ORIGINAL ARTICLE

Impact of deletion of envelope-related genes of recombinant Sendai viruses on immune responses following pulmonary gene transfer of neonatal mice

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We demonstrated previously that the additive-type recombinant Sendai virus (rSeV) is highly efficient for use in pulmonary gene transfer; however, rSeV exhibits inflammatory responses. To overcome this problem, we tested newly developed non-transmissible constructs, namely, temperature-sensitive F-deleted vector, rSeV/dF (ts-rSeV/dF) and a rSeV with all the envelope-related genes deleted (rSeV/dFdMΔHN), for pulmonary gene transfer in neonatal mice, by assessing their toxicity and immune responses. The gene expression in the lungs of neonatal ICR mice peaked on day 2, then gradually decreased until almost disappearing at 14 days after infection in all constructs. Loss of body weight and mortality rate, however, were dramatically improved in mice treated with SeV/dFdMΔHN (mortality = 0%, n = 41) and

ts-rSeV/dF (24.2%, n = 33) compared with additive rSeV (70.7%, n = 58). Although the deletion of envelope-related genes of SeV had a small impact on the production of antibody and cytotoxic T-lymphocyte activity in both adults and neonates, a dramatic reduction was found in the events related to innate responses, including the production of proinflammatory cytokines, particularly in the case of neonates. These results indicate that pulmonary gene transfer using SeV/dFdMΔHN warrants further investigation for its possible use in developing safer therapeutics for neonatal lung diseases, including cystic fibrosis. Gene Therapy (2007) 14, 1017–1028. doi:10.1038/sj.gt.3302955; published online 26 April 2007

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Introduction

Although the lungs are an attractive target for gene therapy due to the large number of patients suffering from intractable pulmonary diseases, including cystic fibrosis (CF),^{1–3} it is hazardous to deliver exogenous genes to this organ.^{1,2,4} In particular, the airway epithelia in the upper airways have evolved multiple barriers to prevent penetration of foreign materials and infectious organisms from the lumen into apical cells, including (1) a mucus layer that traps and washes them up via mucus clearance, (2) a glycocalyx that binds to these materials and inhibits their binding to apical surface receptors and (3) an apical membrane that largely lacks cellular receptors for viral entry.^{4,5} As a result, clinical and laboratory studies in the last decade have revealed that

the efficiency of gene transfer using currently available vectors, including both viral and non-viral vectors, is too low to show clinical benefits or functional corrections of the phenotype.^{6–9}

As a possible candidate for overcoming these serious drawbacks, we have focused on a newly developed powerful gene transfer vector, the recombinant Sendai virus (rSeV) and recently demonstrated that a replication-competent prototype vector, additive rSeV, showed dramatically high gene transfer efficiency in the airway epithelia of mice, ferrets and humans: 3–5 log higher efficiency over cationic lipids or adenoviruses.^{10,11} SeV, a member of the family *Paramyxoviridae*, has a non-segmented negative-strand RNA genome and makes use of sialic acid residues on surface glycoproteins or asialoglycoproteins present as a receptor, leading to a broad spectrum of gene transfer.^{12,13} SeV can mediate gene transfer and expression to a cytoplasmic location using cellular tubulin,¹⁴ avoiding possible malignant transformation due to a genetic alteration of host cells, which is a safety advantage of rSeV. Despite these advantages of rSeV over other systems for use in clinical gene therapy, the immune responses caused by virus administration *in vivo* continue to present a hazard, making it difficult to expand the utility of this mode of vector in a clinical setting.

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As the first generation of clinically available rSeV, we have developed a gene–gene encoding fusion protein (F)-deleted rSeV (rSeV/dF) showing non-transmissible properties.¹⁵ Because this type of rSeV still exhibits powerful gene-transfer and -expression efficiencies in skeletal muscles *in vivo*, it has been employed since 1 February 2006 in an ongoing clinical study of therapeutic angiogenesis at Kyushu University Hospital that was approved by Institutional and Governmental Review Boards. An interesting study in another laboratory has demonstrated that rSeV/dF shows lower toxicity than additive-type rSeV in mouse fetuses;¹⁶ in our laboratory, however, the host immune responses evoked by rSeV/dF via pulmonary gene transfer were almost identical to those of the additive prototype vector in both neonates and adults (unpublished data), suggesting that the first generation is still not appropriate for use in the lungs in a clinical setting. Importantly, pulmonary gene transfer to a fetus and/or newborn is likely to be ideal in the case of CF, because the lung pathology would be rather mild in comparison with that seen in adults, even though the question of whether the CF lung is characterized by developmental abnormalities is currently under debate.¹⁷

