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Activation and Potentiation of Interferon-γ Signaling by 3,3'-Diindolylmethane in MCF-7 Breast Cancer Cells.

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Running title: 3,3'-diindolylmethane activates interferon gamma signaling

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Abbreviations:

IFN, interferon; IFNGR1, interferon gamma receptor 1; GAS, interferon-gamma activated sequence; OAS, oligoadenylate synthase; DIM, 3,3'-diindolylmethane; STAT-1, signal transduction and activator of transcription factor 1; JAK, Janus-activated kinase;

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Abstract

3,3'-diindolylmethane (DIM), a natural autolytic product in plants of the *Brassica* genus including broccoli, cauliflower and Brussels sprouts, exhibits promising cancer protective activities, especially against mammary neoplasia in animal models. We observed previously that DIM induced a G_1 cell-cycle arrest, and strong induction of cell-cycle inhibitor p21 expression and promoter activity in both estrogen responsive and estrogen independent breast cancer cell lines. We recently showed that DIM upregulates the expression of interferon gamma (IFNγ) in human MCF-7 breast cancer cells. This novel effect may contribute to the anticancer effects of DIM since IFNγ plays an important role in preventing the development of primary and transplanted tumors. In this study, we observed that DIM activated the IFNγ signaling pathway in human breast cancer cells. DIM activated the expression of the IFNγ receptor (IFNGR1) and IFNγ responsive genes, p56 and p69-OAS. In cotreatments with IFNγ, DIM produced an additive activation of endogenous p69-OAS and of an OAS-Luc reporter and a synergistic activation of a GAS-Luc reporter. DIM synergistically augmented the IFNγ induced phosphorylation of the transcription factor STAT-1, further evidence of DIM activation of the IFNγ pathway. DIM and IFNγ produced an additive inhibition of cell proliferation and a synergistic increase in levels of major histocompatibility complex class-1 (MHC-I) expression, accompanied by increased levels of mRNAs of MHC-1 associated proteins and transporters. These results reveal novel immune activating and potentiating activities of DIM in human tumor cells that may contribute to the established effectiveness of this dietary indole against various tumors types.

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Introduction

3,3'-diindolylmethane (DIM) is a natural product formed during the autolytic breakdown of glucobrassicin present in food plants of the *Brassica* genus, including the common vegetables, cabbage, Brussels sprouts, cauliflower and broccoli. DIM also is produced following ingestion of indole-3-carbinol (I3C), the immediate precursor of DIM in the plants (Grose and Bjeldanes, 1992). In addition, DIM is slowly produced from I3C under near neutral pH cell culture conditions during extended incubation periods. Results of several studies indicate that DIM exhibits promising cancer protective activities, especially against mammary neoplasia (Wattenberg and Loub, 1978; Shertzer, 1983, 1984). Oral intubation of I3C in a single dose prior to carcinogen treatment reduced the incidence and multiplicity of DMBA-induced mammary tumors in rats by 70-80% (Wattenberg and Loub, 1978; Grubbs et al., 1995). Repeated oral administrations of DIM during the promotion stage of DMBA-induced mammary tumorigenesis inhibited tumor growth in rodents by as much as 95% (Chen et al., 1998). We observed that under conventional cell culture conditions, DIM could inhibit the proliferation of breast tumor cell lines, regardless of estrogen receptor status (Riby et al., 2000). DIM induced a G_1 cell cycle arrest and produced a strong induction of p21 cell cycle inhibitor gene expression and promoter activity in both estrogen responsive and estrogen independent breast cancer cells. The antiproliferative effects of DIM involved Sp1/Sp3 transcription factor activation of p21 as a target for cell cycle control in human breast cancer cells (Hong etal., 2002).

Interferons (IFNs) are a group of immune cytokines with antiviral and cytostatic functions. Type I IFNs, including IFNα and IFNβ, are produced by virus-infected cells. Type II IFN, usually called interferon-γ (IFNγ) or the "immune interferon," promotes B cell differentiation into immunoglobulin-producing cells (Boehm et al., 1997). The recently

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recognized anti-tumor activity of IFNγ includes the priming of macrophages for non-specific tumoricidal activity, the activation of monocytes, natural killer cells and T cells to increase cytotoxicity against tumor cells, and the inhibition of tumor induced angiogenesis (Ikeda et al., 2002). The possible therapeutic use of IFNγ in cancer patients, however, has been limited due to serious side effects (Wimer, 1998).

In a recent report we showed that DIM can upregulate the expression of IFNγ in the human MCF-7 breast cancer cell line (Xue et al., 2005). Using promoter deletions, we showed that the region between -108 and -36 bp in the IFNγ promoter, which contains two conserved and essential regulatory elements, is required for DIM-induced IFNγ expression. DIM activates both JNK and p38 pathways, induces the phosphorylation of c-Jun and ATF-2, and increases the binding of the homodimer or heterodimer of c-Jun/ATF-2 to the proximal AP-1·CREB-ATF-binding element. Moreover, studies with specific enzyme inhibitors showed that upstream Ca^{2+}-dependent kinase(s) is required for the inducing effects of DIM in MCF-7 cells. These results established that DIM-induced IFNγ expression in human breast tumor cells is mediated by activation of both JNK and p38 pathways, which is ultimately dependent on intracellular calcium signaling.

In the present study, we report that DIM activated the IFNγ signal transduction pathway in human breast cancer cells. DIM treatment increased the expression of IFNγ receptor-1 (IFNGR1) and the IFNγ responsive genes, p56 and p69-OAS. In addition, DIM produced a synergistic increase in IFNγ induced activation of phosphorylation of STAT-1 and reporter gene expression. Finally, DIM and IFNγ exhibited additive antiproliferative activities in cultured cells but produced synergistic increases in expression of major histocompatability complex class I (MHC-I). This later result is consistent with a strong immune potentiation activity of DIM.

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Materials and methods

Materials.

Dulbecco's modified Eagles' medium (DMEM), Opti-MEM and Lipofectamine were supplied by Gibco/BRL (Grand Island, NY). Fetal bovine serum (FBS) and human recombinant IFNγ, were supplied by Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP was supplied by New England Nuclear (Boston, MA). DIM was prepared from I3C as described (1) and recrystallized in toluene. All other reagents were of the highest grade available.

Cell Culture.

The human breast adenocarcinoma cell line, MCF-7, obtained from the American Type Culture Collection (ATCC), was grown as adherent monolayers in DMEM, supplemented to 4.0 g/liter glucose , 3.7 g/liter sodium bicarbonate and 10% heat inactivated FBS, in a humidified incubator at 37 °C and 5% CO_2 , and passaged at approximately 80% confluency. Cultures used in subsequent experiments were at less than 25 passages.

Cell Counting.

Cells were harvested by trypsinization and resuspended in complete medium. Aliquots were diluted 50 fold in Isoton II (Coulter Corp. Miami,FL) and 500 µL duplicates were counted in a model Z1 Coulter particle counter and averaged.

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Microarray analysis of differential expression

MCF-7 cells were treated with 50 uM DIM for 24 h or with the DMSO vehicle for controls. Poly-A-RNA was isolated by two cycles of purification on oligo-dT cellulose. Labeled cDNA probes were prepared using the fluorescent dyes cyanine 3 and cyanine 5 for the treated and the control samples, respectively, and hybridized simultaneously to microarrays (Incyte Genomics, Fremont, CA) of 960 selected cDNAs representing human genes involved in cell cycling, apoptosis, signal transduction, motility, adhesion and angiogenesis. Differential expression was measured by the ratio of the fluorescence intensity at the wavelengths corresponding to the two probes. The samples were spiked with known concentrations of various non-human cDNA to serve as positive controls and to correct for variations in hybridization efficiency.

Determination of mRNA by RT-PCR.

Cells were lysed by addition of Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH) and chloroform was used for phase separation. After centrifugation, the aqueous upper phase was collected and total RNA was precipitated by isopropanol, washed with 75% ethanol, and dissolved in DEPC treated water. Levels of specific mRNAs were determined by reverse transcription and polymerase chain reaction (RT-PCR) using Gibco enzymes and reagents. Reverse transcription was done using a 15-mer oligo-dT primer to generate cDNAs from mRNAs only. The sequences of the PCR primer pairs for specific mRNA were as follows:

p69-OAS upper, CGAGCGGCACAAAATCGTCA;

p69-OAS lower, GGCCGGGAGCGAATGAAGTT;

p56 upper, CGCTGGGTATGCGATCTCTGC;

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p56- lower, GCCCTCTAGGCTGCCCTTTTG;

IFNGR-1 upper, CCAGGCATGCATACCGAAGACA;

IFNGR-1 lower, GCGATGCTGCCAGGTTCAGA;

IFNγ upper, TGCAGGTCATTCAGATGTAGCGGATA;

IFNγ lower, TCATGTATTGCTTTGCGTTGGACA.

