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DISCO VERY OF NOVEL ARENAVIRUS RECEPTORS AND ENTRY FACTORS

MORAZ Marie-Laurence

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Institut de Microbiologie

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Marie-Laurence MORAZ

Master en Génomique et Biologie Expérimentale
Université de Lausanne

Jury

Prof. Renaud Du Pasquier, Président
Prof. Stefan Kunz, Directeur de thèse
Prof. Silke Stertz, Experte
Dr. Med. Lukas Flatz, Expert

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DISCOVERY OF NOVEL ARENAVIRUS RECEPTORS AND ENTRY FACTORS

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Finally, I dedicate this thesis to my rest in peace father who has always trusted in me and believed that I could succeed in everything I undertaken to do.

"Il est des douleurs difficiles à consoler
Mais quelques mots peuvent les apaiser
Quand le chagrin aura pu s'enfuir
Il restera les meilleurs souvenirs.»

"There are pains difficult to console
But some words can appease them
When the sorrow will have been able to run away
There will remain the best memories.»

-The thoughts of my dad-

TABLE OF CONTENTS

ABBREVIATIONS

A549	human lung adenocarcinoma epithelial cells
Ab(s)	antibody-(ies)
AdV	adenovirus
NX5	annexin-V
APC	antigen presenting cell
Axl	TAM tyrosine kinase receptor anexeleto
DG	dystroglycan
α-DG	alpha dystroglycan
β-DG	beta dystroglycan
ECM	extracellular matrix
GP	glycoprotein
GP1	glycoprotein subunit 1
GP2	glycoprotein subunit 2
GPC	glycoprotein precursor
DC	dendritic cell
Gas6	growth-arrest-specific protein 6
GFP	green fluorescent protein
HA	hemagglutinin
HEK293	human embryonic kidney 293 cells
HeLa	human cervical cancer cells
HF	hemorrhagic fever
HT1080	human fibrosarcoma cells
HUVEC	human umbilical vein endothelial cells
IFA	immunofluorescence assay
JUNV	Junin virus
LASV	Lassa virus
LCMV	lymphocytic choriomeningitis virus
LCMV WE 54	lymphocytic choriomeningitis virus strain WE 54
mAb	monoclonal antibody
NP	nucleoprotein
NW	New World
OW	Old World
PS	phosphatidylserine
PBS	phosphate buffer saline
ProS	protein S
siRNA	small interfering RNA
SKI-1/S1P	subtilisin/kexin-isozyme-1 or site-1 protease
TAM	Tyro-3, Axl, Mer tyrosine kinases family
VACV	vaccinia virus
VV	vaccinia virus

SUMMARY

Arenaviruses are enveloped negative-strand RNA viruses that contain a bi-segmented genome. They are rodent-borne pathogens endemic to the Americas and Africa, with the exception of lymphocytic choriomeningitis virus (LCMV) that is world-wide distributed. The arenaviruses include numerous important human pathogens including the Old World arenavirus Lassa virus (LASV), the causative agent of a severe viral hemorrhagic fever in humans with several hundred thousand infections per year in Africa and thousands of deaths. Viruses are obligatory intracellular parasites, strictly depending on cellular processes and factors to complete their replication cycle. The binding of a virus to target cells is the first step of every viral infection, and is mainly mediated by viral proteins that can directly engage cellular receptors, providing a key determinant for viral tropism. This early step of infection represents a promising target to block the pathogen before it can take control over the host cell. Old World arenaviruses, such as LASV and LCMV, bind to host cells via attachment to their main receptor, dystroglycan (DG), an ubiquitous receptor for extracellular matrix proteins. The engagement of DG by LASV results in a fast internalization and transfer the virus to late endosomal compartment suggesting that the virus binding to DG causes marked changes in the dynamics of the receptor. These events could result in the clustering of the receptor and subsequent induction of signaling that could be modulated by the virus. Recently, numerous findings also suggest the presence of alternative receptor(s) for LASV in absence of the main DG receptor.

In my first project, I was interested to investigate the effects of virus-receptor binding on the tyrosine phosphorylation of the cytoplasmic domain of DG and to test if this post-translational modification was crucial for the internalization of the LASV-receptor complex. We found that engagement of cellular DG by a recombinant LCMV expressing the envelope GP of LASV in human epithelial cells induced tyrosine phosphorylation of the cytoplasmic domain of DG. LASV GP binding to DG further resulted in dissociation of the adapter protein utrophin from virus-bound DG. Virus-induced dissociation of utrophin and consequent virus internalization were affected by the broadly specific tyrosine kinase inhibitor genistein. We speculate that the detachment of virus-bound DG from the actin-based cytoskeleton following DG phosphorylation may facilitate subsequent endocytosis of the virus-receptor complex.

In the second project, I was interested to characterize the newly indentified LASV alternative receptor Axl in the context of productive arenavirus infection. In a first step, we demonstrated that Axl supports productive infection by rLCMV-LASVGP in a DG-independent manner. In line with previous studies, cell entry of rLCMV-LASVGP *via* Axl was less efficient when compared to functional DG. Interestingly, Axl-mediated infection showed rapid kinetics similar to DG-dependent

entry. Using a panel of inhibitors, we found that Axl-mediated cell entry of rLCMV-LASVGP involved a clathrin-independent pathway that critically depended on actin and dynamin and was sensitive to EIPA but not to PAK inhibitors, compatible with a macropinocytosis-like mechanism of entry. In a next step, we aimed to investigate the molecular mechanism by which rLCMV-LASVGP recognizes Axl. Phosphatidylserine (PS) is the natural ligand of Axl *via* the adaptor protein Gas6. We detected the presence of PS in the envelope of Old World arenaviruses, suggesting that PS could mediate Axl-virus binding, in a mechanism of apoptotic mimicry already described for other viruses. Whether envelope PS and/or the GP of LASV plays any role in virus entry *via* Axl is still an open question.

The molecular mechanisms underlying host cell-virus interaction are of particular interest to answer basic scientific questions as well as to apply key findings to translational research. Understanding pathogen induced-signaling and its link to invasion of the host cell is of great importance to develop drugs for therapeutic intervention against highly pathogenic viruses like LASV.

RÉSUMÉ

Les Arénavirus sont des virus enveloppés à ARN négatifs organisés sous forme de génome bisegmenté. Ils sont véhiculés par les rongeurs et se retrouvent de manière endémique aux Amériques et en Afrique avec l'exception du virus de la chorioméningite lymphocytaire (LCMV) qui lui est distribué mondialement. De nombreux pathogènes humains font parti de la famille des Arénavirus dont le virus de l'Ancien Monde Lassa (LASV), un agent responsable de fièvres hémorragiques sévères chez les humains. Le virus de Lassa cause plusieurs centaines de milliers d'infections par année en Afrique ainsi que des milliers de morts. De manière générale, les virus sont des parasites intracellulaires obligatoires qui dépendent strictement de processus et facteurs cellulaires pour clore leur cycle de réplication. L'attachement d'un virus à sa cellule cible représente la première étape de chaque infection virale et est principalement dirigée par des protéines virales qui interagissent directement avec leur récepteurs cellulaires respectifs fournissant ainsi un indicateur déterminant pour le tropisme d'un virus. Cette première étape de l'infection représente aussi une cible prometteuse pour bloquer le pathogène avant qu'il ne puisse prendre le contrôle de la cellule. Les Arénavirus de l'Ancien Monde comme LASV et LCMV s'attachent à la cellule hôte en se liant à leur récepteur principal, le dystroglycan (DG), un récepteur ubiquitaire pour les protéines de la matrice extracellulaire. La liaison du DG par LASV résulte en une rapide internalisation transférant le virus aux endosomes tardifs suggérant ainsi que l'attachement du virus au DG peut provoquer des changements marqués dans la dynamique moléculaire du récepteur. Ces événements sont susceptibles d'induire un regroupement du récepteur à la surface cellulaire, ainsi qu'une induction subséquente qui pourrait être, par la suite, modulée par le virus. Récemment, plusieurs découvertes suggèrent aussi la présence d'un récepteur alternatif pour LASV en l'absence du récepteur principal, le DG.

Concernant mon premier projet, j'étais intéressée à étudier les effets de la liaison virus-récepteur sur la phosphorylation des acides aminés tyrosines se trouvant dans la partie cytoplasmique du DG, le but étant de tester si cette modification post-translationnelle était cruciale pour l'internalisation du complexe LASV-DG récepteur. Nous avons découvert que l'engagement du récepteur DG par le virus recombinant LCMV, exprimant la glycoprotéine de LASV, dans des cellules épithéliales humaines induit une phosphorylation de résidu(s) tyrosine se situant dans le domaine cytoplasmique du DG. La liaison de la glycoprotéine de LASV au DG induit par la suite la dissociation de la protéine adaptatrice utrophine du complexe virus-DG récepteur. Nous avons observé que cette dissociation de l'utrophine, induite par le virus, ainsi que son internalisation, sont affectées par l'inhibiteur à large spectre des tyrosines kinases, la génistéine. Nous avons donc supposé que le détachement du virus, lié au récepteur DG, du cytosquelette d'actine suite à la phosphorylation du DG faciliterait l'endocytose subséquente du complexe virus-récepteur.

Dans le second projet, j'étais intéressée à caractériser le récepteur alternatif Axl qui a été récemment identifié dans le contexte de l'infection productive des Arénavirus. Dans un premier temps, nous avons démontré que le récepteur alternatif Axl permet l'infection des cellules par le virus LCMV recombinant LASV indépendamment du récepteur DG. Conformément aux études publiées précédemment, nous avons pu observer que l'entrée du virus recombinant LASV *via* Axl est moins efficace que *via* le récepteur principal DG. De façon intéressante, nous avons aussi remarqué que l'infection autorisée par Axl manifeste une cinétique virale d'entrée similaire à celle observée avec le récepteur DG. Utilisant un éventail de différents inhibiteurs, nous avons trouvé que l'entrée du virus recombinant rLCMV-LASVGP *via* Axl implique une voie d'entrée indépendante de la clathrine et dépendant de manière critique de l'actine et de la dynamine. Cette nouvelle voie d'entrée est aussi sensible à l'EIPA contrairement aux inhibiteurs PAK indiquant un mécanisme d'entrée compatible avec un mécanisme de macropinocytose. L'étape suivante du projet a été d'investiguer le mécanisme moléculaire par lequel le virus recombinant rLCMV-LASVGP reconnaît le récepteur alternatif Axl. La phosphatidylsérine (PS) se trouve être un ligand naturel pour Axl *via* la protéine adaptatrice Gas6. Nous avons détecté la présence de PS dans l'enveloppe des Arénavirus du Vieux Monde suggérant que la PS pourrait médier la liaison du virus à Axl dans un mécanisme de mimétisme apoptotique déjà observé et décrit pour d'autres virus. Cependant, il reste encore à déterminer qui de la PS ou de la glycoprotéine de l'enveloppe virale intervient dans le processus d'entrée de LASV *via* le récepteur alternatif Axl.

Les mécanismes moléculaires à la base de l'interaction entre virus et cellule hôte sont d'intérêts particuliers pour répondre aux questions scientifiques de base ainsi que dans l'application de découvertes clés pour la recherche translationnelle. La compréhension de la signalisation induite par les pathogènes ainsi que son lien à l'invasion de la cellule hôte est d'une importance considérable pour le développement de drogues pour l'intervention thérapeutique contre les virus hautement pathogènes comme LASV.

INTRODUCTION

The Arenaviridae family

Viruses are studied for the diseases they cause and can also serve as molecular probes to dissect fundamental biological mechanisms. The *Arenaviridae* family contains a number of viruses including Lassa virus (LASV), Junin (JUNV), Machupo (MACV), Guanarito (GTOV), and Sabia virus (SABV) that are important human pathogens causing hemorrhagic fevers with significant morbidity and high mortality (Botten et al., 2007; Geisbert and Jahrling, 2004). The family comprises more than 20 viral species initially subdivided into two major groups, the Old World (OW) and the New World (NW) arenaviruses, based on geographic relationships, serologic typing, and phylogenetic evidence (Charrel, de Lamballerie, and Emonet, 2008; Emonet et al., 2009) (Fig. 1). The NW arenaviruses, found in the Americas, include Tacaribe virus (TACV), Pichinde virus, JUNV, MACV, SABV and GTOV. The OW arenaviruses, found in Europe and Africa, include LASV, Mopeia, Mobala, Ippy. The prototypic member of the family, lymphocytic choriomeningitis virus (LCMV) has a world-wide distribution.

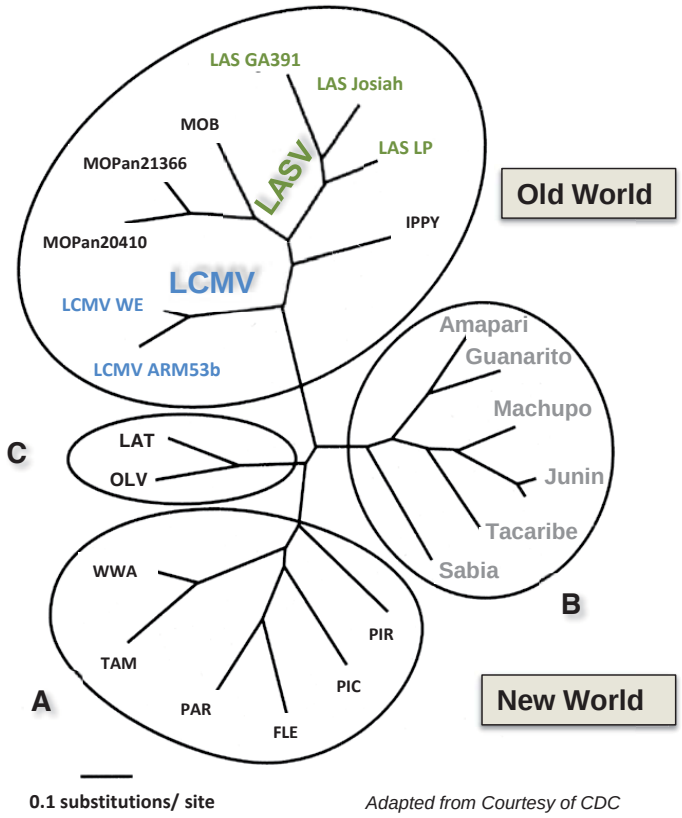


Figure 1. Phylogeny of arenaviruses. Arenaviruses are classified in two main complexes established according to their serologic, genetic and geographic characteristics: The Lassa/LCMV virus complex, called the Old-World arenaviruses, mainly localized in Africa and the Tacaribe virus complex, called the New-World arenaviruses, mainly localized in America.

Apart from being an important human pathogen in its own right, LCMV is a powerful experimental model that had been instrumental in uncovering much of our current knowledge of viral persistence, viral pathogenesis, and several aspects of viral immunobiology (Buchmeier, 2007). LCMV is a neglected human pathogen of clinical significance, especially in pediatric medicine (Barton and Mets, 1999; Barton, Mets, and Beauchamp, 2002) and represents a threat to immune-compromised individuals, as tragically illustrated by recent fatal cases of transplant-acquired LCMV infection (Fischer et al., 2006; Palacios et al., 2008).

For most arenaviruses, the natural reservoirs are specific rodent species as e.g. *Mastomys natalensis* for LASV or *Mus musculus* and *Mus domesticus* for LCMV (Clegg, 2002). The possible exception is TACV, which has only been isolated from bat species. In rodents, arenaviruses are generally maintained via asymptomatic persistent infections, primarily transmitted by congenital and vertical routes. This occurs through transmission of the virus from infected mothers to the fetus maintaining the arenavirus population within their respective host species. This vertical transmission happens before positive and negative selection of the T cell and B cell repertoire. As a consequence, the virus is recognized as "self" by the host's adaptive immunity allowing establishing the so called "carrier state" where host rodents are persistently infected showing high level of virus replication with the adaptive immune system being tolerant for the virus. Immune-competent adults, horizontally infected, manifest a self-limiting disease rapidly counteracted by the anti-viral adaptive immunity, predominantly the anti-viral T cell response (Barton, Mets, and Beauchamp, 2002; Oldstone, 2002).

Arenavirus particle and genome organization

Arenaviruses are enveloped viruses with a nonlytic life cycle confined to the cytoplasm. They contain as genome two negative bisegmented strand RNAs composed of a large L segment (ca 7.3 kb) and a small S one (ca 3.5 kb) (Fig. 2A) (Emonet et al., 2009). Both RNA segments are encoded in an ambisense strategy resulting in two open reading frames in opposite orientation separated by a noncoding intergenic region (IGR) predicted to fold in a stable hairpin structure (Fig. 2B). The S RNA segment encodes for the viral glycoprotein precursor (GPC) and the nucleoprotein (NP) (Fig. 2B). GPC is post-translationally processed by the cellular proprotein convertase subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P) to yield the mature virion glycoproteins (GPs) GP1 and GP2.. Located at the top of mature GP spikes in the viral envelope (Fig. 2A), GP1 mediates attachment of the virus to the host cell, followed by membrane fusion mediated by the transmembrane GP2 that resembles class I viral fusion proteins. The L RNA segment encodes the viral RNA-dependent RNA polymerase (L) and the small RING finger protein Z, the main driving force for arenavirus budding (Perez, Craven, and de la Torre, 2003; Strecker et al., 2003). The L and NP viral proteins are responsible for the first steps of viral transcription and replication (Buchmeier, 2007).

Cell biology of arenaviruses

Viral cell entry is the initial step for all viral infections and represents a target of choice for blocking viruses before they can exploit the cell for their proper needs. The cellular entry pathway of arenaviruses is dependent on virus receptor(s). The precise entry mechanisms are still not clearly understood, however, according to their different receptor use, OW and NW arenaviruses seem to use distinct entry pathways (Rojek and Kunz, 2008).

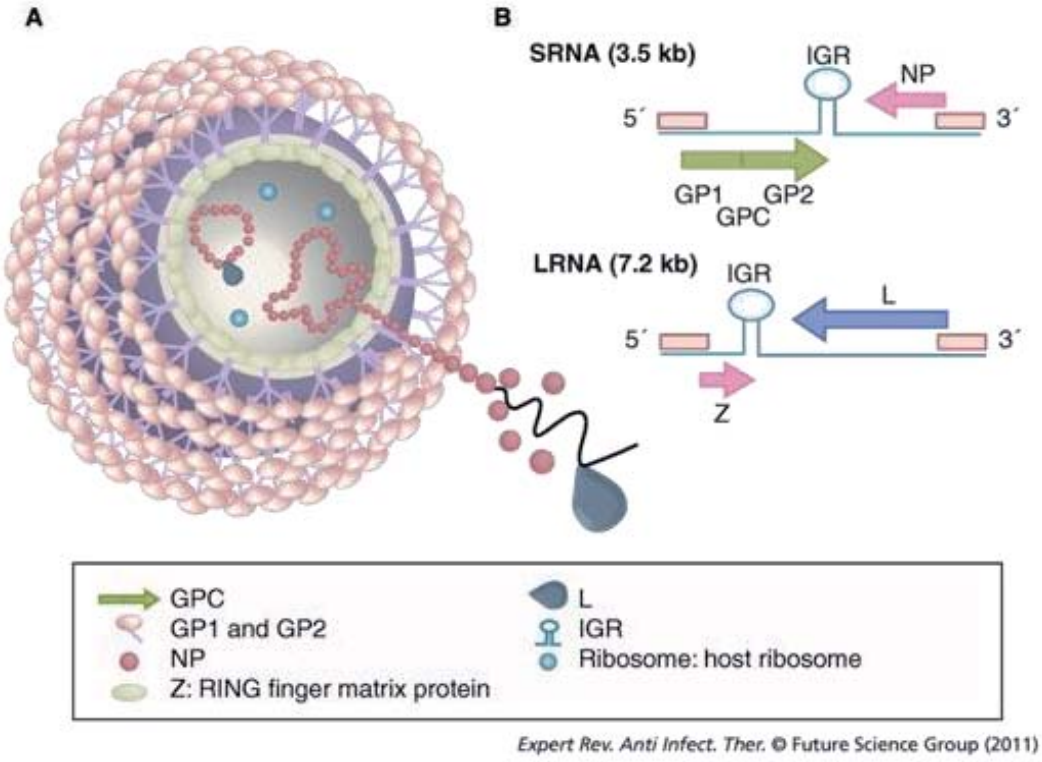


Figure 2. Arenavirus particle and genome organization. **(A)** Schematic of an arenavirus particle. The viral RNA is packaged into ribonucleoparticles (RNPs) containing the viral NP. The RNA-dependent RNA polymerase (L) is associated with RNP and is required for the initial steps of viral transcription. The matrix protein, Z, associates with the inner leaflet of the viral membrane envelope and interacts with the C-terminal part of the transmembrane GP2 moiety of the mature GP1/GP2 complex covering the virion surface. The receptor-binding GP1 forms the top part of the GP virion spikes, whereas the transmembrane GP2 part contains the fusion machinery. In their mature form, arenavirus GPs are fully processed and presumably form trimers, similar to other fusion-active viral membrane proteins. **(B)** The ambisense coding strategy of arenaviruses. Each of the two ssRNA segments, L (ca 7.2 kb) and S (ca 3.5 kb), uses an ambisense coding strategy to direct the synthesis of two polypeptides in opposite orientations and is separated by an IGR predicted to have a stable hairpin structure. The 5' ends of the genomic arenavirus RNA cannot serve as a template for translation and viral protein expression requires prior transcription, as in true negative-strand viruses. (GP: Glycoprotein; GPC: Glycoprotein precursor; IGR: Intergenic region; L: RNA-dependent RNA polymerase; NP: Nucleoprotein) (adapted from (Moraz and Kunz, 2011).

The hemorrhagic NW arenaviruses like JUNV, MACV, GTOV and SABV use the human transferrin receptor 1 (TfR1) (Radoshitzky et al., 2007). The TfR1 is ubiquitously expressed in a wide range of human cells and tissues and is responsible for cellular iron uptake. After viral binding to TfR1, the virus is targeted to the classical clathrin and dynamin-dependent entry pathway, with subsequent delivery to acidified endosomes, where fusion is initiated by low pH (Martinez, Cordo, and Candurra, 2007).

The cellular receptor for LASV, most isolates of LCMV, other OW arenaviruses, and Clade C NW arenaviruses is alpha-dystroglycan (α-DG), a highly conserved and ubiquitously expressed cell surface receptor for extracellular matrix (ECM) proteins (Cao et al., 1998; Kunz, 2009). DG is initially encoded as a single polypeptide cleaved into the extracellular α-DG and transmembrane β-DG (Barresi and Campbell, 2006). DG is expressed in most cells of adult and developing tissues and provides a molecular link between the ECM and the actin-based cytoskeleton (Fig. 5). After binding to the receptor, OW arenaviruses undergo endocytosis and are delivered to late endosomal compartments where low pH is required for the fusion between the viral and cellular membrane (Borrow and Oldstone, 1994). The optimal pH for LASV and LCMV fusion is remarkably low <5.0 indicating possible fusion in late endosomal compartment (Klewitz, Klenk, and ter Meulen, 2007). LASV and LCMV enter the cell mainly through an unusual endocytic pathway having some dependence on membrane cholesterol (Rojek, Perez, and Kunz, 2008), but that seems independent of clathrin, caveolin, dynamin and actin (Quirin et al., 2008; Rojek, Perez, and Kunz, 2008; Rojek et al., 2008). Electron microscopic studies of LCMV cell entry showed uptake of viral particles in smooth vesicles lacking clathrin coat (Borrow and Oldstone, 1994; Quirin et al., 2008).

Based on available evidence, the following working model for cell entry of OW arenaviruses is proposed (Pasqual et al., 2011): (1) Virus-receptor binding induces receptor clustering at the cell surface, possibly followed by receptor-mediated signaling (Fig. 3). (2) After attachment, virus is internalized in smooth vesicles involving a clathrin- and caveolin-independent pathway. (3) Virus-containing vesicles may be delivered either directly to multivesicular bodies (MVB) or passing through a yet unknown intracellular compartment. The MVB is a dynamic organelle giving raise to late endosomes. The delivery of virus-receptor complex within the MVB to late endosomes seems to depend on microtubular transport. (4) As found by Pasqual et al., at the level of the MVB, the virus-receptor complex may undergo sorting into intraluminal vesicles (ILV) mediated by the endosomal sorting complex required for transport (ESCRT) (5), followed by delivery and fusion in late endosomes (6) (Fig. 3).

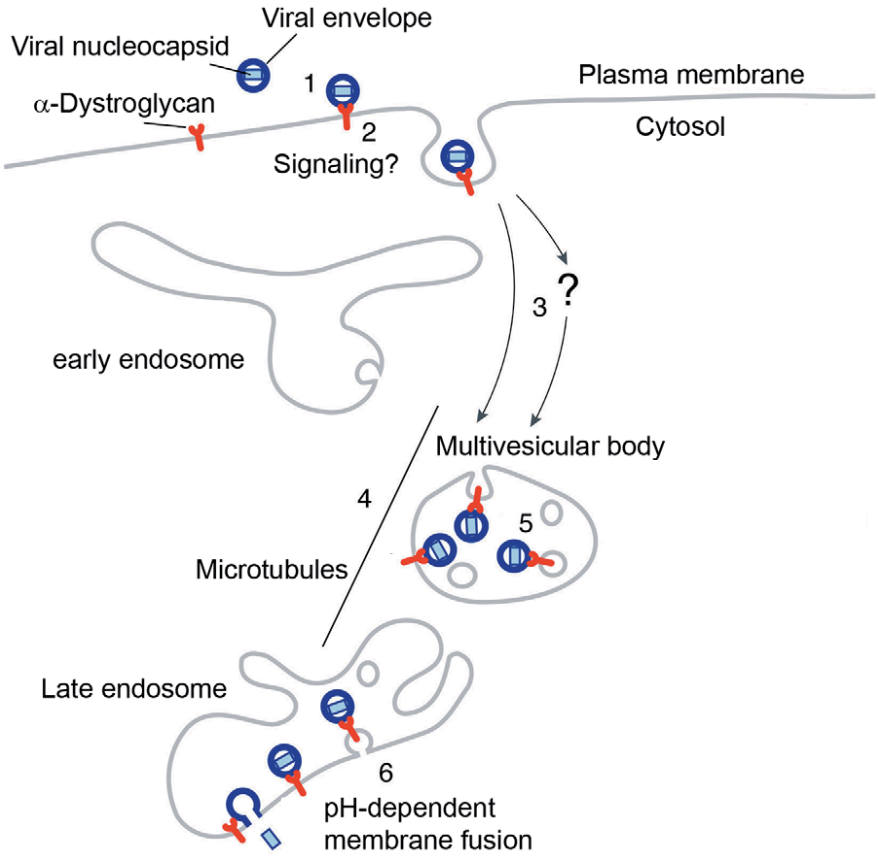


Figure 3. Working model for the cell entry of the OW arenaviruses LASV and LCMV. For details see text (adapted from (Pasqual et al., 2011).

Arenaviruses as human pathogens

The large and diverse family of arenaviruses includes several causative agents of severe viral hemorrhagic fevers (VHFs) that belong to the most devastating emerging human diseases and pose serious public health problems (Buchmeier, 2007; Geisbert and Jahrling, 2004). Arenaviruses responsible for VHFs are LASV, JUNV, MACV and SABV causing Lassa fever, Argentine, Bolivian, Venezuelan and Brazilian hemorrhagic fever, respectively (Rojek, Spiropoulou, and Kunz, 2006). The highest morbidity and mortality among these VHFs is associated with LASV responsible for 300'000 to 500'000 infections per year in Western Africa (McCormick and Fisher-Hoch, 2002). Due to the lack of effective treatments of arenavirus infections, the mortality among hospitalized Lassa fever patients is 15-30% and can reach more than 50% in nosocomial outbreaks (Fisher-Hoch et al., 1995). Available data on the seroprevalence for some endemic regions revealed that 20-50% of the adult population have been infected with LASV (Richmond and Baglole, 2003), making LASV a crucial public health problem. The main routes of human transmission are contact with or inhalation of urine and feces from persistently infected rodents, living in close contact to humans. Human-to-human

transmission occurs mainly by close contact with contaminated blood, body fluids and aerosols making LASV a severe nosocomial pathogen (Fisher-Hoch et al., 1995; McCormick et al., 1987b).

Clinical disease and pathogenesis of Lassa fever

A wide spectrum of clinical manifestations is observed in acute Lassa fever ranging from asymptomatic infection to fatal hemorrhagic fever. The early flu-like symptoms render the diagnosis often difficult (McCormick et al., 1987a). Following an incubation period of 7-18 days, patients develop weakness, fever and general malaise. Most of the patients develop severe headache, sore throat and cough. Frequently, gastrointestinal symptoms such as nausea, vomiting and diarrhea manifest. Poor disease prognosis is indicated by signs of increased vascular permeability such as pleural effusions and facial edema that occur in a minority of patients. In severe cases, decline is rapid with progressive signs and symptoms of pulmonary edema, respiratory distress, shock, signs of encephalopathy followed by seizures, coma and bleeding from mucosa. Survivors usually recover during 2-3 weeks after the disease onset and are able to clear the virus from the blood. Sensorineural deafness is the most commonly encountered complication late in the disease or early in convalescence, making LASV a leading cause of deafness in Western Africa (Cummins et al., 1990).

The extent of viremia is a highly predictive factor for the outcome of the LASV infection: Severe cases of Lassa fever are characterized by high viral load reflecting the inability of the immune system to limit viral replication. During LASV infection, immune function is perturbed by the virus at the level of both innate and adaptive response through different mechanisms. In contrast, survivors have lower initial viral load allowing and control the infection by the unfolding anti-viral immune response (Johnson et al., 1987).

The inability of the patient's immune system to contain and control the virus is a hallmark of human fatal LASV infection. Indeed, arenaviruses are able to subvert the mechanisms of innate pathogen recognition through escape of detection by innate immune receptors or by blocking related signaling pathways. The RNA genome of arenaviruses can be recognized by the cytoplasmic RNA helicases RIG-I and MDA5, leading once activated to the production of type I interferons (IFNs). The viral NP has been shown to mechanistically block signal transduction, by avoiding IFN transcription factor activation preventing IFNs production (Martinez-Sobrido et al., 2009; Martinez-Sobrido et al., 2006).

The marked suppression of the adaptive immune response, including cellular and humoral immunity, is likewise found in fatal Lassa fever. The ability of survivors to control the infection is primarily mediated by cellular immunity, especially the anti-viral CD8 T-cell response (Fisher-Hoch et al., 2000; ter Meulen et al., 2000). In contrast, the humoral response plays a limited role in the

control of acute LASV infection in which a neutralizing antibody response is not necessary for recovering (Johnson et al., 1987). In fatal disease, the development of an efficient adaptive immune response is blunted by the inhibition of viral antigen presentation by antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages (Fig. 3) (Moraz and Kunz, 2011).

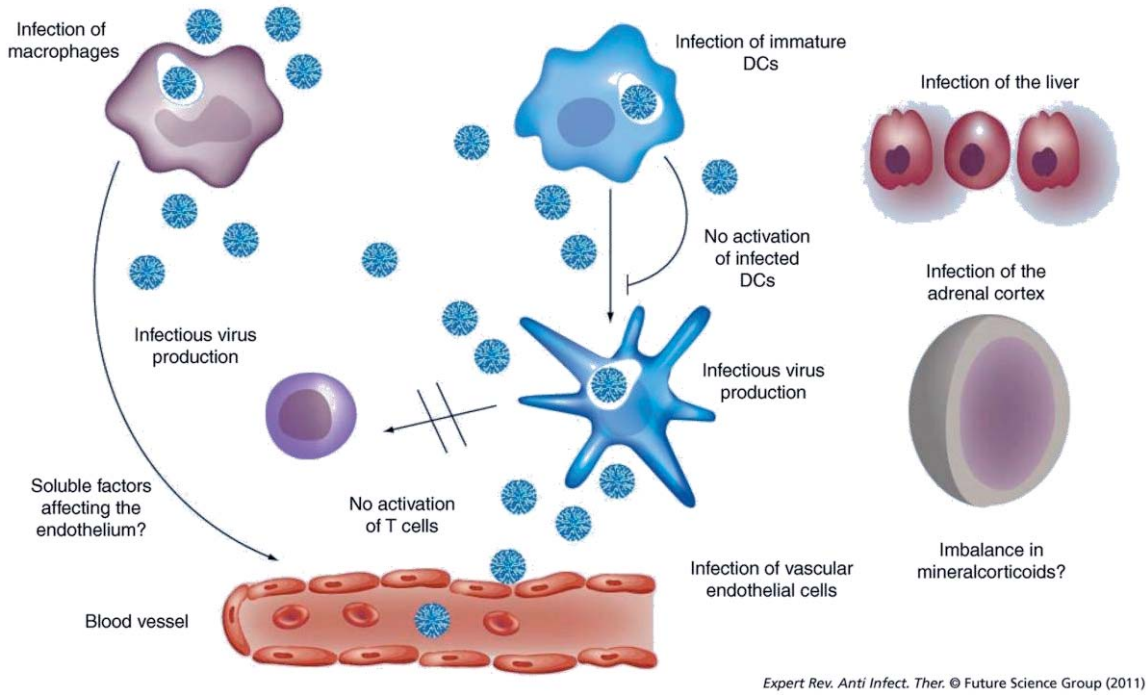


Figure 4. Model for LASV pathogenesis. Macrophages and DCs are early and preferred targets for LASV in human infection. However, instead to being recognized and presented as foreign antigen, LASV establishes productive infection that fails to activate the cells and perturbs their ability to stimulate T cells, contributing to immunosuppression. LASV infection in macrophages and in particular DCs does not result in excessive production of cytokines, but releases significant amount of infectious virus. Infection of the liver results in enhanced levels of transaminases and hepatocellular necrosis. While the histopathology in the liver is too weak to account for liver failure, infection of hepatocytes may alter the production of serum proteins, possibly affecting hemostasis. Extensive viral replication in the adrenal cortex, where high viral loads are detected, may affect the production of mineralcorticoids leading to fluid imbalance. LASV can productively infect vascular endothelial cells without causing overt cytopathology and vascular lesions are mild in fatal Lassa fever. The mechanisms underlying the alterations in endothelial cell functions that precede shock and death in fatal Lassa fever are largely unknown and may involve direct effect of virus infection as well as effects mediated by as yet undefined soluble factors released by other cells (adapted from (Moraz and Kunz, 2011).

The infection of APCs by LASV prevents their maturation and activation, perturbing antigen presentation to CD4 and CD8 T cells (Baize et al., 2004; Mahanty et al., 2003) (Fig. 4). In contrast to other hemorrhagic fever viruses, such as e.g. Ebola, LASV infection of macrophages and DCs is not associated with the release of proinflammatory cytokines (Baize et al., 2004; Klewitz, Klenk, and ter Meulen, 2007). Another salient feature in infected patients is lymphopenia, which is most severe in

fatal cases, and is associated with a marked decrease in the number of CD4, CD8, B and NK cells, suggesting other mechanisms responsible for the virus-induced immunosuppression (McCormick and Fisher-Hoch, 2002).

The pathophysiology of the multi-organs failure in fatal Lassa fever, concomitant with high levels of viral replication, remains still unclear since little cellular damage and only mild infiltration of inflammatory cells have been observed in histological examinations (Walker et al., 1982). A hypothetical mechanism may involve a general virus-induced impairment of the vascular endothelium, the liver (McCormick et al., 1986; Walker et al., 1982) and the adrenal cortex (Fig. 4) (Moraz and Kunz, 2011). In summary, the mechanisms underlying the shock associated with terminal stage of human fatal Lassa fever are still largely undefined. According to the current working model (Fig. 4), infection of several cell types in different tissues may result in a general impairment of the vascular permeability regulation in a direct or indirect way.

Host cell attachment of LASV through dystroglycan

In the host cell, the arenavirus receptor DG plays an important role in cell adhesion and regulation of cell-matrix interactions. Alpha-DG binds with high-affinity and in a calcium-dependent manner to ECM proteins such as laminin (Ervasti and Campbell, 1993), agrin (Sugiyama, Bowen, and Hall, 1994), perlecan (Talts et al., 1999), and to neurexins (Sugita et al., 2001). Alpha-DG is non-covalently associated with the transmembrane β-DG subunit which in turn binds to the intracellular cytoskeletal adaptor proteins dystrophin (in muscle) and utrophin (other cell types), thus bridging the DG complex to the actin-based cytoskeleton (Fig. 5A). This provides a physical connection between the ECM and the cell (Barresi and Campbell, 2006).

DG has a wide tissue distribution being expressed in muscle, in the central and peripheral nervous system, in epithelia, and endothelia (Durbeej and Campbell, 1999). Roles in the development, conservation and migration of several neuronal populations, as well as epithelial morphogenesis from different tissue origins, highlight the functional versatility of DG (Durbeej and Ekblom, 1997; Durbeej et al., 1995; Waite et al., 2009). In many different cell types, DG is found associated with additional cellular proteins including α, β, γ, δ-sarcoglycans (Ervasti and Campbell, 1991), dystrobrevins (Sadoulet-Puccio, Rajala, and Kunkel, 1997), the syntrophins (Peters, Adams, and Froehner, 1997) and sarcospan (Durbeej et al., 2000), forming together a large complex called the dystrophin-glycoprotein complex (DGC) (Fig. 5A). These DG-associated proteins are decisive for the stability and cellular trafficking of the DGC and mutations therein are associated with sometimes severe neuromuscular diseases (Barresi and Campbell, 2006; Durbeej et al., 2000; Kanagawa and Toda, 2006).

In mammals, DG is subject to a complex pattern of post-translational modifications important for its function as an ECM receptor (Barresi and Campbell, 2006; Kanagawa and Toda, 2006). DG is encoded by the *DAG*1 gene as a precursor polypeptide that is directed to the endoplasmic reticulum. The polypeptide is then post-translationally cleaved by an unidentified protease into two subunits, the α-DG and the β-DG subunits (Esapa et al., 2003; Holt et al., 2000). The large mucin-like region of α-DG contains a number of Ser or Thr residues, which were very early identified as sites for extensive modification of numerous heterogeneous O-linked sugar chains (Chiba et al., 1997; Ibraghimov-Beskrovnaya et al., 1992). Specific O-glycans of α-DG's mucin type domain are of particular importance and their biosynthesis involves the glycosyltransferase protein O-mannosyltransferases POMT1 and POMT2, protein O-mannose β1,2-N-GlcNAc transferase (POMGnT1), LARGE, LARGE2, and the proteins fukutin (FKTN), and fukutin-related protein (FKRP) (Fig. 5B and Fig. 6A). In the endoplasmic reticulum, POMT, POMT2 and POMGnT1 catalyze the biosynthesis and attachment of atypical O-mannosyl glycans. Very recently, the isoprenoid synthase domain protein (ISPD) was also shown to play an important role in the DG O-mannosylation process (Willer et al., 2012). Another essential glycan modification of α-DG involves the glycosyltransferases LARGE and LARGE2, which localize in the Golgi (Barresi et al., 2004; Kanagawa et al., 2004; Kanagawa and Toda, 2006) and are implicated in the biosynthesis of a glycan polymers whose building blocks are 3-xylose-α1,3-glucuronic acidβ1 (Inamori et al., 2012) (Fig. 6A). The lack of DG processing (Jayasinha et al., 2003), mutations in components of the DGC as well as in proteins implicated in the correct post-translational modifications are implicated in several congenital muscular dystrophies. Dystroglycanopathies are mainly caused by defective glycosylation of α-DG accounting for its loss of function as an ECM receptor through its impairment of multiple interactions with its extracellular partners (Fig. 6B). Protein O-mannosylation and LARGE-dependent modifications are also necessary for α-DG's function as a receptor for arenaviruses (Imperiali et al., 2005; Kunz et al., 2005) (Fig. 6A). Interestingly, both ECM proteins and arenaviruses recognize the same LARGE-derived sugars on α-DG, suggesting that the viruses closely mimic the mechanisms of receptor recognition by ECM proteins (Rojek et al., 2007). Over-expression of LARGE can functionally bypass defects in other enzymes responsible for α-DG glycosylation by inducing the synthesis of glycan-enriched α-DG with high affinity for ECM ligands, indicating a key role in the receptor biosynthesis (Barresi et al., 2004). Hypoglycosylation causes α-DG to be non-functional as receptor for its common ECM proteins, such as laminin, agrin and neurexin (Michele et al., 2002). In the context of the role of DG as a viral receptor, mutations resulting in milder pathological alterations of the DG complex could somehow provide protection of cells and tissues against viral infection. Mutations that confer this type of resistance e.g. against the highly pathogenic LASV could represent molecular features positively selected for in human populations living in regions where LASV is endemic. Interestingly, a genome-wide screen for positive selection among human populations supported the hypothesis that selective

pressure imposed by LASV may have led to the appearance of particular alleles conferring some resistance to the disease. Indeed, in the genome of the Yoruba population from Nigeria in Western Africa, where LASV is endemic, strong signals of positive evolutionary selection pressure on *LARGE* and interleukin 21 (*IL21*) genes, both involved in infectivity and immunity of LASV, were discovered (Andersen et al., 2012; Sabeti et al., 2007). These observations provide a first hint towards pathogen-driven selection at the level of a viral receptor and entry.

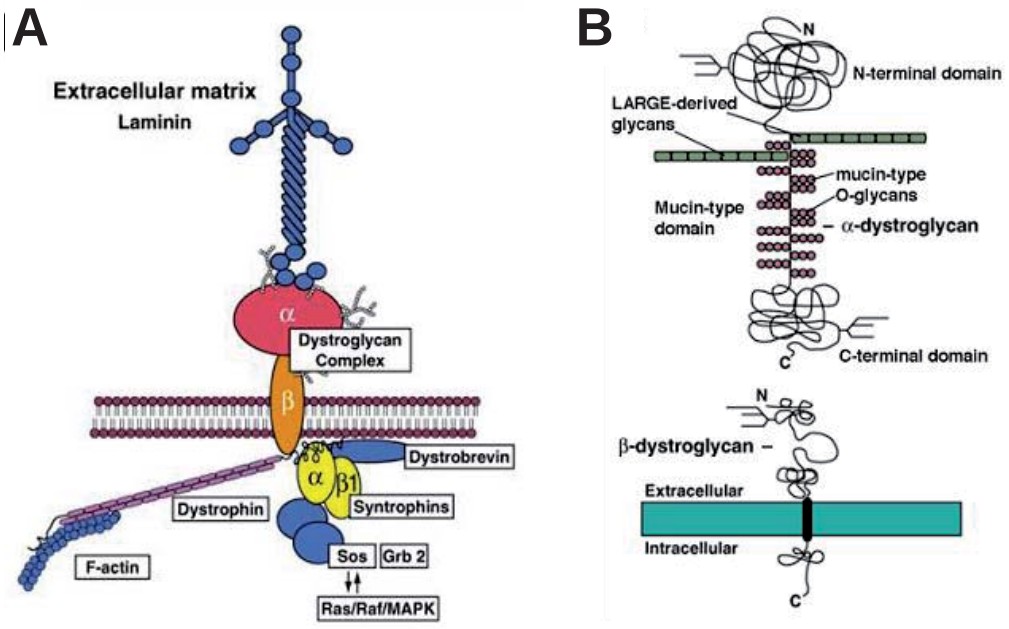


Figure 5. Schematic representation of DG. (A) DG complex with the ECM protein laminin (muscle model). **(B)** DG composed of the extracellular α-DG subunit non-covalently linked to the transmembrane β-DG subunit. (Adapted from Kevin Campbell, Howard Hughes Medical Institute, University of Iowa).

Additionally, recent studies indicate that aberrations in the expression of DG commonly occur in human cancers and may play a role in both tumor progression and the formation of metastasis (Sgambato et al., 2003). In a well-studied paradigm, loss of functional DG and its interaction with the ECM has been correlated with enhanced invasiveness of cancer cells (Henry, Cohen, and Campbell, 2001; Jing et al., 2004; Losasso et al., 2000; Sgambato et al., 2004; Singh et al., 2004). Mechanistically, dysfunctional DG would prevent the formation of robust contacts between basement membranes and the cytoskeleton, promoting tumor development and invasiveness.

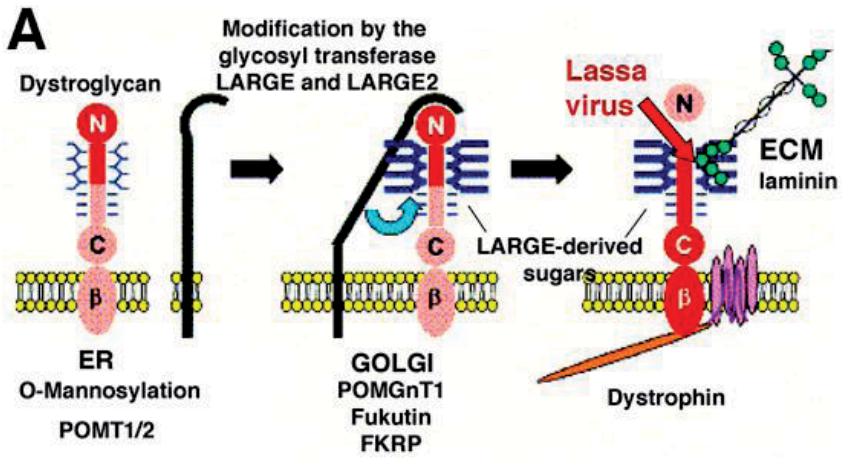
DG was considered for a long time as a relatively static membrane-anchored protein fulfilling mainly a structural role at cell-matrix contacts. However, recent investigations revealed that DG is a highly versatile receptor, interacting with many different partners, and thus represents a multifunctional signaling platform (Gould et al., 2010). Specifically, DG has been found to play a role in signaling cascades involved in the regulation of the host cell's interaction with the ECM and the

dynamics of the actin-based cytoskeleton. Moreover, DG was shown to functionally interact with β1 integrins, another important class of ECM receptors. The cytoplasmic domain of β-DG was shown to interact with signaling molecules including the canonical MAP kinases MEK and ERK, the focal adhesion kinase (FAK) (Cavaldesi et al., 1999; Spence et al., 2004; Yang et al., 1995), the signaling adaptor molecule Growth factor receptor-bound protein (Grb2) (Yang et al., 1995), and non-receptor tyrosine kinases of the Src family (James et al., 2000b; Sotgia et al., 2003; Sotgia et al., 2001), (Fig. 5A). Several studies have reported signaling crosstalk between integrins and DG modulating the activity of the canonical MEK/ERK pathway. One example is the signaling crosstalk between DG and the integrin α6Aβ1, which is a common receptor for laminin. DG and integrin α6Aβ1 exert antagonistic roles in the signaling of the MEK/ERK pathway in epithelial cells. Specifically, DG has an inhibitory effect on the α6Aβ1 integrin-mediated activation of the Ras-Raf-MEK-ERK pathway in response-to cell adhesion to laminin (Ferletta et al., 2003). Thus, synchronous binding of laminin to DG and α6Aβ1 integrin results in a regulated balance of MEK/ERK signaling that is crucial for the normal adhesion of cell to the laminin-based ECM.

Earlier studies from our group revealed that OW arenaviruses closely mimic the molecular mechanisms of receptor recognition by ECM ligands as both recognize DG via highly conserved glycan epitope derived from LARGE (Rojek et al., 2007). This mimicry leads to a competition between LASV and the ECM proteins for DG binding, affecting its normal cell function (Kunz et al., 2005). The impact of LASV binding on DG-mediated signal transduction was recently investigated in human lung epithelial cells in our laboratory. We showed that the engagement of LASV altered the interaction of DG with signaling molecules disturbing the signaling cross-talk with integrins (Rojek et al., 2012). More precisely, cell adhesion to laminin in the presence of LASV resulted in significantly reduced activation of MEK and ERK phosphorylation *via* the integrin α6Aβ1 signaling. However, the MEK/ERK signaling and activity are dispensable for the cell attachment and entry of LASV and the perturbation of this type of receptor signaling appears as a "collateral damage" inflicted by the virus. We currently speculate that the perturbation of ECM-induced cellular signaling by the virus, may contribute to functional alterations of vascular endothelial and epithelial cells that precede shock and death in fatal LASV cases (Fisher-Hoch et al., 1987).

It is clear that DG accomplishes multiple and very diverse functions in different cell types and tissues. It remains to be elucidated how DG is regulated both spatially and temporally to fulfill its diverse functions. On the extracellular side, the glycosylation of the α-DG subunit is crucial for its interaction with laminin and other ECM ligands whereas, on the intracellular face, phosphorylation of the β-DG subunit regulates its association with the actin-based cytoskeleton and other interacting molecules (Barresi and Campbell, 2006). Tyrosine phosphorylation frequently represents a controller "molecular switch" through the modification of decisive tyrosine residues located in cytoplasmic

domains of adhesion receptors. As a consequence, the interacting protein networks are modified to ultimately modulate the adhesion-dependent processes. In non-muscle cells, the tyrosine phosphorylation of β-DG plays a crucial regulatory role in determining the cohesion of the DG complex (Moore and Winder, 2010). A Src tyrosine kinase member is responsible for the phosphorylation of the residue Y892 located within the C-terminal PPxY motif of the β-DG subunit. This tyrosine phosphorylation of Y892 prevented the association of β-DG with the cytoskeletal adaptor protein utrophin (James et al., 2000a). This specific phosphorylation has further been shown to induce internalization of DG into large endosomal vesicular structures suggesting a role in targeting DG for its degradation (Miller et al., 2012; Sotgia et al., 2003; Sotgia et al., 2001). In line with these studies, Miller et al. have recently shown that inhibition of DG Y892 phosphorylation results in accumulation of DG on plasma membrane since DG is selectively internalized only when it acquires this post-translational modification at its C-terminus (Miller et al., 2012). Thus, impairment of Y892 phosphorylation ameliorates pathological symptoms associated with dystrophin deficiency in a Duchenne muscular dystrophy (DMD) mouse model. Indeed, in a dystrophin-free biological background, the point mutated DG Y890F was shown to be protected from degradation, participating also in the maintenance of the other DG complex components. These recent discoveries identify DG tyrosine phosphorylation as a potential future therapeutic target for treatments, not only in muscular dystrophies, but in any pathogenic process such as cancer. As main viral entry and cell signal-transducing receptor, changes in DG post-translational modification could also play a role in arenaviruses cell entry and inhibition of the receptor signaling may provide a new avenue for the development of anti-viral therapeutics. In my thesis work, I investigated the role of tyrosine phosphorylation of DG in LASV cell entry.



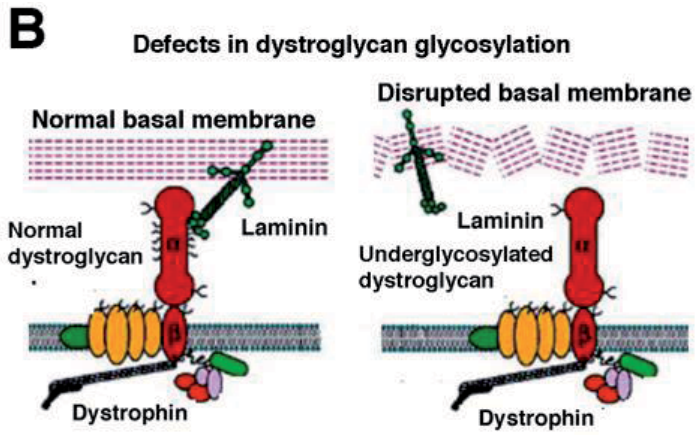


Figure 6. Post-translational modification of DG. (A) Functional glycosylation of α-DG that is crucial for its function as an ECM receptor. The binding of Lassa virus, as for LCMV, is indicated. For details, see text. **(B)** Defects in α-DG glycosylation cause loss of function of the receptor resulting in congenital neuromuscular disorders. (Modified from (Barresi et al., 2004; Kanagawa et al., 2004)).