To overcome these limitations, we recently produced two new types of rSeV/dF (Figure 1); namely, a temperature-sensitive mutant rSeV/dF (ts-rSeV/dF)^{18,19} and an rSeV lacking all three envelope-related genes, F, M and HN (rSeV/dFdM dHN).²⁰ Since a reduction in the number of vector-related proteins may alter host-immune responses, as suggested in our recent study,²⁰ we here examined the efficacy of these two vector constructs for pulmonary gene transfer. We assessed

innate- (natural killer cell (NK cell) activity and proinflammatory cytokine production) and acquired- (cytotoxic T-lymphocytes (CTL) activity and antibody production) immunity-related events following pulmonary gene transfer in neonatal and young adult mice.

Results

Reduced toxicity of newly developed rSeV vectors to neonatal mice

Ten microliters of PBS solution containing 2.5×10^6 (cell infectious units (CIU)) of either wild SeV (Z-strain) (Figure 2b), additive rSeV expressing enhanced green fluorescent protein (rSeV-EGFP; Figure 2c), ts-rSeV/dF-EGFP (Figure 2d), or rSeV/dFdM dHN-EGFP (Figure 2e) was intranasally inoculated into newborn ICR mice on day 0, and their body weights (BW) were measured daily. The survival curves of these mice are also shown in Figure 2f.

A high percentage of neonatal mice inoculated with wild SeV (47/55 animals = 85.5%) or additive rSeV-EGFP (41/58 animals = 70.7%) were dead within 14 days after viral load. This toxicity was significantly reduced when using the same amount of ts-rSeV/dF-EGFP (8/33 animals = 24.2%, $P < 0.001$ vs other groups), the finding that this toxicity was significantly reduced when using the same amount of rSeV/dFdM dHN-EGFP (0/41 animals = 0%, $P < 0.001$ vs other groups). None of the young adult (8-week-old) mice died when inoculated with $100 \mu\text{l}$ of vector solution containing 2.5×10^7 CIU of each vector (data not shown).

Figure 1 Schematic representation of the structures of rSeV vectors used in this study. All recombinant viruses were based on the Z-strain of SeV, which encodes six genes (M, F and HN for envelope-related proteins and NP, P/V/C and L for negative-stranded genomic ribonucleotide-protein complex: RNP). Additive-type rSeV was simply inserted with an exogenous gene between the leader sequence and the open reading frame of the NP gene.^{10–12} The two bottom panels demonstrate the structures of the newly developed rSeVs (ts-rSeV/dF and rSeV/dFdM dHN). ts-rSeV/dF loses the expression of rested envelope-related genes (M and HN), and has some substitution of ribonucleotide sequences on the M, HN, and L genes, as indicated by arrowheads.¹⁸ rSeV/dFdM dHN is the further advanced design without any envelope-related genes in the vector genome.^{19,20} These rSeVs were titrated by assessing CIU (cell infectivity units).^{18–20}

Figure 2 (a), Vector-related toxicity in neonatal mice assessed by body weight gain (BW) (a–e) and survival (f). (a–e) Ten microlitres of PBS solution containing 2.5×10^6 CIU of either wild SeV (Z-strain) (b), additive rSeV expressing enhanced green fluorescent protein (rSeV-EGFP: c), ts-rSeV/dF-EGFP (d), or rSeV/dFdMdhN-EGFP (e) was intranasally inoculated to newborn ICR mice on day 0, and body weight (BW) was measured daily. The blue line indicates the time course of each live animal, and the red line that of each dead animal. (f) The survival curves of these mice are also shown. The curves were analyzed using Kaplan–Mayer’s method. The statistical significance of the survival was determined using the log–rank test.

These findings clearly indicate a significant reduction in the pulmonary toxicity of rSeVs in neonatal mice by deletion of envelope-related genes.

