

## Suicidal erythrocyte death in sepsis

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**Abstract** Sequelae of sepsis include anemia 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  $\text{Ca}^{2+}$  activity and ceramide both trigger suicidal erythrocyte death (i.e., eryptosis), which is characterized by lipid scrambling of the cell membrane leading to phosphatidylserine exposure at the erythrocyte surface. Phosphatidylserine exposing erythrocytes may adhere to vascular walls or may be engulfed by macrophages equipped with phosphatidylserine receptors. To explore whether sepsis leads to eryptosis, erythrocytes from healthy volunteers were exposed to plasma of patients

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suffering from sepsis, or to supernatants from sepsis producing pathogens. Then, phosphatidylserine exposure (annexin V binding), cell volume (forward scatter), cytosolic  $\text{Ca}^{2+}$  activity (Fluo3 fluorescence), and ceramide formation (anti-ceramide antibody) were determined by

flow cytometry. Challenge of erythrocytes with plasma from the patients but not with plasma from healthy individuals triggered annexin V binding. The effect of patient plasma on erythrocyte annexin V binding was paralleled by formation of ceramide and a significant increase of cytosolic  $\text{Ca}^{2+}$  activity. Exposure of erythrocytes to supernatant of pathogens similarly induced eryptosis, an effect correlating with sphingomyelinase activity. The present observations disclose a novel pathophysiological mechanism leading to anemia and derangement of microcirculation during sepsis. Exposure to plasma from septic patients triggers phosphatidylserine exposure leading to adherence to the vascular wall and clearance from circulating blood.

**Keywords** Apoptosis · Ceramide · Calcium · Microcirculation · Anemia

## Introduction

Sepsis is a life-threatening condition during overwhelming infection with a variety of pathogens [1, 2]. Characteristic sequelae of sepsis include rapidly developing anemia which cannot be accounted for by decreased formation of erythrocytes but must involve accelerated clearance of erythrocytes from circulating blood [1].

The clearance of circulating erythrocytes is at least in part the result of hemolysis [3, 4]. Recent in vitro experiments disclosed a novel mechanism affecting erythrocyte survival. Erythrocytes exposed to oxidative stress, hyperosmotic shock, or energy depletion activate a  $\text{Ca}^{2+}$ -

permeable cation channel [5] with subsequent entry of  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$  then activates  $\text{Ca}^{2+}$ -sensitive  $\text{K}^+$  channels leading to cell shrinkage [6]. Moreover, the entry of  $\text{Ca}^{2+}$  triggers  $\text{Ca}^{2+}$  sensitive scrambling of the cell membrane [7] with transbilayer movement of plasma membrane phospholipids and exposure of phosphatidylserine at the erythrocyte surface [5]. The erythrocytes are sensitized towards  $\text{Ca}^{2+}$  by the sphingolipid metabolite ceramide, which is also released after erythrocyte injury [8].

Phosphatidylserine exposing erythrocytes may adhere to endothelial cells of the vascular wall and thus impede microcirculation [9]. On the other hand, macrophages are equipped with receptors specific for phosphatidylserine [10], and erythrocytes exposing phosphatidylserine at their surface are recognized, engulfed, and degraded [11]. Thus, erythrocytes exposing phosphatidylserine at their surface are prone to be eliminated from circulating blood. Erythrocytes destroyed by suicidal death (i.e., eryptosis) [12] may undergo similar but not necessarily identical changes as those undergoing erythrocyte senescence [12–16] or neocytolysis [15].

Eryptosis may be triggered by complement activation in the course of hemolytic–uremic syndrome [17] or by bacterial toxins, such as hemolysin Kanagawa [18]. Several sepsis-inducing bacterial pathogens are known to produce sphingomyelinases, which in turn could stimulate ceramide formation [8]. Along those lines, the  $\beta$ -toxin of *Staphylococcus aureus*, one of the most frequent causative agents of sepsis, is a secretory sphingomyelinase [19].

The present study has been performed to explore whether erythrocyte phosphatidylserine exposure participates in the pathophysiology of sepsis.

**Table 1** Clinical data of patients included in the study

Number	Age	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	<i>S. epidermis</i>	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	<i>E. coli</i>	3	27	Survived
8	39	M	Non-Hodgkin-lymphoma, Sepsis in aplasia	<i>S. epidermis</i>	1	30	Exitus letalis
9	54	M	Pacemaker infection	<i>S. aureus</i>	1	21	Survived
10	49	M	Toxic shock syndrome	<i>S. aureus</i>	35	13	Survived
11	64	M	Unknown focus	<i>B. fragilis</i>	3	14	Survived

APACHE II score is given in points.

