

SHORT COMMUNICATION

Restricted tissue tropism and acquired resistance to Sindbis viral vector expression in the absence of innate and adaptive immunity

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Our previous studies suggest that replication-defective Sindbis vectors might be promising agents for specific tumor targeting and detection. However, the effects of innate and/or adaptive anti-viral immunity, in particular, the IFN-I/STAT1 signaling pathway, may impact their therapeutic potential. Using a bioluminescent imaging system, we demonstrate that although most normal cells are not permissively transduced by replication-defective Sindbis vector, transduction of liver non-sinusoidal endothelial occurs the first time IFN-I/STAT1 signaling deficient mice are inoculated with the vector. Transduction of some cells is not surprising since STAT1 knockout animals show significant delay in IFN responses such as the production of IFN- α/β and transcriptional activation of several anti-viral genes (IRF7, RIG-I,

PKR, TLR3, USP18, ISG15). However, beyond the initial vector transduction, which resolves rapidly, secondary inoculums of Sindbis vectors do not transduce any liver cells, suggesting that an alternative antiviral pathway may protect against further transduction. Other known signaling pathways were examined using mice lacking functional TLR3, tumor necrosis factor α R or nuclear factor-kappa B (p50). Surprisingly, none of those pathways seem to play a significant role in anti-Sindbis responses. Thus it appears that *in vivo*, in contrast to the ready transduction of tumor cells, transduction of normal cells by replication-defective Sindbis vector is limited, possibly by a novel mechanism. Gene Therapy advance online publication, 17 May 2007; doi:10.1038/sj.gt.3302973

Keywords: viral vector; Sindbis virus; interferon; molecular imaging

Introduction

A major goal of cancer gene therapy is to be able to deliver specifically therapeutic genes to target tumors. To achieve this goal, several viral and non-viral gene therapy vector systems have been developed. One major advantage of viral vectors over non-viral vectors is their higher transduction efficiency. However, compared with non-viral vector systems, viral vectors are more likely to raise concerns about potential deleterious effects associated with the parental viruses, especially for vectors derived from known human pathogens. For this reason, several strategies have been developed to reduce the likelihood of vector toxicities associated with the parental virus. The most common approach involves partial deletion of viral genomes that are required for viral replication (replication-defective), or alternatively, conditioning the vector's replication to certain cellular parameters (conditional replication), such as the expression of the Ras oncogene.

Our research group has focused on the development of replication-defective Sindbis viral vectors for cancer gene therapy, because these have a number of positive attributes. First, the genome of Sindbis virus is a sense, single-stranded RNA virus and there is no DNA phase in its life cycle, precluding risks associated with genomic integration.¹ Second, with a relatively small genome (~12 kbp), the vector system can be easily modified to express different therapeutic genes.^{2,3} Third, the vector is capable of systemic dissemination throughout the body via the bloodstream.⁴ The latter property makes Sindbis vectors among the few that have the capability of systemic tumor targeting. Fourth, the vector targets cancer cells via the 67-kDa high-affinity laminin receptor (LAMR),^{5,6} which is substantially upregulated and associated with invasiveness in numerous human cancers.⁷ A significant number of LAMRs on tumor cells appear not to be occupied by laminin, and hence available for Sindbis vector binding. By contrast, unoccupied receptors are likely to be scarce on normal cells, providing a means for differential targeting of tumors versus normal cells^{8,9} and also limiting permissive transduction of most normal cells.

Taking advantage of these special features, we have demonstrated specific tumor targeting of Sindbis vectors in several *in vivo* tumor models.^{10,11} Consecutive vector treatments are safe and have shown no morbidity and mortality in our experimental tumor models. Several

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additional factors may contribute to the safety of the vector system. First, in comparison with other oncolytic vectors, Sindbis vectors cause tumor apoptosis after transduction instead of lysis and necrosis that are more inflammatory to surrounding normal tissues.^{12,13} Second, transduction of most healthy adults by the wild-type, replication-competent Sindbis virus typically causes no symptomatology or may cause mild self-limited symptoms such as rash, arthralgia and moderate fever that are not life threatening to human.⁴ Even so, we currently use replication-defective Sindbis vectors to enhance safety in our tumor models.¹⁴ The fact that minimal, if any, adverse effects are observed contrasts with the strong anti-tumor results observed in several of our cancer models and suggest an inherent difference *in vivo* between tumor cells and normal cells in sensitivity to Sindbis vector transduction. One such difference, as discussed above, is the availability of LAMRs in tumor and normal cells.

To determine further the potential of replication defective Sindbis vectors for *in vivo* therapy, we examined some potential host responses that might limit vector use. The innate immune responses of the host, involving cytokines such as IFNs, might be expected to play important roles in the safety of Sindbis vectors but also pose limitations to their use. In particular, IFN-I and JAK-STAT signaling pathways are known to establish an 'alarming' state that suppresses replication and propagation of several viruses during the early stage of infection.¹⁵ IFN-Is, such as IFN- α and IFN- β , are important anti-viral cytokines and serve as the first line of innate immunity against viral infection. Both IFN- α / β share the same cellular receptor (IFNAR); binding activates a signaling cascade that is mediated by the Janus kinase/signal transducer and activator of transcription (STAT) pathway.¹⁵ IFNAR activation triggers the downstream Janus kinase (JAK)-mediated tyrosine phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 and STAT2 molecules form heterodimers and translocate to the nucleus in association with another transcription factor, interferon regulatory factor 9 (IRF9). This trimolecular complex (STAT1-STAT2-IRF9), named interferon-stimulated gene factor 3 (ISGF3), binds to a specific DNA recognition element, interferon-stimulated response element, to modulate the transcription of a large number of genes that contribute to the antiviral function of these cytokines.

