Peripheral Burst of Tumor-specific Cytotoxic T Lymphocytes and Infiltration of Metastatic Lesions by Memory CD8+ T Cells in Melanoma Patients Receiving Interleukin 12

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ABSTRACT

Systemic effects on T-cell-mediated antitumor immunity, on expression of T-cell adhesion/homing receptors, and on the promotion of T-cell infiltration of neoplastic tissue may represent key steps for the efficacy of immunological therapies of cancer. In this study, we investigated whether these processes can be promoted by s.c. administration of low-dose (0.5 μg/kg) recombinant human interleukin-12 (rHuIL-12) to metastatic melanoma patients. A striking burst of HLA-restricted CTL precursors (CTLp) directed to autologous tumor was documented in peripheral blood by a high-efficiency limiting dilution analysis technique within a few days after rHuIL-12 injection. A similar burst in peripheral CTLp frequency was observed even when looking at response to a single tumor-derived peptide, as documented by an increase in Melan-A/Mart-127–35-specific CTLp in two HLA-A*0201 patients by limiting dilution analysis and by staining peripheral blood lymphocytes (PBLs) with HLA-A*0201-melanoma antigen-A/melanoma antigen recognized by T cells (Melan-A/Mart-1) tetrameric complexes. The CTLp burst was associated, in PBLs, with enhanced expression of T-cell adhesion/homing receptors CD11a/CD18, CD49d, CD44, and with increased proportion of cutaneous lymphocyte antigen (CLA)-positive T cells. This was matched by a marked increase, in serum, of soluble forms of the endothelial cell adhesion molecules E-selectin, vascular cell adhesion molecules (VCAM)-1 and intercellular adhesion molecules (ICAM)-1. Infiltration of neoplastic tissue by CD8+ T cells with a memory and cytolytic phenotype was found by immunohistochemistry in eight of eight posttreatment metastatic lesions but not in five of five pretreatment metastatic lesions from three patients. Increased tumor necrosis and/or fibrosis were also found in several posttherapy lesions of two of three patients in comparison with pretherapy metastases. These results provide the first evidence that rHuIL-12 can boost the frequency of circulating antitumor CTLp in tumor patients, enhances expression of ligand receptor pairs contributing to the lymphocyte function-associated antigen-1/ICAM-1, very late antigen-4/VCAM-1, and CLA-selectin adhesion pathways, and promotes infiltration of neoplastic lesions by CD8+ memory T cells in a clinical setting.

INTRODUCTION

IL-12 is a heterodimeric cytokine acting as key regulator of cell-mediated immunity (see Refs. 1 and 2 for review) and as potent inhibitor of angiogenesis (3). Immune regulation by IL-12 involves induction of T Helper 1 (TH1) differentiation, as well as activation of cytokine secretion, proliferation and cytolytic activity in NK and T cells (1–2). These functions of IL-12 have prompted its evaluation as a potential modulator of antitumor responses. Experimental studies of systemic administration of the cytokine (i.v., i.p., or s.c.) have indicated that IL-12 exerts antitumor activity against pulmonary and hepatic metastases (4–6) and can even prevent spontaneous tumor development in HER-2/neu transgenic mice (7). In addition, models based on intratumor cytokine delivery, or in vivo transfer of cytokine-secreting tumors have indicated that IL-12 has significant and dose-dependent antitumor activity against a wide spectrum of murine tumors including melanoma, breast, ovarian, and bladder tumors (8–12). All of these studies have contributed to showing that IL-12 can inhibit tumor growth, improve the survival of tumor-bearing animals, and induce a long-lasting state of tumor-specific immunity.

The efficacy of IL-12 as antitumor cytokine, documented in experimental models, has opened the way to clinical studies based on systemic administration of rHuIL-12 to cancer patients (13–18). The clinical studies conducted thus far have been designed differently in terms of cytokine dosage (fixed versus escalating dose), route (i.v. versus s.c.), and schedule of administration, as well as clinical characteristics of enrolled patients. Despite these differences among the trials, immunological monitoring has indicated common effects of IL-12, such as transient lymphopenia (13, 14, 16, 17) and induction of IFN-γ in serum (13–17). In addition, increased levels of IL-10 in serum (14) and the enhancement of NK activity and T-cell proliferation in vitro after therapy have been described (17) in some studies. However, it is still unknown whether IL-12 administration in cancer patients induces systemic effects on T-cell-mediated antitumor immunity, affects expression of adhesion/homing receptors regulating T-cell homing/migration, and promotes T-cell infiltration of neoplastic tissue. To this end, we looked for evidence of modulation of CTL-mediated antitumor response in vivo in melanoma patients enrolled in a rHuIL-12 clinical study (14). In that study, 10 metastatic melanoma patients received s.c. injections of low-dose (0.5 μg/kg) rHuIL-12 once a week in three weekly doses (no dose in week 4) in each of two 28-day cycles. That regimen was well tolerated by all of the patients, and antitumor activity was clinically documented, as shown by regression of s.c. nodules, superficial adenopathies, and hepatic metastases in 3 of 10 patients (14).

