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IFN Regulatory Factor 8 Mediates Apoptosis in Nonhemopoietic Tumor Cells via Regulation of Fas Expression

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IFN regulatory factor 8 (IRF8) is a transcription factor that was originally identified in myeloid cells and has been shown to be essential for differentiation and function of hemopoietic cells. Mice with a null mutation of IRF8 exhibit uncontrolled expansion of the granulocytic and monocytic lineages that progress into a phenotype resembling human chronic myelogenous leukemia. In human patients with chronic myelogenous leukemia, IRF8 transcript levels are frequently diminished. Therefore, IRF8 is a key regulator of myeloid tumor development. In this study, we report that IRF8 is a critical regulator of apoptosis in nonhemopoietic tumor cells. Disruption of IRF8 function with IRF8 dominant-negative mutants diminished Fas-mediated apoptosis in sarcoma tumor cells. Both constitutively expressed and IFN-γ-activated IRF8 were involved in regulation of apoptosis. Furthermore, it was found that constitutively expressed IRF8 is associated with the Fas promoter to activate Fas transcription. In addition, disruption of constitutively expressed IRF8 function diminished JAK1 expression and thereby inhibited IFN-γ-induced Fas up-regulation. Interestingly, the constitutively expressed IRF8 was also essential for TNF-α sensitization of Fas-mediated apoptosis because disruption of IRF8 function also inhibited TNF-α sensitized and Fas-mediated apoptosis. Taken together, our data suggest that IRF8 is an essential mediator of Fas-mediated apoptosis and that IRF8 mediates apoptosis through regulation of Fas expression in nonhemopoietic tumor cells. The Journal of Immunology, 2007, 179: 4775–4782.

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2 Abbreviations used in this paper: IRF, IFN regulatory factor; CML, chronic myelogenous leukemia; PI, propidium iodide; ChIP, chromatin immunoprecipitation; GAS, IFN-γ-activation site; shRNA, short hairpin RNA.

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IRF8 is an essential regulator of Fas-mediated apoptosis. Overall, our findings suggest that IRF8 is an essential regulator of Fas-mediated apoptosis and mediates apoptosis through regulation of Fas expression.

Materials and Methods

Cell lines and tissue collection

The mouse sarcoma cell line CMS4, provided by Dr. A. Delge (University of Pittsburgh, Pittsburgh, PA), is of BALB/c (H-2d) origin. Human tumor tissues were harvested from freshly resected surgical specimens at the Medical College of Georgia (MCG) and M. D. Anderson Cancer Center. For immunohistochemical staining of IRF8 protein, tissues were fixed with formalin and embedded in paraffin. For RT-PCR analysis of gene expression, portions of the tissues were processed for total RNA isolation using TRizol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. All experiments involving human tissues were conducted in accordance with protocols approved by the MCG and M. D. Anderson Cancer Center institutional review boards.

Measurement of apoptotic cell death

Apoptotic cell death was measured by propidium iodide (PI) staining as described earlier (28, 29). Briefly, tumor cells were seeded in 6-well culture plates (5 £ 10^5 cells/well) and treated with rIFN-γ (100 U/ml; R&D Systems) at 37 °C overnight. The pretreated cells were then incubated for 24 h in the absence of presence of recombinant human FasL (PeproTech). Adherent and suspended cells were then harvested and incubated with a PI/RNase solution (R&D Systems) for 5 min at room temperature, and analyzed immediately by flow cytometry. The percentage of cell death was calculated by the formula: percent cell death = percent PI-positive cells with FasL – percent PI-positive cells without FasL.

Immunohistochemistry

Tissue sections were cut from formalin-fixed, paraffin-embedded tissues. Specimens were blocked with normal goat serum (1%, and then incubated with a goat anti-IRF8 polyclonal Ab (Santa Cruz Biotechnology) at 1/50 dilution for 30 min, followed by rinsing and staining with anti-goat biotinylated Ab (1/2000) for another 30 min. Color was developed by incubation with 3',3'-diaminobenzidine solution (Sigma-Aldrich), followed by rinsing and counterstaining with hematoxylin. Positive and negative controls were performed in parallel. For negative controls, tumor sections were stained as above except that the primary Ab was omitted from the staining solution. Human tonsils were used as positive control samples. The labeling intensity was graded by two independent observers as none, weak, moderate, or strong for both the cytosolic and nuclear compartments; percentage of positive tumor cells was estimated.