F Female, M male

**Materials and methods**

**Patients and plasma**

Heparinized plasma was isolated from healthy volunteers or from patients suffering from severe sepsis who have been treated in the intensive care unit of the University Hospital of Tübingen. Table 1 lists the clinical data of the patients included in this study. The heparinized plasma was added to erythrocytes with identical blood group from healthy volunteers in vitro. The relatives of the patients and the volunteers providing erythrocytes gave informed consent. The study has been approved by the ethics committee of the University of Tübingen.

**Solutions and chemicals**

Experiments were performed at 37°C either in a CO<sub>2</sub> free (Ringer) or in a 5% CO<sub>2</sub> atmosphere (plasma) with blood group matched isolated erythrocytes drawn from healthy volunteers. Ringer solution contained (in millimolar) 125 NaCl, 5 KCl, 1 MgSO<sub>4</sub>, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl<sub>2</sub>; pH 7.4. Ionomycin was used at a concentration of 1 μM; D-erythro-N-hexanoylsphingosine and ionomycin were purchased from Sigma (Taufkirchen, Germany), the Ca<sup>2+</sup> dye Fluo-3/AM from Calbiochem (Bad Soden, Germany).

**Isolation of pathogen supernatant**

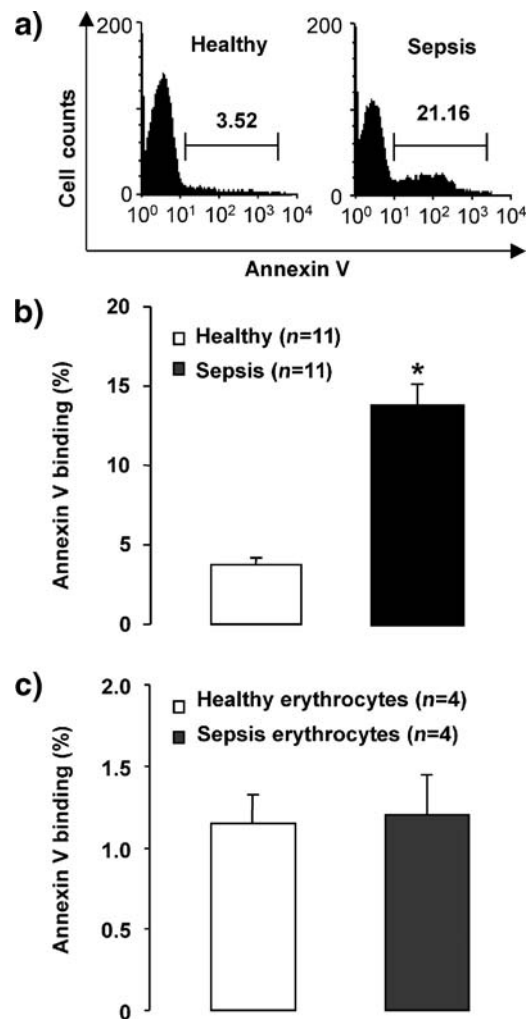
Bacteria were grown in TSB (Difco Laboratories; Sparks, MI, USA) medium; pH 7.2, with 180 rpm shaking at 37°C.

**Table 2** Blood cell count in healthy individuals and patients with sepsis

Cells	Controls	Patients	<i>p</i> -value
Leukocytes (/μl)	6,018±357	10,162±2,363	ns
Lymphocytes (%)	31.12±2.13	12.6±6.63	<0.01
Monocytes (%)	6.4±0.43	2.24±0.48	<0.001
Erythrocytes (million/μl)	4.72±0.11	3.33±0.17	<0.001
Hematocrit (%)	42.01±0.96	30.05±1.48	<0.001
Hemoglobin (g/100 ml)	14.5±0.34	10.48±0.57	<0.001
MCH (pg/Ery)	30.74±0.36	33.0±1.12	<0.05
MCHC (g/100 ml)	34.49±0.1	34.65±0.39	ns
MCV (fl/Ery)	89.14±1.08	93.04±3.02	ns
Thrombocytes (1,000/μl)	292±12.38	141.7±23.37	<0.001

Arithmetic means±SEM (*n*=25 control individuals, 11 septic patients) of leukocyte number and classification, thrombocytes number, of erythrocyte number, packed cell volume (hematocrit), hemoglobin concentration, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC).

