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Assessment of the immunogenicity of different interferon beta-1a formulations using *ex vivo* T-cell assays

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Abstract

Recombinant homologues of human proteins have the potential to induce an immunogenic response when used therapeutically. Each of the three interferon (IFN) beta therapies currently approved for multiple sclerosis can induce the development of neutralising antibodies, the full effects of which on IFN beta therapy remain unclear. To investigate the immunogenicity of the currently licensed formulation of subcutaneous IFN beta-1a, 22 or 44 μg three times weekly (Rebif®), two new formulations of IFN beta-1a (Rebif® New Formulation [RNF]1 and RNF2) have been developed. In this study, the immunogenicity of the current formulation was investigated against RNF1, RNF2 and an IFN beta standard using *ex vivo* T-cells. Dendritic cells, isolated from peripheral blood monocytes donated by 26 healthy volunteers, were matured *in vitro* and incubated with the test antigens for 6 h. Autologous $CD4^+$ T-cells from the same donors were added and further incubated before cytokine release was assessed by ELISpot assay and proliferation by [^{3}H]thymidine pulse. Secretion of T-cell-derived interleukin-2 was 79%, 66% and 105% in incubations with RNF1, RNF2 and the current formulation, respectively (normalised to secretion with the IFN standard; $p < 0.05$, RNF2 versus the current formulation). Secretion of IFN gamma was highly variable between donors, with no significant difference observed between formulations. Normalised values for T-cell proliferation were 56%, 44% and 88% with RNF1, RNF2 and the current formulation, respectively ($p < 0.05$, RNF2 versus the current formulation). The immunogenic potential of RNF2 is significantly lower than that of the current formulation when tested *ex vivo*. This result is now being confirmed through ongoing clinical trials.
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1. Introduction

Recombinant homologues of human proteins have the potential to induce an immunogenic response when used therapeutically [1–3]. The structure of the protein, particularly any lack of glycosylation, can provoke the production of binding antibodies (BAbs) and neutralising antibodies (NAbs), as can aggregates or foreign epitopes that are present in the injection solution [1,4]. The formation of antibodies can also be influenced by the dosing schedule or means of administration [1].

In common with other recombinant proteins administered therapeutically, a minority of patients develop BAbs or NAbs while using recombinant interferon (IFN) beta for the long-term treatment of multiple sclerosis (MS) [3]. However, there is conflicting evidence on the effects of NAbs on clinical efficacy: some studies have reported that efficacy is reduced in some patients, whereas other studies have proved inconclusive or reported no effect [5–8]. This uncertainty and the absence of a standardised assay to measure NAbs has led to the recognition that treatment decisions should be based on clinical grounds alone. Nonetheless, the negative impact of persistent, high-titre NAbs on clinical efficacy needs to be acknowledged, and new strategies to limit the formation of NAbs need to be explored.

Rebif® (Serono International, Geneva, Switzerland), IFN beta-1a, is one of three IFN beta formulations currently licensed for the treatment of MS, and is the only formulation to be administered subcutaneously (sc) at a dose of 22 or 44 μg three times weekly (tiw). To retain the efficacy associated with this high-dose, high-frequency sc IFN beta-1a treatment but to reduce immunogenicity, two new formulations of IFN beta-1a have been developed. Aside from the active IFN molecule, the Rebif® New Formulations (RNF1 and RNF2) are free from the

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addition of human- and animal-derived components. Further process improvements have also been made using state-of-the-art technologies, with a major focus on the structural and functional integrity of the molecule. By altering the composition of the injection solution, while retaining the composition of the active ingredient, there is the potential to reduce overall immunogenicity, thereby limiting the formation of NAbs and improving tolerability.

The aim of this study was to investigate the immunogenicity of these new formulations compared with both the current formulation and an IFN beta standard (the recombinant IFN beta-1a molecule in acetate buffer) as a control. The immunogenic response was measured *ex vivo* using dendritic cells (DC) and T-cells derived from peripheral blood mononuclear cells (PBMCs) from healthy donors. Secretion from the T-cells of two cytokines, interleukin (IL)-2 and IFN gamma, as well as cellular proliferation, were measured in response to the presentation of each of the test antigens.

