REVIEW ARTICLE

Increasing Efficacy of Enveloped Whole-Virus Vaccines by In situ Immune-Complexing with the Natural Anti-Gal Antibody

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Abstract
The appearance of variants of mutated virus in course of the Covid-19 pandemic raises concerns regarding the risk of possible formation of variants that can evade the protective immune response elicited by the single antigen S-protein gene-based vaccines. This risk may be avoided by inclusion of several antigens in vaccines, so that a variant that evades the immune response to the S-protein of SARS-CoV-2 virus will be destroyed by the protective immune response against other viral antigens. A simple way for preparing multi-antigenic enveloped-virus vaccines is using the inactivated whole-virus as vaccine. However, immunogenicity of such vaccines may be suboptimal because of poor uptake of the vaccine by antigen-presenting-cells (APC) due to electrostatic repulsion by the negative charges of sialic-acid on both the glycan-shield of the vaccinating virus and on the carbohydrate-chains (glycans) of APC. In addition, glycan-shield can mask many antigenic peptides. These effects of the glycan-shield can be reduced and immunogenicity of the vaccinating virus markedly increased by glycoengineering viral glycans for replacing sialic-acid units on glycans with α-gal epitopes (Galα1-3Galβ1-4GlcNAc-R). Vaccination of humans with inactivated whole-virus presenting α-gal epitopes ($virus_{\alpha\text{-gal}}$) results in formation of immune-complexes with the abundant natural anti-Gal antibody that binds to viral α-gal epitopes at the vaccination site. These immune-complexes are targeted to APC for rigorous uptake due to binding of the Fc portion of immunocomplexed anti-Gal to Fcγ receptors on APC. The APC further transport the large amounts of internalized vaccinating virus to regional lymph nodes, process and present the virus antigenic peptides for the activation of many clones of virus specific helper and cytotoxic T-cells. This elicits a protective cellular and humoral immune response against multiple viral antigens and an effective immunological memory. The immune response to $virus_{\alpha\text{-gal}}$ vaccine was studied in mice producing anti-Gal and immunized with inactivated influenza-$virus_{\alpha\text{-gal}}$. These mice demonstrated 100-fold increase in titer of the antibodies produced, a marked increase in T-cell response, and a near complete protection against challenge with a lethal dose of live influenza-virus, in comparison to a similar vaccine lacking α-gal epitopes. This glycoengineering can be achieved *in vitro* by enzymatic reaction with neuraminidase removing sialic-acid and with recombinant α1,3galactosyltransferase (α1,3GT) synthesizing α-gal epitopes, by engineering host-cells to contain several copies of the *α1,3GT* gene (*GGTA1*), or by transduction of this gene in a replication-defective adenovirus vector into host-cells. Theoretically, these methods for increased immunogenicity may be applicable to all enveloped viruses with N-glycans on their envelope.

Keywords: Inactivated whole-virus vaccine, vaccine immunogenicity, α-gal epitope, natural anti-Gal antibody, glycan-shield, enveloped virus vaccines, variants.

The variants conundrum in gene-based vs. whole-virus vaccines

The current Covid 19 pandemic illustrates a new conundrum in the area of vaccine preparation against enveloped viruses that cause present and future pandemics: The use of gene-based vaccines or of vaccines made of inactivated whole-virus (referred to as inactivated virus vaccine). Inactivated virus vaccines in the form of whole-virus or as virus disrupted by a detergent have been the traditional types of viral vaccines. The efficacy of such vaccines and the duration of the immune protection varies from one type of vaccine to the other and accordingly, the frequency of boosts required for maintaining the protective anti-viral immune response. In contrast, gene-based vaccines which recently have been widely used for Covid-19 vaccines are comprised of nucleic acid (DNA or mRNA) of the S-protein gene (the gene encoding the major envelope glycoprotein). The gene is delivered into muscle tissue as DNA within a replication defective viral vector, such as replication defective adenovirus, or as mRNA within lipid nanoparticles. This gene encodes for production of the vaccinating S-protein by myotubes. These vaccines have been produced in large amounts within a period of less than one year and have demonstrated very good efficacy in protecting vaccinated individuals against infection by SARS-CoV-2. However, these gene-based vaccines seem to be associated with the appearance of mutated virus strains (called variants) with higher infectivity (transmissibility) and/or virulence, thereby increasing their ability to survive and spread in populations.[1] Appearance of such variants has been a very rare event in populations vaccinated by more traditional methods, such as whole-virus vaccines or vaccines containing the virus disrupted by detergents. Previous examples for viral variants have been reported in HIV patients in whom the virus mutated in course of the infection.[2,3] One amino acid mutation in the envelop glycoprotein gp160 was found to enable virus escape from cytolytic T lymphocyte (CTL) activity which causes the lysis of cells infected with the non-mutated virus.[2] Another HIV variant acquired an additional carbohydrate chain (glycan) on its envelope gp120 portion of gp160.[3] Gp120 has 24 asparagine (N) linked carbohydrate chains (referred to as N-glycans) which "camouflage" (mask) a large proportion of the antigenic peptides and are referred to as the "glycan-shield".[3-6] Synthesis of glycans due to mutations that form more glycosylation sites (i.e., the sequon N-X-S/T) contributes to additional masking of gp120 antigens and thus, to evasion from the anti-HIV neutralizing antibodies.[3]

The S-protein of SARS-CoV-2 has 22 N-glycans.[7,8] Since the virus has the tendency to acquire random mutations in the course of replication, it may be able to undergo selective "mutating evolution", similar to that observed in HIV. Such a selective process may result in appearance of variants with increased number of glycans, or in changes in antigenic structure of the S-protein. In vaccinated individuals, both types of mutations may enable evasion of the variant from neutralizing anti-S-protein antibodies or from anti-S-protein CTL. Variants that can evade the protective immune response against the S-protein may accidentally appear in unimmunized individuals and subsequently infect individuals vaccinated with gene-based vaccines containing only the S-protein gene. Variants resistant to the protective anti-S-protein immune response may selectively expand in infected immunized individuals and further spread in immunized and non-immunized populations. The appearance of such detrimental variants may be avoided by including several antigens in the vaccine (multi-antigenic vaccine). The production of antibodies and virus specific CTL against several viral antigens will markedly decrease the probability for appearance of variants with mutations evading the immune response against a single vaccinating viral antigen. Such mutated SARS-CoV-2 viruses will be

destroyed by the protective immune response against additional viral antigens, e.g., antibodies against other envelope proteins and CTL recognizing antigenic peptides of the virus, other than those of the S-protein. As discussed below, a relatively simple way to prepare multi-antigenic vaccine against various enveloped viruses, including SARS-CoV-2, is to use an inactivated whole-virus vaccine processed to have increased immunogenicity by glycoengineering its glycan-shield.

