Induction of Suicidal Erythrocyte Death by Listeriolysin from *Listeria monocytogenes*

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Key Words

Cation channel • Calcium • Apoptosis • Cell death • Cell volume

Abstract

Listeriolysin, the secreted cytolysin of the facultative intracellular bacterium *Listeria monocytogenes*, is its major virulence factor. Previously, non-lytic concentrations of listeriolysin were shown to induce Ca^{2+} -permeable nonselective cation channels in human embryonic kidney cells. In erythrocytes, Ca^{2+} entry is followed by activation of K^+ channels resulting in K^+ -exit as well as by membrane scrambling resulting in phosphatidylserine exposure at the cell surface. Phosphatidylserine-exposing erythrocytes are recognized by macrophages, engulfed, degraded and thus cleared from circulating blood. Phosphatidylserine exposure is a key event of eryptosis, the suicidal death of erythrocytes. The present study utilized patch-clamp technique, Fluo3-fluorescence, and annexin V-binding in FACS analysis to determine the effect of listeriolysin on cell membrane conductance, cytosolic free Ca^{2+} concentration, and phosphatidylserine exposure, respectively. Within 30 minutes, exposure of human peripheral blood erythrocytes to low concen-

trations of listeriolysin (which were non-hemolytic for the majority of cells) induced a Ca^{2+} -permeable cation conductance in the erythrocyte cell membrane, increased cytosolic Ca^{2+} concentration, and triggered annexin V-binding. Increase of extracellular K^+ concentration blunted, but did not prevent, listeriolysin-induced annexin V-binding. In conclusion, listeriolysin triggers suicidal death of erythrocytes, an effect at least partially due to depletion of intracellular K^+ . Listeriolysin induced suicidal erythrocyte death could well contribute to the pathophysiology of *L. monocytogenes* infection.

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Introduction

Listeria monocytogenes, a pathogen leading to potentially life-threatening disease [1], releases listeriolysin [2, 3], a protein capable of inducing ion channels in host cells [4]. Listeriolysin O (LLO) is a member of the family of cholesterol-dependent (CDCs) pore-forming toxins, the largest class of cytolytic toxins expressed by gram-positive pathogens [5, 6]. Cholesterol is the common ligand known to be bound by these toxins and appears also to be crucial

in promoting toxin oligomerization, probably as an allosteric effector molecule [7]. An interesting variant toxin is produced by the pathogen *Streptococcus intermedius* where changes in the region of the toxin required for cholesterol binding have resulted in a binding domain that binds specifically to the human complement binding protein inhibitor CD59 [8]. Other members of this toxin family include pneumolysin produced by *Streptococcus pneumoniae*, streptolysin by *Streptococcus pyogenes*, and perfringolysin by *Clostridium perfringens*. The latter two toxins represent the best biochemically and structurally characterized toxins of this family on which the putative functions of the other members have been modelled. Despite structural similarities, the biological functions exhibited by the respective toxins are diverse. The toxin streptolysin produced by *S. pyogenes* is required to block uptake of this pathogen into lysosomes thereby prolonging extracellular survival, while LLO enhances uptake of the bacterium, delays vacuole fusion with lysosomes [9, 10], promotes its egress from the endocytic vesicle to the host cytoplasm [11, 12] and fosters the transfer of bacterial DNA [13]. Indeed, LLO possesses unique properties that prevent it from being cytotoxic when produced by cytosolic bacteria [14]. It is highly active at the acidic pH levels present in the phagosomal vacuole [15], but less active in the cytosol where neutral pH predominates. Also within the vacuole, LLO is cleaved at its C-terminal end by the host cell protease cathepsin D, rendering the toxin inactive [16]. Other studies have provided evidence for a short cytosolic half-life of the molecule [17].

Exposure of human embryonic kidney cells to LLO has previously been shown to trigger Ca^{2+} entry [4]. LLO is a crucial pathogenetic factor required for the intracellular survival of the pathogen [18, 19]. The oligomerization of LLO reportedly forms lipid rafts which induce signalling through co-aggregation of raft's associated receptors / kinases [20]. Effects of LLO in nucleated host cells include stimulation of phospholipase C with formation of inositoltrisphosphate and diacylglycerol [21, 22], triggering of MAP kinase pathways [23], activation of NF- κ B [24], expression of cytokines [25, 26], MUC genes [27, 28], and cell adhesion molecules [29], antigen processing [30], exocytosis [31, 32], and host cell apoptosis [33], effects in part attributed to Ca^{2+} entry [4].

Ca^{2+} entry into erythrocytes leads to activation of Ca^{2+} -sensitive K^+ channels [34-36] which, together with Cl^- channels [37], allow the exit of KCl and thus lead to cell shrinkage [38]. Increased intracellular Ca^{2+}

concentration further leads to Ca^{2+} -sensitive scrambling of the cell membrane [39, 40] and subsequent phosphatidylserine exposure at the cell surface [38, 41-46]. Phosphatidylserine-exposing erythrocytes are recognized by macrophages equipped with phosphatidylserine receptors [47] and are thus engulfed and degraded [48, 49]. Accordingly, phosphatidylserine exposure leads to suicidal erythrocyte death or eryptosis [50]. Mechanisms mediating eryptosis are similar but not necessarily identical to erythrocyte senescence [50-53] and neocytolysis [54].

During the early phases of listerial infection and during systemic spread, encounter of the toxin with erythrocytes is inevitable, and is thought to trigger cell lysis. However, for pore formation and erythrocyte lysis, high concentrations of the toxin are required [55]. The present study thus explored whether non-lytic concentrations of LLO could influence Ca^{2+} entry and trigger eryptosis in human erythrocytes.

Materials and Methods

Solutions

Erythrocytes were drawn from healthy volunteers. Experiments were performed at 37°C in Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 CaCl_2 . Where indicated, 125 mM KCl have been added to replace NaCl.

