IFN-β1a Inhibits the Secretion of Th17-Polarizing Cytokines in Human Dendritic Cells via TLR7 Up-Regulation[1]

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IFN-β, an effective therapy against relapsing-remitting multiple sclerosis, is naturally secreted during the innate immune response against viral pathogens. The objective of this study was to characterize the immunomodulatory mechanisms of IFN-β targeting innate immune response and their effects on dendritic cell (DC)-mediated regulation of T cell differentiation. We found that IFN-β1a in vitro treatment of human monocyte-derived DCs induced the expression of TLR7 and the members of its downstream signaling pathway, including MyD88, IL-1R-associated kinase 4, and TNF receptor-associated factor 6, while it inhibited the expression of IL-1R. Using small interfering RNA *TLR7* gene silencing, we confirmed that IFN-β1a-induced changes in MyD88, IL-1R-associated kinase 4, and IL-1R expression were dependent on TLR7. TLR7 expression was also necessary for the IFN-β1a-induced inhibition of IL-1β and IL-23 and the induction of IL-27 secretion by DCs. Supernatant transfer experiments confirmed that IFN-β1a-induced changes in DC cytokine secretion inhibit Th17 cell differentiation as evidenced by the inhibition of retinoic acid-related orphan nuclear hormone receptor C and *IL-17A* gene expression and IL-17A secretion. Our study has identified a novel therapeutic mechanism of IFN-β1a that selectively targets the autoimmune response in multiple sclerosis. *The Journal of Immunology,* 2009, 182: 3928–3936.

Multiple sclerosis (MS)[3] is a chronic inflammatory CNS disease initiated by sensitization of the immune system to CNS myelin Ags (1). IFN-β1a has been used for many years as a first-line therapy for releasing relapsing (RR) MS. Although its efficacy in suppressing disease activity is well documented in large randomized placebo-controlled clinical trials (2), its in vivo operative mechanisms of action are not completely elucidated. IFN-β1a's reported mechanisms of action include inhibition of MHC class II expression on monocytes and microglia, suppression of T cell proliferation, regulation of IL-12/IL-10 cytokine transcription, and inhibition of inflammatory cell migration into the CNS via interference with VLA-4-mediated cell adhesion and matrix metalloproteinase 9 activity (3). However, IFN-β's effects are complex and cellular responses to IFN-β involve >500 genes (4), suggesting that some of the relevant mechanisms of action may still not be identified. Although IFN-β is secreted during the antiviral innate immune response, the physiological role of endogenous type I IFNs in the regulation of the adaptive immune response is poorly understood. We propose that the administration of high-dose recombinant IFN-β1a, which provides a strong therapeutic effect, may enhance the naturally operative IFN-β-mediated control of the autoimmune response. We studied the effects of IFN-β in the context of recent advances in our understanding of the role of the innate immune response (5) and Th17 cells (6) in the development of the autoimmune response.

The discovery of the Th17 cell lineage marked a new era in the studies of the autoimmune response, as it resolved many controversial findings not compatible with the Th1 paradigm of autoimmune response (7). Similarly, the therapeutic effect of IFN-β in MS, which was associated with the up-regulation of IL-12Rβ2 and CCR5 (8), was difficult to reconcile with the previously proposed IFN-β's mechanism of action targeting the Th1-mediated immune response. Recent studies have indicated that Th17 cells play a critical role in the development of the autoimmune response in MS. IL-17 gene and protein expression is elevated in active MS brain lesions (9, 10), as well as in the mononuclear cells derived from the blood and cerebrospinal fluid of MS patients in comparison to healthy controls (11). Th17 cell differentiation in humans is orchestrated by multiple cytokines, including IL-6, IL-1β, TGF-β, IL-21, and IL-23, which stimulate. and IFN-γ, IL-4, IL-12, IL-10, and IL-27, which inhibit the differentiation of this cell subset (6, 12).

Although significant progress in the understanding of the pathogenesis of MS has been achieved over the past decades, the character of the initial disease-triggering event is still poorly understood. Most studies propose that autoreactive T cells, which are represented in the normal TCR repertoire (13), initiate an autoimmune response upon activation in the peripheral circulation. It is proposed that bacterial and viral infections can trigger the onset and/or relapses of the disease via mechanisms of molecular mimicry (14) and via induction of TLR signaling (5). However, only recently was the innate immune response, involved in the first-line defense against microbial pathogens, found to play a role in the regulation of the Ag-specific adaptive immune response implicated

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[3] Abbreviations used in this paper: MS, multiple sclerosis; RR, relapsing remitting; DC, dendritic cell; IRAK4, IL-1R-associated kinase 4; TRAF6, TNFR-associated factor 6; RORC, retinoic acid-related orphan nuclear hormone receptor C; CIS, clinically isolated syndrome suggestive of MS; qRT-PCR, quantitative RT-PCR; IRF, IFN regulatory factor; MRI, magnetic resonance imaging; Pen/Strep, penicillin/streptomycin; siRNA, small interfering RNA.

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in the pathogenesis of the autoimmune response in MS (15). The direct immunoregulatory properties of TLR signaling pathways should be explored, since they may explain the old observation that bacterial and parasitic infections induce protection against subsequent development of autoimmune diseases (16). This "hygiene hypothesis" offers an explanation for the high prevalence of the autoimmune diseases in developed countries with low exposure to infections in childhood (17).

TLR signaling modifies costimulatory molecule expression and cytokine secretion in dendritic cells (DCs). The TLR family is the best-characterized class of pathogen-associated molecular pattern-recognizing receptors, encompassing 10 TLRs in humans (5). TLR1, 2, 4, 5, 6, and 10 recognize bacterial products, while TLR3, 7, 8, and 9 detect viral nucleic acids (18). TLRs activate two signaling pathways: the MyD88-dependent pathway that leads to the NF-κB activation and inflammatory cytokine transcription and the TIR domain-containing adapter-inducing IFN-β-mediated pathway leading to type I IFN production and the induction of IFN-mediated genes (19). Although TLR signaling provides the strongest stimulus for IFN-β secretion during the antimicrobial innate immune response (20), IFN-β in turn induces the expression of TLR1, 2, 3, 4, and 7 (21), which enhance endogenous IFN-β secretion through an autocrine loop.

