

Impaired STAT Phosphorylation in T Cells from Melanoma Patients in Response to IL-2: Association with Clinical Stage

Roberta Mortarini,¹ Claudia Vegetti,¹ Alessandra Molla,¹ Flavio Arienti,² Fernando Ravagnani,² Andrea Maurichi,³ Roberto Patuzzo,³ Mario Santinami,³ and Andrea Anichini¹

Abstract Purpose: To assess the extent of signal transducer and activator of transcription (STAT) activation in response to interleukin 2 (IL-2) in melanoma patients' T cells, along with clinical stage of tumor progression.

Experimental Design: T lymphocytes from peripheral blood of healthy donors and of American Joint Committee on Cancer stage I to IV melanoma patients, as well as from metastatic lymph nodes of patients, were evaluated for responsiveness to IL-2. CFSE assays and single-cell phospho-STAT – specific flow cytometry screening were used.

Results. T cells from advanced melanoma patients, in comparison with healthy donors, showed reduced proliferation to IL-2 and IL-15, but not to anti-CD3 monoclonal antibody. Impaired response occurred in CCR7⁺ and CCR7⁻ T-cell subsets, but not in CD3⁻ CD8⁺ natural killer (NK) cells, and was not explained by induction of apoptosis, increased cytokine consumption, or altered IL-2R subunit expression in patients' T lymphocytes. By phospho-specific flow cytometry, defective STAT1 and STAT5 activation in response to IL-2 was found mainly in T lymphocytes from peripheral blood and/or tumor site of American Joint Committee on Cancer stage III and IV patients, compared with stage I and II patients and to donors, and in melanoma antigen-specific T cells isolated from metastatic lymph nodes. At tumor site, impaired STAT activation in T cells did not correlate with frequency of CD4⁺ CD25⁺ Foxp3⁺ T cells. Serum from advanced melanoma patients inhibited IL-2 – dependent STAT activation in donors' T cells and a neutralizing monoclonal antibody to transforming growth factor β 1 counteracted such inhibition.

Conclusions: These results provide evidence for development of impaired STAT signaling in response to IL-2, along with clinical evolution of the disease, in melanoma patients' T cells.

Members of the cytokine family whose receptors share the common γ chain (CD132) play a central role in the regulation of immune responses (1, 2). These cytokines include interleukin 2 (IL-2), IL-4, IL-7, IL-9, IL-15, and IL-21 (1). All the cytokines of this family use γ chain as one subunit of receptors made of two or three chains (1, 2). IL-4, IL-7, IL-9, and IL-21 have a two chain receptor, with the α chain contributing the specificity of cytokine binding, whereas IL-2 and IL-15 receptors

have an additional common β chain (1, 2). The relevance of the γ chain cytokines in immune regulation and of their signal transducer and activator of transcription (STAT) dependent signal transduction pathway has been underscored by the identification of mutations of γ chain as the basis of X-linked severe combined immunodeficiency (3).

Among the known γ chain cytokines, IL-2, IL-7, IL-15, and IL-21 have been shown to control homeostasis, activation, and functional differentiation of T-cell subsets at several stages of the immune response (4–9). Such immunomodulatory functions of the γ chain cytokines play a key role in antitumor T cell – mediated immunity. This has been documented by experimental models and clinical trials of adoptive immunotherapy (10, 11) and by studies based on *in vivo* administration of cytokines to tumor bearing hosts (12). A significant implication of these results is that the ability of cancer patients' T cells to respond to cytokines of the γ chain family (upon *ex vivo* activation) is a key requisite for antigen-independent expansion and differentiation of T-cell populations to be used in adoptive immunotherapy settings (11). Retained T-cell responsiveness to cytokines as IL-2, IL-15, and IL-21 may be essential even for the generation of T-cell effectors *in vivo*, in the context of active immunotherapy, as well as for the efficacy of therapy based on the administration of high dose IL-2 to patients with advanced melanoma (13). This possibility is also in agreement with experimental models, indicating that tumor

Authors' Affiliations: ¹Human Tumors Immunobiology Unit and ²Laboratory Medicine Unit, Department of Experimental Oncology; and ³Melanoma and Sarcoma Unit, Department of Surgery, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

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Requests for reprints: Andrea Anichini, Human Tumors Immunobiology Unit, Department of Experimental Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, Via Venezian 1, 20133 Milan, Italy. Phone: 39-0223902817; Fax: 39-0223902630; E-mail: andrea.anichini@istitutotumori.mi.it.

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Translational Relevance

The identification of melanoma patients with tumor progression – related immune dysfunction may improve the clinical efficacy of current treatment protocols requiring administration of interleukin 2 (IL-2). In fact, objective clinical responses, even in advanced melanoma patients, have been documented in immunotherapy or biochemotherapy studies that include this cytokine. By looking at phosphorylation of STAT1 and STAT5 proteins, a key signaling event triggered by IL-2, evidence for defective responsiveness to this cytokine was found in T cells from peripheral blood or tumor site of American Joint Committee on Cancer Stage III and IV patients, compared to stage I and II patients and to normal donors. These results indicate that melanoma progression is associated with development of immune dysfunction affecting the γ chain cytokine signaling pathway. In addition, these findings suggest that assessment of STAT phosphorylation at the single-cell level by phospho – flow cytometry may be an effective approach to identify patients with retained responsiveness to cytokines as IL-2.

reactive IL-2R α ^{-/-} CD8⁺ T cells fail to control established tumors (14) and that IL-15 and IL-21 have cooperative effects in CD8⁺-mediated tumor regression (12).

However, available evidence suggests that T-cell responsiveness to cytokines as IL-2 may become impaired in at least a fraction of cancer patients, as indicated, for example, by studies in renal and prostate cancer (15, 16). In addition, in B-cell lymphoma and melanoma patients (17, 18), T cells from periphery and/or tumor site frequently show lack of differentiation to effector stages (such as T_{EM} or T_{TD}), a finding consistent with impaired T-cell responsiveness to γ chain cytokines that can promote such functional differentiation (8).

In the instance of melanoma, the characterization of patients for T-cell responsiveness to γ chain cytokines as IL-2 would be relevant to identify subsets that may benefit from clinical use of this cytokine (13). To this end, in this study, we evaluated proliferation and STAT activation upon stimulation with IL-2 in T-lymphocyte subsets from melanoma patients at different stages of disease. The results indicated that melanoma progression is associated with development of immune dysfunction affecting the γ chain cytokine signaling pathway.

Materials and Methods

Lymphocytes from melanoma patients and healthy donors. Lymphocytes were isolated by Ficoll density gradient centrifugation (Ficoll-Paque, Amersham Biosciences) from peripheral blood of healthy donors and of melanoma patients in American Joint Committee on Cancer (AJCC) stages I to IV. Lymphocytes were also isolated from tumor-invaded or tumor-free lymph nodes of AJCC stage IIIc and IV melanoma patients immediately after surgical removal, as described (18). Written informed consent was obtained from patients and healthy donors. Patients were admitted to our institute for surgical treatment of melanoma, and none of them had received chemotherapy or any other therapy with immunosuppressive activity before isolation of lymphocytes.

CFSE assay. Lymphocytes were stained with CFSE (Molecular Probes), as described (19), with minor modifications. Lymphocytes from peripheral blood or tumor-invaded lymph node were cultured at 1×10^6 /mL in RPMI 1640 containing 10% pooled human serum from healthy donors in the presence of IL-2 (Chiron) at 15 or 150 ng/mL or of IL-15 (PeproTech) at 5 or 50 ng/mL for 5 d. Cultures were also set up with CFSE-stained lymphocytes cultured (at 1×10^6 /mL) in 24-well plates pre-coated or not with anti-CD3 monoclonal antibody (mAb; 0.25 μ g/mL) through cross-linking mediated by 10 μ g/mL goat anti-mouse immunoglobulin G (Sigma-Aldrich), as described (19). Lymphocytes were then stained with mAbs to cell surface antigens (CD3, CD4, CD8, CCR7) and evaluated by flow cytometry for the fraction of proliferating cells in different T-cell subsets.

