Viral apoptotic mimicry

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Abstract | As opportunistic pathogens, viruses have evolved many elegant strategies to manipulate host cells for infectious entry and replication. Viral apoptotic mimicry, defined by the exposure of phosphatidylserine — a marker for apoptosis — on the pathogen surface, is emerging as a common theme used by enveloped viruses to promote infection. Focusing on the four best described examples (vaccinia virus, dengue virus, Ebola virus and pseudotyped lentivirus), we summarize our current understanding of apoptotic mimicry as a mechanism for virus entry, binding and immune evasion. We also describe recent examples of non-enveloped viruses that use this mimicry strategy, and discuss future directions and how viral apoptotic mimicry could be targeted therapeutically.

Through millions of years of coexistence with their hosts, viruses have evolved to exploit the essential functions of the cells that they invade, such as endocytosis, secretion, cell division and apoptosis. Viral pathogens use many mechanisms to evade or subvert apoptosis, including inactivation of apoptotic sensors, molecular mimicry of apoptotic regulators and perturbation of caspase cleavage (reviewed in REF. 1). It was recently shown that viruses also hijack apoptotic recognition and clearance mechanisms for their own means. By eliminating abnormal or harmful cells, dampening immune responses and assuring normal tissue and organ formation during development, the clearance of apoptotic cells serves an essential function in all multicellular organisms (reviewed in REF. 2). Not surprisingly, this process is highly conserved and, importantly, actively anti-inflammatory[3].

To distinguish dead and dying cells from their healthy counterparts, phosphatidylserine, which is a negatively charged phospholipid, is exposed on the external leaflet of the plasma membrane as a hallmark of programmed cell death[4]. There are five key steps involved in the clearance of apoptotic debris: phosphatidylserine exposure on the apoptotic cell; engagement of phosphatidylserine receptors on a phagocyte; endocytic engulfment of the apoptotic cell; intracellular trafficking of the phagosome for degradation; and induction of an anti-inflammatory response. The externalization of phosphatidylserine on apoptotic cells was recently shown to depend on caspase-mediated cleavage of the phospholipid flippase ATP11C[5]. When externalized, phosphatidylserine can be recognized by subsets of receptors on the surface of both professional and non-professional phagocytes (reviewed in REF. 6). These specialized receptors perform two functions: they trigger the signalling cascades and actin rearrangements that are required for the endocytic engulfment, trafficking and degradation of apoptotic debris, and they concomitantly initiate the production of anti-inflammatory cytokines while suppressing the transcription of pro-inflammatory cytokines[7–9] (BOX 1).

Given these properties, it is easy to envision why a virus would evolve to use a strategy of apoptotic mimicry. By masquerading as apoptotic debris, viruses could directly engage the apoptotic clearance machinery. This would in turn trigger uptake by endocytosis and simultaneously dampen the host immune response. As many (perhaps all) cell types are capable of clearing apoptotic cells[10–12], an apoptotic mimicry strategy may enable a virus to expand its cell type tropism without the need to encode specific receptor-binding proteins. The idea that viruses might subvert cellular apoptotic clearance machinery was first proposed by Vanlandschoot and Leroux-Roels in 2003 (REF. 13), when they hypothesized that viral apoptotic mimicry is an immune evasion mechanism used by hepatitis B virus (HBV). The premise being that during chronic HBV infection, large-scale production of non-infectious subviral particles, composed of phosphatidylserine-rich host membranes and viral membrane proteins, would be able to suppress adaptive immunity through interactions between phosphatidylserine and cellular apoptotic clearance receptors.

Since Vanlandschoot and Leroux-Roels' prediction more than a decade ago, viral apoptotic mimicry has been experimentally confirmed for several enveloped viruses. These include alphaviruses, flaviviruses, filoviruses, some arenaviruses, baculoviruses, poxviruses and rhabdoviruses[14–21] (TABLE 1). By containing phosphatidylserine within their membranes, these viruses effectively mimic apoptotic cells, thereby subverting apoptotic clearance mechanisms to facilitate virus entry or infection. For many viruses that use apoptotic mimicry, their cellular phosphatidylserine receptors have been identified, and the role of this strategy in virus infection has been characterized (reviewed in REF. 22).

Interestingly, accumulating evidence suggests that apoptotic mimicry can facilitate infection by several different mechanisms. In this Progress article, we discuss recent studies indicating that viral apoptotic mimicry promotes virus entry, enhances virus binding to host cells or dampens host immune responses, depending on the virus and the phosphatidylserine receptor or receptors that are engaged. Recent findings suggest that both enveloped and non-enveloped viruses can use this strategy, so we introduce the terms classic and non-classic apoptotic mimicry, respectively. Focusing on the best described examples to date, we review phosphatidylserine-mediated entry of vaccinia virus (VACV), Ebola virus (EBOV) and dengue virus (DENV), phosphatidylserine-enhanced binding of lentivirus vectors and the dampening of innate immune responses by phosphatidylserine-containing pseudotyped lentiviral particles. We also discuss how a non-enveloped virus, simian virus 40 (SV40), engages phosphatidylserine receptors for internalization. As all of

Box 1 | **Apoptotic clearance**

In a healthy adult, approximately 50 billion cells undergo apoptosis on a daily basis[69]. Given these vast numbers, it is not surprising that defects in the clearance of apoptotic cells can result in various diseases. The major hallmark of apoptosis is exposure of phosphatidylserine on the cell surface[70]. This negatively charged phospholipid is found on the inner leaflet of the plasma membrane in healthy cells[26], but during apoptosis, phosphatidylserine is exposed on the external leaflet of the plasma membrane. This change in phosphatidylserine localization is mediated by the inactivation of lipid flippases in combination with activation of calcium-dependent phospholipid scramblases.

Clearance of apoptotic cells is initiated when these phosphatidylserine-enriched membranes engage phosphatidylserine receptors. Two types of phosphatidylserine receptor have been described: those that bind the phospholipid directly and those that use bridging molecules to associate with it. Direct phosphatidylserine-binding receptors include T cell immunoglobulin and mucin receptor (TIM) proteins (TIM1, TIM3 and TIM4); the CD300 family members CD300a and CD300f (also known as CLM1); and the seven-transmembrane spanning receptors brain-specific angiogenesis inhibitor 1 (BAI1), stabilin 1 and receptor for advanced glycosylation end products (RAGE) (reviewed in REFS 2,71,72). The phosphatidylserine-bridging molecule MFGE8 is used for apoptotic clearance through αvβ3 and αvβ5 integrins[73], which are indirect phosphatidylserine receptors. Similarly, GAS6 and protein S (PROS) are the bridging molecules that link the indirect phosphatidylserine receptors of the tyrosine protein kinase receptor 3 (TYRO3)–AXL–MER (TAM) family to phosphatidylserine to mediate apoptotic clearance[72].

