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„Development of a multivalent universal influenza virus vaccine based on induction of antibodies against conserved epitopes"

verfasst von / submitted by

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Abstract

Influenza viruses remain a severe threat to human health. Current seasonal influenza virus vaccines are only sufficiently protective against well matched strains, but not against mutated or novel pandemic viruses. Therefore, there is a great need for the development of a universal influenza virus vaccine that would confer broad protection. At the Icahn School of Medicine at Mount Sinai, a vaccination approach that uses sequential vaccination with chimeric hemagglutinins (HAs) has been developed and successfully tested. These constructs contain an exotic head domain, but the same conserved HA stalk domain. By repeated vaccination with divergent hemagglutinin head domains, but the same stalk domain, a strong response against the conserved, immuno-subdominant HA stalk can be induced. Antibodies directed against the stalk domain of the hemagglutinin show broad protection but are often restricted to binding hemagglutinins from either influenza A group 1, group 2, or influenza B viruses. Therefore, to protect against all influenza A and B viruses, a trivalent influenza virus vaccine which covers all groups of HAs will be required. Live-attenuated influenza virus vaccines (LAIVs) are an interesting vaccine platform since they elicit mucosal immunity and show high efficacy, especially in children. Chimeric HA expressing LAIVs have already been successfully tested for influenza A viruses. To extend the testing to influenza B viruses, we aimed to generate reverse genetics systems based on licensed vaccine strains that would allow the production of reassortant viruses expressing the HAs of choice. Plasmids for the B/USSR/60/1969 and B/Ann Arbor/1/1966 were produced. A B/USSR/60/1969 reassortant virus was successfully rescued and experiments indicated a cold-adapted phenotype. Further work will be required for the B/Ann Arbor/1/1966 rescue system. The reverse genetics systems could enable the production of reassortant viruses for use in a universal influenza virus vaccine.

Introduction

Influenza viruses are enveloped, negative-sense RNA viruses belonging to the *Orthomyxoviridae* family[1]. The genome consists of eight segments, which encode for the trimeric RNA polymerase complex (PB2, PB1, PA), the matrix protein (M), the nucleoprotein (NP), the non-structural protein (NS1), as well as two surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA)[1]. Influenza viruses are classified into four different genera - influenza A, B, C and D viruses. Influenza A, B and C viruses circulate in humans, but only influenza A and B viruses cause severe disease[2,3]. Based on the identity of the HA segment, influenza A viruses are further divided into two major groups (group 1 and group 2) and many different subtypes, while influenza B viruses are divided into two distinct lineages (Yamagata and Victoria)[1,4]. Influenza A viruses are generally associated with more severe symptoms than influenza B viruses, but recent studies from the Centers for Disease Control and Prevention (CDC) have shown no significant differences in either the length of hospitalization caused by or the proportion of patients infected with these two viruses[5]. Both genera of influenza viruses are the prevalent circulating types in humans and cause seasonal outbreaks in the Northern and Southern hemispheres every year[6]. The World Health Organization (WHO) estimates there are up to 650,000 deaths annually associated with influenza viruses[7]. Seasonal influenza virus outbreaks are mainly caused by changes in the surface glycoproteins through an event known as antigenic drift[8,9]. Antigenic drift is the result of the ability of influenza viruses to continuously undergo mutations which can allow viruses to escape the human herd immunity[10,11]. Through this mechanism, modified viruses arise and evade prior host immune responses[1].

Current seasonal influenza virus vaccines are protective against viruses that match the vaccine strains well[12,13]. However, because of the ever-changing nature of the virus, vaccines must be updated each year and humans must be re-vaccinated[14]. During pandemic outbreaks, new effective vaccines need to be produced and quickly distributed throughout the entire population. Therefore, there is a substantial public health and economic interest in the research of influenza virus vaccines that elicit a strong long-term protection against all existing and future circulating strains[15,16].

Current influenza virus vaccines mainly elicit an antibody response against the immuno-dominant head domain of the influenza virus surface glycoprotein HA[17].

However, these antibodies are largely strain-specific, and often do not recognize mutated viruses. To achieve broader long-term protection, the immune response can be re-directed towards more conserved epitopes, like the immuno-subdominant HA stalk domain, which is similar between different influenza virus strains[18–21]. One strategy is based on sequential vaccination with so-called chimeric HAs (cHAs). cHAs contain exotic head domains from avian influenza viruses, but the conserved stalk domain of currently circulating viruses (Fig. 1A). Repeated vaccination with cHAs that contain different exotic heads but the same stalk induces a weak head-specific antibody response but elicits potent recall responses against the HA stalk domain (Fig. 1A)[21–30]. These HA stalk domain antibodies can broadly cross-react with different subtypes of influenza viruses[21–30]. This approach has been successfully tested in mouse and ferret models[21–30].

While the antibody response against the stalk domain is broadly reactive within phylogenic groups of HAs, only few antibodies show cross-group reactivity (Fig. 1B)[22,27]. Therefore, it will be necessary to create a trivalent influenza virus vaccine that contains cHAs with stalk domains from influenza A group 1 and 2 viruses and influenza B viruses (Fig. 1B)[18].

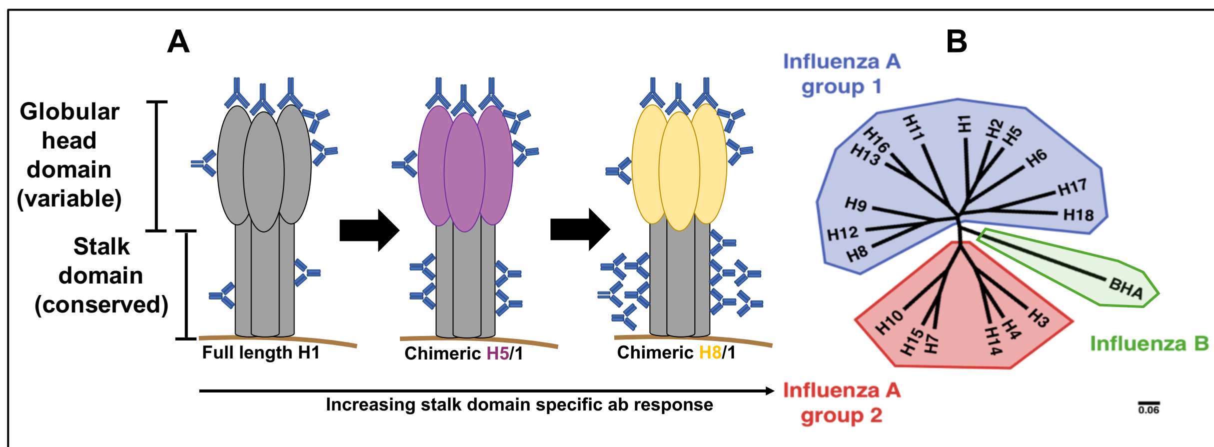


Figure 1│ A: The universal influenza virus vaccination strategy based on chimeric hemagglutinins. Through a sequential vaccination with influenza A group cHAs, which consist of exotic head domains (violet, yellow) on top of a conserved stalk domain, the adaptive immune response (blue) will be re-directed towards the more conserved stalk domain. The exposure to exotic head domains to which humans are naïve induces only low primary head responses but allows for a potent recall response against previously encountered epitopes, namely in the HA stalk domain. Since influenza B virus cHAs are biochemically not stable, four major antigenic sites in the head domain were exchanged with influenza A avian antigenic sites to remain the general principal of re-directing the immune response. The schematic is not to scale. **B: Phylogenetic tree based on the HA sequences of influenza A and B viruses.** HAs of influenza A viruses are separated in two distinct groups (blue, red) whereas

HAs of influenza B viruses are more distantly related and distinct (green). The chimeric vaccination strategy primarily induces HA stalk domain antibodies within one group of HAs but only elicits low cross-group responses. For a universal influenza vaccine, a trivalent formulation which contains HAs from all three different groups is essential. The scale bar indicates 6% amino acid difference. The figure was adapted from Nachbagauer, R., 2016[22]

Studies have shown significantly greater efficacy of live-attenuated influenza virus (LAIV) vaccines compared with inactivated influenza virus (IIV) vaccines in young children aged between 12 to 59 months without a history of asthma or wheezing[31]. Besides this, similar efficacy of LAIVs and IIVs have been observed in adults[32]. For the development of a multivalent universal vaccine, LAIVs containing cHAs have already been generated for influenza A viruses. These candidates have the added benefit of eliciting both a potent cellular immune response and a localized antibody response on mucosal surfaces, and are currently being tested in clinical trials[21–30,33]. LAIVs expressing influenza B cHAs still need to be developed.

The aim of this study was the generation of novel reverse genetics systems for the rescue of cold-adapted influenza B viruses for the development of a universal influenza virus vaccination approach. Through these novel reverse genetics systems, reassortants of the B/USSR/60/1969 master donor virus (MDV) and the B/Ann Arbor/1/1966 MDV should be rescued and compared with a B/Malaysia/2506/2004 wild type virus for the verification of cold-adapted phenotypes.

Material and methods

Cloning of influenza B virus rescue plasmids

For the cloning of the influenza B virus rescue plasmids, novel bi-directional pDZ[34] rescue plasmids were designed and engineered (Fig. 2). RNA of wild type strains was isolated (High Pure Viral RNA Kit, Roche), and all eight individual segments of each virus amplified through RT-PCR (SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase, Invitrogen, Thermo Fisher Scientific)[34]. All genes were separately cloned into SapI-digested pDZ rescue plasmids (Fig. 2). Through PCR, optimized B/Malaysia/2506/2004 gene-specific untranslated regions (UTRs) from an existing highly efficient influenza B rescue system (Fulton, B.) were constructed before and after each open reading frame (ORF) for improved virus replication (Fig. 2). The plasmids were sequenced and the B/USSR/60/1969 and B/Ann Arbor/1/1966 internal wild type genes were aligned with published sequences of the corresponding cold-adapted strains[35,36]. Through site-directed mutagenesis PCR, mutations in the wild type backbone genes were introduced to generate sequences identical to the published sequences. The number of potential cold-adapted mutations varied from 2 to 24 DNA base pair changes within the six individual segments of both strains[35,36]. The B/Malaysia/2506/2004 wild type plasmids as well as the novel potential cold-adapted plasmids were sequenced and used for a recombinant virus rescue through a reverse genetics system (Tab. 1, Fig. 2, 3).