Antibodies and flow cytometry analysis. Four color stainings of lymphocytes were done as described (17, 18). T cells were characterized with APC- or PerCp-conjugated anti-CD8, anti-CD4 (BD Biosciences), APC-conjugated anti-CCR7 (R&D Systems), FITC-conjugated anti-CD45RA (BD Biosciences), and PE- or PerCp-conjugated anti-CD3 (BD Biosciences). To evaluate IL-2 and IL-15 receptor subunit expression, lymphocytes were stained with FITC-conjugated anti-CD25 (IL-2R α chain), with PE-conjugated anti-CD122 (IL-2/IL-15R β chain), or anti-CD132 (common γ chain; BD Biosciences). To detect intracellular perforin, cells were permeabilized with Cytotfix/Cytoperm (BD Biosciences) and then stained with FITC-conjugated antiperforin (BD Biosciences) in the presence of Perm/Wash solution (BD Biosciences). To detect intracellular JAK3, cells were fixed with 2% paraformaldehyde for 10 min and then washed in 2 mL of staining buffer (PBS supplemented with 2% FCS and 0.01% sodium azide). After centrifugation, the cells were incubated for 30 min with 90% methanol at 4°C and then were stained with mouse anti-human JAK3 (Abcam plc), followed by FITC-conjugated goat anti-mouse immunoglobulin G + M (Jackson Immunoresearch Laboratories). To detect total (i.e., regardless of phosphorylation status) STAT1, cells were permeabilized as described for JAK3 detection and then stained with Alexa Fluor 647-conjugated mouse anti-STAT1 (N-terminus; BD Biosciences). As control, cells were also stained with Alexa Fluor 647-conjugated mouse immunoglobulin G1 k isotype control (BD Biosciences). To detect total STAT5, cells were permeabilized as described for JAK3 detection and then stained with mouse anti-STAT5 (BD Biosciences), followed by Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (Molecular Probes). To evaluate expression of Foxp3, lymphocytes were permeabilized and stained with the APC anti-human Foxp3 staining set (eBioscience) according to manufacturer's instructions. To evaluate induction of apoptosis in T cells cultured with IL-2 or IL-15, lymphocytes were stained with Annexin V-FITC and propidium-iodide (BD Biosciences), as described (19). All samples were analyzed by a dual-laser fluorescence-activated cell sorting cytofluorimeter (FACSCalibur, BD Biosciences) using CellQuest software (BD Biosciences).

Single-cell phospho-STAT-specific multicolor flow cytometry analysis. Lymphocytes from patients and donors were kept in RPMI 1640 medium supplemented with 5% pooled human serum and stimulated or not with IL-2 (150 ng/mL) or IL-15 (50 ng/mL) for 15 min to 24 h or, in some experiments, with IL-4 (20 ng/mL; Peprotech). Lymphocytes were then stained with phospho-STAT-specific mAbs according to published methods (20). Briefly, cells were fixed with 2% paraformaldehyde for 10 min to arrest signaling activity and then washed in 2 mL of staining buffer (PBS supplemented with 2% FCS and 0.01% sodium azide). After centrifugation, cells were incubated for 30 min with 90% methanol at 4°C. After two washes with staining buffer, cells were incubated for 1 h at room temperature with Alexa Fluor 488-conjugated mAbs specific for STAT1 phosphorylated at Y701, STAT5 phosphorylated at Y694, STAT6 phosphorylated at Y641 (BD Biosciences), and different combinations of directly conjugated mAbs to cell surface antigens (CD3, CD4, CD8, CCR7) selected according to validated BD Phosflow protocols (BD Biosciences) for multiplexed activation state analysis in human PBMCs. In some experiments,

lymphocytes from melanoma patients were also stained as described (17, 19) with PE-labeled tetramers of HLA-A*0201 (Prolimmune) containing peptides from Melan-A/Mart-1₂₆₋₃₅ (21) and gp100₂₀₉₋₂₁₇ (22) and with mAbs to cell surface antigens (CD8, CCR7, and CD45RA). Permeabilized cells were also stained with Alexa Fluor 488-conjugated mouse immunoglobulin G1 k isotype control (BD Biosciences) to determine background staining. Results were expressed as percentage of cells staining with phospho-STAT mAbs in cytokine-stimulated cultures after subtraction of staining detected in control cultures kept without cytokines. In each experiment involving cytokine stimulation and phospho-STAT staining of lymphocytes from patients, peripheral blood from donors were also included. This provided an internal control for the efficacy of cytokine stimulation and of the staining procedure.

Culture of lymphocytes from healthy donors with serum from melanoma patients. Peripheral blood from healthy donors were cultured for 48 h at 1×10^6 /mL in the presence of pooled human serum or of serum from AJCC stage IV melanoma patients. Such serum samples had been characterized for the content of transforming growth factor β 1 (TGF- β 1) by ELISA (DRG Instruments GmbH). In some experiments, pooled human serum and patients' serum were preincubated or not with 10 μ g/mL of neutralizing mAb to TGF- β 1 (Anogen-YES Biotech Laboratories Ltd.). Alternatively, pooled human serum was supplemented with 500 pg/mL of recombinant TGF- β 1 (R&D Systems). After culture for 48 h with pooled human serum or patients' serum, lymphocytes were stimulated with/without IL-2 (150 ng/mL). Extent of STAT1 or STAT5 phosphorylation in T-cell subsets was evaluated 24 h later by phospho-STAT flow cytometry.

Data analysis and statistics. Experiments assessing extent of proliferative response to γ chain cytokines, expression of γ chain cytokine receptors in T-cell subsets from patients versus donors, and IL-2-induced phosphorylation of STAT proteins in T cells from patients versus donors and from patients at different AJCC clinical stages were evaluated by ANOVA, followed by Student-Newman-Keuls multiple comparison test. Spearman correlation analysis (with two-tailed *P*s) was used to (a) correlate extent of T-cell proliferation with STAT phosphorylation in response to IL-2 and (b) correlate extent of cytokine-induced STAT phosphorylation in T cells from tumor-invaded lymph node with proportion of CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells (Treg) cells in the same tissues.

Results

Reduced proliferative response to IL-2 and IL-15 in T cells from advanced melanoma patients compared with healthy donors. High-dose bolus IL-2 therapy and adoptive cell transfer of T cells activated *ex vivo* with this cytokine have been shown to induce significant clinical responses in advanced melanoma patients (13, 23, 24). However, development of immune dysfunction affecting T-cell responsiveness to γ chain cytokines as IL-2 may hamper the efficacy of such therapeutic approaches. To address this issue, we initially compared, by the CFSE assay, the proliferative response to IL-2 and to the related γ chain cytokine IL-15 of T cells from peripheral blood of 23 advanced melanoma patients (AJCC stages IIIc and IV) and from healthy donors. CD3⁺ CD8⁺ and CD3⁺ CD4⁺ T cells from patients showed a significantly reduced response to different doses of IL-2 and IL-15 in comparison with healthy donors (Fig. 1A for representative CFSE data on four patients versus one donor and Fig. 1B for all results comparing 23 patients and 19 donors). Lymphocytes from patients retained competence for proliferation because extent of T-cell response to immobilized anti-CD3 mAb (Fig. 1A and B) and response of CD3⁻ CD8⁺ NK cells to IL-2 and IL-15 (Fig. 1B) were not reduced

compared with healthy donors (with the exception of patients' NK response to low dose of IL-15; Fig. 1B). Compared with donors, T lymphocytes from patients showed a reduced proliferative response to γ chain cytokines in the immature CCR7⁺ subset, which includes T_{Naive} and T_{Central Memory} cells, and in the more differentiated CCR7⁻ fraction, which includes the T_{Effector Memory} and T_{Terminally Differentiated} cells (Supplementary Fig. S1). Compared with donors (*n* = 6), advanced melanoma patients (*n* = 6) also showed a lower induction of perforin in CD8⁺ T cells upon culture with IL-2 or IL-15 (data not shown).

