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Research Article

Multiplexed Gene Expression as a Characterization of Bioactivity for Interferon Beta (IFN-β) Biosimilar Candidates: Impact of Innate Immune Response Modulating Impurities (IIRMIs)

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Abstract. Recombinant human interferon-β (rhIFN-β) therapy is the first-line treatment in relapsing-remitting forms of multiple sclerosis (MS). The mechanism of action underlying its therapeutic activity is only partially understood as IFN-βs induce the expression of over 1000 genes modifying multiple immune pathways. Currently, assessment of potency for IFN-β products is based on their antiviral effect, which is not linked to its therapeutic effect. Here, we explore the use of a multiplexed gene expression system to more broadly characterize IFN-β bioactivity. We find that MM6 cells stimulated with US-licensed rhIFN-βs induce a dose-dependent and reproducible pattern of gene expression. This pattern of gene expression was used to compare the bioactivity profile of biosimilar candidates with the corresponding US-licensed rhIFN-β products, Rebif and Betaseron. While the biosimilar candidate for Rebif matched the pattern of gene expression, there were differences in the expression of a subset of interferon-inducible genes including CXCL-10, CXCL-11, and GBP1 induced by the biosimilar candidate for Betaseron. Assessment of product impurities in both products suggested that the difference was rooted in the presence of innate immune response modulating impurities (IIRMIs) in the licensed product. These studies indicate that determining the expression levels for an array of reporter genes that monitor different pathways can be informative as part of the demonstration of biosimilarity or comparability for complex immunomodulatory products such as IFN-β, but the sensitivity of each gene to potential impurities in the product should be examined to fully understand the results.

KEY WORDS: bioactivity; biosimilarity; comparability; gene expression; impurities; innate immune response modulating impurities; interferon beta.

INTRODUCTION

IFN-β is an endogenous protein produced by most cells in response to viral, bacterial, and parasitic infections as well as stress and inflammation, and has important antiviral, anti-proliferative, and immunomodulatory activities (1,2). In the clinic, the recombinant version of IFN-β (rhIFN-β) is used as the first-line treatment of relapsing forms of multiple sclerosis (3). Given the extensive use of IFN-β, there are several preparations commercially available around the world and, now as the intellectual property (IP) expire, a growing number of biosimilar candidates (3,4). In the USA, an application for a biosimilar product generally includes, among other things, data derived from analytical studies that demonstrate that the biological product is highly similar to the reference product notwithstanding minor differences in clinically inactive components. Characterization of the bioactivity is also a critical part of comparability studies undertaken to demonstrate that changes to the manufacturing process do not affect the safety or efficacy of a product. Whenever possible, the assays used to characterize bioactivity are selected to reflect the mechanism of action of the product (5).

In the case of rhIFN-β, the mechanism of action underlying the therapeutic effect is not completely understood. Type I interferons (IFN-1) are cytokines with pleiotropic activities (1,6). Upon binding to its heterodimeric cell receptor complex (IFNAR), IFN-β initiates a complex signaling cascade mediated by the phosphorylation of several members of the Janus-activated kinase (JAK)/signal transducer and activator of

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transcription (STAT) signaling pathways that culminates with the transcriptional activation of over 1000 genes (interferon-stimulated genes or ISGs) (1). The exact pattern of gene expression depends on the cell type and the binding affinity of the product for its receptor (6–8). In immune cells, IFN-β reduces antigen presentation and T cell proliferation, alters cytokine and matrix metalloproteinase (MMP) expression, and enhances regulatory T cell function; however, the precise role of each of these effects on the therapeutic effect in MS is not well understood (1,9,10).

Currently, there are four licensed products in the USA: two rhIFN-β-1b (Extavia and Betaseron) produced in *Escherichia coli* and two rhIFN-β-1a (Avonex and Rebif) produced in Chinese hamster ovary (CHO) cells. In the rhIFN-β-1b, the N-terminal methionine has been deleted, there is a substitution of serine for a cysteine residue in position 17, and they are not glycosylated, whereas the two rhIFN-β-1a have the naturally occurring sequence in humans and are glycosylated (9). The transcriptional response to rhIFN-β-1b and rhIFN-β-1a is generally considered to be similar, although subtle differences in their clinical effect and immunogenicity have been described (11–13). Importantly, in a recent study, Haile *et al.* showed that the two products produced in *E. coli* contain trace levels of innate immune response modulating impurities (IIRMIs) that were not evident in the rhIFN-β-1a products and raising the possibility that they could contribute to their immunogenicity (14).

Despite no clear links to its therapeutic activity in MS (1,15), the potency of rhIFN-β is most frequently measured using its antiviral activity (AVA). AVA assays are subject to high intra- and inter-test variations and require virus manipulation under biosafety level two conditions (16). To circumvent these issues, several studies have suggested replacing the AVA assays with gene expression reporter assays monitoring the activation of the interferon-sensitive response element (ISRE), or the expression of an interferon inducible gene such as CXCL10 or MX1, as these would be easier to validate and could be more sensitive to differences in bioactivity (Yang *et al.* 2016; Smilovic, 2008 #5767) (17,18). However, given the complexity of the rhIFN-β response and the lack of understanding of the determinants of therapeutic efficacy in MS, a broader characterization of the gene expression induced by the product could provide additional information on multiple activation paths and strengthen the characterization of the product's bioactivity. As a proof of concept, we explored whether the assessment of a broader gene expression pattern using a cell line responsive to rhIFN-β would provide a useful tool in characterizing the bioactivity of rhIFN-β. Such a tool could be used for characterization of bioactivity or be validated as a potency assay. To this end, we selected an array of genes that would report on different immunomodulatory activities of rhIFN-β and monitor their expression in MM6 cells, a human monocytic cell line responsive to rhIFN-β. We show that rhIFN-β-1a and rhIFN-β-1b induce a reproducible pattern of gene expression and then used to characterize and compare the bioactivity of rhIFN-β biosimilar candidates. Importantly, the pattern of gene expression was modified by the presence of trace levels of innate immune response modulating impurities (IIRMIs) in the product, highlighting the importance of assessing IIRMI in therapeutic proteins.

MATERIALS AND METHODS

Interferon-β Samples

Betaseron® (lyophilized, Bayer Health Care Pharmaceuticals, Montville, NJ) (lots# 33937A, 44036A, 52143A, 52044A) and Rebif (Serono Inc., Randolph, MA) (lots# AU013278, AU010438, AU015150, AU0146341) were purchased from a pharmacy. The biological activity for the biosimilar candidate products was estimated by their protective effect on WISH cells infected with vesicular stomatitis virus (VSV) per recommendation of the European Pharmacopeia. All rhIFN-β products were stored and handled according to label instructions and used before expiry. All calculations for test product concentration were based on the specific activity in international units (IU) stated in the product insert or Certificate of Analysis (IBO and IBA) provided by the manufacturer. For all the experiments, IFN concentrations in IU/mL were assessed based on nominal label concentrations. Endotoxin levels for all products were below the level of detection using the kinetic LAL assay.

