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

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Abstract

The innate immune system senses infection by detecting evolutionarily conserved molecules essential for microbial survival or abnormal location of molecules. Here we demonstrate the existence of a novel innate detection mechanism, which is induced by fusion between viral envelopes and target cells. Virus-cell fusion specifically stimulated a type I interferon (IFN) response with expression of IFN-stimulated genes (ISGs), *in vivo* recruitment of leukocytes, and potentiation of Toll-like receptor 7 and 9 signaling. The fusion dependent response was dependent on stimulator of interferon genes (STING) but 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.

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AUTHOR CONTRIBUTIONS

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

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The authors declare no competing financial interests.

Keywords

Virus; Fusion; type I IFN; STING

The current understanding of viral detection by the innate immune system is dominated by recognition of nucleic acids[1, 2]. Such recognition is attended to by endosomal Toll-like receptors (TLRs), by RIG-I like receptors or by DNA receptors located in the cytosol. TLR signaling depends on the adaptor proteins myeloid differentiation primary response gene 88 (MyD88) and/or TIR-domain-containing adaptor-inducing interferon-β (TRIF), whereas receptors in the cytosol depend on either mitochondrial antiviral-signaling protein (MAVS also known as IPS-1, VISA or Cardif) or on stimulator of interferon genes (STING) for RNA and DNA recognition pathways. STING is an ER resident protein that re-localizes to uncharacterized punctate structures in response to virus 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 is however unresolved[4]. Detection of virus initiates increased self-sampling by autophagy, increased transcription of anti-viral genes such as type I interferons (IFNs), and activation of pro-inflammatory processes such as assembly of the inflammasome. Expression of type I IFNs is pivotal in the innate immune response against virus. Type I IFNs initiate the transcription of a large group of IFN stimulated genes (ISGs) through the type I IFN receptor (IFNAR). ISGs are important in establishing a cellular anti-viral state[5] and for alerting and recruiting leukocytes. The inflammasome is a multi protein complex and assembles in response to detection of virus. One of its constituents, caspase-1, is subsequently activated to cleave pro-interleukin-1β (IL-1β) into IL-1β which is a highly inflammatory cytokine. The inflammasome has, together with autophagy, recently been shown to participate in host protection against virus infection[6, 7].

It has previously been proposed that membrane disturbances can elicit an antiviral response independent of viral nucleotides[8]. In the present study, we showed that Virus-like particles (VLPs), which lack capsid and genomic material induced a type I IFN response in primary murine and human cells which had no apparent effect on the expression of inflammatory genes, autophagy or inflammasome activation. A similar response was seen with cell-cell membrane fusion or following exposure to fusogenic liposomes. 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 complex formation of STING with Tank Binding Kinase 1 (TBK1) and also involved IRF3, which was essential for the subsequent induction of ISG gene expression. Thus, virus-cell fusion is sensed by the innate immune system and activates a STING-dependent signaling pathway leading to production of type I IFN and ISGs.

RESULTS

VLPs are detected by the innate immune system

This work aimed at identifying potential novel principles of innate recognition of viruses not dependent on sensing of viral nucleic acids. For this purpose we prepared herpes simplex

virus (HSV)-1 derived VLPs by using either an UL36 HSV-1 deletion mutant, which is unable to assemble infectious virus particles, or by treating target cells with a DNA polymerase inhibitor prior to infection with HSV-1. VLPs prepared using the UL36 mutant are also referred to as light particles (L-particles)[9], whereas those that are prepared using the DNA polymerase inhibitor are referred to as pre-viral replication enveloped particles (PREPs)[10]. The absence of capsid and DNA in VLPs in comparison with infectious HSV-1 virions was clearly visualized by electron microscopy (EM) and by immunoblotting for capsid component VP5 and the envelope residing glycoprotein D (gD) (Fig. 1a). We treated murine peritoneal cells (PCs) with isolated VLPs for 4 hours and harvested RNA for subsequent analysis by nCounter™ Nanostring technology, a multiplex platform for gene expression analysis. Both L-particles and PREPs induced a profile with increased mRNA levels of certain ISGs, in particular the chemokine CXCL10 (Fig. 1b). By contrast tumor necrosis factor (TNF) mRNA levels were unchanged. The Nanostring data were confirmed by QPCR using primers specific for IFNβ, CXCL10 and TNF (Fig. 1c–d). In comparison, treatment with a preparation of total HSV-1 (L-particles and infectious virions) induced expression of IFNβ and CXCL10 as well as TNF (Supplementary Fig. 1). These results showed that PREPs and L-particles both stimulate a low-grade type I IFN response together with increased expression of ISGs in particular CXCL10. L-particles and PREPs are thus collectively termed VLPs from here on. The response was not restricted to murine PCs as bone marrow derived dendritic cells (BMDCs) (Fig. 1e) and also primary human monocyte derived macrophages (hMDMs) (Fig. 1f) responded to VLP treatment. The increase in CXCL10 was also seen with the protein as shown by confocal microscopy on VLP stimulated PCs (Supplementary Fig. 2) and by ELISA on supernatants from VLP stimulated BMDCs (Fig. 1g). Importantly, since the total HSV1 preparation contains both infectious virus and VLPs, the latter data indicate that VLPs could be responsible for as much as 25 % of the ISG response to HSV1 infection, hence suggesting mechanisms of ISG induction independent of DNA sensing[11, 12].

To investigate whether the responses induced by the VLPs could be caused by contamination with DNA-containing virions we conducted a further series of experiments. Contents of HSV1 genomic DNA in the preparations were measured by QPCR. The L-particle preparations contained trace amounts of DNA (5000 fold less than the unpurified HSV1 stock). This DNA, however, did not reside within the particles, since it was efficiently removed by DNase treatment. The PREPs contained no detectable viral DNA (Fig. 1h). Importantly, VLP-induced CXCL10 expression was not affected by DNase treatment and was therefore also independent of DNA (Fig. 1i). Lastly, by measuring plaque formation in permissive cell lines we established the contamination of infectious DNA containing virions to be less than $1:10^6$ (**data not shown**). Infectious HSV1 diluted to an MOI of 0.001, which corresponds to >60 fold the maximum possible HSV1 contamination when stimulating with VLPs was not sufficient to induce CXCL10 secretion in BMDCs (Fig. 1g). To determine if the induction of CXCL10 was dependent on type I IFN signaling, as suggested by the Nanostring profile, we investigated the effect of VLPs on PCs from mice deficient in the IFN receptor alpha chain (*Ifnar1*$^{-/-}$). In these knockouts CXCL10 production was abolished in response to VLPs (Fig. 1j). Thus, VLPs induce a low-grade type I IFN response triggering expression of a subset of ISGs.

