# A β-lactamase with reduced immunogenicity for the targeted delivery of chemotherapeutics using antibody-directed enzyme prodrug therapy

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## Abstract

Antibody-directed enzyme prodrug therapy (ADEPT) delivers chemotherapeutic agents in high concentration to tumor tissue while minimizing systemic drug exposure. β-Lactamases are particularly useful enzymes for ADEPT systems due to their unique substrate specificity that allows the activation of a variety of lactam-based prodrugs with minimal interference from mammalian enzymes. We evaluated the amino acid sequence of β-lactamase from *Enterobacter cloacae* for the presence of human T-cell epitopes using a cell-based proliferation assay using samples from 65 community donors. We observed a low background response that is consistent with a lack of preexposure to this enzyme. β-Lactamase was found to contain four $CD4^+$ T-cell epitopes. For two of these epitopes, we identified single amino acid changes that result in significantly reduced proliferative responses while retaining stability and activity of the enzyme. The β-lactamase variant containing both changes induces significantly less proliferation in human and mouse cell assays, and 5-fold lower levels of IgG1 in mice were observed after repeat administration of β-lactamase variant with adjuvant. The β-lactamase variant should be very suitable for the construction of ADEPT fusion proteins, as it combines high activity toward lactam prodrugs, high plasma stability, a monomeric architecture, and a relatively low risk of eliciting an immune response in patients. [Mol Cancer Ther 2005;4(11):1791–800]

## Introduction

Chemotherapy is the most common treatment option for patients with metastatic cancers. However, the dose of most chemotherapeutic agents is limited by their severe side effects resulting in a very narrow therapeutic window. Antibody-directed enzyme prodrug therapy (ADEPT) has the potential to overcome this limitation of systemic chemotherapy. ADEPT systems are based on fusion proteins or chemical conjugates that combine two domains: (*a*) an antibody fragment that binds selectively to tumor tissue and (*b*) an enzyme domain that catalyzes the conversion of a nontoxic prodrug to an active drug at the tumor site. This site-specific activation of prodrug results in a high local concentration of the chemotherapeutic agent in tumor tissue while minimizing exposure of healthy tissues. ADEPT systems have been described based on a variety of different enzymes (1–3).

One of the main obstacles for the clinical application of ADEPT is the risk of eliciting an immune response to the enzyme-antibody fusion protein. Several clinical trials of ADEPT systems based on the bacterial enzyme carboxypeptidase G2 have been done. These trials showed the clinical benefit of the ADEPT method (4–6). However, all patients formed antibodies against the protein components that limited the number of treatment cycles. One approach toward reducing the effect of an immune response has been to use human enzymes within the ADEPT constructs. This can be achieved by engineering human-derived enzymes to give them a unique substrate specificity (7, 8). Alternatively, one can use intracellular enzymes thereby relying on the limited ability of prodrugs to reach these enzymes in healthy tissues (9, 10). These approaches require carefully designed prodrugs to avoid the risk of prodrug conversion in healthy tissues. In addition, the use of human enzymes fused to an antibody fragment carries the risk of triggering an autoimmune response, and even fully human proteins are known to trigger immune responses (11). Engineering of human enzymes may increase this risk, as it can introduce novel T-cell or B-cell epitopes. Furthermore, human enzymes are often unstable and difficult to manufacture. An interesting recent report describes the engineering of a stabilized variant of human prolyl endopeptidase that has been constructed for the use in ADEPT (12). It remains to be proven if human enzymes can be used to construct robust fusion proteins that can be manufactured in large quantity and will have low immunogenicity.

An alternative to the use of human enzymes is to use bacterial enzymes for ADEPT that have been engineered to have a low risk of eliciting an immune response. Bacterial enzymes have several properties that make them very attractive for ADEPT systems. They tend to be stable, have

high catalytic rates, and are generally easier to manufacture than mammalian enzymes. A particular advantage is that one can use enzymes with specificities that are unique to bacteria. This allows the design of prodrugs that are not converted by any human enzyme. Of particular interest is β-lactamase, which has been reported to activate a variety of prodrugs to release commonly used cancer drugs, such as doxorubicin (13), vinblastine (14), Taxol (15), paclitaxel (16), and melphalan (17). Lactamases are produced by bacteria in response to treatment with lactam antibiotics. Lactam-based drugs have been used for many years to treat bacterial infections. In general, lactams have low toxicities, and a significant cleavage of lactams by other human hydrolases has not been reported. Finally, lactamases are relatively small single domain enzymes that tend to be very stable and easy to purify (18). Although all these properties make lactamases ideal candidates for ADEPT applications, bacterial proteins are likely to be immunogenic in humans.

The goal of our present research was the construction of a β-lactamase mutant that is associated with a reduced risk of eliciting an immune reaction in human patients. β-Lactamase from *Enterobacter cloacae* was selected as the starting enzyme, as it has been shown to activate prodrugs based on multiple commonly used chemotherapeutic agents. The amino acid sequence of β-lactamase was assessed for the presence of CD4^{+} T-cell epitopes using an *in vitro* assay (19). Subsequently, several CD4^{+} T-cell epitopes from the β-lactamase sequence were modified to reduce its immunogenic potential while retaining sufficient protein expression and stability to preserve the utility of the enzyme for use in ADEPT. Our data suggest that the human population has not been preexposed to β-lactamase from *E. cloacae*. Furthermore, we describe a variant of β-lactamase with significantly reduced *in vitro* and *in vivo* immunogenicity that retains functional properties suitable for application in ADEPT.

## Materials and Methods

### Materials

Cephalosporin-melphalan was kindly provided by Peter Senter and Brian Toki (Seattle Genetics, Inc., Bothell, WA). Tumor cell line MBA-MB-231 (HTB-26) and recommended culture medium were obtained from American Type Culture Collection (Manassas, VA). Nitrocefin was from Oxoid (Ogdensburg, NY). PBS and octylglucopyranoside were purchased from Sigma (St. Louis, MO). Human plasma was obtained from the Stanford University Blood Center (Palo Alto, CA). Synthetic 15-mer peptides overlapping by 12 amino acids were purchased from Mimotopes (San Diego, CA). Peptides were constructed using the Multi-Pin synthesis technique (20). The sequence of *E. cloacae* β-lactamase (accession no. X07274) was used to design peptides. Epitope variant peptides were also constructed using the Multi-Pin synthesis technique. Peripheral blood buffy coat samples were purchased from the Stanford University Blood Center and BloodSource (Sacramento, CA). All materials were handled in accordance with the Genencor International Bloodborne Pathogen Handling Guidelines (Palo Alto, CA).

