Assessment of F/HN-pseudotyped lentivirus as a clinically relevant vector for lung gene therapy

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At a glance commentary:

Scientific Knowledge on the Subject

Gene transfer to the airway epithelium is more difficult than originally anticipated. Until now viral gene transfer agents have not been useful for the treatment of chronic lung disease such cystic fibrosis (CF), due to immunogenicity which prevent successful repeat administration. Lentivirus-based vectors are a notable exception.

What This Study Adds to the Field

Moving novel therapies to the clinic requires that relevant evidence for both safety and efficacy is gathered in appropriate models. Here, we begin to place this vector onto a translational pathway to the clinic and provide a body of supportive evidence for F/HN-pseudotyped SIV as a potential gene transfer agent for CF including a) life-time gene expression and efficient repeat administration in mouse lung, b) lack of chronic toxicity, c) persistent gene expression in human *ex vivo* models.

Running Title: F/HN pseudotyped lentivirus for airway gene transfer

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Abstract

Rational: Our ongoing efforts to improve pulmonary gene transfer thereby enabling gene therapy for the treatment of lung diseases such as cystic fibrosis (CF) has led to the assessment of a lentiviral vector (SIV) pseudotyped with the Sendai virus envelope proteins F and HN. **Objectives**: Here, we begin to place this vector onto a translational pathway to the clinic, by addressing some key milestones that have to be achieved. **Main results:** These include: (1) a single dose produces lung expression for the life-time of the mouse (approximately 2 years), (2) only brief contact time is needed to achieve transduction, (3) repeated daily administration leads to a dose-related increase in gene expression, (4) repeated monthly administration to mouse lower airways is feasible without loss of gene expression, (5) there is no evidence of chronic toxicity during a 2 year study period, (6) F/HN-SIV transduction generates persistent gene expression in human differentiated airway cultures, and human lung slices and transduces freshly obtained primary human airway epithelial cells. **Conclusions:** The data support F/HN-pseudotyped SIV as a promising vector for pulmonary gene therapy for a number of diseases including CF and we are now undertaking the necessary refinements to progress this vector into clinical trials.

Abstract word count: 201

Keywords: lentivirus, cystic fibrosis, gene therapy, lung, gene transfer

Introduction

Gene transfer to the airway epithelium has been more difficult than originally anticipated, largely because of significant extra- and intracellular barriers in the lung [1]. In general, viral vectors are more adapted to overcoming these barriers than non-viral gene transfer agents. Viral vectors that have a natural tropism for the airway epithelium such as those derived from adenovirus (Ad), adeno-associated virus (AAV) and Sendai virus (SeV), have been evaluated for cystic fibrosis (CF) gene therapy. SeV vector, in particular, leads to log orders higher gene expression than non-viral formulation when applied to the apical surface of airway epithelial cells [2;3]. However, gene expression is transient and repeated administration is inefficient [4;5]. These vectors are, therefore, unlikely to solve the challenge of life-long gene therapy treatment for CF.

Lentiviral vectors are commonly pseudotyped with the G-glycoprotein from the vesicular stomatitis virus (VSV-G) allowing for a broad tissue tropism. However, VSV-G-pseudotyped vectors are comparatively inefficient at transducing airway epithelial cells and require the addition of tight junction openers such as lyso-phosphatidylcholine (LPC) to allow virus entry into airway cells.[6;7] Several groups have attempted to further improve lentiviral vector uptake into airway epithelium by changing the viral envelope proteins. Glycoproteins from Ebola or Marburg virus that naturally transfect airway epithelial cells via the apical membrane showed early promise, [8] but have more recently been superseded by viral vectors pseudotyped with the influenza M2 envelope glycoprotein, [9] baculovirus protein GP64 [10] or the Sendai virus-derived F and HN envelope proteins. [11;12]

The F/HN-pseudotyped simian immunodeficiency viral vector (F/HN-SIV) transduces rodent airway epithelial cells *in vitro.* [12] Recently, we have shown that F/HN-SIV leads to

persistent expression in the mouse nose (more than 1 year) importantly allowing for monthly repeat administration without significant loss of efficacy [11]. It is currently unclear, whether the prolonged expression is due to vector integration into pulmonary stem or progenitor cells, or due to the long life-span of airway epithelial cells, which as recently reported, may have a half-life of up to 17 months [13]. It is also unclear how upon repeated administration the viral vector evades the immune system, although interestingly liposome which contain Sendai virus proteins (HVJ-liposome) can also be repeatedly administered [14]. This feature, as well as the efficient and prolonged expression profile and the ability to administer through the apical surface of the respiratory epithelium without pre-conditioning, makes the vector an attractive candidate for treating CF which is a chronic disease and will require life-long correction of the genetic defect in airway epithelial cells.

Here, we have further developed the F and HN pseudotyped SIV towards clinical evaluation. We show that repeated administration to the mouse lower airways is feasible. We also confirm the long-term safety profile of this viral vector and show that it transduces the relevant human airway epithelial cells required for CF gene therapy.

Material and Methods

Viral vector production

Purified and concentrated F/HN-SIV expressing firefly luciferase (F/HN-SIV-Lux) or enhanced green fluorescent protein (F/HN-SIV-GFP) under the transcriptional control of the CMV enhancer/promoter, were prepared and titrated as previously described [11] (see online supplement for further details).

Mouse lung transfection

Female C57BL/6N mice (6–8 weeks old) were used (see online supplements for additional details). 100 μl viral vector in D-PBS was administered to the mouse nose and "sniffed" into the lung as previously described [15] (see Results and Figures for details about vector titers used) and gene expression quantified (see online supplement for further details).

For the daily repeat administration experiments, groups of mice were treated over 10 days with either nine daily doses of D-PBS followed by a single dose of F/HN-SIV-Lux, five daily doses of D-PBS followed by five daily doses of F/HN-SIV-Lux or ten daily doses of F/HN-SIV-Lux. Gene expression was analyzed 28 days after the final F/HN-SIV-Lux administration.

For the monthly repeat administration experiments groups of mice were transduced with either one dose of F/HN-SIV-Lux (single dose group), or two doses of F/HN-SIV-GFP (day 0, day 28), followed by F/HN-SIV-Lux on day 56 (repeat dose group). Importantly, mice receiving F/HN-SIV-Lux (single dose group) and F/HN-SIV-Lux on day 56 (repeat dose group) were of similar age and were transduced at the same time. Gene expression was analyzed 28 days after F/HN-SIV-Lux administration.

Toxicology

Over the 24 months study period mice were carefully observed daily and were given a full clinical examination every 2 weeks (including palpation of the abdomen for tumors) by an experienced animal technician, and bodyweight was recorded. Histological assessment was performed in mice that showed signs of illness throughout the study period. In addition lungs from asymptomatic mice culled at the end of the 24 month study period were also analysed (see online supplement for more details).

Histological assessment of GFP expression

Mouse lungs were transduced with F/HN-SIV-GFP (10^8 TU/mouse in 100 μl) by nasal sniffing. Animals receiving D-PBS only were used as negative controls (n=4/group). One month after transduction animals were culled and GFP expression was assessed in lung tissue (see online supplement for more details).

Gene transfer into relevant pre-clinical model

Gene transfer into human ALI cultures, human nasal brushings and lung slices was performed as described in the online supplements.

Statistical analysis

ANOVA followed by a Bonferroni post-hoc test or Kruskal-Wallis test followed by Dunns-multiple comparison post-hoc test was performed for multiple group comparison after assessing parametric and non-parametric data distribution with the Kolmogorov-Smirnov normality test, respectively. An independent student t-test or a Mann-Whitney test was performed for two group parametric and non-parametric data as appropriate. Pearsons

correlation was performed for parametric data. All analyses were performed using GraphPad Prism4 and the null hypothesis was rejected at $p<0.05$.

Results

Gene Expression in mouse lung persists and is stable

We first assessed if F/HN-SIV produced measurable levels of luciferase in murine lower airways (lungs), and if this expression was dose-related. Mice were transduced with F/HN-SIV-Lux (10^7 or 10^8 TU/mouse in 100 μl total volume) by nasal sniffing (n=8/group) or received D-PBS (n=8). Two days after transduction mice, were culled and luciferase expression quantified in lung homogenates. Luciferase expression was detectable and dose-related (10^7 TU/mouse: 2.0±0.3 relative light units (RLU)/mg protein, 10^8 TU/mouse: 27.0±6.4 RLU/mg protein, $p<0.01$, D-PBS: 0.01±0.03 RLU/mg). However, gene expression was also production batch-related (see online supplement).

We next assessed if expression in mouse lungs persisted. Mice were transduced with F/HN-SIV-Lux ($5x10^8$ TU/mouse in 100 μl total volume) by nasal sniffing (n=8 in two independent experiments) or received D-PBS (negative controls, n=6) and luciferase expression was quantified using *in vivo* bioluminescence imaging at regular intervals for up to 22 months. Luciferase-mediated photon emission was detectable in all treated mice 2 months after transduction (SIV: 362,660±63,922 photons/sec/cm^2, n=8, D-PBS: 66,535±4868 photons/sec/cm^2, n=6, $p<0.005$). Seven out of eight mice survived for 16 months and four out of the eight mice survived until termination of the experiment at 22 months. All of the SIV transduced mice had detectable luciferase expression at all time points. Photon emission increased modestly, but significantly ($p<0.005$), over time (SIV at 2 months: 381,123 ±70665 photons/sec/cm^2, at 16 months: 543,156 ±65234 photons/sec/cm^2, n=7 mice with data for both time-points). This was maintained at 22 months (2 months: 367,485±115923 photons/sec/cm^2, 22 months: 1,407.000±435,790 photons/sec/cm^2, n=4 mice with data for

both time-points). In contrast, photon emission in D-PBS-treated mice remained stable over the same time period (**Figure 1 A+B**).

An increase in photon emission may indicate an increase in luciferase expression potentially caused by an increase in the number of luciferase expressing cells over time or may be a peculiarity of BLI. We, therefore, repeated the experiment comparing BLI and post-mortem quantification of luciferase expression in lung tissue homogenates in the same animals. Mice were transduced with F/HN-SIV (10^7 TU/mouse, n=8-12/time-point) and at regular intervals over a 24 months period (for technical reasons BLI could only be performed between 6 and 24 months after gene transfer) and post-mortem quantification of luciferase expression in lung tissue homogenate was performed in cohorts of mice. Similar to results described above photon emission significantly ($p<0.001$) increased over the study period (BLI 6 months: 87,915±8871 photons/sec/cm^2, BLI at 24 months: 310,334±36274 photons/sec/cm^2) (**Figure 1C**), whereas detectable levels of luciferase in tissue homogenate remained stable (**Figure 1D**). There was no correlation between *in vivo* photon emission and luciferase expression quantified in tissue homogenates (Spearman r=0.205, p=0.11, n=63 paired data points from 6 to 24 months). However, luciferase expression quantified in tissue homogenates correlated well with photon emission from the same animal when lungs were extracted before BLI (**Figure 1E**) (Spearman r=0.75, p=0.01, n=11 paired data points at 24 months), which implies that photon quenching may occur *in vivo*. Additional technical considerations relating to limitations of BLI and virus batch-to-batch variability became apparent in this study (see online supplement).

To determine what cell types were transduced in the lung we transfected mice with F/HN-SIV-GFP (10^8 TU/mouse) or D-PBS (n=4/group) by nasal sniffing and assessed GFP expression one months after transduction. GFP expression was detectable in airway epithelial cells, and also in the alveolar region (**Figure 1F**).

F/HN-SIV requires only short contact time to achieve efficient transduction

We compared prolonged contact time of the vector with the nasal epithelium (via perfusion) with very brief contact time (sniffing). The latter led to equally efficient transduction of the nasal epithelium as the former (**Figs 2A+B**). This suggests that a short contact time between the viral vector and the target cell may be sufficient for efficient vector uptake into the cells, of potential importance in future clinical trials.

