

Virus-cell fusion as a trigger of innate immunity dependent on the adaptor STING

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The innate immune system senses infection by detecting either evolutionarily conserved molecules essential for the survival of microbes or the abnormal location of molecules. Here we demonstrate the existence of a previously unknown innate detection mechanism induced by fusion between viral envelopes and target cells. Virus-cell fusion specifically stimulated a type I interferon response with expression of interferon-stimulated genes, *in vivo* recruitment of leukocytes and potentiation of signaling via Toll-like receptor 7 (TLR7) and TLR9. The fusion-dependent response was dependent on the stimulator of interferon genes STING but was independent of DNA, RNA and viral capsid. We suggest that membrane fusion is sensed as a danger signal with potential implications for defense against enveloped viruses and various conditions of giant-cell formation.

The present understanding of the detection of viruses by the innate immune system is that it is dominated by the recognition of nucleic acids^{1,2}. Such recognition is attended to by endosomal Toll-like receptors (TLRs), by RNA helicase RIG-I-like receptors or by DNA receptors located in the cytosol. TLR signaling depends on the adaptors MyD88 and/or TRIF, whereas receptors in the cytosol depend on either the signaling adaptor MAVS (also known as IPS-1, VISA or Cardif) or the membrane-associated adaptor STING for RNA- and DNA-recognition pathways. STING is an endoplasmic reticulum-resident protein that relocates to uncharacterized punctate structures in response to viral infection^{3,4}. Whether STING is a genuine signaling molecule or a protein that governs membrane-reorganization processes that are essential for certain virus-detection pathways, however, remains unresolved⁴. The detection of virus initiates more sampling of self by autophagy, more transcription of genes encoding antiviral molecules such as type I interferons and the activation of proinflammatory processes such as assembly of the inflammasome. The expression of type I interferons is pivotal in the innate immune response to virus. Type I interferons initiate the transcription of a large group of interferon-stimulated genes (ISGs) through the receptor for type I interferon. The products of ISGs are important in establishing a cellular antiviral state⁵ and for alerting and recruiting leukocytes. The inflammasome is a multiprotein complex and assembles in response to many stimuli, including the detection of virus. One of its constituents, caspase-1, is subsequently activated to cleave pro-interleukin 1 β (pro-IL-1 β) into IL-1 β , a highly inflammatory cytokine.

The inflammasome, together with autophagy, has been shown to participate in host protection against viral infection^{6,7}.

It has been proposed that membrane disturbances can elicit an antiviral response independently of viral nucleic acids⁸. In the present study, we found that virus-like particles (VLPs), which lack capsid and genomic material, induced a type I interferon response in primary mouse and human cells but had no apparent effect on the expression of genes encoding inflammatory molecules, autophagy or inflammasome activation. We noted a similar response to cell-cell membrane fusion or exposure to fusogenic liposomes. The responses to VLPs and fusogenic liposomes were dependent on STING but did not require TLR or RIG-I-like pathways. In addition, treatment with either VLPs or fusogenic liposomes induced the formation of a complex of STING with the kinase TBK1 and also involved the transcription factor IRF3, which was essential for the subsequent induction of ISGs. Thus, virus-cell fusion is sensed by the innate immune system and activates a STING-dependent signaling pathway that leads to the production of type I interferon and molecules encoded by ISGs.

RESULTS

Detection of VLPs by the innate immune system

We aimed this work at identifying potential new principles of innate recognition of viruses not dependent on the sensing of viral nucleic acids. For this, we prepared VLPs derived from herpes simplex virus type 1 (HSV-1) either with a deletion mutant of the HSV-1 multifunctional polypeptide UL36 that is unable to assemble infectious virus particles or by treating target cells with an inhibitor of

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DNA polymerase before infecting the cells with HSV-1. VLPs prepared with the UL36 mutant are also referred to as 'light particles' (L-particles)⁹, whereas those prepared with the DNA-polymerase inhibitor are referred to as 'pre-viral replication enveloped particles' (PREPs)¹⁰. We visualized the absence of capsid and DNA in VLPs, in contrast to their presence in infectious HSV-1 virions, by electron microscopy and by immunoblot analysis for the capsid component VP5 and the envelope-resided glycoprotein gD (Fig. 1a). We treated mouse peritoneal cells (PCs) for 4 h with isolated VLPs and collected RNA for subsequent analysis by a multiplex platform for gene-expression analysis. Both L-particles and PREPs induced a profile of a greater abundance of mRNA products of certain ISGs, in particular the chemokine CXCL10 (Fig. 1b). In contrast, mRNA encoding tumor necrosis factor (TNF) remained unchanged. We confirmed the multiplex platform data by quantitative PCR with primers specific for genes encoding interferon- β (IFN- β), CXCL10 and TNF (Fig. 1c,d). In comparison, treatment with a preparation of total HSV-1 (L-particles and infectious virions) induced expression of IFN- β and CXCL10 as well as of TNF (Supplementary Fig. 1). These results showed that PREPs and L-particles both stimulated a low-grade type I interferon response together with higher expression of ISGs, in particular the gene encoding CXCL10. Thus, we collectively call L-particles and PREPs 'VLPs' here. The response was not restricted to mouse PCs, as bone marrow-derived dendritic cells (BMDCs; Fig. 1e) and also primary human monocyte-derived macrophages (MDMs; Fig. 1f) responded to treatment with VLPs. We also noted more CXCL10 protein, as shown by confocal microscopy of VLP-stimulated PCs (Supplementary Fig. 2) and by enzyme-linked immunosorbent assay of supernatants of VLP-stimulated BMDCs (Fig. 1g). Notably, as the total HSV-1 preparation contained both infectious virus and VLPs, these data indicated that VLPs could have been responsible for as much as 25% of the ISG response to

infection with HSV-1, which suggested mechanisms of ISG induction independent of DNA sensing^{11,12}.

To investigate whether the responses induced by the VLPs could have been caused by contamination with DNA-containing virions, we did a further series of experiments. We measured HSV-1 genomic DNA in the preparations by quantitative PCR. The L-particle preparations contained low, detectable amounts of DNA (0.02% as much as that in the unpurified HSV-1 stock). However, this DNA did not reside inside the particles, as it was efficiently removed by treatment with DNase. The PREPs contained no detectable viral DNA (Fig. 1h). Notably, VLP-induced expression of CXCL10 was not affected by treatment with DNase and was therefore also independent of DNA (Fig. 1i). Finally, by measuring plaque formation in permissive cell lines, we established that the contamination of the VLP preparations with infectious DNA-containing virions was less than 1 virion per 1×10^6 VLPs (data not shown). Infectious HSV-1 diluted to a multiplicity of infection (MOI) of 0.001, which corresponds to >60-fold the maximum possible HSV-1 contamination, for use during stimulation with VLPs was not sufficient to induce the secretion of CXCL10 by BMDCs (Fig. 1g). To determine if the induction of CXCL10 was dependent on signaling via type I interferon, as suggested by the multiplex platform profile, we investigated the effect of VLPs on PCs from mice deficient in the receptor for type I interferon. In these mutant mice, CXCL10 production was abolished in response to VLPs (Fig. 1j). Thus, VLPs induced a low-grade type I interferon response that triggered the expression of a subset of ISGs.

