The phagocytic fitness of leucopatches may impact the healing of chronic wounds

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Summary

Chronic non-healing wounds are significantly bothersome to patients and can result in severe complications. In addition, they are increasing in numbers, and a challenging problem to the health-care system. Handling of chronic, non-healing wounds can be discouraging due to lack of improvement, and a recent explanation can be the involvement of biofilm infections in the pathogenesis of non-healing wounds. Therefore, new treatment alternatives to improve outcome are continuously sought-after. Autologous leucopatches are such a new, adjunctive treatment option, showing promising clinical effects. However, the beneficial effect of the patches are not understood fully, although a major contribution is believed to be from the release of stimulating growth factors from activated thrombocytes within the leucopatch. Because the leucopatches also contain substantial numbers of leucocytes, the aim of the present study was to investigate the activity of the polymorphonuclear neutrophils (PMNs) within the leucopatch. By means of burst assay, phagocytosis assay, migration assay, biofilm killing assay and fluorescence in-situ hybridization (FISH) assay we showed significant respiratory burst in PMNs, active phagocytosis and killing of Pseudomonas aeruginosa by the leucopatch. In addition, bacterial-induced migration of PMNs from the leucopatch was shown, as well as uptake of P. aeruginosa by PMNs within the leucopatch. The present study substantiated that at least part of the beneficial clinical effect in chronic wounds by leucopatches is attributed to the activity of the PMNs in the leucopatch.

Keywords: autologous platelet-rich fibrin patch, phagocytosis, Pseudomonas aeruginosa, wound-healing

Introduction

Chronic wounds can be classified as failing to heal spontaneously within 3 months. As 1% of the western population will suffer from non-healing wounds, they are considered a significant health problem. Socioeconomically, the management of chronic wounds reaches a total and rising cost of 2–4% of the health budget in western countries [1,2]. The major chronic types of wounds are venous stasis wounds (70% of all chronic wounds) [3,4], followed by wounds associated with ischaemia, diabetes or pressure. Complications to non-healing wounds are vast, and patients are at risk of severe pain, sepsis, hospitalization and, in some cases, amputations. The pathophysiology of chronic wounds is not understood fully, and several theo-

ries have been provided [5–8]. An explanation for the various and partly conflicting hypotheses for establishment of chronic wounds could be that different categories of non-healing wounds are described as a whole [9].

However, one important event in the establishment of a non-healing wound is the emergence of a defective skin barrier caused by structural damage, which increases the susceptibility of a wound to infection [10]. Substantial evidence now supports that the establishment of chronic infections residing deep in the wounds in the form of biofilms is a pivotal contributor to the impaired healing of chronic wounds [11,12]. The mechanism is probably a continuous induction of suboptimal host responses unable to eliminate the biofilms and the continuous oxidative milieu

caused by activated polymorphonuclear neutrophils (PMNs), resulting in collateral damage of the host tissue, and thereby contributing to the pathophysiology [13].

Handling and design of therapy depends partly on the distinct pathophysiology of the chronic wounds. In addition, the choice of treatment is multi-modal and is characterized by a relative lack of randomized controlled trials [9]. The implementation of novel alternative treatment modalities is compromised by the equivocal interpretation of the evidence due to heterogenic study designs and populations [14,15]. One explanation could be missing acknowledgement of the significance of biofilms within the wounds. Another reason could be the inclusion of chronic wounds of different pathophysiology in the studies, and thereby possibly important different responses to the tested treatments. In addition, the group of patients with chronic wounds are often further heterogenic due to factors such as obesity, diabetes, smoking, malnutrition and age, to mention a few. However, by careful grouping of patients with different backgrounds for their chronic wounds and taking biofilms into account there is an improved potential for testing new treatment options of chronic wounds.

One relatively recent treatment option for non-healing wounds is the so-called platelet-rich plasma or platelet-rich fibrin, which has been introduced for treatment of various recalcitrant wounds [16,17]. A major effect of the those products has been attributed to the cytokines and growth factors from activated platelets inducing tissue repair, angiogenesis and inflammation, especially platelet-derived growth factor (PDGF) [18].

The recent development of a multi-layered platelet-rich fibrin patch (LeucoPatch®) including leucocytes on the wound-contacting surface and its positive effect in treating hitherto recalcitrant wounds has led us to investigate further the actual mechanisms of these leucopatches on chronic wounds [19].

The present study was designed to analyse the activity of phagocytes within LeucoPatch®, generated using the LeucoPatch® system by a 3CP™ three-step process to prepare autologous patches at the point of care (Reapplix Aps, Birkeroed, Denmark) [18,19]. As *Pseudomonas aeruginosa* has been shown to be one of the most important pathogens of biofilms deep in chronic wounds [20,21], the leucopatches were tested for their respiratory burst, bacterial killing of *P. aeruginosa*, phagocytic effect as well as their effect on alginate-embedded *P. aeruginosa*-resembling biofilm mode of growth.

