Regulation of Interferon-Inducible Proteins by Doxorubicin via Interferon γ-Janus Tyrosine Kinase-Signal Transducer and Activator of Transcription Signaling in Tumor Cells[S]

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ABSTRACT

Activation of the immune system is a way for host tissue to defend itself against tumor growth. Hence, treatment strategies that are based on immunomodulation are on the rise. Conventional cytostatic drugs such as the anthracycline doxorubicin can also activate immune cell functions of macrophages and natural killer cells. In addition, cytotoxicity of doxorubicin can be enhanced by combining this drug with the cytokine interferon-γ (IFNγ). Although doxorubicin is one of the most applied cytostatics, the molecular mechanisms of its immunomodulation ability have not been investigated thoroughly. In microarray analyses of HeLa cells, a set of 19 genes related to interferon signaling was significantly overrepresented among genes regulated by doxorubicin exposure, including signal transducer and activator of transcription (STAT) 1 and 2, interferon regulatory factor 9, N-myc and STAT interactor, and caspase 1. Regulation of these genes by doxorubicin was verified with real-time polymerase chain reaction and immunoblotting. An enhanced secretion of IFNγ was observed when HeLa cells were exposed to doxorubicin compared with untreated cells. IFNγ-neutralizing antibodies and inhibition of Janus tyrosine kinase (JAK)-STAT signaling [aurintricarboxylic acid (ATA), (E)-2-cyano-3-(3,4-dihydrophenyl)-N-(phenylmethyl)-2-propenamide (AG490), STAT1 small interfering RNA] significantly abolished doxorubicin-stimulated expression of interferon signaling-related genes. Furthermore, inhibition of JAK-STAT signaling significantly reduced doxorubicin-induced caspase 3 activation and desensitized HeLa cells to doxorubicin cytotoxicity. In conclusion, we demonstrate that doxorubicin induces interferon-responsive genes via IFNγ-JAK-STAT1 signaling and that this pathway is relevant for doxorubicin's cytotoxicity in HeLa cells. Immunomodulation is a promising strategy in anticancer treatment, so this novel mode of action of doxorubicin may help to further improve the use of this drug among different types of anticancer treatment strategies.

Introduction

Since its discovery in 1969, doxorubicin has been one of the most effective anticancer drugs used in a wide variety of malignancies (Di Marco et al., 1969). Despite its longstanding clinical application, the mechanism of action of doxorubicin has not yet been completely revealed. At the cellular level, the cytotoxic effects of doxorubicin involve direct DNA intercalation with impaired DNA and RNA synthesis, topoisomerase II inhibition resulting in DNA strand breaks, generation of free oxygen radicals, lipid peroxidation, alterations in membrane structure and function (Minotti et al., 2004) as well as induction of apoptosis (Gamen et al., 1997). As early as 1973, Schwartz and Grindey proposed that immune defense mechanisms of the host against tumor antigens may contribute to the effectiveness of doxorubicin. This suggestion was later proven by several groups who showed that doxorubicin induces specific immune functions and cytokine expression, which leads to increased tumoricidal potential of cells of the macrophage and natural killer type in mice (Eh-

ABBREVIATIONS: IL, interleukin; IFN, interferon; PCR, polymerase chain reaction; ATA, aurintricarboxylic acid; Dox, doxorubicin; AG490, (E)-2-cyano-3-(3,4-dihydrophenyl)-N-(phenylmethyl)-2-propenamide; siRNA, small interfering RNA; JAK, Janus tyrosine kinase; STAT, signal transducer and activator of transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; qRT-PCR, quantitative RT-PCR; IRF, interferon regulatory factor; ISGF3G, interferon-stimulated gene factor 3γ; IFI, interferon-inducible protein; IFIT4, interferon-induced protein with tetratricopeptide repeats; IFITM1, interferon-induced transmembrane protein 1; NMI, N-myc and STAT interactor; NK, natural killer.

rke et al., 1984; Maccubbin et al., 1992). Ujhazy et al. (2003) could demonstrate that the greater antitumor potential of peritoneal cells from doxorubicin-treated mice is accompanied by enhanced production of cytokines such as tumor necrosis factor, IL-1, and interferon-γ (IFNγ). Subsequently, it was shown that cytotoxic lymphocytes used in conjunction with doxorubicin are effective in treating murine renal cell cancer (Salup et al., 1987). In addition, in patients receiving doxorubicin, increased cytotoxic T-cell function, IL-2 levels, immature monocyte count, and CD8$^+$ T-cell count were found (Arinaga et al., 1986). However, knowledge about molecular mechanisms involved in the doxorubicin-induced immunomodulation caused by alterations on the tumor cells themselves is still very limited.

In addition to the immunomodulatory effects of doxorubicin described above, an enhancement of the antitumoral efficacy of doxorubicin can be achieved by its combination with the cytokine IFNγ without increased toxicity (Eppstein et al., 1988). IFNγ itself is well known to play crucial roles in several aspects of the immune response or immunomodulation as well as in immunosurveillance of malignant cells (Kaplan et al., 1998). Considering immune regulatory roles of IFNγ, doxorubicin-induced immunomodulation, and the synergistic antitumoral effect of doxorubicin and IFNγ, we performed transcriptome analyses in the tumor cell line HeLa to investigate whether doxorubicin influences expression of genes with immune function. Using this microarray approach, we revealed upregulation of a cluster of interferon-regulated genes with partially known immunomodulatory functions that was verified by real-time PCR and immunoblotting and compared with tumor cells of different origin. Pretreatment of cells with aurintricarboxylic acid (ATA) and (*E*)-2-cyano-3-(3,4-dihydrophenyl)-*N*-(phenylmethyl)-2-propenamide (AG490), inhibitors of JAK-STAT signaling (Wang et al., 1999; Chen et al., 2002), as well as neutralizing antibodies against IFNγ and IFNγ receptor 1 and silencing of STAT1 by the use of siRNA reduced the upregulation of most of the investigated genes. In addition, we demonstrate that doxorubicin treatment results in enhanced secretion of IFNγ as well as JAK1 and STAT1 activation. Therefore, we conclude that the IFNγ-JAK1-STAT1 cascade mediates doxorubicin-induced regulation of interferon-inducible genes and immunomodulatory effects that are associated with the administration of this cytostatic drug.

Materials and Methods

Chemicals and Antibodies. Doxorubicin hydrochloride, ATA, and recombinant human IFNγ were obtained from Sigma-Aldrich (Deisenhofen, Germany). AG490 was purchased from Calbiochem (Darmstadt, Germany). Trypsin and RPMI 1640 medium were from PAN (Aidenbach, Germany), and fetal bovine serum was obtained from Invitrogen (Carlsbad, CA). Neutralizing anti-human IFNγ receptor chain 1 and neutralizing anti-human INFγ were from Biomol (Hamburg, Germany). The following unconjugated primary antibodies were used for immunoblotting: anti-STAT1, anti-caspase 1 (both Santa Cruz Biotechnolofy, Santa Cruz, CA), anti-IFI35, anti-NMI, anti-ISGF3G (all from Abnova, Heidelberg, Germany), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Biodesign Int., Saco, ME), and for detection of phosphorylated forms of STAT1, STAT2, and STAT3 the Phospho-STAT Antibody Sampler Kit (Cell Signaling Technology, Frankfurt am Main, Germany) was used.

Cell Culture. The human cervix carcinoma cell line HeLa, the colon carcinoma cell line Caco2, the breast cancer cell line MCF-7, and the hepatocarcinoma cell line HepG2 were obtained from the American Type Culture Collection (Manassas, VA) and maintained by culture in RPMI 1640 medium or Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. For expression analysis, cells were seeded 24 h before drug treatment in six-well plates (3 × 10^5 cells per well) and were cultured for the respective time points with doxorubicin or IFNγ, with DMSO or phosphate-buffered saline (PBS) as solvent. In some experiments, cells were preincubated for 1.5 h with ATA, AG490, or neutralizing antibodies.

