Immunochemical termination of self-tolerance

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The ability to selectively induce a strong immune response against self-proteins, or increase the immunogenicity of specific epitopes in foreign antigens, would have a significant impact on the production of vaccines for cancer, protein-misfolding diseases, and infectious diseases. Here, we show that site-specific incorporation of an immunogenic unnatural amino acid into a protein of interest produces high-titer antibodies that cross-react with WT protein. Specifically, mutation of a single tyrosine residue (Tyr[86]) of murine tumor necrosis factor-α (mTNF-α) to p-nitrophenylalanine (pNO$_2$Phe) induced a high-titer antibody response in mice, whereas no significant antibody response was observed for a Tyr[86] $\rightarrow$ Phe mutant. The antibodies generated against the pNO$_2$Phe are highly cross-reactive with native mTNF-α and protect mice against lipopolysaccharide (LPS)-induced death. This approach may provide a general method for inducing an antibody response to specific epitopes of self- and foreign antigens that lead to a neutralizing immune response.

TNF-α | unnatural amino acids | vaccination | immunogenicity

A major challenge in modern vaccinology is the development of robust methods to selectively induce a strong immune response against self-proteins or to increase the immunogenicity of specific epitopes in foreign antigens that can elicit neutralizing antibodies but that are not immunodominant. A number of strategies are being pursued to address this challenge including the development of improved adjuvants, the introduction of foreign helper peptides into chimeric antigens, and the use of DNA vaccines (1–4). Interestingly, almost 50 years ago, Weigle (5) showed that rabbits immunized with a rabbit thyroglobulin that had been nonspecifically labeled with a diazonium derivative produced cross-reactive antibodies to native thyroglobulin. Although these early experiments produced a highly heterogeneous antigen, one interpretation is that chemical modification results in immunogenic epitopes that induce high-titer cross-reactive antibodies. Similarly, there is anecdotal evidence that T cell tolerance can be broken by autoreactive B cells, which are readily elicited by immunization with cross-reactive foreign antigens that differ from the self-antigen by one or a few amino acids (6).

In contrast to the relatively nonselective chemical methods for modifying proteins, it is now possible to make highly precise "chemical mutations" to protein structure by means of genetically encoded unnatural amino acids. More than 50 unnatural amino acids have been encoded in bacteria, yeast, or mammalian cells including metal-binding and posttranslationally modified amino acids, fluorescent and redox-active amino acids, and photo- and chemically reactive amino acids (7–9). More specifically, the phenylalanine derivative p-nitrophenylalanine (pNO$_2$Phe, Fig. 1A) has been incorporated into proteins in bacteria in response to the amber nonsense codon with high fidelity and good efficiency for use as a spectroscopic distance probe (10). Nitroaryl groups have historically been used as highly immunogenic haptens (11), most likely because of the propensity of the electron-deficient pi system to interact with the Tyr and Trp side chains common to germ-line antibody combining sites. Because of their close structural similarity, we postulated that proteins containing either Phe $\rightarrow$ pNO$_2$Phe or Tyr $\rightarrow$ pNO$_2$Phe

mutations might generate a robust immune response that would be cross-reactive with the native protein. Here, we show that immunization of mice with a Tyr[86] $\rightarrow$ pNO$_2$Phe mutant of murine tumor necrosis factor-α (mTNF-α) generates a high-titer antibody response to WT mTNF-α that efficiently protects mice against a lipopolysaccharide (LPS) challenge.

Results and Discussion

Murine TNF-α as a Model System. mTNF-α was chosen as the target protein for this study because: (*i*) it is a well characterized cytokine involved in the regulation of infectious, inflammatory, and autoimmune phenomena (12); (*ii*) the biological properties of this protein have been extensively studied including its structure, function, and signaling mechanisms (12–16); and (*iii*) mTNF-α knockout mice are viable and show no apparent phenotypic abnormalities (15), suggesting that mice will survive a neutralizing immune response against TNF-α. In addition, anti-TNF-α antibodies (17, 18) and soluble chimeric TNF-α receptors (19, 20) are widely used in the treatment of autoimmune disease, and a number of approaches are being pursued to develop TNF-α-specific vaccines for clinical use. The latter include recombinant TNF-α molecules containing foreign immunodominant T-helper epitopes, TNF-α fusions to virus-like particles of the bacteriophage Q$^\beta$, and keyhole limpet hemocyanin-TNF-α heterocomplexes (4, 21, 22).

