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Characterization of the role of N-glycosylation sites in the respiratory syncytial virus fusion protein in virus replication, syncytium formation and antigenicity

Annelies Leemans[1], Marlies Boeren[1], Winke Van der Gucht[1], Wim Martinet[2], Guy Caljon[1], Louis Maes[1], Paul Cos[1], Peter Delputte[1*]

[1]Laboratory of Microbiology, Parasitology and Hygiene, University of Antwerp, Antwerp, B-2610, Belgium; annelies.leemans@uantwerpen.be, marlies.boeren@uantwerpen.be, winke.vandergucht@uantwerpen.be, guy.caljon@uantwerpen.be, louis.maes@uantwerpen.be, paul.cos@uantwerpen.be, peter.delputte@uantwerpen.be
[2]Laboratory of Physiopharmacology, University of Antwerp, Antwerp, B-2610, Belgium; wim.martinet@uantwerpen.be

*Corresponding author: peter.delputte@uantwerpen;be; Laboratory of Microbiology, Parasitology and Hygiene, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium; Tel.: +32 3 265 26 25

Abstract

Respiratory syncytial virus (RSV) is a leading cause of infant hospitalization worldwide each year and there is presently no licensed vaccine to prevent severe RSV infections. Two major RSV glycoproteins, attachment (G) and fusion (F) protein, regulate viral replication and both proteins contain potential glycosylation sites which are highly variable for the G protein and conserved for the F protein among virus isolates. The RSV F sequence possesses five N-glycosylation sites located in the F2 subunit (N27 and N70), the p27 peptide (N116 and N126) and the F1 subunit (N500). The importance of RSV F N-glycosylation in virus replication and immunogenicity is not yet fully understood, and a better understanding may provide new insights for vaccine development. By using a BAC-based reverse genetics system, recombinant viruses expressing F proteins with loss of N-glycosylation sites were made. Mutant viruses with single N-glycosylation sites removed could be recovered, while this was not possible with the mutant with all N-glycosylation sites removed. Although the individual RSV F N-glycosylation sites were shown not to be essential for viral replication, they do contribute to the efficiency of *in vitro* and *in vivo* viral infection. To evaluate the role of N-glycosylation sites on RSV F antigenicity, serum antibody titers were determined after infection of BALB/c mice with RSV expressing the glycomutant F proteins. Infection with recombinant virus lacking the N-glycosylation site at position N116 (RSV F N116Q) resulted in significant higher neutralizing antibody titers compared to RSV F WT infection, which is surprising since this N-glycan is present in the p27 peptide which is assumed to be absent from the mature F protein in virions. Thus, single or combined RSV F glycomutations which affect virus replication and fusogenicity, and which may induce enhanced antibody responses upon immunization could have the potential to improve the efficacy of RSV LAV approaches.

Keywords

Orthopneumovirus – fusion protein – N-glycosylation – recombinant virus recovery – antigenicity

1 Introduction

The respiratory syncytial virus (RSV) is a major cause of infant morbidity and mortality related to lower respiratory tract disease. The disease burden in children younger than 5 years is estimated at 33.8 million infections annually from which 10% requires hospitalization (Nair et al., 2010). A vaccine to control the RSV disease burden remains elusive and treatment options are mainly supportive. Palivizumab, a humanized monoclonal antibody which targets a conserved epitope of the RSV fusion (F) protein, is able to reduce RSV-related hospitalizations when prophylactically administered. However, its use is restricted to high-risk children due to the high cost and the requirement of monthly intramuscular injections throughout the RSV season (Homaira et al., 2014). Re-infections are very common which is assumed to be the consequence of an incomplete and short-lived immunity upon natural infection.

RSV is an enveloped virus with a non-segmented negative-stranded RNA genome belonging to the family *Pneumoviridae* and genus *Orthopneumovirus* (Afonso et al., 2016). From the 11 proteins which the RSV genome encodes, three are membrane-bound proteins including the small hydrophobic (SH) protein, attachment (G) protein and F protein which are subjected to the addition of glycan structures during their synthesis (Collins et al., 1984; Ding et al., 1987; Fuentes et al., 2007). A wide range of viral proteins is modified by the attachment of glycan structures co- and post-translationally. The most common type is N-glycosylation which is characterized by attachment of the glycan structure to an asparagine (N) residue of the polypeptide chain within the consensus sequence N-X-S/T (Kornfeld and Kornfeld, 1985). The SH protein, known as a pore-forming protein that enhances membrane permeability of the host cells, is expressed in glycosylated forms but predominantly in non-glycosylated forms (Fuentes et al., 2007). The G protein, important in the RSV entry process by regulating host cell attachment, contains both potential N-glycosylation and O-glycosylation sites (S or T residues within the polypeptide chain) which determine its high molecular weight (McLellan et al., 2013b). Variation within the RSV G O-glycosylation profile is responsible for its high degree of variability among virus strains and may provide an immune evasion strategy (Palomo et al., 2000; Rawling and Melero, 2007). In contrast, the F protein possesses five potential N-glycosylation sites which are highly conserved. Two sites (N27, N70) are located at the F2 subunit, one site (N500) at the F1 subunit and two remaining sites (N116, N126) within p27, a short amino acid sequence positioned between both subunits and released after cleavage to form the mature RSV F protein (Gonzalez-Reyes et al., 2001; Zimmer et al., 2001b). The RSV F protein regulates binding and fusion during host cell entry. Moreover, it is the only required membrane protein for infection in cell cultures (Karron et al., 1997). However, RSV G enhances *in vitro* infectivity and is required for optimal *in vivo* replication (Techaarpornkul et al., 2001; Teng et al., 2001).

Virus glycosylation plays a direct role in protein processing such as protein folding and cleavage, in intracellular trafficking of the protein and in biological functions of the protein in question (Varki and Lowe, 2009; Vigerust and Shepherd, 2007). All these factors are indirectly or directly related to viral replication. Removal of N-linked glycans can result either in enhanced or reduced replication of the involved virus and can differ between N-glycans within a viral glycoprotein (Beyene et al., 2004; Hanna et al., 2005b; Lee et al., 2010; Mossenta et al., 2017; Wang et al., 2013). In this context, glycosylation is often an important determinant of viral pathogenicity and additionally, virus-specific pathogenic characteristics can be determined by the glycosylation profile of viral proteins (de Brogniez et al., 2015; Montefiori et al., 1988; Shirato et al., 2004; Zhao et al., 2017). Previous studies already investigated RSV glycosylation either by chemical or enzymatic deglycosylation or by site-directed mutagenesis of specific glycosylation sites in plasmids encoding the RSV F protein (Collins and Mottet, 1991; Lambert, 1988; McDonald et al., 2006; Zimmer et al., 2001b). By this means, no requirement of glycosylation was observed in proteolytic cleavage and cell surface transport of the RSV F protein (Collins and Mottet, 1991; Zimmer et al., 2001a). Virus infectivity was significantly reduced after enzymatic removal of the N-glycans attached on the RSV glycoproteins (Lambert, 1988). Additionally, inhibition of RSV glycan maturation by alpha-mannosidase inhibitor deoxymannojirimycin affected RSV infectivity remarkably (McDonald et al., 2006). Since both RSV glycoproteins F and G are responsible for efficient replication and contain N-linked glycans, it remains questioned to which extent N-glycosylation of the individual glycoproteins affects RSV replication. Besides the importance of N-glycosylation in RSV replication, it was shown that the N-glycan positioned at N500 of the RSV F protein is important for its fusion activity, whereas removal of other N-linked glycans had no impact (Leemans et al., 2018; Li et al., 2007; Zimmer et al., 2001b). The functional role of the F2 subunit and p27 N-glycans remains to be determined. Moreover, our recently published work showed that DNA immunization with plasmids encoding RSV F in which N-sequons were removed affects the induction of neutralizing antibody responses. More specifically, removal of the N116 sequon results in enhanced neutralizing antibody responses upon DNA immunization of mice and these mice are better protected against challenge with wild type (WT) virus (Leemans et al., 2018).