Gene expression in the nose also persisted for 15-22 months in seven out of eight mice, but, in contrast, to the lung gradually declined by approximately 60% over this period (SIV month 2: 1,309,000±316,612 photons/sec/cm^2, month 15: 583,951±228,804 photons/sec/cm^2, $p<0.05$, n=7/group, D-PBS month 15: 53,021±2325 photons/sec/cm^2, $p<0.005$) which may be due to different cell types being transduced in nose and lung or different turn-over rates of the cells at these two sites. Consistent with our previous data using non-viral gene transfer agents [16], intranasal (IN) administration of luciferin (the substrate for luciferase) boosted F/HN-SIV-Lux derived photon emission in mice that were negative after intraperitoneal (IP) administration of luciferin (**Figure 2C**).

Daily repeat administration to the lung is feasible

Although gene expression after a single dose of F/HN-SIV persists for the life-time of the animal, a single dose may not be sufficient to achieve therapeutic benefit in man. It is, for

example, conceivable that the total volume required for delivery the optimal vector titre to the human lung may be too large for a single dose and administration may have to be split into several doses to accommodate the volume. We, therefore, assessed if repeated daily administration of the viral vector is feasible and if this leads to incrementally increased gene expression compared to a single dose. Mice (n=8/group) were treated with either F/HN-SIV-Lux daily for 10 days (10^6 TU/day in 100 μl) or with PBS (100 μl) for 5 days followed by 5 days of F/HN-SIV-Lux (10^6 TU/day) or with D-PBS for 9 days followed by one day of F/HN-SIV-Lux (10^6 TU/day); Lux expression was quantified 28 days after the last dose. Gene expression was significantly correlated (Pearsons r^2=0.61, p>0.0001) with the number of F/HN-SIV-Lux doses given (**Figure 3A**) and indicated that daily repeat administration is feasible and significantly increases transduction efficiency.

Monthly repeat administration to the lung is feasible

Although a single dose of F/HN-SIV generates persistent gene expression for approximately 2 years in mice (life-time of the animal) a single dose will unlikely be sufficient to achieve clinical benefit for the life-time of a CF patient. Therefore, a crucial question is whether the vector can produce gene expression upon repeated administration to the lung. As shown above daily administration was feasible, but this timeframe is unlikely to be sufficient for the development of effective immune responses to the viral vector. We, therefore, conducted repeat administration experiments at monthly dosing intervals, a period we have shown to be sufficient for the development of an immune response to other viral vectors [5;17]. Mice were either treated with one dose of F/HN-SIV-Lux (single dose group) or two doses of F/HN-SIV-GFP (day 0, day 28), followed by F/HN-SIV-Lux on day 56 (repeat dose group). All mice received 10^7 TU in 100 μl per dose (n=20/group) and gene expression was analyzed 28 days after F/HN-SIV-Lux administration. All mice received only one dose of vector carrying

a luciferase reporter gene to avoid interference of anti-luciferase antibodies. As shown in **Figure 3B** luciferase expression levels after one and three doses of the viral vector were identical and at this vector titer 4 log orders above background levels ($p<0.01$).

No evidence of chronic toxicity was seen during a 2 year follow-up period

Chronic toxicity due to insertional mutagenesis is a potential concern with this vector. Here, we compared 24 months survival of mice treated with F/HN-SIV-Lux (10^7 TU/mouse, n=99 at start of the experiment) or D-PBS (n=48 at start of the experiment). Mortality (**Figure 4A**) and weight (**Table 1**) in both groups was similar. We also assessed the lungs of D-PBS or F/HN-SIV-Lux (10^7 TU/mouse, n=12/group) treated mice histologically 24 months after nasal sniffing; there were no differences in any of the key histological markers (**Figure 4B-D**). Eleven D-PBS and 7 F/HN-SIV treated mice were culled due to showing signs of illness or found dead during the study. Histological examination revealed a range of pathology commonly found in aging C57BL/6N mice equally distributed between the two groups (**Table 2**).

F/HN-SIV transduction of human *ex vivo* models is feasible

A. F/HN-SIV achieves persistent gene expression in differentiated human air liquid interface (ALI) cultures

To assess the efficiency of the viral vector in relevant human tissues we transduced human ALIs with F/HN-SIV-Lux at MOI 25 or 250 (n=5/group), with D-PBS control (n=3) or with GL67A, the most efficient non-viral gene transfer agent for airway epithelium. The latter was complexed with a eukaryotic expression plasmid expressing luciferase (10 μg pCIKLux per ALI equivalent to $3x10^{12}$ plasmids and an approximate MOI of $7x10^6$) (n=3) and luciferase expression followed using BLI for 3 months (representative images are shown in **Figure 5A**).

F/HN-SIV mediated expression was dose-related and persisted for at least 3 months without the loss of activity. In contrast transfection with GL67A/pCIKLux was only barely detectable at an early time-point (day 2) after transfection (**Figure 5B**). Lipid-mediated gene transfer was associated with cell damage as indicated by the appearance of small holes in the ALIs; this was not seen with exposure of the ALIs to the lentiviral vector (data not shown)**.**

B. F/HN-SIV transduces primary human pulmonary cells

We assessed if F/HN-SIV transduces primary human airway epithelial cells obtained from nasal brushings or human lung slices generated from lung resection tissue (**Figure 6A**). Nasal brushings were transduced with F/HN-SIV-Lux at an MOI of 25 and 250 or treated with D-PBS (n=6/group) and luciferase expression was quantified 24 hr after transduction (cell viability precludes longer time scales). Luciferase expression was significantly ($p<0.005$) increased compared to PBS controls (**Figure 6B**). These results were confirmed in an independent experiment (MOI 25:$9.7x10^6 \pm 0.9x10^6$, MOI 250: $1.5x10^7 \pm 1.6x10^6$, Negative Control: 2762±241 RLU/mg protein, n=4/group)

C. F/HN-SIV achieves persistent gene expression in human and sheep lung slices

Precision cut human lung slices were transduced with F/HN-SIV-Lux ($2x10^7$ TU/slice), or remained untransduced, and luciferase expression quantified for 14 days after transduction. Assessment of later time-points is currently not feasible due to limited tissue viability (**Figure 6C)**. Two independent experiments were performed (see open and closed symbols in figure) but data were pooled to allow for robust statistical analysis. Significant ($p<0.001$) and stable gene expression was detectable for at least 14 days ($p<0.05$) after gene transfer.

We also repeated these experiments in sheep lung slices (two independent experiments), which generally survive longer than human lung slices when cultured (unpublished data). Significant ($p<0.001$ to $p<0.05$) and stable luciferase expression was detectable at all time-points for at least 26 days after transduction (**Figure 6D**). These results indicate that F/HN–SIV is able to transduce primary human and sheep pulmonary cells and provides persistent expression.

Discussion

The efforts, of one of our team, to improve pulmonary gene transfer and enable gene therapy for the treatment of lung diseases such as CF have led to the development of a lentiviral vector pseudotyped with the Sendai virus envelope proteins F and HN [12]. Moving novel therapies to the clinic requires that relevant evidence for both safety and efficacy is gathered in appropriate models. Here, we begin to place this vector onto a translational pathway to the clinic and provide a body of supportive evidence for F/HN-pseudotyped SIV as a potential gene transfer agent for CF. We show that (1) a single dose produces lung expression for the life-time of the mouse (approximately 2 years), (2) only brief contact time (seconds) is needed to achieve transduction, (3) repeated daily administration leads to a dose-related increase in gene expression, (4) repeated monthly administration to mouse lower airways is feasible without loss of gene expression, (5) there is no evidence of chronic toxicity during a 2 year study period, (6) F/HN-SIV transduction generates persistent gene expression in human differentiated airway cultures and freshly obtained human lung slices and transduces freshly obtained primary human airway epithelial cells.

At least two previous studies have shown that lentiviral vector-mediated transduction of mouse lung leads to persistent (15 and 24 months) gene expression in the murine lower airways [18;19]. However, both studies used a VSV-G pseudotyped lentiviral vector which almost exclusively transduces alveolar macrophages, rather than lung epithelial cells. In addition to the airway epithelium, the target for CF gene therapy, cells in the peripheral lung (a mixture of pneumocytes and macrophages) are transduced after bolus administration of the viral vector by nasal sniffing which may lead to pooling of liquid in the peripheral lung. To determine transduction efficiency of airway epithelium more accurately the viral vector needs to be administered by nebulisation. Further work is now warranted to determine whether

F/HN-SIV is stable in clinically approved nebulisers and that virus production can be scaled up to the extent required for clinical studies. We are currently (a) assessing if F/HN-SIV is stable in clinically approved nebulisers and preliminary results indicate that infectivity after nebulisation is retained (data not shown) and (b) scaling up vector production to allow us to move into *in vivo* nebulisation experiments in the near future.

Whilst expression levels in the lung are completely stable over the 2 year study period, those in the nasal epithelium declined by approximately 60%. The latter is consistent with our previous data [11] and may relate to different cell types being transduced in the lung (airway epithelium and alveolar cells) and nose (mainly ciliated airway epithelial cells) [11] as well as different turn-over rates of these cells at these two sites. Although, we do not have information about turn-over of nasal airway epithelium, Rawlins *et al* have shown that the half-life of ciliated airway epithelial cells in the mouse trachea and lung differs (trachea: average half-life 6 months, lung: average half-life of 17 months) [13].

We have previously shown that prolongation of contact time between non-viral gene transfer agents and the airway epithelium significantly increases gene expression [20]. In our previous study, we therefore slowly perfused F/HN-SIV (1.3 μl/min) onto the mouse nasal epithelium to maximise transduction [11]. Transduction efficiency in the nasal epithelium after administration of a bolus of fluid which is rapidly (seconds) sniffed into the lung led to similar levels of photon emission. This implies that brief contact time between F/HN-SIV and the target cell may be sufficient for efficient vector uptake into the cells, which is an important factor for clinical translation. F/HN-SIV, therefore, mimics Sendai virus [2] from which the F and HN envelope proteins were derived.

We and others have previously shown that repeated administration of lentiviral vectors to the mouse nasal epithelium is feasible [10;11]. To move our translational research a step closer to clinical relevance we now assessed repeat administration of the vector in the lung. Two types of repeat administration experiments were performed; (1) daily and (2) monthly. The former was assessed because it is conceivable that the total volume required for delivery of a specific vector titre to the human lung may be too large for a single dose and administration may have to be split into several doses to accommodate the volume. Here, we show that daily repeat administration is feasible and leads to a dose-related increase in gene expression. Although important, we have not yet addressed the question if "split-dose" delivery of a specific vector titre (e.g 10 doses of 10^7 TU) offers advantages over "single-dose" delivery of the same vector titre (e.g 1 dose of 10^8 TU) which may also be a relevant translational research question. Although efficient daily-repeat administration is encouraging, this time-frame is generally not considered to be sufficient to induce robust immune responses to the viral vector. To confirm that long-term F/HN-SIV repeat administration to the lower airways is feasible viral vector was administered monthly over a 3-month period. This dosing interval may be of clinical relevance and is also an accepted timeframe for the induction of robust immune responses. Uniquely for a viral vector (in our hands) luciferase expression levels after one and three doses were identical and more than 4 log orders higher than in untransduced control mice.

Another important consideration for translational development of a gene transfer agent is safety. The occurrence of leukaemia and myelodysplasia in some patients with primary immunodeficiencies after treatment with gamma retroviral vector-transduced bone marrow [21] raises concerns about the safety of integrating viral vectors. The risk of insertional mutagenesis in slowly turning over differentiated lung epithelium is unknown, but is likely to be lower than in rapidly dividing bone marrow cells. In addition the improved design of self-

inactivating lentiviral vectors has improved safety [21]. Here we compared survival, weight and lung histology during a 24 month study period and did not see any differences between F/HN-SIV and PBS treated mice. We included only female mice in the study as they can be more readily housed together for prolonged periods of time. It is unlikely that the toxicology profile in male mice would be significantly different. However, before progression into clinical trial a GLP-toxicology study, including male and female animals as well as dose-ranging, will have to be performed. Despite the encouraging toxicology profile of our current vector configuration we plan to assess further modifications before final vector selection for clinical trials, including the use of mammalian promoters as well as ubiquitous chromatin opening elements (UCOE). The former have been shown to reduce the risk of insertional mutagenesis compared to strong viral promoters [22]; whilst the latter may reduce gene silencing [22]. The ability to administer this vector repeatedly and efficiently also opens the possibility of using non-integrating lentiviral vectors [23] which may further reduce the genotoxic risk.

