Multiparametric Flow Cytometric Analysis of Signal Transducer and Activator of Transcription 5 Phosphorylation in Immune Cell Subsets \textit{In vitro} and following Interleukin-2 Immunotherapy

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Abstract Purpose: Treatment with interleukin (IL)-2 (Proleukin) yields a 10\% to 20\% response rate in patients with metastatic melanoma or metastatic renal cell carcinoma. IL-2 is known to activate distinct signals within lymphocytes, including the Janus-activated kinase – signal transducer and activator of transcription (STAT) pathway. We examined the phosphorylation of STAT5 (P-STAT5) in IL-2-stimulated immune cells of normal subjects and in patients receiving IL-2 therapy using a novel flow cytometric assay to characterize the pattern and level of activation within immune subsets.

Experimental Design: Normal peripheral blood mononuclear cells (PBMC) were treated \textit{in vitro} with IL-2 and analyzed for P-STAT5 using an intracellular flow cytometric assay. PBMC were simultaneously evaluated for the induction of STAT5-regulated genes at the transcript level. PBMC were also obtained from patients immediately before and 1 hour after treatment with high-dose IL-2 and analyzed for the presence of P-STAT5 within immune cell subsets by dual-variable intracellular flow cytometry.

Results: \textit{In vitro} IL-2 treatment produced a rapid and dose-dependent increase in P-STAT5 within normal PBMC that correlated with the induction of transcript for the IL-2-responsive genes CIS, Pim-1, and SOCS1 (correlation coefficients 0.8628, 0.6667, and 0.7828, respectively). Dose-dependent induction of P-STAT5 was detected in PBMC for up to 18 hours following \textit{in vitro} pulse stimulation with IL-2. P-STAT5 was detected within a subset of normal donor CD4+ T cells (52.2 \pm 15.0\%), CD8+ T cells (57.6 \pm 25.8\%), and CD56+ natural killer (NK) cells (54.2 \pm 27.2\%), but not CD14+ monocytes or CD21+ B cells, following \textit{in vitro} IL-2 treatment. The generation of P-STAT5 within immune cell subsets after the therapeutic administration of IL-2 varied significantly between individuals. NK cells were noticeably absent in the posttreatment sample, a finding that was consistent for all patients examined. Surprisingly, activated STAT5 persisted within CD4+ and CD8+ T lymphocytes, as well as CD56+ NK cells, for up to 3 weeks post-IL-2 treatment in three patients who exhibited a clinical response to therapy and in a fourth who exhibited a significant inflammatory response after 11 doses of therapy (first cycle).

Conclusions: The flow cytometric assay described herein is a highly efficient and reliable method by which to assess the cellular response to IL-2 within PBMC and specific immune effector subsets, both \textit{in vitro} and in the clinical setting. Assessment of P-STAT5 in patient PBMC in response to therapeutic IL-2 administration reveals disparate responses between immune cell subsets as well as interpatient variation. Persistent activation of STAT5 within NK and T cells was an unexpected observation and requires further investigation.

Interleukin (IL)-2 is a 15-kDa glycoprotein that functions as a growth factor for T cells and natural killer (NK) cells (1, 2). It is produced by activated CD4+ T cells during the T helper type 1 immune response (3). On binding to its receptor [IL-2 receptor (IL-2R)\textsuperscript{2}], IL-2 activates Janus-activated kinases (JAK) 1 and 3, which in turn associate with the cytoplasmic domains of the IL-2R\textsuperscript{β} and IL-2R\textsuperscript{γc} subunits. IL-2R\textsuperscript{β} subsequently undergoes tyrosine phosphorylation, thereby creating docking sites for several intracellular proteins, including the signal transducer and activator of transcription (STAT) 5 transcription factor (4). On phosphorylation, STAT5 forms a heterodimer and translocates to the cell nucleus, where it directs the transcription of multiple IL-2-responsive genes.
STAT5 plays a crucial role in normal immune function (5–7). STAT5 is required for IL-2-induced cell cycle progression in T cells and for NK cell–mediated proliferation and cytolytic activity (8, 9). Both STAT5a and STAT5b are required for antigen-induced T-cell recruitment into tumor tissue (10). STAT5 is known to mediate antiapoptotic signals, and inhibition of STAT5a/STAT5b promotes the apoptosis of IL-2-responsive primary and tumor-derived lymphoid cells (5, 11, 12). Mice deficient in STAT5a and STAT5b exhibit alterations in NK cell function and decreased T-cell and B-cell proliferation in response to chemokines (13). Constitutive activation of STAT proteins has been observed in numerous hematologic and solid malignancies (14, 15). In particular, STAT3 and STAT5 are strongly associated with tumor development and progression (14).

