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Supplemental file:

Biophysical mechanisms of endotoxin neutralization by cationic amphiphilic peptides

Synthesis of the peptides

The peptides were synthesized with an amidated C-terminus by the solid-phase peptide synthesis technique in an automatic peptide synthesizer (model 433 A; Applied Biosystems) on Fmoc-Rink amide resin according to the 0.1mmol FastMoc synthesis protocol of the manufacturer, including the removal of the N-terminal Fmoc-group. The peptidyl-resin was deprotected and cleaved with a mixture of 90 % trifluoroacetic acid (TFA), 5 % anisole, 2 % thioanisole, and 3 % dithiothreitol for 90 min at room temperature. After cleavage the suspension was filtered through a syringe filter into ice-cold diethylether. The precipitated peptides were separated by centrifugation and repeatedly washed with cold ether. The final purification was done by RP-HPLC. Purity levels up to 98% were achieved using an Aqua-C18 column (Phenomenex) in combination with dedicated gradients of acetonitrile in 0.1 % TFA, checked by Maldi-TOF mass spectroscopy and RP-HPLC at 214 nm. The sequences of the peptides was submitted as International Patent (published in October 2009 by the European Patent Office, 2108 372 A1).

Fourier-transform infrared spectroscopy

The infrared spectroscopic measurements were performed on an IFS-55 spectrometer (Bruker). Samples, dissolved in 20 mM Hepes buffer, pH 7.0, were placed in a CaF_2 cuvette with a 12.5 µm teflon spacer. Temperature-scans were

performed automatically between 10 and 70°C with a heating-rate of 0.6 °C/min. Every 3°C, 50 interferograms were accumulated, apodized, Fourier transformed, and converted to absorbance spectra.

Differential Scanning Calorimetry (DSC)

DSC measurements were performed with a VP-DSC calorimeter (MicroCal, Inc., Northhampton, MA, USA) at a heating and cooling rate of 1 °C·min^{-1}. The DSC samples were prepared by dispersing a known ratio of LPS to peptide in 10 mM PBS buffer at pH 7.4. The samples were hydrated in the liquid crystalline phase by vortexing. The measurements were performed in the temperature interval from 5 °C to 95 °C. Five consecutive heating and cooling scans checked the reproducibility of the DSC experiments of each sample (13). The accuracy of the DSC experiments was $\pm$ 0.1 °C for the main phase transition temperatures and $\pm$ 1 kJ·mol^{-1} for the main phase transition enthalpy. The DSC data were analysed using the Origin software. The phase transition enthalpy was obtained by integrating the area under the heat capacity curve.

Fluorescence resonance energy transfer spectroscopy (FRET)

The ability of the peptides to intercalate into phospholipid liposomes or into LPS R60 aggregates was investigated as described earlier (14). Briefly, phospholipid liposomes from phosphatidylserine (PS), phosphatidylcholine (PC), or LPS R60 were doubly labelled with the fluorescent phospholipid dyes N-(7-nitrobenz-2-oxa-1,3-diazol-4yl)-phosphatidyl ethanolamine (NBD-PE) and N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (Rh-PE) (Molecular Probes). Intercalation of unlabeled molecules into the doubly labeled liposomes leads to probe dilution and with that to a lower FRET efficiency: the emission intensity of the donor I_D increases and that of the acceptor I_A decreases (for clarity, only the quotient of the donor and acceptor emission intensity is shown here).

In all experiments, the peptides (100 μl of 100 μM) were added to doubly labelled PS phospholipid liposomes or LPS R60 (900 μl of 10 μM) at 50 s after equilibration. NBD-PE was excited at 470 nm and the donor and acceptor fluorescence intensities were monitored at 531 and 593 nm, respectively, and the fluorescence signal I_D/I_A was recorded for further 250 s.

Alamar blue Assay

To specify the vitality of eukaryotic cells and detect toxic effects of chemicals and other substances on the cell membrane, the Alamar blue (Invitrogen) agent was used. It is also known as resazurin (7-Hydroxy-3-H-phenoxazin-3-one-10-oxide), a blue dye used as an oxidation-reduction indicator for cell viability. First, the cells were treated for a period of time with different concentrations of test substances. Subsequently the blue colored Resazurin was added. In the presence of living cells, the Resazurin is reduced to the pink fluorescent compound resorufin. Via photometric measuring (OD at 620 nm), the metabolic activity was monitored in a microtiter plate reader. Each independent experiment was performed in triplicate.

