Aus dem Institut für Physiologie der Universität Tübingen
Abteilung Physiologie I
Abteilungsleiter: Professor Dr. F. Lang

Sepsis-induced erythrocytes apoptosis

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard-Karls-Universität
zu Tübingen

vorgelegt von
Ahmad Akel
aus
Damaskus, Syrien

2007

Dekan	Professor Dr. I. B. Autenrieth
1. Berichterstatter	Professor Dr. F. Lang
2. Berichterstatter	Professor Dr. M. Bamberg

Dedicated to two hearts of love and
four hands of gold, to my parents

أبي الغالي, أمي الحنون

Zusammenfassung

Hintergrund

Anämie ist eine der hämatologischen Dysfunktionen bei Sepsis; sie ist vermutlich die Folge einer beschleunigten Clearance der Erythrozyten aus dem Blutstrom. Die zugrunde liegenden Mechanismen wurden bisher nicht aufgeklärt. Kürzlich durchgeführte Untersuchungen ergaben, dass sowohl eine erhöhte zytosolische Ca2+-Konzentration als auch eine erhöhte Ceramid-Konzentration das „Scrambling“ der Erythrozytenzellmembran auslösen, das zur Expression von Phosphatidylserin auf der Erythrozytenoberfläche führt.

Methoden

Erythrozyten gesunder Probanden wurden mit Plasma von Sepsispatienten bzw. dem Überstand verschiedener pathogener Bakterienstämme inkubiert. Anschließend wurden die Phosphatidylserinexpression (Annexinbindung), das Zellvolumen („forward scatter“), die intrazelluläre Ca2+-Aktivität (Fluo3-Fluoreszenz) sowie die Ceramidbildung (Anti-Ceramid-Antikörper) mittels FACS-Analyse bestimmt.
Die Aktivität der Sphingomyelinase in den Bakterienüberständen wurde mit Hilfe radioaktiver Methoden gemessen.

Ergebnisse

Die Inkubation normaler Erythrozyten der Blutgruppe 0 mit Plasma von Sepsispatienten, nicht aber mit Plasma gesunder Probanden, löste eine Annexinbindung aus. Der Effekt des Patientenplasmas auf die Annexinbindung an die Erythrozyten ging mit der Bildung von Ceramid sowie einem signifikanten Anstieg der zytosolischen Ca2+-Aktivität einher. Daraus ergibt sich, dass das Plasma von Sepsispatienten eine oder mehrere Komponenten enthält, die Apoptose bei Erythrozyten (Eryptose) induzieren.
Die Inkubation von Erythrozyten mit dem Überstand von Sepsis-auslösenden Pathogenen induzierte in ähnlicher Weise Eryptose, ein Effekt, der mit der Aktivität der Sphingomyelinase im Überstand korrelierte.

Schlussfolgerung

Inkubation mit Plasma von Sepsispatienten löst in Erythrozyten einen Ca2+-Einstrom und Ceramid-Bildung aus. Dies führt zur Schrumpfung der Erythrozyten, dem „Scrambling“ der Membranlipide und anschließender Oberflächenexpression von Phosphatidylserin.

Die Phosphatidylserin-exprimierenden Erythrozyten können an die Gefäßwände adherieren und werden wahrscheinlich aus dem Blutstrom entfernt. Die vorliegenden Ergebnisse zeigen einen neuen pathophysiologischen Mechanismus auf, der bei Sepsis zu Störung der Mikrozirkulation und zu Anämie führt.

Index

1.Summary

1.1 Background

Anaemia is one of the haematological dysfunctions of sepsis, which presumably results from accelerated clearance of erythrocytes from circulating blood. The underlying mechanisms, however, remained hitherto elusive. Most recent studies disclosed that increased cytosolic Ca^{2+} and ceramide both trigger erythrocyte cell membrane scrambling leading to phosphatidylserine exposure at the erythrocyte surface.

Phosphatidylserine exposing erythrocytes may either adhere to vascular walls or may be engulfed by macrophages equipped with phosphatidylserine receptors.

1.2 Methods

Erythrocytes from healthy volunteers were exposed to plasma of patients suffering from sepsis or supernatant from different strains of pathogens, and phosphatidylserine exposure (annexin binding), cell volume (forward scatter), cytosolic Ca^{2+} activity (Fluo3 fluorescence) and ceramide formation (anti-ceramide antibody) were determined by FACS analysis.

The sphingomyelinase activity in the bacterial supernatants was measured by radioactive technique.

1.3 Findings

Exposure of healthy, blood group zero erythrocytes to plasma from sepsis patients but not from healthy individuals triggered annexin binding. The effect of patient's plasma on erythrocyte annexin binding was paralleled by formation of ceramide and a significant increase of cytosolic Ca^{2+} activity. Thus, the plasma from sepsis patients contains one or more components that trigger erythrocyte apoptosis (eryptosis).

Exposure of erythrocytes to supernatant of sepsis-inducing pathogens

similarly induced eryptosis, an effect correlating with the sphingomyelinase activity in the supernatant.

1.4 Interpretation

Exposure of erythrocytes to plasma from septic patients triggers Ca^{2+} entry and ceramide formation in erythrocytes leading to erythrocyte shrinkage, cell membrane lipid scrambling and subsequent phosphatidylserine exposure.

The phosphatidylserine exposing erythrocytes may adhere to the vascular wall and are presumably cleared from circulating blood. The present observations disclose a novel pathophysiological mechanism leading to derangement of microcirculation and anaemia during sepsis.

2. Introduction

2.1 Apoptosis

Apoptosis (programmed cell death) is an important phenomenon that plays important physiological and pathophsiological rules, such as the physiological rule during the embryonic development in the sculpturing organ shape and carving out the interdigital webs of the fingers and toes (Renehan, Booth, & Potten 2001). Apoptosis has an important role in fetal malformation (Norimura et al. 1996) in addition to the known role of apoptosis in the development of the nervous system and the immune system.

The pathophysiological role of apoptosis is clear through the relationship between much diseases and apoptosis, such as neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis); ischemic injury after myocardial infarction, stroke, and reperfusion; and in autoimmune diseases such as hepatitis and graft versus host disease (Renehan, Booth, & Potten 2001), not to forget that the cell apoptotic disorders are often observed in much malignancies and cancer diseases (Wyllie et al. 1999).

Abundant, defective or potentially harmful nucleated cells are disposed by apoptosis (Bergamo, Luongo, & Rossi 2004;Brand et al. 2003;Green & Reed 1998;Gulbins et al. 2000;Long et al. 2003;Sturm et al. 2004;Wenzel & Daniel 2004), which is triggered either by stimulation of respective death receptors such as CD95 (Daniel et al. 2001;Lang et al. 1999), or by cell exposure to stressors such as oxidants, cytostatic drugs, radiation or osmotic shock ((Bortner & Cidlowski 1999;Green & Reed 1998;Rosette & Karin 1996;Wieder et al. 2001), see also Figure.1).

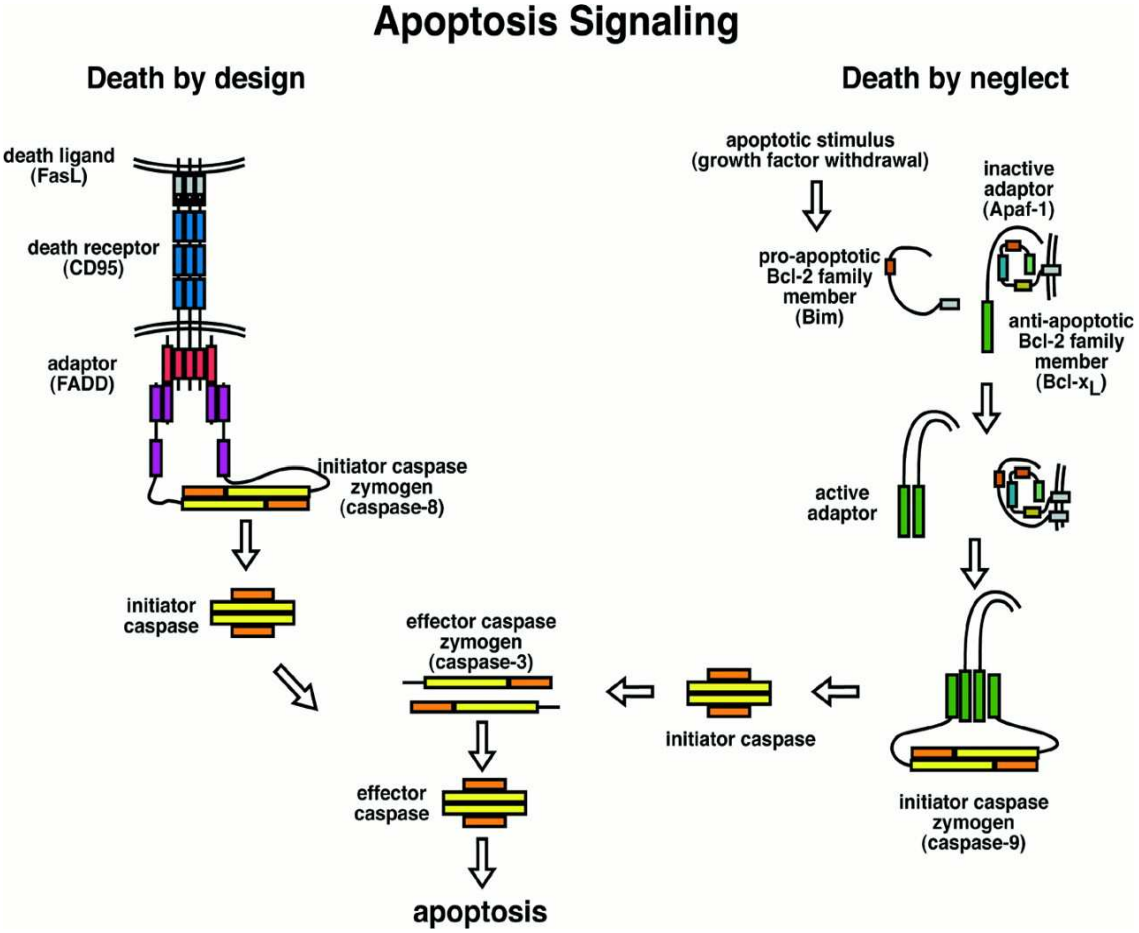


Figure.1: Scheme of apoptotic signaling from (Strasser, O'Connor, & Dixit 2000) The scheme shows the activation of the caspase cascade after stimulation of death receptors at the cell membrane (left) or after stimulation of the cytosolic apoptosome (right). For details see text.

Typical apoptosis is paralleled by cell shrinkage, nuclear condensation, DNA fragmentation, mitochondrial depolarization, cell membrane blebbing and breakdown of phosphatidylserine asymmetry of the plasma membrane, and associated with a loss of intracellular potassium (Bortner & Cidlowski 2002;Bortner & Cidlowski 2004;Grassme et al. 2000;Green & Reed 1998;Gulbins, Jekle, Ferlinz, Grassme, & Lang 2000;Han et al. 2004;Lang et al. 1998;Lang et al. 2000;Maeno et al. 2000;Myssina et al. 2004a;Okada et al. 2001;Yu 2003;Yu, Canzoniero, & Choi 2001;Yurinskaya et al. 2005a;Yurinskaya et al. 2005b) (Figure.2)**.** Cells exposing phosphatidylserine at the cell surface are recognized

by macrophages which are equipped with receptors specific for phosphatidylserine (Fadok et al. 2000b) and rapidly engulf and degrade the affected cells (Boas, Forman, & Beutler 1998;Eda & Sherman 2002). Accordingly, apoptosis allows the elimination of those cells without release of intracellular proteins which would otherwise cause inflammation (Gulbins, Jekle, Ferlinz, Grassme, & Lang 2000).

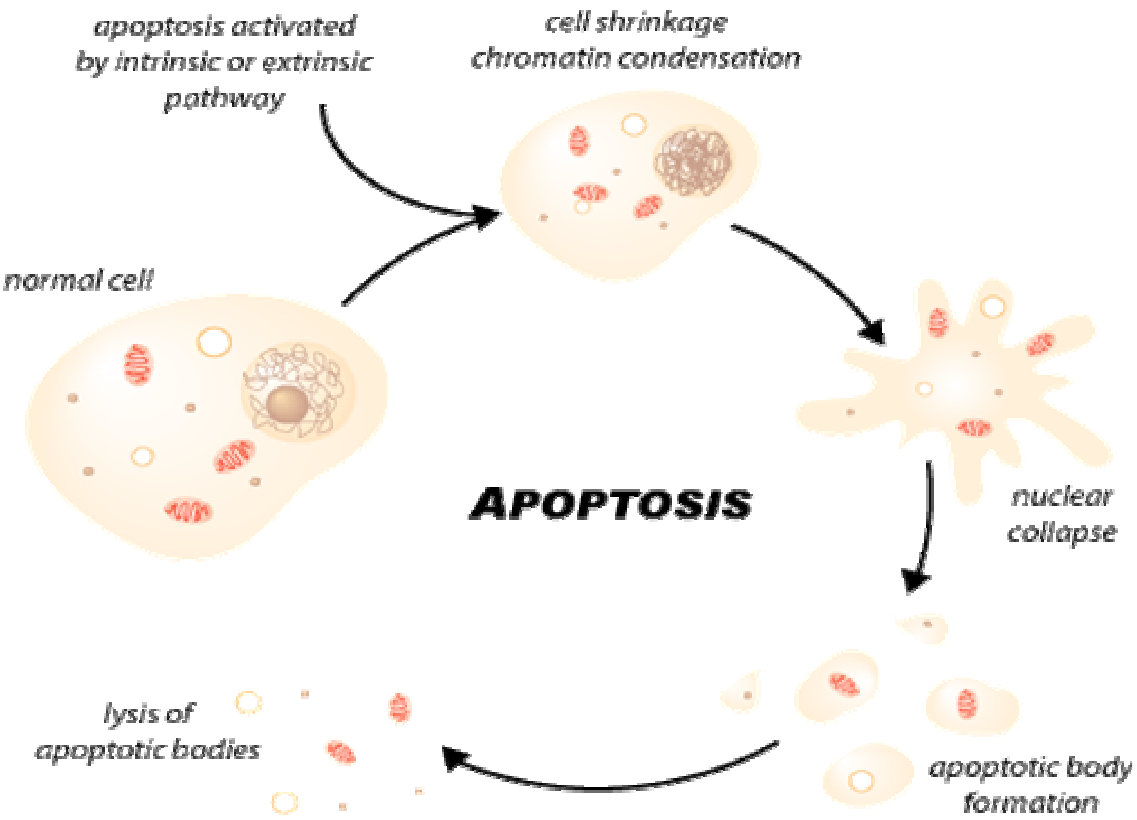


Figure.2: The morphological changes in apoptotic nucleated cells (from Bio Teach, Phillip Yau, and graphics: Jen Philpot, http://www.bioteach.ubc.ca/CellBiology/Apoptosis/index.htm). The scheme shows the different stages of apoptotic cell death in vitro. In vivo, apoptotic bodies are immediately cleared by phagocytosis. For details see text.

2.2. Erythrocytes apoptosis (eryptosis)

Erythrocytes are devoid of nuclei and mitochondria and thus lack crucial

elements in the apoptosis machinery. Thus, until recently, dying erythrocytes have been considered to be eliminated by mechanisms other than apoptosis. Exposure of erythrocytes to the Ca^{2+} ionophore ionomycin, however, triggers cell shrinkage, membrane blebbing and phosphatidylserine exposure, all typical features of apoptotic, nucleated cells (Berg et al. 2001;Bratosin et al. 2001;Daugas, Cande, & Kroemer 2001). The cell shrinkage results from activation of the Ca^{2+}-sensitive K^+ channels, i.e. "Gardos channels" (Lang et al. 2003c), and the phosphatidylserine exposure has previously been thought to result from the activation of a Ca^{2+}-sensitive scramblase (Dekkers et al. 2002;Woon et al. 1999;Zhou et al. 2002) and/or inhibition of a Ca^{2+}-sensitive and ATP-dependent aminophospholipid translocase, i.e. ”flippase” (Seigneuret & Devaux 1984).

In view of the similarities to and differences from the apoptosis program of nucleated cells (for details see also Table 1), the term eryptosis has been coined to describe the suicidal death of erythrocytes (Lang et al. 2005a). Eryptosis may be distinct from the mechanisms involved in erythrocyte ageing (Arese, Turrini, & Schwarzer 2005;Bosman, Willekens, & Werre 2005;Kiefer & Snyder 2000) or neocytolysis, the death of newly formed erythrocytes (Rice & Alfrey 2005).

Recent in vitro experiments disclosed a novel mechanism affecting erythrocyte survival. Erythrocytes exposed to oxidative stress, osmotic shock or energy depletion activate a Ca^{2+}-permeable cation channels which are inhibited by amiloride (1 mM), which further blunts erythrocytes annexin binding following osmotic shock, oxidative stress and energy depletion (Lang, Duranton, Poehlmann, Myssina, Bauer, Lang, Wieder, & Huber 2003a). The activation of these channels will subsequently allow the entry of Ca^{2+} (Fig.3). Ca^{2+} then activates Ca^{2+}-sensitive K^+ channels, i.e. "Gardos channels", leading to cell shrinkage by the efflux of K^+ which drives Cl^- out of the cell. The loss of KCl with the osmotically obliged water then leads to the observed cell shrinkage, an effect that can be blunted by increasing the extracellular K^+ concentration and exposing the cells to Gardos channel inhibitors, such as charybdotoxin or clotrimazole (Lang, Kaiser, Myssina, Wieder, Lang, & Huber 2003c). Increasing of cytosolic Ca^{2+} activity also leads to Ca^{2+}-sensitive scrambling of the cell membrane (Woon, Holland, Kable, & Roufogalis 1999).

Table.1: Comparison between apoptosis in nucleated cells and erythrocyte programmed cell death (eryptosis).

Apoptosis	Eryptosis	Literature
Nuclear condensation, DNA fragmentation	Nothing equivalent	(Wyllie 1980)
Dissipation of the mitochondrial membrane potential	Nothing equivalent	(Green & Kroemer 2004;Martinou & Green 2001)
Cellular shrinkage	Cellular shrinkage	(Lang, Ritter, Gamper, Huber, Fillon, Tanneur, Lepple-Wienhues, Szabo, & Gulbins 2000)
Apoptotic bodies	Vesiculation	(Weedon, Searle, & Kerr 1979) (Willekens et al. 2005)
Activation of caspases	In most cases caspase-independent Activation of μ-calpain	(Berg, Engels, Rothbart, Lauber, Renz, Schlosser, Schulze-Osthoff, & Wesselborg 2001;Bratosin, Estaquier, Petit, Arnoult, Quatannens, Tissier, Slomianny, Sartiaux, Alonso, Huart, Montreuil, & Ameisen 2001;Lang et al. 2004b;Wieder, Essmann, Prokop, Schmelz, Schulze-Osthoff, Beyaert, Dorken, & Daniel 2001)1)
Phosphatidylserine exposure on the outer leaflet of the cell membrane	Phosphatidylserine exposure on the outer leaflet of the erythrocyte membrane	(Bratosin, Estaquier, Petit, Arnoult, Quatannens, Tissier, Slomianny, Sartiaux, Alonso, Huart, Montreuil, & Ameisen 2001;Fadok et al. 1992)
Expression of different death receptors	Expression of CD95/FAS	(Daniel, Wieder, Sturm, & Schulze-Osthoff 2001;Mandal et al. 2005)
Accumulation of ceramide by	Sphingomyelinase-induced ceramide	(Bose et al. 1995;Hannun 1996;Lang et al.

sphingomyelinase-mediated sphingomyelin breakdown or enhanced ceramide synthesis	formation	2005b;Wieder, Orfanos, & Geilen 1998)
Increase of intracellular Ca^{2+} by release from the endoplasmic reticulum	Activation of Ca^{2+}-permeable cation channels in the erythrocyte membrane	(Lang et al. 2003a;Scorrano et al. 2003)

The scrambling causes transbilayer movement of plasma membrane phospholipids with exposure of phosphatidylserine at the erythrocyte surface (Lang, Duranton, Poehlmann, Myssina, Bauer, Lang, Wieder, & Huber 2003a). The erythrocytes are sensitized towards Ca^{2+} by ceramide (acylsphingosine), which is released following erythrocyte injury such as hyperosmotic shock which activates an erythrocyte sphingomyelinase which causes breakdown of sphingomyelin and formation of ceramide. Treatment of erythrocytes with cell-permeable ceramides induces annexin binding of erythrocytes and cell shrinkage. However, ceramide does not increase Ca^{2+} uptake into erythrocytes, but sensitizes the erythrocyte scramblase for cytosolic Ca^{2+}, thus triggering phosphatidylserine exposure even in the absence of Ca^{2+} entry (Lang, Myssina, Brand, Sandu, Lang, Berchtold, Huber, Lang, & Wieder 2004b).

Enhanced eryptosis leading to a shorter life span has been found in different diseases accompanied with anaemia, e. g. sickle cell anaemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency, iron deficiency and Pb^{+} intoxication (Kempe et al. 2005a;Kempe et al. 2005b;Lang et al. 2002).

Phosphatidylserine exposing erythrocytes may adhere to endothelial cells of the vascular wall and thus impede microcirculation (Closse, Dachary-Prigent, & Boisseau 1999b). On the other hand, macrophages are equipped with receptors specific for phosphatidylserine (Fadok, Bratton, Rose, Pearson, Ezekewitz, & Henson 2000b) and erythrocytes exposing phosphatidylserine at their surface are recognized, engulfed and degraded (Boas, Forman, & Beutler 1998). Thus, erythrocytes exposing phosphatidylserine at their surface are prone to be eliminated from circulating blood (Lang, Lang, Bauer, Duranton, Wieder, Huber, & Lang 2005a) and may undergo similar but not necessarily identical changes as those undergoing senescence (Arese, Turrini, & Schwarzer 2005;Barvitenko,

Adragna, & Weber 2005;Bosman, Willekens, & Werre 2005;Lang, Lang, Bauer, Duranton, Wieder, Huber, & Lang 2005a;Rice & Alfrey 2005) or neocytolysis (Rice & Alfrey 2005)

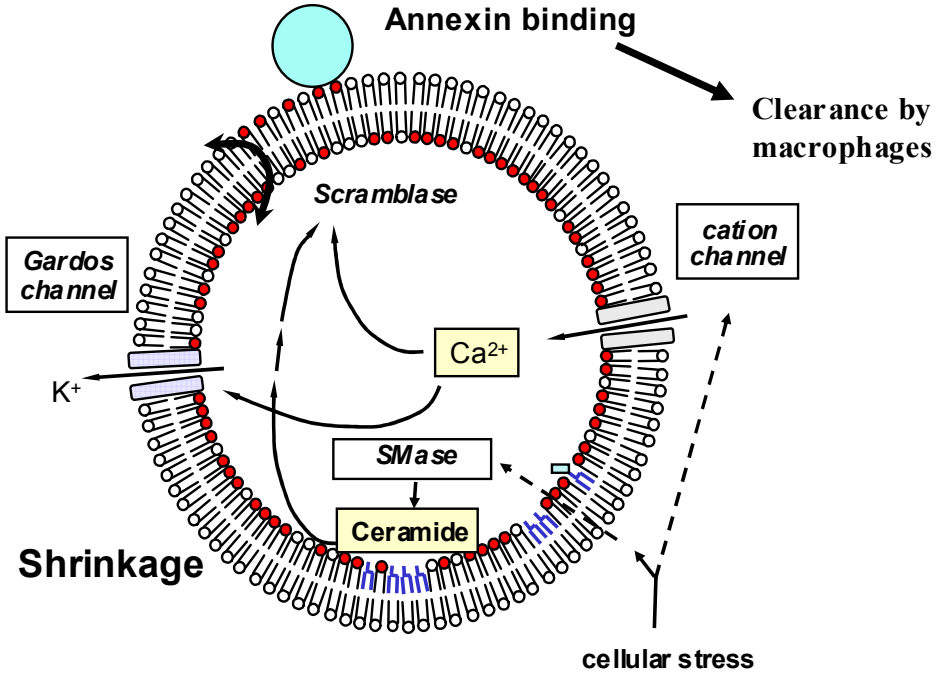


Figure.3: Eryptosis after cellular stress. *The exposing of erythrocytes to different types of stress (oxidative stress, osmotic stress, and energy depletion) causes an increase of cytosolic Ca^{2+} activity leading to activation of a scramblase and breakdown of phosphatidylserine asymmetry, thereby rendering the cell membrane adhesive for annexin V. Ca^{2+} further activates Gardos channels leading to K^{+} efflux (cellular shrinkage). Some types of stress activate an erythrocytic sphingomyelinase leading to ceramide formation which increases phosphatidyserine exposure and sensitizes the Gardos channels for the Ca^{2+} effect. See details (SMase, sphingomyelinase).*

.