The mechanism used for the engulfment of apoptotic cells by phagocytes most closely resembles macropinocytosis[74]. This endocytic mechanism is largely actin driven and regulated by the RHO GTPases RAC1 and CDC42 (REF. 75). Hallmarks of macropinocytosis include vigorous membrane ruffling, uptake into loose-fitting fluid-filled vacuoles and bystander endocytosis[36]. Although, the molecular details remain incomplete[76], genetic screens in *Caenorhabditis elegans* have identified components of the signalling pathways that drive RHO GTPase activation and the subsequent cytoskeletal rearrangements needed for clearance[64]. Once engulfment is complete, the apoptotic corpse is digested; akin to classic endosomes, the vacuole containing the apoptotic cell undergoes stepwise maturation involving a switch of RAB GTPases, exchange of phosphoinositides, acidification and movement towards the nucleus (reviewed in REF. 76). These steps occur in preparation for fusion with lysosomes, which contain the lytic enzymes, peptidases and DNases needed to break down the incoming apoptotic debris.

In addition, binding of apoptotic cells by professional phagocytes initiates the production of the anti-inflammatory cytokines transforming growth factor-β (TGFβ) and interleukin-10 (IL-10)[7,77]. The interaction of apoptotic cells and phagocytes also modulates the inflammatory response at the transcriptional level by suppressing the transcription of genes encoding pro-inflammatory cytokines, including IL-12 (REFS 8,9,78). Both professional and non-professional phagocytes produce a subset of cell survival and growth factors following apoptotic clearance[3]. Collectively, apoptotic clearance prevents inflammation, shapes the immune response and maintains tissue homeostasis.

these mechanisms rely on ligand binding to phosphatidylserine receptors, a comprehensive understanding of the various apoptotic mimicry strategies used by these viruses may aid the development of new broad-spectrum antiviral agents.

Phosphatidylserine acquisition by virions

During apoptosis, the asymmetrical distribution of phospholipids in the plasma membrane is disrupted, which results in the externalization of phosphatidylserine to the outer leaflet[5]. As phosphatidylserine exposure is essential for apoptotic clearance[4], the acquisition of this membrane phospholipid during viral assembly is critical for viral apoptotic mimicry. The mechanisms by which viruses acquire phosphatidylserine are poorly understood, but possible strategies include hijacking phosphatidylserine-rich cellular membranes by budding from intracellular organelles or the plasma membrane[23] (FIG. 1). DENV and other flaviviruses derive their membrane from the endoplasmic reticulum (ER) via budding[24]. The luminal leaflet of the ER membrane is enriched for phosphatidylserine[25,26], which suggests a potential mechanism by which flaviviruses acquire this phospholipid. It has been reported that the membrane of VACV is derived from membrane sheets that are generated by the rupture of ER cisternae[27]. A recent study of a VACV mutant arrested during membrane formation shows that viral membranes were contiguous with the ER and oriented such that the external leaflet of the viral membrane faced the ER lumen[28]. These findings further indicate that the phosphatidylserine-rich luminal leaflet of the ER becomes the external leaflet of the viral membrane. Such a mechanism would explain how VACV and other poxviruses obtain a phosphatidylserine-rich membrane. Several enveloped viruses that use apoptotic mimicry, such as EBOV and Marburg virus (MARV), have been suggested to bud from plasma membrane microdomains called lipid rafts, which are highly enriched for phosphatidylserine in the external leaflet[29,30]. Infection by many viruses triggers apoptosis of the target cell; thus, it is likely that these viruses acquire phosphatidylserine by budding from the surface of apoptotic cells, as has been suggested for the arenavirus Pichinde virus (PICV)[31]. In lieu of inducing apoptosis, cell surface phosphatidylserine exposure may be the consequence of a virus-induced increase in intracellular calcium concentrations[32]. This rise in calcium levels can inactivate lipid flippases — proteins that translocate phospholipids between membrane leaflets and thus maintain phosphatidylserine asymmetry — and result in phosphatidylserine externalization[33]. It is likely that enveloped and non-enveloped viruses alike would be able to exploit such a mechanism; however, as there is no literature to support this theory, it remains to be determined whether viruses use this strategy to acquire phosphatidylserine.

Host cell entry via apoptotic mimicry

Many of the viruses that have been reported to use apoptotic mimicry do so to facilitate host cell entry (TABLE 1). As mentioned above, divergent mechanisms of apoptotic mimicry have been described, so we introduce the terms classic and non-classic apoptotic mimicry. Classic apoptotic mimicry refers to an enveloped virus that exposes phosphatidylserine on the external leaflet of its membrane to engage phosphatidylserine receptors for uptake and/or immunomodulatory purposes. Conversely, non-classic apoptotic mimicry refers to a non-enveloped virus that, through the use of mimicry or host membrane hijacking, engages apoptotic clearance receptors to infect cells.

Classic apoptotic mimicry. Viruses hijack various endocytic mechanisms of a host cell for internalization (reviewed in REF. 34). Clathrin-mediated endocytosis (CME) and macropinocytosis (reviewed in REF. 35) are the two most common host mechanisms that are subverted for virus entry. CME is a constitutive process driven by the formation of clathrin-coated vesicles with a diameter of 100 nm. During macropinocytosis, which is not constitutive and requires induction, a large volume of extracellular material is endocytosed in macropinosomes, which are vesicles with a diameter of up to 10 μm that

