Modulation of Signal Transducers and Activators of Transcription 1 and 3 Signaling in Melanoma by High-Dose IFNα2b

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Abstract **Purpose:** The Janus-activated kinase/signal transducers and activators of transcription (STAT) pathway of IFN signaling is important to immunoregulation and tumor progression. STAT1 plays a prominent role in the effector immune response, whereas STAT3 is implicated in tumor progression and down-regulation of the response to type I IFNs. The goal of this study was to understand the effects of high-dose IFNα2b (HDI) in relation to the balance of pSTAT1 and pSTAT3.

Experimental Design: We evaluated STAT1 and STAT3 jointly as mediators of IFNα effects in the setting of a prospective neoadjuvant trial of HDI, in which tissue samples were obtained before and after 20 doses of HDI therapy. Double immunohistochemistry for pSTAT1 and pSTAT3 was done on paired fixed (9 patients) or frozen (12 patients) biopsies.

Results: HDI was found to up-regulate pSTAT1, whereas it down-regulates pSTAT3 and total STAT3 levels in both tumor cells and lymphocytes. Higher pSTAT1/pSTAT3 ratios in tumor cells pretreatment were associated with longer overall survival ($P = 0.032$). The pSTAT1/pSTAT3 ratios were augmented by HDI both in melanoma cells ($P = 0.005$) and in lymphocytes ($P = 0.022$). Of the immunologic mediators and markers tested, TAP2 was augmented by HDI (but not TAP1 and MHC class I/II).

Conclusion: IFNα2b significantly modulates the balance of STAT1/STAT3 in tumor cells and host lymphocytes, leading to up-regulation of TAP2 and augmented host antitumor response. The pSTAT1/pSTAT3 ratio in tumor cells at baseline may serve as a useful predictor of clinical outcome in cutaneous melanoma; the modulation of this ratio may serve as a predictor of therapeutic effect.

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High-dose IFNα2b (HDI) is the only therapy that has shown a reproducible ability to prolong both relapse-free and overall survival of patients with resected high-risk, deep, primary, or lymph node metastatic melanoma. This therapy has consistently shown a capacity to reduce the hazard of relapse and mortality by 22% to 33% in multiple U.S. intergroup studies done over the past 20 years (1–3). However, in the past decade, no further progress in the adjuvant therapy of melanoma has occurred, and the gap in our knowledge of the IFNα2b mechanism has proven to be a major impediment to further progress. A better understanding of the mechanism(s) of HDI in the adjuvant therapy of melanoma would enable more effective application of this therapy, and more intelligent strategies building upon this modality.

The antitumor effects of IFNα2b include direct inhibition of tumor cell growth and the modulation of host immune response to tumor. The Janus-activated kinase/signal transducers and activators of transcription (STAT) signal pathway is one of the major mechanisms of IFNα2b action. Both human type I IFNα receptor chains 1 and 2 are required for the type I IFN–dependent signaling pathways. The intracellular domains of these receptors are associated with Janus-activated kinases, which are activated upon IFNα2b binding to its receptors. Janus-activated kinases then phosphorylate and activate STATs (pSTAT), which, in turn, translocate to the nucleus and activate gene expression. The Janus-activated kinase/STAT pathway has been shown to be associated with immunoregulation, and the balance of opposing regulators such as STAT1 and STAT3 seems to be central to the effects induced by type I IFNs (IFNα; refs. 4–8). Prior investigations have focused predominantly upon either STAT1 or STAT3, independently of one another (9–12). We propose here for the first time, on the basis of studies in human melanoma lymph node metastases, that the ratio of pSTAT1tyr^{701}/pSTAT3tyr^{705}

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affords us a biomarker of melanoma prognosis, and a predictor of therapeutic effect for IFNα2b.

Immunosuppression associated with STAT3 activation has been reported in several tumor systems, and the inhibition of dendritic cell function is mediated by STAT3 via vascular endothelial growth factor, interleukin-10, and other unknown factors (4, 8). The biological functions of STAT1 and STAT3 seem to be opposed to one another in terms of cell growth regulation and the induction of an immune response to tumor antigens (4, 6, 8, 12 – 14). STAT1, as a tumor suppressor, restrains cell growth and mediates the antitumor effects of IFNα. STAT3 is associated with tumor progression in melanoma and squamous carcinoma of head and neck and mediates host immunosuppression. Recent studies of other investigators have documented that STAT1 and STAT3 are up-regulated simultaneously after administration of IFNγ or interleukin-6 to mice (15). Therefore, coanalysis of pSTAT1 and pSTAT3 signaling may more accurately reveal the dynamic mechanism of melanoma progression and host response. The clinical relevance of IFNα2b effects on the STAT1/STAT3 system has not been well documented in the context of clinical trials to date. We therefore also evaluated the changes in STAT1 and STAT3 during a neoadjuvant trial of HDI for patients with advanced regional lymph node metastasis of melanoma.

