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The blood platelets contribution to innate host defense – what they have learned from their big brothers

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Keywords: blood platelets, thrombin, reactive oxygen species, plasma, innate immunity

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Abbreviations:

BC - buffy coat; DPI - Diphenylene iodonium chloride; NADP - Nicotinamide adenine dinucleotide phosphate; OCS - open canalicular system; PAR - protease activated receptor; PC - platelet concentrate; PMA - Phorbol 12-myristate 13-acetate; ROS - reactive oxygen species

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Abstract

Bactericidal effects of blood platelets have been known for more than 120 years, but the underlying mechanisms are largely obscure. Keeping in mind structural and functional analogies of platelets to neutrophiles, three different mechanisms are thinkable: Engulfment of pathogens, release of microbicidal proteins, and production of reactive oxygen species (ROS). Here, we focus on the release of ROS and a possible contribution of blood plasma and thrombin to the bactericidal effects.

Killing of bacteria was evaluated by DNA fluorescence labeling and electron microscopy. Release of ROS by platelets was measured photometrically by cytochrome C and phenolred/peroxidase assays and was further evaluated by topological methods.

We found that (1) platelets produce 1500 times less O_2^- and 4000 times less H_2O_2 compared to neutrophils, (2) ROS do not effect the killing rates, (3) no local enrichment of ROS was detectable. On the other hand, thrombin and plasma proteins with a MW >100 kDa are essential for bactericidal effects.

We suggest that platelets contribute to the innate host defence by providing a catalytical surface for synthesis of thrombin. In the presence of a heat-instable plasma protein, thrombin may generate a strong bactericidal complex, which is only effective in close vicinity to the platelet membrane.

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Introduction

Platelets are primarily known for their crucial role in haemostatic processes but they do have far more functions. They play a role in inflammation by quickly reacting to various specific signals and releasing inflammatory mediators [1]. They modulate vascular reactions, release mediators for chemotaxis and stimulation of leukocytes, and participate in wound repair by release of PDGF, Angiopoietin or EGF [1]. In this way, platelets chip into all stages of inflammation.

Inflammation is often the consequence of invading pathogens, and it seems to be logical that platelets participate in antimicrobial host defense as they are the first cellular elements in the blood that accumulate at any site of a vascular lesion [2-4]. Various bacteria bind to platelets [5-7] and are embedded and killed in platelet aggregates [8;9]. The binding of bacteria can be a direct one via receptors or can be indirect via a bridging molecule like fibrinogen [10]. Although a bactericidal function of platelets has been described for more than 120 years now, the underlying mechanisms are not clear [11]. Analogous to neutrophils, there are 3 potential killing mechanisms that may be proposed to operate in platelets: 1. platelets store and process bactericidal peptides, 2. they have a phagocytic capacity, and 3. they synthesize reactive oxygen species (ROS). As summarized below, mechanisms 1 and 2 have been under intensive investigation for longer times, but the possible role of ROS in killing bacteria by platelets has not been studied so far and stimulated the present study.

In 1972 Nachman and Weksler detected bactericidal peptides in platelet granules [4]. They were identified and described in detail in rabbits by Yeaman et al.[12] and later on in humans by Krijgsveld et al.[13] and Tang et al.[14]. But Tang et al. reported about conflicting data on bactericidal activities of purified peptides, possibly due to inhibitory effects of sodium phosphate [14], which is also used in our experiments. Moreover, Krijgsveld et al.[13] needed 100-fold higher platelet concentrations to extract thrombocidins in bactericidal amounts than we used in our killing model. Therefore we asked if additional mechanisms exist that are responsible for the observed strong killing effects and one of them could be phagocytosis. Platelets show three main phagocytic features that are typical for leucocytes [4;15]: they interact with particulate material, they contain lysosomes in the cytoplasm and they store inflammatory mediators. But true phagocytosis of bacteria, which means that the vacuoles have no permanent contact to the extracellular space, is not established in platelets yet. Platelets enclose bacteria trapped within an aggregate or act as “covercytes” by covering the bacterial surface [16]. They also may take up bacteria into the open canalicular system [3;16].

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White [16] concluded that bacteria-platelet interaction occurs spontaneously after prolonged incubation times or prestimulation of platelets. Since enclosing by platelets does not seem to alter bacteria morphologically, it is questionable if this mechanism of ingestion is bactericidal. And finally, platelets could have the possibility to kill bacteria by synthesis of ROS [17-24], which act as potent killing agents with particularly high efficacy in leukocytes [25]. In this way, neutrophils and macrophages increase oxygen consumption during phagocytosis in what has been termed respiratory burst [26]. It was observed that ROS production in neutrophils and osteoclasts may be locally restricted to areas of “ruffled membranes” [27;28]. Since we found these ruffled membranes also in platelets, especially at platelet-bacteria binding sites [9], we assumed that platelets could kill bacteria by the local release of ROS. Keeping in mind the analogies to neutrophils, we always investigated the ROS release by platelets and neutrophils simultaneously, taking the latter "big brothers" as the positive control.

Thus, we asked if ROS production in platelets is high enough to kill bacteria in general and if not so, if locally restricted ROS release might explain the observed killing effects. Since the amount of ROS released by platelets was not sufficient alone to explain the bactericidal effects, we further investigated the role of plasma proteins and the effect of thrombin in the observed killing model.

We tested the killing potential of gel-filtered platelets (plasma-free) in buffer and determined the killing rates after adding increasing plasma portions, furthermore after addition of heat-inactivated plasma and after the addition of high (> 100 kDa) and low (<100 kDa) molecular weight plasma protein fractions.

Thrombin performs its platelet stimulation in humans via the protease activating receptors (PAR) 1 and 4 on the platelet surface [29;30] but also cleaves plasma proteins to generate fibrin [31].

Because the addition of thrombin was crucial to get bactericidal effects, we dissected the action of thrombin by selecting the stimulation of the protease activating receptors (PAR) 1 and 4 on the platelet surface. We activated PAR 1 with the synthetic thrombin receptor agonist SFLLRN [32] and the PAR 4 with GYPGKF [33], and determined the killing rates. Furthermore, we mimicked the proteolytical function of thrombin by the addition of trypsin, we inhibited thrombin activity by hirudin, and compared all killing rates.

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Materials and Methods

Platelet and neutrophil preparation

Buffy coats (BC) or platelet concentrates (PC) were collected from healthy donors after informed consent and were kindly provided by Dr. Peter Schlenke from the Department of Immunology and Blood Transfusion, University of Luebeck.

For photometric assays and fluorescence microscopy, BC were prepared by centrifugation (Optipac PL-146/Optipress, Baxter). Platelet-rich plasma was isolated by centrifugation of BC for 15 min at 160 x g and was allowed to rest for 20 min to recover from the trauma of centrifugation and compaction. PC were then obtained by centrifugation of platelet-rich plasma for 10 min at 1000 x g. Platelets were resuspended in Tyrode buffer, containing 138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 5 mM HEPES, 0.4 mM Na_2HPO_4, 1 mM $MgCl_2$ and 5 mM glucose [19]. The number of contaminating leukocytes was < 0.1 %.

