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Low-dose IL-2 induces cytokine cascade, eosinophilia, and a transient Th2 shift in melanoma patients

Received: 11 October 2004 / Accepted: 7 March 2005 / Published online: 12 May 2005
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Abstract *Purpose:* To assess changes in serum cytokine levels in patients treated concomitantly with or without systemic low-dose IL-2. Vaccination targeted CTL responses to peptide antigens, and IL-2 was coadministered to expand activated CTL. Paradoxically, CTL responses were diminished in patients after 2 weeks of IL-2. We hypothesized that changes in the cytokine milieu may have contributed to this result. *Experimental design:* Serum samples were studied from 37 patients enrolled in two clinical trials of a melanoma peptide vaccine administered with or without low-dose IL-2 therapy. Twenty-two patients enrolled in the MEL36 trial received six weekly vaccinations with the four-peptide mixture and were randomized to receive subcutaneous IL-2 (3×10^6 IU/m²/day) daily for 6 weeks beginning either at week 1 (upfront group) or at week 4 (delayed group) of vaccine therapy. Fifteen patients on

the MEL39 trial were treated with the same vaccine without concurrent IL-2 administration. *Results:* Circulating levels of IL-5 peaked 1 week after starting IL-2, followed 2 weeks later by a marked eosinophilia, correlating in magnitude with peak IL-5 serum levels. Levels of IFN γ , GM-CSF, IL-4, IL-10, and IL-12 had no observed relationship to IL-2 administration. At the time of the IL-5 serum peak, PBL responses to mitogen suggested a transient shift to Th2-dominance. *Conclusions:* Low-dose IL-2 appears to have induced a transient Th2-dominant secondary cytokine cascade at the time of vaccination, for which eosinophilia is a surrogate marker. For future vaccine therapies targeting cytotoxic T-cell responses, delaying IL-2 until after initiation of immune responses may be more effective.

Keywords Immunotherapy · Cancer · IL-5

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Introduction

Spontaneous T-cell responses against tumor have been found in melanoma survivors [1–3], and cases of tumor regressions have been observed in patients on vaccine trials [4–6]. However, spontaneous and therapeutic tumor regressions occur only in a minority of patients. Studies have demonstrated that lymphocytes from patients experiencing tumor regressions exhibit a dominant Th1 phenotype, in contrast to patients with progressing disease whose lymphocytes exhibit a Th2 phenotype [7, 8]. Therefore, the ability to promote efficient immune responses to tumor may depend, in part, on the cytokine milieu at the time of immune response development. Many biotherapy protocols and vaccination regimens incorporate immunomodulating adjuvants with the intent of promoting Th1 responses and CTL [9–11]. Interleukin-2 is used in biotherapy protocols and vaccination therapies [4, 5, 12–16] based on its ability to

expand and to augment proliferation of natural killer (NK) and activated T cells in vitro and in vivo [17–19]. However, the influence of IL-2 on the cytokine environment during immune therapy and on the Th1/Th2 balance in developing immune responses has not been studied systematically.

Treatment with high-dose IL-2 can induce IL-5 secretion, leading to marked peripheral eosinophilia and extravascular eosinophil degranulation [20, 21]. The release of toxic eosinophil products at extravascular sites and in the circulation may contribute to the pathogenesis of the capillary leak syndrome complicating high-dose IL-2 therapy. However, immunological effects of low-dose IL-2 treatment in bone marrow transplantation [18] or in cancer immunotherapy [15, 19, 22] have not been investigated in detail. As far as we know, only one study has addressed the Th1/Th2 cytokine balance in tumor-associated mononuclear cells upon treatment with cytokines [23]. Takeuchi et al. [23] demonstrated that treatment of tumor infiltrating lymphocytes with IL-2 induces deviation of lymphocyte responses toward type 2 predominance. However, this has not been evaluated directly in serum or circulating mononuclear cells of patients receiving low-dose IL-2.

In this study, we evaluated serum cytokine levels in patients enrolled in two clinical trials of a melanoma peptide vaccine administered with or without low-dose IL-2 therapy (MEL36 and MEL39). The vaccine consisted of four melanoma peptides derived from melanocyte differentiation proteins, plus a tetanus helper peptide, administered in an emulsion of GM-CSF in incomplete Freund's adjuvant (Montanide ISA-51, Seppic, Inc., Paris, France). Forty patients enrolled in the MEL36 trial received six weekly vaccinations with the four-peptide mixture and were randomized to receive subcutaneous IL-2 (3×10^6 IU/m²/day) daily for 6 weeks beginning either at week 1 (upfront group) or at week 4 (delayed group) of vaccine therapy. Twenty-six patients on the MEL39 trial were treated with the same vaccine without concurrent IL-2 administration. Low-dose IL-2 had been administered to patients on the MEL36 trial with the intent of expanding activated T cells responding to the vaccine. Paradoxically, T-cell responses, measured in a lymph node draining the vaccine site at week 3, were significantly decreased in the group that had received IL-2 for 2 weeks, compared to those who had not yet received IL-2 [24].

We noted that the patients enrolled in MEL36 developed clinical toxicities associated temporally with eosinophilia soon after beginning the IL-2 therapy [25]. It has been suggested previously that IL-5 may play a role in IL-2-induced eosinophilia [26, 27]. We hypothesized secondary cytokine changes induced by IL-2 may have affected the immunologic milieu in these patients. We evaluated changes in the systemic cytokine environment in patients during immunization by measuring serum levels of several Th1 and Th2 cytokines, including IL-5, in patients' serum collected every week during the vaccine protocols. Serum samples from 22 patients enrolled in the MEL36 trial and from 15 patients on

MEL39 were tested. We further examined if changes in the cytokine milieu were accompanied by changes in Th1/Th2 profile of patients' lymphocytes during the course of immunization.