Administration of IL-2 to patients with metastatic malignant melanoma or renal cell carcinoma induces a clinical response in 10% to 20% of patients, one third of whom experience a durable complete response (16–21). However, toxicity involving multiple organ systems is common with the administration of high-dose IL-2, and careful patient selection is required (16, 17, 21). It is not clear how to predict which patients will exhibit severe toxicities, and researchers continue to search for clinical or molecular markers that can predict the response to IL-2 therapy (22–28). However, previous studies have suggested that the clinical response to IL-2 immunotherapy is mediated through the in vivo expansion and activation of cytotoxic lymphocytes and/or enhanced migration of cytotoxic lymphocytes within tumor tissues (29).

The activation of STAT5 proteins is one of the earliest signaling events mediated by the IL-2 family of cytokines, resulting in the expression of many genes involved in T-cell proliferation and activation (4). We hypothesized that flow cytometric analysis of STAT5-mediated signaling events within immune cell subsets could provide further insight into the mechanism of action of IL-2 in cancer patients and also serve as a surrogate marker of gene regulation within IL-2-sensitive cells (30). In the present report, we show that JAK-STAT signal transduction was rapidly stimulated within NK cells and T lymphocytes following IL-2 treatment. STAT5 activation in response to IL-2 was minimal in B lymphocytes and monocytes. Dose-dependent phosphorylation of STAT5 (P-STAT5) was detected up to 18 hours following pulse stimulation with 8 nmol/L IL-2 and correlated with the induction of transcript for IL-2-responsive genes. Analysis of peripheral blood mononuclear cells (PBMC) from patients undergoing immunotherapy with IL-2 revealed marked variability in the generation of P-STAT5 within immune cell subsets. Surprisingly, activated STAT5 persisted within CD4 and CD8 T lymphocytes, as well as CD56+ NK cells, for up to 3-week post-IL-2 treatment in a subset of patients.

Materials and Methods

Reagents. Recombinant human IL-2 was obtained from Roche Pharmaceuticals (Nutley, NJ). Anti-P-STAT5 (Tyr 694) polyclonal antibody was purchased from Cell Signaling Technologies, Inc. (Beverly, MA). Anti-STAT5 antibody was obtained from BD Biosciences PharmMingen (San Jose, CA). APC–conjugated mouse anti-human monoclonal antibody to CD4, CD8, and CD14 and NKH-1 RD1 mouse anti-human monoclonal antibodies were obtained from Immunotech Beckman Coulter (Fullerton, CA). Alexa Fluor 488–conjugated goat anti-mouse IgG was obtained from Molecular Probes (Eugene, OR). FITC-conjugated goat anti-rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Normal mouse and normal goat IgG were obtained from Sigma-Aldrich (St. Louis, MO).

Procurement of PBMC. Normal PBMCs were obtained from healthy adult blood donors (source leukocytes, American Red Cross, Columbus, OH) or from patients with metastatic melanoma or metastatic renal cell carcinoma (n = 11) receiving treatment at the Ohio State University Comprehensive Cancer Center (Columbus, OH) under an Institutional Review Board–approved protocol. Patient blood samples were drawn just before and 1 hour after treatment with IL-2. Two of the patients were treated on a phase II trial (Ohio State University Institutional Review Board #2003C0046) of high-dose IL-2 with or without a peptide vaccine containing gp 100:209-217 (210M). These patients received 720,000 IU/kg of IL-2 administered i.v. every 8 hours for up to 12 doses. Cycles were repeated at 3-week intervals in the event of a clinical response or stable disease. Patients treated off protocol (n = 9) received 600,000 IU/kg of IL-2 i.v. every 8 hours for up to 12 doses. Cycles were repeated at 3-week intervals or more according to the physician’s discretion. PBMC were isolated by density gradient centrifugation (Ficoll-Paque, Amersham Pharmacia Biotech, Uppsala, Sweden).

