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Enhanced efficacy of mesothelin-targeted immunotoxin LMB-100 and anti-PD-1 antibody in patients with mesothelioma and mouse tumor models

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Abstract

LMB-100 is an immunotoxin targeting the cell surface protein mesothelin, which is highly expressed in many cancers including mesothelioma. Having observed that patients receiving pembrolizumab off protocol after LMB-100 treatment had increased tumor responses; we characterized these responses and developed animal models to study whether LMB-100 made

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Author contributions: Q.J., I.P., and R.H. designed the project. Q.J. performed all experiments. R.H. performed clinical trials. A.G., I.M., A.T., and C.A. assisted with clinical trials. M.A.A. performed analysis of PET imaging. S.M.S. performed statistical analysis. D.R., J.Z., and B.M. assisted with laboratory experiments. Q.J., A.G., M.S., I.P., and R.H. analyzed the data. Q.J., A.G., and R.H. wrote the paper.

Competing interests: I.P. holds patents related to immunotoxins that are assigned to the NIH. U.S. patent title “Anti-Mesothelin Antibodies Having High Binding Affinity” and number #7,081,518. The other authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

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tumors more responsive to antibodies blocking programmed cell death protein 1 (PD-1). The overall objective tumor response in the 10 patients who received PD-1 inhibitor (pembrolizumab, 9; nivolumab, 1) after progression on LMB-100 was 40%, and the median overall survival was 11.9 months. Of the seven evaluable patients, four had objective tumor responses, including one complete response and three partial responses, and the overall survival for these patients was 39.0+, 27.7, 32.6+, and 13.8 months. When stratified with regard to programmed death ligand 1 (PD-L1) expression, four of five patients with tumor PD-L1 expression had objective tumor response. Patients with positive tumor PD-L1 expression also had increased progression-free survival (11.3 versus 2.1 months, $P = 0.0018$) compared with those lacking PD-L1 expression. There was no statistically significant difference in overall survival (27.7 versus 6.8 months, $P = 0.1$). LMB-100 caused a systemic inflammatory response and recruitment of CD8⁺ T cells in patients' tumors. The enhanced antitumor effects with LMB-100 plus anti-PD-1 antibody were also observed in a human peripheral blood mononuclear cell-engrafted mesothelioma mouse model and a human mesothelin-expressing syngeneic lung adenocarcinoma mouse model. LMB-100 plus pembrolizumab is now being evaluated in a prospective clinical trial for patients with mesothelioma.

INTRODUCTION

Immune checkpoint inhibitors including cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed death ligand 1 (PD-L1) blocking antibodies have shown antitumor activity in patients with cancer and are approved for many solid tumors, including advanced melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma, and bladder cancer (1, 2). Despite this, a large proportion of patients do not respond to these therapies (3, 4) because of multiple factors, including insufficient infiltration of activated CD8⁺ T cells into the tumor (5). A major focus of many laboratory and clinical studies is to improve the effectiveness of immune checkpoint inhibitors by combining them with agents that could make tumors more sensitive to these therapies.

Malignant mesothelioma is a rare cancer with poor prognosis, and patients have limited treatment options after frontline chemotherapy with pemetrexed and cisplatin (6). Although immune checkpoint inhibitors are not approved by the U.S. Food and Drug Administration (FDA) for treatment of mesothelioma, several clinical trials of these agents have demonstrated a response rate of 9 to 20% in this disease (7–9). Other clinical trials are evaluating mesothelin-targeted therapies in patients with mesothelioma (10). Mesothelin, a glycosylphosphatidylinositol-anchored membrane glycoprotein, is highly expressed in many malignancies, including mesothelioma, NSCLC, pancreatic cancer, and ovarian cancer (11, 12), while only present in a very restricted manner on normal adult tissues, such as the mesothelial lining of the pleura, peritoneum, and pericardium (13). This expression pattern makes mesothelin an attractive target antigen, resulting in clinical trials of a variety of therapeutics targeting mesothelin, including antibody-based therapies, vaccines, and chimeric antigen receptor (CAR) T cells (14, 15).

LMB-100 is a next-generation recombinant immunotoxin consisting of a humanized anti-mesothelin Fab fused to a 24-kDa truncated *Pseudomonas* exotoxin A (PE) fragment

with mutations that suppress B and T cell epitopes to reduce the immunity against the immunotoxin (16, 17). PE is specifically delivered into mesothelin-positive cancer cells after binding to mesothelin. The toxin portion reaches the cytosol, where it ADP (adenosine diphosphate)–ribosylates elongation factor 2, arrests protein synthesis, and induces immunogenic cell death (18). This mechanism of cell killing differs from that of currently approved chemotherapies or targeted agents used to treat solid tumors (19). The antitumor efficacy of LMB-100 has been demonstrated in several mesothelin-expressing tumor models, including mesothelioma patient–derived xenograft (PDX) models (20). A phase 1 clinical trial has established the safety of LMB-100 in patients ([ClinicalTrials.gov NCT02798536](https://clinicaltrials.gov/ct2/show/study/NCT02798536)).

During the clinical trial of LMB-100, we noted that some patients with mesothelioma treated with the immunotoxin who subsequently received pembrolizumab had major and long-lasting tumor regressions. Here, we have described these clinical responses in detail and sought to understand their mechanistic basis by evaluating changes in patients' tumors after LMB-100 therapy. We also conducted preclinical studies to validate the clinical observations. Because the human mesothelin (hMSLN)–expressing syngeneic mouse tumor model we developed previously is sensitive to CTLA-4 blockade but not to PD-1/PD-L1 blockade (21, 22), we generated additional animal models to characterize the antitumor efficacy of LMB-100 plus anti–PD-1 antibodies.

RESULTS

Characteristics of patients who received pembrolizumab or nivolumab after LMB-100 treatment

Twenty-one patients with malignant mesothelioma were treated with LMB-100 at the National Cancer Institute (NCI) on a phase 1 study to determine the maximum tolerated dose (MTD) and safety of LMB-100 as a single agent ($n = 10$) or in combination with nab-paclitaxel ($n = 11$) ([ClinicalTrials.gov NCT02798536](https://clinicaltrials.gov/ct2/show/study/NCT02798536)) from July 2016 to April 2018. Nine of these patients subsequently went on to receive immunotherapy off protocol with pembrolizumab, and one patient received nivolumab at the time of disease progression. Of these 10 patients, 7 were evaluable for treatment response, because 2 patients died before restaging scans due to rapid disease progression, and for 1 patient treated with nivolumab by an outside oncologist, posttreatment scans were not available. The baseline demographic and disease characteristics of these 10 patients are listed (Table 1). Six patients had pleural and four had peritoneal mesothelioma. Nine patients had epithelioid subtype, and one had biphasic (epithelial plus sarcomatoid) tumor. All patients had received prior platinum plus pemetrexed combination chemotherapy but were immune checkpoint therapy naïve. Cell surface expression of mesothelin on the tumor cells was present in all cases as determined by immunohistochemistry (IHC) analysis (Table 1). Tumor PD-L1 expression (percentage of tumor cells with membranous positivity) by IHC was 1 to 20% in five patients and absent in the other five patients. Of the 10 patients, 3 received LMB-100 immunotoxin alone, and the others received LMB-100 in combination with nab-paclitaxel as per protocol. All 10 patients started either pembrolizumab or nivolumab within 1 month after progressing on LMB-100,

but the time interval from the last dose of LMB-100 to the start of pembrolizumab ranged from 0.7 to 9.3 months.