Materials and methods

Study design

Whole blood from normal human volunteers was used for all the experiments. Seven donors participated (four males), aged between 30 and 50 years. All donors were physically well and not taking any medication. Informed consent was obtained from each donor and guidance for the approval process for the study was received from the local ethics committee for research in human subjects, in accordance with the Declaration of Helsinki.

Bacterial preparation

P. aeruginosa PAO1 strain was cultured overnight in Luria–Bertani (LB) broth at 37°C on a platform shaker to attain stationary phase organisms. Exponential phase bacteria were obtained from diluting overnight culture in fresh LB broth and shaking at 37°C to reach optical density $(OD)_{600} = 0.5$ [approximately 10^8 colony-forming units (CFU)/ml]. Bacteria were washed twice in phosphate-buffered saline (PBS) before application.

Leucopatch preparation

Peripheral whole blood (18 ml) from healthy volunteers was drawn directly into LeucoPatch® devices (Reapplix Aps) using a winged blood sampling set (Terumo Quick Fit; Terumo, Leuven, Belgium) fitted with the LeucoPatch® needle-holder [18]. The device was transferred directly to the centrifuge holder provided and was centrifuged in a two-step process separated by 10 min. The initial centrifugation for 8 min at 3000 *g* was followed by a secondary centrifugation at 3000 *g* for 2 min. After removing the device lid, the patches formed were used immediately or stored at 5°C or room temperature in Krebs–Ringer buffer supplemented with 10 mM glucose (KRB) until later use.

Neutrophil isolation

PMNs were isolated from whole blood. Erythrocytes were allowed to sediment in 5% dextran solution and the subsequent leucocyte-enriched plasma was layered on Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged. To remove any remaining erythrocytes, the formed pellets enclosing PMNs were lysed with hypertonic saline and the purified PMNs were resuspended in KRB and adjusted to 10^7 cells/ml.

Respiratory burst assay

The leucopatches were cut into 6-mm circular fragments; 25 μl of freshly isolated PMNs (10^7/ml) or leucopatch fragments were transferred onto a 96-well microtitre plate and phagocytosis was initiated by adding 50 μl of *P. aeruginosa* PAO1 (5×10^7 CFU/ml), zymosan-activated serum (ZAS) (10 mg/ml of zymosan opsonized in 30% AB+ serum) or the protein kinase C agonist phorbol myristate acetate (10 mM PMA), and finally 175 μl luminol (30 nM) to the wells. Ten μl of diphenylene iodonium (DPI) (50 μM DPI), a specific inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, was employed as control in one experiment. The luminol-enhanced chemiluminescence analysing phagocyte-derived reactive oxygen species

(ROS) was measured using a luminometer (Wallac 1420 Victor2; Perkin Elmer, Waltham, MA, USA) at 37°C for 1 h.

Bactericidal assay

Leucopatch-mediated bacterial killing was evaluated by mixing 500 µl of serum-opsonized *P. aeruginosa* PAO1 with leucopatches and determining the loss of bacterial viability over time by plating diluted samples overnight followed by colony counting. Washed *P. aeruginosa* PAO1 were diluted in KRB to a final concentration, in 10% human AB-positive serum, of 5×10^7 CFU/ml or 5×10^8 CFU/ml. The mixture was rotated on an orbital microplate shaker with a speed of 120 rpm for 30 min at 37°C and transferred to six-well plates containing freshly prepared leucopatches. Samples were removed at 0, 20, 60 and 90 min for bacterial killing examination, where 50 µl of the reaction mixture was removed and added to 2·45 ml H_2O pH 11, thus initiating cellular lyses for 5 min before vortexing vigorously and plating onto modified Conradi–Drigalski plates (Statens Serum Institute, Copenhagen, Denmark) and incubated overnight at 37°C.

Alginate biofilm bactericidal assay

Protanal LF 10/60 (FMC BioPolymer N-3002; Drammen, Norway) extracted from brown seaweed was dissolved in 0·9% NaCl to an alginate concentration of 1% and sterile-filtered. The bacterial culture was diluted 1 : 20 in seaweed alginate solution and transferred to a 10-ml syringe and placed into a syringe pump (Graseby 3100; Ardus Medical Inc., Watford, UK). A vertically orientated tube was connected to the syringe and the syringe pump fed the alginate through the exit orifice of the tube into a gelling bath (0·1 M, pH 7·0 Tris HCL buffer containing 0·1 M $CaCl_2$) creating alginate beads of approximately 50 µl. A magnetic stirrer (IKA® RCT Basic; IKA-Werke GmbH & Co. KG, Staufen, Germany) was placed underneath the gelling bath to prevent the beads from sticking together during gelling. The distance between the tube exit and gelling bath was 8 cm. The magnetic stirrer was kept constant (280 rpm) and the beads were left for stabilization in the gelling bath for 1 h and finally washed twice in 0·9% NaCl containing 0·1 M $CaCl_2$. After washing, 20 ml 0·9% NaCl containing $CaCl_2$ was added.

The alginate discs (volume = 250 µl) were created by pipetting the alginate/bacterial solution into a 24-well microtitre plate. The formed discs were transferred immediately to a 50-ml tube containing gelling medium (0·1 M, pH 7·0 Tris HCL buffer containing 0·1 M $CaCl_2$) for stabilization overnight.

