Selective Elimination of Human Regulatory T Lymphocytes In Vitro With the Recombinant Immunotoxin LMB-2

Peter Attia[*], **Daniel J. Powell Jr**[*], **Ajay V. Maker**[*], **Robert J. Kreitman**[†], **Ira Pastan**[†], and **Steven A. Rosenberg**[*]

[*] *From the Surgery Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD; and*

[†] *Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD.*

Summary

CD4$^+$CD25$^+$ T-regulatory cells (T$_{reg}$) can inhibit the proliferation and cytokine secretion of CD4$^+$CD25$^-$ helper T cells in mice and humans. In murine tumor models, the presence of these T$_{reg}$ cells can inhibit the antitumor effectiveness of T-cell transfer and active immunization approaches. We have thus initiated efforts to eliminate T$_{reg}$ cells selectively from human peripheral blood mononuclear cells (PBMCs) to potentially bolster antitumor responses. LMB-2 is a recombinant immunotoxin that is a fusion of a single-chain Fv fragment of the anti-Tac anti-CD25 monoclonal antibody to a truncated form of the bacterial *Pseudomonas* exotoxin A. In vitro incubation of human PBMCs with LMB-2 reduced the levels of CD4$^+$CD25$^+$ and *Foxp3*-expressing cells without impairing the function of the remaining lymphocytes. The short in vivo half-life of LMB-2 makes it an attractive candidate for reducing human T$_{reg}$ cells in vivo before the administration of cancer vaccine or cell transfer immunotherapy approaches.

Keywords

human; immunotoxin; LMB-2; CD25; regulatory T cell; depletion; *Pseudomonas exotoxin*

In humans, naturally occurring CD4$^+$ CD25$^+$ T-regulatory (T$_{reg}$) cells, which represent approximately 5% of the peripheral CD4$^+$ T-cell compartment, maintain homeostatic peripheral self-tolerance by suppressing autoreactive T cells.[1] T$_{reg}$ cells constitutively express the α-chain of the interleukin (IL)-2 receptor (CD25), cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), and transcription factor Forkhead box P3 (*Foxp3*).[2] Compared with CD25$^-$CD4$^+$ T cells, CD25$^+$ T$_{reg}$ cells exhibit a hypoproliferative capacity and possess the ability to suppress CD8$^+$ and CD25$^-$ CD4$^+$ T-cell activation in vitro through a largely unknown mechanism. That T$_{reg}$ cells are key mediators of self-tolerance is evidenced in individuals genetically predisposed to the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), a recessive and often fatal disorder of early childhood caused by loss of function mutations of *Foxp3* and the resultant lack of T$_{reg}$ cells.[3]

Peripheral blood lymphocytes from melanoma patients contain functional $CD4^+CD25^+$ T_{reg} cells, and melanoma antigen-specific T_{reg} cells have been described.[4,5] Further, $CD4^+$ $CD25^+$ T_{reg} cells in human metastatic melanoma lymph nodes have been reported to inhibit the function of infiltrating T cells.[6] T_{reg} cells are also reported to contribute to growth of human ovarian carcinomas in vivo by suppressing tumor-specific T-cell immunity and to be associated with reduced survival of patients with ovarian cancer.[7] Thus, the immune inhibitory effects of T_{reg} cells may account, in part, for the poor clinical response rates reported in cancer patients receiving immunotherapy. The impact of $CD4^+CD25^+$ T_{reg} cells on adoptive immunotherapy has recently been evaluated in a mouse melanoma model. Adoptive transfer of tumor/self-reactive $CD8^+$ T cells with vaccination and IL-2 led to regression of large established B16 melanoma in lymphodepleted hosts.[8] In cotransfer experiments, adoptive transfer of $CD25^-CD4^+$ T helper cells with tumor/self-reactive $CD8^+$ T cells and vaccination into $CD4^+$ T-cell–deficient recipients resulted in regression of established tumor and concomitant autoimmunity.[9] In contrast, cotransfer of $CD25^+$ T_{reg} cells alone or combined with $CD25^-CD4^+$ T helper cells inhibited effective immunotherapy. Based on preclinical findings suggesting that selective depletion of T_{reg} cells and enrichment of $CD4^+$ T helper cells may improve cancer therapy, we sought to neutralize the suppressive effects of T_{reg} cells to bolster antitumor immune responses.

