Original Paper

Lipopeptide-Induced Suicidal Erythrocyte Death Correlates with the Degree of Acylation

Abdulla Al Mamun Bhuyan^a Minh-Thu Nguyen^b Rosi Bissinger^a Florian Lang^{a,c}
Friedrich Götz^b

Departments of ^aCardiology, Vascular Medicine and Physiology and ^bMicrobial Genetics, Eberhard-Karls-University of Tuebingen, Tuebingen, ^cDepartment of Molecular Medicine II, Heinrich Heine University Duesseldorf, Germany

Key Words

Lipopeptide • Phosphatidylserine • Eryptosis • Apoptosis • Pam

Abstract

Background/Aims: Consequences of bacterial infection include anemia, which could result from stimulation of suicidal erythrocyte death or eryptosis, characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Bacterial components known to stimulate eryptosis include lipopeptides. Signaling mediating the triggering of eryptosis include increased cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress and cellular accumulation of ceramide. The present study aimed to define the molecular requirements for lipopeptide-induced cell membrane scrambling. **Methods:** Human erythrocytes were incubated for 48 hours in the absence and presence of 1 or 5 $\mu\text{g/ml}$ of the synthetic lipopeptides Pam1 (lipopeptide with one fatty acid), Pam2 (lipopeptide with two fatty acids), or Pam3 (lipopeptide with three fatty acids). In the following phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, ROS formation from DCF dependent fluorescence, and ceramide abundance utilizing specific antibodies. **Results:** Pam1 (5 $\mu\text{g/ml}$), Pam2 (5 $\mu\text{g/ml}$) and Pam3 (1 and 5 $\mu\text{g/ml}$) significantly increased the percentage of annexin-V-binding to erythrocytes in a dose dependent manner, which was largely independent of Ca^{2+} . Pam1-3 increased the percentage of both, swollen and shrunken erythrocytes without significantly modifying the average forward scatter. They also increased reactive oxygen species (ROS) and ceramide abundance. In all assays the effect on eryptosis increased with increasing number of fatty acids, with Pam3 showing always the strongest effect. In contrast, a comparison of the effect of Pam1-3 on TLR2 dependent immune stimulation showed that not Pam3 but Pam2 displayed the strongest activity, and that immune stimulation was triggered at much lower concentrations than eryptosis. **Conclusions:** Lipopeptides are not only important activators of the immune system; at higher concentrations they also drive host cells into apoptosis thus aggravating a bacterial infection.

© 2017 The Author(s)
Published by S. Karger AG, Basel

A. Al Mamun Bhuyan and M.T. Nguyen contributed equally and thus share first authorship

Friedrich Götz
and Florian Lang

Microbial Genetics, Interfaculty Institute of Microbiology and Infection Medicine (IMIT), University of Tuebingen, Auf der Morgenstelle 28, 72076 Tuebingen, and Department of Internal Medicine III, University of Tuebingen, Gmelinstr. 5, 72076 Tuebingen (Germany)
E-Mail friedrich.goetz@uni-tuebingen.de / florian.lang@uni-tuebingen.de

Introduction

The lipopeptides (Lpp) used in this study are structural components found in almost all bacterial membranes. They represent the membrane anchor group of the lipoproteins that play a crucial role in transport processes as reviewed recently [1, 2]. As they are so characteristic for bacteria it does not astonish that the mammalian immune system responds to the Lpp moiety of bacterial lipoproteins by inducing an innate immune response via the TLR2 receptor [3]. Lpp are not only potent stimulators of the innate immune system [1, 4], they also have strong adjuvant activity [5, 6]. Their TLR2 activation leads via a cascade of intermediary steps to NF-kappaB activation [7]. Dependent on the degree of acylation, Lpp are recognized by different TLR2 heterodimers. Diacylated Lpp are recognized by TLR2 and TLR6 [8, 9], while triacylated Lpp are recognized by TLR2 and TLR1 heterodimers [3, 10]. The signal transduction from Lpp-bound TLR2 to the activation of the nuclear factor NF-kappaB involves a cascade of phosphorylation events [11]. Besides triggering the innate immune response they also induce apoptosis in THP-1 monocytic cells through TLR2 and also initiate apoptosis in an epithelial cell line transfected with hTLR2 [4].

Because of this activity it would be interesting to investigate the effect of Lpp on other host cells such as the enucleated mammalian erythrocytes. They represent the most abundant cells in the bloodstream and transport oxygen to and CO₂ from peripheral tissues. Any injury of erythrocytes affecting their quantity, shape, size, structure or life cycle can affect the oxygen-carrying capacity of the blood. Bacterial sepsis for example may cause anemia, which partially results from eryptosis, the suicidal death of erythrocytes characterized by cell membrane scrambling with phosphatidylserine (PS) exposure at the erythrocyte surface [12]. Signaling of eryptosis includes increase of cytosolic Ca²⁺ activity ([Ca²⁺]_i) [13], ceramide [14], oxidative stress [13], energy depletion [13], heterotrimeric G-protein subunit Gαi2 [15], caspases [13, 16, 17], casein kinase 1α [13], Janus-activated kinase JAK3 [13], protein kinase C [13], and p38 kinase [13]. Eryptosis is inhibited by AMP activated kinase AMPK [13], cGMP-dependent protein kinase [13], PAK2 kinase [13], mitogen- and stress-activated kinase MSK1/2 [18], as well as sorafenib/sunitinib sensitive kinases [13]. Enhanced eryptosis contributes to anemia in a variety of clinical conditions [15, 19-27] and is enhanced following blood storage for transfusion [28]. Eryptosis is triggered by many xenobiotics [13, 16, 20, 21, 25, 29-71] including bacterial Lpp [72]. However, the structural requirements for bacterial Lpp-induced eryptosis are ill-defined.