Conventionally, IFNα2b is administered as a postoperative adjuvant therapy for patients with high-risk melanoma, after all evidence of tumor has been surgically resected. In hopes that it would improve the antitumor effects of this therapy, and to better understand the effects of IFNα2b upon signal transduction, a neoadjuvant approach was adopted, in which HDI induction therapy was delivered after a diagnostic biopsy, before completion of definitive surgery. This trial was conducted in a series of 20 patients with regionally advanced stage IIIB or recurrent regional lymph node involvement by melanoma, as previously reported [University of Pittsburgh Cancer Institute (UPCI) 00-008] by our group (16 – 18). This report presents further studies done in the context of UPCI 00-008, building on our initial report of the clinical and immunologic findings of this trial (18). We now report the assessment of signal transduction molecules in pre-HDI and post-HDI tumor tissue biopsy specimens from this clinical trial, and the association between these potential biomarkers and the clinical outcome of disease and the clinical response to IFNα2b. Activated pSTAT1-tyr701 (pSTAT1) exerts a biological function that opposes that of activated pSTAT3tyr705 (pSTAT3; 4, 6, 8, 12 – 14). Therefore, the pSTAT1/pSTAT3 ratio was evaluated as a potential composite biomarker of relevance to the effects of treatment and the outcome of melanoma. The effects of HDI upon the balance of STAT1 and STAT3 seem to be associated with the immunologic effects of IFN upon transporters associated with antigen processing (TAP), particularly TAP2, as observed in this study. The enhancement of host immunity to a range of autoantigens and the clinical appearance of findings of autoimmunity induced by HDI was recently reported to be correlated with the benefit of postoperative HDI, in the context of a large clinical trial of the Hellenic Oncology Group (19). These observations and our observation of augmented density of dendritic cells and T cells in tumor tissues of those patients who have exhibited clinical response to neoadjuvant HDI (18) argue that the antitumor effects of IFNα2b depend on altered antigen presentation and T-cell polarization in melanoma patients receiving IFNα (20 – 22). The evaluation of the STAT system may define these upstream factors that are central to the polarization of the immune response, and the development and maturation of effective antitumor immunity.

Materials and Methods

Surgical specimens and patient treatment. Patient eligibility, treatment plan, and surgical biopsy schema were described in our first report upon the clinical trial UPCI-00-008 (18). The clinical protocol UPCI-00-008 was approved by the University of Pittsburgh Institutional Review Board, and all patients who entered this trial gave written informed consent. The patient demographic details and clinical response information have been reported (18) and are shown in Table 1. Eligible patients with palpable regional lymph node disease underwent a pretreatment tumor biopsy after written informed consent. A portion of the biopsy was evaluated to confirm the diagnosis, and the remaining portions of the biopsy were evaluated in research described in protocol 00-008. Patients were treated with IFNα2b according to the HDI regimen developed in the E1684/E1690/E1694 Eastern Cooperative Oncology Group and intergroup trials and as approved in 1995 by the Food and Drug Administration (3). IFNα2b, 20 MU/m^2/d, was administered i.v. for 5 consecutive days of 7 for 4 weeks, followed by 10 MU/m^2/d s.c. every other day (Monday, Wednesday, Friday) thrice weekly for 48 weeks. Patients underwent definitive surgery with completion of lymphadenectomy after the induction i.v. therapy and before beginning maintenance s.c. therapy. All study interventions and assessments were done at consistent time points as specified in the protocol UPCI 00-008 for this neoadjuvant trial. At the time of surgery, additional tumor and regional lymph node tissues were obtained for routine pathology and the research studies described in the protocol.

Monoclonal and polyclonal antibodies used for immunohistochemistry. The monoclonal antibody HC-10, which recognizes a determinant expressed on virtually all β$_2$m-free HLA-B heavy chains and on β$_2$m-free HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32, and HLA-A33 heavy chains, was developed and characterized as described (23, 24). The monoclonal antibody LGII-612.14, which recognizes a monomorphic determinant expressed on the β chain of HLA-DR, and on -DQ and -DP antigens, was developed and characterized as described (25). The TAP1 (NOB1) and TAP2 (NOB2) monoclonal antibodies were developed and characterized as previously described (26). Rabbit polyclonal anti-human pSTAT1tyr701, pSTAT3tyr705, and mouse polyclonal anti-human STAT3 antibody, which detects endogenous levels of total STAT3 protein, were purchased from Cell Signaling Technology (Beverly, MA). Unconjugated goat anti-rabbit IgG (H+L) antibody was used as a blocking antibody (Vector Laboratories, Burlingame, CA).

Immunohistochemistry. Paraffin-embedded, formalin-fixed sections preserve morphology optimally, but because the reagents available to detect pSTAT1 react well only in frozen sections, we used frozen sections (for both pSTAT1 and pSTAT3) and paraffin-embedded, formalin-fixed sections (for total STAT3 and pSTAT3) in this study. Fixed sections were used for the balance of other biomarkers studied here. Snap-frozen tissues were fixed in ice-cold acetone for 10 min. Formaldehyde-fixed and paraffin-embedded tissues were unmasked with antigen retrieval reagent (DAKO, Carpinteria, CA). Indirect immunohistochemistry was done to detect the specific antigens of concern with Vector Laboratories Vectastain ABC system (peroxidase and alkaline phosphatase system), and double immunostains were done according to the manufacturer's instructions.

