SUPPLEMENTAL METHODS

Fluorescence RSV Plaque Reduction Assay: Anti-RSV neutralizing antibodies were quantified using a microneutralization assay, as previously described [1, 2], in batches of 10 to 23 samples equally divided among comparison groups. In brief, freshly grown Hep-2 cells were seeded in 96-well plates (20,000 vital cells per well in a 96 well flat bottom tissue culture microtiter plate) overnight at 37°C and 5% CO2. The subsequent day, equal volumes of GFP-expressing RSV-A strain (rgRSV30) were combined with heat-inactivated (30 minutes at 56° C) 2-fold serially diluted sera starting at a 1:4 dilution, or a negative/positive control (media without RSV/palivizumab with RSV respectively) to provide ~100 plaque-forming units (PFU) per 50 µl serum-virus mixture. Serum-virus mixtures were incubated for 60 minutes at 37°C and 5% CO2. Post-incubation, 50 µl serum-virus mixtures were added in duplicates to monolayers of Hep-2 cells and incubated for 2 hours at 37° C and 5% CO2. Two hours post-infection, serum-virus mixtures were replaced by fresh media and incubated for 48 hours at 37° C and 5% CO2. Post-incubation, GFP-stained RSV syncytia were counted using an inverted fluorescence microscope (Olympus IX71). Endpoint of this assay was the reciprocal of the highest serum dilution where more than 95% of viral infection was inhibited, designated as the neutralizing titer 95 (NT95). NT95 was determined by the mean titer of each serum multiplied by two, since diluted sera with equal volumes of RSV were used. For internal controls, we used serial two-fold dilutions of palivizumab as a positive control, beginning at 25 µg/mL, virus-free media as a negative control and performed an RSV back titration to validate the initial infection dose. The lower limit of detection of this assay was a 1:8 dilution. Data below the lower limit of detection was set to a 1:4 dilution. Between experiments, NT95 for palivizumab showed consistency at 1:128 or 1:256 and RSV back titration showed consistency between 3520 and 3840 plaque-forming units (PFU)/mL (**Supplemental Figure 1**).

Results were also externally validated (AS, MG and DM) using a similar fluorescence plaque reduction assay on a subset of paired sera samples from women. Hep-2 cells grown in DMEM +

10% FBS were seeded at 2.0 x 104 vital cells per well in clear 96-well microtiter plates (Greiner Bio-one, 655090 Germany) and incubated for 24h. Serum samples were diluted 5-fold starting at a 1:4 dilution and then in 2-fold serial dilutions. Recombinant RSV-A (rgRSV30) virus expressing GFP during active infection was added to the serum and the mixture was incubated for 1h. The volume of virus used was adjusted to provide ~2000 foci of infection per well in serum-free positive control wells, additionally, RSV-free media was used as a negative control for baseline fluorescence. 100µL of serum-virus mixture was added in triplicate to the Hep-2 cell monolayers and incubated for 48h, and the serum-virus mixture was replaced with fresh media 4h after initial inoculation. After the infection period, the media was replaced with FluoroBriteTM DMEM (ThermoFisher, A1896701) and GFP-expression indicating RSV infection was measured by the GloMax® Explorer plate reader (PromegaTM, GM3500 USA). The excitation filter was set to blue 475 nm and the emission filter to 500-550 nm, and inhibitory concentration 50 (IC50) and PRNT50 values were calculated by GraphPad PRISM software. DMEM + 10% FBS + 1X Pen/Strep was used for media changes and dilutions unless otherwise specified, and all incubations were carried out in a humidified incubator at 37°C with 5% CO2. Serum neutralizations were checked for their accuracy by regular comparison to an RSV blue spot immune assay described below.