As we show here for the first time, rHuIL-12 administration has a systemic impact on T-cell-mediated antitumor immunity, enhances expression of T-cell and endothelial cell adhesion/homing molecules, and promotes infiltration of neoplastic lesions by CD8+ T-cells with a memory phenotype. In addition, in comparison with pretherapy metastases, enhanced tumor necrosis and/or fibrosis was documented in some posttherapy lesions.
PERIPHERAL BURST OF CTL BY IL-12 IN MELANOMA PATIENTS

MATERIALS AND METHODS

Patients and Study Design. Ten patients with advanced metastatic melanoma were enrolled in a pilot study of rHuIL-12 given s.c. Study design, patients characteristics, selection criteria, and clinical course have been reported in details elsewhere (14). All but one of the patients (patient 6 was removed from the study after the first cycle) received 2 identical 28-day cycles, with s.c. injections of rHuIL-12 being given on days 1, 8, and 15 of each cycle. A fixed dose of 0.5 μg/kg of rHuIL-12 was used throughout the study. The treatment protocol was approved by the Ethical and Scientific Committees of our Institute, and a written informed consent was obtained from each patient. rHu IL-12 was supplied by Hoffmann-La Roche (Milan, Italy). The patients who showed clinically significant tumor regressions were patients 1, 5, and 10 (14). Two of the enrolled patients (patients 1 and 3) expressed the HLA-A*0201 allele. An additional panel of metastatic melanoma patients, matched for disease stage but not enrolled in the rHuIL-12 clinical study, was selected as control group for LDA and ELISA assays.

Melanoma Cells. Melanoma cells were isolated from surgical specimens from 3 of 10 patients enrolled in the rHuIL-12 clinical study and from additional patients not enrolled in the clinical study. The tumor cells were established as cell lines and kept in culture as described previously (19). Tumor cells to be used as stimulators and targets for LDA were cultured in 10% pooled human serum (PHS)-RPMI 1640 (BioWhittaker, Verviers, Belgium).

T-Cell Phenotype. Lymphocyte phenotype was evaluated after isolation on Ficoll gradients by means of immunofluorescence followed by flow cytometry analysis using a FACScan instrument (Becton Dickinson, Sunnyvale, CA). The following mAbs were used: Leu4 (anti-CD3), WT31 (anti-CD4), and 11F2 (anti-CD8 TCR; Becton Dickinson). Expression of adhesion/homing receptors on CD3 + T cells was evaluated by two-color immunofluorescence. To this end, cells were first stained with mAbs to CLA (HECA452 mAb, Becton Dickinson), or integrin α4 chain, or CD44, or CD11a, or CD18 (Immunotech, Marseille, FR) followed by incubation with FITC-conjugated goat antimouse or antirat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) depending on the primary mAb, and finally by staining with PE-conjugated anti-CD3 mAbs (Becton Dickinson). Expression of signal transduction molecules (ζ chain and lck) in CD3 + T cells was evaluated by simultaneous membrane (for CD3 detection) and intracytoplasmic (for ζ chain and lck detection) immunofluorescence as recently described (20). Statistical comparison of fluorescence histograms was performed by Kodmogorov-Smirnov statistics.

Cell Cycle Analysis. Cell cycle analysis in patients’ CD3 + T cells was carried out by two-color immunofluorescence as described by Lakhani et al. (21) on cells stained first with FITC-labeled anti-CD3 mAbs (Becton Dickinson) and then, after treatment with paraformaldehyde and ethanol, with propidium iodide (Sigma Chemical Co. LTD., St. Louis, MO). One hundred thousand events were acquired, and an analysis of cell cycle on CD3 + cells was performed with the aid of the Modfit software (Becton Dickinson).

Tetramer Staining. T-cell staining was performed with PE-conjugated HLA-A*0201/Melan-A/Mart-126–35 tetramers and HLA-A*0201/influenza matrix Mrx56–66 tetramers. HLA-peptide tetrameric complexes were synthesized as described previously (22–23), and specificity of tetramer staining was reported previously (22–23). Staining of PBLs was performed by incubating cells for 30 min at 4°C with PE-conjugated tetramers. As control, two-color fluorescence analysis was performed in some instances by staining T cells with FITC-conjugated anti-CD8 mAbs (Becton Dickinson) and PE-conjugated tetramers.

T-cell staining included PBLs from HLA-A*0201-negative healthy donors, whereas a Melan-A/Mart-126–35-specific CTL clone A83 (25) and fresh TILs, previously shown to contain a high frequency of Melan-A/Mart-126–35-specific T cells (22), were used as positive controls. An influenza-matrix 58–66-specific T cell line (25), selected from PBLs after culture for 3 weeks with peptide-MHC complexes, was used as positive control for the influenza matrix 58–66-specific tetramer staining.