HLA-A upper, GACGACACGCAGTTCGTGC

HLA-A lower, CATGTCCGCCGCGGTCCAA

HLA-B upper, ACCAGAGCGAGGCCGGG

HLA-A lower, GTGTCCGCSCGGTCCAG

HLA-C upper, CGCGCGGAGTCCRAGAGG

HLA-A lower, GTGTCCGCSCGGTCCAG

β_2-microglobulin upper, CTCGCGCTACTCTCTCTTTCTGG

β_2-microglobulin lower, GCTTACATGTCTCGATCCCACTTAA

TAP-1 upper, TCTCCTCTCTTGGGGAGATG

TAP-1 lower, GAGACATGATGTACCTGTCTG

TAP-2 upper, CTCCTCGTTGCCGCCTTCT

TAP-2 lower, TCAGCTCCCCTGTCTTAGTC

Expected amplicon sizes from these PCR primer pairs are as follows: p69-OAS, 330 bp; p56, 404 bp; IFNGR-1, 185 bp; IFNγ, 288 bp; HLA-A, 331 bp; HLA-B, 156 bp; HLA-C, 301 bp; β_2-microglobulin, 335 bp; TAP-1, 280 bp; TAP-2, 300 bp. After the appropriate number of cycles, the PCR products were dyed with ethidium bromide and separated on a 15% agarose gel and photographed under UV light to verify the size of the amplicons and relative abundance of the specific mRNA templates.

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Reporter assays.

The interferon inducible luciferase reporter pGL2-25AS(-972) (OAS-Luc) was a gift from Dr. Georgia Floyd-Smith (Arizona State University at Tempe) (Floyd-Smith et al., 1999). OAS-Luc contains the promoter and 5'-flanking region (-972) of p69-OAS. The consensus GAS reporter, GAS-Luc containing 4 repeats of the GAS element (5'-GATCAGTGATTTCTCGGAAAGAGAG-3') from the IFNγ consensus sequence binding protein (ICSBP), was a gift from Dr. Keiko Ozato (National Institute of child Health and Human Development, Bethesda, MD) (Kanno et al., 1993). Reporter plasmids were transiently transfected in MCF-7 cells by lipofection (Lipofectamine, Gibco) and after treatments as indicated in the Results section, luciferase activity in cell lysates was measured as described previously (Riby et al., 2000).

Gel Mobility Shift Assay.

Nuclear extracts of cells were prepared as described previously (7) at the end of the treatment periods indicated in the Results section. The following two complementary 25-mer oligonucleotides,
5'-GATCAGTGATTTCTCGGAAAGAGAG-3' and 5'-GATCCTCTCTTTCCGAGAAATCACT-3' containing the palindromic gamma activated sequence (GAS) consensus motif (underlined), were annealed and 5'end-labelled with γ^{32}P-ATP using T4 nucleotide kinase. The resulting labeled double stranded DNA probe was purified on a Sephadex G50 spin-column, precipitated in ethanol, dissolved in TE buffer and diluted in 25 mM Hepes, 1 mM DTT, 10% glycerol, 1 mM EDTA to contain approximately 25,000 cpm of ^{32}P/µL.

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Nuclear extracts (7 µg of proteins), were mixed with 90 ng poly-dIdC, 25 mM Hepes, 1 mM DTT, 10% glycerol, 1 mM EDTA, 160 mM KCl) in a total volume of 21 µL. For antibody supershift experiments, 0.5 µg of monoclonal mouse-IgG anti-human- IFNγ (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the incubation mixture. After incubation for 15-20 min at room temperature, 4 µL (100,000 cpm) end-labeled ^{32}P-ERE probe was added and incubated for another 15 min at room temperature. After addition of 2.8 µL of 10X Ficoll loading buffer (0.25% bromophenol blue, 25% ficoll type 400), 22 µL aliquots were loaded unto a pre-run, non-denaturing 4.0% polyacrylamide gel in TAE (67 mM tris, 33 mM sodium acetate, 10 mM EDTA, pH 8.0) at 120 V for 2 hours. The gel was then dried and autoradiographed using BioMax MR film (Kodak).

Flow cytometry analysis of cell cycle measurements.

MCF-7 cells were plated at 10,000 cells/well of a 6-well tissue culture dish and treated for 6, 24, 48, 72, 96, 120 and 144 h in complete medium. DIM was added to a final concentration of 10 and 30 µM and IFNγ to 10 ng/ml. Medium was changed every 24 h. Following treatment, cells were washed with phosphate-buffered saline and hypotonically lysed in 1 ml of DNA staining solution (0.5 mg/ml propidium iodide, 0.1% sodium citrate, 0.05% Triton X-100). Cell debris was removed by filtration through 60-µm nylon mesh (Sefar America Inc., Kansas City, MO). Nuclear-emitted fluorescence with wavelengths of >585 nm was measured with a Coulter Elite instrument. Ten thousand nuclei were analyzed from each sample at a rate of 300-500 nuclei/s. The percentages of cells within the G1, S, and G2/M phases of the cell cycle were determined by analysis with the Multicycle software MPLUS (Phoenix Flow Systems) in the Cancer Research Laboratory Microchemical Facility of the University of California, Berkeley.

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Flow Cytometry analysis of MHC-I expression.

Flow cytometric analysis of membrane immunofluorescence was performed as follows: MCF-7 cells were pretreated with 30 μM DIM separately for 6 h, 24 h, or 48 h, and then treated with different concentrations of IFNγ for another 16 h. Cells were harvested, washed with phosphate-buffered saline (PBS), and then 10^6 cells in 90 μl of PBS were incubated with 10 μL of FITC-conjugated HLA-ABC mouse monoclonal antibody or negative control FITC-conjugated IgG2b antibody (Chemicon) on ice for 30 min. After washing twice with PBS, the fluorescence density was measured using a Coulter Elite instrument and analyzed with WinMDI 2.8 software provided by Duke University. The expression of HLA-1complex was calculated as the percentage of antibody positive cells (%PC) and mean fluorescence value (MFV).

Western Blot Analysis

After the indicated treatments, cells were harvested in RIPA buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% Nonidet P-40, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (50 μg/ml PMSF, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 μg/ml NaF, 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate and 0.1 mM -glycerophosphate). Equal amounts of total cellular protein were mixed with loading buffer (25% glycerol, 0.075% SDS, 1.25 ml of 14.4 M 2-mercaptoethanol, 10% bromphenol blue, 3.13% stacking gel buffer) and fractionated by electrophoresis on 10% polyacrylamide, 0.1% SDS resolving gels. Rainbow marker (Amersham Pharmacia Biotech) was used as the molecular weight standard. Proteins were electrically transferred to nitrocellulose membranes (Micron Separations, Inc., Westborough, MA) and blocked overnight at 4 °C with 5% non-fat dry milk in 1X Western wash

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buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Blots were subsequently incubated with antibodies against IFNGR1, phosphorylated STAT1 (P-STAT1) or STAT1 protein purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody concentration was 2 µg/ml in Western wash buffer. Immunoreactive proteins were detected after 1 h of incubation at room temperature with horseradish peroxidase-conjugated secondary antibodies. Goat anti-rabbit antibodies were used as secondary antibodies (Bio-Rad) after being diluted 1:3000 in wash buffer. Blots were treated with ECL reagents (PerkinElmer Life Sciences), and fluorescence was detected using BioMax MR film (Kodak). Equal protein loading was ascertained by Ponceau S staining of blotted membranes, and reprobing of the membranes with anti-tubulin antibody.

Statistical analyses.

Statistically significant differences were determined by two-way analysis of variance (ANOVA), using the SigmaStat software.

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Results

DIM activates transcription of IFNγ and related genes in MCF-7 human breast tumor cells.

