Supplemental information

SI Materials and Methods

Generation of Nanobodies A *Pichia pastoris* expression system was developed, based on the commercially available pPICZαA system from Invitrogen, using X-33 as a host strain, the AOX1 promoter controlling the expression and the alpha mating factor secretion peptide for secretion of the Nanobody into the medium (Figure S1). After transformation the clones were selected on zeocin-containing plates, streaked on a new zeocin-containing plate and a qPCR was performed to rank the clones according to their copy numbers. Single colonies were generated from a single clone with high copy number. A research cell bank was generated using only non-animal derived raw materials

Microneutralisation assay. HEp-2 cells (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS, Sigma) and 1% penicillin/streptomycin (Invitrogen). They were kept at sub-confluent growth conditions until the start of the assay. On the day before the assay HEp-2 cells were seeded in 96 well culture plates at a density of 22,500 cells/well and kept overnight at 37°C in a 5% CO_2 incubator. On the day of the assay RSV-Long (ATCC VR-26™) or RSV-B 18537 (ATCC VR-1580™) (0.05-0.15 MOI) was pre-incubated 30 min with various concentrations of ALX-0171 or palivizumab. The mixtures were then overlaid on the HEp-2 cells and incubated for 1 h at 37°C following which 100 µL of DMEM assay medium was added to each well to give a total volume of 150 µL. Subsequently, the HEp-2 cells were incubated for 72 h at 37°C in a 5% CO_2 incubator, washed twice with D-PBS and fixed with cold 80% acetone for 20 min at 4°C. After blocking with D-PBS containing 2% BSA, the viral F-protein was detected using an

anti-F-protein monoclonal antibody (Synagis®, Abbott) followed by horse-radish peroxidase-labelled Goat anti-human IgG Fcγ-specific monoclonal antibody. Colorimetric revelation was performed using sTMB (SDT-reagents) and stopped after 10 min with 1N HCl. The F protein was then indirectly quantified by measuring the absorbance at 450 nm with a reference wavelength at 620 nm. The IC_{50} values were calculated from the dose-response curves using a 4 parameter logistic nonlinear regression model with GraphPad Prism 5 software.

Viral neutralisation assay. Serial 3-fold dilutions of ALX-0171 or palivizumab were prepared in triplicate in Dulbecco's MEM with 1% foetal bovine serum (Gibco, UK) and were added to 96-well flat-bottomed tissue culture plates (Nunc). Approximately 100 $TCID_{50}$ of RSV-A or –B in 50 µL of medium was added to each well, and plates were incubated at 37°C for 1 h in a 5% CO_2 incubator after which 10^4 HEp-2 cells (kindly provided by Ralph Tripp, University of Georgia, USA) were added to each well. After incubation for 6–8 days at 37°C, cytopathic effect was recorded for each well and the VN_{50} titers were calculated using the Reed and Muench method [1].

Plaque reduction assay for clinical isolate testing. A semi-quantitative plaque assay in a 24-well plate with complete HEp-2 cell monolayer was used to calculate the neutralization capacity of ALX-0171 and palivizumab at 40 µg/mL against the RSV isolates. The 24-well plates were seeded with 20 x 10^4 HEp-2 cells/mL 1 to 2 days prior to the assay and used when a complete monolayer had formed. Briefly, the RSV working pool was diluted to generate a virus concentration of approximately 10^5 PFU/mL. RSV working pools with concentrations of less than 10^5 PFU/mL were tested neat without further dilution. ALX-0171 and palivizumab were diluted in MEM to make a final concentration of 40 µg/mL. One mL (10^5 PFU) of virus was mixed with an equal volume of MEM, ALX-0171 or palivizumab so that three different virus-antibody mixtures were generated; virus-only (RSV & MEM), virus + ALX-0171 and virus + palivizumab (reference). These virus-antibody mixtures were incubated at 36°C at 5%

