we found that the transductions of mixed populations were equivalent to the average transduction of the MLD lengths transfected, suggesting that no synergy was occurring.

**The specific sequence of MLD is nonessential.**

Although a sufficient length MLD is required for TIM-1 enhancement of transduction, we wanted to determine whether specific sequences within the MLD were required in addition to a minimal length. Our first approach to address this question was to replace the 167 aa that compose the TIM-1 MLD with 157 aa of an unrelated MLD from murine alpha-dystroglycan (αDG) to generate the αDG-MLD chimera. While both MLD sequences are rich in threonines, serines, and prolines, there is only 19.9% identity between the two sequences (Figure 3.7A). αDG-MLD expression was comparable to that of WT TIM-1 (Figure 3.7B) and mediated WT levels of EBOV pseudovirion transduction (Figure 3.7C). These results suggested that a specific MLD sequence is not required.
Although we found transduction enhancement plateaued at a MLD length of ~120 aa, we also assessed whether a longer MLD would be more effective. Insertion of the TIM-1 MLD downstream of the αDG MLD present in the αDG-MLD chimera resulted in a new TIM-1 chimera with an MLD length roughly twice that of WT TIM-1, 317 aa (2xMLD). Our 2xMLD mutant mediated transduction of EBOV pseudovirions equivalently to WT TIM-1 (Figure 3.7C).

**Structural requirements of the MLD.**

Protein sequences containing a high frequency of O-linked glycosylations and prolines such as MLDs are believed to form long extended, beta-turn helix conformations (269). Therefore, we hypothesized that the role of the MLD in PVEERs is to provide an extended stalk structure that positions the IgV domain of TIM-1 a sufficient distance from the host cell surface for interaction with virions. In order to test this hypothesis, we replaced the TIM-1 MLD with the well-studied murine leukemia virus (MuLV) SU glycoprotein proline-rich region (PRR) that is not considered to be an MLD, but acts as a flexible hinge (270) and likely forms a beta-turn helix (271). As the PRR is only half the minimal length required for a PVEER MLD, two head-to-tail tandem repeats of the PRR consisting of a total of 142 aa were substituted for the TIM-1 MLD in the h1PRR chimera. This h1PRR chimera also is predicted to contain 35 O-linked glycosylations as detected by NetOGlyc 4.0 Server, about half as many the predicted 67 for WT TIM-1s (272). Expression of this chimera on the surfaces of cells was considerably lower than that of WT TIM-1. In order to examine transduction using cells with comparable surface expression of h1PRR and WT TIM-1, the amount of TIM-1 DNA transfected was reduced until the surface expression of the TIM-1 IgV domain as detected by ARD5 was equal to that of the h1PRR-transfected cells (Figure 3.7D). After compensating for expression, h1PRR supported transduction similar to WT TIM-1 (Figure 3.7E).
Next, we sought to determine whether a more structured stalk of equivalent or longer length would functionally replace the TIM-1 MLD. We previously showed that another PtdSer-binding protein, RAGE, does not enhance virus entry (57). Like TIM-1, RAGE contains an N-terminal IgV domain, but the stalk region of RAGE is composed of two structured Ig-like C2 domains, each composed of more than 90 aa (273). We generated several TIM-1/RAGE chimeras and compared transduction efficiency to that of WT TIM-1. Similar to h1PRR, the TIM-1/RAGE constructs had reduced expression compared to WT TIM-1 and for these mutants we compensated in a similar manner (Figure 3.7D). For our initial TIM/RAGE chimera, we replaced the IgV domain of RAGE with that of TIM-1 (h1RAGE). The presence of the TIM-1 IgV domain on the remainder of RAGE minimally enhanced virus entry above that of the empty vector, resulting in ~25% of WT TIM-1 transduction levels (Figure 3.7E). Replacement of the RAGE derived transmembrane and cytoplasmic domains of the h1RAGE chimera with that of TIM-1 (h1RAGEh1cyto) did not further enhance transduction. Although we have found RAGE expression did not enhance transduction above empty vector (57), the minimal increase by h1RAGE and h1RAGEh1cyto were significantly above background as determined by a two-sample t-test (p<0.01), suggesting the IgV domain of TIM-1 in the context of these chimeras conferred some PVEER activity. More significantly, insertion of 86 aa from the TIM-1 MLD between the RAGE Ig-like C2 domains and the TIM-1 transmembrane domain of h1RAGEh1cyto (h1RAGEmucin) rescued transduction to WT TIM-1 levels. A final chimera in which the IgV domain of TIM-1 was replaced with the RAGE IgV (RAGE-IgV), retaining the TIM-1 mucin-like, transmembrane, and cytoplasmic domains, was expressed (Figure 3.8A) but not sufficient to enhance EBOV entry (Figure 3.8B), a finding consistent with the inability of WT RAGE to enhance transduction.
Mimicking functions of TIM-1 domains produces an artificial PVEER.

In this and previous studies we individually substituted the IgV domain (57), MLD (Figure 3.7C), and cytoplasmic domains of TIM-1 (Figure 3.2I) and retained PVEER functionality. Together, we interpret these results to indicate that PVEER function is derived from the combination of a PtdSer binding domain linked to the host cell membrane by an MLD of at least 120 residues. If this interpretation is correct, then it should be possible to generate a PVEER chimera without any TIM-1 sequence. We created AnxV-αDG-GPI by attaching annexin V, a PtdSer-binding protein, to a GPI-anchored MLD of αDG. This chimera was detected by an annexin V specific antibody but not TIM-1 specific mAbs (Figure 3.7F) and enhanced EBOV pseudovirion entry as effectively as WT TIM-1 (Figure 3.7G). In addition, like TIM-1, AnxV-αDG-GPI enhanced entry of VSV pseudovirions bearing Sindbis 2.2 1L1L env (SINV) (254-256), Ross River virus (RRV) GP, Chikungunya virus (CHIKV) env, or baculovirus Autographa californica nucleopolyhedrovirus GP64. Interestingly, we also found that AnxV-αDG-GPI was more effective at enhancing entry than substitution of the TIM-1 IgV domain with AnxV alone (Figure 2.9B). This may be attributed to the MLD of αDG expressing more effectively than that of TIM-1 as our αDG-MLD mutant expressed slightly better than WT TIM-1 (Figure 3.7B). Alternatively, the N-terminal sequences of the AnxV-αDG-GPI and AnxVΔIgV-TIM-1 MLDs may differently orient AnxV such that it is more effectively positioned in AnxV-αDG-GPI.

Discussion

TIM-1 and TIM-4, along with other PVEERs such as Gas6/Axl, have recently been identified as broad enhancers of virus entry through interaction with PtdSer on the viral envelope (47, 54-56). We and others found that mutations which alter the TIM-1 IgV PtdSer binding pocket decreased virion binding and transduction, providing compelling evidence that the IgV domain is critical for PVEER function (55-57). However, we confirm
here that TIM-3 is poor at enhancing virus entry, as others have found (47, 55, 56), despite the conservation of PtdSer-binding activity (62). In the present study, we extended this understanding by characterizing the features of the other TIM-1 domains required for enhancing virus entry and determining those that account for the differences in efficacy among TIM family members. Through extensive analysis of chimeric proteins and deletion mutants, we established several conclusions regarding the characteristics of PVEER domains. First, neither the cytoplasmic tail nor the transmembrane domain is needed for PVEER function, provided the protein is still membrane bound. Second, a MLD of sufficient length is required for PVEER function and additional length beyond ~120 aa is extraneous. Third, a specific MLD sequence is not required, and proline/serine/threonine rich sequences from other proteins can substitute for that of TIM-1 MLD. However, some structural constraints on this region are apparent, as evidenced by the failure of Ig-like C2 domains containing similar numbers of residues to substitute for the TIM-1 MLD. Finally, an artificially generated PVEER that mimics the functional domains of TIM-1 mediates transduction as efficiently as WT TIM-1 even though it lacks sequences from any TIM family member.

The mechanism of TIM-mediated enhancement of virus entry and uptake of apoptotic bodies remains unknown. The cytoplasmic tails of TIM-1 and TIM-3 are functionally important in T-cells for signaling through pathways associated with activation of the T-cell receptor signaling complex (65-68, 274). However, these TIM family members have not been reported to be involved with uptake of apoptotic bodies or viruses in T cells and these signaling events may be required for other T-cell specific functions. Consistent with this possibility, we and others have demonstrated that the intracellular domains of the TIM family members are dispensable for enhancement of virus entry (55). However, we cannot rule out that these domains do contribute during endogenous expression. In addition, our observations that the TIM-1 IgV domain can be replaced with annexin V (57) and the MLD with that of αDG or Pro/Ser/Thr rich sequences from MuLV Env suggest that specific, direct, intracellular, and extracellular protein-protein interactions are nonessential
for TIM family PVEER function. On one hand, our findings are consistent with previous studies that suggest the TIM family members enhance uptake of apoptotic bodies or virus by functioning as attachment factors (55, 56, 69). However, such a model of virion entry must account for the ability of PVEERs to stimulate rapid uptake of viruses lacking a glycoprotein (56, 57). If PVEERs do not directly mediate virus internalization into endosomes, additional receptors or cofactors responsible for internalization must also bind the virion envelope. Alternatively, binding of multiple PVEERs may result in local changes to plasma membrane structure, such as membrane curvature, that induce cargo internalization in response.

Members of the TAM family of receptor kinases (Tyro3, Axl and Mer) are PVEERs that when complexed with Gas6 enhance binding of virions to cells (54, 55). In contrast to the TIM proteins, the TAM receptors require signaling through their cytoplasmic domains for robust enhancement of virus infection (37, 54, 55, 110). However, this signaling inhibits innate immune responses and has not been shown to directly enhance entry (110). This is supported by the ability of a kinase-dead Axl to enhance virus internalization equivalently to WT Axl (55, 110). Thus, although signaling events of the TAM kinases contribute significantly to infection, signaling likely does not affect virus internalization and therefore are unlikely to contribute to PVEER function, which is consistent with our conclusions for TIM-1.

We would predict that TIM-1 expression in the host has both beneficial and deleterious effects; although TIM-1 is important for immune regulatory functions and clearance of dying and apoptotic cells, it also enhances virus entry. Thus, it is not surprising that high sequence variability and evidence of positive selection within the TIM-1 MLD is found in human populations (275). There is also some evidence for variability within Old World monkeys (265). In both cases, this variation is due to amino acid substitutions or insertions/deletions at multiple sites within the MLD. For instance, in humans, a frequent TIM-1 MLD polymorphism, 157ins/delMTTTVP, results in a 6 aa insertion or deletion. However, we and others (56) have found that larger MLD sequence deletions are required
for significant effects on TIM-1 uptake of virus than seen within natural variation. Large
TIM-1 MLD deletions may have profound consequences for immune function. In mice,
deletions within the TIM-1 MLD lead to defects in IL-10 production by B cells and
subsequent development of autoimmune disorders (276). This is attributed to the inability
of B cells to interact with apoptotic cells and induce IL-10 secretion (277). Even the smaller
157ins/delMTTTVP polymorphism found in humans has been associated with autoimmune
problems of atopy, eczema, and asthma (275, 278). However, the linkage between these
diseases and TIM polymorphisms remains controversial, perhaps due to very modest
alteration of MLD length. In total these studies suggest that a MLD of sufficient length is
essential for efferocytosis and maintenance of homeostasis. Significant deletions to evade
viruses would be expected to come at the price of considerable detrimental effects.
Interestingly however, TIM-1 is nonfunctional in many New World monkeys (265),
suggesting either that compensatory changes can overcome these effects or that the function
of TIM-1 can differ in some species.

While the specific sequences of the MLD are not essential for enhancement of
transduction, some constraints are apparent. Noting that our MLD deletion mutations
encompassed about three-quarters of the MLD residues in a region that contains repetitive
threonine, serine, and proline-rich sequences and predicted O-linked glycosylation sites, we
chose to construct chimeras using two glycoproteins that lack sequence homology to TIM-1,
but are enriched in similar residues, and a third protein, RAGE, that has more condensed
structure (Figure 3.7A). Although the 225 residues IgC-2 domains of RAGE are longer than
the TIM-1 MLD, these sequences fold into rigid and relatively compact beta-barrels
stabilized by two disulfide bonds (230, 273). In contrast, while the structures of the TIM-1
and αDG MLDs and the PRR of MuLV have not been solved, these domains are thought to
have an extended structure. Interestingly, similar domains were recently shown by using
small angle x-ray scatter, circular dichromism, and nuclear magnetic resonance to fold into
multiple conformations between which they readily interconvert, producing intrinsic
disorder that evades structure solution by conventional X-ray crystallography (279). In organic solvents, they fold into extended polyproline II helices in which all the proline residues are trans, but in water cis isomerization of one or more proline residues creates turns and generates multiple conformers (279), lending a degree of flexibility to shape and size that has been shown to be essential to domain function of several proteins (280-283). In our model (see Figure 3.9A), key chimeras are depicted as stalks of extended, rod shaped, polyproline-like helices. However, we speculate that this is one of many conformations in which these domains can fold and that interconversion provides flexibility to a substantial area pivoting around the carboxy-terminal most proline residues (Figure 3.9B).