Leukocyte recruitment *in vivo* and TLR7/9 responsiveness

The innate immune response to virus infections involves, in addition to the IFN response, other activities. This includes both activation of inflammatory responses and cell-intrinsic antiviral effector mechanisms. We were interested in exploring whether other well-characterized innate immune responses to viruses were activated by VLPs. IL-1β production is a two-step process requiring expression of pro-IL-1β and proteolytic cleavage by the inflammasome. Treatment of lipopolysaccharide (LPS)-pretreated macrophages with VLPs had no effect on IL-1β in the supernatant, whereas infection with HSV-1 modestly induced the cytokine (Fig. 2a). By contrast, Poly-dAdT, which activates the AIM2 inflammasome[13], potently stimulated release of IL-1β. HSV-1 also induced a detectable cleavage of pro-IL-1β, which was not seen in response to VLP treatment (Fig. 2b). Autophagy is now appreciated as an important antimicrobial effector mechanism during infections, including viral infections[6]. Autophagy is characterized by conversion of LC3-I to the lipidated form LC3-II and formations of punctuate LC3-positive structures in the cytoplasm. HSV-1 infection but not VLPs induced detectable increases in LC3-II formation by immunoblot and increases in LC3-positive foci in the cytoplasm by confocal microscopy (Fig. 2c). To investigate if VLPs were able to alter cellular sensitivity to subsequent stimulus with pattern recognition receptor (PRR) ligands we pretreated PCs with VLPs for two hours and stimulated with suboptimal concentrations of the TLR9 (CpG, ODN1829) or TLR7 (ssRNA40) ligands. Pretreatment with VLPs did indeed increase the sensitivity to these ligands as measured by CXCL10 mRNA (Fig. 2d). Finally, we wanted to examine if VLPs were able to evoke early immune activities *in vivo*. To do this, fusion competent VLPs and fusion defective VLPs (ΔgB) were injected into the peritoneal cavity of mice. Cells from the peritoneal cavity were then harvested 24 h later and analyzed by flow cytometry. Injection with normal VLPs but not with fusion defective ΔgB VLPs induced a significant increase in NK cells, which are important anti-viral effector cells, in the peritoneal cavity (Fig. 2e–f). In addition, we wanted to investigate if VLP treatments altered the activation status of the peritoneal leukocytes. To do this we stained harvested PCs for the surface expression of the broad activation marker CD69. There was a significant increase in PC CD69 expression in VLP-treated but not in ΔgB VLP-treated mice (Fig. 2g–h). Thus, VLPs selectively induce IFN responses, increase TLR7 and TLR9 sensitivity, and stimulate early immune reactions *in vivo*.

Fusion induces type I IFN and CXCL10 expression

The immune system has evolved to recognize features from pathogenic microorganisms that are not easily lost or altered without also losing infectivity[14, 15]. One feature that VLPs retain, which is essential to infection, is the ability to fuse with cellular membranes[16]. We wanted to investigate if fusion between VLPs and cellular membranes is necessary for the induction of IFNβ and CXCL10. To investigate this we prepared VLPs from two fusion deficient HSV-1 mutant strains, which lack glycoproteins B and H respectively (ΔgB and ΔgH). To ensure that mutant and wild-type (wt) VLPs were added in equal amounts their concentrations were estimated by both EM counting and by immuoblotting against glycoprotein D. Both methods gave rise to similar concentration estimates (**data not shown**). Both ΔgB and ΔgH VLPs were unable to induce IFNβ and CXCL10 in PCs *in vitro* (Fig. 3a–c) and in hMDMs (Fig. 3d). These data suggested that immune recognition of VLPs

depends on fusion. To test if the release of viral material from the VLPs into the target cell cytoplasm was necessary for the immune recognition we decided to use a different experimental approach. HEK293 cells expressing the HIV fusion protein Env were co-cultured with hMDMs. The expression of Env on the surface of HEK293 cells causes these to fuse with the primary macrophages in the co-culture[17]. As fusion deficient controls we used a dysfunctional variant of Env (ΔKS) or a variant with a mutation in the gp41 domain (F522Y), which enables the cells to bind target cells but not to complete membrane fusion. HEK293 cells transfected with wt Env induced robust expression of IFNβ and CXCL10 mRNA in the hMDMs, whereas those transfected with the ΔKS or F522Y mutants were either unable to or induced greatly reduced responses respectively (Fig. 3e,f). Similar results were obtained using Env-transfected HeLa cells to stimulate hMDMs (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 co-cultures of wt-Env transfected HEK293 cells and primary macrophages. T20 inhibited IFNβ expression in a dose dependent manner (Supplementary Fig. 4). Thus, fusion between HEK293 or HeLa cells and primary macrophages induces IFNβ and CXCL10. However, transfer of cellular material from cell to cell could potentially trigger production of IFN. To rule out this possibility we used a system based on fusogenic liposomes. Liposomes are bipolar lipid membrane vesicles and depending on the molecular properties of the lipids, they can fuse with cellular membranes. Cationic liposomes, which are widely used to deliver e.g. DNA plasmids into live cells, are able to fuse with cellular membranes and have previously been shown to induce immune responses through at least two separate pathways, one of which is MyD88 dependent[18]. We therefore decided to test if cationic liposomes could induce CXCL10 expression in PCs from wt and *Myd88*$^{-/-}$ mice and in immortalized BM-derived *Myd88* and *Trif* double deficient macrophages. Treatment with liposomes that are able to fuse with cellular membranes (Supplementary Fig. 5) induced CXCL10 mRNA in both wt PCs and *Myd88*$^{-/-}$ PCs, and in *Myd88*$^{-/-}$ and *Trif*$^{-/-}$ double deficient macrophages (Fig. 3g–h). By contrast, liposome-induced TNF expression was observed only in wt cells (Fig. 3g). This did not seem to be dependent on apoptotic release of DNA as liposomes were also able to induce CXCL10 in the presence of a pan-caspase inhibitor (Supplementary Fig. 6). Importantly, when we compared liposomes with differential fusogenic potential there was a clear correlation between this potential and the ability to induce expression of CXCL10. Phosphatidylcholine deriviate (PtdCho) liposomes with low fusogenic potential did not induce CXCL10 mRNA. In contrast, DOTAP liposomes with intermediate fusogenic potential induced some CXCL10, whereas highly fusogenic LR-DOPE-DOTAP liposomes[19] induced CXCL10 mRNA to the highest levels (Fig. 3i). To investigate if liposomes could induce an mRNA expression profile comparable to those induced by the VLPs we used RNA from liposome-treated PCs for analysis by Nanostring technology. Here we found that in addition to an ISG profile with increases of e.g. CXCL10, IFI205, and viperin, there were also increases in mRNAs from NF-κB stimulated genes such as CXCL1[20] possibly induced through the MyD88 pathway (Supplementary Fig. 7). We also found that the transfection reagent Lipofectamine 2000 induced CXCL10 in BMMs and that this response was abrogated when using BMMs from IRF3 deficient mice (data not shown). Thus, VLP-cell, cell-cell, and liposome-cell fusion induce expression of IFN-β and ISGs.

