

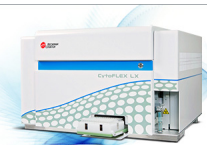


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# Enhancement of Human Melanoma Antigen Expression by IFN- $\beta$ <sup>1</sup>

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Although many immunotherapeutic investigations have focused on improving the effector limb of the antitumor response, few studies have addressed preventing the loss of tumor-associated Ag (TAA) expression, associated with immune escape by tumors. We found that TAA loss from human melanomas usually results from reversible gene down-regulation, rather than gene deletion or mutation. Previously, we showed that inhibitors of MAPK-signaling pathways up-regulate TAA expression in melanoma cell lines. We have now identified IFN- $\beta$  as an additional stimulus to TAA expression, including Melan-A/MART-1, gp100, and MAGE-A1. IFN- $\beta$  (but neither IFN- $\alpha$  nor IFN- $\gamma$ ) augmented both protein and mRNA expression of melanocytic TAA in 15 melanoma lines (irrespective of initial Ag-expression levels). Treatment of low Ag melanoma lines with IFN- $\beta$  increased expression of melanocyte-lineage Ags, inducing susceptibility to lysis by specific CTLs. Treatment with IFN- $\beta$  also enhances expression of class I HLA molecules, thereby inducing both nominal TAA and the presenting HLA molecule. Data from fluorescent cellular reporter systems demonstrated that IFN- $\beta$  triggers promoter activation, resulting in augmentation of Ag expression. In addition to enhancing TAA expression in melanomas, IFN- $\beta$  also stimulated expression of the melanocytic Ag gp100 in cells of other neural crest-derived tumor lines (gliomas) and certain unrelated tumors. Because IFN- $\beta$  is already approved for human clinical use in other contexts, it may prove useful as a cotreatment for augmenting tumor Ag expression during immunotherapy. *The Journal of Immunology*, 2007, 179: 2134–2142.

Successful immune recognition of tumors depends on both induction of effector mechanisms, such as CTL, and specific recognition of peptide/HLA complexes on the tumor cells. Immune eradication of tumors can thus be thwarted by either failure to induce and implement effective immunity and/or loss of tumor Ag expression. Tumors progressing to an increasingly malignant phenotype frequently display significant alterations in their gene expression profiles (1). Genetic instability in tumors may lead to mutations and/or loss of gene sequences that may alter or prevent protein expression. Many melanoma tumor Ags are encoded by melanocyte-specific genes involved with melanocyte differentiation and melanin biosynthesis such as Melan-A/MART-1, tyrosinase, and gp100 (2, 3). These autosomal genes are located on different chromosomes, greatly diminishing the probability of simultaneous loss of gene expression of all such markers by irreversible deletion or mutation. However, we have noted previously (4, 5) that a series of target melanocytic genes remains structurally intact in melanoma cells even when their transcription is very low, suggesting that genetic regulatory perturbations can influence their

expression. Melanomas which lose expression of any one melanocytic marker have a high probability of associated expression loss of a whole suite of other melanocytic genes (4–6). Such linkage of expression is consistent with a coordinated program for gene regulation within differentiated melanocytic cells. Although expression of such genes is often essentially undetectable by protein-based measures such as flow cytometry, basal transcription of intact mRNAs can be routinely demonstrated with high-sensitivity RT-PCR, indicating that the Ag-expression deficit does not result from physical genetic alterations. Such observations are the basis for the proposal that these changes result from alterations in gene regulation relevant to the melanocytic lineage, which is consistent with the available data (4, 5). The melanocytic gene master regulator MITF-M is also consistently down-regulated in the great majority of Ag-loss melanoma cells we have examined (4, 5, 7). Furthermore, we have reported previously that manipulation of the MAPK-signaling pathway with MEK inhibitors results in significant up-regulation of Ag gene expression in initially low-expressor cells, thereby demonstrating that such expression defects are amenable to intervention (7). Ironically, MITF-M expression is actually down-modulated by this MEK inhibitor-driven enhancement of expression of melanosome Ags (7).

Accordingly, we are developing strategies focused on restoration of gene expression to improve therapeutic targeting of tumor cells. Following screening of a panel of chemical and biological mediators, we identified IFN- $\beta$  as a candidate tumor Ag-augmenting agent. Notably, IFN- $\alpha$  did not enhance melanocyte differentiation Ag expression. IFN- $\beta$  is also known to up-regulate expression of the HLA class I genes essential for presentation of Ag to effector CTLs. This dual capability of IFN- $\beta$  to enhance expression of both components of peptide/HLA class I complexes could make it a useful adjunct therapy for improving T cell targeting of tumor cells.

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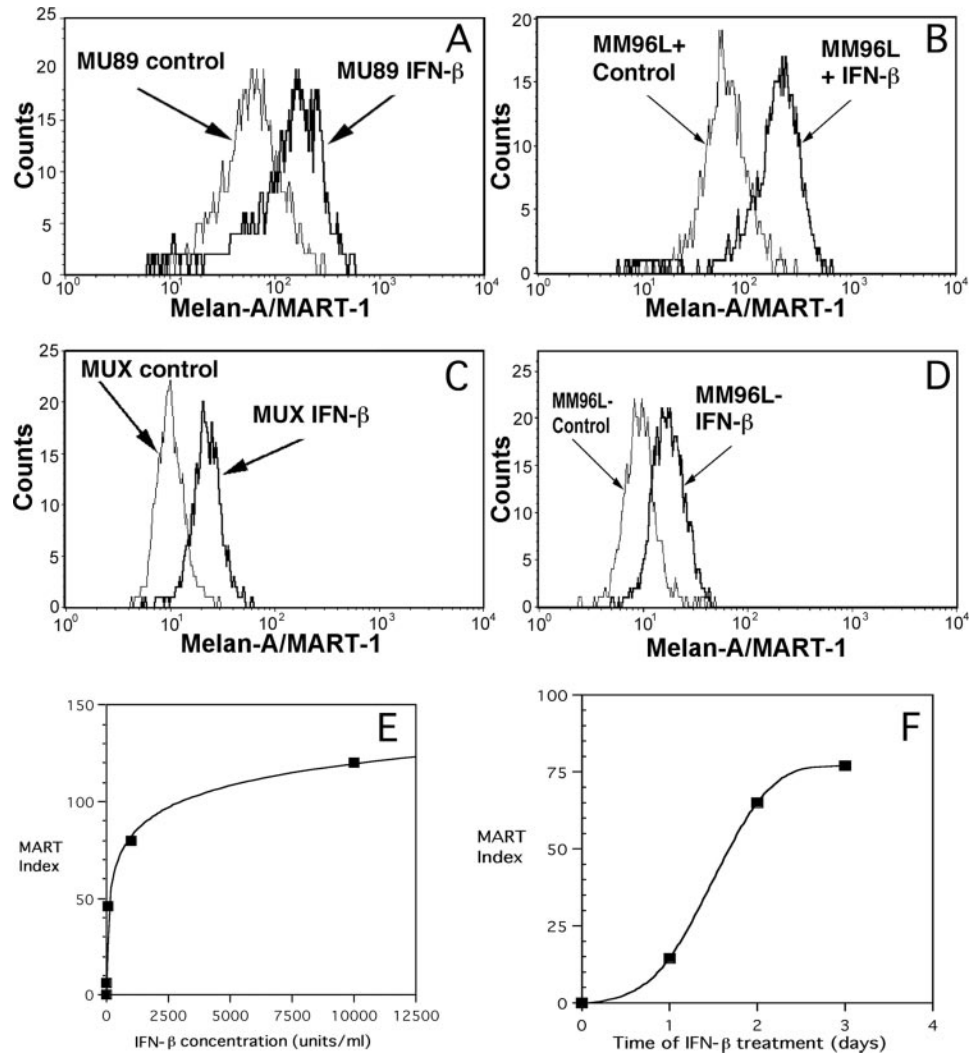
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**FIGURE 1.** A–F, Up-regulation of Melan-A/MART-1 expression after 3 day treatment of melanoma cell lines with IFN- $\beta$ . High expressor cells (MU89, MM96L<sup>+</sup>; A and B) and low expressor cells (MUX, MM96L; C and D) were treated with 10,000 U/ml IFN- $\beta$ , or were untreated (Control). They were stained with A103 Ab for cytoplasmic Melan-A/MART-1 expression on day 3. All show IFN- $\beta$ -induced augmentation of Melan-A/MART-1 expression, as demonstrated by FACS analysis. E, Dose response of IFN- $\beta$  on MU89 cells for Melan-A/MART-1 (MART), assayed 3 days after initiation of treatment. F, Time course of effect of IFN- $\beta$  on MU89 cells for Melan-A/MART-1 (MART), assayed with 5,000 U/ml; y-axes show expression indices as for Table I.