A longer MLD could provide several benefits for enhancing virus entry over a shorter MLD. The enhanced length may potentially project the PtdSer binding pocket of the IgV, necessary for interaction with PtdSer on the virus, above other cell surface proteins and sugars, thereby reducing steric hindrance (Figure 3.9A). This might explain why the additional length of the 2xMLD mutant provides no additional benefit once the IgV has already cleared the majority of cell surface milieu. Similarly, steric hindrance may also be caused by proteins present on the virion surface such as the GP. In addition, the increased length of the MLD might provide a greater area of interaction with the viral membrane by expanding the circumference of rotation around the anchor or transmembrane domain (Figure 3.9B). Finally, the longer MLD may provide some flexibility for interaction with and passage of virus to other proteins. Although our studies did not find synergy between MLD of various lengths that would suggest transfer of virus between TIM proteins, flexibility may still allow for multiple TIM molecules to bind to the same viral envelope by bending as needed to the curvature of the viral envelope (Figure 3.9C). Consistent with this possibility, both adenoviruses and reoviruses have been shown to require flexibility and length in the stalk of their attachment proteins for receptor binding (284, 285). Taken together these results suggest that adequate distance and flexibility on either the host or virus side are necessary for effective receptor/virus interactions and infection.
**Figure 3.1** Insertion of cut sites into TIM-4 and TIM-1 introduces mutations. Transduction of VSV virions pseudotyped with EBOV GP or LASV GPC into cells expressing TIM-4/TIM-4 mutant A134G (A), TIM-1/TIM-1 mutant YF320GS (B), or empty vector (Empty). Transductions are shown as percent of GFP positive cells. Cells were transduced with an MOI of 0.005 (EBOV) or 0.06 (LASV). Data are shown as mean ± SD for a single replicate.
**Figure 3.2** Cytoplasmic and transmembrane domains are nonessential for PVEER activity of TIM proteins. (A) Surface expression of human TIM-1, TIM-3, and TIM-4 and murine TIM-1 in HEK 293T cells. At 48 h after transfection, HEK 293T cells were incubated with polyclonal antisera against the appropriate TIM family member. Cells transfected with the empty vector were stained with anti-TIM-1 polyclonal antisera. (B, D to F, and I) Transduction of VSV virions pseudotyped with EBOV GP or LASV GPC into cells expressing TIM family members (B), IgV domain chimeras (D), cytoplasmic tail switch (E) or deletion (F) mutants, or GPI-anchored TIMs (I). Schematics of WT and chimeric proteins are shown below respective transduction data. IgV domains are represented by a crescent, MLDs are represented by a rectangle, and transmembrane/cytoplasmic domains are represented by a triangle. (C, G and H) Surface expression of IgV domain chimeras (C), TIM cytoplasmic deletion mutants (G) and GPI-anchored TIMs (H) in HEK 293T cells 48 hours after transfection. (C) IgV chimeras were detected using polyclonal antisera specific to the WT proteins from which either the IgV domain or MLD were derived. (G) Comparison of expression of TIM family members (filled grey line) and cytoplasmic tail deletions (solid black line). (H) Surface expression of WT and GPI-anchored TIM family members with (dotted black line) or without (solid black line) PI-PLC treatment. A filled grey line represents background antisera binding. Transductions were normalized to transduction of WT TIM-1 (D and E) or equivalent WT TIM (F and I). Cells were transduced with an MOI of 0.03 (B,D, F, and I) or 0.01 (E) as titered in empty vector HEK 293T cells. Data are shown as mean ± SEM for at least three replicates. For (D-F and I) significance was calculated using a one-sample t-test comparison to 100 (normalized value for EBOV transduction in WT transfected cells (**) p<0.001, *=p<0.01).
Figure 3.3  Length of the MLD affects binding of EBOV virions. (A) CLUSTAL V alignment of a region of TIM-1 and mutant MLD amino acid sequences (aa 128 to 250 of WT TIM-1). Alignment was performed using MegAlign (DNASAR). Mutants were named according to whether they retained amino-terminal (N), middle (M) or carboxy-terminal (C) sequences of the MLD and the length of their MLDs. (B) Binding of FITC-labeled VSV virions pseudotyped with EBOV GP to HEK 293T cells expressing WT TIM-1 or MLD mutants as assessed by flow cytometry. All mutants shown retain carboxy-terminal sequence of the MLD. Representative histograms are shown with black lines representing virus binding and grey-filled lines representing background fluorescence. (C) Representative contour plots of FITC-labeled virion binding versus TIM-1 expression. Transfected cells were stained with MLD specific, mAb AKG7, after binding virus. (D) Dot plot of virus binding to TIM-1 versus estimated mucin length. Data were quantified from binding studies in panel B by using mean fluorescent intensity (MFI) of virus-positive cells. (E) Immunoblot demonstrating equivalent amounts of HA-tagged TIM protein after normalization of HEK 293T supernatants. (F) Binding of soluble WT TIM-1 and MLD mutants to PtdSer liposomes. Serial dilutions of normalized TIM-1 protein were incubated with ELISA plates prebound overnight with PtdSer liposomes (50 μM). Data are shown as mean ± SEM for at least three replicates.
Figure 3.4  MLD mutants express equivalently to WT TIM-1. Histograms showing surface expression of MLD mutants and TIM-1 on surface of transiently transfected HEK 293T cells. TIM-1 or TIM-1 mutant transfected cells (solid black line) were surface stained 48 h after transfection using mAbs ARD5 or AKG7 which detect either the TIM-1 IgV or MLD domains, respectively. Empty vector transfected cells were similarly stained for background binding (filled grey line).
Soluble TIM-1 and MLD mutants bind PtdSer and pseudovirions, but not PtdChl. Binding of soluble WT TIM-1 and TIM-1 MLD mutants to pseudovirions (A) or PtdSer or PtdChl liposomes (B). ELISA plates were prebound overnight with roughly 5.2x10^6 transducing units (as determined from titers on Vero cells) of concentrated EBOV pseudovirions (A) or PtdSer or PtdChl liposomes (50 μM) (B). Supernatants containing soluble, HA-tagged WT TIM-1 or MLD mutants were normalized to protein amounts (Figure 3E) and added to plates with serial dilution (A) or without (B). Background represents absorbance in the absence of any supernatant being added. Data are shown as mean ± SD for a single replicate.
Figure 3.6  EBOV transduction correlates with TIM-1 MLD length. (A to C) Transduction of MLD mutant or WT TIM-1 transfected HEK 293T cells with VSV pseudotyped with EBOV GP (A and B) or LASV GPC (C). Transduction relative to WT TIM-1 is shown as bar graph (A) or plotted against mucin length (B and C). Trend lines were fitted for panels B and C with a one-site specific binding nonlinear regression with Hill Slope and linear regression, respectively, using Prism software (GraphPad). Cells were transduced with an MOI of 0.01 as titered in empty-vector HEK 293T cells. (D) Virus binding (MFI) from Figure 3D plotted against percent transduction data. (E) Transduction of pseudovirions into cells expressing a single MLD mutant (●) or various mixtures of MLD mutants (○). Transduction for mixed MLD populations is plotted using the average MLD length of the MLD mutants transfected. Transduction data for panel A, B, and D are normalized to TIM-1 expression as assessed by surface staining (% TIM-positive cells) and all data are shown as mean ± SEM for at least three replicates. For panel A, significance was calculated using a one-sample t-test comparison to 100 (**= p<0.001).
Figure 3.7   Domains of TIM-1 required for PVEER activity can be functionally substituted. (A) The amino acid sequences of TIM-1 MLD switch chimeras are aligned with the MLD of TIM-1 (aa 128 to 298) (CLUSTAL V). Terminal amino acids of the aligned sequences are noted. (B) Expression of TIM-1 and TIM-1/αDG chimeras as detected using TIM-1 IgV and MLD specific mAbs ARD5 and AKG7 (black line) compared to background staining of HEK 293T cells (filled grey line). (C, E, and G) Transduction of transfected HEK 293T cells with VSV virions pseudotyped with viral glycoprotein as noted in each panel. Schematics of chimeras are shown below transduction data (C and E) or adjacent to expression data (F). (C) Transduction of cells expressing TIM/αDG chimeras. The MLD of murine αDG replaced or added in addition to TIM-1 MLD generated αDG-MLD or 2xMLD constructs, respectively. (D) Representative histograms of TIM-1/PRR and TIM-1/RAGE chimeras (black line) and WT TIM-1 (filled grey line) surface expression as determined using ARD5. (E) Transduction of cells expressing TIM-1/PRR and TIM-1/RAGE chimeras compared to transduction into cells expressing equivalent amounts of wild-type TIM-1 IgV domain. As empty-vector transfected cells lack the ARD5 epitope and cannot be compared to cells expressing equivalent amounts of TIM-1, Empty vector transduction data are compared to the same TIM-1 transduction data as h1RAGE and h1RAGEh1cyto as these chimeras had the lowest expression levels. (F) Expression of WT TIM-1 and AnxV-αDG-GPI as detected using ARD5 and AKG7 mAbs and anti-AnxV antisera (black line) compared to background IgG binding (filled grey line). (G) Transduction of cells expressing either TIM-1 or AnxV-αDG-GPI. Cells were transduced with an MOI of 0.02 (EBOV and LASV in panels C and G) or 0.03 (EBOV and LASV in panel E and all other viruses in panel G) as titered in empty vector transfected HEK 293T cells. Data are shown as mean ± SEM for at least three replicates. Significance was calculated using a two-sample t-test comparison to empty vector (C) or a one-sample t-test comparison to 100 (E) (**= p<0.001).
Figure 3.8  IgV domain of RAGE does not substitute for that of TIM-1 (A) Representative histograms of RAGE-IgV expression as detected by ARD5, AKG7, or an anti-RAGE polyclonal (black line) compared to binding of these antibodies to HEK 293T cells (filled grey line). (B) Transduction of EBOV GP and LASV GPC pseudovirions into HEK 293T cells transfected with TIM-1, RAGE-IgV, RAGE, or empty expression vectors. Data are shown as mean ± SEM for a single replicate.
Figure 3.9  Model of MLD role in PVEER-mediated entry. (A) Accessibility of PtdSer on the surface of virions to IgV domain of TIMs is dependent on length of MLD. Short MLDs or structured stalks cannot project the IgV domain above the majority of cell surface proteins. Mid-length MLDs in some cases are blocked by adjacent proteins, but in others partially accessible if the local environment is shorter. Full-length MLDs however are fully accessible and project the IgV above the majority of cell surface proteins. MLDs containing structured regions in addition to extended conformations or with additional length function similarly. (B) Potential flexibility of the MLD provides a larger area of interaction for longer MLD proteins than shorter MLD proteins. (C) Flexibility of the MLD may also allow for TIMs to conform to curvature of virions and promote binding of multiple TIMs. Crescent shapes represent PtdSer binding domains, ovals represent the IgC domains from RAGE or similar structured domains, and coils represent serine/threonine/proline-rich beta-turn helices.
CHAPTER IV
TARGETING PTDSER-EXPOSING CELLS USING TIM-1

Introduction

The family of T-cell immunoglobulin and mucin domain (TIM) proteins are type 1 cell surface glycoproteins that have been shown to enhance entry of a broad range of viruses including members of the picornavirus, filovirus, flavivirus, alphavirus, arenavirus, and baculovirus families (47, 55-57, 238, 239). For enveloped viruses, this enhancement is believed to occur through binding of the TIM family members to phosphatidylserine (PtdSer) on the viral envelope (55-57), specifically through a binding pocket formed by the CC’ and FG loops of the IgV domain (60-63).

PtdSer is an anionic phospholipid present within the inner leaflet of the plasma membrane of healthy cells (144). However, exposure of PtdSer to the outer leaflet by scramblases can be induced by several mechanisms, including apoptosis and elevated levels of intracellular calcium (145). The exposure of PtdSer on apoptotic cells acts as an indicator for uptake by phagocytotic cells (147, 148). For non-apoptotic cells, exposure of PtdSer can be important for biological functions such as platelet coagulation (152). This is mediated by the calcium-sensitive scramblase, Ano6 (also known as TMEM16F) (153).

The presence of PtdSer on the outer leaflet has also been implicated as a marker for tumors. Anti-PtdSer antibodies and the PtdSer binding protein annexin V (AnxV) have been used intravenously to successfully to label a variety of model tumors in mice and rats (211-213). There is also evidence some cancer cell lines endogenously have elevated levels of exposed PtdSer (214, 215). Interestingly, this exposure of PtdSer by tumors may enhance the binding of PtdSer binding receptors such as CD300a and inhibit their killing by natural killer cells, thereby promoting tumor growth (214).

As a potential marker for tumors, PtdSer binding has been used as a mechanism for targeting therapeutics. PtdSer binding antibodies alone are sufficient to inhibit tumor growth
as they enhance recruitment of monocytes and infiltration of macrophages (216). Anti-PtdSer antibodies have also been shown to enhance the efficacy of other treatments as evidenced with the chemotherapeutic docetaxel or radiation therapy (217, 218). Fusion of AnxV to cytotoxic enzymes has been shown to be effective in killing cancer cells \textit{in vitro} (221-223). However, these treatments, like PtdSer binding antibodies, have significant downsides. For example, AnxV accumulates in the heart during damage (226), potentially leading to off-target effects, and anti-PtdSer antibodies can lead to the development of anti-phospholipid syndrome (286). Nonetheless, these studies suggest PtdSer is a viable target for specific delivery of chemotherapeutics to cancer cells.

Some of the limitations of PtdSer-binding delivered chemotherapeutics could be alleviated through the use of viruses that have intrinsic cell killing ability and whose specificity can be modulated by the introduction of tissue specific miRNA target sites or promoters. As of yet, limited studies have been done to target viruses to tumors using PtdSer binding activity. Adenovirus has been successfully delivered to tumors using a bispecific antibody against the adenovirus fiber protein and PtdSer (287). However, this treatment requires not only the association between antibody and virus but also appropriate relative concentrations between the two. A potential alternative would be the incorporation of a PtdSer binding protein into the virus itself.

In this study we have explored the use of PtdSer to target enveloped viruses to cancer cells. In order to do so, we attempted to generate pseudoviruses that incorporate TIM proteins into their envelope. We found that coexpression of TIMs in virus-producing cells with EBOV GP enhanced virus titers. This enhancement of entry was dependent on the extent of PtdSer exposure on target cells and was seen with several cancer cell lines. However, we also discovered that TIMs were not being directly incorporated into viral membranes. In addition, expression of TIMs in virus-producing cells cotransfected with various non-ebolavirus GPs resulted in both a reduction in the amount of virus released from cells and
virus titer. Thus, expression of TIMs affects several aspects of pseudovirion production both positively and negatively.

**Materials and Methods**

**Cell lines and viruses**

HEK 293T cells, a human embryonic kidney cell line were maintained in Dulbecco modified Eagle medium (DMEM) (Gibco BRL) with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin. A selection of cell lines from the NCI-60 panel (A549, Hop62, SF-295, SF-539, SK-MEL-5, A498, ACHN, PC-3, MCF-7, and HT-1080), Hec1A and Hec50 endometrial cell lines, and murine 4T1 and NT3 cancer cell lines were maintained in DMEM with 10% FBS and 1% penicillin-streptomycin. Hec1A and Hec50 cell lines were kindly provided by Kimberly Leslie (University of Iowa) and 4T1 and NT3 cell lines by Weizhou Zhang (University of Iowa).

Vesicular stomatitis virus (VSV) pseudovirions were produced as previously described (11, 16, 47, 240). We used VSV (strain Indiana) pseudovirions whose genomes had the G glycoprotein gene replaced with enhanced green fluorescent protein (VSVΔG-EGFP). To produce VSV pseudovirions, HEK 293T cells were transfected with plasmids expressing either EBOV GP lacking the mucin domain of GP1, Sudan virus (SUDV) GP, Marburg virus (MARV) GP, Bundibugyo virus (BDBV) GP, full-length EBOV (FL EBOV) GP, Machupo virus (MACH) glycoprotein precursor (GPC), Junín virus (JUNV) GPC, Lassa virus (LASV) GPC, Ross River virus (RRV) GP, GP64, or Chikungunya virus (CHIKV) env (strain OPY1) and transduced 24 h later with VSVΔG-EGFP pseudovirions. Cells were also cotransfected with various TIM protein expressing plasmids or an empty vector with a DNA ratio of 2 μg TIM or empty vector:1 μg GP vector unless indicated otherwise. After 4 h of virus uptake, the plates were washed and refreshed with media. Pseudotyped virions were collected in supernatant 48 and 72 h after transduction, pooled, and filtered through a 0.45 μm pore-size filter. For removal of cell-associated virus, virus producing cells were treated
with 5 mM EGTA for 10 minutes. Cells were resuspended and pelleted by centrifugation. Supernatant was removed and filtered through a 0.45 µm pore-size filter. A portion of this filtered supernatant was dialyzed into 1x PBS using a 100,000 molecular-weight cutoff Slide-E-lyzer dialysis cassette (Thermo). Virus aliquots were stored at -80°C until use.

Purified virus stocks were generated by centrifugation of supernatants through a 20% sucrose cushion at 80,000 × g for 2 h at 4°C. Pellets were resuspended in 1x PBS, filtered through a 0.45-µm syringe filter, aliquoted, and stored at −80°C. Concentrated virus stocks were generated by centrifugation of supernatants through a 100,000 MWCO Ambion ultra centrifugation column (Thermo). After all supernatant collections were passed through the filter (roughly 200 fold concentration of medium), virus stocks were aliquoted and stored at −80°C.

Transductions

HEK 293T cells were transfected with plasmids as indicated in figures using polyethylenimine (PEI) according to standard protocol (267). Twenty-four hours following transfection the cells were transferred to a 48 well tissue culture plate and transduced 24 h later with pseudovirions at the multiplicities of infection (MOIs) noted in the figure legends. If indicated, ARD5 was added to virus at a concentration of 0.5 µg/mL immediately before incubation with cells. At 24 h after transduction, the cells were detached with Accutase solution (Millipore), and EGFP expression was assessed by flow cytometry as the percent positive cells in the FL-1 channel. Transduction data were normalized as indicated in the figure legends.

Generation of Ano6 mutant

An Ano6 expression vector was obtained from the ASU plasmid repository. The sequence encoding Ano6 was then transferred to a eukaryotic expression vector, replacing soluble HA-tagged TIM-1 (57), by using Gibson assembly cloning techniques. This transfer resulted in the insertion of single glycine-serine linker and an HA tag downstream of and in-
frame with the Ano6 coding sequence. A constitutively active Ano6 mutant was generated by insertion of a D409G mutation using site-directed mutagenesis as previously described (57).