TAM kinases as alternative receptors for arenaviruses

Tyro-3, Axl, and Mer compose the TAM family of receptor tyrosine kinases (RTKs). The extracellular domain of TAM receptors is comprised of tandems of two immunoglobulin (Ig) domains and two fibronectin type II domains. The transmembrane domain is linked to a cytoplasmic tyrosine kinase domain (Fig. 7). Similarly to DG, TAM kinases are subject to post-translational modifications, such as glycosylation and phosphorylation (Lu et al., 1999; O'Bryan et al., 1991; Sather et al., 2007). Tyro-3, Axl and Mer are expressed in a broad range of cell types, with the exception of lymphocytes and granulocytes, and have overlapping but unique expression patterns. Mer is predominantly expressed in cell of the hematopoietic lineage, such as monocytes/macrophages, DCs and NK cells but high expression levels are also found in kidney, brain, lung, skeletal muscle, retina and sexual organs (Angelillo-Scherrer et al., 2001; Behrens et al., 2003; Graham et al., 1994; Prasad et al., 2006). Tyro-3 is primarily expressed in the nervous system and is also detected in lung, kidney, retina and sexual organs, in addition to hematopoietic cell lines (Angelillo-Scherrer et al., 2001; Lai, Gore, and Lemke, 1994; Lu and Lemke, 2001; Mark et al., 1994; Prasad et al., 2006). Axl is ubiquitously expressed (O'Bryan et al., 1991) and detected in monocytes/macrophages, platelets, endothelial cells, heart, liver, kidney, skeletal muscle, testis (Angelillo-Scherrer et al., 2001; Graham et al., 1995; Neubauer et al., 1994), as well as in the brain (Bellosta et al., 1995).

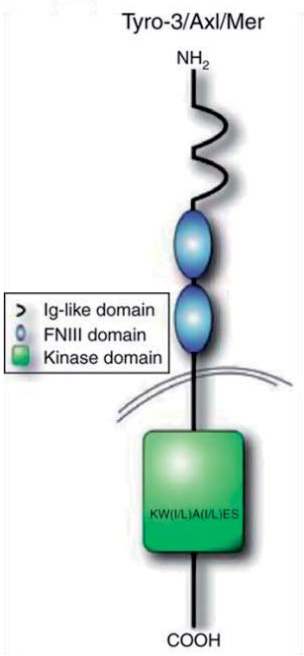


Figure 7. Structure of TAM receptors. Domain organization of Tyro-3, Axl and Mer are depicted (adapted from (Linger et al., 2008)).

The first ligand identified for TAM kinases was the growth-arrest-specific protein 6 (Gas6) followed by the discovery of the anticoagulation factor, Protein S (ProS) (Stitt et al., 1995; Varnum et al., 1995). Usually, activation of the TAM receptors is mediated through ligand binding that induces dimerization and subsequent autophosphorylation of tyrosine residues located within the cytoplasmic domain (Schlessinger, 2000). These phosphorylation events allow other substrates to be phosphorylated, as well as the recruitment of signaling molecules to form elaborated molecular signaling complexes (Braunger et al., 1997; Ling, Templeton, and Kung, 1996).

Upon stimulation, TAM kinases regulate a variety of cellular processes, including cell proliferation and survival, clearance of apoptotic cells (Lu and Lemke, 2001), regulation of the innate immune response (Rothlin et al., 2007; Rothlin and Lemke, 2010), cell adhesion (McCloskey et al., 1997) and survival/migration of DCs (Scutera et al., 2009), blood clot stabilization through platelet aggregation (Angelillo-Scherrer et al., 2005), regulation of inflammatory cytokine release (Sharif et al., 2006) and differentiation/maturation of natural killer cells (NKs) (Caraux et al., 2006). Moreover, increasing evidence suggest a role for TAM receptors in oncogenesis, as family members are over-expressed in a wide range of human cancers (Graham et al., 2006; Greenman et al., 2007; Keating et al., 2006).

The role of TAM receptors in apoptotic cell clearance

The maintenance of normal and healthy cell numbers is mediated trough cell death *via* the apoptosis process. The clearance of apoptotic cells and membranes is crucial for many biological processes, such as homeostasis and tissue development, as well as pathological responses, including inflammation. Uncontrolled accumulation of apoptotic cell debris causes tissue necrosis and the discharge of intracellular contents that can lead to inflammation if not cleared by phagocytic cells. Although a number of professional phagocytes can engulf infectious particles and microorganisms, the removal of apoptotic cells is mostly mediated by macrophages and, to a lesser extent, DCs. Cell surfaces of phagocytes and apoptotic cells are both negatively charged and consequently need proteins that mediate the processes of cell identification and engulfment. Apoptotic cells specifically contain phosphatidylserine (PS) on their outer membrane that mediates direct binding to phagocytes through PS-receptors e.g. of the TIM family. Phagocytes can also indirectly bind to apoptotic cells *via* TAM kinases, using the TAM ligands Gas6 or Protein S as bridging factors (Anderson et al., 2003; Nakano et al., 1997; Stitt et al., 1995) (Fig. 8). Gas6 and ProS are soluble proteins characterized by an N-terminal gamma-carboxylated glutamic acid (GLA) domain. The specific modification of glutamic acid by carboxylation at the γ-position depends on vitamin K and is crucial for binding to PS and thus the ability to mediate uptake of apoptotic debris. Following the N-terminal GLA-domain, Gas6 contains four epidermal growth factor (EGF)-line domains that are followed by a C-terminal sex hormone binding globulin (SHBG)-like module, which is involved in TAM receptor activation. Gas6 serves as ligand for all three TAM receptors, albeit with different affinity: Axl > Tyro3/Dtk >> Mer.

A well-known example of TAM receptor-mediated removal of apoptotic cells is represented by the phagocytosis of the photoreceptor outer segment membranes, observed in retinal pigment epithelium cells. Mer-deficient mice develop a degeneration of the photoreceptor cell layer of the retina due to the incapacity of phagocytotic cells of the retinal pigment epithelia to remove apoptotic cell debris (Lu et al., 1999) and a similar phenotype is observed in RCS rats that lack functional Mer (Duncan et al., 2003; Nandrot et al., 2000). In humans, mutations in the Mer gene are associated with rare forms of the degenerative disease retinitis pigmentosa (McHenry et al., 2004; Thompson et al., 2002). Knockout mice deficient for all three TAM receptors are viable at birth, however, after several weeks, mice start to develop degenerative phenotypes. Germ cells in the testis die due to the incapacity of Sertoli cells to engulf and remove apoptotic cells (Lu et al., 1999) leading to altered spermatogenesis and progressive infertility.

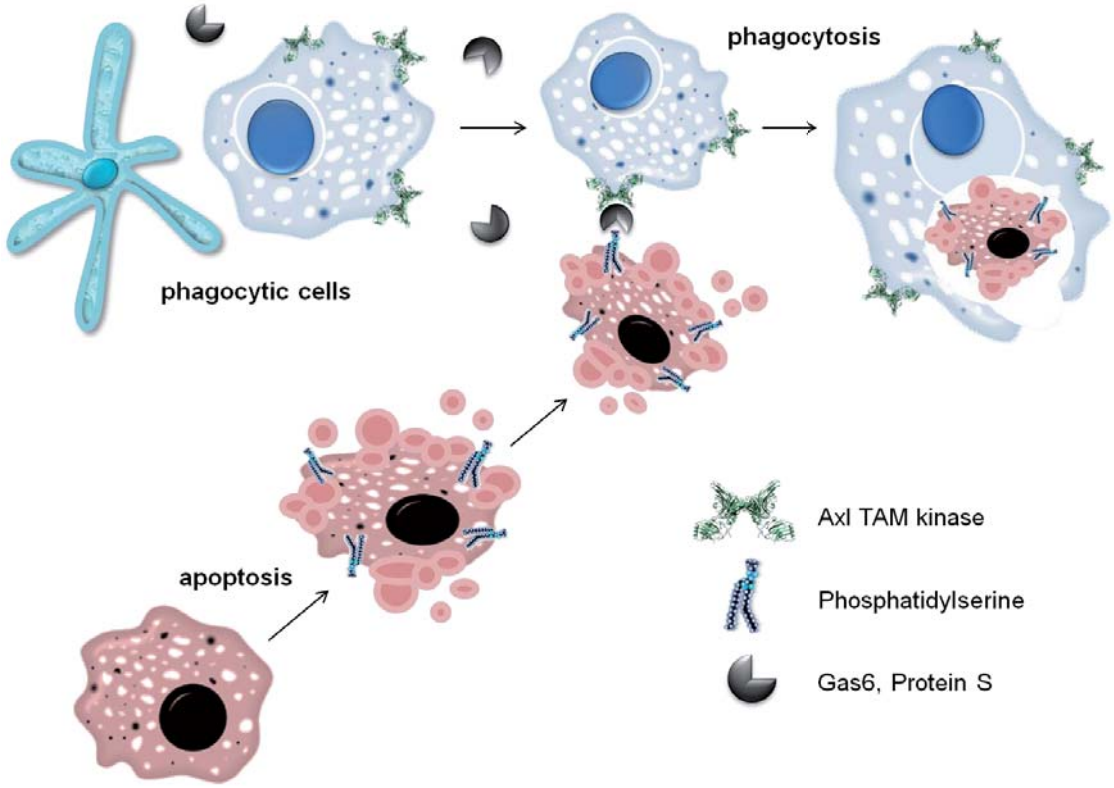


Figure 8. Phagocytosis of apoptotic cells by bridging. Model that proposes the mediation of phagocytosis of apoptotic cells through TAM receptors, expressed on professional phagocytic cells (macrophages and DCs) (Seitz et al., 2007). A "molecular" bridge is created by an interaction between PS-expressing cells and TAM receptor-expressing macrophages (Anderson et al., 2003).

These two debilitating phenotypes are actually linked since both are the consequence of the incapacity of TAM-deficient local phagocytotic cells to correctly engulf the high amount of apoptotic cells and debris generated in these organs. In contrast to the testis and the retina that represent organs sequestered by a blood-organ barrier, removal of apoptotic cells from other organs seems to be mediated mainly by macrophages and DCs implicating TAM receptors as well (Scott et al., 2001; Seitz et al., 2007).

TAM receptors are regulators of innate immunity

Upon recognition of viruses and other microbes by sensors of the innate immune system like RIG-I helicases and Toll-like receptors (TLRs), antigen presenting cells (APCs) like macrophages and DCs, secrete cytokines that drive APC maturation and help priming the unfolding adaptive immune response. Secreted cytokine levels, including those of type I interferons (IFNs) and pro-inflammatory cytokines like TNF-α, are subject to tight regulation to avoid excessive immune responses and constitutive activation that can lead to immunopathology and autoimmunity. In murine APCs, Gas6

functions as a strong inhibitor of TLR-induced activation and production of type I IFNs and pro-inflammatory cytokines (Rothlin et al., 2007). A similar observation has been made in human monocyte-derived DCs (MDDC) differentiated in the presence of type I IFNs (Scutera et al., 2009). TAM receptors function as inhibitors of the signaling pathways linked to TLR3, TLR4, and TLR9 (Abbrederis et al., 2008). Mechanistically, exposure of DCs to type I IFNs induces expression of TAM receptors, in particular Axl (Abbrederis et al., 2008; Scutera et al., 2009). The induced TAM receptor seems to undergo a direct interaction with the type I IFN receptor (IFNAR), resulting in the induction of inhibitory transcription factors of cytokine signaling, the suppressors of cytokine signaling (SOCS)-1 and SOCS-3. The up-regulation of TAM receptors in response to type I IFNs and their subsequent inhibition of cytokine signaling via induction of SOCS revealed the existence of a negative feedback mechanism ensuring the tight control of cytokine activity in the context of the unfolding anti-viral immune response.

From the evidence at hand, the TAM receptors appear thus as major receptors involved in the removal of apoptotic cells and important negative regulators of the pro-inflammatory cytokine response. Accordingly, mice knockout for the TAM receptors are prone to develop autoimmune diseases, probably due to a combination of dysregulated cytokine secretion and accumulation of apoptotic cells (Cohen et al., 2002; Lemke and Lu, 2003). The accumulation of apoptotic debris likely leads to prolonged exposure of intracellular material, including nuclear antigens to the immune system. In combination with the hyperactivation of APCs, autoreactive B cells become activated and produce antibodies able to recognize nuclear antigens, like ribonucleoproteins or DNA, similar to the situation observed in the human autoimmune disorder systemic lupus erythematosus (Cohen et al., 2002; Lu and Lemke, 2001).

Other functions of the TAM receptor Axl

Historically, the TAM receptor Axl, from the Greek word for uncontrolled “anexeleto” (O'Bryan et al., 1991), was first discovered as an unknown transforming gene in patients suffering from chronic myelogenous leukemia (Liu, Hjelle, and Bishop, 1988). Axl over-expression and its increased activity have been observed in other cancers (Linger et al., 2010) and in several chronic immune disorders (Fig. 8) (Lemke and Rothlin, 2008). As mentioned above, compared with other TAM family members, Axl has the highest affinity for the soluble ligand Gas6, activating the so-called Gas6/Axl pathway, known to promote growth and survival of several cell types (Allen et al., 1999; Scutera et al., 2009; Shankar et al., 2006; Stenhoff, Dahlback, and Hafizi, 2004). Axl is further implicated in cellular functions such as neuronal cell migration. The Gas6/Axl pathway has been shown to confer protection of cells from apoptosis *via* multiple mechanisms (Demarchi et al., 2001; Goruppi et al., 1999; Hasanbasic et al., 2004; Lee et al., 2002), including activation of pathways participating in cell

proliferation (Fridell et al., 1996). Under pathological conditions, the Gas6/Axl pathway is also important for the cardiovascular system (Fig. 9), since its activation is implicated in cardiovascular disease progression (Berk, 2001). Indeed, Axl knockout mice suggest a role of Axl in vascular remodeling (Konishi et al., 2004; Korshunov et al., 2006) and Axl was shown to regulate thrombosis, important for cardiovascular homeostasis (Angelillo-Scherrer et al., 2005).

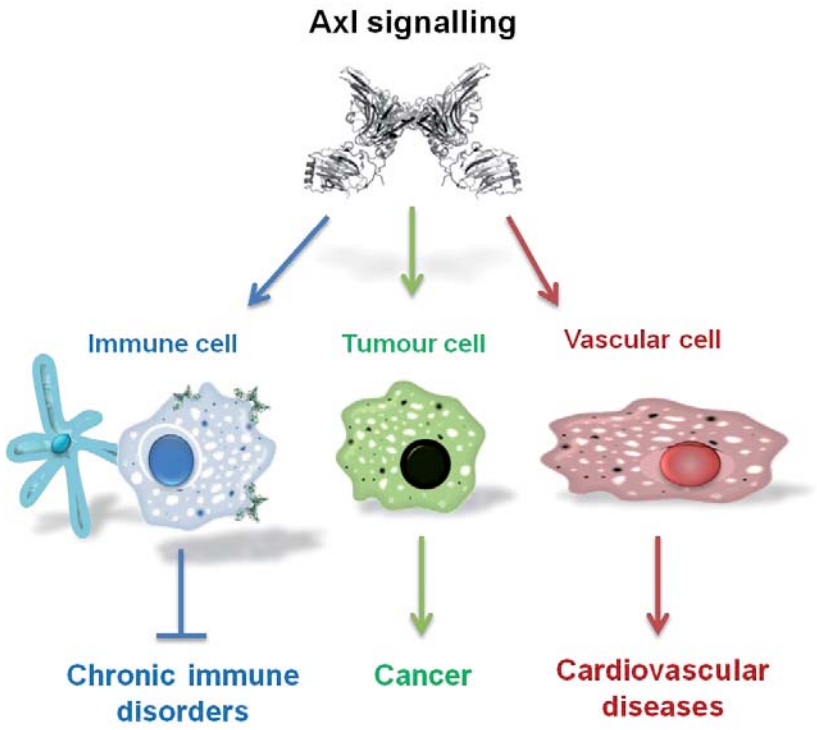


Figure 9. Axl signaling involved in pathological conditions. Axl is implicated in immune cell functions inhibiting chronic immune activation. Axl has been shown to alter tumor cell function and to promote the development of cancer. Its activation is also critically involved in advanced cardiovascular diseases.

Given the role of the Gas6/Axl pathway in a several disorders, therapeutic potential through the inhibition of Axl receptor is under investigation. Administration of Axl-Fc recombinant protein has been shown to impede the interaction of Gas6 with Axl, as illustrated by the protection of mice against thrombosis (Angelillo-Scherrer et al., 2005). Highly specific anti-Axl antibodies have also been used to target Axl, preventing its signaling. Antibodies are able to act on tumor cells and immune cell functions (Li et al., 2009; Smolock and Korshunov, 2010). However, because of poor pharmacokinetics in humans, anti-Axl antibodies are limited in their applications. Actually, the favorite therapeutic strategy targeting Axl in the context of cancer therapy is the use of Axl-specific small-molecule inhibitors (SMIs), (Linger et al., 2010). The inhibitor R428 from Rigel Pharmaceuticals was shown to be selective and effective in Axl signaling inhibition in multiple cell types, showing appropriate pharmacokinetic profiles (Holland et al., 2010). Detection and targeted

treatment of Axl-expressing tumors represent a promising new therapeutic strategy for breast cancer, where the presence of Axl predicts patient survival trough the metastasis progression (Gjerdrum et al., 2010).

In the co-evolution of virus and host, viruses have evolved several strategies to hijack and manipulate necessary cellular functions. Recently, studies revealed how viruses are able to hijack the host's apoptotic clearance machinery for cell entry (Mercer and Helenius, 2008; Mercer and Helenius, 2010; Morizono et al., 2011). As mentioned, apoptotic cells expose PS on their surface, acting as an "eat me" message for the endocytosis and removal of apoptotic debris (Fig. 8) *via* the TAM family members. Viruses have developed several strategies to profit from existing endocytic mechanisms including their ability to hijack the apoptotic debris clearance system. One of these strategies, called "apoptotic mimicry", is the exposure of PS on the pathogen surface, allowing the virus' uptake. This mimicking strategy was first suggested for hepatitis B virus (Vanlandschoot and Leroux-Roels, 2003), and afterwards for vaccinia virus (VACV) (Mercer and Helenius, 2008), possibly HIV (Callahan et al., 2003), and more recently for Dengue virus (Meertens et al., 2012). Recently, Morizono and colleagues proposed a more generalized model in which a range of enveloped viruses displaying PS at their surface can hijack the Gas6/Axl system for cell entry in absence of specific cellular receptors recognized by their glycoproteins. The abuse of the host's evolutionary conserved Gas6/Axl-dependent apoptotic clearance mechanisms for cell invasion would thus broaden the cellular tropism of a virus.

The use of the Gas6/Axl system for cell entry by "apoptotic mimicry" may be of particular relevance for viruses that have recently broken through species barriers. Examples are the filoviruses Ebola virus and Marburg virus that cause severe hemorrhagic fever in humans. Although filoviruses can replicate in diverse tissues and cell types, their broad tropism remains poorly understood. TAM kinases, especially Axl, have been implicated in cell entry of filoviruses. Ectopic expression of Axl and Tyro-3 renders resistant cells susceptible to infection with Ebola and Marburg pseudotypes and live Ebola virus (Shimojima et al., 2006). The susceptibility was reduced by antibodies to TAM members and soluble recombinant proteins. Axl was shown to enhance uptake of Ebola virus via macropinocytosis (Hunt et al., 2011). Interestingly, viral uptake did not appear to be due to direct Axl interaction with Ebola pseudotypes (Brindley et al., 2011). Recently, studies using a panel of Axl mutants demonstrated the need of Axl's physiological functions to allow Ebola virusto enter cells (Shimojima, Ikeda, and Kawaoka, 2007). However, the exact role of PS in the viral envelope and "apoptotic mimicry" for Axl-mediated filovirus cell entry is still not clear.

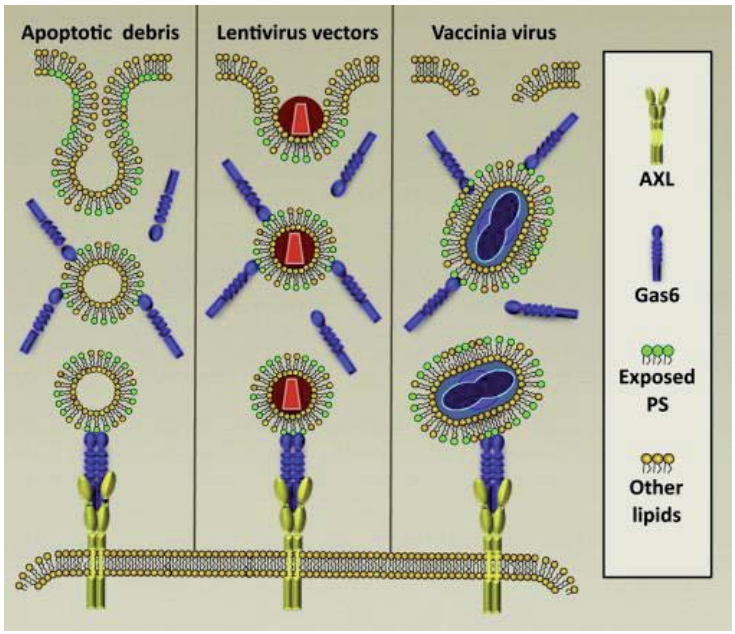


Figure 9. Model of Gas6/Axl-mediated virus apoptotic mimicry. Apoptotic cells expose PS in their outer leaflet of the plasma membrane serving as an "eat-me" signal for their removal. The uptake of apoptotic particles is mediated by Axl together with Gas6 that bridges PS with the receptor. Lentivirus vectors acquire PS at the cell surface during the budding process. VACV gain PS in the cytosol from a yet unknown source and leaves the cells by lysis or fusion. Both lentiviruses and VACV, that are mimicking apoptotic debris, can be recognized by secreted Gas6, linking the viral particles to Axl receptor, allowing an increase in the infectivity (adapted from (Mercer, 2011)).

Very recently, Shimojima and colleagues identified Axl and Tyro-3 as alternative receptors for LASV retroviral pseudotypes (Shimojima et al., 2012). These molecules have been shown to enhance LASV binding to cells and to mediate infection in a DG-independent manner. In my thesis work, I sought to further characterize Axl-mediated cell entry of LASV in the context of productive arenavirus infection.

SPECIFIC AIMS

Project 1: "Post-translational modification of the LASV receptor DG"

Dystroglycan (DG) is best known for its functions in muscle where it mainly provides a molecular link between proteins of the ECM and the cellular actin-based cytoskeleton. The rather static role of DG in muscle cells as a structural component of cell-extracellular matrix contacts contrasts with the expected dynamics required in the context of LASV cell entry. Indeed, engagement of DG by LASV results in rapid internalization of the virus-receptor complex and transfer of the virus to endosomal compartments where fusion occurs. This rapid internalization and delivery suggests that virus binding to α-DG causes marked changes in the trafficking dynamics of DG. Considering these dynamic processes, we hypothesize that virus binding to α-DG results in the clustering of the receptor and the subsequent induction of receptor-mediated signaling. As a consequence, the cytoplasmic tail of the β-DG subunit may undergo post-translational modifications, which could serve as signals triggering the rapid internalization of the virus-receptor complex into vesicles that are then targeted to the endosomal pathway. Previous studies showed that the cytoplasmic tail of β-DG is subject to phosphorylation at tyrosine residue 892 and that this modification affects binding of DG to the cytoskeletal adapters, resulting in receptor internalization. In my first project, I investigated the effect of virus-receptor binding on the tyrosine phosphorylation of β-DG and tested if this post-translational modification was linked to the internalization of LASV-receptor complex. We found that engagement of cellular DG by LASV GP in human epithelial cells induced tyrosine phosphorylation of the cytoplasmic domain of DG. LASV GP binding to DG further resulted in dissociation of the adapter protein utrophin from virus-bound DG. This virus-induced dissociation of utrophin was affected by the tyrosine kinase inhibitor genistein, suggesting a role of receptor tyrosine phosphorylation in the process.

Project 2: "Characterization of LASV cell entry *via* the TAM receptor Axl"

Very recently, Shimojima and colleagues identified the TAM receptors Axl and Tyro3 (Dtk) as candidate receptor for LASV, allowing infection of cells in a DG-independent manner (Shimojima et al., 2012). Axl is the widest expressed member of the TAM receptor family and is present on human cells known to be targeted by LASV *in vivo*. Compared with other members of TAM family, Axl has been shown to have the strongest affinity for the serum human protein Gas6 in the so called Gas6/Axl pathway. This pathway plays a role in two crucial physiological processes, the degradation of apoptotic cell debris and the negative regulation of cytokine signaling in antigen presenting cells like macrophages and DCs. So far, studies on the role of Axl and Tyro3 as candidate LASV receptors have been limited to retroviral pseudotypes, which pose some limitations. In my second project, I used a recombinant form of the prototypic OW arenavirus LCMV expressing the GP of LASV (rLCMV-LASVGP) to study the role of TAM receptors, in particular Axl in LASV cell entry. Considering the close structural and genetic relationship between LASV and LCMV, this recombinant chimera represents a powerful tool to investigate the role of Axl in the context of a productive arenavirus infection. In line with previous studies, I found that Axl mediated LASV cell entry in a DG-independent manner, albeit less efficient than DG. However, Axl-mediated productive infection with rLCMV-LASVGP showed kinetics similar to DG-dependent entry. Axl-mediated cell entry of LASV involved a clathrin-independent pathway that critically dependent on actin and dynamin, was sensitive to EIPA, but not to PAK inhibitors. Lastly, I addressed a possible role of PS displayed in the envelope of Old World arenaviruses in Axl-mediated cell entry.

RESULTS

Project 1: Original manuscript

"Cell entry of Lassa virus induces tyrosine phosphorylation of dystroglycan"

Marie-Laurence Moraz[1], Christelle Pythoud[1], Rolf Turk[2], Sylvia Rothenberger[1], Antonella Pasquato[1], Kevin P. Campbell[2], and Stefan Kunz[1*]

[1] Institute of Microbiology, University Hospital and University of Lausanne, Lausanne, Switzerland
[2]Howard Hughes Medical Institute, Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center, Department of Molecular Physiology and Biophysics, Department of Neurology, and Department of Internal Medicine, Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, Iowa 52242, USA

* Corresponding author. Mailing address: Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne CH-1011, Switzerland. Phone: +41-21 314 7743, Fax: +41-21 314 4060, E-mail Stefan.Kunz@chuv.ch

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Author contributions

Conceived and designed the experiments: **M-L. Moraz**, S. Rothenberger, A. Pasquato, S. Kunz.

Performed the experiments: **M-L Moraz**, Christelle Pythoud, S. Kunz.

Analyzed the data: **M-L Moraz**, S. Rothenberger, A. Pasquato, S. Kunz.

Contributed reagents/materials/analysis tools: R. Turk, K. P. Campbell.

Wrote the paper: S. Kunz, **M-L Moraz**.

ABSTRACT

The extracellular matrix (ECM) receptor dystroglycan (DG) serves as a cellular receptor for the highly pathogenic arenavirus Lassa virus (LASV) that causes a hemorrhagic fever with high mortality in man. In the host cell, DG provides a molecular link between the ECM and the actin cytoskeleton via the adapter proteins utrophin or dystrophin. Here we investigated post-translational modifications of DG in the context of LASV cell entry. Using the tyrosine kinase inhibitor genistein, we found that tyrosine kinases are required for efficient internalization of virus particles, but not virus-receptor binding. Engagement of cellular DG by LASV envelope glycoprotein (LASV GP) in human epithelial cells induced tyrosine phosphorylation of the cytoplasmic domain of DG. LASV GP binding to DG further resulted in dissociation of the adapter protein utrophin from virus-bound DG. This virus-induced dissociation of utrophin was affected by genistein treatment, suggesting a role of receptor tyrosine phosphorylation in the process.

INTRODUCTION

The Old World arenavirus Lassa virus (LASV) is the causative agent of a severe viral hemorrhagic fever in humans with several hundred thousand infections per year in Africa and thousands of deaths annually (McCormick and Fisher-Hoch, 2002). Fatal LASV infection is characterized by rapid viral replication and spread, resulting in uncontrolled viral infection with progressive signs and symptoms of hemorrhagic disease and shock (Geisbert and Jahrling, 2004). The death toll of LASV infection among hospitalized patients can reach 15-30%. There is no licensed vaccine against LASV and current therapeutic options are limited, making LASV arguably one of the most neglected tropical pathogens.

Arenaviruses are enveloped negative-strand RNA viruses with a bi-segmented genome, whose replication takes place in the cytoplasm (Buchmeier, de la Torre, and Peters, 2007; de la Torre, 2009). The two viral RNA segments, L and S, include each two open reading frames. The S segment encodes the envelope glycoprotein precursor (GPC) and the nucleoprotein (NP) and the L segment codes for the matrix protein (Z) and the viral polymerase (L). LASV GPC is synthesized as a single polypeptide and undergoes processing by the host cell protease subtilisin kexin isozyme-1(SKI-1)/site-1 protease (S1P) (Beyer et al., 2003; Lenz et al., 2001; Rojek et al., 2008a), yielding the N-terminal GP1 and the transmembrane GP2. LASV GP1 is involved in binding to cellular receptors (Borrow and Oldstone, 1992), whereas the GP2 part mediates fusion and resembles the membrane-proximal regions of other viral fusion proteins (Eschli et al., 2006; Igonet et al., 2011).

Binding of a virus to its cellular receptor(s) and subsequent entry into target cells are the first steps of virus infection and a fundamental aspect of the virus-host cell interaction. These initial steps of infection are also promising targets to block the pathogen before it can take control over the host cell. Therapeutic intervention at the level of cell entry is of particular interest for highly pathogenic viruses like LASV. The first cellular receptor discovered for LASV and the prototypic Old World arenavirus lymphocytic choriomeningitis virus (LCMV) is dystroglycan (DG) a ubiquitous receptor for extracellular matrix (ECM) proteins (Cao et al., 1998; Oldstone and Campbell, 2011). More recently, additional candidate receptors for LASV have been reported, including the C-type lectins DC-SIGN, LSECtin, and the Tyro3/Axl/Mer (TAM) receptor tyrosine kinases Axl and Tyro3 (Shimojima et al., 2012). However, in DG-expressing cells, DG appears to be the preferred receptor for LASV (Shimojima et al., 2012).

Dystroglycan is a highly conserved protein initially translated as a single polypeptide chain that is cleaved into the extracellular α-DG, and transmembrane β-DG (Barresi and Campbell, 2006). DG is expressed in most developing and adult tissues in cells that adjoin basement membranes (Durbeej et al., 1998) and is crucial for normal cell-matrix interactions (Henry and Campbell, 1998; Henry et al., 2001). Alpha-DG has a central, highly glycosylated mucin-type domain that connects the

globular N-and C-terminal domains. At the extracellular site, α-DG undergoes high-affinity interactions with the ECM proteins laminin, agrin, perlecan, and neurexins (Barresi and Campbell, 2006). Alpha-DG is non-covalently associated with β-DG, which binds intracellular to the adaptor proteins dystrophin or utrophin that link DG to the actin cytoskeleton. DG is also associated with a number of signaling molecules, including the adaptor molecule grb2 (Yang et al., 1995), the canonical MAP kinases MEK and ERK (Spence et al., 2004), and the focal adhesion kinase (Cavaldesi et al., 1999). Upon receptor binding, LASV and LCMV are internalized via an endocytotic pathway that is independent of clathrin, caveolin, and dynamin and bypasses classical Rab5-dependent incoming routes of vesicular trafficking (Quirin et al., 2008; Rojek et al., 2008b). During cell entry, LASV and LCMV pass through the multivesicular endosome, where the virus-receptor complex undergoes sorting by the endosomal sorting complex required for transport (ESCRT) delivering the viruses to late endosomes, where low pH-induced fusion occurs after 20-30 minutes (Pasqual et al., 2011; Quirin et al., 2008; Rojek et al., 2008b).

In the host cell, DG appears as a structural component of cell-matrix contacts, which suggest a rather static role. Considering the receptor dynamics expected in the context of LASV cell entry, we hypothesized that virus binding may alter DG trafficking, e.g. by inducing post-translational modifications that target the DG complex towards rapid endocytosis. Studies in prototypic primate and human cells had demonstrated that cell adhesion to ECM proteins can induce phosphorylation of tyrosine Y892 located within a PPxY motif present at the C-terminus of the cytoplasmic domain of β-DG by non-receptor tyrosine kinases of the src family (James et al., 2000; Sotgia et al., 2003; Sotgia et al., 2001). This tyrosine phosphorylation of Y892 prevented the association of β-DG with the cytoskeletal adaptor protein utrophin (James et al., 2000) and resulted in a redistribution of the DG complex from the plasma membrane to intracellular compartments (Sotgia et al., 2003), linking tyrosine phosphorylation of β-DG to receptor internalization. More recent studies confirmed a role of tyrosine phosphorylation of β-DG at Y892 for the endocytosis of DG in myoblasts and provide evidence for a role of tyrosine phosphorylation of DG in the development of muscle pathophysiology in an animal model for muscular dystrophy (Miller et al., 2012). In the present study we investigated the role of receptor tyrosine phosphorylation for cell entry of LASV.

RESULTS

Tyrosine kinases are involved in endocytosis of rLCMV-LASVGP

Recent studies using the broadly specific tyrosine kinase inhibitor genistein revealed a role for tyrosine kinases in cell entry of LASV (Kolokoltsov et al., 2012). However, the specific step(s) of the viral entry process that depend on tyrosine kinases had not yet been defined. In a first step, we sought to confirm and extend these earlier studies and tried to distinguish effects of genistein on virus-cell attachment from endocytosis. Since LASV is a BSL4 pathogen, work with live virus is restricted to laboratories with high security containment. To circumvent these biosafety restrictions, we used a recombinant form of the prototypic LCMV expressing the envelope GP of LASV (rLCMV-LASVGP) (Rojek et al., 2008b). The chimera rLCMV-LASVGP does not show significant attenuation *in vitro* when compared to the parental LCMV strain and grows to robust titers. Since receptor binding and host cell entry of arenaviruses are mediated exclusively by the viral GP, rLCMV-LASVGP adopts the receptor binding characteristics of LASV (Rojek et al., 2008b) and represents a suitable BSL2 surrogate for our studies on LASV-receptor interaction and cell entry. As a cell culture model, we chose the human lung epithelial cell line WI-26 VA4, which had previously been utilized for studies on DG signaling (Ferletta et al., 2003) and the interaction of LASV with its receptor DG (Rojek et al., 2012).

First, we verified that cell attachment and entry of LASV into WI-26 VA4 cells was mediated by DG. Cells were pre-treated with increasing concentration of the monoclonal antibody (mAb) IIH6 that recognizes a functional glycan epitope on α-DG (Kanagawa et al., 2004) and competes with virus binding (Kunz et al., 2005a). Cells were then infected with rLCMV-LASVGP and infected cells detected after 16 hours by immunofluorescence (IF) staining of the viral nucleoprotein (NP). Pre-treatment with mAb IIH6, but not an IgM isotype control significantly blocked infection with rLCMV-LASVGP in a dose-dependent manner, confirming DG as a major receptor for LASV in these cells (Fig. 1A), as shown previously (Rojek et al., 2012).

To exclude unspecific cytotoxicity caused by genistein, WI-26 VA4 cells were treated with increasing concentrations of the drug for 4 hours, followed by a wash out and incubation for a total of 16 hours. Cell viability was assessed by Cell Titer Glo® assay, which measures intracellular levels of ATP. WI-26 VA4 cells tolerated genistein up to a concentration of 100 μM with only mild loss of cell viability (Fig. 1B). To test the effect of genistein on early infection with LCMV-LASVGP, WI-26 VA4 cells were pre-treated with increasing concentrations of genistein for one hour and infected with rLCMV-LASVGP at multiplicity of 1. At four hours post infection, the drug was removed by wash out. To prevent further infection, the lysosomotropic agent ammonium chloride was added to the fresh medium. When added to cells ammonium chloride raises the endosomal pH rapidly and blocks low

pH-dependent membrane fusion without causing overall cytotoxicity (Ohkuma and Poole, 1978; Ohkuma and Poole, 1981). After 12 hours in presence of ammonium chloride, cells were fixed and infection detected by IF staining for LCMV NP. As shown in Fig. 1C, genistein blocked infection with rLCMV-LASVGP in a dose-dependent manner.

Using expression of LCMV NP in infected cells as readout for infection did not allow discriminating between effects of genistein on LASVGP-mediated cell entry and post-entry steps of early viral infection. To specifically validate the effects of genistein on LASV cell entry in our system, we used recombinant vesicular stomatitis virus (VSV) pseudotyped with the envelope GPs of LASV (rVSV-LASVGP) and VSV (rVSV-VSVG). These pseudotypes are replications deficient and contain a green fluorescent protein (GFP) reporter in their genome. Previous studies demonstrated that rVSV-LASVGP closely mimic the receptor binding and entry characteristics of LASV (Kunz et al., 2005b; Pasqual et al., 2011). Increasing concentrations of genistein blocked infection of rVSV-LASVGP more efficient than rVSV-VSVG (Fig. 1D), consistent with previous reports (Kolokoltsov et al., 2012). Notably, the dose-response characteristic of rVSV-LASVGP closely matched the one of rLCMV-LASVGP (Fig. 1C, D), indicating that at least some of the inhibitory effect of genistein on early infection with rLCMV-LASVGP was due to perturbation of LASVGP-mediated cell entry.

To differentiate between effects of genistein on LASVGP-mediated cell attachment from perturbation of endocytosis, we used a well-established assay previously used to study virus internalization (Pelkmans, Puntener, and Helenius, 2002; Rojek, Perez, and Kunz, 2008), schematically shown in Fig. 1E. Briefly, rLCMV-LASVGP was purified over a renografin gradient and purified virus labeled with the reagent NHS-SS-biotin, resulting in a biotin label that is cleavable by reducing agents. As long as the virus stays bound to the cell surface, the biotin label can be cleaved efficiently with the potent, membrane-impermeable reducing agent Tris(2-carboxyethyl)phosphine (TCEP) (Fig. 1E). Once internalized via endocytosis, the biotin-labeled virus is protected from TCEP and retains its biotin moiety after exposure of cells to TCEP. To assess possible effects of genistein on virus cell attachment and internalization, WI-26 VA4 cells were pre-treated with 50 μM of genistein or vehicle control. After one hour, cells were cooled on ice and incubated with biotin-labeled rLCMV-LASVGP (100 particles/ cell) for one hour in the cold. Unbound virus was removed and cells shifted to 37 °C in presence of genistein. After the indicated time points, cells were rapidly chilled on ice and immediately treated with cold TCEP or reaction buffer only. After quenching of residual TCEP with iodacetamide, cells were lysed and the viral GP isolated by immunoprecipitation (IP) with mAb 83.6 to GP2. Proteins were separated by SDS-PAGE and biotinylation of GP2 detected by Western-blot under non-reducing conditions. In specimens treated with reaction buffer only, similar amounts of cell-associated biotinylated virus were detected in presence and absence of genistein (Fig. 1F), indicating that genistein treatment did not affect virus attachment to the receptor. In control samples treated with TCEP, biotinylated virus became detectable after circa 5 min, with an increase over the

next 20 min. In cells treated with genistein and TCEP, the signals for biotinylated GP2 were markedly reduced, indicating a block in an early step of virus internalization in presence of the inhibitor. In sum, our studies revealed that genistein does not affect virus-cell attachment, but inhibits the subsequent early steps of virus internalization.

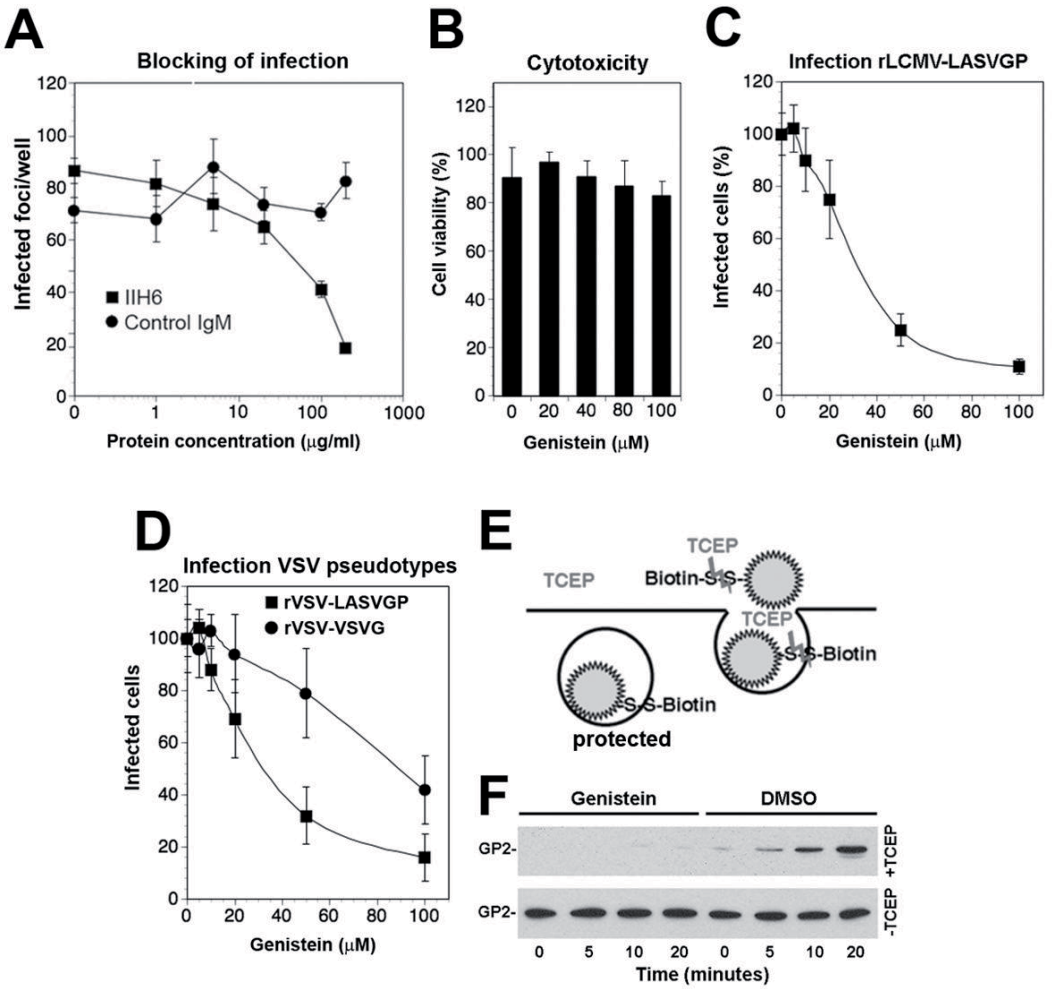

Fig. 1. Genistein inhibits internalization of rLCMV-LASVGP. (A) LASV infection in WI-26 VA4 cells is mediated by DG. Monolayers of WI-26 VA4 cells in M96 plates were blocked with MAb IIH6 or an unrelated mouse IgM (Control IgM) at the indicated concentrations for 2 h at 4°C. Next, 200 PFU of rLCMV-LASVGP was added for 45 min. Infection was assessed after 16 hours by immunofluorescence (IF) staining for LCMV NP. Infected foci were counted in each well (means ± SD, n = 3). (B) Cytotoxicity of genistein. WI-26 VA4 cells were treated with the indicated concentrations of the drug for 4 hours, followed by a wash out and incubation for a total of 16 hours. Cell viability was assessed by Cell Titer Glo® assay. Data are triplicates ± SD. (C) Inhibition of rLCMV-LASVGP infection with genistein. WI-26 VA4 cells were treated with the indicated concentrations of genistein for one hour, followed by infection with rLCMV-LASVGP at multiplicity of 1, followed by wash out of the drug at 4 hours. After 12 hours of culture in presence of 20 mM ammonium chloride, cells were fixed and infection detected by IF for LCMV NP. Data are triplicates ± SD. (D) Inhibition of infection of VSV pseudotypes with genistein. WI-26 VA4 cells were treated with the indicated concentrations of genistein for one hour, followed by infection with rVSV-LASVGP and rVSV-VSVG (200 PFU/well). After 24 hours, infection was assessed by detection of GFP positive cells in direct fluorescence microscopy (n = 3 ± SD). (E) Schematic

of the virus internalization assay (for details, please see text). (F) Genistein treatment prevents internalization of rLCMV-LASVGP. WI-26 VA4 cells were pre-treated with 50 μM of genistein or vehicle control (DMSO). After one hour, cells were chilled on ice and incubated with biotin-S-S-labeled rLCMV-LASVGP (100 particles/cell) for one hour in the cold. Unbound virus was removed, cells shifted to 37 °C in presence or absence of genistein. After the indicated time points, cells were chilled on ice and treated with TCEP (+TCEP) or reaction buffer only (-TCEP). After quenching of residual TCEP, cells were lysed, viral GP isolated by IP with mAb 83.6 to GP2. Biotinylated GP2 was detected with streptavidin-HRP in Western-blot under nonreducing conditions using enhanced chemiluminescence (ECL). The Upper blot (+TCEP) was exposed for 10 minutes, the lower blot (-TCEP) was exposed for 1 minute.

Binding of LASV to cellular DG induced tyrosine phosphorylation of β-DG

Since the binding of LASV to DG is of high affinity and virtually irreversible under neutral pH (Kunz et al., 2005b), we speculated that virus-bound DG may be internalized during endocytosis of the virus. The apparent block of virus internalization in presence of genistein at an early time point (5-20 min) (Fig. 1E) opened therefore the possibility that uptake of the virus-receptor complex could involve tyrosine phosphorylation of the receptor and/or receptor-associated cellular factors. Since phosphorylation of β-DG at residue Y892 had previously been linked to internalization of the DG complex (Miller et al., 2012; Sotgia et al., 2003), we monitored phosphorylation of DG at Y892 during LASV cell entry. For this purpose, we applied mAb cl14a that specifically recognizes β-DG phosphorylated at Y892 (Sotgia et al., 2003). In a first step, we confirmed the specificity of mAb cl14a in our system. To this end, we co-expressed recombinant full-length DG containing a C-terminal HA-tag (DGHA, Fig. 2A) with recombinant c-src in HEK293 cells. Previous studies demonstrated that C-terminal tagging of β-DG had no influence on the biosynthesis, transport, and function of DG (Rojek et al., 2007a). Cells were either treated with the specific src tyrosine kinase family inhibitor PP2 (20 μM) or mock treated. After 48 hours, DGHA was isolated by IP with HA matrix and phosphorylation of Y892 detected with mAb cl14a in Western-blot. As expected, DGHA isolated from cells over-expressing c-src was specifically recognized by mAb cl14a, whereas treatment with PP2 markedly reduced the signal, confirming the specificity of the assay (Fig. 2B).

To examine the effect of LASV binding on tyrosine phosphorylation of DG at Y892, monolayers of WI-26 VA4 cells were incubated with rLCMV-LASVGP (100 particles per cell). Virus attachment was performed in the cold to allow virus binding but preventing lateral movement of receptor molecules in the membrane. As a control, we used the New World arenavirus Pichinde (PICV) that does not bind to α-DG (Spiropoulou et al., 2002). After removal of unbound virus, cells were rapidly shifted to 37°C to restore membrane fluidity. At the indicated time points, cells were lysed in presence of the phosphatase inhibitor sodium orthovanadate. Cleared cell lysates were subjected to lectin purification with wheat germ agglutinin (WGA). Precipitated proteins were separated by SDS-PAGE and probed with mAb cl14a anti-β-DGPY892 and mAb 8D5 to β-DG that

binds independently of phosphorylation. Binding of rLCMV-LASVGP, but not PICV resulted in transient phosphorylation of β-DG at Y892 (Fig. 2C) with maximal signals observed after 5-10 minutes. To confirm the role of src family kinases in the apparent virus-induced receptor phosphorylation at Y892, we treated cells with the src kinase inhibitor PP2 for 30 minutes prior to addition of virus. As shown in Fig. 2D, pretreatment with PP2 markedly reduced virus-induced tyrosine phosphorylation of β-DG at Y892, indicating a direct or indirect involvement of src kinases. In some experiments, we observed an apparent decrease in total β-DG at later time points (Fig. 2C, D). However, this was not observed consistently.

To address a possible role of the observed virus-induced receptor phosphorylation at Y892 for viral entry, we monitored the entry kinetics of LASV in presence of PP2. Upon receptor binding, LASV is taken up by clathrin- and caveolin-independent endocytosis and rapidly delivered to late endosomes, where low pH-dependent membrane fusion occurs (Borrow and Oldstone, 1994; Quirin et al., 2008; Rojek, Perez, and Kunz, 2008; Rojek et al., 2008b). To assess how fast receptor-bound rLCMV-LASVGP trafficked to late endosomes in presence and absence of PP2, we determined the time required for the virus to become resistant to ammonium chloride. Briefly, WI-26 VA4 cells were either pretreated with PP2 for one hour, or mock treated with vehicle (DMSO) only. Cells were then incubated with rLCMV-LASVGP in the cold, allowing virus attachment without internalization. Unbound virus was removed and cells quickly shifted to 37 ºC to allow virus internalization in presence or absence of PP2. After different time points, 20 mM ammonium chloride was added to cells and kept throughout the experiment. At 16 h post infection, cells were fixed and infection assessed by IF detection NP. As shown in Fig. 2E pretreatment of cells with PP2 had no significant effect on the entry kinetics of rLCMV-LASVGP, suggesting that tyrosine phosphorylation of β-DG at Y892 was dispensable for virus cell entry. To complement these inhibitor studies, we examined the entry kinetics of rLCMV-LASVGP in murine embryonic fibroblasts (MEFs) derived from mice deficient for the src-family kinases src, fyn, and yes (Newsome et al., 2006). When compared to wild-type MEFs, src/fyn/yes-deficient cells were infected with similar kinetics (data not shown), in line with our src kinase inhibitor studies (Fig. 2E).

