Administration of a CD25-Directed Immunotoxin, LMB-2, to Patients with Metastatic Melanoma Induces a Selective Partial Reduction in Regulatory T Cells In Vivo[1]

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CD25$^+$CD4$^+$ T regulatory (Treg) cells regulate peripheral self tolerance and possess the ability to suppress antitumor responses, which may in part explain the poor clinical response of cancer patients undergoing active immunization protocols. We have previously shown that in vitro incubation of human PBMC with LMB-2, a CD25-directed immunotoxin, significantly reduced CD25$^+$FOXP3$^+$CD4$^+$ Treg cells without impairing the function of the remaining lymphocytes. In the current study, eight patients with metastatic melanoma were treated with LMB-2 followed by MART-1 and gp100-specific peptide vaccination. LMB-2 administration resulted in a preferential, transient reduction in mean circulating CD25$^+$CD4$^+$ T cell number, from 83 ± 16 cells/μl to a nadir of 17 ± 5 cells/μl, a 79.1% reduction. FOXP3 analysis revealed a less robust depletion with mean FOXP3$^+$CD4$^+$ Treg cell number decreasing from 74 ± 15 cells/μl to 36 ± 8 cells/μl, a 51.4% reduction. FOXP3$^+$CD4$^+$ Treg cells that survived LMB-2-mediated cytotoxicity expressed little or no CD25. Similar to the peripheral blood, immunohistochemical analysis showed a 68.9% mean reduction in FOXP3$^+$CD4$^+$ Treg cell frequency in evaluable lesions. Despite inducing a reduction in Treg cell numbers in vivo, LMB-2 therapy did not augment the immune response to cancer vaccination and no patient experienced an objective response or autoimmunity. These data demonstrate the capacity of a CD25-directed immunotoxin to selectively mediate a transient partial reduction in circulating and tumor-infiltrating Treg cells in vivo, and suggest that more comprehensive Treg cell elimination may be required to bolster antitumor responses in patients with metastatic melanoma. *The Journal of Immunology,* 2007, 179: 4919–4928.

T he goal of vaccination for the treatment of patients with cancer is the in vivo induction or amplification of functionally active, tumor Ag-specific immune cells capable of eradicating tumor cells. In patients with metastatic melanoma, current vaccine approaches can induce the generation of circulating tumor Ag-specific T cells, generally at frequencies <3% (1–5), though objective clinical responses are seldom observed in these immunized patients (6–8). A recent summary of 1306 cancer vaccine treatments for patients with solid tumors reported an overall objective response rate of 3.3% (9). Even in the adjuvant setting, where multiple course peptide vaccination can induce frequencies of tumor Ag-specific T cells as high as 44% of CD8 T cells (10–12), the induction of high levels of antitumor T cells alone appears insufficient to alter tumor progression (11). Together, these findings emphasize the need for new, combinatorial approaches to the study of cancer vaccines.

CD8 T cells are known mediators of antimelanoma responses, however, the role of CD4$^+$ T cells in tumor treatment is less de-fined. Although CD4$^+$ T cells can provide help to enhance CD8-mediated responses through the production of soluble factors such as IL-2 and the ability to activate APCs (13), a subset of these cells, CD25$^+$CD4$^+$ T regulatory (Treg)[3] cells, possesses the ability to suppress T cell responses and regulate tolerance to self proteins (14). Treg cells are phenotypically characterized by the expression of CD25 (IL-2Rα), CTLA-4, glucocorticoid-induced TNFR, and the transcription factor FOXP3 (14). The importance of FOXP3-expressing cells in mediating tolerance to self tissues is observable in humans with the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, a recessive and often fatal autoimmune disorder of early childhood resulting from FOXP3 mutations and a consequential lack of functional Treg cells in vivo (15, 16). In patients with metastatic melanoma, functional CD25$^+$CD4$^+$ Treg cells are present in the peripheral blood and tumor-infiltrating, melanoma Ag-specific Treg cells have been described (17–19). Furthermore, CD25$^+$CD4$^+$ Treg cells are reportedly overrepresented in human metastatic melanoma lymph nodes and can inhibit the function of infiltrating T cells (20). In mouse studies, Treg cells can inhibit the ability to vaccinate against self/tumor Ags. In their absence, organ-specific destruction of tissue expressing a novel "self" Ag can be augmented with self Ag vaccination or through the provision of inflammatory signals (21), tumor protection to tumor-associated Ags expressed as self Ags can be enhanced (22), and latent pools of high-avidity tumor Ag-specific CD8$^+$ T cells can be recruited with vaccination to produce

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[3] Abbreviations used in this paper: Treg, T regulatory; ALC, absolute lymphocyte count; TIL, tumor-infiltrating T cell; IHC, immunohistochemical; GVHD, graft-vs-host disease; QOD, every other day.

effective antitumor responses if Treg cells are blocked or removed before vaccination (23). Treg cells can also suppress antitumor responses directed against self/tumor Ags during adoptive cell transfer therapy for bulky, well-established B16 melanoma in mice (24). These findings suggest that one factor that might hinder the antitumor response in patients undergoing vaccination with cancer Ags is Treg cell-mediated immunosuppression and provides the rationale for the development of novel immunotherapeutic strategies to neutralize Treg cells in vivo to strengthen local antitumor immune responses.

In clinical trials, we and others have sought to eliminate the negative immunoregulatory effects of Treg cells in vivo and augment immune responses to tumor/self Ags (25). Clinical studies using administration of anti-CTLA-4 Ab in patients with metastatic melanoma have demonstrated a 13% response rate, however, the antitumor effects of CTLA-4 blockade were due to increased T cell activation rather than inhibition or depletion of Treg cells which express CTLA-4 (26–28). $DAB_{389}IL-2$ (Denileukin diftitox, ONTAK), a fusion protein of diphtheria toxin and the IL-2 cytokine that targets cells expressing high-affinity IL-2Rs (29), has been used in multiple trials as a Treg cell-directed cytotoxic agent with conflicting results (25, 30–32). In our study, there was no evidence that Treg cells were eliminated or consistently reduced in patients receiving $DAB_{389}IL-2$ and no patient experienced a clinical response (30). In another report, however, administration of $DAB_{389}IL-2$ to eight cancer patients yielded a single objective response in a patient with ovarian carcinoma and paralleled a reduction in circulating Treg cells (25). Similarly, a single dose of $DAB_{389}IL-2$ before vaccination with dendritic cells transfected with total tumor RNA reportedly reduced circulating Treg cells and improved stimulation of tumor-specific effector T cells compared with vaccination alone in patients with renal cancer (31), although the impact on tumor response was not reported. Whether the selective depletion of Treg cells in vivo before the administration of cancer vaccination can bolster the antitumor response and elicit objective tumor regression in vivo requires further evaluation.