**AnxV staining**

Recombinant FITC-labeled AnxV (FITC-AnxV) was generated as previously described (249). Briefly, HIS-tagged AnxV was expressed in *Escherichia coli* and purified from lysates on a nickel column. AnxV was then reacted with fluorescein isothiocyanate (FITC) isomer (Sigma) and dialyzed into 1x PBS using a 10,000 MWCO Slide-E-lyzer dialysis cassette (Thermo). Transfected HEK 293T cells were incubated 48 h after transfection with 1 μg/mL of FITC-AnxV diluted in AnxV binding buffer (10 mM of HEPES-NaOH (pH 7.4), 150 mM of NaCl, 5 mM of KCl, 1 mM of MgCl2, 1.8 mM of CaCl2) at room temperature for 15 minutes. FITC-AnxV binding was assessed by flow cytometry as the percent positive cells in the FL-1 channel.

**Dot and Western blots**

Transfected HEK 293T cells (roughly 5x10⁶ cells/ml) or pseudovirions were lysed in 1% SDS solution. For detection of JUNV and EBOV GPs, supernatants were prepared in 0.025% NP40. Proteins from cell lysates or pseudovirions were separated by SDS-PAGE and transferred to nitrocellulose membranes (Western blot). Virus lysates were also applied directly to nitrocellulose in a dot blot apparatus (dot blot). After blocking nitrocellulose for 1 h with 10% nonfat dry milk in 1x PBS, blots were incubated for 2 h with a mixture of goat anti-TIM polyclonal antisera (0.2 μg/mL; R&D systems), rabbit anti-HA antisera (1:4000; Sigma), or unpurified anti-VSV matrix monoclonal antibody (mAb) 23H12 (1:10) as indicated diluted in 1x PBS with 10% nonfat dry milk and 0.15% Tween 20. EBOV or JUNV GPs were detected using 1H3 mAb (1:200) (288) or QC03-BF11 and GB03-BE0 mAbs (1:500 each), respectively. Protein expression was imaged using a LI-CORE Odyssey CLx and quantified by measuring the total fluorescent signal and subtracting background signal using Image Studio (LI-CORE).
**Immunoprecipitations of pseudovirions**

Prior to pull-down, quantities of matrix were equalized by diluting GP alone virus in Protein A-binding buffer (150 mM NaCl, 20 mM Na₂HPO₄). Pseudovirion supernatant collected from cells expressing EBOV GP or both EBOV GP and TIM-4 GPI were incubated with or without anti-EBOV GP antisera (1:800) overnight at 4°C. Antiserum was kindly provided by Anthony Sanchez (CDC). After incubation with antiserum, 25 μL of equilibrated and washed Protein A agarose resin (GenScript) was added to each sample and incubated at 4°C for 2 hours. Protein A beads were pelleted by centrifugation at 450 x g and washed 4 times with Protein A-binding buffer. After final wash, beads were resuspended in SDS loading buffer and boiled for 10 min before supernatant was run on an SDS-PAGE gel.

**Flow cytometry**

Hec1A cells were detached with 1x PBS plus 5 mM EDTA, washed with 1x PBS plus 5% FBS, and incubated for 1 h with goat polyclonal antiserum specific to TIM-1, TIM-3, TIM-4, Axl, or Mer as indicated (R&D Systems) at a concentration of 4 μg/mL. Normal goat serum (R&D Systems) was used as a negative control. Cells were washed and incubated for 20 min with anti-goat secondary antiserum conjugated to either Cy5 (Invitrogen) or DyLight 649 (Jackson ImmunoResearch). After incubation, cells were washed and protein expression was assessed by measuring the percentage of positive cells in the FL-4 channel using a FACSCalibur flow cytometer (BD Biosciences). All flow cytometry data were analyzed using FlowJo software (TreeStar, Inc).

**Results**

**Presence of TIM-1 in viral stocks enhances entry.**

Expression of TIM-1 in HEK 293T cells greatly enhances entry of viruses pseudotyped with a variety of viral envelope proteins (47, 55-58). Of these viruses, we and others have shown that entry of EBOV GP pseudovirions is more significantly enhanced by
TIM-1 expression than entry of other pseudovirions (56, 57). This enhancement occurs due to TIM-1 on the cell surface membrane binding to PtdSer on the viral envelope. However, we were curious whether this interaction would still occur if reversed, such that TIM-1 was present on the viral envelope for interaction with PtdSer exposed on the cell membrane and whether this would subsequently enhance virus entry. Further, this could also provide insight as to whether or not signaling is required for TIM-mediated uptake as TIM-1 would not be on the cell surface to interact with or induce recruitment of cellular signaling proteins.

In the following studies we used vesicular stomatitis virus (VSV) pseudovirions that express EGFP as reporter molecule in place of native G (VSVΔG). Further, in all cases, VSV pseudovirions were pseudotyped with a viral GP as this is a necessary component for fusion within the endosome and for subsequent transgene expression. In these initial studies we used an EBOV GP that has a deleted mucin domain. This EBOV GP lacks the GP1 mucin domain, but confers the same tropism as the full-length Ebola virus GP and produces higher pseudovirus titers (21, 240, 250).

In order to incorporate TIM-1 into pseudovirions, we exogenously expressed both TIM-1 and EBOV GP in HEK 293T virus-producing cells. After collection of supernatant, we assessed for the presence of TIM-1 in virus stocks by Western blot (Figure 4.1A). We detected VSV matrix protein in both virus stocks generated with EBOV GP alone (GP alone) or EBOV GP and TIM-1 (+TIM-1), while TIM-1 was detected only in the +TIM-1 virus. TIM-1 has a longer cytoplasmic tail than the native glycoprotein of VSV G. We hypothesized that replacement of the TIM-1 transmembrane domain with that of VSV G (TIM-1 VSVtm) would enhance incorporation into pseudovirions by reducing steric hindrance or promoting favorable interactions with the VSV matrix protein. After generation of virus (+TIM-1-VSVtm), we found that more TIM-1 was present in +TIM-1-VSVtm stocks than in +TIM-1 stocks (Figure 4.1A). However, given that TIM-1 expression was assessed in unpurified virion stocks, we cannot determine from these data if TIM-1 is
directly incorporated into virions, associated with virions or cell debris, or cleaved from the cell surface as a soluble protein.

We next wanted to determine if the presence of TIM-1 on the viral envelope would enhance virus entry into HEK 293Ts. The presence of TIM-1 or TIM-1-VSVtm in virus stocks only moderately enhanced transduction into empty-vector transfected cells (Empty) compared to transduction of EBOV GP alone, roughly 1.5 fold enhancement (Figure 4.1B). While the presence of TIM-1 on the plasma membrane of target cells enhances virus entry, the additional presence of TIM-1 in virus stocks did not further enhance, but slightly decreased, entry. We reasoned the minimal enhancement of entry into HEK 293Ts by the presence of TIM-1 on the viral envelope was due to low levels of PtdSer present in the outer leaflet of the cell membrane.

In order to assess entry into cells with more outer leaflet-exposed PtdSer, we expressed a mutant of the scramblase, anoctamin 6 (Ano6), which constitutively disrupts PtdSer asymmetry due to increased sensitivity to intracellular calcium caused by a D409G point-mutation (Ano6 mut) (153). Transfection of this mutant into HEK 293T significantly enhanced the exposure of PtdSer as measured by AnxV binding (Figure 4.1C). Interestingly, expression of TIM-1 also enhanced exposure of PtdSer and, to a lesser extent, so did expression of WT Ano6. We assessed entry of our +TIM-1 and +TIM-1-VSVtm viruses into these cell populations and found that entry into Ano6 mut-expressing cells was enhanced compared to entry into WT Ano6-expressing cells (Figure 4.1B). Further, these data suggest that the presence of TIM-1 in virus stocks enhances virus entry in a manner dependent on the amount of exposed PtdSer on target membranes.

Expression of Ano6 mutant in producer cells does not affect virus titer.

Given that the Ano6 mut enhances PtdSer exposure on the cell membrane, we wanted to determine if pseudovirions produced in cells expressing this mutant would have
enhanced titers due to greater a concentration of PtdSer in their viral envelopes. We found that coexpression of either Ano6 or Ano6 mut with EBOV GP in virus-producing cells did not enhance pseudovirion titers compared to virus produced from cells expressing EBOV GP alone (Figure 4.1D). Further, entry of these viruses into HEK 293T’s were unaffected by the presence of TIM-1 on the cell surface. Thus, production of virus in these Ano6 mut-expressing cells either does not enhance PtdSer incorporation into viral envelopes or concentrations of PtdSer present in viral envelopes are sufficient for maximal TIM-1 binding.

**Determining the ideal ratio of EBOV GP to TIM-1 in virus-producing cells.**

In our initial studies, we transfected TIM-1 and EBOV GP expression plasmids into our producer HEK 293T cells using a DNA mass ratio of 1:1. However, we wanted to determine the ideal ratio between the two genes for maximum enhancement of entry as insufficient EBOV GP or TIM-1 will reduce fusion or binding efficiency, respectively. VSV pseudovirions were produced in cells cotransfected with various ratios of TIM-1-VSVtm and EBOV GP expression plasmids. These virus stocks were normalized for equivalent amounts of matrix and then used to transduce cells transiently transfected with an empty vector, TIM-1, Ano6, or Ano6 mut (Figure 4.2). Optimum titers were achieved when using virus produced from cells transfected with TIM-1-VSVtm and EBOV GP expression plasmids at a ratio of 2 µg to 1 µg, respectively. However, little variation in transduction efficacy occurred when transfecting virus-producing cells with DNA ratios between 1:2 µg and 2.5: 0.5 µg of TIM-1 VSVtm to EBOV GP.

**Presence of any TIM family member in virus stocks enhances virus entry.**

In addition to TIM-1, TIM-4 is effective at enhancing virus entry when expressed on the cell surface while TIM-3 is not (55-58). The reduced efficacy of TIM-3 is attributed to it
having a shorter mucin-like domain (MLD) (289). While the length of the MLD is important for binding virus, the cytoplasmic tails of TIMs are nonessential for PVEER function and replacement of the transmembrane and cytoplasmic domains of the TIM family members with a glycoprophosphoinositol (GPI) anchor does not reduce their PVEER efficacy (289). We wanted to determine if the other human TIM family members and their GPI anchored equivalents would also incorporate or associate with pseudovirion membranes and enhance entry. In order to confirm the role of PtdSer for the enhancement of entry, we also tested PtdSer binding mutants of TIM-1 and TIM-4 in which key aspartate and asparagine residues present within the pocket are mutated (57). In all cases, the TIM family members were detected in virus stocks, although the presence of GPI-anchored proteins was slightly reduced compared to their WT counterparts (Figure 4.3A). Interestingly, of the WT TIM family members we found that the presence of TIM-4 in virus stocks most significantly enhanced virus entry (Figure 4.3B) compared to TIM-1 and TIM-3. WT TIM-3 enhanced virus entry to the lowest extent. This might be explained by the reduced MLD length; however replacement of the cytoplasmic domains with a GPI anchor further enhanced entry of TIM-3 and also TIM-1. Thus, the reduced enhancement of entry by TIM-3 cannot be explained by it having a shorter MLD. Enhancement of entry by TIM-1 and -4 in virus stocks is dependent on PtdSer-binding activity as mutants that are defective in this activity do not enhance virus entry.

We next examined how the exposure of additional PtdSer on the cell membrane would affect entry of our TIM-containing virus stocks. Transduction into Ano6 expressing cells was slightly enhanced (Figure 4.3C) compared to empty vector transfected HEK 293Ts (Figure 4.3B). This is likely due to the slight increase in PtdSer present due to expression of WT Ano6 (Figure 4.1C). Consistent with our previous results, transduction into Ano6 mut expressing cells was dramatically enhanced above the baseline and was increased roughly 2 to 3 fold above that found with WT Ano6. Thus, our results suggest that the enhancement of virus entry by TIMs in virus stocks is dependent on PtdSer binding.
Virus entry into select cancer cell lines is enhanced by the presence of TIM-4 GPI.

The ability of TIMs in virus stocks to enhance EBOV pseudovirion entry appears to be dependent on the amount of PtdSer present on the target-cell membrane (Figure 4.1B). There is strong evidence that tumor vessels and several cancer cell lines endogenously expose PtdSer on the outer leaflet of the plasma membrane to higher levels than healthy cells (211-215). These data suggest that virus produced in TIM-expressing cells might more effectively enter cancer cells than virus produced with GP alone. In the subsequent studies, unless indicated, we produced EBOV GP pseudovirions in the presence of TIM-4 GPI (+TIM-4 GPI) as these virions had the most significantly enhanced entry in our earlier studies (Figure 4.3B and 3C). We assessed the ability of stocks of EBOV GP alone or +TIM-4 GPI pseudovirions to enter several cancer cell lines and compared fold increase in entry due to the presence of TIM-4 GPI (Figure 4.4A). Entry into several of the cancer cell lines was enhanced either significantly (SF-539, SK-MEL-5) or more modestly (Hop62, MCF-7, and HT-1080). However, there were many cell lines for which entry was unaffected (SF-295, A498, ACHN, and PC-3), likely due to minimal exposure of PtdSer, or reduced PtdSer (A549, Hec50, and Hec1A), although this has not been tested.

We had previously seen reduction of +TIM-1 virus entry into TIM-1-expressing cells compared to entry of GP alone virus into these same cells (Figure 4.1B). Consequently we hypothesized that the reduction of entry into A549, Hec50, and Hec1A cells might be due to robust TIM-1 expression on these cell lines. TIM-1 expression has already been detected on A549s (55) and we were also able to detect expression on Hec1A cells (Figure 4.4B). We also tested the sensitivity of GP alone virus entry into Hec1A and Hec50c cells to inhibition by the anti-TIM-1 mAb ARD5 and found pseudovirion entry was reduced in both cell lines (Figure 4.4C). These data suggest both cell lines express TIM-1. Thus, the reduction of entry seen in these cell lines is consistent with the reduction seen previously with exogenous TIM-1 on the surface of 293T cells. We also assessed the ability of TIM-4 in the virus stock
to enhance virus entry into two murine cancer cell lines, 4T1 and NT3 cells, and found that in neither case was +TIM-4 GPI virus entry enhanced above the entry of GP alone virus (Figure 4.4D).

**TIMs are not present in viral envelopes.**

While we saw that TIMs were present in the supernatant collected from virus-producing cells coexpressing TIMs and GPs, we wanted to determine if TIMs were incorporated directly into the viral envelopes. In order to do so, we first generated EBOV GP alone and +TIM-4 GPI pseudovirions and confirmed the presence of TIM-4 in virus stocks (Figure 4.5A). Next, we assessed for the presence of TIM-4 on EBOV GP pseudovirions immunoprecipitated (IP) from supernatants using an EBOV GP-specific antisera. In our IP studies, we were able to co-immunoprecipitate VSV matrix, but not TIM-4 (Figure 4.5A). These data suggest TIM-4 is not being directly associated with viral envelopes. In order to confirm these results, we purified EBOV GP alone and +TIM-4-GPI virus from supernatants through a 20% sucrose cushion by ultracentrifugation and assessed the pelleted virions for the presence of TIM-4 (Figure 4.5B). Consistent with our previous results, we were able to detect TIM-4 in the unpurified virus-containing media but not in purified virus stocks. After centrifugation, TIM-4 remained in the supernatant above the virus pellet. Consistent with the loss of TIM-4 GPI from purified virus stocks, we found the enhancement of virus entry seen due to TIM-4 GPI in the raw supernatants was lost (Figure 4.5C). These data suggest expression of TIM-4 GPI in virus-producing cells leads to PtdSer-binding-dependent enhancement of virus entry without incorporating in the virion itself.

