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A pathogenic trace of *Tannerella forsythia* – shedding of soluble fully active tumor necrosis factor alpha from the macrophages surface by karilysin

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Summary

Tannerella forsythia is implicated as a pathogen causing chronic and aggressive periodontitis. However, its virulence factors, including numerous putative proteases, are mostly uncharacterized. Karilysin is a newly described matrix metalloprotease-like enzyme of *T. forsythia*. Since pathogen-derived proteases may affect the host defense system via modulation of the cytokine network, the aim of this study was to determine the influence of karilysin on tumor necrosis factor-alpha (TNF- α). The results showed that karilysin cleaved the membrane form of TNF- α on the surface of macrophages, and that this led to an increased concentration of soluble TNF- α in the conditioned medium. Importantly, despite partial degradation of soluble TNF- α by karilysin, the released cytokine retained its biological activity, inducing apoptosis and stimulating autocrine pathway pro-inflammatory gene expression. Notably, the observed effect required proteolytic activity by karilysin, since a catalytically inactive mutant of the enzyme did not affect TNF- α secretion. The shedding was independent of the activity of ADAM17, a major endogenous TNF- α converting enzyme (TACE). Karilysin-dependent TNF- α release from the cell surface is likely to occur *in vivo* since human plasma, the main constituent of gingival crevicular fluid, only slightly affected the sheddase activity of karilysin. Taken together, these results indicate that karilysin modulates the host immune response through regulation of TNF- α secretion, and should therefore be considered as a new virulence factor of *T. forsythia*.

Keywords

karilysin; TNF- α ; macrophages; periodontitis; *T. forsythia*

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Introduction

Periodontitis is a bacterial infection-driven chronic inflammatory disease of tooth-supporting tissues, including the periodontal ligament and alveolar bone, which may eventually lead to tooth loss (Flemming, 1999; Hajishengallis and Lamont, 2012; Wright et al., 2013). A so-called red complex bacteria, composed of *Tannerella forsythia* (previously *Bacteroides forsythus*), *Porphyromonas gingivalis*, and *Treponema denticola*, are strongly associated with human periodontal disease (Socransky et al., 1998). In contrast to the two other periodontal pathogens, the virulence factors of *T. forsythia* are still poorly characterized (Stafford et al., 2012; Tanner and Izard, 2006). Pathogenicity of this bacterium is predominantly attributed to BspA, a protein expressed on the bacterial surface that binds extracellular matrix components such as fibrinogen and fibronectin (Inagaki et al., 2005; Sharma et al., 1998). Apart from BspA, lipoprotein (Hasebe et al., 2004), protease with trypsin-like activity (Grenier, 1995), and a PrtH cysteine protease with hemolytic activity (Saito et al., 1997) have been implicated in *T. forsythia* virulence. The released sequence of the whole *T. forsythia* genome (Human Oral Microbiome Database, <http://www.homd.org>) revealed several other potential virulence factors. Among these are putative proteases encoded by numerous open reading frames. As in the case of gingipains of *P. gingivalis* (Wilensky et al., 2013), these enzymes may play an important role in the development of periodontitis. Recently, we characterized a matrix metalloprotease-like enzyme encoded by a TF0367 locus (<http://www.oralgen.org>), which we refer to as karilysin (Karim et al., 2010).

Inactivation of complement (Potempa and Potempa, 2012) and modulation of the cytokine network by proteases derived from microbial pathogens affect host innate immune mechanisms and contribute to uncontrolled development of inflammation (Potempa and Pike, 2009). Induction of *de novo* synthesis, posttranslational modification, or degradation of major pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), IL-1 β , IL-6, and IL-8 by bacterial proteases has recently been shown. On the host side, among the enzymes involved in cytokine processing, the most crucial roles are played by host matrix metalloproteases, which modify several cytokines and their receptors (including TNF- α , IL-1 β , TGF- β , and sIL-1RII) as well as some chemokines (CXCL8, CXCL12, and CX3CL1) (Van Lint and Libert, 2007). Phylogenetic and sequence similarity (N Cerda-Costa and Ruth, 2011) studies have revealed that 18 kDa molecular form of karilysin (Kly18) is evolutionarily closely related to mammalian matrix metalloproteinases (MMPs), and these findings have been confirmed by structural similarity. This suggests that the virulence-associated function of the *T. forsythia* enzyme may be related to interference with networks of cytokines, including TNF- α .