Leukocyte recruitment *in vivo* and TLR7-TLR9 responsiveness

The innate immune response to viral infection involves other activities in addition to the interferon response. These include both the activation of inflammatory responses and cell-intrinsic antiviral effector mechanisms. We were interested in exploring whether other

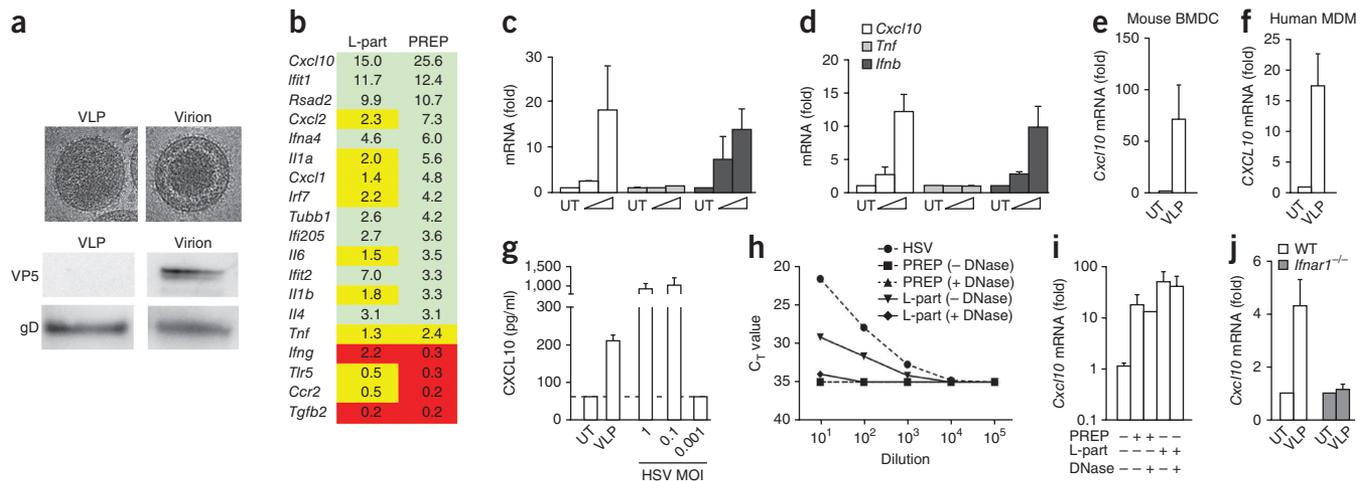


Figure 1 VLPs induce a type I interferon response. (a) Structural contents of VLPs and virions visualized by cryo-electron microscopy (top) and immunoblot analysis of VP5 and gD (below). Original magnification (top), $\times 10,000$. (b) Multiplex platform analysis of RNA expression (genes, left margin) by PCs treated for 4 h with L-particles (L-part) or PREPs (MOI, 15), presented relative to expression in untreated control cells (green, >300%; yellow, between 33% and 300%; red, <33%). (c,d) Quantitative PCR analysis of mRNA encoding CXCL10 (*Cxcl10*), TNF (*Tnf*) and IFN- β (*Iffnb*) in PCs left untreated (UT) or treated for 4 h with PREPs (c) or L-particles (d) at an MOI of 5 or 15 (wedges); results are presented relative to those of untreated cells. (e,f) Expression of mRNA encoding CXCL10 in cultures of mouse BMDCs (e) and human MDMs (f) left untreated or treated for 4 h with VLPs (MOI, 15); results are presented relative to those of untreated cells. (g) Enzyme-linked immunosorbent assay of CXCL10 in supernatants of BMDCs treated for 24 h with VLPs or dilutions of infectious HSV-1 stock. (h) Quantitative PCR analysis of HSV-1 DNA in serial dilutions (horizontal axis) of HSV-1 stock (HSV) or PREPs and L-particles left untreated (- DNase) or treated for 20 min with DNase I (+ DNase), presented as the threshold cycle (C_T). (i) Expression of mRNA encoding CXCL10 in PCs left untreated or treated with PREPs and L-particles treated as in h, presented relative to expression in untreated cells. (j) Expression of mRNA encoding CXCL10 in PCs from wild-type mice (WT) and mice deficient in the IFN- α receptor chain 1 (*Iffnar1*^{-/-}), treated (and presented) as in c (MOI, 15). Data are from one experiment representative of two independent experiments (a) or are from two (b,g-j) or five (c-f) independent experiments (mean and s.e.m.).

well-characterized innate immune responses to viruses were activated by VLPs. The production of IL-1 β is a two-step process that requires the expression of pro-IL-1 β and proteolytic cleavage of this cytokine proform by the inflammasome. The treatment of lipopolysaccharide-pretreated mouse PCs with VLPs had no effect on IL-1 β in the supernatant, whereas infection with HSV-1 induced the cytokine modestly (Fig. 2a). In contrast, the synthetic double-stranded DNA poly(dA:dT), which activates the AIM2 inflammasome¹³, potently stimulated the release of IL-1 β . HSV-1 also induced detectable cleavage of pro-IL-1 β , which did not occur in response to treatment with VLPs (Fig. 2b). Autophagy is now appreciated as an important antimicrobial effector mechanism during infection, including viral infection⁶. Autophagy is characterized by conversion of the autophagy marker LC3-I to its lipidated form, LC3-II, and the formation of punctuate LC3⁺ structures in the cytoplasm. Infection with HSV-1 induced more formation of LC3-II, as noted by immunoblot analysis, and more LC3⁺ foci in the cytoplasm, as assessed by confocal microscopy, but treatment with VLPs did not (Fig. 2c). To determine if VLPs were able to alter cellular sensitivity to subsequent stimulation with ligands of pattern-recognition receptors, we pretreated PCs with VLPs for 2 h and stimulated the cells with suboptimal concentrations of the TLR9 ligand CpG (oligodeoxynucleotide 1826) or the TLR7 ligand single-stranded RNA (ssRNA40). Pretreatment with VLPs did indeed result in greater sensitivity to these ligands, as assessed by measurement of mRNA encoding CXCL10 (Fig. 2d). Finally, we sought to determine if VLPs were able to elicit early immune activities *in vivo*. For this, we injected fusion-competent VLPs and fusion-defective VLPs from mutant HSV-1 that lacks glycoprotein gB (Δ gB VLPs) into the peritoneal cavities of mice. We then collected cells from the peritoneal cavities 24 h later and analyzed them by flow cytometry. The injection of normal VLPs induced a significantly greater abundance of natural killer cells (which are important antiviral effector cells) in the peritoneal cavity than did the injection of medium alone, but the injection of fusion-defective Δ gB VLPs did not (Fig. 2e,f). In addition, we investigated whether treatment with VLPs altered the activation status of the peritoneal leukocytes. For this we stained collected PCs for surface expression of the broad activation marker CD69.