Neutrophils were separated from other cells in the BC using a Histopaque1077® gradient centrifugation (Sigma, Schnelldorf, Germany) for 30 min at 400 x g followed by a subsequent ammonium chloride haemolysis [34]. Neutrophils were resuspended in RPMI 1640® medium (Sigma) and were counted in a Neubauer chamber.

For Killing assays, PC were prepared by plateletpheresis (Amicus® cell separator, Baxter). Platelet counts were adjusted to $6x10^8$/ml. PC were stored for a maximum of 3 days at 22°C under constant agitation for better gas exchange and prevention of clumping.

Gel-filtered platelets were obtained by spinning down PC for 12 min at 1200 x g onto a 50 % albumin cushion [35]. Platelets were collected and resuspended in HEPES buffer containing 0.35 % BSA, 137 mM NaCl, 2.7 mM KCl, 3 mM Na_2HPO_4, 1 mM $MgCl_2$, 5.5 mM glucose and 3.5 mM HEPES and then applied to a Sepharose 2B column (Sigma).

Bacteria suspensions

Bacillus subtilis (B. subtilis), Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus) were grown on tryptone-soya agar plates (Oxoid, Wesel, Germany) at 37°C for 16 h. For killing experiments, bacteria were collected and suspended in 0.9 % sodium chloride to10^9 bacteria/ml.

Plasma preparation and fractionation

Human plasma was obtained by centrifugation of PC for 90 sec at 14 000 x g. To prepare heat-inactivated plasma, the obtained plasma were incubated for 20 min at 56°C. To

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fractionate the plasma proteins, the plasma were centrifuged through a Microcon® filter (Millipore) YM-10 for 30 min at 14 000 g x and YM-100 for 12 min at 14 000 x g. We got 4 plasma protein fractions: < 10 kDa, > 10 kDa, < 100 kDa and > 100 kDa.

Serum preparation

Whole blood without any anticoagulants was allowed to clot until retraction of the fibrin clot. After 30 min at 20 °C, the samples were centrifuged for 10 min at 3000 x g and the supernatant was collected.

Platelet stimulation and inhibition

To achieve maximum killing rates, platelets were stimulated with thrombin (Sigma) at a concentration of 2 U/ml for 10 min at 37°C. Inhibition of thrombin was performed by hirudin (Sigma) at a concentration of 5 antithrombin units/ml.

To stimulate the NAD(P)H-oxidase, we used phorbol myristate acetate (PMA, Sigma) at concentrations up to 1 µM. To block the NAD(P)H-oxidase, we used the unspecific NAD(P)H-oxidase inhibitor Diphenylene iodonium chloride (DPI; Sigma, 50-500 nM). Cells were preincubated with DPI for 30 min before any stimulation, and DPI was also present in the incubation medium.

For selective stimulation of the thrombin receptors, we stimulated PAR 1 with SFLLRN (SF, Sigma, 75 µM) and PAR 4 with GYPGKF (GYP, Sigma, 750 µM). To mimic a proteolytical action of thrombin, we used trypsin (Sigma, 64 000 U/ml).

Superoxide assay

The superoxide (O_2^-) production was measured by the reduction of cytochrom C as described by Krötz et al. [19]. Platelets were stimulated with PMA and thrombin or inhibited by DPI. After incubation times of 15 min (neutrophils) or 30 min (platelets) at 37°C, the absorbance of the supernatants was measured with the spectrometer (Spectronic 20 Genesis) at 550 nm. The O_2^- dependent part of cytochrome C reduction was calculated from the absorbance difference between samples with or without addition of superoxide dismutase (SOD, 380 U/ml). The tests were performed in triplicates. The data were translated into nanomole (nM) of O_2^- using the extinction coefficient of $E_{550nM} = 29.5\ mM^{-1} \times cm^{-1}$.

Hydrogen peroxide assay

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The rate of hydrogen peroxide (H_2O_2) production was detected by the peroxidase/phenolred assay as described by Cabanis et al. [34]. Cells were incubated for 30 min (neutrophils) or 45 min (platelets) at 37°C in the presence of 17 U/ml horseradish-peroxidase (Sigma) and 0.2 mg/ml phenolred in buffer. After addition of NaOH the absorbance in the supernatant was measured at 610 nm. The tests were performed in triplicate. Concentrations of H_2O_2 were calculated by means of a standard curve ranging till 0.6 µmol/l. To additionally detect released O_2^-, we applied superoxide dismutase (SOD), which catalyzes the conversion of O_2^- into H_2O_2.

Topological detection of H_2O_2 by fluorescence microscopy

Cells were dropped on prewarmed slides to adhere. After washing with phosphate buffered saline (PBS), 10 µM of the fluorescence dye Oxyburst Green H_2HFF ® (Molecular Probes, Eugene, Oregon) was added. Cells were stimulated by 0.5 µM of PMA (Sigma), 160 mg/l horseradish-peroxidase were added, and samples were incubated for 30 min. Samples were washed with PBS and were imaged using the confocal laser scanning microscope (LSM 510 Meta, Zeiss, Jena, Germany). The microscope was operated at 488 nm excitation, and a 510-580 nm emission filter was used.

Topological detection of H_2O_2 by electron microscopy

Cerium chloride reacts with H_2O_2 to form ceriumperhydroxide, which precipitates in the form of electron dense crystals at the side of H_2O_2 synthesis [36]. Neutrophils or platelets were incubated on Thermanox® Plastic Coverslips (Nunc, Wiesbaden, Germany) for 30 min in the presence of cerium chloride ($CeCl_3$,10 mM, Sigma), with or without 1 µM PMA and *B. subtilis*. Cells were diluted in ringer lactate solution (Fresenius, Bad Homburg, Germany) with addition of 5 mM glucose according to Telek et al. [37]. Samples were fixed with 2.5 % glutaraldehyde for 2 h and were washed in 0.1 M cacodylate puffer, pH 7.2. Postfixation was performed with 1 % osmiumtetroxide (OsO_4). Samples were dehydrated in ethanol and embedded in araldite (Fluka, Buchs, Switzerland). Ultrathin sections were stained with uranyl acetate and lead citrate and were viewed with a Philips EM 400.

Killing assays

For quantitative detection of dead bacteria, we used DNA double labelling with the fluorescence dye LIVE/DEAD® *Bac*Light ™ (Molecular Probes, Eugene, USA). The rate of

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dead bacteria was calculated by dividing the number of red stained bacteria by the sum of red and green stained bacteria and was expressed as % killing.

