Effects of High-Dose IFNα2b on Regional Lymph Node Metastases of Human Melanoma: Modulation of STAT5, FOXP3, and IL-17

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Abstract

Purpose: Signal transducer and activator of transcription 5 (STAT5) and STAT3 oppose one another in regulation of the reciprocal development of CD4+CD25+FOXP3+ regulatory T cells (Treg) and T helper 17 (Th17). A reduction in STAT3 is associated with up-regulation of Treg, and STAT5 activation promotes Treg differentiation or function while constraining Th17 generation. The effects of IFNα on STAT signaling in relation to tumor tissue Treg and Th17 have not been documented in humans beyond the observations that IFNα2b down-regulates STAT3. Experimental Design: Following diagnostic biopsy and before definitive surgery, 20 doses of high-dose IFNα2b (HDI) were administered to patients with stage IIIb melanoma who gave written informed consent. Lymph node biopsies, in which both total STAT3 and phosphorylated STAT3 were down-regulated by HDI, were probed with STAT5, FOXP3, CD4, and interleukin 17 (IL-17) with immunohistochemistry and/or immunofluorescence techniques. Results: The percentage of FOXP3+ lymphocytes determined by immunohistochemistry was up-regulated from 3.06 ± 0.65% to 9.86 ± 1.27% (n = 13, P = 0.0002), and this observation was confirmed by immunofluorescence evaluation of CD4+FOXP3+ Tregs. HDI induced STAT5 up-regulation (five cases observed) in melanoma cells and lymphocytes but did not induce the generation of IL-17–expressing lymphocytes. Increased STAT5 expression was associated with increased FOXP3 expression among lymphocytes, and STAT5 was constitutively activated among both melanoma cells and lymphocytes. Conclusion: IFNα2b up-regulates STAT5 and down-regulates STAT3, in conjunction with up-regulation of Treg and inhibition of IL-17–expressing lymphocytes in melanoma tissues. These findings suggest that the effects of IFNα may be potentiated through interference with the response of Tregs and/or STAT5.

High-dose interferon has been shown to result in the significant improvement of relapse-free survival in all studies performed to date; a significant effect on overall survival has been reported in the Eastern Cooperative Oncology Group trial E1684 and the intergroup trial E1694 (compared with observation and GMK vaccine, respectively). These benefits on relapse-free and overall survival represent an absolute observation and GMK vaccine, respectively). These benefits on relapse-free and overall survival represent an absolute

results for melanoma (1–3). The JAK (Janus tyrosine kinase)/STAT (signal transducers and activators of transcription) signaling pathway is one of the major mechanisms of IFNα2b action. The balance of opposing regulators, STAT1 and STAT3, appear to be central to the effects induced by type I IFNs (IFNα; refs. 4–9). Down-regulation of STAT3 by high-dose IFNα2b (HDI) is associated with the enhancement of host antitumor immunity (9). The enhancement of host immunity to a range of autoantigens and the clinical appearance of findings of autoimmunity induced by HDI has recently been reported to be correlated with the therapeutic benefit of HDI, in the context of the Hellenic Oncology Group Adjuvant Trial (10). We have also reported that the pretreatment cytokine profile of patients entering treatment for high-risk melanoma supports the existence of elevated numbers of Tregs in solid tumors (15, 16). Tregs are overrepresented in lymph nodes containing metastatic melanoma, where they seem to inhibit the function of effector T cells infiltrating the tumor (17). Treg depletion leads to restoration of antitumor immunity (15, 16). CD4+FOXP3+ Tregs are the best characterized
Translational Relevance

IFNα2b is the only adjuvant therapy approved for use following surgery for high-risk cutaneous melanoma. However, the benefits of IFNα2b therapy in a fraction of <33% of patients leave considerable room for improvement of this adjuvant therapy. The roles of signal transducers and activators of transcription (STAT) signaling and immunoregulatory responses mediated by T regulatory cells have become increasingly clear over the past few years. In the context of a neoadjuvant study evaluating IFNα2b administered before definitive surgery and post diagnostic biopsy, we have evaluated the signaling mechanisms of IFNα2b action in lymph nodes containing metastatic melanoma. IFNα2b up-regulated STAT5 while down-regulating STAT3. Those responses are associated with the up-regulation of Treg and inhibition of IL-17-expressing lymphocytes in the node. This clinical translational study observation suggests that the effects of IFNα may further be potentiated through interference with the response of Tregs and/or STAT5 in the future.