An initial cDNA gene expression microarray screen of human breast cancer MCF-7 cells treated with DIM, followed by confirmation of results using semi-quantitative RT-PCR, established the transcriptional activation of IFNγ, IFNGR1, oligoadenylate-synthase (OAS) family member protein 69 (p69-OAS), and interferon-inducible protein 56 (p56) (Figure 1). The expressions of IFNγ, p69-OAS and p56 were fully induced following 6 h of treatment, whereas maximum IFNGR1 induction was reached following 48 h of treatment. As shown previously (12), the level of mRNA for IFNγ returned to near control level following 48 h of DIM treatment. The mRNA levels for p56, IFNGR1 and p69-OAS remained elevated after 48 h of treatment. Maximum induction for IFNγ, IFNGR1, p69-OAS and p56 were 4.6, 3.8, 3.2 and 4.0 fold, respectively. The mRNAs for IFNα and IFNβ were not detectable by RT-PCR analysis in DIM treated or untreated MCF-7 cells (data not shown).

DIM induces expression of IFNGR1 protein in MCF-7 cells.

Western blot analysis (Figure 2) showed that levels of IFNGR1 protein were strongly upregulated by DIM treatments. IFNGR1 protein levels increased by approximately 7 fold after 24 h and persisted to 48 h compared to DMSO-treated controls. These results are consistent with the increase in mRNA levels shown in Figure 1 and suggest that the effect of DIM on the regulation of IFNGR1 expression is primarily through transcriptional control.

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DIM augments IFNγ-induced expression of the endogenous p69-OAS gene and associated reporter gene constructs.

A. Endogenous genes.

We next examined in more detail the effects of DIM alone and in combination with IFNγ on expression of endogenous p69-OAS. The results of RT-PCR analyses of p69-OAS transcripts (Figure 3A) showed that separate treatments with DIM (50 μM) and IFNγ (10 ng/mL) produced inductions of approximately 3.5- and 5.0-fold, respectively, over the control, whereas treatment with a combination of the two produced an increase of nearly 12-fold. These results suggest a synergistic interaction of DIM and IFNγ on induced expression of endogenous p69-OAS.

We determined next whether the inducing effects of DIM on endogenous p69-OAS gene expression required concurrent protein synthesis. Results presented in Figure 3B indicate that co-treatments with the translation inhibitor, cycloheximide, blocked the inducing effects of DIM treatments, as well as, the enhancing effects of DIM on IFNγ-induced expression of endogenous p69-OAS.

B. Transfected reporter gene constructs.

To examine whether the inducing effects of DIM are at the transcriptional level, the activities of two luciferase reporter constructs, OAS-Luc and 4GAS-Luc were measured following treatment with DIM. OAS-Luc contains the promoter and 5'-flanking region (-972) of p69-OAS and GAS-Luc contains four repeats of the consensus GAS element. MCF-7 cells transiently transfected with these reporter constructs were treated with IFNγ, DIM, or a combination of both for 24 h. As shown in Figure 3C, DIM treatment induced expression of the OAS-Luc reporter to a maximum of about 2.3 fold, a level of induction that was somewhat less

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than the level we observed for induction of the corresponding endogenous gene of over 3-fold. IFNγ induced transcriptional activity of this reporter in a concentration-dependant manner to a maximum of only about 1.8 fold, which is considerably less than the response of the endogenous gene of about 5.0 fold. In marked contrast to the maximum effects of combined treatments of the inducers on endogenous gene expression (nearly 12-fold increase compared to vehicle treated cells), the reporter gene construct responded with less than a 3-fold increase over the controls. These results show that whereas the reporter and endogenous gene responded similarly to treatment with DIM by itself, the reporter was considerably less responsive than the endogenous gene to IFNγ.

Finally, studies with cells transfected with the 4GAS-Luc reporter (Figure 3D) showed an inducing effect of DIM that was similar to the responses of the p69-OAS gene, but a more robust response to IFNγ that was accompanied by a consistent synergistic response to co-treatments with the two inducers. Thus, treatment with DIM (50 μM) by itself again produced a 2 to 3-fold increase in activity of this reporter. IFNγ treatments, however, produced a robust concentration dependent increase in reporter activity to approximately 40-fold induction compared to vehicle treated controls. Co-treatments with DIM and IFNγ produced a roughly 2-fold synergistic activation of the 4GAS-Luc reporter at all IFNγ concentrations examined, reaching a maximum induction of approximately 75 fold.

Taken together, these results show that DIM by itself produced similar levels of activation of endogenous and transfected reporter genes that are differentially responsive to IFNγ. The results show further that the inducing effects of DIM are mediated by a short lived transcription factor or require de novo synthesis of an intermediate regulatory protein.

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Synergistic effects of DIM on IFNγ-mediated signal transduction.

To probe further the role of GAS element activation in transcriptional activation by DIM, we examined the effect of this indole on downstream events in the GAS signaling pathway. For this purpose, the effects of DIM were examined on phosphorylation of the signal transduction and activator of transcription factor 1 (STAT-1), and the binding of the activated STAT-1 dimer to the cognate GAS responsive element were examined. Phosphorylation of STAT-1 by Janus-activated kinase (JAK) is the first step in IFNγ signaling following binding to IFNGR1. For these studies, cells were pretreated with DIM for 6 or 24 h followed by a 15 min. exposure to IFNγ. STAT-1 phosphorylation levels in lysates of DIM-treated cells were compared to controls that received IFNγ but had not been treated with DIM. Phosphorylation was measured by Western blot analysis using an antibody specific to STAT-1 phosphorylated on Tyr-701 residue. Results presented in Figure 4 show that whereas DIM treatment by itself for 6 or 24 h did not induce STAT-1 phosphorylation, pretreatment with DIM augmented IFNγ-induced STAT-1 phosphorylation by over 4 fold after 24 h DIM pretreatment. These treatments did not affect the levels of inactive (total) STAT-1 significantly.

An electrophoretic gel-mobility-shift assay was used to verify that the phosphorylated STAT-1 was effectively activated to a dimer that could bind to the GAS element. The ^{32}P-labeled DNA probe containing a GAS sequence was incubated with nuclear extracts from cells pretreated with DIM for various times as indicated, and with IFNγ for 30 min. As shown in Figure 5, IFNγ treatment produced a band-shift (Band 1) that increased in intensity with the duration of the DIM pretreatment. DIM treatments in the absence of IFNγ did not produce a shifted band at any time (data not shown). When samples were incubated with an antibody

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specific to STAT-1, the band was super-shifted (Band 2), confirming the identity of the protein binding to the labeled GAS probe.

Taken together, these results show that DIM can augment IFNγ-induced STAT-1 activation, dimerization and binding to the GAS element in DNA. We obtained no evidence for STAT-1 activation by DIM by itself, however, under these assay conditions.

Effects of DIM and IFNγ on proliferation and cell cycling.

Possible functional consequences of the synergistic effects of DIM and IFNγ on gene expression were examined at the level of proliferation rates in MCF-7 cells. Cell proliferation was measured over a 4-day treatment period with treatments with DIM, IFNγ, or both substances, and compared to a DMSO control. As shown in Figure 6, with heat-treated serum, IFNγ alone reduced cell proliferation by as much as 70% compared to controls. DIM treatments reduced proliferation by as much as 60%. With the exception of the highest concentration of IFNγ, combined treatments with DIM and IFNγ resulted in roughly additive increases in the cytostatic activity compared to treatment with DIM by itself. These effects were accompanied by morphological changes characteristic of apoptosis (data not shown). We observed no significant effect of IFNγ on proliferation of cells grown in unheated serum (data not shown).

Flow cytometry was used to determine the effects of DIM and IFNγ on cell cycling. Data presented in Figure 7 show the proportion of the cell population in the G1 phase of the cell cycle at different intervals up to 3 days. In control groups approximately 50 % of the cells were in G1. Treatment with IFNγ alone caused a small progressive increase in G1 blocked cells of up to 10 % above the DMSO control, after 3 days. DIM had a more immediate effect that increased over time up to 30% above the DMSO control. The combination of DIM and IFNγ had an additive

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effect leading to more than 90% of the cells in G1 arrest after three days, correlating with the absence of proliferation shown in Figure 6. Taken together, these results indicate that the effects of DIM and IFNγ on cell proliferation are additive and are consistent with separate cytostatic mechanisms for DIM and IFNγ.

DIM potentiates IFNγ induced expression of MHC-I complex in MCF-7 cells.

Induced expression of the MHC-I complex is well established and an important down-stream target of IFNγ-mediated signal transduction. To examine the possible synergistic effects of DIM on this highly significant metabolic product of IFNγ signaling, we tested the effects of co-treatment with DIM on IFNγ induced expression of MHC-I complex in MCF-7 cells.