Deletion of envelope-related genes resulted in the delayed elimination of rSeV vectors in the lungs of neonatal as well as adult mice

Next, we assessed the persistence of each vector in the lungs by monitoring the *firefly* luciferase expression. The data are expressed as the standardized percentage of relative light units (RLU) on day 2.

As shown in Figure 3a, luciferase expression in the lungs on day 4 after additive vector inoculation had already declined by approximately 50%; in contrast, that following administration of either ts-rSeV/dF-luciferase or rSeV/dFdMHN-luciferase showed no significant decrease. On day 7, however, luciferase expression by all these vectors markedly declined, and had almost disappeared 14 days after inoculation. These results suggest that the deletion of envelope-related genes of rSeVs might contribute to the early elimination of vectors; it did not, however, result in persistent infection in young adult mice *in vivo*.

In contrast, in the case of neonatal mice, there was no significant decrease in luciferase expression in the lungs on day 4 in any of the vectors tested (Figure 3b). Even though a significant amount of luciferase expression was detected on day 7, unlike in young adult mice, no apparent transgene expression was observed in neonatal mice on day 14 and later.

These results indicate that deletion of envelope-related genes contributes to the delayed elimination of vectors

in vivo, an effect that might be more pronounced in the case of rSeV/dFdMHN. Furthermore, comparison of the data between adults and neonates suggested that the early response related to the elimination of rSeV vectors was significantly impaired in neonatal mice.

Lack of NK cell activity in neonatal mice

To determine the immune mechanisms related to vector elimination, we first assessed the natural killer (NK) cell activity after vector inoculation. We used wild SeV instead of additive rSeV according to Japanese law, because the Cartagena Protocol of Biosafety did not allow us to use the replication-competent recombinant virus at that time (the Law was ratified on first February, 2005).

Two days after vector inoculation or poly I:C administration, the splenocytes were isolated and used for NK cell activity assay. In the case of adult mice, mild and significant upregulation of NK cell activity was found in each of the vector-treatment groups (Figure 4a). In contrast, significant NK cell activity was not observed in both wild SeV and rSeVs (ts-rSeV/dF-luciferase and rSeV/dFdMHN) in the case of neonatal mice (Figure 4b). An additional experiment assessing NK cell activity by poly I:C in adult mice showed a positive result, but a negative result was seen in neonates (Figure 4c), indicating the lack of NK cell response in neonatal mice. The loss of NK cell responses have also been demonstrated in the case of other organisms, including *Candida albicans*, *Escherichia coli*, *Listeria monocytogenes*, etc.²¹

Figure 3 Monitoring of the persistence of each rSeV in the lungs of young adult (a: 8-week-old) or neonatal (b: day 0 after born) mice. Each rSeV expressing *firefly* luciferase was intranasally inoculated (adult: 100 μ l PBS with 2.5×10^7 CIU/head and newborn: 10 μ l PBS with 2.5×10^6 CIU). At each time point, the lungs were excised and subjected to luciferase activity measurement. The data are expressed as the percentage of RLU standardized by the mean value of each RLU on day 2 after inoculation. Note that a significant reduction of luciferase expression was seen on day 4 in adult mice, while no such decrease was observed in neonatal mice. In both groups, the delay in vector elimination was most pronounced in the use of rSeV/dFdMHN.

Figure 4 Lack of NK cell responses following pulmonary gene transfer of rSeVs in neonatal mice. Two days after vector inoculation or poly I:C administration, the splenocytes were isolated and subjected to NK cell activity. The following experiments were repeated three or more times with similar results. (a) NK cell activity in adult mice. Mice inoculated with each rSeV showed a moderate increase in NK cell activity; however, no significant difference among vector types was found. (b) NK cell activity in neonatal mice. Mice inoculated with each rSeV showed no apparent increase in NK cell activity, except for the positive control adult mice immunized by poly I:C. (c) Confirmation of a lack of NK cell activation by poly I:C. Adults, but not neonates, that were administered poly I:C demonstrated an apparent increase in NK cell activity.