Immune responses to fusion depend on STING

To investigate if responses evoked by VLPs depend on known HSV-sensing pathways we treated PCs from a series of animals each with one or more deficiencies in these pathways. VLPs retained the capacity to induce CXCL10 in PCs from *Tlr2*$^{-/-}$ and *Tlr9*$^{-/-}$ double knockouts, *Tlr3*$^{-/-}$, *Myd88*$^{-/-}$, TRIF deficient *Ticam1*$^{-/-}$, and *Mavs*$^{-/-}$ mice (Fig. 4a–b). To exclude the possibility of redundant mechanisms we also monitored responses in *Myd88*$^{-/-}$, *Ticam1*$^{-/-}$, and *Mavs*$^{-/-}$ triple knockout mice (Supplementary Fig. 8), which responded normally. In contrast to this, we found that the induction of CXCL10 was abolished in both PCs and BMDCs from STING deficient *Tmem173*$^{-/-}$ mice. This was also observed in response to both VLP and liposome treatment (Fig. 4c–f) strongly suggesting that fusion of membranes leads to an innate immune response through DNA-independent sensing mechanisms that depend on STING. To further test the involvement of STING, we investigated intracellular localization of STING in response to liposomes and VLPs in hMDMs, since STING has previously been reported to re-localize following HSV-1 infection[3]. Treatment of primary macrophages with liposomes or VLPs did indeed induce re-localization of STING, which could be observed in >40% of the cells. Moreover, in both cases, STING also co-localized with TBK1 (Fig. 4g–h). Stimulation with liposomes and VLPs of BMDCs from either TBK1 or IRF3 deficient mice indicated that TBK1 was involved but not essential (Fig. 4i), whereas IRF3 was both involved and essential for the induction of CXCL10 expression by both VLPs and liposomes (Fig. 4j).

VLPs activate PLC-PI3K and induce ER calcium release

HSV infection has previously been shown to induce a very early increase in free intracellular calcium using the cervical cancer cell line CaSki[21], which could represent an early signaling event stimulating IFN responses following detection of fusion. Like HSV, VLPs were able to induce increases in free intracellular calcium in CaSki cells, although not to the same extent as the infectious virus (Fig. 5a). Fusion deficient ΔgB VLPs failed to stimulate calcium flux. This was also the case in murine BMDCs (Fig 5b) and in human DCs (**data not shown**). The increase could be abrogated by the chemical inhibitor 2-APB, which inhibits IP3 dependent calcium release from the ER (Fig. 5c). Despite this, 2-APB treatment did not interfere with the induced CXCL10 mRNA expression (Fig. 5d). Other signaling molecules described as being involved in membrane proximal signaling include Phospholipase C gamma (PLCγ) and Phosphoinositide 3-Kinase (PI3K). We therefore wanted to examine the involvement of PLCγ and PI3K in activation of the ISG response to VLPs. VLPs and liposomes were indeed able to induce the phosphorylation of the PI3K substrate AKT (Fig. 5e–f). Moreover, this response was inhibited in the presence of both PLCγ inhibitor (U73122) and PI3K inhibitor (Ly294002), hence placing PLCγ upstream of PI3K in the fusion-activated pathway (Fig. 5e). Both inhibitors blocked VLP induced CXCL10 mRNA expression (Fig. 5g) and also VLP increased free intracellular calcium (Fig. 5h). Altogether the data presented suggest that cells are capable of detecting membrane fusion, which leads to selective induction of IFN and ISG expression through a pathway dependent on STING and involving TBK1, IRF3 and the PLC-PI3K pathway.

DISCUSSION

In the present study we demonstrate the existence of a mechanism for innate immune detection, which detects fusion between viral envelopes and target cells. Membrane fusion specifically induces type I IFN responses and expression of ISGs, and this proceeds through a mechanism dependent on STING. Although STING is associated with DNA sensing during viral infections[3, 11], the mechanism described here is independent of the presence of both DNA and RNA. The principle of innate immune sensing described here applies to virus-cell fusion, liposome-cell fusion, and to cell-cell fusion. The unscheduled fusion between a viral envelope and a cellular membrane is in sharp contrast to scheduled and highly regulated fusion events such as natural syncytia formation and ER-membrane fusion, which do not elicit an immune reaction. We therefore suggest the existence of cellular mechanism to distinguish between scheduled and unscheduled fusion events. These present data expand on earlier studies, which show that cationic liposomes induce expression of co-stimulatory molecules in dendritic cells through MyD88 controlled pathways[18] and on other data showing that cell-cell fusion enforced by overexpression of the reoviral fusogenic protein FAST leads to a type I IFN response[8]. By showing that liposomes stimulate an IFN response through a MyD88 independent and STING dependent mechanism similar to what is observed with VLPs, we demonstrate the existence of a membrane-fusion-activated pathway leading to type I IFN responses.

Many viruses and members of the α-herpesvirus family in particular naturally produce particles that lack genomic material and capsid[16]. The particles are produced in almost equal numbers to infectious genome-containing virus particles but can, due to their lower density, be separated by gradient centrifugation[16]. 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 observed response was mediated by membrane fusion independent of genomic DNA or RNA. The finding that synthetically prepared liposomes also induced IFN and ISG expression in a STING-dependent manner provides significant additional evidence for the existence of cellular mechanisms to sense lipid membrane fusion induction of IFN responses. This conclusion is further strengthened by the observed IFN response in the HIV Env cell-cell fusions assay, which also indicates that this phenomenon is not restricted to detection of virus infections but could represent a mechanism to alert the cell of other unscheduled cellular membrane fusion events.

The response of the innate immune system to virus infections is a coordinated process involving many activities including IFN 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 innate control of viruses[6]. We found that the cellular response to membrane fusion was restricted to a type I IFN response with limited effect on inflammatory gene expression, inflammasome activation, and autophagy. To our knowledge, this is the first example of an innate recognition mechanism that selectively activates IFN responses. This suggests that cellular sensing of viral entry serves the purpose to restrict the virus without activating excessive inflammation. In addition, the finding that the VLPs synergistically enhanced the response to TLR7 and TLR9 agonists suggests that cellular sensing of viral entry also served the

purpose of conditioning the cell for optimal innate immune responses to viral nucleic acid recognition in intracellular compartments. The mechanism for this latter phenomenon is unknown but may involve reactive oxygen species, which we found induced following VLP treatment of PCs (data not shown), and are recently reported to amplify PRR-induced IFN responses[23].