Intracellular staining for STAT5 and P-STAT5. Cells for analysis were resuspended in 100 µL RPMI 1640 supplemented with 10% human AB serum, fixed with Fix and Perm Reagent A (Caltag Laboratories, Burlingame, CA) for 2 to 3 minutes at room temperature, and then incubated for 10 minutes in 3 mL cold methanol. Cells were then washed in flow buffer (PBS supplemented with 5% fetal bovine serum) and incubated in 100 µL Fix and Perm Reagent B (Caltag Laboratories) containing 4 µL of a mouse anti-human STAT5 antibody (BD Transduction Laboratories, San Jose, CA) or 2.7 µL of a rabbit anti-human P-STAT5 antibody (Cell Signaling Technologies) or an appropriate isotype control antibody for 30 minutes at room temperature according to the manufacturer's specifications. Nonspecific binding was blocked with goat IgG and/or mouse IgG, as appropriate. Cells were then washed with flow buffer and incubated with an Alexa Fluor 488–conjugated goat anti-mouse secondary antibody (STAT5) or a FITC-conjugated goat anti-rabbit secondary antibody (P-STAT5) for 30 minutes at room temperature. For subset analysis, cells were also incubated with the appropriate extracellular antibodies as a final step in the staining protocol. Cells were then washed with flow buffer, fixed in 1% formalin, and stored at 4°C until flow cytometric analysis.

Flow cytometric analysis. Analyses were done as described previously using a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with a 488-nm air-cooled argon laser and a 633-nm helium-neon laser, using at least 10,000 PBMC gated in the region of the lymphocyte population, as determined by light scatter properties (30). To analyze monocyte (CD14+) populations, cells were gated in both lymphocyte and monocyte regions. For multiparametric analysis, amplified fluorescence signals were displayed on four-decade log scales and expressed as specific fluorescence (Fsp): Fsp = Fs − Fs − Fsp, where Fs represents the median value of total staining and Fs − Fs represents the median value of background (isotype control) staining. In addition, the percentage of positive cells was determined from quadrants set with isotype control antibodies. Data files were analyzed using the WinMDI software. A minimum of three data files were analyzed for each condition to control for interassay variability.

Immunoblot analysis. PBMC were cultured for 15 minutes in RPMI 1640 supplemented with 10% human AB serum and either PBS or IL-2. Cell lysates were prepared and subjected to immunoblot analysis,

5 Created by Joseph Trotter; http://flowcyt.salk.edu/software.html.
as described previously, using the same anti-P-STAT5 antibody used for flow cytometry (31). A β-actin antibody was used as a loading control (Sigma-Aldrich). P-STAT5 levels were quantified by densitometry using Optimas 6.51 image analysis software (Media Cybernetics, Carlsbad, CA).

**Measurement of IL-2-stimulated gene expression by real-time reverse transcription-PCR.** Total RNA was isolated from cultured PBMC with the RNeasy RNA Isolation kit (Qiagen, Valencia, CA) and quantitated using the Uitrospec 3100 Pro spectrophotometer (Amersham Pharmacia Biotech). Reverse transcription was done using 2 μg total RNA and random hexamers (Perkin-Elmer, Norwalk, CT) as primers for first-strand synthesis of cDNA under the following conditions: 42°C for 60 minutes and 94°C for 5 minutes followed by 4°C. Resulting cDNA (2 μl) was used as a template to measure the levels of mRNA for the CIS (cytokine-inducible SH2 domain-containing protein), SOCS1 (suppressor of cytokine signaling 1), and Pim-1 genes by real-time PCR using predesigned primer/probe sets (Applied Biosystems, Foster City, CA) and 2× Taqman Universal PCR Master Mix (Applied Biosystems). Predesigned primer/probe sets for human β-actin, a housekeeping gene, were used as an internal control in each reaction well.

**Statistical analysis.** For each of the end points, basic summary statistics were calculated, and random effects models were applied to assess dosing trends, trends over time, or differences between subsets, where appropriate. For the in vitro dose response data, a two-slope model was applied to the data with a cut point at 0.2 nmol/L. Due to the differences in variability between several of the subsets, the P-STAT5 measurements following IL-2 stimulation were log transformed before comparisons were made. The correlation between the P-STAT5 and the transcription of IL-2-responsive genes was calculated using Pearson and Spearman nonparametric correlations, where appropriate. An α = 0.05 level of significance was used for all comparisons.