Defective proliferative response of patients' CD4⁺ and CD8⁺ T lymphocytes to IL-2 and IL-15 was not associated with higher induction of T-cell apoptosis (as evaluated by propidium iodide/Annexin-V assays up to 96 hours; Supplementary Table S1) compared with donors. In addition, lymphocyte cultures from patients did not show evidence of increased cytokine consumption compared with normal donors (as determined by evaluating residual growth promoting activity in supernatants of T-cell cultures fed with IL-2, by an IL-2-dependent cell line; Supplementary Fig. S2).

We then compared T cells from peripheral blood of the 23 advanced melanoma patients and 17 donors for the expression of γ chain cytokine receptor subunits. To this end, percentage of CD8⁺, CD4⁺ T cells, or CD3⁻ CD8⁺ NK cells expressing IL-2R α , IL-2/IL-15R β , IL-2R $\alpha\beta$, and γ chain were evaluated. This analysis provided evidence for a moderate albeit significant reduction in IL-2/IL-15R β subunit expression in the CD4⁺ and CD8⁺ subsets of patients' T cells compared with donors (Fig. 2). However, Spearman correlation analysis failed to show significant correlation between extent of proliferative response to high and low doses of IL-2 or IL-15 and IL-2/IL-15R β subunit expression on patients' T cells (Supplementary Fig. S3).

Taken together, these data suggest that the impaired T-cell response to γ chain cytokines in patients compared with donors cannot be explained by induction of apoptosis, increased cytokine consumption, or differential expression of IL-2/IL-15R β subunit. These results also support the hypothesis that an intracellular mechanism may be responsible for the defective response in patients' T cells.

Phospho-flow cytometry identifies defective STAT1 and STAT5 activation in response to IL-2 in T cells from melanoma patients. To evaluate possible defects in the γ chain cytokine signaling pathway in patients' T cells, lymphocytes from stage IIIc and IV patients and donors were initially compared for the expression of JAK3 by flow cytometry after intracellular staining. JAK3 is an apical tyrosine kinase in the IL-2/IL-15R signaling cascade, and previous studies have indicated suppression of its expression by neoplastic cells (25). However, JAK3 was always expressed in T cells from patients (*n* = 17) and donors (*n* = 15), and no significant differences were found, even in terms of mean fluorescence intensity (CD3⁺CD4⁺ subset, JAK3 mean fluorescence intensity = 38.1 ± 14.5 in patients versus 34.1 ± 18.6 in donors; CD3⁺CD8⁺ subset, JAK3 MFI = 20.6 ± 5.3 in patients versus 19.2 ± 4.1 in donors). Moreover, in the same sets of patients and donors, comparison by flow cytometry for expression of total (i.e., regardless of phosphorylation status) STAT1 and STAT5 in T cells did not provide evidence for significant differences (data not shown).

We then used phospho-specific flow cytometry to evaluate phosphorylation of STAT1 and STAT5 in CD4⁺ and CD8⁺

T cells either untreated and after exposure to IL-2 or IL-15. Preliminary time course experiments with lymphocytes from healthy donors indicated that phosphorylation of STAT1 and STAT5 in response to IL-2 and IL-15 could be detected in T cells after 15 minutes (Supplementary Fig. S4). STAT5 showed a peak of response at 15 minutes with subsequent retention of sustained phosphorylation, whereas STAT1 showed a peak of response at 18 to 24 hours. Initial evaluation of lymphocytes, from peripheral blood of stage IV patients, indicated that T cells were defective for STAT1 and STAT5 phosphorylation in response to high (150 ng/mL) and low (15 ng/mL) doses of IL-2 (in the CCR7⁺ and CCR7⁻ subsets) compared with the response seen in donors and in stage I patients (data not shown). Figure 3 shows representative results comparing response to 150 ng/mL IL-2 of two patients in stage I and IV. The impairment in IL-2-induced STAT activation, in stage IV patients' T cells, was observed at all time points after cytokine stimulation between 15 minutes and 24 hours (data not shown). Phosphorylation of STAT4 in T cells in response to IL-4 was also evaluated but was similar in patients with early or late stage tumors (Fig. 3).

Moreover, in melanoma patients' peripheral blood ($n = 16$, AJCC stage IIIc and IV), a positive and significant correlation was found between extent of T-cell proliferation (in the CD4⁺

and CD8⁺ subsets), by the CFSE assay, and extent of STAT1 and STAT5 phosphorylation at 24 hours, in response to IL-2 (at 150 ng/mL). P s for proliferation versus P-STAT1 were 0.03 (CD4⁺ T-cell subset) and 0.01 (CD8⁺), whereas P s for proliferation versus P-STAT5 were <0.001 (CD4⁺) and 0.008 (CD8⁺).

Impaired STAT activation in melanoma patients' T cells in response to IL-2 is associated with advanced disease. To assess whether the reduced STAT activation in response to IL-2 occurred in association with advanced stage of disease, we carried out a phospho-STAT-specific flow cytometry screening of response to IL-2 in a large panel of lymphocytes from patients and donors. This panel included lymphocytes from peripheral blood of patients ($n = 102$), covering AJCC clinical stages I to IV, as well as from tumor-invaded ($n = 39$) and tumor-free lymph nodes ($n = 13$; removed from the same nodal basin as the tumor-invaded lymph node) of metastatic patients. Peripheral blood from healthy donors ($n = 16$) were used as control. Results of this analysis indicated a significant reduction in the fraction of CD4⁺ and CD8⁺ T cells from peripheral blood of metastatic patients (AJCC stage III and IV) responding to IL-2 with phosphorylation of STAT1 compared with healthy donors and with patients in early stages of disease (AJCC stage I and II; Fig. 4A). Lymphocytes from tumor-invaded lymph node were the most defective when compared with peripheral blood of