Recent research on STING function has been dominated by describing its role in DNA sensing. Our results, however, suggest a more complex role for STING possibly by interacting with complexes that govern membrane dynamics. This is in line with earlier work where it was shown that STING associates with the ER translocon and the octameric Sec6-Sec8 complex. This complex is involved in governing dynamic membrane events such as tethering secretory vesicles to the ER[4]. Moreover, RNAi knockdown of Sec5 and Sec6 abolished STING dependent IFNβ expression in response to Sendai virus[4]. It is noteworthy here that Sec5 seems to accumulate in areas where membrane material is added to the plasma membrane during cellularization in embryonic cells from *Drosophila* [24]. Other proteins that are involved in membrane dynamics also seem to be important in innate immune responses to enveloped virus. For example, murine embryonic fibroblasts deficient in the phospholipid membrane protein scramblase 1 (PLSCR1) have been reported to exhibit reduced type I IFN response to vesicular stomatitis virus infection and elevated viral replication[25]. However, we could find no evidence for PLSCR1 being responsible for the innate response to membrane fusion as determined by unaltered CXCL10 expression by *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 the PLCγ-PI3K pathway, which stimulated Ca^{2+} release from ER stores and a separate pathway to drive the fusion-activated IFN-ISG response, which was dependent on STING and IRF3 and also involved TBK1. The PLCγ-PI3K pathway is well-described as playing important roles in membrane proximal signaling[26, 27], and is also activated during the entry process of many viruses[28, 29]. For several viruses, this pathway is in fact essential for successful completion of viral entry[29, 30]. In the present study we did not mechanistically describe the link between the PLCγ-PI3K and the downstream response, including STING translocation and assembly of the STING-TBK1 complex. However, we believe that PLCγ-PI3K signaling does act in the same pathway as STING, since inhibition of PI3K lead to significant elevation in the number of VLP-induced STING foci (**data not shown**). This suggests that the PLCγ-PI3K pathway plays a critical role in stimulating mature STING signalosomes, which signal to IRF3 but are also subject to degradation.

Membrane fusion is essential for entry of enveloped viruses and is also seen in several non-infectious conditions with giant cell formation. Interestingly, measles virus strains capable of forming syncytia are more potent inducers of IFN than strains without this function[31], and giant cell arteritis involves a yet unexplained type I IFN response[32]. This suggests that membrane fusion is a danger signal of importance during both infections and non-infectious conditions. The innate immune system detects evolutionarily conserved microbe-specific molecules essential for both the survival and mis-location of molecules utilized by both microbes and host cells. Innate immune sensing depending on fusion is not easily placed into either category, and hence it seems likely that host cells have sensing systems to detect

dynamic pathogen-associated signals and in this way reveal ongoing microbial activity to the immune system.

METHODS

Reagents, mice, cells and VLPs

Tlr2/9$^{-/-}$ and *Tlr3*$^{-/-}$ mice were bred at M&B Taconic (Denmark) and kept at the animal facility at the Faculty of Health Sciences, University of Aarhus. *Tbk1*$^{-/-}$, *Myd88*$^{-/-}$, *Ticam1*$^{-/-}$, *Mavs*$^{-/-}$, *Tmem173*$^{-/-}$ and triple KO mice were bred and maintained in the animal facilities of the University of Massachusetts Medical School. *Ifnar1*$^{-/-}$ were bred and maintained at the animal facility at the Panum Institute, University of Copenhagen. PCs were isolated from mice either untreated or pre-treated with 5mL PBS with 3% thioglycollate. Differentiation of BM-DCs from bone-marrow cells was achieved by culturing for 7 days in the presence of GM-CSF (Sigma). PBMCs were isolated from healthy donors by Ficoll Paque (GE Healthcare) gradient centrifugation. Monocytes were separated from PBMCs by plastic adherence using UpCell plates (Nunc) pre-coated with Poly-L-Lysine (Cultrex). Differentiation of monocytes to hMDMs was achieved by culturing for 6–8 days in the presence of M-CSF (Sigma). Murine BMDCs were obtained by harvesting non-adherent cells from bone marrow cell cultured for 7 days in the presence of GM-CSF (R&D systems). Preparation and isolation of L-particles and PREPs was performed as described by others[9, 10]. In brief, the UL-36 HSV-1 deletion mutant was used to prepare L-particles in the cell line HAUL-36-1, whereas PREPs were prepared using BHK-21 cells pre-treated with Acycloguanosine (Sigma). Fusion deficient VLPs were prepared as PREPs using gB or gH deficient HSV-1 using respective supporting cells lines (Vero D6 and Vero F6). In VLP treatments of mammalian cells MOIs of 10–30 were used. For DNase treatment of VLPs we used DNase (Sigma) dissolved in DNase buffer (PBS with 1mM $CaCl_2$ and 6mM $MgCl_2$ but without denaturizing agents) or this buffer alone for control. VLPs were then used for gD QPCR (5´-3´; fp: 5CCATACCGACCACACCGACGA, rp: CATACCGGAACGCACCACAC), EM, or cell assays. PCs and BM-DCs were cultured in RPMI 1640, BHK-21 cells in GMEM and HEK293T and hMDMs in DMEM. For all cells growth medium was supplemented with glutamine, penicillin and streptomycin. DMEM for culturing hMDMs was also supplemented with 10% (v/v) human serum albumin (Invitrogen). All other media were supplemented with 10% (v/v) fetal calf serum (Biological Industries). Liposomes were prepared from either 100% DOTAP or a lipid mix of DOTAP/DOPE/LR (Lissamine Rhodamine)-DOPE in a w/w/w of 1/1/0.1. Lipid blends were purchased in chloroform (Avanti Lipids) and liposomes produced according to Avanti guidelines with mini-extruder and 0.2μm membranes (Avanti Lipids). We assessed liposome quality by dynamic light scattering using a ZetaSizer (Malvern). Caspase inhibitor II was purchased from Santa Cruz Biotechnology (USA). We thank Professor Allan R. Thomsen for providing *Ifnar1*$^{-/-}$ mice. The following reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: T-20, Fusion Inhibitor from Roche. We thank J. Sodroski for providing the pSVIII-ADA Env plasmid (obtained through P. Gorry). We also thank P. Gorry for providing the fusion-deficient construct pSVIII-ADA Y552F.

RNA analysis

RNA was collected using High Pure RNA Isolation kit (Applied Bioscience). RNA analysis for human CXCL10, TNFα, IFNβ, beta-actin and murine IFNβ were performed using premade Taqman assays and the RNA to Ct one step kit (Applied Biosystems). RNA analysis for murine CXCL10, TNFα and beta-actin was performed using the Brilliant II QPCR 1 step kit (Agilent) and the following primers (5´-3). CXCL10 fp: CGATGACGGGCCAGTGAGAATG, CXCL10 rp: TCAACACGTGGGCAGGATA GGCT, TNFα fp: ATCGGCTGGCACCACTAGTT, TNFα rp: GTAGCCCACGTCG TAGCAAAC, beta-actin fp: TAGCACCATGAAGAT CAAGAT, beta-actin rp: CCGATCCACACAGAGTACTT. QPCR was performed on an MX3005 (Stratagene). RNA expression was normalized to β-actin and to the relevant untreated control. Analysis by nCounter™ Nanostring was performed on RNA samples as described previously[33].

