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Type I IFNs Downregulate Myeloid Cell IFN-γ Receptor by Inducing Recruitment of an Early Growth Response 3/NGFI-A Binding Protein 1 Complex That Silences ifngr1 Transcription

Staci J. Kearney,* Christine Delgado,† Emily M. Eshleman,† Krista K. Hill,* Brian P. O’Connor,*†,‡,1 and Laurel L. Lenz*‡,†,1

The ability of type I IFNs to increase susceptibility to certain bacterial infections correlates with downregulation of myeloid cell surface IFNGR, the receptor for the type II IFN (IFN-γ), and reduced myeloid cell responsiveness to IFN-γ. In this study, we show that the rapid reductions in mouse and human myeloid cell surface IFNGR1 expression that occur in response to type I IFN treatment reflect a rapid silencing of new ifngr1 transcript by repressive transcriptional regulators. Treatment of macrophages with IFN-β reduced cellular abundance of ifngr1 transcripts as rapidly and effectively as actinomycin D treatment. IFN-β treatment also significantly reduced the amounts of activated RNA polymerase II (pol II) and acetylated histones H3 and H4 at the ifngr1 promoter and the activity of an IFNGR1-luc reporter construct in macrophages. The suppression of IFNGR1-luc activity required an intact early growth response factor (Egr) binding site in the proximal ifngr1 promoter. Three Egr proteins and two Egr/NGFI-A binding (Nab) proteins were found to be expressed in bone macrophages, but only Egr3 and Nab1 were recruited to the ifngr1 promoter upon IFN-β stimulation. Knockdown of Nab1 in a macrophage cell line prevented downregulation of IFNGR1 and prevented the loss of acetylated histones from the ifngr1 promoter. These data suggest that type I IFN stimulation induces a rapid recruitment of a repressive Egr3/Nab1 complex that silences transcription from the ifngr1 promoter. This mechanism of gene silencing may contribute to the anti-inflammatory effects of type I IFNs. The Journal of Immunology, 2013, 191: 3384–3392.

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; ChIP, chromatin immunoprecipitation; Egr, early growth response; HDAC, histone deacetylase; hPBMC, human PBMC; hps, hour poststimulation; MFI, mean fluorescence intensity; Nab, NGFI-A binding protein; qPCR, quantitative RT-PCR; wt, wild-type.

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Address correspondence and reprint requests to Dr. Laurel L. Lenz, Integrated Department of Immunology, National Jewish Health, 1400 Jackson Street, Suite K510, Denver, CO 80260. E-mail address: lenzl@njhealth.org

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Address correspondence and reprint requests to Dr. Laurel L. Lenz, Integrated Department of Immunology, National Jewish Health, 1400 Jackson Street, Suite K510, Denver, CO 80206. E-mail address: lenzl@njhealth.org

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sought to investigate how type I IFNs negatively regulate myeloid cell *ifngr1* expression.

The silencing of basally transcribed genes often involves recruitment of repressive transcription factors to the target gene promoter. The early growth response (Egr) family of transcription factors comprises four members (Egr1, Egr2, Egr3, and Egr4). DNA-binding domains in these Egr proteins are formed by three zinc-finger motifs that bind to the consensus sequence CGCCC-CGCC (18). Egr proteins were originally recognized for their role in the genetic regulation of cell growth and differentiation in response to extracellular stimuli, particularly in the context of the nervous system (19). They are now also known to promote expression of a diverse group of genes, including several with important immunological functions (20–24). Egr family members can also repress the transcription of certain target genes, particularly in response to external stimuli such as cytokines (25, 26). The mechanisms for gene repression include interference with transcriptional activators such as Sp1 (25–29) and TATA binding protein (30, 31) and recruitment of a family of Egr corepressors known as NGFI-A binding proteins (Nab) (32, 33). Egr1, Egr2, and Egr3 proteins (but not Egr4) contain a repression domain (R1) that binds to the highly conserved NCD1 domain present in both Nab family members, Nab1 and Nab2 (34–38). Nab proteins are unable to bind to DNA alone (38) and thus suppress transcription upon recruitment to a DNA-bound Egr family member (32, 33, 38). The repressive Egr–Nab complexes often silence or maintain repression of gene expression by recruiting factors that can induce epigenetic gene silencing, such as HDACs (33, 39).

In this study, we showed that type I IFN treatment rapidly silences *ifngr1* transcription in mouse and human macrophages, but not T cells, and describe a mechanism contributing to this silencing. We identified putative Egr binding sites in the mouse and human *ifngr1* promoters and showed that a proximal Egr site is required for silencing of *ifngr1* transcription in mouse myeloid cells treated with IFN-β. Chromatin immunoprecipitation analysis further indicated that type I IFNs induce rapid recruitment of Egr3 to a region of the *ifngr1* promoter containing this proximal Egr binding site in myeloid but not T cells. Recruitment of Egr3 correlated with reductions in activated RNA polymerase II (pol II) and preceded recruitment of Nab1. Nab1 recruitment coincided with and was required for deacetylation of the *ifngr1* promoter and downregulation of cell surface IFNGR. These data demonstrate involvement of a Egr3/Nab1 complex in the silencing of *ifngr1* transcription and downregulation of IFNGR by type I IFNs. Putative Egr binding sites were also identified in the promoters of other constitutively expressed genes known to be repressed by type I IFNs, suggesting Egr3 and Nab1 may play a general role in negative regulation of myeloid cell gene expression.