Table 1 | **Viruses using apoptotic mimicry**

Virus genus*	Phosphatidylserine function in viral life cycle	Phosphatidylserine receptors	Form of apoptotic mimicry	Refs
Alphavirus (CHIKV, EEEV, RRV and SINV)	Binding, endocytosis and infection	AXL, CD300a, MFGE8-binding integrins, TIM1 and TIM4	Classic	15,19,20,52
Arenavirus (AMAV, LASV, LCMV, PICV and TCRV)	Binding, endocytosis and infection	AXL, TIM1 and TYRO3	Classic	15,21,31,48
Alphabaculovirus (AcMNPV)	Binding, endocytosis and infection	AXL and TIM1	Classic	19,52
Ebolavirus and Marburgvirus (EBOV and MARV)	Binding, endocytosis, infection and immune evasion	AXL, TIM1, TIM4 and TYRO3	Classic	14–16,19, 44,79
Flavivirus (DENV, WNV and YFV)	Binding, endocytosis, infection and immune evasion	AXL, TIM1, TIM3, TIM4 and TYRO3	Classic	14,15,17
Orthopoxvirus (VACV; both mature virions and extracellular virions)	Signalling, endocytosis and infection	AXL	Classic	18,20, 39,40
Vesiculovirus (VSV)	Binding, endocytosis and infection	AXL and TIM1	Classic	19,52
Enterovirus (PV)	Infection	Unknown	Non-classic (autophagosome-like vesicle hijacking)	56
Hepatovirus (HAV)	Unknown	TIM1	Non-classic (budding into MVBs)	54,80,81
Polyomavirus (SV40)	Binding, endocytosis and infection	AXL	Non-classic (GAS6 mimicry)	53

AcMNPV, Autographa californica multicapsid nucleopolyhedrovirus; AMAV, Amapari virus; CHIKV, Chikungunya virus; DENV, dengue virus; EBOV, Ebola virus; EEEV, eastern equine encephalitis virus; HAV, hepatitis A virus; LASV, Lassa virus; LCMV, lymphocytic choriomeningitis virus; MARV, Marburg virus; MBVs, multivesicular bodies; PICV, Pichinde virus; PV, poliovirus; RRV, Ross River virus; SINV, Sindbis virus; SV40, simian virus 40; TCRV, Tacaribe virus; TIM, T cell immunoglobulin and mucin receptor; TYRO3, tyrosine protein kinase receptor 3; VACV, vaccinia virus; VSV, vesicular stomatitis virus; WNV, West Nile virus; YFV, yellow fever virus.
*Listed are the virus genera (and viruses in parenthesis) that have been shown to use apoptotic mimicry. The respective viral families represented by these genera are *Togoviridae* (Alphavirus), *Baculoviridae* (Alphabaculovirus), *Filoviridae* (Ebolavirus and Marburgvirus), *Flaviviridae* (Flavivirus), *Poxviridae* (Orthopoxvirus), *Rhabdoviridae* (Vesiculovirus), *Picornaviridae* (Enterovirus and Hepatovirus) and *Polyomaviridae* (Polyomavirus).

form without a coat[35,36]. After endocytosis, clathrin-coated vesicles feed into the classic endosome trafficking pathway, whereas macropinosomes traffic and mature in parallel. In both cases, maturation of the vesicular compartments provide the virus with a plethora of cues that facilitate infection, including decreasing pH and the presence of proteases and inorganic ions. However, there are differences between classic endosomes and macropinosomes, including intraluminal vesicle formation, and the presence of RAB effectors, major histocompatibility complex (MHC) class II molecules and Toll-like receptors (TLRs)[37]. Together, these two pathways facilitate the internalization of most viruses, regardless of their varying size and intracellular trafficking requirements.

Apoptotic cells are phagocytosed by a mechanism that closely resembles macropinocytosis. Some viruses that use classic apoptotic mimicry, such as poxviruses and filoviruses, have been shown to enter cells by inducing this endocytic pathway. However, as we describe below, other viruses, including flaviviruses and alphaviruses, enter host cells using CME (FIG. 1). In either case, phosphatidylserine exposed on the virion membrane initiates viral uptake by binding to direct phosphatidylserine receptors, such as T cell immunoglobulin and mucin receptor 1 (TIM1; also known as HAVCR1) and other members of the TIM family, or, via bridging molecules, to indirect phosphatidylserine receptors, such as the receptor tyrosine kinase AXL (also known as UFO) and other members of the TYRO3–AXL–MER (TAM) family (BOX 1).

The first experimental evidence of a virus using apoptotic mimicry for entry was provided in 2008 for VACV mature virions[18]. Previous studies had indicated that the VACV membrane is phosphatidylserine rich and that phosphatidylserine can reactivate VACV virions that have been delipidated[38]. More recently, detailed analysis of the entry mechanism of VACV mature virions showed that the virus enters host cells by macropinocytosis. Masking phosphatidylserine using annexin V (also known as annexin A5; a protein that binds to phosphatidylserine with very high affinity) indicated that phosphatidylserine on the viral surface is required for the induction of macropinocytosis and, thus, for productive infection[18,36]. A subsequent study confirmed that phosphatidylserine is the only biologically relevant, naturally occurring lipid constituent of VACV mature virions that can rescue infection[39], and further work implicated AXL as the phosphatidylserine receptor for these viruses[40] (FIG. 1). Overexpression of the TAM ligand GAS6, which acts as a bridging molecule between phosphatidylserine and AXL (BOX 1), was also reported to enhance infection of VACV extracellular virions[20], a second form of infectious VACV that is produced when mature virions acquire additional membranes from host cell organelles. Although it has not been demonstrated that the membranes of extracellular virions contain exposed phosphatidylserine (as the mature-virion membrane does), these virions do enter host cells using macropinocytosis[41], suggesting that they too rely on apoptotic mimicry for infection.

Similarly to VACV and apoptotic cells, EBOV uses macropinocytosis for host cell entry[42,43]. The finding that ectopic expression of the phosphatidylserine receptors tyrosine protein kinase receptor 3 (TYRO3), AXL or

