Heliyon 9 (2023) e14670

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Specific sequence mutations in a long-lasting rhIFN-α2b version reduce *in vitro* and *in vivo* immunogenicity and increase *in vitro* protein stability

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ARTICLE INFO

Keywords:
IFN alpha
Immunogenicity
De-immunization
EpiMatrix
In vivo assays
Anti-viral therapy

ABSTRACT

For decades, recombinant human interferon alpha (rhIFN-α2b) has been used to treat emerging and chronic viral diseases. However, rhIFN-α2b is immunogenic and has a short *in vivo* half-life. To solve these limitations, two long-lasting hyperglycosylated proteins with reduced immunogenicity were developed and designated as 4N-IFN(VAR1) and 4N-IFN(VAR3).

Here, we continue to study the relevant characteristics of these therapeutic candidates. Thus, we demonstrated that both de-immunized IFN versions elicited significantly lower neutralizing antibody responses than the original molecule in HLA-DR1 transgenic mice, confirming our previous *in vitro* protein immunogenicity data. Also, we found that these biobetters exhibited remarkable stability when exposed to different physical factors that the protein product may encounter during its production process and storage, such as low pH, thermal stress, and repeated freezing/thawing cycles. Taking into consideration our previous and present results, 4N-IFN (VAR1) and 4N-IFN-4N(VAR3) appear to be valuable candidates for the treatment of human viral diseases.

1. Introduction

Human IFN alpha (hIFN-α) constitutes a family of signaling proteins that induce a variety of biological actions. In particular, recombinant hIFN-α2b is widely used for the treatment of viral diseases such as chronic Hepatitis B and C [1,2]. Additionally, in recent years, the use of this therapeutic protein alone or in combination with other antiviral agents was also proposed for the treatment of emergent viral infections. For instance, the combination regimen of favipiravir plus rhIFN-α2b was shown to inhibit Zika virus infection in Vero cells without exhibiting cytotoxic effects, at concentrations that are achievable in humans, highlighting its potential use as antiviral therapy [3].

An additional study showed that rhIFN-α2b also inhibited Dengue virus (DENV) replication when used as a single agent in Huh-7 cells. Moreover, ribavirin (RBV) plus rhIFN-α2b showed an additive effect for viral suppression in both Huh-7 and Vero cell lines.

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https://doi.org/10.1016/j.heliyon.2023.e14670
Received 31 August 2022; Received in revised form 8 March 2023; Accepted 15 March 2023
Available online 20 March 2023

Clinical exposures to RBV plus rhIFN-α2b suppressed DENV replication by 99% even when the treatment was initiated 24 h post-infection in a hollow fiber infection model (HFIM) system. Further investigation revealed that the therapeutic effectiveness of this treatment could mainly be attributed to IFN activity [4].

Beyond ZIKV and DENV, the potential for using IFN as a treatment strategy against chikungunya virus (CHIKV) infections has been considered and tested. For example, rhIFN-α2b and RBV were effective against viral replication as single agents and also produced a synergistic effect when used in combination [5,6].

The promise of rhIFN-α2b therapy is limited by the fact that robust immune responses against the cytokine have been observed in treated patients [7–10], even though it is identically conserved with the native gene product.

These immune responses contribute to the short half-life of the drug, which may lead to rapid elimination of the drug from circulation. Unrelated to immune responses, the drug has a very short half-life. For these reasons, repeated high doses are often required to maintain the required therapeutic window.

It is widely accepted that an anti-drug antibody response, such as the response observed to rhIFN-α2b, is closely related to helper T-cell responses to the drug-protein [11–14]. Indeed, the essential role of $CD4^{+}$T cells in *anti*–IFN–α2b antibody (ADA) formation has already been reported by Jones and collaborators [15]. As may be anticipated, the impact of anti-drug antibodies can vary from patient to patient. For instance, ADA may stably bind to rhIFN-α2b without having any detectable effect on the biological activity of the drug. However, it has also been shown that *anti*-IFN antibodies may alter the pharmacokinetic parameters of the drug in clinical use, and in certain cases, they may even neutralize its activity by disrupting the interaction between rhIFN-α2b and its cell-surface receptor on target cells [16,17]. On the other hand, rhIFN-α2b has also been associated with an increased risk of induction of autoimmune thyroiditis in Hepatitis C virus (HCV)-infected patients, revealing a pro-inflammatory role of the drug [18,19].

For these reasons, several groups have attempted to predict and reduce rhIFN-α2b immunogenicity in preclinical studies [15,20,21].