Cell surface marker analysis

For cell surface Fas protein measurement, tumor cells were stained with an FITC-conjugated anti-mouse Fas mAb (BD Pharmingen) or isotype-matched control. For cell surface Fas protein measurement in short hairpin RNA (shRNA)-expressing tumor cells, tumor cells were incubated with a biotin-conjugated anti-mouse Fas mAb (BD Pharmingen) for 30 min, washed three times, and then incubated with Tri-Color-conjugated streptavidin (Caltag Laboratories) for 30 min. The stained cells were then analyzed by flow cytometry. For IFN-γ receptor measurement, tumor cells were incubated with a biotin-conjugated anti-mouse CD119 Ab for 30 min (clone GR20; BD Pharmingen). The cells were then washed three times, followed by incubation with Tri-Color-conjugated streptavidin (Caltag Laboratories) for 30 min. Cells were then analyzed by flow cytometry.

RT-PCR analysis

Total RNA was isolated from cells using RNA STAT-60 reagent (Tel-Test) according to the manufacturer’s instructions, and was used for first-strand cDNA synthesis using the ThermoScript RT-PCR system (Invitrogen Life Technologies). The cDNA was then used as template for PCR amplification of the indicated transcripts as previously described (25, 30). The PCR primer sequences are as follows: Fas: forward: 5'-AGTCTGTTGGATCG GTGCTATAGGC-3'; reverse: 5'-AGGAAACCAATGTCCATCC-3'; STAT1: forward: 5'-CTTCCCTCTGAACCCCCC-3'; reverse: 5'-CCCATATTCCAGGGCACACG-3'; and β-actin: forward: 5'-ATGTTTACACTGG ACCGACATG-3'; reverse: 5'-CTTCAAGTTGATGTCTGACGGT-3'. The PCR cycle number is 27 or 30 for Fas; 25 for Jak1 and Stat1; 30 for IRF8, and 23 for β-actin.

Western blotting analysis

Western blotting analysis was conducted as previously described (31). Briefly, tumor cells were lysed in lysis buffer containing 20 mM HEPES (pH 7.4), 20 mM NaCl, 10% glycerol, 1% Triton X-100, and a protease inhibitor mixture (Calbiochem). Cellular proteins were separated by 4–20% SDS-PAGE gradient gels, transferred to Immobilon-P membranes (Millipore), and probed with primary Abs. Anti-IRF8 Ab (C-19; Santa Cruz Biotechnology) was used at a 1/200 dilution; anti-STAT1 Ab (BD Biosciences) at 1/1500; anti-STAT1 (BD Biosciences) at 1/2000; anti-JAK1 (BD Biosciences) at 1/250; and anti-β-actin (Sigma-Aldrich) at 1/5000. Blots were detected using the ECL Plus Western detection kit (Amersham Pharmacia Biotech).

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted essentially according to protocols from Upstate Biotechnology. Briefly, tumor cells were fixed in 10% formaldehyde for 10 min at 37 °C. The cells (~1 £ 10^6 cells/assay) were lysed in SDS lysis buffer on ice for 10 min, and sonicated with a 60 Soni Dismembrator (Fisher Scientific). The lysate was precleared with a mixture of 0.04% sheared salmon sperm DNA, 0.1% BSA, and agars and protein-A beads (Pierce) for 60 min at 4 °C. The precleared supernatants were then incubated with anti-STAT1 Ab (BD Biosciences) or anti-IRF8 (Santa Cruz Biotechnology) Ab (2 µg of Ab/assay) overnight at 4 °C. A mixture (~25 µl/assay) of shared DNA, BSA, and agars and protein-A beads was then added to the lysate and incubated at 4 °C for 60 min. The beads were then extensively washed. The protein-DNA complexes were eluted from beads and the cross-linking was reversed. The DNA was purified from the eluted solution and used for PCR. The forward primer is 5'-GACACCTTCGCAACCTTG-3'. The reverse primer is 5'-GGACGCACAAACCCGCTT-3'.