Influenza B virus rescue utilizing a reverse genetics system

For the influenza B virus rescue, human embryonic kidney 293T (HEK 293T) cells were transfected with eight individual virus rescue plasmids encoding for all virus segments (HA and NA of B/Malaysia/2506/2004 were used for all rescues) and Madin-Darby Canine Kidney (MDCK) cells were infected with rescued virions for an additional round of viral replication (Fig. 3). For the transfection, $1.25x10^6$ HEK 293T cells were seeded in 2 ml of growth media (1x Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% 1M Hepes, 1% Pen/Strep (Gibco, Thermo Fisher Scientific)) on Poly-L-lysine (Sigma-Aldrich) coated plates and incubated at 37°C with 5% CO_2 20 h before transfection. At the next day, for the transfection mixture, 100 µl of 1x optimized-Minimal Essential Medium (Opti-MEM) (Gibco, Thermo Fisher Scientific) was mixed with 0.5 µg of each individual rescue plasmid (Tab. 1). Afterwards, 20 µl of the transfection reagent TransIT-LT1 (Mirus) was carefully added and the mixture incubated at room temperature (RT) for 30 min. The

growth media of HEK 293T cells was replaced with 1 ml of serum-free transfection media (1x MEM supplemented with 1% 1M Hepes, 1% Pen/Strep (Gibco, Thermo Fisher Scientific)). After incubation, the transfection mixture was added dropwise to 70-80% confluent HEK 293T cells with swirling, and the cells were incubated at 37°C with 5% CO_2 for 20 h. The next day, transfected HEK 293T cells were moved to 33°C with 5% CO_2 and incubated for 24 h. Furthermore, $2.5x10^5$ MDCK cells were seeded in 2 ml of growth media at 37°C with 5% CO_2 24 h before infection. On the day of infection, 100% confluent MDCK cells were washed once with 1x Phosphate Buffered Saline (PBS) (Gibco, Thermo Fisher Scientific). Supernatant of transfected HEK 293T cells was mixed with 1 µg/ml of TPCK-trypsin for proteolytic cleavage of the HA in rescued virions. Afterwards, 250 µl of the supernatant was added onto MDCK cells and the infected cells were incubated at either 37, 33, 29.5 or 27.5°C with 5% CO_2 for 1 h depending on the virus being rescued (Tab. 2). Wild type viruses were incubated at higher temperatures, while more temperature-sensitive cold-adapted viruses were incubated at lower temperatures (Tab. 2). The plates containing infected cells were agitated every 10-15 min to equally distribute viruses and prevent the monolayer from drying out. Afterwards, the infection mixture was aspirated, and cells were washed twice with PBS. PBS was replaced with 1.25 ml of UltraMDCK serum-free media (Lonza) with 1 µg/ml of TPCK-trypsin and the infected cells were incubated at either 37, 33, or 29.5°C with 5% CO_2 for 48-72 h until cytopathic effect (CPE) was observed (Tab. 2). Afterwards, the supernatant of infected MDCK cells was used for a hemagglutination assay as well as for a plaque purification assay (Fig. 3, 4).

Table 1│ Overview of attempted recombinant influenza B virus rescues. Rescues were attempted for B/Malaysia/2504/2004 wild type, a 6+2 potentially cold-adapted B/USSR/60/1966 reassortant and a 6+2 cold-adapted B/Ann Arbor/1/1966 reassortant virus. All rescue plasmids contained optimized gene-specific B/Malaysia/2506/2004 UTRs before and after each ORF. The segments encoding for the internal proteins of the B/USSR/60/1969 and B/Ann Arbor/1/1966 wild type strains were mutagenized to confer a cold-adapted phenotype based on published literature[35,36].

ORFs	B/Malaysia/2506/2004	6+2 B/USSR/60/1969	6+2 B/Ann Arbor/1/1966
PB2	**wild type**	**cold-adapted**	**cold-adapted**
PB1	**wild type**	**cold-adapted**	**cold-adapted**
PA	**wild type**	**cold-adapted**	**cold-adapted**

M	wild type	cold-adapted	cold-adapted
NP	wild type	cold-adapted	cold-adapted
NS	wild type	cold-adapted	cold-adapted
HA	wild type	wild type	wild type
NA	wild type	wild type	wild type

Testing for presence of influenza B viruses by hemagglutination assay

Presence of rescued recombinant influenza B viruses was confirmed by hemagglutination assay with supernatants of infected MDCK cells (Fig. 3, 4). The presence of virus particles causes hemagglutination of chicken red blood cells (RBCs) while the absence of virus particles results in an accumulation of RBCs in a V-bottom plate. For the assay, 2-fold serial dilutions of the infected MDCK supernatants in PBS were prepared in a 96-well V-bottom plate. For a negative control, only PBS was used. Afterwards, 50 µl of 0.5% chicken RBCs in PBS were added. The plate was incubated at 4°C for 30-45 min and the results assessed (Fig. 4).

Plaque purification and plaque assay of recombinant influenza B viruses

The rescued influenza B viruses were plaque purified from a clonal population for a reduction of genetic diversity and viral titers in PFU/ml were determined by plaque assay. For this, $6.5x10^5$ MDCK cells per well were seeded in 2 ml of growth media onto 12-well plates and incubated at 37°C with 5% CO_2 24 h before infection. The MDCK supernatant containing the rescued viruses was serially diluted 10^{-1} to 10^{-9} in PBS. 100% confluent MDCK cells were washed once with PBS and infected with 250 µl of each dilution. Afterwards, the cells were incubated at the same temperature conditions as described earlier with 5% CO_2 for 1 h and swirled every 10-15 min. During infection, 2% Oxoid agar (Thermo Fisher Scientific) was boiled and maintained at 55°C. Furthermore, an overlay (2x MEM supplemented with 16% H_20 and 1% DEAE Dextran) was prepared and warmed up to 37°C. After the diluted virus infection, the Oxoid agar as well as 1 µg/ml of TPCK-trypsin was added to the overlay. The infected cells were washed with PBS and covered with warm overlay. After the overlay solidified at RT, the cells were incubated at the same temperature conditions with 5% CO_2 for 48 h until plaques were observed (Fig. 3). Clearly separated plaques were isolated from the

overlay and resuspended in 400 µl of ice-cold PBS. The plaque-purified viruses were diluted 1:1000 in PBS and 200 µl were injected into 8-day old eggs for virus propagation (Fig. 3). The eggs were incubated at 33°C for 72 h and the allantoic fluid was harvested under sterile conditions. For determination of the viral titers of plaque purified viruses, naïve MDCK cells were infected with 250 µl of the allantoic fluid as described above. After 48 h post infection, the cells were fixed with 4% formaldehyde at 4°C for 24 h. Afterwards, the overlay was removed under running water and the fixed cells blocked with 5% milk powder in Phosphate Buffered Saline with Tween (PBS-T) for 1 h. The blocking solution was replaced with 1% milk powder in PBS-T containing the broadly cross-reactive anti-HA human antibody CR9114. After 2 h of incubation, the primary antibody solution was removed, and the cells were washed three times with PBS-T. Then, the cells were incubated with a secondary anti-human IgG-horseradish peroxidase antibody (Sigma-Aldrich) for another 2 h. Afterwards, the secondary antibody solution was removed, and the cells were washed again three times with PBS-T before plaques were detected using a developing reagent (TrueBlueTM Peroxidase Substrate, Sera care). Plaques were counted, and viral titers determined (Eq. 1).

Equation 1│ Calculation of viral titers in plaque forming units per ml (PFU/ml). For the calculation of the viral titers in PFU/ml, the visualized number of plaques was divided by the dilution of the allantoic fluid and multiplied by the infected sample volume in ml.

Sequence confirmation of rescued influenza B viruses

For sequence confirmation of the rescued influenza B viruses, RNA extraction and RT-PCR of all eight individual segments was performed as described above. The resulting DNA fragments were sequenced, and the resulting sequences aligned with the corresponding published sequences.

Temperature dependent growth of reassortant viruses

For the verification of a potential cold-adapted phenotype of the rescued 6+2 B/USSR/60/1966 reassortant virus, temperature depended viral growth was analyzed by plaque assay. For both B/Malaysia/2506/2004 and the reassortant virus, three 8-day old eggs were injected with 100 µl of 1000 PFU/ml and grown at 37, 33, 29.5 or 27.5°C for 72 h. For influenza B viruses, a cold-adaption is defined when the mutant virus has a greater replication at 25°C than the wild type virus and a temperature-sensitivity is considered when a comparatively lower viral reproductive rate is seen at higher temperatures[37]. The allantoic fluid was collected and 250 µl was used for plaque assays. Plaque assays were performed at 33°C as described earlier.

Results

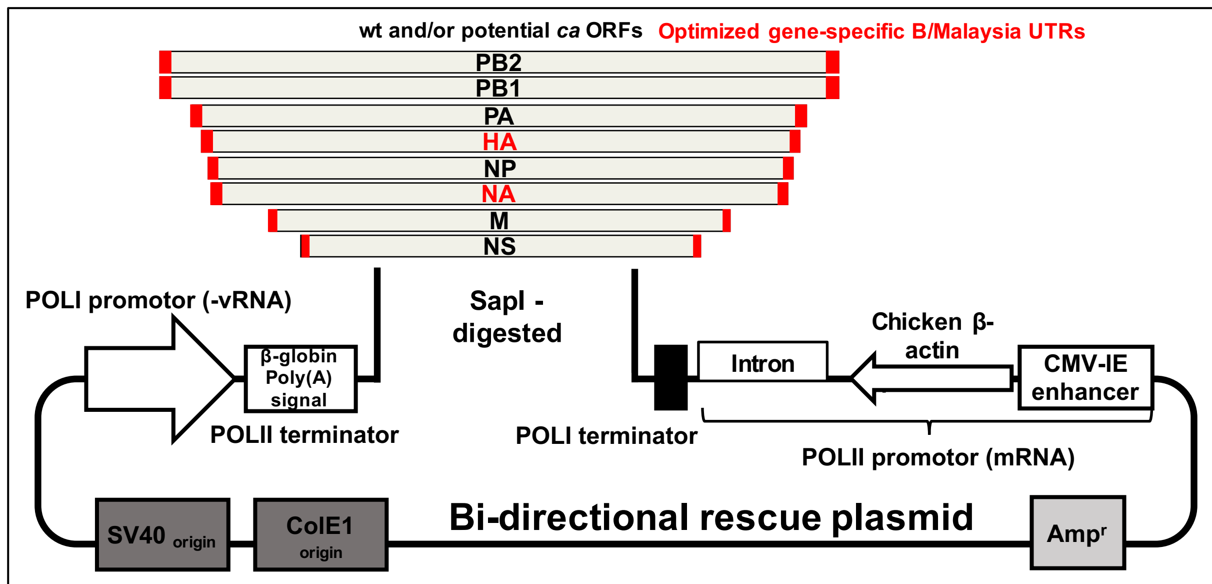


Figure 2│ Generation of different influenza B virus rescue plasmids. All eight influenza B virus genes were separately cloned into SapI-digested pDZ rescue plasmids. Virus genes were wild type and potentially cold-adapted sequences based on published literature[35,36]. For a 6+2 reassortant virus rescue, all backbone genes encoding for internal proteins were cold-adapted sequences (black), whereas the HA and NA genes were B/Malaysia/2506/2004 wild type sequences (red). All influenza rescue plasmids contained optimized gene-specific B/Malaysia/2506/2004 UTRs (red). The plasmid is not to scale. The figure was adapted from Dr. L. Martínez-Sobrido and Dr. A. García-Sastre, 2010[34].

