

SUPPORTING INFORMATION

Redox-Responsive Polycondensate Neoepitope for Enhanced Personalized Cancer Vaccine

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Materials
All the synthetic long peptides (SLPs) were purchased from GenScript (Piscataway, New Jersey, USA). Pam₃CysSer-(Lys)₄ (Pam) was purchased from InvivoGen (San Diego, California, USA). Bis-amine-CpG (5'–/5AmMC6/TCCATGACGTTTCCGT/GCTT/3AmMO/–3', amine-functionalization at 5'– and 3'–positions) was purchased from Integrated DNA Technologies (Coralville, Iowa, USA). Montanide™ ISA 51 VG was a gift from SEPPIC (Paris, France). Murine granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from PeproTech (London, UK). Novex™ 10% - 20% tricine gel and Tricine SDS sample buffer (2X) were purchased from Invitrogen (California, United States). Lipopolysaccharide (LPS), anhydrous dimethysulfoxide (DMSO), triethylamine (TEA), fluoresceinamine and other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Unless otherwise noted, all chemical and biological reagents were used as received. Pierce™ quantitative fluorometric peptide assay, ProLong™ diamond antifade mountant, Alexa Fluor™ 647 N-hydroxysuccinimide (NHS) ester, Alexa Fluor™ 488 N-hydroxysuccinimide (NHS) ester, LysoTracker™ Red DND-99, Hoechst 33342, CellTrace™ carboxyfluorescein succinimidyl ester (CFSE) cell proliferation kit, and Zombie Aqua™ Fixable Viability Kit were purchased from BioLegend (San Diego, California, USA). eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors) was purchased from Invitrogen (Carlsbad, California, USA). Cytofix/Cytoperm™ fixation/permeabilization kit was purchase from BD Bioscience (San Jose, CA, USA). Mouse CD8⁺ T cell isolation kit was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).
Antibodies for fluorescence-activated cell sorting (FACS) including anti-CD16/32 (Clone: 93), anti-CD3 (Clone: 17A2), anti-CD8α (Clone: 53-6.7), anti-CD11c (Clone: N418), anti-CD40 (Clone: 3/23), anti-CD44 (Clone: IM7), anti-CD62L (Clone: MEL-14), anti-CD80 (Clone: 16-10A1), anti-CD86 (Clone: GL-1), anti-Granzyme B (anti-GrzmB, Clone: GB11), anti-IFN-γ (Clone: XMG1.2), and anti-TNF-α (Clone: MP6-XT22) were purchased from BioLegend (San Diego, California, USA). iTAg Tetramer/PE-H-2 Kb OVA (SIINFEKL) was purchased from MBL (Woburn, Massachusetts, USA).

**Instruments**

Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker AVANCE NEO 400 MHz spectrometer (Billerica, Massachusetts, USA). Electrospray ionisation mass spectra (ESI-MS) was acquired on a LTQ Orbitrap ELITE ETD (Thermo Fisher Scientific). Matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-TOF-MS) was acquired on an Autoflex Speed (Bruker, Billerica, Massachusetts, USA). Polycondensate neoepitope (PNE) was characterized by PL-gel permeation chromatography (GPC) 50+ Integrated GPC/SEC System (Agilent, Santa Clara, California, USA) and UltiMate™ 3000 ultra high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific) equipped with a Hypersil Gold™ C18 selectivity LC column or a BioBasic™ SEC 300 LC column, and detectors of diode array detector (UV, DIONEX UltiMate™ 3000) and charged aerosol detector (CAD, DIONEX Corona ultra RS). The fluorescent image of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were taken by Bio-Rad ChemiDoc MP imaging system (Hercules, California, USA). The size of particle was measured by dynamic light scattering (DLS) on Malvern NanoZS (Worcester, UK). The transmission electron microscopy (TEM) images of PNEs were acquired on FEI Tecnai Osiris TEM instrument (FEI, Oregon, USA). The atomic force microscopy (AFM) images of free LEQ and Pam were acquired on a Park NX-10 analytical system (Suwon, South Korea) with non-contact amplitude modulation (PPP-NCHR, Park system) in ambient condition. The fluorescence intensity of samples was measured with a Varioskan® Lux microplate reader (Thermo Fisher Scientific). All the flow cytometry data were acquired using an Attune NxT flow cytometer (Thermo Fisher Scientific). Confocal fluorescent microscope images were acquired with a LSM 700 with 40X or 63X oil objectives (Zeiss, Oberkochen, Germany). Mouse tissue imaging was conducted with an in vivo imaging system (IVIS, PerkinElmer, Waltham, Massachusetts, USA).
Mice and cells