To solve the issue of *in vivo* protein stability (half-life), a long-lasting derivative of the cytokine was developed, referred to as 4N-IFN (21). 4N-IFN showed a 25-fold longer plasma half-life than the commercial rhIFN-α2b, which is even greater than the pegylated derivative Intron-A PEG. However, in silico and preclinical analysis using human peripheral blood mononuclear cells (PBMC) revealed a high immunogenicity risk for this protein [22]. For this reason, we attempted to reduce immunogenicity using a stepwise protein de-immunization process known as de-immunization for functional therapeutics or "DeFT" [23].

The DeFT strategy is based on the identification and modification of specific residues to disrupt the interaction of T cell epitopes to MHCII. These mutations are also selected taking into account protein function, ensuring the retention of biological activity. Then, these in silico predictions are validated through HLA binding assays for eight prevalent HLA class II alleles. Applying this method to IFN, we developed two de-immunized versions of 4N-IFN that were produced in CHO cells. These new IFN variants were designated as 4N-IFN (VAR1), carrying the following mutations: L9A, F47A, L117A, F123A, and L128A and 4N-IFN(VAR3) containing only three modifications: L9A, F47A, and L18A. A detailed description of the method used to de-immunize 4N-IFN can be found in our previous work [24]. The antiviral activity of these IFN-α versions was determined by their ability to inhibit the cytopathic effect caused by vesicular stomatitis virus on MDBK cells. Thus, whereas 4N-IFN(VAR1) retained 28.4% of the original biological activity, 4N-IFN(VAR3) evidenced a residual antiviral activity of 16.9% when compared with the originator [24]. In agreement with in silico predictions, both de-immunized variants showed reduced immunogenicity in ex vivo experiments using human PBMC-derived T cells. However, until now, an *in vivo* immunogenicity analysis for these molecules had not been carried out.

Numerous reports revealed that peptides displayed to T cells in humanized transgenic mice matched those identified in patients that expressed those specific HLA alleles [25–27]. Therefore, in this study, we used humanized HLA-DRB1*0101 mice as we previously found that PBMC samples from donors expressing this HLA-DRB1 allele (and some other alleles) responded to rhIFN-α2b versions *in vitro* [24]. After intraperitoneal injection of IFN in HLA-DRB1*0101 mice, we observed a marked decrease in neutralizing antibody (NAb) titers for mice that had been exposed to either of the de-immunized variants when compared with those treated with the original molecule. These results are in agreement with data from *in vitro* experiments using human PBMC and confirm the success of our strategy to de-immunize 4N-IFN.

In addition, previous studies showed that the incorporation of additional glycosylation sites into the rhIFN-α2b sequence could improve its biological characteristics such as elimination half-life and *in vivo* bioactivity, and enhance the cytokine's stability towards physical factors that may be encountered during its production process and storage [28]. Hence, in this study, we also assessed protein stability of 4N-IFN de-immunized variants exposing these proteins and the original molecule to different challenges such as *in vitro* incubation with human plasma, thermal stability, repeated freezing and thawing cycles, and exposure to acid pH.

2. Materials and methods

2.1. Gene expression in mammalian cells

2.1.1. Cell culture

Chinese hamster ovary (CHO·K1) cells were grown in basal culture medium previously described [29] supplemented with 5% (v/v) fetal calf serum (FCS) (PAA, Argentina). Madine Darby bovine kidney (MDBK) cells were grown in minimum essential medium (MEM; Gibco, USA) supplemented with 10% (v/v) FCS. Bioassays were performed using MEM supplemented with 2% (v/v) FCS (assay medium).

2.1.2. Production and purification of IFN-α2b variants

For large-scale protein production, IFN, 4N-IFN, 4N-IFN(VAR1), or 4N-IFN(VAR3) producing cell clones were grown in multi-layer flasks until reaching cell confluence. Growth medium was replaced by production medium (basal medium supplemented with 0.5% (v/v) FCS. Then, the IFN-enriched medium was carefully collected every 48 h and replaced by fresh medium. This procedure was repeated between 8 and 10 times. Harvests were cryopreserved until protein purification. Every harvest was assayed for IFN concentration by sandwich ELISA, following a protocol previously described [22].

Proteins were purified by immunoaffinity chromatography following a strategy previously described [22]. The concentration of purified 4N-IFN and its de-immunized variants was analyzed by reverse phase HPLC, including the non-glycosylated IFN version as standard, and confirmed by spectrophotometric quantification.

2.2. In vivo assays

2.2.1. Humanized mice

Mutant HLA-DRB1*0101mice humanized for both HLA-A2 and HLA-DR1 and deleted for both H-2 class 1 and 2 molecules (β2m−/− H-2Db−/− IAβ−/− IAα−/− IEβ−/−) were provided by Institut Pasteur, and developed by Francois Lemonnier.