## Materials and Methods

### Cell culture and cytokines

General culture conditions for cell propagation and the origins of most of the specific cell lines used in this article have been previously described (4, 5). Briefly, melanoma cell lines were established from patient tumors (4). Ag<sup>-</sup> derivatives were established by limiting dilution cloning with or without exposure to CTLs (4). These CTLs were isolated from tumor-infiltrating lymphocytes from a melanoma patient as described (8). In brief, a bulk culture was generated by growing the tumor-infiltrating lymphocytes in the presence of IL-2, and IL-2 responsiveness was maintained by periodic restimulation of the activated T cells using an irradiated feeder layer of PBMC and the polyclonal activator, PHA. Clones were generated by limiting dilution and specificity for Melan-A/MART-1 was confirmed (8). Glioma cell lines U87 and U118, the osteosarcoma cell line U2-OS, HeLa cells, and HEK293 cells were obtained from the American Type Culture Collection. Although gliomas have been reported to express melanosome-associated Ags, as an indication of their shared neural crest origins, and can even be targeted by CTL specific for gp100 (9), the finding of gp100 on the U2-OS cells prompted us to confirm the osteosarcoma origin of this cell line. Accordingly, the osteosarcoma-specific Runx2 transcription factor (10) was clearly expressed in the osteosarcoma cells, as demonstrated by RT-PCR, but was not detectable in several melanoma cell lines (data not shown). Tumor lines were cultured in DMEM containing 10% FBS at 37°C in 5% CO<sub>2</sub>. IFN- $\beta$ -1a (Avonex) was obtained from Biogen-Idec; all IFN- $\alpha$  subtypes were purchased from PBL Biomedical Laboratories; IFN- $\gamma$  was provided by Genzyme. Cytokines were reconstituted according to the manufacturer's recommendations.

### Flow cytometry

Staining and flow cytometric analyses of cytoplasmic Melan-A/MART-1 and gp100 expression were performed as described previously (4). Briefly,

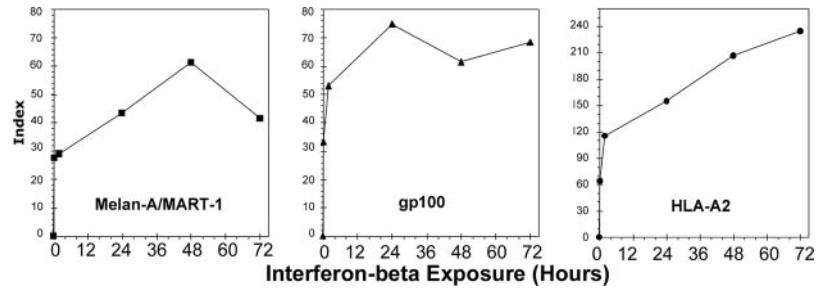
the adherent cell lines were trypsinized, fixed with paraformaldehyde, and permeabilized with mild detergent. Cells were stained appropriately and read in a FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest (BD Biosciences). Live cells were gated and the geometric mean fluorescence of Ab histograms was used for generating response indexes. In most cases, Ag levels were assayed after 3-day culture with or without treatments.

### Quantitative and normal RT-PCR

Primers and probe sequences for Melan-A/MART-1 and gp100 have been previously described (7), with the exception of: cyclin-dependent kinase 2 (CDK2)<sup>3</sup>: CDK2 forward (F), CGC TGG CGC TTC ATG GAG AAC T; CDK2 reverse (R), CTC CTG GCC ACA CCA CCT CAT CT; diacylglycerol kinase (DGKA): DGKA.F, GAA GCT GCC TTT CTG GCC ATC CT; DGKA.R, GCA GGA AAC ATC ATT GAG ACC ACC ACA; HLA-A quantitative PCR (Q-PCR): HLAA-Q.F, GAG TAT TGG GAC CAG GAG ACA C; HLAA-Q.R, ACG TCG CAG CCA TAC ATT ATC; HLA-A probe FAM, FAM-AAT GTG AAG GCC CAG TCA CAG ACT G-BHQ1; gp100 Q-PCR: SILV-Q.F, TCT GGG CTG AGC ATT GGG; SILV-Q.R, AGA CAG TCA CTT CCA TGG TGT GTG; SILV probe FAM, FAM-CAG GCA GGG CAA TGC TGG GC-TAMRA. All PCR procedures were detailed previously (7). Briefly, for Q-PCR, the reactions were performed and optimized (five replicates) using Brilliant QPCR Master Mix and the Mx4000 Multiplex Quantitative PCR System (Stratagene). Data were analyzed with the Mx4000 Software Package, normalized relative to the level of  $\beta$ -actin mRNA control, and adjusted such that values are relative to that untreated cells with a value of 1.0.

<sup>3</sup> Abbreviations used in this paper: CDK2, cyclin-dependent kinase 2; DGKA, diacylglycerol kinase; Q-PCR, quantitative PCR; CTA, cancer testis Ag; TAA, tumor-associated Ag; TIL, tumor-infiltrating lymphocyte.