In order to retain TIM-4 GPI in the supernate, while still concentrating virus stocks, we concentrated virus via a 100,000 MWCO filter. This filter has pore sizes small enough to prevent loss of TIM-4 and consequently we were still able to detect TIM-4 GPI in virus stocks after concentration (Figure 4.5D). In addition, these concentrated stocks were still
able to enhance virus entry in a PtdSer-concentration dependent manner as +TIM-4 GPI virus transduction into Ano6 mut-expressing HEK 293Ts was greater (Figure 4.5E).

**Enhancement of entry by presence of TIMs in virus stocks is unique to ebolavirus GP pseudovirions.**

In our preceding studies we used EBOV GP pseudovirions as entry of these viruses is very significantly enhanced by expression of TIM-1 or TIM-4 on target cells. However, entry of pseudovirions bearing other GPs is enhanced by TIM-1 and TIM-4 expression. We wanted to assess whether or not titers of pseudovirions bearing these GPs would also be enhanced by expression of TIMs in virus-producing cells. First we examined other filovirus GPs, evaluating the impact of the presence of TIM-4 GPI in virus stocks of stocks of Sudan (SUDV), Bundibugyo (BDBV), full-length EBOV (FL EBOV), and Marburg (MARV) virus GP pseudovirions on transduction. The FL EBOV GP contains the mucin-like domain that was deleted from the EBOV GP used in previous figures. Entry of all three ebolaviruses into HEK 293T cells was enhanced by coexpression of TIM-4 GPI in virus-producing cells, SUDV GP pseudovirion entry being most significantly enhanced (Figure 4.6A). Interestingly, entry of MARV GP pseudovirions was not affected despite entry of these virions being enhanced when TIM-1 is expressed on the target-cell surface (47, 57).

Expression of TIM-1 on the cell surface of recipient cells enhances entry of Ross River (RRV), Machupo (MACH), Chikingunya (CHIKV), Junín (JUNV), and Autographa californica multicapsid nucleopolyhedro- (GP64) GP pseudovirions (56, 57). TIM-1, however, does not enhance entry of Lassa virus (LASV) GPC pseudovirions into HEK 293T cells (47, 57). These pseudovirions were normalized for VSV matrix concentration. Interestingly, we consistently found there was a reduction in the quantity of pseudovirions released into the supernatant from virus-producing cells coexpressing TIM-4 GPI in addition to their respective GPs as assessed by a reduction in the presence of VSV matrix in supernatants (Figure 4.6B). In order to control for the reduced amounts of virus in +TIM-4
GPI stocks, GP alone virus stocks were diluted until the amounts of matrix protein were equivalent to that of +TIM-4 GPI virus stock. After normalization, transduction efficacy of +TIM-4 GPI viruses into HEK 293T cells was compared to that of GP alone virus (Figure 4.6C). With all viruses, transduction efficiency was reduced by the expression of TIM-4 GPI in virus-producing cells, in some cases more than 5 fold. Pseudovirions with GPs from New World arenaviruses, MACH and JUNV, and alphavirus CHIKV, were most significantly affected. Thus, expression of TIM-4 GPI results in significant inhibition of both the quantity and titer of matrix-normalized pseudovirions.

We initially hypothesized that the mechanism of virus uptake being utilized by our +TIM pseudovirions might be different from the mechanism used when TIMs are present on the cell surface. This is evidenced by the ability of TIM-3 GPI to enhance virus entry when present in virus stocks (Figure 4.3B) but not on the cell surface (Figure 3.2I). This mechanism of uptake might traffic virus to a cellular compartment favorable for fusion of ebolavirus GPs but inhibitory for other GPs. If this was occurring, we reasoned that adding an inhibitor of TIM binding would prevent virus uptake through this pathway and rescue infectivity. ARD5 is a mAb that binds to TIM-1 and significantly inhibits PtdSer binding and, consequently, TIM-1 mediated enhancement of virus entry (47, 57). Addition of ARD5 to EBOV +TIM-1 GPI pseudoviruses significantly reduced transduction compared to that of virus bearing EBOV GP alone (Figure 4.6D), confirming a role of TIM-1 in enhancing entry of +TIM-1 virus. However, using ARD5 to block the potential TIM-1-mediated delivery of virus to a noncompatible compartment of the cell did not rescue the inhibition of entry seen with MACH, LASV, or GP64 pseudovirions. These results suggest that the inhibition of transduction efficacy mediated by TIM-1 GPI expression occurs during pseudovirion production and not entry. Interestingly, addition of ARD5 reduced entry of GP64 +TIM-1 GPI pseudovirions, suggesting that, similar to EBOV pseudovirions, the presence of TIM-1 GPI in GP64 pseudovirion virus stocks partially enhances transduction despite the reduction in titer caused by its expression on producer cells.
After cotransfection of cells with both GP and TIM expression plasmids, many cells are likely to express both genes. We hypothesized that the TIM proteins might be binding virus as it is being released from the cell surface, reducing the amount of virus released in the supernatant (Figure 4.6B). Consequently, as after cotransfection many cells expressing GP would also be expressing TIMs, much of the GP containing virus may be tethered to the cell surface by TIMs. In order to determine if the titer of cell-associated +TIM-4 GPI virus was greater than the titer of cell-associated GP alone virus, we compared transduction of these viruses into HEK 293T cells. In this study we used JUNV GPC for our virus-producing cells as we saw significant reduction in JUNV pseudovirion titers due to TIM-4 GPI coexpression and we had GPC specific antibodies available. Cell-associated virus was removed using ethylene glycol tetraacetic acid (EGTA), which chelates free calcium required for TIM binding of PtdSer (57). As EGTA might affect virus entry, we also dialyzed cell-associated virus into 1x PBS (Dialysis). Despite adding equal amounts of virus to cells as assessed by matrix quantities (Figure 4.6E, below), cell-associated +TIM-4-GPI virus had a lower titer than GP alone virus (Figure 4.6E). Thus, expression of TIMs not only reduces the amount of virus released, but also the titer.

One explanation for this reduced titer of virus released from TIM-expressing cells might be because coexpression of TIMs affects GP incorporation. Indeed, JUNV pseudovirions produced from TIM-4 GPI-expressing cells have reduced amounts of JUNV GP incorporated (Figure 4.6F). Interestingly, EBOV pseudovirions also have reduced GP incorporation (Figure 4.6F), despite having increased titers (Figure 4.3B). The reduction of JUNV GP might account for the reduction in titer seen upon TIM expression in virus-producing cells.

**Discussion**

The goal of this study was to determine whether incorporation of TIM proteins into the viral envelope would enhance virus entry into PtdSer-exposing cells. Our initial results
confirmed that, indeed, the expression of TIM proteins in virus-producing cells resulted in the presence of TIM proteins in virus stocks. As hypothesized, the presence of these TIMs enhanced EBOV GP pseudovirion entry, the extent of which was dependent on the amount of PtdSer on the target cell membrane. TIM-4 GPI was the most effective of the TIMs at enhancing entry of this virus. Further, viruses produced from cells expressing both TIM-4 GPI and EBOV GP had higher titers on several cancer cell lines compared to viruses produced from cells expressing EBOV GP alone. However, contrary to what we had anticipated, TIM-4 GPI was not incorporated into viral particles and purification of virus resulted in a loss of TIM-4 GPI. Interestingly, the enhancement of virus titer by coexpression of TIMs with a GP was limited to ebolavirus GP as in all other cases expression of TIM-1 and TIM-4 GPI in virus-producing cells prevented the release and reduced the titer of virus. Thus, our studies show the effect of TIM expression in virus-producing cells on virus titers is specific for the viral glycoprotein present on VSV pseudovirions.

Expression of TIM-1 and TIM-4, but not TIM-3, on the cell surface of target cells enhances entry of several enveloped viruses (47, 55-57). Further, both TIM-1 and TIM-4, and their GPI anchored equivalents, enhance entry to the same extent. However, expression of TIM-1, TIM-3, and TIM-4 in virus-producing cells results in different improvements of virus titer. We found the presence of TIM-4 in virus stocks to most effectively enhance entry into HEK293T cells. This might be because TIM-4 has additional residues within the IgV domain for interaction with PtdSer that are absent in TIM-1 and TIM-3 (64). Alternatively, there may be more TIM-4 protein present in the virus stocks compared to the other TIMs. While our western blot of virus stocks might support this, the various TIMs were detected using different polyclonal antisera that are likely binding different numbers of epitopes. The use of tagged TIM proteins would more accurately assess the relative amounts of TIM protein in the virus stocks.

The expression of GPI-anchored TIM-1 and TIM-3 in virus-producing cells enhanced virus titers further than expression of WT TIM-1 and TIM-3. Most surprisingly,
GPI-anchored TIM-3 was as effective as TIM-1 GPI, despite being unable to enhance virus entry when expressed on the cell surface (289). This reduced PVEER activity is attributed to TIM-3 having a shorter MLD that cannot propel the IgV domain from the cell surface. However, we have shown that the MLD length does not factor into PtdSer binding if the TIMs are soluble. Thus, this result, in addition to the data showing that the TIMs do not appear to be directly incorporated into virions, suggest TIMs are present as soluble proteins. Indeed, both TIM-1 and TIM-4 have been shown to be shed from the cell surface after cleavage by metalloproteinases, a disintegrin and metalloproteinase (ADAM) 10 and 17 (290). This would also explain the reduced entry of TIM-containing virus stocks into cells expressing TIM-1 as soluble TIMs would compete for binding of virus. Further, GPI-anchored proteins may be more accessible to proteases for cleavage from the cell or virion surface and release into the supernatant, explaining why they enhance entry further than their WT counterparts.

The ability of TIMs in EBOV GP virus stocks to enhance virus entry without directly incorporating into viral membranes is difficult to explain. Nonetheless, our data suggest binding of the IgV domain to PtdSer plays a critical role as +TIM virus entry is enhanced into ano6 mut-expressing cells, PtdSer-binding mutants do not affect titer, and ARD5 inhibits the enhanced transduction of +TIM1 GPI virus over GP alone virus. It is possible that soluble TIMs are bridging virus to the cell surface. However, this would require multiple TIMs to be associated together to bind both the virus membrane and cellular membrane. This might be occurring due to dimerization of TIMs or TIMs associating with or incorporating into exosomes/cell debris. Interestingly, the IgV domain of murine TIM-1 was crystallized as an antiparallel dimer (243) although no functional significance of this interaction has been confirmed. Either mechanism of bridging would result in enhanced binding of virus to cells, something that remains to be determined.

TIMs may not actually interact with virions themselves, but rather bind to target cells and, in these cells, triggering signaling that promotes virus entry or transduction. The
TIM-4 IgV domain, bound to beads, has been shown to trigger signaling in T-cells, leading to their expansion (291) and both the TIM-1 and TIM-4 IgV domains trigger T-cell proliferation in vivo (78). While these T-cell-associated signaling events do not explain how TIMs enhance virus entry into cell lines, it is possible that the binding of TIMs induces other signal pathways. Alternatively, it may be that overexpression of TIMs in virus-producing cells stimulates production of a cellular factor that is mediating enhanced virus entry. In either case, signaling would require PtdSer-binding by TIMs.

Given that the presence of TIMs in virus stocks enhanced EBOV, SUDV, and BDBV GP pseudovirion entry in a PtdSer-binding dependent manner, it was surprising to find that expression of TIMs was inhibitory to production of other pseudovirions. We found reduced release of pseudovirions into supernatant from virus-producing cells expressing TIM-4 GPI. These results are consistent with data suggesting that overexpression of TIM-1 tethers HIV-1 virions to the cell surface through binding of PtdSer on the viral envelope (Unpublished data; Shan-Lu Liu, University of Missouri). However, we also found that even when titering equivalent amounts of virus, +TIM-4 GPI virus transduced fewer cells than GP alone virus and had less GP incorporated into virions. Thus, expression of TIMs might be detrimental to GP production. The transfer of PtdSer from the inner to outer cytosolic leaflet is important for vesicle transport (292) and may be impeded by overexpression of TIMs, resulting in reduced transport of GP to the cell surface. While it is not clear that the reduced virus entry we see is related to fewer GPs being incorporated onto virions, the extent of both vesicular stomatitis virus and amphotropic murine leukemia virus GP-mediated entry is dependent on the amount of GP incorporation (293). This study also showed that variations in EBOV GP incorporation have minimal effects on virus titers. The reduced sensitivity of the EBOV GP pseudovirion titer to the amount of GP incorporation might explain why these pseudovirions are uniquely enhanced by expression of TIMs in virus-producing cells.
We had originally envisioned using TIMs on viral membranes to target cancer cells with exposed PtdSer. While we found that in some cases, +TIM-4 GPI virus had enhanced entry into SF-539, SK-MEL-5, Hop62, MCF-7, and HT-1080 cells compared to GP alone virus, this was not true for all cancer cell lines. However, in vivo the exposure of PtdSer occurs in tumor vessels due to exposure of these cells to hypoxia/reoxygenation, inflammatory cytokines, thrombin, and acidity (211). Exposure of PtdSer can also be induced by chemotherapeutics and radiation therapy (217, 218). Thus, in an in vivo tumor model, although the cancer cells may not endogenous expose more PtdSer on the outer leaflet, apoptosis triggered by host-induced stresses/proteins and/or use of chemotherapeutics can result in PtdSer flipping. In this case, +TIM-4 GPI virus might be expected to have enhanced entry in vivo.

