Vesicular stomatitis virus glycoprotein G activates a specific antiviral Toll-like receptor 4-dependent pathway

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Abstract

We have previously shown that mutations of CD14 or TLR4 impair type I interferon (IFN) production and macrophage survival during infection with vesicular stomatitis virus (VSV). We now report that VSV glycoprotein G (ppG) is essential for the induction of a previously unrecognized CD14/TLR4-dependent response pathway in which the adapter TRAM has predominant importance, absent any need for MyD88 or Mal, and with only a partial requirement for TRIF. Downstream of TRAM, IRF7 activation leads to a type I IFN response. The pathway is utilized by myeloid dendritic cells (mDCs) and macrophages rather than plasmacytoid DCs. This new mode of TLR4 signal transduction, which does not stimulate NF-κB activation, reveals the importance of viral protein recognition by mDCs and shows that TLR4 can drive qualitatively different events within the cell in response to different ligands.

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Introduction

Toll-like receptor 4 (TLR4) is best known as the transmembrane component of the mammalian LPS sensor (Poltorak et al., 1998), reviewed in Beutler et al. (2006). However, it is also well placed to respond to viral infection. Like TLRs 3, 7, 8, and 9, which sense nucleic acids (Alexopoulou et al., 2001; Heil et al., 2004; Hemmi et al., 2000; Lund et al., 2004) and are known to participate in defense against viral infections (Alexopoulou et al., 2001; Krug et al., 2004; Tabeta et al., 2004), TLR4 is capable of stimulating the production of type I IFNs (Hoebe et al., 2003; Yamamoto et al., 2003), which in many viral infections seem to be secreted primarily by IFN-producing cells (Diebold et al., 2003) (IPCs), also called plasmacytoid dendritic cells (pDCs).

TLR4 utilizes four adapter proteins to signal, and until now, these adapters have been believed to operate in functional pairs (MyD88 with Mal, and TRIF with TRAM). The IFN response has been thought to depend strictly upon activation of the TRIF/TRAM adapter pair (Fitzgerald et al., 2003), reviewed in Beutler et al. (2006), and where responses to LPS are concerned, TRIF and TRAM are recruited only in the presence of CD14; hence the LPS receptor can signal in two qualitatively distinct modes (utilizing either MyD88/Mal only or MyD88/Mal and TRIF/TRAM) (Jiang et al., 2005). In the absence of MyD88 and TRIF, it has been thought that both arms of the TLR4 signaling pathway are inactivated along with signaling from all other TLRs, as well as the IL-1 and IL-18 receptors (Hoebe et al., 2003; Yamamoto et al., 2003).
We recently observed that vesicular stomatitis virus (VSV) signals in a CD14-dependent, TLR4-dependent manner to elicit production of type I IFN (Jiang et al., 2005). We now show that the viral trigger is the envelope glycoprotein G (gpG). gpG activates a third and previously unknown signaling pathway downstream from TLR4. Represented chiefly in myeloid dendritic cells (mDCs) rather than pDCs, the new pathway does not activate NF-κB. It is fully dependent upon the adapter TRAM, only partially dependent upon TRIF, and independent of Mal and MyD88. TRAM recruitment in turn activates IRF7, leading to type I IFN production.

Results

The viral gpG activates the TLR4–CD14 complex to induce a type I IFN response, but does not activate NF-κB

