Melanoma Cells Exhibit Variable Signal Transducer and Activator of Transcription 1 Phosphorylation and a Reduced Response to IFN-α Compared with Immune Effector Cells

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Abstract

Purpose: IFN-α is administered to melanoma patients and its endogenous production is essential for immune-mediated tumor recognition. We hypothesized that a reduced capacity for signal transducer and activator of transcription (STAT) 1 activation allows melanoma cells to evade the direct actions of IFN-α.

Experimental Design: Tyr^{701}-phosphorylated STAT1 (P-STAT1) was measured by flow cytometry in IFN-α–stimulated human melanoma cell lines, melanoma cells derived from patient tumors, and peripheral blood mononuclear cells (PBMC). Expression of other Janus-activated kinase (Jak)-STAT intermediates (STAT1, STAT2, Jak1, tyrosine kinase 2, IFN-α receptor, STAT3, and STAT5) was evaluated by flow cytometry, immunoblot, or immunohistochemistry.

Results: Significant variability in P-STAT1 was observed in human melanoma cell lines following IFN-α treatment ($P < 0.05$) and IFN-α–induced P-STAT1 correlated with the antiproliferative effects of IFN-α ($P = 0.042$). Reduced formation of P-STAT1 was not explained by loss of Jak-STAT proteins or enhanced STAT5 signaling as reported previously. Basal levels of P-STAT3 were inversely correlated with IFN-α–induced P-STAT1 in cell lines ($P = 0.013$). IFN-α–induced formation of P-STAT1 was also variable in melanoma cells derived from patient tumors; however, no relationship between P-STAT3 and IFN-α–induced P-STAT1 was evident. Because IFN-α acts on both tumor and immune cells, we examined the ability of IFN-α to induce P-STAT1 in patient-derived melanoma cells and PBMCs. IFN-α induced significantly lower levels of P-STAT1 in melanoma cells compared with matched PBMCs ($P = 0.046$). Melanoma cells and human melanocytes required 10-fold higher IFN-α doses to exert P-STAT1 levels comparable with PBMCs.

Conclusions: Melanoma cells are variable in their IFN-α responsiveness, and cells of the melanocytic lineage exhibit a lower capacity for IFN-α–induced Jak-STAT signaling compared with immune cells.

IFN-α is used in the treatment of patients with metastatic malignant melanoma and is currently the only therapy approved for use as an adjuvant following surgical resection of high-risk melanoma lesions (1–5). Endogenous production of this cytokine has also been shown to regulate the ability of immune cells to recognize developing tumors (6). The Janus-activated kinase (Jak)-signal transducer and activator of transcription (STAT) signal transduction pathway is activated in both immune effector cells and tumor cells in response to IFN-α stimulation (7, 8). On binding to its receptor, IFN-α activates Jak1 and tyrosine kinase 2 (Tyk2), which phosphorylate tyrosine residues within the cytoplasmic portion of the IFN-α receptor (IFNAR). These phosphotyrosine residues provide docking sites for the STAT family of cytoplasmic transcription factors that are phosphorylated by the Jaks (9). These signal transduction events result in the formation of IFN-stimulated gene factor 3, a DNA-binding complex that consists of STAT1α (or STAT1β), STAT2, and IFN regulatory factor 9 (IRF9; ref. 8).

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IFN-stimulated gene factor 3 translocates to the cell nucleus and binds to IFN-stimulated response elements located in the promoter regions of IFN-stimulated genes (10).

We have shown that STAT1 signal transduction in host immune cells is important for mediating the antitumor effects of exogenous IFN-α; however, this cytokine can also act directly on tumor cells to exert antiproliferative, proapoptotic, and antiangiogenic effects (11–14). Importantly, STAT1 is thought to play a key role in promoting these direct effects of IFN-α on the tumor cell, and defects in Jak-STAT signal transduction intermediates have been associated with an IFN-α–resistant phenotype in melanoma cells (15–17). IFN-α action can also be regulated by other transcription factors. For example, constitutive phosphorylation of STAT3 is prevalent in human tumors of multiple origin and may influence the response to chemotherapy-induced apoptosis (18) and STAT1-dependent inflammatory gene activation (19). In addition, overexpression of STAT5 may contribute to IFN resistance of melanoma cells (20). Thus, activation of signal transduction in melanoma cells by IFN-α is complex and regulated by multiple factors. We hypothesized that a reduced capacity for STAT1 activation allows melanoma cells to evade the antiproliferative properties of endogenously produced or exogenously administered IFN-α.

We examined STAT1 phosphorylation (Tyr^{701}) as a direct marker of IFN-α action in melanoma cells. IFN-α–induced phosphorylated STAT (P-STAT) 1 levels were highly variable in melanoma cells following IFN-α stimulation and correlated with the antiproliferative properties of this cytokine. Interestingly, the variable induction of P-STAT1 by IFN-α was not explained by loss of Jak-STAT signaling protein expression or enhanced STAT5 signaling as reported in previous studies of IFN-α–resistant melanoma cell lines. Basal STAT3 phosphorylation was inversely correlated with IFN-α–induced P-STAT1 in a panel of melanoma cell lines but not in melanoma cells derived from patient tumors. Because IFN-α acts on both the tumor and immune cell compartments, the induction of P-STAT1 by IFN-α was examined in patient-derived melanoma cells and peripheral blood mononuclear cells (PBMC) from the same patients. These studies revealed that IFN-α induced significantly lower levels of P-STAT1 in melanoma cells compared with matched PBMCs. Together, these data highlight the heterogeneous response of melanoma cells to the direct actions of IFN-α and show that cells of the melanocytic lineage require higher doses of IFN-α to achieve a level of signal transduction comparable with IFN-α–stimulated immune cells.

Materials and Methods

Cell lines and reagents. The HT144, A375, SK-MEL-2, SK-MEL-5, Hs294T (human), and B16F1 (murine) cell lines were obtained from the American Type Culture Collection. The SK-MEL-33, 1259 MEL, 18105 MEL, 1106 MEL, MEL 39, 1174 MEL, and FO1 and human melanoma cell lines were a gift from Dr. Soldano Ferrone (Roswell Park Cancer Institute, Buffalo, NY). Primary human melanocytes from two individual donors were a kind gift from Dr. Z. Abdel-Malek (University of Cincinnati, Cincinnati, OH) and were maintained as described previously (21). Recombinant human IFN-α2b was purchased from Schering-Plough, Inc. Universal IFN-α (IFN-A/D) was purchased from R&D Systems, Inc. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to evaluate cell proliferation (American Type Culture Collection).

Patient samples and normal donors. All studies were approved by The Ohio State University Institutional Review Board (OSU99H0348). Tumor specimens were obtained from six consecutive patients with malignant melanoma that had undergone wide local resection of a recurrent, subcutaneous melanoma tumor. Peripheral blood was obtained from these same melanoma patients at the time of surgery. PBMCs from healthy donors were isolated directly from fresh peripheral blood Leukopacks (American Red Cross). All PBMCs were isolated via density gradient centrifugation with Ficoll-Paque Plus (Amersham Pharmacia Biotech).