Immunogenicity of enveloped whole-virus vaccines

One of the major factors determining the efficacy of whole-virus vaccines is the extent of uptake at the vaccination site of the vaccinating inactivated virus, by antigen presenting cells (APC) such as dendritic cells and macrophages. Once the vaccinating virus is internalized by APC, it is transported to regional lymph nodes and processed for presentation of antigenic viral peptides on class I and class II MHC molecules for the activation of $CD8^+$ T cells and $CD4^+$ T cells, respectively. Activated $CD8^+$ T cells become CTL that kill cells infected with the replicating virus, thereby prevent progression of the viral infection. Activated $CD4^+$ T cells function as helper T cells that help in the activation and proliferation of virus specific B cells producing neutralizing antibodies and of CTL. Low uptake of the vaccinating virus results in poor protective immune response whereas rigorous uptake of multiple vaccinating virions results in activation of many virus specific T and B cell clones and generation of an effective anti-viral immune protection. Another reason for poor immunogenicity of whole-virus is masking of antigens on the envelope of the virus which decreases the ability of the vaccinating virus to activate B cells producing the corresponding antibodies.

Two of the mechanisms that contribute to the generation of a poor protective immune response in individuals immunized with inactivated whole-virus vaccine are associated with the glycans on the viral envelope glycoproteins. These are: 1. Generation of a negative electrostatic charge that causes electrostatic repulsion from APC membranes. 2. Masking of viral antigens by glycans of the viral glycan-shield.

Electrostatic repulsion- Internalization of vaccinating virus into APC is mediated by random pinocytosis of small droplets containing the virus reaching close to the surface area of these cells. Many of the N-glycans on viral envelope glycoproteins are capped by the negatively charged carbohydrate sialic-acid (SA). O-glycans (linked to serine or threonine) on the viral glycoproteins also carry SA.[4-9] An example of SA on N-glycans of a glycoprotein is illustrated as the left glycan in Figure 1. The same glycans are present on APC and on other cells in the body. Thus, many vaccinating virions that reach near the APC, close enough to be pinocytosed, are deflected because of the electrostatic repulsion (called ζ [zeta]-potential) between the negative charges on the virus and those on the APC. This repulsion decreases the amount of vaccinating virus internalized by APC.[10]

Antigen-masking by the glycan-shield- It is estimated that the glycan-shields on viruses such as HIV and SARS-CoV-2 cover ~65% of the surface of the gp120 and S-protein, respectively.[3-8] In addition, there are O-glycosylated glycan on viral glycoproteins which together with the N-glycans mask many of the antigenic peptides from recognition by B cell receptors and by the corresponding antibodies.

The combination of negative charge repulsion by sialic acids and masking of antigenic peptides by the glycan-shield results in suboptimal efficacy of some inactivated virus vaccines for stimulation of the immune system in vaccinated individuals. Thus, the two characteristics are likely to contribute to the suboptimal efficacy of Covid-19 inactivated whole-virus vaccine which was recently reported in public news media. The

low immunogenicity of HIV vaccines[11,12] may be associated with the negative effects of the glycan-shield on the virus immunogenicity, as well. This review describes a method for modifying glycans (glycoengineering) of any viral glycan-shield in a way that eliminates the electrostatic repulsion between vaccinating inactivated viruses and APC. Instead, the engineered glycan-shield actively targets the immunizing virus for rigorous uptake by APC. This is achieved by glycoengineering the glycan-shield to present a carbohydrate antigen called the "α-gal epitope" with the structure Galα1-3Galβ1-4GlcNAc-R. The α-gal epitope is illustrated on the right glycan in Figure 1. Injection of vaccinating inactivated viruses presenting α-gal epitopes (virus$_{\alpha\text{-gal}}$) results in binding of the natural anti-Gal antibody to these epitopes on the virus and formation of immune-complexes which are targeted for rigorous uptake by APC. The studies described below demonstrated a ~100-fold increase in anti-virus antibody production and marked increase in anti-virus T cell response following vaccination with anti-Gal/virus$_{\alpha\text{-gal}}$ immune-complexes. The review further describes several methods for glycoengineering of present and future whole-virus vaccines to achieve presentation of multiple α-gal epitopes in order to produce highly immunogenic vaccines that prevent "evolution" of detrimental variants.

The natural anti-Gal antibody binding to mammalian and viral α-gal epitopes

Anti-Gal is produced in all humans throughout life as a natural antibody (i.e., without any vaccination),[13,14] because of antigenic stimulation by bacteria of the natural GI flora.[15-17] Studies quantifying anti-Gal reported on its production in all humans at high levels that correspond to 0.1-1.0% of serum IgG, IgM, and IgA.[14,18-21] Anti-Gal is also present as IgA and IgG antibodies in body secretions such as milk, colostrum, saliva, and bile.[19]

The main antigen (ligand) interacting with anti-Gal is the α-gal epitope (right glycan in Figure 1).[22-24] This carbohydrate antigen is found on cell surface glycans and on secreted glycoproteins, glycolipids, and proteoglycans in all nonprimate mammals (both marsupial and placental mammals that are not monkeys or apes), in lemurs and in monkeys of South America (New-World monkeys).[25-29] The α-gal epitope in all these mammals is synthesized by the glycosylation enzyme α1,3galactosyltransferase (α1,3GT), which transfers galactose (Gal) from the high energy sugar-donor uridine-diphosphate-galactose (UDP-Gal) to Galβ1-4GlcNAc-R (N-acetyllactosamine- LacNAc) of N-glycans in an α1,3 linkage as illustrated in Figure 1 (conversion of the center glycan into the right glycan).[26,30-33]. The α-gal epitope is completely absent in monkeys of Asia and Africa (Old-World monkeys), apes and humans, all of which produce the natural anti-Gal antibody.[14,25,26,34] Accordingly, an active α1,3GT gene (*GGTA1*) is found in mammals synthesizing α-gal epitopes[35-37] whereas this gene is inactivated in Old-World primates (monkeys and apes) and in humans.[38-42]