Patients and methods

Patients

For the MEL 36 trial, 40 patients (25 male, 15 female), with a mean age of 55 (range 25–77) were diagnosed, cytologically or histologically, with resected AJCC stage IIB, IIC, III, or IV melanoma. In arm 1 of the MEL39 trial, 26 patients (14 male, 12 female) with a mean age of 49 were similarly diagnosed with resected AJCC stage IIB, IIC, III, or IV melanoma. Both protocols required patient's informed consent and had Food and Drug Administration approval [IND #7593 (MEL36), #9847 (MEL39)] and IRB approval [HIC #8515 (MEL36), #8878 (MEL39)]. Protocol eligibility requirements included an ECOG performance status of 0 to 1, normal hematologic parameters, sufficient liver and renal function, and expression of HLA-A1, -A2, or -A3. The exclusion criteria included cytotoxic chemotherapy or radiation in the preceding 4 weeks, known or suspected allergies to any component of the vaccine, significant cardiac disease, hyperthyroidism, and patients currently receiving corticosteroids, growth factors, IL-2, or allergy desensitization injections. We tested cytokines in serum samples of 22 patients treated on MEL 36 (11 per group) and 15 patients treated on MEL 39.

Immunization protocol

All patients received a total of six vaccinations, with each vaccine comprised of 100 µg of each of four melanoma peptides: YMDGTMSQV (tyrosinase_{369-377D} with post-translational change of N to D at 371) [28], YLEPGPVTA (gp100₂₈₀₋₂₈₈) [29], DAEKSDICTDEY (tyrosinase₂₄₀₋₂₅₁ with substitution of S for C at 244) [30], and ALLAVGATK (gp100₁₇₋₂₅) [31]. Each vaccine also included 190 µg of a tetanus toxoid peptide (AQ-YIKANSKFIGITEL) [32] and 225 µg of GM-CSF Leukine (sargramostim), Berlex, Seattle, WA, USA) in 1 ml of Montanide ISA-51 (Seppic Inc, Paris, France). The vaccines were administered at weeks 0, 1, 2, 4, 5, and 6, subcutaneously and intradermally as described [24]. A biopsy of a lymph node draining a vaccine site (sentinel immunized node, SIN) was performed at week 3 [24].

The patients on the MEL36 trial were randomized to receive subcutaneous injections of 3×10^6 IU/m²/day of systemic low-dose IL-2 (Proleukin, Chiron Corporation, Emeryville, CA, USA) daily for 6 weeks beginning either at week 1 on the day the second vaccine was administered (group 1) or at week 4 on the day the

fourth vaccine was administered (group 2). Patients enrolled on MEL 39 were immunized with the same peptide-based vaccine on the same schedule, but did not receive IL-2.

Serum and lymphocyte collection

Peripheral venous blood samples were drawn at least once prior to treatment, at weeks 0, 1, 2, 3, 4, 5, 6, 12, and at months 6, 12, and 24. Serum was cryopreserved from each collection date for testing purposes. The lymphocytes were isolated by Ficoll density gradient centrifugation and were cryopreserved in 10% DMSO as previously described [33].

Eosinophilia values

Eosinophil levels were determined using established standards at the University of Virginia Clinical Laboratory. The normal reference range for eosinophils is 0–6% of total white blood cells.

Serum cytokine levels

Serum levels of IL-4, IL-5, IL-10, GM-CSF, and IFN γ were measured by enzyme linked immunosorbent assay (ELISA). Serum samples of 11 patients treated on MEL 36 with upfront IL-2, 11 patients treated on MEL 36 with delayed IL-2, and 15 patients treated on MEL 39, who had not received IL-2, were tested. IL-12 levels were determined for eight upfront group patients and eight delayed group patients on MEL 36. ELISAs for the cytokines tested were performed using paired human primary and biotin-labeled secondary antibodies (Endogen, Cambridge, MA, USA). 96-well Immulon 2HB flat bottom microtiter plates (Dynatech, Chantilly, VA, USA) were coated with the appropriate primary antibody. All protocols were completed according to the manufacturer's instructions, with one slight modification: the incubation time with serum dilution was increased from 1 h to 2 h. The plates were washed using the Ultrawash Plus Automatic washer (Dynex Technologies, Chantilly, VA, USA). Serum samples were diluted 1:2 and assayed in duplicate.

Cytokine release assay

Lymphocytes from melanoma patients collected within a week of the IL-5 peak, and from normal donors, were cultured in a 96-well plate at 5×10^5 cells/well, in complete RPMI 1640 media with 10% heat-inactivated human AB serum (Sigma, St. Louis, MO, USA), 2 mM L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin (Life Technologies, Inc., Grand Island, NY, USA), and IL-2 (100 units/ml) at 37°C and 5% CO $_2$

for a total of 3 days. Media was collected from the wells on days 1, 2, and 3. The concentration of cytokines in the collected media was measured by ELISA as described.