Cell-Cell fusion assay

HEK293T or HeLa cells seeded in 6-well tissue culture plates were co-transfected with Env-expressing plasmid (psvIII-ADA^{wt}; psvIII-ADA^{deltaKS}; psvIII-ADA^{F522Y}) and pSVL-Tat using Lipofectamine 2000 (Invitrogen). At 48h post-transfection, 293T effector cells were added to monolayers of hMDMs and incubated at 37°C for 8 hours. The co-cultures were washed 2 times with PBS to remove unfused HEK293T cells, after which the cells were lysed and RNA was extracted for RNA analysis. For the T-20 inhibition experiment hMDMs were allowed to incubate with T-20 (NIH HIV and AIDS reagent program) in concentrations ranging form 0–20ug/ml for 1 hour prior to addition of HEK293T effector cells.

Analysis by confocal and electron microscopy

For CXCL10 detection PCs were treated with either VLPs, Poly I:C or left untreated for 6 hours. Of these, the last four were with brefeldin A (Sigma). Cells were then stained with DAPI and anti-CXCL10 Ab (G15, Santa Cruz). For STING analysis hMDMs were treated with liposomes or VLPs for 4 hours. Cells were then stained with DAPI and anti-STING Ab (Imgenex). Cells were cultured on coverslips and mounted in pro-long gold (Molecular Probes). All images were taken using the Zeiss LSM710. For cryoEM freshly prepared virions or PREPs were applied to holey carbon, Quantifoil grids and plunge-frozen in liquid ethane using a Vitrobot (FEI, Eindhoven). The grids were imaged in a JEM-2200FS microscope (JEOL, Tokyo, Japan) operating at 200 kV. Images were recorded on a Gatan Ultrascan 4k × 4k SlowScan charge-coupled-device camera at a nominal magnification of ×10,000.

Flow cytometry

Flow-cytometry was performed on a FC500 (Beckman Coulter). NK1.1 APC and CD45 PECy7 antibodies were from BD Pharmingen. Count beads for calculation of cell numbers were from Beckman Coulter. Unspecific staining was blocked with mouse IgG and tested using the relevant APC or PECy7 conjugated isotype controls.

Immunoblotting

Whole-cell extracts were denatured in XT Sample Buffer and XT Reducing Agent and subjected to SDS PAGE (Bio-Rad). LC3 was visualized with anti-mouse LC3 (PM036, MBL). Antibodies for IL-1β was from R&D systems. Antibodies for AKT and phospho-AKT were from Cell Signaling.

Calcium release assay

Cells were seeded in 96 well (Corning) and incubated with 25 μM Fura-2/AM (Invitrogen) for 60 min at 37oC, rinsed with PBS thrice, and then placed on ice and exposed to purified HSV-2 (MOI≈5) or VLP (MOI ≈ 15). Cells were transferred to SpectraMaxMFe (Molecular Devices) and photometric data for $[Ca^{2+}]$ was generated. The mean $[Ca^{2+}]$ was determined from four wells according to the manufacturer's recommendations.

Supplementary Material

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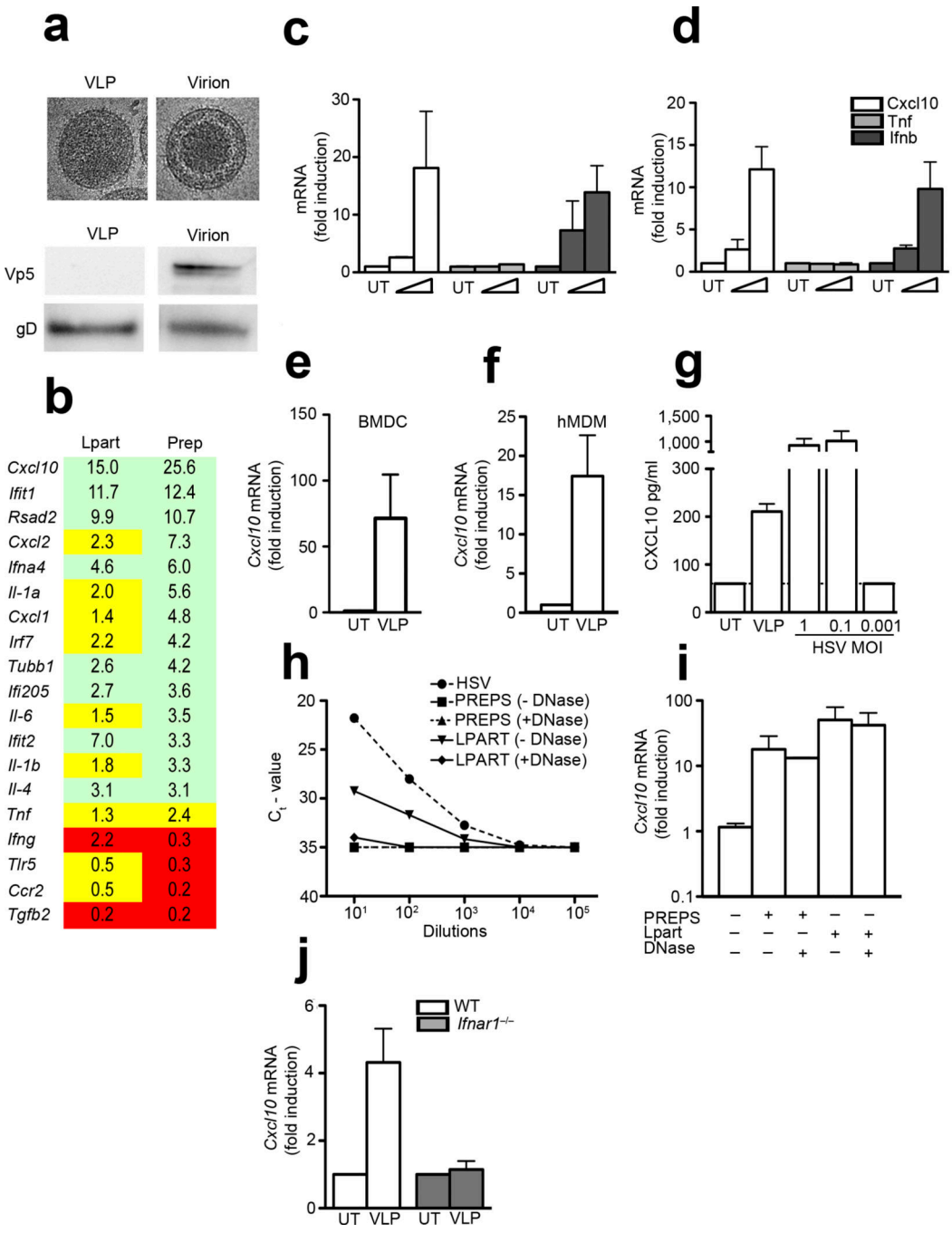

Figure 1.

