Macrocyclic θ-defensins suppress tumor necrosis factor-α (TNF-α) shedding by inhibition of TNF-α converting enzyme

Justin B. Schaal1, Thorsten Maretzky2,3, Dat Q. Tran1, Patti A. Tran1, Prasad Tongaonkar1, Carl P. Blobel2, André J. Ouellette1,4, and Michael E. Selsted1,4*

Running title: θ-defensin suppression of TACE mediated TNF shedding

1Department of Pathology & Laboratory Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California, USA

2Hospital for Special Surgery at Weill Cornell Medicine, New York, New York, USA

3Inflammation Program and Department of Internal Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA

4Norris Comprehensive Cancer Center of the University of Southern California, Los Angeles, California, USA

*To whom correspondence should be addressed: Michael E. Selsted, Department of Pathology and Laboratory Medicine, Keck School of Medicine, University of Southern California, 2011 Zonal Ave. Hoffman Medical Research Room 204, Los Angeles, CA, 90089-9092, USA, Telephone: 1-323-442-1180, E-mail: selsted@usc.edu

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ABSTRACT

Theta-defensins (θ-defensins) are macrocyclic peptides expressed exclusively in granulocytes and selected epithelia of Old World monkeys. They contribute to anti-pathogen host defense responses by directly killing a diverse range of microbes. Of note, θ-defensins also modulate microbe-induced inflammation by affecting the production of soluble tumor necrosis factor (sTNF) and other proinflammatory cytokines. Here, we report that natural rhesus macaque θ-defensin (RTD) isoforms regulate sTNF cellular release by inhibiting TNF alpha converting enzyme (TACE; also known as a disintegrin and metalloprotease 17; ADAM17), the primary pro-TNF sheddase. Dose-dependent inhibition of cellular TACE activity by RTDs occurred when leukocytes were stimulated with live E. coli cells as well as numerous Toll-like receptor agonists. Moreover, the relative inhibitory potencies of the RTD isoforms strongly correlated with their suppression of TNF release by stimulated blood leukocytes and THP-1 monocytes. RTD isoforms also inhibited ADAM10, a sheddase closely related to TACE. TACE inhibition was abrogated by introducing a single opening in the RTD-1 backbone, demonstrating that the intact macrocycle is required for enzyme inhibition. Enzymologic analyses showed that RTD-1 is a fast binding, reversible, non-competitive inhibitor of TACE. We conclude that θ-defensin-mediated inhibition of proTNF proteolysis by TACE represents a rapid mechanism for the regulation of sTNF and TNF-dependent inflammatory pathways. Molecules with structural and functional features mimicking those of θ-defensins may have clinical utility as TACE inhibitors for managing TNF-driven diseases.

INTRODUCTION

Mammalian defensins are host defense peptides composed of three structural families, designated as α-, β- or θ-defensins. The three defensin families are genetically related, but are distinguished by amino acid chain length and distinctive trisulfide motifs that are characteristic of each family (1-4). Isolation and characterization of each of the defensin families was the result of studies aimed at identifying...
antimicrobial substances in granulocytes or epithelial cells implicated in host defense (5,6). Among defensins, θ-defensins are unique in that they are 18-amino acid macrocyclic peptides known to be expressed only in cells of Old World monkeys and are the only cyclic polypeptides known in animals (7).

Multiple θ-defensin isoforms are expressed in neutrophils and monocytes of rhesus monkeys (six; (8)) and olive baboons (ten; (9)), each of which is a macrocyclic octadecapeptide that includes six disulfide linked cysteines (Fig. 1). The sequence diversity of θ-defensins results from the binary combinations of gene-encoded nonapeptides that are ligated head-to-tail to form the θ-defensin backbone (Fig. 1) (10). Humans and great apes lack θ-defensins due to stop codon interruptions in the signal peptide coding regions of the respective θ-defensin precursors (11).