Antitumor activity of pembrolizumab or nivolumab in patients pretreated with LMB-100

Out of seven evaluable patients, one had complete response, three had partial responses (PRs), one had stable disease (SD), and two had progressive disease (PD) after pembrolizumab treatment (Fig. 1A). The overall response rate for all patients (seven evaluable and three nonevaluable) was 40% (4 of 10). The median progression-free survival (PFS) on immune checkpoint inhibitor (pembrolizumab or nivolumab) was 3.3 months [95% confidence interval (CI), 1.1 to 11.3 months] (Fig. 1B), and the median overall survival (OS) from the start of LMB-100 was 11.9 months (95% CI, 2.5 months to not estimable) (Fig. 1C). Median PFS on LMB-100 was 3.2 months (95% CI, 1.3 to 5.5 months) (fig. S1). Four of the five patients with positive tumor PD-L1 expression had objective tumor response (Table 1), and the median PFS was significantly prolonged in patients whose tumors were PD-L1 positive versus those with PD-L1-negative tumors [11.3 months (95% CI, 3.4 months to not estimable) versus 2.1 months (95% CI, 1.1 to 3.2 months); $P = 0.0018$] (Fig. 1D). The median OS was not significantly different in patients with positive tumor PD-L1 expression versus those without [27.7 months (95% CI, 9.8 to not estimable) versus 6.8 months (95% CI, 2.5 months to not estimable); $P = 0.1$] (Fig. 1E). Although limited by the small number of patients, these results demonstrate a high response rate and longer PFS and OS than would be expected with pembrolizumab alone in PD-L1-positive patients. These results also suggest that basal tumor PD-L1 expression may be an important determinant of antitumor efficacy of LMB-100 plus anti-PD-1 therapy.

Representative positron emission tomography (PET) and computed tomography (CT) images of the four patients with extensive tumor burden before and at different time points after pembrolizumab treatment are shown in Fig. 1 (F to I), indicating response to PD-1 checkpoint inhibitor. Patient P02 achieved a PR at 4.4 months after start of pembrolizumab and progressed after 11.4 months of treatment. Patient P05 also obtained a PR after 4.4 months on pembrolizumab and is disease free as of the last follow-up at 39 months after initiation of LMB-100 treatment. P12, with widely metastatic peritoneal mesothelioma, obtained a PR after 3.9 months on pembrolizumab and had disease progression at 11.2 months from the start of therapy. This patient is alive at 32.6 months from the start of LMB-100. Patient P15, with extensive pleural mesothelioma, had a PR at 2.2 months and disease progression at 8.7 months.

Treatment with LMB-100 results in systemic inflammatory response and recruitment of CD8⁺ T cells in tumors of some patients with mesothelioma

To understand whether treatment with LMB-100 could make patient tumors more sensitive to pembrolizumab, we studied the ability of LMB-100 to induce a systemic inflammatory response and its effect on immune cell populations within the tumor. To do so, we analyzed the C-reactive protein (CRP) concentrations ($n = 7$ patients), the cytokine profile ($n = 10$ patients), and the gene expression in tumor biopsies of patients ($n = 6$) who were treated on the phase 1 clinical trial with LMB-100 alone (table S1). As shown in Fig. 2A, the concentration of CRP, an acute-phase reactant, in the serum increased substantially in

six of seven patients on cycle 1 day 5 after two doses of LMB-100 ($P = 0.031$). After completion of LMB-100 therapy, there was a gradual decrease in CRP to baseline values, and a representative example is shown in Fig. 2B. Next, we analyzed the cytokine profile of the 10 patients before and after LMB-100 treatment. Interferon- γ (IFN- γ) concentrations increased within 6 hours of LMB-100 treatment in five of eight patients with detectable baseline values. In addition, proinflammatory cytokines interleukin-8 (IL-8), IL-6, and monocyte chemoattractant protein-1 (MCP-1) were increased in 9 of 10 patients, and IL-18 was increased in 7 of 10 patients on day 5 after two doses of LMB-100 treatment on days 1 and 3 (Fig. 2C). Together, these results show that treatment with LMB-100 results in a systemic inflammatory response.

We used NanoString nCounter Gene Expression Assay to detect cancer immune-related gene expression in pre- and posttreatment tumor biopsies of six patients with mesothelioma treated with LMB-100 (before they received pembrolizumab). Because lymphocyte infiltration is a key factor for effective anti-PD-1 and anti-PD-L1 therapy (23), we evaluated different cell type signatures using NanoString Advanced Analysis (24), which included CD45, T helper 1 (T_H1) cells, CD8⁺ T cells, exhausted CD8⁺ cells, dendritic cells (DCs), and macrophages. Treatment with LMB-100 increased scores of CD45, CD8⁺ T cells, exhausted CD8⁺, DCs, and macrophages in four of six patients, and T_H1 cell score in three of six patients (Fig. 2D). However, only the increase in exhausted CD8⁺ cell signature (gene set includes *LAG3*, *CD244*, *EOMES*, and *PTGER4*) was statistically significant ($P = 0.031$). Natural killer cell gene signature score was very low in most of the biopsies and was automatically excluded by the NanoString Advanced Analysis. With the cell type signature correlation analysis, we observed that cytotoxic cell-related gene expression was correlated with CD8⁺ T cells' signature gene expression (fig. S2A), suggesting that CD8⁺ T cells were major antitumor mediators in these patients.

We next analyzed the expression of the antitumor immunity-related genes that have been used to predict efficacy of PD-1 inhibitors (25). These include checkpoint markers, T cell markers, cytolytic activity markers, antigen presentation markers, antitumor inflammation chemokines, and tumor markers. The expression of selected genes in pre- and post-LMB-100 patient biopsies is shown as fold change of normalized gene expression (as log₂ RNA counts per 100 ng of total RNA) in fig. S2B. Most antitumor immunity-related genes were up-regulated in P04, P05, and P10 patient biopsies after LMB-100 treatment, indicating that they might benefit from pembrolizumab. In all six patients, T cell-recruiting chemokine *CCL21* was up-regulated after LMB-100 treatment, and *CX3CL1* (Fractalkine), a potent chemoattractant for PD-1 therapy-responsive CD8⁺ T cells, was up-regulated in five of six patients after LMB-100. We observed a marked decrease in *CXCL1* expression in four of six LMB-100-treated patient biopsies. A recent study showed that CXCL1 is a determinant of the non-T cell-inflamed microenvironment, and ablation of CXCL1 promoted T cell infiltration and sensitivity to immunotherapy (26). Together, the above results suggest that LMB-100 treatment triggered immune infiltration in some patient tumors.