The present study explored, whether differentially acylated Lpp, Pam1-3, stimulates eryptosis and to which extent. We tested human erythrocytes from healthy volunteers and exposed them to Pam1-3 and determined PS surface abundance, cell volume, [Ca²⁺]_i, ROS formation, and ceramide abundance. Interestingly, the extent of eryptosis increased with increasing number of fatty acids bound to the lipid moiety of the Lpp, while for immune stimulation Pam2, the diacylated Lpp, showed the strongest effect and was triggered at much lower concentrations than eryptosis.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Li-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003 V). The blood was centrifuged at 120 g for 20 min at 21 °C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 CaCl₂, at 37°C for 48 hours. Where indicated, erythrocytes were exposed for 48 hours to the synthetic Lpp Pam1, Pam2 and Pam3 (for their structure see Fig. 1). In order to estimate the impact of Ca²⁺ entry, erythrocytes were exposed in the presence and nominal absence (0 CaCl₂, 1 mM EGTA) of Ca²⁺.

Fig. 1. Chemical structure of Pam1, Pam2 and Pam3.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 μ l cell suspension was washed in Ringer solution containing 5 mM CaCl_2 and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 15 min under protection from light. The annexin-V-abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin-V-binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin-V-binding cells and control cells. The same threshold was used for untreated and Pam1-3 treated erythrocytes. Light scattering is an empirical technique employed to measure rapid changes in cell volume [73]. Here we used a dot plot of forward scatter (FSC) vs. side scatter (SSC) that was set to linear scale for both parameters. The threshold of forward scatter was set at the default value of “52”.

Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl_2 and 5 μ M Fluo-3/AM. The cells were incubated at 37°C for 30 min. Ca^{2+} -dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein (DCF) diacetate. After incubation, a 150 μ l suspension of erythrocytes was washed in Ringer solution and stained with DCF (Sigma, Schnelldorf, Germany) in Ringer solution containing DCF at a final concentration of 10 μ M. Erythrocytes were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. The DCF-loaded erythrocytes were resuspended in 200 μ l Ringer solution and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Ceramide abundance

For the determination of ceramide abundance at the erythrocyte surface, a monoclonal antibody-based assay was used. To this end, cells were stained for 1 hour at 37°C with 1 μ g/ml anti ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. The samples were washed twice with PBS-BSA. The cells were stained for 30 minutes with polyclonal fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. The samples were analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As a control, secondary antibody alone was used.

HEK-TLR2 signaling assay

Human Embryonic Kidney (HEK 293) cells, stably transfected with the human TLR2 gene, were purchased from Invivogen. The cultivation was performed following the previous study [74]. For the signaling stimulation assay, HEK-TLR2 cells were seeded 5×10^4 cells/ 200 μ l well into 96 well cell culture

plates and incubated at 37°C with 5% CO₂. After one-day incubation, the HEK-TLR2 cells were used for the stimulation test. 100 ng of different synthetic Lpp including Pam1C, Pam2C and Pam3C were dissolved in 10 µl of H₂O and transferred to the 200 µl medium containing approximately 1x 10⁵ HEK-TLR2 cells. The negative controls (unsti.) were used with 10 µl of H₂O. Stimulation was carried out for 18h at 37°C with 5% CO₂. The supernatants were collected and stored at -20 °C. Human IL-8 secretion was measured in cellular supernatants using the BD OptEIA ELISA kits according to the manufacturer's instructions.

Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and *t* test as appropriate. N denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

In the present study we investigated the stimulating effect of the differently acylated Lpp (Pam1, Pam2 and Pam3) on eryptosis, which is the suicidal erythrocyte death characterized by cell shrinkage and phospholipid scrambling of the cell membrane triggered by PS translocation to the cell surface. In the following we analyzed various parameters known to be typical for eryptosis.

Lpp induced translocation of PS to the cell surface

Surface exposed PS was identified by annexin-V-binding and subsequent flow cytometry. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with Pam1-3 (1 – 5 µg/ml). All three Lpp triggered annexin binding, which was consecutively increased with increasing acylation of the Lpp (Fig. 2). Pam3 even revealed a sixfold higher annexin binding than Pam1 and the binding with Pam2 was in between. The increase in annexin binding was also concentration dependent; 5 µg/ml triggered an almost fivefold stronger binding than 1 µg/ml.

Lpp modulated cell volume

Erythrocyte volume was estimated by forward scatter and flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or

Fig. 2. Effect of Pam1-3 on phosphatidylserine exposure. (A) Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas, control) and in the presence of 5 µg/ml Pam1 (light grey line), Pam2 (deep grey line), or Pam3 (black line). (B) Arithmetic means ± SEM (n = 14) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with 1 and 5 µg/ml Pam1-3 (black bars). * (p<0.05), *** (p<0.001) indicate significant difference from the absence of Pam's.

with Pam1-3 (1 and 5 $\mu\text{g/ml}$). Pam1-3 in a concentration of 1 $\mu\text{g/ml}$ did not appreciably alter cell volume (Fig. 3). However, at a concentration of 5 $\mu\text{g/ml}$ there was a clear cell shrinkage observed with Pam1, with Pam2 the control volume was reached, and with Pam3 a clear increase beyond the control volume was observed (Fig. 3). We assume that the observed cell shrinkage with 5 $\mu\text{g/ml}$ of Pam1 was due to an increase of cytosolic Ca^{2+} concentration which further activates the Gardos channel, resulting in hyperpolarization of the cell membrane resulting in loss of K^+ , Cl^- ions and H_2O from the cell [75]. Pam3 increased cell volume [76].

Lpp increased intracellular Ca^{2+} concentration.

Cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) was determined by Fluo3 fluorescence. As shown in Fig. 4, a 48 hours exposure to Pam1-3 increased the Fluo3 fluorescence, an effect reaching statistical significance at 5 $\mu\text{g/ml}$ Pam2 and at 1 and 5 $\mu\text{g/ml}$ Pam3; Pam1 showed no effect.

