

In Vivo Interferon Regulatory Factor 3 Tumor Suppressor Activity in B16 Melanoma Tumors¹

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Abstract

Delivery of transcription factors to cancer cells to reprogram gene expression may represent a novel strategy to augment the production of immune stimulatory cytokines and trigger a more potent antitumor response. In the present study, a bicistronic retroviral vector (AP2) was used to transduce B16-F0 melanoma cells with IFN regulatory factor (IRF)-3, which has been shown to activate type I IFN genes (*IFN-β* and *IFN-α*) as well as other cytokines. Gene-modified B16 melanoma cells were inoculated s.c. into *C57BL/6* syngeneic mice. In animals receiving IRF-3 B16 melanoma cells, tumors grew at a 4- to 5-fold reduced rate, and tumors that developed from these mice had a moderate-to-dense infiltration of inflammatory cells, whereas only low levels of lymphocyte infiltration were observed in mock-transduced B16 tumors. Furthermore, tumor growth was not inhibited in severe-combined immunodeficient mice after inoculation of IRF-3-expressing B16 cells, which suggested that IRF-3-mediated antitumor responses were dependent on a functional adaptive lymphocyte response. Interestingly, these *in vivo* effects on tumor growth correlated with higher mRNA expression of chemokines such as *MIP-1β*, *RANTES*, and *IP-10*, as well as dramatic increases *in vitro* in the inducibility of cytokine mRNA such as *IFN-β*, *TNF-α* and *interleukin 6*. Our results demonstrate that with weakly antigenic tumors such as B16 melanoma, IRF-3 gene transfer can mediate important antitumor responses. These findings suggest a novel role for IRF-3 as a potential molecular target for gene therapy of cancer.

Introduction

Melanoma is the prototype of an immunogenic tumor to which various types of immunotherapy have been applied over the past decade (1, 2). An alternative strategy for the treatment of advanced melanoma has been an *in vivo* gene therapy approach. The genetic manipulation of tumor cells to express immunostimulatory molecules provides a means to analyze immune reactions against tumor cells *in vivo*. Studies using animal models have demonstrated that the transduction of different cytokine genes into tumor cells results in a local recruitment of inflammatory cells that, in turn, inhibit tumor growth (3–5). A potential alternative strategy is to use transcription factors to reprogram gene expression in cancer cells; such an approach could augment the production of a multitude of immune stimulatory cytokines and enhance the antitumor response by modulating the local expression of cytokines at the tumor site.

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IFNs are cytokines endowed with potent antiviral and immunomodulatory activities, capable of exerting marked antitumor effects in both animal models and patients (6), and have served as an important treatment modality for a number of solid tumors and hematological malignancies (7). IRF-3⁴ is a member of the IRF transcription factor family that plays an essential role in the regulated expression of type I IFN genes, *RANTES* and ISGs (8–12). Production of IFN-α and IFN-β in turn can stimulate both innate and acquired arms of the immune response (13). Substitution of serine and threonine residues in the amino acid 396–405 region of IRF-3 with aspartic acid (IRF-3-5D) creates a constitutively active form of IRF-3 that is able to stimulate the transcription of target genes in the absence of virus induction (9). In addition, the constitutive expression of IRF-3-5D alone is sufficient to induce apoptosis in human embryonic kidney 293 and human Jurkat T cells (14, 15). Because of its critical role in the activation of the IFN cascade, IRF-3 was selected as a prototype gene to modify B16 melanoma cells to determine whether ectopic IRF-3 expression could enhance cytokine production and trigger an antitumor response in syngeneic mice. We found that a gene transfer of IRF-3 into B16 melanoma cells modulated the cytokine profile of the tumors and inhibited tumor development by recruiting inflammatory cells to the site of the B16 tumor.

Materials and Methods

Cell Lines and Culture Medium. B16.F0 is a murine melanoma cell line of *C57BL/6* origin and was maintained in DMEM (Wisent) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics.

Retroviral Transduction. Construction and characterization of the bicistronic AP2 to GFP retroviral vector has been described previously (16). cDNA of human IRF-3 was inserted into multiple cloning site of the retrovector AP2 (*EcoRI*blunt/*XhoI*). Pantropic retroviral supernatant was generated by transfection of the retrovirus vector pAP2-GFP or pAP2 IRF-3-GFP into 293 GPG packaging cells using LipofectAMINE (Life Technologies, Inc.) and sorted by FACS for GFP expression. For retroviral transduction, 10⁶ B16 melanoma cells were seeded in 10-cm plates and incubated with retroviral supernatants filtered from virus-producing cultures in the presence of LipofectAMINE (6 μl/ml). The stable transfectants, IRF-3-GFP B16 and AP2-GFP B16 (AP2-B16) cells were selected by FACS and tested for IRF-3 expression by immunoblot analysis.