Unlike replication-defective vectors, replication-competent wild-type Sindbis virus has been shown to infect

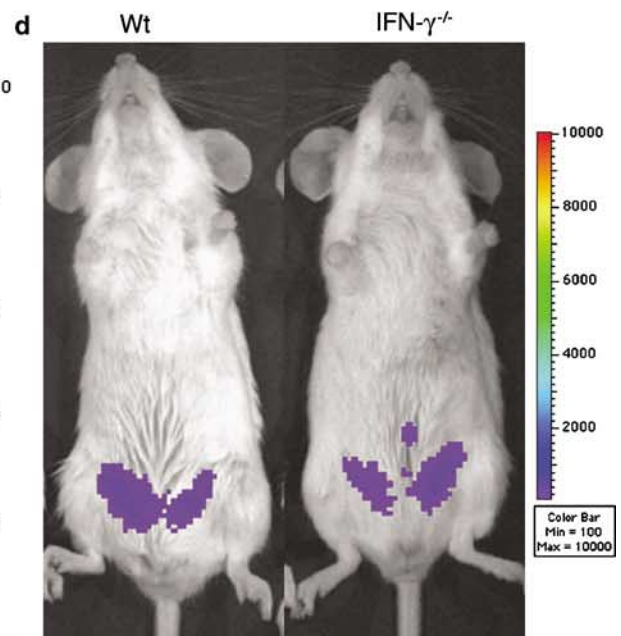
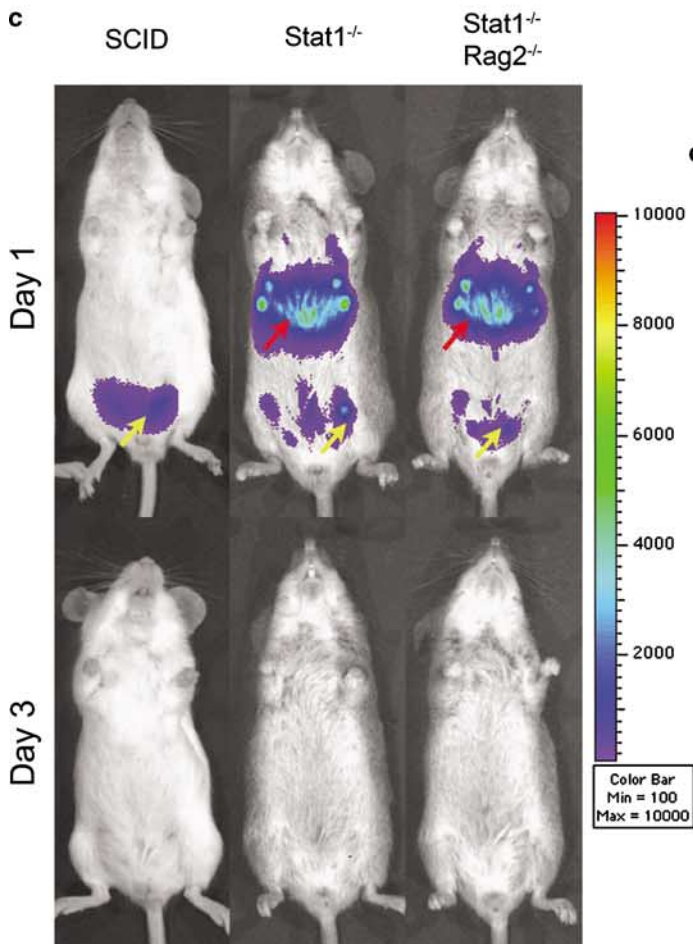
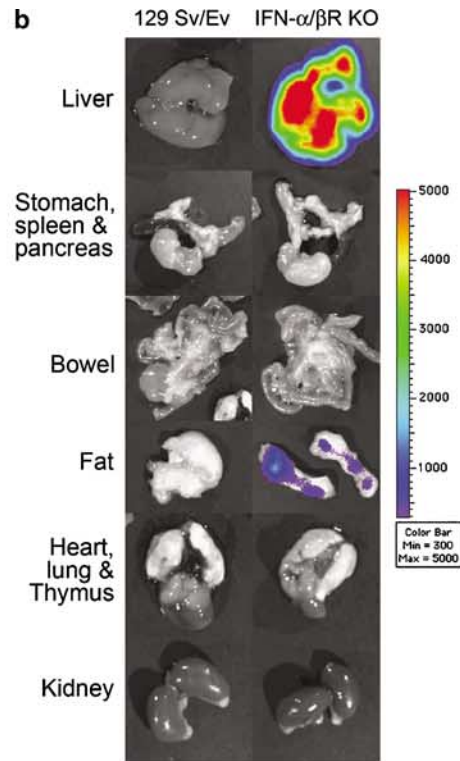
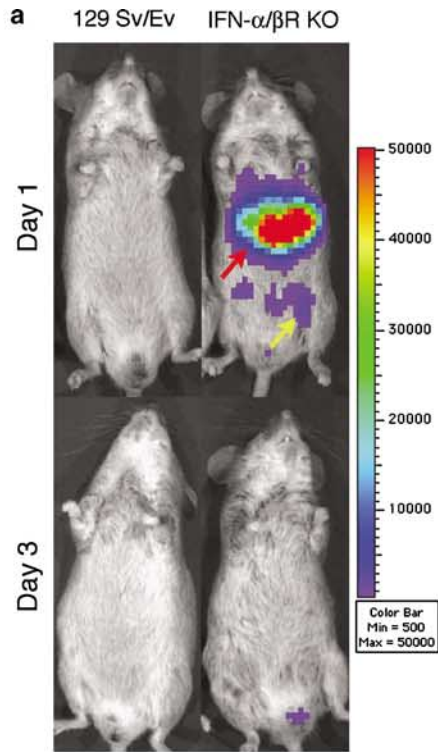
macrophage-dendritic cell-like cells in the draining lymph nodes causing viremia within 24 h in mice lacking functional receptors for IFN- α / β . In IFNAR-deficient mice the virus results in widespread infection of monocyte-lineage cells throughout the body and mortality at later stages (>72 h) of infection, while wild-type mice experience no morbidity or mortality.¹⁶ However, the toxicity of replication-defective Sindbis vector in mice lacking a functional IFN-I signaling pathway has not been addressed. One of the challenges of such a study is that of measuring early vector delivery and transduction. Since the vector is replication defective, traditional plaque assays that require longer time (>18 h) for viral propagation are not feasible. To overcome this limitation, one can use *in vivo* bioluminescent imaging methods to detect the early transduction of the replication-defective vector, as measured by expression of a reporter transgene, such as luciferase. Non-invasive bioluminescent imaging does not require viral propagation for analysis and therefore enable us to observe the early stages of vector transduction, before the onset of viremia usually occurs in mice treated with replication-competent wild-type virus. We have used this method to examine vector transduction in wild-type mice and in mice lacking functional IFN-I/STAT1 signaling.