Lpp induced translocation of PS to the cell surface in the absence of Ca^{2+}

A next series of experiments explored whether the Pam1-3-induced translocation of PS required the entry of extracellular Ca^{2+} . Therefore, erythrocytes were incubated for 48

Fig. 3. Effect of Pam1-3 on erythrocyte forward scatter. (A) Original histograms of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas, control) and in the presence of 5 $\mu\text{g/ml}$ Pam1 (light grey line), Pam2 (dark grey line), or Pam3 (black line). (B) Arithmetic means $\pm$ SEM ($n = 14$) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with 1 or 5 $\mu\text{g/ml}$ Pam1-3 (black bars). * ($p < 0.05$), *** ($p < 0.001$) indicate significant difference from the absence of Pam's.

Fig. 4. Effect of Pam1-3 on Fluo3 fluorescence. (A) Original histograms of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and in the presence of 5 $\mu\text{g/ml}$ Pam1, Pam2, or Pam3 (black lines). (B) Arithmetic means $\pm$ SEM ($n = 19$) of Fluo3 fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with 1 and 5 $\mu\text{g/ml}$ Pam1-3 (black bars). * ($p < 0.05$), *** ($p < 0.001$) indicate significant difference from the absence of Pam's.

hours in the absence or presence of 5 $\mu\text{g/ml}$ Pam1-3 in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 5, removal of extracellular Ca^{2+} significantly blunted the effect of Pam1, of Pam2 and of Pam3 on annexin-V-binding. However, even in the absence of extracellular Ca^{2+} , Pam1, Pam2 and Pam3 significantly increased annexin-V-binding of erythrocytes. Thus, the annexin-V-binding following exposure to Pam1, Pam2 and Pam3 was in part but not fully dependent on entry of extracellular Ca^{2+} (Fig. 5). The presence of Ca^{2+} led to an increase in annexin V binding following administration of all three Lpp. Compared to Pam1, Pam2 caused roughly 2-times higher and Pam3 even 6-times higher annexin V binding. A similar correlation, but less pronounced was observed in the absence of Ca^{2+} , which was strange as it is known that Ca^{++} is one of the most important activators of the TMEM16F scramblase [77].

Lpp induced oxidative stress

Indeed, eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) were quantified utilizing 2',7'-dichlorodihydrofluorescein (DCF) diacetate. DCF itself is a fluorogenic substance and when it crosses the cell membrane, the intracellular esterases deacetylate DCF to an impermeable, non fluorescent product. But later the reactive oxygen species convert this DCF into 2',7'-dichlorofluorescein (DCF) by oxidation which is a highly fluorescent compound that can be detected easily by flow cytometry. As shown in Fig. 6, a 48 hours exposure to 5 $\mu\text{g/ml}$ Pam2 and Pam3, but not Pam1, increased significantly the DCF dependent fluorescence, with Pam3 exerting the strongest effect.

Fig. 5. Ca^{2+} sensitivity of Pam1-3 induced phosphatidylserine exposure. (A-C) Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution in the presence (left panels) and absence (right panels) of extracellular Ca^{2+} . Black line indicates the presence of each 5 $\mu\text{g/ml}$ of Pam1 (A), Pam2 (B) or Pam3 (C). (D) Arithmetic means $\pm$ SEM ($n = 4$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with 5 $\mu\text{g/ml}$ Pam1-3 (black bars) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . **($p < 0.01$), ***($p < 0.001$) indicates significant difference from the absence of PAM1, ###($p < 0.001$) indicate significant difference from presence of Ca^{2+} (ANOVA).

Lpp increased ceramide abundance

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As shown in Fig. 7, a 48 hours exposure to 5 $\mu\text{g}/\text{ml}$ Pam2 and Pam3, but not Pam1, significantly increased the ceramide abundance. The effect of Pam3 was even 5-times stronger than that of Pam2.

Lpp Pam2 exerted the strongest immune stimulation

Finally, we investigated the TLR2 dependent signaling of Pam1-3 in Human Embryonic Kidney (HEK 293) cells, stably transfected with the human TLR2 gene. IL-8 production was determined after stimulation of the cells with 0.5 $\mu\text{g}/\text{ml}$ of Pam1, Pam2 or Pam3 for 18 h. While Pam1 showed no signaling activity at all, Pam2 showed the highest activity which was even twofold higher than that of Pam3 (Fig. 8).

Fig. 6. Effect of Pam1-3 on DCF fluorescence. (A) Original histograms of DCF fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and in the presence of 5 $\mu\text{g}/\text{ml}$ Pam1, Pam2, or Pam3 (black lines). (B) Arithmetic means $\pm$ SEM (n = 14) of DCF fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with 5 $\mu\text{g}/\text{ml}$ Pam1-3 (black bars). *(p<0.05), *** (p<0.001) indicate significant difference from the absence of Pam's.

Fig. 7. Effect of Pam1-3 on ceramide abundance. (A) Original histograms of ceramide abundance at the surface of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and in the presence of 5 $\mu\text{g}/\text{ml}$ Pam1 (light grey line), Pam2 (deep grey line), and Pam3 (black line). (B) Arithmetic means $\pm$ SEM (n = 8) of ceramide abundance at the surface of erythrocytes following incubation for 48 hours to Ringer solution without (control) or in the presence of 5 $\mu\text{g}/\text{ml}$ Pam1, Pam2, and Pam3. *** (p<0.001) indicate significant difference from the absence of Pam's.