2. Materials and methods

2.1. Donors

The use of all blood supplies was approved by the Addenbrooke's Hospital Local Research Ethics Committee, Cambridge, UK. PBMCs were isolated from each healthy donor by Ficoll (Amersham Biosciences, Chalfont St. Giles, UK) density centrifugation and were stored under liquid nitrogen. HLA-DR allotypes were determined by sequence-specific polymerase chain reaction (PCR; Dynal, Wirral, UK) and donors were selected according to the expression of individual major histocompatibility complex (MHC) class II allotypes (Table 1).

Table 1
HLA-DR allotypes of the healthy donors

Donor	Allotype
1	DRB1*04, DRB1*13, DRB4*01
2	DRB1*01, DRB1*07, DRB4*01
3	DRB1*03, DRB1*16, DRB3
4	DRB1*11, DRB1*13 or DRB1*14, DRB3
5	DRB1*03, DRB1*09, DRB3, DRB4*01
6	DRB1*13, DRB1*15, DRB3, DRB5
7	DRB1*01, DRB1*07, DRB4*01
8	DRB1*01, DRB1*03, DRB3
9	DRB1*08, DRB1*15, DRB5
10	DRB1*01, DRB1*13, DRB3
11	DRB1*07, DRB1*15, DRB4*01, DRB5
12	DRB1*08, DRB1*11 or DRB1*13 and DRB3
13	DRB1*03, DRB1*07, DRB3, DRB4*01
14	DRB1*04, DRB1*15, DRB4*01, DRB5
15	DRB1*04, DRB1*13, DRB3, DRB4*01
16	DRB1*03, DRB1*15, DRB3, DRB5
17	DRB1*07, DRB1*15, DRB4*01, DRB5
18	DRB1*01, DRB1*04, DRB4*01
19	DRB1*04, DRB1*09, DRB4*01
20	DRB1*03, DRB1*04, DRB3, DRB4*01
21	DRB1*07, DRB1*13, DRB3, DRB4*01
22	DRB1*04, DRB4*01
23	DRB1*03 or DRB1*03 or 13, DRB3
24	DRB1*03, DRB1*11, DRB3
25	DRB1*04, DRB1*16, DRB4*01, DRB5
26	DRB1*01, DRB1*0103
27	DRB1*03, DRB1*04, DRB3, DRB4*01

2.2. Test antigens and controls

Three test antigens were studied: IFN beta-1a current formulation, RNF1 and RNF2. Each was suspended in 0.5 mL formulation buffer at 44 μg/mL. Their excipients before suspension are listed in Table 2. The two control antigens were keyhole limpet hemocyanin (Pierce, Cramlington, UK), and the recombinant molecule before formulation (referred to as the IFN beta standard (Table 2)). Both were suspended in 0.5 mL PBS (plus 0.01% Tween 20) to represent the composition of the test formulations. Growth medium was used as the non-antigen control; this was mixed with PBS (plus 0.01% Tween 20) to represent the composition of the test formulations.

2.3. Ex vivo T-cell assays

The methodology for *ex vivo* T-cell assays has been reported previously [9] and is summarised in Fig. 1.

2.3.1. Isolation of DC

Monocytes were isolated from PBMCs from each donor blood sample. Monocytes (>90% $CD14^+$, as determined by flow cytometric analysis; data not shown) were cultured in growth medium containing AIM V medium (Gibco, Paisley, UK) with 3% heat-inactivated human AB serum (Autogen Bioclear, Calne, Wiltshire, UK) at an approximate density of 1×10^6 cells per well in 24-well plates. To induce an immature phenotype of monocyte-derived DC ($CD40^+$, $CD80^{lo}$, $CD83^+$, MHC class II^+), monocytes were incubated in growth medium containing 1000 U/mL each of human IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) (both from Peprotech, Rocky Hill, NJ, USA) for 4 days.

2.3.2. Antigen pulsing/loading of DC

On day 4, test antigens, control antigens and non-antigen control (see Section 2.2) were added. All solutions were diluted

Table 2
Excipients of the three interferon (IFN) beta-1a formulations and the IFN beta-1a standard

	Stabilisers	Buffer
Current IFN formulation	D-Mannitol Human serum albumin	0.01 M sodium acetate
RNF1	Poloxamer 188 Lysine	0.01 M sodium acetate
RNF2	D-Mannitol Benzyl alcohol L-Methionine Poloxamer 188	0.01 M sodium acetate
IFN beta standard	None	50 mM sodium acetate

RNF, Rebif® New Formulation.

