Research Article

Molecular Cancer Therapeutics

IFNγ Restores Breast Cancer Sensitivity to Fulvestrant by Regulating STAT1, IFN Regulatory Factor 1, NF-κB, BCL2 Family Members, and Signaling to Caspase-Dependent Apoptosis

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Abstract

Antiestrogens are effective therapies for the management of many estrogen receptor-α (ER)–positive breast cancers. Nonetheless, both *de novo* and acquired resistance occur and remain major problems in the clinical setting. IFNγ is an inflammatory cytokine that induces the expression and function of IFN regulatory factor 1 (IRF1), a tumor suppressor gene that can increase antiestrogen responsiveness. We show that IFNγ, but not IFNα, IFNβ, or fulvestrant (ICI; ICI 182,780; Faslodex), induces IRF1 expression in antiestrogen-resistant MCF7/LCC9 and LY2 cells. Moreover, IFNγ restores the responsiveness of these cells to fulvestrant. Increased IRF1 activation suppresses NF-κB p65 (RELA) activity, inhibits the expression of prosurvival (BCL2, BCL-W), and induces the expression of proapoptotic members (BAK, mitochondrial BAX) of the BCL2 family. This molecular signaling is associated with the activation of signal transducer and activator of transcription 1 and leads to increased mitochondrial membrane permeability; activation of caspase-7 (CASP7), CASP8, and CASP9; and induction of apoptosis but not autophagy. Whereas antiestrogen-resistant cells are capable of inducing autophagy through IFN-mediated signaling, their ability to do so through antiestrogen-regulated signaling is lost. The abilities of IFNγ to activate CASP8, induce apoptosis, and restore antiestrogen sensitivity are prevented by siRNA targeting IRF1, whereas transient overexpression of IRF1 mimics the effects of IFNγ treatment. These observations support the exploration of clinical trials combining antiestrogens and compounds that can induce IRF1, such as IFNγ, for the treatment of some ER-positive breast cancers. *Mol Cancer Ther; 9(5); 1274–85. ©2010 AACR.*

Introduction

Breast cancer is the second most common cause of cancer death in women, with >40,000 women dying of breast cancer in the United States each year. Over 180,000 new cases of invasive breast cancer will be diagnosed in the United States within the next 12 months (1); almost 70% of which will express detectable levels of the estrogen receptor-α (ER) protein (2). Whereas antiestrogen or aromatase therapies are effective treatments for many of these ER-positive breast cancers, a significant proportion either will fail to respond initially or will eventually recur.

Antiestrogens inhibit the function of ER, a nuclear transcription factor that directs the expression of genes controlling cell proliferation and cell fate (2, 3). The most frequently prescribed antiestrogen is the nonsteroidal selective estrogen receptor modulator tamoxifen, which is highly effective in both the treatment of ER-positive breast tumors and in reducing breast cancer incidence in women at high risk for the disease. However, many initially sensitive ER-positive tumors acquire resistance to antiestrogen therapy, whereas the remainder exhibits *de novo* or intrinsic resistance (4). The steroidal antiestrogen fulvestrant (ICI; ICI 182,780; Faslodex) induces significant clinical responses in patients whose tumors are tamoxifen resistant (5). Indeed, the effectiveness of ICI in patients with tamoxifen-resistant disease is similar to that of the aromatase inhibitor anastrozole (6). Several clinical trials have shown ICI to be a viable alternative to nonsteroidal antiestrogens and aromatase inhibitors as a first-line endocrine treatment (7).

ICI is a selective estrogen receptor downregulator that prevents receptor dimerization and stimulates degradation of the ERα protein (8, 9). As a pure antagonist of ER action, ICI is not associated with the increased risk

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for endometrial cancer seen with tamoxifen (10). However, resistance to ICI can also arise in ER-positive breast tumors (3). Understanding the mechanisms of resistance to antiestrogens and developing novel strategies to restore sensitivity to antiestrogen therapy once resistance has developed are essential to improving survival for many patients with ER-positive breast cancer.

Breast cancer cells can acquire antiestrogen resistance through changes in the molecular signaling that controls cell proliferation and apoptosis (4). To test this hypothesis, several ER-positive variant cell lines have been derived from estrogen-dependent and antiestrogen-sensitive MCF-7 breast cancer cells. MCF7/LCC1 cells are estrogen independent but remain responsive to antiestrogens (11); MCF7/LCC9 cells are derivatives of MCF7/LCC1 that were selected for acquired resistance to ICI (12). LY2 cells were derived by selecting MCF-7 cells against the raloxifene analogue LY117018 (13, 14). Similar to what is seen in breast cancer patients with multiple endocrine resistance, MCF7/LCC9 and LY2 cells are estrogen independent and have also acquired cross-resistance to the nonsteroidal antiestrogen tamoxifen (12, 13).

Analysis of the transcriptomes of the MCF7/LCC1 and MCF7/LCC9 breast cancer models by serial analysis of gene expression and gene expression microarrays identified several differentially expressed genes likely to contribute to the endocrine-resistant phenotype, including upregulation of p65 NF-κB (RELA) and downregulation of IFN regulatory factor 1 (IRF1; ref. 15).

IRF1 is a nuclear transcription factor that promotes apoptosis following DNA damage (16). IRF1 can signal to apoptosis with (17) or without induction of $p21^{cip1}$ (18) or $p27^{kip1}$ (19) and through caspase-1 (CASP1; ref. 16), CASP3 (20), CASP7 (20, 21), CASP8 (20), and/or FasL (22). IRF1 also induces apoptosis in a p53-dependent or p53-independent manner (16, 18). Whereas loss of p53 activity is common in breast cancer (23), this disease remains one of the most initially responsive solid tumors to systemic therapies (24). Thus, IRF1, which can signal through both p53-dependent and p53-independent mechanisms (16, 18), may be an important signaling molecule for integrating and regulating breast cancer cell survival. A dominant-negative IRF1, which lacks the carboxyl-terminal transcriptional activation domain, reduces antiestrogen sensitivity in MCF7 and T47D breast cancer cells by suppressing apoptosis (25) and altering the activity of CASP3 and/or CASP7 (20).

IRF1 was initially identified because of its transcriptional activation of type I IFN genes. IFNs are a family of cytokines that have multiple biological effects, including immunomodulatory, antiviral, antiproliferative, antigen modulation, cell differentiation, and apoptotic effects (26). Once activated by binding IFN, their specific cell membrane receptors activate the Janus-activated kinase–signal transducer and activator of transcription (STAT) pathway, which results in the regulation of IFN-stimulated genes, including IRF1 (27, 28). Early reports described the ability of some IFNs to enhance the growth inhibitory actions of tamoxifen (29–31), but the precise mechanisms through which this occurs have remained largely unclear. Furthermore, whether or how IFNγ affects sensitivity to ICI in antiestrogen-sensitive and antiestrogen-resistant breast cancer cells is unknown.

The principal goals of this study were to assess whether the restoration of IRF1 expression by IFNγ can reverse ICI resistance by enabling ICI to again induce breast cancer cell death and to investigate the molecular mechanism by which ICI sensitivity is restored. We show here that treatment with a low dose of IFNγ is sufficient to restore sensitivity to ICI and synergistically enhances the induction of apoptosis in antiestrogen-resistant cells. With IFNγ treatment, IRF1 protein expression and transcriptional activity are significantly increased whereas those of NF-κB p65 are reduced. IRF1 siRNA blocks both IFNγ-mediated repression of NF-κB p65 activity and impairs ICI responsiveness in sensitive MCF7/LCC1 cells. These observations imply that upregulating IRF1 may be a successful approach in the treatment of ER-positive breast cancers that have acquired resistance to antiestrogen therapy. These data provide support for considering the design of clinical studies combining antiestrogens and compounds that can induce IRF1, such as IFNγ, for the treatment of ER-positive breast cancers.