P. aeruginosa PAO1 embedded in alginate beads were positioned on a modified Conradi–Drigalski agar plate (Statens Serum Institute) and covered with prepared leucopatches. After 2 h the leucopatches were removed and the

alginate beads transferred to tubes containing PBS and homogenized (Heidolph Silent Crusher M; Heidolph Instruments, Schwabach, Germany), serially diluted and plated onto Conradi–Drigalski plates (Statens Serum Institute) for quantification by colony-counting the next day.

The alginate discs containing *P. aeruginosa* PAO1 were added to the bottom of a 24-well microtitre plate and covered with prepared leucopatches (alginate disc to leucopatch ratio 1 : 1). After 2 h the leucopatches were removed and the quantification of bacterial content were determined as described previously.

Leucocyte chemotaxis assay

Transwell chambers were used to mimic chemotactic leucocyte migration. Leucopatches in 200 µl KRB were added to the upper wells of 5-µm pore size polycarbonate filters in 6·5-mm diameter 24-well Transwell chambers (Corning/Sigma, St Louis, MO, USA), with 600 µl chemoattractant or medium (KRB) in the lower wells. Formyl–methionyl–leucyl–phenylalanine (fMLP) or *P. aeruginosa* PAO1 were used as leucocyte activators/chemoattractants. At specified time-points, filters were removed from the wells and the number of cells migrated into the bottom chambers were counted with a flow cytometer (FACSCanto; BD Biosciences, San Jose, CA, USA). The identification of leucocytes was performed using fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD15, allophycocyanin (APC)-conjugated mouse anti-human CD14 and peridinin chlorophyll (PerCP)-conjugated mouse anti-human CD45 monoclonal antibodies (all purchased from BD Biosciences).

PNA fluorescence *in-situ* hybridization (FISH) imaging

Prepared leucopatches were mixed with *P. aeruginosa* PAO1 in a microtitre plate to commence phagocytosis. The microtitre plate was placed on a platform rocker for 60 min at 37°C; subsequently, the leucopatches were transferred to 10% formalin (formaldehyde 4% aqueous-buffered solution). The fixated patches were paraffin-embedded and cut into ultrathin (3–5 µm) sections using a microtome and placed on microscope slides, dewaxed and processed through xylene and ethanol to water before probe hybridization. The deparaffinized leucopatch sections were examined by FISH using peptide nucleic acid (PNA) probes. A Texas Red-labelled *P. aeruginosa*-specific PNA probe in hybridization solution (AdvanDx, Inc., Woburn, MA, USA) was added to each section and hybridized in a PNA FISH workstation covered by a lid at 55°C for 90 min. The slides were washed for 30 min at 55°C in wash solution (AdvanDx) and the slides were covered with a coverslip. Slides were read using a fluorescence microscope equipped with Texas Red and 4',6-diamidino-2-phenylindole (DAPI) filters.

Statistics

GraphPad Prism (version 6·0d) software was used for statistical work. Chemiluminescence data were logged automatically into Microsoft Excel. The non-parametric Mann–Whitney U-test was used to determine differences between groups if the D'Agostino–Pearson test demonstrated that not all data sets were distributed normally. In the case of normally distributed data sets the parametric t-test was applied. A level of $P \leq 0.05$ was used for assigning statistical significance.

Results

The production of reactive oxygen species (ROS) is sustained in PMNs residing in leucopatches

Reactive oxygen species (ROS) are anti-microbial metabolites produced during phagocytosis or equivalent stimulation of PMNs. The respiratory burst describes the generation of ROS by the electron-transfer system NADPH oxidase, where electrons are delivered to oxygen present in phagosomes or the extracellular milieu generating bactericidal oxygen metabolites. The oxidation of luminol generates excited state metabolites and emits light on dropping to the ground state. Thus, the luminol-enhanced chemiluminescence assay quantifies ROS production during microbial phagocytosis, activation of PMNs by zymosan or the simulation of phagocytosis by stimulating PMNs with PMA.

Figure 1 depicts the level of chemiluminescence detected during stimulation of PMNs residing in leucopatches. When leucopatches are challenged with increasing concentrations of *P. aeruginosa* there is a prominent chemiluminescence response [6094 counts per second $(cps)_{max}$ to 29886 cps_{max}] in a concentration-dependent manner. Moreover, the chemiluminescence is reduced significantly by addition of DPI, which inhibits NADPH oxidase and impedes the respiratory burst ($P < 0.0005$). Thus, mixing *P. aeruginosa* and leucopatches prompts a noticeable ROS production, indicating strongly that the inherent PMNs in leucopatches have preserved their phagocytic competence. ZAS and PMA are employed as activators of PMNs and are indeed capable of generating a chemiluminescence response in leucopatches (Fig. 1); consequently, various stimulants of PMN function seem to provoke a conspicuous response in leucopatches.