As part of our translational pathway we next determined whether F/HN-SIV is able to transduce human tissues. We have previously shown that F/HN-SIV can transduce fully differentiated human air liquid interface (ALI) cultures when analysed 5 days after transduction [11]. These cultures mimic human airway epithelium and are difficult to transfect [24]. Here, we have extended these studies, and show that F/HN-SIV-mediated expression in ALIs is stable, persists for at least 3 months (the length of the experiment) and is higher than GL67A-mediated expression, which in our hands is currently the most efficient non-viral vector. Preliminary experiments indicated that in contrast to lipid-mediated gene transfer, F/HN-SIV does not appear to damage the ALI cultures, but more extensive studies are required to determine acute toxicity *in vitro* and *in vivo*.

To further evaluate the viral vector in even more relevant tissues we assessed freshly obtained human primary airway cells and showed that F/HN-SIV-Lux transduction leads to significant levels of luciferase expression. We performed only short-term (24 hr) experiments to avoid potential cell division which would have affected interpretation of the results. Collection of primary cells by nasal brushings exposes the basolateral membrane of these cells and we cannot exclude the possibility that the vector may have entered the cells via this route. However, experiments performed in mice *in vivo* and in ALI cultures clearly show that F/HN-SIV can transduce airway epithelial cells when applied to the apical membrane without the need to co-administer agents to open tight junctions. There was no difference in gene expression when cells were transduced with an MOI of 25 or 250. In future experiments the MOI will be further reduced to determine the minimum MOI for efficient transduction.

We also assessed F/HN-SIV in precision cut human lung slices. Expression in both experiments was stable and persisted for at least 14 days, after which the experiment was terminated because of reduced tissue viability. Although we have not determined which cells express the recombinant protein these studies show that persistent SIV-mediated transduction into human pulmonary cells is feasible and is not prevented by viral restriction factors. We also transduced ovine lung slices, which are generally more resilient when cultured (Nikki Newman personal communication); stable luciferase expression was detectable for at least 26 days. These data support the future use of these models in the clinical development of this vector.

Sendai-virus shares high sequence homology with human parainfluenza virus (HPIV) and pre-existing immunity may be a factor. Interestingly, Slobod KS *et al* assessed Sendai virus-vaccination against HPIV and reported evidence of immunogenicity in three of nine vaccinees despite pre-existing, cross-reactive immunity presumably induced by previous exposure to human PIV-1 [25]. Although this initial study is encouraging the effects of pre-

existing humoral immunity on virus transduction efficiency is something we will address as a high priority.

Gene transfer to the airway epithelium is more difficult than originally anticipated. Until now viral gene transfer agents have not been useful for the treatment of chronic lung disease such cystic fibrosis (CF), due to immunogenicity which prevent successful repeat administration. Lentivirus-based vectors are a notable exception. Moving novel therapies to the clinic requires that relevant evidence for both safety and efficacy is gathered in appropriate models. Here, we begin to place this vector onto a translational pathway to the clinic and provide a body of supportive evidence for F/HN-pseudotyped SIV as a potential gene transfer agent for CF including a) life-time gene expression and efficient repeat administration in mouse lung, b) lack of chronic toxicity, c) persistent gene expression in human *ex vivo* models.

The data presented here support F/HN-SIV as a promising vector for pulmonary gene therapy, with potential for many diseases including CF. We are currently undertaking the next critical steps (vector optimisation and virus production) in this developmental process towards clinical evaluation and are improving vector optimisation and viral production methods to support progression into early phase trials.

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Figure Legends

Figure 1: F/HN-SIV transduction leads to persistent gene expression in mouse lung

Mice were transduced with F/HN-SIV-Lux ($5x10^8$ TU/mouse) by nasal sniffing (n=8 in two independent experiments) or received PBS (negative controls, n=6). Luciferase expression was quantified using bioluminescence imaging or in tissue homogenate at regular intervals for up to 24 months. (**A**) Bioluminescence *in vivo* imaging 2 to 22 months after transduction. Representative images of two mice reaching the 22 months time point are shown. (**B**) Quantification of *in vivo* bioluminescence over time (black lines, n=8) or PBS (red lines, n=6). Each line represents photon emission over time in one animal. Solid and dotted lines represent independent experiments. ***=$p<0.005$ when compared to month 2. Bioluminescent *in vivo* imaging (BLI) (**C**) and luciferase expression in lung tissue homogenate (**D)** was repeated at regular intervals over 24 month in a third independent experiment (10^7 TU/mouse, n=8-12/time-point). For technical reasons BLI could only be performed between 6 and 24 months after gene transfer. ***=$p<0.005$ when compared to month 6. Quantification of luciferase expression in lung tissue homogenate was performed at regular intervals over the 24 months study period. Each dot represents one animal. Horizontal bars represent the group median. (**E**) Correlation between luciferase expression quantified in tissue homogenates and photons emitted from extracted lungs (representative images from 11 extracted lungs are shown). Each dot represents one animal. (**F**) Detection of green fluorescent protein (GFP) expression in lung after transduction with F/HN-SIV-GFP (10^8 TU/mouse). Transduced GFP positive cells were identified using fluorescent microscopy (original magnification x20). (**i**) PBS treated negative control. (**ii** and **iii**) Tissue sections from

F/HN-SIV-GFP treated mice showing GFP positive cells in airways (arrows) and alveoli. Representative images from four mice/group are shown.

Figure 2: F/HN-SIV requires only short contact time to achieve efficient transduction

Luciferase expression was compared in mice receiving F/HN-SIV-Lux (3-4x10^8 TU/mouse in 100 μl) by slow perfusion (1.3 μl/min, n=10) or as bolus administration by nasal sniffing (n=8). (**A**) Bioluminescence *in vivo* signal in the mouse nose. Representative images of three mice are shown. (**B**) Quantification of *in vivo* bioluminescence in mouse nose after slow perfusion or nasal sniffing of the vector. Each dot represents one animal. Horizontal bars represent the group median. (**C**) Bioluminescence *in vivo* signal in mouse nose after intraperitoneal (IP) or intranasal (IN) administration of luciferin. Representative images of two of five mice are shown. Luciferase expression after slow perfusion of the F/HN-SIV-Lux has been published previously [11].

Figure 3: Daily and monthly repeat administration to the lung is feasible

(**A**) Daily re-administration: Mice (n=8/group) were treated with either F/HN-SIV-Lux for 10 days (10^6 TU/day) or with D-PBS for 5 days followed by 5 days of F/HN-SIV-Lux (10^6 TU/day) or with D-PBS for 9 days followed by 1 day of F/HN-SIV-Lux (10^6 TU). Luciferase expression was analyzed 28 days after F/HN-SIV-Lux administration. (**B**) Monthly re-administration: Mice were either treated with one dose of F/HN-SIV-Lux (single dose group) or two doses of F/HN-SIV-GFP (day 0, day 28), followed by F/HN-SIV-Lux on day 56 (repeat dose group). All mice receive 10^7 TU/dose (n=20/group) and gene expression was analyzed 28 days after F/HN-SIV-Lux administration. Each dot represents one animal.

Horizontal bars represent the group median. Dotted line represents negative control values, *** $p<0.0001$ when compared to 5 and 10 doses, **$p<0.01$ when compared to 10 doses.

Figure 4: No evidence of chronic toxicity during 2 year follow-up period

(**A**) 24 months survival of mice treated with F/HN-SIV-Lux (10^7 TU/mouse, n=99 at start of the experiment) or PBS (n=48 at start of the experiment) by nasal sniffing. (**B-D**) Comparison of lymphocytes (B), oedema (C) and inflammation (D) in mouse lung 24 months after F/HN-SIV-Lux or PBS treatment. Each dot represents one animal. Horizontal bars represent the group median.

Figure 5: Persistent gene expression in differentiated ALI cultures

Differentiated human air liquid interface cultures were transduced with F/HN-SIV-Lux at MOI 25 or 250 (n=5/group), transfected with GL67A complexed to a eukaryotic expression plasmid expressing luciferase (10 μg pCIKLux per ALI equivalent to $3x10^{12}$ plasmids and an approximate MOI of $7x10^6$) (n=3) or treated with PBS (negative control, n=3). Luciferase expression was followed using BLI for 3 months. (**A**) Representative images are shown. (B) Quantification of luciferase expression over 3 months.

Figure 6: F/HN-SIV transduces primary human pulmonary cells

(**A**) Microscopic section showing primary human airway epithelial cells obtained from nasal brushings (left, GC=goblet cell, CC=ciliated airway epithelial cell) or human lung slices (right, AW=airway, P=parenchyma). (**B**) Nasal brushings were transduced with F/HN-SIV-Lux at an MOI of 25 and 250 or treated with PBS (negative control) and luciferase expression

was quantified 24 hr after transduction. Each dot represents one sample. Horizontal bars represent the group median. ***=p<0.005 when compared to negative controls. (**C**) Human lung slices were transfected with F/HN-SIV-Lux ($2x10^7$ TU/slice) or remained untransfected (negative control) and luciferase expression quantified 2, 7 and 14 days after transduction. Two independent experiments were performed (open and closed symbols). (**D**) Sheep lung slices were transfected with F/HN-SIV-Lux ($2x10^7$ TU/slice) or remained untransfected (negative control) and luciferase expression quantified up to 26 days after transduction. Two independent experiments were performed (open and closed symbols). Each dot represents one animal. Horizontal bars represent the group median. *=p<0.05, **=p<0.01, ***=p<0.005 when compared to negative control.

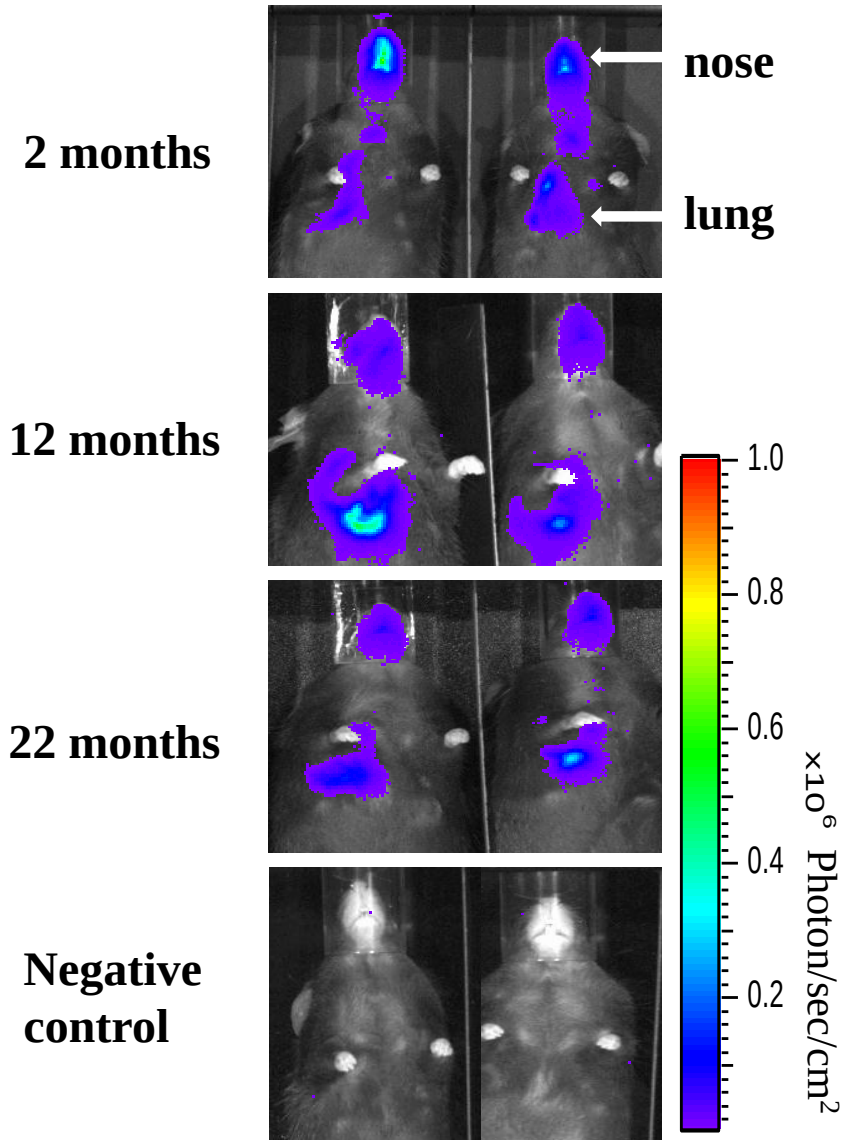

2 months
12 months
22 months
Negative control
nose
lung
1.0
0.8
0.6
0.4
0.2
×10⁶ Photon/sec/cm²

