Antibacterial and anti-inflammatory activity of a Temporin B peptide analogue on a in vitro model of cystic fibrosis

Journal:	*Journal of Peptide Science*
Manuscript ID:	PSC-14-0084.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Bezzerri, Valentino; Università di Verona, Dipartimento di Patologia e Diagnostica Avitabile, Concetta; Università di Napoli "Federico II", Dipartimento di Farmacia Dechecchi, Maria; Università di Verona, Dipartimento di Patologia e Diagnostica Lampronti, Ilaria; Università di Ferrara, Dipartimento di Scienze della Vita e Biotecnologie Borgatti, Monica; Università di Ferrara, Dipartimento di Scienze della Vita e Biotecnologie Montagner, Giulia; Università di Ferrara, Dipartimento di Scienze della Vita e Biotecnologie Cabrini, Giulio; Università di Verona, Dipartimento di Patologia e Diagnostica Gambari, Roberto; Università di Ferrara, Dipartimento di Scienze della Vita e Biotecnologie Romanelli, Alessandra; Università di Napoli "Federico II", Dipartimento di Farmacia
Keywords:	temporin, antimicrobial, anti-inflammatory, Pseudomonas aeruginosa, pro-inflammatory, peptide

Antibacterial and anti-inflammatory activity of a Temporin B peptide analogue on a *in vitro* model of cystic fibrosis

Valentino Bezzerri,[1] Concetta Avitabile,[2] Maria Cristina Dechecchi,[1] Ilaria Lampronti,[3] Monica Borgatti,[3] Giulia Montagner,[3] Giulio Cabrini, [1] Roberto Gambari[3] and Alessandra Romanelli[2]

1 Università di Verona, Dipartimento di Patologia e Diagnostica, 37134 – Verona

2 Università di Napoli "Federico II" , Dipartimento di Farmacia, 80134 – Napoli

3 Università di Ferrara, Dipartimento di Scienze della Vita e Biotecnologie, 44100 – Ferrara

Corresponding author: Alessandra Romanelli

e-mail: alessandra.romanelli@unina.it
tel: +39 0812532037

Abstract

Natural peptides with antimicrobial properties are deeply investigated as tools to fight bacteria resistant to common antibiotics. Small peptides, as those belonging to the temporin family, are very attractive since their activity can easily be tuned after small modification to their primary sequence. Structure-activity studies previously reported by us allowed the identification of one peptide, analogue of temporin B, TB_KKG6A, showing, unlike temporin B, antimicrobial activity against both Gram positive and Gram negative bacteria. In this paper we investigated the antimicrobial and anti-inflammatory activity of the peptide TB_KKG6A against *Pseudomonas aeruginosa*. Interestingly we found that the peptide exhibits antimicrobial activity at low concentrations, being able to down-regulate the pro-inflammatory chemokines and cytokines IL-8, IL-1β, IL-6 and TNF-α produced downstream in infected human bronchial epithelial cells. Experiments were carried out also with Temporin B, which was found to show pro-inflammatory activity. Details on the interaction between TB_KKG6A and the *P. aeruginosa* LPS were obtained by CD and fluorescence studies.

1. Introduction

Cystic fibrosis (CF) is a severe and diffuse recessive genetic disease due to defects of the CF Transmembrane conductance Regulator (CFTR) gene.[1] CF affects several organs, with the chronic pulmonary disease being the major cause of reduction of the quality and expectancy of life. The hallmark of CF lung disease is chronic infection generally sustained by the Gram-negative bacterium *Pseudomonas aeruginosa (P.aeruginosa)* and excessive lung inflammation with a huge infiltrate of neutrophils in the bronchial lumen, mainly due to the release of the chemokine interleukin IL-8.[2-5] The identification of innovative drugs, exhibiting strong antibacterial activity and thereby able to reduce the excessive lung inflammation in CF patients, is considered a promising therapeutic strategy to prevent the progressive lung tissue deterioration. Unfortunately, many of the known antibacterial molecules targeting *P. aeruginosa* have important undesired side effects. The ability of bacteria as *P. aeruginosa* to adapt themselves to the CF pulmonary environment and to form biofilms resistant to commonly used antibiotics renders the research of new molecules against such bacteria compelling.[6]

To this aim, antimicrobial peptides have been proposed as a tool to overcome bacterial insusceptibility. The search of drugs able to kill strains resistant to common antibiotics led to the discovery and design of several peptides, derived from natural fonts, with improved antibacterial and anti-inflammatory activities. Peptides derived from thrombin were found able to inhibit the inflammatory response and reduce mortality in a mouse model of *P. aeruginosa* induced sepsis[7]; the peptide Api88 derived from aepidaecin shows strong antibacterial activity against Gram negative bacteria including several isolates of *P. aeruginosa*, without evidences of immunomodulatory activity.[8] Several peptides between those isolated from frog skin have been tested and found active against *P. aeruginosa* strains[9-12]: the esculentin derivative Esc(1-21) was reported to be highly active against *P. aeruginosa* isolated from CF patients, having the ability to prolong the survival of animals in models of *P. aeruginosa* infections.[13]

In order to identify novel molecules active against *P. aeruginosa*, we have focused our attention on antimicrobial peptides belonging to the temporin family. Temporins are short peptides secreted by the granular glands of the European frog Rana Temporaria, mainly active against Gram positive bacteria[14, 15]. The only member of the temporin family showing activity also against Gram negative bacteria is temporin L, whose activity and structure in membrane like environment has been deeply investigated[16, 17]. The antimicrobial activity of the peptide Temporin-1Tb (TB) has been investigated on multidrug resistant clinical isolates of *P. aeruginosa* and on a *C. elegans* model[9]: it has been found that although TB promotes the survival of infected nematodes, it does not

display antimicrobial activity *in vitro*. Studies focused on Temporin B analogues demonstrated that subtle changes of the peptide primary structure result in new and interesting biological properties: addition of a tripeptide KKY at the N-terminus of temporin B produces the peptide TB-KK which acts in synergy with temporin A against Gram positive and Gram negative bacteria also *in vivo*[18]. TB-KK in combination with an analog of royal jellein I (RJI-C), an antimicrobial peptide isolated from the bee jelly, is strongly active against *S. epidermidis*; the combination of TB-KK and RJI-C does not kill probiotic bacteria and *in vivo*, in cells stimulated with LPS, down regulates the level of the pro-inflammatory cytokines TNF−α and IFN-γ while enhancing the expression of the anti-inflammatory cytokine IL-10, to an extent comparable with gentamicin[19].

The recently developed TB analogue TB_KKG6A, unlike TB, shows activity against Gram positive and Gram negative bacteria at low concentrations[20]. Compared to TB, this peptide has glycine 6 replaced by alanine and two extra lysines at the N-terminus. Fluorescence, CD and NMR data demonstrated that this peptide strongly interacts with the *E. coli* LPS and folds into a helix upon binding. Unlike TB, TB_KKG6A does not aggregate on LPS, probably due to the high number of positive charges and interestingly does not show hemolytic activity. The features discovered for TB_KKG6A encouraged us to explore the antimicrobial activity of this peptide against microorganisms as *P. aeruginosa*.

The aim of the present study was to determine the activity of TB_KKG6A on *P. aeruginosa* growth and downstream biological effects on the cystic fibrosis IB3-1 cell line (see Table 1 for peptide sequences). This cell line, after exposure to *P. aeruginosa* activates several pro-inflammatory cytokines and chemokines, as published by some of us.[21, 22] We investigated the antimicrobial activity of TB_KKG6A against *P. aeruginosa* strain PAO1 and the amount of IL-8, IL-1β, IL-6 and TNF-α produced in IB3-1 cells in different experimental conditions. Interactions of TB_KKG6A with bacterial LPS were investigated by Circular Dichroism with the aim to determine the secondary structure assumed by the peptide on bacterial cells and by fluorescence to gain information on the peptide-LPS binding.

