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ABSTRACT

Several human autoimmune diseases are characterized by increased expression of type I IFN-stimulated genes in both the peripheral blood and tissue. The contributions of different type I IFNs to this gene signature are uncertain as the type I IFN family consists of 13 alphas and one each of β, ε, κ, and ω subtypes. We sought to investigate the contribution of various IFNs to IFN signaling in primary human cell types. We stimulated primary skin, muscle, kidney, and PBMCs from normal healthy human donors with various TLR ligands and measured the expression of type I IFN subtypes and activation of downstream signaling by quantitative PCR. We show that IFNB1 is the dominant type I IFN expressed upon TLR3 and TLR4 stimulation, and its expression profile is associated with subsequent MX1 transcription. Furthermore, using an IFN-β–specific neutralizing Ab, we show that MX1 expression is inhibited in a dose-dependent manner, suggesting that IFN-β is the primary driver of IFN-stimulated genes following TLR3 and TLR4 engagement. Stimulation with TLR7/8 and TLR9 ligands induced IFNB1 and IFNA subtypes and MX1 expression only in PBMCs and not in tissue resident cell types. Concordantly, IFN-β neutralization had no effect on MX1 expression in PBMCs potentially because of the combination of IFNB1 and IFNA expression. Combined, these data highlight the potential role for IFN-β in driving local inflammatory responses in clinically relevant human tissue types and opportunities to treat local inflammation by targeting IFN-β.


INTRODUCTION

Type I IFNs are secreted pleiotropic cytokines that play a pivotal role in innate and adaptive immune responses against pathogen invasion. Among the various type I IFNs known, the most well studied are IFN-α (13 IFN-α subtypes exist, each encoded by a different gene) and IFN-β (1). Although these cytokines play a crucial role in pathogen defense, their sustained production can result in inflammation (2). Type I IFNs are linked to the pathogenesis of autoimmune disorders, including dermatomyositis (DM), lupus nephritis (LN), cutaneous lupus erythematosus (CLE), and systemic lupus erythematosus (SLE) (3–6) as well as monogenic autoimmune disorders such as STING-associated vasculopathy of infancy and Aicardi-Goutièrès syndrome (7, 8). Specifically, correlation of type I IFN gene signature with disease severity has been reported in DM, LN, and SLE (6, 9–11). One study found that ectopic stimulation of cultured muscle cells with purified IFN-α or IFN-β phenocopied the molecular

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Abbreviations used in this article: CLE, cutaneous lupus erythematosus; Ct, cycle threshold; DM, dermatomyositis; GMC, glomerular mesangial cell; LN, lupus nephritis; NHDF, normal human dermal fibroblast; poly I:C, polyinosinic-polycytidylic acid; SLE, systemic lupus erythematosus; SMC, skeletal muscle cell.

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signature found in DM muscle biopsies with stronger correlation to IFN-β stimulation than IFN-α (12). Elevated blood IFN-β levels, but not IFN-α, also correlate with high type I IFN gene signature and DM disease activity (5, 9, 13). Elevated skin IFNB1 transcript, but not IFNA, IFNK, or IFNO transcripts, also correlate with skin type I IFN gene signature in DM (14).

The correlation of IFN-stimulated genes with disease severity has led to recent clinical studies to treat SLE by neutralizing IFN-α subtypes (sifalimumab, rontalizumab) or by neutralizing IFNAR1 (anifrolumab) (15–17). Phase IIb clinical studies with the various mAbs showed mixed results, with rontalizumab failing to meet its primary and secondary endpoints (18). Sifalimumab met its primary, but not secondary endpoints, with modest therapeutic effect (19). Anifrolumab met both primary and secondary endpoints, and treated patients showed improvement over patients receiving placebo, although the result was not dose dependent and only observed in patients with a high IFN gene signature at baseline (20, 21). Combined, these results suggest that targeting IFN-α alone may not be sufficient to reduce modest to severe SLE.