Materials and Methods

Cell culture and reagents. MCF-7 cells were routinely grown in improved minimal essential media (IMEM; Mediatech, Inc.) with phenol red and supplemented with 5% fetal bovine serum. MCF7/LCC1 (11), MCF7/LCC9 (12), and MCF7/LY2 cells (13, 14) were routinely cultured in phenol red–free IMEM supplemented with 5% charcoal-stripped calf serum (CCS-IMEM). All cells were maintained in a humidified atmosphere at 37°C and 95% air/5% CO_2. IFNα and IFNβ were purchased from Calbiochem. Human recombinant IFNγ was purchased from Roche, and ICI 182,780 (ICI, Faslodex) was from Tocris Bioscience. All cells were shown to be free of *Mycoplasma* spp. contamination. We confirmed the genetic lineage of the three variant cell lines as being derived from the original MCF-7 cell line by DNA fingerprinting using genetic markers at nine different loci (CSF1PO, TPOX, TH01, vWA, D16S539, D7S820, D13S317, D5S818, and the Y chromosome–specific amelogenin).

Cell proliferation. Cell proliferation assays were done using the Cell Counting Kit-8 (Dojindo Laboratories). Cells were seeded at a density of 5 to 6 × 10^3 per well in 96-well plates and, 24 hours later, were treated with the indicated concentrations of drug in CCS-IMEM. Cells were incubated with the drugs for 6 days, with fresh media containing either drug and/or vehicle being replaced on day 3 where appropriate. After transfection with IRF1 siRNA by Amaxa, cells were seeded at a density of 1 × 10^4 per well in 96-well plates and, 24 hours later, were treated with the indicated concentrations of ICI or vehicle

in CCS-IMEM. Cell numbers were measured as the absorbance (450 nm) of reduced WST-8.

Mitochondria membrane potential. Changes in mitochondrial membrane potential (MMP) were measured using the MitoProbe JC-1 assay kit for flow cytometry (Invitrogen). Cells were first transfected with control or IRF1 siRNA by Amaxa and, 24 hours later, seeded at a density of 2×10^5 per well in six-well plates. Twenty-four hours after seeding, cells were treated with the indicated concentrations of drug and/or vehicle in CCS-IMEM for 3 days, media were removed, and the cells were trypsinized and resuspended in PBS (Invitrogen). Mitochondria were stained by JC-1 according to the manufacturer's instructions, and the fluorescence was detected and measured by fluorescence-activated cell sorting (LCCC FACS Shared Resource). Where appropriate, cells were also treated with 20 μmol/L CASP8 inhibitor II (Merck KGaA) or DMSO vehicle, with the drugs indicated but without IRF1 siRNA, for 2 days before analysis.

Immunostaining and confocal microscopy. Cells (1×10^5) were seeded onto 18 × 18 mm glass coverslips and, on the following day, treated with 100 nmol/L ICI 182,780 and/or 100 IU/mL IFNγ in CCS-IMEM for 48 hours. Cells were then fixed in 4% paraformaldehyde for 20 minutes at room temperature and then permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate for 5 minutes at room temperature. Cells were sequentially incubated with primary antibody (anti-p65 at 1:200 dilution from Upstate Biotechnology; anti-IRF1 at 1:100 dilution from BD Biosciences) and fluorophore-conjugated secondary antibody Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG (Invitrogen Molecular Probes). 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) were obtained from Molecular Probes, Inc. Where appropriate, DAPI was added to visualize the nucleus, and nonconfocal DAPI images were acquired using Hg lamp excitation and a UV filter set. Confocal microscopy was done using an Olympus IX-70 confocal microscope with 405, 488, and 543 nm excitation lasers. Fluorescence emission was separately detected for each fluorophore in optical sections of <1 μm in thickness (pinhole set to achieve 1 airy unit).

Suppression of IRF1 with siRNA. To downregulate IRF1 mRNA expression, we transfected a Silencer Select Predesigned siRNA specific for IRF1 and a noncoding control siRNA (Applied Biosystems, Inc.) using the Nucleofector kit V from Amaxa, Inc. Two million cells were electroporated with 0.1 nmol of siRNA according to the manufacturer's protocol. Twenty-four hours later, cells were split into either 96-well plates, 12-well plates, or 6-well plates, treated, and used for measuring cell proliferation, gene expression (promoter-reporter activity), MMP, or protein expression.

Transcriptional reporter assays. Heterologous promoter constructs containing the luciferase gene driven by tandem IFN stimulated response elements (3 × ISRE-Luc reporter plasmid) or NF-κB p65/RELA sites (the p65-Luc reporter plasmid) were purchased from Stratagene. The BCL2 promoter-Luc plasmids (LB322, full length; LB124, promoter-1 and upstream elements; LB334, only promoter-1; LB335, promoter-2; and LB556, minimal promoter-2) were a kind gift from Dr. Linda Boxer (Stanford University). The sequence of each construct can be found in the original studies (32, 33). Cells were seeded at a density of 1×10^5 per well in 12-well plates. The following day, cells were transfected with 0.65 μg of luciferase reporter plasmid and 0.02 μg phRL-SV40-*Renilla* (Promega) per well using the FuGENE 6 transfection reagent (Roche). Five hours post-transfection, media were changed and cells were treated with either drug and/or vehicle for 48 hours. Subsequently, cells were lysed, and activation of the luciferase constructs was measured using the Dual-Luciferase Assay kit (Promega). Luminescence was quantified using a Lumat LB 9501 luminometer (EG&G Berthold). Luciferase values were normalized to *Renilla* luminescence. In some experiments, cells were cotransfected before drug treatment with wild-type IRF1 plasmid (pcDNA3-IRF1; a generous gift from Dr. Taniguchi, University of Tokyo) or pretransfected with IRF1 siRNA.

Real-time reverse transcription–PCR. Cells were seeded at 2×10^6 in T-25 cm^2 flasks and, 24 hours later, treated with the drugs and/or vehicles as indicated for a further 48 hours. Total RNA was extracted using the TRIzol reagent (Life Technologies, Inc.). Total RNA (1 μg) was used to synthesize the first-strand cDNA by SuperScript II First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's instructions.

Primers were purchased from Applied Biosystems, Inc.; the ribosomal protein, large, P0 (RPLP0) was used as an internal standard. The genes and Assay ID of the primers used in this experiment are as follows: IRF1 (Hs00233698_m1); NF-κB p65/RELA (Hs00153294_m1); survivin (Hs00153353_m1); BCL2 (Hs00608023_m1); BCL-W (BCL2L2; Hs00187848_m1); BAX (Hs00180269_m1); BAK (Hs00832876_g1); BAD (Hs00188930_m1); BID (Hs00609632_m1); BIK (Hs00609635_m1); RPLP0 (Hs99999902_m1). Probes were labeled with the reporter fluorescent dye FAM (6-carboxyfluorescein) at the 5′ end and a quencher fluorescent dye 6-carboxy-tetramethylrhodamine at the 3′ end.

PCR reactions were done using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Inc.) in a total volume of 10 μL reaction mixture in 384-well plates. Real-time PCR data were plotted as the ΔRn fluorescence signal versus the cycle number. Relative quantification of gene expression using the $2^{-\Delta\Delta Ct}$ method was analyzed as previously described (34).

Immunoblotting. Cells were grown in either 10-cm^2 dishes or T-75 cm^2 tissue culture flasks before lysis. To determine the effects of IFNs on the expression of IRF1 protein, cells were treated with 0, 10, 100, and 1,000 IU/mL of IFNα, IFNβ, or IFNγ for 24 hours and lysates were harvested for analysis. To determine the effects of IFNγ and ICI on protein expression, cells were treated with drug and/or vehicle for 24 and 48 hours.

