Supporting Information

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SI Text

Analytical Size-Exclusion Chromatography. For quaternary structure analysis of WT and mutant mTNF-α, a Superdex 75 10/300 GL gel filtration column (GE Healthcare) was used. Size-exclusion chromatography was performed in PBS buffer at 25°C and using a flow rate of 0.3 ml/min. The column was calibrated with a molecular mass gel-filtration standard from Bio-Rad containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44.0 kDa), myoglobin (17.0 kDa), and vitamin B_{12} (1.35 kDa). Protein elution was followed by measuring the absorption at 280 nm.

NFκB-Luciferase Reporter Gene Assay. HEK293 cells stably expressing NFκB-luciferase were used for the reporter gene assay. The stable cells were dissociated with trypsin, resuspended in DMEM containing 10% FBS at 5×105 cells per ml, and plated at 20 μl per well in a 384-well white plate (Greiner). After 2-h incubation at 5% CO_2 in a 37°C tissue culture incubator, 20 μl of mTNF-α was added to the cells. The cells were continuously incubated for 24 h. Luciferase activities were measured by the addition of 20 μl of Bright-Glo (Promega), and the plate was read by using a luminescence plate reader.

ELISA. Maxisorp 384-well plates (Nunc) were coated with 30 μl of 0.5 μg/ml protein overnight at 4°C. The coated plates were washed with PBS + 0.05% Tween 20 (PBST), blocked with 80 μl of 1% BSA in PBS, and washed again with PBST. The plates were sequentially incubated with 20 μl of primary antibody or serum diluted in 1% BSA in PBS, 20 μl of HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch), and 20 μl of TMB substrate (KPL) and read at an absorbance of 650 nm. The plates were washed with PBST between incubations.