Fig. 8. Induction of IL8 production with HEK-TLR2 cells triggered with Pam1-3. HEK-TLR2 cells were stimulated for 18 h with each 0.5 µg/ml of either Pam1, Pam2 or Pam3. The human IL8 cytokine was used as readout for immune stimulation and was measured by Elisa kit. The un-stimulated sample (unsti.) was used as negative control by the application of 10 µl of H₂O. The experiments were independently carried out three times. Statistical significances were calculated by using Student's t-tests of different samples versus the Pam1: not significant $P > 0.05$, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

Discussion

Bacterial lipopeptides (Lpp) represent not only a major class of membrane-anchored surface proteins in Gram-positive bacteria, they also play a crucial role in innate immune stimulation via TLR2 activation [1]. Until recently it was not clear whether low-GC Gram-positive bacteria produce di- or triacylated Lpp. However, Kurokawa and colleagues showed that in *S. aureus* the 33 kDa SitC, a component of the proposed iron ABC transporter SitABC, is triacylated and induces IL-6 and TNF- α release in human monocytes and NF- κ B-activation in TLR2-transfected HEK293 cells [78]. However, recently it has been shown that some other bacterial species produce only diacylated Lpp showing a much stronger immune stimulation and inflammation response than triacylated Lpp [79]. The question we raised here was whether diacylated Lpp have also a stronger impact on mammalian cell damage than triacylated ones. To facilitate the task we have chosen synthetic Lpp that were mono-, di- and triacylated, each with palmitate as fatty acid (Fig. 1). As model cells we have chosen human erythrocytes from volunteers. Like in other mammalian cells in the normal erythrocyte membrane PS are located exclusively in the inner leaflet [80]. The loss of membrane asymmetry and the exposure of PS affects the hemostatic balance in a number of ways [81]: PS-presenting erythrocytes adhere to endothelial cell monolayers [82], they are prone to eryptosis with the consequence that they are removed by macrophages like many other senescent or virus- and bacteria-infected cells [83, 84]; surface-exposed PS represents an 'eat-me' signal [85].

Exposure of Pam1, Pam2 and Pam3 to erythrocytes induced all the classical features of eryptosis such as increased exposure of PS on the surface, which was largely Ca^{2+} independent, an increased cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), increased oxidative stress, and an increased ceramide abundance. The important result of this study was that the degree of all the eryptosis signs correlated with the degree of the acylation of the Lpp; Pam3 always exerted the strongest effect.

The question remains how Pam1-3 mediate eryptosis. Do they activate the Ca-dependent phospholipid scramblase (TMEM16F), or distort the erythrocyte membrane in such a way that the scramblases get activated even in the absence of Ca^{2+} ? As the annexin V binding increased with increasing degree of acylation of the Lpp we assume that the lipophilicity plays a crucial role in scramblase activation even in the absence of Ca^{2+} . As in Pam3 the lipophilicity of the Lpp is highest we speculated that Pam3 might act like a detergent that disintegrates the erythrocyte membrane.

We also wondered why there was a relatively high annexin binding in the absence of Ca^{2+} . An explanation could be that Pam1-3-induced lipid peroxidation was due to oxidative stress [86]. Reduced glutathione may be oxidized and thus be unable to protect the cell from oxidative stress [87]. Indeed, we found that Lpp induced oxidative stress and ceramide production, which might explain why they are able to activate the scramblase in the absence of Ca^{2+} .

The observation that increasing degree of Lpp-acylation correlated with increasing eryptosis indicates that increasing lipophilicity causes more damage to the plasma membrane. Cyclic lipopeptides constitute a structurally diverse group of metabolites produced by various bacterial and fungal genera via non-ribosomal pathways [88]. Structurally they are amphiphilic molecules that comprise both hydrophobic and hydrophilic moieties; in this respect they resemble our Lpp. However, they are built up as cyclic peptide that carries one alkyl chain. Most of them attack cell membranes and are used as antimicrobials, antitumor drugs or surfactants. One of the most aggressive cyclic lipopeptides is surfactin. It has a high detergent effect by inserting its fatty acid chain into the lipid bilayer [89, 90]. Its cell destruction is indiscriminate, and it attacks also erythrocytes with deadly efficiency. Pseudofactins inhibit bacterial adhesion (the first stage of biofilm formation) but have also antiproliferative effects on mammalian cells [88]. Polymyxin B has antibacterial activity against Gram-negative bacteria by binding selectively to lipopolysaccharide (LPS) [91]. Daptomycin shows high antibacterial activity in the presence of Ca^{2+} ; the mode of action is still unknown but it induces permeabilization and depolarization of the bacterial cell membrane [92]. Iturin [93] and fengycin [94] strongly inhibit growth of filamentous fungi; the mode of action of fengycin is dose dependent: at low dose it induces apoptosis and at high dose necrosis [95].

Finally, there is the question whether the anucleated mammalian erythrocytes possess functional immune receptors that are responsive to microbe-associated molecular patterns (MAMPs). The nucleated erythrocytes of fish and chicken express specific PRRs (pathogen recognition receptors) including members of the toll-like receptors (TLRs) and peptidoglycan recognition protein (PGRP) receptors and are also responsive to corresponding stimuli [96, 97]. Therefore, an active role in pathogen recognition has been considered. However, mammalian erythrocytes lack DNA, cannot synthesize RNA, and in addition they also lack organelles such as mitochondria or Golgi apparatus and gain their energy essentially from glycolysis. It is therefore unlikely that they are responsive to immune stimulation. Nevertheless, they represent excellent model cells to study the impact of compounds on eryptosis and membrane damage.

Conclusion

If we compare the various activities of the Lpp (Pam1-3) in immune stimulation and induction of eryptosis one can observe at least two fundamental differences. Immune stimulation occurs already at very low concentrations ($0.5 \mu\text{g}/\text{m}$), a concentration where we see no signs for eryptosis. The highest immune stimulation was observed with Pam2, which is sensed by the TLR2/TLR6 heterodimer [9]. On the other hand the highest activity in eryptosis was mediated by Pam3, the higher the degree of acylation the stronger the eryptosis. Finally, the concentration of Pam1-3 to induce eryptosis is roughly ten times higher than that required for immune stimulation. The results also show that Pam1-3 as representative of bacterial Lpp not only contribute to immune stimulation but damage at higher concentration also the host cells, thus aggravating an infection.