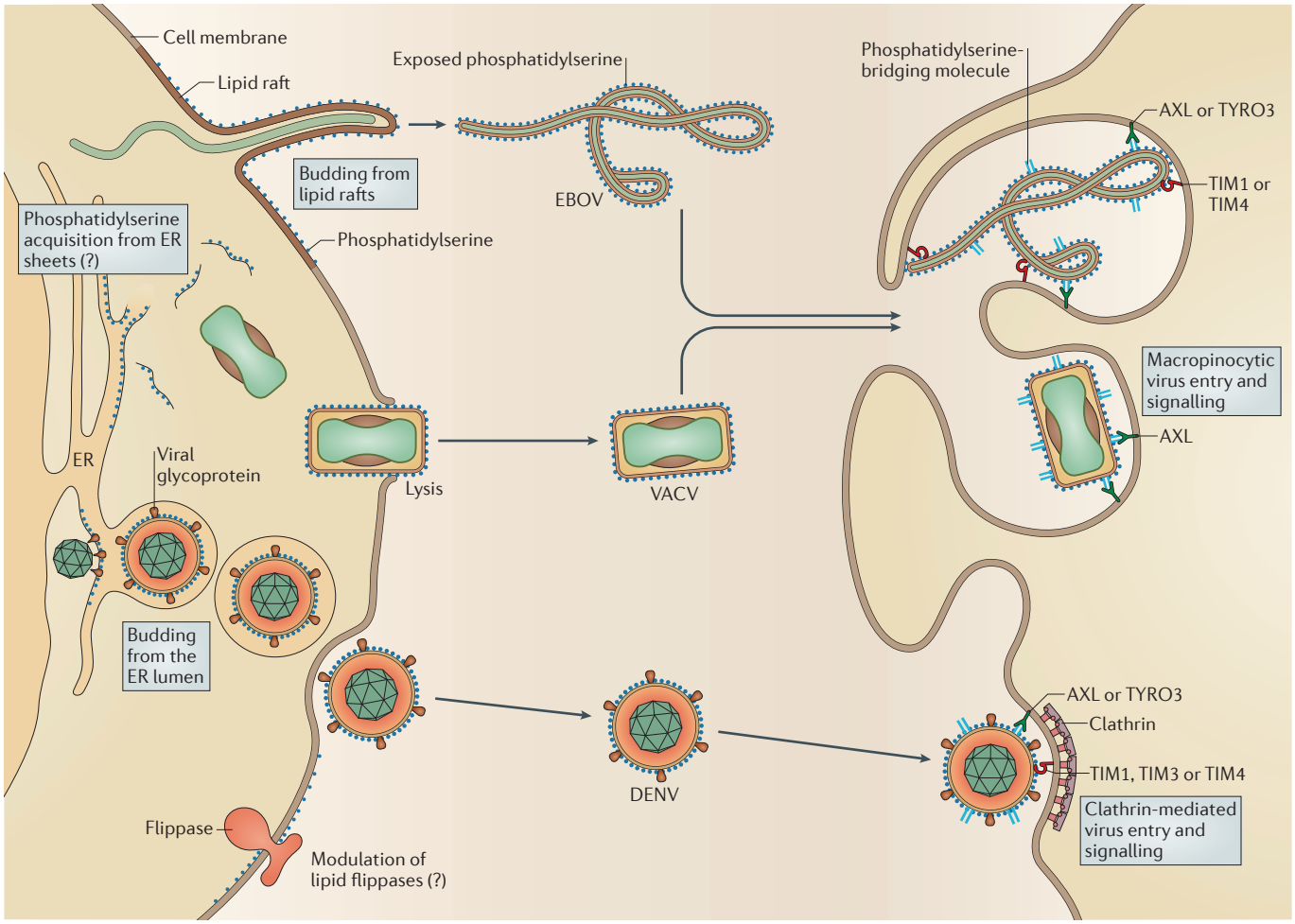

Figure 1 | **Classic apoptotic mimicry.** During classic apoptotic mimicry, a virus acquires host cell phosphatidylserine and incorporates it into the viral membrane. Exposed phosphatidylserine on the viral surface binds directly or indirectly to phosphatidylserine receptors, which facilitate virus entry or infection. Shown are several potential strategies that viruses may use to acquire phosphatidylserine in their membranes during assembly. Ebola virus (EBOV) has been shown to bud from plasma membrane microdomains, or lipid rafts, that are highly enriched for phosphatidylserine in the external leaflet. Furthermore, it has been proposed that vaccinia virus (VACV), which acquires its membrane within the host cytoplasm and exits host cells by inducing cell lysis, derives its membrane from endoplasmic reticulum (ER) sheets generated by the rupture of ER cisternae. Finally, dengue virus (DENV) and other flaviviruses derive their membrane via ER budding. Although these examples cover a range of mechanisms, it is also possible that phosphatidylserine enrichment is facilitated by the viral modulation of lipid flippases or of apoptosis (not illustrated). Recent evidence indicates that phosphatidylserine exposed on the viral surface binds to both direct phosphatidylserine receptors, such as T cell immunoglobulin and mucin receptor (TIM) proteins, and indirect phosphatidylserine receptors, such as AXL and tyrosine protein kinase receptor 3 (TYRO3), which require phosphatidylserine-bridging molecules. Both EBOV and DENV have been shown to use both direct and indirect phosphatidylserine receptors, whereas VACV has only been shown to use the indirect receptor AXL. Whether EBOV and DENV can engage these various receptors simultaneously or whether VACV can use other phosphatidylserine receptors has not been determined. For some viruses, such as EBOV and VACV, engagement of phosphatidylserine receptors triggers their internalization by macropinocytosis. For other viruses, including DENV, binding of phosphatidylserine to receptors on the host cell surface induces clathrin-mediated uptake, which is an alternative mechanism of endocytosis. After internalization, downstream signalling cascades promote additional steps of infection.

TIM1 enhanced macropinocytic virus entry provided the first link between apoptotic mimicry and EBOV[16,44,45]. It was originally proposed that EBOV glycoproteins mediate the association with TIM1 (REF. 16). However, recent studies showed that exposure of phosphatidylserine on viral particles is essential for EBOV binding to TIM1 and subsequent virus entry[15,19] (FIG. 1), demonstrating that phosphatidylserine within the EBOV membrane was responsible for the phosphatidylserine receptor-mediated enhancement of infection. Consistent with this, phosphatidylserine-containing liposomes and annexin V can both inhibit TIM1-mediated enhancement of EBOV infection, as they compete for phosphatidylserine receptors and mask phosphatidylserine, respectively[15,19].

Members of the Flavivirus genus, such as DENV, West Nile virus (WNV) and yellow fever virus (YFV), are mosquito-borne enveloped RNA viruses that cause various medically relevant human diseases, including haemorrhagic fever and encephalitis[46]. It was recently shown that ectopic expression of either TIM receptors (TIM1, TIM3 (also known as HAVR2) and TIM4 (also known as TIMD4)) or TAM receptors (AXL and TYRO3) enhanced flavivirus endocytosis through CME (A.A., unpublished observations) (FIG. 1) and that subsequent infection by all DENV serotypes, WNV and YFV was

also enhanced[14,15,17]. Moreover, it was shown that phosphatidylserine is incorporated into the membrane of flavivirus particles and is essential for infection of TIM- or TAM-expressing cells, suggesting that flaviviruses use apoptotic mimicry to invade host cells. Characterization of the interactions revealed that virions bind directly to the TIM receptors TIM1 and TIM4 and require the metal ion-dependent ligand-binding site in these receptors. By contrast, binding of TAM receptors occurred indirectly via the bridging molecules GAS6 or protein S (PROS). These data strongly argue for a tripartite model, whereby TAM ligands bind to phosphatidylserine exposed on DENV particles and bridge virions to TAM receptors.