### Determination of CD4^{+} T-Cell Epitopes

CD4^{+} T-cell peptide epitope determinants were identified using community donor blood as described (19). Briefly, dendritic cells were differentiated *in vitro* from adherent monocytes. CD4^{+} T cells from the autologous donor were cocultured with activated dendritic cells and peptide for 5 days in AIM V medium (Life Technologies, Carlsbad, CA). Proliferation was assessed using tritiated thymidine incorporation. The CD4^{+} T cell-to-dendritic cell ratio was 10:1. Peptides were dissolved in DMSO and diluted to a concentration of 5 μmol/L. DMSO was present at 0.25% volume in the final assay and in all positive and negative controls. Positive control was tetanus toxoid (List Biologicals, Campbell, CA). For each donor, peptides were tested in duplicate. Responses were averaged, and the average counts/min (cpm) for each peptide was divided by the control cpm (DMSO only). A stimulation index of ≥2.95 was considered a positive response. The stimulation index values were compiled for a large replicate of community donors. The percentage of donors responding to each peptide is reported. Epitope designations were determined as described previously (19). *HLA-DRB1* and *HLA-DQB1* alleles were determined for each donor using a commercially available kit (Dynal, Brown Deer, WI). Low stringency results are reported. HLA associations were determined for responsiveness to any given peptide using a $\chi^2$ analysis (1 degree of freedom). Where an allele was present in both the responder and the nonresponder population, a relative risk value was reported.

### *In vitro* Peripheral Blood Mononuclear Cell Proliferation

Peripheral blood mononuclear cells (PBMC) from community donors were cultured at $2 \times 10^6$ per mL in RPMI 1640 supplemented with 5% heat-inactivated human AB-negative serum. β-Lactamase and β-lactamase variants were purified as described. All proteins were provided at 2 mg/mL, with <0.3 EU/mL endotoxin. The optimum concentration for parametric testing was determined in pilot studies to be 20 μg/mL (data not shown). Control cultures contained no added protein. Cultures were incubated at 37°C for 5 days. On day 5, cultures were resuspended and 100-μL aliquots were transferred to 96-well plates. Tritiated thymidine was added at 0.5 μCi/well. Tritiated thymidine incorporation was assessed after 6 hours. Each PBMC culture was tested in replicates of at least eight wells. Proliferation data were collected for 30 individual donors. A positive response was indicated if the proliferative response reached a stimulation index (average experimental cpm / average control cpm) of ≥1.99. A parametric, two-tailed Student's *t* test was done. Significance was determined at $P < 0.05$.

### Mouse Model for *In vitro* Proliferation

Female CB6F1 mice (6–8 weeks of age) were immunized i.p. with 20 μg β-lactamase or β-lactamase variant in alum on days 1, 3, and 10. Splenocytes were prepared on day 15. Splenocytes were cocultured at $5 \times 10^5$ per well in 96-well plates with 5 μg/mL β-lactamase 15-mer peptides. Cultures were pulsed on day 5 with 0.5 μCi tritiated