**Results**

**Flow cytometric analysis of P-STAT5 levels in IL-2-stimulated PBMC.** The utility of a flow cytometric assay for the detection of P-STAT5 was evaluated using freshly isolated PBMC from normal donors (n = 6) that had been treated for 20 minutes with increasing doses of IL-2 (Fig. 1A, inset). Results from a representative donor show the ability of increasing IL-2 doses to stimulate the P-STAT5 as measured by Fsp (Fig. 1A). There was a significant increase in the level of P-STAT5 (as measured by Fsp) at the 20-minute time point compared with baseline (P < 0.0001). A statistically significant
A dose-dependent response was noted for IL-2 concentrations between 0.02 and 16 nmol/L IL-2 ($P = 0.0148$). In addition, basal P-STAT5 was routinely identified in PBS-treated cells, further showing the sensitivity of this technique. However, when the effects of IL-2 are evaluated in terms of the percent of cells exhibiting any level of P-STAT5, it is clear that lower doses of IL-2 (e.g., 0.4 nmol/L) were nearly as effective as higher concentrations. Time course studies revealed that maximal induction of P-STAT5 in PBMC occurred at 30 minutes posttreatment in response to a pulse dose of 8 nmol/L IL-2 ($P = 0.0172$; Fig. 1B). Levels of P-STAT5 in IL-2-treated cells decreased over the first 4 hours poststimulation but remained elevated over baseline, even after 18 hours ($P < 0.0001$). Importantly, this assay required only $5 \times 10^5$ cells per condition, and acquisition of meaningful data was possible using as few as $1 \times 10^5$ cells (data not shown).

**Validation of IL-2-stimulated P-STAT5 formation.** To confirm the results obtained with the flow cytometric assay, freshly isolated normal donor PBMC were stimulated with increasing concentrations of IL-2 and processed simultaneously for flow cytometric and immunoblot analysis using the same anti-P-STAT5 antibody. This analysis revealed a dose-dependent increase in the levels of activated STAT5 for both assays ($P < 0.0001$; Fig. 1C). Importantly, the flow cytometric technique requires 10-fold fewer cells than the immunoblot technique, a major advantage when assaying patient PBMC.

**Association between P-STAT5 levels detected by flow cytometry and IL-2-stimulated gene expression.** To correlate the induction of P-STAT5 with the activation of IL-2-responsive genes, PBMC from a healthy donor were stimulated with various doses of IL-2 and processed simultaneously for flow cytometric analysis of P-STAT5 (20-minute time point) and reverse transcription-PCR analysis. Real-time reverse transcription-PCR data represents the results from triplicate wells with all values relative to $\beta$-actin (housekeeping gene).

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**Fig. 2.** Dose-dependent activation of STAT5 and expression of IL-2-responsive genes. PBMC were isolated from a single healthy donor and analyzed for P-STAT5 levels by flow cytometry and for gene expression by reverse transcription-PCR analysis. A. PBMCs were stimulated with IL-2 for 20 minutes, and P-STAT5 levels were quantitated by flow cytometry. B-D. Total RNA was isolated following stimulation of cells for 4 hours with increasing doses of IL-2 and converted to cDNA. Reverse transcription-PCR analysis revealed dose-dependent expression of the IL-2-responsive genes CIS (B), Pim-1 (C), and SOCS1 (D). Real-time reverse transcription-PCR data represents the results from triplicate wells with all values relative to $\beta$-actin (housekeeping gene).

**Fig. 3.** Measurement of P-STAT5 in immune cell subsets. PBMCs were isolated from healthy adults, stimulated with PBS or 8 nmol/L IL-2 for 20 minutes, and immediately stained for both intracellular P-STAT5 and extracellular molecules (NK-RD1 (NK), CD3, CD4, CD8, CD14, or CD21). Quadrants were set using appropriate isotype controls for each intracellular and extracellular antibody. Dual-variable histograms represent only cells gated on the lymphocyte population or lymphocyte and monocyte populations (for CD14). A. $P$-STAT5 levels are shown for 10 normal donors. Dash, average value. B, staining for P-STAT5 from a single normal adult donor.
real-time reverse transcription-PCR analysis of CIS, Pim-1, and SOCS1 gene expression (4-hour time point). CIS and SOCS1 are members of the SOCS family that are activated in response to the binding of P-STAT5 to the promoter region (32–34). Pim-1 is a STAT5-dependent cytoplasmic serine/threonine kinase that is induced in response to IL-2 stimulation of T lymphocytes (35). We chose the 4-hour time point to evaluate the expression of IL-2-induced genes based on previous reports in the literature and on our own experience with the induction of gene expression in cytokine-treated PBMC (30). A dose-dependent increase in the induction of transcription that correlated with the P-STAT5 was observed for CIS (Pearson correlation, 0.8628; \(P = 0.0028\)), Pim-1 (Spearman rank correlation, 0.6667; \(P = 0.0499\)), and SOCS1 (Pearson correlation, 0.7828; \(P = 0.0126\)) following stimulation of PBMC with IL-2 (Fig. 2). The levels of transcript for the genes Pim-1 and SOCS1 appear to follow a biphasic pattern in response to increasing IL-2 doses. It is not clear why this pattern occurs; however, it may reflect the overall expression of negative regulatory proteins at higher doses of IL-2. This gene expression pattern was a consistent observation that was seen in multiple donors.

