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Trimer-to-Monomer Disruption Mechanism for a Potent, Protease-Resistant Antagonist of Tumor Necrosis Factor- α Signaling

Jiani Niu¹, Annika J. Cederstrand¹, Geoffrey A. Eddinger¹, Boyu Yin¹, James W. Checco^{1,‡}, Craig A. Bingman², Victor K. Outlaw^{1,†}, Samuel H. Gellman¹

¹Department of Chemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA.

²Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA.

Abstract

Aberrant tumor necrosis factor- α (TNF α) signaling is associated with many inflammatory diseases. The homotrimeric quaternary structure of TNF α is essential for receptor recognition and signal transduction. Previously, we described an engineered α/β -peptide inhibitor that potently suppresses TNF α activity and resists proteolysis. Here we present structural evidence that both the α/β -peptide inhibitor and an all- α analogue bind to a monomeric form of TNF α . Calorimetry data support a 1:1 inhibitor:TNF α stoichiometry in solution. In contrast, previous co-crystal structures involving peptide or small-molecule inhibitors have shown the antagonists engaging a TNF α dimer. The structural data reveal why our inhibitors favor monomeric TNF α . Previous efforts to block TNF α -induced cell death with peptide inhibitors revealed that surfactant additives to the assay conditions cause a more rapid manifestation of inhibitory activity than is observed in the absence of additives. We attributed this effect to a loose association surfactant-TNF α association that lowers the barrier to trimer dissociation. Here, we used the new structural data to design peptide inhibitors bearing a surfactant-inspired appendage intended to facilitate TNF α trimer dissociation. The appendage modified the time course of protection from cell death.

Graphical Abstract

Corresponding Author: Samuel H. Gellman – gellman@chem.wisc.edu.

[‡]Present Addresses

Department of Chemistry, University of Nebraska – Lincoln, Lincoln, Nebraska 68588, USA

[†]Department of Chemistry, University of Missouri, Columbia, Missouri 65211, USA

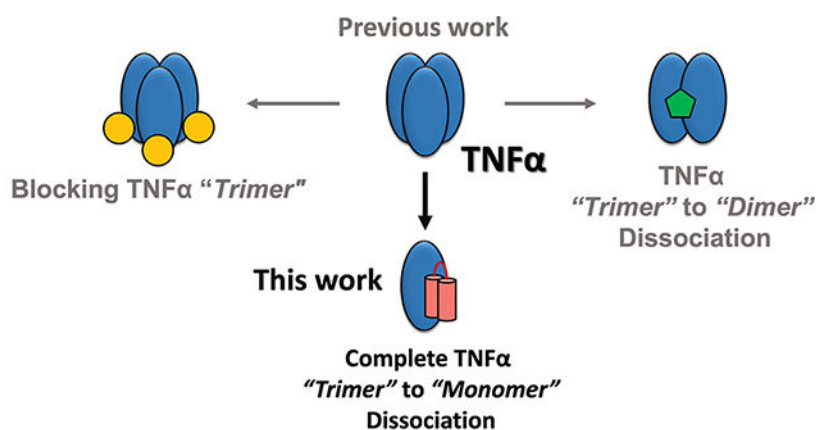
The authors declare the following competing financial interest(s): S.H.G. is a co-founder of Longevity Biotech, Inc., which is pursuing biomedical applications of α/β -peptides.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Materials and instrumentations (Section I), method descriptions (Section II), additional figures and tables (Section III), and characterization data for all synthesized peptides (Section IV) (PDF).

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INTRODUCTION

Excessive signaling mediated by cytokines, growth factors and other proteins is associated with many human diseases.^{1,2} These processes require that the soluble "message" protein engage with cell-surface receptors, and agents that block this engagement can be effective as drugs.³ Because contact between signaling proteins and their receptors usually involves large surfaces on each partner, therapeutically useful antagonists in current clinical use are themselves proteins, most commonly engineered antibodies.^{4,5} Antagonists of lower molecular weight would be appealing because antibodies are challenging to produce and store, and sustained use of engineered proteins can lead to adverse immunological responses.^{6,7} Efforts to inhibit protein-protein interactions with small molecules, however, have seldom led to clinical success, presumably because most small molecules cannot bind tightly enough to a target protein surface to prevent engagement of a much larger partner protein.^{3,8,9} This challenge has inspired many creative approaches based on peptides and related oligomers.^{10–24}

Aberrant tumor necrosis factor- α (TNF α) signaling occurs in many inflammatory diseases, and engineered proteins that inhibit TNF α binding to cognate receptors have been widely adopted in human medicine.^{1,4} TNF superfamily proteins function as non-covalent homotrimers; protein drugs that block signaling act by binding to the trimeric cytokine in a manner that prevents association with cell-surface receptors.^{25,26} An alternative mechanism of antagonism would be to disrupt the trimeric TNF α quaternary structure, which is required for signal transduction. The small molecule SPD304, a modest inhibitor of TNF α signaling in cell-based assays, was originally proposed to function in this way.²⁷ A co-crystal structure of SPD304 and TNF α revealed a dimeric form of the protein, with the small molecule engaging a surface exposed by the absence of the third protomer.²⁷ However, a comparable co-crystal structure was subsequently reported for another small molecule inhibitor, and in this case several copies of the inhibitor could be resolved in association with the TNF α dimer.²⁸ Retrospective analysis suggested a similar possibility for the structure containing SPD304. Other studies indicate that inhibition of TNF α signaling by SPD304 does not depend on trimer dissociation.²⁹ A series of benzimidazole derivatives has recently been shown to block TNF α signaling by binding to the trimer and stabilizing an unsymmetrical

quaternary structure that cannot properly engage with cell-surface receptors; the impressive qualities of these compounds include oral activity in mice.^{30,31} Luzi et al. have reported a chemically crosslinked 19-mer peptide, designated M21, that inhibits TNF α signaling by stabilizing a dimeric form, as established by a co-crystal structure.³²

