

Impaired neutrophil killing precedes infectious complications in severely injured patients

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Abstract

Background

Severe traumatic injury is often accompanied by late onset infectious complications, possibly driven by trauma-induced immune dysregulation involving the neutrophil compartment. Severe trauma initiates the release of distinct neutrophil subsets into the circulation, characterized by their CD16 and CD62L surface expression. The phenotype and function of these subsets in severely injured patients remains poorly defined, despite their role in post-trauma infectious complications.

Methods

A prospective observational cohort study was conducted where blood samples were collected longitudinally from severely injured patients during the first 15 days after trauma. Blood neutrophil counts, surface marker expression, phagocytosis, phagolysosomal acidification and bacterial killing capacity were analyzed over time. Neutrophil subsets were FACS-sorted for single-cell killing assays to assess intracellular killing. Patients with and without infectious complications were compared.

Results

15 male severely injured patients (ISS $\geq$ 32) were included, of whom 8 developed at least one infectious complication (earliest onset: day 4; median onset: day 7). Banded neutrophils (CD16^{low}) were present during initial leukocytosis immediately after trauma. Progenitors and hypersegmented (CD62L^{low}) neutrophils predominated during the second leukocytosis after the first week. Phagocytic capacity per neutrophil was increased in patients compared to controls at all time points, while intracellular acidification was decreased. Notably, the infection group showed decreased killing capacity already from day 1, while the no-infection group did not. Significant differences in killing capacity were found between different neutrophil subsets in all patients.

Conclusions

This study demonstrates quantitative, phenotypical, and functional changes in the circulating neutrophil compartment occurring within the first hours after severe trauma and persisting for up to 15 days. Patients who developed infectious complications exhibited reduced intracellular killing from day 1, preceding the onset of infections. These findings suggest that extensive tissue damage triggers DAMP-driven innate immune dysregulation, impairing neutrophil killing capacity and predisposing severely injured patients to infectious complications.

Background

Traumatic injury is a major cause of death and disability worldwide (1). Improvements in trauma resuscitation and acute care have led to large reductions in initial mortality rates (2). In turn, this increase in survival has led to new challenges; management of complications and a focus on functional recovery have become increasingly important (2). Frequently observed complications after severe trauma are respiratory failure, neurological dysfunction, thrombosis/embolism and infections (3–5). It is apparent that the immune system plays a major role in the development of these complications, particularly infections (6).

Neutrophils are the most abundant white blood cells and function as first responders to infection and inflammation. They instantaneously respond to the release of damage associated molecular patterns (DAMPs) following traumatic injury by increasing their numbers and displaying specific phenotypic subsets (6, 7). Neutrophils are important in regaining homeostasis through clearance of damage, tissue repair and microbial containment (8, 9). However, their recruitment also carries detrimental potential, as delayed healing and pulmonary damage can result in Acute Respiratory Distress Syndrome (ARDS) (8). Injury severity correlates with both the extent of acute neutrophil subset recruitment and the risk of infectious complications (10), yet the specific functional contributions of individual subsets remain inconclusive.

The neutrophil subsets recruited after trauma can be identified by differential CD16 (FcγRIII) and CD62L (L-selectin) expression (11). In homeostasis, the CD16^{high}CD62L^{high} mature phenotype is dominant in the blood, while CD16^{low} banded neutrophils quickly appear after trauma. In the subsequent days to weeks, CD16^{low}-banded neutrophils disappear and CD62L^{low}-hypersegmented neutrophils emerge in the circulation, often accompanied by neutrophil progenitors (7). These subsets are functionally distinct: in an experimental human endotoxin challenge model, CD16^{low}-banded neutrophils showed increased bacterial containment whereas CD62L^{low}-hypersegmented neutrophils showed decreased containment and an immunosuppressive profile (11, 12). Complementary findings from trauma patients demonstrated increased phagolysosomal acidification in CD16^{low}-banded neutrophils and decreased capacity in the CD62L^{low}-hypersegmented subset (13). However, the antibacterial function of neutrophil subsets in trauma patients over time and their relationship with infectious complications remain unknown.