Hemolysis Assay

The activity of the peptides to lyse freshly isolated human erythrocytes was determined in PBS, pH 7.4 at 37 °C. Dilutions were prepared in duplicate in a round bottom microtiterplate. For that, 20 µl erythrocytes (5 x 10^8 cells/ml) was incubated with 80 µl of a peptide sample at 37 °C for 30 min in a humidified box. The hemolytic activity after incubation was measured by transferring the supernatents into another empty microtiter plate. This plate was read on a microtiter plate reader at 405 nm. Hemolytic activity was expressed as percentage of released hemoglobin with respect to water controls (100 % release) or controls processed without peptides (0 % release).

Results

Gel to liquid crystalline phase transition of the acyl chains

It was shown that effective antibacterial peptides such as polymyxin B cause a strong fluidization of the acyl chains of LPS (15).

The phase transition behavior of the acyl chains of the lipid A part of LPS R60 was monitored by applying FTIR and DSC (Figs. S1,S2). In Fig. S1, the peak position of the symmetric stretching vibrational band of the methylene groups of the hydrocarbon chains is plotted versus temperature for LPS R60 at different concentrations of Pep19-2.5 (A) and 19-8 (B). As can be seen, for LPS a phase transition from the ordered gel phase into an unordered liquid crystalline phase is found at T_c= 35 °C, and the addition of the peptides to LPS leads to an increase in the wavenumber

values in particular in the gel phase corresponding to a fluidization of the acyl chains. Interestingly, this is much stronger for the biologically less active Pep19-8. In the DSC experiment (file Fig. S2A), however, for both peptides the transition disappears completely at sufficiently high peptide concentrations which can be deduced in particular from the vanishing of the phase transition enthalpy (exemplarily shown in Fig. S2B for Pep19-2.5).

Peptide incorporation into phospholipid and LPS membranes

The ability of the peptides Pep19-2.5, Pep19-2.5KO, and Pep19-8 to intercalate into phospholipid membranes from phosphatidylcholine (PC), phosphatidylserine (PS), and into LPS was determined by applying fluorescence resonance energy spectroscopy (FRET) (see Figs. S3A-C). The results for the incorporation into PC shows nearly equal capacity for all three peptides (A), whereas a succession from Pep19-2.5KO to Pep19-2.5, and Pep19-8 is observed for PS (B). However, the FRET signal of the latter is much higher than that for PC. For LPS, all three peptides intercalate nearly with the same amplitude (C). Thus, these data show a clear membrane activity of the peptides with a predominance for negatively charged (PS, LPS) compounds.

The incorporation of the peptides was also studied with a mimetic of a human immune cell membrane , DOPC, sphingomyelin, and cholesterol in a molar ratio of 9:9:2. The data were very similar as found for the pure PC membrane (Fig. S3A) except the fact that the measuring curves were much more noisy.