### Materials and Methods

#### Mouse

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). IFNAR-−/− crossed to C57BL/6 (The Jackson Laboratory) for >10 generations were described previously (14). Mice were housed in the National Jewish Health Biological Resource Center. The National Jewish Health Institutional Animal Care and Use Committee approved all studies.

#### Cell culture and IFN-β treatment

To culture bone marrow–derived macrophages (BMDMs), cells were flushed from the femurs, tibias, and fibulas of mice and cultured for 6 d in bone marrow macrophage medium (DMEM supplemented with 10% FBS, 1% sodium pyruvate, 1% l-glutamine, 1% penicillin/streptomycin, 2-ME, plus 10% L-cell conditioned media). Media components were Life Technologies (Carlsbad, CA). Fresh medium was added at day 3, and BMDMs were used for experiments on day 7. RAW264.7 murine macrophage cells and EL4 murine T cells were cultured in DM10 medium (DMEM supplemented with 10% FBS, 1% sodium pyruvate, 1% l-glutamine, and 1% penicillin/streptomycin). THP-1 cells were cultured in suspension with RPMI 1640 supplemented with 10% FBS, 1% sodium pyruvate, 1% l-glutamine, and 1% penicillin/streptomycin. Twenty-four hours prior to experimentation, THP-1 cells were stimulated with 0.1 µg/ml PMA (P-8139; Sigma-Aldrich, St. Louis, MO) to obtain adherent cells. To obtain human PBMCs (hPBMCs), de-identified blood from donors was collected in heparin-containing vacuum tubes, and WBCs were separated from whole blood by Ficoll–Paque gradient (Histoaque-1077; Sigma-Aldrich). Isolated cells were incubated overnight in 6-well culture plates in DMEM supplemented with human serum. BMDMs, RAW264.7, and EL4 cells were treated at various time points with 100 U/ml murine IFN-β (PBL IFN Source, Piscataway, NJ). THP-1 cells and hPBMCs were treated at various time points with 100 U/ml human IFN-β (PBL IFN Source).

#### Flow cytometry

BMDMs and adherent cell lines were lifted from culture dishes with cold PBS. Adherent hPBMCs were lifted from culture dishes with cold PBS and added to nonadherent cell lines. Murine FcRs were blocked before staining using supernatant from hybridoma 2.4G2 (rat anti-CD16/32), and human FcRs were blocked using pooled human serum in PBS. To detect murine FcRs, cells were stained with biotinylated Abs to IFNGR1/CD119 (BD Biosciences, San Jose, CA), followed by streptavidin–APC secondary Ab (eBioscience, San Diego, CA). To detect human IFNGR1, cells were stained with biotinylated Abs to IFNGR1/CD119 (Caltag Laboratories, Life Technologies, Carlsbad, CA), followed by streptavidin–APC–secondary primary human T cells and monocytes were detected using CD3–FITC and CD14–PE Abs (eBioscience), respectively. To detect MHC class I, Egr3 antibodies were stained with FITC–FITC (eBioscience). Abs were diluted in surface staining buffer (PBS/1%BSA/0.01% NaN3). The mean fluorescence intensities (MFIs) for each of three treated samples per time point were normalized to mean MFI for three untreated samples using the following formula: relative surface staining = (MFI treated)/(MFI untreated). For statistical analyses, we pooled the relative MFI values from three separate experiments each using at least three control and three treated samples.

#### Real-time quantitative PCR

Preparation and analysis of samples for quantitative RT-PCR (qPCR) was described previously (14). Briefly, 9 × 10^6 BMM or RAW264.7 cells were distributed into 3 wells of a 6-well plate (Cell Star; Sigma-Aldrich) for each treatment time point. Cells were pooled from 3 wells, and the RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). DNAD synthesis was conducted with random primers, subtracting oligo(dT) from the reaction (both with and without IFN treatment), and preceded by DNase to eliminate any contaminating DNA. cDNA synthesis was conducted with random primers, subtracting oligo(dT) from the reaction (both with and without IFN treatment), and preceded by DNase to eliminate any contaminating DNA. cDNA was reverse-transcribed and real-time quantitative PCR (qPCR) was performed on 1/100 cDNA in 15-mL reaction volumes using LightCycler 480 III SYBR Green PCR Master Mix (Roche) with primers specific for each gene quantified. Each sample was run in triplicate and the average Cq value was used for quantification. Mean expression levels were normalized to the expression of β-actin and calculated using the 2^ddCt method. For each time point, 3 separate experiments were performed with at least 3 technical replicates.

#### Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) experiments were performed according to the protocol provided for the Active Motif ChIP Express Kit (Active Motif, Carlsbad, CA). Briefly, after treatment with IFN-β, RAW 264.7, EL4, and BMDMs were cross-linked with 1% methanol-free formaldehyde for 5 min at room temperature. Fixed cells (7 × 10^6 in 300 µl) were resuspended in kit lysis buffer plus protease inhibitors and incubated at 30 min at 4°C. Cell nuclei were pelleted and resuspended in 300 µl kit shearing buffer plus protease inhibitors. A Covaris S2 sonicator was used to shear the samples, using a 27-cycle treatment. To ensure that the shearing process consistently produced fragments of 200–500 bp, 5 µl of all supernatants was run on a 1% agarose gel (Supplemental Fig. 3A). An additional 10 µl supernatant was saved for use as total input DNA. All samples were stored at −80°C until use. Immunoprecipitations were performed with protein G magnetic beads overnight at 4°C, using an estimate 7 µg sheared chromatin and Abs specific for pS5-RNA pol II (ab5131; Abcam, Cambridge, MA), acetyl-histone H3 (39139; Active Motif), pan-acetyl-histone H4 (39925; Active Motif), total histone H3 (ab1791; Abcam)
Egr1 (number 4135; Cell Signaling Technology, Danvers, MA), Egr2 (PRB-236P; Covance, Princeton, NJ), Egr3 (ab75461; Abcam), and Nab1 (NPBI-71838; Novus, Littleton, CO). Equal amounts of chromatin were also immunoprecipitated using equivalent amounts of a control IgG Ab (ab46540; Abcam). Following immunoprecipitation, beads were washed, and the immune complexes were eluted with ketyl elution buffer. Reverse cross-linking buffer was added to each eluted supernatant at 1:1, and the samples and input DNA were heated for 1 h at 95 °C. After treatment with 10 μg/ml proteinase K for 1 h at 37 °C, samples were purified using Qiagen PCR purification kit and then used for qPCR. The promoter primer sequences used to analyze chromatin immunoprecipitations for p53-RNA pol II, acetylated histone H3, total histone H3, and acetylated histone H4 were sense, 5'-GCAAATGGTGGCTCTCCGGCGAGGATGGGCC-3', and antisense, 5'-GCAGCTCTCAAGCTCACTCCCCGCC-3'. The primer sets used to analyze Egr and Nab immunoprecipitations were sense, 5'-CCTAGGCACATGATGCCCGG-3', and antisense, 5'-GGGACAGCTGCCTCGGG-3'. Real-time quantitative PCR was performed using an Absolute qPCR Sybr GREEN PCR ROX Mix (Thermo, Waltham, MA) and an ABI Prism 7900 Sequence detector. The equation used for determining percent input is 

\[ \text{percent input} = \frac{2^{\Delta \text{IP Ct}} - 2^{\Delta \text{input Ct}}}{2^{\Delta \text{IP Ct}}} \]  

The equation used for determining fold enrichment over isotype is 

\[ 2^{\Delta \text{IP Ct}} - 2^{\Delta \text{input Ct}} \]  

Luciferase constructs

The wild-type (wt) ifngr1 promoter luciferase reporter construct (IFNGR1pr-luc) was created by amplification of the proximal portion of the ifngr1 promoter from ~2320 to +1 using C57BL/6 genomic DNA as template. Primers were as follows: sense, 5'-GGGGTACCCAGACTAGATGACCTCCCTGCCACC-3', and antisense, 5'-CAGTCTCCTACAGGAGCGGCTTGAGCTCCGG-3'. KpnI and XhoI restriction sites were included in the primers and used to clone the amplified sequence into the multiple cloning site of pGL3-Basic (Promega). The hygromycin resistance gene from pGL4.15[Hygro] (Promega) was linearized, and the inserts were ligated into the BamHI and SalI sites of pGL3 to generate IFNGR1pr-luc. For mutagenesis, the Stratagene QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used. The primer set for mutagenesis of the Egr site in mEgr-luc was sense, 5'-CTAGTCCACCCCTTCTCC-3', and antisense, 5'-GAGGCGGCTCTGTCGCGG-3'. The two independent IFNGR1pr-luc and mEgr-luc cell lines were obtained from two separate transfections.

Creation of putative transcription factor binding maps

To construct a putative transcription factor binding site map of the mouse and human ifngr1 promoters, the DNA sequences for the first 2320 bp were entered into the TFSearch program (40). Results were limited to consensus sequences with a score of 85.0 or more according to the program’s algorithm. The presence and location of these binding sites were confirmed using TESS (41) and ENCODE (42).

Reporter cell lines and luciferase assay

To make stable cell lines, 5 × 10⁶ RAW 264.7 cells were transfected with 1 μg linearized IFNGR1pr-luc or mEgr-luc plasmid by electroporation (250 V, 950 μF) using a Bio-Rad electroporator. Following electroporation, the cells were resuspended in 10 ml DM10 and plated at 100 μl/well in a 96-well plate. The transfected cells were selected for using 250 μg/ml hygromycin. To determine the luciferase activity, expanded cell lines were obtained from two separate transfections. To determine IFNGR1 staining when BMDMs were treated with IFN-β, the reduction in cell surface IFNGR1 staining in both wt BMDMs and RAW264.7 cells was found to be significant as early as 2 h poststimulation (hps) with IFN-β (Fig. 1C, 1D). These data indicate that both primary mouse macrophages and RAW264.7 cells respond to type I IFNs by rapidly down regulating cell surface IFNGR1, although the reduction in IFNGR1 staining seen with RAW264.7 cells (40–50%) was consistently less than that seen with wt C57BL/6 BMDMs (55–65%).

