Synergistic Antitumor Activity of Anti-CD25 Recombinant Immunotoxin LMB-2 with Chemotherapy

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Abstract

Purpose: Although anti-CD25 recombinant immunotoxin LMB-2 is effective against CD25⁺ hairy cell leukemia, activity against more aggressive diseases such as adult T-cell leukemia (ATL) is limited by rapid disease progression between treatment cycles. Our goal was to determine in vivo whether rapid growth of CD25⁺ tumor is associated with high levels of tumor interstitial soluble CD25 (sCD25) and whether chemotherapy can reduce tumor sCD25 and synergize with LMB-2.

Experimental Design: Tumor xenografts expressing human CD25 were grown in mice, which were then treated with LMB-2 and chemotherapy either alone or in combination, and sCD25 level and antitumor activity were measured.

Results: CD25⁺ human xenografts growing rapidly in nude mice had intratumoral sCD25 at levels that were between 21- and 2,200 (median 118)-fold higher than in serum, indicating that interstitial sCD25 interacts with LMB-2 in tumors. Intratumoral sCD25 levels were in the range 21 to 157 (median 54) ng/mL without treatment and 0.95 to 6.1 (median 2.6) ng/mL (P < 0.0001) 1 day after gemcitabine administration. CD25⁺ xenografts that were too large to regress with LMB-2 alone were minimally responsive to gemcitabine alone but completely regressed with the combination. Ex vivo, different ratios of gemcitabine and LMB-2 were cytotoxic to the CD25⁺ tumor cells in an additive, but not synergistic, manner.

Conclusions: Gemcitabine is synergistic with LMB-2 in vivo unrelated to improved cytotoxicity. Synergism, therefore, appears to be related to improved distribution of LMB-2 to CD25⁺ tumors, and is preceded by decreased sCD25 within the tumor because of chemotherapy. To test the concept of combined treatment clinically, patients with relapsed/refractory ATL are being treated with fludarabine plus cyclophosphamide before LMB-2.

Introduction

Recombinant immunotoxins are fusion proteins that contain a bacterial toxin and a targeting Fv fragment of an antibody, and because of the high potency of the toxin, one or a few molecules in the cytosol are sufficient for catalytic activity (ADP ribosylation of elongation factor 2), which leads to apoptotic cell death (1–3). Recombinant immunotoxins have shown potent activity in leukemias including hairy cell leukemia (HCL) without causing immunosuppression similar to that observed with chemotherapy (4–8). The first recombinant immunotoxin described was anti-Tac (Fv)-PE40 (9, 10), which contained a single-chain Fv fragment of anti-Tac (11) and a monoclonal antibody (MAb) directed against CD25, the alpha subunit of the interleukin-2 (IL2) receptor. The toxin was Pseudomonas exotoxin A containing a deletion of the binding domain, such that the recombinant immunotoxin would bind selectively to CD25, internalize, and induce cell death in CD25⁺ cells. A derivative of this molecule, called anti-Tac(Fv)-PE38 or LMB-2, containing the toxin fragment PE38, was tested in patients with relapsed and refractory hematologic malignancies, and major responses were observed in HCL, chronic lymphocytic leukemia (CLL), Hodgkin’s lymphoma, cutaneous T-cell lymphoma (CTCL), and adult T-cell leukemia (ATL; refs. 4, 5). Important limitations of clinical benefit included immunogenicity and rapid progression, both observed particularly in ATL. Another recombinant immunotoxin, BL22, targeting CD22, as a single-agent showed significant activity against HCL (6, 8); however, it showed more limited activity in less indolent leukemias and lymphomas (7), particularly acute lymphoblastic leukemia (12).

A solid tumor antigen, mesothelin, was also targeted with MAb therapy (13), including an anti-mesothelin immunotoxin containing PE38 (14). The anti-mesothelin recombinant immunotoxin SS1P, which also contains PE38, has limited clinical activity as a single agent (15, 16), and was reported, in murine xenografts, to exhibit synergistic activity with either taxol (17, 18), or gemcitabine (19). In the tumor...
Translational Relevance

Recombinant anti-CD25 immunotoxin LMB-2 has had limited efficacy in patients with rapidly growing tumors. In this study, we determined that CD25+ tumor xenografts contain levels of sCD25, within the tumor xenograft interstitial space, which are >100-fold higher than in serum. We were able to reduce both intratumoral and serum sCD25 levels with chemotherapy and observed in vivo synergy with LMB-2. When the tumor cells were treated ex vivo with chemotherapy and LMB-2, no synergy was observed, indicating that the in vivo synergy was due to improved distribution of LMB-2 to the tumor, rather than improved cytotoxicity. Thus, clinical results were brought to the laboratory for study, and a combination treatment was developed that was, in turn, translated back to the clinic. At this time, a clinical trial using chemotherapy and LMB-2 is ongoing in patients with adult T-cell leukemia, a rapidly growing and fatal leukemia/lymphoma with inadequate standard therapeutic options.