Having earlier established that TLR4 and CD14 are essential components of a pathway for response to VSV, each required for type I IFN-mediated survival of macrophages infected with the virus ex vivo (Jiang et al., 2005), we sought to identify the viral component recognized by the host. We noted that virus inactivated by UV irradiation was also capable of inducing Ifnb1 gene transcription, albeit at a lower level when compared to intact, live virus (data not shown); hence, dsRNA produced during viral replication of the ssRNA virus was deemed an unlikely candidate. We reasoned that surface gpG would be a likely activator, given its accessibility to TLR4 of the host. To test the importance of gpG while avoiding any possibility of LPS contamination, we produced a pseudotyped murine leukemia virus (MLV), expressing either VSV gpG or gpG from Lassa fever virus (LFV; an arenavirus). Peritoneal macrophages isolated from wild-type (C57BL/6) or CD14-deficient (heedless) mice were exposed to VSV, MLV core particle, MLV-gpG<sup>VSV</sup> or MLV-gpG<sup>LFV</sup> (Figs. 1A and B). VSV itself is capable of inducing IFNβ through both CD14-dependent and CD14-independent processes. However, only MLV-gpG<sup>VSV</sup> (and not MLV-gpG<sup>LFV</sup> nor MLV core particles) is able to induce Ifnb1 gene activation in a CD14-dependent manner, revealing that VSV gpG possesses activating properties of a CD14-dependent response pathway. VSV infection also stimulates upregulation of the Il-6 gene, which is not observed when macrophages are stimulated by MLV-gpG<sup>VSV</sup>. IL-6 gene activation is known to be NF-κB dependent, suggesting that the
MLV-gpGVSV activated pathway is directed toward activation of type I IFN production and not toward the activation of NF-κB dependent cytokine production. This result extends our previous observation (Jiang et al., 2005) that VSV infection does not induce IκB phosphorylation.

We further characterized the transcriptional response of VSV-infected cells and showed that CD14-deficient (hdl) as well as Tlr4 mutant (HeJ) macrophages have a pronounced defect in transcriptional activation of Ifr7, consistent with the conclusion that IRF7 is responsible for TLR4-dependent, CD14-dependent type I IFN gene activation (Fig. 2A).

Ifnb1 gene transcription was augmented in Cd14hdl and Tlr4Lps mutant cells following stimulation by VSV, beyond levels observed in wild-type cells (Figs. 2A and B). Nonetheless, the extreme VSV susceptibility of macrophages with genetic lesions in the CD14/TLR4 signaling axis (Jiang et al., 2005) is mirrored by susceptibility of mutant mice infected with VSV by intravenous inoculation. C3H/HeJ mice showed markedly enhanced mortality as compared to C3H/HeN mice (P<0.0001; Fig. 2C). To investigate in more detail the discrepancy between undetectable IFN production at the protein level (upon the bio-assay used in this study or during ELISA quantification; Jiang et al., 2005) and high transcriptional activity of the Ifnb1 gene in VSV-infected macrophages isolated from Cd14 and Tlr4 mutant mice, we performed polysome analysis after sucrose gradient separation of the different mRNA species (Pillai et al., 2005). As seen in Fig. 3, polysomes isolated from VSV-infected wild-type macrophages (C57BL/6, panel A) are detected in fractions 5–9 as judged by the peak of vsg-g transcripts. The same fractions also contain detectable amounts of actin and Ifnb1 mRNAs. In contrary, the same analysis performed on mRNAs isolated from heedless macrophages (panel B) indicates that actin and Ifnb1 transcripts are mostly present in low-sucrose fractions (fractions 2–5), indicating that these mRNAs are poorly translated. This analysis demonstrates that high viral replication in the susceptible Cd14 mutant macrophages favors viral gene expression and lowers cellular mRNA translation, including translation of the immunologically important mRNA encoding IFNβ.

VSV-dependent TLR4 activation triggers a specific signaling cascade

LPS-induced TLR4 signaling involves recruitment of MyD88/Mal and TRIF/TRAM heterodimers, and the selective activation of type I IFN production by VSV gpG suggested selective activation of the TRIF/TRAM pathway. Surprisingly in view of their functional association with one another, TRAM was far more important for macrophage resistance to VSV (20% survival of TRAM-deficient cells vs. 70% survival of C57BL/6 cells; P<0.001) than TRIF (40% survival, C57BL/6 vs. TrifLps2, P<0.05, Fig. 4A). Moreover, a high level of VSV-gpG transcription was detected in the TRAM-deficient cells, reflecting uncontrolled viral replication (Fig. 4B). VSV-dependent activation of the Ifr7 gene was impaired in TRAM-deficient macrophages (as judged by semi-quantitative PCR experiment illustrated in Fig. 4B), and confirmed by real-time quantitative PCR (P<0.05 between wild-type and Tram-deficient macrophages, Fig. 4C) thus establishing a specific VSV-responsive TLR4→TRAM→IRF7 pathway. As observed in Tlr4- and Cd14-mutant macrophages, TRAM-deficient cells exhibit ifna and -b1 gene transcription when infected with intact VSV (Figs. 4B and C), but not when macrophages are exposed to pseudoviruses expressing the VSV gpG (Fig. 4D). Finally, we observed that macrophages from 3d homozygotes (in which signaling via TLRs 3, 7 and 9 is abolished (Tabeta et al., 2006)) and from mice lacking TLR3 are fully resistant to VSV infection (Fig. 5A). Further, chloroquine and 2-aminopurine fail to increase macrophage susceptibility (Fig. 5B). These observations exclude TLRs 3, 7, and 9 as well as PKR as important contributors to VSV resistance in macrophages.
Differential responses of dendritic cell subsets infected with VSV