VLPs induce a type I IFN response. (**a**) Structural contents of VLPs and virions visualized with cryo-EM (top) and immunoblotting against VP5 and gD (bottom). Images are from one representative of two independent experiments. (**b**) PCs from C57BL6 mice were treated with either L-particles or PREPs (MOI 15) for 4 hours. RNA from two independent experiments was collected and analyzed by Nanostring technology. Numbers indicate fold increase against untreated controls. Data represent means from two independent experiments. Green indicates an increase of >3 fold, red a decrease of >3 fold, and yellow

no change (<3 fold). (**c,d**) RNA from samples prepared as in (**b**) was analyzed by QPCR after treatment with PREPs (**c**) or L-particles (**d**) (5–15 MOI). (**e,f**) CXCL10 expression in cultures of BMDCs (**e**) and hMDMs (**f**) treated with VLPs (MOI 15) for 4 hours. Data in **c, d, e**, and **f** represent means and s.e.m. of five independent experiments. (**g**) BMDCs were treated with VLPs or dilutions of infectious HSV1 stock for 24 hours. Culture supernatants were analyzed for CXCL10 using ELISA. (**h, i**) Vials of PREPs and L-particles were treated with DNase I for 20 min or left untreated. Part of each vial was analyzed for viral DNA content by QPCR (**h**), while the remaining were used to treat PCs followed by analysis of CXCL10 mRNA expression (**i**). (**j**) PCs from *Ifnar1*$^{-/-}$ mice were treated as described above and analyzed for CXCL10 expression. Data in **g–j** represent means and s.e.m. of two independent experiments.

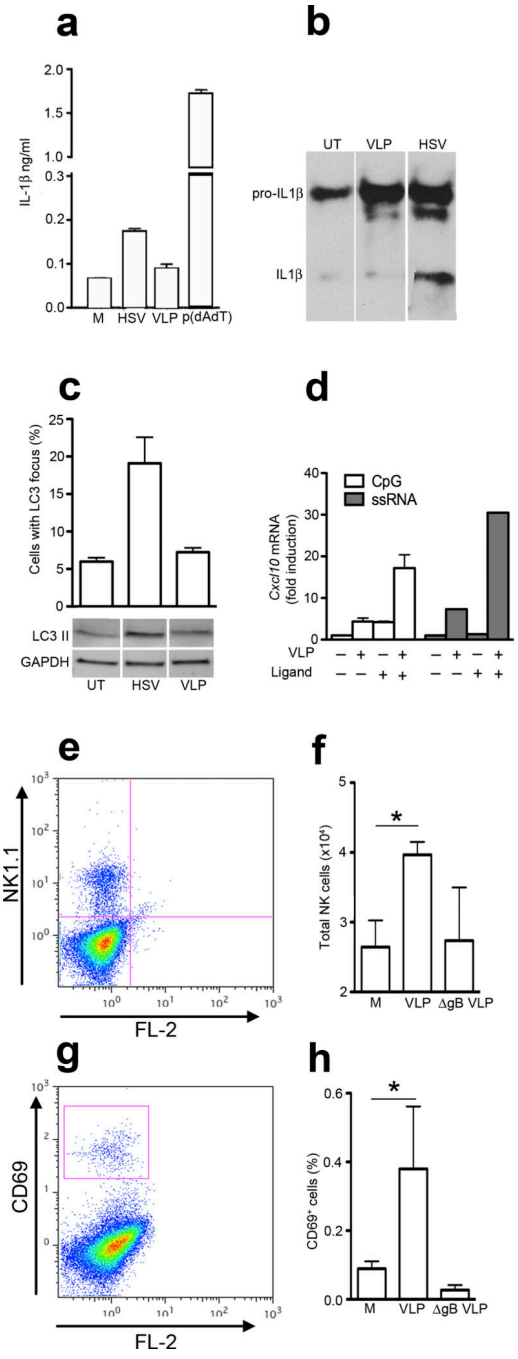

Figure 2.

VLPs increase TLR7 and TLR9 sensitivity and induce leukocyte recruitment and activation *in vivo*. (**a**) PCs from C57BL6 mice were pretreated with LPS (20 ng/ml) and subsequently treated with either media (M), HSV (MOI 3), VLP (MOI 15), or p(dAdT) (2 μg/ml). Supernatants were harvested 24 h later and analyzed for IL-1β protein. (**b**) BMDCs were pre-stimulated with LPS for 3 hours. Cells were then either left untreated or stimulated with either VLPs or HSV-1 for 16 hours. Supernatants were subsequently analyzed for IL-1β by immunoblotting. (**c**) PCs were treated with VLPs or HSV-1 for 6 hours. Whole-cell extracts

were analyzed for LC3 II by immunoblotting (bottom) or for LC3 foci formation by confocal microscopy (top). (**d**) PCs were pre-stimulated with VLPs for two hours. Cells were then treated with suboptimal concentration of ODN1829 (0.5μM) or ssRNA40 (1μg/mL) for an additional 4 hours. Cells were then analyzed for CXCL10 expression by QPCR. Data in **a–d** show one representative of two independent experiments. (**e–h**) Eight-week-old mice were injected intraperitoneally either with PBS (M), VLPs, or ΔgB VLPs both suspended in PBS (25×10^6 particles per animal). Injections were repeated after 12 hours. After 24 hours cells were harvested from the peritoneal cavity. Cell numbers and the expression of surface markers were quantified by flow cytometry using count beads. Flow-charts show representative stainings using cells from mice injected with VLPs. Data in **f–h** show one representative of two independent experiments each with 7 mice in each experimental group. *Indicates $P<0.05$ using a students t-test.

