Submitted to ADVANCED HEALTHCARE MATERIALS

Supporting Information

for *Adv. Healthcare Mater.*, DOI: 10.1002/adhm.201200435

A self-adjuvanting supramolecular vaccine carrying a folded protein antigen

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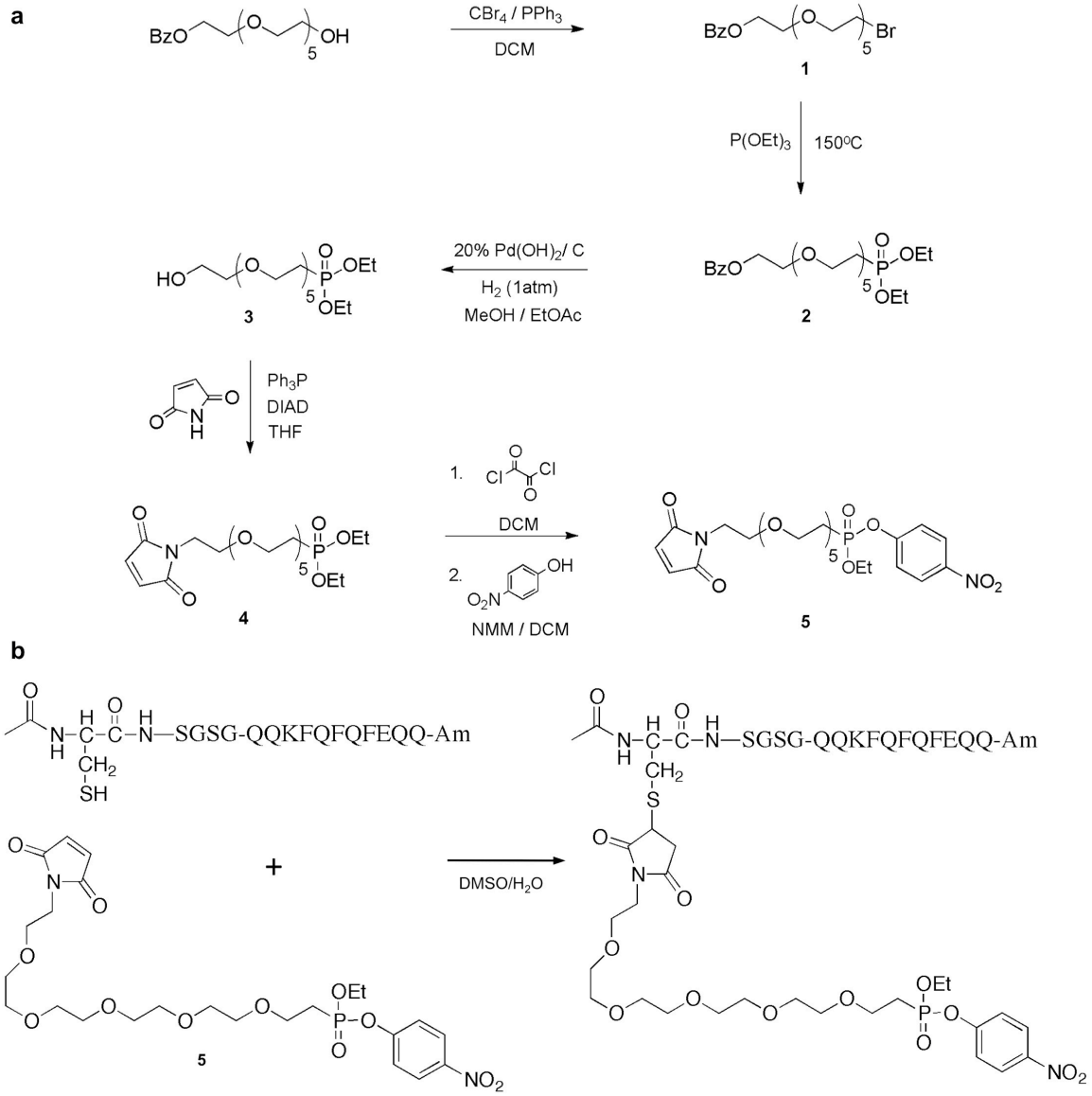
DETAILED METHODS