Assessment of Th1/Th2 balance

Lymphocyte samples collected before vaccination, at the time of the IL-5 peak, and after treatment from two patients in group 1 and two patients in group 2, were thawed into complete media with 10% human AB serum. Lymphocytes were stimulated with phorbol myristate acetate (1 ng/ml) and ionomycin (1 μ M) (PMA/Ionomycin), or a mixture of anti-CD3, anti-CD28, and protein G (2 μ g/ml each). The lymphocytes were cultured in a 96-well plate at 0.5×10^6 cells/well. Media was collected at 48 h, and concentrations of IL-4, IL-5, and IFN γ were measured by ELISA. Samples were diluted 1:5 and assayed in duplicate. The Th1/Th2 ratio in lymphocyte responses was evaluated by dividing the concentration (pg/ml) of secreted IFN γ by the concentration of IL-5 and IL-4 [34].

Statistical methods

Summary statistics were calculated on the peak cytokine and eosinophil measurement of each patient. The Wilcoxon rank-sum test was used to determine whether peak cytokine and eosinophil levels differed between patients in the upfront and delayed groups. Patient profiles of cytokine and eosinophil levels over time were summarized using the 'supsmu' function in Splus 6.1 (S-Plus 6.1, Insightful Inc.) resulting in smoothed curves describing overall trends in cytokine and eosinophil levels through the treatment period. These curves are generated by locally weighted regression with local cross-validation to choose the span. This nonparametric regression approach was chosen due to the extreme nonlinearity present in the data and the descriptive nature of the analyses.

To further examine possible temporal relationships, for each cytokine the amount of time elapsed between peak cytokine level, and both the onset of IL-2 therapy and time of eosinophil peak, were measured. The mean time elapsed was calculated and bootstrapping was used to produce 95% confidence intervals for the mean elapsed time, both overall and separately by treatment group. Differences between time quantities of interest were calculated and bootstrap samples were generated 1,000 times, with replacement, and the 2.5th and 95.5th quantiles of the distributions were obtained for use as empirical 95% confidence limits. Likewise, the observed means along with the means and standard errors of the bootstrap distributions are provided in Tables 2 and 3. Spearman correlations were computed between peak eosinophil level and cytokine levels at various timepoints prior to time of eosinophil peak.

Results

Serum cytokine levels

We evaluated the concentrations of IL-4, IL-5, IL-10, IL-12, GM-CSF, and IFN γ in patient serum samples collected before, during, and after immunization for the MEL36 and MEL39 trials. Individual cytokine profiles of four patients (two per group) treated on the MEL36 protocol are shown in Fig. 1. Serum levels of IL-4, IFN γ , and GM-CSF remained essentially unchanged during immunization (Fig. 1 and data not shown). The results of statistical modeling of IL-5, IL-10, and IL-12 cytokine levels for all patients for each study arm on MEL36 are depicted in Fig. 2. In some patients treated with IL-2, concentrations of IL-10 and IL-12 increased during the course of vaccine therapy; however, their levels and timing did not correlate with the beginning of IL-2 therapy (Figs. 1, 2). The levels of IL-10 are very low, and the change from baseline is of doubtful clinical significance (Figs. 1, 2). The increases in IL-12 levels were substantial, but the changes differed for groups 1 and 2 of the MEL36 trial, without a consistent relationship to IL-2 administration (Figs. 1, 2). Serum levels of IL-5 increased noticeably immediately

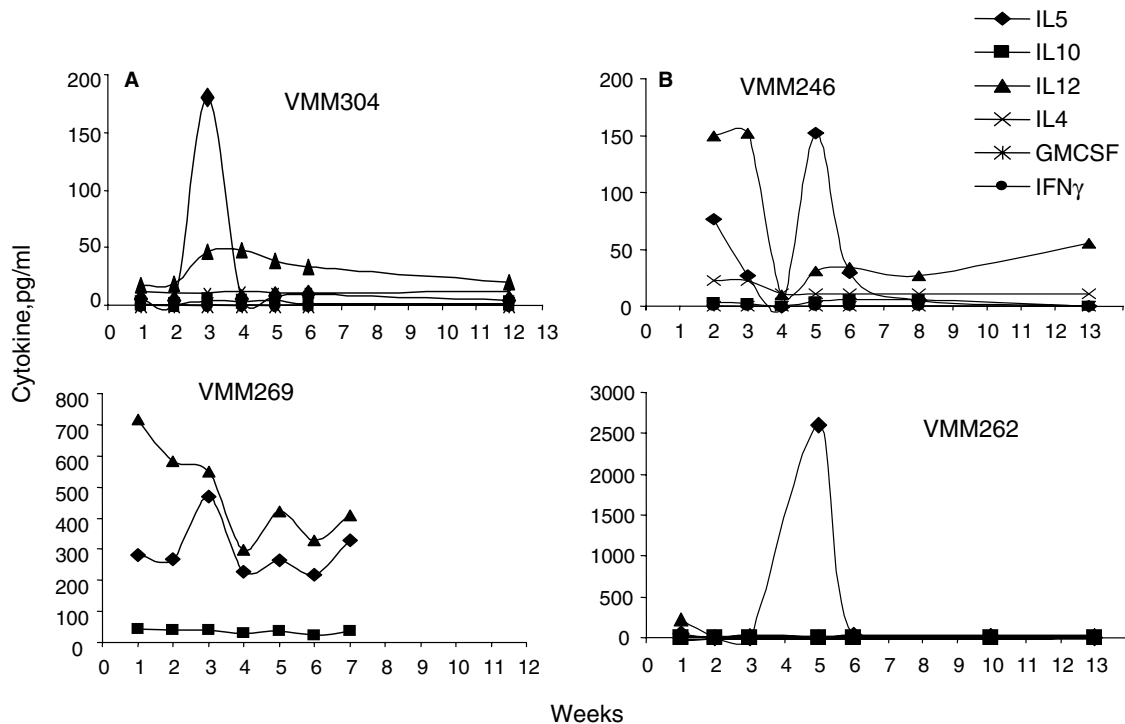
following the start of IL-2 therapy in both groups of patients (Figs. 1, 2). The mean maximal level of IL-5 for all 22 patients studied was 393.4 pg/ml, with the highest level reaching up to 2,600 pg/ml (Table 1A, B). We were unable to detect significant differences in maximal levels of IL-5 between groups 1 and 2 ($p=0.90$).