Figure 1A

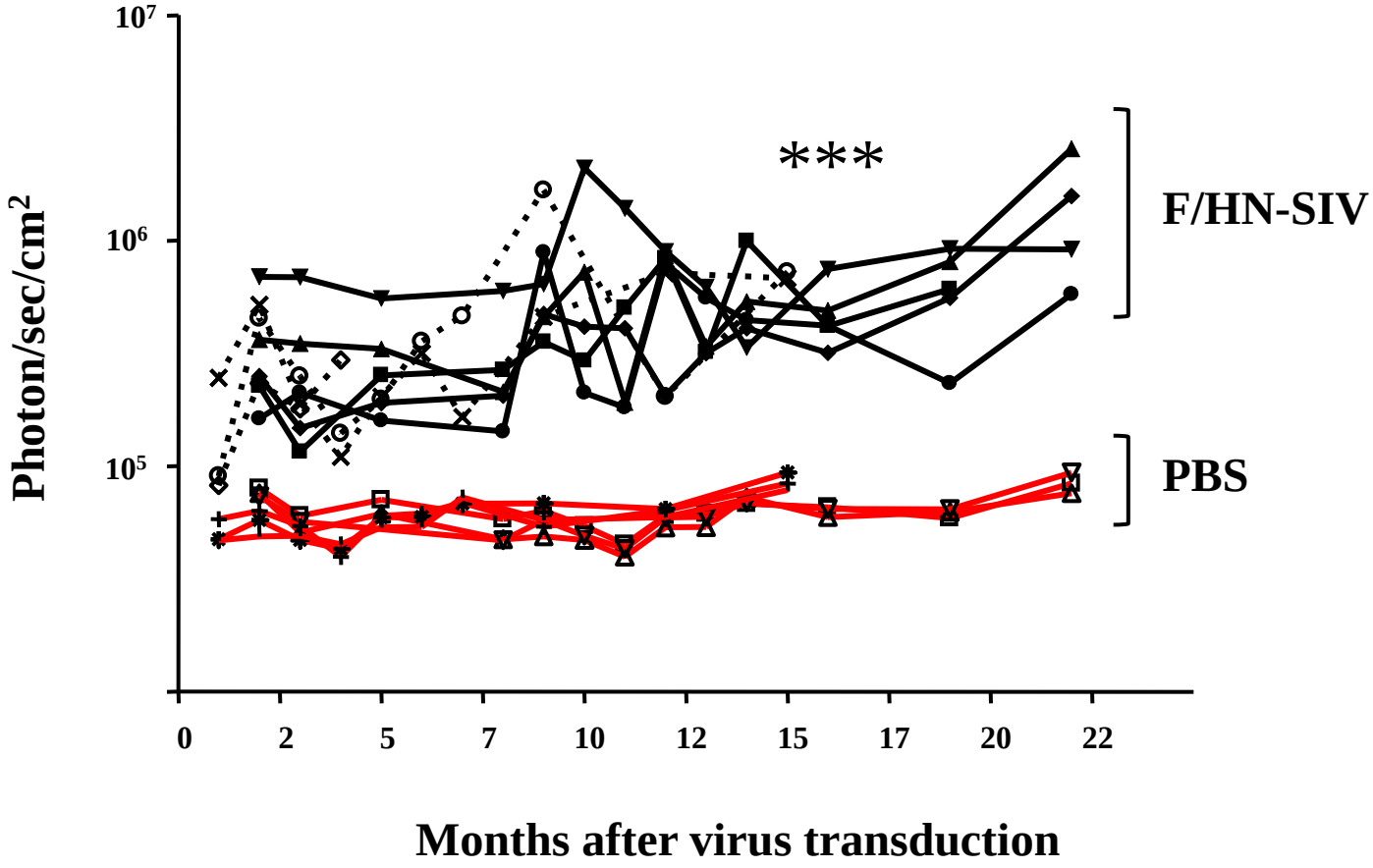

10^7
10^6
10^5
Photon/sec/cm^2
***
F/HN-SIV
PBS
0
2
5
7
10
12
15
17
20
22
Months after virus transduction

Figure 1B

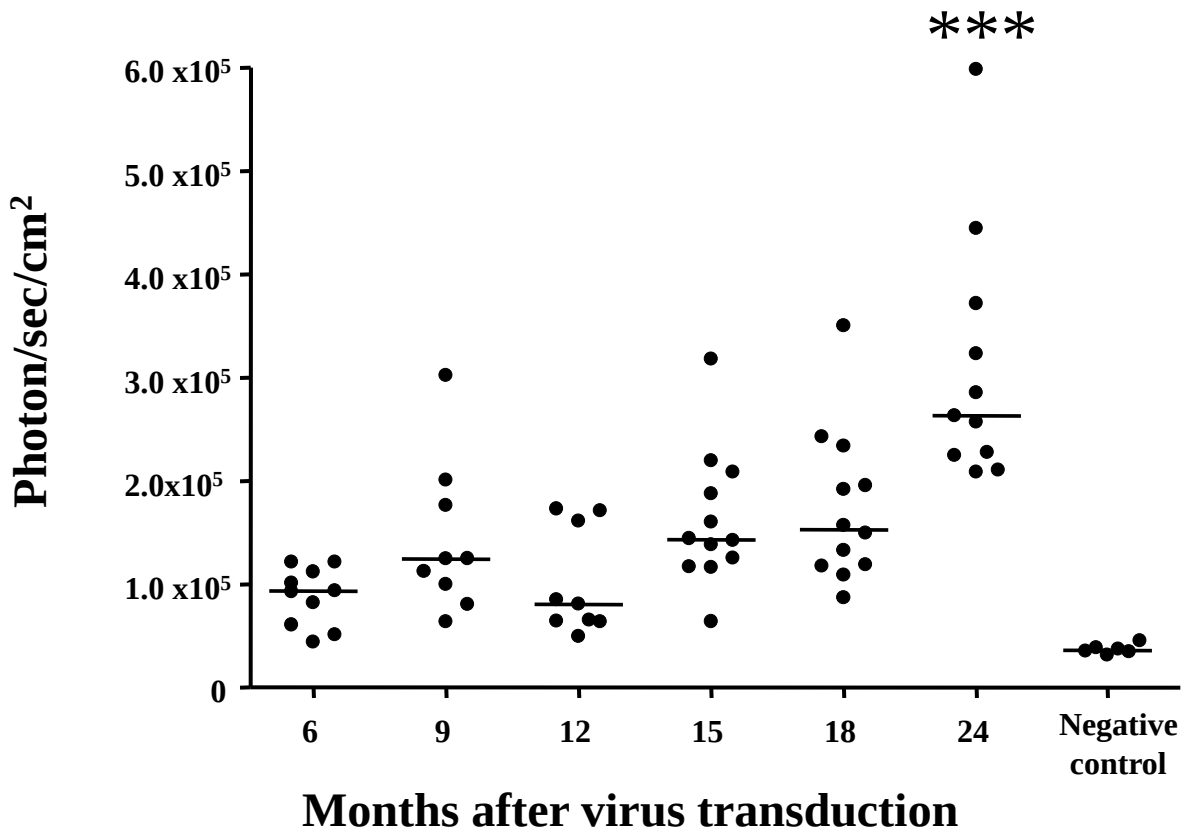

***
6.0 x10^5
5.0 x10^5
4.0 x10^5
3.0 x10^5
2.0x10^5
1.0 x10^5
0
Photon/sec/cm^2
6
9
12
15
18
24
Negative control
Months after virus transduction

Figure 1C

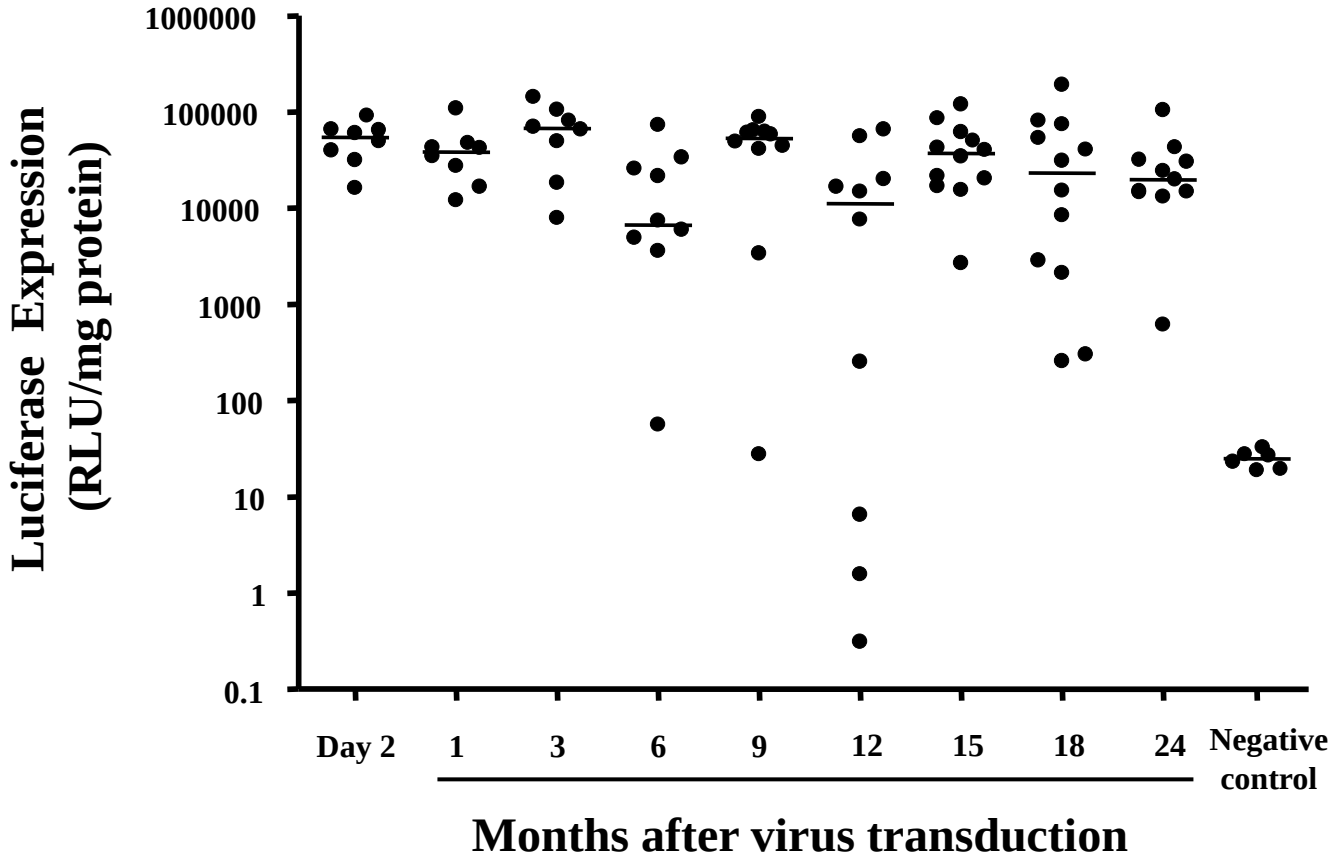

Luciferase Expression
(RLU/mg protein)
1000000
100000
10000
1000
100
10
1
0.1
Day 2
1
3
6
9
12
15
18
24
Negative control
Months after virus transduction