Mitochondrial fractions were isolated using the Mitochondria Isolation Kit for Cultured Cells (Pierce). Briefly, cells were seeded in T-75 cm^2 tissue culture flasks at 2×10^6 and, 24 hours later, treated with indicated drugs and/or vehicle. Forty-eight hours later, cells were trypsinized and pelleted by centrifugation, and mitochondria fractions were isolated according to the manufacturer's instructions.

Lysate preparation and analysis were done as previously described (35). Proteins were probed with the following antibodies overnight at 4°C: STAT1 (1:1,000, Cell Signaling), phosphorylated STAT1 Tyr^{701} or Ser^{727} (1:1,000, Cell Signaling), extracellular signal-regulated kinase 1/2 (ERK1/2; 1:1,000, Cell Signaling), phosphorylated ERK1/2 Thr^{202}/Tyr^{204} (1:1,000, Cell Signaling), IKKγ (1:200, Santa Cruz Biotechnology), NF-κB p65 (RELA; 1:1,000), IRF1 (1:500), HSP27 (1:5,000, Abcam, Inc.), phosphorylated HSP27 Ser^{78} (1:2,000, Abcam), BCL2 (1:1,000, Stressgen), BCL-xL (1:1,000, Cell Signaling), BCL-W (1:1,000, Cell Signaling), BAX (1:1,000, Millipore), CASP7 (1:1,000, Cell Signaling), cleaved CASP7 (1:1,000, Cell Signaling), CASP8 (1:1,000, Cell Signaling), cleaved CASP8 (1:1,000, Cell Signaling), p62/SQSTM1 (1:2,000, Abcam), and LC3B (1:1,000, Cell Signaling). To confirm equal loading of the gels, membranes were reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:2,000, Santa Cruz Biotechnology) or β-actin (1:5,000, Sigma) for the whole lysates and cytochrome *c* oxidase IV (1:1,000, Cell Signaling) for mitochondrial fractions. Semiquantitative analysis of protein expression was done by densitometry using NIH ImageJ software (36).

Caspase activity assay. Cells were seeded at 1×10^4 in white 96-well plates and, 24 hours later, treated with indicated drug/vehicle for 16 hours (CASP9) or 72 hours (CASP7, CASP8). Caspase activity was detected using the Apo-ONE Homogeneous CASP-3/7 Assay, CASP-Glo 8 Assay, and CASP-Glo 9 Assay from Promega according to the manufacturer's instructions.

Statistical analyses. Data are reported as mean ± SEM. Two-tailed Student's *t* tests were used for the comparison of two groups for immunoblot, cell proliferation, and MMP assays as indicated. For luciferase reporter assays, Dunnet's post hoc *t* test was used to compare all treatment groups following one-way ANOVA. Statistical significance is defined as follows: *, $P < 0.05$ versus vehicle/control; #, $P < 0.05$ versus ICI; ^, $P < 0.05$ versus IFNγ; &, $P < 0.05$ versus CTRLsi or empty vector transfection.

Results

IFNγ induces IRF1 expression and increases antiestrogen responsiveness. Supplementary Fig. S1A shows that IFNγ treatment induces a significant increase in IRF1 protein expression, whereas IFNα or IFNβ treatment has no significant effect (Supplementary Fig. S1A, top and middle). In contrast, IRF1 expression increases 15-fold following treatment with 10 IU/mL IFNγ; a ~20-fold increase is induced by 100 and 1,000 IU/mL IFNγ treatment (Supplementary Fig. S1B; $P < 0.05$).

Low doses of IFNγ (0–100 IU/mL) do not inhibit cell proliferation (Fig. 1A), but a dose of 1,000 IU/mL significantly inhibits the proliferation of MCF7/LCC9 cells ($P < 0.05$). Because a dose of 100 IU/mL IFNγ is maximally effective at inducing IRF1 (20-fold) but has no effect on cell proliferation, we used this concentration of IFNγ in subsequent studies. We first combined 100 IU/mL IFNγ with increasing concentrations of ICI (0, 100, and 1,000 nmol/L) and measured the effects on cell proliferation. As shown in Fig. 1B, a dose of 100 IU/mL IFNγ increases the antiproliferative effects of ICI at all concentrations tested. We used 100 nmol/L ICI for further studies because this concentration approximates the IC_{50} for ICI in the parental, antiestrogen-sensitive MCF7/LCC1 cells (12).

To confirm that restoration of ICI sensitivity by IFNγ occurs in other models of antiestrogen resistance, we did a similar experiment in MCF7/LY2 cells. LY2 cells, which were originally selected for resistance to LY117018, an analogue of the nonsteroidal antiestrogen raloxifene (13, 29), are also cross-resistant to ICI. ICI has no effect on cell proliferation at concentrations of up to 1 μmol/L (Fig. 1C, top curve, closed circles). However, when combined with 100 IU/mL IFNγ, which induce IRF1 expression (inset), ICI produces a significant decrease in cell proliferation ($P < 0.05$ versus ICI alone; Fig. 1C, bottom curve, open circles).

To test directly whether a reduction in IRF1 expression affects sensitivity to ICI, we introduced IRF1 siRNA into the parental, ICI-sensitive MCF7/LCC1 and MCF7 cells, which have higher levels of IRF1 expression than their paired, ICI-resistant MCF7/LCC9 cells. MCF7/LCC1 and MCF7 cells are markedly less sensitive to growth inhibition by ICI treatment following IRF1 siRNA transfection compared with no siRNA (Mock) or control siRNA (CTRL siRNA) transfection (Fig. 1D and E; $P < 0.05$). The insets show representative immunoblots for IRF1 protein, which is undetectable after IRF1 siRNA transfection.

IRF1 is required for the antiproliferative effects of combined IFNγ and ICI treatment. To elucidate the mechanism by which the combination of IFNγ and ICI reduces the proliferation of antiestrogen-resistant cells, MCF7/LCC9 cells were transfected with control or IRF1 siRNA before treatment with 100 IU/mL IFNγ and 100 or 1,000 nmol/L ICI. The growth inhibitory effect of the combination of IFNγ and either concentration of ICI is completely and significantly reversed by IRF1 siRNA (Fig. 2A; $P < 0.05$). MMP, an early measure of cell death signaling, is also significantly increased by the combination treatment of IFNγ and ICI, whereas this effect is blocked by IRF1 siRNA (Fig. 2B; $P < 0.05$). Western blot analysis confirms that transfection with IRF1 siRNA before treatment prevents IRF1 induction by IFNγ in antiestrogen-resistant MCF7/LCC9 cells (Fig. 2C).