There was significantly higher CD69 expression on PCs from mice treated with normal VLPs than on those from mice treated with medium, but not on those from mice treated with Δ gB VLP (Fig. 2g,h). Thus, VLPs selectively induced interferon responses, enhanced the sensitivity of TLR7 and TLR9, and stimulated early immune reactions *in vivo*.

Induction of type I interferon and CXCL10 by fusion

The immune system has evolved to recognize features of pathogenic microorganisms that are not easily lost or altered without also resulting in loss of infectivity^{14,15}. One feature that VLPs retain that is essential to infection is the ability to fuse with cellular membranes¹⁶. We investigated whether fusion between VLPs and cellular membranes was necessary for the induction of IFN- β and CXCL10. For this we prepared VLPs from two fusion-deficient HSV-1 mutant strains that lack glycoprotein gB (Δ gB) or glycoprotein gH (Δ gH). To ensure that we added mutant and wild-type VLPs in equal amounts, we estimated their concentrations by counting with electron microscopy and by immunoblot analysis of glycoprotein gD. Both methods resulted in similar estimates of concentration (data not shown). Neither Δ gB VLPs nor Δ gH VLPs were able to induce IFN- β or CXCL10 in PCs *in vitro* (Fig. 3a–c) or in human MDMs (Fig. 3d). These data suggested that the recognition of VLPs by the immune system depends on fusion. To determine if the release of viral material from the VLPs into the target-cell cytoplasm was necessary for recognition by the immune system, we used a different experimental approach. We cultured human MDMs together with HEK293 human embryonic kidney cells expressing the human immunodeficiency virus (HIV) fusion protein Env. The expression of Env on the surface of HEK293 cells causes these cells to fuse with primary macrophages in the coculture¹⁷. As fusion-deficient controls we used a dysfunctional variant of Env (Δ KS) or a variant with a substitution (F522Y) in the glycoprotein gp41 domain of Env that enables the cells to bind target cells but not to complete membrane fusion. HEK293 cells transfected with wild-type Env induced robust expression of mRNA encoding IFN- β and CXCL10 in the human MDMs, whereas those transfected with the Δ KS or F522Y mutant either were unable to induce such a response or

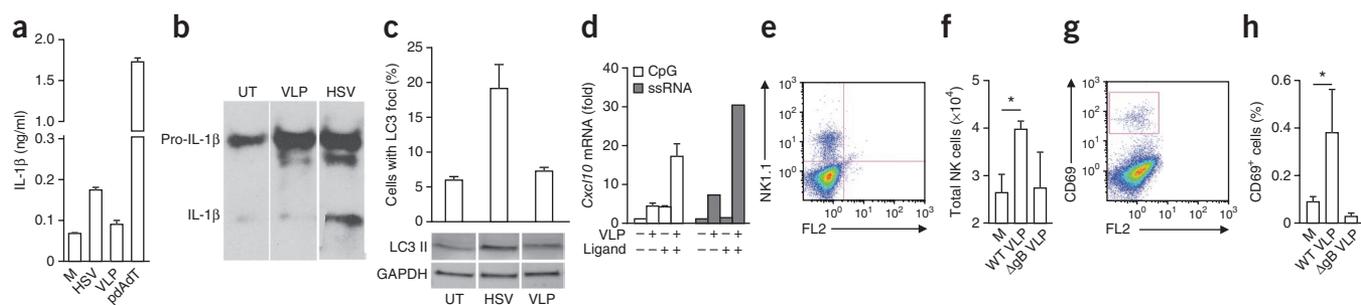
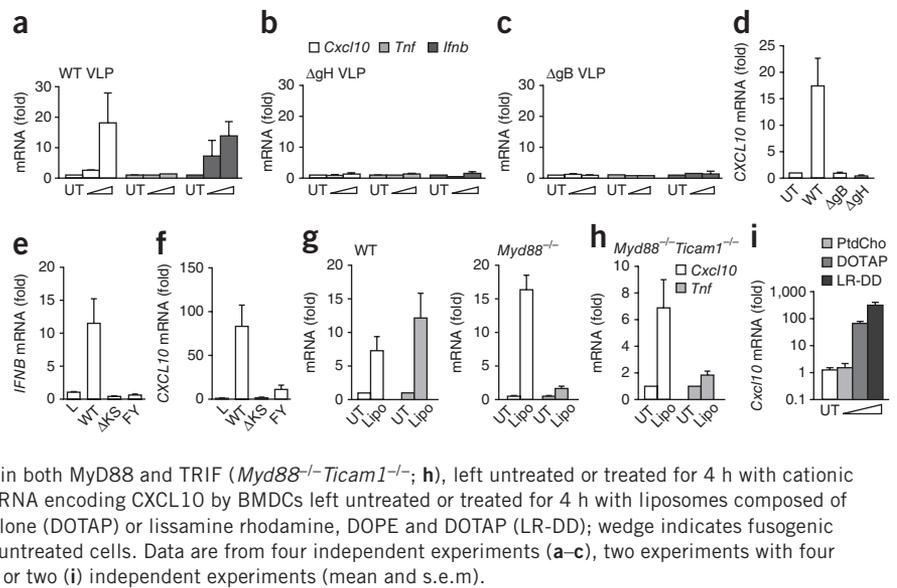