For killing assays, platelets were mixed with *B. subtilis* in a ratio of 1:1. After addition of stimulators or inhibitors (thrombin, PMA, DPI), samples were incubated on glass slides for 10 up to 45 min. Cells were labelled with LIVE/DEAD® and were imaged using the fluorescence microscope (Axiophot®, Zeiss, Oberkochen, Germany) at 630 x magnifications for every sample. Three optical fields containing a continuous layer of platelets were chosen. 300 bacteria were counted with respect to their labels. Baseline percentages of dead bacteria were determined by LIVE/DEAD® labelling of *B. subtilis* after suspension in sodium chloride without any additives. Maximum controls were performed by 10 min coincubation of LIVE/DEAD®-labelled *B. subtilis* and thrombin-stimulated platelets (2 U/ml).

The validity of the LIVE/DEAD® staining method was proven by the conventional colony counting method: mixed bacteria suspensions with 0, 25, 50, 75 and 100 % of dead bacteria were checked both by manual counting following LIVE/DEAD® staining and by counting of the colony forming units (CFU) after plating bacteria on agar and incubation for 24 h at 37°C. In agreement with the LIVE/DEAD® data sheet, we found an excellent correlation between growing on agar plates and LIVE/DEAD® staining.

To appraise the direct hurtful effect of H_2O_2 on *B. subtilis* we developed an automated method. Killing of bacteria was achieved by adding increasing concentrations of H_2O_2. 96-well microtiter plates were loaded with 50 µl of bacteria suspension and H_2O_2 in concentration between 50 µM to 50 mM. After 10 min incubation time, bacteria were labelled with 5 µM LIVE/DEAD® and killing was determined by measuring the emitted fluorescence signal with the LSM 510 Meta.

Three images per well were recorded using the multitrack mode. Numbers of green and red pixels between intensities 50- 255 were counted automatically. We calculated the killing rates by dividing the number of red pixels by the sum of red and green pixels. Double coloured pixels (colocalised) were excluded.

Statistics

All data are expressed as mean ± standard deviation (SD). Statistical significance ($p<0.05$) was determined by using the Wilcoxon rank sum test performed by JMP® software (SAS Institute) and was characterized at the figures by an asterisk (*).

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Results

Killing rates of *B. subtilis, E. coli* and *S. aureus* diverge

Thrombin-stimulated platelets in plasma are capable to kill *B. subtilis* and *E. coli* up to 97 %, but were not able to kill *S. aureus*. All following killing tests were performed with the most susceptible bacteria, *B. subtilis*, which also yielded the brightest fluorescence labeling.

O_2^-- production in platelets is very low compared to neutrophils

To see if platelets release ROS in bactericidal amounts, we first investigated the O_2^- release photometrically. Platelets produce a small and hardly detectable amount of superoxide. Resting platelets show a basal production of 0.2 ± 0.27 µM O_2^- /min/7x10^8 platelets (n=9). Stimulation with 1 µM PMA led to a 2.4-fold increase of O_2^- release on average (0.47 ± 0.46 µM O_2^-). Thrombin (2 U/ml) led to an even lower increase of O_2^- production up to 1.5-fold, again in only some preparations (0.33 ± 0.14 µM O_2^-, n=5).

In general, no stimulators were able to induce platelet O_2^- formation significantly, since platelets of some donors did not respond. If platelet stimulation with PMA was performed in the presence of the unspecific NAD(P)H-oxidase inhibitor DPI, we observed a tendency of decreasing O_2^- production from 0.47 ± 0.46 µM to 0.34 ± 0.51 µM O_2^-.

In contrast, neutrophils strongly respond to PMA. One neutrophil produced about 1500-fold more O_2^- after PMA stimulation compared to one platelet. By addition of DPI, the maximum O_2^- release was reduced from 1.58 ± 0.17 µM O_2^-/min/10^6 neutrophils to 0.66 ± 0.08 µM (p<0,001, Wilcoxon, n=5). Thrombin had no effect on neutrophils (Fig.1).

Low H_2O_2-formation in platelets in contrast to neutrophils

O_2^- is unstable and has a short half live time in the millisecond range. It is converted into H_2O_2, which is detectable with the phenolred/peroxidase assay. Resting platelets produce 80 ± 50 nM H_2O_2/min/10^9 platelets (n=5). Following stimulation with 1 µM PMA, platelets release significantly more H_2O_2 (115 ± 30 nM H_2O_2, p= 0.03, Wilcoxon). Addition of DPI holds the H_2O_2 basal release to 64 ± 41 nM. SOD converts generated O_2^- into H_2O_2. Thus the addition of SOD in this assay can detect indirectly the O_2^- release, too. In the presence of SOD, PMA stimulation fails to further enhance the H_2O_2 production (Fig. 2) These findings correspond to the low detection of O_2^- in the cytochrome C assay (Fig. 1).

In contrast to platelets, neutrophils produce about 4000-fold more H_2O_2 on a single cell basis. 1 µM PMA increases the H_2O_2 production from 134 ± 36 nM H_2O_2/min/10^5 neutrophils up to 219 ± 22 nM (p<0.001, Wilcoxon). DPI reduces the basal production to 25 ± 15 nM.

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Coincubation with SOD further enhances the maximum H_2O_2 production from 219 ± 22 nM to 260 ± 15 nM (Fig.2).

Topological fluorescence analysis of H_2O_2-production by platelets

Since the level of ROS in the supernatant is not able to reflect a possible local enrichment of ROS, we tried to identify ROS topologically by fluorescence microscopy. PMA-stimulated neutrophils showed very bright fluorescent dots, likely phagolysosomes (Fig. 3A). If platelets were stimulated with PMA, we found a very weak fluorescence signal surrounding some aggregated platelets (Fig. 3B). At bacteria-platelet-binding sites, no particular H_2O_2 signal was detectable.

No colocalization of H_2O_2 - production and killing of bacteria

Since fluorescence detection was unable to demonstrate any locally restricted ROS-synthesis in platelets, we further tried to detect H_2O_2-release by ceriumperhydroxide precipitation as visualized by electron microscopy.

As reported by Repine et al. [38], in PMA-stimulated neutrophils the external surface of the plasma membrane was coated with a thick layer of electron dense material representing this precipitation. To assure about the validity of the H_2O_2 detection by the $CeCl_3$ method, we added catalase to the activated neutrophils. Catalase abolishes the released H_2O_2. Following addition of catalase, PMA-activated neutrophils showed no ceriumperhydroxide crystals on the plasma membrane. Unstimulated neutrophils displayed ceriumperhydroxide crystals only at a few sites.

In coincubation experiments of PMA-stimulated neutrophils with *B. subtilis*, most bacteria were phagocytosed. Within vacuoles of some neutrophils, ceriumperhydroxide crystals are seen, which indicates the local synthesis of H_2O_2 (Fig. 4 A-D).