Treg population among the large and heterogeneous group of CD4 and CD8 T cells with suppressive functions (18). The effect of FOXP3 is to suppress the activation of target genes promoting T-cell stimulation, and the suppression of FOXP3 targets seems to be crucial for the normal function of Tregs (19). Evidence has also recently documented that Treg generation is reciprocally associated with differentiation of Th helper 17 (Th17) cells (20–24), which plays an important role in inflammatory responses. It is now believed that STAT5 and STAT3 oppose one another in regulation of the reciprocal development and functions of Treg and Th17 (25–30); the STAT5 signal promotes Treg generation and function and constrains the development of Th17, whereas the STAT3 signal inhibits Treg development and promotes Th17 development or function.

IFNα has immunoregulatory functions that seem to be important for its adjuvant therapeutic effects on melanoma, and we have therefore studied the alterations in the Treg compartment of nodal tumor tissue in samples obtained before and following initiation of IFNα. We evaluated Treg and other related markers in the biopsy specimens of regional lymph nodes taken pretreatment and 29 days after initiation of therapy in the context of a prospective neoadjuvant trial of high-dose IFNα2b, as we have previously reported (9, 31). We hypothesized that Treg populations might be modulated by IFNα; we also equally considered that the immunopotentiation induced by IFNα might be accompanied by Treg elevations similar to the responses following interleukin 2 (IL-2) therapy (32, 33). Conventionally, IFNα2b is administered as a postoperative adjuvant therapy for patients with high-risk melanoma after all evidence of tumor has been surgically resected. To assess the potential for the earlier administration of this agent in hopes that earlier preoperative administration would improve antitumor effects of this therapy and to better understand the effects of IFNα2b on tumor tissues, a neoadjuvant approach was adopted in which HDI induction therapy was delivered after a diagnostic biopsy and before completion of definitive surgery. This neoadjuvant trial [University of Pittsburgh Cancer Institute (UPCI) 00-008] was conducted in a series of patients with regionally advanced stage IIIIB or recurrent regional lymph node involvement by melanoma, as previously reported (9, 31). This report presents studies that have been performed in the context of the UPCI protocol 00-008 and builds on our initial report of the clinical and immunologic findings of this trial (9, 31). We now report the assessment of Treg populations evaluated with both CD4 and FOXP3 markers in pre- and post-HDI tumor tissue biopsy specimens from this clinical trial. HDI down-regulates STAT3 (9) and up-regulates STAT5 associated with up-regulation of CD4+FOXP3+ Treg, whereas the IL-17-expressing lymphocytes observed were not altered in response to HDI therapy.

Materials and Methods

Surgical specimens and patient treatment. Patient eligibility, treatment plan, and surgical biopsy schema were described in our earlier reports on the clinical trial UPCI 00-008 (9, 31). 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 (31). 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 the research described in protocol 00-008. Patients were treated with IFNα2b according to the HDI regimen developed in the Eastern Cooperative Oncology Group and Intergroup trials E1684/E1690/E1694, using the regimen approved by the Food and Drug Administration in 1995 (3). IFNα2b, 20 MU/m2/d, was administered i.v. for 5 days per week for 4 wk, followed by 10 MU/m2/d s.c. every other day (M, W, F) thrice each week × 48 wk. 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 and immunofluorescence. Goat polyclonal anti-human FOXP3 was purchased from Abcam. Mouse monoclonal anti-human CD4, mouse monoclonal anti-human total STAT5, and rabbit polyclonal anti-human pSTAT5tyr694 were purchased from BD Pharmingen. Alexa Fluor 488–conjugated donkey anti-goat IgG, Alexa Fluor 568–conjugated donkey anti-mouse, Alexa Fluor 555–conjugated donkey anti-rabbit IgG, and Hoechst 33342 were purchased from Molecular Probes.

Immunohistochemistry and immunofluorescence. FOXP3 expression was probed first because the reported data support FOXP3 as a lineage marker for a broader regulatory population of Tregs than for other phenotypic markers (33). The serially biopsied tumor specimens of 13 cases were available for evaluation of FOXP3 expression. Among the 13 cases with tissue available for study, 1 case experienced complete response, 6 cases experienced partial response, and 6 cases were nonresponders.

Snap-frozen tissue sections fixed with acetone or paraffin-embedded formalin-fixed sections were used in this study. Indirect immunohistochemistry was done to detect FOXP3 (in snap-frozen sections) or STAT5 (in paraffin-embedded, formalin-fixed sections) with the Vectastain ABC system (peroxidase system) from Vector Laboratories. Methyl green (Vector Laboratories) or hematoxylin was used as the counterstain. Under bright-field examination, red represented FOXP3 or STAT5, whereas blue or green represented the counterstain.