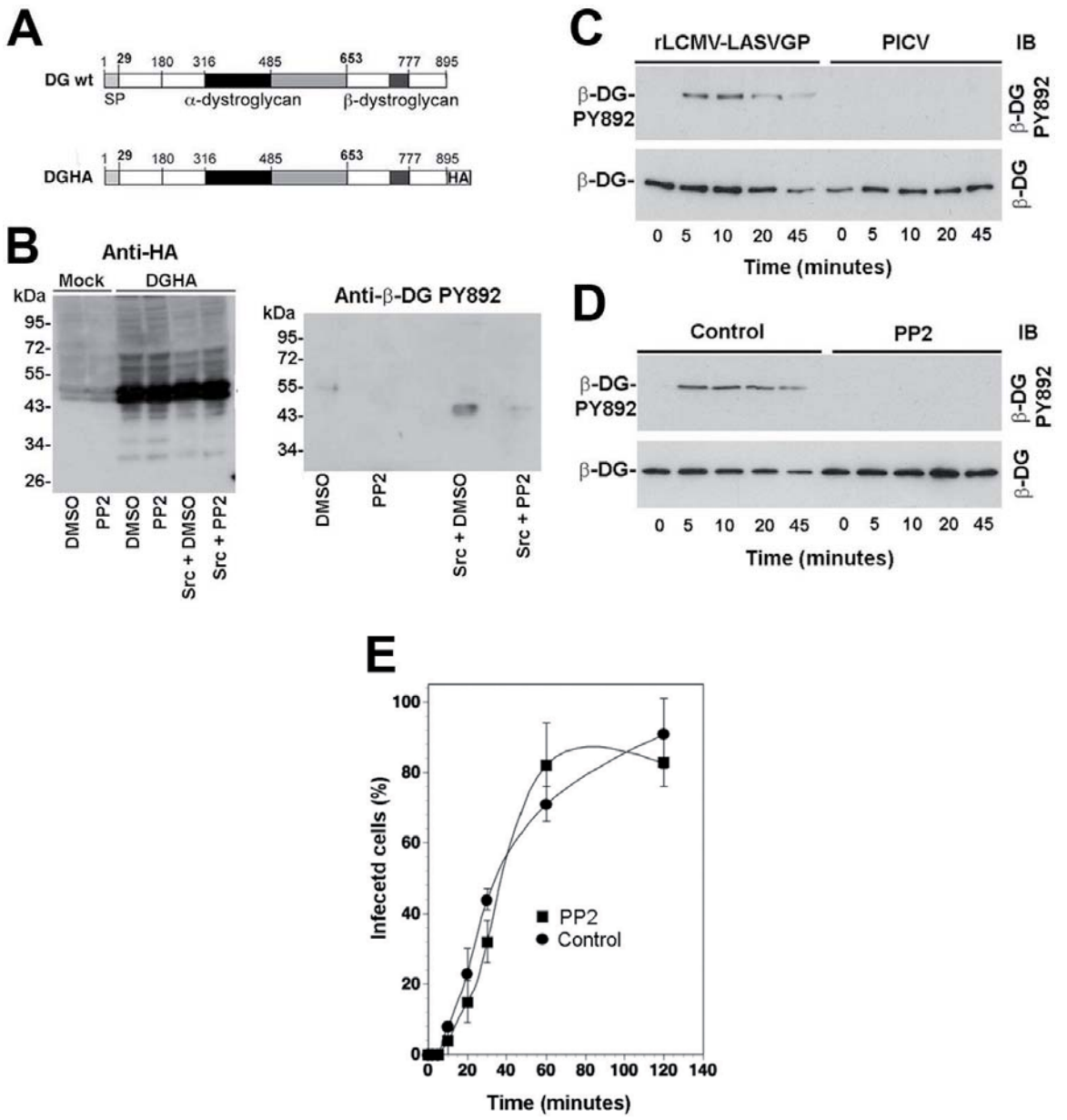

Fig. 2. Binding of LASV to cellular DG induces tyrosine phosphorylation of β-DG by src-family kinases
(A) Schematic representation of C-terminally tagged DG (DGHA). The N-terminal domain (white), the mucin-type domain (black) and the C-terminal domain (gray) of α-DG, β-DG, and the C-terminal HA tag are indicated. (B) Detection of tyrosine phosphorylation at residue Y892 with mAb cl14a. DGHA was transiently expressed either alone or in combination with c-src. Parallel specimens were pretreated with 20 μM PP2 or mock treated with vehicle (DMSO). After 48 hours, DGHA was isolated by pull-down with HA matrix. Proteins were separated and probed in Western blot with an antibody to HA (anti-HA) or mAb cl14a to β-DG phosphorylated at tyrosine 892 (anti-β-DG PY892). Apparent molecular masses and the positions of β-DG are indicated. (C) Attachment of rLCMV-LASVGP to cells induces tyrosine phosphorylation of β-DG. Monolayers of WI-26 VA4 cells were incubated with rLCMV-LASVGP or PICV (100 particles/cell) for 1 hour in the cold. Unbound virus was removed and cells shifted to 37°C. At the indicated time points, cells were lysed and DG enriched by WGA affinity purification. WGA-bound glycoproteins were probed in Western-blot with mAb cl14a (anti-β-DG PY892) and antibody 8D5 to β-DG. The positions of β-DG and β-DG PY892 are indicated. (D) Virus induced

tyrosine phosphorylation of β-DG is blocked by PP2. Monolayers of WI-26 VA4 cells were pretreated with 20 μM PP2 or DMSO (Control) for 1 hour prior to exposure to rLCMV-LASVGP. Virus-induced phosphorylation of β-DG at Y892 was assessed as in (C). (E) The phosphorylation of β-DG at PY892 is not required for LASV cell entry. Monolayers of WI-26 VA4 cells were pretreated with 20 μM PP2 or DMSO (Control) for 1 hour as in (D), followed by incubation with rLCMV-LASVGP (MOI = 1) in the cold in presence of the drug. After 1 hour, unbound virus was removed and pre-warmed (37 °C) medium containing the drug added. At the indicated time points, 20 mM ammonium chloride was added and left throughout the experiment. At 16 hours post infection, cells were fixed and infection detected by intracellular staining for LCMV NP (means ± SD, n = 3). The apparent differences in infection at 60 minutes were not statistically significant.

Despite the marked inhibition of rLCMV-LASVGP internalization by genistein (Fig. 1F), treatment of cells with the src inhibitor PP2 did not affect LASV cell entry, which was rather unexpected. Examination of the sequence of the cytoplasmic tail of β-DG revealed the presence of four putative sites of tyrosine phosphorylation, in addition to Y892 (Fig. 3A). To assess tyrosine phosphorylation of β-DG in response to LASV binding at sites other than Y892, WI-26 VA4 cells were pretreated with PP2 or vehicle, followed by exposure to rLCMV-LASVGP as described above. After the indicated time points, β-DG was isolated by IP using mAb 8D5 that does not discriminate between phosphorylated and unphosphorylated β-DG. Immunocomplexes were separated by SDS-PAGE and probed in Western blot using mAb cl14a to β-DGPY892 and mAb 4G10 to tyrosine phosphate. Total β-DG was detected with polyclonal antibody AP83. As expected, treatment with PP2 prevented virus-induced phosphorylation at Y892 (Fig. 3B). However, immunoblotting with the broadly specific anti-phosphotyrosine mAb 4G10 revealed significant tyrosine phosphorylation of β-DG in response to virus binding that was not affected by PP2 (Fig. 3B). This suggested that binding of rLCMV-LASVGP to cellular DG induced tyrosine phosphorylation of β-DG at sites other than Y892, possibly implicating non-src family tyrosine kinases. Pre-treatment of cells with genistein (50 μM) for 30 minutes reduced virus-induced tyrosine phosphorylation of β-DG altogether (Fig. 3C).

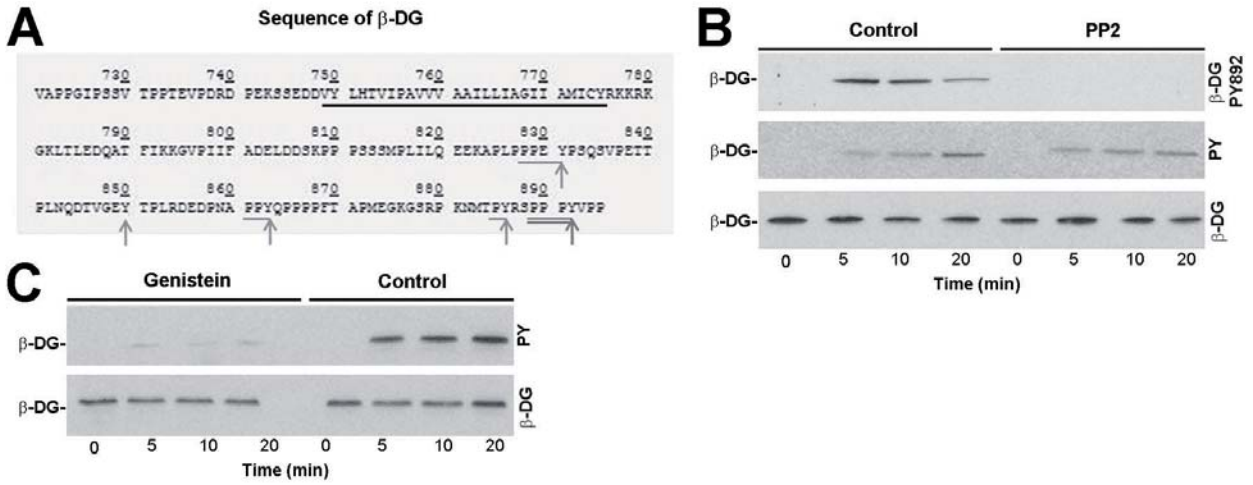

Fig. 3. Binding of LASV to cellular DG induces tyrosine phosphorylation of β-DG by non-src tyrosine kinases (A) Sequence of the cytoplasmic domain of human β-DG. The putative transmembrane domain is underlined in black and tyrosine residues indicated with gray arrows. Putative phosphorylation sites for src-family kinases are underlined and Y residues marked (arrows). The PPxY motif including Y892 is double underlined. (B) Detection of virus-induced tyrosine phosphorylation β-DG in presence of PP2. WI-26 VA4 cells were pretreated with 20 μM PP2 or vehicle, followed by exposure to rLCMV-LASVGP as in Fig. 2D). After the indicated time points, β-DG was isolated by IP using mAb 8D5 against β-DG covalently coupled to Sepharose 4B. Immunocomplexes were eluted under non-reducing conditions, separated by SDS-PAGE and probed in Western blot using mAb cl14a to β-DGPY892 and mAb 4G10 to tyrosine phosphate (pY). For detection of bound mouse IgG a TrueBlot® detection system was used to avoid cross-reaction with the murine IgG heavy and light chains. Total β-DG was detected with rabbit polyclonal antibody AP83. (C) Genistein blocks virus-induced tyrosine phosphorylation of β-DG. Cells were pre-treated with 50 μM genistein or vehicle (DMSO) only. After 30 minutes, rLCMV-LASVGP was added (100 particles/cell) for the indicated time points and tyrosine phosphorylation of β-DG was assessed as in (B).

Engagement of DG by LASV detaches the DG complex from the adaptor utrophin

In the host cell, DG provides a molecular link between the ECM and the actin cytoskeleton by anchorage of the cytoplasmic domain of β-DG to the cytoskeletal adapter proteins dystrophin or utrophin. Cell entry of LASV occurs independently of the dynamics and stability of the actin cytoskeleton (Rojek et al., 2008b). We hypothesized that virus binding, possibly involving receptor clustering and signaling, may somehow detach DG from the cytoskeletal adaptors, allowing subsequent actin-independent endocytosis. A major challenge to test this hypothesis was to assess specific changes in utrophin binding to the relatively small fraction of virus-bound DG as compared to total cellular DG. To overcome this problem, we used recombinant retroviruses bearing the recombinant GP of LASV. Retroviral pseudotypes containing the GP of the New World virus Amapari (AMPV), which does not use DG as a receptor (Spiropoulou et al., 2002), served as a negative control. These arenavirus pseudotypes, which have been previously generated and extensively characterized in

our laboratory, adopt the receptor binding characteristics and cellular tropism of the viruses from which the GPs are derived (Rojek et al., 2007b). Importantly, the use of retroviral pseudotypes allowed the insertion of a C-terminal FLAG-tag into LASV GP and AMPV GP (Fig. 4A) allowing co-IP of the viral GP with associated cellular receptor proteins. The C-terminal FLAG-tag had no adverse effect on the function of the viral GPs in host cell attachment and entry (Rojek et al., 2008a). Retroviral pseudotypes containing FLAG-tagged LASV GP and AMPV GP were produced and purified as described (Rojek et al., 2008a; Rojek et al., 2007b) and detection of GP in purified pseudotypes by ELISA revealed more efficient incorporation of LASV GP when compared to AMPV GP (Fig. 4B). As previously shown, infection of cells with LASV pseudotypes, but not AMPV pseudotypes or pseudotypes of VSV depended on DG (Fig. 4C), confirming their receptor specificity.

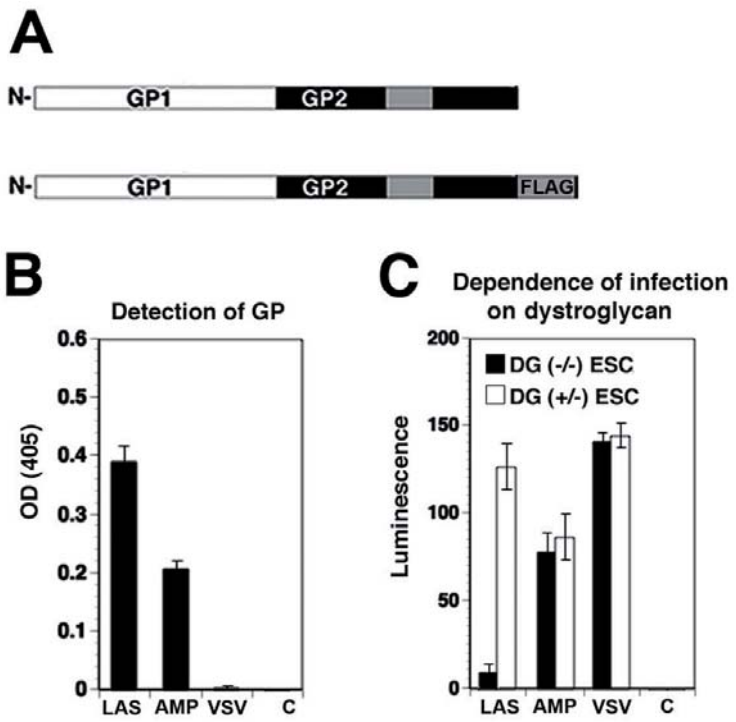

Fig. 4. Retroviral pseudotypes. (A) Schematic representation of Flag-tagged LASV GP. The receptor-binding GP1 and transmembrane GP2 parts are indicated. The transmembrane domain of GP2 is represented as a grey box and the C-terminal FLAG-tag indicated. (B) Detection of GP in retroviral pseudotypes. Retroviral pseudotypes were generated by co-transfection of packaging cell line GP2293 expressing retroviral Gag/Pol with a GP expression plasmid and an expression plasmid for a packable retroviral genome bearing a luciferase and a GFP reporter gene. Pseudotypes were purified by ultracentrifugation through a sucrose cushion and re-suspended in HBSS. Purified pseudotypes of LASV (LAS), AMPV (AMP), and VSV, as well as pseudotypes lacking GP (C) were immobilized in microtiter plates and the viral GP detected with mAb 83.6, combined with an HRP-conjugated secondary antibody in a colour reaction (means $\pm$ SD, n = 3). (C) Infection of cells with LASV pseudotypes depends on DG. DG (-/-) murine embryonic stem (ES) cells and parental DG (+/-) ES cells were infected with pseudotypes of LASV (LAS), AMPV (AMP), and VSV, as well as pseudotypes lacking GP (C). After 48 hours, infection was detected by luciferase assay (means $\pm$ SD, n = 3).

To assess the impact of LASV GP binding on the association of cellular DG with utrophin, we incubated LASV or AMPV pseudotypes with monolayers of WI-26 VA4 cells. A parallel set of cells was incubated with medium only. This step was carried out in the cold to allow receptor binding without clustering or internalization. Cells were either kept on ice or shifted to 37 °C for 10 minutes, followed by cell lysis in the cold. Lysates prepared from cells incubated with pseudotypes were subjected to IP with FLAG matrix. Lysates of untreated control cells were incubated with mAb 16G4 to α-DG, combined with protein G sepharose. Immunocomplexes and total cell lysates were probed for β-DG and utrophin in Western blot. As shown in Fig. 5A, IP anti-FLAG in specimens incubated with LASV, but not AMPV pseudotypes resulted in the detection of β-DG and co-IP of utrophin, suggesting specific pull-down of LASV GP-associated DG complex. As expected, IP of α-DG resulted in robust co-IP of utrophin under all conditions. To quantify possible changes in the ratio of utrophin/β-DG upon exposure of cells to LASV pseudotypes, we performed densitometric analysis of the signals for both conditions and normalized to the utrophin/β-DG detected in the IP of α-DG (Fig. 5B). In cells kept at 4°C, we consistently observed a significantly lower utrophin/β-DG ratio in the LASV GP-associated receptor fraction when compared to the DG-utrophin complexes isolated by IP with anti-α-DG antibody. This suggested that the LASV pseudotypes associate preferentially with cellular DG that shows a weaker or more transient association with utrophin. As shown in Fig. 5B, the temperature shift to 37 °C, which allows clustering of the receptor and signaling to occur, resulted in a reduction of the utrophin/β-DG ratio in LASV GP-associated DG when compared to cells kept in the cold. This suggests that virus-induced receptor clustering and/or signaling promotes the dissociation of the DG complex from utrophin.

To address the role of tyrosine phosphorylation in the observed virus-induced dissociation of utrophin from DG, we tested the effect of genistein and PP2. To this end, cells were pre-treated with genistein and PP2 for one hour, followed by exposure to LASV pseudotypes. After removal of unbound virus, cells were shifted to 37 °C in presence of the drugs. Virus-associated DG was isolated by IP with FLAG matrix and the ratio of utrophin/β-DG assessed as described above. Treatment with genistein significantly reduced virus-induced dissociation of utrophin from DG, whereas PP2 had only a weak effect (Fig. 5C, D). This suggested that the phosphorylation of β-DG at Y892 was dispensable for the virus-induced dissociation of utrophin from the DG complex.

To complement our inhibitor studies, we examined LASV cell entry into murine ES cells expressing a DG variant lacking the last 15 C-terminal amino acids of β-DG (DGΔC), including the PPxY motif containing Y892. Briefly, ES cells expressing either wild type DG or DGΔC were infected with rLCMV-LASVGP at low multiplicity. To exclude differences at the level of post-entry steps in LCMV replication, a recombinant LCMV expressing VSVG was used as a control. At four hours of infection, 20 mM ammonium chloride was added to prevent secondary infection. After 16 hours, cells were fixed and infection detected by IF for LCMV NP. As shown in Fig. 5E, ES cells

expressing DGΔC were as permissive as wild-type cells, suggesting that the last 15 amino acids of β-DG are dispensable for LASV cell entry.

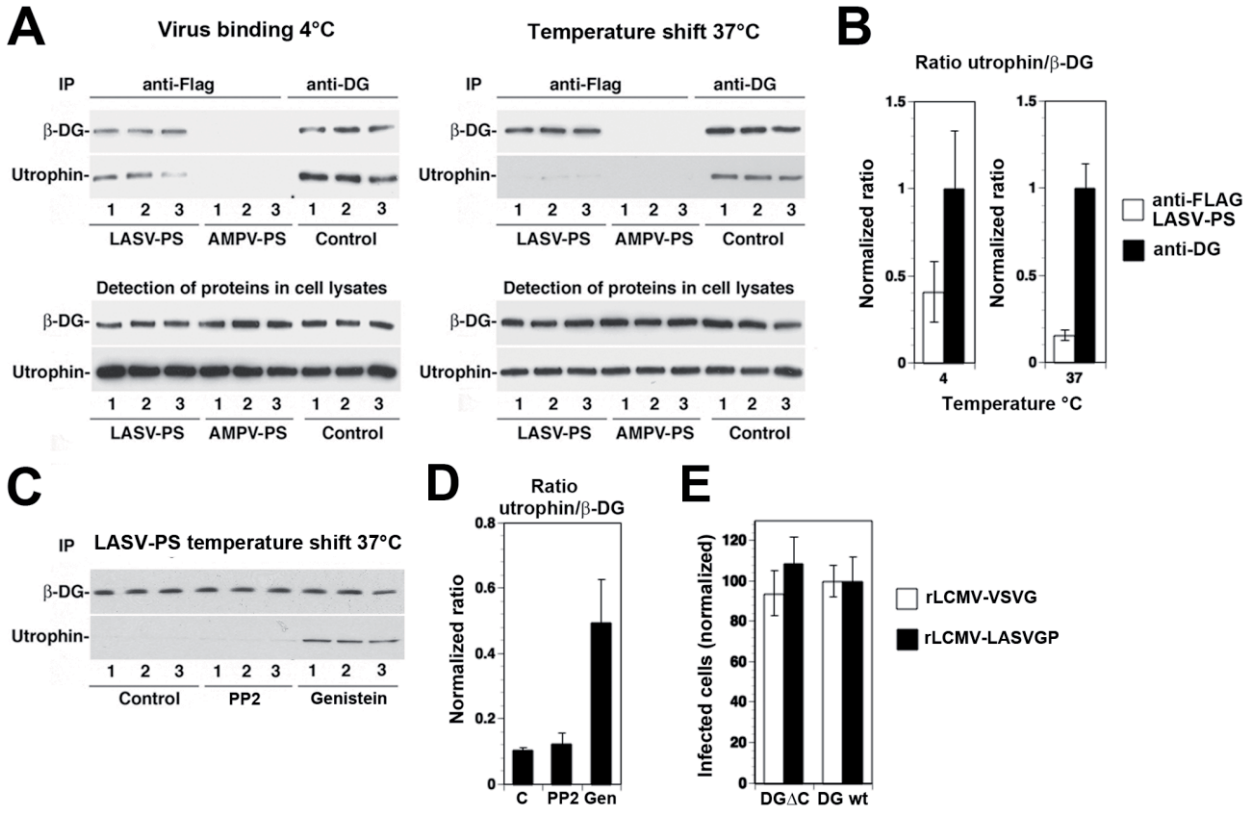

Fig. 5. Effect of LASV pseudotype binding on the association of DG with utrophin. Monolayers of WI-26 VA4 cells were chilled on ice and incubated with either LASV or AMPV pseudotypes (LASV-PS, AMPV-PS) at a multiplicity of infection (MOI) of 50 transforming units (TU)/cell. Parallel specimens were incubated with mAb 16G4 to α-DG (anti-DG). After one hour, unbound virus or mAb were removed by washing. Cells were either kept on ice (virus binding 4°C) or shifted to 37 °C for 10 minutes (temperature shift 37°C). Cells were quickly chilled on ice, lysed and subjected to IP using FLAG matrix or protein G-conjugated Sepharose 4B. Immunocomplexes were separated by SDS-PAGE using 100% of the IPs anti-FLAG and 5% of the IP anti-DG. Beta-DG and utrophin were detected on Western-blot using monoclonal antibodies 8D5 and combined with HRP-conjugated secondary antibodies in a TrueBlot® detection system to avoid cross-reaction with the IgG heavy chain. For the detection of total protein in cell lysates, 1/20 of the lysate were separated by SDS-PAGE and subjected to Western-blot detection. (B) Quantification of the signals in (A). Blots were scanned in a densitometer and the ratios of the signals for utrophin normalized to β-DG (utrophin/β-DG) for the IPs anti-FLAG (LASV pseudotypes only) and the IP anti-DG. For each series, the utrophin/β-DG ratio detected in the IP anti-DG was defined as 1.0. (C) Pre-treatment with genistein, but not PP2 reduced virus-induced dissociation of utrophin from DG. Monolayers of WI-26 VA4 cells were pre-treated with DMSO only (control), 20 μM PP2 and 50 μM genistein for one hour. Cells were then chilled on ice and incubated with LASV pseudotypes (LASV-PS) for one hour in the cold in presence of drugs. Cells were then quickly shifted to 37 °C, lysed, and subjected to IP with FLAG matrix as in (A). Precipitated β-DG and utrophin were detected in Western blot and the ratios utrophin/β-DG determined as in (B). (D) Quantification of the data in (C). (E) The 15 C-terminal amino acids of β-DG are dispensable for LASV cell entry. Murine ES cells expressing either wild-type DG (DG wt) or DG lacking the C-terminal 15 amino acids of β-DG (DGΔC) were infected with rLCMV-LASVGP or rLCMV-

VSVG at a multiplicity of 0.1. Infection of the cells expressing wild-type DG was set at 100% (means ± SD, n = 3).

DISCUSSION

In the present study we investigated the role of tyrosine phosphorylation for cell entry of LASV. We show that tyrosine kinases are required for endocytosis of the virus-receptor complex, but dispensable for virus-receptor binding. Binding of LASV to cellular dystroglycan (DG) induced phosphorylation of DG at Y892 by src family kinases and other tyrosine residues of β-DG by non-src kinases. Virus-induced receptor phosphorylation was accompanied by dissociation of DG from the cytoskeletal adaptor protein utrophin, which might facilitate virus endocytosis.

In the host cell, DG provides a molecular link between the ECM and the actin-based cytoskeleton and has a slow turnover. However, engagement of DG by LASV results in rapid internalization of the virus and delivery to the late endosome (Quirin et al., 2008; Rojek et al., 2008b), suggesting marked changes in trafficking dynamics of DG induced by virus binding. In our study, we investigated if attachment of LASV affects post-translational modifications of DG, altering receptor trafficking in the membrane. Extending previous studies (Kolokoltsov et al., 2012; Vela et al., 2008), we found that treatment of cells with the broadly-specific tyrosine kinase inhibitor genistein prevented LASV cell entry at an early step of virus internalization without affecting virus-receptor binding, providing a first link between tyrosine phosphorylation and viral endocytosis. Since tyrosine phosphorylation of β-DG at Y892 by src family kinases has previously been implicated in internalization of DG (Miller et al., 2012; Sotgia et al., 2003), we tested the effect of LASV binding on phosphorylation of the receptor at Y892. Engagement of cellular DG by rLCMV-LASVGP rapidly induced phosphorylation of β-DG at Y892 that was blocked by the src kinase inhibitor PP2. The observed kinetics of Y892 phosphorylation was compatible with a role in viral endocytosis. However, rather unexpected, treatment of cells with PP2 had no effect on the entry kinetics of the virus. Since the cytoplasmic tail of β-DG contains four additional putative sites of tyrosine phosphorylation, in addition to Y892, we examined virus-induced phosphorylation at tyrosines. We found that LASV binding induced tyrosine phosphorylation of β-DG at sites other than Y892 with similar kinetics. These tyrosine phosphorylation events were not sensitive to PP2 and likely involved non-src tyrosine kinases. Virus-induced tyrosine phosphorylation of β-DG was entirely abrogated by genistein.

Using a pseudotype platform, we assessed the composition of DG complexes associated with LASV GP upon virion attachment. We found that LASV pseudotypes associated with a fraction of cellular DG that showed less association with utrophin. The reasons for this are currently unclear, but may be related to different accessibility of DG as a function of its association with utrophin and the actin cytoskeleton. In cells, DG associated with cell-substrate adhesion structures at the lower plasma membrane tends to co-localize with utrophin (Belkin and Smalheiser, 1996). Due to the close association with the substratum at the basal face of the cell, DG located in such substrate-adhesion complexes may be less accessible to the virus.

Our analysis further revealed that engagement of DG by LASV GP results in dissociation of utrophin from β-DG. This virus-induced dissociation of utrophin could be perturbed by genistein, but not PP2, suggesting that phosphorylation of β-DG at Y892 by src kinases was dispensable. The efficient virus-induced dissociation of utrophin from DG in presence of PP2 is in line with the inability of the inhibitor to perturb viral entry. However, our findings are different from reports supporting a role for phosphorylation of β-DG at Y892 by src kinases in the regulation of utrophin binding and endocytosis of DG in prototypic primate cells (James et al., 2000; Sotgia et al., 2003) and myoblasts (Miller et al., 2012) in response to endogenous substrates. The reasons for this discrepancy are currently unclear. One possibility is that, contrary to our initial assumption, DG may not stay associated with the virus during the entry process. In this scenario, DG would serve as an attachment factor rather than a true entry receptor. One would have to postulate the existence of another, yet unknown receptor molecule that would mediate viral endocytosis. The recently discovered alternative LASV receptors Axl and Dtk (Shimojima et al., 2012) appear as interesting candidates in this context. Alternatively binding of the multivalent virion particle to cellular DG may induce extensive clustering and activate signaling pathways that are not triggered by DG's ECM ligands, allowing the virus to "bypass" a block in Y892 phosphorylation. To address these possibilities, studies aiming at tracking the virus-DG complex in live cells are currently launched in our laboratory.

Since virus-induced phosphorylation of β-DG is blocked by genistein, which inhibits internalization of the virus, it is tempting to speculate that virus-induced phosphorylation of β-DG is necessary for virus entry. However, in the host cell, DG is found associated with a number of cellular proteins that are required for the correct assembly and stability of the DG complex in the membrane, as well as its anchorage to the actin cytoskeleton (Barresi and Campbell, 2006). Such "pre-formed" DG complexes represent the *functional units* of virus attachment. Our data have shown that virus binding to DG results in receptor signaling. Such virus-induced signaling may affect the composition of the virus-receptor complex by recruiting new proteins into the virus-DG complex and/or excluding others. During the entry process, the "interactome" of the virus-DG complex may therefore change in a dynamic manner resulting in sorting at the plasma membrane required for subsequent cell entry. Candidate cellular proteins that interact with the virus-DG complex during the entry process and are part of this "interactome" would represent potential substrates for tyrosine phosphorylation. We cannot exclude the possibility that tyrosine phosphorylation of such receptor-associated proteins, and not β-DG itself, is the actual target of genistein in the viral entry process.

In sum, the data at hand suggest that attachment of LASVGP to cellular DG induces tyrosine phosphorylation of β-DG at Y892 and other tyrosine residues accompanied by the dissociation of DG from utrophin. The consequent detachment of virus-bound DG from the actin-based cytoskeleton may facilitate subsequent endocytosis of the virus-receptor complex, providing a possible link between virus-induced post-translational modification of DG and virus entry.

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MATERIALS AND METHODS

Cell lines and viruses

WI-26 VA4 cells (ATCC CCL-95.1) were cultured in DMEM, 10 % (vol/vol) FBS, supplemented with glutamine, and penicillin/streptomycin. Embryonic stem (ES) cells DG (+/-), DG (-/-) have been described (Henry and Campbell, 1998). Transgenic ES cells expressing DG lacking the last 15 amino acids (DGΔC) were generated through introduction of a triple premature stop codon affecting all possible reading frames via targeted homologous recombination (gift from Kevin P. Campbell).
The recombinant virus rLCMV-LASVGP has been described elsewhere (Rojek et al., 2008b) and was produced and the titers determined as previously described (Dutko and Oldstone, 1983). Recombinant LASV GP and AMPV GP containing a C-terminal FLAG-tag have been described (Rojek et al., 2008a). Retroviral pseudotypes expressing GFP and luciferase reporters were produced and concentrated, and titers determined as described (Rojek, Spiropoulou, and Kunz, 2006). Concentrated pseudotypes were diluted in HBSS at 10^7 transforming units per ml. For detection of viral GP in ELISA, purified pseudotypes were immobilized in microtiter plates at 10^6 TU/ml and the viral GP detected as described (Rojek, Perez, and Kunz, 2008). Recombinant VSV pseudotyped with LASV GP (rVSV□G-LASVGP), and VSV GP (rVSV□G -VSVG) were generated as reported previously (Kunz et al., 2005b). Virus titers were determined by the infection of Vero E6 cell monolayers and detection of GFP-positive cells by fluorescence microscopy.

Antibodies and reagents

Monoclonal antibodies (mAbs) 113 (anti-LCMVNP) and 83.6 (anti-LCMVGP) have been described (Buchmeier et al., 1981; Weber and Buchmeier, 1988), as has mAb IIH6 anti-□-DG (Ervasti and Campbell, 1991). Other mAbs included mouse IgG 8D5 anti-β-DG (Novocastra) and mouse IgG 16C4 to α-DG (provided by Kevin P. Campbell), mouse IgG anti-utrophin from St. Cruz Biotechnology (St. Cruz, CA), mAb cl14a to phospho-β-DG PY982 (BD Bioscience), and mAb 4G10 to phospho-tyrosine (St. Cruz, CA) . Rabbit anti-influenza HA (Y11) was from St. Cruz Biotechnology (St. Cruz, CA). Polyclonal rabbit anti-mouse secondary antibodies conjugated to HRP were from Dako (Glostrup, Denmark) and goat anti-mouse antibody conjugated with Rhodamin Red X were from Jackson Immuno Research Laboratories. Genistein and PP2 were purchased from Calbiochem. The Bright-Glo® luciferase assay and Cell Titer Glo® assay systems were obtained from Promega (Madison WI).

Immunoblotting

Proteins were separated by gel electrophoresis using 12% polyacrylamide gels and transferred to nitrocellulose. After blocking in 5% (wt/vol) skim milk in PBS, membranes were incubated with Abs used at following concentrations: mAb 8D5, mAb cl14a, mAb 4G10, and polyclonal Abs AP83, and Y11 (10 µg/ml) in 2% (wt/vol) skim milk, PBS for 12 h at 6 ºC. Secondary Abs coupled to HRP were applied 1: 5, 000 in PBS, 0.1 % (wt/vol) Tween for 1 hour at room temperature. Blots were developed by enhanced chemiluminescence (ECL) using Super Signal West Pico ECL Substrate or TrueBlot® detection system (Pierce), where indicated.

Infection of cells with retroviral pseudotypes

Cells were plated in 96-well plates in a density of 10^4 cells/well. After 24 hours, retroviral pseudotypes were added at the indicated MOI and incubated for 1 hour at 37°C. The viral particles were removed, cells washed twice with DMEM, and fresh medium added. Infection was quantified by Bright Glo® luciferase assay. Luminescence was calculated as fold-increase over background signals obtained from uninfected cells.

Infection of cells with rLCMV-LASVGP

Cells were plated in 96-well plates in a density of 10^4 cells/well. After 24 hours, cells were pre-treated with genistein or PP2 as indicated, followed by infection with rLCMV-LASVGP at the indicated MOI for 1 hour at 37°C. Unbound virus were removed, cells washed twice with DMEM, and fresh medium added. Infection was quantified by detection of LCMV NP in IF as described (Kunz et al., 2004). Cell entry kinetics of rLCMV-LASV in presence and absence of PP2 were performed as described (Rojek et al., 2008b). Blocking of infection with mAb IIH6 was done as reported (Kunz et al., 2005a).

Virus internalization assay

Purification of rLCMV-LASVGP was performed by ultracentrifugation on a renografin gradient. Purified LCMV was labeled with the thiol-cleavable reagent NHS-SS-biotin (Pierce). The cleavage of the biotin label was verified by reaction with the membrane-impermeable reducing agent Tris (2-carboxyethyl) phosphine (TCEP) (10 mM) (Pierce) for 30 min, which resulted in a loss of >95% of the biotin label. Internalization assay was performed as described previously (Rojek, Perez, and Kunz, 2008). Briefly, cells were cultured in 10-cm dishes to obtain closed monolayers. Medium was removed, and cells were washed twice with cold HBSS and chilled on ice for 5 min. Cold solution containing NHS-SS-biotinylated rLCMV-LASVGP (10^7 PFU/ml) in HBSS was added. After incubation for 1 h on ice, unbound virus was removed and cells were washed with cold HBSS. For internalization, cells were shifted to 37°C. After the indicated incubation times, medium was removed and cells chilled on ice. TCEP (15 mM) in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$,

1 mM $MgCl_2$ was added (5 ml/dish) and applied twice for 30 min on ice. Cells were washed three times with cold HBSS, and the remaining TCEP was quenched with 100 mM iodoacetamide for 10 min, and cells were lysed immediately. LCMV GP2 was isolated by IP as described (Rojek, Perez, and Kunz, 2008). Immunocomplexes were separated by nonreducing SDS-PAGE. Biotinylated LCMV GP2 was detected by Western blotting with HRP-conjugated streptavidin as described above.

Detection of DG-utrophin complexes associated with LASV GP

Triplicate cultures of WI-26 VA4 cells were cultured in 10 cm plates over night to obtain closed monolayers. Retroviral pseudotypes of LASV and AMPV (10^7 TU/ml in HBSS) were added at 50 TU/cell. Parallel specimens were incubated with 10 µg/ml mAb 16C4 to α-DG in HBSS. After incubation for one hour in the cold, cells were washed 3 times with cold HBSS. One series of cultures was kept on ice whereas the other was quickly shifted to 37°C in a water bath. After 10 minutes, cells were chilled on ice. Cells were lyzed for 30 minutes in 1% (wt/vol) β-octylglucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, 50 mM Hepes pH 7.5, 10% (wt/vol) glycerin supplemented with protease inhibitor cocktail Complete® (Roche) and 1 mM PMSF. Cleared lysates were subjected to IP with FLAG matrix (Sigma) or protein G-conjugates sepharose 4B (Sigma) for 2 hours at 6°C. After three short washes in lysis buffer, the matrix was eluted with non-reducing SDS-PAGE sample buffer to minimize elution of IgG from the FLAG matrix. After addition of 100 mM DTT (final concentration) and boiling for another 5 minutes Western blot performed as described above. For quantification, X-ray films were scanned with a Storm densitometer, and acquired data were processed using Image Quant software.

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Project 2: Original manuscript

"Characterization of the cell entry of Lassa virus via the TAM receptor Axl"

Marie-Laurence Moraz[1], Ana-Rita Goncalves[1], Antonella Pasquato[1], and Stefan Kunz[1*]

[1] Institute of Microbiology, University Hospital and University of Lausanne, Lausanne, Switzerland

* Corresponding author. Mailing address: Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne CH-1011, Switzerland. Phone: +41-21 314 7743, Fax: +41-21 314 4060, E-mail Stefan.Kunz@chuv.ch

Manuscript in preparation.

Author contributions

Conceived and designed the experiments: **M-L Moraz**, A. Pasquato, S. Kunz.

Performed the experiments: **M-L Moraz**.

Analyzed the data: **M-L Moraz**, A. Pasquato, S. Kunz.

Contributed reagents/materials/analysis tools: A. Pasquato, S. Kunz.

Wrote the paper: S. Kunz, **M-L Moraz**.

ABSTRACT

The Old World arenavirus Lassa (LASV) is the causative agent of a severe hemorrhagic fever in humans causing several hundred thousand infections per year in Western Africa. The first cellular receptor discovered for LASV is dystroglycan (DG) a versatile receptor for proteins of the extracellular matrix (ECM). Recognition of DG by LASV critically depends on functional glycosylation of DG involving the glycosyltransferase LARGE, which occurs in a tissue-specific manner. Recent studies identified the TAM-family receptor tyrosine kinases Axl and Dtk as alternative cellular receptors for LASV in cells lacking functional DG. Here we further characterized Axl-mediated cell entry of LASV in the context of productive arenavirus infection using a chimera of the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) expressing the LASV envelope glycoprotein (rLCMV-LASVGP). In line with previous studies, we found that cell entry of rLCMV-LASVGP *via* Axl was less efficient when compared to functional DG. However, Axl-mediated productive infection with rLCMV-LASVGP showed kinetics similar to DG-dependent entry. Axl-mediated cell entry of LASV involved a clathrin-independent pathway that critically depended on actin and dynamin, was sensitive to EIPA, but not to PAK inhibitors.

INTRODUCTION

The Old World arenavirus Lassa (LASV) is the causative agent of a severe hemorrhagic fever with high mortality in humans (McCormick and Fisher-Hoch, 2002). LASV is endemic in Western Africa from Senegal to Cameroon and causes several hundred thousand infections per year with thousands of deaths. Considering the number of people affected, the current lack of a licensed vaccine and the limited therapeutic options at hand, LASV represents a serious public health problem. LASV is an enveloped negative strand RNA virus with a non-lytic life cycle (Buchmeier, de la Torre, and Peters, 2007). The genome of LASV consists of two single-stranded RNA species, a large segment encoding the virus polymerase (L) and a small zinc finger motif protein (Z), and a small segment encoding the virus nucleoprotein (NP) and glycoprotein precursor (GPC). GPC is processed into GP1, implicated in receptor binding, and the transmembrane GP2, which contains the viral fusion machinery, allowing fusion of the viral and the cellular membrane during viral entry (Nunberg and York, 2012).

The first cellular receptor discovered for LASV and the prototypic Old World arenavirus lymphocytic choriomeningitis virus (LCMV) is dystroglycan (DG) an ubiquitously expressed receptor for extracellular matrix (ECM) proteins (Cao et al., 1998; Oldstone and Campbell, 2011). Binding of LASV and LCMV to DG critically depends on the functional glycosylation of the α-DG subunit that critically depends on the glycosyltransferase LARGE (Kunz et al., 2005). Upon binding to DG, LASV and LCMV enter the host cell *via* an endocytotic pathway that is independent of clathrin, caveolin, dynamin, and actin (Quirin et al., 2008; Rojek, Perez, and Kunz, 2008; Rojek et al., 2008). Upon internalization, the virus is delivered to acidified endosomes *via* a pathway that is independent of the small GTPases Rab5 and Rab7, suggesting an unusual route of incoming vesicular trafficking. Recent studies revealed that LASV passes through the multivesicular body (MVB), where it undergoes sorting into intraluminal vesicles (ILV) by hijacking the host cell's endosomal sorting complex required for transport (ESCRT) machinery (Pasqual et al., 2011). Upon sorting into ILV, the virus is delivered to late endosomes, where fusion occurs at an unusually low pH (Klewitz, Klenk, and ter Meulen, 2007). Using an expression cloning approach, Shimojima and colleagues identified the C-type lectins DC-SIGN and LSECtin, as well as the Tyro3/Axl/Mer (TAM) receptor tyrosine kinases Axl and Tyro3 (Dtk) as alternative LASV receptors (Shimojima et al., 2012). The data at hand convincingly show that Axl and Tyro-3 enhance LASV binding to DG-deficient cells and were able to mediate infection in a DG-independent manner.

Tyro-3, Axl, and Mer compose the TAM family of receptor tyrosine kinases (RTKs), whose extracellular domain is comprised of tandems of two immunoglobulin (Ig) domains and two fibronectin type II domains (Linger et al., 2008). Tyro-3, Axl and Mer are expressed in a wide variety of cell types, with overlapping but unique patterns. Among the TAM receptors implicated in LASV

cell entry, Axl shows the widest expression pattern (O'Bryan et al., 1991) and is found on epithelial cells, platelets, endothelial cells, in the heart, liver, kidney, skeletal muscle, testis (Angelillo-Scherrer et al., 2001; Graham et al., 1995; Neubauer et al., 1994), and the brain (Bellosta et al., 1995). Tyro-3 is primarily expressed in the nervous system and is also detected in lung, kidney, retina and sexual organs, as well as hematopoietic cells (Angelillo-Scherrer et al., 2001; Lai, Gore, and Lemke, 1994; Lu and Lemke, 2001; Mark et al., 1994; Prasad et al., 2006). TAM receptors undergo interactions with serum protein S, and growth-arrest-specific protein 6 (Gas6), and proteins of the tubby family (Caberoy, Zhou, and Li, 2010; Lemke and Burstyn-Cohen, 2010; Lemke and Rothlin, 2008). Gas6 serves as ligand for all three TAM receptors, albeit with different affinity: Axl > Tyro3/Dtk >> Mer. Ligand binding induces homo- or hetero-dimerization of TAM receptors with consequent activation of their cytoplasmic tyrosine kinase domain. TAM-receptors are involved in many cellular functions, including chemotaxis (Fridell et al., 1998), cell survival (Lee et al., 1999), the modulation of innate immunity (Lemke and Rothlin, 2008; Rothlin and Lemke, 2010), and clearance of apoptotic debris (Lemke and Burstyn-Cohen, 2010). Apoptotic cells display the phospholipid phosphatidylserine (PS) that is recognized by Gas6 and ProS *via* their N-terminal gamma-carboxylated glutamic acid (GLA) domain. Subsequent engagement of TAM receptors by the C-terminal domains of Gas6 and ProS results in the endocytosis of apoptotic cell debris.

In the co-evolution with their hosts, viruses developed several strategies to hijack and manipulate cellular functions. Recent studies revealed that viruses are able to hijack the host's apoptotic clearance machinery for cell entry (Mercer and Helenius, 2008; Mercer and Helenius, 2010; Morizono et al., 2011). Enveloped viruses display PS at their surface, which can act as an "eat me" signal triggering endocytosis. This strategy of "apoptotic mimicry" to gain access to the host cell was initially described for vaccinia virus (Mercer and Helenius, 2008). Subsequent studies demonstrated that a variety of enveloped viruses expose PS and can hijack the Gas6/TAM system to enter the host cell (Morizono et al., 2011), providing an entry mechanism that is not dependent on specific interaction between viral envelope proteins and cellular receptors, thus enlarging the tropisms of viruses. TAM kinases have been implicated in cell entry of the filoviruses Ebola and Marburg virus (Shimojima et al., 2006), as well as Dengue virus (Meertens et al., 2012). Axl was shown to enhance uptake of Ebola virus *via* macropinocytosis (Hunt et al., 2011) and cell entry *via* Axl did not involve a direct interaction with the viral glycoprotein (Brindley et al., 2011). Mutagenesis of Axl highlighted the importance of a functional ligand-binding domain and signal transduction for Ebola virus entry (Shimojima, Ikeda, and Kawaoka, 2007), however, the exact underlying mechanism remains unclear. In the present study, we sought to characterize the unknown endocytotic pathway involved in Axl-mediated cell entry of LASV.

RESULTS

Axl mediates cell entry of rLCMV-LASVGP independently of DG

LASV is a BSL4 pathogen and work with the live virus requires high containment laboratories. To circumvent the biosafety restrictions associated with live LASV, we used a recombinant LCMV expressing the LASV envelope GP (rLCMV-LASVGP) (Rojek et al., 2008). Based on the close structural and genetic relationship between LASV and LCMV and the fact that receptor binding and entry of arenaviruses are mediated exclusively by the viral envelope, rLCMV-LASVGP represents a suitable BSL2 surrogate for studies on LASV cell entry in the context of productive arenavirus infection (Pasqual et al., 2011; Rojek et al., 2012; Rojek et al., 2008).

Previous studies had shown that Axl can function as a cellular receptor for lentiviral pseudotypes of LASV in the human fibrosarcoma cell line HT1080 (Shimojima et al., 2012). Consistent with earlier studies (Shimojima et al., 2012; Shimojima et al., 2006), Western-blot analysis revealed abundant Axl, but only low amounts of functionally glycosylated α-DG in HT1080 cells, whereas HEK293H cells expressed high amounts of functional DG, but lacked Axl (Fig. 1A, B). To exclude a possible contribution of residual functional DG to rLCMV-LASVGP entry into HT1080 cells, we depleted the DG core protein by RNA interference (RNAi). For this purpose, HT1080 cells were stably transduced with a lentiviral vector expressing a small hairpin (sh)RNA targeting DG or a scrambled control siRNA. Selected cells stably transfected with DG shRNA showed a reduction in DG expression of > 95%, as assessed in Western-blot (Fig. 1C). DG-depleted and control HT1080 were infected with rLCMV-LASVGP at low multiplicity (0.1). At 16 hours post infection, cells were fixed and infection detected by immunofluorescence assay (IFA). As shown in Fig. 1D, depletion of DG in HT1080 cells did not affect infection of rLCMV-LASVGP (Fig. 1B), indicating a negligible role for residual functional DG.

In line with published data, HT1080 cells expressed only Axl, but lacked the candidate LASV receptors Dtk, DC-SIGN (Fig. 2A and B), and LSECtin (data not shown). Two consecutive transfections of HT1080 cells with Axl-specific siRNA resulted in depletion of > 95% of the protein after 72 hours (Fig. 2C). Axl-depleted and control cells treated with scrambled siRNA were infected with rLCMV-LASVGP and a recombinant LCMV expressing the G protein of vesicular stomatitis virus (rLCMV-VSVG) (Pinschewer et al., 2003), which mediates infection in an Axl-independent manner (Morizono et al., 2011). As expected, depletion of Axl markedly reduced infection with rLCMV-LASVGP, but not rLCMV-VSVG, confirming a crucial role of Axl in LASV entry into HT1080 cells (Fig. 2D). To address the relative efficiency of Axl and DG as cellular receptors for LASV, functional DG was rescued in HT1080 cells by over-expression of LARGE (Fig. 2E).

Expression of functional DG greatly enhanced infection with rLCMV-LASVGP (Fig. 2F), confirming that DG acts as preferred receptor (Shimojima et al., 2012).

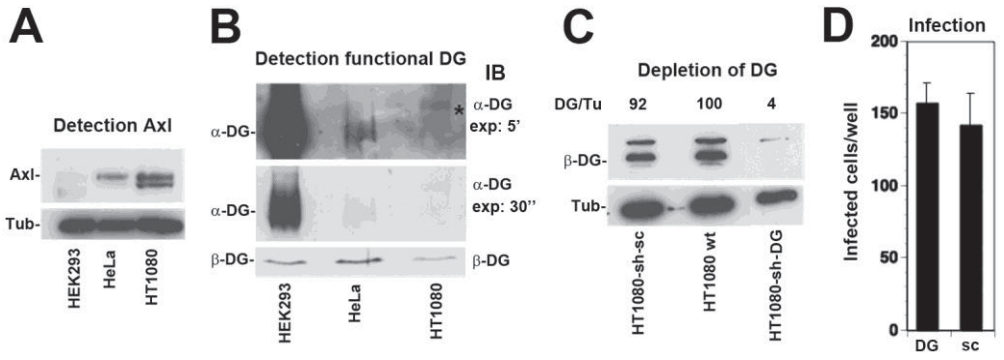

Fig. 1. DG is dispensable for infection of rLCMV-LASVGP in HT1080 cells. (A) Detection of Axl in HT1080 cells. HT1080, HeLa, and HEK293H cells were lysed, total proteins separated by SDS-PAGE, and blotted to nitrocellulose. Blots were probed with a polyclonal rabbit antibody to Axl and an HRP-conjugated secondary antibody using enhanced chemiluminescence (ECL) for development. As a loading control, α-tubulin was detected. The positions of Axl and α-tubulin (Tub) are indicated. (B) Detection of functional DG. HT1080, HeLa, and HEK293H cells (5 x 10^6 cells) were lysed and subjected to jacalin affinity purification as described (Rojek et al., 2007b). Lectin-bound proteins were eluted by boiling in reducing SDS-PAGE sample buffer and analyzed in Western-blot (IB) using mAb IIH6 to functionally glycosylated α-DG (Kanagawa et al., 2004) and mAb 8D5 to β-DG. Prolonged exposure time (exp) of blots for glycosylated α-DG revealed a weak, but specific band for HT1080 cells (asterisk) indicating the presence of residual functional DG. The positions of glycosylated α-DG in HeLa and HEK293H, as well as β-DG are indicated. (C) Depletion of DG core protein by RNAi. HT1080 cells were transduced with a lentiviral vector expressing a shRNA targeting human DG (sh-DG) or a scrambled control shRNA that does not target any known human gene (sh-sc). After 48 hours of transfection, transduced cells were subjected to antibiotic selection with puromycin for a total of ten days. Resistant colonies were isolated and expanded. Depletion of DG by RNAi was validated by Western-blot for β-DG. Shown are selected clones for HT1080 cells expressing DG-specific shRNA (HT1080-sh-DG), scrambled shRNA (HT1080-sh-sc), and wild-type HT1080 cells. Beta-DG and α-tubulin (loading control) were detected as in (B). Prolonged exposure of β-DG blots lead to the detection of an unspecific band of circa 50-55 kDa (*). The positions of β-DG and α-tubulin are indicated. The efficiency of DG depletion was quantified by normalization of the β-DG signals with α-tubulin in densitometry. The rate of β-DG/α-tubulin (DG/Tub) in parental HT1080 cells was set as 100. (D) Infection of DG-depleted HT1080 cells with rLCMV-LASVGP. HT1080-sh-DG cells (DG) and HT1080-sh-sc cells (Sc) were infected with rLCMV-LASVGP at multiplicity of 0.1. After 4 hours, 20 mM ammonium chloride was added to prevent secondary infection. At 16 hours post infection, cells were fixed and infection detected by intracellular staining of LCMV NP using mAb 113 combined with a Rhodamine-conjugated secondary antibody as described (Pasqual et al., 2011). Data are total numbers of infected foci per well (means $\pm$ DG, n = 3).

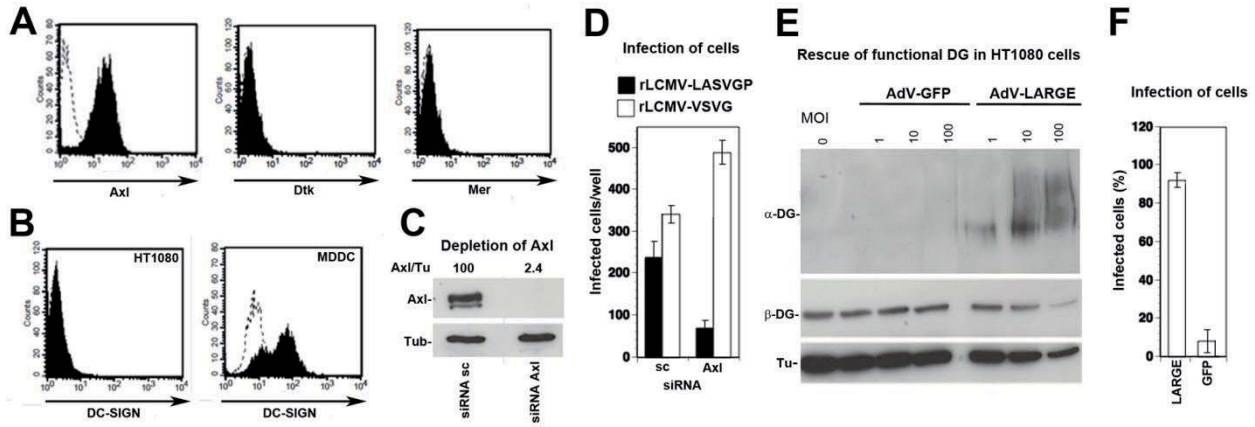

Fig. 2. Detection of the TAM receptors Axl, Mer, and Dtk in HT1080 cells. Live non-permeabilized HT1080 cells were stained with a polyclonal rabbit antibody to Axl, combined with a PE-conjugated secondary antibody in the cold. Dtk and Mer were detected with PE-conjugated mAb to Dtk and mAb to Mer, respectively. Cells were fixed and analyzed by flow cytometry in a FACSCalibur flow cytometer (Becton Dickinson) using the CellQuest Pro® acquisition and analysis software. Empty peaks: secondary antibody only, shaded peaks: primary and secondary antibody. (B) Detection of DC-SIGN in HT1080 cells. Live non-permeabilized HT1080 cells and human monocyte-derived dendritic cells (MDDC) were stained with mAb 120507 to DC-SIGN and a PE-conjugated secondary antibody. Cells were fixed and analyzed by flow cytometry as in (A). Empty peaks: secondary antibody only, shaded peaks: primary and secondary antibody. (C) Depletion of Axl by RNAi. HT1080 cells were transfected with Axl-specific siRNA (siRNA Axl) and a scrambled control RNA (siRNA sc) by reverse transfection as described (Pasqual et al., 2011). After 24 hours, cells were subjected to a second round of transfection with the same siRNAs (forward transfection). At 72 hours after the first transfection, cells were lysed and Axl detected by Western-blot as in 1A, using α-tubulin for normalization. The efficiency of Axl depletion was assessed by normalization of the Axl signals with α-tubulin in densitometry and the rate of Axl/α-tubulin (Axl/Tub) in HT1080 cells transfected with scrambled RNA set at 100. (D) Infection of Axl-depleted cells with rLCMV-LASVGP. HT1080 cells transfected with siRNA to Axl (Axl) or scrambled siRNA (sc) were infected with rLCMV-LASVGP and rLCMV-VSVG at multiplicity of 0.1 and infection detected after 16 hours as in 1D. Data represent total numbers of infected foci per well (means ± DG, n = 3). (E) Rescue of functional DG in HT1080 cells. HT1080 cells were infected with adenoviral vectors expressing GFP (AdV-GFP) or LARGE (AdV-LARGE) at the indicated multiplicities of infection (MOI). After 48 hours, DG was extracted by jacalin affinity purification as in (B). Functionally glycosylated α-DG and β-DG were detected as in 1B. The broad band in cells transfected with AdV-LARGE at MOI = 100 corresponds to glycosylated α-DG. (F) Infection with rLCMV-LASVGP. HT1080 cells were infected with AdV-LARGE and AdV-GFP at MOI = 100 as in (E). After 48 hours, cells were infected with rLCMV-LASVGP at multiplicity of 1 and infection detected after 16 hours as in (D). Shown are percentages of infected cells (means ± DG, n = 3).