Like α- and β-defensins, numerous θ-defensin isoforms have antimicrobial activities against bacteria, fungi, and viruses (2-4,7-10,12,13). However, among the three peptide families, θ-defensins are distinguished by their unique immunomodulating properties. For example, the prototype θ-defensin RTD-1 reduced lethality in murine models of polymicrobial sepsis, E. coli bacteremia, and SARS coronavirus infection, and the therapeutic effects in each model were associated with significant reductions in tissue proinflammatory cytokine and chemokine levels (14,15). Studies in the mouse SARS coronavirus model strongly implicated host-directed anti-inflammatory effects of RTD-1 because the peptide had no direct antiviral activity (15). RTD-1 was also effective in reducing pulmonary pathology in murine models of endotoxic lung injury (16) and cystic fibrosis (17) by moderating inflammatory responses. Also, RTD-1 arrested joint inflammation in a rat model of rheumatoid arthritis (RA), pristane-induced arthritis (PIA), an autoimmune disease characterized by dysregulated pro-inflammatory cytokines and erosive joint changes similar to those associated with RA (18). Parenteral administration of RTD-1 to rats with established PIA rapidly induced arrest of disease progression and resolution of arthritis that correlated with significant reductions in proinflammatory cytokines in joint tissues (in press).

sTNF is produced when pro-TNF, a type-II transmembrane protein, is cleaved at the cell surface by TACE (ADAM17) (19-21). TACE is a membrane-anchored zinc-metalloprotease and is responsible for “shedding” the ectodomain of TNF and many additional cytokines, growth factors, receptors, and adhesion molecules (22,23). Dysregulated TACE activity has been associated with disruption of cytokine homeostasis, elevating levels of TNF in chronic and acute inflammatory diseases including RA, sepsis, and colitis (24-29) as well as cancer progression (22,30). Inhibition of TACE activity with broad-spectrum metalloprotease inhibitors prevents TNF release from cell surfaces, suppressing levels of sTNF (31-33). In a previous study on the kinetics of RTD-1 inhibition of TNF release by E. coli-stimulated leukocytes we found that that suppression of sTNF occurred rapidly upon peptide addition to the bacteria-blood incubation mixture (14). We hypothesized that the blockade of sTNF release is mediated by inhibition of proTNF ectodomain shedding. Thus, we performed studies to evaluate the effects of RTD isoforms on the TNFα convertase TACE and the related sheddase, ADAM10.

RESULTS

RTD-1 suppresses TNF release by human blood leukocytes stimulated by diverse TLR agonists. In a previous study we showed that RTD-1 suppressed the release of several pro-inflammatory cytokines, including TNF, IL-1α, IL-1β, IL-6, IL-8, CCL2, CCL3, and CCL4 by human buffy coat leukocytes stimulated with agonists of TLRs 2, 4, 5, and 8 (14). TNF release was markedly suppressed irrespective of the stimulus (14). In the current study we analyzed the effects of 5 or 15 µM RTD-1 on TNF secretion by human blood leukocytes stimulated with an expanded panel of TLR ligands including agonists of TLRs 1/2, 2, 4, 5, 2/6, 8, and 9. In the absence of peptide, stimulated cells secreted 30-1100 pg/ml of TNF (Fig. 2). Consistent with our previous report, RTD-1 dose-dependently reduced TNF release by agonists for TLRs 2, 4, 5, and 8 and also had similar effects on cells stimulated with ligands for TLRs 1/2, 2/6, and 9 (Fig. 2). These findings, and the rapid blockade of TNF release observed when blood leukocytes were stimulated with E. coli cells in the presence of
RTD-1, suggested that the peptide regulates proteolytic release of TNF.

**RTD-1 inhibits TNF release by THP-1 cells but does not affect downstream signaling of sTNF in colonic epithelial cells.** We previously showed that RTD-1 dose-dependently suppressed TNF release by LPS stimulated THP-1 monocytes (14). To determine whether RTD-1 pretreatment of THP-1 cells blocked LPS-induced TNF secretion, cells were incubated for 60 min with 5 µM RTD-1 or vehicle, washed, and stimulated with LPS in the presence or absence of 5 µM RTD-1. As shown in Figure 3A, suppression of LPS-induced TNF secretion only occurred if RTD-1 was present when the cells were stimulated, as washout of the peptide prior to LPS stimulation had no effect on TNF release. This result was not due to neutralization of LPS as we showed previously that RTD-1 does not block the endotoxic properties of LPS (14).

Cellular responses to soluble TNF are mediated by paracrine and autocrine signaling, resulting in further TNF release and activation of other downstream inflammatory effects (34). To determine whether RTD-1 affects downstream TNF signaling, we tested whether the peptide alters the response of HT-29 cells to TNF. HT-29 cells express TNF receptor I (TNFRI) and release IL-8 in response to sTNF (35). Consistent with previous reports, TNF-stimulated HT-29 cells induced robust IL-8 release (Fig. 3B). This was unaffected by the co-incubation with RTD-1 (Fig. 3B), and RTD-1 alone had no effect on IL-8 release by HT-29 cells (data not shown). Thus, RTD-1 does not affect TNF signaling by direct neutralization of sTNF nor does the peptide appear to interfere with sTNF/TNFRI signaling in HT-29 cells.