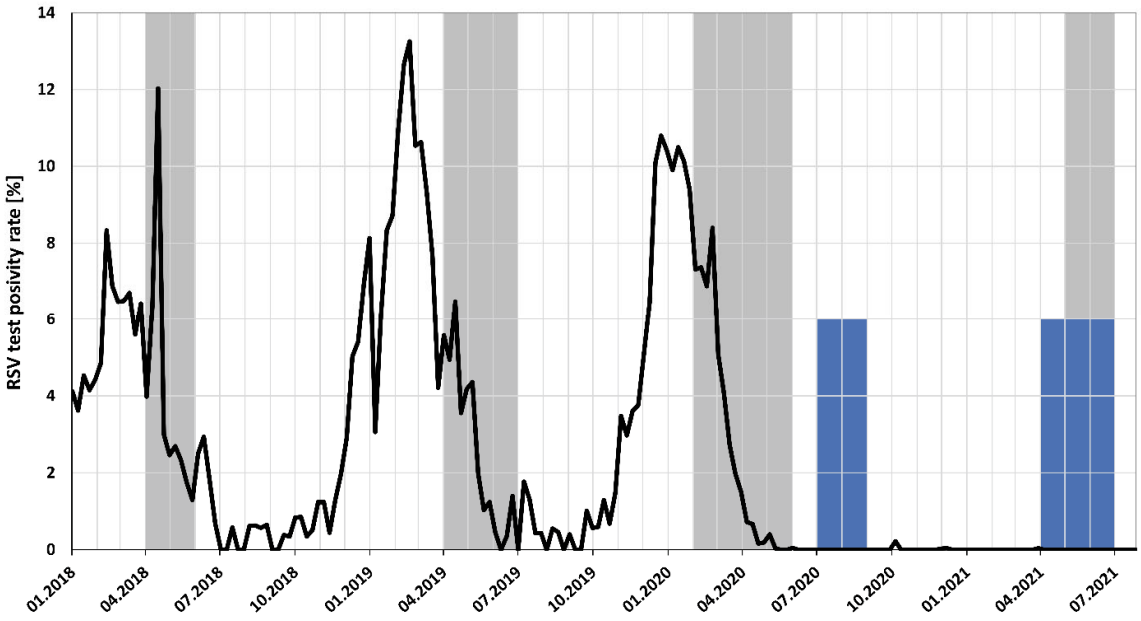
RSV Blue Spot Immune Assay: To validate the results of the fluorescence RSV plaque reduction assay, described above, a blue spot immuno assay [3] was used. Briefly, sub-confluent Hep-2 cells were inoculated with a 10-fold serial dilution of rgRSV30 in DMEM + 10% FBS + 1X Pen/Strep and incubated for 4h at 37°C with 5% CO2. The virus media was replaced with fresh growth media and incubated for another 20h. Hep-2 monolayers were treated with methanol:acetone (1:1 volume) and incubated for 10 min at RT to fix and permeabilize the cells. Cells were subsequently blocked with 300µL of PBS + 5% FBS and incubated for 30 min at RT. Following blocking, cells were stained with 200µL of primary polyclonal goat α RSV antibody (Meridian, #B65860G) diluted at 1:1000 in PBS + 1% FBS and incubated overnight at 4°C. The next day, an equal volume of

secondary rabbit α goat antibody conjugated to β-galactosidase (Abcam, #ab136712) diluted at 1:2000 in PBS + 1% FBS was added to the cells and they were incubated away from light for 1h at RT. In order to identify RSV infected cells, cells were treated with 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (ThermoFisher, BP1615-1) diluted to 0.5mg/mL in PBS containing 3mM potassium ferricyanide III, 3mM potassium ferrocyanide trihydrate, and 1mM magnesium chloride hexahydrate ("Yellow Substrate"). The cells were incubated away from light for 2-4 hours in a humidified incubator at 37°C with 5% CO2 to allow for blue spot development. After the blue spots had adequately developed, cells were washed and stored in distilled water (dH2O) at 4°C. RSV infection was quantified by counting the number of blue stained cells per well using the EVOS® Fl Auto Imaging System (ThermoFisher, AMAFD1000) and calculating the viral titre in focus-forming units (FFU/mL). Note that the cells were washed with PBS following all blocking and staining treatments.