Axl-mediated cell entry of rLCMV-LASVGP occurs rapidly

In a next step, we compared the entry kinetics of rLCMV-LASVGP *via* Axl and DG. To assess how fast receptor-bound rLCMV-LASVGP trafficked to late endosomes, we assessed the time required for the viruses to become resistant to ammonium chloride a lysosomotropic agent that raises

the endosomal pH rapidly and prevents low pH-dependent membrane fusion without causing overall cytotoxicity (Ohkuma and Poole, 1978; Ohkuma and Poole, 1981). The virus was bound to HEK293H cells that express functional DG, but lack Axl, and HT1080 cells in the cold, allowing receptor attachment without internalization. Unbound virus was removed and cells rapidly shifted to 37°C to restore membrane mobility. Ammonium chloride was added at different time points post infection and kept throughout the experiment. Cells were fixed and infection assessed by IFA. In line with published data, DG-mediated infection allowed rLCMV-LASVGP to escape from late endosomes after circa 20 minutes (Fig. 3) (Rojek et al., 2008). The kinetics of the infection of HT1080 cells mediated by Axl followed a similar kinetics with escape from the late endosome after only 15-20 minutes. In sum, the data confirm and extend published results by showing that Axl can serve as a cellular receptor in DG-deficient cells. While Axl seems significantly less efficient in mediating LASV cell entry when compared to DG, the two receptors mediate productive infection with similar kinetics.

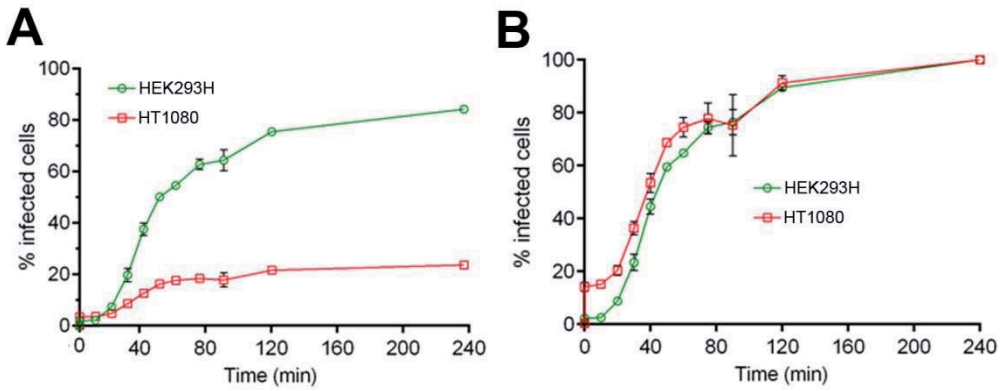

Fig. 3. Entry kinetics of rLCMV-LASVGP in HEK293H and HT1080 cells. (A) Cells were incubated with rLCMV-LASVGP at multiplicity of 3 for one hour in the cold. Unbound virus was removed and cells shifted to 37°C. At the indicated time points, 20 mM ammonium chloride was added and left throughout the experiment. After 16 hours cells were fixed and infection detected by FACS. The percentage of infected cells was plotted against time. Data are triplicates means ± SEM. (B) Data from (A) normalized with the 240 minutes time point set as 100%.

Axl-dependent cell entry of rLCMV-LASVGP resembles macropinocytosis

Next, we sought to characterize the Axl-mediated entry pathway for LASV using our rLCMV-LASVGP chimera in HT1080 cells. To characterize the Axl-dependent LASV entry pathway, we first perturbed clathrin-mediated endocytosis (CME) using chlorpromazine (CPZ), which prevents assembly of clathrin-coated pits at the plasma membrane. As control, we used a recombinant LCMV expressing the glycoprotein of vesicular stomatitis virus (VSV) that enters cells *via* CME (Johannsdottir et al., 2009). Treatment of HT1080 cells with up to 8 µM CPZ did not significantly

affect infection with rLCMV-LASVGP, but diminished infection with rLCMV-VSVG in a dose-dependent manner (Fig. 4A). Next, we addressed the role of dynamin employing the dynamin inhibitor dynasore. As shown in Fig. 4B, infection of rLCMV-LASVGP in HT1080 was blocked by dynasore in a dose-dependent manner, similar to rLCMV-VSVG, which depends on dynamin for cell entry (Johannsdottir et al., 2009). In contrast, infection of rLCMV-LASVGP in HEK293H cells was not inhibited by dynasore, suggesting a specific role of dynamin in LASV entry *via* Axl, but not DG.

To address a possible role of actin in Axl-mediated LASV cell entry, HT1080 and HEK293H cells were treated with cytochalasin B or latrunculin A, which disrupt actin fibers, as well as jasplakinolide, an actin-polymer stabilizing drug that blocks the dynamics of actin filaments. Staining of actin fibers in drug-treated HT1080 cells with fluorescence-labeled phalloidin revealed the characteristic disruption of F-actin by cytochalasin B or latrunculin A, whereas jasplakinolide reduced phalloidin staining, as expected (Fig. 4C). Drug treatment did not affect cell viability as assessed by Cell titer Glo® assay (Fig. 4D). Perturbation of actin by all three inhibitors significantly reduced infection of rLCMV-LASVGP in HT1080 cells, whereas DG-mediated infection in HEK293H cells was unaffected.

The marked actin- and dynamin-dependence of Axl-mediated LASV provided first hints towards a possible role of macropinocytosis. To address this possibility, HT1080 cells were treated with ethylisopropyl amiloride (EIPA), an inhibitor of Na^+/H^+ exchangers (Mercer and Helenius, 2009). Pre-treatment with EIPA reduced infection with rLCMV-LASVGP, but did not affect infection with a recombinant Murine Mooney leukemia virus (MMLV) pseudotypes bearing an amphotropic envelope that mediated fusion at the cell membrane (Fig. 4F). Using the inhibitor IPA-3, we blocked PAK1, which plays a central role in macropinocytosis of some viruses (Mercer and Helenius, 2008). As shown in Fig. 4G, IPA-3 had no significant effects on infection with rLCMV-LASVGP, but significantly reduced infection with vaccinia virus, in line with published data (Mercer and Helenius, 2008). Although by no means comprehensive, our first characterization of Axl-dependent rLCMV-LASVGP entry revealed the involvement of a pathway that shares some characteristics of macropinocytosis (Mercer and Helenius, 2009; Mercer, Schelhaas, and Helenius, 2010) and is strikingly different from DG-mediated entry (Rojek et al., 2008).

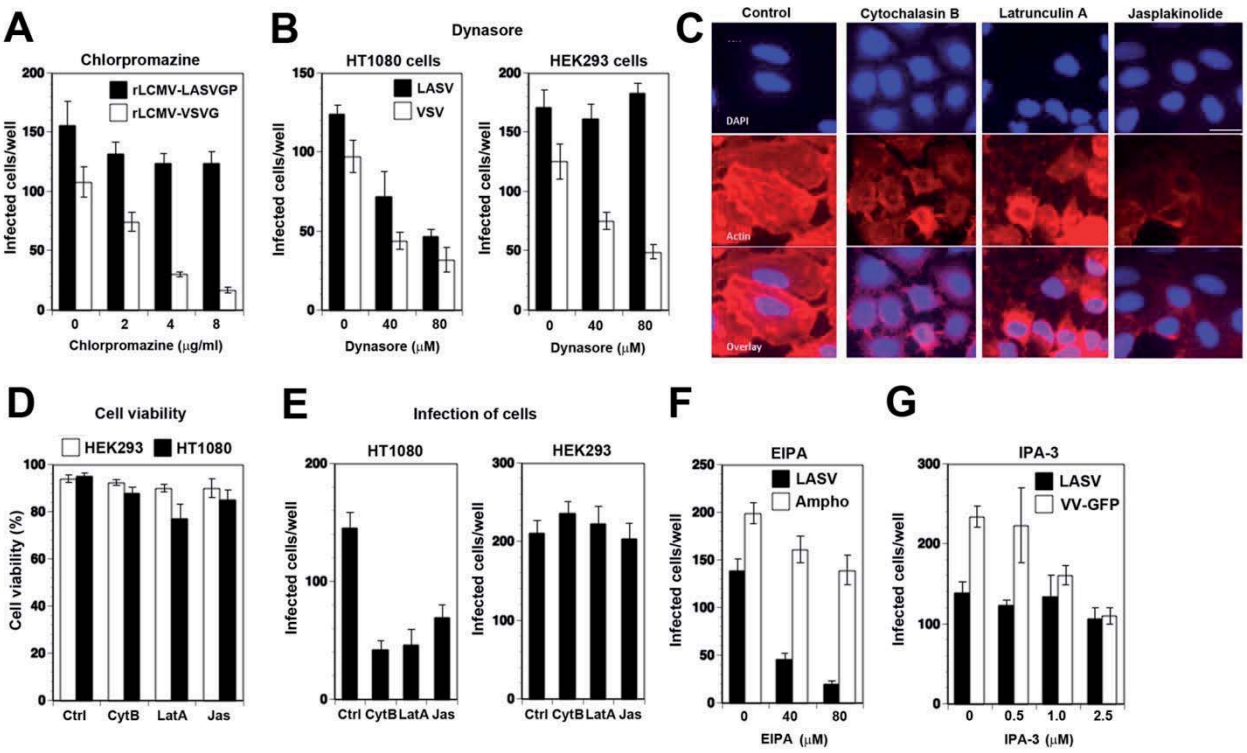

Fig. 4. Characterization of the Axl-associated cell entry pathway of rLCMV-LASVGP in HT1080 cells. (A) Effect of CPZ on the infection of rLCMV-LASVGP. HT1080 cells were treated with the indicated concentrations of CPZ or PBS only (0) for one hour, followed by infection with rLCMV-LASVGP or rLCMV-VSVG at a multiplicity of 0.01. After 4 hours, 20 mM ammonium chloride were added to prevent secondary infection, and infected cells detected after 16 hours by IFA as in 1D (means ± SEM, n = 3). (B) Blocking of infection of rLCMV-LASVGP with dynasore. HT1080 and HEK293H cells were pre-treated with the indicated concentrations of dynasore or vehicle control (0), followed by infection with rLCMV-LASVGP (LASV) or rLCMV-VSVG (VSV). Infection was detected after 16 hours as in (A) (means ± SEM, n = 3). (C) Treatment of HT1080 cells with actin inhibitors. HT1080 cells were treated with cytochalasin B (20 μM), latrunculin A (5 μM), and jasplakinolide (1 μM) for 30 minutes. Cells were fixed and permeabilized. F-actin was stained with Rhodamine-conjugated phalloidin (red) and nuclei counter-stained with DAPI (blue). Please note the reduced phalloidin staining in cells treated with jasplakinolide due to a competition of the drug for the phalloidin binding site. Bar = 20 μm. (D) Cytotoxicity of actin inhibitors. HT1080 cells were treated with 20 μM cytochalasin D (Cyto), 5 μM latrunculin A (Lat), and 1 μM jasplakinolide (Jas), or solvent control (Ctrl) for 4 hours, followed by a wash out and incubation for a total of 16 hours. Cell viability was assessed by Cell Titer Glo® assay according to the manufacturer's recommendations. Data are triplicates ± SD. (E) Axl-mediated infection of rLCMV-LASVGP in HT1080 cells is actin-dependent. HT1080 and HEK293H cells were treated with 20 μM cytochalasin D (Cyto), 5 μM latrunculin A (Lat), and 1 μM jasplakinolide (Jas), or solvent control (Ctrl) for 30 minutes, followed by infection with rLCMV-LASVGP. After 4 hours, drugs were washed out and fresh medium containing 20 mM ammonium chloride added. Infection was detected after a total of 16 hours post-infection as in (A) (means ± SEM, n = 3). (F) Inhibition of rLCMV-LASVGP infection in HT1080 cells by EIPA. HT1080 cells were pre-treated with the indicated concentrations of EIPA or vehicle control (0) for 30 minutes, prior to infection with rLCMV-LASVGP as in (A) (means ± SEM, n = 3). As a control, we employed a recombinant Moloney murine leukemia virus expressing an Amphotropic (Ampho) envelope and a GFP reporter in its genome generated in the Retro-X^{TM} Universal Packaging System (Clontech) as described (Rojek, Spiropoulou,

and Kunz, 2006). Infection by the Ampho retrovirus was detected by direct fluorescence at 24 hours post infection. (G) Effect of the PAK1 inhibitor IPA-3 on infection of rLCMV-LASVGP. HT1080 cells were pre-treated with the indicated concentration of IPA-3 or vehicle control (0) for 30 minutes, followed by infection with rLCMV-LASVGP (LASV) or recombinant vaccinia virus expression GPF (VV) (Mercer and Helenius, 2008). Infection with rLCMV-LASVGP was assessed as in (A) and infection with VV by detection of the GFP reporter in direct fluorescence microscopy at 12 hours post infection (means $\pm$ SEM, n = 3).

DISCUSSION

In line with previous studies (Shimojima et al., 2012), our results confirmed the TAM receptor Axl as an entry receptor for LASV in absence of DG. However, the molecular mechanisms of Axl recognition by LASV are largely unknown. Recently, a general model for cell entry of enveloped viruses *via* TAM receptors has been proposed based on the concept of "apoptotic mimicry". According to this model, viral entry *via* Axl critically depends on PS of the viral envelope, which is bound by the serum proteins Gas6 or ProS that provide a bridge to cellular TAM receptors (Morizono et al., 2011). Previous studies detected PS in the envelope of the Clade A New World arenavirus Pichinde (Soares, King, and Thorpe, 2008), and studies in our laboratory revealed that virions of LCMV and rLCMV-LASVGP also display PS at their surface (Moraz et al., unpublished results). Current efforts in our laboratory investigate the exact role of virus-derived PS, Gas6, and proS in Axl mediated cell entry of LASV and Old World arenaviruses in general. Alternatively, LASV GP may be able to directly bind to cellular Axl, a possibility we are likewise testing.

Previous work on cell entry of the Old World arenaviruses, revealed that these viruses use an unusual pathway to invade the host cell. Upon DG binding, LASV is internalized *via* a cholesterol-dependent endocytic pathway that seems independent of classical regulatory proteins like clathrin, caveolin, and dynamin and apparently bypasses the classical Rab5-dependent incoming pathways of vesicular trafficking, suggesting an unusual way of delivery to late endosomes (Quirin et al., 2008; Rojek, Perez, and Kunz, 2008; Rojek et al., 2008; Vela et al., 2007). Here we performed an initial characterization of the LASV entry pathway linked to Axl. We found that Axl-mediated cell entry of rLCMV-LASVGP was clathrin-independent, but critically depended on actin and dynamin. Combined with the exquisite sensitivity to EIPA, these initial findings suggest the involvement of a macropinocytosis-like pathway that has recently been implicated in cell entry of several viruses, including poxviruses and filoviruses (Mercer and Helenius, 2009; Mercer, Schelhaas, and Helenius, 2010). Comparison of the entry kinetics of rLCMV-LASVGP *via* DG or Axl revealed similar rapid escape of the virus from the late endosome after less than 20 minutes. Rescue of functional DG in HT1080 cells revealed much higher efficiency of cell entry of rLCMV-LASVGP when compared to Axl. This suggests that in cells co-expressing the two receptors, like e.g. epithelial or endothelial cells, LASV cell entry occurs predominantly *via* DG.

A hallmark of LASV infection in most cell types is the inability of the infected host cell to induce a type I interferon (IFN) response. One reason for this is the ability of the arenavirus NP to counteract the activation of the IFN regulatory factor (IRF)-3 (Martinez-Sobrido et al., 2009; Martinez-Sobrido et al., 2006) and NF-κB (Rodrigo et al., 2012). The NP contains a 3'-5' exonuclease

activity that is linked to its immunosuppressive activity (Hastie et al., 2011; Qi et al., 2010) and targets the non-canonical interferon regulatory factor-activating kinase IKKε (Pythoud et al., 2012). Since the viral NP acts as an IFN antagonist in the cytoplasm, the suppression of IFN production by NP depends on productive viral infection with expression of sufficiently high levels of NP. During viral entry, the virus is located in extracellular space where NP cannot act as an IFN antagonist. It is thus possible that incoming arenaviruses could be detected by pathogen recognition receptors (PRRs) located in endosomal compartments, e.g. Toll-like receptors (TLRs). Upon DG binding, Old World arenaviruses are internalized *via* a pathway of endocytosis that bypasses the early endosome where PRRs like TLRs localized (Rojek et al., 2008), possibly allowing the virus to evade innate detection. It will be of interest to investigate if LASV entry *via* Axl likewise allows the virus to evade innate detection and if its entry *via* Axl results in innate detection with the consequent induction of IRF3 and NF-κB or not.

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MATERIALS AND METHODS

Cell lines and viruses

HT1080, HEK293H and A549 cells were cultured in DMEM, 10 % (vol/vol) FBS, supplemented with penicillin/streptomycin. The recombinant virus rLCMV-LASVGP and rLCMV-VSVG have been described (Pinschewer et al., 2003; Rojek et al., 2008). Viruses were produced and titers determined as previously described (Dutko and Oldstone, 1983). Recombinant vaccinia virus expressing GFP (VV-GFP) was kindly provided by Jason Mercer and produced as described (Mercer and Helenius, 2008). A recombinant Moloney murine leukemia virus expressing an Amphotropic envelope was generated as reported (Rojek, Spiropoulou, and Kunz, 2006). Recombinant adenoviral vector (AdV)-Ad5-LARGE-enhanced green fluorescent protein (EGFP) and (AdV)-Ad5-EGFP have been described and were produced and titered as previously defined (Barresi et al., 2004).

Virus infection

For virus infection, 5 x 10^4 HT1080 cells per well were seeded in 96-well microtiter plates and cultured overnight. For infection of cells with rLCMV-LASVGP and rLCMV-VSVG, seed stocks were diluted to the indicated MOI and added to cells for 1 hour at 37°C. After 1 hour of incubation, the inoculum was removed; cells were washed once with serum-free medium and replaced with normal medium. To prevent secondary infection, 20 mM ammonium chloride (NH_4Cl) was added to the cells 4 hours post-infection. Cells were fixed at 16 hours post-infection and infected cells quantified by immunofluorescence assay (IFA) for LCMV NP using mAb 113 combined with fluorescence-labeled secondary antibodies (Kunz et al., 2004).

Antibodies and reagents

Monoclonal antibody (mAb) 113 (anti-LCMVNP) and mAb clone 83.6 (anti-LCMVGP) have been previously described (Buchmeier et al., 1981; Weber and Buchmeier, 1988). The mAb IIH6 anti-□-DG was used to detect functional DG (Ervasti and Campbell, 1991). To detect the DG core protein, the mouse mAb anti-β-DG clone 56 from BD Biosciences was used (NJ, USA). TAM kinases were detected using the polyclonal goat anti-Axl Ab, anti-Dtk and anti-Mer PE-conjugated mouse mAbs from R&D Systems® (AF154, FAB859P, FAB8912P, MN, USA). DC-SIGN was detected using the mAb DCN46 anti-CD209 (DC-SIGN)-PE purchased from BD Pharmingen. Mouse mAb anti-α-tubulin was purchased from Sigma-Aldrich (MO, USA). The rabbit polyclonal anti-Adenovirus Type 5 was purchased from Abcam® (ab6982, UK) and directed against all capsid proteins. To detect Vaccinia virus (VV), an anti-A27L viral protein rabbit polyclonal antibody was used from LifeSpan BioSciences (Cat. No. LS-C19415, WA, USA). Horseradish peroxidase (HRP) conjugated polyclonal rabbit secondary antibodies anti-goat and anti-mouse IgG were purchased from Dako (P0449, P0260,

(Glostrup, Denmark). The HRP-conjugated polyclonal goat anti-mouse IgM antibody was purchased from Thermo Scientific (PA1-85999). Rhodamine Red-X-AffiniPure goat anti-mouse IgG from Jackson ImmunoResearch (EU) and Alexa Fluor 488 goat anti-mouse IgG2b were from Molecular Probes (A11017, Eugene, OR). Streptavidin HRP-conjugated secondary antibody was used to detect biotinylated annexin-V (ANX5) purchased from Thermo Fisher Scientific Inc. Dharmacon (Lafayette, CO).

Cytochalasin B, jasplakinolide, latrunculin A, Phalloidin-FITC, chlorpromazine hydrochloride, dynasore hydrate, p21-activated kinase (PAK)-1 inhibitor 2,2'-dihydroxy-1, 1'-dinaphthyldisulfide (IPA-3) and 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) were purchased form Sigma-Aldrich. LIVE/DEAD® fixable dead cell stain kit was from Molecular probes® Invitrogen. The annexin-V-Biotin conjugated from Roche-applied-science (Cat. No. 11 828 690 001) was used to detect the phosphatidylserine on purified viral particles. HT1080 cells transduced with lentiviral vectors were then selected using the Puromycin antibiotic from Calbiochem® (Merck Millipore, MA, USA). For all experiments, HT1080 cells were plated in poly-L-lysine-coated wells purchased from Sigma-Aldrich® (P8920).

Lentivirus shRNA production and transduction of HT1080 cells

To deplete DG core protein in HT1080 cells, lentiviral vectors expressing validated shRNA targeting human DG (sh-DG) (ID clone: TRCN0000056191, Thermo Scientific RHS3979-9623375) or a scrambled control shRNA (sh-sc) were generated following the manufacturers recommendations. Briefly, 3 x 10^6 HEK293T cells were cultured in 10-cm-diameter dishes in serum-free 293 SFM II medium (Gibco™, Cat. No. 11686-029). At 24 hours post-seeding, fresh medium was added to cells 4 hours before the transfection. $CaCl_2$ (250 mM $CaCl_2$, ultrapure H_2O) and HBS (50 mM Hepes, 1.5 mM Na_2HPO_4, 140 mM NaCl, ultrapure H_2O) solutions were used to co-transfect the four required plasmids: pLP1 helper (gag/ pol), pLP2 helper (rev), pCAGGS/ VSVGP, with sh-DG 56191 (human pLKO.1) or with sh-sc (human pLKO.1) shRNA control. Transfected cells were incubated for16 hours at 37°C, 5% CO_2. After 16 hours, the transfection medium was replaced by fresh serum-free 293 SFM II medium and cells incubated for circa 24 hours more at 37°C, 5% CO_2. Circa 40 hours post-transfection, cell supernatants were collected and centrifuged for 5 min at 500 g to remove cellular debris. Each lentivirus was concentrated using the Amicon® Ultra-15 Centrifugal Filter Devices by ultra-centrifugation according to the manufacturer's instructions (Millipore, Ultracel® 100KREF. UFC910024). Then, 5 x 10^5 HT1080 cells/well were seeded in 6-well plate format and cultured for 24 hours at 37°C, 5% CO_2. The day after, cell medium was replaced by classical medium supplemented with [6 µg/ml] Polybrene® in which 100 µl of each crude lentivirus stock was added. The cells were spinoculated at 2600 rpm for 3 hours at 23°C. Cells were then washed twice with supplemented classical medium to remover Polybrene®. The transduced cells were then incubated for 72 hours at

37°C, 5% CO_2. After 72 hours, cells were washed once with 1 x PBS and [2 µg/ml] puromycin selective medium added. After 48 hours, cell death was checked and the selective medium replaced each two days by fresh one during all the selective process. Depletion of DG was verified by Western-blot detecting the β-DG core protein.

Immunoblotting

Proteins were separated by gel electrophoresis using 6% or 8% polyacrylamide gels and transferred to nitrocellulose. After blocking in 3% (wt/vol) skim milk in PBS, membranes were incubated with primary Abs used at 5-10 µg/ml in 3% (wt/vol) skim milk in PBS for 1 hour at room temperature. After several washes in PBS, 0.1 % (wt/vol) Tween, secondary Abs coupled to HRP were applied 1:6000 in PBS, 0.1% (wt/vol) Tween for 1 hour at room temperature. Blots were developed by enhanced chemiluminescence (ECL) using Super Signal West Pico ECL Substrate or TrueBlot® detection system (Pierce).

Rescue of functional DG in HT1080 cells

Functional DG was rescued in HT1080 cells by over-expression of LARGE using recombinant adenoviral vector (AdV)-Ad5-LARGE-EGFP and (AdV)-Ad5-EGFP as control (Barresi et al., 2004). Briefly, 3 x 10^5 HT1080 and A549 cells per well were seeded in poly-L lysine-coated 6-well plate format and incubated for 24 hours at 37°C, 5% CO_2. At 24 hours post-seeding, AdV-LARGE and AdV-EGFP vectors were added to cells at indicated MOI and incubated for 4 hours at 37°C, 5% CO_2. 4 hours post-transduction, inoculums were removed, cells washed with fresh normal medium and incubated overnight at 37°C, 5% CO_2. Twenty-four hours post-transduction, transduced cells were re-seeded in 96-well plate format at 5 x 10^4 cells/well. At 48 hours post-transduction, cells were either infected with rLCMV-LASVGP at MOI = 1 and infection quantified by detection of LCMV NP in IFA, or lysed to extract functional DG using Jacalin affinity purification as described (Rojek et al., 2007b). Lectin-bound proteins were then subjected to SDS-PAGE and Western-blotting using mAb IIH6 to functionally glycosylated α-DG (Kanagawa et al., 2004) and mAb anti-β-DG clone 56 to β-DG core protein.

Flow cytometry analysis

For extracellular staining with enzyme-free cell dissociation solution, resuspended in FACS buffer (1% (vol/vol) FCS, 0.1% (wt/vol) sodium azide, PBS), and plated in conical 96-well plates, followed by one hour on ice with FACS buffer diluted with corresponding primary antibody. Cells were then washed twice in FACS buffer and labeled with secondary antibodies (as needed) for 1 hour on ice in the dark. After two wash-steps in 1% (vol/vol) FBS in PBS, cells were fixed with 1:10 CellFix® solution for 10 minutes at room temperature. Cells were washed twice with PBS, and fluorescence

intensity assessed using a FACS Calibur flow cytometer (Becton Dickinson) using the CellQuest Pro® acquisition and analysis software. Intracellular FACS staining of LCMV NP antigen was performed as described (Rojek et al., 2007a).

RNA interference (RNAi)

RNA interference (RNAi) was performed using validated small interfering RNAs (siRNAs) ON-TARGETplus SMARTpool for Axl (L-003104-00-0005) and scrambled siRNA (D-001820-10-05) as control from Thermo Scientific Dharmacon (Lafayette, CO). Briefly, 3 x 10^6 HT1080 cells were reverse transfected with 0.72 μM siRNA using a10-cm-diameter dish and Lipofectamine RNAiMAX (Invitrogen, Paisley, United Kingdom) according to the manufacturer's recommendation. Twenty-four hours after transfection, cells were replated in 96-well plate format, and 48 hours post-transfection, cells were infected with rLCMV-LASVGP (MOI = 0.1) and rLCMV-VSVG (MOI = 0.02) or mock infected or lysed to confirm by Western-blotting Axl knock-down using specific antibodies. To prevent secondary infection, 20 mM ammonium chloride (NH_4Cl) was added to the cells 4 hours post-infection. Cells were fixed 16 hours post-infection and infected cells quantified by immunofluorescence assay (IFA) detection of LCMV NP using mAb 113 (anti- LCMVNP) and combined with fluorescence-labeled secondary antibody.

Inhibitor studies in HT1080 cells

HT1080 cells were seeded in round bottom 96-well plates (Costar) pretreated for 2 hours in the presence of chlorpromazine or for 30 minutes in presence of the actin inhibitors cytochalasin B, latrunculin A, and jasplakinolide, dynasore, EIPA, and IPA-3 at the indicated concentrations at 37°C. Cells were then infected in the presence of inhibitors for 1 hour with rLCMV-LASVGP at 37°C. At 4 hours post-infection, 20 mM NH_4Cl was added to prevent secondary infection. Infection was detected 16 hours post-infection by IFA as mentioned above. The *via*bility of drug-treated cells was determined by staining of single-cell preparations with Live and dead staining.

Entry kinetics of rLCMV-LASVGP in HT1080 cells

The kinetics of rLCMV-LASVGP cell entry by NH_4Cl treatment was performed as described (Rojek et al., 2008). Briefly, 3 x 10^5 HEK293 and HT1080 cells per well were seeded in 24-well plate format coated with poly-L-lysine and incubated for 24 hours. The day after, seeded cells were incubated on ice for 30 min and fresh ice cold medium containing rLCMV-LASVGP (MOI 3; 10) was added and cells incubated on ice for 1 hour to allow virus attachment. Unbound virus was removed by washing with cold medium. Pre-warmed complete medium was added and cells rapidly shifted to 37°C at 5% CO_2. 20 mM NH_4Cl was added at 0, 10, 20, 30, 40, 50, 60, 75, 90, 120 and 240 min. After 16 hours,

rLCMV-LASVGP infection was quantified by intracellular staining of the viral proteins by flow cytometry as described above.

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Diagnostic activities: Serology: Detection of anti-LCMV antibodies in patient serum

BACKGROUND AND RATIONALE

The worldwide distributed prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) is a neglected human pathogen of clinical relevance (Barton and Mets, 1999; Barton, Mets, and Beauchamp, 2002; Bonthius, 2009). In adult immunocompetent individuals, LCMV causes aseptic meningitis and encephalitis, which is usually self-limiting and rarely leads to complications. However, LCMV is a severe human pathogen in cases of congenital infection. LCMV infection during the first trimester of pregnancy is associated with an increased risk of spontaneous abortion. Infection during the second and third trimester has been linked to severe brain abnormalities including hydrocephalus, psychomotor retardation, macrocephaly or microcephaly, and chorioretinitis. In addition, LCMV poses special threat to immuno-compromised individuals, as tragically illustrated by recent cases of transplant-associated infections by LCMV with fatal outcome (Fischer et al., 2006; Palacios et al., 2008). Immunofluorescence assay (IFA) is currently the preferred serological method for the diagnosis of human arenavirus infections, including LCMV. However, this method is time consuming, labour intensive, and the interpretation of results requires significant experience in the field. Over past years, faster and reliable serological tests based on enzyme-linked immunosorbent assay (ELISA) have been developed for other arenaviruses, in particular Lassa virus (Bausch et al., 2000; Emmerich et al., 2006; Saijo et al., 2007) and Junin virus (Ure et al., 2008). ELISA-based tests for Lassa virus have recently been successfully used for serological survey of Lassa in Africa, proving the power of this method (Emmerich, Gunther, and Schmitz, 2008). Recent efforts by veterinary research centers in Japan resulted in the development of an ELISA for the detection of LCMV antibodies in sera of mice, hamsters, cotton rats (*Mastomys sp.*), and gerbils, which has however not yet been licensed (Takimoto et al., 2008).

STUDIES AND RESULTS

During my thesis, I participated to diagnostic activities concerning the serology of LCMV in serum of hospitalized newborns (mainly TORCH negative), young children and mothers (CHUV). I was in charge to detect anti-LCMV antibodies in the patient serum using IFA. A few days after primary LCMV infection in patients, IgM directed against LCMV NP and to a lesser extent GP can be detected. A few weeks after the primary IgM response, anti-viral IgG against NP and GP become detectable. Specific antibody titers in early acute infection (mainly IgM) should be at least 4-fold over the background control and in convalescent patients or late infection (IgM + IgG), at leat 10-fold over background. However, in each case, the observation of a significant above-background signal in IFA

was hardly detectable. Even with our experience in the field, no significant above-background signal in IFA was detected in any of our patient's samples.

To circumvent this lack of sensitivity and specificity, we contributed to the elaboration of a specific LCMV real-time RT-PCR assay, based on the detection of genomic sequences of the viral nucleoprotein NP to assess the presence of LCMV in cerebrospinal fluids (CSF). This work was done in collaboration with the diagnostic platform from the CHUV and the Swiss National Reference Center for Emerging Viral Diseases from the University of Geneva. Collaborators from Geneva validated the specificity and sensitivity of the real-time RT-PCR assay for the diagnosis of LCMV infections. First results showed that LCMV infections are extremely rare in hospitalized patients Western in Switzerland. The results of these studies have been published in: Cordey S., Sahli R., **Moraz M.L.**, Estrade C., Morandi L., Cherpillod P., Charrel R..N., Kunz S., and Kaiser L. (2011) Analytical validation of a lymphocytic choriomeningitis virus real-time RT-PCR assay. *J. Virol. Methods. 177(1):118-22.*

In addition, I have been involved in the development of an ELISA-based test for the detection of antibodies to LCMV in human sera using recombinant LCMV NP produced in insect cells. This has been established and optimized and first quality control experiments revealed a high degree of sensitivity and specificity. This novel ELISA-based test for serology of LCMV will be applicable in human medicine and epidemiological studies to investigate the seroprevalence of LCMV in patient groups of interest.

PERSPECTIVES

Project 1: "Post-translational modification of the LASV receptor DG"

Binding of pathogens to their receptors often induces signaling that facilitates the entry process into the host cell. The entry step is a promising target for therapeutic intervention against viruses before they are able to take control over the host cell. The molecular mechanisms underlying this fundamental aspect of virus-host cell interaction are therefore of particular interest from a basic science standpoint as well as for translational research aiming at the development of novel anti-viral therapeutics. Research over the past decades revealed that viruses can hijack virtually all known existing pathways of endocytosis that are used in the cell for transport of proteins, lipids and other cargo (Mercer and Helenius, 2010). The study of pathogen entry greatly contributed to our current understanding of these fundamental cellular processes. Interestingly, in recent years, evidence started to accumulate that, in addition to using existing pathways, pathogens can manipulate the host cell's endocytotic machinery in order to "create" their own pathways of entry specifically tailored to their needs. This novel concept in pathogen-host cell interaction is illustrated by several lines of evidence, including recent work on the toxin of the bacterium *Bacillus anthracis,* the causative agent of anthrax (Abrami et al., 2010; Abrami, Kunz, and van der Goot, 2010). Previous work on cell entry of the Old World arenaviruses, including the studies performed in the context of my thesis revealed that these viruses likewise use an unusual pathway to invade the host cell. Upon DG binding, LASV is internalized via a cholesterol-dependent endocytic pathway that seems independent of regulatory proteins like clathrin, caveolin, and dynamin and apparently bypasses the classical Rab5-dependent incoming pathways of vesicular trafficking, suggesting an unusual way of delivery to late endosomes (Quirin et al., 2008; Rojek, Perez, and Kunz, 2008; Rojek et al., 2008; Vela et al., 2007). DG is well known for its function as a molecular bridge between the ECM and the actin cytoskeleton of the cell. This apparently static role as a structural component of cell-matrix contacts seems to contrast with the dynamics observed upon the engagement of LASV with DG. Indeed, the binding of LASV to DG results in a fast endocytosis of the virus-receptor complex, followed by targeting to the multivesicular endosome, where the complex undergoes sorting by the endosomal sorting complex required for transport (ESCRT), and is delivered to late endosome within circa 20 minutes (Pasqual et al., 2011; Rojek et al., 2008). Considering the experimental evidence at hand, we hypothesize that virus binding may induce receptor-mediated signaling resulting in subsequent internalization of the virus-receptor complex *via* either an existing pathway linked to degradation of DG or a novel pathway "created" *de novo* by the pathogen, which does not exist in uninfected cells. The virus-induced signaling events uncovered in my work may serve as a "knock on the door" resulting in the modification and assembly of receptor-associated proteins that form the functional receptor complex that mediates viral entry. In follow-up studies to my thesis, I propose two lines of further research: 1) the identification of DG-

associated cellular proteins required for LASV cell entry and 2) the investigation of cellular signaling pathways involved in the regulation of DG-mediated LASV cell entry.

Identification of DG-associated cellular proteins involved in viral entry

In our studies, we provide evidence that LASV binding to DG affects receptor signaling and its interaction with cellular proteins, illustrated by the observed virus-induced tyrosine phosphorylation of DG's cytoplasmic domain and the dissociation of DG from utrophin. We hypothesize that this virus-induced-detachment of virus-bound DG from the actin cytoskeleton, in a direct or indirect way, facilitates the subsequent endocytosis of the virus-receptor complex. So far, our studies focused mainly on the post-translational modification of DG itself. However, in most cell types, DG is associated with several other cellular proteins forming molecular complexes that likely represent the functional "receptor units" for the attachment and internalization of arenaviruses (Barresi and Campbell, 2006). In some cell types, DG is found associated with the sarcoglycans (Yoshida et al., 1994), a family of transmembrane proteins that play important roles in the stability of the DG complex. Abnormalities of sarcoglycans are a common molecular feature in several muscular dystrophies (Bonnemann, McNally, and Kunkel, 1996; Campbell, 1995). Thus, it will be interesting to address the role of sarcoglycans and other DG-associated proteins in LASV cell entry. It is conceivable that specific DG-associated host cell proteins may function as co-receptors or auxiliary factors in the viral entry process. Considering the multivalent nature of the virion particles and the experimental evidence at hand, attachment of LASV to cellular DG likely results in receptor clustering and induces receptor-mediated signal transduction. In addition to DG, virus binding may affect post-translational modification of other cellular proteins that comprise the functional DG complex and modulate their interactions. We expect that during LASV attachment and entry, the composition of the virus-associated DG complex may change in a dynamic manner.

In the context of human LASV infection, epithelial cells of the lung represent the first barrier for aerosol transmission and are the initial site of replication. Efficient productive infection of lung epithelium is likely crucial for the subsequent invasion of the lymphatic system and the bloodstream resulting in dissemination of the virus to lymphatic organs and other sites of secondary replication, like the liver, adrenal gland, and vascular endothelium. Our initial studies on LASV-receptor interactions and signaling, as well as endocytosis focused therefore on human lung epithelial cells (Pasqual et al., 2011; Rojek et al., 2012). Many of the DG-associated molecules found in skeletal muscle are also expressed in other tissues, including most sarcoglycans, sarcospan, synthophins and several dystrophin isoforms (Crosbie et al., 1997; Grady, Merlie, and Sanes, 1997; James et al., 1996; Jung et al., 1996; Peters, Adams, and Froehner, 1997; Vainzof et al., 1996). This broad pattern of expression suggests that distinct DG complexes may occur in different tissues,

compatible with the possible roles of DG outside of skeletal muscle. A previous study investigating the DG complexes in epithelial cells from lung and kidney revealed that the "epithelial" DG complexes apparently lack sarcoglycans, suggesting a distinct biochemical composition when compared to the classical DG complex from muscle (Durbeej and Campbell, 1999). As a first step, I propose to define the cellular binding partners or the "interactome" of DG in human lung epithelial cells.

Conventional affinity purification protocols used in the past (Durbeej and Campbell, 1999) were limited to the detection of known interacting partners of DG and thus represented "closed systems" approaches. Moreover, the stringent washing conditions used restricted the analysis to components that bind DG with relative high affinity bearing the risk of losing more transient interaction partners. In contrast to stably associated proteins, more transiently interacting proteins may associate with and dissociate from DG and in response to virus-induced signals. Upon LASV binding and virus-induced receptor clustering, such transiently interacting proteins may be recruited to or dissociate from the DG complex in a dynamic manner. To characterize the dynamic DG "interactome" during the process of LASV cell entry in a comprehensive manner, I propose the use of a novel sensitive technology of proteomic analysis based on quantitative mass spectroscopy involving stable isotope labeling by amino acids in cell culture (SILAC). The SILAC system is a straightforward approach for *in vivo* incorporation of a label into proteins for mass spectrometry (MS)-based quantitative proteomics (for more details see *http://silac.org/index_html*). SILAC combined with quantitative MS has recently been used to identify dynamic components of multiprotein complexes (Wang and Huang, 2008). In a first step, we will define the DG "interactome" in resting lung epithelial cells, prior to addition of virus. For this purpose, human lung epithelial cells will be stable transfected with DG containing a C-terminal HA-tag separated by a flexible spacer to prevent interference with interacting partners. Cells will be differentially labeled using media supplemented with amino acids containing heavy isotopes (e.g. Arg13C615N4 or Lys13C615N2) or normal medium. DG complexes will be isolated by immunoprecipitation (IP) with a mAb to HA or an isotype control. IP conditions should be optimized to allow the detection of weaker and more transient interactions. After washing, IPs will be combined and isolated proteins analyzed by MS. Due to the differential labeling by SILAC, unspecifically bound proteins can be subtracted, minimizing the background "noise" of the method. After setting the "baseline" with the identification of the composition of the DG complex in uninfected cells, we will look at possible changes in the composition of DG complexes in cells subjected to attachment of rLCMV-LASVGP. For this purpose, SILAC labeled and unlabelled cells will be exposed to rLCMV-LASVGP at a virus/particle ratio of 100 in the cold, to allow receptor binding without signaling and internalization. Unbound virus will be removed and cells rapidly shifted to 37°C. At different time points, cells will be chilled on ice, lysed and IP performed with anti-HA (heavy amino acid labeled

cells) or isotype controls (normal medium). Differential MS analysis will be performed for each time point and specifically bound proteins for the distinct points compared to identify candidates whose association with DG is altered upon viral attachment and/or during entry. Candidate proteins that are found to be specifically associated with DG at any point of viral attachment and internalization will then be tested for their role in viral entry. For this purpose, candidate proteins will be depleted by RNA interference (RNAi) using specific siRNAs and effects on early infection tested by infection with rLCMV-LASVGP. We expect that some of the DG-associated proteins will affect the stability of the DG complex, whereas others may be specifically required for viral entry but not expression of functional DG *per se*. It will be of particular interest to see if virus binding results in the recruitment of cellular proteins that are not normally associated with DG and that may play a role in the pathway that the virus "creates" to invade human cells.

Detection of changes in protein phosphorylation during LASV entry

We and others showed that treatment of cells with the broadly-specific tyrosine kinase inhibitor genistein affects LASV cell entry into at an early step of virus internalization without affecting the binding. Since tyrosine phosphorylation of β-DG at Y892 by src family kinases has previously been linked to the internalization of DG (Miller et al., 2012; Sotgia et al., 2003), we tested the impact of a src family kinase specific inhibitor (PP2) on rLCMV-LASVGP infection. Unexpectedly, the treatment of cells with PP2 had no effect on the entry of LASV, thus excluding phosphorylation at Y892 as an event crucial for entry. Since the broad phosphorylation inhibitor genistein significantly blocks LASV cell entry, we suppose that non-src tyrosine kinases play a role in the entry process of LASV by modifying β-DG and possibly other DG-associated proteins. Specifically, we hypothesize that virus induced-phosphorylation of DG-associated proteins could affect receptor trafficking, possibly targeting the receptor for lysosomal degradation via the MVB/late endosome. In a next step, I propose a comprehensive examination of tyrosine phosphorylation of DG and DG-associated proteins in function of virus-cell attachment and entry. The SILAC technology introduced above has been efficiently used by Olsen and colleagues to study temporal dynamics of signaling pathways by exploiting phosphorylation-based enrichment methods coupled to mass spectrometry (Olsen et al., 2006). This method has been successfully used to perform temporal dynamic studies of the EGFR pathway. In our context, a similar approach can be used to investigate the dynamic phosphorylation state of candidate proteins comprising the "interactome" of the virus-DG complex during the viral entry process (see above). Moreover, by using double-isotope labeling, the SILAC system allows the identification and mapping of novel phosphorylation sites within proteins (Ibarrola et al., 2003). Despite its promise, there are obstacles for the SILAC system, including the culture process that is time consuming, some samples cannot

be obtained through culture processes and some cell types cannot accommodate certain amino acids. Thus, many aspects have to be considered for this type of study.

An alternative approach to the SILAC-based "phosphoproteomic" strategy is provided by antibody arrays that allow the detection of hundreds of phosphorylated motifs in cellular proteins on the basis of immunoblot with specific antibodies. Powerful platforms are the Kinex™ antibody microarrays and the Kinetworks™ multi-immunoblotting systems, provided by Kinexus Inc. The two approaches use phosphorylation site-specific antibodies. In a first step, antibody microarrays are performed with non-denatured, native proteins in lysates to detect phosphorylated candidate proteins. Hits are then validated using Kinetworks™ multi-immunoblotting in which antibody leads from the Kinex™ Antibody microarray can be confirmed using an immunoblot approach. Human lung epithelial cells will be exposed to rLCMV-LASVGP for different time points and cell lysates scanned sequentially by both Kinexus approaches. This approach will allow the detection of changes in phosphorylation of cellular proteins induced by the virus covering major kinases and signaling pathways. Although by no means comprehensive, this screen could identify novel candidate cellular proteins affected by the virus that may play a role in the entry process.

Identification of the tyrosine kinases involved in LASV cell entry

Phosphorylation by cellular protein is crucial for the regulation of cellular signaling cascades and it is known that the binding of pathogens to their receptors often induces cell signaling that facilitate subsequent internalization. A requirement for kinase activity for LASV cell entry had already been shown with broadly active kinase inhibitors (Vela et al., 2008). This requirement has also been shown for the alternative LASV receptor Axl (Shimojima et al., 2012). We confirmed that tyrosine kinases are required for the endocytosis of LASV, but that they are dispensable for the binding of the virus to DG. We demonstrated that the binding of LASV to cellular DG induced phosphorylation of DG at Y892 by src family kinases and other tyrosine residues of β-DG by yet unknown kinases. Cellular kinases specifically required for LASV entry would represent promising targets for therapeutic intervention.

To identify specific cellular candidate kinases implicated in LASV cell entry, several approaches can be used. One of them is the screening available libraries of kinase inhibitors. Screening of such defined kinase libraries may allow to identify cellular kinases required for the early steps of LASV infection and may give us at the same time potential inhibitors of LASV infection with drug-like properties. Candidate compounds showing an effect on LASV entry steps or infection could then be assessed for their mechanism of action and which step of viral infection they perturb. We have already launched in our laboratory the screening of a small library of 80 kinase inhibitors (Screen-Well™, Enzo® Life Sciences). Some interesting hits have been identified

and are currently validated. In a next step, larger libraries may be tested (e.g. InhibitorSelect™ 384-Well Protein Kinase, Millipore). However, we have to keep in mind that we could have off-target effects and toxicity due to the use of inhibitors and adequate dose-response studies as well as necessary controls are imperative for the validation of the hits obtained by screening.

A broad screening technique using RNAi silencing technology can be employed to identify cellular kinases involved in LASV cell entry. Predicted human kinases that are implicated in endocytic pathways will be systematically silenced by specific siRNAs and consequences of the silencing on LASV entry will be tested using infection with rLCMV-LASVGP as readout. To identify kinases specifically affecting LASV GP-mediated cell entry, we will use rLCMV-VSVG for a counter-screen. Only candidates whose silencing affects rLCMV-LASVGP, but not rLCMV-VSVG will be retained. Since the human genome comprises hundreds of known and putative kinases, it will be necessary to focus on kinases already known to play a role in cell entry pathways. Pelkmans and colleagues identified several candidate kinases implicated in clathrin-mediated endocytosis as caveolae/raft endocytosis using high-throughput RNA interference technology (Pelkmans et al., 2005). We could base our study on the library of validated siRNAs characterized by Pelkmans and colleagues.

Another approach to identify candidate cellular kinases implicated in LASV entry is to use correspondent kinases' substrates. To this end, a high-throughput kinase substrate arrays can be applied to perform a systematic analysis of changes in cellular kinase activity induced by LASV binding and entry. Pepscan Inc. developed the PepChip kinase assay (Peptide arrays Pepchip™, *http://www.pepscan.com/presto/products-services/kinase-profiling/*) that is based on arrays of kinase substrates making this tool very potent for our application since it allows the analysis of pathogen-induced changes in cell signaling in response to the infection. We can then compare LASV infected cells versus uninfected ones at different time points. The identification of cellular kinases activated by LASV may also allow pinpointing possible altered signaling pathways upon LASV infection.

Candidate kinases obtained from each approach will be then compared to identify a "short list" of candidates identified by both methods. It will be of interest to compare the kinase candidate list with the signaling pathways identify in our phosphoproteomic studies using SILAC outlined above. Promising candidates will be validated using corresponding inhibitors and dominant negative mutants, if available. If candidate kinases are specifically able to block LASV infection, their role in the different viral entry steps will be investigated, including attachment of the virus to the cell, the endocytosis process, the intracellular transport as well as the fusion process. If an effect is observed, virus-receptor internalization could be then tracked by microscopy in co-localization experiments, using specific antibodies to see which step of the entry pathway is affected. The

identification of cellular kinases, involved in the entry pathway of LASV entry would represent promising “druggable” targets for therapeutic intervention.

Project 2: "Characterization of LASV cell entry *via* the TAM receptor Axl"

Molecular characterization of the interaction of LASV with cellular Axl

In line with previous studies (Shimojima et al., 2012), our results confirmed Axl as an entry receptor for LASV in a DG-independent manner. However, the molecular mechanism of Axl recognition by LASV needs to be further investigated. Recently, Morizono et al. (Morizono et al., 2011) proposed a general model for cell entry of enveloped viruses *via* TAM receptors. In this model, the serum proteins Gas6 or protein S attach to the lipid phosphatidylserine (PS) present in the membrane of the viral envelope and provide a bridge to cellular TAM receptors. This model is proposed as a possible mechanism of internalization for enveloped viruses in cell that lack specific receptors. Shimojima and colleagues showed that binding of LASV pseudovirion particles to Axl was detectable in cells expressing Axl but not with purified recombinant Axl protein (Shimojima et al., 2012). This observation suggested that LASV pseudotypes may bind Axl through an indirect mechanism, possibly via Gas6 or protein S. To test this possibility, a mutant of Axl (Axl E59R) which is deficient in Gas6 binding (Sasaki et al., 2002) was expressed in otherwise refractory cells. When compare to the wild-type, Axl E59R was apparently not impaired in its function as a LASV receptor (Shimojima et al., 2012), suggesting that Gas6 is not involved in LASV cell entry via Axl. However, the Axl mutant E59R is not a complete loss-of-function mutant and has not been fully characterized (Sasaki et al., 2006). Furthermore, the binding capacity of Axl E59R to bovine protein S present in the experiment was not investigated. Considering the limited data at hand, a possible role for PS, Gas6, and protein S in Axl-mediated LASV entry can in my opinion not be categorically excluded and need to be further investigated.