Because of the variability in Treg cell-depletion efficacy reported in prior $DAB_{389}IL-2$ trials, we investigated the use of the recombinant immunotoxin LMB-2 for the depletion of $CD25^+$ Treg cells in vivo before cancer vaccination. LMB-2 is a fusion of a single-chain Fv fragment of the CD25-specific, anti-Tac mAb to a truncated form of the bacterial *Pseudomonas* exotoxin A (33). In clinical trials, LMB-2 induced responses in patients with $CD25^+$ chemotherapy-resistant hematologic malignancies (34). The short in vivo half-life (~4 h) and CD25 specificity of LMB-2 makes it an attractive candidate for reducing Treg cells in vivo before the administration of cancer vaccine or cell transfer immunotherapy approaches (34). In a preclinical study, we demonstrated that in vitro incubation of human PBMCs with LMB-2 significantly reduced the levels of Treg cells without impairing the function of the remaining lymphocytes (35). The mechanism of this Treg cell-depleting capacity appeared mediated through both the cytotoxic effect of *Pseudomonas* exotoxin and the ability of LMB-2 to block IL-2 activity because anti-Tac alone, which possesses IL-2-blocking activity, and LMB-2 containing a functionally mutated exotoxin reduced Treg cells in vitro, but were inferior to intact LMB-2 alone (35). Because melanoma patients often have naturally circulating anti-melanoma effector cells that can be bolstered by vaccination (3, 4), we hypothesized that transient elimination of T-regulatory cells will enhance the clinical effectiveness of immunotherapy. In the current study, we evaluated immune responses and the clinical effectiveness of immunization with the

$gp100_{209-217(210M)}$ and $MART-1_{27-35}$ peptide vaccine following LBM-2 administration in patients with metastatic melanoma.

Materials and Methods

Treatment regimen

All patients in this study expressed HLA-A*0201, had metastatic melanoma, and were entered on institutional review board-approved protocols in the Surgery Branch of the National Cancer Institute. Informed consent was obtained from all subjects. Starting on day 0, all patients received 30 mcg/kg of LMB-2 i.v. every other day for a total of three doses. One day after the last dose of LMB-2 (day 5), patients were vaccinated for 4 days s.c. with $gp100_{209-217(210M)}$ vaccine in MONTANIDE ISA 51 or Montanide ISA 51 VG and, independently, the $MART-1_{27-35}$ vaccine in MONTANIDE ISA 51 or Montanide ISA 51 VG (cycle 1). Three weeks after the first peptide vaccination, patients received a second cycle of peptide vaccines in MONTANIDE ISA 51 or Montanide ISA 51 VG (cycle 2) given for 4 consecutive days (days 26–29). This constituted one treatment course. Three weeks after the last peptide vaccination, patients were evaluated for tumor response and toxicity.

Patient eligibility

$HLA-A201^+$ patients who were 18 years of age or greater, with measurable metastatic melanoma which was not responsive to standard therapy, who met standard laboratory safety criteria, and who did not have concomitant major medical illnesses, require steroid therapy, or had previously received LMB-2 were eligible for enrollment. Patients had clinical Eastern Cooperative Oncology Group performance status of 0, 1, or 2. Eligibility criteria required serum creatinine levels <1.4 mg/dl and bilirubin <2.0 mg/dl. Serum eligibility criteria included serum albumin >3.0 g/dl, aspartate aminotransferase/alanine aminotransferase <2.5 times normal, and serum LMB-2-neutralizing activity of <75% of the activity of 1 $\mu g/ml$ LMB-2. Blood eligibility criteria included WBC >3000/mm^3, absolute lymphocyte count (ALC) >500/mm^3, and platelets ≥90,000/mm^3. Excluded from the protocol were patients who received LMB-2 on another trial or mAb therapy within 12 wk of enrollment, patients with a resting left ventricular ejection fraction of <45%, and patients with autoimmune disease, immunodeficiency, HIV infection, or other concurrent malignancies.

Immunotoxin

LMB-2 (anti-Tac (Fv-PE38)) is a single-chain Fv fragment of the anti-CD25 mAb (Daclizumab, Zenapax) fused to a truncated form of the bacterial *Pseudomonas* exotoxin A. Clinical grade LMB-2 was produced as previously described in detail elsewhere (33, 34, 36) by the Monoclonal Antibody and Recombinant Protein Production Facility (National Cancer Institute (NCI), Frederick, MD). The Investigational New Drug application is held by the Cancer Therapy and Evaluation Program (CTEP) of the NCI.

Immunotoxin administration

As described previously (34), patients received fluid prophylaxis, consisting of 1 L of fluid over 2–4 h before LMB-2 infusion and another liter over 2–4 h after the infusion was completed. Patients were premedicated with 25 mg of hydroxyzine and 150 mg of ranitidine orally 1 h before and 8 h after each dose. Acetaminophen 650 mg orally was given every 6 h for four doses starting 1 h before each LMB-2 dose. Vials of LMB-2, stored at −70°C, were thawed in a room-temperature water bath. LMB-2 (30 $\mu g/kg$) was infused through a peripheral i.v. or central line in 50 ml of 0.9% NaCl and 0.2% albumin via a partial additive container (PAB; McGraw) over 30 min every other day for three doses.

Peptides

The nonamer-modified $gp100_{209-217(210M)}$ peptide (g209-2M, IMDQ VPFSV) and the $MART-1_{27-35}$ peptide (AAGIGILTV) were produced to Good Manufacturing Practice grade by solid-phase synthesis by Ben Venue Laboratories and provided by the NCI CTEP. The $gp100_{209-217}$ peptide (g209, ITDQVPFSV), $MART-1_{26-35(27L)}$ peptide (ELAGIGILTV), influenza$_{58-66}$ peptide (Flu, GILGFVFTL), and the $gp100_{280-288}$ peptide (g280, YLEPGPVTA) were used for in vitro studies.

Patient samples and culture media

Patient PBMC were isolated by Ficoll-Hypaque separation after obtaining an informed consent and were cryopreserved in heat-inactivated human AB serum (HSA; Gemini Bioproducts) with 10% DMSO and stored at −180°C until the time of study. Serum samples were collected and stored at 4°C until study. Complete medium consisted of RPMI 1640 (Invitrogen Life

Technologies) supplemented with 2 mM glutamine (Biofluids), 25 mM HEPES buffer (Biofluids), 100 U/ml penicillin (Biofluids), 100 μg/ml streptomycin (Biofluids), 50 μM 2-ME (Invitrogen Life Technologies), and 10% heat-inactivated FBS (Gemini Bioproducts). For patients with nonvisceral lesions, pre- and posttreatment 4-mm punch biopsy specimens of representative skin lesions were obtained while the patient was under local anesthesia for immunostaining.

mAbs and flow cytometric immunofluorescence analysis

FITC-conjugated anti-human CD4 and CD8 Abs and allophycocyanin-conjugated CD3 Ab were all obtained from BD Biosciences. PE- or allophy-cocyanin-labeled gp100$_{209-217}$ and MART-1$_{26-35(27L)}$ peptide/HLA-A*0201 tetramer complexes were obtained from Beckman Coulter Immunomics. For CD25 detection, PE-conjugated CD25 Ab (4E3 clone; Miltenyi Biotec) and allophycocyanin-conjugated CD25 Ab (2A3 clone; BD Biosciences) were used. FOXP3 (clone PCH101) and control rat IgG2a Abs were obtained from eBioscience. Thawed PBMC were resuspended in FACS buffer consisting of PBS with 2% FBS (Gemini Bioproducts) at 10^7 cells/ml and blocked with 10% normal mouse Ig (Caltag Laboratories) for 10 min on ice. A total of 10^6 cells in 100 μl were stained with fluoro-chrome-conjugated mAbs at 4°C for 40 min in the dark. In some cases, cells were briefly stained with propidium iodide for nonviable cell exclusion after washing twice and subsequently analyzed in a FACSCalibur (BD Biosciences). FOXP3 staining was performed according to the manufacturer's instructions (eBioscience).