Transgenic mice, aged 6–10 weeks, were used in this study. All animals were housed and bred in pathogen-free micro isolator cages at the animal facilities operated by the Faculty of Veterinary Sciences of Universidad Nacional del Litoral (UNL). Handling procedures and animal protocols were previously approved by the UNL Research Ethics Committee, code number: CE2018-36 (Santa Fe, AR).

2.2.2. Mouse immunizations

Groups of 6 mice were immunized three times via intraperitoneal (i.p.) injection with PBS or 25 μg of 4N-IFN, 4N-IFN(VAR1), or 4N-IFN(VAR3) emulsified in CFA (Sigma, St. Louis, MO) at a final volume of 200 μl. Blood was collected 14 days post-immunization and plasma was isolated.

2.2.3. Detection of NAbs to IFN-α

Antibody titers were determined by a neutralization test against 4N-IFN, 4N-IFN(VAR1), or 4N-IFN(VAR3). Serial dilutions (starting from 1:10 and increasing twofold) of sera from HLA-DR1 transgenic mice immunized with each IFN species or unimmunized controls were incubated at 37 °C with 20 IU/ml of each type of IFN-α2b, considering the specific antiviral activity previously reported for each protein [24]. After 1 h of incubation, 200 μl of each of the individual mixtures were added to quintuplicate monolayers of MDBK cells in 96-well microtiter plates. Then antiviral assays were carried out as described below.

Titers were expressed as the dilution of serum that reduces to 50% (IC50) the initial IFN antiviral activity (IFN proteins incubated with control serum). The serum samples were found to be free of endogenous or residual IFN activity.

2.3. Antiviral assay

The biological antiviral activity of rhIFN-a2b was determined by its ability to inhibit the cytopathic effect caused by the vesicular stomatitis virus (VSV, Indiana strain) on MDBK cells (30,31). 2.5×10^4 MDBK cells were grown at 37 °C overnight. Then, culture supernatants were removed and 1:2 serial dilutions of rhIFN-a2b WHO international standard (NIBSC 95/566) from 20 U ml^{-1} to 0.16 U ml^{-1} or 1:2 serial dilutions of 4N-IFN variants were added to samples in the assay medium. After 6 h of incubation, we removed the supernatants, and cells were infected with VSV (ratio: 1.6 PFU/cell). Viral treatment was stopped when the cytopathic effect was evident in the control cell culture (no IFN). Cells were stained by incubation with a solution of crystal violet (0.75% w/v) in methanol (40% v/v) for 15 min. After successive washing steps with water, stained cells were treated with acetic acid (20% v/v). Finally, signal intensity was read at 540 nm and determinations were performed in quintuplets.

2.4. Stability assays

2.4.1. Stability of IFN variants in human plasma

4N-IFN, 4N-IFN(VAR1), and 4N-IFN(VAR3) were diluted (final concentration of 50 ng/ml) in human plasma previously obtained under informed consent from healthy volunteers and then incubated at 37 °C. Samples were taken at specific incubation times (0, 24, 48, 72, 96, 120, 144, and 168 h) and immediately cryopreserved at −20 °C. Then, the sample residual antiviral activity was determined as described above. The experiment was performed in triplicates.

2.4.2. Thermal stability

Stability against heat treatment of 4N-IFN and its de-immunized variants was determined by evaluating the remaining antiviral activity after treatment at different temperatures. Protein samples were prepared in PBS buffer (pH 7.4) at a concentration of 1 μg/ml and then incubated for 10 min at different temperatures using a heat block (Biometra Biosciences, Germany). After treatment samples were immediately stored at −20 °C. Protein stability was evaluated by measuring the residual antiviral activity after heating. The experiment was carried out in triplicate.

2.4.3. Influence of repeated freezing and thawing cycles on protein stability

The stability of 4N-IFN and its de-immunized variants was investigated by measuring the residual biological activity after repeated

freezing and thawing cycles. IFN samples were diluted in PBS to reach a concentration of 50 ng/ml. Protein samples were subjected to a treatment consisting of freezing at −70 °C and then heating at 37 °C in a thermal block, until complete thawing. This procedure was repeated 15 times and then samples were stored at −20 °C until assessed for IFN antiviral activity. The experiment was performed in triplicate.

2.4.4. Stability at acid pH

To investigate the effect of acid pH on protein stability, 4N-IFN, 4N-IFN(VAR1), and IFN(VAR3) were firstly diluted in PBS buffer at a concentration of 70 μg/ml. Protein samples were then diluted in glycine solution (pH 2) to reach a concentration of 50 ng/ml. Incubations were made at room temperature for different times and then a solution of Tris-HCl (1 M, pH 9) was added to neutralize the protein solution. Residual antiviral activity was measured for each sample and all samples were tested in triplicate.