**FIGURE 2.** Effect of transient exposure to IFN- $\beta$  on melanoma Ag expression. MM96L<sup>+</sup> cells were exposed to IFN- $\beta$  for the times indicated, and then fresh medium without IFN- $\beta$  was added. All of the cells were plated at the same time and density and were assayed on day 3 of the experiment. Cells were analyzed by flow cytometry and the index was calculated from the geometric mean of fluorescence for each Abs as for table 1. Cells were stained with Abs to Melan-A/MART-1, gp100, and HLA class I as indicated.



### Reporter assays/GFP reporter cell lines

A 1200-bp segment of human genomic DNA, containing core and extended regions of Melan-A/MART-1 promoter was obtained by PCR with a proof-reading polymerase system and cloned upstream of enhanced GFP coding sequence (BD Clontech) with a downstream SV40 polyadenylation signal. A control plasmid had the same structure except for replacement of the Melan-A/MART-1 promoter by the SV40 early promoter. Constructs were confirmed by sequencing and transfected into desired cell lines by means of Fugene-6 (7), under selection for G418 resistance conferred by a separate cotransfected plasmid. Antibiotic-resistant transfected cells were tested for expression of GFP by flow cytometry. Transfected cells were purified by limiting-dilution cloning and screened for GFP activity. Amplified populations of these cloned cells were confirmed as deriving from the original parental cell line by examining expression of a panel of melanocytic markers.

### Preparation of cell extracts and Western blotting

Melanoma cell extracts (PhosphoSafe Extraction Buffer; Novagen) were electrophoresed in reducing 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad) saturated with StartingBlock Blocking Buffer (Pierce) containing 0.5% Tween 20. Blots were incubated with 1/2000 dilution of primary Ab to phosphorylated Tyr<sup>701</sup> STAT1 (Cell Signaling Technologies) and then further incubated with HRP-conjugated secondary Ab (Pierce). Bound Abs were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

### Cytotoxicity assays

The assay for T cell-mediated cytotoxicity using <sup>51</sup>Cr release from tumor target cells has been described in detail elsewhere (4). Briefly, tumor cells were labeled with <sup>51</sup>Cr and cocultured for 4 h with varying numbers of CTLs, including a clone specific for Melan-A/MART-1 as previously described (4). The supernatants were removed and counted in a gamma counter for released radioactivity. To assess the effect of cytokines on cell lysis, tumor target cells were cultured in the presence of 5000 U/ml IFN- $\beta$  or IFN- $\gamma$  for 3 days before labeling with <sup>51</sup>Cr in parallel with unstimulated cells. Percent lysis is calculated using the formula: (lysed experimental – spontaneous lysis)/(maximal lysis – spontaneous lysis)  $\times$  100%. Data were collected for three replicates of experimental and six replicates of spontaneous and maximal lysis.

## Results

### Augmentation of melanocytic Ag expression by IFN- $\beta$ but not IFN- $\alpha$

Melanoma cell lines were classified for Ag expression status by Ab staining and flow cytometry (7). When the Ag-positive melanoma lines MU89 and MM96L<sup>+</sup> were treated with IFN- $\beta$ , substantial increases in expression of Melan-A/MART-1 were demonstrated by flow cytometric analysis (Fig. 1, A and B; a significant effect was defined as an expression increase of  $\geq$ 25%). This effect was not limited to cells with high levels of Ag expression, as the very low Ag expressors MUX and MM96L<sup>-</sup> also showed pronounced shifts mediated by IFN- $\beta$  in the mean channel fluorescent peaks for melanocyte differentiation Ags (Fig. 1, C and D). The dose response to IFN- $\beta$  reached 80% of the maximal level above 1000 U/ml and plateaued between 5,000 and 10,000 U/ml (Fig. 1E). Higher levels of IFN- $\beta$  induced significant toxicity, precluding effective measurement of Ag status. The Ag augmentation elicited

by IFN- $\beta$  was insignificant at 24 h, detectable at 48 h, and reached a plateau beyond 72 h (Fig. 1F). Although full Ag induction thus took several days, transient (15 min) treatments of target melanoma cells with IFN- $\beta$  followed by removal of the cytokine still allowed marked Ag up-regulation when flow assays were performed at 72 h (Fig. 2). This was observed for HLA class I as well as melanocytic Ags (Fig. 2).

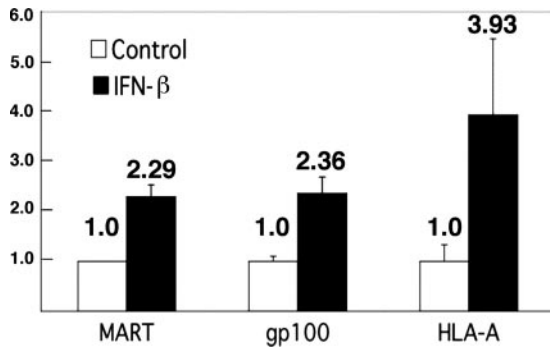
IFN- $\beta$  responses were assessed in a panel of 15 cell lines, including six lines each corresponding to high and low melanocytic Ag expression, and three lines which were negative for Melan-A/MART-1 expression but positive for gp100. All but one of these cell lines (K2) showed significant up-regulation of HLA class I in response to IFN- $\beta$  treatment (Table I). Although K2 cells showed no Melan-A/MART-1 response mediated by IFN- $\beta$ , they did exhibit elevated gp100, suggesting that type I IFN signaling was still taking place despite certain defects in downstream target genes. Significant responses for gp100 were seen with 11 of 15 cell lines in this panel, and 9 of 15 of these cell lines showed significant Melan-A/MART-1 responses (Table I). However, only two cell lines in the panel (H59-44T and 435A) failed to show significant augmentation of either Ags (Table I). Therefore, in a large majority of cell lines, IFN- $\beta$  was able to increase the expression of at least one melanoma differentiation Ag known to be recognized by the immune system.