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  1 TPVSEKQLAEVVANTITPLMKAQSVPGMAVAVIYQGKPHYYTFGKADIAA  50
    | | | | | | | | | | | | | | | | | | | | | | | |
    1           5             10              15
 51 NKPVTPQTLFELGSISKTFTGVLGGDAIARGEISLDDAVTRYWPQLTGKQ 100
    | | | | | | | | | | | | | | | | | | | | | | | |
         20          25            30
101 WQGIRMLDLATYTAGGLPLQVPDEVTDNASLLRFYQNWQPQWKPGTTRLY 150
    | | | | | | | | | | | | | | | | | | | | | | | |
       35          40            45            50
151 ANASIGLFGALAVKPSGMPYEQAMTTRVLKPLKLDHTWINVPKAEEEAHYA 200
    | | | | | | | | | | | | | | | | | | | | | | | |
            55            60            65
201 WGYRDGKAVRVSPGMLDAQAYGVKTNVQDMANWVMANMAPENVADASLKQ 250
    | | | | | | | | | | | | | | | | | | | | | | | |
         70          75            80
251 GIALAQSRYWRIGSMYQGLGWEMLNWPVEANTVVEGSDSKVALAPLPVAE 300
    | | | | | | | | | | | | | | | | | | | | | | | |
       85          90            95           100
301 VNPPAPPVKASWVHKTGSTGGFGSYVAFIPEKQIGIVMLANTSYPNPARV 350
    | | | | | | | | | | | | | | | | | | | | | | | ||
              105           110           115
351 EAAYHILEALQ     361
```

**Figure 1.** Amino acid sequence of β-lactamase from *E. cloacae*. The first amino acid of each synthetic peptide used for epitope mapping is indicated. Residues K21 and S324 that were replaced with alanine to remove T-cell epitopes are underlined.

thymidine and harvested on day 6. cpm data were averaged and are presented as cpm ± SD, or the cpm results were normalized to the control well response (DMSO vehicle only) and are presented as stimulation indices. Typically, peptides were tested in triplicate and controls were tested in six to nine replicates. For whole protein proliferation, mice were immunized with 50 μg β-lactamase or β-lactamase variant in alum on days 1, 8, and 15. Splenocytes were tested *in vitro* on day 20. Splenocytes were cultured at $5 \times 10^5$ per well in 96-well plates with varying concentrations of protein. Cultures were pulsed with 0.5 μCi tritiated thymidine on day 2 and were harvested on day 3.

### Mouse Model for Antibody Responses

Female CB6F1 mice were immunized with 20 μg/mL β-lactamase or β-lactamase variant i.p. with alum on days 1, 3, and 10. Serum samples were obtained on day 15 via the retro-orbital route. Costar enzyme immunoassay plates (Corning, Acton, MA) were coated with a β-lactamase-containing fusion protein. Plates were blocked using RDI diluent. Serum samples from β-lactamase-immunized mice were diluted in RDI diluent and allowed to incubate on the coated plates for 2 hours at room temperature on a plate rotator. Plates were subsequently washed thrice using PBS containing 0.05% Tween 20 (Sigma). Plates were then incubated with 1 μg/mL biotinylated antimouse IgG1 (clone A85-1, PharMingen, San Jose, CA) diluted in RDI diluent for 1 hour. Plates were again washed thrice followed by incubation with horseradish peroxidase-streptavidin (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) at a 1:5,000 dilution. Plates were developed for 15 minutes using TMB substrate (PharMingen) and read at 450 to 570 nm absorbance.

### Construction and Evaluation of β-Lactamase Variants

The expression system used to produce β-lactamase variants as well as the procedure for stability testing has been described recently (21). A pBR322-based vector was used carrying a chloramphenicol resistance marker. Expression was driven by a lac promoter, and the majority of lactamase activity was found in the periplasm of the host TOP10F′ (Invitrogen, Carlsbad, CA). All variants were constructed using the QuikChange procedure (Stratagene, La Jolla, CA) as suggested by the manufacturer.

Variants were cultured at 37°C in microtiter plates with 180 μL/well Luria-Bertani medium containing 5 mg/L chloramphenicol and 0.1 mg/L cefotaxime. Expression of β-lactamase activity was monitored after 1, 2, and 3 days of culture. For most variants, we observed the highest expression after 2 days of culture and we used these samples to assess temperature stability of the variants. The variants were incubated for 1 hour in 100 mmol/L citrate-200 mmol/L phosphate buffer (pH 7.0) at 56°C. The wild-type lactamase retained ~6% of its catalytic activity after heat stress and the stability of the variants was calculated relative to the stability of the wild-type enzyme. Reported stability and expression values are average values for two to four wells.

### Expression and Purification of β-Lactamase and β-Lactamase Variant

β-Lactamase was expressed in TOP10F′ cells in 600 mL Terrific Broth supplemented with 10 mg/L chloramphenicol at 30°C for 48 hours. The culture was centrifuged and the pellets were treated with B-PER (Pierce, Rockford, IL) to release soluble protein. The supernatants were collected by centrifugation and loaded onto column packed with 10 mL phenylboronic acid resin (Sigma) in gravity mode. The column was packed and equilibrated in 20 mmol/L TEA and 0.5 mol/L NaCl at pH 7. After loading the sample, the column was washed in the equilibration buffer and eluted with 0.5 mol/L borate and 0.5 mol/L NaCl at pH 7. β-Lactamase was further purified using a Superdex 75 column (Amersham Biosciences, Piscataway, NJ) to ~99% purity by SDS gel. Samples used for *in vitro* PBMC assays or *in vivo* studies were passed through a 10 mL Detoxi-Gel column (Pierce) according to the manufacturer's