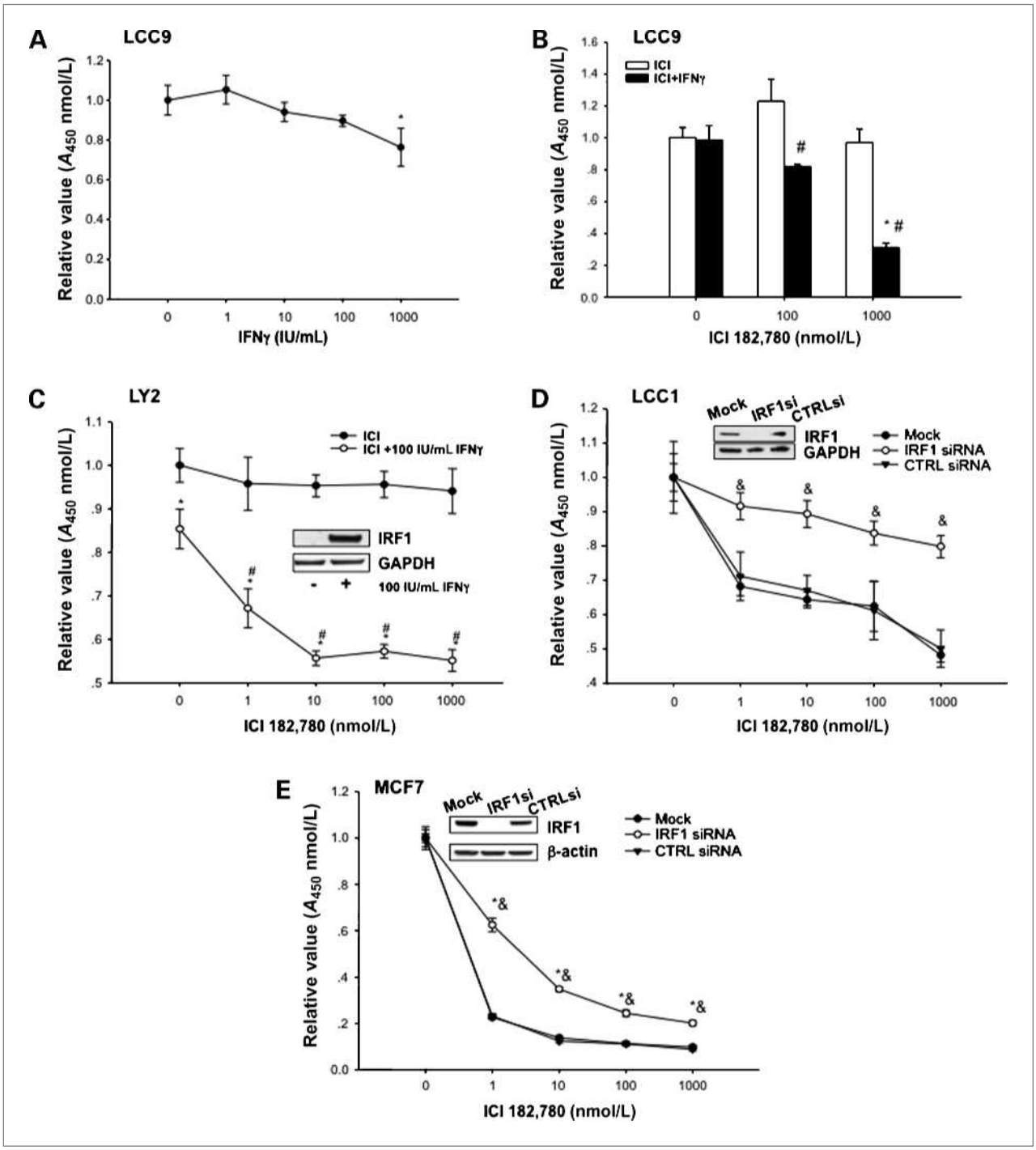

Figure 1. IFNγ restores and IRF1 siRNA transfection impairs ICI sensitivity in breast cancer cells. A, antiestrogen-resistant MCF7/LCC9 cells were seeded in 96-well tissue culture dishes and treated with 0 to 1,000 IU/mL IFNγ for 6 d, at which time cell proliferation was measured using the Cell Counting Kit-8 reagent. B, MCF7/LCC9 cells were seeded in 96-well tissue culture dishes and treated with 100 or 1,000 nmol/L ICI in the presence or absence of 100 IU/mL IFNγ for 6 d before measuring cell proliferation as in A. C, antiestrogen-resistant MCF7/LY2 cells were seeded in 96-well tissue culture dishes and treated with 0 to 1,000 nmol/L ICI in the presence (○) or absence (●) of 100 IU/mL IFNγ for 6 d before measuring cell proliferation as in A. D and E, antiestrogen-sensitive MCF7/LCC1 (D) and MCF7 (E) cells were mock-transfected (●) or transfected with either nonsilencing control (CTRLsi,▲) or IRF1-specific (IRF1si,○) oligonucleotides 1 d before seeding in 96-well tissue culture dishes. Cells were then treated with 0 to 1,000 nmol/L ICI for 6 d before measuring cell proliferation as in A. Each inset in C to E shows a representative Western blot for expression of IRF1 and GAPDH or β-actin loading control. In all panels, data are presented as relative absorbance (*A* 450 nm) and represent the mean ± SEM for a representative experiment; at least three independent experiments were done. *, $P < 0.05$ versus control/vehicle treatment; #, $P < 0.05$ versus ICI treatment; &, $P < 0.05$ versus CTRLsi transfection.