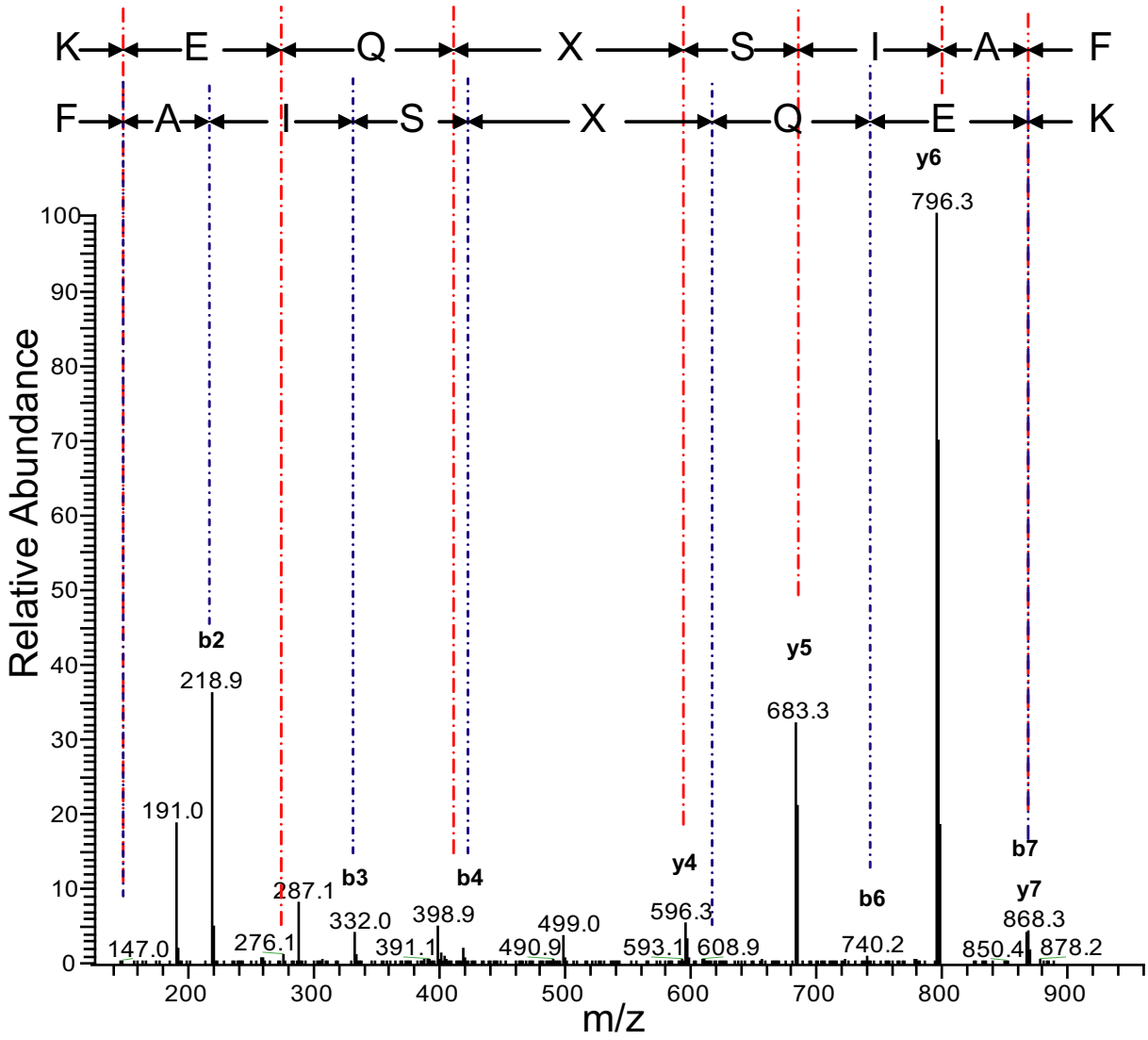
Purification of WT and Mutant mTNF-α Under Native Conditions. All purification steps were performed at 4°C. After thawing the cell pellet for 15 min on ice, the cell paste was resuspended in lysis buffer [50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 10% (vol/vol) glycerol] at 5 ml per gram of wet weight. After addition of Complete Protease Inhibitor Mixture (Roche), 10 ml of cell suspension was treated with 150 μl of lysozyme (100 mg/ml; MP Biomedicals), 50 μl of DNase I (5 mg/ml; Roche), 5 μl of RNase A (100 mg/ml; Sigma–Aldrich), and 125 units of benzonase nuclease (Novagen). The cell suspension was stirred at room temperature for 20 min to allow lysis to occur. The prelysed cells were then flash-frozen in liquid nitrogen and thawed in a 37°C water bath. This freeze–thaw cycle was repeated once. Complete lysis was then achieved by sonication on ice for 2 min. After centrifugation at $18,000 \times g$ for 20 min, 1 ml of Ni-NTA His-Bind Resin (Novagen) was added to the supernatant and mixed on a rotary shaker for 30 min. The lysate–resin mixture was loaded onto a 5-ml polypropylene column (Qiagen) and washed twice with 20 ml of lysis buffer. Protein was eluted by the addition of 2 ml of elution buffer [50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 250 mM imidazole, 10% (vol/vol) glycerol], concentrated with a 10K molecular mass cut-off Amicon Ultra-15 centrifugal filter device (Millipore), and further purified by a Superdex 75 10/300 GL column (flow rate of 0.3 ml/min) preequilibrated with PBS. All proteins were characterized by MALDI-TOF mass spectrometry, which was performed on a Voyager-DE-STR instrument (Applied Biosystems) with sinapinic acid as a matrix at The Scripps Center for Mass Spectrometry, The Scripps Research Institute (La Jolla, CA).

MS/MS Sequencing of Tryptic Fragment Containing pNO$_2$Phe. The excised gel slice containing the pNO$_2$Phe^{86}mTNF-α was diced into small pieces and mixed with 100 μl of 25 mM NH_4HCO_3/50% acetonitrile. After vortexing for 10 min, the supernatant was discarded. This step was repeated twice, before the gel pieces were dried in a Speed Vac for $\approx$20 min. The protein sample was reduced by addition of 25 μl of 10 mM DTT in 25 mM NH_4HCO_3. The reaction was allowed to proceed at 56°C for 1 h. After removal of the supernatant, the gel pieces were mixed with 25 μl of 55 mM iodoacetamide. After incubation in the dark for 45 min at room temperature, the gel pieces were subjected to tryptic in-gel digestion. The resultant peptide mixture was purified by C18 ZipTip (Millipore) and subjected to MS/MS fragmentation on a Thermo Finnigan LTQ mass spectrometer (Thermo Scientific), which was run in positive-ion mode using the nanospray source at The Scripps Center for Mass Spectrometry.