Stable transfecion of tumor cells

CMS4 cells were transfected with mammalian expression plasmid pcDNA containing mouse IRF8 mutants. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. The transfected cells were propagated and maintained in culture medium containing genetin (0.75 mg/ml; Invitrogen Life Technologies). shRNA sequences were designed with the siRNA Wizard program (Invivogen). The shRNA sequence-containing double-stranded oligonucleotides were cloned into pSilencer1-7KSz vector (Invivo- gen). The cloned sequences were verified by DNA sequencing. The IRF8-specific shRNA sequence is: 5'-GACTTGTTCGGATATGCTGTA-3'. The scramble control shRNA sequence is: 5'-ATACGCACTAACA CATCAGA-3'. CMS4 cells were stably transfected with the shRNA-expressing plasmid using the Lipofectamine 2000 reagent (Invitrogen Life Technologies). Transfected cells were selected in culture medium containing zeocin (2 mg/ml; Invivogen). The selected cells were then sorted using a FACs Vantage SE cell sorter (BD Biosciences) based on GFP intensity. The sorted cells were maintained and propagated under zeocin selection.

Results

IRF8 is an essential regulator of Fas-mediated apoptosis in nonhemopoietic tumor cells

IRF8 is a transcription factor of myeloid cells and was originally identified only in hemopoietic cells (8). We have recently identi- fied IRF8 expression in solid tumor (nonhemopoietic) cell lines (25, 27). To further analyze IRF8 expression in nonhemopoietic cells, we examined IRF8 protein in human tumor tissues. Immunohistochemical staining of a cohort of human soft tissue sarcoma specimens of a variety of histological subtypes, location, and stage revealed IRF8 protein expression in all specimens (Fig. 1A). Ex- pression level was weak to moderate, and ~30–50% positive cells could be found in all cases. Staining the tumor sections in the absence of the primary Ab gave no signal (Fig. 1A, ac and dc),
indicating the specificity of the positive staining in the tumor sections. In addition, human tonsil sections were used as positive controls. Specific nuclear staining of immune cells was observed (data not shown). Interestingly, while most specimens (liposarcoma, malignant fibrous histiocytoma, rhabdomyosarcoma, and alveolar soft part sarcoma) exhibited both nuclear and cytoplasmic staining, while specimen f (leiomyosarcoma) shows diffuse cytoplasmic IRF8 expression but no nuclear staining. B, RT-PCR analysis of IRF8 transcript levels in human primary colon and ovarian cancers. Total RNA was isolated from tumor tissues obtained from 18 colorectal (left panel) and 5 ovarian (right panel) cancer patients. C, Western blot analysis of IRF8 protein levels in CMS4 tumor cells. CMS4 tumor cells were transfected with the empty expression vector (CMS4.Vector), or vector containing IRF8 cDNA with point mutations in either the DNA-binding domain (CMS4.K79E) or IFN-association domain (CMS4.R289E) and stable sublines were selected. The tumor cell sublines were treated with IFN-γ (100 U/ml) overnight and analyzed for IRF8 protein levels. D, Measurement of cell death in CMS4 tumor cells. Tumor cells were treated with IFN-γ (100 U/ml) overnight, followed by incubation with rFasL (200 ng/ml) for ~24 h. Cell death was determined by PI staining and flow cytometry analysis. Shown is one of three representative experiments. E, Quantification of apoptotic cell death as shown in D. Column, mean; bars, SD.

IRF8 has been shown to be a key regulator of apoptosis in myeloid tumors (4, 21). To examine the role of IRF8 in apoptosis in nonhemopoietic tumor cells, we introduced two IRF8 mutants, termed K79E and R289E (provided by Dr. K. Ozato, National Institute of Child Health and Human Development/National Institutes of Health, Bethesda, MD), respectively, into the sarcoma cell line. CMS4.K79E contains a point mutation at amino acid position 79 in the DNA-binding domain, whereas R289E contains a point mutation in the IFN-association domain of IRF8. Both mutants have been well-characterized and shown to specifically disrupt IRF8 function in myeloid cells (32, 33). CMS4 sarcoma tumor cells were transfected with the expression vector (CMS4.Vector), or vector containing either K79E (CMS4.K79E) or R289E (CMS4.R289E), respectively, and selected for stable transfectants. The control cell lines and transfectants were then analyzed for IRF8 protein expression (Fig. 1C) and sensitivity to apoptosis (Fig. 1, D and E). CMS4 and CMS4.Vector cells expressed low level of IRF8 protein, and expressed higher levels of Fas protein after IFN-γ treatment (Fig. 1C). The CMS4.K79E cells exhibited a high level of IRF8 protein before IFN-γ treatment and remained at a high IRF8 protein level after IFN-γ treatment (Fig. 1C). The parent CMS4 cells and vector-transfected CMS4 cells showed considerable sensitivity to IFN-γ-sensitized and Fas-mediated apoptosis (Fig. 1D). In contrast, CMS4.K79E and CMS4.R289E cells exhibited significantly higher degree of resistance to Fas-mediated apoptosis as compared with CMS4.Vector cells (Fig. 1, D and E) (p = 0.03 and 0.003 for CMS4.K79E and CMS4.R289E, respectively).