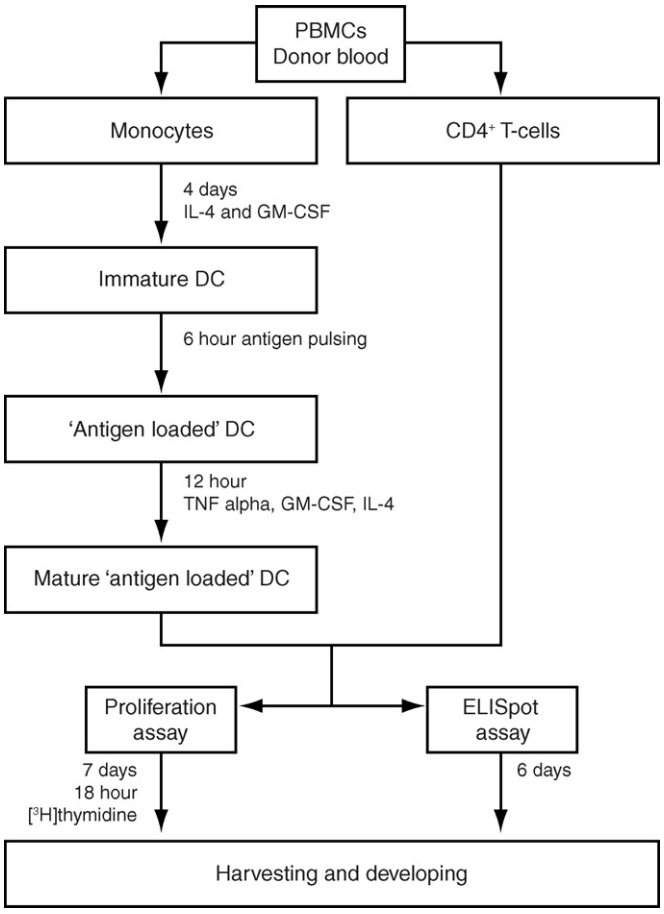

Fig. 1. Study design. DC, dendritic cells; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; PBMCs, peripheral blood mononuclear cells; TNF, tumour necrosis factor.

in 0.5 mL growth medium containing 25 mM HEPES, pH 7.4, immediately before adding to the immature DC (final culture volume, 1 mL). This level of buffering was necessary as the low pH of the test formulations inhibited the assay. The pH of the formulations was adjusted immediately prior to their addition to the DC.

After a minimum of 6 h of incubation, DC were washed to remove exogenous antigen and resuspended overnight in growth medium containing tumour necrosis factor (TNF) alpha, GM-CSF and IL-4 (all from Peprotech) to induce a mature DC phenotype ($CD40^+$, $CD80^+$, $CD83^{hi}$, $CD86^{hi}$, MHC class II^{hi}); 'antigen-loaded' DC were incubated for 24 h in medium containing 1000 U/mL IL-4, 1000 U/mL GM-CSF and 20 ng/mL TNF alpha. Viable DC were then counted and gamma-irradiated (4000 rad, ^{137}Cs source) before being plated out at 1×10^4 cells per well in 100 μL growth medium. PVDF ELISpot assay plates (Millipore, Billerica, MA, USA) were used for both IL-2 and IFN gamma ELISpot assays (BD-Biosciences, Oxford, UK); 1 μg/mL capture antibody (IL-2 or IFN gamma) was used to pre-coat ELISpot assay plates before adding DC (according to the manufacturer's instructions). Triplicate cultures were established for each test condition in ELISpot and proliferation assays.

2.3.3. Isolation of autologous T-cells

Autologous (i.e. from the same donor) $CD4^+$ T-cells were isolated by negative selection from PBMCs (using the Human $CD4^+$ Negative Isolation Kit, Dynal) and added at a concentration of 1×10^5 cells per well to the 96-well proliferation and ELISpot plates.

2.3.4. In vitro priming of autologous T-cells with antigen-pulsed DC

$CD4^+$ T-cells were added at a concentration of 1×10^5 cells per well to the 96-well proliferation and ELISpot plates. Proliferation plates were incubated for a further 7 days before [^{3}H]thymidine was added (1 μCi per well) and incubated for 18 h. Cultures were harvested onto glassfibre filter mats (Perkin Elmer, Wellesley, MA, USA) using a Tomtec Mach III plate harvester (Hamden, CT, USA). Cpm were determined by scintillation counting using a Wallac Microbeta TriLux (Wellesley, MA, USA) plate reader.