In patients on MEL39, the serum levels of IL-4, IL-5, IL-10, IL-12, IFN γ and GM-CSF in serum did not change dramatically during the vaccination sequence (Fig. 3 and data not shown). The actual level of IL-5 measured in MEL39 patients was higher at baseline than for patients on the MEL36 trial, but did not change significantly during vaccination. The analyses of serum samples from MEL39 were performed separately from those of MEL36, and this may explain the differences at baseline.

Eosinophilia

The percent of eosinophils (EOS) in 22 patients on the MEL 36 trial increased dramatically with low-dose IL-2 therapy (Fig. 2). In group 1, eosinophil levels peaked approximately 3 weeks after IL-2 therapy began on week 1, and then spontaneously returned to prestudy levels (Fig. 2). Similarly, group 2 had a peak rise in eosinophil counts three weeks after their IL-2 therapy began on week 4 (Fig. 2). The mean eosinophil peak value for the 22 patients enrolled on MEL36, as a percent of total white blood cells, was 46.5%, with 48.8 and 44.1% for groups 1 and 2, respectively (Table 1). These values did not differ significantly ($p=0.48$). The 26 MEL39 patients, who were vaccinated but did not re-

Fig. 1 Representative kinetics of serum cytokine levels in patients on the MEL36 trial. Concentrations of IL-5, IL-4, IL-10, GM-CSF and IL-12 in serum samples collected weekly were determined by ELISA as described in Patients and methods. Data from four patients show representative kinetics of these cytokines in serum. **a** patients VMM304 and VMM269 treated with IL-2 upfront; **b** patients VMM246 and VMM262 treated on delayed IL-2 schedule



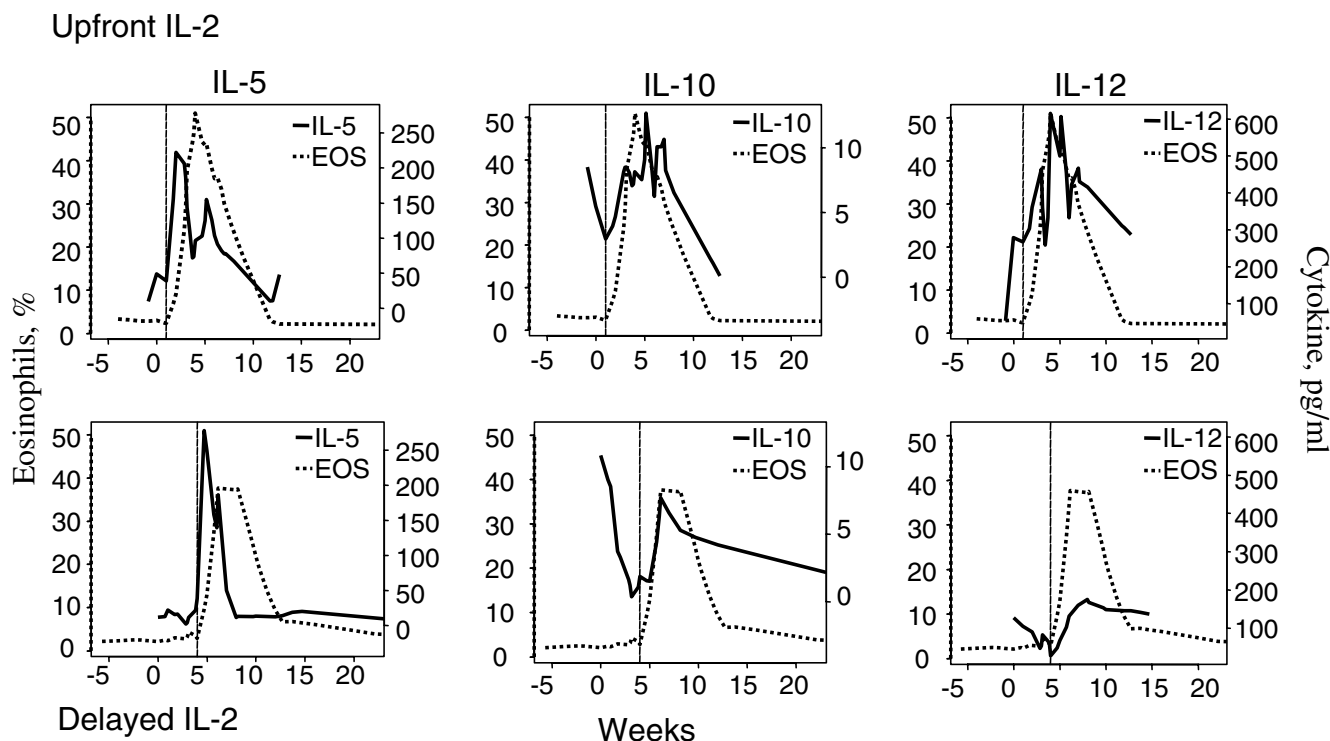


Fig. 2 Changes in eosinophil and cytokine levels in patients on the MEL36 trial. Eosinophil levels in peripheral blood are shown as a percentage of circulating leukocytes (*dotted line*). Concentrations of IL-5, IL-10, and IL-12 in serum samples collected weekly were determined by ELISA as described in Patients and methods (*solid line*). Average values for 11 patients treated with IL-2 upfront are shown in the *top three panels*, for the group treated on delayed IL-2 schedule on the *bottom three panels*. Cytokine and eosinophil levels are shown as nonparametric regression lines representing the mean of 11 patients per group (eight patients per group for IL-12 evaluations)