2. Material and Methods

2.1 Peptide synthesis

Peptides were synthesized on solid phase by Fmoc chemistry on the MBHA (0.54 mmol/g) resin by consecutive deprotection, coupling and capping cycles. [19 20] Deprotection: 30% piperidine

in DMF, 5 min (2×). Coupling: 2.5 equivalents of amino acid+2.49 equivalent of HOBT/ HBTU (0.45 M in DMF)+3.5 equivalents NMM, 40 min. Capping: acetic anhydride/DIPEA/DMF 15/15/70 v/v/v, 5 min. Peptides were cleaved off the resin and deprotected by treatment of the resin with a solution of TFA/TIS/H_2O 95/2.5/2.5 v/v/v, 90 min. TFA was concentrated and peptides were precipitated in cold ethylic ether.

Analysis of the crudes was performed by LC–MS using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 30 to 70% in 30 min. Purifications were performed by semipreparative RP-HPLC using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 30 to 70% in 30 min.

The conjugation of the peptide to NBD was carried out on solid phase, on the peptide derivatized with a 6 amino-hexanoic acid (Ahx) linker at the N-terminus. NBD-Cl was reacted with the free amino group of Ahx in the presence of NMM.[20] . NBD-Cl (5 eq.) was dissolved in DMF, NMM (7 eq) was added; the solution was reacted with the peptide 3 hours at r.t. and double couplings were performed.

Peptides were cleaved off the resin and deprotected by treatment of the resin with a solution of TFA/TIS/H_2O 95/2.5/2.5 v/v/v, 90 minutes. TFA was concentrated and the peptides were precipitated in cold ethylic ether. Analysis of the crudes was performed by LC-MS using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 30 to 70% in 30 minutes. Purification was performed by semipreparative RP-HPLC using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 30 to 70% in 30 minutes. Characterization of the peptides by LC-MS confirmed previously reported results [20]. Peptides sequences and names are reported in Table 1.

2.2. Cell lines and bacteria

IB3–1 cells, derived from a CF patient with a ΔF508/W1282X mutant genotype and immortalized with adeno12/SV40, were grown in LHC-8 supplemented with 5% FBS in the absence of gentamicin, at 37 °C/ 5% CO_2.[23] The effects of active principles were analyzed as elsewhere described.[24] The nonmucoid laboratory strain of *P. aeruginosa*, PAO1, has been donated by A. Prince (Columbia University, New York, NY). Bacteria colonies from overnight cultures on trypticase soy agar (Difco, Detroit, MI) plates were grown with shaking in 20 ml trypticase soy broth (Difco) at 37°C until an OD (A660 nm wavelength), corresponding to 1.5 x 10^7 CFU/ml, was reached. Bacteria were washed twice with PBS and diluted in each specific serum-free medium before infection and added to cells at the concentration indicated as CFUs per cell.

2.3. Anti-microbial activity assay

The anti-microbial activity of peptides was determined following the procedure for the Minimum Inhibitory Concentration (MIC) of the National Committee for Clinical Laboratory Standards. In brief, *P. aeruginosa* was cultured on plates of Tryptic Soy Agar (TSA) overnight at 37 °C. The colonies were harvested, suspended in sterile saline, and adjusted to a concentration of a 0.5 McFarland standard. The range of TB and derivatives concentrations tested (as indicated in the figure) was prepared in 15 ml tubes containing 5 ml of Tryptic Soy Broth (TSB) starting from a 1000-fold concentrated of each compound stock solution. McFarland 0.5 standard of *P. aeruginosa* (20 µl) was added to each tube, and samples were incubated at 37 °C for 24 h. MIC is defined as the lowest concentration of compound at which there is no visible growth of the organism. In addition, the samples were read at 660 nm wavelength for quantitative analysis with a Beckman DU 640 spectrophotometer.

2.4. Quantification of mRNA content

Total RNA was extracted using TRIzol Reagent (Sigma, St. Louis, MO) following the manufacturer's instructions. Reverse transcription (RT) was performed using Reverse Transcription System kit (Promega, Madison, WI): 1 µg of total RNA was reverse transcribed in the presence of 5 mM $MgCl_2$, 1× Reverse transcription Buffer (10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100), 1 mM each dNTPs, 20 U recombinant Rnasin Ribonuclease Inhibitor, 15 U AMV Reverse Transcriptase, 0.5 µg $Oligo(dT)_{15}$ primers in a total volume of 20 µl for 10 min at 70 °C and 60 min at 42 °C. The resulting cDNA was quantified by relative quantitative real-time PCR (real-time qPCR). For the Real-time qPCR, 5 µl of cDNA were used for each Sybr Green real-time PCR to quantify the relative IL-8 expression. The cDNA (5 µl) was then amplified for 40 PCR cycles using the SYBR Green PCR Master Mix (Applied Biosystems) in a 25 µl reaction using 7900HT Fast Real-Time PCR apparatus (Applied Biosystems, Foster City, CA). In order to perform the PCR reaction QuantiTect Primer assays (Qiagen, Hilden, Germany) for IL-8 (Hs_IL8_1_SG, NM_000584), IL-1β (Hs_IL1B_1_SG, NM_000576), IL-6 (Hs_IL6_1_SG, NM_000600), TNF-α (Hs_TNF_1_SG, NM_000594) and Actin-beta (ACTB) (Hs_ACTB_1_SG, NM_001101) were purchased. The quantified real-time PCRs were performed in duplicates for both target and normalizer genes. Relative quantification of gene expression was performed utilizing the comparative threshold (C_T) method. Changes in mRNA expression level were calculated following normalization with the ACTB calibrator gene and expressed as fold change over untreated samples.

2.5. Statistics

Results are expressed as mean ± standard error (SEM). Comparisons between groups were made by using paired Student's *t* test and a one-way analysis of variance (ANOVA). Differences were considered significant when $p<0.05$ and highly significant when $p<0.01$.

2.6. Bio-Plex-analysis

IL-8 in tissue culture supernatants released from the cells under analysis, was measured by Bio-Plex cytokine assay (Bio-Rad Laboratories, Hercules, CA).[25, 26] IL-8 standards or samples (supernatants recovered from treated cells) were incubated with anti-IL-8 conjugated beads in 96-well filter plates for 30 min at RT with shaking. Plates were then washed with Bio-Plex wash buffer, diluted detection antibody was added and were incubated for 30 min at RT with shaking. After washes, streptavidin-phycoerythrin was added and the plates were incubated for 10 min at RT with shaking. Finally, plates were washed by vacuum filtration three times, beads were suspended in Bio-Plex assay buffer, and samples were analyzed on a Bio-Rad 96-well plate reader using the Bio-Plex Suspension Array System and Bio-Plex Manager software (Bio-Rad Laboratories).

2.7. Circular dichroism

Circular dichroism (CD) spectra were recorded at 25 °C using a 1 cm quartz cell with the Jasco-715 spectropolarimeter, a 260–198 nm measurement range, 100 nm/min scanning speed, 1 nm bandwidth, 4 s response time, 1.0 nm data pitch. LPS from *P. aeruginosa* 10 (Sigma, purified by phenol extraction) was employed for the experiments. The peptide TB_KKG6A was dissolved in phosphate buffer 10 mM pH 7.0 at a 5 μM concentration; the LPS was dissolved in phosphate buffer 10 mM pH 7.0 at a 0.75μg/μL concentration, before use it was subjected to temperature cycles between 4° and 70 °C, interrupted by vortexing (10 min). The sample was stored at 4 °C overnight. LPS titrations were carried out recording the CD spectra of the peptide in the presence of increasing concentrations of LPS at 25 °C, adding aliquots of 18.7 μg LPS.

