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Rational design of low immunogenic anti CD25 recombinant immunotoxin for T cell malignancies by elimination of T cell epitopes in PE38

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Abstract

LMB-2, is a potent recombinant immunotoxin (RIT) that is composed of scFv antibody that targets CD25 (Tac) and a toxin fragment (PE38). It is used to treat T cell leukemias and lymphomas. To make LMB-2 less immunogenic, we introduced a large deletion in domain II and six point mutations in domain III that were previously shown to reduce T cell activation in other RITs. We found that unlike other RITs, deletion of domain II from LMB-2 severely compromised its activity. Rather than deletion, we identified T cell epitopes in domain II and used alanine substitutions to identify point mutations that diminished those epitopes. The novel RIT, LMB-142 contains a 38 kDa toxin and nine point mutations that diminished T cell response to the corresponding peptides by an average of 75%. LMB-142 has good cytotoxic activity and has lower nonspecific toxicity in mice. LMB-142 should be more efficient in cancer therapy because more treatment cycles can be given.

Keywords

Immunogenicity; T cell epitopes; *Pseudomonas exotoxin A*; domain II; deimmunization; TAC; IL2 receptor; RIT

1. Introduction

Immunotoxins are recombinant proteins designed to treat cancer. They are composed of a targeting antibody fragment that targets cancer cells and a toxin fragment that kills these

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Author Contributions

Conceptualization, R.M. and I.P.; Methodology, R.M., I.P., D.P., G.K. and R.K.; Investigation, R.M., D.P., J.Y. and F.L.; Writing – Original Draft, R.M. and I.P.; Writing – Review & Editing, R.M. and I.P.; Supervision, R.M. and I.P.

cells. Immunotoxins that employ bacterial toxins, whose cytotoxic activity targets the protein translation mechanism like Diphtheria toxin and *Pseudomonas* exotoxin A (PE38), were shown to be very effective in treatment of some hematological malignancies and are either approved or pending approval by the FDA [1, 2]. LMB-2 is an immunotoxin consisting of a 38 kDa fragment of PE38 and the variable fragment (Fv) of anti-Tac antibody that targets CD25 (the α chain of the IL2 receptor) [3]. LMB-2 was very cytotoxic towards CD25+ leukemic cells from patients with adult T cell leukemia (ATL), hairy cell leukemia, and T-cell leukemias (reviewed in [4]) and produced significant tumor regression in patients with chronic lymphocytic leukemia, ATL, cutaneous T-cell lymphoma and Hodgkin's disease [4].

One of the most frequently observed limitations to retreatment with bacterial toxin fragments in patients, is immunogenicity, namely the formation of anti-drug antibodies (ADA) [5–7]. While 100% of patients with solid tumor diseases like mesothelioma or breast cancers exhibit a rapid immune response after the first or second treatment cycles with PE38 immunotoxins, patients with hematological malignancies have a lower overall rate of ADA development, and many of the patients can receive more than two cycles. This is probably due to the immune status of the patients that was affected by the disease and previous chemotherapies [4]. Nevertheless, many of the patients treated with LMB-2 had ADA formation that neutralized the activity of the immunotoxin and prohibited further treatments, which prevented complete eradication of the tumor cells.

Recent efforts to reduce immunogenicity in patients included a combining LMB-2 with fludarabine and cyclophosphamide, two chemotherapy drugs shown to be effective in both tumor reduction [8] and in prevention of ADA formation [9]. This combination treatment was found to be very effective and reduced the rate of immunogenicity from 60% to 29% [10]. Another approach to reduce the immunogenicity of LMB-2 is to modify its bacterial fragment to escape recognition by the immune system.