In contrast to neutrophils, platelets never showed any ceriumperhydroxide crystals at the plasma membrane, regardless of any stimulation. Although a lot of viable and dead *B. subtilis* were enclosed in platelet aggregates or in platelets themselves, no electron dense material was detectable at binding sites of *B. subtilis*, suggesting that no H_2O_2 was produced at these sites (Fig.4 E-H).

Promoting platelet ROS-generation does not lead to higher killing rates

B. subtilis is highly susceptible to thrombin-stimulated platelets with killing rates to nearly 100 % within 10 min. We compared these killing rates with those achieved following PMA-stimulation of platelets. PMA does not yield any significant killing effect within 10 min

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(13 ± 6 % versus baseline 8 ± 3 %, p>0.05, Wilcoxon). After 20, 30 and 45 min incubation time, the PMA-stimulated platelets only reached a moderate killing up to 50 ± 20 %, which was significantly higher than the control but did not reach the killing efficacy of stimulation with thrombin (Fig. 5, p<0.001, Wilcoxon, n=5).

Inhibition of O_2^- does influence killing rates partially

To determine if the killing is ROS dependent, we also inhibited the superoxide generating NAD(P)H-oxidase by preincubation of cells with DPI. When thrombin was used as a stimulator, the preincubation with DPI (0.1mM) leads to a tendency of decreased killing of *B. subtilis* within 10 min (80 ± 20 % versus 92 ± 5 %, p= 0.2, Wilcoxon) and was significantly decreased after 20 min (68 ± 24 % versus 87 ± 10 %, p= 0.009, Wilcoxon) and after 45 min (74 ± 20 % versus 95 ± 6 %, p= 0.0006, Wilcoxon, n=5).

If DPI-preincubated platelets are stimulated with PMA, the rate of dead bacteria vary slightly from 13 ± 6 % (PMA) to 21 ± 7 % (DPI/PMA) after 10 min (n=5). After 20 and 30 min incubation, there was the same killing rate with or without DPI. Only after 45 min the killing rate decreased significantly from 50 ± 20 % (PMA) to 40 ± 16 % (DPI/PMA; p= 0.03, Wilcoxon, n= 5).

H_2O_2-addition at platelet synthesis levels is not bactericidal to *B. subtilis*

To determine at which H_2O_2-concentrations *B. subtilis* is killed, we established a dose-response relationship in the range from 0.05 to 50 mM H_2O_2. Since *B. subtilis* is catalase positive [39], it is relatively resistant against ROS. At concentrations up to 5 mM we observed no bactericidal effect on *B. subtilis* (11 ± 5 % versus 11 ± 3 % of dead bacteria in controls). At concentrations of 50 mM H_2O_2 we observed only 25 ± 1 % killed bacteria (n=3). With a synthesis of 1 µM H_2O_2 in 10 min, platelet concentrates only produce less than $1/10^5$ of H_2O_2 that would be needed for effective killing of this bacteria.

Killing rates are dependent on addition of plasma

Blood plasma contains many adhesive proteins, which could enhance the binding of bacteria to platelets and thereby change the rate of killing. To determine the role of plasma components in the bactericidal mechanism of platelets, we used gel-filtered platelets with addition of increasing plasma portions.

Thrombin-stimulated platelets in buffer killed only 22 ± 22 % of the added bacteria, which was significantly less than the maximum control in plasma (p<0.001, Wilcoxon, n=3). But

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already a proportion of 20 % plasma completely regains the bactericidal effect (100 %). If the plasma was heat-inactivated, no killing was observed (8 ± 3 % versus baseline 11.1 ± 2.8 %).

Plasma but not Serum is bactericidal after addition of thrombin

Because plasma seemed to be crucial for yielding high killing rates of *B. subtilis*, we tested if plasma can kill these bacteria without the addition of platelets.

Plasma without platelets showed no significant killing (19 ± 4 %). The addition of thrombin to plasma resulted in a high killing rate of 84 ± 17 %, which was still significantly below the maximum control shown in fig.5 (p=0.0011, Wilcoxon). The inhibition of thrombin activity by hirudin completely aborted bactericidal effects, also the incubation of *B. subtilis* with thrombin alone, without addition of plasma, did not show any significant killing (17 ± 8 %). To exclude complement or antibody effects in the killing assay, we also tested the bactericidal effects of serum. But in the presence of serum there was no killing observed with (18 ± 7 %) or without the addition of thrombin (19 ± 6 %).

Plasma protein fraction > 100 kDa mediates the high killing rates

To determine the plasma component, which cause the high killing rates, we fractionated the plasma proteins with two filters in the MW ranges > 10 kDa, < 10 kDa, > 100 kDa and < 100 kDa and tested the killing rates after addition of these fractions to gel-filtered platelets. It was determined, that the fraction > 100 kDa was responsible for a killing as high as 91 ± 8 %. The effects of the fractions > 100 kDa and > 10 kDa were not significantly different from killing rates in normal plasma (max-control), which reached 95 ± 5 % (p= 0.34, Wilcoxon). In contrast, the fractions < 100 kDa showed a decreased killing of 33 ± 8 % and the fraction < 10 kDa reached only 19 ± 8 % (Fig. 6).

Thrombin interaction with platelet receptors is not responsible for killing *B. subtilis*

In our experiments thrombin was always crucial for killing of *B. subtilis*. Therefore we studied which effect of thrombin was responsible for the observed killing. Thrombin activates human platelets via the protease activating receptors (PAR) 1 and 4. We dissected these effects by using the synthetic thrombin receptor agonists SFLLRN (SF) for activating the platelet PAR 1 and GYPGKF (GYP) for activating PAR 4. SF reached a maximum killing of 39 ± 19 %, whereas GYP achieved a maximum killing of 34 ± 20 %. Both activators together yielded a killing rate of 41 ± 20 %. To mimic the proteolytical function of thrombin, we also used trypsin in killing experiments. Trypsin reached a significant higher killing rate compared

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to GYP or SF with a killing of 56 ± 16 %, but did not achieve the killing rate that was observed upon thrombin stimulation (83 ± 19 %).

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Discussion

While the critical role of platelets in haemostasis, thrombosis and inflammation is clearly identified, their contribution to the innate host defense has received much less attention though the bactericidal properties of blood and platelets have been described for the first time as early as one century ago [11].

In analogy to leukocytes, 3 basic bactericidal mechanisms are thinkable to exist in platelets: 1. storage and processing of bactericidal proteins, 2. phagocytosis and 3. synthesis of reactive oxygen species (ROS).