Immunotoxins, which couple the specificity of monoclonal antibodies (mAbs) with a highly lethal cellular toxin, have been used to selectively eliminate cell subpopulations in vivo.[10] 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 toxin *Pseudomonas* exotoxin (PE). [10–12] LMB-2 administered to mice bearing $CD25^+$ human tumors penetrated into the tumors and produced complete tumor regression.[13] Similarly, in phase I trials, LMB-2 administration induced clinical responses in patients with $CD25^+$ hematologic malignancies, including cutaneous T-cell lymphoma, hairy cell leukemia, chronic lymphocytic leukemia, Hodgkin disease, and adult T-cell leukemia.[14,15] In the current study, LMB-2 was evaluated for the ability to eliminate $CD25^+$ T_{reg} cells selectively from peripheral blood mononuclear cells (PBMCs) in vitro. Here, we demonstrate that treatment of human PBMCs with a recombinant immunotoxin, LMB-2, can specifically target and diminish $CD25^+$ T_{reg} cells in vitro without jeopardizing the functional and proliferative potentials of the remaining effector T-cell precursors.

MATERIALS AND METHODS

Patient Samples

PBMCs were isolated by Ficoll-Hypaque separation from normal donors after obtaining informed consent and were used fresh or were cryopreserved at 10^8 cells per vial in heat-inactivated human antibody serum with 10% dimethyl sulfoxide and stored at $-180°C$.

Immunotoxins

LMB-2 [anti-Tac(Fv)-PE38] is a single-chain Fv fragment of the anti-CD25 mAb (Zenapax) fused to a truncated form of the bacterial PE A. Clinical grade LMB-2 was produced as previously described in detail elsewhere[12,15,16] by the Monoclonal Antibody and Recombinant Protein Production Facility (National Cancer Institute [NCI], Frederick, MD). The investigation new drug (IND) application is held by the Cancer Therapy and Evaluation Program (CTEP) of the NCI. LMB-9 is a disulfide, stabilized, recombinant immunotoxin composed of the variable regions of the light and heavy chains of mAb B3 fused to PE38, which directs the cytotoxic potential of PE38 toward cells expressing the Lewis Y antigen, which is not known to be expressed on normal human PBMCs. LMB-2^{Asp553} is an immunotoxin composed of the Fv portion of anti-Tac fused to a mutated and thus inactivated

PE38 [anti-Tac(Fv)-PE38^{Asp553}]. LMB-9 and LMB-2^{Asp553} were supplied by Ira Pastan and Robert Kreitman. Anti-Tac antibody (Zenapax) was purchased from Roche Pharmaceuticals (Hoffmann-LaRoche, Nutley, NJ).

Media

PBMCs were cultured in complete media (CM) consisting of RPMI 1640 (Invitrogen Corporation, Carlsbad, CA) supplemented with 2 mM glutamine (Biofluids, Rockville, MD), 25 mM HEPES buffer (Biofluids), 100 U/mL penicillin (Biofluids), 100 μg/mL streptomycin (Biofluids), and 20% heat-inactivated fetal bovine serum (Gemini Bioproducts, Woodland, CA).

Flow Cytometry

Flow cytometry was used to assess the surface expression of selected T-cell markers and was performed as previously described.[17] Briefly, cryopreserved PBMCs were thawed into ice-cold buffer, washed, and Fc-receptor blocked with mouse IgG (Caltag Laboratories, Burlingame, CA). CD25 staining was performed using a PE-conjugated 4E clone (Miltenyi Biotec, Auburn, CA). Cells were then incubated with appropriate fluorochrome-labeled antibodies (BD Biosciences, San Diego, CA) and relevant isotype controls and washed twice subsequently. FACSCalibur and CellQuest software (BD Biosciences) was used for acquisition and analysis.

In Vitro Sensitization

The 10-day in vitro sensitization was carried out as previously described.[18] Briefly, after 48 hours of exposure to LMB-2, PBMCs were washed and plated at 3×10^6 per well in 24-well plates with 1 μM soluble peptide (Flu$_{58-66}$ GILGFVFTL or gp100$_{280-288(288V)}$ YLEPGPVTV) for 10 days. Cells were harvested, washed, and plated in 96-well plates with T2 cells alone or pulsed with peptide at 1 μM. After 24 hours, the supernatant from each well was harvested and interferon-γ (IFNγ) was measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Pierce Endogen, Rockford, IL).