Rational design of recombinant proteins that delete or mutate T cell epitopes is becoming a well-accepted approach in order to reduce immunogenicity. This approach was utilized to design less immunogenic therapeutic proteins for various indications [11–14]. We previously used this approach to delete domain II and mutate T cell epitopes in domain III of PE38 which allowed us to construct next generation immunotoxins with low immunogenicity against CD22 and mesothelin [15, 16]. Here, we used alanine scanning mutagenesis and T cell activation assays to identify point mutations in domain II that diminish T cell activation. We have constructed a new immunotoxin that consists of a disulfide stabilized Fv (dsFv) of anti-Tac antibody and PE38 with 9 point mutations in domain II and III. We found that unlike CD22-mediated internalization, CD25-mediated cell killing by immunotoxins requires the presence of domain II. Compared with LMB-2, the new immunotoxin LMB-142 has a potent cytotoxic activity *in vitro* and 5-fold lower nonspecific toxicity in mice.

2. Materials and Methods

2.1. Cytotoxicity assays

2.1.1 WST8 assay—For cytotoxicity assays CD25+ cell lines (HUT102, KARPAS 299, SUDHL, ATAC4 [17] and CA46 transfected with CD25 were seeded in a 96 well plate at a concentration of 10,000 cells/well and treated on the same day with different concentrations of various CD25+ immunotoxin variants or cycloheximide (Sigma) in quadruplicates. Cell viability was evaluated 72 hours later using a WST8 counting kit (Dojindo Laboratories) and read at 650–450 nm. For every immunotoxin variant, viability was normalized between the cycloheximide and no treatment controls. Cytotoxicity curves were fitted to a four parameter curve and IC₅₀ was calculated.

2.1.2 ATP assay—Viability of leukemia cells obtained from five ATL patients was measured by the ATP levels using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to manufacturer's protocol. Each sample was evaluated in three replicas. Values were averaged and normalized as described above and IC₅₀ was calculated.

2.2. Transfection of CA46 cell line with CD25

CA46 Burkitt lymphoma cells were transfected with pcDNA3.1(+) (Invitrogen) vector containing aCD25 cDNA by Lipofectamine LTX/PLUS reagents (Invitrogen) according to manufacturer's protocol. The transfected cells were sub-cultured with selection medium (complete RPMI with G-418) for 3 weeks.

2.3. FACS sorting for CA46/CD25+ cells

1×10⁸ CA46/CD25+ Cells were labeled with R-PE (phycoerythrin)-tagged anti-CD25 antibody and sorted using a BD FACS Aria to select CD25-hi cells.

2.4. Binding of immunotoxin variants to CD25 expressing cells

To evaluate the binding affinity of the variant RITs, 5×10⁵ CA46/CD25 cells were allowed to bind to various concentrations of immunotoxin. Rabbit anti-*Pseudomonas* exotoxin antibody and R-PE labeled anti-rabbit IgG as secondary. The fluorescence associated live cells were analyzed using a FACSCallibur flowcytometer (Becton Dickinson), and analyzed using FlowJo.

2.5. Peptide synthesis

Peptides (15-mer or 21 mer) spanning the amino acid sequence of domain II and alanine variants were synthesized by American Peptides. Peptides spanning the sequence of domain II were 15-mers having an overlap of 11 residues. Peptides used for alanine scanning were either 15-mers, or 21-mers for cases where adjacent or overlapping epitopes were identified (Supplementary Table S1). All peptides were purified to >95% homogeneity by HPLC and confirmed by mass spectrometry. They were dissolved in DMSO at 10 mM and stored at −20°C.

2.6. Human PBMCs samples

Peripheral mononuclear cells (PBMCs) were collected from apheresis samples of 40 naïve donors, four patients that were treated with immunotoxin containing PE38 and five ATL patients under protocols approved by the National Institutes of Health Institutional Review Board (99-CC-0168) and (08-C-0026), respectively with informed consent. Samples were isolated using the gradient-density separation by Ficoll-Hypaque (GE Healthcare) and frozen in liquid nitrogen using 10% human AB serum (Gemini) RPMI media (Lonza) and 7.5% DMSO (Sigma Life Science). Class II HLA typing was performed using PCR sequence-specific primers/sequence-specific oligonucleotides-based tissue typing by the HLA typing unit in the Warren G. Magnuson Clinical Center, NIH or by the Texas BioGene Molecular Typing Laboratory.