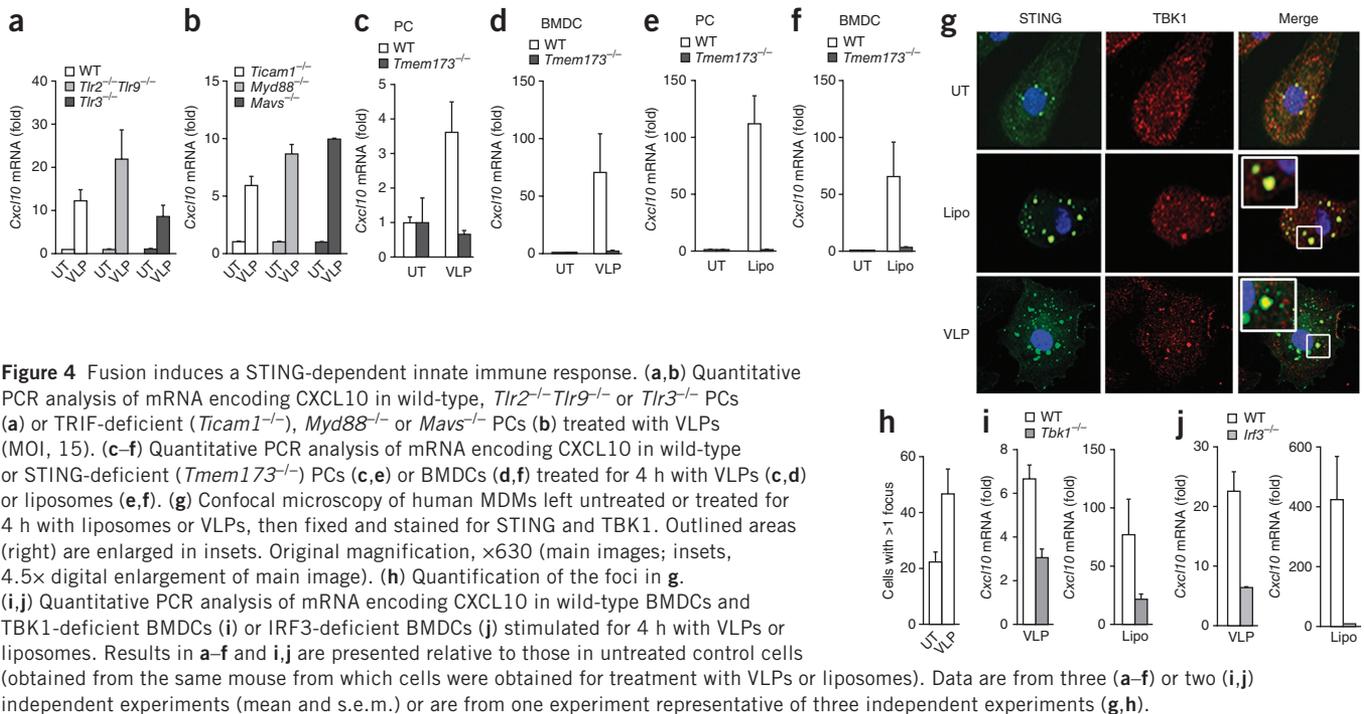
Figure 2 VLPs enhance the sensitivity of TLR7 and TLR9 and induce the recruitment and activation of leukocytes *in vivo*. (a) IL-1 β protein in supernatants of C57BL/6 mouse PCs pretreated with lipopolysaccharide (20 ng/ml), then treated for 24 h with medium alone (M), HSV (MOI, 3), VLPs (MOI, 15) or poly(dA:dT) (pdAdT; 2 μ g/ml). (b) Immunoblot analysis of pro-IL-1 β and IL-1 β in supernatants of BMDCs prestimulated for 3 h with lipopolysaccharide, then left untreated or stimulated for 16 h with VLPs or HSV-1. (c) Formation of LC3 foci in PCs left untreated or treated for 6 h with HSV-1 or VLPs, assessed by confocal microscopy (top) and immunoblot analysis of LC3 II in whole-cell extracts (below). GAPDH (glyceraldehyde phosphate dehydrogenase) serves as a loading control. (d) Quantitative PCR analysis of mRNA encoding CXCL10 in PCs without prestimulation (VLP –) or prestimulated for 2 h with VLPs (VLP +), then left untreated (Ligand –) or treated for an additional 4 h (Ligand +) with a suboptimal concentration of CpG (oligodeoxynucleotide 1826; 0.5 μ M) or ssRNA40 (1 μ g/ml); results are presented relative to those of untreated cells. (e) Flow cytometry of cells from the peritoneal cavity of an 8-week-old mouse injected intraperitoneally VLPs suspended in PBS (25 \times 10⁶ particles), followed by another injection after 12 h and collection of cells after 24 h, to assess expression of the natural killer cell marker NK1.1 through the use of count beads (FL2, fluorescence channel 2). (f) Total natural killer (NK) cells in the peritoneal cavities of 8-week-old mice injected intraperitoneally (as in e) with PBS (M), wild-type (WT) VLPs or Δ gB VLPs (suspended as in e). (g) Flow cytometry of cells from the peritoneal cavity of an 8-week-old mouse injected as in e, to assess expression of the activation marker CD69 (as in e). (h) Frequency of CD69⁺ cells in the peritoneal cavities of 8-week-old mice injected as in f. **P* < 0.05 (Student's *t*-test). Data are from one experiment representative of two independent experiments (a–d) or two independent experiments with seven mice per group (e–h).

Figure 3 Fusion induces the expression of type I interferon and CXCL10. (**a–c**) Quantitative PCR analysis of mRNA encoding CXCL10, TNF and IFN- β (key) by C57BL/6 mouse PCs left untreated or treated for 4 h with wild-type VLPs (**a**) or fusion-deficient Δ gH VLPs (**b**) or Δ gB VLPs (**c**); MOI of VLPs, 5 or 15 (wedges). (**d**) Quantitative PCR analysis of mRNA encoding CXCL10 in human MDMs treated with VLPs as in **a–c**. (**e,f**) Quantitative PCR analysis of mRNA encoding IFN- β (**e**) and CXCL10 (**f**) in human MDMs cultured for 8 h with HEK293 cells treated with Lipofectamine 2000 (L) or transfected 2 d earlier with expression plasmids for wild-type Env or the Env mutant Δ KS or F522Y (FY). (**g,h**) Quantitative PCR analysis of mRNA encoding CXCL10 and TNF (key) in PCs from wild-type or *Myd88*^{-/-} mice (**g**) or an immortalized macrophage cell line doubly deficient in both MyD88 and TRIF (*Myd88*^{-/-}*Ticam1*^{-/-}; **h**), left untreated or treated for 4 h with cationic liposomes (Lipo). (**i**) Quantitative PCR analysis of mRNA encoding CXCL10 by BMDCs left untreated or treated for 4 h with liposomes composed of a phosphatidylcholine derivative (PtdCho), DOTAP alone (DOTAP) or lissamine rhodamine, DOPE and DOTAP (LR-DD); wedge indicates fusogenic potential. Results are presented relative to those of untreated cells. Data are from four independent experiments (**a–c**), two experiments with four independent human MDM donors (**d–f**), or five (**g,h**) or two (**i**) independent experiments (mean and s.e.m.).