Plasmacytoid dendritic cells (pDC) are considered to be the main source of type I IFN during viral infection (Colonna et al., 2002; Diebold et al., 2003). However, in a first set of experiments we determined that myeloid (GM-CSF derived) dendritic cells (mDC) are highly resistant to VSV infection, whereas FLT3-derived plasmacytoid dendritic cells (pDC) are comparatively susceptible (Fig. 6A). The resistance of mDC to VSV infection is correlated with type I IFN secretion by these cells in response to viral stimulation (Fig. 6B) and with low viral replication (Fig. 6C), while FLT-3L derived pDC support VSV replication to high titers and produce no detectable type I IFN.

To identify a molecular difference between these two cell populations, we performed a series of semi-quantitative RT-PCR experiments to follow the expression of several genes potentially involved in viral resistance. As shown in Fig. 6D, both cell types show induction of Ifnb1 gene transcription, but only the FLT-3L-derived DCs exhibit a high level of transcripts encoding VSV gpG, consistent with the high viral titer observed in these infected cells. Strikingly, strong and sustained induction of IRF7 mRNA is observed in VSV-infected mDC and cannot be detected in FLT-3L-derived dendritic cells.

To precisely measure type I IFN mRNA induction, we performed quantitative PCR on Ifna4 (not shown) and Ifnβ1 transcripts. The results presented in Fig. 6E are in agreement with our previous semi-quantitative RT-PCR experiments and show that both cell types respond to VSV by activating Ifnb1 gene transcription; hence, the difference in IFN production cannot be explained on the basis of a difference in transcriptional activation, and must be sought at the post-transcriptional level, as suggested by our polysome analysis of heedless macrophages (see above).

Because FLT3-L induced cells constitute a heterogeneous population, in a second series of experiments we used magnetic beads to separated B220− from B220+ cells (Fig. 7A) which have distinct properties and give rise to different mature DC populations in vivo (Brawand et al., 2002; Diao et al., 2004). We observed striking differences in the susceptibility of these
two populations after \textit{ex vivo} VSV infection. Whereas B220\(^{-}\) cells permit high viral replication, as judged by viral RNA amplification measured by real-time quantitative PCR (Fig. 7B), B220\(^{+}\) cells are totally resistant and only a limited amount of viral transcripts can be detected 18 h after VSV infection. Again, GM-CSF-derived cells show a moderate level of viral genome amplification. B220\(^{+}\) cells represent only 15% of the FLT3L-derived bone marrow cells. Therefore, we suspect that our analysis of the mixed population (B220\(^{-}\) and B220\(^{+}\) cells) was biased and essentially represented the results for the B220\(^{-}\) subtype. Finally, we observed highest \textit{Ifnb1} mRNA expression by B220\(^{+}\) cells upon viral infection and moderate \textit{Ifnb1} transcription by B220\(^{-}\) cells, as well as GM-CSF-derived DCs (Fig. 7C).