2.3. Sepsis

Sepsis is a life threatening condition during overwhelming infection with a variety of pathogens (Afessa et al. 2001;Aird 2003;Cobb & O'Keefe 2004;Moore, Schrag, & Schuchat 2003;Napolitano 2004;Rice & Bernard 2004;Sessler, Perry, & Varney 2004;Singer et al. 2004;Terpos et al. 2004), defined in 1989 by Bone as the systemical hyperinflammatory reaction to the presence of the microorganisms and/or its toxins in the blood (Bone et al. 1989), and by the American College of Chest Physicians/Society of Critical Care Medicine as SIRS (Systemic inflammatory Reaction Syndrome) resulting from infection (bacterial, viral, fungal, or parasitic) (1992). That the prescence of microorganisms (bacteria, viruses, fungi, parasites) or their products such as toxins, cell wall components as lipopolisaccharide (LPS), known also as endotoxin (Bouchon et al. 2001;Dinges & Schlievert 2001), peptidoglycan and lipoteichoic acids (Majcherczyk et al. 1999;Morath, Geyer, & Hartung 2001), and DNA fragments (Sparwasser et al. 1997). These substances activate the cells of the immune system to produce interleukins (IL-1, IL-12, IL-6), tumor necrosis factor alpha (TNF-α) (Michie et al. 1988;Thijs & Hack 1995;van Deventer et al. 1990;Wang et al. 2000), Interferon gamma (INF-γ) (Dinarello 1997;Dinges & Schlievert 2001), which in turn mediate the activation of complement cascade (Haeney 1998) and coagulation cascade (Joost C.M; Sepsis 1999) leading to coagulation disorders, e. g. DIC (Disseminated intravascular coagulation) (Baglin 1996;Gando et al. 1996;Levi et al. 1993;Thijs et al. 1993;Ulevitch et al. 1975), evoking ARDS (Adult Respiratory Distress Syndrome) (Brandtzaeg et al. 1989;Brigham & Meyrick 1986;Macnaughton & Evans 1992;Martin & Silverman 1992;Parsons et al. 1989) and causing direct myocardial depression leading to vasodilatory shock (Landry & Oliver 2001), in addition to activation of production of inflammatory factors (prostaglandins, leukotrines) (van der 2001;van der & van Deventer 1999) which explains the high inflammatory state in sepsis patients. In addition, there is an increase of sphingomyelinase (Smase) activity in the plasma of sepsis patients as compared with healthy plasma (Claus et al. 2005). Sphingomyelinase is the key

enzyme in the initiation of the sphingomyelin/ceramide signaling pathway (Pettus, Chalfant, & Hannun 2002) which has been implicated in the regulation

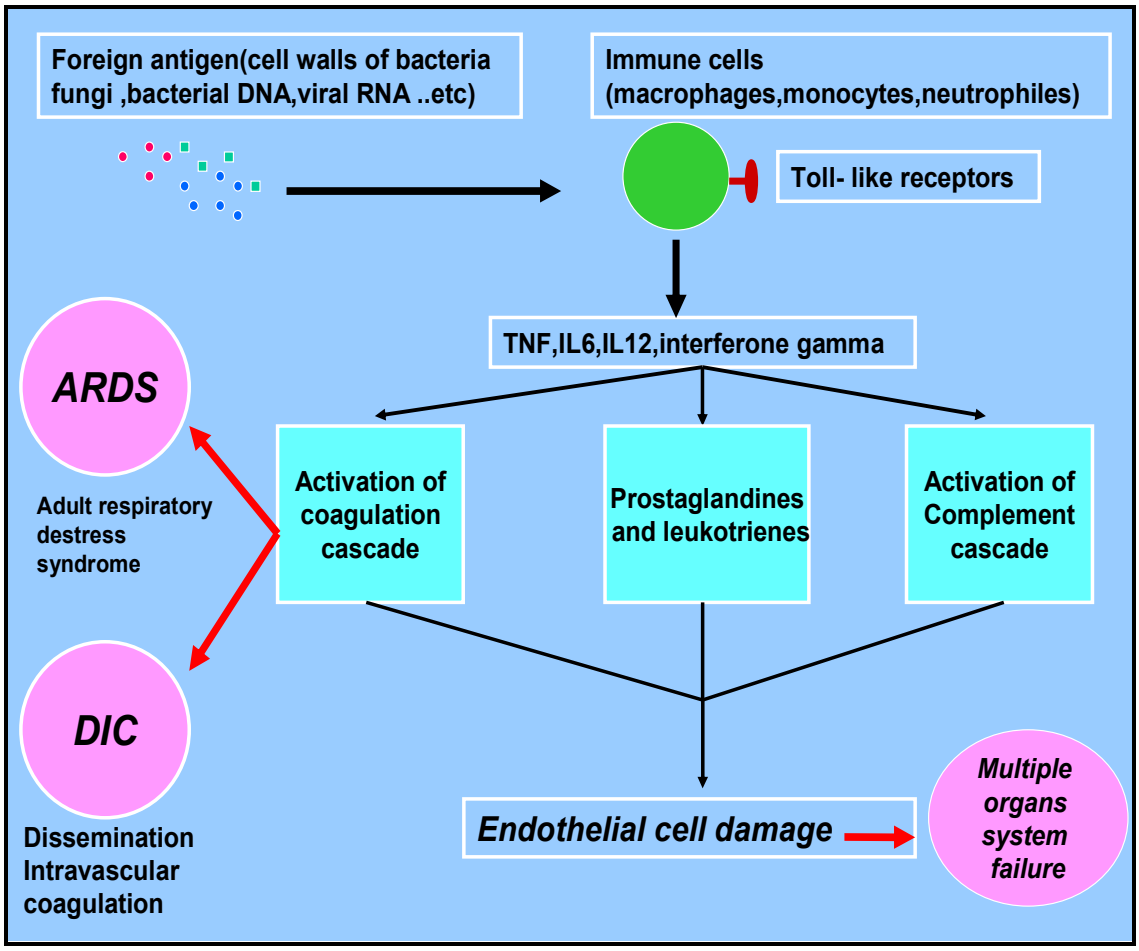


Figure.4: The immunological haematosis and inflammatory reactions in sepsis patients.

The simplified scheme shows these reactions in sepsis patients leading to the multiorgan damage. The pathogens or their products such as (DNA fragments or RNA, cell wall components such as peptidoglycan) induce immunological reaction leading to increasing of inflammatory factors and activation of coagulation and complement cascade, these reaction leads in conclusion to organ damage. See details (TNF, tumor necrosis factor; IL, interleukine; ARDS, adult respiratory distress syndrome; DIC, disseminated intra vascular coagulation).

of immunological processes during inflammation (Baumruker & Prieschl 2002) (see also Figure 4).

According to the excessive inflammatory reaction, the damage of endothelial cells in the different organs (Curzen, Griffiths, & Evans 1994;Cybulsky, Chan, & Movat 1988;Hack & Zeerleder 2001;Lehr, Bittinger, & Kirkpatrick 2000;Mutunga et al. 2001) leads to multiple organs system failure (Beal & Cerra 1994;Bell et al. 1983;Fry et al. 1980). The high death percentage among sepsis patients reaches 30-50%, in spite of the standard supportive care (Angus et al. 2001;Angus & Wax 2001) (Table.2.).

Table.2: Comparison of deaths associated with severe sepsis to those from other diseases in the United States of America (Anderson et al. 2004;Minino et al. 2002;Minino & Smith 2001).

Disease Mortality Chart	Number Of Death
Severe sepsis (Augus 2001)	215.000
Acute Myocardial Infarction (Minino, 2002)	193.000
Lung Cancer (Minino, 2002)	156.000
Colon Cancer (Minino, 2002)	57.000
Breast Cancer (Minino, 2002)	42.000

2.4. Haematological sequelea of sepsis

Haematologic changes are present in virtually every patient with severe sepsis, and it is known that patients with haematologic disorders have increased morbidity and mortality. The faster the identification and treatment of haematologic dysfunction the better is the improvement of survival (Aird 2003).
One characteristic of haematological dysfunction of sepsis includes rapidly developing anaemia which cannot be accounted for by decreased formation of erythrocytes but must involve accelerated clearance of erythrocytes from

circulating blood (Aird 2003;Napolitano 2004).

The pathophysiology of anaemia in sepsis patients have been explained with the following reasons:

- Blood loss (gastrointestinal tract bleeding, phlebotomy and clinical procedures) (Vincent et al. 2002;Zimmerman et al. 1997)
- disseminated intravascular coagulation (DIC), pathogen-associated hemolysis, hypoadrenalism, and nutritional deficiency (Batge et al. 1992;Campillo, Zittoun, & de Gialluly 1988;Rodriguez et al. 2001).
- Chronic disease anemia in some sepsis patients with clinical story of chronic diseases (Krantz 1994)
- reduced production of erythropoietin, impaired bone marrow response to erythropoietin, and decreased red blood cell survival (Jelkmann et al. 1992;Jurado 1997;Krafte-Jacobs et al. 1994;Krafte-Jacobs 1997;Krantz 1994;Rogiers et al. 1997;van Iperen et al. 2000).

However, the decreased survival of erythrocytes may also be due to enhanced eryptosis and accelerated clearance of affected cells from the circulation.

Several sepsis-inducing bacterial pathogens are known to produce ß–toxin (secretory sphingomyelinases) such as *Streptococcus pyrogenic* and *Staphylococcus aureus* (Barsumian et al. 1978;Bohach 1997), which might affect phosphatidylserine exposure of erythrocytes. In addition, data were provided showing an increase of SMase activity in sepsis plasma as compared with healthy plasma (Claus, Bunck, Bockmeyer, Brunkhorst, Losche, Kinscherf, & Deigner 2005). The source of this SMase, however, and whether the bacterial secretory SMases play a role in this increase of enzyme activity are still a matter of debate.

Staphylococcus aureus, one of the most frequent causative agents of sepsis, produces ß–toxin but its role in infections and septic shock has remained unclear (Claus, Bunck, Bockmeyer, Brunkhorst, Losche, Kinscherf, & Deigner 2005)

The present study has been performed to explore whether erythrocyte

phosphatidylserine exposure participates in the pathophysiology of sepsis. The experimental data should further shed some light on the role of bacterial SMase in virulence. Enzymes such as the *S. aureus* ß-toxin might contribute to the severity of sepsis by promoting erythrocyte death and anaemia.

3. Materials and methods

3.1. Materials

3.1.1 Notes on Suppliers

Baxter: Unterschleissheim, Germany
Biochrom AG: Berlin, Germany
Abbott GmbH: Wiesbaden, Germany
Beckman Coulter: Krefeld, Germany
Becton Dickinson: Heidelberg, Germany
Medical Diagnostics Marx: Butzbach, Germany
Wescor: Logan, Utah, USA
Sigma: Taufkirchen, Germany
Calbiochem: Schwalbach, Germany
Roche Diagnostics: Mannheim, Germany
Alexis: Grünberg, Germany
Ancell: Bayport, MN, USA
Pharmingen: Hamburg, Germany
Amersham Biosciences: Freiburg, Germany
Wallac: Freiburg, Germany
Tecan: Crailsheim, Germany
Heraeus, Germany
Eppendorf, Hamburg, Germany
Sorvall, Langenselbold, Germany
Jouan, Germany
Roth, Karlsruhe, Germany
Knick, Germany
Kern, Germany
Sartorius, Germany
Heidolph, Germany

Greiner bio-one, Frickenhausen, Germany
Millipore, Cork, Ireland
Sarstedt, Germany
Abimed, Germany
Labnet, Germany
Fresenius Kabi, Homburg, Germany
Gib co, United Kingdom
Nikon, Düsseldorf, Germany
AHF Analysentechnik, Tübingen, Germany
Visitron Systems, Puchheim, Germany
Invitrogen GmbH, Karlsruhe, Germany
PAA Laboratories, Cölbe, Germany

3.1.2. Equipments

Coulter Epics XL (Beckman Coulter: Krefeld, Germany)
FACS-Calibur: (Becton Dickinson: Heidelberg, Germany)
MDM 905 electronic hematology particle counter (Medical Diagnostics Marx: Butzbach, Germany)
VAPRO 5520 vapor pressure osmometer: (Wescor: Logan, Utah, USA)
ß-Scintillation counter (Wallac: Freiburg, Germany)
Microplate reader.Tecan Sunrise (Tecan: Crailsheim, Germany)
Incubator (Heraeus, Germany)
Centrifuge 5417R (Eppendorf, Hamburg, Germany)
Centrifuge RT 6000 B (Sorvall, Langenselbold, Germany)
Centrifuge MR 1812 (Jouan, Germany)
Mini shaker MS1 (Roth, Karlsruhe, Germany)
PH meter 761 Calimatic (Knick, Germany)
Digital-PH-meter 646 (Knick, Germany)
Balance: (Kern, Germany), (Sartorius, Germany).
Magnetic stirrer MR 3001 (Heidolph, Germany)

3.1.3. Laboratory ware

OptiPure RC quadruple blood pack set (Baxter: Unterschleissheim, Germany)
Fetal calf serum (FCS) (Biochrom AG: Berlin, Germany)
Suspension culture plate 96 W (Greiner bio-one, Frickenhausen, Germany)
Sterile PS-tube 4.5 ml 12.4/75 MM (Greiner bio-one, Frickenhausen, Germany)
Falcon tubes (Greiner bio-one, Frickenhausen, Germany)
Sterile filter (Millipore, Cork, Ireland)
LI-Heparin tubes (Sarstedt, Germany)
Plastic tubes (Eppendorf, Hamburg, Germany)
Pipettes (Eppendorf, Hamburg, Germany. Abimed, Germany. Labnet, Germany)
Tips for pipettes (Greiner bio-one, Frickenhausen, Germany)
Scintillation tubes (Roth, Karlsruhe, Germany)

3.1.4. Chemicals

Ficoll (Biochrom AG: Berlin, Germany)
TrueCount kit, Retic-COUNT (Thiazole orange) reagent (Becton Dickinson: Heidelberg, Germany)
Ionomycin, acetylsalicylic acid, diclophenac, thromboxane B_2, purified sphingomyelinase from *Streptomyces sp* (Sigma: Taufkirchen, Germany)
Annexin-Fluos, ethylenediamine tetraacetic acid (EDTA) (Roche Diagnostics: Mannheim, Germany)
Anti-ceramide antibody, clone MID 15B4 (Alexis: Grünberg, Germany)
Polyclonal fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG + IgM specific antibody (Pharmingen: Hamburg, Germany)
[^{3}H] Scintillation Proximity Assay (SPA) System (Amersham Biosciences: Freiburg, Germany)
Ampuva: sterile water for injection (Fresenius Kabi, Homburg, Germany)
PBS (Phosphate buffer salin) solution (Gibco, United Kingdom)

3.1.5. Solutions

A. Ringer solution

- 125 mM sodium chloride NaCl.
- 5 mM potassium chloride KCl.
- 1 mM magnesium sulphate $MgSO_4$.
- 32 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES).
- 5 mM glucose.
- 1 mM calcium chloride$CaCl_2$.
- pH=7.4.
- Osmolarity is measured by the use of a VAPRO 5520 vapor pressure osmometer
- The solution was passed through a sterile filter (Millipore, Cork, Ireland).

B. Fluo3AM buffer solution

- 123 mM NaCl, 5mM KCl.
- 1 mM $MgSO_4$.
- 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES).
- 10 mM Glucose.
- 2 mM $CaCl_2$.
- 10 mM pyruvate.
- pH = 7.4.

C. Annexin- binding buffer

- 125 mM NaCl.
- 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES).
- 5 mM $CaCl_2$.
- pH=7.4.

D. SMase assay buffer (10 fold)

- 1 M Tris-HCl.

- 50 mM $MgCl_2$.
- 0.05 % NaN_3.
- pH=7.4.

E. CPD buffer

- 3.27 mg/ml citric acid
- 26.30 mg/ml sodium citrate
- 2.50 mg/ml sodium dihydrogenphosphate dihydrate
- 25.50 mg/ml dextrose monohydrate.

F. SAG-M stabilizer solution

- 8.77 mg/ml NaCl
- 9.00 mg/ml dextrose monohydrate
- 0.17 mg/ml adenine
- 5.25 mg/ml mannitol

3.2. Methods

3.2.1. What is flow cytometry?

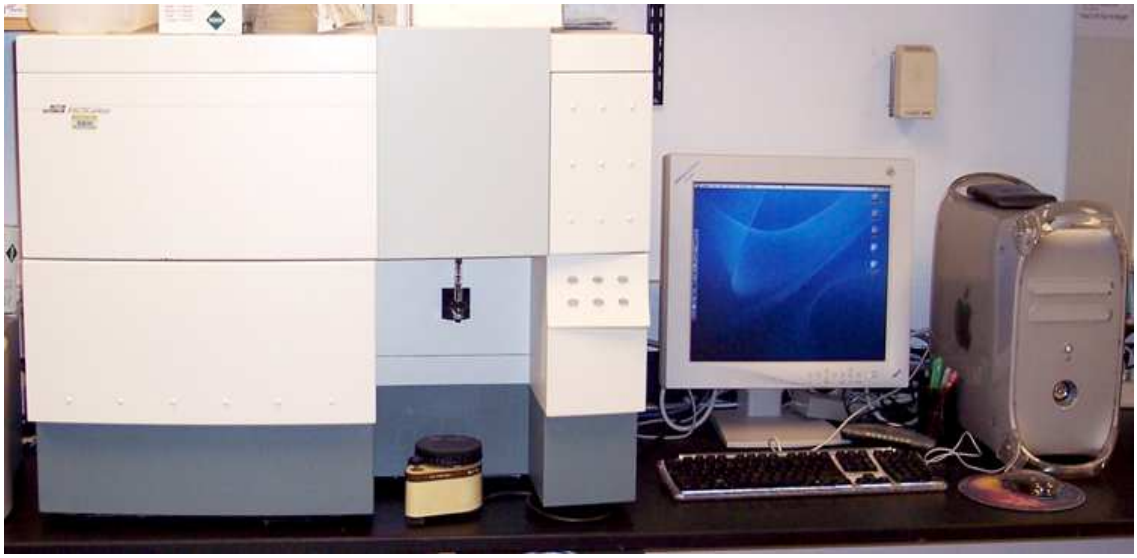


Figure.5. BD FACSCalibur Flow Cytometer from Becton Dickinson.

Flow cytometry is the measurement and characterization of cells as they are flowing in a stream, and to determine their cellular constituents. This is achieved by focusing a laser beam on the cells.

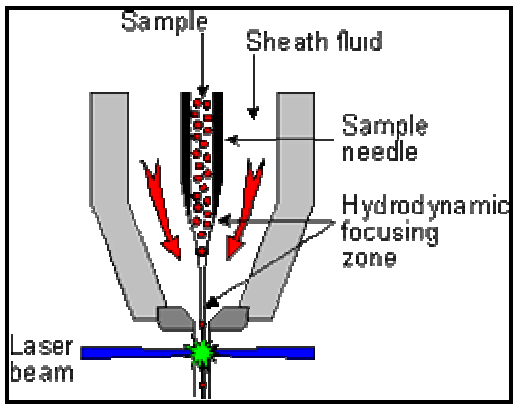
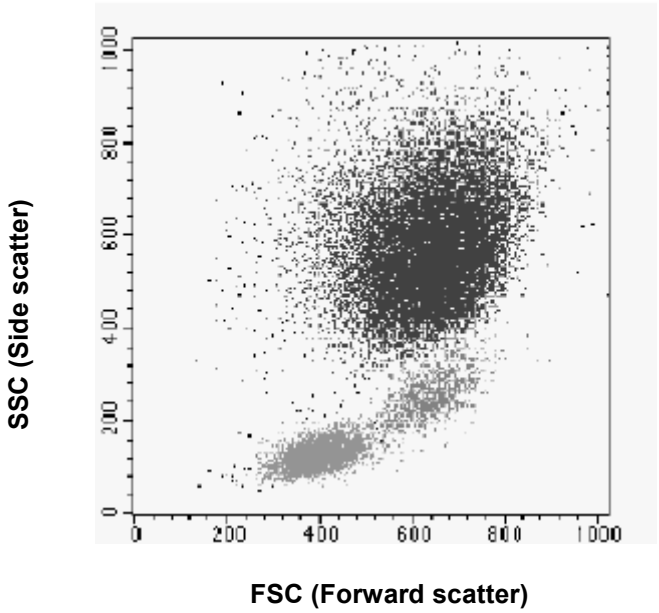


Figure.6 Hydrodynamic Focusing. *The picture shows the flow of cells thereby passing the laser beam. For details see text.*

The laser beam of the fluorescence activated cell sorter is focused on the cells stream flowing as a single file. The cells must be measured one at a time and to have accurate measurements therefore the cells should travel single-file through the stream at the point of laser interrogation. The method of achieving this ordered stream is known as hydrodynamic focusing (Fig.6).

A. Histogram plot



B.