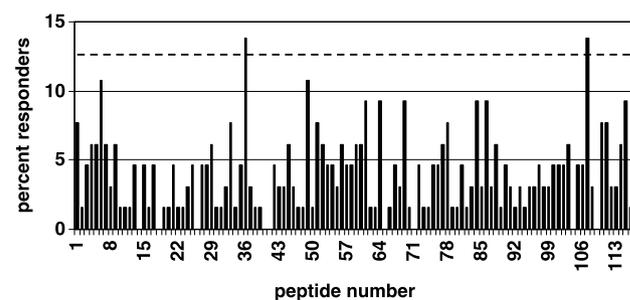


**Figure 2.** Compiled human CD4[+] T-cell proliferative responses to β-lactamase peptides. Sixty-five community donor samples were tested for proliferative responses to 15-mer peptides based on the sequence of β-lactamase as described in Materials and Methods. *Y axis,* percentage of donors responding with a proliferative response greater than 2.95 stimulation index; *dotted line,* a response greater than background plus 3 SDs.

recommendation to remove endotoxin. Endotoxin levels in these samples were <0.3 EU/mg using the LAL assay (Cambrex, East Rutherford, NJ). A typical purification run yielded ~ 50 mg purified protein from 1 liter of culture broth.
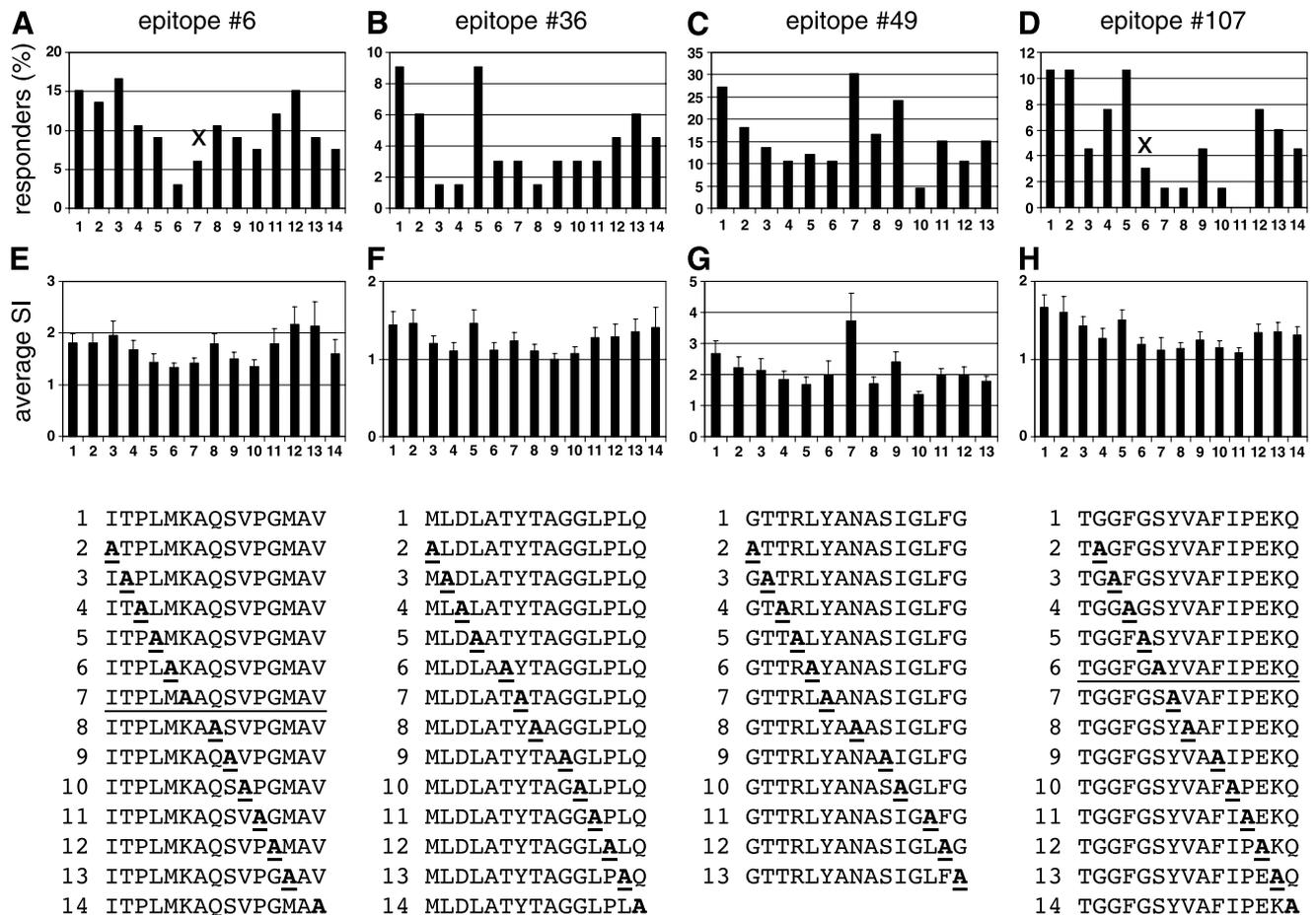
### β-Lactamase Activity Assay and Plasma Stability

The activity of β-lactamase was monitored by adding 20 μL β-lactamase solution into 180 μL nitrocefin working solution at 200 μmol/L in PBS containing 0.125% octylglu-copyranoside. Hydrolysis of nitrocefin results in increase of absorbance at 490 nm (absorbance coefficient at 490 nm, $1.95 \times 10^{-4}$ mol/$L^{-1}$ $cm^{-1}$), which was monitored every 12 seconds for 5 minutes. Kinetic constants of β-lactamase or β-lactamase variant toward nitrocefin were determined by measuring nitrocefin hydrolysis in a range of 0.2 to 400 μmol/L at 25°C with enzyme concentrations of 1 to 2 ng/mL. Kinetic constants for cephalosporin-melphalan were determined by measuring cephalosporin-melphalan hydrolysis in a range of 0.8 to 100 μmol/L by the loss of absorbance at 260 nm (absorbance coefficient at 260 nm, $-2.0 \times 10^{-4}$ mol/$L^{-1}$ $cm^{-1}$). Rate constants were calcu-lated based on Lineweaver-Burk plots. Kinetic constants

are averages of three to six independent experiments. β-Lactamase and β-lactamase variant samples were added to human plasma at 1 μg/mL and incubated at 37°C for 12 days. Samples were taken at various time points over that period and β-lactamase activity was measured as described above. Three samples were incubated for each variant and the resulting activities were averaged.

### *In vitro* Cytotoxicity

MDA-MB-231 cells were grown in monolayer cultures in Leibovitz's L-15 medium with 2 mmol/L L-glutamine containing 10% fetal bovine serum. The cells were seeded into 96-well plates at 50,000 per well and allowed to adhere for 18 hours at 37°C. β-Lactamase and β-lactamase variant were added at a final concentration of 10 nmol/L. Melphalan and cephalosporin-melphalan stock solutions were prepared directly before addition to the cultures. Melphalan was dissolved in 20% polyethylene glycol 400, 0.1 mol/L NaCl, and 0.2% Povidon by sonication. Cepha-losporin-melphalan was dissolved in DMSO at 100 mg/mL. Melphalan and cephalosporin-melphalan were diluted in medium and tested at final concentrations from 2 to



| 1 | ITPLMKAQSVPGMAV | 1 | MLDLATYTAGGLPLQ | 1 | GTTRLYANASIGLFG | 1 | TGGFGSYVAFIPEKQ |
|---|---|---|---|---|---|---|---|
| 2 | **A**TPLMKAQSVPGMAV | 2 | **A**LDLATYTAGGLPLQ | 2 | **A**TTRLYANASIGLFG | 2 | T**A**GFGSYVAFIPEKQ |
| 3 | I**A**PLMKAQSVPGMAV | 3 | M**A**DLATYTAGGLPLQ | 3 | G**A**TRLYANASIGLFG | 3 | TG**A**FGSYVAFIPEKQ |
| 4 | IT**A**LMKAQSVPGMAV | 4 | ML**A**LATYTAGGLPLQ | 4 | GT**A**RLYANASIGLFG | 4 | TGG**A**GSYVAFIPEKQ |
| 5 | ITP**A**MKAQSVPGMAV | 5 | MLD**A**ATYTAGGLPLQ | 5 | GTT**A**LYANASIGLFG | 5 | TGGF**A**SYVAFIPEKQ |
| 6 | ITPL**A**KAQSVPGMAV | 6 | MLDLA**A**YTAGGLPLQ | 6 | GTTR**A**YANASIGLFG | 6 | TGGFG**A**YVAFIPEKQ |
| 7 | ITPLM**A**AQSVPGMAV | 7 | MLDLAT**A**TAGGLPLQ | 7 | GTTRL**A**ANASIGLFG | 7 | TGGFGS**A**VAFIPEKQ |
| 8 | ITPLMKA**A**SVPGMAV | 8 | MLDLATY**A**AGGLPLQ | 8 | GTTRLYA**A**ASIGLFG | 8 | TGGFGSY**A**AFIPEKQ |
| 9 | ITPLMKAQ**A**VPGMAV | 9 | MLDLATYT**A**AGLPLQ | 9 | GTTRLYANA**A**IGLFG | 9 | TGGFGSYV**A**AIPEKQ |
| 10 | ITPLMKAQS**A**PGMAV | 10 | MLDLATYTAG**A**LPLQ | 10 | GTTRLYANAS**A**GLFG | 10 | TGGFGSYVAF**A**PEKQ |
| 11 | ITPLMKAQSV**A**GMAV | 11 | MLDLATYTAGG**A**PLQ | 11 | GTTRLYANASIG**A**FG | 11 | TGGFGSYVAFI**A**EKQ |
| 12 | ITPLMKAQSVP**A**MAV | 12 | MLDLATYTAGGL**A**LQ | 12 | GTTRLYANASIGL**A**G | 12 | TGGFGSYVAFIP**A**KQ |
| 13 | ITPLMKAQSVPG**A**AV | 13 | MLDLATYTAGGLP**A**Q | 13 | GTTRLYANASIGLF**A** | 13 | TGGFGSYVAFIPE**A**Q |
| 14 | ITPLMKAQSVPGMA**A** | 14 | MLDLATYTAGGLPL**A** | | | 14 | TGGFGSYVAFIPEK**A** |

**Figure 3.** Alanine scanning of β-lactamase epitopes. *Bottom,* sequences of the alanine variants of epitope peptides; *sequence 1,* original sequence. **A – H,** CD4[+] T-cell proliferative responses that were observed for alanine variants of peptides 6, 36, 39, and 107. Mutations K21A and S324A that were incorporated into the final β-lactamase variant are marked in **A** and **D** and the peptide sequences containing these mutations are underlined.

**Table 1.** Expression and stability of β-lactamase variants

| Epitope | Mutation | Expression | Stability |
|---------|----------|-----------|-----------|
| 6 | M20A | 0.05 | ND* |
| 6 | K21A | 1.73 | 0.53 |
| 6 | S24A | 2.23 | 1.11 |
| 6 | V30A | 0.12 | 7.6 |
| 36 | L107A | 0.10 | ND* |
| 36 | D108A | 0.09 | ND* |
| 36 | T113A | 0.21 | ND* |
| 36 | P118A | 0.16 | ND* |
| 49 | T146A | 0.47 | 0.42 |
| 49 | T147A | 0.43 | 0.38 |
| 49 | L149A | 0.73 | 0.2 |
| 49 | I155A | 0.19 | ND* |
| 107 | S324A | 0.80 | 0.65 |
