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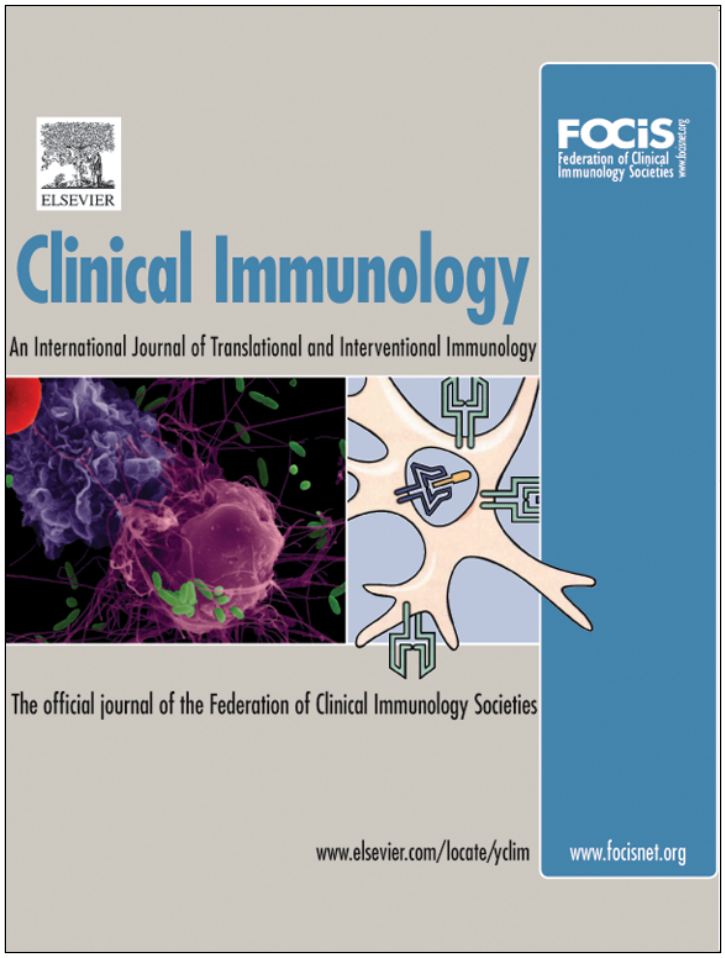

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De-immunized and Functional Therapeutic (DeFT) versions of a long lasting recombinant alpha interferon for antiviral therapy

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ABSTRACT

Interferon α (IFN-α) exerts potent antiviral, immunomodulatory, and antiproliferative activity and have proven clinical utility in chronic hepatitis B and C virus infections. However, repeated IFN-α administration induces neutralizing antibodies (NAb) against the therapeutic in a significant number of patients. Associations between IFN-α immunogenicity and loss of efficacy have been described.

So as to improve the *in vivo* biological efficacy of IFN-α, a long lasting hyperglycosylated protein (4N-IFN) derived from IFN-α2b wild type (WT-IFN) was developed. However, *in silico* analysis performed using established *in silico* methods revealed that 4N-IFN had more T cell epitopes than WT-IFN. In order to develop a safer and more efficient IFN therapy, we applied the DeFT (De-immunization of Functional Therapeutics) approach to producing functional, de-immunized versions of 4N-IFN.

Using the OptiMatrix *in silico* tool in ISPRI, the 4N-IFN sequence was modified to reduce HLA binding potential of specific T cell epitopes. Following verification of predictions by HLA binding assays, eight modifications were selected and integrated in three variants: 4N-IFN(VAR1), (VAR2) and (VAR3). Two of the three variants (VAR1 and VAR3) retained anti-viral function and demonstrated reduced T-cell immunogenicity in terms of T-cell proliferation and Th1 and Th2 cytokine levels, when compared to controls (commercial NG-IFN (non-glycosylated), PEG-IFN, WT-IFN and 4N-IFN).

It was previously demonstrated that *N*-glycosylation improved IFN-α pharmacokinetic properties. Here, we further reduce immunogenicity as measured *in vitro* using T cell assays and cytokine profiling by modifying the T cell epitope content of a protein (de-immunizing). Taking into consideration the present results and previously reported immunogenicity data for commercial IFN-α2b variants, 4N-IFN(VAR1) and 4N-IFN-4N(VAR3) appear to be promising candidates for improved IFN-α therapy of HCV and HBV.

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1. Introduction

Human alpha interferons (hIFN-α) are a multigene family of inducible cytokines that exert a variety of biological actions. Three allelic variants, hIFN-α2a, hIFN-α2b and hIFN-α2c were described, but only the first ones are used in clinical settings [1]. Recombinant human interferon alpha (rhIFN-α) is widely used for the treatment of viral diseases such as chronic Hepatitis B and C (CHC), AIDS-related Kaposi's sarcoma and several types of cancer [2,3].

Although rhIFN-α is a 'self' derived protein therapeutic, anti-IFN-α immune responses have been reported in treated patients. For instance, in treatment with rhIFN-α2a in patients with malignant midgut carcinoid tumors, 41% of patients developed anti- IFN-α antibodies [4]. Moreover 32–42% of patients with metastatic renal cell carcinoma (RCC) treated intramuscularly developed neutralizing antibodies to IFN-α (NAB) [5–7].

Prolonged half-life is one of the most prominent features of pegylated IFN-α. However, patients with chronic hepatitis C who developed anti-IFN-α antibodies during pegylated IFN-α treatment failed the treatment [8,9]. Moreover, it was reported that PEG-IFN-α preparations are immunogenic when administered to CHC patients who previously received conventional IFN-α therapy [10].

Given the clinical data from IFN-α therapy, it is not surprising that a number of attempts to predict and mitigate its immunogenicity in preclinical studies have been carried out. In this context, T cell epitopes recognized by therapy-induced human anti-interferon-α neutralizing antibodies have been identified [11,12]. In addition, a strategy to reduce IFN-α immunogenicity was carried out by Jones and collaborators. The authors identified, using a human T cell proliferation assay and peptides spanning the IFN-α2b sequence, three potentially immunogenic regions and corresponding mutations that remove the T-cell epitopes.

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However, they were not able to test the modified whole proteins in their T cell assays because IFN-α itself has antiproliferative properties [13].

An additional inconvenience of unmodified IFN-α2 monotherapy is the frequency of administration. In general, it requires daily or thrice weekly injections over the course of treatment due to the rapid clearance of the cytokine from blood [14]. To improve bioavailability, a hyperglycosylated derivative of the cytokine (4N-IFN) was developed, through the introduction of four potential *N*-glycosylation consensus sequences by site-directed mutagenesis [15]. The strategy was based on the knowledge that glycans can prolong the circulation half-lives of proteins, which may lead to a reduced dosing frequency and consequently decrease side effects in patients. Although the hyperglycosylated protein was found to have a 25-fold longer plasma half-life than non-glycosylated IFN, which is even greater than the commercial pegylated derivative Intron-A PEG, the potential immunogenicity of the molecule was not assessed.

Therefore, we performed an *in silico* immunogenicity screen followed by a stepwise de-immunization process of 4N-IFN. The strategy was based on the concept that T cell epitope modifications can be identified to reduce binding of T cell epitopes to MHCII, while preserving protein function. This process is called de-immunization for functional therapeutics or "DeFT" [16].

An *in silico* analysis of 4N-IFN using EpiMatrix and ClustiMer [17] revealed that the hyperglycosylated protein had more T cell epitopes than the original sequence. Further analysis revealed promiscuous T cell epitopes that could be modified using the OptiMatrix program, to reduce MHC binding across multiple HLA alleles, as originally described in Moise et al. [16].

To validate the computational predictions, peptides representing the original predicted immunogenic regions (ORG) and their defined modifications (MOD) were chemically synthesized. HLA binding assays for eight prevalent HLA class II alleles were performed to screen the modified peptides and confirm reduced binding to MHC class II as predicted. Eight modifications were selected for further studies and incorporation into three "de-immunized" variants (designated as 4N-IFN(VAR1–3)). The originator and variants were produced in CHO cells. Once tested for anti-viral activity, two of the variants were found to be still functional; these were analyzed in T-cell proliferation assays with Th1 and Th2 cytokine profiling. Markedly reduced immunogenicity was observed for both de-immunized versions when compared with the original molecule, with a commercially available non-glycosylated IFN-α and with a pegylated variant of the cytokine.

Additionally, an isoelectric focusing assay showed a higher degree of glycosylation for one of the de-immunized proteins (4N-IFN(VAR3)) in comparison to the original molecule. This feature is usually correlated with benefits in stability, protease resistance and solubility. Taking these results altogether, the 4N-IFN de-immunized variants developed in this study appear to be promising candidates for antiviral therapy in patients.

2. Materials and methods

A stepwise approach followed in this study is illustrated in Fig. 1.

3. De-immunization tools

To de-immunize 4N-IFN, the strategy described in Moise et al. [16] was carried out. Briefly, the sequence of human 4N-IFN was parsed into overlapping 9-mer frames and the immunogenic potential of each frame was assessed against a panel of eight HLA class II supertype alleles (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301 and DRB1*1501) that represent >90% of MHC diversity in the human population [18]. A "Z" score for each 9-mer is assigned by EpiMatrix. Any 9-mer scoring above 1.64 on the EpiMatrix "Z" scale (approximately the top 5% of the random peptide set) has a significant chance of binding to the MHC II molecule and is designated as a "hit". 9-mers scoring above 2.32 on the scale (the top 1%) are extremely likely to bind. In general most published T cell epitopes fall within this category. These EpiMatrix results were then screened for the presence of concentrated regions of MHC binding potential using ClustiMer. Regions of high immunogenic potential, defined as having a score above 10 (including multiple 'hits' against many different HLA DR), were identified, and the corresponding "original" (ORG) peptides were synthesized [19].