The intensity of chemiluminescence during phagocytosis differs between freshly isolated PMNs and leucopatches

Comparison of the detected chemiluminescence during phagocytosis of *P. aeruginosa* by freshly isolated PMNs and leucopatches originating from equivalent donors is represented in Fig. 2. When freshly isolated PMNs are
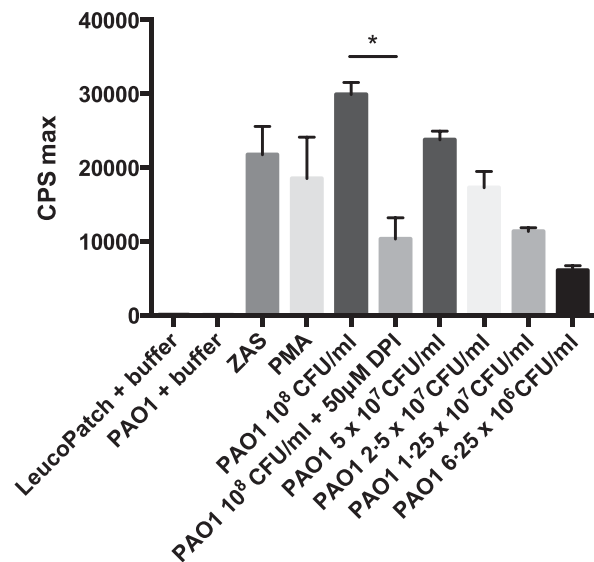

Fig. 1. Chemiluminescence assay represents the production of reactive oxygen species (ROS) in leucopathes during 60 min of stimulation with *Pseudomonas aeruginosa* PAO1, zymosan-activated serum (ZAS) or the protein kinase C agonist phorbol myristate acetate (PMA). Diphenylene iodonium (DPI) a specific inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, was employed as control in one experiment. The luminol-enhanced NADPH oxidase is employed as control. Results are expressed as counts per second (cps) max ± standard deviation (s.d.). Experiments were performed in triplicate. *$P < 0.0005$.

phagocytizing *P. aeruginosa* the subsequent mean chemiluminescence signal (cps_{max} 78095) is twice as high compared to the mean chemiluminescence (cps_{max} 34205) detected
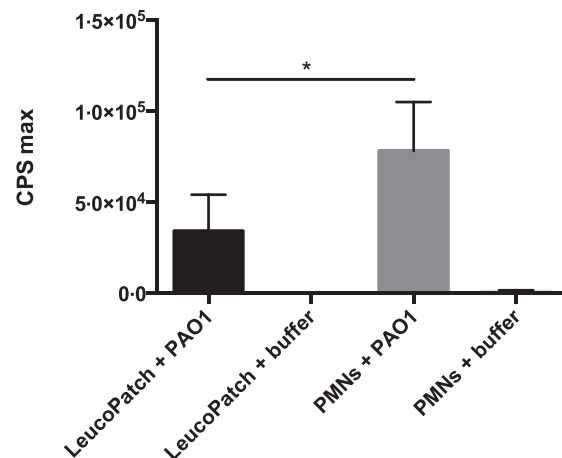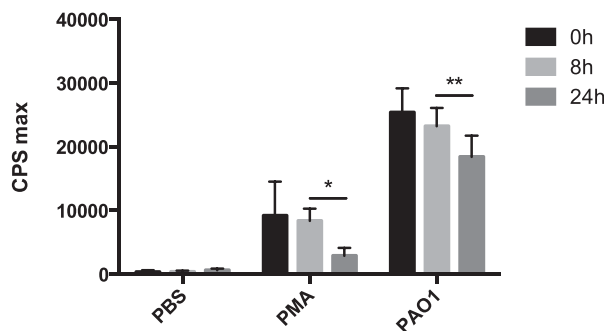

Fig. 2. Chemiluminescence assay compares the production of reactive oxygen species (ROS) during phagocytosis of *Pseudomonas aeruginosa* PAO1 [5×10^7 colony-forming units (CFU)/ml] in leucopathes and isolated polymorphonuclear neutrophils (PMNs) (10^7 cells/ml) from identical donor blood. Results are expressed as counts per second (cps) max ± standard deviation (s.d.). Experiments were performed in triplicate. *$P < 0.005$.

from leucopatches ($P < 0.005$). However, the difference in chemiluminescence between free PMNs and leucopatches may be attributable to the relative reduction in phagocytosis-primed cells in the leucopatch, as probably only a fraction of the absolute amount is conditioned to initiate phagocytosis.
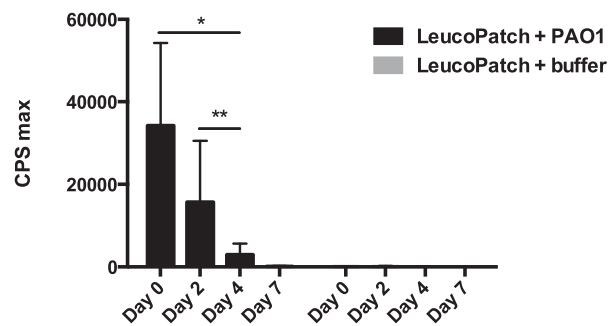
The durability of ROS production in leucopatches is maintained