In the present study, recombinant RSV strains expressing glycomutant F proteins were developed using an BAC-based RSV rescue system (Hotard et al., 2012). This allowed us to study the role of the single RSV F N-glycosylation sites in the context of the virus instead of the RSV F protein only. The recombinant viruses were evaluated for their *in vitro* and *in vivo* replication, the RSV F antigenicity and mucogenicity.

2 Material and methods

2.1 Cells and virus

The human epidermoid carcinoma larynx (HEp-2) and Vero cell lines were obtained from the ATCC. The cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% inactivated fetal bovine serum (iFBS). BSR T7/5 cells were a gift of K.K. Conzelmann (Max-von-Pettenhofer-Institut, Munich, Germany) and grown in Glasgow's minimal essential medium (GMEM) supplemented with 3% iFBS, 2% minimal essential amino acids and 1 mg/mL geneticin. Cell culture media and supplements were obtained from Thermo Fisher Scientific. The mKate2-expressing BAC-RSV construct, named pSynkRSV-line19F, and helper plasmids pcDNA3.1 RSV L, M2.1, N and P were kindly provided by M.L. Moore (Emory University of School of Medicine, Children's Healthcare of Atlanta, Atlanta, Georgia, USA).

2.2 Construction and recovery of recombinant viruses

The RSV F glycosylation mutants were obtained by switching the asparagine (N) codon (AAT/AAC) at the conserved positions N27, N70, N116, N126 or N500 into a glutamine (Q) codon (CAA/CAG). WT and recombinant RSV line19F sequences were synthetized by Genscript and delivered in pUC57 simple. Subcloning into vector pSynkRSV-line19F was performed using appropriate restriction enzymes (New England Biolabs) to excise insert DNA from vector pUC57 simple and ligate the insert into vector pSynkRSV-line19F using T4 DNA ligase (New England Biolabs). Ligation products were transformed into electrocompetent *E. coli* cells and plasmid DNA was recovered using PureLink® HiPure Plasmid Midiprep Kit according to the manufacturer's instructions (Thermo Fisher Scientific). The sequences of the recombinant vectors were confirmed by DNA sequencing (VIB Genetic Service Facility, University of Antwerp).

Recombinant virus was recovered as described previously (Hotard et al., 2012). Briefly, BSR T7/5 cells, passaged with 1 mg/mL geneticin (Thermo Fisher Scientific), were seeded in 6 well plates to be 100% confluent at the time of transfection. The appropriate concentrations of the recombinant BAC constructs (0.8 µg), helperplasmids pcDNA3.1 RSV L (0.2 µg), RSV N (0.4 µg), RSV P (0.4 µg) and RSV M2.1 (0.4 µg) and 6,6 µL Lipofectamine 2000 (Thermo Fisher Scientific) were diluted in 100 µL opti-MEM (Thermo Fisher Scientific) and mixed. After a 20 min incubation, transfection complexes of 600 µL were added to the cells, incubated for 2 h at room temperature on a shaking plate and further incubated with an additional 600 µL GMEM supplemented with 3% iFBS overnight. Then, transfection complexes were replaced by medium and sub-passed in T25 flasks two days post-transfection. Every 2 or 3 days the cells were sub-cultured until cytopathic effects were evident throughout the flask and subsequently scraped and snap frozen. Subconfluent HEp-2 cell cultures were used to propagate recovered virus for three passages to minimize adaptation to HEp-2 cells. Virus stocks were titrated by a conventional plaque assay in HEp-2 cells as described previously (Schepens et al., 2014). RSV RNA of the final stocks was isolated using a viral RNA isolation kit (Qiagen) according the manufacturer's protocol. A reverse-transcriptase-PCR kit (Agilent Technologies) was used to synthesize cDNA that was further analyzed by sequencing (VIB Genetic Service Facility, University of Antwerp) to verify the presence of the N-glycosylation mutations in the final virus stocks and after serial passage.

2.3 Western blot analysis

For Western blot analysis, RSV-infected HEp2 cell cultures were scraped and supernatant was collected and centrifuged (1000 x g, 10 min, 4°C). Virus in the supernatant was pelleted by ultracentrifugation (90 min, 20,000 rpm, 4°C) (Optima™ XPN, SW32) and resuspended in HBSS. Aliquots were mixed 1:1 with Laemmli sample buffer (Bio-Rad) with or without β-mercaptoethanol. After boiling, the mixtures were loaded and separated on 4-20% polyacrylamide gels (Bio-Rad) and transferred to an Immobilon-P transfer membrane (Millipore). RSV F proteins were stained with palivizumab and HRP-conjugated goat anti-human IgG (Thermo Fisher Scientific). Palivizumab leftovers were provided by the Department Pediatrics of the Antwerp University Hospital (S. Verhulst). RSV N protein was detected by using bovine RSV N-specific monoclonal antibodies (mAbs), cross-reactive with hRSV N, kindly provided by J-J. Letesson (Université de Namur) and HRP-conjugated goat anti-mouse IgG (Thermo Fisher Scientific). Protein bands were visualized with chromogenic 3,3' diaminobenzidine (DAB) (Sigma-Aldrich) or ECL substrate (Biorad).

2.4 *In vitro* infection

HEp-2 cells were seeded in 96-well black µClear® flat bottom microtiter plates (Greiner Bio-one) to be subconfluent after overnight incubation. Cells were infected with WT or recombinant RSV expressing glycomutant F proteins at a multiplicity of infection (MOI) of 0.1 in 50 µL basal growth medium (DMEM) and incubated for 2 h at 37°C. Unbound virus was washed away and pre-warmed medium was added to the cells for further incubation. After 24 h the cells were fixed with 4% paraformaldehyde (PF), permeabilized with 0,5% Triton-X100 (Thermo Fisher Scientific) and nuclei were stained with DAPI (Sigma). Cells were analyzed by fluorescence microscopy (Axio Observer, Zeiss). Infection percentages were determined by imaging 10 random fields whereby mKate2 expression served as a marker for RSV-infected cells.

2.5 Indirect immunofluorescence staining of RSV F surface proteins

Infection of subconfluent HEp-2 cells seeded on coverslips in 24 well plates was performed as described before (Leemans et al., 2017). After a 24 h incubation, the cells were fixed with 4% PF. To visualize the surface-expressed RSV F proteins, the cells were stained with palivizumab and AF 488 anti-human IgG (Thermo Fisher Scientific). Nuclei were stained with DAPI. The images were obtained using a Leica SP8 confocal microscope and Velocity 3D Image Analysis Software.