IgG Preparation. Murine serum was loaded onto a 10-ml Sepharose-conjugated protein G affinity column (GammaBind Plus Sepharose; Amersham Pharmacia Biotech). The column was washed with three column volumes of PBS (pH 7.4). Elution was carried out with two column volumes of 0.1 M acetic acid (pH 3.0). The eluent was then neutralized with 1 M Tris·HCl (pH 9.0) and dialyzed into PBS (pH 7.4).

T Cell Proliferation Assay. $CD4^+$ T cells from immunized mice were isolated from lymph nodes by magnetic depletion with MACS beads (Miltenyi Biotec). T cells were then placed into culture with irradiated splenocytes from naïve Bcl-2 mice and increasing amounts of antigen. The cultures were incubated for 48 h and then pulsed with [^{3}H]thymidine overnight. The culture plates were harvested onto filter mats and radioactivity was quantified with a TopCount scintilltion counter (PerkinElmer).

(A)



(B)

Sample	Retention time (min)	Observed mass (calculated mass of trimer) (kDa)
pNO_2Phe^{86}mTNF-α without His_6tag	33.00	55.2 (51.9)
Phe^{86} mTNF-α without His_6tag	33.20	53.8 (51.7)
pNO_2Phe^{42}mTNF-α with His_6tag	32.64	58.0 (57.7)
Phe^{42} mTNF-α with His_6tag	32.01	63.3 (57.6)
wt mTNF-α without His_6tag	32.97	55.5 (51.8)
pNO_2Phe^{11}mTNF-α with His_6tag	32.55	58.8 (57.6)

Fig. S1. Characterization of the pNO_2Phe^{86}mTNF-α mutant. (*A*) Tandem mass spectrum of the octamer fragment FAISXQEK, where X denotes pNO_2Phe, produced from trypsin digestion of pNO_2Phe^{86}mTNF-α. The partial sequence of the octamer containing pNO_2Phe can be read from the annotated b (blue) or y (red) ion series. (*B*) The quaternary structures of pNO_2Phe^{86}mTNF-α, Phe^{86} mTNF-α, pNO_2Phe^{42}mTNF-α, Phe^{42} mTNF-α, pNO_2Phe^{11}mTNF-α, and WT mTNF-α were determined based on a plot of the logarithm of the molecular mass of the protein standards versus the retention time on a Superdex 75 10/300 GL gel filtration column.