With the intention of evaluating the durability of PMN-mediated ROS production, the prepared leucopatches were left in their devices at room temperature for 24 h. The chemiluminescence detected by stimulating leucopatches with either PMA or *P. aeruginosa* after 8 and 24 h were compared to the initial chemiluminescence of freshly prepared leucopatches (Fig. 3). After 8 h the chemiluminescence is reduced, although not significantly, by 9% (PMA stimulation) and 8% (bacterial stimulation) compared to that of recently prepared leucopatches. Twenty-four h post-producing the leucopatches the chemiluminescence is reduced by 68% (PMA stimulation) and 27% (bacterial stimulation) compared to the preliminary quantity. Although the respiratory burst elicited by stimulated leucopatches appears to diminish over time, the chemiluminescence level is preserved within 8 h after generation of the leucopatches, indicating that the phagocytic capacity of PMNs in the leucopatches remains sufficient beyond several hours after preparation.

The conserved chemiluminescence in PMNs during a longer time-period of 7 days was assessed on leucopatches maintained in a cellular buffer (KRB) and stored at 5°C (Fig. 4). In these conditions the respiratory burst by PMNs exposed to *P. aeruginosa* was reduced by 54% after 2 days ($P < 0.04$) and 91% after 4 days ($P < 0.002$). The chemilu-
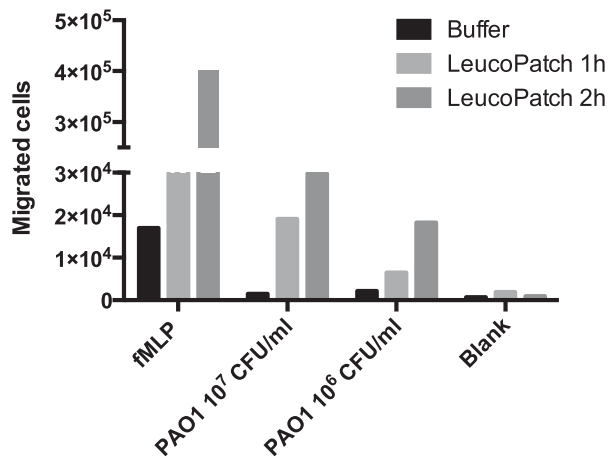

Fig. 4. Chemiluminescence assay compares the production of reactive oxygen species (ROS) in leucopatches at different time-points after preparation. The leucopatches were used immediately after preparation or stored in Krebs–Ringer buffer supplemented with 10 mM glucose (KRB) at 5°C prior to stimulation with *Pseudomonas aeruginosa* PAO1 [5×10^7 colony-forming units (CFU)/ml] for 60 min. Results are expressed as counts per second (cps) max ± standard deviation (s.d.). Experiments were performed in triplicate and leucopatches were prepared from seven donors. *$P < 0.04$; **$P < 0.002$.

minescence after 7 days of storage was undetectable. The phagocytic activity of PMNs is obviously influenced by several factors, and optimal cellular milieu to ensure appropriate metabolism is imperative. Nevertheless, the respiratory burst activity of leucopatches remained detectable several days after their production.

The PMNs in leucopatches are able to detach and migrate towards leucocyte attractants

Possible chemotactic properties of the PMNs in leucopatches were evaluated by their ability to migrate towards the chemoattractants fMLP and *P. aeruginosa* in a membrane-separated two-phase system (Transwell chambers). Cells able to migrate through the membrane towards a chemoattractant were quantified by flow cytometry (Fig. 5). fMLP is a powerful chemoattractant, inducing a migratory response in more than 10^5 cells/ml. The efficacy of fMLP to attract PMNs is time-dependent, thus after 2 h the quantity of attracted cells are 13·3 times higher than after 1 h. Bacteria are also potent chemoattractors; however, the amount of cells migrated towards *P. aeruginosa* is more than 10 times lower compared to fMLP. As observed previously, time is an important determinant of chemotaxis and the extent of migrated cells after 2 h is 2·8 (10^6 CFU/ml) and 1·5 times higher compared to the quantity after 1 h. The present chemotaxis assay shows that PMNs are able to detach their fixation in the leucopatches and migrate towards chemotactically active factors, which is a fundamental consequence to ensure sufficient anti-microbial effects in tissues.

Fig. 3. Chemiluminescence assay compares the production of reactive oxygen species (ROS) in leucopathes at various time-points after preparation. The leucopatches were stored at room temperature in the device for 8 or 24 h before stimulation with *Pseudomonas aeruginosa* (PAO1) or the protein kinase C agonist phorbol myristate acetate (PMA) for 60 min. Results are expressed as counts per second (cps) max ± standard deviation (s.d.). Experiments were performed in triplicate. *Not significant (n.s.); **n.s.

Fig. 5. Chemotaxis assay describe the chemotactic migration of leucocytes residing in the leucopatch. Formyl-methionyl-leucyl-phenylalanine (fMLP) and *Pseudomonas aeruginosa* (PAO1) were applied as leucocyte chemoattractants/activators. The identification and the quantity of leucocytes migrated through 5-μm pore size polycarbonate filters were determined by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD15, allophycocyanin (APC)-conjugated mouse anti-human CD14 and peridinin chlorophyll (PerCP)-conjugated mouse anti-human CD45 monoclonal antibodies. Experiments were performed twice.