TNF- α is a pleiotropic, pro-inflammatory cytokine expressed mainly by macrophages as a 26 kDa transmembrane protein that is converted to a 17 kDa soluble protein by a membrane bound secretase, ADAM17 (Black et al., 1997). Among the cytokines, TNF- α plays a fundamental role in periodontitis, causing bone resorption by osteoclast activation and degradation of extracellular matrix protein via the induction of MMP1, MMP2, and MMP9 expression in macrophages, fibroblasts, and osteoclasts (Azuma et al., 2000). In addition, TNF- α increases vascular permeability and influences the chemotaxis of neutrophils to the

site of infection (Graves et al., 2001). Together, this destructive potential of TNF- α may lead to the development of pathological conditions and, consequently, chronic inflammatory diseases (Masi et al., 2013). On the other hand, however, excessive reduction of TNF- α expression tilts the host's immunological balance in favor of the pathogen and is considered a poor prognostic marker in infectious diseases as in case of *Yersinia enterocolitica* or *Brucella suis* infection (Wilson et al., 1998). In the case of periodontitis, there is little doubt that the biological activity of TNF- α is significantly associated with pathological changes associated with development of this disease. Therefore, the influence of periodontopathogens and their virulence factors on TNF- α turnover is of significant importance. To analyze this interaction, we investigated the role of the *T. forsythia* metalloprotease karilysin in TNF- α shedding, and how this virulence factor influences inflammatory responses.

Materials and Methods

Reagents

Recombinant human tumor necrosis factor alpha (TNF- α), recombinant human interferon γ (IFN- γ), gentamycin, *o*-phenanthroline (OF), Griess reagents (1% sulfanilic acid and 0.1% *N*-(1-naphtyl) ethylenediamine dihydrochloride), Thiazolyl Blue Tetrazolium Bromide (MTT), dimethylformamide (DMF) were from Sigma. Alexa Fluor 488 F(ab)₂ fragment of goat anti mouse IgG (H+L) antibody were obtained from Invitrogen. Murine anti-human TNF α monoclonal antibody was purchased from Hycult biotech. DMEM, RPMI-1640, fetal calf serum (FBS) and lymphocyte separation medium (LSM) were obtained from PAA.

Recombinant karilysin

Wild type and catalytically inactive Glu136Ala mutant forms of *T. forsythia*-derived karilysin were expressed in *Escherichia coli*. Purification was performed as described previously (Karim et al., 2010). To confirm the absence of LPS in recombinant proteins the LAL (Lymulus Amebocyte Lysate) test purchase from Lonza was performed. Where indicated an inhibitor of karilysin, 0.3 mM *o*-phenanthroline (OF) was added. Biochemical studies carried out on the purified enzyme have shown that karilysin is synthesized as an inactive precursor, which undergoes autoactivation by the sequential proteolytic events. To obtain individual discrete forms of karilysin, the fully active enzyme Kly48 (47.9 kDa) was allowed to undergo autoproteolysis. First it was incubated at 37°C for 72h to Kly38 (38 kDa). A mature form of karilysin (Kly18) (18 kD) was obtained by Kly38 incubation at 50°C for 48h (Karim et al., 2010). Since enzymatic activity of karilysin increases in the presence of Ca²⁺ the enzymes was preincubated in 100 mM Tris-HCl, 5 mM CaCl₂ (pH 8.0) at room temperature for 15 min before being used.

Gingipain RgpB

RgpB was purified from *P. gingivalis* as previously described (Pike et al., 1994; Potempa et al., 1998). The purified enzymes were activated by incubation at 37°C for 15 min in 100 mM Tris, 150 mM NaCl, 5 mM CaCl₂ and 20 mM cysteine (pH 7.5). Activated gingipain was diluted to the desired concentrations in culture medium supplemented with 10 mM cysteine.

Cell culture and treatment

Blood was obtained from the Red Cross, Krakow, Poland. The Red Cross de-identified blood materials as appropriate for human subjects confidentiality assurance. Thus, the current paper adheres to appropriate exclusions from human subjects approval. The fraction of peripheral blood mononuclear cells (PBMCs) highly enriched in monocytes (90% CD14-positive) was obtained from human blood using a Lymphocyte Separation Medium density gradient as described previously (Koziel et al., 2013). The PBMCs were plated at 3×10^6 and 2×10^7 cells/well in 24- or 6-well plates, respectively. PBMCs were cultured in RPMI-1640, supplemented with 10% autologous heat-inactivated human serum, 2 mM L-glutamine, and 50 mg/mL gentamicin at 37° C in a humidified atmosphere of 5% CO₂. The following day, nonadherent cells were removed by washing with PBS, and the adherent monocytes were differentiated to human monocyte-derived macrophages (hMDMs) over 7–10 days, with fresh medium changes every second day.