Cell Culture

Human monocytic cell line macrophage-like-MonoMac6 (MM6) cells (Germany Culture Collection) (19) were a generous gift from Marina Zaitseva (CBER, FDA) and used as previously described (20). Briefly, cells were grown in RPMI 1640 containing 15% FBS, 2 mM glutamate, 1 mM non-essential amino acid, and 1 mM sodium pyruvate, 100 IU/mL penicillin-streptomycin, and 10 μg/mL insulin-transferrin-selenium (Life Technologies, Carlsbad, CA) with 1 mM oxaloacetic acid purchased from Sigma-Aldrich (St. Louis, MO). Cell passage was limited to 25. The murine Raw 264.7 macrophage cell line carrying a SEAP reporter construct inducible by NF-κB (RAW-Blue cells) was purchased from InvivoGen. Cells were grown in DMEM supplemented with 10% FCS, 2 mM L glutamine, and 100 μg/mL Normocin in the presence of selection antibiotic 200 μg/mL Zeocin and passaged until 70% confluency was reached per manufacturer's recommendation. Cells were scraped and resuspended in RAW-Blue test media (DMEM, 10% heat-inactivated FBS, 100 μg/mL Normocin, and 2 mM L glutamine) for testing. HEK 293 cells purchased from InvivoGen. HEK-BLUE-hTLR2 and HEK-BLUE-hTLR4 cells were cultured in DMEM 10% FCS with 50 μg/mL penicillin-streptomycin, 100 μg/mL Normocin, and 2 mM L glutamine supplemented with 1X HEK-BLUE selection. Whereas HEK-BLUE-hTLR5, HEK-BLUE-hTLR7, and HEK-BLUE-hTLR9 cells were maintained with growth media supplemented with blasticidin and Zeocin as per manufacturer instructions.

Testing of IFN-β Products Using MM6 Cells

MM6 cells were plated at 1×10^6 cells/mL in flat bottom 96-well plates and incubated with each IFN-β product at the concentration and incubation time indicated on the corresponding figure legend. Total RNA and cDNA were prepared as described below. Changes in gene expression were assessed using qPCR or custom-designed TaqMan® Micro Fluidic Cards and included known interferon-stimulated genes,

cytokines, chemokines, and chemokine receptors. Fold changes were calculated by normalizing mRNA expression using a housekeeping gene and then expressed as fold increase over unstimulated/untreated samples (2–ΔΔCt method).

Testing of Products on NF-κB Reporter Cell Lines

For testing the products, RAW-BLUE cells were suspended at 1×10^6 cells/mL in test media. Then, each product was diluted in test media at a volume of 100 μL and added to the wells. After 2, 4, 6, or 24 h of incubation, supernatants were collected, and NF-κB activation was determined using detection medium QUANTI-Blue prepared per manufacturer recommendations. Briefly, 150 μL of QUANTI-Blue was added to a 96-well flat bottom plate together with 50 μL of cell culture supernatant. After 2 h of incubation at 37 °C, the SEAP levels were determined colorimetrically at 620 nm by spectrophotometry. For mRNA analysis of specific pro-inflammatory genes, RAW-BLUE cells were also collected, and total RNA and cDNA were prepared as described below.

qRT-PCR Analysis by Taqman Low-Density Array

Total RNA was prepared from cells lysate using TRIzol (Invitrogen, Carlsbad, CA) as per manufacturer instructions. Subsequently, RNA (1 μg/mL) was reverse transcribed into cDNA using high-capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA) as per manufacturer recommendation. Custom-made human or mouse-specific TaqMan® Micro Fluidic Cards containing panels of 96 gene expression assays were loaded with a sample volume of cDNA solution equivalent to 250 ng of the original RNA, mixed with 2x Universal Master Mix (ThermoFisher, Carlsbad, CA). The Ct values obtained for each gene were directly normalized to housekeeping values (GAPDH or 18S) in order to obtain ΔCt. The difference in ΔCt values between stimulated and media controls was calculated to get ΔΔCt. The fold change in the expression of these genes was obtained using 2(-ΔΔCt) method. The radial figures provided exclude housekeeping genes as well as genes that were not modified by any of the products in the cell lines tested and/or had responses that were not reproducible between plates.

Statistical Analysis

Experiments were repeated at least twice and run in triplicate. Individual data points represent the mean ± SD. For statistical comparisons with multiple groups, we used one-way ANOVA followed by Tukey's multiple comparison analysis post-test, and for studies comparing 2 multiple genes of two treatment groups, we used the Holm-Sidak method, with an alpha of 0.1, where each row was analyzed individually without assuming consistent SDs. Statistical analyses were performed with GraphPad Statistical Software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was defined as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

Use of MM6 Cells as a Platform for the Comparison of Bioactivity of rhIFN-β Preparations

Evaluating comparability or similarity in terms of bioactivity for proteins that have complex mechanisms of action is challenging. Given the breadth of biological effects of rhIFN-β, we reasoned that monitoring the expression of a panel of ISG genes would provide a more comprehensive approach to assessing bioactivity than a single-gene assay. Using PBMC to generate a bioactivity characterization assay was not adequate since the response varied significantly between donors (supplementary Fig. 1). Further, it is known that freeze-thawing PBMC may modify the rhIFN response suggesting that PBMC is not a good platform to monitor rhIFN-β activity (Yang *et al.* 2016). Therefore, we selected a human monocytic cell line (MM6), which has functional IFNA/BRs, to monitor the expression of 92 genes linked to the antiviral, pro-inflammatory, immune-modulatory, and pro-apoptotic effects upon stimulation with rhIFN-β. Stimulation of MM6 cells for 24 h with commercial rhIFN-β products Rebif and Betaseron at a concentration of 1000 IU/mL led to a robust increase in the expression of multiple ISGs including CXCL-10, CXCL-11, ISG15, IFIT1, IFIT2, MX1, and GBP1 as well as OAS1, IRF7, STAT1, CSF1, TNFSF13b (BAFF), and XIAF1. In addition, the rhIFN-β products stimulated several genes linked to immune and inflammatory responses CD69, AIM2, CCL2, CD80, and CD86. Lastly at 24 h, the MM6 cells showed modest increases in the expression of IL-10 and IL-6, but no increase in IFN-γ, TNF-α, LTa, IL-8, or IL-1β. The response of MM6 cells was dose-dependent (supplementary Fig. 2A and B) and consistent between lots (supplementary Fig. 3). Indeed, as shown in Fig. 1, the gene expression pattern induced by Betaseron and Rebif at 24 h showed significant overlap. This suggested that MM6 cells respond to rhIFN-β products and can be used to monitor their bioactivity. Further, since the assay monitors the expression of multiple ISGs, including genes linked to inflammation and cellular activation, it provides a more granular assessment of the bioactivity of rhIFN-βs than the singular expression of ISRE, MX1, or CXCL10.