Because IFN- $\beta$  and the family of IFN- $\alpha$  cytokines (expressed from a gene cluster of 13 separate members expressing 12 unique

Table I. Responses of melanoma cell lines to IFN- $\beta$ <sup>a</sup>

Melanoma Cell Line	Ag Status	MART Index	gp100 Index	HLA Class I Index
MU89	+	175.6	112.8	278.2
ML	-	93.8	140.0	532.8
MUX	-	90.8	175.4	168.2
MM455	M <sup>-</sup> /G <sup>+</sup>	61.1	52.3	101.2
A375	-	60.5	92.5	109.2
WM164	+	47.3	5.8	114.6
MM96L <sup>-</sup>	-	31.2	49.7	53.5
MM96L <sup>+</sup>	+	30.2	17.8	98.4
Roth	M <sup>-</sup> /G <sup>+</sup>	27.9	4.9	96.7
Mel-Juso	M <sup>-</sup> /G <sup>+</sup>	19.7	51.1	36.5
LH	-	3.4	45.4	84.5
Chollar	-	3.1	59.6	138.4
H59-44T	+	-0.7	20.2	235.8
453A	+	-6.3	7.4	143.2
K2	+	-9.1	77.8	-7.5

<sup>a</sup> An index was calculated to reflect the protein changes observed and is equivalent to the percent change in protein level, such that a 2-fold increase in protein level compared to untreated control equals an index of 100. Index = (IFN- $\beta$  response – untreated control response)/(untreated control response)  $\times$  100; where “response” = geometric mean fluorescence value from fluorescence histogram plots generated by flow cytometry with the indicated Ab. Indices are ranked by magnitude of Melan-A/MART-1 (MART) values. Control and treated cells were assayed by flow cytometry for Melan-A/MART-1, gp100, and HLA class I. M<sup>-</sup>/G<sup>+</sup> = positive for gp100 but not Melan-A/MART-1. Underlined values show responses that fail to reach the 25% increase judged to be significant.



**FIGURE 3.** Effect of IFN- $\beta$  on mRNA levels of melanoma Ags (Melan-A/MART-1 and gp100) and HLA-A, assessed by quantitative PCR. Shown are measurements of mRNA steady-state levels in MUX cells following 3-day treatments with control medium or with 5000 U/ml IFN- $\beta$ . Results are calculated from the threshold cycle number in each case, normalized to the corresponding values for  $\beta$ -actin; and then shown relative to untreated control values which were assigned a baseline of 1.00. Averages and SDs are calculated from triplicate samples.

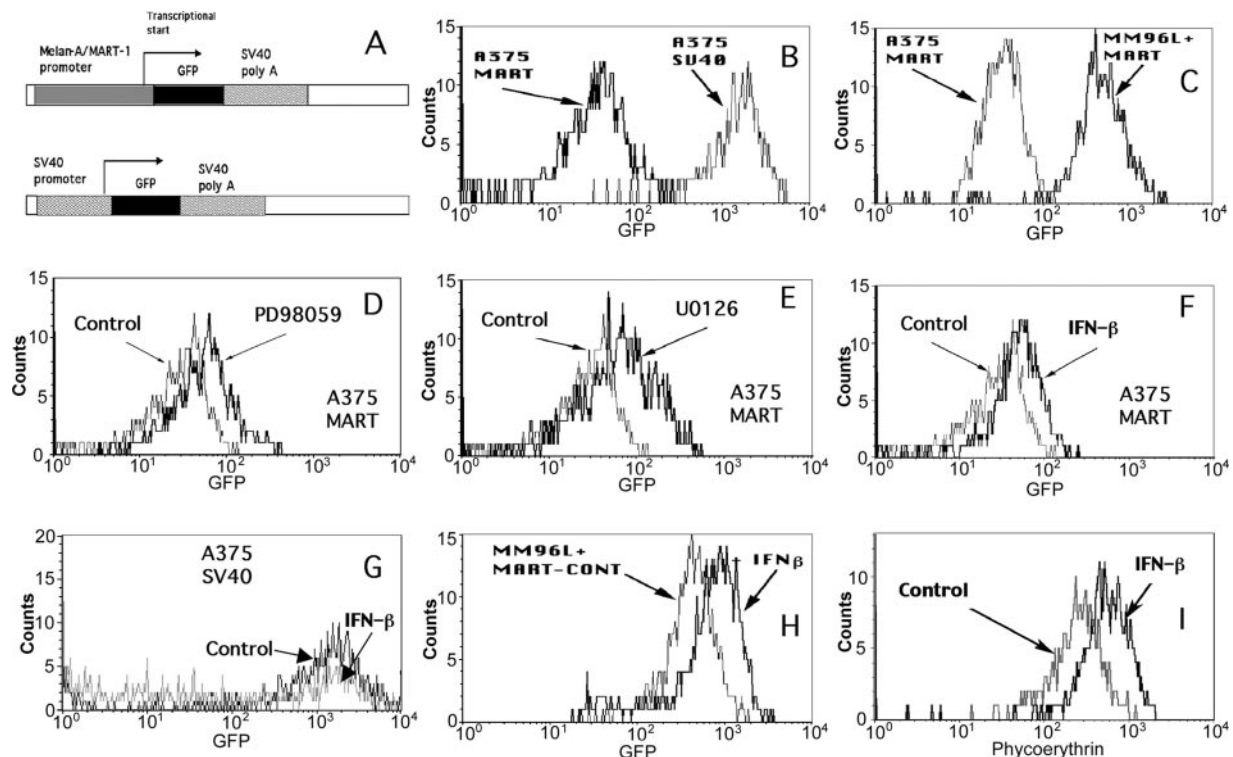
proteins (11)) use the same type I receptor (albeit with divergent signaling effects, (12, 13)), we tested whether IFN- $\alpha$  elicited comparable effects on melanocyte differentiation Ag expression. In assays using conditions where IFN- $\beta$  elicited strong activity, no significant effects were seen with any of the 12 human IFN- $\alpha$  isoforms (IFN- $\alpha$  A, 4b, B2, C, D, F, G, H, I, J, K, WA; data not

shown). Five of the tumor lines were stimulated with IFN- $\gamma$ , and all of the cell lines showed >2-fold enhancement of HLA class I (data not shown). However, with respect to Melan-A/MART-1 and gp100, none showed significant increases in Melan-A/MART-1, and only MU89 cells showed a strong response with respect to gp100 (enhanced 68%). In fact, other work has shown that IFN- $\gamma$  can reduce Ag expression in melanomas (14).

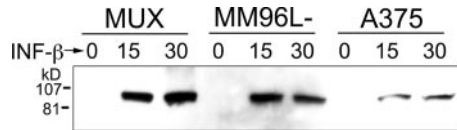
In addition to enhancing protein expression, treatment of low Ag MUX cells with IFN- $\beta$  resulted in consistent increases in steady-state levels of mRNAs for both Melan-A/MART-1 and gp100, as assessed by quantitative RT-PCR (Fig. 3). As a positive control, we demonstrated the known ability of IFN- $\beta$  to stimulate class I HLA mRNA expression (15, 16) (Fig. 3).