Results and discussion

IFN- α / β signaling protects liver from Sindbis vector transduction

To investigate if the IFN signaling pathway protects normal tissues from Sindbis *in vivo*, we intraperitoneally (i.p.) administered Sindbis/Fluc vectors into mice that lack functional type-I interferon (IFN-I) receptor (IFN- α / β R^{-/-}) on day 0. We chose i.p. delivery of Sindbis vector in IFN- α / β R^{-/-} mice since i.p. injection of Sindbis vector enables systemic delivery via the bloodstream and has been shown to target tumors in living animals.¹¹ Replication-defective Sindbis/Fluc vector was produced using the defective helper DHBB and a SinRep5 replicon,¹⁴ which carries a firefly luciferase gene for bioluminescent IVIS imaging.² The vector treatment resulted in extensive transduction of IFN- α / β R^{-/-} mice in the upper region of the abdomen 24 h after vector injection (Figure 1a, day 1). In some cases, minor transduction was also observed in the lower region of the abdomen (Figure 1a, yellow arrow). By contrast, the vector caused no detectable permissive transduction in

Figure 1 IFN-I/STAT1 signaling pathway protects mouse liver from Sindbis transduction. Replication-defective Sindbis vectors were produced as described previously¹⁰ and all animal experiments were performed in accordance with NIH and institutional guidelines. (a) 129Sv/Ev wild-type and IFN- α / β R KO mice receive i.p. injection of Sindbis/Fluc vectors (0.5 ml, $\sim 10^7$ vector particles) on day 0 and day 2. The bioluminescent signals generated by vector transduction were determined using the IVIS Imaging System Series 100 (Xenogen Corp., Alameda, CA, USA) on day 1 and day 3. For imaging, each mouse was i.p. injected with 0.3 ml of 15 mg/ml beetle luciferin in PBS 5 min before acquiring. An integration time of 1 min was used for luminescent image acquisition for all animal tumor models. On day 1, none of wild-type mice (0/5) showed any signals, while 4 out of 5 IFN- α / β R KO mice showed significantly strong liver signals. No significant signals observed on day 3 in both animals. (b) IVIS imaging of the organs on day 1. Strong liver signals were observed in IFN- α / β R KO mouse and lower signals in the peritoneal fat. (c) Similar to experiment in (a), SCID, Stat1 KO (129S6/SvEvTac-Stat1^{tm1Rds}) and Stat1/Rag2 double KO mice (129S6.B6-Stat1^{tm1Rds}-Rag2^{tm1Fluc}), both obtained from Taconic, Germantown, NY, USA) were i.p. injected with Sindbis/Fluc vector on day 0 and day 2 and imaged on day 1 and day 3. As in IFN- α / β R KO mice, strong liver signals were observed on day 1 in both Stat1 KO (5 out of 5 mice) and Stat1/Rag2 double KO (5 out of 5 mice), but not in SCID mice (0 out of 5 mice). No signals were observed in all animals on day 3 after second vector challenge on day 2. Red arrow indicates the liver transduction and the yellow arrow indicate transduction in the peritoneal fat. (d) Wild-type (Balb/c) and IFN- γ KO mice (C.129S7(B6)-Ifng^{tm1Ts}/J), Jackson Laboratory, Bar Harbor, ME, USA) received i.p. Sindbis/Fluc injection on day 0 and imaged on day 1. No signal was observed in all five mice of each group except low signals in the peritoneal fat.



background 129 Sv/Ev mice. By imaging individual organs, we confirmed that the stronger signal is clearly associated with the liver of IFN- α / β R $^{-/-}$ mice (Figure 1b), and the weaker signals correspond to the peritoneal fat of IFN- α / β R $^{-/-}$ animals. No signal is present in the stomach, spleen, pancreas, bowel, heart, lung, thymus and kidney in either knockout or wild-type mice. Interestingly, an additional Sindbis/Fluc treatment on day 2 did not lead to detectable re-transduction of the liver on day 3 (Figure 1a) and subsequently all treated animals remained signal-free. No observable morbidity or mortality in IFN- α / β R $^{-/-}$ animals was associated with these systemic Sindbis/Fluc treatments. These results suggest that in the absence of IFN-I responses, Sindbis vector initially transduces some liver cells, but this transduction appears to resolve within 3 days. It is surmized that in wild-type mice, IFN-I responses are involved in suppressing Sindbis vectors for permissive transduction.

To determine if STAT1, a downstream mediator of IFN-I signaling, is involved in the anti-Sindbis effects in the liver, similar experiments were performed in mice lacking STAT1. Transduction of some liver cells was observed only on day 1 but not subsequently, which is consistent with observations in IFN- α / β R $^{-/-}$ mice (Figure 1c). One explanation for these findings might be that after the first injection, adaptive immunity rather than IFN-I innate immunity is involved in liver protection. To test this notion, we performed the same experiment using SCID and Stat1 $^{-/-}$ /Rag2 $^{-/-}$ double knockout mice (Figure 1c). The same level of initial liver transduction and subsequent suppression of transduction was observed in Stat1 $^{-/-}$ /Rag2 $^{-/-}$ double knockout mice as in Stat1 $^{-/-}$ mice. Thus, the absence of persistent liver transduction in SCID and Stat1 $^{-/-}$ /Rag2 $^{-/-}$ mice (Figure 1c) is not likely the result of adaptive immunity, since mice lacking RAG2, which is required for generation of T and B cells, and STAT1 show the same transduction pattern as STAT1 KO mice; that is, transduction after the initial inoculum, but not transduction after subsequent injections. As Sindbis transduction is transient because no integration of the viral genome occurs, and the vectors used are replication defective, no super-transduction block appears likely.