Figure 1D

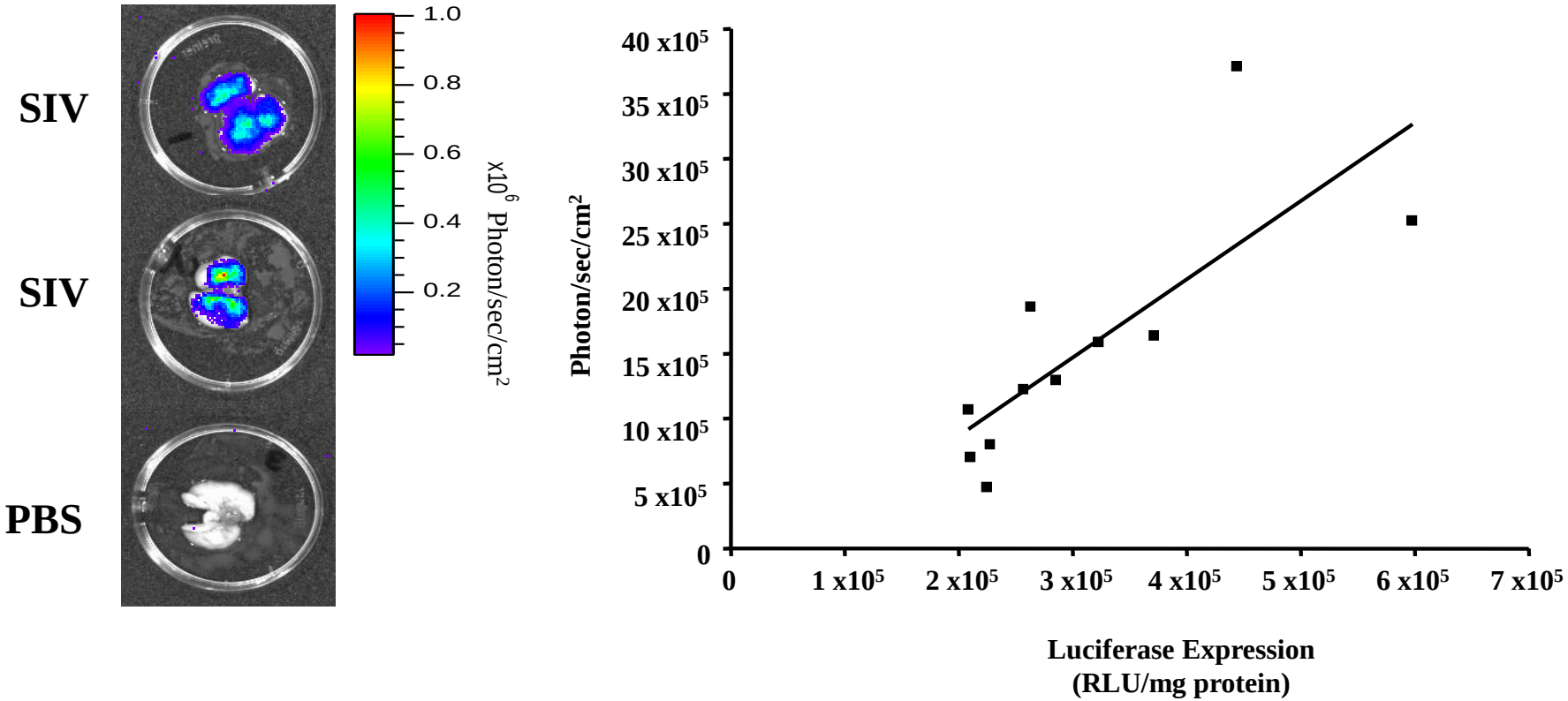

SIV
SIV
PBS
1.0
0.8
0.6
0.4
0.2
x10^6 Photon/sec/cm²
Photon/sec/cm²
40 x10^5
35 x10^5
30 x10^5
25 x10^5
20 x10^5
15 x10^5
10 x10^5
5 x10^5
0
0
1 x10^5
2 x10^5
3 x10^5
4 x10^5
5 x10^5
6 x10^5
7 x10^5
Luciferase Expression
(RLU/mg protein)

Figure 1E

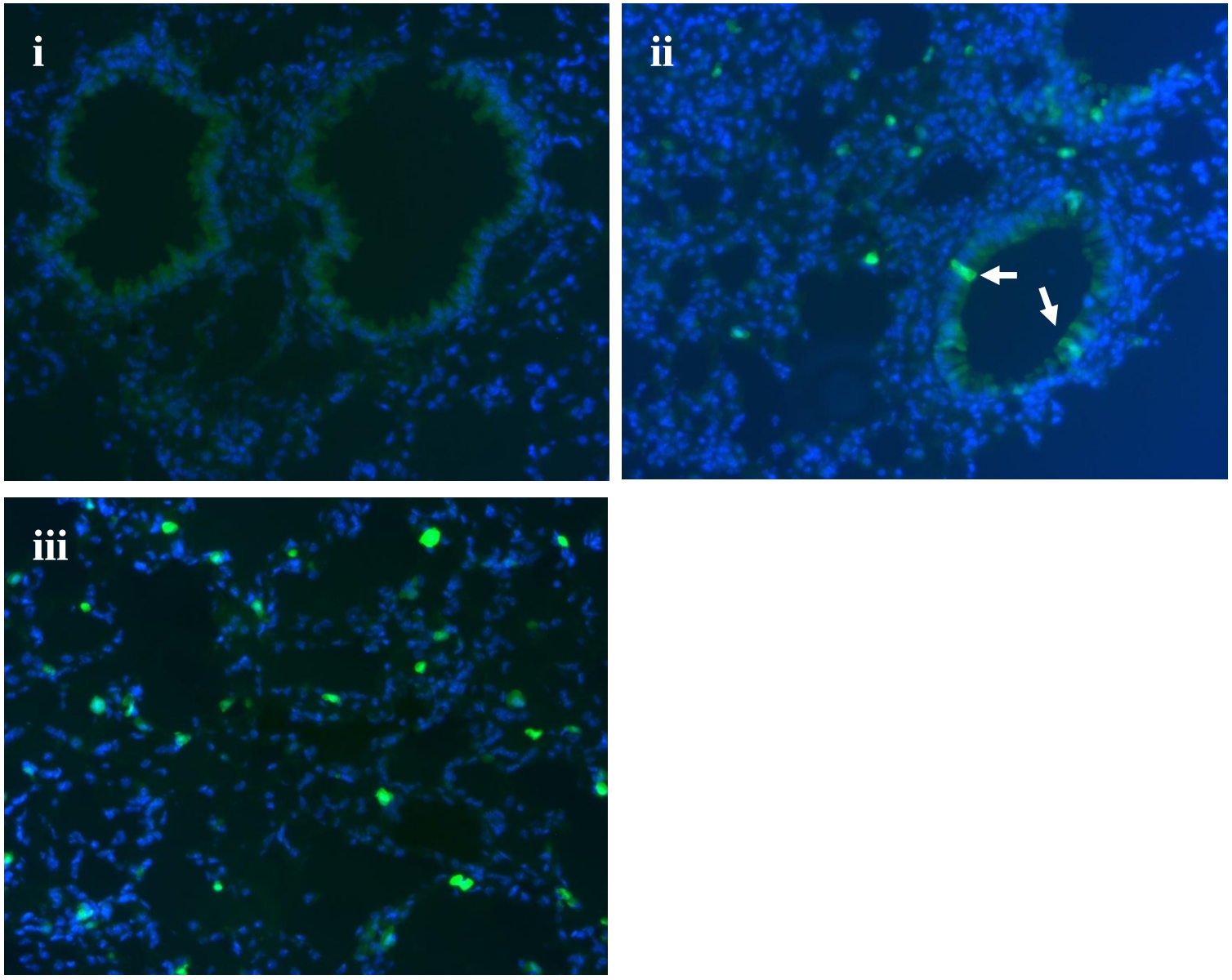


Figure 1F

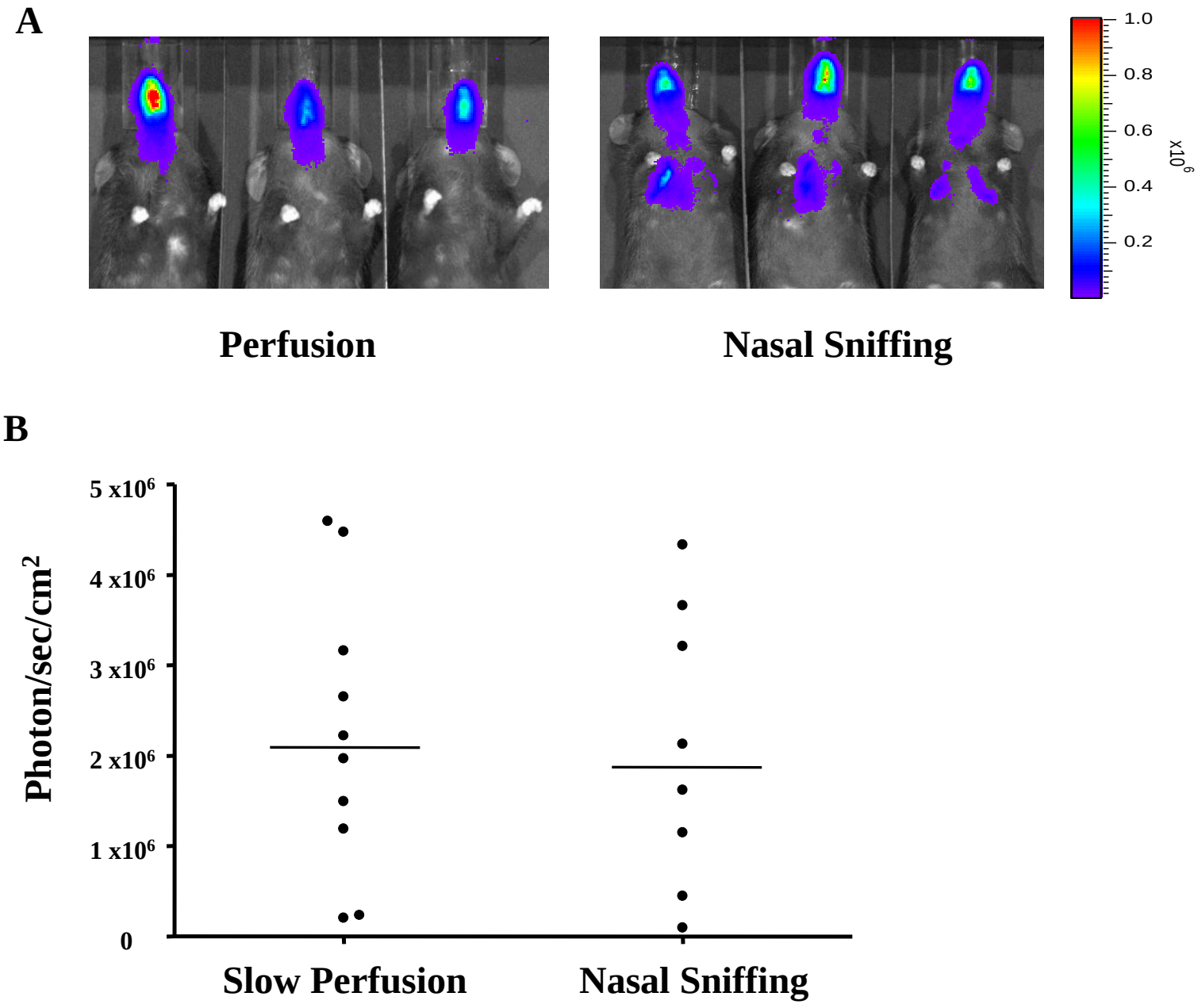

A
1.0
0.8
0.6
0.4
0.2
x10^6
Perfusion
Nasal Sniffing
B
5 x10^6
4 x10^6
3 x10^6
2 x10^6
1 x10^6
0
Photon/sec/cm^2
Slow Perfusion
Nasal Sniffing

