Supporting Information

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SI Methods

General Experimental. All chemicals were from commercial sources and were used as received. Fmoc-Lys(Mtt)-OH, Fmoc-Gly-OH, Fmoc-Thr-OH, Fmoc-Pro-OH, Fmoc-Glu-OH, Fmoc-Leu-OH, O-benzotriazole-N,N,N′,N′-tetramethyl-uronium hexafluorophosphate (HBTU), and benzotriazol-1-yl oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from EMD Biosciences/Novabiochem. Rink amide resin was purchased from Advanced Chemtech. Cyclooctyne reagents were purchased from Click Chemistry Tools. Water used in biological procedures or as a reaction solvent was purified using a MilliQ purification system (Millipore). DriSolv anhydrous CH2Cl2, DriSolv anhydrous MeOH, and DriSolv anhydrous DMF were purchased from EMD Chemicals. Redistilled, anhydrous N,N′-disopropylethylamine (DiPEA), trifluoroacetic acid (TFA), triisopropylsilane (TIS), and N-methylpyrrolidone (NMP) were obtained from Sigma-Aldrich.

Mass Spectrometry. Liquid chromatography (LC)-ESI (electrospray ionization)-MS analysis was performed using a Micromass LCT mass spectrometer (Micromass MS Technologies) and a Paradigm MG4 HPLC system equipped with an HTC PAL autosampler (Michrom BioResources) and a Waters Symmetry 5-μm C8 column (2.1 × 50 mm, MeCN:H2O 0.1% formic acid) gradient mobile phase, 150 μL/min.

HPLC/FPLC. HPLC purifications were achieved using an Agilent 1100 Series HPLC system equipped with a Waters Delta Pak 15-μm, 100-Å C18 column (7.8 × 300 mm, MeCN:H2O gradient mobile phase, 3 mL/min) as indicated below. Size exclusion and cation exchange chromatography were performed on a Pharmacia AKTA Purifier system equipped with a HiLoad 16/60 Superdex 75 column (Amersham) or a Mono S 5/50 GL column (Amersham), respectively.

UV-Vis Spectroscopy. UV-vis spectroscopy was performed on a Nanodrop ND-1000 spectrophotometer (Thermo Scientific).

In-Gel Fluorescence. Fluorescent gel images were obtained using a Typhoon 9200 Variable Mode Imager (GE Healthcare).

General Procedure for the Solid-Phase Peptide Synthesis of the Probes. Rink-amide resin was solvated in NMP and after removal of the Fmoc group by treating the resin with 20% (vol/vol) piperidine in NMP, the resin was loaded and elongated using consecutive steps: (i) The resin was washed with NMP (three times), CH2Cl2 (three times), and NMP. (ii) Fmoc-protected amino acids were condensed using HOBt [3 equivalents (equiv.)], PyBOP (3 equiv.), and DiPEA (6 equiv.). (iii) The resin was washed again using the same conditions as in step i. (iv) The coupling was monitored using a Kaiser test and, if complete, (v) the Fmoc-protective group was removed using 20% (vol/vol) piperidine in NMP.

In the final step, the peptides were cleaved off resin by agitating the resin in the presence of 95% TFA, 2.5% TIS, 2.5% H2O (vol/vol) for 3 h. Ice-cold Et2O was added to the cleavage solution and the formed precipitate was collected by centrifugation of the solution for 30 min at 4 °C. The crude pellet was purified by reverse-phase HPLC purification [buffers used: A, H2O; B, MeCN; C, 10% (vol/vol) TFA in H2O].

N-Terminal Probes. Azidohexanoic acid-LPETGG-CO NH2 (1). Rink amide resin (60 μmol) was loaded with Fmoc-Glyc-OH, elongated with the appropriately protected amino acids, and cleaved off the resin as described in the general method. For the final coupling azidohexanoic acid was used. RP-HPLC [26–35% (vol/vol) B in 12 min (3 column volumes)] gave the title compound (9.5 mg, 13 μmol, 13%) as a white solid.

LC/MS: Rf = 6.34 min; linear gradient 5→45% (vol/vol) B in 10 min. ESI/MS: m/z = 711.1 [M+H]+. 1H NMR (400 MHz, CDCl3) 6 ppm 4.45 (dd, J = 10.0, 4.4 Hz, 1H), 3.42 (dd, J = 8.4, 6.0 Hz, 1H), 3.45 (dd, J = 9.2, 5.2 Hz, 1H), 4.30–4.24 (m, 2H), 4.00 (m, 2H), 3.96 (s, 2H), 3.91–3.84 (m, 4H), 3.70–3.63 (m, 4H), 2.48 (t, J = 7.2 Hz, 2H), 2.26–2.19 (m, 4H, 3H, 1.78–1.70 (m, 1H), 1.69–1.56 (m, 7H), 1.44–1.38 (m, 3H), 1.21 (d, J = 6.4 Hz, 3H), 0.97 (t, 6.4 Hz, 6H).