CO_2 for 1 h to allow neutralisation of virus to occur. In order to determine the reduction in viral infectivity that occurred during the incubation period, serial ten-fold dilutions of the virus-antibody mixtures were made in MEM. Undiluted, 10^{-1}, 10^{-2} and 10^{-3} dilutions were dispensed in duplicate into a 24-well plate with a complete HEp-2 cell monolayer. Infectious virus in the virus-antibody mixture would be able to attach and infect the HEp-2 cells during the subsequent 2 hour incubation onto the cell monolayer. After the 2 hour incubation, the virus-antibody mixture was aspirated, replaced by a methylcellulose overlay, and incubated for 6 to 7 days at 36°C at 5% CO_2 when viral plaques are easily visualised. The cell-virus monolayer was fixed overnight, air dried and plaques were counted using a plaque reader. The lower limit of detection was 5 pfu/ml. Samples with no visual plaques were assigned a value of 2.5 PFU/mL for statistical analysis.

Nebuliser set-up for cotton rat study. Akita² Apixneb nebulizer (Vectura GmbH, Germany) was used for whole body exposure. Air flow was supplied by an Aridyne 2000 compressor with a flow meter set at 15 L/min. Cotton rats were housed 2 per cage with 3 cages linked together in series (a total of 6 cotton rats were treated per run) (Figure S2). They were exposed to an aerosol from a single nebulizer containing 9 mL of formulation buffer or ALX-0171 at 5 mg/mL, 15 mg/mL or 50 mg/mL. Aerosol was collected from the exhaust using an all glass impinger to calculate estimated delivered dose. The nebulizer was allowed to run for 10-13 minutes until the nebulizer reservoir ran dry.

Determination of viral titers in lung lavage and nasal wash in cotton rats. Viral titers were determined by a plaque assay. These were performed using 24-well tissue cultures plates containing nearly confluent monolayers (seeded at $2x10^5$ cells/well) of HEp-2 cells prepared in 10% Foetal bovine serum (FBS) 24 h prior to the start of the assay. Dilutions (serial $\log_{10}$) of the nasal or lung lavage fluids were then made and 0.2 mL of each sample was added in duplicate to the wells. Adsorption was allowed for 90

min in a 36°C, 5% CO_2 incubator with occasional gentle agitation before removal of the inoculum and overlay of the monolayers with 0.75% methylcellulose in 2% FBS-MEM containing antibiotics, vitamins and other nutrients (100 units penicillin, 100 µg/mL streptomycin, 0.25 µg/mL Fungizone and 2 mM glutamine from GIBCO). Tissue culture and positive virus controls were included in each assay. After culturing for 6 $\pm$ 1 days in a 36°C, 5% CO_2 incubator, the plates were stained with 0.01% crystal violet/10% formalin solution (1.5 mL/well) and allowed to sit for 24-48 h at RT before being rinsed. All wells containing between ~20 and ~100 plaques were enumerated, averaged and the virus titers calculated as $\log_{10}$ total PFU for nasal wash fluid or $\log_{10}$ PFU/g of tissue for lungs. As the lung titers are calculated as “per g of lung tissue” there is a range of values for both minimum detection levels and the assigned value for no plaques as slightly different amounts of lung tissue is removed from each animal. Minimum detection (i.e., only 1 PFU in either duplicate well) is therefore estimated to be in the range of 1.03-1.21 PFU/g lung. For statistical analysis, no detection (0 plaques) in the lung tissue was assigned a value that was the mean of minimal detection values per experiment. For the nose, 0 plaques were assigned a value of 0.40 $\log_{10}$ total PFU for nose.