Intracellular flow cytometry. Total levels of intracellular STAT1, STAT2, and Tyr^{701} P-STAT1 were measured by flow cytometry as described previously (22). Data were expressed as specific fluorescence (Fsp = Ft - Fb), where Ft represents the median value of total staining and Fb represents the median value of background staining with an isotype control antibody (22).

Patient-derived melanoma cell cultures. Patient tumor specimens were processed immediately following surgical excision for cell culture or immunohistochemical analyses. Fresh tumor tissue was minced in RPMI 1640 and 10% human AB serum (Pel Freez Clinical Systems) and processed to obtain a single-cell suspension. Tumor cells were cultured in complete RPMI 1640 to select for actively growing cells and eliminate contaminating stromal tissue that may have been present in the preparations before conducting flow cytometric or immunoblot analysis.

Immunohistochemical analysis. Freshly isolated patient tumor tissue was fixed in 10% formalin, paraffin-embedded and sectioned into 5 micron slices. A melanotic phenotype was confirmed by staining with S-100 and Melan-A antibodies (DAKO). Tumor sections were stained with antibodies against Jak1 (Cell Signaling Technology), STAT1 (BD Biosciences), STAT2 (Biosource International), or isotype control antibodies as described (23).

Immunoblot analysis. Lysates were prepared from normal PBMCs, melanoma cell lines, or melanoma cells derived from patients and assayed for the expression of Jak-STAT proteins by immunoblot as described previously (22, 24) with antibodies to STAT1, Jak1, IRF9, Tyk2, STAT5 (BD Biosciences), STAT2 (Biosource International), IFNAR (R&D Systems), STAT3, P-STAT3 (Cell Signaling Technology), or β-actin (Sigma).

Statistical analysis. Results were considered significant if improbable ($P < 0.05$) under the null hypothesis. The Kruskal-Wallis nonparametric procedure tested for significant variability between cell lines. The Levene test then compared variances within different cell lines. Hochberg's GT2 post-hoc comparison tested differences in means between cell lines or IFN-α dose levels while constraining the family-wise error rate ($P < 0.05$). Pearson correlations examined associations between STAT1, STAT2, and P-STAT1 levels and between P-STAT1 levels in IFN-α–stimulated PBMCs and tumor cells. Nonparametric Spearman's ρ correlated IFN-α–induced P-STAT1 with the antiproliferative effects of IFN-α. For comparison of IFN-α–induced P-STAT1 levels in cell lines with or without basal P-STAT3, log transformation was used to remove the skewness of the data and stabilize the variance across the cell lines. Linear effect models were used to determine whether there was a significant difference in the level of P-STAT1 in cell lines with or without basal P-STAT3. Paired sample t tests or nonparametric Wilcoxon tests as appropriate compared IFN-α sensitivity of PBMCs versus patient melanoma cells.

Results

Variable IFN-α–induced STAT1 phosphorylation in human melanoma cell lines. Phosphorylation of the Tyr^{701} residue on STAT1 is a critical event for its dimerization with other STAT proteins and its subsequent downstream effects (8, 10). To characterize the activation of Jak-STAT signal transduction in response to IFN-α, a panel of human melanoma cell lines was stimulated *in vitro* with IFN-α2b for 15 min and analyzed for

the formation of Tyr^{701} P-STAT1 by flow cytometry (See Supplementary Table S1 for further information on these cell lines). IFN-α stimulated the phosphorylation of STAT1 in each of the 12 cell lines that were tested (Fig. 1A and B). Maximal activation of STAT1 was observed following a 15-min stimulation with 10^4 to 10^5 units/mL IFN-α (Fig. 1A; data not shown). Multiple comparisons indicated that there was no significant difference between the two highest doses of IFN-α (10^4 and 10^5 units/mL) with respect to their ability to induce the phosphorylation of STAT1 ($P = 0.565$). However, both of these doses generated significantly greater levels of P-STAT1 than did IFN-α at 10^1 to 10^3 units/mL (Ps < 0.001). There was significant variance (χ^2s ≥ 22.90; $df = 11$; Ps ≤ 0.018) between cell lines with respect to the ability of IFN-α to induce the formation of P-STAT1 at each dose tested. The variable induction of P-STAT1 in response to IFN-α was validated in representative melanoma cell lines (SK-MEL-5, 1106 MEL, and 1259 MEL) by immunoblot analysis (Fig. 1C). Time course analysis indicated that activation of STAT1 occurred within minutes of exposure to IFN-α in both highly responsive and poorly responsive melanoma cell lines, began to decline at 60 min, and returned to near basal levels by 4 h (Fig. 1D; data not shown). The validity of P-STAT1 as an upstream marker of IFN-α action on melanoma cells was evaluated via an analysis of cell proliferation (Fig. 1E). A consistent pattern was observed in which cell lines displaying low IFN-α–induced STAT1 phosphorylation were least sensitive to the antiproliferative effects of IFN-α (i.e., SK-MEL-5 and 1174 MEL). Conversely, the cell lines with high levels of P-STAT1 following IFN-α treatment (i.e., SK-MEL-2 and Hs294T) were most sensitive to the antiproliferative effects of IFN-α (for 10^3 units/mL, $P = 0.042$; for 10^4 units/mL, $P < 0.001$). An evaluation of the ability of IFN-α to inhibit cell proliferation at the 10^2 units/mL dose level showed that only the Hs294T human melanoma cell line showed a marked inhibition of cell proliferation at the 48-h time point (42% growth inhibition versus PBS-treated cells). All other cell lines showed <20% inhibition of cell growth versus PBS-treated cells at this time point and dosage of IFN-α (data not shown).

Baseline levels of intracellular STAT1 and STAT2. We next investigated whether the variable IFN-α responsiveness of melanoma cell lines was due to reduced expression of STAT1 or STAT2. Total intracellular levels of STAT1 and STAT2 protein were measured in the 12 human melanoma cell lines via flow cytometry (Fig. 2). Comparison of STAT1 levels showed that there was statistically significant variance between individual cell lines ($\chi^2 = 31.75$; $df = 11$; $P < 0.001$). The variance in STAT2 levels was also statistically significant ($\chi^2 = 24.23$; $df = 11$; $P = 0.012$) but less than that of STAT1 [Levene test; W (1, 68) = 11.63; $P = 0.001$]. Of note, total baseline intracellular levels of STAT1 correlated with P-STAT1 levels only following stimulation of cell lines with high doses of IFN-α (10^5 units/mL; $r = 0.54$; $P = 0.035$), whereas at moderate doses (10^3-10^4 units/mL), total intracellular levels of STAT2 were correlated with P-STAT1 levels (for 10^3 units/mL, $r = 0.62$, $P = 0.016$; for 10^4 units/mL, $r = 0.51$, $P = 0.046$). These data indicate that baseline STAT protein levels in melanoma cells influence the degree to which P-STAT1 is induced following treatment with high doses of IFN-α.