Microscopic imaging visualizes interaction between leucopatch and *P. aeruginosa*

Adjacent interface between the fraction of PMNs in the leucopatch and bacterial constituents is a premise to concede phagocytic killing. In order to visualize such interaction we utilized fluorescence microscopy. Leucopatches were challenged with planktonic *P. aeruginosa* and fixed subsequently in formalin prior to paraffin-embedding. The presence of bacteria in the resulting slides was examined by PNA-FISH technology by addition of a Texas Red-labelled

P. aeruginosa-specific PNA probe. Figure 6 presents microscopic images of the leucopatch–bacteria interaction. The red-fluorescent *P. aeruginosa* are localized in close contact with the subjacent layer in the leucopatch and are accordingly accessible to PMN mediated phagocytosis.

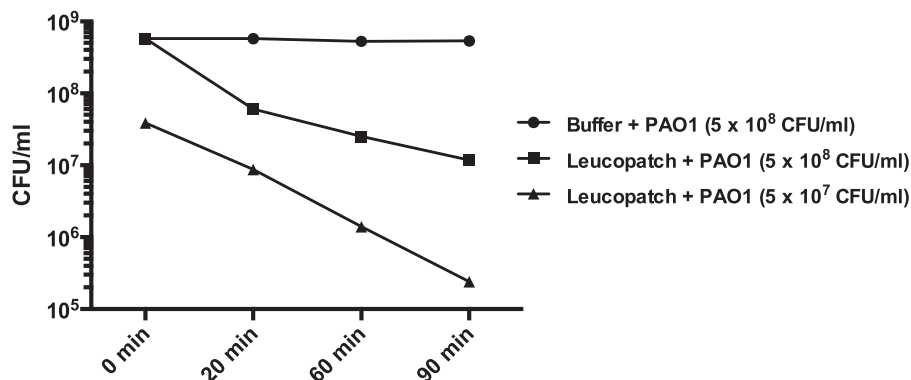## The bactericidal activity of leucopatches is prominent

The PMN-mediated killing of planktonic bacteria was determined by exposing leucopatches to serum-opsonized *P. aeruginosa* PAO1 followed by plating diluted samples overnight and colony-counting the next day. The loss of bacterial viability during 90 min of phagocytosis is depicted in Fig. 7. When mixing leucopatch and 5×10^8 CFU/ml of *P. aeruginosa* the bacterial viability was reduced by 89% after 20 min of leucopatch-facilitated phagocytosis and decreased further by 98% after 90 min compared to control. The PMN-mediated phagocytic killing is even more distinct when a 10-times reduced bacterial concentration (5×10^7 CFU/ml) is subjected to phagocytosis, where the bacterial viability is reduced by more than 99% with respect to the initial inoculum. The lowering of bacterial concentration renders the relation between phagocytic cells and bacteria more favourable and ensures an optimal exploitation of the phagocytic competence of PMNs.

The contribution of bacterial biofilms to the pathogenesis of chronic wounds necessitates proper simulation *in vitro* of the anti-microbial activity of leucopatches. Thus, we employed alginate-embedded *P. aeruginosa*, mimicking the biofilm mode of growth, in our bactericidal assays. Figure 8a shows the efficacy of leucopatches to control bacterial growth in alginate beads. During 2 h of growth the inoculum of 8×10^7 CFU/ml is increased by 111%; however, leucopatches inhibited bacterial growth completely compared to non-leucopatch control ($P < 0.002$).

To ensure optimal physical contact between the PMN fraction of leucopatches and bacterial biofilms, we employed alginate discs encompassing *P. aeruginosa*; see
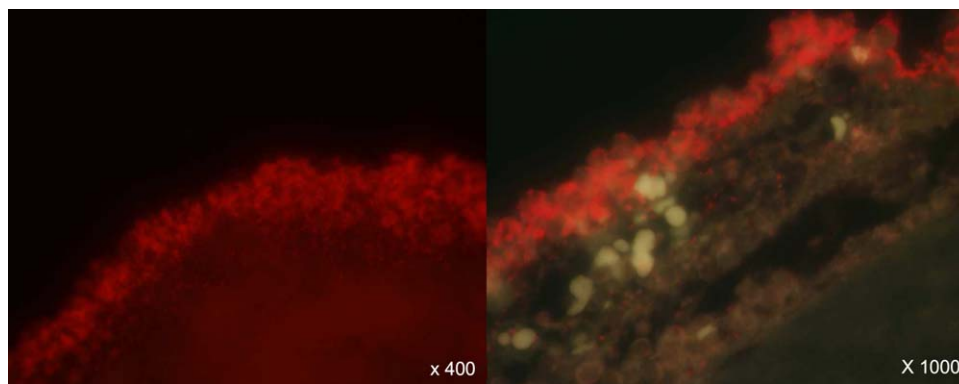
Fig. 6. The fluorescent microscopic images visualize the interaction between *Pseudomonas aeruginosa* and leucopatch constituents. Preformed leucopatches were mixed with *P. aeruginosa* PAO1 and eventually fixated in formalin and subsequently paraffin-embedded. Tissue sections were processed by peptide nucleic acid–fluorescence *in-situ* hybridization (PNA–FISH) technology allowing the *P. aeruginosa* to appear fluorescent by hybridization with a Texas Red-labelled *P. aeruginosa*-specific PNA probe.