Transcriptional Profiling Experiments. Total RNA of one batch of doxorubicin-treated and untreated (DMSO) HeLa cells for two timepoints (24 and 48 h) was extracted using TRIzol PeqGold RNA Pure (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. T7-RNA polymerase-mediated linear amplification was performed starting with 1 μg of RNA (One cycle Target labeling protocol; Affymetrix, Santa Clara, CA). Size-distribution of biotin-labeled amplified RNA was analyzed with an Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA), and concentrations were photometrically determined. For transcriptome analysis, Human Genome U133 Plus 2.0 Arrays (Affymetrix) were used. After hybridization and washing, arrays were scanned using the GeneChip System Confocal Scanner 3000 (Affymetrix). Expression data have been submitted to the Gene Expression Omnibus.

Gene Chip operating software with MAS5.0 was used for data extraction. Expression raw data were transferred to GeneSpring GX version 7.3.1. (Agilent Technologies) and per-chip normalized. Genes that were flagged as present or marginal and exceeding a fold change of 2 compared with control were analyzed in Ingenuity Pathway Analysis release 8.5 (Ingenuity Systems, Redwood City, CA) for functional classification.

Quantitative Real-Time PCR. After treatment with the respective substances, cells were harvested, and total RNA was isolated using the PeqGold RNAPure reagent protocol. Five hundred nanograms of total RNA was used for cDNA synthesis in a 20-μl reaction volume. Real-time PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). cDNA was amplified using Assays on Demand for STAT1 (Hs00234829_m1), STAT2 (Hs00237139_m1), IFI6 (Hs00242571_m1), IFI27 (Hs00271467_m1), IFI30 (Hs00173838_m1), IFI35 (Hs00413458_m1), IFIT4 (Hs00382744_m1), IFITM1 (Hs01652522_g1), ISGF3G/IRF9 (Hs00196051_m1), NMI (Hs00190768_m1), and caspase 1 (Hs00354832_m1), all conjugated with fluorochrome 5-carboxyfluorescein and 18S rRNA (Predeveloped TaqMan Assay Reagent) conjugated with fluorochrome VIC (Applied Biosystems). Quantitation was performed with the comparative $\Delta\Delta C_T$-method.

Immunoblot Analysis. After treatment with the respective substances, cells were solubilized in lysis buffer, pH 7.4, consisting of 1% Triton X-100, 100 mM NaCl, 50 mM Tris, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 mM aprotinin. The amount of protein was determined by the bicinchoninic acid method using bovine serum albumin. Fifty micrograms of protein was separated on a 10% SDS-polyacrylamide gel electrophoresis followed by electrophoretic transfer to nitrocellulose membranes. The membranes were blocked using 5% nonfat milk in 150 mM NaCl, 10 mM Tris, pH 8.0 with 0.05% Tween 20 for 1 h followed by incubation with the primary antibodies for 1 h at room temperature (anti-GAPDH or overnight at 4°C (anti-STAT1, anti-caspase 1, anti-NMI, anti-IFI35, anti-ISGF3G or anti-phosphoSTATs; see *Chemicals and Antibodies*). After washing, the membranes were incubated with peroxidase-conjugated secondary antibodies and developed using enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Determination of JAK1 Activation. For measurement of active JAK1, we used the JAK1 phospho (Tyr1022/Tyr1023) fast-activated cell-based ELISA Kit (FACE; Active Motif, Carlsbad, CA).

Determination of Transcriptional Binding Activity of Nuclear STAT1. For assessment of binding ability of nuclear STAT1 to

its respective consensus sequence, we used the DuoSet IC STAT1 p91 activity assay (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol with preparation of nuclear extracts. Activity values were normalized to the nuclear protein content.

Measurement of IFNγ. Detection of IFNγ in supernatants of HeLa cells was performed by using the human IFNγ Quantikine ELISA kit (R&D Systems) according to the manufacterer's protocol. Values were normalized to the respective protein content of HeLa lysates.

siRNA-Mediated Silencing of STAT1 Expression. For siRNA transfection, cells were seeded at 75×10^3 cells/well in a 12-well multiplate. After reaching a confluence of 70 to 80%, cells were transfected with either SignalSilence Control siRNA (100 nM) or the specific SignalSilence Stat1 siRNA (100 nM; both from Cell Signaling Technology) by using the Lipofectamine RNAiMAX reagent according to the manufacturer's protocol. Forty-eight hours later, cells were treated with either DMSO (solvent) or 1 μM doxorubicin for a further 30 h followed by RNA isolation of the harvested cells, reverse transcription and qRT-PCR.

Assays for Caspase 3 Activity. Caspase 3 activity was measured in cell lysates with a commercially available Colorimetric assay kit (R&D Systems) according to the manufacturer's instructions. Activity was normalized to the respective protein content and is shown as fold increase relative to DMSO control.

Cell Viability Assay. HeLa cells were seeded at 5×10^3 cells/well in a 96-well multiplate. The growth medium was changed after 1 day and replaced with DMSO- or doxorubicin-containing media with or without preincubation with ATA or AG490. After the respective incubation period, 1/10 of Alamar Blue (BioSource International, Camarillo, CA) was added, and analysis was performed according to the manufacturer's instructions. Data were expressed as percentage of viability of DMSO-treated control cells.

Statistical Analysis. Data represent at least three independent experiments and were expressed as the mean ± S.D. Data were analyzed using Student's *t* test or one-way analysis of variance. Statistical significance was defined as $p < 0.05$.

Results

Functional Categorization of Differentially Expressed Genes upon Doxorubicin Treatment. For screening of changes at the transcriptome level, we have performed comparative DNA microarray analysis of control and doxorubicin (1 μM)-treated HeLa cells. After doxorubicin application for 48 h, 2102 regulated genes were identified (at 24 h, 1316 genes), displaying at least a 2-fold change. Functional classification of regulated genes at 48 h by Ingenuity pathway analysis revealed the following most over-represented categories: 1) interferon signaling; 2) hepatocyte growth factor signaling; 3) cell division control protein 42 homolog signaling; 4) activation of interferon regulatory factor (IRF) signaling by cytosolic pattern recognition receptors; and 5) production of nitric oxide and reactive oxygen species in macrophages (Fig. 1, A and B; Supplemental Table 1). Obviously, many of the identified genes are related to immunomodulation and/or play a role in interferon signaling. In detail, among those genes related to interferon signaling, 3 genes were down-regulated and 16 were up-regulated. Likewise, among those genes related to activation of IRF signaling, 6 genes were down-regulated and 24 were up-regulated (Supplemental Table 1).

Effect of Doxorubicin, IFNγ or Doxorubicin + IFNγ on IFN Signaling Regulatory Genes. Because IFN-dependent signaling pathways were strongly affected upon doxorubicin (1 μM) treatment, we analyzed regulation of selected

genes of this functional category in detail. Expression data for these genes, determined by microarray analysis of HeLa cells after treatment with doxorubicin for 24 or 48 h, are displayed in Supplemental Table 2.