Figure 2

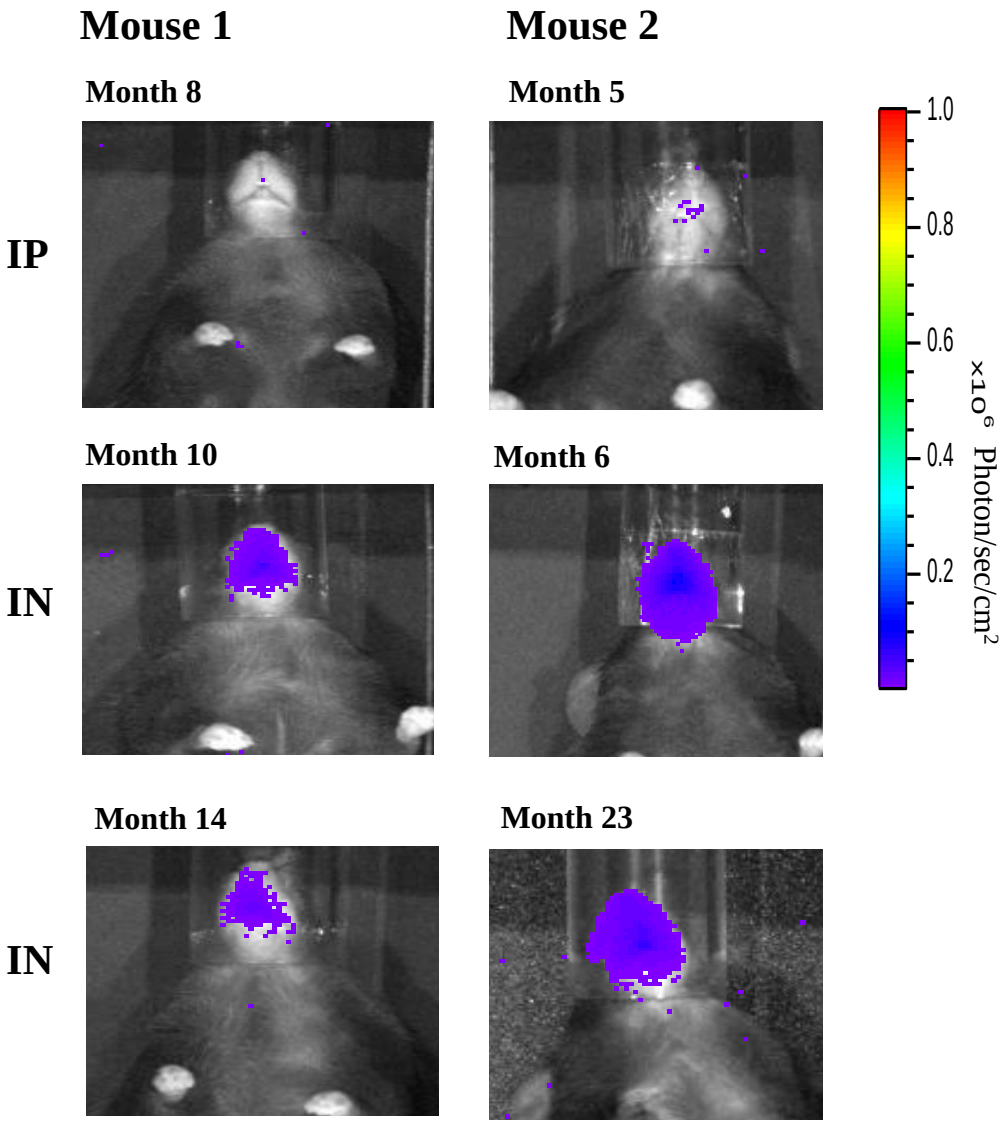

Mouse 1
Mouse 2
Month 8
Month 5
IP
Month 10
Month 6
IN
Month 14
Month 23
IN
1.0
0.8
0.6
0.4
0.2
×10⁶ Photon/sec/cm²

Figure 2C

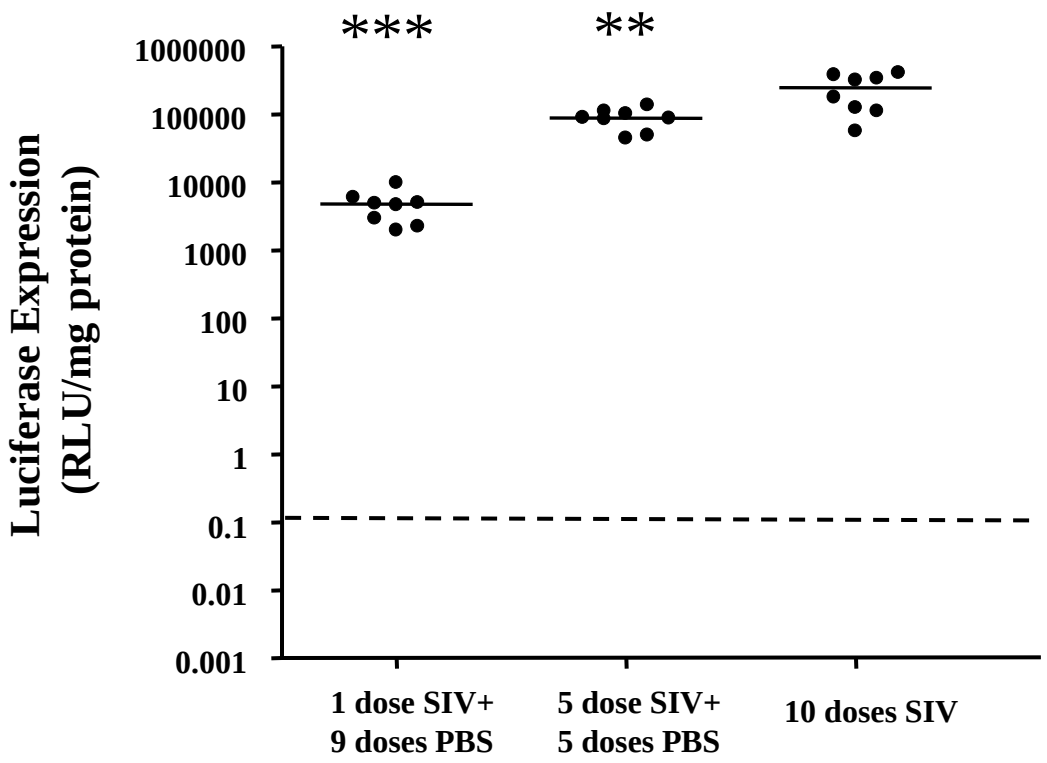

***
**
1000000
100000
10000
1000
100
10
1
0.1
0.01
0.001
Luciferase Expression
(RLU/mg protein)
1 dose SIV+
9 doses PBS
5 dose SIV+
5 doses PBS
10 doses SIV

Figure 3A

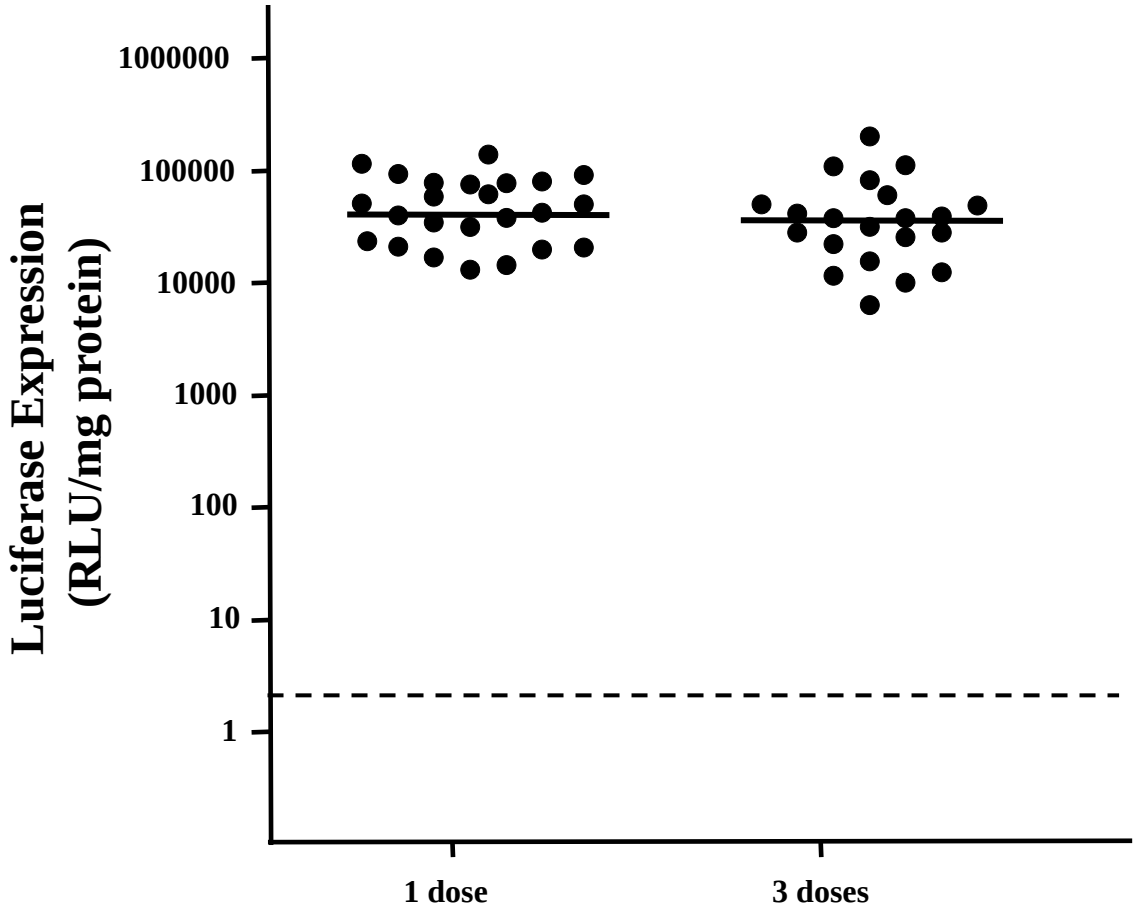

Luciferase Expression
(RLU/mg protein)
1000000
100000
10000
1000
100
10
1
1 dose
3 doses

Figure 3B

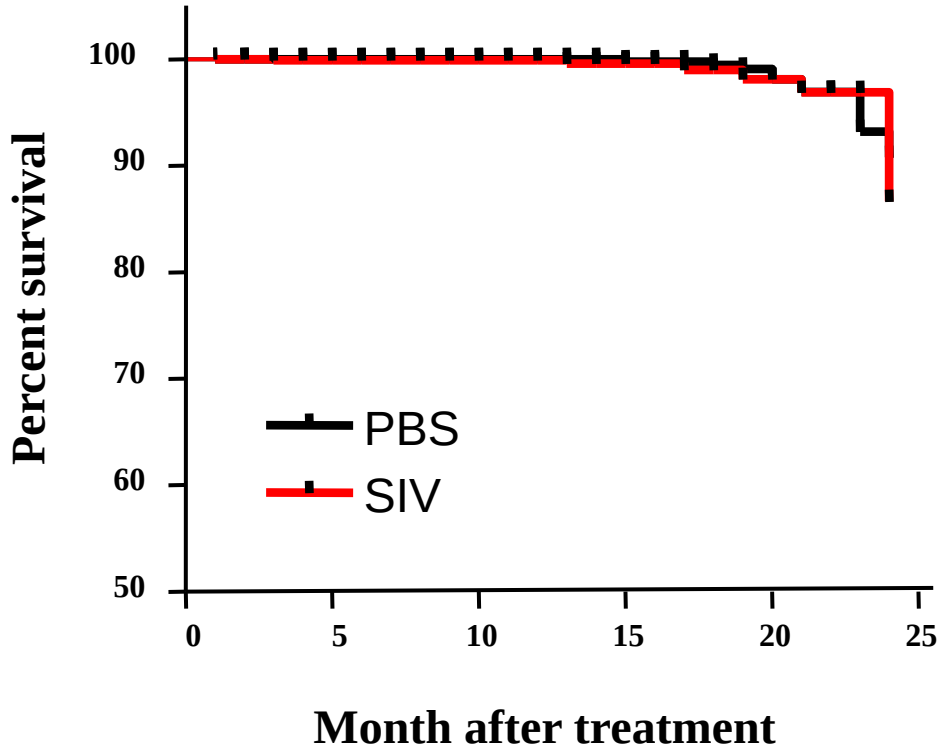

Percent survival
100
90
80
70
60
50
0
5
10
15
20
25
PBS
SIV
Month after treatment