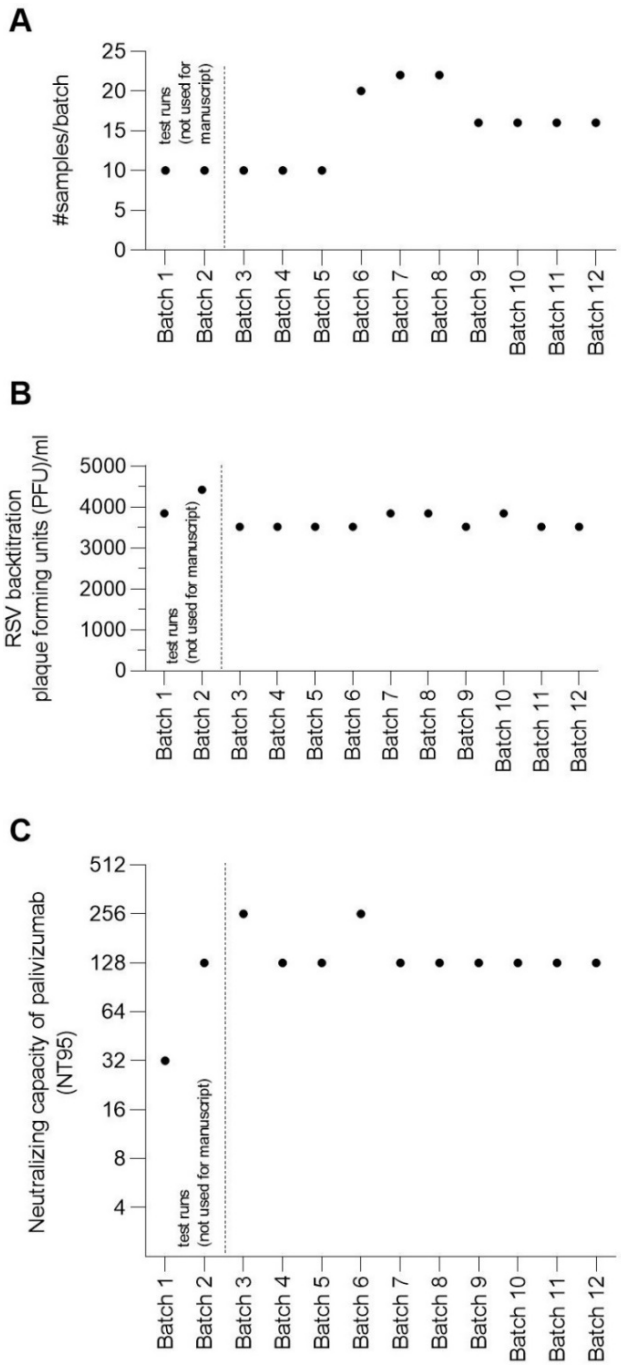
SUPPLEMENTAL TABLE & FIGURES

Characteristic	**N = 20**
Gestational age (weeks), mean ± SD	31 ± 4.7
Chronological age (months), mean ± SD	9.1 ± 2.9
Received palivizumab, %	100
Time between last dose palivizumab and blood sample (days), mean ± SD	144 ± 19
Comorbidities (primary indication for immunoprophylaxis), N =	20
Premature 29 to 35 weeks	13
Premature <29 weeks	5
Chronic lung disease	1
Congenital Heart disease	4
Trisomy 21	1

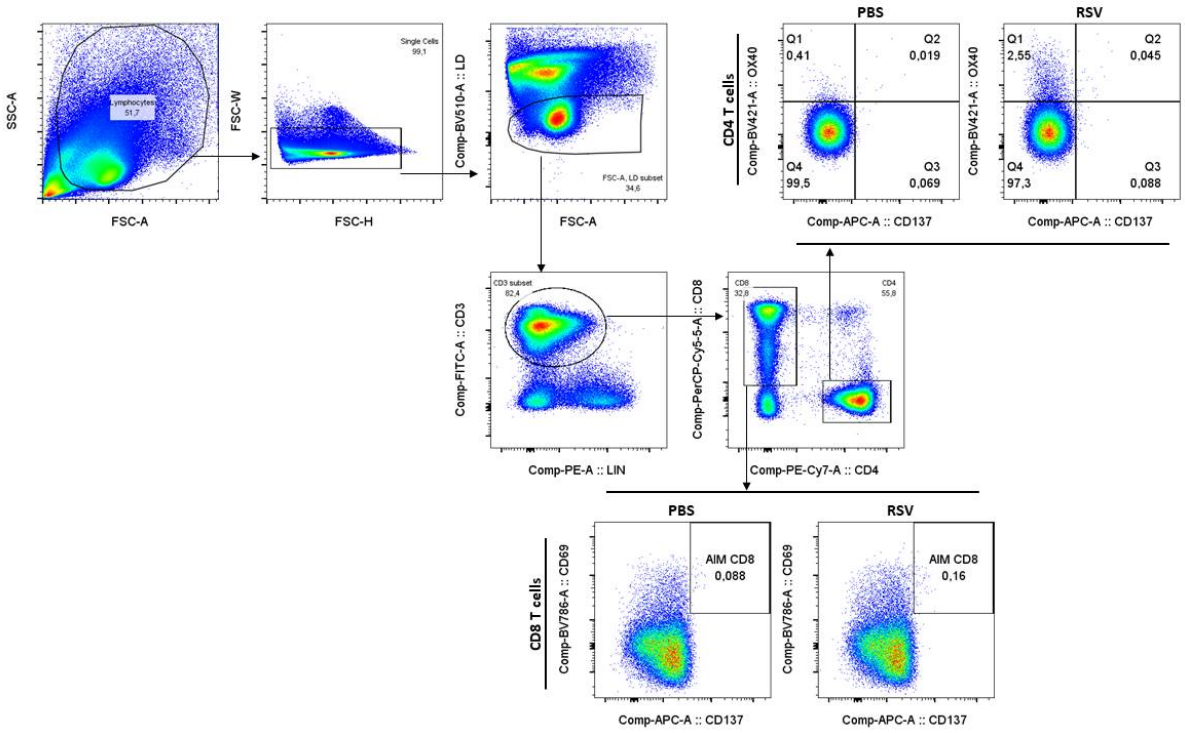
Supplemental Table 1. Clinical characteristics of study infants enrolled in the British Columbia Immunoprophylaxis program.