Based on the x-ray crystal structure of trimeric mTNF-α (13), a single Tyr[86] $\rightarrow$ pNO$_2$Phe mutant mTNF-α (pNO$_2$Phe[86]mTNF-α) was selected as an immunogen for our initial studies (Fig. 1B). Tyr[86] is highly conserved among different mammalian TNFs, and it has been determined that mutations at this site have no effect on protein folding and trimer formation but lead to a significant loss of cytotoxicity (23, 24) (which is advantageous for vaccination purposes).

Expression and Characterization of Mutant mTNF-α Proteins. To express mTNF-α in *Escherichia coli*, plasmid pET26-mTNF was constructed that consists of an N-terminal His$_6$ tag, a factor Xa cleavage site, and the *mTNF-α* gene downstream of the T7-lac promoter. The pNO$_2$Phe[86]mTNF-α mutant was generated by substitution of the codon for Tyr[86] with a TAG amber codon in plasmid pET26-mTNF. This mutated gene was then expressed in the presence of an orthogonal, amber suppressor tRNA$_{CUA}$/aminoacyl-tRNA synthetase pair derived from *Methanococcus jannaschii* that specifically inserts pNO$_2$Phe into proteins in *E.*

Fig. 1. Incorporation of pNO$_2$Phe into mTNF-α. (*A*) Structure of pNO$_2$Phe. (*B*) X-ray crystal structure of mTNF-α trimer with Tyr-86, Asp-42, and Lys-11 indicated (PDB ID code 2TNF). (*C*) Expression of the Tyr^{86} amber mutant of mTNF-α in the absence (lane 2) and presence (lane 3) of 1 mM pNO$_2$Phe with the pNO$_2$Phe-specific mutRNA$_{CUA}$/aminoacyl-tRNA synthetase pair. Protein samples were purified by Ni-NTA affinity column under denaturing conditions and analyzed by SDS/PAGE with SimplyBlue staining. Lane 4 contains WT mTNF-α, and lane 1 is a molecular mass standard.

coli in response to the amber codon (10). The mutant protein ($\approx$1 mg/liter in GMML minimum medium) was purified by Ni^{2+} affinity chromatography either under denaturing or native conditions, followed by cleavage of the His$_6$ tag and size-exclusion chromatography. The composition and homogeneity of the mutant protein were subsequently analyzed by SDS/PAGE and mass spectrometry.

pNO$_2$Phe^{86}mTNF-α purified under denaturing conditions has a similar mobility on SDS/PAGE as WT mTNF-α (Fig. 1*C*); no full-length mTNF-α was observed when the mutant gene was expressed in the absence of pNO$_2$Phe, indicating that there is no detectable incorporation of endogenous amino acids at position 86. The MS/MS analysis of an 8-mer tryptic fragment exactly matches the pattern for the incorporation of pNO$_2$Phe at residue 86 [supporting information (SI) Fig. S1*A* and Table S1]. The MALDI-TOF spectrum (Table 1 and Fig. S2*A*) also shows a peak ([M-H]$^+$:17287) that matches the expected molecular mass of pNO$_2$Phe containing full-length mTNF-α ([M-H]$^+$:17286). These results confirm the selective incorporation of pNO$_2$Phe into the mutant mTNF-α.