This prospective observational study explores how the antibacterial functions of circulating neutrophils and their subsets in the first weeks after severe trauma relate to subsequent infectious complications. We analyzed activation marker expression, phagocytosis and phagolysosomal acidification as well as bacterial killing capacity over time in severely injured trauma patients, comparing outcomes between those who developed infectious complications and those who did not.

Methods

Study design and patient selection

We performed a prospective observational cohort study at a level one trauma center (UMC Utrecht; The Netherlands). Severely injured trauma patients were screened for eligibility according to the inclusion and exclusion criteria listed in Table 1. All included patients or their next of kin provided written informed consent. Blood samples from six healthy volunteers (four females, two males; age 25–32 years) were obtained via the Mini Donor Service in the UMCU. Volunteers reported no illness at the time of donation.

Table 1
Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
Severely injured trauma patients defined by having: - Severe injury: (AIS > 3) in at least 2 body regions - Physiological disturbances during hospital admission: o heart rate > 100 beats/minute and/or o systolic blood pressure < 100mmHg and/or o base excess < -6 mmol/L and/or o pH < 7.20 and/or o hemoglobin < 5.5	Present or recent (< 3 months) use of immunosuppressive medication
Admission to the MCU or ICU of the UMCU with an expected stay of at least 48 hours	Any known severe immunocompromising disease as per the International Classification of Disease [15]
Age 18–80 years	
Informed consent from patient or legal representative	

Patient characteristics

Baseline patient characteristics were extracted from electronic health records. Injury characteristics and resuscitation/surgery details, including the number of packed red blood cells (PRBCs) administered, were recorded. Data from the first 15 days post-injury were analyzed for the occurrence, timing, and type of infectious complications.

Blood sampling

Blood was sampled after trauma bay presentation within 0–12 hours (day 0), 12–24 hours (day 0.5) or 24–48 hours (day 1), with follow-up samples taken on days 2–4, 5–7, 8–12 and 13–16. When multiple measurements fell within the same window, the one closest to the midpoint was used; if no midpoint could be determined, the earliest sample was selected. Not all patients were sampled at every timepoint

due to logistical reasons (e.g. discharge, flow cytometer availability). Blood samples were collected via arterial line or venipuncture into 4 mL or 9 mL VACUETTE® sodium heparin tubes (Greiner Bio-One, Austria) and subjected to automatic and manual assays. Automatic flow cytometric assays (neutrophil phenotyping, phagocytosis and acidification) were performed on the AQUIOS CL “Load & Go” flow cytometer (Beckman Coulter, CA, USA), as previously described (10, 13), and detailed in **Supplementary Text 1**. Manual sample processing was used to perform single-cell killing assays with live *S. aureus* on FACS-sorted neutrophil populations, as described below.

Manual flow cytometric and functional assays

Blood erythrocyte lysis

Erythrocytes were removed from 9 mL whole-blood samples by lysis using an ammonium chloride-based buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4) as described in Duebel et al. (14) Following the lysis procedure, cells were washed and resuspended in ice-cold PBS²⁺ (0.32% trisodium citrate, 4 g/L human albumin) for further processing. Samples were subsequently maintained on ice.

FACS sorting of subsets

Total leukocytes were stained for 30 min at 4°C with a cocktail of fluorochrome-conjugated antibodies (**Suppl. Table 1**) and sorted on a BD FACS Aria™ III Cell Sorter at 4°C using an 85 µm nozzle. The neutrophil population was gated and sorted according to Duebel et al. (14), and neutrophil subsets (progenitor, CD16^{low}-banded, mature and CD62L^{low}-hypersegmented) were sorted based on differential CD16 and CD62L expression (**Suppl. Figure 2**). All sorted populations were collected in separate tubes containing PBS²⁺.