For independent validation of the genes with potential regulatory function in interferon signaling and immunomodulation, we performed qRT-PCR analyses of the selected 11 genes that were differentially expressed in the presence of doxorubicin in our microarray analysis. When HeLa cells were incubated with 1 μM doxorubicin, mRNA expression increased in a time-dependent manner, with a maximum at 48 h for most of the investigated genes except NMI, IFI35 (maxima at 24 h), and IFI27 (maximum at 72 h) (Fig. 1C; Supplemental Table 2). Furthermore, qRT-PCR assays demonstrated that the mRNA levels of all selected genes were dose-dependently up-regulated by IFNγ with the exception of IFI30, which was slightly reduced upon 50 U/ml IFNγ and unchanged upon 100 U/ml IFNγ (Supplemental Fig. 1).
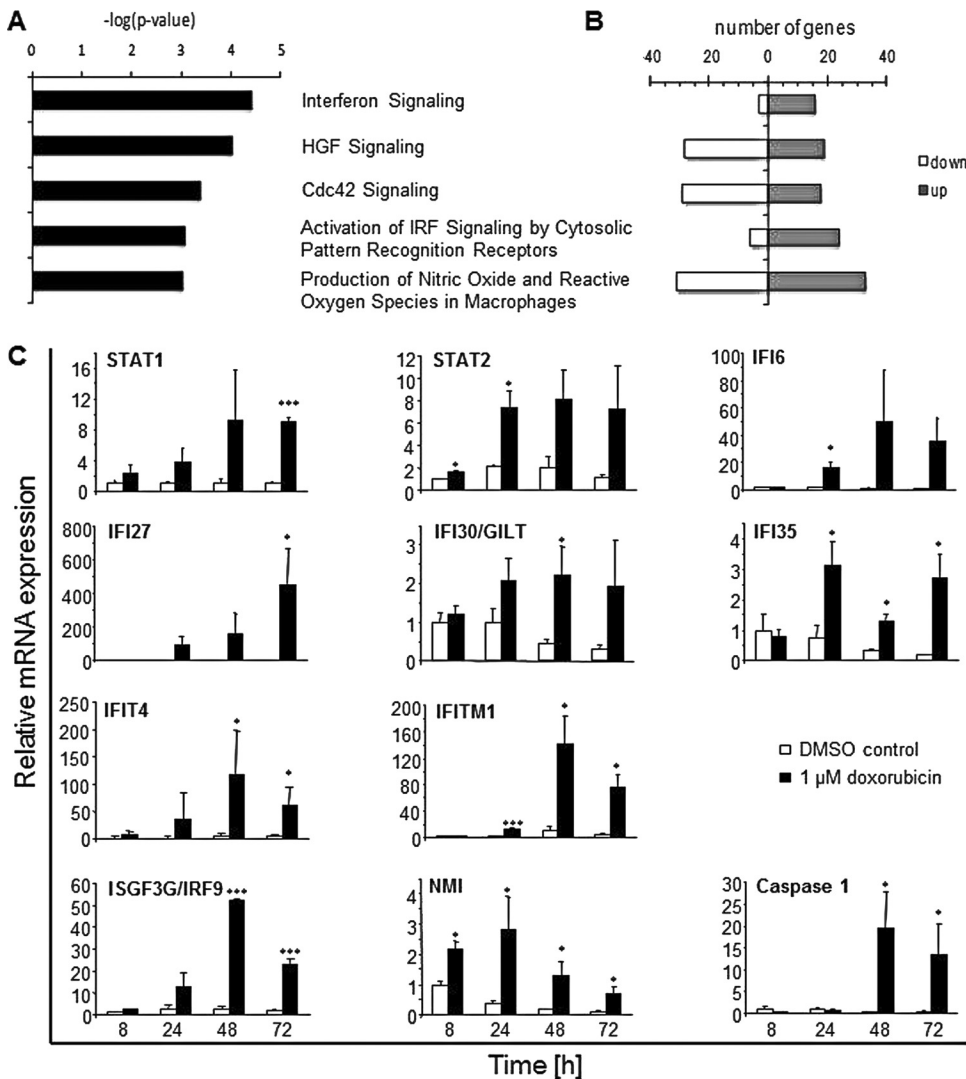
Furthermore, we checked the consequence of coadministration of doxorubicin and IFNγ on mRNA expression of the genes of interest (Table 1). For all genes investigated, the combination of doxorubicin and IFNγ resulted in significantly higher mRNA expression than each of the compounds alone.

Release of IFNγ upon Doxorubicin Exposure and Effects of Neutralizing anti-IFNγ and anti-IFNγRI Antibodies. Because IFNγ functions as a secreted cytokine by binding to its specific receptors, we tested whether exposure of HeLa cells with doxorubicin results in the release of IFNγ into the supernatant, too. As shown in Fig. 2A, within 4 h of doxorubicin exposure, the release of IFNγ into supernatant was significantly enhanced from 15.8 ± 0.62 (control) to 23.5 ± 2.89 pg/mg of protein. Furthermore, using immunoblot analysis we were able to detect the IFNγ receptors 1 (54 kDa) and 2 (37 kDa) in HeLa as well as in Caco2, HepG2, and MCF-7 cells (Fig. 2D).

To determine whether doxorubicin triggers gene expression via the IFNγ/IFNγ-receptor pathway, we performed inhibition experiments for selected genes using neutralizing antibodies against IFNγ and IFNγR1 (raised against the IFNγ receptor subunit 1) followed by RNA extraction and qRT-PCR. As shown in Table 2, addition of neutralizing antibodies 1.5 h before doxorubicin application resulted in significantly diminished doxorubicin-mediated mRNA induction of the investigated genes, with the exception of STAT1, STAT2, and IFI30. While doxorubicin-induced STAT1 and IFI30 mRNA expression was reduced by the anti-IFNγ antibody only, STAT2 mRNA expression was not influenced by either of the neutralizing antibodies. As expected, IFNγ-mediated induction of selected immunomodulatory genes in HeLa cells was also clearly and significantly inhibited by the use of the neutralizing IFNγ antibody (data not shown).

Involvement of the JAK1-STAT1 Pathway in the Transcriptional Response to Doxorubicin. It is known that IFNγ signaling involves activation of JAKs and STATs such as JAK1 and STAT1. To test whether treatment with IFNγ (50 U/ml) or doxorubicin (1 μM) resulted in activation of JAK1 as primary target kinase of the interferon receptors in our HeLa cell model, we performed JAK1-specific ELISA. Using this assay, we were able to determine both total and phosphorylated JAK1 protein in HeLa cells. Although no changes in regulation of total JAK1 were observed (data not

A
-log(p-value)

Interferon Signaling

HGF Signaling

Cdc42 Signaling

Activation of IRF Signaling by Cytosolic Pattern Recognition Receptors

Production of Nitric Oxide and Reactive Oxygen Species in Macrophages

B
number of genes

□ down
■ up

C

Relative mRNA expression

STAT1

STAT2

IFI6

IFI27

IFI30/GILT

IFI35

IFIT4

IFITM1

ISGF3G/IRF9

NMI

Caspase 1

□ DMSO control
■ 1 μM doxorubicin

Time [h]

Fig. 1. Functional categorization of differentially expressed genes identified in HeLa cells upon doxorubicin treatment for 48 h. Functional classification was performed for genes displaying ≥2-fold change in doxorubicin-treated compared with control cells (DMSO) by Ingenuity pathway analysis. A, display of categories over-represented among the genes exhibiting differential expression in doxorubicin versus DMSO treated cells (p-value <0.001). B, number of repressed and induced genes. C, time dependence of transcriptional regulation of IFN-inducible genes by doxorubicin. HeLa cells were treated with DMSO (solvent) or 1 μM doxorubicin for the indicated times and RNA was extracted, reverse-transcribed, and analyzed by qRT-PCR Mean + S.D., $n = 3$, *, $p < 0.05$; ***, $p < 0.001$.