Peptide synthesis. Dimethylformamide, diethyl ether, trifluoroacetic acid (TFA), and dichloromethane were purchased from Fisher Scientific. Piperidine, p-nitrophenyl butyrate, and acetic acid were purchased from Sigma-Aldrich. All amino acids and Rink Amide AM resin were purchased from Novabiochem. The peptides Q11 (QQKFQFQFEQQ) and Cys-SGSG-Q11 were synthesized using a standard Fmoc solid-phase peptide synthesis protocol involving DIEA/HOBt/HBTU activation. Q11 was cleaved from the resin using 95% TFA/2.5% TIS/2.5% DI H2O and precipitated from the TFA cocktail using cold diethyl ether. Peptides were collected by centrifugation and washed five times in ether. The resulting peptide pellet was dried over vacuum, dissolved in deionized water, frozen, and lyophilized to dryness.

Synthesis of pNP-Q11.



Supplemental Figure 1. Synthesis of (a) Ethyl (4-nitrophenyl) (17-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3,6,9,12,15-pentaoxaheptadecyl)phosphonate (5) and (b) pNP-Q11.

19-bromo-1-phenyl-2,5,8,11,14,17-hexaoxanonadecane (1). Monobenzyl hexaethylene glycol (2.0 g, 5.37 mmol), and CBr_4 (3.56 g, 10.74 mmol) were dissolved in CH_2Cl_2 (30 mL) and cooled to 0°C in an ice water bath. To this solution was added triphenylphosphine (2.82 g, 10.74 mmol), the reaction allowed to warm to room temperature, and stirred for 14 hr. After this period, the reaction was concentrated on a rotary evaporator to yield a crude white oil. This oil was resuspended in Et_2O to precipitate triphenylphosphine oxide, filtered, concentrated, and then purified via flash chromatography first using a gradient of 10:1 → 3:1 hexane : ethyl acetate to remove non-polar impurities then by

elution with 1:1 hexane : EtOAc to yield a colorless oil (1.98 g, 85%): ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.47 (t, 2H), 3.67 (m, 20H), 3.81 (t, 2H), 4.57 (s, 2H), 7.28 (m, 1H), 7.35 (m, 4H). MALDI-MS: *m/z* 458.4077 ([M + Na]$^+$, calcd for $C_{19}H_{31}BrO_6Na$ 458.3396).

Diethyl 1-phenyl-2,5,8,11,14,17-hexaoxanonadecan-19-yl-phosphonate (2). Compound 1 (2.61 g, 6.00 mmol) was dissolved in triethyl phosphite (20 mL, excess), the reaction flask fitted with a reflux condenser and the mixture heated to 150°C for 4 hr. After this period, the mixture was allowed first to cool to room temperature and then the excess triethyl phosphite removed by short path distillation under reduced pressure at 70°C. The resulting clear and colorless oil was then purified via flash chromatography on silica by using EtOAc first to remove non-polar impurities then by elution with 10:1 EtOAc : MeOH to afford the product as a light yellow oil (2.57 g, 87%): ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.32 (t, 6H), 2.13 (dt, 2H), 3.66 (m, 20H), 3.72 (m, 2H), 4.10 (m, 4H), 4.57 (s, 2H), 7.28 (m, 1H), 7.33 (m, 4H). MALDI-MS: *m/z* 515.5947 ([M + Na]$^+$, calcd for $C_{23}H_{41}O_9PNa$ 515.5298).