**Fig. 4.** Differential levels of unphosphorylated STAT5 protein in T lymphocytes and NK cells. PBMC were isolated from normal healthy adults (\(n = 6\)) and stained with antibodies against total STAT5 (intracellular) and extracellular molecules (NK-RD1, CD3, CD4, CD8, CD14, or CD21). Quadrants were set using appropriate isotype controls for each intracellular and extracellular antibody. Dual-variable histograms represent only cells gated on the lymphocyte population or lymphocyte and monocyte populations (for CD14). Fsp for STAT5 in various immune cell subsets for six normal donors.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Diagnosis</th>
<th>Site of metastasis</th>
<th>Trial arm</th>
<th>IL-2 dose (IU/kg every 8 h)</th>
<th>No. cycles</th>
<th>CD3(^*) cells expressing P-STAT5 (%) (^\text{a})</th>
<th>Autoimmune toxicity</th>
<th>Clinical response</th>
<th>Toxicity</th>
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<td>Severe inflammatory reaction</td>
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<td>Expected (^1)</td>
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<td>C(^1)</td>
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<td>Pulmonary</td>
<td>IL-2 plus vaccine</td>
<td>720,000</td>
<td>7</td>
<td>50.8</td>
<td>Hypothyroidism</td>
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<td>Expected (^1)</td>
</tr>
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<td>Bladder, pulmonary alone</td>
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Abbreviations: PD, progressive disease; SD, stable disease; PR, partial response.

\(^a\)(Percent positive cells post-IL-2) – (Percent positive cells pre-IL-2).

\(^1\)Expected toxicities may include hypotension, mild creatinine elevation, tachycardia, rigors, fluid shifts/capillary leak, anemia, lymphopenia, hypocalcemia, and hypophosphatemia.

\(^2\)On a trial of high-dose IL-2 (700,000 IU/kg every 8 hours) with or without peptide vaccine.

\(^3\)These patients had persistent signaling at 3 weeks post-IL-2 therapy.
**P-STAT5 levels in subsets of immune cells.** A dual-variable flow cytometric technique that combines intracellular and extracellular antibody staining was developed to measure the activation of STAT5 within individual immune subsets. This technique was applied to the analysis of STAT5 activation within immune subsets of normal donors \((n = 10; \text{Fig. 3A})\). Ex vivo stimulation of PBMC with 8 nmol/L IL-2 for 20 minutes led to significant increases in P-STAT5 levels compared with PBS-stimulated cells in the CD56+ NK cell, CD3+ T lymphocyte, CD4+ T helper lymphocyte, and CD8+ CTL compartments \((P < 0.0001 \text{ for all conditions; Fig. 3A})\). Both CD56dim and CD56bright NK cells exhibited activation of STAT5 post-IL-2 treatment. However, CD14+ monocytes and CD21+ B lymphocytes (each representing a small percentage of total PBMC in all patients) did not show appreciable activation of STAT5 in response to IL-2. In this study, the range of CD14+ cells as a percentage of total PBMC was 1.0% to 15.3% (mean, 7.3%), and the range of CD21+ cells was 0.9% to 10.2% (mean, 4.23%). Pairwise comparisons revealed a significant increase in the percentage of cells expressing P-STAT5 after exposure to 8 nmol/L IL-2 compared with PBS treatment: 53.3% for CD56+ NK cells [95% confidence interval (95% CI), 42.9-63.75; \(P < 0.0001\)], 51.3% for CD4+ cells (95% CI, 40.91-61.75; \(P < 0.0001\)), 52.7% for CD8+ cells (95% CI, 42.25-63.1; \(P < 0.0001\)), and 57.4% (95% CI, 46.94-67.78; \(P < 0.0001\)) for CD3+ T cells overall. Thus, a significant percentage of NK cells and T lymphocytes did not exhibit activation of STAT5 posttreatment with IL-2. The average increase in P-STAT5 compared with PBS-treated cells was 1.6% for CD14+ cells (95% CI, –8.87 to 11.98; \(P < 0.7675\)) and 2.2% for CD21+ cells (95% CI, –8.2 to 12.65; \(P < 0.6732\)). There was also considerable variation between donors with respect to the induction of P-STAT5 in CD56+ NK cells (10-98% positive cells), CD4+ T cells (33-75%), and CD8+ T cells (17-91% positive cells) in response to IL-2. Results from a single donor are shown in Fig. 3B. Multiparametric staining indicated that the increased levels of activated STAT5 seen in IL-2-treated NK cells and T lymphocytes was not due to greater overall levels of STAT5 protein within these immune subsets compared with the other immune compartments (described below). These results also show that there is significant interpatient variation within the NK and T-cell compartments with respect to the IL-2-induced activation of JAK-STAT signal transduction. It is not clear why only a proportion of NK and T cells exhibit activation following exposure to IL-2, although IL-2R expression patterns may play a role. Studies to answer this question are ongoing.