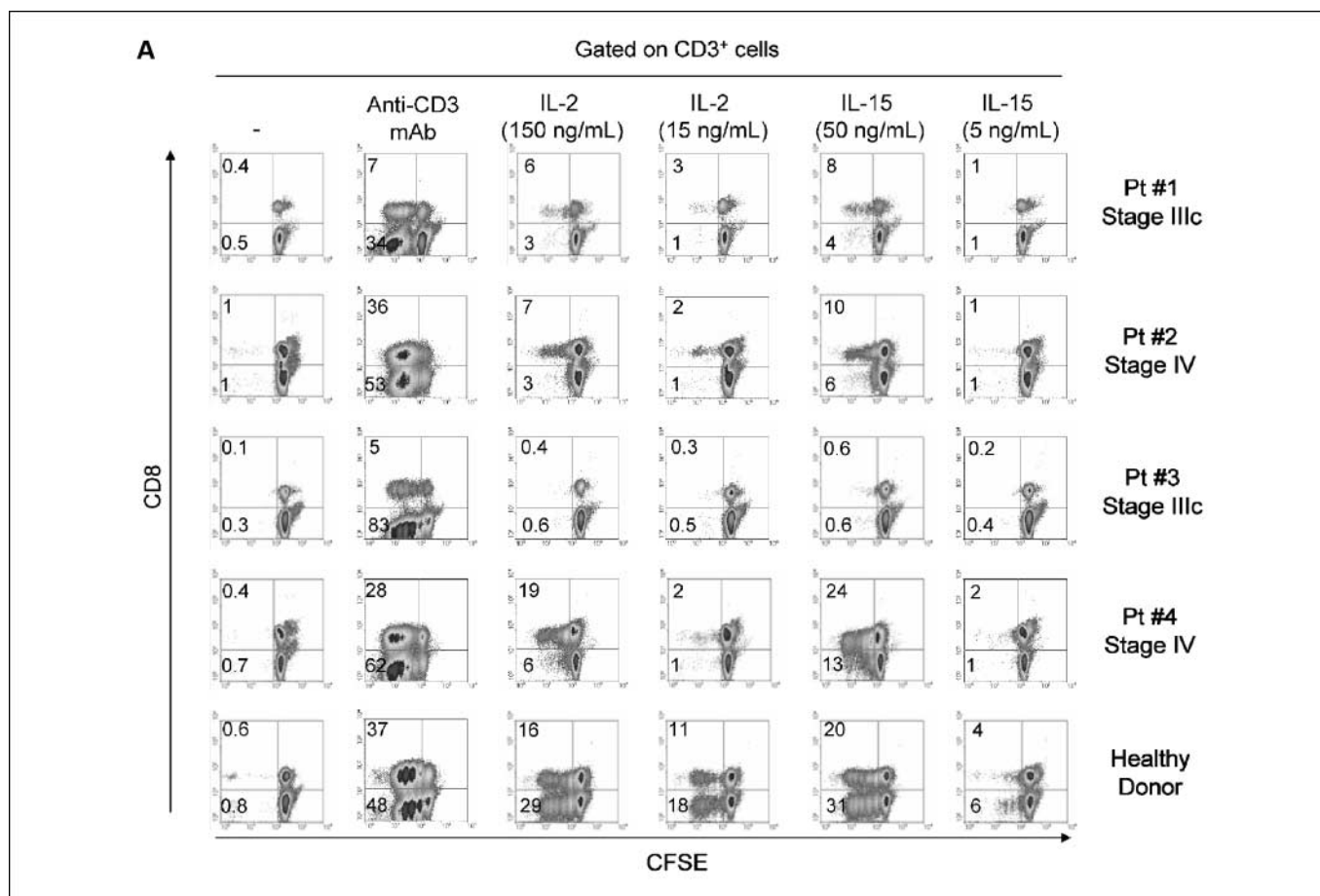


Fig. 1. Reduced proliferative response to IL-2 and IL-15 in T cells from peripheral blood of advanced melanoma patients compared with healthy donors. **A**, CFSE-stained lymphocytes isolated from peripheral blood of four melanoma patients in AJCC stage IIIc or IV and from a healthy donor were cultured for 5 d in the presence of IL-2 or IL-15 at the indicated concentrations or on immobilized anti-CD3 mAb. Extent of proliferation was evaluated by flow cytometry after staining for CD3 and CD8. Numbers in the upper and lower left quadrants of each dot plot, the % of proliferating CD8⁺ or CD8⁻ T cells, respectively.

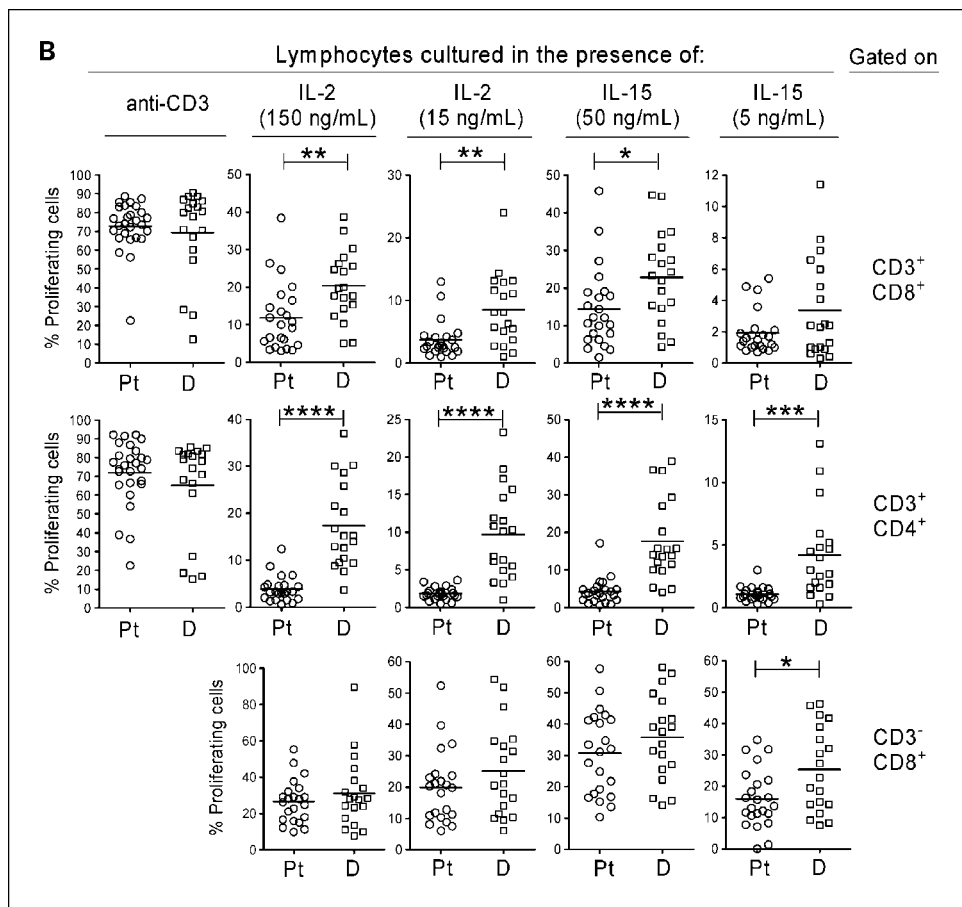


Fig. 1. (cont'd) *B*, comparison of proliferative response to IL-2 and IL-15 by the CFSE assay, carried out as described for (A), in T cells from peripheral blood of 23 patients with advanced melanoma and of 19 healthy donors. Results expressed as percentage of proliferating cells [determined as described for (A)] in each of the indicated lymphocyte subsets. Statistical analysis (by ANOVA followed by Student-Newman-Keuls test) was annotated as follows. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

normal donors (Fig. 4A). Interestingly, even lymphocytes from tumor-free lymph node were strongly deficient in STAT1 phosphorylation in response to IL-2. When looking at STAT5 phosphorylation after stimulation with IL-2, in the same panel of lymphocytes, a defective response was mainly found in $CD4^+$ T cells from tumor-invaded lymph node and in $CD8^+$ T cells from tumor-invaded lymph node, tumor-free lymph node, and peripheral blood compared with donors (Fig. 4A). In the latter T-cell subset, the most significant impairment of STAT 5 activation, compared to donors, was observed in peripheral blood from stage IV patients and in tumor-invaded lymph node ($P < 0.001$; Fig. 4A, lower right). Phosphorylation of STAT6 in response to IL-4 was also evaluated in the whole panel of lymphocytes from donors and patients. Defective STAT6 phosphorylation in response to IL-4 was found in T cells from tumor-invaded lymph node (in the $CD4^+$ subset; Fig. 4B, top) and peripheral blood of patients (in the $CD8^+$ subset; Fig. 4B, bottom) compared with donors.

Lack of STAT1 and STAT5 phosphorylation in response to IL-2 in melanoma antigen-specific T cells at tumor site. Lymphocytes from tumor-invaded lymph nodes of HLA-A*0201⁺, AJCC stage IIIc patients ($n = 7$) were stimulated with or without IL-2 and then evaluated for phosphorylation of STAT1 and STAT5. Tumor-invaded lymph node from these patients contained a small but detectable fraction of $CD4^+$ and $CD8^+$ T cells that

could activate STAT1 (representative results from one patient shown in Fig. 5A) and STAT5 (Fig. 5C) in response to IL-2. However, in these tumor-invaded lymph node, phosphorylation of STAT1 (Fig. 5B, arrows) or STAT5 (Fig. 5D, arrows) in response to IL-2 was almost completely inhibited in the subset of melanoma antigen-specific T cells identified by tetramers of HLA-A2 complexed with Melan-A/Mart-1₂₆₋₃₅ and gp100₂₀₉₋₂₁₇ peptides. Such impaired response occurred despite the predominant antigen experienced (CCR7⁺ CD45RA⁺) T_{EM} phenotype of these cells from these patients (Fig. 5B).