There are two major limitations preventing the use of +TIM-4 GPI virus for cancer therapeutics trials in vivo. First, virus cannot be purified without the loss of TIM-4. This is due to the second limitation, which is that the TIMs do not incorporate into the virions and therefore would dilute out into the blood stream. The first limitation can be partially addressed through concentration of virus with a MWCO filter. However, this would still leave, in addition to the TIMs, some contaminating proteins. Both these limitations could be addressed by reengineering how TIMs might be incorporated directly into virions, which is discussed in Chapter V.
Figure 4.1  Expression of TIM-1 in virus-producing cells enhances virus titer. (A) Representative western blot of TIM-1 and VSV matrix expression in GP alone, +TIM-1, and +TIM-1-VSVtm virus stocks. (B) Transduction of GP alone, +TIM-1, and +TIM-1-VSVtm virus into HEK 293T cells transfected with either an empty (Empty), TIM-1, Ano6, or Ano6 mutant (Ano6 mut) expression vector. (C) Representative histograms of FITC-AnxV binding to transfected (black line) or empty vector transfected HEK 293T cells (filled grey line) at 48 h. (D) Transduction of EBOV GP alone virus or EBOV GP pseudovirions produced in cells cotransfected with Ano6 or Ano6 mut and EBOV GP expression vectors. Transductions are shown as percent of GFP positive cells. Cells were transduced with an MOI of 0.02 (EBOV GP alone) as titered on HEK 293T cells. Equivalent amounts of +TIM-1, +TIM-1-VSVtm, Ano6, and Ano6 mut viruses as EBOV GP alone were added to cells as determined by quantity of matrix protein. Data in panels B and D are shown as mean ± SD for a single replicate.
Figure 4.2 Optimization of transfected-DNA ratios for virus production. Transduction of GP alone (circles) and +TIM-1-VSVtm (squares) pseudovirions into HEK 293T cells transfected with empty (Empty), TIM-1, Ano6, or Ano6 mutant (Ano6 mut) expression vectors. GP alone and +TIM-1-VSVtm pseudovirions were produced in HEK 293T cells transfected with various ratios of EBOV GP to TIM-1-VSVtm expression vectors. Transductions are plotted against the mass of +TIM-1-VSVtm or empty expression vector DNA transfected into virus-producing cells. The total mass of DNA transfected was brought to 3 μg by adding GP expression vector DNA. Data are shown as mean ± SD for a single replicate.
Figure 4.3  Expression of various TIMs in virus-producing cells enhances virus titer. (A) Representative western blot of TIM-1, TIM-3, TIM-4, and VSV matrix expression in virus stocks collected from producer-cells cotransfected with various TIM or empty expression vectors. GPI-anchored TIMs have their transmembrane and cytoplasmic domains replaced with a GPI anchor. PtdSer-binding mutant TIM-1 and TIM-4 proteins (PtdSer mutant) contain mutations of IgV-domain aspartate and asparagine residues, resulting in abolished PtdSer binding. Molecular weight markers are shown on the right for TIM bands. (B and C) Transduction of pseudovirions into HEK 293T cells transfected with either an empty vector (B) or Ano6 or Ano6 mutant (Ano6 mut) expression vectors (C). Transduction data for viruses produced in TIM-expressing cells are normalized to transduction data for GP alone virus to generate a fold increase in entry. Cells were transduced with an MOI of 0.01 (EBOV GP alone) as titered on HEK 293T cells. The quantity of +TIM viruses use for transductions was equivalent to the quantity of EBOV GP alone virus used as determined by amount of matrix protein (dot blot). Data in panels B and C are shown as mean ± SEM for 3 replicates.
Figure 4.4  Enhanced entry of +TIM-4-GPI virus into cancer cell lines. (A and D) Fold increase in transduction of +TIM-4-GPI virus over GP alone virus into human (A) or murine (D) cancer cell lines. After normalizing the virus stocks to equivalent amounts of matrix protein, 10 µL of GP alone or +TIM-4-GPI virus was added to the cell lines. The tissues from which the cancer cell lines originate are indicated in white text. (B) Representative histograms of TIM-1, TIM-3, TIM-4, Axl, and Mer expression (black line) in Hec1A cells compared to binding of NGS (filled grey line). (C) Transduction of GP alone virus into Hec1A or Hec50c cells in the presence or absence of ARD5 (0.5 µg/mL). Data for panels A and D are shown as mean ± SEM for at least 2 replicates while data for panel C is shown as mean ± SD (no mAb) or mean (ARD5) for a single replicate.
Figure 4.5 TIM-4 GPI is not incorporated into the viral envelope. (A) Pull-down of EBOV GP alone and +TIM-4 GPI pseudovirions. Virus in supernatants were incubated with or without anti-EBOV GP antibody and bound to Protein A beads. TIM-4 GPI and VSV matrix were detected in onput supernatant and beads after immunoprecipitation. (B) Expression of TIM-4 GPI and VSV matrix in EBOV GP alone and +TIM-4 GPI pseudovirions before (Unpurified) and after (Pellet) purification and in supernatant above purified virus (Supernatant). (C) Fold increase in transduction of purified +TIM-4 GPI virus into HEK 293Ts over transduction of purified GP alone virus. (D) Expression of TIM-4 GPI and VSV matrix in concentrated EBOV GP alone or +TIM-4 GPI virus. (E) Transduction of concentrated EBOV GP alone virus and +TIM-4 GPI virus into HEK 293T cells exogenously expressing either Ano6 (left) or Ano6 mut (right). Transduction data in panels A and E are shown as mean ± SD for a single replicate.
Figure 4.6 Expression of TIMs in virus-producing cells can inhibit virus release and titers. (A) Fold increase in transduction of filovirus +TIM-4-GPI pseudovirions over GP alone virus into HEK 293Ts. Sudan (SUDV), Bundibugyo (BDBV), full-length Ebola (FL EBOV), and Marburg (MARV) virus GPs were expressed in virus-producing cells with or without TIM-4 GPI coexpression. Cells were transduced at an MOI of 0.01. (B) Dot blot showing expression of VSV matrix in supernatants from virus-producing cells. Ross River (RRV), Lassa (LASV), Chikungunya (CHIKV), Machupo (MACH), Junin (JUNV), and baculo- (GP64) virus GPs were expressed in virus-producing cells with or without TIM-4 GPI coexpression. The quantity of virions released into supernatant was assessed by dot blot and staining for VSV matrix. (C) Fold increase in transduction of +TIM-4-GPI virus over GP alone virus into HEK 293Ts. Cells were transduced with an MOI of 0.05. (D) Transduction of MACV, EBOV, LASV, and GP64 pseudovirions, either GP alone or +TIM-1 GPI, into HEK 293T cells in the presence or absence of ARD5 (0.5 μg/mL). (E) Transduction of cell-associated JUNV GP alone and +TIM-4 GPI pseudovirions into HEK 293Ts. Western blots of VSV matrix protein present in onput virus are shown below the graph. Cell-associated virus was removed from the surface using EGTA (EGTA) and a portion was further dialyzed into 1x PBS (Dialysis). (F) Incorporation of JUNV or EBOV GP into GP alone and +TIM-4 GPI pseudovirions. Dot blots were prepared by directly applying equal quantities of GP alone and +TIM-4 GPI virus to nitrocellulose. Quantities of virus were normalized by previously determined matrix amounts. The presence of JUNV and EBOV GPs were detected by staining the dot blot with respective anti-GP antibodies. Transduction data in panels A and C are shown as mean ± SEM for three replicates and data in panels D and E are shown as mean ± SD for a single replicate.
CHAPTER V
DISCUSSION

It has only been over the last three years that several PtdSer-binding proteins/complexes were identified as receptors for enveloped virus-entry. While TIM-1 was discovered by our group as a receptor for EBOV before I began my graduate career, it was not until my studies that we appreciated the importance of PtdSer-binding for interaction with virions. We and others have made several advances in this new field of study and my studies have provided a major contribution to the field. Consequently, no information on PVEERs and their mechanism of action were known when I began my work. This discussion is written not only to place my contributions into the context of the field, but provide a current overview of this important and emerging field of virology as of June, 2014.

PVEERs

Overview

We and others have identified several receptors and receptor complexes that enhance entry of a diverse range of enveloped viruses including members of the flavivirus, filovirus, New World arenavirus, baculovirus, and alphavirus families/genera (55-58). This group of viral receptors shares the ability to bind to PtdSer present on the viral envelope and, consequently, we have termed them PtdSer-mediated virus entry enhancing receptors or PVEERs. The broad expression of these receptors and their ability to interact with PtdSer on a wide array of enveloped viruses has huge potential implications for virus infection. Most importantly, PVEERs enhance virus binding to cells and facilitate internalization. These receptors also contribute to immune evasion through both anti-inflammatory signaling and a mechanism of entry that doesn’t require key receptor-binding domain and fusion epitopes to be exposed for cell surface receptor binding and potential neutralization from antibodies. However, many aspects of PVEER biology during viral infection remain to be elucidated.
**Identified PVEERS**

Six PVEERs have been identified to date: TIM-1, TIM-4, Gas6 or Protein S/Axl, Mer, and Tyro3, and MFG-E8/integrin αvβ3 or αvβ5 (Figure 1.1). The key property that these proteins or complexes utilize to enhance virus entry is their ability to bind PtdSer, a function these receptors natively use to bind and clear apoptotic bodies (294). Mutation of residues involved in PtdSer binding or complex formation results in inhibition of PVEER function (54, 55, 57, 58). Further, competition with PtdSer liposomes, but not phosphatidylcholine liposomes, inhibits entry enhancement by PVEERs (56, 57). Phosphatidylethanolamine liposomes are also somewhat inhibitory, likely due to sharing similar structure to PtdSer (56). In some cases, prebinding virus with the PtdSer-binding protein, Annexin V (AnxV), has been shown to inhibit PVEER enhancement of entry (49, 53, 55), but not in others (58). Consistent with the ability of an array of PtdSer receptors to serve as PVEERS, we found an artificially generated PVEER containing the PtdSer-binding domain from AnxV was highly effective at mediating uptake of vesicular stomatitis virus (VSV) pseudovirions bearing a filovirus, alphavirus or baculovirus glycoprotein (289).

**Approaches to identifying new PVEERs**

The proteins that function as PVEERs are very diverse and, except within protein families, do not share similar structure. Thus, it becomes difficult to identify new PVEERs on structural similarity to those already identified. PVEERs do, however, share a function, binding to PtdSer. Consequently, one approach to identify new PVEERs would be to test previously discovered PtdSer-binding proteins for their ability to enhance virus entry. CD36, for example, has been shown to bind oxidized PtdSer for uptake of apoptotic cells into macrophages (295, 296). Two major drawbacks of this approach are that many of the identified PtdSer-binding receptors have already been tested and that PtdSer-binding activity is not sufficient for a protein to have PVEER activity. Thus, trying to identify new PVEERs through their shared function will provide limited results.
New PtdSer-binding receptors could also be identified through high-throughput genetic screening. Using lentiviruses to deliver CRISPR guide-RNA libraries and Cas9 for gene deletion allows for effective genetic screening (297). After generation of a cell population with mixed gene deletions, cells could be incubated with FITC-labeled virus and assessed for virus internalization. PVEER-null cells would be expected to be negative for internalization. By using labeled virus as a readout rather than reporter gene expression, the number of potential hits related to processes downstream of internalization, such as fusion, could be reduced. The selected pool may be further narrowed by using several different PVEER-utilizing viruses. After several rounds of selection, potential hits can be obtained through high-throughput sequencing of the guide-RNAs integrated in the selected cells. Cell lines that endogenously express high levels of multiple PVEERs may not be suitable for selection as compensation may occur between PVEERs. However, this could be circumvented by targeting identified PVEERs for deletion before screening. This broad screening approach may also identify non-receptor genes involved in internalization processes.

**Expression of PVEERs and implications for tropism**

The presence of PVEERs in various tissues permissive to infection may contribute to the tropism of some viruses, particularly for flaviviruses and filoviruses, whose entries are most enhanced by PVEER expression. PVEERs are expressed in a variety of tissues and cell types (summarized in Table 1.1) that are key targets of infection by enveloped viruses. TIM-1, TIM-4, and Axl (298-300) may enhance uptake of Dengue virus into Langerhans dendritic cells (DCs), one of the first targets during flavivirus infection (301, 302). TIM-1 is also present on the mucosal epithelia of the airway and eye (47), both of which may be routes of infection for filoviruses (303). Early during infection, flaviviruses and filoviruses replicate in antigen-presenting cell (APC) populations: DCs and macrophages (9, 304, 305). Similarly, alphaviruses infect DCs (306-309) and establish persistent infections in
macrophages (306, 310). Interestingly, these APCs express a variety of PVEERs including TIM-4 (60, 78-81), TAM receptors (97-99), and MFG-E8/integrin αvβ3 or αvβ5 (121, 123, 126-128). However, little is known about whether these PVEERs play critical roles in entry of virus into APC populations. Knock out of TAM receptors from bone marrow-derived DCs significantly reduces infection by West Nile virus or lentiviruses pseudotyped with Ebola, Marburg, vesicular stomatitis, or murine leukemia virus entry proteins (110). In addition, PtdSer liposomes compete for uptake of EBOV virus-like particles (VLPs) in mouse peritoneal macrophages (56). These data suggest these receptors are relevant for uptake into early infection targets.

PVEERs are also expressed in tissues that are later targets of virus spread. After infection by flaviviruses, Langerhans cells traffic to the lymph nodes (311). Once there, flaviviruses infect lymph node DCs (312, 313), which have been shown to express TIM-4 (60, 78). The alphavirus, Chikungunya virus, and filoviruses also replicate in lymph nodes (9, 304, 306), where TIM-4, in conjunction with MFG-E8/integrin αvβ3 and αvβ5 (121, 129), may mediate infection. Infected APCs also traffic flaviviruses and filoviruses to the liver and spleen, two major sites of replication (9, 305, 312, 314, 315). Expression of MFG-E8/integrin αvβ5 and TIM-4 has been detected in spleen (78, 121, 130). In particular, TIM-4 is expressed on splenic macrophages (60, 89), a target of flaviviruses (312). TIM-1 and integrin αvβ5 have been detected on hepatocellular Huh7 cells (47, 55) and Kupffer cells (316), respectively, and may contribute to flaviviruses and filovirus infection of the liver (304, 312, 317-320). Flaviviruses also infect bone marrow myeloid cells (305, 312), which were recently shown to express TIM-4 after stimulation (81).

PVEER expression does not appear to affect the tropism of every virus for which they enhance entry. For instance, Ross River, Chikungunya, and Sindbis virus infect muscle cells, which do not express any currently identified PVEERs (321-323), suggesting that either other unidentified PVEERs exist or virus entry into muscle cells is independent of PVEER expression. Interestingly, the PtdSer-binding receptor BAI1 has been shown to be important
for myoblast fusion (324). Although BAI1 does not effectively enhance virus entry in vitro (55), it may contribute to virus entry in vivo in its native cell type and environment. This may also be the case for other PtdSer receptors that do not function as PVEERs in cell culture as the efficacy of these receptors was assessed after exogenous expression in cell types that do not endogenously express these proteins. Interestingly, PVEERs may also not function effectively in every cell population in which they are endogenously expressed as TIM-1 is present on some populations of T-cells (70, 75, 78), but, T cells are refractory to EBOV infection (12). However, this may also be due to defects in virion internalization machinery or subsequent steps in the viral life cycle and not PVEER activity. Vaccinia virus does infect activated T-cells (325) and in this case TIM-1 may contribute to entry. Nonetheless, the effect of PtdSer binding protein expression on virus tropism has not been thoroughly explored in tissue culture and has yet to be assessed in vivo.

**In vivo relevance of PVEERs**

A major question remaining regarding PVEER-mediated enhancement of virus entry is its relevancy in vivo. The efficacy of PVEERs on wild-type infectious virus has been demonstrated in vitro with virus harvested from both cell culture and mice (56, 57). Additionally, entry of Ebola virus VLPs into mouse peritoneal macrophages is inhibited by PtdSer liposomes, providing evidence of PVEER importance for filovirus entry into relevant primary cell populations (56). However, to date only limited studies have been done in mice to determine the effect of PtdSer-binding inhibitors and none using PVEER knock down or knock out. Difficulty of testing in vivo relevancy of PVEERs using knockout mice arises from the potential for compensation. Additionally, many PVEERs play critical roles in regulating adaptive immunity and single or combination knockout of genes could make interpretation of results difficult and/or lead to development of autoimmunity (79, 121, 276, 326, 327). Nonetheless, in a single study, a chimeric antibody that recognizes PtdSer was shown to inhibit infection by the New World arenavirus, Pichinde (52). Future studies will
benefit from the development of a broad PtdSer-binding inhibitor that can target multiple PVEERs.

**Approaches to studying in vivo relevance**

Due to an interest in the role of many of these receptors in autoimmune diseases and immune regulation, several PtdSer-receptor-knockout mouse models have been developed. These include mice deficient in TIM-1\(^{(84)}\), TIM-1\(^{\text{Δmucin}}\) (276), TIM-4 (79), MFG-E8 (121), both MFG-E8 and TIM-4 (326), TAM receptors (101, 328), Protein S (329), and Gas6 (100). Assessing whether or not deletion of these genes results in a reduction of viral titers or pathogenicity during virus infection will be a first step in determining the importance of PVEERs for replication \textit{in vivo}. However, deletion of these genes individually or in combination can lead to the development of autoimmunity (79, 121, 276, 326, 328, 330), asthma (84), or other defects (100, 101, 329, 331). In some cases, complications due to these defects, such as autoimmunity, can be avoided by infecting younger mice before symptoms develop (121, 276, 326). Also as many of these receptors may have redundant function, loss of individual receptors or ligands may not provide insights.

An alternative to using knockout mice would be the use of PtdSer-binding inhibitors. A major benefit of inhibitors is their transient effect. We have not identified any mAbs that effectively inhibit mTIM-1-mediated enhancement of virus entry. While we have shown that ARD5 and other TIM-1-specific mAbs effectively inhibit virus entry into several cell lines (47, 57), these would not be not be effective in mice due to their specificity for human TIM-1. However, our group and collaborators have recently developed a human-TIM-1-expressing mouse model for which we can test the efficacy of these mAbs. This model, in parallel with our mTIM-1-null mouse model, will allow for the assessment of TIM-1-mediated virus entry \textit{in vivo}.