Flow cytometry analysis of cell surface MHC-I expression was conducted using FITC-conjugated HLA-ABC antibody. In an initial control experiment, cultured MCF-7 cells were pre-treated with 30 μM DIM for 48 hours and then with or without 10 ng/mL IFNγ for another 16 hours. Analyses using a negative control antibody, FITC-conjugated mouse IgG2b, indicated no significantly induced signal (data not shown). In a subsequent experiment in which cells were treated with a range of IFNγ concentrations and analyzed with the anti-MHC-I antibody, a strong signal indicative of IFNγ-induced MHC-I expression (Figure 8) was detectable at 0.1 ng/mL treatment and the level appeared to plateau at 10 ng/mL. In cells treated with vehicle control only, MHC-I expression was not detected in greater than 99% of cells. In another control experiment, treatment of cells with 30 μM DIM induced mean fluorescence value (MFV) of approximately 60 after 48 h in only 2% of the cells. Pretreatment with 30 μM DIM for 48 h followed by treatment with 0.1 ng/mL IFNγ produced a further strong increase in the percentage of MHC-I positive cells (%PC) from 12.97% to 40.98%, but had no significant effect on MFV

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(Figure 8D). Exposure to DIM of cells treated with 10 ng/mL of IFNγ, about 95% of which expressed MHC-I produced a strong increase in MFV from 72 to 131. As expected, addition of an IFNγ blocking antibody into the medium before the treatments abrogated the inducing effects of co-treatments of IFNγ and DIM on MHC-I expression (data not shown), confirming the requirement of the cytokine for the observed effects. In an analogous experiment, we observed no effect of DIM or IFNγ on MHC-II expression in the MCF-7 cells (data not shown). These results show that whereas DIM by itself produces little or no effect on expression of MHC-I, this indole strongly augments the proportion of cells that express this protein complex, as well as the maximum level of MHC-I per cell, in response to IFNγ.

DIM enhances IFNγ-induced transcription of MHC-I components and transporters in MCF-7 cells.

Since the level of the IFNγ-induced MHC-I protein complex on the surface of MCF-7 cells could be enhanced by pretreatment with DIM, we examined whether expressions of the corresponding genes for the complex and its transporters were also increased. Results of RT-PCR analyses of human MHC-I, including HLA-A, HLA-B, HLA-C, and ß-microtubulin, and of two important associated transporters, TAP1 and TAP2 are presented in Figure 9. The expression levels of all six genes were not significantly enhanced by DIM compared to vehicle treated MCF-7 cells. As expected, the expressions of these genes were increased 2-8 fold by treatment with IFNγ. The results show that pretreatment of cells with DIM further increased the mRNA levels of these genes by at least 2 fold above the levels induced by IFNγ by itself, which equates to increases in mRNA levels of 4-16 fold above background levels.

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These results show that DIM synergistically enhances the level of IFNγ-induced expression of the MHC-I protein complex and associated mRNAs. The roughly 2-fold augmentation by DIM in levels of both MHC-I protein/cell and in the associated mRNAs, suggests that MHC-1 expression induced by DIM is regulated at the level of gene transcription.

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Discussion

The results of these studies indicate that DIM is an immunomodulator that not only can induce expression of IFNγ in breast tumor cells as shown in our previous report (Xue et al., 2005), but also can modify the response of the cells to exogenous exposure to this cytokine. We measured the levels of secreted IFNγ protein in conditioned medium, using a very sensitive ELISA assay and found that after three days of DIM treatment IFNγ had accumulated to a concentration of less than 100 pg/mL, while it was undetectable in DMSO controls (data not shown). Therefore, the inducing effects on the IFNγ responsive genes and reporters do not appear to result from DIM-induced secretion of IFNγ, since this concentration is below the effective concentration of IFNγ treatments. Furthermore, it is unknown whether the immunoreactive material found in the conditioned medium had retained biological activity. The results of the present study show that in MCF-7 cells DIM can 1) induce the expression of IFNγ, the IFNγ receptor, and two IFNγ-inducible genes, 2) induce the expression of IFNγ-inducible reporter gene constructs, 3) synergistically augment IFNγ-mediated activation of the STAT-1 signal transduction pathway, 4) additively augment the cytostatic effects of IFNγ in cultured cells, and 5) synergistically augment the expression of IFNγ-induced MHC-I protein complex and associated mRNAs.

The signaling cascade regulated by IFNγ has been examined in considerable detail in many laboratories. Binding of IFNγ to the highly specific IFNγ receptor (IFNGR1) on the membrane of target cells activates a phosphorylation cascade involving JAK1 and JAK2 and STAT-1. Phosphorylated STAT-1 homodimerizes, translocates to the nucleus, and binds to the IFNγ activated sequence (GAS) in the promoter of IFNγ-inducible genes, and activates transcription. Activation, of one such gene, p69-OAS, plays an important role in the prevention

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of replication of viral RNA in infected cells. In addition, p69-OAS has been identified recently as an inhibitor of cell growth and a pro-apoptotic protein related to Bcl-2 (Ghosh et al., 2001). Our results confirm that IFNγ can activate the JAK/STAT pathway in breast tumor cells, and show that each of the steps in IFNγ-induced gene activation is synergistically augmented by treatments with DIM.

The effects of DIM are clearly distinguishable from the effects of other cytostatic agents that induce IFNγ expression. In one series of studies, the synergistic effects of retinoic acid (RA) and IFNγ in breast cancer cells were described. Initially, a synergistic antiproliferative effect was observed following sequential treatment of cells with IFNγ and RA. The effect appeared to result from an IFNγ-mediated augmentation of RA cytostatic activity. IFNγ was shown to increase RA cytostatic potency by increasing levels of expression of retinoic acid receptor – gamma (RARγ) and decreasing expression of cellular RA-binding proteins (CRABP) (Widschwendter et al. 1995). Subsequently, the effects of RA on IFNγ signaling were also examined in breast tumor cells. The results showed that RA could synergistically augment the effects of IFNγ on gene transcription in MCF-7 cells by a mechanism that involved RA-mediated augmentation of STAT1 expression (Kolla et al., 1996). In another series of studies the effects of combined treatments of breast cancer cells with IFNs and the estrogen antagonist, tamoxifen, were examined. Co-treatments with these substances caused an augmentation of the expression of certain IFN-stimulated genes, including the transcription factors ISGF-3 and GAF (Lindner et al., 1997). Tamoxifen by itself, however, produced no effect on the expression of these transcription factors. Thus, our results with DIM show significant differences from the reported effects of RA and tamoxifen on IFNγ action. We observed only an additive inhibitory effect of DIM and IFNγ on MCF-7 cell proliferation, whereas the effect with RA was synergistic. Also in

contrast to RA, although DIM synergistically augmented IFNγ signal transduction, this indole did not increase STAT1 expression in the absence of IFNγ treatment. Finally, DIM treatment by itself clearly induced expression of IFNGR1, IFNγ and OAS-related genes, effects that were not reported for RA or tamoxifen.

Our observation of a synergistic effect of DIM on IFNγ-induced expression of MHC-I is potentially of considerable importance since this complex plays a central role in tumor immunosurveillance. MHC-I molecules are required for the presentation of tumor-associated antigen (TAA) to cytotoxic T-lymphocytes (CTLs). Decreased expression of MHC antigen is thought to protect tumor cells from immunosurveillance (Marincola et al., 2000; Goodenow et al, 1985). Accordingly, loss or down-regulation of MHC-I has been shown to be a frequent event in breast tumorigenesis. Indeed, several studies with murine models of induced carcinogenesis have confirmed the role of down-regulation of MHC-I antigens in increasing the tumorigenic and metastatic potential of tumors (Tanaka et al., 1988). Conversely, induced MHC-I expression is thought to be important in antitumorigenic properties of IFNγ (David-Watine et al., 1990) and certain small molecule cancer therapeutic agents, including the nucleotide analog, 5-azacytidine cystosine arbinoside, 5-flurouracil, retinoids, vitamin D3, as well as the plant alkaloid, vincristine (Ohtsukasa et al., 2003, Geissmann et al., 2003). In contrast to the activities of these other MHC-I inducers, we observed little or no effect of DIM by itself, whereas we observed a clear synergistic augmentation by DIM of IFNγ-induced MHC-I expression. Significant also was our observation that following treatments with the higher concentrations of DIM, the level of MHC-I expressed/cell was increased to levels that were unattainable by treatments with IFNγ by itself. The roles in these synergies of DIM-induced increases in expression of IFNGR1 or

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possibly of modified MHC-I protein processing and trafficking are under investigation in our laboratories.