To determine whether human myeloid cells also downregulate IFNGR1 in response to type I IFNs, cell surface IFNGR1 staining was evaluated in human THP-1 macrophage–like cells and in primary PBMCs. Similar to mouse myeloid cells, IFNGR1 staining was selectively and significantly reduced in THP-1 macrophages (Fig. 1B). However, downregulation of IFNGR1 was not seen in mouse EL4 T cells (Fig. 1B), even though they responded to IFN-β with upregulation of MHC class I (Supplemental Fig. 1B) and phosphorylation of STAT1 (Supplemental Fig. 1C). Increasing the concentration of IFN-β used for stimulation did not further reduct IFNGR1 downregulation for either BMDMs or RAW 264.7 cells (Supplemental Fig. 1D). We also observed similar reductions in IFNGR1 staining when BMDMs were treated with IFN-α (Supplemental Fig. 1E). To facilitate statistical comparisons of cell surface IFNGR1 staining across multiple experiments and time points, the MFIs of staining on IFN-β–treated cells were normalized to those of mock-treated control cells. When normalized data from three independent experiments were plotted and analyzed, the reduction in cell surface IFNGR1 staining in both wt BMDMs and RAW264.7 cells was found to be significant as early as 2 h poststimulation (hps) with IFN-β (Fig. 1C, 1D). These data indicate that both primary mouse macrophages and RAW264.7 cells respond to type I IFNs by rapidly down regulating cell surface IFNGR1, although the reduction in IFNGR1 staining seen with RAW264.7 cells (40–50%) was consistently less than that seen with wt C57BL/6 BMDMs (55–65%).

Type I IFNs silence transcription of ifngr1

The observed reductions in cell surface IFNGR1 staining in mouse macrophages correlated with similar magnitude reductions in the abundance of ifngr1 transcripts as measured by quantitative real-time RT-PCR (qPCR) (Fig. 2A, Supplemental 2A). The effects of

Stable knockdown of Egr and Nab family members

Sigma-Aldrich Mission short hairpin RNA constructs Egr3 (TRCN number 96139), Nab1 (TRCN number 96134), and Nab2 (TRCN number 96349) were obtained from the University of Colorado Cancer Center (University of Colorado-Anschutz Medical Campus, Aurora, CO). To make stable cell lines, each construct was linearized, and 1 μg was used for transfection of

5 × 10⁶ RAW 264.7 cells. Transfected cells were selected using 5 μg/ml puromycin. The two independent Nab1-KD and Nab2-KD cell lines used for experiments were obtained from two separate transfections.

Results

Type I IFNs decrease surface expression of IFNGR1 on mouse and human macrophages

Consistent with our previous studies (14), we observed that cell surface IFNGR1 staining was reduced at 6 h after IFN-β treatment (100 U/ml) in BMDMs from wt C57BL/6, but not IFNAR−/− mice (Fig. 1A). The intensity of IFNGR1 staining on cells from IFNGR1−/− mice was similar to that of the secondary reagent alone, demonstrating specificity of the stain (Supplemental Fig. 1A). The ability of IFN-β treatment to reduce IFNGR1 staining was also observed with RAW264.7 macrophages (Fig. 1B). However, downregulation of IFNGR1 was not seen in mouse EL4 T cells (Fig. 1B), even though they responded to IFN-β as judged by upregulation of MHC class I (Supplemental Fig. 1B) and phosphorylation of STAT1 (Supplemental Fig. 1C). Increasing the concentration of IFN-β used for stimulation did not further reduce IFNGR1 downregulation for either BMDMs or RAW 264.7 cells (Supplemental Fig. 1D). We also observed similar reductions in IFNGR1 staining when BMDMs were treated with IFN-α (Supplemental Fig. 1E). To facilitate statistical comparisons of cell surface IFNGR1 staining across multiple experiments and time points, the MFIs of staining on IFN-β–treated cells were normalized to those of mock-treated control cells. When normalized data from three independent experiments were plotted and analyzed, the reduction in cell surface IFNGR1 staining in both wt BMDMs and RAW264.7 cells was found to be significant as early as 2 h poststimulation (hps) with IFN-β (Fig. 1C, 1D). These data indicate that both primary mouse macrophages and RAW264.7 cells respond to type I IFNs by rapidly down regulating cell surface IFNGR1, although the reduction in IFNGR1 staining seen with RAW264.7 cells (40–50%) was consistently less than that seen with wt C57BL/6 BMDMs (55–65%).

To determine whether human myeloid cells also downregulate IFNGR1 in response to type I IFNs, cell surface IFNGR1 staining was evaluated in human THP-1 macrophage–like cells and in primary PBMCs. Similar to mouse myeloid cells, IFNGR1 staining was selectively and significantly reduced in THP-1 cells and CD3+CD14+, but not CD3+CD14−, PBMCs at 6 hps with 100 U/ml human IFN-β (Fig. 1E, 1F; gating shown in Supplemental Fig. 1F). The similar effect of type I IFNs on IFNGR expression in mouse and human myeloid cells is consistent with the known ability of type I IFNs to suppress activation of both mouse and human macrophages (43, 44) and the ability of IFN-β treatment to ameliorate mouse and human neuroinflammatory diseases (45). In addition, the lack of IFNGR1 downregulation in mouse EL4 T cells (Fig. 1B, 1D), mouse splenic CD3+ T cells (14), and human CD3+ PBMCs (Fig. 1E, 1F) suggests that this conserved response to type I IFNs primarily occurs in myeloid cells.
IFN-β on *ifngr1* transcript abundance were significant as early as 2 hps. A similarly rapid decrease in transcript abundance was seen upon chemical inhibition of the transcription machinery using inhibitory concentrations of actinomycin D (Fig. 2A). The actinomycin D concentration used (1 μg/ml) did not affect BMDM viability but was sufficient to block de novo expression of IFN-β in BMDM treated with poly I:C (Supplemental Fig 2B). On the basis of the changes in transcript abundance over time, we calculated the half-life of *ifngr1* mRNA in the BMDM to be 2.74 h (± 0.257 SD) following IFN-β treatment, and 2.70 h (± 0.169 SD) following actinomycin D treatment. These results suggest that IFN-β treatment rapidly and fully silences de novo transcription of *ifngr1*.