The PtdSer binding domains of the identified PVEERs vary greatly between families. However, within the TIM family there is a conserved PtdSer binding pocket within the IgV-
domain. The crystal structures of both the murine TIM-1 and TIM-4 IgV domains have been solved (61, 243). The use of in silico screening of chemical libraries might identify some potential hits for testing efficacy of inhibition. The crystal structure of the interface between Gas6 and Axl has also been solved (95). Chemical inhibitors, antibodies, or Gas6-mimics that disrupt this interaction might prove to be effective at reducing virus binding. In addition, the RTK domain of the TAM receptors also represents a viable target to limit anti-immune signaling. Anti-Axl antibodies and several small-molecule inhibitors of Axl and have already been developed as potential chemotherapeutics (332) and been used effectively in mice (333, 334). Similarly, peptides mimicking the RGD recognition sequence of αvβ3 and αvβ5 integrin have also been developed as chemotherapeutics and can block the interaction of these integrins with their ligands, such as MFG-E8 (335, 336). However, the efficacy of these inhibitors on virus infection remains to be determined in vitro before in vivo studies can be initiated. Nonetheless, they could prove to be valuable tools for assessing the role of PVEERs play during infection in vivo.

**Mechanism of PVEER enhancement of virus entry**

**Overview**

The key factors required for proteins to function as PVEERs are PtdSer-binding activity and attachment to the plasma membrane (54-57). While TIM-1 and TIM-4 individually contain both of these factors, the TAM receptors and αvβ3 or αvβ5 integrins provide membrane attachment and their ligands bind to PtdSer. In the case of the TIM family PVEERs, the spacer domain between the PtdSer-binding pocket and plasma membrane attachment is a MLD. We found the MLDs from other molecules can substitute for the TIM MLDs, provided the MLD is of sufficient length, while the more compact structure of RAGE IgC2 domains cannot (289). These studies led us to propose that an extended structure was needed in the spacer region, perhaps to extend the PtdSer binding-
pocket above the extracellular matrix that surrounds the cell (289). Thus, sufficient distance is necessary between the two essential functions of PVEERs for virus binding.

While we understand how PVEERs bind virus, much less is currently known about the pathways and/or mechanisms for PVEER mediated internalization of virus are unknown and there is no evidence for direct internalization. Ebola, baculo-, and vaccinia virus enter predominantly or at least partially through macropinocytosis (26-29, 49, 337) and these viruses are amongst those whose entry is most significantly enhanced by PVEER expression. However, entry of flaviviruses and alphaviruses is enhanced by PVEER expression, but primarily occurs through clathrin-mediated endocytosis (338-343). Thus, if PVEERs directly mediate virus internalization, there are currently no data to support entry via a single pathway. It is certainly possible that different PVEERs mediate uptake through different pathways. Alternatively, in some cases, PVEERs may function only as attachment factors to facilitate interaction of viral proteins with other cell surface receptors that trigger internalization. This may be true for New World arenaviruses that are known to enter cells using transferrin receptor 1 (344) and whose entry is only moderately enhanced by PVEER expression (56). However, this mechanism does not account for the ability of PVEERs to stimulate rapid uptake of viruses lacking a glycoprotein (12, 23).

**Incorporation of PtdSer into viral envelopes**

Several viruses have been shown to expose PtdSer on the outer leaflet of their membrane (52, 54, 55, 57). However, it remains to be determined how PtdSer, which is normally present within the inner leaflet of the cell membrane, is exposed on the outer leaflet of viral membranes. On cellular membranes the asymmetrical distribution of PtdSer is maintained by the activity of flippases and floppases that transfer phospholipids unidirectionally from either the extracellular side to the cytosolic side or the reverse, respectively (145). Meanwhile, scramblases disrupt asymmetry by mediating random bidirectional transfer of phospholipids. In healthy cells, exposure of PtdSer on the outer
leaflet of the plasma membrane can be induced by several mechanisms, including apoptosis and elevated levels of intracellular calcium (145, 345). Some viruses encode proteins that increase cytosolic calcium levels such as Nef of HIV-1 (346) and p7 of hepatitis C virus (347), reviewed in (348), which may contribute to PtdSer exposure. Entry of flaviviruses into cells also increases intracellular calcium levels (349). It is also possible that cellular stresses associated with virus infection trigger apoptosis as is seen with influenza A (350) and flaviviruses (351-353). There is evidence that West Nile, Sindbis, and Chikungunya virus actively activate apoptosis to their advantage (258, 354, 355). In contrast, however, apoptosis is not induced in cells that are infected by Ebola viruses (356, 357), although internalization of Ebola VLPs and infection of Ebola virus is still enhanced by TIM-1 expression (47, 56, 57). Further, some viruses for which entry is enhanced by PVEERs also encode anti-apoptotic genes, such as NS1 of vaccinia virus (358, 359) and P35 of baculovirus (360). Thus, there is not a clear correlation between induction of apoptosis and incorporation of PtdSer on viral envelopes.

Viruses may not utilize cellular functions to induce PtdSer flipping, but rather, concentrate the limited PtdSer present on the outer leaflet. PtdSer may be incorporated into the outer leaflet of viral envelopes during budding for promotion of favorable membrane curvature (361). Alternatively, viruses may associate with PtdSer in the inner leaflet due to its anionic charge as has been suggested for the matrix proteins of Ebola virus (362) and human immunodeficiency virus-1 (363, 364). The VSV matrix has also been shown to associate with PtdSer enriched domains (365). Thus, the inner leaflet would be enriched with PtdSer and, after budding, the absence of flippases that normally regulate PtdSer asymmetry may lead to equilibration of PtdSer between the outer and inner leaflets. Nonetheless, the expression of the matrix protein alone results in sufficient PtdSer accumulation on the outer leaflet of Ebola virus VLPs to allow for TIM-1 enhancement of internalization (56, 57). These data suggest that regardless of the mechanism, the activity of the matrix protein is sufficient for PtdSer incorporation into viral envelopes.
Approaches to studying PtdSer incorporation into the outer leaflet of the virion envelope

In order to examine how PtdSer becomes exposed on the viral membrane, initial studies would need to determine whether PtdSer is present on viral envelopes prior to or post virus budding. These studies would suggest incorporation of PtdSer in viral membranes occurs due to the favorable membrane curvature, activity of the viral matrix protein, or the absence of flippase activity to maintain asymmetric distribution after budding. Labeling virus budding from cells with anti-PtdSer antibodies and using fluorescence microscopy might determine whether matrix proteins are being recruited to or associating with PtdSer-rich domains. Transmission electron microscopy with immunogold labeling might further these studies by determining which leaflet contains PtdSer.

If there is exchange of phospholipids between the inner and outer bilayers after budding there should be a disruption of PtdSer asymmetry and other phospholipids in viral membranes. Techniques used to characterize lipid distribution in liposomes may also work on viruses, although the presence of viral proteins may complicate results (366, 367). An alternative approach would be to assess the increase in PtdSer amounts on the outer leaflet of virions as the distribution of phospholipids reaches equilibrium. Previously we have used ELISA-based approaches to detect PtdSer (57), however this requires an overnight binding of virions to plates. In order to assess PtdSer amounts on the outer leaflet before their accumulation, either a shorter binding step would be required or alternative approaches such as bio-layer interferometry could be used. Bio-layer interferometry uses differences in wavelength to detect binding of ligands to antibody immobilized on a surface. One benefit of this technique is that raw supernatants can be used to detect virus binding to immobilized anti-PtdSer antibodies and thus could be used in time-lapse studies with virus-producing cells. Additionally, as binding is assessed by the application of light, measurements can be made in real-time without the addition of conjugated secondary antibodies. This is a critical advantage if the translocation of PtdSer occurs rapidly after budding.
Signaling pathways for virus internalization

In theory, phagocytosis would best explain the mechanism of PVEER-mediated internalization with PVEERs sequentially binding PtdSer on the virion surface. This is particularly fitting for TIM-1- and TIM-4-mediated internalization of apoptotic bodies, as they have been shown to form phagocytic cups around cargo (85, 89). However, both PtdSer and VLPs have been shown to induce macropinocytosis (26, 134). Additionally, amiloride and its derivative 5-(N-Ethyl-N-isopropyl) amiloride (EIPA), considered specific inhibitors of macropinocytosis (368) but not phagocytosis (369), inhibit Ebola virus uptake into and infection of Vero cells or SNB19 cells that express TIM-1 and Axl respectively (25, 27, 29, 47). These inhibitor studies suggest that Ebola viruses utilize macropinocytosis in PVEER expressing cells. Thus, there exists disparity between mechanisms of apoptotic body and virus uptake. Some of this confusion may be attributed to many components being shared between phagocytosis and macropinocytosis. Both require phosphoinositide 3-kinase signaling (370), phospholipase Cγ (371, 372), Rac1 (373, 374), and dynamin (375-378), making differentiation between the two processes by targeting components difficult. The only macropinocytosis specific inhibitor, EIPA, functions by deregulating intracellular pH and thus may disrupt additional aspects of the viral life cycle that contribute to inhibition of virus infection (379).

Neither phagocytosis nor macropinocytosis have been studied in the context of PVEERs. Phagocytosis is associated with phagocytic cells such as macrophages and dendritic cells (380), while all cells can initiate macropinocytosis. It is possible that both mechanisms are being used and are cell-type dependent. For example Ebola virus initially infects macrophages and dendritic cells, which may occur through phagocytosis, and subsequently spreads and enters a broad variety of tissues and cell types, perhaps by macropinocytosis (8). Interestingly, receptors that induce either phagocytosis or macropinocytosis require phosphorylation and recruitment of kinases and adaptor proteins (381, 382), but several
PVEERs have been shown not to require signaling through cytoplasmic tails for internalization of virus (55, 110, 289).

Neither phagocytosis nor macropinocytosis have been shown to be directly elicited by PVEERs. While Axl has been associated with macropinocytosis in some cells (25), it is unknown whether Axl triggers macropinocytosis. There are, however, several studies that show PtdSer receptors coordinate to induce uptake signaling. In macrophages there is evidence that Mer induces internalization of apoptotic bodies, but requires TIM-4 for initial attachment due to a higher binding affinity for PtdSer (383). Mer has also been shown to work synergistically with integrin αvβ5 (384). Similarly, TIM-4 and MFG-E8 have been implicated as partners for uptake of apoptotic bodies in which MFG-E8/integrin signaling triggers uptake (385). Several details of these mechanisms remain to be elucidated, such as whether or not TIM-4 actively associates with signaling partners. TIM-4 has also been shown to interact with adenosine monophosphate activating kinase (AMPK) (81), a protein important for macropinocytosis of Ebola and vaccinia virus (386, 387). However, this interaction is believed to occur after phagocytosis and does not explain how initial internalization events are triggered. This is further complicated by evidence that the cytoplasmic tail of TIM-4, the only domain accessible to AMPK, is unnecessary for internalization (69). Further, these mechanisms do not explain why entry of viruses that utilize clathrin-coated pits is enhanced by PVEER expression. Thus, additional studies are required to determine if and how PVEERs contribute directly to internalization.

**Approaches to studying the mechanism of PVEER-mediated entry**

PVEERs enhance binding of several viruses (54-57), indicating that they function as attachment factors. However, there is also evidence that PVEERs might also mediate internalization as evidenced by the uptake into cells of virions without GPs (56, 57). Insight into these potential mechanisms of internalization or entry will be obtained through the
studying these mechanisms in the context of PVEER expression. Initial studies might utilize confocal microscopy to determine the relative localization of virions and PVEERs within the cell. New, super-resolution microscopy techniques such as stochastic optical reconstruction microscopy, photo activated localization microscopy, and stimulated emission depletion, significantly enhance the clarity of cellular interactions visualized by microscopy compared to previous techniques. These technologies would allow for not only assessment of whether or not PVEERs follow virus into endosomes but perhaps also provide insight into whether or not PVEERs bind virus individually or in groups/clusters.

It remains to be determined whether or not PVEERs enhance entry by driving virus interaction with a native receptor or by mediating entry directly. Some insight into this gap in knowledge might be gained by comparison of virus entry pathways in the presence or absence of PVEER expression. The cell lines for comparison could be prepared by either transient expression of PVEERs in cell line that is not known to express any PVEERs, such as HEK 293T cells, or through deletion of gene expression from a cell line that endogenously expresses a PVEER, such as TIM-1 in Vero cells. Complete deletion of gene expression can be achieved using clustered regularly interspaced short palindromic repeats (CRISPRs) and Cas9 (388). Interrogation of the pathways used for internalization, such as phagocytosis, macropinocytosis, clathrin-coated pits, or caveolin-mediated, can be achieved through use of various inhibitors and dominant-negative proteins commercially available. Utilization of disparate pathways between PVEER positive or negative cell lines would suggest a role of PVEERs in internalization.

Absence of or independence from PVEER activity

PtdSer-binding proteins without PVEER function

While some PtdSer-binding receptors function effectively as PVEERs, this is not true for all. Receptor for advanced glycation endproducts (RAGE), brain-specific angiogenesis inhibitor 1 (BAI1), CD300a, Stabilin-1 and -2, and TIM-3 have all been shown to bind to
phosphatidylserine and enhance engulfment of apoptotic cells (Figure 5.1 and Table 5.1) (62, 229, 231, 253, 324, 389-392). However, expression of these proteins does not enhance virus entry (55, 57, 58). Of these proteins, we elucidated the mechanism responsible for reduced PVEER efficacy of TIM-3 (289). Although all human TIM family members can bind PtdSer and enhance the uptake of apoptotic bodies (60, 62), TIM-3 does not effectively enhance virus entry (<2 fold) (47, 55, 56, 289). While the IgV domain of TIM-3 has reduced affinity for PtdSer (62), we found that replacing the TIM-1 or TIM-4 IgV domain with that of TIM-3 results in a functional PVEER (289). Thus, the reduced efficacy is not due to the TIM-3 IgV domain. Rather, the inability of TIM-3 to function effectively as a PVEER is likely due to the short length of the TIM-3 MLD which is ~2.5-fold shorter than that of TIM-1 and -4 (Figure 1.1). This is supported by our studies and others showing that deletions within the MLD of TIM-1 reduce PVEER efficacy (56, 289).

Stabilin-1/-2 and BAI1 bind PtdSer through epidermal growth factor (EGF)-like domain repeats (392-394) and type 1 thrombospondin repeats (TSRs), respectively (389) (Figure 1.2), although the residues within these domains mediating PtdSer binding have yet to be identified. While it is unknown why these motifs are able to bind apoptotic cells, but not virions, none of the PtdSer binding receptors that function as PVEERs bind PtdSer using repeat regions. One explanation may be that the repeats require a larger or flatter surface area to bind PtdSer than a virion envelope allows. This biological conundrum requires further examination and may provide insight for identification of other PVEERs.

RAGE is the only PtdSer receptor listed above for which the PtdSer-binding-domain is unidentified. The extracellular portion of RAGE consists of an N-terminal IgV domain followed by two IgC2 domains (Figure 5.1) (230). The IgV domain of RAGE, shown in Figure 1.2, has been suggested to be responsible for PtdSer binding although this has not yet been experimentally shown (231). The lack of a similar PtdSer-binding pocket to that of the CD300a and TIM IgVs and inability to functionally replace the IgV domain of TIM-1(289) would suggest at least the IgV domain of RAGE cannot bind to PtdSer on viral envelopes.
Additionally, our demonstration that the condensed structure of the RAGE stalk provided by the IgC2 domains likely also further reduces PVEER efficacy by reducing overall length (289). Thus, both the absence of a clear PtdSer-binding pocket in the IgV domain and the short stalk likely explain the absent PVEER activity of RAGE.