In addition to IFN- α / β signaling, STAT1 also participates in the IFN- γ signaling, a type II interferon pathway in which IFN- γ signaling utilizes phosphorylated STAT1 homodimer (STAT1-STAT1, GAF) to mediate its transcription activity instead of STAT1-STAT2 heterodimer used for IFN- α / β signaling. For this reason, we used IFN- γ knockout mice to investigate whether IFN- γ plays a role in the protection of Sindbis vector transduction. One day after i.p. Sindbis/Fluc treatment, no liver transduction was observed in IFN- γ null mice, except for low levels of transduction in the peritoneal fat (Figure 1d), suggesting that the protection of transduction against liver cells is mediated by the IFN-I signaling but not by the IFN-II signaling, even though STAT1 is a shared component for both signaling pathways.

To confirm that adaptive immunity does not contribute to early protection in the liver we studied the transduction kinetics in Stat1 $^{-/-}$ and Stat1 $^{-/-}$ Rag2 $^{-/-}$ mice. Wild-type 129 Sv/Ev mice were used as control. After Sindbis/Fluc vector i.p. injection, mice were imaged to follow its transduction at different time points

(Figure 2a). As early as 6 h after transduction, we were able to detect transduction in the lymph nodes (by the neck) and peritoneal fat (lower abdomen) along with lower, but considerable liver transduction signals in both Stat1 $^{-/-}$ and Stat1 $^{-/-}$ Rag2 $^{-/-}$ mice. The bioluminescent signals in the lymph nodes were likely caused by monocyte-dendritic cell transduction.¹⁶ Lymph signals reached highest levels around 12 h after transduction and mostly resolved in 1 day. On the other hand, the signals in liver reach the highest levels 20–25 h after vector treatment and no liver signals were observed after 46 h. Wild-type animals only showed minor transduction in the peritoneal fat that generally resolved within 1 day. A second vector inoculation was given at 118 h (~5 days after the first vector injection) and no significant liver transduction was observed in Stat1 $^{-/-}$ or Stat1 $^{-/-}$ Rag2 $^{-/-}$ double knockout mice. The lack of retransduction is not likely due to induced adaptive immunity, such as neutralizing antibody, since no liver signal was observed in Stat1 $^{-/-}$ Rag2 $^{-/-}$ mice (Figure 2a and b). Therefore, in wild-type mice, at early stages of transduction, the vector is suppressed in liver by a STAT1-dependent manner without T or B cells, although a STAT1-independent innate immunity, such as NK cell, may protect mice from second Sindbis/Fluc challenge.¹⁷ Arguing against this possibility is our finding that the absence of NK cells is slightly detrimental to the therapeutic potential of Sindbis vectors in animal tumor models.¹⁰

Sindbis vector transduces non-sinusoidal endothelial cells in the liver of Stat1 $^{-/-}$ mice

Since the liver is a vital organ, it is of interest to determine which cell type in the liver was transduced by Sindbis vector and if the transduction causes any pathological effects to the liver. We performed immunohistology staining of liver sections harvested from Stat1 $^{-/-}$ mice 24 h after transduction by Sindbis/LacZ vector, which expresses a bacterial β -galactosidase. β -gal staining indicated that the vector transduced vascular endothelial cells but not sinusoidal Kupffer cells which are of monocyte lineage (Figure 2c, upper left panel). In addition, we observed no hepatocyte transduction and no histopathological effects associated with Sindbis transduction in all liver sections examined. Neither necrosis nor inflammation was associated with vector treatment in the liver, consistent with the observation that the treatment caused no morbidity or mortality. This finding is in contrast to the apoptotic and cytopathic effects on tumor cells we have reported previously.^{2,10}

As determined by immunohistochemistry (Figure 2c), vascular endothelial cells express less LAMR than the sinusoidal endothelial cells (Figure 2c), suggesting that, unlike tumor targeting, the specificity of vector transduction of the vascular endothelial cells is not completely dependent upon higher expression level of LAMR, and other factors may be involved in transduction. One such possibility involves a role for heparan sulfate, which has been shown to mediate Sindbis virus attachment to some mammalian cells.^{18,19}

STAT1 $^{-/-}$ mice display diminished and delayed IFN-I responses

Deficiency of STAT1 should affect the host anti-viral responses in term of IFN-I production. It is known that