TABLE 1

Effects of doxorubicin and IFNγ either alone or in combination on the expression levels of IFNγ-regulated genes

HeLa cells were incubated for 48 h with DMSO, PBS, 1 μM doxorubicin, 50 U/ml IFNγ, or 1 μM doxorubicin plus 50 U/ml IFNγ. Total RNA was extracted, reverse transcribed, and analyzed by qRT-PCR. Presented values are normalized to the respective control (DMSO or PBS). Mean ± S.D., $n = 3$.

	Dox	IFNγ	Dox + IFNγ
STAT1	3.99 ± 0.95*	7.57 ± 1.10**	16.52 ± 5.87#
STAT2	7.99 ± 1.97*	3.14 ± 0.55**	58.89 ± 9.97#
IFI6	85.86 ± 20.74*	45.28 ± 3.28***	551.16 ± 58.39###
IFI27	1648 ± 359*	98 ± 142**	9424 ± 2036#
IFI30/GILT	3.52 ± 0.12***	0.41 ± 0.50	5.56 ± 0.48###
IFI35	9.12 ± 2.81*	7.23 ± 1.86*	35.82 ± 6.08##
IFIT4	384.81 ± 141*	122.41 ± 11.35***	1652.16 ± 162###
IFITM1	4.73 ± 0.81*	2.55 ± 0.52*	37.51 ± 10.28#
ISGF3G/IRF9	8.11 ± 0.70***	7.76 ± 2.58*	20.57 ± 7.33
NMI	5.45 ± 1.71*	14.77 ± 2.88*	23.74 ± 4.23##
Caspase 1	4.2 ± 0.46***	1.42 ± 0.11*	8.77 ± 2.99

* $P < 0.05$ versus respective control.
** $P < 0.01$ versus respective control.
*** $P < 0.001$ versus respective control.
# $P < 0.05$ versus doxorubicin.
$P < 0.01$ versus doxorubicin.
$P < 0.001$ versus doxorubicin.

shown), JAK1 was clearly phosphorylated 1 h after IFNγ application and 4 h after doxorubicin treatment (Fig. 2B). This increase in phosphorylated JAK1 induced by doxorubicin and IFNγ was significantly diminished in the presence of ATA (30 μM), an inhibitor of JAK-STAT signaling (Fig. 2B).

JAK1-regulated STAT1 phosphorylation is a prerequisite for STAT dimerization, nuclear translocation, and finally gene activation. Therefore, we performed nuclear extraction and measured the phosphorylation of STAT1 on tyrosine 701, which is necessary for dimerization and nuclear transloca-
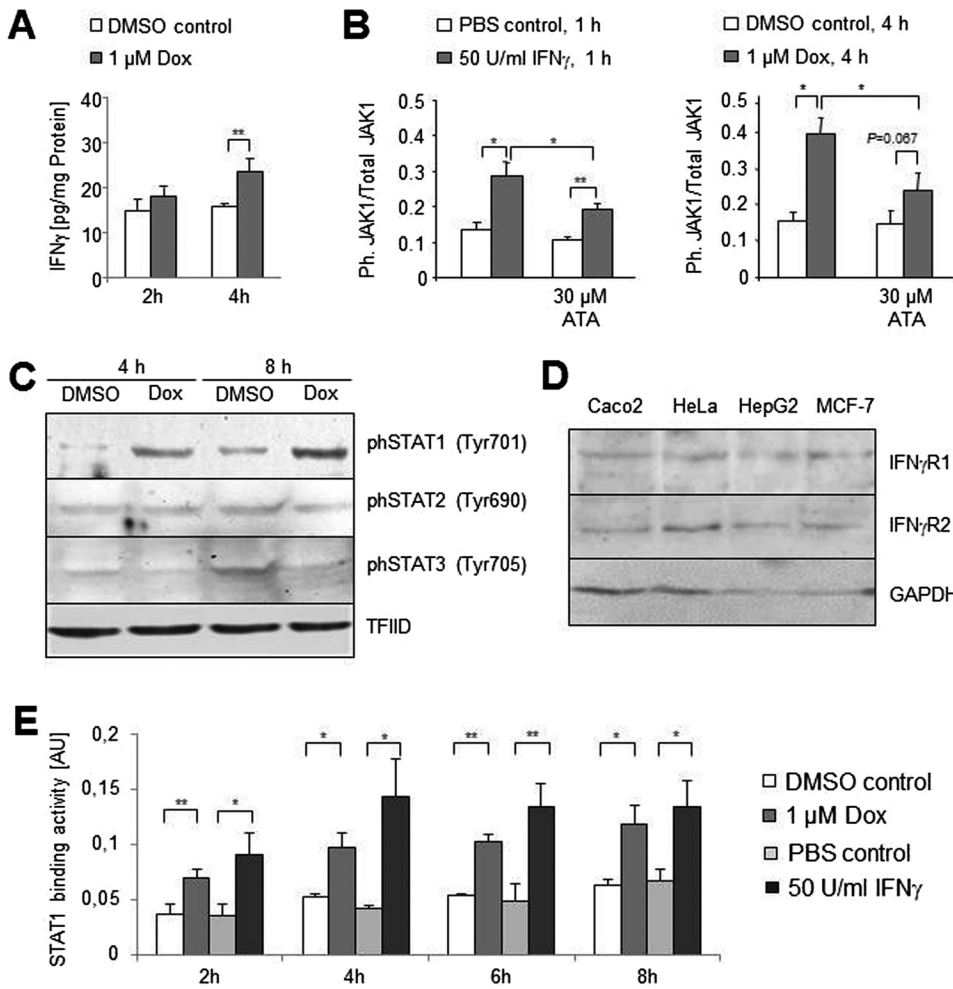
Fig. 2. Involvement of the IFNγ-JAK1-STAT1 pathway in the transcriptional response to doxorubicin. A, measurement of IFNγ secretion. HeLa cells were treated for 2 and 4 h with 1 μM doxorubicin, and the supernatant was used for determination of IFNγ levels with a specific ELISA. Mean ± S.D., $n = 3$; ∗, $p < 0.05$. B, determination of phospho-JAK1 and total JAK1 was performed in HeLa lysate using a specific ELISA. Cells were pretreated for 1.5 h with or without ATA followed by incubation with PBS, DMSO, 50 U/ml IFNγ or 1 μM doxorubicin (Dox) for the indicated time points. Mean ± S.D., $n = 3$; ∗, $p < 0.05$; ∗∗, $p < 0.01$. C, detection of phospho-STAT1, -2, and -3 was done by immunoblotting using nuclear extracts of DMSO or doxorubicin (Dox) treated HeLa cells. D, detection of the interferon-γ receptor system in Caco2, HeLa, HepG2, and MCF-7 cells by immunoblot analysis. E, assessment of transcriptional binding activity of nuclear STAT1 to its specific consensus sequence. Mean ± S.D., $n = 3$; ∗, $p < 0.05$; ∗∗, $p < 0.01$. AU, arbitrary units.

TABLE 2

Involvement of the IFNγ-JAK1-STAT1 pathway in the transcriptional response to doxorubicin

HeLa cells were pretreated for 1.5 h with or without neutralizing antibodies against IFNγ or INFγ receptor 1, ATA (30 μM), or AG490 (50 μM) followed by treatment with 1 μM doxorubicin for 48 h. RNA was extracted, reverse-transcribed, and analyzed by qRT-PCR. Gene expression is calculated as the ratio of 18S normalized fluorescence intensities measured in the respective DMSO controls. Gene expression levels upon doxorubicin exposure was set to 100%, and assays containing inhibitors were normalized to this value. Mean ± S.D., $n = 3$.