**STAT5 expression in PBMC from normal donors.** Basal levels of STAT5 in normal PBMC were analyzed by flow cytometry. PBMC from healthy donors \((n = 6)\) were procured from

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**Fig. 5.** Detection of P-STAT5 in lymphocytes of patients undergoing IL-2 immunotherapy. PBMC were isolated from the peripheral venous blood of patients with metastatic melanoma or renal cell carcinoma immediately before and 1 hour following systemic IL-2 immunotherapy. Quadrants were set using appropriate isotype controls for each intracellular and extracellular antibody. Dual-variable histograms represent only cells gated on the lymphocyte population or lymphocyte and monocyte populations (CD14). A, distribution of P-STAT5 levels in immune cell subsets of patients \((n = 11)\) 1 hour after in vivo IL-2 immunotherapy. Dash, average value. B, representative staining for P-STAT5 within immune cell subsets in an individual patient prior to, 1 hour following, and 1 week following in vivo IL-2 immunotherapy. C, P-STAT5 levels in pre-IL-2 blood for seven patients who had received prior cycles of IL-2 therapy 2 to 7 weeks previously.
Peripheral blood samples and examined for levels of unphosphorylated STAT5 by intracellular flow cytometry (Fig. 4). Interestingly, the CD14+ monocyte compartment exhibited the highest Fsp for STAT5 of all of the subsets examined (mean Fsp, 17.3 ± 10.4; P < 0.0002; Fig. 4). Levels of STAT5 were not significantly different between the remaining cell compartments.

Detection of P-STAT5 in PBMC from patients undergoing IL-2 immunotherapy. Flow cytometry was used to analyze P-STAT5 in patient immune cells following therapy with IL-2 (Table 1). Peripheral venous blood was obtained from patients (n = 11) with metastatic melanoma or renal cell carcinoma before and 1 hour following the systemic administration of the first dose of high-dose IL-2. Levels of P-STAT5 were strongly induced in the CD3+, CD4+, and CD8+ T-cell subsets following the administration of IL-2 (Fig. 5A). As in the normal donors shown earlier, IL-2 administration had little effect on the activation of STAT5 in the monocyte and B-cell populations. NK cells were noticeably absent in the posttreatment sample in all patients analyzed. P-STAT5 levels in the nine patients for whom complete T-cell subset data were available pretherapy and posttherapy revealed STAT5 activation in 33.9 ± 17.3% of CD4+ cells and 44.3 ± 21.1% of CD8+ cells (data not shown). P-STAT5 levels in CD3+ lymphocytes varied considerably among patients (7.9-50.8% after subtraction of background; see Table 1).

Clinical outcomes. Among the 11 patients from whom data from the initial cycle of therapy was available, three have exhibited partial responses, four have achieved stable disease (defined as the absence of progression of known lesions and the absence of new lesions at 12 weeks), and four had progressive disease (Table 1). Two of the three patients exhibiting a partial response (patients C and D; Table 1) were treated on the Institutional Review Board–approved clinical trial of high-dose IL-2 with or without a peptide vaccine (Ohio State University #2003C0046) and received 720,000 IU IL-2 i.v. every 8 hours. The other patients (including patient I with a partial response) received 600,000 IU IL-2 i.v. every 8 hours (see Table 1). Four patients continue to receive treatment.