We found that IFN-β1a treatment induced the expression of TLR7 and the members of its downstream signaling pathway. TLR7 is primarily expressed on plasmacytoid DCs, which produce high levels of IFN-β, but also on monocyte-derived DCs, which secrete multiple cytokines involved in T cell differentiation (21, 22). TLR7 is expressed in the endosomal vesicles, where its response is restricted to the guanosine and uridine-rich ssRNA that is produced during viral replication (23). TLR7 signals via MyD88, a cytosolic protein that is recruited to the receptor complex, where it functions as an adaptor that recruits IL-1R-associated receptor kinase 4 (IRAK4) (23). Upon phosphorylation, IRAK4 leaves the complex and interacts with TNFR-associated factor 6 (TRAF6) in the cytoplasm, which then leads to the activation of NF-κB-mediated cytokine transcription. We report here that IFN-β1a therapeutic manipulation of the TLR7/IL-1R signaling pathway inhibited IL-1β, TGF-β, and IL-23, while it induced IL-27 secretion by monocyte-derived DCs that collectively inhibited DC-mediated Th17 cell differentiation. Our study has identified a novel therapeutic mechanism of IFN-β1a, characterized by the TLR7-mediated regulation of DC cytokine secretion, which inhibits Th17 cell differentiation in the context of the autoimmune response in MS.

Materials and Methods

Study subjects

Fifteen patients with a clinically isolated syndrome suggestive of MS (CIS) and 31 RR MS patients were enrolled in the study upon signing an institutional review board-approved informed consent form. CIS patients experienced their first clinical presentation consistent with demyelinating disease within 1 year from blood sample collection and had at least two magnetic resonance imaging (MRI) lesions consistent with MS (24). All RR MS patients had a confirmed diagnosis according to McDonald's diagnostic criteria (25) and did not receive immunomodulatory therapy at the time of blood sample collection. The treatment-free period was at least 6 wk for i.v. methylprednisolone and 3 mo for IFN-β and glatiramer acetate. The patients were not previously treated with immunosuppressive therapies.

Affymetrix gene arrays

Gene expression changes induced by IFN-β1a were tested using Affymetrix Human Genome U133 (HG-U133) arrays that contain 45,000 probe sets representing 39,000 transcripts derived from ~33,000 human genes. In brief, 10^7 PBMCs per condition derived from 15 CIS patients were stimulated with plate-immobilized anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) mAb (BD Biosciences) in the absence or presence of IFN-β1a (1000 U/ml; EMD Serono) for 24 h in serum-free medium (Life Technologies). Cells were harvested and the total RNA was isolated using a RNeasy kit (Qiagen). Arrays were hybridized for 16 h at 45ºC in the GeneChip Hybridization Oven 640 (Affymetrix). The arrays were washed and stained with R-PE-streptavidin in the GeneChip Fluidics Station 400 (Affymetrix). The arrays were scanned with a Hewlett Packard GeneArray Scanner. Affymetrix GeneChip Microarray Suite 5.0 software was used for washing, scanning, and basic analysis. To detect differential gene expression profiles between the untreated and IFN-β1a-treated samples, a two-class paired test of significance analysis of microarrays was used. For the differentially expressed genes, the mean inhibitory effect on gene expression between two groups was estimated using a Welch two-sample t test. A $p < 0.05$ was considered significant.

Protein arrays

Supernatants from the above-described cultures from five CIS patients were collected at 24 h, and quantitative analysis of 120 secreted proteins was performed using human cytokine protein arrays (Ray Biotech) according to the manufacturer's instructions. Briefly, after blocking, the membranes were incubated with 1 ml of SN sample in 1/250 diluted biotin-conjugated primary Abs, followed by incubation with 1/1000-diluted HRP-conjugated streptavidin and the detection buffer. Membranes were exposed to Premium Autoradiography Film (Denville Scientific). The films were scanned with an Epson America Expression 1680 Scanner and the signal intensities were quantified using Meta Imaging Series 5.0 software (Molecular Devices). The lowest measurement on the membrane was subtracted from all measured proteins as the background and the results were normalized by the sum of the OD values for all proteins on the membrane. The results were expressed as a fold change of the normalized protein level in the SNs of IFN-β1a-treated PBMCs in comparison to the untreated cultures.

Quantitative RT-PCR (qRT-PCR) and PCR arrays

$CD14^+$ monocytes were isolated from the peripheral blood of 16 RR MS patients using an EasySep Negative Selection Monocyte Enrichment Kit (StemCell Technologies). One $\times$ 10^6/ml monocytes were cultured with GM-CSF (1000 U/ml) and IL-4 (500 U/ml; R&D Systems) in complete RPMI 1640 medium containing RPMI 1640, 1% HEPES, 2% L-glutamine, 1% sodium pyruvate, 1% essential amino acids, 1% penicillin/streptomycin (Pen/Strep), and 5% human serum for 7 days to generate DCs. After washing, 3×10^6 DCs per condition were cultured in the absence or presence of IFN-β1a (1000 U/ml) for 24 h in serum-free medium and then harvested or matured with LPS (1 μg/ml; Sigma-Aldrich) or loxoribine (100 μM; InvivoGen) for 2 h before the RNA extraction for qRT-PCR and for 48 h before the SN collection and cell harvesting for ELISA and FACS. Total RNA was isolated and cDNA was synthesized using a High Capacity cDNA Archive Kit (Applied Biosystems). The primers for *TLR7*, *MyD88*, *IRAK4*, *TRAF6*, *IL-1*α, *IL-1*β, *IL-1R1*, and *18S* were purchased from Applied Biosystems and gene expression was measured by qRT-PCR using TaqMan Gene Expression Assays (Applied Biosystems). The results are expressed as the average relative gene expression normalized for the *18S* mRNA expression for each subject.

For the PCR array, cDNA was synthesized using a Reverse Transcriptase First Strand Kit (SuperArray Bioscience) from the RNA samples derived from the DCs from 4 RR MS patients. Changes in gene expression in the TLR signaling pathways upon IFN-β1a treatment were measured using a PCR array human TLR signaling pathway followed by data analysis with an online Reverse Transcriptase Profiler PCR Array Data Analysis System (SuperArray Bioscience).

ELISA

Supernatants from the DC cultures were collected and stored at -70ºC until the cytokine measurements by ELISA. IL-1β, IL-10, IL-12p70, and TGF-β1 were measured with an ELISA kit purchased from BD Biosciences, IL-27 with an ELISA kit from R&D Systems, and IL-23 (p19/p40) with an ELISA kit from eBioscience in duplicate wells according to the manufacturer's recommendations.

The cytokine production of IL-17A, IFN-γ, and IL-4 from $CD45RA^+$ cells was measured by ELISA using a Human IL-17A ELISA Construction Kit (Antigenix America), IFN-γ and IL-4 ELISA kits (BD Biosciences), an IL-21 ELISA kit (eBioscience), and an IL-22 ELISA kit (R&D Systems).