Real-Time Polymerase Chain Reaction

Levels of messenger RNA (mRNA) *Foxp3* were analyzed using the quantitative real-time polymerase chain reaction (PCR) with the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA) as described previously.[19] In each experiment, 500 ng total RNA was isolated from CD4-purified lymphocytes using the RNeasy Mini Kit (Qiagen USA, Valencia, CA) and reverse-transcribed to prepare complementary DNA (cDNA) using the ThermoScript reverse transcriptase (RT)–PCR system (Invitrogen) according to the manufacturer's instructions. The TaqMan Universal Master Mix (Applied Biosystems) was also used as a template. For β-actin, the forward primer used was 5′-GCGAGAAGATGACCCAGATC-3′, the reverse primer used was 5′-CCAGTAGGTACGGCCAGAGG-3′, and the probe used was 5′-FAM-CCAGCCATGTACGTTGCTATCCAGGC-TAMRA-3′. For *Foxp3*, the combined primer, probe reagent was used (Assay-on-demand gene expression assay, Applied Biosystems). The ABI Prism 7700 detection system (Applied Biosystems) was used with the following settings: 50°C for 2 minutes, followed by 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Lymphocyte Separation

CD4$^+$ cells were separated from whole PBMCs by magnetic bead selection using the Dynal CD4 negative isolation kit (Dynal Biotech; Invitrogen) according to the manufacturer's instructions. In indicated experiments, CD4$^+$ T cells were enriched using the Dynal CD4

positive isolation kit (Dynal Biotech; Invitrogen). For suppression assays, CD4$^+$ cells were further purified into CD25$^−$ and CD25$^+$ fractions using the Dynal T$_{reg}$ kit according to the manufacturer's instructions. Separations were performed in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin.

Proliferation Assay

PBMCs treated with 0 or 100 ng/mL LMB-2 for 48 hours were plated in 96-well plates coated with anti-CD3 (1 μg/mL) at a cell concentration of 50×10^3 PBMCs per well. On days 2 and 4 of cell culture, 1 μCi [^{3}H]-thymidine incorporation was added per well and further cultured for 18 hours before harvesting for measurement on days 3 and 5. Plates were harvested onto nylon filters using the Betaplate system, and radioactivity was quantified using a Betaplate counter. Results are expressed as the mean counts per minute of 24 cultures ± standard error of measure (SEM) per condition.

RESULTS

Impact of LMB-2 on Resting Lymphocytes

Doses of LMB-2 ranging from a final concentration of 0 to 1000 ng/mL were incubated in vitro with resting PBMCs for 48 hours. The surface expression of CD25 on CD3$^+$CD4$^+$ lymphocytes within this population decreased by 75% to 90% (Fig. 1A), which was paralleled by the decrease in *Foxp3* expression (see Fig. 1B). Resting PBMCs were sensitive to LMB-2 at 1 ng/ mL, but a maximum impact was observed between 100 and 1000 ng/mL. This experiment was representative of several dose titrations performed. For the remainder of our in vitro experiments, 100 ng/mL was chosen as the treatment dose.

To determine the optimal exposure time, PBMCs were harvested at 12, 24, 48, and 72 hours (data not shown). Based on the decrease in CD25 expression and reduction in *Foxp3* expression, 48- or 72-hour exposure was deemed equally appropriate and superior to the shorter exposures after a single administration of LMB-2 at time 0. For the remainder of our in vitro experiments, a 48-hour harvest was used.

Specificity of LMB-2

Several immunotoxin controls were used to examine the specificity of LMB-2 cytotoxicity toward resting PBMCs. LMB-2^{Asp553} is identical to LMB-2 except for a single modification (E553D) of PE38 that nearly eliminates adenosine diphosphate (ADP) ribosylation activity. Unmodified anti-Tac antibody,[20,21] which is not fused to toxin, was also used as a control. Finally, PBMCs mechanically depleted of CD25$^+$ cells were also used as a control to assess the nadir of *Foxp3* expression. Figure 2 shows the impact of LMB-2, LMB-9, LMB-2^{Asp553}, and anti-Tac (each at 100 ng/mL) on CD25 surface expression and *Foxp3* expression, respectively. LMB-9 had no impact on PMBCs, suggesting that the cytotoxicity of LMB-2 was directed toward cells expressing CD25 rather than killing because of nonspecific internalization of PE38. LMB-2^{Asp553} and anti-Tac had a similar impact on resting CD3$^+$CD4$^+$ PBMCs, reducing CD25 expression from 19.1% to 6.2% and 6.2%, respectively, and reducing *Foxp3* expression from 16.7 to 11.1 and 10.3 copies per 10^4 β-actin copies. LMB-2 had a superior depleting effect, reducing CD25 expression to 2.2% and *Foxp3* expression to 7.2 copies. This experiment was performed twice with similar results. The observed reductions in CD25 and *Foxp3* expression by LMB-2^{Asp553} and anti-Tac suggest that blockade of the α-chain of the IL-2 receptor on resting CD4$^+$CD25$^+$ cells may be detrimental to their survival, although not to the extent resulting from internalization of the toxin. Finally, the *Foxp3* expression in PBMCs mechanically depleted of CD25$^+$ cells showed even greater depletion, underscoring the partial depletion by LMB-2.