Persistent signaling in a subset of patients. In addition to the pretreatment and 1-hour posttreatment samples, peripheral venous blood was also obtained 1-week post-IL-2 treatment in two patients (patients A and C; Fig. 5B; data not shown). Unfortunately, blood was not available at the 1-week time point for the other patients who experienced a partial response (patients D and I). In both patients A and C, significant levels of P-STAT5 could still be detected in CD4+ and CD8+ T cells 1 week after the first dose of IL-2 had been administered. NK cells (uniformly absent in the 1-hour posttreatment sample) were once again present in the peripheral blood at 1-week post-IL-2 and exhibited high levels of P-STAT5 in the CD56bright NK cell subset (Fig. 5B). Levels of P-STAT5 in CD14+ and CD21+ cells remained negligible 1-week post-IL-2 treatment (data not shown). In another set of patients (n = 7), blood was obtained immediately before and just after subsequent treatment cycles (i.e., cycles 2-8; Fig. 5C). Three of these patients (C, D, and L) showed persistent P-STAT5 pre-IL-2 that was presumably an effect of the previously administered IL-2. The clinical histories of the patients with persistent activation of STAT5 are notable. Patient A received one cycle of IL-2 therapy (11 doses) and exhibited a severe inflammatory response characterized by a capillary leak syndrome and massive fluid shifts that resulted in pneumonitis. This patient received no further doses of IL-2. Patients C and D exhibited partial responses, and patient L had what might be termed a mixed response (significant decrease in the diameter of the central nervous system metastasis and stable bilateral axillary adenopathy that was ≤1 cm). Because not all patients were monitored at 3-week post-IL-2 treatment for persistent STAT5 activation, additional patients must be studied to further clarify the clinical implications of this phenomenon.

Discussion

We hypothesized that the study of IL-2-induced signal transduction within immune cell subsets would provide important information about the identity and activation of the immune compartments that mediate the antitumor effects of exogenous IL-2. Using an intracellular flow cytometric assay to analyze the P-STAT5 at Tyr694, we have shown that this transcription factor is rapidly activated in a subset of CD56+ NK cells and CD4+ and CD8+ T cells following in vitro treatment of normal PBMC with IL-2. A distinct dose-dependent response was observed that correlated well with the transcription of STAT5-regulated genes. Analysis of patient PBMC following the administration of high-dose IL-2 immunotherapy confirmed that only a portion of NK and T cells were activated in response to this cytokine. In addition, interpatient variability was observed with respect to the percentage of cells that exhibited increased levels of STAT5 at the 1-hour time point posttreatment. Persistent activation of STAT5 was observed in the NK and T-cell compartments 7 days or more following the first dose of IL-2 in a subset of patients. This technique provides a rapid, quantitative, and highly reproducible method for the analysis of IL-2-induced signal transduction events in specific immune cell compartments.

We have described a flow cytometric technique for the analysis of P-STAT5 in immune cell subsets that can be used to monitor the immune response to exogenous IL-2. This technique uses 10-fold less cells than standard assays, thus permitting the simultaneous analysis of signal transduction within multiple cellular compartments. Activation of STAT5 was observed in NK cells and CD4+ and CD8+ T cells, but not in B cells or monocytes, despite the presence of high basal levels of STAT5 within the latter two cell types. The correlation of IL-2-induced JAK-STAT signal transduction with downstream transcriptional events suggested that this technique could be used as a surrogate marker of NK and T-cell activation post-IL-2 therapy. It is generally believed that several signaling pathways are induced in T and NK cells following IL-2 stimulation, including the mitogen-activated protein kinases, phosphatidylinositol 3-kinase, and STAT5. Interestingly, although activation of STAT5 was readily detectable in CD4+ and CD8+ T cells, significant induction of phosphorylated extracellular signal-regulated kinase in T cells post-IL-2 therapy was not observed. These findings suggest that the signaling profile of IL-2-activated T cells is dominated by JAK-STAT signaling events.