induced a much lower response, respectively (**Fig. 3e,f**). We obtained similar results when we used Env-transfected HeLa human cervical cancer cells to stimulate human MDMs (**Supplementary Fig. 3**). In a parallel series of experiments, we instead inhibited fusion by adding increasing amounts of the synthetic HIV fusion inhibitor T20 to cocultures of HEK293 cells transfected with wild-type-Env and primary macrophages. T20 inhibited IFN- β expression in a dose-dependent manner (**Supplementary Fig. 4**). Thus, fusion between HEK293 cells or HeLa cells and primary macrophages induced IFN- β and CXCL10. However, transfer of cellular material from cell to cell could have potentially triggered the production of interferon. To rule out that possibility, we used a system based on fusogenic liposomes. Liposomes are bipolar lipid membrane vesicles and, depending on the molecular properties of the lipids, can fuse with cellular membranes. Cationic liposomes, which are widely used to deliver, for example,

DNA plasmids into live cells, are able to fuse with cellular membranes and have been shown to induce immune responses through at least two separate pathways, one of which is MyD88 dependent¹⁸. We therefore determined whether cationic liposomes were able to induce CXCL10 expression in PCs from wild-type and MyD88-deficient (*Myd88*^{-/-}) mice and in immortalized bone marrow-derived macrophages doubly deficient in both MyD88 and TRIF (*Myd88*^{-/-}*Ticam1*^{-/-} macrophages). Treatment with liposomes able to fuse with cellular membranes (**Supplementary Fig. 5**) induced mRNA encoding CXCL10 in wild-type and *Myd88*^{-/-} PCs and *Myd88*^{-/-}*Ticam1*^{-/-} macrophages (**Fig. 3g,h**). In contrast, we observed liposome-induced expression of TNF only in wild-type cells (**Fig. 3g**). This did not seem to be dependent on the apoptotic release of DNA, as liposomes were also able to induce CXCL10 in the presence of a pan-caspase inhibitor (**Supplementary Fig. 6**). Notably, when we



compared liposomes with differences in fusogenic potential, there was a clear correlation between that potential and the ability to induce expression of CXCL10. Liposomes composed of a phosphatidylcholine derivative with low fusogenic potential did not induce mRNA encoding CXCL10. In contrast, liposomes composed of the cationic lipid DOTAP, which have intermediate fusogenic potential, induced some CXCL10, whereas liposomes composed of lissamine rhodamine, the neutral lipid DOPE and DOTAP, which are highly fusogenic¹⁹, induced the highest expression of mRNA encoding CXCL10 (Fig. 3i). To investigate whether liposomes induced an mRNA expression profile similar to that induced by VLPs, we used RNA from liposome-treated PCs for analysis by multiplex platform technology. Here we found that in addition to an ISG profile with higher expression of genes encoding CXCL10, IFI205 and viperin, there was also a greater abundance of mRNA from NF- κ B-stimulated genes such as the gene encoding CXCL1 (ref. 20), possibly induced by the MyD88 pathway (Supplementary Fig. 7). We also found that the transfection reagent Lipofectamine 2000 induced CXCL10 in BMDCs and that this response was abrogated in BMDCs from IRF3-deficient mice (data not shown). Thus, VLP-cell, cell-cell and liposome-cell fusion induced expression of IFN- β and ISGs.

STING-dependent immune responses to fusion

To determine if the responses elicited by VLPs depended on known HSV-sensing pathways, we treated PCs from mice with a series of one deficiency or more deficiencies in these pathways. VLPs retained the ability to induce CXCL10 in PCs from *Tlr2*^{-/-}*Tlr9*^{-/-} mice, *Tlr3*^{-/-} mice, *Myd88*^{-/-} mice, *Ticam1*^{-/-} mice and *Mavs*^{-/-} mice (Fig. 4a,b). To exclude the possibility of redundant mechanisms, we also monitored responses in *Myd88*^{-/-}*Ticam1*^{-/-}*Mavs*^{-/-} mice and found that these mice responded normally (Supplementary Fig. 8). In contrast to that, we found that the induction of CXCL10 by VLPs or liposomes was abolished in both PCs and BMDCs from STING-deficient mice (Fig. 4c-f), which suggested that the fusion of membranes led to an innate immune response through DNA-independent sensing

mechanisms that depended on STING. To further assess the involvement of STING, we investigated the intracellular localization of STING in response to liposomes or VLPs in human MDMs, as STING has been reported to relocate after HSV-1 infection³. The treatment of primary macrophages with liposomes or VLPs did indeed induce the relocalization of STING in >40% of the cells (Fig. 4g,h). Moreover, in both cases, STING also localized together with TBK1 (Fig. 4g,h). The stimulation of TBK1-deficient or IRF3-deficient mouse BMDCs with liposomes or VLPs indicated that TBK1 was involved but not essential (Fig. 4i), whereas IRF3 was both involved in and essential for the induction of CXCL10 expression by either VLPs or liposomes (Fig. 4j).

VLPs activate a phosphatidylinositol-3-OH pathway

Infection of the human cervical cancer cell line CaSki with HSV has been shown to induce a very early increase in free intracellular calcium²¹, which could represent an early signaling event that stimulates interferon responses after fusion is detected. Like HSV, VLPs were able to induce an increase in free intracellular calcium in CaSki cells, although not to the same extent as the infectious virus (Fig. 5a). Fusion-deficient Δ gB VLPs failed to stimulate calcium flux. This was also true for mouse BMDCs (Fig. 5b) and human DCs (data not shown). The flux was abrogated by the chemical inhibitor 2-APB, which inhibits inositol-1,4,5-trisphosphate-dependent release of calcium from the endoplasmic reticulum (Fig. 5c). Despite that finding, treatment with 2-APB did not interfere with the induced expression of mRNA encoding CXCL10 (Fig. 5d). Other signaling molecules described as being involved in membrane-proximal signaling include phospholipase C- γ (PLC- γ) and phosphatidylinositol-3-OH kinase (PI(3)K). We therefore examined the involvement of PLC- γ and PI(3)K in activation of the ISG response to VLPs. VLPs and liposomes were indeed able to induce phosphorylation of the PI(3)K substrate Akt (Fig. 5e,f). Moreover, this response was inhibited in the presence of either the PLC- γ inhibitor U73122 or the PI(3)K inhibitor Ly294002 (Fig. 5e), which placed PLC- γ upstream of PI(3)K in the fusion-activated pathway. Each inhibitor blocked the