**Inhibition of TACE by θ-defensins.** RTD-1 blockade of TNF release by human leukocytes stimulated with E. coli cells is extremely rapid (14), and the peptide down-regulates TNF release by leukocytes irrespective of the stimulating TLR ligand (Fig. 2). Based on these findings, we hypothesized that RTD-1 inhibits TNF release by inhibition of its mobilization from the cell surface by its principal convertase, TACE (ADAM17). RTD-1 dose-dependently inhibited recombinant human TACE (rhTACE) cleavage of its fluorogenic substrate (Fig. 4A) with an IC₅₀ = 0.11 µM +/- 0.04. Of note, the inhibition by RTD-1 did not require pre-incubation of the peptide and the TACE enzyme. As discussed later, we found that RTD-1 rapidly bound to and inhibited the enzyme.

*The sigmoidal inhibitory response of RTD-1 was approximately 10-fold less potent than that of marimastat (MRM), a small molecule inhibitor of zinc matrix metalloproteinases (36) (Fig. 4A). A single chain opening in the RTD-1 backbone, producing the S7 analog (Fig. 1), reduced inhibitory potency >99% (Fig. 4A). We also tested the TACE inhibitory activities of human θ-defensins (human neutrophil peptides, HNPs 1-4) that have proinflammatory and/or immune activating properties (37-43). Neither HNP-2 nor HNP-4 inhibited TACE (Fig. 4A). We then evaluated the effects of RTD-1, S7, marimastat, and human θ-defensins for their inhibition of TNF release by LPS stimulated THP-1 cells. None of the HNPs, nor S7 was effective in blocking TNF release, whereas RTD-1 and marimastat dose-dependently inhibited sTNF release (Fig. 4B), consistent with the TACE inhibitory activity of each compound (Fig. 4A).

In a previous study, we showed that natural RTD isoforms (RTDs 1-5) varied markedly in their inhibition of TNF release by stimulated blood leukocytes or THP-1 monocytes (14). Therefore we evaluated RTDs 1-5 for their relative inhibition of rhTACE. Each peptide dose-dependently inhibited TACE, but with IC₅₀ values that ranged from 50 - 265 nM, giving an inhibitory hierarchy of RTD-2 > RTD-5 > RTD-4 > RTD-1 > RTD-3 (Fig. 4 C; Table 1). These peptides were then evaluated for their inhibition of sTNF release by LPS-stimulated THP-1 cells. The hierarchy of dose-dependent inhibition of sTNF release (Fig. 4D) was the same as that obtained for TACE inhibition by RTDs 1-5. Indeed, the TACE IC₅₀ values of the RTDs were highly correlated with their TNF-release IC₅₀ values in assays using LPS-stimulated THP-1 cells or E. coli-stimulated blood leukocytes (Fig. 5).

**θ-Defensins inhibit TACE activities of THP-1 and HT-29 cells.** To test whether the effects of θ-defensins on rhTACE are replicated with TACE-expressing cells, we incubated RTDs 1-5 with THP-1 macrophages and HT-29 epithelial cells in the presence of a fluorogenic substrate that mimics the cleavage site of membrane-bound TNF, allowing for continuous, real-time measurement of TACE activity in live cells.
(44,45). After a brief lag, substrate conversion in RTD-free controls was linear for 60 min. RTDs dose-dependently inhibited substrate hydrolysis by both THP-1 macrophages and HT-29 cells (Fig. 6). Nearly maximum inhibition of THP-1 cell TACE was achieved with 5 µM of RTDs 1, 3, and 5 while RTDs 2 and 4 were slightly less inhibitory at this concentration (Fig. 6A). The absence of complete inhibition at the highest RTD levels tested is similar to findings reported for the small molecule TACE inhibitors GM6001 and BB94 (45).

\[ \theta \text{-defensins inhibit TACE and ADAM10 expressed on COS7 cells.} \]

To further evaluate the effect of \( \theta \)-defensins on sTNF mobilization, we analyzed the inhibitory activities of RTDs 1-5 on TACE and the related ADAM10 in COS7 cells (46,47) co-expressing their respective AP-TGFα and AP-BTC reporter substrates (48,49). PMA-stimulation increased TACE shedding of TGFα 2-4-fold compared to unstimulated cells and RTD 1-5 inhibited PMA-stimulated TACE activity in a dose-dependent manner (Fig. 7 A, B). Of note, as observed with THP-1 cells (Fig. 4 A, B), acyclic S7 and \( \theta \)-defensin HNP-4 were ineffective in inhibiting TACE activity (Fig. 7B), providing further evidence that macrocyclic RTDs uniquely interact with TACE to inhibit enzymatic activity.