We sought to identify which type I IFNs are induced in clinically relevant primary human cells when challenged with TLR ligands. Heightened expression of TLRs in local tissue and infiltrating cells has been observed in DM and polymyositis (PM), highlighting a potential feedback loop for inflammation (22). In this study, we used primary human cells from healthy donors that represent tissues involved in DM, CLE, LN, and SLE pathologies: dermal fibroblasts, skeletal muscle cells (SMC), glomerular mesangial cells (GMC), and PBMCs. We measured the expression profile of IFNB1 and IFNA subtypes in response to TLR3, TLR4, TLR7/8, and TLR9 activation. To evaluate downstream IFNAR signaling, we measured expression of MX1, a clinical marker for type I IFN activity that is overexpressed in DM, CLE, LN, and SLE patients (23–26). In addition, we used a high-affinity anti–IFN-β Ab to assess the specific role of IFN-β in driving MX1 expression in the various cell types. This study identifies the expression pattern of type I IFN species in human disease-relevant tissues and suggests a novel role for IFN-β in driving local inflammation and tissue injury that may contribute to the autoimmune disease phenotype.

**MATERIALS AND METHODS**

**Reagents**

Polyinosinic:polycytidylic acid (poly I:C; P9582) and LPS (L2755) were purchased from Sigma-Aldrich. R848 (Tlr1-r848) and ODN2216 (Tlr7-2216) were purchased from Invivogen. Anti–IFN-β Ab was generated in-house using licensed commercially available IFN-β (Peprotech) as Ag. Anti-human IFN-β Abs were generated by immunizing BALB/c mice with IFN-β and fusing mouse splenocytes with NS-1 myeloma cells. Hybridomas were screened for IFN-β binders, and leading candidates were humanized and underwent affinity maturation to improve affinity. The final IgG1 candidate was shown to have a neutralizing affinity <20 pM to human IFN-β and no cross-reactivity to mouse or rat IFN-β. Isotype control Ab was generated in-house and showed no binding to any IFN. All procedures performed on animals were in accordance with regulations and established guidelines and were reviewed and approved by an institutional animal care and use committee.

**Specificity of anti–IFN-β Ab**

IFN-β (300-02BC; Peprotech) and IFN-α2 (11100-1; PBL Assay Science) were diluted with anti–IFN-β in RPMI. The final Ab concentrations ranged from 100 to $1 \times 10^3$ nM. The IFNs were diluted to a final concentration of 20 pM (IFN-β) or 50 pM (IFN-α2) with medium alone or a dose curve of the anti–IFN-β Ab in a final volume of 50 μl and incubated for 2 h at 37°C. U-937 cells were cultured in RPMI supplemented with 10% FBS, 1× Glutamax (35050061; Thermo Fisher Scientific) and harvested just before stimulation. Cells were counted and resuspended at 2 × 105 cells/ml in RPMI. Fifty microliters of cells was added to 50 μl of diluted α–IFNAR Ab and incubated for 2 h. After the incubations, the IFNs and diluted Abs were mixed with cells (100,000 per well) and incubated at 37°C for 15 min.

At the conclusion of the incubation, an equal volume of Cytofix (554655; BD Biosciences) was added per well, and the plate was returned to 37°C for 15 min. After fixation, plates were centrifuged at 1500 rpm for 5 min to pellet cells. Cells were washed with 200 μl of PBS and resuspended in 100 μl of 1× Phosflow Perm Buffer IV (612597; BD Biosciences) for 15 min at room temperature. After incubation, plates were centrifuged and washed as described above. Cells were resuspended in 100 μl of PBS with 5% FBS (FACS buffer) containing TruStain FcX (422302; BioLegend) and incubated for 10 min at 4°C. After incubation, 10 μl of AF647 mouse anti–STAT1 (pY701) Ab (612597; BD Biosciences) was added per well and incubated for 20 min. Next, 100 μl of FACS buffer was added per well and centrifuged as described above. The wash was repeated, and samples were resuspended in a final volume of 120 μl of FACS buffer for data acquisition on the Fortessa flow cytometer (BD Biosciences).

Data were analyzed using FlowJo software (TreeStar); forward scatter (light) channel versus side scatter (light) channel was used to identify cells, and the geometric mean fluorescence intensity of the pSTAT1 signal was determined. EC50, EC95, and IC50 analyses were done using GraphPad Prism v 6.0. Data were transformed (concentration X = log [x]), then a nonlinear regression analysis using a 4-parameter slope of log (inhibitor/stimulator) versus response (geometric mean fluorescence intensity) was performed (Supplemental Fig. 1).