Impaired T-cell responsiveness to IL-2 in tumor-invaded lymph node does not correlate with frequency of $CD4^+$ $CD25^+$ Foxp3⁺ T cells at tumor site. $CD4^+$ $CD25^{\text{high}}$ Foxp3⁺ regulatory T cells, known to increase in melanoma patients (26, 27), may inhibit proliferative responses of other T-cell subsets at tumor site (27). Following this evidence, we tested the hypothesis that frequency of Tregs in tumor-invaded lymph node should negatively correlate with extent of cytokine-induced STAT1 or STAT5 phosphorylation in T cells isolated from the same tumor-invaded lymph node. To this end, extent of IL-2-induced STAT1 and STAT5 phosphorylation in T cells from tumor-invaded lymph node of 22 patients (Supplementary Fig. S5A) was correlated with frequency, in the same tissues, of $CD4^+$, $CD25^{\text{high}}$, Foxp3⁺ regulatory T cells ($n = 22$; range of Treg frequency = 1.17% to 13.45%/ $CD4^+$; Supplementary

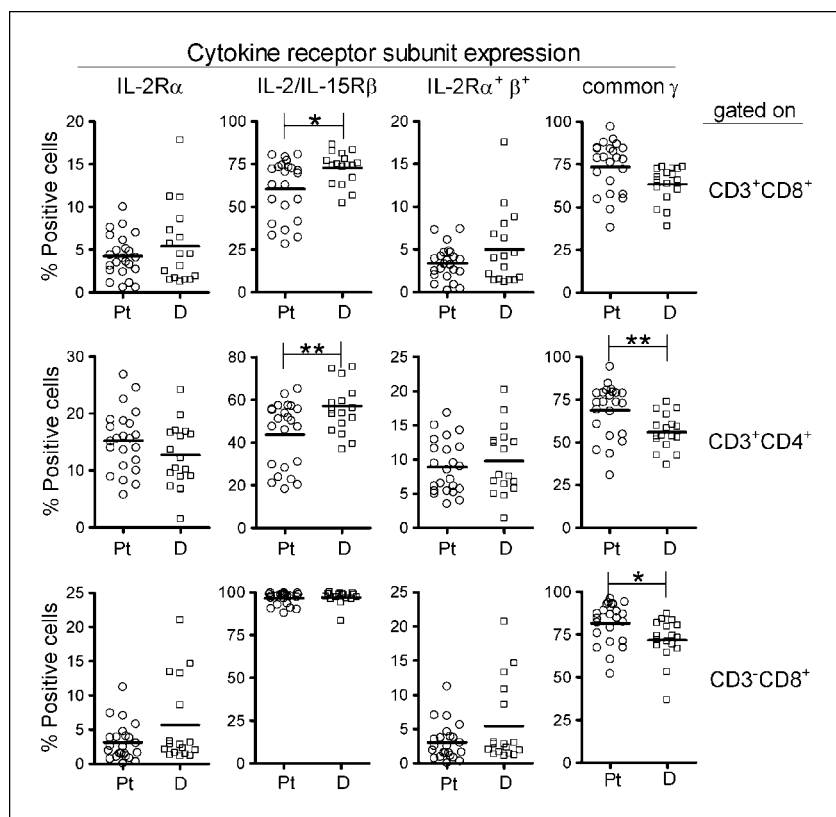


Fig. 2. Expression of IL-2R and IL-15R subunits in T cells from peripheral blood of advanced melanoma patients and donors. Percentage of CD4⁺ and CD8⁺ T cells and of CD3⁺CD8⁺ NK cells expressing IL-2R α , IL-2/IL-15R β , the α and the β subunits (indicated as IL-2R α ⁺ β ⁺), and common γ chain in peripheral blood of 23 metastatic melanoma patients and 17 healthy donors, as evaluated by flow cytometry. Statistical analysis (by ANOVA followed by Student-Newman-Keuls test) was annotated as follows. *, $P < 0.05$; **, $P < 0.01$.

Fig. S5B). Correlation analysis (exemplified in Supplementary Fig. S5C) was carried out to evaluate the significance of all 24 possible combinations, two at a time, between Treg frequency and extent of STAT phosphorylation in T-cell subsets in tumor-invaded lymph node (Supplementary Fig. S5D). In contrast with the initial hypothesis, all of the possible correlations had a positive r , not a negative one, and moreover, none had a significant P (Supplementary Fig. S5D).

Inhibition of IL-2-dependent STAT phosphorylation in T cells by soluble factors present in serum of patients. We then tested the hypothesis that soluble factors present in serum of patients could contribute to inhibit IL-2-dependent STAT phosphorylation in T cells. To this end, lymphocytes from normal donors were precultured for 48 hours with serum from stage IV melanoma patients or from a pool of healthy donors (pooled human serum) and then extent of IL-2-dependent STAT phosphorylation was compared. Preculture of lymphocytes with serum from AJCC stage IV patients led to >50% reduction in the extent of IL-2-dependent STAT1 and STAT5 activation compared with cells kept with normal donors' serum (Fig. 6A). The CCR7⁺ and CCR7⁻ subsets showed reduced STAT phosphorylation in response to IL-2 when cells had been preincubated with patients' serum. Interestingly, preincubation of patients' serum with a neutralizing mAb to TGF- β 1 mAb restored IL-2-dependent STAT5 activation in T cells (Fig. 6B). Instead, preincubation of normal donors' serum with the same mAb did not affect subsequent IL-2-dependent STAT activation in T cells (Fig. 6B). Similarly, by pretreatment of such serum with anti-TGF- β 1 mAb, even STAT1 phosphorylation was rescued (completely in the CCR7⁺ subset and partially in the CCR7⁻ subset; Supplementary Fig. S6A). To corroborate the evidence for involvement of TGF- β 1 in the suppressive effect, we first analyzed by ELISA the sera from 27 stage IIIc and IV melanoma patients and found a concentration of 445 \pm

110 pg/mL of TGF- β 1 (in contrast to <100 pg/mL in healthy donors and stage I and II patients; data not shown). Then, serum from normal donors was supplemented with 500 pg/mL of recombinant TGF- β 1 and used to preculture lymphocytes for 48 hours. We found that such TGF- β 1-supplemented serum could inhibit IL-2-dependent STAT1 and STAT5 activation in donors' T cells to a similar extent, as previously seen with serum from patients (Fig. 6C). Control experiments indicated that preculture of donors' lymphocytes with advanced melanoma patients' serum did not affect STAT6 phosphorylation induced by IL-4 in comparison with cells precultured with normal donors' serum (Supplementary Fig. S6B).

Taken together, these results suggest that soluble factors such as TGF- β 1 present in advanced melanoma patients' serum may contribute to induce defective STAT phosphorylation in T cells in response to IL-2.

Discussion

Our findings provide evidence for defective proliferative response associated with an impairment in STAT phosphorylation in T cells from advanced melanoma patients, in response to IL-2. Defective STAT signaling in patients' T lymphocytes occurred mostly in advanced/metastatic disease and affected the CD4⁺ and the CD8⁺ T cells. In addition, the CCR7⁺ and CCR7⁻ T-cell subsets from patients were less responsive (compared with healthy donors), indicating that T cells at all maturation stages along the naive to effector and memory pathway were impaired in the response to these cytokines.