Discussion

We have previously shown that LPS-activated Toll-like receptor 4 (TLR4) can signal in two distinct modes depending upon the presence or absence of CD14, activating either MyD88/Mal and TRIF/TRAM together, or MyD88/Mal alone, respectively (Jiang et al., 2005). We have now described a third signaling pathway, initiated by VSV gpG, that is qualitatively distinct from either of the others. This pathway is chiefly represented in mDCs. It entails CD14- and TLR4-dependent recruitment of TRAM and leads to dramatic induction of IRF7, culminating in the activation of type I IFN secretion. Notably, IFNa and IFNb encoding mRNAs are strongly induced by VSV even when components of this pathway are eliminated by mutation (Figs. 1AB and 2AB). However, IFN protein [monitored by ELISA (Jiang et al., 2005) or with an ISRE-reporter bioassay (this study)] is not effectively produced, suggesting that translational or post-translational events are involved. Certain features of the system, such as the presence of a class II AU-rich element in the 3’ UTR of the \textit{Ifnb1} gene (Caput et al., 1986), and the pleiotropic effects of IRF7 overexpression [not only on gene transcription but also on RNA processing and intracellular trafficking (Barnes et al., 2004)], might explain this observation. To determine whether \textit{Ifnb1} mRNA translation was actually suppressed, we separated different fractions of mRNA isolated from wild-type and \textit{Cd14} mutant macrophages infected with VSV using sucrose gradient ultracentrifugation. This experiment clearly demonstrated that high viral replication and gene expression in
susceptible cells displaces the pool of cellular transcripts in the non-transcribed (not associated to ribosomes) fraction. Therefore, despite normal expression of genes encoding type I IFN, competition for ribosomes in the susceptible heedless macrophages prevents normal IFN production and secretion. These data highlight the importance of measuring both transcripts and proteins when studying IFN production during viral infection.

Recently, attention has been focused on the cytoplasmic sensor of double-stranded RNA, RIG-I, in the innate immune defense against single-stranded RNA viruses (Kato et al., 2005b; Yoneyama et al., 2004). Several new components of this pathway have been described, including MDA5 (Yoneyama et al., 2005) and IPS-1 (Kawai et al., 2005) [also called MAVS (Seth et al., 2005), VISA (Xu et al., 2005) or Cardiff (Meylan et al., 2005)], all of which share with RIG-I the capacity to induce both IRF- and NK-κB-dependent reporter genes when over-expressed in fibroblasts. Our study shows that, in addition to this double-stranded RNA-based detection system, recognition of a viral protein (directly or indirectly) by the TLR4/CD14 complex on the cell surface specifically induces IFN production and therefore, constitutes an essential aspect of the antiviral defense mechanism. This means of viral sensing makes a key contribution to host defense, as indicated by the marked susceptibility of thioglycolate-elicited peritoneal macrophages isolated from Tlr4- or Cd14-mutant mice to VSV infection ex vivo, and by the pronounced susceptibility of these animals to a lethal outcome when inoculated with VSV in vivo. The CD14/TLR4 and RIG-I pathways seem to be independent and non-redundant at the receptor/adaptor level, but our data do not exclude the possibility that both transduction cascades utilize common factors, such as IRF7. In addition, both pathways seem to function in the GM-CSF-derived dendritic cells subpopulation, which, as recently reported (Janssen et al., 2006) are strongly LPS responsive, whereas FLT3L-derived cells are not. In these cells, VSV-dependent type I IFN gene expression depends upon TLR7-MyD88 in CD11c+B220+ cells and upon RIG-I for CD11c+B220− cells (Kato et al., 2005a). At this stage however, the reason for high viral susceptibility of the B220− subpopulation despite Ifnb1 gene transcription remains unexplained. Non-redundancy of TLR4- and IPS-1/RIG-I-dependent IFN production is also illustrated at the functional level by the high susceptibility of both IPS-1 KO mice (Kumar et al., 2006) and Tlr4 mutants to VSV (this study).