Purification of listeriolysin and its mutant derivative

Listeriolysin O (LLO) was expressed and purified from the wild type *L. innocua* 6a strain as previously described [56] with some modifications. An overnight bacterial culture grown at 37°C in BHI broth was used to inoculate one litre of the chemically defined minimal medium [57]. Following 48 h incubation at 30°C, bacteria were removed by centrifugation and the supernatant fluid was concentrated to 50 ml using a Millipore filtration apparatus with a cut-off point of 10 kDa. The crude supernatant was then batch-absorbed with Q-sepharose (Pharmacia, Freiburg, Germany) and pre-equilibrated with loading buffer (50 mM NaH_2PO_4 , pH 6.2) for 60 min. The non-absorbed fraction was centrifuged and desalted by transferring through a super loop to a HiPrep™ 26/10 desalting column (Pharmacia, Freiburg, Germany), where loading buffer (50 mM NaH_2PO_4 , pH 6.2) was used to elute the desalted fraction. This fraction was subsequently filtered through a Millipore filter (0.22 μm) and loaded onto a Resource-S column previously equilibrated with 50 mM NaH_2PO_4 , pH 6.2. The pure toxin eluted reproducibly from the column at 0.21 to 0.28 M NaCl using elution buffer (50 mM NaH_2PO_4 , 1M NaCl). Fractions were collected and individually tested for hemolytic activity. Protein desalting and purification processes were carried out using

Fig. 1. Listeriolysin-induced cation currents in human erythrocytes: A. Purified listeriolysin (LLO) was used in this study. 1 µg of the toxin was loaded on a 10% SDS-PAGE and subsequently stained with Coomassie Blue to examine for purity of the sample used. B. Current traces recorded in whole-cell mode of cells preincubated with LLO (LLO, 45–60 min, 240 ng/ml), with KCl/K-gluconate pipette solution combined with standard NaCl (left) or NMDG-Cl (right) bath solutions. C. Mean whole-cell current-voltage (I/V) relationships ($\pm$ SEM) of non-stimulated erythrocytes (control, open diamonds, $n = 4$), or erythrocytes treated with LLO (LLO, closed triangles, $n = 5$) recorded as in (A) with standard NaCl bath solution. D. Mean whole-cell current-voltage (I/V) relationships ($\pm$ SEM, $n = 5$) of LLO-stimulated erythrocytes recorded as in (A) with NaCl (closed triangles), CaCl_2 (open circles) or NMDG-Cl (open triangles) bath solutions. E. Mean whole-cell conductance ($\pm$ SEM, $n = 5$) obtained from LLO-stimulated erythrocytes as calculated from (C) by linear regression at negative membrane potentials ($-100/-40$ mV) recorded with NaCl, CaCl_2 , or NMDG-Cl bath solutions. F. Mean reversal potential ($\pm$ SEM, $n = 5$) obtained from LLO-stimulated erythrocytes recorded with NaCl, CaCl_2 , or NMDG-Cl bath solutions.

the high performance chromatography system ÄKTA explorer and UNICORN™ control system (Pharmacia, Freiburg, Germany). LLO showed a high purity on SDS-PAGE (Fig. 1A), and was recognised with LLO-specific mAb M275 [58].

Patch-clamp recordings

Patch-clamp experiments have been performed as described previously in detail [59]. Currents were recorded at room temperature from freshly acquired human erythrocytes. Recordings were obtained in whole-cell voltage-clamp mode using patch pipettes made of borosilicate glass (150 TF-10, Clark Medical Instruments) with resistances ranging from 9 to 15 MΩ. Currents were recorded (10 kHz sampling rate) and 3-kHz low-pass-filtered by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, NY, USA). The cells were held at -20 mV holding potential. Whole-cell currents were elicited by

400 ms square pulses to voltages between -100 and $+80$ mV (20 mV increments). Data were analysed by averaging the currents between 100 and 350 ms. Applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. Liquid junction potentials between bath and pipette solution and between the bath solutions and the salt bridge were calculated and data were corrected according to Barry and Lynch [60]. The inward currents, defined as flow of positive charge from the bath into the cell are negative currents and depicted as downward deflections of the original current traces. Current traces are depicted upon 500 Hz low-pass-filtering; the currents of the individual square pulses are superimposed. The pipette solution contained (in mM): 80 KCl, 60 K-D-gluconate, 10 HEPES/KOH, 1 ethylene glycol-bis (aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 Mg-ATP, 1 MgCl_2 (pH 7.2). The recorded cells were superfused with standard NaCl bath solution containing

(in mM): 145 NaCl, 5 KCl, 10 HEPES/NaOH, 5 D-glucose, 2 MgCl₂, 1 CaCl₂ (pH 7.4). Further bath solutions contained (in mM): 180 N-methyl-D-glucamine (NMDG)-Cl, 10 HEPES/NMDG, 1 MgCl₂, 1 CaCl₂ (pH 7.4) or 100 mM CaCl₂, 10 HEPES/Ca(OH)₂ (pH 7.4).

FACS analysis

FACS analysis was performed as described [38]. After incubation, cells were washed in annexin-binding buffer containing (in mM): 125 NaCl, 10 HEPES/NaOH (pH 7.4), 5 CaCl₂. Erythrocytes were resuspended in a solution composed of Annexin-V-Fluos (Böhringer Mannheim, Germany) and annexin buffer (dilution of 1:500). After 15 minutes of incubation, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson). Annexin-fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca²⁺

Intracellular free Ca²⁺ measurements were performed as described [41]. Briefly, erythrocytes were washed in NaCl Ringer solution and then loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) in 2 mM CaCl₂- and 2 μM Fluo-3/AM-containing NaCl Ringer solution. The cells were incubated at 37°C for 20 minutes under shaking and washed twice in 2 mM CaCl₂-containing NaCl Ringer solution. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. In the following, Ca²⁺-dependent fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of hemolysis

Erythrocytes were washed in Ringer solutions of three different pH values (6.5, 7.0, 7.4) and further incubated for 30 minutes at 37°C in the presence of 10 ng/ml, 100 ng/ml, or 250 ng/ml LLO. The incubation was stopped by centrifugation (2 minutes at 1000 g), and the supernatants were harvested. As a measure of hemolysis the hemoglobin (Hb) concentrations of the supernatant were determined photometrically at 546 nm upon transformation of Hb into cyanomet Hb as described earlier [61].