Materials and Methods

Cell lines and cytotoxicity assays

Cell lines tested for sCD25 expression included the Hodgkin’s cell lines L540 (22) and L540cy (23), and cell lines transfected with human CD25, including murine plasmacytoma SP2/Tac (24) and the human epidermoid carcinoma line ATAC-4 (25). All cell lines were grown in suspension, with the exception of ATAC-4. For cytotoxicity assays, suspension cells were cultured at 4 x 10^5 cells/mL (4 x 10^5 per well), whereas ATAC-4 was plated at 1 x 10^4 per well in 96-well plates. Cytotoxicity assays were carried out as described previously (21, 25).

Determination of interstitial sCD25

As previously reported (20), tumor extracellular fluid was isolated by excising tumors and centrifuging at 4°C over 297-μm mesh (Spectra/Mesh; Spectrum) in a 1.5-mL microfuge tube. An initial centrifugation cycle for 10 minutes at 1,500 r.p.m. was done to remove fluid on the outside of the tumor, followed by a second centrifugation for 10 minutes at 5,000 r.p.m. to obtain the tumor extracellular (or interstitial) fluid. Additional interstitial fluid with similar sCD25 could be obtained in mouse tumors with a third 10-minute centrifugation at 15,000 r.p.m., although, in human tumors, the sCD25 level in the third centrifugation cycle was higher than in the second. sCD25 levels of serum and tumor fluid were determined using the high-sensitivity kit obtained from R&D Systems.

Antitumor experiments

Animal experiments were conducted under an approved protocol. ATAC-4 cells [1 x 10^6 cells in 0.1 mL Dulbecco’s Modified Eagle’s Medium (DMEM)] were injected subcutaneously in ~20-g athymic nude mice (Frederick, MD) and established tumors usually appeared by day 4. Mice were treated with LMB-2 and/or gemcitabine diluted in PBS containing 0.2% human serum albumin (HSA-PBS) at 10 μL/g body weight, intravenously via tail vein as previously described (21, 25) for LMB-2, and intraperitoneally for gemcitabine (19). When treated with gemcitabine and LMB-2 on the same day, the gemcitabine was administered first.

Statistical analysis

Statistical analysis was carried out with SAS version 8.02 using Wilcoxon nonparametric comparison of tumor sizes, or Fisher exact comparison of regression rates. Combination index (CI) values to determine in vitro synergy were calculated using CalcuSyn 2.0 (Biosoft).

Clinical trial data

Preliminary data were obtained from a phase II clinical trial (NCT00117845) using fludarabine, cyclophosphamide, and LMB-2 for ATL. The trial and consent forms used were approved by the Investigators Review Board of the NCI. In addition, the human samples tested were covered by the protocol.

Results

Although recombinant immunotoxins are markedly effective against HCL, where most of the tumor burden is suspended in the peripheral blood or spleen (4–8), clinical activity in more aggressive malignancies with solid collections of tumor cells has been more limited. To determine whether sCD25, like soluble mesothelin (17, 19), can concentrate within tumors and potentially block immunotoxin distribution, we decided to measure sCD25 within CD25+ ATAC-4 xenografts before and after administration of gemcitabine chemotherapy. Mice bearing the xenografts were treated with LMB-2 and gemcitabine alone and in combination to establish whether these agents would display in vivo synergy. Finally, different combinations of gemcitabine and LMB-2 were incubated with the ATAC-4 cells in tissue culture to determine whether these agents...
would display antagonistic, additive, or synergistic cytotoxicity *ex vivo*.