The involvement of TLR4 in viral infections has previously been mentioned. The mouse mammary tumor virus (MMTV) envelope glycoprotein has been shown to engage TLR4 (Burzyn et al., 2004; Rassa et al., 2002), eliciting the production of IL-10 that permits persistent viral infection (Jude et al., 2003). In this example, TLR4 activation is considered to be a vehicle for evasion of innate immune responses, and the signaling pathways that are triggered are not known. It was elsewhere reported that purified preparations of the F-protein encoded by respiratory syncytial virus (RSV) activate the CD14/TLR4 complex, and that TLR4 mediates a protective response to RSV infection (Haynes et al., 2001; Kurt-Jones et al., 2000). However, this study did not address the role of CD14, and the conclusion that TLR4 mediates protection was challenged by genetic experiments that ascribed protection to a difference at the IL-12Rβ2 locus (Ehl et al., 2004). The present study provides strong evidence that TLR4/CD14 engagement by VSV gpG activates a specific anti-viral pathway. This property of the VSV envelope protein might explain the strong stimulatory (adjuvant) effect of fusion-active VSV gpG when incorporated within immunodeficiency virus-derived particles (Kuate et al., 2006). Indeed, such modified vaccines induce a 100 fold increase in gag antibody titers, an effect which can be at least partially mediated by the innate immune response induced by VSV gpG on antigen presenting cells such as myeloid DCs. Recently, viral glycoprotein from Herpesviridae (Human Cytomegalovirus and Herpes Simplex Virus) have been shown to promote innate resistance mediated by TLR2 activation (Boehme et al., 2006; Sato et al., 2006) and HCMV proteins gB and gH physically interact with human TLR1/2 heterodimer. Our observations, combined with the unexpected structural homology between gB from Herpes-
viruses and gpG of VSV (Roche et al., 2006) raise the possibility that viral detection by multiple TLRs expressed at the cell surface is a key element for the overall antiviral innate response. Further additional work will be required to decipher the details of viral envelope proteins/TLR interactions in general, and more particularly, in the case of VSV gpG and TLR4.

Finally, we note that TLR4 orthologs are expressed in most vertebrate species (Jault et al., 2004; Smirnova et al., 2000). However, despite the presence of a bone fide Tlr4 gene in fish, amphibians and reptiles, these vertebrate families are highly resistant to LPS toxicity (Berczi et al., 1966) and are not known to exhibit cellular responses to LPS. This raises the possibility that TLR4 might be dedicated to viral sensing in LPS-unresponsive species, which are known to be prey to the Rhabdoviridae (Hoffmann et al., 2005; Monath et al., 1979).

Materials and methods

Mice

Thioglycolate (TG)-elicited macrophages were harvested 3 days after TG injection by peritoneal lavage and resuspended at 10⁶ cells/ml. MyD88−/− mice were provided by S. Akira, Osaka University, Osaka, and backcrossed to C57BL/6 mice. C3H/HeN mice were obtained from Charles River and C3H/HeJ mice from the Jackson Laboratories. Tram-deficient mice were generated by Xenogen in a pure C57BL/6 background. Tirap knockout animals were provided by R. Medzhitov, Yale University, New Haven, CT. All experiments were carried out in compliance with the rules of the TSRI Animal Use Committee.
Viruses

VSV (Indiana Strain) was propagated and amplified by infection of a monolayer of Vero cells. 24 h after infection, the supernatant was harvested and clarified by centrifugation. Viral titer was determined by plaque assay on Vero cells. For the VSV cytolytic assay, 100,000 cells were plated and infected at m.o.i 1, 10 and 50. 40 h post-infection, cell survival was quantified by MTT staining. Recombinant Moloney murine leukemia virus (MoMuLV) pseudotyped with gpG of VSV or with a Lassa fever virus (LFV) glycoprotein, were produced by transient transfection of the package cell line GP2-293 (BD Biosciences) with the packable MoMuLV genome pLXRN, which contains a luciferase reporter gene and a neomycin resistance cassette (BD Biosciences). LFVGP and VSVGP were provided in trans by co-transfection with the expression plasmid pC-LFVGP, containing the full-length cDNA of LFV Josiah, and the VSVGP expression construct pVSV-G (BD Biosciences), respectively. Briefly, 1.2×10^7 GP2-293 cells were plated in poly-L-lysine coated T175 tissue culture flasks. After 16 h, cells were co-transfected with 20 μg each of pLXRN and pC-LFVGP or pVSV-G using calcium phosphate. Forty hours after transfection, cell supernatants were harvested and cleared by centrifugation for 15 min at 3000 rpm. Retroviral pseudotypes were then concentrated by ultracentrifugation at 25,000 rpm at 4 °C for 2 h using a SW28 rotor. Supernatants were discarded after centrifugation and pellets resuspended for 16 h in DMEM, 20 mM Hepes, pH 7.5 at 4 °C. For determination of titers, monolayers of HEK293 cells were infected with serial dilutions of pseudotypes and cells subsequently subjected to antibiotic selection using G418 according to the manufacturer’s recommendations. Clusters of resistant cells were counted and titers calculated.