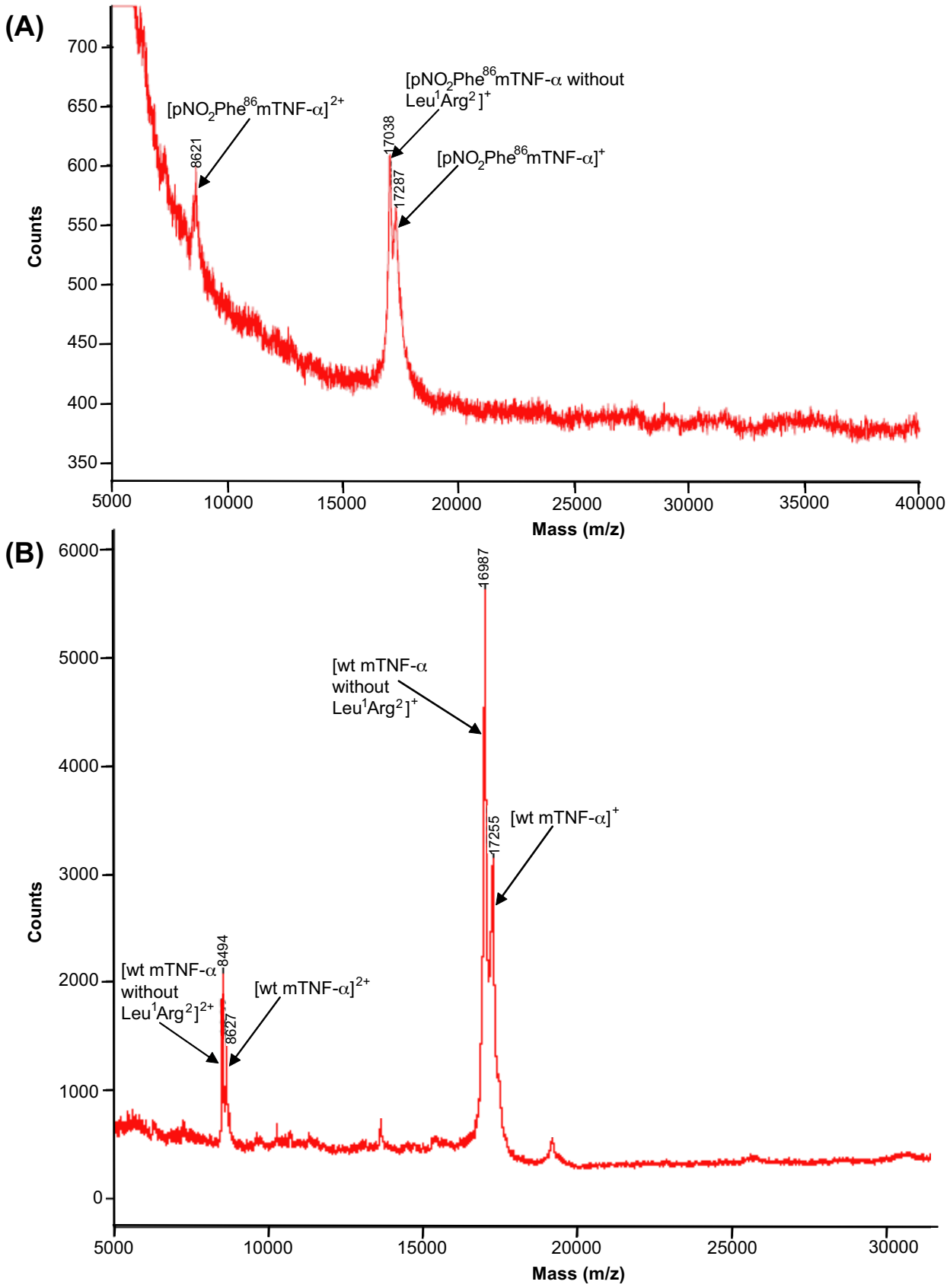
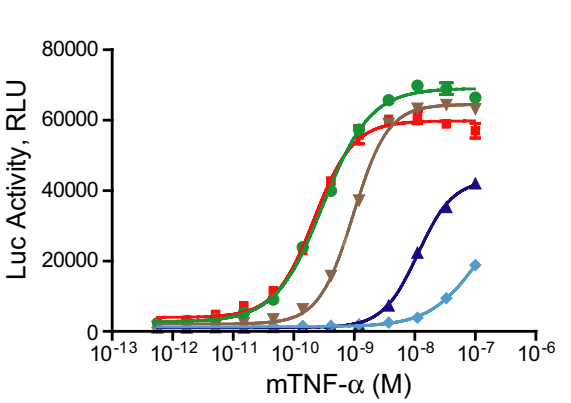


Fig. S2. Mass spectrometric analysis of pNO_2Phe^{86}mTNF-α. (*A*) MALDI-TOF mass spectrometric analysis of pNO_2Phe^{86}mTNF-α. (*B*) MALDI-TOF mass spectrometric analysis of WT mTNF-α.



Protein	EC_{50} (M)
wt mTNF-α	2.163×10^{-10}
pNO_2Phe^{86}mTNF-α	1.124×10^{-8}
pNO_2Phe^{42}mTNF-α	9.668×10^{-10}
Phe^{86} mTNF-α	1.165×10^{-7}
Phe^{42} mTNF-α	3.044×10^{-10}

Fig. S3. NF-κB-luciferase activity analysis of WT mTNF-α, pNO_2Phe^{86}mTNF-α, pNO_2Phe^{42}mTNF-α, Phe^{86}mTNF-α, and Phe^{42}mTNF-α.