Infrared fingerprint spectral region

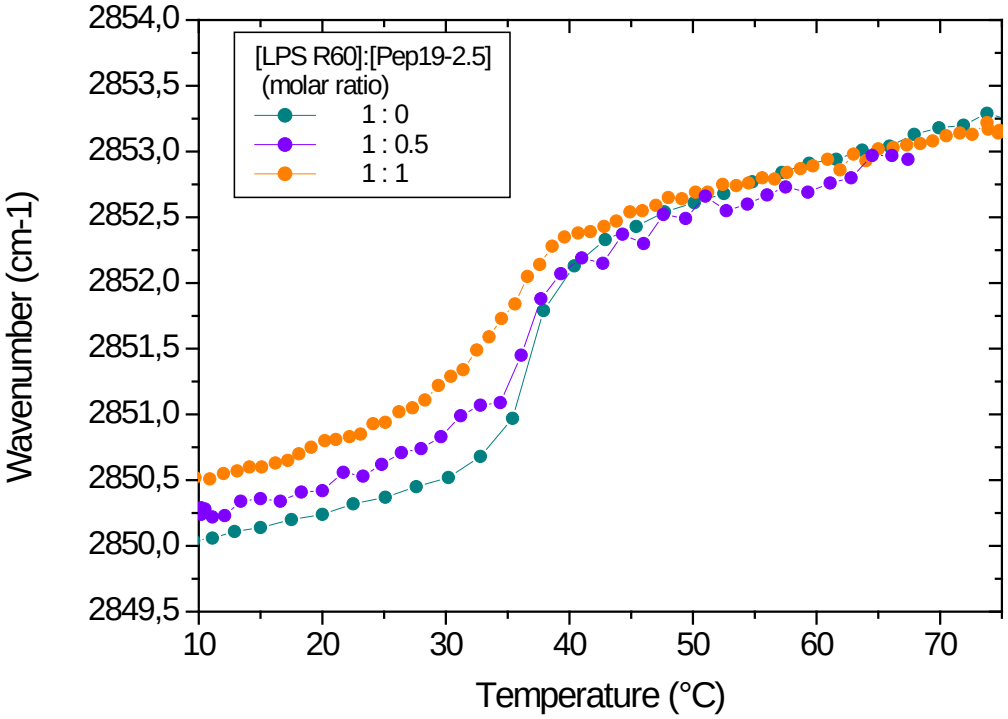
To determine the influence of the Pep19-2.5 on the phosphate groups of LPS, infrared spectra of the fingerprint spectral region 1320 to 1180 cm^{-1} of LPS were evaluated. The spectra in the absence of the peptide show the appearance of two band components at 1255-1260 and 1220 cm^{-1} corresponding to the weakly hydrated 4'-phosphate and highly hydrated 1-phosphate, respectively (16). In the presence of the peptide both bands are changed, due to the binding of the peptide (not shown). In particular, there is a strong decrease of the band intensity of the band at 1260 cm^{-1} which indicates that this phosphate group is immobilized via e.g. the formation of a hydrogen bond interaction with the peptide.

Cytotoxicity

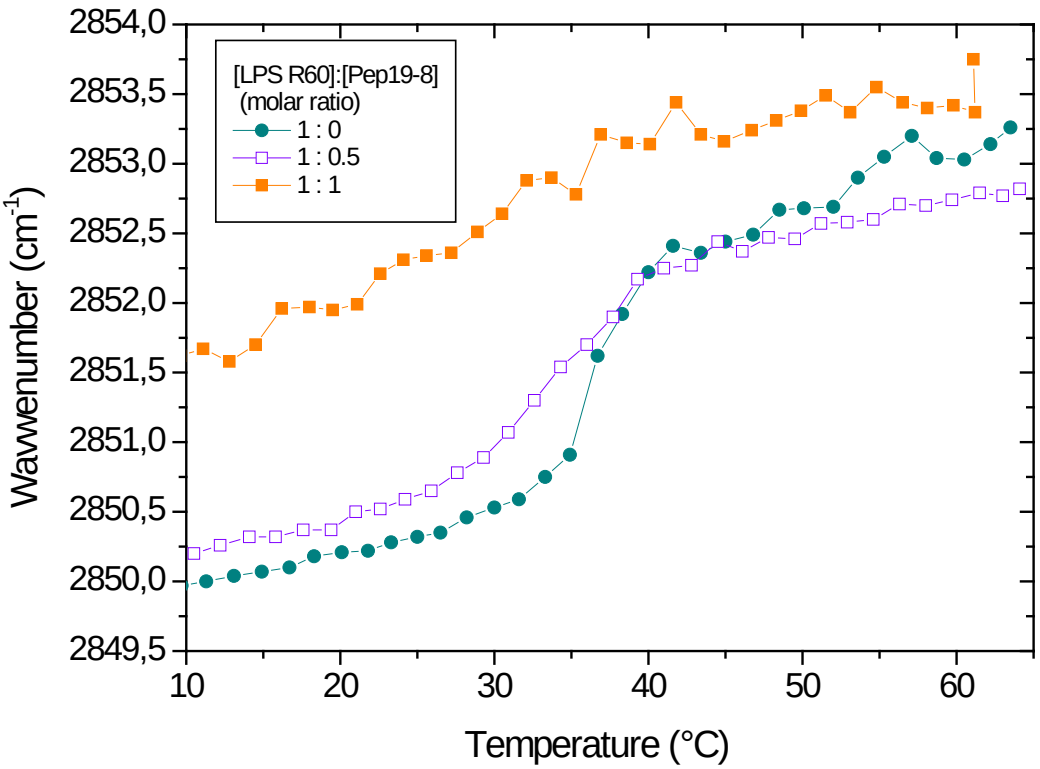
For the use of peptide as antiseptic agents, cytotoxic effects have to be carefully excluded. For this, two different test systems were investigated, human macrophages and red blood cells (RBC). Furthermore, beside the peptides described above further peptides with some modifications in the amino acid sequence were also monitored.
The results of these experiments are shown in Fig.S4A for macrophages using the Alamar blue test, and for red blood cells in Fig. S4B by using the efflux of haemoglobin. The measurements indicate for the macrophages that some cytotoxic effects start in the concentration range above 30 μg/ml, except for peptides Pep19-4 and Pep19-2.5OH (Fig. S4B), and of course the control peptide melittin. For RBC, the peptide induce a slight increase in lysis up to approximately 50 μg/ml, and then a stronger increase.