Single-cell killing assay

Neutrophil killing was assessed as recently described by Duebel et al. (14) Briefly, sorted neutrophils were incubated with serum-opsonized GFP-expressing *S. aureus* MW2 at a multiplicity of infection (MOI) of 3. GFP-expressing *S. aureus* MW2 were cultured as previously described (15–17) After incubation, neutrophils were gated and GFP-positive cells were identified by FACS (**Suppl. Figure 3**). The proportion of GFP-positive neutrophils and the MFI of GFP of this population were recorded. As previously shown by Duebel et al. (14), GFP MFI correlates with the number of bacteria internalized per neutrophil. We confirmed this correlation in the sorted neutrophil subsets used for downstream assays (**Suppl. Figure 4**).

The GFP-positive neutrophils were single-cell sorted by FACS into black Falcon® 384-well Optilux plates (Corning, NY, USA), with each well containing 40 µL of medium composed of HEPES³⁺ (20mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid buffer, 132 mM NaCl, 6 mM KCl, 1mM MgSO₂, 1.2mM

KH₂PO₄, 1.0 mM CaCl₂, 5 mM glucose, 5 mg/mL human albumin [Sanquin, the Netherlands]; pH 7.4; osmolarity 280–300 mOsm) supplemented with 40% human pooled serum (Sanquin, The Netherlands). Sorted plates were incubated in a FLUOstar Omega plate reader (BMG Labtech, France) to measure GFP signal over time. Wells with increasing GFP signal indicated failure to kill bacteria, whereas wells without signal indicated successful killing. Plates were scanned hourly for at least 100 hours, and the proportion of bacterial killing was calculated for each neutrophil subset.

Statistical analyses

Patient characteristics were compared between the patients who did and did not develop infectious complications using Mann-Whitney test or Chi-square test. Data of sorted neutrophil populations were compared over time and with controls using generalized estimating equations (GEE) in SPSS Statistics v27.0 (IBM, NY, USA). This was also applied in the analysis of the killing assays of neutrophil subsets. Phagocytosis and bacterial killing were further analyzed by comparing ≤ 3 days versus ≥ 6 days post-trauma. Neutrophil subsets were compared using a mixed-effects model (REML) with Fisher's LSD test, whereas infection versus no-infection group comparisons were made by Kruskal-Wallis with Dunn's test. All statistical analyses and data visualization were performed using GraphPad Prism software v9 (GraphPad Software Inc., CA, USA). Data were presented as median (IQR), mean ($\pm$ SD), or individual data points.

Results

Patient characteristics and infection outcomes

A total of 15 male patients were included in this study with a median age of 42 (IQR: 26–61) (Table 2). Eight patients (53%) developed at least one infectious complication within 15 days post-trauma. Inflicted injuries were primarily due to blunt trauma mechanisms. Age, sex, mechanism of injury, Injury Severity Score (ISS), the day of the first surgical procedure and the number of surgical procedures performed did not significantly differ between both groups. Each patient underwent index surgery on the same day as the traumatic injury (day 0).

Table 2

Baseline and outcome characteristics during the first 15 days after trauma of all included patients. Data are shown as n (%) or median (IQR). Day 0 is defined as the day of traumatic injury. M = male, F = female, ISS = Injury Severity Score. Statistical differences were tested between the infection and no-infection group using a Mann-Whitney test or a Chi-square test. Statistical significance was defined as $P < 0.05$, ns = not significant.