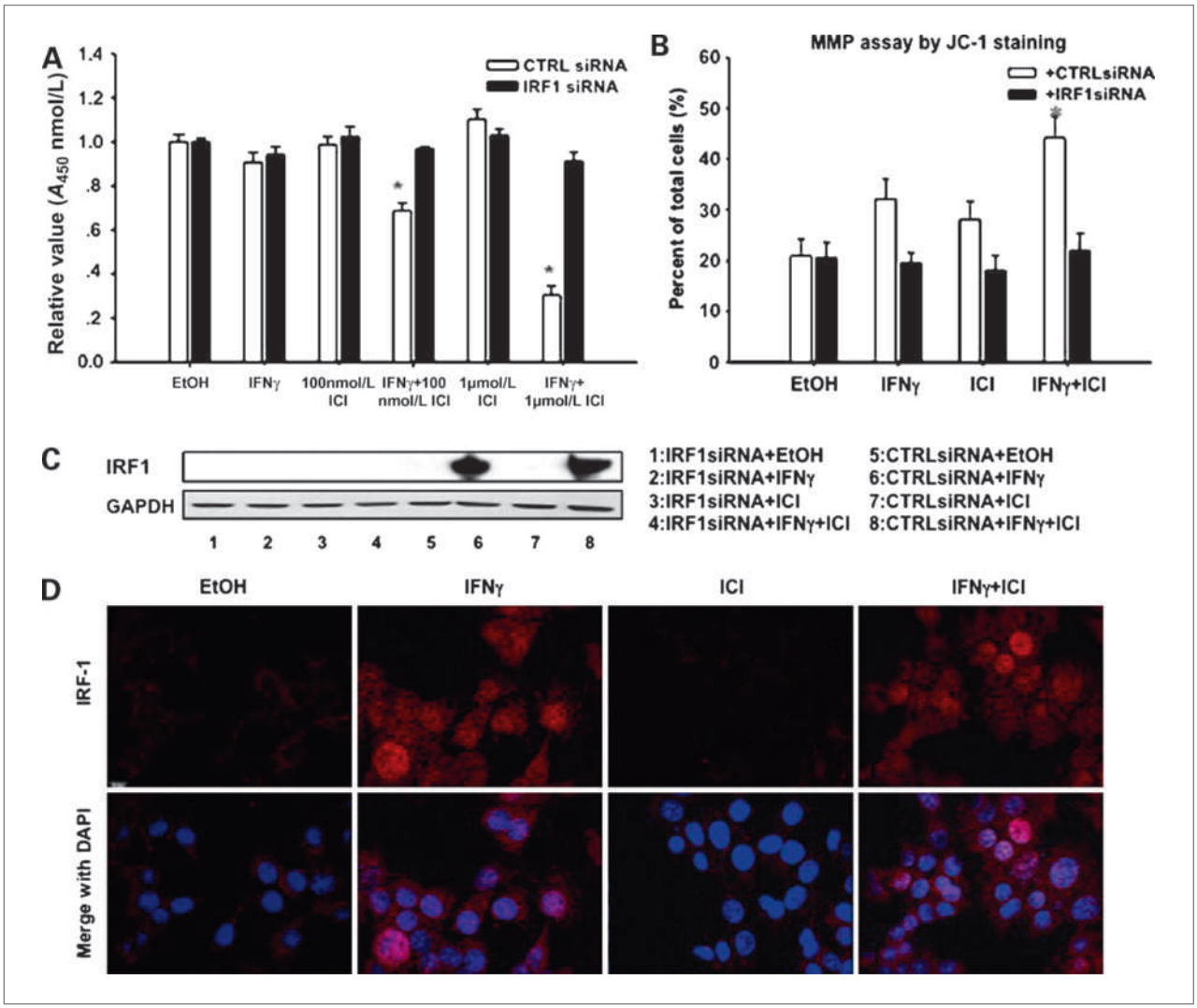

Figure 2. Restoration of ICI sensitivity by IFNγ induces apoptosis, requires the induction of IRF1, and induces nuclear localization of IRF1. A, MCF7/LCC9 cells were transfected with either nonsilencing control (CTRLsi, white columns) or IRF1-specific (IRF1si, black columns) oligonucleotides 1 d before seeding in 96-well tissue culture dishes. Cells were then treated with ethanol (EtOH) vehicle, 100 or 1,000 nmol/L ICI in the presence or absence of 100 IU/mL IFNγ for 6 d before measuring cell proliferation. Data are presented as relative absorbance (*A* 450 nm) and represent the mean ± SEM for a representative experiment; at least three independent experiments were done. *, $P < 0.05$ versus control/vehicle treatment. B, MCF7/LCC9 cells were transfected with either nonsilencing control (CTRLsi, white columns) or IRF1-specific (IRF1si, black columns) oligonucleotides 1 d before seeding in six-well tissue culture dishes. Cells were then treated with EtOH vehicle or 100 nmol/L ICI in the presence or absence of 100 IU/mL IFNγ for 3 d before detecting apoptosis by measuring MMP. Data are presented as percentage of total cells positive for green fluorescence (indicative of MMP) and represent the mean ± SEM for three independent experiments. *, $P < 0.05$ versus control/vehicle treatment. C, MCF7/LCC9 cells were transfected with CTRLsi or IRF1si 1 d before seeding in six-well tissue culture dishes and treating with EtOH vehicle or 100 nmol/L ICI in the presence or absence of 100 IU/mL IFNγ for 48 h. Cells were lysed, and IRF1 expression was detected by immunoblot. GAPDH serves as the loading control. D, MCF7/LCC9 cells were seeded onto 18 mm^2 glass coverslips 1 d before treatment with EtOH vehicle or 100 nmol/L ICI in the presence or absence of 100 IU/mL IFNγ for 48 h. The cells were then fixed, permeabilized, stained for IRF1 and DAPI, and visualized by confocal microscopy. Nuclear IRF1 expression (red staining) is observed in cells treated with IFNγ.

Combined IFNγ and ICI treatment increases the expression and nuclear translocation of IRF1 but has no effect on NF-κB p65. The subcellular localization of IRF1 and NF-κB p65 was detected by immunofluorescence using confocal microscopy in MCF7/LCC9 cells treated with 100 IU/mL IFNγ, 100 nmol/L ICI alone, or in combination (Fig. 2D). DAPI staining shows the location of the nuclei; when merged with IRF1 staining (Fig. 2D, bottom), the levels of IRF1 in IFNγ with or without ICI-treated cells are seen to be higher than those in vehicle and ICI-treated cells. IRF1 translocates to the nuclei following IFNγ treatment (red staining), whereas the location of NF-κB p65 is not affected by IFNγ (not shown).

IFNγ and ICI treatment increases IRF1 and decreases NF-κB p65 transcriptional activation. To further explore the mechanism of IFNγ-mediated restoration of ICI sensitivity, we measured the transcriptional activity of IRF1 using promoter-reporter luciferase assays. As expected, the transcriptional activity of IRF1 is significantly increased by IFNγ treatment, whether given alone or in combination with ICI (Fig. 3A). We then measured the transcriptional activity of NF-κB p65 (RELA), which is upregulated in MCF7/LCC9 cells (where basal IRF1 expression and activity are low); NF-κB p65 may play a key functional role in driving antiestrogen-resistant breast cancer cell growth and survival (15, 35). The transcriptional activity of NF-κB p65 is reduced following treatment with IFNγ and is reduced significantly further by the combination of IFNγ and ICI (Fig. 3A; $P < 0.05$). Basal and IFNγ-induced ISRE-Luc activities are substantially reduced in MCF7/LCC9 cells (Fig. 3B) when transfected with IRF1 siRNA before treatment. Similarly, transfection of IRF1 siRNA partially reverses both the effect of IFNγ alone and the effect of a combination of IFNγ and ICI on NF-κB p65-luciferase activity (Fig. 3C; $P = 0.001$ and $P = 0.004$, respectively). To show that IRF1 can directly repress NF-κB p65 transcriptional activity, we transiently transfected MCF7/LCC9 cells with an expression plasmid encoding the wild-type IRF1 cDNA. Transcriptional activity of NF-κB p65 is significantly reduced by ectopic IRF1 expression, whether or not the cells are treated with ICI (Fig. 3D). These data suggest that restoration of IRF1 expression may improve ICI responsiveness, likely at least partly through the downregulation of prosurvival NF-κB activities (35, 37).

IFNγ and ICI treatment decreases prosurvival gene expression while increasing that of proapoptotic mitochondrial BAX. Consistent with increased IRF1 protein expression following IFNγ stimulation (Supplementary Fig. S1), IRF1 mRNA expression is significantly increased by IFNγ treatment (Fig. 4A). In contrast, the mRNA of several key prosurvival genes such as BCL2, BCL-W (BCL2L2), and survivin (BIRC5) is significantly reduced by 60%, 75%, and 73%, respectively, with IFNγ and ICI treatment. Conversely, the expression of proapoptotic genes including BAK and BAX is increased 2-fold with IFNγ and ICI treatment (Fig. 4B); the expression of