To identify amino acids within the ORG sequences that were suitable for modification, we evaluated the contribution of each amino acid in these regions to HLA binding using OptiMatrix (part of the EpiVax ISPRI toolkit for de-immunization). OptiMatrix begins with looking at "critical" residues, which contribute most to MHC binding affinity across multiple 9-mer frames and multiple HLA alleles. The program then iteratively substitutes all 19 alternative amino acids in any given position of a protein sequence (with operator-defined input that may limit the list to naturally conserved variants) and then re-analyzes the predicted immunogenicity of the sequence, following that change. To avoid a negative effect on protein structure and consequently in biological activity a comprehensive search in literature for critical residues was also conducted, which identified amino acids that were not candidates for modification. The predicted disruptive impact on the overall immunogenic potential of the modified (MOD) peptides was verified by confirming that the modifications reduced binding to HLA in HLA binding assays.

3.1. Synthetic peptides

ORG and MOD peptides corresponding to the predicted epitopes were synthesized (New England Peptide, Gardner, MA, USA) by 9-fluoronylmethoxy-carbonyl (Fmoc) method on an automated Rainen Symphony/Protein Technologies synthesizer. The peptides were purified to 85% or higher by reversed phase HPLC.

3.2. HLA binding assay

Class II HLA binding assays were used to estimate the affinity of predicted epitope sequences for multiple HLA alleles and their de-immunized variants. We performed a competition-based HLA binding assay that was initially described by Steere and collaborators [20]. In 96-well plates, non-biotinylated test peptides at five different concentrations (100 μM, 50 μM, 10 μM, 1 μM and 0.1 μM) competed for binding to purified Class II molecules (50 nM) against a biotinylated standard peptide at a fixed concentration (0.1 μM) for 24 h at 37 °C to reach equilibrium. Class II complexes were then captured on ELISA plates using pan anti-Class II antibodies (L243, anti-HLA-DR). Plates were washed and incubated with Europium-labeled Streptavidin for 1 h at room temperature. The Europium activation buffer was added to develop the plates for 15–20 min at room temperature before they were read on a Time Resolved Fluorescence (TRF) plate reader. All assays were performed in triplicate. Binding assays were performed for 8 alleles: DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301 and DRB1*1501, which provided a broad representation of class II HLA allele binding pockets [18].

3.3. Gene expression in mammalian cells

3.3.1. Cell culture

Cell culture Chinese hamster ovary (CHO·K1) cells were grown in basal culture medium previously described [21] 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).

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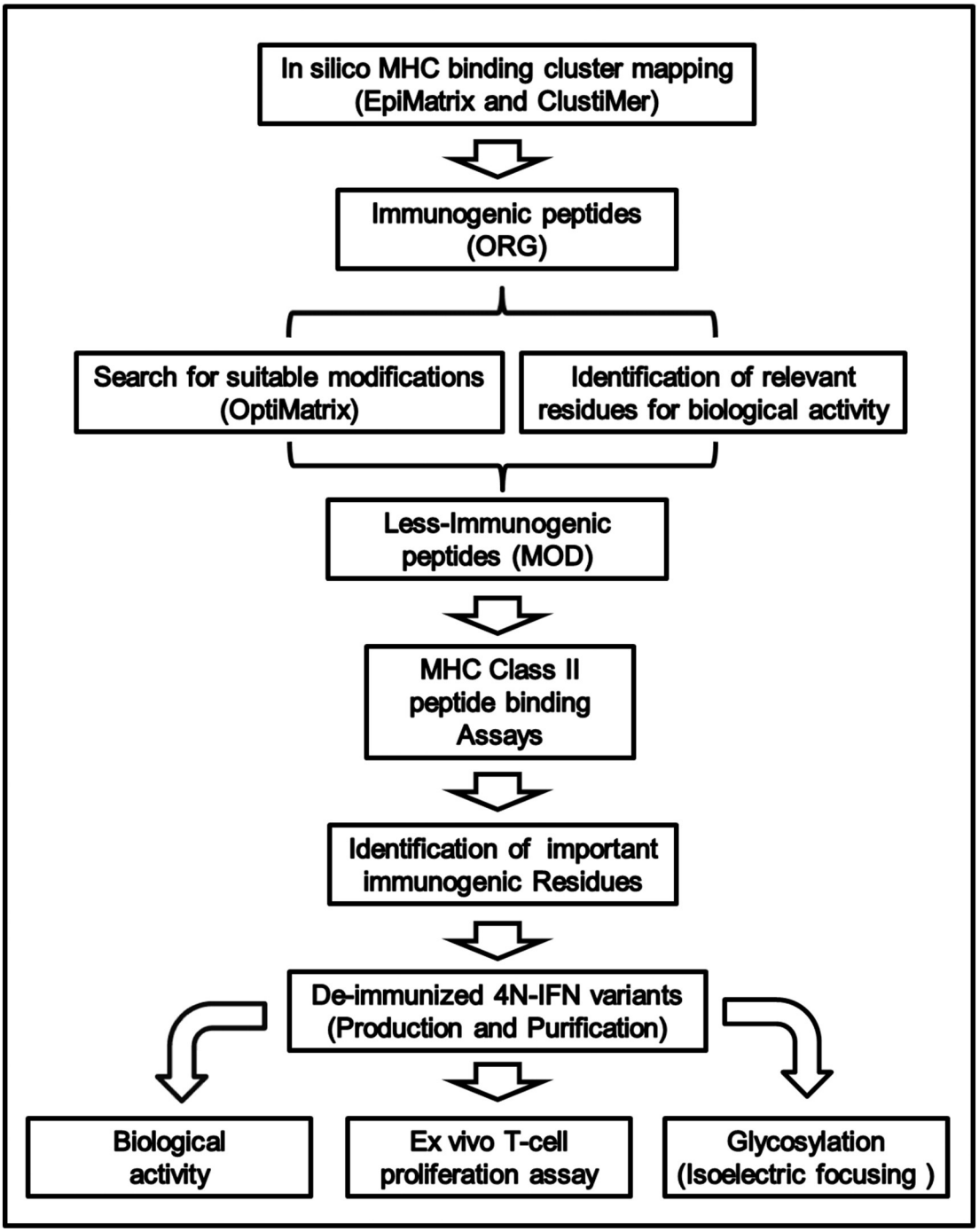

Fig. 1. Flow diagram illustrating the main steps in this study.

3.3.2. Production and purification of IFN-α variants

In order to produce large amount of protein for further analysis, a third-generation lentiviral strategy was carried out which included the packaging construct (pMDLg/pRRE), VSV-G expressing construct (pMD.G) and the Rev. expressing construct (pRSV-Rev) (Addgene, USA; Plasmid numbers #12251, #12259, #12253, respectively) [22,23].

3.3.3. Vector construction

The 4N-IFN de-immunized variants were synthetically generated by GeneArt™ (USA) and cloned into a self-inactivating (SIN) promoter-containing expression vector. Sequences of all constructions were verified by DNA sequencing.

3.3.4. LV particles production and titration

Research grade HIV-based lentiviral particles containing IFN variants transgenes were manufactured following the protocol suggested by Naldini et al. [23] and Dull et al. [22]. Following a lipofection protocol, adherent HEK293T cells (1.2×10^7 cells per well) were simultaneously co-transfected with four plasmids: the packaging construct (pMDLg/pRRE) [22], the VSV-G-expressing construct (pMD.G) [23], the Rev.-expressing construct (pRSV-Rev) [22], and the self-inactivating (SIN) promoter-containing LV. Lentiviral particles (LVPs) containing supernatant were collected 72 h after transfection. LVPs were titered using the QuickTiter™ Lentivirus Titer Kit (Lentivirus-Associated HIV p24) following instructions suggested by the manufacturer (Cell Biolabs Inc.,

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USA) and the titer (TU ml^{-1}) was determined. The titer was used to calculate the necessary supernatant dilutions to transduce cells at the same multiplicity of infection (MOI).

3.3.5. Stable expression (lentiviral transduction)

Transductions were carried out by incubating 6.0×10^4 cells per well seeded onto 6-well plates (Greiner) with LVPs at a final MOI of sixty. Twenty-four hours post-transduction, media were replaced with fresh media. In order to eliminate the remaining wild type cells, 96 h post-transduction supernatants were replaced by fresh growth medium containing 10 $\mu g \cdot ml^{-1}$ puromycine (Sigma Aldrich, USA). Selective medium was changed every 3–4 days until control cell death. Transduced cells were expanded for IFN-α production. 4N-IFN producing cell lines were cloned by the limit dilution method [24]. The productivity of individual clones was evaluated by determination of expression levels and cell counting. The highest expressing clones of each 4N-IFN variant were cultivated for large-scale production. Cells were grown until confluence in 500 cm^2 triple flasks using growth medium. The medium was then changed to basal medium supplemented with 0.5% (v/v) FCS (production medium). Every 48 or 72 h, conditioned medium was harvested and replaced with fresh production medium. Harvests were clarified by centrifugation and stored at −20 °C. Protein was purified by immunoaffinity chromatography following a strategy previously described [15]. The concentration of purified 4N-IFN variant was determined by reverse-phase HPLC, using the non-glycosylated cytokine as standard and confirmed by spectrophotometric quantification.