Assessment of Early Response to rhIFN-β Products

It is well established that IFNs have feedback loops that amplify the effect of the initial stimulation (21). In addition, the rapid turnover of mRNA could reduce the sensitivity of the assay when tested after 24 h. To get a better sense of the direct effect of rhIFN-β on MM6 cells, we next explored the response to rhIFN-β by MM6 cells after 2, 4, 6, or 24 h of treatment with Rebif or Betaseron in culture (supplementary Fig. 4). As shown in Fig. 2, a significant induction of ISG was evident in MM6 cells after 4 h in culture for both products including most ISGs in the panel (CXCL-10, CXCL11, GBP1, ISG15, MX1, IFIH1, IFITM1 and IFIT2, TNFSF10/Trail, and IRF7). The changes in ISG expression were modest compared to 24 h; however, the cells exposed to rhIFN-β products showed a clear increase in most ISGs tested as well as several genes linked to modulation of inflammation and apoptosis of immune cells including IL-6, Ido, Fas, and several genes in

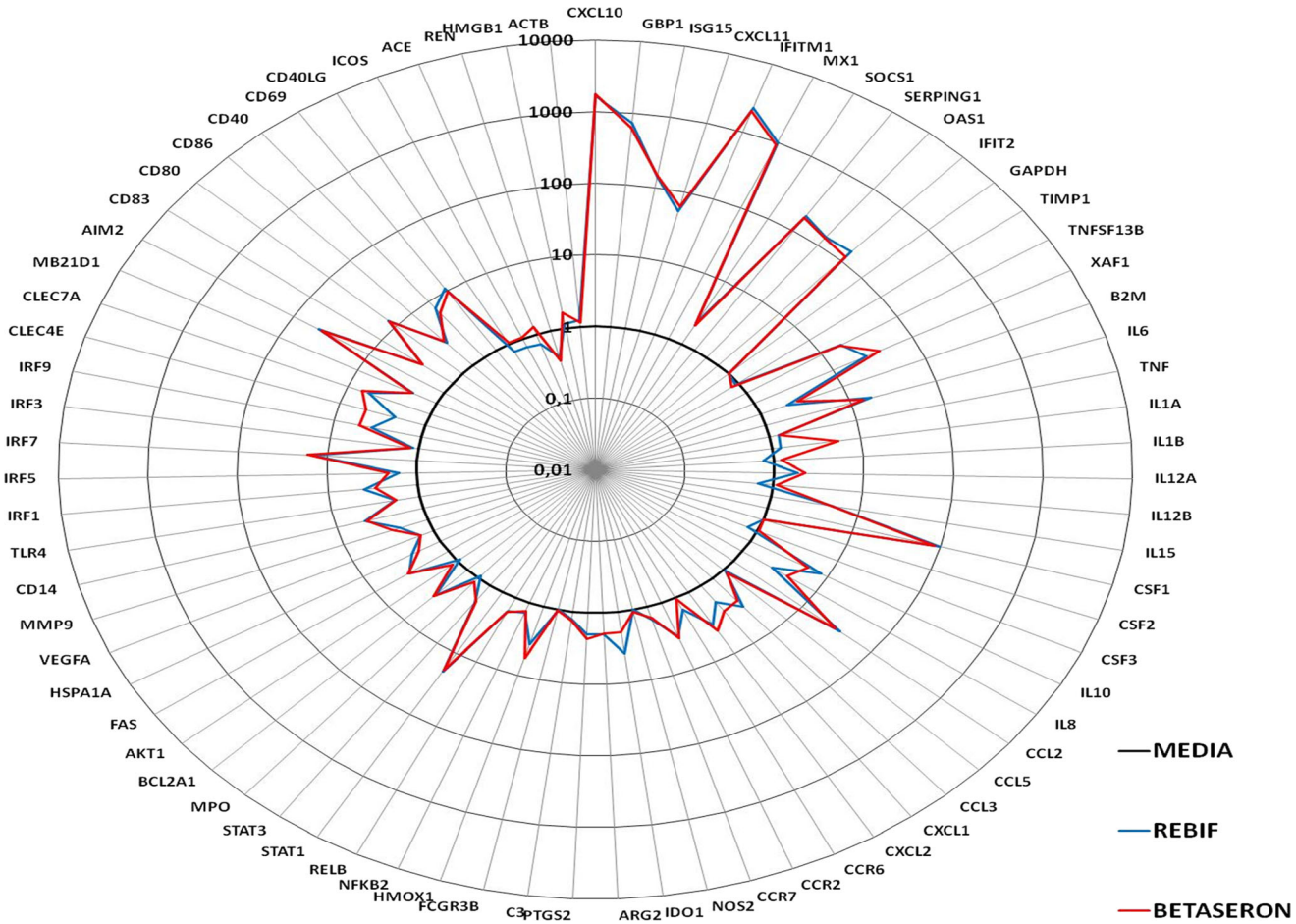

Fig. 1. MM6 cells induce a defined pattern of gene expression when stimulated with rhIFN-β. Message RNA expression in MM6 cells stimulated with 1000 IU/mL of Rebif or Betaseron for 24 h. Gene expression changes were assessed using custom-designed TaqMan® Micro Fluidic Cards that included known ISGs, cytokines, chemokines, chemokine receptors, co-stimulatory molecules, and pro-inflammatory genes. Fold changes were calculated by normalizing mRNA expression using a HKG and then expressed as fold increase over unstimulated/untreated samples (2–ΔΔCt method). Both products elicited a robust activation of multiple immune genes with significant overlap. The result shows mean from two experiments run in triplicate

the TNF family. Interestingly, at 4 h, the magnitude of the change in expression of ISG was different between the two products for a subgroup of genes, with Betaseron inducing higher levels of CXCL11, GBP1, and IFITM1. In addition, the cells stimulated with Betaseron had significantly higher mRNA levels for IL-6 and Ido1. This indicated that there are differences in the pattern of gene expression induced by different IFN-β products, and assessing a large number of genes on MM6 cells provides a sensitive platform to evaluate subtle differences in bioactivity between rhIFN-β products.