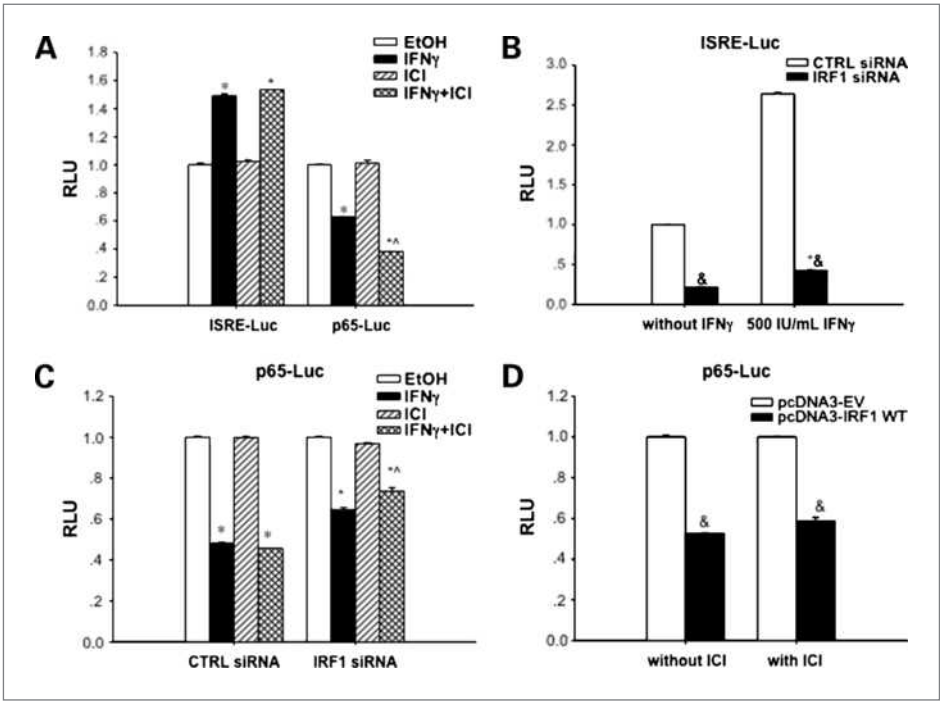

Figure 3. Combined treatment with IFNγ and ICI increases ISRE transcriptional activity and reduces NF-κB transcriptional activity in an IRF1-dependent manner. A, MCF7/LCC9 cells were seeded in 12-well tissue culture dishes 1 d before transfection with plasmids encoding ISRE-Luc or p65-Luc in combination with the control phRL-SV40-*Renilla*. Cells were then treated with EtOH vehicle or 100 nmol/L ICI in the presence or absence of 500 IU/mL IFNγ for 48 h before lysis and analysis by dual-luciferase promoter-reporter assay. B, MCF7/LCC9 cells were transfected with CTRLsi or IRF1si 1 d before seeding in 12-well tissue culture dishes, followed by transfection with ISRE-Luc and phRL-SV40-*Renilla*, treatment with vehicle or 500 IU/mL IFNγ for 48 h, and lysis/analysis as in A. C, MCF7/LCC9 cells were transfected with CTRLsi or IRF1si 1 d before seeding in 12-well tissue culture dishes, followed by transfection with ISRE-Luc and phRL-SV40-*Renilla*, treatment with EtOH vehicle or 100 nmol/L ICI in the presence or absence of 500 IU/mL IFNγ for 48 h, and lysis/analysis as in A. D, MCF7/LCC9 cells were seeded in 12-well tissue culture dishes 1 d before cotransfection with plasmids encoding p65-Luc, phRL-SV40-*Renilla*, and either pcDNA3-IRF1 wild-type (WT) or the empty vector (EV) control. Cells were then treated with EtOH vehicle or 100 nmol/L ICI for 48 h before lysis/analysis as in A. A-D, data are presented as the ratio of luciferase to *Renilla* signal (RLU, relative light units) and represent the mean ± SEM for a representative experiment done in quadruplicate. At least three independent experiments were done. *, $P < 0.05$ versus control; ^, $P < 0.05$ versus IFNγ; &, $P < 0.05$ versus CTRLsi or empty vector transfection.

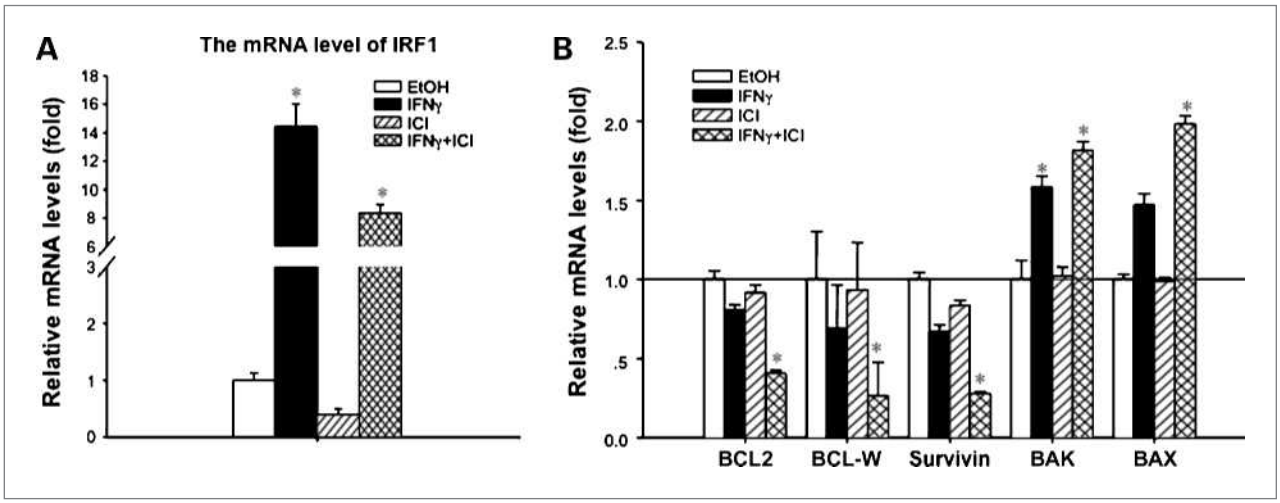

Figure 4. Combined treatment with IFNγ and ICI decreases the mRNA expression of prosurvival genes while increasing expression of proapoptotic genes in MCF7/LCC9 cells. A, IRF1 mRNA expression is induced by IFNγ. MCF7/LCC9 cells were seeded in T-25 cm^2 tissue culture flasks 1 d before treatment with EtOH vehicle or 100 nmol/L ICI in the presence or absence of 100 IU/mL IFNγ for an additional 48 h. Total RNA was extracted, reverse transcribed, and assayed for IRF1 expression by quantitative reverse transcription–PCR. RPLP0 serves as the housekeeping gene. Data are presented as a ratio of IRF1/RPLP0 expression (relative mRNA levels) and represent the mean ± SEM for a representative experiment; at least three independent experiments were done. *, $P < 0.05$ versus control/vehicle treatment. B, BCL2, BCL-W, and survivin are reduced, whereas BAK and BAX are increased by IFNγ. MCF7/LCC9 cells were seeded, treated, and assayed as in A. Data are presented as a ratio of target gene/RPLP0 expression (relative mRNA levels) and represent the mean ± SEM for a representative experiment; at least three independent experiments were done. *, $P < 0.05$ versus control/vehicle treatment.

BIK and BAD does not change (not shown). To validate the changes in mRNA expression, whole-cell lysates were prepared and the proteins were detected by Western blot. The protein levels of NF-κB p65, BCL2, BCL-XL (BCL2L1), and BCL-W (BCL2L2) are each significantly reduced with IFNγ and ICI treatment (Fig. 5A and B; $P < 0.05$).

To explore the most likely upstream regulator of pathway affected by IFNγ, the levels of total STAT1 and phosphorylated STAT1 were measured by immunoblotting. Protein levels of total STAT1 and phosphorylated STAT1 (Tyr^{701} or Ser^{727}) are substantially increased by IFNγ treatment as expected (38), with or without concurrent ICI treatment. We also detect expression of IKKγ, a key regulator of NF-κB p65 (RELA) expression and activity. As shown in Fig. 5A and B, the protein level of IKKγ is reduced at ≤50% with IFNγ and ICI treatment. We then measured the levels of total and phosphorylated ERK1/2 and phosphorylated HSP27, which are strongly associated with proliferative status. Protein levels of phosphorylated ERK1/2 (Thr^{702}/Tyr^{704}) and phosphorylated HSP27 (Ser^{78}) are reduced by combined IFNγ and ICI treatment, whereas the total protein levels do not change (Fig. 5A and B). BAX mRNA is increased by IFNγ and ICI, but there is no significant change in BAX protein expression in the whole-cell lysates. However, BAX expression in the mitochondrial fraction increases significantly with IFNγ ± ICI treatment, whereas there is no change in cytosolic BAX levels (Fig. 5C and D; $P < 0.05$).

Combined IFNγ and ICI treatment inhibits the BCL2 P1 but not P2 promoter. BCL2 expression is induced by 17β-estradiol (estrogen; E2) and decreased by antiestrogens (39, 40). Transcription from the BCL2 gene is driven by two promoters (P1 and P2; ref. 33). Analysis of the proximal promoter region (−3 kb) of BCL2 by the MatInspector algorithm (41) reveals three consensus ISREs (one in P1 and two in P2). To determine whether the inhibition of BCL2 mRNA expression by IFNγ/ICI treatment is due to reduced BCL2 transcription, we transfected MCF7/LCC9 cells with various BCL2 promoter-reporter constructs. Activity of the full-length BCL2 5′ untranslated region (contains both P1 and P2 sequences) is significantly reduced by IFNγ and ICI treatment (Fig. 6A; $P < 0.05$). Activity of the BCL2 P1 promoter is also significantly reduced by IFNγ and ICI treatment, whereas the activity of the BCL2 promoter P2 is not affected.