Abbreviations

PS, (phosphatidylserine); Lpp, (lipopeptides(s)); Pam1, ((Pam-Dhc-CSK4) synthetic monopalmitoylated lipopeptide); Pam2, ((Pam₂CSK₄) synthetic dipalmitoylated lipopeptide that mimicks the acylated amino terminus of bacterial lipoproteins); Pam3, ((Pam₃CSK₄) synthetic tripalmitoylated lipopeptide); Ros, (Reactive oxygen species); TLR, (toll-like receptor).

Acknowledgements

The authors acknowledge Tanja Loch in helping to prepare the manuscript. The Deutsche Forschungsgemeinschaft TR-SFB34 supported the study and Open Access Publishing Fund of Tuebingen University.

Disclosure Statement

All authors declare that there are no conflicts of interest.

References

- 1 Nguyen MT, Götz F: Lipoproteins of Gram-Positive Bacteria: Key Players in the Immune Response and Virulence. *Microbiol Mol Biol Rev* 2016;80:891-903.
- 2 Shahmirzadi SV, Nguyen MT, Götz F: Evaluation of *Staphylococcus aureus* Lipoproteins: Role in Nutritional Acquisition and Pathogenicity. *Front Microbiol* 2016;7:1404.
- 3 Takeda K, Takeuchi O, Akira S: Recognition of lipopeptides by Toll-like receptors. *J Endotoxin Res* 2002;8:459-463.
- 4 Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, Klimpel GR, Godowski P, Zychlinsky A: Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science* 1999;285:736-739.
- 5 Deres K, Schild H, Wiesmuller KH, Jung G, Rammensee HG: In vivo priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine. *Nature* 1989;342:561-564.
- 6 Spohn R, Buwitt-Beckmann U, Brock R, Jung G, Ulmer AJ, Wiesmüller KH: Synthetic lipopeptide adjuvants and Toll-like receptor 2--structure-activity relationships. *Vaccine* 2004;22:2494-2499.
- 7 Rawadi G, Garcia J, Lemerrier B, Roman-Roman S: Signal transduction pathways involved in the activation of NF-kappa B, AP-1, and c-fos by *Mycoplasma fermentans* membrane lipoproteins in macrophages. *J Immunol* 1999;162:2193-2203.
- 8 Bulut Y, Faure E, Thomas L, Equils O, Arditi M: Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and *Borrelia burgdorferi* outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. *J Immunol* 2001;167:987-994.
- 9 Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, Takeda K, Akira S: Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int Immunol* 2001;13:933-940.
- 10 Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, Akira S: Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol* 2002;169:10-14.
- 11 Takeda K, Akira S: TLR signaling pathways. *Semin Immunol* 2004;16:3-9.
- 12 Kempe DS, Akel A, Lang PA, Hermle T, Biswas R, Muresanu J, Friedrich B, Dreischer P, Wolz C, Schumacher U, Peschel A, Götz F, Döring G, Wieder T, Gulbins E, Lang F: Suicidal erythrocyte death in sepsis. *J Mol Med (Berl)* 2007;85:273-281.
- 13 Alzoubi K, Calabro S, Bissinger R, Abed M, Faggio C, Lang F: Stimulation of suicidal erythrocyte death by artesunate. *Cell Physiol Biochem* 2014;34:2232-2244.
- 14 Abed M, Towhid ST, Mia S, Pakladok T, Alesutan I, Borst O, Gawaz M, Gulbins E, Lang F: Sphingomyelinase-induced adhesion of eryptotic erythrocytes to endothelial cells. *Am J Physiol Cell Physiol* 2012;303:C991-999.
- 15 Bissinger R, Lang E, Ghashghaeinia M, Singh Y, Zelenak C, Fehrenbacher B, Honisch S, Chen H, Fakhri H, Umbach AT, Liu G, Rexhepaj R, Liu G, Schaller M, Mack AF, Lupescu A, Birnbaumer L, Lang F, Qadri SM: Blunted apoptosis of erythrocytes in mice deficient in the heterotrimeric G-protein subunit Galphai2. *Sci Rep* 2016;6:30925.
- 16 Lau IP, Chen H, Wang J, Ong HC, Leung KC, Ho HP, Kong SK: In vitro effect of CTAB- and PEG-coated gold nanorods on the induction of eryptosis/erythroptosis in human erythrocytes. *Nanotoxicology* 2012;6:847-856.