Immunoblot analysis of Jak-STAT intermediates. To determine if the expression of other Jak-STAT intermediates could also contribute to the variability in IFN-α–induced P-STAT1, immunoblot analysis of IFNAR, Jak1, Tyk2, and IRF9 was conducted. Overall, no cell line tested lacked the expression of any target protein, and the expression of both the IFNAR and Jak1 proteins were relatively equal across the cell lines. In contrast, there was variable expression of Tyk2 and IRF9 in these cell lines (Fig. 3). The 1259 MEL, A375, and SK-MEL-33 cell lines had elevated levels of the Tyk2 protein, whereas levels of the IRF9 protein were lower in the FO1, SK-MEL-33, and Hs294T cell lines. However, across all cell lines, the expression of Tyk2 and IRF9 as measured by quantitative densitometry did not correlate with the ability of IFN-α to stimulate the phosphorylation of STAT1 ($P = 0.444$; data not shown). These data indicate that the variable response to IFN-α in this panel of melanoma cell lines was not a result of defective Jak-STAT protein expression.

Phosphorylation of STAT5 in melanoma cell lines. Overexpression of STAT5 has been shown to contribute to the IFN resistance of melanoma cells via the induction of negative regulatory proteins, such as cytokine-inducible SH2-containing protein (20). An examination of total STAT5 protein and IFN-α–induced STAT5 phosphorylation (at the Tyr^{694} residue) in the panel of melanoma cell lines revealed no relationship between the phosphorylation of STAT1 by IFN-α and STAT5 expression level, basal STAT5 phosphorylation, or IFN-α–induced P-STAT5 (Fig. 4A). These data do not exclude a role for STAT5 in regulating the actions of IFN-α on melanoma cells but they do suggest that additional factors are likely involved in regulating the level of STAT1 activation following exposure to IFN-α.

Phosphorylation of STAT3 in melanoma cell lines. Recent studies have identified a role for STAT3 in limiting the downstream effects of IFN-α–induced gene activation. Studies by Ho and Ivashkiv (19) in the THP-1 monocytic cell line showed that STAT3 sequestered STAT1 and suppressed the formation of DNA-binding STAT1 homodimers on IFN-α stimulation. Furthermore, STAT3 has been reported to be constitutively active in several cancers, including melanoma (25–27). We hypothesized that an altered phosphorylation profile of STAT3 in these melanoma cell lines may contribute to the variable level of IFN-α–induced P-STAT1. Basal P-STAT3 was observed in 8 of 12 melanoma cell lines (Fig. 4B). Six cell lines in this panel were derived from metastatic melanoma lesions (Supplementary Table S1) and all six displayed basal phosphorylation of STAT3 (Fig. 4B, *arrows*). This finding is in accordance with other studies implicating a role for STAT3 in regulating metastasis (25, 28–30). We next tested whether cell lines with basal phosphorylation of STAT3 exhibited differences in the level of IFN-α–induced P-STAT1 (as measured by flow cytometry). Using linear mixed effect models, we showed that P-STAT1 levels were significantly lower among cell lines with basal P-STAT3 compared with cell lines without basal P-STAT3 following high doses of IFN-α ($P = 0.0135$ at 10^4 units/mL; $P = 0.005$ at 10^5 units/mL). These data should be interpreted with caution as the MEL 39 cell line (derived from a metastatic lesion) was highly responsive to IFN-α despite exhibiting basal phosphorylation of STAT3. Of note, rapid phosphorylation of STAT3 was observed in 10 of 12 melanoma cell lines following IFN-α stimulation (10^4 units/mL, 15 min; Fig. 4B), but activation of this transcription factor also did not correlate with the level of IFN-α–induced P-STAT1.

IFN-α–induced STAT1 phosphorylation in melanoma cells derived from patient tumors. It was postulated that the inherent