Comparison between our current results with DIM and our recently published studies with I3C (Chatterji et al., 2004) show some distinct differences in the activity of DIM and its in vivo precursor in their effects on IFNγ signaling. Similar to our current results with DIM, we showed in the previous work that I3C also can affect IFNγ signaling in MCF-7 cells by a mechanism that involves a strong and rapid increase in expression of IFNGR1 and synergistic activation of STAT1 signaling. Similar effects of DIM and I3C were also observed on cell proliferation and cell cycling. In contrast to the present results with DIM, however, we did not observe in the previous studies increased expression by I3C of the IFNγ-stimulated genes (p56 and p69-OAS). These results are consistent with the hypothesis that the effects of I3C on IFNGR1 expression are mediated by its slow conversion to DIM during incubation with the cells. Indeed, we have shown previously that DIM accumulates in the nucleus of cultured MCF-7 cells treated with I3C (Staub et al., 2002). We speculate that our failure to detect I3C-induced expression of p56 and p69-OAS in the previous studies, may be due to insufficient intracellular concentrations of DIM, or more interestingly, to a requirement for extracellular exposure to DIM for induced expression of these genes. Further studies are in progress to test these possibilities.

Our results may provide a rationale for explaining the clinical effectiveness of indole treatments in the control of recurrent respiratory papillomatosis (RRP). I3C and DIM are said to be the most popular adjunct therapies for this disorder because of their effectiveness and low level of toxicity (Auborn., 2002; Wiatrak, 2003). RRP is caused by certain types of human papilloma viruses (HPVs) (Coll et al., 1997; Rosen et al.,1998), and a hallmark of this disease is the tendency of the papillomas to recur after surgical removal (Gissmann et al., 1982; Kashima et

al., 1996). One report indicated that most patients (55.4%) respond to the treatment of I3C/DIM by slowing down the recurrence rate, and recurrence of the disease is completely inhibited in 19% of patients (Yuan et al., 1999). Previously suggested modes of action of I3C/DIM in the control of RRP include induction of a better estrogen metabolite balance (Yuan et al., 1999), inhibition of cell proliferation (Cover et al., 1998), and induction of apoptosis (Hong et al., 2002). Our results suggest that I3C/DIM may function by yet another mechanism, that of immune potentiation. If confirmed by on going studies in our laboratories, this activity may not only be useful in the prevention of recurrent papillomas, but also could be useful in treatment of papillomatosis and the prevention of malignant conversion of a broad range of tumor types.

In summary, our studies have shown that DIM can induce the expression of IFNγ, IFNGR1, and two IFNγ responsive genes in cultured human breast tumor cells. The inducing effects on the IFNγ responsive genes do not appear to result from DIM-induced secretion of IFNγ, but they are dependent on de novo protein synthesis. DIM synergistically augments IFNγ-induced STAT-1 signaling, which, in combination with the increased expression of IFNGR1, may be responsible for an increase in MHC-I expression per cell that is not attainable by treatment with IFNγ by itself. However, the antiproliferative effects of DIM and IFNγ in MCF-7 cells are only additive, suggesting that these substances may affect cell cycling by unrelated mechanisms. Thus, these studies provide evidence that DIM is a novel immune potentiator that can induce IFNγ expression and potentiate IFNγ activities in breast tumor cells. Further studies of the immune function of DIM are in progress.

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Footnotes

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Legends to figures

Figure 1. Transcriptional activation of IFNγ and related genes by DIM. Cells were treated with 50 μM DIM for the indicated times. The mRNA levels were measured by RT-PCR. Quantitative detection of amplicons required 40 PCR cycles for IFNγ and 17 cycles for the other genes. Images of the ethidium bromide-stained gels were inverted (negative black/white) for presentation. Molecular weights are compared to a 100 bp ladder (darker band is 600 bp) to confirm identity of the amplicons. GAPDH was used as the control. Densitometry results are presented as fold induction over the 0 hour treatment after correction for GAPDH. Results are presented as the mean ± SD of three separate experiments. Statistically significant difference with time zero control is noted * ($p<0.05$).

Figure 2. Western blot analysis of INFGR1. Cells were treated with DMSO (control) or 50 μM DIM for 6, 24 or 48 hours. The relative abundance of IFNGR1 was corrected for variations in tubulin, as a loading control between samples. A representative Western blot from three separate experiments is shown.

Figure 3. DIM activates transcription of IFNγ-responsive genes. (A and B) Transcriptional activation of endogenous p69-OAS was measured by RT-PCR. Cells were treated with DIM 10, 25 or 50 μM alone or in combination with IFNγ 10 ng/mL for 24 hours. In addition, (B) some plates received cycloheximide (CHX) 50 μM one hour before the beginning of the treatments to inhibit protein synthesis. Results are presented as the mean ± SD of three separate experiments. Statistically significant difference with the corresponding DMSO control for each concentration

of DIM is noted * (p<0.05) and significant difference with the IFNγ alone is noted ** (p<0.05). (C) Cells transfected with the p69OAS-Luc reporter were treated with IFNγ (1.0 to 30 ng/mL) alone or in combination with DIM 50 μM, for 24 hours. Transcriptional activity is expressed as fold induction over the DMSO treated control. Results are presented as the mean ± SD of three separate experiments. Statistically significant difference with the DMSO control is noted * (p<0.05). (D) Cells transfected with the 4xGAS-Luc reporter were treated with IFNγ (10 pg/mL to 1 ng/mL) alone or in combination with DIM 50μM, for 24 hours. Transcriptional activity is expressed as fold induction over the DMSO treated control. Results are presented as the mean ± SD of three separate experiments. Statistically significant difference with the DMSO control is noted * (p<0.05).

Figure 4. Effects of DIM on STAT-1 activation. Cells were pre-treated with DIM 50 μM for 0, 6 or 24 hours. Following treatments, some plates were also treated with IFNγ 1.0 ng/mL for 15 minutes. Phosphorylated STAT-1 and total STAT-1 were measured by Western analysis. Three replicate blots were analyzed by densitometry. Results are presented as the mean ± SD of three separate experiments. A representative Western blot from three separate experiments is shown. Statistically significant difference with the IFNγ alone without pretreatment with DIM is noted * (p<0.05) and significant difference between 6 and 24 hours of DIM pretreatments is noted ** (p<0.05).

Figure 5. Gel mobility shift analysis of STAT-1 binding to GAS. Cells were pre-treated with DIM 50 μM for the indicated periods and then with IFNγ 10 ng/mL for 30 minutes. Nuclear extracts were incubated with a ^{32}P-labelled DNA probe containing a GAS consensus sequence.

The identity of the DNA binding protein was confirmed by super-shifting with a STAT-1 antibody. Results are representative of three separate experiments.

Figure 6. Cell proliferation. Plates were seeded with $4x10^4$ cells and incubated overnight to allow attachment before starting the treatments. Treatments were DMSO as a control, DIM 10 or 25 μM, and IFNγ 1, 10 or 100 ng/mL, alone or a combination of DIM in triplicate wells. (A) Cells were counted 4 days after the beginning of treatments. (B) Thymidine incorporation was measured after 24 hours from the beginning of treatments. Results are presented as the mean ± SD of three separate experiments. Statistically significant difference with the corresponding DMSO control for each concentration of IFNγ is noted * ($p<0.05$).

Figure 7. Flow cytometry. The percentage of cells in the G1 phase of the cell cycle was measured in cells treated with DIM, IFNγ or combinations of the two for 6 hours, 1, 2 or 3 days. Results are presented as the mean ± SD of three separate experiments. Statistically significant difference with the corresponding DIM treatment without IFNγ at the same time point is noted * ($p<0.05$).

Figure 8. The expression level of MHC-I complex on the MCF-7 cell surface. (A) Cells were treated with different concentrations of IFNγ for 16 hours. (B,C) Cells were pretreated with DMSO (control) or 30 μM DIM for 48 hours, followed by 0.1 ng/mL of IFNγ (B) or 10 ng/mL of IFNγ (C) for another 16 hours. One million cells were incubated in 90 μl of PBS with 10 μl of FITC-conjugated anti-HLA-ABC mouse monoclonal antibody. The fluorescence intensity was measured by flow cytometry using a Coulter Elite instrument and analyzed by WinMDI 2.8

software provided by Duke University. (D) The expression of HLA class I complex was calculated as the percentage of antibody positive cells (%PC in open bars) and mean fluorescence value (MFV in crossed hatched bars).