**Determination of sCD25 production from tumor cells**

To determine the extent to which CD25<sup>+</sup> tumors produce sCD25, the tumor cells were cultured *ex vivo* and the supernatants tested for sCD25. As shown in Table 1, ATAC-4 produced the highest levels of sCD25, 34 ng/mL. The second highest was SP2/Tac, 24 ng/mL, but this cell line did not grow reproducibly as subcutaneous tumors in nude mice. The Hodgkin’s lymphoma lines L540 and L540CY are able to grow in mice but had sCD25 levels of <1 ng/mL in supernatants. Thus, among the cell lines tested, ATAC-4 was best able to serve as a model for targeting ATL tumors with LMB-2, where the cells produce high levels of sCD25.

**Levels of sCD25 in serum versus interstitial space of tumor xenografts**

To determine the levels of sCD25 within the extravascular space of tumors, mice were injected subcutaneously with 1 x 10<sup>6</sup> ATAC-4 cells on day 0, and subcutaneous tumors measuring between 126 and 1,170 (median 330) mm<sup>3</sup> were harvested. As shown in Fig. 1A, sCD25 levels in the untreated tumor interstitial fluid were 21 to 157 (median 54) ng/mL, which is much higher than the serum levels shown in Fig. 1B, and between 0.02 and 1.6 (median 0.38) ng/mL (*P* < 0.0001). For 11 mice with detectable serum sCD25, the ratios of tumor to serum sCD25 levels were 43–2,200 (median 118). In the 10 mice with tumor sCD25 <100 ng/mL, the tumor sCD25 correlated directly with serum sCD25 levels (*r* = 0.81, *P* = 0.004). No correlation was observed between tumor size and either tumor or serum levels of sCD25, indicating that other factors such as tumor architecture may influence levels of sCD25 in tumor and serum. Thus, like mesothelin (20), sCD25 was present in much higher levels in tumors than in serum of tumor-bearing mice. However, instead of the ~10-fold tumor-to-serum ratio observed with soluble mesothelin (20), the ratio with sCD25 was usually >100.

**Effect of gemcitabine on tumor interstitial sCD25**

To determine whether gemcitabine would decrease concentrations of sCD25 within the interstitial space of the CD25<sup>+</sup> tumors, nude mice bearing CD25<sup>+</sup> ATAC-4 tumors were treated with gemcitabine 100 mg/kg and interstitial concentrations of sCD25 were determined 1 and 2 days later. As shown in Fig. 1A, just 1 day after gemcitabine administration, tumor sCD25 levels in 33 mice were between 0.95 and 6.1 ng/mL, which is much lower than tumor sCD25 in untreated mice (*P* < 0.0001), and the median tumor sCD25 level of 2.6 ng/mL was <5% of the 54 ng/mL median for untreated mice. In this period of time, there was no significant decrease in the tumor size. Tumors from 10 other mice were harvested 2, rather than 1, days after treatment with gemcitabine, and as shown in Fig. 1A, the tumor interstitial sCD25 levels, between 17 and 118 ng/mL (median 50 ng/mL), were much higher than on 1 day (*P* < 0.0001) and no different from those of untreated mice (*P* = 0.5). Thus, gemcitabine depleted sCD25 from the interstitial space of tumors within 1 day, but the interstitial sCD25 recovered after an additional day, possibly because of recovery of cellular and microenvironmental characteristics of the tumor. As shown in Fig. 1B, serum levels of sCD25 were undetectable in most mice 1 day after gemcitabine administration and significantly lower than serum sCD25 in untreated mice (*P* < 0.0001); however, serum sCD25 levels 2 days after gemcitabine administration were similar to those of untreated mice (*P* = 0.11).

**Antitumor activity of LMB-2 given after gemcitabine in CD25<sup>+</sup> tumor-bearing mice**

To determine whether gemcitabine is synergistic *in vivo* with LMB-2, nude mice were injected with 1 x 10<sup>6</sup> to 2 x 10<sup>6</sup> ATAC-4 cells on day 0 and treated with each of these agents alone or in combination, as described in Table 2. In the first experiment, mice were first treated with gemcitabine chemotherapy on days 4 and 6, followed by LMB-2 on days 6, 8, and 10. This design was modeled from an ongoing clinical trial in which patients with ATL were treated with fludarabine plus cyclophosphamide (FC) on days 1, 2, and 3, followed by LMB-2 on days 3, 5, and 7. In the clinical trial,

### Table 1. sCD25 levels in supernatant of cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>sCD25, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP2/Tac</td>
<td>24</td>
</tr>
<tr>
<td>L540</td>
<td>0.96</td>
</tr>
<tr>
<td>L540CY</td>
<td>0.525</td>
</tr>
<tr>
<td>ATAC-4</td>
<td>34</td>
</tr>
</tbody>
</table>