ns Not significant



**Fig. 1** Stimulation of phosphatidylserine exposure at the erythrocyte surface by plasma of septic patients **a** Histograms of annexin V binding in a representative experiment of erythrocytes from a healthy volunteer incubated for 24 h in plasma from a septic patient (*right panel*) or from a healthy volunteer (*left panel*). **b** Arithmetic means±SEM (*n*=11) of annexin V binding at erythrocytes incubated for 24 h in plasma from septic patients (*right, black column*) or from healthy volunteers (*left white column*). The asterisk 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 V binding of erythrocytes from septic patients (*right, black column*) or from healthy volunteers (*left white column*) both incubated for 24 h in Ringer

Media were supplemented with glucose to a final concentration of 0.5% (*w/v*). All ingredients were mixed before 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 optical density (OD<sub>578</sub>) was adjusted to 0.1. After a growth period of 14–16 h, cells were harvested by centrifugation at 10,000 rpm for 30 min at 4°C. The supernatants were sterilized by passing through a 0.22 μm filter (Millipore; Schwalbach, Germany), preserved at 4°C and then used for further studies.

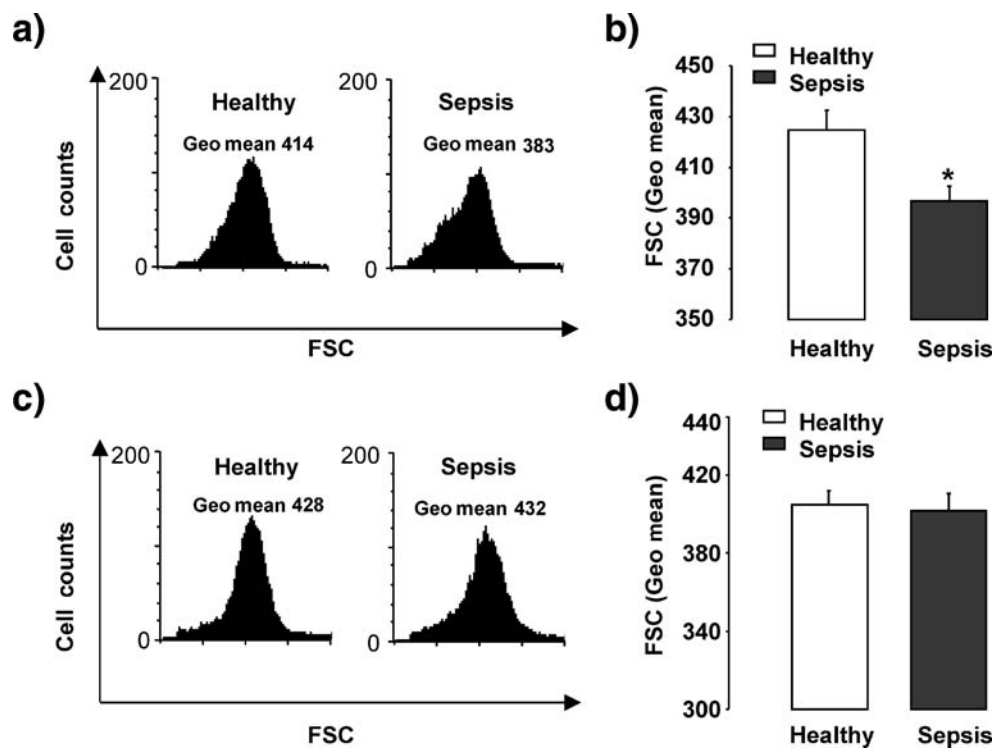
### Measurements of intracellular $\text{Ca}^{2+}$

For intracellular  $\text{Ca}^{2+}$  measurements, erythrocytes were loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) by addition of 2  $\mu\text{l}$  of a Fluo-3/AM stock solution (2.0 mM in DMSO) to 1 ml erythrocyte suspension (0.3% hematocrit in Ringer). Cells were incubated at 37°C for 15 min under protection from light. Subsequently, an additional 2  $\mu\text{l}$  aliquot of Fluo-3/AM was added, and cells incubated for further 25 min. Fluo-3-AM-loaded erythrocytes were centrifuged at 1,000 $\times g$  for 5 min at 22°C and washed two times with Ringer solution containing 1% fetal calf serum (Sigma) and one time with Ringer. Fluo-3/AM-loaded erythrocytes were resuspended in 0.5 ml plasma from patients with sepsis or from healthy volunteers (0.3% hematocrit) and incubated for different time periods at 37°C. Then,  $\text{Ca}^{2+}$ -dependent fluorescence intensity was measured by fluorescence-activated cell sorter (FACS) analysis on a FACS-Calibur from Becton Dickinson (Heidelberg, Germany) in the fluorescence channel FL-1 with an

excitation wavelength of 488 nm and an emission wavelength of 530 nm. As a positive control, labeled erythrocytes were incubated for 5 min in Ringer solution containing 1  $\mu\text{M}$  of ionomycin.