Reduced production of proinflammatory cytokines following newly developed rSeVs in lung homogenates of neonatal mice

To assess the early response against vectors after pulmonary gene transfer, we next examined the time course of expression of typical proinflammatory cytokines in the lung, namely, IL-6, IL-1 β and TNF- α , after vector inoculation.

In the case of adult mice, a mild but significant elevation of IL-6 and TNF- α was observed within 7 days in mice inoculated with wild SeV, but not in those inoculated with the newly developed rSeVs (Figure 5, left). In contrast, marked and sustained elevation of IL-6

(within 7 days) and IL-1 β /TNF- α (within 14 days) were noted in the lung homogenates of surviving neonatal mice who received wild SeV (Figure 5, right).

Next, we focused on the pulmonary expression of typical chemoattractants of neutrophils, that is macrophage-inflammatory protein-2 (MIP-2/CXCL-2/3) and keratinocyte-derived chemokine (KC/CXCL1)^{22,23} because we previously identified that persistent infiltration of neutrophils into the peribronchial area and lung parenchyma was a representative pathology after pulmonary inoculation of additive-type rSeV vector.¹⁰

We first assessed the protein expression level of these chemokines in lung homogenates of adult mice by enzyme-linked immunosorbent assay system (ELISA)

Figure 5 Time course of the proinflammatory cytokine content in the lungs following pulmonary gene transfer of rSeVs. Each rSeV expressing *firefly* luciferase was intranasally inoculated (adult: 100 μ l PBS with 2.5×10^7 CIU/head and newborn: 10 μ l PBS with 2.5×10^6 CIU). At each time point, the lungs were excised and subjected to specific ELISA for mIL-6, mIL-1 β , and mTNF- α . The data were expressed as pg/g. Note the marked and sustained upregulation of these cytokines in the lungs of neonatal mice that were treated with wild SeV, while there was a mild and transient increase in mIL-6 and mTNF- α in adult mice. In neither was a significant increase in these cytokines detected with the use of ts-rSeV/dF-luciferase or rSeV/dFdMdhN-luciferase.

on day 2 and 7 after vector inoculation. As shown in Figure 6a, an apparent increase of MIP-2/CXCL-2/3 (Figure 6a, left), but not of KC/CXCL-1 (Figure 6a, right), was observed in the lungs of wild SeV-inoculated adult mice on day 2 under the experimental conditions of the current study; the expression returned to baseline on day 7. Interestingly, another set of experiments demonstrated that the number of neutrophils in BAL from mice treated with wild SeV did not decline (mean = 20 918 cells on day 2 and mean = 21 111 cells on day 7; $n = 6$, respectively), probably owing to the time lag between the

expression of that chemoattractant and neutrophil recruitment. None of the animals died during these experiments.

Next, we assessed the expression of these chemokines, but not in bronchoalveolar lavage (BAL), in lungs of neonatal mice after inoculation of various viruses, because the neonatal mice were too small for BAL sampling. As shown in Figure 6b, a dramatic enhancement of both MIP-2/CXCL-2/3 (Figure 6b, left) and KC/CXCL-1 (Figure 6b, right) expression was seen in mice inoculated with wild SeV. In contrast, no apparent

Figure 6 Expression of chemotactic factors for neutrophil recruitment, MIP-2/CXCL-2/3 and KC/CXCL-1 in lungs of young adult (a) or neonatal (b) mice inoculated with wild SeV or envelop-related gene-deleted rSeVs. On 2 or 7 days after virus inoculation, the lungs were homogenized and subjected to specific ELISA. * $P < 0.01$. (a) MIP-2/CXCL-2/3 and KC/CXCL-1 expression in lungs of adult mice. (b) MIP-2/CXCL-2/3 and KC/CXCL-1 expression in lungs of neonatal mice.

induction of these chemokines was seen in envelope-related gene-deficient vectors, implying that there was a reduction in toxicity due to excess accumulation of neutrophils.

Together with the data presented in Figures 2–6, these findings strongly suggested that (1) in the lungs of young adult mice, inoculated replication-competent rSeVs (wild SeV and additive rSeV) begin to be attacked by NK cells, resulting in their prompt clearance; (2) in contrast, in neonatal mice there is persistent virus replication following the loading of replication-competent rSeVs, resulting in a sustained upregulation of proinflammatory cytokines, often leading to death due to pulmonary inflammation, which in turn probably involves the prominent accumulation of neutrophils; and (3) deletion of envelope-related genes of rSeVs (ts-rSeV/dF and rSeV/dFdmHN) markedly attenuates the severity of such early responses, probably due to the delayed elimination of vectors in the lung.