The bi-directional pDZ plasmid was used for recombinant influenza B virus rescue through a reverse genetics system. It is derived from the protein expression plasmid pCAGGs and contains a human RNA polymerase I promotor with a mouse terminator region, which encodes negative sense RNA. The opposite reading direction includes a polymerase II transcription cassette (CMV-IE enhancer, chicken β-actin promoter, intron) with a β-globin Poly(A) terminator signal, which encodes viral mRNA from the same gene. Both, negative sense RNA as well as viral mRNA is essential for the formation and replication of recombinant virions. For the rescue of a B/Malaysia/2506/2004 wild type virus, eight individual plasmids with PB2, PB1, PA, M, NP, NS, HA and NA wild type genes were generated from a laboratory stock (Fulton, B.) (Tab. 1). For the rescue of a cold-adapted 6+2 B/USSR/60/1969 and B/Ann Arbor/1/1966 reassortant virus, six individual plasmids for PB2, PB1, PA, M, NP, and NS genes were generated for each virus (Tab. 1). All plasmids contained optimized gene-specific UTRs for improved viral replication of the rescued recombinant viruses (Tab. 1). All novel plasmids were sequence confirmed.

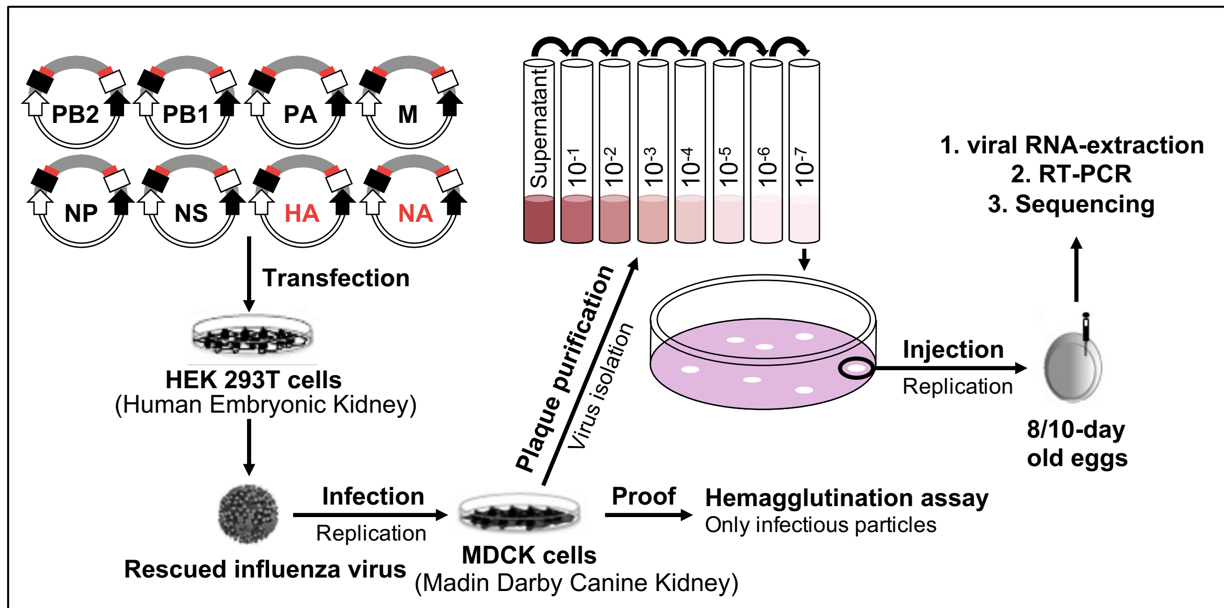
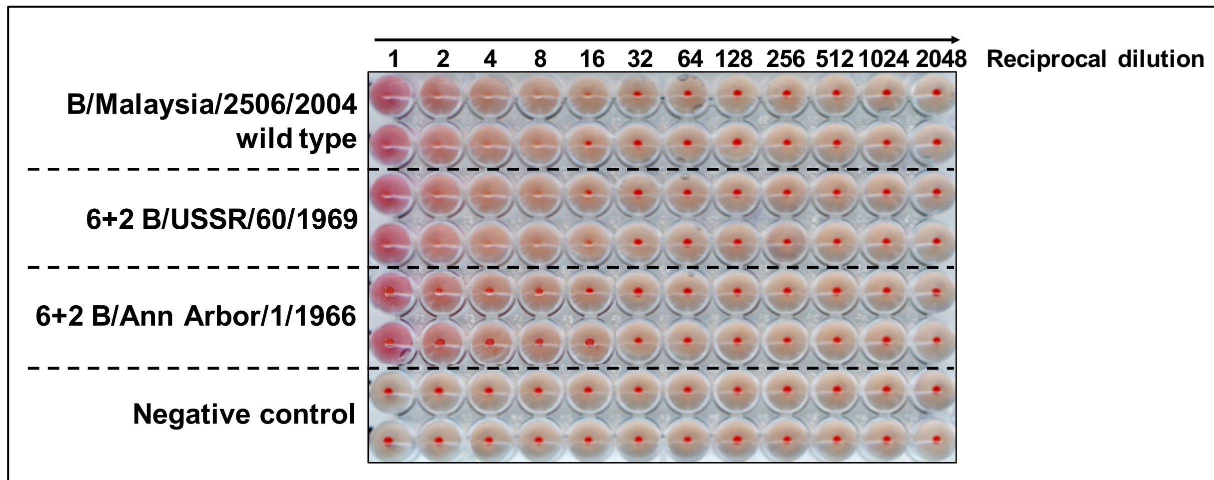


Figure 3│ Principle of influenza virus rescue through a reverse genetics system. All eight individual virus rescue plasmids were transfected into HEK 293T cells for the rescue of recombinant influenza B viruses. Transfected HEK 293T tissue culture supernatants were used to infect MDCK cells for viral replication. Through a hemagglutination assay, infectious virus particles were confirmed. MDCK tissue culture supernatants were serial diluted and rescued viruses were plaque purified before being passaged in 8-day old eggs for further viral replication. The figure was adapted from Dr. L. Martínez-Sobrido and Dr. A. García-Sastre, 2010[34].

For the rescue of a B/Malaysia/2506/2004 wild type virus, all eight individual rescue plasmids with the appropriate wild type sequences were transfected into HEK 293T cells (Tab 1.) In contrast, for the rescue of 6+2 cold-adapted B/USSR/60/1969 and B/Ann Arbor/1/1966 reassortant viruses, plasmids for the six internal proteins from the viruses were transfected with the B/Malaysia/2506/2004 wild type HA and NA (Tab. 1, Fig. 2). Transfected HEK 293T cells produce negative sense RNA and express recombinant proteins through viral mRNA. Through this mechanism, infectious particles are generated. For a selection of infectious virus particles through viral replication, MDCK cells were infected with HEK 293T tissue culture supernatants until CPE was observed. The MDCK tissue culture supernatants were used to confirm rescued recombinant influenza B viruses through a hemagglutination assay. The viruses were further plaque purified to reduce clonal diversity and the genetic composition confirmed through RT-PCR.



positive hemagglutination units were observed. In contrast, no hemagglutination was observed for the cold-adapted 6+2 B/Ann Arbor/1/1966 reassortant virus. PBS was used as a negative control.

Hemagglutination was detected for the recombinant B/Malaysia/2506/2004 wild type and the potential cold-adapted 6+2 B/USSR/60/1969 reassortant virus in infected MDCK supernatants at 33°C 48 h post infection. For both viruses, 4-5 positive hemagglutination units were observed. In contrast, no hemagglutination was detected for the cold-adapted 6+2 B/Ann Arbor/1/1966 reassortant virus under the same conditions. The hemagglutination assay was done in duplicates with very similar results for each sample. The negative control with PBS showed no hemagglutination.