1. Storage and processing of bactericidal proteins

In 1960 Hirsch et al. [40] demonstrated bactericidal activities of rabbit serum that are not present prior to clotting. He noticed that plasma regains its bactericidal effect after addition of platelets but not after addition of leukocytes or erythrocytes. Investigations on that subject were intensified in the 1970`s. Donaldson et al. [41-43] isolated β-lysin as one bactericidal protein, which is stored in rabbit platelets at high concentrations but also occurs to a lower extend in human platelets. Since then several authors focussed on isolating the supposed bactericidal proteins derived from platelets. Nachman et al. [4;44] and the group leaded by Yeaman [45-48] isolated and characterized platelet microbicidal proteins in rabbits. Later on Krijgsveld et al. described analogous bactericidal proteins in human platelets that are released upon thrombin stimulation [13]. Surprisingly, these proteins differ from not bactericidal proteins by a proteolytical truncation of only 2 amino acids. The possibility exist, that thrombin or other extracellular proteases cleave a precursor protein, which contributes to the generation of bactericidal proteins [2]. According to these findings, Tang et al. [14] could identify more bactericidal proteins in human platelets, but two of them are only present in stimulated platelets.

In our model of killing of *B. subtilis*, however, several lines of evidence militate against a main role of bactericidal proteins. Platelet releasates, in our hands, only exert moderate killing effects [9], and we observed a massive killing of bacteria with thrombin-stimulated platelets at about 100fold lower platelet concentrations in comparison to Krijgsveld et al.[13]. Moreover, in earlier investigations we could show, that even completely degranulated platelets have bactericidal capacity [9]. A possible explanation for these conflicting data on bactericidal activity of platelet releasates is presented by Tang et al. [14], who supposed an inhibiting effect of sodium phosphate which is used in most buffer systems.

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2. Phagocytosis

Lewis et al. [49] demonstrated for the first time that platelets really phagocytose small particles (<0.1 µm), which means that lysosomes fuse with the phagosome and no luminal continuity exists to the outer space. Latex particles were endocytosed through the OCS and reached the cytoplasm in small vesicles. But this way of ingestion is doubtful for bacteria with its much larger size (2 µm). Resuming the works of decades, Clawson found no evidences for a real phagocytosis of bacteria [3]. He observed that stimulated platelets recruit other platelets and the growing platelet aggregate surrounds initially adhered bacteria. There they are well separated from the surrounding media and usually appear within interspaces. But really engulfed bacteria are rarely to be seen in platelets [50]. Although granule secretion at platelet-bacteria binding sites was observed, no killing of bacteria was described [3]. That is contrary to our investigations. Youssefian et al. [51] postulated an engulfment of *Staphylococcus aureus* in platelets, which is enhanced after stimulation. But he did not point out that killing occurs after engulfing. From White's updated point of view, bacteria are too big to be actively and completely enclosed within platelets. Therefore platelets may just cover bacteria as "covercytes" to separate this foreign material from the blood stream in vivo [16].

3. Synthesis of reactive oxygen species

After phagocytosis by neutrophils, ROS play an important role in the definitive killing of pathogens. Several clues exist that also platelet-derived ROS could be engaged in host defense: ROS-dependent cytocidal effects were described in the platelet-mediated killing of helminths [52], ruffled platelet membranes were observed at bacteria binding sites [9], and a decreased killing of bacteria was observed to occur in the presence of antioxidants (own unpublished data). Thus we tried to establish the role of ROS in killing *B. subtilis* by platelets. The term ROS contains several molecular species with different properties. The key role has superoxide anion (O_2^-) as the first molecule in the complete reaction chain. Being unstable, it is converted to peroxynitrite or by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). H_2O_2 itself is strongly bactericidal and can be further converted into other bactericidal oxygen species. In 1977, Marcus et al.[53] found superoxide (O_2^-) production and in 1982 Finazzi-Agro et al. [54] could detect hydrogen peroxide (H_2O_2) release from platelets.

First hints that platelet-derived ROS have cytocidal effects was given by Joseph et al. in IgE-mediated killing of helminths [52;55]. This hypothesis is aided by our findings of ruffled membranes at platelet-bacteria binding sites which suggested similarities to ROS generating neutrophils [27] and osteoclasts [28]. In these cells, ruffled membranes are generated by actin

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polymerization and become sites of assembly of the NAD(P)H-oxidase complex. In this way, neutrophils and osteoclasts produce locally restricted large quantities of ROS to kill bacteria or to remodel the bone matrix. In addition to ruffled membranes, we observed a decreased killing rate by addition of the antioxidant vitamin E (α-tocopherol) in earlier investigations in our laboratory (unpublished data).

Based on these reports and observations, we assumed a possible role for platelet-derived ROS in our bactericidal model. We focussed in our experiments on detecting the release of O_2^- and H_2O_2 quantitatively as well as topologically, and we studied the influence of stimulation and inhibition of ROS-synthesis on the resulting killing rates.

B. subtilis is highly susceptible to the killing abilities of thrombin-stimulated platelets. It is a Gram- and catalase-positive bacterium, which has 3 catalases [39], and it should be well protected against the cytocidal effects of H_2O_2. In our experiments we detected that one platelet does produce about 1500 times less superoxide and 4000 times less H_2O_2 compared to one neutrophil. Although the concentration of platelets in native blood is 30 times higher and under our experimental conditions even 100- 500fold higher than the physiological concentration of neutrophils, the detected ROS release does not reach bactericidal dimensions for *B. subtilis* at all. Consistent with your observation that 50 mM H_2O_2 kills 25 % of *B. subtilis* within 10 min, Murphy et al. demonstrated that 10 mM H_2O_2 is enough to kill almost all *B. subtilis* by a longer incubation time of 1 h [56].

The detection of very low levels of ROS with subsequent high standard deviations and the disappointing weak reaction to stimulators led us to the assumption, that aggregated platelets can not release all generated ROS into the supernatant. In this way, freshly synthesized ROS inside a platelet aggregate will be converted before it will be detected in the supernatant. That might explain the low ROS detection when strong aggregating agents like thrombin were used.

Another critical factor in the ROS assay is that the isolation process prestimulates platelets in a donor-dependent fashion, so that it is difficult to further increase ROS production with stimulators. Low amounts and prestimulated, aggregated platelets hamper the exact quantification of ROS release. Another delicate point is that platelet concentrates are not totally free of neutrophils. To assure about the cellular source of ROS (neutrophils or platelets), we used a direct local detection of ROS in addition to the cytochrom C or phenolred/peroxidase assays. Both electron and fluorescence microscopy revealed that no locally restricted ROS synthesis could be found at platelet-bacteria binding sites. In contrast

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to that, neutrophils show a strong ROS production as visualized with both microscopical methods, whereas in platelets nearly no detection of H_2O_2 is possible.

Finally we investigated and compared the killing effects of PMA- and thrombin-stimulated platelets to determine the role of ROS in killing *B. subtilis*. Stimulation by PMA yields only a moderate killing rate after prolonged time compared to platelet stimulation by thrombin, which kill with high efficiency within a few minutes. Furthermore we preincubated platelets with DPI, an unspecific inhibitor of NAD(P)H-oxidase in neutrophils. DPI preincubated platelets do change their thrombin-dependent killing of bacteria partially. Hence neither PMA did increase the killing of *B. subtilis* nor did DPI inhibit the enormous thrombin-mediated killing clearly.