We assessed the effect of RTDs on ADAM10, a TACE-related metalloprotease, by monitoring processing of AP-BTC. Ionomycin stimulation of COS7 cells increased ADAM10 cleavage of AP-BTC by 10-20-fold more than unstimulated cells (Fig. 7C). RTD-1 dose-dependently reduced ionomycin-stimulated ADAM10 activity, but acyclic S7 had little inhibitory activity. HNP-4 enhanced ADAM10 activity by an unknown mechanism that was not investigated further. The effect of \( \theta \)-defensins on ADAM10 was analyzed further by testing for inhibition rADAM10 by RTDs 1-5. While each RTD inhibited ADAM10 as a function of peptide concentration, (Fig. 7D), the inhibitory potency was lower, and the isoform-specific hierarchy differed, compared to inhibition of RTDs of rTACE and cellular TACE expressed by THP-1 cells (Figs. 4C and 5).

\[ \text{RTD-1 is a fast-binding non-competitive inhibitor of TACE.} \]

As noted above, the hypothesis that RTDs inhibit TACE was based in part on the exceedingly rapid blockade of sTNF release by E. coli-stimulated leukocytes exposed to RTD-1 (14). To further study the kinetics of inhibition, we analyzed the temporal effect of RTD-1 addition to a steady state reaction of fluorogenic substrate cleavage by rTACE. As shown in Fig. 8A, addition of RTD-1 very rapidly inhibited enzyme activity and the degree of blockade was dose-dependent. These findings are consistent with rapid binding of RTD-1 to TACE. Additionally, a large molar ratio of RTD-1 (0.05-25 µM) to TACE (2 nM) is required for proteolytic inhibition, indicating that RTD-1 is not a tight-binding inhibitor.

Michaelis-Menten kinetics of RTD-1 inhibition of rhTACE was determined by measuring substrate conversion in reactions with varied substrate and peptide concentrations (Fig. 8B). RTD-1 inhibition of TACE could not be overcome by increasing concentrations of substrate, thus \( V_{\text{max}} \) decreased with increasing RTD-1 concentrations while \( K_m \) remained unaffected, kinetics consistent with classical non-competitive inhibition. Best fit modeling of enzyme inhibition kinetics was performed (see Methods) disclosing that RTD-1 is indeed a non-competitive TACE inhibitor which likely binds to an exosite that modifies interaction of the enzyme with proTNF and related soluble substrates. As the Ki value for a non-competitive inhibitor is the same as its \( IC_{50} \) value, the values reported in Table 1 reflect both the measured \( IC_{50} \) and apparent Ki values for each peptide.

\[ \text{DISCUSSION} \]

ADAMs are key regulators of growth factor signaling, development, tumor progression and inflammation (22,50,51), and among known ADAMs TACE plays a central role in both EGFR signaling and proinflammatory pathways (52). TACE-mediated shedding of EGFR ligands is required for normal organogenesis (53-57). TACE is also critical in generating sTNF (19-21), the ectodomain of proTNF, as well as the production of other proinflammatory cytokines and their receptors (58). Regulation of TACE activation is rapid and reversible and recent studies have demonstrated the important role of two membrane proteins, inactive Rhomboid 1 and 2, which selectively regulate activation of TACE in different mouse tissues (29,59-61). The current study discloses a previously unknown mechanism
of sTNF regulation wherein macrocyclic θ-defensins allosterically inhibit TACE. To our knowledge, θ-defensins are the only known endogenous inhibitors of TACE sheddase activity other than tissue inhibitor of matrix metalloproteinases 3 (TIMP3), which has been shown to play a critical role in regulating proTNF shedding (62-65).

Enzymology experiments demonstrate that θ-defensin inhibition of TACE is rapid, non-tight binding, reversible, and non-competitive. Inhibition of TACE by RTDs explains how RTD-1 is able to suppress sTNF release by blood leukocytes stimulated by an array of TLR agonists (Fig. 2). Moreover, the correlation between potency of θ-defensin isoforms in blocking TACE enzymatic activity and inhibition of sTNF release supports the conclusion that θ-defensins regulate sTNF production by TACE inhibition (Fig. 5). This was further validated using cell-based assays that showed that RTDs inhibit TACE and ADAM10 shedding of the ectodomains of their respective membrane bound substrates.