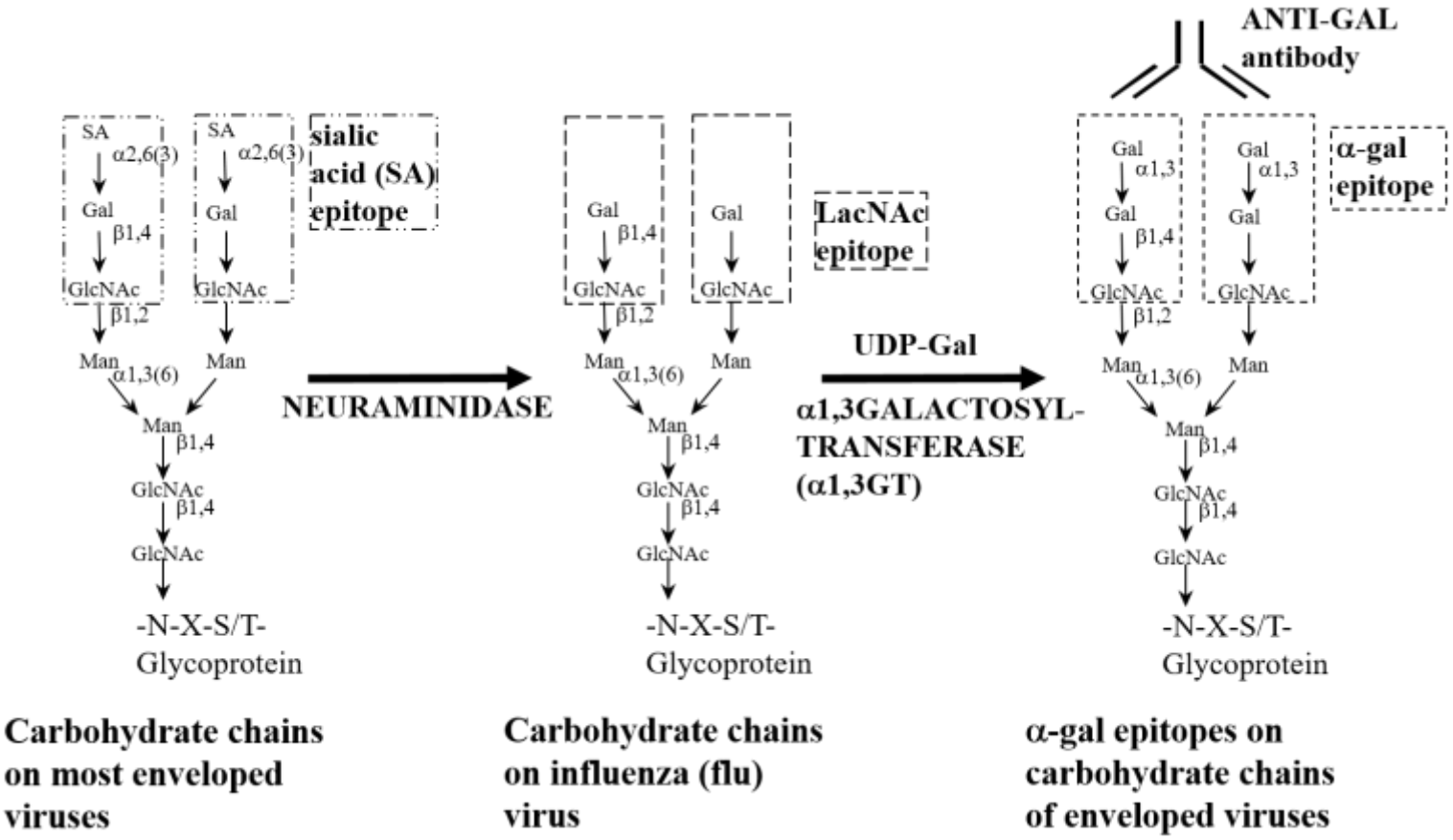

Figure 1. Enzymatic synthesis of α-gal epitopes on the glycan-shield of enveloped viruses by α1,3galactosyltransferase (α1,3GT). Left chain- N-glycans of the complex type on the glycan-shield synthesized in the host-cell on asparagine (N) in amino acid sequences (sequon) N-X-S/T-. In most viruses these glycans are capped by sialic acid (SA). Center chain- Sialic acid is removed from the glycan by neuraminidase to expose the penultimate Galβ1-4GlcNAc-R called N-acetyllactosamine (LacNAc). In influenza virus, SA is absent from the glycan-shield. Right chain- α1,3GT links to LacNAc the galactose (Gal) provided by the sugar donor uridine diphosphate galactose (UDP-Gal), resulting in synthesis of α-gal epitopes (Galα1-3Galβ1-4GlcNAc-R) on the carbohydrate chain. These epitopes readily bind the anti-Gal antibody. Adapted with permission from ref. 63.

Since glycans on envelope glycoproteins of viruses are synthesized by the host-cell glycosylation machinery, α-gal epitopes are found on envelope glycoproteins of viruses replicating in host-cells containing active α1,3GT. This was demonstrated with Eastern Equine Encephalitis virus replicating in mouse cells,[43] influenza virus produced in bovine and canine cells,[44] Friend murine leukemia virus replicating in mouse cells,[45,46], porcine endogenous retrovirus replicating in porcine cells,[47] rhabdo-, lenti-, and spumaviruses replicating in murine, hamster and mink cells,[48] Newcastle disease virus, Sindbis virus and vesicular stomatitis virus replicating in murine and hamster cells,[49,50] and measles virus replicating in human cells transfected with α1,3GT cDNA.[51] Accordingly, when these viruses were incubated in human serum, anti-Gal binding to their α-gal epitopes resulted in their destruction following activation of the complement system which bores holes in the envelope of these viruses.[46-51] These observations also suggest that the natural anti-Gal antibody is likely to function as a first line barrier, contributing to the protection against infections by zoonotic viruses replicating in

nonprimate mammals.[46-53] Such zoonotic viruses carry α-gal epitopes because their natural hosts have active α1,3GT in their cells.

Hypothesis on increased immunogenicity of anti-Gal immunocomplexed vaccines

The vast experience with whole-virus vaccines that present a glycan-shield has suggested that some of these vaccines display suboptimal efficacy, such as HIV vaccine[12] and influenza virus vaccines.[54,55] As discussed above, it has been suggested that the suboptimal efficacy may be associated to the glycan-shield masking antigenic epitopes[3,6-9] and causing electrostatic repulsion from APC.[10] The review proposes conversion of the glycan-shield from an obstacle for induction of a protective immune response, to a part of the vaccinating virus that increases the immunogenicity and efficacy of viral vaccines. This is achieved by using the converted glycan-shield for targeting the vaccinating virus to APC that effectively internalize large amounts of the vaccinating virions which form immune-complexes with the natural anti-Gal antibody at the vaccination site.