2.6 FACS analysis of RSV F surface expression

HEp-2 cells were seeded in 6-well plates to be subclonfluent after overnight incubation at 37°C. Subsequently the cells were infected at an MOI of 0.1 in 750 µL of basal growth medium (DMEM) and incubated for 2 h at 37°C. Cells were washed with pre-warmed medium and incubated overnight at 37°C. Infected cells were washed and resuspended in ice-cold PBS and pelleted by centrifugation (210× g, 10 min, 4 ◦C) and cells incubated with palivizumab at a concentration of 5 µg/mL for 1 h at 4°C to stain RSV F surface proteins. To remove unbound antibodies the cells were washed two times with ice-cold PBS. Then, goat anti-human AF647-conjugated secondary antibodies (Thermo Fisher Scientific) were added to the cell pellets for 1 h at 4°C and cells were washed with ice-cold PBS two times and analyzed by flow cytometry with a MACSQuant analyzer 10 (Miltenyi Biotec, Germany). Mean fluorescence intensity (MFI) of the cells infected with glycomutant virus was calculated and presented as values relative to the MFI of the RSV WT F infected cells (100%).

2.7 Fusion assay

HEp-2 cells were seeded in 96-well black µClear® flat bottom microtiter plates (Greiner Bio-one) to be subconfluent at the time of infection. Cells were infected with WT or recombinant RSV expressing glycomutant F proteins at an MOI of 0.5. After 36 h infection, the cells were fixed with 4% PF, permeabilized with 0.5% Triton X-100 and stained with DAPI. Syncytium frequency (cells with more than two nuclei) and mean syncytium size was determined of 100 mKate-positive cells by fluorescence microscopy. Cells were analyzed by fluorescence microscopy (Axio Observer, Zeiss).

2.8 Determination of particle/PFU ratios

Stocks of WT or recombinant RSV expressing glycomutant F proteins, for which PFU titers were determined by plaque assay, were diluted in basal growth medium (DMEM) and 2 µL of the dilution was dried overnight in a 96-well black µClear® flat bottom microtiter plate (Greiner Bio-one). Subsequently the virus particles were fixed with 4% PF, blocked with 1% BSA for 1 h and stained with a polyclonal goat anti-RSV antibody (Virostat). The particles were visualized with AF 555 donkey anti-goat IgG (Thermo Fisher Scientific). Images were acquired by fluorescence microscopy (Axio Observer, Zeiss) and further semi-quantitative analyzed using ImageJ software (Schindelin et al., 2012). Particle/PFU ratios were calculated as particles/ml divided by PFU/ml.

2.9 *In vitro* virus growth curve

Subconfluent HEp-2 cells in 6-well plates were infected with WT or recombinant RSV expressing glycomutant F proteins at an MOI of 0.1 in 750 µL basal growth medium (DMEM). After 2 h incubation at 37°C, the cells were washed twice with pre-warmed medium to remove unbound virus and fresh medium (+10% iFBS) was added for further incubation. Samples of supernatant were collected 16, 24,

48, 72 and 96 h post-infection (p.i.), clarified by centrifugation, snap-frozen and stored until plaque titration by an immunodetection plaque assay in HEp-2 cells (Schepens et al., 2014).

2.10 BALB/c mice studies

Female 7-8 weeks old BALB/c mice (Janvier, France) were randomly allocated to individually ventilated cages of 9 animals each. Food (Carfil, Belgium) and drinking water were available ad libitum. Prior to RSV challenge, the mice were anesthetized with 5% isoflurane (Halocarbon®, New Jersey, USA) and subsequently intranasal inoculated with $2x10^5$ PFU of pelleted WT or glycomutant RSV diluted in 100 µL HBSS. Mice were sacrificed by CO_2 at 4, 6 and 8 days p.i. and the lungs were excised. The left lung was homogenized in HBSS containing 20% sucrose and clarified by centrifugation (4°C, 15 min, 1000 x g) for further titration by plaque assay in Vero cells as described (Schepens et al., 2014). The right lung was fixed by formaldehyde (PF) for the preparation of paraffin slides (see below). To study antibody responses after infection, 6 animals/recombinant virus were included and blood was collected 3 and 5 weeks p.i. by retro-orbital bleeding after anesthetization with 5% isoflurane. The blood was left to clot in a serum clot activator tube (Sarstedt, Nümbrecht, Germany) at room temperature for 30 min and supernatant was collected after centrifugation (5 min, 10,000 x g). The animal studies were approved by the Animal Ethical Committee of the University of Antwerp (UA-ECD 2015-63).

2.11 Antibody responses and neutralization assay

To determine serum antibody levels, 96-well microtiter plates (Falcon) were coated with RSV-infected HEp-2 cells (MOI=0.5) (Leemans et al., 2018). Two-fold dilutions of the heat-inactivated mice serum were added to the cells and incubated for 1 h at 37°C. Afterwards, the cells were stained with HRP conjugated goat anti-mouse IgG (Thermo Fisher Scientific). 3,3'diaminobenzidine (DAB) (Sigma) was added to the cells as a substrate for HRP. Light microscopy analysis was performed to determine the antibody titers of the serum and are displayed as log 2 of the lowest concentration were staining of RSV-infected cells was observed.

To determine serum neutralizing antibody titers, plaque reduction neutralization tests (PRNT) were performed. Prior to inoculation of subconfluent HEp-2 monolayers, 2-fold dilutions of heat-inactivated serum in duplicate were incubated with virus inoculum (RSV A2L19F, MOI: 0,1) for 1 h at 37°C. Binding of virus was allowed for 2 h at 37°C and afterwards an overlay of DMEM + 0.6% Avicel (FMC Biopolymer) was added to the cells. After 3 days incubation at 37°C, cells were fixed with 4% PF, permeabilized with Triton X-100 and blocked with 1% BSA. Plaques were stained with palivizumab and HRP-conjugated goat anti-human IgG. Chloronapthol (Thermo Fisher Scientific) was used to visualize the plaques. Neutralization titers were calculated by the concentration resulting in a 50% reduction compared to control wells.

2.12 Prefusion F ELISA

Prefusion F ELISAs were performed as described previously (Stobart et al., 2016). High-binding ELISA microplates (Greiner) were coated with viral stocks diluted in DMEM and incubated overnight at room temperature. Coated plates were blocked with 5% bovine serum albumin (BSA) for 2 h and washed three times with PBS-Tween (PBS-T) before incubation with two-fold dilutions of MPE8 or D25 (prefusion F-specific mAbs) and motavizumab (pre- and posfusion F-specific mAb) in 1% BSA for 2 h at room temperature. RSV F-specific mAbs were provided by J.A. Melero (Centro Nacional de Microbiología and CIBER de Enfermedades Respiratorias, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain), J.S. McLellan (Department of Biochemistry, Geisel School of Medicine at Dartmouth, Hanover, NH, USA) and B.S. Graham (Vaccine Research Centre, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA). Thereafter, plates were washed again three times with PBS-T and incubated with goat anti-human HRP (Thermo Fisher Scientific) diluted in 1% BSA for 1 h at room temperature. After three final washes with PBS-T, a chromogenic substrate reagent, 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma) was added to the plates and incubated for 30 min at room temperature. The colorimetric reaction was quenched with a stop solution (2N sulfuric acid) and absorbance was measured at 450 nm using a spectrophotometer (GloMax Discover, Promega). To determine the ratio prefusion F to total F expression, the ratio of area under the curve was calculated for MPE8 or D25 and motavizumab.

2.13 Histochemistry

Eight days p.i. right lungs were excised. The lungs were fixed in 4% formaldehyde (pH 7.4), transferred to 60% isopropanol and paraffin embedded. Sections of 5 µm thickness were stained with periodic acid-Schiff (PAS) to evaluate pulmonary mucin expression and scored as previously described (Moore et al., 2009).