Figures and legends:

FigureS1: Gel to liquid crystalline phase transition behaviour of LPS R60 by Fourier-transform infrared spectroscopy.
The peak position of the symmetric stretching vibrational band of the methylene groups is plotted versus temperature for LPS R60 in the presence of Pep19-2.5 (A) and Pep19-8 (B).


[LPS R60]:[Pep19-2.5]
(molar ratio)
1 : 0
1 : 0.5
1 : 1
Wavenumber (cm-1)
2854,0
2853,5
2853,0
2852,5
2852,0
2851,5
2851,0
2850,5
2850,0
2849,5
10
20
30
40
50
60
70
Temperature (°C)

FigS1A


[LPS R60]:[Pep19-8]
(molar ratio)
1 : 0
1 : 0.5
1 : 1
Wavenumber (cm-1)
Temperature (°C)
2854,0
2853,5
2853,0
2852,5
2852,0
2851,5
2851,0
2850,5
2850,0
2849,5
10
20
30
40
50
60

FigS1B

FigureS2: Gel to liquid crystalline phase transition behaviour of LPS R60 by differential scanning calorimetry.

(A) DSC heating-scan of LPS:Pep19-2.5 mixtures at various concentration ratios.

(B) Phase transition enthalpies and temperatures in dependence on time.