Figure 4A

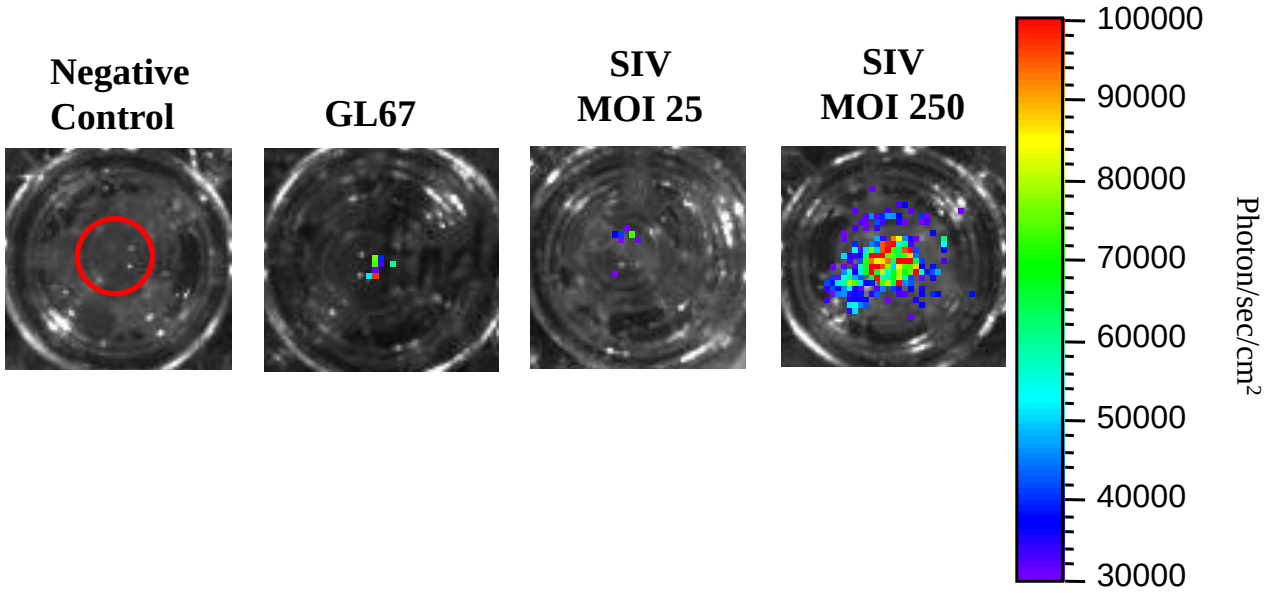

Negative Control
GL67
SIV MOI 25
SIV MOI 250
100000
90000
80000
70000
60000
50000
40000
30000
Photon/sec/cm2

Figure 5A

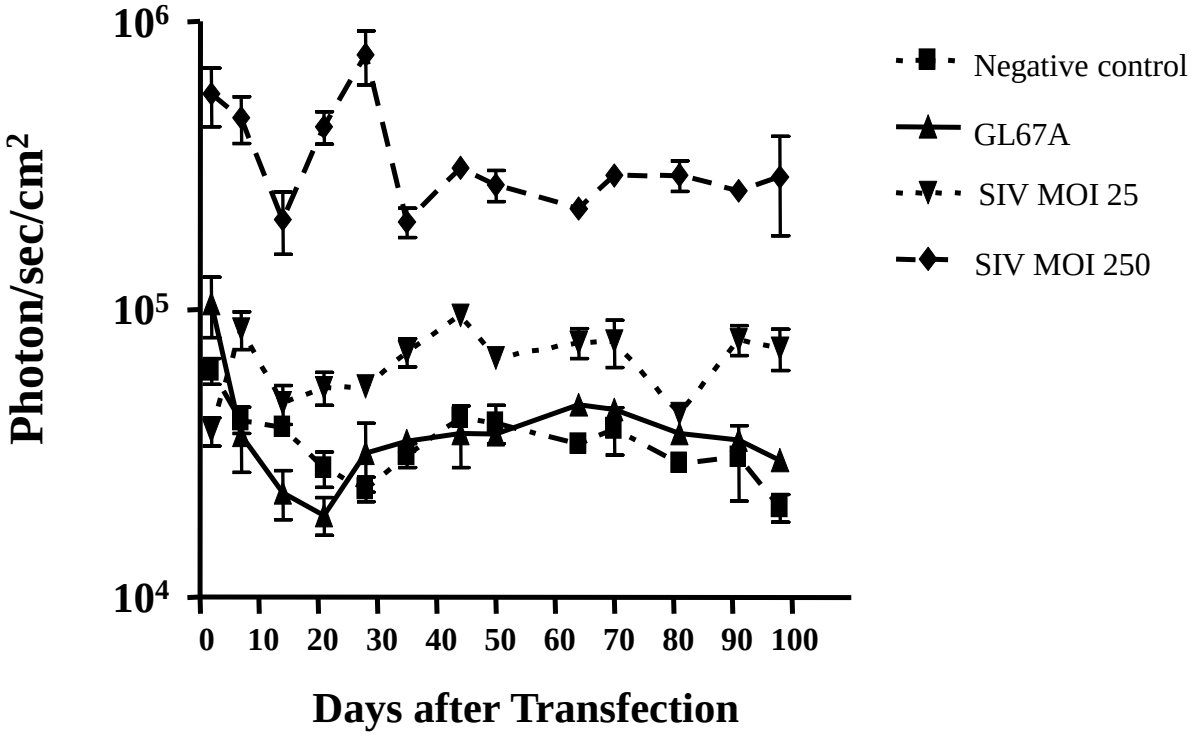
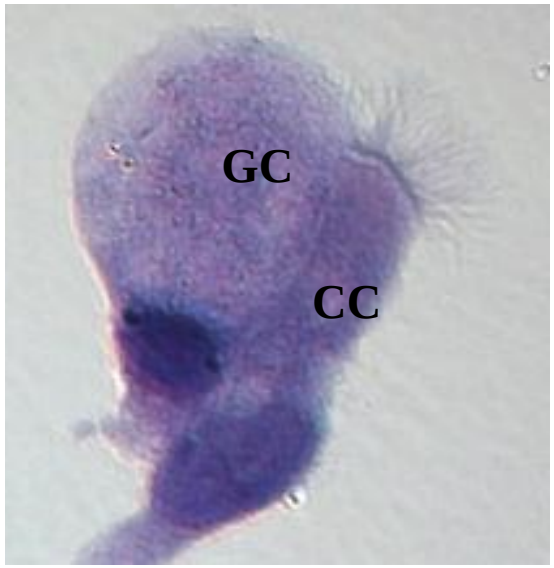

10^6
10^5
10^4
Photon/sec/cm^2
0 10 20 30 40 50 60 70 80 90 100
Days after Transfection
Negative control
GL67A
SIV MOI 25
SIV MOI 250