CTL activity against rSeVs was not impaired in neonatal mice

To assess acquired immune responses to rSeV, we first examined CTL activities against rSeV using splenocytes 2 weeks after inoculation. We used C57BL/6J strain mice (H-2^b) for assessing CTL activity, since it is well known that the target amino-acid residue of CTL of this strain is NP321–336 of SeV.

We expected that the CTL response against SeVs in neonatal mice might be impaired, since a number of studies have indicated that immature phenotypes of Th1-cytokine production and naïve/killer T-cells function in both human and murine neonates.²⁴ Contrary to our expectations, however, repeated (more than five) experiments consistently demonstrated strong CTL activity in neonatal mice, irrespective of envelope-related gene depletion, compared to that seen in young adult mice (Figure 7a). This result may have been due to the

Figure 7 Induction of acquired immunity assessed by CTL activity (a) and anti-SeV antibody (b) following pulmonary administration of rSeVs. (a) CTL activities against rSeV in adults (left graph) and neonates (right graph) using splenocytes 2 weeks after vector inoculation. No significant difference could be found irrespective of the types of vectors in both animals. Note the higher levels of CTL activity in neonatal mice than those seen in adult mice. These experiments were repeated three or more times with similar results. (b) Serum levels of anti-SeV antibody 4 weeks after inoculation using commercially available ELISA. No vector-type-related difference in anti-SeV antibody was found in adult mice (left graph); in contrast, a mild but significant decrease in antibody production was observed in neonatal mice inoculated with rSeV/dFdMdhN-luciferase (right graph). The data represent a total of two independent experiments.

elimination of all these SeVs after 14 days inoculation, as suggested in Figure 3.

Production of anti-SeV antibody against rSeVs was slightly decreased in neonatal mice

Finally, we measured the serum levels of anti-SeV antibody 4 weeks after inoculation using a commercially available ELISA.

As shown in Figure 7b (left graph), deletion of the envelope-related genes of rSeV did not contribute to a

reduction in the serum levels of anti-SeV antibody in adult mice, suggesting that primary infection, but not virus replication, is important for producing this antibody. In contrast, the use of envelope-related gene-deleted vectors resulted in a slight decrease in the serum anti-SeV antibody levels in neonatal mice, and the decrease was most pronounced when rSeV/dFdMdhN was used (Figure 7b, right graph).

These findings suggest that the deletion of envelope-related genes of rSeV had a limited impact (neonates) or

no impact (adults) on the acquired immune responses after pulmonary gene transfer.

Discussion

In light of the recent development of highly efficient techniques for gene transfer to the airway epithelium, rSeV could have great potential for the treatment of CF and other intractable lung diseases.^{10,11} However, the clinical utility of rSeV is limited owing to the immune responses induced by such treatment.

Using two newly developed envelope-related gene-deleted rSeVs, ts-rSeV/dF and rSeV/dFdMdHN, we examined the details of the toxicity and related immune responses following pulmonary gene transfer, directly comparing young adult and neonatal mice. Key observations obtained in this study were as follows: (1) the use of each of these new vectors resulted in a reduction of toxicity compared to that by the additive-type rSeV, and this effect was especially pronounced in the case of rSeV/dFdMdHN; (2) a delay in vector elimination in the lungs was observed around 4–7 days after gene transfer in both adults and neonates; however, transgene expression almost disappeared 14 days later in all vector types tested; (3) elimination of replication-competent virus was seriously impaired in neonatal mice, in association with a complete lack of NK cell activity, probably contributing to the sustained elevation of proinflammatory cytokines and toxicity; and (4) parameters indicating acquired immunity, namely CTL response and antibody production, were not seriously impaired in neonatal mice. These results are the first clear demonstration of the impact of deletion of envelope-related genes of rSeVs on immune responses following pulmonary gene transfer; in other words, these envelope proteins of SeV are important in evoking an early defense response of NK cell activity, but not acquired immunity.