The principle of amplifying viral vaccine immunogenicity by immunocomplexing the inactivated virus with a specific antibody has been demonstrated in a number of vaccines, including Eastern Equine Encephalitis virus,[56] hepatitis virus[57] and Simian Immunodeficiency virus.[58] Immunization with vaccines prepared prior to injection as immune-complexes increases titers of the antibodies produced by 10-1000-fold in comparison with the same vaccines that were not immunocomplexed. This increased immunogenicity is the result of binding the Fc "tail" of the immunocomplexed anti-virus antibody to Fcγ receptors on APC. This binding stimulates APC to actively endocytose the vaccinating inactivated virus, resulting in much larger amounts of internalized virus than in the absence of immunocomplexing antibody. The internalized viral antigens are processed by the APC and transported to regional lymph nodes, where presented antigenic peptides activate many more virus specific $CD8^+$ and $CD4^+$ T cells than regular vaccines that are not immunocomplexes.[58-60] This Fc/Fcγ receptors interaction further generates a signal that induces maturation of APC into professional APC which effectively present immunogenic peptides on cell surface MHC molecules for activation of the virus specific $CD8^+$ and $CD4^+$ T cells.[61,62] Administration of immunocomplexed viral vaccines has not been practiced in the clinic, possibly because of the inability to control the affinity of the immunocomplexing antibody to the vaccinating virus and the difficulties associated with using human or mammalian antibodies for injection in large populations. These difficulties can be overcome by using anti-Gal as a universal endogenous antibody forming immune-complexes at the vaccination site with any injected vaccine, provided that the vaccinating antigen presents multiple α-gal epitopes.[60] We hypothesized that vaccination with inactivated $virus_{\alpha\text{-gal}}$ will result in formation of immune-complexes with the natural anti-Gal antibody at the vaccination site. [44,63,64] Activation of the complement system by anti-Gal/$virus_{\alpha\text{-gal}}$ interaction will be followed by formation of complement cleavage chemotactic peptides which recruit dendritic cells and macrophages to the vaccination sites (Step 1 in Figure 2). The anti-Gal/$virus_{\alpha\text{-gal}}$ immune-complexes will be further targeted for rigorous uptake by APC as a result of anti-Gal Fc/Fcγ receptors interaction on the APC (Step 2 in Figure 2). The increased uptake may be mediated also by C3b on the $virus_{\alpha\text{-gal}}$ binding to CR1 receptor on APC. The APC will transport the large amounts of internalized vaccinating $virus_{\alpha\text{-gal}}$ to regional lymph nodes, process the viral antigens and present them on cell surface MHC class I and class II molecules (Step 3 in Figure 2). Ultimately, the effective presentation of many processed viral antigens by APC matured into professional APC will

result in activation and proliferation of many more virus specific CTL, helper T cell and B cell clones leading to a much higher and longer anti-virus protective immune response and stronger immunological memory in comparison to vaccination with virus lacking α-gal epitopes. Preliminary *in vitro* studies with influenza virus[44] and with measles virus[65] presenting α-gal epitopes by propagation in mammalian cells containing active α1,3GT confirmed the basic assumption of this hypothesis. Inactivated virus presenting α-gal epitopes and immunocomplexed with anti-Gal was internalized much more effectively by APC, as indicated by higher ability to activate virus specific T cells than virus lacking α-gal epitopes.