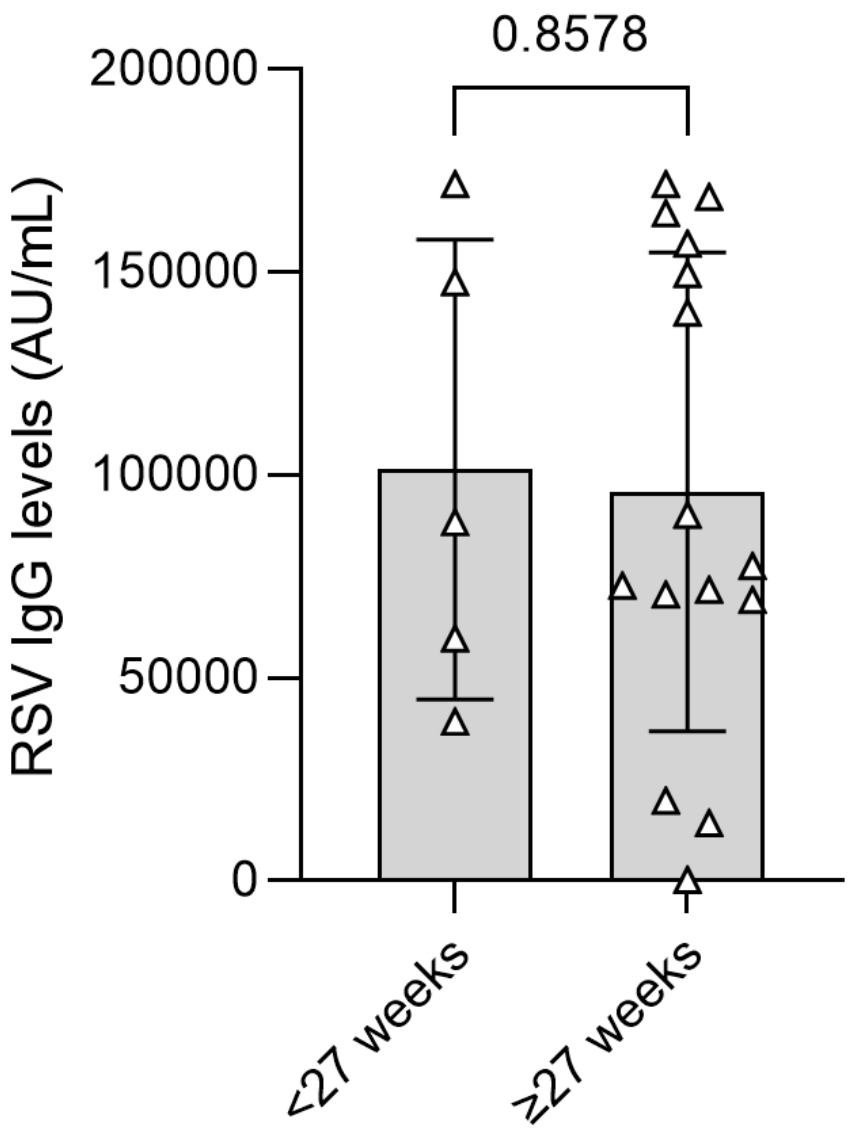
Supplemental Figure S1: Blood sampling in women of childbearing age (shaded grey area) and infants (shaded blue area) in relation to RSV positive testing rates (% of RSV tests) in British Columbia (Canada) between January 1st 2018 to July 31st 2021. Data were obtained from: https://www.canada.ca/en/public-health/services/surveillance/respiratory-virus-detections-canada.html and included all cases in the province of British Columbia, Canada.