3.3.6. Human PBMC HLA-DR typing

All blood extraction and handling procedures were approved by the Universidad Nacional del Litoral Research Ethics Committee (Santa Fe, AR). Blood was obtained from healthy donors by venipuncture after obtaining informed consent. PBMCs were isolated by Ficoll-Paque™ PLUS (GE Healthcare Bio-Science, SE) density-gradient separation according to manufacturer's instructions, and the buffy coat was collected and washed twice with PBS. PBMCs were cryopreserved in liquid nitrogen at a concentration of $1–3 \times 10^7$ cells/ml. Previously, an aliquot of blood was separated and HLA-DR allotypes were determined by Luminex Sequencing Technology (PRICAI, Buenos Aires, AR). Typing results were compared to publicly available HLA-DR frequencies in the world population: (www.allelefrequencies.net).

3.3.7. Ex vivo T-cell assays

For *ex vivo* T-cell assays a strategy suggested by Jaber and Baker [25] with modifications was performed. Monocytes were isolated from PBMCs from each donor blood sample by differential adherence to plastic [26]. The adherent cells were retained for differentiation and the non-adherent cells were collected and cryopreserved for further use. To induce an immature phenotype of monocyte-derived DC, monocytes were incubated in growth medium containing 1000 U/ml each of human IL-4 (Milipore, USA) and granulocyte macrophage colony-stimulating factor (GM-CSF, GemaBiotech, AR) for 6 days with a change of media at day 3. On day 6, immature dendritic cells were collected, counted and incubated with IFN-α variants or non-antigen (medium or excipients). Test antigens that were included in this study were: non-glycosylated IFN (NG-IFN), wild type IFN produced in CHO cells (WT-IFN), pegylated IFN (PEG-IFN), 4N-IFN and its de-immunized variants. After an overnight incubation, DC were washed to remove exogenous antigen and resuspended in growth medium containing recombinant human tumor necrosis factor (rhTNF, ProsPec, USA) alpha, GM-CSF and IL-4 for 4 days to induce DC maturation. Ag pulsed-DCs were then incubated with autologous cells for 48 h in medium containing 2 ng/ml human IL-2 (Thermo, USA). Supernatants were collected and evaluated for IFN-γ and IL-4 quantification by sandwich ELISA. Negative controls (medium or excipients), and positive controls (phytohemagglutinin, Sigma Aldrich, USA) were also included.

3.3.8. IFN-γ and IL-4 sandwich ELISA

96-well plates were coated with 100 μl primary IFN-γ mAb (clone 2G1) (Thermo, USA), or primary IL-4 mAb (clone E10023, Endogen, USA) at a concentration of 1.5 μg/ml, first for 1 h at 37 °C and then overnight at 4 °C. After blocking 1 h at 37 °C with 1% (w/v) BSA in phosphate-buffered saline (PBS), culture supernatants were added and incubated for 1 h at 37 °C. Serial 1:2 dilutions of rhIFN-γ or rhIL-4 (Thermo, USA) from 1 ng/ml were also included. Then, 100 μl/well of IFN-γ pAb (Catalog Number P700, Thermo, USA) 150 ng/ml or biotinylated IL-4 secondary mAb (clone E10021, Endogen, USA) at a concentration of 300 ng/ml was added to the plates and incubated for 1 h at 37 °C. Then, plates were incubated with a peroxidase-labeled goat anti-rabbit immunoglobulins (DAKO, Denmark) diluted 1:1000 or Streptavidin horseradish peroxidase conjugate (RPN4401-AMDEX, USA) diluted 1:5000. After 1 h, plates were incubated with substrate solution (0.5 mg ml^{-1} o-phenylenediamine, 0.015% (v/v) H_2O_2 in 50 mM phosphate citrate buffer). Reactions were stopped by the addition of 2 N H_2SO_4 and the absorbance was measured at 492 nm with a microtiter plate reader Labsystems Multiskan MCC/340, Finland). Between every step, plates were washed with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Dilutions were prepared in PBS-T containing 0.1% (w/v) BSA. The assay was performed in triplicate.

A similar acceptance criterion to that suggested by Wullner et al. [51] to define a positive response for a given donor was used. The Stimulation Index (SI) was defined as a ratio of the cytokine concentration from protein challenged samples divided by cytokine concentration from excipient treated samples. A geometric mean (GM) of the SI was then calculated and a positive donor was defined when SI > GM.

3.3.9. IFN-α sandwich ELISA

rhIFN variants yields from culture supernatants were quantified by a specific sandwich ELISA assay previously described by Ceaglio and collaborators [15].

3.3.10. Antiviral assay

The biological antiviral activity of rhIFN-a2b was determined by its ability to inhibit the cytopathic effect caused by vesicular stomatitits virus (VSV, Indiana strain) on MDBK cells [27,28]. To evaluate the impact of modifications on the anti-viral activity of the IFN-α variants, MDBK cells were seeded into culture microtiter plates in growth medium (2.5×10^4 cells per well) and incubated at 37 °C overnight. After removing culture supernatants, we added 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 test samples in assay medium. The plates were then incubated for 6 h at 37 °C, and after removal of supernatants, the monolayers were infected with 1.6 PFU of VSV virus per cell. Viral replication was allowed to proceed until the cytopathic effect was clearly observable in control wells (no IFN-α). The medium was discarded and cells were fixed and stained simultaneously with a solution of 0.75% (w/v) crystal violet in 40% (v/v) methanol. After 10 min, plates were washed with water and the remaining dye was solubilized in 20% (v/v) acetic acid. The plates were read at 540 nm with a microtiter plate reader and the signal intensity of each dilution was reported as the mean of the absorbance measured in five wells.

3.3.11. SDS-PAGE and Western blotting

SDS-PAGE analysis was performed according to the standard method using 15% (w/v) polyacrylamide resolving gels and 5% (w/v) stacking gels. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (BioRad). Blots were blocked for 1 h with 5% (w/v) non-fat milk in Tris-buffered saline (TBS) and then probed with rabbit anti-rhIFN- a2b polyclonal antibodies. After 1 h, blots were incubated with the same peroxidase-conjugated described in the ELISA. Immunoreactive bands were visualized using an ECL™ Chemiluminescent

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Western Blotting Analysis System (GE Healthcare). Washes between steps were performed with TBS containing 0.05% (v/v) Tween 20 (TBS-T). Dilutions were prepared in TBS-T containing 0.5% (w/v) non-fat milk.

3.3.12. Isoelectric focusing (IEF)

IEF was performed in 1 mm thick 8% (w/v) polyacrylamide gels containing 7 M urea, 20% (w/v) 4.0–6.5 ampholytes and 80% (w/v) 2.5–5 ampholytes. The gel was prefocused at 10 W, 2000 V and 100 mA for 1 h. Then, 5–20 ml samples were applied at 1 cm from cathode and electrophoresis was carried out using the same conditions as the prefocusing step for 30 min. The IEF-separated components were transferred to PVDF membranes and Western blotting proceeded as described above.

3.3.13. Statistical analysis

Differences between treatments were evaluated through a one-way analysis of variance (ANOVA). When the ANOVA produced significant differences ($p < 0.05$), a post-hoc Tukey's multiple comparison test was applied.

4. Results

4.1. Immunogenicity prediction and experimental validation

In silico assessment for potential immunogenicity using EpiMatrix revealed that 4N-IFN had more T cell epitopes per unit amino acid than the unmodified protein (WT-IFN), suggesting the generation of new epitopes and/or the immunogenicity enhancement of those previously existent ones, as a consequence of the incorporation of *N*-glycosylation sites (Fig. 2A). Further analysis using the EpiMatrix and ClustiMer algorithms allowed us to identify regions containing concentrated putative 9-mer MHC binding known as clusters in the 4N-IFN sequence. As shown in Fig. 2B, regions of putative MHC binding were located at the following positions: 1–19, 11–32, 41–62, 57–78, 114–141 and 140–165. Five out of six predicted MHC binding clusters overlapped with T cell epitopes previously defined [13].

We then identified modifications that disrupted or reduced MHC II binding affinity using OptiMatrix. Among the changes suggested by OptiMatrix, we selected those ones that were not identified as critical for biological activity or receptor binding (Table 1). In order to reduce perturbations to protein structure, mutations were made by substituting alanine (except for one modification that was made to tryptophan).