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Triple-color immunofluorescence was done to evaluate the presence of CD4+FOXP3+ Treg cells and to observe the correlation between FOXP3 expression and STAT5 or pSTAT5tyr694 expression. Under fluorescence microscope examination (Olympus Fluoview 1000 or Provis I), blue (Hoechst 33342) represented nuclear counterstain, whereas green (Alexa Fluor 488) represented FOXP3. Red (Alexa Fluor 568 or Alexa Fluor 555) represented CD4 or pSTAT5tyr694 and STAT5, respectively. The MetaMorph or MagnaFire Imaging systems were used to process all images. The area with the highest FOXP3+ expression was documented photographically before and after treatment.

**Data and statistical analysis.** Whole tissue sections of lymph nodes were screened and evaluated at ×20 magnification by the study investigator and pathologists who were blinded with regard to treatment assignment and the sequence of the biopsy samples from each patient. The overall score (the percentage of cells positive for relevant antigen) was assigned to the whole section observed. Quantitation was accomplished by consensus of the two pathologists and the research faculty investigator. The percentage of cells that stained positive in each sample was evaluated and is presented as the score for each section. Cells stained with an intensity from 1+ (the lowest intensity) to 3+ (the highest intensity) were considered as positive; cells without staining (intensity 0), compared with negative control, were considered as negative. Lymph node lymphocytes rather than tumor were evaluated for staining. Lymphocytes observed in this study of lymph node metastases included tumor-infiltrating lymphocytes although the scoring was done for the entire node section. The percentage of positive cells in the same population of cells was assigned as zero. Triple-color immunofluorescence to probe CD4+FOXP3+ Treg cells was used to confirm the FOXP3 immunohistochemistry data.

Statistical analysis was done by a member of the UPCI Biostatistical Facility (H.W.): Mean values of the percentage of positive cells are presented with SE. Comparisons of the percentages of positively stained cells between pretherapy and posttherapy samples were done using the Wilcoxon signed rank test.

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**Fig. 1.** A, FOXP3 expression in lymphocytes in response to HDI therapy. Blue columns, baseline of FOXP3 expression; red columns, FOXP3 expression posttreatment. FOXP3 expression is up-regulated in response to HDI neoadjuvant therapy in all of the 13 patient biopsy samples studied. The percentage of FOXP3+ lymphocytes in the lymph node increases from 3.06 ± 0.65% pretreatment to 9.86 ± 1.27% posttreatment (n = 13, P = 0.0002). Columns, mean; bars, SE. CR, complete responder; PR, partial responder; NR, nonresponder. B, FOXP3 expression in lymphocytes was up-regulated in response to HDI therapy. a, pretreatment section; b, posttreatment section. Red, FOXP3 expression; green, counterstain. A ×20 objective was used to take the photographs. C, CD4+FOXP3+Treg cells were up-regulated in response to HDI therapy. The area with the highest expression of FOXP3+ cells was selected for both pretreatment and posttreatment sections. CD4+ was probed with red fluorescence, FOXP3 was probed with green fluorescence, and blue fluorescence represents Hoechst counterstaining. a1 and b1, nuclear Hoechst staining before and after treatment, respectively. a2 and b2, pretreatment and posttreatment FOXP3 immunostaining, respectively. a3 and b3, pretreatment and posttreatment CD4 immunostaining, respectively. a4 and b4, overlay of the triple-color staining pretreatment and posttreatment, respectively. b4 has more CD4+FOXP3+ Treg cells compared with a4. A ×40 objective was used to take the photographs.
Results

**CD4+FOXP3+ Tregs among regional tumor-involved lymph nodes are up-regulated in response to HDI therapy in vivo.** FOXP3 expression was increased in response to the HDI neoadjuvant therapy in all of the 13 cases studied. The mean percentage of FOXP3+ lymphocytes in the nodal biopsy tissues was increased from 3.06 ± 0.65% pretreatment to 9.86 ± 1.27% posttreatment (n = 13, P = 0.0002), as shown in Fig. 1A. Figure 1B shows that FOXP3 expression was increased in response to HDI neoadjuvant therapy. No significant differences were observed either at baseline or in the changes induced by treatment, between those patients in which objective clinical response was observed and those without clinical response, in terms of FOXP3 expression. No correlation was observed between the levels of FOXP3 expression documented at baseline, or the changes in FOXP3, and the survival of the subjects who have participated in this trial.