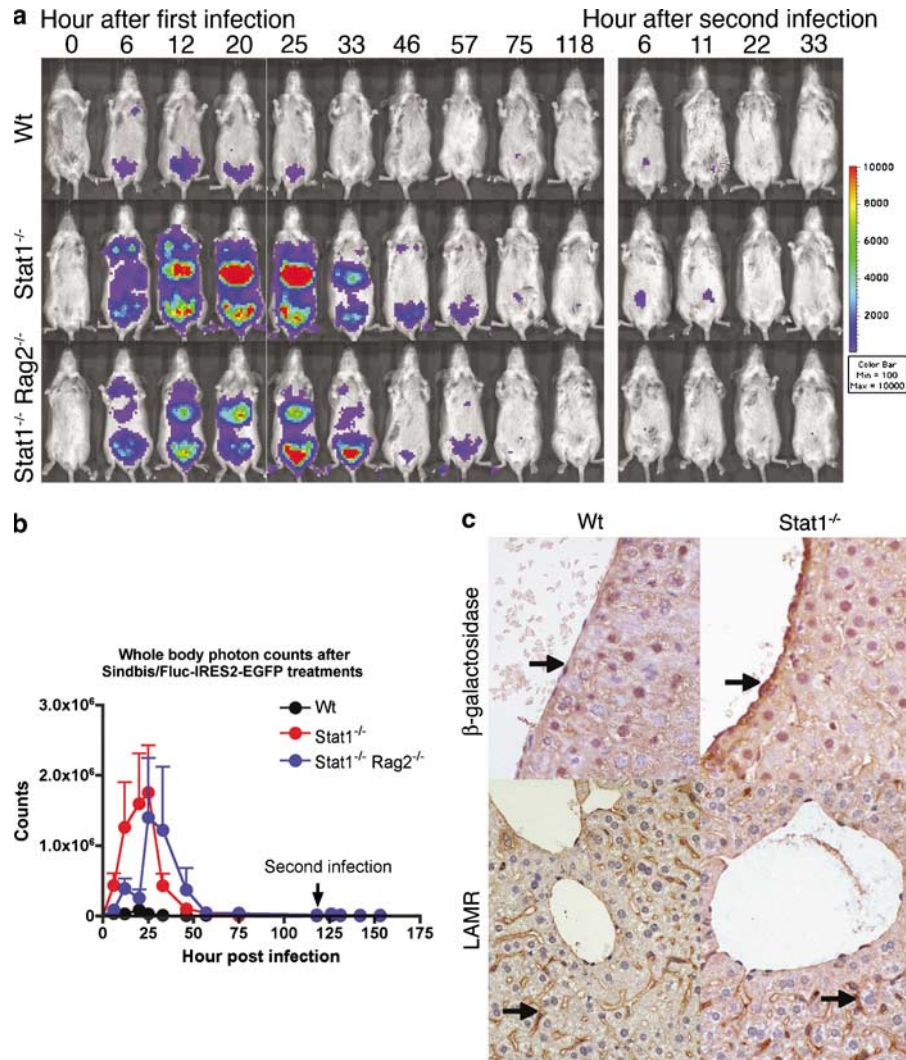
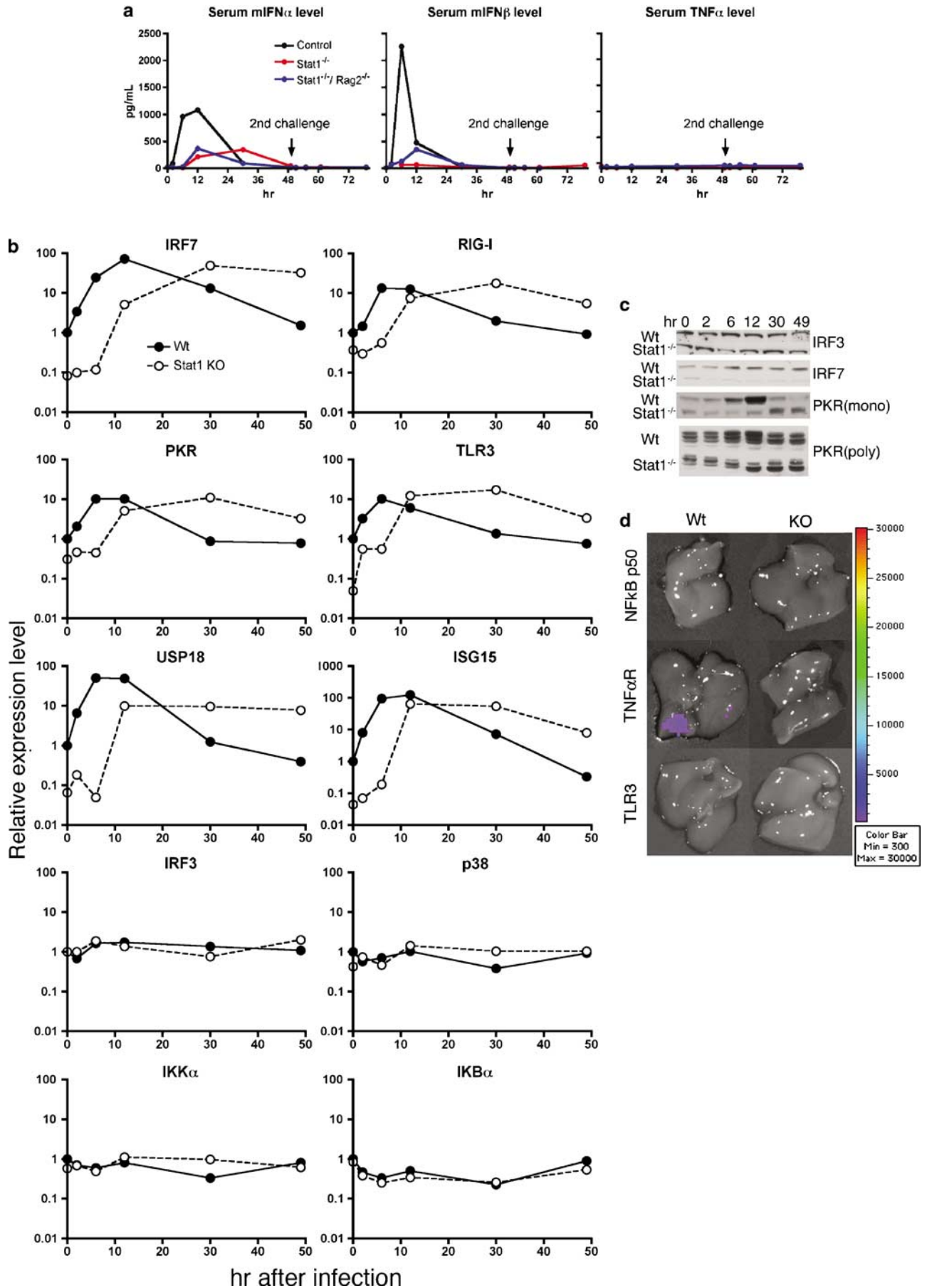


Figure 2 Sindbis vector transduces non-sinusoidal endothelial cells in the liver of Stat1^{-/-} mice. (a) Kinetics of Sindbis/Fluc transduction in WT ($n=5$), Stat1 KO ($n=5$) and Stat1/Rag2 double KO mice ($n=5$) after Sindbis/Fluc transduction. Sindbis/Fluc (0.5 ml, $\sim 10^7$ vector particles) was i.p. injected at 0 h and bioluminescent signals were followed using the IVIS system. (b) We use Living Image software to integrate the total bioluminescence signals (in terms of photon counts) obtained from animals. A second Sindbis/Fluc injection was performed at 118 h and no significant signals were observed in all mice. The error bars represent the s.e.m. of five animals. (c) Immunohistological staining of Stat1 KO liver sections after Sindbis/LacZ transduction. Stat1 KO mice were treated with i.p. injection of Sindbis/LacZ on day 0. Liver tissues were harvested on day 1 and subject to IHC staining using antibodies specific to β -galactosidase (BioDesign International, Kennebunk, ME, USA) or high-affinity laminin receptor (LAMR) (Abcam Ltd., Cambridge, UK). Positive β -gal staining was observed in the non-sinusoidal endothelial cells (indicated by arrows), while higher LAMR staining was observed in the sinusoidal endothelial cells (indicated by arrows).