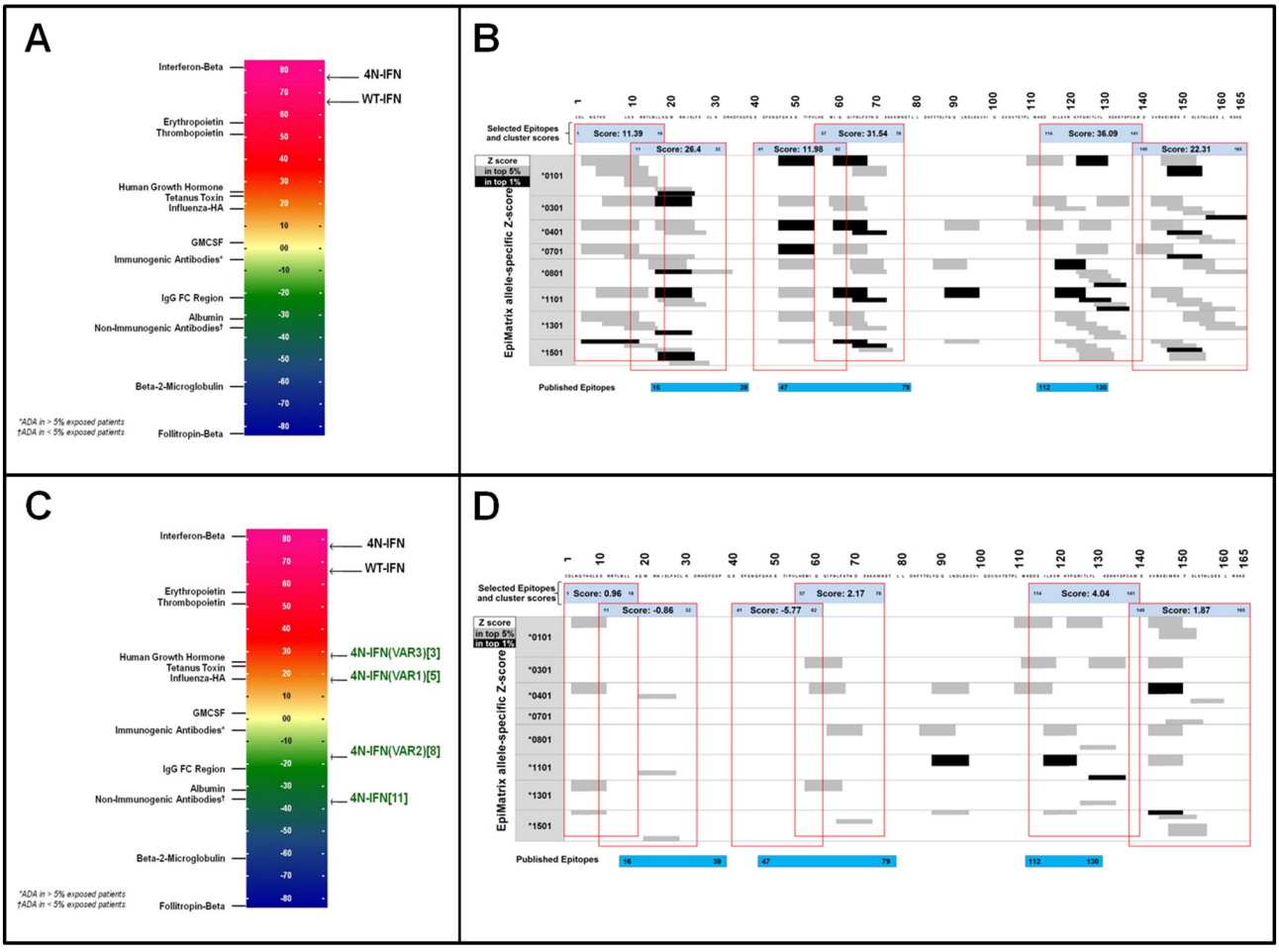

Fig. 2. *In silico* immunogenicity analysis of 4 N–IFN. A) EpiMatrix MHC binding cluster immunogenicity scale. Peptides are mapped onto a cluster immunogenicity scale according to their individual EpiMatrix scores. The EpiMatrix cluster immunogenicity score represents the deviation in putative epitope content from baseline expectation based on a random peptide standard. MHC binding clusters scoring above +10 are considered to be potentially immunogenic. Some positive control peptides and proteins are also arranged by EpiMatrix score, from highest (red) to lowest (blue). B) MHC Class II binding cluster map of 4N-IFN as predicted by EpiMatrix. EpiMatrix-predicted 9-mer hits for 8 prevalent HLA class II alleles are aligned along the 4N-IFN sequence. Any peptide scoring above 1.64 on the EpiMatrix "Z" scale (top 5%) is considered to be a potential epitope (gray bars). Peptides scoring above 2.32 on the scale (top 1%) are extremely likely to bind MHC (black bars). Clusters identified by EpiMatrix with the respective scores indicated above are framed in red. Published epitopes (blue bars) determined by experimental methods overlapped with those defined here. C) Location of and D) Impact of selected mutations on the overall immunogenic potential of 4N-IFN. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Table 1
Critical residues for IFN-α structure/biological function previously identified and reported in the literature.

Function	Residues	Reference
Biological activity	Leu30, Lys31, Arg33, His34, Phe36, Arg120, Lys121, Gln124, Tyr122, Tyr129, Lys131, and Glu132	Mitsui et al., 1993 Uzé et al., 1995 Radhakrishnan et al., 1996
	Arg144	Senda et al., 1995
	Glu146	Korn et., 1994
	Arg22, Leu26 and Phe27	Weber et al., 1987
	Ser68, Thr79, and Tyr85	Rehberg et. Al, 1982
	Functional hot-spot residues (fully conserved) Leu30, Arg33, Arg144, Ala145, Met148, and Arg149	Piehler et al., 2000; Roisman et al., 2001
Presumably important for protein structural integrity	Phe36, Tyr122 and Tyr129	Radhakrishnan et al., 1996
Interaction with IFN-αR1	AB loop (Arg22, Leu26, Phe27, Leu30, Lys31, Arg33,	Uzé et al., 1994
	His34), the bend in helix B (Ser68) and helices C (Thr79, Lys83, Tyr85, and Tyr89), D (Arg120, Lys121, Gln124, Lys131, and Glu132) and E (Arg144, and Glu146) and Arg149	Radhakrishnan et al., 1996
Interaction with IFN-αR2	Leu15, Leu26, Phe27, Leu30, Lys31, Asp32, Arg33, His34; Asp35, Lys133, Arg144, Ala145, Met148, Arg149, Ser152, Leu153, Arg12	Piehler et al., 2000 Roisman et al., 2001
Dimerization	Arg33, His34, Asp35, Gly38, Gln41, Glu42, Glu 43, Gly45, Arg114, Ser115, Ala118, Lys121, Arg125 and Glu132	Radhakrishnan et al., 1996
Stabilization of the conformation of the AB loop	(Cys29–Cys138, Phe36, Tyr122, Arg125 and Tyr129	Radhakrishnan et al., 1996
Cys involved in disulfide bond	Cys29 with Cys138 and Cys1 with Cys98	Radhakrishnan et al., 1996
Zinc-binding site	Glu41 and Glu42	Radhakrishnan et al., 1996

A total of eleven mutations were identified and their impact on potential immunogenicity is shown Fig. 2C and D. For each original (ORG) cluster one to three modifications (MOD) were designed, these sequence are described in greater detail in Table 2.

To validate the computational predictions, HLA Class II binding assays were performed for the DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301 and DRB1*1501 alleles at five different peptide concentrations. IC_{50} values for each peptide and HLA allele were calculated. IC_{50} values and the corresponding EpiMatrix Z score are shown in Supplemental material (Fig. 1). To identify those modifications that effectively produced a relevant impact on HLA binding, we plotted the IC_{50} average for each peptide (ORG and MOD) across all alleles (IC_{50} = 100 μM were adopted for non-binders for calculation purposes). As shown in Fig. 3, L9A, F47A, L117A, F123A and L128A mutations in peptides 1a (ORG), 2 (ORG) and 4–5 (ORG) reduced HLA binding affinity and were considered to produce 4N-IFN(VAR1). Discrepancies between predictions and binding assays may be attributable to peptide aggregation or folding that interferes in the binding assay or to less than accurate predictions (EpiMatrix is 85% accurate, as described in previous publications). Even though L18A, I60A and F64A did not disrupt peptide:HLA interactions in binding assays, these substitutions were still considered for synthesis, because they showed a marked reduction in Z score according to OptiMatrix. A total of eight mutations were introduced into the 4N-IFN sequence to develop 4N-IFN(VAR2). In addition, we decided to produce an additional variant carrying a lower number of mutations so as to reduce the potential for compromising IFN-α function. Therefore, we designed 4N-IFN(VAR3) carrying only three mutations that were predicted to have the highest impact on HLA binding disruption according to binding assays (L9A, F47A) and EpiMatrix predictions (L18A). 4N-IFN variant sequences are shown in Fig. 2 (Supplemental material) and their EpiMatrix scores are shown in Fig. 2C.

Table 2
Original (ORG) and modified (MOD) 4N-IFN peptides sequences. Highlighted residues are considered essential for HLA binding and consequently optimal for substitution. For each original cluster one to three modifications were introduced.

Peptide ID	Sequence
1a (ORG)	C D L P Q T H S L G S R R T L M L L A
1a (MOD)	C D L N Q T H S **A** G S R R T L M L L A
1b1 (ORG)	S R R T L M L L A Q M R N I S L F S C L K D
1b1 (MOD1)	S R R T L M L **A** A Q M R N I S L F S C L K D
1b1 (MOD2)	S R R T L M **A A** A Q M R N I S L F S C L K D
2 (ORG)	E E F G N Q F Q K A E T I P V L H E M I Q
2 (MOD)	E E F G N Q **A** Q K A E T I P V L H E M I Q
3 (ORG)	L H E M I Q Q I F N L F S T N D S S A A W N
3 (MOD1)	L H E M **A** Q Q I F N L F S T N D S S A A W N
3 (MOD2)	L H E M **A** Q Q I **A** N L F S T N D S S A A W N
4–5 (ORG)	E D S I L A V R K Y F Q R I T L Y L K E K K Y S P C A W
4–5 (MOD)	E D S I **A** A V R K Y **A** Q R I T **A** Y L K E K K Y S P C A W
6 (ORG)	A W E V V R A E I M R S F S L S T N L Q E S L R S K E
6 (MOD)	A W E V V R A E **A** M R S F S L S T **W** L Q E S L R S K E

4.2. 4N-IFN de-immunized variants: Production, purification and characterization

4N-IFN de-immunized variants were synthesized and cloned into a third generation lentiviral vector and then the variants were expressed in CHO cells. In a preliminary analysis, cell culture supernatants were characterized for antiviral activity. Despite all cell lines produced high levels of each IFN variant (data not shown) no residual activity was observed for 4N-IFN(VAR2), which was then eliminated from further study. It is likely that this variant was not functional due to the number of modifications that had been introduced in the sequence.