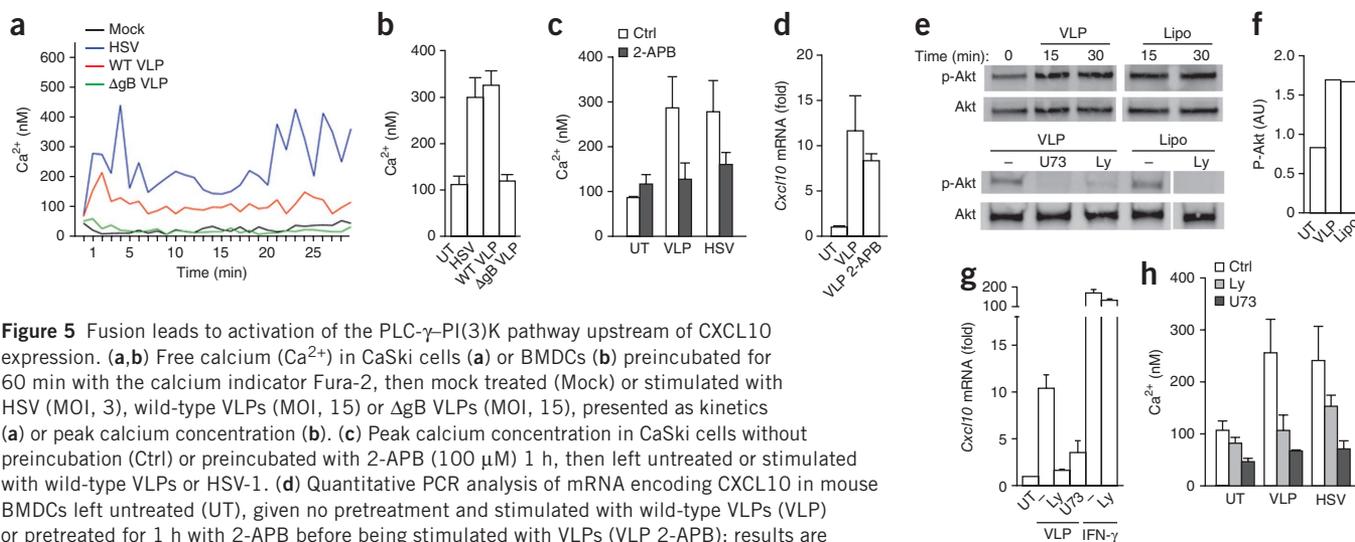


Figure 5 Fusion leads to activation of the PLC- γ -PI(3)K pathway upstream of CXCL10 expression. **(a,b)** Free calcium (Ca²⁺) in CaSki cells **(a)** or BMDCs **(b)** preincubated for 60 min with the calcium indicator Fura-2, then mock treated (Mock) or stimulated with HSV (MOI, 3), wild-type VLPs (MOI, 15) or Δ gB VLPs (MOI, 15), presented as kinetics **(a)** or peak calcium concentration **(b)**. **(c)** Peak calcium concentration in CaSki cells without preincubation (Ctrl) or preincubated with 2-APB (100 μ M) 1 h, then left untreated or stimulated with wild-type VLPs or HSV-1. **(d)** Quantitative PCR analysis of mRNA encoding CXCL10 in mouse BMDCs left untreated (UT), given no pretreatment and stimulated with wild-type VLPs (VLP) or pretreated for 1 h with 2-APB before being stimulated with VLPs (VLP 2-APB); results are presented relative to those of untreated cells. **(e)** Immunoblot analysis of phosphorylated (p-) and total Akt in lysates of BMDCs left unstimulated (0) or stimulated for 15 or 30 min with VLPs or liposomes (0.1 mg/ml; top) or given no pretreatment (-) or preincubated with U73122 (U73; 50 μ M) or Ly294002 (Ly; 5 μ M), then stimulated with VLPs or liposomes (bottom). **(f)** Quantification of phosphorylated Akt in BMDCs left untreated or stimulated with VLPs or liposomes. **(g)** Quantitative PCR analysis of mRNA encoding CXCL10 in BMDCs left untreated (UT) or given no pretreatment (-) or preincubated for 45 min with Ly294002 or U73122, then stimulated for 4 h with VLPs or IFN- γ ; results are presented relative to those of untreated cells. **(h)** Peak calcium concentration in CaSki cells given no pretreatment (Ctrl) or preincubated with Ly294002 or U73122 (key), then left untreated or stimulated with VLPs or HSV-1. Data are from one experiment representative of two independent experiments (mean and s.e.m.).

VLP-induced expression of mRNA encoding CXCL10 (Fig. 5g) and also the VLP-induced increase in free intracellular calcium (Fig. 5h). Together these data suggested that cells are able to detect membrane fusion, which leads to the selective induction of expression of interferon and ISGs through a pathway that depends on STING and involves TBK1, IRF3 and the PLC- γ -PI(3)K pathway.

DISCUSSION

In the present study we have demonstrated the existence of a mechanism for detection by the innate immune system that senses the fusion of viral envelopes with target cells. Membrane fusion specifically induced type I interferon responses and expression of ISGs, and this proceeded through a mechanism dependent on STING. Although STING is associated with the sensing of DNA during viral infections^{3,11}, the mechanism described here was independent of the presence of both DNA and RNA. The principle of sensing by the innate immune system described here applies to virus-cell fusion, liposome-cell fusion and cell-cell fusion. The 'unscheduled' fusion between a viral envelope and a cellular membrane is in contrast to 'scheduled' and highly regulated fusion events such as natural syncytia formation and endoplasmic reticulum-membrane fusion, which do not elicit an immune reaction. We therefore suggest the existence of cellular mechanisms to distinguish between scheduled and unscheduled fusion events. Our data here expand on those of published studies showing that cationic liposomes induce the expression of costimulatory molecules in dendritic cells through MyD88-controlled pathways¹⁸ and on other data showing that cell-cell fusion enforced by overexpression of the reoviral fusogenic protein FAST leads to a type I interferon response⁸. By showing that liposomes stimulated an interferon response through a MyD88-independent and STING-dependent mechanism similar to that observed with VLPs, we have demonstrated the existence of a membrane fusion-activated pathway that leads to type I interferon responses.

Many viruses, and members of the α -herpesvirus family in particular, naturally produce particles that lack genomic material and capsid¹⁶. Such particles are produced in numbers almost equal to those of infectious genome-containing virus particles but can, because of their lower density, be separated by gradient centrifugation¹⁶. The demonstration that the VLP preparations were not contaminated by genome-containing particles was critical for our line of experiments and the conclusion that the response observed was mediated by membrane fusion independently of genomic DNA or RNA. The finding that synthetically prepared liposomes also induced the observed expression of interferon and ISGs in a STING-dependent manner has provided substantial additional evidence for the existence of cellular mechanisms for sensing lipid membrane fusion to induce interferon responses. That conclusion was further strengthened by the observed interferon response in the HIV Env cell-cell fusions assay, which also indicated that this phenomenon was not restricted to detection of viral infection but could represent a mechanism for alerting the cell of other unscheduled cellular membrane fusion events.