Collectively, these studies suggest that viruses which use classic apoptotic mimicry to enter cells undergo macropinocytosis or CME. Currently, no clear pattern linking the choice of endocytic mechanism to receptor preference can be delineated, nor can alternative endocytic mechanisms be ruled out. For viruses using CME, many of the studies are gain-of-function assays performed in non-permissive cell lines; therefore, additional experimental evidence is needed to determine whether phosphatidylserine receptor engagement by enveloped viruses is required for CME. Thus, it may be important to reinvestigate the endocytic mechanism of virus internalization in the context of apoptotic mimicry.

The molecular mechanisms by which phosphatidylserine receptors mediate virus entry are poorly understood. It remains to be clarified whether phosphatidylserine receptors mediate virus internalization directly or act in concert with unknown molecules to coordinate viral endocytosis. In addition, as these receptors are used by various viruses with different entry mechanisms, it is plausible that the receptors are exploited for different purposes, depending on the virus. For instance, several enveloped viruses enter host cells by receptor-mediated endocytosis and deliver their genome into the host cytosol through low-pH-triggered fusion of the viral and endosomal membranes[47]. For flaviviruses and alphaviruses, this membrane fusion reaction is exclusively dependent on endosome acidification. The fact that phosphatidylserine receptors are not required for this step suggests that they act as bona fide entry receptors that are necessary and sufficient to promote infection. Conversely, for the filoviruses MARV and EBOV, TIM1 overexpression does not enhance virus entry in cells lacking the cognate entry receptor for these viruses, NPC1 (REF. 15), suggesting that these viruses use an entry mechanism that requires interactions with both phosphatidylserine receptors and virus-specific entry receptors. Infectivity of the arenavirus Lassa virus (LASV) is also enhanced by phosphatidylserine[48]. LASV binds to well-described extracellular (α-dystroglycan) and intracellular (lysosomal-associated membrane protein 1 (LAMP1)) receptors on host cells to facilitate entry[49–51], but it is not known whether these receptors are required for phosphatidylserine-mediated enhancement of LASV infection or whether phosphatidylserine receptors are sufficient for infection in the absence of these virus-specific receptors. It is possible that, in some cases, phosphatidylserine receptors act as a scaffold, recruiting additional virus-specific receptors or cell factors that are required to assemble an entry complex for viral endocytosis. However, these proposed mechanisms of apoptotic mimicry have yet to be experimentally tested.

Classic apoptotic mimicry may also enhance virus binding to host cells. The first link between cellular phosphatidylserine receptors and enhanced virus binding was provided by a study investigating the mechanism of residual transduction of pseudotyped lentiviruses in which the natural envelope proteins had been substituted to ablate receptor-binding activity[20]. Although the endocytic pathway used by these pseudotyped lentiviruses when engaging phosphatidylserine receptors was not determined, the authors showed that the TAM ligands GAS6 and PROS enhanced lentiviral binding to target cells expressing AXL and TYRO3, thus leading to increased transduction. Staining of pseudovirus particles with the phosphatidylserine-binding protein annexin V showed that the lentiviral vectors contained phosphatidylserine regardless of the viral envelope protein used for pseudotyping. This suggests that phosphatidylserine acquisition, and thus apoptotic mimicry, is an intrinsic feature of lentiviruses. Overexpression of the TAM receptors TYRO3 and AXL in a non-permissive cell line enhanced GAS6-mediated transduction[20]. The link between lentiviral phosphatidylserine, GAS6 and AXL was confirmed by showing that either mutation of the phosphatidylserine-binding domain of GAS6 or incubation with AXL-specific antibodies could abrogate viral transduction[20]. Apoptotic mimicry by lentiviruses does not seem to be limited to the use of TAM receptors, as bridging of viral phosphatidylserine to integrin by the phosphatidylserine receptor MFGE8 (also known as lactadherin) has also been observed[52]. In addition, transduction of a baculovirus was enhanced by the expression of TIM1 in non-permissive cell lines, and this enhancement was shown to occur in a phosphatidylserine-dependent manner[19]. Both baculoviruses and lentiviruses are used as viral vectors for gene delivery. The fact that these unrelated viral vectors were found to use apoptotic mimicry for enhanced transduction suggests that it is important to consider the consequences of phosphatidylserine-mediated residual transduction before the use of these vectors for gene delivery.

Non-classic apoptotic mimicry. Recent findings indicate that even non-enveloped viruses have evolved strategies to engage apoptotic clearance receptors for internalization (FIG. 2), underscoring the adaptive advantage offered by apoptotic mimicry to a wide range of viruses. Non-enveloped viruses do not have a membrane in which to incorporate phosphatidylserine and so are not able to engage in classic apoptotic mimicry. In the most divergent viral apoptotic mimicry mechanism, SV40, a non-enveloped polyomavirus, displays its own ligand for AXL in lieu of a bridging molecule, thereby circumventing the requirement for phosphatidylserine[53]. An elegant predictive approach was taken to identify this SV40 receptor, based on the principle that pathogens have evolved to exploit the cellular machinery of target cells and on the resultant hypothesis that proteins from pathogens mimic the structure of host proteins which are recognized by cellular receptors. *In silico* ligand–receptor modelling of the SV40 major capsid protein VP1 against a library of known receptor structures suggested that VP1 structurally mimics the bridging molecule GAS6 to bind TAM receptors. This prediction was experimentally confirmed[53] by binding studies between SV40 and both TYRO3–Fc and AXL–Fc soluble recombinant proteins, and also by infection experiments in which SV40 colocalized with AXL-expressing cells immediately after viral adsorption. Apoptotic mimicry seems to be required for SV40 infectivity, as AXL knockdown in SV40-permissive cells inhibits infection[53]. The fact that SV40 usurps the apoptotic clearance pathway by mimicking the phosphatidylserine-bridging molecule GAS6 suggests that the use of phosphatidylserine receptors and the concept of viral apoptotic mimicry is not restricted to enveloped viruses harbouring exposed phosphatidylserine on their membranes.