mice lacking STAT1 are unable to mount positive feedback amplification and are limited to production of the immediate-early IFN subtype $\alpha 4$ and β when challenged with virus.²⁰ To verify this, we used enzyme-linked immunosorbent assay (ELISA) assays to determine serum level of IFN- α/β after i.p. Sindbis/Fluc treatments (Figure 3a). Both Stat1^{-/-} and Stat1^{-/-}/Rag2^{-/-} mice showed diminished and delayed expression of IFN- α/β . In particular, IFN- β , the predominant IFN-I in response to viral infection, was dramatically reduced in mice with STAT1 deficiency. Lack of RAG2 seems to have little impact on the expression of IFN-I. Interestingly, the second challenge of Sindbis/Fluc at 49 h did not evoke a second wave of IFN responses suggesting that the host is still in an anti-viral state. We

also checked the levels of tumor necrosis factor (TNF)- α to see if the transduction causes serious inflammation or toxicity. Surprisingly, in contrast to infection of wild-type Sindbis virus in IFN- α/β R knockout mice,¹⁶ very low levels of TNF- α were induced by the transduction of replication defective vector (Figure 3a).

In addition to reduced and delayed expression levels of IFN-I in sera, the lack of STAT1 should affect the expression of other anti-viral gene products in the liver, which may contribute to establishment of the anti-viral state. We used real-time PCR analysis to determine the expression levels of several genes in the liver (Figure 3b). Expression of transcription factor IRF7 is reduced and delayed in a manner similar to IFN- α production (Figure 3b), reflecting the fact that IRF7 can mediate the positive



feedback loop of IFN- α expression.²⁰ On the other hand, IRF3 expression remained unchanged in both wild-type and knockout mice. Other anti-viral genes such as RNA helicase RIG-I, dsRNA protein kinase PKR, dsRNA receptor TLR3, ubiquitin-like modifier ISG15 and ISGylation deconjugating enzyme USP18 genes showed similar reduced and delayed expression patterns in Stat1^{-/-} liver after Sindbis/Fluc transduction (Figure 3b). By contrast, expression levels of genes involved in TLR pro-inflammatory signaling such as p38, IKK α and IKB α showed no difference after transduction (Figure 3b). In addition, the transcription levels of TNF α did not increase in wild-type or knockout mice. To verify the delayed expression pattern of real-time PCR results, we examined the protein expression levels of IRF3, IRF7 and PKR in wild-type and Stat1^{-/-} liver (Figure 3c). Similar to the reverse transcriptase (RT)-PCR result, expression of IRF3 was not affected by Sindbis vector transduction in wild-type and knockout mice. Using a monoclonal antibody as probe, PKR protein expression in the liver was significantly induced by vector transduction in wild-type mice while in the Stat1^{-/-} liver the induction is delayed and reduced as the RT-PCR result. On the other hand, blotting using a polyclonal antibody capable of detecting multiple phosphorylation forms revealed that, although the expression level increased, PKR was not fully activated in Stat1^{-/-} liver. Interestingly, the protein levels of IRF7 in wild-type liver increased after vector transduction, as was the case for transcript levels. However, in Stat1^{-/-} liver, IRF7 protein levels are much lower and no significant expression increases are seen after vector transduction, which is in marked contrast to the expression pattern determined by RT-PCR. The reduced protein level of IRF7 might be due to the translational shut-off mediated by activation of PKR and/or GCN2 resulting phosphorylation of eIF2 α .^{21,22} In summary, our results indicate that the lack of Stat1, in most case, only delays the expression of anti-viral genes but does not completely prevent their activation. Other alternative mechanisms, such as RIG-I, Mda5 and/or TLR7, might be involved in initiating and establishing an antiviral state in the absence of functional STAT1.

We also used *in vivo* imaging to investigate if other signaling mechanisms, such as nuclear factor-kappa B (NF- κ B), directly participate in the observed protection of liver cells from vector transduction. Classic NF- κ B pathways play important roles in cell survival, inflammation and innate immunity. There are three major ways

for virus to activate classic NF- κ B pathway: TNF α , TLR and PKR. Mice lacking TNF- α receptor (TNF α R) did not show liver transduction after i.p. injections of Sindbis/Fluc vector (Figure 3d), which is consistent with the observation that no significant increase in serum TNF- α level after Sindbis vector transduction (Figure 3a). In addition, mice lacking TLR3 also showed resistance to Sindbis transduction in liver (Figure 3d). Despite the fact that TLR3 was induced in wild-type liver after Sindbis/Fluc treatment (Figure 2b), TLR3 dose not seem to play a role in the early defense of Sindbis vector. Similar negative results were observed in NF- κ B (p50) knockout mice (Figure 3d), suggesting that protection of transduction of liver cells by replication-defective Sindbis vectors does not require TLR-NF- κ B inflammatory response. The fact that TLR3 KO mice show no difference in liver protection also indicates that, even if NF- κ B is activated, it is not likely via the TNF pathway. However, activation of NF- κ B could be achieved by PKR. Although it may not be the case for Stat1 KO mice, since the vector did not induce hyperphosphorylation of PKR for NF- κ B activation (Figure 3c). In conclusion, the classic NF- κ B pathway is not likely to be activated by Sindbis vector transduction. Even activated, it may not play an important role of early protection of Sindbis transduction.

These studies demonstrate that even in mice lacking function IFN-I/STAT1 signaling most normal tissues, with the salient exception of the liver, are not susceptible to transduction by Sindbis vectors. The expression of the Sindbis transduced luciferase gene in the liver of knockout mice, but not wild type mice, indicates that IFN-I/STAT1 signaling protects mice from the initial Sindbis vector transduction of liver cells. This liver transduction is limited to the non-sinusoidal endothelium cells. Several IFN-responsive genes were activated in Stat1 knockout mice after the initial Sindbis vector transduction, although at a slower rate than in wild-type animals. However, after the initial transduction other antiviral mechanism must be responsible for control of vector transduction, since subsequent Sindbis vector injections in IFNAR or STAT1 knockout mice fail to transduce liver cells. Despite susceptibility in the knockout mice, no adverse effects from the initial or subsequent injections were observed.