Experimental procedures in mouse studies were approved by the Swiss authorities (Canton of Vaud, animal protocol ID 3206) and performed in accordance with École Polytechnique Fédérale de Lausanne (EPFL) CPG guidelines. Six- to eight-week-old female Thy1.2+ C57BL/6 mice, TCR-transgenic OT-I mice (B6-Tg(TcraTcrb)1100Mjb/J) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) or Charles River Laboratories (Lyon, France) and maintained in the animal facility. Ovalbumin (OVA)-expressing YUMM1.7 (YUMM1.7-OVA), a murine melanoma cell line, was a generous gift from Prof. Ping-Chih Ho (University of Lausanne, Lausanne, Switzerland) and cultured in DMEM complete medium supplemented by fetal bovine serum (FBS, 10%), Penicillin (100 U/mL), Streptomycin (100 µg/mL). Immature bone marrow-derived dendritic cells (BMDCs) were isolated from C57BL/6 mice (Charles River laboratory, Wilmington, Massachusetts, USA), and cultured in RPMI 1640 complete medium containing HI-FBS (10%), L-glutamine (2 mM), Penicillin (100 U/mL), Streptomycin (100 µg/mL), and 2-mercaptoethanol (β-ME, 50 µM) with GM-CSF (20 ng/mL) for 6 days at 37 ºC with CO₂ (5%) before use. Naïve OT-I CD8+ T cells were isolated from splenocytes of OT-I mice with a mouse CD8+ T cell isolation kit.

Synthesis of bi-functional Monomer B<sub>red</sub> (Mon<sub>red</sub>)

The synthesis of Mon<sub>red</sub> was reported previously with minor modification. 1 2-Hydroxyethyl disulphide (1.329 g, 8.616 mmol) was dissolved in anhydrous tetrahydrofuran (THF, 20 mL) in a 100-mL round-bottom flask, followed by addition of phosgene solution (12.5 mL, 15% w/w, 18.95 mmol) in anhydrous THF (10 mL). The reaction mixture was stirred under room temperature (rt) for 2 hours under the protection of N₂, and then concentrated under vacuum. The remaining residue was dissolved again with anhydrous dichloromethane (DCM, 10 mL) and mixed with N-hydroxysuccinimide (NHS, 2.182 g, 18.95 mmol) and anhydrous triethylamine (TEA, 1.918 g, 18.95 mmol) in DCM solution (45 mL). The reaction was stirred...
at rt overnight under the protection of N\textsubscript{2}. The solvent was removed by rotary evaporator. The crude product was purified with silica chromatography (DCM:Methanol=10:1) and recrystallized with icy petroleum. The acicular crystal (2.14 g, yield: 57\%) was dried under vacuum and characterized by \textsuperscript{1}H NMR and ESI-MS (Figure S1). \textsuperscript{1}H NMR (400 MHz, chloroform-d, 25 °C, TMS): \textbf{δ}=4.61 (t, 3\textit{J}(H, H)=4 Hz, 4H, CH\textsubscript{2}), 3.08 (t, 3\textit{J}(H,H)=4 Hz, 4H, CH\textsubscript{2}), 2.88 (s, 8H, CH\textsubscript{2}). ESI (m/z): [M+Na]\textsuperscript{+}=459.0).

**Preparation of polycondensate neoepitopes (PNEs)**

Depending on the solubility of peptides and adjuvants, synthesis of PNEs can be done in anhydrous DMSO or aqueous solution. The detailed information of composition and conditions for the syntheses of each PNE has been summarized in Table S1.