2.14 Statistical analysis

Data are presented as means of two, three or four independent repeats. RSV F WT data was compared with the mutant RSV viruses using a student *t*-test, one-way or two-way ANOVA by GraphPad Prism 6. P values <0.05 were considered statistically significant.

3 Results

3.1 Recovery of recombinant RSV expressing glycomutant F proteins using an BAC-based RSV rescue system

The RSV F protein conserves 5 potential N-glycosylation sites at positions N27, N70, N116, N126 and N500 from which the asparagine codons were individually, or all together substituted by a glutamine (Q) codon. Since the side chains of Q are very similar to N, Q is a good substitute to limit interfering effects on F protein function. To study the impact of N-glycosylation site removal at the level of infectious virus, we attempt to rescue viable virus by a BAC-based RSV rescue system (Hotard et al., 2012). An RSV-BAC clone, encoding the complete RSV genome, was used as vector for the recombinant RSV F sequences. After subcloning, the recombinant BAC clones were transfected in BSR T7/5 cells together with RSV helperplasmids (RSV L, P, N and M2.1) to recover recombinant virus. Viable virus was rescued for all single glycosylation mutants indicating no essential role of the individual N-glycans in viral replication. However, no viable virus could be rescued from cDNA where all conserved N-sequons were substituted. After three passages in HEp-2 cells, RNA was isolated of the different recombinant RSV stocks and further processed to cDNA for sequence analysis to confirm the presence of the N-glycosylation substitutions. Expression of the recombinant viruses was confirmed by immunofluorescence staining of infected HEp-2 (data not shown). To assess the stability of the introduced mutations, serial passage of the viruses was performed in HEp-2 cells. After 16 passage, the stability of the mutations was confirmed for all glycomutant viruses (data not shown).

3.2 Recombinant RSV with single N-sequon deletions on the mature RSV F protein have a reduced molecular weight

The molecular weight of glycoproteins is determined by the amount and extent of the attached N-glycan structures. Pelleted virus was denatured under non-reducing or reducing conditions and subsequently separated by molecular weight by gel electrophoresis. Staining of the membrane with palivizumab after protein transfer visualized the RSV F proteins of the recombinant viruses. Non-reducing conditions resulted in protein bands around 70 kDA, corresponding to the non-cleaved RSV F protein. Loss of N-sequons N27Q, N70Q and N500Q resulted in a reduced molecular weight compared with WT F whereas deletion of p27 N-sequons N116 or N126 did not change the molecular weight of RSV F (**Fig.1A**). Reduction by β-mercaptoethanol resulted in cleavage of the disulphide-linked RSV F subunits F1 (50 kDa) and F2. In reducing conditions, the N500Q mutant showed a lower molecular weight as this is the only glycan positioned at the F1 subunit which is recognized by palivizumab (**Fig.1B**). Western blots were also developed with a RSV N-specific monoclonal antibody (mAb) as a reference for the amount of virus particles to assess differences of F incorporation into virus particles (**Fig.1C**). Here, no remarkable differences could be observed compared to RSV F WT.

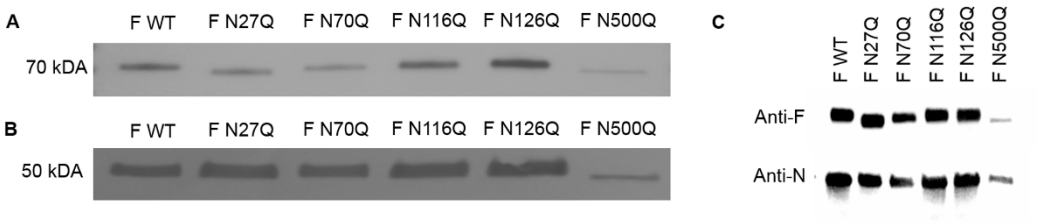

Figure 1: Western blot analysis. After infection of HEp-2 cells with the indicated virus, the supernatant was pelleted by ultracentrifugation and subsequently separated by SDS-PAGE under non-reducing conditions (A) or reducing conditions (B). Transfer of the proteins to an PVDF membrane was followed by staining of the RSV F proteins by palivizumab. The protein bands were visualized by staining the membrane with HRP-conjugated goat anti-human IgG and subsequently adding the chromogenic substrate DAB. (C) Western blot was developed by palivizumab and anti RSV N-specific antibody.

Surface expression was assessed by immunofluorescence staining of surface-expressed RSV F proteins. After infection of HEp-2 cells, the cell surface was stained with palivizumab and AF 488-conjugated secondary antibody. Microscopic analysis showed expression at the cell surface for all glycomutant RSV F proteins (**Fig.2A**). FACS analysis with palivizumab showed, however, a significant reduction in F surface expression for all recombinant viruses compared to RSV F WT (**Fig.2B**). Comparable observations were obtained after staining the cells with human RSV antiserum (BEI resources) (data not shown). This indicates that binding efficiency of palivizumab was not affected after N-sequon removal and that the observed reductions were due to reduced F surface expression.

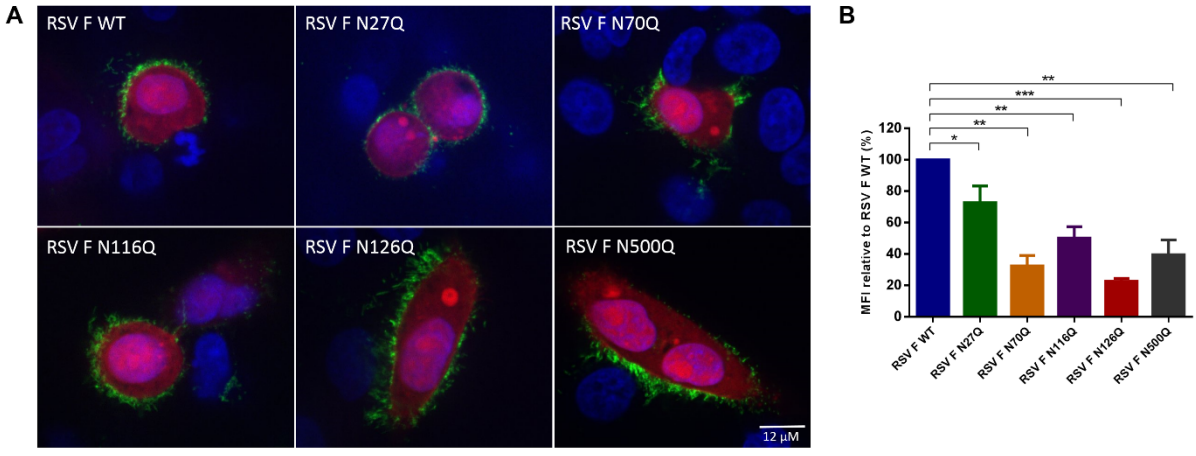

Figure 2: F surface expression analysis. Subconfluent HEp-2 cells were infected overnight with the indicated virus. (A) RSV F protein was detected by indirect immunofluorescence staining with palivizumab and AF 488 goat anti-human IgG. Nuclei were visualized with DAPI. RSV F protein (green), mKate (red), nuclei (blue). (B) Infected cells were washed and detached with ice-cold PBS, stained with palivizumab and AF 647 goat anti-human IgG at 4°C to assure surface staining only and analyzed by flow cytometry. Surface expression is expressed as the MFI relative to RSV F WT expression. Data represents the means (±SD) from 3 independent repeats. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Student's unpaired two-tailed *t* test).

