NB protein does not affect Influenza B virus replication *in vitro* and is not required for replication in or transmission between ferrets.

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Short title: NB is not required for replication or transmission of influenza B virus.

Abstract 196 words

The influenza B virus encodes a unique protein, NB, a membrane protein whose function in the replication cycle is not, as yet, understood. We engineered a recombinant influenza B virus lacking NB expression with no concomitant difference in expression or activity of viral neuraminidase protein, an important caveat since NA is encoded on the same segment and initiated from a start codon just 4 nucleotides downstream of NB. Replication of the virus lacking NB was not different to wild type virus with full length NB in clonal immortalized or complex primary cell cultures. In the mouse model, virus lacking NB induced slightly lower IFN α levels in infected lungs but this did not affect virus titres or weight loss. In ferrets infected with a mixture of viruses that did or did not express NB, there was no fitness advantage for the virus that retained NB. Moreover, virus lacking NB protein was transmitted following respiratory droplet exposure of sentinel animals. These data suggest no role for NB in supporting replication or transmission in vivo in this animal model. The role of NB and the nature of selection to retain it in all natural influenza B viruses remain unclear.

Introduction

Seasonal influenza illness results from infection of the upper respiratory tract epithelium by influenza A or B viruses. Influenza B viruses are generally less well studied than influenza A viruses and do not give rise to pandemics as they have no sustained animal reservoir (Jackson *et al.*, 2011). Nonetheless influenza B viruses cause a significant number of annual influenza cases and the divergence of two antigenically distinct lineages of influenza B virus has necessitated the recent inclusion of two influenza B strains into the annual vaccine, resulting in a quadrivalent product (Belshe, 2010; Rota *et al.*, 1990).

Influenza A and B viruses are orthomyxoviruses; both have two glycoproteins on the surface of the viral membrane, the Haemagglutinin (HA) and the Neuraminidase (NA), involved in viral entry by binding sialic acid receptors and release of progeny viruses by cleaving sialic acid respectively. The M2 protein is a third, minor membrane component of the influenza A virus particle. M2 is an ion channel protein and has several roles in the influenza A replication cycle, including disassembly, assembly and scission as well as modification of the autophagy pathway and activation of the inflammasome (Gannagé *et al.*, 2009; Hughey *et al.*, 1995; Ichinohe *et al.*, 2010; Iwatsuki-Horimoto *et al.*, 2006; McCown & Pekosz, 2005; Rossman *et al.*, 2010). Influenza B viruses encode a homologue known as BM2 with an ion channel similar in function to that of influenza A M2 required for uncoating and with a role in assembly of the influenza B virus particle (Horvath *et al.*, 1990; Imai *et al.*, 2004; Pinto *et al.*, 1992). Influenza B viruses uniquely encode a fourth surface glycoprotein, the NB protein (Shaw *et al.*, 1983).

NB is encoded by segment 6 of the influenza B virus, the same segment that encodes NA. The NB open reading frame overlaps that of NA, and the two AUG start codons are separated by only four nucleotides, with the NB start codon being closer to the 5' end of the mRNA (Shaw *et al.*, 1983).

NB is an integral membrane protein of 100 amino acids, found on the surface of infected cells (Brassard *et al.*, 1996). It has an 18 amino acid extracellular N-terminus, a 22 amino acid transmembrane domain, and a 60 amino acid cytoplasmic C-terminus. There are two glycosylation sites on the extracellular

domain that acquire high mannose sugars during post-translational processing. Multiple N-acetyl-lactosamine residues (Galβ1→4-GlcNAcβ1→3) are added to these carbohydrates to form complex structures, termed polylactosaminoglycans (Williams & Lamb, 1986, 1988). The NB glycoprotein forms a tetramer at the infected cell surface, and is incorporated in the virion during budding but only in relatively small numbers (~15-100 molecules per virion) (Betakova *et al.*, 1996; Brassard *et al.*, 1996).

The function of NB is currently unknown. The similarity of domain organization of NB and M2 proteins originally led to suggestions that NB may act as the functional homologue for the influenza A virus M2 ion channel, and data from voltage conductance studies supported this notion (Betakova *et al.*, 1996; Fischer *et al.*, 2000, 2001; Premkumar *et al.*, 2004; Sunstrom *et al.*, 1996). However the discovery that BM2, a protein encoded on influenza B virus RNA segment 7, served as the influenza B virus ion channel discounted this. Therefore it is likely that NB serves an alternative function for influenza B virus. Hatta and Kawaoka used reverse genetics to produce recombinant influenza B viruses that lacked NB, indicating that the protein is not essential for virus viability. Indeed viruses lacking NB replicated as well as wild-type (WT) virus in MDCK cells. Conversely, in BALB/c mice the replication of the mutant viruses was restricted compared to the WT (Hatta & Kawaoka, 2003).