θ-defensins are expressed at high levels in neutrophils and monocytes of rhesus macaques (8), olive baboons (9), and African green monkeys (unpublished data). Despite the high degree of sequence conservation among RTDs 1-5 (identity in 11 of 18 residue positions), the TACE inhibitory potency of RTDs varied by more than 5-fold. Of note, the hierarchy of TACE inhibition of RTDs 1-5 strongly correlated with the suppression of TNF release by each isoform from stimulated THP-1 monocytes and whole blood leukocytes.

θ-defensins are readily measurable in plasma of baboons with experimental E. coli bacteremia, and are released by granulocytes challenged with E. coli in vitro (66) Moreover, RTDs are present in saliva of healthy macaques (unpublished data). In clinical sepsis, high levels of human neutrophil α-defensins are released into the circulation (67). In baboons, θ-defensins are secreted by blood granulocytes stimulated with E. coli in vitro, and θ-defensins are rapidly released into baboon plasma following experimental E. coli bacteremic challenge (66). We hypothesize that θ-defensins are endogenous regulators of TACE, ADAM10, and potentially other matrix metalloproteases in Old World monkeys. Since α-defensins stimulate proinflammatory pathways (38-43) and lack inhibitory activities against TACE or ADAM10, TACE inhibition by θ-defensins may underlie the differential susceptibility of humans and other great apes to endotoxin, compared to endotoxin resistance of Old World monkeys that express θ-defensins (68,69).

RTD-1 markedly suppressed mRNA expression and release of TNF, IL-1β, and IL-8 by LPS-stimulated THP-1 macrophages (70). Suppression of transcriptional activation was associated with suppression of NF-κB and MAPK signaling with a concomitant increase in pAkt which negatively regulates these pathways (70). Thus θ-defensins are pleiotropic effectors of host defense and inflammation. Like α- and β-defensins, they are potent, broad spectrum microbicides. However, unlike their acyclic counterparts, θ-defensins also function as anti-inflammatory molecules that moderate proinflammatory stimuli at both transcriptional and post-translational levels.

Given the importance of TACE as a regulator of sTNF, numerous laboratories have sought to identify selective TACE inhibitors for pharmacologic regulation of the enzyme’s sheddase activity to antagonize TNF expression in diseases such as rheumatoid arthritis, inflammatory bowel diseases, diabetes, and sepsis. Small molecules evaluated for this purpose have included succinates, hydroxamates, sulfonamides, γ-lactams, β-benzamido compounds, benzothiadiazepine inhibitors, and zinc chelators (31,71-74), each designed to competitively target the zinc-containing catalytic site (31). To date none of these programs has produced an approved TACE-targeted drug due to lack of efficacy and/or off target toxicities (27,31,75,76). Perhaps molecules with structural and functional features embodied in θ-defensins may provide a new avenue for development of TACE inhibitors for treatment of TNF-driven diseases.

EXPERIMENTAL PROCEDURES

Human subjects. All studies involving human subjects were approved by an Institutional Review Board at the Keck School of Medicine, University of Southern California, (IRB #HS-09-00280) and were fully compliant with the Declaration of Helsinki principles.

Peptides. The hydrochloride salts of RTD 1-5 were produced by solid-phase synthesis and
purified (≥98%) (4), and human neutrophil α-
defensins I-4 (HNPs; ≥ 98%) were purified from human buffy coat leukocytes as described previously (77). Peptides were dissolved in 0.01% (v/v) acetic acid or HPLC-grade water, and peptide concentrations were confirmed by coupled liquid chromatography and mass spectrometry (LC-MS) using previously quantified standards.

**Bacteria and TLR agonists.** E. coli #UCI 9021, a human blood isolate, was obtained from the clinical laboratory at the University of California Irvine Medical Center. Bacteria were cultured from single colonies, harvested by centrifugation, washed, and suspended in phosphate buffered saline (PBS) (14). Bacterial density was determined by absorbance at 620 nm and correlated with colony forming units (CFU) grown on tryptic soy agar (TSA). The following toll-like receptor (TLR) agonists were from Invivogen (San Diego, CA): Pam3CSK4 synthetic triacylated lipopeptide (TLR 1/2), heat-killed L. monocytogenes (TLR 2), E. coli K12 LPS (TLR 4), S. typhimurium flagellin (TLR 5), FSL1 synthetic (TLR2/6), ssRNA40 (TLR 8), and ODN2006 unmethylated CpG oligonucleotide (TLR 9).