Diethyl 17-hydroxy-3,6,9,12,15-pentaoxaheptadecylphosphonate (3). Compound 2 (1.98 g, 4.02 mmol) was dissolved in nitrogen-sparged 1:1 EtOAc : MeOH (20 mL). To this solution was added 20% $Pd(OH)_2$ on carbon (wet) (100 mg) and the reaction was stirred under an atmosphere of H_2 (1 atm) for 14 hr. After this period, the reaction was filtered through a pad of celite, and concentrated in vacuo to yield a colorless oil that was used without further purification (1.49 g, 92%): ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.31 (t, 6H), 2.13 (dt, 2H), 3.65 (m, 19H), 3.72 (m, 4H), 4.09 (m, 4H). MALDI-MS: *m/z* 425.5341 ([M + Na]$^+$, calcd for $C_{16}H_{35}O_9PNa$ 425.4072).

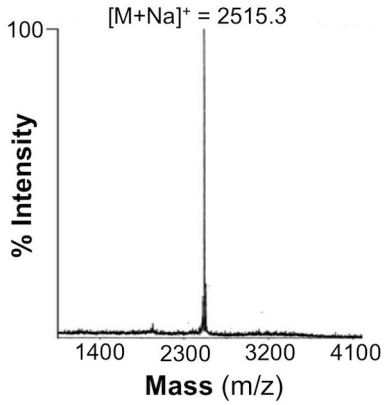
Diethyl (17-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3,6,9,12,15-pentaoxaheptadecyl)phosphonate (4). PPh_3 (325 mg, 1.24 mmol) was dissolved in CH_2Cl_2 (5 mL). To this solution was added diisopropylazodicarboxylate (DIAD, 95%, 264 mg, 1.24 mmol) dropwise over 1 min. Upon addition of DIAD, a slight reflux occured. At the completion of the addition, the solution went from orange to light yellow. This mixture was allowed to stir 10 min. After this period, the reaction was cooled to 0°C in an ice water bath followed by addition of compound 3 (500 mg, 1.24 mmol) dissolved in CH_2Cl_2 (1 mL). The reaction was then allowed to stir at 0°C for an additional 10 min. After this period, maleimide (120 mg, 1.24 mmol) was added and the reaction allowed to come to room temperature over 1 hr. After this period, the mixture was concentrated, and the deep yellow oil purified by flash chromatography on silica by using EtOAc first to remove non-polar impurities, then by elution with 10:1 EtOAc : MeOH to afford the product as a light yellow oil (393 mg, 66%): ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.32 (t, 6H), 2.13 (dt, 2H), 3.63 (m, 18H), 3.72 (m, 2H), 3.81 (m, 2H), 4.20 (m, 4H), 6.70 (s, 2H). MALDI-MS: *m/z* 504.9013 ([M + Na]$^+$, calcd for $C_{20}H_{36}NO_{10}PNa$ 504.4641).

Ethyl (4-nitrophenyl) (17-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3,6,9,12,15-pentaoxaheptadecyl)phosphonate (5). Compound 4 (390 mg, 0.81 mmol) was dissolved in CH_2Cl_2 (10 mL) and to the solution was added oxalyl chloride (424 μL, 617 mg, 4.86 mmol) resulting in an immediate and rapid evolution of gas. The reaction was vented and stirred for 5 hr. After this period, the reaction was concentrated on a rotary evaporator and excess volatiles removed with stirring under high vacuum overnight (~14 hr). The resulting orange oil was redissolved in CH_2Cl_2 (15 mL) and cooled to 0°C in an ice water bath. To this solution was then added dropwise over 2 min a solution of 4-nitrophenol (113 mg, 0.81 mmol) and N-methylmorpholine (89 μL, 82 mg, 0.81 mmol) in CH_2Cl_2 (5 mL). The reaction was allowed to come to room temperature with stirring and continued until TLC of the reaction showed no more progress, approximately 2 hr. The reaction was then concentrated on a rotary evaporator to yield a deep orange / brown oil. This oil was then purified via flash chromatography on a short column of silica using CH_2Cl_2 first to remove unreacted 4-nitrophenol and other yellow impurities and then by elution with 10:1 CH_2Cl_2 : MeOH to yield the product as an orange oil (260 mg, 56%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.33 (t, 3H), 2.33 (dt, 2H), 3.60 (m, 18H), 3.65 (m, 2H), 3.75 (m, 2H), 4.19 (m, 2H), 6.71 (s, 2H), 7.40 (d, 2H), 8.23 (d, 2H): MALDI-MS *m/z* 597.3625 ([M + Na]$^+$, calcd for $C_{24}H_{35}N_2O_{12}P$ 597.5044).