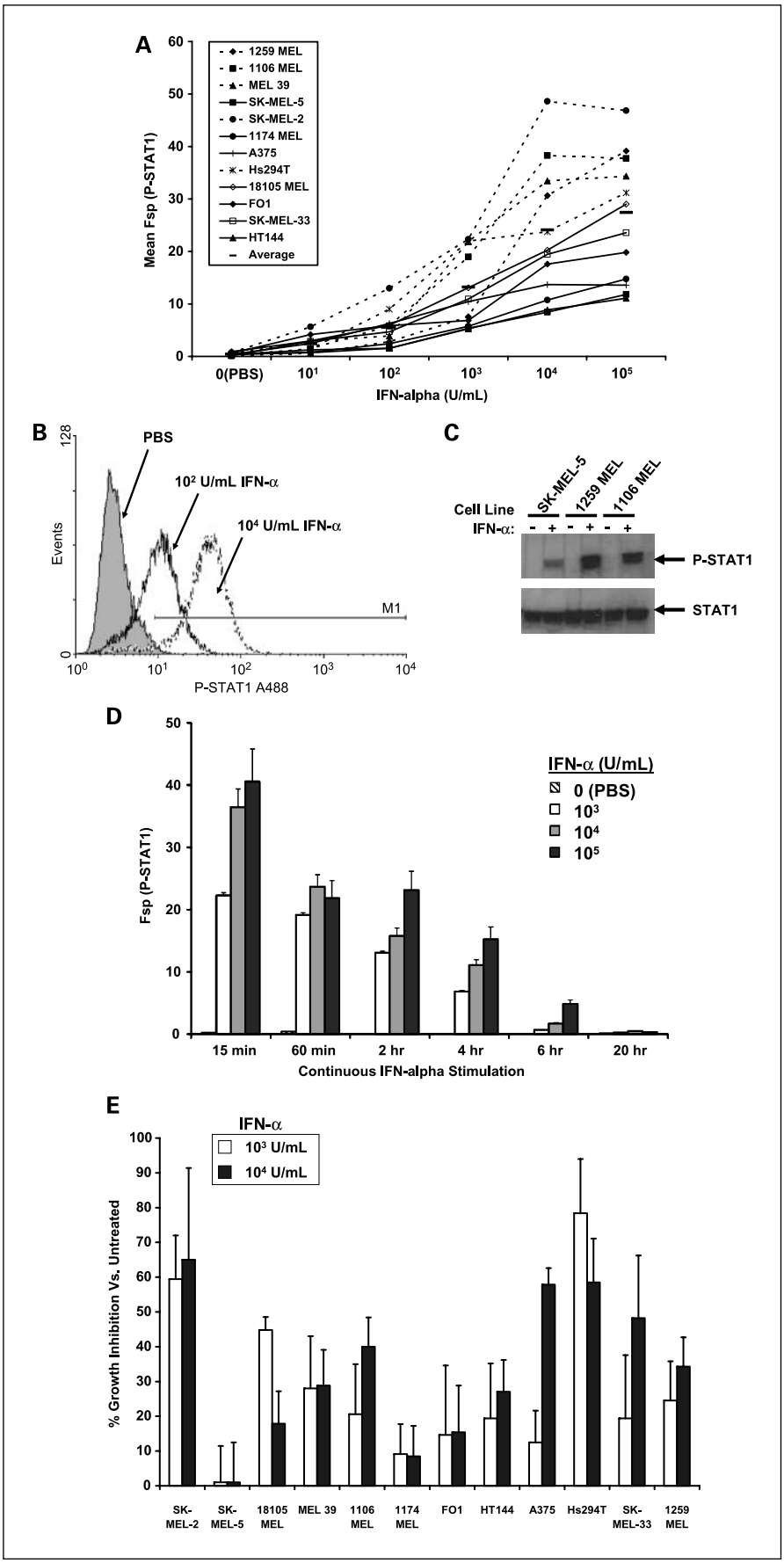


Fig. 1. STAT1 phosphorylation and the antiproliferative properties of IFN-α in melanoma cell lines. *A,* human melanoma cell lines ($n = 12$) were stimulated with IFN-α or PBS (15 min); P-STAT1 levels were measured by flow cytometry. Data are presented as specific fluorescence intensity of P-STAT1 staining (Fsp). Individual curves were derived from the mean of three replicates per cell line. Horizontal bars, mean Fsp of P-STAT1 at each dose level across all cell lines measured. *B,* representative histograms from 1106 MEL melanoma cells following a 15-min stimulation with IFN-α. X-axis, fluorescence of P-STAT1. Shaded histogram, PBS-stimulated cells (negative control). Voltage was set using an isotype control antibody (M1). *C,* lysates were prepared from representative IFN-α–insensitive (SK-MEL-5) or IFN-α–sensitive (1259 MEL and 1106 MEL) melanoma cell lines following a 15-min stimulation with PBS (-) or IFN-α (+; 10^3 units/mL) and subjected to immunoblot analysis with an antibody directed against P-STAT1. Nitrocellulose membranes were stripped and reprobed with total STAT1 antibody to control for equal loading. *D,* representative time course of STAT1 phosphorylation following IFN-α stimulation. Human 1106 MEL cells were analyzed for P-STAT1 levels at several time points following stimulation with PBS or IFN-α. *E,* human melanoma cell lines were cultured with IFN-α (10^3-10^4 units/mL) for 48 h. Cell proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay. Data are the percentage of growth inhibition compared with PBS-treated cells (set at 100%).

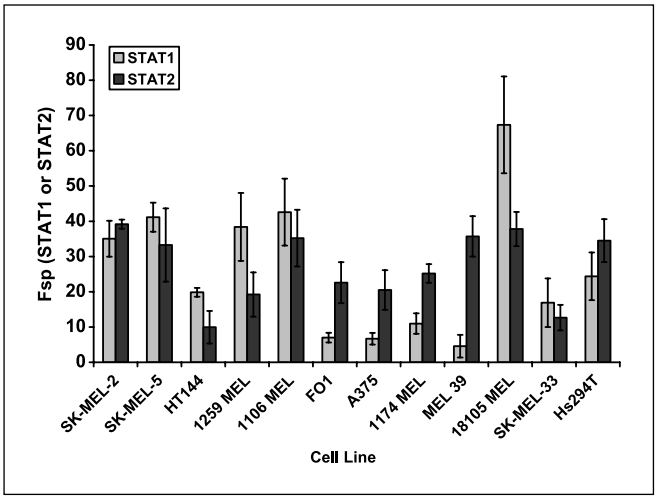


Fig. 2. STAT1 and STAT2 protein levels in human melanoma cell lines. Human melanoma cell lines were stained for intracellular STAT1 or STAT2 protein and analyzed by flow cytometry. Columns, mean STAT1 and STAT2 levels (Fsp) from triplicate experiments; bars, SD.

differences in IFN-α–induced P-STAT1 would also be evident in melanoma cells derived from patient tumors. To this purpose, cells derived from recurrent, cutaneous melanoma tumors were evaluated for levels of P-STAT1 following a 20-min *ex vivo* stimulation with IFN-α. A summary of tumor characteristics and the prior treatment history of each patient ($n = 6$) is presented in Table 1. Four of the six patients had received prior therapy with IFN-α. The diagnosis of malignant melanoma was confirmed by histologic analysis of paraffin-embedded tumor sections (HMB-45$^+$, S100$^+$, and Melan-A$^+$; data not shown) and nested PCR analysis of tyrosinase expression in cell lines derived from these tumors (Supplementary Fig. S1). Immediately following surgical excision, tumors from each patient were processed into single-cell suspensions and established in culture. Within 1 week of establishing these initial cultures, flow cytometry was done on the resulting cell suspensions to examine IFN-α–induced STAT1 phosphorylation (Fig. 5A). Of note, IFN-α induced very low levels of P-STAT1 in five of the six patient tumor cell lines.

Analysis of Jak-STAT intermediates in melanoma cells derived from patient tumors. To determine whether low levels of IFN-α–induced P-STAT1 observed in patient tumor preparations were the result of defective expression of Jak-STAT proteins, we examined the tumoral expression of STAT1, STAT2, IFNAR, Jak1, Tyk2, and IRF9 (Fig. 5B and C). Due to limited sample material, analyses of tumor cells from patients 1 and 2 were restricted to initial flow cytometric and immunohistochemical analysis of tumor tissue for STAT1, STAT2, and Jak1 (Fig. 5A and B). These tumor cell preparations displayed a strong cytoplasmic expression of STAT1, strong nuclear and cytoplasmic expression of STAT2, and positive staining for Jak1 (Fig. 5B). Consistent with data in melanoma cell lines (please see Figs. 2 and 3), no gross defects in the expression of Jak-STAT signal transduction intermediates were observed in any of the remaining tumors. The levels of IFNAR, STAT2, Tyk2, and IRF9 were consistent across all tumors, whereas the expression of Jak1 and STAT1 were slightly higher in the tumors from patients who exhibited greater levels of activated STAT1 (patients 3, 4, and 6; Fig. 5C). These data suggest that defective expression of Jak-STAT signaling proteins was not the reason for reduced sensitivity of patient tumor cells to IFN-α.

Basal phosphorylation of STAT3 in melanoma cells derived from patient tumors. The levels of basal and IFN-α–induced P-STAT3 were next examined in the 2- to 3-week expanded cultures of melanoma cells derived from patient tumors. Basal levels of P-STAT3 were detected only in cultures derived from patients 4 and 6, whereas cells from all four patients displayed further increases in P-STAT3 following stimulation with IFN-α (10^4 units/mL; Fig. 5D). In contrast to the data obtained in the 12 established melanoma cell lines, no direct relationship between basal levels of P-STAT3 and IFN-α–induced P-STAT1 was observed in patient cell lines (Fig. 5D). Moreover, no consistent relationship between levels of IFN-α–induced P-STAT3 and P-STAT1 was evident in patient tumor cells under study. Interestingly, on the 2- to 3-week expansion in culture, one patient melanoma cell line (derived from patient 6) regained sensitivity to lower doses of IFN-α (10^2 units/mL) as determined by immunoblot analysis (Fig. 5D). These results underscore the limitations associated with data obtained from cell lines and the need for studies on freshly isolated clinical samples. Based on these additional data, the role of STAT3 in regulating the level of IFN-α–induced signal transduction in melanoma cells remains unresolved.