2.4.5. Statistical analysis

To evaluate the differences between treatments we used a one-way analysis of variance (ANOVA). When this analysis produced significant differences ($p < 0.05$), we applied a post-hoc Tukey's multiple comparison test. Both statistical analyses were performed using GraphPad Prism for Windows, version 5.01.

3. Results

3.1. 4N-IFN(VAR1) and 4N-IFN(VAR3) exhibited reduced in vivo immunogenicity

It was previously shown that human HLA-DR alleles are involved in antigen presentation and required for T-cell responses against therapeutic proteins [30,31]. In particular, we have recently demonstrated the presence of T-cell epitopes in the 4N-IFN sequence that bind to multiple HLA-DR alleles. We also identified specific mutations that disrupted the interaction between those peptides with individual HLA-DR molecules through *in vitro* and ex vivo assays using human PBMCs [24].

However, the use of regular PBMC assays for testing protein immunogenicity has some limitations, such as the inability to evaluate neutralizing anti-drug antibody formation.

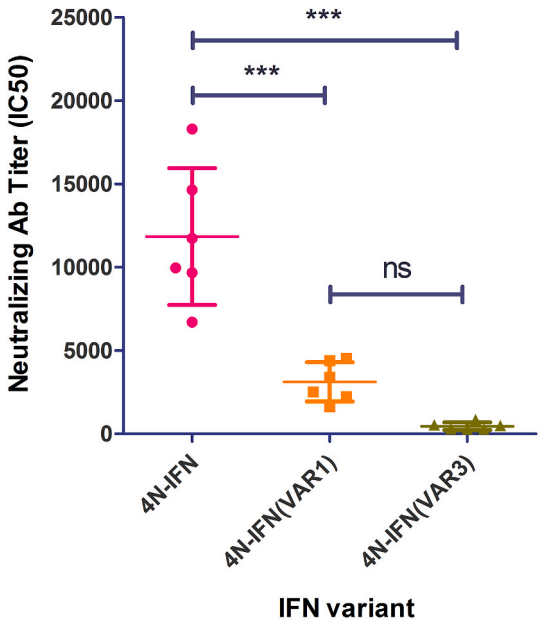

Fig. 1. 4N-IFN de-immunized variants showed a markedly reduced *in vivo* immunogenicity in comparison with the original molecule. Six HLA-DR1 transgenic mice per group were inoculated with PBS (control) or the indicated IFN variant emulsified with CFA. Neutralizing antibody titers were determined by a neutralization test against 4N-IFN, 4N-IFN(VAR1), or 4N-IFN(VAR3). Serial dilutions (starting from 1:10 and increasing twofold) of sera from HLA-DR1 transgenic mice immunized with each IFN species or unimmunized controls were incubated at 37 °C with 20 IU/ml of each type of IFN-α2b, considering the specific antiviral activity previously reported for each protein.
Then, the individual mixtures were added to monolayers of MDBK cells to assay the antiviral activity. Titers were expressed as the dilution of serum that reduces to 50% (IC50) the initial IFN antiviral activity (IFN proteins incubated with control serum). While sera from mice treated with PBS alone did not develop detecqtable neutralizing antibodies against IFN (data not shown) high Nab titers were observed for those mice inoculated intraperitoneally with 4N-IFN. In contrast, a marked reduction in Nab titers was detected in mice treated with both de-immunized variants. Horizontal bars represent mean ± SD. (***$p < 0.001$).

An attractive alternative to *in vitro* human PBMC assays is to use HLA-transgenic mice. This animal model can be used to generate immune responses that are more closely related to the responses expected from humans. Certain HLA-DR transgenic mice do not express any murine MHCII genes, and therefore T cell responses are restricted by the transgenic human HLA-DR molecules [32,33]. In this study, we used HLA-DR1 transgenic mice because we previously found that the HLA-DRB1*0101 allele was involved in IFN-derived peptide presentation to T cells [24]. As shown in Fig. 1, HLA-DR1 mice developed high titers of neutralizing anti-4N-IFN antibodies (NAb) when treated intraperitoneally with IFN. In contrast, NAb titers in sera from mice injected with 4N-IFN(VAR1) protein were significantly lower ($p < 0.001$) and the magnitude of the effect was even more pronounced for mice inoculated with 4N-IFN(VAR3), where Nabs were barely detected. However, the difference between neutralizing antibody titers from mice treated with each of the de-immunized variants was not statistically significant. These experiments were repeated twice with reproducible results. It is noteworthy to note that control mice did not develop detectable *anti*-IFN neutralizing antibodies. The results described here are in agreement with our previous study using human PBMCs and highlight the successful introduction of the modifications in the 4N-IFN sequence to reduce its immunogenicity.