2.7. In vitro expansion of PBMCs

PBMCs were stimulated with whole immunotoxins containing PE38 (LMB9) as previously described [18]. LMB9 immunotoxin was used because we had a large amount of this protein available with low endotoxin <1EU [19]. Briefly, cells were thawed, resuspended in RPMI-1640 media (Lonza) supplemented with L-glutamine, Penicillin/Streptomycin, and 5% human serum A/B in a concentration of 4×10^6 /ml. Cells were stimulated with 5 µg/mL LMB9, media control or 0.5 µg/ml CEFT peptide pool (Axxora) and supplemented with human IL2 (Millipore) every four days for 14 or 17 days. Cells were incubated at 37°C with 5% CO₂.

2.8. ELISpot assay

After the *in vitro* expansion step, cells were harvested, washed and plated in ELISpot plates that were pre-coated with anti-human IL2 antibody (Mabtech). Cells were re-stimulated with 5 nM peptides and incubated for 24 hours at 37°C. Negative controls were treated with media, and positive controls were treated with either CEFT or phytohemagglutinin (PHA, Sigma). We used the computer software (Immunospot 5.0; Cellular Technology Limited) to measure the number of IL2 spot forming cells (SFC). The assay was performed in quadruplicate, and repeated at least once for each donor. In Alanine scanning experiments, T cell response to wild-type peptides was set to 100%, and the response to the mutated peptides was normalized to that wild-type response.

2.9. Construction, expression, and purification of RIT

LMB-2 is composed of an anti CD25 antibody fragment fused to a two-domain 38 kDa fragment of PE38 (Fig. 1). All protein-encoding sequences were constructed by ordering double stranded DNA fragments (gBlocks, Integrated DNA Technologies) codon optimized for *E. coli* and cloning them into our standard protein production lab vector [20] using Gibson Master Mix (New England Biolabs, Ipswich, MA, USA). Sequences were verified by DNA sequencing. All RIT variants were expressed and purified from *E. coli* inclusion bodies as previously described [21]. -

2.10. In vivo toxicity

Nonspecific toxicity was evaluated by single IV tail injection of immunotoxin into female Swiss mice. Mice were treated with increasing doses of immunotoxin (n = 4 per treatment group), starting with 0.35mg/Kg, and followed by 0.4mg/Kg, 0.5mg/Kg, 0.75mg/Kg, 1mg/Kg, 1.5mg/Kg, 2mg/Kg and 3mg/Kg. When a toxic dose was observed in 4/4 mice, dose escalation was stopped. A dose was considered toxic if mice appeared morbid, lost >10% body weight or died. The animals were handled under the approved protocol from National Cancer Institute Animal Care and Use Committee (protocol LMB-014).

3. Results

3.1. Rational design of anti TAC immunotoxin variants

To improve the stability of the antibody fragment, we introduced a disulfide bond in the conserved framework regions, because anti-Tac-dsFv was previously shown to be more stable in human serum and more resistant to thermal and chemical denaturation than the single chain (sc)Fv immunotoxin [22, 23]. Anti-Tac scFv and dsFv immunotoxins have very similar cytotoxic activity *in vitro* (Supplementary Fig. S1). To reduce nonspecific toxicity and immunogenicity, other immunotoxins that target mesothelin or CD22 were previously modified by a deletion of the majority of domain II (PE24), retaining just 11 amino acids that are required for furin cleavage [24]. To reduce the immunogenicity in domain III, six point mutations that were previously shown to reduce T cell activation by 81% were inserted (aTac-dsFv-PE24 domain III 6x) (Fig. 1) [25]. Because we found that domain II is critical for anti-Tac mediated cytotoxic activity, domain II was included in the final construct, and three novel point mutations in domain II were inserted; E270A, Q283A and L299A, to reduce T-cell activation (LMB-142) (Fig. 1).