Aza-dibenzo[cyclocyclotyne (DIBAC)-LPETGG-CO NH2 (2). Rink amide resin was loaded with Fmoc-Glyc-OH, elongated with the appropriately protected amino acids, and cleaved off the resin as described in the general method. Purification from Et2O afforded crude H2N-LPETGG-CO NH2 (17.9 mg, 31.3 μmol), which was dissolved in DMF (0.5 mL). DIBAC-OsU (14 mg, 20 μmol) was added and the reaction was stirred overnight. The solution was diluted before being purified by RP-HPLC [25–34% (vol/vol) B in 12 min (3 column volumes)], which gave the title compound (13.1 mg, 12.3 μmol, 39%) as an off-white solid.

LC/MS: Rf = 9.42 min; linear gradient 5→45% (vol/vol) B in 10 min. ESI/MS: m/z = 1066.14 [M+H]+. 1H NMR (400 MHz, CDCl3) 6 ppm 7.65 (dd, J = 13.2, 7.2 Hz, 1H), 7.46–7.28 (m, 7H), 5.05 (d, J = 14.4 Hz, 1H), 4.72–4.65 (m, 1H), 4.60–4.50 (m, 1H), 4.64–4.38 (m, 2H), 4.36 (d, J = 4 Hz, 1H), 4.26–4.23 (m, 1H), 4.04–3.87 (m, 5H), 3.73–3.62 (m, 2H), 3.52–3.38 (m, 1H), 3.10–2.92 (m, 1H), 2.82–2.67 (m, 1H), 2.56–2.39 (m, 3H), 2.34–2.09 (m, 6H), 2.07–1.98 (m, 4H), 1.94–1.85 (m, 2H), 1.72–1.52 (m, 6H), 1.50–1.40 (m, 1H), 1.20 (d, J = 6.0 Hz, 3H), 0.98–0.90 (m, 6H).

C-Terminal Probes. H2N-GGGK(N3)(TAMRA)-CO NH2 (3). Rink amide resin (60 μmol) was loaded with Fmoc-Lys(Mtt)-OH and elongated with Fmoc-Acidolysine-OH and Fmoc-GGG-OH as described in the general method. After washing the resin with CH2Cl2, the Mtt protective group was removed by treating the resin twice with 1% TFA, 1% TIS in CH2Cl2 (vol/vol) for 30 min (or until the yellow color completely disappeared). The resin was washed with CH2Cl2 (five times), NMP (five times), and NMP containing DiPEA (43.5 μL, 250 μmol, 5 equiv.). 5(6)-Carboxyhexylaminophosphonic acid (77 mg, 180 μmol, 3 equiv.) was condensed using PyBOP (94 mg, 180 μmol, 6 equiv.) and DiPEA (50 μL, 370 μmol, 6 equiv.). After 16 h shaking, the Kaiser test showed complete conversion. The N-terminal Fmoc group was removed and the peptide was cleaved off resin as described in the general method. Reverse-phase HPLC purification [25–34% (vol/vol) B in 12 min (3 CV)] gave the title compound (41.4 mg, 50.5 μmol, 81%) as a purple solid.

LC/MS: Rf = 5.50 and 6.10 min; linear gradient 5→45% (vol/vol) B in 10 min. ESI/MS: m/z = 883.3 [M+H]+. 1H NMR (400 MHz, CDCl3) 6 ppm 8.78 (d, J = 1.6 Hz, 1H), 8.28 (dd, J = 7.6, 1.6 Hz, 1H), 7.53 (d, J = 8.0 Hz, 7H), 7.14 (d, J = 9.6 Hz, 2H), 7.06 (dd, J = 9.6, 2.4 Hz, 2H), 6.98 (d, J = 2.4 Hz, 2H), 4.34 (dd, J = 9.2, 5.2 Hz, 2H), 3.98 (d, 14.8 Hz, 1H), 3.96 (s, 2H), 3.82 (d, 18.4 Hz, 1H), 3.80 (s, 2H), 3.54–3.46 (m, 2H), 3.32–3.28 (m, 14H), 1.94–1.45 (m, 12H).