Constitutive IRF8 is essential for constitutive and IFN-γ-induced Fas expression

The molecular mechanisms underlying IRF8 function in apoptosis in solid tumor cells are unknown. Because IFN-γ simultaneously activates IRF8 and Fas (25) and IRF8 is a transcription factor (11, 34, 35), we reasoned that IRF8 might mediate IFN-γ-activated Fas expression. To test this possibility, we first examined the effects of IRF8 mutant K79E on the components of the IFN-γ signaling pathway in CMS4 tumor cells. K79E was chosen because of its higher expression levels in CMS4 cells and its greater effect on

FIGURE 1. Disruption of IRF8 function inhibits tumor cell sensitivity to Fas-mediated apoptosis. A, IRF8 is expressed in human soft tissue sarcoma. Sections were prepared from paraffin-embedded tissues. The sections were stained with anti-IRF8 Ab. a, Primary liposarcoma; b, primary malignant fibrous histiocytoma (MFH); c, metastatic rhabdomyosarcoma; d, recurrent alveolar soft tissue sarcoma (ASPS); e, recurrent MFH; and f, primary leiomyosarcoma. Negative control staining of liposarcoma (no primary Ab in the staining procedure); d, negative control of ASPS. Specimens a–e exhibit both nuclear and cytoplasmic staining, while specimen f (leiomyosarcoma) shows diffuse cytoplasmic IRF8 expression but no nuclear staining. B, RT-PCR analysis of IRF8 transcript levels in human primary colon and ovarian cancers. C, Western blot analysis of IRF8 protein levels in CMS4 tumor cells. CMS4 tumor cells were transfected with the empty expression vector (CMS4.Vector), or vector containing IRF8 cDNA with point mutations in either the DNA-binding domain (CMS4.K79E) or IFN-association domain (CMS4.R289E) and stable sublines were selected. The tumor cell sublines were treated with IFN-γ (100 U/ml) overnight and analyzed for IRF8 protein levels. D, Measurement of cell death in CMS4 tumor cells. Tumor cells were treated with IFN-γ (100 U/ml) overnight, followed by incubation with rFasL (200 ng/ml) for ~24 h. Cell death was determined by PI staining and flow cytometry analysis. Shown is one of three representative experiments. E, Quantification of apoptotic cell death as shown in D. Column, mean; bars, SD.
apoptosis inhibition (Fig. 1, D and E). Ectopic expression of IRF8 mutant K79E in CMS4 cells exhibited no effect on IFN-γ receptor expression (Fig. 2A). However, The JAK1 transcript and protein were diminished in CMS4.K79E cells (Fig. 2B), suggesting that IRF8 is involved in regulation of constitutive expression of JAK1. Because JAK1 expression was diminished, as expected, STAT1 phosphorylation was dramatically decreased (Fig. 2B). Disruption of constitutively expressed IRF8 function also increased total STAT1 protein levels (Fig. 2B), suggesting that IRF8 is a negative regulator of STAT1 transcription. Because phosphorylated STAT1 is an essential effector of IFN-γ receptor-initiated signals (36, 37), we reasoned that diminished STAT1 phosphorylation should decrease IFN-γ-regulated gene expression, including Fas expression. To test this hypothesis, we measured Fas protein levels on the surface of these tumor cells. As expected, Fas protein levels were significantly decreased in CMS4.K79E cells as compared with CMS4.Vector cells (Fig. 3, A–C). Interestingly, both constitutively expressed and IFN-γ-activated Fas expression levels were significantly decreased in CMS4.K79E cells as compared with CMS4 and CMS4.Vector cells (Fig. 3, A–C) \((p < 0.001)\). To confirm the results obtained with the IRF8 mutant, we took a complementary approach to investigate the effect of loss of IRF8 expression and function on Fas expression. IRF8 expression was silenced in CMS4 cells with IRF8-specific shRNA, both constitutively expressed and IFN-γ-activated Fas expression levels were also significantly decreased in CMS4 psiRNA.IRF8 cells as compared with CMS4 psiRNA scramble cells (Fig. 3, E and F) \((p < 0.001)\).