We recently described a 29-mer peptide (**1**; Figure 1a) that potently inhibits TNF α signal transduction, an activity that was correlated with disruption of the native trimeric quaternary structure.³³ This inhibitor contains six non-proteinogenic residues, three derived from β -amino acids and three from 2-aminoisobutyric acid (Aib) (Figure 1b), and displays considerable resistance to proteolytic degradation.³⁴ Here we demonstrate that α/β -peptide **1** stabilizes a *monomeric* form of TNF α . To our knowledge, this work provides the first demonstration of complete dissociation of the trimeric TNF α to a monomeric form. High-resolution structural data elucidate the basis of this unique inhibitory mode of action.

RESULTS AND DISCUSSION

α/β -Peptide **1** stabilizes a *monomeric* form of TNF α .

The design of α/β -peptide **1** was based on a 59-mer polypeptide (Z-TNF α , Figure S1) that had been selected and optimized by Jonsson et al. via phage display for binding to TNF α .³⁵ This 59-mer has a Z domain scaffold, which adopts a three-helix bundle tertiary structure; the TNF α -binding surface is displayed by two of the three helices.^{7,36,37} Our previous development of **1** involved eliminating the third helix, stabilizing the necessary helix-loop-helix conformation with a disulfide, and introducing nonproteinogenic residues, among other modifications.^{33,35–42} Size-exclusion chromatography (SEC) data showed that **1** caused dissociation of the trimeric state,³³ but we could not determine from this analysis whether a dimer or monomer was formed.

To elucidate the interaction of **1** with TNF α , we determined a cocrystal structure, which reveals a 1:1 ratio of α/β -peptide **1** and TNF α monomer (Figure 2). The crystallization solution contained 0.8 M sodium potassium tartrate, 0.1 M Tris (pH 8.5), and 4% ethylene glycol. (No surfactant was included.) The asymmetric unit contains eight independent copies of each component. The TNF α monomers are arranged in an octameric ring, with the α/β -peptides engaged by surfaces oriented toward the center of the ring (Figure 2a). All eight TNF α -peptide copies are very similar structurally (Figure S2a, Table S1). Based on data described below and the well-established significance of the trimeric TNF α quaternary structure, we hypothesize that the octameric arrangement has no biological significance and is a consequence of crystal packing forces.

In the crystal structure, α/β -peptide **1** is bound to a TNF α surface that would pack against neighboring monomers in the native trimeric quaternary assembly (Figure 2b, 3a). The side chains of His¹⁷ and Phe²⁰ on α/β -peptide **1** are oriented toward complementary TNF α surface cavities. The side chains of Leu², Ile⁶, Arg²¹, Ile²⁴, and Leu²⁵ make other close contacts with the TNF α monomer surface (Figure 2c). The side chain guanidinium group of Arg²¹ forms hydrogen bonds with the main chain carbonyl of TNF α residue Ala¹⁵⁶ (Figure 2d). As intended in the original design of **1**, all of the β - and Aib-substitution sites are oriented toward solvent rather than toward the TNF α protomer surface (Figure

2b,c). The loop of the helix-loop-helix structure (Figure 2c) does not directly interact with the TNF α surface, as expected from our previous design. This structure thus validates the design strategy that produced α/β -peptide **1**, which sought to incorporate non-proteinogenic residues to protect the peptide from proteolytic degradation without affecting the molecular surface required for engagement of TNF α .^{33,37}

Trimer-to-monomer dissociation of TNF α has not been previously demonstrated, despite multiple efforts to inhibit TNF α signaling with small molecules and peptides.^{27,28,31–33} To understand how α/β -peptide **1** could stabilize a monomeric form of TNF α , we overlaid a representative TNF α -peptide pair from our new co-crystal structure with the native TNF α trimer structure (Figure 3a). In this overlay, the TNF α monomer from our structure was aligned with one of the monomers from the TNF α trimer (gray A and A' in Figure 3a) with an average RMSD of 0.54 Å (Table S2). This structural similarity supports our hypothesis that **1** inhibits TNF α -mediated signaling by disrupting the trimeric quaternary structure rather than distorting monomer tertiary structure.