To determine the effect of the pNO$_2$Phe^{86} mutation on the quaternary structure of this protein, both WT mTNF-α and pNO$_2$Phe^{86}mTNF-α samples were analyzed by fast protein liquid chromatography (Fig. S1*B*). Both WT mTNF-α and pNO$_2$Phe^{86}mTNF-α show a similar retention time that corresponds to the molecular mass of the trimeric forms. Furthermore, both proteins were completely soluble at >10 mg/ml in PBS buffer (pH 7.5) at 25°C. As expected, WT mTNF-α activates

Table 1. MALDI-TOF mass spectrometry analysis of mTNF-α variants

Protein	Species	Observed mass (calculated mass), Da	
		Full-length protein without His$_6$ tag	Protein without Leu^{1}Arg^2
pNO$_2$Phe^{86}mTNF-α	[M+H]$^+$	17,287 (17,286)	17,038 (17,017)
Phe^{86}mTNF-α	[M+H]$^+$	17,237 (17,241)	16,972 (16,972)
WT mTNF-α	[M+H]$^+$	17,255 (17,257)	16,987 (16,988)

NFκB signaling in a NFκB-luciferase reporter cell line. In contrast the pNO$_2$Phe^{86} mutant (Fig. S3) has only 2% of the activity of WT mTNF-α in this assay, consistent with previous reports that Tyr^{86} is essential for receptor binding and that a variety of mutations at residue 86 lead to a significant decrease in activity (23, 24). One additional peak was also found in the MALDI-TOF spectrum that corresponds to the deletion of first 2 aa of pNO$_2$Phe^{86}mTNF-α (Table 1, Fig. S2*A*), presumably because of overdigestion during the factor Xa cleavage step. Because it is difficult to separate this truncated protein from full-length protein, and the deletion of the first two N-terminal amino acids only slightly affects TNF activity (24), the mixture was used directly to immunize mice both for the mutant mTNF-α and WT control. Additional experiments also indicated that the presence or absence of an N-terminal His$_6$ tag had no influence on the immunization results.

Immunization Studies with WT and Mutant mTNF-α. To determine the immunogenicity of the pNO$_2$Phe^{86}mTNF-α mutant, 32 C57BL/6 mice were randomized into three groups and injected with pNO$_2$Phe^{86}mTNF-α, WT mTNF-α, and PBS buffer by using the RIMMS (repetitive immunization at multiple sites) protocol (25). To avoid adverse effects due to cytotoxicity of mTNF-α, a dose of 5 μg of mTNF-α per injection was used throughout this study (26). Briefly, mice were injected eight times over 17 days. In each injection, 5 μg of protein in 200 μl of PBS was mixed 1:1 with complete Freund's adjuvant (CFA) for the first injection or with incomplete Freund's adjuvant (IFA) for the remaining injections at six specific sites proximal to peripheral lymph nodes. On day 21, antibody titers against pNO$_2$Phe^{86}mTNF-α and WT mTNF-α were determined by ELISA using a horseradish peroxidase conjugate of goat anti-mouse IgG secondary antibody. Mice immunized with either WT mTNF-α or PBS buffer alone had insignificant serum IgG titers against both pNO$_2$Phe^{86}mTNF-α and WT mTNF-α (Fig. 2). This is expected because WT mTNF-α is a self-protein and should be tolerated by the murine immune system. In contrast, mice immunized with pNO$_2$Phe^{86}mTNF-α were found to display markedly high serum IgG titers reacting against both pNO$_2$Phe^{86}mTNF-α (Fig. 2*C*, blue bars) and WT mTNF-α (Fig. 2*C*, red bars). Thus, a single pNO$_2$Phe mutation (which alters the monomer molecular mass by 29 Da) induces a strong immunological response that results in antibodies that are highly cross-reactive with WT mTNF-α. Similar results were obtained with Bcl-2 transgenic mice (Fig. S4).

We also examined the immunogenicity in the absence of strong immunopotentiators and found that immunization with pNO$_2$Phe^{86}mTNF-α in the absence of any adjuvant also elicited significant anti-TNF-α titers (Fig. S5), suggesting that this approach may be applicable to therapeutic settings in which strong adjuvants are not desirable. Furthermore, the antibody response after immunizations with pNO$_2$Phe^{86}mTNF-α, remains quite robust after 19 weeks (Fig. 3). Such a long sustainability is highly desirable for clinical use, because current strategies often suffer from rapidly decreasing autoantibody titers when immunization ceases.