3.3 RSV F N-glycosylation is important for the efficiency of *in vitro* RSV syncytium formation

Previous studies with glycomutant RSV F proteins showed the importance of the N-glycan positioned at N500 for efficient *in vitro* RSV F fusion activity (Leemans et al., 2018; Li et al., 2007; Zimmer et al., 2001b). In the present study, it was assessed whether these findings could be confirmed at the level of infectious virus. Syncytia (cells with more than two nuclei) in infected cell cultures were analyzed and nuclei were quantified manually by fluorescence microscopy after 36 h infection (**Fig.3**). WT virus developed large syncytia in HEp-2 cells, a well-described feature of *in vitro* RSV infection (Shahrabadi and Lee, 1988; Shigeta et al., 1968). All recombinant viruses showed significantly smaller syncytia compared to RSV F WT (**Fig.3A**). Mean syncytium frequency showed no remarkable differences between RSV F WT and the glycomutant viruses except for RSV F N116Q and in particular for RSV F N500Q (**Fig.3B**). Our data confirm that the N-glycan at position N500 is important for efficient *in vitro* syncytium formation of RSV-infected HEp-2 cells.

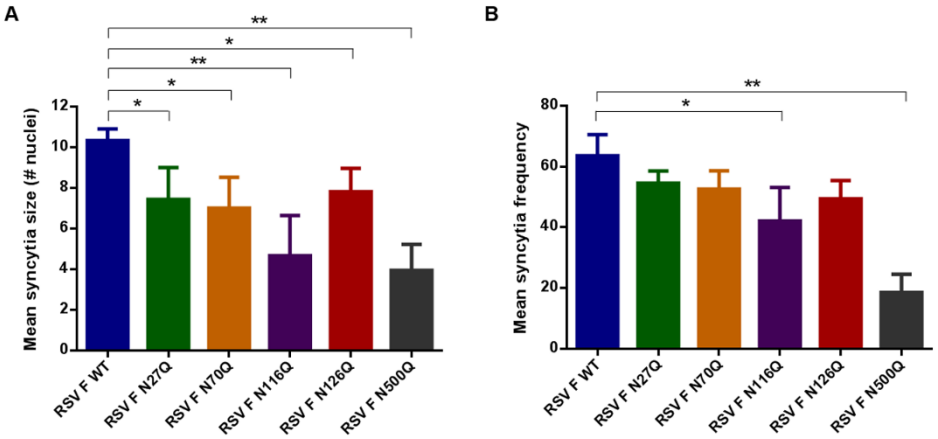

Figure 3: RSV syncytium formation efficiency. Thirty-six h after infection of HEp-2 cells with the recombinant RSV mutants, the cells were fixed and permeabilized. Syncytia were visualized by staining the nuclei with DAPI and further analyzed by fluorescence microscopy. Mean syncytia size (A) and syncytium frequency (B) were determined of 100 mKate-positive HEp-2 cells. Data represents the mean (± SD) of three independent repeats. *, $P < 0.05$; **, $P < 0.01$ (Student's unpaired two-tailed *t* test).

3.4 Removal of the N-sequon at position N500 results in impaired *in vitro* growth

Glycosylation of viral proteins is known as an important determinant of virus infectivity for numerous viruses (Beyene et al., 2004; Hanna et al., 2005b; Lee et al., 2010; Mossenta et al., 2017; Wang et al., 2013). Previous studies showed reduced infectivity after enzymatic or chemical deglycosylation of all RSV glycoproteins. Here we examined the effect of removal of the individual RSV F N-glycosylation sites at DNA level on RSV growth in HEp-2 cells. Prior to infection, viral titers were determined by an immunodetection plaque assay to ensure the same MOI for the different strains. Infection was performed at a MOI of 0.5 and supernatant was collected and titrated by plaque assay at different time points (**Fig.4A**). Approximately 18 h p.i., infected cells start to release new virus particles, which is characterized by a progressive increase in viral titers (Van der Gucht et al., 2017). At each time point, the highest titers were obtained for the WT virus, followed by mutants F N126Q, N70Q, N27Q, N116Q and lastly N500Q that showed significantly lower titers compared to F WT.

Virus particles were quantified after indirect immunofluorescence staining to determine the particle/PFU ratio (**Fig.4B**). Lower ratios indicate that more virus particles are infectious and are able to yield plaques in a plaque assay whereas higher ratios correspond with a higher number of particles that are not infectious (Carpenter et al., 2009; Schwerdt and Fogh, 1957). The mean particle/PFU ratio of recombinant virus N500Q was significantly higher as compared to the WT virus, showing that RSV F N500Q produces much more non-infectious virus particles. No significant differences could be observed for the other mutant viruses. These observations are consistent with the results of the virus growth curve and could explain the slower growth of mutant N500Q.

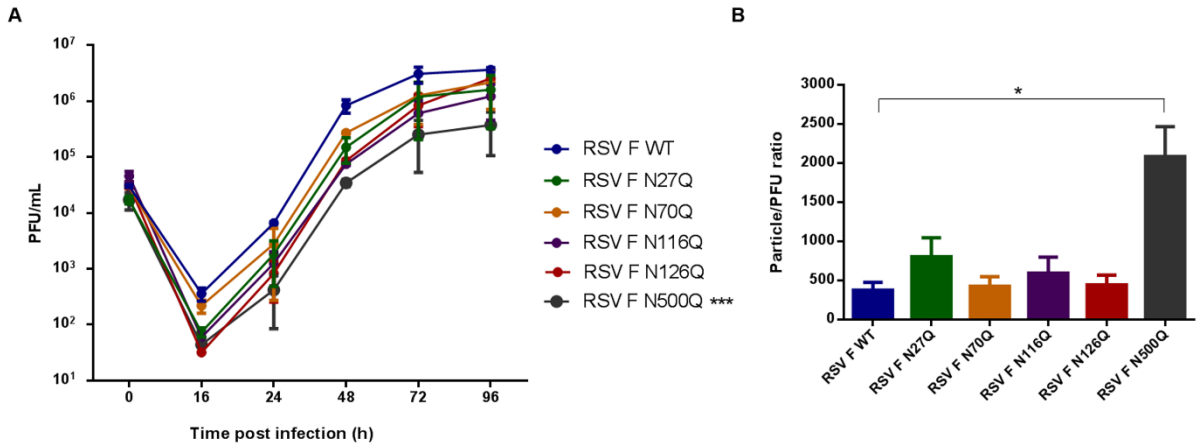

Figure 4: Effect of loss of single RSV F N-glycosylation sites on *in vitro* growth. (A) HEp-2 cells were infected with the indicated virus at an MOI of 0.5. After 2 h incubation, the cells were washed and fresh medium was added to the cells for further incubation. At the indicated time points, supernatant was collected and titrated by plaque assay. ***, $P < 0.001$ One-way ANOVA, compared to RSV F WT. (B) Two µL of virus stocks with known viral titers (PFU/mL) were dried overnight, fixed with PF and stained with polyclonal goat anti-RSV. To visualize the particles, staining with AF 555 donkey anti-goat IgG was performed. Particles were semi-quantified by fluorescence microscopic analysis and expressed as mean particles/mL of three independent repeats. Particle/PFU ratios were calculated by the following equitation: [mean particles/mL] / [PFU/mL]. Data represents the mean (± SEM) of two (A) or three (B) independent repeats. *, $P < 0.05$ Student's unpaired two-tailed *t* test).