CysQ11-EG6-PNP-phosphonate (pNP-Q11) (6). Cys-Q11 (60 mg, 31.3 μmol) was suspended in a mixture of DMSO and water (3 mL / 1.5 mL). To this cloudy suspension was added compound 5 (36 mg, 62.6 μmol) and the reaction allowed to stand at ambient temperature overnight (14 hr). After this period, no starting CysQ11 was detectable by MALDI-MS. This solution was then diluted with 2 mL iPrOH and then poured into ice cold Et_2O (40 mL) in a conical centrifuge tube. This resulted in the immediate formation of an off-white precipitate. This precipitate was pelleted via centrifugation and shaken with room temperature Et_2O. The precipitate was then pelleted by centrifugation. This

washing procedure was repeated two additional times. Excess Et_2O was removed in vacuo. The resulting off-white precipitate was dissolved in 50% aqueous DMSO and purified via Waters Delta 400 HPLC on a C18 reversed phase column with a linear gradient of 75% aqueous acetonitrile + 0.1% trifluoroacetic acid (TFA) as the eluent and 0.1% aqueous TFA as the mobile phase running at 1 mL / min over 1 hr. Fractions corresponding to peaks in the HPLC traces were collected and assayed by MALDI to determine the presence of product (Supplemental figure 2). Fractions containing product were pooled and lyophilized to yield a white powder (21 mg, 30%): MALDI-MS *m/z* 2492.4578 ([M + H]$^+$, calcd for $C_{107}H_{156}N_{26}O_{39}PS$ 2492.0476).

Purification. Q11 and phos-Q11 were purified using a Varian ProStar HPLC system, Grace-Vydac C18 reverse-phase columns, and water-acetonitrile + 0.1% TFA gradients to greater than 90% purity. Peptide molecular weight was verified using MALDI-TOF-MS on an Applied Biosystems Voyager system 6187 with α-cyano-4-hydroxycinnamic acid as the matrix.



Supplemental Figure 2. MALDI-TOF MS of phos-Q11 (expected m/z [M+Na]$^+$ = 2515.04).

Protein expression and purification. A pET-21d vector encoding cutinase-$(GGGGS)_3$-GFPuv with a His6-tag was transformed into Origami B (DE3) E. coli (EMD Biosciences, MA). Ten milliliters of 2XTY media with 100 μg/mL ampicillin and 50 μg/mL kanamycin A was inoculated with E. coli and maintained overnight at 37°C, 220 rpm. The 10 mL culture was subcultured into 1 L 2XTY with 100 μg/mL ampicillin and 50 μg/mL kanamycin A and maintained at 37°C, 220 rpm until an optical density of 0.6 at λ = 600 nm was reached. Protein expression was then induced by adding 0.5 mM IPTG to the culture and maintaining the culture at 18°C, 220 rpm for 16-18 h. Cells were collected by centrifugation, washed, and lysed into 1x PBS containing 1x BugBuster Protein Extraction Reagent (Novagen, CA), 1 protease inhibitor tablet, 300 units DNAse I from bovine pancrease (Sigma-Aldrich, MO), and 100 μg lysozyme for 20 min at room temperature. The lysis buffer was cleared by centrifugation and His6-tagged Cut-GFP was purified from the supernatant using metal-affinity chromatography on HisPur cobalt resin (Thermo Scientific, IL). Column-bound protein was washed twice with 20 mL 1x PBS containing 0.1% Triton X-100 to remove endotoxin. Protein was eluted from the column and concentrated into 1x PBS using centrifugal filter units with a 10,000 DA MWCO (Millipore).