The differences in gene expression patterns could be rooted in structural or manufacturing differences (1). Indeed, we recently showed that Betaseron lots may contain trace levels of impurities capable of triggering TLR2 and TLR4 (14). We reasoned that IIRMI present in the product could influence the expression levels of specific genes within the array distorting the bioactivity measurements. To test whether the lots of Betaseron we were using contained IIRMIs that might modify the pattern of gene expression, we used mouse RAW cells, which can be activated by multiple TLR but do not have receptors for human type I IFNs. We observed that the cells cultured with Betaseron had increased levels of NF-kB activation, as well as increased expression of Nos2 and IL-1b as early as 2–4 h after stimulation, while the cells stimulated with Rebif did not (supplementary Fig. 4) suggesting that the difference in gene expression between Rebif and Betaseron could be due to trace levels of IIRMI as previously reported. Whether due to trace levels of impurities or to intrinsic differences between rhIFN-β-1a and rhIFN-β-1b, the assay monitoring the expression of an array of IFN-inducible genes on MM6 cells provided a very sensitive platform that detected subtle differences in rhIFN-β bioactivity, but also raised concern that trace levels of IIRMI in the products could impact a bioactivity assessment.

Use of MM6 Cells to Assess Bioactivity of rhIFN-β Products

Following the expiry of the patent protection for the rhIFN-β products, several non-innovator products have been licensed around the world (4,22) and more are under development. To examine whether the MM6-based assay could be useful in characterizing and comparing the bioactivity of rhIFN-β products, we obtained a biosimilar candidate for Rebif (IBA) and a biosimilar candidate for Betaseron

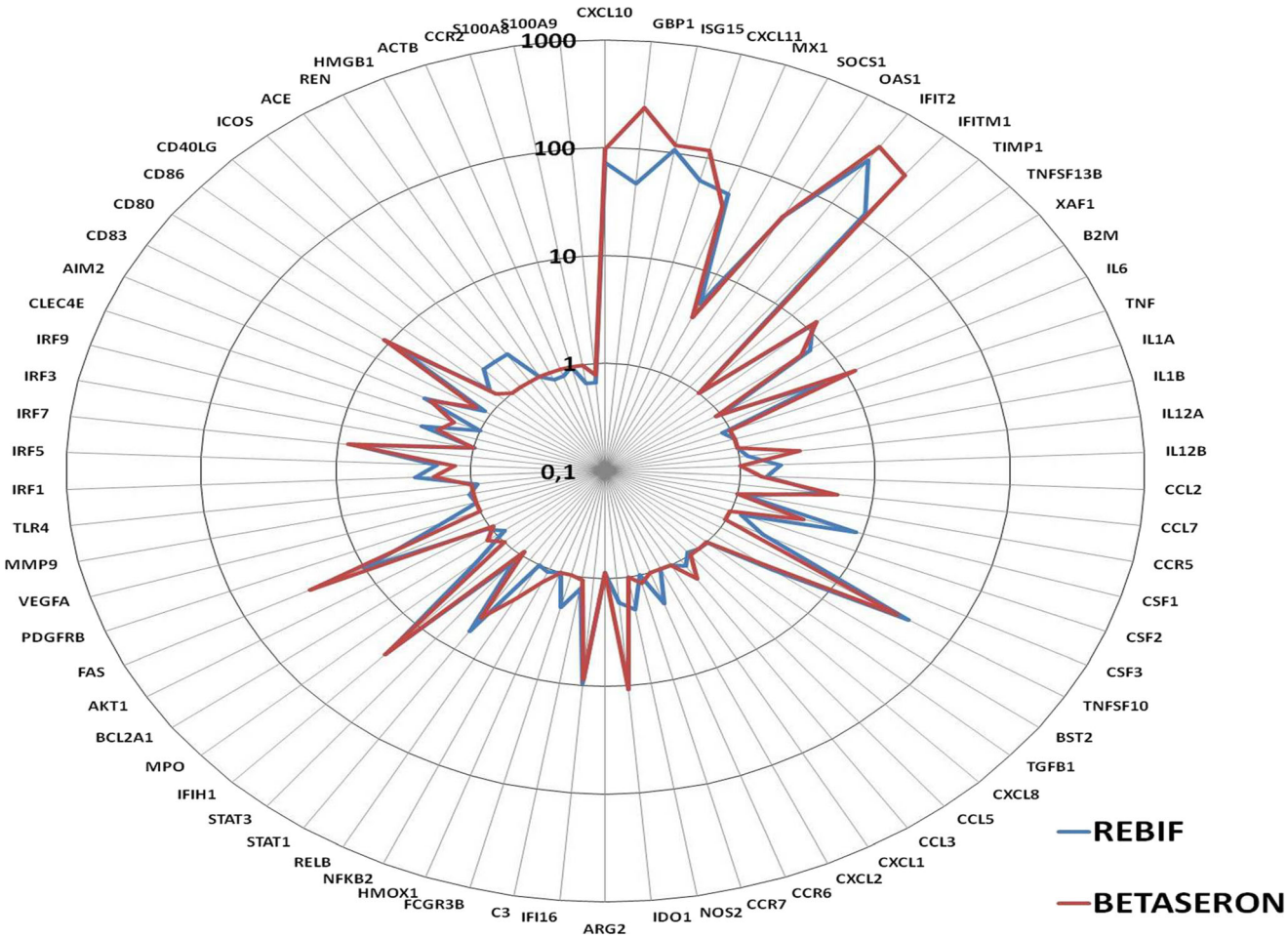

Fig. 2. Differential gene pattern expressed in MM6 cells stimulated with Rebif and Betaseron for 4 h. mRNA expression of MM6 cells stimulated for 4 h with 1 million international units (1 MIU) of Rebif or Betaseron, as described in Fig. 1. The result shows mean from two experiments run in triplicate and each point represents mean for each gene. Statistical analysis was carried out using one-way ANOVA followed by post-test multiple comparison. Note the differences in the magnitude of the change in gene expression for some specific ISGs including GBP1, IFITM1, CXCL11, and IDO1,between the two products

(IBO) as well as their licensed counterparts (23). The bioactivity of IBO and IBA had been adjusted by the manufacturer to match that of the corresponding innovator products by the manufacturer based on the results of the AVA assay.