### Determination of ceramide formation

For determination of ceramide, cells were stained for 1 h at 4°C with anti-ceramide antibody or isotype matched pure mouse antibody in phosphate buffered saline (PBS) containing 1% fetal calf serum (FCS) at a dilution of 1:5 as described recently [8]. After three washes with PBS/1% FCS, cells were stained with polyclonal fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig specific antibody (Pharmingen, Hamburg, Germany) in PBS/1% FCS at a dilution of 1:50 for 30 min. Unbound secondary antibody was removed by washing the cells two times with PBS/1% FCS and samples were analyzed by flow cytometric analysis on a FACS-Calibur. FITC-fluorescence intensity was measured in FL-



**Fig. 2** Decrease of erythrocyte forward scatter after exposure to plasma from septic patients. **a** Histograms of forward scatter in a representative experiment of erythrocytes from a healthy volunteer incubated for 24 h in plasma from a septic patient (right panel) or from a healthy volunteer (left panel). Numbers depict the respective geo mean values. **b** Arithmetic means  $\pm$  SEM ( $n=10$ ) of forward scatter of erythrocytes incubated for 5 h in plasma from septic patients (right, black column) or from healthy volunteers (left white column). The asterisk indicates significant

difference between the plasma from septic patients and plasma from healthy volunteers (unpaired  $t$  test  $P < 0.05$ ). **c** Histograms of forward scatter in a representative experiment of erythrocytes from a healthy volunteer (left panel) or a septic patient (right panel) each incubated for 24 h in Ringer. **d** Arithmetic means  $\pm$  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 h in Ringer

1. As a positive control, erythrocytes were treated for 5 min with 1 U/ml *Streptomyces* sp. sphingomyelinase from Sigma.

#### Sphingomyelinase assay

Sphingomyelinase (SMase) content in bacterial supernatants was measured using the Sphingomyelinase [ $^3\text{H}$ ] SPA (scintillation proximity assay) enzyme assay (code TRK Q7140) from Amersham Bioscience (Freiburg, Germany) according to the manufacturer's protocol. Briefly, 10  $\mu\text{l}$  bacterial supernatant, 10  $\mu\text{l}$  of 10-fold enzyme buffer containing 1 M Tris-HCl (pH 7.4), 50 mM  $\text{MgCl}_2$  and 0.05%  $\text{NaN}_3$ , 70  $\mu\text{l}$  distilled water, and 10  $\mu\text{l}$  tracer solution (biotinylated [ $^3\text{H}$ ]-labeled sphingomyelin) were mixed. After addition of the radioactive tracer, the samples were incubated at 37°C with moderate shaking for 30–60 min. Then, the reaction was stopped by adding 20  $\mu\text{l}$  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*)  $\text{NaN}_3$  and streptavidin-coated Yttrium silicate SPA beads. Finally, the samples were counted in a  $\beta$ -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. 5c).

#### Annexin V binding in FACS analysis

FACS analysis was performed essentially as described [5]. After incubation of erythrocytes (0.3% hematocrit) with plasma from sepsis patients or with plasma from healthy individuals, the cells were washed in annexin V-binding buffer containing (in millimolar) 125 NaCl, 10 HEPES; pH 7.4 and 5  $\text{CaCl}_2$ . Erythrocytes were stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:50 dilution. After 10 min, samples were diluted 1:5 and measured by flow cytometric analysis on a FACS-Calibur. Cells were analyzed by forward scatter and Annexin V-fluorescence intensity was measured in FL-1.

#### Statistics

Data are expressed as arithmetic means $\pm$ SEM and statistical analysis was performed by paired student's *t* test or analysis of variance (ANOVA), as appropriate.

## Results

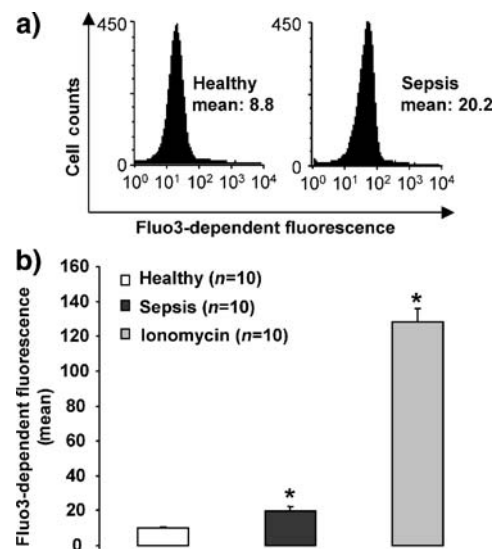
Eleven patients with sepsis were included in the study (seven male, four female, 55.6 $\pm$ 4.1 years, see Table 1). As shown in Table 2, the patients suffered from severe anemia,

which may result from decreased formation of new erythrocytes or from accelerated clearance of damaged cells from circulating blood.