Comparison of IFN-α–induced P-STAT1 between melanoma cells and immune effector cells. To date, a direct comparison of IFN-α–induced STAT1 activation between the melanoma cell and immune effector cell populations has not been reported previously. This is an important comparison given the ability of IFN-α to exert both potent antiproliferative effects on melanoma cells, the stimulatory properties of IFN-α on immune effector cells, and the apparent need for high doses of IFN-α in effective antitumor regimens (31). To this purpose, we conducted a matched comparison of tumor cells and PBMC derived from each of the six melanoma patients in this study

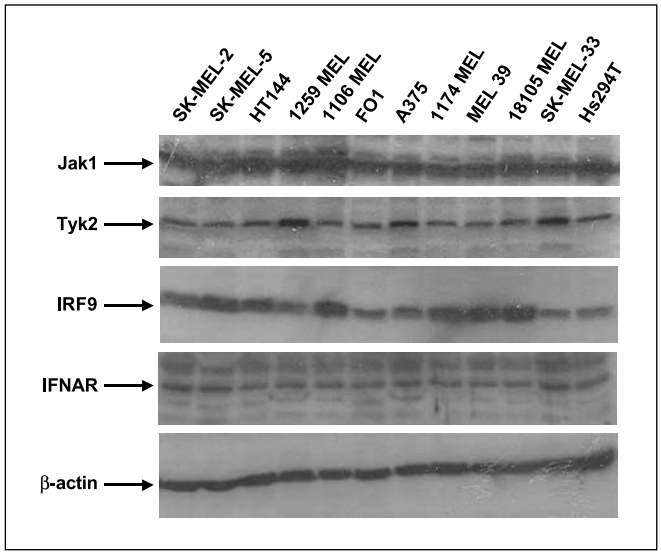
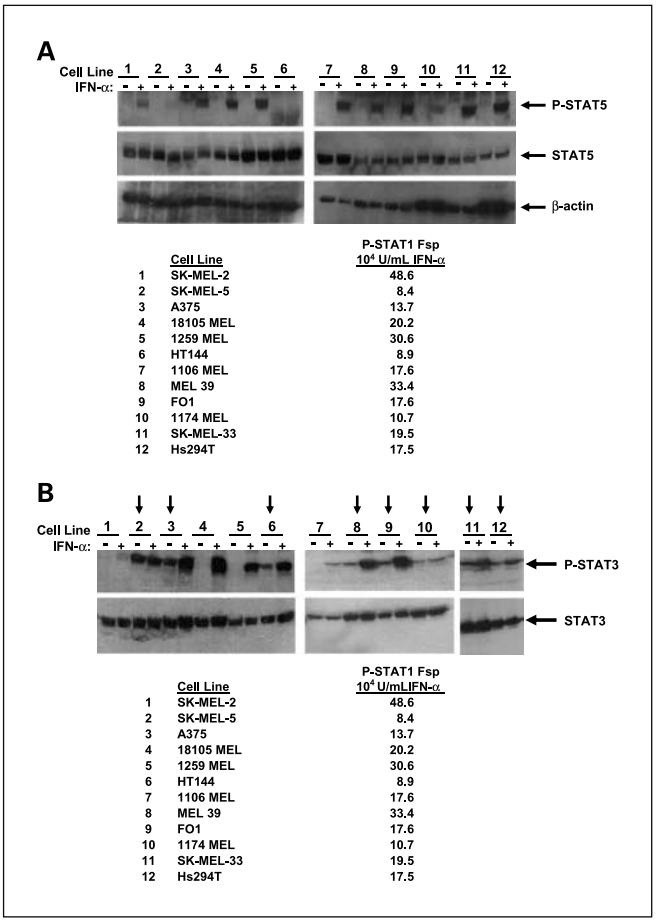


Fig. 3. Expression of Jak-STAT signal transduction intermediates in melanoma cell lines. Lysates were prepared from human melanoma cell lines and subjected to immunoblot analysis with antibodies directed against Jak1, Tyk2, IRF9, IFNAR. Nitrocellulose membranes were stripped and reprobed with a β-actin antibody to control for equal loading.



	Cell Line	P-STAT1 Fsp 10^4 U/mL IFN-α
1	SK-MEL-2	48.6
2	SK-MEL-5	8.4
3	A375	13.7
4	18105 MEL	20.2
5	1259 MEL	30.6
6	HT144	8.9
7	1106 MEL	17.6
8	MEL 39	33.4
9	FO1	17.6
10	1174 MEL	10.7
11	SK-MEL-33	19.5
12	Hs294T	17.5

	Cell Line	P-STAT1 Fsp 10^4 U/mLIFN-α
1	SK-MEL-2	48.6
2	SK-MEL-5	8.4
3	A375	13.7
4	18105 MEL	20.2
5	1259 MEL	30.6
6	HT144	8.9
7	1106 MEL	17.6
8	MEL 39	33.4
9	FO1	17.6
10	1174 MEL	10.7
11	SK-MEL-33	19.5
12	Hs294T	17.5

Fig. 4. P-STAT5 and P-STAT3 in melanoma cell lines. Top, P-STAT5 (*A*) or P-STAT3 (*B*) levels were evaluated in lysates from melanoma cell lines following a 15-min PBS (-) or IFN-α (+; 10^4 units/mL) stimulation. Membranes were stripped and reprobed with STAT5, STAT3, or β-actin antibodies to control for equal loading. Bottom, mean specific fluorescence (Fsp) values of P-STAT1 following a 15-min IFN-α stimulation (10^4 units/mL) as measured by flow cytometry are listed for comparison.

(Table 1). PBMCs were isolated from each patient immediately before surgical excision of their melanoma lesions, and the level of P-STAT1 in the PBMCs was measured by flow cytometry following *ex vivo* stimulation with IFN-α. Patient PBMCs routinely displayed high levels of P-STAT1 in response to IFN-α at 10^2 units/mL (Fig. 5E). As had been noted in previous studies, each patient exhibited a unique dose-response curve for IFN-α–induced P-STAT1 in PBMCs (22). A paired sample *t* test revealed that mean levels of P-STAT1 were significantly higher in patient PBMCs following stimulation with 10^3 and 10^4 units/mL IFN-α ($P = 0.046$ and 0.038, respectively; $n = 6$) than in the corresponding tumor cells. In fact, little activation of STAT1 was observed at any dose of IFN-α in tumor cells from patients 1, 2, and 5, whereas levels of P-STAT1 comparable with those seen in matched PBMCs were not achieved in tumor cells from patients 4 and 6 unless very high doses of IFN-α (e.g., 10^5 units/mL) were used (Fig. 5A and E). Only tumor cells from patient 3 displayed levels of P-STAT1 in response to intermediate doses of IFN-α (10^2-10^4 units/mL) that were comparable with those in PBMCs. A combination of highly responsive tumor cells and relatively unresponsive PBMCs in patient 3 resulted in a unique profile where IFN-α signal transduction exceeded that of immune effector cells. These data suggest that immune cells are inherently more sensitive to the direct effects of exogenously administered or endogenously produced IFN-α than melanoma cells.