2.8. Fluorescence studies

LPS from *P. aeruginosa* 10 (Sigma, purified by phenol extraction) was employed for the experiments. The peptide TB_KKG6A-NBD was dissolved in phosphate buffer 10 mM pH 7.0 at a 0.5 μM concentration, the LPS was dissolved in phosphate buffer 10 mM pH 7 at a 0.075μg/μL concentration. LPS titrations were carried out monitoring the fluorescence intensity at 550 nm of the peptide in the presence of increasing concentrations of LPS, from 1.8 to 28 μg. The excitation wavelength was set at 487 nm. The maximum emission wavelength vs the excitation wavelength were monitored for the peptide TB_KKG6A-NBD and for the mixture TB_KKG6A-NBD +18.7 μg

of *P. aeruginosa* LPS to detect possible red edge excitation shift effects. All experiments were repeated in duplicate.

3. Results and discussion

3.1. Inhibition of *P. aeruginosa* cell growth after exposure to Temporin derivatives.

P. aeruginosa strain PAO1 was exposed for 24 hours to increasing amounts of the peptides TB, TB_G6A , TB_KKG6A and gentamicin (Figure 1) as positive control, since this compound is one of the gold standards in the current antibiotic therapy on cystic fibrosis patients.[27] As clearly evident, no inhibitory effects on PAO1 were displayed by TB and TB_G6A, even when administered at 25-50 μM concentrations (Figures 1A and 1B). Interestingly, TB_KKG6A displayed anti-PAO1 activity at 5 μM concentrations (Figure 1C), confirming the ability of the modified peptide to kill Gram negative bacteria; gentamicin was more active (full PAO1 suppression obtained at 1 μM concentration) (Figure 1D), as elsewhere reported.[27]

3.2. Effects of Temporin analogues on *P. aeruginosa* induced upregulation of IL-8 gene expression.

The results of this experiment are shown in Figure 2. Cystic fibrosis IB3-1 cells were exposed to the analyzed compound for 24 hours before infection with PAO1 for 4 hours and RNA isolation for IL-8 mRNA content analysis. We found that TB and TB_G6A were inactive (Figure 2, A and B). On the contrary, TB_KKG6A displayed inhibitory activity, but only at high concentrations (10 and 50 μM) (Figure 2C). As expected, gentamycin displayed inhibitory activity at 5-10 μM concentration (Figure 2D). These data indicate that TB_KKG6A, as gentamicin, exerts anti-inflammatory activity, by inhibiting the PAO1 induced up-regulation of IL-8 gene expression.

3.3. Effects of pre-incubation of PAO1 with Temporin analogues on IL-8 gene expression.

When PAO1 cells were pre-incubated for 24 hours with the peptides and then mixed with IB3-1 cells, only TB_KKG6A exhibited strong inhibitory effects on accumulation of IL-8 mRNA (Figure 3, panels A-C). Very strong effects were found at 5 μM concentration. As expected, gentamicin displayed inhibitory effects with higher efficiency (Figure 3D).

3.4. Effects of temporin analogues on expression of IL-1β, IL-6 and TNF-α pro-inflammatory genes.

The effects of pre-treatment of IB3-1 cells with the synthetic peptides before 4 hours PAO1 infection were also determined by RT-PCR on other pro-inflammatory genes, such as IL-1β, IL-6 and TNF-α. Surprisingly, TB was found to up-regulate the expression of two pro-inflammatory genes, IL-1β and IL-6 (Figure 4, A and B), while was inactive on TNF-α (Figure 4C). On the contrary, TB_G6A, as found for IL-8 (see Figure 2) exhibited no effect on the expression of IL-1β and TNF-α and only minor effects on IL-6 (Figure 4, D-F). By sharp contrast TB_KKG6A was found to significantly inhibit the expression of IL-1β, IL-6 and TNF-α (Figure 4, G-I). This suggests that TB_KKG6A is a strong inhibitor of PAO1 induced expression of pro-inflammatory genes. Fully in agreement with the results shown in Figure 3C, TB_KKG6A was found to abolish PAO1 induction of IL-1β, IL-6 and TNF-α gene expression when pre-incubated with PAO1, before infection of IB3-1 cells (Figure 5). This suggests that TB_KKG6A is a strong inhibitor of PAO1 induced expression of pro-inflammatory genes, working with an efficiency similar to that exhibited by gentamicin, extensively used as antibacterial drug on cystic fibrosis patients.

3.5. Effects of TB_KKG6A on IL-8 release.

In order to confirm that the effects of TB_KKG6A measured with RT-PCR (and therefore measuring IL-8 mRNA levels, see Figures 2 and 3) are accompanied by inhibition of IL-8 protein secretion, the levels of IL-8 protein were analyzed in the medium of cultured IB3-1 cells, following the protocols described for Figure 6A in the legends of Figures 2 and 4 and for Figure 6B in the legends of Figures 3 and 5. Fully in agreement with the RT-PCR data, Figure 6 shows that TB_KKG6A inhibits IL-8 secretion either when added to IB3-1 before PAO1 infection (Figure 6A) or when added to PAO1 before treatment of IB3-1 cells (Figure 6B).

3.6. Effects of TB_KKG6A on expression of IL-1β, IL-6 and TNF-α pro-inflammatory genes in uninfected IB3-1 cells.

In order to exclude an effect of TB_KKG6A on the basic levels of IL-8, IL-1β, IL-6 and TNF-α, the experiment reported in Figure 7 was performed. IB3-1 cells were treated for 24 hours with 50 μM TB, TB_G6A and TB_KKG6A and the expression of the pro-inflammatory genes IL-8, IL-1β, IL-6 and TNF-α was determined by RT-PCR. The results obtained demonstrated that the peptides

TB_G6A and TB_KKG6A do not affect the expression of pro-inflammatory genes in uninfected IB3-1 cells. The only inhibitory effect was found when TB was employed and the expression of IL-8 and IL-6 genes analyzed (Figure7, A and C). When these data are considered together with the results shown in Figures 2-5, they suggests that TB_KKG6A is a strong inhibitor of PAO1 induced expression of pro-inflammatory genes and its activity is mainly due to antibacterial effects without alteration of cellular pathways involved in inflammatory processes.