3.5 Reduced *in vivo* replication after removal of single N-sequons of the RSV F protein

To date, the importance of RSV F glycosylation in virus infectivity was mainly studied in *in vitro* systems (Lambert, 1988; McDonald et al., 2006). Moreover, the role of the individual N-linked glycans of RSV F in this process remains unknown. Therefore, the impact of deletion of N-linked glycans of the RSV F protein individually on virus replication was assessed by infection of BALB/c mice with the RSV F glycomutant strains and determination of viral lung viral loads at 4, 6 and 8 d.p.i. (**Fig.5**). Using a conventional plaque assay no viral plaques could be observed in the lung homogenates of any of the infected animals at day 8. Both at day 4 and 6 d.p.i., no remarkable differences in lung viral load were observed between WT virus and mutants N116Q and N126Q, corresponding with the results of the *in vitro* growth assay. As observed in the *in vitro* assay, N500Q showed significant lower lung titers at 4 and 6 d.p.i. in comparison with WT virus. Unexpectedly, also mutant viruses N27Q and N70Q showed remarkable lower lung titers 4 and 6 days p.i. which was not observed *in vitro*. Taken together, our data indicate an important role for glycosylation in *in vivo* RSV replication.

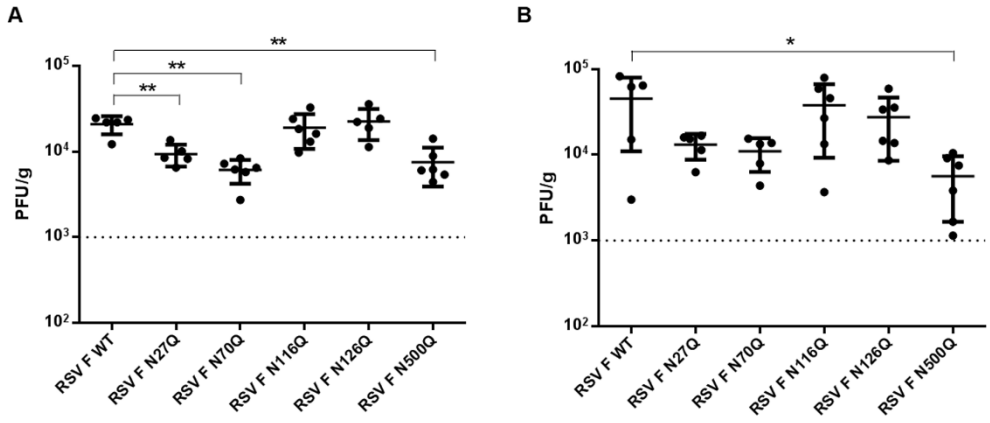

Figure 5: Effect of loss of single RSV F N-glycosylation sites on *in vivo* replication. BALB/c mice were infected with $2x10^5$ PFU/mL of indicated virus by intranasal inoculation. Lungs were collected and weighed 4 d.p.i (A) and 6 d.p.i. (B) to determine viral loads in lung homogenates by an immunodetection plaque assay. *, $P < 0.05$; n=5-6 animals/group (Student's unpaired two-tailed *t* test).

RSV disease is characterized by exacerbated mucus production causing airway hyperresponsiveness and airway constriction (Aherne et al., 1970). Moore et al identified differences in the capacity to cause

abundant mucus production between RSV F proteins of different RSV strains (Moore et al., 2009). In this study, strain RSV A2-K-line19F was used containing the mucogenic F protein of strain L19 (Hotard et al., 2012; Moore et al., 2009). Eight days p.i. lungs were excised, fixed and stained with PAS to visualize and score mucus production (**Fig.6**). Compared with mock-infected mice, more PAS-positive bronchi were observed in the RSV-infected mice, except for mutants RSF F N27Q and N70Q. Interestingly, RSF F N500Q induced the highest mucin expression, suggesting an important role of the glycan at this position in mucus production in BALB/c mice.

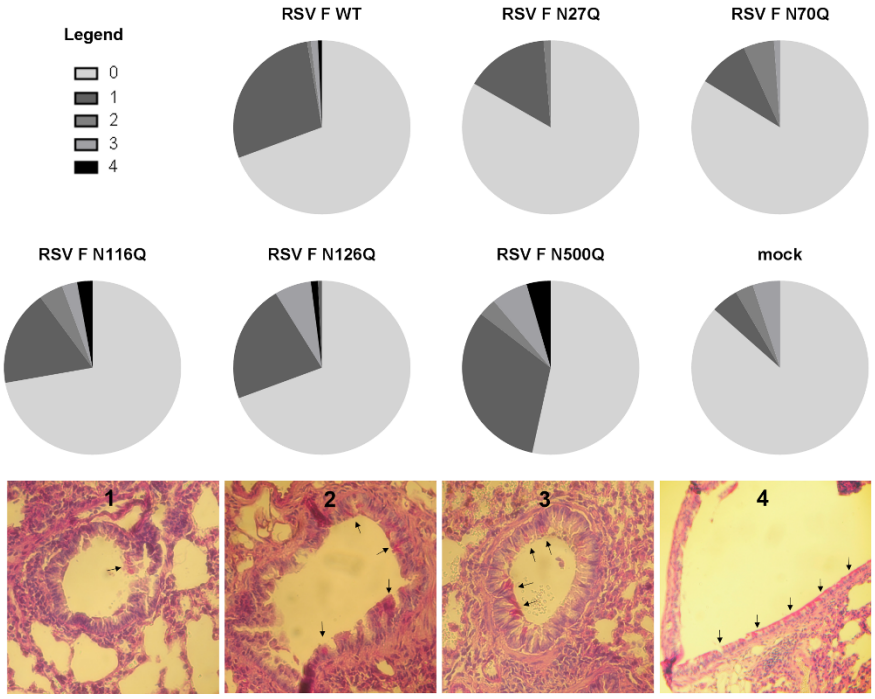

Figure 6: Pulmonary mucin expression after infection. Right lungs of mice infected with the indicated virus were collected 8 days p.i., paraffin-embedded and stained with PAS. Within the group, 30 airways of each mouse were scored (n=5-6 animals/group) 0-4 for PAS-positive cells by light microscopic analysis (representative images are shown).

3.6 Induction of enhanced neutralizing serum antibody responses upon infection with RSV F N116Q

For many viruses humoral immunity was shown to be dependent on the glycosylation profile of viral proteins since glycosylation sites can determine the antigenicity of viral proteins. Our previous research already showed the importance of RSV F N-glycosylation for its antigenicity since improved neutralizing antibody responses were observed upon F N116Q DNA immunization and lower responses after F N70Q DNA immunization compared to F WT DNA immunization (Leemans et al., 2018). To study the effect of removal of single N-glycosylation sites on the induction of antibody responses in the context of virus infection, blood was collected 3 and 5 weeks p.i. to determine total and neutralizing serum antibody titers (**Fig.7**). Comparable total antibody titers were observed after RSV F WT infection and infection with the different recombinant RSV mutants both after 3 and 5 weeks. Mice infected with RSV containing the F N116Q mutation showed significant higher neutralizing antibody responses compared to RSV F WT infection at both time points of serum collection. Five weeks p.i., neutralizing antibody titers in RSV F N500Q infected mice were significantly lower compared to RSV F WT infection which could be explained by the impaired *in vivo* growth of the virus.