Fig. 8b. The surface of the discs was sheeted with a size-approximated leucopatch to allow proper interaction for phagocytosis. When simulating bacterial biofilm in the form of discs, the leucopatches were not only repressing bacterial growth but were indeed able to eradicate the biofilm by reducing the bacterial content by 70% after 2 h ($P < 0.005$).

Discussion

Chronic wound patients often have systemic and local ischaemia in the affected area, and therefore display a decreased early inflammatory cell infiltration [22–24]. Polymorphonuclear neutrophil granulocytes (PMNs) are the first line of defence upon recognition of invading bacteria. Damage to the protective skin surface and underlying tissue facilitates bacterial colonization in the wound and the subsequent establishment of bacterial biofilms and induction of a suboptimal chronic inflammation unable to clear the infection [12,25]. The biofilm infections cannot be eliminated due to the resilience of the biofilms to host immune response and antibiotic treatment [26]. Platelet-rich fibrin has already been included in wound care of various kinds, and by the inclusion of leucocytes into patches, e.g. as LeucoPatch® promising effects in the treatment of chronic wounds have been observed ([19] and Löndahl 2015, submitted for publication). The present study shows an additional mechanism of leucocyte-containing platelet-rich patches, apart from the already suggested immune modulating and healing support effect [18].

The observation of biofilms deep in non-healing wounds has added significant knowledge to the recalcitrant nature of these wounds [12]. As the biofilms are not eliminated by the host responses collateral tissue damage occurs instead, and thereby host responses contribute eventually to the chronic nature of the non-healing wounds [27]. Additionally, the necrotic tissue in the wounds may provide a wound environment which limits the migratory skills of the recruited PMNs [28]. Furthermore, the release of anti-microbial host factors in the wound milieu is probably degraded in the proteolytic environment [6]. However,

with the supplementing of host immune cells from the surface, biofilms are challenged from more sites and on a larger surface. Therefore, part of the effect of leucocyte-containing patches may be due to oxidative as well as non-oxidative defence mechanisms, in addition to the growth factors and cytokines from the platelets. Although lower in leucopatches compared to free PMNs, our results showed a substantial oxidative burst as well as phagocytic activity and bacteria-killing. Accordingly, the respiratory burst and subsequent phagocytic activity of freshly isolated PMNs is superior compared to that of PMNs derived from leucopatches. Such a comparison is only legitimate if the concentration of activated PMNs is equivalent in the two set-ups. The content of PMNs in leucopatches is estimated theoretically to be approximately 10^7 cells/ml; however, it is unlikely that the absolute amount is conditioned to accomplish phagocytosis. Because approximately a fraction of 1/17 of one patch is used for respiratory burst assay, presumably around 600 000 PMNs are used. For comparison, approximately 250 000 free PMNs are used as control respiratory burst assay. This means that, even though far from all PMNs in the leucopatch are being exposed to stimulation, the measured respiratory burst is still approximately half of what is observed from free PMNs.

The emigration of circulating leucocytes from the blood into inflamed tissues is a crucial event in the anti-microbial activity of PMNs. Chemotaxis is a complex process; integrating several components of the host response, however, is finalized by the migratory response to a chemoattractant gradient. Thus, we observed migration of PMNs from the leucopatch both by stimulation with fMLP and *P. aeruginosa*, suggesting that in the patients PMNs can invade into the wound from the leucopatch and attack the biofilms from two sides. These observations indicate that at least part of the beneficial effect of the leucopatches is due to anti-bacterial effects.