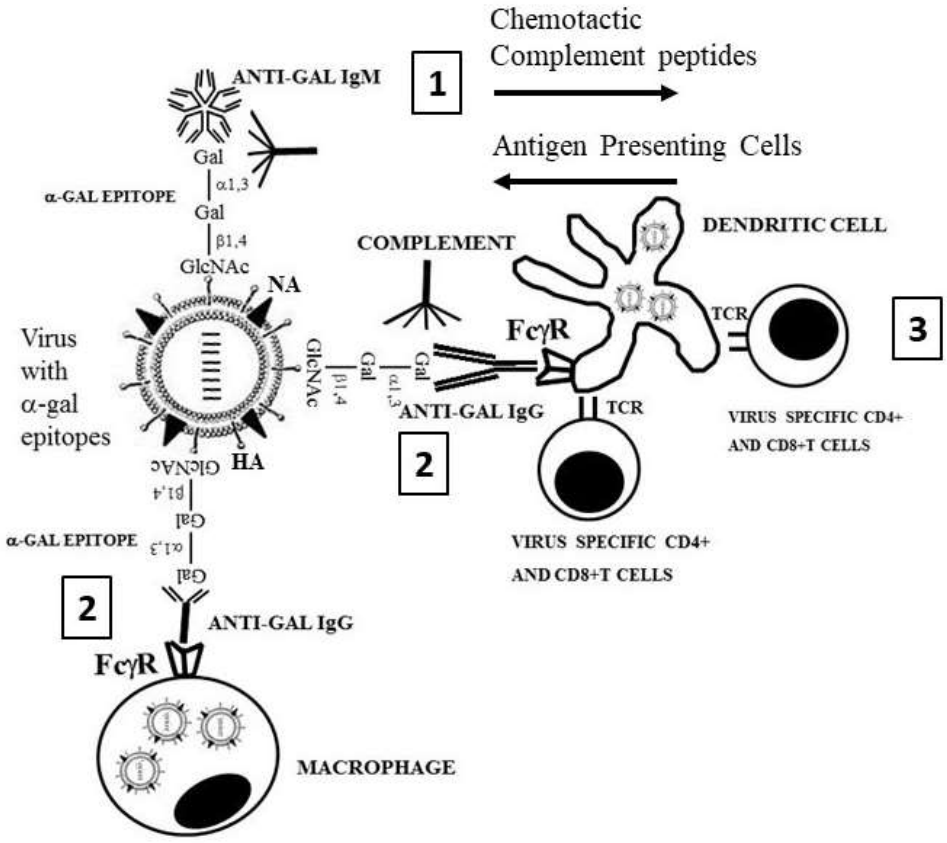

Figure 2. Hypothesis on mechanisms for amplification of inactivated whole-virus vaccine immunogenicity by glycoengineering the glycan-shield to present α-gal epitopes (virus$_{\alpha\text{-gal}}$). Inactivated influenza virus presenting α-gal epitopes is used as vaccine example. Step 1- Anti-Gal IgM and IgG bind to α-gal epitopes on virus$_{\alpha\text{-gal}}$ at the vaccination site and activate the complement system to form complement cleavage chemotactic peptides that recruit APC such as dendritic cells and macrophages. Step 2- Anti-Gal IgG immunocomplexed with the virus$_{\alpha\text{-gal}}$ targets it for rigorous uptake by the recruited dendritic cells and macrophages via Fc/Fcγ receptors (FcγR) interaction. Step 3- The APC transport the large amounts of internalized virus$_{\alpha\text{-gal}}$ to regional lymph nodes, process and present virus antigenic peptides on MHC molecules for the activation of many virus-specific $CD8^+$ and $CD4^+$ T cells. HA, hemagglutinin; NA, neuraminidase; TCR, T cell receptor. Modified with permission from "Galili U. *The natural anti-Gal antibody as foe turned friend in medicine*. Academic Press/ Elsevier Publishers, London, 2018, page 153".

The various stages in the hypothesis illustrated in Figure 2 were demonstrated in the experimental animal model of mice lacking α-gal epitopes (called GT-KO mice) as a result of disruption (knockout) of the *α1,3GT* gene (*GGTA1*).[66] These mice produce anti-Gal following immunization with xenogeneic tissues such as porcine kidney membrane (PKM) homogenate which contains high concentration of α-gal epitopes.[67,68] Chicken ovalbumin (OVA) was used as an antigen because it provides the means for tracing it following processing at various stages post immunization.[69] Since OVA lacks glycans, it was simulated as an antigen in an virus$_{\alpha\text{-gal}}$ vaccine by encapsulating it in small liposomes that present multiple α-gal epitopes (OVA-liposomes) and which were prepared from a mixture of extracted rabbit red blood cell phospholipids and glycolipids carrying this epitope.[70,71] OVA was used as a tracing antigen since the most immunogenic peptide of OVA for $CD8^+$ T cells is the 8-amino acid peptide SIINFEKL.[69] When presented in association with class I MHC molecules on APC, SIINFEKL can be readily detected by activation of the T hybridoma cell line B3Z to proliferate, because these hybridoma cells have a T cell receptor (TCR) specific for SIINFEKL.[72,73] As detailed in ref. 71, the studies with OVA-liposomes indicated that immunocomplexing of these liposomes with anti-Gal greatly amplified the uptake and processing by APC and the transport of OVA to regional lymph nodes was 7-8-fold higher than in the absence of anti-Gal. Activation of the vaccine specific T cells increased by 15-fold and anti-OVA antibody production increased by 30-100-fold in comparison to vaccination with OVA-liposomes in the absence of the immunocomplexing anti-Gal antibody.

Anti-Gal mediated amplification of influenza virus vaccine immunogenicity

Demonstration of the extent of increased immunogenicity by glycoengineering of an actual virus vaccine to present multiple α-gal epitopes was achieved with inactivated influenza whole-virus vaccine. The vaccine was prepared from the strain A/Puerto Rico/8/34-H1N1 (PR8 virus) which was propagated in embryonated chicken eggs.[64] The virus was inactivated by incubation for 45 min at 65°C. Synthesis of α-gal epitopes on PR8 virus was achieved by the use of recombinant (r) α1,3GT produced in the yeast *Pichia pastoris* expression system. [37,74] The general reaction for replacement of sialic acid with α-gal epitopes on the glycan-shield of viruses is illustrated in Figure 1 in which the inactivated virus is incubated with neuraminidase, rα1,3GT and UDP-Gal. However, since the main influenza virus glycoprotein - hemagglutinin (HA), uses sialic acid on cell glycans as "docking" receptor, the sialic acid synthesized on the 5-7 N-glycans of HA is removed by the viral neuraminidase on the envelope in order to prevent HA mediated adhesion between the virions. Thus, the N-glycans on HA have a structure as that in the center glycan illustrated in Figure 1 and no neuraminidase is included in the enzymatic solution. Quantification studies indicated that ~3000 α-gal epitopes are synthesized by this reaction on the glycan-shield of each PR8 virion, upon the conversion to PR8$_{\alpha\text{-gal}}$.[75]