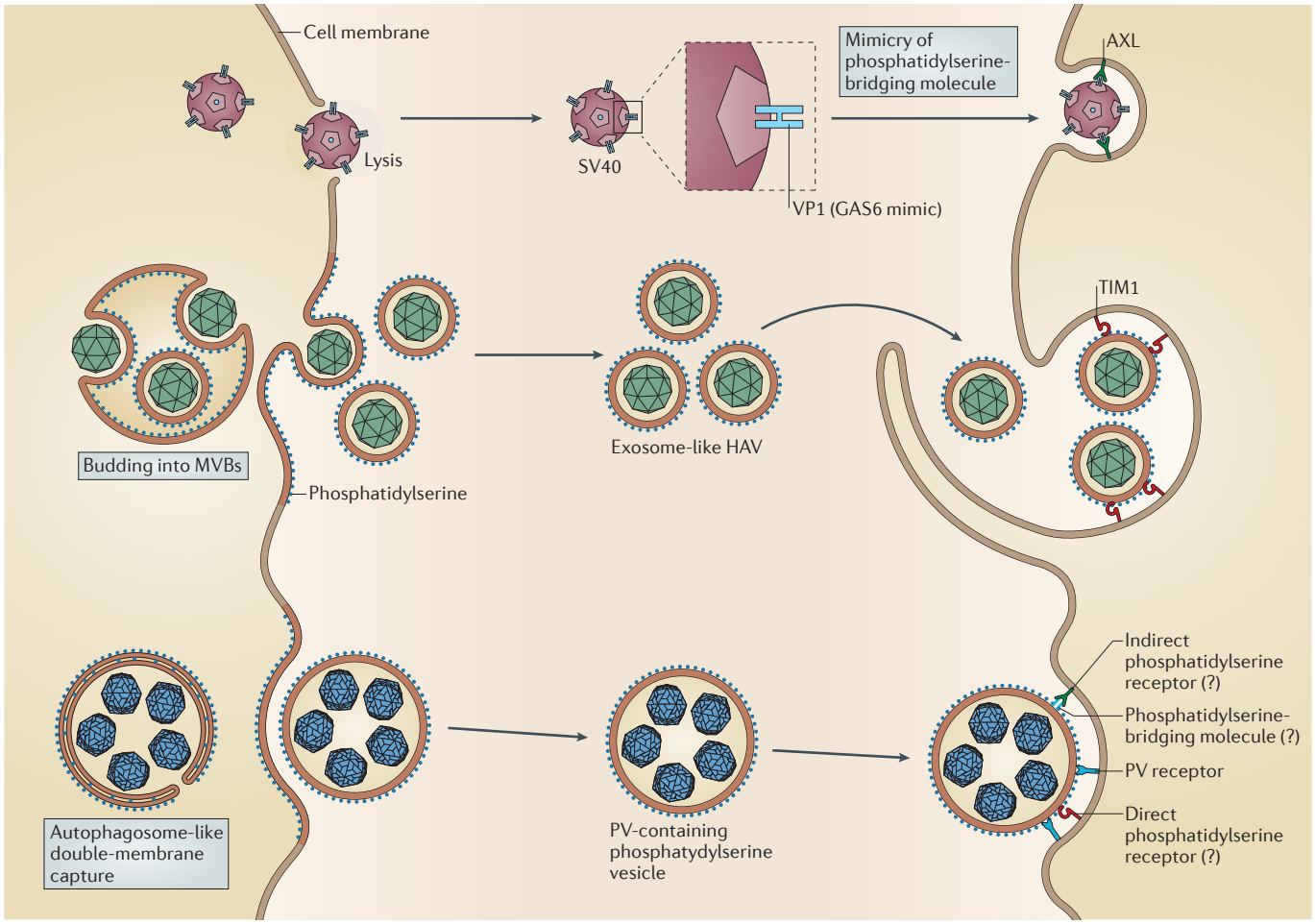

Figure 2 | **Non-classic apoptotic mimicry.** In non-classic apoptotic mimicry, non-enveloped viruses use alternative means to engage phosphatidylserine receptors. For instance, the non-enveloped polyomavirus simian virus 40 (SV40), which exits cells by lysis, mimics the phosphatidylserine-bridging molecule GAS6 to engage tyrosine protein kinase receptor 3 (TYRO3)–AXL–MER (TAM) family receptors. By sharing structural homology with GAS6, the SV40 major structural protein VP1 engages the indirect phosphatidylserine receptor AXL to initiate internalization. Another non-enveloped virus, hepatitis A virus (HAV), probably hijacks phosphatidylserine-containing membranes by budding into cellular organelles known as multivesicular bodies (MVBs). When the MVBs fuse with the plasma membrane, the HAV particles cloaked in the cell-derived envelope are released in a process thought to be akin to exosome egress. The phosphatidylserine-enriched exosome-like particles bind to T cell immunoglobulin and mucin receptor 1 (TIM1) on target cells. To facilitate bulk virus transfer, poliovirus (PV) virions are captured by autophagosome-like double-membrane vesicles. The outer membrane of these vesicles fuses with the cell surface to release phosphatidylserine-rich vesicles containing multiple PV virions. Both PV receptors and phosphatidylserine in these vesicles are required for subsequent infection. However, the phosphatidylserine receptors required remain undefined.

Hepatitis A virus (HAV), which is a member of the *Picornaviridae* family, uses TIM1 as an entry receptor[54], suggesting that this virus uses apoptotic mimicry for infection (FIG. 2). As HAV is a non-enveloped virus and thus has no membrane, it is possible that it exploits TIM1 by mechanisms other than phosphatidylserine binding. However, it has been proposed that HAV particles become enveloped in host cell membrane when they bud into host cell organelles known as multivesicular bodies (MVBs)[55]. These organelles are thought to release vesicles known as exosomes into the extracellular space on fusion with the plasma membrane. HAV particles that bud into MVBs may exit cells as exosome-like membrane vesicles[55]. It is therefore plausible that phosphatidylserine within this acquired membrane engages TIM1 directly, thereby facilitating an apoptotic mimicry mechanism.

Another recent example of non-classic apoptotic mimicry posits that the spread of enteroviruses, including poliovirus (PV), is facilitated by bulk transfer of virions within phosphatidylserine vesicles[56]. After assembly, non-enveloped cytoplasmic PV virions are captured by large double-membrane autophagosome-like vesicles. These vesicles then bud from the cell, leaving behind the outermost membrane, resulting in extracellular PV-containing vesicles with phosphatidylserine exposed on their outer leaflet. Blocking experiments using annexin V indicated that, in addition to the PV receptors, phosphatidylserine on these vesicles was required for infection[56]. Although the phosphatidylserine receptors and endocytic mechanism exploited for entry remain to be determined, infection by PV within phosphatidylserine vesicles was more efficient than infection by free PV, emphasizing the advantage of this non-classic apoptotic mimicry strategy.