**PNE of SLP only [PNE(LEQ)]:** LEQLESIINFEKLK\textsubscript{5} (LEQ, 50 μg, 16.6 nmol) in anhydrous DMSO (17.24 μL) was mixed with Mon\textsubscript{red} (40 mgmL\textsuperscript{-1} in anhydrous DMSO, 0.76 μL, 69.7 nmol). The mixture was shaken in an Eppendorf ThermoMixer at 25 °C for 30 min at 800 rpm followed by the addition of TEA (2 μL) and another 60-min incubation at 25 °C, 800 rpm. The resultant product was lyophilized overnight to remove DMSO and TEA, and characterized with DLS, HPLC and TEM.

**PNE of SLP with adjuvant [PNE(LEQ-Pam)]:** In an anhydrous DMSO solution (17.24 μL) of LEQ (50 μg, 16.6 nmol) and Pam\textsubscript{3}CSK\textsubscript{4} (4.18 μg, 5.5 nmol), Mon\textsubscript{red} (40 mgmL\textsuperscript{-1} in anhydrous DMSO, 0.76 μL, 69.7 nmol) was added. The mixture was shaken at 25 °C for 30 min at 800 rpm by the addition of TEA (2 μL) and another 60-min incubation at 25 °C, 800 rpm. The resultant product was purified following the same procedure as described above, and characterized with DLS, GPC, HPLC, TEM, and SDS-PAGE.

**Preparation of non-degradable PNE (non-deg. PNE):** Non-degradable PNE(LEQ-Pam) was prepared similarly as described above by replacing Mon\textsubscript{red} with a non-degradable monomer B, bis(sulfosuccinimidyl)suberate (Mon\textsubscript{BS3}, 112.75 nmol). The other procedures were the same as PNE(LEQ-Pam).

Synthesis of PNEs could be performed in aqueous solution with a similar procedure. In a typical synthesis of PNE, same amount of SLP and adjuvant were dissolved in phosphate-buffered saline (PBS, adjusted to pH = 8.5 with Na\textsubscript{2}CO\textsubscript{3}) and mixed with Mon\textsubscript{red} (40 mgmL\textsuperscript{-1} in anhydrous DMSO). The mixture was shaken at 25 °C, 600 rpm, for 30 min. The resultant product was purified by PBS (pH 7.4, 200 μL × 3) with Amicon\textsuperscript{®} centrifugal filters (Merck
Millipore, Burlington, Massachusetts, USA) with molecular weight cut-off (MWCO) of 3 kDa, to get rid of unreacted peptides or Mon-red.

Characterizations of PNEs

NMR: Free LEQ (200 μg) and PNE(LEQ) (200 μg) were ultrafiltered with D₂O (200 μL × 3) using Amicon® centrifugal filters (MWCO 3 kDa, 10000 rpm) to get rid of H₂O, DMSO, salts, and other possible impurities. The trace of water and protons from acid phosphate was further removed by repeating lyophilizing and dissolving with D₂O. The purified free LEQ and PNE(LEQ) were re-dissolved in D₂O (400 μL) respectively, and measured by ¹H NMR spectrometer.

UHPLC: PNE(LEQ) and PNE(LEQ-Pam) were lypholized to get rid of DMSO and TEA. LEQ, Pam, and PNE each was diluted in ultrapure water with a concentration of 0.5 mgmL⁻¹. For LEQ, a mobile phase gradient (A=acetonitrile, B=H₂O+0.1% trifluoroacetic acid (TFA), B at 90% for 0-1 min, 90-10% for 1-18 min, 10-90% for 18-19 min, and 90% for 19-20 min at flow rate of 0.4 mLmin⁻¹) was used on a Hypersil Gold™ C18 selectivity LC column at 40 ºC. For the detection of Pam, a mobile phase gradient (B at 5% for 0-2 min, 5-95% for 2-18 min, 95-5% for 18-19 min, and 5% for 19-20 min at flow rate of 0.4 mLmin⁻¹) was used on the same C18 column. The loading efficiency of LEQ and Pam in PNE(LEQ-Pam) was calculated from the integral area of CAD curves. To verify the increased MW of PNE(LEQ) and PNE(LEQ-Pam) comparing with free PNE, the lypholized PNE(LEQ) and PNE(LEQ-Pam) were dissolved in PBS (pH = 7.4), a mobile phase of NaCl solution (200 mM, pH 7.4 at flow rate of 0.5 mLmin⁻¹) was used on BioBasic™ SEC 300 LC column.