Exposure of mouse erythrocytes to different strains of *Listeria monocytogenes*

Erythrocytes were drawn from healthy 5 weeks old female Balb/c mice kept under hygienic conditions in our breeding facilities. Erythrocytes were incubated with the isogenic deletion Δhly mutant containing the pSOG304 vector or its derivatives expressing either the wild type LLO or LLO^{-PEST} toxins at MOI of 0.5 in Ringer solution for 30 minutes. Thereafter, FACS analysis of annexin V-binding was performed as described above. No hemolysis was detected after incubation of erythrocytes with bacteria under the above mentioned conditions.

Statistics

Data are expressed as arithmetic means ± SEM and statistical analysis was made by unpaired t-test or ANOVA, p<0.05 was considered statistically significant.

Fig. 2. Effect of listeriolysin on Fluo3 fluorescence in erythrocytes: A. Original histogram of Fluo3 fluorescence of human erythrocytes following a 30 minute treatment with 0 ng/ml (control) or 250 ng/ml listeriolysin (LLO). B. Arithmetic means ± SEM (n =9-22) of normalized Fluo3 fluorescence (logarithmic scale) following a 30 minute treatment with listeriolysin (LLO, 0-250 ng/ml). Asterisks indicate significant (p<0.05) *, (p<0.001) *** difference from control (0). C. Arithmetic means ± SEM (n = 4) of the Fluo3 fluorescence after a 30 minute treatment with LLO (0-250 ng/ml) at pH 6.5 (black bars), pH 7.0 (grey bars), or pH 7.4 (white bars).

Results

In patch-clamp experiments, human erythrocytes exhibited very low whole-cell currents when recorded with KCl/K-gluconate pipette and standard NaCl bath solution (Fig. 1C) suggesting low channel activity in unstimulated human erythrocytes. Preincubation with LLO (240 ng/ml; Fig. 1A) added to the standard NaCl bath solution for 45-60 minutes increased whole-cell currents (Fig. 1B, 2nd trace, 1C,D,E). The recorded current was cation-selective since substitution of Na⁺ in the bath by the larger cation NMDG⁺ decreased the inward currents and shifted the current reversal potential towards -37 mV (Fig. 1D,E,F). From the reversal potential recorded with NaCl in the bath (-15.4 ± 1.8 mV, n=5, Fig. 1F) a permeability ratio of $P_{K}/P_{Na} = 2:1$ can be deduced for the

Fig. 3. Effect of listeriolysin on hemolysis: A. Arithmetic means $\pm$ SEM ($n = 3$) of the percentage of hemolysed erythrocytes after a 30 minute treatment with LLO (0-250 ng/ml) at pH 6.5 (black bars), pH 7.0 (grey bars), or pH 7.4 (white bars). B. Time-dependence of the arithmetic means $\pm$ SEM ($n = 4-5$) of the percentage of hemolysed erythrocytes at pH 7.4 following treatment with LLO (0-250 ng/ml).

LLO-induced current. A similar reversal potential (-18.8 ± 4.6 mV, $n=5$, Fig. 1F) was observed with Ca^{2+} as a principal cation in the bath, corresponding to relatively high Ca^{2+} permeability ($P_{\text{K}}/P_{\text{Ca}} = 4:1$). In addition, Ca^{2+} seemed to block the LLO-induced current (Fig. 1D, open circles and 1E).

Formation of a Ca^{2+} permeable cation conductance is expected to increase cytosolic free Ca^{2+} concentration. Similar to its effect on nucleated cells [4], LLO indeed increased erythrocyte cytosolic free Ca^{2+} concentration (Fig. 2). Within 30 minutes treatment with LLO led to an increase of Fluo3 fluorescence (Fig. 2B). LLO increased erythrocyte cytosolic free Ca^{2+} concentration at pH 6.5, pH 7, and pH 7.4. However, the strongest effect was observed at pH 6.5 (Fig. 2C). The low con-

centrations of LLO leading to an increased erythrocyte cytosolic Ca^{2+} concentration did not induce excessive hemolysis within 30 minutes. As shown in Fig. 3A, almost no hemolysis was observed at 10 ng/ml and about 15% at 100 ng/ml in solutions of pH 6.5, 7.0, and 7.4 respectively after 30 min. The time-dependence of hemolysis at pH 7.4 with various concentrations of LLO is displayed in Fig. 3B.

An increase of cytosolic Ca^{2+} concentration is expected to trigger cell membrane scrambling with subsequent phosphatidylserine exposure at the cell surface [38]. Phosphatidylserine binds annexin V, which has thus been utilized to detect phosphatidylserine-exposing erythrocytes. As illustrated in Fig. 4A, annexin V-binding was not paralleled by disruption of the cell membrane integrity. Within 30 min, exposure to LLO was followed by a significant increase in the percentage of annexin V-binding erythrocytes (Fig. 4C). The effect of LLO on phosphatidylserine exposure was observed at non-hemolytic concentrations of LLO (10 ng/ml or 100 ng/ml). The percentage of phosphatidylserine-exposing erythrocytes stimulated by LLO was not significantly influenced by pH variations within the range from 6.5 to 7.4 (Fig. 4D). Annexin V-binding of erythrocytes was also enhanced upon treatment with live bacteria. Exposure of mouse erythrocytes to the wild type LLO producing strain of EGD-e Δ hly induced annexin V-binding (Fig. 4E), an effect blunted in the LLO^{-PEST} strain. In contrast, exposure to the isogenic Δ hly mutant strain lacking LLO production did not affect annexin V-binding of mouse erythrocytes.