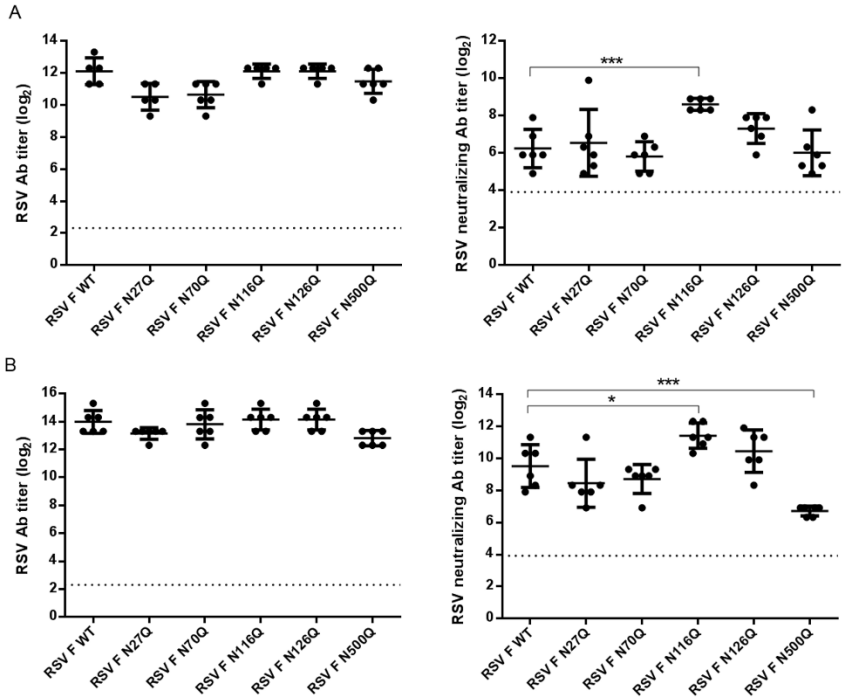

Figure 7: Antibody responses induced by recombinant RSV strains expressing glycomutant RSV F proteins. Mice were immunized by intranasal inoculation with the indicated recombinant viruses and serum was collected three weeks post immunization (A) and 5 weeks post immunization (B). (Left panel) Serum antibody titers were determined by titration of 2-fold serial dilutions of heat-inactivated serum. Binding of the antibodies was detected by HRP-conjugated goat anti-mouse IgG. Endpoint titers were determined by light microscopic analysis. (Right panel) PRNT were performed to determine neutralizing antibody responses. Serial 2-fold dilutions of heat-inactivated serum were incubated with RSV A2-K-line19F for 1 h at 37°C prior to inoculation of HEp-2 monolayers. Plaques were visualized by immunostaining with palivizumab and HRP-conjugated secondary antibodies. The 50% endpoint titers were determined by manual plaque counting. The dotted line represents the detection limit. * $p < 0.05$; *** $p < 0.001$; n = 5–6 animals/group (Student's unpaired two-tailed *t* test).

Like other class I fusion protein, the RSV F protein exist in a metastable, high-energy prefusion state that undergoes a major rearrangement to a stable, postfusion state by an irreversible and complex process that initiates fusion with host cells (McLellan et al., 2013b). Neutralizing antibodies in human serum of naturally infected RSV patients are targeted to both envelope proteins, F and G, whereby RSV F prefusion-specific antibodies are known to account for the majority of the neutralizing activity (Ngwuta et al., 2015). To evaluate differences in prefusion F expression between RSV F WT and RSV F N116Q, the glycomutant virus showing enhanced neutralizing antibodies, an ELISA-based approach was used to compare the relative amount of prefusion F on the virion surface in viral stocks (**Fig.8**). Two different prefusion F-specific mAbs MPE8 and D25 recognizing antigenic sites III and site Ø, respectively, and conformationally non-specific motavizumab were used (Corti et al., 2013; McLellan et al., 2013a; Wu et al., 2007). Significant higher ratios were observed for RSV F N116Q compared to RSV F WT with both mAbs MPE8 and D25, indicating that higher levels of prefusion F are expressed on RSV F N116Q virions.

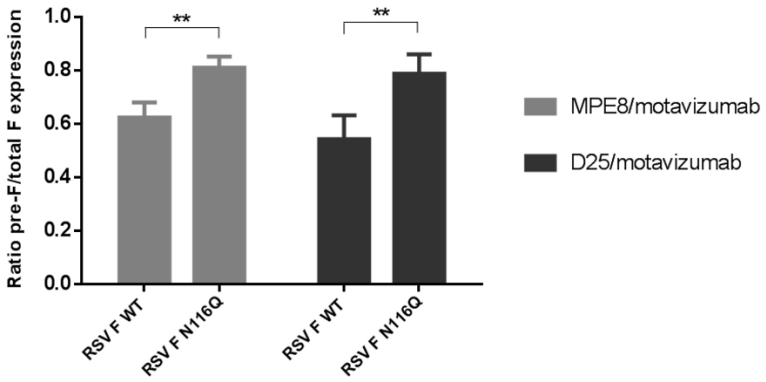

Figure 8: Relative amount of prefusion F expression compared between RSV F WT and RSV F N116Q. Virus-coated plates were incubated with 2-fold dilutions prefusion F-specific mAbs MPE8 or D25 and pre-and postfusion F-specific mAb motavizumab. After 2 h incubation, plates were incubated with goat anti-human HRP and a colorimetric reaction was catalyzed with TMB substrate reagent. Absorbance was measured at 450 nm and the ratio of binding of pre-F to total F was determined. Data represents the mean (± SD) of 3 or 4 independent repeats. **, $P < 0.01$ (Two-way ANOVA).

4 Discussion

Although current research mainly focuses on the development of a vaccine to control the burden of RSV disease, gaps remain in the knowledge about the virus itself, such as the roles of N-glycans. The RSV envelope consists of two major glycoproteins, RSV F and G, important for viral replication. Both proteins are glycosylated, and for the F protein, 5 N-glycosylation sites spread across the RSV F polypeptide chain are highly conserved, suggestive of the importance of these glycans in the structure and/or function of the protein. The importance of RSV F N-glycosylation is not yet fully understood, and a better understanding may provide new insights for vaccine development. So far, studies regarding the role of RSV F glycosylation at the level of the virus are rather limited. Incorporation of glycomutant RSV F sequences in an RSV-BAC system allowed us to study the importance of N-glycosylation in the context of replication-competent virus (**Table 1**). Substitution of the N codon into an Q codon at the individual N-sequons was performed to prevent post-translational attachment of the glycan structure. Recombinant virus was recovered after incorporation of the glycomutant RSV F sequences into an RSV-BAC clone and transfection in BSR T7/5 cells.

Table 1: Table summarizing the analysis of glycomutant viruses with different assays. (Relative assessment indicated by =, comparable to F WT; +, higher than F WT; -, lower than F WT).

	RSV F N27Q	RSV F N70Q	RSV F N116Q	RSV F N126Q	RSV F N500Q
F incorporation	=	=	=	=	=
F surface expression	-	--	--	---	--
Fusion capacity	-	-	--	-	--
***In vitro* growth**	=	=	=	=	--
Particle/PFU ratio	=	=	=	=	+
***In vivo* replication**	--	--	=	=	--
Pulmonary mucin expression	-	-	=	=	+
Total Ab response	=	=	=	=	=
Neutralizing antibody response	=	=	++	=	--

N-glycan structures can account for a significant proportion of the molecular weight of the protein, as analyzed by Western blot analysis. Non-reducing conditions of F detected with F1 subunit-specific palivizumab showed a shift for RSV F N27Q, N70Q and N500Q whereas reducing conditions showed

only a shift for RSF F N500Q due to its unique location on the F1 subunit. The molecular weight of RSV F remained unchanged after deletion of the p27 N116 and N126, confirming their absence on the mature RSV F protein (Zimmer et al., 2001b).