We found almost no response of NK cells either to poly I:C, a well-known NK stimulator, or to rSeV in neonatal mice (Figure 4). Although similar findings regarding human neonatal/perinatal cells against human viruses and bacterias have been reported,^{21,25} this is to our knowledge the first report indicating a defect of NK responses against replication-competent SeVs in neonatal mice. In addition, these results suggest that the lack of NK cell response against viruses may be ubiquitous in neonates of any mammalian species. These findings, together with the findings of the present study, which included death with sustained elevation of proinflammatory cytokines and persistence of virus-derived luciferase expression, strongly suggest that NK cells might play a major role in the virus elimination after SeV infection in neonatal mice. This possibility is well supported by a study, in which induction of NK cells conferred protection against wild-type SeV infection following inoculation with anti-CD3 antibody. A conflict, however, seems to be present in the case of adult mice; the luciferase activity expressed by the two non-transmissible vectors was not decreased on day 4, in clear contrast to that seen in use of additive rSeV (Figure 3), even though mice inoculated with these vectors showed similar levels of NK activity (Figure 4). A possible explanation is that NK cells activated by rSeVs could eliminate secondary viral particles but not vector-

infected cells; this hypothesis may be supported by data showing that F-defective rSeV (rSeV), a first-generation rSeV producing secondary particles without infectious activity,¹⁵ has an expression pattern similar to that of additive rSeV (unpublished data). However, further studies will be needed before drawing a definitive conclusion.

At the initial stage of the current study, prolonged transgene expression in the use of ts-rSeV/dF and/or rSeV/dFdMdHN was expected, particularly in the case of neonates, because several studies have indicated the lower proliferative response of CTLs against viruses.^{25,26} The results obtained in this study, however, demonstrated neither apparent prolongation of luciferase expression (Figure 3) nor a significant reduction in CTL activities (Figure 7a), suggesting that envelope-related components of SeV may not play a major role in the induction of acquired immunity in either adults or neonates. When taken together with the present finding that there was only a slight reduction in antibody production against these new vectors in neonatal mice (Figure 7b), it is clear that there are still practical problems to be overcome before instituting clinical trials for CF. Some studies have identified the protein residue in NP of SeV as a major target of CTL,²⁷ and modification of this protein residue may be an option for reducing the CTL response against rSeV.

In turn, an apparent advance of the current study is that a dramatic reduction in neonatal toxicity was evident following pulmonary administration of vectors from which the envelope-related genes had been deleted, associated with a markedly reduced induction of proinflammatory cytokines. This finding suggests the elimination of a possible risk of systemic inflammatory response syndrome (SIRS), which is widely known as a potentially adverse effect induced by adenoviral gene transfer in a clinical setting.²⁸ Further assessments including those utilizing non-human primates should be carried out to confirm that these findings are applicable to other animal species.

One unexpected result of this study was that we could find no significant difference in immunological parameters, including proinflammatory cytokine levels in the lung, between ts-rSeV/dF and rSeV/dFdMdHN. Since the use of rSeV/dFdMdHN resulted in a better prognosis than the use of ts-rSeV/dF, these results may suggest that neither a cytokine storm nor immune responses against the vectors were likely to have caused these discrepant results. We repeatedly confirmed the vector titers, and the final products were also highly purified via chromatography; therefore, these are not likely to have been causes of the difference in animal survival. One possible explanation may be the immune responses against the reporter (EGFP), as flow cytometry analyses showed a moderately lesser fluorescent intensity of cells treated with rSeV/dFdMdHN than that seen with ts-rSeV/dF (unpublished data). However, this result is not definitive because the absolute value of luciferase expression via rSeV/dFdMdHN was mildly higher than that seen in the use of ts-rSeV/dF (Figure 3). We must determine, therefore, whether there are more sensitive parameters to predict the vector-related toxicity in future studies.

In summary, we have developed new types of non-transmissible rSeVs, ts-rSeV/dF and rSeV/dFdMdHN, that show markedly reduced toxicity even to neonatal

animals, a finding that was more pronounced in the use of rSeV/dFdMdHN. Therefore, these new constructs warrant further investigation from the perspective of clinical gene therapy to treat intractable pulmonary diseases, including CF.