Increased antitumor efficacy of LMB-100 plus anti-PD-1 antibody in a humanized mouse model using tumor cells from a patient with complete response to this combination

To confirm the enhanced efficacy of combination treatment with LMB-100 plus anti-PD-1 antibody, we established a humanized mesothelioma xenograft tumor model using NCI-Meso63 tumor cells derived from patient P05 and human peripheral blood mononuclear cells (hPBMCs) from an HLA (human leukocyte antigen)-A2 matched healthy donor (Fig. 3A). We confirmed hMSLN expression by flow cytometry and PD-L1 up-regulation in response to IFN- γ stimulation in NCI-Meso63 cells (Fig. 3B). NCI-Meso63 cells were sensitive to LMB-100 with a median inhibitory concentration (IC₅₀) of 13.5 ng/ml (Fig. 3C). NCI-Meso63 tumor cells were subcutaneously injected into NSG mice. When these tumors reached an average tumor volume of 90 mm³, hPBMCs were intravenously injected. We confirmed the successful engraftment of human T cells by flow cytometry using blood collected from NCI-Meso63-bearing humanized mice on days 5, 10, and 15 after hPBMC injection (Fig. 3D). The human immune cell (hCD45⁺mCD45⁻) percentage was increased from 0.49% on day 5 to 11.1% on day 10 (composed of 61.9% CD4⁺ and 17.3% CD8⁺ T cells) and to 31.2% on day 15 (composed of 64.1% CD4⁺ and 35.8% CD8⁺ T cells). The mice were treated with LMB-100 alone, anti-PD-1 antibody alone, or the two drugs in combination 3 days after hPBMC injection. Tumor growth was measured until the mice developed graft-versus-host disease (GVHD) (about 4 weeks after hPBMC injection; symptoms included weight loss >15%, hunched posture, fur loss, reduced mobility, and tachypnea). The median tumor volume was 350, 306, 248, 216, and 135 mm³ in untreated, hPBMC-transplanted, LMB-100-treated, hPBMC plus anti-PD-1-treated, and hPBMC plus LMB-100 and anti-PD-1 combination-treated groups, respectively, on day 41 after tumor inoculation (day 21 after start of treatments) ($P < 0.01$) (Fig. 3E). These results show that in this model, tumor growth in the mice treated with LMB-100 and anti-PD-1 antibody was decreased compared with that in mice treated with either agent alone.

Establishing a syngeneic immunocompetent mouse model (531LN2-hMSLN) to investigate LMB-100 plus anti-PD-1 antibody efficacy

Because the development of GVHD in the PBMC-humanized mouse model limits assessment of duration of antitumor efficacy, we developed a syngeneic immunocompetent mouse model to study the treatment efficacy for a longer period of time as well as evaluate the mechanism of antitumor efficacy. Because LMB-100 specifically targets hMSLN, we established an hMSLN-expressing immunocompetent syngeneic mouse tumor model. We stably transfected the PD-L1-positive mouse lung adenocarcinoma cell line 531LN2 (27) with an hMSLN-expressing vector encoding the membrane-bound fragment of hMSLN to generate 531LN2-hMSLN cells. As shown in Fig. 4, we confirmed a human *MSLN* mRNA expression by reverse transcription polymerase chain reaction (RT-PCR) (Fig. 4A) and cell surface hMSLN protein expression by flow cytometry (Fig. 4B) in the 531LN2-hMSLN cell line. These cells were sensitive to LMB-100, whereas the control plasmid-transfected 531LN2-Ctr cells and untransfected 531LN2 cells, which lacked hMSLN, were not (Fig. 4C). Furthermore, when we treated 531LN2-hMSLN cells with BL22 (an immunotoxin against human CD22) (28) as a negative control, there was no decrease in cell viability, and IC₅₀ was not reached even at 100 ng/ml concentration (fig. S3). Our data show that the cytotoxicity of LMB-100 against 531LN2-hMSLN cells is specifically due to hMSLN

expression. In addition, hMSLN expression did not affect PD-L1 expression on 531LN2 cells (Fig. 4D). As shown in Fig. 4E, we observed similar subcutaneous tumor growth in the mice inoculated with 531LN2-hMSLN cells compared with mice inoculated with untransfected or control plasmid-transfected cells. The 531LN2-hMSLN primary tumor had similar morphologic and pathologic characteristics as lung adenocarcinoma, and hMSLN expression was detected by IHC (Fig. 4F). As shown in fig. S4A, 531LN2-hMSLN growth in vitro was also inhibited by LMB-100. In addition, repeated doses of anti-PD-1 antibody alone substantially reduced tumor growth in the 531LN2-hMSLN subcutaneous tumor model (fig. S4B), whereas anti-CTLA-4 antibody did not (fig. S4C). We also confirmed that the antitumor effect of anti-PD-1 antibody treatment in the 531LN2-hMSLN tumor model was not associated with hMSLN expression, because anti-PD-1 antibody reduced tumor growth similarly in both 531LN2-hMSLN and control 531LN2-Ctr tumor models (Fig. 4G). Similar to the recently reported findings that mesothelin-targeted immunotoxin SS1P induced immunogenic cell death (18), we also observed cell surface translocation of calreticulin (CRT), an immunogenic cell death marker, in LMB-100-treated 531LN2-hMSLN cells, including both 7-AAD⁻ live cells and 7-AAD⁺ dead cells (Fig. 4H), which supports the rationale for combining LMB-100 with immunotherapy.

LMB-100 plus anti-PD-1 therapy increases antitumor efficacy and survival of 531LN2-hMSLN tumor-bearing mice

Using the established 531LN2-hMSLN mouse model, we evaluated the antitumor efficacy of systemic administration of LMB-100 combined with anti-PD-1 antibody. Because this tumor is very sensitive to anti-PD-1 therapy, we used a lower dose of anti-PD-1 to evaluate the efficacy of the combination. LMB-100 (2.5 mg/kg) was given intravenously every other day, three doses per cycle for two cycles, with a cycle interval of 3 days. Anti-PD-1 antibody (10 mg/kg) was administered intraperitoneally twice with an interval of 4 days between the two doses. In mice bearing 531LN2-hMSLN tumors, tumor growth was inhibited by LMB-100 plus anti-PD-1 treatment compared with mice treated with either drug alone. The median tumor volume was 865, 420, 277, and 65 mm³ in untreated, LMB-100-treated, anti-PD-1-treated, and combination-treated groups, respectively, on day 34 after tumor inoculation ($P < 0.001$) (Fig. 5A). The median OS was 38 days without treatment, 52 days with either LMB-100 or anti-PD-1 antibody alone, and 74 days with the combination ($P < 0.05$) (Fig. 5B). In contrast, LMB-100 had no additional antitumor effect when combined with anti-PD-1 antibody against hMSLN-negative 531LN2 parental tumors (fig. S5). In addition, LMB-92, a recombinant immunotoxin with similar structure to LMB-100 but targeting the B cell maturation antigen, as a negative control for LMB-100, had no increased antitumor activity when combined with anti-PD-1 antibody against 531LN2-hMSLN tumors (fig. S6). These results demonstrate that the increased antitumor efficacy of LMB-100 combined with anti-PD-1 antibody is due to specific targeting of mesothelin-expressing tumor cells by LMB-100.