**FIGURE 2.** Disruption of IRF8 function diminished JAK1 expression in CMS4 tumor cells. A, Cell surface IFN-γ receptor expression levels by the indicated tumor line/sublines. Tumor cells were stained with biotin-conjugated anti-IFN-γ receptor mAb, followed by staining with Tri-Color-conjugated streptavidin. The stained cells were analyzed by flow cytometry. Gray area: staining with the isotype-matched control Ab. Solid line: IFN-γ receptor-specific staining. B, RT-PCR and Western blot analysis of JAK1 and STAT1. Tumor cells were treated with IFN-γ (100 U/ml) and lysed for mRNA and protein analysis at the indicated time point. RT-PCR analysis of JAK1 and STAT1 is shown at the left. Western blot images of JAK1, phosphorylated STAT1 (pSTAT1) and STAT1 protein levels in indicated cell line/sublines are shown at the right. β-actin was used as normalization standard.

**FIGURE 3.** IRF8 regulates Fas expression. A, RT-PCR analysis of Fas transcript levels in CMS4 tumor cells and sublines. Tumor cells were treated with IFN-γ (100 U/ml) and used for RNA isolation and PCR analysis of Fas expression at the indicated time point. The Fas amplification was conducted with 30 PCR cycles (a) or 27 PCR cycles (b). β-actin was used as normalization standard. B, Cell surface Fas expression levels by the indicated tumor line/sublines. Tumor cells were treated with IFN-γ (100 U/ml) for ~24 h. The untreated and IFN-γ-treated cells were then stained with FITC-conjugated anti-Fas mAb and analyzed by flow cytometry. Dotted lines: histograms of untreated cells; solid lines: histograms of IFN-γ-treated cells. Gray area: staining with the isotype-matched control Ab. C, Relative Fas protein levels in untreated and IFN-γ-treated (24 h) cells as shown in B were quantified by mean fluorescence intensity (MFI). Columns: mean of three replicated experiments; bars: SD. D, RT-PCR analysis of Fas transcript levels in CMS4 tumor cells expressing scramble (CMS4ψpsiRNA.Scramble) or IRF8-specific shRNA (CMS4ψpsiRNA.IRF8). Tumor cells were treated with IFN-γ (100 U/ml) for ~24 h and used for RNA isolation and PCR analysis of IRF8 at the indicated time point. β-actin was used as normalization standard. E, Cell surface Fas expression levels in CMS4ψpsiRNA.Scramble and CMS4ψpsiRNA.IRF8 cells. Tumor cells were treated with IFN-γ (100 U/ml) for ~24 h and used for RNA isolation and PCR analysis of Fas expression at the indicated time point. β-actin was used as normalization standard. F, Relative Fas protein levels as shown in E were quantified by MFI. Columns: mean of three replicated experiments; bars: SD.

Both IRF8 and STAT1 are associated with the Fas promoter
It has been well-established that binding of IFN-γ to IFN-γ receptor leads to JAK1 phosphorylation, followed by phosphorylation of preformed cytosolic STAT1, which are then translocated to the
nucleus as an active homodimer. Active STAT1 binds to IFN-γ-activation site (GAS) in the promoter of primary IFN-γ-response genes (36, 38). IRF8 is also capable of binding to certain GAS elements to regulate transcription, at least in myeloid cells (35). Because both IFN-γ-activated STAT1 and constitutively expressed IRF8 activate Fas transcription, we reasoned that the Fas promoter might be a direct target of these two transcription factors. To test this possibility, we first used the MacVector program to analyze the mouse Fas promoter DNA sequence (39) and identified a GAS element (TTCTGGGAA) (35) that is located between −100 and −92 in the mouse Fas promoter region (Fig. 4A).