Impact of LMB-2 on CD4+CD25+ Regulatory T Cells

To quantify the impact of LMB-2 on resting T_{reg} cells, large numbers of freshly isolated PBMCs were treated with or without LMB-2. After 48 hours of incubation, PBMCs were counted and mechanically sorted into CD4+ fractions by negative isolation and then into CD4+CD25− and CD4+CD25+ fractions. Table 1 shows the absolute cell counts for each cell subset from 2 independently performed experiments on separate patient samples. In the first experiment, 1.5×10^9 PMBCs were treated in flasks with no treatment or 100 ng/mL LMB-2 for 48 hours. The impact of LMB-2 on the absolute CD4+ count and the absolute CD4+CD25− count was minimal (decrease of 14.4% and 1.9%, respectively). The decrease in CD4+CD25+ cells was more profound (98.7% reduction), however. In the second experiment, starting with one fifth the total number of PBMCs compared with the first experiment, a greater reduction in was seen in the treated CD4+ fraction (47.9%), whereas an increase was observed in the absolute count of CD4+CD25− cells (27.4%). A similar reduction in absolute CD4+CD25+ cell count as seen in the first experiment was observed (98.7%), however.

Using flow cytometry, surface expression of CD25 was measured on the treated PBMCs in addition to posttreated purified subsets (CD4+ cells isolated by negative selection, CD4+ cells isolated by positive selection, and CD4+CD25+ cells). Figure 3A shows the reduction in CD25 expression on PBMCs, negatively isolated CD4+ cells, and CD4+CD25+ cells. Figure 3B shows the reduction in CD25 expression on PBMCs as well as on negatively and positively isolated CD4+ cells from a second representative experiment. In both experiments, the reduction in CD25 expression, regardless of the method of posttreatment isolation, was between 80% and 95%.

Total RNA was isolated from the cells from these experiments, and real-time quantitative PCR was used to quantify *Foxp3* expression, relative to β-actin, in the treated PBMCs and posttreated isolated cell subsets (Fig. 4). In both experiments, the reduction in *Foxp3* expression in whole PBMCs was approximately 50%, whereas in CD4+ subsets, the reduction varied from 50% to 75%.

Impact of LMB-2 on Non-CD25+ Bystander Cells

The impact of LMB-2 on the surviving non-CD4+CD25+ cells was examined using proliferation (Table 2) and in vitro peptide sensitization assays (Table 3). PBMCs were harvested after 48 hours of exposure to 0 or 100 ng/mL LMB-2 and plated in 96-well plates that had been coated with anti-CD3 (1 μg/mL) at a cell concentration of 50×10^3 PBMCs per well. On days 3 and 5 of cell culture, [^{3}H]-thymidine incorporation was measured (see Table 2). On day 3, the untreated PBMCs seemed to have a proliferative advantage compared with the treated PBMCs; however, LMB-2–treated and –untreated PBMCs had proliferated equivalently by day 5 of culture. It is unclear whether the recovery of day 5 LMB-2–treated PBMCs reflects a continued growth of non-CD25+ PBMCs or the efficient incorporation of [^{3}H]-thymidine by remnant CD25+ cells not eliminated by LMB-2.

To examine the ability of surviving PBMCs to respond to peptide stimulation, LMB-2–treated and –untreated PBMCs were cultured with relevant (Flu_{58-66}), irrelevant ($gp100_{280-288\,(288V)}$), or no soluble peptide for 10 days. The cells were then washed and plated with T2 cells pulsed with and without each peptide overnight, and IFNγ secretion in the supernatant was measured (see Table 3). LMB-2–treated PBMCs responded to relevant peptide stimulation to a similar extent as the untreated PBMCs, suggesting that LMB-2 had no deleterious effects on the remaining CD4+ and CD8+ non-CD25+ cells.