3.2. Protein stability

3.2.1. 4N-IFN de-immunized variants retained the in vitro stability of 4N-IFN in human plasma

Several mechanisms have been identified that are associated with rhIFN-α2b inactivation *in vivo*, such as plasma proteases, culminating with the therapeutic protein elimination from blood circulation [34,35]. Moreover, *in vitro* experiments revealed that the commercial non-glycosylated IFN lost more than 70% of its original antiviral bioactivity after treatment with human plasma for 7 days [28]. Altogether, these studies partially explain the short-half life of this product and the requirement for repeated dosing to reach the therapeutic window.

The attachment of O-glycans to rhIFN-2b protein is a successful strategy to increase its resistance to human plasma protease degradation [28]. Therefore, to evaluate whether protein *N*-glycosylation would also confer increased stability to proteolytic degradation, we treated 4N-IFN and its de-immunized versions with human plasma for 7 days. While parental IFN exhibited a successive loss of antiviral activity, all 4N-IFN variants retained full antiviral activity until the end of the treatment with no significant differences between them (Fig. 2). This result indicates that *N*-glycans attached to the IFN molecule may provide additional resistance to those destabilizing components present in human plasma. In addition, these data also revealed that the mutations introduced into the 4N-IFN sequence, to reduce protein immunogenicity, did not disturb protein stability under these stress conditions.

3.3. Thermal stability

Glycosylation engineering has also been shown to be a powerful strategy that can improve other protein properties. Indeed, this was the case for 4N-IFN, where the introduction of additional *N*-glycosylation sites improved resistance to heating at high temperatures [29].

To investigate whether the modifications introduced to generate 4N-IFN(VAR1) and 4N-IFN(VAR3) could have an impact on this protein feature, we evaluated the thermal stability for these proteins by measuring residual protein antiviral activity after heat treatment at different temperatures for 10 min.

As shown in Fig. 3 all 4N-IFN proteins maintained nearly intact antiviral activity after 10 min of incubation at 65 °C. However, heat

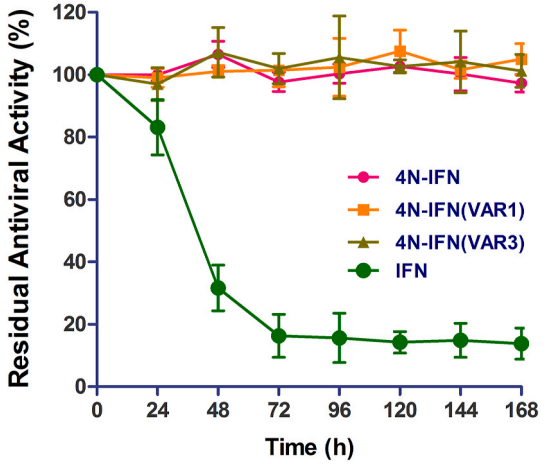

Fig. 2. De-immunized 4N-IFN variants retained the prolonged *in vitro* human plasma stability of 4N-IFN. IFN variants were incubated with human plasma for 7 days at 37 °C. After treatment, residual antiviral biological activity was evaluated at the indicated times. While a gradual loss of activity was observed for parental IFN, no significant differences were detected for all 4N-IFN proteins. This reflects the potential protective effect of *N*-glycans attached to the IFN molecule to proteases present in human plasma. Data points represent mean ± SD of triplicate samples.

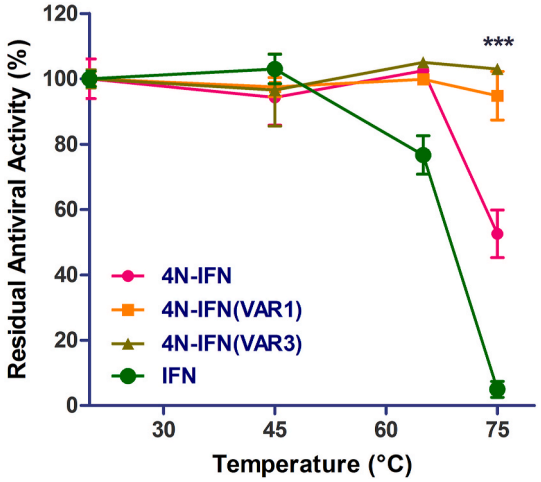

Fig. 3. 4N-IFN(VAR1) and 4N-IFN(VAR3) showed higher thermal stability than the original molecule. IFN variants were exposed to different temperatures for 10 min. Protein stability was assayed by measuring the residual antiviral activity in each sample. All 4N-IFN proteins retained nearly intact their biological function after incubation at 65 °C. However, when treated at 75 °C, 4N-IFN lost nearly 50% of the initial antiviral activity. Contrarily, 4N-IFN(VAR1) and 4N-IFN(VAR3) were not affected by this stress condition as both proteins maintained intact their original biological function. These results highlight the additional stabilizing effect of specific mutations introduced in the 4N-IFN sequence to reduce its immunogenicity. Experiments were performed in triplicate and each data point represents mean ± SD. (***$p < 0.001$).

treatment above this temperature led to a loss of almost 50% of the 4N-IFN initial antiviral activity and a complete loss of activity for parental IFN. Interestingly, 4N-IFN(VAR1) and 4N-IFN(VAR3) were less affected by this stress condition, as both proteins retained their full original biological function even at very high temperatures.