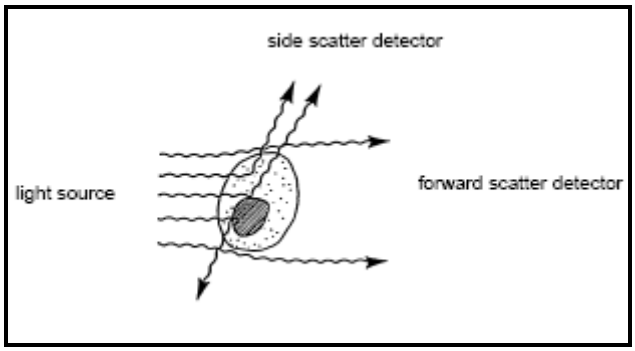


Figure.7. The forward scatter and side scatter phenomenon. *As the photons strike the cells in the stream, the light is scattered in different angles*

Within the flow cell, a slow-moving sample stream is injected into a faster moving "sheath" stream (sheath fluid or FACS Flow).

The laser stream passes through the stream and the majority of its photons will pass through unobstructed. Some of these photons will diverge slightly, primarily via light diffraction, when these photons contact the membranes of passing cells. These scattered photons are then received and collected by a detector placed in line with the laser path (on the opposite side of the stream). Because of the nature of its collection, this parameter is referred to as Forward Scatter (FSC) (Fig. 7). The FSC is proportional to the cell size; the big cells will scatter more light than the small ones causing higher detected signal.

On the other side, many photons will pass through the cytoplasm. When they hit cellular organelles (e. g. the nucleus, the endoplasmic reticulum) they will be reflected at a bigger angle than the forward scatter. For that reason there is a second detector placed perpendicular to the laser path to collect light scattered in this way called as Side Scatter (SSC) (Fig. 7), a parameter proportional to the cell components. The cells with more organelles inside the cytoplasm would cause higher Side Scatter values (SSC).

This measurement is improved by using fluorescent labelling of the cells by fluorescence labelled substances such as: Annexin-V FITC. As these cells pass through the stream, the fluorescent conjugated part, or fluorochrome is excited with the laser light causing emission of photons of a higher wavelength (FITC emits light at ~530 nm when excited by a 488 nm laser) This light is collected to get more information about the cellular fluorescence (a high Annexin-V label of the cells indicates phosphatidylserine exposure at the cell surface).

All FACS measurements were done on a BD FACSCalibur Flow Cytometer from Becton Dickinson (see also Fig. 5).

3.2.2. Blood cell preparation

A. Blood cells and purification of erythrocytes

For erythrocyte signaling experiments highly purified erythrocyte concentrates were used. Human whole blood was drawn from healthy volunteers and erythrocyte concentrates were obtained by filtration (Lang, Kempe, Tanneur, Eisele, Klarl, Myssina, Jendrossek, Ishii, Shimizu, Waidmann, Hessler, Huber, Lang, & Wieder 2005b).

In any case, the purity of the erythrocyte concentrates was measured by different automated procedures as outlined in the protocol section.

Human whole blood was drawn from healthy volunteers and erythrocyte concentrates were obtained using the OptiPure RC quadruple blood pack set equipped with a soft housing red cell filter (Baxter: Unterschleissheim, Germany).

- 500 ml of whole blood was automatically mixed with 70 ml CPD buffer.
- Blood components were separated by centrifugation at 4795 g for 10 min at 22°C.
- Plasma, buffy coat and erythrocytes were then pressed into the respective blood bags.
- During this process SAG-M stabilizer solution was added to the erythrocytes.
- The erythrocytes were passed through the integrated leukocyte depletion filter at room temperature.
- Aliquots of the erythrocyte concentrates were stored at 4°C until usage.
- Alternatively (e. g. for Western blot analyses or in animal experiments), erythrocytes were purified by centrifugation of whole blood for 25 min., 2000 g over Ficoll (Biochrom AG), washed 3 times in phosphate buffered saline (PBS) and centrifuged at 450 g for 5 min.
- Purified erythrocytes should be immediately used for experimental purposes.

B. Analysis of blood cell numbers in whole blood and in erythrocyte concentrates

Platelet numbers in whole blood and in erythrocyte concentrates as well as leukocytes in whole blood were measured using an automated blood cell counter (CellDyn3000) (Abbott GmbH: Wiesbaden, Germany).

- The cells were identified by a combination of their optical and electrical properties in the appropriate counter medium.
- Representative erythrocyte concentrates should not contain more than 3 % of the original platelet number of the respective whole blood samples.
- Leukocyte numbers in erythrocyte concentrates were quantified by flow cytometric analysis on a Coulter Epics XL (Beckman Coulter: Krefeld, Germany) using the internally normalized TrueCount kit from Becton Dickinson. The kit is based on the detection of nucleated cells by the fluorescent DNA-intercalator propidium iodide.
- Erythrocyte concentrates should contain less than 0.1 % of the original leukocyte number of the respective whole blood samples.
- Furthermore, thrombocyte numbers in whole blood and in erythrocyte concentrates were determined by flow cytometric analysis of thiazole orange-stained cells (Kienast & Schmitz 1990) using the Retic-COUNT (Thiazole orange) reagent from Becton Dickinson according to the manufacturer´s instructions.
- Measurements are performed on a FACS-Calibur from Becton Dickinson, and the number of cells in the thrombocyte gate of the respective forward scatter (FSC) versus thiazole orange-fluorescence intensity (FL-1H) dot plots is determined using the CellQuest^{TM} software.
- Erythrocytes, thrombocytes and leukocytes of whole blood and erythrocyte concentrates were further quantified by the use of a MDM 905 electronic hematology particle counter (Medical Diagnostics Marx: Butzbach, Germany).
- For determination of relative reticulocyte numbers 5 µl of whole blood was added to 1 ml Retic-COUNT (Thiazole orange) reagent from Becton Dickinson.

- Samples were stained for 30 min. at room temperature in the dark.
- Then, FACS analysis of stained samples was performed according to the manufacturer´s instructions.

Forward scatter (FSC), side scatter (SSC) and thiazole orange-fluorescence intensity (in the fluorescence channel FL-1) of the blood cells was measured and the percentage of Retic-COUNT positive, gated erythrocytes was calculated.

- Gating of erythrocytes was achieved by analysis of FSC vs SSC dot plots using the CellQuest™ software.

3.2.3. Plasma of healthy volunteers and sepsis patients

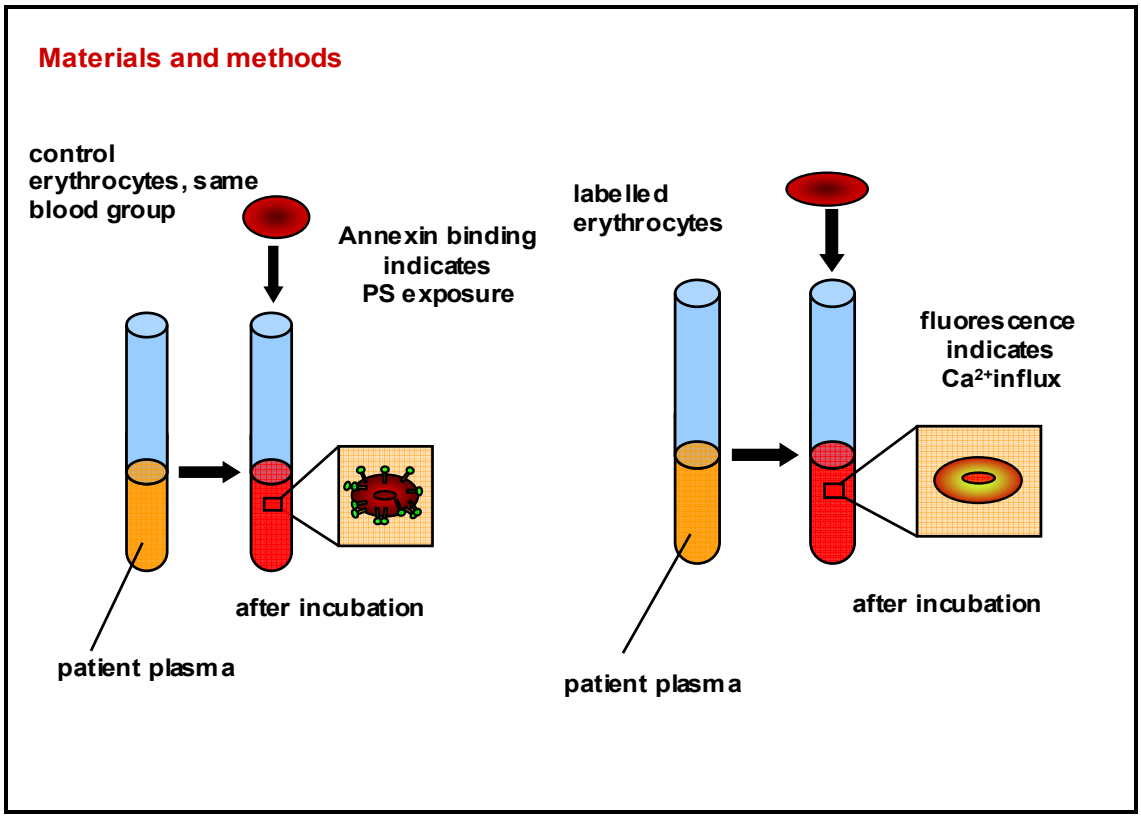


Figure 8. Design of the study. *Sepsis and/or healthy heparinized blood (from the same blood group) was drawn, and then centrifuged to get the heparinized plasma samples. Then, healthy control erythrocytes from the same blood group were incubated in sepsis patient's or healthy volunteer plasma at 37°C in a 5 % CO_2 atmosphere. After incubation, Annexin V binding, Forward scatter, Ca^{2+}*

influx and ceramide were measured as described. For details see text (PS, phosphatidylserine).

Heparinized plasma was isolated from healthy volunteers and from patients suffering from severe sepsis and maintained in the intensive care unit of the University hospital. Table. 3 lists the patients included in the study. The heparinized plasma was added to erythrocytes from healthy volunteers *in vitro.* The relatives of the patients and the volunteers providing erythrocytes gave informed consent. The ethics committee of the University of Tübingen has approved the study.
The scheme of the experiments with sepsis patients is shown in Fig. 8.

3.2.4. Bacterial supernatant preparation

The preparation of bacterial supernatant was done according to one of the two following protocols:

Protocol A

- Different bacterial strains were grown in TSB (Difco Laboratories) medium; pH 7.2, with 180 rpm shaking at 37°C.
- Media were supplemented with glucose to a final concentration of 0.5 % (wt/vol).
- All ingredients were mixed prior to autoclaving, and the medium pH did not change after autoclaving.
- Late-exponential-phase cultures were used to inoculate 50 ml of the same medium.
- The initial $O.D_{578}$ was adjusted to 0.1.
- After over night growth, the cells were harvested by centrifugation at 10,000 rpm for 30 min at 4°C.
- The culture supernatants were filter sterilized by passing through 0.22 µm filter (Millipore) and used for further studies.

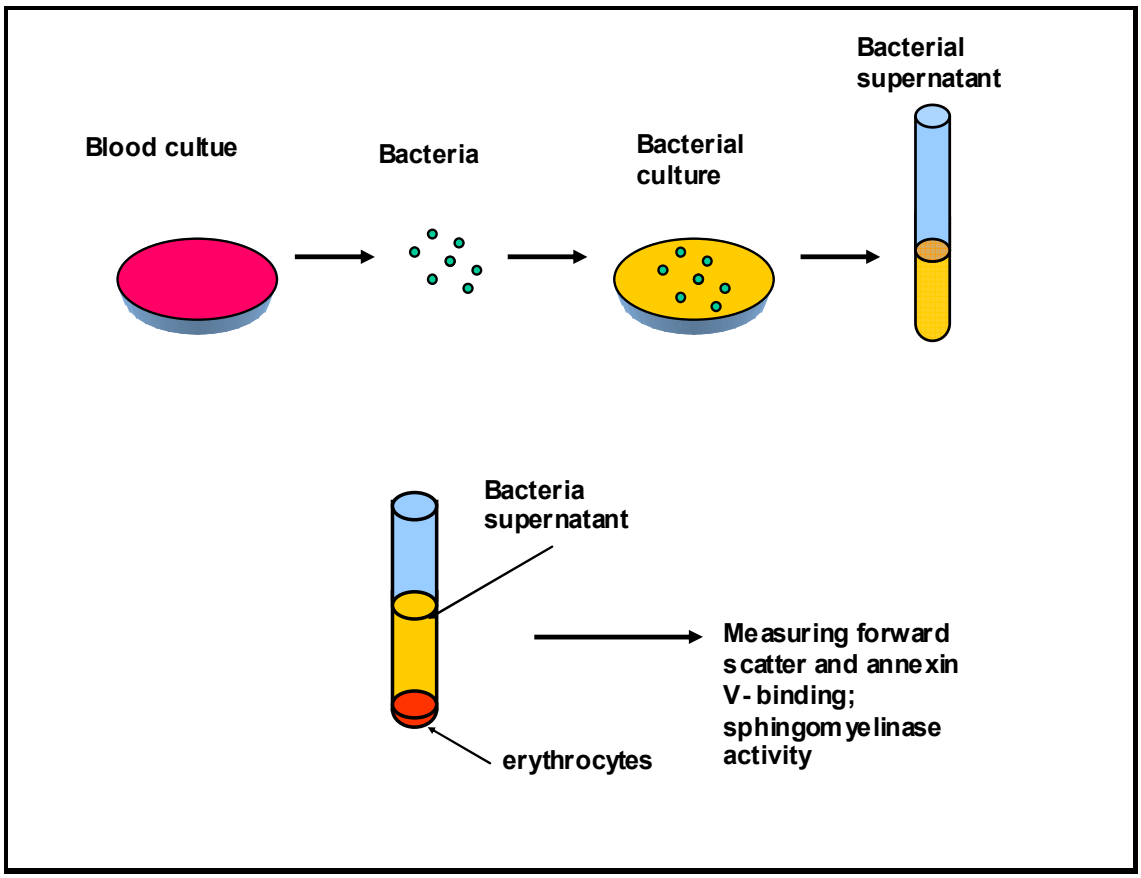


Figure 9. Isolation of the pathogens from patient's blood. *The pathogens were grown in bacterial culture to get the supernatant that has been used later in the annexin binding, haemolysis and sphingomyelinase assay measurements.*

Protocol B

- The isolated bacteria from the sepsis patients as shown in Fig. 9, and special strains of *Staphylococcus aureus* Wild type (8325-4) and Sphingomyelinase deficient *S. aureus* (8325-4Φ13) an isogenic mutant strain whose sphingomyelinase gene (*hlb*) is disrupted because of the integration of a prophage in the *hlb* gene (Goerke, Koller, & Wolz 2006).
- The bacteria strains were grown in soy broth (CM0129; Oxid Inc).

- After overnight cultivation, bacterial supernatants were isolated by centrifugation (10 min, 5,000 rpm, 4°C) and subsequently filtrated (pore size 0.2 µm).
- Bacterial supernatants were stored at -20 °C until usage.
- Supernatants were then assayed for sphingomyelinase activity and for their ability to induce erythrocyte phosphatidylserine exposure as described below.

3.2.5. Endothelial cells

The effect of plasma on annexin binding of human endothelial cells (HMEC-1) was studied using immunofluorescence microscopy.

- HMEC-1 cells were grown in MCDB131 medium (Invitrogen GmbH, Karlsruhe, Germany) supplemented with 15 % fetal calf serum (PAA Laboratories, Cölbe, Germany), 50 µg/ml endothelial cell growth supplement (Becton Dickinson: Heidelberg, Germany), 1 µg/ml hydrocortisone (Becton Dickinson: Heidelberg, Germany) and 0.5 % gentamicin (Sigma: Taufkirchen, Germany).
- Cells were seeded on Poly-L-lysine-coated glass cover slips (0.01 % Poly-L-lysine solution (Sigma: Taufkirchen, Germany).
- The cells were then treated for 24 h in HMEC-1 growth medium containing either 50 % healthy control plasma or 50 % sepsis plasma.
- The cells were incubated for 24 h at 37°C in a 5 % CO_2 atmosphere.
- Tthe cells were washed twice with annexin-binding buffer, stained with Annexin-V-Fluos in annexin-binding buffer (1:50 dilution), and washed again.
- Finally, the cells were analyzed using a fluorescence microscope (Nikon, Düsseldorf, Germany. AHF Analysentechnik, Tübingen, Germany) with 440/480 nm excitation and 535/550-nm emission wavelength.
- Digital pictures were taken using a digital imaging system (Visitron Systems, Puchheim, Germany) equipped with the Metaview software.

3.2.6. Sphingomyelinase (SMase) assay

SMase content in bacterial supernatants was measured using the Sphingomyelinase [^{3}H] SPA (scintillation proximity assay) enzyme assay (code TRKQ7140) from Amersham Biosciences according to the manufacturer´s protocol. Briefly,

- Every sample was prepared by adding:
  - 10 µl bacterial supernatant (or 10 µl of medium)
  - 10 µl of 10-fold- enzyme buffer containing 1 M Tris-HCl (pH 7.4), 50 mM $MgCl_2$ and 0.05 % NaN_3 (the final volume of the sample will be 100 µl, this means 10 times dilution of this 10-fold buffer)
  - 70 µl distilled water
  - 10 µl tracer solution (biotinylated [^{3}H]-labelled sphingomyelin)
  - The samples were mixed carefully using the pipette (no vortexing).
  - All the last steps were done on ice (4 C°)
- After addition of the tracer (the radioactive substance), the samples were incubated at 37˚C with soft shaking for 30 - 60 min.
- Then, the reaction was stopped by adding 20 µl of stop reagent containing
  - 2 M Glycin-HCl (pH 3.6)
  - 0.05 M ethylenediamine tetraacetic acid (EDTA),
  - 0.2 % Triton X-100 and
  - 0.05 % (w/v) NaN_3
  - Streptavidin-coated tritium silicate SPA beads
  - Before adding the stop reagent it should be shaken carefully to achieve a homogenous cloudy form of the suspended beads.
  - When adding this stop reagent to the samples it should be mixed carefully using the pipette.
- Finally, the samples were counted in a ß-scintillation counter (Wallac: Freiburg, Germany)
- The activity of SMase in the samples was calculated from a SMase

calibration curve (0.3 - 80 mU/tube), which was run in parallel (Fig. 11).

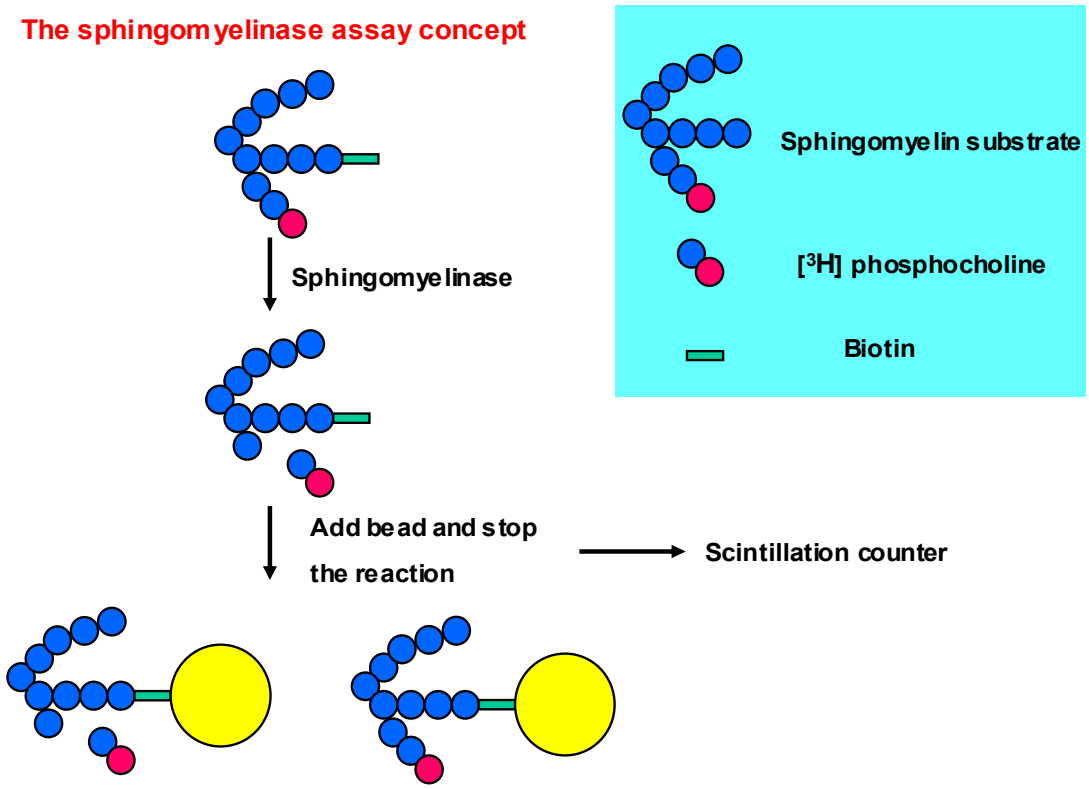


Figure.10 Sphingomyelinase assay technique used to determine the sphingomyelinase activity in the bacterial supernatant.

The binding of the [^{3}H] biotinylated sphingomyelin substrate to the streptavidin-coated tritium silicate SPA bead brings the isotope into close enough proximity to allow b-particles released from the tritium to excite the bead. The cleaved product of the sphingomyelinase activity containing the tritiated/labelled component is not close enough to the scintillant to allow this energy transfer, so no signal is generated. In addition, nonbiotinylated substrate does not bind to the bead. For details see the text.

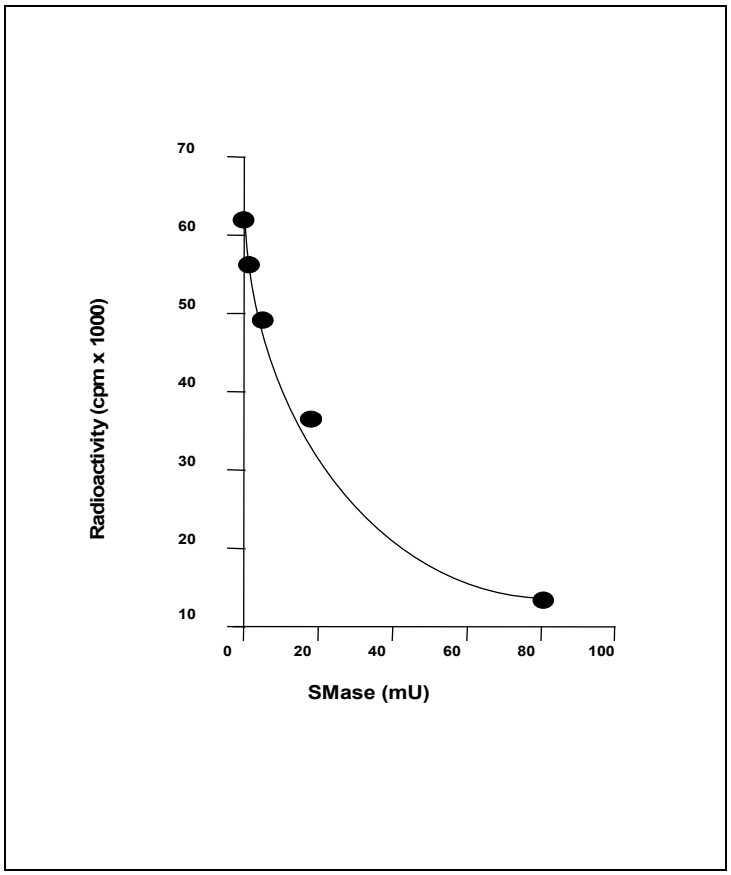


Figure.11 The Sphingomyelinase standard curve measured using different concentrations of Sphingomyelinase (0.3 - 80 mU/tube). See Fig.10.