While previous studies detected PS in the envelope of the Clade A New World arenavirus Pichinde (Soares, King, and Thorpe, 2008), no data were available on the PS content of Old World arenaviruses. To address this issue, we tested the binding of the prototypic LCMV to ANX5, a high affinity ligand for PS. As positive control, we used vaccinia virus (VACCV) that uses "apoptotic mimicry" to infect cells and whose envelope is rich in PS (Mercer and Helenius, 2008). As a negative control, we used human adenovirus (AdV)-5, which lacks a lipid envelope. In a first approach, viruses were incubated with biotinylated ANX5, followed by ultracentrifugation through a sucrose cushion, separating virion particles from the supernatant. Pellets were lysed in SDS-PAGE sample buffer and virion-associated ANX5 detected in Western blot using HRP-conjugated streptavidine. As shown in Fig. 1A, LCMV and VACCV, but not AdV-5 associated with ANX5, indicating the presence of PS in the outer leaflet of the LCMV envelope. The marked reduction of ANX5 binding in presence of the chelator EGTA underscored the specificity of the Ca^{2+}-dependent PS-ANX5 interaction. In a complementary experiment, biotinylated ANX5 was incubated with viruses, followed by pull-down with streptavidin-conjugated magnetic beads. Bound material was

eluted and examined in Western-blot using specific antibodies to viral proteins. Consistent with the previous experiment, ANX5 was able to pull down LCMV and VACCV, but not AdV-5 (Fig. 1B). Comparison of ANX5 binding between LCMV and rLCMV-LASVGP revealed similar specific binding, indicating comparable content of PS in the outer leaflets of LCMV and the rLCMV-LASVGP chimera. To exclude unlikely differences between LCMV and LASV, we will perform detection of PS via ANX5 with inactivated LASV Josiah that we will receive from the Centers of Disease Control in Atlanta. Our preliminary data show that the prototypic Old World arenavirus and the rLCMV-LASVGP chimera display PS in their envelope. Since an arenavirus particle has a size comparable to that of an apoptotic body this suggest that Old World arenaviruses may very well be capable of "apoptotic mimicry", hijacking Axl or other TAM receptors in cells where DG is absent. We therefore started to re-evaluate the molecular mechanism underlying the interaction of rLCMV-LASVGP with Axl addressing this possibility.

Recognition of PS in viral envelopes by Gas6/protein S is dependent on divalent cations, in particular Ca^{2+}. In a first step, we performed infection of HT1080 cells with rLCMV-LASVGP in presence of the chelators EDTA/EGTA and observed a marked reduction in infection, suggesting dependence on divalent cations (data not shown). In a next step, we will assess serum-dependence to determine whether Axl-mediated infection of rLCMV-LASVGP requires the presence of protein S for its entry into HT1080 cells. In a complementary approach to address the role of PS in the viral envelope in Axl mediated entry, we will pre-treat rLCMV-LASVGP with recombinant ANX5, prior to addition to HT1080 cells. The combination of these experiments addressing cation-dependence, serum-dependence, and blocking by ANX5 will provide evidence for a possible involvement of PS and an "apoptotic mimicry"-like mechanism for LASV cell entry *via* Axl.

In case we find no evidence for an involvement of PS or serum proteins in Axl-mediated LASV cell entry, we will pursue the possibility of a direct binding of the viral GP to Axl. Productive infection of LASV mediated by the viral GP requires binding of GP1 to cellular receptors to allow subsequent pH-induced membrane fusion via GP2. In a first step, to assess the role of GP1 in binding to Axl, we will perform solid phase binding assays of rLCMV-LASVGP to immobilized Axl in microtiter plates. rLCMV-LASVGP produced in serum-free medium will be treated with 1 M NaCl to remove GP1 and then tested for Axl binding. Bound virus will be detected with a mAb to GP2. A reduction of virus binding to Axl after high salt treatment would suggest a role for GP1, but not PS, in binding.

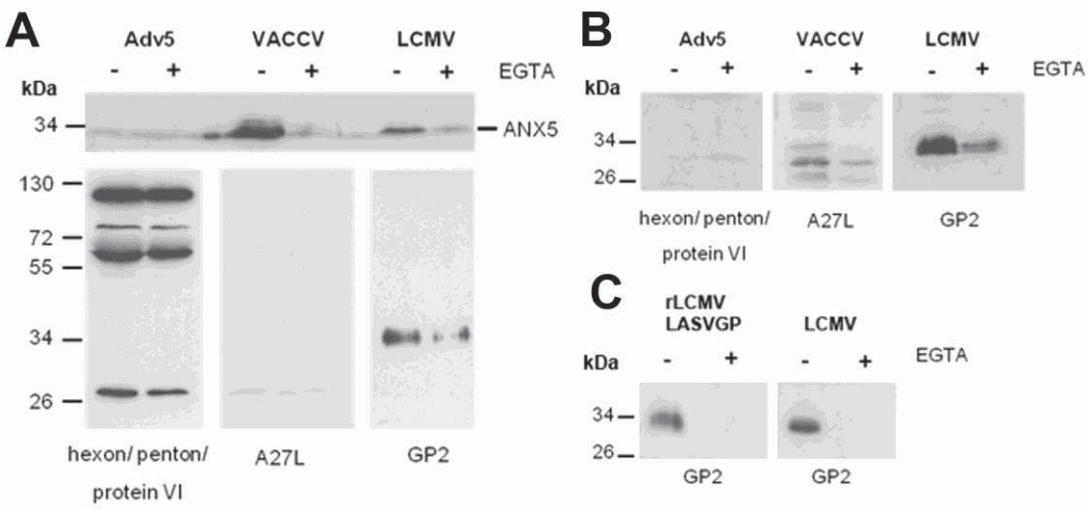

Figure 1. Old World arenaviruses display PS in the outer leaflet of their envelope. (A) ANX5 binds to PS in the envelope of LCMV. Equal amounts of LCMV, VACCV, and AdV-5 were incubated with biotinylated ANX5 (25 µg/ml) in presence and absence of 5 mM EGTA. Samples were subjected to ultracentrifugation through a sucrose cushion and pellets subjected to Western-blot analysis. Biotinylated ANX5 was detected by HRP-conjugated streptavidine (top). The presence of viruses was detected in pellets by detection of hexon/penton proteins of Ad-V, protein A27L of VACCV, and LCMV GPs. (B) Pull-down of viruses with biotinylated ANX5. LCMV, VACCV, and AdV5 were incubated with biotinylated ANX5 as in (A) followed by pull-down with streptavidine conjugated to magnetic beads. After several washes, bound material was eluted and viral proteins detected in Western-blot as in (A). (C) ANX5 binding to LCMV and rLCMV-LASVGP. Viruses were incubated with biotinylated ANX5, followed by pull-down as in (B). Bound virus was eluted and LCMV GP2 and LASV GP2 detected in Western-blot with mAb 83.6.

A complementary way to investigate the implication of LASV GP in Axl-mediated productive infection is the detection of a direct molecular interaction between LASV GP1 and Axl. With the help of the laboratory of Prof. Erica Ollman-Saphire (Scripps Research Institute, La Jolla), we produced soluble recombinant fusion proteins of LASV GP1 with the IgG Fc moiety of rabbit Fc (LASV GP1-Fc). These LASV GP1 "immunoadhesins" are fully functional and bind to DG with nanomolar affinity (Ana Rita Gonçalves, unpublished results). To assess a direct binding between LASV GP1 and Axl, we will perform solid-phase binding assays of LASV GP1-Fc to immobilized recombinant human Axl fused to human IgG Fc (Axl-Fc). Bound LASV GP1-Fc will then be detected with an HRP-conjugated secondary antibody in a color reaction. This ELISA-format solid-phase binding assays will allow the detection of interactions that are not detectable in classical pull-downs performed by Shimojima et al. However, limited sensitivity remains a possible concern, in particular when we consider a possibly low affinity binding of LASV GP1 to Axl, as suggested by the poor efficiency of Axl as a LASV receptor. In case we are unable to detect an interaction by solid-phase binding assay, we envisage the use of surface plasmon resonance (SPR) that allows detection of weaker interactions (Kd < 10^5 mol/l). For SPR, we will have access to a BiaCore platform available at the Institute of Pathology (IPA) of UNIL/CHUV.

In a third approach, we will use our LASV GP1-Fc immunoadhesins to block cellular Axl in HT1080 cells prior to the addition of rLCMV-LASVGP. A dose-dependent reduction of viral infection by pre-treatment of cells with LASV GP1-Fc would further support a role for GP1 in Axl-mediated LASV cell entry.

Together, these studies will shed light on the molecular recognition of rLCMV-LASVGP by Axl and help to distinguish if the virus uses "apoptotic mimicry" via PS-Gas6/protein S or if a direct interaction of GP1 with Axl is involved. In the latter case, it would be of interest to map the Axl binding site on GP1 and to see to what extent it overlaps with the binding site for DG in future work. Such studies could help to understand the interaction of LASV GP with Axl and DG on cells co-expressing the two receptors, like e.g. epithelial and endothelial cells, which are important *in vivo* targets of LASV.

The role of Axl-mediated signaling in LASV cell entry

Does binding of LASV activate Axl tyrosine kinase activity?

Binding of Gas6 induces the dimerization of Axl with consequent auto-phosphorylation of tyrosine residues of its intracellular domain. Recently, a study demonstrated that Y702 and Y703 undergo phosphorylation upon Gas6 stimulation (Pao-Chun et al., 2009). However, none of these sites has been shown to directly regulate or interact with downstream effectors of Axl. Three other tyrosines, Y779, Y821, and Y866, within the Axl cytoplasmic domain were implicated in binding of several substrates, highlighting them as potential candidates for auto-phosphorylation sites (Braunger et al., 1997; Fridell et al., 1996). The residue Y779 provides a docking site for PI3K, while Y866 plays a role in binding of phospholipase C (PLC). Using Axl mutants deficient in tyrosine kinase activity, Shimojima and colleagues demonstrated that Axl-mediated infection of LASV pseudotype virus in Jurkat cells requires intracellular Axl signaling (Shimojima et al., 2012). We will extend these studies looking at the interaction of LASV GP and Axl in cells that normally express Axl.

In a first step, we will investigate if attachment of rLCMV-LASVGP induces activation of Axl. To examine activation of Axl signaling in response to virus attachment, HT1080 cells will be subjected to serum starvation to reduce endogenous Axl signaling. Cell will be chilled on ice and rLCMV-LASVGP added in the cold to allow virus binding without signaling and/or internalization. Unbound virus will be removed and cells shifted to 37°C. At different time points, cells will be lysed and tyrosine phosphorylation of Axl monitored over time using specific antibodies directed against phosphorylated forms of the receptor. These studies will show if virus binding to cellular Axl induces its activation and auto-phosphorylation and may reveal quantitative or qualitative differences with respect to activation *via* Gas6. Virus-induced autophosphorylation of specific Y residues on Axl may give first hints towards signaling pathways that are activated upon virus binding. In case we are able to

detect virus-induced tyrosine phosphorylation of Axl in HT1080 cells, it will be interesting to extend the analysis to other cell types, which co-express Axl with functional DG. It will be of particular interest to see if the presence of functional DG can prevent virus-induced autophosphorylation of Axl, suggesting that binding of the virus to DG prevents the interaction with Axl.

Is Axl activation and/or signaling required for LASV entry?

In case we are able to detect virus-induced tyrosine phosphorylation and thus activation of Axl, it will be interesting to investigate whether this receptor activation is needed for viral entry. Previous studies using the broadly specific tyrosine kinase inhibitor genistein provided evidence for tyrosine phosphorylation to be involved in Axl-mediated LASV cell entry (Shimojima et al., 2012). However, neither the kinases nor the substrates involved were characterized. To address the role of tyrosine phosphorylation of specific Y residues of Axl, we will employ a panel of point mutations of Axl (Y702F, Y703F, Y779F, Y821F, and Y866F). These mutations will be introduced in a recombinant form of human Axl containing silent mutations in the target sequence of the shRNA that was used to deplete Axl in my studies. HT1080 cells will be depleted of endogenous Axl by siRNA, followed by transfection with the siRNA-resistant mutants and wild-type Axl. Cells will then be infected with our rLCMV-LASVGP chimera and early infection assessed by IFA. These experiments could give first hints towards Axl-associated signaling pathways may be implicated in LASV cell entry.

Targeting of Axl is a novel strategy to combat human arenavirus infection

According to the role of Axl-dependent signaling in several disorders, the inhibition of its activation is already under investigation. Indeed, extracellular targeting of Axl with specific inhibitory antibodies has been already shown to prevent downstream signaling mediated by Gas6, especially in the context of cancer (Li et al., 2009; Ye et al., 2010). Thus, we could use these available antibodies and evaluate them in the context of LASV entry. A second approach will be the use of Axl-specific small-molecule inhibitors able to block its signaling. The majority of studies on Axl inhibition by small-molecule inhibitors have been performed in the context of cancer (Linger et al., 2010). The candidate inhibitor R428 (Rigel Pharmaceuticals) has been shown to be effective and has favorable pharmacokinetic profiles. This inhibitor potently blocks auto-phosphorylation of Axl on the multiple docking site Tyr821, stimulated by either antibody-mediated cross-linking or Gas6 (Holland et al., 2010). In addition, R428 shows specificity to Axl and strong inhibitory effect in multiple cell lines (Smolock and Korshunov, 2010). Thus, treatment of cells with R428 followed by infection with rLCMV-LASVGP could reveal whether the inhibitor affects viral entry. Successful inhibition of LASV cell entry *via* Axl by antibodies or drugs like R428 would provide

proof-of-concept identifying Axl as a novel therapeutic target to combat LASV infection. However, considering the roles that Axl is playing in the host cell, potential side effects may be a concern. Moreover, given the preeminent role of DG as a LASV receptor in many important human cell types involved in infection *in vivo*, antibodies or drugs targeting Axl would represent only one component of an anti-viral drug cocktail.

A role of Axl in innate immune evasion or immunosuppression by LASV

Does LASV cell entry *via* Axl allow the virus to evade innate detection?

Pathogenic arenaviruses are able to subvert the cellular mechanisms of innate pathogen recognition. The genomic RNA of arenaviruses can be recognized by the cytoplasmic RNA helicases of the retinoic acid-inducible gene I (RIG-I) family, which represent principal innate sensors for viral RNA and, as a consequence, induce the production of type I interferons (IFNs) (Habjan et al., 2008; Zhou et al., 2010). However, cells infected with arenaviruses fail to induce type I IFN because the viral NP acts as a potent IFN antagonist and prevents activation of the IFN regulatory factor (IRF)-3 (Martinez-Sobrido et al., 2009; Martinez-Sobrido et al., 2006) and NF-κB (Rodrigo et al., 2012). The NP contains a 3'-5' exonuclease activity that is linked to its immunosuppressive activity (Hastie et al., 2011; Qi et al., 2010) and targets the non-canonical interferon regulatory factor-activating kinase IKKε (Pythoud et al., 2012). Since the viral NP act as an IFN antagonist in the cytoplasm, the suppression of IFN production by NP depends on productive viral infection with expression of sufficiently high levels of NP. During viral entry, the virus is located in extracellular space where NP cannot act as an IFN antagonist. It is thus possible that incoming arenaviruses could be detected by pathogen recognition receptors (PRRs) located in endosomal compartments, e.g. Toll-like receptors (TLRs). Upon DG binding, Old World arenaviruses are internalized *via* a pathway of endocytosis that apparently bypasses the early endosome where PRRs like TLRs mostly localize (Rojek et al., 2008), possibly allowing the virus to evade innate detection. It will be of interest to investigate if LASV entry *via* Axl likewise allows the virus to evade innate detection or its entry *via* Axl results in innate detection with the consequent induction of IRF3 and NF-κB.

The major PRRs that can detect viral RNAs leaking out of damaged virions during entry are TLR3 and TLR7/8 located in endosomal compartments. TLR3 recognizes double-stranded viral RNA whereas TLR7/8 recognizes viral single-stranded RNAs (Boehme and Compton, 2004). After stimulation, TLR3 recruits the adapter protein TRIF which then activates TBK1 and the canonical IKK complex, resulting in activation of IRF3 and NF-κB, respectively. TLR7/8 requires MyD88 to activate the signaling proteins TRAF6 and IRAK4 inducing the activation of IRF7 (Kawai et al.,

2004). In a proof-of-concept study, we will use HT1080 cells to investigate if LASV cell entry *via* Axl evades innate detection by endosomal TLRs. In case HT1080 cells lack endogenous TLR3 and TLR7, they will be expressed as recombinant proteins using retroviral gene delivery vectors. In a first step, we will examine activation of the downstream transcription factors IRF3/7 and NF-κB upon infection of cells with rLCMV-LASVGP, using Sendai virus (SeV) as a positive control. Activation of IRF3/7 could be detected by its nuclear translocation, which is indispensable for the activation of the INF-β promoter (Martinez-Sobrido et al., 2007; Martinez-Sobrido et al., 2006). The nuclear translocation of IRF3/7 critically depends on its phosphorylation, which could be assessed by Western-blot using an antibody that specifically recognizes the phosphorylated form as described (Pythoud et al., 2012). Activation of NF-κB could be monitored using NF-κB luciferase reporter constructs and nuclear translocation assessed by cell fractionation (Rodrigo et al., 2012). Consequent up-regulation of IFN-β could be monitored by RT-PCR. In case Axl-mediated cell entry of rLCMV-LASVGP induces IRF3/7 and/or NF-κB, we will confirm the roles of TLR7/8 and TLR3. For this purpose, we will employ specific siRNAs that target and efficiently silence TLR genes.

As mentioned above, when rLCMV-LASVGP uses DG as receptor, it enters and infects cells *via* an unusual pathway that seems to bypass the early endosome, which may allow the virus to escape from detection by endosomal PRRs like TLRs. It will be interesting to compare the innate immune response between HT1080 cells expressing functional DG and cells expressing only Axl. For this purpose, we would compare the induction of IFN-β upon cell entry of rLCMV-LASVGP into either wild-type HT1080 cells expressing TLRs or the same cells in which functional DG has been rescued by over-expression of LARGE using an adenoviral vector (LARGE-AdV). If the rescue of functional DG prevents induction of INF-β in response to infection, the results would indicate that high affinity binding of LASV to DG "sequesters" the virus away from the TAM receptors and allows it to bypass recognition by endosomal TLRs. This may suggest that selection of DG as a high affinity receptor by arenaviruses may have allowed them to escape from the innate detection by endosomal PRRs in cells co-expressing DG and TAM receptors.

Can LASV suppress the function of antigen presenting cells *via* Axl?

Autoimmunity phenotypes have been observed in TAM-knockout mice, revealing the importance of the TAM family in the regulation of the host's immune response (Lu and Lemke, 2001). A proposed mechanism is the negative regulation of pro-inflammatory signaling by Axl in innate immune cells (Fig. 2). Specifically, it has been demonstrated that the activation of the Gas6/Axl pathway leads to inhibition of TLR and cytokine receptor signaling in antigen presenting cells (APC). Mechanistically, Gas6/Axl co-activates the INFAR (IFNα/β receptor)/STAT1 (signal

transducer and activator of transcription 1) pathway, which increases the expression of pro-inflammatory signal suppressors, such as Twist 1, SOCS1 and SOCS3 (Fig. 2) (Rothlin et al., 2007; Sharif et al., 2006).

Human DCs, like for example monocyte-derived DCs (MDDC) are highly susceptible to LASV infection, but fail to be activated and do not produce type I IFNs or cytokines in response to infection (Baize et al., 2004; Mahanty et al., 2003). Recent studies in our laboratory and by others showed that MDDC lack functional DG and Axl. However, Axl, but not DG were induced upon exposure of MDDCs to type I IFNs (Scutera et al., 2009). This scenario is of interest regarding human LASV infection *in vivo*, since experimental studies in primates revealed early infection of plasmacytoid DCs (pDCs) resulting in production of high levels of type I IFNs. Type I IFN released from pDCs early in infection could act upon classical DCs, resulting in the up-regulation of Axl. We speculate that the interaction of LASV with Axl on DCs may induce inhibitory signals and may contribute to the down-modulation of the DC's cytokine production. We are currently testing this hypothesis in the laboratory.

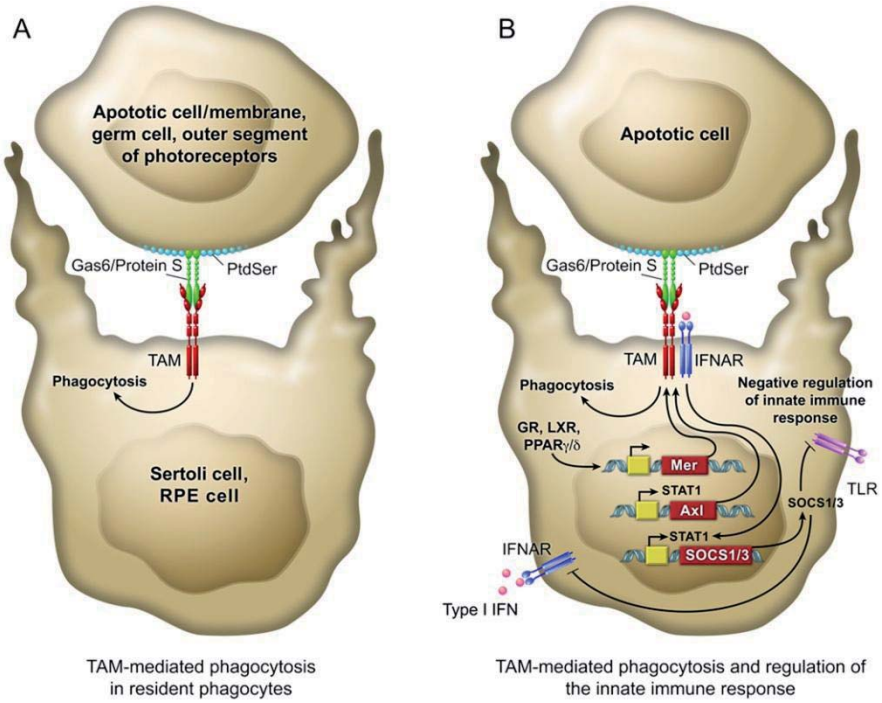

Figure 2. Overview of TAM signaling. (A) Phagocytic removal of apoptotic cells. Phagocytic cells use TAM receptors (red) to identify and engulf apoptotic cells and membranes. TAM ligands, such as Gas6 and protein S (green), bind to phosphatidylserine (PtdSer; light blue) that is expressed on the surface of apoptotic cells. The carboxy-terminal domains of these ligands then interact with and activate TAM receptors on the phagocyte. This induces a signal transduction cascade that results in mobilization of the actin cytoskeleton, and the phagocytosis of the apoptotic cell. (B) Regulation of the inflammatory response. This same scheme of engagement, when it occurs in the context of the joint expression of TAM and type I IFN receptors (IFNARs; dark blue) in macrophages and DCs, also inhibits the inflammatory response of the innate immune system. Induced-expression of SOCS proteins inhibit the signaling downstream of both IFNARs and Toll-like receptors (TLRs, violet) (Rothlin and Lemke, 2010).

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APPENDIX

Original article

"Cell entry of Lassa virus induces tyrosine phosphorylation of dystroglycan"

Marie-Laurence Moraz[1], Christelle Pythoud[1], Rolf Turk[2], Sylvia Rothenberger[1], Antonella Pasquato[1], Kevin P. Campbell[2] and Stefan Kunz[1*]

[1] Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne, Switzerland

[2] Howard Hughes Medical Institute, Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center, Department of Molecular Physiology and Biophysics, Department of Neurology, Department of Internal Medicine, and Roy J and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA

* Corresponding author. Mailing address: Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne CH-1011, Switzerland. Phone: +41-21 314 7743, Fax: +41-21 314 4060, E-mail Stefan.Kunz@chuv.ch

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Cell entry of Lassa virus induces tyrosine phosphorylation of dystroglycan

Marie-Laurence Moraz,[1] Christelle Pythoud,[1] Rolf Turk,[2] Sylvia Rothenberger,[1] Antonella Pasquato,[1] Kevin P. Campbell[2] and Stefan Kunz[1]*

[1]*Institute of Microbiology, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland.*
[2]*Howard Hughes Medical Institute, Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center, Department of Molecular Physiology and Biophysics, Department of Neurology, Department of Internal Medicine, and Roy J and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA.*

Summary

The extracellular matrix (ECM) receptor dystroglycan (DG) serves as a cellular receptor for the highly pathogenic arenavirus Lassa virus (LASV) that causes a haemorrhagic fever with high mortality in human. In the host cell, DG provides a molecular link between the ECM and the actin cytoskeleton via the adapter proteins utrophin or dystrophin. Here we investigated post-translational modifications of DG in the context of LASV cell entry. Using the tyrosine kinase inhibitor genistein, we found that tyrosine kinases are required for efficient internalization of virus particles, but not virus–receptor binding. Engagement of cellular DG by LASV envelope glycoprotein (LASV GP) in human epithelial cells induced tyrosine phosphorylation of the cytoplasmic domain of DG. LASV GP binding to DG further resulted in dissociation of the adapter protein utrophin from virus-bound DG. This virus-induced dissociation of utrophin was affected by genistein treatment, suggesting a role of receptor tyrosine phosphorylation in the process.

Introduction

The Old World arenavirus Lassa virus (LASV) is the causative agent of a severe viral haemorrhagic fever in humans with several hundred thousand infections per year in Africa and thousands of deaths annually (McCormick and Fisher-Hoch, 2002). Fatal LASV infection is characterized by rapid viral replication and spread, resulting in uncontrolled viral infection with progressive signs and symptoms of haemorrhagic disease and shock (Geisbert and Jahrling, 2004). The death toll of LASV infection among hospitalized patients can reach 15–30%. There is no licensed vaccine against LASV and current therapeutic options are limited, making LASV arguably one of the most neglected tropical pathogens.

Arenaviruses are enveloped negative-strand RNA viruses with a bisegmented genome, whose replication takes place in the cytoplasm (Buchmeier *et al.*, 2007; de la Torre, 2009). The two viral RNA segments, L and S, include each two open reading frames. The S segment encodes the envelope glycoprotein precursor (GPC) and the nucleoprotein (NP) and the L segment codes for the matrix protein (Z) and the viral polymerase (L). LASV GPC is synthesized as a single polypeptide and undergoes processing by the host cell protease subtilisin kexin isozyme-1 (SKI-1)/site-1 protease (S1P) (Lenz *et al.*, 2001; Beyer *et al.*, 2003; Rojek *et al.*, 2008b), yielding the N-terminal GP1 and the transmembrane GP2. LASV GP1 is involved in binding to cellular receptors (Borrow and Oldstone, 1992), whereas the GP2 part mediates fusion and resembles the membrane-proximal regions of other viral fusion proteins (Eschli *et al.*, 2006; Igonet *et al.*, 2011).

Binding of a virus to its cellular receptor(s) and subsequent entry into target cells are the first steps of virus infection and a fundamental aspect of the virus–host cell interaction. These initial steps of infection are also promising targets to block the pathogen before it can take control over the host cell. Therapeutic intervention at the level of cell entry is of particular interest for highly pathogenic viruses like LASV. The first cellular receptor discovered for LASV and the prototypic Old World arenavirus lymphocytic choriomeningitis virus (LCMV) is dystroglycan (DG), a ubiquitous receptor for extracellular matrix (ECM) proteins (Cao *et al.*, 1998; Oldstone and Campbell, 2011). More recently, additional candidate receptors for LASV have been reported, including the C-type lectins DC-SIGN, LSECtin, and the Tyro3/Axl/Mer (TAM) receptor tyrosine kinases Axl and Tyro3 (Shimojima *et al.*, 2012). However, in DG-expressing cells, DG appears to be the preferred receptor for LASV (Shimojima *et al.*, 2012).

Received 29 August, 2012; revised 14 November, 2012; accepted 19 November, 2012. *For correspondence. E-mail stefan.kunz@chuv.ch; Tel. (+41) 21 314 7743; Fax (+41) 21 314 4060.

Dystroglycan is a highly conserved protein initially translated as a single polypeptide chain that is cleaved into the extracellular α-DG, and transmembrane β-DG (Barresi and Campbell, 2006). DG is expressed in most developing and adult tissues in cells that adjoin basement membranes (Durbeej *et al*., 1998) and is crucial for normal cell–matrix interactions (Henry and Campbell, 1998; Henry *et al*., 2001). Alpha-DG has a central, highly glycosylated mucin-type domain that connects the globular N- and C-terminal domains. At the extracellular site, α-DG undergoes high-affinity interactions with the ECM proteins laminin, agrin, perlecan and neurexins (Barresi and Campbell, 2006). Alpha-DG is non-covalently associated with β-DG, which binds intracellularly to the adaptor proteins dystrophin or utrophin that link DG to the actin cytoskeleton. DG is also associated with a number of signalling molecules, including the adaptor molecule grb2 (Yang *et al*., 1995), the canonical MAP kinases MEK and ERK (Spence *et al*., 2004) and the focal adhesion kinase (Cavaldesi *et al*., 1999). Upon receptor binding, LASV and LCMV are internalized via an endocytotic pathway that is independent of clathrin, caveolin and dynamin and bypasses classical Rab5-dependent incoming routes of vesicular trafficking (Quirin *et al*., 2008; Rojek *et al*., 2008c). During cell entry, LASV and LCMV pass through the multivesicular endosome, where the virus–receptor complex undergoes sorting by the endosomal sorting complex required for transport (ESCRT) delivering the viruses to late endosomes, where low pH-induced fusion occurs after 20–30 min (Quirin *et al*., 2008; Rojek *et al*., 2008c; Pasqual *et al*., 2011).

In the host cell, DG appears as a structural component of cell–matrix contacts, which suggests a rather static role. Considering the receptor dynamics expected in the context of LASV cell entry, we hypothesized that virus binding may alter DG trafficking, e.g. by inducing post-translational modifications that target the DG complex towards rapid endocytosis. Studies in prototypic primate and human cells had demonstrated that cell adhesion to ECM proteins can induce phosphorylation of tyrosine Y892 located within a PPxY motif present at the C-terminus of the cytoplasmic domain of β-DG by non-receptor tyrosine kinases of the src family (James *et al*., 2000; Sotgia *et al*., 2001; 2003). This tyrosine phosphorylation of Y892 prevented the association of β-DG with the cytoskeletal adaptor protein utrophin (James *et al*., 2000) and resulted in a redistribution of the DG complex from the plasma membrane to intracellular compartments (Sotgia *et al*., 2003), linking tyrosine phosphorylation of β-DG to receptor internalization. More recent studies confirmed a role of tyrosine phosphorylation of β-DG at Y892 for the endocytosis of DG in myoblasts and provided evidence for a role of tyrosine phosphorylation of DG in the development of muscle pathophysiology in an animal model for muscular dystrophy (Miller *et al*., 2012). In the present study we investigated the role of receptor tyrosine phosphorylation for cell entry of LASV.

Results

Tyrosine kinases are involved in endocytosis of rLCMV–LASVGP

Recent studies using the broadly specific tyrosine kinase inhibitor genistein revealed a role for tyrosine kinases in cell entry of LASV (Kolokoltsov *et al*., 2012). However, the specific step(s) of the viral entry process that depend on tyrosine kinases had not yet been defined. In a first step, we sought to confirm and extend these earlier studies and tried to distinguish effects of genistein on virus–cell attachment from endocytosis. Since LASV is a BSL4 pathogen, work with live virus is restricted to laboratories with high security containment. To circumvent these biosafety restrictions, we used a recombinant form of the prototypic LCMV expressing the envelope GP of LASV (rLCMV–LASVGP) (Rojek *et al*., 2008c). The chimera rLCMV–LASVGP does not show significant attenuation *in vitro* when compared to the parental LCMV strain and grows to robust titres. Since receptor binding and host cell entry of arenaviruses are mediated exclusively by the viral GP, rLCMV–LASVGP adopts the receptor binding characteristics of LASV (Rojek *et al*., 2008c) and represents a suitable BSL2 surrogate for our studies on LASV–receptor interaction and cell entry. As a cell culture model, we chose the human lung epithelial cell line WI-26 VA4, which had previously been utilized for studies on DG signalling (Ferletta *et al*., 2003) and the interaction of LASV with its receptor DG (Rojek *et al*., 2012).

First, we verified that cell attachment and entry of LASV into WI-26 VA4 cells were mediated by DG. Cells were pretreated with increasing concentration of the monoclonal antibody (mAb) IIH6 that recognizes a functional glycan epitope on α-DG (Kanagawa *et al*., 2004) and competes with virus binding (Kunz *et al*., 2005b). Cells were then infected with rLCMV–LASVGP and infected cells detected after 16 h by immunofluorescence (IF) staining of the viral nucleoprotein. Pretreatment with mAb IIH6, but not an IgM isotype control, significantly blocked infection with rLCMV–LASVGP in a dose-dependent manner, confirming DG as a major receptor for LASV in these cells (Fig. 1A), as shown previously (Rojek *et al*., 2012).

To exclude unspecific cytotoxicity caused by genistein, WI-26 VA4 cells were treated with increasing concentrations of the drug for 4 h, followed by a wash out and incubation for a total of 16 h. Cell viability was assessed by Cell Titer Glo® assay, which measures intracellular levels of ATP. WI-26 VA4 cells tolerated genistein up to a

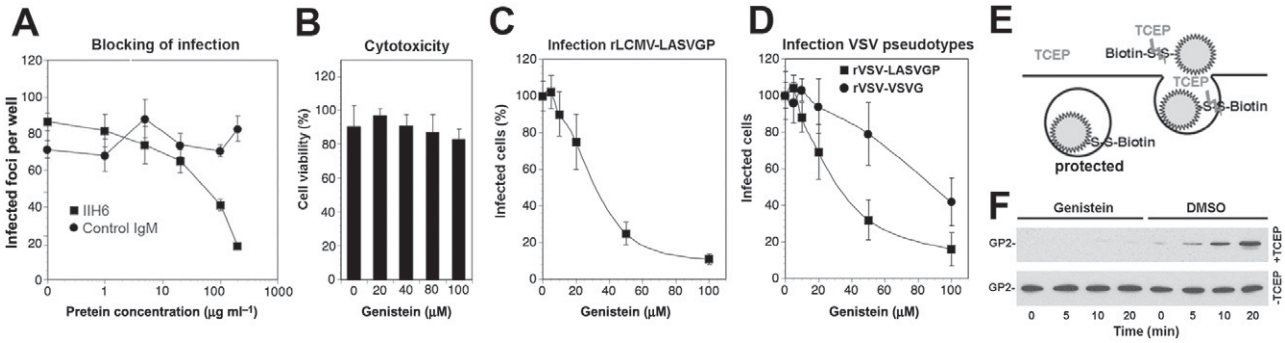

Fig. 1. Genistein inhibits internalization of rLCMV–LASVGP.
A. LASV infection in WI-26 VA4 cells is mediated by DG. Monolayers of WI-26 VA4 cells in M96 plates were blocked with mAb IIH6 or an unrelated mouse IgM (control IgM) at the indicated concentrations for 2 h at 4°C. Next, 200 pfu of rLCMV–LASVGP was added for 45 min. Infection was assessed after 16 h by immunofluorescence (IF) staining for LCMV NP. Infected foci were counted in each well (means ± SD, $n = 3$).
B. Cytotoxicity of genistein. WI-26 VA4 cells were treated with the indicated concentrations of the drug for 4 h, followed by a wash out and incubation for a total of 16 h. Cell viability was assessed by Cell Titer Glo® assay. Data are triplicates ± SD.
C. Inhibition of rLCMV–LASVGP infection with genistein. WI-26 VA4 cells were treated with the indicated concentrations of genistein for 1 h, followed by infection with rLCMV–LASVGP at multiplicity of 1, followed by wash out of the drug at 4 h. After 12 h of culture in presence of 20 mM ammonium chloride, cells were fixed and infection detected by IF for LCMV NP. Data are triplicates ± SD.
D. Inhibition of infection of VSV pseudotypes with genistein. WI-26 VA4 cells were treated with the indicated concentrations of genistein for 1 h, followed by infection with rVSV–LASVGP and rVSV–VSVG (200 pfu per well). After 24 h, infection was assessed by detection of GFP-positive cells in direct fluorescence microscopy ($n = 3$ ± SD).
E. Schematic of the virus internalization assay (for details, please see text).
F. Genistein treatment prevents internalization of rLCMV–LASVGP. WI-26 VA4 cells were pretreated with 50 μM genistein or vehicle control (DMSO). After 1 h, cells were chilled on ice and incubated with biotin-S-S-labelled rLCMV–LASVGP (100 particles per cell) for 1 h in the cold. Unbound virus was removed, cells shifted to 37°C in presence or absence of genistein. After the indicated time points, cells were chilled on ice and treated with TCEP (+TCEP) or reaction buffer only (–TCEP). After quenching of residual TCEP, cells were lysed, viral GP isolated by IP with mAb 83.6 to GP2. Biotinylated GP2 was detected with streptavidin-HRP in Western blot under non-reducing conditions using enhanced chemiluminescence (ECL). The upper blot (+TCEP) was exposed for 10 min; the lower blot (–TCEP) was exposed for 1 min.

concentration of 100 μM with only mild loss of cell viability (Fig. 1B). To test the effect of genistein on early infection with LCMV–LASVGP, WI-26 VA4 cells were pretreated with increasing concentrations of genistein for 1 h and infected with rLCMV–LASVGP at multiplicity of 1. At 4 h post infection, the drug was removed by wash out. To prevent further infection, the lysosomotropic agent ammonium chloride was added to the fresh medium. When added to cells ammonium chloride raises the endosomal pH rapidly and blocks low pH-dependent membrane fusion without causing overall cytotoxicity (Ohkuma and Poole, 1978; 1981). After 12 h in presence of ammonium chloride, cells were fixed and infection detected by IF staining for LCMV NP. As shown in Fig. 1C, genistein blocked infection with rLCMV–LASVGP in a dose-dependent manner.

Using expression of LCMV NP in infected cells as readout for infection did not allow discriminating between effects of genistein on LASVGP-mediated cell entry and post-entry steps of early viral infection. To specifically validate the effects of genistein on LASV cell entry in our system, we used recombinant vesicular stomatitis virus (VSV) pseudotyped with the envelope GPs of LASV (rVSV–LASVGP) and VSV (rVSV–VSVG). These pseudotypes are replications-deficient and contain a green fluorescent protein (GFP) reporter in their genome. Previous studies demonstrated that rVSV–LASVGP closely mimics the receptor binding and entry characteristics of LASV (Kunz *et al.*, 2005a; Pasqual *et al.*, 2011). Increasing concentrations of genistein blocked infection of rVSV–LASVGP more efficiently than rVSV–VSVG (Fig. 1D), consistent with previous reports (Kolokoltsov *et al.*, 2012). Notably, the dose–response characteristic of rVSV–LASVGP closely matched the one of rLCMV–LASVGP (Fig. 1C and D), indicating that at least some of the inhibitory effect of genistein on early infection with rLCMV–LASVGP was due to perturbation of LASVGP-mediated cell entry.

To differentiate between effects of genistein on LASVGP-mediated cell attachment from perturbation of endocytosis, we used a well-established assay previously used to study virus internalization (Pelkmans *et al.*, 2002; Rojek *et al.*, 2008a), schematically shown in Fig. 1E. Briefly, rLCMV–LASVGP was purified over a renografin gradient and purified virus labelled with the reagent NHS-SS-biotin, resulting in a biotin label that is cleavable by reducing agents. As long as the virus stays bound to the cell surface, the biotin label can be cleaved efficiently with the potent, membrane-impermeable reducing agent Tris(2-carboxyethyl)phosphine (TCEP) (Fig. 1E). Once

internalized via endocytosis, the biotin-labelled virus is protected from TCEP and retains its biotin moiety after exposure of cells to TCEP. To assess possible effects of genistein on virus cell attachment and internalization, WI-26 VA4 cells were pretreated with 50 μM genistein or vehicle control. After 1 h, cells were cooled on ice and incubated with biotin-labelled rLCMV–LASVGP (100 particles per cell) for 1 h in the cold. Unbound virus was removed and cells shifted to 37°C in presence of genistein. After the indicated time points, cells were rapidly chilled on ice and immediately treated with cold TCEP or reaction buffer only. After quenching of residual TCEP with iodacetamide, cells were lysed and the viral GP isolated by immunoprecipitation (IP) with mAb 83.6 to GP2. Proteins were separated by SDS-PAGE and biotinylation of GP2 detected by Western blot under non-reducing conditions. In specimens treated with reaction buffer only, similar amounts of cell-associated biotinylated virus were detected in presence and absence of genistein (Fig. 1F), indicating that genistein treatment did not affect virus attachment to the receptor. In control samples treated with TCEP, biotinylated virus became detectable after circa 5 min, with an increase over the next 20 min. In cells treated with genistein and TCEP, the signals for biotinylated GP2 were markedly reduced, indicating a block in an early step of virus internalization in presence of the inhibitor. In sum, our studies revealed that genistein does not affect virus–cell attachment, but inhibits the subsequent early steps of virus internalization.

Binding of rLCMV–LASVGP to cellular DG induces tyrosine phosphorylation of β-DG

Since the binding of LASV to DG is of high affinity and virtually irreversible under neutral pH (Kunz *et al*., 2005a), we speculated that virus-bound DG may be internalized during endocytosis of the virus. The apparent block of virus internalization in presence of genistein at an early time point (5–20 min) (Fig. 1E) opened therefore the possibility that uptake of the virus–receptor complex could involve tyrosine phosphorylation of the receptor and/or receptor-associated cellular factors. Since phosphorylation of β-DG at residue Y892 had previously been linked to internalization of the DG complex (Sotgia *et al*., 2003; Miller *et al*., 2012), we monitored phosphorylation of DG at Y892 during LASV cell entry. For this purpose, we applied mAb cl14a that specifically recognizes β-DG phosphorylated at Y892 (Sotgia *et al*., 2003). In a first step, we confirmed the specificity of mAb cl14a in our system. To this end, we coexpressed recombinant full-length DG containing a C-terminal HA tag (DGHA, Fig. 2A) with recombinant c-src in HEK293 cells. Previous studies demonstrated that C-terminal tagging of β-DG had no influence on the biosynthesis, transport and function of DG (Rojek *et al*., 2007b). Cells were either treated with the specific src tyrosine kinase family inhibitor PP2 (20 μM) or mock treated. After 48 h, DGHA was isolated by IP with HA matrix and phosphorylation of Y892 detected with mAb cl14a in Western blot. As expected, DGHA isolated from cells overexpressing c-src was specifically recognized by mAb cl14a, whereas treatment with PP2 markedly reduced the signal, confirming the specificity of the assay (Fig. 2B).

To examine the effect of LASV binding on tyrosine phosphorylation of DG at Y892, monolayers of WI-26 VA4 cells were incubated with rLCMV–LASVGP (100 particles per cell). Virus attachment was performed in the cold to allow virus binding but prevent lateral movement of receptor molecules in the membrane. As a control, we used the New World arenavirus Pichinde (PICV) that does not bind to α-DG (Spiropoulou *et al*., 2002). After removal of unbound virus, cells were rapidly shifted to 37°C to restore membrane fluidity. At the indicated time points, cells were lysed in presence of the phosphatase inhibitor sodium orthovanadate. Cleared cell lysates were subjected to lectin purification with wheat germ agglutinin (WGA). Precipitated proteins were separated by SDS-PAGE and probed with mAb cl14a anti-β-DGPY892 and mAb 8D5 to β-DG that binds independently of phosphorylation. Binding of rLCMV–LASVGP, but not PICV, resulted in transient phosphorylation of β-DG at Y892 (Fig. 2C) with maximal signals observed after 5–10 min. To confirm the role of src family kinases in the apparent virus-induced receptor phosphorylation at Y892, we treated cells with the src kinase inhibitor PP2 for 30 min prior to addition of virus. As shown in Fig. 2D, pretreatment with PP2 markedly reduced virus-induced tyrosine phosphorylation of β-DG at Y892, indicating a direct or indirect involvement of src kinases. In some experiments, we observed an apparent decrease in total β-DG at later time points (Fig. 2C and D). However, this was not observed consistently.

To address a possible role of the observed virus-induced receptor phosphorylation at Y892 for viral entry, we monitored the entry kinetics of LASV in presence of PP2. Upon receptor binding, LASV is taken up by clathrin- and caveolin-independent endocytosis and rapidly delivered to late endosomes, where low pH-dependent membrane fusion occurs (Borrow and Oldstone, 1994; Quirin *et al*., 2008; Rojek *et al*., 2008a,c). To assess how fast receptor-bound rLCMV–LASVGP trafficked to late endosomes in presence and absence of PP2, we determined the time required for the virus to become resistant to ammonium chloride. Briefly, WI-26 VA4 cells were either pretreated with PP2 for 1 h, or mock treated with vehicle (DMSO) only. Cells were then incubated with rLCMV–LASVGP in the cold, allowing virus attachment without internalization. Unbound virus was

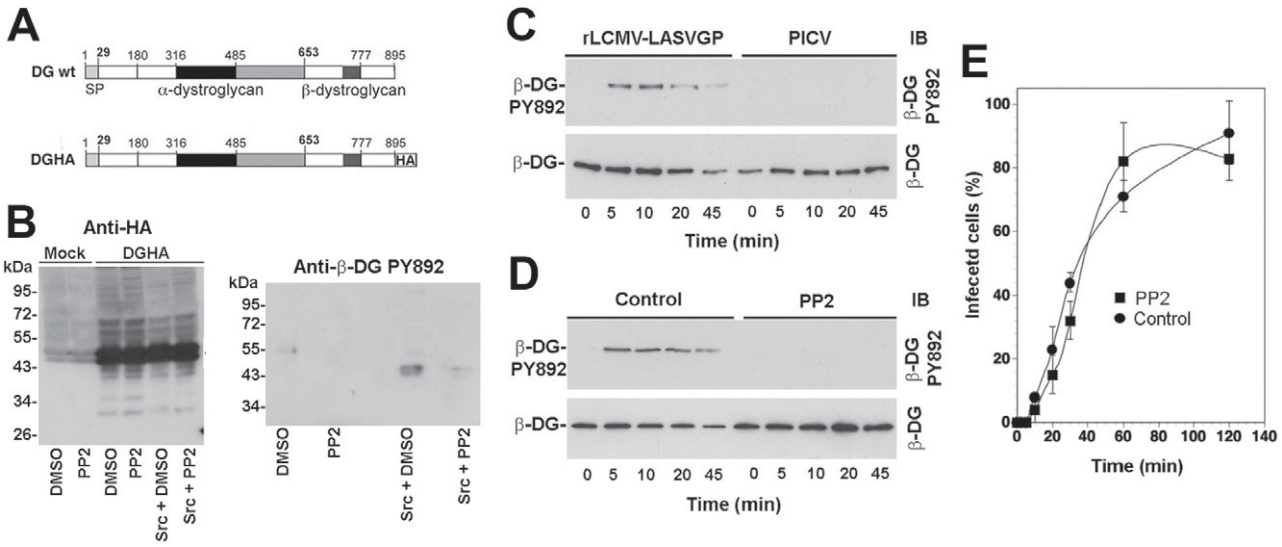

Fig. 2. Binding of LASV to cellular DG induces tyrosine phosphorylation of β-DG by src family kinases.
A. Schematic representation of C-terminally tagged DG (DGHA). The N-terminal domain (white), the mucin-type domain (black) and the C-terminal domain (gray) of α-DG, β-DG and the C-terminal HA tag are indicated.
B. Detection of tyrosine phosphorylation at residue Y892 with mAb cl14a. DGHA was transiently expressed either alone or in combination with c-src. Parallel specimens were pretreated with 20 μM PP2 or mock treated with vehicle (DMSO). After 48 h, DGHA was isolated by pull-down with HA matrix. Proteins were separated and probed in Western blot with an antibody to HA (anti-HA) or mAb cl14a to β-DG phosphorylated at tyrosine 892 (anti-β-DG PY892). Apparent molecular masses and the positions of β-DG are indicated.
C. Attachment of rLCMV–LASVGP to cells induces tyrosine phosphorylation of β-DG. Monolayers of WI-26 VA4 cells were incubated with rLCMV–LASVGP or PICV (100 particles per cell) for 1 h in the cold. Unbound virus was removed and cells shifted to 37°C. At the indicated time points, cells were lysed and DG enriched by WGA affinity purification. WGA-bound glycoproteins were probed in Western blot with mAb cl14a (anti-β-DG PY892) and antibody 8D5 to β-DG. The positions of β-DG and β-DG PY892 are indicated.
D. Virus-induced tyrosine phosphorylation of β-DG is blocked by PP2. Monolayers of WI-26 VA4 cells were pretreated with 20 μM PP2 or DMSO (control) for 1 h prior to exposure to rLCMV–LASVGP. Virus-induced phosphorylation of β-DG at Y892 was assessed as in C.
E. The phosphorylation of β-DG at PY892 is not required for LASV cell entry. Monolayers of WI-26 VA4 cells were pretreated with 20 μM PP2 or DMSO (control) for 1 h as in D, followed by incubation with rLCMV–LASVGP (MOI = 1) in the cold in presence of the drug. After 1 h, unbound virus was removed and prewarmed (37°C) medium containing the drug added. At the indicated time points, 20 mM ammonium chloride was added and left throughout the experiment. At 16 h post infection, cells were fixed and infection detected by intracellular staining for LCMV NP (means ± SD, $n = 3$). The apparent differences in infection at 60 min were not statistically significant.

removed and cells quickly shifted to 37°C to allow virus internalization in presence or absence of PP2. After different time points, 20 mM ammonium chloride was added to cells and kept throughout the experiment. At 16 h post infection, cells were fixed and infection assessed by IF detection of NP. As shown in Fig. 2E, pretreatment of cells with PP2 had no significant effect on the entry kinetics of rLCMV–LASVGP, suggesting that tyrosine phosphorylation of β-DG at Y892 was dispensable for virus cell entry. To complement these inhibitor studies, we examined the entry kinetics of rLCMV–LASVGP in murine embryonic fibroblasts (MEFs) derived from mice deficient for the src family kinases src, fyn and yes (Newsome *et al*., 2006). When compared to wild-type MEFs, src/fyn/yes-deficient cells were infected with similar kinetics (data not shown), in line with our src kinase inhibitor studies (Fig. 2E).