The response of the innate immune system to viral infection is a coordinated process that involves many activities, including interferon expression and function as well as inflammatory gene expression and effector functions. In addition, cell-intrinsic antiviral effector mechanisms such as autophagy have been reported to contribute to the innate control of viruses⁶. We found that the cellular response to membrane fusion was restricted to a type I interferon response with limited effect on the expression of genes encoding inflammatory molecules, inflammasome activation and autophagy. To our knowledge, this is the first example of an innate recognition mechanism that selectively activates

interferon responses. This suggests that the cellular sensing of viral entry serves the purpose of restricting the virus without activating excessive inflammation. In addition, the finding that the VLPs synergistically enhanced the response to agonists of TLR7 and TLR9 suggested that the cellular sensing of viral entry also serves the purpose of conditioning the cell for optimal innate immune responses to the recognition of viral nucleic acids in intracellular compartments. The mechanism for this last phenomenon is unknown but may involve reactive oxygen species, which we found were induced after the treatment of PCs with VLPs (data not shown) and have been reported to amplify interferon responses induced by pattern-recognition receptors²².

Research on STING function has been dominated by descriptions of its role in DNA sensing. Our results, however, suggest a more complex role for STING, possibly through interactions with complexes that govern membrane dynamics. This is in line with published work showing that STING associates with the endoplasmic reticulum translocon and the octameric Sec6-Sec8 complex. This complex is involved in governing dynamic membrane events such as the tethering of secretory vesicles to the endoplasmic reticulum⁴. Moreover, knockdown of Sec5 and Sec6 by RNA-mediated interference abolishes STING-dependent IFN- β expression in response to Sendai virus⁴. It is noteworthy that Sec5 seems to accumulate in areas in which membrane material is added to the plasma membrane during cellularization in embryonic cells from *Drosophila*²³. Other proteins involved in membrane dynamics also seem to be important in innate immune responses to enveloped viruses. For example, mouse embryonic fibroblasts deficient in the membrane phospholipid PLSCR1 are reported to have diminished type I interferon responses to infection with vesicular stomatitis virus and enhanced viral replication²⁴. However, we found no evidence that PLSCR1 was responsible for the innate response to membrane fusion, as shown by the unaltered CXCL10 expression in *Plscr1*^{-/-} DCs treated with VLPs or liposomes (data not shown). Hopefully, future work will identify which proteins upstream of STING are involved in sensing the actual fusion.

Membrane fusion activated both the PLC- γ -PI(3)K pathway, which stimulated the release of Ca²⁺ from endoplasmic reticulum stores, and a separate pathway that drove the fusion-activated interferon-ISG response, which was dependent on STING and IRF3 and also involved TBK1. The PLC- γ -PI(3)K pathway is well described as having important roles in membrane-proximal signaling^{25,26} and is also activated during the entry process of many viruses^{27,28}. For several viruses, this pathway is in fact essential for the successful completion of entry into the cell^{28,29}. In the present study we did not mechanistically describe the link between the PLC- γ -PI(3)K and the downstream response, including translocation of STING and assembly of the STING-TBK1 complex. However, we believe that PLC- γ -PI(3)K signaling does act in the same pathway that STING acts in, as inhibition of PI(3)K led to considerably more VLP-induced STING foci (data not shown). This suggests that the PLC- γ -PI(3)K pathway has a critical role in stimulating mature STING signalosomes, which signal to IRF3 but are also subject to degradation.

Membrane fusion is essential for the entry of enveloped viruses and is also found in several noninfectious conditions with giant-cell formation. Notably, measles virus strains able to form syncytia are more potent inducers of interferon than are strains without this function³⁰, and giant-cell arteritis involves an as-yet-unexplained type I interferon response³². This suggests that membrane fusion is a danger signal of importance during both infections and noninfectious conditions. The innate immune system detects evolutionarily conserved microbe-specific molecules essential for survival and also detects the mislocation of molecules used by both microbes and host cells. Sensing of membrane

fusion by the innate immune system is not easily placed into either category, and hence it seems likely that host cells have systems for the detection of dynamic pathogen-associated signals and in this way display ongoing microbial activity to the immune system.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.K.H., K.A.F. and S.R.P. conceived of the project; C.K.H. designed the experimental setup, organized the project, did most of the experiments, drafted the manuscript and, together with S.R.P. and K.A.F. edited and finalized the manuscript; S.B.J., M.R.J., K.A.H., M.H.C., R.G.-D., S.B.R., H.B.M. and N.C. did experiments; S.R.P., K.A.F. and B.C.H. supervised experiments; and F.J.R. and T.O.Y. provided reagents.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Reagents, mice, cells and VLPs. *Tlr2*^{9-/-} and *Tlr3*^{1-/-} mice were bred at M&B Taconic and were kept at the animal facility at the Faculty of Health Sciences, University of Aarhus. *Tbk1*^{1-/-}, *Myd88*^{1-/-}, *Ticam1*^{1-/-}, *Mavs*^{1-/-}, STING-deficient (*Tmem173*^{1-/-}) and *Myd88*^{1-/-}*Ticam1*^{1-/-}*Mavs*^{1-/-} mice were bred and maintained in the animal facilities of the University of Massachusetts Medical School. Mice deficient in IFN- α receptor chain 1 (from A.R. Thomsen) were bred and maintained at the animal facility at the Panum Institute of the University of Copenhagen. PCs were isolated from mice left untreated or pretreated with 5 ml PBS with 3% thioglycollate. Differentiation of BMDCs from bone-marrow cells was achieved by culture for 7 d in the presence of granulocyte-macrophage colony-stimulating factor (Sigma). PBMCs were isolated from healthy donors by Ficoll Paque gradient centrifugation (GE Healthcare). Monocytes were separated from PBMCs by adherence to plastic on UpCell plates (Nunc) precoated with poly-L-lysine (Cultrex). Differentiation of monocytes to human MDMs was achieved by culture for 6–8 d in the presence of macrophage colony-stimulating factor (Sigma). Mouse BMDCs were obtained by collection of nonadherent cells from bone marrow cell cultured for 7 d in the presence of granulocyte-macrophage colony-stimulating factor (R&D Systems). L-particles and PREPs were prepared and isolated as described^{9,10}. The UL36 HSV-1 deletion mutant was used for the preparation of L-particles in the rabbit skin cell line HAUL36-1, whereas PREPs were prepared with BHK-21 hamster kidney cells pretreated with acycloguanosine (Sigma). Fusion-deficient VLPs were prepared as PREPs with gB- or gH-deficient HSV-1 with the supporting cell line Vero D6 or Vero F6 (derived from the African green monkey epithelial cell line Vero). For the treatment of mammalian cells with VLPs, an MOI of 10–30 was used. For treatment of VLPs with DNase, the DNase (Sigma) was dissolved in DNase buffer (PBS with 1 mM CaCl₂ and 6 mM MgCl₂ but without denaturizing agents) or the buffer alone was used as a control. VLPs were then used for quantitative PCR analysis of gD (5'→3': forward primer, CCATACCGACCACCGACGA; reverse primer, CATACCGAACGACCACAC), electron microscopy or cell assays. PCs and BMDCs were cultured in RPMI-1640 medium, BHK-21 cells were cultured in Glasgow modified essential medium, and HEK293T and human MDMs were cultured in DMEM. For all cells, the growth medium was supplemented with glutamine, penicillin and streptomycin. DMEM for the culture of human MDMs was also supplemented with 10% (vol/vol) human serum albumin (Invitrogen). All other media were supplemented with 10% (vol/vol) FCS (Biological Industries). Liposomes were prepared from either 100% DOTAP or a lipid mixture of DOTAP, lissamine rhodamine and DOPE (dioleoylphosphatidyl-ethanolamine) at a ratio of a 1/1/0.1 (wt/wt/wt). Lipid blends were in chloroform (Avanti Lipids) and liposomes were produced according to Avanti guidelines with a mini-extruder and membranes with a pore size of 0.2 μ m (Avanti Lipids). Liposome quality was assessed by dynamic light scattering with a ZetaSizer (Malvern). Caspase inhibitor II was from Santa Cruz Biotechnology. The fusion inhibitor T20 from Roche was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, US National Institutes of Health). The pSVIII-ADA Env plasmid was from J. Sodroski (obtained through P. Gorry). The fusion-deficient construct pSVIII-ADA Y552F was from P. Gorry.