ELISpot plates were incubated for 6 days before developing according to the manufacturer's instructions. Spot analysis was performed using ImmunoScan (Cellular Technologies, Cleveland, OH, USA) and ImmunoSpot software (Version 3.2) to determine the number of spots per well (spw). Spot parameters were set for each donor using test and untreated control wells to establish sensitivity, background balance, spot separation and spot size gate settings.

2.4. Data analysis

To ensure low intra-assay variability for both proliferation and IL-2 ELISpot assays, the data were analysed to determine the coefficient of variance, standard deviation and significance ($p < 0.05$) using a parametric statistical one-tailed, two-sample, Student's *t*-test (assuming unequal variances). Positive responses were defined by donors who produced a significant ($p < 0.05$) response with a stimulation index (SI) ≥ 2. Analyses of the normally distributed variables (SIs) were performed by comparing the mean values using ANOVA to determine significant differences ($p < 0.05$) between the various test formulations and the IFN beta standard.

3. Results

3.1. Donors

Blood samples were taken from 26 healthy donors aged between 18 and 60 years. Comparison of the allotypes expressed in the cohort against those expressed in the world population revealed that coverage of >80% was achieved and that all major HLA-DR alleles (individual allotypes with a frequency >5% expressed in the world population) were well represented (Fig. 2).

3.2. DC recovery

Similar numbers of viable cells were recovered in treated and control cultures, with marginally higher recoveries in the

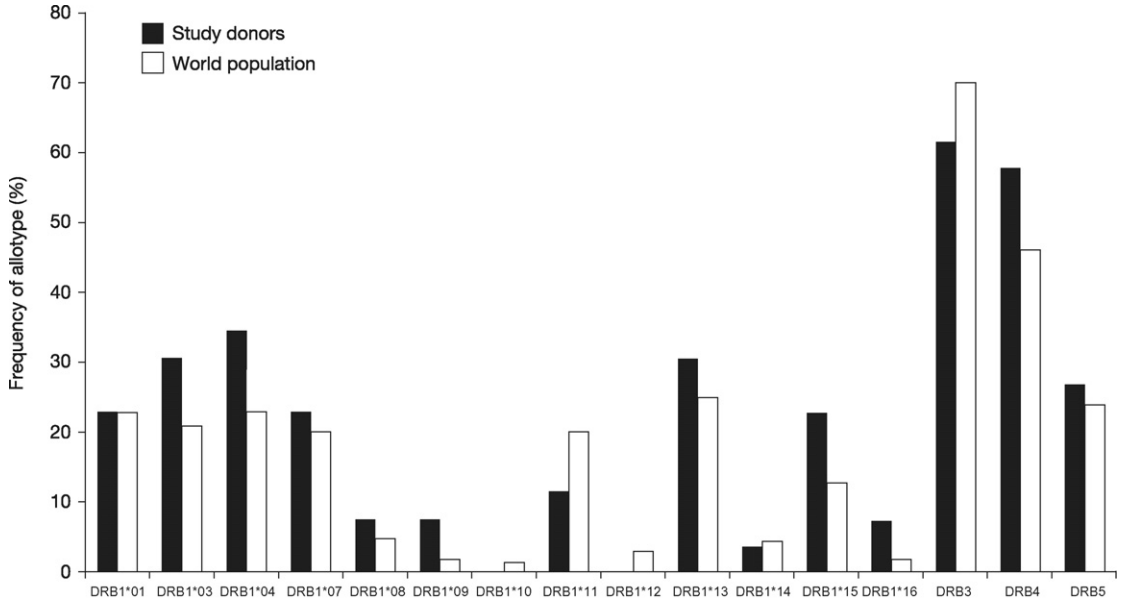

Fig. 2. Expression of HLA-DR allotypes in the 26 study donors compared with the world population.

antigen incubations than the non-antigen control incubations (recovery was 106%, 103%, 101% and 108% of non-antigen control with RNF1, RNF2, current formulation and the IFN beta standard, respectively). Visual inspection of the cells under a light microscope showed similar morphology of DC between treatments.