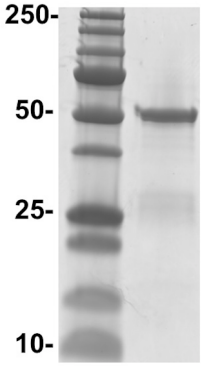
Cut-GFP nucleotide sequence:
ATG *GGC CTG CCT ACT TCT AAC CCT GCC CAG GAG CTT GAG GCG CGC CAG CTT GGT AGA ACA ACT CGC GAC GAT CTG ATC AAC GGC AAT AGC GCT TCC TGC GCC GAT GTC ATC TTC ATT TAT GCC CGA GGT TCA ACA GAG ACG GGC AAC TTG GGT ACC CTC GGT CCT AGC ATT GCC TCC AAC CTT GAG TCC GCG TCC GGC AAG GAC GGT GTC TGG AAT CAG GGC GTT GGC GGT GCC TAC CGT GCC ACT CTT GGA GAC AAT GCT CTC CCT CGC GGA ACC TCT AGC GCC GCA ATC AGG GAG ATG CTC GGT CTC TTC CAG CAG GCC AAC ACC AAG TGC CCT GAC GCG ACT TTG ATC GCC GGT GGC TAC AGC CAG GGT GCT GCA CTT GCA GCC GCC TCC ATC GAG GAC CTC GAC TCG GCC ATT CGT GAC AAG ATC GCC GGA ACT GTT CTG TTC GGC TAT ACC AAG AAC CTA CAG AAC CGT GGC CGA ATC CCC AAC TAC CCT GCC GAT AGG ACC AAG GTC TTC TGC AAT ACA GGG GAT CTC GTT TGT ACT GGT AGC TTG ATC GTT GCT GCA CCT*

CAC TTG GCG TAT GGT CCT GAT GCT CGT GGC CCT GCC CCT GAG TTC CTC ATC GAG AAG GTT CGG GCT GTC CTG GGT TCT GCT GGA TCC GGC GGT GGC GGT TCT GGT GGC GGT GGC TCT GGC GGT GGC GGC TCT AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT GAA TTA GAT GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG GGT GAA GGT GAT GCA ACA TAC GGA AAA CTT ACC CTT AAA TTT ATT TGC ACT ACT GGA AAA CTA CCT GTT CCA TGG CCA ACA CTT GTC ACT ACT TTC TCT TAT GGT GTT CAA TGC TTT TCC CGT TAT CCG GAT CAT ATG AAA CGG CAT GAC TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA CGC ACT ATA TCT TTC AAA GAT GAC GGG AAC TAC AAG ACG CGT GCT GAA GTC AAG TTT GAA GGT GAT ACC CTT GTT AAT CGT ATC GAG TTA AAA GGT ATT GAT TTT AAA GAA GAT GGA AAC ATT CTC GGA CAC AAA CTG GAA TAC AAC TAT AAC TCA CAC AAT GTA TAC ATC ACG GCA GAC AAA CAA AAG AAT GGA ATC AAA GCT AAC TTC AAA ATT CGC CAC AAC ATT GAA GAT GGG TCG GTT CAA CTA GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT GTC CTT TTA CCA GAC AAC CAT TAC CTG TCG ACA CAA TCT GCC CTT TCG AAA GAT CCC AAC GAA AAG CGT GAC CAC ATG GTC CTT CTT GAG TTT GTA ACT GCT GCT GGG ATT ACA CAT GGC ATG GAT GAG CTC TAC AAA CTC GAG CAC CAC CAC CAC CAC CAC TGA

Cut-GFP amino acid sequence:

M *G L P T S N P A Q E L E A R Q L G R T T R D D L I N G N S A S C A D V I F I Y A R G S T E T G N L G T L G P S I A S N L E S A S G K D G V W N Q G V G G A Y R A T L G D N A L P R G T S S A A I R E M L G L F Q Q A N T K C P D A T L I A G G Y S Q G A A L A A A S I E D L D S A I R D K I A G T V L F G Y T K N L Q N R G R I P N Y P A D R T K V F C N T G D L V C T G S L I V A A P H L A Y G P D A R G P A P E F L I E K V R A V L G S A* G S G G G G S G G G G S G G G G S S K G E E L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T Y G K L T L K F I C T T G K L P V P W P T L V T T F S Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K L E H H H H H H Stop

Italics = cutinase (F. solani); Underline = GFPuv

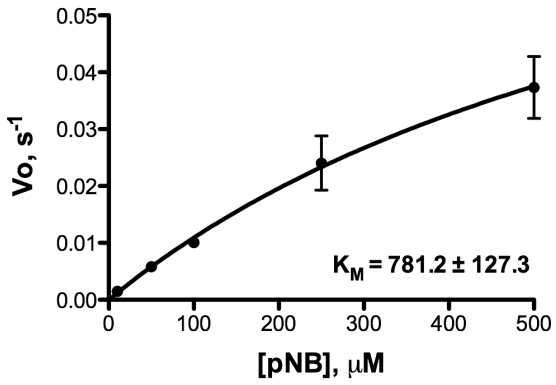


Supplemental Figure 3. SDS-PAGE gel of cutinase-GFP (expected MW 51,248 Da).

Nanofiber assembly. Lyophilized Q11 and pNP-Q11 were dissolved in deionized water at a concentration of 10 mM and 1 mM, respectively, by vortexing and sonication. Aqueous Q11 and pNP-Q11 solutions were then mixed at the appropriate volumetric ratio to give the desired molar ratio. For example, a mixture containing 10 µL Q11 and 1 µL pNP-Q11 would give a Q11/pNP-Q11 molar ratio of 99:1. These solutions were then mixed by vortexing and sonicated to facilitate peptide intermixing. Aqueous Q11/pNP-Q11 solutions were then diluted 1:10 with 1x phosphate-buffered saline (1x PBS) containing cut-GFP. For cutinase and pNP-Q11 reaction characterization, these solutions were then incubated overnight at room temperature on a Barnstead/Thermolyne LabQuake rotator to reduce the diffusion required for cut-GFP to react with pNP-bearing nanofibers. For immunization experiments, solutions of Q11/pNP-Q11 and cut-GFP were incubated on a Barnstead/Thermolyne LabQuake rotator

at 4°C. For fluorimetry experiments, unreacted cut-GFP was removed from the nanofibers at the end of the reaction by: 1) sedimenting the nanofibers via centrifugation at 12000xg for 5 min on an Eppendorf MiniSpin; 2) removing 75% of the supernatant by pipet; 3) adding fresh 1x PBS to the pellet to return to the initial volume; and 4) resuspending the nanofibers by pipetting. This process was then repeated 3 additional times to reduce residual, unreacted protein levels. For cutinase activity assays, samples were analyzed without removal of unreacted protein, as described below.