Neutralizing Ab analysis

The cytotoxicity of neutralized LMB-2 was measured by incubation of a serum-LMB-2 mixture with SP2/Tac cells (37). In brief, patient serum was mixed with different concentrations of LMB-2 diluted in HSA-PBS, incubated at 37°C for 15 min, and diluted into DMEM (Invitrogen Life Technologies) plus 10% FBS. In triplicate, 50-μl aliquots of diluted serum-toxin mixtures are added to 150-μl aliquots of SP2-Tac cells (40,000/well) in 96-well flat-bottom plates to final toxin concentrations of 0, 0.04, 0.2, or 1 μg/ml. After the 16–20 h incubation at 37°C, cells were pulsed for 4–5 h with [^{3}H]leucine, harvested, and counted to determine inhibition of protein synthesis. Percent neutralization is calculated by determining the percent inhibition of protein synthesis of toxin in HSA-PBS, minus the percent inhibition in serum, divided by the percent inhibition in HSA-PBS, times 100.

Plasma pharmacokinetics

Blood samples were obtained in sodium-heparin-containing tubes before and at 30, 60, 90, 120, 240, and 360 min after the first dose for patients 5 and 7, and at 30, 60, 90, 210, 330, and 690 min after the first dose for patients 6 and 8. Plasma levels of LMB-2 were determined by incubating dilutions of plasma with CD25$^+$ SP2/Tac cells (37) and comparing cyto-toxicity as assessed by decreased [^{3}H]leucine incorporation to that obtained by an LMB-2 standard. Plasma lifetimes of LMB-2 were determined by fitting the plasma levels to monoexponential or biexponential decay curves using a weighting factor of 2 (1/y2).

Immunohistochemical analysis

For immunostaining, 4-μm-thick paraffin-embedded sections were cut, incubated at 58°C for 1 h, and deparaffinized in xylene and graded alcohols. Endogenous peroxidase activity was blocked with 0.03% H$_2$O$_2$ in methanol for 5 min. Slides were immersed in Tris/EDTA buffer (pH 9.0) in a high-pressure cooker and subjected to heat-induced Ag retrieval at 98°C for 20 min using a microwave oven. Slides were cooled to room temperature and rinsed in PBS 0.1% Tween 20 for 5 min followed by a nonspecific blocking step with 0.25% casein in PBS for 20 min. Slides were incubated with rat affinity-purified Ab anti-human FOXP3 (clone PCH101, 1/1000 dilution; eBioscience) for 30 min, followed by rabbit anti-rat Ab, and the ABC peroxidase detection system (Vectastain Elite kit; Vector Laboratories) using 3-3′-diaminobenzidine (DakoCytomation) as a brown peroxidase substrate. After another nonspecific blocking step with 0.25% casein in PBS (DakoCytomation) for 20 min, the slides were incubated for an additional 30 min with the second primary Ab either against CD4 (clone 1F6, 1/25 dilution; Novocastra) or CD8 (clone C8/144B, 1/100 dilution; DakoCytomation), followed by detection using a horse anti-mouse secondary Ab and the ABC alkaline phosphatase system (Vector Laboratories). Vector red (Vector Laboratories) was used as a chromogen in the second immunostaining. Slides were washed in deionized water, stained with Meyer's hematoxylin, and mounted. Tonsil tissues slides were used as positive controls. Negative controls were obtained by using tonsil tissues slides substituting the primary Ab with either rat (IgG2a; eBioscience) or

Table I. *Patient characteristics, clinical response, and toxicity*[a]

Patient	Age/Sex	Sites of Evaluable Disease	Prior Therapies
1	60/F	Intraperitoneal, breast, abdominal, spleen	I, S
2	61/F	Brain, lung, liver	I, R, S
3	59/F	Cutaneous	S
4	51/M	Inguinal nodes	C, I, S
5	51/M	Liver, lung, spleen	I, S
6	64/F	Brain, lung, cutaneous	I, S
7	45/M	Hilum, lung, liver, bone, subcutaneous	C, I, R, S
8[b]	52/M	Lung	I, S

[a] One treatment course consisted of infusion of LMB-2 administered every other day for three doses, followed by daily vaccination beginning the next day with gp100$_{209-217(210M)}$ and MART-1$_{27-35}$ peptides for 4 days, and a second vaccination cycle 3 wk after the end of the last vaccination cycle. C, chemotherapy; F, female; I, immunotherapy; M, male; R, radiotherapy; S, surgery.

[b] Patient did not receive second cycle of peptide vaccines due to a grade II injection site reaction.

mouse (IgG1; DakoCytomation) isotype controls at similar dilutions used for the primary Abs. For quantization, 10 independent high-powered fields with the most abundant tumor-infiltrating T cells (TIL) from each entire tumor section were selected, digitally photographed using a ×40 objective lens at a size of 0.0355 mm^2, and semiautomated counted using Adobe Photoshop 7.0 software (Adobe Systems). Because CD68$^+$ macrophages occasionally stain with CD4 Ab, only CD4$^+$ cells with morphological features compatible with lymphocytes were counted. Lymphocytes in the fibrous tissue were counted as stromal and lymphocytes between at least two tumor cells were counted as intratumoral. The number of CD8$^+$, CD4$^+$, FOXP3$^+$, and double-staining cells indicated were expressed by 10 high-power fields for each case.

Results

Patient characteristics

Eight HLA-A*0201-positive patients with progressive metastatic melanoma (four male/four female) received LMB-2 and peptide vaccine therapy (Table I); six patients (75%) had visceral metastases. Patients age ranged from 45 to 64 years old (mean of 55.4 ± 2.3). All had undergone excision of the primary lesion, two had received prior radiation (25%), and two patients (25%) had received prior chemotherapy. Seven patients (87.5%) had received prior immunotherapy that included IFN-α (patients 1, 2, 4, 7, and 8), high-dose IL-2 (patients 2, 5, 6, and 8), low-dose IL-2 (patient 7), gp100$_{209-217(210M)}$ (g209-2M) and MART-1$_{26-35(27L)}$ peptide vaccination (patient 5), g209-2M and tyrosinase peptide vaccination (patient 8), or IL-2 gene therapy (patient 7).