Several factors might contribute to the acquired resistance to Sindbis transduction we observed in mouse liver. One simple explanation is that the vector transduces and eliminates the initial transduced sinusoidal

Figure 3 STAT1^{-/-} mice display diminished and delayed IFN-I responses. (a) The kinetics of serum levels of IFN- α , IFN- β and TNF- α was determined by enzyme-linked immunosorbent assay (ELISA) after Sindbis/Fluc i.p. injection. After vector injection, mouse sera were collected at different time points as indicated. Each time point represents the pooled serum cytokine level of three mice. The data points represent the average of two independent assays of the pooled serum. Mouse IFN- α and IFN- β ELISA kits obtained from PBL Biomedical Laboratories (Piscataway, NJ, USA) and TNF- α ELISA kit was purchased from R&D systems (Minneapolis, NE, USA). (b) Real-time PCR analysis of gene expression in liver after Sindbis/Fluc transduction. Total liver RNA was isolated using the TRIzol reagent and cDNA was generated using of ThermoScript RNase H⁻ Reverse Transcriptase (Invitrogen Co., Carlsbad, CA, USA). Real-time quantitative PCR was performed on a iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA, USA). GAPDH was chosen as the housekeeping gene for comparative analysis. (c) Western analysis of IRF3, IRF7 and PKR expression levels in the liver after vector injection. We used polyclonal rabbit anti-IRF3 or anti-IRF7 antibodies (ZYMED Laboratories, South San Francisco, CA, USA) to detect IRF3 or IRF7 and used a mouse monoclonal (B-10) or a rabbit polyclonal (D-20) anti-PKR antibody for PKR detection (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). (d) IVIS imaging of livers of various genetic knockout mice 24 h after Sindbis/Fluc transduction. No significant signals were observed in all livers (five animals for each group). NF- κ B-p50 knockout (B6;129P2-Nfkb1^{tm1Bal}/J), TNFR1/2 double knockout (B6;129S-Tnfrsf1a^{tm1Imx}Tnfrsf1b^{tm1Imx3}/J), TLR3 knockout (B6;129S1-Tlr3^{tm1Fto}/J) and their background wild-type mice (Balb/c for IFN- γ KO, B6129PF2/J for NF- κ B-p50 KO and B6129SF2/J for TNFR1/2 KO and TLR3 KO) were obtained from Jackson Laboratory.

endothelial cells, although no noticeable tissue damage and inflammation is observed in liver tissue section after transduction. Liver regeneration is rapid and would argue that this type of protection would not be long-lasting, contrary to what is observed. The second possibility is that initial transduction causes the downregulation of LAMR, or other molecules involved in vector binding and transduction of endothelial cells, and therefore reduces the likelihood of subsequent transduction. This mechanism could directly protect the initial transduced cells from secondary transduction provided that they survive the transduction, or indirectly protect neighboring cells from the second wave of vector challenge via soluble signaling factors. However, our histology data suggest that vascular sinusoidal endothelial cells do not express very high levels of LAMR and therefore it is not likely that the cells are protected from subsequent transduction via downregulation of LAMR, although we cannot rule out the involvement of other accessory molecules involved in vector binding and entry. Again, arguing against this possibility is the long-term nature of subsequent resistance. The third possibility is that the cells protect themselves from subsequent transduction via an IFN-independent antiviral mechanism, whose full activation requires a longer time period than IFN-I/Stat1 signaling. If such mechanism exists, it may fill in the window between immediate innate immunity (the first ~1–2 days) and adaptive immunity (after ~5 days). One such possible mechanism is via IRF3, whose expression levels in liver were not affected by Sindbis transduction (Figure 3). IRF3 has been shown to mediate IFN-independent antiviral responses in cell culture and its activation does not require replication active virus.²³ An IRF3-dependent antiviral mechanism would likely be cell autonomous in the absence of IFN signaling. For this to be the mechanism mediating Sindbis resistance *in vivo*, only cells transduced from the first inoculum would be protected from subsequent challenge. Although we doubt that our initial transduction efficiently targets all cells that could be subsequently permissive, this notion will require further investigation. On the other hand, activation of such mechanism may need help from other immune cell types, such as NK cells or monocyte lineage. More studies are required to elucidate the mechanism behind the acquired resistance.

In the present studies, our imaging data provide evidences, for the first time, suggesting the existence of IFN/STAT1-independent antiviral mechanism(s) in live animals. IFN-I/STAT1 signaling plays an important role in the initial protection of non-sinusoidal endothelial cells in blood vessels of the mouse liver from Sindbis viral vector transduction, but probably plays no role in protection of other normal cells. In addition, the facts that the initial liver transductions are self-limited, and the vector causes no observable morbidity or mortality even after repetitive injection in animals lacking functional IFN/STAT1 signaling, increase the likelihood that replication-defective Sindbis vectors may be able to show therapeutic benefits.

Unlike the IFN responses triggered in some normal cells that protect the host from unwanted toxicity after Sindbis vector treatment, the IFN responses raised in tumor cells would be expected to reduce the vectors' therapeutic efficacy. However, it has been shown that some cancer cells acquire genetic defects during

tumorigenesis to diminish IFN responses,^{24–28} suggesting that IFN signaling pathways may regulate cell growth and mediate apoptosis in addition to providing innate immunity against viral infection. Some viral vector systems, such as one based on vesicular stomatitis virus (VSV), have been genetically engineered for selective replication and lysis in tumor cells that lack functional IFN responses.^{29,30} In the case of replication-defective Sindbis vector, it is still not clear whether IFN responsiveness plays any role in specific tumor targeting. Any innate immunity in transduced tumors may suppress the replication of viral vectors and reduce their therapeutic efficacy against cancer cells. The flip side is that, if cancer cells downregulate IFN-I responses to obtain growth advantage, Sindbis vectors, like VSV vectors, may gain increased tumor targeting specificity. Such specificity would build on the exquisite tumor targeting resulting from differential unoccupied LAMR expression between normal and tumor cells. Normal cells would be expected to retain their IFN responsiveness and resist viral replication, whereas tumor cells, defective in IFN-mediated responses, would more readily support viral replication.