To engineer the mutations that abrogated NB expression, Hatta and Kawaoka disrupted sequences around and including the NB AUG start codon. However given the close proximity of the start codon of NB to that of the essential viral protein NA, it was not clear whether the attenuated phenotype observed in mice for the NB deletions might result from an effect on NA expression (Williams & Lamb, 1989).

We therefore wanted to pursue the investigation of the role of the NB protein by using a NB mutant with intact NA gene expression. To achieve that, a novel mutant virus, Δ5NB, was generated. This virus had a premature stop codon after the fifth amino acid of the NB sequence leaving the sequence of NA and the upstream nucleotides that control NA translation levels unchanged. Using this viral mutant we sought to determine a role for NB *in vitro* or *in vivo*.

Results

Generation of a recombinant influenza B virus lacking expression of the NB protein.

Segment six of a 12 plasmid reverse genetics system based on B/Florida/04/2006 (Elderfield *et al.*, 2015) was modified to create a truncated NB protein. The Δ5NB virus was engineered by the introduction of a premature stop codon after the fifth amino acid of NB coding sequence by site directed mutagenesis. The NB open reading frame was reduced to five amino acids whilst the NA amino acids remained unchanged (Table 1). Both wild type and Δ5NB viruses were rescued and propagated in MDCK cells. We confirmed the lack of NB expression in cells infected with the Δ5NB mutant virus by western blot of lysates containing infected cells using an NB specific monoclonal antibody. This detected a heterogeneous range of NB proteins in lysates of cells infected with wild type virus ranging in size from 15 to 46 kDa as previously described (Williams and Lamb 1988) that were lacking in similar lysates from Δ5NB mutant virus-infected cells. Detection of equivalent amounts of BM2 and NP showed that levels of other viral proteins did not differ between the two viruses (Fig.1a).

The Δ5NB mutation did not affect virus neuraminidase activity.

To confirm that the mutations introduced in Δ5NB virus did not affect NA, enzyme activity associated with the mutant virus was measured using three different assays.

For the standard NA enzyme assay that employs a soluble substrate, the MuNANA assay, the wild type and mutant viruses were standardized to equivalent PFU and assessed over a range of dilutions for their ability to cleave the MuNANA reagent. There was no significant difference between the two viruses (Fig.1b).

In the red blood cell elution assay, we assessed the virus' ability to desialylate red blood cells. The two viruses were mixed with human red blood cells at equal HA titres and incubated at 4^{o}C for 1 hour to allow agglutination. The plates were

then shifted to 37^{o}C for 6 hours to permit NA activity and observed at regular intervals for the reversal of haemagglutination, which would indicate desialylation of RBCs by the viral NA. If virus eluted from the RBCs they formed a pellet. This effect was more evident at high titres of virus where more NA was present in the well. An end point titre for elution was measured. The two viruses exhibited the same degree of elution at all time points (Fig. 1c).

The mucus inhibition assay measured the ability of the viral NA to cleave abundant sialic acids found in mucus, which can act as decoy receptors, delaying viral infection (Blumenkrantz *et al.*, 2013). Mucus was harvested from the apical surface of well differentiated Human Airway Epithelial (HAE) cell cultures. Diluted mucus was mixed with 100 pfu of each virus prior to inoculating MDCK cells. During incubation at 37^{o}C on the MDCK cells, the viral NA cleaved sialic acids in mucus enabling infection of the cell monolayer. The infectivity remaining was determined by comparing the number of plaques formed by virus in the presence and absence of mucus. No significant difference in ability to overcome mucus inhibition was observed between the wild-type and ΔNB virus (Fig. 1d). Collectively, these results demonstrate that the Δ5NB mutation did not affect NA activity.

Δ5NB influenza B virus displayed no growth attenuation in a variety of cell types.