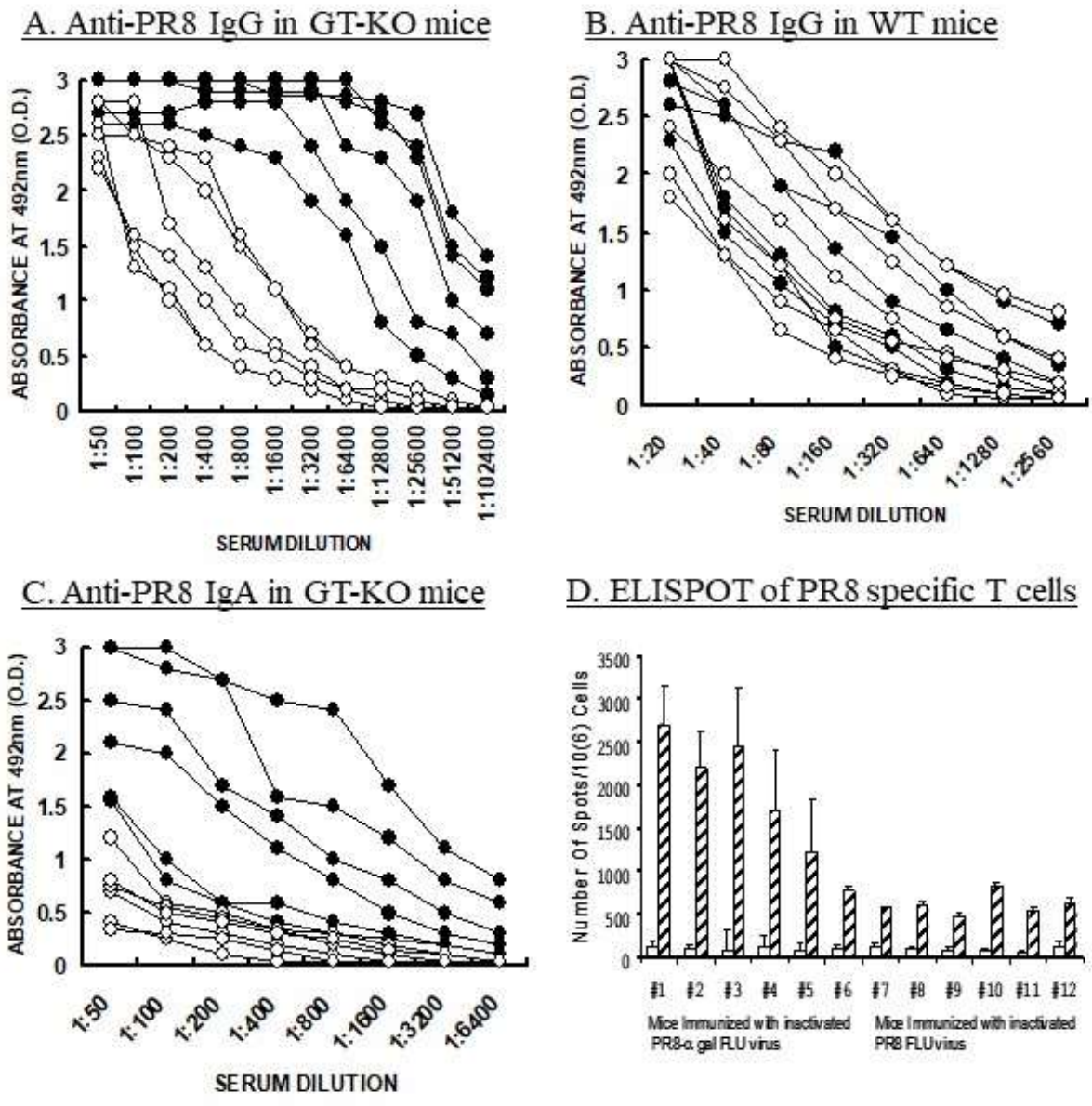

Figure 3. Amplification of antibody and T cell response to PR8 influenza virus in mice immunized twice with 1μg inactivated $PR8_{\alpha\text{-gal}}$ virus (●), or with inactivated PR8 virus (○) (n=6 per group). Antibody activity in the serum was measured by ELISA with PR8 virus attached to ELISA wells as solid-phase antigen. T cell activation was analyzed by ELISPOT for IFNγ secretion, by splenocytes incubated with PR8 pulsed dendritic cells. Immune response was evaluated 14 days following the second immunization. A. Anti-PR8 IgG production in GT-KO mice. B. Anti-PR8 IgG production in WT mice. C. Anti-PR8 IgA production in GT-KO mice. D. Spots per well in ELISPOT (mean of triplicates) of secreted IFNγ. Hatched columns- splenocytes incubated with PR8 pulsed dendritic cells, open columns- Splenocytes incubated with non-pulsed dendritic cells. $PR8_{\alpha\text{-gal}}$ immunized mice (#1 to #6), PR8 immunized mice (#7 to #12). Modified from ref. 64, with permission.

The immunogenicity of $PR8_{\alpha\text{-gal}}$ vs. that of PR8 was evaluated following two immunizations of anti-Gal producing GT-KO mice in two-week interval with either 1μg of $PR8_{\alpha\text{-gal}}$ vaccine, or with a similar amount of PR8 vaccine. The vaccines were delivered with Ribi (trehalose dicorynomycolate) adjuvant. Production of anti-PR8 antibodies in

the immunized mice was determined, two weeks after the second immunization, by ELISA with inactivated PR8 virus as solid-phase antigen. The activity of both IgG and IgA anti-PR8 antibodies in the blood of four of the six PR8$_{\alpha\text{-gal}}$ immunized GT-KO mice was ~100-fold higher than that in PR8 immunized mice (Figure 3A and 3C).[64] Anti-PR8 IgA antibodies were also found in the lungs of PR8$_{\alpha\text{-gal}}$ immunized GT-KO mice, but not in lungs of PR8 immunized mice (not shown, see ref. 64). This increased anti-PR8 antibody production was associated with presence of anti-Gal in the immunized mice. This was indicated by the low production of anti-PR8 IgG antibody in WT mice (mice lacking anti-Gal) immunized with either PR8$_{\alpha\text{-gal}}$ or with PR8 vaccine (Figure 3B). A marked difference in T cell activation between in GT-KO mice receiving PR8$_{\alpha\text{-gal}}$ vs. PR8 vaccine, was observed in ELISPOT assays measuring interferon-γ (IFNγ) secretion (Figure 3D). Spots representing this secretion by activated T cells were quantified with splenocytes co-incubated with dendritic cells pulsed with inactivated PR8 virus. In four of the six mice immunized with PR8$_{\alpha\text{-gal}}$, the number of spots was 4-5-fold higher than in ELISPOT wells of splenocytes from PR8 immunized mice. The immunization of anti-Gal producing GT-KO mice with PR8$_{\alpha\text{-gal}}$ vaccine also resulted in a much higher T cell response than immunization of these mice with PR8 vaccine when assayed by flow cytometry measuring intracellular cytokine staining for IFNγ both in $CD8^+$ T cells and in $CD4^+$ T cells.[64]

The differences in anti-PR8 antibody production and T cell activation were further reflected in the resistance to challenge with live virus in the mice immunized with PR8$_{\alpha\text{-gal}}$ vs. PR8 vaccine. The mice in the two groups were challenged with a lethal dose of live PR8 virus (2000 plaque forming units [PFU]), by intranasal infection, two weeks after the second immunization. This dose resulted in 100% death of unimmunized mice. Challenge of PR8 immunized mice with the live virus resulted in the survival of only 11% of the mice and the rest died within 10 days post infection (Figure 4A). In contrast, similar challenge in PR8$_{\alpha\text{-gal}}$ immunized mice resulted in survival of 89% of the mice, implying a much more potent immune protection than in PR8 immunized mice (Figure 4A).[64] Survival on Day 30 was the same as that on Day 15 post challenge. In a second challenge study, mice immunized with PR8$_{\alpha\text{-gal}}$ or with PR8 vaccines were euthanized 3 days after challenge with the lethal dose of live PR8 virus. The lungs of these mice were harvested, homogenized, the membranes spun, the supernatants collected, and the virus in these supernatants was quantified by evaluating the virus cytopathic tissue culture infection dose (TCID) in cultured Madin Darby Canine Kidney (MDCK) cell monolayers. The virus amount in lungs of PR8$_{\alpha\text{-gal}}$ immunized mice was 10 to 100-fold lower than that in lungs of PR8 immunized GT-KO mice (Figure 4B).