Comparison of the gene expression pattern for IBA and Rebif using the MM6 cell–based assay showed that IBA replicated the gene expression pattern of Rebif after 4 h in culture (Fig. 3a). Although minor differences were evident in individual mean gene expression, none reached statistical significance. In contrast, when we compared the pattern of expression of Betaseron with IBO, significant differences became evident in the expression of a subset of ISGs (Fig. 3b). As shown in Fig. 3b, Betaseron had significantly higher levels of GBP1, CXCL11, and CXCL10, as well as increased mRNA expression of IL-6, CCL7, and CD80 than the biosimilar rhIFN-β-1b candidate, IBO. These results were confirmed by individual PCR reactions (Fig. 3c). This data suggested that even though the bioactivity of IBO had been matched to that of Betaseron based on the AVA, there were differences in some aspects of the bioactivity of the biosimilar candidate for rhIFN-β-1b which were evident when using the MM6 cell–based reporter assay.

Impurities in Betaseron and Potential Implications for Comparative Analytical Studies

Given that IBO, like Betaseron, is an rhIFN-β-1b manufactured on an *E. coli* platform, we next explored whether the difference in gene expression pattern could be rooted in a difference of impurities. Thus, we tested the IBA and IBO lots for IIRMI using the RAW Blue cells. As shown in Fig. 4 a and b, IBO, IBA, and Rebif did not induce NF-κB translocation to the nucleus, and IBO induced only minimal increase in mRNA levels for pro-inflammatory genes in RAW cells when compared to Betaseron, suggesting that the biosimilar candidate did not contain the same IIRMI as Betaseron. In previous studies, the IIRMI in Betaseron were shown to stimulate TLR2 and TLR4. To confirm that IBO did not contain IIRMI, we next determined whether the products would stimulate HEK-293 cells expressing individual TLR. As previously reported (14) and shown in Fig. 4c, Betaseron induced NF-κB activation in cells expressing TLR2 and TLR4, but not in the other TLR-expressing cells. Importantly, this product did not show any presence of endotoxin when tested using the LAL test (14). In contrast, IBA and IBO did not induce a similar response in these cells. Together, this

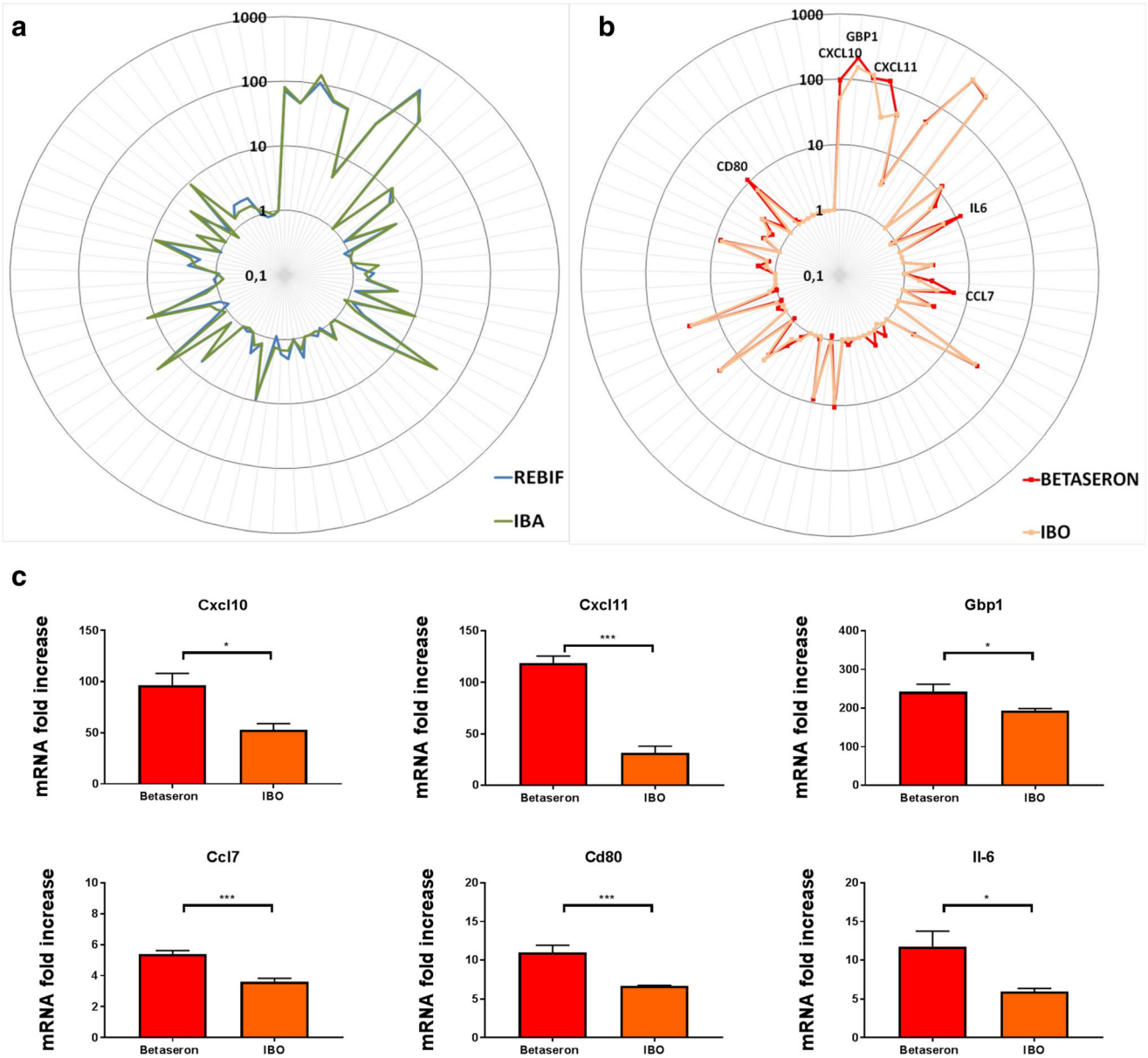

Fig. 3. Comparison of gene expression pattern induced by biosimilar candidates to Rebif and Betaseron. MM6 cells were stimulated *in vitro* with 1 MIU of **a** Rebif, and IBA, a biosimilar candidate to Rebif, or **b** Betaseron and IBO, a biosimilar candidate to Betaseron, for 4 h. The cells were harvested, and mRNA quantified using Taqman low-density array (TLDA) arrays as in Fig. 1. The label for individual genes was omitted for lack of space but can be found in Fig. 2. Note in **a** that Rebif and IBA induced an overlapping pattern of gene expression at 4 h. In contrast, in **b**, Betaseron and IBO differed in the magnitude of the mRNA expression for GBP1, CXCL10, CXCL11, IL6, CD80, and CCL7 genes. **c** Expression of GBP1, CXCL10, CXCL11, IL6, CD80, and CCL7 by cells stimulated with Betaseron or IBO as assessed by qPCR. Each bar represents mean from 3 different separate experiments performed in triplicate. Statistical significance was established using the Holm-Sidak test where $*p < 0.5$, $**p < 0.01$, and $***p < 0.001$

indicated that despite being produced in *E. coli*, the biosimilar candidates for rhIFN-β-1b did not contain trace levels of IIRMI and suggested that differences in impurities may underlie the differences in the pattern of gene expression by MM6 cells.