3.2.7. FACS

A. Determination of phosphatidylserine exposure by flow cytometry

Fluorescence activated cell sorting (FACS) analysis is performed essentially as described elsewhere (Andree et al. 1990) with some modifications to adapt the method to erythrocyte research (Lang, Roll, Myssina, Schittenhelm, Scheel-Walter, Kanz, Fritz, Lang, Huber, & Wieder 2002;Lang, Duranton, Poehlmann, Myssina, Bauer, Lang, Wieder, & Huber 2003a).

- Erythrocyte concentrates (3µl erythrocytes in 1ml plasma, 0.3 % hematocrit) are incubated with healthy and /or sepsis volunteer's plasma for 24 hours.

- After incubation, cells are washed in annexin-binding buffer containing (in mM) 125 NaCl, 10 HEPES; pH 7.4 and 5 $CaCl_2$ as following.
- The tubes containing the erythrocytes incubated in plasma were vortexed, and then 50µl of the suspension was added to 350µl annexin-binding buffer and centrifuged 5 minutes, 3500 rpm at room temperature.
- The supernatant was removed after that.
- The erythrocytes pellet was stained with the fluorescein-conjugated anticoagulant Annexin-Fluos® (Roche Diagnostics: Mannheim, Germany) at a 1:50 dilution (20µl of Annexin-Fluos® was diluted with 980µl annexin-binding buffer and vortexed, then for every sample 50µl of this diluted Annexin-Fluos® was added).
- After 15 min**,** samples were diluted 1:5 by adding annexin-binding buffer (200µl to each sample) and measured by flow cytometric analysis on a FACS-Calibur.
- Annexin-fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

B. Determination of cell volume by flow cytometry

To determine the cell volume of stressed erythrocytes, the forward scatter of unlabelled cells is measured and analysed on a FACS-Calibur (Lang, Duranton, Poehlmann, Myssina, Bauer, Lang, Wieder, & Huber 2003a).
In case that the cells have been incubated in healthy or sepsis patient plasma, forward scatter analysis can also be accomplished with Annexin-stained erythrocytes.

C. Determination of cellular ceramide levels by flow cytometry

For determination of ceramide, a monoclonal antibody-based assay was used (Bieberich et al. 2003;Grassme et al. 2002).
The measurement was done as follows:

- 3 µl of erythrocyte concentrate were incubated for 24 hours in 1 ml plasma

of healthy and/or sepsis patient (0.3 % hematocrit)

- The positive control was prepared by incubation the erythrocytes for 24 hours in Ringer. Then, the cells were treated with Ringer solution containing 0.1 U/ml purified sphingomyelinase from *Streptomyces sp.* (Sigma: Taufkirchen, Germany) for 5 min.

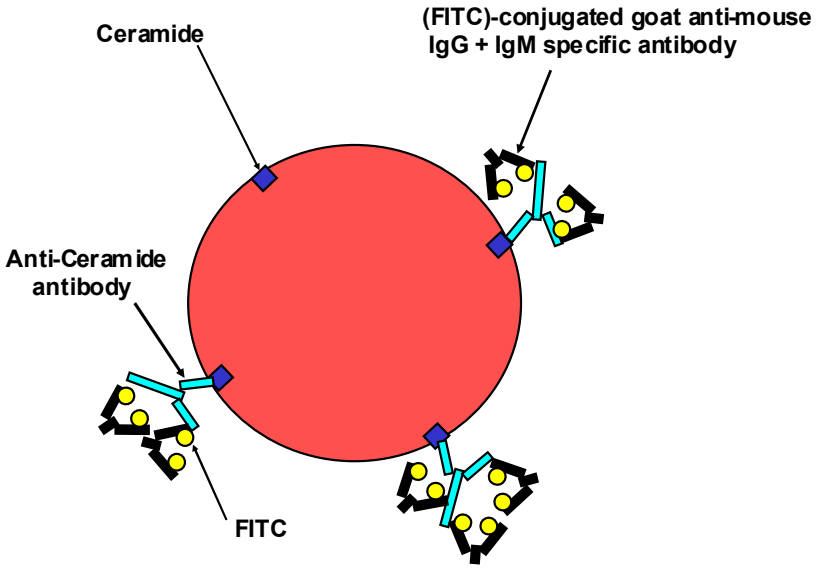


Figure 12: Principle of ceramide measurement by flow cytometry.
The anti-ceramide antibody (first antibody) binds to ceramide in the outer leaflet of the membrane and the secondary FITC-conjugated (fluorescent) antibody then binds to the F_c part of the anti-ceramide antibody. FITC, (Fluorescein Isothiocyanate).

- 150 µl of the erythrocyte suspension was transferred to a plastic tube containing 1 ml PBS.
- The erythrocytes were washed 3 times with PBS by centrifuging at 3000 rpm for 5 min at 4C° and removing the supernatant.
- The pellet was suspended in 40 µl PBS and incubated for 1 hour at 4°C

with 1 µg/ml anti-ceramide antibody (clone MID 15B4) (Alexis: Grünberg, Germany) or 1 µg/ml isotype matched pure mouse IgM antibody (Ancell: Bayport, MN, USA) in PBS containing 1 % fetal calf serum (FCS) at a dilution of 1:5 as described (Lang et al. 2004a).

- After the incubation, the erythrocytes were washed three times with PBS/1% FCS and the supernatant was removed.
- The cells were stained with polyclonal fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG + IgM specific antibody (Pharmingen: Hamburg, Germany) in PBS/1% FCS at a dilution of 1:50 for 30 min.
- After the second incubation, the unbound secondary antibody was removed by repeated washing with PBS/1% FCS three to four times.
- The samples were analysed by flow cytometric analysis on a FACS-Calibur. Mean values of FITC-fluorescence intensity in the fluorescence channel FL-1 were determined using the CellQuest^{TM} software.
- Isotype matched pure mouse antibody should not display increased fluorescence intensity in FL-1 as compared with the negative control (which was prepared by adding PBS containing 1% FCS instead of the anti-ceramide antibody). In contrast, SMase-treated erythrocytes should show enhanced fluorescence (positive control). Figure 12 depicts a simplified scheme of the principles of the assay.

D. Measurement of intracellular Ca^{2+} activity by flow cytometry

1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)]-2-(2′-amino-5′ -methylphenoxy) ethane-N, N, N′, N′-tetraacetic acid pentaacetoxymethyl ester (Fluo3AM) is a Ca^{2+} indicator which is membrane permeable until it is hydrolyzed inside the cell by cellular esterases. Fluo3AM is almost non-fluorescent at resting levels of calcium but the fluorescence becomes 40-times more intense when it binds to intracellular calcium, and its excitation maximum 506 nm and emission maximum is 526 nm is in the prescence of high and low concentrations of calcium. In the present study, intracellular Ca^{2+} measurements were performed as described earlier (Andrews, Yang, & Low 2002).

- 3 µl erythrocytes were suspended in 1 ml Fluo-3/AM buffer (0.3 % hematocrit).
- The erythrocytes were loaded with Fluo-3/AM (Calbiochem: Schwalbach, Germany) by addition of 2 µl of a Fluo-3/AM stock solution (1 mM in dimethyl sulfoxide [DMSO]) to 1 ml erythrocyte suspension.
- The cells were incubated at 37 °C for 15 min under vigorous shaking and protection from light.
- An additional 2 µl of Fluo-3/AM stock solution (1 mM) was added, and incubation was carried out for 25 min.
- Fluo-3-AM-loaded erythrocytes were centrifuged at 1800 rpm for 5 min. at 22 °C and the supernatant was removed.
- The erythrocytes were washed two times with Ringer solution containing 1 % FCS and once with Ringer.
- For flow cytometry, 1.5 µl of Fluo-3/AM-loaded erythrocytes were resuspended in 0.5 ml plasma (0.3 % hematocrit) and incubated in a CO_2 incubator at 37°C.
- As a positive control, Fluo3-labelled erythrocytes were suspended in 1 ml Ringer (3µl erythrocytes in 1 ml, 0.3 % hematocrit) supplemented with the Ca^{2+} ionophor ionomycin (1 µM) (Sigma: Taufkirchen, Germany). As a negative control, erythrocytes were incubated in Ringer solution containing vehicle (0.1 % DMSO) alone and incubated for different time periods at 37°C in a CO_2 incubator with light protection.
- For the measurement 100 µl of the erythrocyte suspension was diluted with 200 µl of plasma (when it is suspended plasma) or 200 µl Ringer for the positive control **.**
- Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

3.2.8. Statistics

Erythrocytes from different donors show a relatively high degree of variability. Thus, all experiments were repeated with several blood samples from different individuals as indicated. Data are expressed as arithmetic means ± S.E.M. and statistical analyses were made by paired or unpaired t-test, or ANOVA using Dunnett´s or Tukey´s test as post hoc test, where appropriate.

4. Results

4.1. Age, sex and clinical condition of the patients included in the study

Eleven patients who had septic shock diagnosed according to the recommendations of the German society of sepsis and accepted in the intensive care unit of the Hospital of Tübingen University have been included in the study. As shown in Table 3, these patients had differences and diverse degrees of concomitant diseases, variable sources of infection, a broad range of sepsis severity and clinical background. Those parameters predisposed them to have septic shock. 4 patients were female and 7 patients were male, their age varied between 36-79 years.

Table.3

Clinical data of patients

No.	Age (Years)	Sex	Septic focus	Microbiology	ICU stay (days)	APACHE II score *	Outcome
1	51	F	Crohn disease, abdominal abscess	Not identified	2	17	Survived
2	36	F	Pneumonia	Mycoplasma pneumoniae	3	21	Survived
3	35	M	Crohn disease, Port infection	Staph. Epi	3	11	Survived
4	70	F	Agranulocytosis, necrotic fasciitis	Not identified	23	17	Survived
5	70	F	Necrotic fasciitis	Not identified	1	26	Survived
6	65	M	Pneumonia	Not identified	9	24	Exitus

							letalis
7	79	M	Urosepsis	E. coli	3	27	Survived
8	39	M	Non-Hodgkin-Lymphome, Sepsis in aplasia	Staph. epi,	1	30	Exitus letalis
9	54	M	Pacemaker infection	Staph. aureus	1	21	Survived
10	49	M	Toxic shock syndrome	Staph. aureus	35	13	Survived
11	64	M	Unknown focus	Bact. fragilis	3	14	Survived

******APACHE II score is given in points***.**

4.2. Blood cell counts in healthy individuals and sepsis patients

The blood cell count of these patients showed that they suffered from severe anemia: the patients haemoglobin was 10.48±0.57 g/dl (n=11) comparing to the healthy volunteers 14.5±0.34 g/dl (n=12), and the hematocrit was 42±0.97% (n=12) and 30.5±1.48% (n=11) for healthy volunteers and sepsis patients, respectively (see Table 4).

Table 4:

Blood cell count in healthy individuals and patients with sepsis.

Arithmetic means ± SEM (n = 12 control, 11 patients) of leukocyte number and classification, thrombocytes number, of erythrocyte number, packed cell volume (hematocrit), haemoglobin concentration, mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), and reticulocyte count.

	Controls	Patients	P
Leukocytes *1000	6018±357	10162±2363	n.s
Neutrophils%	57.88±2.37	76.84±12.77	n.s
Eosinophils%	1.99±0.43	0.64±0.15	<0.05
Basophils%	0.74±0.11	0.67±0.41	n.s
Lymphocytes%	31.12±2.14	12.6±6.63	<0.01
Monocytes%	6.4±0.43	2.24±0.48	<0.001
Unclassified%	1.86±0.14	6.8±5.88	n.s
Erythrocytes*million	4.72±0.12	3.33±0.17	<0.001
Hematocrit%	42±0.97	30.5±1.48	<0.001
HB (g/dl)	14.5±0.34	10.48±0.57	<0.001
MCH (pg)	30.74±0.37	33±1.12	<0.05
MCHC (g/dl)	34.49±0.1	34.65±0.39	n.S
Hypochrom erys(%)	1.4±0.2	1	n.S
MCV(fl)	89.14±1.09	93.04±3.03	n.S
Thrombocytes*1000	292±12.38	141.7±23.37	<0.001

4.3. Sepsis patient plasma induces apoptotic morphology and PS exposure in human endothelial cells

Because sepsis induces endothelial cells damage leading to endothelial cells apoptosis (Curzen, Griffiths, & Evans 1994;Cybulsky, Chan, & Movat 1988;Hack & Zeerleder 2001;Lehr, Bittinger, & Kirkpatrick 2000;Mutunga, Fulton, Bullock, Batchelor, Gascoigne, Gillespie, & Baudouin 2001) the apoptotic effect of sepsis patient plasma was investigated. For this, the effect of plasma on annexin binding of human endothelial cells (HMEC-1) was studied using immunofluorescence microscopy.

The endothelial cells were incubated for 24 h in HMEC-1 growth medium containing either 50 % healthy control plasma or 50 % sepsis plasma. Exposure

to the sepsis plasma led to a high apoptosis incidence as demonstrated by the appearance of apoptotic morphology (shrinkage, detachment and rounding up of the cells) in light microscopy (Fig. 13A). Additionally, the sepsis plasma-treated cells were Annexin V positive when analysed by fluorescence microscopy (Fig. 13B).

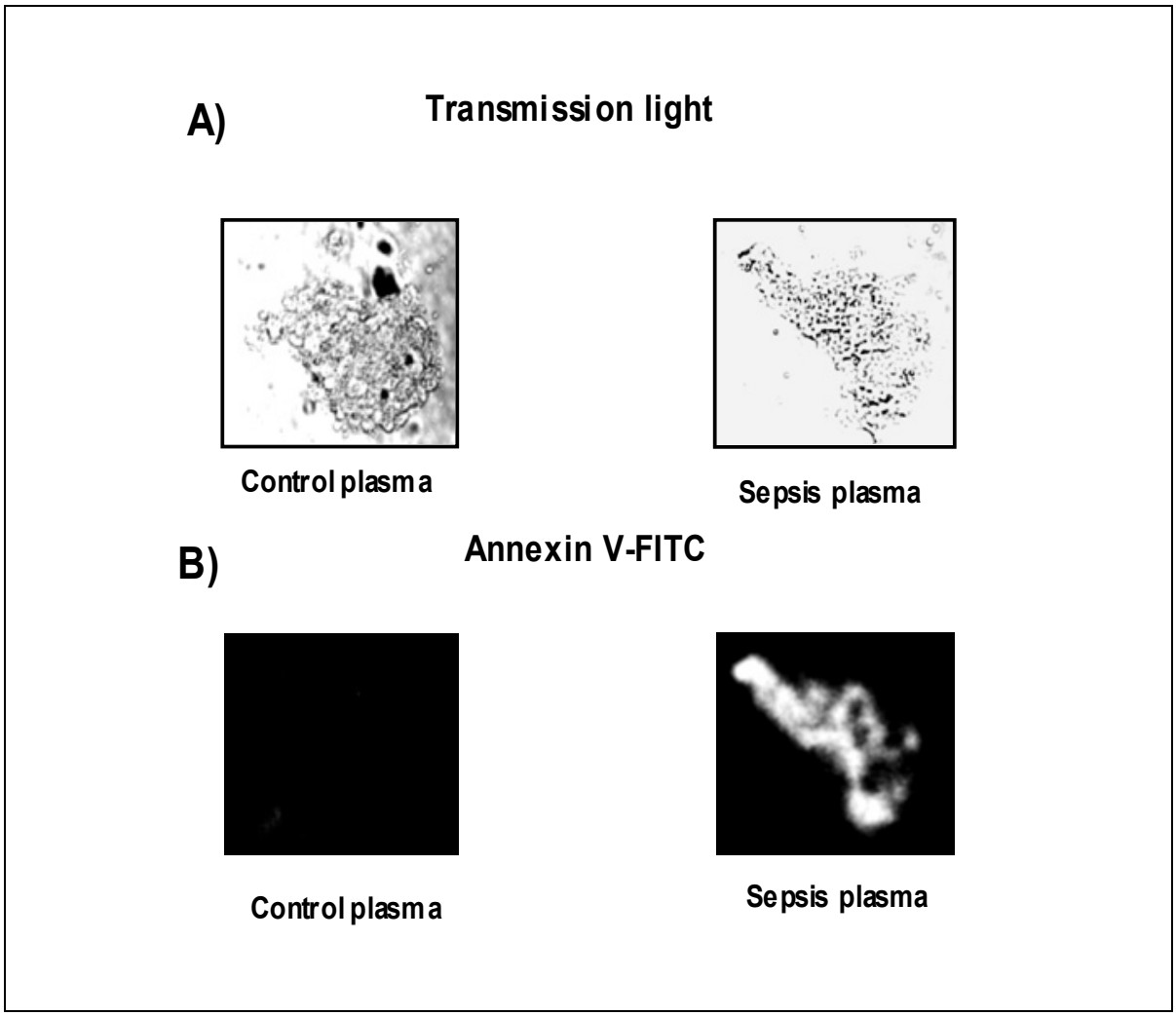


Figure.13. Sepsis patient plasma induces apoptotic morphology and PS exposure in human endothelial cells

Analysis of morphological changes (A) and annexin binding (B) in endothelial cells incubated in healthy plasma (left panel) or in sepsis patients plasma (right panel). Pictures were taken under a transmission light (A) or fluorescence microscope (B).

4.4. Stimulation of phosphatidylserine exposure at the erythrocyte surface by plasma of septic patients

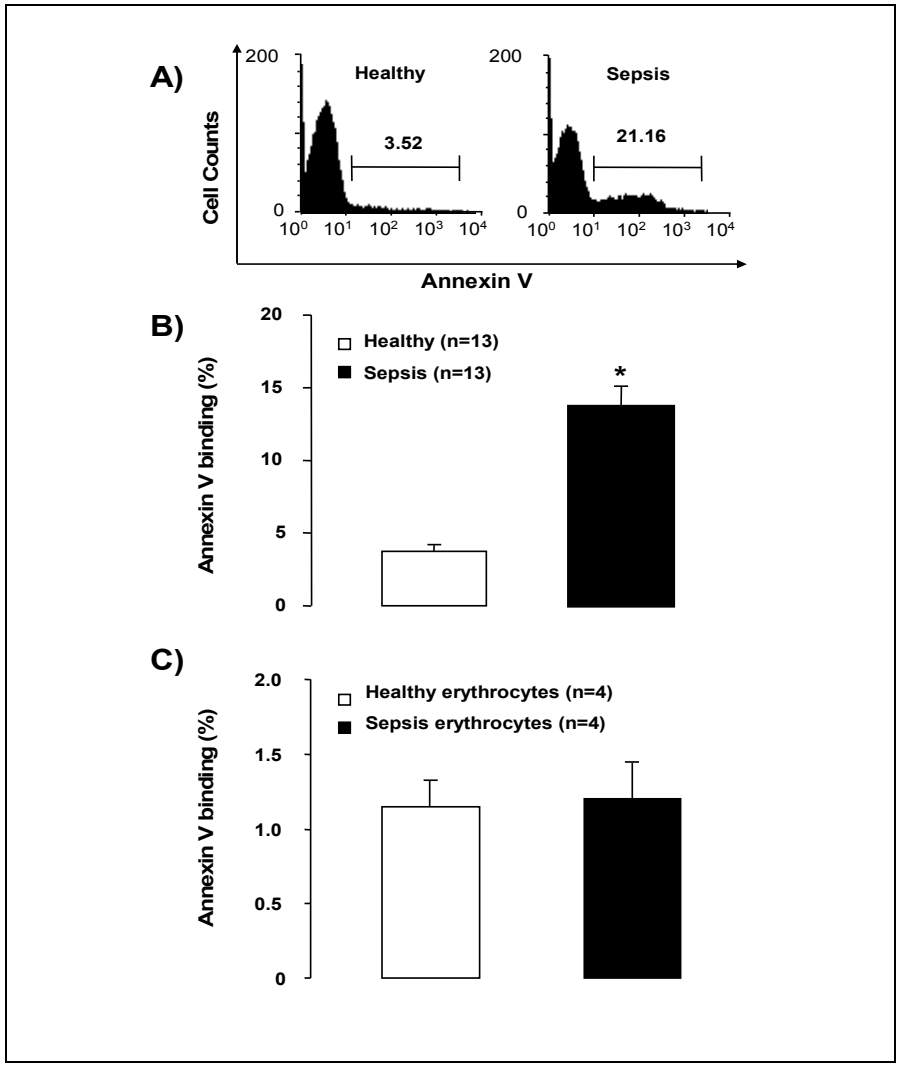


Fig.14. Stimulation of phosphatidylserine exposure at the erythrocyte surface by plasma of septic patients

A. Histograms of annexin binding in a representative experiment of erythrocytes from healthy volunteers incubated for 24 hours in plasma from a septic patient (right panel) or from a healthy volunteer (left panel).

*B. Arithmetic means ± SEM (n = 12) of annexin binding of erythrocytes incubated for 24 hours in plasma from septic patients (right, black column) or from healthy volunteers (left white column). * Indicates significant difference between the plasma from septic patients and plasma from healthy volunteers (unpaired t-test P ≤ 0.05).*

C. Arithmetic means ± SEM (n = 4) of annexin binding of erythrocytes from a septic patient (right, black column) or from a healthy volunteer (left white column) both incubated for 24 hours in Ringer.

Accelerated clearance of erythrocytes could result from increasing of the phosphatidylserine exposure of erythrocytes. Phosphatidylserine exposing erythrocytes are then recognized by macrophages, thus leading to phagocytosis and engulfment of affected erythrocytes (Kempe, Lang, Eisele, Klarl, Wieder, Huber, Duranton, & Lang 2005b;Lang, Lang, Bauer, Duranton, Wieder, Huber, & Lang 2005a).

As shown in Fig. 14, erythrocytes of healthy volunteers from the same blood group were exposed to the plasma of healthy individuals or sepsis patients' plasma for 24 hours. This incubation led to marked stimulation of phosphatidylserine exposure at the erythrocyte surface. The values were 3.75% ± 0.4% (n=12) and 13.7% ± 1.4% (n=11) in healthy and sepsis plasma respectively, which is significantly different (Fig. 14 A, B).

To exclude defects in the erythrocyte populations of sepsis patients, phosphatidylserine exposure of their erythrocytes was analysed. However, following exposure of erythrocytes from either healthy individuals or sepsis patients to Ringer solution, the percentage of annexin binding erythrocytes remained low (1.15%± 0.18% and 1.2%± 0.23% for healthy and sepsis erythrocytes, respectively) (Fig. 14C). Moreover, no significant difference of annexin binding was observed between erythrocytes from healthy people and erythrocytes from sepsis patients.