Decreased T-cell response may be due to increased cytokine consumption by some cell subsets in patients' cultures. This mechanism would be consistent with the described high frequency, in cancer patients, of regulatory T cells expressing

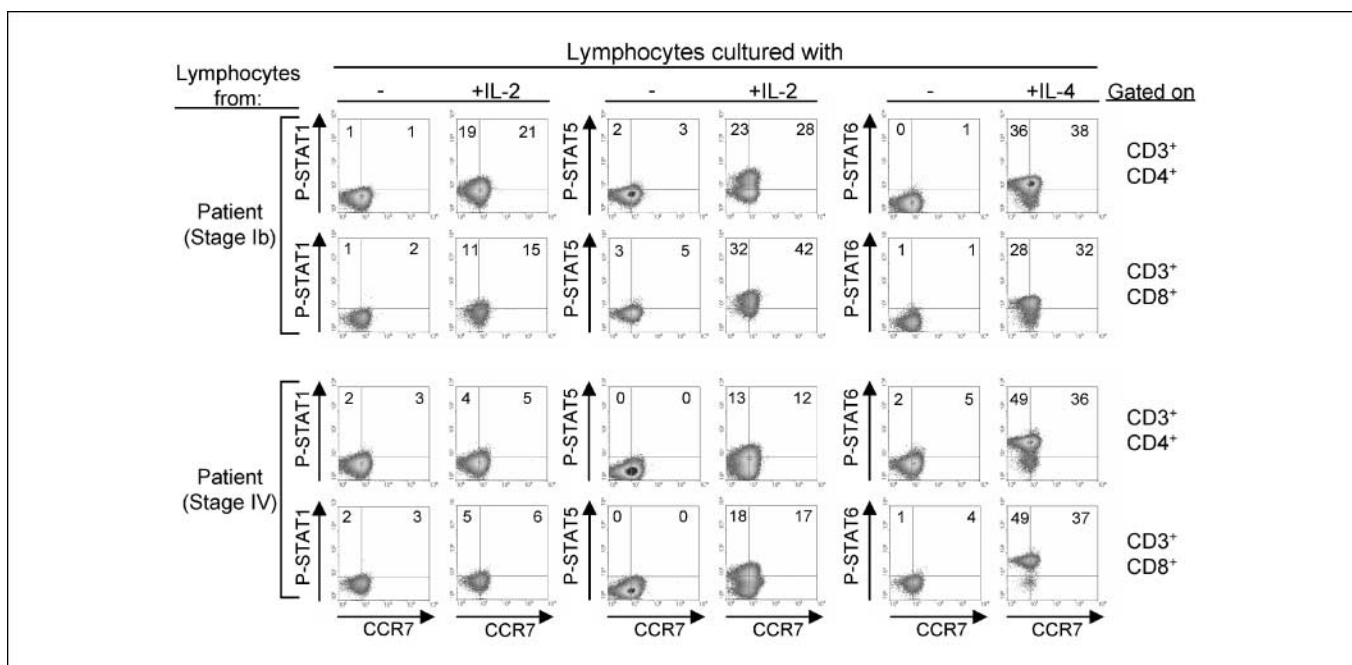


Fig. 3. STAT activation in T cells from melanoma patients in response to IL-2 or IL-4. Peripheral blood from two melanoma patients (AJCC Stage Ib and IV) were cultured for 24 h with or without IL-2 (150 ng/mL) or IL-4 (20 ng/mL). Phosphorylation of STAT1 and STAT5 (in response to IL-2) and STAT6 (in response to IL-4) was evaluated by intracellular staining with phospho-STAT – specific mAbs. Cells were fixed at 24 h after stimulation with cytokines, permeabilized, and stained with phospho-STAT – specific mAbs and with other mAbs to cell surface markers. Numbers in each dot plot, percentage of cells staining with phospho-STAT mAbs in the CCR7⁺ (upper left quadrant) or CCR7⁺ (upper right quadrant) subsets. Isotype control antibodies were used to determine background staining.

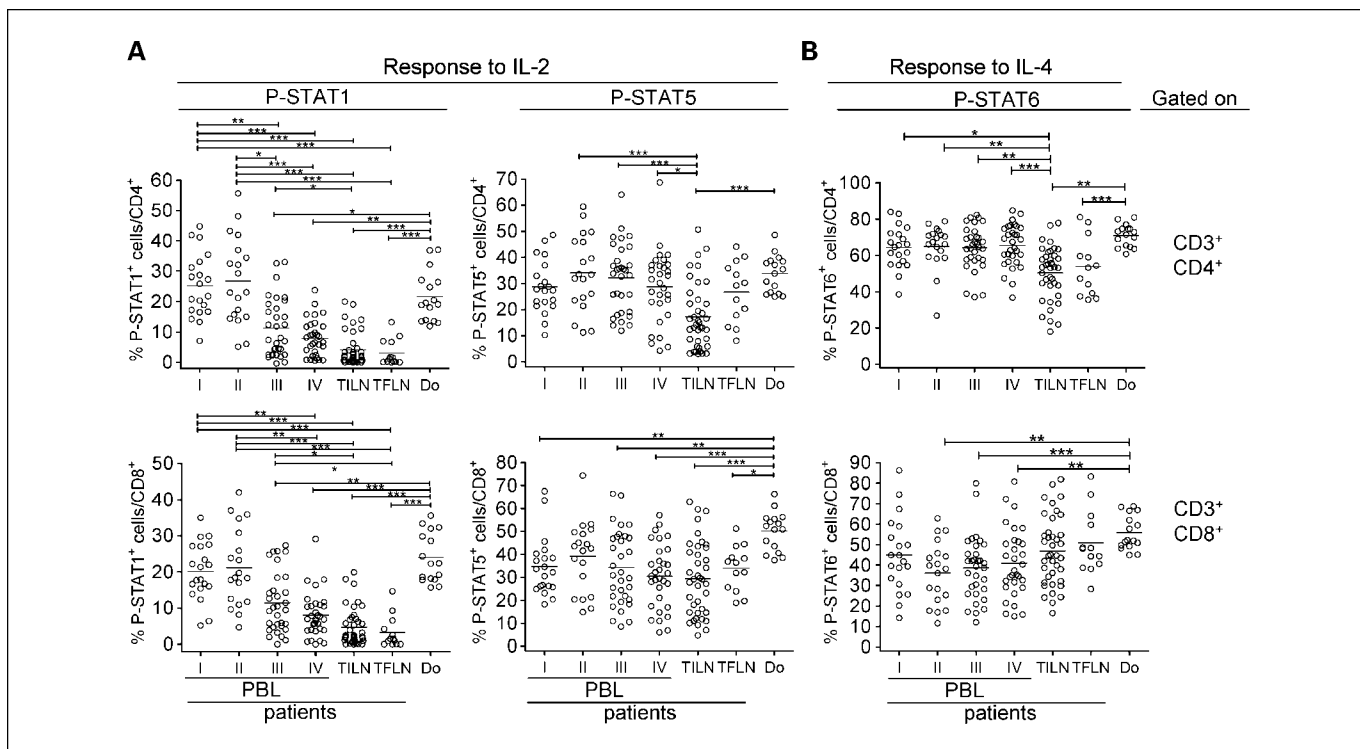


Fig. 4. Defective STAT activation in patients' T cells stimulated with IL-2: association with clinical stage of disease. *A* and *B*, lymphocytes from peripheral blood of healthy donors ($n = 16$), melanoma patients in AJCC stage I ($n = 20$), II ($n = 19$), III ($n = 32$), IV ($n = 31$), from tumor-invaded lymph nodes ($n = 39$), and tumor-free lymph nodes ($n = 13$) were evaluated by flow cytometry for activation of STAT1 and STAT5 in response to IL-2 (*A*; 150 ng/mL) or for activation of STAT6 in response to IL-4 (*B*; 20 ng/mL). Cells were cultured for 24 h with or without cytokines and then analyzed as described in the legend to Fig. 3. Results expressed as percentage of CD4⁺ or CD8⁺ T cells staining with phospho-STAT mAbs in cytokine-stimulated cultures after subtraction of staining detected in control cultures kept without cytokines. Isotype control antibodies were used to determine background staining. Statistical analysis (by ANOVA followed by Student-Newman-Keuls test for all comparisons) was annotated as follows. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