Pharmacokinetics and neutralizing Ab induction

The pharmacokinetics of LMB-2 after administration to heavily pretreated patients with CD25$^+$ hematologic malignancies are well-established (34); however, the pharmacokinetics of immunotoxin when administered to patients with metastatic melanoma who have small numbers of circulating CD25$^+$ lymphocytes is not known. In the study of serum from four patients, the plasma disappearance of LMB-2 was monoexponential with a mean half-life ($T_{1/2}$) of 135.1 ± 23.6 min after the first dose and a range of 74.4–179 min (Table II). The serum $T_{1/2}$ was nearly half of that previously measured in patients with hematologic cancer (260 min) (34), although with the small number of patients studied here, the significance of this finding is not evaluable. Immunotoxin levels in the plasma were below the detectable threshold (0.16 ng/ml) in all tested patients 24 h after the first LMB-2 infusion. It is not known whether the Ab component of LMB-2 had a longer plasma half life after LMB-2 has lost its immunotoxic activity in these

Table II. *Pharmacokinetics of LMB-2 and neutralizing Ab development[a]*

| | Pharmacokinetics | | | | | | % LMB-2 nAb Activity | |
| | Peak level | C$_{max}$ | T$_{1/2}$ | AUC | Vd | Clearance | | |
Patient	(ng/ml)		(minutes)	(μg $\times$ min/mL)	(L)	(ml/min)	Pretreatment	Posttreatment
1							26	104
2							0	81
3							0	102
4							0	99
5	536 $\pm$ 57	801	74.4	66	4.3	40	1	96
6	324 $\pm$ 10	328	165	77.8	7.02	29.6	0	4
7	454 $\pm$ 16	412	122	72.6	6.17	35	0	91
8	498 $\pm$ 7	422	179	109	7.76	30	0	91
Mean:	378 $\pm$ 52	491 $\pm$ 106	135 $\pm$ 24	81 $\pm$ 10	6.3 $\pm$ 0.7	33.7 $\pm$ 2.4		

[a] LMB-2 neutralizing Ab (nAb) activity was quantified by measuring the degree to which coincubation of patient serum with LMB2 at 1000 ng/ml blocked the cytotoxic activity of the immunotoxin on SP2/Tac cells. Values represent percent neutralization calculated by determining the percent inhibition of protein synthesis of toxin in HSA-PBS, minus the percent inhibition in serum, divided by the percent inhibition in HSA-PBS, times 100. Values represent the mean of duplicate cultures. C$_{max}$, maximum concentration of drug; T$_{1/2}$, half-life; AUC, area under the curve; Vd, volume of distribution.

patients; however, in preclinical study, unbound radio-labeled anti-Tac Fv fragment was rapidly cleared from the blood with apparently biphasic pharmacokinetics ($\alpha t_{1/2} \leq 10$ min; $\beta t_{1/2} = \sim 5.5$ h) (38). By all other measures, including peak level of drug, maximum concentration, area under the curve, volume of distribution, and clearance, the pharmacokinetics of LMB-2 in patients with metastatic melanoma was similar to those measured in patients with CD25$^+$ hematologic cancers.

To determine the degree to which immune responses had been elicited against LMB-2 and whether patients were eligible for retreatment, serum from treated patients was assayed for neutralizing Ab activity by measuring the ability of the serum to inhibit the cytotoxic activity of LMB-2 on the CD25-expressing cell line, SP2/Tac, in vitro (Table II). Before LMB-2 therapy, neutralizing Ab activity was not detectable in serum samples from all patients, except patient 1 whose serum inhibited LMB-2-mediated cytotoxicity by 26%. By contrast, seven of eight (87.5%) of the patient serum samples collected at the time of tumor evaluation, nearly 2 mo after the initial LMB-2 dose infusion, were capable of neutralizing LMB-2 activity in vitro at levels >80%. The sole exception was patient 6 whose serum did not possess substantial neutralizing activity. Although eligible, patient 6 was not retreated due to the development of two new brain lesions.

*Selective elimination of circulating CD25$^+$ Treg cells after LMB-2 infusion*

In the resting peripheral blood, Treg cells represent ∼5–10% of all CD4 T cells and are the principal T cell subset that expresses CD25. One day after administration of the first dose of LMB-2 immunotoxin, a significant reduction in mean lymphocyte count, from 1788 $\pm$ 262 to 1216 $\pm$ 148 cells/μl, was observed ($p \leq 0.01$, two-tailed; Fig. 1*A*), which persisted until day 3 (day 2; 1282 $\pm$ 215 cells/μl; $p \leq 0.001$) when pretreatment cell counts were restored. Beginning on day 6, 1 day after the first dose of peptide vaccine, the mean lymphocyte count dropped to 1239 $\pm$ 255 cells/μl and remained low until day 9. Two weeks after the first dose of LMB-2, mean cell counts were restored to pretreatment levels. Thus, a transient reduction in total lymphocyte count followed both the first dose of LMB-2 and the first cycle of peptide vaccination.

Longitudinal T cell subset analysis indicated that CD4$^+$ and CD8$^+$ T cell numbers were similarly impacted by LMB-2 infusion, suggesting a nonspecific effect of the CD25-directed immunotoxin because circulating CD8 T cells seldom express high lev-

els of CD25 in the resting peripheral blood (Fig. 1*B*). To determine the impact of LMB-2 on CD25$^+$ Treg T cells, CD4$^+$ T cells were initially measured for expression of CD25. Fig. 1*C* shows representative kinetic data of CD25 expression by CD4 T cells from patient 5. To measure the mean expression of CD25 by CD4$^+$ T cells after LMB-2, two distinct CD25-specific Abs were used. Although clone 2A3 binds CD25 at a site similar to anti-Tac (from which the single-chain Fv fragment portion of LMB-2 is derived), clone 4E3 binds a distinct epitope on CD25. Before treatment, nearly 10% of CD4 T cells expressed a high level of CD25 using either Ab (Fig. 1*D*). Staining with either CD25-specific Ab demonstrated a significant reduction in CD25 expression by CD4$^+$ T cells (from 9.9 $\pm$ 0.1% to a 0.7 $\pm$ 0.2% nadir on day 3 for 2A3; from 10.0 $\pm$ 0.1% to a 2.5 $\pm$ 0.4% nadir on day 3 for 4E3) which was maintained until day 8 after the first LMB-2 infusion. Staining with the 2A3 Ab suggested a more complete depleting potency by LMB-2 than did 4E3 clone staining, which may result from competition between residual LMB-2 bound to CD25 on Treg cells and the 2A3 clone for the same epitope. It is unknown whether residual LMB-2 remained bound to CD25 on Treg cells after infusion. Regarding the CD8 T cell population, only a small frequency of circulating CD8 T cells expressed high levels of CD25 before therapy (mean; 0.62% $\pm$ 0.15). Although this may represent a small population of activated T cells in vivo, their frequency was not significantly reduced at any time point after LMB-2 administration.