FACS

One × 10^6/ml DCs derived from 16 RR MS patients were stained with PE-Cy5.5-conjugated CD11c, allophycocyanin-conjugated CD1a mAb (BD Biosciences), and a primary Ab for IL-1R1 (Abcam). For IL-1R1 surface staining, the cells were stained with FITC-conjugated goat anti-rabbit IgG secondary Ab (Abcam). For TLR7 intracellular staining, the cells were fixed, permeabilized and stained with TLR7 primary Ab and with FITC-conjugated goat anti-rabbit IgG secondary Ab (Abcam). Isotype IgG FITC-conjugated Ab (Abcam) was used as a control. The percentage of gated cells expressing each molecule was determined using CellQuest software (BD Biosciences).

Western blotting

Three × 10^6 monocyte-derived DCs per condition were derived from three RR MS patients, pretreated with IFN-β1a for 24 h in serum-free medium, and then matured with LPS for 5 h before protein extraction. Proteins from cell lysates were denatured in SDS, resolved by 7.5% SDS-PAGE for TLR7 and IL-1R1 detection and by 12.5% SDS-PAGE for MyD88 and IRAK4 detection, and transferred on polyvinylidene difluoride membranes prewetted by 100% methanol. Nonspecific binding was blocked for 1 h in the blocking buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 2% fish gelatin, and 1% OVA in double-distilled water) and incubated overnight with rabbit anti-human Ab for TLR7 (Abcam), MyD88, TRAF6 (Santa Cruz Biotechnology), and IRAK4 (ProSci) and with mouse anti-human Ab for IL-1R1 and β-actin (Santa Cruz Biotechnology). The blots were washed with TBST and incubated with IRDye 680-conjugated anti-mouse IgG or anti-rabbit IgG secondary Ab (LI-COR Biosciences) for 1 h. The blots were washed and protein bands were quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences).

Small interfering RNA (siRNA) treatment

Three × 10^6/well monocyte-derived DCs from six RR MS patients were plated in antibiotic-free medium (RPMI 1640 plus 10% FBS) in 6-well plates. After a 24-h incubation at 37ºC, the DCs were treated with *TLR7* siRNA or control siRNA (Santa Cruz Biotechnology) according to the manufacturer's recommendations. Briefly, DCs were washed with Transfection Medium (Santa Cruz Biotechnology) and incubated with siRNA, which was premixed with Transfection Reagent (Santa Cruz Biotechnology) and Transfection Medium, and incubated for 45 min at room temperature. After a 7-h incubation at 37ºC, the growth medium (RPMI 1640 plus 20% FBS plus 2% Pen/Strep) was added for an additional 24-h incubation. After removing the medium, culture medium (RPMI 1640 plus 10% FBS plus 1% Pen/Strep) was added to the cells and they were incubated for an additional 48 h. Harvested siRNA-treated and control siRNA-treated cells were cultured in serum-free medium in the absence or presence of IFN-β1a for 24 h, followed by LPS maturation for 5 h before protein extraction, and for 48 h before SN collection. The expression of TLR7, MyD88, IRAK4, TRAF6, IL-1R1, and β-actin was measured by Western blotting, and the secretion of IL-1β, TGF-β1, IL-23/p19, IL-27, IL-12p70, and IL-10 was measured by ELISA.

SN transfer experiments

$CD45RA^+$ T cells were isolated from the peripheral blood of six RR MS patients using a Human Naive $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec). Two × 10^6 $CD45RA^+$ T cells per condition were cultured with the SNs from the siRNA TLR7 or control siRNA-treated allogenic DCs that were cultured in the absence or presence of IFN-β1a treatment and matured by LPS. CD45RA naive T cells were stimulated with plate-immobilized anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) mAb (BD Biosciences). After 12 days of culture, the cells were harvested for RNA extraction and the SNs were collected for cytokine measurement. The gene expression of *IL-17A*, *IL-17F*, *IFN-γ*, *IL-4*, retinoic acid-related orphan nuclear hormone receptor C (RORC), *T-bet*, *GATA3*, and *18S* was measured by qRT-PCR using TaqMan Gene Expression Assays (Applied Biosystems). The cytokine production of IL-17A, IL-21, IL-22, IFN-γ, and IL-4 was measured by ELISA.

Statistics

Statistical analysis of the paired results of the qRT-PCR, ELISA, and FACS experiments was performed using Student's paired *t* test with SigmaPlot 10.0 software (Systat Software). The statistical analysis for correlation between gene array and protein array data was performed by linear correlation analysis using GraphPad InStat software. Statistical analyses of gene expression in DCs, cytokine secretion in siRNA experiments, and the gene expression and cytokine secretion by $CD45RA^+$ cells in SN transfer experiments were performed using repeated measures ANOVA with GraphPad InStat software. A $p < 0.05$ was considered significant.

Results

IFN-β1a selectively modulates the TLR7 signaling pathway and regulates the gene expression of inflammatory cytokines and chemokines

We used Affymetrix gene arrays to capture the complex changes in gene expression in the PBMCs derived from 15 patients with CIS upon IFN-β1a in vitro treatment. Our study identified 1728 IFN-β1a-induced differentially expressed genes (935 up-regulated and 793 down-regulated; $p < 0.05$). Among the differentially expressed genes, there were 199 immune response genes (141 up-regulated and 58 down-regulated).

In this report, we focus on the IFN-β-induced changes in the expression of genes involved in the innate immune response that characterize the effect of the endogenous IFN-β secreted during the antimicrobial response (supplemental Table I[4]), while the complete gene array data set will be presented in another manuscript (X. Zhang, J. Jin, Y. Tang, and S. Markovic-Plese, manuscript in preparation). We identified a significantly increased *TLR7* expression ($p < 0.0001$) and its downstream signaling molecules *MyD88* ($p = 0.0249$), *IRAK4* ($p = 0.0033$), *TRAF6* ($p = 0.0290$), *IFN regulatory factor* (*IRF*) *7* ($p < 0.0001$), and *IRF2* ($p = 0.0043$) (Fig. 1*A*).