**Cells and cell culture**

Normal human dermal fibroblasts (NHDF) (CC-2511; Lonza) were cultured in FGM-2 media (CC-3146; Lonza), and GMC (CC-2559; Lonza) were cultured in MsGM media (CC-3146; Lonza). SMC (SK-1111; Cook Myosite) were cultured in Myotonic Growth Medium (MK-4444; Cook Myosite). At least five donors of each cell type were purchased for experiments. PBMCs were isolated from healthy donor blood using Ficoll-Paque Plus separation medium (17-1440-03; GE Healthcare) in Leucosep tubes (227290;
Greiner Bio-One) and cultured in RPMI 1640 (1875-093; Life Technologies) supplemented with 10% FBS (16140-071; Life Technologies). Informed consent was obtained from all human subjects in accordance with Pfizer Institutional Review Board guidelines.

Real-time quantitative PCR, data analyses, and heatmap generation
RNA was isolated using RNeasy Micro Kit (74004; Qiagen), and cDNA was synthesized using a high-capacity cDNA reverse transcription kit (4368813; Applied Biosystems). TaqMan assays were purchased from Applied Biosystems. For heatmap generation, four independent donors of each cell type were assayed and the results were averaged. For neutralization studies, at least five independent donors were used except where noted. Samples from each donor were run in duplicate and averaged. Data are displayed as the average with each individual donor plotted. Relative quantification was obtained using the ΔΔCt method. Individual primer efficiency was tested and ranged from 91.99 to 109.37%.

Neutralization studies
Primary human cells were stimulated with LPS, poly I:C, R848, or ODN2216 at the minimal dose that generated robust MX1 expression (determined empirically for each cell line and stimulant). Neutralizing IFN-β Ab (100 nM) or a dose curve of 0.1–100 nM was added to cells concurrently with TLR ligands and incubated with cells for varying times (see figures for details). RNA was isolated and analyzed by TaqMan quantitative PCR as described above. Expression of MX1 was measured as a relative amount per 10 ng of RNA per sample.

RESULTS
IFNB1 is the dominant type I IFN produced in somatic cell types
Primary human cells were chosen based on their relevance to a variety of autoimmune disorders. We obtained primary human cells from normal healthy donors and exposed them to a range of stimulatory ligands to mimic various pathogen- or danger-associated molecular patterns that may trigger the production of an immune response. In NHDF, GMC, and SMC samples, both the TLR3 ligand poly I:C and the TLR4 ligand LPS induced a robust IFNB1 response, whereas IFNA expression was mostly unaffected (Fig. 1). The highest expression of IFNA was in the NHDF, where one α subtype, IFNA5, showed a 6-fold expression increase over medium alone, whereas the corresponding IFNB1 increase was 2200-fold (Fig. 1A). Following poly I:C and LPS stimulation, the IFNB1 transcript was upregulated and trailed at later time points by a concomitant increase in MX1 expression. With the TLR 7/8 and 9 ligands, R848 and ODN2216, respectively, little IFNB1 or IFNA expression was seen, and therefore, no MX1 expression was seen either.

To assess the contribution of IFN-β protein to drive expression of MX1, we stimulated all the cell types in the presence of a neutralizing anti–IFN-β Ab (0.1–100 nM). The time frame and

IFN ELISA
Cell supernatants were collected and run neat (IFN-β) or diluted 1:10 (IFN-α). IFN-β ELISA (41415-1; PBL Assay Science) and IFN-α multisubtype (41105-1; PBL Assay Science) were run according to the manufacturer’s specifications.

FIGURE 1. IFNB1 is the primary IFN generated in nonhematopoietic cells.
Heat map depiction of IFNB1, MX1, and IFNA subtype transcript expression on a log scale in (A) donor-derived NHDF, (B) GMC, and (C) SMC. Cells were stimulated with the following: 25 μg/ml (NHDF, GM) or 125 μg/ml (SMC) of poly I:C, 5 ng/ml (NDHF, GMC) or 25 ng/ml (SMC) of LPS, 5 μg/ml of R848, and 5 μM of ODN 2216. The number following the ligand name is the length of stimulation. Each cell in the heat map represents the average result of at least four individual donors. Results are displayed as log2 of ΔΔCt of the gene of interest compared with the geometric mean of GUSB and B2M as endogenous controls.