#### Mechanism of IFN- $\beta$ effects

To extend our study of the mode of action of IFN- $\beta$  on melanocytic genes, we initially used a GFP reporter system to investigate the effect of IFN- $\beta$  on the Melan-A/MART-1 promoter. A 1200-bp segment of the Melan-A/MART-1 promoter was therefore used to drive expression of GFP in stable melanoma cell lines. As a control, the Melan-A/MART-1 promoter was replaced by the SV40 early promoter (Fig. 4A). This SV40 promoter is constitutively expressed, and allowed the efficient production of GFP in the low-Ag cell line A375. In contrast, the production of the GFP reporter from the Melan-A/MART-1 promoter in the same cell line was far weaker, although still detectable (Fig. 4B). The



**FIGURE 4.** Responses of GFP reporter driven by the Melan-A/MART-1 promoter in stably transfected melanoma cells: effects of IFN- $\beta$  and MEK inhibitors on GFP reporter expression. *A*, Structures of transfected constructs with GFP expressed from a 1.2-kb Melan-A/MART-1 promoter segment or the SV40 early promoter. *B*, Fluorescence levels of A375-Melan-A/MART-1-GFP reporter cells compared with A375 cells transfected with GFP under the control of the constitutively active SV40 promoter. *C*, Fluorescence levels of A375-Melan-A/MART-1-GFP cells compared with MM96L<sup>+</sup> Melan-A/MART-1-GFP cells. *D* and *E*, Augmentation of GFP fluorescence from A375 Melan-A/MART-1-GFP reporter cells treated with 40  $\mu$ M PD98059 and 20  $\mu$ M U0126, respectively. *F*, Enhancement of GFP fluorescence from A375-Melan-A/MART-1-GFP cells treated with IFN- $\beta$ . *G*, Unchanged GFP fluorescence after treatments of A375 reporter cells, where GFP expression is driven by the SV40 promoter. *H*, Enhanced GFP fluorescence from MM96L<sup>+</sup> Melan-A/MART-1-GFP cells treated with IFN- $\beta$ . *I*, Augmentation of endogenous Melan-A/MART-1 expression by IFN- $\beta$  in the same transfected MM96L<sup>+</sup> cells as in *H*, using flow cytometry with PE. All treatments were for 3 days; all IFN- $\beta$  treatments used 5000 IU. MART, Melan-A/MART-1; CONT, control.



**FIGURE 5.** Effect of IFN- $\beta$  on JAK/STAT signaling pathways. Cells as shown were treated with IFN- $\beta$  (5000 U). For each lane of the gel, 15  $\mu$ g of total cellular protein was run on 10% SDS-PAGE and subjected to Western blotting with Ab against activated STAT1 (phosphorylated Y701). Lanes for each cell line show samples from untreated cells (0), and IFN- $\beta$ -treated samples (15 and 30 min).

activity of the Melan-A/MART-1 promoter in the reporter construct closely paralleled the endogenous levels of the protein itself, as demonstrated by the widely divergent levels of Melan-A/MART-1-reporter expression observed between low Ag A375 and high Ag MM96L<sup>+</sup> cells (Fig. 4C). The MEK inhibitors PD98059 and U0126 were previously shown to augment Melan-A/MART-1 expression (7), and these inhibitors also augmented GFP levels driven by the Melan-A/MART-1 promoter in the A375 reporter cells at previously established doses (7) (Fig. 4, D and E). Treatment of the latter cells with IFN- $\beta$  produced a comparable level of enhancement (Fig. 4F). In contrast, GFP expression driven by the SV40 promoter was unaffected by IFN- $\beta$  (Fig. 4G).

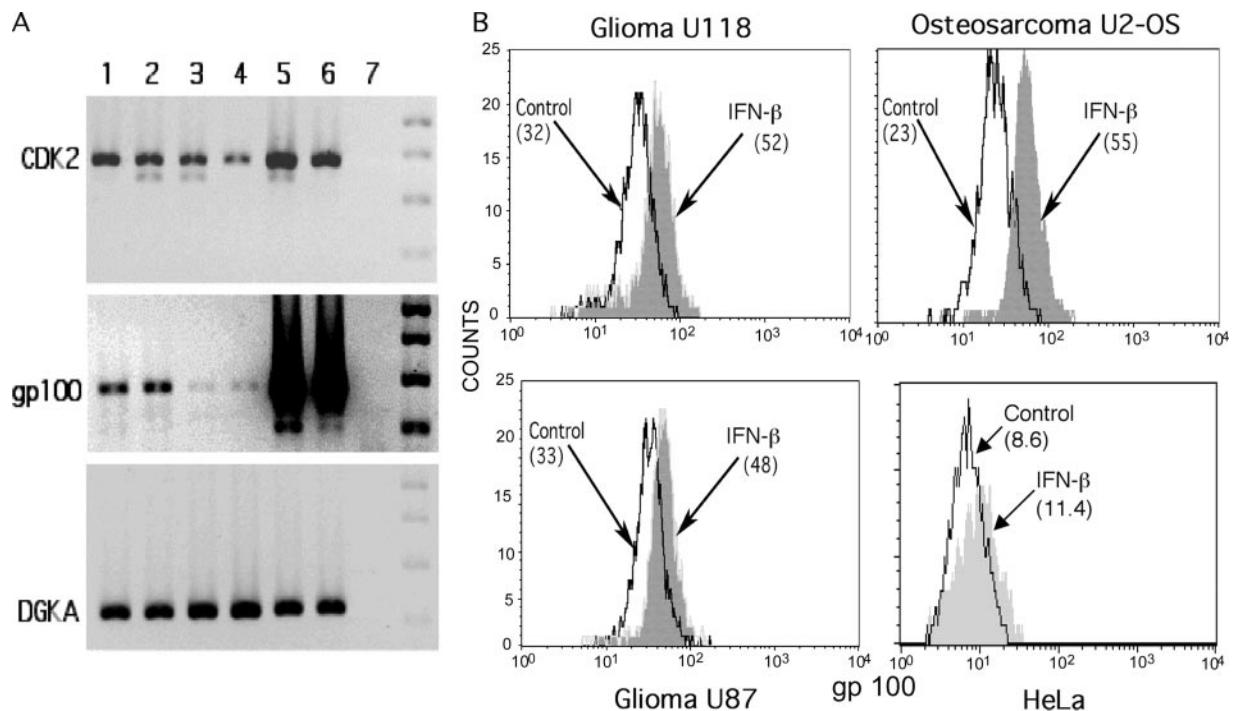
To address the possibility that the transfected cell lines had been altered in their responsiveness to IFN- $\beta$ , we also tested whether the endogenous *Melan-A/MART-1* gene was enhanced concomitantly with the reporter gene after cytokine treatment. As evidenced by the Ag-positive MM96L<sup>+</sup> transfectants with the Melan-A/MART-1-GFP construct, both the reporter system and the endogenous gene responded equally well to stimulation with IFN- $\beta$  (Fig. 4, H

and D). This confirmed that the cellular reporter systems recapitulated the responses of the endogenous *Melan-A/MART-1* gene.

IFN- $\beta$  signaling has been linked with activation of the JAK-STAT pathway (13). Therefore, we tested for activation of this pathway in melanoma cells known to respond to IFN- $\beta$  with Ag up-regulation. Activation of STAT1 was clearly detectable after short exposure of cells to IFN- $\beta$  (Fig. 5). The cells used as examples in Fig. 5 are Ag negative; Ag-positive cells responded in a similar manner (data not shown).