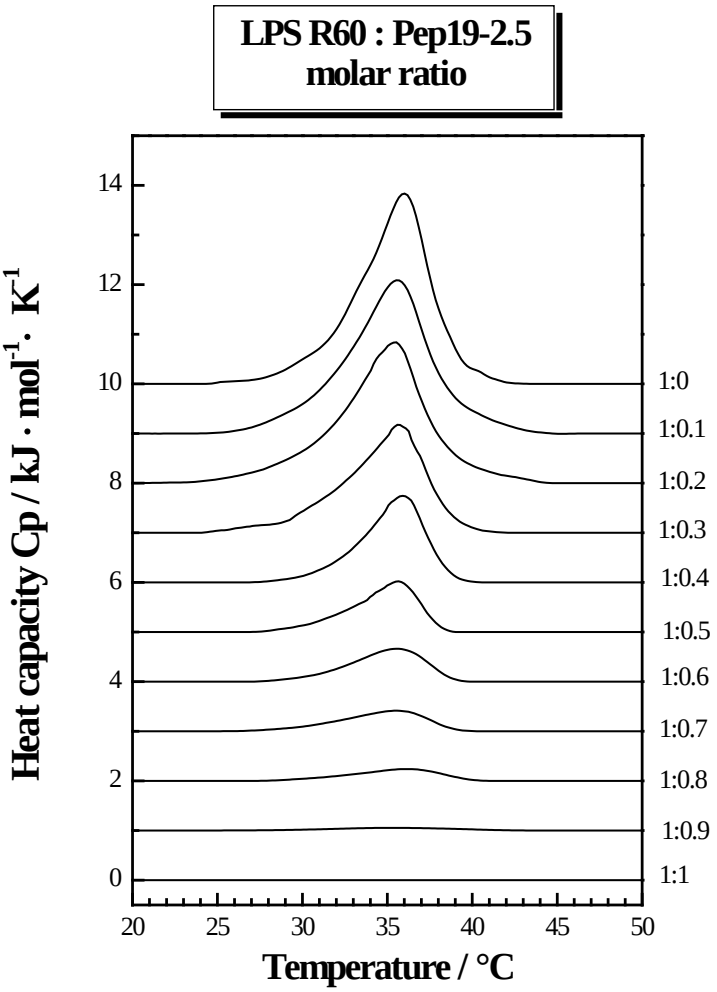


Fig. S2A

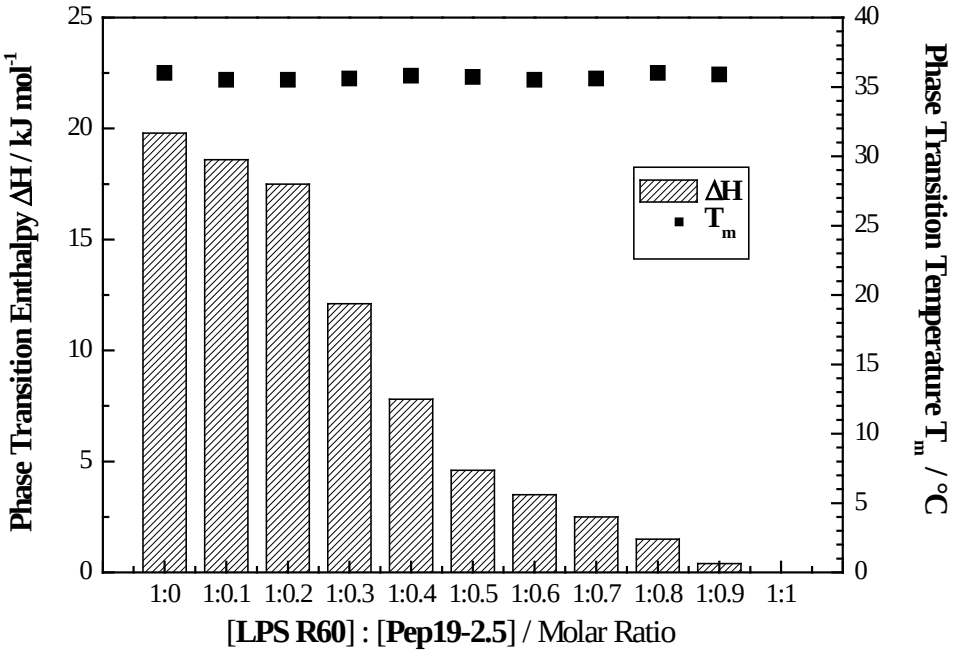


Fig. S2B

Peptide incorporation into phospholipid and LPS membranes

FigureS3: Intercalation of peptides into different lipid systems.

Incorporation of the peptides Pep19-2.5, Pep19-2.5KO, and Pep19-8 into liposomes made from phosphatidylcholine (A), phosphatidylserine (B), and into LPS R60 aggregates (C). The FRET signal (ratio of the donor NBD-PE to the acceptor RhoPE intensity) is plotted versus time.