We chose to test the anti-bacterial effect of the leucopatches on *P. aeruginosa* as this pathogen has been shown to be an important contributor to the pathophysiology of chronic wounds [21]. Whether the effect is similar against other wound pathogens is unknown. However, Moojen
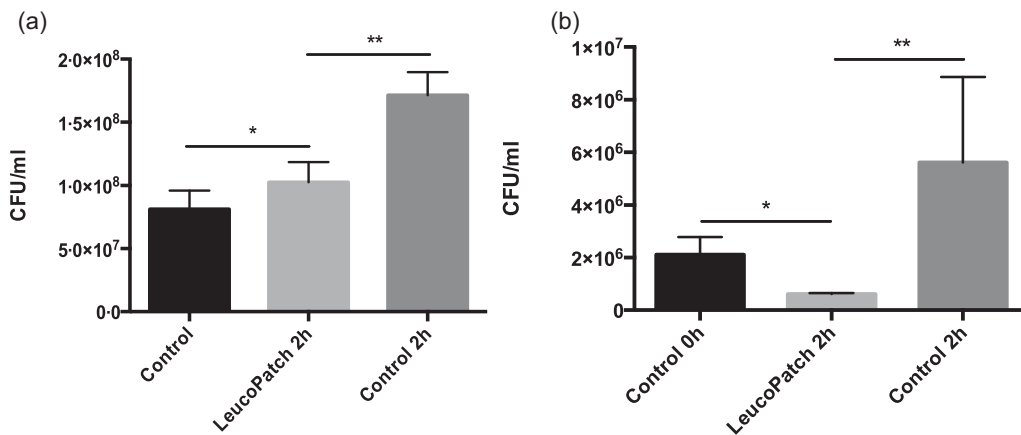
Fig. 8. Bacteriostatic activity of leucopatches challenged with alginate-embedded *Pseudomonas aeruginosa* (PAO1) in beads mimicking biofilm mode of bacterial growth (a). The alginate beads enclosing PAO1 were positioned in direct contact with leucopatches and transferred subsequently to tubes containing phosphate-buffered saline (PBS) and homogenized, serially diluted and plated onto Conradi–Drigalski plates followed by colony counting the next day. *Not significant (n.s.); **$P < 0.002$. Bactericidal activity of leucopatches challenged with a biofilm model of *Pseudomonas aeruginosa* (PAO1) embedded in alginate discs (b). The freshly prepared leucopatches were layered carefully on the surface, in direct contact with the alginate disc containing PAO1. After 2 h the alginate discs were transferred to tubes containing phosphate-buffered saline and homogenized, serially diluted and plated onto Conradi–Drigalski plates followed by colony-counting the next day. *$P < 0.005$; **$P < 0.02$.

et al. showed a bacterial killing effect against *Staphylococcus aureus* by a comparable platelet-leucocyte gel [29]. In addition, the platelet-leucocyte gels used in that study had substantial levels and activity of myeloperoxidase (MPO) compared to controls. The number of leucocytes in the platelet-leucocyte gels ($17.9 \pm 2.62 \times 10^6$/ml) and in the leucopatch (55×10^6/patch) is difficult to compare due to the differences in three-dimensional structures; however, both contain viable leucocytes [18,29].

The clinical standard use of LeucoPatch® is application for 1 week in the chronic wound and replacement of the patch with a new patch produced just before replacement [19]. Therefore, we also investigated the activity of patches over several days, and although the activity was reduced over time the oxidative burst could still be observed after 7 days. This observation is interesting, as it is generally accepted that the lifespan of PMNs is short (< 1 day), although a recent study using *in-vivo* labelling in humans have estimated the average circulatory neutrophil lifespan to be 5.4 days [30]. In addition, a relatively large interindividual variation could be observed between patches from the different donors. Whether this can also be correlated with interindividual treatment effects or whether it would actually be possible to stimulate the patches further to improve their effect still remains to be studied.

As mentioned above, a significant characteristic of chronic wounds are the presence of biofilms. Because bacteria are significantly more resistant to host response when growing in biofilms compared to planktonic growth, we used our biofilm model of embedding bacteria in seaweed alginate to resemble the extracellular matrix in biofilms [31]. Using this model system, we observed a substantial reduction in bacterial reduction compared to the experiments using planktonic growing bacteria. However, and more importantly, we still showed a reduction in the bacterial load in the model biofilms where *P. aeruginosa* is embedded in seaweed alginate. The 40 times lower baseline bacterial burden in the alginate discs, compared to the equivalent concentration in the alginate beads, probably balances the ratio between PMNs and bacteria in favour of phagocytic killing. The kinetics of phagocytosis is a saturable process. Consequently, the effect is dependent upon initial bacterial burden to allow the PMNs to contribute optimally to bacterial clearance. This indicates that the anti-bacterial effects of the LeucoPatch® can also be expected against the biofilm-growing bacteria in the chronic wounds. Surgical debridement is the traditional remedy to remove or break up formed biofilm; however, these are often reformed within 48–96 h [32].This study confirms the potential of leucopatches to kill bacteria and inhibit the reformation of biofilm.

Previously, it was shown that production and release of rhamnolipids from a quorum sensing competent *P. aeruginosa* could lyse PMNs effectively *in vitro* and thereby adds significantly to the virulence of biofilm infections [33]. Although to a lesser extent, this was confirmed *in vivo* in a lung infection mouse model as well as in sputum from patients with cystic fibrosis [33]. Rhamnolipid release from *P. aeruginosa* biofilms in non-healing wounds could probably contribute to the pathogenesis. As rhamnolipids effect

is mediated through binding to the cell membranes of the host cells, one could speculate that part of the beneficial effects of the leucopatch may be due to neutralization of rhamnolipids by the high presence of thrombocytes and their membranes.

Finally, modifications can be performed, including activation of the patches for augmented effect. In addition, potential anti-inflammatory effects have not been studied until now. Nevertheless, and in conclusion, the LeucoPatch® system is an easily obtained treatment modality, using the patient's own material, and is therefore well tolerated.

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Disclosures

Author contributions

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