As shown in Fig. 1, the exposure of erythrocytes from healthy volunteers to heparinized plasma of septic patients for 24 h led to marked stimulation of phosphatidylserine exposure at the erythrocyte surface (Fig. 1a,b; unpaired *t* test  $P<0.05$ ). On the other hand, exposure of erythrocytes from either healthy individuals or septic patients to Ringer solution did not significantly increase the percentage of annexin V binding erythrocytes (Fig. 1c).

As shown in Fig. 2, exposure of erythrocytes from healthy volunteers to heparinized plasma of septic patients for 24 h further led to a marked decrease of the forward scatter (Fig. 2a,b; unpaired *t* test  $P<0.05$ ). Similar to what was demonstrated for annexin V binding, exposure of erythrocytes from either healthy individuals or septic patients to Ringer solution did not significantly modify the forward scatter (Fig. 2c,d).

In search for a possible stimulator of phosphatidylserine exposure, we tested whether plasma of septic patients enhances the intraerythrocytic  $\text{Ca}^{2+}$  activity. Fluo3-fluorescence revealed that exposure to the plasma of septic patients led to a slight but significant increase of the mean fluorescence of the erythrocytes pointing to increased cytosolic

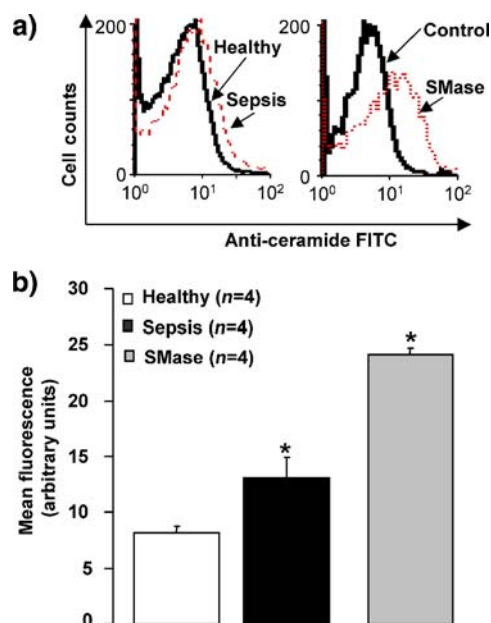


**Fig. 3** Increase of cytosolic  $\text{Ca}^{2+}$  activity in erythrocytes exposed to plasma from septic patients. **a** Representative original histograms of  $\text{Ca}^{2+}$ -dependent Fluo3 fluorescence from erythrocytes incubated for 360 min in plasma from a septic patient (right panel) or from a healthy volunteer (left panel). **b** Arithmetic means $\pm$ SEM ( $n=10$ ) of Fluo3 fluorescence in erythrocytes incubated for 360 min in plasma from septic patients (black column) or from healthy volunteers (white column), and of erythrocytes exposed to the  $\text{Ca}^{2+}$  ionophore ionomycin (1  $\mu\text{M}$ , gray column) in Ringer. The asterisk indicates significant difference between the value to erythrocytes exposed to plasma of healthy individuals (ANOVA using Dunnett's test as post hoc test.  $P<0.05$ )



$\text{Ca}^{2+}$  activity (Fig. 3a,b; ANOVA using Dunnett's test as post hoc test  $P<0.05$ ). As a positive control, the  $\text{Ca}^{2+}$  ionophore ionomycin (1  $\mu\text{M}$ ) led to a marked increase of the cytosolic  $\text{Ca}^{2+}$  concentration in virtually all erythrocytes (Fig. 3b).

We further tested whether plasma of septic patients stimulates ceramide formation. According to binding of a FITC-labeled anti-ceramide antibody, challenge of erythrocytes with plasma from septic patients significantly stimulated the formation of ceramide (Fig. 4a, left histogram, and Fig. 4b; ANOVA using Dunnett's test as post hoc test  $P<0.05$ ). As a positive control, purified sphingomyelinase (1 U/ml) was added to the erythrocyte suspension. As expected, treatment of the cells with sphingomyelinase for 5 min led to an increase of the mean cellular fluorescence, i.e., to an increase of the