3.7 CD and fluorescence studies

As the antibacterial activity of peptides belonging to the temporin family is supposed to be mediated by the interactions of the peptides with the bacterial outer membrane, we performed CD and fluorescence studies of TB_KKG6A in the presence of *P. aeruginosa* LPS. The peptide TB_KKG6A was titrated with the lipopolysaccharide from *P. aeruginosa* in phosphate buffer at pH 7.0 . CD spectra of the peptide in buffer show one minimum at 200 nm which clearly indicates that the peptide is in an unordered conformation in buffer, while in the presence of LPS two minima around 207 and 224 nm appear, suggesting that the peptide assumes the conformation of a helix upon binding to LPS. (Figure 8) The interaction of the peptide with LPS was also assessed by fluorescence, monitoring the intensity of the fluorescence emission of the NBD labeled TB_KKG6A, TB_KKG6A_NBD, titrated with increasing amounts of LPS. A sigmoidal curve was obtained plotting the fluorescence intensity at 550 nm vs μg of LPS added.(Figure 9) This result is very similar to what reported in the literature for titrations carried out for this and other NBD labeled peptides with LPS from other bacteria, as *E.coli*.[20, 28] The binding of the peptide to LPS causes an increase in the hydrophobicity of the peptide environment which is sensed by the NBD probe. As it is reported in the literature that the LPS from *P. aeruginosa* has a high degree of heterogeneity[29], we could not calculate its molar concentration and therefore we could not express the binding constant of the peptide to LPS. In order to detect the position of the fluorophore with respect to the LPS, we also monitored how the maximum in the emission wavelength varies with the excitation wavelength. It has been observed, in fact, that membrane bound peptides show red edge excitation shift (REES), due to the location of the fluorophore in a membrane region which is motionally restricted[30]. No REES was detected for the peptide in solution and for the peptide in the presence of LPS (data not shown), suggesting that the N-terminus of the peptide is solvent exposed. CD and fluorescence data demonstrate that the peptide interacts with the bacterial membrane and that its folding is mediated by the interaction with the bacterial LPS. It has recently been demonstrated that CD spectra recorded for antimicrobial peptides in the presence of LPS recall CD

spectra recorded in the presence of cells[31]; based on this we might hypothesize that the peptide TB_KKG6A also assumes an helical structure upon interaction with *P.aeruginosa* cells.

Conclusions

The major conclusion of this work is that TB_KKG6A exhibits strong antimicrobial activity on *P.aeruginosa* PAO1 cells, likely mediated by the interactions of the peptide with the bacterial membranes. Experiments aimed to evaluate the antimicrobial activity of TB_KKG6A were carried out in parallel also on TB, TB_G6A and gentamycin. Interestingly TB_KKG6A was found active against *P. aeruginosa* at 10 μM concentration, unlike the other peptides which were found inactive. Gentamicin shows activity at lower concentration. These results confirm the ability of TB_KKG6A to kill Gram negative bacteria at low concentrations. In addition, TB_KKG6A was found to strongly inhibit the PAO1 induced upregulation of the pro inflammatory genes IL-8, IL-1β, IL-6 and TNF-α in IB3-1 cystic fibrosis cells infected by *P. aeruginosa* PAO1 in different conditions. This effect is mainly associated with the antibacterial activity of TB_KKG6A, and in fact it is particularly evident when PAO-1 was pre-treated with TB_KKG6A before infection of the IB3-1 cells (Figure 5); moreover no inhibitory effects of TB_KKG6A were found on the expression of IL-8, IL-1b, IL-6 and TNF-a genes in uninfected IB3-1 cells (Figure 7). In any case inhibitory effects of TB_KKG6A are clearly detectable in IB3-1-infected cells in the protocol mimicking the pathological situation and based on pre-treatment of IB3-1 cells with the inhibitory peptide before infection. TB_KKG6A. The effects of TB_KKG6A on IL-8 gene expression are of relevance in consideration of the key role of this protein in cystic fibrosis inflammatory process. On the other hand TB was found to be inactive on IL-8 and TNF-α gene expression, but to exert induction effects on pro-inflammatory IL-1β and IL-6 genes, suggesting that TB should be considered as a potential pro-inflammatory compound.

Acknowledgments

We are grateful to Alice Prince for donating the *P. aeruginosa* strain PAO1, to Valentina Lovato for excellent technical support. This work was supported by grants from the Italian Cystic Fibrosis Research Foundation (grants # 15/2004 and # 17/2010 to R.G; grant # 14/2012 to IL as external collaborator). VB is a fellow of Italian Cystic Fibrosis Research Foundation. CA is granted by Programma Merit RBNE08YFN3.

References

1. Welsh MJ, Tsui LC, Boat TF, Beaudet AL. Cystic fibrosis. The Metabolic and Molecular Bases of Inherited Disease
1995: 3799-876.
2. Becker MN, Sauer MS, Muhlebach MS, Hirsh AJ, Wu Q, Verghese MW, et al. Cytokine secretion by cystic fibrosis airway epithelial cells. Am J Respir Crit Care Med. 2004; **169**: 645-53.
3. Black HR, Yankaskas JR, Johnson LG, Noah TL. Interleukin-8 production by cystic fibrosis nasal epithelial cells after tumor necrosis factor-alpha and respiratory syncytial virus stimulation. Am J Respir Cell Mol Biol. 1998; **19**: 210-5.
4. Bonfield TL, Panuska JR, Konstan MW, Hilliard KA, Hilliard JB, Ghnaim H, et al. Inflammatory cytokines in cystic fibrosis lungs. Am J Respir Crit Care Med. 1995; **152**: 2111-8.
5. Tamanini A, Borgatti M, Finotti A, Piccagli L, Bezzerri V, Favia M, et al. Trimethylangelicin reduces IL-8 transcription and potentiates CFTR function. Am J Physiol Lung Cell Mol Physiol. 2011; **300**: L380-90.
6. Hancock RE, Speert DP. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. Drug Resist Updat. 2000; **3**: 247-55.
7. Kalle M, Papareddy P, Kasetty G, Morgelin M, van der Plas MJ, Rydengard V, et al. Host defense peptides of thrombin modulate inflammation and coagulation in endotoxin-mediated shock and Pseudomonas aeruginosa sepsis. PLoS One. 2012; **7**: e51313.
8. Czihal P, Knappe D, Fritsche S, Zahn M, Berthold N, Piantavigna S, et al. Api88 is a novel antibacterial designer peptide to treat systemic infections with multidrug-resistant Gram-negative pathogens. ACS Chem Biol. 2012; **7**: 1281-91.
9. Uccelletti D, Zanni E, Marcellini L, Palleschi C, Barra D, Mangoni ML. Anti-Pseudomonas activity of frog skin antimicrobial peptides in a Caenorhabditis elegans infection model: a plausible mode of action in vitro and in vivo. Antimicrob Agents Chemother. 2010; **54**: 3853-60.
10. Conlon JM, Sonnevend A, Patel M, Al-Dhaheri K, Nielsen PF, Kolodziejek J, et al. A family of brevinin-2 peptides with potent activity against Pseudomonas aeruginosa from the skin of the Hokkaido frog, Rana pirica. Regul Pept. 2004; **118**: 135-41.
11. Simmaco M, Mignogna G, Barra D, Bossa F. Antimicrobial peptides from skin secretions of Rana esculenta. Molecular cloning of cDNAs encoding esculentin and brevinins and isolation of new active peptides. J Biol Chem. 1994; **269**: 11956-61.
12. Giacometti A, Cirioni O, Barchiesi F, Fortuna M, Scalise G. In-vitro activity of cationic peptides alone and in combination with clinically used antimicrobial agents against Pseudomonas aeruginosa. J Antimicrob Chemother. 1999; **44**: 641-5.
13. Luca V, Stringaro A, Colone M, Pini A, Mangoni ML. Esculentin(1-21), an amphibian skin membrane-active peptide with potent activity on both planktonic and biofilm cells of the bacterial pathogen Pseudomonas aeruginosa. Cell Mol Life Sci. 2013; **70**: 2773-86.
14. Simmaco M, Mignogna G, Canofeni S, Miele R, Mangoni ML, Barra D. Temporins, antimicrobial peptides from the European red frog Rana temporaria. Eur J Biochem. 1996; **242**: 788-92.
15. Wade D, Silveira A, Silberring J, Kuusela P, Lankinen H. Temporin antibiotic peptides: A review and derivation of a consensus sequence. Protein Peptide Lett. 2000; **7**: 349-57.
16. Rinaldi AC, Mangoni ML, Rufo A, Luzi C, Barra D, Zhao H, et al. Temporin L: antimicrobial, haemolytic and cytotoxic activities, and effects on membrane permeabilization in lipid vesicles. Biochem J. 2002; **368**: 91-100.
17. Bhunia A, Saravanan R, Mohanram H, Mangoni ML, Bhattacharjya S. NMR structures and interactions of temporin-1Tl and temporin-1Tb with lipopolysaccharide micelles:

mechanistic insights into outer membrane permeabilization and synergistic activity. J Biol Chem. 2011; **286**: 24394-406.
18. Capparelli R, Romanelli A, Iannaccone M, Nocerino N, Ripa R, Pensato S, et al. Synergistic antibacterial and anti-inflammatory activity of temporin A and modified temporin B in vivo. PLoS One. 2009; **4**: e7191.
19. Romanelli A, Moggio L, Montella RC, Campiglia P, Iannaccone M, Capuano F, et al. Peptides from Royal Jelly: studies on the antimicrobial activity of jelleins, jelleins analogs and synergy with temporins. J Pept Sci. 2011; **17**: 348-52.
20. Avitabile C, Netti F, Orefice G, Palmieri M, Nocerino N, Malgieri G, et al. Design, structural and functional characterization of a Temporin-1b analog active against Gram-negative bacteria. Biochim Biophys Acta. 2013; **1830**: 3767-75.
21. Bezzerri V, Borgatti M, Nicolis E, Lampronti I, Dechecchi MC, Mancini I, et al. Transcription factor oligodeoxynucleotides to NF-kappaB inhibit transcription of IL-8 in bronchial cells. Am J Respir Cell Mol Biol. 2008; **39**: 86-96.
22. Gambari R, Borgatti M, Bezzerri V, Nicolis E, Lampronti I, Dechecchi MC, et al. Decoy oligodeoxyribonucleotides and peptide nucleic acids-DNA chimeras targeting nuclear factor kappa-B: inhibition of IL-8 gene expression in cystic fibrosis cells infected with Pseudomonas aeruginosa. Biochem Pharmacol. 2010; **80**: 1887-94.
23. Nicolis E, Lampronti I, Dechecchi MC, Borgatti M, Tamanini A, Bezzerri V, et al. Modulation of expression of IL-8 gene in bronchial epithelial cells by 5-methoxypsoralen. Int Immunopharmacol. 2009; **9**: 1411-22.
24. Gambari R, Borgatti M, Lampronti I, Fabbri E, Brognara E, Bianchi N, et al. Corilagin is a potent inhibitor of NF-kappaB activity and downregulates TNF-alpha induced expression of IL-8 gene in cystic fibrosis IB3-1 cells. Int Immunopharmacol. 2012; **13**: 308-15.
25. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol. 2003; **10**: 133-9.
26. Penolazzi L, Lambertini E, Tavanti E, Torreggiani E, Vesce F, Gambari R, et al. Evaluation of chemokine and cytokine profiles in osteoblast progenitors from umbilical cord blood stem cells by BIO-PLEX technology. Cell Biol Int. 2008; **32**: 320-5.
27. Hodson ME, Penketh AR, Batten JC. Aerosol carbenicillin and gentamicin treatment of Pseudomonas aeruginosa infection in patients with cystic fibrosis. Lancet. 1981; **2**: 1137-9.
28. Rosenfeld Y, Sahl HG, Shai Y. Parameters involved in antimicrobial and endotoxin detoxification activities of antimicrobial peptides. Biochemistry. 2008; **47**: 6468-78.
29. Pier GB. Pseudomonas aeruginosa lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. Int J Med Microbiol. 2007; **297**: 277-95.
30. Raghuraman H, Chattopadhyay A. Orientation and dynamics of melittin in membranes of varying composition utilizing NBD fluorescence. Biophys J. 2007; **92**: 1271-83.
31. Avitabile C, D'Andrea LD, Romanelli A. Circular Dichroism studies on the interactions of antimicrobial peptides with bacterial cells. Sci Rep. 2014; **4**: 4293.

Table 1. Name and sequences of the peptides employed.

Sequence	Name
LLPIVGNLLKSLL	TB
LLPIVANLLKSLL	TB_G6A
KKLLPIVANLLKSLL	TB_KKG6A
NBD-Ahx-KKLLPIVANLLKSLL	TB_ KKG6A _NBD

Legend to Figures

Figure 1. Anti-microbial assay of Temporin B and its derivatives against *P. aeruginosa*. Effects of increasing amounts of TB (A), TB_G6A (B), TB_KKG6A (C) and Gentamicin (positive control) (D) on *P. aeruginosa* growth in TSB after 24 hours at 37°C.

Figure 2. Effects of pre-incubation of Temporin B and its derivatives in human bronchial epithelial cells before infection with *P. aeruginosa*. IB3-1 cells were pre-incubated with increasing amounts of TB, TB derivatives or Gentamicin for 24 hrs before *P. aeruginosa* (PAO1 strain) infection sustained for further 4 hours. Total RNA was finally extracted and qRT-PCR was performed in order to quantify IL-8 mRNA expression. Effects of pre-incubation of TB (A), TB_G6A (B), TB_KKG6A (C), Gentamicin (D).

Figure 3. Effect of pre-incubation of Temporin B and its derivatives with *P. aeruginosa* on PAO1-mediated IL-8 mRNA expression in human bronchial epithelial cells. Equal amounts of *P. aeruginosa*, PAO1 strain, were pre-incubated for 24 hours in TSB culture medium containing increasing amounts of TB, TB derivatives or Gentamicin. Then, the resulting bacterial suspension was washed with sterile PBS and IB3-1 cells were infected with the bacterial suspension obtained after PBS washes for 4 hours. Total RNA was extracted and mRNA was quantified by qRT-PCR. Effect of pre-incubation of TB (A), TB_G6A (B), TB_KKG6A (C), Gentamicin (D).

Figure 4. Effect of pre-incubation of Temporin B and its derivatives in human bronchial epithelial cells before infection with *P. aeruginosa*: expression of pro-inflammatory IL-1β, IL-6 and TNF-α genes. IB3-1 cells were pre-incubated with increasing amounts of TB and TB derivatives for 24 hrs before PAO1infection sustained for further 4 hours. Total RNA was finally extracted and qRT-PCR was performed in order to quantify IL-1β, IL-6 and TNF-α mRNAs. A-C. Effect of pre-incubation with TB on IL-1β (A), IL-6 (B) and TNF-α (C); D-F. Effect of TB_G6A on IL-1β (D), IL-6 (E) and TNF-α (F); G-I) effect of TB_KKG6A on IL-1β (G), IL-6 (H) and TNF-α (I).

Figure 5. Effect of pre-incubation of *P.aeruginosa* with TB_KKG6A (A-C) or Gentamycin (D-F) on PAO1-mediated IL-1β (A,D), IL-6 (B,E) and TNF-α (C,F) mRNA expression in IB3-1 cells. Equal amounts of *P. aeruginosa*, PAO1 strain, were pre-incubated for 24 hours in TSB culture medium containing increasing amount of TB_KKG6A or Gentamicin. Then, the resulting bacterial suspension was washed with sterile PBS and IB3-1 cells were infected with the bacterial suspension obtained after PBS washes for 4 hours. Total RNA was extracted and mRNA was quantified by qRT-PCR.