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A dose of stimulant were chosen empirically for each cell type, based on minimum dose of agonist required to observe maximal MX1 induction (Supplemental Fig. 2A, 2B). Addition of the neutralizing Ab resulted in a dose-dependent decrease in MX1 expression, whereas IFNB1 expression levels were unchanged (data not shown). A nonneutralizing control IgG had no effect on MX1 levels (Fig. 2, Supplemental Fig. 3A–C, 3E–G).

**PBMCs generate IFNB1 and IFNA in a ligand-dependent manner**

As PBMCs have been widely studied and validated as a major source of IFN-α in blood (27), we investigated IFNA, IFNB1, and MX1 transcript levels following TLR activation. We stimulated PBMCs with all four TLR ligands in a dose- and time-dependent manner to measure IFNA, IFNB1, and MX1 expression. Of all cell types tested, only PBMCs responded to R848 and ODN stimulation and upregulated IFNB1 and IFNA transcripts in response to the ligands. These data show that among the cell types tested, only PBMCs respond to TLR7/8/9 ligands and express both IFNB1 and IFNA subtypes (Fig. 3A, Supplemental Fig. 2C, 2D).

When PBMCs were stimulated with poly I:C and LPS, however, it was predominately IFNB1 that was upregulated with little to no expression of the IFNA subtypes. To explore the contribution of IFN-β or IFN-α subtypes to MX1 expression following stimulation with all the ligands, we inhibited IFN-β with a dose curve of anti–IFN-β neutralizing Ab. With both TLR3 and 4 stimulation, we observed a significant reduction of MX1 expression, further demonstrating the significance of IFN-β expression following TLR3 and 4 engagement (Fig. 3B, 3C, Supplemental Fig. 3D, 3H). However, we were unable to alter MX1 expression in PBMCs stimulated with R848 or ODN2216, likely because of the abundant expression of IFN-α subtypes (Fig. 3D, 3E, Supplemental Fig. 3I, 3J). To confirm expression of IFN-β and IFN-α subtypes, we measured protein concentration by ELISA (Fig. 3F). Supernatant from stimulated cells of two donors was collected after 8 h and analyzed by an IFN-β–specific ELISA and a pan–IFN-α ELISA. IFN-β was only detected in cultures from one donor stimulated with ODN2216 and was present at 1/50th of the pan–IFN-α levels observed with the same donor. IFN-α was observed from both donors with both stimuli. The contribution of individual IFN-α subtypes is difficult to determine because of the lack of selective reagents. This result, combined with the inability of the anti–IFN-β Ab to impact MX1 expression, suggests that IFN-α is the primary type 1 IFN driving MX1 expression following TLR7/8/9 stimulation in PBMCs.

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**FIGURE 2. Anti–IFN-β Ab blocks IFN signaling in nonhematopoietic cells.**

NHDF (A and B), GMC (C and D), and SMC (E and F) were stimulated with LPS for 5 h (A, C, and E) or poly I:C for 6 h (B, D, and F) in the presence of medium, control IgG, or anti–IFN-β Ab. The TLR ligands and Ab were given at the same time. MX1 expression was measured, and the relative quantification (RQ) was calculated by transforming the log2 value of ΔΔCt of MX1 compared with the geometric mean of GUSB and B2M as endogenous controls. As there was donor-to-donor variation, the response of each donor to ligand only was set to 1, and the results are displayed as a percentage of the ligand-only response. Each data point represents the average of two to three independent biological replicates of five independent healthy donors per condition (except LPS-stimulated GMC, which only had three donors).

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Differential gene expression of TLRs in primary human cells

We assessed TLR transcript levels in unstimulated cells to determine if TLR expression correlated with response (Supplemental Fig. 4). Consistent with the capacity of all these cell types to respond to poly I:C and LPS, TLR3 and TLR4 transcript was detected in all cells. In contrast, basal TLR7 was only detected in PBMCs, and a small amount was detected in GMC. TLR8 was undetectable in all the cell types except PBMCs. Whereas a response to ODN2216 was observed only in PBMCs, TLR9 transcripts were detectable in all cell types, albeit at levels far reduced compared with PBMCs.