As an independent method to confirm whether IFN-β treatment silenced new *ifngr1* transcription, we used ChIP to evaluate accumulation of active RNA pol II to a 100-bp region adjacent to the predicted transcriptional initiation start site (TSS) of the *ifngr1* gene (Fig. 2B, *inset*). Chromatin isolated from BMDMs 0, 2, 4, and 6 hps with IFN-β was immunoprecipitated with an Ab specific for a phosphorylated isoform of the RNA pol II complex (pS5-RNA pol II). This Ab specifically recognizes RNA pol II phosphorylated at serine 5 within the C-terminal domain heptapeptide repeat, a modification that is necessary for the initiation of transcription (46). The relative abundance of promoter DNA immunoprecipitated with anti–pS5-RNA pol II was determined using qPCR (Fig. 2B). The data showed that IFN-β treatment significantly reduced association of activated RNA pol II with the *ifngr1* promoter by 2 hps. Consistent with the conclusion that IFN-β prevents new *ifngr1* transcription, the association of activated RNA pol II and the *ifngr1* promoter remained low for at least 6 hps.

The silencing of *ifngr1* transcription suggested that type I IFN stimulation might alter the epigenetic structure of the *ifngr1*.
promoter. Epigenetic changes associated with transcriptionally inactive condensed chromatin include deacetylation of lysine residues in histones H3 and H4 (47, 48). We used Abs specific for lysine N-acetylation of histones H3 (H3Ac) and H4 (H4Ac) to conduct ChIP assays on BMDMs stimulated with IFN-β for 0, 2, 4, and 6 h. Type I IFN stimulation induced a transient increase in H3Ac signal at 2 hps, presumably reflecting rapid changes in the structure of the promoter. However, starting at 4 hps, the stimulation significantly reduced acetylation of histones H3 and H4 at the ifngr1 promoter (Fig. 2C). Taken together, the pS5-RNA pol II, H3Ac, and H4Ac ChIP results indicate that stimulation of primary macrophages and cell lines with type I IFNs induces a rapid and sustained silencing of ifngr1 transcription.

An Egr site is required for silencing of transcription from the proximal ifngr1 promoter

To determine whether the proximal ifngr1 promoter was responsive to transcriptional repression by type I IFNs, we stably transfected RAW 264.7 macrophages with a luciferase construct containing the 2320-bp region from the proximal mouse ifngr1 promoter (IFNGR1pr-luc). The type I IFN–responsive region of the mouse promoter contained three putative Sp1 binding sites adjacent to a TATA box and a putative Egr binding site near the ifngr1 transcriptional start site (Fig. 3A). Putative binding sites for Sp1 and Egr1 were also present in the proximal region of the human ifngr1 promoter (Supplemental Fig. 4A). A previous study implicated Sp1 as a positive regulator of ifngr1 transcription (49), and others suggested that Egr family members repress Sp1–dependent transcriptional activity (26–29). We thus engineered a mutated version of IFNGR1pr-luc with three point mutations at the putative Egr binding site (Fig. 3A). The selected mutations were previously shown to disrupt the ability of DNA to bind Egr1 protein (50). The IFNGR1pr-luc reporter cells experienced a rapid and significant decrease in luciferase activity following treatment with IFN-β, demonstrating a decrease in ifngr1 promoter activity (Fig 3B). In contrast, the reporter activity in RAW 264.7 cells stably transfected with the mutated construct (mEgr-luc) was not affected by treatment with IFN-β. These data suggested that the presence of a functional Egr binding site is essential for the silencing of transcription from the proximal ifngr1 promoter.

Egr3 and Nab1 are recruited to the proximal Egr site of the ifngr1 promoter in response to type I IFN stimulation

Previous studies reported expression of Egr1, Egr2, and Egr3 mRNAs in BMDMs (51). We thus used immunobots to investigate expression of these Egr proteins in our BMDMs, and whether IFN-β treatment affected such expression (Fig. 4A). Egr1 protein was present at low or undetectable levels in the unstimulated cells but was strongly induced within 2 hps with IFN-β (Fig. 1C). Egr2 protein was present in unstimulated cells and at late times poststimulation but appeared to undergo a transient depletion at 2 hps. Egr3 was readily detected in unstimulated cells, and its expression was reduced cell surface IFNGR1 staining in IFN-β–treated BMDMs (Fig. 1C).

Nab proteins are known to interact with DNA-bound Egr family members as corepressors of target gene expression (32, 33). We thus asked whether Egr3 binding to the ifngr1 promoter might recruit Nab proteins to mediate silencing of ifngr1. Both Nab1 and Nab2 showed detectable levels of protein expression in unstimulated BMDMs (Fig. 4A). Stimulation with IFN-β modestly reduced Nab2 expression at late times (6 hps) but did not alter Nab1 protein amounts. ChIP assays were thus used to evaluate whether Nab1 was recruited to the ifngr1 promoter. By 4 hps, Nab1 was detected at a region of the ifngr1 promoter containing the Egr site (Fig. 4C, Supplemental Fig. 4C). This result established that IFN-β treatment of BMDMs stimulates the recruitment of Nab1 subsequent to recruitment of Egr3 (Fig. 4B) and at a time when there is deacetylation of the ifngr1 promoter (Fig. 2C). Recruitment of Egr3 and Nab1 to the ifngr1 promoter also occurred in RAW 264.7 cells following stimulation with IFN-β for 4 h (Fig. 4D). However, Egr3 was not recruited to the ifngr1 promoter in EL4 cells (Supplemental Fig. 4D), suggesting that these events selectively occur in myeloid cells as they silence ifngr1 transcription.