The observations on increased immunogenicity of PR8$_{\alpha\text{-gal}}$ vaccine have been supported by a recent study in which anti-Gal producing GT-KO mice were immunized with an attenuated influenza virus in which the mouse *α1,3GT* gene (*GGTA1*) was introduced as part of the viral genome.[76] The virus released from infected cells in these mice presented α-gal epitopes synthesized by α1,3GT introduced by the *α1,3GT* gene within the infecting virions. GT-KO mice immunized with the mutated virus displayed a much higher resistance to intranasal challenge with a lethal dose of the original virus than mice immunized with attenuated virus lacking the *α1,3GT* gene.[76]

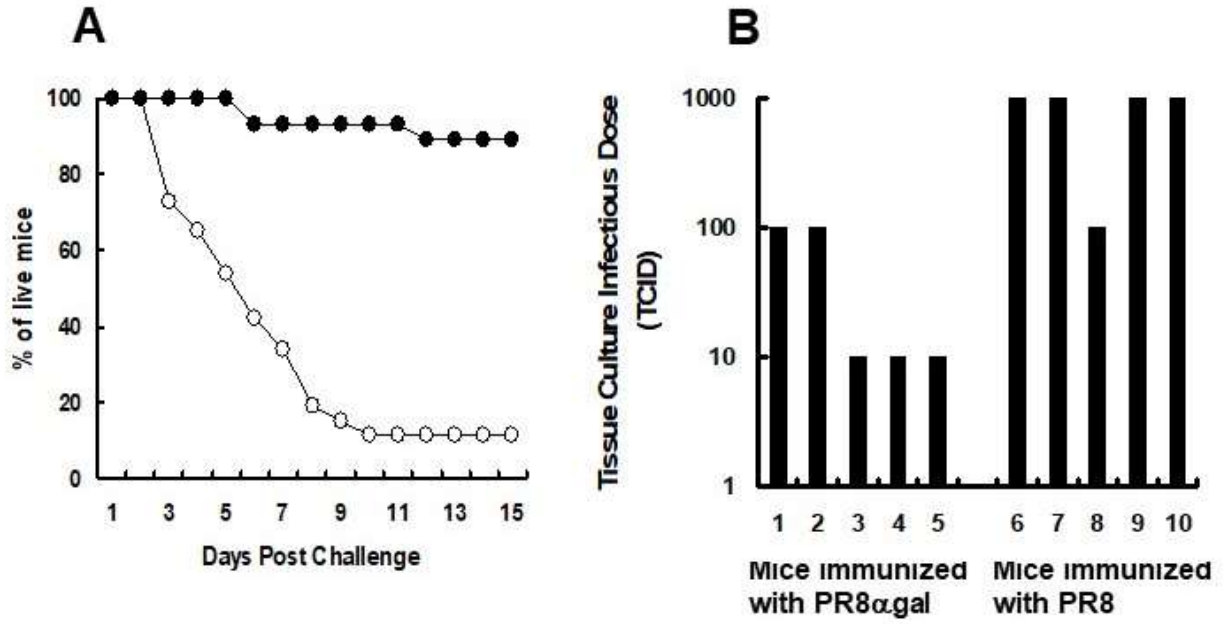

Figure 4. Survival and lung infection in mice immunized twice with inactivated PR8 or PR8$_{\alpha\text{-gal}}$ and challenged intranasal with a lethal dose of live PR8 virus. **A.** PR8 vaccine (○), or PR8$_{\alpha\text{-gal}}$ vaccine (●) (n=25/group). Survival at various days post challenge presented as proportion (%) of live mice. The survival on Day 30 was similar to Day 15. **B.** Measurement of virus titer as tissue culture infectious dose (TCID) in lungs of the immunized mice, 3 days post challenge (n=5/group). Cytopathic effects were determined in Madin Darby Canine Kidney (MDCK) cell monolayers cultured for 4 days (n=5/group). From ref. 64 with permission.

Vaccinating glycoproteins glycoengineered to present α-gal epitopes resulted in a similar increase in antibody and T cell response, as well as in neutralizing activity of antibodies. Such increased immunogenicity was demonstrated in anti-Gal producing mice immunized with recombinant gp120 of HIV vaccine glycoengineered to present α-gal epitopes (i.e., gp120$_{\alpha\text{-gal}}$),[63,77] and in GT-KO mice immunized with bovine serum albumin (BSA) presenting α-gal epitopes.[78] All these studies support the hypothesis illustrated in Figure 2 and strongly suggest that glycoengineering inactivated virus vaccine to present multiple α-gal epitopes will results in a much higher efficacy of whole-virus vaccines than vaccination with virus lacking α-gal epitopes. It should be stressed that non-enveloped viruses usually do not have carbohydrate chains on their capsid proteins. Therefore, linking α-gal epitopes covalently to the capsid proteins[79] may cause changes in antigenicity of viral peptides, making the inactivated virus vaccine unsuitable for use. In contrast, synthesizing α-gal epitopes on N-glycans of the enveloped virus does not affect the structure of the protein portion of the envelope glycoprotein.

Suggested methods for glycoengineering of whole-virus vaccines to present multiple α-gal epitopes

This section describes suggested methods for achieving high number of α-gal epitopes on vaccinating virus$_{\alpha\text{-gal}}$. Some of these methods were previously presented for increasing immunogenicity of whole-virus Covid-19 vaccines.[10] However, these methods may be applicable for increased immunogenicity of any strain of enveloped virus that has a glycan-shield.

Use of neuraminidase and recombinant α1,3galactosyltransferase for in vitro synthesis of α-gal epitopes on inactivated

virus- The section on the influenza virus vaccine describes the use of rα1,3GT for synthesizing α-gal epitopes on glycans of hemagglutinin lacking sialic acid. Thus, there is no need for neuraminidase in glycoengineering of this virus. However, N-glycans in most enveloped viruses carry sialic acid which has to be removed by neuraminidase to enable the exposure of the LacNAc required for synthesis of α-gal epitopes by rα1,3GT, as illustrated in Figure 1. Neuraminidase can be obtained from commercial sources. rα1,3GT can be produced as described in studies in which the *α1,3GT* gene (*GGTA1*) was cloned from a cDNA library and truncated for deleting both cytoplasmic and trans-membrane domains. [37] The truncated gene also carried a $(His)_6$ tag and the recombinant enzyme produced in a yeast expression system was isolated on a nickel-Sepharose column and eluted with imidazole.[74] The efficacy of this method in synthesis of α-gal epitopes by rα1,3GT was demonstrated with human tumor cells,[80-82] influenza virus,[64,75] and gp120 of HIV.[63,77]