Figure 6. Effects of TB_KKG6A of IL-8 secretion. A. Effects of pre-incubation of human bronchial epithelial IB3-1 cells with the peptide before infection with *P.aeruginosa* (for details on the experimental protocol, see legend to Figs. 2 and 4). B. Effects of pre-incubation of the peptide with *P. aeruginosa* before infection of IB3-1 cells (for details on the experimental protocol, see legend to Figs. 3 and 5). IL-8 was quantified by Bio-plex analysis. Open symbols: control uninfected cells.

Figure 7. Effect of TB, TB_G6A and TB_KKG6A (A-C) on accumulation of IL-8 (A), IL-1β (B), IL-6 (C) and TNF-α (D) in uninfected IB3-1 cells. The treatments were carried out for 24 hours at 50 μM concentration of the peptides. Total RNA was extracted and mRNA was quantified by qRT-PCR.

Figure 8. Superimposition of CD spectra obtained titrating *P. aeruginosa* LPS into the TB_KKG6A (5μM) solution, in phosphate buffer pH7. The direction of the arrow indicates increasing LPS concentration.

Figure 9. Plot of the fluorescence intensity at 550 nm vs μg *P.aeruginosa* LPS obtained titrating the LPS into the TB_KKG6A_NBD (0.5 μM) solution.

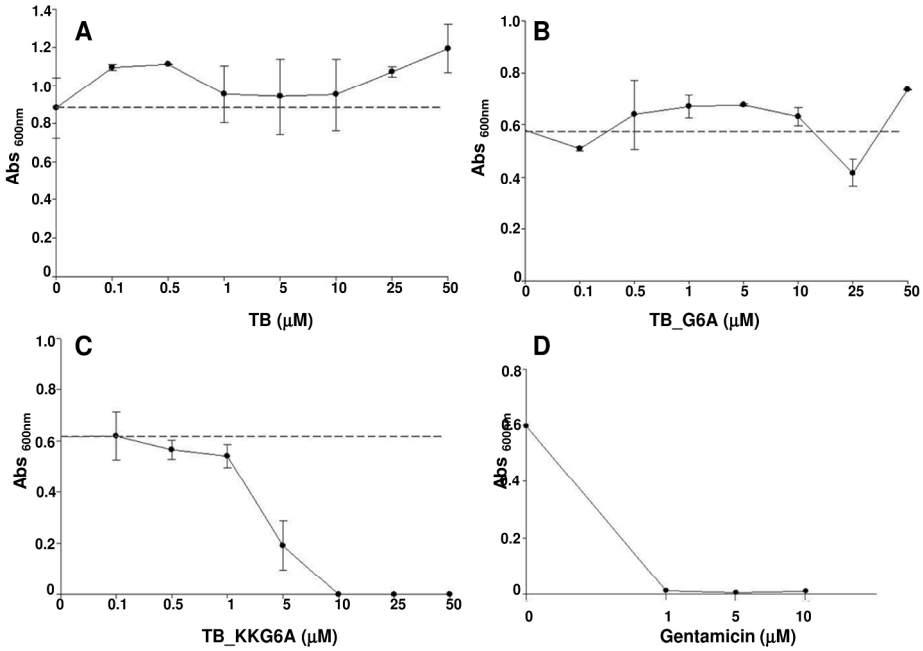

Figure 1. Anti-microbial assay of Temporin B and its derivatives against P. aeruginosa. Effects of increasing amounts of TB (A), TB_G6A (B), TB_KKG6A (C) and Gentamicin (positive control) (D) on P. aeruginosa growth in TSB after 24 hours at 37°C.
126x86mm (600 x 600 DPI)

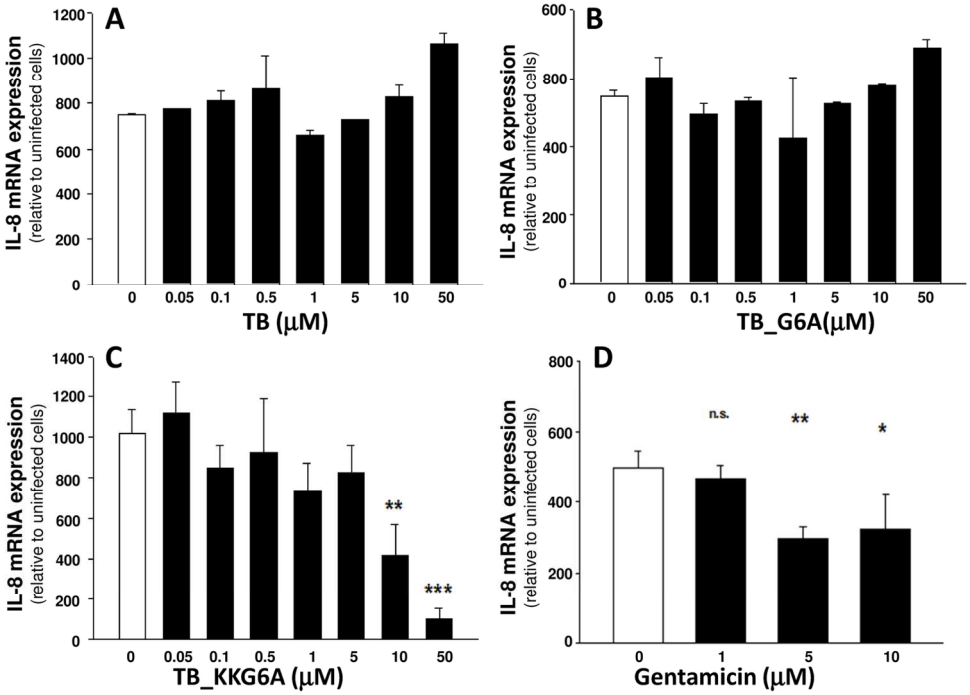

Figure 2. Effects of pre-incubation of Temporin B and its derivatives in human bronchial epithelial cells before infection with P. aeruginosa. IB3-1 cells were pre-incubated with increasing amounts of TB, TB derivatives or Gentamicin for 24 hrs before P. aeruginosa (PAO1 strain) infection sustained for further 4 hours. Total RNA was finally extracted and qRT-PCR was performed in order to quantify IL-8 mRNA expression. Effects of pre-incubation of TB (A), TB_G6A (B), TB_KKG6A (C), Gentamicin (D).
133x95mm (300 x 300 DPI)