ADAM17^{-/-}TNF⁺ cell line established from ADAM17^{-/-} Ras-Myc-immortalized murine fibroblasts stably transfected with human pro-TNF- α cDNA (Mezyk-Kopeck et al., 2005), murine brain microvascular endothelial cells (MBE-SV) and murine fibrosarcoma L929 cell line were obtained from Prof. Joanna Bereta from the Department of Cellular Biochemistry, Jagiellonian University, Cracow, Poland. All cell lines were maintained in DMEM supplemented with 10% FBS, at 37°C, in a humidified 5% CO₂ atmosphere. For all experiments cells were cultured without supplements if not stated otherwise.

For cytokine measurement hMDMs (3×10^5 cells/well of 24-well plate) and ADAM17^{-/-}TNF⁺ cells (0.2×10^6 cells/well of 24-well plate) were incubated for indicated time points with different doses of karilysin (1- 100 nM) then the medium was harvested and analyzed by ELISA.

Neutralization of biological activity of TNF- α was exerted by 15 min preincubation of hMDMs cells with anti-TNF- α antibodies (100 ng/ml) with subsequent karilysin treatment for 1h or 6h. After indicated time cultured media were collected and total RNA was isolated.

ELISA

The concentration of TNF- α , IL-6, IL-8 in the culture media of hMDMs or ADAM17^{-/-}TNF⁺ cell line was measured by ELISA carried out according to the manufacturer's instructions (BD Biosciences).

FACS

For analysis of plasma membrane bound TNF- α ADAM17^{-/-}TNF⁺ cells (10^6 cells/ml) were incubated for 1h with 100 nM karilysin or 100 nM gingipain RgpB as a positive control. After desired time, cells were washed twice with PBS and incubated for 1h with fluorescence labeled antibodies recognizing the membrane form of cytokine. Then, cells were collected, resuspended in complete medium containing Alexa Fluor 488 F(ab)₂ fragment of goat anti- mouse IgG and analyzed by a FACScan flow cytometer (Becton Dickinson). The analysis was performed using the CellQuest software to determine the percentage and mean fluorescence intensity of positive, labeled cells.

SDS-PAGE electrophoresis and immunoblotting

Recombinant human TNF- α (1 μ g) was incubated at 37°C with karilysin (10 ng - 10 μ g) in 100 mM Tris- HCl and 5 mM CaCl₂, pH 8.0, at different E:S molar ratios ranging from 1:0.1 to 1:100. After 12h of incubation samples were separated on 12% polyacrylamide gel and stained with silver or electrotransferred onto nitrocellulose membranes (nitrocellulose BioRad) in buffer composed of 25 mM Tris, 0.2 M glycine, 20% methanol (30 V, overnight). Membranes were stained with Ponceau S to visualize the efficiency of transfer and confirm the loading precision (same amount of protein loaded per well). Non-specific binding sites were blocked with 3% BSA in TTBS buffer (20 mM Tris, 0.5 M NaCl, pH 7.5 with 0.05% Tween 20) for 1h, followed by 1-2h incubation with the relevant primary antibody: 100-fold diluted murine anti-human TNF- α or 10,000-fold diluted anti- β -actin (BD Transduction Lab.). Membranes were washed extensively in TTBS buffer and incubated with secondary horseradish peroxidase (HRP)-conjugated antibodies, 10,000-fold diluted rabbit anti-mouse IgG (Sigma), for 1h in TTBS buffer containing 1.5% BSA. Membranes were washed (4 $\times$ 15 min) in TTBS buffer and blots were developed using ECL detection (Western Blotting Detection Reagents; Amersham Biosciences).

Cytotoxicity assay

Bioassay for the activity of TNF- α were performed by the method of Mathews and Neale (Matthews N, 1987), using L929 cells, which were placed in 96-well plates at the concentration 4×10^5 cells/well. Cells were stimulated with conditioned media collected from hMDMs after stimulation with karilysin (100 nM) for 6h. The concentration of TNF- α in conditioned medium determined by ELISA was estimated in a range from 10 ng/ml to 0.625 ng/ml. L929 were incubated for 24h in the presence of 125 ng/ml actinomycin D. As a positive control rhTNF- α at the concentration 10 ng/ml was used. Viability was examined by MTT reduction assay according to vendor's instructions.