Thus, plasma of sepsis patients indeed contains a component, which triggers phosphatidylserine exposure of healthy erythrocytes.

4.5. Decrease of erythrocyte forward scatter following exposure to plasma of septic patients

The phosphatidylserine exposure of suicidal erythrocytes is usually paralleled by cellular shrinkage which should lead to a corresponding decrease of the forward scatter in flow cytometry (Lang, Kaiser, Myssina, Wieder, Lang, &

Huber 2003c;Myssina, Lang, Kempe, Kaiser, Huber, Wieder, & Lang 2004a). As shown in Fig. 15, the exposure of erythrocytes from healthy volunteers to heparinized plasma of septic patients for 24 hours indeed led to marked decrease of forward scatter (424.8± 7.7 arbitrary unit and 396.6± 5.99 arbitrary unit, in healthy and sepsis plasma, respectively) (Fig. 15 A, B).

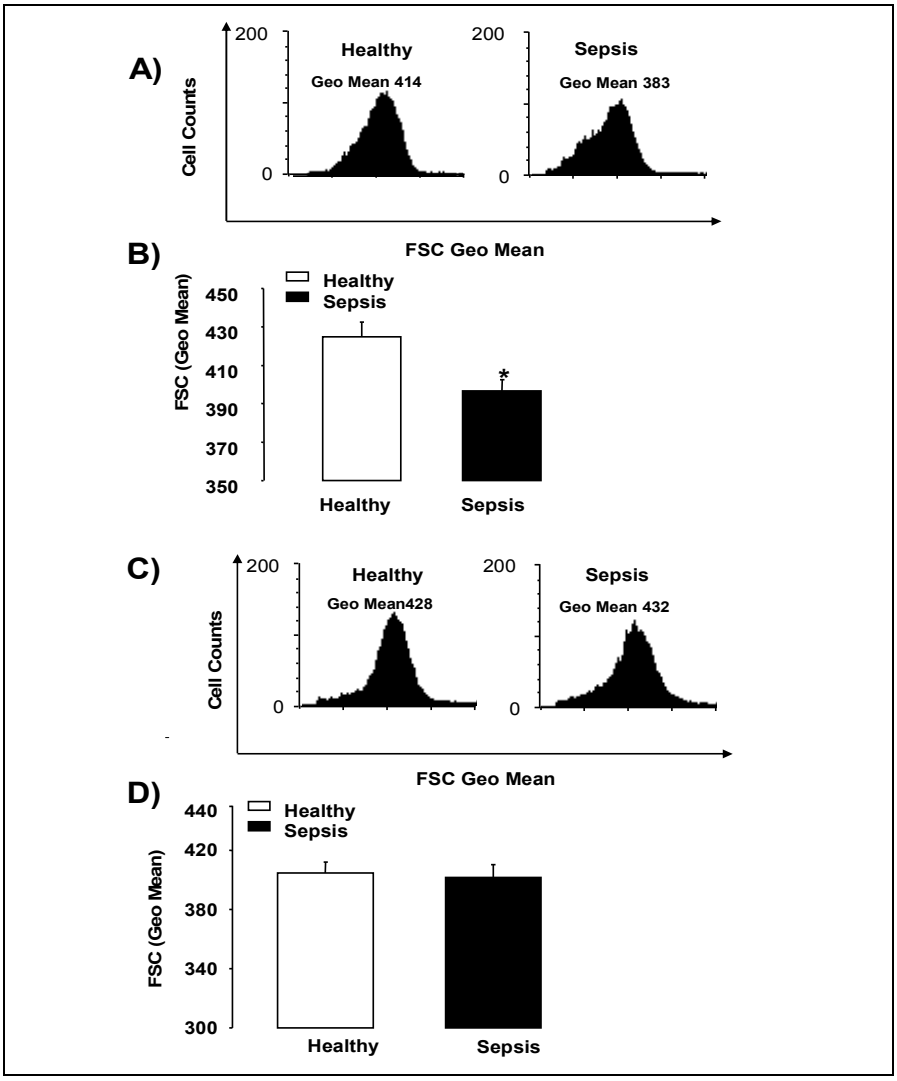


Fig. 15: ***Decrease of erythrocyte forward scatter following exposure to plasma of septic patients***

A. Histograms of forward scatter in a representative experiment of erythrocytes from a healthy volunteer incubated for 24 hours in plasma from a septic patient (right panel) or from a healthy volunteer (left panel).

B. Arithmetic means ± SEM (n = 10) of forward scatter of erythrocytes incubated

*for 24 hours in plasma from septic patients (right, black column) or from healthy volunteers (left white column). * Indicates significant difference between the plasma from septic patients and plasma from healthy volunteers (unpaired t-test $P \leq 0.05$).*

C. Histograms of forward scatter in a representative experiment of erythrocytes from a healthy volunteer (left panel) or a septic patient (right panel) incubated for 24 hours in Ringer.

D. Arithmetic means ± SEM (n = 6) of forward scatter of erythrocytes from septic patients (right, black column) or from healthy volunteers (left white column) both incubated for 24 hours in Ringer.

Again, the exposure of erythrocytes from healthy individuals or sepsis patients to Ringer solution did not significantly modify the forward scatter, and no significant differences between both groups of erythrocytes were measured (404.75± 7.23 arbitrary unit and 401.54± 8.84 arbitrary unit in healthy and sepsis erythrocytes, respectively) (Fig. 15 C, D).

Thus, whereas the sepsis plasma contains a component that causes the shrinkage of healthy erythrocytes, the volume of circulating erythrocytes of sepsis patients is not significantly altered as compared with the volume of healthy erythrocytes.

4.6. Increase of cytosolic Ca^{2+} activity in erythrocytes exposed to plasma of septic patients.

The increase of intracellular Ca^{2+} activity is one of the stimulators of phosphatidylserine exposure, as the exposure of erythrocytes to the Ca^{2+} ionophore ionomycin led to breakdown of phosphatidylserine asymmetry and annexin binding in addition to the shrinkage of the erythrocytes. Hyperosmotic stress, oxidative stress, and energy depletion activate non-selective Ca^{2+}-permeable cation channels, triggering the exposure of phosphatidylserine and erythrocyte shrinkage. These channels are inhibited by amiloride, which further blunts annexin binding following osmotic shock, oxidative stress and energy

depletion (Lang, Duranton, Poehlmann, Myssina, Bauer, Lang, Wieder, & Huber 2003a).

Thus, the effect of sepsis patient's plasma on erythrocyte cytosolic Ca^{2+} activity was tested. The Fluo3-fluorescence measurements revealed that exposure of erythrocytes to the plasma of the sepsis patients led to a slight but significant increase of the number of erythrocytes with increased cytosolic Ca^{2+} activity (10.05± 0.47 arbitrary units

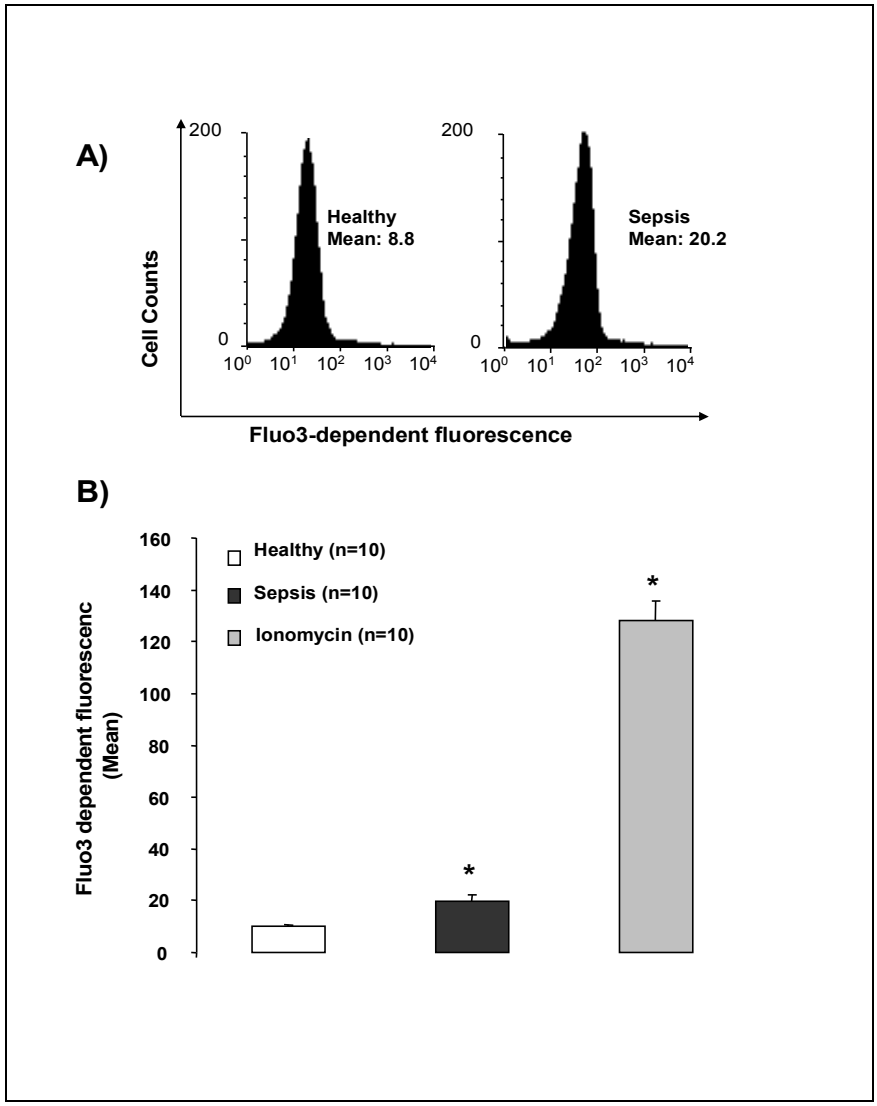


Figure.16: ***Increase of cytosolic Ca^{2+} activity in erythrocytes exposed to plasma of septic patients.***

A*. Representative original histograms of Fluo3 fluorescence from erythrocytes incubated for 360 minutes in plasma of a septic patient (right panel) or from a*

healthy volunteer (left panel).

***B.** Arithmetic means ± SEM (n = 7) of Fluo3 fluorescence in erythrocytes incubated for 360 minutes in plasma of septic patients (black column) or from healthy volunteers (white column), and of erythrocytes exposed to Ca^{2+} ionophore ionomycin (1 µM, grey column) in Ringer. * Indicates significant difference between the value to erythrocytes exposed to plasma of healthy individuals (ANOVA using Dunnett´s test as post hoc test. P ≤ 0.05).*

and 19.71± 2.37 in healthy and sepsis plasma, respectively) (Fig.16). As a positive control, the Ca^{2+} ionophore ionomycin (1 µM) led to a marked increase of cytosolic Ca^{2+} activity in virtually all erythrocytes (127.99± 7.7 arbitrary unit).

4.7. Stimulation of ceramide formation in erythrocytes exposed to plasma of septic patients.

A further stimulator of phosphatidylserine exposure in erythrocytes is ceramide which is produced by sphingomyelinase activity. Thus, the effect of plasma of sepsis patients on ceramide formation was tested. According to flow cytometric ceramide measurements, exposure of erythrocytes to plasma from sepsis patients indeed stimulated the formation of ceramide (8.18±0.53 rbitrary unit and 13.08±1.82 arbitrary unit in healthy and sepsis plasma, respectively) (Fig. 17). As positive control, erythrocytes were incubated in Ringer solution containing 0.1 U/ml purified sphingomyelinase from *Streptomyces sp.* or from *S. aureus* for 5 min, and a significant increase of the fluorescence intensity was observed (Fig. 17B, grey column).

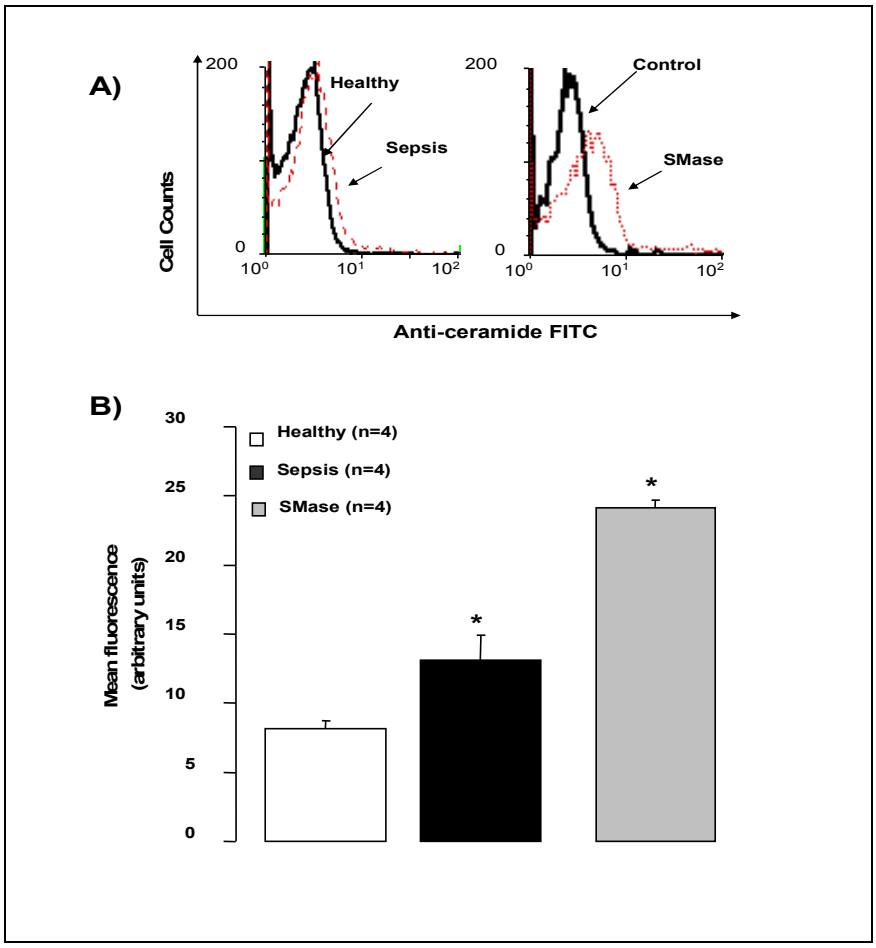


Figure.17: ***Stimulation of ceramide formation in erythrocytes exposed to plasma of septic patients.***

A. Left: Histograms of ceramide presenting erythrocytes in a representative experiment of erythrocytes incubated for 24 hours in plasma of a septic patient (red line, left panel) or in plasma from a healthy volunteer (black line). Right: Histograms of ceramide presenting erythrocytes in a representative experiment of erythrocytes incubated for 24 hours in Ringer without (black line) and with 0.1 mU bacterial Sphingomyelinase (red line right panel).

*B. Arithmetic means ± SEM (n = 4) of ceramide formation in erythrocytes incubated for 24 hours in plasma of septic patients (black middle column), plasma from healthy volunteers (white left column) and with 0.1 mU bacterial Sphingomyelinase (grey left column). * Indicates significant difference between the plasma from septic patients and plasma from healthy volunteers (unpaired t-test. P≤0.05).*

4.8. Stimulation of phosphatidylserine exposure at the erythrocyte surface by supernatant from Sphingomyelinase-producing Staphylococcus aureus

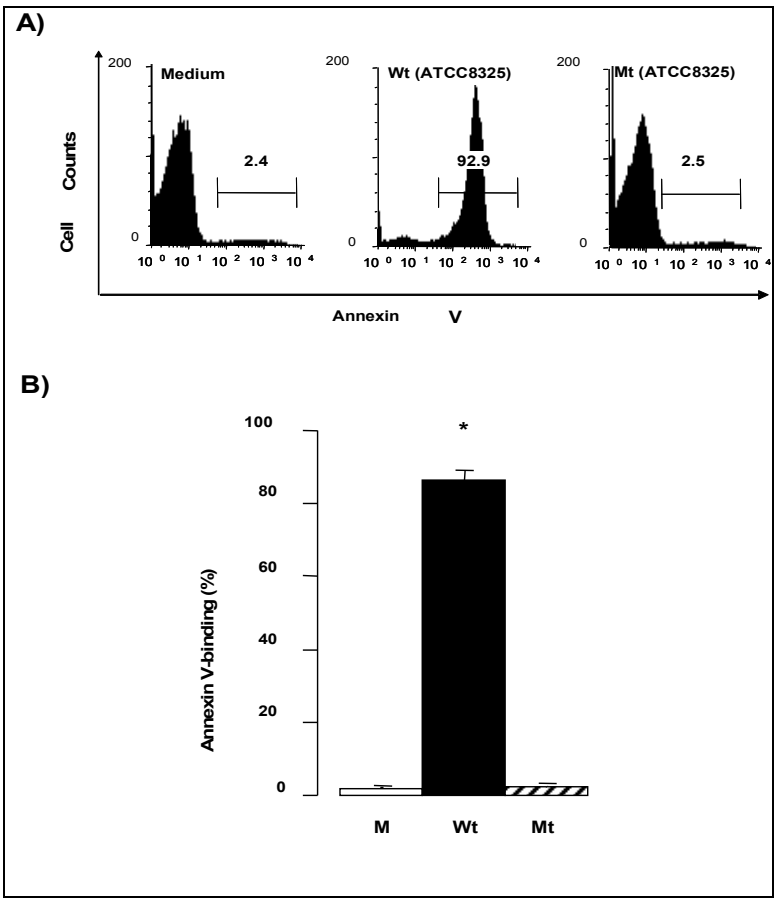


Figure.18: ***Stimulation of phosphatidylserine exposure at the erythrocyte surface by supernatant from Sphingomyelinase-producing Staphylococcus aureus***

A. Histograms of annexin binding in a representative experiment of erythrocytes incubated for 60 minutes in bacterial growth medium without pathogen (left panel) or in supernatant (middle and right panels) from wild type Staphylococcus aureus ATCC 8325 (middle panel) or from mutated Staphylococcus aureus ATCC 8325 lacking functional Sphingomyelinase (right panel).

B. Arithmetic means ± SEM (n = 3) of the percentage annexin binding erythrocytes after incubation for 60 minutes in pathogen free medium (M) or in

*supernatant from wild type Sphingomyelinase expressing Staphylococcus aureus ATCC 8325 (Wt) or from mutated Staphylococcus aureus ATCC 8325 lacking functional Sphingomyelinase (Mt). * Indicates significant difference from the annexin binding cells incubated in pathogen free medium (ANOVA using Dunnett´s test as post hoc test. P≤0.05).*

Further experiments have been performed to explore whether sphingomyelinase released from pathogens could trigger phosphatidylserine exposure of erythrocytes. To this end, erythrocytes from healthy volunteers have been exposed to culture medium (negative control) or to supernatant from sphingomyelinase-producing *Staphylococcus aureus* Wild type (8325-4) and sphingomyelinase-deficient *S.aureus* (8325-4Φ13)(mut), an isogenic mutant strain whose sphingomyelinase gene (*hlb*) has been knocked out. Incubation of erythrocytes for 60 min in supernatant from wild type *S aureus* (8325-4F) was followed by a marked phosphatidylserine exposure, while incubation in bacterial growth medium or in supernatant from mutated *S aureus* (8325-4Φ13) lacking functional sphingomyelinase (Goerke, Koller, & Wolz 2006) were without any appreciable effect (Fig.18A, B). Thus, sphingomyelinase specifically released from living bacteria is able to trigger phosphatidylserine exposure in previously healthy human erythrocytes.

4.9. Annexin binding of erythrocytes and hemolysis after incubation in bacterial supernatant.

To investigate whether the enhanced annexin binding was due to erythrocytes hemolysis, the cells were incubated with supernatant from *S aureus* (8325-4F) (Wt) (Fig. 19), and annexin V binding and hemolysis was measured in parallel. After 60 min of incubation of erythrocytes in bacterial supernatant, the dramatic annexin binding (86.3%±2.87) was not accompanied by hemolysis (0 %). However, after 24 hours of incubation hemolysis was almost 100 % and reached the same value as in the annexin binding assay (Fig. 19). Thus, the early effect on annexin binding after one hour was not due to hemolysis. Under

physiological conditions, these annexin-positive erythrocytes are recognized and engulfed by macrophages. After longer incubation periods (24 hours) and in the abscence of macrophages, however, all annexin-positive cells were hemolysed, resulting in 100% of annexin binding due to disrupture and exposure of the intracellular leaflet of the erythrocyte membrane.

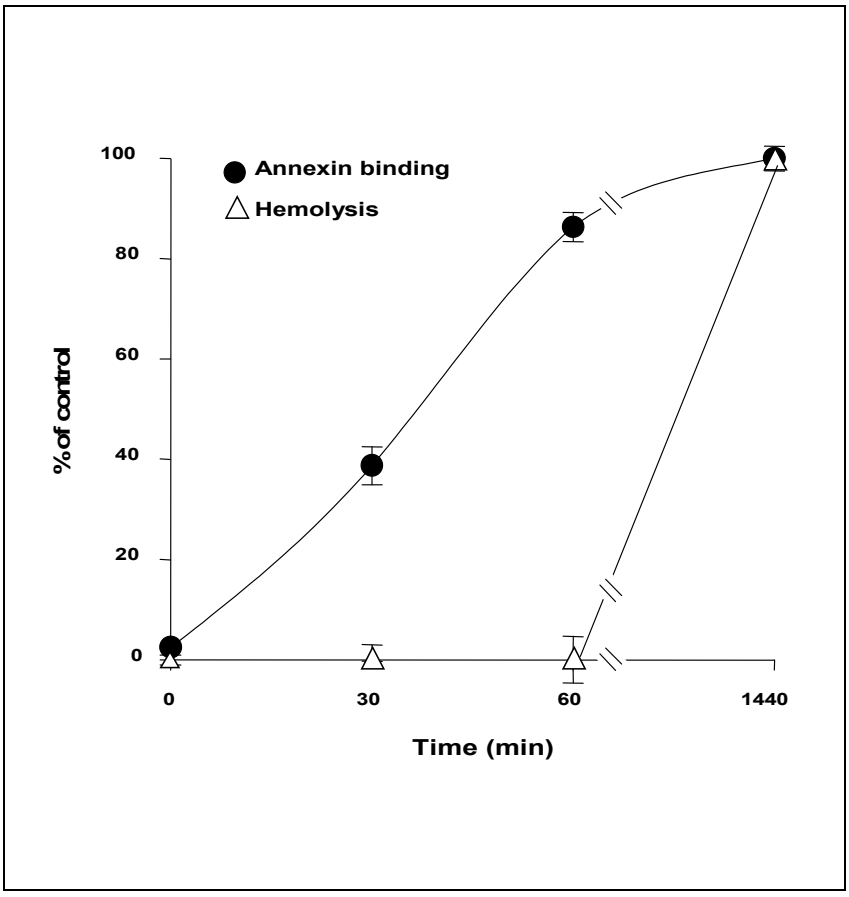


Figure.19. Arithmetic means ± SEM (n=3) of the percentage of annexin binding erythrocytes (black circle) and percentage of hemolysis (open white triangle) after incubation for up to 24 hours in bacterial supernatant.