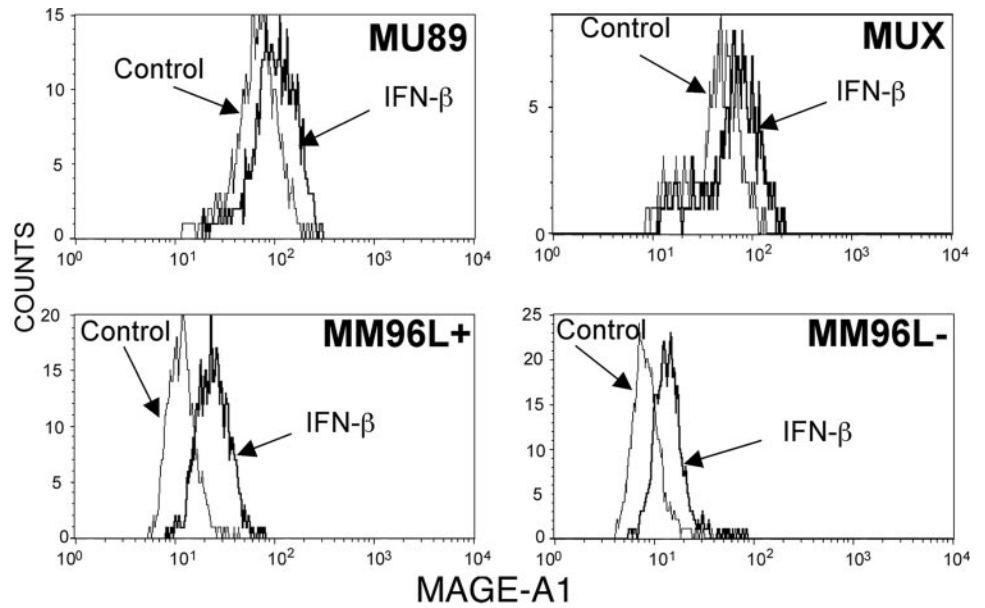
#### Enhancement of a melanocytic Ag in nonmelanocytic tumor cell lines

Differentiation-related genes are normally tightly regulated, with expression in inappropriate cell lineages effectively silenced (17), but these control mechanisms are often compromised or relaxed in transformed cells. We have noted that low but detectable expression of gp100 is more often observed in nonmelanocytic cells than for other melanocytic-associated genes (Fig. 6A and our unpublished data). One contributing factor to this effect may be the local configuration of the gp100 gene on chromosome 12, where it is sandwiched between two other genes (*CDK2* and *DGKA*) transcriptionally active in a wide range of cell types (Fig. 6A). The promoters for the *CDK2* and gp100 gene overlap, and despite data that did not confirm a correlation of their mutual expression (18, 19), a common influence of the melanocytic transcription factor MITF-M has been proposed (20). Regardless of the specific control mechanisms involved, the chromatin region encompassing the *gp100* gene may therefore persist in a more accessible state than would otherwise be the case for melanocytic genes in nonmelanocytic lineages. Combined with dysregulation of normal gene control processes, this might contribute to the more widespread aberrant basal transcription of



**FIGURE 6.** A, Expression of CDK2, gp100, and DGKA assessed by RT-PCR and gel electrophoresis. (Note: equal quantities of RNAs were processed in each case). Lanes: 1, U2-OS; 2, MG63; 3, SAOS-2; 4, MM96L<sup>-</sup>; 5, MM96L<sup>+</sup>; 6, 453A; 7, no-template control. Lanes 1–3 are osteosarcoma lines, 4 is a low Ag melanoma line, and 5 and 6 are high Ag-expressing melanomas. (The gp100 panel is shown at a darker exposure relative to the others to help visualize the fainter bands). B, Enhanced gp100 expression of glioma, osteosarcoma, and HeLa cell lines treated with IFN- $\beta$ . Indicated cell lines were stained with HMB45 (gp100) Ab after 3 days with and without IFN- $\beta$  treatment (5000 IU; shaded and open histograms, respectively). Numbers in parentheses indicate geometric mean of fluorescence values.

**FIGURE 7.** Effects of IFN- $\beta$  on MAGE-A1 expression in two Ag-positive (MU89, MM96L<sup>+</sup>) and Ag-negative (MUX, MM96L<sup>-</sup>) cell lines, as demonstrated by FACS analysis. Cells were treated with 5000 U/ml IFN- $\beta$  or untreated (Control), and stained with Abs for cytoplasmic MAGE-A1 expression on day 3.



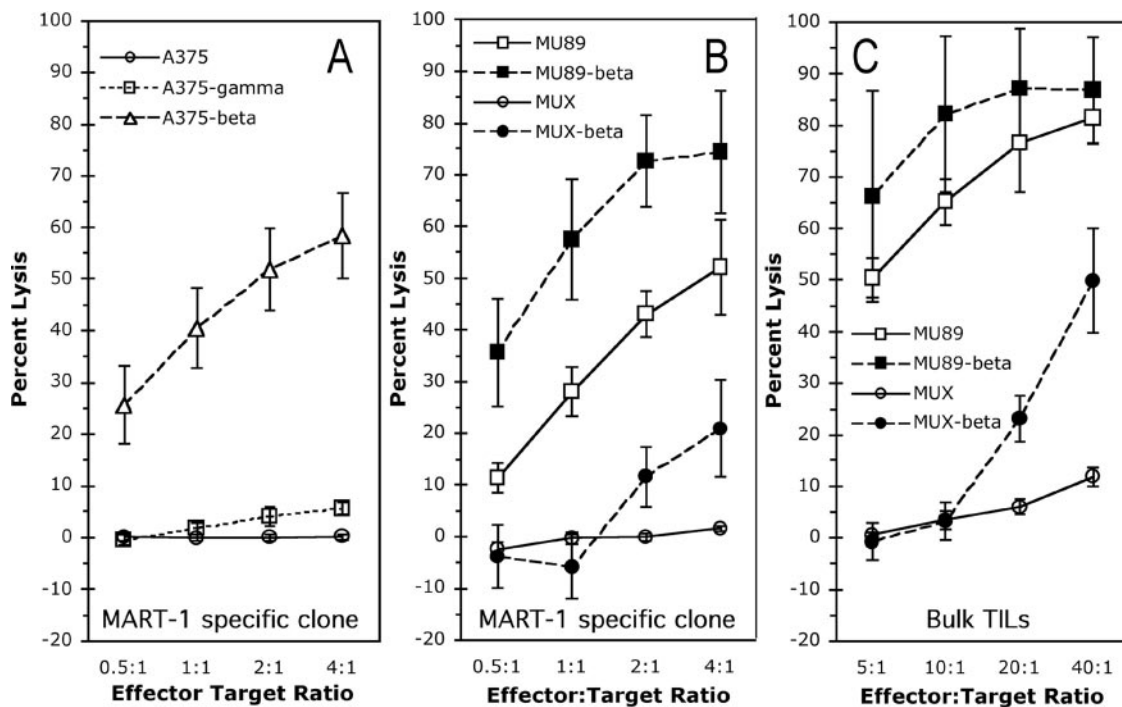
gp100 in diverse tumors, which in turn could render gp100 susceptible to agents already known to up-regulate its expression in melanocytic cells. Accordingly, we tested additional nonmelanocytic tumor types: gliomas, an osteosarcoma (U2-OS), the cervical carcinoma line HeLa, and the transformed human kidney cell line HEK293, for augmentation of melanocytic genes by IFN- $\beta$ .