3.2. Effect of domain II on cytotoxic activity

We evaluated the cytotoxic activity of anti-Tac immunotoxin that is deficient of domain II (aTac-dsFv-PE24) in five cell lines derived from human malignancies; HUT102 (cutaneous T cell lymphoma), KARPAS 299 and SUDHL (human non-Hodgkin's large cell lymphoma), ATAC-4 (A431 epidermoid carcinoma cells transfected with CD25, the α subunit of the IL2 receptor) [17] and a Burkitt lymphoma cell line (CA46) that was transfected with CD25. Cells were treated with various concentrations of either anti-Tac-dsFv-PE38 or anti-Tac-dsFv-PE24 and IC₅₀s were determined. We found that while aTac-dsFv-PE38 was very potent, anti-Tac-dsFv-PE24 was much less active in all five cell lines ($p < 0.002$), with a relative activity less than 5.4% in five cell lines (Fig. 2A). This result was unexpected, because previous studies with immunotoxins that target mesothelin or CD22 did not usually show such a decrease in activity after deletion of domain II and often showed an increase in activity [24, 26].

Because we found that deletion of domain II induced a significant reduction in cytotoxic activity in multiple CD25 expressing cell lines, we tested if B cells transfected with CD25 will have a similar loss in cytotoxic activity. CA46 (a Burkitt lymphoma cell line with high expression of CD22 [27]) was transfected with CD25 and selected using FACS for high expression of the CD25. Cytotoxic activity of PE24 and PE38 immunotoxins is shown in

Figure 2B. While anti-CD22-dsFv-PE38 (Moxetumomab Pasudotox) and anti-CD22-dsFv-PE24 (HA22-LR) have similar cytotoxic activities (IC_{50} of 0.02 ng/ml for both immunotoxins), aTac-dsFv-PE38 (IC_{50} of 0.05 ng/ml) is 8-fold more active than anti-Tac-dsFv-PE24 (0.41 ng/ml).

One possible cause of reduced cytotoxic activity is that deletion of domain II decreases the binding of the immunotoxin to CD25. To test this, we measured the affinity of anti-Tac-dsFv-PE38 and anti-Tac-dsFv-PE24 to CD25 expressing cells and found very little difference (Fig. 2C). The IC_{50} of anti-Tac-dsFv-PE38 is 0.82 nM and that of anti-Tac-dsFv-PE24 is 0.55 nM.

3.3. T cell epitopes in domain II

We previously mapped the T cell epitopes in PE38, and identified three epitopes in domain II and five in domain III [15]. To currently focus on domain II, more PBMC samples were collected and pre-screened for responsiveness to the 40 peptides in domain II. PBMCs from 41 donors were expanded *in vitro* for 14 days using PE38 immunotoxin and restimulated with eight peptide pools that span the amino acid sequence of domain II. Pools that had a response higher than 3-fold of the “no peptide” negative control and >85 SFC/million cells were considered positive and were screened for the individual peptides that compose those pools. The normalized responses of the 40 peptides that span the amino acids of domain II are shown in Figure 3. We found that peptides 14 and 15 had 25 and 26 positive responses, respectively. These peptides, along with peptide 13 that had 13 responses, represent the strongest T cell epitope in domain II (epitope 1). Peptides 8 and 9 that had 12 and 11 positive responses (epitope 2) and peptides 5 and 6 that had 13 and 10 positive responses make epitope 3. To insure coverage of various HLA populations, we analyzed the DNA of 40/41 PBMC samples used in this analysis for HLA DRB1, DR3/4/5, DQB1, and DPB1 (Supplementary Table S2). We found that the HLA class II haplotypes of the PBMC used for the analysis included a great variety of HLA DR and DP (including DRB1*101, 301, 401, 405, 701, 802, 1101, 1201, 1302, 1501 and DPB1*0101, 0201, 0401, 0402, 0501 and 1401) which provides a great coverage of more than 65% of DRB alleles and 94.5% of DPB1 alleles in the world's population [28].