H2N-GGGG(DIBAC)-CO NH2 (4). Rink amide resin (167 mg, 100 μmol) was loaded with Fmoc-Cys(Trt)-OH, elongated with Fmoc-GGG-OH, and cleaved off the resin as described in the general method, affording crude tetrapeptide, H2N-GGGG-CO NH2, in
quantitative yield. This peptide (38 mg, 83 μmol, 2 equiv.) was dissolved in PBS (0.25 mL) and to this was added DIBAC-maleimide (17 mg, 40 μmol, 1 equiv.) in DMF (0.25 mL). The reaction was stirred overnight, acidified with TFA, and purified by RP-HPLC [20-35% (vol/vol) B in 20 min (5 CV), giving the title compound (15.3 mg, 22 μmol, 27%) as a white solid.

LC/MS: R retention time [min] = 6.90 min; linear gradient 5–45% (vol/vol) B in 10 min. ESI/MS: m/z = 719.3 [M+H]+. 1H NMR (400 MHz, M δ ppm: 7.66 (d, J = 7.2 Hz, 1H), 7.55–7.51 (m, 1H), 7.48–7.45 (m, 3H), 7.38 (dt, J = 7.6, 1.4 Hz, 1H), 7.37–7.33 (m, 1H), 7.28 (d, J = 7.2 Hz, 1H), 5.14 (d, J = 14 Hz, 1H), 4.69–4.64 (m, 1H), 4.01–3.85 (m, 6H), 3.77 (d, J = 4.8 Hz, 1H) 3.73 (s, 1H), 3.70 (s, 1H), 3.67–3.63 (m, 2H), 3.39 (dd, J = 14.0, 5.2, 2.8 Hz, 1H), 3.27–3.05 (m, 5H), 2.97 (dld, J = 14, 8.4, 5.2 Hz, 1H), 2.48–2.41 (m, 3H), 3.33–2.87 (m, 2H) 2.08–1.99 (m, 1H).

H-N-GGGK(Azidoheaxanionic acid)-CONH2 (5). Rink amide resin (100 mg, 50 μmol) was loaded with Fmoc-Lys(Mtt)-OH and elongated with Fmoc-GGG-GH as described in the general method. After washing the resin with CH2Cl2, the Mitotic group was removed by treating the resin twice with 1% TFA, 1% TIS in CH2Cl2 (vol/vol/vol) for 30 min (or until the yellow color completely disappeared). The resin was washed with CH2Cl2 (five times), NMP (five times), and NMP containing DiPEA (43.5 μL, 250 μmol, 5 equiv.). Azidoheaxanionic acid (31 mg, 200 μmol, 4 equiv.) was added using PyBOP (104 mg, 200 μmol, 4 equiv.) and DiPEA (70 μL, 400 μmol, 8 equiv.). After 2 h shaking, the Kaiser test showed complete conversion. The N-terminal group was removed and the peptide was cleaved off resin as described in the general method. Reverse-phase HPLC purification [15–24% (vol/vol) B in 12 min (3 CV)] gave the title compound (15.4 mg, 33 μmol, 67%) as an off-white solid.

LC/MS: R = 2.77 min; linear gradient 5–45% (vol/vol) B in 10 min. ESI/MS: m/z = 456.3 [M+H]+. 1H NMR (400 MHz, CDCl3) δ ppm: 4.35 (dd, J = 9.2, 4.8 Hz, 1H), 3.98 (d, J = 16.8 Hz, 1H), 3.97 (s, 2H), 3.86 (d, J = 16.8 Hz, 1H), 3.78 (s, 2H), 3.29 (t, J = 6.8 Hz, 2H), 3.17 (dt, J = 6.8, 2.0 Hz, 2H), 2.20 (t, J = 7.2 Hz, 2H), 1.86–1.81 (m, 1H), 1.73 (dd, J = 18.4, 9.4, 5.0 Hz, 1H), 1.67–1.57 (m, 4H), 1.55–1.47 (m, 2H), 1.43–1.38 (m, 4H).

Cloning and Expression of Proteins. Ubiquitin N-terminally fused to N-terminal his tag followed by a thrombin cleavage site (MGSSHHHHHHHSSGLVPRGGGSGH) was cloned into a pET28 vector. The vector was transformed into BL21(DE3)pLysS and a starter culture was grown overnight at 37 °C. The starter culture was added to the expression culture (3 L, 2YT) and grown until the OD600 reached 0.6. To induce expression, 1 mM IPTG (final concentration) was added and the bacteria were grown at 37 °C for 4 h. The bacteria were collected by centrifugation at 6,000 × g for 15 min at 4 °C. The bacteria were lysed by sonification in lysis buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 50 μg/mL DNaseI (Roche), and 1 tablet/25 mL complete protease inhibitor (Roche)]. The inclusion bodies were collected by centrifugation (12,000 × g for 15 min at 4 °C). Before being dissolved in 50 mM Tris, pH 7.4, 150 mM NaCl, 6 M guanidine, the inclusions were first washed by resuspending the pellet in lysis buffer (one time), l-butanol (one time), and 50 mM Tris, pH 7.4, 150 mM NaCl, and 1 M guanidinium HCl (two times) and subsequent centrifugation. The unfolded protein (6 mg/mL, 0.7 mL) was pretreated with tris(2-carboxyethyl)phosphine (1 mM) and subsequently added (0.1 mL/h) to refolding buffer (200 mL, 50 mM Tris, pH 7.4, 150 mM NaCl, 10% (vol/vol) glycerol, 5 mM glutathione, 0.5 mM oxidized glutathione) at 25 °C. The reaction was stirred for 2 d, concentrated on a Ni-NTA column, and subsequently purified by size-exclusion chromatography.