Purification of the secreted 4N-IFN variants from individual harvests was achieved in one step by immunoaffinity chromatography using a monoclonal antibody (CA5E6). CA5E6 mAb binds with high affinity to both 4N-IFN variants (a representative chromatogram is shown in Supplemental material, Fig. 3) and was adsorbed on CNBr-activated Sepharose as ligand for immunoaffinity chromatography purification. Supernatants containing different IFN variants were loaded onto the matrix, without exceeding 40% of its theoretical capacity. No loss of the cytokine was observed during washing steps, which were useful to remove a high content of contaminants. The achieved purity level was above 95% as judged by RP-HPLC-C4 chromatography (data not shown).

As shown in Fig. 4A, a comparison of electrophoretic migration by SDS-PAGE showed similar patterns for both 4N-IFN and 4N-IFN(VAR1). However, although no modifications were introduced into N-glycosylation sites, 4N-IFN(VAR3) demonstrated a marked electrophoretic retardation in comparison to the original protein. The increased size of this variant may be attributed to the presence of multiple N-linked glycans as a consequence of more efficiently occupied consensus sites.

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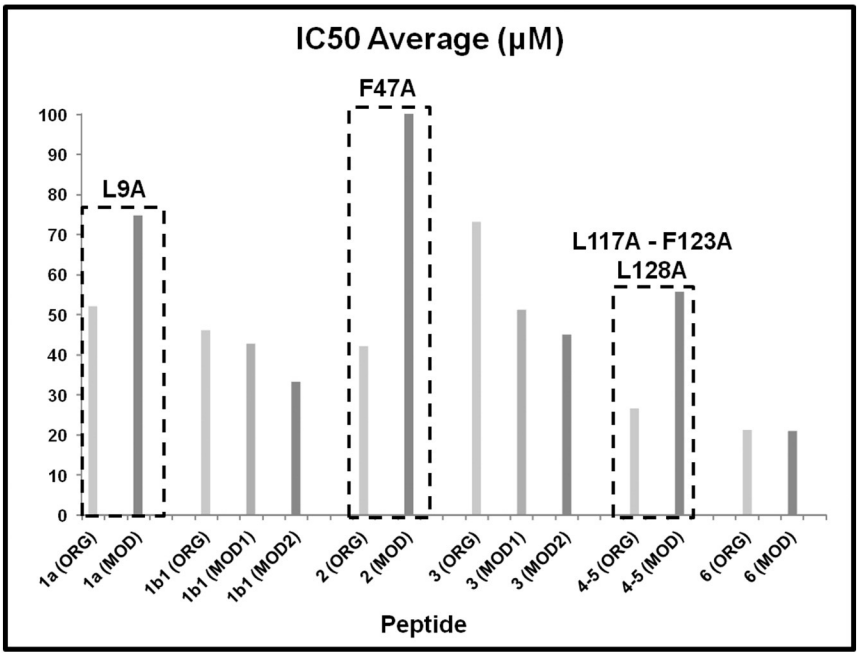

Fig. 3. Experimental validation of 4N-IFN immunogenicity predictions. IC_{50} average for each peptide (ORG and MOD) across eight relevant alleles that represent >90% of MHC diversity in the human population. Bars represent averages. Mutations that effectively produced an impact on the binding to HLA alleles are correlated with an increase of the bar height and are highlighted in the figure.

In order to confirm this hypothesis, the charge-based heterogeneity of the 4 N–IFN variants was analyzed by IEF (Fig. 4B). The non-glycosylated IFN-α consisted in a unique band of a pI close to the lower limit of the pH gradient (6.5) as previously reported in the literature [29]. As suggested by the SDS-PAGE result, 4N-IFN(VAR1) demonstrated a similar IEF pattern to 4N-IFN, but with a lower proportion of O-glycosylated isoforms. In contrast, 4N-IFN(VAR3) showed a markedly different pattern of bands with the majority of them concentrated in the upper part of the pH range, suggesting that 4N-IFN(VAR3) was enriched in highly sialylated isoforms.

4.3. Biological activity

In order to evaluate the impact of the modifications on IFN biological activity, an antiviral activity assay was carried out using MDBK cells as target cells for viral infection by VSV virus.

Although the immunization strategy was aimed to change the minimum number of amino acids without varying residues directly involved in biological activity (see Table 1), a reduction in antiviral activity was observed for the modified variants (Table 3). For 4N-IFN(VAR1) the residual activity was 28.4% and even lower for 4N-IFN(VAR3) (16.9%). Frequently, excessive sugar attachment might result in steric hindrances that negatively impact the cytokine-receptor interaction. Additionally, enhanced sialic acid content could also explain the observed reduction in antiviral activity.

4.4. Immunogenicity

4.4.1. Donor samples

Blood samples were taken from 26 healthy donors aged between 24 and 60 years. Previous studies showed that HLA-DRB1 are involved in antigen presentation and required for the T-cell response [30,25]; hence, donor HLA-DRB1 types were determined.

Comparison of the allotypes expressed in the cohort against those expressed in the world population revealed that coverage of nearly 85% of HLA DR types was achieved and that all major HLA-DRB1 alleles (allotypes with frequencies higher than 5% expressed in the world population) were well represented (Fig. 5).

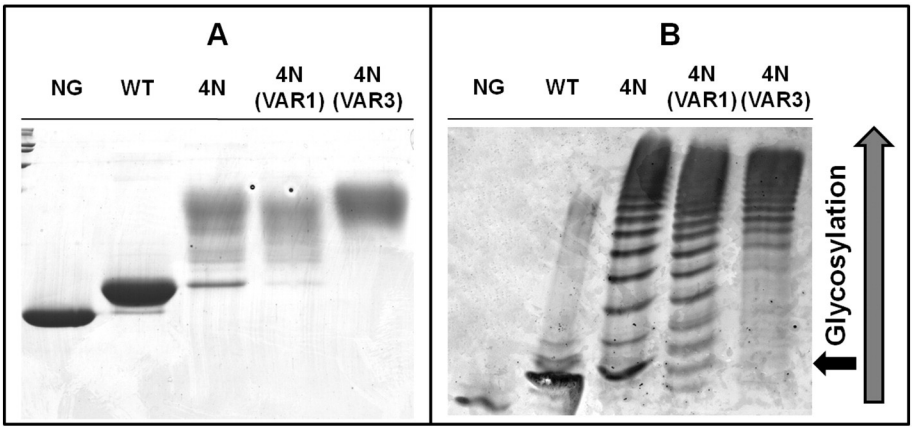

Fig. 4. Mutations introduced into 4N-IFN(VAR3) produced an enrichment in highly glycosylated isoforms in comparison with 4N-IFN. (A) Glycosylation patterns of purified IFN analogs were analyzed by denaturing PAGE. (B) The charge-based heterogeneity of the IFN variants was analyzed by IEF and differently sialylated forms were distinguished.

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Table 3
The antiviral activity of IFN-α variants was determined by their ability to inhibit the cytopathic effect caused by vesicular stomatitis virus on MDBK cells and normalized to the activity of 4N-IFN.

Protein	% Residual Activity
4N-IFN	100.0
4N-IFN(VAR1)	28.4
4N-IFN(VAR3)	16.9

4.4.2. T-cell proliferation assay

Because of antiproliferative effect of IFN-α on T-cells (especially on *naïve* T-cells) it was not possible to test T-cell proliferation by direct incubation of IFN (antigen) with PBMCs. For this, an alternative protocol, involving monocyte isolation and further differentiation into dendritic cells, was considered.

Immature DCs were loaded with Ag (IFN), matured and then incubated with monocyte-depleted autologous PBMCs. Cytokine secretion was then evaluated by sandwich ELISA and used to calculate the SI and the number of positive donors. SI for each donor and protein are shown in Supplemental material (Fig. 4).

As shown in Fig. 6A, a Th1 profile analysis showed that almost 54% of donors responded to the non-glycosylated commercial version (NG-IFN) produced in *E. coli*. Surprisingly, almost all of the donors responded to WT-IFN, despite only an additional O-glycosylation. In addition, 4N-IFN was, in this analysis, more immunogenic than the non-glycosylated cytokine (73% of positive responses). A markedly reduced immune response was detected for both 4N-IFN de-immunized variants, with 38% and 27% positive responses for 4N-IFN(VAR1) and 4N-IFN(VAR3), respectively. Relatively low immunogenicity was also observed for PEG-IFN (38%).

However, a different scenario was observed when IL-4 secretion (Th2 profile) was analyzed (Fig. 6B). NG-IFN and PEG-IFN showed similar immunogenicity (nearly 60% positive responses). In contrast with the results from the Th1 analysis, a lower frequency of donors responded to WT-IFN (50%). In addition, while low immunogenicity was detected for 4N-IFN (19%), the lowest proportion of responders was observed for both de-immunized versions (4% and 8%). Then, it is important to highlight that although a similar Th1 immunogenicity was observed for PEG-IFN and both de-immunized 4N-IFN variants, the Th2 profile was significantly higher for PEG-IFN.