In addition, CD4+FOXP3+ Treg cells were probed with triple-color immunofluorescence. In three cases randomly selected, CD4+FOXP3+ Treg cells were uniformly up-regulated in response to HDI. Figure 1C (b4) shows that CD4+FOXP3+ Treg cells were up-regulated in response to HDI therapy. Panels a1 and b1 represent nuclear Hoechst (blue) staining before and after treatment, respectively. Panels a2 and b2 represent pretreatment and posttreatment distribution of FOXP3 (green), respectively. Panels a3 and b3, STAT5 (red); a4 and b4, Hoechst counterstain. A >20 objective was used to take the photographs.

**HDI up-regulates STAT5 both in lymphocytes and in melanoma cells in vivo but does not induce IL-17-positive cells.** CD4+FOXP3+ Treg cells were up-regulated in response to HDI neoadjuvant therapy; thus, we asked whether STAT5 was up-regulated, as associated with the up-regulation of FOXP3 expression following IFNα, as much as IL-2–induced FOXP3 expression is STAT5 dependent (34, 35). STAT5 activation has previously been shown to be IFNα dependent (36). Total STAT5 was up-regulated in both melanoma cells and lymphocytes in response to IFNα2b neoadjuvant therapy in the five cases studied, as shown in Fig. 2A. Figure 2A panels a1-a5 and b1-b5 are pretreatment and posttreatment lymph node sections, respectively. Red represents total STAT5 staining; blue, hematoxylin counterstain. A >20 objective was used to take the photographs.

Panel a5 shows increased CD4+FOXP3+ Treg compared with panel a4.
the total level of STAT5, as shown in Fig. 2B. Figure 2B panels a1-a4 and b1-b4 are pretreatment and posttreatment sections, respectively. Red represents total STAT5; green represents FOXP3; blue represents Hoechst nuclear staining. pSTAT5-tyr694 and FOXP3 were also probed simultaneously with the immunofluorescence technique. STAT5 activation at site Tyr694 (pSTAT5tyr694) was observed to be associated in part with expression of FOXP3 in the lymph node biopsies of patients treated in vivo, as shown in Fig. 3. This photomicrograph shows that FOXP3 expression is partially connected with pSTAT5-tyr694. Panel A shows FOXP3 immunostaining (green); panel B shows pSTAT5tyr694 immunostaining (red); panel C represents nuclear Hoechst staining (blue); panel D is the overlay of the triple staining; and panel E is the magnification of two lymphocytes with FOXP3 expression alone and with both FOXP3 and pSTATtyr694 expression. The yellow arrows (A-B) illustrate the area of panel E that has been magnified. The white arrow in panel D denotes pSTAT5tyr694 expression in endothelial cells as the positive control. STAT5 was found to be constitutively activated in melanoma cells (Fig. 4). The baseline expression level of pSTAT5tyr694 in tumor cells and lymphocytes was very high. IFNα did not alter pSTAT5tyr694 expression levels between pretreatment and posttreatment sections, in either tumor cells or lymphocytes.

Recent studies have shown that IL-2 constrains Th17 generation mediated by STAT5 (27). In addition, Th17 cells are reciprocally correlated with Treg development (21, 23, 27, 37). Therefore, IL-17 was probed with immunofluorescence and

Fig. 4. STAT5 is constitutively activated in melanoma cells in vivo. Photomicrograph demonstrating STAT5 constitutive activation in melanoma cells documented with biopsy samples obtained from the trial of IFNα administered in vivo. A, nuclear Hoechst staining (blue). B, pSTAT5tyr694 immunostaining (red). C, overlay of A and B. A × 40 objective was used to take the photograph.
Discussion

The evaluation of changes induced by systemic interventions such as high-dose IFNα2b may be considered using several investigative designs. We considered partial biopsy of large lymph nodes before high-dose IFNα and resection of the remainder posttreatment, but felt that the surgical violation of the lymph node would potentially induce artifacts in the nodal tissue. We therefore chose to evaluate different lymph nodes or portions of a single basin, or lymph nodes affecting different basins, in the same patient to avoid artifacts in our studies of this neoadjuvant intervention. No differences observed in the course of this study seemed to be associated with the surgical removal of one node of a basin when these samples were compared with lymph node samples drawn from different nodal basins. The alterations in signaling molecules associated with response to HDI did not differ in the nodes sampled from a single basin and from different nodal basins.