Total	All patients	Infectious complications	No infectious complications	P-value
	15	8 (53%)	7 (47%)	-
Age	42 (26–61)	42 (31–68)	45 (22–61)	ns
Sex (M/F)	15/0	8/0	7/0	ns
ISS	34 (34–41)	36 (34–44)	34 (18–41)	ns
Mechanism of injury				
<i>Traffic accident</i>	9 (60%)	5 (63%)	4 (57%)	ns
<i>Fall from height</i>	4 (27%)	3 (38%)	1 (14%)	ns
<i>Gunshot</i>	1 (7%)	0	1 (14%)	ns
<i>Crush incident</i>	1 (7%)	0	1 (14%)	ns
Day of first surgical procedure	0 (0–0)	0 (0–0)	0 (0–0)	ns
Number of surgical procedures	2 (2–3)	3 (2–3)	2 (2–4)	ns
Day of first infectious complication	-	7 (4–7)	-	-
Number of infectious complications				
<i>1</i>	-	3 (38%)	-	-
<i>2</i>	-	3 (38%)	-	-
<i>3</i>	-	2 (25%)	-	-
Type infectious complication				
<i>Pneumonia</i>	-	6 (75%)	-	-
<i>Bacteremia</i>	-	5 (63%)	-	-
<i>Empyema</i>	-	2 (25%)	-	-
<i>Meningitis</i>	-	1 (13%)	-	-
<i>Wound infection</i>	-	1 (13%)	-	-

Total	All patients	Infectious complications	No infectious complications	P-value
	15	8 (53%)	7 (47%)	-
Mortality	0 (0%)	0 (0%)	1 (14%)	ns

Patients who developed infectious complications had a median time to first infection of 7 days (IQR 4–7), with the earliest onset at day 4. The number of infectious complications per patient varied from 1 to 3 and included pneumonia (75%), bacteremia (63%), empyema (25%), meningitis (13%) and wound infection (13%). The timing and cumulative number of each infectious complication over time is visualized in Fig. 1.

CD16^{low}-banded blood cell counts peak first, followed by CD62L^{low}-hypersegmented cells

Counts of total white blood cells (WBC), neutrophils and neutrophil subsets (progenitor, CD16^{low}-banded, mature and CD62L^{low}-hypersegmented neutrophils) were measured for each patient over time (Fig. 2A). Figure 2B shows the counts of each circulating cell population separately, distinguishing between patients with and without infectious complications.

The WBC counts of all trauma patients were elevated compared to controls values (mean: $6.9 \times 10^6/\text{mL}$; SD: 1.8×10^6) throughout the 15-day period, with peaks at days 0–1 (mean: $19.2 \times 10^6/\text{mL}$; SD: 13.6×10^6 ; $P = 0.001$) and days 8–12 (mean: $18.5 \times 10^6/\text{mL}$; SD: 4.8×10^6 ; $P < 0.001$). Total neutrophil and mature neutrophil counts followed a similar pattern, while other subsets showed distinct dynamics. CD16^{low}-banded neutrophil counts were significantly increased at days 0–1 (mean: $4.3 \times 10^6/\text{mL}$; SD: 3.4×10^6) compared to controls (mean: $0.02 \times 10^6/\text{mL}$; SD: 0.02×10^6 ; $P < 0.001$) and subsequently decreased. As banded cell counts declined, progenitor counts increased at 5–7 days (mean: $3.3 \times 10^5/\text{mL}$; SD: 2.6×10^5) and remained elevated compared to controls (mean: $0.81 \times 10^5/\text{mL}$; SD: 0.57×10^5 ; $P < 0.001$). CD62L^{low}-hypersegmented neutrophil counts were initially low, began to rise around days 5–7 and peaked between days 8–12 (mean: $3.7 \times 10^6/\text{mL}$; SD: 4.5×10^6) compared to controls (mean: $0.62 \times 10^6/\text{mL}$; SD: 0.52×10^6 ; $P = 0.011$).

Decreased expression of CD10, increased CD64 expression on days 2–4

Surface marker expression on the total neutrophil population was assessed over time in infection and no-infection groups (Fig. 2C). Activation marker CD11b showed minimal changes, with a slight but significant dip at days 2–4 (mean: 2.8×10^5 AU; SD: 1.2×10^5) versus control (mean: 4.0×10^5 AU; SD: 1.3×10^5 ; $P = 0.039$). Maturation marker CD10 decreased significantly early after trauma, reaching a nadir at days 2–4 (mean: 0.8×10^4 AU; SD: 0.67×10^4) versus control (mean: 4.6×10^4 AU; SD: 2.7×10^4 ; $P < 0.001$) and partially recovered thereafter. Neutrophil CD64 expression was elevated post-trauma,

reaching a peak at days 2–4 (mean: 7.1×10^3 AU; SD: 4.9×10^3) versus control (mean: 1.1×10^3 AU; SD: 0.41×10^3 ; $P < 0.001$) before gradually declining.