Studies of the inflammatory cytokine gene expression revealed that IFN-β1a significantly inhibited *IL-17F* gene expression ($p = 0.0290$) and the expression of multiple cytokines and their receptors that mediate Th17 cell differentiation, including *IL-1R1* ($p < 0.0001$), *IL-1α* ($p = 0.0019$), *IL-1β* ($p = 0.0387$), and *IL-12Rβ1* ($p = 0.0308$) (Fig. 1*A*). IFN-β1a also inhibited the expression of chemokines involved in the inflammatory cell migration across the blood-brain barrier, including *CXCL1*, *CXCL2*, *CXCL5*, *CXCL8*, *CCL17*, and *CCL22* (Fig. 1*A*). These effects of IFN-β1a were confirmed by qRT-PCR; *TLR7* gene expression was significantly up-regulated (3.0-fold, $p = 0.0001$) and *IL-1α* (−3.1-fold, $p = 0.0040$) and *IL-17F* (−1.6-fold, $p = 0.0038$) were down-regulated in the same RNA samples as those used for the Affymetrix gene arrays (data not shown). Our protein array results correlated with the gene expression changes ($r = 0.6223$, $p = 0.0307$) and confirmed changes in secretion of 12 chemokines and cytokines, including decreased secretion of IL-1α and IL-1β from IFN-β1a-treated PBMCs derived from five CIS patients tested in the Affymetrix study (Fig. 1*B*).

IFN-β1a up-regulates the expression of TLR7 and multiple molecules involved in its signaling pathway, while it down-regulates the expression of IL-1R and IL-1α/β in the DCs derived from RR MS patients

According to our previous studies (26), the in vitro effects of immunomodulatory therapies on the activated cells reflect their mechanisms of action in inflammatory disease. Although the anti-CD3+anti-CD28 mAb used in our gene array studies stimulate T cells, activated T cells subsequently activated other cell subsets. Thus, the above gene array results reflect IFN-β1a's effect on multiple cell subsets and their interactions, which are relevant for its in vivo effect. Following the identification of IFN-β-differentially expressed genes, further studies were focused on DCs, as this cell subset exhibits the highest expression of TLRs. To confirm the gene array results in DCs, whose cytokine secretion regulates T cell differentiation, we performed qRT-PCR studies on LPS-matured DCs. In addition, to examine whether our initial results in CIS patients apply to later phases of the disease, qRT-PCR studies

[4] The online version of this article contains supplemental material.

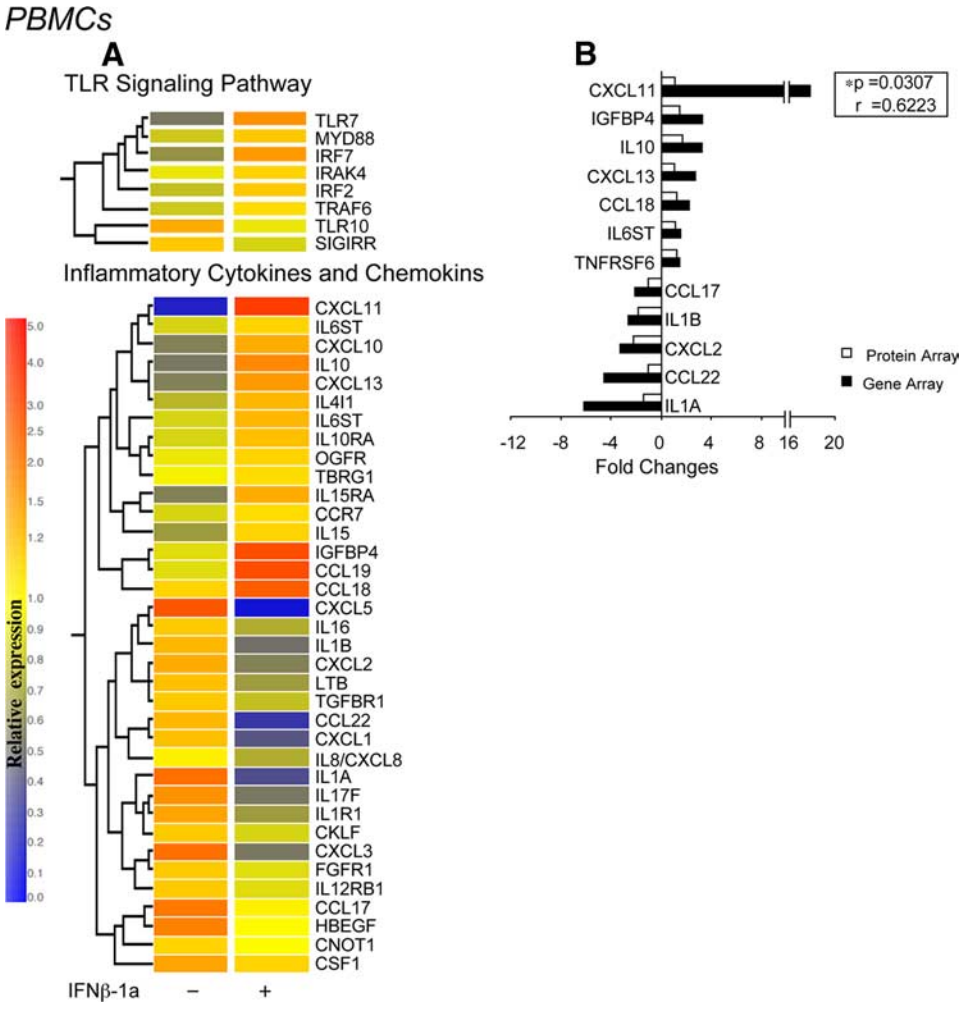

FIGURE 1. IFN-β1a selectively modulates the TLR7 signaling pathway and regulates the gene expression of inflammatory cytokines and chemokines. *A*, In brief, 10^7 PBMCs per condition from 15 CIS patients were cultured in the absence or presence of IFN-β1a and stimulated with plate-immobilized anti-CD3 and anti-CD28 mAb for 24 h. Cells were harvested and the total RNA was isolated and used for gene array hybridization. Tree graphs present genes with statistically significant ($p < 0.05$) differences in the expression between the IFN-β1a-treated and untreated samples. *B*, Protein array measurements of 12 secreted proteins in the SNs of PBMCs treated with IFN-β1a confirmed significant gene expression changes from the same PBMCs. The results are expressed as fold changes between the normalized protein levels in the supernatants of PBMCs cultured in the presence and absence of IFN-β1a. The correlation between the fold changes in gene expression and the protein levels was statistically significant ($r = 0.6233$; $p = 0.0307$).