In order to measure any effect of loss of NB protein on virus replication kinetics we used human airway-derived Calu-3 cell cultures or primary human airway epithelium (HAE) cell cultures, the latter containing a mixture of ciliated and non-ciliated cells types, with an air interface at the apical surface onto which mucus is secreted. In initial experiments each cell system was inoculated by each virus type in separate wells at an equal MOI and the comparative growth was assessed at set time points by plaque assay on MDCK cells. Both systems were highly permissive for influenza B virus replication with peak titres in excess of 10^{7} pfu/ml in Calu-3 and 10^{8} pfu/ml in HAE cultures. Δ5NB virus was not attenuated in either cell system (Figure 2 a and b). Consensus whole genome sequencing of virus generated during multi-step replication confirmedno

sequence variation between the wt and Δ5NB viruses except for the specifically introduced truncation.

Competition assays have increasingly been used to assess small variations in fitness between two influenza viruses not observable by traditional viral growth curves (Brookes *et al.*, 2010; Guarnaccia *et al.*, 2013; Yen *et al.*, 2014). To establish whether lack of NB has a fitness cost for the Δ5NB mutant virus compared to the WT virus, we infected HAE cells with a mixture of 20% WT and 80% Δ5NB virus at a final moi of 0.001. After multi-cycle replication over several days, the relative abundance of the two genotypes in virus harvested from the apical surface was measured using a pyrosequencing reaction. At no time point was there a statistically significant difference in the ratio of WT to Δ5NB from the ratio of genotypes in the input inoculum, after 4 days replication the Δ5NB genotype was still present at 80% of the RNA population (Fig. 2c). These results indicate that the NB protein does not offer a selective advantage *in vitro* even in a complex cell culture system in which deficiency in NA is readily measured (Brookes *et al.*, 2010).

The Δ5NB recombinant virus is not attenuated in mice *in vivo*.

C57BL/6 mice were infected intranasally with $4.5x10^5$ pfu of either WT or Δ5NB virus. The weight loss of the mice was monitored daily. Viral titre, IFN-α and IL-1β in the lungs were determined on day 2, 3 and 4 post infection. As shown in Fig. 3a, the two viruses replicated well in mouse lungs, with the Δ5NB displaying slightly higher growth 3 dpi ($p=0.007$) (Multiple T-test [Holm-Sidak method]).
As a gross measurement of pathology, weight loss was monitored (Fig. 3b). On all days mice infected with the wild-type virus displayed a slightly greater weight loss but the difference between the two groups was not significant (Multiple T-test [Holm-Sidak method]).
As an indication of the innate immune response, IFN-α in the mouse lungs was measured at days 2,3, and 4 post infection. Significant production of IFN-α in the murine lungs was only evident on day 2 after infection. There was no difference

202 in the interferon levels in the mice infected with WT virus or those infected with
203 the Δ5NB virus, except a slight increase for the Δ5NB infected mice on day 4
204 (p=0.005) (Multiple T-test [Holm-Sidak method]) (Fig. 3c). Activation of the
205 inflammasome was assessed by measuring levels of IL-1β. Levels of pro-IL1β and
206 total IL-1β (pro form plus activated form) were measured. Both sets of infected
207 mice had higher total IL-1β levels in their lungs than the mock infected animals
208 and this was significant at all days measured (Multiple T-test [Holm Sidak
209 method]). Total IL-1β protein levels tended to be higher in the lungs of mice
210 infected by Δ5NB virus, but this difference was not significant.
211
212 **Virus lacking NB expression replicates in ferrets and is transmitted by the**
213 **respiratory droplet route.**
214 We tested whether the loss of NB affected replication within a single host or
215 transmission onwards to a new host by inoculating ferrets with a mixture of wild
216 type and Δ5NB viruses, and monitoring the ratio of genotypes within and
217 between hosts using pyrosequencing. Four donor ferrets were inoculated with a
218 1:1 ratio of a total of 10^4 pfu wild-type and Δ5NB virus. All four donors were
219 robustly infected with the virus mixture and shed infectious virus in their nasal
220 wash over the following 6 or 7 days. Peak viral titres were shed on day 2 and
221 were between 10^5 and 10^6 pfu/ml nasal wash. Shedding followed a biphasic
222 kinetic profile similar to that we have previously reported for ferrets infected
223 with influenza A viruses (Roberts *et al.*, 2011) (Figure 4a). Pyrosequencing of
224 RNA isolated from the daily nasal washes showed that the relative proportions of
225 the two virus genotypes did not vary by more than 11% from the starting mix in
226 any of the four directly inoculated hosts and the direction of the small variation
227 was sometimes to favour wild type genomes encoding full NB and sometimes
228 Δ5NB mutant genomes (Figure 4 b and c).
229 Only one of the four respiratory droplet (RD) exposed sentinel animals acquired
230 infection following exposure to the infected donor. Pyrosequencing of the nasal
231 washes from this animal revealed that the virus it had acquired was uniquely the
232 Δ5NB genotype (Figure 4b). This infected recipient continued to shed Δ5NB
233 virus for 4 days from day 6 to day 9 after it was first exposed. Peak titre in nasal
234 wash from this animal was 10^5 pfu/ml .