3.4. Alanine substitution to identify key amino acids for T cell activation

To identify amino acids in epitopes 1, 2 and 3 that can be mutated and reduce the T cell activation, we synthesized three sets of peptides that have alanine substitutions for each and every one of the amino acids in epitopes 1, 2 and 3 and their ability to activate T cells was evaluated.

Epitope 1 is composed of three peptides (13, 14, and 15), so a 21 mer peptide that spans the sequence of all three peptides was synthesized. Cells from 19 PBMC donors that had a positive response in this epitope were expanded *in vitro* and restimulated with alanine variant peptides (Fig. 4A) with single point mutations. We identified three point mutations that can diminish the response by more than 50%: L297A, L294A and L299A diminished the average response by $62\% \pm 36\%$, $60\% \pm 27\%$, and $50\% \pm 39\%$, respectively.

Epitope 2 is composed of peptides 8 and 9, with peptide 8 having the stronger response. Peptide 8 that spans the 12 overlapping amino acids was used as a template for the alanine variants. PBMCs from eight donors that had a positive response to this epitope were expanded *in vitro* and restimulated with the alanine variant peptides (Fig. 4B). We identified three point mutations that can diminish the response by more than 50%: R279A, W281A and Q283A diminished the average response by $76\% \pm 21\%$, $71\% \pm 28\%$ and $71\% \pm 23\%$, respectively.

Epitope 3 is composed of peptides 5 and 6, with peptide 5 having the stronger response. Peptide 5 that spans the 12 overlapping amino acids was used as a template for the alanine variants. PBMCs from five donors that had a positive response to this epitope were expanded *in vitro* and restimulated with the alanine variant peptides (Fig. 4C). We identified two point mutations that can optimally diminish the response by more than 85%: E270A and R274A diminished the average response by $88\% \pm 9\%$ and $87\% \pm 8\%$, respectively.

3.5. Construction and activity of deimmunized RIT variants

Based on the alanine scan of domain II and based on analysis of the domain II structure, mutant RITs with point mutations were constructed, and their activity examined. Each alanine mutation found to be effective to prevent T cell activation was cloned into V_H anti-Tac-PE38 plasmid. Point mutations that are located in the center of the furin cleavage site (amino acids 273–285) like R279A and Q281A were not tested in this study due to previous data demonstrating that such mutations severely compromise the catalytic activity [29]. The cytotoxic activity of each single mutant variant was evaluated using HUT-102 cells (Supplementary Table S3). We found that most variants had very good yield and cytotoxic activity (IC₅₀ were lower than 80 pg/ml), though all had lower activity than wild type (WT) aTac-dsFv-PE38. Because Q283A had a lower yield (1.1mg) compared to the other constructs, we also made Q283T. However, Q283T did not have a better yield. Finally, point mutations L299A, Q283A and E270A were chosen as optimal for cytotoxic activity and T cell activity and were used in the final contracts.

To combine the different point mutations, the three mutations in domain II were introduced into V_H anti-Tac-PE38 plasmid (Table 1), the six alanine substitutions in domain III that were identified in previous work [15] were also cloned and a final deimmunized immunotoxin with nine point mutations was evaluated for cytotoxic activity in two T cell lines. We found that most combination variants had very potent cytotoxic activity in the range of 5–44 pg/ml in HUT-102 cells and 15–290 pg/ml in KARPAS 299 cells (Table 1 and Fig. 5). The intermediate RIT, aTac-dsFv-PE38 domain II 3x, that has three point mutations in domain II (and no change in domain III) had a 2-fold decrease in activity compared to WT in both cell lines. The intermediate RIT, aTac-dsFv-PE38 domain III 6x, that has six point mutations in domain III (and no change in domain II) had a 3-fold decrease in activity compared to WT in both cell lines. LMB-142 that combines all nine mutations had a 9-fold and 19-fold decrease in activity compared to WT in HUT102 and KARPAS 299 cells, respectively.