ceive IL-2, had no notable change in eosinophil levels during immunization (Fig. 3).

Timing of IL-2 therapy, cytokine peaks, and eosinophilia

The peak in IL-5 followed the start of IL-2 therapy by 1.05 weeks on average (95% CI: 0.46, 1.40) for both groups combined on the MEL36 trial (Table 2A). Peak IL-5 levels followed the start of IL-2 therapy by 1.35 weeks (0.99, 2.27) in group 1 and 0.75 weeks (−0.60, 1.17) in group 2 (Table 2B). We observed highly variable lag times for both IL-10 and IL-12. The peak in IL-10 levels followed IL-2 therapy by 0.93 weeks (−0.33, 2.41) and the peak in IL-12 levels followed IL-2 therapy by 1.53 weeks (0.00, 2.95) for both groups combined (Table 2A).

The peak in eosinophils followed the peak in IL-5 on average by 2.22 weeks, (95% CI 1.81, 2.96) (Table 3A). The average lag times between IL-5 and eosinophil peak for group 1 and group 2 patients were similar (Table 3B).

The times between peak eosinophil and peak IL-10 and IL-12 were much more variable. The peak in eosinophils tended to follow the IL-10 peak by 2.34 weeks (95% CI 0.61, 3.57) and the IL-12 peak by 1.65 weeks (0.11, 2.95) (Table 3A).

Peak eosinophil level correlated with the IL-5 level in serum 2 weeks prior

We found a significant correlation between peak eosinophilia in PBL and IL-5 level in serum at 2 weeks before the peak of eosinophilia (Spearman correlation coefficient (ρ)=0.58, p value=0.02). Correlations at other times for IL-5 were not found to be significant. Significant correlations were also seen between peak eosinophil and IL-10 measurements taken 1 (ρ =0.48) and 2 (ρ =0.64) weeks prior (p =0.03, <0.01, respectively), although the magnitude of the changes in IL-10 level are of uncertain biological significance. No other significant correlations were noted for the same day or lagged values of IL-10 or IL-12.

IL-5 is released by patients' lymphocytes treated with IL-2 in vitro

To assess whether direct effects of IL-2 on circulating PBL may explain increases in IL-5 levels in serum, PBL from normal donors and patient PBL samples collected at the time of IL-5 peak or 1 week before the peak were cultured in complete media supplemented with 100 units/ml of IL-2 for 3 days. Media was collected at

Table 1 Comparison of maximal values (interpreted as peaks) of cytokines and eosinophil levels for patients on MEL36

A. Overall maximum peak values							
Variable	Number of patients	Mean peak value	Std deviation	Median	Minimum	Maximum	
IL-5pg/ml	22	393.4	583.6	158.1	10.0	2600.0	
IL-10 pg/ml	22	12.7	15.4	7.6	0.5	64.3	
IL-12 pg/ml	16	448.7	590.3	223.2	20.1	2350.3	
EOS ^a %	22	46.5	15.3	48.2	22.0	77.9	
B. Maximum peak values by treatment group on MEL36 trial							
Tx ^b	Variable	Number of patients	Mean peak value	Std deviation	Median	Minimum	Maximum
1	IL-5 pg/ml	11	300.7	347.4	149.9	21.6	1078.7
	IL-10 pg/ml	11	14.6	20.4	6.0	0.5	64.3
	IL-12 pg/ml	8	661.9	775.4	365.6	48.1	2350.3
	EOS ^a %	11	48.8	12.2	53.5	23.5	62.9
2	IL-5 pg/ml	11	486.1	758.7	164.4	10.0	2600.0
	IL-10 pg/ml	11	10.8	8.6	8.0	1.3	32.2
	IL-12 pg/ml	8	235.6	203.6	223.2	20.1	669.8
	EOS ^a %	11	44.1	18.2	37.0	22.0	77.9

^aEOS eosinophils % of total leukocytes

^bTx treatment group (1 IL-2 up-front; 2 IL-2 delayed)

days 1, 2, and 3, and the concentration of IL-5 was determined by ELISA. Lymphocytes collected from patients undergoing IL-2 treatment produced more IL-5 in vitro than lymphocytes from normal donors (Fig. 4).

Th1/Th2 balance

To assess the Th1/Th2 balance of PBL responses to mitogen in patients receiving IL-2, we evaluated the cytokine responses to activation signals at several time points during immunization. PBL were stimulated

through the T-cell receptor (anti-CD3/CD28), or non-specifically with PMA/ionomycin. Samples collected before immunization, at the time of the IL-5 peak in blood, and after completion of immunization for two patients from each treatment group of the MEL36 trial were evaluated. The ratios of IFN γ to IL-5 and IL-4 were used to calculate the Th1/Th2 ratio [34]. For all four patients, the Th1/Th2 ratios decreased for PBL samples collected at the time of IL-5 peak, in comparison to the ratio prevaccination (Fig. 5). This shift to a Th2 pattern was transient in three of the four patients evaluated, such that Th1/Th2 ratios returned to pre-study values after the treatment was finished and IL-5 levels were back to baseline for those patients.