![Figure 1. Soluble CD25 (sCD25) measured in tumor-bearing mice. ELISA-determined sCD25 levels are shown in the interstitial space of ATAC-4 tumors (A) and in the serum (B) of mice. Tumors were excised from mice that were either untreated (Untx) or 1 to 2 days after administering gemcitabine 100 mg/kg intraperitoneally. Serum sCD25 levels shown as ≤0.001 ng/mL in (B) were undetectable (Undet).](#)
FC was given before LMB-2 not only to decrease tumor burden and sCD25 in ATL tumors but also to decrease immunogenicity. In the mouse experiment shown in Fig. 2A, LMB-2 and gemcitabine have similar activity alone, and are much more effective when combined. The combination of gemcitabine 80 mg/kg intraperitoneally × 2 and LMB-2 100 μg/kg intravenously × 3 produced greater tumor regression than gemcitabine alone (P = 0.009–0.04 on days 13 and 14; P = 0.001 on days 16 and 19; and P < 0.001 on days 17 and 20) or LMB-2 alone (P = 0.015–0.024 on days 13–20). In fact, as much as half of each agent combined was more effective than gemcitabine (P = 0.003–0.04 on days 13 and 14; P < 0.001 on days 16–20) or LMB-2 (P = 0.03–0.045 on days 12–17 and day 20) alone. Thus, LMB-2 given after 2 doses of gemcitabine was synergistic, because the antitumor activity was significantly greater than with either LMB-2 or gemcitabine alone even when half the dose of each drug was administered in the combination therapy. However, the group receiving LMB-2 alone may have had larger tumors because treatment in this group was delayed by more than 2 days than in the other groups.

Antitumor activity of gemcitabine and LMB-2 given on the same days

To determine the synergistic activity of gemcitabine with LMB-2 more accurately, both agents were given on the same
gemcitabine (i.p.) & LMB-2 (i.v.)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Gemcitabine (i.p.)</th>
<th>LMB-2 (i.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1</td>
<td>9</td>
<td>None</td>
<td>100 μg/kg; d6, 8, 10</td>
</tr>
<tr>
<td>A 2</td>
<td>9</td>
<td>80 mg/kg; d4, 6</td>
<td>None</td>
</tr>
<tr>
<td>A 3</td>
<td>9</td>
<td>80 mg/kg; d4, 6</td>
<td>100 μg/kg; d6, 8, 10</td>
</tr>
<tr>
<td>A 4</td>
<td>10</td>
<td>40 mg/kg; d4, 6</td>
<td>50 μg/kg; d6, 8, 10</td>
</tr>
<tr>
<td>A 5</td>
<td>10</td>
<td>20 mg/kg; d4, 6</td>
<td>25 μg/kg; d6, 8, 10</td>
</tr>
<tr>
<td>B 0</td>
<td>5</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>B 1</td>
<td>10</td>
<td>None</td>
<td>160 μg/kg; d6, 8, 10</td>
</tr>
<tr>
<td>B 2</td>
<td>9</td>
<td>80 mg/kg; d6, 8, 10</td>
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<tr>
<td>B 3</td>
<td>10</td>
<td>40 mg/kg; d6, 8, 10</td>
<td>80 μg/kg; d6, 8, 10</td>
</tr>
<tr>
<td>B 4</td>
<td>10</td>
<td>20 mg/kg; d6, 8, 10</td>
<td>40 μg/kg; d6, 8, 10</td>
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<tr>
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<td>None</td>
<td>120 μg/kg; d7, 9, 11</td>
</tr>
<tr>
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</tr>
<tr>
<td>C 3</td>
<td>10</td>
<td>60 mg/kg; d7, 9, 11</td>
<td>120 μg/kg; d7, 9, 11</td>
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<tr>
<td>C 4</td>
<td>10</td>
<td>30 mg/kg; d7, 9, 11</td>
<td>60 μg/kg; d7, 9, 11</td>
</tr>
<tr>
<td>C 5</td>
<td>10</td>
<td>15 mg/kg; d7, 9, 11</td>
<td>30 μg/kg; d7, 9, 11</td>
</tr>
</tbody>
</table>