3.6. Estimation of T cell immunogenicity based on alanine scan

To estimate the reduction in T cell responsiveness of the final molecule, we calculated the weighted average of the normalized relative T cell response to the nine mutated peptides. Values were calculated after background subtraction and are shown in Supplementary Table S4. Based on this calculation, we estimate that LMB-142 has a reduction of 74.8% compared to the WT peptides.

3.7. Cytotoxic activity of LMB-142 on patient's cells

To determine the cytotoxic activity of LMB-142 on patient's cells, we used cells from five ATL patients. Figure 6 shows that both variants had good cytotoxic activity. aTac-dsFv-PE38 had a slightly better cytotoxic activity (though not significantly $p=0.18$) than LMB-142 in all five samples, with a IC_{50} median of 6.9 pg/ml and 22 pg/ml, respectively.

3.8. Off target toxicity in mice

To evaluate the non-specific toxicity of LMB-142, we treated small groups of mice with variable single doses of aTac-dsFv-PE38 and LMB-142. Table 2 shows that aTac-dsFv-PE38 is tolerated at 0.35 mg/kg and toxic at 0.4 mg/kg. This maximal tolerated dose is in agreement with the toxic dose previously published for the scFv RIT (LMB-2) [30]. LMB-142 was significantly better tolerated. A dose of 0.4 mg/kg of LMB-142 did not affect the health of the mice, and neither did a 3.5-fold higher dose of 1.5 mg/kg. A dose of 2mg/kg caused mortality in 4/8 mice and 3 mg/kg was toxic in 4/4 mice.

4. Discussion

In this study we used a rational design approach to make a next generation immunotoxin that targets CD25 and has lower immunogenicity by eliminating as many T cell epitopes as possible in domain II and III of the toxin. This is the first time that domain II of PE38 is engineered with point mutation replacement in order to diminish immunogenicity.

Previous efforts to eliminate the T cell epitopes in domain II of PE38 involved a deletion of 103/114 amino acids in domain II, which resulted in very effective and cytotoxic immunotoxins that target CD22 and mesothelin [15, 24–26]. However, this approach was not effective when we constructed an immunotoxin that targets CD25. We found that aTac-ds-PE24 was 20–100 fold less active in multiple cell lines (Fig. 2A) and that this reduction in activity is not a result of binding interference (Fig. 2C) or unique to specific cells. The finding that PE24 was 20-fold less active than PE38 in CA46 cells when conjugated to aTac antibody but no difference was observed when conjugated to a CD22 antibody indicates that the reduction in activity is receptor-or antibody-mediated. This demonstrates the complexity of rational design of immunotoxins; the deimmunized toxin cannot be “cut and pasted” interchangeably into any antibody, and specific engineering is required to match the toxin to the antibody.

We used alanine mutagenesis because alanine substitutions reduce the binding of a peptide to an HLA molecule [31, 32]. Substitutions to other amino acids than alanine can also be useful in deimmunization [33–35]. We previously used alanine scanning to identify

mutations that can reduce T cell activation in epitope 1 [18]. Here, we repeated the alanine scanning for epitope 1 with two changes; 1) the peptides used to stimulate the cells were longer and inclusive for the three peptides that compose the epitope (21 mer, spanning peptides 13, 14 and 15), and 2) different PBMC samples were used for the most part. The results presented in this work are in agreement with the previous work, with two minor differences. First, in the previous alanine scan, we found four mutations that can diminish the T cell response (L297A, Y298A, L294A and L299A). Similarly, the alanine scan results shown in Figure 4A also identified three of those mutations (L297A, L294A and L299A) as the best mutations to diminish the epitope, however Y298A was not identified. Furthermore, the alanine substitution in the previous work led to a greater reduction in T cell activation (80%-90%), whereas the reduction observed here was not as impressive (50%-60%). The difference in the results can be explained by the longer peptide length and inclusion of PBMC samples that activate peptides 13 and possibly have different HLA haplotypes. Those samples that activate peptide 13 but not peptide 15 can potentially have a different binding core, and require different mutations to prevent the activation.