Percentage of Dox	Dox + Anti-IFNγ-Ab	Dox + Anti-IFNγR1-Ab	Dox + 30 μM ATA	Dox + 50 μM AG490
STAT1	48.3 ± 3.0***	83.4 ± 23.5	178.5 ± 32.8	41.1 ± 4.8*
STAT2	86.2 ± 39.1	88.5 ± 43.9	61.9 ± 4.6**	53.0 ± 16.0*
IFI6-16	43.3 ± 16.8*	62.7 ± 14.4*	31.7 ± 2.7*	29.5 ± 7.8*
IFI30	43.9 ± 20.8*	55.5 ± 28.1	42.1 ± 17.9*	27.8 ± 7.3*
IFI35	34.6 ± 2.8*	56.2 ± 13.6*	22.0 ± 2.1**	48.0 ± 13.2*
IFITM1	23.0 ± 7.1*	32.7 ± 6.3*	37.0 ± 8.8*	48.7 ± 9.6**
ISGF3G/IRF9	36.3 ± 10.2**	39.4 ± 17.2*	63.4 ± 30.5*	195 ± 45.7
NMI	35.5 ± 12.6**	46.9 ± 9.2*	41.5 ± 5.6*	39.2 ± 5.9*
Caspase 1	24.2 ± 12.4*	45.6 ± 5.2*	32.6 ± 8.3**	74.2 ± 5.0*

* $P < 0.05$ versus doxorubicin.
** $P < 0.01$ versus doxorubicin.
*** $P < 0.001$ versus doxorubicin.

tion. Results shown in Fig. 2C clearly demonstrate that nuclear extracts obtained from doxorubicin-treated cells after 4 and 8 h contained higher amounts of tyrosine-phosphorylated STAT1 than those from DMSO-treated control cells. In contrast, phosphorylation of STAT2 and STAT3 was not affected by doxorubicin treatment. In addition, the transcriptional binding activity of nuclear STAT1 to the respective consensus sequence was significantly enhanced approximately 2-fold after treatment of HeLa cells with doxorubicin (Fig. 2E). Application of IFNγ also resulted in a clearly am-

plified and significant STAT1 binding activity, serving as a kind of positive control. An unlabeled competitor oligonucleotide demonstrated the specificity of the assay (data not shown).

To further analyze whether the doxorubicin-mediated regulation actually involves the activation of JAK-STAT cascade, we performed inhibition experiments with AG490 and ATA as inhibitors of JAK-STAT signaling (Wang et al., 1999) and determined the mRNA content of selected genes by real-time RT-PCR. AG490 pretreatment significantly suppressed

doxorubicin-induced gene expression with the exception of ISGF3G. Pretreatment with ATA also resulted in a significantly diminished doxorubicin-mediated induction of all investigated genes with the exception of STAT1 (Table 2).

In addition, we investigated the effect of ATA and AG490 on doxorubicin-mediated changes of caspase 1, STAT1, NMI, IFI35, and ISGF3G levels by immunoblot analysis (Fig. 3A). Densitometric analysis of protein levels is shown in Fig. 3B and demonstrates that treatment with doxorubicin for 48 h triggered significant increases in levels of STAT1 (DMSO, 0.20 ± 0.14, versus Dox, 2.35 ± 0.40), NMI (DMSO, 0.13 ± 0.06; versus Dox, 0.84 ± 0.11), ISGF3G (DMSO, 0.14 ± 0.10; versus Dox, 0.58 ± 0.13) and of caspase 1 (DMSO, 0.43 ± 0.40; versus Dox, 1.48 ± 0.44). For IFI35, protein levels did not significantly change (DMSO, 0.48 ± 0.03; versus Dox, 0.91 ± 0.65) despite significantly increased mRNA levels. Pretreatment with AG490 and ATA significantly reduced the doxorubicin-mediated increases of STAT1 and caspase 1 protein levels. For NMI, only pretreatment with ATA prevented increase in protein levels upon doxorubicin, and no significant influences of either AG490 or ATA were seen on doxorubicin-mediated effects on IFI35 and ISGF3G. The findings above suggest that doxorubicin is a potent activator of the JAK1-STAT1 pathway, resulting in downstream regulation of interferon-responsive genes, which is in part also reflected on the protein level.

To further support the role of STAT1 in doxorubicin-mediated signaling and gene induction, we decided to knock down STAT1 expression by using a specific siRNA against STAT1. After 78 h, STAT1 expression was silenced to approximately 25% in DMSO-treated cells and to 43% in doxorubicin-exposed cells in STAT1 siRNA transfected cells compared with transfection with a nontargeting control siRNA. In addition, with the exception of IFI30, the doxorubicin-mediated induction of NMI, caspase 1, IFI6, and IFI27 was clearly reduced in STAT1-silenced cells but did not reach the control level maybe because the remaining STAT1 expression is sufficient to mediate the transcriptional activation of the genes.

Effect of ATA and AG490 on Doxorubicin-Mediated Cell Death. To test whether the doxorubicin-mediated changes have any consequences for cell viability, we performed an Alamar Blue assay and determined caspase 3 activity as a marker of apoptotic cell death.

As shown in Fig. 4A, cytotoxicity of doxorubicin exposure for 72 h resulted in cell viability of $41.9 \pm 3.3\%$. However, when HeLa cells were pretreated with 30 and 50 μM ATA, cell viability significantly increased to 73 ± 5.3 and $82 \pm 6.6\%$, respectively. In contrast, pretreatment with 30 and 50 μM AG-490 did not significantly reduce cytotoxicity of doxorubicin (39 ± 4.8 and $50 \pm 5.6\%$, respectively). In addition, treatment with IFNγ sensitized HeLa cells to doxorubicin-induced loss of cell viability ($41.9 \pm 3.3\%$ for doxorubicin and $24.2 \pm 3.8\%$ for doxorubicin plus IFNγ; Fig. 4A). In contrast, cells treated with IFNγ alone ($125.4 \pm 1.4\%$) seemed to even increase proliferation compared with untreated control cells.