**Viruses that do not utilize PVEERs**

PVEER expression does not enhance entry of all enveloped viruses (Table 1.1). In the case of arenaviruses, there exists an interesting dichotomy between the ability of PVEERs to enhance entry of New World but not Old World arenaviruses. Unlike for filo-, baculo-, alpha-, vaccinia, and flaviviruses, definitive cellular receptors have been identified for both Old World and New World arenaviruses: α-dystroglycan (261) and transferrin receptor 1 (344, 395), respectively. These viruses also use different entry pathways as New World arenaviruses such as Junin virus enter using clathrin coated pits (396) while Old World arenaviruses such as Lassa and lymphocytic choriomeningitis virus use an unknown pathway that is clathrin, caveolin, and dynamin independent (397, 398). Compared to filoviruses and flaviviruses, PVEER-mediated enhancement of New World arenaviruses entry is modest (56, 57). New World arenaviruses can bind to host cells and mediate efficient entry through interaction with transferrin receptor 1, and this is likely supplemented by PVEER expression. For Old World arenaviruses, expression of and/or affinity to α-dystroglycan may be sufficiently robust that PVEER expression does not notably enhance entry further. Indeed, in cells lacking α-dystroglycan, other attachment factors, including Axl, can enhance Old World arenavirus entry (262). However, PVEERs are likely not biologically relevant for Old World arenavirus entry as α-dystroglycan expression is ubiquitous (261, 399).

Entry of severe acute respiratory syndrome (SARS) corona-, influenza A, and herpes simplex 1 viruses is not enhanced by PVEER expression (55, 56). Influenza A and SARS-CoV have viral envelope proteins that bind effectively to sialic acid (400) and ACE2 (401) respectively. Expression of TIM-1 on HEK 293T cells enhances internalization of
pseudovirions bearing either envelope protein, but does not result in enhancement of transduction (56). These results indicate that PVEER-mediated or -enhanced internalization results in unproductive infection by these viruses. This could occur due to virions being delivered to compartments with incompatible conditions for fusion, i.e. lacking correct pH or fusion triggers. An alternative possibility is that PVEERs enhance internalization of defective SARS coronavirus or influenza A virions with low or negligible levels of glycoprotein on the viral envelope. These particles would not effectively bind cellular receptors or be able to fuse, and thus their enhanced internalization would not contribute to infection. Internalization into endosomes may similarly inhibit herpes simplex virus 1 entry which fuses at the cell surface (402). However, as the effects of PVEER expression on herpes simplex virus 1 internalization have not been tested, it is possible that, in a manner similar to that proposed for Old World arenaviruses, strong association with cellular receptors masks any enhancement. Interestingly in all cases, PVEER overexpression on permissive cells does not appear to inhibit overall virus entry, suggesting that either a majority of these viruses either escape these compartments or enters through natural productive routes.

**Conclusions for PVEER studies**

Utilization of PtdSer-binding proteins by viruses to enhance internalization provides a broad mechanism of viral entry with little investment by the virus itself. This mechanism may allow some viruses to attach to cells, thereby making viral glycoprotein/cellular receptor interactions more probable. Alternatively, other viruses may rely entirely on PVEERs for internalization into endosomes. This latter mechanism would eliminate the need of viruses to expose sensitive viral epitopes extracellularly, thereby protecting critical receptor binding or membrane fusion motifs from neutralizing antibodies. Once virions are internalized into endosomes, glycoprotein structural alterations that lead to membrane fusion can occur unhindered by antibodies. Additionally, the broad expression of PVEERs might contribute to the extensive tropism of viruses such as flaviviruses and alphaviruses that infect a broad
array of insect and mammalian hosts. Further research on these receptors will elucidate these gaps in knowledge and determine feasibility of broad-spectrum antivirals to target them.

Expression of TIMs on virus-producing cells

Overview

PtdSer has shown to be a surface marker of tumors and cancer cells (211-215). Consequently, we hypothesized that expression of TIM proteins on viral envelopes would enhance binding of these viruses to cancer cells. This could prove to be an effective method for enhancing the specificity and binding efficiency of oncolytic viruses to tumors without modifying viral glycoproteins. Unlike previous attempts to target adenovirus to PtdSer using soluble bispecific antibodies (287), direct incorporation of TIMs into the viral envelope could bypass the need for a soluble PtdSer-binding protein to associate with the virus.

Our initial results were promising as we found that expression of TIM proteins in virus-producing cells enhanced the titer of EBOV GP pseudovirions. In addition, this enhancement was dependent on the amount of PtdSer exposed on the envelope of target cells. However, we found that while TIMs were present in the virus stocks, the TIMs did not directly incorporate into viral envelopes. Consequently, purification of viruses resulted in a loss of TIMs and PtdSer-binding enhancement of entry. Virus produced from TIM-expressing cells could be concentrated using a MWCO filter, while still maintaining enhanced entry into PtdSer-exposing cells. However, use of this virus in vivo would still be problematic due to contaminating proteins. Thus, significant further work is required before TIMs can be used to deliver oncolytic viruses to tumors.

Approaches to determining the mechanism of virus enhancement

TIMs appear to not to be associated with virions, perhaps either due to cleavage from the cell/virion membranes or expression in the membrane of exosomes or cell debris. The
first step to clarifying the mechanism of enhanced entry would be to determine whether or not TIMs are soluble or membrane-bound. This might be achieved by using TIMs with distinct tags on the termini. We have already successfully tagged TIMs on both the N-termini (HA tag on GPI-anchored TIMs) and C-termini (GFP tag after the cytoplasmic tail). The C-terminal tag would be expected to be absent from the supernatant if TIMs are being cleaved from membranes. Further, exosomes and cell debris could be separately purified from supernatants to determine if TIMs are associated with them.

The presence of TIMs in supernatant might enhance entry through either bridging of virus to cells or triggering signaling events in target cells that promote entry or transduction. The former can be assessed by binding of FITC-labeled virions or GFP-VLPs to HEK 293T cells. The triggering of internalization events such as macropinocytosis, clathrin-mediated endocytosis, and caveloin-mediated uptake by the presence of TIMs in virus stocks can be evaluated using fluorescently labeled dextran, transferrin, and albumin, respectively. However, TIMs may not affect internalization pathways, but rather, induce anti-immune responses. In this case, production of cytokines or activation of transcription factors associated with dampening the immune system may be affected and can be monitored by ELISA and/or qPCR.

**Approaches to determining mechanism of virus inhibition**

We and others (Shan-Lu Liu, University of Missouri) have found that expression of TIMs in virus-producing cells reduces the release of pseudovirions into the supernatant, acting as BST-2 does to tether virions to the outer membrane of infected cells. Given that TIMs enhance binding of virus to cells (47, 55-57), it is reasonable that TIMs also bind virus after budding from cells, thereby tethering them to the membrane. In addition, we found that expression of TIMs reduced virus titers. This may simply be because of a reduction in GP expression in virus-producing cells either due to the presence of two CMV-promoter-driven plasmids competing for transcription factors or TIM overexpression affecting general
protein synthesis, processing, glycosylation, and/or vesicle transport. Producing GP alone
virus with reduced amounts of GP incorporation, similar to +TIM-4 GPI virus, would
determine whether or not reduced quantities of GP explain the reduction in titer.
Alternatively, expression of TIMs might be affecting the quality of GPs that make it to the
plasma membrane and incorporate into virions. This might be assessed by fusion assays,
comparison of GP sizes by western blot, or use of conformational specific antibodies.

**Approaches to utilizing membrane-bound TIMs for enhancement of virus entry**

The lack of TIM incorporation into virions represents a significant hurdle to future
*in vivo* studies as virions cannot be purified without losing TIMs. The critical domain for the
binding activity of TIMs is the IgV domain. The MLDs of TIM proteins are highly
glycosylated and extended, which may cause steric hindrance, preventing packaging onto
viral envelopes. Interestingly, removal of the highly-glycosylated mucin domain of the
EBOV GP enhances incorporation (250). The TIMs may also lack effective signal peptides,
transmembrane domains, or cytoplasmic tails for appropriate incorporation into budding
virions. These problems may be alleviated with a chimeric protein made of the IgV domain
of TIM-1 and stalk, transmembrane, and cytoplasmic regions from a protein known
effectively incorporate into virions, such as a viral GP. Thus, further engineering is required
generate a virus-bound TIM.

Soluble TIMs are not ideal as an *in vivo* therapeutic as TIMs would be diluted upon
delivery and therefore less likely to associate with virus. However, this effect may limited by
direct injection of virions into tumors. Further, a greater understanding of how TIM
expression in virus-producing cells enhances virus entry may allow for the separation of
virus-entry enhancing factors, presumably soluble TIMs, from virus production. For
example, if soluble TIMs enhance virus entry by bridging virus to cells, soluble dimeric
TIMs could be generated, purified, and then mixed with virus stocks rather than expressing
TIMs in virus-producing cells. This might also circumvent the inhibition of virus titers seen with other GPs when TIMs are expressed in virus-producing cells.

Beyond determining the therapeutic potential of TIMs for delivery of oncolytic viruses, further study of the mechanisms described above may provide additional discoveries. For example, understanding why only pseudovirions with filovirus GPs are enhanced by TIM expression in virus-producing cells might provide insight into biology of the GP. If soluble TIMs should bridge virus to cells through dimerization, this might have implications for functional relevance of IgV dimers seen with purified proteins (243). Alternatively, should TIMs instead be inducing signaling pathways that affect virus internalization or dampen the immune response, this would be a novel finding and could provide insight into the function of soluble TIMs in vivo.
Figure 5.1  PtdSer-binding receptors that do not function as PVEERs. Cartoon representations of Stabilin-1, BAI1, CD300a, RAGE, and TIM-3 are shown. Estimations of N-linked glycan sites are indicated with blue tridents and O-link glycosylation sites (TIM-3 only) are indicated with a green line. The binding pocket of the RAGE IgV domain is only hypothesized, as indicated by the presence of a dotted line. Domains for which calcium-binding is necessary for interaction with PtdSer are shown with calcium ions (orange spheres).
<table>
<thead>
<tr>
<th>Protein Name(s)</th>
<th>PtdSer Binding Domain</th>
<th>Tissue/Cell Type Expression</th>
<th>Pseudoviruses for which entry is enhanced (&gt;2 fold)</th>
<th>Pseudoviruses for which entry is unaffected (&lt;2 fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD300a</td>
<td>IgV (229)</td>
<td>Immune cells: CD8+ T cells (403), CD4+ T cells (404-406), B cells (407), and NK cells (408, 409)</td>
<td>Alphavirus: Sindbis (58)*</td>
<td>Alphavirus: Ross River and Sindbis (58) Baculovirus: Autographa californica multicapsid nucleopolyhedrovirus (58) Rhabdovirus: Vesicular stomatitis virus (58)</td>
</tr>
<tr>
<td>BAI1</td>
<td>Type 1 thrombospondin repeats (TSRs) (389)</td>
<td>Tissues: Brain (410, 411), muscle(324), bone marrow, and spleen (389) Immune cells: Macrophages (389)</td>
<td>Alphavirus: Ross River and Sindbis (58) Baculovirus: Autographa californica multicapsid nucleopolyhedrovirus (58) Rhabdovirus: Vesicular stomatitis virus (58) Flavivirus: Dengue (55)</td>
<td></td>
</tr>
<tr>
<td>RAGE</td>
<td>IgV (231)*</td>
<td>Immune cells: CD8+ and CD4+ T cells (412, 413), DCs (414), macrophages (415, 416), and monocytes (417) Tissues: Smooth muscle (415, 418), cartilage chondrocytes (419), skin keratinocytes (420), and vascular system (421, 422)</td>
<td>Old World Arenavirus: Lassa (57) Filovirus: Ebola virus (57)</td>
<td></td>
</tr>
<tr>
<td>MFG-E8/ αvβ3-5 integrin</td>
<td>C2 domain (112, 113)</td>
<td>Immune cells: Macrophages (114, 121, 126, 127), Immature DCs (123, 128) Tissues: Mammary glands (124, 125), spleen, lymph node, brain (121, 129, 130), and vascular system (122, 131)</td>
<td>Alphavirus: Ross River and Sindbis (58) Baculovirus: Autographa californica multicapsid nucleopolyhedrovirus (58)</td>
<td>Rhabdovirus: Vesicular stomatitis virus (58)</td>
</tr>
</tbody>
</table>

*A Enhances binding only, but not infection

*Hypothesized but not shown
APPENDIX
MAPPING EPITOPES OF ANTI-TIM MONOCLONAL ANTIBODIES

Introduction

T-cell immunoglobulin and mucin domain protein 1 (TIM-1) has been identified as a receptor for multiple groups of enveloped viruses including members of the filovirus, flavivirus, alphavirus, arenavirus, and baculovirus families/genera (47, 55-57, 238, 239). Critical to this enhancement is the phosphatidylserine (PtdSer)-binding-pocket containing, N-terminal IgV domain of TIM-1 that interacts with PtdSer present in viral envelopes. This pocket is located between the FG and CC' loops and each contains critical residues involved in cation coordination and binding of PtdSer (61). Mutation of this pocket inhibits the binding of PtdSer and subsequently the enhancement of virus entry (55-57).

The importance of this pocket was identified through site-directed mutagenesis of residues throughout the IgV domain (57). Critical to determining whether or not mutation of these residues were affecting entry or structure of the protein was to confirm proper folding and expression of the mutants on the cell surface. In order to assess expression we used a selection of mouse monoclonal IgG2a antibodies (mAb) that are specific for human TIM-1: ARD5, A6G2, AKG7, or A8E5. Previous studies determined that ARD5, A6G2, and A8E5 bind to the IgV domain of TIM-1 while AKG7 binds to the mucin-like domain (MLD) that projects the IgV domain from the cell surface (47, 66, 247). The IgV specific antibodies also require correct conformation, and thus can be used to determine the appropriate structure as well as stability of the IgV domain.

In order to inhibit TIM-mediated enhancement of entry, several groups have used PtdSer liposomes (56, 57). These function by competing with virus for binding to TIM-1. A number of anti-TIM-1 monoclonal antibodies (mAbs) have also been identified that partially or completely block EBOV entry. Five mAbs produced as anti-asthmatic therapies were
evaluated for their ability to block virus infection or transduction (47). Of the five, A6G2, A8E5, and most effectively, ARD5, were found to be potent inhibitors of TIM-1-mediated entry (47, 57). The following studies have been done to identify the epitopes of ARD5 and our other IgV-specific mAbs, A6G2 and A8E5. In addition, we have also determined the relative efficacy of these mAbs to inhibit TIM-1-mediated enhancement entry of Ebola virus (EBOV) pseudovirions.

**Materials and Methods**

**Cell lines and viruses**

HEK 293T cells, a human embryonic kidney cell line, H3 cells, and Vero cells, an African green monkey kidney epithelial cell line, were maintained in DMEM (Gibco BRL) with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. H3 cells are a clonal population of HEK 293T cells selected for stable TIM-1 expression as previously described (47).

Vesicular stomatitis virus (VSV) pseudovirions were produced as previously described (11, 16, 47, 240). We used VSV (strain Indiana) pseudovirions whose genomes had the G glycoprotein gene replaced with EGFP (VSVΔG-EGFP). To produce VSV pseudovirions, HEK 293T cells were transfected with plasmids expressing either EBOV GP lacking the mucin domain of GP1 or Lassa virus (LASV) glycoprotein precursor (GPC) and transduced 24 h later with VSVΔG-EGFP pseudovirions. After 4 h of virus uptake, the plates were washed and refreshed with media. Pseudotyped virions were collected in supernate 48 and 72 h following transduction, pooled, and filtered through a 0.45 μm pore-size filter. Virus aliquots were stored at -80°C until use.