**Cell culture.** THP-1 human monocytes (ATCC TIB-202) cells were cultured in RPMI-1640 containing 10% FBS and 100 U/ml Pen/Strep in 5% CO₂. Cells (5x10⁵ /well in 24-well plates) were treated with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma) for 48 h, washed with warm PBS, suspended in fresh medium and incubated for 24 h prior to use in assays described below. Human monic epithelial HT-29 cells (ATCC HTB-38) were grown to confluence in 24-well tissue culture dishes in DMEM supplemented with 10% FBS and 100 U/ml Pen/Strep, washed and cultured in RPMI-1640, 100 U/ml Pen/Strep plus 5% human EDTA plasma. HT-29 cells were incubated with 0 - 5 µM RTD-1 plus 500 pg/ml recombinant human TNF (Invivogen, San Diego, CA). After 4 h incubation at 37 °C in 5% CO₂ culture supernatants were analyzed for soluble IL-8 by ELISA (Invivogen).

**TNF release assays.** Kinetics of TNF release by stimulated leukocytes was determined as described previously (14). Briefly, THP-1 cells (5x10⁵ cells/ml) were stimulated for 4 h with indicated concentrations of TLR agonist in the presence of 0 – 5 µM of RTDs 1-5 at 37 °C in 5% CO₂. TNF in cell-free supernatants was determined by ELISA (Life Technologies). For studies using peripheral blood leukocytes, EDTA-anticoagulated blood was obtained from healthy adult volunteers, and buffy coat leukocytes were harvested by centrifugation at 200 × g. Cells were washed twice with 3-5 ml of RPMI, counted with a hemocytometer, and suspended at 5x10⁵ cells/ml in RPMI + 5% human EDTA plasma. Leukocytes were incubated for 4 h with the indicated concentrations of TLR ligands or 100 CFU/ml E. coli #UCI 9021, and TNF in clarified supernatants was quantified by ELISA.

**TACE and ADAM10 inhibition assays.** Proteolytic activities of recombinant human TACE (R&D Systems, 930-ADB, 52 kDa, full ectodomain) and ADAM10 (R&D Systems, 936-AD, 52 kDa, full ectodomain) were measured using fluorogenic substrates Mca-PLAQAV-Dpa-RSSSR-NH₂ (R&D Systems #ES003) and Mca-KPLGL-Dpa-AR-NH₂ (R&D Systems #ES010), respectively. Assays were performed in F16 black Maxisorp 96-well plates in 25 mM TRIS, 2.5 µM ZnCl₂, 0.005% Brij35 vol/vol (pH 9.0). Individual RTDs were added to TACE (0.1 µg/ml, 2 nM) or ADAM10 (0.05 µg/ml, 1 nM) at final concentrations of 0 – 1.5 µM, followed by addition of 10 µM fluorogenic substrates. Marimastat (Sigma-Aldrich #154036-60-8) was used as the control metalloproteinase inhibitor. Substrate conversion was measured every 30 sec for 30 min at 22 °C (TACE) or 37 °C (ADAM10) in a SpectraMax M5e fluorometer (Molecular Devices; 320em/405sm). Vₘₐₓ was calculated for each sample and transformed into percent change in product formation rate (mean +/- SEM, n = 6). Inhibition curves were fitted with a non-linear variable slope curve using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Half-maximal inhibition concentrations (IC₅₀) were obtained from fitted curves.

**Cellular sheddase assays.** PMA-differentiated THP-1 cells and HT-29 cells were grown in Black μClear CELLSSTAR 96-well plates (Greiner Bio-one, Monroe, NC), washed and suspended in assay buffer (20 mM Tris, 154 mM NaCl, 1% human serum, pH 7.4). At t=0, 0 – 15 µM of individual θ-defensins (RTDs 1-5) and
θ-Defensin suppression of TACE mediated TNF shedding

10 μM fluorogenic TNF substrate (Mca-PLAQAV-Dpa-RSSSR-NH₂) were added to cells and incubated at 37 °C for 60 min. Fluorescence (320/ex/405/em) was measured at 30 sec intervals. V_max was calculated for each sample and transformed into percent sheddase activity relative to solvent controls.