DISCUSSION

Murine models have demonstrated that that elimination of T_{reg} cells can augment the antitumor efficacy of the adoptive transfer of antitumor T cells.[9,22] Conversely, the addition of T_{reg} cells can inhibit the antitumor impact of cell transfer.[9] In humans, adoptive immunotherapy strategies that involve a nonmyeloablative lymphodepleting chemotherapy regimen before cell transfer induce an objective response rate of approximately 50% in patients with metastatic melanoma.[23,24] One plausible explanation for the success of this treatment lies in the elimination of host T_{reg} cells by lymphodepletion before cell infusion. A host homeostatic environment devoid of T_{reg} cells and competing cells can influence the efficacy of tumor vaccination as well. Mice immunized with tumors in the absence of $CD4^+CD25^+$ cells generated immunity against these tumors.[25] Further, tumor-specific T cells preferentially expanded in the lymphopenic environment after a melanoma vaccine was administered to RAG1-deficient mice reconstituted with naive T cells from normal mice.[26] The fact that simply removing T_{reg} cells can allow effector T cells to display their innate antitumor capabilities suggests that T_{reg} cells may paradoxically protect tumors from the host by inhibiting effector T cells from recognizing or responding to the antigenic stimulus. Because many cancer antigens are derived from nonmutated self-proteins,[27] T_{reg} cells that may be playing a role in protection from autoimmunity may also be inhibiting antitumor responses.

The ability to surmount peripheral self-tolerance may be important to mediate in vivo antitumor responses in cancer patients. Overcoming the immunoregulatory influences of T_{reg} cells in vivo requires clinical-grade reagents with specific targeting and elimination of T_{reg} cells.[19] Recombinant immunotoxins and cytotoxins have been shown to deliver a cytotoxic signal targeted by the specificity of mAbs or growth factor ligands, respectively.[28] In phase I trials, immunotoxins and cytotoxins have demonstrated clinical effectiveness against malignancies expressing CD22, CD122, granulocyte macrophage colony-stimulating factor receptor (GM-CSFR), IL-13 receptor, IL-2 receptor, and CD25.[10] Because CD25, CTLA-4, and GITR represent cell surface markers of resting T_{reg} cells to which clinical reagents can be targeted, we have initiated the evaluation of clinical-grade reagents that target these molecules on T_{reg} cells.

We have recently treated patients with a fusion protein, Denileukin Diftitox (ONTAK), which enzymatically links the active portion of the diphtheria toxin to IL-2.[29] Because T_{reg} cells constitutively express the high-affinity α-chain CD25, it was hypothesized that treatment with Denileukin Diftitox would specifically expose these resting CD25-expressing cells to the potent toxicities of diphtheria toxin. Thirteen patients with metastatic cancer (12 with melanoma, 1 with renal cell carcinoma) were treated with the approved low dose (9 μg/kg) or high dose (18 μg/kg) of Denileukin Diftitox without any regression of cancer or impact on T_{reg} cells.[30] In some patients, the frequency of $CD4^+CD25^+$ cells increased, as did normalized levels of *Foxp3* expression. In addition, there was no evidence that the suppressor function of posttreatment T_{reg} cells had decreased compared with pretreatment T_{reg} cells. Because IL-2 is a homeostatic cytokine for T_{reg} cells,[31] the inability of Denileukin Diftitox to eliminate T_{reg} cells may result from the ligand triggering effect of the IL-2 portion of the fusion protein. Indeed, in previous studies, treatment of IL-2 receptor-positive T cells with IL-2–based toxin in vitro initially mimicked the stimulatory effects of IL-2 on gene transcription and DNA synthesis, yet concomitant inhibition of protein synthesis was evident.[32,33] Based on our efforts with Denileukin Diftitox, we hypothesized that an antibody rather than a stimulating ligand may be a more suitable means to target $CD25^+$ cells.