**Transductions**

HEK 293T cells were transfected with plasmids as indicated in figures using polyethylenimine (PEI) following standard protocol (267). Twenty-four hours following
transfection, one portion of the cell population was transferred to a 6 well tissue culture plate and stained for surface expression 24 h later as described below. A second portion of the transfected cells were transferred to a 48 well tissue culture plate and transduced 24 h later with pseudovirions at MOIs noted in figure legends. Twenty-four hours after transduction, cells were detached with Accutase solution (Millipore) and EGFP expression was assessed by flow cytometry as percent positive cells in FL-1 channel. Transduction data were normalized as indicated in figure legends. For antibody inhibition studies, mAbs were added to media upon addition of virus at concentrations indicated in figure legends.

**Surface expression**

Transfected HEK 293T cells were detached with 1xPBS + 5 mM EDTA 48 h after transfection, washed with 1xPBS + 5% FBS, and incubated for 1 h with mouse mAbs ARD5, A6G2, AKG7, or A8E5 (47, 66, 247) as indicated at a concentration of 5 μg/mL. Polyclonal antisera specific for mTIM-1 (R&D Systems) was used in epitope transfer studies for surface staining. Normal goat serum or purified mouse IgG2a (R&D Systems) were used as negative controls. Cells were washed and incubated for 20 minutes with anti-mouse secondary antisera conjugated to either Cy5 (Invitrogen) or DyLight 649 (Jackson ImmunoResearch). After incubation, cells were washed and protein expression was assessed by measuring the percentage of positive cells in the FL-4 channel using a FACSCalibur flow cytometer (BD Biosciences). All flow cytometry data was analyzed using FlowJo software (TreeStar, Inc).

**Generation of TIM point mutants**

TIM-1 and mTIM-1 mutants were generated as previously described (57). Briefly, point mutations were introduced into a TIM-1 or mTIM-1 expression plasmid by amplifying using primers with targeted nucleotide changes and flanking base pairs of homology. Mutations were confirmed by DNA sequencing.
Protein Structures

The predicted TIM-1 IgV structures were generated by threading the TIM-1 amino acid sequence on the mTIM-1 (2OR8) crystal structure using the protein homology/analogy recognition engine 2 (phyre2) (245). Crystal structures were manipulated and rendered using PyMOL software (246).

**Results**

Identification of epitopes for ARD5, A6G2, and A8E5

During our search for mutations that inhibit virus entry, we generated a panel of IgV domain point mutants (57). In total, 45 individual residues of the IgV domain were mutated. Those IgV residues identified for mutagenesis were in general surface exposed or within the PtdSer binding cleft. While staining for surface expression of our mutants, we identified several mutants, for which binding of one of our mAbs but not the others was inhibited, suggesting local disruption of a single epitope rather than the full structure (Figure A.1). Antibody binding was quantified by surface staining mutant and WT TIM-1 transfected HEK 293T cells with the mAbs and comparing the relative percent positive-stained cells between a mutant and WT TIM-1.

The mAb AKG7 bound equivalently to all the IgV mutants, except for D115A, as to WT TIM-1. As AKG7 is a MLD specific mAb and binds a linear epitope (268), the reduced detection of the D115A mutant is likely due to reduced protein stability and/or expression and not changes in local conformation. Binding of AKG7 to TIM-1 mutants with alternative substitutions of D115 was not reduced. For instance, AKG7 bound as well to D115E or D115N as WT TIM-1. D99A had reduced binding by all IgV-specific antibodies, but not AKG7, suggesting an IgV conformational change rather than altered stability of the whole protein.

We identified two arginines (R85 and R86) important for ARD5 binding located in a loop on the face opposite the PtdSer binding pocket (Figure A.2). Mutation of these
residues does not affect the ability of TIM-1 to enhance entry of vesicular stomatitis virus (VSV) pseudotyped with EBOV GP compared to WT TIM-1 (Figure A.3A). While the presence of ARD5 (1.7µg/mL) significantly inhibits enhancement of EBOV entry by WT TIM-1, individual mutations of R85 or R86 or in combination (AA and LL) prevent this inhibition. However, the expression of TIM-1 or the presence of ARD5 has no effect on entry of VSV pseudotyped with LASV GPC (Figure A.3B).

Similarly, we were able to find two mutants that affected the ability of A8E5 to bind to TIM-1, G29A and P30A (Figure A.1). These residues are also located on the face opposite the PtdSer binding pocket, near the bottom of the IgV domain (Figure A.2).

A6G2 binding was abrogated by mutating several residues within the FG loop of the PtdSer binding pocket (R110, G111, W112, N114) with mutation of glycine 111 having the most significant effect (Figure A.1 and 2). Unlike for ARD5, we were also able to identify mutations that appeared to enhance A6G2 binding compared to WT: F55A, R106A, K117A (Figure A.1). R106 and K117 are located within the F and G beta sheets respectively of the PtdSer binding pocket while F55 is present on the CC’ loop (Figure A.3C). Due to the overlap of the A6G2 epitope with the PtdSer binding pocket, mutation of many of these residues that alter A6G2 binding also partially or significantly inhibit EBOV entry (Figure A.3D). Incubation of WT TIM-1 expressing cells with A6G2 (0.5µg/mL) inhibits EBOV transduction ~30%. This inhibition was lost with mutations that reduce A6G2 binding: R110A, W112A, and N114D. Interestingly, mutations that enhance A6G2 binding (F55A, R106A, and K117A) also enhanced the efficacy of A6G2 to inhibit EBOV transduction.

**Attempts to reconstitute ARD5, A6G2, and A8E5 epitopes in mTIM-1**

Mutation of residues within the IgV domain could be having distal structural effects on the mAb epitopes rather than directly altering the sequence of the epitope itself. We
wanted to examine whether or not we could reconstitute these mAb epitopes in murine TIM-1 (mTIM-1) through introduction of the mAb epitope amino acid sequences. Human TIM-1 and mTIM-1 have 52% sequence identity (Figure A.4A), although there are sequence discrepancies at the sites of mAb binding. Subsequently, ARD5, A6G2, and A8E5 do not bind to mTIM-1 (Figure A.4B). We introduced IP113HR, HP32PS, and EG89RR mutations into mTIM-1 in an attempt to generate A6G2, A8E5, and ARD5 epitopes respectively. While IP113HR and HP32PS allowed for weak A6G2 binding, in no other cases were binding to mTIM-1 enhanced. Further, the HP32PS mutations would not have been predicted to enhance A6G2 binding, but rather A8E5 binding. In all cases, binding of a polyclonal anti-mTIM-1 antiserum was not reduced (Figure A.4C), indicating lack of mAb binding was not due to reduced expression. These results suggest that introduction of the mAb epitopes into mTIM-1 was not successful.

**Inhibition by mAbs**

In our studies, while all three IgV binding mAbs inhibit TIM-1 mediated enhancement of virus entry, ARD5 is the most efficacious mAb (47, 57). However, we wanted to more extensively compare these mAbs and determine their relative efficacy. For these studies we used a clonal HEK 293T population, H3 cells, that was selected after transfection for stable human TIM-1 expression (47). Serial dilutions of antibodies were added to H3 cells immediately prior to addition of EBOV pseudovirions and assessment of transduction was performed at 24 h (Figure A.5). Similar to our previous studies, ARD5 was the most effective with a 50% infective dose (ID₅₀) of 0.014 μg/mL, followed by A8E5 with an ID₅₀ of 0.38 μg/mL, and A6G2 with an ID₅₀ of 1.02 μg/mL. At higher concentrations we did see some inhibition by AKG7 above our IgG2a control antibody, but inhibition was still below 50% at our highest concentration of 33.33 μg/mL. The inhibition curves of ARD5 and A6G2 both plateaued at roughly 80% inhibition. However,
interestingly A8E5 inhibition plateaued lower at ~60%. Thus despite having a higher ID\textsubscript{50} than A8E5, A6G2 was more effect at higher concentrations.

**Discussion**

TIM-1 has recently been shown by several groups to enhance entry of multiple groups of enveloped viruses including members of the filovirus, flavivirus, alphavirus, arenavirus, and baculovirus families/genera (47, 55-57, 238, 239). Several mAbs that target the IgV domain of human TIM-1, ARD5, A6G2, and A8E5, have been shown to inhibit TIM-1 binding to PtdSer and enhancement of virus entry (47, 57). Here, we have used a panel of IgV domain point mutants to characterize their binding sites. We also assessed the impact that IgV mutations had on the ability of the mAbs to inhibit EBOV GP pseudovirion transduction. Mutations of residues R85 and R86 on the opposite face of the IgV domain from the PtdSer binding pocket reduced binding of ARD5. Similarly, mutation of two residues, G29 and P30, on the same face of the IgV domain inhibited binding of A8E5. We also found that mutation of multiple residues within the FG loop of the PtdSer binding pocket (R110, G111, W112, N114) inhibited A6G2 binding while mutation of residues F55, R106, and K117 enhanced A6G2 binding. In addition, the effects these mutations have on mAb binding correlated with their ability to inhibit transduction as mutations that reduced binding also reduced inhibition and those that enhanced binding had increased inhibition.

The ARD5 and A8E5 binding sites are located on the opposite face of the PtdSer binding pocket. Although these binding sites don’t overlap with the PtdSer binding pocket, mAbs are composed of multiple domains, including 4 IgV domains, and could provide steric hindrance. This could occur by mAbs either obscuring access to the PtdSer binding pocket or impeding the targeted PtdSer-containing membrane from reaching the pocket. Interestingly, unlike ARD5, the inhibition of TIM-1-mediated virus entry by A8E5 plateaus much earlier at 60% inhibition. These data suggest that binding of A8E5 does not provide
complete steric hindrance, perhaps due to the epitope being more distal from the PtdSer-binding pocket. As an alternative to inhibition by steric hindrance, it is possible that binding of ARD5 and A8E5 induces local conformational changes that prevent accessibility of the PtdSer-binding pocket. This latter hypothesis might be tested using solvent accessibility techniques such as hydrogen-deuterium exchange.

The presence of either ARD5 or A6G2 reduces virus entry, with their efficacy plateauing at ~80% inhibition. These data suggest that these antibodies strongly block entry once bound. The relative binding affinities of the IgV-specific mAbs for TIM-1 rank as follows: ARD5>A6G2>A8E5 (247, 427). Thus, the lower ID_{50} of ARD5 than A6G2 is likely due to its stronger affinity. While ARD5 and A8E5 significantly inhibit TIM-1, low levels of virus entry are still occurring due to the background permissivity of HEK 293T cells to EBOV transduction in the absence of TIM-1 expression.

The A6G2 binding site is located on the FG loop of the PtdSer binding pocket. Thus, it stands to reason that binding of A6G2 inhibits through direct competition with PtdSer or alters local structure to prevent interaction with PtdSer. The latter is likely occurring as we found mutation of several residues present below the epitope enhanced binding and inhibition, perhaps due to stabilization of the FG loop.

We were unsuccessful in reconstituting the mAb epitopes in mTIM-1. Although there is good identity between the two IgV domains in general, and specifically adjacent to the epitopes, it is possible that sequence and conformational differences in structurally adjacent sequences alter the positioning or exposure of the epitope. This might also explain why, despite mTIM-1 and TIM-1 sharing similar amino acid sequences at the location of some of the epitopes, such as A6G2, we do not see binding of our mAbs to mTIM-1. While mutation of our mapped epitopes reduces mAb binding, we cannot rule out that our mutations are disrupting distal portions of the IgV domain and thus the mAb epitope is not located at the site of mutation. In this case, introduction of similar sequences at the site of mutation would not restore the distal sequence that forms the epitope. However, this seems
unlikely as our panel of point mutations covered a majority of the surface exposed residues of the IgV domain, reducing the possibility we missed the distal epitope.

We were able to slightly enhance A6G2 binding to mTIM-1 through introduction of the A6G2 epitope sequence and inadvertently through A8E5 epitope sequence. This is likely due to the high conservation of the PtdSer binding pocket between human and murine TIM-1 (243). It is possible that alteration of the CC' loop, as these residues affect A6G2 binding to human TIM-1, might further enhance binding. Intriguingly, our HP32PS mutation, intended to enhance A8E5 binding, actually enhanced A6G2 binding. This suggests that introduction of these sequences could have distal effects on either the overall stability of the IgV domain or the PtdSer binding pocket. Nonetheless, these epitope transfer studies have only been repeated twice and thus no concrete conclusions can be made.

From these studies we were able to determine the binding sites of our IgV-specific mAbs. These will provide a valuable tool for studies on the human TIM-1 IgV domain as they allow for determination of conformation integrity. These mAbs may also prove to be useful inhibitors of TIM-1-mediated enveloped virus entry and thus an understanding of their epitopes may provide valuable information for designing additional inhibitors or refining and enhancing antibody binding.
Figure A.1  Surface staining of TIM-1 mutants with mAbs. HEK 293T were surface stained with TIM-1-specific mAbs ARD5, A6G2, A8E5, and AKG7 48 h after transfection with WT TIM-1 or TIM-1 point mutants. Data are shown as relative mAb binding as determined by surface staining point-mutant and WT TIM-1 expressing cells with mAbs and comparing the percent of mAb positive cells. Values below 1 indicate reduced binding while values greater than 1 indicate increased binding. Data are shown as mean ± SD of at least 2 replicates.
Comparison of mAb epitopes on predicted model of TIM-1. A predicted model of the human TIM-1 IgV domain was generated by threading the human TIM-1 IgV amino acid sequence on the crystal structure of the mTIM-1 IgV domain (2OR8) (243). The epitopes for ARD5 (red), A6G2 (blue), and A8E5 (yellow) are highlighted in color on the structure and key residues required for binding are labeled as determined by our point mutations (Figure 1). The structure of the face containing the PtdSer binding pocket is shown on the left while the opposite face is on the right.
Figure A.3  Effect of epitope mutation on mAb inhibition. Transduction of EBOV (A and D) or LASV (B and E) pseudovirions into HEK 293T cells expressing WT TIM-1, an empty vector (Empty), or ARD5-(A) or A6G2-(B) binding mutants. (A and B) ARD5-binding double mutants, RR86AA and RR86LL, are labeled as AA and LL, respectively. Transductions were done in the absence (black) or presence (grey) of ARD5 (1.7 µg/ml) (A and B) or A6G2 (0.5 µg/ml) (D and E). (C) Location on the predicted TIM-1 structure of residues that enhance binding of A6G2 when mutated. EBOV pseudovirions were added at an MOI of 0.01 and LASV at an MOI of 0.1 as titered on HEK 293T cells. Data are shown as mean ± SEM for at least three replicates.
Figure A.4  mAb epitopes cannot be reconstituted in mTIM-1. (A) Clustal V alignment of mTIM-1 and human TIM-1 IgV domain amino acid sequences. Residues identified as mAb epitopes are highlighted with taller, bold letters. The sequences that were mutated in mTIM-1 and the corresponding reference sequence of human TIM-1 are indicated in boxes. Numbers above the amino acid sequence indicate residue number of mTIM-1 sequence. (B) Percent antibody binding by ARD5, A6G2, and A8E5 to human TIM-1, mTIM-1, empty vector (empty), and mTIM-1 epitope-transfer mutant transfected cells. Binding to cells was assessed 48 h after transfection, was quantified using percent of positively stained cells and normalized to human TIM-1. (C) Binding of mTIM-1 specific antisera to transfected cells normalized was to WT mTIM-1. Data from one representative experiment with no replicates are shown.
Figure A.5  Dose dependent inhibition of EBOV transduction by mAbs. H3 cells were transduced with a constant amount of EBOV pseudovirions (MOI 0.2, as titered in H3 cells) in the presence of increasing quantities of mAbs ARD6, A6G2, AKG7, or A8E5 or an isotype control IgG. EGFP reporter expression was assessed 24 h following transduction by flow cytometry. One-site binding nonlinear fit was estimated using PRISM software with R² values of 0.99, 0.97, 0.70, 0.93, and 0.77 for ARD5, A6G2, AKG7, A8E5, and mIgG respectively. Data are shown as mean ± SEM for three replicates.
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