Differential IFN-α responsiveness of immune and melanocytic cells. To evaluate whether established melanoma cell lines also displayed a reduced level of IFN-α–induced P-STAT1 relative to immune effector cells, the levels of IFN-α–induced P-STAT1 in melanoma cells ($n = 12$ cell lines) and PBMCs isolated from healthy donors ($n = 9$) were compared by flow cytometry. Melanoma cell lines exhibited significantly lower levels of P-STAT1 following IFN-α stimulation compared with normal human PBMCs ($\alpha = 0.05$; $P < 0.001$, Student's *t* test; Fig. 5F). In fact, melanoma cells typically required 10-fold higher doses of IFN-α than PBMCs (10^3-10^4 units/mL) to achieve maximal phosphorylation of STAT1 (data not shown). The reduced responsiveness of human melanoma cell lines compared with PBMCs was confirmed via immunoblot analysis comparing P-STAT1 induction in PBMCs from healthy donors with representative melanoma cell lines (Fig. 5G). In a similar manner, melanoma cells displayed a reduced induction of the IFN-α–responsive gene, *IFN-stimulated gene-15*, compared with PBMCs from healthy donors (Supplementary Fig. S2). These data confirm that differences in STAT1 activation also lead to functional consequences downstream of STAT1. Two separate cultures of primary human melanocytes were also evaluated by flow cytometry as a control. These data showed that melanocytes displayed a lower phosphorylation of STAT1 compared with immune effector cells (PBMCs) and suggest that cells of the melanocytic lineage are less responsive to IFN-α than are immune cells.

Discussion

We have shown that IFN-α–induced STAT1 phosphorylation was highly variable in melanoma cells and that it correlated with the antiproliferative properties of this cytokine. Surprisingly, this variability was not explained by defective expression of Jak-STAT signaling proteins as has been reported in previous studies of IFN-α–resistant melanoma cells (15–17). The variable level of IFN-α–induced P-STAT1 was not associated with altered phosphorylation or overexpression of STAT5, suggesting that additional factors regulate the direct response of melanoma cells to IFN-α. Analysis of STAT3 phosphorylation in melanoma cell lines suggested an inverse relationship between basal P-STAT3 and IFN-α–induced P-STAT1; however, this inverse relationship was not evident in melanoma cells derived from patient tumors. Finally, we show that IFN-α induces a more robust activation of STAT1 within immune cells compared with melanoma cells or primary human melanocytes.

Previous reports have shown that Jak1, STAT1, or IRF9 deficiency could confer an IFN-α–resistant phenotype to human melanoma cell lines and/or patient tumors (16, 32, 33). In patient-derived melanoma cells, the levels of STAT1 and Jak1 were elevated in cells that showed the highest degree of IFN-α–induced P-STAT1. Surprisingly though, immunoblot and histologic analysis revealed no gross defects in the expression of Jak-STAT signal transduction intermediates or the IFNAR in cell lines or patient tumor cells that exhibited very low activation of STAT1 in response to IFN-α. These results

agree with other reports which suggest that IFN resistance in melanoma cells can occur in cells with intact expression of Jak-STAT intermediates (34).

Melanoma cells with activated STAT3 may have increased metastatic potential. We observed that all six cell lines derived from metastatic melanoma lesions displayed basal P-STAT3, a finding that is consistent with the proposed role of STAT3 in promoting a metastatic phenotype in tumor cells (25, 28–30). Recent studies have also shown that STAT1 and STAT3 can play opposing roles in regulating the cellular response to IFN-α. In fact, STAT3 has been shown to sequester STAT1 and suppress the formation of STAT1 homodimers and downstream gene regulation in the THP-1 monocytic cell line (19). In melanoma cells, STAT3 may also influence the proliferative response to IFN-α or drive the expression of other proteins (e.g., SOCS3) that mediate IFN-α–induced signal transduction (35, 36). Based on this data, we postulated that the phosphorylation state of STAT3 may influence the response of melanoma cells to IFN-α. The majority of melanoma cell lines and 50% of patient-derived tumor cells exhibited basal P-STAT3 and robust STAT3 phosphorylation in response to IFN-α. A pooled analysis of all 12 established melanoma cell lines indicated that the direct actions of IFN-α were greatest in cell lines without basal P-STAT3. However, this inverse relationship was not evident in patient tumor lines or in the IFN-α–responsive, metastatic MEL 39 cell line. In contrast to other reports, our data revealed no relationship between STAT5 expression and the IFN-α responsiveness of melanoma cell lines. Therefore, these results suggest that additional factors are present, which regulate the magnitude of the melanoma cell response to IFN-α. These observations highlight the complexity of IFN-α signaling in melanoma cells and the need to study tissues from multiple sources when evaluating potential biomarkers that influence the ability of IFN-α to activate tumor cells.

IFN-α is known to exert potent effects on both melanoma cells and immune effector cells. However, a direct comparison of IFN-α–induced STAT1 activation between these two cellular populations has not been reported previously. Of note, recent findings have shown that clinical responders to IFN-α immunotherapy had significantly greater amounts of tumor-infiltrating immune cells compared with nonresponders (31). Using murine models, our group has shown that the ability of IFN-α to induce the activation of STAT1 in immune effector cells (but not melanoma tumor cells) is critical to its antitumor actions (11). Preliminary studies by Wang et al. (37) have also suggested that a high ratio of P-STAT1 to P-STAT3 in both melanoma cells and immune effector cells is associated with a favorable clinical outcome to IFN-α immunotherapy. These findings implicate an indirect immunomodulatory mechanism action for IFN-α rather than a direct antitumor effect. Interestingly, in the present study, melanocytes and melanoma cell lines were 10-fold less

Table 1. Patient characteristics

Patient no.	Age	Sex	Site of recurrent tumor	Histology	Prior IFN-α2b (dose; route; duration)	Time interval from prior IFN-α2b until biopsy	Prior therapies/ response
1	47	F	Neck	Melanoma, epithelioid, frequent tumor cell necrosis.	None	Not Applicable	Cisplatin, vinblastine, dacarbazine, no response. Gamma knife therapy for brain metastases.
2	83	M	Axillary skin	Melanoma, epithelioid with pleomorphic nuclei having prominent nucleoli.	3 MU/m^2; s.c. 3×/wk (adjuvant); duration, 1 mo	11 wks	Radiation therapy to axilla, disease recurred.
3	46	M	Chest	Partially pigmented epithelioid melanoma with pleomorphic nuclei having prominent nucleoli.	20 MU/m^2; i.v. 5×/wk (adjuvant); duration, 12 mo	3 y	Disease recurred after IFN-α2b therapy.
4	71	F	Thigh	Melanoma, epithelioid, involving subcutaneous soft tissue.	3 MU/m^2; s.c. 3×/wk (for recurrent disease); duration, 13 mo	2 y	Failed treatment on the C-Vax trial. Flattening of recurrent skin lesions was observed in response to IFN-α. However, tumor recurred at the surgical site 13 mo following completion of IFN-α.
5	51	M	Back	Melanoma, epithelioid, extensive infiltrate into the dermis and adipose tissue.	20 MU/m^2; i.v.; 5×/wk (adjuvant); duration, 12 mo	Recurred while on IFN-α2b	Recurrent disease while patient was receiving IFN-α2b therapy.
6	71	M	Back	Melanoma involving the deep dermis and subcutaneous adipose tissue. Neural invasion by tumor was noted.	None	Not applicable	C-Vax trial. Recurrence within the resection scar during therapy on the trial.