Fig. 1. Frequency of HLA-restricted, tumor-antigen-specific CTLp in PBLs of patients before and during rHuIL-12 therapy. Frequency of CTLp directed to autologous tumor and inhibited by anti-CD3 (left panels) or by anti-HLA-class I mAbs (right panels) was evaluated by LDA in PBLs from patients 5, 10, and 6. In patients 5 and 10, PBLs for LDA analysis were taken before therapy (day –4) and 4 and 7 days after the first IL-12 administration, as well as 1 and 7 days after the last IL-12 administration. In patient 6, PBLs were taken at day –4, and then 1 and 7 days after the first IL-12 administration. Patient 6 was removed from the trial after the first treatment cycle. Arrows, rHuIL-12 was given at day +1, +8, +15, +29, +36, and +43; close to each experimental point, observed values of CTLp frequency; *, CTLp frequencies are significantly different from pretherapy (day –4 values) on the basis of the 95% confidence intervals of the regression lines.
Determination of Tumor-specific and Peptide-specific CTLp Frequency. Frequency of CTLp directed to autologous tumor or to a peptide from the melanoma antigen Melan-A/Mart-1 (Melan-A/Mart-127–35) was evaluated by a high-efficiency LDA (22, 25) that, as recently described (22), provides frequency estimation in fresh and activated T-cell populations in the same range as by HLA-peptide tetramer staining. Split-well analysis for HLA-restricted precursors was performed at day +28 of culture by comparing lysis of autologous tumor in the presence or absence of mAbs with CD3 (OKT3, American Type Culture Collection, Manassas, VA) or to HLA-class I antigens (w6/32; Ref. 27). The final cytolytic assay was performed in the presence of 1.5 × 10^5 tumor targets/well, and a threshold of 10% lysis was used. Comparison of two populations of lysis values from the whole LDA set was performed using the Student-Newman-Keuls multiple-range test, as well as by ANOVA followed by the Tukey multiple comparison test.

RESULTS
rHuIL-12 Administration Induces a Burst of Tumor-specific, HLA-restricted CTLp and of Melan-A/Mart-127–35-specific CTLp in Peripheral Blood. To evaluate possible systemic effects of rHuIL-12 administration on frequency of CTLp directed to tumor antigens, LDA was performed on PBLs from five of the enrolled patients (patients 1, 3, 5, 6, and 10). In three of these patients (patients 5, 6, and 10), the autologous tumor line was available, thus allowing us to evaluate the frequency of HLA-restricted CTLp directed to autologous melanoma. As shown in Fig. 1, in all of the three patients, a marked and significant increase in overall tumor-specific CTLp frequency (as evaluated by inhibition of tumor lysis with anti-CD3 mAbs), as well as in HLA-class-I-restricted antitumor CTLp frequency (as evaluated by inhibition with w6/32 mAbs) was observed between 4 and 7 days after the first rHuIL-12 administration. In patient 5, tumor-specific CTLp frequency values peaked at day +4, after the first rHuIL-12 administration, and dropped below threshold of detection at day +7 (Fig. 1). In blood samples taken 1 and 7 days after the last rHuIL-12 administration (patients 5 and 10, Fig. 1), frequency of CTLp inhibited by anti-CD3 or by anti-HLA class-I mAbs was again higher than in pretreatment PBLs, although lower than the highest values seen between day +4 and +7 of the first cycle. CTLp determination in patient 6 could be performed in a blood sample taken even 1 day after the first rHuIL-12 administration, and at that time, frequency of CTLp inhibited by anti-CD3 mAbs showed statistically significantly higher values compared to the pretherapy value.

Fig. 2. Frequency of peptide-specific CTLp directed to Melan-A/Mart-127–35 peptide in two HLA-A*0201+ melanoma patients before and during rHuIL-12 therapy. PBLs from patients 1 and 3 were taken before therapy (day −4), 4 and 7 days after the first rHuIL-12 administration, as well as 1 and 7 days after the last IL-12 administration. *, CTLp frequencies are significantly different from pretherapy (day −4 values) on the basis of the 95% confidence intervals of the regression lines; arrows, timing of rHuIL-12 injections.
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Staining with HLA-A*020!-Melan-A/Mart-126–35 tetrameric complexes

Table 1 Staining of Melan-A/Mart-1-specific T cells by HLA-A*0201-Melan-A/Mart-126–35 tetrameric complexes