Viral envelope proteins play an essential role in the virus life cycle. Despite the high degree of conservation, N-linked glycosylation sites of these proteins may be dispensable for viral replication, as demonstrated for the human immunodeficiency virus type 1 (HIV-1) gp41 and the Newcastle disease virus (NDV) hemagglutinin-neuraminidase protein (Johnson et al., 2001; Panda et al., 2004). Here, we show that single mutants of each of the five conserved RSV F N-glycosylation sites resulted in the recovery of replication-competent virus. However, differences in *in vitro* and *in vivo* growth between the single mutants were observed indicating that RSV F glycosylation affects replication efficiency. Moreover, no viable virus could be rescued after removal of all RSV F conserved N-glycosylation sites, suggesting that the cumulative effect of removal of several N-glycans is lethal. Attempting to recover all combinations should reveal the combination of N-linked glycosylation sites indispensable for RSV replication, but this requires further research.

The efficiency of viral replication is determined by different steps of the replication cycle, from host cell entry to assembly and release of new virus particles, which are often dependent on the glycosylation profile of viral proteins. For example, influenza A virus entry depends on the N-glycans flanking the receptor-binding site of hemagglutinin (Sun et al., 2013). Furthermore, enhanced West Nile virus assembly and release was observed when the envelope protein is glycosylated (Hanna et al., 2005a). In this report, previous findings about the impact of single mutation of RSV F N-glycosylation sites on cell surface transport were confirmed in the context of a viral infection since RSV F surface expression was observed for all single mutants (Zimmer et al., 2001b). Additionally, dramatic disturbance of syncytium formation after mutation of site N500 was also seen after infection of HEp-2 cells with recombinant RSV expressing F N500Q (Leemans et al., 2018; Zimmer et al., 2001b). In contrast with previous research, our results showed a significant decrease in syncytium size for all single RSV F glycomutant viruses. Moreover, syncytium frequency of RSV F N116Q infected cells was also significantly reduced, suggesting a role of the N-glycosylation at N116 in the efficiency of syncytium formation in infected HEp-2 cells. The formation of multinucleated cells is a typical characteristic of RSV growth in cell lines, in particular HEp-2 cells. Our results suggest that this decreased syncytium formation impacts the efficiency of *in vitro* growth since a good correlation was observed between the level of syncytium formation and *in vitro* growth. After infection of BALB/c mice, significant reductions in lung viral load were observed for mutants RSV F N27Q, N70Q and N500Q, in comparison with F WT virus. For RSV F N500Q an explanation is provided by the increased particle/PFU ratio indicating that more non-infectious particles are produced after removal of F N500 as well as by its disturbed capacity to form syncytia. No dramatic impairment of syncytium formation was observed for mutants RSV F N27Q and F N70Q, indicating that efficient RSV *in vivo* growth is ensured by other mechanisms which are affected after removal of glycans N27, N70 and N500.

Due to the importance of viral glycosylation in determining and maintaining the antigenic conformation of viral proteins, removal of N-glycans can affect virus-specific antibody responses. As demonstrated before with recombinant F sequences lacking single N-glycosylation sites, deletion of N-glycosylation sites at position N116 can enhance antibody responses significantly upon immunization (Leemans et al., 2018). The impact of removal of this site on antibody elicitation was confirmed here. Since the position of N116 within p27, that is assumed to be released from the mature F protein, unmasking of a neutralizing epitope is less likely. However, it has been suggested that p27 is not always removed from the mature F protein and that a second cleavage event may occur at host cell entry which does suggests exposure of p27 to the immune system (Krzyzaniak et al., 2013). Alternatively, higher prefusion F expression levels observed for RSV F N116Q could be responsible for the enhanced neutralizing responses. Removal of N116 could induce conformational changes or interfere with proteolytic processing of F and affect its antigenic conformation. Whereas RSV F N116Q *in vivo* replication was comparable to RSV F WT, a reduced lung viral load after infection with RSV F N500Q is probably the cause of the lower neutralizing antibody responses induced by this recombinant virus. For decades, many attempts have been made to develop a vaccine to control the RSV burden. The most recommended approach for the pediatric population is live attenuated vaccination (LAV) since this was shown to be safe in RSV-naïve infants and children (Karron et al., 2013). Nonetheless, it remains challenging to find an optimal balance between sufficient attenuation and immunogenicity. In this

context, combined RSV glycomutations which attenuate the virus and induce enhanced antibody responses may provide insights in LAV approaches. However, single amino acid mutations may be more susceptible to reversion and the influence of the mutations on pathogenicity needs to be considered. For multiple viruses the role of glycosylation in viral pathogenicity was already demonstrated (de Brogniez et al., 2015; Montefiori et al., 1988; Shirato et al., 2004; Zhao et al., 2017). For example, West Nile virus (WNV) envelope protein glycosylation was shown to be related to the neuroinvasiveness of the virus (Shirato et al., 2004). Moreover, combined glycomutations of WNV proteins showed no neuroinvasiveness and enhanced neutralizing titers in a mouse model (Whiteman et al., 2010). RSV-associated lower respiratory tract disease in children is characterized by excessive mucus production. Since RSV strains with different F sequences were identified that induced varying levels of airway mucin expression in mice, it was suggested that RSV F is an important mediator for this process (Hotard et al., 2015; Moore et al., 2009; Stokes et al., 2013). In our study, a mucogenic strain was used and enhanced mucin expression was observed 8 d.p.i by PAS staining, compared with mock-infected mice. Mutants RSV F N27Q and N70Q showed comparable levels of PAS-positive cells with mock-infected mice whereas the levels of mutants RSV F N116Q and N126Q coincided with these of WT-infected mice. For these mutants the levels of PAS-positive cells were consistent with the *in vivo* virus growth. Surprisingly, N500Q showed the highest levels mucin-expressing cells despite its low lung viral loads, suggesting no correlation between lung viral load and mucin expression, as observed previously (Hotard et al., 2015).

In summary, previous observations about the role of RSV F N-glycosylation sites in RSV F cell surface transport and fusion capacity were confirmed in the context of replication-competent virus. We showed that complete removal of RSV F glycosylation resulted in replication-incompetent virus particles, indicating an indispensable role of RSV F glycosylation for RSV replication. Additionally, the importance of the individual sites of the mature RSV F protein in *in vivo* growth was demonstrated. Moreover, more evidence was obtained for the removal of the N-glycosylation site at position N116 to enhance the antigenicity of the F protein, further supporting its application in potential vaccine approaches.

5 Acknowledgements

We would like to thank Pim-Bart Feijens for performing all mice experiments and Rita Van den Bossche for the histochemical staining of the lung samples. We also thank K.K. Conzelmann for providing BSR T7/5 cells, M.L. Moore and A.L. Hotard for the pSynkRSV-line19F construct and associated RSV helper plasmids, J-J Letesson for the RSV N-specific mAbs and J.A. Melero, J.S. McLellan and B.S. Graham for providing RSV F-specific mAbs. This work was supported by project 36378 (University of Antwerp Industrial Research Fund).

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