| 107 | V326A | 0.53 | 0.50 |
| 107 | I329A | 0.05 | ND* |
| 107 | P330A | 0.06 | ND* |

NOTE: Variants were grown in Luria-Bertani medium at 37°C and lactamase expression was measured after 1 day. Thermostability was measured after incubation at 56°C for 1 hour. The parent enzyme β-lactamase retains ~6% of its activity after heat stress. Expression and stability data are average of two to four replicates and shown relative to the parent enzyme β-lactamase, which was set to 1.
*Expression was too low to allow stability assessment.

1,000 μmol/L. The cells were incubated with the drug for 3 hours at 37°C. Subsequently, the cells were washed thrice with medium and incubation was continued in fresh medium for 20 hours at 37°C. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carbo-xymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium reagent (Promega, Madison, WI) was added to each well and survival was measured after 2 hours based on absorbance at 490 nm. Each data point was measured in triplicate. The entire experiment was done thrice, giving very similar results.

## Results

### CD4[+] T-Cell Epitope Mapping of β-Lactamase

The parent gene for our work was a mutant of β-lactamase from *E. cloacae* that has shown good activity in a published ADEPT system (22, 23). The amino acid sequence of β-lactamase is shown in Fig. 1. A set of 117 synthetic 15-mer peptides, overlapping by 12 amino acids, was constructed. The peptides were tested in a human CD4[+] T-cell–based proliferative assay (19) using peripheral blood samples from 65 community donors. The results are shown in Fig. 2. A proliferative response was considered positive for a given peptide if the average stimulation index was ≥2.95. The percentage of positive responses within the 65-donor set for each peptide is shown on the *Y* axis. The *X* axis indicates the β-lactamase peptides. The average percent response for all the peptides tested was 4.04 ± 2.86. This background rate is low, consistent with a lack of donor preexposure to β-lactamase (19).

Four CD4[+] T-cell epitopes were identified in β-lactamase. Peptides were designated as epitopes if the percent response was equal to or greater than the background plus 3 SDs

(12.62%), which is very unlikely to occur by chance. The peptides at positions 36 and 107 meet this criteria. The peptides at positions 6 and 49 displayed responses slightly <12.62% but were selected for modification due to their prominence in the data set. The response rates to all four peptides were significantly different from the background at *P* < 0.05.

To identify amino acid variants that reduce the rate of proliferative responses by donor CD4[+] T cells, we created sets of variant peptides for each of the four selected epitopes. Each of these alanine scan peptides has one amino acid replaced with alanine. The sequences of these peptides are shown in Fig. 3. Alanine scan peptides and parent sequence (sequence 1 in Fig. 3) were synthesized and tested in the CD4[+] T-cell proliferation assay with a set of 66 community donors. The percent responses (Fig. 3A-D) and the average stimulation indices (Fig. 3E-H) are shown. The percent response rate for the resynthesized parent epitope peptides 6, 36, and 107 were approximately equivalent to the rates seen in the original data set. The response rate for the resynthesized parent epitope 49 peptide was found to be considerably higher, 27.3% versus 10.8% in the original data set. Proliferative responses to this peptide epitope within the original data set displayed a strong association with the presence of the *HLA-DRB1*09* allele (*P* = 0.009). The HLA types of the donors in the alanine scan retest were not available. Although present at ~1% gene frequency in Caucasian populations, the *HLA-DRB1*09* allele has an estimated gene frequency of 9.7% in Asian populations. Because our donor numbers are small, it is likely that the HLA content varies in each set of tested donors. Therefore, a likely explanation for the discrepant percent response data is a skewed *HLA-DRB1*09* content in the donor set that was used to generate the alanine scan data. Peptide 49 was tested a third time using a third group of 66 donors, and a percent response rate of 12.12% was measured.
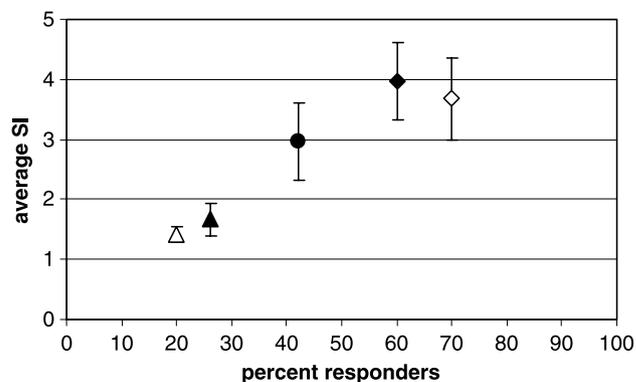


**Figure 4.** Reduced human PBMC proliferative responses to the β-lactamase variant. Unfractionated PBMC from 30 donors were tested with 20 μg/mL streptokinase (*diamonds*), 20 μg/mL unmodified β-lactamase (*circle*), or β-lactamase variant (*triangles*). *Y* axis, average stimulation index (*SI*) ± SE; *X* axis, percentage of donors who mounted a stimulation index of >1.99; *open and closed symbols,* two separate experiments; *open circle,* data done parametrically; *closed symbols,* done parametrically in a second experiment with a different set of 30 donors.