Both glial cells and melanocytes are derived from the neural crest (21), consistent with the increased gp100 expression in two tested glioma cell lines treated with IFN- $\beta$  (Fig. 6B). However, some tumor cells with a completely different embryonic origin (U2-OS, HeLa) also responded (Fig. 6B), but the transformed kidney cell line HEK293 did not (data not shown), despite showing

detectable baseline gp100 expression at a higher level than HeLa cells. It was notable that HEK293 cells expressed readily detectable HLA class I which also failed to show augmentation by IFN- $\beta$  (data not shown), indicating a possible defect in the IFN signal transduction pathway in this cell line.

*Effects of IFN- $\beta$  on cancer testis Ags*

Cancer testis Ags (CTAs) are X-linked tumor-associated Ags (TAAs) which are expressed in many tumor types, including melanomas (22). It was of interest to test whether IFN- $\beta$  modulated expression of CTAs in melanoma cells, because the regulatory pathways controlling the CTA expression are likely to be divergent



**FIGURE 8.** Effect of IFN- $\beta$  on the killing of melanoma cells by CTL. Cells as indicated (A375, A; MU89 and MUX, B and C) were treated with 1000 U of IFN- $\beta$  for 3 days, and then labeled with <sup>51</sup>Cr and tested as targets in cytotoxicity assays. Both a Melan-A/MART-1-specific T cell clone (A and B) and IL-2 propagated tumor (melanoma) infiltrating lymphocytes (“Bulk TILs”; C) were used as the effector cells. Percent lysis = 100 × (experimental – spontaneous)/(maximum – spontaneous). The average and SD of three replicates is shown.

from those controlling melanocytic genes. Accordingly, we also tested whether IFN- $\beta$  affected the expression of a representative CTA, MAGE-A1, and found that IFN- $\beta$  significantly up-regulated MAGE-A1 by levels approximating those seen for Melan-A/MART-1 (Fig. 7).

#### *Augmentation of T cell cytotoxicity against melanoma Ags by IFN- $\beta$*

Our principal aim is to augment Ag expression in low-expressor tumor cells in the hopes that we can restore immune recognition by cytotoxic T effector cells. Thus, we assessed the impact of IFN- $\beta$  treatment on specific T cell-mediated cytotoxicity. For this purpose, we used a cytotoxic T cell clone derived from tumor-infiltrating lymphocytes (TILs) which recognizes a Melan-A/MART-1 peptide (AAGIGILTV) in the context of HLA-A2 (8). Enhancement of killing of both low Ag cells A375 (Fig. 8A) and MUX (Fig. 8B), and high Ag MU89 (Fig. 8B), was observed as a consequence of pretreatments with IFN- $\beta$  (Fig. 8). Treatment of the same A375 tumor target cells with IFN- $\gamma$  did not increase their recognition by MART-1-specific CTLs recognition of the treated A375 targets (Fig. 8A), although IFN- $\gamma$  did enhance expression of class I-MHC (data not shown). In addition to Melan-A/MART-1-specific killing, the "bulk" culture from which the clone was derived showed even greater enhancement of killing against IFN- $\beta$ -treated targets than did the clone (Fig. 8C), although at different target to effector ratios. Indeed, while the bulk culture contains Melan-A/MART-1-specific CTL (8), there are additional undefined specificities present. Because we have demonstrated up-regulation of several TAA following IFN- $\beta$  treatment, it is to be expected that killing against additional target Ags would be further enhanced if the CTL contained multiple specificities.

## Discussion

Successful antitumor immunotherapy is contingent upon a number of independent factors, including the induction of a vigorously effective immune response against relevant tumor Ags (23, 24). Recent studies have also stressed the importance of manipulating homeostatic mechanisms to circumvent down-regulation of the antitumor response (25, 26). Likewise, the loss of target Ags on tumor cells will render them invisible to even the strongest and most specific immune responses. Previous results have indicated that apparent Ag loss from melanomas is indeed frequent and a major immunotherapeutic hurdle (6, 27–29), but we have found that this loss is most commonly associated with gene regulatory changes which do not alter the structural genes for the Ags involved (4, 5). Because the coding sequences and promoters for such Ags remain intact, the possibility exists for reconstituting the immune recognition of tumor cells by up-regulating Ag expression. Inhibition of the MAPK-signaling pathway with MEK inhibitors can enhance Ag expression and T cell-mediated recognition of treated tumors (7). A search for additional agents which enhance tumor Ag expression revealed that IFN- $\beta$  exhibited these desired properties with the further advantage of simultaneous up-regulation of HLA class I Ag expression.

Although originally discovered through their antiviral effects, type I IFNs (IFN- $\alpha$ , IFN- $\beta$ , IFN- $\epsilon$ , IFN- $\kappa$ , IFN- $\delta$ , IFN- $\omega$ , IFN- $\tau$ , limitin/IFN- $\zeta$ ) have a diverse range of biological activities (11). A common property of each type I member, however, is their engagement with the same two proteins which constitute the IFN type I receptor, IFN- $\alpha$ R1 and IFN- $\alpha$ R2 (11, 12). At the same time, the signaling processes which ensue following engagement of type I IFNs with their common receptors diverge among different members of this class, resulting in overlapping, but nonredundant, biological consequences (13). Specific residues (which vary between

respective type I IFNs) interfacing with the type I receptor evidently determine the precise nature of the downstream signaling response (30, 31), and the affinity of the specific type I IFN-receptor engagement may be the crucial factor dictating signaling outcome (32). Receptor-ligand complexes for different type I IFNs in turn show different induction patterns of potential target genes (33, 34), consistent with the finding that the Ag-enhancing properties of IFN- $\beta$  are not shared by the IFN- $\alpha$  subfamily of type I IFNs.

In vitro modulation of cultured tumor cells by type I IFNs has been previously documented. Treatment of melanoma cells with IFN- $\beta$  elicits antiproliferative effects (35, 36) and apoptosis (37). Some early reports noted increased melanogenesis in cultured melanomas in response to IFN- $\beta$  (14, 38), but did not further characterize these effects. More recently, both IFN- $\alpha$  and IFN- $\gamma$  have been shown to be effective in inducing class I HLA, but with limited effectiveness for Ag induction (39). Furthermore, IFN- $\gamma$  sometimes down-regulates Ag expression (40), consistent with our own results.