**Fig. 4** Stimulation of ceramide formation in erythrocytes exposed to plasma from septic patients. **a** *Left* Histogram overlay of ceramide presenting erythrocytes in a representative experiment of erythrocytes incubated for 24 h in plasma of a septic patient (*dotted line, left panel*) or in plasma from a healthy volunteer (*solid line*). *Right* Histogram overlay of ceramide presenting erythrocytes in a representative experiment of erythrocytes incubated for 24 h in Ringer without (*solid line*) and with 0.1 mU bacterial sphingomyelinase (*dotted line right panel*). **b** Arithmetic means $\pm$ SEM ( $n=4$ ) of ceramide-dependent fluorescence in erythrocytes incubated for 24 h in plasma from septic patients (*black, middle column*), plasma from healthy volunteers (*white, left column*), or with 1 U/ml bacterial sphingomyelinase (*gray, right column*). The *asterisk* indicates significant difference of the plasma from healthy volunteers (ANOVA using Dunnett's test as post hoc test.  $P<0.05$ )

cellular ceramide levels (Fig. 4a, right histogram, and Fig. 4b).

Further experiments were performed to explore whether sphingomyelinase activity released from pathogens triggers phosphatidylserine exposure of erythrocytes. To this end,

erythrocytes from healthy volunteers were exposed to culture medium (as a negative control) or to supernatant from sphingomyelinase-producing *S. aureus*. In additional control experiments, erythrocytes were exposed to supernatants from an isogenic mutant strain whose sphingomyelinase gene (*hly*) is disrupted because of the integration of a prophage in the *hly* gene [20]. As illustrated in subpanels a and b of Fig. 5, incubation of erythrocytes for 60 min with the supernatant of sphingomyelinase-producing *S. aureus* 8325-4 (for sphingomyelinase determination see Fig. 5c,d) resulted in a marked increase of annexin V-positive erythrocytes (ANOVA using Dunnett's test as post hoc test;  $P<0.05$ ), while incubation in bacterial growth medium or in supernatant from mutated *S.aureus* 8325-4 lacking functional sphingomyelinase was without any appreciable effect. The analysis of different sepsis-inducing pathogens disclosed a positive correlation between the released sphingomyelinase activity and the potency of the supernatant to trigger eryptosis (Fig. 5e;  $R^2=0.891$ ).

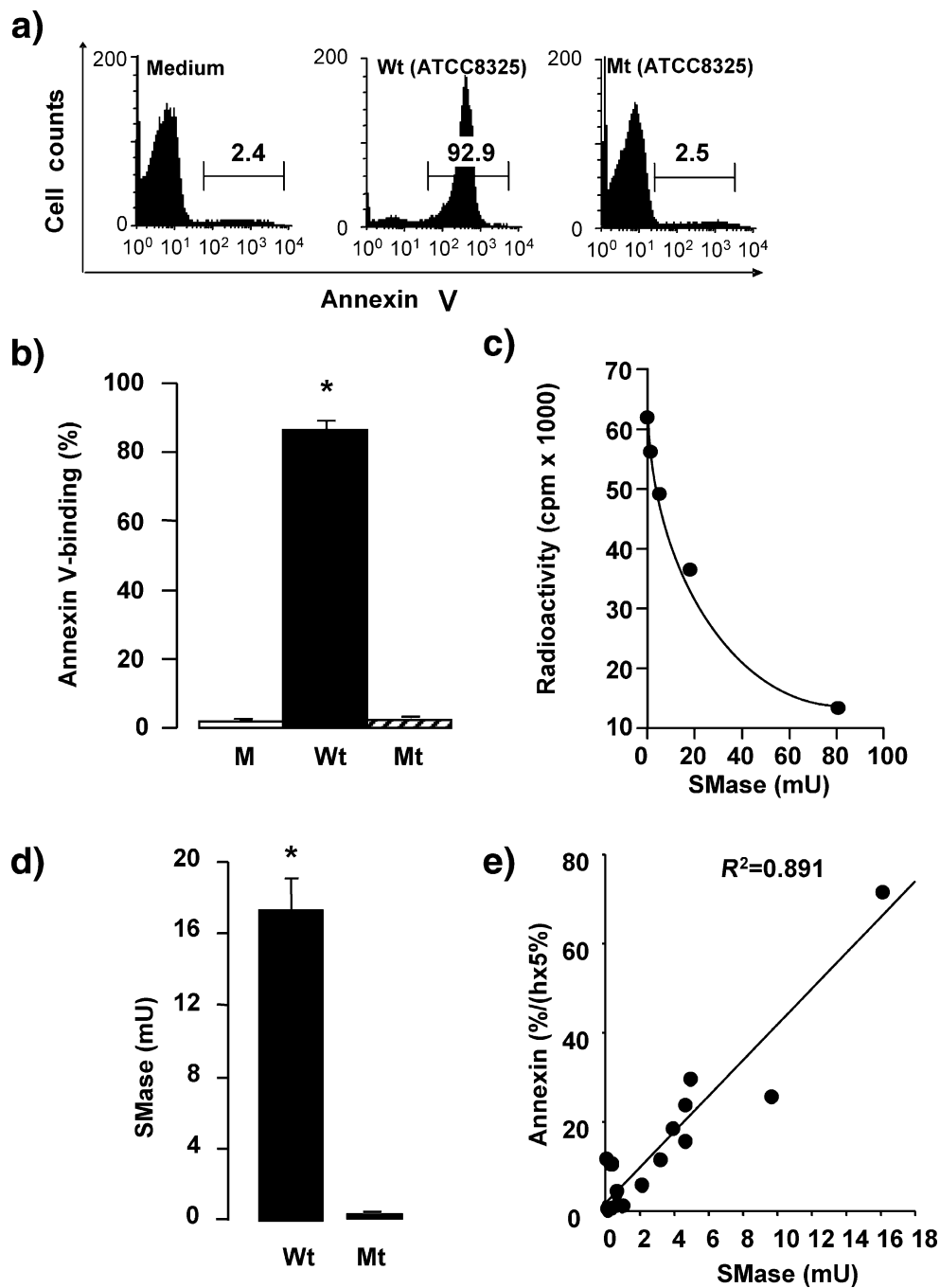
In another set of experiments, we investigated whether eryptosis occurred before hemolysis. In contrast to annexin V binding, which already reached more than 80% of the erythrocyte population after 60 min, no hemolysis was observed after exposure of the cells to sphingomyelinase-containing, bacterial supernatant during the first 60 min of incubation (Fig. 6). Within 24 h, however, almost complete hemolysis was observed.