Figure 9. DIM further increases IFNγ-induced transcription of MHC-I components and two associated transporter proteins. Cells were pretreated with 30 μM DIM for 48 hours, and 10 ng/mL of IFNγ was added into the medium for another 16 hours. The mRNA levels were measured by RT-PCR. GAPDH was used as the control. Densitometry results are presented as fold induction over the DMSO treatment after correction for GAPDH. Results are presented as the mean $\pm$ SE of three separate experiments. Statistically significant difference with DMSO control is noted * ($p<0.05$).

Figure 1

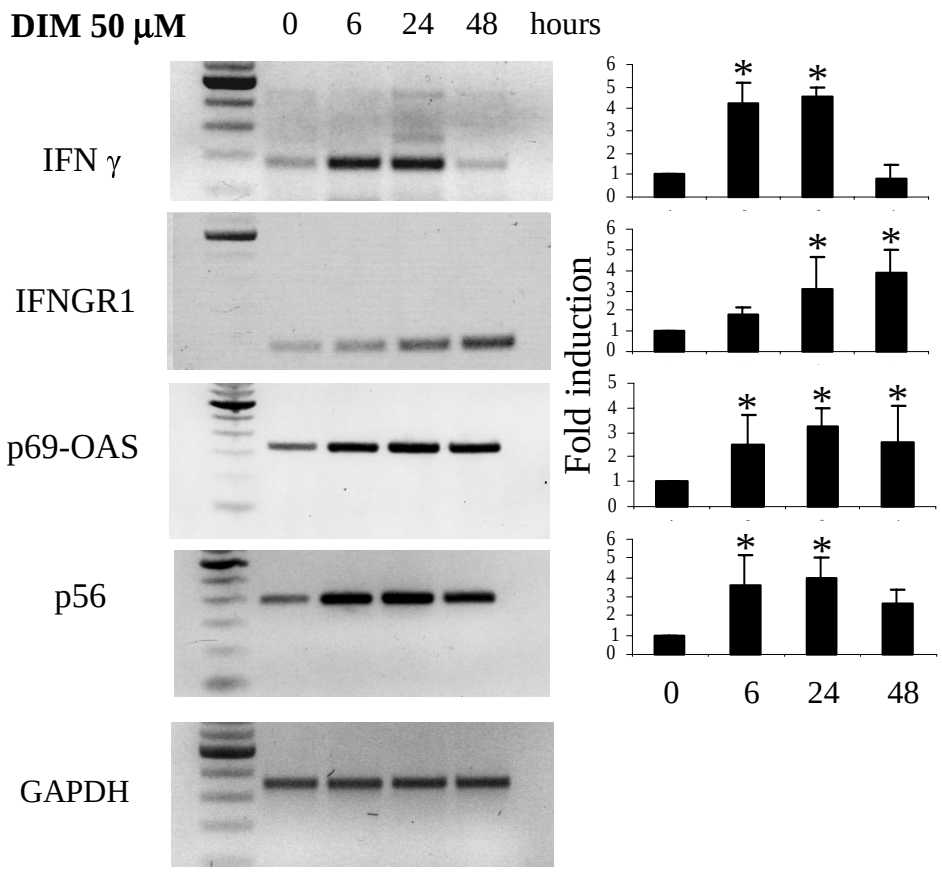
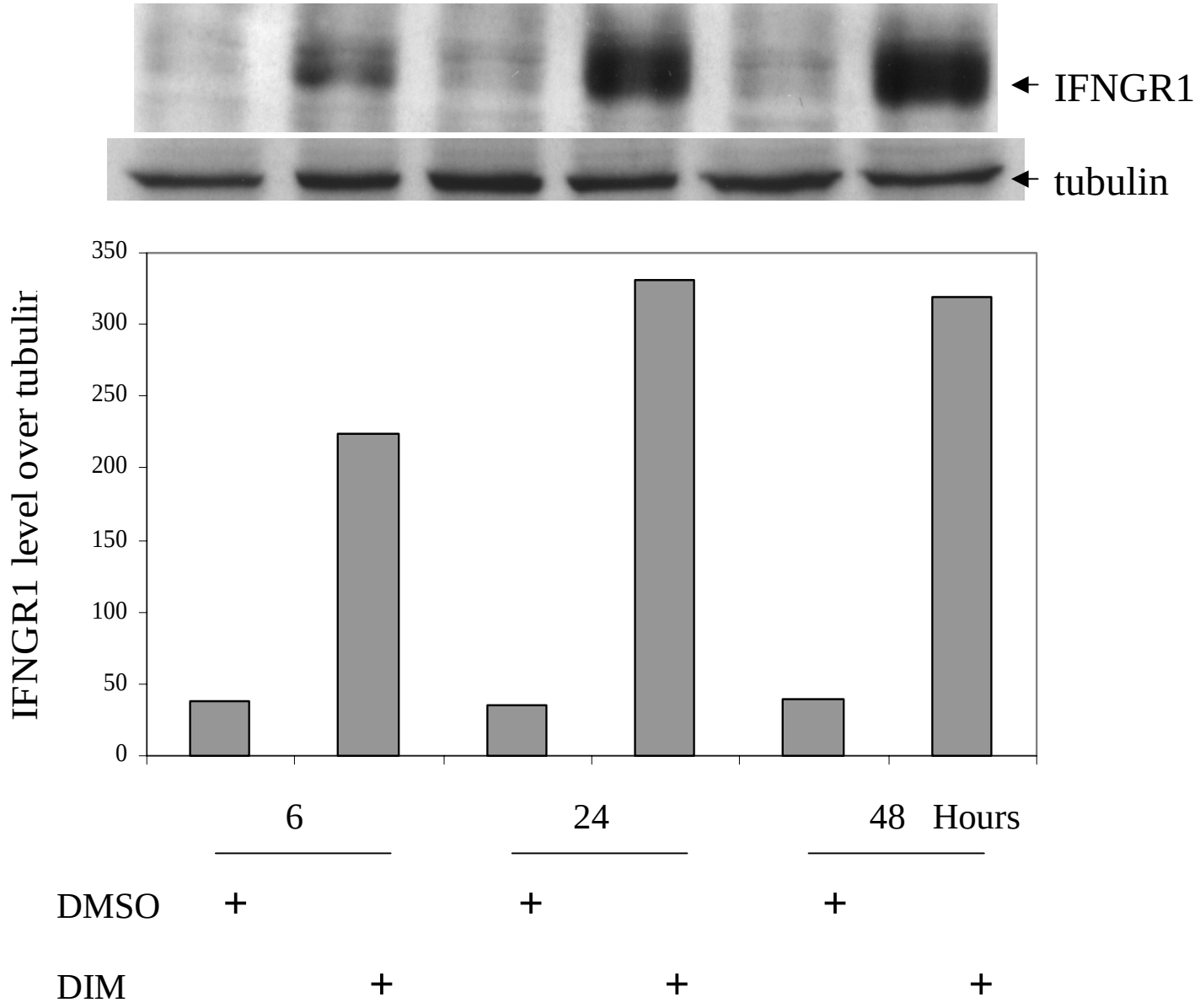