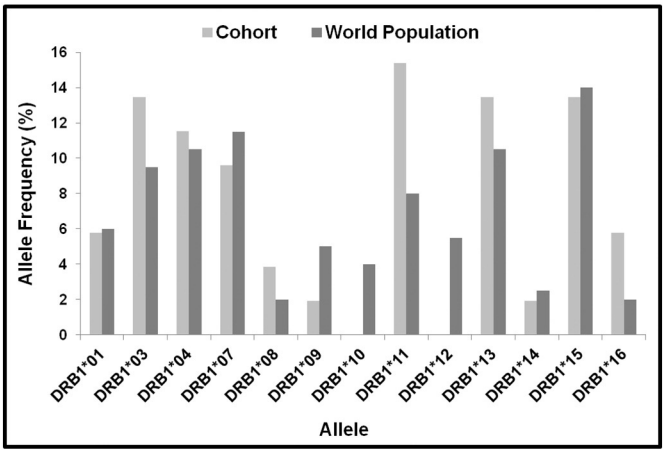

Fig. 5. The HLA-DRB1 allelic distribution of our donors is representative of the world-wide population. HLA allelic frequency in our cohort was compared with public available frequencies of HLA-DRB1 alleles in the world population (data extracted from www.allelefrequencies.net).

4.4.3. HLA-DR restriction for antigen presentation

To confirm that cytokine responses were mediated through antigen presentation in the context of HLA-DR molecules, WT-IFN-pulsed dendritic cells derived from five responsive donors were treated with an anti-DR antibody (in two different concentrations) before incubation with autologous T-cells. As shown in Fig. 7 a successive reduction of IFN-γ SI with increasing amounts of antibody was observed in all donors, demonstrating the essential role of HLA-DR in mediating T cell responses to IFN.

5. Discussion

During IFN-α treatment a significant number of patients develop anti-IFN-α antibodies. The impact of the antibodies can vary: They may bind to the molecule but have no effect on the efficacy of the drug, or they may alter its pharmacokinetics, or in certain cases they may neutralize its activity by preventing IFN-α from binding to its cell-surface receptor on target cells [31,32]. Additionally, IFN-α has been associated with an increase in certain autoimmune diseases. Initially it was suspected that impurities in the leukocyte-derived medication were driving increased expression of MHC class II molecules and subsequently inducing autoimmune thyroiditis [33,34]. However, even after leukocyte-derived IFN-α was replaced with recombinant IFN, patients continued to develop autoimmune diseases [35,36], suggesting that the drug can be pro-inflammatory. This pro-inflammatory effect may contribute to its immunogenicity in the clinic.

It is now known that anti-drug antibody formation is closely related with T-cell help [37–40]. In particular, the critical role of CD4 + T cells in anti-IFN-α antibody development was evidenced by Jones and collaborators (2004) [13]. The authors evaluated fifteen-mer peptides staggered by three amino acids and spanning the entire IFN-α2b sequence. When these regions were synthesized as single peptides and tested in time course assays both in healthy individuals and in IFN-α2b-treated HCV patient PBMCs, they were able to identify three immunogenic regions into the molecule: Region 1 (amino acids 22–39); Region 2 (amino acids 52–79) and Region 3 (amino acids 112–130). Region 3 was reported to be the most immunogenic region in terms of proliferative responses *in vitro*.

In this study, we used an *in silico* approach to identify immunodominant T-cell epitopes in 4N-IFN and to modify them so as to generate functional de-immunized variants. Several MHC binding clusters were identified in the 4N-IFN sequence using EpiMatrix and ClustiMer. Four out of five regions overlapped with the immunogenic regions previously identified in IFN-α2b, demonstrating the accuracy of the predictions (in retrospect). Also, consistent with previous experimental data, Region 5 (amino acids 114–141) was the most potentially immunogenic (Z score = 36.09). After identifying the key immunogenic regions, we used OptiMatrix to modify those amino acids that contributed the most to MHC Class II binding. We substituted alanine (one mutation to tryptophan) to reduce the potential for HLA binding, to reduce the chance of having a negative impact on protein structure and/or biological activity [30]. When predictions were put to the test in HLA binding assays, we observed a correlation between both analyses. These results are consistent with reports previously published for the EpiMatrix algorithm [16,17,41].

Based on these results we selected a total of eight mutations that were introduced into the 4N-IFN sequence in different combinations, to generate three 4N-IFN de-immunized variants in CHO cells. Although the mutations were chosen with the aim of reducing the impact on protein structure, one of the variants (designated here as 4N-IFN(VAR2)) was nonfunctional and was not included in further studies. This result is not surprising considering that mutations affected as much as 5% of the protein sequence.

We found a reduction in the *in vitro* antiviral activity for 4N-IFN(VAR1) and 4N-IFN(VAR3) in comparison with the original molecule. One reason for this may be that the modifications produced a

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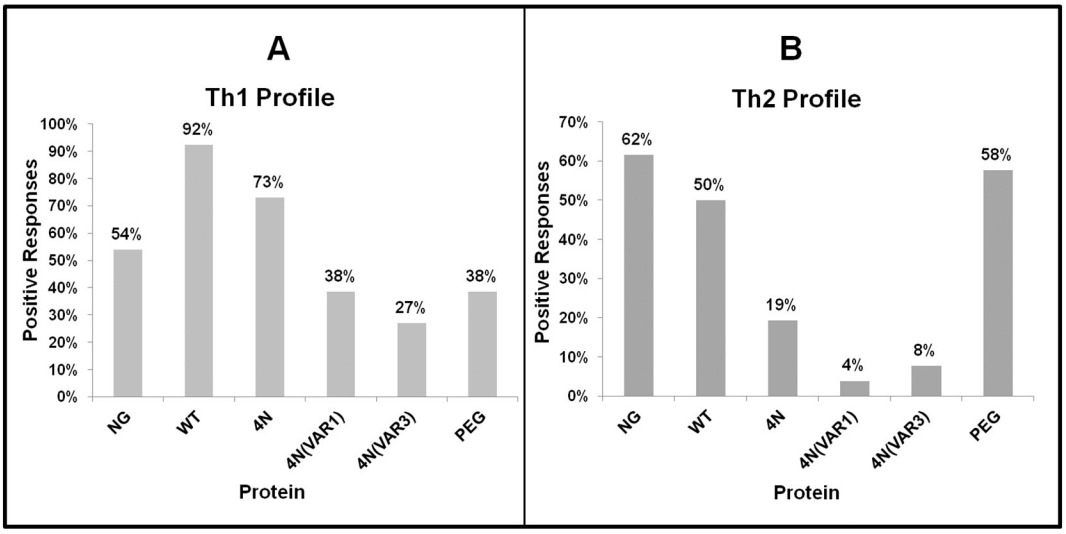

Fig. 6. 4N-IFN de-immunized variants showed a marked reduced immunogenicity in comparison with other IFN versions. *Ex vivo* cytokine secretion by T-cells after incubation with IFN-pulsed dendritic cells. Data were obtained from 26 donors. A Stimulation Index (SI) was defined as a ratio of the cytokine concentration (IFN-γ (A) and IL-4 (B)) from protein challenged samples divided by cytokine concentration from excipient treated samples. A geometric mean (GM) of the SI was then calculated and a positive donor was defined when SI > GM.

direct impact on protein structure or binding to its receptor on MDBK cells. Another plausible explanation could be that mutations in these proteins increased the site accessibility for *N*-glycosylation. This is especially true for 4N-IFN(VAR3) where a markedly different glycosylation pattern was observed when compared with 4N-IFN. Taking into account that each glycoform could be considered a subset of IFN-α molecules provided with a particular charge due to its sialic acid content, the IEF analysis suggests that 4N-IFN(VAR3) would be significantly enriched in more sialylated forms. This change could also explain the reduction of *in vitro* antiviral activity. A concern with reduced *in vitro* activity is the potential need for higher doses. However, changes in glycosylation patterns may be useful for a prolonged *in vivo* action [42]. Sialic acids contribute to extend the glycoproteins half-life limiting the binding to hepatic asialoglycoprotein receptors specific for terminal galactose or *N*-acetylgalactosamine residues [43], a receptor-mediated clearance mechanism. In addition, carbohydrates may also play an important role in protecting from proteolytic enzymes in the extra cellular space and so, reducing protein elimination from the circulatory system [44]. Also, it has been reported for other therapeutics that a reduction of the binding affinity for their receptor would contribute to potentially minimize negative side effects associated with therapy [48]. On the other hand, a reduced IFN antiviral activity could be solved through combined therapies. Recently, a screening of chemical libraries for compounds that enhanced cellular responses to IFN-α allowed identifying a triterpenoid, known as toosendanin (TSN). Pretreatment with TSN prior to IFN-α treatment was more effective in suppressing HCV replication than treatment with either drug alone. Although TSN alone did not activate the IFN-α pathway, it significantly enhanced the IFN-α induced increase of phosphorylated STATs, interferon-stimulated response element activation, and interferon-stimulated gene expression [49]. Additionally, efforts have been recently made to identify compounds from natural and synthetic libraries that can modulate the activity of IFNs [50]. Nevertheless, the potential effect of modifications on 4N-IFN de-immunized variants stability and pharmacokinetic parameters remains to be determined.