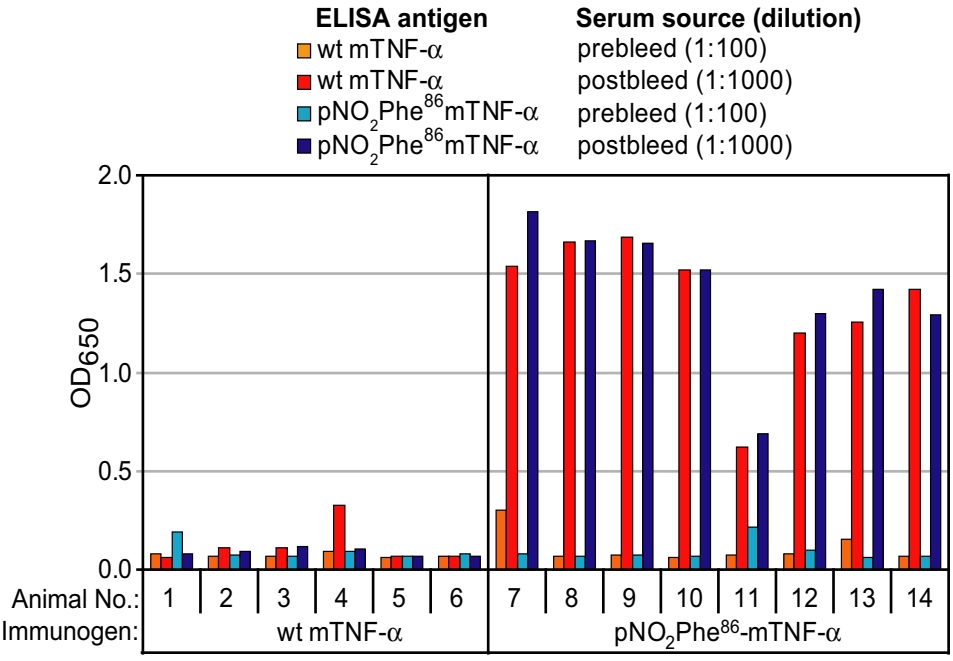


Fig. S4. Serum titers against WT mTNF-α and pNO_2Phe^{86}mTNF-α for Bcl2 mice immunized with WT mTNF-α or pNO_2Phe^{86}mTNF-α. The RIMMS protocol involved eight injections (5 μg of protein per injection) over a period of 17 days in the presence of CFA for the initial injection and IFA for the remaining seven injections. ELISAs were measured against WT mTNF-α (red and orange bars) or pNO_2Phe^{86}mTNF-α (blue and light blue bars). Before measurement, serum samples were diluted either 1:100 or 1:1,000 with 1% BSA in PBS buffer.

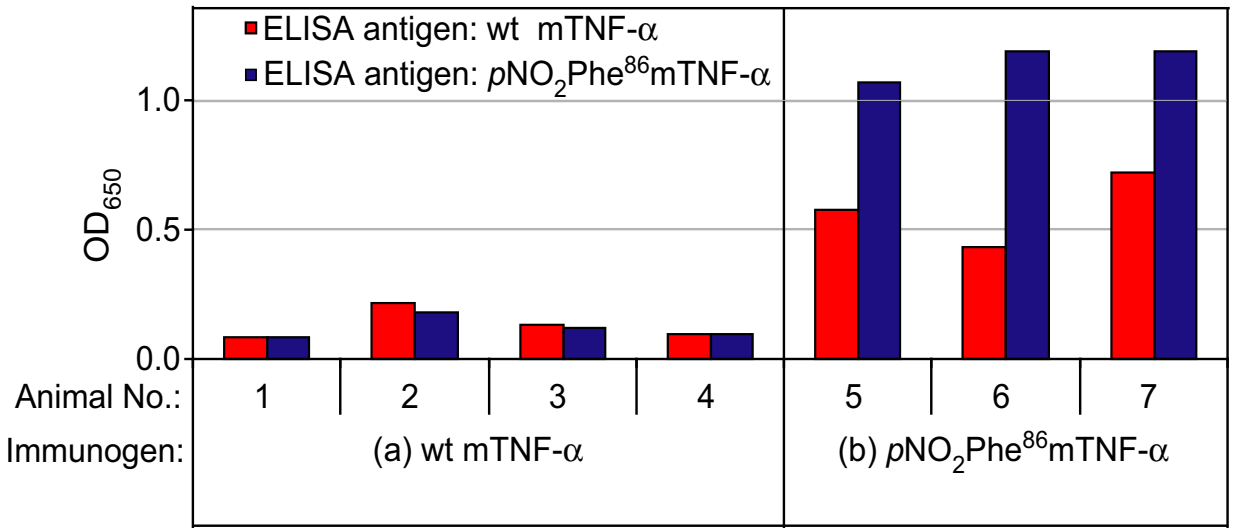


Fig. S5. Immunization with pNO_2Phe^{86}mTNF-α in the absence of adjuvant. Serum titers for Bcl-2 mice immunized with WT mTNF-α (*a*), or pNO_2Phe^{86}mTNF-α (*b*) for eight injections (5 μg of protein per injection) over a period of 17 days in the absence of either CFA or IFA. ELISAs were measured against WT mTNF-α (red bar) or pNO_2Phe^{86}mTNF-α (blue bar). Before measurement, serum samples were diluted 1:1,000 with 1% BSA in PBS buffer.