Discussion

The NB protein is absolutely conserved across influenza B viruses, a finding that might indicate it is required for effective replication or transmission. However our ability to rescue and propagate a recombinant influenza B virus that lacks expression of NB supports the previous conclusion from Hatta et al., (Hatta & Kawaoka, 2003) that NB is dispensable for virus replication *in vitro*. The design of our mutant virus allowed us to investigate the unique role of NB because we abrogated NB expression with no concomitant effect on the NA function or activity. Although the set of experiments we describe here does not elucidate the function of NB, it does show that NB is not required for replication or transmission of influenza B virus *in vitro* or *in vivo* in mice or ferret models. Previously loss of NB was correlated with attenuation of replication *in vivo* in the mouse lung and nasal turbinate and an increase in MLD_{50}, but it could not be excluded that this phenotype arose from a change in NA expression. In our study with a NB mutation that did not compromise NA, there were minimal differences in viral titre and weight loss and only slightly higher levels of IFN-α in the mice infected with the truncated NB protein. We looked carefully at the levels of IL-1β that result from activation of the inflammasome response because other small hydrophobic viral proteins have been implicated in regulation of inflammasomes (Triantafilou *et al.*, 2013). For example, deletion of the SH protein of respiratory syncytial virus, RSV, which is similar in size and domain distribution to NB, led to increased induction of proinflammatory cytokines including IL-1β, and deletion of SH is an attenuation strategy for bovine and human RSV vaccines under development (Karron *et al.*, 2005; Taylor *et al.*, 2014). On the other hand the ion channel proteins of other viruses such as M2 of influenza A and E protein of the coronavirus SARS, appear to stimulate the inflammasome and their deletion leads to a decreased IL-1β response (Ichinohe *et al.*, 2010; Nieto-Torres *et al.*, 2014). In our experiments, loss of NB expression had a marginal effect on IL-1β expression in the mouse lung. We cannot exclude that a role for NB in modulating inflammasome activation was masked in the mouse model by other factors, or that the effects of NB are mouse strain, host species or cell type specific for this human pathogen.

Very recently, Kim et al. published the first study of transmission of influenza B virus in ferrets (Kim *et al.*, 2015). In line with our data, they also report low transmission efficiency of the B/Florida/04/2006 virus in the ferret model, . in contrast to a more efficient influenza B virus respiratory droplet transmission previously reported between guinea pigs (Pica *et al.*, 2012). The frequency of transmission events we recorded between RD exposed ferrets was surprisingly low, at just one of 4 exposed animals acquiring infection. We do not yet know if this observation will prove common to a number of influenza B viruses or whether transmission efficiency varies amongst different influenza B virus strains. The influenza B virus we used replicated well in the upper respiratory tract of ferrets and in well differentiated cultures of ferret airway cells (Elderfield *et al.*, 2015). The low transmission efficiency of a seasonal influenza virus suggests either that this animal model does have limitations for the study of influenza B viruses, or that some successful human influenza viruses are not necessarily transmitted primarily by this RD route. As far as the NB protein is concerned, we found that in the single transmission event we did record, the virus that transmitted was uniquely of the genotype that lacked expression of NB. Because we set up mixed infections and both mutant and wild type virus were replicating simultaneously in the donor animals we cannot absolutely exclude that the Δ5NB virus genome was not complemented by wild type NB protein during the transmission event. However our data unequivocally show that NB is not required for replication in the ferret model.

A recent paper also used a reverse genetics approach to probe for a role for NB. Rather than creating a completely null mutant, they chose to mutagenize the cytoplasmic tail of the protein and in particular abrogate the post translational modification of palmitoylation at residue 49 from cysteine to serine (Demers *et al.*, 2014) . Surprisingly in view of our data and that of Hatta et al., that suggested no role *in vitro* for NB, Demers et al., found that their palmitoylation mutant was attenuated for replication in MDCK cells. It may be that the continued expression of an unnecessary and mutated membrane protein interfered with the normal assembly of virus particles, but whether this result

verifies a role for the non-mutated NB protein in wild type influenza virus infections *in vitro* is not clear.