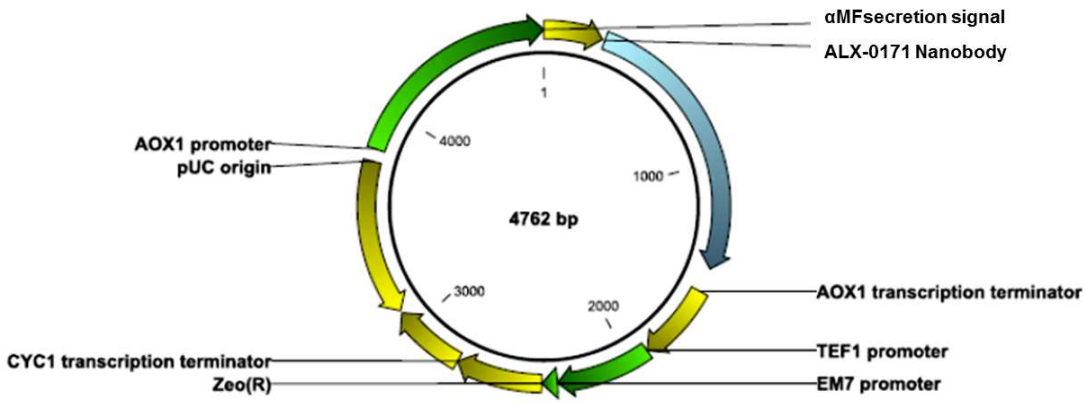

Figure S1. Map of the expression construct used for transformation of *Pichia pastoris* X 33.

Table S1. RSV strains by year of isolation (selected from the RSV bank at Baylor College of Medicine, Houston Texas) [2]

RSV-A Virus name	Genotype classification	Month/Year of Isolation	Age of patient at time of Collection
A-TX-Tracy	GA1	Jan-89	
A-TX-66125	GA5	Mar-94	6 mos.
A-TX-37425	GA3/GA6/SAA1	Dec-87	
A-TX-79312	GA2	Jan-05	8 mos
A-TX-79258	GA2	Dec-04	8 mos.
A-TX-79218	GA5	Nov-04	1 yr.
A-TX-79257	GA5	Dec-04	6 mos.
A-TX-79321	GA2	Jan-05	2 mos
A-TX-79228	GA5	Nov-04	9 mos
A-TX-79309	GA2	Jan-05	9 mos.
A-TX-79248	GA5	Nov-04	2 mos.
A-TX-79219	GA5	Nov-04	1 yr.
A-TX-79308	GA2	Jan-05	6 mos.
A-TX-50437	GA5	Jan-91	59 yrs.
A-TX-68357	GA5	Dec-94	2 mos.
A-TX-79365	GA5	Feb-05	4 mos.
A-TX-79306	GA5	Jan-05	7 mos.
A-TX-79254	GA5	Dec-04	4 mos.
A-TX-79240	GA5	Nov-04	9 mos.
A-TX-61245	GA5	Feb-93	7 mos.
A-TX-79299	GA2	Jan-05	5 mos.
A-TX-79326	GA5	Jan-05	2 mos.
A-TX-50106	GA5	Jan-91	1 yr.
A-TX-79216	GA5	Nov-04	10 mos.
A-TX-79285	GA5	Dec-04	3 mos.
A-TX-79286	GA5	Dec-04	1 yr.
A-TX-79230	GA5	Nov-04	10 mos.
A-TX-79303	GA7	Jan-05	4 mos.
A-TX-79310	GA5	Jan-05	8 mos.
A-TX-79334	GA2	Jan-05	5 mos.
A-TX-79223	GA2	Nov-04	1 yr.
A-TX-79256	GA2	Dec-04	1 mo.

