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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

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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 transcription 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.
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 CCGCC-
CCGC (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 IFNGR1. These data demonstrate involvement of a Egr3/Nab1 complex in the silencing of ifngr1 transcription and downregulation of IFNGR1 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

Mice

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-ML, 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 RPM1 medium (RPMI 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 (Histopaque-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 human 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 cells. MurineFcRs were blocked before staining using supernatant from hybridoma 2.4GZ (rat anti-CD16/32), and human FcRs were blocked using pooled human serum in PBS. To detect murine IFNGR1, 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, Egr family members were stained with biotinylated-PE Egr2 (eBioscience, San Diego, CA). All 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 using each 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 × 106 BMM or RAW264.7 cells were distributed into 3 wells of a 6-well plate (CellStar; 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). cDNA synthesis was conducted with 1 μl total RNA, reverse oligo(dT)18 primer (0.5 μl), and Superscript II (Life Technologies, Carlsbad, CA). Commercial oligonucleotide primer sets from Applied Biosystems (Life Technologies) were used to quantify mouse ifngr1 and human ifngr1 transcripts and the primer set, sense, 5'-GACCTGGTGGAATGAGACTATTG-3' and antisense, 5'-GACCTGTCAAGTGATGCCCTAGAA-3', was used to quantify mouse IFN-β transcripts. Real-time quantitative PCR was performed using Sybr Green PCR MasterMix (Applied Biosystems) and an ABI Prism 7300 Sequence detector. The equation used to determine relative transcript abundance is $2^{(-\Delta\Delta Ct)}=\frac{\text{mean GAPDH Ct}_{\text{sample}} - \text{mean GAPDH Ct}_{\text{reference}}}{\text{mean unsupplemented Ct}_{\text{sample}}}$. Transient half-life was calculated using the equation: half-life = (elapsed time) log2/\log (beginning amount/ending amount); SD was determined by pooling values from three separate experiments.

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, RAW264.7, EL4, and BMDMs were cross-linked with 1% methanol-free formaldehyde for 7 min at room temperature. Fixed cells (7 × 106 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 estimated 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 4153; Cell Signaling Technology, Danvers, MA), Egr2 (PRB-236P; Covance, Princeton, NJ), Egr3 (ab75461; Abcam), and Nab1 (NBPI-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 kit 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'–GCGAATGTGCTCTGGCAGAAATGGGCCG-3’, and antisense, 5’–GC GCCGGAAGCTCCACCTGCAGGAC-3’. Real-time quantitative PCR was performed using an Absolute qPCR Syber Green PCR ROX Mix (Thermo, Waltham, MA) and an ABI Prism 7900 Sequence detector. The equation used for determining percent input is 2^ΔΔCT = (immunoprecipitation Ct) - (input Ct). The equation used for determining fold enrichment over isotype was 2^ΔΔCT (isotype ΔCt – input ΔCt) – (isotype ΔCt – input ΔCt).

Luciferase constructs

The wild-type (wt) ifngr1 promoter luciferase reporter construct (IFNGR1 promoter-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’–GCGGATCCAGACTAAGCCAATCCTGCCCC-3’ and antisense, 5’–CAGTCTCCACAGGGAGCGCTGCTAGCTCG-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 subsequently cloned into the BamH I and Sall sites of pGL3 to generate IFNGR1 promoter-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’–CCAACCTTTGAAACTCTCAGTACCCAGGC-3’ and antisense, 5’–GGTTGATCCATTTGAGAGTCTGTTGTCG-3’. The two independent IFNGR1 promoter-luc and mEgr-luc cell lines used for experiments 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 better 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 × 10⁴ RAW 264.7 cells were transfected with 1 μg linearized IFNGR1 promoter-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 placed at 1 × 10⁶ in a 24-well plate and stimulated with 100 U/ml IFN-β at various time points. Following stimulation, lysates were harvested using lysis buffer from the Promega Luciferase Assay System. Luminescence was measured with a Synergy 2 plate reader (Bio-Tek, Winooski, VT).