Bearing in mind the absolute conservation of NB in all influenza B viruses isolated from clinical cases and even in strains such as influenza B/Lee/40 that have been passaged in eggs and cell culture for more than 75 years, it is highly likely that this protein does serve a role for the virus. However using current technologies we have been unable to measure a fitness cost in viruses engineered to lack the protein either *in vitro* or *in vivo*.

Materials and Methods

Cells: Madin Darby Canine Kidney (MDCK) cell line and the Human lung adenocarcinoma derived cell line (Calu-3) were maintained in DMEM (Gibco-Life technologies) supplemented with 10% foetal bovine serum, non-essential amino acids and penicillin/ streptomycin (Gibco- Life technologies) at 37C with 5% CO2 and originated from the ATCC. The Human Airway Epithelial cells (Mucilair™ HAE) were purchased from Epithelix, and were maintained with the proprietary Mucilair medium.

Viruses: The B/Florida/04/2006 virus was rescued by reverse genetics from plasmids containing cDNA synthesized de novo directly from the database sequence (GenBank Accessions: CY033876, CY033877, CYO33878, CY033879, CY0330880, CY033881, CY033882, CY033883)(Elderfield et al., 2015); the Δ5NB mutant was generated by site-directed mutagenesis of the segment 6 plasmid. The viruses were cultured and titred in triplicate by plaque assay on MDCK cells.

Cell infections: For growth kinetic experiments in CALU-3 cells, the cells were washed with PBS, virus was added at an MOI of 0.001 in DMEM. The inoculated cells were incubated for 1 hour at 37oC prior to removal of the inoculum, washed with PBS and overlayed with DMEM containing NEAA, P/S and 1µg/ml TPCK-trypsin (Worthington Biosciences). Time points were taken at 24, 48 and 72 hours post infection. For the growth kinetics and competition assays in human airway epithelial cultures, the apical surfaces of the HAE cell cultures were washed by a 30 min incubation in 250µl of DMEM. After removal of the medium, virus was added at an MOI of 0.001 (for the competition assay with a mixture of

20% WT and 80% ΔNB virus) in a volume of 200µl of DMEM. After one hour, the inoculum was removed and the cell layer washed with DMEM. The cultures were incubated at 37°C at 5% CO2 and at 24, 48, 72 and 96 hours post infection, virus released from apical surface was collected in 200µl DMEM

Virus Sequencing: Viral RNA was extracted using the Qiagen QIAmp RNA kit. Reverse transcription was conducted using random hexamers and Superscript III (Invitrogen). DNA amplification was conducted using KOD polymerase (Novagen) and non-coding region segment specific oligomers. DNA bands generated were excised from agarose and cleaned using a Qiagen gel extraction kit. Sequencing was conducted by the GATC sequencing service using the previously mentioned oligomers and additional internal oligomers. The data generated was analysed by alignment using Geneious software. All oligomer sequences are available on request.

Ferret infections: Animal studies were conducted as previously described (van Doremalen et al., 2011) Female ferrets (14–16 weeks old) were used. Body weight was measured daily. After acclimatization the ferrets were anaesthetized with ketamine (22 mg kg−1) and xylazine (0.9 mg kg−1) then inoculated intranasally with 10^4 PFU of a mix of the two RG variants of the B/Florida/04/2006 virus diluted in DMEM (0.1 ml per nostril). All animals were nasal washed daily, while conscious, by instilling 2 ml PBS into the nostrils, and the expectorate was collected in 250 ml centrifuge tubes. Infectious virus was titrated by plaque assay of the nasal wash on confluent MDCK cells.

Mouse infections: 6-8 week-old female C57BL/6 mice, anesthetized with isoflurane, were intranasally infected with 40µl containing $4.5x10^5$ pfu of WT or Δ5NB virus or with 40µl of PBS. Lungs were isolated and homogenized in 1 ml of PBS using the Minilys homogenizer (Bertin Technologies) and the Precellys Ceramic Kit 1.4mm (PeqLab). Virus titres in the lungs were determined by plaque assay on MDCK cells.

ELISA: To detect the cytokines in the homogenized mouse lungs, the VeriKine™ Mouse Interferon Alpha ELISA Kit (Pbl Interferon Source) was used to determine IFN-α levels, and R & D systems Quantikine ELISA kit for IL-1β.