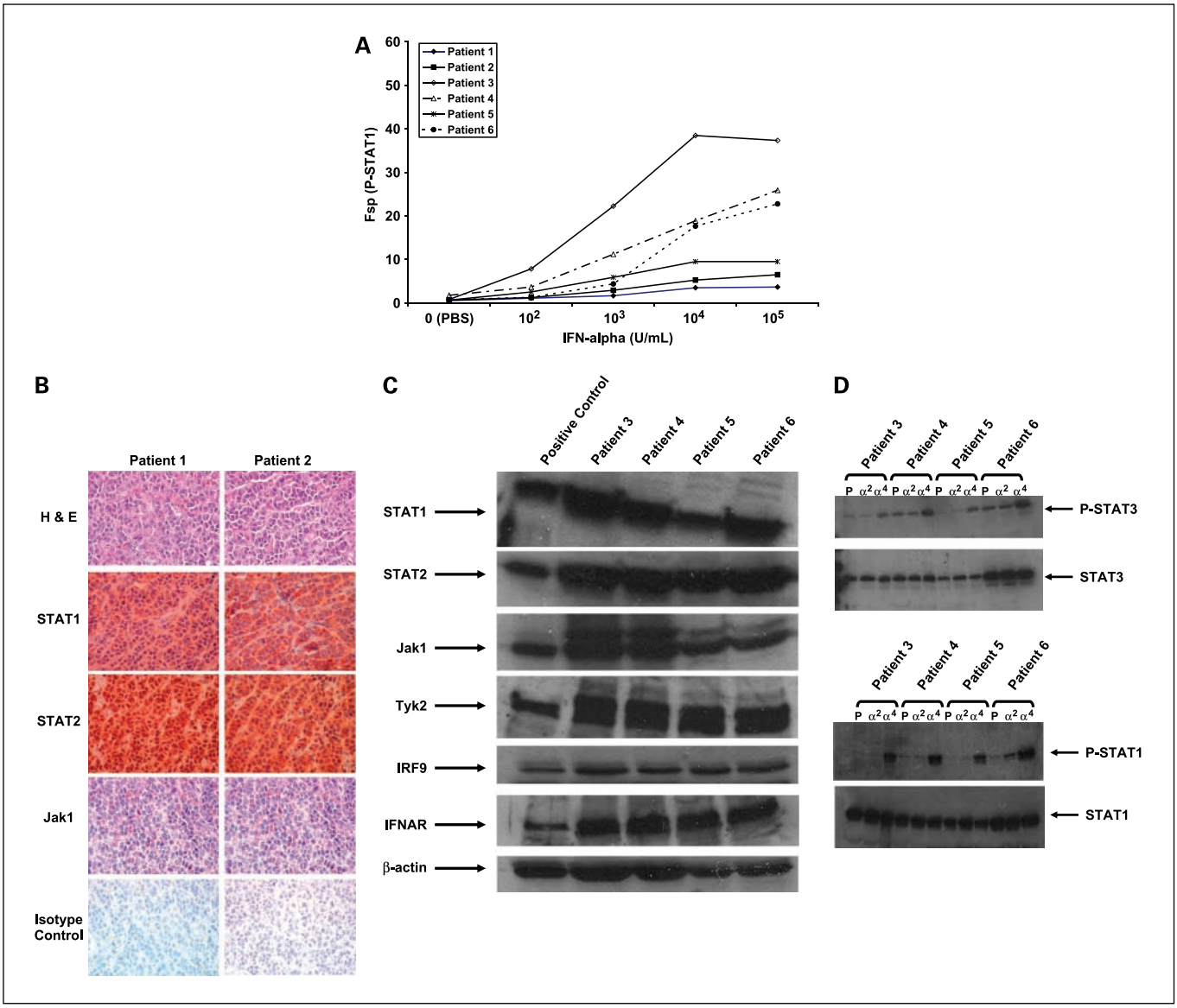
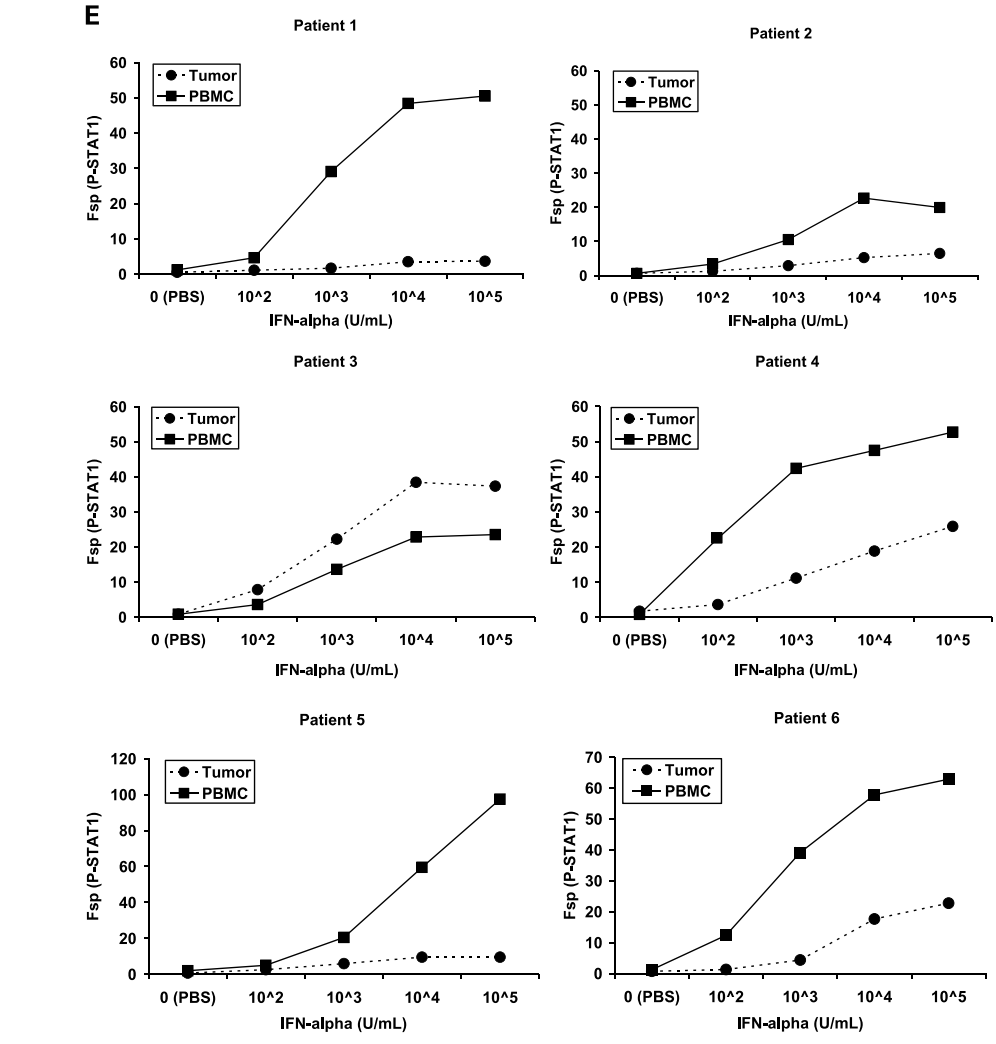
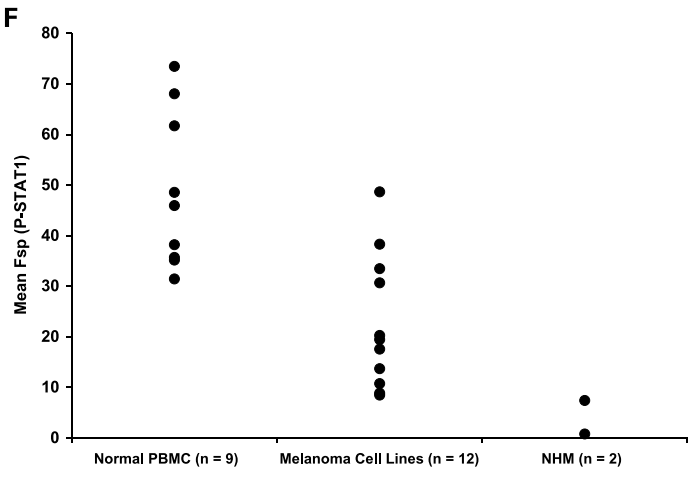