were performed on the DCs derived from 10 RR MS patients. Specifically, our goal was to determine to what extent IFN-β1a-mediated up-regulation of the TLR7/MyD88/IRAK4/TRAF6 signaling pathway inhibits IL-1R, IL-1α, and IL-1β expression. The results confirmed that IFN-β1a significantly increased the gene expression of *TLR7* (6.0-fold) and the *MyD88-IRAK4* cascade (3.7-fold, and 2.0-fold, respectively), but decreased the expression of *IL-1R1* (1.2-fold), *IL-1*α (2.0-fold), and *IL-1*β (2.1-fold) in LPS-matured DCs, which is consistent with the Affymetrix gene array results. *TRAF6* gene expression was increased upon IFN-β1a treatment, but the difference was not significant in DCs (Fig. 2*A*). The IFN-β1a treatment of immature DCs revealed the same pattern of gene expression changes, but the differences did not reach statistical significance (data not shown). To confirm the effect of IFN-β1a on DCs matured with other TLR ligands, we matured the DCs with the TLR7 ligand loxoribine. IFN-β1a treatment of loxoribin-matured DCs induced the same pattern of *TLR7*, *MyD88*, *IRAK4*, *TRAF6*, *IL-1R1*, and *IL-1*α/β gene expression changes that were observed in the LPS-matured DCs (data not shown).

To obtain greater insight into the TLR signaling pathway changes induced by IFN-β1a treatment in DCs, we used a PCR array of the human TLR signaling pathway, which includes 84 TLR signaling pathway genes. The results for the RNA derived from DCs from four RR MS patients confirmed the significant up-regulation of *TLR7* (17.1-fold) and *MyD88* (2091.8-fold). IFN-β1a treatment increased the expression of *TLR3* (32.2-fold), which binds viral dsRNA and induces IFN-β secretion via an autocrine loop (20), as indicated by the 117.5-fold up-regulation of *IFN-*β. Consistent with previous reports, IFN-β1a increased the *IL-10* expression (64.3-fold) in human DCs (Ref. 8 and data not shown).

To confirm that changes in TLR7 and IL-1R1 gene expression translate to the protein level in a cohort of patients, we performed intracellular staining for TLR7 and surface staining for IL-1R1 on the monocyte-derived DCs generated from 16 RR MS patients. As presented in the representative experiment in Fig. 2*B*, IFN-β1a significantly increased the percentage of TLR7-positive cells (1.25-fold, $p = 0.006$), consistent with a previous report on IFN-β-induced functionally active TLR7 in DCs (21), while it reduced the percentage of IL-1R1-positive cells (−1.30-fold, $p = 0.001$).

Furthermore, the Western blots presented in Fig. 2*C* confirmed the up-regulation of TLR7 and its signaling components MyD88, IRAK4, and TRAF6 and the down-regulation of IL-1R1 induced by IFN-β1a treatment in LPS-matured DCs derived from three RR MS patients.

IFN-β1a modifies the production of Th17-polarizing cytokines in DCs

Cytokine measurements in the SNs of DCs derived from 10 RR MS patients revealed that IFN-β1a significantly inhibited the secretion of IL-1β (−5.1-fold), TGF-β1 (−2.1-fold), and IL-23 (−1.4-fold), while it induced the production of IL-27 (1.4-fold; Fig. 3). IL-1β, TGF-β, and IL-23 were reported to induce and IL-27 to inhibit Th17 cell differentiation (6), indicating that the IFN-β1a-mediated changes may create an inhibitory milieu for the differentiation of Th17 cells. In addition, consistent with previous reports (3), IFN-β1a modified the