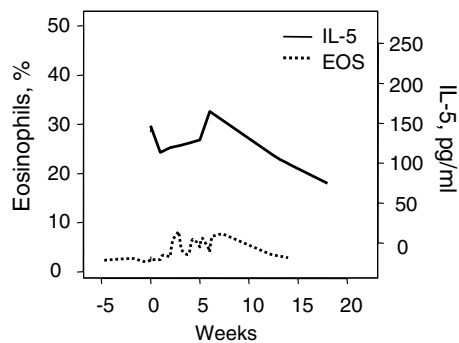


Fig. 3 Changes in eosinophil and IL-5 levels in patients on the MEL36 trial, vaccinated without IL-2. Eosinophils levels in peripheral blood are shown as a percentage of circulating leukocytes (dotted line). Concentrations of IL-5 in serum samples collected weekly were determined by ELISA as described in Materials and methods (solid line). IL-5 and eosinophil levels are shown as nonparametric regression lines representing the mean of 15 patients

Discussion

In murine models, as well as in experiments with human T cells in vitro, low-dose IL-2 expands activated NK and T cells [11, 17]. For the MEL36 trial, we hypothesized that addition of systemic low-dose IL-2 to vaccination would expand T-cell responses to the peptide immunogens. However, we found T-cell responses were lower in magnitude and frequency among patients receiving IL-2 early than in those patients receiving it on a delayed schedule [24]. In particular, the T-cell response in a vaccine-draining lymph node (SIN) was lower in the upfront-IL-2 group at week 3, when IL-2 had been administered for 2 weeks to patients in the upfront group, and IL-2 had not been administered to patients in the delayed group [24]. Clinical relevance of these findings was suggested by a trend toward prolonged disease

Table 2 Analyses of the difference in time between the start of IL-2 therapy and the peak levels of IL-5, IL10, and IL-12, for patients on MEL 36

A. Overall time between the start of IL-2 therapy and the peaks in cytokine levels						
Time (weeks) between	Observed mean	Bootstrap mean	SE	95% Confidence interval		
				Lower	Upper	
Start of IL-2 & IL-5 peak	1.05	1.04	0.23	0.46	1.40	
Start of IL-2 & IL-10 peak	0.93	0.91	0.70	-0.33	2.41	
Start of IL-2 & IL-12 peak	1.53	1.53	0.75	0.00	2.95	
B. Time difference between the start of IL-2 therapy and the peaks in cytokine levels for both groups separately						
Tx ^a	Time (weeks) between	Observed mean	Bootstrap mean	SE	95% Confidence interval	
					Lower	Upper
1	Start of IL-2 & IL-5 peak	1.35	1.35	0.27	0.99	2.27
	Start of IL-2 & IL-10 peak	1.66	1.66	0.70	0.47	3.22
	Start of IL-2 & IL-12 peak	2.98	2.99	0.78	1.45	4.55
2	Start of IL-2 & IL-5 peak	0.75	0.74	0.37	-0.60	1.17
	Start of IL-2 & IL-10 peak	0.19	0.16	1.19	-1.84	2.69
	Start of IL-2 & IL-12 peak	0.07	0.01	1.06	-1.83	2.29

^aTx treatment group (1 IL-2 up-front, 2 IL-2 delayed)

free survival at 2 years in the group with delayed IL-2 [24]. These paradoxical findings prompted the evaluations of the present report.

Patients on the MEL36 and MEL39 trials received the same four-peptide vaccine plus GM-CSF and

Montanide ISA-51 as local adjuvants. The regimens differed by the inclusion, exclusion, or timing of systemic low-dose IL-2 administration. Thus, comparisons of cytokine responses in peripheral blood for these patient groups reflect effects of low-dose IL-2.

Table 3 Analyses of the difference in time between the peak levels of IL-5, IL-10, and IL-12 and the peak level of eosinophils for patients on MEL 36

A. Overall time between the peak levels in cytokines and the peak level of eosinophils						
Time (weeks) between	Observed mean	Bootstrap mean	SE	95% Confidence interval		
				Lower	Upper	
IL-5 peak & EOS peak	2.22	2.23	0.28	1.81	2.96	
IL-10 peak & EOS peak	2.34	2.37	0.75	0.61	3.57	
IL-12 peak & EOS peak	1.65	1.67	0.70	0.11	2.95	
B. Time difference between the peak levels in cytokines and the peak level of eosinophils for both groups separately						
Tx ^a	Time (weeks) between	Observed mean	Bootstrap mean	SE	95% Confidence interval	
					Lower	Upper
1	IL-5 peak & EOS peak	2.34	2.34	0.22	1.91	2.79
	IL-10 peak & EOS peak	2.03	2.02	0.80	0.24	3.37
	IL-12 peak & EOS peak	0.63	0.62	0.91	-1.29	2.22
2	IL-5 peak & EOS peak	2.10	2.11	0.50	1.43	3.67
	IL-10 peak & EOS peak	2.66	2.69	1.33	-0.75	4.77
	IL-12 peak & EOS peak	2.68	2.73	0.95	0.59	4.31