Data and statistical analysis. The ×20 objective was used to evaluate the whole section by two pathologists who were blinded with regard to treatment assignment. Quantitation was decided by consensus of the two pathologists and a research faculty member. The percentage of cells and

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Table 1. Patient clinical information

Patient no.	Age (y)	Sex	Disease status	Disease stage at original diagnosis	HDI completed	Response		Duration of disease-free interval (mo)	Current status
						Clinical	Pathologic		
801	50	M	I	$T_{4a}N_{2b(cl)}$	1/3DR	CR	RMiD	32	Deceased
803	42	M	I	$T_{4b}N_{x}$	Yes	NR	RMaD	6	Deceased
804	65	F	R	$T_{3a}N_{0(s)}$	Yes	NR	RMaD	1	Deceased
805	41	M	R	$T_{1b}N_{0(cl)}$	Yes	PR	RMiD	49	NED
806	56	M	R	$T_{3a(al)}N_{0(cl)}$	Yes	NR	CR	34	MET
807	66	F	R	$T_{3b}N_{0(s)}$	Yes	NR	RMaD	32	NED
809	56	F	I	$T_{4b}N_{3(cl)}$	Yes	NR	RMaD	6	Deceased
810	60	M	I	$T_{4b}N_{2b(cl)}$	Yes	NR	RMaD	6	Deceased
811	72	F	R	$T_{3a}N_{0(cl)}$	Yes	PR	RMaD	30	NED
812	76	M	R	$T_{x}N_{0(s)}$	Yes	PR	RMaD	15	MET
814	57	F	I	$T_{x}N_{3(cl)}$	1/3DR	PR	RMaD	4	Deceased
817	62	M	IP	$T_{x}N_{3(cl)}$	1/3DR	PR	RMaD	10	NED

NOTE: Adapted from ref. 18.
Abbreviations: M, male; F, female; I, initial presentation; R, regional lymph node recurrence; IP, isolated palpable nodal metastases arising from an unknown primary; cl, nodal staging based on clinical exam; s, nodal staging based on sentinel lymph node mapping and pathologic interpretation; DR, dose reduction; CR, complete response; PR, partial response; NR, no response; RMiD, residual microscopic disease; RMaD, residual macroscopic disease; NED, no evidence of disease; MET, metastatic disease.

intensity (0-4+) of staining were evaluated for all samples. The intensity of staining was assessed for the entire cell, because the pSTAT reagent we used also detected a minor component of pSTAT in the cytoplasm. However, the localization of pSTAT within the nucleus has been documented in the literature. For accuracy, we tabulated pSTAT, including the cytoplasm as well as nucleus. Cells with staining intensity 1+ to 4+ were considered as positive cells; cells without staining (intensity 0) were considered as negative. The numbers of tumor cells and lymphocytes were separately evaluated. Lymphocytes observed in this study of lymph node metastases included tumor-infiltrating lymphocytes although the scoring was done for the entire node. The percentage of positive cells in the same population of cells was enumerated. Cell fractions of <1% were considered as zero. The double staining of pSTAT3tyr705 and total STAT3 was done on paraffin sections because fixed sections preserve morphology better than snap-frozen sections. These antigens are formalin-fixation stable, as is TAP2. For evaluation of pSTAT1/pSTAT3, snap-frozen sections were required because the reagents for pSTAT1 do not react on formalin-fixed, paraffin-embedded sections.

The statistical analysis was done by the UPCI Biostatistical Facility. Mean values of the percentage of positive cells are presented with SEs. Comparisons of the percentages of positive cells between pretherapy and posttherapy samples were done using paired-sample permutation tests (27) with a two-sided significance level of 0.05. Two-sample permutation tests were used to compare those with objective clinical response (partial and complete) to those without clinical response, and to compare patients who had subsequent relapse and those without subsequent relapse at 1 year of follow-up, both in terms of the baseline percentages of positive cells and the change from pretherapy to posttherapy. No P value adjustment has been done to account for multiple comparisons in this exploratory study.

The correlation of STAT signaling molecule expression and activation with disease-free and overall survival was estimated using the log-rank test and graphically explored using Kaplan-Meier plots. For the purpose of these comparisons, signaling molecule levels of expression (pretreatment and change from pretreatment to posttreatment) were dichotomized around the median of the data observed.

Results

Both pSTAT3tyr705 and total unphosphorylated STAT3 are down-regulated by HDI. Because systemic IFNα2b treatment leads to STAT3 inactivation in melanoma precursor lesions (7), total endogenous STAT3 expression levels were probed, and the ratio of activated pSTAT3tyr705 to unphosphorylated STAT3 was determined. Indirect double immunostaining was done using formaldehyde-fixed, paraffin-embedded tissues. Vector blue (pSTAT3tyr705), Vector red (total STAT3), and methyl green (counterstain) were used. Blue, red, and light green colors represent pSTAT3tyr705, STAT3, and nuclear counter stains, respectively. Among the nine cases studied, seven cases yielded tissue that was adequate to evaluate STAT expression among tumor cells, whereas eight cases were adequate to evaluate expression among lymphocytes. HDI down-regulated the percentage of pSTAT3tyr705-positive tumor cells from 20 ± 8.45% to 10.29 ± 6.75% (P = 0.016) in all of the seven cases, as shown in Fig. 1A. HDI down-regulated STAT3 in tumor cells in six of seven cases, with the mean percentage of positive cells decreased from 67.86 ± 7.23% to 48.57 ± 7.62% (P = 0.032), as shown in Fig. 1A. No significant differences at baseline or in changes after treatment were observed between clinical responders and nonresponders to IFNα2b therapy, considering the pSTAT3-tyr705 level, STAT3 level, or pSTAT3tyr705/STAT3 ratios studied.