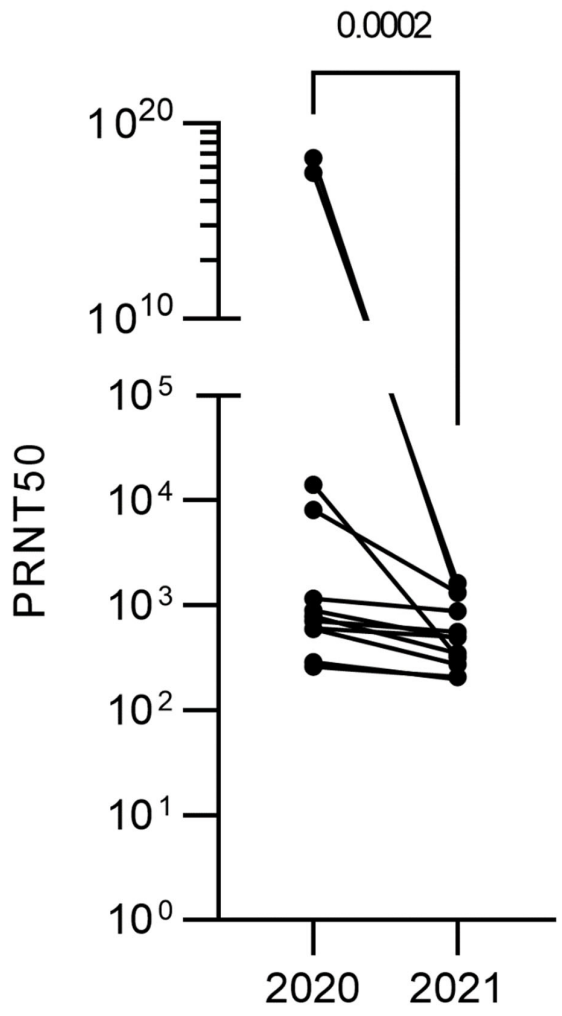
Supplemental Figure S2: Internal controls from RSV antibody neutralization assay (live virus). Data from batch 1 and 2 were test runs (data not used for the manuscript). Batches 3-12 were used to assess the neutralizing capacity of the samples used for the manuscript, balanced between comparison groups in each batch. (**A**) Number of samples, tested in duplicates, per batch; (**B**) RSV back titration (plaque forming units per ml) per batch to validate the initial infection dose; (**C**) Neutralizing capacity of palivizumab (NT5) per batch as an internal positive control.