Materials and methods

Animals

Female 8-week-old ICR (CD-1) pregnant/non-pregnant mice of Charles River grade were obtained from KBT Orientals Co., Ltd. (Tosu, Saga, Japan) and kept under specific pathogen-free and humane conditions. These mice were used for all experiments except the CTL assay. For CTL analysis, female 8-week-old pregnant/non-pregnant C57BL/6J mice were purchased from the same suppliers. We here used the C57BL/6J strain for assessing CTL activity. All animal experiments were carried out according to the protocols approved by the Institutional Committee for Animal Experiments and by the Institutional Committee for Recombinant DNA and Infectious Pathogen Experiments, Kyushu University. These experiments were carried out in accordance with recommendations for the proper care and use of laboratory animals and according to The Law (No. 105) and Notification (No. 6) of the Japanese Government. We used wild SeV instead of additive rSeV, according to Japanese law, because the Cartagena protocol has not allowed the use of replication-competent recombinant virus since 1 February, 2005. In pilot studies, mice inoculated with these viruses consistently showed similar immune responses (data not shown).

Wild and recombinant Sendai viruses

Wild SeV. The Z-strain of wild SeV was a kind gift from Professor Yasufumi Kaneda (Osaka University, Osaka, Japan). The seed virus was amplified by injection into the chorioallantoic cavity of 10-day-old embryonated chicken eggs, as described previously.^{29,30}

Additive rSeV. Additive-type rSeVs were constructed as described previously.^{10,11} In brief, the entire cDNA encoding *jellyfish* enhanced green fluorescent protein (for additive rSeV-GFP) and luciferase (for additive rSeV-luciferase) were inserted into the *NotI* site of the cloned genome. Template SeV genomes with an exogenous gene and plasmids encoding the N, P and L proteins (plasmids pGEM-N, pGEM-P and pGEM-L, respectively) were conjugated with commercially available cationic lipids, then cotransfected with UV-inactivated vaccinia virus vT7-3 into LLMCK2 cells. Forty hours later, the cells were disrupted by three cycles of freezing and thawing and injected into the chorioallantoic cavity of 10-day-old embryonated chicken eggs. Subsequently, the virus was recovered and the vaccinia virus was eliminated by a second propagation in eggs.

Additive rSeVs were used only in the early stage of the study (Figures 2 and 3), because Japanese law according to the Cartagena Protocol of Biosafety did not allow us to use the replication-competent recombinant virus at that time (the Law was ratified on 1 February, 2005).

ts-rSeV/dF and rSeV/dFdMdHN. Insertion of temperature-sensitive mutations into the genome template

of rSeV and the recovery procedures of F-deficient SeV vectors from cDNAs were described previously.^{18–20} Briefly, approximately 10^7 LLC-MK2 cells were transfected with template pSeV and pCAG-plasmids each carrying the NP, P, M, F, HN, or L gene.²⁰ The cells were maintained in MEM containing trypsin, and 24 h later, they were overlaid with LLC-MK2/F7/M#33/HN7 cells after induction of M, F and HN proteins by AxCANCre infection at an MOI of five and cultured for another 48 h. The cell lysate was infected into new LLC-MK2/F7/M#33/HN7 cells after AxCANCre infection. These cells were then cultured at 32°C in MEM containing trypsin for 10 to 20 days. The viral vectors were further amplified by several rounds of propagation.

The titers of recovered viral vectors were expressed as CIU.¹⁵

Vector inoculation

The vector intranasal inoculation was carried out as described previously.¹⁰ Briefly, phosphate-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄ and 1 mM KH₂PO₄, pH 7.2) or each vector solution (2.5×10^6 CIU in 10 μ l PBS for neonates or 2.5×10^7 CIU in 100 μ l PBS for adults) was placed into the nasal cavity, and the solution was sniffed into the lungs of mice.