Similar observations were made with regard to tissue lymphocytes. HDI down-regulated pSTAT3tyr705 expression in lymphocytes in seven of eight cases. The mean percentage of positive cells was decreased from 19.38 ± 6.37% to 12.88 ± 5.64% (P < 0.015), as shown in Fig. 1B. HDI down-regulated STAT3 in lymphocytes in six of the eight cases with paired biopsy tissues available. The mean percentage of positive cells was decreased from 41.25 ± 8.70% to 15.63 ± 3.05% (P = 0.062), as shown in Fig. 1B. The pSTAT3tyr705/STAT3 ratio was reduced by HDI in three of the eight cases studied. The mean change in the ratio was not significant (P = 0.908). There were no significant differences at baseline or in changes associated with treatment between clinical responders and nonresponders in terms of pSTAT3tyr705, STAT3, or pSTAT3tyr705/STAT3 ratio.

pSTAT1tyr701 is up-regulated, whereas pSTAT3tyr705 is down-regulated by HDI; the pSTAT1/pSTAT3 ratio is a potential predictive factor. Because STAT1 and STAT3 are biological

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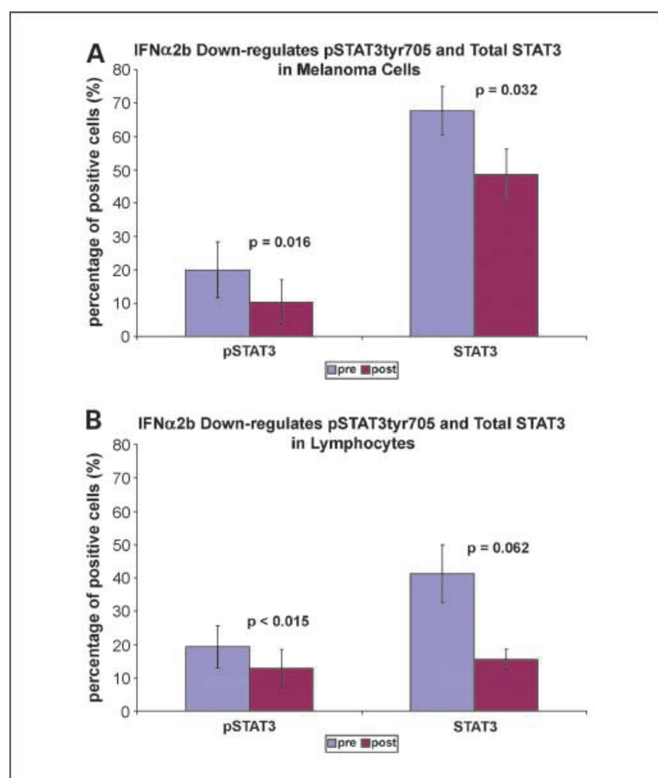

Fig. 1. *A,* pSTAT3tyr705/STAT3 double immunostaining of melanoma cells in the lymph node metastases. Formaldehyde-fixed, paraffin-embedded tissues of nine cases with adequate biopsy samples pre-HDI and post-HDI therapy were available for evaluation of pSTAT3tyr705 and STAT3. Only seven cases had adequate biopsy samples available before and after HDI for analysis of tumor and eight cases for analysis of lymphocytes. Columns, mean; bars, SE. *B,* pSTAT3tyr705/STAT3 double immunostaining of lymphocytes in the lymph node metastases. Similar phenomena were observed among lymphocytes as in tumor cells. Columns, mean; bars, SE.

regulators that balance one another (4, 6, 12–14), these markers were evaluated simultaneously. Double immunostaining for the activated pSTAT1 and pSTAT3 species was done on snap-frozen tissue sections to evaluate the two transcriptional factors in the same nodal tumor biopsies obtained from patients before and after 20 doses of IFNα2b. Vector blue (pSTAT1tyr701), Vector red (pSTAT3tyr705), and methyl green (counterstain) were used. The specimens of 12 patients (one complete responder, five partial responders, and six nonresponders) were available for this study. Eleven of 12 cases studied proved to be adequate to evaluate tumor cells, and 10 of 12 cases were adequate to evaluate lymphocytes. The quantitative data of pSTAT1/pSTAT3 in the tumor compartment of the lymph nodes infiltrated by metastatic disease is described in Fig. 2. HDI up-regulated pSTAT1tyr701 in tumor cells in 8 of 11 cases. The percentage of pSTAT1tyr701-positive tumor cells was increased from 20.55 ± 6.59% to 28.45 ± 7.60% (P = 0.043), as shown in Fig. 2A. HDI down-regulated pSTAT3tyr705 in tumor cells in 9 of 11 cases. The percentage of pSTAT3tyr705-positive tumor cells was decreased from 19.18 ± 4.63% to 9.19 ± 3.99% (P = 0.055), as shown in Fig. 2A. The pSTAT3 data for melanoma cells obtained from snap-frozen sections are consistent with the data obtained from formaldehyde-fixed, paraffin-embedded sections as stated above. Individual patient data for pSTAT1 or pSTAT3 in tumor cells is shown as Fig. 2B and C, respectively. Among those cases, there were studies done of both pSTAT1 and pSTAT3 for seven cases. To be precise, in the same patient, pSTAT1 was up-regulated, whereas pSTAT3 was down-regulated. The value of the pSTAT1tyr701/pSTAT3tyr705 ratio was augmented significantly by HDI in all 11 cases studied here, increasing from 5.07 ± 3.54 to 16.93 ± 5.81 (P = 0.005). There were no significant differences at baseline or in changes associated with treatment between those with clinical response and those without, in terms of pSTAT1tyr701, pSTAT3tyr705, or pSTAT1tyr701/pSTAT3tyr705 ratio. However, when the population of patients studied was dichotomized according to the pSTAT1/pSTAT3 ratio in tumor cells, patients with higher pSTAT1/pSTAT3 ratios in the pretreatment tumor samples had an improved overall survival (P = 0.032; Fig. 3A). Neither pSTAT1 (P = 0.12) nor pSTAT3 (P = 0.66) alone showed a correlation with overall survival. The pSTAT3/STAT3 ratios were not correlated with overall survival either (P = 0.92). We also analyzed the correlations of overall survival with the other markers studied here, finding no correlations between the pretreatment and posttreatment changes of pSTAT1 or pSTAT3, and overall survival.