- 17 Maellaro E, Leoncini S, Moretti D, Del Bello B, Tanganelli I, De Felice C, Ciccoli L: Erythrocyte caspase-3 activation and oxidative imbalance in erythrocytes and in plasma of type 2 diabetic patients. *Acta Diabetol* 2013;50:489-495.
- 18 Lang E, Bissinger R, Fajol A, Salker MS, Singh Y, Zelenak C, Ghashghaieina M, Gu S, Jilani K, Lupescu A, Reyskens KM, Ackermann TF, Foller M, Schleicher E, Sheffield WP, Arthur JS, Lang F, Qadri SM: Accelerated apoptotic death and in vivo turnover of erythrocytes in mice lacking functional mitogen- and stress-activated kinase MSK1/2. *Sci Rep* 2015;5:17316.
- 19 Bissinger R, Kempe-Teufel DS, Honisch S, Qadri SM, Randrianarisoa E, Haring HU, Henes J, Lang F: Stimulated Suicidal Erythrocyte Death in Arteritis. *Cell Physiol Biochem* 2016;39:1068-1077.
- 20 Egler J, Lang F: Licochalcone A Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2015;37:2060-2070.
- 21 Lang E, Pozdeev VI, Gatidis S, Qadri SM, Haussinger D, Kubitz R, Herebian D, Mayatepek E, Lang F, Lang KS, Lang PA: Bile Acid-Induced Suicidal Erythrocyte Death. *Cell Physiol Biochem* 2016;38:1500-1509.
- 22 Lang E, Qadri SM, Lang F: Killing me softly - suicidal erythrocyte death. *Int J Biochem Cell Biol* 2012;44:1236-1243.
- 23 Lupescu A, Bissinger R, Goebel T, Salker MS, Alzoubi K, Liu G, Chirigiu L, Mack AF, Qadri SM, Lang F: Enhanced suicidal erythrocyte death contributing to anemia in the elderly. *Cell Physiol Biochem* 2015;36:773-783.
- 24 Qadri SM, Mahmud H, Lang E, Gu S, Bobbala D, Zelenak C, Jilani K, Siegfried A, Foller M, Lang F: Enhanced suicidal erythrocyte death in mice carrying a loss-of-function mutation of the adenomatous polyposis coli gene. *J Cell Mol Med* 2012;16:1085-1093.
- 25 Ran Q, Xiang Y, Liu Y, Xiang L, Li F, Deng X, Xiao Y, Chen L, Chen L, Li Z: Eryptosis Indices as a Novel Predictive Parameter for Biocompatibility of Fe3O4 Magnetic Nanoparticles on Erythrocytes. *Sci Rep* 2015;5:16209.
- 26 Bissinger R, Schumacher C, Qadri SM, Honisch S, Malik A, Gotz F, Kopp HG, Lang F: Enhanced eryptosis contributes to anemia in lung cancer patients. *Oncotarget* 2016;7:14002-14014.
- 27 Lang E, Gatidis S, Freise NF, Bock H, Kubitz R, Lauer mann C, Orth HM, Klindt C, Schuier M, Keitel V, Reich M, Liu G, Schmidt S, Xu HC, Qadri SM, Herebian D, Pandya AA, Mayatepek E, Gulbins E, Lang F, Haussinger D, Lang KS, Foller M, Lang PA: Conjugated bilirubin triggers anemia by inducing erythrocyte death. *Hepatology* 2015;61:275-284.
- 28 Lang E, Pozdeev VI, Xu HC, Shinde PV, Behnke K, Hamdam JM, Lehnert E, Scharf RE, Lang F, Haussinger D, Lang KS, Lang PA: Storage of Erythrocytes Induces Suicidal Erythrocyte Death. *Cell Physiol Biochem* 2016;39:668-676.
- 29 Bissinger R, Barking S, Alzoubi K, Liu G, Liu G, Lang F: Stimulation of Suicidal Erythrocyte Death by the Antimalarial Drug Mefloquine. *Cell Physiol Biochem* 2015;36:1395-1405.
- 30 Bouguerra G, Aljanadi O, Bissinger R, Abbes S, Lang F: Embelin-Induced Phosphatidylserine Translocation in the Erythrocyte Cell Membrane. *Cell Physiol Biochem* 2015;37:1629-1640.
- 31 Bouguerra G, Bissinger R, Abbes S, Lang F: Stimulation of Eryptosis by Narasin. *Cell Physiol Biochem* 2015;37:1807-1816.
- 32 Bissinger R, Bouguerra G, Stockinger K, Abbes S, Lang F: Triggering of Suicidal Erythrocyte Death by Topotecan. *Cell Physiol Biochem* 2015;37:1607-1618.
- 33 Briglia M, Fazio A, Faggio C, Laufer S, Alzoubi K, Lang F: Triggering of Suicidal Erythrocyte Death by Ruxolitinib. *Cell Physiol Biochem* 2015;37:768-778.
- 34 Briglia M, Calabro S, Signoretto E, Alzoubi K, Laufer S, Faggio C, Lang F: Fucoxanthin Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2015;37:2464-2475.
- 35 Alzoubi K, Egler J, Abed M, Lang F: Enhanced eryptosis following auranofin exposure. *Cell Physiol Biochem* 2015;37:1018-1028.
- 36 Fazio A, Briglia M, Faggio C, Alzoubi K, Lang F: Stimulation of Suicidal Erythrocyte Death by Garcinol. *Cell Physiol Biochem* 2015;37:805-815.
- 37 Briglia M, Fazio A, Faggio C, Lang F: Triggering of Suicidal Erythrocyte Death by Zosuquidar. *Cell Physiol Biochem* 2015;37:2355-2365.
- 38 Officioso A, Alzoubi K, Manna C, Lang F: Clofazimine Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2015;37:331-341.