Whereas the anti-Tac mAb can target $CD25^+$ alloreactive and malignant lymphocytes in vivo, the long half-life of the antibody in vivo (2–3 weeks) prevents its application in cancer immunotherapy, where developing activated antitumor T cells may also express CD25 and be

destroyed.[34] In a phase I trial of LMB-2 administration, the median peak plasma level of LMB-2 was approximately 600 ng/mL, with a mean half-life of 4.5 hours at the maximum tolerated dose.[15] The short half-life of the LMB-2 immunotoxin in vivo provides the possibility of eliminating T_{reg} cells and then administering agents such as IL-2 or cancer antigen vaccines once the immunotoxin has been cleared.[15]

In the current study, we have shown that treatment with the LMB-2 immunotoxin targeting the CD25 molecule on T_{reg} cells can selectively eliminate T_{reg} cells in vitro without impairing the function of the remaining cells. An 8.5-fold reduction in CD25-expressing CD4^{+} T cells and a 3-fold reduction in *Foxp3* mRNA expression by enriched CD4^{+} T cells were seen after 48 hours of LMB-2 incubation compared with LMB-9 control. The disconnect between CD25 and *Foxp3* reduction levels may reflect the coelimination of a small fraction of activated CD25-expressing CD4^{+} T cells that do not express *Foxp3*, although selective survival of CD25^{+} cells with higher *Foxp3* levels is also possible. Indeed, the depletion of T_{reg} cells is not complete based on the evaluation of CD25 and the surrogate marker *Foxp3*. Given that LMB-2 can reduce T_{reg} cells in vitro, however, LBM-2 administration may provide meaningful improvement to current immunotherapeutic approaches, particularly with repeated dosing. These studies have provided the preclinical framework for the design of clinical trials to evaluate the ability of LMB-2 to eliminate T_{reg} cells in vivo in humans.