^aTx treatment group (1 IL-2 up-front, 2 IL-2 delayed)

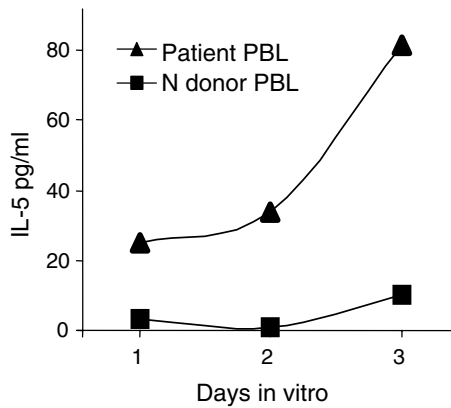


Fig. 4 PBL samples from five normal donors and from seven melanoma patients on the trial collected within a week of IL-5 peak in serum were cultured in a 96-well plate at 5×10^5 cells/well in complete media supplemented with 100 units/ml of IL-2. Media was collected from the wells on days 1, 2, and 3. Concentration of IL-5 was measured by ELISA as described

The immunologic data from the MEL36 trial are consistent with another reported observation that high-dose IL-2 inhibited CTL responses to a peptide vaccine [4]. As in the upfront group of MEL36, patients on that study received IL-2 during the vaccine regimen; however, the dose and schedule of IL-2 in that study was very different from that used in the MEL36 trial. High-dose IL-2 may decrease the number of peptide-reactive T cells in the blood while increasing T-cell trafficking to tumors [4], but in MEL36, patients were clinically free of disease, so trafficking to tumor was largely removed as a variable. Furthermore, in the MEL36 study, the greatest measured impact on T-cell responses was in the sentinel immunized node, which should reflect immunogenicity

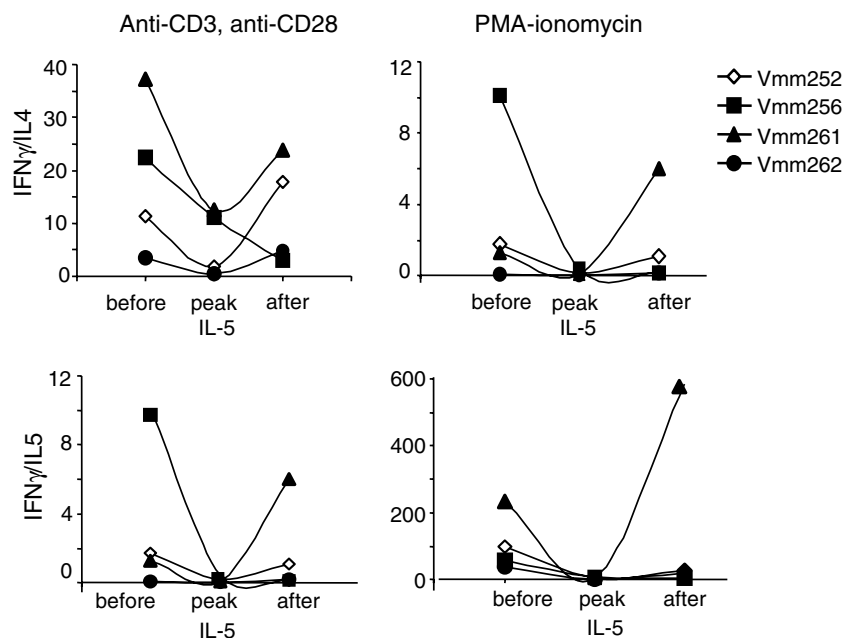
and be less affected by dilution and trafficking than measures of responses in the blood.

Studies in mice demonstrate prolonged administration of IL-2 can lead, not only to expansion of activated T cells, but also to activation-induced apoptosis of T cells [35]. We have not studied that phenomenon in this study, and cannot rule out that as a factor in the observed immunologic results. Instead, the present study was designed to identify changes in serum levels of cytokines associated with daily administration of low-dose IL-2, to address the hypothesis that low-dose IL-2 may induce a secondary and/or compensatory release of cytokines including IL-5. The availability of serum drawn weekly during the vaccine trials permitted measurements of serum cytokine levels over time, which were correlated with the IL-2 schedule.

Serum levels of IL-4, IL-10, $\text{IFN}\gamma$, and GM-CSF were very low and hardly detectable. We observed changes in IL-12 (Figs. 1, 2); however, the timing of IL-12 peak values did not track consistently with the administration of IL-2 or with the time of the eosinophil peak (Tables 2, 3). Increases in IL-12 levels were not observed in the MEL39 patients (data not shown), but were observed in some patients on the MEL36 trial. The higher levels in group 1 patients are puzzling, especially since that group had lower CTL responses. These findings are likely to be independent of the IL-2 dosing, are probably multifactorial, and deserve further study.