<table>
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<th>Lymphocytes from</th>
<th>Experiment no.</th>
<th>HLA-A*0201-Melan-A/Mart-126–35</th>
<th>HLA-A*0201-influenza-matrix58–66</th>
</tr>
</thead>
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<tr>
<td>HLA-A*0201 donor</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Influenza-matrix58–66 specific T cell line</td>
<td>0</td>
<td>450,669</td>
<td>0</td>
</tr>
<tr>
<td>CTL A83</td>
<td>977,500</td>
<td>0</td>
<td>1,937</td>
</tr>
<tr>
<td>Fresh TILs</td>
<td>57,340</td>
<td>239</td>
<td>120</td>
</tr>
<tr>
<td>PBLs of Pt 1, Day -4</td>
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<td>252</td>
<td>208</td>
</tr>
<tr>
<td>PBLs of Pt 1, Day +4</td>
<td>1</td>
<td>239</td>
<td>120</td>
</tr>
<tr>
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<tr>
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<td>1,172</td>
</tr>
<tr>
<td>PBLs of Pt 1, Day +7</td>
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<td>0</td>
<td>1,048</td>
</tr>
</tbody>
</table>

In two patients (patients 1 and 3) expressing the restricting element (HLA-A*0201) for the tumor antigen Melan-A/Mart-127–35, the frequency of CTLp directed to this immunodominant epitope was evaluated in PBLs from the LDA sets from the three patients, including those showing tumor-specific CTLp frequency < 5 CTLp/10^6 lymphocytes (Table 1). Analysis of day -4 and day +7年间T cells in the experimental samples from patient (Pt) 1. Frequency of tetramer^* T cells obtained by single staining with tetramers was confirmed by two-color analysis on cells stained with PE-labeled tetramers and FITC-labeled anti-CD8 mAbs (data not shown). PBLs of patient 1 were taken before (day -4) and after (day +1, +4, +7) the first rHuIL12 injection. One × 10^6 cells were analyzed for each sample.

A drop in comparison with pretherapy values (day -4) and to the subsequent burst (day +7).

The changes in CTLp frequency detected in patients 5, 6, and 10 were confirmed by repeated LDA assays on the same blood samples (data not shown). Furthermore, a frequency of precursors nonspecifically lysing the autologous tumor (i.e., lysis not blocked by either anti-CD3 or anti-HLA mAbs) was evaluable in all of the LDA sets from the three patients, including those showing tumor-specific CTLp frequency < 5 CTLp/10^6 lymphocytes (data not shown).

In two patients (patients 1 and 3) expressing the restricting element (HLA-A*0201) for the tumor antigen Melan-A/Mart-126–35, the frequency of CTLp directed to this immunodominant epitope was evaluated in PBLs by LDA. In comparison with pretherapy values (day -4) in both patients (Fig. 2), a marked increase in peptide-specific CTLp in peripheral blood was observed between day +4 and day +7 after the first rHuIL12 administration, as well as between 1 and 7 days after the last rHuIL12 injection. To corroborate the LDA results, T cells from patient 1 were stained with HLA-A*0201-Melan-A/Mart-126–35 tetramers or, as control, with HLA-A*0201-influenza-matrix58–66 tetramers (Table 1). Analysis of day -4 and day +7 PBLs from patient 1 (Table 1) did not detect T cells staining with HLA-A*0201-Melan-A/Mart-126–35 tetramers, in agreement with LDA (< 5 CTLp/10^6 PBLs; see Fig. 2). However, in day +1 PBLs, the T cells staining with HLA-A*0201-Melan-A/Mart-126–35 tetramers T cells were 252 CTLp/10^6 PBLs and at day +4 tetramer^* T cells were 239 CTLp/10^6 and 255 CTLp/10^6 lymphocytes (in two distinct experiments) in good agreement with LDA data in patient 1 (200 CTLp/10^6 PBL at day +4; see Fig. 2). By contrast, staining of patient-1 PBLs with a control tetramer (HLA-A*0201 complexed with influenza matrix58–66, peptide) revealed a reduction in frequency of influenza-matrix-specific T cells at days +1 and +4 in comparison with day -4, followed by a rebound at day +7. Control experiments, in HLA-A*0201+ patients matched for disease stage but not enrolled in the rHuIL12 study, indicated that the frequency of T cells staining with HLA-A*0201-Melan-A/Mart-1 tetramers was constant in independent blood samples taken weekly during 1 month (data not shown). Thus, the experiments with HLA-A*0201-Melan-A/Mart-1 tetramers confirmed the LDA data and indicated that rHuIL12 administration transiently boosts the frequency of peptide-specific CTLp directed to a melanoma antigen peptide in peripheral blood.

Additional experiments were performed, before and during rHuIL12 therapy, to characterize the peripheral T-cell population from 8 of 10 patients (patients 1 and 3–9). These experiments indicated that, at the same time points used for LDA and tetramer analysis, rHuIL12 administration did not affect, in each patient, the proportion of circulating T lymphocytes nor the proportion of CD3^+ cells expressing aβ or γδ TCRs (data not shown). Furthermore, the expression of two TCR signaling molecules (ζ chain and Iκκ that can be defective in cancer patients was normal in the treated patients and did not change after rHuIL12 administration (data not shown). In addition, cell cycle analysis of patient^* T cells in PBLs did not show any difference in CD3^+ T-cell proliferation in posttherapy (day +4 and +7) samples in comparison with pretherapy (day -4) T cells in any of the patients (see Fig. 3 for representative data from one patient).