Tumor Formation. Young (6–8-week-old) female *C57BL/6* or immunodeficient mice (SCID) were purchased from Charles River (Montreal, Quebec, Canada) and handled under the guidelines of the Canadian Council on Animal Care. Mice were shaved in the right flank area and were given injections s.c. with either 1 × 10⁶ mock-transfected AP2-B16 cells or IRF-3 B16 in a total volume of 100 μl of PBS. Tumor growth was followed by Vernier caliper measurement every other day from day 7 after injection. All of the experiments included seven mice/group. Tumor volume was calculated according to the

⁴ The abbreviations used are: IRF, IFN regulatory factor; ISG, IFN-stimulated gene; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; SCID, severe combined immunodeficient/immunodeficiency; TNF, tumor necrosis factor.

formula $V = (a \times b^2)/2$, where a = the largest superficial diameter and b = the smallest superficial diameter. Tumors were excised at day 19 (day of sacrifice) and were used for subsequent RNA extraction or fixed in formaldehyde, embedded in paraffin, and stained for histological evaluation (H&E staining).

RNAse Protection Analysis. Total RNA was isolated from various tumors (AP2-B16 and IRF-3-B16) at 19 days after injection (day of sacrifice) and from AP2-B16 and IRF-3 B16 melanoma cells after Sendai virus infection (40HAU) by using Trizol reagent (Life Technologies, Inc.) RNAse protection assays were performed using two Riboquant multiprobe template sets from PharMingen. The mCK-3b and mCK-5 template was used for the T7 polymerase-directed synthesis of high specific activity [³²P]UTP-labeled antisense RNA probes. The probe set contained 12 probes for mCK-3b and 11 probes for mCK-5, including two housekeeping genes, *GAPDH* and *L32*. Probe (3×10^5 cpm) was hybridized with each RNA (10 μg) sample overnight at 56°C. RNA samples were digested with RNase A and T1, purified, and resolved on 5% denaturing polyacrylamide gels. Internal housekeeping genes were analyzed to confirm equal RNA loading.

Results and Discussion

Injection of IRF-3-transduced B16 Cells Leads to Suppression of Tumor Growth *in Vivo*. Because B16 is a well-characterized, poorly immunogenic s.c. tumor (17, 18), we focused our studies on this model to evaluate whether ectopic expression of IRF-3 could be used as an immunomodulatory transcription factor. Retroviral trans-

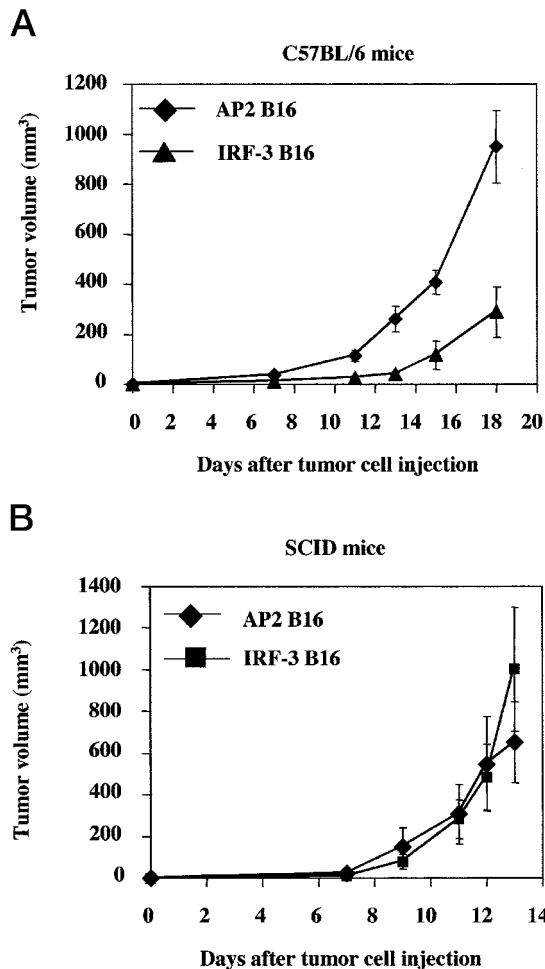


Fig. 1. Expression of *IRF-3* inhibits B16 tumor growth *in vivo*. In A, IRF-3 B16 melanoma cells (▲) and mock-transduced AP2 B16 (◆) cells (1×10^6) were injected with s.c. into female *C57BL/6* syngeneic mice (seven mice/group). Tumor growth was followed by perpendicular caliper measurement every other day from day 7 after injection. In B, SCID mice were injected with IRF-3-transduced (■) and mock-transduced AP2 B16 melanoma cells (◆; 1×10^6). Tumor growth was followed by perpendicular caliper measurement every other day from day 7 after injection.