4.10. Characterization of the pathogens used for the SMase/Annexin binding correlation

The isolated pathogens from the sepsis patients, and special strains of

Staphylococcus aureus Wild type and sphingomyelinase-deficient species, in addition to other different laboratory pathogens which were used in this measurement, are shown in Table 5.

Table.5

List of the pathogens used for the SMase/Annexin binding correlation

1	*Streptococcus pneumoniae*
2	*Streptococcus pyogenes*
3	*Staphylococcus aureus ATCC 8325*
4	*Pseudomonas aeruginosa*
5	*E.coli*
6	*Staphylococcus aureus ATCC 8325FΦ13*
7	*Salmonella*
8	*Staphylococcus carnosus TM 300*
9	*Staphylococcus aureus Strain Newman*
10	*S. aureus SA 113*
11	*Yersinia pestis*
12	*Pseudomonas aeruginosa*
13	*Staphylococcus haemolyticus*
14	*Shigella flexneri*
15	*Shigella dysenteria*
16	*Staphylococcus epidermidis*
17	*Staphylococcus lugdunensis*
18	*Klebsiella pneumoniae*
19	*Staphylococcus lentus*
20	*Serratia liquefaciens*

4.11. Correlation between the annexin binding of treated erythrocytes and the sphingomyelinase activity of bacterial supernatants

The SMase (Sphingomyelinase) activity and the eryptosis-inducing activity in the supernatant from wild type *S aureus* (8325-4F) and from mutated *S aureus* (8325-4Φ13) lacking functional sphingomyelinase (Goerke, Koller, & Wolz 2006) and in the supernatant from the other different pathogens listed in Table 5 were determined in parallel. As shown in Figure 20, graphical analysis of the data of the different pathogens disclosed a significant correlation (R^2=0.891) between sphingomyelinase activity and the potency to trigger eryptosis.

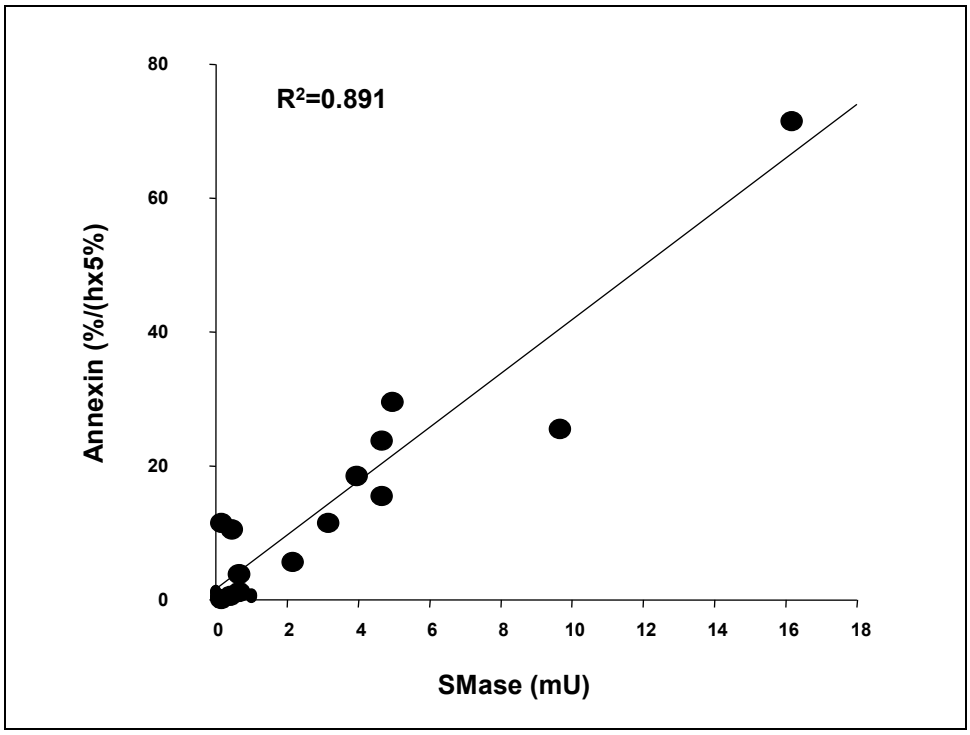


Figure 20. Correlation between the percentage of annexin binding erythrocytes and sphingomyelinase activity after incubation with bacterial supernatants from 20 different bacterial strains.

5. Discussion

5.1. Erythrocytes cytoskeleton

The erythrocytes have biconcave shape with a diameter of ~7.5 μm, and have a lipid bilayer membrane consisting of phospholipids, cholesterol, carbohydrates, glycolipids and proteins. Phospholipids are approximately 40 % by weight, mostly: phosphatidylcholine, phosphatidylserine, phosphatidyl-ethanolamine and phosphatidylinositol. Carbohydrates are about 10 % by weight, linked to lipid or protein. Glycolipids are on the outer leaflet in small concentrations. The proteins are about 50 % by weight, mostly glycoproteins(Mohandas & Evans 1994;Stokke, Mikkelsen, & Elgsaeter 1986;Svetina & Iglic 1996).

Passing through the narrow capillaries that have a diameter of less than 4 μm (almost half of the erythrocyte diameter), the erythrocytes are squeezed. This normally does not lead to membrane disrupture due to the red blood cells strong and flexible plasma membrane depending on the cytoskeleton network that underlies the entire membrane and is attached to it at many points. The erythrocytes cytoskeleton is a cross-linked brushy polymer network, and the protein spectrin is the primary component of this network. The membrane proteins interactin plays an important role in its stability, as spectrin attaches to actin filament plus adducin, tropomyosin, and tropomodulin which makes the actin filaments stable by preventing their depolymerising, in addition to that spectrin attaches to peripheral membrane proteins. Ankyrin, another cytoskeleton protein connects the center of spectrin to band 3 protein, the anion-transporter protein in the membrane, and to Band 4.1 protein(Bennett & Baines 2001;Bennett & Gilligan 1993;Palek & Lambert 1990;Peters LL & BarkerJE 2001;Tse & Lux 1999) (Fig. 21).

5.2. Sepsis plasma and erythrocytes

The present experiments demonstrate that plasma from septic patients triggers

phosphatidylserine exposure of erythrocytes finally leading to suicidal erythrocyte death or eryptosis.

The phosphatidylserine exposure is partially due to an increase of cytosolic Ca^{2+} activity. Besides its effect on phosphatidylserine exposure by increasing

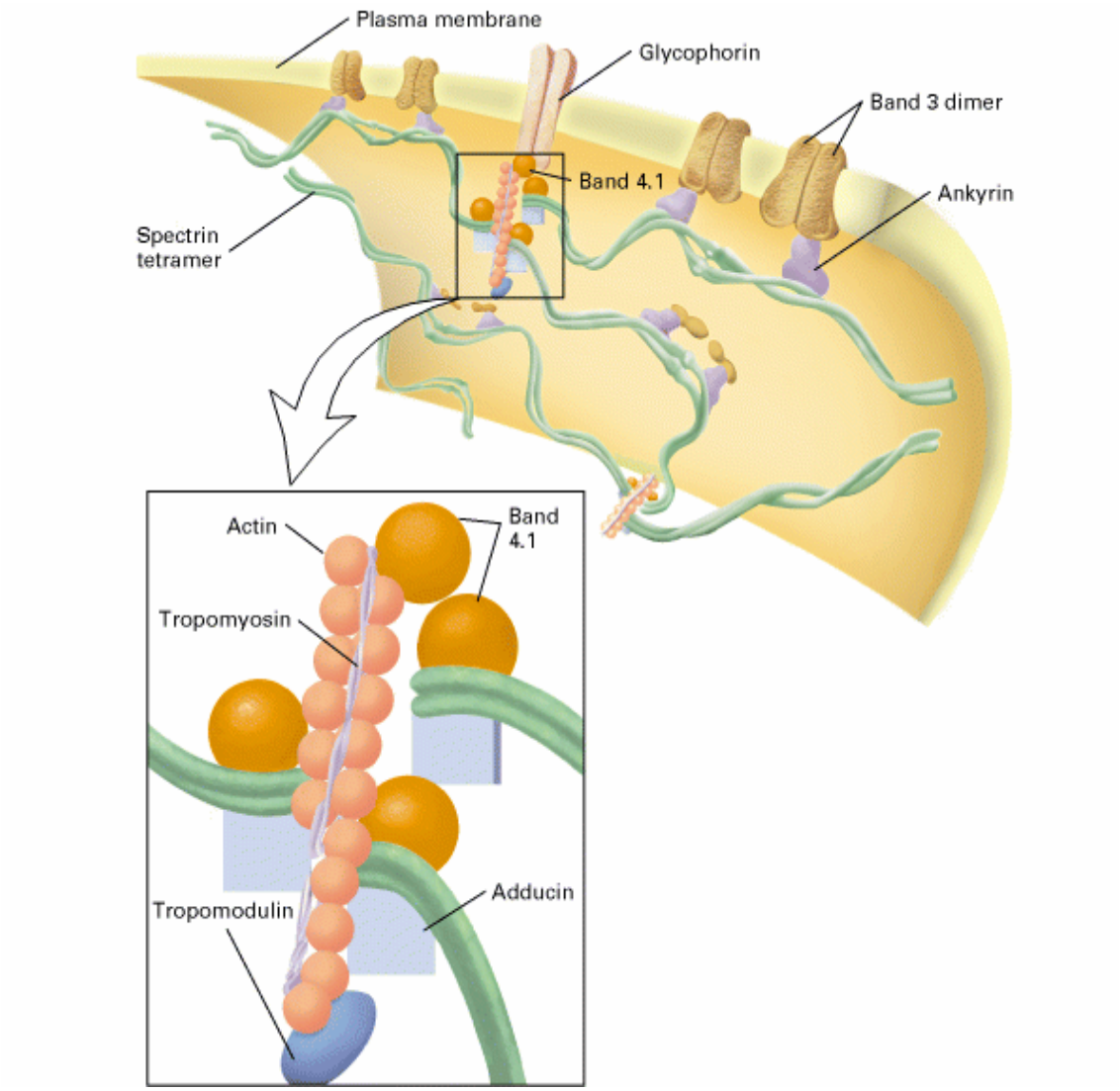


Figure. 21: The organization of the major erythrocyte cytoskeletal proteins and their interactions with integral membrane proteins. *Hypothetical arrangement of the components of a junctional complex and their interactions with the termini of spectrin tetramers. (from "Molecular cell biology" 18.1: adapted from (Luna & Hitt 1992;Lux 1979).*

erythrocyte membrane scrambling. The increase of cytosolic Ca^{2+} activity could also modify the cytoskeleton and this modification could affect the resistance and elasticity of the erythrocytes leading to deformation when they are squeezed in the capillaries. The membrane stability is regulated by membrane protein interactions, as demonstrated for the Ca^{2+}-calmodulin induced modulation of the interaction of protein 4.1 and spectrin-like proteins. The increasing of Ca^{2+} causes a reduction of the binding between protein 4.1 and band 3 proteins. This binding in turn modulates ankyrin interaction with band 3, leading to instability of the erythrocytes cytoskeleton (Nunomura et al. 1997;Takakuwa & Mohandas 1988). In addition, Ca^{2+} activates a transglutaminase leading to protein aggregate formation and crosslinking of proteins in erythrocytes membrane (Anderson, Davis, & Carraway 1977), as well as calpain (non-lysosomal intracellular proteinases) (Anderson, Davis, & Carraway 1977). The degradation of membrane proteins by calpain may participate in the machinery eventually leading to erythrocyte death. Ca^{2+} stimulates phospholipases leading to production of 1,2-diacylglycerol (Allan & Michell 1976), protein kinases and phosphatases (Cohen & Gascard 1992;Minetti et al. 1996) The role of protein kinases in erythrocytes apoptosis was approved, as glucose depletion-induced eryptosis was shown to be triggered by the activation of protein kinase C alpha (PKCα) (Klarl et al. 2006).

Moreover, Ca^{2+} entry activates Ca^{2+}-sensitive K^+ channels (Gardos channels) (Bookchin, Ortiz, & Lew 1987;Brugnara, de Franceschi, & Alper 1993;Franco et al. 1996), which together with Cl^- channels (Myssina et al. 2004b) allow the efflux of KCl and thus lead to cell shrinkage (Lang et al. 2003b).

The effect of plasma from septic patients on cytosolic Ca^{2+} activity is, however, modest and probably does not account for the strong stimulation of phosphatidylserine exposure. Rather, the stimulation of ceramide formation and its role in apoptosis signaling might be more important. In this context, the plasma activity of sphingomyelinase has previously been shown to be elevated in septic patients (Claus, Bunck, Bockmeyer, Brunkhorst, Losche, Kinscherf, & Deigner 2005). The sphingomyelin pathway is an ubiquitous signaling system starting at

the plasma membrane, where both acid and neutral sphingomyelinase (SMase) isoforms under stress might hydrolyse the sphingomyelin leading to ceramide formation (Pena, Fuks, & Kolesnick 1997). Intracellular ceramides play an important role as second messengers in proliferation, differentiation and apoptosis (Hannun 1996;Spiegel, Foster, & Kolesnick 1996), and experimental evidence for the role of sphingomyelinase-mediated ceramide formation in apoptosis has been provided in different cell types, such as macrophages (Steinbrecher, Gomez-Munoz, & Duronio 2004), keratinocytes (Geilen et al. 1996), melanoma cell lines(Raisova et al. 2000), prostate and colon carcinoma cells (von Haefen et al. 2002) and erythrocytes (Lang, Myssina, Brand, Sandu, Lang, Berchtold, Huber, Lang, & Wieder 2004b).

In addition to the apoptotic effect following exposure of cells to *Staphylococcus aureus* by inducing the activation of cellular caspases and acid sphingomyelinase, the release of cytochrome c and stimulation of Jun NH2-terminal kinase (JNK) was demonstrated (Esen et al. 2001). Another example of bacteria which cause apoptosis accompanied by generation of ceramide through activation of acid sphingomyelinase is *Escherichia coli (Falcone et al. 2004)*. Thus, the sphingomyelinase activation and ceramide formation could well account for the stimulation of phosphatidylserine exposure in erythrocytes.

The present study does not allow any conclusions as to the origin of the sphingomyelinase in the sepsis plasma. It is noteworthy, however, that the supernatant of pathogens contains sufficient sphingomyelinase activity to trigger marked phosphatidylserine exposure. The perfect linear correlation between phosphatidylserine exposure and sphingomyelinase activity on the one hand and the disappearance of phosphatidylserine exposure following loss of function by mutation of the bacterial Sphingomyelinase clearly demonstrate that the activity in the supernatant is largely due to sphingomyelinase. This observation does not rule out, however, that the pathogens express additional factors triggering phosphatidylserine exposure, which are not released into the supernatant. These bacterial components or other produced factors than the sphingomyelinase, e. g. lipopolysaccharide (LPS), bacterial lipoproteins (BLPs), peptidoglycan, pyocyanin and haemolysin, have been proved to induce cellular apoptosis and

phosphatidylserine exposure (Lang et al. 2004d;Navarre & Zychlinsky 2000). The observations further do not exclude the possibility that sphingomyelinase could be from other origin, such as leukocytes, released into the plasma of septic patients and contributes to the stimulation of phosphatidylserine exposure.

The exposure of phosphatidylserine at the cell surface favors the binding to respective phosphatidylserine receptors expressed by macrophages (Fadok et al. 2000a). Binding to those receptors triggers engulfment and subsequent degradation of the affected erythrocytes (Boas, Forman, & Beutler 1998). Thus, erythrocytes exposing phosphatidylserine at their surface will be cleared from circulating blood. Moreover, the erythrocytes may bind to receptors in the vascular wall and thus impede microcirculation (Andrews & Low 1999a;Closse, Dachary-Prigent, & Boisseau 1999a;Closse, Dachary-Prigent, & Boisseau 1999b). Along those lines, we observed enhanced trapping of annexin binding erythrocytes in renal medulla following ischemia of the mouse kidney (Lang et al. 2004c). Phosphatidylserine exposing cells may further participate in hemostasis (Andrews & Low 1999b). This increased clearance of annexin positive erythrocytes might be one of the mechanisms inducing anemia in sepsis patients by shorting the lifespan of erythrocytes, in addition to the possible roles of many other factors, such as blood loss, low erythrocytes formation by bone marrow because of erythropoietin deficiency or impaired response, hemolysis, coagulation disorders and nutrition deficiency.

Plasmapheresis is a nonselective method by which plasma is separated from the blood and replaced with donor plasma and/or albumin. The theoretical rationale is that plasmapheresis removes the harmful mediators and replenishes the consumed plasma factors, thus restoring the homeostatic milieu and blood purification. The clinical outcome of sepsis patients, including the haematological disorder has been improved by removing the inflammatory factors and toxins in plasmapheresis (Berlot et al. 2004;Kellum & Venkataraman 2005;Kyles & Baltimore 2005). Thus, the plasma factor that induced erythrocytes apoptosis and which is possibly identical with sphingomyelinase could as well be removed as the plasma is exchanged.

The present observations may not only be relevant for anemia but as well

for the stimulation of thrombocytes and the pathophysiology of organ damage. Ceramide (Kolesnick & Golde 1994;Kolesnick & Kronke 1998;Obeid et al. 1993;Unger 2002) and Ca^{2+} (Perretti & Solito 2004) have similarly been implicated in the triggering of apoptosis of nucleated cells and the pathogenic plasma component may similarly trigger apoptosis of endothelial, renal or hepatic cells thus leading to the pleiotropic clinical features of sepsis.

In conclusion, the present observations provide evidence for the stimulation of erythrocyte phosphatidylserine exposure by plasma of septic patients. It demonstrates that the effect of sepsis plasma is at least partially due to stimulation of Ca^{2+} entry and ceramide formation. The study thus reveals a novel pathophysiological mechanism and may open new therapeutic advents of this severe, life threatening disease.

6. References

1992, "American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis", *Crit Care Med.*, vol. 20, no. 6, pp. 864-874.

Afessa, B., Green, B., Delke, I., & Koch, K. 2001, "Systemic inflammatory response syndrome, organ failure, and outcome in critically ill obstetric patients treated in an ICU", *Chest*, vol. 120, no. 4, pp. 1271-1277.

Aird, W. C. 2003, "The hematologic system as a marker of organ dysfunction in sepsis", *Mayo Clin.Proc.*, vol. 78, no. 7, pp. 869-881.

Allan, D. & Michell, R. H. 1976, "Production of 1,2-diacylglycerol in human erythrocyte membranes exposed to low concentrations of calcium ions", *Biochim.Biophys.Acta*, vol. 455, no. 3, pp. 824-830.

Anderson, D. R., Davis, J. L., & Carraway, K. L. 1977, "Calcium-promoted changes of the human erythrocyte membrane. Involvement of spectrin, transglutaminase, and a membrane-bound protease", *J.Biol.Chem.*, vol. 252, no. 19, pp. 6617-6623.

Anderson, R. N., Minino, A. M., Fingerhut, L. A., Warner, M., & Heinen, M. A. 2004, "Deaths: injuries, 2001", *Natl.Vital Stat.Rep.*, vol. 52, no. 21, pp. 1-86.

Andree, H. A., Reutelingsperger, C. P., Hauptmann, R., Hemker, H. C., Hermens, W. T., & Willems, G. M. 1990, "Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers", *J.Biol.Chem.*, vol. 265, no. 9, pp. 4923-4928.

Andrews, D. A. & Low, P. S. 1999a, "Role of red blood cells in thrombosis", *Curr.Opin.Hematol.*, vol. 6, no. 2, pp. 76-82.

Andrews, D. A. & Low, P. S. 1999b, "Role of red blood cells in thrombosis", *Curr.Opin.Hematol.*, vol. 6, no. 2, pp. 76-82.

Andrews, D. A., Yang, L., & Low, P. S. 2002, "Phorbol ester stimulates a protein kinase C-mediated agatoxin-TK-sensitive calcium permeability pathway in human red blood cells", *Blood*, vol. 100, no. 9, pp. 3392-3399.

Angus, D. C., Linde-Zwirble, W. T., Lidicker, J., Clermont, G., Carcillo, J., & Pinsky, M. R. 2001, "Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care", *Crit Care Med.*, vol. 29, no. 7, pp. 1303-1310.

Angus, D. C. & Wax, R. S. 2001, "Epidemiology of sepsis: an update", *Crit Care Med.*, vol. 29, no. 7 Suppl, p. S109-S116.

Arese, P., Turrini, F., & Schwarzer, E. 2005, "Band 3/complement-mediated recognition and removal of normally senescent and pathological human erythrocytes", *Cell Physiol Biochem.*, vol. 16, no. 4-6, pp. 133-146.

Baglin, T. 1996, "Disseminated intravascular coagulation: diagnosis and treatment", *BMJ*, vol. 312, no. 7032, pp. 683-687.

Barsumian, E. L., Cunningham, C. M., Schlievert, P. M., & Watson, D. W. 1978, "Heterogeneity of group A streptococcal pyrogenic exotoxin type B", *Infect.Immun.*, vol. 20, no. 2, pp. 512-518.

Barvitenko, N. N., Adragna, N. C., & Weber, R. E. 2005, "Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance", *Cell Physiol Biochem.*, vol. 15, no. 1-4, pp. 1-18.

Batge, B., Filejski, W., Kurowski, V., Kluter, H., & Djonlagic, H. 1992, "Clostridial sepsis with massive intravascular hemolysis: rapid diagnosis and successful treatment", *Intensive Care Med.*, vol. 18, no. 8, pp. 488-490.

Baumruker, T. & Prieschl, E. E. 2002, "Sphingolipids and the regulation of the immune response", *Semin.Immunol.*, vol. 14, no. 1, pp. 57-63.

Beal, A. L. & Cerra, F. B. 1994, "Multiple organ failure syndrome in the 1990s. Systemic inflammatory response and organ dysfunction", *JAMA*, vol. 271, no. 3, pp. 226-233.

Bell, R. C., Coalson, J. J., Smith, J. D., & Johanson, W. G., Jr. 1983, "Multiple organ system failure and infection in adult respiratory distress syndrome", *Ann.Intern.Med.*, vol. 99, no. 3, pp. 293-298.

Bennett, V. & Baines, A. J. 2001, "Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues", *Physiol Rev.*, vol. 81, no. 3, pp. 1353-1392.

Bennett, V. & Gilligan, D. M. 1993, "The spectrin-based membrane skeleton and micron-scale organization of the plasma membrane", *Annu.Rev.Cell Biol.*, vol. 9, pp. 27-66.

Berg, C. P., Engels, I. H., Rothbart, A., Lauber, K., Renz, A., Schlosser, S. F., Schulze-Osthoff, K., & Wesselborg, S. 2001, "Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis", *Cell Death.Differ.*, vol. 8, no. 12, pp. 1197-1206.