HDI up-regulated pSTAT1tyr701 and down-regulated pSTAT3tyr705 in the lymphoid cells of all regions evaluated in the nodal biopsy samples, including the tumor-infiltrating, peritumoral, and perivascular regions. HDI up-regulated pSTAT1tyr701 in the lymphoid component of 8 of the 10 cases studied. The percentage of pSTAT1tyr701-positive cells was increased from 15.6 ± 2.67% to 23.6 ± 5.22% (P = 0.061), as shown in Fig. 2D. HDI down-regulated pSTAT3-tyr705 in the lymphoid component of nodal biopsy samples from all of the10 cases studied. The percentage of pSTAT3-tyr705-positive cells decreased from 8.2 ± 3.95% to 2.9 ± 1.96% (P = 0.003), as shown in Fig. 2D. The pSTAT3 data for lymphocytes obtained from snap-frozen sections are consistent with the data obtained from formaldehyde-fixed, paraffin-embedded sections as stated above. Patient individual data of pSTAT1 or pSTAT3 in lymphocytes are shown as Fig. 2E or F, respectively. The ratio of pSTAT1tyr701/pSTAT3tyr705 was up-regulated in 8 of 10 cases. The ratio was increased from 7.43 ± 1.87 to 22.71 ± 5.55 (P = 0.022). There was no significant difference between clinical responders and nonresponders in terms of pSTAT1tyr701, pSTAT3tyr705, or pSTAT1tyr701/pSTAT3tyr705 ratio. The baseline pSTAT1/pSTAT3 ratio in lymphocytes was not found to be correlated with overall survival (P = 0.226; Fig. 3A).

TAP2 is up-regulated by HDI. Reduction in the expression of TAP molecules has been correlated with progression of melanoma and associated with reduced immune response to melanoma (28). Host immune response to melanoma depends in part on antigen presentation (29), which is, in turn, related to the expression of TAP1 and TAP2. Thus, we were interested in determining the influence of HDI on TAP1 and TAP2 expression, as well as ascertaining whether inactivation of pSTAT3 by HDI in tumor cells might be accompanied by evidence of reversal of the immunosuppression of the host responses to the previously described tumor antigens (4). HDI up-regulated TAP2 in all of the five cases studied from 55.2 ± 14.13% to 86.0 ± 9.80% (n = 5, P = 0.063) in tumor cells, as shown in Fig. 4. No similar effects on TAP1 or MHC class I and II antigen expression were observed

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in this study, although these have previously been implicated in the immunomodulatory effects of IFN. Melanoma samples studied here expressed TAP1 only weakly, compared with the internal positive controls, including plasmacytoid-derived dendritic cell– and dendritic cell–like cells, lymphocytes, and macrophages within the tissue biopsies. Figure 4 further shows that HDI not only increases TAP2 expression in melanoma cells but also prominently increases TAP2 expression in lymphoid cells that infiltrate regional lymph node metastases. The immunohistochemistry evaluation of the five cases is illustrated in Fig. 4. A1 to A5 are pretreatment biopsies and B1 to B5 are posttreatment biopsies, showing consistent up-regulation of TAP2 in each of these paired biopsy samples. In B1, B3, and B5, the cells designated with the arrow are strongly positive plasmacytoid-derived dendritic cell–like cells. In case 5, melanoma cells are necrotic in the pretreatment section, and TAP2-positive cells are <1%. Melanin (appearing as yellowish brown tiny particles) is evident in this pretreatment section as well.

Discussion

High-dose IFNα2b has shown consistent and significant relapse-free survival benefits in multiple randomized controlled trials of the U.S. Cooperative Groups over the past 20 years. This therapy has been controversial due to its toxicity and its cost in relation to the incremental benefits of treatment upon relapse-free and overall survival (1). The identification of molecular mechanisms that mediate the benefit of high-dose IFNα2b therapy is vital to our efforts to improve this treatment modality. Molecular markers of treatment effect would improve both our ability to select patients for treatment and our ability to rationally combine this therapy with other agents that might have greater therapeutic effect on melanoma. Together, these would allow us to improve the therapeutic index of adjuvant treatment for melanoma.