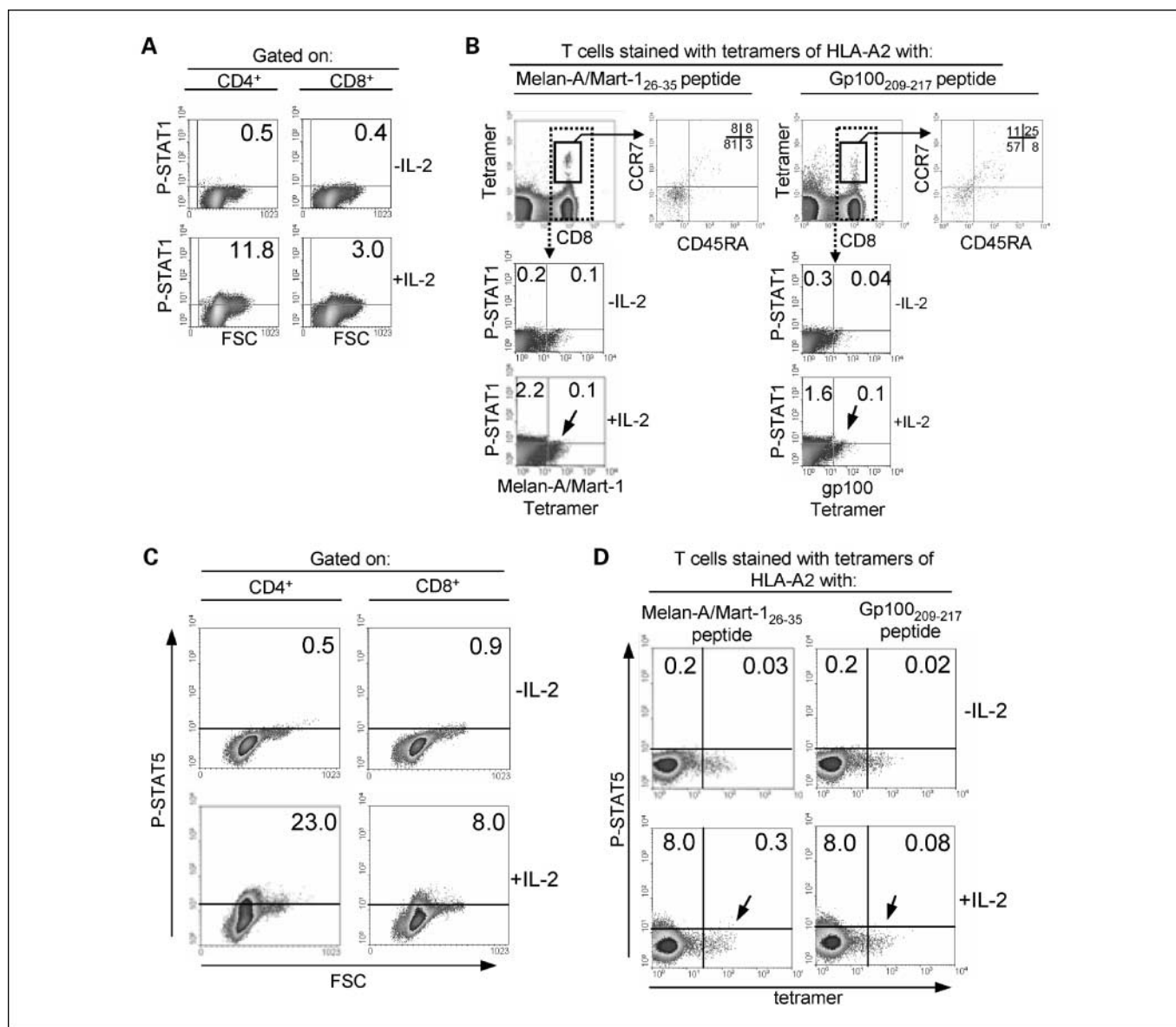


Fig. 5. Impaired STAT1 and STAT5 phosphorylation in response to IL-2 in tumor-antigen-specific T cells isolated from metastatic lymph nodes. **A** and **C**, lymphocytes from tumor-invaded lymph node of an HLA-A2.1⁺ metastatic melanoma patient (representative of seven patients investigated) were cultured with (+IL-2) or without (-IL-2) 150 ng/mL IL-2 for 24 h. Phosphorylation of STAT1 (**A**) or STAT5 (**C**) in CD4⁺ and CD8⁺ T cell subsets was then evaluated by flow cytometry, as described in the legend to Fig. 3. Cells were gated on CD3⁺ CD4⁺ or CD3⁺ CD8⁺, and results plotted as forward side scatter versus P-STAT1 (**A**) or forward side scatter versus P-STAT5 (**C**). **B**, top, cells from the same tumor-invaded lymph node sample as in (**A**) were stained with tetramers of HLA-A2 containing peptides Melan-A/Mart-1₂₆₋₃₅ or gp100₂₀₉₋₂₁₇ and evaluated for expression of CCR7 and CD45RA (after gating on tetramer⁺ T cells, identified by the inner rectangular gate). **B**, middle and bottom, tumor-invaded lymph node were also cultured with or without IL-2 (150 ng/mL; 24 h), and then characterized for STAT1 phosphorylation. Dot plots in middle and bottom (P-STAT1 versus tetramer) were gated on CD8⁺ (identified by the dotted gate in the upper). **D**, Tetramer⁺ T cells were evaluated for phosphorylation of STAT5 in response to IL-2, as described for panel (**B**). Numbers in dot plots, the percentage of positive cells in the corresponding quadrant. Arrows point to impaired STAT activation in tetramer⁺ T cells.

high affinity IL-2 receptors (28). Such cells may in fact efficiently compete with other T cell subsets for IL-2 use. However, analysis, by an IL-2 – dependent cell line, of residual growth promoting activity in supernatants of T-cell cultures fed with IL-2 failed to provide support for increased cytokine consumption in patients' cultures compared with donors. Selective induction of T-cell apoptosis in patients' T cells in response to γ chain cytokines is another possibility. As shown initially by Refaeli et al. (5), IL-2 has a specific role in activation-induced cell death (AICD) in T cells by increasing transcription and expression of CD95L and by suppressing the inhibitor of apoptosis FLIP. Experimental models have also shown that administration of IL-2 *in vivo* to

tumor bearing mice can promote apoptosis of previously activated tumor-infiltrating lymphocytes (29). Interestingly, T cells from cancer patients with solid tumors and hematologic malignancies often develop an activated apoptosis-prone phenotype characterized by increased expression of CD95 (17, 30). Despite these potential mechanisms, apoptosis assays failed to show increased T-cell death in patients' cultures fed with IL-2 or IL-15 compared with donors' lymphocytes. We then assessed whether responsiveness to γ chain cytokines was associated with differential expression of γ chain cytokine receptor subunits. A significant reduction was indeed found for the expression of IL-2/IL-15R β subunit in patients' T cells compared with donors, but

such reduction did not correlate with the defective proliferative response to IL-2 and IL-15.

Engagement of receptors of γ chain cytokines activates an array of tyrosine kinases, including those associated with the extracellular signal-regulated kinase pathway and the JAK/STAT pathway (31). Within the STAT pathway, early studies indicated that in cells that express the appropriate receptors, cytokines such as IL-2 and IL-15 activate multiple STAT proteins, including STAT1, STAT3, and STAT5 (32–34). In agreement, in T cells from healthy donors, by phospho-STAT-specific flow cytometry, we found that IL-2 and IL-15 induced phosphorylation of STAT1 and STAT5. When we looked at components of the γ chain cytokine signaling, we found that JAK3, STAT1 and STAT5 expression were similar in patients and donors. However, evaluation of STAT1 and STAT5 activation by IL-2 in peripheral blood from patients with advanced melanoma revealed a marked reduction compared with healthy donors. Moreover, lack of STAT1 and STAT5 phosphorylation in response to IL-2 was found in melanoma antigen-specific T cells at tumor site of metastatic patients. Furthermore, analysis for response to IL-2 in T cells from a large panel of patients indicated that impaired STAT1 phosphorylation (and, to a lesser extent, even STAT5 phosphorylation) in T cells from peripheral blood mainly occurred at late stages (AJCC stage III and IV). In agreement, even T cells from tumor-invaded lymph nodes (as well as from tumor-free lymph node removed from the same nodal basin as the tumor-invaded lymph node) showed marked reduction of STAT phosphorylation in response to IL-2.