3.3. Cytokine response

DC treated with RNF2 had a reduced immunogenic potential to stimulate T-cell-mediated secretion of IL-2 compared with those incubated with either the current IFN beta formulation or the IFN beta standard ($p < 0.05$). The mean number of cells that secreted IL-2 by incubation condition is shown in Table 3, and is shown normalised to the IFN beta standard in Fig. 3. The IFN gamma response was highly variable between donors, with 17 producing <10 IFN gamma spw in medium control cultures. Differences in responses between incubation conditions did not reach significance (Table 3).

3.4. Proliferative response

T-cell proliferation showed a similar pattern to cytokine release, and mean cpm and SI values differed depending on the IFN beta formulation (Table 4). Although the differences were not as marked as those observed with the IL-2 cytokine analysis, the ability to induce T-cell proliferation was greater with the current formulation than with RNF1; RNF1 was, in turn, greater than RNF2 in eliciting a response.

4. Discussion

The use of *ex vivo* T-cell assays to investigate the immunogenicity of two new formulations of IFN beta-1a, 22 or 44 μg sc tiw, suggests an improved immunogenic profile of RNF1 and RNF2 compared with the current IFN beta-1a formulation. RNF2 was significantly less immunogenic in terms of T-cell proliferation and IL-2 secretion than the current IFN beta-1a for-

Table 3
Ex vivo cytokine secretion by T-cells after incubation with antigen or medium control-pulsed dendritic cells (data from 26 donors)

	RNF1	RNF2	Current formulation	IFN beta standard	KLH	Medium control
Interleukin-2 secretion						
Mean spw ± S.D.	33.52 ± 6.55	22.72 ± 8.24	43.16 ± 10.8	37.54 ± 9.44	42.53 ± 8.32	35.76 ± 8.63
Mean SI	0.95	0.80*,†	1.27	1.21	1.43	–
Normalised SI (%)	78.73	66.17	108.15	100	–	–
IFN gamma secretion						
Mean spw ± S.D.	35.74 ± 9.24	22.92 ± 10.43	52.57 ± 12.14	43.47 ± 14.21	37.99 ± 12.88	30.50 ± 8.85
Mean SI	1.90	0.85	2.19	1.89	2.36	–
Normalised SI[a] (%)	100.34	44.92	115.80	100	–	–

IFN, interferon; KLH, keyhole limit haemocyanin; RNF, Rebif® New Formulation; S.D., standard deviation; SI, stimulation index; spw, spots per well.
[a] SI were normalised to the SI of the IFN beta standard.
* $p < 0.05$ vs. the IFN beta standard.
† $p < 0.05$ vs. the current IFN beta-1a formulation.

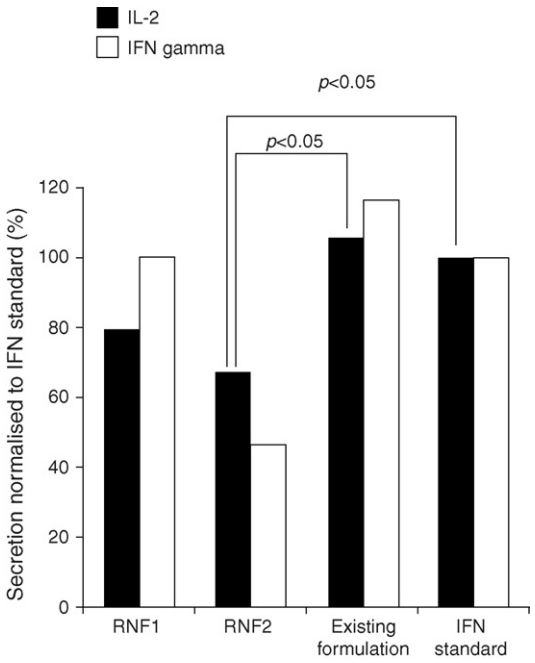

Fig. 3. *Ex vivo* cytokine secretion by T-cells after incubation with antigen or medium control-pulsed dendritic cells (data from 26 donors): average stimulation index values normalized to IFN beta standard. IFN, interferon; IL, interleukin; RNF, Rebif® New Formulation.

mulation; RNF1 also offered improvements in immunogenicity, but to a lesser extent than RNF2.

The combined proliferative and cytokine secretory response to RNF2 showed that this formulation induced the least T-cell activation of all the antigens tested. Within these parameters, RNF2 therefore has a lower immunogenic potential than the current IFN beta-1a formulation.