Figure 2. Antitumor activity of LMB-2 and gemcitabine. For experiments A, B, and C, mice were injected subcutaneously with ATAC-4 cells (2 × 10^6 in A and 1 × 10^6 in B and C) on day 0 and treated with gemcitabine and LMB-2 at the doses and time points indicated in Table 2. Group 0 (●) received neither, group 1 (○) LMB-2 only, group 2 (+) gemcitabine only, group 3 (△) in A and C, both at full doses, and groups 4 (▲) and 5 (◇) received both at half and quarter doses, respectively.
day. As shown in Table 2 and Fig. 2B, mice receiving gemcitabine 40 mg/kg intraperitoneally plus LMB-2 80 µg/kg intravenously on days 6, 8, and 10 (group 4) had smaller tumors than mice receiving twice this dose of either gemcitabine \((P = 0.025 \text{ on day } 9; \ P = 0.001–0.007 \text{ on days } 10–12 \text{ and } 16–20; \text{ and } P < 0.001 \text{ on days } 13–15)\) or LMB-2 \((P = 0.003 \text{ on day } 12; P < 0.001 \text{ on days } 13–20)\) alone. Moreover, even one-fourth of the dose in the combination of gemcitabine and LMB-2 (group 5) showed higher antitumor activity than either untreated mice \((P = 0.028–0.05 \text{ on days } 8 \text{ and } 9; P = 0.003–0.008 \text{ on days } 10–13; \text{ and } P < 0.001 \text{ on days } 15 \text{ and } 16)\), or mice receiving gemcitabine \((P = 0.02 \text{ on days } 8 \text{ and } 18; P = 0.0036–0.009 \text{ on days } 9, 10, 16, \text{ and } 20; \text{ and } P < 0.001 \text{ on days } 12–15)\) or LMB-2 \((P = 0.003 \text{ on day } 12; P < 0.001 \text{ on days } 13–20)\) alone. To evaluate synergy more accurately using larger tumors and lower doses, mice received gemcitabine 60 mg/kg intraperitoneally and/or LMB-2 120 µg/kg intravenously on days 7, 9, and 11, as well as one-half and one-fourth doses of the combination (Table 2). As shown in Fig. 2C, gemcitabine and LMB-2 were again synergistic, achieving complete regression in all mice that received the full dose combination, persisting in 9 of 10 mice past day 60, compared with no complete regressions in mice receiving gemcitabine or LMB-2 alone \((P < 0.001)\). The time to reach a mean tumor size of 150 mm³ was 51 days for the full-dose combination (group 3), compared with 7 days for untreated mice and 12 or 15 days for mice receiving LMB-2 (group 1) or gemcitabine (group 2) alone, respectively. Thus, LMB-2 and gemcitabine each prolonged tumor progression by 5 and 8 days, respectively, making 13 days the expected prolongation of progression for additive antitumor activity. The observed prolongation by the combination of 44 days (51–7 days) thus confirms the \textit{in vivo} synergy. Moreover, even when combined at one-fourth of the dose levels (group 5), gemcitabine and LMB-2 were more effective than either gemcitabine \((P = 0.016–0.04 \text{ on days } 9 \text{ and } 26; P < 0.001 \text{ on days } 11–20; \text{ and } P = 0.005–0.008 \text{ on days } 22 \text{ and } 24)\) or LMB-2 \((P = 0.002 \text{ on days } 11–20)\) alone. In addition, \textit{in vivo} synergy relative to toxicity could be expressed in this experiment by dividing days of prolongation of tumor progression by the ratios of dose used to the LD10 dose. By this definition, the tumor progression values were between 37 and 47 for the combination compared with 9 for LMB-2 compared with 11 for gemcitabine alone.

### Dose response of gemcitabine combined with LMB-2

To determine dose response, tumor sizes and complete regression rates in mice receiving 60 mg/kg intraperitoneally plus LMB-2 120 µg/kg intravenously on days 7, 9, and 11 (group 3) were compared with half (group 4) and one-fourth (group 5) doses of the combination (Table 2 and Fig. 2C). Complete regression rates in these 3 respective groups of 10 mice (groups 3, 4, and 5 in Table 2) were 100%, 60%, and 10% \((P < 0.001)\). Permanent complete regression rates in groups 3 to 5 were 90%, 30%, and 10% \((P = 0.001)\), respectively. Tumor sizes in group 3 were smaller than either group 4 \((P = 0.03–0.05 \text{ on days } 12, 22, \text{ and } 24; P = 0.001–0.007 \text{ on days } 16–20, 28, \text{ and } 30)\), or group 5 \((P = 0.001–0.007 \text{ on days } 11–16, 20–30; \text{ and } P < 0.001 \text{ on day } 18)\), and the 2 smaller groups 4 and 5 also differed from each other with regard to tumor sizes \((P = 0.004–0.015 \text{ on days } 15–28; P = 0.025 \text{ on day } 30)\). Thus, by either measurement of regression rates or tumor sizes, a dose response was observed in the 3 different dose levels of gemcitabine and LMB-2 combinations.