Despite the marked inhibition of rLCMV–LASVGP internalization by genistein (Fig. 1F), treatment of cells with the src inhibitor PP2 did not affect LASV cell entry, which was rather unexpected. Examination of the sequence of the cytoplasmic tail of β-DG revealed the presence of four putative sites of tyrosine phosphorylation, in addition to Y892 (Fig. 3A). To assess tyrosine phosphorylation of β-DG in response to LASV binding at sites other than Y892, WI-26 VA4 cells were pretreated with PP2 or vehicle, followed by exposure to rLCMV–LASVGP as described above. After the indicated time points, β-DG was isolated by IP using mAb 8D5 that does not discriminate between phosphorylated and unphosphorylated β-DG. Immunocomplexes were separated by SDS-PAGE and probed in Western blot using mAb cl14a to β-DGPY892 and mAb 4G10 to tyrosine phosphate. Total β-DG was detected with polyclonal antibody AP83. As expected, treatment with PP2 prevented virus-induced phosphorylation at Y892 (Fig. 3B). However, immunoblotting with the broadly specific anti-phosphotyrosine mAb 4G10 revealed significant tyrosine phosphorylation of β-DG in response to virus binding that was not affected by PP2 (Fig. 3B). This suggested that binding of rLCMV–LASVGP to cellular DG induced tyrosine phosphorylation of β-DG at sites other than Y892, possibly implicating non-src family tyrosine kinases. Pre-

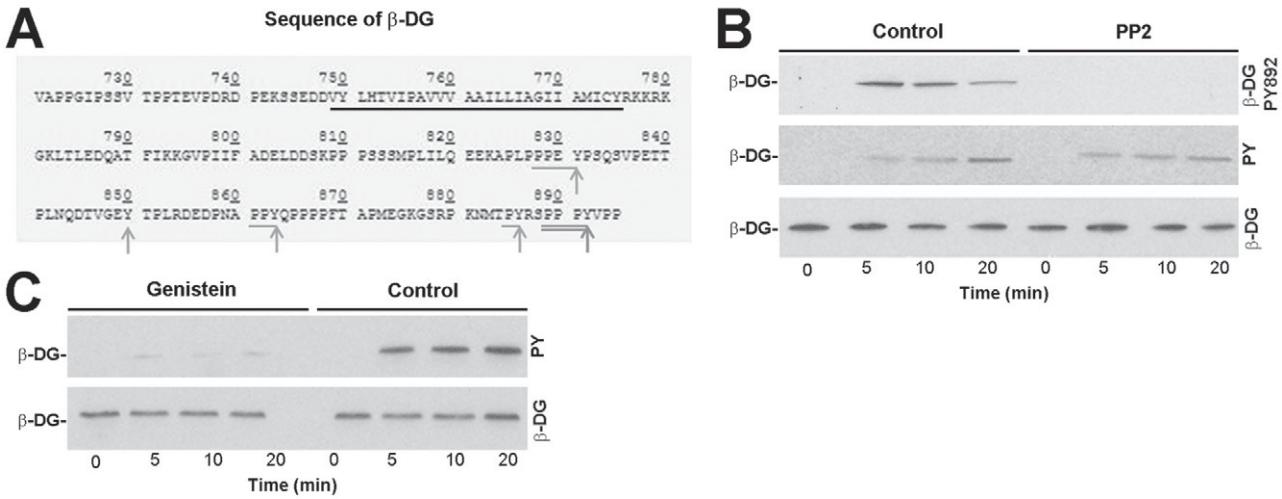

Fig. 3. Binding of LASV to cellular DG induces tyrosine phosphorylation of β-DG by non-src tyrosine kinases.
A. Sequence of the cytoplasic domain of human β-DG. The putative transmembrane domain is underlined in black and tyrosine residues indicated with gray arrows. Putative phosphorylation sites for src family kinases are underlined and Y residues marked (arrows). The PPxY motif including Y892 is double underlined.
B. Detection of virus-induced tyrosine phosphorylation β-DG in presence of PP2. WI-26 VA4 cells were pretreated with 20 μM PP2 or vehicle, followed by exposure to rLCMV–LASVGP as in Fig. 2D. After the indicated time points, β-DG was isolated by IP using mAb 8D5 against β-DG covalently coupled to sepharose 4B. Immunocomplexes were eluted under non-reducing conditions, separated by SDS-PAGE and probed in Western blot using mAb cl14a to β-DGPY892 and mAb 4G10 to tyrosine phosphate (pY). For detection of bound mouse IgG a TrueBlot® detection system was used to avoid cross reaction with the murine IgG heavy and light chains. Total β-DG was detected with rabbit polyclonal antibody AP83.
C. Genistein blocks virus-induced tyrosine phosphorylation of β-DG. Cells were pretreated with 50 μM genistein or vehicle (DMSO) only. After 30 min, rLCMV–LASVGP was added (100 particles per cell) for the indicated time points and tyrosine phosphorylation of β-DG was assessed as in B.

treatment of cells with genistein (50 μM) for 30 min reduced virus-induced tyrosine phosphorylation of β-DG altogether (Fig. 3C).

Engagement of DG by LASV GP detaches the DG complex from the adaptor utrophin

In the host cell, DG provides a molecular link between the ECM and the actin cytoskeleton by anchorage of the cytoplasmic domain of β-DG to the cytoskeletal adapter proteins dystrophin or utrophin. Cell entry of LASV occurs independently of the dynamics and stability of the actin cytoskeleton (Rojek *et al*., 2008c). We hypothesized that virus binding, possibly involving receptor clustering and signalling, may somehow detach DG from the cytoskeletal adaptors, allowing subsequent actin-independent endocytosis. A major challenge to test this hypothesis was to assess specific changes in utrophin binding to the relatively small fraction of virus-bound DG as compared to total cellular DG. To overcome this problem, we used recombinant retroviruses bearing the recombinant GP of LASV. Retroviral pseudotypes containing the GP of the New World virus Amapari (AMPV), which does not use DG as a receptor (Spiropoulou *et al*., 2002), served as a negative control. These arenavirus pseudotypes, which have been previously generated and extensively characterized in our laboratory, adopt the receptor binding characteristics and cellular tropism of the viruses from which the GPs are derived (Rojek *et al*., 2007a). Importantly, the use of retroviral pseudotypes allowed the insertion of a C-terminal FLAG tag into LASV GP and AMPV GP (Fig. 4A) allowing co-IP of the viral GP with associated cellular receptor proteins. The C-terminal FLAG tag had no adverse effect on the function of the viral GPs in host cell attachment and entry (Rojek *et al*., 2008b). Retroviral pseudotypes containing FLAG-tagged LASV GP and AMPV GP were produced and purified as described (Rojek *et al*., 2007a,b) and detection of GP in purified pseudotypes by ELISA revealed more efficient incorporation of LASV GP when compared to AMPV GP (Fig. 4B). As previously shown, infection of cells with LASV pseudotypes, but not AMPV pseudotypes or pseudotypes of VSV, depended on DG (Fig. 4C), confirming their receptor specificity.

To assess the impact of LASV GP binding on the association of cellular DG with utrophin, we incubated LASV or AMPV pseudotypes with monolayers of WI-26 VA4 cells. A parallel set of cells was incubated with medium only. This step was carried out in the cold to allow receptor binding without clustering or internalization. Cells were either kept on ice or shifted to 37°C for 10 min, followed by cell lysis in the cold. Lysates prepared from cells incu-

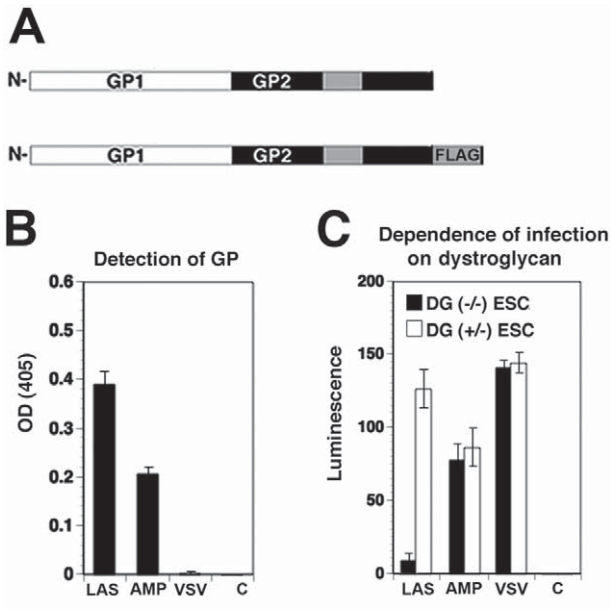

Fig. 4. Retroviral pseudotypes.
A. Schematic representation of Flag-tagged LASV GP. The receptor binding GP1 and transmembrane GP2 parts are indicated. The transmembrane domain of GP2 is represented as a grey box and the C-terminal FLAG tag indicated.
B. Detection of GP in retroviral pseudotypes. Retroviral pseudotypes were generated by cotransfection of packaging cell line GP2293 expressing retroviral Gag/Pol with a GP expression plasmid and an expression plasmid for a packable retroviral genome bearing a luciferase and a GFP reporter gene. Pseudotypes were purified by ultracentrifugation through a sucrose cushion and resuspended in HBSS. Purified pseudotypes of LASV (LAS), AMPV (AMP) and VSV, as well as pseudotypes lacking GP (C), were immobilized in microtitre plates and the viral GP detected with mAb 83.6, combined with an HRP-conjugated secondary antibody in a colour reaction (means ± SD, $n = 3$).
C. Infection of cells with LASV pseudotypes depends on DG. DG (–/–) murine embryonic stem (ES) cells and parental DG (+/–) ES cells were infected with pseudotypes of LASV (LAS), AMPV (AMP) and VSV, as well as pseudotypes lacking GP (C). After 48 h, infection was detected by luciferase assay (means ± SD, $n = 3$).

bated with pseudotypes were subjected to IP with FLAG matrix. Lysates of untreated control cells were incubated with mAb 16G4 to α-DG, combined with protein G sepharose. Immunocomplexes and total cell lysates were probed for β-DG and utrophin in Western blot. As shown in Fig. 5A, IP anti-FLAG in specimens incubated with LASV, but not AMPV, pseudotypes resulted in the detection of β-DG and co-IP of utrophin, suggesting specific pull-down of LASV GP-associated DG complex. As expected, IP of α-DG resulted in robust co-IP of utrophin under all conditions. To quantify possible changes in the ratio of utrophin/β-DG upon exposure of cells to LASV pseudotypes, we performed densitometric analysis of the signals for both conditions and normalized to the utrophin/β-DG detected in the IP of α-DG (Fig. 5B). In cells kept at 4°C, we consistently observed a significantly lower utrophin/β-DG ratio in the LASV GP-associated receptor fraction when compared to the DG–utrophin complexes isolated by IP with anti-α-DG antibody. This suggested that the LASV pseudotypes associate preferentially with cellular DG that shows a weaker or more transient association with utrophin. As shown in Fig. 5B, the temperature shift to 37°C, which allows clustering of the receptor and signalling to occur, resulted in a reduction of the utrophin/β-DG ratio in LASV GP-associated DG when compared to cells kept in the cold. This suggests that virus-induced receptor clustering and/or signalling promote(s) the dissociation of the DG complex from utrophin.

To address the role of tyrosine phosphorylation in the observed virus-induced dissociation of utrophin from DG, we tested the effect of genistein and PP2. To this end, cells were pretreated with genistein and PP2 for 1 h, followed by exposure to LASV pseudotypes. After removal of unbound virus, cells were shifted to 37°C in presence of the drugs. Virus-associated DG was isolated by IP with FLAG matrix and the ratio of utrophin/β-DG assessed as described above. Treatment with genistein significantly reduced virus-induced dissociation of utrophin from DG, whereas PP2 had only a weak effect (Fig. 5C and D). This suggested that the phosphorylation of β-DG at Y892 was dispensable for the virus-induced dissociation of utrophin from the DG complex.

To complement our inhibitor studies, we examined LASV cell entry into murine ES cells expressing a DG variant lacking the last 15 C-terminal amino acids of β-DG (DGΔC), including the PPxY motif containing Y892. Briefly, ES cells expressing either wild-type DG or DGΔC were infected with rLCMV–LASVGP at low multiplicity. To exclude differences at the level of post-entry steps in LCMV replication, a recombinant LCMV expressing VSVG was used as a control. At 4 h of infection, 20 mM ammonium chloride was added to prevent secondary infection. After 16 h, cells were fixed and infection detected by IF for LCMV NP. As shown in Fig. 5E, ES cells expressing DGΔC were as permissive as wild-type cells, suggesting that the last 15 amino acids of β-DG are dispensable for LASV cell entry.

Discussion

In the present study we investigated the role of tyrosine phosphorylation for cell entry of LASV. We show that tyrosine kinases are required for endocytosis of the virus–receptor complex, but dispensable for virus–receptor binding. Binding of LASV to cellular dystroglycan (DG) induced phosphorylation of DG at Y892 by src family kinases and other tyrosine residues of β-DG by non-src kinases. Virus-induced receptor phosphorylation was accompanied by dissociation of DG from the cytoskeletal adaptor protein utrophin, which might facilitate virus endocytosis.

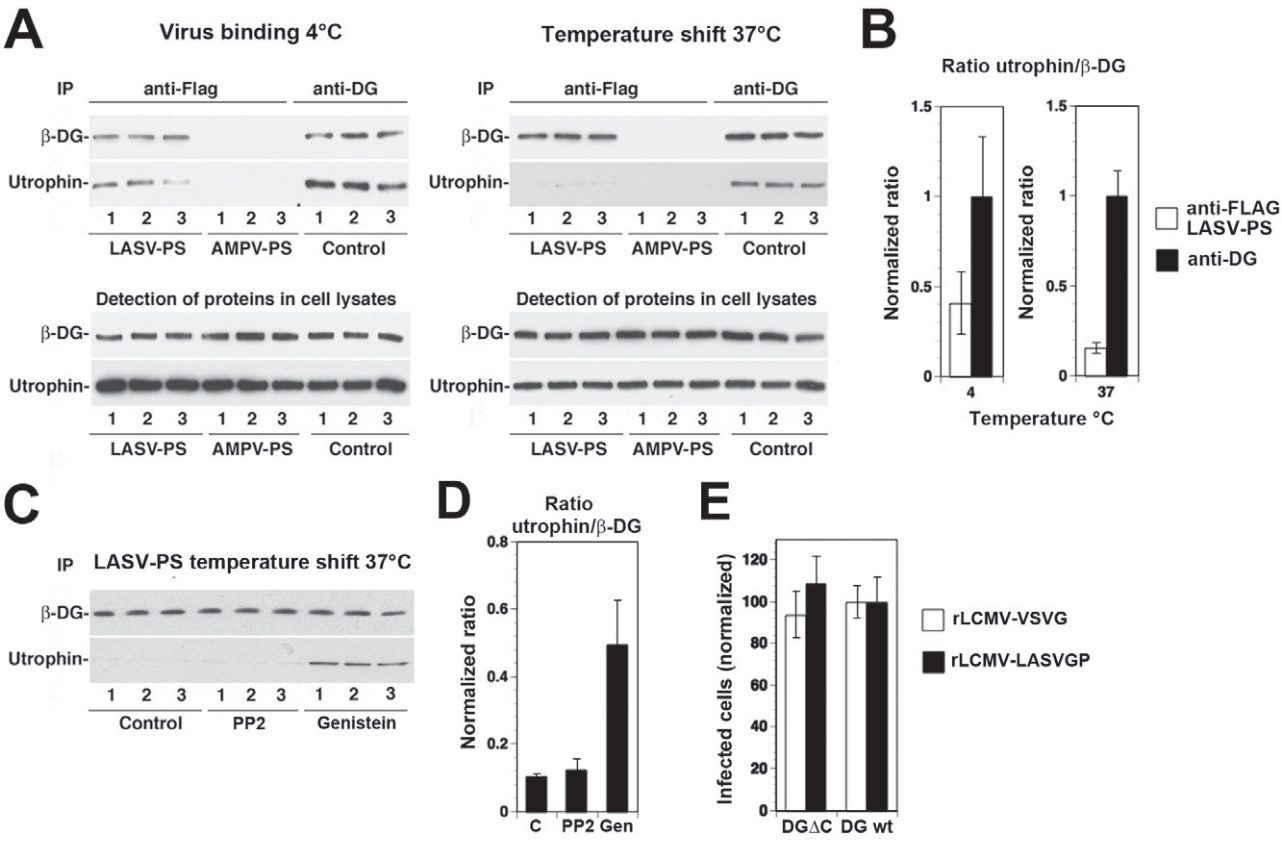

Fig. 5. Effect of LASV pseudotype binding on the association of DG with utrophin.
A. Monolayers of WI-26 VA4 cells were chilled on ice and incubated with either LASV or AMPV pseudotypes (LASV-PS, AMPV-PS) at a multiplicity of infection (MOI) of 50 transforming units (TU) per cell. Parallel specimens were incubated with mAb 16G4 to α-DG (anti-DG). After 1 h, unbound viruses or mAb were removed by washing. Cells were either kept on ice (virus binding 4°C) or shifted to 37°C for 10 min (temperature shift 37°C). Cells were quickly chilled on ice, lysed and subjected to IP using FLAG matrix or protein G-conjugated sepharose. Immunocomplexes were separated by SDS-PAGE using 100% of the IP anti-FLAG and 5% of the IP anti-DG. Beta-DG and utrophin were detected on Western blot using monoclonal antibodies 8D5 and combined with HRP-conjugated secondary antibodies in a TrueBlot® detection system to avoid cross reaction with the IgG heavy chain. For the detection of total protein in cell lysates, 1/20 of the lysates were separated by SDS-PAGE and subjected to Western blot detection.
B. Quantification of the signals in A. Blots were scanned in a densitometer and the ratios of the signals for utrophin normalized to β-DG (utrophin/β-DG) for the IP anti-FLAG (LASV pseudotypes only) and the IP anti-DG. For each series, the utrophin/β-DG ratio detected in the IP anti-DG was defined as 1.0.
C. Pretreatment with genistein, but not PP2, reduced virus-induced dissociation of utrophin from DG. Monolayers of WI-26 VA4 cells were pretreated with DMSO only (control), 20 μM PP2 and 50 μM genistein for 1 h. Cells were then chilled on ice and incubated with LASV pseudotypes (LASV-PS) for 1 h in the cold in presence of drugs. Cells were then quickly shifted to 37°C, lysed and subjected to IP with FLAG matrix as in A. Precipitated β-DG and utrophin were detected in Western blot and the ratios utrophin/β-DG determined as in B.
D. Quantification of the data in C.
E. The 15 C-terminal amino acids of β-DG are dispensable for LASV cell entry. Murine ES cells expressing either wild-type DG (DG wt) or DG lacking the C-terminal 15 amino acids of β-DG (DGΔC) were infected with rLCMV–LASVGP or rLCMV–VSVG at a multiplicity of 0.1. Infection of the cells expressing wild-type DG was set at 100% (means ± SD, $n = 3$).

In the host cell, DG provides a molecular link between the ECM and the actin-based cytoskeleton and has a slow turnover. However, engagement of DG by LASV results in rapid internalization of the virus and delivery to the late endosome (Quirin *et al.*, 2008; Rojek *et al.*, 2008c), suggesting marked changes in trafficking dynamics of DG induced by virus binding. In our study, we investigated if attachment of LASV affects post-translational modifications of DG, altering receptor trafficking in the membrane. Extending previous studies (Vela *et al.*, 2008; Kolokoltsov *et al.*, 2012), we found that treatment of cells with the broadly specific tyrosine kinase inhibitor genistein prevented LASV cell entry at an early step of virus internalization without affecting virus–receptor binding, providing a first link between tyrosine phosphorylation and viral endocytosis. Since tyrosine phosphorylation of β-DG at Y892 by src family kinases has previously been implicated in internalization of DG (Sotgia *et al.*, 2003; Miller *et al.*, 2012), we tested the effect of LASV binding on phosphorylation of the receptor at Y892. Engagement of

cellular DG by rLCMV–LASVGP rapidly induced phosphorylation of β-DG at Y892 that was blocked by the src kinase inhibitor PP2. The observed kinetics of Y892 phosphorylation was compatible with a role in viral endocytosis. However, rather unexpected, treatment of cells with PP2 had no effect on the entry kinetics of the virus. Since the cytoplasmic tail of β-DG contains four additional putative sites of tyrosine phosphorylation, in addition to Y892, we examined virus-induced phosphorylation at tyrosines. We found that LASV binding induced tyrosine phosphorylation of β-DG at sites other than Y892 with similar kinetics. These tyrosine phosphorylation events were not sensitive to PP2 and likely involved non-src tyrosine kinases. Virus-induced tyrosine phosphorylation of β-DG was entirely abrogated by genistein.

Using a pseudotype platform, we assessed the composition of DG complexes associated with LASV GP upon virion attachment. We found that LASV pseudotypes associated with a fraction of cellular DG that showed less association with utrophin. The reasons for this are currently unclear, but may be related to different accessibility of DG as a function of its association with utrophin and the actin cytoskeleton. In cells, DG associated with cell–substrate adhesion structures at the lower plasma membrane tends to colocalize with utrophin (Belkin and Smalheiser, 1996). Due to the close association with the substratum at the basal face of the cell, DG located in such substrate adhesion complexes may be less accessible to the virus.

Our analysis further revealed that engagement of DG by LASV GP results in dissociation of utrophin from β-DG. This virus-induced dissociation of utrophin could be perturbed by genistein, but not PP2, suggesting that phosphorylation of β-DG at Y892 by src kinases was dispensable. The efficient virus-induced dissociation of utrophin from DG in presence of PP2 is in line with the inability of the inhibitor to perturb viral entry. However, our findings are different from reports supporting a role for phosphorylation of β-DG at Y892 by src kinases in the regulation of utrophin binding and endocytosis of DG in prototypic primate cells (James *et al*., 2000; Sotgia *et al*., 2003) and myoblasts (Miller *et al*., 2012) in response to endogenous substrates. The reasons for this discrepancy are currently unclear. One possibility is that, contrary to our initial assumption, DG may not stay associated with the virus during the entry process. In this scenario, DG would serve as an attachment factor rather than a true entry receptor. One would have to postulate the existence of another, yet unknown receptor molecule that would mediate viral endocytosis. The recently discovered alternative LASV receptors Axl and Dtk (Shimojima *et al*., 2012) appear as interesting candidates in this context. Alternatively, binding of the multivalent virion particle to cellular DG may induce extensive clustering and activate signalling pathways that are not triggered by DG's ECM ligands, allowing the virus to 'bypass' a block in Y892 phosphorylation. To address these possibilities, studies aiming at tracking the virus–DG complex in live cells are currently launched in our laboratory.

Since virus-induced phosphorylation of β-DG is blocked by genistein, which inhibits internalization of the virus, it is tempting to speculate that virus-induced phosphorylation of β-DG is necessary for virus entry. However, in the host cell, DG is found associated with a number of cellular proteins that are required for the correct assembly and stability of the DG complex in the membrane, as well as its anchorage to the actin cytoskeleton (Barresi and Campbell, 2006). Such 'preformed' DG complexes represent the *functional units* of virus attachment. Our data have shown that virus binding to DG results in receptor signalling. Such virus-induced signalling may affect the composition of the virus–receptor complex by recruiting new proteins into the virus–DG complex and/or excluding others. During the entry process, the 'interactome' of the virus–DG complex may therefore change in a dynamic manner resulting in sorting at the plasma membrane required for subsequent cell entry. Candidate cellular proteins that interact with the virus–DG complex during the entry process and are part of this 'interactome' would represent potential substrates for tyrosine phosphorylation. We cannot exclude the possibility that tyrosine phosphorylation of such receptor-associated proteins, and not β-DG itself, is the actual target of genistein in the viral entry process.

In sum, the data at hand suggest that attachment of LASVGP to cellular DG induces tyrosine phosphorylation of β-DG at Y892 and other tyrosine residues accompanied by the dissociation of DG from utrophin. The consequent detachment of virus-bound DG from the actin-based cytoskeleton may facilitate subsequent endocytosis of the virus–receptor complex, providing a possible link between virus-induced post-translational modification of DG and virus entry.

Experimental procedures

Cell lines and viruses

WI-26 VA4 cells (ATCC CCL-95.1) were cultured in DMEM, 10% (vol/vol) FBS, supplemented with glutamine, and penicillin/streptomycin. Embryonic stem (ES) cells DG (+/–), DG (–/–) have been described (Henry and Campbell, 1998). Transgenic ES cells expressing DG lacking the last 15 amino acids (DGΔC) were generated through introduction of a triple premature stop codon affecting all possible reading frames via targeted homologous recombination (gift from Kevin P. Campbell).

The recombinant virus rLCMV–LASVGP has been described elsewhere (Rojek *et al*., 2008c) and was produced and the titres determined as previously described (Dutko and Oldstone, 1983). Recombinant LASV GP and AMPV GP containing a C-terminal

FLAG tag have been described (Rojek *et al.*, 2008b). Retroviral pseudotypes expressing GFP and luciferase reporters were produced and concentrated, and titres determined as described (Rojek *et al.*, 2006). Concentrated pseudotypes were diluted in HBSS at 10^7 transforming units (TU) ml^{-1}. For detection of viral GP in ELISA, purified pseudotypes were immobilized in microtitre plates at 10^6 TU ml^{-1} and the viral GP detected as described (Rojek *et al.*, 2008a). Recombinant VSV pseudotyped with LASV GP (rVSVΔG–LASVGP) and VSV GP (rVSVΔG–VSVG) were generated as reported previously (Kunz *et al.*, 2005a). Virus titres were determined by the infection of Vero E6 cell monolayers and detection of GFP-positive cells by fluorescence microscopy.

Antibodies and reagents

Monoclonal antibodies (mAbs) 113 (anti-LCMVNP) and 83.6 (anti-LCMVGP) have been described (Buchmeier *et al.*, 1981; Weber and Buchmeier, 1988), as has mAb IIH6 anti-α-DG (Ervasti and Campbell, 1991). Other mAbs included mouse IgG 8D5 anti-β-DG (Novocastra) and mouse IgG 16C4 to α-DG (provided by Kevin P. Campbell), mouse IgG anti-utrophin from St Cruz Biotechnology (St Cruz, CA, USA), mAb cl14a to phospho-β-DG PY982 (BD Bioscience) and mAb 4G10 to phosphotyrosine (St Cruz, CA, USA). Rabbit anti-influenza HA (Y11) was from St Cruz Biotechnology (St Cruz, CA, USA). Polyclonal rabbit anti-mouse secondary antibodies conjugated to HRP were from Dako (Glostrup, Denmark) and goat anti-mouse antibodies conjugated with Rhodamin Red X were from Jackson Immuno Research Laboratories. Genistein and PP2 were purchased from Calbiochem. The Bright-Glo® luciferase assay and Cell Titer Glo® assay systems were obtained from Promega (Madison, WI, USA).

Immunoblotting

Proteins were separated by gel electrophoresis using 12% polyacrylamide gels and transferred to nitrocellulose. After blocking in 5% (wt/vol) skim milk in PBS, membranes were incubated with Abs used at following concentrations: mAb 8D5, mAb cl14a, mAb 4G10 and polyclonal Abs AP83, and Y11 (10 μg ml^{-1}) in 2% (wt/vol) skim milk, PBS for 12 h at 6°C. Secondary Abs coupled to HRP were applied 1:5000 in PBS, 0.1% (wt/vol) Tween for 1 h at room temperature. Blots were developed by enhanced chemiluminescence (ECL) using Super Signal West Pico ECL Substrate or TrueBlot® detection system (Pierce), where indicated.

Infection of cells with retroviral pseudotypes

Cells were plated in 96-well plates in a density of 10^4 cells per well. After 24 h, retroviral pseudotypes were added at the indicated MOI and incubated for 1 h at 37°C. The viral particles were removed, cells washed twice with DMEM and fresh medium added. Infection was quantified by Bright Glo® luciferase assay. Luminescence was calculated as fold increase over background signals obtained from uninfected cells.

Infection of cells with rLCMV–LASVGP

Cells were plated in 96-well plates in a density of 10^4 cells per well. After 24 h, cells were pretreated with genistein or PP2 as indicated, followed by infection with rLCMV–LASVGP at the indicated MOI for 1 h at 37°C. Unbound virus were removed, cells washed twice with DMEM and fresh medium added. Infection was quantified by detection of LCMV NP in IF as described (Kunz *et al.*, 2004). Cell entry kinetics of rLCMV–LASV in presence and absence of PP2 were performed as described (Rojek *et al.*, 2008c). Blocking of infection with mAb IIH6 was carried out as reported (Kunz *et al.*, 2005b).

Virus internalization assay

Purification of rLCMV–LASVGP was performed by ultracentrifugation on a renografin gradient. Purified LCMV was labelled with the thiol-cleavable reagent NHS-SS-biotin (Pierce). The cleavage of the biotin label was verified by reaction with the membrane-impermeable reducing agent Tris(2-carboxyethyl)phosphine (TCEP) (10 mM) (Pierce) for 30 min, which resulted in a loss of > 95% of the biotin label. Internalization assay was performed as described previously (Rojek *et al.*, 2008a). Briefly, cells were cultured in 10 cm dishes to obtain closed monolayers. Medium was removed, and cells were washed twice with cold HBSS and chilled on ice for 5 min. Cold solution containing NHS-SS-biotinylated rLCMV–LASVGP (10^7 pfu ml^{-1}) in HBSS was added. After incubation for 1 h on ice, unbound virus was removed and cells were washed with cold HBSS. For internalization, cells were shifted to 37°C. After the indicated incubation times, medium was removed and cells chilled on ice. TCEP (15 mM) in 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ was added (5 ml per dish) and applied twice for 30 min on ice. Cells were washed three times with cold HBSS, and the remaining TCEP was quenched with 100 mM iodoacetamide for 10 min, and cells were lysed immediately. LCMV GP2 was isolated by IP as described (Rojek *et al.*, 2008a). Immunocomplexes were separated by non-reducing SDS-PAGE. Biotinylated LCMV GP2 was detected by Western blotting with HRP-conjugated streptavidin as described above.

Detection of DG–utrophin complexes associated with LASV GP

Triplicate cultures of WI-26 VA4 cells were cultured in 10 cm plates over night to obtain closed monolayers. Retroviral pseudotypes of LASV and AMPV (10^7 TU ml^{-1} in HBSS) were added at 50 TU per cell. Parallel specimens were incubated with 10 μg ml^{-1} mAb 16C4 to α-DG in HBSS. After incubation for 1 h in the cold, cells were washed three times with cold HBSS. One series of cultures was kept on ice whereas the other was quickly shifted to 37°C in a water bath. After 10 min, cells were chilled on ice. Cells were lysed for 30 min in 1% (wt/vol) β-octylglucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, 50 mM Hepes pH 7.5, 10% (wt/vol) glycerin supplemented with protease inhibitor cocktail Complete® (Roche) and 1 mM PMSF. Cleared lysates were subjected to IP with FLAG matrix (Sigma) or protein G-conjugated sepharose 4B (Sigma) for 2 h at 6°C. After three short washes in lysis buffer, the matrix was eluted with non-reducing SDS-PAGE sample buffer to minimize elution of IgG from the FLAG matrix. After addition of 100 mM DTT (final concentration) and boiling for another 5 min Western blot was performed as described above. For quantification, X-ray films were scanned with a Storm densitometer, and acquired data were processed using Image Quant software.

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Original article

"Binding of Lassa virus perturbs extracellular matrix-induced signal transduction *via* dystroglycan"

Jillian M. Rojek[4], Marie-Laurence Moraz[1], Christelle Pythoud[1], Sylvia Rothenberger[1], F. Gisou Van der Goot[2], Kevin P. Campbell[3] and Stefan Kunz[1*]

[1] Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne, Switzerland

[2] Global Health Institute, Ecole Polytechnique Fédérale de Lausanne, Faculty of Life Sciences, Lausanne, Switzerland

[3] Howard Hughes Medical Institute, Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center, Department of Molecular Physiology and Biophysics, Department of Neurology, and Department of Internal Medicine, Roy J and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, IA 52242, USA.

[4] Department of Immunology and Microbial Science, The Scripps Research Institute La Jolla, CA 92037, USA

* Corresponding author. Mailing address: Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne CH-1011, Switzerland. Phone: +41-21 314 7743, Fax: +41-21 314 4060, E-mail Stefan.Kunz@chuv.ch

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Binding of Lassa virus perturbs extracellular matrix-induced signal transduction via dystroglycan

Jillian M. Rojek,[4†] Marie-Laurence Moraz,[1] Christelle Pythoud,[1] Sylvia Rothenberger,[1] F. Gisou Van der Goot,[2] Kevin P. Campbell[3] and Stefan Kunz[1]*

[1]*Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne, Switzerland.*
[2]*Global Health Institute, Ecole Polytechnique Fédérale de Lausanne, Faculty of Life Sciences, Lausanne, Switzerland.*
[3]*Howard Hughes Medical Institute, Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center, Department of Molecular Physiology and Biophysics, Department of Neurology, and Department of Internal Medicine, Roy J and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, IA 52242, USA.*
[4]*Department of Immunology and Microbial Science, The Scripps Research Institute La Jolla, CA 92037, USA.*

Summary

The arenavirus Lassa virus (LASV) causes a severe haemorrhagic fever with high mortality in man. The cellular receptor for LASV is dystroglycan (DG). DG is a ubiquitous receptor for extracellular matrix (ECM) proteins, which cooperates with β1 integrins to control cell–matrix interactions. Here, we investigated whether LASV binding to DG triggers signal transduction, mimicking the natural ligands. Engagement of DG by LASV resulted in the recruitment of the adaptor protein Grb2 and the protein kinase MEK1 by the cytoplasmic domain of DG without activating the MEK/ERK pathway, indicating assembly of an inactive signalling complex. LASV binding to cells however affected the activation of the MEK/ERK pathway via α6β1 integrins. The virus-induced perturbation of α6β1 integrin signalling critically depended on high-affinity LASV binding to DG and DG's cytoplasmic domain, indicating that LASV–receptor binding perturbed signalling cross-talk between DG and β1 integrins.

Received 9 January, 2012; revised 21 February, 2012; accepted 5 March, 2012. *For correspondence. E-mail Stefan.Kunz@chuv.ch; Tel. (+41) 21 314 7743; Fax (+41) 21 314 4060.
†Present address: Genomics Institute of the Novartis Research Foundation, La Jolla, CA 92037, USA.

Introduction

Lassa virus (LASV) is the causative agent of a severe haemorrhagic fever with high mortality in humans that is endemic to West Africa and infects several hundred thousand individuals per year with thousands of deaths (Geisbert and Jahrling, 2004). There is neither a licensed vaccine nor an efficacious treatment for this disease, resulting in 15–30% mortality in hospitalized patients (McCormick and Fisher-Hoch, 2002). Despite the widespread viral replication in fatal Lassa fever cases, histological analysis revealed only modest infiltration of inflammatory cells (Walker *et al.*, 1982). The terminal shock syndrome associated with fatal Lassa fever occurs without evidence of massive cellular necrosis, vascular damage or bleeding, suggesting that direct perturbation of host cell function by LASV contributes to disease (McCormick and Fisher-Hoch, 2002; Kunz, 2009; Moraz and Kunz, 2011). The analysis of the virus–host cell interaction and the consequences for host cell function are therefore important to understand the viral pathogenesis underlying fatal Lassa fever.

LASV belongs to the family *Arenaviridae*, enveloped negative strand RNA viruses with a non-lytic life cycle confined to the cytoplasm (Buchmeier *et al.*, 2007). The bisegmented genome of LASV codes for only four proteins, an RNA-dependent RNA polymerase (L), the viral nucleoprotein (NP), the matrix protein (Z) and the precursor of the viral envelope glycoprotein (GPC) (Buchmeier *et al.*, 2007). The GPC precursor is processed by the cellular protease subtilisin kexin isozyme-1(SKI-I)/site-1 protease (S1P) yielding the mature envelope proteins GP1 and GP2. (Lenz *et al.*, 2001; Beyer *et al.*, 2003; Rojek *et al.*, 2008c). The GP1 part is implicated in binding to cellular receptor(s), whereas GP2 resembles fusion-active portions of other viral GPs (Eschli *et al.*, 2006; Igonet *et al.*, 2011).

The first cellular receptor discovered for LASV and other Old World arenaviruses is α-dystroglycan (α-DG), the peripheral subunit of DG, a ubiquitously expressed versatile receptor for extracellular matrix (ECM) proteins

(Cao *et al*., 1998; Oldstone and Campbell, 2011). Very recently, additional candidate receptors for LASV have been reported: the C-type lectins DC-SIGN, LSECtin, as well as the Tyro3/Axl/Mer (TAM) receptor tyrosine kinases Axl and Tyro3 (Shimojima *et al*., 2012). While the C-type lectins have expression patterns limited to specific cell types, the TAM receptors Tyro3 and in particular Axl are widely expressed, including cell types positive for DG. Notably, in cells coexpressing DG and Axl, DG seems to be the preferred receptor for LASV (Shimojima *et al*., 2012), suggesting a role for TAM receptor kinases as LASV receptors primarily in cells lacking functional DG.

Dystroglycan is expressed in most developing and adult tissues, typically in cell types that adjoin basement membranes (Durbeej *et al*., 1998) and is crucial for normal cell–matrix adhesion (Henry and Campbell, 1998; Henry *et al*., 2001). Mutations affecting DG in humans result in severe congenital diseases (Barresi and Campbell, 2006). Encoded as a single protein, DG is proteolytically processed into α-DG, which is extracellular, and β-DG which is membrane anchored (Barresi and Campbell, 2006). DG provides a molecular link between the ECM and the actin-based cytoskeleton. Alpha-DG binds with high affinity to the ECM proteins laminin, agrin, perlecan and neurexins and is non-covalently associated with β-DG that interacts with the cytoskeletal adaptor proteins dystrophin and utrophin, which anchor the DG complex to the actin-based cytoskeleton (Ervasti and Campbell, 1991; 1993). The cytoplasmic domain of β-DG was found to interact with the signalling adaptor molecule Grb2 (Yang *et al*., 1995), the protein kinases MEK, ERK and focal adhesion kinase (FAK) (Yang *et al*., 1995; Cavaldesi *et al*., 1999; Spence *et al*., 2004), suggesting a role of DG in cellular signal transduction.

Several lines of evidence support a functional interaction between DG and β1 integrins in the assembly of laminin-based ECM structures (Henry *et al*., 2001). Laminins recognize α-DG and β1 integrins via distinct LG modules located in the C-terminal G domain of their α-chain, LG4 and LG1–3 respectively (Hohenester *et al*., 1999). Interestingly, DG and α6β1 integrins seem to have opposing roles in the regulation of the canonical MEK/ERK MAP kinase pathway in response to laminin (Ferletta *et al*., 2003). Binding of laminin LG1–3 to α6β1 integrins activates MEK/ERK signalling, whereas attachment of LG4 to α-DG resulted in a dose-dependent reduction of MEK/ERK activation (Ferletta *et al*., 2003). Simultaneous binding of laminin to the two receptors likely results in a defined equilibrium of MEK/ERK signalling critical for normal cell–matrix adhesion.

In mammals, α-DG undergoes complex post-translational modifications that are crucial for its function as an ECM receptor (Barresi and Campbell, 2006; Kanagawa and Toda, 2006). Of particular importance are specific O-glycan modifications of α-DG's mucin-type domain, which involve protein O-mannosylation and modifications by the glycosyltransferases LARGE and LARGE2 (Barresi *et al*., 2004). We and others have shown that protein O-mannosylation and LARGE-dependent modifications are also crucial for α-DG's function as a high-affinity receptor for arenaviruses (Kunz *et al*., 2005b) and that the viruses closely mimic the molecular mechanisms of receptor recognition by ECM proteins (Rojek *et al*., 2007a). As a consequence LASV competes with ECM proteins for receptor binding, resulting in displacement of DG-bound ECM (Kunz *et al*., 2005a), likely affecting DG's function in the host cell. In the present study, we investigated the impact of LASV binding to α-DG on DG-mediated signal transduction. We found that engagement of LASV affected the interaction of DG with signalling molecules and perturbed the signalling cross-talk with β1 integrins.

Results

Binding of inactivated LASV to cellular DG triggers recruitment of Grb2 and MEK1 without activating MEK1

Considering the high binding affinity of LASV for α-DG and the multivalent nature of the virus particles, we hypothesized that virus attachment may result in extensive receptor clustering with possible effects on DG-mediated signal transduction. Since LASV is a BSL4 pathogen, work with live virus is restricted to laboratories with high security containment. To circumvent this problem, we used either inactivated virus or recombinant retroviruses bearing the envelope glycoprotein of LASV (Reignier *et al*., 2006; Rojek *et al*., 2006). Since receptor binding and host cell entry of arenaviruses are mediated exclusively by the viral GP, recombinant retroviruses displaying LASV GP adopt the receptor binding characteristics of LASV (Reignier *et al*., 2006). They represent a suitable BSL2 surrogate for LASV and have been widely used to study LASV–receptor interactions (Kunz *et al*., 2005a,b; Reignier *et al*., 2006; Rojek *et al*., 2007a). As a cell culture model, we chose the human lung epithelial cell line WI-26 VA4, which had previously been utilized for studies on DG signalling (Ferletta *et al*., 2003). Alpha-DG isolated from WI-26 VA4 cells by lectin affinity purification bound inactivated LASV with high affinity, as assessed by virus overlay protein binding assay (Fig. 1A). In a next step, we verified if cell attachment and entry of LASV into WI-26 VA4 cells was indeed mediated by α-DG. For this purpose, cells were pre-treated with increasing concentration of the monoclonal antibody (mAb) IIH6 that recognizes a functional glycan epitope on α-DG (Kanagawa *et al*., 2004) and competes with virus binding (Kunz *et al*., 2005b). Cells were then infected with LASV pseudotypes

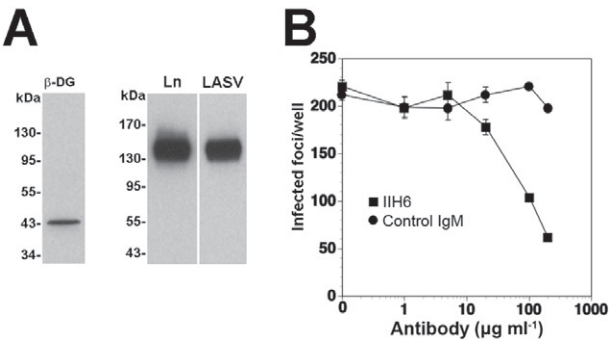

Fig. 1. Cell entry of LASV pseudotypes in WI-26 VA4 human lung epithelial cells is mediated by DG.
A. WI-26 VA4 cells express DG that is functional as LASV receptor. WI-26 VA4 cells were lysed and glycoproteins enriched by affinity purification with the lectin wheat germ agglutinin. Functional α-DG was detected by laminin overlay assay (Ln) and virus overlay protein binding assay using inactivated virus (LASV).
B. Blocking of infection with mAb IIH6. Monolayers of WI-26 VA4 cells were blocked with mAb IIH6 or an unrelated mouse IgM (Control IgM) at the indicated concentrations for 2 h at 4°C. Next, LASV pseudotypes (200 PFU) were added for 45 min. Infection was assessed after 24 h by immunofluorescence staining for GFP. Infected foci were counted in each well (means ± SD, $n = 3$).

containing a green fluorescent protein (GFP) reporter in their genome at a multiplicity of infection (moi) of 0.01 infectious virus particle per cell. Infected cells were detected after 24 h by immunofluorescence (IF) detection of the GFP reporter. Pre-treatment with mAb IIH6, but not an IgM isotype control significantly blocked infection with LASV pseudotypes in a dose-dependent manner, confirming DG as a major receptor for LASV in these cells (Fig. 1B). The only partial inhibition of LASV pseudotype infection in presence of 200 μg ml^{-1} mAb IIH6 is consistent with earlier studies (Kunz *et al*., 2005b) and is likely due to the high binding affinity of LASV GP to α-DG.

The cytoplasmic tail of β-DG can associate with signalling molecules, including Grb2, MEK, ERK and FAK, suggesting a role in cellular signal transduction (Yang *et al*., 1995; Cavaldesi *et al*., 1999; Spence *et al*., 2004). We next assessed the impact of LASV binding to cellular α-DG on the association of β-DG with these signalling molecules. Since available anti-β-DG antibodies recognize the cytoplasmic domain, interference with the binding of signalling molecules was a concern. To circumvent this problem, we made use of a recombinant full-length DG containing a C-terminal spacer sequence of four amino acids (GGGS), followed by an HA-tag (DGHA) (Fig. 2A). Previous studies demonstrated that this C-terminal tagging of β-DG had no influence on the biosynthesis, transport and function of DG (Rojek *et al*., 2007b). Considering the biosafety restrictions of work with live LASV and to avoid any possible effect due to virus replication, we used inactivated LASV to study the effect of virus binding on DG signalling. For this purpose, LASV (strain Josiah) was grown in a BSL4 facility of the Centers for Disease Control and inactivated by gamma irradiation as described (Spiropoulou *et al*., 2002). As a control, we used inactivated Amapari virus (AMPV), a New World arenavirus that does not use DG as a receptor (Spiropoulou *et al*., 2002). Previous studies had shown that our inactivation protocol abrogated virus replication, but did not affect receptor binding (Spiropoulou *et al*., 2002). In a first step, we determined binding of LASV and AMPV to WI-26 VA4 cells. Briefly, monolayers of WI-26 VA4 cells were cultured in microtitre plates, chilled on ice and incubated with increasing concentrations of inactivated virus. After removing unbound virus, cells were fixed. Bound virus was detected with mAb 83.6 that recognizes a highly conserved epitope in LASV and AMPV GP2 (Weber and Buchmeier, 1988), combined with a HRP-labelled secondary antibody in a colour reaction. Both LASV and MAPV bound WI-26 VA4 cells to a similar extent in a dose-dependent manner (Fig. 2B). Next, WI-26 VA4 cells were transfected with DGHA, using un-tagged DG as a control. After 48 h, cells were incubated with inactivated LASV and AMPV (100 particles per cell) in the cold, allowing virus attachment without receptor clustering. After removal of unbound virus, the temperature was shifted to 37°C for 10 min. Cells were rapidly chilled, cold detergent extracts prepared and cleared lysates subjected to immunoprecipitation (IP) with anti-HA matrix. Immunocomplexes were probed for the presence of DGHA, Grb2, Sos, FAK, MEK1/2 and ERK1/2 in Western blot. Similar amounts of DGHA were detected in IPs from cells incubated with LASV, AMPV and mock-treated cells (Fig. 2C), excluding receptor downregulation and/or degradation as a consequence of virus binding. In line with published data (Yang *et al*., 1995; Spence *et al*., 2004), we detected an association of β-DG with Grb2 and MEK1/2, whereas neither FAK, Sos nor ERK was detected in anti-HA immunocomplexes in this cell type under our experimental conditions (Fig. 2D and E). Binding of LASV, but not AMPV resulted in a significant increase in the amounts of β-DG-associated Grb2 (Fig. 2D and F) and MEK1 (Fig. 2E and F), indicating recruitment of Grb2 and MEK1 upon virus binding. In a next step, we investigated if the observed LASV-induced recruitment of Grb2 and MEK1 resulted in activation of MEK1. For this purpose, we exposed cells to LASV and AMPV, isolated HA-tagged β-DG at different time points as described above. Immunocomplexes were probed with a mAb specific for phosphorylated (activated) MEK1 in Western blot and signals normalized to total MEK1. At no time point did we observe significant phosphorylation of MEK1 (data not shown), suggesting that the observed virus-induced recruitment of Grb2 and MEK1 results in assembly of an inactive signalling complex associated with β-DG.

 Cellular Microbiology, **14**, 1122–1134

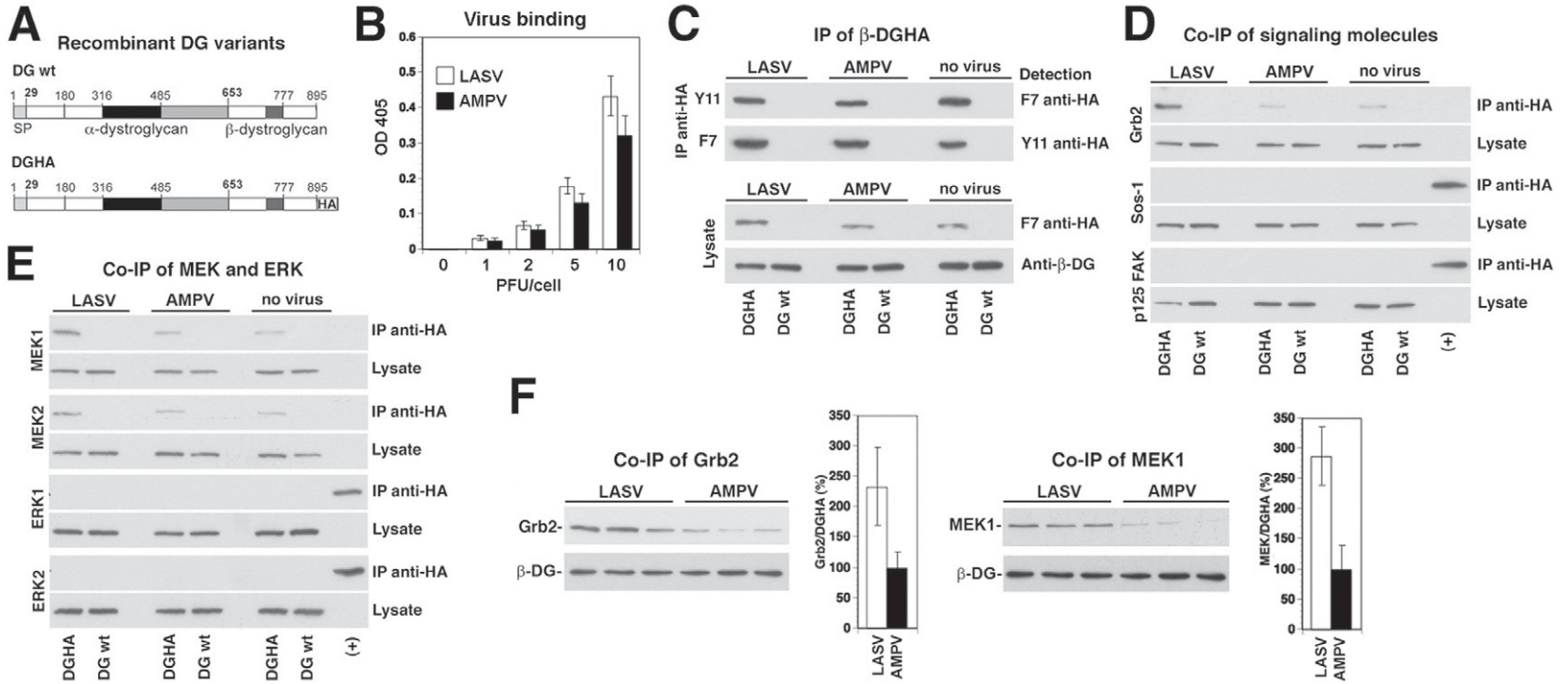

Fig. 2. Binding of inactivated LASV to cells promotes association of β-DG with Grb2.

A. Schematic representation of C-terminally tagged DG (DGHA). The N-terminal domain (white), the mucin-type domain (black) and the C-terminal domain (grey) of α-DG, β-DG and the C-terminal HA tag are indicated.

B. Binding of inactivated LASV and AMPV to WI-26 VA4 cells: cell monolayers were incubated with the indicated concentrations of inactivated virus in the cold. Bound virus was detected with mAb 83.6 to arenavirus GP2, combined with a HRP-conjugated secondary antibody in a colour reaction (means ± SD, $n = 3$).

C. IP of β-DGHA: WI-26 VA4 cells transiently transfected with either DGHA or wild-type DG (DG wt) were seeded on poly-L-lysine and incubated with inactivated LASV, AMPV, or no virus at a particle per cell ratio of 100. After 20 min, cells were lysed and DGHA precipitated with either a polyclonal rabbit antibody anti-HA Y11 or mouse mAb F7 anti-HA. Immunocomplexes were probed for HA in Western blot using the indicated antibodies. Total-cell lysates were probed for DGHA with mAb F7 anti-HA and for β-DG with pAb AP83 anti-β-DG.

D and E. Co-immunoprecipitation (co-IP) of β-DGHA with signalling molecules: immunocomplexes and total lysates (C) were probed for the presence of Grb2, Sos-1, FAK, MEK1/2 and ERK1/2 in Western blot. In case of Sos-1, FAK and ERK1/2, a positive control corresponding to 0.1% of total-cell protein was included (+).

F. Binding of inactivated LASV increases the association of β-DG with Grb2 and MEK1: triplicate specimens of WI-26 VA4 cells transiently transfected with DGHA were exposed to inactivated viruses, lysed and DGHA precipitated as in (C). DGHA, Grb2 and MEK1 were detected in Western blot as in (D) and (E). For quantitative analysis, X-ray films were scanned with a densitometer and the ratios of Grb2/DGHA and MEK1/Grb2 calculated. For normalization, signals obtained with the AMPV-negative control were defined as 100% ($n = 3$, ±SD).

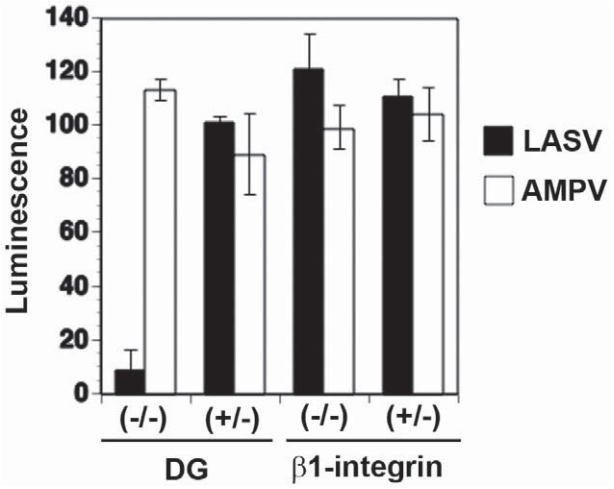

Fig. 3. Infection of LASV pseudotypes is independent on β1 integrins. DG (–/–) and DG (+/–), β1 integrin (–/–) and β1 integrin (+/–) mouse ES cells cultured in 96-well plates were infected with the retroviral pseudotypes bearing the GPs of LASV or AMPV (moi = 10). Infection was assessed after 48 h by luciferase assay ($n = 3$, ±SD).