The structural overlay shows that the bulk of α/β -peptide **1** occupies a surface on the TNF α monomer that would engage one of the two neighbors in the trimer (yellow B' in Figure 3a). The N- and C-termini of **1**, however, extend onto a surface required to engage the other neighbor in the trimer (blue C' in Figure 3a). In the native TNF α trimer, β -strands formed by segments 55–67 and 112–123 mediate contacts between subunits.²⁵ Key residues from these strands, Leu⁵⁷ and Val¹²³, contribute side chains that are buried in the core of the native trimer (Figure S3b,c). The overlay in Figure 3b shows that these two side chains from the TNF α monomer in blue clash with the volume of α/β -peptide **1** bound to the TNF α monomer in gray. In contrast, the co-crystal structure of bicyclic peptide M21 engaged with a TNF α dimer³² shows that inter-subunit contacts mediated by the side chains of Leu⁵⁷ and Val¹²³ can be maintained when M21 is bound (Figure S4). Therefore, we hypothesize that the slightly larger size of **1** relative to M21 is responsible for the unique trimer-to-monomer TNF α dissociation achieved by the α/β -peptide. This hypothesis is consistent with the observation of dimeric TNF α in co-crystal structures with small molecules SPD304 and JNJ525, even though multiple small molecules appear to associate with the dimer in each case.^{27,28}

α -Peptide 3 stabilizes a monomeric form of TNF α .

Because the features observed in a single crystal structure might not correspond to behavior in solution, we sought to co-crystallize TNF α with other peptide antagonists we had identified during the development of α/β -peptide **1** (α -peptides **2** and **3** in Figure 1 and α/β -peptides **4–8** in Figure S1).³³ Most efforts failed, but co-crystallization of **3** with TNF α yielded a high-resolution structure. (Peptide **3** and α/β -peptide **1** are comparable in their ability to inhibit binding of TNF α to TNF receptor 1 (Figure S1, S5).) Peptide **3** co-crystallized with TNF α from a solution containing 1.5 M (NH₄)₂SO₄, 0.1 M Tris (pH 8.5), and 12% glycerol. (No surfactant was included.) This crystal contained a 1:1 ratio of **3** and the TNF α protomer, matching the stoichiometry observed for the crystal containing α/β -peptide **1** and TNF α . The asymmetric unit contains two TNF α protomers, each bound to a molecule of **3**. The relative orientations of these two TNF α protomers are quite distinct

from the parallel packing in the native trimer (Figure S6a vs Figure S3a). Despite the considerable difference in the asymmetric units of the TNF α +1 and TNF α +3 structures, the interface between α -peptide 3 and the TNF α monomer is very similar to the interface between α / β -peptide 1 and the TNF α monomer (Figure S6a, Figure S2c and Table S1).

The mechanism by which α -peptide 3 induces trimer-to-monomer disruption of TNF α appears to be similar to that described above for α / β -peptide 1. Overlaying the TNF α +3 structure with the native TNF α trimer (Figure 4) reveals that the termini of 3 occupy the region that is required by Leu⁵⁷ and Val¹²³ from a neighboring protomer in the native trimer. This portion of 3 further extends into the region occupied by the Tyr⁵⁹ and Tyr¹¹⁹ side chains of the neighboring protomer in the native trimer (Figure 4b). Overall, the co-crystal structure containing TNF α and inhibitor 3 supports our mechanistic explanation for the unique ability of helix-loop-helix peptides to induce TNF α trimer disassembly to an inactive monomeric form and thereby block signaling mediated by this cytokine.

Evidence for 1:1 binding stoichiometry in solution.

Our previous SEC analysis of interactions between TNF α and peptide inhibitors showed disruption of the native cytokine trimer but could not distinguish between dimer and monomer as the resulting form.³³ Additional SEC data that support this conclusion are provided in Figure S8. To determine whether the stoichiometries of the TNF α -inhibitor complexes observed in the crystal structures correspond to behavior in solution, we used isothermal titration calorimetry (ITC) to characterize the interaction between the α -peptide inhibitor 3 with TNF α (Figure 5). Each addition of peptide solution caused an instantaneous heat absorption followed rapidly by heat release. Control ITC studies (Figure S7) showed that the initial endothermic effect is not related to TNF α and arises instead from peptide dilution. The exothermic effect results from interaction between peptide 3 and TNF α . Analysis of the ITC data revealed a 1:1 binding stoichiometry ($N = 0.93 \pm 0.09$), and an apparent K_D of $2.1 \pm 0.5 \mu\text{M}$. Very similar results were obtained with α -peptide 2 ($N = 0.93 \pm 0.04$; K_D of $3.3 \pm 0.2 \mu\text{M}$).

The 1:1 stoichiometry indicated by the ITC analysis is consistent with a final complex in which one molecule of peptide binds to one TNF α protomer, or a complex in which three molecules of peptide bind to the native TNF α trimer. However, our SEC data demonstrating the disruption of the TNF α trimer by 2 are provided in Figure S8, and since the crystal structures of TNF α +3 and TNF α +1 show single peptide molecules bound to TNF α protomer, we conclude that the complex formed in solution involves a single molecule of peptide bound to a TNF α protomer. The exotherm that results from formation of this complex must include contributions from dissociation of the TNF α trimer and from binding of the inhibitor to monomeric TNF α .

Evaluation of hydrophobically modified peptides for inhibition of TNF α -induced cell toxicity.