To verify that the immunological response is a result of the immunogenic nitroaryl group of the unnatural amino acid, a Tyr^{86} → Phe mutant mTNF-α (Phe^{86} mTNF-α) was generated. After confirmation of its trimeric quaternary structure by size-exclusion chromatography, mice were immunized with this mutant either in the presence or absence of CFA/IFA. In both cases no significant anti-TNF-α titers were generated, indicating that the pNO$_2$ group is required to break immunological tolerance (Fig. 2*D* and Fig. S7). Furthermore, CD4$^+$ T cells specific for pNO$_2$Phe^{86} mTNF-α were elicited only when mice were immunized with this mutant protein and not when mice were immunized with WT mTNF-α or Phe^{86} mTNF-α (Fig. S6*A*). In
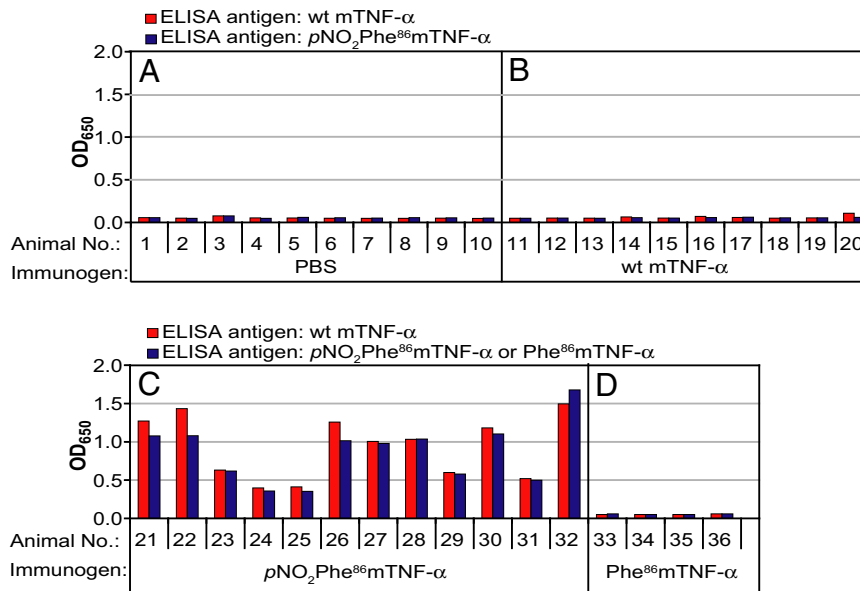
Fig. 2. Serum titers for C57BL/6 mice immunized with PBS (*A*), WT mTNF-α (*B*), pNO₂Phe⁸⁶mTNF-α (*C*), or Phe⁸⁶ mTNF-α (*D*). The protocol involved eight injections (5 µg of protein per injection) over a period of 17 days in the presence of complete Freund's adjuvant (CFA) for the initial injection and incomplete Freund's adjuvant (IFA) for the remainder. ELISAs were measured against WT mTNF-α (red bar) or pNO₂Phe⁸⁶mTNF-α (blue bar). For mice immunized with Phe⁸⁶ mTNF-α, ELISAs were measured against WT mTNF-α (red bar) or Phe⁸⁶ mTNF-α (blue bar). Before measurement, serum samples were diluted 1:1,000 with 1% BSA in PBS buffer.