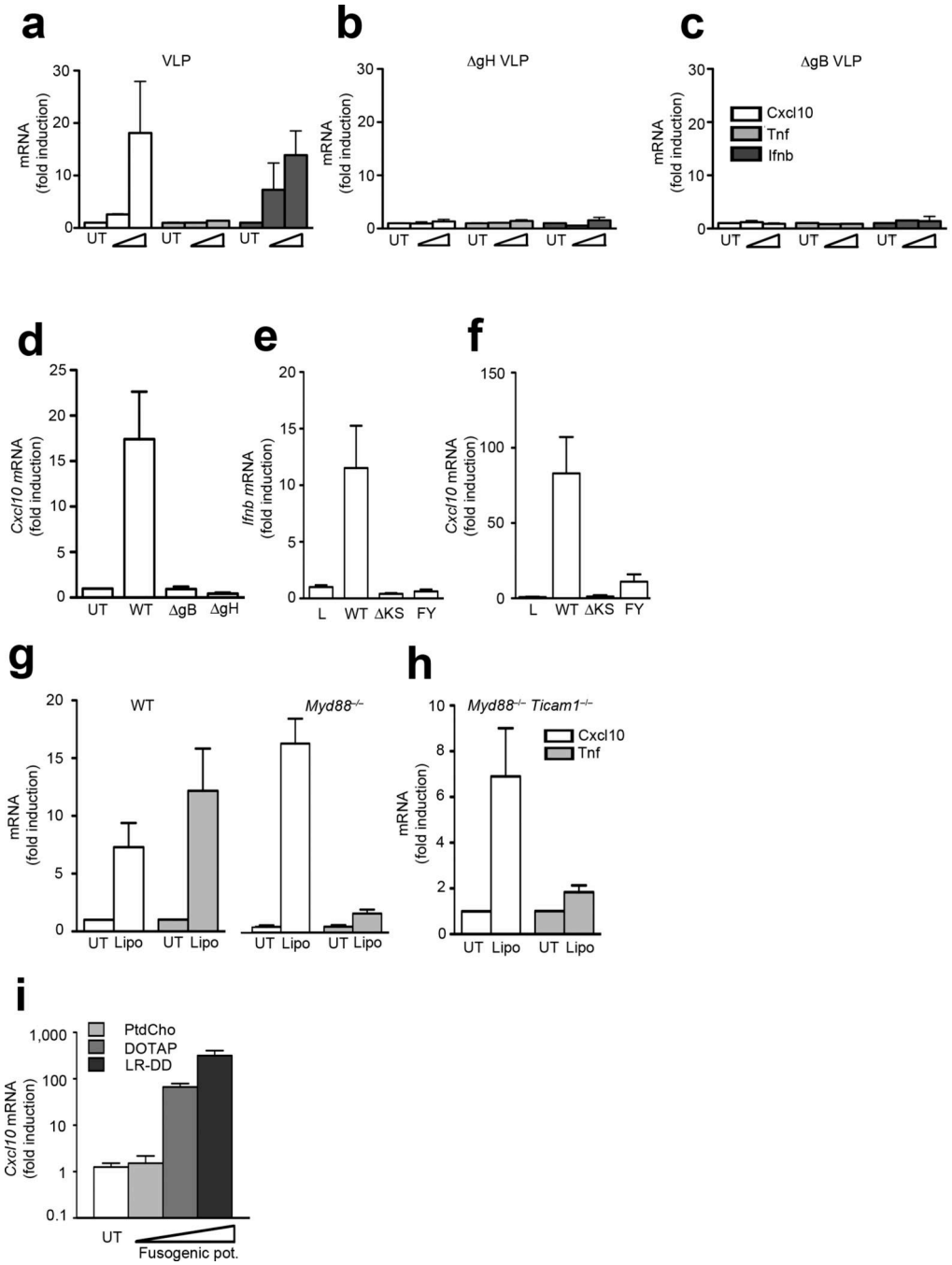

Figure 3.

Fusion induces type I IFN and CXCL10 expression. (**a–c**) VLPs prepared from wt (**a**) or the fusion deficient HSV-1 mutants ΔgH (**b**) and ΔgB(**c**) were used to treat PCs (MOI 15) from C57BL6 mice for 4 hours. Expressions of CXCL10, TNF and IFNβ were analyzed by QPCR. Data in **a–c** represent means and s.e.m. of four independent experiments. (**d**) Wt, ΔgH and ΔgB VLPs were used to treat hMDMs as in panel **a** and analyzed for CXCL10 expression by QPCR. (**e–f**) HEK293 cells were transfected with expression plasmids for wt HIV Env, F522 Env (FY), or the ΔKS Env. After two days, the HEK293 cells were

transferred to cultures with hMDMs. RNA was isolated 8 h later and analyzed for IFNβ and CXCL10 by QPCR. Data in **d–f** represent means and s.e.m. of data obtained using four independent hMDM donors. (**g–h**) PCs from wt or *Myd88*$^{-/-}$ mice (**g**) or an immortalized MyD88 and TRIF double deficient macrophage cell-line (**h**) were treated with cationic liposomes for 4 hours. RNA was isolated and analyzed for CXCL10 and TNF by QPCR. Data in **g–h** represent means and s.e.m. of five independent experiments. (**i**) BMDCs were treated with PtdCho, DOTAP, or LR-DOPE-DOTAP liposomes for 4 hours. BMDCs were then analyzed for CXCL10 expression by QPCR. Data in **i** are represent means and s.e.m of two independent experiments.