We previously showed that α / β -peptide 1 can interfere with TNF α -mediated killing of WEHI-13VAR cells.³³ TNF α -induced cell death results from binding of the cytokine to cell-surface receptors; therefore, inhibitory efficacy in this cellular assay is a measure of a

peptide's ability to block TNF α -receptor interaction. We found that if the antagonist peptide and TNF α were pre-incubated together for one hour before being added to the cells, then the peptide had no effect, and the toxic effects of TNF α were fully manifested. However, if a low concentration of the surfactant TWEEN20 was included during the pre-incubation, then significant inhibition of TNF α -induced cell killing was observed with low concentrations of the antagonist (IC₅₀ for **1** < 10 nM).

Based on these and related findings,³³ and a reconsideration of results from other groups,^{28,29,32,43,44} we concluded that peptide inhibitors such as **1** must overcome a significant kinetic barrier to dissociation of the TNF α trimer in order to block cell toxicity by stabilizing an inactive and dissociated form of the cytokine. Our data suggested that TWEEN20 can interact with TNF α in a way that facilitates but does not induce trimer dissociation. This interaction was attributed to the hydrophobic moiety within TWEEN20, a dodecanoyl group (Figure 6a); similar behavior was observed with other amphiphilic or hydrophobic additives, including the small molecule SPD304.³³

These prior results led us to hypothesize that a peptide antagonist such as **1** might display more rapid inhibition of TNF α signaling if a hydrophobic unit were appended. This hydrophobic unit would be intended to lower the barrier to TNF α trimer dissociation and thereby facilitate capture of a TNF α protomer by the linked peptide inhibitor. The co-crystal structures described above show that the N-termini of α/β -peptide **1** and α -peptide **3** do not make direct contact with the TNF α monomer surface (Figure 2b, S6b), which suggested that N-terminal modification of these peptide antagonists might be tolerated with minimal loss of antagonist activity.

We tested our hypothesis with a design featuring an N-terminal Lys residue bearing dodecanoyl groups on both the α and ϵ nitrogens (Figure 6b). A flexible oligo-ethylene glycol spacer was placed between this modified Lys residue and the N-terminus of the antagonist segment (Figure 6b). The resulting peptide, (C₁₂)₂-PEG₁₆-**1** (Figure 6c), was compared with **1** itself in the ELISA assay for inhibition of TNF α binding to TNFR1 (Table S3; Figure S9). These measurements were carried out in the presence of 0.05% TWEEN20, to avoid complications from non-specific binding to ELISA plate or reagents. The comparison revealed that the inhibitory potency of (C₁₂)₂-PEG₁₆-**1** was substantially diminished relative to that of **1** itself: IC₅₀ for (C₁₂)₂-PEG₁₆-**1** was ~44-fold higher than IC₅₀ for **1**.

The difference in ELISA results for **1** vs. (C₁₂)₂-PEG₁₆-**1**, in conjunction with the crystal structures of **1** and **3** bound to TNF α , led us to conclude that the large N-terminal appendage might be too close to the peptide surface that engages the TNF α protomer to allow optimal binding of (C₁₂)₂-PEG₁₆-**1**. We therefore extended the hydrophobic modification strategy to longer α/β -peptide **4** (Figure 1), which is an analogue of α -peptide **2**. In unmodified form, **4** is slightly more effective than **1** or **2** in blocking TNF α -TNFR1 association, as indicated by ELISA (Table S3; Figure S9). We hypothesized that lengthening of the N-terminal region (**4** vs. **1**) might diminish interference of the hydrophobic appendage with binding to TNF α . Comparison of **4** vs. (C₁₂)₂-PEG₁₆-**4** via ELISA, in the presence of 0.05% TWEEN20,

supported this structure-based hypothesis: IC_{50} for $(C_{12})_2$ -PEG₁₆-**4** was only ~7-fold higher than IC_{50} for **4** (Table S3; Figure S9).

To ask how the hydrophobic appendage affects the protection of cells from TNF α -induced toxicity, we compared **4** and $(C_{12})_2$ -PEG₁₆-**4** in a time course assay with WEHI-VAR13 cells in the absence of TWEEN20 (Figure 7). Almost no cells survived when 0.08 ng/mL TNF α was added without any antagonist; this condition was used to define 0% viability. Cell survival in the absence of TNF α was used to define 100% viability. These experiments explored two parameters: (1) the concentration of the antagonist (**4** or $(C_{12})_2$ -PEG₁₆-**4**) with which TNF α was pre-incubated, before addition to the cells; and (2) the length of the pre-incubation period, which varied between 1 hr and 24 hr at 37°C.