contrast, no significant proliferation was observed when CD4⁺T cells from pNO₂Phe⁸⁶mTNF-α-immunized Bcl-2 mice were stimulated *in vitro* with WT mTNF-α (Fig. S6*B*). Preliminary epitope mapping experiments with mTNF-α mutants and peptide fragments of WT mTNF-α (data not shown) indicate that the polyclonal response to pNO₂Phe⁸⁶mTNF-α involves multiple protein epitopes. Together, these results suggest that insertion of pNO₂Phe into the sequence of mTNF-α creates a T cell epitope, which enchances T cell help to trigger an effective immune response against this disease-associated self protein. It is possible that other immunization protocols (e.g., sequential immunization with the mutant and WT TNF-α) will also yield high-titer cross-reactive antibodies. These results are consistent with those of Dalum *et al*. (4), who incorporated immunodominant T-helper cell epitopes into mTNF-α to break immune tolerance. The current strategy, however, results in minor pertubations in a protein and should not disrupt its tertiary fold or dramatically affect expression, solubility or stability.
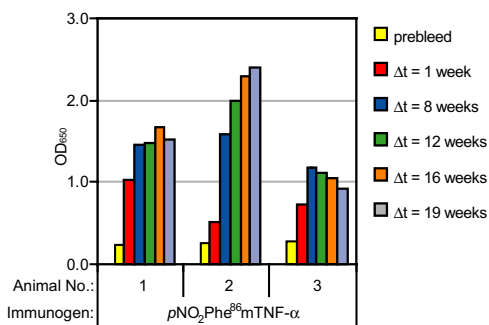

Fig. 3. Determination of serum titer durability. Three Bcl-2 transgenic mice were immunized with pNO₂Phe⁸⁶mTNF-α. After a sequence of eight immunizations, bleeds were taken for ELISA analysis against pNO₂Phe⁸⁶mTNF-α at defined time points. Before each measurement, serum samples were diluted 1:100 with 1% BSA in PBS buffer. Δt corresponds to the time period between the last immunization and the bleed.

Extension to Mutations at Other Surface Sites. The polypeptide sequence surrounding Tyr⁸⁶ is not predicted to be a T cell epitope based on *in silico* sequence-based analysis of potential MHC class II DR epitopes in TNF-α (27). Nonetheless, to begin to explore the generality of this approach, we determined whether substitution of pNO₂Phe at other sites might have a similar effect. The surface-exposed residue Asp⁴², which is not involved in trimerization or receptor binding, was therefore mutated to pNO₂Phe. After confirming the mutation by SDS/PAGE and mass spectrometry, two groups of C57BL/6 mice were immunized with the pNO₂Phe⁴²mTNF-α and the Phe⁴² mTNF-α mutant (Fig. S8). Again, significant anti-TNF-α titers were elicited only by immunization with the pNO₂Phe mutant protein. A similar result was obtained with mutation of another surface-exposed residue, Lys¹¹, to pNO₂Phe. These results suggest that pNO₂Phe mutagenesis may be a fairly general approach to render specific self- or foreign antigens highly immunogenic and may not be limited to substitutions at surface-exposed Tyr or Phe residues. However, preliminary results indicate that incorporation of pNO₂Phe is less effective at positions 104 and 19. Immunization of C57BL/6 mice with pNO₂Phe¹⁰⁴mTNF-α or pNO₂Phe¹⁹mTNF-α resulted in the generation of antibodies that lacked significant cross-reactivity with native mTNF-α. Thus, context effects likely play a role in determining the nature of the immune response. Finally, it is likely that other immunogenic unnatural amino acids can also be used; alternatively for smaller antigens, immunogenic unnatural amino acids can be incorporated by semisynthesis or total peptide synthesis.