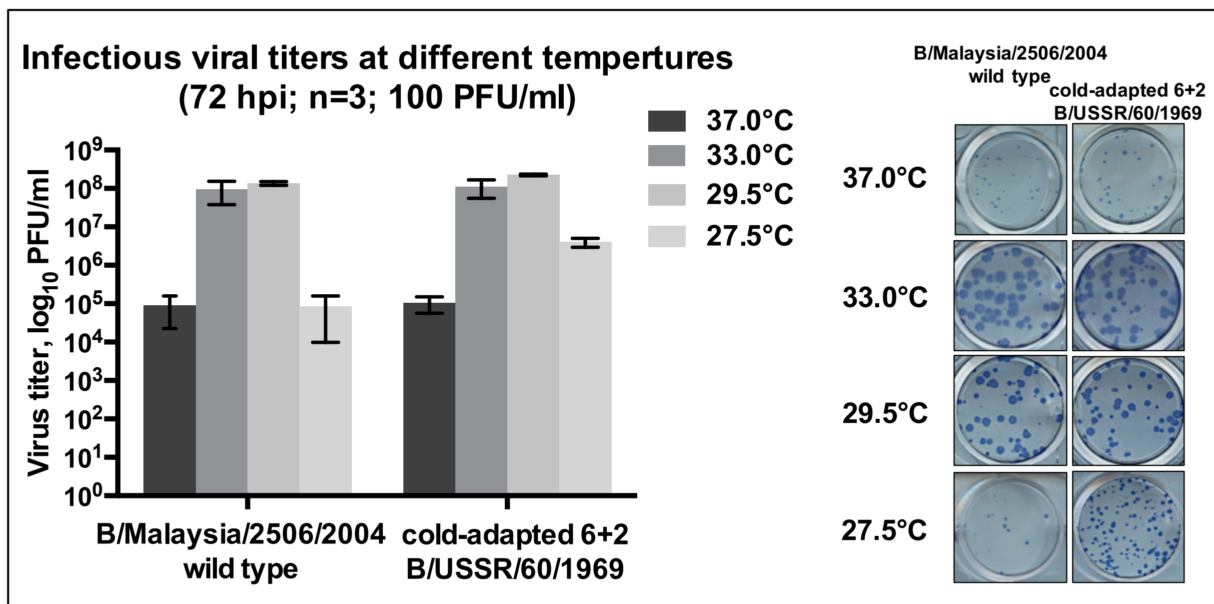


Figure 5│ Verification of a cold-adapted influenza B virus phenotype. In plaque assays, viral titers and plaque sizes of the potential cold-adapted 6+2 B/USSR/60/1969 reassortant virus and the B/Malaysia/2506/2004 wild type virus were compared. Both viruses showed similar viral titers and plaque sizes at 37, 33 and 29.5°C. In contrast, the potential cold-adapted virus showed an almost 2-log increase in the viral titers at 27.5°C compared to the wild type virus at the same temperature condition. Similar results were observed in plaques sizes, where the potential cold-adapted virus showed slightly larger plaques compared to the wild type virus at the same temperature condition.

For the verification of a cold-adapted influenza B virus phenotype, plaque assays were compared between the potential cold-adapted 6+2 B/USSR/60/1969 reassortant virus and the B/Malaysia/2506/2004 wild type virus. For both viruses, 100 µl of 1000 PFU/ml were injected into eggs and grown at 37, 33, 29.5 and 27.5°C for 72 h. Three eggs were used for each different temperature condition. Plaque assays were performed with the harvested allantoic fluid.

Both viruses achieved similar viral titers of 10^5 PFU/ml at 37°C and 10^8 PFU/ml at 33 and 29.5°C. In contrast, the potentially cold-adapted virus showed levels of 10^6 to 10^7 PFU/ml at 27.5°C, which is close to 2-fold higher compared to the wild type virus at the same temperature condition. These results show a higher viral reproductive rate at 27.5°C compared with the wild type virus and a comparatively similar reproduction rate at higher temperatures. Furthermore, plaque sizes of the potential cold-adapted virus were slightly larger compared to the wild type virus at 27.5°C.

As a result of troubleshooting for the cold-adapted B/Ann Arbor/1/1966 virus, different temperature adjustments in the transfection and infection process during virus rescue were made (Tab. 2). The transfection temperature was performed at either 37 or 33° for an efficient uptake of the transfected plasmids by HEK 293T cells. The infection temperature of the MDCK cells was adapted to the suggested optimal viral replication temperature of the viruses. For temperature-sensitive or cold-adapted viruses, the optimal viral replication temperature was assumed between 25 and 27.5°C.

To see if the cold-adapted B/Ann Arbor/1/1966 plasmids with optimized gene-specific UTRs are able to rescue independently in a well-established influenza B rescue system, all six plasmids were transfected individually in an attempt to rescue a 7+1 B/Malaysia/2506/2004 reassortant virus. A transfection temperature of 37 or 33°C was used with an infection temperature of 33°C. While the cold-adapted B/Ann Arbor/1/1966 PB1, PA, M and NS genes were rescued, the PB2 and NP genes could not be rescued at different temperature conditions (Tab. 2). The non-rescuable cold-

adapted PB2 and NP genes showed more changes in amino acid sequence than the other rescuable cold-adapted genes. A 4+4 B/Malaysia/2506/2004 reassortant virus with the rescuable cold-adapted PB1, PA, M and NS genes was rescued under a transfection temperature of 33°C and an infection temperature of 27.5°C (Tab. 2). Since a 7+1 with the missing PB2 or NP genes as well as a cold-adapted 6+2 reassortant virus rescue had not been achieved, optimized gene-specific B/Malaysia/2506/2004 UTRs were exchanged with published gene-specific B/Ann Arbor/1/1966 UTRs and more virus rescues were attempted. In comparison, the previously rescued cold-adapted PB1, PA, M and NS genes with optimized gene-specific UTRs could not be rescued with published UTRs in the same backbone and under the same temperature conditions (Tab. 2). The desired cold-adapted 6+2 B/Ann Arbor/1/1966 MDV could not be rescued under several transfection and infection temperatures (Tab. 2).

Table 2│ Overview of rescue attempts of B/Malaysia/2506/2004 reassortant viruses with cold-adapted B/Ann Arbor/1/1966 genes. Reassortant virus rescues were attempted at different transfection and infection temperatures. UTR-M stands for optimized gene-specific B/Malaysia/2506/2004 UTRs, whereas UTR-A stands for published cold-adapted B/Ann Arbor/1/1966 UTRs.

<table>
<tr><th>Reassortant virus</th><th>UTRs [M/A]</th><th>Cold-adapted ORFs</th><th>Rescue</th><th>Transfection Temp. [°C]</th><th>Infection Temp. [°C]</th></tr>
<tr><td rowspan="12">7+1 B/Malaysia/2506/2004 backbone</td><td rowspan="6">M</td><td>PB2</td><td>No</td><td colspan="2">37, 33, 29.5, 27.5</td></tr>
<tr><td>PB1</td><td rowspan="3">Yes</td><td rowspan="3">37</td><td rowspan="3">33</td></tr>
<tr><td>PA</td></tr>
<tr><td>M</td></tr>
<tr><td>NP</td><td>No</td><td colspan="2">37, 33, 29.5, 27.5</td></tr>
<tr><td>NS</td><td>Yes</td><td>37</td><td>33</td></tr>
<tr><td rowspan="6">A</td><td>PB2</td><td rowspan="6">No</td><td colspan="2">37, 33, 29.5, 27.5</td></tr>
<tr><td>PB1</td><td rowspan="3">37</td><td rowspan="3">33</td></tr>
<tr><td>PA</td></tr>
<tr><td>M</td></tr>
<tr><td>NP</td><td colspan="2">37, 33, 29.5, 27.5</td></tr>
<tr><td>NS</td><td>37</td><td>33</td></tr>
<tr><td>4+4 B/Malaysia/2506/2004 backbone</td><td>M</td><td>PB1/PA/M/NS</td><td>Yes</td><td>33</td><td>27.5</td></tr>
</table>

2+6 B/Malaysia/2506/2004 (≙ cold-adapted 6+2 B/Ann Arbor/1/1966)	M	PB2/PB1/PA/ M/NP/NS	No	37, 33, 29.5, 27.5
2+6 B/Malaysia/2506/2004 (≙ cold-adapted 6+2 B/Ann Arbor/1/1966)	A	PB2/PB1/PA/ M/NP/NS	No	37, 33, 29.5, 27.5

Discussion

In the present study, several novel pDZ plasmids have been designed and engineered, which allowed the rescue of a potentially cold-adapted 6+2 B/USSR/60/1966 reassortant virus. Furthermore, a B/Malaysia/2506/2004 wild type virus was rescued for the verification of a cold-adapted phenotype.

All six individual B/USSR/60/1969 backbone rescue plasmids with potential cold-adapted mutations and optimized gene-specific UTRs as well as eight additional B/Malaysia/2506/2004 wild type rescue plasmids were sequence confirmed (Tab. 1, Fig. 2). Both viruses were rescued in HEK 293T cells and passed through MDCK cells and eggs for viral replication (Fig. 3, 4).

For the verification of a cold-adapted influenza B MDV as a potential component of a universal influenza virus vaccine, several plaque assays were performed (Fig. 5). Based on the literature, a verification of cold-adapted influenza B viruses is evaluated at temperatures of 37, 32 and 25°C[37]. However, because of temperature limitations in the available laboratory equipment, the phenotype was compared with the B/Malaysia/2506/2004 wild type virus at 37, 33, 29.5 and 27.5°C.

Both viruses have achieved similar viral titers of 10^8 PFU/ml at 33 and 29.5°C, which cover the optimal replication temperature of influenza B viruses (Fig. 5). A 3-log decrease in the viral titers of both viruses was observed at 37°C, which is the optimal replication temperature for a number of influenza A viruses (Fig. 5). This moderate decrease in the viral titers of these influenza B viruses might be explained through the different origins of the both viruses. Interestingly, a viral titer of 10^6 to 10^7 PFU/ml was seen for the 6+2 B/USSR/60/1969 reassortant virus at 27.5°C, which is almost 2-log higher compared with the wild type virus at the same temperature condition (Fig. 5). Similar results were observed in the plaque sizes of both viruses under the same temperature conditions (Fig. 5).

In general, for influenza A viruses, a temperature-sensitive phenotype is described as a greater than 2-log difference in the viral titer from 39 to 33°C, whereas a cold-adapted phenotype is described as a greater than 2-log difference in the viral titer from 33 to 25°C. In comparison, for the published cold-adapted B/USSR/60/1969 strain, a temperature-sensitive and cold-adapted phenotype is characterized with a 2.5-log difference in the viral titer from 33 to 25°C[37]. Under the caveat of not using the correct measurement temperatures, it seems that the rescued reassortant virus meets the literature-based characteristics and can therefore be considered as temperature-

sensitive and cold-adapted (Fig. 5). However, it has to be confirmed that the difference is maintained at lower temperatures.

The B/USSR/60/1969 virus sequence was chosen for the virus rescue due to its publication by Dr. A. Egorov, who was majorly involved in the development of the cold-adapted MDV strain for the current LAIV vaccine sold by BioDiem Ltd. Since the rescued virus contains both a temperature-sensitive and a cold-adapted phenotype this virus may be present in the LAIV vaccine.

Similar to the authors of the B/USSR/60/69 virus, the authors of the published B/Ann Arbor/1/1966 sequence claimed a cold-adaptation of their strain. All mutations contributing to this phenotype were clearly described and compared with the wild type strain. The cold-adapted B/Ann Arbor/1/1966 MDV is used by MedImmune LLC for their current quadrivalent LAIV Flumist® vaccine[38].