The cell survival assays showed that the hydrophobic modification influences the time course over which the α/β -peptide antagonist manifests its protective effect. After 2 hr pre-incubation of TNF α and antagonist **4**, no protection was detected even at the highest α/β -peptide concentration (10 μ M initial concentration, which was diluted 350-fold upon addition to cells; see SI sec II 7c for details). In contrast, at this time point 10 μ M $(C_{12})_2$ -PEG₁₆-**4** led to survival of ~25% of the cells (Figure 7a). The maximum level of cell survival achieved with $(C_{12})_2$ -PEG₁₆-**4** after 2 hr pre-incubation exceeded that achieved with unmodified **4** up to 6 hr of pre-incubation (Figure 7b). At 12 hr, 10 μ M $(C_{12})_2$ -PEG₁₆-**4** protected nearly all cells from TNF α -induced death, while only ~2/3 cells survived with 10 μ M **4** (Figure 7c). After 24 hr pre-incubation, however, both **4** and the hydrophobically modified analogue protected nearly all cells from the toxic effects of TNF α (Figure 7c). The dose-response data obtained after 24 hr pre-incubation suggested that the IC_{50} for **4** (4.0 nM) is approximately 5-fold higher than the IC_{50} for $(C_{12})_2$ -PEG₁₆-**4** (Table S4). This modest difference in the abilities of **4** and $(C_{12})_2$ -PEG₁₆-**4** to block TNF α -induced cell death is very similar to the difference in their abilities to inhibit TNF α -TNFR1 interaction as measured by ELISA (Table S3).

The cell assay data in Figure 7 support our design hypothesis that the two portions of hybrid molecule $(C_{12})_2$ -PEG₁₆-**4** can work synergistically to affect the time course over which protection of WEHI VAR13 cells from TNF α -induced toxicity is manifested. We propose that the dodecanoyl segments of this inhibitor associate with the TNF α trimer in a manner that loosens the quaternary structure and facilitates full-fledged trimer disruption mediated by the α/β -peptide portion. These observations raise interesting questions about the effects of hydrophobically modified TNF α antagonists *in vivo*, where the overall effect is likely to result from an interplay among thermodynamic and kinetic factors.

CONCLUSIONS

We have shown that α/β -peptide **1**, a proteolysis-resistant 29-mer, disrupts the native trimeric quaternary structure of TNF α and stabilizes a monomeric form. This disruptive activity presumably explains how **1** protects cells from TNF α -induced death.³³ Previous structural studies have shown that small molecules, apparently acting in clusters, and a cross-linked 19-mer peptide can induce trimer-to-dimer dissociation of TNF α .^{27,28,32} Our structural analysis of the complex formed by α/β -peptide **1** and monomeric TNF α and

of the analogous complex formed by α -peptide **3** reveals that these peptides occupy a moderately larger surface area on a TNF α protomer relative to the other inhibitors (Figure S10), which presumably explains the high potencies of **1** and **3** and the trimer-to-monomer dissociation induced by these inhibitors. Comparisons between the structure of α/β -peptide **1** and α -peptide **3** bound to TNF α show how strategic replacement of proteinogenic residues by β residues or Aib at non-contact sites can preserve a broad and specific binding surface displayed by a mini-protein tertiary structure while conferring resistance to proteolysis.

Dissociation of the native TNF α trimer by our peptides or other smaller agents requires that the antagonist overcome a substantial kinetic barrier.^{28,29,32,33} We previously discovered that additives such as TWEEN20 or SPD304 shorten the time course of antagonism manifested by peptides such as **1**. The additive apparently lowers the barrier to TNF α trimer dissociation.³³ The two co-crystal structures reported here suggested that appending a lipophilic group to the N-terminus of an α/β -peptide antagonist might improve the time course of cell rescue from TNF α -induced death. The anticipated improvement was observed for (C₁₂)₂-PEG₁₆-**4**, relative to the unmodified α/β -peptide, **4**.

These studies provide a basis for future efforts to block aberrant oligomeric cytokine signaling *in vivo* with backbone-modified peptides that bear a hydrophobic appendage and resist proteolysis. Such molecules might ultimately be useful alternatives to the engineered antibodies and receptor fragments that are currently used to treat inflammatory diseases mediated by TNF α . Peptides that bear a hydrophobic appendage and activate the glucagon-like peptide-1 receptor (GLP-1R), such as liraglutide and semaglutide, are widely used to treat type 2 diabetes.⁴⁵ In these cases, the appendage apparently promotes peptide binding to albumin in the bloodstream, which hinders peptide excretion. Attaching a lipid moiety enhances the potency of peptide inhibitors of enveloped virus infection.⁴⁶ The lipid is believed to localize such peptides in the cell membrane and thereby enhance their potency in blocking the function of viral fusion proteins. Pharmacokinetic benefits, such as those seen with GLP-1R agonists, and/or localization benefits observed for antiviral lipopeptides, might be manifested *in vivo* for an agent such as (C₁₂)₂-PEG₁₆-**4**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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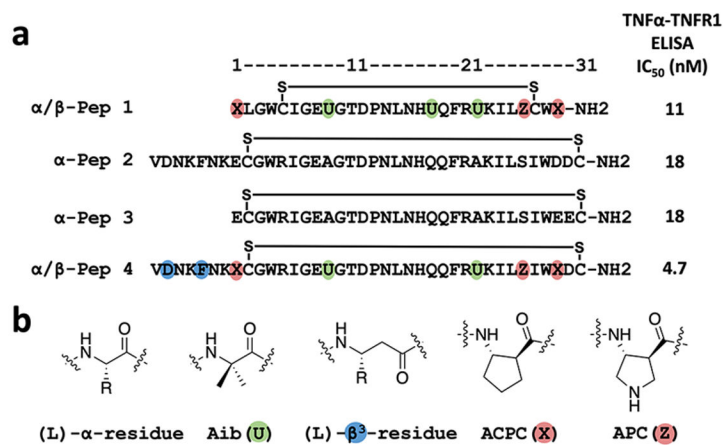
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**Figure 1.**

(a) Sequences of TNF α peptide inhibitors (1–4) used in this study. The two cysteines are engaged in the intramolecular disulfide bond. Unnatural amino acids are indicated by colored ovals. (b) Structures of a generic (L)- α -residue, an Aib residue (U) (green), a (L)- β^3 -residue (blue), a cyclic β -residue ACPC residue (X) (red), and a cyclic β -residue APC residue (Z) (red).