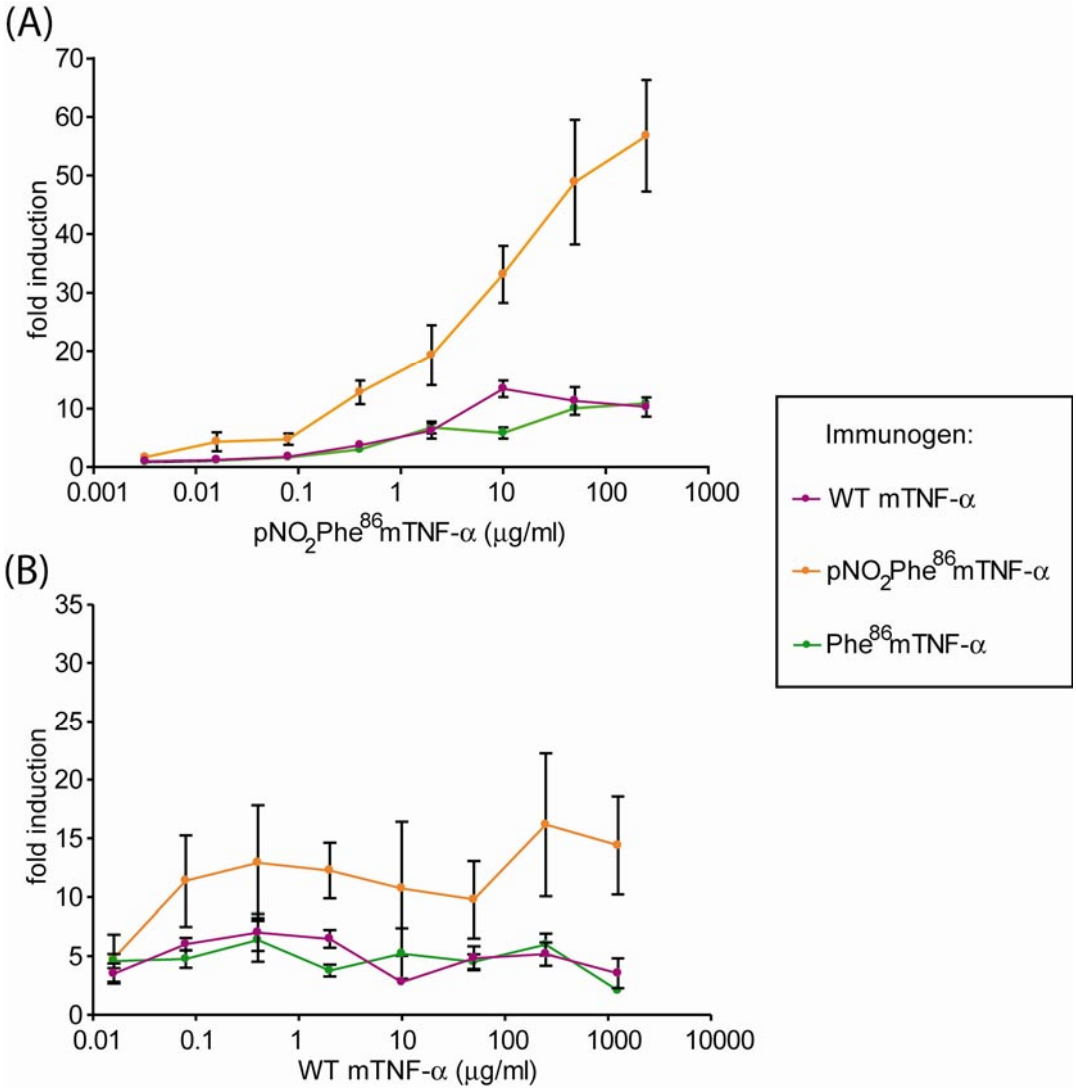


Fig. S6. T cell proliferative assay. (*A*) Proliferation of $CD4^+$ T cells from Bcl-2 transgenic mice immunized with WT mTNF-α (pink line), pNO_2Phe^{86}mTNF-α (orange line), and Phe^{86}mTNF-α (green line) and stimulated *in vitro* with serial dilutions of pNO_2Phe^{86}mTNF-α. (*B*) Proliferation of $CD4^+$ T cells from Bcl-2 transgenic mice immunized with WT mTNF-α (pink line), pNO_2Phe^{86}mTNF-α (orange line), and Phe^{86}mTNF-α (green line) and stimulated *in vitro* with serial dilutions of WT mTNF-α.