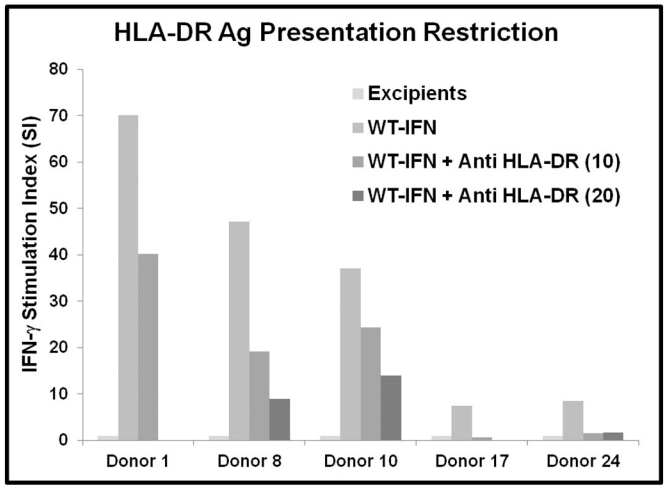

Fig. 7. HLA-DR restriction. Blocking antibody assay to determine the HLA restriction of IFN presentation by DC. A successive decrease in IFN-γ SI was observed when two different blocking Ab concentrations were evaluated. SI were normalized to the untreated control (excipients).

IFN-α has natural antiproliferative properties. Therefore, we could not use standard immunogenicity assays (T cell proliferation) and had to identify alternative methods for measuring immunogenicity. We obtained blood from healthy donors that possessed HLA DR types representing the majority of HLA-DR allotypes expressed in the world population. This allowed for detection of anti-IFN-α T-cell responses restricted to a specific HLA-DR allotype. In this analysis we included the original molecule (4N-IFN), a commercial non-glycosylated IFN (NG-IFN) produced in bacteria, a pegylated version (PEG-IFN), the O-glycosylated molecule (WT-IFN) produced in CHO cells and both 4N-IFN de-immunized variants. The aim of this analysis was to confirm the impact of those changes introduced into the 4N-IFN sequence on immunogenicity. In this study, we compared the novel deimmunized variants with a commercially available molecule and with PEG-IFN, a molecule that has with extended bioavailability like 4N-IFN.

A marked difference in the *in vitro* immunogenicity was observed between the IFN-α versions analyzed here. NG-IFN showed a very high immunogenicity for both Th profiles. This result is in good agreement with previous data that have revealed an immune response in patients after IFN-α therapy [45]. WT-IFN also demonstrated high immunogenicity, especially in the Th1 assays; almost all the donors developed an immune response. This result is surprising if we consider that this protein is analogous to that endogenously produced. A possible explanation for this could be the presence of contaminants in the sample that may escape the single purification step used here. It was previously reported that these undesirable products, known as host cell

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proteins (HCP) may produce, even at very low levels, an increase in the overall immunogenicity of the product [46]. An algorithm designed to predict the potential immunogenicity of residual CHO proteins has been developed by our group [47]. As the present work was focused on the development of de-immunized versions of 4N-IFN, we did not further identify possible HCP contaminants.

When 4N-IFN was analyzed, a high proportion of responders was observed, especially with a Th-1-polarized immunogenicity profile, and was even higher than the number of responders for NG-IFN. This result is in accordance with that previously predicted by EpiMatrix and would suggest the generation of new and/or more immunogenic epitopes as a consequence of *N*-glycosylation site addition. However, further assays should be addressed to confirm this hypothesis. In contrast, 4N-IFN(VAR1) and 4N-IFN(VAR3) have shown a markedly lowered immunogenicity for both cytokine profiling, highlighting the success of the modifications in terms of reducing IFN-α immunogenicity. The magnitude of this effect was even more pronounced for the Th2 profile. Similarly, PEG-IFN has reduced Th1 immunogenicity, which is similar to 4N-IFN(VAR1). However, when IL-4 secretion was analyzed, more than half of the donors responded to the pegylated version. It is well known that T cell help is necessary for adaptive humoral immune response, inducing B cells to produce antibodies. Therefore, the IL-4 response to PEG-IFN observed in this study is consistent with previous studies reporting antibody formation in patients with HCV [8,9]. The high correlation between the results showed here and those previously reported from clinical studies highlight the usefulness of this experimental platform as a screening tool to predict potential susceptibility of patients to develop undesired immune responses to biologics. In particular, for PEG-IFN a careful evaluation for IL-4 production and monitoring of these patients for hypersensitivity reactions in the clinic should be considered.

In conclusion, using a step-wise "DeFT" approach to reducing the immunogenicity of proteins, we first identified immunogenic regions into 4N-IFN, a long-lasting variant of IFN-α2b, and then selected and introduced the most suitable mutations in order to reduce its immunogenicity. Two functional 4N-IFN variants were successfully produced in CHO cells. These de-immunized 4N-IFN variants had significantly reduced immunogenicity, when compared with a commercial non-glycosylated and a pegylated IFN, as measured *in vitro* using T cell assays and cytokine profiling. This study also reconfirms the utility of *in silico* approaches to deimmunization that have been previously described by our [20]. Considering the impact of immunogenicity on the efficacy of IFN-α in the clinic, ranging from reduced to non-effective therapy, the 4N-IFN variants developed in the course of this study appear to be promising candidates for antiviral therapy of HCV and HBV.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2017.01.003.

References

[1] P. Ferenci, Historical treatment of chronic hepatitis B and chronic hepatitis C, Gut 34 (2 Suppl) (1993) S69–S73.

[2] A.U. Neumann, N.P. Lam, H. Dahari, D.R. Gretch, T.E. Wiley, T.J. Layden, A.S. Perelson, Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy, Science 282 (5386) (Oct. 1998) 103–107.

[3] D. Goldstein, J. Laszlo, Interferon therapy in cancer: from imaginon to interferon1, Cancer Res. 46 (1986) 4315–4329.

[4] L.E. Rönnblom, E.T. Janson, A. Perers, K.E. Oberg, G.V. Alm, Characterization of anti-interferon-alpha antibodies appearing during recombinant interferon-alpha 2a treatment, Clin. Exp. Immunol. 89 (3) (Sep. 1992) 330–335.

[5] J.R. Quesada, A. Rios, D. Swanson, P. Trown, J.U. Gutterman, Antitumor activity of recombinant-derived interferon alpha in metastatic renal cell carcinoma, J. Clin. Oncol. 3 (11) (Nov. 1985) 1522–1528.

[6] L.M. Itri, M.I. Sherman, A.V. Palleroni, L.M. Evans, L.L. Tran, M. Campion, R. Chizzonite, Incidence and clinical significance of neutralizing antibodies in patients receiving recombinant interferon-alpha 2a, J. Interf. Res. 9 (Suppl. 1) (Sep. 1989) S9–15.

[7] P.V. Wussow, D. Jakschies, M. Freund, R. Hehlmann, F. Brockhaus, H. Hochkeppel, M. Horisberger, H. Deicher, Treatment of anti-recombinant interferon-alpha 2 antibody positive CML patients with natural interferon-alpha, Br. J. Haematol. 78 (2) (Jun. 1991) 210–216.

[8] T. Santantonio, M. Milella, G. Antonelli, C. Scagnolari, Neutralizing antibodies to interferon alpha in a chronic hepatitis C patient non-responder to pegylated interferon, J. Hepatol. 45 (5) (Nov. 2006) 759–761.

[9] A.A. van der Eijk, J.M. Vrolijk, B.L. Haagmans, Antibodies neutralizing peginterferon alfa during retreatment of hepatitis C, N. Engl. J. Med. 354 (12) (Mar. 2006) 1323–1324.

[10] C. Scagnolari, S. Trombetti, A. Soldà, M. Milella, G.B. Gaeta, G. Angarano, G. Scotto, N. Caporaso, F. Morisco, R. Cozzolongo, G. Giannelli, M. Fasano, T. Santantonio, G. Antonelli, Development and specificities of anti-interferon neutralizing antibodies in patients with chronic hepatitis C treated with pegylated interferon-α, Clin. Microbiol. Infect. 18 (10) (Oct. 2012) 1033–1039.

[11] K.-U. Nolte, G. Günther, P. von Wussow, Epitopes recognized by neutralizing therapy-induced human anti-interferon-α antibodies are localized within the N-terminal functional domain of recombinant interferon-α2, Eur. J. Immunol. 26 (9) (Sep. 1996) 2155–2159.

[12] I. Bello-Rivero, M. Cervantes, Y. Torres, J. Ferrero, E. Rodríguez, J. Pérez, I. García, G. Díaz, P. López-Saura, Characterization of the immunoreactivity of anti-interferon alpha antibodies in myasthenia gravis patients. Epitope mapping, J. Autoimmun. 23 (1) (Aug. 2004) 63–73.

[13] T.D. Jones, M. Hanlon, B.J. Smith, C.T. Heise, P.D. Nayee, D.A. Sanders, A. Hamilton, C. Sweet, E. Unitt, G. Alexander, K.-M. Lo, S.D. Gillies, F.J. Carr, M.P. Baker, The development of a modified human IFN-alpha2b linked to the Fc portion of human IgG1 as a novel potential therapeutic for the treatment of hepatitis C virus infection, J. Interf. Cytokine Res. 24 (9) (Sep. 2004) 560–572.