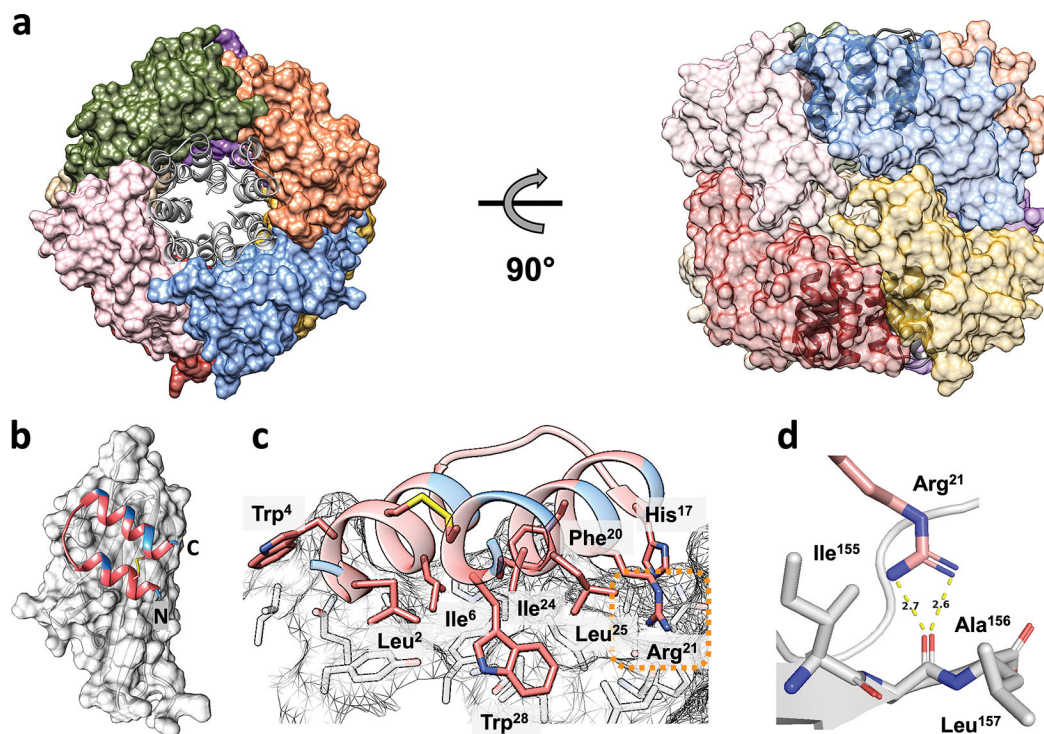


Figure 2.

Crystal structure of α/β -peptide **1** bound to TNF α with a 1:1 stoichiometry (PDB ID: 7TA6). **(a)** Top (left) and side (right) views of the asymmetric unit, which contains eight copies α/β -peptide **1** (grey) and eight copies of TNF α monomer (colored). **(b)** α/β -Peptide **1** (pink, with unnatural residues in blue and intramolecular disulfide in yellow) bound to TNF α monomer (light grey). The N- and C- termini of α/β -Peptide **1** are labeled. **(c)** Close-up showing side chains of the α/β -peptide **1** that contact the TNF α surface. **(d)** The side chain of residue Arg²¹ on α/β -peptide **1** interacts with the backbone of Ala¹⁵⁶ on TNF α through hydrogen bonding.