Fig. 5. IFN-α–induced P-STAT1 in human cells derived from recurrent cutaneous melanoma tumors and patient-matched PBMCs. *A,* P-STAT1 was measured in recurrent cutaneous melanoma cells derived from six individual patients by flow cytometry following a 20-min *in vitro* stimulation with IFN-α. *B,* immunohistochemical analysis of Jak-STAT intermediates in tumors from patients 1 and 2. *C,* following the initial flow cytometry experiments, the patient-derived tumor cells were expanded in culture for an additional 2 to 3 wks to generate sufficient cell numbers for immunoblot analysis of these markers. Expression of STAT1, STAT2, Jak1, Tyk2, and IFNAR was measured by immunoblot analysis of lysates from recurrent cutaneous tumor cells derived from patients 3 to 6. Membranes were stripped and reprobed with β-actin antibody to control for equal loading. *D,* P-STAT3 and P-STAT1 levels were evaluated in lysates from recurrent patient tumors following a 20-min *in vitro* stimulation with PBS (*P*) or two doses of IFN-α ($\alpha^2 = 10^2$ units/mL; $\alpha^4 = 10^4$ units/mL). Membranes were stripped and reprobed with STAT3, STAT1, or β-actin antibodies to control for equal loading.

sensitive to IFN-α in terms of the induction of P-STAT1 than were normal PBMCs. Of note, IFN-α stimulation also resulted in significantly greater levels of P-STAT1 in murine splenocytes when compared with B16 tumor cells derived from subcutaneous tumors maintained concurrently in the same host (Supplementary Fig. S3). These data support the notion that immune cells are inherently better equipped to respond to exogenously administered or endogenously produced IFN-α compared with melanoma cells.

The fact that four of six patient tumors in this study were obtained from individuals who had failed IFN-α immunotherapy raises the question of whether a phenotype of reduced STAT1 activation can be shaped over time in tumor cells following selective pressures exerted by the tumor microenvironment (38–42). This would explain the reduced sensitivity of these melanoma cells to IFN-α. Recent studies suggest that endogenously produced IFN-α and IFN-γ play a role in cancer immunoediting by enhancing the ability of host immune cells to recognize and destroy developing tumors (39, 42, 43). Thus, the patient tumors that were studied may represent aggressive variants that had 'escaped' innate immune recognition and the effects of endogenously produced IFNs (38–41). It remains to be determined whether this tumor phenotype of reduced IFN-α–induced signal transduction could influence the patient's response to exogenous IFN-α.





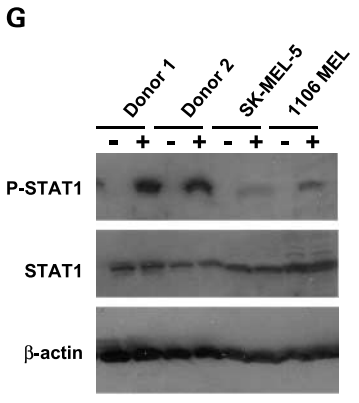


Fig. 5 *Continued. E,* comparison of IFN-α–induced P-STAT1 in matched melanoma tumor cells and PBMCs derived from patients. Following a 20-min *ex vivo* stimulation with IFN-α, P-STAT1 was measured by flow cytometry. *F,* comparison of IFN-α–induced P-STAT1 in immune effectors, established melanoma cell lines, and normal human melanocytes. PBMCs from healthy donors, established human melanoma cell lines, or normal human melanocytes (*NHM*) were stimulated for 15 min with IFN-α (10^4 units/mL) and P-STAT1 was measured by flow cytometry. Data are the mean specific fluorescence (Fsp) of P-STAT1. *G,* measurement of IFN-α–induced P-STAT1 by immunoblot. PBMCs isolated from healthy donors ($n = 2$) or representative human melanoma cell lines were stimulated for 15 min with PBS or IFN-α (10^4 units/mL) and P-STAT1 was evaluated by immunoblot analysis. The membrane was stripped and reprobed with antibodies targeting total STAT1 and β-actin proteins to control for equal loading.

In summary, we show that treatment of melanoma cells with IFN-α results in a variable level of STAT1 phosphorylation, despite the presence of intact signal transduction machinery. Of note, IFN-α–induced STAT1 phosphorylation was significantly lower in cells of the melanocytic lineage compared with immune cells. Data obtained from established melanoma cell lines suggested that P-STAT3 may play a role in regulating the level of IFN-α–induced P-STAT1. In contrast, an inverse relationship between STAT1 and STAT3 phosphorylation was not evident in patient-derived melanoma cells. These data suggest that the response of melanoma cells to IFNs is complex and regulated by multiple factors.

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