[14] E. Chatelut, L. Rostaing, N. Grégoire, J.L. Payen, A. Pujol, J. Izopet, G. Houin, P. Canal, A pharmacokinetic model for alpha interferon administered subcutaneously, Br. J. Clin. Pharmacol. 47 (4) (Apr. 1999) 365–371.

[15] N. Ceaglio, M. Etcheverrigaray, R. Kratje, M. Oggero, Novel long-lasting interferon alpha derivatives designed by glycoengineering, Biochimie 90 (3) (Mar. 2008) 437–449.

[16] L. Moise, C. Song, W.D. Martin, R. Tassone, A.S. De Groot, D.W. Scott, Effect of HLA DR epitope de-immunization of factor VIII in vitro and in vivo, Clin. Immunol. 142 (3) (Mar. 2012) 320–331.

[17] A.S. De Groot, W. Martin, Reducing risk, improving outcomes: bioengineering less immunogenic protein therapeutics, Clin. Immunol. 131 (2) (May 2009) 189–201.

[18] S. Southwood, J. Sidney, A. Kondo, M.F. del Guercio, E. Appella, S. Hoffman, R.T. Kubo, R.W. Chesnut, H.M. Grey, A. Sette, Several common HLA-DR types share largely overlapping peptide binding repertoires, J. Immunol. 160 (7) (Apr. 1998) 3363–3373.

[19] A.S. De Groot, Immunomics: discovering new targets for vaccines and therapeutics, Drug Discov. Today 11 (5–6) (Mar. 2006) 203–209.

[20] A.C. Steere, W. Klitz, E.E. Drouin, B.A. Falk, W.W. Kwok, G.T. Nepom, L.A. Baxter-Lowe, Antibiotic-refractory Lyme arthritis is associated with HLA-DR molecules that bind a Borrelia burgdorferi peptide, J. Exp. Med. 203 (4) (Apr. 2006) 961–971.

[21] R.B. Kratje, R. Wagner, Evaluation of production of recombinant human interleukin-2 in fluidized bed bioreactor, Biotechnol. Bioeng. 39 (2) (Jan. 1992) 233–242.

[22] T. Dull, R. Zufferey, M. Kelly, R.J. Mandel, M. Nguyen, D. Trono, L. Naldini, A third-generation lentivirus vector with a conditional packaging system, J. Virol. 72 (11) (Nov. 1998) 8463–8471.

[23] L. Naldini, U. Blömer, P. Gallay, D. Ory, R. Mulligan, F.H. Gage, I.M. Verma, D. Trono, In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector, Science 272 (5259) (Apr. 1996) 263–267.

[24] R.I. Freshney, in: R.I. Freshney (Ed.), Culture of Animal Cells. A Manual of Basic Technique, fourth ed.Wiley-Liss Inc., Hoboken, N.J. 2000, pp. 195–215.

[25] A. Jaber, M. Baker, Assessment of the immunogenicity of different interferon beta-1a formulations using ex vivo T-cell assays, J. Pharm. Biomed. Anal. 43 (4) (Mar. 2007) 1256–1261.

[26] E. Elkord, P.E. Williams, H. Kynaston, A.W. Rowbottom, Human monocyte isolation methods influence cytokine production from in vitro generated dendritic cells, Immunology 114 (2) (Feb. 2005) 204–212.

[27] P.C. Familletti, S. Rubinstein, S. Pestka, A convenient and rapid cytopathic effect inhibition assay for interferon, Methods Enzymol. 78 (Pt A) (1981) 387–394.

[28] S. Rubinstein, P.C. Familletti, S. Pestka, Convenient assay for interferons, J. Virol. 37 (2) (Feb. 1981) 755–758.

[29] P. Kontsek, Human type I interferons: structure and function, Acta Virol. 38 (6) (Dec. 1994) 345–360.

[30] R. Mazor, A.N. Vassall, J.A. Eberle, R. Beers, J.E. Weldon, D.J. Venzon, K.Y. Tsang, I. Benhar, I. Pastan, Identification and elimination of an immunodominant T-cell epitope in recombinant immunotoxins based on pseudomonas exotoxin A, Proc. Natl. Acad. Sci. U. S. A. 109 (51) (Dec. 2012) E3597–E3603.

[31] F. Deisenhammer, Neutralizing antibodies to interferon-beta and other immunological treatments for multiple sclerosis: prevalence and impact on outcomes, CNS Drugs 23 (5) (2009) 379–396.

[32] B.A. Cohen, J. Oger, A. Gagnon, G. Giovannoni, The implications of immunogenicity for protein-based multiple sclerosis therapies, J. Neurol. Sci. 275 (1–2) (Dec. 2008) 7–17.

[33] T. Vial, J. Descotes, Clinical toxicity of the interferons, Drug Saf. 10 (2) (Feb. 1994) 115–150.

[34] P. De Somer, V.G. Edy, A. Billiau, Interferon-induced skin reactivity in man, Lancet 310 (8027) (Jul. 1977) 47–48.

[35] Y. Oppenheim, Y. Ban, Y. Tomer, Interferon induced Autoimmune Thyroid Disease (AITD): a model for human autoimmunity, Autoimmun. Rev. 3 (5) (Jul. 2004) 388–393.

[36] L.E. Rönnblom, G.V. Alm, K.E. Oberg, Possible induction of systemic lupus erythematosus by interferon-alpha treatment in a patient with a malignant carcinoid tumour, J. Intern. Med. 227 (3) (Mar. 1990) 207–210.

Author's Personal Copy

[37] E.A. Clark, J.A. Ledbetter, How B and T cells talk to each other, Nature 367 (6462) (Feb. 1994) 425–428.
[38] G.G. Klaus, M.S. Choi, E.W. Lam, C. Johnson-Léger, J. Cliff, CD40: a pivotal receptor in the determination of life/death decisions in B lymphocytes, Int. Rev. Immunol. 15 (1–2) (1997) 5–31.
[39] E. Koren, A.S. De Groot, V. Jawa, K.D. Beck, T. Boone, D. Rivera, L. Li, D. Mytych, M. Koscec, D. Weeraratne, S. Swanson, W. Martin, Clinical validation of the "in silico" prediction of immunogenicity of a human recombinant therapeutic protein, Clin. Immunol. 124 (1) (Jul. 2007) 26–32.
[40] J.D. Reveille, The genetic basis of autoantibody production, Autoimmun. Rev. 5 (6) (Jul. 2006) 389–398.
[41] A.S. De Groot, D.W. Scott, Immunogenicity of protein therapeutics, Trends Immunol. 28 (11) (Nov. 2007) 482–490.
[42] M.J. Koury, Sugar coating extends half-lives and improves effectiveness of cytokine hormones, Trends Biotechnol. 21 (11) (Nov. 2003) 462–464.
[43] A.G. Morell, G. Gregoriadis, I.H. Scheinberg, J. Hickman, G. Ashwell, The role of sialic acid in determining the survival of glycoproteins in the circulation, J. Biol. Chem. 246 (5) (Mar. 1971) 1461–1467.
[44] N. Jenkins, E.M. Curling, Glycosylation of recombinant proteins: problems and prospects, Enzym. Microb. Technol. 16 (5) (May 1994) 354–364.
[45] H. Schellekens, J.C. Ryff, P.H. van der Meide, Assays for antibodies to human interferon-alpha: the need for standardization, J. Interf. Cytokine Res. 17 (Suppl. 1) (Jul. 1997) S5–S8.
[46] E. S., K. Champion, H. Madden, J. Dougherty, Defining your product profile and maintaining control over it, part 2, BioProcess Int. 3 (2005) 52–57.
[47] C. Bailey-Kellogg, A.H. Gutiérrez, L. Moise, F. Terry, W.D. Martin, A.S. De Groot, CHOPPI: a web tool for the analysis of immunogenicity risk from host cell proteins in CHO-based protein production, Biotechnol. Bioeng. 111 (11) (Nov. 2014) 2170–2182.
[48] J. Cleland, N.C. Geething, J.A. Moore, B.C. Rogers, B.J. Spink, C. Wang, S.E. Alters, W.P.C. Stemmer, V. Schellenberger, et al., A novel long-acting human growth hormone fusion protein (VRS-317): enhanced in vivo potency and half-life, J. Pharm. Sci. 101 (8) (2012).
[49] T. Watanabe, N. Sakamoto, M. Nakagawa, S. Kakinuma, Y. Itsui, Y. Nishimura-Sakurai, M. Ueyama, Y. Funaoka, A. Kitazume, S. Nitta, K. Kiyohashi, M. Murakawa, S. Azuma, K. Tsuchiya, S. Oooka, M. Watanabe, Inhibitory effect of a triterpenoid compound, with or without alpha interferon, on hepatitis C virus infection, Antimicrob. Agents Chemother. 55 (2011) 2537–2545.
[50] M. Bürgi, V.A. Zapol'skii, B. Hinkelmann, M. Köster, D.E. Kaufmann, F. Sasse, H. Hauser, M. Etcheverrigaray, R. Kratje, M. Bollati-Fogolín, M. Oggero, Screening and characterization of molecules that modulate thebiological activity of IFNs-I, J. Biotechnol. 233 (2016) 6–16.
[51] D. Wullner, L. Zhou, E. Bramhall, A. Kuck, T.J. Goletz, S. Swanson, N. Chirmule, V. Jawa, Considerations for optimization and validation of an in vitro PBMC derived T cell assay for immunogenicity prediction of biotherapeutics, Clin. Immunol. 137 (2010) 5–14.