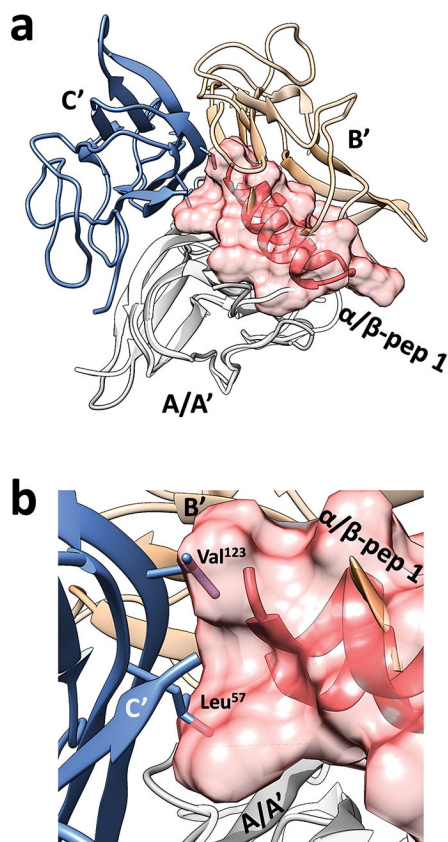


Figure 3. Overlay of a representative TNF α monomer and the bound α/β -peptide **1** from the TNF α +1 crystal structure with the native TNF α trimer crystal structure (PDB ID: 1TNF, denoted as A', B' and C'). (a) The TNF α monomer (A; dark grey ribbons) bound to **1** is aligned to TNF α monomer (A'; light gray ribbons) from the native trimer. The α/β -peptide **1** is shown as a pink volume, and the other two TNF α monomers (B' and C') from the native homotrimer are shown as blue and yellow ribbons. This overlay shows that binding to α/β -peptide **1** prevents association of a TNF α monomer with either of the other subunits that would constitute a native trimer. (b) Close-up showing that the surface of α/β -peptide **1** bound to the TNF α monomer (A; dark gray) occupies space that would be occupied by key interface side chains from the TNF α monomer in blue in the native homotrimer.

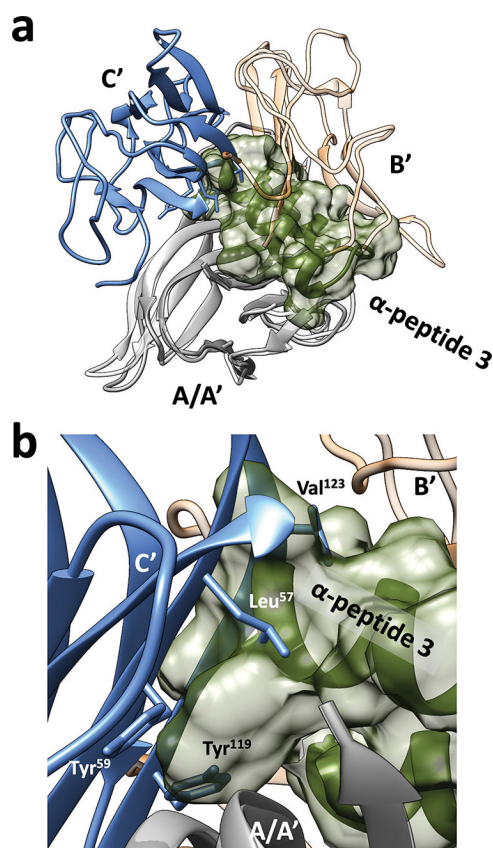


Figure 4. (a) Overlay one TNF α and α -peptide **3** pair in TNF α +**3** crystal structure (PDB ID: 7TA3) with the native TNF α trimer crystal structure (PDB ID: 1TNF) as in Figure 3. The α -peptide **3** (green volume) prevents association of the TNF α monomer with either of the other subunits (B' and C') that would constitute a native trimer. (b) Zoom in showing how the N- and C-termini of **3** occupy space that would be occupied by key interface side chains from the TNF α monomer in blue in the native homotrimer.

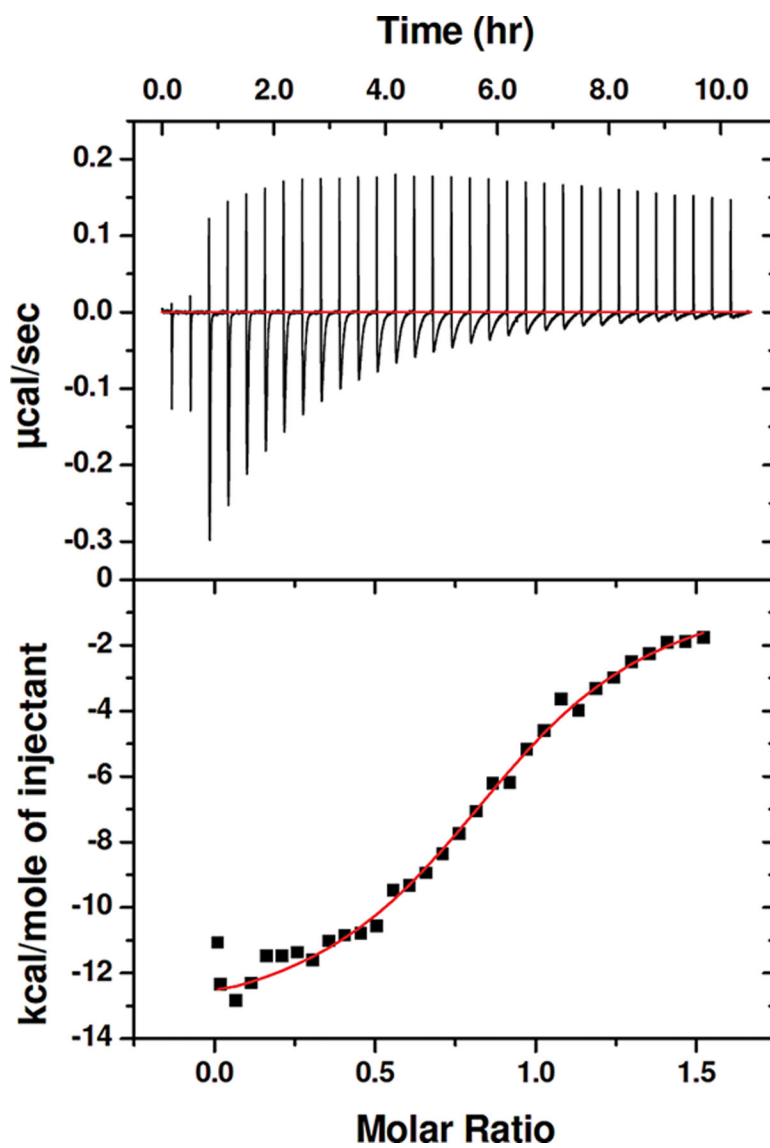
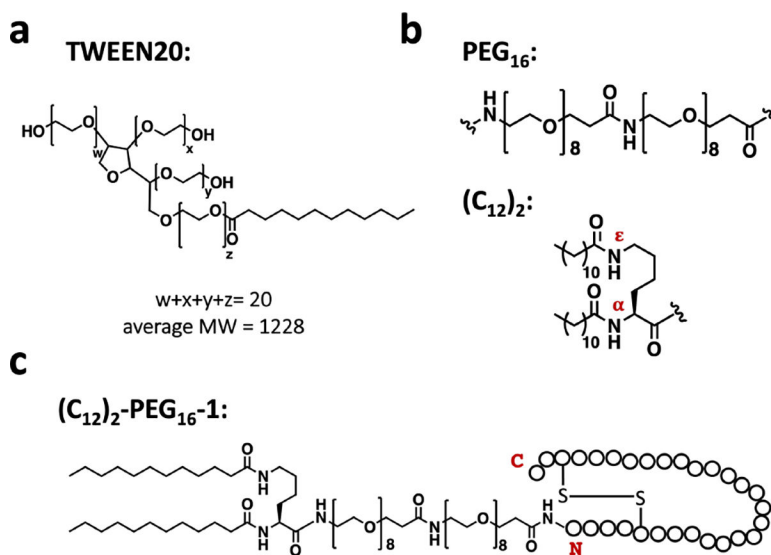