So until now, neither bactericidal peptides nor phagocytosis or ROS release can explain the mechanism of platelet-mediated bactericidal action, and we have to conclude that the platelets obviously have learned not enough from their big brothers, the granulocytes. But what establishes their bactericidal activity, which is clearly observed both *in vitro* and *in vivo*? Platelets might have found their own way, to participate in host defense.

4. Role of Thrombin and Plasma Proteins

In all experiments it was remarkable that the presence of plasma as well as a stimulation by thrombin was essential to kill *B. subtilis*. Gel-filtered and thrombin-stimulated platelets showed no killing effects until at least a portion of 20 % plasma was added. In contrast, heat-inactivated plasma, a plasma protein fraction < 100 kDa, serum or thrombin alone offered no killing potential. Therefore complement or antibody action in that killing can be excluded, because these components are also present in the serum.

Furthermore, the action of thrombin was mimicked by the synthetic thrombin receptor agonists SFLLRN or GYPGKF, which stimulated the platelets without having any proteolytic effects [32;33]. Although the platelets were activated and degranulated, no remarkable killing of *B. subtils* occured.

In conclusion, the observed bactericidal effects do not seem to be mainly due to an active contribution of platelets, like bactericidal peptides, the production of ROS or phagocytosis. The present work rather suggests that platelets contribute to the innate host defense by providing a catalytical surface for synthesis of thrombin. Thrombin was always added in our *in-vitro* experiments, but can be generated *in vivo* only in close contact to the platelet surface. Inhibition of thrombin by hirudin completely abolishes all bactericidal activity of plasma in our experiments. In the presence of a heat-instable plasma protein with a MW > 100 kDa, the

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protease thrombin seems to generate a bactericidal compound, which is responsible for the observed killing.

Distinct evidence from animal and clinical studies exist, that platelets are effectively engaged in innate host defense. Several *in vitro* and animal studies have shown that the susceptibility of bacteria to platelet-mediated defense mechanisms influences the induction and progression of endovascular infections [57-60]. For example, Sullam et al. tested the effects of an induced thrombocytopenia in rabbits on heart valve endocarditis after infection with *Streptococcus sanguis*. Compared to controls, thrombocytopenic rabbits had higher densities of streptococci per valve [57].

In several clinical studies it was described, that thrombocytopenia in man is also accompanied by higher morbidity and mortality. An increased incidence and severity of lobar pneumonia in elderly individuals with thrombocytopenia was described [61], thrombocytopenia was found to be a significant predictor of mortality in pneumococcal meningitis [62], and in liver transplant recipients thrombocytopenia is an independent and significant predictor associated with increased infection and related morbidity and mortality [63].

However, the less pathogenic bacteria species *B. subtilis* and *E. coli* were steadily killed in our experiments, whereas *S. aureus* could not be killed. Thus, the effect of platelet-mediated antibacterial defense mechanisms seems to be limited to less pathogenic species, or vice versa, the ability to escape from the platelet-mediated defense makes bacteria much more pathogenic.

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Figure legends

Fig. 1

Release of superoxide anion by platelets (left y axis) and neutrophils (right y axis) following stimulation with PMA and inhibition with DPI. Whereas neutrophils clearly respond to both stimulation and inhibition, the platelets did not react significantly. Calculated onto a per cell basis, a maximum stimulation with PMA induces one neutrophil to release appr. 1500-fold more O_2^- than one platelet. Thus, release of superoxide anion will not substantially contribute to bactericidal activity of platelets.

Fig. 2

Release of hydrogen peroxide by platelets (left y axis) and neutrophils (right y axis) following stimulation with PMA or inhibition with DPI. Both cells significantly react on the maximum stimulus (Asterisks; $p<0.05$; Wilcoxon), but platelets fail to further enhance their hydrogen peroxide release after addition of SOD. Calculated onto a per cell basis, a maximum stimulation with PMA induces one neutrophil to release appr. 4000-fold more H_2O_2 than one platelet. Thus, release of hydrogen peroxide will not substantially contribute to bactericidal activity of platelets.

Fig. 3

Topological fluorescence analysis of hydrogen peroxide release following PMA stimulation of neutrophils (A) and platelets (B). In neutrophils, the circumferentially located phagolysosomes release a strong signal, but the fluorescence intensity produced by aggregated platelets is very weak and does not show local enrichment of the signal, thus not supporting a role of hydrogen peroxide in the killing of bacteria by platelets.

Bars: 5 µm

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Fig. 4 A - D

Electron microscopical detection of neutrophil hydrogen peroxide release by precipitation of cerium perhydroxide. Stimulation of neutrophils with PMA induces precipitation on the outer cell membran and also on the inner aspect of vacuoles (arrows in A). Addition of catalase totally abolishes the precipitation, thereby confirming the specificity of the reaction (B). Unstimulated neutrophils display crystals only at a few sites (C). At higher magnification, precipitation (arrow) can be detected close to ingested bacteria (Bs).

Bars: 1 µm

Fig. 4 E - H

Electron microscopical detection of platelet hydrogen peroxide release by precipitation of cerium perhydroxide. Neither stimulation with PMA (A), nor stimulation in the presence of catalase (B), control platelets (C) or ingestion of bacteria (D; Bs) lead to any precipitation of cerium perhydroxide. Thus, the assumption of a local enrichment of reactive oxygen species is not supported.

Bars: 1 µm

Fig. 5

Killing of bacteria by stimulated platelets. Whereas stimulation of platelets with thrombin leads to nearly complete killing already after 10 min, stimulation with PMA only induces moderate killing rates after 20 and more min.

Asterisks: Significantly higher killing rates compared to the baseline value ($p<0.01$; Wilcoxon)

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Fig. 6

Killing of bacteria by thrombin-stimulated platelets is dependent from the presence of blood plasma components. Gel-filtered platelets resuspended in buffer excert a comparable low killing activity like platelets in serum or platelets in plasma without high molecular weight proteins. To achieve a maximum of killing, both the plasma protein fraction above 100 kD and the activity of thrombin are required.