GPC: LEQ and PNE(LEQ-Pam) in PBS (0.1 mgmL⁻¹, pH 7.4) were injected into GPC (100 μL each sample injection) and analyzed with a mobile phase of NaCl (100 mM) and TFA (1%) and flow rate of 1.0 mLmin⁻¹.

DLS: PNEs were diluted with PBS (0.05 mgmL⁻¹, pH 7.4) in Fisherbrand™ PS semi micro cuvettes at rt for DLS measurement with Marven NanoZS. Data represented 3 independent samples for each PNE samples.

TEM: Freshly prepared PNEs was lypholized overnight to get rid of DMSO and TEA. The dry powder was then redissolved with ultrapure water (0.5 mgmL⁻¹, calculated by the stoichiometry of LEQ) and loaded onto a copper grid. After washing with ultrapure water, the sample-loaded copper grid was negatively stained with uranyl acetate (1% w/v in ultrapure water). The TEM
image was taken by FEI Tecnai Osiris TEM instrument (200 kV) equipped with 4k × 2.6k Gatan Orius CCD camera.

**SDS-PAGE:** Freshly prepared PNEs in PBS (0.5 mgmL⁻¹, 7.5 μL, pH = 7.4) was mixed fluoresceinamine in acetone (1 mM, 7.5 μL), and then mixed with Novex™ Tricine SDS sample buffer (2X). The samples were then loaded onto Novex™ 10-20% Tris-Glycine Gels and run in MES buffer at 110V for 60 min. The image of the gel were taken by Bio-Rad microwell plate reader equipped with a gel imager (ex: 590 nm, em: 700 nm).

**Release kinetics and characterization of LEQ from PNE(LEQ-Pam)**
Freshly prepared PNE(LEQ-Pam) (50 µg) diluted in PBS (0.1 mgmL⁻¹, 490 µL, pH 7.4), was added with DL-dithiothreitol (DTT) (10 µL, 100 mM in PBS, pH 7.4). The mixture was aliquot and incubated at rt or 37 ºC. At set time points (0, 2, 4, 8, 12, and 24 hours), the aliquot (10 µL) was collected and analyzed by Pierce™ quantitative fluorometric peptide assay according to the kit protocol. The mixture was kept in dark for 10 min and the fluorescent intensity was quantified with a microplate reader (Ex 390 nm, Em 475 nm). The released LEQ was also characterized with MALDI-TOF-MS to measure the molecular weight (MW) and compare with original LEQ.

**Fluorescent labelling of LEQ, Pam and PNE(LEQ-Pam)**
Solutions of LEQ, Pam, or the mixture of two were added with Alexa Fluor™ 647 NHS ester (10 mgmL⁻¹ in anhydrous DMSO) with equal stoichiometry and then shaken with an Eppendorf ThermoMixer at 25 °C (600 rpm, 10 min). The “labeled” mixture was used for the next step without purification. Additional LEQ, Pam, or both (unlabeled) were then added the labeled mixture followed by the similar procedures for preparation and purification of PNEs as described above. For *in vitro* studies, 10% of LEQ or Pam was fluorescently labeled; for *in vivo* studies, 50% was labeled.