Nitrite assay

MBE-SV cells were seeded in 96-well plate at a concentration 4×10^4 cells/well. The next day cells were stimulated with conditioned medium containing TNF- α (1 - 10 ng/well) gathered from hMDMs stimulated with karilysin. In some experiments conditioned medium was supplemented by IFN- γ (10 ng/ml). Following 20h incubation, the concentration of nitrite in the media was measured by Griess assay as described previously (Koziel et al., 2014). Sodium nitrite was used as a standard.

Quantitative Real-Time PCR (qRT-PCR)

Total cellular RNA was extracted from 2×10^6 cultured hMDMs using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA samples were DNase treated, and cDNA was prepared by reverse transcription using Revert AidTM First Strand cDNA Synthesis Kit (Fermentas). Five hundred nanograms of RNA from each sample were used for cDNA synthesis reaction with oligo(dT) primers according to the manufacturer's instructions. Quantitative PCR reaction was performed with an SYBR Green method in a reaction volume of 20 μ L, containing 1 μ L of cDNA sample, 0.5 μ M of each primer (as shown in Table 1), and 1 $\times$ SYBR Green JumpStart Taq Ready Mix (Sigma). qRT-PCR

forward and reverse primers for *TNF- α* , *IL-6*, and *IL-8* genes and for the housekeeping *EF-2* gene (used for normalization) are listed in Table 1. After 5 min of initial denaturation at 95°C, reactions were carried out for 40 cycles at the given conditions: denaturation, 95°C, 20 sec; annealing, 56°C, 60 sec; extension, 72°C, 60 sec; followed by a final elongation step at 72°C for 10 min. All the reactions were performed in duplicates. Means for threshold cycle (Ct) values were calculated and analyzed using the “delta-delta Ct” quantification method (Livak and Schmittgen, 2001). Routinely, for the evaluation of quality of qRT-PCR reactions, samples were resolved on nondenaturing 1.5% agarose gels and visualized by staining with ethidium bromide.

Statistical Analysis

Results were analyzed for statistical significance using the nonparametric Student's *t*-test. Differences were considered significant when $P < 0.05$.

Results

Catalytic activity of karilysin regulates secretion of soluble TNF- α from human macrophages

TNF- α is one of the major pro-inflammatory cytokines expressed by macrophages in response to pathogens. As such, it is a sensitive marker of infection. Since karilysin is a newly described protease with unknown function, we first tested whether it is capable of affecting the secretion of TNF- α . Human monocyte-derived macrophages were stimulated with recombinant karilysin (Kly48) for 6 h, and the level of the soluble cytokine in conditioned medium was then determined by ELISA. The obtained data revealed a dose-dependent effect of Kly48 on TNF- α secretion, which was observed even at concentrations of the enzyme as low as 1 nM (Fig. 1A). To confirm that the process of TNF- α release from macrophages depends on the catalytic activity of Kly48, the metal chelator *o*-phenanthroline (OF) and the proteolytically inactive form of the enzyme with a point mutation in the catalytic Glu residue (Kly_{Glu156Ala}) were used. Both approaches revealed that the release of TNF- α depends on the catalytic activity of *T. forsythia* karilysin; *o*-phenanthroline completely abolished the level of secreted cytokine, and no release of the cytokine was observed when the mutant Kly_{Glu156Ala} was used (Fig. 1B). Importantly, the generation of soluble TNF- α by karilysin was also noted in human plasma, which mimics *in vivo* conditions in the gingival crevicular fluid (GCF) (Fig. 1C).