Fig. 3. Cell cycle analysis of peripheral blood T cells before and during rHuIL12 treatment. A. PBLs of a healthy donor were stimulated for 3 days with phytohemagglutinin followed by culture for 2 days in the presence 500 units/ml IL-2 (positive control for cell cycle analysis). Uncultured PBLs from a patient were taken before (day -4, B) and at day +4 (C, and at day +7 (D) after the first rHuIL12 administration. Analysis of cells stained with anti-CD3 mAbs and propidium iodide was performed after gating on FL2-Area versus FL2-Width plots to exclude cell doublets and aggregates. The position of the vertical marker to discriminate cells in G1 from those in S and G2-M phases of the cell cycle was set with the aid of the Modfit software. In each dot plot, the upper left panel contains CD3^+ T cells in G1 phase of the cell cycle, whereas the upper right panel contains CD3^+ T cells in S and G2-M phases. A total of 100,000 cells were analyzed for each dot plot. Propidium iodide staining (FL2-Area) is reported on a linear scale, whereas staining for CD3 (FL1-height) is reported on a log scale.
Enhanced Expression of T-Cell Adhesion/Homing Molecules and Increased Serum Levels of Endothelial Cell Adhesion Molecules in Patients Receiving rHuIL-12. Changes in expression of adhesion receptors regulating T-cell interaction with endothelial cells may have an impact on T-cell migratory patterns and, thus, promote T-cell infiltration of neoplastic tissues. To document evidence consistent with a systemic effect of rHuIL-12 on adhesion/homing molecules, analysis of expression of such receptors on circulating CD3⁺ T cells was performed. Within 24 h of the first rHuIL-12 administration, enhanced expression of the brightest fraction of T cells expressing the CD1a and CD18 subunits of LFA-1 and of the ω4 subunit of VLA-4 (CD49d), as well as a clear increase in fluorescence intensity for CD44, was observed (Fig. 4). The increased expression was transient as, at day +7, fluorescence intensity for CD49d and CD44 dropped to values lower than those seen in pretherapy samples. In most patients a new transient increase in expression of LFA-1, VLA-4, and CD44 on circulating T cells took place between 1 and 4 days after the last rHuIL-12 administration of the second cycle (data not shown). In addition (see Table 2), a clear increase in the proportion of T cells expressing CLA, the skin homing receptor, was documented, mainly after the first rHuIL-12 administration. Within 24–48 h after the first rHuIL-12 administration, the phenotype changes for T-cell adhesion/homing receptors were matched by striking increases in the levels of soluble forms of three adhesion molecules (sICAM-1, sE-selectin, and sVCAM-1) that can be shed by activated endothelial cells (Fig. 5). In many instances, additional increases took place even at day +4 and +7 (Fig. 5). Soluble endothelial cell adhesion molecule levels declined after the first cycle of rHuIL-12 administration, but a new increase was detected in all of the patients at the second cycle (data not shown). Control experiments were performed by monitoring for 1 week the serum samples taken from 10 metastatic melanoma patients matched for disease stage with the patients receiving rHuIL-12. These experiments indicated that sICAM-1, sE-selectin, and sVCAM-1 levels were either constant or showed a maximum range of fluctuation within ±30% of the initial value (data not shown).

Multicycle rHuIL-12 Administration Promotes Infiltration of Neoplastic Lesions by CD8⁺ T Cells with a Memory Phenotype. To document possible evidence of enhanced T-cell infiltration of neoplastic lesions as a result of rHuIL-12 administration, metastatic lesions removed before and after rHuIL-12 therapy were compared by immunohistochemistry. All of the clinically regressing lesions (in patients 1, 5, and 10, as described previously in Ref. 14) could not be surgically excised. Nevertheless, another eight posttherapy metastases (clinically judged as nonregressing lesions) could be removed within 1–2 months after the last rHuIL-12 administration from three patients (patients 2, 3, and 10). From the same three patients, five pretherapy metastatic lesions could also be studied. All of these lesions were analyzed for patterns of infiltrating lymphocytes according to the “brisk/non-brisk/absent” code proposed by Clark et al. (28), for evidence of tumor necrosis and/or regression, and for expression of two melanoma antigens (Melan-A/Mart-1 and gp100). As shown in Table 3 and exemplified in Fig. 6A (lesion 1 of patient 10), none of the five pretherapy lesions from the three patients contained infiltrating CD3⁺ T cells and, thus, were classified as “absent.” In contrast, from the same patients, 8 of 8 posttherapy metastatic lesions contained infiltrating T cells with either a “brisk” or a “non-brisk” pattern (Table 3). None of the posttherapy s.c. lesions showed an enhanced infiltration of T cells in the areas of normal skin adjacent or overlaying the tumor tissue (data not shown). In addition, as shown in Table 3 and exemplified in Fig. 6B (lesion 2, patient 10), all of the T cells infiltrating the posttherapy lesions were CD8⁺ and expressed a memory phenotype (CD45RO⁺). Furthermore, a proportion between 10 and 100% of the T lymphocytes that infiltrated the lesions expressed the cytolytic granule-associated protein recognized by the TIA-1 antibody (Ref. 29; Table 3). Histological evidence of increased tumor necrosis and/or regression was documented in posttherapy lesions from two patients (patients 10 and 2). In these instances, tumor necrosis areas were of the coagulative type, as defined by nuclear loss and marked cytoplasmic eosinophilia. In patient 2, histological evidence of tumor necrosis was observed only in posttherapy lesions (Table 3), whereas in patient 10, posttherapy samples showed larger areas of tumor necrosis in comparison with the pretherapy metastasis (Table 3; Fig. 6B) and presence of fibrosis in two lesions (lesions 2 and 4 in Table 3). In patient 3, no differences in the extent of tumor necrosis were observed between pre- and posttherapy lesions. Interestingly, none of the lesions in this