**In vivo lymph node targeting and dendritic cell internalization**
Fluorescently labeled LEQ, Pam, or PNE(LEQ-Pam) in PBS solution (50 µL, pH 7.4) containing equivalent LEQ (4.51 nmol) and/or Pam (1.12 nmol) were subcutaneously injected into tail base (25 µL for each side, both sides) of C57BL/6 mice (female, 7 weeks old, n=3 per group). PBS (50 µL, pH 7.4) was used as a negative control. The mixture of equivalent LEQ and Pam (25 µL) was pipetted vigorously with Montanide ISA 51 VG (25 µL) to generate a
stable emulsion for injection. The mice were sacrificed 24 hours later and inguinal lymph nodes (LNs) were harvested and imaged with IVIS to measure the total fluorescent intensity in LNs (Ex 640 nm, Em 680 nm, exposure time 0.5 s). The LNs were then ground through a 70-μm cell strainer and the collected cells were washed with FACS buffer (0.2% BSA in PBS, 200 μL × 2). The cells were blocked with anti-CD16/32 at 4 ºC for 15 min, and then stained with anti-CD11c (PE/Dazzle™ 594) at 4 ºC for another 20 min followed by washing with FACS buffer (200 μL × 3). The stained cells were resuspended in a 4,6-diamidino-2-phenylindole (DAPI) solution (0.1 μgmL⁻¹, 200 μL) and analyzed with flow cytometry.

\textit{In vitro BMDC internalization}
Immature BMDCs were prepared as described above. On Day 6, the BMDCs were plated in 24-well plates (5×10⁵ cells per well) with RPMI 1640 medium (1 mL, FBS free, penicillin/streptomycin free) and incubated with fluorescently labeled PNE(LEQ-Pam) or other control formulations containing equivalent LEQ (2.25 nmol) and/or Pam (0.56 nmol) at 37 ºC with CO₂ (5%). After 6-hour incubation, BMDCs were harvested, washed with FACS buffer (200 μL × 2), and incubated with anti-CD16/32 at 4 ºC for 15 min. The BMDCs were then stained with PE/Dazzle 594-anti-CD11c at 4 ºC for 20 min, washed with FACS buffer (200 μL × 2), and resuspended in a DAPI solution (0.1 μgmL⁻¹, 200 μL) for flow cytometry analyses.

\textit{In vitro BMDC activation}
Immature BMDCs were prepared as described above. On Day 6, immature BMDCs were plated in 24-well plates (5 × 10⁵ cells per well) with RPMI 1640 complete medium (1 mL) supplemented with GM-CSF (20 ngmL⁻¹) and incubated with PNE(LEQ-Pam) or other control formulations containing equivalent LEQ (2.25 nmol) and/or Pam (0.56 nmol) at 37 ºC with CO₂ (5%). After 48-hour incubation, BMDCs were harvested, washed with FACS buffer (200 μL × 2), and incubated with anti-CD16/32 at 4 ºC for 15 min. The cells were then stained with a mixture of antibodies of PE-anti-CD11c, PE-Cy7-anti-CD80, BV510-anti-CD86, PerCP-Cy5.5-anti-CD40 at 4 ºC for 20 min. The cells were then washed with FACS buffer (200 μL × 2) and resuspended in a DAPI solution (0.1 μgmL⁻¹, 200 μL) for flow cytometry analyses.

\textit{In vitro cross-priming of OT-I CD8+ T cells}
Immature BMDCs were prepared as described above. On Day 6, immature BMDCs were plated in 24-well plates (5 × 10^5 cells per well) with RPMI 1640 complete medium (1 mL) supplemented with GM-CSF (20 ngmL⁻¹). PNE(LEQ-Pam) and other control formulations containing equivalent LEQ (2.25 nmol) and/or Pam (0.56 nmol) were then added to pulse the BMDCs. SIINFEKL (SIIN, 2.25 nmol) with or without Pam (0.56 nmol) were also added as control samples. The BMDCs were cultured at 37 °C with CO₂ (5%) for 24 hours. The pulsed BMDCs (2 × 10^6) were first washed with PBS (200 μL × 3) and then co-cultured with naïve OT-I CD8⁺ T cells (1 × 10⁵) labelled with CFSE (1 μM for 10 million cells in PBS, 5 min, 37 °C) in complete RPMI 1640 medium (200 μL) at 37 °C with CO₂ (5%). The RPMI complete medium was supplemented with FBS (10%), L-glutamine (2 mM), Penicillin (100 UmL⁻¹), Streptomycin (100 μgmL⁻¹), β-ME (50 μM), sodium pyruvate (1 mM), and HEPES (0.02 M). After a 72-hour co-incubation, the cells were harvested, washed with FACS buffer (200 μL × 2), and incubated with anti-CD16/32 at 4 ºC for 15 min. The cells were then stained with PE/Dazzle 594-anti-CD8α at 4 ºC for 20 min, washed with FACS buffer (200 μL × 2), and processed similarly for flow cytometry analyses.