Previous studies have demonstrated that enhanced cytosolic Ca^{2+} concentrations stimulate phospholipid scrambling in part by activation of the Ca^{2+} -sensitive K^{+} channels and the resulting cellular K^{+} loss [46]. Thus, additional experiments have been performed in the presence of 125 mM K^{+} , which prevents cellular K^{+} loss upon K^{+} channel activation. As illustrated in Fig. 5, the stimulation of phosphatidylserine exposure by LLO is significantly blunted, but not abrogated, at increased extracellular K^{+} concentration, indicating that cellular K^{+} loss contributes to but does not fully account for the stimulation of phospholipid scrambling by LLO.

Discussion

The present observations demonstrate that treatment of human erythrocytes with low concentrations of listeriolysin O (LLO) leads to cation channel activation, Ca^{2+} entry, and subsequent phospholipid scrambling of

Fig. 4. Effect of listeriolysin on phosphatidylserine exposure of erythrocytes: A. Light micrograph (left) and corresponding immunofluorescence (right) showing phosphatidylserine exposure of an intact erythrocyte following a 30 minute treatment with LLO (10 ng/ml). B. Original histogram of annexin V-binding of human erythrocytes following a 30 minute treatment with 0 ng/ml (control) or 250 ng/ml listeriolysin (LLO). C. Arithmetic means $\pm$ SEM (n = 9-23) of the percentage of annexin V-binding erythrocytes following a 30 minute treatment with listeriolysin (LLO, 0-250 ng/ml). Asterisks indicate significant (p<0.05)*, (p<0.001) *** difference from control. D. Arithmetic means $\pm$ SEM (n = 4) of the percentage of annexin V-binding cells after a 30 minute treatment with LLO (0-250 ng/ml) at pH 6.5 (black bars), pH 7.0 (grey bars), or pH 7.4 (white bars). E. Original histogram of annexin V-binding of mouse erythrocytes following a 30 minute exposure to the isogenic Δ hly mutant strain (LLO-^{HLY}) or to the same mutant producing either the wild type listeriolysin (LLO) or listeriolysin lacking the PEST-like sequence (LLO-^{PEST}) at MOI of 0.5. Untreated erythrocytes were included as a control.

the cell membrane with exposure of phosphatidylserine at the erythrocyte surface. Notably, not all erythrocytes appear similarly sensitive to treatment with listeriolysin. While channel activity was observed invariably in each erythrocyte studied, excessive phosphatidylserine exposure was observed only in a portion of LLO treated erythrocytes. Thus, the erythrocytes are not homogeneous in their susceptibility to LLO-induced Ca²⁺ entry. The susceptibility presumably depends on the age of the erythrocytes, whereby both, senescent [50-53] and newly formed [54] erythrocytes may be particularly sensitive to suicidal death.

Phosphatidylserine-exposing cells are engulfed by macrophages [47], leading to trapping of affected erythrocytes and subsequent clearance from circulating blood. Accordingly, accelerated phosphatidylserine

exposure has previously been demonstrated in several anemic conditions, including sickle-cell anemia, beta-thalassemia or glucose-6-phosphate dehydrogenase (G6PD)-deficiency [44], in phosphate depletion [62], iron deficiency anemia [63], Hemolytic Uremic Syndrome [64], sepsis [65], malaria [66], and Wilson disease [67]. Moreover, enhanced eryptosis has been observed following treatment of erythrocytes with hemolysin Kanagawa [68], PGE₂ [69], lipoxygenase inhibitor Bay-Y5884 [70], platelet activating factor [71], paclitaxel [72], methylglyoxal [73], chlorpromazine [74], cyclosporine [75], curcumin [76], amyloid peptides [77], lead [78] or mercury [79].

Interaction of bacteria or their components with erythrocytes and subsequent suicidal erythrocyte death may thus underly anemia, a common and important

Fig. 5. Contribution of erythrocytic K⁺ loss to the effect of listeriolysin on phosphatidylserine exposure: A. Original histograms of annexin V-binding of human erythrocytes following a 30 minute treatment with 0 ng/ml (control) or 250 ng/ml (LLO) LLO at 5 mM (5 mM K⁺, left histogram) and 125 mM (125 mM K⁺, right histogram) extracellular K⁺ concentration. B. Arithmetic means $\pm$ SEM (n = 7-8) of annexin V-binding following a 30 minute treatment with 0 or 250 ng/ml LLO at 5 mM K⁺ concentration (5 mM K⁺, left bars) or at 125 mM K⁺ concentration (125 mM K⁺, right bars). * indicates significant (p<0.05) difference from absence of LLO. # indicates significant (p<0.05) difference from 5 mM K⁺.

complication of sepsis [80, 81]. Specifically, the contact of LLO expressing bacteria with erythrocytes may lead to the triggering of suicidal erythrocyte death. The anemia may lack during infection with mutant bacteria not expressing LLO.

Phosphatidylserine-exposing erythrocytes may further adhere to the vascular wall and thus impair microcirculation [82-84]. Accordingly, annexin V-binding erythrocytes have been identified in the renal medulla following renal ischemia [45, 82-84]. Enhanced adhesiveness of erythrocytes participates in hemostasis [85-89] and enhances the risk to develop thrombosis [90]. Thus, the pathophysiological effects of LLO on erythrocytes during systemic disease are likely to be profound and warrant further study.