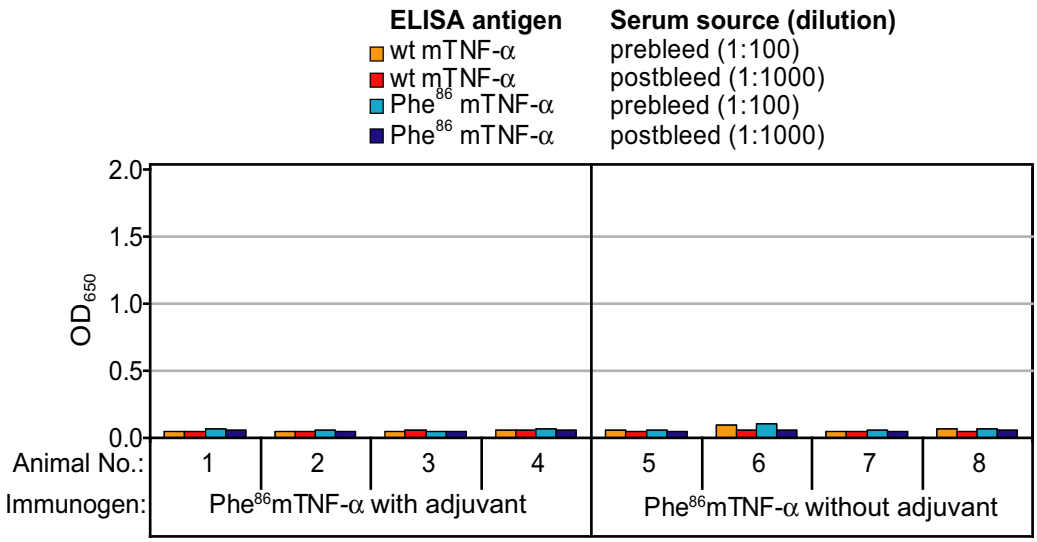


Fig. S7. Serum titers against WT mTNF-α and Phe^{86} mTNF-α for Bcl2 mice immunized with Phe^{86} mTNF-α in the absence or presence of adjuvant. For mice immunized without adjuvant, the RIMMS protocol involved eight injections (5 μg of protein per injection) over a period of 17 days. For mice immunized with adjuvant, CFA was used for the first injection and IFA for the remaining seven injections. ELISAs were measured against WT mTNF-α (red and orange bars) or Phe^{86} mTNF-α (blue and light blue bars). Before measurement, serum samples were diluted either 1:100 or 1:1,000 with 1% BSA in PBS buffer.

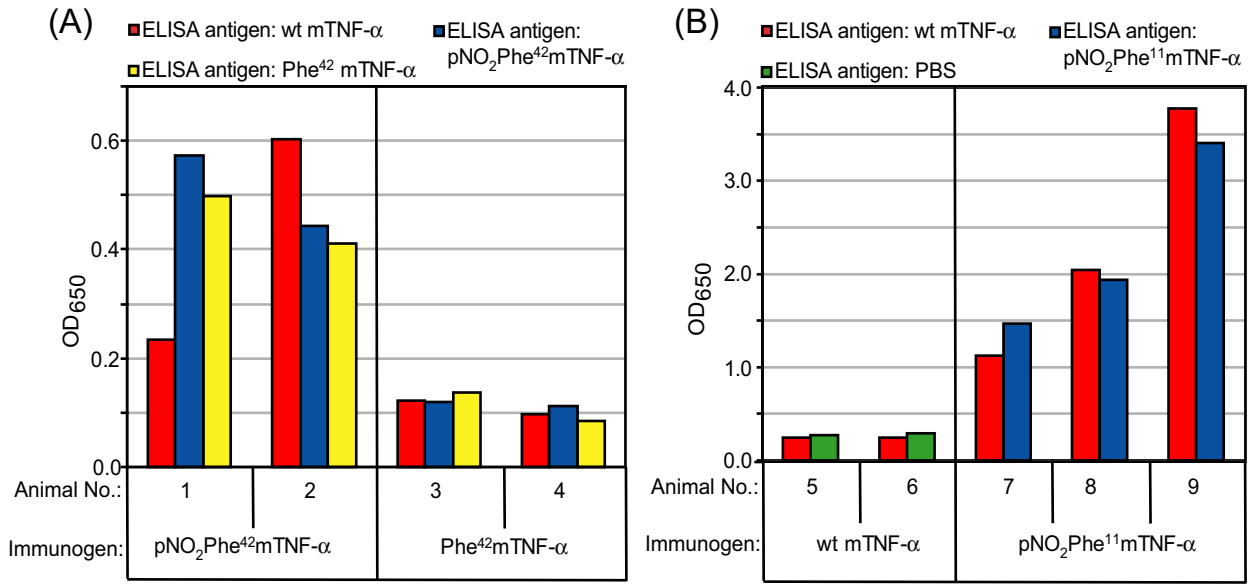
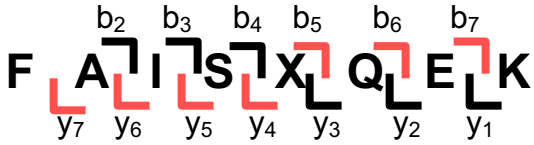