Cutinase activity assay. Cutinase activity in buffered solutions was assayed using hydrolysis of p-nitrophenol butyrate (pNB) to p-nitrophenol, which results in a change in solution color from colorless to yellow that can be measured spectrometrically. [ref: Kolattukudy P.E., Purdy, R.E., and Maiti, I.B. *Cutinase from Fungi and Pollen,* **Methods in Enzymology,** vol. 71, pg 652-664 1981]. 0.125 μM Cut-GFP cleaved pNB, as indicated by the increased absorbance at 405 nm over time, and the amount of pNB cleaved by cut-GFP was dependent on the amount of pNB present at t = 0. Using non-linear regression analysis in GraphPad Prism software, a plot of V_O versus pNB concentration gave a K_m of 781.2 ± 127.3 μM, and a predicted V_{max} of 0.096 ± .01 μM/s (Supplemental figure 4b). The calculated K_m value was consistent with previous reports of cutinase K_m for pNB [(1) 640 ± 40 μM, Zhang, Y., Chen, S., Xu, M., Cavoco-Paulo, A., Wu, J., Chen, J. **Applied and Environmental Microbiology**, 76, pg 6870-6876. (2) 970 ± 200 μM, Dantzig, A.H., Zuckerman, S.H., Andonov-Roland, M.M. **J Bacteriology**, 168, pg 911-916. (3) 350 ± 20 μM, Brissos, S., Melo, E.P., Martinho, J.M.G., Cabral, J.M.S. **Biochimica et Biophysica Acta – Proteins and Proteomics**, 1784, pg 1326-1334.]. To assay for residual cutinase activity in solution following reaction with fibrillized phos-Q11, solutions containing 0-10 μM pNP-Q11 doped into 1 mM Q11 and 5 μM cut-GFP were reacted overnight at room temperature in 1x PBS. At the end of the overnight reaction, these solutions were diluted 40-fold to give a final cut-GFP concentration of 0.125 μM. These solutions were then spiked with 100 μM p-nitrophenyl butyrate in DMSO, and absorbance at 405 nm was measured for 2.5 minutes. Here, 100 μM pNB was chosen because hydrolysis resulted in the most sensitive and reproducible changes in absorbance, while still being well below the K_m for pNB hydrolysis by cut-GFP (Supplemental figure 4). V_O was calculated from the linear portion of plots of A405 vs time in figure 2b. The amount of unreacted, active cutinase remaining in solution presented in figure 2c was calculated from the ratio of V_O at each condition to V_O of the 0 μM pNP-Q11 group.



Supplementary Figure 4: Plot of initial rate of hydrolysis (V_O) of 10-500 μM pNB by 0.125 μM cutinase-GFP. N = 3, mean ± s.d.

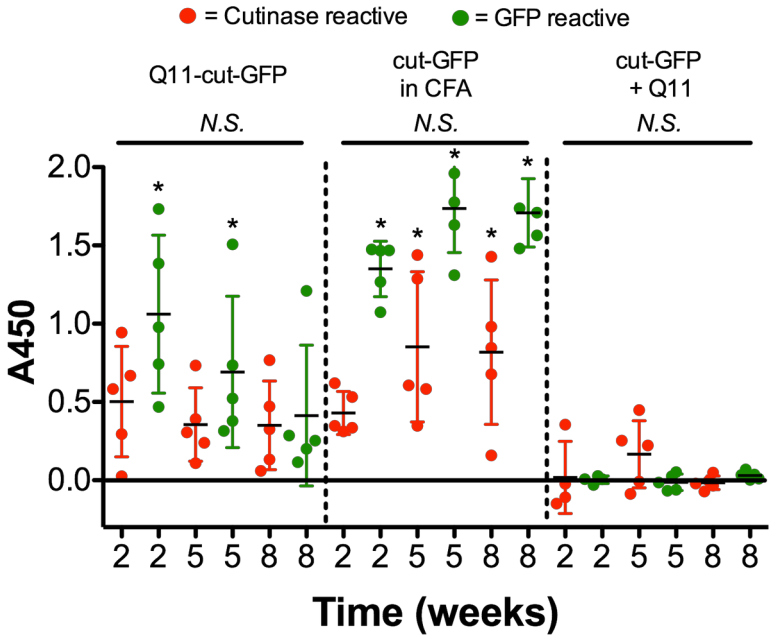
Fluorescence microscopy. Fluorescent photomicrographs of Q11:pNP-Q11 reacted with cut-GFP were collected using a Zeiss Axioscope inverted fluorescent microscope equipped with a FITC filter set. Pseudocolor was applied using ImageJ software (NIH).

ELISAs. ELISA was conducted as previously reported [6], with minimal modifications. Serum anti-cut-GFP Ig titers were determined based on ELISA absorbance values being above or below a cutoff value. The cutoff consisted of the mean plus three times the standard deviation of the negative control group (mice receiving cut-GFP in PBS) for each corresponding dilution. Any sample dilutions having absorbance readings above this cutoff value were considered positive, and the reported titer was the highest dilution for which it and all lower dilutions had positive readings. A titer of 10^2 was reported if no positive dilutions were present. Since mice immunized with cut-GFP in PBS did not raise detectable

IgG, all negative control mice are reported as having titers of 10^2, which is the baseline level of detection for this study.