For a rescue of the cold-adapted 6+2 B/Ann Arbor/1/1966 reassortant virus as an MDV for a potential LAIV candidate, six backbone rescue plasmids were generated based on published literature, which were all sequence confirmed (Tab. 1, Fig. 2).

The desired cold-adapted 6+2 B/Ann Arbor/1/1966 reassortant virus could not be rescued at a transfection temperature of 37, 33°C and an infection temperature of 37, 33, 29.5 and 27.5°C (Fig. 4, Tab. 2). However, the cold-adapted PB1, PA, M, and NS genes were rescued individually in a 7+1 B/Malaysia/2506/2004 wild type backbone and all together in a 4+4 B/Malaysia/2506/2004 reassortant virus (Tab. 2). These results might indicate that certain transfection and infection temperatures are essential for a successful cold-adapted 6+2 reassortant virus rescue.

Other articles described an altered enzymatic activity of internal influenza virus backbone proteins which resulted from the acquired cold-adapted mutations[39]. The non-rescuable cold-adapted PB2 and NP genes contained the most amino acid changes, compared with their respective wild type sequences. More precisely, a 0.52% amino acid change in the PB2 protein and a 1.61% amino acid change in the NP protein was observed[35]. The activity of the expressed PB2 and NP proteins might be inefficient under the attempted temperature conditions. Also, the acquired cold-adapted mutations could potentially have an impact on the folding of the protein.

Since further mutations were observed in the 3`-end of the published UTRs, the optimized gene-specific B/Malaysia/2506/2004 UTRs were exchanged with the published B/Ann Arbor/1/1966 UTRs and additional rescues were attempted (Tab. 2). However, the published full length PB2 and NP genes could still not be rescued in a

7+1 B/Malaysia/2504/2006 wild type backbone (Tab. 2). Interestingly, the previously rescued full length PB1, PA, M and NP segments with the B/Ann Arbor/1/1966 UTRs could also not be rescued in this system, which strongly indicates an expected positive effect of the optimized gene-specific UTRs in the rescue of the novel cold-adapted influenza viruses (Tab. 2).

For a further understanding of the cold-adapted B/Ann Arbor/1/1966 MDV rescue, one of the future directions will be a separate transfection of the respective plasmids with an immunohistochemical detection for investigations of the expression and folding of the cold-adapted proteins at different temperature conditions. Furthermore, a rescue of the cold-adapted PB2 and NP genes in a 7+1 B/Ann Arbor/1/1966 wild type reassortant virus might increase the rescue efficacy since the cold-adapted and wild type strain are closely related. With this information, other important factors can be considered and a successful rescue of the desired cold-adapted B/Ann Arbor/1/1966 will be more likely. Furthermore, it is possible that the published sequence for the PB2 and NP proteins contained errors in the published sequence. Therefore, for a confirmation of the exact sequence, the seasonal Flumist® vaccine will be tested in the future.

In conclusion, in this study it was possible to generate a novel reverse genetics system for the rescue of the B/USSR/60/1969 virus strain. The rescued virus indicated a temperature-sensitive and cold-adapted phenotype and could potentially be used as an influenza B virus backbone in a future formulation of a universal influenza virus vaccine.

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Zusammenfassung

Influenzaviren bleiben eine ernsthafte Bedrohung für die menschliche Gesundheit. Aktuelle saisonale Influenza-Impfstoffe schützen nur ausreichend gegen gut übereinstimmende Influenza-Stämme, aber bieten einen ungenügenden Schutz gegen mutierte oder neuartige Pandemieviren. Dies unterstreicht die Notwendigkeit der Entwicklung eines universellen Influenza-Impfstoffs, der einen breiten Schutz gegen aktuell zirkulierende und neu einstehende Influenza-Stämme bietet. An der Icahn School of Medicine at Mount Sinai Universität in New York wurde ein solcher Impfansatz, welcher eine wiederholte Impfung mit chimären Hämagglutininen (HAs) vorsieht, entwickelt und getestet. Diese künstlichen Konstrukte enthalten eine exotische HA Kopf-Domäne, aber dieselbe konservierte HA Stamm-Domäne. Durch wiederholte Impfung mit unterschiedlichen HA Kopf-Domänen, aber derselben Stamm-Domäne, kann eine starke Reaktion gegen die konservierten immun-subdominanten HA Stamm-Domänen induziert werden. Antikörper, welche gegen diese Stamm-Domäne gerichtet sind weisen zwar einen breiten Schutz auf, sind allerdings oft auf die Bindung von HAs von entweder Influenza A Gruppe 1, Gruppe 2 oder Influenza B-Viren beschränkt. Um einen Schutz gegen alle Influenza A- und B-Viren zu ermöglichen, wird daher ein dreiwertiger Influenza-Impfstoff benötigt, welcher alle Gruppen von HAs abdeckt. Lebend-attenuierte Influenzavirus Impfstoffe (LAIV) sind eine interessante Plattform, weil diese eine mukosale Immunität hervorrufen und insbesondere eine hohe Wirksamkeit bei Kindern zeigen. LAIVs, welche chimäre HAs exprimieren wurden bereits erfolgreich mit Influenza A-Viren getestet. Um diese Tests auf Influenza B-Viren auszuweiten, wurden neue Reverse Genetik Systeme auf der Basis lizenzierter kälteadaptierter Impfstämme entwickelt, welche die Produktion von unterschiedlichen reassortanten Viren, die verschiedene HAs exprimieren, ermöglichen. Für die lizensierten B/USSR/60/1969 und B/Ann Arbor/1/1966 Virus Stämme wurden unterschiedliche Plasmide entworfen und konstruiert. Dadurch konnte ein reassortanter B/USSR/60/1969 Virus, welcher einen deutlichen kälteadaptierten Phänotyp aufwies, erfolgreich produziert werden. Für die Produktion eines zusätzlichen reassortanten B/Ann Arbor/1/1966 Virus sind weitere Studien erforderlich. Diese neu entwickelten Reverse Genetik Systeme könnten die Produktion verschiedener reassortanten Viren, zur Verwendung in einem universellen Influenza-Impfstoffs, ermöglichen.

SIDE PROJECT (Not part of the master's thesis)

ABSTRACT

During this study, I have performed investigations on the glycosylation of antibodies in eukaryotic cells. Therefore, I have successfully cloned two human glycosylation enzymes – the galactosyltransferase (B4GALT1) and the sialyltransferase (ST6GAL1) - into a pIRES plasmid. The plasmid was used to co-transfect human 293F cells. Through co-transfection of the B4GALT1_pIRES_ST6GAL1 plasmid containing these essential glycosylation enzymes as well as other plasmids containing human antibody sequences, our group were able to show differences in antibody binding through altered glycosylation in the Fab-, Hinge- and Fc region.

Moreover, I have successfully cloned the hemagglutinins of A/California/04/2009/(H1N1) and A/Vietnam/1203/2004/(H5N1) into a pFastBacDual plasmid for protein expression using the baculovirus expression system. Therefore, I transformed both plasmids into competent DH10 bacteria, which contained a so-called bacmid plasmid that is used for the transfection of eukaryotic Sf9 insect cells. Through western blot and immunostaining, I confirmed the protein expression of Cal09 and Vn04 in transfected Sf9 cells. A cooperator group purified both proteins through a *c-terminal* strep-tag and used these proteins for further analysis such as ELISAs or vaccination approaches in different animal models.

Introduction

Glycosylation in the Fab- and Fc-region of antibodies

Glycosylation of antibodies is a common post-translational modification event, which is predicted to have a fundamental role in the immune response during infection of different pathogens. Glycosylation in general is the adding of specific sugar rings on very conserved amino acid sequences (single n-linked asparagine) during maturation of proteins [(1)]. These so called glycans have the ability to change the conformation of proteins through diverse glycan-glycan interactions. These interactions can be affected by the chemical structures, distances, sizes as well as lengths of glycans. Different enzymes are essential during the maturation of these glycans such as the galactosyltransferase (B4GALT1) and the sialyltransferase (ST6GAL1) (Figure 1). The B4GALT1 enzyme recognizes the n-acetylglucosamine (GlcNAc) on a glycan structure and adds a galactose whereas the ST6GAL1 enzyme recognizes the pre-added galactose and adds a sialic acid (Figure 1) [(2)]. Glycosylation of antibodies can happen in the Fab-region (fragment antigen binding), the hinge-region (linker between Fab-/Fc-region), the Fc-region (fragment crystalizeable) or in all regions at once. Different glycosylation patterns can significantly alter the conformation and function of antibodies and therefore lead to a positive or negative impact on antibody affinity. The outcome of a positive glycosylation effect on antibodies could result in stronger and longer binding affinity of antibodies to antigens or Fc-receptors [(3)]. This could potentially lead to more efficient signaling pathways or enhanced effector functions such as the humoral immune response, the complement system, agglutination, opsonization, ADCC (antibody dependent cell-mediated cytotoxicity)- and ADCP (antibody dependent cell-mediated phagocytosis) activity [(4, 5)]. Since antibody glycosylation became more are more accessible in academic research in the last couple of years and seem to be an important mechanism of the immune response, glycosylation events of antibodies might be another potential target for future vaccine candidates.