RNA analysis. RNA was collected with a High Pure RNA Isolation kit (Applied Bioscience). RNA for human CXCL10, TNF, IFN- β and β -actin and mouse IFN- β was analyzed with pre-made Taqman assays and an RNA-to-*C_T* 1-step kit (Applied Biosystems). RNA for mouse CXCL10, TNF and β -actin was analyzed with a Brilliant II QPCR 1-step kit (Agilent) and the following primers (5'→3'). CXCL10 forward primer, CGATGACGGGCCAGTGAGAATG, and reverse primer, TCAACACGTGGGCAGGATAGGCT; TNF forward primer, ATCGGCT

GGCACCCTAGTT, and reverse primer, GTAGCCCACGTCGTAGCAAAC; β -actin forward primer, TAGCACCATGAAGATCAAGAT, and reverse primer, CCGATCCACACAGAGTACTT. An MX3005 (Stratagene) was used for quantitative PCR. RNA expression was normalized to β -actin expression and to expression in the relevant untreated control sample. The nCounter multiplex platform (NanoString) was used for analysis of RNA samples as described³².

Cell-cell fusion assay. HEK293T or HeLa cells seeded in six-well tissue culture plates were cotransfected with Env-expressing plasmid (psvIII-ADA^{wt}; psvIII-ADA^{AKS}; psvIII-ADA^{F522Y}) and pSVL-Tat through the use of Lipofectamine 2000 (Invitrogen). At 48 h after transfection, 293T effector cells were added to monolayers of human MDMs, followed by incubation for 8 h at 37 °C. The cocultures were washed twice with PBS for removal of unfused HEK293T cells, after which the cells were lysed and RNA was extracted for analysis. For the T20 inhibition experiment, human MDMs were allowed to incubate with T20 (in concentrations ranging from 0 μ g/ml to 20 μ g/ml) for 1 h before addition of HEK293T effector cells.

Analysis by confocal and electron microscopy. For the detection of CXCL10, PCs were left untreated or were treated for 6 h with VLPs or poly(I:C), and brefeldin A (Sigma) was added during the final 4 h of incubation. Cells were then stained with DAPI (4,6-diamidino-2-phenylindole) and antibody to CXCL10 (G15; Santa Cruz). For analysis of STING, human MDMs were treated for 4 h with liposomes or VLPs, then were stained with DAPI and antibody to STING (IMG-6422A; Imgenex). Cells were cultured on coverslips and mounted in pro-long gold (Molecular Probes). All images were obtained with a Zeiss LSM 710 laser-scanning microscope. For cryoelectron microscopy, freshly prepared virions or PREPs were applied to Quantifoil holey carbon films, and the grids were frozen by being plunged into liquid ethane with a Vitrobot (FEI). The grids were imaged with a JEM-2200FS microscope (JEOL) operating at 200 kV. Images were recorded on a Gatan UltraScan 4k \times 4k SlowScan charge-coupled-device camera at a nominal magnification of \times 10,000.

Flow cytometry. An FC500 (Beckman Coulter) was used for flow cytometry. Allophycocyanin-conjugated anti-NK1.1 (PK136) and phycoerythrin-indotricarbocyanine-conjugated anti-CD45 (30-F11) were from BD Pharmingen. Count beads for cell quantification were from Beckman Coulter. Unspecific staining was blocked with polyclonal mouse IgG (015-000-002; Jackson) and tested with the relevant allophycocyanin- or phycoerythrin-indotricarbocyanine-conjugated isotype-matched control antibodies (G155-178 (for NK1.1) or R35-38 (for CD45); both from BD Pharmingen).

Immunoblot analysis. Whole-cell extracts were denatured in XT Sample Buffer and XT Reducing Agent and separated by SDS PAGE (Bio-Rad). LC3 was visualized with antibody to mouse LC3 (PM036; MBL). Antibody to IL-1 β (AF-401-NA) was from R&D Systems. Antibody to Akt (11E7) and antibody to phosphorylated Akt (D9E) were from Cell Signaling.

Calcium-release assay. Cells seeded in 96-well plates (Corning) were incubated for 60 min at 37 °C with 25 μ M Fura-2 (acetoxymethyl ester form; Invitrogen) and rinsed three times with PBS, then were placed on ice and exposed to purified HSV-2 (MOI, \sim 5) or VLP (MOI, \sim 15). Cells were transferred to a SpectraMaxMFe (Molecular Devices) and photometric data for the concentration of Ca²⁺ were generated. The mean concentration of Ca²⁺ was determined for four wells according to the manufacturer's recommendations (Molecular Devices).

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