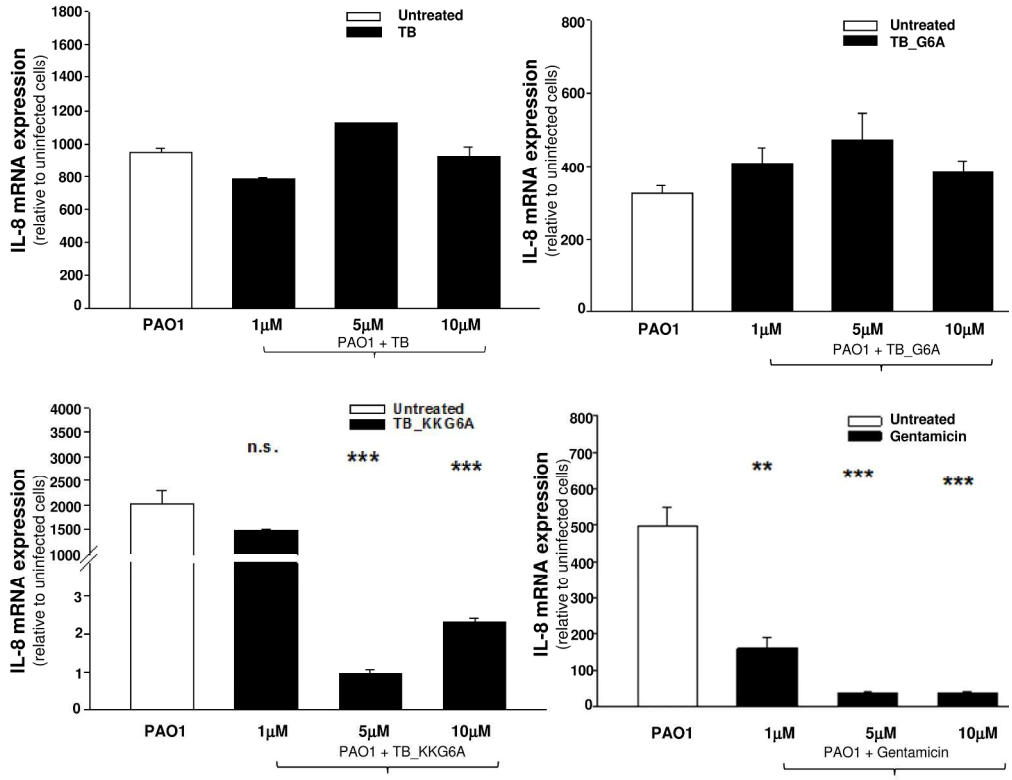

Figure 3. Effect of pre-incubation of Temporin B and its derivatives with P. aeruginosa on PAO1-mediated IL-8 mRNA expression in human bronchial epithelial cells. Equal amounts of P. aeruginosa, PAO1 strain, were pre-incubated for 24 hours in TSB culture medium containing increasing amounts of TB, TB derivatives or Gentamicin. Then, the resulting bacterial suspension was washed with sterile PBS and IB3-1 cells were infected with the bacterial suspension obtained after PBS washes for 4 hours. Total RNA was extracted and mRNA was quantified by qRT-PCR. Effect of pre-incubation of TB (A), TB_G6A (B), TB_KKG6A (C), Gentamicin (D).
152x124mm (600 x 600 DPI)

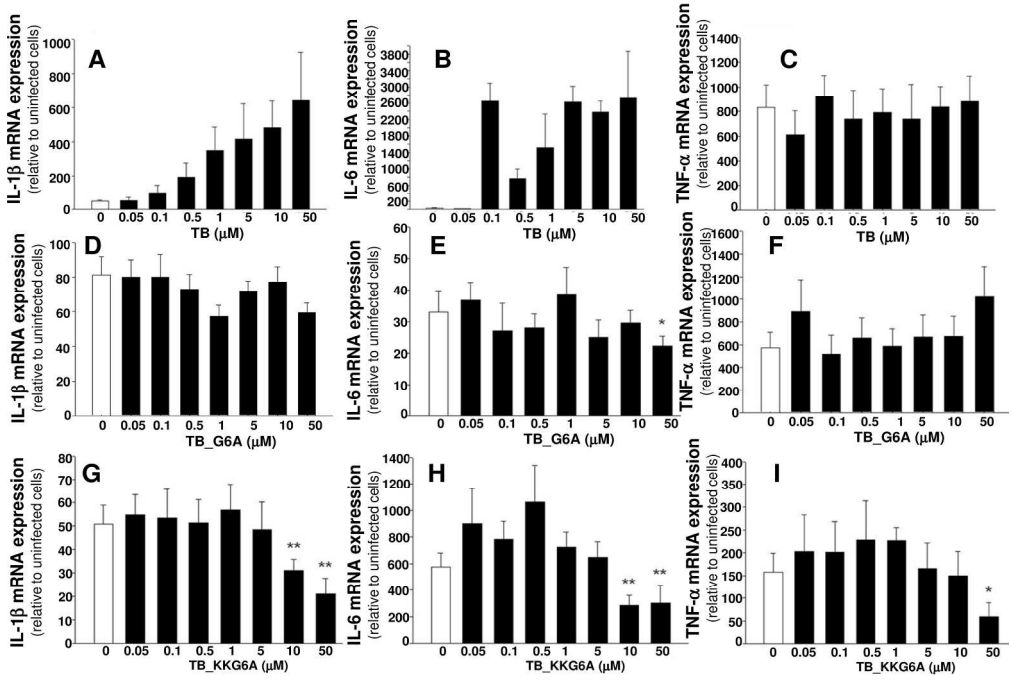

Figure 4. Effect of pre-incubation of Temporin B and its derivatives in human bronchial epithelial cells before infection with P. aeruginosa: expression of pro-inflammatory IL-1β, IL-6 and TNF-α genes. IB3-1 cells were pre-incubated with increasing amounts of TB, TB derivatives or Gentamicin for 24 hrs before PAO1infection sustained for further 4 hours. Total RNA was finally extracted and qRT-PCR was performed in order to quantify IL-1β, IL-6 and TNF-α mRNAs. A-C. Effect of pre-incubation with TB on IL-1β (A), IL-6 (B) and TNF-α (C); D-F. Effect of TB_G6A on IL-1β (D), IL-6 (E) and TNF-α (F); G-I) effect of TB_KKG6A on IL-1β (G), IL-6 (H) and TNF-α (I).
126x86mm (600 x 600 DPI)

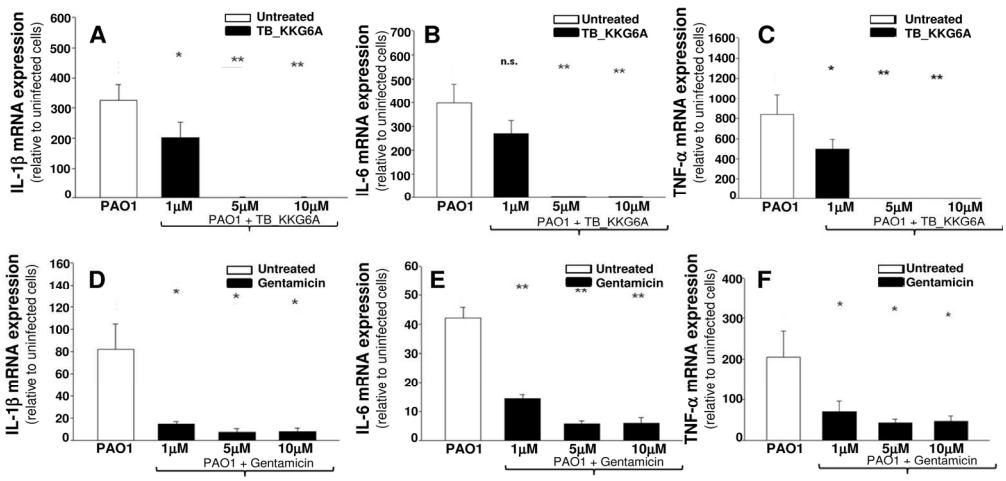

Figure 5. Effect of pre-incubation of P.aeruginosa with TB_KKG6A (A-C) or Gentamycin (D-F) on PAO1-mediated IL-1β (A,D), IL-6 (B,E) and TNF-α (C,F) mRNA expression in IB3-1 cells. Equal amounts of P. aeruginosa, PAO1 strain, were pre-incubated for 24 hours in TSB culture medium containing increasing amount of TB_KKG6A or Gentamicin. Then, the resulting bacterial suspension was washed with sterile PBS and IB3-1 cells were infected with the bacterial suspension obtained after PBS washes for 4 hours. Total RNA was extracted and mRNA was quantified by qRT-PCR.
88x42mm (600 x 600 DPI)