Nab1 knockdown prevents ifngr1 promoter deacetylation and downregulation of IFNGR1 in response to type I IFN stimulation

Repeated attempts to knockdown Egr3 expression in RAW264.7 cells failed, suggesting this factor may be important for macrophage viability. However, we were successful in generating knockdowns of Nab1 and Nab2. Independent, stably transfected cell lines were developed using constructs encoding short hairpin RNAs that targeted the nab1 and nab2 genes. These lines respectively showed large decreases in Nab1 (Nab1-KD) and almost complete elimination of Nab2 (Nab2-KD) protein expression when
FIGURE 4. Egr3 and Nab1 are recruited to the proximal Egr site of the ifngr1 promoter in response to type I IFN stimulation. (A) Representative Western blots of Egr1, Egr2, Egr3, Nab1, and Nab2 to determine protein expression in BMDMs stimulated or not with 100 U/ml IFN-β. Anti-actin Ab was used to determine that equivalent protein concentrations were loaded in each lane. ChIP assay for Egr3 (B) and Nab1 (C) in BMDMs or in RAW 264.7 cells (D) stimulated or not with 100 U/ml IFN-β. Primers denoted in Fig. 3A were used for all qPCR analysis. Graphs depict fold enrichment over isotype normalized to those of the respective untreated cells, calculated as in Fig. 2B. Each bar in the graphs represents the mean ± SD of pooled values from a total of at least three independent experiments. Statistical significance was determined using an unpaired t test: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

compared with control RAW 264.7 cells (Fig. 5A). In response to treatment with IFN-β, cell surface IFNGR1 staining was barely reduced in the Nab1-KD cells (Fig. 5B), whereas IFNGR1 staining in the Nab2-KD cells was reduced to a similar extent as seen in control RAW 264.7 cells (Fig. 5C). These data established that Nab1, but not Nab2, plays a role in the suppression of IFNGR1 expression.

Nab proteins were previously shown to interact with complexes that actively deacetylate histones to silence gene expression (33, 39). We thus asked whether reducing the recruitment of Nab1 to the ifngr1 promoter could prevent the histone deacetylation associated with silencing of ifngr1 transcription. ChIP for Nab1 and H3Ac was performed on Nab1-KD and Nab2-KD cell lines. In contrast to Nab2-KD cells, recruitment of Nab1 to the ifngr1 promoter was not detectable in the Nab1-KD cells after 6 h of stimulation with IFNβ (Fig. 5D). This finding indicated that the knockdown efficiency in the Nab1-KD cells was adequate to prevent detectable Nab1 recruitment and that Nab2 knockdown did not prevent this recruitment. Furthermore, this lack of Nab1 recruitment was associated with a failure of IFNβ stimulation to induce deacetylation of histone H3 in Nab1-KD, but not Nab2-KD, macrophages (Fig. 5E). Thus, Nab1 recruitment is necessary for the histone deacetylation associated with silencing of the ifngr1 promoter in response to type I IFN stimulation (Fig. 6).

Discussion

Although IFN-β has been widely used in treatment of multiple sclerosis and is known to impair resistance to certain bacterial infections, the mechanisms through which type I IFNs suppress inflammatory responses remain poorly understood. Our findings here provide further insight into this mystery by showing how type I IFNs downregulate expression of the IFNGR. Reduced IFNβ expression is known to correlate with reduced myeloid cell responsiveness to the proinflammatory cytokine IFN-γ (14), and thus may itself contribute to the anti-inflammatory effects of type I IFNs. In addition, the mechanism suggested by our findings in this paper may also contribute to the silencing of other genes involved in the proinflammatory responses of myeloid cells.

Transcripts for ifngr1 and ifngr2 are constitutively present in a variety of tissues, and functional IFNGRs are constitutively present at the surface of diverse cell types (2, 52, 53). However, variations in ifngr1 and ifngr2 expression occur in lymphoid and
myeloid cells. Early studies established that the expression of IFNGR2 is selectively downregulated in Th1-type T cells (54, 55), presumably to protect them from killing by the IFN-γ they produce. IFNGR1 is not downregulated in T cells, but we recently reported that both IFNGR1 and IFNGR2 are lost from the cell surface of myeloid and B cells responding to type I IFNs (14). The loss of cell surface IFNGR in myeloid cells, which was associated with reduced abundance of ifngr1 (but not ifngr2) transcripts, correlated with reduced responsiveness to IFNγ and increased susceptibility to infection by the bacterium L. monocytogenes (14). This previous work indicated that type I IFN stimulation reduces both cell surface IFNGR1 and total cellular IFNGR1 in myeloid cells exposed to type I IFN (14). Moreover, the loss of cell surface IFNGR1 occurred with a half-life of ~4 h, matching the previously measured half-life of IFNGR1 protein (56). Silencing of ifngr1 transcription and the consequent reduction in cell surface IFNGR1 has also been observed in breast cancer cells, which enables the tumor cells to evade killing by IFN-γ (57). These findings together argue that constitutive transcription of ifngr1 is necessary to maintain cell surface expression of the IFNGR, and that silencing of new ifngr1 transcription is sufficient to reduce sensitivity of myeloid and other nonlymphoid cell types to IFN-γ.