An increase of cytosolic Ca²⁺ concentration was expected to activate the Ca²⁺-sensitive K⁺ (GARDOS) channel [35]. The subsequent hyperpolarisation of the cell membrane drives Cl⁻ out of the cell [37] and the cellular loss of KCl, with osmotically obliged water, is prone to induce cell shrinkage [38]. Loss of cellular K⁺ is further considered to participate in the triggering of apoptosis in several cell types [91] including erythrocytes [46]. More recently, studies using wild type *L. monocytogenes* and LLO-deficient mutants have led to the suggestion that the depletion of intracellular K⁺ constitutes a cellular “danger signal” for nucleated cells

recognized by components of the caspase-1 “inflammasome” [92]. Here we provide unequivocal evidence that it is LLO itself, in the absence of other bacterial components, that induces the cellular “danger signal” i.e., by inducing cellular K⁺ depletion. Hence, impairing K⁺ exit blunts the stimulation of phosphatidylserine exposure by LLO.

In conclusion, LLO mediates Ca²⁺ entry into erythrocytes and thus triggers phosphatidylserine exposure. As has previously been postulated for nucleated cells, depletion of intracellular K⁺ levels probably provides a signal leading to eryptosis. This effect may lead to clearance of erythrocytes from circulating blood resulting in anemia, and to adherence at the vascular wall resulting in deranged microcirculation. Those effects could play an important role in the pathophysiology of *Listeria monocytogenes* infection.

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References

- 1 Schuchat A, Swaminathan B, Broome CV: Epidemiology of human listeriosis. *Clin Microbiol Rev* 1991;4:169-183.
- 2 Cossart P, Vicente MF, Mengaud J, Baquero F, Perez-Diaz JC, Berche P: Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. *Infect Immun* 1989;57:3629-3636.
- 3 Portnoy DA, Jacks PS, Hinrichs DJ: Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. *J Exp Med* 1988;167:1459-1471.
- 4 Repp H, Pamukci Z, Koschinski A, Domann E, Darji A, Birringer J, Brockmeier D, Chakraborty T, Dreyer F: Listeriolysin of *Listeria monocytogenes* forms Ca²⁺-permeable pores leading to intracellular Ca²⁺ oscillations. *Cell Microbiol* 2002;4:483-491.
- 5 Alouf JE: Cholesterol-binding cytolytic protein toxins. *Int J Med Microbiol* 2000;290:351-356.
- 6 Palmer M: The family of thiol-activated, cholesterol-binding cytolytins. *Toxicon* 2001;39:1681-1689.
- 7 Palmer M, Vulicevic I, Saweljew P, Valeva A, Kehoe M, Bhakdi S: Streptolysin O: a proposed model of allosteric interaction between a pore-forming protein and its target lipid bilayer. *Biochemistry* 1998;37:2378-2383.
- 8 Giddings KS, Zhao J, Sims PJ, Tweten RK: Human CD59 is a receptor for the cholesterol-dependent cytotoxin intermedilysin. *Nat Struct Mol Biol* 2004;11:1173-1178.
- 9 Henry R, Shaughnessy L, Loessner MJ, Alberti-Segui C, Higgins DE, Swanson JA: Cytolysin-dependent delay of vacuole maturation in macrophages infected with *Listeria monocytogenes*. *Cell Microbiol* 2006;8:107-119.
- 10 Shaughnessy LM, Hoppe AD, Christensen KA, Swanson JA: Membrane perforations inhibit lysosome fusion by altering pH and calcium in *Listeria monocytogenes* vacuoles. *Cell Microbiol* 2006;8:781-792.
- 11 Alberti-Segui C, Goeden KR, Higgins DE: Differential function of *Listeria monocytogenes* listeriolysin O and phospholipases C in vacuolar dissolution following cell-to-cell spread. *Cell Microbiol* 2007;9:179-195.
- 12 Hakansson A, Bentley CC, Shakhnovic EA, Wessels MR: Cytolysin-dependent evasion of lysosomal killing. *Proc Natl Acad Sci U S A* 2005;102:5192-5197.
- 13 Grillot-Courvalin C, Goussard S, Courvalin P: Wild-type intracellular bacteria deliver DNA into mammalian cells. *Cell Microbiol* 2002;4:177-186.
- 14 Decatur AL, Portnoy DA: A PEST-like sequence in listeriolysin O essential for *Listeria monocytogenes* pathogenicity. *Science* 2000;290:992-995.
- 15 Cheng LW, Portnoy DA: *Drosophila* S2 cells: an alternative infection model for *Listeria monocytogenes*. *Cell Microbiol* 2003;5:875-885.