The kinetics of Ag enhancement by IFN- $\beta$  are revealing, in that initial triggering can be demonstrated after as little as 15 min, even if the IFN- $\beta$  is removed thereafter (Fig. 2). However, it still takes days for the full expression of the enhanced protein expression (Fig. 1), although the continued presence of IFN- $\beta$  appears to be largely superfluous after the initial stimulatory events elicit a presumptive downstream cascade. Because early events following IFN- $\beta$  receptor engagement could thus determine a relatively prolonged response, we accordingly confirmed that the JAK/STAT pathway is activated in melanoma cells (Fig. 5), as previously found in various cell types (13). Many other details of the mechanism of Ag expression enhancement through IFN- $\beta$  remain to be elucidated. It is noteworthy that the p38 MAPK stress pathway has been found to be of major significance in IFN- $\beta$  signaling (41, 42), while we have demonstrated that inhibition of the MAPK/ERK-signaling pathway promotes Ag up-regulation (7). Investigation of this possible linkage between the MAPK pathway, IFN- $\beta$  signaling, and Ag up-regulation is currently being pursued.

Of particular importance, specific cytotoxic recognition of tumor cells will benefit from renewed expression of target Ags, given functional Ag processing and HLA class I presentation of antigenic peptide epitopes. Type I IFNs have the property of boosting HLA class I expression (15, 16), and IFN- $\beta$  may be a stronger class I up-regulator in melanoma cells than IFN- $\alpha$  (16), although a less potent stimulator of HLA class I and class II than IFN- $\gamma$ . Clearly, the ability of a cytokine treatment to simultaneously augment both Ag and class I HLA increases the likelihood of improved immune recognition and cytotoxic killing of tumor targets. Despite its superior stimulation of HLA class I Ag expression (16), IFN- $\gamma$  is a poor inducer of melanocytic Ags (Ref. 39 and this work), and may even repress Ag expression in some tumors (40). Our own experience has shown that stimulation of A375 cells with IFN- $\gamma$  fails to elicit the enhanced cytotoxicity induced by IFN- $\beta$ .

Enhancement of cytotoxicity toward murine melanoma cells has been claimed by cotreatment with IFN- $\gamma$  and IFN- $\beta$ , which was attributed to induction of B7-1, ICAM-1, and MHC class I (43). Studies with knockout mice indicate a role for IFN- $\beta$  in retarding tumor development (44). However, a recent elegant study in a murine system has provided evidence for an important role of type I IFNs in antitumor responses which eventually select for poorly immunogenic tumor cells ("cancer immunoediting"; Ref. 45). The role of IFN- $\alpha$ /IFN- $\beta$  in this process was found to be at the level of host hemopoietic cell targets rather than tumor cells themselves (45). In this context, it is important to note that human and murine biological responses to IFN- $\beta$  cannot be assumed to be equivalent.



For example, murine IFN- $\beta$  has been found to be less effective in inducing an antiproliferative response on murine tumor cells than human IFN- $\beta$  is on human tumor targets (46).

IFN- $\beta$  is currently approved for use in humans in the treatment of multiple sclerosis (47). Although early clinical experience with IFN- $\beta$  in melanoma treatment was unpromising (48, 49), local or intralesional application of IFN- $\beta$  as an adjuvant melanoma therapy has recently provided more encouraging outcomes (50). Our current results suggest the potential benefit of IFN- $\beta$  as an adjunct to immunotherapy of melanoma to optimize tumor targeting. Beyond melanoma, it is possible that other tumor types will be amenable to immunotherapeutic strategies using IFN- $\beta$  to enhance Ag expression. We have shown that gp100 Ag can be up-regulated by IFN- $\beta$  in neural crest tumors and at least some tumors from other embryonic lineages (Fig. 6). Some earlier evidence also has suggested that the antigenic phenotypes of breast carcinoma and astrocytoma cells can be modified by IFN- $\beta$  treatments (51, 52), and dramatic antitumor activity has been noted in a patient with ovarian cancer treated with immunotherapy and adenovirally expressed IFN- $\beta$  (53). The diverse nature of such cellular IFN- $\beta$  responses suggested that the up-regulatory process was either dependent on normal ubiquitous factors, or factors aberrantly expressed in each of these tumor cell lines. The positive effect of IFN- $\beta$  on expression of the cancer testis Ag MAGE-A1 (Fig. 7) also argues that nonmelanocyte-specific factors are involved. In contrast, unresponsiveness (as seen with HEK293 cells) could result from absence of such cofactors, or loss of IFN type I receptor functional expression.

The proposed use of IFN- $\beta$  in the therapy of tumors would obviously benefit from any direct antiproliferative effect on target cells, but it is important to note that the effectiveness of IFN- $\beta$  as an adjunct to immunotherapy will be dependent on its ability to augment tumor differentiation Ags as well as enhancing HLA class I expression. The activity of IFN- $\beta$  on both melanocytic Ags and at least one CTA (Table I and Fig. 7) is encouraging in this regard. The ability to stimulate an array of TAA indicates that IFN- $\beta$  may be particularly useful when the effector cells contain multiple specificities, as noted in broader reactivity of the bulk vs the cloned cells, giving cause to use multivalent Ags for vaccination, or T cells with multiple reactivities in adoptive immunotherapy approaches. Also, the observation that prolonged exposure of tumor cells to IFN- $\beta$  is not required to achieve Ag augmentation (Fig. 2) is a potential boon to immunotherapy, where drug-related toxicity makes it clinically undesirable to maintain continuous high cytokine levels.

Because type I IFN signal transduction is complex, it is possible that antiproliferative signals are not directly linked with the induction of Ag expression. Of course, "IFN resistance" may limit the therapeutic impact of cytokine therapy, and while loss of type I receptors would negate the effects of cytokine, unresponsiveness of melanoma cells to type I IFNs has been frequently correlated with changes in internal signaling pathway components rather than receptor loss (54). Consequently, lack of antiproliferative effects may not necessarily correlate with negation of other IFN- $\beta$  consequences of type I receptor engagement. Thus, the use of IFN- $\beta$  to enhance immunotherapy is not dependent on the antiproliferative effect of the cytokine, and indeed could operate in the absence of it. We propose that IFN- $\beta$  could have a complementary role in a variety of immunotherapy approaches, including vaccine and adoptive immunotherapy protocols.

## Disclosures

Current employee (part- or full-time) or contractor Consultancies, employment, service on Board of Directors, honoraria, royalties, research support,

grants, or contracts in excess of \$5,000 per year in any of the preceding five years Stock or equity interests (diversified mutual funds or investment trusts do not constitute competing financial interests) Additional Comments: JTK, PJD, and LBR have equity interests in CytoCure, LLC. ISD, LBR, TJH, DB and DBM are or were employees of CytoCure, LLC.

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