Karilysin induces rapid TNF- α release from the cell surface independently of TNF- α converting enzyme (TACE)

TACE/ADAM-17 is the most potent host-derived TNF- α convertase. It truncates an extracellular domain of a transmembrane pro-form of TNF- α to generate the soluble cytokine (Fig. 2A – blue arrow). To determine whether an increased level of soluble TNF- α is the consequence of direct cleavage by the bacterial enzyme, we investigated the ability of karilysin to induce shedding of the soluble form of TNF- α from cells without functional ADAM-17 (ADAM-17^{-/-} TNF₊). Since *in vivo* karilysin may occur in three forms that differ in molecular mass (Kly48, Kly38, and Kly18) (Fig. 2B), we treated ADAM-17^{-/-} TNF₊ cells with purified Kly48, Kly_{38/18}, and Kly₁₈, and determined the level of TNF- α released into

the cell culture medium. The obtained results showed that all tested forms of Kly release soluble TNF- α from ADAM17 $^{-/-}$ TNF $^{+}$ cells, despite the lack of a functional endogenous TACE (Fig. 2C). Moreover, we observed that different molecular forms of karilysin (Kly₄₈, Kly_{38/18}, and Kly₁₈) have similar TNF- α shedding induction potential, since the levels of accumulated soluble cytokine in conditioned media were comparable (Fig. 2C). The cleaved sites corroborate the established substrate specificity of karilysin (Karim et al., 2010), with one cleavage site shared between karilysin and TACE secretase (Ala 76: Val 77) (Fig. 2A – red arrows). Since all forms of karilysin appeared to be equally potent inducers of TNF- α release, all further experiments were routinely performed with the Kly₄₈ form.

Shedding of soluble TNF- α from the surface of cells, which modulates the development of inflammation, is a common virulence mechanism utilized by other bacterial proteases, e.g., gingipains originating from *P. gingivalis* (Mezyk-Kopec et al., 2005). Thus, we compared the efficiency of induction of TNF- α shedding from the surface of ADAM17 $^{-/-}$ TNF $^{+}$ cells by karilysin and gingipain (RgpB) using immunofluorescent staining of TNF- α and subsequent fluorescent-activated cell sorter (FACS) analysis. Our results clearly indicate that incubation of cells with Kly₄₈ and RgpB resulted in decreases in both the number of TNF- α positive cells and TNF- α levels on the cell surface. However, Kly was significantly less potent than RgpB (Fig. 2D). Of note, the observed decrease in the TNF- α level did not result from any deleterious effect of karilysin on the cells, since cell viability was not affected upon karilysin treatment as estimated by propidium iodide staining (data not shown). Taken together, these data indicate that karilysin mediates the release of soluble TNF- α and that this process is independent of ADAM17.

Proteolytic modification of released soluble TNF- α by karilysin

The inflammatory potential of cytokines depends on their stability and the accessibility of specific, corresponding receptors on responding cells. Since soluble TNF- α is further degraded by karilysin (Fig. 2A), we sought to verify the stability of cytokine in the presence of Kly. To this end, macrophages were stimulated with 100 nM Kly₄₈ and the level of soluble TNF- α at various time points was measured in the cells' supernatants. The increase in the soluble form of TNF- α was observed in the culture medium up to 8 h post-stimulation with Kly₄₈. However, 16 h later, a significant decrease in the concentration of the cytokine was noted (Fig. 3A). This result may indicate proteolytic degradation of soluble TNF- α by karilysin. We therefore examined the stability of the cytokine by incubation of rhTNF- α with Kly₄₈ for 12 h at different molar ratios. Western-blot analysis using the polyclonal antibody anti-rhTNF- α revealed the same intensity band corresponding to TNF- α even at a 1:1 karilysin:cytokine molar ratio, arguing against any degradation of the cytokine. Only when rhTNF- α was incubated with a 10-fold molar excess of karilysin (E:S = 10:1) was there an evident decrease in band intensity, indicating cytokine degradation (Fig. 3B). Degradation of rhTNF- α at high concentration of Kly was confirmed by SDS-PAGE (Fig. 3C). Collectively, this data shows that karilysin has a rather limited ability to degrade soluble TNF- α . This is consistent with the suggested virulence-associated function of Kly as an inflammation-inducing sheddase.

The soluble form of TNF- α retains its biological activity

Operating as a pro-inflammatory sheddase, Kly must release biologically active TNF- α . One of the activities of TNF- α is induction of cell death mediated by TNF receptors. Therefore, we sought to verify the cytotoxic effect of Kly-released TNF- α in the L929 cell line. L929 cells were incubated in conditioned medium of human monocyte-derived macrophages (hMDMs) treated with karilysin as the source of truncated, soluble TNF- α . The concentration of TNF- α in the conditioned medium was determined by ELISA. Our results showed that TNF- α present in conditioned media was as effective in cell death induction as rhTNF- α (Fig. 4A).