IFN has diverse effects on the tumor and host that may relate to adaptive and innate defenses against tumors (38). The regulatory function of constitutive IFNα/β signaling has been recognized in terms of cellular responses in murine and other systems. In a previous study, we have shown that IFNα modulates the balance of phosphorylated STAT1 tyr701 and phosphorylated STAT3 tyr705 and that this was correlated with host immunity against melanoma (9). More recently, it has been found that IFNα plays a critical role in the induction and perpetuation of autoimmune responses in human subjects with resected melanoma (39). IFNα induces durable activation and maturation of peripheral dendritic cells, which, in turn, may activate T cells reactive against normal or tumor tissues (40). The benefit of HDI adjuvant therapy of melanoma has been shown to be closely correlated with its induction of clinical manifestations of autoimmunity and the induction of antibodies against multiple tissues, in particular to the thyroid (10). Naturally occurring CD4+CD25+ Foxp3+ regulatory T cells (Tregs) play a critical role in maintaining homeostasis of the immune system and in assuring peripheral tolerance to self-antigens (41). A decrease in the number of circulating CD4+CD25+ Tregs and their suppressive activity have been found in patients suffering from various autoimmune diseases (42). In addition, the tumor microenvironment has been shown to be associated with the induction of immunologic tolerance and increased numbers and functions of Tregs (43, 44). The balance between immune tolerance induced and maintained by Tregs and immunity induced by IFNα and other factors may play a pivotal role in determining tumor progression. The relative balance between IFNα-induced immunity to self-antigens, and CD4+CD25+Foxp3+ Treg-induced immune tolerance is likely to determine the clinical outcome of adjuvant therapy in patients treated with IFN. Our current and previous studies show that HDI down-regulates STAT3 (9) associated with the up-regulation of lymph node CD4+Foxp3+ Tregs, whereas tissue IL-17–expressing lymphocytes were not altered in response to HDI therapy. Recent studies of others show that down-regulation of STAT3 is associated with up-regulation of FOXP3-expressing Tregs and reduction of Th17 (in mice; refs. 27, 29). Similar peripheral Treg responses to high-dose IL-2 were recently reported and are of interest considering that IL-2 is the only other agent currently approved by the Food and Drug Administration for the treatment of melanoma (33). These observations suggest that selective inhibition of IFNα- and IL-2–mediated enhancement of Tregs might enhance the therapeutic efficacy of one or both of these agents. The up-regulation of Treg in response to IFNα and IL-2 appears to represent a negative feedback system that maintains the balance between immunopotential and immunoregulation to assure tolerance in relation to normal organ autoantigens. IFNα negatively regulates CD8+ T-cell responses through IL-10–producing CD4+ regulatory T cells in mice (45). The response of Treg to neoadjuvant HDI affords us a setting in which it will now be readily possible to assess the role of Treg as a potential basis of compromised therapeutic benefit for this agent. Our observations suggest the possibility of new therapeutic combinations targeting CD4+CD25+Foxp3+ Tregs during IFNα therapy, and the neoadjuvant model system in which we have explored this will allow us to answer this question, potentially leading to more effective clinical results.

STAT5 phosphorylation in melanoma is mediated through SRC and JAK1 kinases (46). STAT5 contributes to IFNα resistance of melanoma cells (47). This study has shown that total STAT5 levels are up-regulated by HDI and that up-regulation of total STAT5 is associated with up-regulation of FOXP3 in lymphocytes of patients treated for nodal metastasis of melanoma in vivo. However, we could not document that pSTAT5tyr694 is up-regulated by HDI. The reason for this may lie in the high baseline levels of pSTAT5tyr694 noted in both tumor cells and the associated tumor-infiltrating lymphocytes. pSTAT5tyr694 is an activated form of phosphorylated STAT5. Total STAT5 was found to be up-regulated in response to HDI and this provides an alternative explanation in that STAT5 is phosphorylated at multiple sites of the peptide. FOXP3 up-regulation is STAT5 dependent (30, 34, 35), and STAT5 activation depends on IFNα (36). Therefore, the up-regulation of FOXP3 expression that we have observed to be induced by IFNα is likely to occur through the IFNα/STAT5 pathway. The incomplete and imperfect correlation of pSTAT5tyr694 with the expression of FOXP3 suggests that FOXP3 expression is modulated by other signaling pathways or other phosphorylated species of STAT5 beyond the pSTAT5tyr694 we have studied. On the other hand, IFNα-induced reduction of the STAT3 signal also contributes to the consequence of the up-regulation of CD4+CD25+Foxp3+ Tregs because these data are consistent with the published murine data (27). The lack of up-regulation of IL-17+ lymphocytes seems also to be attributable to STAT5 activation and the down-regulation of STAT3 activation, as it is known that IL-2 constrains Th17 cell induction through up-regulation of STAT5, and STAT3 promotes Th17 differentiation or function (27).

Disclosure of Potential Conflicts of Interest

J. Kirkwood is a member of the speakers’ bureau of Schering Plough.

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References


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