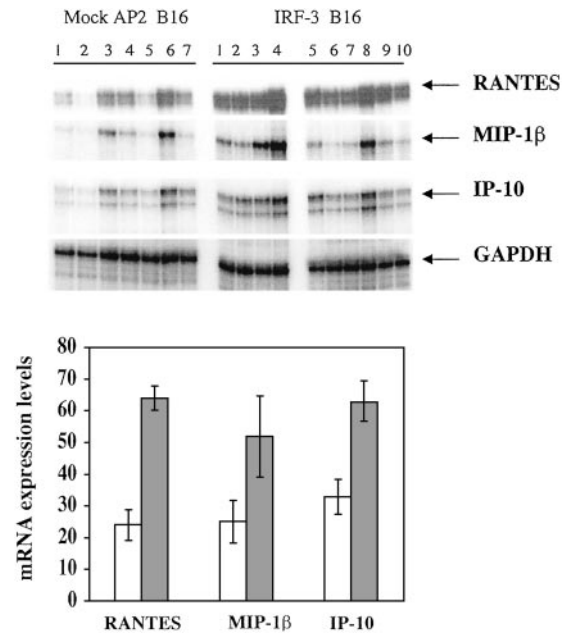


Fig. 2. *IRF-3* overexpression in B16 melanoma cells results in up-regulation of *RANTES*, *IP-10*, and *MIP-1β* mRNA expression *in vivo*. In A, total RNA (10 μg) was isolated from various transduced B16 tumors (mock-transduced AP2 B16, $n = 7$; IRF-3 B16, $n = 10$) and hybridized with the mCK-5 multiple probe before digestion with RNase. Separation of protected fragments was performed by 5% SDS gel electrophoresis. Fragment assignment was determined by migration of protected fragments relative to internal standards. Induction of *RANTES*, *IP-10*, and *MIP-1β* mRNA expression was confirmed by performing two additional RPA analyses. In B, cytokine mRNA expression levels were quantified relative to the internal control *GAPDH*.

duction of IRF-3 was performed *in vitro* with B16-F0 cells, and GFP-positive cells were sorted by FACS to generate a stable IRF-3 transduced population. *IRF-3*-expressing B16 melanoma cells (IRF-3 B16) were inoculated s.c. into syngeneic *C57BL/6* mice, and tumor growth was monitored between the mock AP2- and IRF-3-transduced populations. Expression of *IRF-3* in the B16 melanoma cells resulted in slower tumor growth than injection of vector alone, mock-transduced cells (AP2-B16; Fig. 1A). By day 19, at the time of sacrifice, the mean tumor volume in the AP2 B16-injected animals was ~1000 mm³, whereas in animals inoculated with *IRF-3*- expressing B16 cells, the tumor volume on average was 200–300 mm³. The slower tumor growth rate *in vivo* was of interest because the retrovirally transduced B16 cells had virtually the same *in vitro* growth in terms of doubling time (15 ± 0.7 h). Furthermore, no difference between the AP2-B16 and IRF-3 B16 cells was observed when cells were grown under conditions of lowered serum concentration. Both AP2 and IRF-3 B16 cells reached similar saturation densities in 0.5% serum (2×10^5 cells/35 mm dish), 1.0% serum (4×10^5 cells/35 mm dish) and 10% serum (1×10^6 cells/35 mm dish). Furthermore, flow cytometric analysis indicated that there was no difference in the cell cycle distribution of AP2-B16 and IRF-3-B16 as measured by propidium diiodide staining of nuclear DNA (data not shown).