For all total IgG ELISAs, following overnight coating with 1 μg/mL cut-GFP in PBS, GFP (Vector Labs cat# MB-0752), or a fusion of cutinase and the first 50 amino acids of alpha-hemolysin, an irrelevant fusion domain used to probe anti-cutinase reactivity, all plates were washed 3 times with 0.5% Tween-20 in PBS. Wells were then blocked with 200 μL of 1% BSA/0.5% Tween-20 in PBS for 1 h at room temperature. This solution was removed from the wells, 100 μL of serum diluted $1:10^2$-$1:10^9$ in PBS with 1% BSA was then added directly to the wells without washing, and the plate was incubated for 1 h at room temperature. At the end of serum binding, the solution was removed from the plate, and the plates were washed 5 times with 0.5% Tween-20 in PBS. 100 μL of peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson Immuno Research, cat 115035003) diluted 1:5000 in PBS with 1% BSA was then added to the wells, and the plates were incubated for 45 min at room temperature. At the end of secondary antibody binding, the solution was removed and plates were washed 5 times with 0.5% Tween-20 in PBS. Plates were then developed by adding 100 μL of TMB substrate (eBioscience CA, cat 00-4201-56), incubating for 5 min at room temperature, then quenching the reaction with 50 μL of 1 M H_3PO_4. Absorbance was then measured at 450 nm with a SpectraMax M5 plate reader (Molecular Devices, CA).

For all isotyping ELISAs, following overnight coating with 1 μg/mL cut-GFP or GFP in PBS (Vector Labs cat# MB-0752), all plates were washed 3 times with 0.5% Tween-20 in PBS. Wells were then blocked with 200 μL of 1% BSA/0.5% Tween-20 in PBS for 1 h at room temperature. This solution was removed from the wells, 100 μL of serum diluted $1:10^3$ in PBS with 1% BSA was then added directly to the wells without washing, and the plate was incubated for 1 h at room temperature. At the end of serum binding, the solution was removed from the plate, and the plates were washed 3 times with 0.5% Tween-20 in PBS. 100 μL of goat anti-mouse IgG1 (Sigma cat M5532), IgG2a/c (M5657), IgG2b (M5782), IgG3 (M5907), or IgM (M6157) diluted 1:1000 in PBS with 1% BSA was then added to the wells, and the plates were incubated for 30 min at room temperature. At the end of primary antibody binding, the solution was removed from the plate, and the plates were washed 3 times with 0.5% Tween-20 in PBS. 100 μL of peroxidase-conjugated rabbit anti-goat IgG diluted 1:5000 in PBS with 1% BSA was then added to the wells, and the plates were incubated for 15 min at room temperature. At the end of secondary antibody binding, the solution was removed from the plate, and the plates were washed 3 times with 0.5% Tween-20 in PBS. Plates were then developed by adding 100 μL of TMB substrate, incubating for 5 min at room temperature, then quenching the reaction with 50 μL of 1 M H_3PO_4. Absorbance was then measured at 450 nm with a SpectraMax M5 plate reader.



Supplementary figure 5: Raw ELISA A450 values for Figure 3C. Plates were coated with GFP or cutinase fused to an irrelevant antigen and probed with serum collected from C57BL/6 mice immunized with 9.3 µg cut-GFP conjugated to 0.5 mM Q11 co-fibrillized with 5 µM pNP-Q11 (Q11-cut-GFP), emulsified in CFA, or mixed with 0.5 mM Q11 at weeks 2, 5, and 8. Serum dilution is 1:1000 for all groups. *p<0.05 compared to cut-GFP + Q11 group at the respective time point, ANOVA with Tukey's post-hoc analysis.