Figure 5. An isothermal titration calorimetry (ITC) experiment for addition of α -peptide **3** to TNF α in PBS with 0.05% TWEEN-20. The binding affinity (K_D) and stoichiometry (N) values were calculated from three independent experiments.

**Figure 6.**

(a) Chemical structure of TWEEN 20. (b) Chemical structure of oligo-ethylene glycol (PEG₁₆) and an N-terminal Lys residue bearing two dodecanoyl groups (C₁₂)₂ on the α and ϵ nitrogens. (c) A depiction of the lipid-modified peptide (C₁₂)₂-PEG₁₆-1 where peptide 1 residues are indicated by circles.

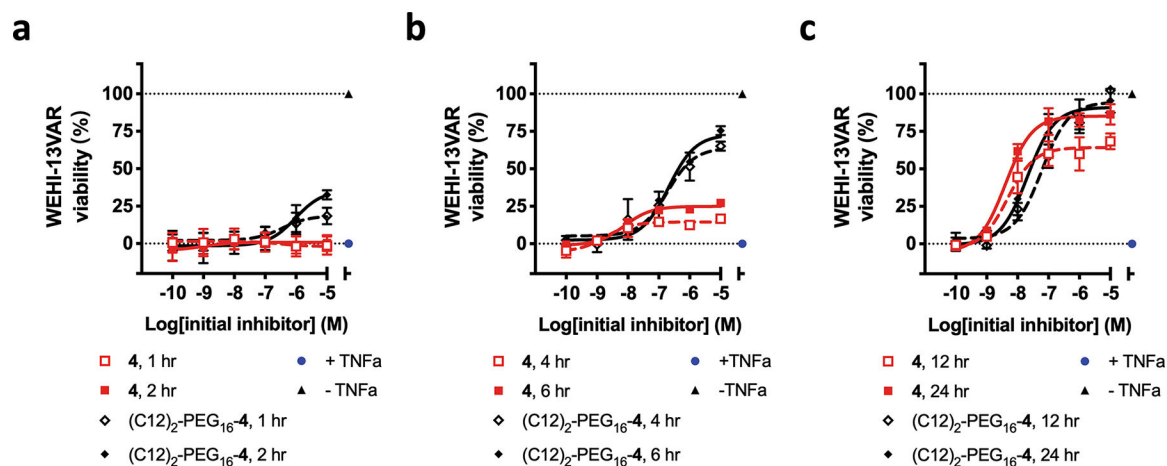


Figure 7.

Protection of WEHI-13VAR cells from TNF α -induced toxicity, by α/β -peptide **4** and a hydrophobically modified analogue, (C₁₂)₂-PEG₁₆-**4**. These studies show the effects of two variables: concentrations of the peptide antagonist (**4** or (C₁₂)₂-PEG₁₆-**4**), and the pre-incubation times of the peptide antagonist with TNF α before the mixture was added to the cells ((**a**) 1 or 2 hr; (**b**) 4 or 6 hr, (**c**) 12 or 24 hr). “+TNF α ”: TNF α + DMSO; “-TNF α ”: PBS + DMSO. All pre-incubation steps were conducted at 37°C in PBS. No amphiphilic additive (e.g., TWEEN20) was used at any point.