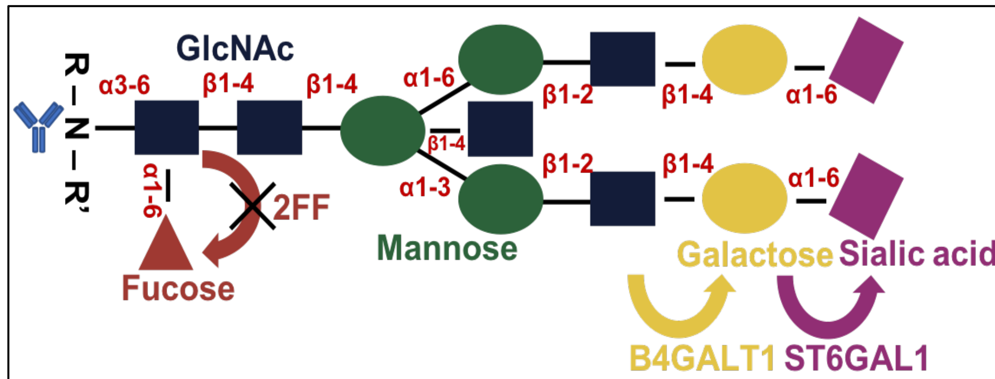


Figure 1: Complex glycosylation structure on antibodies. Shown is a complex glycosylation structure in the Fab-/hinge-/Fc-region of antibodies. Glycosylation only happens on Ns of conserved R-N-R' amino acid repeats. α and β indicate the up- or down-directed binding of a glycan-glycan construct. The first number after α and β indicates the binding position of the left with the right glycan, whereas the second number indicates the binding position of the right with the left glycan. The basic structure of several GlcNAc-Mannose (Man) glycans are essential for a successful glycosylation of antibodies. 2-fluorofucose (2FF) is an enzyme, which regulates the cellular fucosylation through the adding of a fucose on the first GlcNAc in the glycosylation structure. The B4GALT1 enzyme recognizes GlcNAc and adds a galactose (Gal), whereas the ST6GAL1 enzyme recognizes the pre-added galactose and adds a sialic acid (Sial) on top of the glycosylation structure. The adding of these complex structures can happen multiple times at multiple branches.

Generation of recombinant proteins in insect cells

There are currently three different platforms used for the development of different vaccines. Vaccines can be either inactivated vaccines, live-attenuated vaccines or recombinant proteins. Recombinant proteins are artificially produced proteins, which are expressed outside their normal environment in eukaryotic insect cell lines. The generation of recombinant proteins for vaccine development became of high interest since they are known to elicit a very specific immune response towards a conserved epitope of a pathogen [6,7,8,9,10,11].

For the generation of recombinant proteins, a plasmid containing the gene of interest (GOI) with a *c-terminal* his- or strep-tag need to be designed and engineered (Figure 2). After the GOI is sequence-confirmed, it will be transformed into competent DH10 bacterial cells (Figure 2). The transformation happens through a genetic recombination event between the generated plasmid and a so-called bacmid in DH10 bacterial cells. The recombinant bacmid contains the DNA sequence of the baculovirus expressing the protein of interest (POI) as well as three antibiotic resistance cassettes (Gentamycin, Tetracyclin and Kanamycin) for the selection of transformed clones. Transformed clones will be screened through a blue-white screening, which is based on the molecular function and dysfunction of the *lacZ* gene in the bacmid. In case the

GOI was properly transformed into the bacmid of DH10 bacterial cells, it will destroy the *lacZ* gene which leads to a loss of function of the β-galactosidase. This enzyme will not be able to catalyze x-gal, a specific substrate in the agar plates, which results in white colonies. White clones will be further confirmed through PCR-amplification of the GOI and the bacmid will be isolated and purified. Once this is done, the bacmid containing the baculovirus expressing POI will be transfected into eukaryotic SF9 insect cells (Figure 2). After the transfection, a western blot with an immunostaining of the cell pellet for the detection and confirmation of the POI will be done. While the cell pellet can be discarded, the supernatant containing the infectious baculovirus particles will be used to infect naïve SF9 insect cells in order to generate more infectious baculovirus particles expressing the POI (Figure 2). After several passages, the infected insect cells will be harvested, and the POI will be purified based on the c-terminal tag through beads in columns. The purified protein can then be used for ELISAs or as a potential vaccine candidate.

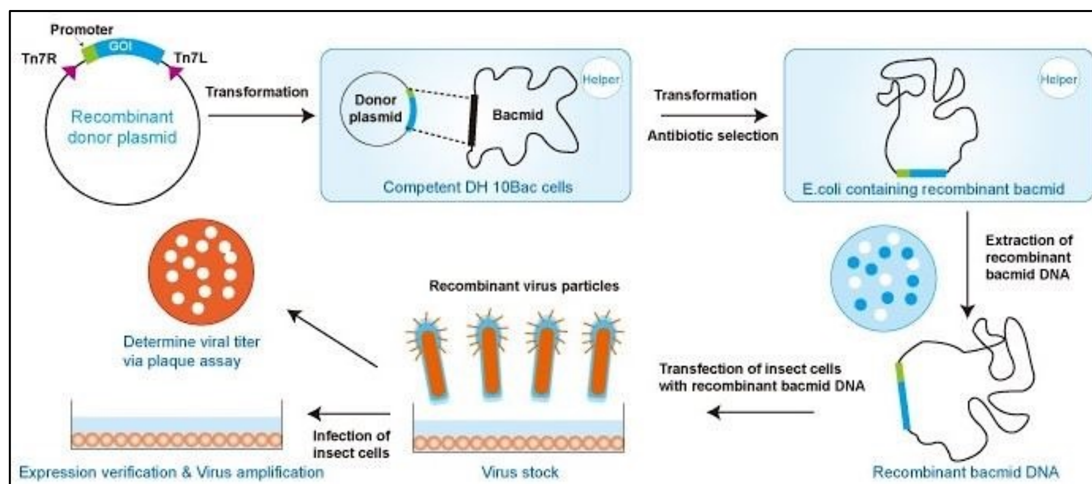


Figure 2: Baculovirus-insect cell expression system. For the generation of recombinant proteins in insect cells, a plasmid containing the gene of interest (GOI) with a *c-terminal* strep-tag and a promotor region (green) need to be designed and engineered. Afterwards, this plasmid will be transformed into competent DH10 bacterial cells, which contain a helper plasmid called bacmid. The transformation happens through genetic recombination between the generated plasmid and the bacmid. The selection of clones is done through a blue-white screening of the colonies. The recombinant DNA will be isolated from white colonies and then transfected into insect cells, which produce recombinant baculovirus particles expressing the protein of interest (POI). The baculovirus will be passaged several times in insect cells to increase the amount of infectious virus particles. Afterwards, the POI will be purified based on the *c-terminal* strep-tag. Source: https://www.creativebiomart.net/baculovirus-insect-cell-expression-systems.htm

Aim of this study

The aim of the first project was to investigate different glycosylation events on antibody binding affinity. Our group wanted to determine if glycosylation in the Fab- and Fc-region have an impact on antigen binding affinity and immune complex effectiveness during infection. Furthermore, we wanted to study the role of antigen shielding or affinity competition caused through altered glycosylation during antibody maturation.

The aim of the second project was to generate two functional plasmids, containing different hemagglutinins. These plasmids can be used for the transfection of eukaryotic insect cells in order to generate recombinant hemagglutinins for further experiments such as ELISAs and vaccination approaches in different animal models.

Material and methods

The cloning and transfection of the B4GALT1_pIRES_ST6GAL1 plasmid

For the generation of the B4GALT1_pIRES_ST6GAL1 plasmid, the B4GALT1 and ST6GAL1 DNA sequences were bought as gBlocks gene fragments from IDT (Integrated DNA technologies). Afterwards, both inserts were PCR-amplified using the overlapping primer combinations listed below. Both forward primers introduced the KOZAK sequence before the 5' start codon for an enhanced transcription of the inserts. The overlapping regions of the primers were used for an in-fusion ligation with the inserts and the pIRES backbone plasmid.

#	Notation	Sequence
1	FW Kozak/B4GALT1 + OL MCS-A pIRES	TCGACCCGGGCGGCCAGCTTGCCG CCACCATGAGA
2	RV B4GALT1 + OL MCS-A pIRES	TAAAGGGAAGCGGCCTCAAGATG TGCCATCCACGGTGG
3	FW Kozak/ST6GAL1 + OL MCS-B pIRES	ATAGGCTAGCCTCGAAGCTTGCC GCCACCATGCAC
4	RV ST6GAL1 + MCS-B pIRES	CGCGTGAATTCTCGATCAACAGTGTG TTCTGCCGGTGG

The premix and the program for the PCR-amplification of the inserts are shown below.

PCR premix for insert amplification

10 mM FW primer 1 or 3	1 µl
10 mM RV primer 2 or 4	1 µl

gBlocks gene fragment (IDT)	1 µl
2x Premix HiFi DNA-Polymerase	12,5 µl
ddH_2O	9,5 µl

PCR program for insert amplification

Initial denaturation	96 °C	2 min	
Denaturation	96 °C	1 min	
Annealing	55 °C	1 min	35 cycles
Elongation	72 °C	1 kb/min	
Final extension	72 °C	10 min	
Hold	4 °C	~	

For the confirmation of the amplified inserts, both DNA samples were separately loaded on a 1% agarose gel and run for 35 min at 120 V. The lengths of both DNA fragments were compared with their calculated lengths and then purified from the agarose gel using the NucleoSpin® Gel and PCR Clean-Up Kit from Takara Bio USA, Inc.

The pIRES plasmid is a common plasmid for the protein expression in eukaryotic cells. It contains an ampicillin resistance cassette for the selection of transformed bacterial cells and a piromycin resistance cassette for the selection of transfected eukaryotic cells. Moreover, the pIRES plasmid contains two distinct multiple cloning sites A and B (MCS-A and B) for the insertion of two DNA sequences. Between both MCS, there is an IRES (internal ribosome entry site) motive, which ensures an efficient transcription of the inserts.

For the first cloning of the B4GALT1 insert into the MSC-A site of the pIRES plasmid, an empty pIRES plasmid was digested using the restriction enzyme XhoI.

Digestion of an empty pIRES plasmid

pIRES plasmid (1.5-2 µg/µl)	X µl
XhoI (New England BioLabs®)	2,5 µl
CutSmart®-buffer (New England BioLabs®)	5 µl
ddH_2O	up to 50 µl

The digestion was done at 37°C overnight. Afterwards, the length of the linearized backbone was analyzed on an agarose gel as mentioned above.