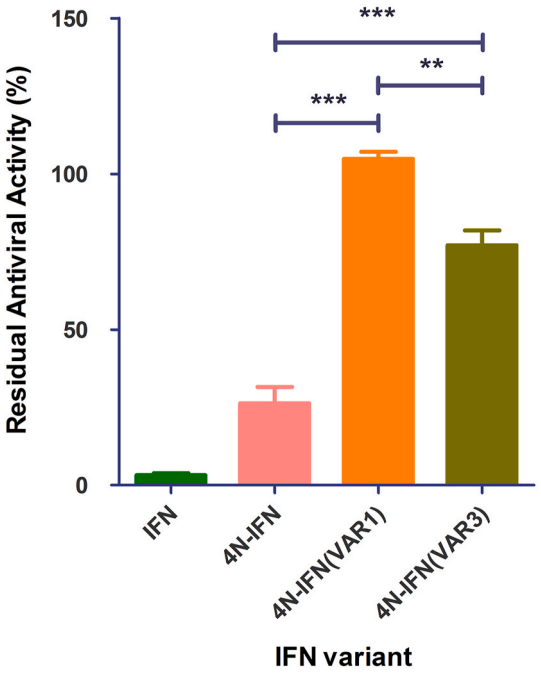

Fig. 4. De-immunized 4N-IFN proteins are more stable than the original molecule after treatment of successive freezing/thawing cycles. IFN variants were exposed to 15 cycles consisting of freezing at −70 °C followed by thawing in a water bath at 37 °C. To evaluate the effect of successive freezing/thawing cycles on the stability of each protein, we measured the antiviral biological activity at the end of the treatment. After this stress condition, 4N-IFN protein was severely affected as only retained 26% of the initial antiviral activity. In contrast, 4N-IFN(VAR3) was less affected by this stress treatment, as this protein retained more than 70% of the initial biological antiviral activity. The magnitude of this effect was even more notorious for 4N-IFN(VAR1) which conserved full residual biological function. Experiments were carried out in triplicate and each bar represents mean ± SD. Statistical comparisons were assessed by One-way ANOVA followed by Tukey (**$p < 0.01$, ***$p < 0.001$).

This shows how the specific mutations introduced into the 4N-IFN protein sequence may also confer resistance to heating, which is one of the most important factors to affect protein stability in clinical use.

3.4. Protein stability against freezing and thawing cycles

Therapeutic proteins are routinely exposed to freeze-thawing stresses during different steps of production and storage. Shipping can expose the protein product to additional freeze-thawing stresses. Freeze/thaw cycles may compromise protein stability [36–38] and efficacy.

4N-IFN was shown to be stable when exposed to 10 consecutive cycles of freezing and thawing but subsequent treatment cycles contributed to a significant reduction in its stability (28).

To evaluate the impact of freeze/thawing on the protein stability of 4N-IFN de-immunized variants, the proteins were exposed to 15 cycles of freezing at −70 °C and thawing in a water bath at 37 °C.

As shown in Fig. 4, and in agreement with previous reports, the original 4N-IFN retained only 26% of residual antiviral activity when exposed to these experimental conditions. In contrast, the modified variant 4N-IFN(VAR1) showed full biological potency and 4N-IFN(VAR3) retained about 70% of residual activity after this freeze-thawing treatment. It is noteworthy that parental IFN completely lost its activity at the end of treatment. These results add to the previous observations of increased *in vitro* protein stability for the de-immunized IFN versions.

3.5. 4N-IFN de-immunized variants are more stable at acid pH than 4N-IFN

Short-term resistance of human type I interferons to acid pH was demonstrated decades ago [39]. However, non-glycosylated IFN does lose about half of its initial antiviral activity when incubated in a solution of 0.1 M glycine, pH 2 for 15 min. In contrast, 4N-IFN retained full bioactivity in such stress conditions [28].

To investigate whether 4N-IFN de-immunized versions retained this favorable feature, we mimicked the experimental conditions by exposing 4N-IFN variants to low pH for 2 h. As expected 4N-IFN retained the original bioactivity for 30 min of incubation and showed a gradual loss of antiviral activity until reaching almost complete inactivation after 2 h of treatment (Fig. 5). A more pronounced decrease in biological activity was observed for parental IFN. In contrast, loss of function did not occur for 4N-IFN de-immunized variants as both proteins were stable for a longer period of time and retained full bioactivity even when treated at low pH for 2 h.