To further analyze whether doxorubicin-triggered cell death indeed involves apoptosis, we measured the activity of caspase 3. Treatment of HeLa cells with doxorubicin alone resulted in significant activation of caspase 3. It is noteworthy that both ATA and AG-490 pretreatment of HeLa cells resulted in a significant reduction of doxorubicin-induced caspase 3 activity, as shown in Fig. 4B.
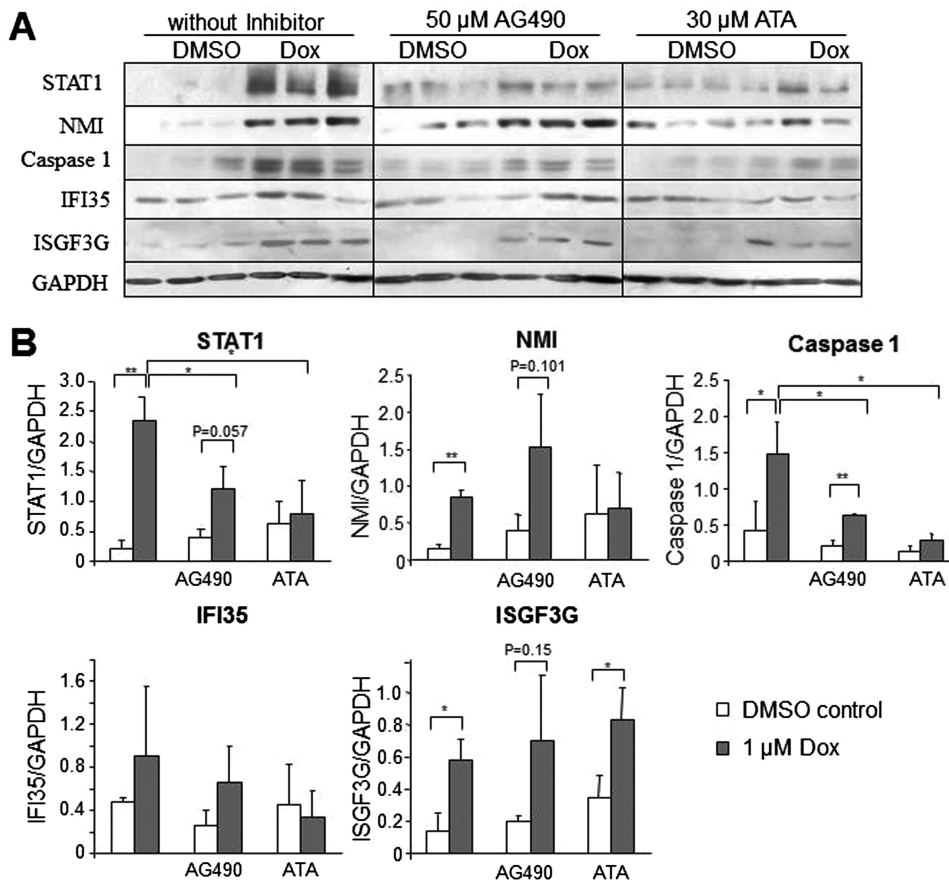
Fig. 3. Influence of ATA and AG490 on doxorubicin-induced changes on the protein level. HeLa cells were pretreated with NH$_4$OH (solvent), 30 μM ATA, or 50 μM AG490 for 1.5 h followed by treatment with 1 μM doxorubicin for 72 h and detection of caspase 1, NMI, IFI35, ISGF3G, STAT1, and GAPDH in whole-cell lysate by immunoblot analysis using specific antibodies. A, Western Blot signals. B, densitometric evaluation of protein levels with normalization to GAPDH as a housekeeping protein. Mean$\pm$ S.D., $n = 3$; *, $p < 0.05$; **, $p < 0.01$.
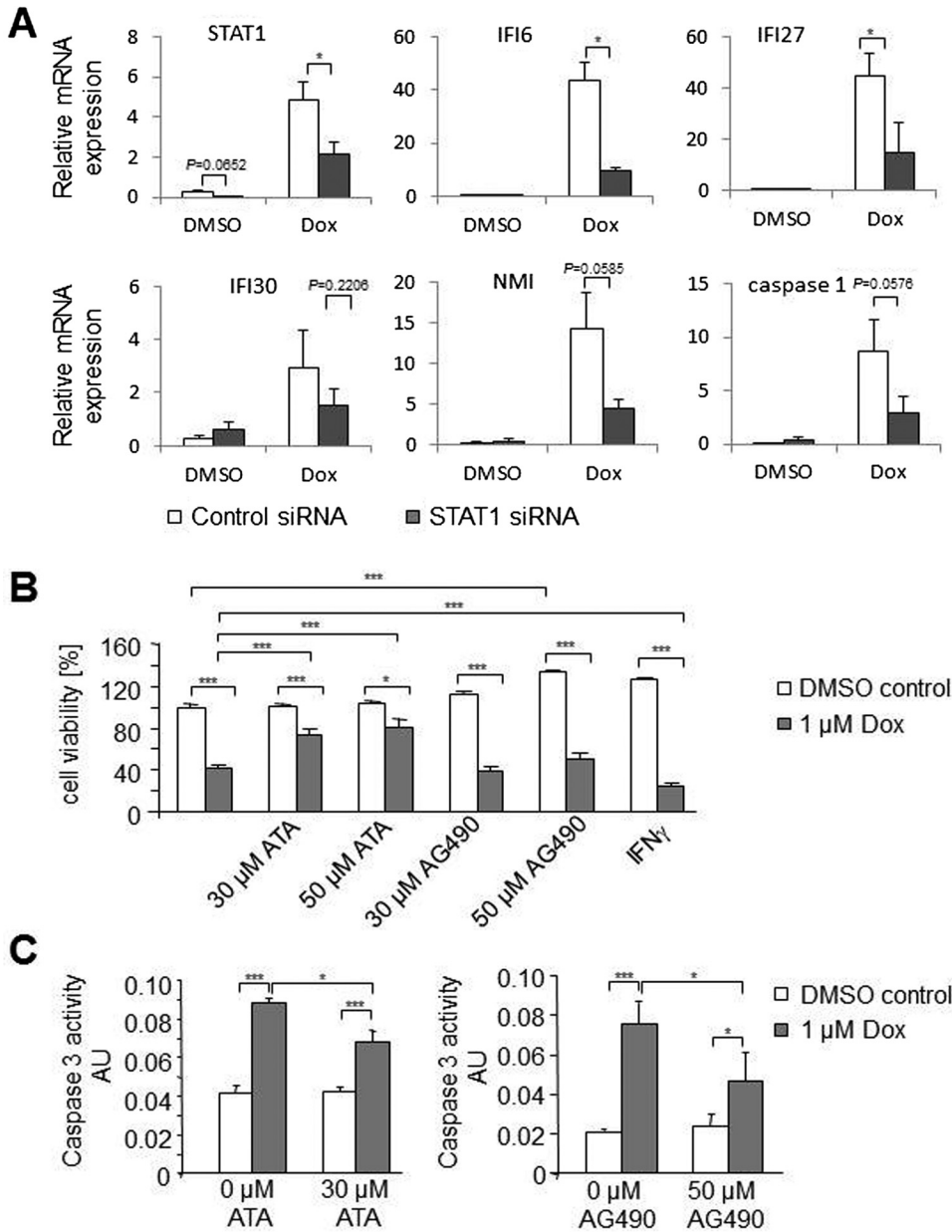
Fig. 4. siRNA-mediated silencing of STAT1 and effects of JAK/STAT blockade on cell viability and caspase 3 activity. A, doxorubicin-mediated gene regulation in STAT1 siRNA silenced cells determined by RT-PCR. Forty-eight hours after siRNA transfection, cells were incubated with 1 μM doxorubicin for a further 30 h. Mean $\pm$ S.D., $n = 3$ (B + C) HeLa cells were pretreated with ATA or AG490 for 1.5 h and then incubated with 1 μM doxorubicin for 72 h. B, cell viability was determined using AlamarBlue assay. Mean $\pm$ S.D., $n = 3$. C, caspase 3 activity was assayed using a commercially available colorimetric test and values were normalized to the protein content of each sample. Mean $\pm$ S.D., $n = 3$. *, $p < 0.05$; ***, $p < 0.001$. AU, arbitrary units.

Effect of Doxorubicin on IFNγ Secretion and Interferon-Inducible Genes in Tumor Cells of Different Origins. To analyze whether the regulation of interferon-inducible genes by doxorubicin is limited to HeLa cells, we further investigated three cell lines of different tumor entities, namely Caco2 (colon carcinoma), MCF-7 (breast cancer), and HepG2 (hepatocarcinoma). First, we checked IFNγ secretion into supernatant upon treatment with doxorubicin (1 μM) in these cells. As shown in Fig. 5A, doxorubicin treatment resulted in a significantly increased IFNγ secretion in all three tumor cell lines after 2 or 4 h.