SDS-PAGE and Western: MDCK cells were inoculated with B/Florida/04/ 2006 WT, Δ5NB or PBS at a MOI of 3 and incubated overnight at 37°C. The cells were

then lysed and the proteins were separated on a 12% SDS-PAGE gel and then transferred to a membrane. The membrane was blocked in Odyssey blocking buffer (LI-COR Biosciences) and then stained with a mouse anti-NB monoclonal antibody (a kind gift from Dhan Samuel PHE Colindale UK) and rabbit anti-BM2 (Jackson et al., 2004) and anti-NP primary antibodies and then with an anti-mouse Dylight 680 and anti-rabbit Dylight 800 conjugated secondary antibodies (Cell signalling technologies). The LI-COR detection system was used, allowing both NB and BM2 to be detected in the same gel lane, the image has been split to display both proteins in grayscale, an equal amount of lysate was loaded onto two additional lanes and stained with anti-NP.

Pyroseqeuncing: Viral RNA was extracted from the HAE or ferret nasal wash using the Qiagen QIAmp RNA kit. A 50-cycle PCR reaction was performed using the following primers: 5' GCCAAAAATGAACAATGCTACCT and 3' CTGATGTGAGAAATAGGGTTAACG. The amplified product was mixed with sepharose beads at 1400 rpm for 10mins. The beads were sequentially washed in 70% ethanol, 0.2M NaOH and Wash Buffer and transferred to a plate containing the primer: AAAATGAACAATGCTACCT for sequencing on the QIAgen PyroMark Q96 ID pyrosequencer.

MuNANA Assay: As described in section 6 of the protocol (Lackenby, 2012) MuNANA substrate (2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid, sodium salt hydrate) (Sigma-Aldrich) was diluted to 100μM in MES buffer and added to a black 96-well plate (Matrix Microplates, Thermo Scientific) containing equal PFU ($4x10^6$ in 50ul) of WT and Δ5NB B/Florida/04/2006 serially diluted two-fold in MES buffer. The reactions were shaken for 60 mins at 37oC prior to the addition of a glycine/ethanol stop solution. The plate was then read using FLUOstar OPTIMA (BMG Labtech) with a UV excitation filter set at 355nm and emission filter at 460nm. This was conducted in triplicate with two different stocks of each virus.

Mucus Inhibition Assay: Mucus was collected from the apical surface of HAE cells by incubating with equilibrated DMEM for 30 mins at 37^oC. 100 PFU of WT or Δ5NB virus were mixed with either 1:70 diluted mucus or virus diluent (PBS+0.35% BSA), these mixtures were used to infect confluent MDCK cells (in triplicate). At one hour post infection the cells were washed with PBS and

overlaid with plaque overlay media. After 72 hours, the cells were fixed and stained with crystal violet and the plaques counted. The infectivity remaining was calculated as the percentage of virus in mucus over the virus in diluent alone.

RBC Elution Assay: 16 HA units of the B/Florida/04/2006 WT or Δ5NB virus were serially diluted 2-fold in PBS in triplicate in a 96-well plate. 50µl of 0.5% fresh human red blood cells were added and the plate was incubated at 4°C for 1 hour before a temperature shift to 37°C for 4 hours, the elutions were read hourly until the end point at 6 hours.

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542 Table 1.

WT Nucleotide Sequence	AATGAACAATGCTACCTTCAACTATA…
Δ5NB Nucleotide Sequence	AATGAACAATGCTACCTAGTACTATA…
WT NB Amino Acid Sequence	M N N A T F N Y...
Δ5NB NB Amino Acid Sequence	M N N A T STOP
WT NA Amino Acid Sequence	M L P S T I...
Δ5NB NA Amino Acid Sequence	M L P S T I...

543

544

545 Figure Legends

546 **Table 1. Nucleotide sequence alterations engineered to generate an**

547 **influenza B virus that lacks NB expression.** The initiating methionines for the

548 NB and NA proteins are single underlined and the mutations that lead to

549 NBtruncation are double underlined.**Figure 1. Characterisation of a virus**

550 **lacking expression of NB.**

551 **(a) Western blot to assess NB expression**.

552 Wild-type (wt) and mutated (Δ5NB) influenza/B/Florida/04/2006 viruses were

553 used to infect MDCK cells at a high MOI (3). After 24 hours the cells were lysed,

554 the proteins separated by SDS-PAGE and transferred by western blot.