Regarding the mechanism of ifngr1 silencing in myeloid cells, our studies here showed that an Egr binding region in the proximal ifngr1 promoter is responsive to type I IFNs. The type I IFN-responsive Egr site in the proximal ifngr1 promoter is near sites for binding of the Sp1 transcription factor. Sp1 promotes transcriptional activation through direct interaction with factors important for assembly of the RNA pol II complex (58), and has been shown to promote basal transcription of human ifngr1 (49). It has also previously been established that Egr family members can interfere with Sp1 binding (26–28), and/or assembly of the RNA pol II transcriptional complex (30, 31). Hence, we speculate that the binding of Egr3 may initially impair new transcription of ifngr1 by blocking Sp1 binding to the promoter. Consistent with this model, decreased binding of Sp1 to the ifngr1 promoter was associated with decreased transcription of ifngr1 in THP-1 cells infected with M. tuberculosis (59), and in breast cancer cells (57).

Prolonged silencing of active promoters often involves recruitment and activity of HDAC enzymes by DNA-bound repressors (60). We found that binding of Egr3 to the ifngr1 promoter preceded recruitment of Nab1 and loss of acetylated histones H3 and H4. Nab family members have been shown to repress gene transcription by interacting with HDACs (33, 39), and our data reveal that Nab1 knockdown largely prevents deacetylation of histone H3 at the ifngr1 promoter and IFNGR1 downregulation. Thus, we speculate that the prolonged and complete silencing of ifngr1 in myeloid cells involves the formation of an Egr3/Nab1 complex that fosters the recruitment of HDACs to deacetylate the ifngr1 promoter (Fig. 6).

Microarray analysis has shown that numerous basally active genes are rapidly repressed by type I IFN in BMDMs (9). The affected genes include several that code for proteins known to be associated with an activated macrophage state, such as ICAM-1, IL-1β, Jagged-1, and IL-12p40. The promoters for these four genes, and numerous other genes repressed by type I IFNs in myeloid cells, contain Egr sites. Thus, the downregulation of these genes may use a similar mechanism to that proposed here for ifngr1. In addition, such downregulation could arguably have anti-inflammatory effects. For example, the induction of Jagged-1 has recently been shown to be important for enhancing the responsiveness to IFN-γ (61), suggesting that its downregulation by type I IFN may synergize with the suppressive effects of IFNGR1 downregulation. Likewise, reductions in IL-12p40 would be expected to reduce the induction of IFN-γ (and IL-17) production by T cells. Interestingly, the ifngr1 silencing in breast cancer cells (which is not known to require type I IFNs) was previously shown to involve the transcription factor AP-2α (57), rather than Egr3. A search of the ImmGen database showed that AP-2α is not expressed in immune cells (62), and our data here indicated that IFN-β stimulation failed to stimulate recruitment of Egr3 to the ifngr1 promoter in EL4 T cells. Thus, Egr3 appears to selectively participate in the silencing of ifngr1 transcription that is triggered by type I IFNs in myeloid cells. The observation that there are distinct mechanisms to permit repression of ifngr1 or ifngr2 transcription in myeloid, lymphoid, and other cell types underscores the biological importance of regulating responsiveness to IFN-γ, and raises the possibility of therapies that selectively increase or reduce such responsiveness in specific cell types.

It remains unclear how stimulation with type I IFNs induces the rapid recruitment of Egr3/Nab1 to the ifngr1 promoter and why only these family members are recruited. As shown in this paper, we failed to observe significant induction of Egr3 or Nab1 expression in response to type I IFN stimulation. We have also failed to see increased nuclear localization of Egr3 in stimulated macrophages (data not shown). We thus hypothesize that recruitment of Egr3 is triggered by phosphorylation or other posttranslational modification of Egr3 or an associated factor. Multiple studies have demonstrated posttranslational modifications that alter the activity of Egr1 in response to exogenous stimuli (63–66). In addition, one study suggests that different modifications to the phosphorylation state of Egr1 alter whether this factor acts as an inducer or repressor of transcription at the same promoter (65). Phosphorylation or other modifications of Egr1 and 2 might also conceivably prevent their recruitment to the proximal ifngr1 promoter. Further
investigation is needed to resolve these issues and determine more precisely how type I IFN stimulation regulates the association of Egr3 with the ifngr1 promoter.

Polymorphisms in the ifngr1 promoter are known to correlate with susceptibility to diverse diseases such as Leishmaniasis, tuberculosis, leprosy, and hepatitis infections (67–70). Suppression of ifngr1 transcription by type I IFNs also correlates with impaired macrophage activation, and compromised resistance to diverse bacterial infections (5–7). Yet, surprisingly little is known about the transcriptional regulation of ifngr1. Our efforts in this study have provided new insight into the mechanisms for silencing of ifngr1 expression by type I IFNs and thus the regulation of IFNGR expression in myeloid cells. These findings may also prove relevant for silencing of other myeloid cell gene expression by type I IFNs. Ultimately, such efforts may reveal new strategies for improving host resistance to a variety of infectious diseases.