### Assessment of synergy \textit{ex vivo}

To determine whether the synergy observed \textit{in vivo} between gemcitabine and LMB-2 was attributable to synergistic cytotoxicity toward the cell line used, instead of other factors like improved immunotoxin distribution, different concentrations of LMB-2 and gemcitabine were studied in cytotoxicity assays \textit{ex vivo}. Isobolograms are shown in Fig. 3, where concave, linear, and convex curves are expected for synergistic, additive, and antagonistic cytotoxicity \textit{ex vivo}, respectively (26, 27). Both IC50 and IC75 isobolograms in Fig. 3A and B, respectively, show additive cytotoxicity, with the regression most consistent with a straight line \((r^2 = 0.78–0.9, P < 0.001)\). Another method for determining synergy is by calculation of Cls from the percentage inhibition \textit{ex vivo} at each combination of the 2 agents tested, with values less than 1 consistent with synergy, particularly with Cl < 0.1 (28). Calculated Cl values for the different ratios of LMB-2 and gemcitabine tested are shown in Table 3. The LMB-2:gemcitabine ratio used \textit{in vivo} was usually 1:500 (Table 2; Fig. 2B and C). Although some variability was observed \textit{ex vivo}, Cl values were usually more than 0.5 and medians were very close to 1.0. The IC75 Cl values as low as 0.267 were calculated for LMB-2:gemcitabine ratios of 1:500 to 1:2,500 (Table 3). However, because the half-life of LMB-2 \((a = 35 \text{ minutes}, \ b = 192 \text{ minutes})\) is longer than that of gemcitabine \((17 \text{ minutes})\) in mice (21, 29), the higher IC75 Cl values \((0.791–3.538)\) from the higher LMB-2:gemcitabine ratios \(1:4–1:100\) are more relevant. Thus, the \textit{in vivo} synergism between gemcitabine and LMB-2 observed in tumor-bearing mice is not consistent with synergistic cytotoxicity against the ATAC-4 cells making up the tumor. Rather, the \textit{in vivo} synergy may be due to...
Synergy of chemotherapy with other immunotoxins

Although SS1P targeting mesothelin is the only other recombinant immunotoxin, to our knowledge, which has been shown to exhibit synergistic in vivo antitumor activity with chemotherapy (17, 19, 20), synergy with chemotherapy has been reported for other immunotoxins and recombinant growth-factor fusion toxins, either in vitro, in vivo, or both. Examples of in vitro synergy were reported using chemotherapeutic agents with an anti-p40 prostate antigen ricin A-chain immunotoxin conjugate (30), the immunotoxin 317G5-rRta directed against ovarian cancer (31), the diphtheria toxin-containing fusion toxin DT338-GM-CSF toward acute myelogenous leukemia (26, 27), the anti-CD20 immunotoxin containing rituximab and saporin-S6 (32), and the anti-erbB2 recombinant immunotoxin HEL-PE38KDEL against breast and gastric cancer (33). Additive toxicity was reported in vitro and in vivo with doxorubicin and anti-transferrin receptor-ricin A chain immunotoxin (34). Chemotherapy with anti-CD19 anti-B4-blocked ricin showed in vitro synergy (35, 36) and improved antitumor activity (35). In vivo synergy was reported with cyclophosphamide and NR-LU-10/PE, an anti-colon carcinoma conjugated with whole Pseudomonas exotoxin (37). Interferon and daunorubicin synergized in vivo with SN1-RA and SN2-RA, ricin A-chain immunotoxins targeting their respective T-cell antigens, TALLA and GP37 (38). The recombinant circularly permuted fusion toxin IL4 (83–37)-PE38KDEL, targeting interleukin (IL)-4–receptor-bearing carcinomas and lymphomas (39), was shown to be synergistic in vitro and in vivo with gemcitabine against pancreatic adenocarcinoma (40). Finally, bexarotene was found to upregulate CD25 in CTCL cells of patients treated with the anti-IL2-receptor recombinant fusion toxin denileukin difitox, and the combination was well tolerated (41). Other than SS1P, we were unable to find any previous reports of chemotherapy–immunotoxin combinations shown in animals to have in vivo without in vitro synergy.