Combined IFNγ and ICI treatment increases CASP7, CASP8, and CASP9 activation. The data above imply that IFNγ and ICI induce an apoptotic, caspase-mediated cell death. Thus, we measured the expression and activity of CASP7, CASP8, and CASP9 by immunoblotting and colorimetric caspase assays. As shown in Fig. 6B, cleaved CASP7 and CASP8 were detected after IFNγ and ICI treatment; cleaved CASP9 was not detected (not shown). The activity of these three caspases is significantly increased by combined IFNγ and ICI treatment (Fig. 6C; $P < 0.05$). CASP7 and CASP9 expressions are also increased by IFNγ alone, but this induction is less than that seen in combination with ICI.

We also measured the induction of autophagy in MCF7/LCC9 cells treated by IFNγ and/or ICI. As shown in Supplementary Fig. S2A, the protein level of SQSTM1/p62 is reduced by IFNγ alone or combined with ICI treatment whereas cleavage of LC3B is increased. However,

there is no significant difference in the levels of autophagy between IFNγ alone and when combined with ICI. These observations indicate that, although antiestrogen resistant cells are capable of inducing autophagy through IFN-mediated signaling, their ability to do so through antiestrogen-regulated signaling is lost, and this is not restored by the combination of IFNγ and ICI. When IFNγ restores antiestrogen sensitivity, the primary increase in cell death seems to result from increased apoptosis (Fig. 2B), despite the ability of cells to induce autophagy through IFNγ. CASP8 activation can occur during extrinsic apoptosis and is a critical mediator of this process (37). To determine further the signaling involved in this regulation of apoptosis, we used a CASP8 inhibitor and measured its effects on MMP and apoptosis. As shown in Supplementary Fig. S2B, the CASP8 inhibitor prevents the apoptosis induced by combined IFNγ and ICI treatment in MCF7/LCC9 cells.

Discussion

IRF1 expression is efficiently induced by the type II IFNγ in many cell types. We and others have shown previously that overexpression of IRF1 in breast cancer cells can induce apoptosis (20, 25, 42–44) and inhibit breast tumorigenesis (20, 45). Furthermore, endogenous IRF1 expression is significantly reduced in antiestrogen-resistant MCF7/LCC9 breast cancer cells (15). Whereas both IRF1 and IRF2 are expressed in breast cancer (46, 47), we detect increased IRF1 expression, but the expression of IRF-2 remains constant (data not shown), maintaining a high ratio of IRF1/IRF-2. Thus, it is unlikely that IRF2 plays a

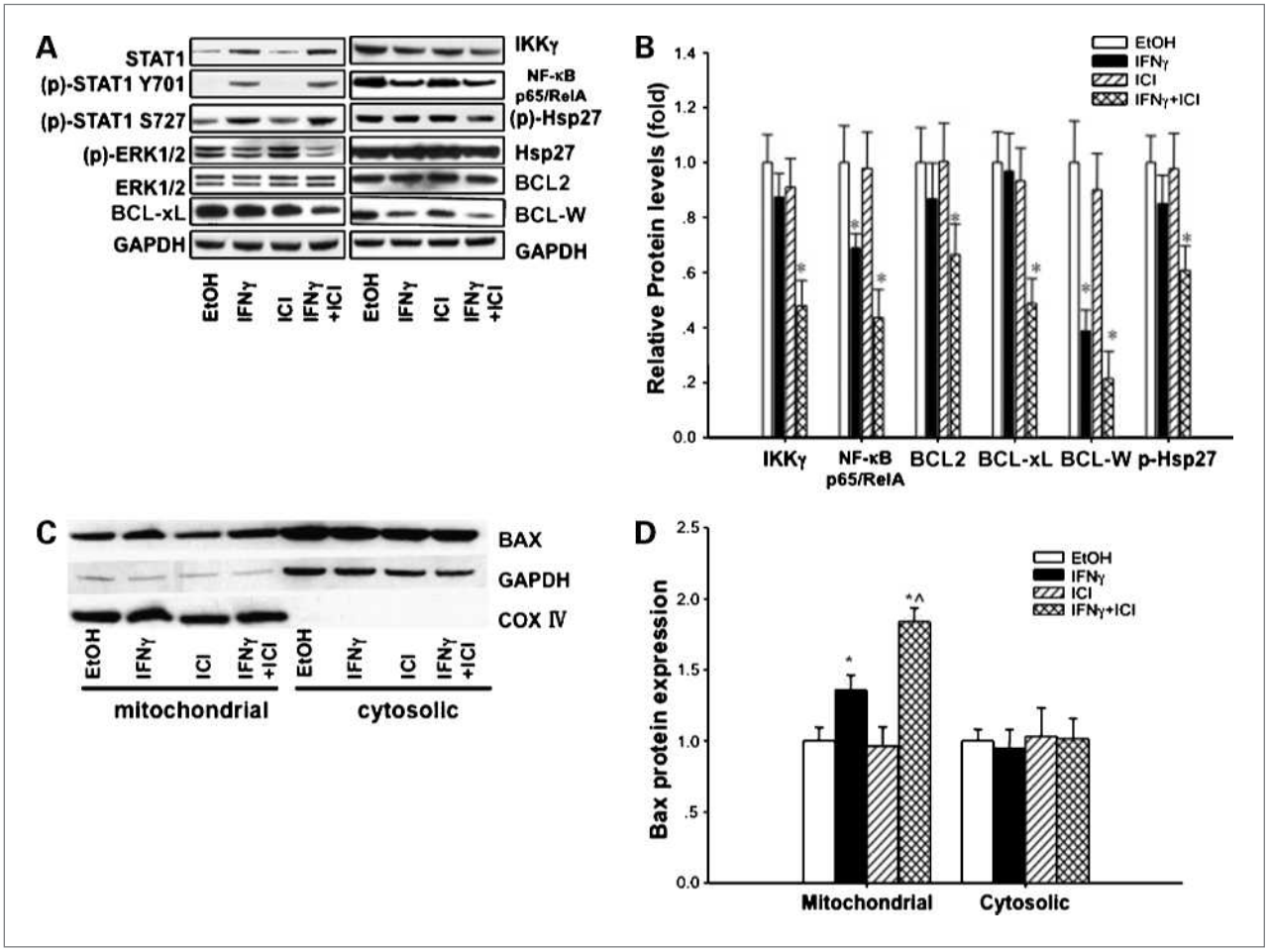

Figure 5. Effects of IFNγ and ICI on the protein expression of proapoptotic and antiapoptotic signaling molecules in MCF7/LCC9 cells. A and B, cells were seeded in T-75 cm^2 tissue culture flasks 1 d before treatment with EtOH vehicle or 100 nmol/L ICI in the presence or absence of 100 IU/mL IFNγ for an additional 48 h. Whole-cell lysates were prepared and analyzed by immunoblot for the indicated proteins; GAPDH serves as the loading control. A, representative images; B, densitometric analysis from at least three independent experiments. *, $P < 0.05$ versus control/vehicle treatment. C and D, IFNγ increases mitochondria-associated BAX. MCF7/LCC9 cells were seeded and treated as described in A. Mitochondrial and cytosolic fractions were isolated and analyzed by immunoblot for BAX, GAPDH (loading control for cytosolic fraction), and cytochrome *c* oxidase IV (COX IV; loading control for mitochondrial fraction). C, representative image; D, densitometric analysis from at least three independent experiments. *, $P < 0.05$ versus control/vehicle treatment; ^, $P < 0.05$ versus IFNγ.

major role in the regulation of responsiveness to antiestrogens in breast cancer.