To better determine the impact of LMB-2 on Treg cells in vivo, CD4$^+$ T cells were measured for FOXP3 protein expression (Fig. 1, *C* and *E*). Before therapy, the mean level of FOXP3 expression by CD4$^+$ T cells from all patients was 9.3 $\pm$ 1.3%. FOXP3 expression was primarily restricted to CD4$^+$ T cells expressing high levels of CD25, although FOXP3 was also detected in CD4$^+$ T cells expressing intermediate levels of CD25 (Fig. 1*C*). LMB-2 administration induced a rapid reduction in FOXP3$^+$CD4$^+$ T cells (down to 4.7 $\pm$ 0.6% of CD4$^+$ T cells on day 3, $p \leq 0.002$) that was sustained for 7 days, 3 days beyond the last dose of immunotoxin (Fig. 1*E*). Although the mean frequency of FOXP3$^+$CD4$^+$ T cells was reduced by up to 50%, LMB-2-mediated elimination was transient and incomplete in nature. Three weeks after the first dose of LMB-2, the mean frequency of FOXP3$^+$CD4$^+$ T cells was mildly elevated (12.2 $\pm$ 1.1%; $p \leq 0.018$) compared with pretreatment levels but had returned to a normal frequency (10.6 $\pm$ 1.8%) by day 50. Because a small number of FOXP3$^+$CD4$^+$ T cells do not express high levels of CD25, we assessed whether the

FIGURE 1. LMB-2 administration causes a selective, transient elimination of circulating CD25$^+$ Treg cells in vivo. *A*, A transient reduction in the mean ALC was induced after the first dose of LMB-2 on day 0. Each dot represents the percent change in ALC relative to pretreatment counts for each patient. The boxed line indicates the mean percent change in ALC for all patient values on each day. *B*, Changes in T cell subset counts following LMB-2 infusion. Each line represents the mean percent change in the number of the indicted T cell subset relative to pretreatment counts for all available patients. CD25 expression was evaluated with the 4E3 Ab clone. *C*, CD25 and FOXP3 expression by circulating CD4$^+$ T cells is reduced after LMB-2 administration. Representative dot plots show the expression of CD25 (4E3 clone; *y*-axis) and FOXP3 (*x*-axis) on CD4$^+$CD3$^+$ gated cells from patient 5 at the indicated time after first infusion. *D*, CD25 expression by CD4$^+$CD3$^+$ gated T cells was transiently reduced following LMB-2 administration in all patients. The mean level of CD25 expression by CD4 T cells from all patients is shown, as detected using Ab clones 2A3 (which recognizes the same epitope region as LMB-2) or 4E3 (which binds a unique region of CD25). *E*, The mean level of FOXP3-expressing CD4$^+$CD3$^+$ T cells was reduced for 1 wk after initiation of LMB-2 therapy. *F*, LMB-2 selectively eliminates FOXP3-expressing CD4$^+$ T cells that express high levels of CD25 in vivo. The frequency CD4$^+$CD3$^+$-gated cells expressing FOXP3 and high levels of CD25 or intermediate/negative CD25 were assessed over time. The mean frequency of CD25^{high}FOXP3$^+$CD4$^+$ T cells and CD25$^{int/negative}$FOXP3$^+$CD4$^+$ T cells is shown. *, Significance ($p \leq 0.05$) on the indicated day; ns, changes that were not significant.
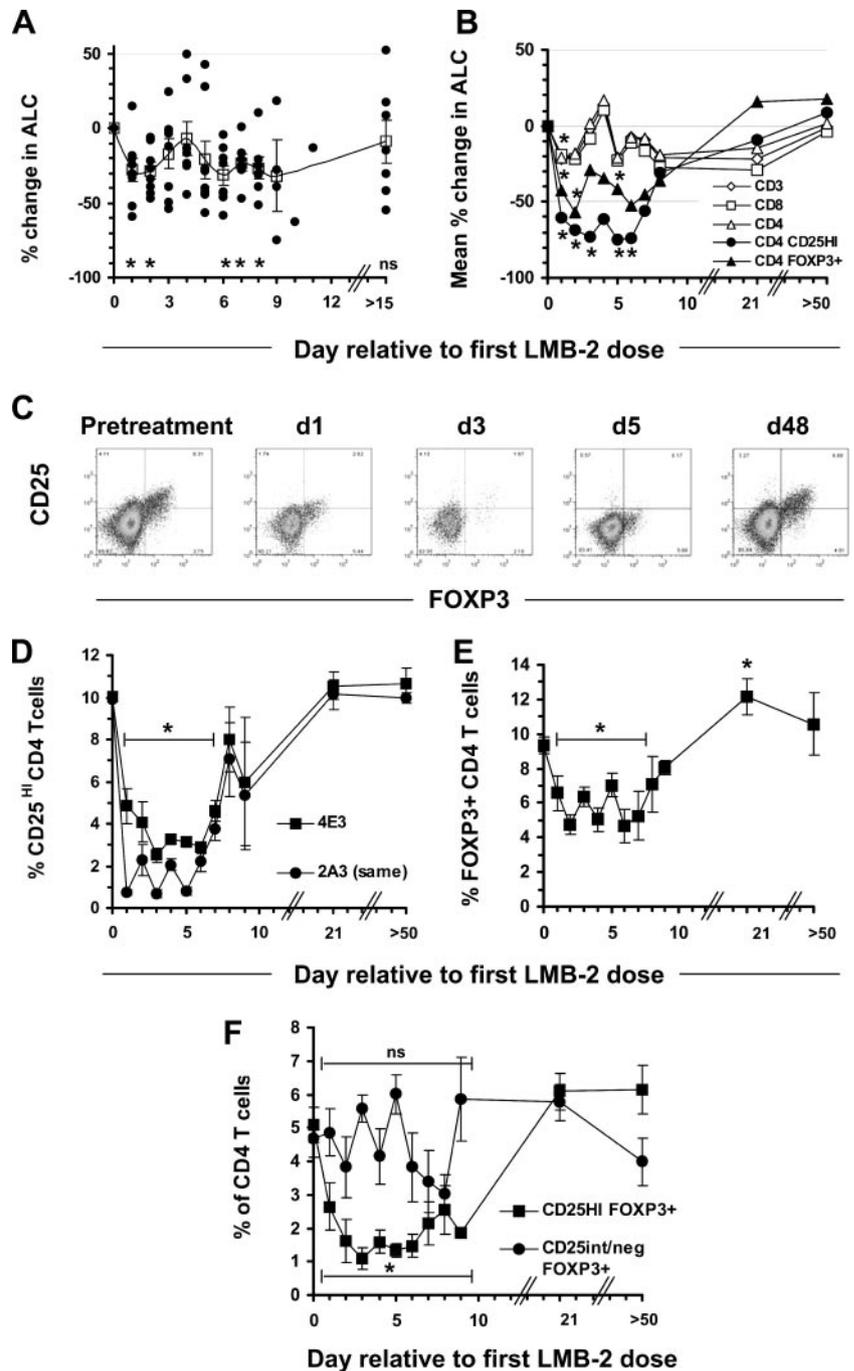
incomplete nature of FOXP3$^+$ Treg cell reductions by CD25-directed immunotoxin therapy resulted from a selective survival of these cells. Fig. 1*F* shows that while the frequency of FOXP3$^+$CD4 T cells expressing high levels of CD25 was significantly reduced from 5.1 ± 0.5% to levels as low as 1.1 ± 0.3% for 6 days following the first dose of LMB-2, similar cells expressing intermediate to low levels of CD25 were not significantly reduced at any time point, indicating their continued survival. The inability to selectively enrich FOXP3-expressing cells and the lack of high CD25 expression by CD4 T cells after immunotoxin inhibited our ability to adequately test the suppressive function of the CD25^{low}, FOXP3$^+$ cells in vitro. Of note, substantial expression of FOXP3 was not detected in CD8$^+$ T cells at any time point, and the low level of CD25 expressed by these cells was not significantly altered by LMB-2 therapy (data not shown).