Figure 2



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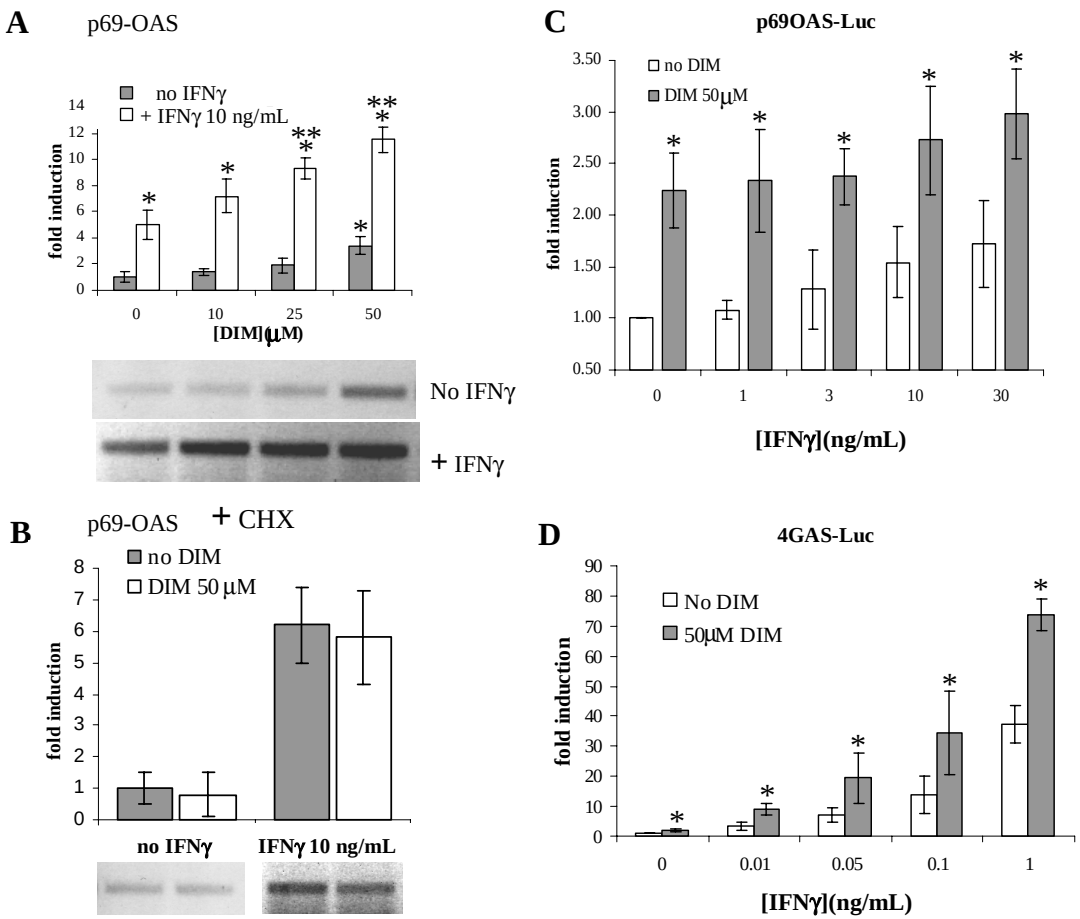

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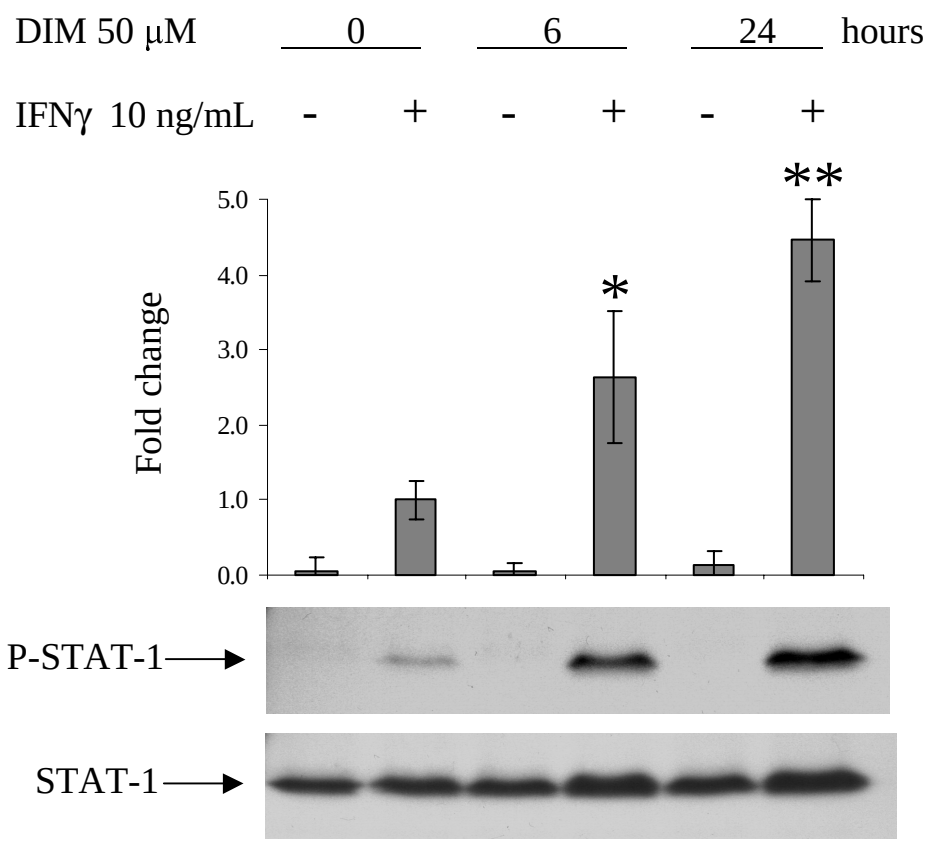