The B4GALT1 insert and the linearized pIRES backbone were together used for an in-fusion ligation. The molecular ratio of insert and plasmid was calculated and

normalized to 2:1. The in-fusion ligation was done using the In-Fusion® HD Cloning Kit from Takara Bio USA, Inc. The newly generated B4GALT1_pIRES plasmid was further used for a transformation into *E. coli* cells. Therefore, competent *E. coli* cells were slowly thawed on ice. Afterwards, 1 µl of the B4GALT1_pIRES plasmid DNA was added to 40 µl of the competent cells and then incubated on ice for 1h. The competent cells were heat shocked at 42°C for 1 min then placed on ice for 1-2 min. Afterwards, 450 µl of SOC-medium (Mediatech) was added to the competent cells, which were then recovered at 37°C for 1 h. After the recovery, 40 µl of the cells were plated on ampicillin imMedia Growth Medium agar plates (Invitrogen). The next day, colonies were picked, and a colony-PCR was performed for the verification of a correct insertion of the DNA sequence. The colony-PCR was done under the same conditions as described above with the exception of picking one clone instead of using purified DNA. Afterwards, PCR-confirmed positive clones were grown in 5 ml of 1xLB-Broth-media (Gibco life technologies^{TM}) for 24 h. The following day, the B4GALT1_pIRES plasmids were isolated through minipreps using the PureLink Quick Plasmid Miniprep kit from Invitrogen Thermo Fisher Scientific. The amplified B4GALT1-pIRES plasmids were then sequenced using the primers listed above. Only the plasmid with the correct B4GALT1 DNA sequence was kept for further cloning.

For the second cloning of the S6GAL1 insert into the MSC-B site of the B4GALT1_pIRES plasmid, the B4GALT1_pIRES plasmid was digested using the restriction enzyme NotI. The digestion as well as a further in-fusion ligation of the linearized B4GALT1_pIRES backbone and the ST6GAL1 insert with a transformation into *E. coli* was done the same way as described earlier.

The plasmid was then transfected into eukaryotic 293F cells for the expression of the glycosylation enzymes. Therefore, $1.25x10^6$ cells were seeded in 2 ml of growth media (880 ml DMEM, 100 ml Fetal Bovine Serum (FBS), 10 ml 1M Hepes, 10 ml Pen/Strep (Gibco life technologies)) 24 h before transfection. The next day, 5 µg of the purified plasmid was carefully mixed with 100 µl of 1xOpti-MEM (Gibco life technologies) and 20 µl of TransIT-LT1 reagent (Mirus). The transfection mixture was then incubated at RT for 30 min. In the meantime, 293F cells were once washed with 1xPBS pH 7.4 (Gibco life technologies) and the growth media replaced with 1 ml of serum-free transfection media (450 ml 1xMEM, 5 ml 1M Hepes, 5 ml Pen/Strep (Gibco life technologies)). The incubated transfection mixture was dropwise added to the 293F

cells while slowly swirling. At the end, the transfected cells were moved to 37°C for 48 h.

The cloning and protein expression of two different influenza A

The cloning process of the hemagglutinins A/California/04/2009/(H1) and A/Vietnam/1203/2004/(H5) into the pFastBacDual plasmid was done the same way as described for the B4GALT1 and ST6GAL1 inserts. Therefore, only differences in the cloning process are described.

Both hemagglutinins contain a trimerization domain with a cleavage site as well as a *c-terminal* strep-tag. The trimerization domain is essential for the correct formation of the proteins, whereas the strep-tag is used for the protein purification. The inserts were PCR-amplified using the overlapping primer combinations listed below.

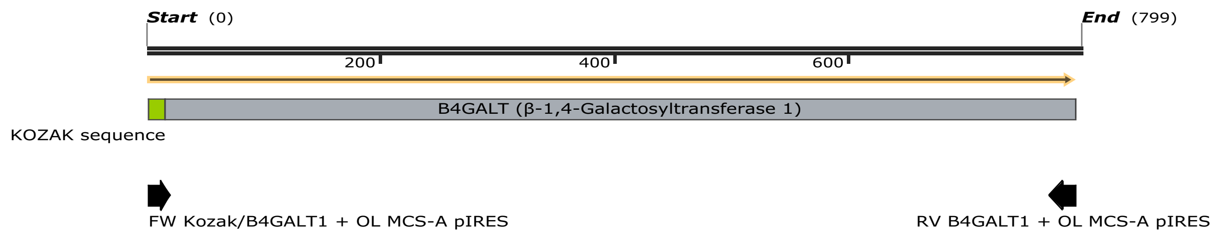
#	Notation	Sequence
1	FW Cal09 + OL pFastBacDual	GTCCCACCATCGGGCGCGGATCCGCCACC <u>ATGAAGGCGATTTTGGTTGT</u>
2	RV Cal09-strep-tag + OL pFastbacDual	CTGGAAGTACAGGTTCTCGCGGCCGCT <u>GACTCCGTCGATCTCCTC</u>
3	FW Vn04 + OL pFastBacDual	GTCCCACCATCGGGCGCGGATCCGCCACC <u>ATGGAGAAAATAGTGCTTCT</u>
4	RV Vn04-strep-tag + pFastBacDual	GGAAGTACAGGTTCTCGCGGCCGCT <u>TACTCCACTTATTTCCTCTC</u>

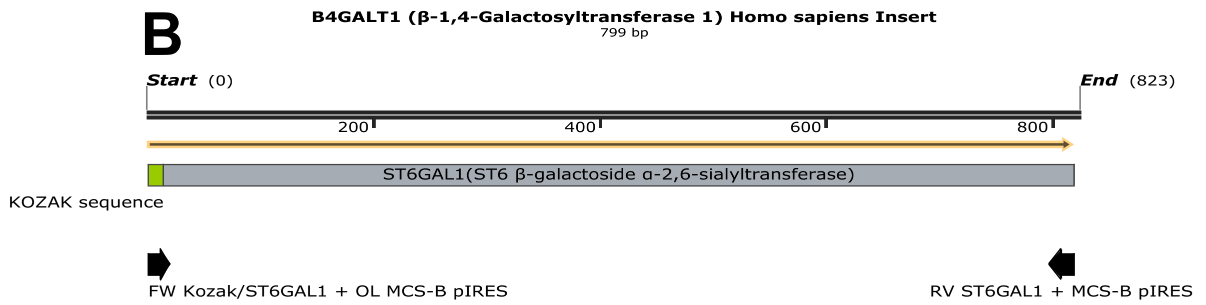
The pFastBacDual plasmid is designed and engineered for the generation of recombinant proteins using the baculovirus expression system. It can undergo a recombination event with the bacmid in DH10 bacterial cells. The pFastBacDual plasmid was digested using the restriction enzymes NotI and BamHI. After ligation of the Cal09 H1- and the Vn04 H5 inserts with the pFastBacDual plamid, the newly generated plasmids were transformed into competent DH10 bacterial cells. The transformation was similar to a conventional transformation into *E. coli* cells. However, the recovery time of transformed E. coli cells was increased to 4 h. Furthermore, the cells were plated on agar-plates containing 400 ml of ddH$_2$O, 2 packages of Kanamycin agar imMedia™ Growth Medium (Invitrogen Thermo Fisher Scientific), 400 µl Tetracylin, 50 µl Gentamycin, 500 µl x-gal and 800 µl IPTG. Only white clones were inoculated in growth media, which contained 500 ml LB-media, 500 µl Kanamycin, 500

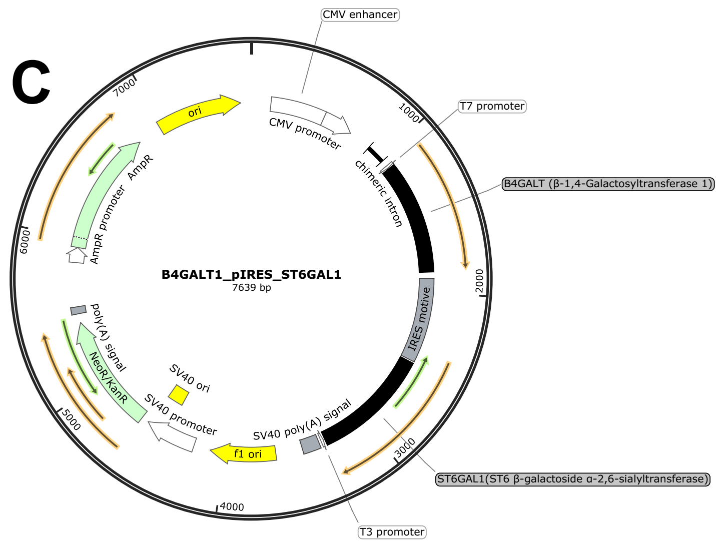
µl Tetracylin and 10 µl Gentamycin. Isolated and purified bacmid plasmids were stored at 4°C until further transfection into eukaryotic insect cells.

For the transfection of the bacmid plasmid into Sf9 insect cells, Sf9 cells were grown in TNM-FH media (Gemini Bio Products) at 27°C without CO2 until 50-80% confluency. Afterwards, cells were equally distributed to a 6-well plate with 70-80% confluency. Next, the cells were incubated the same conditions to attach to the bottom of the flask again. In the meantime, two different rows of Eppendorf-tubes were prepaid for the transfection mixture. In the first row, 100 µl of TNM-FH media was mixed with 2-4 µg bacmid DNA. In the second row, 100 µl TNM-FH media was mixed with 5 µl of Cellfectin® (Invitrogen). Afterwards, both tubes were carefully combined, and the mixture was incubated at RT for 30 min. The solution was dropwise added to the cells and incubated for 6 h. After this time, the media was changed to 3% TNM-FH media (30 ml of FBS, 10 ml of Pluronic (Sigma-Aldrich) and 10 ml of Pen/Strep) and incubated for 7 days. Afterwards, the cells were harvested, and the supernatant stored at 4°C. The cell pellet was used to verify successful protein expression via western blot. Therefore, the cell pellet was washed twice with PBS and then resuspended in 100 µl of PBS. Next, 20 µl of cells were mixed with 20 µl of loading dye (950 µl of 2xLaemmli-buffer and 50 ul of β –mercaptoethanol). 20 µl of the mixture was loaded on a SDS-gel (Bio-Rad Laboratories) and run at 200V for 30 min. The gel was transferred to a nitrocellulose membrane by semi-dry transfer at 18A for 20 min. The nitrocellulose membrane (Thermo Fisher Scientific) was then blocked with 3%-milk/PBST for 1h at RT. Afterwards, the membrane was incubated with a monoclonal α-strep-tag (mouse) antibody in 1%-milk/PBST for 1h. Next, the membrane was washed three times with PBST and incubated with an anti-mouse secondary antibody for 1h at RT. At the end, the membrane was washed again three times with PBST and developed by using the AP Conjugate Substrate Kit (Bio-Rad Laboratories).