LMB-100 plus anti-PD-1 treatment results in increased CD8⁺ T cells in 531LN2-hMSLN tumors

To understand the immune responses induced by LMB-100 plus anti-PD-1 antibody treatments, we used the NanoString technique to analyze cancer immune-related gene

expression in 531LN2-hMSLN tumors and calculated the scores for different cell types and tumor immune response pathways (Fig. 6A). Although the number of mice in each group was small, the cell type gene signatures including CD8⁺ T cells, DCs, total T cells, CD45⁺ cells, and neutrophils were up-regulated in LMB-100 plus anti-PD-1-treated tumors. The cell cycle pathway was substantially inhibited, and apoptosis signature was increased in combination-treated tumors, suggesting that the combination treatment inhibited tumor proliferation and induced cell apoptosis. T cell function score as well as the antigen presentation-related pathways including macrophage and DC functions, antigen processing, and major histocompatibility complex scores were increased in combination-treated tumors compared with tumor treated with anti-PD-1 antibody alone. Among checkpoint biomarkers related to T cell function, we observed a reduction in indoleamine 2,3-dioxygenase 1 (IDO1) in LMB-100 plus anti-PD-1 antibody-treated tumors but not in tumors treated with LMB-100 or anti-PD-1 antibody alone (fig. S7A), suggesting that IDO1-fostered immunosuppressive tumor microenvironment was decreased by the combination therapy. There was no significant difference in expression of other checkpoint markers after treatment with the combination or anti-PD-1 antibody alone. LMB-100 alone did not change checkpoint marker expression in 531LN2-hMSLN mouse tumors as it did in some patient biopsies. Anti-PD-1 antibody alone up-regulated *CD274*, *PDCD1LG2*, and *CTLA4* expression compared with the untreated group.

To better understand how LMB-100 plus anti-PD-1 treatment regulated systemic and local tumor immune responses, we used flow cytometry to detect CD4⁺ T cells and CD8⁺ T cells in the spleens and tumors of untreated, LMB-100, anti-PD-1 monoclonal antibody (mAb), and combination-treated mice. In the spleen, both anti-PD-1 alone and combination increased CD4⁺ T cells compared with the untreated group, whereas only the combination increased CD8⁺ T cells (6.6×10^6) compared with the anti-PD-1 group (3.7×10^6) ($P < 0.05$) (Fig. 6B). In the tumor, LMB-100 plus anti-PD-1 combination increased CD8⁺ T cells (Fig. 6C) compared with all other groups. The median CD8⁺ T cell number per gram tumor weight was 1.7×10^6 , 2.1×10^6 , 2.2×10^6 , and 4.6×10^6 in untreated, LMB-100-treated, anti-PD-1-treated, and combination-treated groups ($P < 0.05$). LMB-100 plus anti-PD-1 treatment slightly increased CD4⁺ T cells in tumors compared with the untreated group but without significant difference between the combination treatment and LMB-100 or anti-PD-1 mAb alone. Among the checkpoint markers on tumor T cells, combination treatment decreased CTLA-4, PD-1, and Tim-3 on CD8⁺ T cells, as well as CTLA-4 on CD4⁺ T cells, compared with the untreated group (fig. S7, B and C), which suggests that the CD8⁺ T cells are more activated in combination-treated tumors. Anti-PD-1 decreased PD-1 but increased CTLA-4 on CD8⁺ T cells, which is consistent with the observation that *CTLA4* expression was up-regulated in anti-PD-1-treated 531LN2-hMSLN tumors (fig. S7A).

The antitumor efficacy of LMB-100 plus anti-PD-1 is CD8⁺ T cell dependent

To further investigate the role of CD8⁺ T cells in the antitumor effects induced by LMB-100 plus anti-PD-1 antibody, we depleted CD8⁺ T cells through intraperitoneal injection with the anti-CD8⁺ T cell mAb (clone 2.43) given 1 day before LMB-100 plus anti-PD-1 treatment and then twice a week for 2 weeks. We found that treating with the anti-CD8⁺ T cell antibody significantly diminished the antitumor effects of LMB-100 plus anti-PD-1

antibody but had no effect in the untreated group ($P < 0.001$) (Fig. 7A). The median tumor volumes on day 34 after tumor inoculation was 1110, 1095, 122, and 560 mm³ (Fig. 7A), and the median OS was 36, 38, 75, and 48 days in untreated isotype control, untreated CD8⁺-depleted, isotype control treated with LMB-100 plus anti-PD-1 antibody, and CD8⁺ depleted with LMB-100 plus anti-PD-1 groups, respectively (Fig. 7B). These results indicate that CD8⁺ T cells play an important role in antitumor efficacy of combination treatment with LMB-100 plus anti-PD-1 antibody.

DISCUSSION

On the basis of our clinical observation of tumor regressions in patients with treatment-refractory mesothelioma who received pembrolizumab after treatment with the anti-mesothelin immunotoxin LMB-100, we studied the clinical responses in these patients and investigated the immune changes in patients' tumors due to LMB-100. In addition, we developed preclinical models to further understand the potential antitumor efficacy of LMB-100 and anti-PD-1 therapy. In these previously treated patients with progressive mesothelioma, the overall response rate was 40%, and the median OS was 11.9 months. LMB-100 treatment in patients induced inflammatory response both systemically as well as in the tumor. Using humanized and syngeneic mouse tumor models, we showed that the combination treatment with LMB-100 plus anti-PD-1 antibody resulted in greater antitumor activity that was mediated by increased intratumoral CD8⁺ T cell infiltration.

LMB-100 is an improved anti-mesothelin immunotoxin with reduced antigenicity compared with the first-generation molecule SS1P (29). The recently completed phase 1 clinical trial of LMB-100 ([NCT02798536](#)) in patients with advanced cancers demonstrated its safety and established its MTD [140 µg/kg, intravenously (iv) given on days 1, 3, and 5 of a 21-day cycle]. On this phase 1 study, 10 patients with mesothelioma were treated with LMB-100 alone, and 11 were treated with LMB-100 in combination with nab-paclitaxel. The latter group was added based on preclinical data showing synergy between LMB-100 plus nab-paclitaxel (20). Of these 21 patients treated with LMB-100, 9 received pembrolizumab, and 1 received nivolumab off-label as their next therapy after progression on LMB-100. The median between the last dose of LMB-100 and the start of anti-PD-1 inhibitor was 1.8 months (range of 0.7 to 9.3 months). Tumor PD-L1 expression was associated with increased tumor response (four of five patients) and increased PFS and OS. Although the sample size is small, there was a high response rate, and the median PFS of 11.3 months and median OS of 27.7 months are higher than those seen with pembrolizumab alone in patients with PD-L1-positive mesothelioma treated on the KEYNOTE-028 study (7). Whether response to LMB-100 followed by pembrolizumab requires some basal PD-L1 expression is being investigated in the ongoing phase 2 clinical trial ([ClinicalTrials.gov NCT03644550](#)), evaluating changes in the tumor immune microenvironment using multiplex immunofluorescence analysis of tumor biopsies obtained at baseline, after LMB-100 treatment, and after pembrolizumab treatment.