### Evaluation of Single Amino Acid Variant β-Lactamase Proteins

Based on the alanine scan data, four alanine substitutions were chosen for each epitope sequence. β-Lactamase mutants were constructed and evaluated for enzyme production and stability using a microtiter plate–based procedure. We did not determine if variants lost activity due to reduced expression or reduced specific activity, as both of these properties are critical for the utility of the enzyme. Functional alanine mutations were identified for two of the four epitopes (Table 1). For epitope 6, mutation K21A was selected as it improves expression of β-lactamase while leading to a small but acceptable loss in thermostability. Mutation S324A was selected for epitope 107, which abolishes the proliferative response to the epitope peptide with only minor losses in stability and expression. All tested mutations in epitope 36 led to significant losses in expression and/or stability. This result was unexpected as two of the positions, L107 and D108, show a low degree of conservation among homologous lactamase sequences. Based on an analysis of the β-lactamase structure and the sequence of homologues, the difficulty of modifying epitope 49 was expected. Therefore, several non-alanine substitutions were tested for this epitope but none of these changes led to sufficiently robust variants. Based on testing all single amino acid variants, it was decided to avoid changes in epitopes 36 and 49 and combine mutations K21A and S324A to yield the variant molecule β-lactamase variant.

### Production and Purification of β-Lactamase Variant

Expression of the engineered β-lactamase variant was compared with the parent protein β-lactamase. Both proteins were expressed at very high levels as soluble proteins and titers of 430 and 600 mg/L were observed in shake flask cultures of β-lactamase and β-lactamase variant, respectively. Both proteins were purified to single-band purity by affinity chromatography using phenylboronic acid agarose followed by size exclusion chromatography.

### *In vitro* Testing of Immunogenicity

To test the comparative immunogenicity of the β-lactamase variant versus the parent protein, a human cell proliferation assay was established. Unfractionated PBMC from 30 community donors were incubated with an optimal concentration of purified, endotoxin-free β-lactamase, β-lactamase variant, or streptokinase. After 6 days, proliferation was assessed by tritiated thymidine incorporation. Donors with a stimulation index of ≥1.99 were considered positive. Stimulation indices from all 30 donors were averaged and are presented in Fig. 4 in relation to the percentage of donors with a positive response. Streptokinase was included as a positive control. The optimal concentration of 20 μg/mL protein was determined by dose response in pilot experiments (data not shown). Results from two separate experiments are shown. The average stimulation index for streptokinase was >3.0 in both experiments, and the percentage of positive donors was ~70%. Forty-two percent of the donors mounted positive responses to the parent enzyme
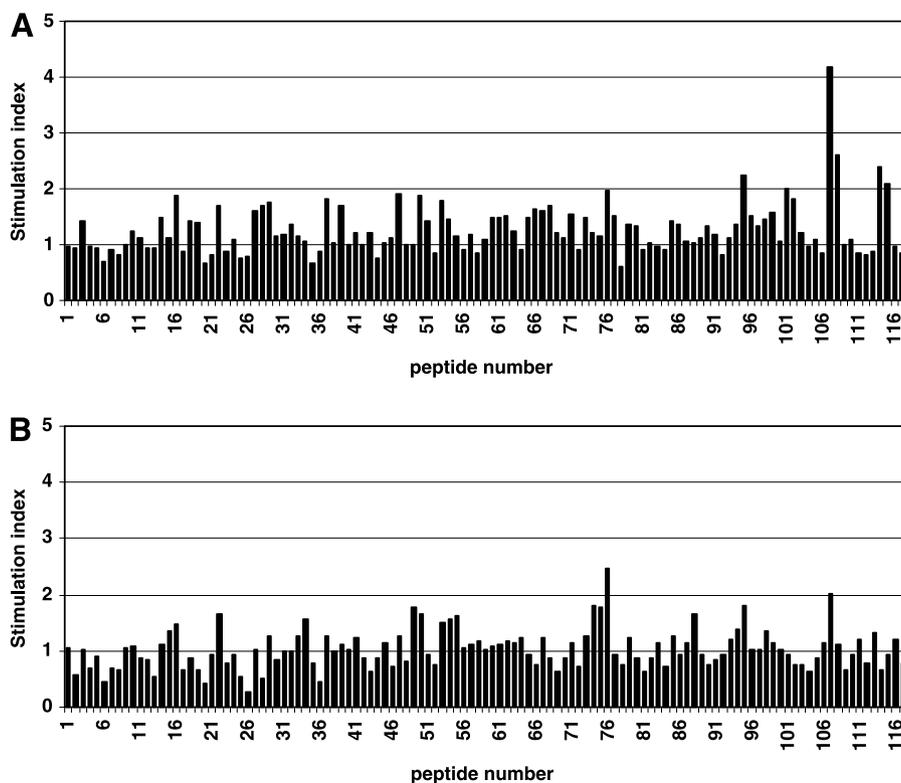


**Figure 5.** The β-lactamase variant removes a major T-cell epitope in CB6F1 mice. Groups of three C57Bl/6 mice were immunized with β-lactamase (**A**) or β-lactamase variant (**B**). Splenocytes were pooled and tested *in vitro* with 15-mer peptides describing the sequence of β-lactamase. Mice have a major epitope response to β-lactamase at peptide 107 and a prominent response to peptide 114. Immunization with the β-lactamase variant did not result in a response to peptide 107 or 114.
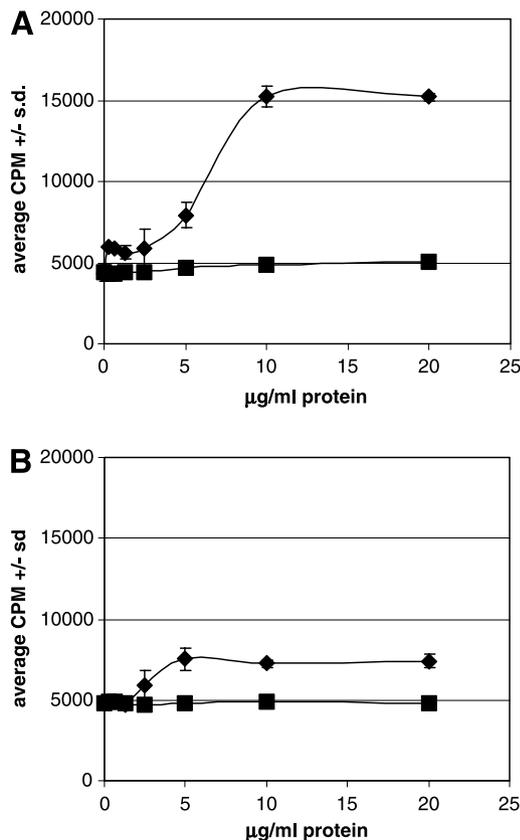
**Figure 6.** The β-lactamase variant induces significantly less proliferation in a mouse model. Groups of four CB6F1 mice were immunized i.p. with 20 μg β-lactamase (**A**) or β-lactamase variant (**B**) in alum on days 1, 8, and 15. Splenocytes were tested *in vitro* on day 20 with β-lactamase (♦) or β-lactamase variant (■). Proliferation was assessed by tritiated thymidine incorporation at 72 h. *X axis,* titration of protein *in vitro*; *Y axis,* average cpm ± SD.

protein, β-lactamase. Supporting the apparent immunogenicity of the parent molecule, the average stimulation index was 3.0. In comparison, the proliferative responses to the β-lactamase variant were much weaker. In the two experiments shown, 20% to 30% of the donors were responders to the protein, and the average stimulation indices were <2.0. This result confirms that the two amino acid substitutions in peptide epitopes 6 and 107 significantly affect the immunogenicity of the whole protein molecule.

### *In vivo* Testing of Immunogenicity

It was of interest to test the β-lactamase variant protein *in vivo*. Because the molecule was modified based on human *in vitro* proliferation data, mouse strains were tested to select a model that most closely aligned with the epitope results from humans. The mouse F1 strain CB6F1 was selected as it displayed a prominent proliferative response *in vitro* to the peptide 107 epitope when immunized with β-lactamase (Fig. 5). The mice also displayed a secondary response to the peptide 114 region of the parent β-lactamase molecule. However, when immunized with β-lactamase variant, the peptide responses to 107 and 114 were significantly reduced.

This result shows that the S324A change in the peptide 107 region reduces the capacity of that peptide to induce a proliferative response and that the reduction of the response to the immunodominant T-cell epitope reduces subsequent responses to subdominant epitopes. This phenomenon has been observed before when the dominating T-cell epitope of β-IFN was removed (24). In response to the β-lactamase variant immunization, it seems that a prominent response to peptide 76 develops. However, the magnitude of this response is quite low.