Infection with LASV pseudotypes depends on DG, but not β1 integrins

Several lines of evidence indicate that DG functionally interacts with β1 integrins in the host cell (Henry *et al*., 2001; Ferletta *et al*., 2003). While the role of DG in host cell attachment and entry of LASV has been well established, a possible function of β1 integrins in LASV cell entry had not yet been addressed. To address this issue, we exploited the fact that LASV efficiently infects wild-type murine embryonic stem (ES) cells (Spiropoulou *et al*., 2002). To define a possible role of β1 integrins in LASV cell entry, we employed a murine ES cell line deficient in β1 integrin (–/–) and the parental hemizygous (+/–) line (Fassler and Meyer, 1995; Fassler *et al*., 1995). To separate virus entry mediated by the envelope GP of LASV from subsequent steps of virus replication, we employed recombinant retroviruses pseudotyped with LASVGP that contained a luciferase reporter in their genome (Rojek *et al*., 2007a). As a control, we used retroviral pseudotypes containing the GP of AMPV, which is independent of DG. Murine ES cell with the genotypes DG (+/–), DG (–/–), β1 integrin (+/–) and β1 integrin (–/–) were infected with retroviral pseudotypes bearing the GPs of LASV and AMPV. Since murine retroviruses show relatively low levels of reporter gene expression in murine ES cells (Reignier *et al*., 2006), pseudotype infection was performed at high moi (10). Infection was assessed after 48 h by luciferase assay. In line with previous reports, cells deficient in DG showed markedly reduced susceptibility to LASV pseudotypes, whereas AMPV pseudotype infection was not affected (Fig. 3). In contrast, cells deficient in β1 integrins showed similar susceptibility to both pseudotypes (Fig. 3) indicating that β1 integrins are dispensable for LASVGP-mediated cell attachment and entry.

Attachment of inactivated LASV and LASV pseudotypes to cells perturbs activation of MEK/ERK signalling by laminin

Previous studies demonstrated that cell adhesion to laminin results in activation of the MEK/ERK pathway via β1 integrins, which is counterbalanced by DG (Ferletta *et al*., 2003). To validate our experimental system, we monitored the activation of the MEK/ERK pathway in WI-26 VA4 cells in response to cell adhesion to laminin employing a cell adhesion assay described previously (Ferletta *et al*., 2003). Briefly, WI-26 VA4 cells were cultured for 16 h under serum starvation, detached and single-cell suspension prepared. Cells were then added to culture dishes coated with purified mouse laminin-1 or left in suspension. At different time points, cells were lysed, total protein extracted and the phosphorylation of MEK and ERK detected by specific antibodies to the phosphorylated forms of the kinases in Western blot. For normalization, total MEK/ERK was detected with antibodies that are insensitive to the phosphorylation state. Adhesion of WI-26 VA4 cells to laminin-1 resulted in the induction of phosphorylation of MEK and ERK with maximal activation after 40–60 min, a kinetic profile consistent with published work (Ferletta *et al*., 2003) (Fig. 4A). Addition of the specific MEK inhibitor PD98059 abrogated phosphorylation of ERK, confirming that the enhanced phosphorylation of ERK was mediated by MEK (Fig. 4B).

Next, we addressed the impact of LASV binding to cellular α-DG on laminin-induced signalling. For this purpose, single-cell suspensions of WI-26 VA4 cells were mixed with inactivated LASV or AMPV (10 particles per cell) immediately before adding to plates coated with laminin-1. The presence of the viruses did not affect the number of adherent cells (data not shown), excluding simple blocking of cell adhesion by the virus. After 40 min, cells were lysed and the phosphorylation of MEK and ERK detected. As shown in Fig. 4C, cell adhesion to laminin-1 in presence of LASV, but not AMPV resulted in significantly reduced activation of MEK and ERK phosphorylation (Fig. 4C and D).

The MEK/ERK kinase pathway is influenced by many cellular signalling cascades including cellular responses to stress. To exclude artefacts due to unknown contaminations that may be present in our inactivated virus preparations, we performed analogous experiments with retroviral pseudotypes for LASV and AMPV. As shown in Fig. 4E and F, adhesion of WI-26 VA4 cells to laminin in

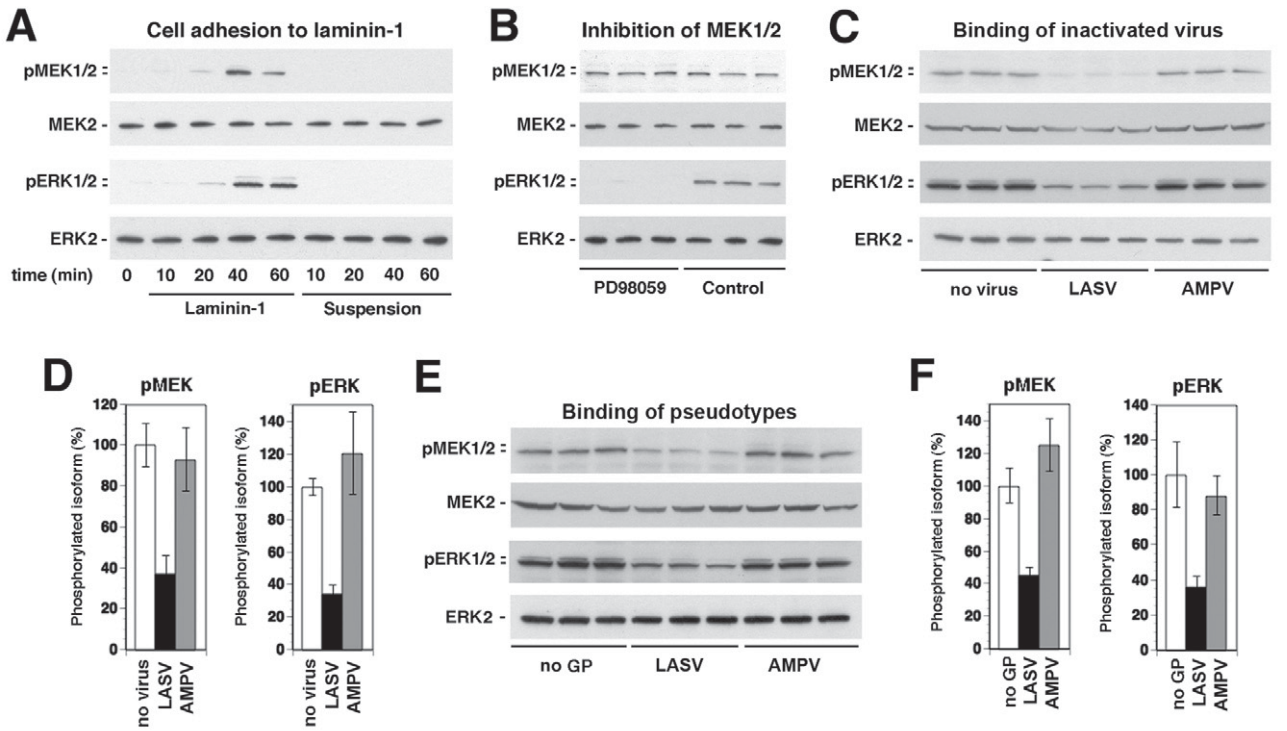

Fig. 4. Binding of inactivated LASV to cells perturbs laminin-induced activation of the ERK-MAP kinase pathway.
A. Phosphorylation of MEK and ERK in WI-26 VA4 cells in response to cell adhesion to laminin-1. Serum-starved cells were detached and seeded onto wells coated with laminin-1 or kept in suspension. At the indicated time points, total-cell lysates were prepared and subsequently analysed for the presence of MEK1/2 and ERK1/2 as well as the phosphorylated forms of the kinases using specific antibodies.
B. ERK is phosphorylated via MEK: suspensions of serum-starved cells were incubated with the MEK-specific inhibitor PD98059 (50 μM) or solvent control for 30 min and then plated onto wells coated with laminin-1 for 40 min. The phosphorylated forms of MEK1/2 and ERK1/2, MEK2 and ERK2 were detected in total-cell lysates.
C. Binding of inactivated LASV to cells reduces laminin-induced activation of MEK and ERK: serum-starved WI-26 VA4 cells were detached, mixed with inactivated LASV or AMPV (10 particles per cell), or no virus, and plated onto wells coated with laminin-1. After 40 min of cell adhesion, total-cell lysates were prepared and analysed for the presence of phosphorylated MEK and ERK as in (A).
D. Quantification of (C): the signal for phosphorylated MEK1/2 and ERK1/2 in the control samples set as 100% ($n = 3$, ±SD).
E and F. Binding of LASV pseudotypes reduces laminin-induced activation of MEK and ERK: experiment was performed as in (C) and (D) using retroviral pseudotypes of LASV and AMPV (10 PFU per cell) and pseudotypes without GP (no GP).

presence of pseudotypes gave similar results than obtained with inactivated viruses. Only the presence of pseudotypes bearing the GP of LASV, but not AMPV significantly reduced the induction of MEK/ERK phosphorylation in response to cell adhesion to laminin. Together, the data suggest that LASV attachment to cells somehow perturbs laminin-induced activation of the MEK/ERK pathway.

Activity of the MEK/ERK pathway is dispensable for cell entry of LASV pseudotypes

The ability of LASV to modulate cellular MEK/ERK signalling raised the possibility that this pathway may be involved in cell entry of the virus. To test this possibility, we pre-treated cells with the MEK inhibitor PD98059 at concentrations that abrogated ERK phosphorylation (Fig. 4B), followed by infection with LASV and AMPV pseudotypes. When assessed after 48 h, pre-treatment with the MEK inhibitor did not significantly affect LASV pseudotype infection, indicating that MEK/ERK signalling is dispensable for LASVGP-mediated cell attachment and entry (Fig. 5A). Since these first experiments detected infection at 48 h post infection, i.e. at a late time point, we next examined the cell entry kinetics of LASV in presence of the MEK inhibitor PD98059. Upon receptor binding, LASV is taken up by clathrin- and caveolin-independent endocytosis and rapidly delivered to late endosomes, where low pH-dependent membrane fusion occurs (Borrow and Oldstone, 1994; Quirin *et al.*, 2008; Rojek *et al.*, 2008a,b). To assess how fast receptor-bound LASV pseudotypes trafficked to late endosomes, we determined the time required for the virus to become resistant to the lysosomotropic agent ammonium chloride. When added to cells, ammonium chloride raises the endosomal pH rapidly and blocks low pH-dependent membrane fusion without causing overall cytotoxicity (Ohkuma and Poole, 1978; 1981). WI-26 VA4 cells were either pre-treated with

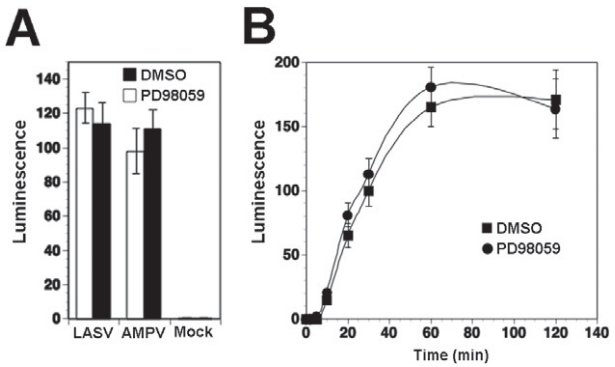

Fig. 5. MEK1 activity is dispensable for cell entry of LASV pseudotypes.
A. Infection of LASV pseudotypes is not affected by the MEK inhibitor PD98059. Monolayers of WI-26 VA4 cells were pre-treated with PD98059 (50 μM) or solvent control (DMSO) for 30 min, followed by infection with LASV and AMPV pseudotypes (moi = 0.1). Infection was detected by luciferase assay and fold increase of luminescence above background is given (means ± SD, $n = 3$).
B. The MEK inhibitor PD98059 does not affect LASV cell entry kinetics. Monolayers of WI-26 VA4 cells were pre-treated with PD98059 as in (A), followed by incubation with LASV pseudotypes (moi = 0.1) in the cold in presence of the drug. After 1 h, unbound virus was removed and pre-warmed (37°C) medium containing the drug added. At the indicated time points, 20 mM ammonium chloride was added and left throughout the experiment. At 24 h post infection was detected by luciferase assay as in (A) (means ± SD, $n = 3$). Please note that the apparent differences in infection at 60 min were not statistically significant.

PD980592 for 1 h or mock treated with vehicle (DMSO) only. Cells were then incubated with LASV pseudotypes in the cold, to allow virus attachment without internalization. Unbound virus was removed and cells quickly shifted to 37°C to allow virus internalization in presence or absence of PD980592. After different time points, 20 mM ammonium chloride was added to cells and kept throughout the experiment. After 24 h, cells were fixed and infection assessed by detection of the luciferase reporter activity. As shown in Fig. 5B, pre-treatment of cells with PD98059 had no significant effects on the cell entry kinetics of LASV pseudotypes, suggesting that activity of the MEK/ERK pathway is dispensable for virus cell entry.

Binding of LASV pseudotypes to cells perturbs activation of MEK and ERK via the integrin α6β1

The major laminin-binding integrins in WI-26 VA4 cells are the integrins α6β1 and α3β1, whereas α6β4 integrin is present only at low levels (Ferletta *et al.*, 2003). Since mouse laminin-1 binds only to human α6β1 but not α3β1 integrins (Delwel *et al.*, 1994), laminin-1-induced activation of MEK and ERK in WI-26 VA4 cells is thought to be mediated by α6β1 integrins (Ferletta *et al.*, 2003). To validate the role of α6β1 integrins in laminin-1-induced MEK/ERK signalling in our system, serum-starved WI-26 VA4 cells were added to tissue culture plates coated with the signalling inducing anti-integrin α6 mAb GoH3, as well as mAb P1B5 anti-α3. Consistent with published reports, only adhesion to the mAb anti-integrin α6, but not anti-integrin α3 resulted in significant activation of MEK and ERK (Fig. 6A), confirming the role of α6β1 integrins in laminin-mediated MEK/ERK activation.

To specifically address the effect of LASV binding to cells on the activation of MEK/ERK via α6β1 integrin-mediated signalling, we performed cell adhesion to mAb anti-α6 in presence of LASV and MAPV pseudotypes.

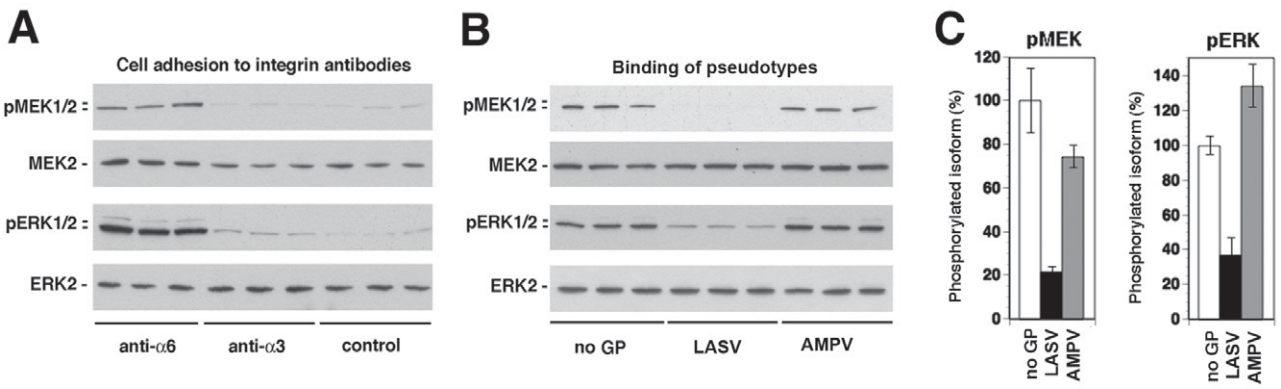

Fig. 6. Binding of LASV pseudotypes to cells blocks α6β1 integrin-mediated activation of MEK and ERK.
A. Induction of MEK and ERK phosphorylation by anti-α6 integrin antibody. Serum-starved WI-26 VA4 cells were detached and plated onto wells coated with antibodies to α6 and α3 integrin or an isotype control antibody for 40 min. Activation of MEK and ERK was determined by Western blot.
B. Binding of LASV pseudotypes reduces MEK and ERK phosphorylation induced by anti-α6 integrin antibody: serum-starved WI-26 VA4 cell suspensions were mixed with pseudotypes of LASV and AMPV at 10 particles per cell or control pseudotypes containing no GP (no GP) and plated onto wells coated with antibodies to α6. After 40 min of cell adhesion total-cell lysate were prepared and phosphorylation of MEK and ERK analysed by Western blot.
C. Quantification of (B), setting the signal for phosphorylated MEK1/2 and ERK1/2 in the control sample (no GP) as 100% ($n = 3$, ±SD).

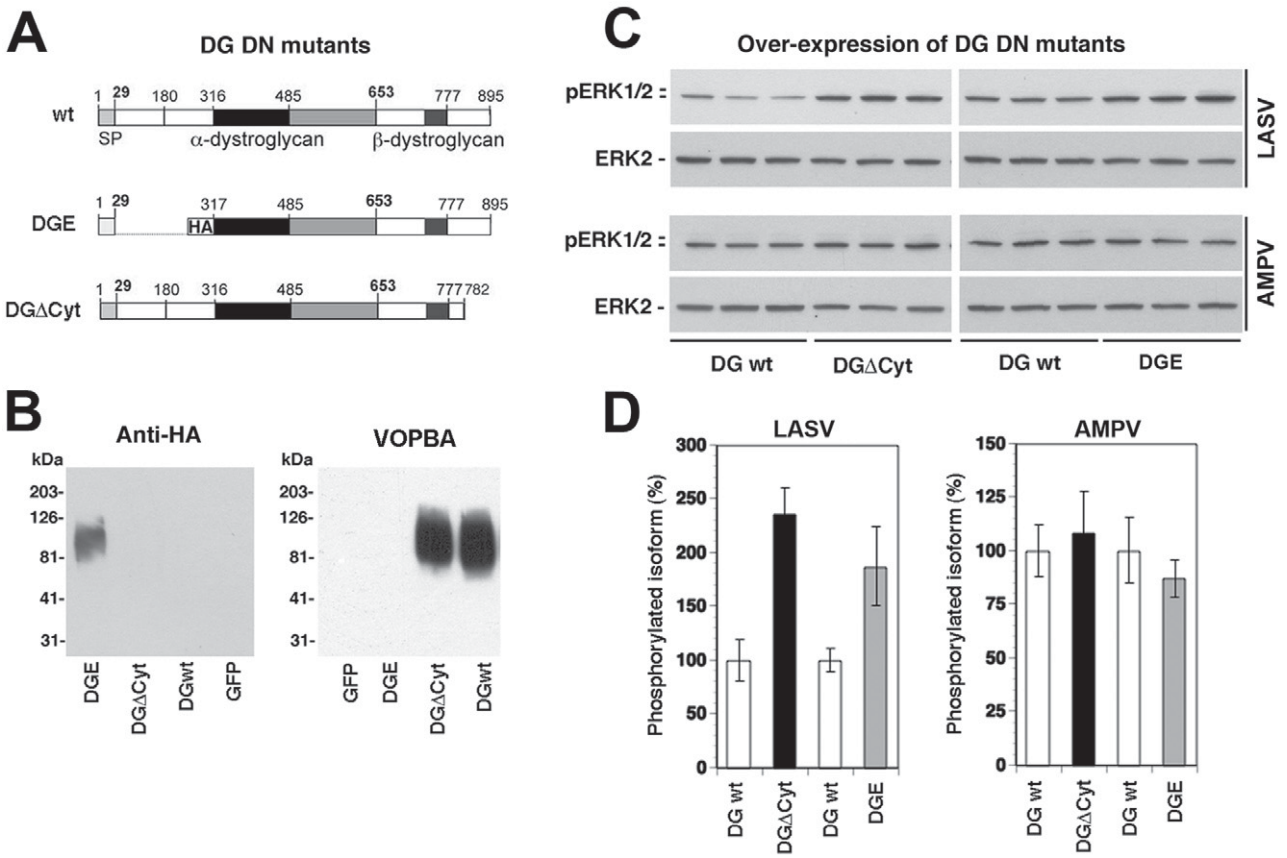

Fig. 7. Functional α-DG and the cytoplasmic domain of β-DG are involved in LASV pseudotype-induced inhibition of α6 integrin-mediated MEK/ERK activation.
A. Schematic representation of the DG mutants.
B. Detection of the α-DG parts of the DG variants. DGE, DGΔCyt, wild-type DG and GFP were expressed in DG (–/–) ES cells using AdV vectors. After 48 h, total membrane extracts were prepared and probed with an antibody to HA and in virus overlay protein binding assay (VOPBA).
C. Overexpression of DG DN mutants releases LASV pseudotype-induced inhibition of α6 integrin mediated MEK/ERK activation. WI-26 VA4 cells were infected with AdV expressing the DG variants. After 48 h, cells were serum-starved and detached. Single-cell suspensions were mixed with LASV and AMPV pseudotypes and plated onto wells coated with antibody to α6. Activation of ERK was determined by Western blot.
D. Quantification of (B), setting the signal for phosphorylated MEK1/2 and ERK1/2 in the control sample (no GP) as 100% ($n = 3$, ±SD).

Single-cell suspensions of serum-starved WI-26 VA4 cells were mixed with LASV and MAPV pseudotypes at 10 particles per cell and immediately added to cell culture plates coated with mAb GoH3 anti-α6 integrin At 40 min, cells were lysed and phosphorylation of MEK and ERK detected by Western blot. Pseudotypes of LASV, but not AMPV specifically perturbed the activation of MEK and ERK induced by mAb anti-α6 (Fig. 6B and C), suggesting that LASVGP binding to cells perturbs activation of MEK and ERK via the integrin α6β1.

LASV pseudotype-induced perturbation of α6β1 integrin-mediated signalling depends on functional DG

To address the involvement of DG in the LASV-induced perturbation of α6β1 integrin signalling, we utilized two well-characterized DG mutants (Fig. 7A). DGE contains the LASV binding site but lacks the N-terminal domain and does not undergo the post-translational modifications required for LASV binding (Kanagawa *et al.*, 2004). DGΔCyt lacks the cytoplasmic tail of β-DG, but shows normal cell surface expression and virus binding (Kunz *et al.*, 2003). First, we verified virus-binding phenotypes of DGE and DGΔCyt. For this purpose, the DG mutants and wild-type DG were expressed in DG (–/–) ES cells using adenoviral (AdV) vectors (Kunz *et al.*, 2001). After 48 h, total-cell lysates were prepared and glycoproteins isolated by wheat germ agglutinin (WGA) affinity purification (Michele *et al.*, 2002). Glycoprotein fractions were separated by SDS-PAGE and virus overlay assay performed with inactivated LASV (Cao *et al.*, 1998). As expected, the α-DG part of DGE lacked detectable virus binding,

whereas α-DG from DGΔCyt and wild-type DG bound LASV with high affinity (Fig. 7B).

To test the effect of the DG mutants on LASV-induced modulation of α6β1 integrin signalling, WI-26 VA4 cells were infected with AdV vectors expressing DGE, DGΔCyt and wild-type DG. After 48 h, cells were serum-starved for 16 h, detached, and added to plates pre-coated with mAb anti-α6 integrin in presence of LASV and AMPV pseudotypes. Cells were lysed and phosphorylation of ERK assessed by Western blot. In cells overexpressing DGE and DGΔCyt the inhibition of anti-α6 integrin-induced ERK phosphorylation by LASV pseudotypes was significantly released (Fig. 7C and D). As expected, no effect was observed in cells exposed to AMPV pseudotypes (Fig. 7C and D). The ability of the DG mutants to partially release the LASV-induced blocking of α6β1 integrin-mediated MEK/ERK activation suggest that LASV-pseudotype induced perturbation of α6β1 integrin-mediated signalling involves functional DG.

Discussion

Viruses have evolved to use a plethora of different cell surface molecules with very different biochemical characteristics and functions for host cell attachment and entry (Smith and Helenius, 2004; Marsh and Helenius, 2006). In some cases, the pathogens evolved to mimic the nature of the natural ligands of their cellular receptors, like, e.g. the integrin binding sites found in some picornaviruses (Baranowski *et al*., 2003). Old World arenaviruses, including LASV provide a striking example for this phenomenon (Rojek *et al*., 2007a; Oldstone and Campbell, 2011). Similar to DG's ECM ligands, high-affinity binding of arenaviruses critically depends on functional glycans present on α-DG. A comparative study between ECM proteins and arenaviruses revealed that the viruses mimic the molecular mechanism of receptor binding of α-DG's ECM ligands in a striking manner as both recognizing a highly conserved glycan epitope derived from the glycosyltransferase LARGE (Rojek *et al*., 2007a). In the present study we investigated the consequences of this 'mimicry' on the normal function of DG in the host cell, in particular DG-mediated signal transduction.

Several lines of evidence indicate that in the host cell, DG can associate with signalling molecules, including components of the MEK/ERK signalling pathway, Grb2, MEK and ERK (Yang *et al*., 1995; Spence *et al*., 2004; Moore and Winder, 2010). However the roles of these interactions for the cellular function of DG are currently unclear. Engagement of cellular α-DG by LASVGP, displayed on either inactivated LASV virions or retroviral pseudotypes, resulted in significant recruitment of Grb2 and MEK1 by the DG complex. Interestingly, we were unable to detect recruitment of the GTP exchange factor Sos, required by Grb2 for the activation of the canonical Ras/Raf/MEK/ERK pathway and found no evidence for activation of MEK/ERK signalling upon virus binding at any point, suggesting recruitment of Grb2 and MEK1 into an inactive signalling complex. An important role for such an inactive signalling complex for viral entry seemed rather unlikely. We therefore investigated the effect of these virus-induced changes on DG's association with Grb2 and MEK1 on the known cross-talk between DG and β1 integrins.

Previous work by us and others showed that attachment and cell entry of LASV critically depends on the high-affinity interaction between LASVGP and cellular α-DG (Cao *et al*., 1998; Kunz *et al*., 2005a; Reignier *et al*., 2006). Our present study revealed that β1 integrins are dispensable for attachment and cell entry of LASV pseudotypes. However, binding of inactivated LASV and LASV pseudotypes to cells markedly reduced the phosphorylation of MEK and ERK in response to laminin-1 and signalling activating antibodies to α6β1 integrins. Using mutants of DG, which either were deficient in virus binding or lacked the cytoplasmic tail of β-DG, we found that the effect of LASV cell attachment on α6β1 integrin signalling involves functional DG. Together, our data provide evidence that high-affinity LASV binding to cellular α-DG perturbs the signalling cross-talk between DG and α6β1 integrins, shifting the normal signalling equilibrium towards inhibition of the MEK/ERK pathway.

It is currently unclear how LASV binding to α-DG modulates MEK/ERK signalling through α6β1 integrins. To activate ERK, integrins can use two distinct pathways, one, involving FAK, and the other, the adaptor protein Shc (Giancotti and Ruoslahti, 1999). In both pathways, tyrosine phosphorylation of the adaptor proteins generates binding sites for the SH2 domain of Grb2, which in turn recruits Sos leading to activation of the Ras/Raf/MEK/ERK cascade. In contrast, binding of Grb2 to β-DG occurs in an SH3-dependent manner (Yang *et al*., 1995). Here we show that virus binding affects the association of DG with Grb2. However, an association of DG with Grb2 and MEK1 is already detected in uninfected cells and the extent of recruitment upon virus binding seems modest (Fig. 2F). It seems therefore rather unlikely that virus–receptor binding can sufficiently sequester signalling molecules like Grb2 and MEK1 to affect α6β1 integrin signalling. One might speculate that LASV binding to DG induces a negative regulatory signal of unknown nature that may affect α6β1 integrin-induced MEK/ERK signalling (Fig. 8).

Intrigued by the observation that binding of LASV to cells can modulate cellular MEK/ERK signalling, we investigated the role of this pathway for LASV cell entry. Inhibition of MEK, which is the only kinase that can phosphorylate ERK, did not affect infection of cells with LASV pseudotypes, making a role of this pathway in LASVGP-

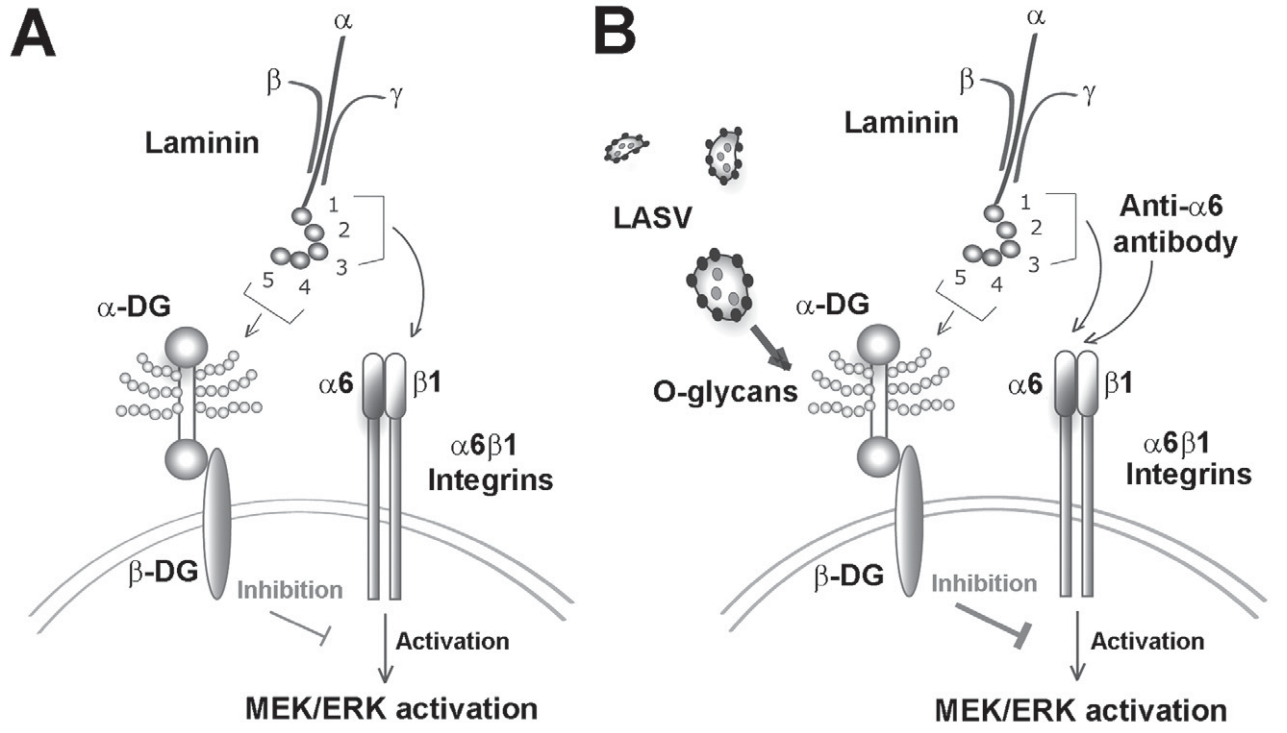

Fig. 8. Working model: binding of LASV to DG perturbs signalling cross-talk with β1 integrins.
A. Binding of laminin to cellular DG modulates MEK/ERK signalling through α6β1 integrins: laminin engages cellular DG via the LG domains 4 and 5 of the α1 chain (Hohenester *et al*., 1999) and binding critically depends on α-DG-linked O-glycans, in particular sugar polymers attached by LARGE (Kanagawa *et al*., 2004). Binding of laminin to α6β1 integrins involves the LG domains 1–3 of α1 (Hohenester *et al*., 1999) and results in activation of MEK/ERK signalling (Ferletta *et al*., 2003). Simultaneous binding of laminin to DG via LG4/5 inhibits activation of MEK/ERK via α6β1 integrins (Ferletta *et al*., 2003).
B. High-affinity binding of LASV to cellular α-DG perturbs the signalling cross-talk between DG and α6β1 integrins (this study): binding of inactivated LASV and LASV pseudotypes to cellular DG inhibits activation of α6β1 integrins via laminin or a signalling-inducing antibody to α6. The effect of LASV on α6β1 integrin signalling depends on the functional O-glycosylation of α-DG and the cytoplasmic domain of β-DG. The nature of the inhibitory signal induced by binding of laminin LG4/5 and LASV is currently unknown.

mediated cell entry rather unlikely. Due to biosafety restrictions associated with work with live LASV, a possible role of MEK/ERK signalling in post-entry steps of LASV replication have not yet been addressed, but will be pursued in future studies.

Since DG and α6β1 integrins are coexpressed on a wide variety of human cell types involved in LASV pathogenesis like epithelial cells, endothelial cells and macrophages (Wei *et al*., 1998; ffrench-Constant and Colognato, 2004) the impact of LASV binding on DG-mediated signalling may affect normal cell function in LASV infected individuals. A possible concern with our *in vitro* studies is the relatively high particle/cell ratios used. However, in late stages of fatal human Lassa fever, virus loads often exceed 10^9 infectious particles per ml of blood and similar virus loads are found in many tissues (Walker *et al*., 1982; McCormick and Fisher-Hoch, 2002; Moraz and Kunz, 2011). In this situation, extensive binding of virus particles to cellular α-DG may result in significant perturbation of DG-mediated signalling that may contribute to cellular dysfunctions that are associated with the Lassa shock syndrome.

In fatal Lassa fever there is surprisingly little inflammation and tissue destruction, vascular damage is mild and disseminated intravascular coagulation (DIC) is rare (Walker *et al*., 1982; McCormick and Fisher-Hoch, 2002). The absence of classical hallmarks of immunopathology in fatal disease suggests that the direct interaction of the virus with host cells may contribute to some aspects of pathogenesis, such as vascular leakage and oedema formation. Among other mechanisms, the virus-induced perturbation of ECM-induced cell signalling shown in this study, may contribute to the functional alterations of epithelial and vascular endothelial cells that precede shock and death (Fisher-Hoch *et al*., 1987). This type of LASV-induced receptor signalling reported here is likely due to the extensive mimicry of endogenous ligand binding by the pathogen. The consequent perturbation of cell signalling appears as a 'collateral damage' inflicted on the cell that may contribute to viral pathogenesis.

Experimental procedures

Proteins and antibodies

Mouse laminin-1 was from Gibco-BRL (Gaithersburg, MD). Monoclonal antibody (mAb) IIH6 anti-α-DG has been described (Ervasti and Campbell, 1991). Polyclonal rabbit anti-laminin-1 was from Sigma (St. Louis, MO) and rabbit anti-influenza HA (Y11) and mouse anti-HA (F7) from St. Cruz Biotechnology (St. Cruz, CA). HRP-conjugated secondary Abs and Streptavidin-HRP were from Pierce. MAbs to human integrins P1B5 anti-α3 and GoH3 anti-α6 were from Chemicon (Temecula, CA) and BD Biosciences (San Jose, CA) respectively. Mouse mAbs specific for MEK1, MEK2, ERK1 and ERK2 were from Transduction Laboratories (Lexington, KY), rabbit polyclonal Abs against phospho-MEK1/2 (S217/221) and phospho-ERK1/2 (T202/Y204) were from New England Biolabs (Beverly, MA). Polyclonal rabbit anti-Sos-1 Abs were from Upstate (Lake Placid, NY), mouse mAb anti-Grb2 from Chemicon, and rabbit polyclonal Ab to p125FAK from St. Cruz. The Steady Glo® and Bright-Glo® luciferase assay systems were obtained from Promega (Madison, WI). The MEK-specific inhibitor PD98059 was obtained from Calbiochem.

Cell lines

WI-26 VA4 cells (ATCC CCL-95.1) were cultured in DMEM, 10% (v/v) FBS, supplemented with glutamine, and penicillin/streptomycin. Embryonic stem (ES) cells DG (+/–), DG (–/–) have been described (Henry and Campbell, 1998). Mouse ES cells β1 integrin (+/–) and β1 integrin (–/–) (Fassler and Meyer, 1995; Fassler *et al*., 1995) were kindly provided by Dr R. Fassler. All ES cell lines were cultured as described (Henry and Campbell, 1998).

Virus strains, purification and quantification

Recombinant AdV DGΔH30-A316 (DGE) and DGΔCyt and wt DG have been described (Kunz *et al*., 2001; 2003). Lassa virus (LASV) strain Josiah and Amapari (AMPV) virus were obtained from the collection at the Special Pathogens Branch, Center for Disease Control and Prevention in Atlanta GA and inactivated viruses produced as reported (Spiropoulou *et al*., 2002). Retroviral pseudotypes expressing GFP and luciferase reporters were produced as described (Rojek *et al*., 2006) and, where indicated, inactivated by UV irradiation for 5 min. Inactivation was verified by luciferase assay.

Immunoblotting and VOPBA

Proteins were separated by gel electrophoresis and transferred to nitrocellulose. After blocking in 5% (w/v) skim milk in PBS, membranes were incubated with Abs used at following concentrations: mAb IIH6, mAb F7, mAb 8D5 and polyclonal Abs FTP, AP83 and Y11 (10 μg ml^{-1}) in 2% (w/v) skim milk, PBS for 12 h at 6°C. Abs to MEK1/2, ERK1/2, Sos-1, Grb-2, p125FAK, phospho-MEK1/2 and phospho-ERK1 at 1:1000 in 2% (w/v) skim milk, TBS for 12 h at 6°C. Secondary Abs coupled to HRP were applied 1:5000 in PBS, 0.1% (w/v) Tween for 1 h at room temperature. Blots were developed by enhanced chemiluminescence (ECL) using Super Signal West Pico ECL Substrate (Pierce). Laminin overlay assay (LOA) and VOPBA with γ-inactivated LASV, and AMPV were performed as described (Kunz *et al*., 2005b).

Virus cell binding assay

Monolayers of WI-26 VA4 cells were cultured in 96-well microtitre plates. For virus binding, medium was removed, cells washed twice with PBS, chilled on ice and blocked for 1 h in 1% (w/v) FBS/PBS containing 0.1% (w/v) of sodium azide. Cells were incubated with the indicated concentrations of inactivated LASV and AMPV in 1% (w/v) FBS/PBS/0.1% (w/v) sodium azide. After 1 h on ice, cells were washed three times with cold PBS and fixed with 4% (w/v) paraformaldehyde supplemented with 0.1% (w/v) glutaraldehyde for 20 min in ice. For detection of bound virus, cells were incubated with mAb 83.6 (20 μg ml^{-1} purified IgG) overnight in the cold. After three washes in PBS, primary antibody was detected with goat anti-mouse IgG conjugated to HRP for 45 min in the cold. After three wash-steps in PBS, HRP-conjugated secondary antibody was detected in a colour reaction using ABTS [2,2′azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] substrate and OD_{405} detected in an ELISA reader.

Infection of cells with retroviral pseudotypes

Cells were plated in 96-well plates in a density of 10^4 cells per well. After 24 h, retroviral pseudotypes were added at the indicated moi and incubated for 1 h at 37°C. The viral particles were removed, cells washed twice with DMEM and fresh medium added. Luciferase activity was determined by Steady-Glo® luciferase assay except in murine ES cells. AdV-mediated gene transfer of wild-type DG and the DG DN mutants DGE (ΔH30-A316) and DGΔCyt was performed as described (Kunz *et al*., 2005b). After 48 h, the indicated infectious units (iu) of pseudotypes were added to AdV transfected DG (–/–) ES cells, as well as untreated control cells and incubated for 1 h at 37°C. The viral particles were removed, cells washed twice with DMEM and fresh medium added. Infection was quantified by Bright Glo® luciferase assay. Luminescence was calculated as fold increase over background signals obtained from uninfected cells. Cell entry kinetics of LASV pseudotypes were performed as described (Rojek *et al*., 2008b). Blocking of infection with mAb IIH6 was done as reported (Kunz *et al*., 2005b).

Co-immunoprecipitation

For virus binding and co-IP of β-DG with signalling molecules, WI-26 VA4 cells were transfected with DGHA and DGwt with Superfect® (QIAGen). After 48 h, cells were exposed to purified, inactivated LASV, AMPV, or LASV– and AMPV pseudotypes at a particle to cell ratio of 1:100 for the indicated time periods. To detect changes in the association of signalling molecules with β-DG, co-IP was performed as described (Rojek *et al*., 2007b) using either mAb F7 or Y11 immobilized on Sepharose 4B for IP of DHHA and the abovementioned Abs for detection of signalling molecules in Western blot. IP with mouse mAb F7 were analysed with polyclonal rabbit Abs and IP with pAb Y11 with mouse mAbs.

Detection of MEK/ERK activation by cell adhesion to laminin and anti-α6 integrin Abs

Cell adhesion assays were performed according to Ferletta *et al*., 2003. MEK/ERK activation was detected from WI-26 VA4 cells that had been seeded to confluence, serum starved for 16–24 h, detached and plated on laminin-1 or wells pre-coated with mAbs to α3 and α6 integrins in presence or absence of purified inactivated LASV, AMPV, or LASV– and AMPV pseudotypes at a particle to cell ratio of 1:10 for the indicated time periods. Phosphorylated and total MEK and ERK were determined by Western blot as described above and the degree of phosphorylation determined as described (Ferletta *et al*., 2003) using densitometry (Kunz *et al*., 2004). For overexpression of the DN DG mutants DGE and DGΔCyt and wt DG in WI-26 VA4 cells we used AdV vectors (moi = 100) for gene transfer as described (Kunz *et al*., 2005b).

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Original article

"Analytical validation of a lymphocytic choriomeningitis virus real-time RT-PCR assay"

Samuel Cordey[a,b,c,*,1], Roland Sahli[d,1], Marie-Laurence Moraz[d], Christine Estrade[d], Laurence Morandi[b,c], Pascal Cherpillod[a,b,c], Remi N. Charrel[e], Stefan Kunz[d] and Laurent Kasier[a,b,c]

[a] Swiss National Reference for Emerging Viral Diseases, University of Geneva Hospitals, Geneva, Switzerland

[b] Laboratory of Virology, University of Geneva Hospitals, Geneva, Switzerland

[c] University of Geneva Medical School, Geneva, Switzerland

[d] Institute of Microbiology of the Centre Hospitalier Universitaire Vaudois and University of Lausanne, Switzerland

[e] Unité des Virus Emergents (UMR190), Université de la Méditerranée – Institut de Recherche pour le Développement, Marseille, France

* Corresponding author. Mailing address: Laboratory of Virology, Division of Infectious Diseases, University of Geneva Hospitals, 4 Rue Gabrielle-Perret-Gentil, 1211 Geneva 14, Switzerland. Phone: +41-2203724079, Fax: +41-2203724097, E-mail samuel.cordey@hcuge.ch

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Short communication

Analytical validation of a lymphocytic choriomeningitis virus real-time RT-PCR assay

Samuel Cordey[a,b,c,*,1], Roland Sahli[d,1], Marie-Laurence Moraz[d], Christine Estrade[d], Laurence Morandi[b,c], Pascal Cherpillod[a,b,c], Remi N. Charrel[e], Stefan Kunz[d], Laurent Kaiser[a,b,c]

[a] Swiss National Reference Centre for Emerging Viral Diseases, University of Geneva Hospitals, Geneva, Switzerland
[b] Laboratory of Virology, University of Geneva Hospitals, Geneva, Switzerland
[c] University of Geneva Medical School, Geneva, Switzerland
[d] Institute of Microbiology of the Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland
[e] Unité des Virus Emergents (UMR190), Université de la Méditerranée – Institut de Recherche pour le Développement, Marseille, France

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A B S T R A C T

Lymphocytic choriomeningitis virus (LCMV) is a rare cause of central nervous system disease in humans. Screening by real-time RT-PCR assay is of interest in the case of aseptic meningitis of unknown etiology.

A specific LCMV real-time RT-PCR assay, based on the detection of genomic sequences of the viral nucleoprotein (NP), was developed to assess the presence of LCMV in cerebrospinal fluids (CSF) sent for viral screening to a Swiss university hospital laboratory.

A 10-fold dilution series assay using a plasmid containing the cDNA of the viral NP of the LCMV isolate Armstrong (Arm) 53b demonstrated the high sensitivity of the assay with a lowest detection limit of ≤50 copies per reaction. High sensitivity was confirmed by dilution series assays in a pool of human CSF using four different LCMV isolates (Arm53b, WE54, Traub and E350) with observed detection limits of ≤10 PFU/ml (Arm53b and WE54) and 1 PFU/ml (Traub and E350).

Analysis of 130 CSF showed no cases of acute infection. The absence of positive cases was confirmed by a published PCR assay detecting all Old World arenaviruses.

This study validates a specific and sensitive real-time RT-PCR assay for the diagnosis of LCMV infections. Results showed that LCMV infections are extremely rare in hospitalized patients western in Switzerland.

Lymphocytic choriomeningitis virus (LCMV), the prototypic member of the *Arenaviridae* family, was isolated from a fatal case of aseptic meningitis during the St. Louis encephalitis epidemic in 1933 (Armstrong and Lillie, 1934).

In nature, LCMV is maintained by congenital transmission within infected populations of the mouse species *Mus domesticus* and *Mus musculus*. LCMV infects readily other rodents, including hamsters and guinea pigs. The LCMV carrier state is characterized by persistent infection with high virus loads in serum and several organs in the absence of a virus-specific immune response and overt pathology. Humans are accidental hosts and the main route of transmission is by contact with infected rodents that shed large quantities of virus in nasal secretions, saliva, milk, semen, urine, and feces.

Although human LCMV cases are observed throughout the year, disease incidence increases in winter, due probably to the movement of infected rodents indoors, thus increasing the risk of human exposure (Barton et al., 1993). In immunocompetent adult individuals, LCMV infection is either asymptomatic or results in a self-limiting febrile illness associated rarely with fatalities. Signs and symptoms are largely non-specific, including fever, myalgia, and malaise. Central nervous system involvement manifests as headache and photophobia, associated with nausea or vomiting. In most cases, infected individuals recover completely, but this may require several months. Severe aseptic meningitis or meningoencephalitis is observed only in a minority of cases (Barton et al., 1993). Human-to-human transmission has been documented through organ donation associated with severe disease in the immunocompromised recipient (Fischer et al., 2006), and congenital infections leading to severe and irreversible brain (Barton et al., 1993; Barton and Mets, 2001; Bonthius et al., 2007; Wright et al.,

* Corresponding author at: Laboratory of Virology, Division of Infectious Diseases, University of Geneva Hospitals, 4 Rue Gabrielle-Perret-Gentil, 1211 Geneva 14, Switzerland. Tel.: +41 22 3724079; fax: +41 22 3724097.
E-mail address: samuel.cordey@hcuge.ch (S. Cordey).
[1] These authors contributed equally to this work.

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1997) and retinal (Bonthius et al., 2007; Mets et al., 2000) injury have been described.

Acute human LCMV infections are diagnosed generally by virus isolation from CSF and the use of serological testing or classical PCR assays (Park et al., 1997; Emonet et al., 2007) offer the possibility to detect pre- and postnatal LCMV infections. More recently, SYBR Green-based quantitative PCR assays were developed to quantify LCMV in infected mice (McCausland and Crotty, 2008; Emonet et al., 2007). Due to their higher sensitivity and specificity compared to both viral culture and immunofluorescence-based detection methods, RT-PCR viral assays are favored for the diagnosis of viral infection in most routine laboratories.

A real-time RT-PCR assay with specific primers and probes adapted to published LCMV sequences available was developed to screen acute LCMV infections in CSF. The use of a specific probe has the main advantage to increase the LCMV-specificity compared to SYBR Green PCR methods. Over a period of two years, all hospitalized pediatric cases and adults less than 25-years-old for whom a screening for viral meningoencephalitis was required by the physician at the University of Geneva Hospitals were screened for LCMV infections using this real-time RT-PCR.

To identify primers and probes, conserved regions specific to LCMV were screened based on an extensive alignment of all LCMV and Old World arenavirus's sequences available in Genbank in August 2010, including all recently discovered arenaviruses (Briese et al., 2009; Gunther et al., 2009; Palacios et al., 2008). This alignment allowed to pinpoint a conserved region within the viral nucleoprotein (NP) gene. Primer pairs were designed to correspond to the least number of combinations matching perfectly each distinct LCMV sequence. Therefore, the primers used in the PCR are constituted of a mix of 9 and 8 selected forward and reverse primers, respectively (Fig. 1). Primers and probe (Eurogentech, Seraing, Belgium) were screened by NCBI nucleotide BLAST (Altschul et al., 1990) to exclude any cross-reactions with human cellular sequences and distantly related viruses. The probe was labelled at the 5′ end with the 6-carboxyfluorescein (FAM) and at the 3′ end with the BHQ1 black hole quencher. In brief, the viral genome was extracted individually from 400 μl of samples using the NucliSENS easyMAG (bioMérieux, Geneva, Switzerland) nucleic acid kit, according to the manufacturer's instructions. In addition, 20 μl of standardized canine distemper virus (CDV) of known concentration was added to each sample before extraction to control for intra- and inter-assay variability as previously described (Cordey et al., 2010). To remove a maximum of DNA contaminant in eluates, a DNAse treatment was done using the DNA-free kit (Ambion, Rotkreuz, Switzerland), according to the manufacturer's instructions. This step was necessary for optimal sensitivity in the presence of more than 5 ng/ml of human DNA in the sample. The synthesis of cDNA was performed with random hexamers (Roche, Rotkreuz, Switzerland) at 42 °C using the Superscript II Reverse Transcriptase (Invitrogen, Basel, Switzerland), according to the manufacturer's instructions. cDNA was amplified using a TaqMan® 7500 (Applied Biosystems, Rotkreuz, Switzerland) thermocycler under the following cycling conditions: 95 °C for 9 min; 50 cycles of 15 s at 95 °C and 1 min at 58 °C. After assessment of optimal primers/probe concentrations, the reaction was performed in 20 μl containing 1× TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.2 μM of each individual LCMV forward primer, 0.2 μM of each individual LCMV reverse primer, 0.2 μM of LCMV probe per reaction, and 5 μl of cDNA. Results were analyzed using the SDS 1.4 program (Applied Biosystems).

The analytical sensitivity of the LCMV real-time RT-PCR was determined using the mammalian expression plasmid pcAGGS containing the cDNA of the NP derived from the LCMV isolate Armstrong (Arm) 53b in 10-fold serial dilutions. The experiment showed a linear range between 50 and 5×10^4 copies of input with the lowest limit of detection being ≤50 copies per reaction (Fig. 2). Of note, 5 plasmid copies were detected only in one of the triplicate wells. Sensitivity was assessed further using crude stocks of the LCMV isolates LCMV-Arm53b, LCMV-WE54, LCMV-Traub, and LCMV-E350 grown in BHK21 cells (Table 1) which were diluted serially in a pool of human CSF negative for LCMV. The four LCMV isolates showed low limits of detection in plaque-forming units (PFU) determined by immunofocus assay (≤10 PFU/ml for Arm53b, ≤10 PFU/ml for WE54, ≤1 PFU/ml for Traub, and ≤1 PFU/ml for E350). Similarly, dilution series assays performed in phosphate buffered saline showed a reproducible detection limit of ≤1 PFU/ml for each LCMV isolate, thus suggesting the presence of defective interfering particles, a well-known phenomenon with LCMV (Martinez Peralta and Lehmann-Grube, 1983; Francis and Southern, 1988; Meyer and Southern, 1997). In addition, the recently identified LCMV-Marseille strain supernatant (Emonet et al., 2007) has also been tested positive. To assess the potential specificity of this real-time RT-PCR, the genetically more distant New World arenaviruses, Junin virus and Pichinde virus isolates, as well as individual pcAGGS plasmids containing the cDNA of the NP derived from Lassa virus, Machupo virus, Tacaribe virus, Latino virus, and Whitewater Arroyo virus were examined also (Table 1). Consistent with their phylogenetic distance from LCMV, none of these arenaviruses was detected by the assay, except Lassa virus for which a weak cross-reaction was observed at a very high concentration, although a minimum of 1 and 4 mismatches are present in the forward and the reverse primer sets, respectively. This cross-reaction can be explained by the extremely high sequence homology between Lassa and LCMV. Therefore, cross-reaction events should be considered for samples with C_T (threshold value) values close to the limit of positivity determined at 40 C_T. Finally, a large panel of unrelated virus isolates, including herpes simplex virus, varicella zoster virus, Epstein–Barr virus, JC virus, measles virus, enterovirus and parechovirus, belonging to other virus families known to lead potentially to meningitis or meningoencephalitis complications, remained undetected with the LCMV real-time RT-PCR.