The improvements in PFS and OS seen in LMB-100-treated patients with PD in this study are also better than would be expected with conventional chemotherapy agents. In the frontline setting, treatment with pemetrexed and cisplatin (the only FDA-approved

therapy for this disease) results in PFS of 5.7 months and OS of 12.1 months (30). The phase 3 Mesothelioma Avastin Cisplatin Pemetrexed Study (MAPS) trial demonstrated that bevacizumab added to cisplatin and pemetrexed improved the median OS from 16.1 to 18.8 months with the combination (31). Vinorelbine, one of the more frequently used treatments in the second-line setting, results in a median PFS and OS of 2.3 and 6.2 months, respectively (32).

Of the seven patients who were evaluable for response, four had objective tumor response noted by CT scan as well as PET. As shown in Fig. 1, all patients had extensive disease burden before treatment, and one of the patients achieved a complete response that is ongoing as of the last follow-up on 31 December 2019. Because single-agent pembrolizumab by itself induces tumor shrinkage in about 20% of patients with mesothelioma and given the small number of patients in our study, the observed tumor response rate of 40% (4 of 10 patients) could be due to pembrolizumab alone; we next studied whether LMB-100 treatment could make these patients more likely to respond to anti-PD-1 therapy.

Our results show that treatment with LMB-100 alone causes systemic inflammation, as evidenced by the increase in the acute-phase reactant CRP that correlated with LMB-100 administration and normalized to baseline values after completion of LMB-100 treatment. Treatment with LMB-100 increased IFN- γ concentrations within 6 hours of a single dose of LMB-100 and increased inflammatory cytokines including IL-6, IL-8, MCP-1, and IL-18 on day 5 after LMB-100 treatment.

Using the NCI-Meso63 humanized mouse model established with tumor cells derived from the patient who achieved complete response to LMB-100 plus anti-PD-1 antibody, we confirmed the enhanced antitumor efficacy with the combination treatment. However, because these mice develop GVHD, we established the hMSLN-expressing 531LN2-hMSLN syngeneic mouse model and observed enhanced antitumor efficacy with LMB-100 plus anti-PD-1 treatment compared with either agent alone. CD8⁺ T cells and the expression of antigen presentation pathway-related genes were increased by LMB-100 plus anti-PD-1 combination treatment as compared with either agent alone. Furthermore, combining LMB-100 with anti-PD-1 antibody induced substantial infiltration and activation of CD8⁺ T cells in the tumor, which played a key role in the antitumor response, whereas depletion of CD8⁺ T cells abrogated the antitumor efficacy. Unexpectedly, using this model, we did not see an increase in immune cell populations, including T cells, in the tumors of mice treated with LMB-100 alone as was the case in patients. This could be related to the fact that the tumors were collected at earlier time points in mice than in patients and had not received sufficient doses of LMB-100 when collected for analysis.

Our results are in agreement with a recent study showing that intratumoral administration of LMB-100 plus CTLA-4 blockade eradicates murine subcutaneous tumors by promoting infiltration of activated CD8⁺ T cells into the tumor (22). Because of the limitations of the tumor model, LMB-100 could only be given intratumorally because the BALB/c transgenic mice used in that study expressed hMSLN in essential organs, resulting in toxicity when the drug was intravenously given. However, the 531LN2-hMSLN syngeneic mouse model

is suitable for systemic administration of LMB-100, similar to patients who receive the drug intravenously. Because anti-CTLA-4 treatment enhances the formation of antidrug antibodies against LMB-100 in vivo (33), whereas anti-PD-1 and anti-PD-L1 do not, LMB-100 plus PD-1 inhibitors represent a more promising combination. Moreover, because anti-CTLA-4 antibodies have very limited single-agent antitumor efficacy in mesothelioma (34), we have focused on studies of LMB-100 plus pembrolizumab in the clinic.

Despite our observation of promising efficacy of the combination of LMB-100 and PD-1 inhibitor in patients with mesothelioma and mouse models, our study has several limitations. Because this was a retrospective analysis of patients who received pembrolizumab or nivolumab after LMB-100 treatment rather than a prospective clinical trial, it could lead to patient selection bias. Also, given the small number of patients treated, it is possible that the high response rate could be due to anti-PD-1 therapy by itself. Although tumor responses were observed in patients who received LMB-100 with or without nab-paclitaxel, we cannot completely rule out any potential beneficial effect of nab-paclitaxel because it has been shown to impair regulatory T cells (35). Another limitation of our study is that although we demonstrate an increased number of CD8⁺ T cells in patient tumors, we did not quantitate tumor mutational burden or neoantigen load before and after LMB-100 treatment or immune changes in the tumor after pembrolizumab therapy. These questions are being addressed in the prospective phase 2 trial ([ClinicalTrials.gov NCT03644550](https://clinicaltrials.gov/ct2/show/study/NCT03644550)) that also includes quantitative immunofluorescence imaging to determine the quantitative changes and spatial localization of immune cells after LMB-100 and pembrolizumab treatments. In our 531LN2-hMSLN model, we observed a decrease in tumor volume and increased survival of mice treated with the combination of LMB-100 plus anti-PD-1 therapy. Because of the limitations of animal models, we tested the combination of LMB-100 plus anti-PD-1 treatment given concurrently rather than sequentially. Although this regimen was effective in mice, for our phase 2 study, we decided to continue with the sequential approach given the previous responses observed in patients. Whether concurrent administration of LMB-100 plus pembrolizumab would offer any advantage over the sequential approach would require further study in the clinic.

In conclusion, our work presents early evidence of clinical activity of mesothelin-targeting immunotoxin LMB-100 and anti-PD-1 antibody combination therapy in patients with malignant mesothelioma. Our study used mouse models to show that combining mesothelin-targeting immunotoxin LMB-100 with anti-PD-1 antibody enhanced CD8⁺ T cell-mediated antitumor responses. These coclinical studies in humans and mice indicate that combining mesothelin-targeting drugs with checkpoint inhibitor immunotherapy may be an option for patients with mesothelin-positive cancers. LMB-100 followed by pembrolizumab is now being studied in a prospective clinical trial for patients with treatment-refractory malignant mesothelioma ([ClinicalTrials.gov NCT03644550](https://clinicaltrials.gov/ct2/show/study/NCT03644550)). Clinical outcomes from this trial will help determine whether anti-mesothelin immunotoxin plus immune checkpoint blockade is a valid treatment strategy for mesothelin-positive solid tumors.