RSV B Virus name	Genotype classification	Month/Year of Isolation	Age at time of Collection
B-CH-18537	GB1	1962	
B-TX-64817	GB1/GB2	Dec-93	4 mos.
B-TX-65859	GB3	Feb-94	2 mos.
B-TX-61326	GB4	Feb-93	20 yrs.
B-TX-79233	BA	Nov-04	8 mos.
B-TX-60687	GB3	Jan-93	8 mos.
B-TX-79247	BA	Nov-04	1 mo.
B-TX-79226	BA	Nov-04	2 mos.
B-TX-65449	GB4	Jan-94	7 mos.
B-TX-60911	GB4	Jan-93	19 days
B-TX-60823	GB1/GB2	Jan-93	9 mos.
B-TX-79307	BA	Jan-05	2 mos.
B-TX-79325	BA	Jan-05	2 mos.
B-TX-60462	GB4	Jan-93	3 mos.
B-TX-61699	GB4	Mar-93	67 yrs.
B-TX-61500	GB3	Feb-93	8 yrs/
B-TX-60188	GB4	Dec-92	1 yr.
B-TX-79362	BA	Feb-05	4 mos.
B-TX-57097	GB1	Dec-91	5 mos
B-TX-61501	GB3	Feb-93	9 mos.
B-TX-60567	GB3	Jan-93	10 days
B-TX-61138	GB1	Feb-93	1 mo.
B-TX-60586	GB4	Jan-93	51 yrs.
B-TX-61406	GB4	Feb-93	5 mos.
B-TX-79222	BA	Nov-04	1 yrs.
B-TX-61512	GB4	Feb-93	57 yrs.
B-TX-61077	GB4	Feb-93	3 mos.
B-TX-61736	GB4	Mar-93	32 yrs.
B-TX-61735	GB4	Mar-93	28 yrs.

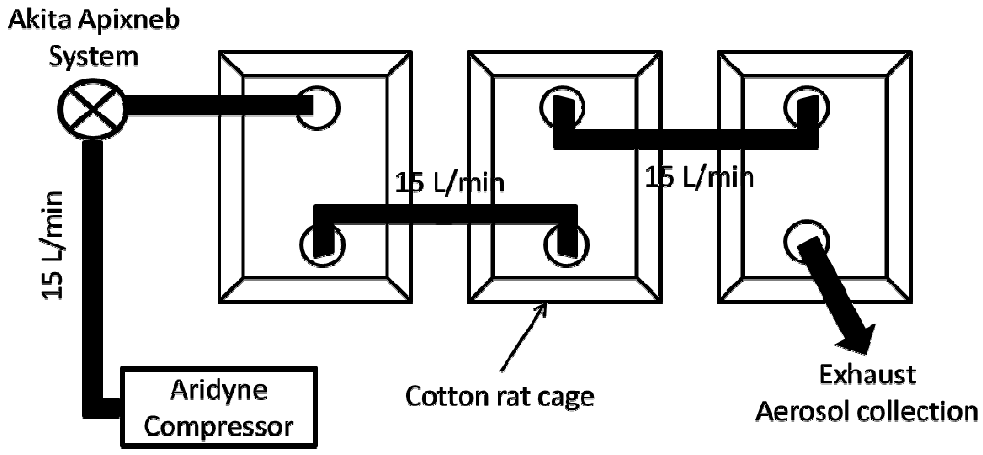

Figure S2. Cage setup for aerosol treatment

Table S2. Stability data of ALX-0171 at long-term storage condition (5°C ± 3°C) before and after nebulisation using an Akita^2 Apixneb nebuliser.

Method	Pre/post Nebulisation	Time points (months)				
		0	6	12	24	36
Content [mg/mL] (OD_{340})	Pre	50.5 (0.012)	52.2 (0.007)	46.6 (0.001)	47.7 (0.005)	47.8 (0.000)
	Post	54.5 (0.035)	45.5 (0.021)	45.0 (0.001)	48.1 (0.004)	46.7 (0.000)
SE-HPLC [% main peak]/ [% HMW]	Pre	99 / 1	99 / 1	99 / 0	100 / 0	99 / 0
	Post	98 / 2	98 / 1	98 / 1	99 / 1	97 / 2
Potency ELISA [% compared to reference standard]	Pre	105	95	106	103	106
	Post	95	121	116	97	111

REFERENCES

1. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. The American Journal of Hygiene **1938**; 27:493-7.

2. Tapia LI, Shaw CA, Aideyan LO, et al. Gene sequence variability of the three surface proteins of human respiratory syncytial virus (HRSV) in Texas. PLoS One **2014**; 9:e90786.