To determine whether phosphorylated STAT1 is associated with the GAS DNA element in solid tumor cells, we conducted a ChIP assay of IFN-γ-treated CMS4 tumor cells with phosphorylated STAT1 antibody and analyzed the coprecipitated genomic DNA by PCR using a pair of Fas promoter DNA-specific primers that flank the GAS site. It is clear that phosphorylated STAT1 is associated with the GAS site in the Fas promoter region of CMS4 cells (Fig. 4B). To determine whether IRF8 is also associated with the GAS site in the Fas promoter in CMS4 cells, CMS4 cells were cultured in the absence (−) or presence (+) of IFN-γ for 12 h and analyzed by ChIP assay for IRF8 and Fas promoter interactions. Fig. 4C clearly indicates that both constitutive and IFN-γ-activated IRF8 protein is associated with the Fas promoter GAS site in CMS4 cells.

IRF8 also mediates TNF-α-dependent apoptosis

The above observations that constitutively expressed IRF8 can regulate Fas and JAK1 expression raise the possibility that IRF8 might regulate both IFN-γ-dependent and -independent apoptosis. To test this possibility, we treated CMS4 tumor cells with TNF-α, a cytokine that has been shown to sensitize tumor cells to apoptosis (30, 40, 41). RT-PCR analysis of TNF-α-treated tumor cells indicated that TNF-α does not activate IRF8 expression, as expected (Fig. 5A). The TNF-α-treated cells were then incubated with FasL, and cell death was analyzed by PI staining. TNF-α-sensitized CMS4 and CMS4 Vector cells exhibited high sensitivity to FasL-induced apoptosis. In contrast, cell death was diminished in CMS4.K79E and CMS4.R289E cells (Fig. 5, B and C). The p values are <0.001 between CMS4, Vector and CMS4.K79E cells, and between CMS4 Vector and CMS4.R289E cells.

Discussion

Among the 10 IRF family members, IRF8, along with IRF4, was originally thought to be exclusively expressed in hematopoietic cells (9, 10). In those early studies, only normal tissues were examined, and it was observed that IRF8 expression was restricted to monocytic and lymphoid lineages (9, 10). In earlier studies (25, 27) and in this study, we detected IRF8 transcript and protein in both human and mouse nonhemopoietic tumor cells. Moreover, these observations were made in tumor cell lines in vitro as well as in human and mouse tumor tissues in vivo. Thus, we conclude that IRF8 is also expressed in solid (nonhemopoietic) tumors.

IRF8 is an apoptosis regulator in myeloid cells (4, 21, 22). It has been shown that IRF8 mediates apoptosis through down-regulation of Bcl-xL (21) or Bcl-2 (4, 22) in myeloid cells. In this study, we observed that disruption of IRF8 function in mouse tumor cells

FIGURE 4. STAT1 and IRF8 are associated with the Fas promoter. A, Genomic organization of the mouse Fas gene promoter region showing the GAS site. B, ChIP analysis of phosphorylated STAT1 association with the GAS-containing Fas promoter region. CMS4 tumor cells were incubated in the absence (−IFN-γ) or presence (+IFN-γ) of IFN-γ (100 U/ml) for 4 h and then used for ChIP assay as described in Materials and Methods. Anti-phosphorylated STAT1 mAb was added to lysates prepared from both untreated (as negative control) and IFN-γ-treated cells. The Fas promoter DNA fragment was amplified from immunoprecipitated genomic DNA by PCR using Fas promoter DNA-specific sequences that flank the GAS site. C, ChIP analysis of IRF8 and mouse Fas promoter interaction. CMS4 tumor cells were cultured in the absence (−IFN-γ) or presence (+IFN-γ) of 100 U/ml IFN-γ overnight and used for ChIP assay as described in Materials and Methods. Anti-IRF8 Ab was added to lysates prepared from both untreated and IFN-γ-treated cells, respectively. The lysates without Ab were used as negative controls. The Fas promoter DNA fragment was amplified from immunoprecipitated genomic DNA by PCR using Fas promoter DNA-specific sequences that flank the GAS site.