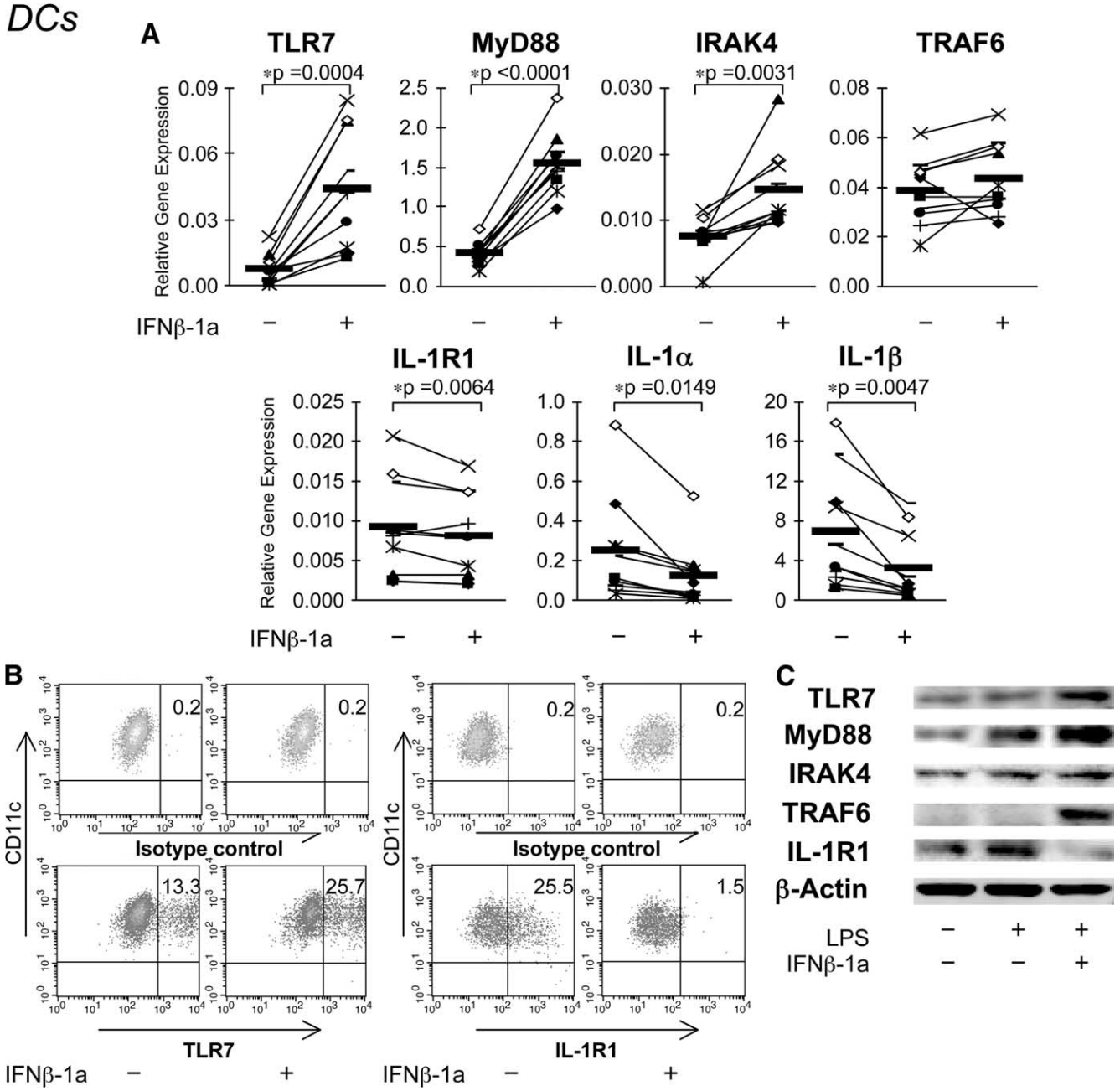

FIGURE 2. IFN-β1a treatment up-regulates TLR7 and its signaling pathway, while it down-regulates the expression of IL-1R and IL-1α/β. *A*, Three × 10^6 DCs per condition derived from 10 RR MS patients were pretreated with IFN-β1a for 24 h and then matured with LPS for 2 h before RNA extraction. *TLR7*, *MyD88*, *IRAK4*, *TRAF6*, *IL-1R1*, *IL-1α*, and *IL-1β* gene expression was measured by RT- PCR. The results are presented as relative gene expression normalized for the *18S* RNA expression. *B*, Three × 10^6 DCs per condition from 16 RR MS patients were pretreated with IFN-β1a for 24 h and then matured with LPS for 48 h. DCs were stained with PE-Cy-CD11c, allophycocyanin-CD1a mAb, and anti-IL-1R1 Ab, followed by FITC-conjugated secondary Ab, anti-TLR7 primary Ab, and FITC-conjugated secondary Ab. FITC-conjugated IgG isotype Ab was used as a control. The percentage of positive cells was determined in the CD1a-gated population. *C*, Three × 10^6 DCs per condition derived from three RR MS patients were pretreated by IFN-β1a for 24 h and then matured with LPS for 5 h before protein extraction. The expression of TLR7, MyD88, IRAK4, TRAF6, IL-1R1, and β-actin were measured by Western blotting. The results present one of three similar experiments.

Th1/Th2 balance by significantly inhibiting the expression of IL-12p70 (−1.7-fold, $p = 0.0013$) a key cytokine for Th1 differentiation, and inducing the expression of the immunoregulatory cytokine IL-10 (2.1-fold, $p = 0.0044$; data not shown).

IFN-β1a-mediated up-regulation of TLR7 and its signaling pathway inhibits the secretion of Th17-polarizing cytokines in DCs

To determine the extent to which are the IFN-β1a-induced MyD88/IRAK4/TRAF6 signaling and the inhibition of IL-1R1 expression mediated through the TLR7 induction, we used *TLR7* siRNA gene silencing in DCs derived from six RR MS patients. *TLR7* siRNA silenced *TLR7* expression by 85% in comparison to the control siRNA-treated cells (Fig. 4*A*). Although the *TLR7* gene silencing had no effect on the baseline expression of MyD88, IRAK4, and IL-1R, the IFN-β1a failed to increase the expression of MyD88 and IRAK4 or to inhibit the expression of IL-1R in TLR7 siRNA-treated cells (Fig. 4*A*), as demonstrated in control siRNA-treated DCs and nonmanipulated cells (Fig. 2*C*). Interestingly, IFN-β1a's induction of TRAF6 was not affected by TLR7

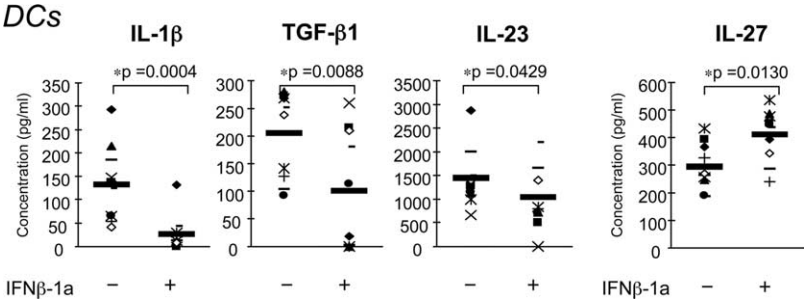

FIGURE 3. IFN-β1a modifies the production of Th17-polarizing cytokines in DCs. Three $\times$ 10^6 DCs per condition derived from 10 RR MS patients were pretreated with IFN-β1a for 24 h and matured with LPS for 48 h before the SN collection. IL-1β, TGF-β1, IL-23, and IL-27 were measured by ELISA in duplicate wells. The results are expressed for each subject as cytokine concentration in pg/ml.

gene silencing, implying that IFN-β-induced TRAF6 expression may be mediated directly through the IFNR signaling. In summary, the results indicate that the IFN-β1a-induced increase in MyD88 and IRAK4 expression, as well as the inhibition of IL-1R1, are mediated via IFN-β1a-induced TLR7 expression in DCs.

To determine the extent to which the effects of IFNβ-1a on DC cytokine secretion are mediated through its induction of TLR7 expression, we measured IL-1β, TGF-β1, IL-23, IL-27, IL-12p70, and IL-10 in the SNs from the TLR7 siRNA and control siRNA-treated DCs. The baseline secretion of IL-1β, TGF-β1, and IL-23 were not changed upon TLR7 gene silencing, while the baseline secretion of IL-27 was significantly decreased (Fig. 4*B*), consistent with the reported role of TLR7 in IL-27 production (27, 28). IFN-β1a failed to inhibit the secretion of IL-1β and IL-23/p19 after TLR7 gene silencing (Fig. 4*B*), from which we infer that the IFN-β1a-mediated inhibition of IL-1β and IL-23 requires TLR7 expression. In addition, IFN-β1a failed to increase the secretion of IL-27 after TLR7 gene silencing (Fig. 4*B*), which indicated that the induction of IL-27 secretion by IFN-β1a is also TLR7 dependent. The secretion of TGF-β1, IL-12p70, and IL-10 was not changed by TLR7 gene silencing (Fig. 4*B* and data not shown).

Previous studies have reported that IFN-β inhibits IL-1R signaling by the induction of IL-1Ra secretion, which blocks the IL-1R signaling via competitive inhibition (29). We confirmed that IFN-β1a induced the secretion of IL-1Ra and further demonstrated that this effect is lost upon siRNA *TLR7* treatment of DCs (data not shown). It is unclear whether the inhibition of IL-1R expression inhibits IL-1β secretion, and studies of the mechanisms of TLR7-induced changes in cytokine production are currently in progress in our laboratory.