The *in vitro* responses to β-lactamase variant protein were also significantly reduced relative to β-lactamase (Fig. 6). Mice were immunized with either β-lactamase or β-lactamase variant, and splenocytes were tested *in vitro* with both proteins. Immunization with the parent β-lactamase molecule resulted in a robust proliferative response (Fig. 6A). However, β-lactamase-primed cells do not proliferate in response to the β-lactamase variant protein. This result suggests that the majority of the T-cell proliferative response in these assays is directed at the peptide 107 epitope. Immunization with the β-lactamase variant induces a low level of proliferative response to β-lactamase *in vitro* but no response to the β-lactamase variant molecule (Fig. 6B). A low level of response that is specific for the variant protein may indicate the presentation of a unique epitope that is not present, or processed, from the parent β-lactamase molecule.

Serum samples were obtained from β-lactamase and β-lactamase variant immunized mice to detect antigen-specific IgG formation (Fig. 7). Consistent with the peptide and protein proliferation results, the level of antigen-specific IgG1 induced in mice immunized with β-lactamase variant was found to be >5-fold reduced relative to β-lactamase.

### Functional Testing of β-Lactamase Variant

To verify that epitope removal did not significantly affect the utility of β-lactamase, we studied the hydrolysis kinetics of the chromogenic lactam antibiotic nitrocefin as well as
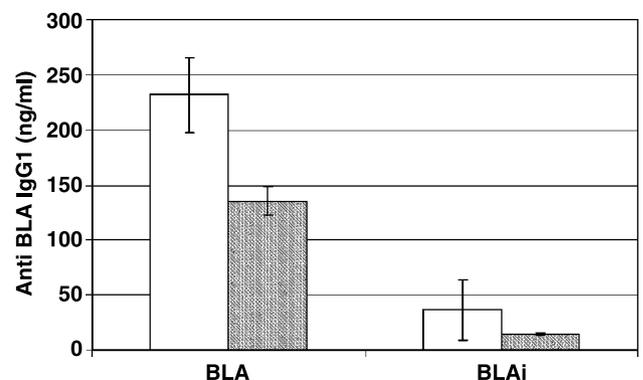


**Figure 7.** β-Lactamase variant induces significantly less antigen-specific IgG1 in CB6F1 mice than β-lactamase. Groups of three mice were immunized i.p. with 20 μg/dose β-lactamase (*BLA*) or β-lactamase variant protein (*BLAi*) on days 1, 3, and 10. Serum samples were taken on day 15. An ELISA was done, and results were standardized to a control curve. *X axis,* the serum sample that was tested. Plates were coated with β-lactamase (*open columns*) or β-lactamase variant (*gray columns*).

**Table 2.** Kinetics of nitrocefin and prodrug hydrolysis by β-lactamase and β-lactamase variant

| Protein | Substrate | $k_{cat}$ (s$^{-1}$) | $K_m$ (μmol/L) | $k_{cat}/K_m$ (mol/L$^{-1}$ s$^{-1}$) |
|---|---|---|---|---|
| β-Lactamase | Nitrocefin | 359 ± 16 | 13.8 ± 2.3 | $2.6 \times 10^7$ |
| β-Lactamase variant | Nitrocefin | 272 ± 17 | 15.7 ± 4.1 | $1.7 \times 10^7$ |
| β-Lactamase | Cephalosporin-melphalan | 904 ± 124 | 294 ± 21 | $3.0 \times 10^6$ |
| β-Lactamase variant | Cephalosporin-melphalan | 656 ± 36 | 336 ± 61 | $2.0 \times 10^6$ |

the prodrug cephalosporin-melphalan (Table 2). Both proteins have identical $K_m$ values and the catalytic rate of β-lactamase variant is only slightly reduced relative to β-lactamase. The rate constants for hydrolysis of cephalosporin-melphalan are in good agreement with previously published data by Kerr at al. (17). Analysis of plasma stability showed that both proteins are extremely stable in human plasma, with >60% of activity remaining after a 12-day incubation in human plasma at 37°C (Fig. 8). To further test the utility of β-lactamase variant for ADEPT, we studied the *in vitro* activation of the melphalan-based prodrug cephalosporin-melphalan that has shown promising *in vivo* activity in a melanoma model of ADEPT (17). Figure 9 shows that both β-lactamase and β-lactamase variant are able to fully activate the prodrug cephalosporin-melphalan.

## Discussion

The β-lactamase enzyme from *E. cloacae* was found to contain four CD4$^+$ T-cell epitopes as determined by a human cell-based proliferation assay. Two of the four epitopes were modified with single amino acid substitutions that were selected based on reduced proliferative responses and acceptable functional activity and expression. Protein β-lactamase variant was made and tested in an *in vitro* human cell proliferation assay and a selected mouse model. The variant induced less proliferation in mouse and human cell assays and 5-fold lower levels of antigen-specific IgG1 antibody in mice compared with the unmodified parent protein. The β-lactamase variant molecule was found to be highly active in the conversion of a prodrug to the toxic drug in an *in vitro* tumor cell cytotoxicity assay.

ADEPT is a powerful approach that allows the delivery of chemotherapeutic drugs directly to tumor tissue. Consequently, one can increase the dose of drug in the tumor tissue while reducing systemic exposure. The tumor-targeted delivery of several drugs has been shown successfully *in vivo* using a variety of enzyme/prodrug combinations (1, 2, 25). However, one of the largest obstacles for the clinical use of ADEPT is the risk of eliciting an immune response to the enzyme-antibody conjugate or fusion protein that is a key component of all ADEPT systems. The present work addresses this issue by constructing a variant of β-lactamase that retains all valuable properties of β-lactamase for ADEPT while significantly reducing the risk of generating an immune reaction in patients.