To evaluate the role of host immunity in IRF-3-mediated antitumor responses; IRF-3- and AP2-transduced B16 tumor cells were injected s.c. into tumor-bearing SCID beige *C57BL/6* mice. In contrast to the results with syngeneic mice, IRF-3 expression in B16 cells did not alter tumor volumes in SCID mice (Fig. 1B). If the lack of growth in AP2-B16 versus IRF-3 B16 melanoma in the syngeneic model was related to changes in the growth pattern of the tumor cell line, slower growth would have been expected in SCID mice. Because growth suppression was observed only in immune competent animals, this observation provides evidence that growth inhibition is not attributable to growth pattern changes of the B16 IRF-3. Together, these

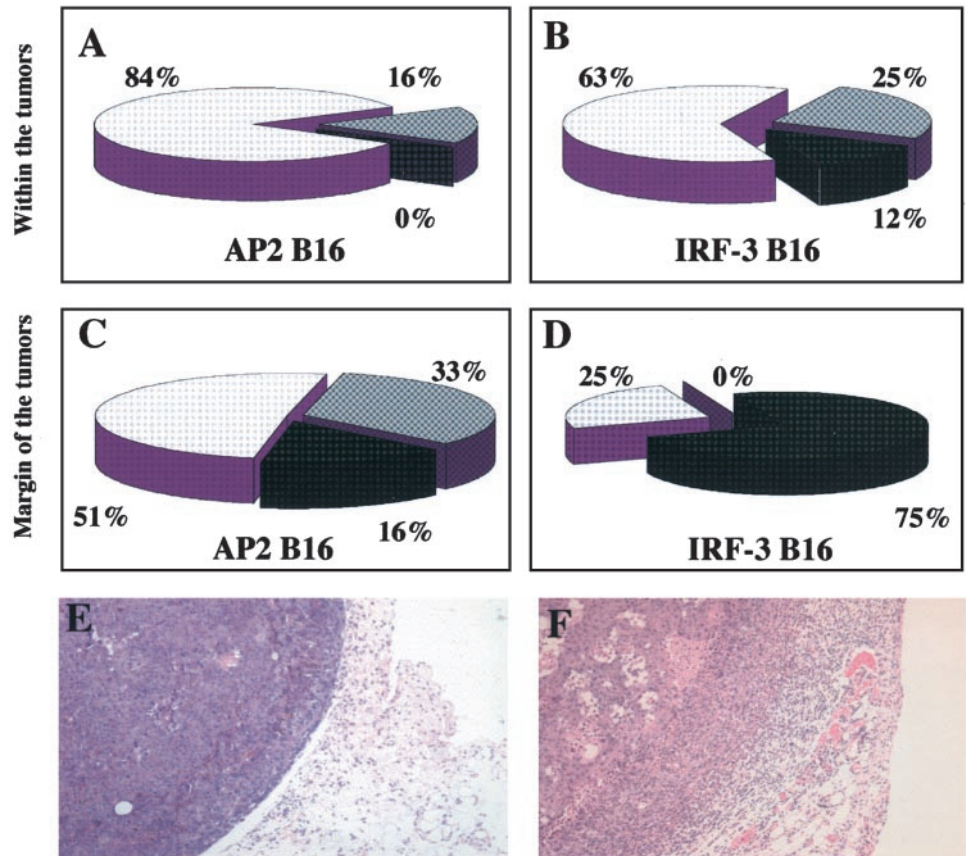


Fig. 3. Recruitment of lymphocytes by IRF-3-transduced B16 melanoma cells. Histograms represent the percentage of infiltrating cells within (A and B) and at the margin (C and D) of the B16 melanoma tumors (AP2 and IRF-3): absence of lymphocyte is indicated by (□), moderate infiltration (▨) and dense infiltration (■). E and F, histology of tumors growing s.c. from mock-transduced AP2 and IRF-3 B16 injected mice (C57BL/6) excised at day 19 (day of sacrifice). H&E stain; magnification $\times 100$.

observations suggest that the antitumor effects of IRF-3 do not involve differences in tumor growth properties, cell cycle, or apoptosis in B16 melanoma cells, but rather indicate the requirement for a functional adaptive immune response as part of the antitumor response.