555 Membranes were immunostained with a mouse anti-NB protein antibody (top

556 panels) and a rabbit anti-BM2 protein antibody (bottom panels), the blot was

557 detected using the LI-cor system and the two panels separated for clarity.

558 **(b) MuNANA enzyme assay to assess NA activity.**

559 Wild-type (wt) or mutated (Δ5NB) influenza B/Florida/04/2006 viruses were

560 serially diluted in MES buffer, MuNANA reagent was added, the reaction was

561 allowed to continue for one hour prior to the addition of stop solution. The plate

562 was read with UV excitation filter of 355nm and emission filter of 460nm.

(c) Mucus inhibition assay to assess NA activity.

100 pfu influenza B/Florida/04/2006 wild-type (wt) or mutant (Δ5NB) virus were incubated in human airway mucus or virus diluent. The inoculants were then used to infect confluentMDCK cell monolayers and incubated under solid overlay to allow plaque formation , . The percentage reduction in plaques formed in the presence ofmucus was calculated.

(d) Red Blood Cell Elution Assay to assess NA activity.

Influenza B/Florida/04/2006 wild-type (wt) or mutant (Δ5NB) virus were serially diluted in PBS. 50µl of 0.5% fresh human red blood cells were added and the plate was incubated at 4^{o}C for 1 hour before a temperature shift to 32^{o}C for 4 hours. The plates were observed hourly for evidence of haemagglutination of RBCs until the end point at 6 hours.

Figure 2. Growth kinetics of virus lacking NB expression on human derived clonal or primary differentiated cell lines.

(a) Influenza B/Florida/04/2006 wt or mutant (Δ5NB) viruses were used to infect Calu-3 cells at a low MOI (0.01). At 24 hour intervals the viral titre released was assessed by titration on MDCK cells by plaque assay.

(b) Influenza B/Florida/04/2006 wt or mutant (Δ5NB) viruses were used to infect Mucilair HAE cell cultures at a low MOI (0.001). Timepoints were taken by the addition of media to the apical surface and after 30 mins the media was removed and the viral titre was assessed by titration on MDCK cells by plaque assay.

(c) Competition assay assessing the growth kinetics of virus lacking NB expression on human differentiated primary cell cultures. Influenza

588 B/Florida/04/2006 wt or mutant (Δ5NB) viruses were used to infect Mucilair
589 HAE cell cultures at an MOI of 0.001 with a mixture of 20% WT and 80% Δ5NB
590 virus. At 24, 48 and 72 hours post infection, virus released from apical surface
591 was collected in 200ul of DMEM and after RNA extraction subjected to
592 pyrosequencing to assess the relative percentages of the wt and truncated NB
593 forms.
594
595 **Figure 3. Infection of mice with influenza B virus lacking NB expression.**
596 (a) Infectious titres of influenza B virus lacking NB expression in lungs of
597 infected C57BL/6 mice. 6-8 week-old female C57BL/6 mice were infected with
598 $4.5x10^5$ pfu of WT B/Florida/04/2006 or Δ5NB virus or inoculated with PBS. At
599 days 2,3, and 4 post infection lungs were isolated and homogenized in 500µl of
600 PBS prior to virus titres being determined by plaque assay on MDCK cells.
601 (b) Weight loss in influenza B virus infected mice.
602 (c, d & e) Cytokine induction in C57BL/6 mice infected with wt orΔ5NB mutant
603 influenza B virus. Interferon αlpha (c), IL-1β pro (d) and IL-1β (e) in the mice
604 lungs were measured by ELISA of the homogenised lungs from (a).
605
606 **Figure 4. Infection of ferrets with influenza B virus.**
607 Four ferrets (D1-D4) were inoculated with 10^4 PFU of an equal mixture wt and
608 truncated NB virus. One day post infection four naive ferrets were placed in
609 adjacent cages as respiratory droplet (RD) sentinels (RD1-RD4). (a) Infectious
610 virus titres shed in nasal wash collected each day after infection or exposure
611 established by plaquing on MDCK cells. (b) The relative proportions of the two
612 virus genotypes in daily nasal washes from a paired donor ferret D4 and

613 infected exposed ferret RD4 assessed by pyrosequencing. (c) The relative

614 proportions of the two virus genotypes in daily nasal washes from the remaining

615 3 donor ferrets D1 D2 and D3.