## Discussion

The present experiments demonstrate that plasma from septic patients triggers phosphatidylserine exposure of erythrocytes in vitro leading to suicidal erythrocyte death (i.e., eryptosis).

The phosphatidylserine exposure is partially due to an increase of the cytosolic  $\text{Ca}^{2+}$  activity. Besides its effect on phosphatidylserine exposure, the increase of cytosolic  $\text{Ca}^{2+}$  could activate  $\text{Ca}^{2+}$ -sensitive  $\text{K}^+$  channels [21], which together with  $\text{Cl}^-$  channels allow the exit of  $\text{KCl}$  and thus lead to cell shrinkage [6]. Further effects of enhanced cytosolic  $\text{Ca}^{2+}$  concentrations include the activation of the protease calpain leading to degradation of membrane proteins and cell membrane blebbing [22, 23].

The effect of plasma from septic patients on cytosolic  $\text{Ca}^{2+}$  activity is, however, modest and probably does not fully account for the strong stimulation of phosphatidylserine exposure. Rather, the stimulation of ceramide formation by sphingomyelinase-mediated sphingomyelin breakdown may be more important. Activation of sphingomyelinase has previously been observed in septic patients [24] and after exposure of cells to *S. aureus* [25] or *Escherichia coli* [26]. Thus, sphingomyelinase-induced accumulation of

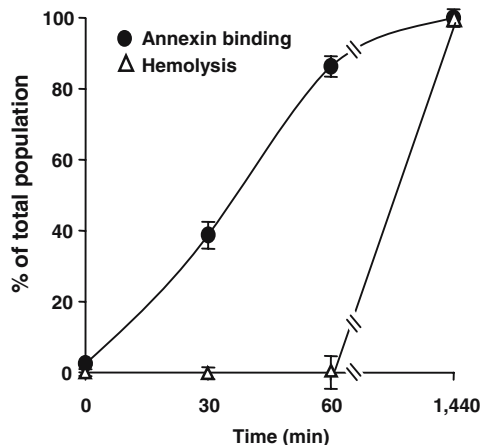


**Fig. 5** Stimulation of phosphatidylserine exposure at the erythrocyte surface by supernatant from sphingomyelinase producing staphylococcus aureus. **a** Histograms of annexin V binding in a representative experiment of erythrocytes incubated for 60 min in bacterial growth medium without pathogen (*left panel*) or in supernatant (*middle and right panels*) from wild type *S. aureus* 8325-4 (*middle panel*) or from mutated *S. aureus* 8325-4 lacking functional sphingomyelinase (*right panel*). **b** Arithmetic means±SEM ( $n=3$ ) of the percentage annexin V binding erythrocytes after incubation for 60 min in pathogen free medium (*M*) or in supernatant from wild-type sphingomyelinase expressing *S. aureus* 8325-4 (*Wt*) or from mutated *S. aureus* 8325-4 lacking functional sphingomyelinase (*Mt*). The *asterisk* indicates