Transfection of host-cells with several copies of the α1,3GT gene- An alternative method for glycoengineering viral glycan-shields to produce $virus_{\alpha\text{-gal}}$ without *in vitro* enzymatic reactions is to use host-cells that were engineered to produce large amounts of α1,3GT. This can be achieved by performing stable transfection of established host-cell line with several copies of the *α1,3GT* gene (*GGTA1*). Such cells are likely to produce large amounts of α1,3GT which will successfully compete with sialyltransferases in capping glycans with α-gal epitopes rather than with sialic acid. This competition is the result of location of α1,3GT and of sialyltransferases in the same trans-Golgi compartment.[83] Therefore, many of the N-glycans that could be capped by α-gal to form α-gal epitopes (right glycan in Figure 1) are capped by sialic acid (left glycan in Figure 1). A further step for increasing α-gal epitopes on $virus_{\alpha\text{-gal}}$ vaccines is to decrease the competition with sialyltransferases within the trans-Golgi compartment by inactivation (knockout) of sialyltransferase genes in the host-cell line. Studies demonstrating the feasibility of this method have been performed with a mouse melanoma cell-line and with CHO cells, both lacking α-gal epitopes.[83-85]

Transduction of host-cells with replication defective adenovirus Adα1,3GT- A second method for conversion of host-cell lines into cells with high α1,3GT activity is transduction of such cells with replication defective adenovirus containing the *α1,3GT* gene (AdαGT),[86,87] prior to infection of the cells with the replicating virus to be used for $virus_{\alpha\text{-gal}}$ vaccine preparation. The replication defective adenovirus lacks the ability to replicate in the host-cells, however, it effectively introduces several copies of the *α1,3GT* gene into the cells. Transduction of human HeLa cells with AdαGT resulted in introduction of ~20 copies of the *α1,3GT* gene in <1h. Appearance of α1,3GT mRNA was observed within 4h post-transduction and maximum production of α-gal epitopes on cell surface glycans (~$4x10^6$ epitopes/cell) within 48h. [86] For the production of vaccinating $virus_{\alpha\text{-gal}}$, it is suggested that cells would be transduced with AdαGT 12-24h prior to the infection of the host-cells with the vaccinating virus. This period is likely to enable the accumulation of large amounts α1,3GT within the host-cells for the synthesis of α-gal epitopes on most N-glycans of the complex type. As argued above, it is possible that inactivation of sialyltransferase genes in the host-cells may further increase the probability of capping these glycans with α-gal epitopes.

Engineering the vaccinating virus to contain the α1,3GT gene- Recent studies have demonstrated the formation of an influenza virus containing the *α1,3GT* gene.[76] Such a virus introduces the α1,3GT gene into the cells it infects, resulting in production of large amounts of α1,3GT that synthesizes many α-

gal epitopes on the glycan-shield of the virus. It is of note that the yield of virus in host-cells infected with influenza virus containing the *α1,3GT* gene was ~1000-fold lower than in cells infected with the wild-type virus.[76] This raises the possibility that in some strains, alteration in carbohydrate composition of the glycan-shield may affect the number of virions released from infected host-cells. This finding further suggests that optimization of the number of virions produced per cell vs. the number of α-gal epitopes per virion should be evaluated also in the *α1,3GT* gene transfection and AdαGT transduction methods, in order to determine the optimal method for preparation of various virus$_{\alpha\text{-gal}}$ vaccines.

virus$_{\alpha\text{-gal}}$ vaccines and the α-gal syndrome

Administration of α-gal epitopes into humans appears to be safe. This assumption is based the many cases of porcine heart valve implantation in humans for replacement of impaired heart valves. Binding of anti-Gal to the many α-gal epitopes on endothelial cells of the porcine valve is not followed by adverse effects. Similarly, immunization of humans with a variety of autologous cancer vaccines presenting α-gal epitopes was found to be safe.[81,82,88-90] All these observations suggest that virus$_{\alpha\text{-gal}}$ vaccines may be safe in humans, as well. However, the safety of virus$_{\alpha\text{-gal}}$ vaccines should be determined in particular in individuals with the "α-gal syndrome" who are allergic to α-gal epitopes in meat such as beef, pork and lamb.[91-93] In these individuals, bites by certain ticks in different continents (e.g., *Amblyomma americanum* in the USA) result in isotype switch for production of anti-Gal IgE. The binding of this IgE antibody to the multiple α-gal epitopes in red meat results in an allergic immune response which appears within several hours following eating red meat. Thus, virus$_{\alpha\text{-gal}}$ vaccine administration to individuals with α-gal syndrome and those with history of tick bites may have to be performed in clinics equipped for prevention of allergic reactions.

Conclusions

The glycan-shield on enveloped viruses assists the virus to evade the protective immune response by decreasing uptake by APC and by masking immunogenic peptides. Glycoengineering the glycan-shield to present α-gal epitopes on N-glycans converts the glycan-shield into a portion of the viral envelope that actively targets the vaccinating virus$_{\alpha\text{-gal}}$ for rigorous uptake by APC, thereby greatly increasing the immunogenicity of the various antigens of the virus. The resulting effective immune response to multiple antigens increases the immune protection against infectious virus, improves the immune memory and prevents the appearance of detrimental variants of the virus. The effective targeting of the vaccinating virus$_{\alpha\text{-gal}}$ to APC is mediated by the natural anti-Gal antibody, which is abundant in all humans. Anti-Gal binds to α-gal epitopes on the vaccinating virus at the injection site and forms immune-complexes. The binding of the immunocomplexed anti-Gal Fc portion to Fcγ receptors on APC induces rigorous uptake of the vaccinating virus$_{\alpha\text{-gal}}$ by the APC, transport of the large amounts of APC internalized virus to regional lymph nodes, processing and presentation of the viral antigens and activation of multiple virus specific T and B lymphocytes. Glycoengineering of viruses into virus$_{\alpha\text{-gal}}$ vaccines is feasible by neuraminidase and rα1,3galactosyltransferase enzyme reaction, or by replication of the virus in host-cells containing multiple copies of the α1,3galactosyltransferase gene. These methods for amplification of vaccine immunogenicity are applicable to all viruses with N-glycans in the glycan-shield. Clinical trials have shown that injection of α-gal epitopes is safe in humans, however individuals with α-gal syndrome and those with multiple tick bites, should receive such vaccines in clinics equipped for prevention of allergic reactions.

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