4. Discussion

Recombinant human IFN-α2b is widely used for the treatment of chronic Hepatitis B and C (1,2,56) and has also recently been proposed as a candidate therapeutic to treat infections by Zika, Dengue, Chikungunya, and SARS-CoV-2 viruses (3–6). The cytokine has also been investigated for use in combination with other agents for the treatment of COVID-19 [40].

The extensive previous and current clinical uses for rhIFN-α2b as an antiviral agent explain the many efforts underway to characterize the drug in terms of structure, stability, biological activities, and clinical therapeutic effects [29,41–43].

Due to the short half-life of rhIFN-α2b in blood circulation and because immunogenicity problems reported in the clinic, the use of IFN alpha in antiviral therapy has been fairly limited [44,45]. For this reason, new rhIFN-α2b versions that have improved stability and reduced immunogenicity are being developed [15,22,24,42,46].

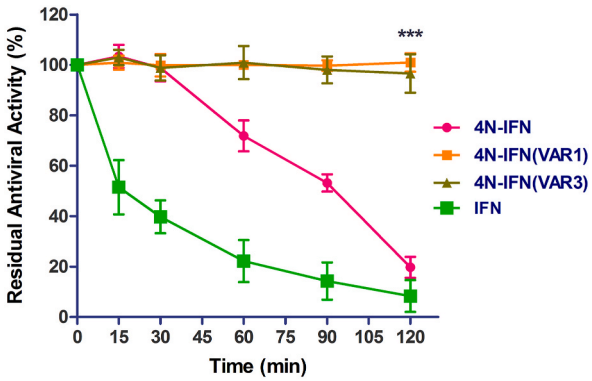

Fig. 5. De-immunized IFN versions are more stable at acid pH **treatment than the original molecule.** IFN variants were exposed at low pH at the indicated times. After each time point, we measured the antiviral biological activity as an indicator of the stability of each protein. 4N-IFN retained the initial bioactivity for 30 min of incubation and then evidenced a gradual loss of antiviral activity until almost complete inactivation after 2 h of treatment. In contrast, both 4N-IFN(VAR1) and 4N-IFN(VAR3) retained full biological function even at the end of treatment. Assays were performed in triplicate and each data point represent mean ± SD. (***$p < 0.001$).

For instance, protein glycosylation, a widespread post-translational modification, has been explored for use in novel interferon versions. Glycosylation has a marked influence on many properties of glycoproteins. Carbohydrates often improve protein solubility and decrease the level of aggregation of proteins, increase thermal stability and protect against protease attack, and may also reduce protein immunogenicity. Consequently, post-translational modification may prolong *in vivo* protein stability [47,48].

We glycoengineered a hyperglycosylated rhIFN-α2b referred to as 4N-IFN, through the attachment of *N*-glycans to the structure of this protein. Pharmacokinetic experiments showed a 25-fold increase in the elimination half-life and a 20-fold decrease in the systemic clearance rate in rats compared with the non-glycosylated molecule [22]. However, further characterization revealed the potential immunogenicity risk for this rhIFN-α2b variant [24].

For this reason, we recently reported the development of new 4N-IFN versions produced in CHO cells. These variants had significantly reduced immunogenicity, when compared with the original molecule (whether unglycosylated or pegylated), as measured *in vitro* using T cell assays and cytokine profiling [24].

During rhIFN-α2b treatment a significant number of patients developed *anti*–IFN–α2b antibodies. The impact of these antibodies is diverse and includes different potential scenarios: They may interact with the molecule but have no effect on the efficacy of the drug, or they may alter its pharmacokinetics, or in certain cases, they can neutralize its activity by preventing IFN-α from binding to its cell-surface receptor on target cells [16,17].

Here, we report the *in vivo* evaluation of protein immunogenicity, through the use of transgenic mice expressing human HLA DR molecules and depleted in murine antigen presentation molecules. These transgenic mouse models have been used previously to uncover mechanisms that drive the immune system's decision whether or not to develop neutralizing antibodies against therapeutic proteins [23,49].

In this study, we used HLA-DRB1*0101 (HLA-A2.1-/HLA-DR1-transgenic H-2 class I-/class II-knockout) mice [50] as we have recently found that this specific human MHC class II allele (among others) was involved in IFN-derived peptide presentation and further T-cell activation in human PBMC samples [24].