- 16 Dominguez-Bernal G, Muller-Altroch S, Gonzalez-Zorn B, Scortti M, Herrmann P, Monzo HJ, Lacharme L, Kreft J, Vazquez-Boland JA: A spontaneous genomic deletion in *Listeria ivanovii* identifies LIPI-2, a species-specific pathogenicity island encoding sphingomyelinase and numerous internalins. *Mol Microbiol* 2006;59:415-432.
- 17 Jones S, Preiter K, Portnoy DA: Conversion of an extracellular cytolysin into a phagosome-specific lysin which supports the growth of an intracellular pathogen. *Mol Microbiol* 1996;21:1219-1225.
- 18 Chakraborty T: Molecular and cell biological aspects of infection by *Listeria monocytogenes*. *Immunobiology* 1999;201:155-163.
- 19 Tilney LG, Tilney MS: The wily ways of a parasite: induction of actin assembly by *Listeria*. *Trends Microbiol* 1993;1:25-31.
- 20 Gekara NO, Jacobs T, Chakraborty T, Weiss S: The cholesterol-dependent cytolysin listeriolysin O aggregates rafts via oligomerization. *Cell Microbiol* 2005;7:1345-1356.
- 21 Goldfine H, Wadsworth SJ, Johnston NC: Activation of host phospholipases C and D in macrophages after infection with *Listeria monocytogenes*. *Infect Immun* 2000;68:5735-5741.
- 22 Sibelius U, Rose F, Chakraborty T, Darji A, Wehland J, Weiss S, Seeger W, Grimminger F: Listeriolysin is a potent inducer of the phosphatidylinositol response and lipid mediator generation in human endothelial cells. *Infect Immun* 1996;64:674-676.
- 23 Tang P, Rosenshine I, Cossart P, Finlay BB: Listeriolysin O activates mitogen-activated protein kinase in eucaryotic cells. *Infect Immun* 1996;64:2359-2361.
- 24 Kayal S, Lilienbaum A, Poyart C, Memet S, Israel A, Berche P: Listeriolysin O-dependent activation of endothelial cells during infection with *Listeria monocytogenes*: activation of NF-kappa B and upregulation of adhesion molecules and chemokines. *Mol Microbiol* 1999;31:1709-1722.
- 25 Kohda C, Kawamura I, Baba H, Nomura T, Ito Y, Kimoto T, Watanabe I, Mitsuyama M: Dissociated linkage of cytokine-inducing activity and cytotoxicity to different domains of listeriolysin O from *Listeria monocytogenes*. *Infect Immun* 2002;70:1334-1341.
- 26 Nishibori T, Xiong H, Kawamura I, Arakawa M, Mitsuyama M: Induction of cytokine gene expression by listeriolysin O and roles of macrophages and NK cells. *Infect Immun* 1996;64:3188-3195.
- 27 Lievin-Le Moal V, Huet G, Aubert JP, Bara J, Forgue-Lafitte ME, Servin AL, Coconnier MH: Activation of mucin exocytosis and upregulation of MUC genes in polarized human intestinal mucin-secreting cells by the thiol-activated exotoxin listeriolysin O. *Cell Microbiol* 2002;4:515-529.
- 28 Lievin-Le Moal V, Servin AL, Coconnier-Polter MH: The increase in mucin exocytosis and the upregulation of MUC genes encoding for membrane-bound mucins induced by the thiol-activated exotoxin listeriolysin O is a host cell defence response that inhibits the cell-entry of *Listeria monocytogenes*. *Cell Microbiol* 2005;7:1035-1048.
- 29 Krull M, Nost R, Hippenstiel S, Domann E, Chakraborty T, Suttrop N: *Listeria monocytogenes* potentially induces up-regulation of endothelial adhesion molecules and neutrophil adhesion to cultured human endothelial cells. *J Immunol* 1997;159:1970-1976.
- 30 Darji A, Chakraborty T, Wehland J, Weiss S: Listeriolysin generates a route for the presentation of exogenous antigens by major histocompatibility complex class I. *Eur J Immunol* 1995;25:2967-2971.
- 31 Coconnier MH, Dliissi E, Robard M, Laboisse CL, Gaillard JL, Servin AL: *Listeria monocytogenes* stimulates mucin exocytosis in cultured human polarized mucosecreting intestinal cells through action of listeriolysin O. *Infect Immun* 1998;66:3673-3681.
- 32 Coconnier MH, Lorrot M, Barbat A, Laboisse C, Servin AL: Listeriolysin O-induced stimulation of mucin exocytosis in polarized intestinal mucin-secreting cells: evidence for toxin recognition of membrane-associated lipids and subsequent toxin internalization through caveolae. *Cell Microbiol* 2000;2:487-504.
- 33 Guzman CA, Domann E, Rohde M, Bruder D, Darji A, Weiss S, Wehland J, Chakraborty T, Timmis KN: Apoptosis of mouse dendritic cells is triggered by listeriolysin, the major virulence determinant of *Listeria monocytogenes*. *Mol Microbiol* 1996;20:119-126.