Bio-assays

Type I IFN activity was measured with reference to a recombinant mouse IFNβ standard using an L-929 cell line (5×10^5 cells/ml) transfected with an IFN-sensitive (ISRE) luciferase construct.

Polysome analysis

Thiglycolate-induced peritoneal macrophages from four control (C57BL/6) or mutant (heedless) mice were pooled, plated in 10 cm dishes and infected with VSV with a multiplicity of infection of 10. After 18 h, cells were harvested, washed in cold PBS containing 100 μg/ml cycloheximide and incubated on
ice for 5 min. After 2 washes in cold PBS, cell lysis was realized with hypotonic buffer containing 0.5% deoxycholate, 0.5% Triton X-100 and 120 U/ml of Rnasin (Promega). Lysates were centrifuged for 8 min at 3000×g at 4 °C and supernatants were then layered onto 10–50% sucrose gradients and spun in an SW40 rotor (Beckman) at 36,000 rpm for 2 h at 4 °C. Fourteen 800 μl fractions were collected and RNA was extracted with phenol/chloroform and precipitated with ethanol. RNA samples were treated with DNaseI, extracted again with phenol/chloroform, precipitated and solubilised in 20 μl RNase free water. Following quantification at O.D. 260 nm, 2 μl of each fraction were used for reverse transcription reactions. Actin, vsv-g and Ifnb1 cDNAs were PCR amplified (30 cycles in all cases, except for vsv-g in control macrophages which required 40 cycles for detection) and 4 μl were loaded on agarose gels.

Bone marrow-derived dendritic cell preparation

Bone marrow cells harvested from 2 femurs of a wild-type mouse were plated at a concentration of 106 cells/ml in IMDM containing 10% FCS and 2% Penicillin/Streptomycin. One flask was supplemented with human FLT-3L (200 ng/ml) and incubated at 10% CO2 for 9 days and the other was supplemented with mouse GMCSF (5 ng/ml) and incubated at 5% CO2. B220+ cells were isolated by positive selection using MACS microbeads (Miltenyi Biotec, Auburn, CA). Cell purity after positive selection was 97% and viability was >97% as determined by FACS analysis using B220-FITC and 7-AAD.

Reverse transcription and PCR

2 μg of total mRNA were used according to the manufacturer’s recommendations (Ambion) in a 20 μl reaction volume for reverse transcription. 1 μl of RT reaction was used for each PCR whose number of cycles was optimized to avoid saturation (25 for Ifn-7, 30 for all the others primer pairs). 3 μl of reaction were loaded on agarose gels.

Quantitative PCR

Quantitative Real-time PCR was performed in a PE Biosystems Gene-Amp 5700 thermocycler using Bio-Rad SyBr Green detection protocol as outlined by the manufacturer. Briefly, 12 ng of total complementary DNA, 50 nM of each primer and 1× SyBr Green mix were used in a total volume of 25 μl. Actin and L32 transcripts were used as internal normalization controls.

Primers:

- Ifna4 forward: 5′ CCT GGT AAT GAT GAG CTA CTA CTG GT
- Ifna4 reverse: 5′ ATT TCT TCC AGG ACT GTC AAG GC
- Ifnb1 forward: 5′ TCC AAG AAA GGA CGA ACA TTC G
- Ifnb1 reverse: 5′ TGA GGA CAT CTC CCA CGT CAA

Statistical analysis

Data were analyzed using ANOVA test with GraphPad software.

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