Bergamo, P., Luongo, D., & Rossi, M. 2004, "Conjugated linoleic acid--mediated apoptosis in Jurkat T cells involves the production of reactive oxygen species", *Cell Physiol Biochem.*, vol. 14, no. 1-2, pp. 57-64.

Berlot, G., Di Capua, G., Nosella, P., Rocconi, S., & Thomann, C. 2004, "Plasmapheresis in sepsis", *Contrib.Nephrol.*, vol. 144, pp. 387-394.

Bieberich, E., MacKinnon, S., Silva, J., Noggle, S., & Condie, B. G. 2003, "Regulation of cell death in mitotic neural progenitor cells by asymmetric distribution of prostate apoptosis response 4 (PAR-4) and simultaneous elevation of endogenous ceramide", *J.Cell Biol.*, vol. 162, no. 3, pp. 469-479.

Boas, F. E., Forman, L., & Beutler, E. 1998, "Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia", *Proc.Natl.Acad.Sci.U.S.A*, vol. 95, no. 6, pp. 3077-3081.

Bohach, G. A. 1997, "Staphylococcal enterotoxins B and C. Structural requirements for superantigenic and entertoxigenic activities", *Prep.Biochem.Biotechnol.*, vol. 27, no. 2-3, pp. 79-110.

Bone, R. C., Fisher, C. J., Jr., Clemmer, T. P., Slotman, G. J., Metz, C. A., & Balk, R. A. 1989, "Sepsis syndrome: a valid clinical entity. Methylprednisolone Severe Sepsis Study Group", *Crit Care Med.*, vol. 17, no. 5, pp. 389-393.

Bookchin, R. M., Ortiz, O. E., & Lew, V. L. 1987, "Activation of calcium-dependent potassium channels in deoxygenated sickled red cells", *Prog.Clin.Biol.Res.*, vol. 240, pp. 193-200.

Bortner, C. D. & Cidlowski, J. A. 1999, "Caspase independent/dependent regulation of K(+), cell shrinkage, and mitochondrial membrane potential during lymphocyte apoptosis", *J.Biol.Chem.*, vol. 274, no. 31, pp. 21953-21962.

Bortner, C. D. & Cidlowski, J. A. 2002, "Apoptotic volume decrease and the incredible shrinking cell", *Cell Death.Differ.*, vol. 9, no. 12, pp. 1307-1310.

Bortner, C. D. & Cidlowski, J. A. 2004, "The role of apoptotic volume decrease and ionic homeostasis in the activation and repression of apoptosis", *Pflugers Arch.*, vol. 448, no. 3, pp. 313-318.

Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuks, Z., & Kolesnick, R. 1995, "Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals", *Cell*, vol. 82, no. 3, pp. 405-414.

Bosman, G. J., Willekens, F. L., & Werre, J. M. 2005, "Erythrocyte aging: a more than superficial resemblance to apoptosis?", *Cell Physiol Biochem.*, vol. 16, no. 1-3, pp. 1-8.

Bouchon, A., Facchetti, F., Weigand, M. A., & Colonna, M. 2001, "TREM-1 amplifies inflammation and is a crucial mediator of septic shock", *Nature*, vol. 410, no. 6832, pp. 1103-1107.

Brand, V. B., Sandu, C. D., Duranton, C., Tanneur, V., Lang, K. S., Huber, S. M., & Lang, F. 2003, "Dependence of Plasmodium falciparum in vitro growth on the cation permeability of the human host erythrocyte", *Cell Physiol Biochem.*, vol. 13, no. 6, pp. 347-356.

Brandtzaeg, P., Kierulf, P., Gaustad, P., Skulberg, A., Bruun, J. N., Halvorsen, S., & Sorensen, E. 1989, "Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease", *J.Infect.Dis.*, vol. 159, no. 2, pp. 195-204.

Bratosin, D., Estaquier, J., Petit, F., Arnoult, D., Quatannens, B., Tissier, J. P., Slomianny, C., Sartiaux, C., Alonso, C., Huart, J. J., Montreuil, J., & Ameisen, J. C. 2001, "Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria", *Cell Death.Differ.*, vol. 8, no. 12, pp. 1143-1156.

Brigham, K. L. & Meyrick, B. 1986, "Endotoxin and lung injury", *Am.Rev.Respir.Dis.*, vol. 133, no. 5, pp. 913-927.

Brugnara, C., de Franceschi, L., & Alper, S. L. 1993, "Inhibition of Ca(2+)-dependent K+ transport and cell dehydration in sickle erythrocytes by clotrimazole and other imidazole derivatives", *J.Clin.Invest*, vol. 92, no. 1, pp. 520-526.

Campillo, B., Zittoun, J., & de Gialluly, E. 1988, "Prophylaxis of folate deficiency in acutely ill patients: results of a randomized clinical trial", *Intensive Care Med.*, vol. 14, no. 6, pp. 640-645.

Claus, R. A., Bunck, A. C., Bockmeyer, C. L., Brunkhorst, F. M., Losche, W., Kinscherf, R., & Deigner, H. P. 2005, "Role of increased sphingomyelinase activity in apoptosis and organ failure of patients with severe sepsis", *FASEB J.*, vol. 19, no. 12, pp. 1719-1721.

Closse, C., Dachary-Prigent, J., & Boisseau, M. R. 1999b, "Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium", *Br.J.Haematol.*, vol. 107, no. 2, pp. 300-302.

Closse, C., Dachary-Prigent, J., & Boisseau, M. R. 1999a, "Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium", *Br.J Haematol.*, vol. 107, no. 2, pp. 300-302.

Cobb, J. P. & O'Keefe, G. E. 2004, "Injury research in the genomic era", *Lancet*, vol. 363, no. 9426, pp. 2076-2083.

Cohen, C. M. & Gascard, P. 1992, "Regulation and post-translational modification of erythrocyte membrane and membrane-skeletal proteins", *Semin.Hematol.*, vol. 29, no. 4, pp. 244-292.

Curzen, N. P., Griffiths, M. J., & Evans, T. W. 1994, "Role of the endothelium in modulating the vascular response to sepsis", *Clin.Sci.(Lond)*, vol. 86, no. 4, pp. 359-374.

Cybulsky, M. I., Chan, M. K., & Movat, H. Z. 1988, "Acute inflammation and microthrombosis induced by endotoxin, interleukin-1, and tumor necrosis factor and their implication in gram-negative infection", *Lab Invest*, vol. 58, no. 4, pp.

365-378.

Daniel, P. T., Wieder, T., Sturm, I., & Schulze-Osthoff, K. 2001, "The kiss of death: promises and failures of death receptors and ligands in cancer therapy", *Leukemia*, vol. 15, no. 7, pp. 1022-1032.

Daugas, E., Cande, C., & Kroemer, G. 2001, "Erythrocytes: death of a mummy", *Cell Death.Differ.*, vol. 8, no. 12, pp. 1131-1133.

Dekkers, D. W., Comfurius, P., Bevers, E. M., & Zwaal, R. F. 2002, "Comparison between Ca2+-induced scrambling of various fluorescently labelled lipid analogues in red blood cells", *Biochem.J.*, vol. 362, no. Pt 3, pp. 741-747.

Dinarello, C. A. 1997, "Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock", *Chest*, vol. 112, no. 6 Suppl, pp. 321S-329S.

Dinges, M. M. & Schlievert, P. M. 2001, "Role of T cells and gamma interferon during induction of hypersensitivity to lipopolysaccharide by toxic shock syndrome toxin 1 in mice", *Infect.Immun.*, vol. 69, no. 3, pp. 1256-1264.

Eda, S. & Sherman, I. W. 2002, "Cytoadherence of malaria-infected red blood cells involves exposure of phosphatidylserine", *Cell Physiol Biochem.*, vol. 12, no. 5-6, pp. 373-384.

Esen, M., Schreiner, B., Jendrossek, V., Lang, F., Fassbender, K., Grassme, H., & Gulbins, E. 2001, "Mechanisms of Staphylococcus aureus induced apoptosis of human endothelial cells", *Apoptosis.*, vol. 6, no. 6, pp. 431-439.

Fadok, V. A., Bratton, D. L., Rose, D. M., Pearson, A., Ezekewitz, R. A., & Henson, P. M. 2000a, "A receptor for phosphatidylserine-specific clearance of apoptotic cells", *Nature*, vol. 405, no. 6782, pp. 85-90.

Fadok, V. A., Bratton, D. L., Rose, D. M., Pearson, A., Ezekewitz, R. A., & Henson, P. M. 2000b, "A receptor for phosphatidylserine-specific clearance of apoptotic cells", *Nature*, vol. 405, no. 6782, pp. 85-90.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., & Henson, P. M. 1992, "Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages", *J.Immunol.*, vol. 148, no. 7, pp. 2207-2216.

Falcone, S., Perrotta, C., De Palma, C., Pisconti, A., Sciorati, C., Capobianco, A., Rovere-Querini, P., Manfredi, A. A., & Clementi, E. 2004, "Activation of acid sphingomyelinase and its inhibition by the nitric oxide/cyclic guanosine 3',5'-monophosphate pathway: key events in Escherichia coli-elicited apoptosis of dendritic cells", *J.Immunol.*, vol. 173, no. 7, pp. 4452-4463.

Franco, R. S., Palascak, M., Thompson, H., Rucknagel, D. L., & Joiner, C. H.

1996, "Dehydration of transferrin receptor-positive sickle reticulocytes during continuous or cyclic deoxygenation: role of KCl cotransport and extracellular calcium", *Blood*, vol. 88, no. 11, pp. 4359-4365.

Fry, D. E., Pearlstein, L., Fulton, R. L., & Polk, H. C., Jr. 1980, "Multiple system organ failure. The role of uncontrolled infection", *Arch.Surg.*, vol. 115, no. 2, pp. 136-140.

Gando, S., Kameue, T., Nanzaki, S., & Nakanishi, Y. 1996, "Disseminated intravascular coagulation is a frequent complication of systemic inflammatory response syndrome", *Thromb.Haemost.*, vol. 75, no. 2, pp. 224-228.

Geilen, C. C., Bektas, M., Wieder, T., & Orfanos, C. E. 1996, "The vitamin D3 analogue, calcipotriol, induces sphingomyelin hydrolysis in human keratinocytes", *FEBS Lett.*, vol. 378, no. 1, pp. 88-92.

Goerke, C., Koller, J., & Wolz, C. 2006, "Ciprofloxacin and Trimethoprim Cause Phage Induction and Virulence Modulation in Staphylococcus aureus", *Antimicrob.Agents Chemother.*, vol. 50, no. 1, pp. 171-177.

Grassme, H., Jendrossek, V., Bock, J., Riehle, A., & Gulbins, E. 2002, "Ceramide-rich membrane rafts mediate CD40 clustering", *J.Immunol.*, vol. 168, no. 1, pp. 298-307.

Grassme, H., Kirschnek, S., Riethmueller, J., Riehle, A., von Kurthy, G., Lang, F., Weller, M., & Gulbins, E. 2000, "CD95/CD95 ligand interactions on epithelial cells in host defense to Pseudomonas aeruginosa", *Science*, vol. 290, no. 5491, pp. 527-530.

Green, D. R. & Kroemer, G. 2004, "The pathophysiology of mitochondrial cell death", *Science*, vol. 305, no. 5684, pp. 626-629.

Green, D. R. & Reed, J. C. 1998, "Mitochondria and apoptosis", *Science*, vol. 281, no. 5381, pp. 1309-1312.

Gulbins, E., Jekle, A., Ferlinz, K., Grassme, H., & Lang, F. 2000, "Physiology of apoptosis", *Am.J.Physiol Renal Physiol*, vol. 279, no. 4, p. F605-F615.

Hack, C. E. & Zeerleder, S. 2001, "The endothelium in sepsis: source of and a target for inflammation", *Crit Care Med.*, vol. 29, no. 7 Suppl, p. S21-S27.

Haeney, M. R. 1998, "The role of the complement cascade in sepsis", *J.Antimicrob.Chemother.*, vol. 41 Suppl A, pp. 41-46.

Han, H., Wang, J., Zhang, Y., Long, H., Wang, H., Xu, D., & Wang, Z. 2004, "HERG K channel conductance promotes H2O2-induced apoptosis in HEK293 cells: cellular mechanisms", *Cell Physiol Biochem.*, vol. 14, no. 3, pp. 121-134.

Hannun, Y. A. 1996, "Functions of ceramide in coordinating cellular responses to stress", *Science*, vol. 274, no. 5294, pp. 1855-1859.

Jelkmann, W., Pagel, H., Wolff, M., & Fandrey, J. 1992, "Monokines inhibiting erythropoietin production in human hepatoma cultures and in isolated perfused rat kidneys", *Life Sci.*, vol. 50, no. 4, pp. 301-308.

Jurado, R. L. 1997, "Iron, infections, and anemia of inflammation", *Clin.Infect.Dis.*, vol. 25, no. 4, pp. 888-895.

Kellum, J. A. & Venkataraman, R. 2005, "Application of blood purification to non-renal organ failure", *Int.J.Artif.Organs*, vol. 28, no. 5, pp. 445-449.

Kempe, D. S., Lang, P. A., Duranton, C., Akel, A., Lang, K. S., Huber, S. M., Wieder, T., & Lang, F. 2005a, "Enhanced programmed cell death of iron-deficient erythrocytes", *FASEB J.*

Kempe, D. S., Lang, P. A., Eisele, K., Klarl, B. A., Wieder, T., Huber, S. M., Duranton, C., & Lang, F. 2005b, "Stimulation of erythrocyte phosphatidylserine exposure by lead ions", *Am.J.Physiol Cell Physiol*, vol. 288, no. 2, p. C396-C402.

Kiefer, C. R. & Snyder, L. M. 2000, "Oxidation and erythrocyte senescence", *Curr.Opin.Hematol.*, vol. 7, no. 2, pp. 113-116.

Kienast, J. & Schmitz, G. 1990, "Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders", *Blood*, vol. 75, no. 1, pp. 116-121.

Klarl, B. A., Lang, P. A., Kempe, D. S., Niemoeller, O. M., Akel, A., Sobiesiak, M., Eisele, K., Podolski, M., Huber, S. M., Wieder, T., & Lang, F. 2006, "Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion", *Am.J.Physiol Cell Physiol*, vol. 290, no. 1, p. C244-C253.

Kolesnick, R. & Golde, D. W. 1994, "The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling", *Cell*, vol. 77, no. 3, pp. 325-328.

Kolesnick, R. N. & Kronke, M. 1998, "Regulation of ceramide production and apoptosis", *Annu.Rev.Physiol*, vol. 60, pp. 643-665.

Krafte-Jacobs, B. 1997, "Anemia of critical illness and erythropoietin deficiency", *Intensive Care Med.*, vol. 23, no. 2, pp. 137-138.

Krafte-Jacobs, B., Levetown, M. L., Bray, G. L., Ruttimann, U. E., & Pollack, M. M. 1994, "Erythropoietin response to critical illness", *Crit Care Med.*, vol. 22, no. 5, pp. 821-826.

Krantz, S. B. 1994, "Pathogenesis and treatment of the anemia of chronic disease", *Am.J.Med.Sci.*, vol. 307, no. 5, pp. 353-359.

Kyles, D. M. & Baltimore, J. 2005, "Adjunctive use of plasmapheresis and intravenous immunoglobulin therapy in sepsis: a case report", *Am.J.Crit Care*, vol. 14, no. 2, pp. 109-112.

Landry, D. W. & Oliver, J. A. 2001, "The pathogenesis of vasodilatory shock", *N.Engl.J.Med.*, vol. 345, no. 8, pp. 588-595.

Lang, F., Busch, G. L., Ritter, M., Volkl, H., Waldegger, S., Gulbins, E., & Haussinger, D. 1998, "Functional significance of cell volume regulatory mechanisms", *Physiol Rev.*, vol. 78, no. 1, pp. 247-306.

Lang, F., Ritter, M., Gamper, N., Huber, S., Fillon, S., Tanneur, V., Lepple-Wienhues, A., Szabo, I., & Gulbins, E. 2000, "Cell volume in the regulation of cell proliferation and apoptotic cell death", *Cell Physiol Biochem.*, vol. 10, no. 5-6, pp. 417-428.

Lang, F., Szabo, I., Lepple-Wienhues, A., Siemen, D., & Gulbins, E. 1999, "Physiology of Receptor-Mediated Lymphocyte Apoptosis", *News Physiol Sci.*, vol. 14, pp. 194-200.

Lang, K. S., Duranton, C., Poehlmann, H., Myssina, S., Bauer, C., Lang, F., Wieder, T., & Huber, S. M. 2003a, "Cation channels trigger apoptotic death of erythrocytes", *Cell Death.Differ.*, vol. 10, no. 2, pp. 249-256.

Lang, K. S., Lang, P. A., Bauer, C., Duranton, C., Wieder, T., Huber, S. M., & Lang, F. 2005a, "Mechanisms of suicidal erythrocyte death", *Cell Physiol Biochem.*, vol. 15, no. 5, pp. 195-202.

Lang, K. S., Myssina, S., Brand, V., Sandu, C., Lang, P. A., Berchtold, S., Huber, S. M., Lang, F., & Wieder, T. 2004a, "Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes", *Cell Death.Differ.*, vol. 11, no. 2, pp. 231-243.

Lang, K. S., Myssina, S., Brand, V., Sandu, C., Lang, P. A., Berchtold, S., Huber, S. M., Lang, F., & Wieder, T. 2004b, "Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes", *Cell Death.Differ.*, vol. 11, no. 2, pp. 231-243.

Lang, K. S., Myssina, S., Lang, P. A., Tanneur, V., Kempe, D. S., Mack, A. F., Huber, S. M., Wieder, T., Lang, F., & Duranton, C. 2004c, "Inhibition of erythrocyte phosphatidylserine exposure by urea and Cl-", *Am.J.Physiol Renal Physiol*, vol. 286, no. 6, p. F1046-F1053.

Lang, K. S., Roll, B., Myssina, S., Schittenhelm, M., Scheel-Walter, H. G., Kanz, L., Fritz, J., Lang, F., Huber, S. M., & Wieder, T. 2002, "Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency", *Cell Physiol Biochem.*, vol. 12, no. 5-6, pp. 365-372.

Lang, P. A., Kaiser, S., Myssina, S., Birka, C., Weinstock, C., Northoff, H., Wieder, T., Lang, F., & Huber, S. M. 2004d, "Effect of Vibrio parahaemolyticus haemolysin on human erythrocytes", *Cell Microbiol.*, vol. 6, no. 4, pp. 391-400.

Lang, P. A., Kaiser, S., Myssina, S., Wieder, T., Lang, F., & Huber, S. M. 2003b,

"Role of Ca2+-activated K+ channels in human erythrocyte apoptosis", *Am J Physiol Cell Physiol*, vol. 285, no. 6, p. C1553-C1560.

Lang, P. A., Kaiser, S., Myssina, S., Wieder, T., Lang, F., & Huber, S. M. 2003c, "Role of Ca2+-activated K+ channels in human erythrocyte apoptosis", *Am.J.Physiol Cell Physiol*, vol. 285, no. 6, p. C1553-C1560.

Lang, P. A., Kempe, D. S., Tanneur, V., Eisele, K., Klarl, B. A., Myssina, S., Jendrossek, V., Ishii, S., Shimizu, T., Waidmann, M., Hessler, G., Huber, S. M., Lang, F., & Wieder, T. 2005b, "Stimulation of erythrocyte ceramide formation by platelet-activating factor", *J.Cell Sci.*, vol. 118, no. Pt 6, pp. 1233-1243.

Lehr, H. A., Bittinger, F., & Kirkpatrick, C. J. 2000, "Microcirculatory dysfunction in sepsis: a pathogenetic basis for therapy?", *J.Pathol.*, vol. 190, no. 3, pp. 373-386.

Levi, M., ten Cate, H., van der, P. T., & van Deventer, S. J. 1993, "Pathogenesis of disseminated intravascular coagulation in sepsis", *JAMA*, vol. 270, no. 8, pp. 975-979.

Long, H., Han, H., Yang, B., & Wang, Z. 2003, "Opposite cell density-dependence between spontaneous and oxidative stress-induced apoptosis in mouse fibroblast L-cells", *Cell Physiol Biochem.*, vol. 13, no. 6, pp. 401-414.

Luna, E. J. & Hitt, A. L. 1992, "Cytoskeleton--plasma membrane interactions", *Science*, vol. 258, no. 5084, pp. 955-964.

Lux, S. E. 1979, "Dissecting the red cell membrane skeleton", *Nature*, vol. 281, no. 5731, pp. 426-429.

Macnaughton, P. D. & Evans, T. W. 1992, "Management of adult respiratory distress syndrome", *Lancet*, vol. 339, no. 8791, pp. 469-472.

Maeno, E., Ishizaki, Y., Kanaseki, T., Hazama, A., & Okada, Y. 2000, "Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis", *Proc.Natl.Acad.Sci.U.S.A*, vol. 97, no. 17, pp. 9487-9492.

Majcherczyk, P. A., Langen, H., Heumann, D., Fountoulakis, M., Glauser, M. P., & Moreillon, P. 1999, "Digestion of Streptococcus pneumoniae cell walls with its major peptidoglycan hydrolase releases branched stem peptides carrying proinflammatory activity", *J.Biol.Chem.*, vol. 274, no. 18, pp. 12537-12543.

Mandal, D., Mazumder, A., Das, P., Kundu, M., & Basu, J. 2005, "Fas-, caspase 8-, and caspase 3-dependent signaling regulates the activity of the aminophospholipid translocase and phosphatidylserine externalization in human erythrocytes", *J.Biol.Chem.*, vol. 280, no. 47, pp. 39460-39467.

Martin, M. A. & Silverman, H. J. 1992, "Gram-negative sepsis and the adult respiratory distress syndrome", *Clin.Infect.Dis.*, vol. 14, no. 6, pp. 1213-1228.

Martinou, J. C. & Green, D. R. 2001, "Breaking the mitochondrial barrier", *Nat.Rev.Mol.Cell Biol.*, vol. 2, no. 1, pp. 63-67.

Michie, H. R., Spriggs, D. R., Manogue, K. R., Sherman, M. L., Revhaug, A., O'Dwyer, S. T., Arthur, K., Dinarello, C. A., Cerami, A., Wolff, S. M., & . 1988, "Tumor necrosis factor and endotoxin induce similar metabolic responses in human beings", *Surgery*, vol. 104, no. 2, pp. 280-286.

Minetti, G., Piccinini, G., Balduini, C., Seppi, C., & Brovelli, A. 1996, "Tyrosine phosphorylation of band 3 protein in Ca2+/A23187-treated human erythrocytes", *Biochem.J.*, vol. 320 (Pt 2), pp. 445-450.

Minino, A. M., Arias, E., Kochanek, K. D., Murphy, S. L., & Smith, B. L. 2002, "Deaths: final data for 2000", *Natl.Vital Stat.Rep.*, vol. 50, no. 15, pp. 1-119.

Minino, A. M. & Smith, B. L. 2001, "Deaths: preliminary data for 2000", *Natl.Vital Stat.Rep.*, vol. 49, no. 12, pp. 1-40.