MATERIALS AND METHODS

Study design

The primary objective of this study was to determine whether combining mesothelin-targeting immunotoxin LMB-100 with PD-1 checkpoint inhibitor would increase antitumor efficacy. Ten patients with mesothelioma who were treated on a phase 1 clinical trial of LMB-100 ([ClinicalTrials.gov NCT02798536](https://clinicaltrials.gov/ct2/show/study/NCT02798536)) and then received pembrolizumab ($n = 9$) or nivolumab ($n = 1$) were retrospectively evaluated for antitumor response and OS. The study was conducted in accordance with the principles of the International Conference on Harmonisation–Good Clinical Practice guidelines. The specimens were collected on Institutional Review Board (IRB)–approved protocol, and all participants provided written informed consent for the use of specimen for research purpose. No randomization or blinding was performed. CRP and cytokine concentrations in serum or plasma and cancer immune-related gene expression were analyzed in tumors from patients with mesothelioma before and after LMB-100 treatment. We also evaluated LMB-100 plus anti-PD-1 combination efficacy in a humanized mouse model and an immunocompetent hMSLN-expressing lung adenocarcinoma syngeneic mouse model (531LN2-hMSLN). Using gene expression analysis and flow cytometry, we characterized the immune changes in 531LN2-hMSLN tumors treated with LMB-100 and anti-PD-1 antibody. Last, to better understand the role of CD8⁺ T cells in the antitumor effects of the combination, we conducted studies in 531LN2-hMSLN-bearing mice whose CD8⁺ T cells were depleted using anti-CD8⁺ T cell antibody.

Phase 1 clinical trial design and specimen collection

Patients with pleural or peritoneal mesothelioma were enrolled on an open-label, single-center phase 1 study of LMB-100 with or without nab-paclitaxel. Patients were eligible if they were 18 years or older, had histologically confirmed epithelial or biphasic mesothelioma (≥ 50% epithelial component), not amenable to potentially curative surgical resection, have had at least one prior chemotherapy regimen that included pemetrexed and cisplatin or carboplatin, and have an Eastern Cooperative Oncology Group performance status score of 0 or 1. Oversight for the protocol was provided by the National Institutes of Health (NIH) Safety Monitoring Committee and reviewed by the NCI-IRB. Informed consent was obtained from all the participants. Patients received intravenous LMB-100 on days 1, 3, and 5 of every 21-day cycle for up to four cycles as monotherapy. After the MTD of single-agent LMB-100 was established, the protocol was amended to allow treatment of patients with mesothelioma with LMB-100 plus nab-paclitaxel. In this part of the protocol, patients received LMB-100 at the same dose and schedule as described above along with nab-paclitaxel at a dose of 100 mg/m², intravenously over 30 min on days 1 and 8 of each 21-day cycle for up to two cycles. In the absence of toxicity or disease progression, these patients could receive up to four additional cycles of nab-paclitaxel alone. Patients were evaluated for response every 6 weeks or two cycles. Response and progression were assessed by the investigator based on physical examinations, CT, and PET scans. For peritoneal mesothelioma, Response Evaluation Criteria in Solid Tumors (RECIST) guideline (version 1.1) (36) was used. For pleural mesothelioma, modified RECIST for malignant pleural mesothelioma (37) was used.

For patients who were treated with LMB-100 alone, serum CRP concentrations were collected before dose on days 1, 3, 5 and 8 for each cycle. Plasma samples for cytokine detection were collected before dose on day 1 and on day 5 after LMB-100. Tumor biopsies were obtained before treatment with LMB-100 and 6 weeks after initiation of LMB-100 treatment after two cycles of LMB-100.

Administration of pembrolizumab or nivolumab after progression on LMB-100

Upon progression on the above study, nine patients received the anti-PD-1 antibody pembrolizumab, and one received nivolumab off-label either at the NIH or through their local provider. These patients had no other therapy after the completion of the LMB-100 treatment. Patients received pembrolizumab intravenously at a dose of 200 mg every 3 weeks until disease progression. Overall tumor responses and survival outcomes were assessed in these patients. Data cutoff for OS was 31 December 2019. Response evaluation varied for each patient, around every 2 to 4 months with PET-CT or CT, and response or progression was determined using modified RECIST for pleural mesothelioma and RECIST for peritoneal mesothelioma. Tumor mesothelin and PD-L1 expression was assessed retrospectively from archival or on-study tissue using IHC analysis performed by the Laboratory of Pathology at NCI.

Mouse tumor models

In the NCI-Meso63 humanized mouse model, 8×10^6 NCI-Meso63 cells were injected subcutaneously into 5- to 6-week-old female NSG mice, and then 6×10^6 PBMCs isolated from healthy donors were intravenously injected into NSG mice when average tumor size reached 90 mm^3 . Both LMB-100 and anti-PD-1 antibody (Keytruda from Merck) treatments were started 3 days after hPBMC injection. LMB-100 (2.5 mg/kg) was administered intravenously every other day, three doses per cycle for two cycles, with a cycle interval of 3 days. Anti-PD-1 antibody was intraperitoneally administered at 10 mg/kg twice a week for 2 weeks. Tumor growth was measured with caliper, and tumor volume was calculated using the formula $\text{volume} = (\text{length} \times \text{width} \times \text{width}) \times 0.4$. Survival time was defined as the time for tumors to reach a volume of 1200 mm^3 .

In the 531LN2 tumor model, 531LN2 cells were injected subcutaneously into 6- to 7-week-old female 129S2/SvPasCrl mice on day 0 at a dose of 1×10^6 for the tumor formation test and 2×10^6 cells for the treatment studies. For drug treatment, LMB-100 or LMB-92 was administered intravenously at 2.5 mg/kg every other day, three doses per cycle for two cycles, with a cycle interval of 3 days. Anti-PD-1 antibody (BioXCell, catalog no. BE0146) was intraperitoneally administered at 10 mg/kg twice a week for either 1 or 2 weeks as indicated. Anti-CTLA-4 antibody (BioXCell, catalog no. BE0131) was administered intraperitoneally at 5 mg/kg twice a week for 2 weeks in the monotherapy study. All drug treatments were started when average tumor size reached 90 mm^3 . For CD8⁺ T cell in vivo depletion study, intraperitoneal injection of 300 μg of anti-mouse CD8 antibody (BioXCell, clone 2.43, catalog no. BE0061) or rat immunoglobulin G2b (IgG2b) isotype control (BioXCell, catalog no. BE0090) was initiated 1 day before LMB-100 plus anti-PD-1 treatment and then continued twice a week for 2 weeks (depletion efficiency was 90%). Control mice received isotype control.

Statistical analysis

For the clinical study, the probability of OS was calculated using the Kaplan-Meier method, starting from the date LMB-100 began until the date of death or date last known alive. The probability of PFS was calculated using the Kaplan-Meier method, starting from the date LMB-100 or pembrolizumab began until the date of progression on that agent, with censoring at the date last known alive without progression. A Wilcoxon signed-rank test was used to determine the significance of the difference in CRP, cytokines, and cell type scores between two time points. All *P* values are two tailed and presented without adjustment for multiple comparisons. Analysis of data was done using SAS version 9.4 (SAS Institute Inc.).

For mouse studies, differences between groups were compared using unpaired Student's *t* test. Mouse survival differences were compared using log-rank (Mantel-Cox) test. Statistical analysis was performed using GraphPad Prism 6.0. *P* < 0.05 was considered statistically significant. Original data are provided in data file S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials availability:

All data associated with this study are present in the paper and/or the Supplementary Materials.