- 39 Peter T, Bissinger R, Enkel S, Alzoubi K, Oswald G, Lang F: Programmed erythrocyte death following in vitro Treosulfan treatment. *Cell Physiol Biochem* 2015;35:1372-1380.
- 40 Stockinger K, Bissinger R, Bouguerra G, Abbes S, Lang F: Enhanced Eryptosis Following Exposure to Carnosic Acid. *Cell Physiol Biochem* 2015;37:1779-1791.
- 41 Waibel S, Bissinger R, Bouguerra G, Abbes S, Lang F: Saquinavir Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2015;37:1973-1982.
- 42 Zierle J, Bissinger R, Egler J, Lang F: Lapatinib Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2015;37:2275-2287.
- 43 Macczak A, Cyrkler M, Bukowska B, Michalowicz J: Eryptosis-inducing activity of bisphenol A and its analogs in human red blood cells (in vitro study). *J Hazard Mater* 2016;307:328-335.
- 44 Officioso A, Alzoubi K, Lang F, Manna C: Hydroxytyrosol inhibits phosphatidylserine exposure and suicidal death induced by mercury in human erythrocytes: Possible involvement of the glutathione pathway. *Food Chem Toxicol* 2016;89:47-53.
- 45 Qadri SM, Donkor DA, Bhakta V, Eltringham-Smith LJ, Dwivedi DJ, Moore JC, Pepler L, Ivetic N, Nazi I, Fox-Robichaud AE, Liaw PC, Sheffield WP: Phosphatidylserine externalization and procoagulant activation of erythrocytes induced by *Pseudomonas aeruginosa* virulence factor pyocyanin. *J Cell Mol Med* 2016;20:710-720.
- 46 Zierle J, Bissinger R, Bouguerra G, Abbes S, Lang F: Triggering of Suicidal Erythrocyte Death by Regorafenib. *Cell Physiol Biochem* 2016;38:160-172.
- 47 Al Mamun Bhuyan A, Bissinger R, Stockinger K, Lang F: Stimulation of Suicidal Erythrocyte Death by Tafenoquine. *Cell Physiol Biochem* 2016;39:2464-2476.
- 48 Al Mamun Bhuyan A, Signoretto E, Bissinger R, Lang F: Enhanced Eryptosis Following Exposure to Dolutegravir. *Cell Physiol Biochem* 2016;39:639-650.
- 49 Al Mamun Bhuyan A, Signoretto E, Lang F: Triggering of Suicidal Erythrocyte Death by Psammaphin A. *Cell Physiol Biochem* 2016;39:908-918.
- 50 Almasry M, Jemaa M, Mischitelli M, Faggio C, Lang F: Stimulation of Suicidal Erythrocyte Death by Phosphatase Inhibitor Calyculin A. *Cell Physiol Biochem* 2016;40:163-171.
- 51 Bissinger R, Bhuyan AA, Signoretto E, Lang F: Stimulating Effect of Elvitegravir on Suicidal Erythrocyte Death. *Cell Physiol Biochem* 2016;38:1111-1120.
- 52 Egler J, Zierle J, Lang F: Stimulating Effect of Manumycin A on Suicidal Erythrocyte Death. *Cell Physiol Biochem* 2016;38:1147-1156.
- 53 Jemaa M, Mischitelli M, Fezai M, Almasry M, Faggio C, Lang F: Stimulation of Suicidal Erythrocyte Death by the CDC25 Inhibitor NSC-95397. *Cell Physiol Biochem* 2016;40:597-607.
- 54 Mischitelli M, Jemaa M, Almasry M, Faggio C, Lang F: Stimulation of Erythrocyte Cell Membrane Scrambling by Quinine. *Cell Physiol Biochem* 2016;40:657-667.
- 55 Mischitelli M, Jemaa M, Almasry M, Faggio C, Lang F: Stimulation of Suicidal Erythrocyte Death by Rottlerin. *Cell Physiol Biochem* 2016;40:558-566.
- 56 Mischitelli M, Jemaa M, Almasry M, Faggio C, Lang F: Triggering of Erythrocyte Cell Membrane Scrambling by Emodin. *Cell Physiol Biochem* 2016;40:91-103.
- 57 Mischitelli M, Jemaa M, Almasry M, Faggio C, Lang F: Triggering of Suicidal Erythrocyte Death by Fascaplysin. *Cell Physiol Biochem* 2016;39:1638-1647.
- 58 Mischitelli M, Jemaa M, Almasry M, Faggio C, Lang F: Ca²⁺ Entry, Oxidative Stress, Ceramide and Suicidal Erythrocyte Death Following Diosgenin Treatment. *Cell Physiol Biochem* 2016;39:1626-1637.
- 59 Peter T, Bissinger R, Lang F: Stimulation of Eryptosis by Caspofungin. *Cell Physiol Biochem* 2016;39:939-949.
- 60 Peter T, Bissinger R, Liu G, Lang F: Anidulafungin-Induced Suicidal Erythrocyte Death. *Cell Physiol Biochem* 2016;38:2272-2284.
- 61 Peter T, Bissinger R, Signoretto E, Mack AF, Lang F: Micafungin-Induced Suicidal Erythrocyte Death. *Cell Physiol Biochem* 2016;39:584-595.
- 62 Pretorius E, du Plooy JN, Bester J: A Comprehensive Review on Eryptosis. *Cell Physiol Biochem* 2016;39:1977-2000.
- 63 Shan F, Yang R, Ji T, Jiao F: Vitamin C Inhibits Aggravated Eryptosis by Hydrogen Peroxide in Glucose-6-Phosphated Dehydrogenase Deficiency. *Cell Physiol Biochem* 2016;39:1453-1462.