To confirm that the presence of IIRMI in the reference product could lead to a shift in gene expression pattern in the MM6 cell–based bioactivity assay, we spiked IBO with low levels of endotoxin (1 EU/mL) and Pam3CSK4 (10 ng/mL). As shown in Fig. 5, the addition of low levels of TLR2 and TLR4 agonists resulted in a change in the gene expression pattern for the product, with increased mRNA levels of CXCL11, CXCL10, and GBP1 as well as IL-6, CD80, and CCL7. Indeed, the pattern of gene expression replicated the one observed for Betaseron. Together, these data show that the assessment of multiple IFN-induced pathways by monitoring the expression of an array of genes provides a sensitive platform to evaluate and compare the bioactivity of IFN-β, detecting differences missed by the AVA assay. Importantly, the results of the assay also showed that trace levels of IIRMI impacted on the magnitude of expression of a subgroup of genes, which could lead to a misallocation of bioactivity,

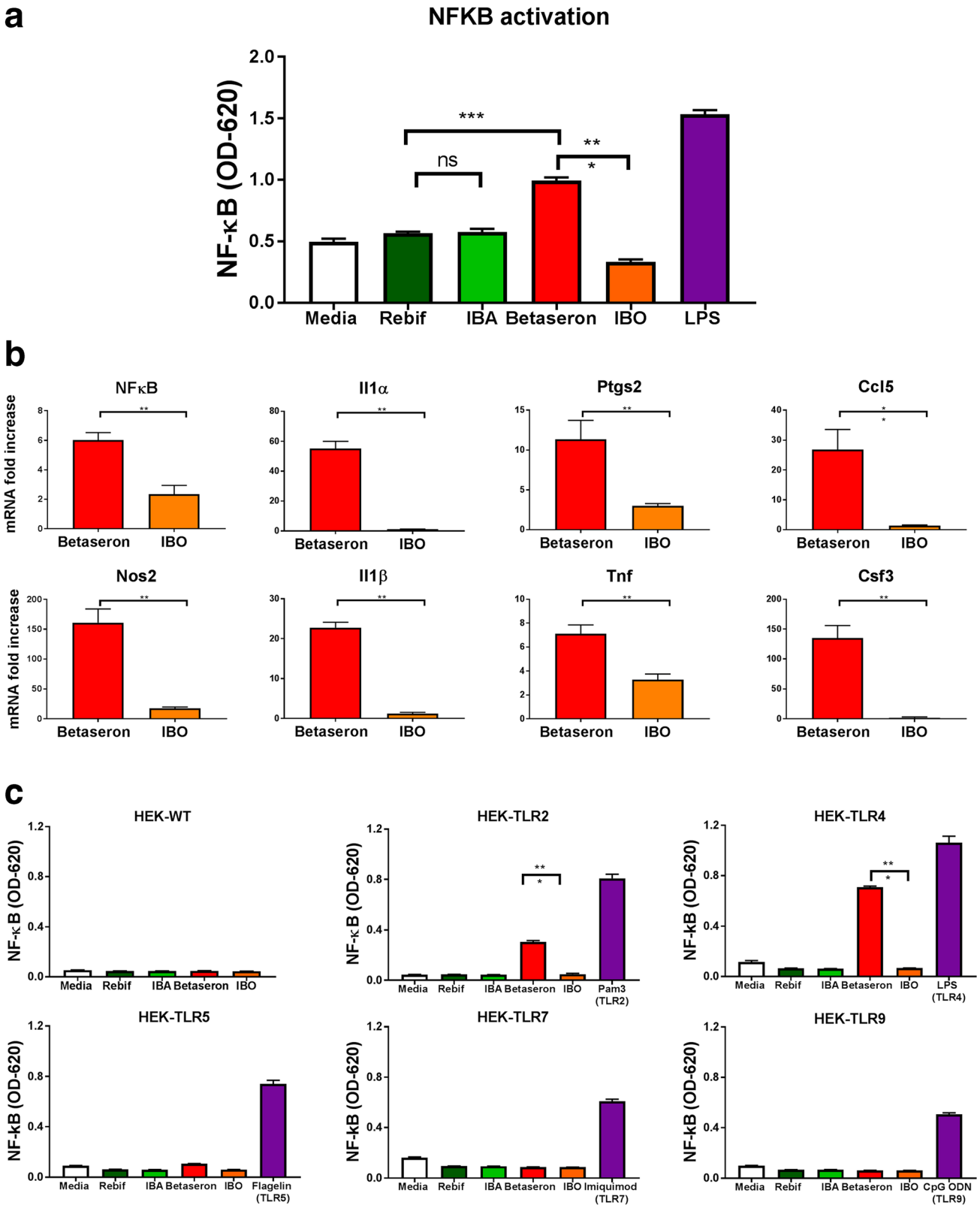

Fig. 4. Betaseron but not IBO has impurities that activate the NF-κB pathway and pro-inflammatory genes. **a** RAW-Blue cells carrying a SEAP-reporter inducible by NF-κB activation were incubated with Rebif, IBA, Betaseron, and IBO (1 MIU/mL) for 4 h. LPS was used as a positive control. Absorbance (OD, 620 nm) was read after 4 h in culture. **b** mRNA expression for specific genes induced by Betaseron and IBO in RAW cells was determined by qPCR. **c** HEK-BLUE cells expressing hTLR2, hTLR4, hTLR5, hTLR7, or hTLR9 were cultured with the Rebif, IBA, Betaseron, or IBO (1 MIU/mL), as well as the corresponding TLR-specific ligand. As shown, Betaseron induced activation of cells expressing TLR2 or TLR4 in HEK293 cells. Experiments were run in triplicate and each bar represents mean ± SD. Statistical differences were defined by ANOVA with Tukey's post-test (**a**) or the Holm-Sidak test (**b**). Statistical significance was defined as $p < 0.01$ (**) or $p < 0.001$ (***)