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Fig. 1. Antitumor efficacy of LMB-100 followed by pembrolizumab in patients with mesothelioma.

(A) The waterfall plot illustrates radiologic tumor response by graphing the percent change from the sum of target lesions at baseline in the seven patients treated with LMB-100 followed by pembrolizumab. Pink bars indicate patients with progressive disease (PD) as defined by 20% or greater increase in the sum of target lesions; blue bars indicate partial response (PR), defined as 30% reduction in the sum of target lesions from pretreatment scan, or complete response (CR); and white bar indicates stable disease (SD). (B to E) Kaplan-Meier plots of median PFS and median OS of patients receiving PD-1 checkpoint inhibitor (pembrolizumab or nivolumab) treatment after progressing on LMB-100 as of the last follow-up on 31 December 2019. (B) Median PFS on checkpoint inhibitor (3.3 months; 95% CI, 1.1 to 11.3 months). (C) Median OS of patients from the beginning of LMB-100 treatment [11.9 months (95% CI, 2.5 months to not estimable)]. (D) Median PFS on immune checkpoint inhibitor treatment for patients with [11.3 months (95% CI, 3.4 months to not estimable)] and without tumor PD-L1 expression [2.1 months (95% CI, 1.1 to 3.2 months); $P = 0.0018$]. (E) OS of patients with [27.7 months (95% CI, 9.8 months to not estimable)] and without tumor PD-L1 expression [6.8 months (95% CI, 2.5 months to not

estimable); $P = 0.1$]. (F to I) PET and CT scans of four patients with mesothelioma treated with LMB-100 and pembrolizumab. Representative [^{18}F] fluorodeoxyglucose PET images and axial CT scans before and at different time points after pembrolizumab treatment are shown. (F) Patient P02. Blue arrows show lateral abdominal wall improvement in maximum standardized uptake value (SUV_{max}) from 7.3 to 4.4 in conjunction with improvement of mesenteric disease throughout the abdomen. (G) Patient P05. Blue arrows show that disease along the liver improved in SUV_{max} from 15.9 to normal background activity. (H) Patient P12. Blue arrows show improvement in retrocrural mass with SUV_{max} change from 18.4 to normal background activity. Red arrows show disease that is improved in SUV_{max} but remains high at $>15 \text{ SUV}_{\text{max}}$. (I) Patient P15. Disease improvement throughout the right hemithorax with pericardiac SUV_{max} changing from 17.9 to 10.9. Representative tumor involvement on CT in (F) to (I) is indicated by red circles or “x” letters.

Fig. 2. LMB-100 alone induced immune responses in patients with mesothelioma.

(A) CRP concentrations in the serum of patients with mesothelioma on days 1 and 5 of cycle 1 of LMB-100 treatment. (B) Change in CRP concentration in patient P05 in response to LMB-100 through cycle 1. Red arrowheads indicate days when LMB-100 was administered. (C) Cytokine concentrations in plasma from patients with mesothelioma on days 1 and 5 of LMB-100 cycle 1. IFN- γ was evaluated before and 6 hours after LMB-100 treatment on day 1. (D) Cancer immune gene expression analyzed by NanoString in tumor biopsies of patients obtained before (day 1) and after (day 42) LMB-100 treatment. Different cell type

gene expression signature sets were scored using NanoString advanced analysis modules. Cell type scores are shown as $\log_2$ scale.

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Fig. 3. Combination therapy with LMB-100 and anti-PD-1 antibody in P05 patient-derived NCI-Meso63 tumor xenograft model transplanted with healthy donor PBMCs.

(A) Study design with tumor cells derived from patient P05, who achieved complete response with LMB-100 and pembrolizumab combination treatment. (B) The expression of hMSLN (red line) and PD-L1 [blue line without IFN- γ and red line with IFN- γ (100 ng/ml) stimulation for 24 hours] by NCI-Meso63 cells was detected by flow cytometry. Isotype control antibodies are shown as black lines. (C) NCI-Meso63 cells were treated in vitro with LMB-100 at different concentrations for 72 hours. The WST-8 cell proliferation assay kit was used to test cell viability. (D and E) NCI-Meso63 tumor cells were subcutaneously injected into NSG mice. hPBMCs (6×10^6) isolated from healthy donor blood were injected intravenously into tumor-bearing mice (brown arrowhead) when average tumor size reached 90 mm³. (D) Mouse blood was collected on days 5, 10, and 15 after hPBMC injection. The whole blood was stained with fluorescent dye-conjugated antibodies against human CD45, mouse CD45, human CD8a, human CD4, and human CD3. The samples were analyzed by flow cytometry. For analysis, the cells were first gated for singlets [forward scatter height (FSC-H) versus forward scatter area (FSC-A)] and then the lymphocytes [side scatter area (SSC-A) versus FSC-A]. Then, hCD45⁺mCD45⁻ cells were gated for hCD4, hCD8a, and hCD3 analysis. (E) Mice ($n = 6$ to 7 for each group) were administered LMB-100 (2.5 mg/kg, iv, every other day, three doses per cycle for two cycles with a cycle interval of 3 days) alone or with anti-PD-1 antibody [10 mg/kg, intraperitoneally (ip), twice a week for 2

weeks]. All drug treatments were started 3 days after hPBMC injection. NCI-Meso63 tumor growth was measured, and tumor volume was calculated.

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Fig. 4. Establishing the syngeneic 531LN2-hMSLN lung adenocarcinoma mouse tumor model expressing hMSLN.

(A) Mouse lung adenocarcinoma 531LN2 cells were transfected with hMSLN or control plasmids, and single-cell clones 531LN2-hMSLN and 531LN2-Ctr were generated through limiting dilution. Ctr: control plasmid-transfected cells. Human *MSLN* mRNA expression in indicated cell lines was detected by RT-PCR. (B) Cell surface hMSLN expression by 531LN2-hMSLN (red) and 531LN2-Ctr cells (gray) was detected by flow cytometry using hMSLN antibody. Open histogram: PE-rat IgG2a isotype control. Solid histogram: PE-hMSLN antibody detection. (C) 531LN2, 531LN2-hMSLN, and 531LN2-Ctr cells were treated with LMB-100 at different concentrations for 72 hours in culture, and then cell viability was detected via WST-8 cell proliferation assay kit. (D) PD-L1 and hMSLN expression on 531LN2 and 531LN2-hMSLN cells were detected by flow cytometry.

(E) 129S2/SvPasCrl mice ($n = 4$ to 6 for each group) were subcutaneously injected with 531LN2, 531LN2-Ctr, or 531LN2-hMSLN cells. Tumor growth was monitored. (F) Hematoxylin and eosin (H&E) and hMSLN IHC detection in 531LN2-hMSLN and 531LN2-Ctr subcutaneous tumors. (G) 531LN2-hMSLN- and 531LN2-Ctr-bearing mice ($n = 5$ for each group) were administered anti-PD-1 antibody (10 mg/kg, ip, twice a week for 1 week, blue arrowheads), starting when tumor reached 90 mm³. Tumor volume was measured and calculated. (H) LMB-100 induces immunogenic death of 531LN2-hMSLN cells. The 531LN2-hMSLN cells were treated with LMB-100 (100 ng/ml) for 72 hours, and immunogenic death marker CRT on the cell surface was detected by flow cytometry. Cell vital dye 7-AAD was used to analyze CRT expression in both live and dead cell gates.