We found that only a proportion of CD56+, CD4+, and CD8+ cells exhibited activation of STAT5 at the 1-hour time point 7 days after the last dose of IL-2, despite the presence of high basal levels of STAT5 within the latter two cell types. The correlation of IL-2-induced JAK-STAT signal transduction with downstream transcriptional events suggested that this technique could be used as a surrogate marker of NK and T-cell activation post-IL-2 therapy. It is generally believed that several signaling pathways are induced in T and NK cells following IL-2 stimulation, including the mitogen-activated protein kinases, phosphatidylinositol 3-kinase, and STAT5. Interestingly, although activation of STAT5 was readily detectable in CD4+ and CD8+ T cells, significant induction of phosphorylated extracellular signal-regulated kinase in T cells post-IL-2 therapy was not observed. These findings suggest that the signaling profile of IL-2-activated T cells is dominated by JAK-STAT signaling events.
posttherapy and that there was considerable variation between patients with respect to the percentage of cells that displayed elevated levels of P-STAT5. The differential activation of JAK-STAT signaling intermediates within effector lymphocytes post-IL-2 may reflect reduced expression of the IL-2R on some cells. However, previous work by Ohashi et al. (36) suggests that the heterodimeric intermediate affinity IL-2R is universally expressed by CD4+ and CD8+ cells. Similarly, our group has shown that the β and γ chains of the IL-2R are also expressed on a majority of CD56dim NK cells (37). Reduced responsiveness of circulating T cells to exogenous IL-2 may therefore reflect the immunosuppressive effects of a high tumor burden, increased expression of negative regulators of the JAK-STAT pathway, such as the suppressors of cytokine signaling proteins (SOCS), limitations of the current technique, or some other factor (34, 38). In addition, it is important to note that the observed levels of lymphocyte activation may not be reflective of the activation state of tumor-infiltrating lymphocytes or immune cells contained within tumor-draining lymph nodes. Persistent activation of STAT5 1 week following the last dose of IL-2 was observed in both NK and T cells, and this event, although not described previously, seemed to correlate with a favorable response to IL-2 therapy. We plan to study this phenomenon further in a large group of patients.

Given the potential toxicity of IL-2 treatment and the relatively low incidence of complete responses, significant effort has been expended in an attempt to identify predictors of response to IL-2 therapy. Multiple clinical variables that have been associated with response to IL-2 include performance status, number of metastatic sites, absence of bony metastasis, prior nephrectomy (for renal cell carcinoma), degree of treatment-related thrombocytopenia, absence of prior IFN therapy, thyroid dysfunction, rebound lymphocytosis, erythrocytopenic production, initial clinical response to high-dose IL-2, and posttreatment serum elevations of tumor necrosis factor α and IL-1 (23–26, 39). In addition, Upton et al. (22) identified recently the presence of clear cell histology with alveolar features and the absence of papillary or granular features as determinants of response to IL-2 therapy. Further work by this group has identified carbonic anhydrase IX, a molecular marker potentially predictive of prognosis in multiple types of cancer, as a potential determinant of response to IL-2 therapy in renal cell carcinoma (27, 28, 40–43). Our identification of individual differences in IL-2-induced signal transduction may represent an important additional layer of complexity that could lead to an altered transcriptional response within immune effectors. In their analysis of the transcriptional profiles of tumor-infiltrating lymphocytes in patients receiving IL-2 immunotherapy, Panelli et al. (44) found that IL-2 induced inflammation at tumor sites, thereby activating antigen-presenting cells, initiating cytokytic mechanisms in monocytes, and stimulating the release of chemokin attractants with the ability to recruit immune cells to the tumor. Thus, the signal transduction and transcriptional profile of tumor-infiltrating lymphocytes may differ significantly from that of circulating immune effectors.

We have described a novel intracellular flow cytometric assay that is an accurate and efficient method to determine the level of activation of STAT5 in PBMC and in immune effector subsets. A dose-dependent response to IL-2 that correlated with the induction of IL-2-responsive genes was identified. The P-STAT5 in immune effector subsets of cancer patients receiving IL-2 immunotherapy was highly variable among patients. Interestingly, in a subset of patients, persistent P-STAT5 activation in CD3+, CD4+, and CD8+ cells was identified up to 3 weeks following IL-2 treatment. Further speculation about the clinical implications of this observation cannot be made at this time.

References


Multiparametric Flow Cytometric Analysis of Signal Transducer and Activator of Transcription 5 Phosphorylation in Immune Cell Subsets \textit{In vitro} and following Interleukin-2 Immunotherapy

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