Despite secretion of IFNγ upon doxorubicin and expression of the IFNγ receptor, the change in expression of the interferon-inducible genes IFI6, IFI30, and STAT1 48 h after doxorubicin application was somewhat different in the analyzed tumor cell lines (Caco2, HepG2, MCF-7) compared with that in HeLa cells (Fig. 5B). In contrast to the doxorubicin-induced up-regulation of STAT1 expression in HeLa cells,

up-regulation of STAT1 was not observed in HepG2, and even a reduced STAT1 mRNA expression was detected in Caco2 (0.5-fold) and MCF-7 cells (0.39-fold) (not statistically significant). This decrease in doxorubicin-mediated STAT1 expression was significantly amplified by pretreatment with the JAK/STAT inhibitor AG490 in MCF-7 cells (0.08-fold of DMSO control). Concerning IFI6, in MCF-7 cells, no significant regulation upon doxorubicin application was seen, whereas in Caco2 and HepG2 cells IFI6 expression was significantly increased 25.3-fold (Caco2) and 3.8-fold (HepG2) by doxorubicin. It is noteworthy that this increase in IFI6 expression was significantly reduced by pretreatment with the neutralizing IFNγ antibody only in Caco2 cells (3.4- compared with 25.3-fold for only doxorubicin) but not in HepG2 cells (4.4- compared with 3.8-fold for only doxorubicin). Furthermore, pretreatment with AG490 showed no effect on the doxorubicin-mediated up-regulation of IFI6 in both Caco2 (21.2- compared 25.3-fold for only doxorubicin) and HepG2
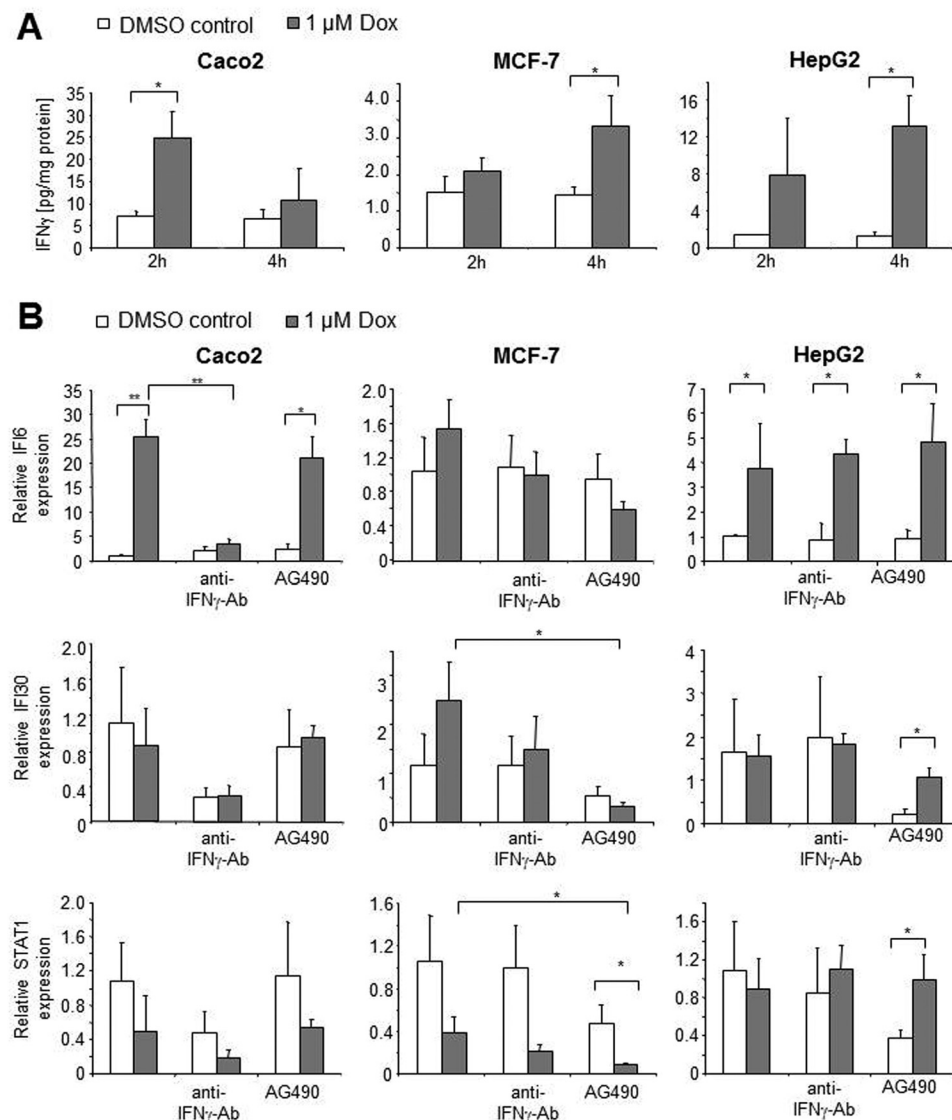
Fig. 5. Influence of doxorubicin on mRNA expression of interferon-inducible genes and IFNγ secretion in different tumor cell lines. A, Caco2 (colon carcinoma), MCF-7 (breast carcinoma), and HepG2 (hepatocarcinoma) cells were pretreated with anti-IFNγ antibody or 50 μM AG490 for 1.5 h and then incubated with 1 μM doxorubicin for 48 h. mRNA expression was analyzed with RT-PCR and normalization to 18S rRNA. B, measurement of IFNγ secretion. Cells were treated with 1 μM doxorubicin for 2 and 4 h, and the supernatant was used for determination of IFNγ levels with a specific ELISA. Mean ± S.D., $n = 3$; ∗, $p < 0.05$.

cells (4.8- compared with 3.8-fold for only doxorubicin), which is not in accordance with the results obtained in HeLa cells. IFI30 mRNA expression was slightly but not significantly elevated upon doxorubicin in MCF-7 cells (2.5-fold), whereas no obvious regulation was seen in Caco2 and HepG2 cells. Despite this, a decrease of IFI30 expression was observed after pretreatment with the neutralizing IFNγ antibody in Caco2 cells (to approximately 0.3-fold for both DMSO or doxorubicin) and after pretreatment with AG490 in MCF-7 (0.54-fold for DMSO and 0.3-fold for doxorubicin) and HepG2 cells (0.22-fold for DMSO) (Fig. 5B).

Discussion

Immune defense against tumors is complex and can be mediated by cells of the innate immune system, such as macrophages or natural killer (NK) cells, as well as by the adaptive immune system, including B and T cells (Bhardwaj, 2007). Hence, immune-based therapeutic strategies that target tumors use these effector cells, particularly because infiltration of malignant tumors with NK or T cells was associated with a better clinical outcome (Ishigami et al., 2000). Experimental studies have demonstrated that doxorubicin

can augment immune responses that involve macrophage and NK cell activity (Ujhazy et al., 2003). It is noteworthy that antitumoral efficiency of some cytostatics, including doxorubicin, could be enhanced by coadministration of IFNγ and an improved antitumor efficacy by coadministration of doxorubicin and IL-12 is dependent on the accumulation of IFNγ in tumors (Wadler and Schwartz, 1990; Zhu et al., 2007). To date, detailed studies on the effects of doxorubicin on the pattern of tumor cell gene expression linked to immunomodulation or interferon signaling are not reported. Therefore, the purpose of this study was to identify genes with potentially immunomodulatory functions regulated by doxorubicin, underlying regulatory mechanisms, and potential consequences for doxorubicin's efficiency.

Screening of the effect of doxorubicin on the gene expression profile of the cervix carcinoma cell line HeLa revealed up-regulation of interferon-inducible and immunomodulatory genes, including the interferon-inducible/stimulated proteins IRF1, IFI6, IFI27, IFI30, IFI35, IFITM1, IFIT4, ISGF3G, the transcription factors STAT1 and NMI, and the protease caspase 1. For all of these genes, an induction by interferons has previously been shown in different cell mod-

els. Results of the DNA microarray analysis were confirmed by RT-PCR and immunoblot analysis of selected genes. Although such regulatory effects have already been reported for STAT1 and caspase 1 (Gupta et al., 2001), regulation of the remaining genes upon doxorubicin is novel.