Luciferase assay

The excised lung tissue was minced in 500 μ l of lysis buffer (Promega, Madison, WI, USA) with 2.5 μ l of protease inhibitor cocktail (31.34 mg benzamidine, 3.484 g phenylmethylsulfonyl fluoride, 20 mg 1,10-phenanthroline chloride monohydrate in 200 ml ethanol) and centrifuged, after which 20 μ l of the supernatant was subjected to luciferase assay, as described previously.^{10,11} Light intensity was measured after 10 s of preincubation at room temperature using a luminometer (Model LB9507; EG&G Berthold, Bad Wildbad, Germany) with 10 s integration. The data are expressed as the percentage of RLU per lung standardized by the mean value of each RLU on day 2 after inoculation, and each sample was measured more than twice.

NK cell activity

On day 2 after SeV inoculation or poly I:C administration, splenocytes were obtained and contaminated erythrocytes were depleted by 0.83% ammonium chloride. The splenocytes were directly used as NK effector cells. Target cells (YAC-1; obtained from the American Type Culture Center, Manassas, VA, USA) were labeled with 100 μ Ci of Na₂ ⁵¹CrO₄ for 1.5 h, and the Cr release assay was performed as described previously.³¹ The percentage of specific ⁵¹Cr release or triplicates was calculated as follows: ((experimental counts per minute (c.p.m.) – spontaneous c.p.m.) / (maximum c.p.m. – spontaneous c.p.m.)) $\times$ 100. Spontaneous release was always <10% of maximal Cr release (target cells in 1% triton-X).

Measurement of protein expression of proinflammatory cytokines and neutrophil-chemoattractant chemokines in lungs

The lungs were harvested and minced in lysis buffer with protease inhibitor cocktail. The supernatant was subjected to measure the concentrations of murine IL-6,

IL-1 β , TNF- α and neutrophil-chemoattractant chemokines (MIP-2 and KC) with a quantitative sandwich enzyme immunosorbent assay kit (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions.

FACS analyses for cell components in bronchoalveolar lavage (BAL)

Two or 7 days after virus inoculation, BAL was obtained by washing with 1.5 ml of PBS through the intratracheally inserted 24 G catheter. Cells were collected by centrifugation, and subjected to FACS analysis to determine the percentage of each cell component using labeled antibodies (the following monoclonal antibodies were from Pharmingen: for T-cells, CD3-APC; for B-cells, CD19-FITC; for NK cells, CD49b/pan-NK-PE; and for granulocytes, Gr-1-PE and CD11b-FITC; Pharmingen, San Diego, CA, USA).

CTL activity

Two weeks after vector inoculation, splenocytes were obtained, and contaminated erythrocytes were depleted by 0.83% ammonium chloride. For the CTL assay, 4×10^6 splenocytes were cultured with 1 μ M of SeV-peptide (H-2^b-restricted SeV NP fragment 321–336; Sigma-Aldrich, St Louis, MO, USA) for NP protein of SeV.³² Two days later, 30 IU/ml of recombinant IL-2 was added to the medium. After 5 days, the cultured cells were collected and used as CTL effector cells. Target cells were labeled with 100 μ Ci of Na₂ ⁵¹CrO₄ for 1.5 h, and Cr release assay was performed as described previously.^{27,31} The percentage of specific ⁵¹Cr release of triplicates was calculated as follows: ((experimental c.p.m. – spontaneous c.p.m.)/(maximum c.p.m. – spontaneous c.p.m.)) $\times$ 100. Spontaneous release was always <10% of maximal Cr release (target cells in 1% Triton-X).

Measurement of serum levels of anti-SeV antibody

Four weeks after vector inoculation, serum samples were obtained and analyzed to determine the concentrations of anti-SeV antibody using a quantitative ELISA (Monilizer for HIVJ, Wakamoto, Tokyo, Japan) according to the manufacturer's instructions. The optical density at 490 nm (OD₄₉₀) was measured and used to determine the level of anti-SeV antibody. These values were determined using a 1:40 dilution of serum and were measured simultaneously.

Statistical analysis

All data are expressed as the mean $\pm$ s.e.m., and were analyzed by one-way ANOVA with Fisher's adjustment, with the exception of the data on animal survival. Survival was plotted using Kaplan–Meier curves, and statistical relevance was determined using a log-rank comparison. $P < 0.05$ was considered significant.

Competing interest statement

Dr Yonemitsu is a member of the Scientific Advisory Board of DNAVEC Corporation.

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