Because frequency analysis does not adequately address cell numbers, circulating CD25$^+$ or FOXP3$^+$CD4$^+$ T cell counts were enumerated (Fig. 1*B*). Total CD3$^+$ T cell counts were significantly reduced compared with pretreatment counts (1391 ± 254 cells/μl) only at day 1 of therapy (959 ± 90 cells/μl; $p \leq 0.047$); however, CD25$^+$CD4$^+$ T cell counts were significantly reduced from 83 ± 16 cells/μl before therapy, for up to 6 days after the first LMB-2 dose, except day 4 (39 ± 7 cells/μl; $p \leq 0.066$). FOXP3$^+$CD4$^+$ T cell counts (74 ± 15 cells/μl) were similarly reduced after LMB-2 infusion with statically significant reductions observed 1, 2, and 5 days after the first dose of immunotoxin (39 ± 8 cells/μl, 36 ± 8 cells/μl, and 45 ± 13 cells/μl, respectively). Circulating CD25$^+$FOXP3$^+$CD4 T cell numbers were therefore significantly reduced at the initiation of peptide vaccination on day 5 ($p \leq 0.05$).
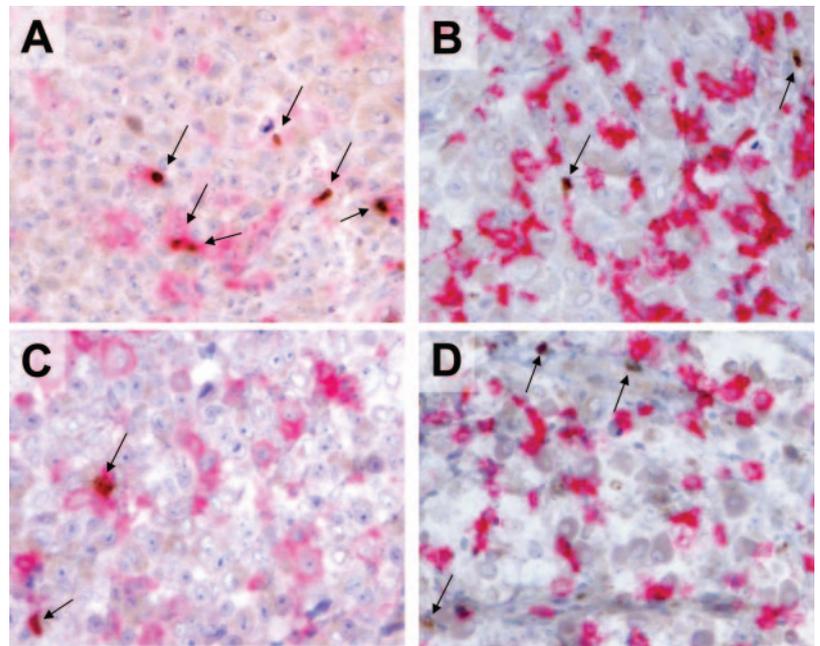
FIGURE 2. A reduction in the frequency of tumor-infiltrating FOXP3+CD4+ T cells in metastatic melanoma lesions following LMB-2 administration. Immunohistochemical analysis for FOXP3 (brown), CD4 (red, in *A* and *C*), and CD8 (red, in *B* and *D*) was performed on pretreatment (*A* and *B*) or day 8 posttreatment (*C* and *D*) metastatic melanoma lesions from patient 1. Arrows denote FOXP3-expressing cells; ×40 magnification was used.

A reduction in FOXP3+CD4+ T cell frequency in metastatic melanoma lesions

To evaluate whether LMB-2 administration induced a selective elimination of tumor-infiltrating Treg cells in vivo, immunohistochemical (IHC) analysis was performed on available tumor biopsies collected immediately before and 1 wk after the first LMB-2 dose from three patients with visceral disease. Fig. 2 shows the result of a representative IHC analysis of s.c. melanoma lesions from patient 1. CD8, CD4, and FOXP3-positive cells were each detected in intratumoral and stromal regions before therapy (Fig. 2 and Table III). Costaining analysis revealed the frequent presence of CD4-positive lymphocytes expressing FOXP3 in the lesion before treatment (Fig. 2*A*), whereas CD8- and FOXP3-expressing lymphocyte populations were completely exclusive (Fig. 2*B*). Eight days after the first dose of LMB-2, IHC analysis of a biopsy from the same lesion showed CD4 and FOXP3 dual-stained lymphocytes in the tumor (Fig. 2*C*). Meanwhile, CD8- and FOXP3-positive cell populations remained mutually exclusive (Fig. 2*D*).

To quantify the number of Treg cells in tumor deposits, 10 high-powered fields containing the greatest number of TIL from each tumor were counted by a pathologist in a blinded manner (Table III). Lymphocytes in the surrounding fibrous tissue were counted as peritumoral/stromal whereas TIL positioned between at least two tumor cells were counted as intratumoral. Before LMB-2 therapy, CD4 and CD8 cells were observed in both the peri- and intratumoral regions of the lesions of patients 1 and 3. Samples from

Table III. *Immunohistochemical analysis of nonvisceral metastatic melanoma lesions after LMB-2 therapy*[a]

		Cell Counts (per 10 HPF)					
		Intratumoral lymphocytes			Peritumoral/stromal lymphocytes		
Patient	Time	CD4 total	CD4+Foxp3+ total	FOXP3+/CD4 (%)	CD4 total	CD4+Foxp3+ total	FOXP3+/CD4 (%)
1	Pre	72	31	43.1	647	102	15.8
	Day 8	97	11	11.3	663	61	9.2
3	Pre	23	5	21.7	516	93	18.0
	Day 6	69	4	5.8	210	9	4.3
	Day 26	NE[b]	NE[b]	NE[b]	719	122	17.0
4	Pre	11	9	81.8	63	17	27.0
	Day 6	21	6	28.6	71	9	12.7
		CD8 total	CD8+Foxp3+ total	FOXP3+/CD8 (%)	CD8 total	CD8+Foxp3+ total	FOXP3+/CD8 (%)
1	Pre	322	0	0.0	692	0	0.0
	Day 8	348	0	0.0	274	0	0.0
3	Pre	60	0	0.0	338	0	0.0
	Day 6	135	0	0.0	91	0	0.0
	Day 26	NE[b]	NE[b]	NE[b]	742	0	0.0
4	Pre	0	0	0.0	1	0	0.0
	Day 6	0	0	0.0	1	0	0.0

[a] Values represent the total number of the indicated cell type counted from 10 independent high-powered fields (HPF) with the most abundant TILs from each entire tumor section. Lymphocytes in the fibrous tissue were counted as stromal and lymphocytes between at least two tumor cells were counted as intratumoral. The number of CD8+, CD4+, FOXP3+, and double-staining cells indicated were expressed by 10 HPF for each case. Time indicates the day relative to the first administered dose of LMB-2 on day 0. Pre indicates pretreatment.
[b] Denotes that intratumoral cell counting was not possible due to scattered tumor cells. NE represents not evaluable.