Effects of RTDs on cellular TACE and ADAM10 activities were also analyzed using transfected COS7 (ATCC) cells expressing alkaline phosphatase (AP)-tagged substrates of the respective enzymes (48,49). Briefly, COS7 cells transiently transfected with AP-tagged transforming growth factor-alpha (AP-TGFα; TACE substrate) or AP-betacellulin (AP-BTC; ADAM10 substrate) were cultured overnight, washed, starved 2 - 4 h in Opti-mem media (Gibco), and incubated for 10 min with 0 - 5 μM of RTDs 1-5 in fresh media. TACE activity was induced by addition of 25 ng/ml PMA for 30 - 45 min, after which culture aliquots (supernatants) were collected and cells were lysed. Samples of cell supernatants and lysates were added to alkaline phosphatase (AP) assay buffer (100 mM Tris, 100 mM NaCl, 20 mM MgCl₂, pH 9.5). After addition of 2% (vol/vol) of 50 mg/ml aqueous p-nitrophenyl phosphate (PNPP) substrate (Thermo Scientific 34045), TACE activity was quantified as AP conversion of PNPP. Cellular ADAM10 activity was determined similarly using COS7 cells transfected with AP-tagged betacellulin. ADAM10 activity was induced with 2.5 μM ionomycin. All assays were performed in triplicate.

Enzyme kinetics. RTD-1 (0 - 960 nM) was incubated with 2.5, 5, 10, and 20 μM Mca-PLAQAV-Dpa-RSSSR-NH₂ (320/ex/405/em) in the presence of 25 ng/ml rTACE and enzyme activity was measured every 30 sec for 90 min. Initial velocity (V₀) was plotted as a function of substrate concentration (Mean +/- SD, n = 3), subjected to Michaelis-Menten kinetics analyses which were compared with best-fit models for competitive, non-competitive, uncompetitive, and mixed modeling inhibition using GraphPad Prism. Inhibition of rTACE by RTD-1 was also determined by addition of 0 – 25 μM RTD-1 to steady-state (Vₚ) TACE-Mca-PLAQAV-Dpa-RSSSR-NH₂ reactions in which substrate conversion was monitored for 10 min prior to addition of peptide, and then for 25 min after the addition of the indicated concentration of RTD-1.

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Conflict of interest: The following authors declare competing interests in relation to their affiliations with Oryn Therapeutics: Dat Q. Tran is Scientific Director, Michael E. Selsted is Chief Scientific Officer, Andre. J. Ouellette is a minority investor. Oryn Therapeutics has licensed technologies disclosed in US Patents 6,335,318; 6,514,727; 6,890,537; 7,119,070; 7,399,823 B1, 7,462,598, and 9,346,866 B2. The affiliation of the authors with Oryn Therapeutics does not alter author adherence to JBC policies on sharing data and materials.

Author Contribution: JBS, DQT, and MES developed the hypothesis. JBS and TM designed and conducted experiments, and analyzed results. TM, DQT, PAT, PT, CPB, AJO, MES analyzed results JBS, AJO, CPB, and MES authored the paper with editorial input from all co-authors.
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Table 1. Relative TACE Inhibitory Activities of RTDs (nM ± SD)

| Peptide | IC₅₀  |
|---------|------|
| RTD-1   | 141 ± 14 |
| RTD-2   | 52 ± 3   |
| RTD-3   | 285 ± 52 |
| RTD-4   | 132 ± 22 |
| RTD-5   | 55 ± 7   |
| S7      | >1000   |
| Marimastat | 7.6 ± 0.2 |
The θ-defensin structural motif is defined by an 18-amino acid backbone stabilized by three conserved disulfide bonds. Other invariant residues are shaded and net charges of RTDs 1-5 are listed. θ-defensin isoforms are produced by homo- or heterodimeric head-to-tail ligation of nonapeptides excised from one or two of three known propeptides. The ribbon structure of RTD-1 (top) shows the substituent nonapeptides (blue and gold) spliced to form the mature macrocycle. S7 is an acyclic version of RTD-1 with an opening between Cys3 and Arg4 (red arrow), one of the two peptide bonds formed during post-translational formation of the macrocyclic backbone.
FIGURE 2. RTD-1 suppresses TNF release from blood leukocytes. Human buffy coat leukocytes cells were stimulated with a panel of TLR agonists and treated with vehicle or 5 or 15 µM RTD-1. sTNF release is shown as a percent of TNF released compared to peptide-free controls for each agonist [pg/ml sTNF]. Bars represent means ± SD of two independent experiments performed in duplicate.
FIGURE 3. RTD-1 inhibition of TNF release is reversible and does not block signaling by sTNF.  