![Image](cancerres.aacrjournals.org)
HLA-A*0201 patient expressed two tumor antigens (Melan-A/Mart-1 and gp100; Table 3) that can be recognized by HLA-A2-restricted CTL, and the posttherapy lesion expressed HLA-A2 on only 30% of tumor cells (as evaluated by immunohistochemistry, data not shown). This suggested a possible mechanism of immune evasion, despite the non-brisk T-cell infiltrate found in the posttherapy lesion of this patient.

Taken together, these data suggest that low-dose s.c. rHuIL-12 treatment in metastatic melanoma patients promotes infiltration of neoplastic tissue by CD8+ T cells with a cytolytic and memory phenotype.

DISCUSSION

The results of this study show that s.c. administration of low-dose rHuIL-12 in metastatic melanoma patients induces a peripheral burst of HLA-restricted, tumor-specific and peptide-specific CTLp, enhances expression of ligand receptor pairs contributing to the LFA-1/ICAM-1, VLA-4/VCAM-1 and CLA/E-Selectin adhesion pathways, and promotes infiltration of neoplastic tissue by CD8+ T cells with a memory and cytotoxic phenotype. Thus, in the clinical setting, rHuIL-12 can impact on three key immunological mechanisms that can regulate T-cell-mediated antitumor responses.

The rHuIL-12 effect on CTLp frequency was not attributable to a change in the proportion and phenotype of circulating CD3+ T cells. In fact, pretherapy values of circulating CD3+ T cells expressing either αβ or γδ TCRs indicated no significant differences in comparison with values detected at day +4 or +7, at times when LDA revealed a marked increase of tumor-specific CTLp. Furthermore, expression of two TCR signal transduction molecules (ζ chain and lck), which can be defective in cancer patients (30–31), was found to be normal in the enrolled patients, and rHuIL-12 treatment did not improve the expression of such molecules. Reversal of T-cell anergy by rHuIL-12 is another possible mechanism that may underlie the CTLp burst. IL-12 has indeed been shown to reverse antigen-specific T-cell anergy in vitro and in vivo (32–34). However, this mechanism is not supported by the data obtained with tetramers. The tetramer experiments, with HLA-A*0201 complexed with Melan-A/Mart-126–35, indicated that an increase in the number of circulating peptide-specific CTLp did take place, as a result of rHuIL-12 administration, as early as 24 h after the first injection, which suggests rapid
recruitment of tumor-specific CTLp into the blood as a possible mechanism of the observed burst.

Interestingly, rHuIL-12 administration did not similarly affect the peripheral frequency of any antigen-specific T-cell precursor. In fact, in patient 1, in contrast to the burst observed for Melan-A/Mart-1-specific precursors, the number of circulating influenza-matrix58-66-specific precursors dropped at day +1 and +4 after rHuIL-12 injection and showed a rebound only at day +7. In addition, evaluation of the frequency of Melan-A/Mart-1-specific T cells in peripheral blood by LDA and by tetramers provided similar values, in agreement with our recent results that indicated that the LDA technique, as modified by us, achieves the same efficiency in detecting antigen-specific T cells as the enzyme-linked immunospot or tetramer staining (22).

Administration of rHuIL-12 may promote rapid clonal expansion of tumor-specific CTLp. This possibility is supported by several lines of evidence. For example, repeated s.c. injections of IL-12 can lead to selective expansion of a CD8+ T-cell subset characterized by high expression of CD18 and by non-MHC-restricted cytotoxicity after treatment with antibodies to VCAM-1 can activate a mechanism that may promote adhesion and then extravasation of activated/memory T cells by the LFA-1/ICAM-1, VLA-4/VCAM-1 and CLA/E-selectin pathways (43). Inasmuch as circulating CLA+ VLA-4+ T cells comprise memory/effector T cells that express each of the three markers.