IFN-β1a-modified DC cytokine secretion inhibits Th17 differentiation

Based on the findings that IFN-β1a inhibited DC secretion of IL-1β, TGF-β, and IL-23 and induced the secretion of IL-27, we predicted that the IFN-β1a-induced changes in DC cytokine production would collectively inhibit Th17 cell differentiation. We confirmed this prediction by transferring SNs from the control siRNA-treated DCs to allogenic CD45RA$^+$ naive T cells. After 12 days of differentiation induced by immobilized anti-CD3 and andti-CD28 mAbs, our results revealed that the SNs from IFN-β1a-treated mature DCs inhibit the gene expression of *RORC* (-2.8-fold, $p < 0.05$), a transcription factor that mediates Th17 cell differentiation, as well as *IL17A* gene expression (-4.8-fold, $p < 0.05$) and protein secretion (-2.2-fold, $p < 0.01$). Consistent with the results on the IFN-β1a's effect on the DC cytokine secretion (Fig. 4*B*), the SNs from TLR7 siRNA-treated DCs failed to inhibit Th17 cell differentiation (Fig. 5), which confirmed that the inhibitory effect of IFN-β1a on Th17 differentiation is mediated through the up-regulation of TLR7 in DCs. Moreover, as the gene expression of *RORC* (-2.9-fold, $p < 0.05$) and *IL-17A* (-5.1-fold, $p < 0.05$) and the secretion of IL-17A (-2.0-fold, $p < 0.001$) were significantly lower in the CD45RA$^+$ cells cultured with SNs from the IFN-β1a-treated control siRNA in comparison to the SNs from the IFN-β1a-treated *TLR7* siRNA DCs, we conclude that the TLR7-mediated changes in DC cytokine production and not the direct effect of IFN-β1a on CD45RA cells inhibit the Th17 cell differentiation. The effect on the additional Th17 cytokines IL-21 and IL-22 was similar to the changes in IL-17A secretion; however these differences were not statistically significant (data not shown).

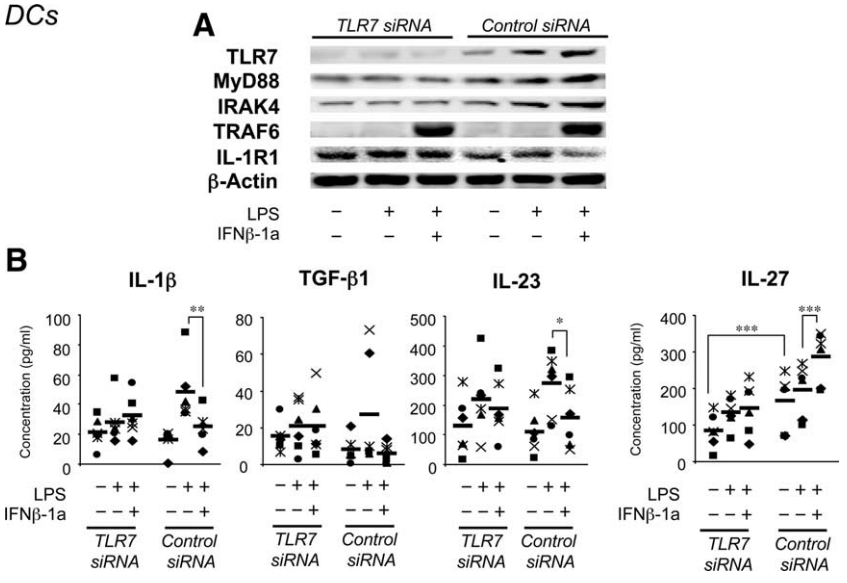

FIGURE 4. IFN-β1a modifies MyD88/IRAK4/TRAF6 signaling and cytokine production in DCs through its induction of TLR7 expression. Three $\times$ 10^6/well DCs generated from six RR MS patients were plated in antibiotic-free medium. After a 24-h incubation at 37°C, the DCs were transfected with TLR7 siRNA or control siRNA. The siRNA-treated cells were harvested and cultured in serum-free medium in the absence or presence of IFN-β1 for 24 h, followed by LPS maturation for 5 h before the protein extraction, and for 48 h before the SN collection. *A*, The expression of TLR7, MyD88, IRAK4, TRAF6, IL-1R1, and β-actin was measured by Western blotting. The results present one of three similar experiments. *B*, The production of IL-1β, TGF-β1, IL-23/p19, and IL-27 was measured by ELISA. The results present six experiments performed in triplicate. Statistical analysis was performed using a repeated measures ANOVA. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

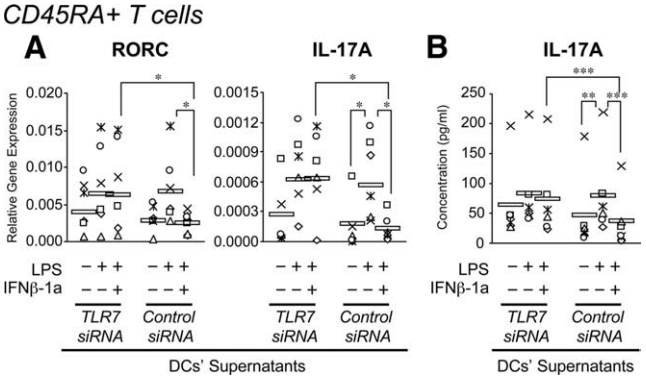

FIGURE 5. IFN-β1a-modified DC cytokine secretion inhibits Th17 differentiation. Two × 10^6 CD45RA$^+$ T cells per condition were incubated with the SNs from TLR7 or control siRNA-treated DCs and cultured in the absence or presence of IFN-β1a before LPS maturation. CD45RA$^+$ naive T cells derived from six RR MS patients were stimulated with plate-immobilized anti-CD3 and anti-CD28 mAb for 12 days, when the cells were harvested for RNA extraction and the SNs were collected for cytokine measurement. *A*, The gene expression of *RORC*, *IL-17A*, and *18S* was measured by qRT-PCR. *B*, The cytokine production of IL-17A was measured by ELISA. Statistical analysis was performed using a repeated measures ANOVA. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

The SNs from IFN-β1a-treated DCs did not change the expression of *T-bet/IFN-γ* and *GATA3/IL-4* in cultured CD45RA naive cells (data not shown).