Vaccination Protects Mice in a LPS Challenge Mode. We next determined whether vaccination of mice with the pNO₂Phe⁸⁶mTNF-α would protect against a LPS challenge in a severe endotoxemia mouse model (28). Septic shock induced by LPS in this model is known to involve the production and release of TNF-α. C57BL/6 mice were immunized with PBS, WT mTNF-α, and pNO₂Phe⁸⁶mTNF-α. These mice were subsequently injected i.p. with LPS (8.5 mg/kg) 3 days after completion of the above immunization regime, and their survival rate was determined. As depicted in Fig. 4*A*, mice immunized with the pNO₂Phe⁸⁶mTNF-α
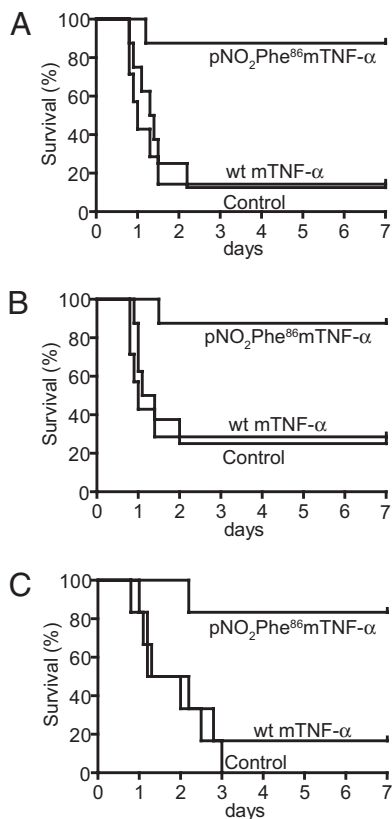
Fig. 4. Immunization with pNO$_2$Phe^{86}mTNF-α improves survival of mice in a TNF-α-dependent severe endotoxemia model. Kaplan–Meier survival plots of mice receiving active or passive immunizations are shown. (*A*) Mice (eight per group) immunized with pNO$_2$Phe^{86}mTNF-α or WT mTNF-α are compared with seven mice receiving sham immunizations. Survival advantage of mice immunized with pNO$_2$Phe^{86}mTNF-α ($P < 0.01$) vs. WT is shown. (*B*) Mice (eight per group) injected with 100 μg of purified IgG from pNO$_2$Phe^{86}mTNF-α or WT immunized mice were compared with controls receiving saline injection. Survival advantage of mice immunized with pNO$_2$Phe^{86}mTNF-α ($P < 0.01$) vs. WT is shown. (*C*) Mice (six per group) received 100 μl of pooled serum from mice immunized with pNO$_2$Phe^{86}mTNF-α or WT mTNF-α. Survival advantage of mice immunized with pNO$_2$Phe^{86}mTNF-α ($P < 0.01$) vs. WT is shown.

mutant show a significantly greater survival advantage (87.5%) than those that received PBS or WT mTNF-α (12.5% survival rate) immunizations. Similarly, C57BL/6 mice receiving either pooled serum (100 μl) or purified IgG antibody (4 mg/kg) collected from Bcl-2 mice preimmunized with pNO$_2$Phe^{86}mTNF-α showed a significantly higher survival rate (83.3–87.5%) than those receiving pooled serum or IgG from Bcl-2 mice immunized with WT mTNF-α (16.7–25.0%) (Fig. 4 *B* and *C*). Hence, these results demonstrate that a single pNO$_2$Phe group incorporated into a self-protein induces a robust cross-reactive antibody response against native protein that is protective in a disease model. We are currently extending these studies to other TNF-α dependent models including the collagen-induced arthritis (CIA) model and KRN transgenic mouse (K/BxN) model (29).

Concluding Comments. We have shown that it is possible to break immunological self-tolerance by the site-specific incorporation of pNO$_2$Phe into a protein epitope. Although it has been known for some time that altered proteins can induce autologous antibodies, the ill-defined nature of the changes that render the proteins immunogenic complicate their production and therapeutic utility (30). For example, the chemically modified thyroglobulin preparations used in the studies of Weigle contained ≈50 azo linkages per molecule of thyroglobulin (31), resulting in

a highly heterogeneous and possibly aggregated or partially unfolded antigen. Similarly, insertion of T cell epitopes at various positions in antigens can create proteins with altered tertiary structure, solubility, and stability compared with native protein. In contrast, the changes made here are chemically defined and confined to single residues. Moreover, these mutations do not appear to affect the overall quaternary structure of the protein nor its solubility. The resulting antibodies are therefore more likely to recognize the corresponding epitopes in the native protein. Finally, the pNO$_2$Phe-containing mTNF-α mutants induced a protective cross-reactive immune response without the need for strong adjuvants and resulted in high titers for at least 4 months, attributes that may facilitate therapeutic applications of this methodology.