The identification of defective STAT phosphorylation in periphery, as well as in tumor-free lymph nodes, suggested that the mechanism supporting the observed defect might not require direct tumor cell–T cell interaction. Instead, these findings were consistent with either the action of regulatory T cells with suppressive activity and/or of soluble immunosuppressive factors. An experiment designed to address the potential involvement of Tregs did not provide evidence for a significant correlation between extent of impaired STAT phosphorylation (in response to IL-2) in T cells from tumor-invaded lymph node and frequency of CD4⁺ CD25⁺ Foxp3⁺ cells in the same tissues.

On the other hand, preincubation of T cells from healthy donors with serum from advanced melanoma patients indicated that soluble factors could be involved in inducing the defective STAT phosphorylation. Moreover, functional assays with recombinant TGF- β 1 and with a neutralizing mAb to TGF- β 1 added to patients' sera were consistent with a role of this factor, known to be present in serum from advanced melanoma patients (35), in inhibition of IL-2-induced STAT phosphorylation in T cells. These results are in agreement with the inhibitory effect of TGF- β 1 on IL-12- and IL-2-induced STAT phosphorylation (36, 37) in T cells. The latter results have not been confirmed in another study by Sudarshan et al. (38), although these authors investigated the effect of TGF- β 1 on cytokine-induced STAT phosphorylation in PHA-activated T cells rather than in freshly isolated T cells.

In conclusion, we found that STAT signaling pathway in T cells from melanoma patients shows a reduced activation in

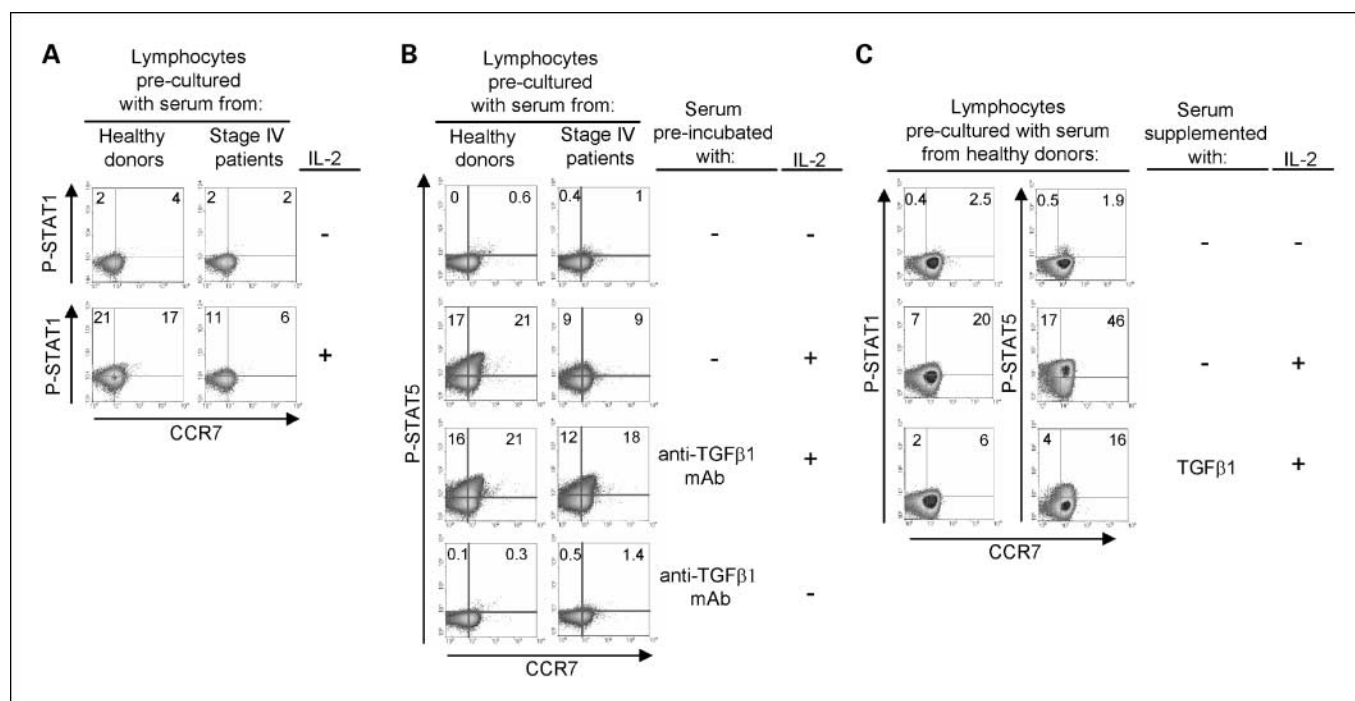


Fig. 6. Inhibition of IL-2-dependent STAT phosphorylation in T cells by serum from advanced melanoma patients. *A* and *B*, peripheral blood from a healthy donor were precultured for 48 h with serum from a pool of healthy donors or from an AJCC stage IV patient. Cultures were then stimulated or not with IL-2 (150 ng/mL) for 24 h. STAT1 (*A*) or STAT5 (*B*) phosphorylation was then evaluated as described in the legend to Fig. 3. *B*, serum from donors or from a stage IV patient was preincubated or not with 10 μ g/mL of a neutralizing mAb to TGF- β 1 before use for preculturing lymphocytes. *C*, serum from a pool of healthy donors was supplemented or not with 500 pg/mL of recombinant TGF- β 1 and then used for culture of lymphocytes from a healthy donor for 48 h. Lymphocytes were then stimulated or not for 24 h with 150 ng/mL of IL-2. STAT1 and STAT5 phosphorylation was then evaluated. All dot plots were gated on CD3⁺ CD4⁺ T cells. Serum from advanced melanoma patient used in experiments shown in (*A*) and (*B*) contained ~500 pg/mL of TGF- β 1, as evaluated by ELISA. Numbers in each dot plot, the percentage of cells staining with phospho-STAT mAbs in the upper left (CCR7⁻ subset) and upper right (CCR7⁺ subsets) quadrants. Data shown are representative of three experiments with sera from different patients.

response to stimulation with IL-2 and that this occurs in association with late stages of disease. These findings provide a further mechanism that could contribute to impairment of T-cell function in advanced stage of disease, in addition to the previously described down-regulation of TCR signaling molecules such as ζ chain (39). The latter is brought about by several mechanisms, including HLA molecule shedding by the tumor, consumption of L-arginine by tumor-associated macrophages and tumor-induced activation of caspases (see ref. 39 for review). Thus, it seems that advanced tumors as melanoma may contribute either directly or indirectly to generate immune suppression by inhibition of expression and/or function of apical signaling molecules in the TCR and γ chain cytokine pathways.

Finally, these results suggest that monitoring of signaling pathways at the single cell level by phospho-flow cytometry (20) may be an effective approach to identify cancer patients with progression-related immune dysfunction and for improv-

ing immunotherapy of cancer, as suggested by recent studies (40–42). For example, monitoring by phospho-flow cytometry of T cells from patients receiving IL-2 *in vivo* may allow finding significant correlations between extent of IL-2-induced STAT activation and patients' response to therapy. Additional investigation, needed to further dissect the mechanism(s) of the impaired STAT activation process described in this study, might also allow identifying effective strategies for restoring T-cell function in patients with advanced tumors (42).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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