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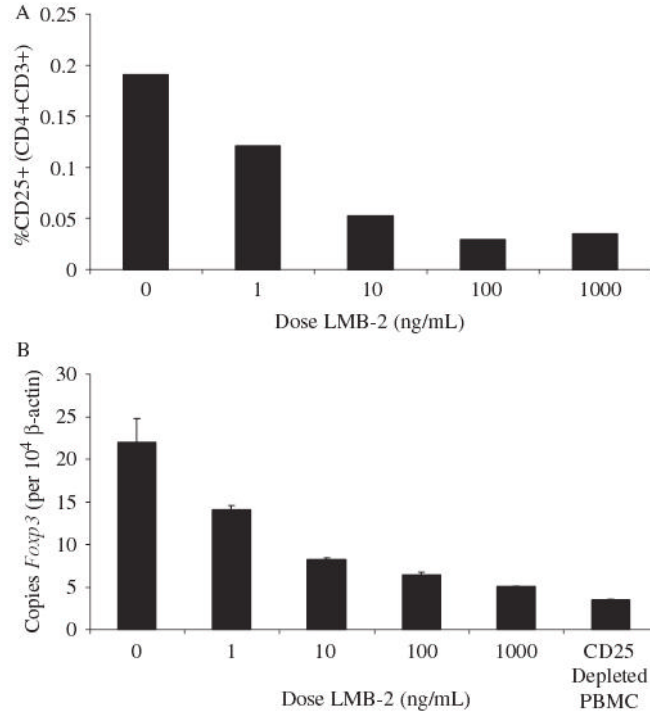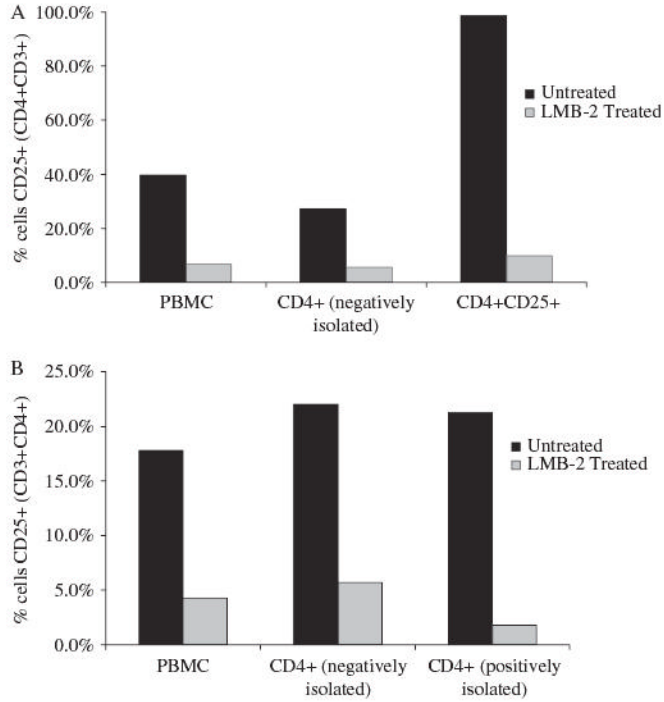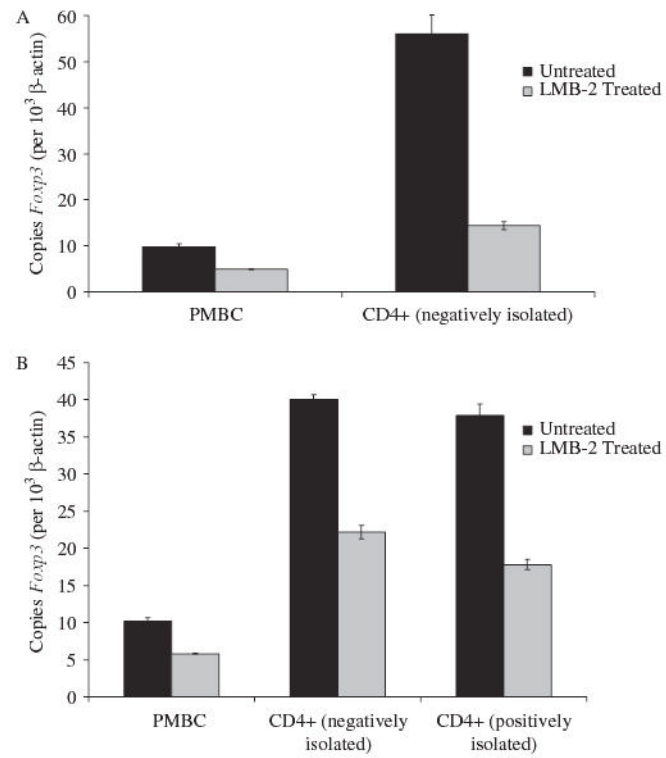
FIGURE 1.
Varying doses of LMB-2 were incubated with resting human PBMCs for 48 hours, and the percentages of residual CD4$^+$CD25$^+$ cells (top) and the copies of *Foxp3* mRNA per 10^4 copies of β-actin mRNA (bottom) were evaluated. A dose-related reduction in these 2 surrogate markers of human T$_{reg}$ cells was seen.
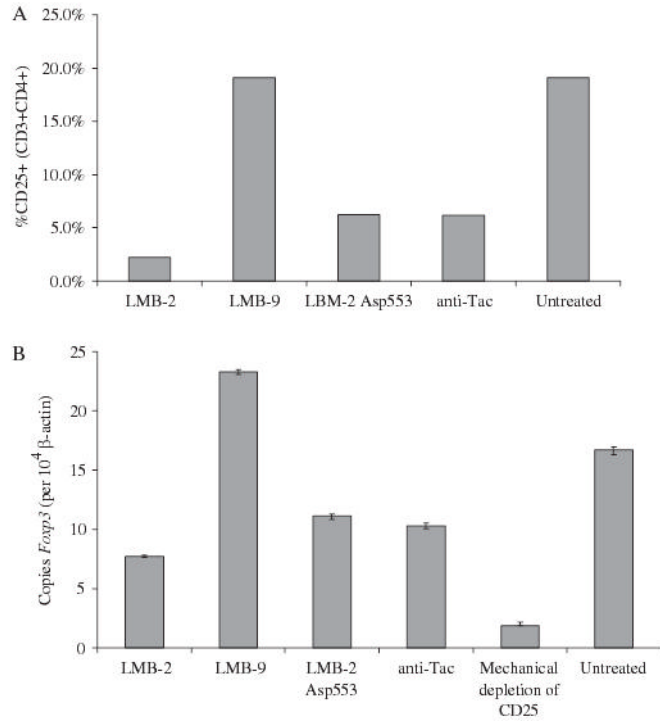
FIGURE 2.
Resting human PBMCs were incubated in vitro for 48 hours with 100 ng/mL of LMB-2, LMB-9 (an immunotoxin that recognized the Lewis Y antigen not expressed on PBMCs), LMB-2^{Asp553} (anti-CD25 fused to an inactivating mutation in the *Pseudomonas* exotoxin), or the intact anti-Tac mAb. Some decrease in the percentage of CD4$^+$ CD25$^+$ cells and/or *Foxp3*-expressing cells was seen with LMB-9, LMB-2^{Asp553}, and anti-Tac. A greater decrease was seen in PBMCs treated with LMB-2, although it did not reach the level of *Foxp3* depletion achieved by ex vivo mechanical depletion using anti-CD25 magnetic leads.