FIGURE 5. Disruption of IRF8 function inhibited TNF-α-sensitized and Fas-mediated apoptosis. A, RT-PCR analysis of IRF8 expression levels in CMS4 cells after IFN-γ or TNF-α treatment. CMS4 tumor cells were treated with IFN-γ or TNF-α overnight, and then used for analysis of IRF8 transcript level. B, Measurement of apoptosis. Tumor cells and sublines were treated with rTNF-α (100 U/ml) overnight, followed by incubation with FasL (200 ng/ml) for ~24 h. Cell death was determined by PI staining and flow cytometry analysis. Shown is one of three representative experiments. C, Quantification of apoptotic cell death as shown in B. Column: mean; bars: SD.

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diminished expression of the death receptor Fas and inhibited tumor cell sensitivity to Fas-mediated apoptosis. Both Bcl-2 and Bcl-xL protein levels were not changed in tumor cells ectopically expressing the mutant IRF8 (data not shown). Our results thus define IRF8 as an essential regulator of Fas-mediated apoptosis in solid tumor cells, and suggest that IRF8 mediates apoptosis in solid tumor cells through a mechanism that is different from that in myeloid cells.

Constitutively expressed IRF8 is abundant in macrophages, other myeloid cells, and B cells, and is low but detectable in T cells (35). Expression of IRF8 can be dramatically up-regulated by IFN-γ. Engagement of IFN-γ receptor with IFN-γ activates JAK1 and JAK2, which induce phosphorylation and dimerization of STAT1. The dimerized STAT1 translocates to the nucleus and binds to the GAS element to activate transcription from IFN-γ-inducible gene promoters (11, 34, 42–46). Because the GAS element is present in the IRF8 promoter region, it is apparent that IFN-γ up-regulates IRF8 transcription through activating STAT1, which binds to the GAS element in myeloid cells. However, the relative roles of constitutively expressed IRF8 and IFN-γ-activated IRF8 are still not fully defined in myeloid cells (47). Some of the IRF8-binding partners, including PU.1, are not inducible by IFNs in myeloid cells, and it has been shown that IRF8/IRF2 binding to DNA is constitutive and IFN-γ independent (48). These observations suggest that constitutively expressed IRF8 might play a primary role in myeloid cells as compared with IFN-γ-induced IRF8 (35).

In solid tumor cells, we observed that IRF8 is also constitutively expressed, albeit at a low level, but can be dramatically up-regulated by IFN-γ. Consistent with what is known in myeloid cells, constitutive IRF8 also plays a primary role in solid tumor cells as compared with IFN-γ-activated IRF8. Disruption of constitutive IRF8 function diminished constitutive Fas expression. Moreover, it is interesting to note that constitutive IRF8 is also required for constitutive expression of Jak1, a key kinase in the IFN-γ-signaling pathway that is essential for activation of IFN-γ-inducible genes including IRF8 (42, 43, 47, 49). Therefore, constitutive IRF8 not only regulates transcription of constitutive Fas, but also controls the expression of a key molecule in the IFN-γ-signaling pathway, thereby regulating both Fas and its own activation during IFN-γ stimulation. Our results thus suggest that although IRF8 is an IFN-γ-inducible gene, constitutive IRF8 plays a primary role in mediation of IFN-γ-dependent and -independent Fas expression and apoptosis.

Gel EMSA with a GAS element-containing DNA probe detected STAT1, but not IRF8, binding to the GAS sequence (43, 47); however, it has been shown that IRF8 strongly stimulates GAS reporter activities and is present in the GAS-bound protein complex along with STAT1 in a macrophage cell line (50). These findings suggest that IRF8 might bind to or compete with STAT1 to associate with the GAS element to promote GAS-dependent transcription in myeloid cells. In this study, we demonstrated that constitutively expressed and IFN-γ-regulated IRF8 are associated with the GAS element in the Fas promoter in a solid tumor cell line as revealed by ChIP assay. The phosphorylated STAT1 also binds to the same GAS element. However, coimmunoprecipitation with phosphorylated STAT1-specific Ab failed to pull-down IRF8 in an immunoprecipitation-Western analysis (data not shown). Thus, whether IRF8 and STAT1 form a complex and simultaneously bind to the GAS element, or IRF8 competes with STAT1 for GAS-sequence binding in the Fas promoter in solid tumor cells, requires further study.