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References

- 1 Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P *et al*. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 2003; **302**: 415–419.
- 2 Tseng JC, Hurtado A, Yee H, Levin B, Boivin C, Benet M *et al*. Using sindbis viral vectors for specific detection and suppression of advanced ovarian cancer in animal models. *Cancer Res* 2004; **64**: 6684–6692.
- 3 Tseng JC, Zanzonico PB, Levin B, Finn R, Larson SM, Meruelo D. Tumor-specific *in vivo* transfection with HSV-1 thymidine kinase gene using a Sindbis viral vector as a basis for prodrug ganciclovir activation and PET. *J Nucl Med* 2006; **47**: 1136–1143.
- 4 Kurkela S, Manni T, Myllynen J, Vaheri A, Vapalahti O. Clinical and laboratory manifestations of Sindbis virus infection: prospective study, Finland, 2002–2003. *J Infect Dis* 2005; **191**: 1820–1829.
- 5 Strauss JH, Wang KS, Schmaljohn AL, Kuhn RJ, Strauss EG. Host-cell receptors for Sindbis virus. *Arch Virol Suppl* 1994; **9**: 473–484.
- 6 Wang KS, Kuhn RJ, Strauss EG, Ou S, Strauss JH. High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. *J Virol* 1992; **66**: 4992–5001.
- 7 Menard S, Tagliabue E, Colnaghi MI. The 67 kDa laminin receptor as a prognostic factor in human cancer. *Breast Cancer Res Treat* 1998; **52**: 137–145.
- 8 Aznavoorian S, Murphy AN, Stetler-Stevenson WG, Liotta LA. Molecular aspects of tumor cell invasion and metastasis. *Cancer* 1993; **71**: 1368–1383.

- 9 Liotta LA. Tumor invasion and metastases – role of the extracellular matrix: Rhoads Memorial Award lecture. *Cancer Res* 1986; **46**: 1–7.
- 10 Tseng JC, Levin B, Hirano T, Yee H, Pampeno C, Meruelo D. *In vivo* antitumor activity of Sindbis viral vectors. *J Natl Cancer Inst* 2002; **94**: 1790–1802.
- 11 Tseng JC, Levin B, Hurtado A, Yee H, Perez de Castro I, Jimenez M *et al*. Systemic tumor targeting and killing by Sindbis viral vectors. *Nat Biotechnol* 2004; **22**: 70–77.
- 12 Jan JT, Griffin DE. Induction of apoptosis by Sindbis virus occurs at cell entry and does not require virus replication. *J Virol* 1999; **73**: 10296–10302.
- 13 Levine B, Huang Q, Isaacs JT, Reed JC, Griffin DE, Hardwick JM. Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene. *Nature* 1993; **361**: 739–742.
- 14 Bredenbeek PJ, Frolov I, Rice CM, Schlesinger S. Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. *J Virol* 1993; **67**: 6439–6446.
- 15 Levy DE, Garcia-Sastre A. The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. *Cytokine Growth Factor Rev* 2001; **12**: 143–156.
- 16 Ryman KD, Klimstra WB, Nguyen KB, Biron CA, Johnston RE. Alpha/beta interferon protects adult mice from fatal Sindbis virus infection and is an important determinant of cell and tissue tropism. *J Virol* 2000; **74**: 3366–3378.
- 17 Shresta S, Sharar KL, Prigozhin DM, Snider HM, Beatty PR, Harris E. Critical roles for both STAT1-dependent and STAT1-independent pathways in the control of primary dengue virus infection in mice. *J Immunol* 2005; **175**: 3946–3954.
- 18 Byrnes AP, Griffin DE. Binding of Sindbis virus to cell surface heparan sulfate. *J Virol* 1998; **72**: 7349–7356.
- 19 Klimstra WB, Ryman KD, Johnston RE. Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. *J Virol* 1998; **72**: 7357–7366.
- 20 Prakash A, Smith E, Lee CK, Levy DE. Tissue-specific positive feedback requirements for production of type I interferon following virus infection. *J Biol Chem* 2005; **280**: 18651–18657.
- 21 Gorchakov R, Frolova E, Williams BR, Rice CM, Frolov I. PKR-dependent and -independent mechanisms are involved in translational shutoff during Sindbis virus infection. *J Virol* 2004; **78**: 8455–8467.
- 22 Berlanga JJ, Ventoso I, Harding HP, Deng J, Ron D, Sonenberg N *et al*. Antiviral effect of the mammalian translation initiation factor 2alpha kinase GCN2 against RNA viruses. *EMBO J* 2006; **25**: 1730–1740.
- 23 Paladino P, Cummings DT, Noyce RS, Mossman KL. The IFN-independent response to virus particle entry provides a first line of antiviral defense that is independent of TLRs and retinoic acid-inducible gene I. *J Immunol* 2006; **177**: 8008–8016.
- 24 Bello MJ, de Campos JM, Kusak ME, Vaquero J, Sarasa JL, Pestana A *et al*. Molecular analysis of genomic abnormalities in human gliomas. *Cancer Genet Cytogenet* 1994; **73**: 122–129.
- 25 Linge C, Gewert D, Rossmann C, Bishop JA, Crowe JS. Interferon system defects in human malignant melanoma. *Cancer Res* 1995; **55**: 4099–4104.
- 26 Lu R, Au WC, Yeow WS, Hageman N, Pitha PM. Regulation of the promoter activity of interferon regulatory factor-7 gene. Activation by interferon and silencing by hypermethylation. *J Biol Chem* 2000; **275**: 31805–31812.
- 27 Sun WH, Pabon C, Alsayed Y, Huang PP, Jandeska S, Uddin S *et al*. Interferon-alpha resistance in a cutaneous T-cell lymphoma cell line is associated with lack of STAT1 expression. *Blood* 1998; **91**: 570–576.
- 28 Wong LH, Krauer KG, Hatzinisiriou I, Estcourt MJ, Hersey P, Tam ND *et al*. Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3gamma. *J Biol Chem* 1997; **272**: 28779–28785.
- 29 Stojdl DF, Lichty B, Knowles S, Marius R, Atkins H, Sonenberg N *et al*. Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat Med* 2000; **6**: 821–825.
- 30 Stojdl DF, Lichty BD, tenOever BR, Paterson JM, Power AT, Knowles S *et al*. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* 2003; **4**: 263–275.