Synergy with recombinant immunotoxins having limited plasma lifetime

Compared with MAbs, which have half-lives up to several weeks in duration, recombinant immunotoxins of ~63 kDa in size are much more limited in half-life, quantified in humans at ~8 hours for SS1P and 4 hours for LMB-2 (5). Although the short half-life of LMB-2 may limit capillary leak syndrome (5), it may also limit distribution of the recombinant immunotoxin to the cells within a tightly packed tumor. Unlike conventional immunotoxins, smaller recombinant immunotoxins like LMB-2 may not remain in the plasma long enough for the immunotoxin molecules to bind to tumor cells, particularly if the immunotoxin encounters a block or delay in reaching the target cell. Thus, synergistic combinations with chemotherapy or other agents may be particularly needed for these smaller targeting agents.

Mechanism of synergy in mice receiving LMB-2 and gemcitabine

The cytotoxicity experiment in Fig. 3 excludes synergistic cytotoxicity in vitro as the mechanism for in vivo synergism of LMB-2 and gemcitabine, making improved distribution of LMB-2 to tumor cells the likely outcome of combined use with chemotherapy. Because murine CD25 and sCD25 do not bind anti-Tac MAb or LMB-2, it was not our goal to further define the exact mechanisms of synergy in our xenograft model. Improved tumor distribution of LMB-2 could be due to mechanisms in addition to or instead of sCD25 depletion, including decreased packing of tumor cells due to partial cytotoxicity of chemotherapy (42). In the latter case, the sCD25 depletion would be a marker of antitumor activity rather than a cause of synergism. Whether or not intratumoral sCD25 can, in fact, prevent to other factors, such as improved distribution of LMB-2 to the tumor cells.

Discussion

Our goal was to determine whether sCD25, like soluble mesothelin, can become concentrated within CD25+ tumors, and whether improved antitumor activity can be achieved by combining the anti-CD25 recombinant immunotoxin with chemotherapy. We measured sCD25 levels within CD25+ xenografts before and after administration of gemcitabine chemotherapy, and then determined whether gemcitabine and LMB-2 would show in vivo or in vitro synergy. We found that sCD25 levels within the interstitial space of tumors were much (median > 100-fold) higher than in the serum, and could be reduced more than 10-fold with gemcitabine. Moreover, gemcitabine and LMB-2 showed synergistic antitumor activity in vivo but only additive cytotoxicity in vitro, consistent with improved targeting of LMB-2 to tumors.

Table 3. Combination index values for LMB-2 and gemcitabine

<table>
<thead>
<tr>
<th>LMB-2:gemcitabine ratio</th>
<th>IC25</th>
<th>IC50</th>
<th>IC75</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:4</td>
<td>1.033</td>
<td>1.018</td>
<td>1.049</td>
</tr>
<tr>
<td>1:5</td>
<td>1.205</td>
<td>0.972</td>
<td>1.499</td>
</tr>
<tr>
<td>1:10</td>
<td>2.269</td>
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<td>3.538</td>
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<tr>
<td>1:20</td>
<td>0.841</td>
<td>0.899</td>
<td>0.791</td>
</tr>
<tr>
<td>1:100</td>
<td>1.504</td>
<td>0.896</td>
<td>2.554</td>
</tr>
<tr>
<td>1:100</td>
<td>1.028</td>
<td>0.659</td>
<td>1.643</td>
</tr>
<tr>
<td>1:500</td>
<td>0.598</td>
<td>0.695</td>
<td>0.520</td>
</tr>
<tr>
<td>1:1,000</td>
<td>0.979</td>
<td>0.893</td>
<td>1.088</td>
</tr>
<tr>
<td>1:2,000</td>
<td>0.515</td>
<td>0.874</td>
<td>0.306</td>
</tr>
<tr>
<td>1:2,500</td>
<td>0.625</td>
<td>1.463</td>
<td>0.267</td>
</tr>
<tr>
<td>Median CI values</td>
<td>1.004</td>
<td>0.897</td>
<td>1.069</td>
</tr>
</tbody>
</table>