Confocal fluorescent microscope imaging

PNE(LEQ-Pam) or the simple mixture containing equivalent LEQ (4.51 nmol) and Pam (1.12 nmol) in PBS solution (5 μL, pH 7.4) were incubated with immature BMDCs (1 × 10⁶) in complete RPMI 1640 culture medium (1 mL) supplemented with GM-CSF (20 ngmL⁻¹). At set time points (6, 24, and 48 hours), the BMDCs were washed with PBS (1 mL × 2) and then stained with LysoTracker™ Red DND-99 (125 nM) for endolysosome staining and Hoechst 33342 (4 µM) for nuclei staining in phenol/serum-free RPMI 1640 medium at 37 °C with CO₂ (5%) for 1.5 hours followed by PBS washing (1 mL × 2). BMDCs were then fixed with 4% paraformaldehyde (PFA, 100 μL) for 10 min, washed with PBS (1 mL × 2), and resuspended in ProLong™ diamond antifade mountant (10 μL). After centrifuging onto a poly-lysine coated glass slide, the BMDCs were imaged with a LSM 700 confocal microscope with a 40X or 63X oil objective.

In vivo immunization study

C57BL/6 mice (female, 7-week old) were immunized subcutaneously at tail base with LEQ (15 nmol) and Pam (5 nmol) in a PBS solution (50 μL) in form of a simple mixture, an emulsion
formulation in Montainide, or PNE(LEQ-Pam) following a experimental timeline that mice were primed on day 0 and boosted on day 14 and 28.

**Tetramer staining:** On day 7, 21 and 35, peripheral blood (50 µL) was collected from the tail veins of immunized mice to assess the antigen-specific CD8+ T cells (tetramer staining) and cytokine secretion (intracellular cytokine staining) respectively. For tetramer staining, the whole blood (50 µL) was treated with ACK lysing buffer (1 mL × 2) at rt for 5 min to lyse the erythrocyte, and then transferred to a U-bottom 96-well plate. After washing with FACS buffer (200 µL × 2), the cells were resuspended in tetramer staining buffer [50 µL, PBS containing BSA (1% w/v), EDTA (5 mM), and dasatinib (50 nM)] with iTAG Tetramer/PE-H-2 Kb OVA (SIINFEKL) (1 µL) and anti-CD16/32, and incubated at rt for 40 min. Antibodies including FITC-anti-CD8α, PerCP-Cy5.5-anti-CD62L, and BV711-anti-CD44 in a solution of tetramer staining buffer (50 µL) were then added on top of each well and the cells were incubated at 4 °C for another 10 min. The stained cells were washed with a DAPI solution (0.1 µg/mL, 150 µL × 2) and resuspended in tetramer staining buffer for flow cytometry analyses.

**Intracellular cytokine staining:** Blood sample (50 µL) was lysated and transferred to a 96-well plate following the similar procedure with that of tetramer staining. After washing with PBS (200 µL × 2), the cells were resuspended in complete RPMI 1640 medium (200 µL) containing eBioscience™ Cell Stimulation Cocktail (0.4 µL) and incubated at 37 °C for 6 hours to restimulate the CD8+ T cells. The cells were then spun down and washed with FACS buffer, and resuspended with FACS buffer (20 µL) containing anti-CD16/32 (0.3 µL). After incubating at 4 °C for 15 min, another portion of FACS buffer (20 µL) containing anit-CD3 and anti-CD8 were added on top of each well and incubate at 4°C for 20 min. After washing with PBS (200 µL × 2), the cells were stained with Zombie Aqua™ Fixable Viability Kit. The cells were fixed and permeabilized with the BD Fixation/Permeabilization Kit. After washing with BD Perm/Wash™ buffer (250 µL × 2), the cells were resuspended with BD Perm/Wash™ buffer (20 µL) containing anti-Granzyme B, anti-IFN-γ, and anti-TNF-α antibodies. After 30-min incubation at 4 °C, the cells were washed with BD Perm/Wash™ buffer buffer (250 µL × 2) and finally resuspended in the FACS buffer (200 µL) for flow cytometry analyses.