Changes in the IL-5 levels were dramatic, with a marked increase in serum concentrations approximately 1 week after the start of IL-2 therapy for patients on the MEL36 trial (Figs. 1, 2). For patients on that study, eosinophilia peaked about 2 weeks after the IL-5 peak, and followed a curve almost identical to the IL-5 curve (Fig. 2). In addition, there was a correlation between IL-

Fig. 5 Lymphocytes collected before vaccination, at the time of the IL-5 peak during treatment, and after treatment from two patients in group 1 (VMM252 and VMM256), two patients in group 2 (VMM261 and VMM262), were thawed into complete media. Lymphocytes were stimulated with phorbol myristate acetate (1 ng/ml) and ionomycin (1 μM) (PMA/ionomycin), or with a mixture of anti-CD3, anti-CD28, and protein G (2 $\mu\text{g}/\text{ml}$ each). The lymphocytes were cultured in a 96-well plate for 48 h at 5×10^5 cells/well. Media was collected, and concentrations of IL-4, IL-5, and $\text{IFN}\gamma$ were measured by ELISA. Th1/Th2 ratio in lymphocyte responses was evaluated by dividing $\text{IFN}\gamma$ concentration by IL-5 or IL-4 concentrations



5 peak levels and eosinophil counts 2 weeks later (Spearman correlation coefficient = 0.58, p value = 0.02). We have observed neither eosinophilia nor an increase in IL-5 levels in patients treated with the same vaccine without IL-2 in the MEL39 trial (Fig. 3 and data not shown). Thus, it appears eosinophilia is not mediated by the GM-CSF incorporated locally in each vaccine injection. Prior reports suggested eosinophilia associated with IL-2 therapy is not caused directly by IL-2 but may be mediated by several cytokines, including IL-5 and GM-CSF [36, 37, 38]. The findings in the current report support the hypothesis that IL-5 likely mediates IL-2-associated eosinophilia. They also suggest eosinophilia may be used as a surrogate marker of an IL-5 dominant Th2 cytokine environment.

Systemic clinical toxicities in the MEL36 trial were temporally associated with rising eosinophil numbers and were attributable to the IL-2 treatment [25]. Since IL-5 levels correlate well with the eosinophils counts, it may be that some of the clinical toxicities of IL-2 therapy may be mediated by IL-5. This suggests the possibility of considering IL-5 inhibition as a means to decrease IL-2 toxicity. This hypothesis could be tested in murine systems and could also be evaluated in humans, as an anti-IL-5 antibody is available for human investigation.

Interestingly, IL-5 levels peaked sharply, and then fell even while IL-2 was still being administered. Possible mechanisms may include a negative feedback mechanism in which IL-5 causes a downregulation of its own production and/or secretion through an auto-regulatory loop, a downregulation caused by another molecule other than IL-5, or apoptosis of activated cells secreting IL-5.

Several possible sources exist for the enhanced production of IL-5 in vivo. Proliferating human NK cells can produce IL-5, with secretion regulated by IL-4, IL-10, and IL-12 [39]. Marked increases in NK cell number have been detected during IL-2 treatment in patients with cancer or with bone marrow transplantation [18, 19]. There is also evidence from murine studies of IL-5 production from splenic CD4⁺ T cells [40, 41]. Our data suggest circulating lymphocytes collected from patients at the time of IL-5 peak in serum secrete IL-5 when exposed to IL-2 (Fig. 4). Thus, activated T cells of the Th2 phenotype are a likely source for the high IL-5 levels in these patients.

In four patients tested, at the time of maximal serum concentration of IL-5, circulating lymphocytes respond to nonspecific stimulation by increased secretion of Th2 type cytokines (Fig. 5). The shift is transient in three of the four patients (Fig. 5). Statistical validity of conclusions from Fig. 5 would require a larger data set; however, the findings are consistent with findings from the remainder of this manuscript. Overall, shifts in lymphocyte response patterns, and of the systemic serum cytokine environment, are toward a Th2 pattern during administration of IL-2. These changes may inhibit induction of cytotoxic T-cell responses. Improvements in IL-2 therapies should focus on ways to enhance favorable Th1 effects while minimizing clinical toxicities and

the Th2 shift. Improvements may be made with changes in dosing or schedules of IL-2.

Patients on the MEL36 trial who received low-dose IL-2 on the delayed schedule had better T-cell responses than those with early IL-2, even later in the vaccine regimen, when patients on both arms were receiving IL-2 [24]. Similarly, in murine studies, the best effect of IL-2 on the adoptive transfer of antigen-specific CTL was achieved in a short 2-day time course of low-dose IL-2 at the peak of clonal CTL expansion or later [35]. These data support consideration of trials in which low-dose IL-2 is administered on a delayed schedule, after initiation of the T-cell response.

In summary, the present manuscript demonstrates that high endogenous production of IL-5 is an indirect effect of low-dose IL-2 administration and that IL-5 is the most likely mediator of IL-2 associated eosinophilia. Interestingly, IL-2-mediated IL-5 production is a self-limited process. This suggests that the cytokine cascade initiated by daily low-dose IL-2 may include a complex sequence of regulatory and counter-regulatory processes. Low-dose IL-2 may be more effective as an adjuvant when administered after a CTL response is initiated so that induction of the CTL response does not occur during the IL-5-dominant systemic cytokine environment. Since enhanced IL-5 secretion is transient, a short course of IL-5 inhibitory therapy could be studied as a possible method to decrease toxicity and to increase Th1 effects of systemic IL-2.

Acknowledgements We thank Melanie Mayer for her assistance with the manuscript preparation and submission. This research was supported in part by NIH/NCI grant R01 CA57653 (to CLS), by Chiron Corporation, by an American Cancer Society Virginia Summer Student Fellowship Grant (to WCC), the University of Virginia Cancer Center Support Grant (NIH/NCI P30 CA44579, Clinical Trials Office, Tissue Procurement Facility, Biomolecular Core Facility); the UVA General Clinical Research Center (NIH M01 RR00847); and the Pratt Fund at the University of Virginia. Cancer Research Institute supported infrastructure of the UVA Human Immune Therapy Center.

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