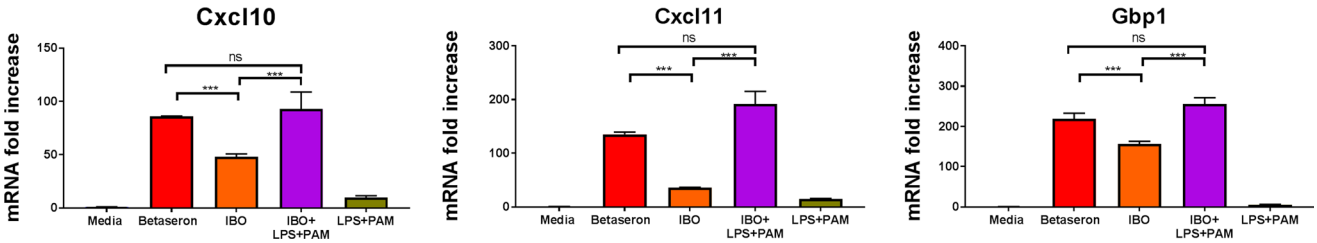

Fig. 5. Spiking of IBO-stimulated MM6 cells with TLR2 and TLR4 agonists reduces the difference in gene expression between Betaseron and IBO. MM6 cells were cultured for 4 h in the presence of Betaseron, IBO, IBO spiked with endotoxin (1 ng) and Pam3CSK4 (10 ng), or endotoxin (1 ng/mL) + Pam3CSK4 (10 ng/mL). mRNA levels were determined by PcR. Increased mRNA levels of ISGs such as CXCL10, CXCL11, and GBP1 were observed upon spiking of IBO. Data derived from mean ± SD from two independent experiments run in triplicate. Statistical significance was tested by ANOVA with Tukey's multiple comparison post-test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

particularly if the expression of a single gene was used as a biomarker of potency.

Trace Levels of IIRMI Do Not Impact on all ISGs

The pattern of gene expression for Betaseron and IBO differed only in the expression of CXC10, CXCL11, GBP1, CCL7, IL-6, and CD80, but the response of other genes in the array appeared similar. To confirm that the expression of these genes was not modified by the presence of IIRMI, MM6 cells were cultured in the presence of Betaseron, IBO, or IBO spiked with levels of endotoxin and Pam3Cys4 capable of inducing NF-κB activation and increased expression of ISGs such as CXCL10, CXCL11, GBP1, and IL-6. As shown in Fig. 6, the presence of spiked endotoxin or Pam3Cys4 did not modify the expression of ISGs MX1, OAS1, IRF7, STAT1, ISG15, IFITM1, TNFS13, or IL-12a, suggesting that these genes might be less sensitive to the presence of the IIRMI found in Betaseron when monitoring the bioactivity of rhIFNs.

DISCUSSION

Evaluating comparability following manufacturing changes or similarity to a reference product in terms of bioactivity for proteins that have complex biological activities is challenging, particularly when the mechanisms underlying the therapeutic effect are unknown. An example of this are the rhIFN-βs, which elicit complex immunomodulatory responses in patients, some that underlie the therapeutic effect and some that may be linked to adverse events as these patients can develop capillary leak syndrome, anaphylactic shock, thrombotic-thrombocytopenic purpura, insomnia, headache, alopecia, and depression. In this study, we propose a rapid and simple method for characterizing the bioactivity of rhIFN-β-1a and rhIFN-1b products by monitoring the expression of multiple genes in MM6 cells, a human monocytic cell line that expresses IFNAR1 and IFNAR2. The arrays used include several genes reported to be modulated by rhIFN-β in patients with RRMS, as well as genes linked to the inflammatory, immunomodulatory, antiviral, and pro-apoptotic activity of the product (7,24). We show that MM6 cells respond to rhIFN-β drugs by rapidly upregulating the expression of multiple genes in a time- and concentration-dependent manner. While rhIFN-β-1a and rhIFN-β-1b products induced a similar pattern of mRNA expression that was dose-dependent and consistent between lots, they showed product-specific differences in the magnitude of the induction for some genes including GBP1 and CXCL11. This indicated that this cell-based gene expression assay could be used to characterize the bioactivity of rhIFN-βs and detect small differences between products. When applied to compare the bioactivity of biosimilar candidates adjusted for potency based on the AVA assay, no differences were evident between the pattern of gene expression induced by Rebif and IBA, a CHO-manufactured rhIFN-β-1a biosimilar candidate. In contrast, the MM6-based method showed that IBO induced weaker expression for a subset of

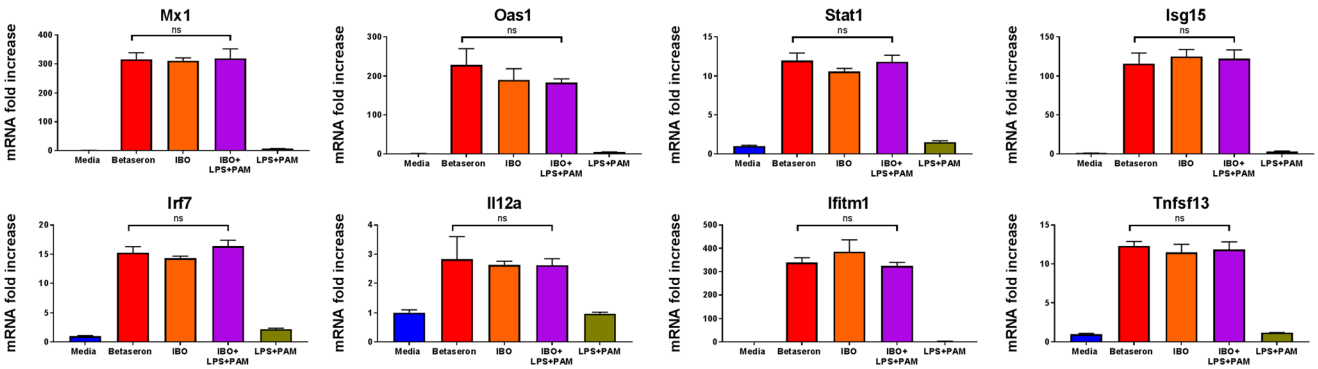

Fig. 6. Spiking IBO with TLR-2 and TLR-4 ligands does not affect the expression of all ISGs. MM6 cells were cultured for 4 h in the presence of Betaseron, IBO, IBO spiked with endotoxin (1 ng) and Pam3CSK4 (10 ng), or endotoxin (1 ng/mL) + Pam3CSK4 (10 ng/mL). mRNA levels were determined by PcR. Note that endotoxin and Pam3CSK4 do not increase the expression of MX1, STAT1, OAS1, IRF7, ISG15, IFITM1, TNFSF13, or IL12a genes in MM6 cells. Each bar represents mean ± SD from independent experiments run in triplicate. Statistical difference was tested by ANOVA; ns: not significant

genes as compared to Betaseron. Investigation into the source of the differences in gene expression pattern suggested a link between the presence of trace levels of TLR2 and TLR4-triggering IIRMI, which had been previously reported in Betaseron, and the difference in expression levels for a subset of ISGs in the MM6 bioactivity assay. Our results indicate that trace levels of IIRMI in the product can distort the expression pattern of a subset of genes, and this could have implications for comparative analytical studies between interferon products. Importantly, it is unlikely that product aggregates contributed to the NF-κB activation observed in RAW Blue or HEK cells incubated with Betaseron, as we showed in a previous study that increasing the aggregation of Betaseron or Rebif does not modify the level of NF-κB activation in RAW-Blue or in HEK-hTLR2 and HEK-hTLR4 cells (14). Together, our studies suggest that determining the expression levels for an array of reporter genes that monitor different pathways can be informative when characterizing a complex immunomodulatory product, but the sensitivity of each gene to potential impurities in the product should be examined to fully understand the results of the comparative analytical studies.