Here, in a trial directed at the most advanced setting of operable regional metastasis (stage IIIB or bulky nodal recurrence) of melanoma, we have altered the sequence of therapy to administer high-dose IFNα2b before definitive surgery (as a neoadjuvant therapy) and have shown significantly increased pSTAT1 expression in tumor cells and a trend of increase in pSTAT1 among lymphocytes (P = 0.061) of regional lymph nodes. Activation of STAT1 within the host immune cells, not the tumor tissues, has been suggested to be responsible for the antitumor effects of IFNα in murine models of melanoma (6, 30–32). STAT1 is one of the signaling molecules associated with tumor growth restraint and

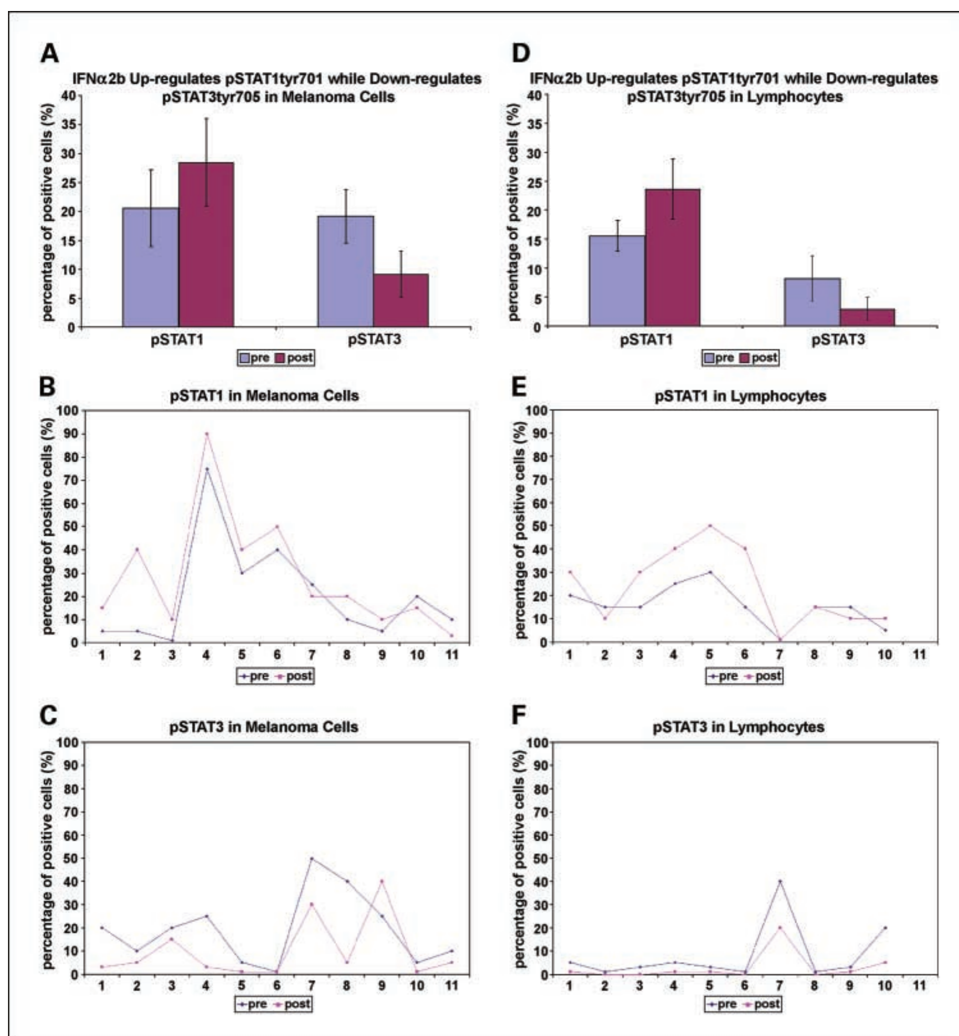

Fig. 2. *A* to *C,* pSTAT1tyr701 and pSTAT3tyr705 double immunostains of nodal melanoma cells. Snap-frozen tissues (total of 12 cases) pre-HDI and post-HDI therapy were stained for pSTAT1tyr701 or pSTAT3tyr705. Columns, mean; bars, SE. *D* to *F,* pSTAT1tyr701 and pSTAT3tyr705 double immunostains of nodal lymphocytes. Similar phenomena were observed among lymphocytes as in tumor cells. Points, mean; bars, SE.

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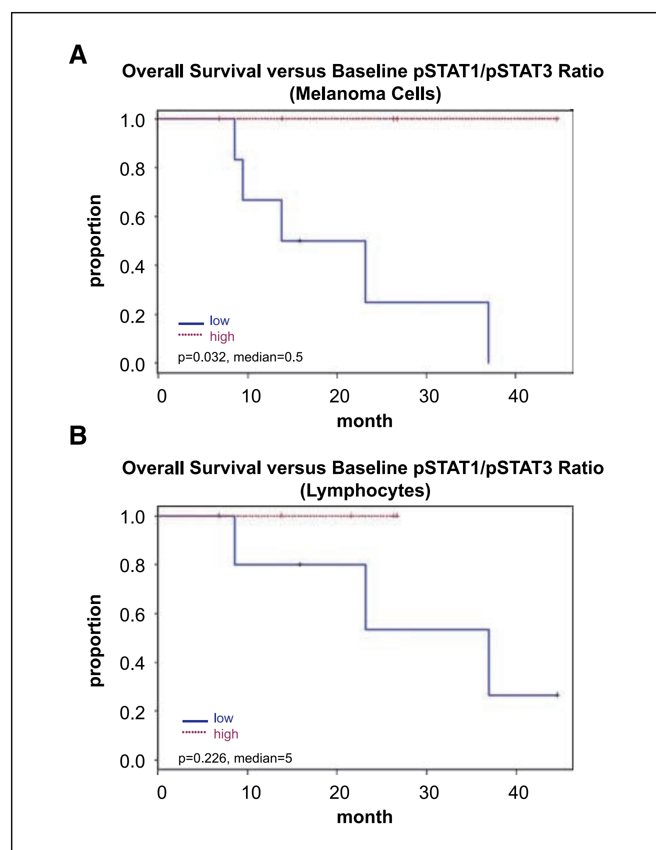