**Tumor challenging:** On day 37 (9 days post the final immunization), the immunized mice (as described above) were challenged subcutaneously with YUMM1.7-OVA cells (5 × 10^5) on the right flank. Tumor growth was monitored every other day from the fifth day post tumor inoculation. Tumor area (product of two measured orthogonal diameters) and body weight were measured every 2 days. Mice were euthanized when the body weight loss was > 15% of the
pre-dosing weight, or the tumor area reached 150 mm$^2$ (as a pre-determined endpoint) or the animal had become moribund.

**In vivo therapeutic study**
Female C57BL/6 mice were subcutaneously inoculated with YUMM1.7-OVA cells ($5 \times 10^5$) on the right flank. The vaccine treatment containing LEQ (15 nmol) and Pam (5 nmol) in a PBS solution (50 μL) in forms of a simple mixture, an emulsion formulation in Montainide, or PNE(LEQ-Pam) were given on day 5, 12 and 19. Tumor growth were monitored every other day following a similar procedure as described above.

**Statistical analysis.**
Statistical analysis was performed using GraphPad Prism 8 (GraphPad software, Inc., La Jolla, CA, USA). Unless otherwise noted, the data are presented as Mean ± SEM. Comparisons of two groups were performed by using two-tailed unpaired Student’s t test. Comparisons of multiple groups at a single time point were performed by using one-way analysis of variance (ANOVA). Comparisons of survival curves were performed by using Log-rank analysis. $P$-values were presented as *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

**Safety consideration**
No unexpected or unusually high safety hazards were encountered.
| Entry | PNE          | Antigen (epitope) | Adjuvant | Monomer A | Monomer B mole ratio (A_{ag} : A_{adj} : B) | Antigen Loading efficiency | Loading capacity[^2] | Preparaiton phase[^3] |
|-------|--------------|-------------------|----------|-----------|-----------------------------------------------|---------------------------|----------------------|-------------------|
| 1     | PNE(LEQ)     | LEQLESIINFEK \_{K_5} | ~        | Mon\text{_{red}} | 1 : 0 : 3                                   | 97.5%                     | 62.9%                | ~                 |
| 2     | PNE(LEQ-Pam) | LEQLESIINFEK \_{K_5} | Pam, CS  | Mon\text{_{red}} | 4 : 1 : 20                                  | > 99%                     | 46.4%                | 7.9%              | DMSO              |
| 3     | Non-deg. PNE | LEQLESIINFEK \_{K_5} | Pam, CS  | Mon\text{_{red}} | 4 : 1 : 80                                  | > 99%                     | 46.4%                | 7.9%              | PBS               |
| 4     | PNE(LEQ)     | LEQLEK\text{_{3}}\text{AAYSI} INFEKL | Pam, CS  | Mon\text{_{red}} | 4 : 1 : 12                                  | 96.5%                     | 59%                  | 9.2%              | PBS               |
| 5     | PNE(K\text{_{5}}LEQ-Pam) | K\text{_{5}}LEQLEAAYSI INFEKL | Pam, CS  | Mon\text{_{red}} | 4 : 1 : 12                                  | > 99%                     | 46.4%                | 7.9%              | DMSO              |
| 6     | PNE(LEQ-Pam) | LEK\text{_{5}}AAYASM TNMELM | Pam, CS  | Mon\text{_{red}} | 4 : 1 : 12                                  | > 99%                     | 46.4%                | 7.9%              | DMSO              |
| 7     | PNE(CSV)     | CSVYDFVWL \_{K_5}  | ~        | Mon\text{_{red}} | 1 : 0 : 3                                   | > 99%                     | 43.1%                | 31.4%             | PBS               |
| 8     | PNE(CLC)     | CLCPGNKYEM \_{K_5} | ~        | Mon\text{_{red}} | 1 : 0 : 1                                   | > 99%                     | 43.1%                | 31.4%             | PBS               |
| 9     | PNE(LEQ-CpG) | LEQLESIINFEK \_{K_5} | CpG      | Mon\text{_{red}} | 4 : 1 : 12                                  | > 99%                     | 43.1%                | 31.4%             | PBS               |