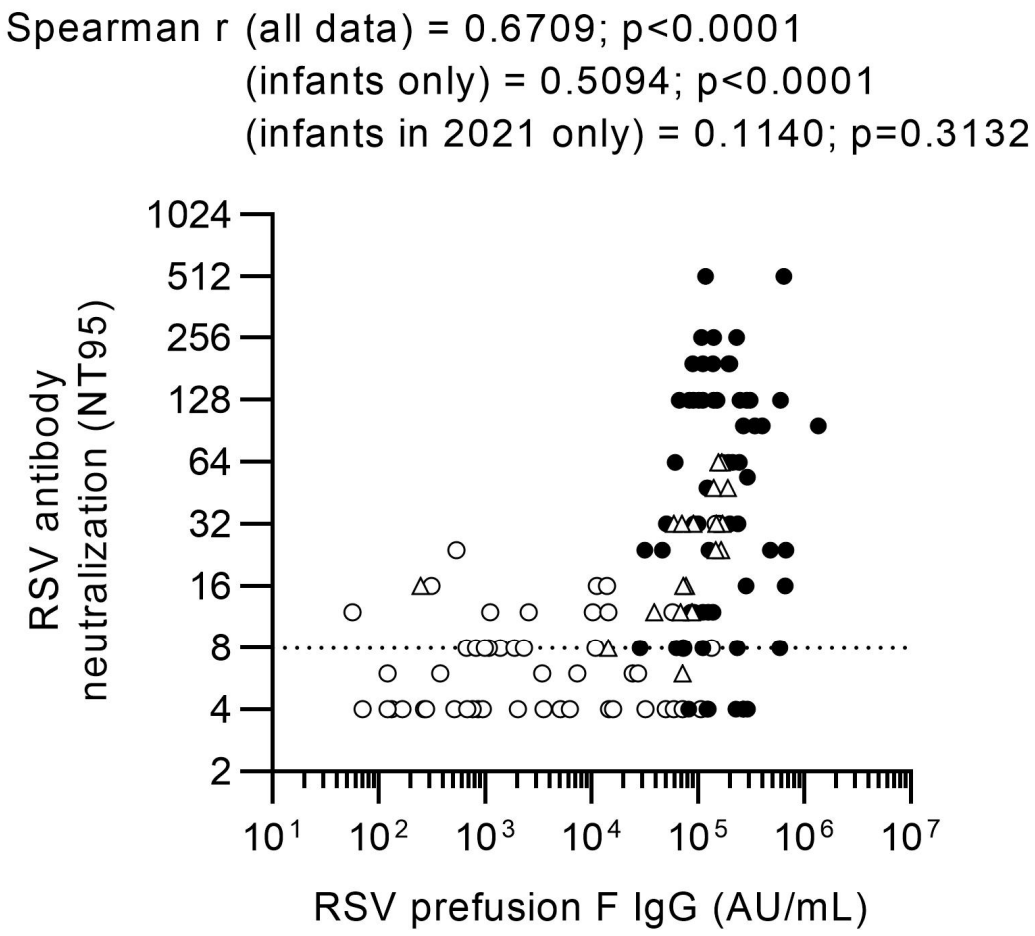
Supplemental Figure S3: Exemplar gating strategy to identify RSV-activated T cells. Events are gated on singlet live cells, and CD137/OX40-positive CD4/CD3-expressing T cells and excluding the combined CD14-PE/CD19-PE-labeled cells (identified as "LIN" marker in panel above).



Supplemental Figure S4: Comparison of RSV prefusion F IgG between infants sampled in 2020 born <27 weeks gestational age and infants sampled in 2020 born ≥27 weeks gestational age; p value determined using a 2-sided unpaired t test; bars represent mean ± SD.



Supplemental Figure S5: External validation of the difference in RSV neutralization from paired sera (n = 10 each year) collected from a subset of women of childbearing age in 2020 and 2021 using a separate plaque reduction assay. Results are expressed as sera concentration that results in 50% plaque reduction compared to viral-free sera (PRTN50). Difference between groups was calculated using a 2-sided Wilcoxon matched-pairs signed rank test.



Supplemental Figure S6: Spearman correlation of RSV prefusion F IgG antibodies (AU/ml) and RSV neutralizing antibody titers (expressed as highest titer to achieve 95% neutralization of live RSV virus infection of cells *in vitro*, NT95) in women of childbearing age sampled in 2018, 2019, 2020 and 2021 (solid dots; n = 64), infants sampled in 2021 (open circles; n = 65) and infants sampled in 2020 (triangles; n = 20). Dotted line shows lower limit of detection of the RSV neutralization assay. Spearman correlations were performed on log-transformed data.

SUPPLEMENTAL REFERENCES

1. Lavoie PM, Solimano A, Taylor R, et al. Outcomes of Respiratory Syncytial Virus Immunoprophylaxis in Infants Using an Abbreviated Dosing Regimen of Palivizumab. JAMA Pediatr **2016**; 170(2): 174-6.
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3. Bilawchuk LM, Griffiths CD, Jensen LD, Elawar F, Marchant DJ. The Susceptibilities of Respiratory Syncytial Virus to Nucleolin Receptor Blocking and Antibody Neutralization are Dependent upon the Method of Virus Purification. Viruses **2017**; 9(8).