- 64 Signoretto E, Bissinger R, Castagna M, Lang F: Stimulation of Eryptosis by Combretastatin A4 Phosphate Disodium (CA4P). *Cell Physiol Biochem* 2016;38:969-981.
- 65 Signoretto E, Castagna M, Al Mamun Bhuyan A, Lang F: Stimulating Effect of Terfenadine on Erythrocyte Cell Membrane Scrambling. *Cell Physiol Biochem* 2016;38:1425-1434.
- 66 Signoretto E, Castagna M, Lang F: Stimulation of Eryptosis, the Suicidal Erythrocyte Death by Piceatannol. *Cell Physiol Biochem* 2016;38:2300-2310.
- 67 Signoretto E, Honisch S, Briglia M, Faggio C, Castagna M, Lang F: Nocodazole Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2016;38:379-392.
- 68 Signoretto E, Laufer SA, Lang F: Stimulating Effect of Sclareol on Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2016;39:554-564.
- 69 Signoretto E, Zierle J, Bissinger R, Castagna M, Bossi E, Lang F: Triggering of Suicidal Erythrocyte Death by Pazopanib. *Cell Physiol Biochem* 2016;38:926-938.
- 70 Wesseling MC, Wagner-Britz L, Huppert H, Hanf B, Hertz L, Nguyen DB, Bernhardt I: Phosphatidylserine Exposure in Human Red Blood Cells Depending on Cell Age. *Cell Physiol Biochem* 2016;38:1376-1390.
- 71 Zierle J, Bissinger R, Lang F: Inhibition by Teriflunomide of Erythrocyte Cell Membrane Scrambling Following Energy Depletion, Oxidative Stress and Ionomycin. *Cell Physiol Biochem* 2016;39:1877-1890.
- 72 Wang K, Mahmud H, Foller M, Biswas R, Lang KS, Bohn E, Götz F, Lang F: Lipopeptides in the triggering of erythrocyte cell membrane scrambling. *Cell Physiol Biochem* 2008;22:381-386.
- 73 Srinivas SP, Bonanno JA, Lariviere E, Jans D, Van Driessche W: Measurement of rapid changes in cell volume by forward light scattering. *Pflugers Arch* 2003;447:97-108.
- 74 Nguyen MT, Hanzelmann D, Hartner T, Peschel A, Götz F: Skin-Specific Unsaturated Fatty Acids Boost the *Staphylococcus aureus* Innate Immune Response. *Infect Immun* 2015;84:205-215.
- 75 Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM: Role of Ca²⁺-activated K⁺ channels in human erythrocyte apoptosis. *Am J Physiol Cell Physiol* 2003;285:C1553-1560.
- 76 Browning JA, Ellory JC, Gibson JS: Pathophysiology of red cell volume. *Contrib Nephrol* 2006;152:241-268.
- 77 Suzuki J, Fujii T, Imao T, Ishihara K, Kuba H, Nagata S: Calcium-dependent phospholipid scramblase activity of TMEM16 protein family members. *J Biol Chem* 2013;288:13305-13316.
- 78 Kurokawa K, Lee H, Roh KB, Asanuma M, Kim YS, Nakyama H, Shiratsuchi A, Choi Y, Takeuchi O, Kang HJ, Dohmae N, Nakanishi Y, Akira S, Sekimizu K, Lee BL: The triacylated ATP binding cluster transporter substrate-binding lipoprotein of *Staphylococcus aureus* functions as a native ligand for the toll-like receptor 2. *J Biol Chem* 2009;
- 79 Skabytska Y, Wolbing F, Gunther C, Koberle M, Kaesler S, Chen KM, Guenova E, Demircioglu D, Kempf WE, Volz T, Rammensee HG, Schaller M, Röcken M, Götz F, Biedermann T: Cutaneous innate immune sensing of Toll-like receptor 2-6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells. *Immunity* 2014;41:762-775.
- 80 Kuypers FA: Phospholipid asymmetry in health and disease. *Curr Opin Hematol* 1998;5:122-131.
- 81 Zwaal RF, Schroit AJ: Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* 1997;89:1121-1132.
- 82 Schlegel RA, Prendergast TW, Williamson P: Membrane phospholipid asymmetry as a factor in erythrocyte-endothelial cell interactions. *J Cell Physiol* 1985;123:215-218.
- 83 Boas FE, Forman L, Beutler E: Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. *Proc Natl Acad Sci U S A* 1998;95:3077-3081.
- 84 Nagata S: Apoptosis by death factor. *Cell* 1997;88:355-365.
- 85 Asano K, Miwa M, Miwa K, Hanayama R, Nagase H, Nagata S, Tanaka M: Masking of phosphatidylserine inhibits apoptotic cell engulfment and induces autoantibody production in mice. *J Exp Med* 2004;200:459-467.
- 86 Calderon-Salinas JV, Munoz-Reyes EG, Guerrero-Romero JF, Rodriguez-Moran M, Bracho-Riquelme RL, Carrera-Gracia MA, Quintanar-Escorza MA: Eryptosis and oxidative damage in type 2 diabetic mellitus patients with chronic kidney disease. *Mol Cell Biochem* 2011;357:171-179.
- 87 Raftos JE, Whillier S, Chapman BE, Kuchel PW: Kinetics of uptake and deacetylation of N-acetylcysteine by human erythrocytes. *Int J Biochem Cell Biol* 2007;39:1698-1706.
- 88 Janek T, Rodrigues LR, Gudina EJ, Czyznikowska Z: Structure and mode of action of cyclic lipopeptide pseudofactin II with divalent metal ions. *Colloids Surf B Biointerfaces* 2016;146:498-506.

- 89 Grau A, Gomez Fernandez JC, Peypoux F, Ortiz A: A study on the interactions of surfactin with phospholipid vesicles. *Biochim Biophys Acta* 1999;1418:307-319.
- 90 Buchoux S, Lai-Kee-Him J, Garnier M, Tsan P, Besson F, Brisson A, Dufourc EJ: Surfactin-triggered small vesicle formation of negatively charged membranes: a novel membrane-lysis mechanism. *Biophys J* 2008;95:3840-3849.
- 91 Velkov T, Thompson PE, Nation RL, Li J: Structure--activity relationships of polymyxin antibiotics. *J Med Chem* 2010;53:1898-1916.
- 92 Taylor SD, Palmer M: The action mechanism of daptomycin. *Bioorg Med Chem* 2016;24:6253-6268.
- 93 Raaijmakers JM, De Bruijn I, Nybroe O, Ongena M: Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiol Rev* 2010;34:1037-1062.
- 94 Wu CY, Chen CL, Lee YH, Cheng YC, Wu YC, Shu HY, Götz F, Liu ST: Nonribosomal Synthesis of Fengycin on an Enzyme Complex Formed by Fengycin Synthetases. *J Biol Chem* 2007;282:5608-5616.
- 95 Tang Q, Bie X, Lu Z, Lv F, Tao Y, Qu X: Effects of fengycin from *Bacillus subtilis* fmbJ on apoptosis and necrosis in *Rhizopus stolonifer*. *J Microbiol* 2014;52:675-680.
- 96 Rodriguez MF, Wiens GD, Purcell MK, Palti Y: Characterization of Toll-like receptor 3 gene in rainbow trout (*Oncorhynchus mykiss*). *Immunogenetics* 2005;57:510-519.
- 97 Morera D, Roher N, Ribas L, Balasch JC, Donate C, Callol A, Boltana S, Roberts S, Goetz G, Goetz FW, MacKenzie SA: RNA-Seq reveals an integrated immune response in nucleated erythrocytes. *PLoS One* 2011;6:e26998.