Discussion

The clinically isolated syndrome suggestive of MS is defined by the first clinical attack and MRI changes consistent with CNS demyelinating lesions (24). Since CIS patients have a high risk of developing clinically definitive RR MS (30), IFN-β treatment is initiated at this early phase of the disease based on clinical trials that have demonstrated its efficacy in postponing disease progression (24). Although several studies have examined the IFN-β in vitro-induced changes in gene expression in the PBMCs derived from RR MS patients (31), this is the first study to examine this effect in CIS patients, whose diagnostic criteria, as well as response to therapy, are still not well characterized. Our Affymetrix gene array data revealed that IFN-β1a treatment of PMBCs derived from CIS patients induced the expression of *TLR7* and the genes in its downstream signaling pathway, including *MyD88*, *IRAK4*, and *TRAF6*. In addition, IFN-β1a treatment inhibited the expression of IL-17F, a cytokine secreted by Th17 cells which are implicated in the development of the autoimmune response. IL-17F is secreted as a homodimer (IL-17F-F) and as a heterodimer with IL-17A (IL-17F-A), with which it shares the highest degree of sequence homology within the IL-17 cytokine family (32, 33). IL-17F and IL17A bind to the same receptor (IL-17RC) and share biological functions, which are not yet completely elucidated (34, 35). IFN-β1a also inhibited the expression of multiple cytokines that mediate Th17 cell differentiation in CIS patients (*IL-1α*, *IL-1β*, *IL1R1*, *IL12Rβ1*). Consistent with the inhibition of Th17 cells, we identified a decrease in the expression of multiple chemokine genes induced by IL-17 (32, 34). The IL-17 target genes *CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, and *CXCL8* were inhibited in the CIS patients. These chemokines mediate neutrophil recruitment, the best-characterized function of IL-17, which has recently been demonstrated to play a critical role in the development of early CNS demyelinating lesions in an animal model of the disease (36). In addition to these neutrophil chemoattractants, *CCL17* and *CCL22* chemokines that mediate the Th17 cell migration (37) were also decreased upon the IFN-β1a in vitro treatment. Thus, our gene profiling study revealed two relevant gene expression changes induced by IFN-β1a in vitro treatment: a selective up-regulation of the TLR7 signaling pathway and the inhibition of multiple cytokines involved in Th17 cell differentiation.

IFN-β1a treatment of DCs significantly inhibited the secretion of IL-1β, TGF-β1, and IL-23 and induced the production of IL-27. Guo et al. (19) have recently reported that IFN-β induces IL-27 secretion, which is further supported by a study by Shinohara et al. (38), who reported that the IFNR-dependent inhibition of osteopontin induces IL-27 secretion. However, the IFN-β inhibitory effects on IL-1β, TGF-β, and IL-23 secretion have not been previously reported.

Using siRNA *TLR7* gene silencing in human DCs, we confirmed that IFN-β1a-induced up-regulation of MyD88 and IRAK4 were dependent on TLR7 expression, while the IFN-β1a-induced TRAF6 up-regulation persisted even in the absence of TLR7 expression. The IFN-β1a-induced inhibition of IL-1R expression was also dependent on TLR7 expression. Multiple TLRs and IL-1R share a common downstream signaling pathway. It has been reported that activation of individual TLRs may induce a cross-tolerance to the activation of other TLR pathways whereby the activation of the proximal signaling pathway becomes unresponsive to subsequent stimulation (39, 40). Based on TLR7 and IL-1R's structural similarities and their common downstream signaling pathway, we propose that the IFN-β1a-induced TLR7 expression may down-regulate the expression of IL-1R, possibly through a mechanism analogous to TLR cross-tolerance induction.

Next, we sought to investigate to what extent the IFN-β1a-mediated effect on the innate immune response TLR7 signaling pathway inhibits cytokines involved in T cell differentiation. Following siRNA *TLR7* gene silencing, the IFN-β1a-induced changes in the secretion of IL-1β, IL-23, and IL-27 were not observed, indicating that the IFN-β1a's immunomodulatory mechanism is mediated via the up-regulation of the TLR7 signaling pathway. Although several studies have reported that activation of TLR signaling pathways have an immunoregulatory potential mediated by the enhanced production of type I IFNs (20, 41, 42), our study for the first time identified an effect of the TLR7 signaling pathway that leads to the changes in DC immunoregulatory cytokine secretion.

Recent studies of cytokines that mediate Th17 cell differentiation have revealed great differences between mice and human system. Although several reports (6, 43) confirmed a central role of IL-1β in the human Th17 cell differentiation, it has been reported that TGF-β, which plays an important role in differentiation of murine Th17 cells, is not required and even inhibits human Th17 cell differentiation (6). However, a recent study by Yang et al. (44) has implicated TGF-β as one of the key cytokines involved in Th17 cell differentiation from naive human CD4$^+$ T cells, suggesting that further studies are needed to definitively characterize the role of TGF-β in human Th17 cell differentiation. In contrast, IL-23, which was reported to contribute only to the maintenance and expansion of murine Th17 cells, plays a key role in the differentiation of human Th17 cell lineage (12, 43). IL-27, another IL-12 family member, was reported to inhibit Th17 cell differentiation in humans through STAT1-mediated mechanisms (45), which counterbalance the IL-23/STAT3-mediated Th17 cell differentiation.

SN transfer experiments confirmed that the effect of the IFN-β1a-induced changes in DC cytokine secretion inhibits Th17 cell

differentiation, as evidenced by the decreased *RORC* gene expression and IL-17A production. Consistent with the requirement of IFN-β-induced TLR7 expression for the changes in the DC-secreted IL-1β, IL-23, and IL-27, the inhibitory effect of DC SNs on Th17 cell differentiation was not observed with SNs from TLR7 siRNA-treated DCs. In contrast, the inhibitory effect of IFN-β1a on the DC TGF-β secretion was not mediated by TLR7 up-regulation, so that it was maintained in the SNs from TLR7 siRNA DCs. Although we did not directly study the effect of TGF-β, the finding from the above experiment implies that TGF-β inhibition did not affect Th17 cell differentiation, consistent with a previous report by Acosta-Rodriguez et al. (6).

In conclusion, we identified a new IFN-β1a's immunomodulatory mechanism mediated via the up-regulation of the TLR7 signaling pathway, which may represent an endogenous IFN-β effect during the innate immune response. We propose that the exogenously administered high-dose IFN-β1a augments this naturally occurring regulatory mechanism and provides a therapeutic effect in patients with RR MS. Further studies are needed to characterize the mechanisms of IFN-β-induced TLR7 expression. We are currently investigating whether IFN-β-induced IRF2 and IRF7 play a role in the TLR7 up-regulation, analogous to the study by Severa et al. (21) who reported that type I IFN-induced TLR7 expression was mediated by IRF1's binding to the TLR7 promoter.

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Disclosures

The authors have no financial conflict of interest.

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