DISCUSSION

Clinical evidence has implicated circulating levels of type I IFNs in the pathogenesis of autoimmune diseases like SLE, CLE, and LN.
(6). However, the potential for IFN-β to contribute to substantial local inflammation and tissue destruction remained unexplored. Measuring IFNs in diseased tissue faces technical and biological challenges that have largely prohibited researchers from identifying IFN-β or distinguishing between IFN-α subtypes in serum and tissues from patients with autoimmune disorders. Given the heterogeneous nature of autoimmune disease, the underlying triggers for the type I IFN response are unknown. Thus, only measuring circulating IFNs may be a poor clinical biomarker for triggers for the type I IFN response are unknown. Thus, only measuring circulating IFNs may be a poor clinical biomarker for determining the contribution of those IFNs to disease. This study identifies IFNβ1 as the major type I IFN produced by primary human disease-relevant cells when challenged with various TLR ligands. Furthermore, we show that neutralizing the production of IFN-β in nonlymphoid primary cells can abrogate downstream IFN signaling.

Of note, the primary cells used in this study were from healthy donors. Cells from diseased tissue may amplify the type I IFN response through upregulation of TLR3, TLR7, and TLR9 as reported in PBMCs from SLE patients (28), overexpression of TLR4 and TLR9 as seen in DM muscle tissues (29), and glomerular expression of TLR9 as observed in LN but not in normal kidneys (30). These changes may render tissues susceptible to additional upregulation of IFN-β. Using anti–IFN-β Ab to block downstream IFNAR signaling (as evidenced by loss of MX1 expression) following stimulation in nonhematopoietic cells may interfere with a cascade of inflammatory signaling (Fig. 2). To address this question in vivo, we have an ongoing phase II clinical trial in humans with DM comparing anti–IFN-β Ab to placebo control with a readout expected in 2021 (ClinicalTrials.gov identifier: NCT03181893).

Stimulation of TLR7/8 and TLR9 with R848 and ODN2216, respectively, resulted in a type I IFN response only in PBMCs, (Fig. 3) which correlated with the expression of their cognate receptors. As we did not isolate individual cell types out of the PBMC population, the differing responses we observed based on ligand could be due to multiple cell types responding to different ligands. Although plasmacytoid dendritic cells likely make up the bulk of the TLR7/8 and 9 signaling, conventional (myeloid) dendritic cells could be responsible for the IFN-β produced following TLR3 and 4 engagement (31). The expression patterns of type I IFN in PBMCs were confirmed by the detection of IFN protein levels, with cumulative IFN-α protein detected at much greater concentrations than IFN-β (Fig. 3F).

Regardless of the stimulation, when IFN-β was the predominant IFN generated, the neutralization of the protein with an anti–IFN-β Ab led to an abrogation of MX1 expression. Given that the underlying causes for DM and lupus have not been established, questions remain as to how IFN-β and IFN-α can initiate or modulate disease. The source of IFN in these diseases could come from the resident tissues where the disease manifests, from infiltrating immune cells, or some combination of the two. If disease is initiated or maintained through resident tissue cells, then neutralization of IFN-β in that tissue may result in both decreased IFN-β–driven local inflammation and concomitant recruitment of immune cells to the tissue. Additionally, a reduction of IFN-β may result in a decrease of pathogen-associated molecular pattern receptors, therefore increasing the threshold of signaling required to initiate or maintain a response. Further profiling of DM and lupus tissues may elucidate the source and effect of localized IFN induction.

In summary, our results indicate that IFN-β is the dominant type I IFN induced in human-derived primary tissue resident cells when challenged with various TLR ligands. These findings suggest that IFN-β may have a critical role in local tissue damage and autoimmune disease modulation that has previously been underappreciated. Furthermore, specific neutralization of IFN-β resulted in an attenuation of MX1 expression, indicating that IFN-β inhibition could be a novel therapy in the treatment of DM, lupus, and other IFNopathies.

DISCLOSURES


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REFERENCES