A. THP-1 macrophages were pre-treated with vehicle (open circles) or 5 μM RTD-1 (closed circles) for 60 min after which medium removed and the cells washed. Cells were then treated with vehicle or RTD-1 (5 μM) with or without LPS, incubated for 4 h and sTNF levels determined.

B. HT-29 cells were treated with 0 - 5 μM RTD-1, stimulated with 500 pg/ml rTNF for 4 h and supernatant IL-8 was quantified. Control incubations (Ctrl) lacked TNF and produced no IL-8 response, as did samples treated with RTD-1 alone (data not shown). Scatter plots represent means ± SD of two independent experiments performed in duplicate (3A) or triplicate (3B).
FIGURE 4. θ-defensins inhibit TACE and suppress TNF release by LPS stimulated THP-1 monocytes. A, C, Inhibition of recombinant TACE proteolytic activity is shown as percent change in rate of product formation relative to peptide-free control. Enzyme reactions were performed for 30 min at 22 °C with 2 nM TACE and 10 µM substrate (R&D Systems ES003). Data represent means ± SEM of 3 independent experiments containing 2 - 3 technical repeats each. B, D, Suppression of TNF release from THP-1 monocytes stimulated with LPS and peptides or MRM. Results are expressed as means ± SD of 2 – 6 individual experiments containing 2 technical repeats each.
FIGURE 5. TACE inhibition correlates with suppression of soluble TNF release from stimulated THP-1 monocytes and whole blood. IC$_{50}$s of RTDs 1-5 for sTNF release by LPS stimulated THP-1 monocytes (●) and *E. coli* stimulated whole blood (○) were plotted against IC$_{50}$s of each peptide for rTACE inhibition. Symbol numbers refer to RTDs 1-5 with correlation coefficients shown (THP-1 $P = 0.0082$, Blood $P = 0.0107$).
FIGURE 6. Real time measurement of RTD inhibition of cellular TACE. RTDs 1-5 were incubated with THP-1 macrophages or HT-29 cells in the presence of 10 µM fluorogenic substrate (R&D Systems ES003). Substrate conversion was monitored continuously for 60 min at 37 °C and plotted as a function of percent product formation rate relative to peptide-free controls. Results are shown as means ± SD of two independent experiments containing 2-3 technical repeats each.
FIGURE 7. RTDs inhibit cellular TACE and ADAM10 sheddase activities. A-B, RTDs, but not acyclic S7 or α-defensin HNP-4, suppress TACE-mediated ectodomain shedding of TGFα in COS7 cells. Data are expressed as fold TGFα shedding relative to constitutive TGFα release. C, RTD-1, but not S7 or HNP-4, inhibits ADAM10 dependent cleavage of BTC in COS7 cells. Data are expressed as fold BTC shedding relative to constitutive BTC release. Data in panels A-C represent means ± SD of a representative experiment containing 3 technical repeats. D, RTDs inhibit rADAM10 proteolysis of its fluorogenic substrate. Enzyme reactions were performed for 30 min at 37 °C with 1 nM ADAM10 and 10 µM substrate (R&D Systems ES010). Enzyme inhibition is expressed as percent change in substrate conversion rate relative to peptide-free controls. Data represent means ± SD of 2 independent experiments containing 2 technical repeats each.
FIGURE 8. RTD-1 is a fast binding, non-competitive inhibitor of TACE. A, Kinetics of RTD-1 inhibition of TACE (2 nM) was evaluated in a steady-state TACE/FRET-substrate (10 μM substrate R&D Systems ES003) cleavage reaction at 22 °C. RTD-1, at the concentrations indicated was added to steady state reaction mixtures at 10 min (dotted line) after reaction initiation. Results shown are from a single representative experiment performed twice containing 2 technical repeats each. B, Michaelis-Menten kinetics for RTD-1 inhibition of rTACE (2 nM) with multiple concentrations of substrate (ES003) at 22 °C. Data represent means ± SD of a single representative experiment performed three times, each performed in triplicate.
Macrocyclic θ-defensins suppress tumor necrosis factor-α (TNF-α) shedding by inhibition of TNF-α converting enzyme
Justin B. Schaal, Thorsten Maretzky, Dat Q Tran, Patti A. Tran, Prasad Tongaonkar, Carl P. Blobel, André J. Ouellette and Michael E. Selsted

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