Sortase A of Staphylococcus aureus and human IFNa2a were expressed and purified as previously described (1, 2).

Modification of Ubiquitin with N2-LPETGG (1) and DIBAC-LPETGG (2). Ubiquitin was modified with 1 and 2 as described for UbVME. N2-Ub: R = 7.17 min; linear gradient 5–45% (vol/vol) B in 10 min. ESI/MS: m/z = 9,542 [M+H]+. DIBAC-Ub: R = 7.37 min; linear gradient 5–45% (vol/vol) B in 10 min. ESI/MS: m/z = 9,898 (M+H)+.

Dimerization of Ubiquitin. Azido-modified ubiquitin (5 μL, 4 μg/μL) and DIBAC-modified ubiquitin (8 μL, 2.5 μg/μL) were mixed (final concentration of the proteins 170 μM) and incubated for 0.5–7 h at 37 °C. The conversion to the dimerized product was analyzed using gel electrophoresis.


Fig. S1. Requirements for dimerization of ubiquitin. (A) Schematic approach. (B) Ubiquitin is sortagged with 1 or 2 for 3 h and analyzed with LC/MS. (C and D) Dimerization of ubiquitin. Azido-modified ubiquitin (2 nmol) is incubated with an equimolar amount of cyclooctyne-equipped ubiquitin in 13 μL H2O. The dimer was resolved on 15% SDS/PAGE and the proteins were detected by Coomassie staining (C) and immunoblotting (D) for ubiquitin. (E) Azido-ubiquitin (0.1 nmol) incubated with DIBAC-ubiquitin (0.1 nmol) for the indicated time was resolved on a Tris-tricine gel and Coomassie stained, and the resulting protein was quantified by ImageJ. The relative amount of monomer and dimer per lane was determined as follows: relative amount of dimer = intensity of dimer/ total intensity; relative amount of monomer = intensity of monomer/ total intensity. (F) Labeling of UCHL3 with either ubiquitin or UbVME. (Left) Coomassie-stained gel; (Right) immunoblotting for the His6 tag.
Fig. S2. (A) Purification of anti-GFP sortagged with probe 4. (B and C) Coomassie brilliant-blue-stained gel (B) and mass spectrum (C) of purified anti-GFP labeled with 4. (D) Dimerization of aGFP-3 and aGFP-4. aGFP-3 (2.5 μg, 0.17 nmol) in Tris (50 mM, pH 7.4, 150 mM NaCl) was incubated with an equimolar amount of aGFP-4 for the indicated time at room temperature. The dimerized product was resolved from the monomer on a Tris-tricine SDS/PAGE. Proteins were visualized by fluorescent imaging (λ<sub>ex</sub> = 532, λ<sub>em</sub> = 580, Left) and Coomassie brilliant blue (Center) and quantified (Right). The relative amount of monomer vs. dimer was determined as described for ubiquitin. (E) Purification of anti-GFP dimer on a Superdex 75 10/30. (F) Analysis of the concentrated purified protein on 15% SDS/PAGE.
Fig. S3. Superdex 200 10/30 elution profile of monomer anti–GFP-3 and anti–GFP-4 incubated in the presence and absence of GFP.
Fig. S4. The peaks eluting at 12.5 mL (1) and 15.5 mL (2) of anti-GFP dimer incubated with 30 μL GFP were concentrated and loaded on a native page.

Fig. S5. (A and B) Dimerization and purification of fluorescent anti-GFP-4-VHH7-3 (A) and nonfluorescent anti-GFP-4-VHH7-5 (B). (C) Structure of S.

Fig. S6. FACs staining of mouse lymph node cells with anti-MHC II-anti-GFP antibodies. (Upper) Staining observed in wild-type cells. (Lower) Staining of MHC class II-deficient cells.
Fig. S7. Production of heterodimers of aGFP with VHH7, IL2, and IFNα.

Other Supporting Information Files

SI Appendix (PDF)