**Immunogenicity during Previous ADEPT Clinical Trials**

Results for three clinical trials of ADEPT have been published, and a fourth trial is currently ongoing (1). Encouraging tumor responses were observed in the first two trials although accompanied by significant systemic toxicity. Immunogenicity seemed to be a key obstacle as all patients generated antibodies against the protein components of the treatment. Immune responses could not be prevented by cyclosporine administration and therefore limited the number of treatment cycles per patient. It is not known if carboxypeptidase G2 contains any T-cell epitopes. However, several other factors could have contributed to the immunogenicity observed in these ADEPT trials. All published trials used chemical conjugates between antibody fragments and carboxypeptidase. It has been observed that chemical conjugates of proteins have relatively high immunogenicity (26). The current clinical trial uses a fusion protein between carboxypeptidase and a single chain antibody fragment, and it will be interesting to see if this measure leads to reduced immunogenicity. Furthermore, carboxypeptidase is a dimeric enzyme. Consequently, fusion proteins or conjugates of carboxypeptidase G2 have relatively slow plasma clearance, and various clearance mechanisms have been explored to accelerate the plasma elimination of ADEPT constructs involving this protein (27). These approaches, particularly the use of a clearing antibody (28), may have aggravated immunogenicity. In contrast, β-lactamase is a monomeric protein and ADEPT fusion proteins between β-lactamase and an antibody fragments showed sufficiently fast plasma clearance when studied *in vivo* (23, 29).
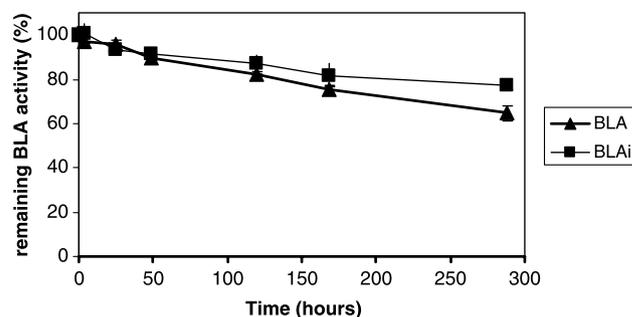
**Figure 8.** Stability of β-lactamase and β-lactamase variant (*BLAi*) in human plasma at 37°C. The β-lactamase activity was monitored >12 d. Average of triplicate experiments.
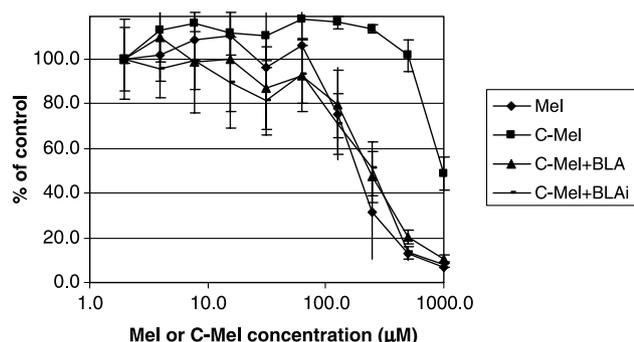
**Figure 9.** *In vitro* activation of cephalosporin-melphalan. Cells were incubated with β-lactamase/β-lactamase variant (10 nmol/L) and melphalan (*Mel*) or cephalosporin-melphalan (*C-Mel*) at 2 to 1,000 μmol/L for 3 h and then washed. Cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium reagent assay. Average of triplicates. The experiment was repeated thrice with variation of <15%.

## Conclusion

The β-lactamase variant described here has several features that make it very attractive for use in ADEPT. β-Lactamase is a very stable monomeric single-domain protein. It has been shown that β-lactamase can be fused to a single chain antibody fragment (23) or a VHH domain (29) and the fusion proteins showed good tumor retention as well as rapid plasma clearance. β-Lactamase has been shown to activate prodrugs based on a variety of chemotherapeutics. Furthermore, β-lactamase can be produced in very high yield as a soluble protein in *Escherichia coli* and it can be effectively recovered by affinity chromatography. The β-lactamase variant described here retains all favorable traits of β-lactamase for ADEPT and the removal of two T-cell epitopes should significantly reduce the risk of eliciting an immune response in patients. The evaluation of fusion proteins between antibody fragments and β-lactamase variant is currently in progress.

## T-Cell Epitopes Are Critical for Immune Response

The induction of high titer antigen-specific IgG responses is dependent on the presence of CD4[+] T helper cell responses (30, 31). In the absence of helper cell priming, antibody responses that develop are of low titer and tend to be limited to IgM. This effect is especially important in the design of vaccines, where the inclusion of degenerate HLA class II–binding peptides is crucial for the activation of protective immunity (32). In addition, the inclusion of a "foreign" CD4[+] T-cell epitope peptide sequence into an autologous protein sequence has been shown to overcome nonresponsiveness in a mouse model (33). It follows that the elimination of helper CD4[+] T-cell epitopes from protein sequences would result in variants with a reduced capacity to induce immune responses (34). Using a variety of techniques to localize and eliminate CD4[+] T-cell epitopes, several groups have published reports on reduced immunogenicity of therapeutic proteins (35, 36, 24). The functional method used here allows for the characterization of immunodominant CD4[+] T-cell epitope responses on a human population basis (19, 37). Elimination of immunodominant responses has been shown to reduce responses to subdominant CD4[+] T-cell epitopes in both epitope spreading and direct priming responses (38, 24). In this report, two of the four identified CD4[+] T-cell epitopes in β-lactamase were modified to reduce their potential for inducing an immune response *in vitro*. Removal of these two epitopes had a profound effect on the overall immunogenicity of the whole protein when tested with human PBMC and in mice. Removal of the remaining two epitopes requires more drastic protein modifications. Considering that *in vitro* and *in vivo* immune responses to β-lactamase variant already approach the limit of detection of available assays, such further modification may not justify the risk of compromising other performance or expression-related properties of β-lactamase variant.

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# Molecular Cancer Therapeutics

# A β-lactamase with reduced immunogenicity for the targeted delivery of chemotherapeutics using antibody-directed enzyme prodrug therapy

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