Western blots

BMDM or RAW cell cultured monolayers were lysed in 100 μl 1× SDS-PAGE buffer (0.0625 M Tris-Cl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-ME, and 0.01% bromophenol blue) containing HALT protease inhibitors (Thermo) at 1× concentration. Cell lysates were scraped from plates and frozen at −20°C. The lysate volume corresponded to 3.5 × 10⁵ cells for all Egr family members and 2 × 10⁶ cells equivalents/lane for all other proteins. Proteins were transferred to nitrocellulose and probed with Abs to total STAT1 (number 9172; Cell Signaling Technology), STAT1pY701 (number 9167; Cell Signaling Technology), Egr1 (number 4153; Cell Signaling Technology), Egr2 (PRB-236P; Covance), Egr3 (ab75461; Abcam), Nab1 (NBPI-71838; Novus), and Nab2 (sc-22815; Santa Cruz Biotechnology, Santa Cruz, CA). As loading control, blots were stripped and reprobed with an anti-actin Ab (MAB1501; Millipore, Billerica, MA).

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 × 10⁵ 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 BMDM 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. 1G). 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.

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
Type I IFNs reduce cell surface IFNGR1 on mouse and human macrophages. Cells were treated or not with 100 U/ml IFN-β and then stained to quantify cell surface IFNGR1. Histograms (A, B, E) illustrate reduced cell surface IFNGR1 staining typically seen on macrophages after 6 h of IFN-β treatment (black lines). Control histograms included unstimulated cells (gray shading) and cells stained with secondary reagent alone (dashed line). Graphs (C, D, F) depict MFI values normalized to those of the respective untreated cells (relative MFI = MFI from treated sample/average of MFI from untreated sample). Cells used included live-gated C57BL/6 and B6.1FNAR−/- mouse BMDMs (A, C), mouse RAW 264.7 macrophages and EL4 thymoma cells (B, D), and THP-1 cells and gated CD14+ or CD3+ human PBMCs (E, F). Each bar in the graphs represents the mean ± SD of the pooled values for each condition from a total of at least three independent experiments. Statistical significance was determined using an unpaired t test: *p ≤ 0.05; ***p ≤ 0.001.

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 at 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 isofrom 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 this region of 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 p55-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. 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 unaffected by type I IFN stimulation. We next used ChIP to ask whether Egr1, 2, or 3 associated with the proximal Egr site in the *ifngr1* promoter before or after type I IFN stimulation of BMDMs. An initial percent input analysis revealed that Egr3 was the primary Egr family member recruited to the *ifngr1* promoter following type I IFN stimulation (Supplemental Fig. 4B). Moreover, the kinetics of Egr3 recruitment to the proximal Egr site (Fig. 4B) correlated well with loss of activated RNA pol II recruitment to the *ifngr1* transcriptional start site (Fig. 2B) and reduced *ifngr1* transcript abundance (Fig. 2A). These events all preceded the 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
Knockdown of Nab1 prevents downregulation of IFNGR1 and deacetylation of ifngr1 promoter by type I IFNs. (A) Representative immunoblots to determine expression of Nab1 and Nab2 proteins in RAW 264.7 cell lines with or without stable knockdown of Nab1 (Nab1-KD) or Nab2 (Nab2-KD). Anti-actin Ab was used to confirm equivalent protein loading. Control RAW 264.7 and Nab1-KD (B) or Nab2-KD (C) cell lines were treated or not with 100 U/ml IFN-β then stained to quantify cell surface IFNGR1. ChIP assays for Nab1 (D) and H3Ac in Nab1-KD or Nab2-KD (E) cell lines stimulated or not for 6 h with 100 U/ml IFN-β. Primers denoted in Fig. 3A were used for Nab1 ChIP qPCR analysis, and primers denoted in Fig. 2B were used for H3Ac qPCR analysis. Graphs depict MFI (B, C) or fold enrichment over isotype (D, E) values normalized to those of the respective untreated cells, calculated as in Figs. 1C and 2B, respectively. Each bar in the graphs represents the mean ± SD of the pooled values for each condition 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.

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 IFNGR 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 (56). 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 IFNGR 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 Egr 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 iifngr1 promoter.

Polymorphisms in the iifngr1 promoter are known to correlate with susceptibility to diverse diseases such as Leishmaniasis, tuberculosis, leprosy, and hepatitis infections (67–70). Suppression of iifngr1 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 iifngr1. Our efforts in this study have provided new insight into the mechanisms for silencing of iifngr1 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.

References