To date, viruses from at least nine different families have been shown to use an apoptotic mimicry strategy to engage host

cell phosphatidylserine receptors (TABLE 1). However, it should be noted that conflicting results have been observed in the case of the Old World arenaviruses LASV and lymphocytic choriomeningitis virus (LCMV). Whether these discrepancies are due to experimental approaches (differences in infection readouts, cell types and virus production protocols) remains to be clarified[15,19,21,48]. The most striking similarity between all these viruses is their broad *in vitro* and *in vivo* cell type specificity. This broad tropism is possibly facilitated by the expression of phosphatidylserine receptors on various different host cell types.

Viral immune evasion

Experimental evidence suggests that in addition to subverting host cell apoptotic clearance machinery for the promotion of virus binding and uptake, viruses subvert this machinery for other purposes. For filoviruses as well as some arenaviruses and some flaviviruses, TAM receptor kinase activity has been implicated in the enhancement of infection[14,17,21,57]. Deletion of the cytoplasmic tail of AXL or mutation of its ATP-binding site — a site that is essential for AXL kinase activity — inhibits DENV and WNV infection without having an effect on endocytosis[14,17]. Although the underlying mechanism remains unknown, these results suggest that activation of AXL facilitates a post-entry step of flavivirus infection. Interestingly, apoptotic clearance is intimately linked with a dampening of inflammatory responses (BOX 1; FIG. 3). On formation of a tripartite complex with phosphatidylserine and GAS6, TAM receptors have been shown to heterodimerize with type I interferon receptor (IFNAR). This leads to activation of IFNAR signalling, which triggers nuclear translocation of signal transducer and activator of transcription 1 (STAT1) and the subsequent transcription of suppressor of cytokine signalling 1 (*SOCS1*) and *SOCS3*, which encode proteins that contribute to the pleiotropic inhibition of inflammatory cytokines and of TLR signalling[58]. The major question that arises from these findings is whether virus-mediated activation of phosphatidylserine receptors also dampens the innate immune response to potentiate infection.

A recent study suggests that TAM ligands complexed to pseudotyped lentiviral particles are 'super TAM agonists' that disable host immune responses and facilitate virus spread[14]. It was shown that TAM-mediated inhibition of type I interferon (IFN) signalling enhances viral infection[14]. Experiments in bone marrow-derived dendritic cells (BMDCs) from knockout mice lacking all three TAM receptors showed that pseudotyped lentiviral particles containing phosphatidylserine on the viral surface produced a strong antiviral response, with high levels of *Ifna4*, *Ifnb* and *Socs1* mRNAs compared with levels in infected BMDCs from wild-type mice. Importantly, inclusion of antibodies against IFNα and IFNβ proteins restored the level of infection in TAM triple-knockout BMDCs from the reduced level caused by the antiviral response to that seen in wild-type cells. Therefore, these data indicate that inhibition of the antiviral type I IFN response is the primary mechanism of TAM-mediated enhancement of lentiviral infection (FIG. 3).

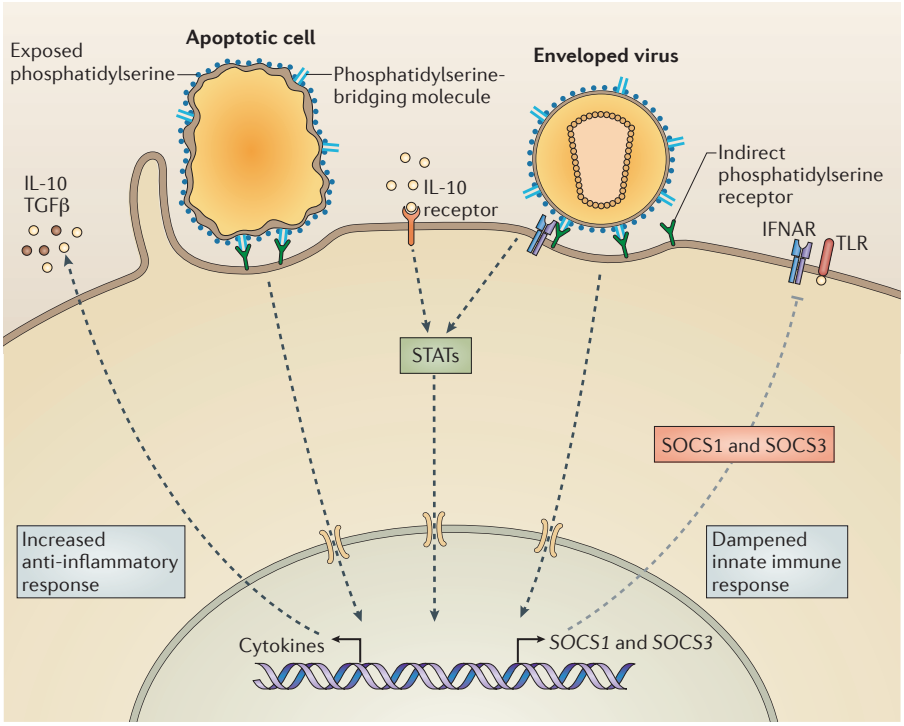

Figure 3 | **Viral apoptotic mimicry and immune evasion.** The clearance of apoptotic cells and debris induces an anti-inflammatory response. Binding of apoptotic cells to phosphatidylserine receptors and the subsequent engulfment of these cells by phagocytes initiates the production of anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGFβ). This initiates a feed-forward suppression of the innate immune response at the level of transcription, and this suppression is dependent on prolonged signalling through signal transducer and activator of transcription (STAT) family proteins. Akin to apoptotic cells, enveloped viruses, including pseudotyped lentivirus vectors and West Nile virus, are thought to use apoptotic mimicry to dampen innate immune responses. Binding of an enveloped virus complexed to bridging molecules (such as GAS6) promotes the activation of tyrosine protein kinase receptor 3 (TYRO3)–AXL–MER (TAM) family receptors, which heterodimerize with type I interferon receptor (IFNAR) to induce suppressor of cytokine signalling 1 (*SOCS1*) and *SOCS3* expression, and this in turn inhibits IFNAR and Toll-like receptor (TLR) signalling. Although phosphatidylserine receptors are often studied individually, it is likely that viruses using apoptotic mimicry can simultaneously engage different phosphatidylserine receptors to modulate various innate immune and anti-inflammatory pathways and thus promote immune evasion. Of note, T cell immunoglobulin and mucin receptor (TIM) family phosphatidylserine receptors are not included in the figure owing to a lack of evidence for their participation in the dampening of immune response by viruses.