Disclosures

The authors have no financial conflicts of interest.

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Transcriptional Repression by Type I IFNs

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La biodiversidad de bacterias en el sedimento.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: Stimulation with 100 U/mL of IFNβ is appropriate to induce decreased IFNGR1 surface staining in myeloid but not T cells. (A) Histogram of BMDMs stained with anti-CD119 + SA-APC (black line), SA-APC alone (dashed line), or unstained (gray shading). (B) EL4 cells treated with 100 U/mL IFNβ for 24 hrs, and stained to measure upregulation of MHC1. Graph depicts MFI values of both treated and untreated cells. Each bar in the graphs represent mean ± STD from at least 3 pooled experiments, and statistical significance was determined using an unpaired t-test with * ≤ 0.05, ** ≤ 0.01, *** ≤ .001. (C) Western Blot for STAT1pY701 or Total STAT1 in EL4 or RAW cells treated or not with 100 U/mL IFNβ. (D) BMDMs or RAW 264.7 cells treated with increasing concentrations of IFNβ for 6 h. (E) BMDMs with increasing concentrations of IFNα. Graphs represent one experiment, and depict MFI values normalized to those of the respective untreated cells that were calculated using the equations previously described in Fig. 1C. Each bar in the graphs represents the mean ± STD. (F) Dot plot showing gating scheme to identify CD14+ and CD3+ human peripheral blood mononuclear cells.

Supplemental Figure 2: Type I IFNs decrease ifngr1 transcript abundance in RAW 264.7 and Actinomycin D treatment blocks de novo synthesis of IFNβ transcripts. (A) Total RNA was isolated from RAW 264.7 cells treated or not with 100 U/mL of IFNβ. Graph depicts ifngr1 transcript abundance values normalized to those of the respective untreated cells from a total of at least three pooled independent experiments. Statistical significance was determined using an unpaired t-test with * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001. (B) Total RNA was isolated from BMDMs pre-treated or not with 1μg/mL of Actinomycin D, followed by a
three hour stimulation with 5 pg/mL of poly I:C (PIC) or not. Graph depicts IFNβ transcript abundance from one experiment. Relative change in normalized expression between treated and untreated cells quantified by qRT-PCR, and the equation described in Fig. 2A was used to calculate the relative transcript abundance values. Each bar in the graphs represents the mean ± STD.

**Supplemental Figure 3: Sheared DNA, and percent input analysis for pS5-RNA pol II, H3Ac, H4Ac, and Total H3 ChIP assays** (A) Sheared DNA from BMDMs treated or untreated with 100 U/mL IFNβ run on a 1% agarose gel. The sheared chromatin in these samples represents similar results for all samples. Representative percent input analyses for (B) pS5-RNA pol II, (C) H3Ac, and (D) Total H3 ChIP assays. Primers denoted in Fig. 2B were used for all qPCR analysis. Percent input=(2^(CT Input – CT IP))*100. Each bar in the graph represents the mean ± STD.

**Supplemental Figure 4: Diagram of human ifngr1 promoter and percent input analysis for Egr1, Egr2, Egr3, and Nab1 ChIP Assays.** (A) Schematic of the proximal region of the human ifngr1 promoter. Representative percent input analyses for (B) Egr1, Egr2, Egr3, and (C) Nab1 ChIP assays in BMDMs stimulated or not with 100 U/mL of IFNβ. (D) Representative percent input analysis for Egr3 ChIP assay in RAW 264.7 or EL4 cells treated or not with 100 U/mL IFNβ for 6 hrs. (D-inset) Immunoblot showing Egr3 expression in RAW and EL4 cells. Primers denoted in Fig. 3A were used for all qPCR analysis. Percent input calculated with the following equation: Percent Input=(2^(CT Input – CT IP))*100. Each bar in the graphs represents the mean ± STD.
Supplemental Figure 1

A

C57BL/6 IFNGR1−/−

Relative Cell #

IFNGR1

B

MHC MFI

***

Hours Post Stimulation

mock 24

C

EL4 RAW 264.7

IFNβ (min) 0 30 60 0 30 60

pY701

STAT1

D

C57BL/6 BMDMs RAW 264.7

Relative IFNGR1 MFI

mock 100 200 500 1000

IFNβ Dose (U/mL)

E

Relative IFNGR1 MFI

mock 100 200 500 1000

IFNα Dose (U/mL)

F

hPBMC

CD14

CD3
Supplemental Figure 2

A

![Bar chart showing IFNA/ transcripts relative abundance over hours post stimulation.](image)

B

![Bar chart showing IFNβ transcripts relative abundance with different treatments.](image)
Supplemental Figure 3

A

B

C

D

H3Ac

H4Ac

Isotype

pS5-RNA pol II

Isotype

Percent Input

Percent Input
Supplemental Figure 4

A

TATA Sp1 Egr Egr Sp1 Sp1
-900 +1

B

Egr1 Egr2 Egr3 Isotype

Percent Input

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5

mock 2 4 6

Hours Post Stimulation

C

Nab1 Isotype

Percent Input

0.0 0.5 1.0 1.5 2.0

mock 2 4 6

Hours Post Stimulation

D

Egr3 Isotype

Percent Input

0.0 0.1 0.2 0.3 0.4

RAW 264.7 EL4

mock 6 mock 6