significant difference to the annexin V binding of cells incubated in pathogen free medium (ANOVA using Dunnett’s test as post hoc test.  $P<0.05$ ). **c** Standard curve of the radioactive measurement of SMase activity. **d** Arithmetic means±SEM ( $n=3$ ) of sphingomyelinase activity in the supernatant from wild-type sphingomyelinase expressing *S. aureus* 8325-4 (*Wt*) or from mutated *S. aureus* 8325-4 (*Mt*). The *asterisk* indicates significant difference to the sphingomyelinase activity of mutated *S. aureus* (unpaired *t* test;  $P\leq 0.05$ ). **e** Correlation between sphingomyelinase activity in bacterial supernatants of 20 different bacterial strains and the percentage of annexin V binding erythrocytes after treatment with those supernatants ( $R^2=0.891$ )

intraerythrocytic ceramide most likely contributes to the stimulation of phosphatidylserine exposure [8]. The present study does not allow any conclusions as to the origin of the sphingomyelinase. It is noteworthy, however, that the supernatant of pathogens contains sufficient sphingomyelinase activity to trigger marked phosphatidylserine exposure in vitro. The perfect linear correlation between phosphatidylserine exposure and sphingomyelinase activity and the disappearance of phosphatidylserine exposure after loss of function mutation of the bacterial sphingomyelinase clearly demonstrates 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. The observations, further, do not address the possibility that a sphingomyelinase from other origin, such as leukocytes, is released into the plasma of septic patients and contributes to the stimulation of phosphatidylserine exposure. Whatever the origin, sphingomyelinases are critically involved in the pathophysiology of sepsis, a conclusion consistent with most recent findings [24] demonstrating that inhibitors of the acid sphingomyelinase protect animals from liver cell apoptosis and death of the animal upon induction of sepsis by intraperitoneal injection of endotoxin.

The exposure of phosphatidylserine at the cell surface favors the binding to respective phosphatidylserine receptors expressed by macrophages [10]. Binding to those receptors triggers engulfment and subsequent degradation



**Fig. 6** Dissociation of annexin V binding and hemolysis after exposure to bacterial supernatant. Time course of phosphatidylserine exposure and hemolysis after incubation of erythrocytes with supernatant from *S. aureus* 8325-4 (*Wt*). Arithmetic means $\pm$ SEM ( $n=3$ ) of the percentage of annexin V binding erythrocytes (●) and hemolysis (△), respectively

of the affected erythrocytes [11]. 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 [9]. Along those lines, we observed enhanced trapping of annexin V-binding erythrocytes in renal medulla after ischemia of the mouse kidney [27].

Pathogen-induced hemolysis may similarly contribute to accelerated erythrocyte clearance and anemia in septic patients, especially after infection with clostridium perfringens [3, 4]. Hemoglobin released from hemolytic erythrocytes is bound to the glycoprotein haptoglobin, which prevents glomerular filtration and intratubular precipitation of circulating free hemoglobin, but may be overridden during excessive hemolysis [28, 29]. As shown in this study, *S. aureus*-induced eryptosis precedes hemolysis, and rapid clearance of phosphatidylserine-exposing erythrocytes [12, 30] serves to remove defective erythrocytes before hemolysis. In vitro eryptotic erythrocytes are not engulfed by macrophages and thus eventually undergo delayed hemolysis. It should be kept in mind, though, that the concentrations of sphingomyelinase in bacterial supernatants are most likely higher than those in septic patients. Moreover, protective mechanisms such as erythropoietin [31] may counteract erythrocyte death in vivo. Thus, the time course of eryptosis and subsequent hemolysis in vitro presumably does not reflect the time course of erythrocyte death in septic patients.

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 [32–34] and  $Ca^{2+}$  [35] have similarly been implicated in the triggering of apoptosis of nucleated cells and the pathogenic plasma components may similarly trigger apoptosis of endothelial, renal, and hepatic cells thus contributing to the pleiotropic clinical features of sepsis. For instance, lymphocyte apoptosis participates in the pathophysiology of sepsis in mice [36]. Our studies therefore shed new light on the role of bacterial sphingomyelinase toxins whose role in virulence had remained elusive. Enzymes such as the *S. aureus*  $\beta$ -toxin might well contribute to the severity of sepsis by promoting erythrocyte death and anemia.

In conclusion, the present observations provide evidence for the stimulation of erythrocyte phosphatidylserine exposure by the plasma of septic patients. It demonstrates that the effect of the plasma is at least partially due to stimulation of  $Ca^{2+}$  entry and ceramide formation. The study thus reveals a novel mechanism which presumably contributes to the pleiotropic pathophysiology of this severe, life-threatening disease.

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