Results







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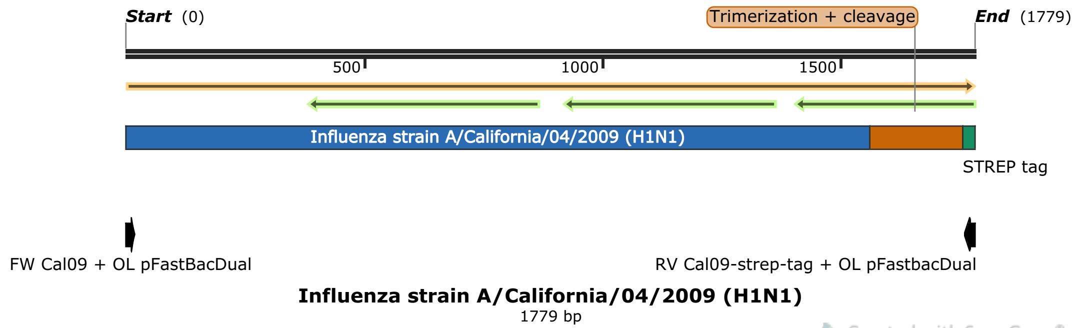
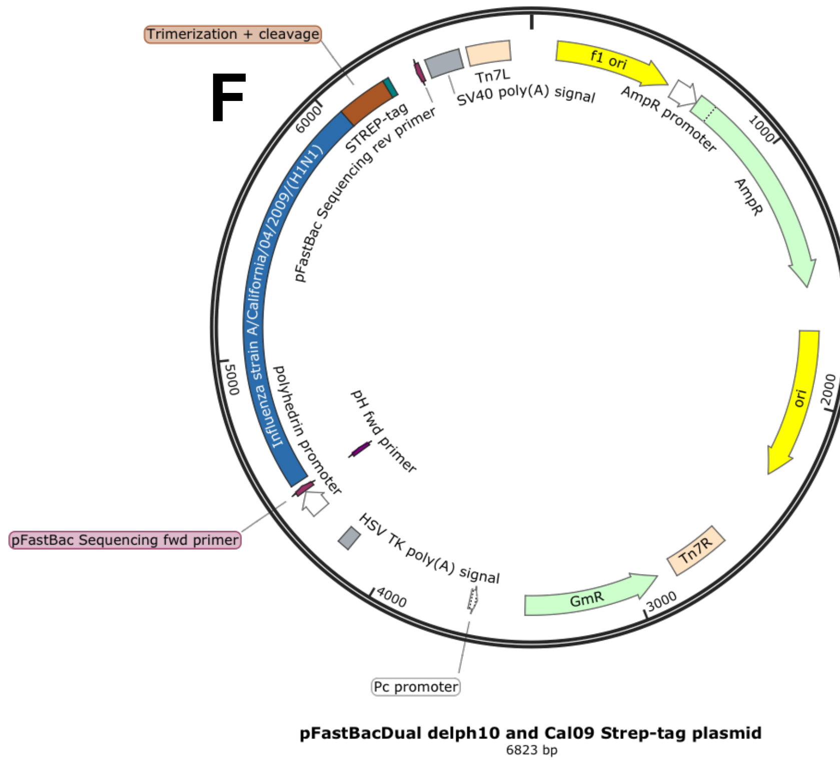
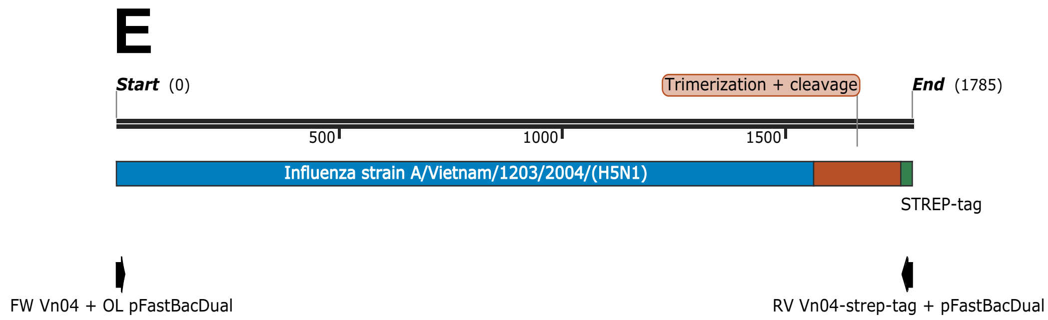
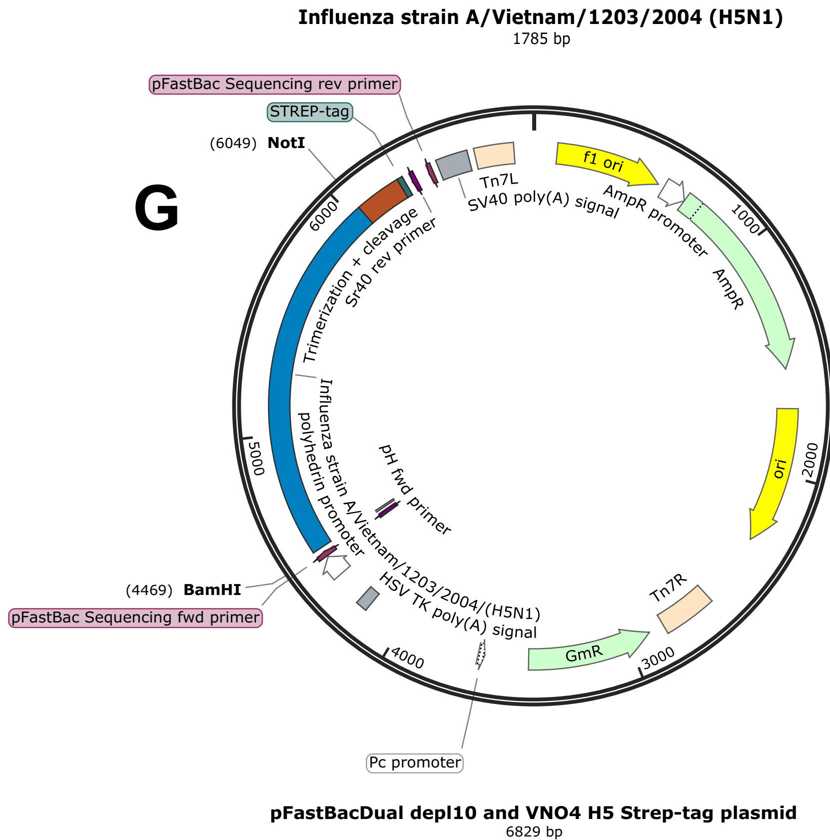


F







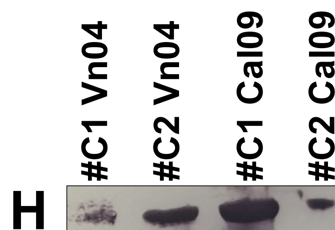


Figure 3: Generation of the B4GALT1_pIRES_S6GALT1, pFastBacDual_Cal09 and pFastBacDual_Vn04 plasmids. A/B: Shown are the B4GALT1 as well as the S6GALT1 homo sapiens inserts. **C:** B4GALT1_pIRES_S6GALT1 plasmid after successful in-fusion ligation of both inserts A/B. **D/E:** Shown are the hemagglutinin inserts of A/California/04/2009 (H1) as well as A/Vietnam/1203/2004 (H5). **F:** pFastBacDual_Cal09 plasmid after successful in-fusion ligation of the insert D. **G:** pFastBacDual_Vn04 plasmid after successful in-fusion ligation of the insert E. **H:**

Confirmation of the Cal09 and Vn04 protein expression in eukaryotic Sf9 insect cells through immunostaining.

In Fig. 3A/B, DNA sequences of the homo sapiens glycosylation enzymes, galactosyltransferase and sialyltransferase, are shown. Both genes are around 800 bp long and contain the KOZAK sequence before the start codon for an enhanced transcription of these genes. In Fig. 3C, the 6823 bp long B4GALT1_pIRES_S6GAL1 plasmid, which contains both glycosylation enzymes, is shown. The plasmid was used for a co-transfection with other protein expression plasmids containing human antibody sequences. These antibodies were glycosylated through both glycosylation enzymes in human 293F cells. In Fig. 3D/E, DNA sequences of the hemagglutinins, A/California/04/2009 (H1) and A/Vietnam/1203/2004 (H5), are shown. Both DNA sequences are around 1780 bp long a contain a trimerization/cleavage site as well as a strep-tag at the *c-terminal* end. The trimerization domain is necessary for an accurate formation of the trimeric protein, whereas the cleavage site is used for a proteolytic cleavage of the strep-tag. The strep-tag is essential for the purification of the expressed proteins in insect cells. In Fig. 3 F/G, the 6820 bp long pFastBacDual_Cal09 and Vn04 plasmids are shown. Both plasmids were used for the protein expression using the baculovirus expression system. In Fig. 3H, the expressed Cal09-H1 and Vn04-H5 proteins were detected in transfected insect clones through immunostaining. The molecular weight of both proteins was about 60-70 kDa.

Discussion

During this project, three novel plasmids were generated. All of these plasmids were sequence confirmed and used for further experiments. The generated B4GALT1_pIRES_S6GAL1 plasmid was used for the glycosylation of antibodies in human 293F cells. Therefore, human antibody sequences were separately cloned into a protein expression plasmid (pCAGGs) and co-transfected into the same cell line. These glycosylated antibodies were purified, and their antibody affinity to hemagglutinins compared with the same non-glycosylated antibodies. In ELISAs, our group were able to show an enhanced binding affinity of glycosylated antibodies. These antibodies will be analyzed based on their glycosylation patterns through flow cytometry. Therefore, our group hopes to find specific glycosylated epitopes on antibodies, which can be artificially glycosylated in therapeutic antibodies. These glycosylation patterns might have the potential to enhance antibody binding during a specific pathogenic infection.

The generated pFastBacDual_Cal09-H1 and Vn04-H5 plasmid was used for the transfection of Sf9 insect cells. The transfection was successful since the expressed proteins were detected through immunostaining. Transfected and expressed proteins normally get purified based on a *c-terminal* his tag. However, our group had both proteins with a his tag, but needed them with a strep tag for vaccination studies. Humans and animals can develop antibodies against these protein tags when they are administered twice. Through a series of vaccinations in mice with the hemagglutinins Cal09-H1/Vn04-H5 his and a strep tag, our group was able to show an enhanced immune response to immune complexes. Both proteins were also used for several ELISAs.

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