Table 3

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Lesion no.</th>
<th>Sitea</th>
<th>Timing of removal, mo</th>
<th>Extent of tumor necrosis and/or regressionb</th>
<th>Lymphocyte infiltratec</th>
<th>Tumor antigen expressionc</th>
<th>Melan-A/Mart-1 % positive cells (staining intensity)</th>
<th>gp100 % positive cells (staining intensity)</th>
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<td>1</td>
<td>L.N.</td>
<td>Pre-, 11</td>
<td>Necr. (5%)</td>
<td>Absent</td>
<td>CD8+ TIA-1+ CD45RO+</td>
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<td>2</td>
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<td>Fibr. (65%)</td>
<td>Necr. (5%)</td>
<td>Brisk</td>
<td>100% 100% 100%</td>
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<td>s.c.</td>
<td>Post-, 1.5</td>
<td>Necr. (10%)</td>
<td>Non-brisk</td>
<td>100% 100% 100%</td>
<td>40 (++)</td>
<td>30 (++)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>s.c.</td>
<td>Post-, 1.5</td>
<td>Necr. (10%)</td>
<td>Fibr. (20%)</td>
<td>Brisk (60%)</td>
<td>100% 100% 100%</td>
<td>20 (++)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>L.N.</td>
<td>Pre-, 18</td>
<td>No</td>
<td>Absent</td>
<td></td>
<td>CD8+ TIA-1+ CD45RO+</td>
<td>60 (10%: +; 50%: +)</td>
<td>60 (++)</td>
</tr>
<tr>
<td>6</td>
<td>s.c.</td>
<td>Pre-, 18</td>
<td>No</td>
<td>Absent</td>
<td></td>
<td>CD8+ TIA-1+ CD45RO+</td>
<td>85 (15%: +; 70%: +)</td>
<td>60 (++)</td>
</tr>
<tr>
<td>7</td>
<td>s.c.</td>
<td>Post-, 1</td>
<td>Necr. (20%)</td>
<td>Non-brisk</td>
<td>100% 100% 100%</td>
<td>100 (70%: +; 30%: +)</td>
<td>50 (++)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>s.c.</td>
<td>Post-, 1</td>
<td>Necr. (30%)</td>
<td>Non-brisk</td>
<td>100% 30% 100%</td>
<td>60 (40%: +; 20%: +)</td>
<td>40 (++)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Mus.</td>
<td>Post-, 1</td>
<td>Necr. (5%)</td>
<td>Non-brisk</td>
<td>100% 50% 100%</td>
<td>100 (90%: +; 10%: +)</td>
<td>10 (++)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Om.</td>
<td>Post-, 1</td>
<td>No</td>
<td>Non-brisk</td>
<td>100% 100% 100%</td>
<td>80 (60%: +; 20%: +)</td>
<td>5 (++)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>L.N.</td>
<td>Pre-, 17</td>
<td>Necr. (15%)</td>
<td>Absent</td>
<td></td>
<td>CD8+ TIA-1+ CD45RO+</td>
<td>0</td>
<td>0 (++)</td>
</tr>
<tr>
<td>12</td>
<td>S.T.</td>
<td>Pre-, 9</td>
<td>Necr. (10%)</td>
<td>Non-brisk</td>
<td>100% 100% 100%</td>
<td>0</td>
<td>0 (++)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>s.c.</td>
<td>Post-, 2</td>
<td>Necr. (10%)</td>
<td>Non-brisk</td>
<td>100% 100% 100%</td>
<td>0</td>
<td>0 (++)</td>
<td></td>
</tr>
</tbody>
</table>

* L.N., lymph nodes; Mus., muscle tissue; Om., omentum; and S.T., soft tissue.
* a Extent of tumor necrosis and/or regression (as fibrosis) in each tissue section was coded as follows: No, absent; Necr., necrosis; Fibr., fibrosis. The number in parentheses refers to the percentage of the area of the neoplastic tissue showing the indicated feature.
* Consecutive sections of paraffin-embedded tumor fragments were conventionally stained with H&E (EE) or subjects immunochemical staining. CD3+ lymphocytes infiltrating the neoplastic tissue were coded using the “brisk/non-brisk/absent” classification (28). Expression of CD8, TIA-1, and CD45RO was evaluated only in lesions showing a brisk or non-brisk CD3+ infiltrate. Results of staining for CD8, TIA-1, and CD45RO were expressed as percentage of the brisk or non-brisk CD3+ lymphocytes that expressed each of the three markers.
* Percentage of positive cells stained with mAb M2-7C10 (anti-Melan-A/Mart-1) or with mAb lMB45 (anti-gp100). Intensity of staining for Melan-A/Mart-1 and gp100 was coded in comparison with negative and positive controls as described in “Materials and Methods”: +, weak; ++, moderate; ++++, strong.

rHuIL-12 did promote rapid CTLp proliferation in the tissues other than blood and before the peripheral burst of CTLp was induced and detected.