One key difference between the new formulations and the current IFN beta-1a formulation that may explain the different immunogenicity was the removal of all human- and animal-derived components from the injection solution. Human serum albumin (HSA) is commonly used as a stabilising agent in IFN injections but it may affect the tertiary and quaternary structure of the IFN molecule, increasing the formation of immunogenic aggregates of IFN–IFN and HSA–IFN [10,11]. Previous studies have demonstrated that alternative technical solutions can be as successful as HSA in stabilising IFN without causing the problem of aggregates [10,11]. The presence of IFN aggregates has been suggested to relate directly to the immunogenic potential of IFN beta [8].

In some patients, the immunogenicity of IFN beta treatment induces the development of NAbs, but the impact of NAbs on the clinical efficacy of treatment in this subset of patients has not been elucidated fully. Some studies have shown a correlation between the development of NAbs and a reduction of therapeutic activity [12–15]. There are also many studies that have shown that the efficacy of IFN beta is identical in patients whether or not they have developed NAbs [5,8,16–21]. To complicate this assessment further, seroconversion or the loss of NAb positivity is a relatively frequent phenomenon with long-term treatment [22]. Even though the overall impact of NAbs on treatment efficacy is unclear, and it seems logical for physicians to make treatment decisions on the basis of clinical outcomes and not on NAbs status alone, it is prudent to investigate ways to limit the immunogenicity of current treatments and thus the proportion of patients who go on to develop NAbs. The data described in this paper suggesting that the incidence of NAbs may be lower with RNF2 compared with the current IFN beta-1a formulation are, therefore, a significant step towards achieving this goal and to maximising the benefits of treatment in the greatest number of patients.

The use of *ex vivo* DC to determine whether the injection formulation can contribute to the overall immunogenicity is a new use for this methodology. To overcome the inhibitory effects of IFN beta-1a on T-cell activation, immature DC were pulsed with the various IFN beta-1a formulations during only a short incubation period, at a relatively high concentration, followed by washing of the cells. During this antigen-loading step, it is hypothesised that IFN beta-1a was endocytosed and processed by the DC. Peptide fragments, bound to MHC class II, would then have been presented on the cell surface, where they could stimulate T-cell responses from the $CD4^+$ T-cells that had been added once the DC had been induced to a mature phenotype using TNF alpha. Future work, including an antigen-restimulation assay, would be necessary, however, to confirm that the T-cell responses detected were indeed antigen specific.

Healthy donors were selected to ensure that all major HLA-DR allotypes expressed in the world population were represented in the study cohort. This wide coverage of HLA allotypes ensured that any IFN beta-1a-specific T-cell responses that were restricted to a particular HLA-DR allotype would be detected. It was clear, based on this relatively small sample

Table 4
Ex vivo proliferation by T-cells after incubation with antigen or medium control-pulsed dendritic cells (data from 26 donors)

	RNF1	RNF2	Current IFN beta-1a formulation	IFN beta standard	KLH
Mean cpm	41,903	35,719	55,612	55,012	65,375
Mean SI	0.9*	0.7*,†	1.4	1.6	2.1
Normalised SI[a] (%)	59.65	47.67	90.83	100	

cpm, counts per minute; IFN, interferon; KLH, keyhole limit haemocyanin; RNF, Rebif® New Formulation; SI, stimulation index.
[a] SI were normalised to SI of IFN beta standard.
* $p < 0.05$ vs. the IFN beta standard.
† $p < 0.05$ vs. the current IFN beta-1a formulation.

size, that antigen-specific T-cell responses to human IFN beta-1a were not restricted by HLA-DR allotypes. This does not, however, exclude that there may be a weak association between T-cell epitopes derived from IFN beta-1a and certain HLA-DR allotypes [23].

The results from this Phase I study provide *ex vivo* evidence of a lower immunogenic potential for RNF2 compared with the current formulation of sc IFN beta-1a. Results are awaited of a Phase IIIb clinical study (protocol 25632) to learn whether the *ex vivo* benefits from RNF2 translate into clinical benefit for patients with MS. The re-formulation of IFN beta-1a, 22 or 44 μg sc tiw, reflects the long-term commitment to patients with MS, in maximising the benefit from this treatment by innovative scientific research and development.

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