Cellular Recruitment into Tumor Tissue Correlates with Increased Expression of Chemokines. Tumor progression has been previously shown to be modified by host cytokine expression, which, in turn, mediates leukocyte migration and activation (19, 20). Because IRF-3 plays an active role in the induction of *IFN* and *RANTES* genes, we, therefore, analyzed the effect of IRF-3 expression on cytokine production. The profiles of several immunomodulatory chemokines were examined in tumor cells derived from mock-transduced AP2 ($n = 7$) or IRF-3-transduced B16 tumors ($n = 10$). Cytokine mRNA expression from solid tumor was determined by RNase protection assay. Interestingly, the IRF-3 B16 tumors displayed a 2- to 3-fold increase in basal mRNA levels of *RANTES*, *IP-10*, and *MIP-1 β* compared with AP2 B16 tumors (Fig. 2). Chemoattractants such as IP-10, RANTES, and MIP-1 β recruit lymphocytes into malignant tissue (21–23), which suggests that IRF-3 transduction into B16 melanoma enhanced cytokine release in the tumor microenvironment. Whether IRF-3 overexpression is directly involved in cytokine secretion or additional inflammatory cells recruited to the microenvironment are required is currently under investigation.

Because inflammatory chemokines such as RANTES, MIP-1 β and IP-10 are specialized to recruit effector cells, including monocytes, granulocytes, and effector T cells, tumor infiltration was next evaluated by histology within the tumor cell mass, as well as at the margin of the tumor. Within the IRF-3 B16 tumor mass, moderate or dense lymphocyte infiltration was detected in 25 and 12%, respectively, of the tumors, compared with mock-transduced AP2 B16 tumors (16%

moderate infiltration; Fig. 3, A and B). Interestingly, dense lymphocyte infiltration was clearly visible at the margins of the IRF-3 B16 tumors (75%) when compared with mock-transduced AP2 B16 tumors (12%; Fig. 3, C and D). Although ectopic expression of IRF-3 had no direct inhibitory effect on B16 melanoma cells growth *in vitro*, a striking effect on local tumor infiltration *in vivo* was observed. Histologically, AP2 B16 tumors demonstrated diffuse growth and a paucity of host inflammatory cells, which was not associated with areas of coagulation necrosis (Fig. 3E). In contrast, IRF-3 B16 tumors possessed a larger capsule, displayed dense infiltration of both neutrophils and lymphocytes, and contained fewer blood vessels. Also apparent were areas of necrosis in which tumor cells close to the inflammatory cells had degenerated. Tumor infiltration of both mononuclear cells and neutrophils may participate in the killing of residual tumor cells, which suggests that one strategy to improve the efficacy of IRF-3-based therapy is to combine it with immunotherapy.

Expression of Cytokine mRNA in IRF-3-transduced B16 Melanoma Cells *in Vitro*. To test whether IRF-3 induced cytokine expression in B16 melanoma cells *in vitro*, RNase protection analysis was performed at various times after infection with Sendai virus, a well-known inducer of cytokine gene expression and activator of IRF-3 function (9, 24). Expression of *TNF- α* , *IL-6*, *IP-10*, and *IFN- β* mRNA was markedly enhanced in B16 cells expressing IRF-3. At their peak, 4–12 h after induction, IFN- β mRNA levels were enhanced more than a 1000-fold, compared with the AP2-B16 population (Fig. 4A, Lanes 10–12, and B), whereas in the AP-2 B16 cells, only a weak response to virus induction was observed (Fig. 4A, Lanes 2–7, and B), which suggests that IRF-3 expression reconstituted a defective IFN response. Although the magnitude of the response was decreased for TNF α and IL-6, the same enhanced production of mRNA was observed in the IRF-3-transduced B16 cells, and, again,