[^1]: Loading efficiency was calculated by conjugated antigen peptides in PNE / total antigen peptides added × 100% based on UHPLC characterizations.
[^2]: Loading capacity was calculated by weight of conjugated antigen peptides or adjuvants in PNE / total PNE weight × 100%. Weight of conjugated peptide antigen was determined by the feeding amount and the loading efficiency; adjuvant and monomer B were assumed for quantitative loading as non-detectable free adjuvant or monomer B was found in UHPLC characterizations.
[^3]: Depending on the solubility of peptides and adjuvants, synthesis of PNEs can be done in DMSO or aqueous solution. The aqueous solution is phosphate-buffered saline (PBS) with adjusted pH = 8.5 with Na\textsubscript{2}CO\textsubscript{3}.}
Figure S1. Characterizations of Mon$_{\text{red}}$. A) $^1$H NMR spectrum of Mon$_{\text{red}}$; B) ESI-MS spectrum of Mon$_{\text{red}}$. 
Figure S2. $^1$H NMR spectrum of LEQ and PNE(LEQ).

Figure S3. Characterizations of PNE(LEQ). A) Size and size distribution of PNE(LEQ) measured by DLS; B) Representative TEM image of PNE(LEQ).
Figure S4. GPC and HPLC chromatographic analyses of PNEs. A) GPC traces showing the MW increase of PNE(LEQ-Pam) comparing with free LEQ; B, C) UHPLC-C18 chromatographic traces showing negligible free LEQ or Pam left post the polycondensation reaction in the synthesis of PNE(LEQ-Pam).

Figure S5. SDS-PAGE analyses of free LEQ, Pam, PNE(LEQ-Pam), and PNE(LEQ).
Figure S6. Schematic illustration of the synthesis of non-degradable PNE(LEQ-Pam).

Figure S7. Fluorescence imaging (A) and quantification (B) of inguinal draining LNs excised from mice treated with PNEs with and without adjuvants.
Figure S8. Flow cytometry analyses of the activation of BMDCs treated with PNE(LEQ-Pam) vaccine or other indicated formulations. Shown are the histogram of CD80 and CD40 expression of BMDCs.

Figure S9. *In vitro* cross-priming of OT-I CD8\(^+\) T cells by BMDCs pulsed with non-deg. PNE(LEQ-Pam). CFSE-labeled naïve OT-I CD8\(^+\) T cells were co-cultured with BMDCs pulsed with PNE(LEQ-Pam) or non-deg. PNE(LEQ-Pam). Proliferated OT-I CD8\(^+\) T cells were counted by flow cytometry analysis. *P* < 0.05.
Figure S10. PNE(ELE-Pam) elicited potent antigen-specific CD8$^+$ T cell response against adpgk. A) C57BL/6 mice were vaccinated with PBS, LEQ+Pam, (LEQ+Pam)-Montanide, or PNE(LEQ-Pam) on day 0, 14, and 28. The peripheral blood was collected 7 days post the last vaccination. B) The average frequencies of adpgk-specific CD8$^+$ T cells in peripheral blood on day 35.
Figure S11. Flow cytometry plots showing the frequencies of SIINFEKL-specific CD8α⁺ T cells (A) and their memory phenotype (B) in PBMC from survival mice immunized by PNE(LEQ-Pam) vaccines (Mouse 1 and Mouse 2) or a naïve mice control. The PBMC was collected 90 days post tumor inoculation.
Figure S12. Anti-tumor efficacy of PNE(LEQ-Pam) vaccine in a therapeutic setting. A) C57BL/6 mice were inoculated with YUMM1.7-OVA tumor cells ($5 \times 10^5$) at the right flank and treated with indicated vaccine formulations on day 5, 12, 19. (n = 5 animals) B) Individual tumor growth curves of each treatment group. C) Survival rate of mice in each group. The data show Mean ± SEM. The statistical analysis between survival curves was performed by Log-rank test; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.
References:
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