FIGURE 3.
Resting human PBMCs were incubated with LMB-2 for 48 hours, and the percentage of CD4$^+$CD25$^+$ cells remaining was evaluated by fluorescence-activated cell sorter analysis. A significant decrease in CD25-expressing cells was seen as a result of LMB-2 treatment in whole PBMCs and negatively isolated purified CD4$^+$CD25$^+$ cells (top). A similar decrease was seen in a second experiment testing negatively and positively isolated CD4$^+$ cells (bottom).

FIGURE 4.
Same cells as in Figure 3. LMB-2 treatment also reduced *Foxp3* levels in PBMCs and in purified CD4^{+} cells.

TABLE 1

LMB-2 Mediated Reduction of CD25 Expressing Cells in Human PBMC

	Patient 1		Patient 2	
	Untreated	LMB-2	Untreated	LMB-2
Fresh PBMC	1500	1500	300	300
48 hour PBMC	1305	1190	230	278
Isolated CD4$^+$	160	137	38.4	20.4
Isolated CD4$^+$ CD25$^-$	106	104	9.9	12.6
Isolated CD4$^+$ CD25$^+$	5.1	0.067	0.15	<0.002
Reduction in CD4$^+$ CD25$^+$ (%)	98.7%	98.7%		

Fresh patient pheresis samples (PBMC) were cultured in T175 flasks at 4×10^6 cells/mL in CM with or without LMB-2 (100 ng/mL). After 48 hours, PBMC were counted and mechanically sorted into CD4$^+$ fractions by negative magnetic selection. Subsequently, CD4$^+$ enriched cells were counted and separated into CD25$^+$ and CD25$^-$ fractions and enumerated. Upper values represent the absolute number ($\times 10^{-6}$) of treated or untreated PBMC before and after subset separations. Percent LMB-2 mediated reduction in CD4$^+$ CD25$^+$ cell number was calculated as $1 -$ (LMB-2 treated CD4$^+$ CD25$^+$ count $\div$ untreated CD4$^+$ CD25$^+$ count).

TABLE 2

Proliferation of LMB-2 Treated PBMC

Age of Culture	Pre-Treatment	
	Untreated	LMB-2
Day 3	73.7 ± 1.2	48.6 ± 2.3
Day 5	74.0 ± 1.2	75.3 ± 1.2

PBMC, pretreated with 0 or 100 ng/mL LMB-2 for 48 hours, were plated at 5×10^4 cells per well in 96-well plates precoated with anti-CD3 (1 µg/mL).

Cells were cultured, in the presence of 1 µCi [^{3}H]-thymidine for the final 18 hours, for 3 and 5 days and harvested onto nylon filters for assessment of proliferation by betacount. Values indicate the mean counts per minute (CPM) of 24 cultures ± SEM per condition.

TABLE 3

The Function of Effector Cell Precursors Is not Affected by LMB-2 Exposure

| | Pre-Treatment | | | | | |
| | Untreated | | | LMB-2 | | |
Stimulation	None	g280	Flu	None	g280	Flu
Flu/T2	104	42	**799**	178	174	**626**
g280/T2	76	31	54	115	107	174
T2 alone	155	112	129	231	210	326
None	119	121	131	102	94	147

PBMC were pretreated with 0 or 100 ng/mL LMB-2 for 48 hours, washed and cultured for 10 days at 3×10^6 cells per well in 24-well plates with IL-2 (50 CU/mL) alone or with 1 μM soluble peptide (Flu: Flu_{58-66}, GILGFVFTL; gp100:$gp100_{280-288(288V)}$, YLEPGPVTV). After 10 days, cells were washed, and co-cultured overnight with an equivalent number (1×10^5) of T2 cells alone or pulsed with 1 μM of the indicated peptide. Values indicate the concentration of gamma-interferon (pg/mL) secreted in response to stimulation as measured by standard ELISA. Values in bold denote concentrations twice background and ≥ 200 pg/mL.