Based on our observations, we propose a model for loss of Fas function on apoptosis resistance in solid tumor cells (Fig. 6). It seems that IRF8 regulates Fas-mediated apoptosis by dual mechanisms; i.e., IRF8 regulates both the expression of Fas receptor and at least another essential unknown molecule in the Fas-apoptosis pathway that is activated by either IFN-γ or TNF-α. The dual mechanisms are of interest. In a recent study, we demonstrated that IRF8 functions as a tumor suppressor and its expression is diminished in apoptotic-resistant metastatic tumors (27). Loss of IRF8 expression results in alteration in more than one molecule of the same death signaling pathway, thereby ensuring the tumor cells to maintain a stable apoptosis resistance phenotype to evade destruction in the host if one defect is reversed by host mechanisms. For example, immune cells often secrete proinflammatory cytokines, such as IFN-γ (19, 51) and TNF-α (52), when encountered with stimuli, such as tumor cells (19). Proinflammatory cytokines often activate the expression of key apoptotic genes, including Fas (18). Alterations of multiple regulators in the Fas-mediated apoptosis pathway enable the tumor cells to resist apoptotic cell death induction even if the defect of one regulator (i.e., Fas) is reversed. Therefore, simultaneous alterations in the expression patterns of multiple apoptosis modulators in the same death pathway dramatically increase the survival capability of the tumor cells. Current data suggest that IRF8 exerts its transcriptional activity through association with other proteins and harbors dual functions as either a transcriptional activator or repressor (53). This dual functionality is in part due to interactions between IRF8 and other proteins that lead to alterations in transcriptional activities of IRF8 complex (53). For example, IRF8 forms protein complexes with transcriptional factors, such as PU.1 and E47, to bind to different composite DNA elements resulting in transcriptional activation (11, 34, 44–46). It also has been well-established that IRF8 interacts with IRF1 or IRF2, two genes that were also regulated by IFN-γ, to form protein complexes that facilitate IRF8 binding to the IFN-stimulated response element (ISRE) element in the promoter regions and thus acts as a transcriptional repressor (24, 48, 54, 55). In addition, IRF8 was identified in multiprotein complexes that include at least CBP/p300 (56), Trip15/CSN2 (34), and NFAT (2). These data suggest that IRF8 functions through associations with other transcriptional factors in hemopoietic cells. Because IRF8 protein alone has very weak DNA-binding affinity and its function requires its association with other proteins (8), it is the IRF8-binding proteins that dictate IRF8 function. Therefore, the IRF8.K79E and IRF8.R289E mutants likely function by competing with endogenous IRF8 to bind to IRF8-interacting proteins. We have shown that both the IRF8 mutants and the IRF8 shRNA-expressing tumor cells exhibit decreased Fas expression, suggesting that the IRF8 mutants act as dominant-negative mutants to

![FIGURE 6. Model for the effects of disruption of IRF8 function on Fas-mediated apoptosis. Disruption of IRF8 function by ectopic expression of IRF8 mutant K79E has at least three effects on the Fas-mediated apoptosis of the tumor cells. First, the constitutive Fas (cFas) is diminished; second, the IFN-γ-induced Fas up-regulation (uFas) is decreased due to diminished JAK1 expression; and third, TNF-α-mediated activation of an essential molecule (X) that is required for the Fas-mediated apoptosis is also blocked. Therefore, IRF8 regulates Fas-mediated apoptosis by multiple mechanisms.](http://www.jimmunol.org/)

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disrupt endogenous IRF8 function by competing with IRF8-bind-
ing proteins from endogenous IRF8. However, as mentioned above, IRF8 is also involved in functions of other proteins, in-
cluding IFN-γ-regulated IRF1 and IRF2. Besides IRF8, IFN-γ also up-regulates IRF1, IRF2, IRF5, IRF7, and IRF9 in CMS4 tumor cells (data not shown). Therefore, our results do not exclude the possibility that IRF8 mutation may disrupt the functions of other IRF8-associated proteins, thus resulting in secondary effects in the tumor cells. Further studies are needed to determine these possible secondary effects of IRF8 in solid tumor cells.

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Disclosures

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References