Table IV. *Frequency of tumor Ag-specific tetramer-positive CD8 T cells after therapy[a]*

Patient	gp100: 209–217/A2 Pre (%)	Post (%)	MART-1: 26–35(27L)/A2 Pre (%)	Post (%)
1	0	0	0.01	0
2	0	0	0	0
3	0	0.01	0	0
4	0	0	0	0
5[b,c]	0.36	0.40	0.27	0.33
6	0.01	0	0.08	0
7	0.30	0.10	1.15	0.67
8[b]	0.15	0.76	0.01	0.06
Control cell lines				
JFK6	0		99.88	
TIL 2102–2	7.97		0.17	

[a] Cryopreserved PBMC were thawed, blocked with normal mouse Ig before gp100$_{209-217}$:HLA-A*0201 or MART-1$_{26-35(27L)}$:HLA-A*0201 tetramer and human CD8 and CD3 Ab staining. Viable T cells were positively selected by propidium iodide-negative and CD3-postive gating. Values correspond to the number of gated CD8^{bright} tetramer$^+$ cells divided by the total number of CD8^{bright} cells minus the CD8$^-$ tetramer$^+$ cells background (number of CD8$^-$tetramer$^+$ cells divided by the total CD8$^-$ cell number) × 100. Percentages ≅0.10% are underlined. JKF6 is a MART-1-reactive cell line. TIL 2102-2 contains in part gp100-reactive T cells.
[b] Previously received gp100$_{209-217(210M)}$ peptide vaccination.
[c] Previously received MART-1$_{26-35(27L)}$ peptide vaccination.

Table V. *Number of patients receiving LMB-2 and peptide vaccine who experienced treatment-related toxicity*

Toxicity		I	II	III	IV
Transaminases	AST	6	2		
	ALT	4	2		
Fever		2			
Edema (capillary leakage syndrome)			5		
Alkaline phosphatase		1			
Thrombocytopenia		4			
Activated partial thromboplastin time		2		2	
Hypophosphatemia			1	1	
Hypoalbuminemia			1		
Hypokalemia				1	
Hypocalcemia			3		
Hypotension		1			
Tachycardia		1			
Nausea/vomiting		1			
Diarrhea		1			
Dermatitis, exfoliative		4	1		
Pruritus		3			
Injection site reaction			1		
Lymphopenia				1	
Neutropenia			3		
Leukopenia			2		
Fatigue		4	1		
Myalgia/pain/aches		2	1		
Dyspnea			1		
Low urine output			1		
Total observed		36	25	5	0

patient 4 contained few CD4 cells in and around the tumor, and no CD8 cells. The numbers of CD4 and CD8 cells (ranges of 647-63 and 692-1 cell, respectively) in the peritumoral/stromal regions was generally greater than within the tumor (CD4 range of 72-11 cells; CD8 range of 322-0 cells). However, in all patients the frequency of intratumoral FOXP3$^+$CD4 cells was higher than the frequency of FOXP3$^+$CD4 cells found in the surrounding stromal tissue (43, 22, and 82% vs 16, 18, and 27%, respectively), suggesting a selective enrichment of Treg cells in the tumor. No cells coexpressing CD8 and FOXP3 were observed in either intratumoral or stromal regions. One week after the initial dose of LMB-2, the frequency of CD4 cells expressing FOXP3 in both the tumor (mean of 15.2 ± 6.9%) and surrounding stromal tissue (8.7 ± 2.4%) was reduced compared with pretreatment frequencies for all patients (48.9 ± 17.6% and 20.3 ± 3.4%, respectively). With the limited number of paired samples available for IHC analysis, these results were not or were barely statistically significant ($p < 0.09$ in tumor and $p < 0.04$ in stromal tissue; two-tailed paired t test), yet support the notion that intratumoral Treg cells are reduced by LMB-2 therapy.

LMB-2-mediated Treg cell reduction does not augment the immune response to peptide vaccination in vivo

Because LMB-2 administration resulted in a significant reduction in Treg cells in the peripheral blood at the initiation of peptide vaccination (day 5), we assessed the immune response to gp100$_{209-217(210M)}$ and MART-1$_{27-35}$ peptide vaccines in all patients' PBMC before and at the completion of therapy (~day 50). Specific peptide/HLA tetramer staining analysis showed low but detectable frequencies (≅0.1%) of native gp100$_{209-217}$ peptide-specific CD8 T cells in three of eight (37.5%) of patients, and low frequencies of MART-1$_{26-35(27L)}$-specific CD8 T cells in two of eight (25%) patients before therapy (Table IV). PBMC from patients 5 and 7 contained CD8 T cells specific for both the gp100$_{209-217}$ and MART-1$_{26-35(27L)}$ peptides. Following LMB-2 and peptide vaccine therapy, gp100$_{209-217}$-specific CD8 T cells

were maintained at frequencies similar to pretreatment levels in the three patients with pre-existent cells. Vaccine-induced gp100$_{209-217}$-specific CD8 T cells were not detected in any other patient. Similarly, MART-1$_{26-35(27L)}$-specific CD8 T cells were maintained in the two patients with pre-existent cells and were not induced in any other patient after LMB-2 and peptide vaccine therapy. The lack of induction of tumor Ag-specific T cells by peptide vaccination was confirmed by direct ex vivo ELISPOT analysis and by 10-day in vitro peptide stimulation and cytokine secretion analysis (data not shown). Pre-existent Ag-specific immune responses were not dampened by LMB-2 therapy because reactivity to influenza was maintained by all patients at similar levels after therapy, as measured using the in vitro stimulation assay (data not shown).

Treatment-related toxicities and clinical response

We have previously shown that LMB-2 at the maximum-tolerated dose (40 µg/kg every other day (QOD) × 3) and at 30 µg/kg QOD × 3 is well-tolerated in patients with CD25$^+$ hematological malignancies with transient toxicity most commonly including transaminase elevations, fever, and hypoalbuminemia (34). In the current study, treatment of patients with metastatic melanoma, who comparatively do not harbor large numbers of CD25$^+$ lymphocytes, with LMB-2 (30 µg/kg QOD × 3) and peptide vaccination induced a similar panel of toxicities (Table V). Toxicities observed in these eight patients were mild to moderate expected side effects and included grade I/II altered transaminases, edema (capillary leakage syndrome), thrombocytopenia, hypocalcemia, leukopenia, fatigue, fever, rash, diarrhea, nausea, muscle pain, dyspnea, and low urine output. Grade III events were rare but included two cases of prolonged activated partial thromboplastin time and individual cases of lymphopenia, hypophosphatemia, and hypokalemia.

Despite the partial reduction in Treg cell frequency in the peripheral blood and at the tumor site, peptide vaccination following

LMB-2-mediated Treg cell reduction did not induce objective cancer regression or overt autoimmune disease in any treated patient.