Fig. 3. *A,* pSTAT1/pSTAT3 ratio in melanoma cells versus overall survival indicates that patients with higher pSTAT1/pSTAT3 ratios in pretreatment tumor samples have prolonged overall survival ($P = 0.032$). *B,* pSTAT1/pSTAT3 ratio in lymphocytes versus overall survival does not show significant correlation ($P = 0.226$). Low, patients whose pSTAT1/pSTAT3 ratio was at or below the median value; high, patients whose pSTAT1/pSTAT3 ratio was above the median value.

tumor-suppressor pathways (13, 14). Here, we report the elevation of pSTAT1 levels in human lymph node biopsy samples taken before and after treatment with IFNα2b in patients with regional lymph node metastasis who participated in a prospective phase II trial of neoadjuvant therapy with HDI. The E1684 regimen of HDI, initially reported to significantly prolong relapse interval and overall survival in patients with high-risk, node-positive, or deep primary cutaneous melanoma in 1996, has used a component of i.v. induction that is now being tested alone in the intergroup international trial E1697. Data reported here show that the pretreatment levels of pSTAT1 in tumor cells and lymphocytes themselves do not correlate with the clinical responses observed in patients at day 29 of treatment with high-dose IFNα2b, consistent with the findings of Lesinski et al. (9) in melanoma.

STAT3 activation seems to be a major determinant of tumor progression in melanoma and is associated with host immunosuppression, one of the major factors implicated in melanoma progression. Systemic high-dose IFNα2b treatment has previously been reported to inhibit pSTAT3 expression in melanoma and precursor atypical nevus lesions (7). Our current observations expand on these previous results, demonstrating both inhibition of constitutively activated pSTAT3-tyr705 and the augmentation of pSTAT1tyr701 expression in tumor tissues. Patients studied in this prospective trial were consistently evaluated and analyzed in terms of treatment, biopsy timing, and molecular pathologic evaluation as outlined in the institutional review board–approved protocol UPCI 00-008 (7). HDI significantly down-regulates both pSTAT3tyr705 and total STAT3 levels in tumor cells and lymphoid cells ($P < 0.05$ and $P = 0.06$) assessed by pSTAT3/STAT3 double immunohistochemistry staining. HDI down-regulates pSTAT3 both in tumor cells ($P = 0.055$) and lymphoid cells ($P < 0.05$). IFNα inhibition of STAT3 expression and activation in tumor cells may allow the expression of proinflammatory cytokines and chemokines to mobilize dendritic and T-cell responses to tumor (4, 8, 12, 33). These results are concordant with other recent results that show a role of STAT3 in tumor progression in other tumor systems (34). We conclude that down-regulation of STAT3 and pSTAT3 by high-dose IFNα2b in melanoma and host immune cells is central to the immunologic mechanism of IFNα, and that the benefits of high-dose IFNα2b are mediated by immunologic effects in addition to direct antitumor effects.

We have found that patients with higher pSTAT1/pSTAT3 ratios in pretreatment tumor biopsy tissues have an improved prognosis and longer overall survival. The utility of this index may also apply to other malignancies, as well as to the precursors of melanoma, in which the pSTAT1/pSTAT3 balance has been observed to be deranged.[7] Baseline levels of pSTAT1 and pSTAT3 measured individually did not correlate with overall survival. Moreover, the ratio of pSTAT1/pSTAT3 measured in the lymphoid compartment of the biopsy samples studied here did not correlate with outcome. The influence of IFNα2b was seen, however, in the ratio of pSTAT1/pSTAT3 among both tumor cells and lymphocytes. The lack of any correlation between pSTAT1/pSTAT3 ratios among lymphocytes and survival may relate to the importance of derangements in the STAT system in the tumor microenvironment (8). Our evaluation of pSTAT1 and pSTAT3 in conjunction with one another (and the pSTAT1/pSTAT3 ratio) may serve as a useful prognostic factor for melanoma. Baseline data here argues that the pSTAT1/pSTAT3 ratio has prognostic utility; the significant up-regulation of the pSTAT1/pSTAT3 ratio during HDI therapy suggests that this may also have predictive utility in relation to HDI therapy. The baseline pSTAT1/pSTAT3 ratio warrants further evaluation in larger patient samples as a potential prognostic and predictive index of disease. The dynamic balance of pSTAT1/pSTAT3 in tumor cells over time and the lymphoid as opposed to tumor cell changes that follow administration of IFNα are under study.

Host immune responses to melanoma depend on antigen presentation in the context of molecules of the TAP system. Deficient expression of TAP molecules has been shown to be one basis on which tumor progression occurs (28, 35, 36). Striking up-regulation of TAP2 expression by IFNα in both tumor and immune cells has been observed in this study. This is noteworthy because TAP2 is crucial for the delivery of peptides from the cytoplasm to the lumen of the endoplasmic

[7] W. Wang et al., unpublished data.