TNF- α and INF- γ complement one another in the inflammatory reaction induced in endothelial cells. We therefore analyzed the generation of nitric oxide (NO) in MBE-SV cells stimulated with conditioned medium from Kly-treated hMDMs containing TNF- α in the presence or absence of rhIFN γ . We observed that conditioned medium alone potently induced NO production by MBE-SV cells (Fig. 4B). Moreover, supplementation of conditioned medium with INF- γ led to a further increase in NO generation (Fig. 4B). Collectively, these findings unambiguously indicate that karilysin releases a biologically active, soluble form of TNF- α from the cell surface.

Kly induces pro-inflammatory gene expression in human macrophages

The observed accumulation of TNF- α (up to 8.5 h) in the conditioned media of Kly-treated cells suggests continuous re-supply of the membrane-associated substrate by induction of TNF- α expression in Kly-treated macrophages. To verify this assumption, we measured the expression level of TNF- α mRNA by qRT-PCR and found very strong induction of TNF- α transcription 1 h post karilysin stimulation (Fig. 5A). Since one of the TNF- α expression pathways involves autocrine signaling, we probed this pathway using blocking antibodies against TNF- α . hMDMs were pretreated with anti-TNF- α antibodies for 15 min and then stimulated with Kly for 1 h. Significantly, anti-TNF- α antibodies blocked karilysin-induced mRNA expression in proinflammatory cytokines (TNF- α , IL-6, and IL-8), confirming that Kly activates an auto-/paracrine amplification loop (Fig. 5A). The proposed mechanism was further confirmed on the protein level; antibodies blocking soluble TNF- α efficiently inhibited pro-inflammatory cytokine production (Fig. 5B). To sum up, soluble TNF- α can be increased by two mechanisms: directly, through release of the active molecule from the plasma membrane of the cytokine by karilysin; and indirectly, through stimulation of the autocrine amplification loop of TNF- α production (Fig. 6).

Discussion

The success of periodontopathogens as infectious agents is determined by the potential of bacterial virulence factors to dysregulate the immune system of the host. An apparent paradox is the fact that immune systems compromised in this way inflict damage on periodontal tissues. Since the host response is initiated and regulated by locally synthesized and released inflammatory mediators, including major inflammatory cytokines, targeting of the cytokine network by pathogens may lead to dysfunction of the immune response. Recently, such a role was proposed for *P. gingivalis*-derived gingipains, since they are

engaged in cytokine induction, membrane shedding and degradation, and/or processing of IL-1 β , IL-6, IL-8, and TNF- α (Fletcher et al., 1998; Mezyk-Kopec et al., 2005; Mikolajczyk-Pawlinska et al., 1998). This observation suggests that proteases expressed by other species involved in periodontitis development might utilize a similar strategy. Among these is *T. forsythia*, the pathogen harboring many genes encoding putative secretory proteases, which have not yet been functionally studied as virulence factors.

The present study clearly showed that karilysin, a matrix metalloprotease-like enzyme secreted by *T. forsythia*, plays an important role in the proteolytic processing of TNF. Accumulation of soluble TNF- α depends on the proteolytic activity of karilysin. The observed phenomenon may be explained by karilysin's specificity, which was found to be similar to that of other MMPs, with preference for Leu/Tyr/Met at P1' and Pro/Ala at P3 (Karim et al., 2010). Our data showed Ala76-Val77 peptide bond as the potential site for karilysin cleavage in membrane-associated TNF- α , shedding the soluble form of the cytokine, mimicking the action of ADAM17 (Karim et al., 2010). Moreover, the intensity of TNF- α shedding was not limited or restricted to a particular form of the enzyme (Kly48, Kly38, or Kly18), suggesting broad activity and high efficiency in soluble cytokine generation.

Metalloproteases are often activated in a cascade-like manner; thus, the possible indirect effect of karilysin on TNF- α , mediated by activation of a host protease(s), must be considered. Our study excluded the contribution of ADAM17, the major secretase responsible for TNF- α shedding; however, the possible role of other secretases, such as ADAM10 or MMP7, has not been verified. Moreover, we found that *T. forsythia* karilysin activates G protein-coupled protease-activated receptors (data not shown). This may suggest the involvement of that pathway in observed TNF- α shedding upon treatment of macrophages with karilysin. However, the exact mechanism/signaling pathway needs to be further analyzed.