Phagocytosis and acidification over time

Figure 3 shows the percentages of PF520+ cells, PF520 signal intensity and acidification ratio across timepoints. The percentage of PF520+ neutrophils was transiently reduced at days 0-1 (mean: 68%; SD: 14) versus control (mean: 81%; SD: 9.1; $P = 0.025$) but normalized thereafter (**Figure 3A**). Despite this, PF520 signal intensity was significantly elevated at all post-trauma timepoints, peaking at days 5-7 (mean: 7.2×10^4 AU; SD: 4.0×10^4 ; $P < 0.001$ vs. control mean: 0.97×10^4 AU; SD: 0.15×10^4), reflecting increased bioparticle content per neutrophil (**Figure 3B**). The pHrodo/PF520 ratio was significantly reduced at all timepoints post-trauma reaching a nadir at days 5-7 (mean: 1.1 ; SD: 0.25; $P < 0.001$ vs. control mean: 3.3; SD: 0.48), indicating persistently impaired intracellular acidification capacity (**Figure 3C**).

Phagocytosis and bacterial killing of *S. aureus* over time

All trauma patients vs. control

Firstly, we assessed the percentage of neutrophils that phagocytosed one or more live bacteria, the intensity of the GFP signal within neutrophils, and the percentage effectively killing phagocytosed bacteria over time (Fig. 4A). The percentage of phagocytosing neutrophils did not differ significantly over time or from controls. Consistent with the automated bioparticles analysis above, GFP signal intensity was overall increased in trauma patients compared to controls. This is a surrogate marker for the number of bacteria internalized per neutrophil, as demonstrated for each sorted subset in **Supplementary Fig. 4**. Bacterial killing capacity was overall decreased in trauma patients, and reaching significance at days 8–12 (mean: 51%; SD: 17) versus control (mean: 68%; SD: 8.1; $P = 0.002$).

Comparison of infection vs. no-infection patient

Secondly, we compared phagocytosis and bacterial killing capacities between patients with and without infectious complications, grouped into early (≤ 3 days) and late (≥ 6 days) periods (Fig. 4B). Phagocytosis rates and GFP signal intensities did not differ between groups at either time points. However, during the early period, patients who later developed infections (median: 50%; IQR: 38–67) showed a significantly lower killing capacity compared to those who did not (median: 73%; IQR: 64–79; $P = 0.0341$) and to controls (median: 69%; IQR: 63–74; $P = 0.045$). In the late period, the infection group (median: 50%; IQR: 41–62) showed a similar though non-significant trend versus the no-infection patients (median: 64%; IQR: 48–87; $P = 0.201$), but did still significantly differ from controls (median: 69%; IQR: 63–74; $P = 0.034$), while the no-infection patients did not.