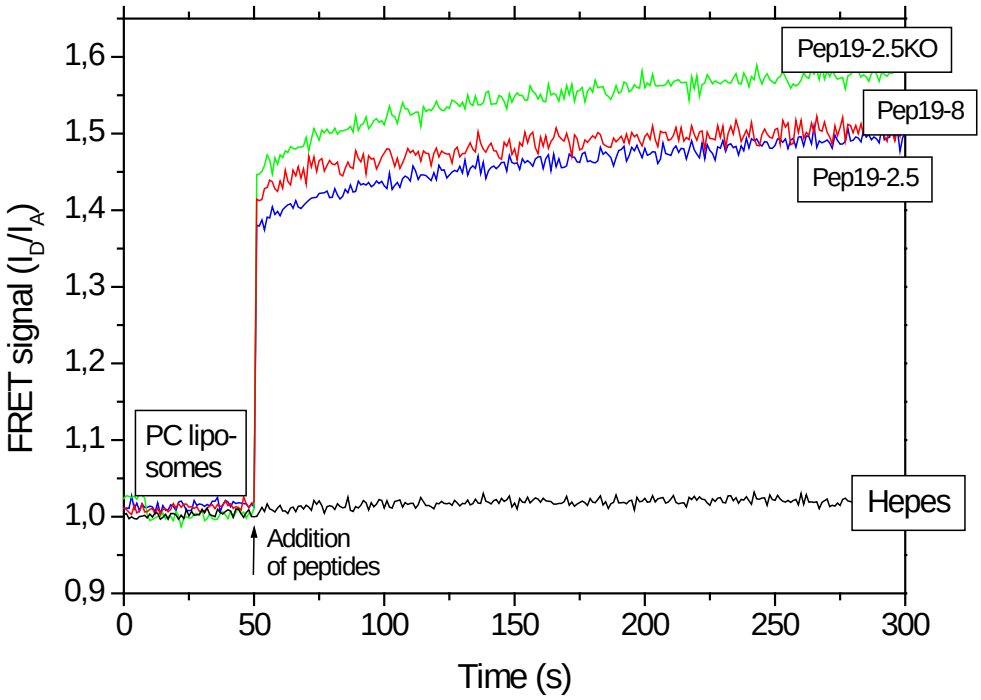


Fig. S3A

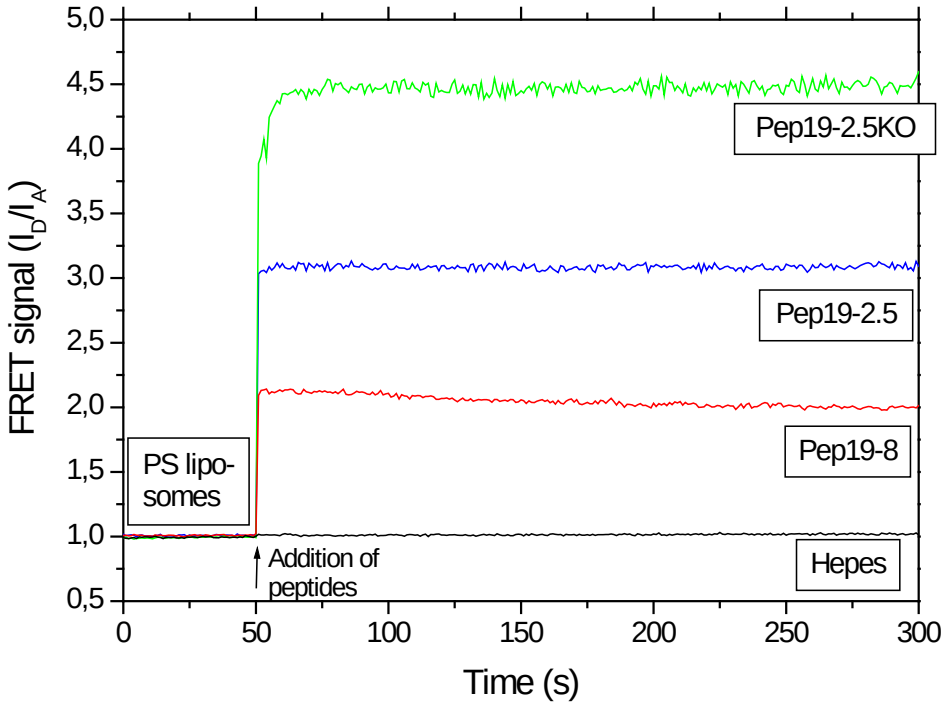


Fig. S3B

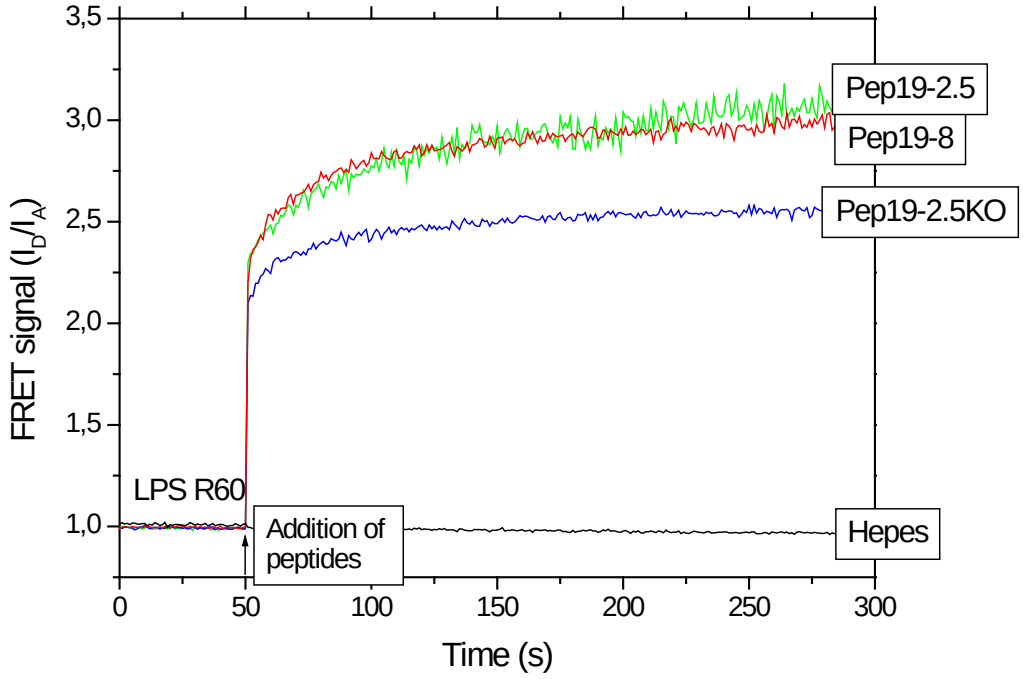


Fig. S3C

Figure S4A: Characterization of peptide-associated cytotoxicity.

(A) Metabolic activity of human macrophages at different concentrations of peptide Pep19-2.5, Pep19-2.5KO, Pep19-8, and the reference compounds polymyxin B (PMB) and bee venom melittin. (B) Red blood cell haemolysis of the same selected peptides as compared to PMB and melittin.