- 34 Bookchin RM, Ortiz OE, Lew VL: Activation of calcium-dependent potassium channels in deoxygenated sickled red cells. *Prog Clin Biol Res* 1987;240:193-200.
- 35 Brugnara C, de Franceschi L, Alper SL: Inhibition of Ca(2+)-dependent K⁺ transport and cell dehydration in sickle erythrocytes by clotrimazole and other imidazole derivatives. *J Clin Invest* 1993;92:520-526.
- 36 Franco RS, Palascak M, Thompson H, Rucknagel DL, Joiner CH: Dehydration of transferrin receptor-positive sickle reticulocytes during continuous or cyclic deoxygenation: role of KCl cotransport and extracellular calcium. *Blood* 1996;88:4359-4365.
- 37 Myssina S, Lang PA, Kempe DS, Kaiser S, Huber SM, Wieder T, Lang F: Cl⁻ channel blockers NPPB and niflumic acid blunt Ca(2+)-induced erythrocyte 'apoptosis'. *Cell Physiol Biochem* 2004;14:241-248.
- 38 Lang KS, Duranton C, Poehlmann H, Myssina S, Bauer C, Lang F, Wieder T, Huber SM: Cation channels trigger apoptotic death of erythrocytes. *Cell Death Differ* 2003;10:249-256.
- 39 Dekkers DW, Comfurius P, Bevers EM, Zwaal RF: Comparison between Ca²⁺-induced scrambling of various fluorescently labelled lipid analogues in red blood cells. *Biochem J* 2002;362:741-747.
- 40 Woon LA, Holland JW, Kable EP, Roufogalis BD: Ca²⁺ sensitivity of phospholipid scrambling in human red cell ghosts. *Cell Calcium* 1999;25:313-320.
- 41 Andrews DA, Yang L, Low PS: Phorbol ester stimulates a protein kinase C-mediated agatoxin-TK-sensitive calcium permeability pathway in human red blood cells. *Blood* 2002;100:3392-3399.
- 42 Berg CP, Engels IH, Rothbart A, Lauber K, Renz A, Schlosser SF, Schulze-Osthoff K, Wesselborg S: Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. *Cell Death Differ* 2001;8:1197-1206.
- 43 Bratosin D, Estaquier J, Petit F, Arnoult D, Quatannens B, Tissier JP, Slomianny C, Sartiaux C, Alonso C, Huart JJ, Montreuil J, Ameisen JC: Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. *Cell Death Differ* 2001;8:1143-1156.
- 44 Lang KS, Roll B, Myssina S, Schittenhelm M, Scheel-Walter HG, Kanz L, Fritz J, Lang F, Huber SM, Wieder T: Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. *Cell Physiol Biochem* 2002;12:365-372.
- 45 Lang PA, Warskulat U, Heller-Stilb B, Huang DY, Grenz A, Myssina S, Duszenko M, Lang F, Haussinger D, Vallon V, Wieder T: Blunted apoptosis of erythrocytes from taurine transporter deficient mice. *Cell Physiol Biochem* 2003;13:337-346.
- 46 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-C1560.
- 47 Henson PM, Bratton DL, Fadok VA: The phosphatidylserine receptor: a crucial molecular switch? *Nat Rev Mol Cell Biol* 2001;2:627-633.
- 48 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.
- 49 Eda S, Sherman IW: Cytoadherence of malaria-infected red blood cells involves exposure of phosphatidylserine. *Cell Physiol Biochem* 2002;12:373-384.
- 50 Lang KS, Lang PA, Bauer C, Duranton C, Wieder T, Huber SM, Lang F: Mechanisms of suicidal erythrocyte death. *Cell Physiol Biochem* 2005;15:195-202.
- 51 Barvitenko NN, Adragna NC, Weber RE: Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance. *Cell Physiol Biochem* 2005;15:1-18.
- 52 Bosman GJ, Willekens FL, Werre JM: Erythrocyte aging: a more than superficial resemblance to apoptosis? *Cell Physiol Biochem* 2005;16:1-8.
- 53 Schwarzer E, Kühn H, Valente E, Arese P: Band 3/COMPLEMENT-mediated Recognition and Removal of Normally Senescent and Pathological Human Erythrocytes. *Cell Physiol Biochem* 2005;16:133-146.
- 54 Rice L, Alfrey CP: The negative regulation of red cell mass by neocytolysis: physiologic and pathophysiologic manifestations. *Cell Physiol Biochem* 2005;15:245-250.
- 55 Jacobs T, Darji A, Frahm N, Rohde M, Wehland J, Chakraborty T, Weiss S: Listeriolysin O: cholesterol inhibits cytolysis but not binding to cellular membranes. *Mol Microbiol* 1998;28:1081-1089.
- 56 Darji A, Chakraborty T, Niebuhr K, Tsonis N, Wehland J, Weiss S: Hyperexpression of listeriolysin in the nonpathogenic species *Listeria innocua* and high yield purification. *J Biotechnol* 1995;43:205-212.
- 57 Premaratne RJ, Lin WJ, Johnson EA: Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. *Appl Environ Microbiol* 1991;57:3046-3048.
- 58 Darji A, Niebuhr K, Hense M, Wehland J, Chakraborty T, Weiss S: Neutralizing monoclonal antibodies against listeriolysin: mapping of epitopes involved in pore formation. *Infect Immun* 1996;64:2356-2358.
- 59 Huber SM, Gamper N, Lang F: Chloride conductance and volume-regulatory non-selective cation conductance in human red blood cell ghosts. *Pflügers Arch* 2001;441:551-558.
- 60 Barry PH, Lynch JW: Liquid junction potentials and small cell effects in patch-clamp analysis. *J Membr Biol* 1991;121:101-117.
- 61 Huber SM, Uhlemann AC, Gamper NL, Duranton C, Kremsner PG, Lang F: Plasmodium falciparum activates endogenous Cl⁻ channels of human erythrocytes by membrane oxidation. *EMBO J* 2002;21:22-30.
- 62 Birka C, Lang PA, Kempe DS, Hoefling L, Tanneur V, Duranton C, Nammi S, Henke G, Myssina S, Krikov M, Huber SM, Wieder T, Lang F: Enhanced susceptibility to erythrocyte "apoptosis" following phosphate depletion. *Pflügers Arch* 2004;448:471-477.
- 63 Kempe DS, Lang PA, Duranton C, Akel A, Lang KS, Huber SM, Wieder T, Lang F: Enhanced programmed cell death of iron-deficient erythrocytes. *FASEB J* 2006;20:368-370.
- 64 Lang PA, Beringer O, Nicolay JP, Amon O, Kempe DS, Hermle T, Attanasio P, Akel A, Schafer R, Friedrich B, Risler T, Baur M, Olbricht CJ, Zimmerhackl LB, Zipfel PF, Wieder T, Lang F: Suicidal death of erythrocytes in recurrent hemolytic uremic syndrome. *J Mol Med* 2006;84:378-388.
- 65 Kempe DS, Akel A, Lang PA, Hermle T, Biswas R, Muresanu J, Friedrich B, Dreischer P, Wolz C, Schumacher U, Peschel A, Gotz F, Doring G, Wieder T, Gulbins E, Lang F: Suicidal erythrocyte death in sepsis. *J Mol Med* 2007;85:269-277.
- 66 Brand VB, Sandu CD, Duranton C, Tanneur V, Lang KS, Huber SM, Lang F: Dependence of Plasmodium falciparum in vitro growth on the cation permeability of the human host erythrocyte. *Cell Physiol Biochem* 2003;13:347-356.
- 67 Lang PA, Schenck M, Nicolay JP, Becker JU, Kempe DS, Lupescu A, Koka S, Eisele K, Klarl BA, Rubben H, Schmid KW, Mann K, Hildenbrand S, Heffter H, Huber SM, Wieder T, Erhardt A, Haussinger D, Gulbins E, Lang F: Liver cell death and anemia in Wilson disease involve acid sphingomyelinase and ceramide. *Nat Med* 2007;13:164-170.
- 68 Lang PA, Kaiser S, Myssina S, Birka C, Weinstock C, Northoff H, Wieder T, Lang F, Huber SM: Effect of Vibrio parahaemolyticus haemolysin on human erythrocytes. *Cell Microbiol* 2004;6:391-400.