When we combined the point mutations in domains II and III we found that the six point mutations in domain III induced a 3-fold decrease in activity. Such a decrease in cytotoxic activity was also observed in other deimmunized RIT that were designed in our lab (targeting CD22 and mesothelin). This loss in activity was suspected to be attributed to one of those mutations (R494A). The three novel point mutations in domain II induced an additional reduction in activity which resulted in a total reduction of 9- and 20-fold in cytotoxic activity in two cells lines. This reduction in cytotoxic activity in LMB-142 may be compensated by the resultant low nonspecific toxicity that was observed in mice, which allowed to give very high doses of LMB-142. Furthermore, the reduction of immunogenicity should allow multiple treatment cycles that otherwise could not be given, and lead to an improved anti-tumor effect.

Anti-tumor experiments in mice are useful to understand the toxicity/activity/immunogenicity relationship and to demonstrate that the deimmunized molecule is superior to the original LMB-2. However, there is currently no good and reliable mouse model that could examine the three parameters of immunogenicity, anti-tumor activity and nonspecific toxicity and their interaction.

In conclusion, we developed a next generation immunotoxin (LMB-142) targeting CD25 expressing cells. LMB-142 has potent cytotoxic activity, low nonspecific toxicity, and low immunogenicity. We found that domain II of the toxin is required for targeting CD25 but not CD22 expressing cells and identified three new mutations that greatly diminish immunogenicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADA	anti-drug antibodies
ATL	adult T cell leukemia
dsFv	disulfide stabilized variable fragment
PE38	<i>Pseudomonas</i> exotoxin A
PBMC	peripheral mononuclear cells
RIT	recombinant immunotoxin
R-PE	phycoerythrin-tagged anti-CD25 antibody
scFv	single chain Fv
SFC	spot forming cells
Tac	scFv antibody that targets CD25
WT	wild type

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Highlights

- Recombinant immunotoxins that target T cell leukemias are immunogenic in patients
- Unlike other RITs, deletion of domain II from LMB-2 severely compromises its activity
- We identified and eliminated T cell epitopes within domain II of PE38
- LMB-142 contains a 38kDa toxin and 9 point mutations to diminished T cell response
- LMB-142 has good cytotoxic activity and has lower nonspecific toxicity in mice

Fig. 1.

Schematic structure of PE38 and derivative RIT. PE38 is composed of three major structural domains; domain I (blue) domain II (yellow) and domain III (red). PE38 is composed of domain I and II. aTac-dsFv-PE38 is composed of a dsFv antibody that targets CD25 (anti-Tac) (green). aTac-dsFv-PE24 includes the antibody moiety, an 11 amino acid-long fragment of domain II and the entire domain III. aTac-dsFv-PE24 domain III 6x has six point mutations in domain III and LMB-142 has three point mutations in domain II and six in domain III (mutations shown in purple).

Fig. 2.