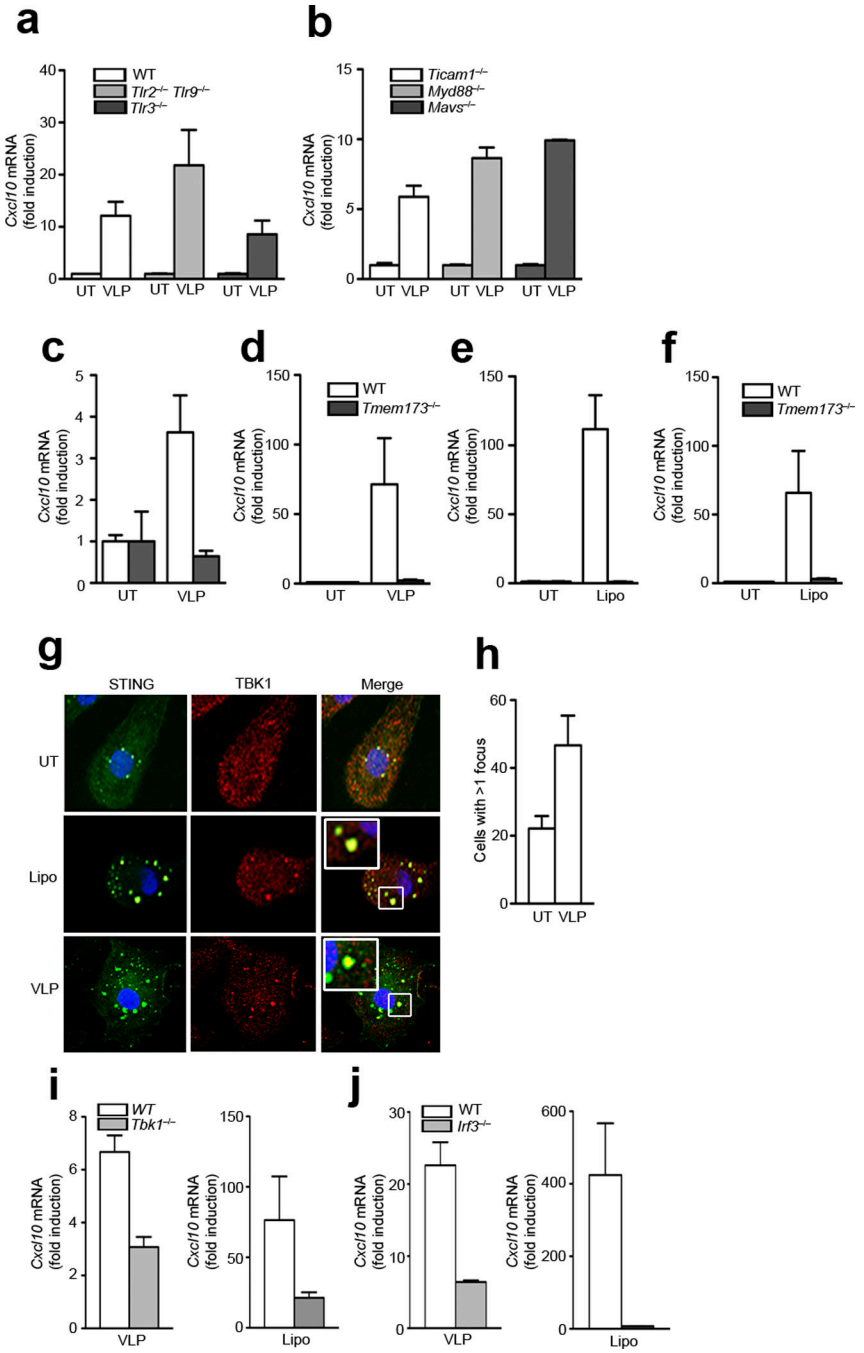

Figure 4.

Fusion induces a STING dependent innate immune response. (**a,b**) PCs from wt and animals with the indicated deficiencies were treated with VLPs (MOI 15). VLPs (**c,d**) or liposomes (**e,f**) were used to treat either PCs (**c,e**) or BM-DCs (**d,f**) from either wt or *Tmem173*$^{-/-}$ mice for 4 hours. RNA was then harvested and analyzed by QPCR. Data in **a–f** reprensent means and s.e.m. from three independent experiments. (**g,h**) hMDMs were treated with liposomes or VLPs for 4 hours. Cells were fixed and stained for STING and TBK1 and subsequently analyzed by confocal microscopy. Data in **g–h** show one representative of three independent

experiments. (**i–j**) BMDCs from TBK1 or IRF3 deficient mice were stimulated with either VLPs or liposomes for 4 hours and analyzed by QPCR. Bars indicate values normalized to untreated controls. Cells used for the untreated controls were from the same animal as cells used for the treated samples. Data in **i–j** represent means and s.e.m. from two independent experiments.

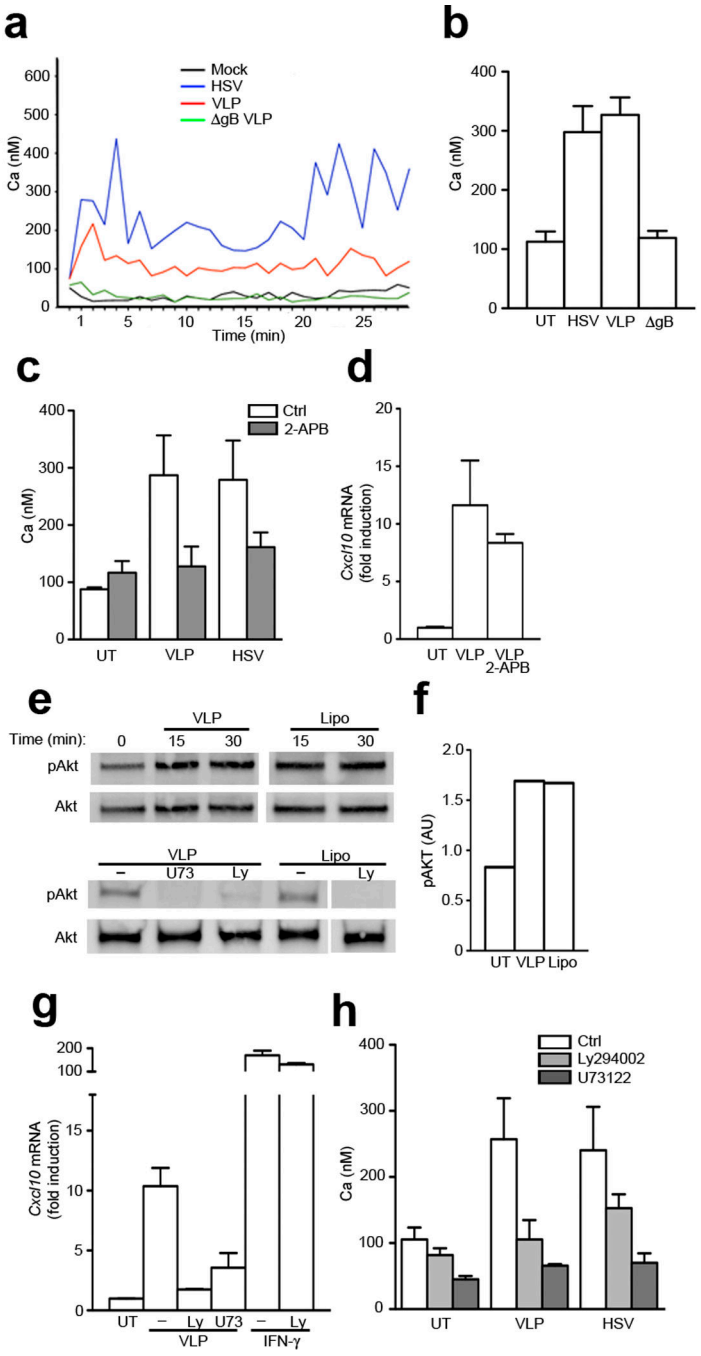

Figure 5.

Fusion leads to activation of the PLC-PI3K pathway upstream CXCL10 expression. CaSki cells (**a,b,h**) or BMDCs (**b**) were pre-incubated with Fura-2 for 60 min. Cells were stimulated with VLPs (MOI 15) and HSV (MOI 3) as indicated and levels of free calcium were measured and presented as either (**a**) kinetics or (**b,h**) peak calcium levels. (**c**) CaSki cells were pre-incubated with 2-APB (100 μM) 1 hour before stimulation. (**d**) BMDCs were pretreated with 2-APB for 1 hour before stimulation with VLPs and analysis of CXCL10 expression by QPCR. (**e**) BMDCs were stimulated with VLPs or liposomes (0.1 mg/ml) for

either 15 or 30 min. Cell lysates were then analyzed for pAKT and total AKT by immunoblotting (top). This was also performed with BMDCs pre-incubated with either U73122 (50 μM) for both VLPs and liposome treatments or with Ly294002 (5 μM) for VLP stimulations. (**g**) BMDCs were pre-incubated with indicated inhibitors for 45 min. before stimulation with VLPs or IFN-γ for 4 hours and subsequent RNA analysis by QPCR. (**h**) CaSKi cells were incubated with indicated inhibitors before stimulation with VLPs. Calcium concentrations were then subsequently analyzed. Data show one representative of two independent experiments. (mean and s.e.m.).