- 69 Lang PA, Kempe DS, Myssina S, Tanneur V, Birka C, Laufer S, Lang F, Wieder T, Huber SM: PGE(2) in the regulation of programmed erythrocyte death. *Cell Death Differ* 2005;12:415-428.
- 70 Shumilina E, Kiedaisch V, Akkel A, Lang P, Hermle T, Kempe DS, Huber SM, Wieder T, Laufer S, Lang F: Stimulation of suicidal erythrocyte death by lipoxygenase inhibitor Bay-Y5884. *Cell Physiol Biochem* 2006;18:233-242.
- 71 Lang PA, Kempe DS, Tanneur V, Eisele K, Klarl BA, Myssina S, Jendrosseck V, Ishii S, Shimizu T, Waidmann M, Hessler G, Huber SM, Lang F, Wieder T: Stimulation of erythrocyte ceramide formation by platelet-activating factor. *J Cell Sci* 2005;118:1233-1243.
- 72 Lang PA, Huober J, Bachmann C, Kempe DS, Sobiesiak M, Akel A, Niemoeller OM, Dreischer P, Eisele K, Klarl BA, Gulbins E, Lang F, Wieder T: Stimulation of erythrocyte phosphatidylserine exposure by paclitaxel. *Cell Physiol Biochem* 2006;18:151-164.
- 73 Nicolay JP, Schneider J, Niemoeller OM, Artunc F, Portero-Otin M, Haik G, Jr., Thornalley PJ, Schleicher E, Wieder T, Lang F: Stimulation of suicidal erythrocyte death by methylglyoxal. *Cell Physiol Biochem* 2006;18:223-232.
- 74 Akel A, Hermle T, Niemoeller OM, Kempe DS, Lang PA, Attanasio P, Podolski M, Wieder T, Lang F: Stimulation of erythrocyte phosphatidylserine exposure by chlorpromazine. *Eur J Pharmacol* 2006;532:11-17.
- 75 Niemoeller OM, Akel A, Lang PA, Attanasio P, Kempe DS, Hermle T, Sobiesiak M, Wieder T, Lang F: Induction of eryptosis by cyclosporine. *Naunyn Schmiedeberg Arch Pharmacol* 2006;374:41-49.
- 76 Bentzen P, Lang E, Lang F: Curcumin induced suicidal erythrocyte death. *Cell Physiol Biochem* 2007;19:153-164.
- 77 Nicolay JP, Gatz S, Liebig G, Gulbins E, Lang F: Amyloid induced suicidal erythrocyte death. *Cell Physiol Biochem* 2007;19:175-184.
- 78 Kempe DS, Lang PA, Eisele K, Klarl BA, Wieder T, Huber SM, Duranton C, Lang F: Stimulation of erythrocyte phosphatidylserine exposure by lead ions. *Am J Physiol Cell Physiol* 2005;288:C396-C402.
- 79 Eisele K, Lang PA, Kempe DS, Klarl BA, Niemoller O, Wieder T, Huber SM, Duranton C, Lang F: Stimulation of erythrocyte phosphatidylserine exposure by mercury ions. *Toxicol Appl Pharmacol* 2006;210:116-122.
- 80 Roth RI, Levin J: Transfusion of soluble hemoglobin. *N Engl J Med* 2000;343:1273.
- 81 Russell JA: Management of sepsis. *N Engl J Med* 2006;355:1699-1713.
- 82 Pawloski JR, Hess DT, Stamler JS: Export by red blood cells of nitric oxide bioactivity. *Nature* 2001;409:622-626.
- 83 Sprague RS, Stephenson AH, Dimmitt RA, Weintraub NL, Branch CA, McMurdo L, Lonigro AJ, Weintraub NA: Effect of L-NAME on pressure-flow relationships in isolated rabbit lungs: role of red blood cells. *Am J Physiol* 1995;269:H1941-H1948.
- 84 Sprague RS, Ellsworth ML, Stephenson AH, Lonigro AJ: ATP: the red blood cell link to NO and local control of the pulmonary circulation. *Am J Physiol* 1996;271:H2717-H2722.
- 85 Andrews DA, Low PS: Role of red blood cells in thrombosis. *Curr Opin Hematol* 1999;6:76-82.
- 86 Bergfeld GR, Forrester T: Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. *Cardiovasc Res* 1992;26:40-47.
- 87 Luthje J, Schomburg A, Ogilvie A: Demonstration of a novel ecto-enzyme on human erythrocytes, capable of degrading ADP and of inhibiting ADP-induced platelet aggregation. *Eur J Biochem* 1988;175:285-289.
- 88 Pawloski JR, Swaminathan RV, Stamler JS: Cell-free and erythrocytic S-nitrosohemoglobin inhibits human platelet aggregation. *Circulation* 1998;97:263-267.
- 89 Valles J, Santos MT, Aznar J, Osa A, Lago A, Cosin J, Sanchez E, Broekman MJ, Marcus AJ: Erythrocyte promotion of platelet reactivity decreases the effectiveness of aspirin as an antithrombotic therapeutic modality: the effect of low-dose aspirin is less than optimal in patients with vascular disease due to prothrombotic effects of erythrocytes on platelet reactivity. *Circulation* 1998;97:350-355.
- 90 Gallagher PG, Chang SH, Rettig MP, Neely JE, Hillery CA, Smith BD, Low PS: Altered erythrocyte endothelial adherence and membrane phospholipid asymmetry in hereditary hydrocytosis. *Blood* 2003;101:4625-4627.
- 91 Bortner CD, Hughes FM, Jr., Cidlowski JA: A primary role for K⁺ and Na⁺ efflux in the activation of apoptosis. *J Biol Chem* 1997;272:32436-32442.