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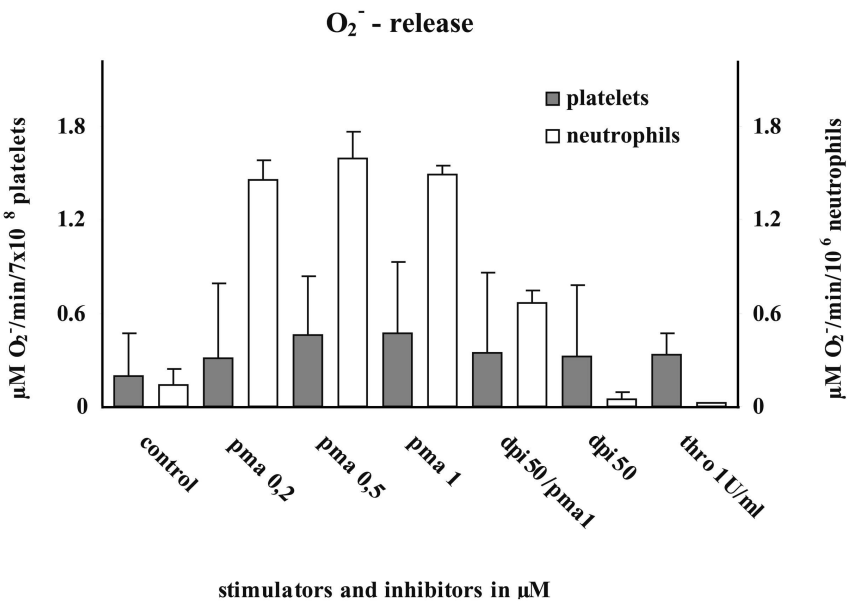

Zander & Klinger: Fig. 1
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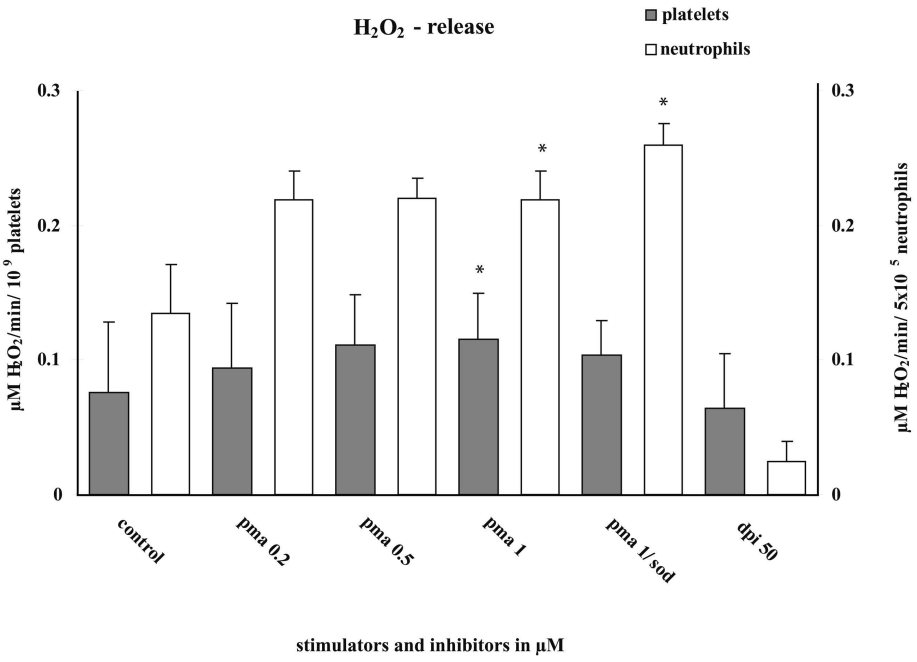

Zander & Klinger: Fig. 2
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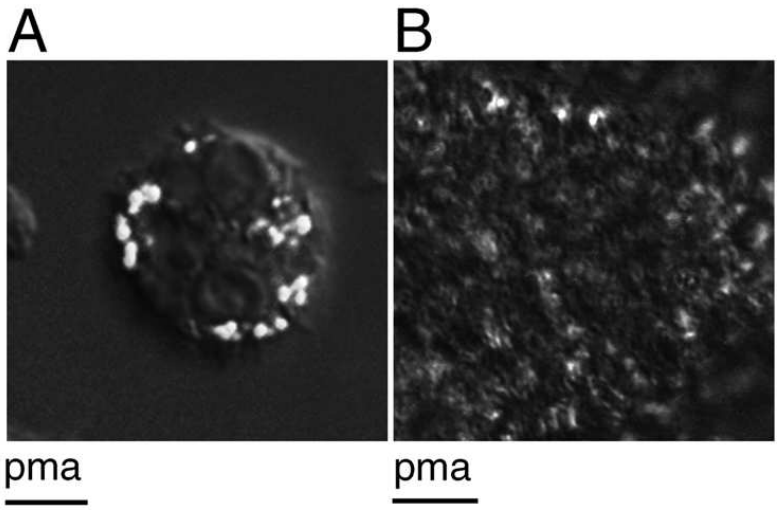

Zander & Klinger: Fig. 3
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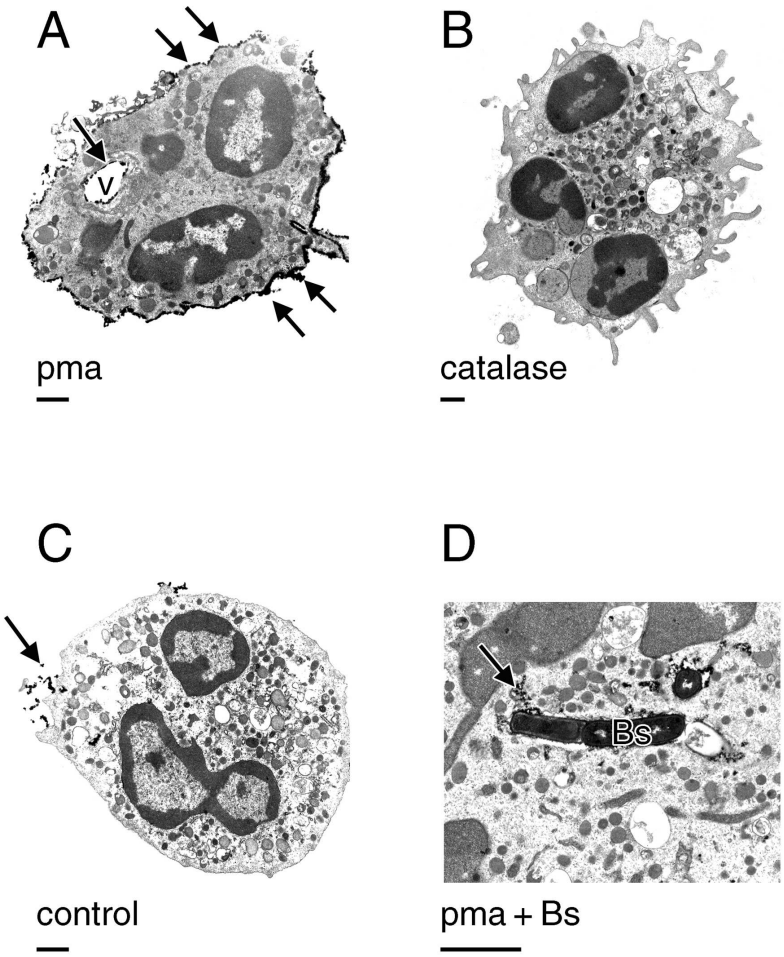
For Review