The relevance of karilysin in TNF- α -dependent disruption of local homeostasis in periodontitis sites is supported by the fact that the karilysin gene and its mRNA transcript are present in GCF at sites infected with *T. forsythia* (Jusko et al., 2012). This indicates that TNF- α shedding and stimulation of expression by karilysin *in vivo* is a highly plausible scenario, especially considering the fact that the enzyme is active in human plasma rich in both metalloproteases inhibitors as well as α 2-macroglobulin with universal activity against endoproteinases. This scenario is further supported by the accumulation of the soluble cytokine in the culture medium of macrophages treated with karilysin, and the finding of soluble TNF- α in GCF and tissue biopsy specimens from periodontal patients (Gorska et al., 2003; Hirose et al., 1997). The latter occurs despite TNF- α degradation by other proteases – gingipains (Calkins et al., 1998) and/or dentilysin (Miyamoto et al., 2006) from *P. gingivalis* and *T. denticola*, respectively, and may be related to karilysin activity, causing continuous shedding of TNF- α .

The known specificity of karilysin (Karim et al., 2010) suggests that the enzyme should recognize and cleave Ala111-Leu112 and/or Ile34-Tyr35 peptide bonds in the soluble TNF- α molecule, thus degrading this cytokine. Nevertheless, the hydrolysis of rhTNF- α by

karilysin occurs only very slowly and at high E:S molar ratios. Karilysin and gingipains are most often present simultaneously at the sites of infection colonized with *T. forsythia* and *P. gingivalis*. It could therefore be hypothesized that their synergistic action, leading to shedding of TNF- α by karilysin, is followed by degradation of the soluble molecule by gingipains. The strong association of *P. gingivalis* and *T. forsythia* in dental subgingival plaque (Jusko et al., 2012; Mezyk-Kopec et al., 2005) supports this hypothesis.

Taken together, we have shown that karilysin sheds an abundant amount of biologically active TNF- α from macrophages. The soluble cytokine may strongly affect inflammatory reactions through the induction of cell death, generation of nitrites, and *de novo* expression of pro-inflammatory cytokines, such as IL-6 and IL-8, apparently as part of the autocrine pathway. Although TNF- α is essential for defense against pathogens, it is well-known that prolonged action of this mediator at high concentration may have deleterious effects on periodontitis. Such a conclusion is supported by the observation that application of a TNF- α antagonist inhibits tissue damage in experimental periodontitis (Graves and Cochran, 2003). Therefore, excessive TNF- α shedding by karilysin can be considered to be a very important virulence trait of *T. forsythia*, ultimately leading to host immune evasion and periodontal tissue destruction, including alveolar bone loss, and may also exert systemic effects (Mueller et al., 1999). To further confirm our hypothesis, the correlation between the presence of karilysin and the level of TNF- α in GCF should be determined. Circulating TNF- α may further induce the production of other cytokines, contributing to the development of systemic diseases (Aggarwal, 2003). This is supported by an expanding body of evidence correlating severe periodontitis with increased risk for atherosclerosis, diabetes, and rheumatoid arthritis (de Pablo et al., 2009; Friedewald et al., 2009).

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Figure 1.

The secretion of soluble TNF-α by human macrophages is mediated by the enzymatic activity of karilysin. (A) The dose-dependent effect of karilysin on TNF-α secretion from human monocyte-derived macrophages (hMDMs). Human macrophages (3×10^5 cells/well) were incubated with karilysin (Kly48) at concentrations ranging from 1 nM to 100 nM for 6 h. (B) hMDMs were stimulated for 6 h with Kly48 alone (100 nM) or in the presence of *o*-phenanthroline (0.3 mM), or a proteolytically inactive form of the enzyme, Kly^{Glu156Ala} (100 nM). (C) hMDMs were incubated with 100 nM karilysin in the absence or presence of different concentrations (0–10%) of autologous human serum for 6 h. The level of TNF-α in the culture medium was estimated by ELISA. Data are shown as the mean ($\pm$ SEM) values of three independent experiments using hMDMs derived from different donors. **, $p < 0.01$; ***, $p < 0.001$.

Figure 2.

Karilysin-catalyzed TNF- α shedding is independent of TNF- α converting enzyme (TACE).