Figure 5

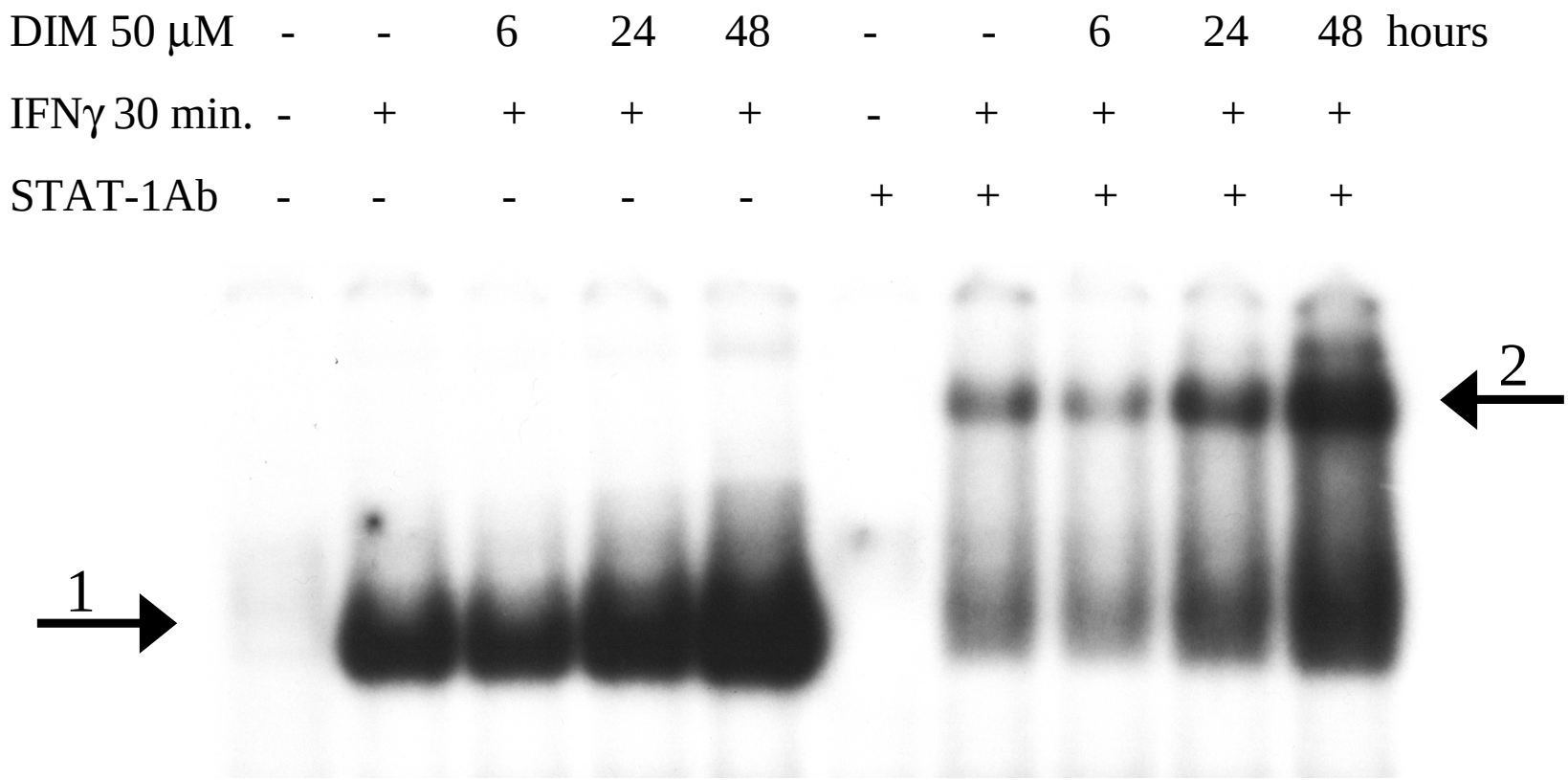

Figure 6

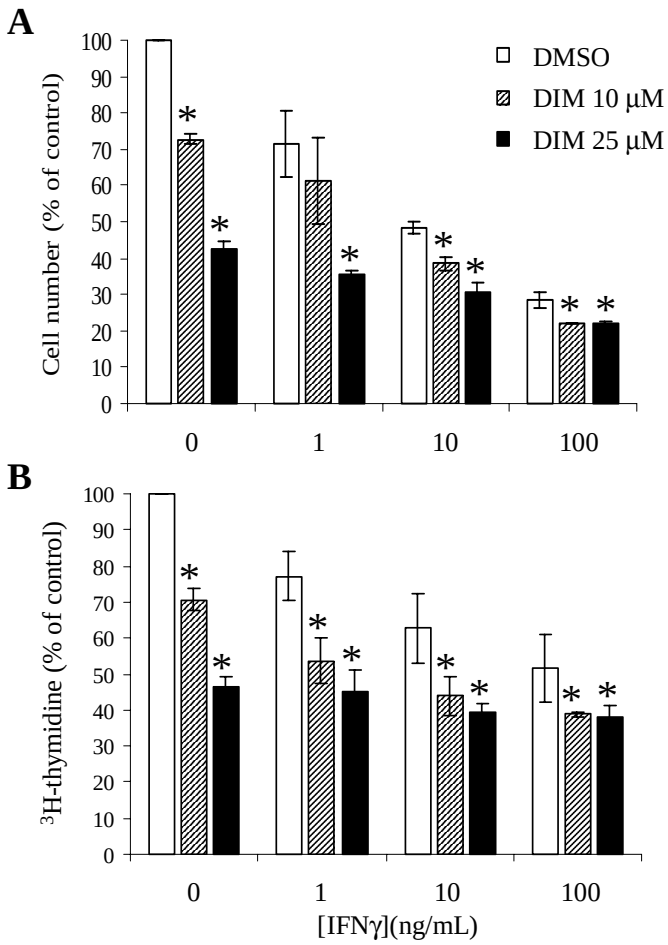

Figure 7

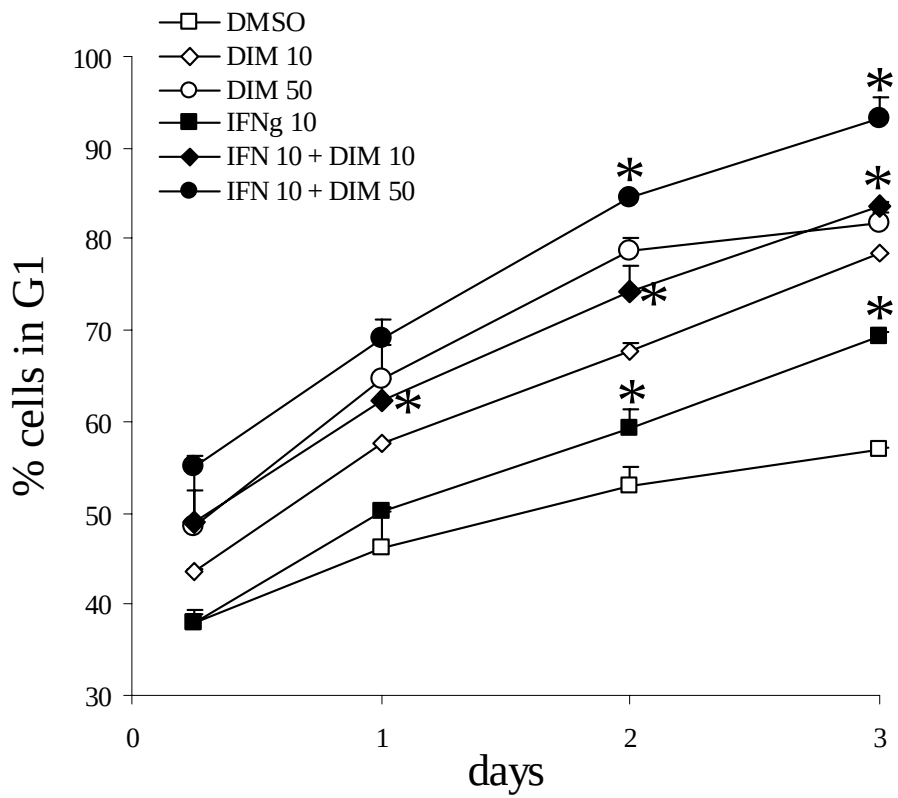

Figure 8

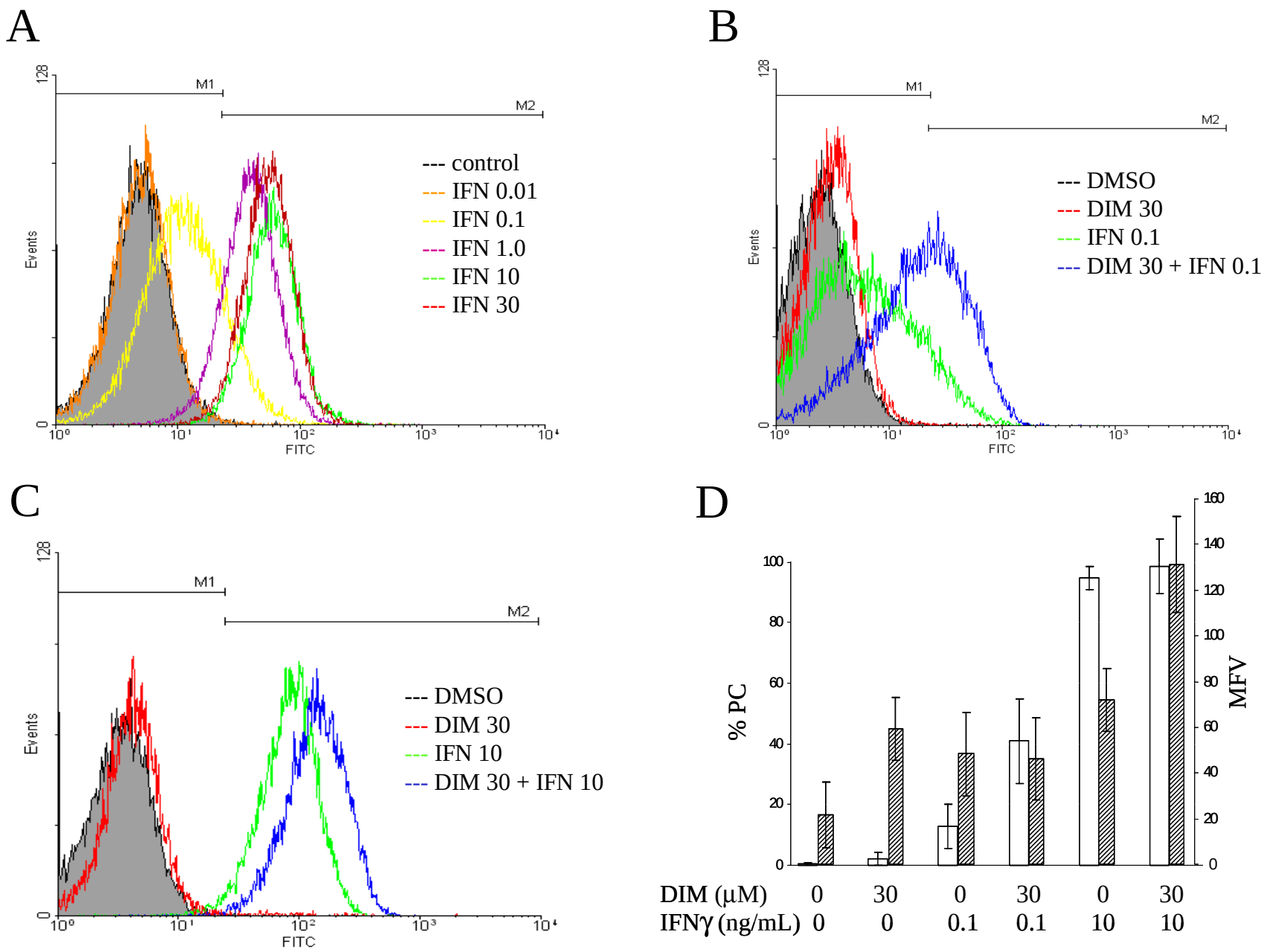

Figure 9