All hospitalized pediatric cases and adults less than 25 years old who underwent a CSF screening with negative results for selected viral infections (enterovirus, parechovirus, herpes simplex virus, varicella zoster virus, Epstein–Barr virus, JC virus, and/or measles virus) between October 2008 and September 2010 were assessed for LCMV with the newly developed real-time RT-PCR assay.

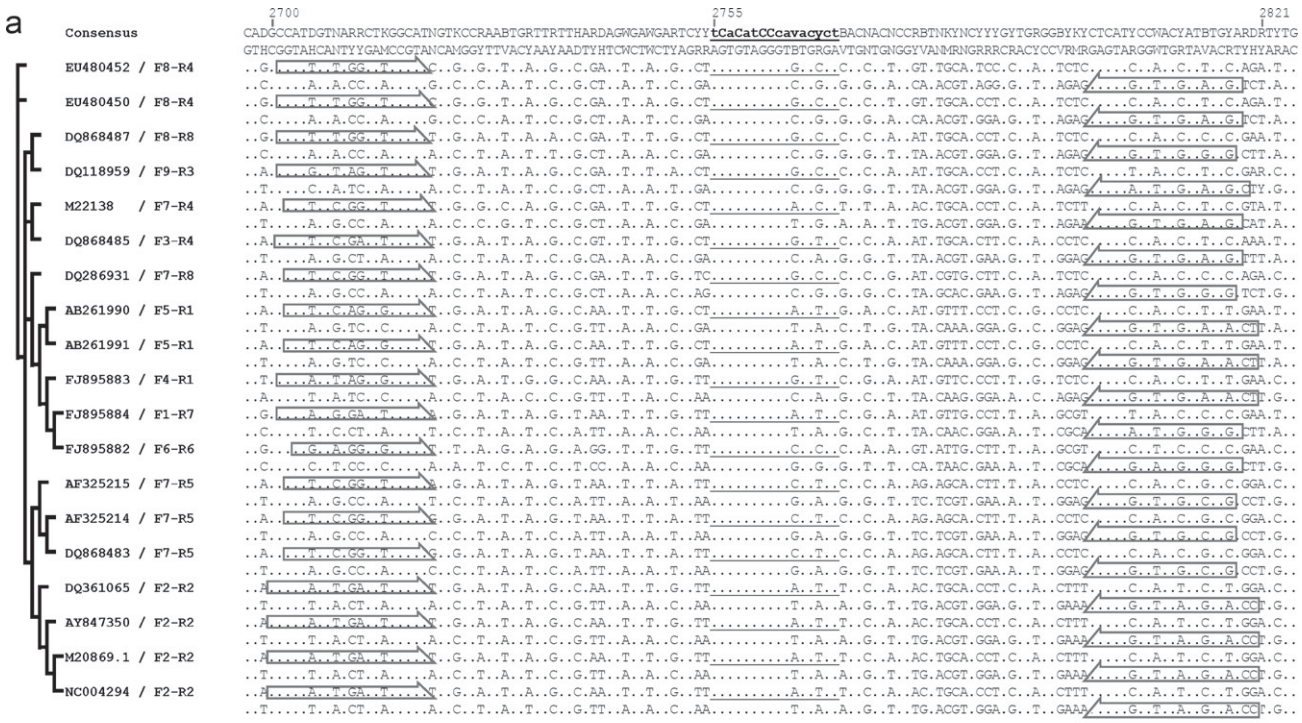
Of a total of 130 CSF samples (56 female [43%]; 74 male [57%]; median age, 2 years old), 51 (39%) were collected in infants less than 1-year-old. The clinical syndromes ranged from meningitis, encephalitis, convulsions, and other central nervous system diseases. The analysis covers two winter seasons known to represent the peak window period of infection.

None of the 130 CSF samples was found positive for LCMV. Since the design of the specific LCMV primers and probe was based on the available sequence information in Genbank at that time, the presence of yet unknown strains of LCMV circulating in rodents and transmitted to humans cannot be ruled out. To address this issue, a previously published pan-arenavirus RT-PCR (Vieth et al., 2007) capable of detecting all currently known Old World arenavirus species, including LCMV, Lassa virus, and Mobala virus, was used. When applied to the 130 CSF samples, none was positive for LCMV, similar to the results obtained with the specific LCMV real-time RT-PCR assay described above.

This report describes the analytical validation of a LCMV real-time RT-PCR assay allowing rapid detection of LCMV strains that have been shown to infect humans.

Despite important efforts to contact most investigators with documented cases of human LCMV infections over the last few years, it was not possible to obtain such rare clinical samples due

a



b

Primer Names	Sequence (5'-3')		Concentration [nM] in reaction
LCMV_NP-For1	CCATAGTGAGACTTGGCAT		200
LCMV_NP-For2	GCCATAGTTAGACTTGGCAT		200
LCMV_NP-For3	CCATTGTCAGACTTGGCAT		200
LCMV_NP-For4	CCATAGTTAAGCTGGGCAT		200
LCMV_NP-For5	CATTGTCAAGCTGGGCAT		200
LCMV_NP-For6	ATGGTAAGGCTGGGCAT		200
LCMV_NP-For7	CATTGTCAGGCTTGGCAT		200
LCMV_NP-For8	CCATTGTTAGGCTTGGCAT		200
LCMV_NP-For9	CCATGGTTAAGCTTGGCAT		200
LCMV_NP-Rev1	TCTACAAATGGTTGGGATGAG		200
LCMV_NP-Rev2	CCTACAGATAGTTGGGATGAG		200
LCMV_NP-Rev3	CTGCAAATGGTTGGAATGAG		200
LCMV_NP-Rev4	TGCAAATGGTTGGGATGAG		200
LCMV_NP-Rev5	GCACATGGTTGGGATGAG		200
LCMV_NP-Rev6	TGCAGATGGTAGGGATGAG		200
LCMV_NP-Rev7	TGCAGATGGTTGGAATGAG		200
LCMV_NP-Rev8	GCAGATGGTTGGGATGAG		200
LCMV_NP-Probe	FAM- tCaCatCCcavacyct-TAMRA	upper case = LNA	200

Fig. 1. Primers and probe for LCMV real-time RT-PCR assay. (a) Nineteen LCMV NP reference sequences indicated by their Genbank accession numbers (EU480452, EU480450, DQ868487, DQ118959, M22138, DQ868485, DQ286931, AB261990, AB261991, FJ895883, FJ895884, FJ895882, AF325215, AF325214, DQ868483, DQ361065, AY847350, M20869.1 and NC004294) were aligned with the MAFFT algorithm in the Geneious software package. Consensus was established with 100% identical residue at each position. Conserved residues are indicated by dots and polymorphic residues by their actual IUPAC code. The most conserved area, shown here as double stranded cDNA, was used as the target for the real-time RT-PCR assay. The primer sequences are boxed in strand-specific arrows. The forward primers (F1–F9) are on the coding (upper) strand and the reverse primers (R1–R8) are on the complementary (lower) strand. The probe is shown in bold fonts in the upper strand of the consensus sequence; capital letters indicate LNA residues used to increase its melting temperature. LNA residues were positioned within this highly conserved area to limit the risk of false negativity with unknown LCMV sequences. Numbers on top refer to the 5′ end of the primer/probe positions within the NP open reading frame using the Armstrong strains as reference. LCMV phylogeny is depicted by the tree on the left-hand side pointing to each individual reference sequence. Primer combination adapted to the amplification of each genotype is shown to the right of the accession number and refers to the primer names indicated in (b) (F, For; R, Rev).

to the very limited amount of CSF collected initially. Nevertheless, serial dilutions of both Arm-derived plasmid and different LCMV strains cultures in a pool of CSF confirmed the high analytical sensitivity of the new assay with limits of detection reaching ≤50 copies per reaction and ≤10 or 1 PFU/ml, respectively, indicating a potential high clinical sensitivity.

Irrespective of the PCR assay used (LCMV specific real-time RT-PCR and classical broad range arenavirus PCR), LCMV was not detected in any CSF samples over the study period. These results show the extremely low incidence of LCMV infections leading to hospital admission for acute meningoencephalitis in western Switzerland and confirm previous prospective surveillance investigations conducted elsewhere. For instance, Park et al. (1997) found no positive case for LCMV among a total of 813 CSF samples collected over a one-year period at two Birmingham (UK) hospitals. Only extensive serological studies at country level may reveal the prevalence of LCMV within the human population.

Table 1
Performance of the LCMV real-time RT-PCR for the detection of different LCMV and other arenavirus strains.

Strain	L CMV real-time RT-PCR	Limit of detection	Slope	R^2
LCMV-ARM53b	Positive	≤10 PFU/ml (in CSF)	3.62	1
LCMV-WE54	Positive	≤10 PFU/ml (in CSF)	3.91	0.99
LCMV-Traub	Positive	≤1 PFU/ml (in CSF)	3.87	1
LCMV-E350	Positive	≤1 PFU/ml (in CSF)	3.65	1
LCMV-Marseille	Positive	ND	ND	ND
Lassa	Weak positive cross-reaction ($C_T > 39$)	Tested for 10^4 plasmid copies/PCR		
Tacaribe	Negative	Tested for 10^4 plasmid copies/PCR		
Machupo	Negative	Tested for 10^4 plasmid copies/PCR		
Latino	Negative	Tested for 10^4 plasmid copies/PCR		
Whitewater Arroyo	Negative	Tested for 10^4 plasmid copies/PCR		
Junin	Negative	Tested for 10^5 copies/ml (in plasma)		
Pichinde	Negative	Tested for 10^5 copies/ml (in plasma)		

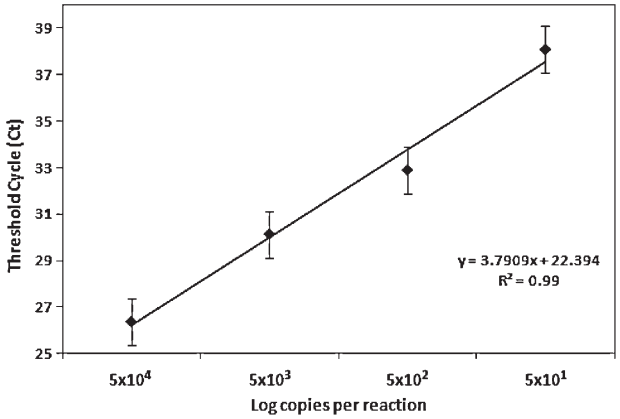


Fig. 2. Analytical sensitivity of the LCMV real-time RT-PCR assay. 10-Fold serial dilutions of Armstrong-derived plasmid (5×10^4–5×10^1 copies per reaction) were tested by real-time RT-PCR assay. The log of copy numbers is plotted versus the threshold cycle (C_T). Each dot represents the average of three independent experiments. Of note, a dilution with 5×10^0 plasmid copies showed positive detection in only one single triplicate. It was not considered for slope calculation. Errors bars indicate standard deviations.

The real-time RT-PCR assay described here provides a sensitive and adapted tool for specialized clinical laboratories.

Although the current study did not reveal the presence of acute LCMV infection in children and young adults over the past two years in western Switzerland, potential infections should not be overlooked as clinical complications related to LCMV have been observed in sporadic cases throughout the world (Asnis et al., 2010; Ceianu et al., 2008; Emonet et al., 2007; Sosa et al., 2009). In addition, this real-time RT-PCR assay could be used in rodents, particularly in pet shops, as they represent the principal LCMV reservoir.

Competing interests

None declared.

Ethical approval

Not required.

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Review

"Pathogenesis of arenavirus hemorrhagic fevers"

Marie-Laurence Moraz[1] and Stefan Kunz[1*]

[1] Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne, Switzerland

* Corresponding author. Mailing address: Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne CH-1011, Switzerland. Phone: +41-21 314 7743, Fax: +41-21 314 4060, E-mail Stefan.Kunz@chuv.ch

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EXPERT REVIEWS

Pathogenesis of arenavirus hemorrhagic fevers

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Marie-Laurence Moraz[1] and Stefan Kunz[†1]

[1]*Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne, Switzerland*
[†]*Author for correspondence:*
Tel.: +41 213 147 743
Fax: +41 213 144 060
stefan.kunz@chuv.ch

Viral hemorrhagic fevers (VHFs) caused by arenaviruses belong to the most devastating emerging human diseases and represent serious public health problems. Arenavirus VHFs in humans are acute diseases characterized by fever and, in severe cases, different degrees of hemorrhages associated with a shock syndrome in the terminal stage. Over the past years, much has been learned about the pathogenesis of arenaviruses at the cellular level, in particular their ability to subvert the host cell's innate antiviral defenses. Clinical studies and novel animal models have provided important new information about the interaction of hemorrhagic arenaviruses with the host's adaptive immune system, in particular virus-induced immunosuppression, and have provided the first hints towards an understanding of the terminal hemorrhagic shock syndrome. The scope of this article is to review our current knowledge on arenavirus VHF pathogenesis with an emphasis on recent developments.

Keywords: arenavirus • hemorrhagic fever • hemorrhagic shock syndrome • immunosuppression • Junin virus • Lassa virus • vascular endothelium • viral pathogenesis

Arenavirus hemorrhagic fevers are devastating emerging human diseases

Viral hemorrhagic fevers (VHFs) are severe human diseases that show different clinical courses and are associated with the cardinal symptoms of fever, hemorrhages and shock. A wide variety of viruses that belong to different virus families can cause VHFs, including the filoviruses (Ebola and Marburg hemorrhagic fever [HF]), the arenaviruses (Lassa fever, Argentine HF [AHF], Bolivian HF, Venezuelan HF and Brazilian HF), bunyaviruses (Crimean Congo HF and Rift Valley fever) and flaviviruses (Yellow fever and Dengue HF) [1]. The arenaviruses are a large and diverse family of enveloped RNA viruses that include several causative agents of severe VHFs, which belong to the most devastating emerging human diseases and serious public health problems [1,2]. Arenavirus VHFs in humans are acute diseases characterized by fever and, in severe cases, different degrees of hemorrhages associated with a shock syndrome in the terminal stage. The most prevalent pathogen among the arenaviruses is Lassa virus (LASV), the causative agent of Lassa fever that is endemic to West Africa from Senegal to Cameroon and causes several hundred thousand infections per year with thousands of deaths [3]. The mortality of hospitalized Lassa fever patients is 15–30% and can reach more than 50% in some outbreaks. There is currently no vaccine available and therapeutic intervention is limited to intensive care and the use of ribavirin, which shows some efficacy when given early in disease. On the South American continent, the arenaviruses Junin virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV) and Sabia virus (SABV) have emerged as etiological agents of severe VHF in Argentina, Venezuela, Bolivia and Brazil, respectively [2]. The prototypic arenavirus lymphocytic choriomeningitis virus (LCMV), which is distributed worldwide, is also a neglected human pathogen of clinical significance, especially in pediatric medicine [4,5] and represents a threat to immunocompromised individuals [6,7]. Considering the number of people affected and the unaddressed need for better therapeutics, arenaviruses arguably belong to the most neglected tropical pathogens. Apart from the severe humanitarian burden in endemic regions, arenavirus HF cases are regularly imported into metropolitan areas around the globe, placing local populations at risk [8]. This article will briefly cover some general aspects of the classification, epidemiology and basic virology of arenaviruses, and will then provide a more detailed overview of the clinical disease, and in particular the pathogenesis of arenavirus VHFs with an emphasis on recent developments in the field.

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Classification & epidemiology of arenaviruses

The *Arenaviridae* are currently subdivided into two major subgroups, the Old World arenaviruses and the New World arenaviruses [9,10]. The Old World lineage contains the prototypic LCMV, the related Dandenong virus and the African viruses LASV, Mopeia, Mobala, Ippy and Morogoro virus. The New World arenaviruses are divided into three Clades, A, B and C. Clade A includes the South American viruses Pichinde virus, Parana virus, Pirital virus, Flexal virus and Allpahuayo virus. A North American Clade A/Rec comprises Whitewater Arroyo, Bear Canyon, and Tamiami, Catarina and Skinner Tank virus. The phylogenetic relationship of these viruses is poorly resolved, but there is evidence that a recombination event between Clade A and B viruses may be at the origin of this Clade as reviewed by Emonet *et al.* [9]. Whitewater Arroyo virus has been associated with three cases of fatal human disease [11,12]. However, a definitive causal relationship has not yet been established. Clade B contains the hemorrhagic viruses JUNV, MACV, GTOV, SABV and the recently emerged Chapare virus, as well as the nonpathogenic viruses Tacaribe (TACV), Amapari and Cupixi. Clade C contains the viruses Oliveros, Latino and Pampa.

In nature, each arenavirus species is carried by one or a limited number of related rodent species, which serve as their natural reservoirs. A possible exception is TACV, which has only been isolated from the fruit-eating bat species *Artibeus*. The present phylogenetic diversity of arenaviruses is the result of long-term co-evolution between viruses and their corresponding host species, involving vertical and horizontal transfer of viruses within and between populations, respectively, and probably occasional genetic recombination events [9,10,13].

A particular concern is the continued emergence of novel arenaviruses that are associated with fatal human disease. New arenaviruses emerge on average every 3 years [9]. In December 2003, a small cluster of fatal VHF cases was reported in a rural area near the Chapare River in Bolivia, Southern America. Examination of patient samples allowed the isolation and identification of Chapare virus, a novel species of arenavirus phylogenetically closely related to SABV [14]. More recently, in September 2008, a nosocomial outbreak of unexplained VHF occurred in Zambia, Southern Africa. Five patients were involved and four of them died with suspected human-to-human transmission. A high-throughput sequencing approach based on unbiased pyrosequencing revealed the presence of an arenavirus-like genome identified as a new member of the *Arenaviridae* family called LuJo virus according to its geographic distribution: Lusaka, Zambia and Johannesburg, South Africa [15]. The high mortality of four out of five hospitalized individuals and the strong evidence for human-to-human transmission are of particular concern regarding the epidemiological and disease potential of this new member of the arenavirus family.

Human arenavirus infections occur principally by aerosol or close contact with rodent excreta, contamination of food and drink or via skin abrasions. AHF caused by JUNV has a marked seasonal incidence, coinciding with the maize harvest between April and June when feral rodent populations reach their peak [16]. Agricultural workers and those in rural communities are thus at greatest risk. It is thought that transmission to humans results from the virus being carried on dust particles or by direct ingestion of contaminated foodstuffs.

Molecular & cell biology of arenaviruses

The basic virology of arenaviruses has been covered by excellent recent reviews [2,17] and only a short summary will be given here. Arenaviruses are enveloped viruses with a bisegmented negative strand RNA genome and a nonlytic life cycle restricted to the cytoplasm (Figure 1A). Each genomic RNA segment, L (circa 7.3 kb) and S (circa 3.5 kb), uses an ambisense coding strategy to direct the synthesis of two polypeptides in opposite orientation, separated by a noncoding intergenic region with a predicted hairpin structure (Figure 1B). The S RNA encodes the viral glycoprotein (GP) precursor, which is synthesized as a single polypeptide chain (circa 75 kDa) and post-translationally cleaved by the cellular proprotein convertase subtilisin kexin isozyme-1/site-1 protease to yield the mature virion GPs GP1 (40–46 kDa) and GP2 (35 kDa) [18–20], and the nucleoprotein (NP; circa 63 kDa). The L RNA encodes the viral RNA dependent RNA polymerase (RdRp or L polymerase; circa 200 kDa) and a small RING finger protein Z (circa 11 kDa).

Attachment to the host cell is mediated by the arenavirus GP1, which is located at the top of the mature GP spike present in the viral envelope. The transmembrane GP2 resembles the fusion-active membrane-proximal parts of other enveloped viruses. The cellular receptor for LASV, most other Old World arenaviruses and Clade C New World arenaviruses is α-dystroglycan (α-DG), a cell surface receptor for proteins of the extracellular matrix [21,22]. The hemorrhagic New World arenaviruses JUNV, MACV, GTOV and SABV can use human transferrin receptor 1 (TfR1) [23]. Upon attachment to the target cell, arenavirus particles are taken up by endocytosis and delivered to acidified endosomes where low pH induces membrane fusion [24]. New World arenaviruses like JUNV that use human TfR1 enter the cell via clathrin-mediated endocytosis [25]. In contrast, the Old World arenaviruses LASV and LCMV, which depend on α-DG, use a distinct and unusual pathway for cell entry that is independent of clathrin, caveolin, dynamin and actin [26–28]. Upon penetration into the cytoplasm, the viral ribonucleoparticles serves as a template for both transcription and replication that is mediated by the arenavirus RdRp L. The L and NP proteins are necessary and sufficient for these initial steps of viral transcription and replication [17]. Formation and release of arenavirus infectious progeny from infected cells occurs by budding during which ribonucleoparticles associate at the cell surface with membranes that are enriched in viral GPs. In arenaviruses, the Z protein functions as the main driving force for viral budding and resembles the matrix proteins of other enveloped RNA viruses [29,30].

Clinical disease & pathogenesis of Lassa fever

LASV was isolated in 1969 after a hospital outbreak in northern Nigeria [31,32], with additional outbreaks in Nigeria, Liberia and Sierra Leone. In 1972, the reservoir of LASV was identified as the rodent *Mastomys natalensis* [33]. Currently, LASV is

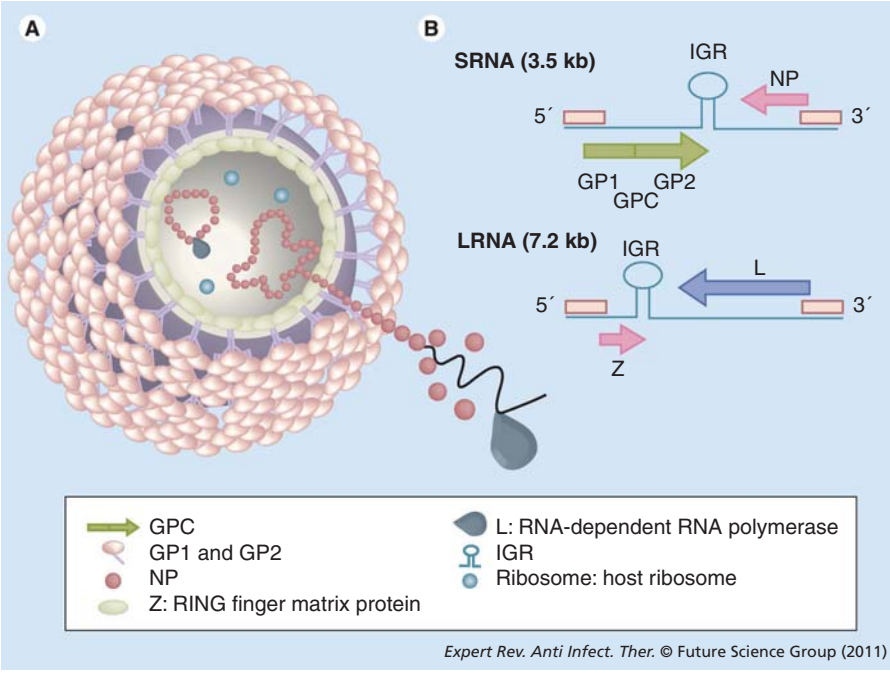

Figure 1. Arenavirus particle and genome organization. (A) Schematic representation of an arenavirus particle. The viral RNA is packaged into ribonucleoparticles (RNPs) containing the viral NP. The RNA-dependent RNA polymerase (L) is associated with RNP and is required for the initial steps of viral transcription. The matrix protein, Z, associates with the inner leaflet of the viral membrane envelope and interacts with the C-terminal part of the transmembrane GP2 moiety of the mature GP1/GP2 complex decorating the virion surface. The receptor-binding GP1 forms the top part of the GP virion spikes, whereas the transmembrane GP2 part contains the fusion machinery. In their mature form, arenavirus GPs are fully processed and presumably form trimers, similar to other fusion-active viral membrane proteins. **(B)** The ambisense coding strategy of arenaviruses. Each of the two ssRNA segments, L (circa 7.2 kb) and S (3.5 kb), uses an ambisense coding strategy to direct the synthesis of two polypeptides in opposite orientations and is separated by an IGR predicted to have a stable hairpin structure. The 5′ ends of the genomic arenavirus RNA cannot serve as a template for translation and viral protein expression requires prior transcription, as in true negative-strand viruses.
GP: Glycoprotein; GPC: Glycoprotein precursor; IGR: Intergenic region; L: RNA-dependent RNA polymerase; NP: Nucleoprotein.

endemic from Senegal to Cameroon [3] with recent emergence in Mali [34]. Reliable epidemiological data are missing from most affected regions, however, available information on seroprevalence for Nigeria, Sierra Leone and Guinea reveal that in some areas, 20–50% of the adult population have been infected with LASV [35], making LASV a major public health problem. Contact with urine or feces of persistently infected rodents invading human dwellings are the main routes of human transmission, and human-to-human spread can occur by contact with contaminated blood or body fluids [36,37]. The fatality rate of Lassa fever in hospitalized patients is between 15 and 20% [38], rising to more than 30% in pregnant women in the third trimester with fetal or neonatal loss of 88% [39]. The fatality ratio can be as high as 50% or greater in nosocomial outbreaks [37].

Acute Lassa fever presents with a wide spectrum of clinical manifestations from asymptomatic infection to fatal HF, and diagnosis based on early clinical symptoms is often difficult [38]. After an incubation period of 7–18 days, patients develop fever, weakness and general malaise. A majority of patients develop cough, severe headache and sore throat. Gastrointestinal manifestations such as nausea, diarrhea and vomiting are frequent. Signs of increased vascular permeability such as facial edema and pleural effusions occur in a minority of patients and indicate a poor prognosis. With severe cases leading to death, deterioration is rapid, occurring between the sixth and tenth day of illness with progressive signs and symptoms of pulmonary edema, respiratory distress, shock, signs of encephalopathy accompanied with seizures and coma, and bleeding from mucosal surfaces. Those recovering, generally 2–3 weeks after disease onset, clear the virus from the blood. A complication late in the course of disease or in early convalescence is sensorineural deafness [40].

A highly predictive factor for the outcome of LASV infection is the extent of viremia. Patients with fatal Lassa fever have higher viral loads at time of hospitalization and are unable to limit viral replication, whereas survivors have lower initial viral load and control the infection [41]. Despite the widespread infection and development of shock in terminal stages of the disease, histological examination of fatal Lassa fever cases showed surprisingly little cellular damage and only a modest infiltration of inflammatory cells [42]. Hepatic lesions in the form of multifocal hepatocellular necrosis were a consistent pathological change [42,43]; however, the degree of hepatic tissue damage was insufficient to cause hepatic failure and only minimal recruitment of inflammatory cells was observed. High viral titers were detected in the lung, spleen, kidney, heart and the adrenal gland [42]. Other histological alterations included interstitial pneumonia and acute myocarditis. In the spleen, necrosis occurred predominantly in the marginal zone of the splenic periarteriolar lymphocytic sheath.

A hallmark of fatal LASV infection in humans is the inability of the patient's innate and adaptive immune system to contain the virus, resulting in uncontrolled fatal infection. Instead of being recognized and contained by the host cell's innate defense system, pathogenic arenaviruses are able to subvert the normal mechanisms of innate pathogen recognition. The elucidation of the innate immune defense against arenaviruses and the mechanisms of viral countermeasures have been a particularly active area of arenavirus research in the past 5 years. As is the case for other RNA viruses, the genomic RNA of arenaviruses can be recognized by the cytoplasmic RNA helicases of the retinoic acid-inducible gene I (RIG-I) family, which represent the

major innate sensors for viral RNA and induce the production of type I interferons (IFNs) [44,45]. However, cells infected with arenaviruses are unable to induce a type I IFN response owing to the ability of the viral NP to act as an IFN antagonist [46,47]. Mechanistically, the NPs of arenaviruses block the activation of the transcription factor IFN regulatory factor 3 (IRF3), a major downstream regulator of type I IFN induction via RIG-I helicases [46]. The ability of arenavirus NP to block type I IFN induction is conserved among arenaviruses, with the exception of TACV, whose NP is much less efficient [48]. In contrast to Old World arenaviruses, where the NP appears to be the only IFN antagonist, the matrix protein Z derived from the New World HF viruses JUNV, MACV, GTOV and SABV is also able to perturb the activation of RIG-I [49]. The Z protein of New World HF viruses directly interacts with RIG-I and blocks the activation of downstream signaling involving IRF3 and NF-κB via the adaptor mitochondrial antiviral signaling protein. Interestingly, arenaviruses selectively prevent the induction of type I IFNs, but do not perturb signaling induced by the addition of exogenous type I IFNs [46]. The role of the type I IFN response in survivors of LASV infection is not clear. Recent studies in nonhuman primates revealed production of type I IFNs early in LASV infection of animals that survived [50], however, the source of IFN has not been clearly identified and it is at present not clear what role this IFN response plays in protection.

Fatal Lassa fever is characterized by a marked suppression of both branches of the adaptive immune response, cellular and humoral immunity. Surviving Lassa fever patients control the infection primarily by cellular immunity, in particular the antiviral T-cell response [51,52]. By contrast, antibodies play a modest role in acute LASV infection as patients can recover in the absence of a neutralizing antibody response [41]. The marked virus-induced immunosuppression in fatal Lassa fever likely involves infection of antigen-presenting cells (APCs), in particular macrophages and dendritic cells (DCs), crucial populations of professional APCs required to induce the adaptive antiviral immune response (Figure 2) [3]. Infection of human monocyte-derived macrophages and DC with LASV *in vitro* fails to activate the cells and results in an impairment of their ability to present antigens to T cells [53,54]. LASV infection of macrophages and DCs is not associated with the release of proinflammatory cytokines [53,54] and fatal Lassa fever in humans and nonhuman primates seems not to be associated with massive inflammatory and T-cell-derived cytokine release [50,55].

Another hallmark of LASV infection in humans and nonhuman primates is lymphopenia, which is most pronounced in fatal cases and associated with tissue damage in lymphoid organs [3]. Recent studies in nonhuman primates revealed that both CD4 and CD8 cells, as well as B cells and NK cells, were affected [50]. Since human T and B cells lack a functional LASV receptor [56,57], they are not infected, suggesting an indirect effect that may involve virus-induced immunoregulatory factors. Possible candidates are type I IFNs implicated in lymphopenia observed in experimental infection of the mouse with the related arenavirus LCMV [58].

Although hemorrhages represent a cardinal symptom of arenavirus VHFs, blood loss does normally not account for the fatal outcome of the disease. This is particularly true for Lassa fever, where bleeding is not a salient feature and does not significantly contribute to the shock syndrome [3]. LASV infection in humans is normally associated with only weak thrombocytopenia and mild perturbation of platelet function [59,60]. The platelet function defect appears to be mediated by a plasma inhibitor that has not yet been characterized [60]. Only mild vascular lesions were observed in postmortem examination of fatal human Lassa fever cases [42]. In contrast to other VHFs, disseminated intravascular coagulation involving activation of fibrin deposition is virtually absent in arenavirus VHFs (Table 1). Virus-induced impairment of vascular function is likely central to fatal Lassa fever (Figure 2) and perturbation of the function of endothelial cells precedes the onset of shock and death [59,61]. The mechanisms by which LASV infection affects endothelial cells are largely unknown and may include direct effects of the virus infection and indirect mechanisms, for example. virus-induced release of soluble factors that impact on endothelial cell function. In other VHFs, in particular Ebola and Marburg HF, alterations of vascular function have been attributed mainly to the virus-induced host responses, in particular excessive production of TNF-α, IFN-γ and nitric oxide, resembling some aspects of septic shock syndrome [62,63]. Initial *in vitro* studies infecting human monocytes/macrophages and human endothelial cells with LASV provided the first evidence for important differences between the pathogenesis of LASV and filoviruses. LASV infection was highly productive in these cell types without causing an overt cytopathic effect. In the absence of cell damage, LASV infection resulted in reduced levels of proinflammatory cytokines, including TNF-α and IL-8 [64]. Detection of proinflammatory cytokines in the sera of patients with fatal Lassa fever and experimental infection in nonhuman primates have so far revealed little evidence for a 'cytokine storm' as observed in filovirus VHF [50,55]. Although a possible role of host-derived soluble factors in LASV-induced perturbation of endothelial cell function *in vivo* can at present not be ruled out, it is conceivable that more direct effects of the virus on vascular endothelial cells as a consequence of viral gene expression may underlie at least some of the vascular dysfunction observed in fatal Lassa fever. In summary, the mechanisms underlying the profound shock associated with the terminal stage of fatal Lassa fever in humans are still largely unknown. According to a working model presented in Figure 2, infection of several cell types in different tissues, including the vascular endothelium, the liver and the adrenal cortex, may result in an overall impairment of the regulation of vascular permeability in a direct or indirect way. The terminal shock syndrome may involve an imbalance of fluid distribution between intravascular and interstitial spaces combined with coagulation abnormalities and perhaps dysregulation of blood pressure.

Clinical disease & pathogenesis of South American hemorrhagic fevers

Among the South American HF viruses, JUNV, the causative agent of AHF, represents the most important public health problem, whereas MACV, GTOV and SABV cause only sporadic

outbreaks. JUNV was isolated from fatal VHF cases in the humid Pampas, the major agricultural area of Argentina, in the 1950s [65,66]. The rodent *Calomys musculinus* serves as the main natural reservoir of the virus. The virus is occasionally isolated from other rodents, possibly because of transmission from the natural host population. Former endemic hot spots are currently cooling off, whereas the overall geographic regions affected by the disease increases progressively [67]. AHF is a severe illness with hemorrhagic and/or neurological manifestations and a fatality rate of 15–30% in untreated cases [16,67,68]. JUNV appears to be infectious as aerosols and deposits in the terminal respiratory bronchioles [69]. Upon early replication in the lung, the virus enters into the lymphoid system and spreads systemically. After an incubation period of 1–2 weeks, AHF starts with rather unspecific symptoms: fever, asthenia, muscular pain, dizziness, skin and mucosal rashes, and lymph node swelling. A total of 6–10 days after disease onset, symptoms worsen with cardiovascular, gastrointestinal, renal and neurological involvement, associated with hematologic and hemostatic alterations. Disease manifestations are mainly neurological and/or hemorrhagic. Those who recover from AHF develop an antiviral immune response including antibodies in the second week of disease and clear the virus [70]. In convalescent plasma from AHF survivors, robust titers of neutralizing antiviral antibodies, mainly IgG, can be detected. In fatal cases, viremia is unchecked and patients succumb to a terminal hemorrhagic shock syndrome with a mortality of 15–30% in the absence of immune plasma therapy [16,68].

Pathological lesions in fatal AHF include generalized vasocongestion with multiple hemorrhages in the gastrointestinal mucosa, different organs, such as the liver, kidney and lungs, as well as subcutaneous tissue. In the kidneys, tubular and papillar necrosis is observed and scattered necrotic hepatocytes are found in the liver. No overt CNS pathology is apparent, despite the frequent manifestation of neurological symptoms. Secondary bacterial infections such as pneumonia are common in AHF. The lesions consistently associated with fatal cases are present

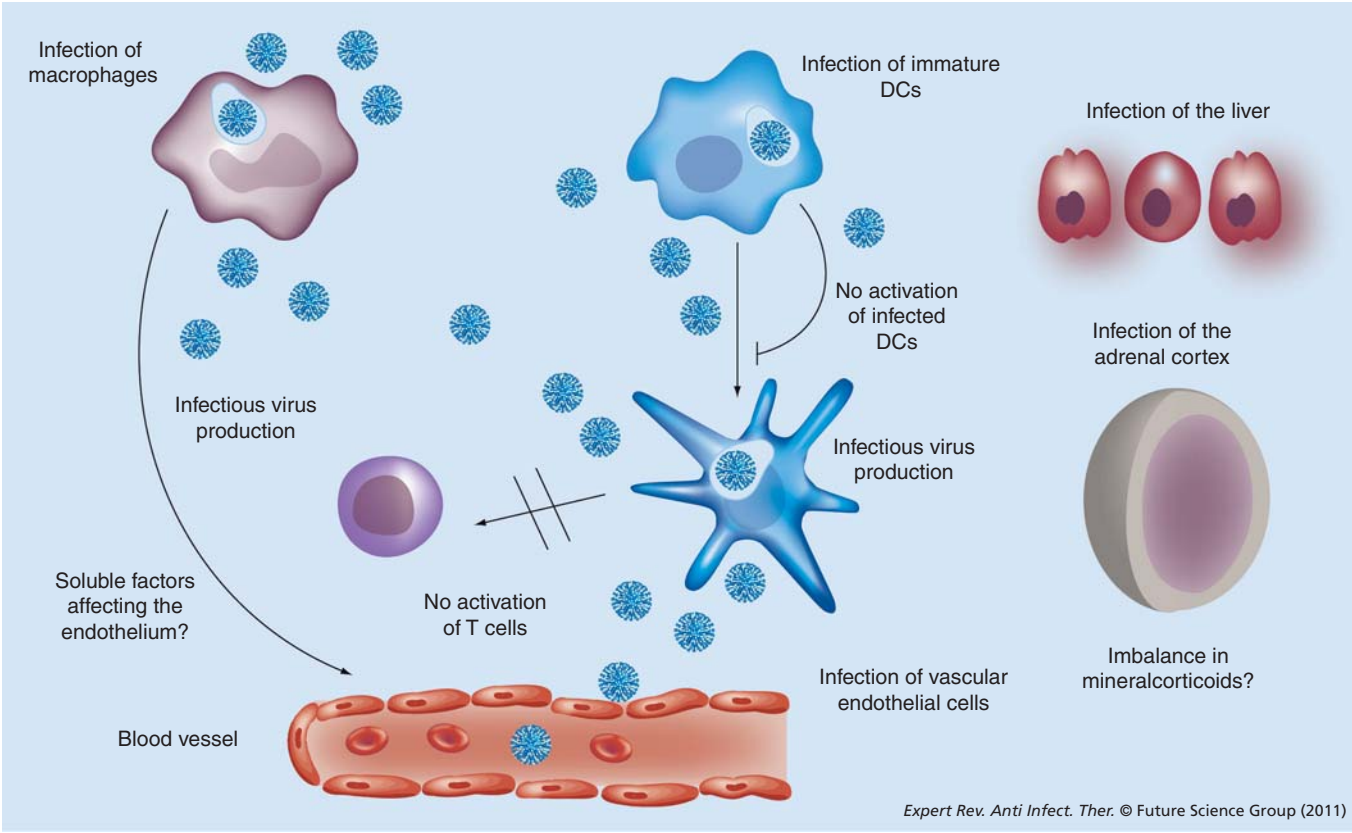

Figure 2. Model for Lassa virus pathogenesis. Macrophages and DCs represent early and preferred targets for Lassa virus (LASV) in human infection. However, instead of being recognized and presented as foreign antigen, LASV establishes productive infection that fails to activate the cells and perturbs their ability to stimulate T cells, contributing to immunosuppression. LASV infection in macrophages and in particular DCs does not result in excessive production of cytokines, but releases significant amounts of infectious virus. Infection of the liver results in enhanced levels of transaminases and hepatocellular necrosis. While the histopathology in the liver is too weak to account for liver failure, infection of hepatocytes may alter the production of serum proteins, possibly affecting hemostasis. Extensive viral replication in the adrenal cortex, where high viral loads are detected, may affect the production of mineralcorticoids leading to fluid imbalance. LASV can productively infect vascular endothelial cells without causing overt cytopathology and vascular lesions are mild in fatal Lassa fever. The mechanisms underlying the alterations in endothelial cell functions that precede shock and death in fatal Lassa fever are largely unknown and may involve direct effect of virus infection as well as effects mediated by as yet undefined soluble factors released by other cells.
DC: Dendritic cell.

Table 1. Comparison of Lassa fever with the South American arenavirus hemorrhagic fevers and Ebola hemorrhagic fever.

Parameter	LASV	JUNV	MACV	Ebola
Fever	Yes	Yes	Yes	Yes
Hypotension	Yes	Yes	Yes	Yes
Hemorrhages	Weak†	Occasional	Occasional	Occasional
Lymphopenia	Yes	Yes	Yes	Yes
Hepatic lesions	Yes	Yes	Yes	Yes
Vascular lesions	No	No	No	No
Thrombocytopenia	Rare	Yes	Yes	Yes
Platelet dysfunction	Mild	Yes	Yes	Yes
DIC	No‡	Rare§	Rare§	Yes
Infection of MP	Yes	Yes	Yes	Yes
Infection of DC	Yes	Yes	Yes	Yes
Elevated interferons	No¶	Yes	Yes	Yes
Elevated cytokines	No¶	Yes	Yes	Yes

†Hemorrhages in LASV are limited to mucosal surfaces and blood loss is mild.
‡Fibrin deposits have been reported in rare cases.
§In isolated cases, there is biochemical evidence for DIC and detection of fibrin deposits.
¶No excessive levels of interferons and cytokines detected in Lassa fever patient sera.
DC: Dendritic cell; DIC: Disseminated intravascular coagulation; JUNV: Junin virus; LASV: Lassa virus; MACV: Machupo virus; MP: Macrophage.

in the lymphatic tissue with widespread necrosis of the splenic white pulp and in the cortical and paracortical areas of the lymph nodes. The bone marrow shows global cell depletion. The highest virus titers are found in the spleen, lymph nodes and lungs, and high levels of viral antigen are found in cells of the monocyte/macrophage lineage in peripheral blood [71], lymphatic tissue, lung and liver [72]. Patients with AHF have very high levels of IFNs, TNF-α, and other inflammatory mediators that correlate with the severity of disease [73–75]. However, at present it is unclear if the enhanced levels of cytokines reflect mainly enhanced levels of viral replication and more widespread infection or if they play a role in immune-mediated pathology similar to fatal filovirus VHFs [62,63].

Virus-induced immunosuppression is also a hallmark of fatal infection with the South American HF viruses. Similar to LASV, JUNV efficiently targets DC early in infection, likely contributing to immunosuppression (Figure 2). In contrast to Lassa fever, AHF manifests with more pronounced hemorrhages, although the extent of vascular damage and blood loss does not account for the fatal outcome [16]. The hemorrhagic syndrome in AHF is attributed to coagulation alterations and marked thrombocytopenia, possibly in combination with virus and cytokine-induced vascular damage and does, in contrast to other VHF, generally not involve disseminated intravascular coagulation (Table 1).

Several lines of evidence implicate infection of vascular endothelial cells in AHF pathogenesis. Infection of cultured endothelial cells with JUNV did not cause overt cytopathic effects but altered expression of cell adhesion molecules ICAM-1 and VCAM-1 and reduced secretion of coagulation factors, such as the prothrombic von Willebrand factor (vWF) [76]. The latter finding seems to contradict clinical data showing increased vWF in sera of AHF patients [77] and the reason for this discrepancy is likely another source of vWF in AHF *in vivo*, such as megakaryocytes/platelets. Of particular interest is the increased production of the vasoactive mediator NO and prostaglandin PGI_2 in endothelial cells infected with a virulent strain of JUNV, but not an avirulent isolate [76], providing a first possible link between viral infection and the increased vascular permeability observed in fatal AHF cases.

A hallmark of fatal AHFs are coagulation abnormalities, including thrombocytopenia and low levels of platelet activity [78]. As in the case of Lassa fever, evidence for an inhibitor of platelet aggregation in plasma of infected patients has been found [60]. However, the exact nature of this inhibitor remains elusive. Recent studies shed light on the mechanism of virus-induced thrombocytopenia employing an *in vitro* model of human $CD43^+$ cells stimulated with thrombopoietin [79]. JUNV infection of $CD43^+$ precursor cells did not affect proliferation and cell viability but markedly perturbed the differentiation of megakaryocytes resulting in reduced thrombopoiesis at the levels of proplatelet formation and platelet release. Interestingly, perturbation of thrombopoiesis was not dependent on direct infection of cells, but seems to be, at least in part, mediated by a bystander effect that involves type I IFNs. A remarkable aspect of AHF is the fact that some patients die in absence of hemorrhagic disease and manifest mainly with neurological symptoms [16]. The pathophysiological basis for the neurological syndrome that is present to a variable degree in AHF patients is currently largely unknown.

MACV is also a rodent-borne pathogen that caused serious outbreaks of HF in Bolivia in the 1960s [80], but the number of cases has declined since. Human-to-human transmission has been reported [81]. GTOV emerged as the cause of Venezuelan HF in the 1990s [82,83]. Based on their close phylogenetic relationship to JUNV, infections with MACV [84,85] and GTOV [82,83,86] resemble AHF in their pathology, clinical manifestations and mortality.

While no specific treatments or vaccines have been established for MACV and GTOV, a live-attenuated JUNV has been developed and is used in high-risk groups in endemic areas [87]. Current therapy of AHF infection involves the administration of immune plasma from convalescent patients [88], which is effective when started during the first week of disease and critically depends on the titers of neutralizing antibodies. Immune plasma therapy reduces mortality from 15–30% to less than 1%. Circa 10% of patients develop a self-limiting late neurological syndrome, whose pathophysiology is unknown.

Insights into the pathogenesis of arenavirus hemorrhagic fevers from new animal models

The investigation of arenavirus pathogenesis critically depends on suitable animal models. Nonhuman primates are still considered the major relevant model for Lassa fever, in particular rhesus and cynomolgus monkeys (*Macaca mulatta* and *Macaca fascicularis*, respectively). Experimental studies in these nonhuman primates largely reproduce the pathology of human Lassa fever at the systemic and histological level, including hepatocellular necrosis, interstitial pneumonia, increased blood transaminase levels, and hemorrhagic signs and symptoms [50,51,89–94]. As in the human disease, the terminal shock syndrome includes hypovolemia, hypotension and acute respiratory distress. A recent study comparing antiviral immune responses between fatal and nonfatal LASV infection in cynomolgus monkeys revealed that fatal infection was associated with only weak activation of antiviral T cells and monocytes, a delayed and weak antibody response resulting in unchecked viremia, shock and death. Nonfatal infection was characterized by a vigorous antiviral T-cell response, activation of monocytes and effective control of viral replication [50]. The similarities with clinical findings in human Lassa fever underline the potential of this model for future studies. Marmosets have recently been described as an alternative nonhuman primate model for LASV infection [95]. Another powerful model for LASV infection in nonhuman primates uses the closely related LCMV, which is a BSL2/3 pathogen [96,97]. This model has recently been used for comprehensive transcriptome analysis in the blood and liver of rhesus monkeys infected with a lethal dose of LCMV that has provided an invaluable source for the search for candidate genes involved in arenavirus VHF pathogenesis and biomarkers for diagnostic purposes [98,99].

A major challenge in LASV research has been the lack of a mouse model for LASV that allows the application of useful mouse genetics for studies on LASV pathogenesis *in vivo*, mainly owing to the fact that normal laboratory mouse strains are resistant to LASV infection. A recent study found that mice expressing humanized MHC class I failed to control LASV infection and developed fatal VHF [100]. Interestingly, in this model, depletion of T cells prevented disease, irrespective of viremia, indicating a role for T cells in VHF pathogenesis. It will be of great interest to compare the observations from this novel small animal model with established nonhuman primate models and human infection. Another important lesson learned from this novel model is to consider the possibility of T-cell-mediated pathology in the development of novel Lassa fever vaccine strategies.

Other important BSL2/3 models for arenavirus VHF are the infection of Pichinde virus in guinea pigs (reviewed in [101]) and the infection of Syrian golden hamsters with Pirital virus [102,103]. Several experimental animal models exist for JUNV pathogenesis, including mice [104–107], guinea pigs [108–110] and rats [111], as well as New and Old World primates [112–116]. A particularly interesting small animal model for JUNV infection has recently been established using the JUNV Romero strain in guinea pigs and largely recapitulates the pathological findings in human AHF [117], holding great potential for basic research on JUNV pathogenesis and evaluation of novel drugs and vaccine candidates.

Expert commentary & five-year view

The past years have seen an impressive progress in our understanding of the basic molecular and cellular biology of arenaviruses. In particular, the advent of a powerful reverse genetic system for arenaviruses [118–120] paves the way for sophisticated structure–function studies and the molecular dissection of the mechanisms of virus–host interaction *in vitro* and also *in vivo*. Over the years, much has been learned about the pathogenesis of arenaviruses at the cellular level, in particular their ability to subvert the host cell's innate antiviral defenses [45–49]. Key IFN antagonists of arenaviruses have been discovered and the mechanisms by which the viruses block innate antiviral signaling elucidated. Other *in vitro* studies have shed light on the impact of arenavirus infection on the differentiation and function of cells targeted by hemorrhagic arenaviruses *in vivo*, including APCs such as macrophages and DCs [53,54], endothelial cells [76], and megakaryocytes [79] involved in platelet formation. At the same time, novel animal models provided important new information about the interaction of hemorrhagic arenaviruses with the host's adaptive immune system, in particular virus-induced immunosuppression, and provided the first hints towards an understanding of the terminal hemorrhagic shock syndrome [50,98–100,117]. However, many questions remain unanswered. In particular, the pathogenesis of Lassa fever appears to be rather atypical with little evidence for immunopathological mechanisms and largely unknown mechanisms underlying the vascular dysfunction involved in the terminal shock syndrome. As more subtle, direct effects of virus replication and gene expression may be responsible for the perturbation of endothelial cell function, future research involving suitable cell culture models for human endothelium, which allow detailed analysis of virus-induced cell biological and biochemical alterations, will be of great importance. Advances in endothelial cell culture, combined with high resolution confocal microscopy and the ability to measure endothelial cell functions such as transendothelial electrical resistance and hydraulic conductivity in live cells will allow monitoring of subtle, virus-induced functional changes as a consequence of arenavirus infection. The availability of a powerful reverse genetic system for arenaviruses [118–120] combined with suitable well-controlled *in vitro* experimental systems may allow the identification of viral proteins and their cellular targets implicated in virus-induced changes in the major cell types targeted by LASV *in vivo*, including APCs such as DCs and vascular endothelial cells. The identification of cellular proteins and signaling pathways affected by virus infection and the characterization of the molecular interactions involved over the next 5 years will open new avenues for the development of drugs that could prevent arenavirus-induced endothelial cell dysfunction associated with fatal arenavirus VHFs. In addition, recently developed novel diagnostic tools, including more sensitive molecular probes for viral nucleic acids and new antibodies against LASV proteins, will allow a better assessment of the extent of viral infection of the endothelium and consequent functional alterations *in vivo*. The recent development of novel and promising small animal models for human Lassa fever and AHF, as well as refinements

of established nonhuman primate models for LASV will greatly facilitate the validation of mechanistic findings obtained *in vitro* in the systemic context and allow evaluation of novel therapeutic strategies in preclinical studies.

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Key issues

- Viral hemorrhagic fevers (VHFs) caused by arenaviruses are among the most devastating emerging human diseases and represent serious public health problems. There is no licensed vaccine against these pathogens and therapeutic options are limited.
- A particular concern with arenaviruses is the continued emergence of new viruses that are associated with severe VHFs, as illustrated by the emergence of Chapare virus in 2003 and Lujo virus in 2008.
- Arenavirus VHFs in humans are acute diseases characterized by fever and, in severe cases, different degrees of hemorrhages associated with a shock syndrome in the terminal stage. Blood loss in arenavirus VHF does not account for the fatal outcome, and the pathophysiology underlying the terminal shock syndrome is not yet fully understood.
- Based on its clinical and pathological features, Lassa fever presents as a rather atypical VHF. Postmortem examination of fatal Lassa fever cases show surprisingly little tissue damage and only modest inflammation with absence of disseminated intravascular coagulation in the terminal stage.
- Fatal arenavirus VHFs are characterized by marked immunosuppression of the host, resulting in uncontrolled viral spread and replication associated with hemorrhagic shock. The immunosuppression associated with fatal disease is likely related to the fact that hemorrhagic arenaviruses efficiently target antigen-presenting cells such as macrophages and, in particular, dendritic cells, and perturb their function in antigen presentation.
- Arenaviruses can efficiently block the host cell's antiviral interferon response with the viral nucleoprotein and, in some species, also the Z protein, acting as interferon antagonists. Efficient blocking of the host innate antiviral defense by arenaviruses may contribute to the inability of the host to contain virus infection in fatal arenavirus VHF.
- A number of novel animal models for VHFs have been developed in past years and hold much promise for future studies on arenavirus pathology *in vivo*.

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