Mohandas, N. & Evans, E. 1994, "Mechanical properties of the red cell membrane in relation to molecular structure and genetic defects", *Annu.Rev.Biophys.Biomol.Struct.*, vol. 23, pp. 787-818.

Moore, M. R., Schrag, S. J., & Schuchat, A. 2003, "Effects of intrapartum antimicrobial prophylaxis for prevention of group-B-streptococcal disease on the incidence and ecology of early-onset neonatal sepsis", *Lancet Infect.Dis.*, vol. 3, no. 4, pp. 201-213.

Morath, S., Geyer, A., & Hartung, T. 2001, "Structure-function relationship of cytokine induction by lipoteichoic acid from Staphylococcus aureus", *J.Exp.Med.*, vol. 193, no. 3, pp. 393-397.

Mutunga, M., Fulton, B., Bullock, R., Batchelor, A., Gascoigne, A., Gillespie, J. I., & Baudouin, S. V. 2001, "Circulating endothelial cells in patients with septic shock", *Am.J.Respir.Crit Care Med.*, vol. 163, no. 1, pp. 195-200.

Myssina, S., Lang, P. A., Kempe, D. S., Kaiser, S., Huber, S. M., Wieder, T., & Lang, F. 2004a, "Cl- channel blockers NPPB and niflumic acid blunt Ca(2+)-induced erythrocyte 'apoptosis'", *Cell Physiol Biochem.*, vol. 14, no. 4-6, pp. 241-248.

Myssina, S., Lang, P. A., Kempe, D. S., Kaiser, S., Huber, S. M., Wieder, T., & Lang, F. 2004b, "Cl- channel blockers NPPB and niflumic acid blunt Ca(2+)-induced erythrocyte 'apoptosis'", *Cell Physiol Biochem*, vol. 14, no. 4-6, pp. 241-248.

Napolitano, L. M. 2004, "Current status of blood component therapy in surgical critical care", *Curr.Opin.Crit Care*, vol. 10, no. 5, pp. 311-317.

Navarre, W. W. & Zychlinsky, A. 2000, "Pathogen-induced apoptosis of macrophages: a common end for different pathogenic strategies", *Cell*

Microbiol., vol. 2, no. 4, pp. 265-273.

Norimura, T., Nomoto, S., Katsuki, M., Gondo, Y., & Kondo, S. 1996, "p53-dependent apoptosis suppresses radiation-induced teratogenesis", *Nat.Med.*, vol. 2, no. 5, pp. 577-580.

Nunomura, W., Takakuwa, Y., Tokimitsu, R., Krauss, S. W., Kawashima, M., & Mohandas, N. 1997, "Regulation of CD44-protein 4.1 interaction by Ca2+ and calmodulin. Implications for modulation of CD44-ankyrin interaction", *J.Biol.Chem.*, vol. 272, no. 48, pp. 30322-30328.

Obeid, L. M., Linardic, C. M., Karolak, L. A., & Hannun, Y. A. 1993, "Programmed cell death induced by ceramide", *Science*, vol. 259, no. 5102, pp. 1769-1771.

Okada, Y., Maeno, E., Shimizu, T., Dezaki, K., Wang, J., & Morishima, S. 2001, "Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD)", *J.Physiol*, vol. 532, no. Pt 1, pp. 3-16.

Palek, J. & Lambert, S. 1990, "Genetics of the red cell membrane skeleton", *Semin.Hematol.*, vol. 27, no. 4, pp. 290-332.

Parsons, P. E., Worthen, G. S., Moore, E. E., Tate, R. M., & Henson, P. M. 1989, "The association of circulating endotoxin with the development of the adult respiratory distress syndrome", *Am.Rev.Respir.Dis.*, vol. 140, no. 2, pp. 294-301.

Pena, L. A., Fuks, Z., & Kolesnick, R. 1997, "Stress-induced apoptosis and the sphingomyelin pathway", *Biochem.Pharmacol.*, vol. 53, no. 5, pp. 615-621.

Perretti, M. & Solito, E. 2004, "Annexin 1 and neutrophil apoptosis", *Biochem.Soc.Trans.*, vol. 32, no. Pt3, pp. 507-510.

Peters LL & BarkerJE 2001, "Spontaneous and targeted mutations in erythrocyte membrane skeleton genes: mouse models of hereditary spherocytosis," in *Clinical Medicine*, Zon LI, ed., Oxford Press, New York, pp. 582-608.

Pettus, B. J., Chalfant, C. E., & Hannun, Y. A. 2002, "Ceramide in apoptosis: an overview and current perspectives", *Biochim.Biophys.Acta*, vol. 1585, no. 2-3, pp. 114-125.

Raisova, M., Bektas, M., Wieder, T., Daniel, P., Eberle, J., Orfanos, C. E., & Geilen, C. C. 2000, "Resistance to CD95/Fas-induced and ceramide-mediated apoptosis of human melanoma cells is caused by a defective mitochondrial cytochrome c release", *FEBS Lett.*, vol. 473, no. 1, pp. 27-32.

Renehan, A. G., Booth, C., & Potten, C. S. 2001, "What is apoptosis, and why is it important?", *BMJ*, vol. 322, no. 7301, pp. 1536-1538.

Rice, L. & Alfrey, C. P. 2005, "The negative regulation of red cell mass by neocytolysis: physiologic and pathophysiologic manifestations", *Cell Physiol Biochem.*, vol. 15, no. 6, pp. 245-250.

Rice, T. W. & Bernard, G. R. 2004, "New additions to the intensive care armamentarium", *Drugs Today (Barc.)*, vol. 40, no. 2, pp. 157-170.

Rodriguez, R. M., Corwin, H. L., Gettinger, A., Corwin, M. J., Gubler, D., & Pearl, R. G. 2001, "Nutritional deficiencies and blunted erythropoietin response as causes of the anemia of critical illness", *J.Crit Care*, vol. 16, no. 1, pp. 36-41.

Rogiers, P., Zhang, H., Leeman, M., Nagler, J., Neels, H., Melot, C., & Vincent, J. L. 1997, "Erythropoietin response is blunted in critically ill patients", *Intensive Care Med.*, vol. 23, no. 2, pp. 159-162.

Rosette, C. & Karin, M. 1996, "Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors", *Science*, vol. 274, no. 5290, pp. 1194-1197.

Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., & Korsmeyer, S. J. 2003, "BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis", *Science*, vol. 300, no. 5616, pp. 135-139.

Seigneuret, M. & Devaux, P. F. 1984, "ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes", *Proc.Natl.Acad.Sci.U.S.A*, vol. 81, no. 12, pp. 3751-3755.

Sessler, C. N., Perry, J. C., & Varney, K. L. 2004, "Management of severe sepsis and septic shock", *Curr.Opin.Crit Care*, vol. 10, no. 5, pp. 354-363.

Singer, M., De, S., V, Vitale, D., & Jeffcoate, W. 2004, "Multiorgan failure is an adaptive, endocrine-mediated, metabolic response to overwhelming systemic inflammation", *Lancet*, vol. 364, no. 9433, pp. 545-548.

Sparwasser, T., Miethke, T., Lipford, G., Borschert, K., Hacker, H., Heeg, K., & Wagner, H. 1997, "Bacterial DNA causes septic shock", *Nature*, vol. 386, no. 6623, pp. 336-337.

Spiegel, S., Foster, D., & Kolesnick, R. 1996, "Signal transduction through lipid second messengers", *Curr.Opin.Cell Biol.*, vol. 8, no. 2, pp. 159-167.

Steinbrecher, U. P., Gomez-Munoz, A., & Duronio, V. 2004, "Acid sphingomyelinase in macrophage apoptosis", *Curr.Opin.Lipidol.*, vol. 15, no. 5, pp. 531-537.

Stokke, B. T., Mikkelsen, A., & Elgsaeter, A. 1986, "The human erythrocyte membrane skeleton may be an ionic gel. I. Membrane mechanochemical properties", *Eur.Biophys.J.*, vol. 13, no. 4, pp. 203-218.

Strasser, A., O'Connor, L., & Dixit, V. M. 2000, "Apoptosis signaling", *Annu.Rev.Biochem.*, vol. 69, pp. 217-245.

Sturm, J. W., Zhang, H., Magdeburg, R., Hasenberg, T., Bonninghoff, R., Oulmi, J., Keese, M., & McCuskey, R. 2004, "Altered apoptotic response and different liver structure during liver regeneration in FGF-2-deficient mice", *Cell Physiol Biochem.*, vol. 14, no. 4-6, pp. 249-260.

Svetina, S. & Iglic, A. K.-I. V. &. Z. B. 1996, "Cytoskeleton and red cell shape", *Cell.Mol.Biol.Lett.*, vol. 1, pp. 67-75.

Takakuwa, Y. & Mohandas, N. 1988, "Modulation of erythrocyte membrane material properties by Ca2+ and calmodulin. Implications for their role in regulation of skeletal protein interactions", *J.Clin.Invest*, vol. 82, no. 2, pp. 394-400.

Terpos, E., Theocharis, S., Panitsas, F., Philippidis, T., Kotronis, E., & Karkantaris, C. 2004, "Autoimmune hemolytic anemia with myelodysplastic features followed by bilateral adrenal non-hodgkin lymphoma: a case report and review of the literature", *Leuk.Lymphoma*, vol. 45, no. 11, pp. 2333-2338.

Thijs, L. G., de Boer, J. P., de Groot, M. C., & Hack, C. E. 1993, "Coagulation disorders in septic shock", *Intensive Care Med.*, vol. 19 Suppl 1, pp. S8-15.

Thijs, L. G. & Hack, C. E. 1995, "Time course of cytokine levels in sepsis", *Intensive Care Med.*, vol. 21 Suppl 2, p. S258-S263.

Tse, W. T. & Lux, S. E. 1999, "Red blood cell membrane disorders", *Br.J.Haematol.*, vol. 104, no. 1, pp. 2-13.

Ulevitch, R. J., Cochrane, C. G., Henson, P. M., Morrison, D. C., & Doe, W. F. 1975, "Mediation systems in bacterial lipopolysaccharide-induced hypotension and disseminated intravascular coagulation. I. The role of complement", *J.Exp.Med.*, vol. 142, no. 6, pp. 1570-1590.

Unger, R. H. 2002, "Lipotoxic diseases", *Annu.Rev.Med*, vol. 53, pp. 319-336.

van der, P. T. 2001, "Immunotherapy of sepsis", *Lancet Infect.Dis.*, vol. 1, no. 3, pp. 165-174.

van der, P. T. & van Deventer, S. J. 1999, "Cytokines and anticytokines in the pathogenesis of sepsis", *Infect.Dis.Clin.North Am.*, vol. 13, no. 2, pp. 413-26, ix.

van Deventer, S. J., Buller, H. R., ten Cate, J. W., Aarden, L. A., Hack, C. E., & Sturk, A. 1990, "Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways", *Blood*, vol. 76, no. 12, pp. 2520-2526.

van Iperen, C. E., Gaillard, C. A., Kraaijenhagen, R. J., Braam, B. G., Marx, J. J., & van de, W. A. 2000, "Response of erythropoiesis and iron metabolism to

recombinant human erythropoietin in intensive care unit patients", *Crit Care Med.*, vol. 28, no. 8, pp. 2773-2778.

Vincent, J. L., Baron, J. F., Reinhart, K., Gattinoni, L., Thijs, L., Webb, A., Meier-Hellmann, A., Nollet, G., & Peres-Bota, D. 2002, "Anemia and blood transfusion in critically ill patients", *JAMA*, vol. 288, no. 12, pp. 1499-1507.

von Haefen, C., Wieder, T., Gillissen, B., Starck, L., Graupner, V., Dorken, B., & Daniel, P. T. 2002, "Ceramide induces mitochondrial activation and apoptosis via a Bax-dependent pathway in human carcinoma cells", *Oncogene*, vol. 21, no. 25, pp. 4009-4019.

Wang, J. E., Jorgensen, P. F., Almlof, M., Thiemermann, C., Foster, S. J., Aasen, A. O., & Solberg, R. 2000, "Peptidoglycan and lipoteichoic acid from Staphylococcus aureus induce tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model", *Infect.Immun.*, vol. 68, no. 7, pp. 3965-3970.

Weedon, D., Searle, J., & Kerr, J. F. 1979, "Apoptosis. Its nature and implications for dermatopathology", *Am.J.Dermatopathol.*, vol. 1, no. 2, pp. 133-144.

Wenzel, U. & Daniel, H. 2004, "Early and late apoptosis events in human transformed and non-transformed colonocytes are independent on intracellular acidification", *Cell Physiol Biochem.*, vol. 14, no. 1-2, pp. 65-76.

Wieder, T., Essmann, F., Prokop, A., Schmelz, K., Schulze-Osthoff, K., Beyaert, R., Dorken, B., & Daniel, P. T. 2001, "Activation of caspase-8 in drug-induced apoptosis of B-lymphoid cells is independent of CD95/Fas receptor-ligand interaction and occurs downstream of caspase-3", *Blood*, vol. 97, no. 5, pp. 1378-1387.

Wieder, T., Orfanos, C. E., & Geilen, C. C. 1998, "Induction of ceramide-mediated apoptosis by the anticancer phospholipid analog, hexadecylphosphocholine", *J.Biol.Chem.*, vol. 273, no. 18, pp. 11025-11031.

Willekens, F. L., Werre, J. M., Kruijt, J. K., Roerdinkholder-Stoelwinder, B., Groenen-Dopp, Y. A., van den Bos, A. G., Bosman, G. J., & van Berkel, T. J. 2005, "Liver Kupffer cells rapidly remove red blood cell-derived vesicles from the circulation by scavenger receptors", *Blood*, vol. 105, no. 5, pp. 2141-2145.

Woon, L. A., Holland, J. W., Kable, E. P., & Roufogalis, B. D. 1999, "Ca2+ sensitivity of phospholipid scrambling in human red cell ghosts", *Cell Calcium*, vol. 25, no. 4, pp. 313-320.

Wyllie, A. H. 1980, "Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation", *Nature*, vol. 284, no. 5756, pp. 555-556.

Wyllie, A. H., Bellamy, C. O., Bubb, V. J., Clarke, A. R., Corbet, S., Curtis, L.,

Harrison, D. J., Hooper, M. L., Toft, N., Webb, S., & Bird, C. C. 1999, "Apoptosis and carcinogenesis", *Br.J.Cancer*, vol. 80 Suppl 1, pp. 34-37.

Yu, S. P. 2003, "Regulation and critical role of potassium homeostasis in apoptosis", *Prog.Neurobiol.*, vol. 70, no. 4, pp. 363-386.

Yu, S. P., Canzoniero, L. M., & Choi, D. W. 2001, "Ion homeostasis and apoptosis", *Curr.Opin.Cell Biol.*, vol. 13, no. 4, pp. 405-411.

Yurinskaya, V., Goryachaya, T., Guzhova, I., Moshkov, A., Rozanov, Y., Sakuta, G., Shirokova, A., Shumilina, E., Vassilieva, I., Lang, F., & Vereninov, A. 2005a, "Potassium and sodium balance in U937 cells during apoptosis with and without cell shrinkage", *Cell Physiol Biochem.*, vol. 16, no. 4-6, pp. 155-162.

Yurinskaya, V. E., Moshkov, A. V., Rozanov, Y. M., Shirokova, A. V., Vassilieva, I. O., Shumilina, E. V., Lang, F., Volgareva, E. V., & Vereninov, A. A. 2005b, "Thymocyte K+, Na+ and water balance during dexamethasone- and etoposide-induced apoptosis", *Cell Physiol Biochem.*, vol. 16, no. 1-3, pp. 15-22.

Zhou, Q., Zhao, J., Wiedmer, T., & Sims, P. J. 2002, "Normal hemostasis but defective hematopoietic response to growth factors in mice deficient in phospholipid scramblase 1", *Blood*, vol. 99, no. 11, pp. 4030-4038.

Zimmerman, J. E., Seneff, M. G., Sun, X., Wagner, D. P., & Knaus, W. A. 1997, "Evaluating laboratory usage in the intensive care unit: patient and institutional characteristics that influence frequency of blood sampling", *Crit Care Med.*, vol. 25, no. 5, pp. 737-748.

7.Publications

A part of the present thesis has already been published:

7.1. Original publications

2005 Inhibition of erythrocyte "apoptosis" by catecholamines.
Naunyn Schmiedebergs Arch Pharmacology 372 :228-235.
Lang PA, Kempe DS, **Akel A**, Klarl BA, Eisele K, Podolski M, Hermle T, Niemoeller OM, Attanasio P, Huber SM, Wieder T, Lang F, Duranton C.

2006 Stimulation of erythrocyte phosphatidylserine exposure by chlorpromazine.
Eur J Pharm. 532: 11-17.
Ahmad Akel, Tobias Hermle, Olivier M. Niemoeller, Daniela S. Kempe, Philipp A. Lang, Philipp Attanasio, Marlis Podolski, Thomas Wieder, Florian Lang.

2006 Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion.
Am J Physiol Cell Physiol. 290: C244-53.
Klarl BA, Lang PA, Kempe DS, Niemoeller OM, **Akel A**, Sobiesiak M, Eisele K, Podolski M, Huber SM, Wieder T, Lang F.

2006 SGK1 Kinase Upregulates GLUT1 Activity and Plasma Membrane Expression.
Diabetes 55: 421-7.
Monica Palmada, Christoph Boehmer, **Ahmad Akel**, Jeyaganesh Rajamanickam, Sankarganesh Jeyaraj, Konrad Keller and Florian Lang.

2006 Enhanced programmed cell death of iron-deficient erythrocytes.
FASEB J 20: 368-370.
Kempe DS, Lang PA, Duranton C, **Akel A**, Lang KS, Huber SM, Wieder T, Lang F.

2006 Decreased cation channel activity and blunted channel-dependent eryptosis in neonatal erythrocytes
Am J Physiol Cell Physiol 291: C710 - C717.
Tobias Hermle, Ekaterina Shumilina, Philipp Attanasio, **Ahmad Akel**, Daniela S. Kempe, Philipp A. Lang, Marlies Podolski, Robert Bachmann, Cornelia Bachmann, Harald Abele, Thomas Wieder, Stephan Huber, Florian Lang.

2006 Suicidal death of erythrocytes in recurrent hemolytic-uremic syndrome.
J Mol Med 84: 378-388.
Philipp A. Lang, Ortraud Beringer, Jan P. Nicolay, Oliver Amon, Daniela S. Kempe, Tobias Hermle, Philipp Attanasio, **Ahmad Akel**, Richard Schäfer, Björn Friedrich, Teut Risler, Matthias Baur, Christoph J. Olbricht, Lothar Bernd Zimmerhackl, Peter F. Zipfel, Thomas Wieder, Florian Lang.

2006 Stimulation of erythrocyte phosphatidylserine exposure by paclitaxel.
Cell Physiol Biochem 18: 151-164.
Lang, P. A., Huober, J., Bachmann, C., Kempe, D. S., Sobiesiak, M., **Akel, A.,** Niemoeller, O. M., Dreischer, P., Eisele, K., Klarl, B. A., Gulbins, E., Lang, F., and Wieder, Th.

2006 Suicidal erythrocyte death in sepsis.
J Mol Med. 2006 Dec 16.
Kempe D. S., **Akel A.,** Lang P. A., Hermle T., Biswas R., Muresanu J., Friedrich B., Dreicher P., Wolz C., Schumacher U., Peschel A., Goetz F., Döring G., Wieder Th., Gulbins E., and Lang F

2006 Enhanced eryptosis of erythrocytes from gene-targeted mice lacking the Cl-/HCO3- exchanger AE1
Am J Physiol Cell Physiol. 2007 Jan 24 .
Akel A, Wagner CA, Kovacikova J, Kasinathan RS, Kiedaisch V, Koka S, Alper SL, Bernhardt I, Wieder T, Huber S, Lang F.

2006 Induction of eryptosis by cyclosporine.
Naunyn Schmiedebergs Arch Pharmacol. 2006 Oct;374(1):41-9. Epub 2006 Sep 22..
Olivier M. Niemoeller, **Ahmad Akel**, Philipp A. Lang, Philipp Attanasio, Daniela S. Kempe, Tobias Hermle, Malgorzata Sobiesiak, Thomas Wieder[1] and Florian Lang.

7.2. Abstracts and posters

14-17.03. 2004 Deutsche Physiologische Gesellschaft, 83 Annual meeting, 2004 Leipzig (Poster).
The retinal glutamate transporter EAAT3 is stimulated by the serum and glucocorticoid inducible kinase SGK1 and the related kinase PKB.

6-9. 03. 2005 Deutsche Physiologische Gesellschaft, 84 Annual meeting, 2005 Göttingen (Poster).
Stimulation of RBC apoptosis by paclitaxel.

26-29.03. 2006 Deutsche Physiologische Gesellschaft, 85 Annual meeting, 2006
München (Poster).
Enhanced eryptosis of red blood cells from gene targeted mice lacking the $Cl^-/HCO3^-$ exchanger AE1.

8. Acknowledgements

This work was carried out and completed under the supervision of Prof. Dr. med. Florian Lang at the Institute of Physiology at the University of Tübingen from October 2003 to December 2006.

First of all, I would like to express my sincere gratitude to Prof. F. Lang who gave me the chance to join his institute, and for his helpful guidance, support and understanding in every aspect throughout my project program.

Especially, I would like to thank PD Dr. Thomas Wieder for his help in finishing and reviewing my dissertation. I am gratefully aware that without his helpful engagement and great knowledge my work could have never been successfully accomplished.

I am grateful to all my laboratory colleagues for the comfortable work atmosphere, for their support and friendship.

9. Curriculum Vitae

Ahmad Akel

Nationalität:	syrisch
Familienstand:	verheiratet
geb. am	28. Mai 1975, Damaskus, Syrien

1993	Abiturprüfung naturwissenschaftlicher Fachrichtung in Homs, Syrien („Ausgezeichnet“)
1993-1999	Studium an der Fakultät für Humanmedizin der Universität Damaskus (“sehr gut“)
1999-2001	Assistenzarzt am Zentrum für Nuklearmedizin, Damaskus
03/2002-09/2002	Assistenzarzt am privaten Krankenhaus “Al-Shifa Hospital“, Abteilung für Innere Medizin
2001-07/2003	Fakultäts-Assistent an der Fakultät für Humanmedizin der Universität Damaskus, Abteilung Physiologie
10/2003-jetzt	Promotion an der medizinischen Fakultät der Universität Tübingen, Abteilung Physiologie
4/2006-1/2007	Gastarzt in der Abteilung für Radioonkologie der Universitätsklinik Tübingen
1/2007- jetzt	Gastarzt in der Klinik für Kinder- und Jugendmedizin der Universitätsklinik Tübingen, Abteilung I .

Besondere Sprachkenntnisse

- Arabisch Muttersprache
- English sehr gut
- Deutsch sehr gut (Sprachkurse am Goethe-Institut in Damaskus und am Zentrum-Institut in Frankfurt/M)

- Französisch Grundkenntnisse (Kurse im Institut Culturel Franco-Allemand (ICFA) in Tübingen)