This strategy should be applicable to other self-proteins, including those associated with protein-misfolding diseases or cancer. In addition, by introducing the pNO$_2$Phe group at weakly immunogenic or otherwise silent epitopes, this approach may also enable the generation of a strong antibody response against regions of a pathogen that are predicted to result in neutralizing antibodies against viral, bacterial, or parasitic infections. Furthermore, the selective introduction of immunogenic amino acids into proteins may facilitate the generation of functional antibodies, e.g., agonists or antagonists of G protein-coupled receptors and other membrane-bound receptors for which it has historically been difficult to generate strong antibody responses. The structural basis for this phenomenon and exploration of its application to human disease remain to be elucidated.

Materials and Methods

Bacterial Strains and Plasmids. *E. coli* XL1-Blue and BL21(DE3) were used as hosts for cloning and expression, respectively. pET26b was obtained from Novagen. *E. coli* strains were grown in minimal medium containing 1% glycerol and 0.3 mM leucine (GMML medium) or 2× YT medium. Restriction enzymes, T4 DNA ligase, dNTPs, and factor Xa protease were obtained from NEB. IPTG and 4–12% Bis-Tris Gels for SDS/PAGE were purchased from Invitrogen. pNO$_2$Phe was purchased from Advanced ChemTech. Primers were purchased from Integrated DNA Technologies. DNA polymerase was obtained from Stratagene. The anti-TNF-α antibody was from R & D Systems, and recombinant mTNF-α was obtained from BioSource). Plasmid DNA was isolated by using Qiagen Plasmid Purification Kits and DNA purification after restriction digestion was performed by using QiaQuik PCR or gel purification kit.

Construction of mTNF-α Expression Vector, pET26-mTNF. The murine *tnf*-α gene was amplified from plasmid pMuTNF (cat. no. 63169; American Type Culture Collection) by using PCR with the following primers: 5′-ATATACATATGCT CAGATCATCTTCTCAAAATTCG and 5′-AACAACCTCGAGTTATCACAGAGCA ATGACTCCAAAGTAGACC. The resulting PCR product was digested with NdeI and XhoI restriction enzymes and ligated into a pET26b vector (Novagen). The recombinant vector was then modified to append an N-terminal hexahistidine-tag (His$_6$-tag), followed by a proteolysis site for factor Xa immediately before the first codon for mature WT mTNF-α. Site-specific incorporation of pNO$_2$Phe into mTNF-α was carried out by mutating the codon for Tyr^{86}, Lys^{11}, or Asp^{42} to a TAG amber codon by using the Quick Change Mutagenesis Kit (Stratagene). The same kit was also used to prepare the mTNF-α mutants Phe^{86} mTNF-α and Phe^{42} mTNF-α. The sequences of all mTNF-α constructs were confirmed by DNA sequence analysis.

Expression of WT and Mutant mTNF-α. To express the pNO$_2$Phe^{86}mTNF-α, pNO$_2$Phe^{11}mTNF-α, and pNO$_2$Phe^{42}mTNF-α mutants, *E. coli* BL21(DE3) cells were cotransformed with mutNO$_2$PheRS, mutRNA$_{CUA}$ and the respective mutant *mTNF*-α gene. The transformed cells were grown in the presence of 1 mM pNO$_2$Phe in GMML medium at 37°C and induced with 1 mM IPTG when the OD$_{600}$ reached 0.5. The cells were then continually shaken at 37°C for 12–16 h and then harvested. The cell pellet was stored at −80°C until use. WT mTNF-α, Phe^{86} mTNF-α, and Phe^{42} mTNF-α were expressed by essentially the same procedure. However, in contrast to the pNO$_2$Phe mTNF-α mutants, these proteins were expressed in rich medium (2× YT medium) in the absence of pNO$_2$Phe.