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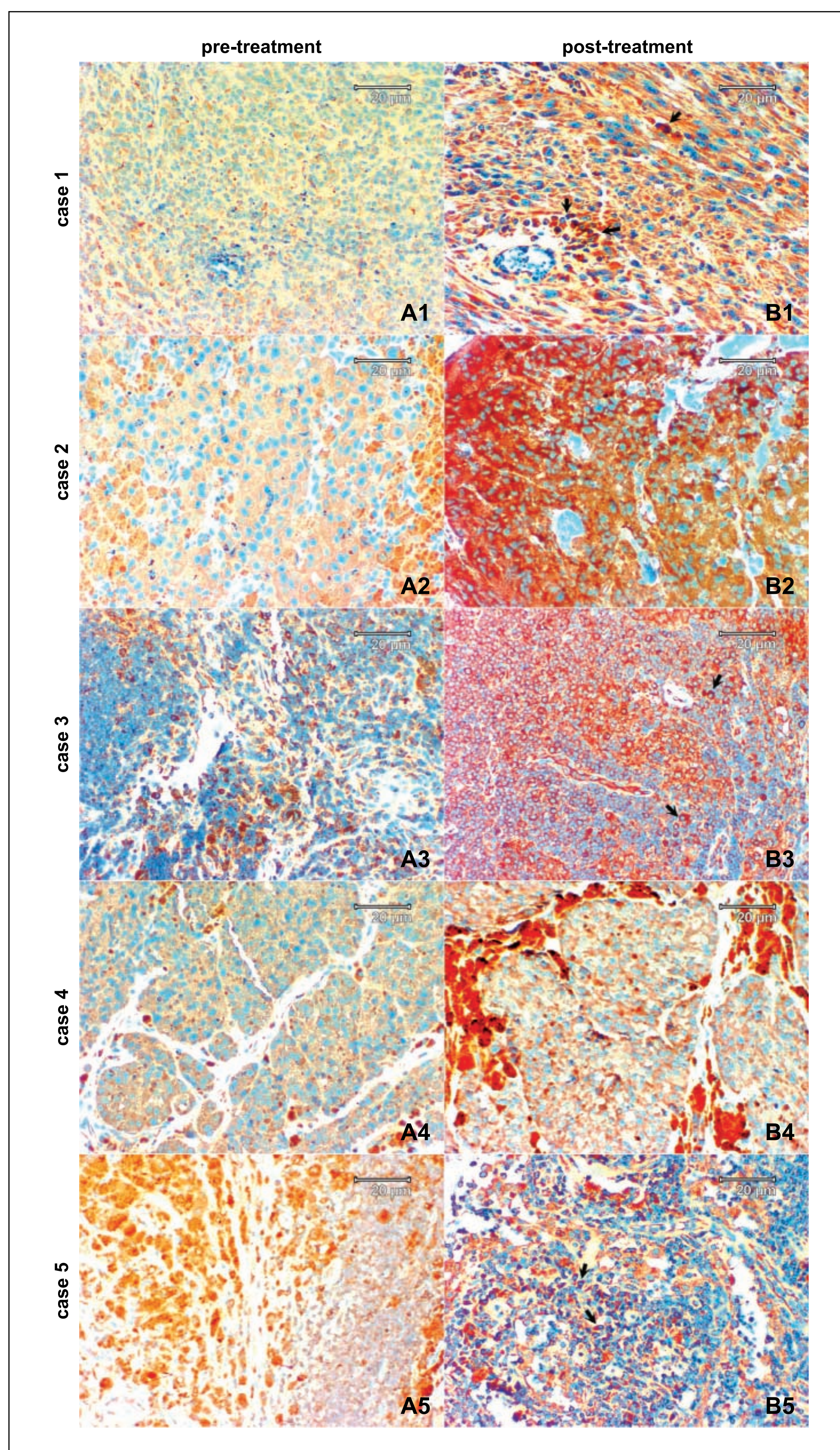

Fig. 4. High-dose IFNα2b up-regulates TAP2 in tumor cells and immune cells. Magnification, ×20. Red, TAP2; blue, counterstain. *A1* to *A5,* pretreatment sections; *B1* to *B5,* posttreatment sections; the red color (representing TAP2) is strongly increased in intensity and percentage of cells observed. Small yellowish brown particles, melanin. Black arrows, lymphoid cells.

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reticulum, where peptides are loaded onto MHC class I molecules for presentation to CTLs (37).

Activation of the cellular *TAP2* gene occurs through IRF-7, the master regulator of type I IFN–dependent immune responses (38, 39). STAT1-dependent induction of *IRF7* and *IFN* gene expression has been reported as well (40–42). The expression of TAP1, TAP2, and proteasome activator 28 have recently been reported to be up-regulated in peripheral

blood mononuclear cells during IFNα2b adjuvant therapy at dosages of 10×10^6 IU/m^2 (43). Additional studies indicate that expression of TAP1 depends on functional cooperation between STAT1 and IRF-2 in relation to the TAP1 promoter (44). Both STAT1 and IRF-2 are up-regulated by IFNα (45, 46). This study has shown that IFNα down-regulates pSTAT3, whereas it up-regulates pSTAT1 and the pSTAT1/pSTAT3 ratio, associated with the stimulation of TAP2 but neither TAP1 nor MHC class I and II antigens of tumor and lymphoid cells. Inactivation of the *TAP1/LMP2* (low molecular mass polypeptides) genes has been associated with malignant transformation and disease progression (47). Both TAP1 and TAP2 are required for MHC class I function (48–53), although no effect on TAP1 was observed in this study. Our data also suggest that further evaluation of specific STAT1 and/or STAT3 downstream signaling are warranted in relation to the broader immunoregulatory effects of type I IFNs in human melanoma.

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