We used Antiviral Activity Assays (AVAs) to evaluate IFN-α biological activity neutralization after incubation with mice sera as this is the test currently recommended by the European and U.S. Pharmacopoeia to determine IFN-α potency. Based on previous data exploring the incidence of T-cell help on anti-drug antibody formation, we expected to see a higher titer of neutralizing antibodies in mice inoculated with 4N-IFN rather than for the de-immunized variants. The results obtained here confirmed our hypothesis. After peritoneal application of 4N-IFN, all mice developed a high titer of neutralizing antibodies. A significant reduction in NAb titers was observed for both 4N-IFN de-immunized variants, confirming the success of our de-immunization strategy.

Other aspects of the proposed drug also improved. Since therapeutic protein instability is a major concern during every step in product manufacturing, from host cell production to final packaging, we evaluated the impact of environmental stresses on our novel IFN alpha.

To further characterize the stability of both de-immunized 4N-IFN variants, we exposed the modified IFN-alpha proteins to severe stresses that resemble those that may occur during manufacturing, transport, or after administration to the patient. We included treatments such as heating, freezing/thawing cycles, and acid pH. We also assayed the *in vitro* stability against human plasma protease inactivation. The residual antiviral activity was then monitored as a measure of protein integrity/stability after stress treatment. Improved protein stability was observed for both of the de-immunized variants when heated to 75 °C, suggesting that point mutations introduced to reduce 4N-IFN immunogenicity also had a positive impact on protein thermal stability.

Therapeutic protein handling may expose drugs to several freezing and thawing cycles that can eventually promote protein instability and aggregation [36,37,51]. The formation of microscopic ice crystals can result, which can be accompanied by changes in solute concentration and pH [51]. This can contribute to protein denaturation which is reflected, in the case of rhIFN-α2b, in a loss of antiviral function. Here, we showed that the mutations introduced to generate 4N-IFN(VAR1) and 4N-IFN(VAR3) were also beneficial increasing the resistance to several repetitive freezing/thawing cycles. This behavior was also observed when exposing the 4N-IFN de-immunized variants to low pH. Consequently, the specific point mutations that allowed reducing both *in vitro* and *in vivo* 4N-IFN immunogenicity also had a stabilizing effect on 4N-IFN structure/function. However, further experiments will be needed to elucidate whether these mutations induced changes in protein structure and/or glycosylation that could ultimately explain the enhanced stability observed for these proteins.

Although the de-immunization strategy has been successfully used in the development of new versions of FVIII and IFN-alpha with reduced immunogenicity [23,24], the immuno-informatics algorithms used in this study have mainly two limitations. First, although the in silico toolkit is one of the most reliable among those currently available, it exhibits approximately 80% of accuracy. This limitation was evident in HLA binding experiments in which not all EpiMatrix predictions reflected changes in peptide antigenicity [24]. Second, the number and nature of mutations proposed by EpiMatrix were restricted because some changes proposed by the algorithm involved residues directly involved in cytokine structure/function [24,52]. Moreover, despite not including any changes involved in the biological function of the protein nor in the interaction of the protein with the receptor, the *in vitro* antiviral activity of 4N-IFN(VAR1) and 4N-IFN(VAR3) showed a significant reduction with respect to the original biological potency. This highlights the need for further exploration of protein characteristics, such as secondary and tertiary protein structure by circular dichroism, pharmacokinetic parameters in model animals, and glycosylation patterns, among other additional studies. In conclusion, a major achievement of this work was to demonstrate that rational protein mutations are not only useful for reducing rhIFN-α2b immunogenicity but can also improve the product's stability and resistance to different physicochemical factors that the protein may encounter during its manufacturing process, packaging, handling, and storage.

Considering the limitations associated with rhIFN-α2b therapy, such as protein instability and immunogenicity, the data shown here suggest that the two 4N-IFN de-immunized variants are valuable therapeutic candidates for the treatment of chronic and

emerging viral diseases.

Further clinical trials will be needed to validate the *in vitro* antiviral effectiveness and safety of these new rhIFN-α2b versions in humans.

Author contribution statement

Eduardo Federico Mufarrege: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Anne De Groot: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Marina Etcheverrigaray, William Martin: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Lucía Carolina Peña: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement

Dr Eduardo Mufarrege was supported by Agencia Nacional de Promoción Científica y Tecnológica [PICT-2014-2343].

Data availability statement

Data included in article/supplementary material/referenced in article.

Additional information

No additional information is available for this paper.

Declaration of comepting interest

The authors declare the following conflict of interests: Author name; [Two of the contributing authors, Anne S. De Groot and William Martin are senior officers and shareholders at EpiVax, Inc., a privately owned biotechnology company located in Providence, RI. These authors acknowledge that there is a potential conflict of interest related to its relationship with EpiVax and attest that the work contained in this research report is free of any bias that might be associated with the commercial goals of the company.]

Acknowledgments

We acknowledge Institut Pasteur as the supplier of the mutant humanized mice. ME and EFM are members of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). LCP is a fellow of CONICET.

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