Multiple sclerosis (MS) is a chronic immune-modulated disease of the central nervous system (CNS), characterized by inflammation, demyelination, and axonal injury. Most patients with RRMS evolve overtime to the chronically progressive form. Disease exacerbations are characterized by increased blood brain barrier (BBB) permeability that allows activated cells to migrate into the CNS, leading to cytolytic lesions that affect primarily myelinated cells. rhIFN-βs, first approved in 1993, continue to be the first-line treatment for RRMS, as they reduce the number of lesions in CNS and the frequency of relapses (1). The proposed therapeutic effects of rhIFN-β in RRMS include the modulation of APCs, T cells, and B cell function, a direct anti-inflammatory effect on the BBB that reduces the influx of immune cells to the CNS, down-modulation of MHC-II expression and antigen presentation by astrocytes and microglia, cross-regulation with the IL-1-inflammasome pathway, and increase in apoptosis of pro-inflammatory T cells via upregulation of fas, TRAIL/TNFSF10, and XAF1, as well as IL-10 mediated fostering of Treg populations (1,6,25,26). To achieve its effect, rhIFN-β is known to bind to IFNAR1 and IFNAR2 to activate the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways leading to the phosphorylation and dimerization of STAT1 and STAT2 and recruitment of IRF3 or IRF7, which and are then translocated to the nucleus where they bind to ISREs initiating a chain of events that can result in the transcription of over 1000 genes. The precise combination of genes induced heavily dependent on the cell types stimulated and underlying immune status of the patient (27). Recent studies assessing the gene expression pattern in responders *vs* non-responders suggest that rhIFN-β induces a reproducible gene expression pattern in individual patients, and the balance between the pro- and anti-inflammatory effects of rhIFN-β appear to be critical, as non-responders tend to make lower IL-10 and have reduced ratios of IL-10 or IL-4 to IFN-γ, as well as a relative increase in the expression of ISGs, as compared to those who respond to therapy (1,28). Importantly, type I IFNs can also foster a pro-inflammatory response, increasing the expression of several chemokines and facilitating the activation of inflammasome via AIM1, NK cells via IL-15, and B cell antibody production via BAFF (1). Lastly, ISGs such as STAT1, IRF7, and IRF9 are involved in feedforward loops that further increase not only IFN-β but other type I IFNs levels as well. Thus, given the extraordinary multiplicity of rhIFN-β on and off target effects, a comprehensive characterization of the product's bioactivity may be useful in characterizing the product's bioactivity.

As described above, rhIFN-β products have traditionally used the AVA assay to estimate biological potency. While these assays test only a small fraction of rhIFN-β activity and the link between the antiviral and therapeutic effects is weak, the results are supported by many years of experience with the assay and a record of safety and efficacy established in extensive clinical studies. In the absence of supporting extensive clinical experience, such as following a substantial manufacturing change or for biosimilar candidates, having an additional assay that provides a broad and granular evaluation of rhIFN-β's bioactivity may strengthen the characterization of rhIFN-β products. While it may not be possible or practical to monitor all possible ISGs induced in multiple cell types, the use of a cell line that elicits a consistent pattern of response and the advent of multiplexed PCR, microarrays, and other arrays has made it possible and cost-effective to conduct detailed and informative studies monitoring the product's activity on multiple pathways. Indeed, these types of study could be validated in the GLP space to serve as informative potency assays for the products. Our data builds on the original gene reporter assays that monitored the individual expression of ISRE, MX1,or CXCL10, using 78 genes known to be sensitive to changes in IFN-β level, genes linked to the immunomodulatory and pro-apoptotic effects of rhIFN-β and genes that have been described as modulated by rhIFN-β in MS patients. As shown above, the pattern of gene expression in MM6 cells stimulated with Rebif largely overlapped with that of IBA, its biosimilar candidate, confirming that these products induce a reproducible gene expression pattern. While it is not possible to ensure that the subset of genes we selected is representative of expression levels of all possible ISGs, the broad multi-gene assessment of bioactivity suggests that the two products have analogous bioactivity. In contrast, the studies comparing IBO to Betaseron identified 6 genes with significantly lower fold increase in mRNA expression. Thus, the assay identified differences between the innovator and its biosimilar candidate that had not been evident when using the AVA assay and would not have been captured by monitoring the expression of MX1, which is not different between the products. Importantly, the differences were evident in only a subset of genes, underscoring the importance of testing multiple different responses when characterizing the bioactivity of rhIFN-β. The subset of genes differentially expressed in Betaseron and IBO-stimulated cells included CXCL10, CXCL11, GBP1, CCL7, IL-6, and CD80. These genes are associated with increased inflammation. CCL7, CXCL10, and CXCL11 are thought to play a key role in recruiting T cells to the CNS; CXCL10 and CXCL11 are ligands of CXCR3, which is increased in TH1/17 responses in MS patients; and GBP1, IL-6, and CD80 are markers of monocyte/macrophage activation. Without understanding the impact of potential impurities, the relatively lower level of expression of these genes could confound the comparison of bioactivity suggesting that the biosimilar candidate has decreased bioactivity compared to the reference product.

In previous studies, we had shown that cell-based assays that monitor cytokine expression can detect IIRMI that could impact on the immunogenicity risk of therapeutics including IFN-β (29,30). Here, our studies show that assessing the expression levels for an array of reporter genes that monitor different drug-induced activation pathways can be informative when characterizing or comparing the bioactivity of complex immunomodulatory product; however, the presence of IIRMI can confound the assessment of product bioactivity.

CONCLUSION

Monitoring the transcriptomic profile can be informative when characterizing or comparing the bioactivity of complex products; however, the presence of IIRMI can confound the assessment of product bioactivity, particularly in immunomodulatory products.

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