Fig. 5. Combining LMB-100 and anti-PD-1 antibody enhanced antitumor effects in 531LN2-hMSLN syngeneic mouse tumor model.

(A) 531LN2-hMSLN-bearing mice ($n = 6$ to 10 for each group) were administered LMB-100 (2.5 mg/kg, iv, every other day, three doses per cycle for two cycles with a cycle interval of 3 days, blue arrowheads) or anti-PD-1 antibody (10 mg/kg, ip, twice with an interval of 4 days, magenta arrowheads) or both. All drug treatments were started when tumor size reached 90 mm³. Tumor volume was measured and calculated. (B) Survival time of mice treated with LMB-100 plus anti-PD-1 antibody and control groups was defined as the time when their tumors reached a volume of 1200 mm³, at which point the mice were euthanized.

Fig. 6. Cancer-associated immune regulation induced by LMB-100 plus anti-PD-1 antibody combination in 531LN2-hMSLN tissues.

(A) Subcutaneous tumors were harvested from mice on day 11 after the start of LMB-100 plus anti-PD-1 treatments. Cancer immune gene expression in untreated ($n = 2$), LMB-100-treated ($n = 3$), anti-PD-1 antibody-treated ($n = 3$), and LMB-100 plus anti-PD-1 antibody-treated ($n = 4$) tumors was analyzed by NanoString. Signature sets of gene expression associated with different cell types or pathways were scored with NanoString Advanced Analysis modules. z score values were calculated from cell type or pathway scores ($\log_2$ scaled) across 12 samples. The heat map of z scores for each sample is shown. Spleen (B) and subcutaneous tumors (C) were harvested from mice on day 10 after the start of LMB-100 plus anti-PD-1 antibody treatments ($n = 3$ for each group; each sample contained one tumor for the untreated group, whereas in the treatment groups, two to five tumors

were pooled as one sample because of their small size). Cells were isolated and analyzed by flow cytometry. A singlet gate was used to exclude any doublets, followed by a viability gate to exclude any dead cells. (B and C) CD45⁺ cells were gated, and CD4⁺ and CD8⁺ cells were quantified. T_{reg}, regulatory T cell; NK cells, natural killer cells; MHC, major histocompatibility complex.

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Fig. 7. CD8⁺ T cells play important roles in antitumor effects induced by LMB-100 plus anti-PD-1 in 531LN2-hMSLN tumors.

531LN2-hMSLN-bearing mice ($n = 8$ for each group) were administered LMB-100 (2.5 mg/kg, iv, every other day, three doses per cycle for two cycles with a cycle interval of 3 days) and anti-PD-1 antibody (10 mg/kg, ip, twice with interval of 4 days). All drug treatments were started when tumor size reached 90 mm³. CD8⁺ T cell depletion treatment was initiated 1 day before LMB-100 plus anti-PD-1 treatment and then continued twice a

week for 2 weeks. **(A)** Tumor volume was determined. **(B)** Survival time was defined as the time for tumors to reach a volume of 1200 mm³.

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Table 1.

Clinical characteristics, tumor pathology, and treatment responses of patients with mesothelioma treated with pembrolizumab or nivolumab after LMB-100 treatment. Pt, patient; Peri, peritoneal mesothelioma; Pleu, malignant pleural mesothelioma; E, epithelioid; B, biphasic; MSLN, mesothelin.

Pt. ID	Age/sex	Site	Prior therapy*	Histology	Tumor MSLN (% of cells)	Tumor PD-L1 (% of cells)	Treatment cycles LMB-100 (with nab-paclitaxel)	Start of pembrolizumab after LMB-100 [†] (months)	Overall response [‡] (% change in tumor)	PFS [§] on pembrolizumab (months)	OS (months)
P02	72/M	Peri	HIPEC, C + P	E	3+ (100%)	15%	4 (0)	9.3	PR (-40%)	11.4	27.7
P05	68/M	Pleu	Ca + P, mP, V	E	3+ (95–100%)	1–5%	2 (0)	1.1	CR	36.9 [¶]	39.0 [#]
P08 ^{**}	68/M	Pleu	Ca + P, P/D, BMS 986148	E	3+ (>50%)	0%	4 (0)	1.1	NE ^{‡‡}	3.2	6.8
P12	60/M	Peri	C + P, Ca + P, HIPEC, Ca + P, HIPEC	E	2–3+ (100%)	5–7%	2 (6)	5.4	PR (-30%)	11.3	32.6 [#]
P15	61/M	Pleu	C + P, mP	E	3+ (100%)	1%	2 (2)	1.1	PR (-67%)	8.7	13.8
P16	25/F	Peri	C + M, C + P, mP	E	2+ (40%)	0%	2 (2)	2.4	NE ^{‡‡}	1.8	5.0
P18	52/M	Pleu	Ca + P	E	3+ (100%)	0%	2 (6)	5.1	SD (+0.65%)	2.1	10.1
P19	62/F	Pleu	P/D, Ca + P	B	3+ (50%) ^{§§}	0%	2 (4)	2.8	PD (+26%)	3.0	21.6 [#]
P20	24/F	Peri	C + P, HIPEC (C + D)	E	2+ (5%), 1+ (10%)	0%	2 (2)	0.5	NE	1.1	2.5
P21	63/F	Pleu	Ca + P, Ca + P + B, mB	E	3+ (>90%)	20%	2 (3)	0.7	PD (+35.9%)	3.5	9.8

* Prior therapy: B, bevacizumab mB, maintenance bevacizumab; BMS, mesothelin-directed antibody drug conjugate; C, cisplatin; Ca, carboplatin; D, doxorubicin; M, mitomycin; P, pemetrexed; mP, maintenance pemetrexed; V, vinorelbine; HIPEC, hyperthermic intraperitoneal chemotherapy; P/D, pleurectomy and decortication.

[†]Time interval in months between the last dose of LMB-100 and the first dose of pembrolizumab.

[‡]PR, partial response; CR, complete response; SD, stable disease; PD, progression of disease.

[§]PFS, progression-free survival in months; time interval from start of pembrolizumab until PD.

^{||}Overall survival, time interval in months from start of LMB-100 until the last follow-up or death.

[¶]Patient continuing on pembrolizumab until the last follow-up.

[#]Patient alive.

^{**}Treated with nivolumab.

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Nonevaluable due to unavailability of postnivolumab scans that were performed by outside oncologist.

Nonevaluable; received two cycles of pembrolizumab after progressing on LMB-100 but died within 5 weeks before radiological assessments could be performed.

Of epithelioid component.

Nonevaluable; received one cycle of pembrolizumab but died within 5 weeks before the scans could be done.