(A) Sequence of pro-TNF- α with the indicated cleavage site for TACE (

↓

) and potential cleavage sites for karilysin (↓). The shaded amino acids constitute the sequence of the soluble form of the cytokine; (B) protein profile of different forms of karilysin (Kly48, Kly38/18, and Kly18) 5 μ g per lane, obtained by SDS-PAGE stained with Coomassie Blue. (C) ADAM17^{-/-}, TNF-negative murine fibroblasts (0.2×10^6 cells/well) were incubated with different forms of karilysin (100 nM) for 1 h. The level of soluble TNF- α in the cells' supernatant was then measured by ELISA. (D) ADAM17^{-/-}, TNF-positive cells were stimulated with 100 nM Kly48 and RgpB for 1 h. After washing, the cells were labeled using fluorescent anti-TNF- α antibody, and the level of remaining cytokine on the plasma membrane was determined by flow cytometry. Data represent average mean fluorescence intensity (MFI) values for the TNF- α -positive cells. Mean data ($\pm$ SEM) from three separate experiments are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 3.

Karilysin induces degradation of soluble TNF- α . (A) The levels of soluble TNF- α in the conditioned medium of human monocyte-derived macrophages (hMdmS) (3×10^5 cells/well) measured at different time points (1-24 h) post-stimulation with 100 nM karilysin (Kly48). Cytokine levels were determined by ELISA. Mean data ($\pm$ SEM) from three separate experiments are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (B, C) One microgram of recombinant human TNF- α (rhTNF- α) was incubated with 10 ng-10 μ g of karilysin, at different E:S molar ratios ranging from 1:0.1 to 1:100, for 12 h. Samples were separated by SDS-PAGE. (B) The recognition of TNF- α by specific antibodies was determined by Western-blot analysis. (C) The profile of separated protein was visualized by silver staining. A representative silver stained immunoblot from one of the three separate experiments is shown.

Figure 4.

Karilysin releases a biologically active form of soluble TNF-α. Conditioned medium (CM) was gathered from human monocyte-derived macrophages (hMDMs) stimulated with Kly48 for 6 h. The concentration of soluble TNF-α was determined by ELISA. (A) L929 cells were incubated in CM supplemented with 125 ng/ml actinomycin D. Cell death was estimated by MTT assay after 24 h of incubation. (B) MBE-SV cells were treated with CM in the presence or absence of 10 ng/ml interferon-γ (INFγ). After 20 h of incubation, the accumulated NO₂⁻ was measured in the culture medium by the Griess reaction. (A, B) Mean data (±SEM) from three separate experiments are shown. *, p<0.05; ***, p<0.001.

Figure 5.

Karilysin regulates pro-inflammatory gene expression in macrophages in an autocrine manner, by a positive amplification loop. Human monocyte-derived macrophages (hMDMs) were preincubated with 100 ng/ml anti-TNF- α antibodies for 15 min and then treated with Kly48 (100 nM) for 1 or 6 h. After 1 h (A) total RNA was isolated and the levels of mRNA for TNF- α , IL-6, and IL-8 were estimated using qRT-PCR. (B) The levels of released TNF- α , IL-6, and IL-8 in the culture medium were determined by ELISA 6 h post-stimulation with karilysin. Mean data ($\pm$ SEM) from three separate experiments, using hMDMs from different donors, are shown. *, $p < 0.05$; **, $p < 0.01$.

Figure 6.

Schematic representation of the putative role of karilysin in TNF- α induction by macrophages. Karilysin is secreted by *T. forsythia* (1). Kly sheds the membrane form of TNF- α (2), thus generating the soluble and biologically active (3) form of the cytokine (sTNF- α). The released cytokine activates a pro-inflammatory signaling cascade via the autocrine pathway (4), leading to local accumulation of TNF- α and other cytokines (5). Figures were produced using Servier Medical Art.

Table 1
Oligonucleotide sequences used in qRT-PCR

Oligonucleotide	Sequence
EF-2 F	5' – TCAGCACACTGGCATAGAGGC – 3'
EF-2 R	5' – GACATCACCAAGGGTGTGCAG – 3'
TNF- α F	5' – GTCAGATCATCTTCTCGAACCCCGA – 3'
TNF- α R	5' – CAGGGCAATGATCCCAAAGTAGA – 3'
IL-6 F	5' – AAATTCGGTACATCCTCGACGGCA – 3'
IL-6 R	5' – AGTGCCTCTTTGCTGCTTTCACAC – 3'
IL-8 F	5' – ATGACTTCCAAGCTGGCCGTGGCT – 3'
IL-8 R	5' – TCTCAGCCCTCTTCAAAAATTCT – 3'