Differences between neutrophil subsets

Thirdly, we assessed functional capacities across circulating neutrophil subsets over time after trauma (Fig. 4C). The percentage of phagocytosis and GFP signal intensity showed a transient increase followed by a decline over time in the mature and CD62L^{low}-hypersegmented subsets, while bacterial killing was not significantly influenced by time in any individual subset. Subset-specific phagocytosis and killing were then compared between early (≤ 3 days) and late (≥ 6 days) periods (Fig. 4D). Phagocytosis was comparable across subsets in the early period but diverged later, with progenitors showing a significant decrease (mean: 16%; SD: 6.6) compared to other subsets. The mature subset showed higher phagocytosis percentages in the early (mean: 77%; SD: 7.0) versus late period (mean: 71%; SD: 6.3; $P = 0.040$). GFP signal intensity followed a similar pattern: the mature subset had a lower GFP signal in the late period, and progenitors exhibited a significantly decreased signal at ≥ 6 days. Subset differences were most pronounced for bacterial killing: in the early period, the CD62L^{low}-hypersegmented neutrophils showed the lowest killing capacity (mean: 49%; SD: 17%), significantly below the mature subset (mean: 58%; SD: 15; $P = 0.013$) and the CD16^{low}-banded subset (mean: 65%; SD: 13; $P < 0.001$). In the late period, progenitors exhibited the highest killing capacity (mean: 67%; SD: 7.9) among the three subsets, particularly compared to CD62L^{low}-hypersegmented neutrophils (mean: 55%; SD: 16; $P = 0.005$), which remained the lowest.

Discussion

In this proof-of-principle study, we investigated the antibacterial function of circulating neutrophils and their subsets over the first 15 days after severe traumatic injury, and explored how this related to infectious complications. Blood cell counts and surface marker expression followed patterns consistent with prior reports (6, 7, 10, 18). Severely injured patients showed an increased phagocytic capacity (bacteria per cell) but diminished killing compared to controls. Killing capacity was further reduced in patients who developed infectious complications, with this deficit already present within the first 3 days – preceding the majority of infections, which occurred toward the end of the first week. Functional differences were also observed between subsets: CD16^{low}-banded neutrophils demonstrated enhanced bacterial killing of *S. aureus*, CD62L^{low}-hypersegmented neutrophils a decreased killing capacity. Together, these findings suggest intricate functional shifts in the circulating neutrophil population following severe traumatic injury.

The antibacterial capacity of neutrophil subsets in acute inflammation has been described before by Leliefeld et al. (12), who assessed bacterial containment in volunteers undergoing experimental endotoxemia (LPS challenge). Our findings were complementary: CD16^{low}-banded neutrophils showed the highest killing capacity, followed by mature neutrophils, with CD62L^{low}-hypersegmented neutrophils showing the lowest. This suggests that neutrophil subsets generated in diverse acute inflammatory contexts share comparable functional characteristics. We additionally assessed the killing capacity of progenitor neutrophils, which are released into the circulation during the sub-acute phase after severe

trauma. Unexpectedly, progenitor neutrophils demonstrated a high killing capacity comparable to CD16^{low}-banded neutrophils, but exhibited significantly lower phagocytosis. One possible explanation is that fewer phagocytosed bacteria per cell allows antibacterial machinery to concentrate on a smaller number of phagolysosomes, resulting in more effective killing per bacterium (14).

These functional differences between subsets were largely independent of the sampling time after trauma, whereas subset counts varied considerably over time. CD16^{low}-banded and mature neutrophils predominated early after trauma, while progenitors and CD62L^{low}-hypersegmented neutrophils increased toward the end of the first week alongside persistent mature neutrophils. This suggests that both the intrinsic quality of the subsets and their quantitative changes over time shape the functional composition of the overall neutrophil compartment. Notably, the rise of progenitors and CD62L^{low}-hypersegmented neutrophils – which are less effective in antibacterial defense – and the concurrent decline of the highly antibacterial CD16^{low}-banded subset coincide with the peak occurrence of infectious complications. However, subset kinetics appeared similar in both infection and no-infection groups (13). Together, this suggests that additional processes beyond subset kinetics contribute to infectious complications after severe injury.