Interestingly, expression of CD300a (also known as CLM8), a recently identified direct phosphatidylserine receptor expressed mainly on myeloid and mast cells[59,60], has been shown to increase the binding of pseudotyped lentiviral particles to host cells, but without any change in transduction efficiency[52]. Of note, engagement of CD300a by apoptotic cells does not promote their clearance; instead, on binding to phosphatidylserine, the cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM) of CD300a recruits the phosphatase SHP1 (also known as PTPN6), which blocks the release of inflammatory cytokines and chemokines[61]. Thus, it is plausible that binding of a virus to CD300a modulates the inflammatory and innate

immune responses of the host rather than facilitating viral uptake by endocytosis.

Although direct evidence for using apoptotic mimicry to dampen the host immune response is thus far limited to pseudotyped lentiviruses, similarities have been noted between immunosuppression during apoptotic cell clearance and that during *in vivo* VACV infections; VACV infections lead to the upregulation of anti-inflammatory cytokines, including transforming growth factor-β (TGFβ) and interleukin-10 (IL-10), which prevent macrophage infiltration and inhibit T cell maturation[62]. It has been proposed that the suppression of innate immunity facilitates unchecked viral replication, but whether this is due to the engagement of phosphatidylserine receptors — and hence to apoptotic mimicry — remains to be determined.

These studies do suggest that phosphatidylserine receptors are activated by viruses during binding and/or entry to modulate innate immunity, but additional studies are required to determine the exact molecular mechanisms involved and to extend the findings to other viral systems. In addition, the concept of viral apoptotic mimicry being a means of immune evasion has to date been reported only in infection studies performed with mouse BMDCs and awaits confirmation in additional cell types and, more importantly, *in vivo.* Furthermore, as several viruses can engage both direct and indirect phosphatidylserine receptors, whether apoptotic mimicry for the purpose of immune evasion can be extended to direct phosphatidylserine receptors, such as TIMs, should be explored.

Perspectives

Apoptotic cell death and clearance are essential functions within all multicellular organisms[63,64]. Owing to their importance in the maintenance of tissue homeostasis, defects in these processes often result in disease. Like many other indispensible host processes, apoptosis is subverted by viruses[1], and recent studies have shown that viruses disguised as apoptotic debris hijack the apoptotic clearance machinery. In the past few years, the list of enveloped viruses that use apoptotic mimicry has expanded (TABLE 1). Experimental evidence indicates that phosphatidylserine is displayed on the external leaflet of viral membranes and is required for binding, endocytosis or infectivity. The broad use of viral apoptotic mimicry strategies to enhance entry and infection suggests that it is a general mechanism that is exploited by many enveloped viruses.

As the field of viral apoptotic mimicry is still young, many important questions have not yet been answered. How many and which viruses exploit apoptotic mimicry? For many of the enveloped viruses described, we still do not know how they acquire phosphatidylserine in their membrane nor whether this is an active process. For viruses that bud from the plasma membrane, timed induction of apoptosis or modulation of cellular ATP-dependent phospholipid flippases[5] could possibly provide a means of enriching phosphatidylserine on the membrane before virion exit from the cell. Once a virus displays phosphatidylserine, does the distribution or concentration of this phospholipid in the viral membrane dictate binding to a distinct phosphatidylserine receptor and, by extension, the cell type specificity of the virus? To better understand viral apoptotic mimicry, it may be important to elucidate why some enveloped viruses, including herpes simplex virus 1 (HSV-1), severe acute respiratory syndrome coronavirus (SARS-CoV), influenza A virus and some arenaviruses, do not use apoptotic mimicry for infection[17,19] and why some phosphatidylserine receptors, such as brain-specific angiogenesis inhibitor 1 (BAI1) and receptor for advanced glycosylation end products (RAGE), do not mediate virus entry[52]. Also, the possibility that viruses using apoptotic mimicry simultaneously engage different phosphatidylserine receptors to promote virus entry and modulate the immune response at the same time should be investigated.

In addition, the *in vivo* relevance of viral apoptotic mimicry needs to be explored. Established mouse pathogenesis models exist for several of the viruses described in this article, and knockout mice are available for TIM and TAM receptors, as well as for the integrins that bind the bridging molecule MFGE8. At the clinical level, it could be interesting to evaluate cohorts of patients infected with these viruses, to determine whether any correlation exists between disease severity and polymorphisms in phosphatidylserine receptors or phosphatidylserine-bridging molecules.

Finally, and perhaps most importantly, can viral apoptotic mimicry be targeted therapeutically? Treatment of PICV infection with a humanized antibody against phosphatidylserine showed a protective effect against viral infection *in vivo*, indicating that targeting of phosphatidylserine may hold promise as an effective antiviral strategy[31]. Several small-compound inhibitors and TAM decoy receptors directed against AXL and MER are in various stages of preclinical development as cancer therapeutics[65,66]. In light of the use of these receptors by highly pathogenic viruses, including EBOV and DENV, these compounds should be tested for their potential antiviral efficacy. In support of this, a recent report showed that AXL-specific antibodies increased innate immune responses and could attenuate influenza virus and respiratory syncytial virus (RSV) lethality *in vivo*[67]. Although these particular viruses do not use apoptotic mimicry, this study serves as a proof of concept for the therapeutic potential of *in vivo* AXL targeting. Conversely, it was demonstrated that TIM receptors on virus-producing cells can capture HIV-1 virions through phosphatidylserine exposed on the HIV-1 membrane, thus restricting virion release and spread of infection[68]. Perhaps for some viruses, therapeutic overexpression (rather than inhibition) of phosphatidylserine receptors could be considered. Collectively, these results suggest that future efforts should be directed at exploiting viral apoptotic mimicry, phosphatidylserine exposure and phosphatidylserine receptors for the development of antiviral therapies.

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doi:10.1038/nrmicro3469
Published online 8 June 2015

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Acknowledgements

The authors apologize to all whose important contributions could not be cited owing to space constraints. A.A. is supported by grants from the Fondation pour la Recherche Médicale, the US National Institutes of Health (grant R01 N°AI101400), and the French Agence Nationale de la Recherche (grant TIMTAMDEN CE2014 and the Investissements d'Avenir programme reference ANR-10-IHUB -0002). J.M. is supported by the UK Medical Research Council (MRC) core funding to the MRC Laboratory for Molecular Cell Biology, University College London.

Competing interests statement

The authors declare no competing interests.