Discussion

In the current study, administration of a CD25-directed immunotoxin, LMB-2, to patients with metastatic melanoma resulted in a selective, transient, partial elimination of circulating Treg cells in vivo. LMB-2 mediated a significant reduction in Treg cell number which endured for nearly 1 wk following the initial dose of immunotoxin as measured by both CD25 and FOXP3 protein expression on CD4$^+$ T cells. Enumeration of Treg cells based on CD25 expression revealed a more robust Treg cell depletion efficiency than when measured by FOXP3 expression in CD4$^+$ T cells (79.1 vs 51.4%, respectively). This discrepancy appeared to result from the inability of a CD25-directed immunotoxin to selectively eliminate the subset of FOXP3$^+$ Treg cells which expressed low levels of CD25, a population observed by others (16). Indeed, the mean number of FOXP3$^+$ CD25^{low}CD4$^+$ T cells was not significantly reduced after LMB-2 infusion at any time point tested. Thus, while administration of LMB-2 eliminated nearly 80% of CD25$^+$FOXP3$^+$ CD4$^+$ Treg cells in vivo, FOXP3$^+$CD25^{low}CD4$^+$ T cells, by virtue of their low CD25 expression, were resistant to the cytotoxic activity of this CD25-directed immunotoxin. Whether human FOXP3$^+$CD25^{low}CD4$^+$ T cells possess suppressive function in vivo is not known, largely due to the inability to selectively enrich these cells for study. Recent studies using Foxp3^{gfp} reporter mice have shown Foxp3$^+$CD25^{low}CD4$^+$ T cells to be hypoproliferative and exhibit suppressive function in vitro (39). However, CD25$^{-/-}$ and IL-2$^{-/-}$ mice, despite having Foxp3$^+$ T cells, produce autoantibodies, amass activated CD4$^+$ T cells in vivo, and develop autoimmunity (40, 41). In addition, CD25$^{-/-}$Foxp3$^+$ T cells, unlike CD25$^{+/+}$Foxp3$^+$ Treg cells, do not suppress the expansion or antitumor function of CD8$^+$ T effector cells in vivo (42), suggesting that mouse Foxp3$^+$ Treg cells lacking CD25 do not exhibit functional suppression activity in vivo.

Analogous to the blood, the frequency of CD4$^+$ T cells expressing FOXP3 in metastatic lesions was reduced by 68.9% on average following LMB-2 administration, compared with pretreatment levels in three paired patient lesions available for study. Thus, the tumor does not appear to be impervious to the Treg cell-depleting effects of LMB-2 in vivo. Despite recent demonstrations that activation can induce FOXP3 expression in human CD8$^+$ and CD4$^+$CD25$^-$ T cells (16, 43–45), no CD8$^+$ cells in any patient tumor sample tested expressed FOXP3 and the frequency of CD4$^+$ T cells expressing FOXP3 was consistently reduced after LMB-2 infusion. Interestingly, the frequency of tumor-infiltrating FOXP3$^+$CD4$^+$ T cells from three patients (mean 48.9 ± 17.6%) appeared increased compared with their peripheral blood counterparts (mean 9.93 ± 0.8%) before treatment, however, this difference was not statistically significant ($p < 0.16$, two-tailed paired t test). These results parallel a previous report of elevated Treg cell frequencies in tumor-involved lymph nodes compared with noninvolved lymph nodes or peripheral blood of patients with metastatic melanoma (20).

Despite reductions in circulating and tumor-infiltrating Treg cells following LMB-2 administration, initiation of peptide vaccination under this Treg cell-reduced condition did not elicit robust immune response to gp100 and MART-1 tumor Ags as measured by tetramer staining or cytokine secretion analysis performed ex vivo or after in vitro stimulation. Although we cannot rule out the possibility that the lack of a strong vaccine response was the product of LMB-2-mediated elimination of tumor Ag-specific T cells activated by vaccination, which are susceptible to elimination by immunotoxins directed against the high-affinity IL-2R complex (31, 46), LMB-2 has a short half-life in vivo and was undetectable in the plasma 24 h after dosing, when vaccination was initiated. Furthermore, vaccination was continued for 4 consecutive days followed by a second vaccine cycle 3 wk later, making it improbable that LMB-2 sustained a negative affect on vaccine-induced effector cells over the duration of the vaccination schedule. Rather, the limited response to vaccination might result from the small number of peptide vaccine cycles that did not induce high frequencies of tumor Ag-specific T cells, similar to our previous findings in immune-replete patients with metastatic melanoma (1, 2). Overall, the level of Treg cell reduction achieved in the current study was not sufficient to substantially bolster the immune response to peptide vaccination in vivo. Our data differ from other clinical reports of Treg cell reduction enhancing vaccine-induced immune responses in vivo (31, 32). Dannull et al. (31) reported that administration of a single dose of DAB$_{389}$IL-2, a cytotoxin which targets the IL-2R complex through the binding of the IL-2 cytokine portion of the agent (29), was sufficient to augment the immunizing potency of a renal cell carcinoma RNA-transfected DC vaccine by partial Treg cell reduction and suggested that CD25$^+$ FOXP3-expressing Treg cells surviving DAB$_{389}$IL-2 exposure had lost their immunosuppressive activity in vivo (31). A similar study reported enhanced immune function and generation of vaccine-induced gp100 and MART-1-specific CD8 T cells after DAB$_{389}$IL-2-mediated Treg cell reduction in vivo, although the impact of vaccination alone was not assessed (32). In neither study was the impact on clinical response evaluated.

CD25-directed recombinant immunotoxins have been successfully used for the treatment of patients with CD25$^+$ hematologic malignancies (34, 47), but until now, their function as agents for targeted elimination of Treg cells has been limited to preclinical studies (35). However, in one clinical study testing the use of the RFT5-dgA immunotoxin for the prevention of graft-vs-host disease (GVHD) in patients undergoing allotransplantation, patients receiving the CD25-directed immunotoxin had a higher incidence of grade III/IV GVHD than historical controls, suggesting that CD25$^+$ Treg cells may have been targeted, thereby increasing GVHD (48). In our study of patients with metastatic melanoma, LMB-2-mediated Treg cell reduction did not provide a sufficient shift in the balance between self/tumor Ag-specific activation and tolerance because no patient experienced a significant antitumor response or developed autoimmunity. It is unclear whether the lack of clinical responses observed in these patients was related to their HLA-A2$^+$ status; however, in our recent clinical trials using nonspecific immunomodulating agents, such as IL-2 cytokine (F. O. Smith, S. G. Downey, J. A. Klapper, J. C. Yang, R. E. Royal, U. S. Kammula, M. S. Hughes, C. L. Levy, D. E. White, S. M. Steinberg, and S. A. Rosenberg, manuscript in preparation) and CTLA-4 Ab (49), HLA-A2$^+$ status had no significant impact on response. Because not all Treg cells appear to express high levels of CD25, alternative and combinatory approaches to control Treg cell frequency and function in vivo deserve consideration, including addition of TLR agonists to vaccine regimens (50), targeting of suppressive molecules (27, 51), selective elimination of FOXP3-expressing cells (52), or altering Treg cell migration to the tumor site (53).

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Disclosures

The authors have no financial conflict of interest.

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