We now show that treatment with IFNγ rescues or enhances IRF1 expression in MCF7/LCC9 and MCF7/LY2 cells and restores their sensitivity to ICI-mediated apoptosis. This restoration of antiestrogen sensitivity is driven by an IRF1-dependent increase in mitochondrial outer membrane permeability and activation of the intrinsic (mitochondrial) apoptotic pathway in the absence of any change in the level of autophagy. Mechanistically, the effects of IFNγ in resistant cells are mediated by differential alterations in the signaling of both prosurvival (BCL2 and BCL-W) and proapoptotic BCL2 family members (BAK and BAX) and other prosurvival signaling, including that affected by survivin, NF-κB p65, phosphorylated ERK1/2, and phosphorylated HSP27.

The upstream regulation of these events is most likely the consequence of IFNγ receptor activation of STAT1, a potent regulator of IRF1 transcription. However, it is also evident that NF-κB, a transcription factor implicated in the regulation of cell proliferation and in resistance to cytotoxic drugs (48), is also a key player in this signaling. Both the mRNA and protein expressions of NF-κB are increased in MCF7/LCC9 cells when compared with its parental antiestrogen-sensitive MCF7/LCC1 cells (15, 35). Sustained NF-κB activity is necessary for maintenance of the antiestrogen-resistant phenotype (37). IRF1 and NF-κB form productive heterodimers and cooperate in regulating gene expression (49, 50). Furthermore, upregulation of NF-κB expression is associated with E2 independence (51, 52). We show that the combination of IFNγ and ICI reduces both NF-κB protein expression and transcriptional activation and that this requires the induction of IRF1 by IFNγ. A significant inverse association between nuclear IRF1n and NF-κB is evident in some ER-positive breast cancers (47). Future studies will focus on this inverse relationship and its consequences for predicting endocrine responsiveness.

The BCL2 gene family comprises both prosurvival proteins (such as BCL2, BCL-XL, and BCL-W) and proapoptosis proteins (including BAD, BAK, BAX, BID, and BIK) and is functionally involved in the regulation of cell fate (53). A central output from the sum of prosurvival and proapoptotic signaling through this family is an alteration in MMP, which affects cytosolic cytochrome *c* concentrations and the level of apoptosis as executed by selected caspases. The regulation of several members of the BCL2 family is affected by NF-κB (35, 37), consistent with the regulation of NF-κB described above. Expression of the prosurvival genes BCL2, survivin, and BCL-W is reduced by the combination of IFNγ and ICI, whereas that of the proapoptotic genes BAK and BAX is increased. The ratio of BCL2 to BAX can alter the rate of apoptosis in response to treatment (54). For BAX protein expression, there is no change in total expression in whole-cell lysates, but BAX expression is significantly increased in the mitochondrial fraction, consistent with the modifications seen in MMP. Reduced expression of BCL2,

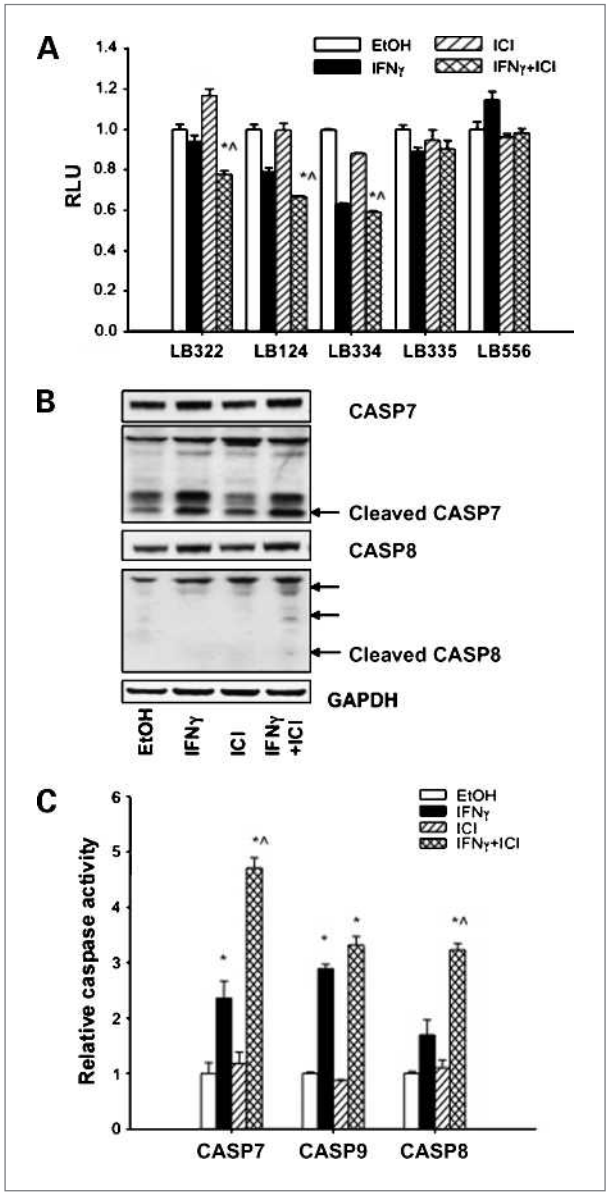

Figure 6. Combined treatment with IFNγ and ICI inhibits the BCL2 P1 promoter and increases the activation of CASP7, CASP8, and CASP9 in MCF7/LCC9 cells. A, MCF7/LCC9 cells were seeded in 12-well plates 1 d before transfection with different BCL2 promoter-reporter and phRL-SV40-*Renilla* constructs and treatment with 100 IU/mL IFNγ or 100 nmol/L ICI (singly or in combination) or with EtOH vehicle for 48 h. Data are presented as relative light units (RLU) and represent mean ± SEM for a representative experiment; three independent experiments were done. *, $P < 0.05$ versus control; ^, $P < 0.05$ versus IFNγ. B, protein levels of CASP7, cleaved CASP7, CASP8, and cleaved CASP8 were detected by immunoblot in MCF7/LCC9 cell lysates after 48 h of treatment with EtOH vehicle or 100 nmol/L ICI in the presence or absence of 100 IU/mL IFNγ. A representative image is shown. C, MCF7/LCC9 cells were seeded in white, 96-well tissue culture dishes 1 d before treatment with EtOH vehicle or 100 nmol/L ICI in the presence or absence of 100 IU/mL IFNγ. At 16 h (CASP9) or 72 h (CASP7, CASP8) later, caspase activity was detected using colorimetric or luminescent assay as described in the Materials and Methods. Data are normalized to the vehicle-treated control and represent the mean ± SEM for three independent experiments. *, $P < 0.05$ versus control; ^, $P < 0.05$ versus IFNγ.

BCL-W (55), and survivin, in the presence of increased BAK and BAX expression, likely generates a dominant signal in favor of cell death.

In conclusion, our studies show that treatment with IFNγ restores ICI-induced apoptosis in breast cancer cells that have acquired resistance to this antiestrogen. IFNγ action is dependent on its induction of IRF1 expression and involves regulation of NF-κB, specific BCL2 family members, and downstream caspases. Whereas antiestrogen-resistant cells retain the ability to induce autophagy in response to IFN, the restoration of antiestrogen responsiveness by IFN seems to be driven primarily through increasing apoptosis. Overall, these observations suggest that a combination of antiestrogens and compounds that can induce IRF1, such as low-dose IFNγ, may be useful for the treatment of some ER-positive breast cancers that have acquired resistance to antiestrogen therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

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