Fig. S8. Immunogenicity of other surface sites on mTNF-α. (*A*) Serum titers against WT mTNF-α, pNO_2Phe^{42}mTNF-α, and Phe^{42} mTNF-α for C57BL/6 mice immunized with either pNO_2Phe^{42}mTNF-α or Phe^{42} mTNF-α. (*B*) Serum titers against WT mTNF-α, PBS, and pNO_2Phe^{11}mTNF-α for C57BL/6 mice immunized with either pNO_2Phe^{11}mTNF-α or WT mTNF-α. The RIMMS protocol involved eight injections (5 μg of protein per injection) over a period of 17 days in the absence of adjuvant. ELISAs were measured against WT mTNF-α (red bars), pNO_2Phe^{42}mTNF-α/pNO_2Phe^{11}mTNF-α (blue bars), Phe^{42}mTNF-α (yellow bars), or PBS (green bars). Before measurement, serum samples were diluted 1/100 (*A*) or 1/800 (*B*) with 1% BSA in PBS buffer.

Table 1. MS/MS sequencing of a tryptic fragment of pNO_2Phe^{86}mTNF-a



	Observed mass, Da	Calculated mass, Da
y-series ions		
$[y_1]^+$	147.0	147.1
$[y_2]^+$	276.1	276.2
$[y_2\text{-}H_2O]^+$	258.1	258.1
$[y_2\text{-}NH_3]^+$	259.2	259.1
$[y_3]^+$	404.4	404.2
$[y_3\text{-}H_2O]^+$	386.2	386.2
$[y_4]^+$	596.3	596.3
$[y_5]^+$	683.3	683.3
$[y_5\text{-}H_2O]^+$	665.4	665.3
$[y_5\text{-}H_2O]^{2+}$	333.1	333.1
$[y_5\text{-}NH_3]^+$	666.3	666.3
$[y_6]^+$	796.3	796.4
$[y_6]^{2+}$	398.9	398.7
$[y_6\text{-}H_2O]^+$	778.3	778.4
$[y_6\text{-}NH_3]^+$	779.5	779.4
$[y_7]^+$	867.3	867.4
$[y_7]^{2+}$	434.2	434.2
$[y_7\text{-}H_2O]^+$	849.6	849.4
b-series ions		
$[b_2]^+$	218.9	219.1
$[b_3]^+$	332.0	332.2
$[b_4]^+$	419.1	419.2
$[b_4\text{-}H_2O]^+$	401.1	401.2
$[b_4\text{-}H_2O]^{2+}$	201.1	201.1
$[b_5]^+$	611.3	611.3
$[b_5]^{2+}$	305.9	306.1
$[b_5\text{-}H_2O]^+$	593.2	593.3
$[b_6]^+$	739.4	739.3
$[b_6\text{-}H_2O]^+$	721.3	721.3
$[b_7]^+$	868.3	868.4
$[b_7\text{-}H_2O]^+$	850.3	850.4
$[b_7\text{-}NH_3]^+$	851.1	851.4
$[b_7]^+$	868.3	868.4
$[b_7\text{-}H_2O]^+$	850.4	850.4

The sequence of the tryptic fragment containing pNO_2Phe is shown in single letter code (X, pNO_2Phe). Observed fragment ions of the y and b series are indicated. Key y and b ions proving the incorporation of pNO_2Phe are represented in red. All masses are reported as monoisotopic masses.