NOTE: Ratios are based on ng/mL of LMB-2 and gemcitabine added simultaneously to ATAC-4 cells ex vivo. IC25, IC50, and IC75 are defined as the calculated concentrations necessary for 25%, 50%, and 75% inhibition, respectively.
distribution of LMB-2 to tumor cells might depend on the off- and on-rates of LMB-2 for sCD25, the stability of LMB-2 in tumor interstitial space compared with time course of LMB-2 in the serum, the 3-dimensional relationships between sCD25 and tumor-associated CD25 molecules within the tumor, and complex interactions among LMB-2, sCD25, and other contents of the tumor interstitial microenvironment. It is unlikely that synergistic tumor regression was due to nonspecific toxicity of chemotherapy to the animals, resulting in decreased tumor size as animals lose weight, because animals remained healthy during the experiment. In addition, it is unlikely that gemcitabine creates synergy by increasing CD25 on the malignant cells, because expression levels are already $\sim 2 \times 10^3$ sites/cell (25). Indirect mechanisms, for example, involving cytokines liberated by either agent, are unlikely because murine factors would be inactive against human xenografts. LMB-2 can eliminate Tregulatory (Treg) cells expressing CD4, CD25, and Foxp3 of human but not of murine origin (43), and results of previous experiments indicate that this would be unlikely to lead to synergy in patients due to rapid reconstitution of Treg cells from CD25-negative T cells (44). Thus, we believe that gemcitabine induces in vivo synergy with LMB-2 by allowing better distribution of LMB-2 to tumor cells. Although these animal experiments cannot further define the mechanism of synergy with LMB-2 and chemotherapy, they do indicate sCD25 as an important marker to follow in serum and tumors of patients being treated.

**Time course of sCD25 decrease and synergy**

We found that intratumoral and serum sCD25 were significantly decreased at 1, but not 2, day(s) after gemcitabine dosing in mice (Fig. 1), but that synergy was achieved by dosing gemcitabine and LMB-2 every 2 days (Fig. 2B and C). Biodistribution studies of LMB-2 using the ATAC-4 model previously reported persistence of LMB-2 in the tumor interstitial space at $>2,000$ ng/mL for 15 minutes to 6 hours after intravenously injection, and 460 ng/mL at 24 hours (21). These earlier experiments indicate that significant levels of LMB-2 are present in the tumor 24 hours after gemcitabine dosing, the time point when sCD25 is depleted. Thus, chemotherapy administered on the same day as LMB-2 could allow improved distribution of LMB-2 to tumor cells 24 hours later when the chemotherapy effect has occurred.

**Relevance to clinical testing of LMB-2 in ATL**

At this time, a clinical trial is ongoing in which FC chemotherapy is administered before LMB-2 to patients with aggressive (leukemic or lymphomatous) forms of ATL. Patients receive 3 daily doses of FC and, 2 weeks later, begin cycles every 3 weeks in which FC is administered on days 1 to 3, followed by LMB-2 on days 3, 5, and 7. The goal of this trial using FC is not only to improve LMB-2 distribution to solid ATL tumors by use of chemotherapy but also to help prevent rapid progression of ATL between cycles and to block immunogenicity. FC was selected for the clinical trial on the basis of transplant data in humans showing safe reductions of normal T and B cells (45, 46) and prevention of human anti-murine antibodies (47), but could not be used in mice because of unfavorable pharmacokinetics. Rather than the purine analog fludarabine, we therefore used the pyrimidine analog gemcitabine, which has activity in both hematologic and solid human tumors (48, 49) and has shown synergistic antitumor activity in mice with recombinant immunotoxin SS1P (19). Because prevention of immunogenicity was not a goal of the xenograft model, gemcitabine was an appropriate substitute for FC in mice. In the clinical trial of FC/LMB-2, lack of immunogenicity has allowed up to 5 cycles of combination FC and LMB-2 to be administered, and in 9 evaluable patients, a response rate of 56% has been observed, including 33% complete remissions. Although sCD25 data is limited from excisional biopsies, in 1 patient before treatment, the sCD25 was 32.9 ng/mL in the tumor compared with 7.8 ng/mL in the serum. We believe that both clinical and laboratory data from combined use of chemotherapy and immunotoxins will lead to expansion of the successful use of these biologic agents in the treatment of hematologic and solid tumors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

R. Singh carried out the research and analyzed data. Y. Zhang and I. Pastan analyzed data. I. Pastan also helped design research, and R.J. Kreitman designed research, analyzed data, and wrote the manuscript.

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