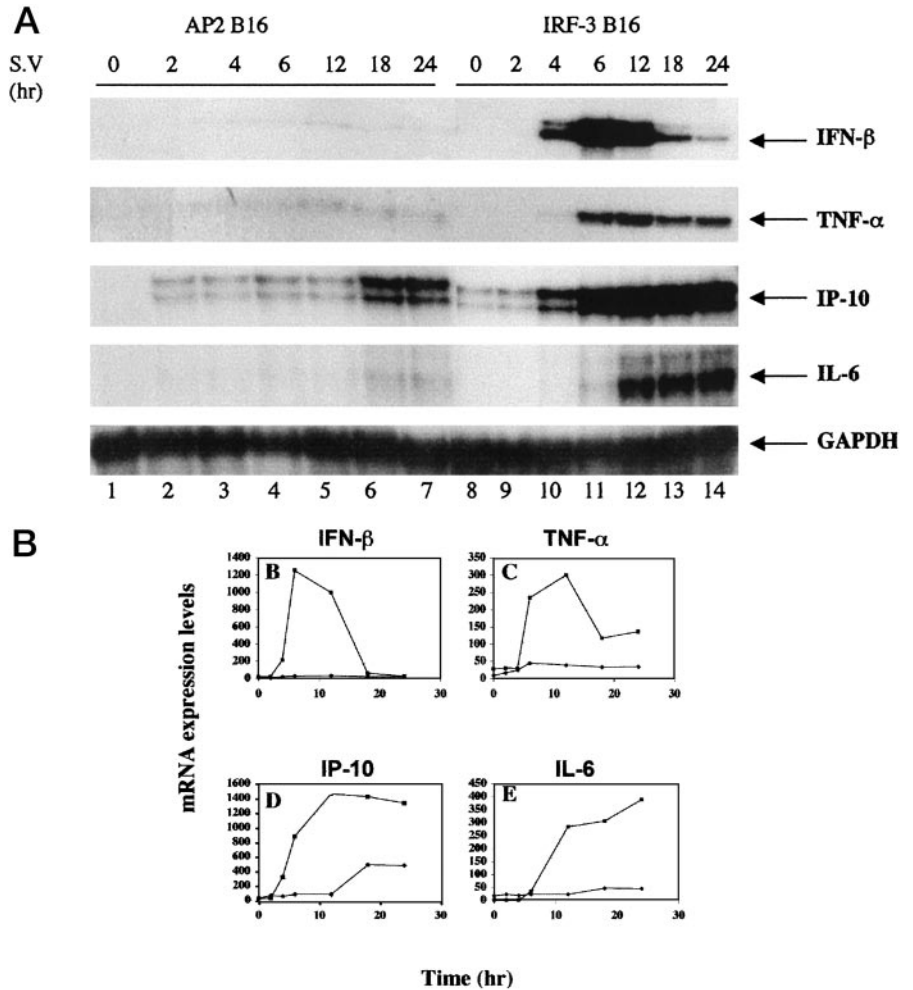


Fig. 4. Kinetics of cytokine mRNA expression in IRF-3-transduced B16 melanoma cells *in vitro*. In A, mock-transduced AP2 and IRF-3 B16 melanoma cells were infected with Sendai virus (40 HAU), and total RNA was prepared at different times after infection (0–24 h) as indicated above the lanes. Total RNA (10 μg) was subjected to RNase protection analysis using the mCK-3b and mCK-5 probe sets. The intensity of the bands was measured using NIH Image v1.6, and GAPDH protected probes were used for normalization. In B, the fold induction of IFNβ (section B), TNFα (section C), IP-10 (section D), and IL-6 (section E) mRNA was plotted for AP-2 B16 and IRF-3 B16 cells at each time point.

IRF-3 expression appeared to restore an otherwise deficient TNF and IL-6 response (Fig. 4, A, C, and E). The expression of Sendai-induced IP-10 was also enhanced in IRF-3-expressing B16 melanoma cells; IP-10 was induced at 18–24 h after virus induction in control B16 cells, (Fig. 4A, Lanes 6 and 7), whereas in IRF-3-expressing B16 cells, the kinetics and magnitude of IP-10 mRNA expression were dramatically enhanced, with mRNA induction beginning as early as 4-h postinfection and reaching a peak at 12 h postinfection (Fig. 4A, Lanes 10–14, and D). Together, these data indicate that IRF-3 expression reconstitutes a defective cytokine response pathway in B16 melanoma cells. The potential of IRF-3-transduced B16 cells to secrete these cytokines on *in vivo* stimulation could modulate the microenvironment of the tumor by attracting and activating inflammatory cell infiltration. Recent studies have demonstrated that in addition to viruses, multiple activators including lipopolysaccharide, cellular stress, and DNA damage can activate IRF-3 function (25). Therefore, it is conceivable that IRF-3 activation, albeit at a low level, may occur *in vivo* as a consequence of stress within the tumor microenvironment. Alternatively, simply increasing the intranuclear concentration of IRF-3 may be sufficient to increase the effect of IRF-3 on IFN and cytokine gene expression.

In summary, we used a syngeneic murine B16 tumor model to evaluate the capacity of transcription factor reprogramming of cytokine gene expression to modulate the tumorigenicity of a poorly immunogenic tumor model. Recruitment of inflammatory effector cells to the tumor microenvironment as a consequence of cytokine release may inhibit tumor cell growth and metastasis by directed

cell-mediated killing (26–28). The reconstitution of cytokine profiles in IRF-3-expressing B16 melanoma contributes to the enhanced recruitment of lymphocytes to the local tumor site compared with AP2-B16 in which cytokine induction remains defective. Our results demonstrate that transcription factor reprogramming of gene expression may be a potential therapeutic strategy to augment the host antitumor response.

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