With the exception of STAT1 and caspase 1, the functions of most of the regulated genes in infection, inflammation, and innate immune system (Lamkanfi et al., 2007; Matsukawa, 2007) are to date only partly described. IFI35 and NMI are similarly induced by IFNγ, with IFI35 being stabilized by NMI (Zhou et al., 2000). NMI itself can potentiate STAT-dependent transcription (Zhu et al., 1999). Although no antitumoral function has been shown for IFI35 by now, NMI inhibits Wnt/β-catenin signaling and retards tumor growth (Fillmore et al., 2009). Hence, up-regulation of these interferon-induced proteins by doxorubicin could be involved in its antitumoral effect. Concerning therapy of malignancies, STAT1 is of potential interest because it mediates growth inhibitory signals and contributes to the host's rejection of tumors (Levy and Gilliland, 2000). Nuclear STAT1 was directly correlated with intratumoral T-cell levels in colorectal cancer accompanied by a better patient prognosis, suggesting that doxorubicin-mediated increase in STAT1 expression and nuclear localization could result in enhanced T-cell migration into the tumor and may lead to recognition of tumor cells by the immune system. Besides, doxorubicin potentiates STAT1 activation in response to IFNγ resulting in enhanced apoptosis in breast cancer cells (Thomas et al., 2004), which is also supported by our findings. Otherwise, it was shown that constitutive overexpression of STAT1 correlates with the selection of doxorubicin- and IFNγ-resistant tumor cells, raising the question of growth advantages for cells with increased STAT1 expression upon doxorubicin (Khodarev et al., 2009).

Because most genes shown to be regulated by doxorubicin are also stimulated by IFNγ via JAK-STAT signaling (Darnell, 1998), we investigated whether doxorubicin used this signaling axis for gene induction. Igarashi et al. (1994) demonstrated that binding of IFNγ to HeLa cells initiated a series of events that resulted in phosphorylation of not only JAKs but also the IFNγ receptor, suggesting fully intact IFNγ signaling in HeLa cells. Beside detection of the IFNγ receptor system, we were able to determine a substantial induction of IFNγ secretion upon doxorubicin treatment in HeLa cells. Until now, the doxorubicin-dependent increase in IFNγ has been described only for peritoneal cells of doxorubicin-treated mice (Ujhazy et al., 2003), not for tumor cells. Using neutralizing antibodies against the IFNγ cascade, we could show that regulation of all investigated genes beside STAT2 was dependent on IFNγ.

To date, four mammalian JAKs and seven STAT family members have been identified; they may be activated individually or in combination (Darnell, 1997; Leonard and O'Shea, 1998). Upon doxorubicin treatment, activation of JAK1 was observed in HeLa cells that was inhibited by ATA, underlining the role of ATA as JAK inhibitor. Furthermore, enrichment of nuclear phosphorylated STAT1 and increased binding to its consensus sequence was seen in HeLa cells after doxorubicin application, whereas no enhanced phosphorylation was seen for STAT2 and STAT3. These results indicate that in HeLa cells, doxorubicin treatment results in activation of IFNγ signaling and has no influence on IFNα/β

cascade, which would lead to phosphorylation of both STAT1 and STAT2. This notion is supported by the doxorubicin-mediated up-regulation of IFN-regulatory factor 1, which is preferentially induced by IFNγ, whereas hypoxia-inducible factor 1 is primarily induced by IFNβ (Der et al., 1998) and was not increased upon doxorubicin. However, one has to be aware of cell type-dependent differences in STAT1 signaling upon doxorubicin treatment, because STAT1 phosphorylation was not seen after doxorubicin exposure in the breast cancer cell line MDA-MB 435 (Thomas et al., 2004).

In HeLa cells, pretreatment with the JAK inhibitor AG490 significantly reduced doxorubicin-mediated gene induction with the exception of ISGF3G, and pretreatment with ATA, another inhibitor of JAK-STAT signaling, also resulted in a diminished induction of all investigated genes with the exception of STAT1. The discrepancy between the results obtained with ATA and AG490 could be based on different intervention in additional signaling pathways distinct from JAK-STAT signaling, which are in fact described in other cell models (Tsi et al., 2002; Kwak et al., 2008). However, the involvement of STAT1 in doxorubicin-mediated gene regulation was further evidenced by silencing STAT1 expression using a specific siRNA, resulting in a strong reduction of doxorubicin induced gene regulation.

To analyze whether regulation of interferon-inducible genes by doxorubicin is limited to HeLa cells, we further investigated three cell lines of different tumor entities: Caco2 (colon carcinoma), MCF-7 (breast cancer), and HepG2 (hepatocarcinoma). Despite an increased secretion of IFNγ upon doxorubicin in all three cell lines, the influence of doxorubicin on expression of IFI6, IFI30, and STAT1 was somewhat different compared with HeLa cells. Therefore, it seems that induction of IFNγ secretion is not cell type-specific, whereas activation of the downstream JAK-STAT cascade and regulation of interferon-inducible genes is dependent on the intracellular repertoire of signaling mediators, such as kinases or transcription factors. Therefore, immunomodulation upon doxorubicin treatment occurs in a cell-type-specific manner. Complementarily, it has to be noted that Zhu et al. (2007) could demonstrate a doxorubicin-mediated increased immigration of IFNγ-secreting immune cells into tumor and a role of IFNγ/STAT signaling of the invaded immune cells in tumor growth inhibition. This finding argues for a dual modulation of the IFNγ cascade in both immune and tumor cells, suggesting a potential interaction between these two cell types. However, in peripheral blood mononuclear cells from healthy volunteers, we could not find such a regulation of immunomodulatory genes upon doxorubicin treatment as seen in HeLa cells (data not shown).

The IFNγ-JAK-STAT signaling is described to induce cell inhibitory effects. Therefore, the effects of JAK-STAT inhibitors on doxorubicin-mediated cell death were investigated, yielding some interesting results. Whereas both inhibitors caused significantly reduced caspase 3 activation, only treatment with ATA improved cell viability upon doxorubicin treatment. In fact, for AG490 both pro- (Wu et al., 2009) and antiapoptotic (Zhao et al., 2009) mechanisms are described. In contrast, for ATA, only antiapoptotic actions are delineated (Cho et al., 2004), which is in agreement with the observations made in this study. Failure of AG490 to rescue viability of HeLa cells could be caused by its pleiotropic action [e.g., blocking not only JAK1 and -2 but also JAK3

signaling, which leads to inhibition of the growth promoting STAT3 (Bromberg and Darnell, 2000)]. Hence, JAK1/2-STAT1-mediated proapoptotic functions and JAK3-STAT3 antiapoptotic components could leverage each other, and thus in sum no influence on overall cell survival was observed. In addition to these direct cytotoxic effects of the JAK1-STAT1 signaling, it seems possible that regulation of genes with potential immunomodulatory function could enhance the recognition by or activation of immune cells and thereby tumor defense mechanisms. This is in line with observations that doxorubicin-treated cells are more prone to attack by peripheral mononuclear cells than the respective control cells (Supplemental Fig. 2). However, further work is needed to clarify this question.

Taken together, in this study we demonstrate that doxorubicin induces the secretion of IFNγ and modulates several interferon-responsive and immunomodulatory genes via IFNγ-JAK1-STAT1 signaling, leading to apoptosis. These findings contribute to a better understanding of molecular mechanisms involved in the destruction of tumor cells by immune cells and chemotherapeutic drugs, which could facilitate the selection of protocols with effective tumor killing.

Authorship Contributions

Participated in research design: Hammer, Schroeder, Kroemer, Ritter, Völker, and Bien.

Conducted experiments: Hussner, Ameling, Hammer, Herzog, Steil, Schwebe, Niessen, and Bien.

Performed data analysis: Hussner, Ameling, Hammer, Herzog, Steil, and Bien.

Wrote or contributed to the writing of the manuscript: Hammer, Kroemer, Ritter, Völker, and Bien.

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