Domain II is necessary for activity of aTac RIT. **A.** Deletion of domain II inhibits cytotoxic activity by >95% in five cell lines. Cells were treated in quadruplicates with various concentrations of aTac-dsFv-PE38 or aTac-dsFv-PE24 and evaluated for cell viability using the WST-8 cell proliferation kit. Cytotoxicity curves were created and IC_{50} calculated. Averaged relative IC_{50} are shown (n = 3 assays for each cell line). Error bars indicate SEM. **B.** Deletion of domain II reduces activity in anti-Tac RITs but not anti-CD22 RIT. CA46 cell line that naturally expresses CD22 was transfected with CD25 and selected using FACS for high expression of CD25. Cells were treated with variable concentrations of aTac-dsFv-PE38 (solid red), aTac-dsFv-PE24 (broken red), aCD22-dsFv-PE38 (solid blue) or aCD22-dsFv-PE24 (broken blue) and evaluated for cell viability using the WST-8 cell proliferation kit. Data points represent mean and error bars represent SD. **C.** Deletion of domain II does not impact binding of the aTac moiety. HUT-102 cells were labeled with PE conjugated anti CD25 antibody. aTac-dsFv-PE38 and aTac-dsFv-PE24 were added at various concentrations. PE labeled cells were detected using Flow cytometry and affinity was calculated.

Fig. 3.

T cell epitopes in domain II. PBMC from 41 donors were expanded *in vitro* for 14 days using PE38 immunotoxin and restimulated with eight peptide pools that span the amino acid sequence of domain II. T cell activation was assayed using IL2 ELISpot. Pools that had positive responses were de-convoluted for the individual peptides that compose those pools. The accumulative normalized responses are shown. Different shades of gray in each bar represent responses of different donors, and the height of the bar represents the sum of the responses. The dotted line represents 3-fold of the accumulative normalized responses observed in the no-peptide negative control.

Fig. 4.

Alanine scanning mutagenesis for epitopes 1, 2 and 3 in domain II of PE38. T-cell cumulative response for alanine variant peptides was evaluated using IL-2 ELISpot, and the SFC responses of each donor were normalized to the response of parent peptide (WT). Different shades of gray in each bar represent responses of different donors, the sum of the responses. Small bars indicate mutants that diminish the epitope. Alanine variants of: (A) Epitope 1, (B) Epitope 2 and (C) Epitope 3. Alanine scans for all epitopes were run in quadruplicates and repeated once.

Fig. 5.

Cytotoxic activity of intermediate aTac RITs. HUT-102 and KARPAS299 Cells were treated in quadruplicates with various concentrations of aTac-dsFv-PE38 (blue), aTac-dsFv-PE38 domain II 3x (red), aTac-dsFv-PE38 domain III 6x (green) and LMB-142 (purple). Cell viability was evaluated by the WST-8 cell proliferation kit. Data points represent mean and error bars represent SD.

Fig. 6.

Cytotoxic activity in cells obtained from ATL patients. Cells from five ATL patients were treated with variable concentrations of aTac-dsFv-PE38 and LMB-142. Cell viability was evaluated using the ATP cell titer glow kit. Cytotoxicity curves were created and IC₅₀s were calculated. Center values are medians.

Table 1

Cytotoxic activity of aTAC RITs

Name	Domain II substitutions	Domain III substitutions	Cytotoxic activity (IC ₅₀ pg/ml)	
			HUT 102 ^I	KARPAS 299
aTac-dsFv-PE38	WT		5±1.8	15.2±7.1
aTac-dsFv-PE38 domain II 3x	E270A, L299A, Q283A	WT	9±2	35.7±19.5
aTac-dsFv-PE38 domain III 6x	WT	R427A, F443A, L477H, R494A, R505A, L552E,	14.5±1.6	58.2±53.5
LMB-142	E270A, L299A, Q283A	R427A, F443A, L477H, R494A, R505A, L552E,	44±12	298.9±241

^I Variability indicates SD values

Table 2

Off target toxicity of aTac-dsFv-PE38 and LMB-142

	Dose (mg/kg)	Frequency	Response	% Change in body weight	Number of mice
aTac-dsFv-PE38	0.35	1	Healthy	-2	4
	0.4	1	4/4 dead	-3	4
LMB-142	1.5	1	Healthy	-3	4
	2.0	1	4/8 Healthy	-1	8
	3.0	1	4/4 dead	-3	4