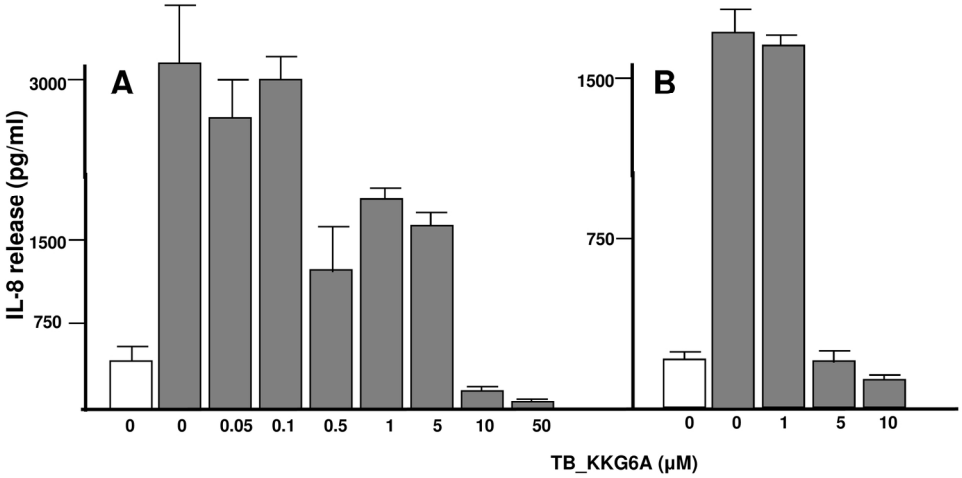

Figure 6. Effects of TB_KKG6A of IL-8 secretion. A. Effects of pre-incubation of human bronchial epithelial IB3-1 cells with the peptide before infection with P.aeruginosa (for details on the experimental protocol, see legend to Figs. 2 and 4). B. Effects of pre-incubation of the peptide with P. aeruginosa before infection of IB3-1 cells (for details on the experimental protocol, see legend to Figs. 3 and 5). IL-8 was quantified by Bio-plex analysis. Open symbols: control unifected cells.
85x43mm (600 x 600 DPI)

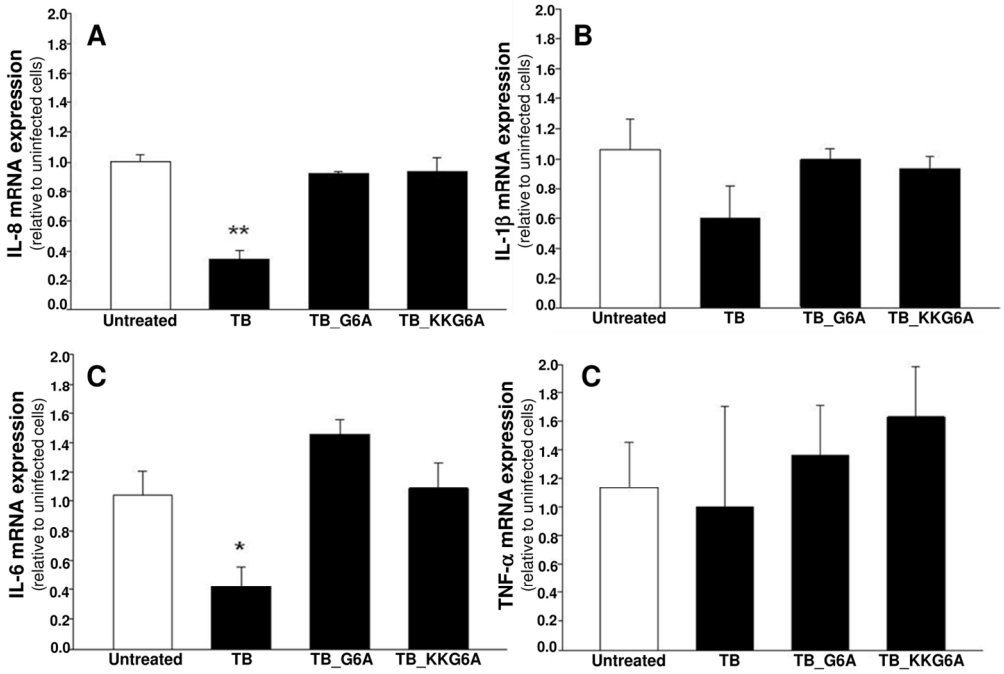

Figure 7. Effect of TB, TB_G6A and TB_KKG6A (A-C) on accumulation of IL-8 (A), IL-1β (B), IL-6 (C) and TNF-α (D) in uninfected IB3-1 cells. The treatments were carried out for 24 hours at 50 μM concentration of the peptides. Total RNA was extracted and mRNA was quantified by qRT-PCR.
137x95mm (300 x 300 DPI)

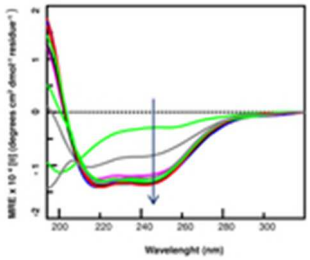

Figure 8. Superimposition of CD spectra obtained titrating P. aeruginosa LPS into the TB_KKG6A (5μM) solution, in phosphate buffer pH 7. The direction of the arrow indicates increasing LPS concentration.
6x5mm (600 x 600 DPI)

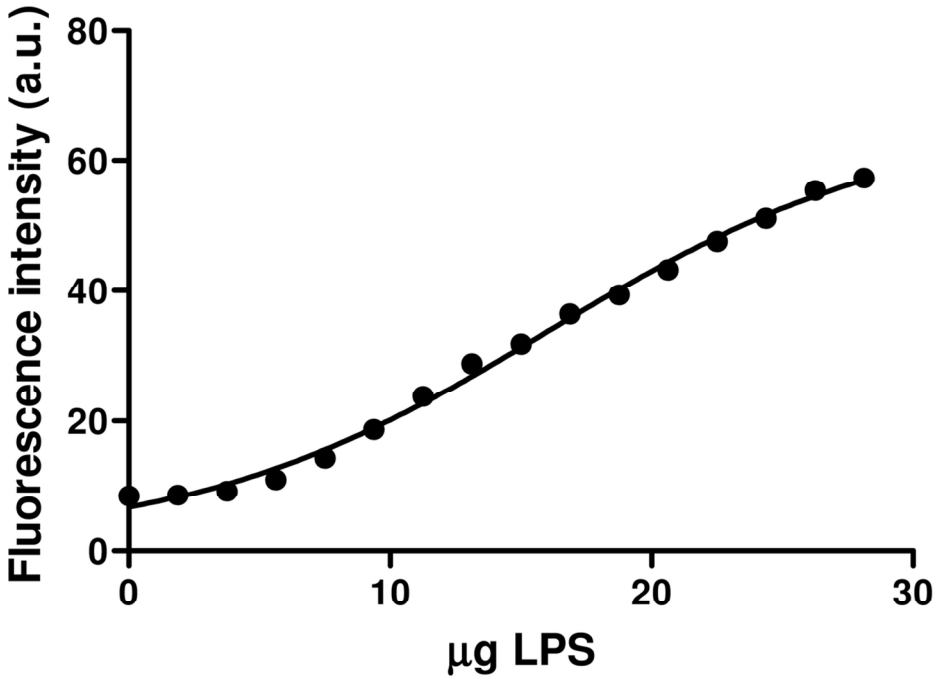

Figure 9. Plot of the fluorescence intensity at 550 nm vs µg P.aeruginosa LPS obtained titrating the LPS into the TB_KKG6A_NBD (0.5 µM) solution.
57x41mm (600 x 600 DPI)