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Purification of WT and Mutant mTNF-α Under Denaturing Conditions. All purification steps were performed at room temperature. After thawing the cell pellet for 15 min on ice, the cell paste was resuspended in lysis buffer [100 mM NaH_2PO_4 (pH 8.0), 10 mM Tris·HCl, 8 M urea] at 5 ml per gram of wet weight. The cell suspension was sonicated on ice for 3 min. After centrifugation at $10,000 \times g$ for 25 min, 10 ml of Ni-NTA His-Bind Resin (Novagen) was added to the supernatant and mixed on a rotary shaker for 60 min. The lysate–resin mixture was loaded onto a 5-ml polypropylene column (Qiagen) and washed twice with 40 ml of wash buffer A [100 mM NaH_2PO_4 (pH 6.3), 10 mM Tris·HCl, 8 M urea]. After another two washing steps with 10 ml of wash buffer B [100 mM NaH_2PO_4 (pH 5.9), 10 mM Tris·HCl, 8 M urea], elution was carried out with 100 mM NaH_2PO_4 (pH 4.5), 10 mM Tris·HCl, 8 M urea. The protein was concentrated with a 10K molecular mass cut-off Amicon Ultra-15 centrifugal filter device (Millipore) and loaded onto a HiPrep 26/10 desalting column (GE Healthcare) preequilibrated with factor Xa cleavage buffer [20 mM Tris·HCl, 200 mM NaCl, 1 mM EDTA (pH 7.4)]. Turbid fractions containing inclusion bodies were concentrated by several rounds of diafiltration using a 10K molecular mass cut-off Amicon Ultra-15 centrifugal filter device before addition of factor Xa (5% WT/WT). Quantitative removal of the N-terminal His_6-tag was achieved within ≈3 days at room temperature as verified by SDS/PAGE analysis. After protease digestion, soluble factor Xa protease and the His_6-tag peptide were separated from the inclusion bodies by centrifugation. The protein was then dissolved in ≈1 ml of solubilization buffer [8 M urea, 50 mM Tris·HCl (pH 8.0), 10 mM DTT] and injected onto a Superdex 75 10/300 GL column (GE Healthcare) preequilibrated with solubilization buffer. Two rounds of size-exclusion chromatography were carried out on an ÁKTA purifier instrument (GE Healthcare) at a flow rate of 0.3 ml/min. For refolding, the protein sample was dialyzed against renaturation buffer [240 mM NaCl, 10 mM KCl, 0.5% Triton X-100, 50 mM Tris·HCl, 1 mM EDTA (pH 8.0)] using a 10K molecular mass cut-off Slide-A-Lyzer dialysis cassette (Pierce). After refolding, the renatured protein was dialyzed against PBS.

Passive Immunization Protocols. Mice were passively immunized 24 h before the endotoxin challenge. In the first experiment, mice received an i.p. injection of 100 μl of pooled serum from mice immunized with either $pNO_2Phe^{86}mTNF-α$ or WT mTNF-α. A second cohort received 4 mg/kg of IgG purified from serum of mice immunized with either $pNO_2Phe^{86}mTNF-α$ or WT mTNF-α. Control mice were injected with equal volumes of physiological saline.

Mouse Model of Severe Endotoxemia. All experiments were performed in accordance with the National Institutes of Health Animal Protection Guidelines and were approved by The Scripps Research Institute Animal Care and Use Committee. Lipopolysaccharide (LPS, *E. coli* O111:B4; Calbiochem/EMD Biosciences) was dissolved in 37°C normal saline (0.9% wt/vol NaCl) by vortexing for 30 s before and after 2 minutes of sonication. Male C57BL/6 mice from The Jackson Laboratories were injected i.p. under 2% isoflurane at the age of 9 weeks with 7.5 mg/kg LPS for the passive immunizations or 15 weeks with 8.5 mg/kg LPS for the active immunization. All experiments were carried out in a room with alternating 12-h light/dark cycles under stable conditions of temperature (20°C-22°C) and relative humidity (40–60%). Kaplan–Meier curves were plotted, and survival differences were analyzed by using a log rank test. For additional materials and methods, see *SI Text*.

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