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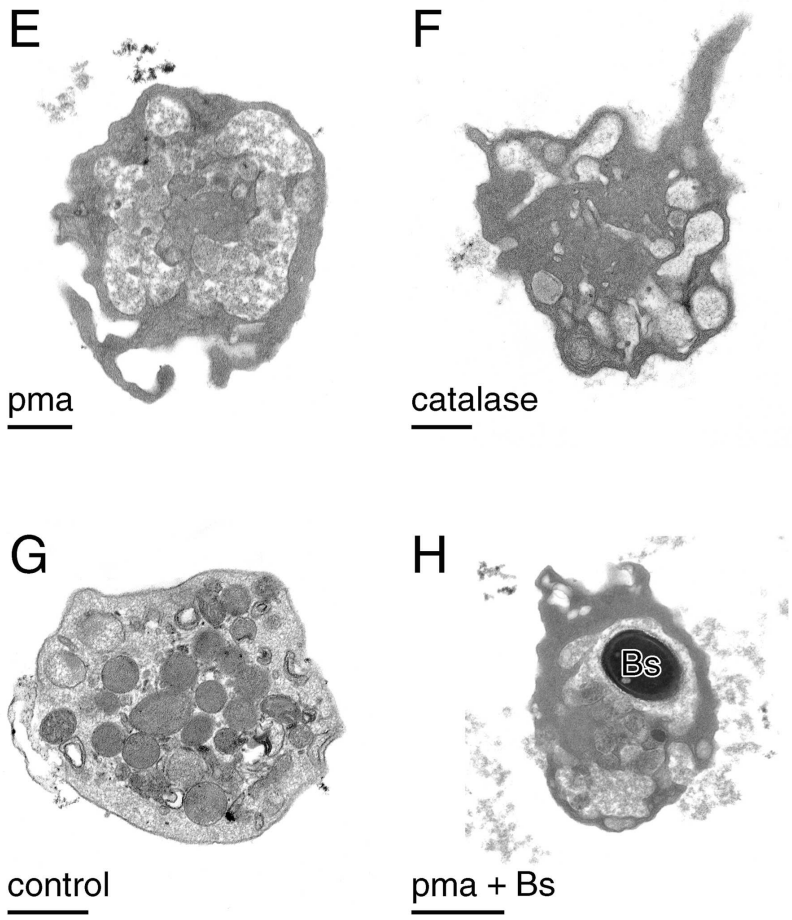

Zander & KLinger: Fig. 4e-h
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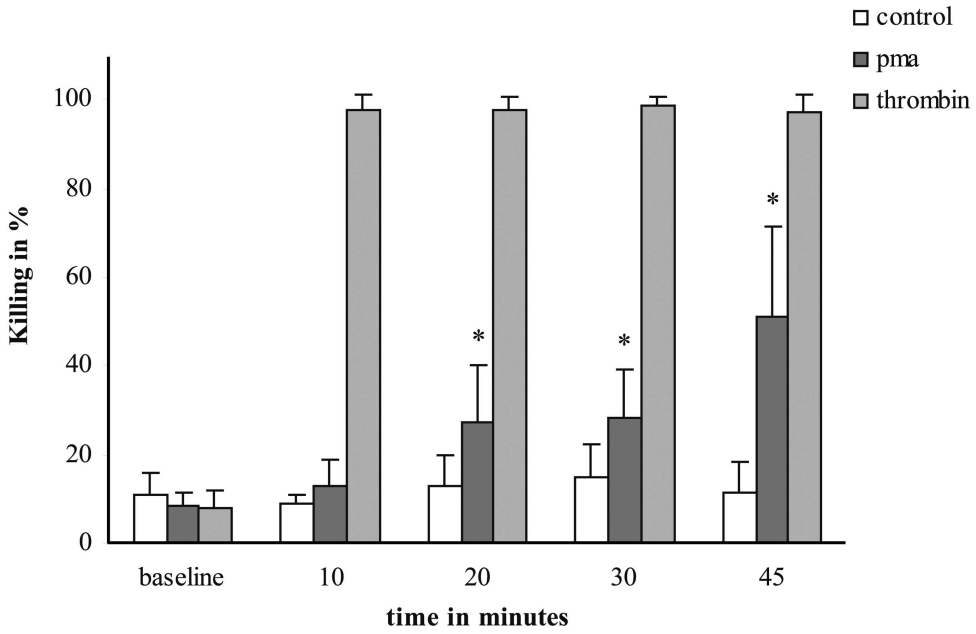

Zander & Klinger: Fig. 5
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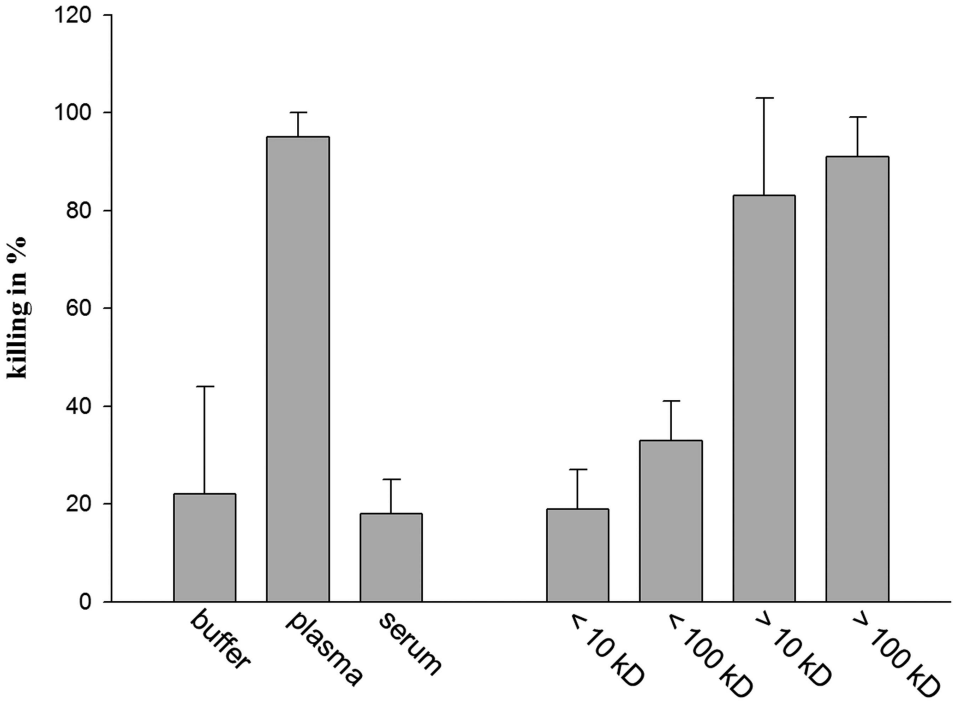

Zander & Klinger: Fig. 6
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