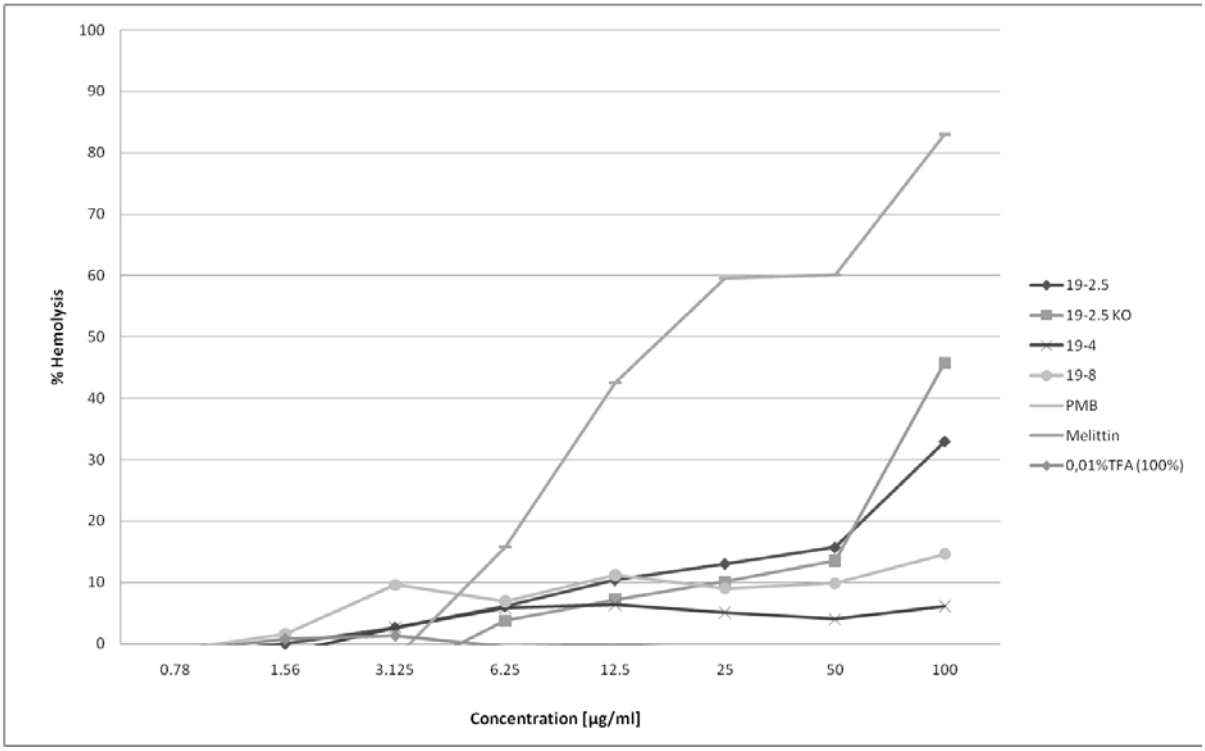

100
90
80
70
60
50
40
30
20
10
0
% Hemolysis
0.78
1.56
3.125
6.25
12.5
25
50
100
Concentration [µg/ml]
19-2.5
19-2.5 KO
19-4
19-8
PMB
Melittin
0,01%TFA (100%)

Fig. S4A

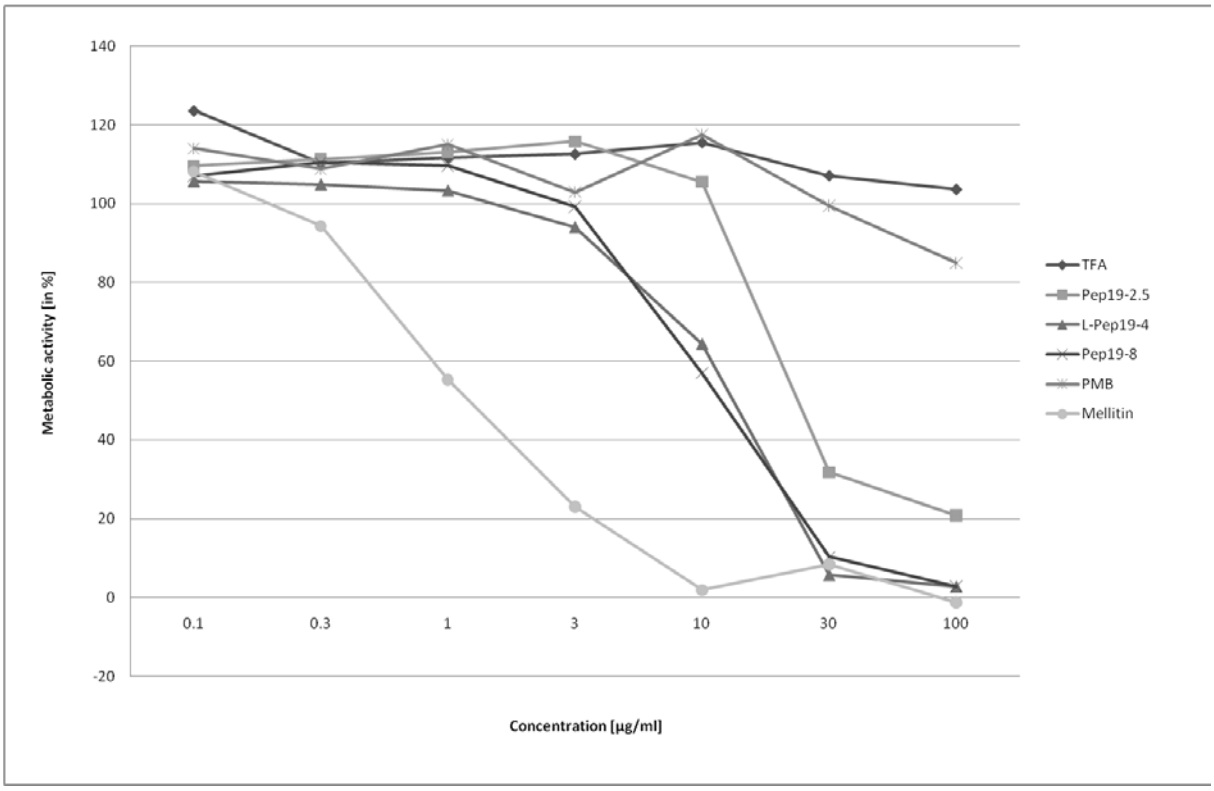

140
120
100
80
60
40
20
0
-20
Metabolic activity [in %]
0.1
0.3
1
3
10
30
100
Concentration [µg/ml]
TFA
Pep19-2.5
L-Pep19-4
Pep19-8
PMB
Mellitin

Fig.S4B