Figure 5B



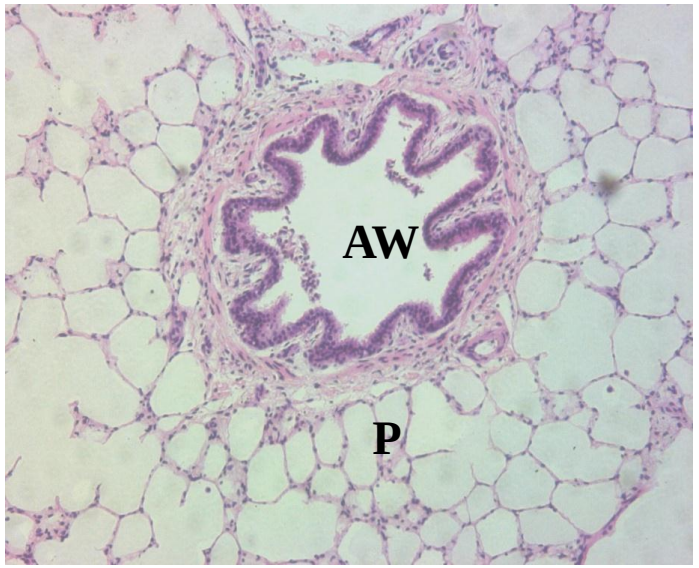


Figure 6A

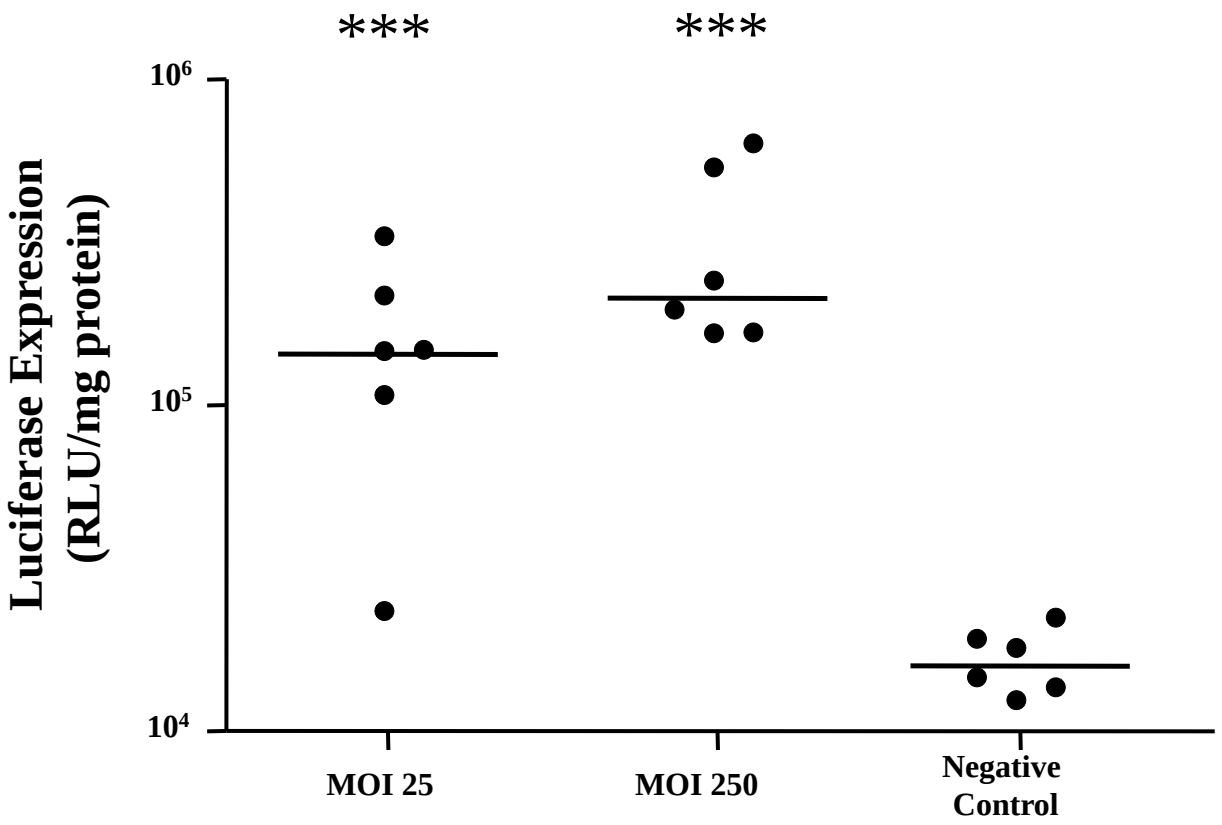

***
***
Luciferase Expression
(RLU/mg protein)
10^6
10^5
10^4
MOI 25
MOI 250
Negative
Control

Figure 6B

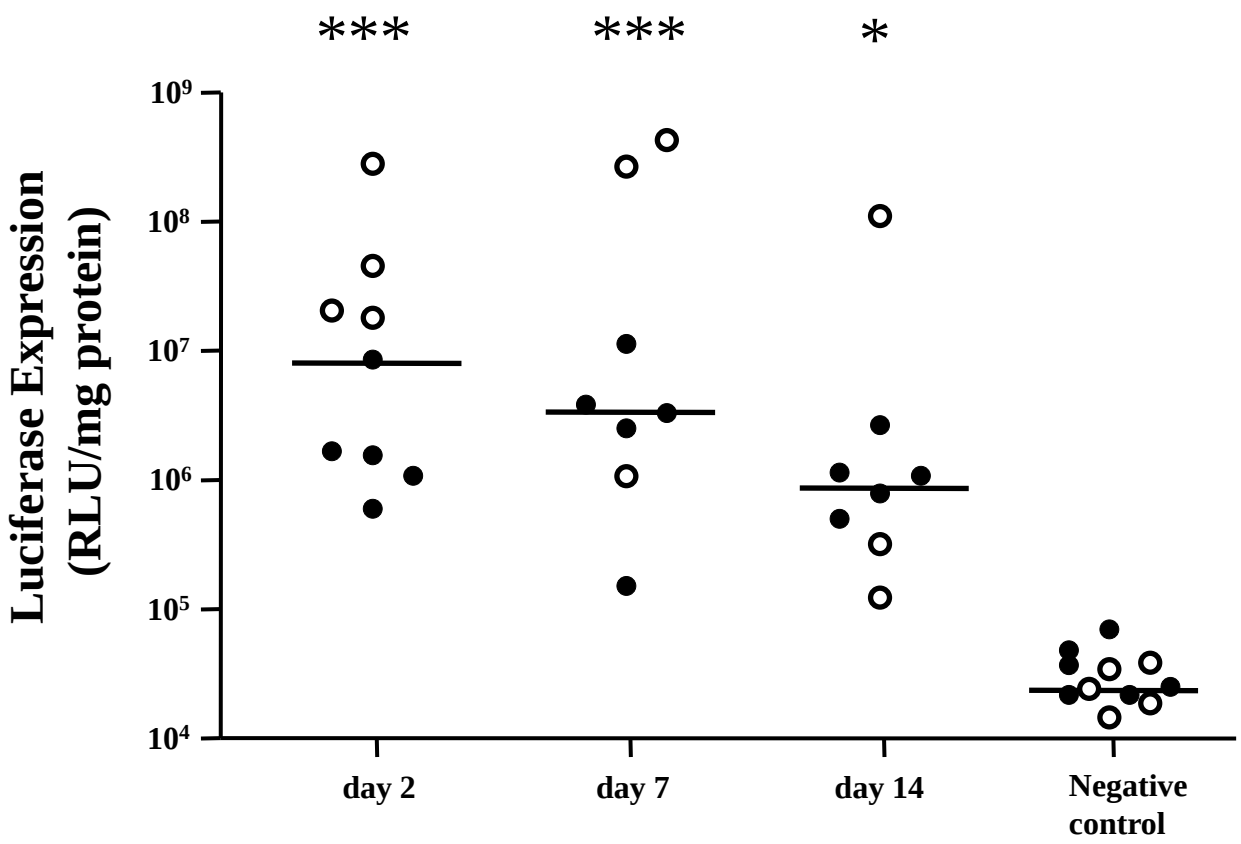

***
***
*
Luciferase Expression
(RLU/mg protein)
10^9
10^8
10^7
10^6
10^5
10^4
day 2
day 7
day 14
Negative
control

Figure 6C

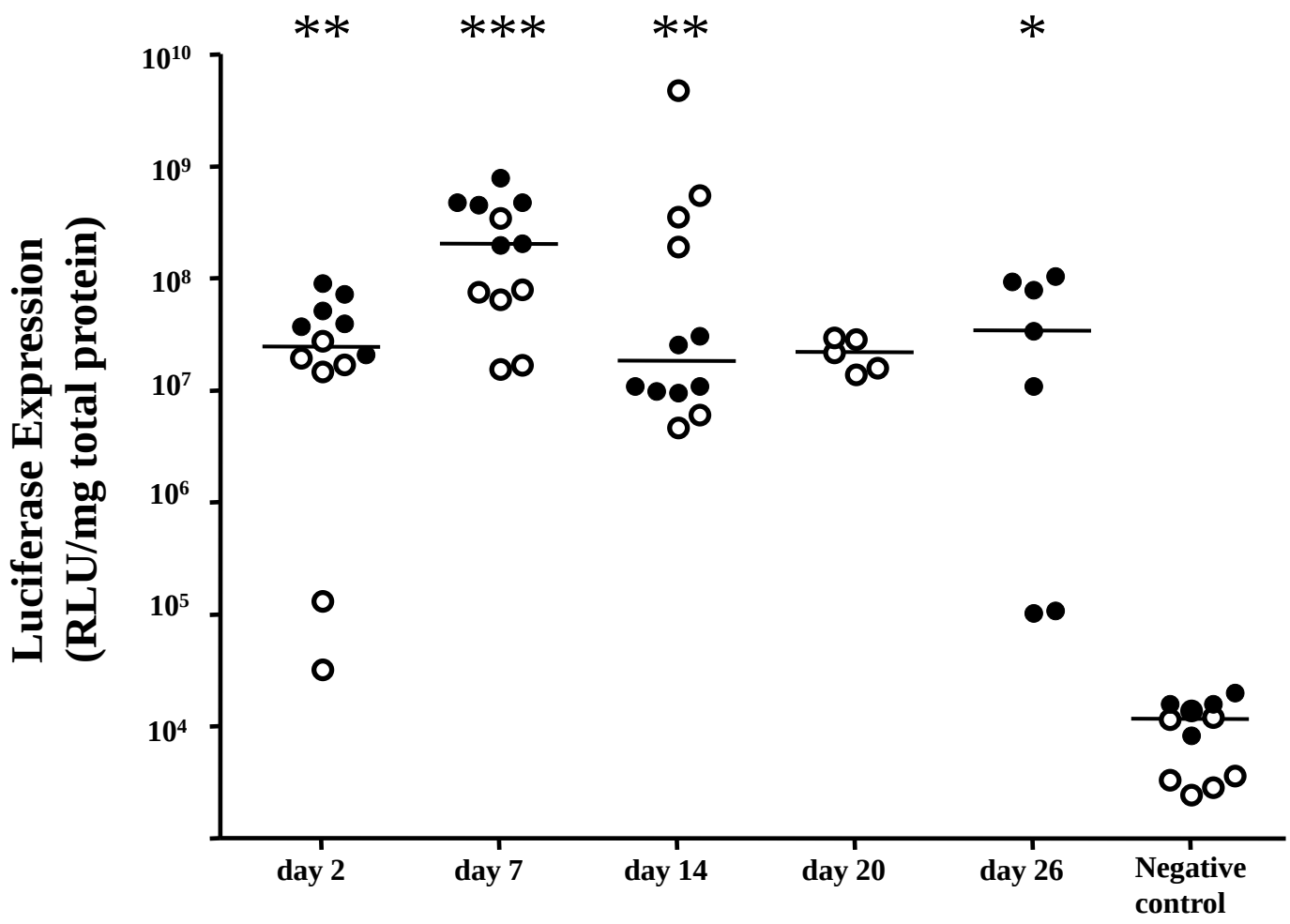

**
***
**
*
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
Luciferase Expression
(RLU/mg total protein)
day 2
day 7
day 14
day 20
day 26
Negative control

Figure 6D

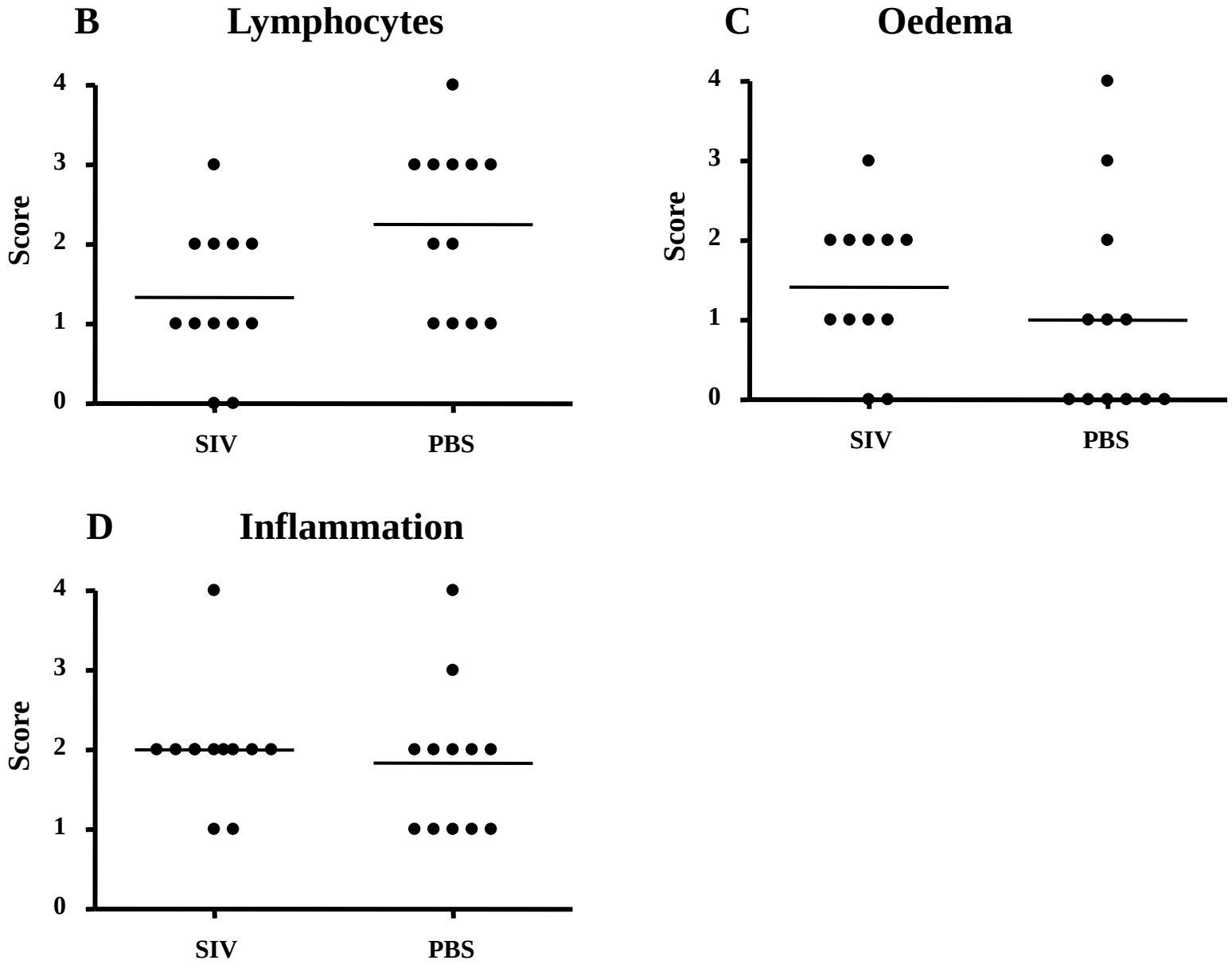

B
Lymphocytes
Score
4
3
2
1
0
SIV
PBS
C
Oedema
Score
4
3
2
1
0
SIV
PBS
D
Inflammation
Score
4
3
2
1
0
SIV
PBS

Figure 4 B,C,D

Months after treatment	PBS treated mice Weight (g)	SIV treated mice Weight (g)
6	24.9 (0.5) n=48	25.4 (0.3) n=71
15	30.7 (0.7) n=47	31.4 (0.8) n=35
24	30.1 (1.4) n=36	29.1 (1.1) n=12

Table 1: Mouse weights 6, 15 and 24 months after lung transduction with F/HN-SIV-Lux or PBS administration. Mean weight (±SEM) are shown. There were no differences between PBS and SIV treated mice.

Treatment	Death (Months after treatment	Diagnosis
PBS	15	Found dead
PBS	18	Lymphoma
PBS	19	Lymphoma
PBS	20	Lymphoma
PBS	20	Inconclusive
PBS	21	No pathology found
PBS	21	Lymphoma
PBS	23	No pathology found
PBS	23	Found dead
PBS	23	Lymphoma
PBS	24	Found dead
SIV	3	Peritonitis
SIV	13	Found dead
SIV	17	Found dead
SIV	19	Pancreatic tumour
SIV	21	Multi-organ inflammation
SIV	24	Found dead
SIV	24	Multifocal lymphocytic inflammation

Table 2: Cause of mortality of PBS and F/HN-SIV treated mice over a 24 months period