Serum levels of soluble adhesion molecules and phenotype analysis of circulating CD3+ T cells provided evidence for an early and marked effect of rHuIL-12 on several receptor/ligand pairs involved in regulating interaction of T cells with endothelial cells and T-cell homing. The data suggested that s.c. rHuIL-12 treatment could activate a mechanism that may promote adhesion and then extravasation of activated/memory T cells by the LFA-1/ICAM-1, VLA-4/VCAM-1 and CLA/E-selectin pathways. This possibility is in agreement with experimental models that show that IL-12 therapy can induce T-cell infiltration of tumor tissue associated with enhanced VCAM-1 expression on tumor blood vessels (7). Furthermore, in mice, IL-12-induced T-cell migration to tumor tissue can be inhibited by treating tumor-bearing animals with antibodies to either VLA-4 and LFA-1 (39). Additional experimental evidence indicates that IL-12 can promote adhesion-dependent homing of T cells to both skin and liver (40–41), and in vivo treatment with antibodies to VCAM-1 can abrogate leukocyte recruitment, in mice, of T and NK cells induced by systemic IL-12 (41).

An increased proportion of CLA+ T cells was found in peripheral blood after rHuIL-12 administration. Interestingly, IL-12 has been shown to induce expression of this homing receptor (42), and it is known that trans-endothelial migration of CLA+ T cells requires the VLA-4/VCAM-1 and LFA-1/ICAM-1 adhesion pathways (43). Inasmuch as circulating CLA+ T cells comprise memory/effector T cells (44), then it is possible that rHuIL-12 treatment may promote adhesion-dependent extravasation of CLA+ activated/memory T cells. This possibility is in agreement with the memory phenotype (CD45RO+) expressed by T cells infiltrating posttherapy lesions in

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the present study. Furthermore, the increased expression of CLA antigens was more pronounced after the first cytokine injection than after the last one. This reduced biological effect of the last rHuIL-12 administration was observed also when looking at CTLp frequency and may be attributable to a reduced systemic availability of the cytokine, as previously described in these patients at the end of the therapy cycles (14).

The immunohistochemical analysis of metastatic tissues that were...
removed from patients indicated the presence of a brisk, or non-brisk infiltrate of CD8+ T cells with a cytolytic (TIA-1+) and memory (CD45RO+) phenotype in posttherapy lesions. In addition, although all of the clinically regressing lesions could not be analyzed, other available posttherapy metastases showed increased histological evidence of tumor necrosis and/or regression in comparison with pretherapy tumor tissues. These data suggest that the antitumor activity of rHuIL-12, as evaluated at the histological level, may be greater than previously assessed at the clinical level in the same patients (14). As suggested by some experimental models (45) and by clinical data obtained in cutaneous T-cell lymphoma patients treated with rHuIL-12 (18), it is possible that the infiltrating CD8+, TIA-1+, CD45RO+ T cells may be involved in the observed tumor regressions. However, we cannot exclude the possibility that additional mechanisms, such as inhibition of angiogenesis and/or induction of endothelial wall injury, may contribute to the antitumor activity of rHuIL-12 in the clinical setting.

Finally, the increase in peripheral antitumor CTLp frequency was transient and subjected to attenuation after the last rHuIL-12 administration, in comparison with the peak value detected after the first injection. A similar “adaptive response,” was previously observed by us and others when looking at the serum levels of IL-12 and at induction of serum IFN-γ (13–14) and IL-10 (14) after rHuIL-12 administration. In particular, in patients, a burst of IFN-γ was detected in serum within 1–2 days after the first rHuIL-12 injection, whereas lower levels were induced after the last rHuIL-12 administration (14). The adaptive response could reduce the toxic effects associated with prolonged IL-12 therapy, but it might also contribute to reducing the antitumor efficacy of the cytokine. In support of this possibility, it has been shown (16) that a single IL-12 dose, given weeks before consecutive doses, can abrogate IL-12-associated toxicity but can also block induction of IFN-γ, an important mediator of IL-12 biological effects (1). At least two mechanisms have been described that could explain the adaptive response. On one hand, IL-12 administration to either cancer patients or mice, by fixed weekly doses, promotes enhanced IL-12 receptor expression in lymphoid cells, which may favor increased IL-12 clearance but leads also to reduced IFN-γ mRNA expression (46). On the other hand, murine models of tumor immunotherapy have indicated the involvement of nitric oxide, produced by macrophages in response to IFN-γ, in the immune suppression induced by IL-12 given i.p. (47). Additional studies are clearly needed to fully understand the mechanism of the adaptive response, thus improving the clinical efficacy of rHuIL-12 in anticancer therapy.

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Roberta Mortarini, Alessandra Borri, Gabrina Tragni, et al.


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