Beyond subset-level differences, broader functional changes in the whole neutrophil population in response to DAMPs due to tissue damage may also drive the onset of infectious complications. We observed an initial leukocytosis upon severe injury that rapidly declined within hours to days following injury, consistent with previous research (7, 19, 20). This likely reflects early bone marrow mobilization followed by neutrophil migration toward damaged tissue – the extent of which correlates with injury severity and DAMP release (19, 21–23). Importantly, activation and extravasation enhance neutrophil function essential for bacterial killing such as NADPH oxidase activity (24). Thus, neutrophils remaining in the circulation may represent a functionally inferior subset, unable to sufficiently respond to inflammatory signals (6). In addition, DAMP release and induction of chemokines and cytokines have been previously associated with immune suppression in trauma patients (25). Consistent with this, deficits in phagocytosis, acidification were already observed at day 0–1 post-trauma in all patients and killing capacity was already decreased within three days in patients who later developed infections, compared to controls and the no-infection group. These changes preceded the first infectious complications. Impaired intracellular killing preceding infection has also been found in critically ill ICU patients and has been linked to elevated levels of immunomodulatory cytokine IL-10 (26). It is likely that extensive release of DAMPs can elicit an immunosuppressive cascade that may be more pronounced in patients developing infections.

Toward the end of the first week, CD62L^{low}-hypersegmented neutrophils appear in blood in increasing numbers, potentially decreasing the overall antibacterial functionality of the circulating pool (7). These cells display a more activated phenotype (CD62L^{low}CD11b^{high}) possibly reflecting activation in circulation or reverse-transmigration from damaged tissues (11, 27–29). We tested the latter by assessing reverse transmigration markers CD54 and CXCR1 (29), but found no distinct

CD54^{high}CXCR1^{low} population and thus no significant differences between mature and hypersegmented subsets (**Suppl. Figure 5**). While these cells have been suggested to represent older or “used” neutrophils ready to be cleared, LPS-challenge in humans found no difference in cellular age (30). In fact, these cells have been proposed to constitute a functionally distinct neutrophil subset specialized in immune modulation rather than bacterial killing (11, 12, 30, 31). Supporting an alternative role in tissue repair, Bastian et al. showed that trauma patients with non-union fractures lacked the second week neutrophil influx – which contain a large proportion of CD62L^{low} cells – seen in normal healers (32). Regardless of their alternative functions, the predominance of the “less-antibacterial” CD62L^{low}-hypersegmented neutrophils during the second week following trauma may increase the likelihood of pathogen invasion and systemic dissemination via Trojan Horse-like mechanisms (33). This risk is further compounded by compromised tissue barriers due to injury and medical interventions such as drains and/or intravenous access (34–36).

To explain the observed decrease in bacterial killing capacity, we examined phagocytosis parameters more closely. After trauma, the number of ingested bioparticles per cell increased while the acidification ratio decreased. A similar effect was also seen for phagocytosis of live GFP expressing *S. aureus*, especially during the first days after trauma. Notably, higher bacterial ingestion was associated with lower killing capacity, consistent with previous reports that a higher bacteria-to-cell ratio impairs killing efficiency and that ingestion of more bacteria per neutrophil diminishes intracellular killing capacity (14, 37). Increased phagocytosis has also been linked to several cytokine combinations prominent in sepsis and trauma (38, 39). Together, this suggests that the “overeating” observed here may overwhelm the intracellular killing mechanisms – particularly phagolysosomal acidification – potentially contributing to the increased infection risk observed in trauma patients.

This study has several limitations. Incomplete sampling at designated time points – due to logistical reasons, patient status, or early discharge – resulted in missing values and reduced statistical power, which we addressed by bundling data into day ranges. Additionally, the inherent heterogeneity of severely injured patients is a limitation: individuals sustained varied injury types and mechanisms, and trauma severity is highly variable. Currently available scoring systems such as the ISS do not capture the extent of DAMP released or the resulting immune response (40, 41), making precise quantification of tissue damage and its biological consequences challenging.

Conclusion

Our results show clear alterations in the antibacterial functionality of circulating neutrophils following severe injury, initiated immediately after trauma and persisting for at least two weeks. These shifts reflect changing cell counts, inflammatory cues and the balance between functionally distinct neutrophil subsets, potentially contributing to the infectious complications seen at the end of the first week. Validation in larger patient cohorts is needed, but the early neutrophil response after trauma could serve as a predictor of infectious complications and a target for improving patient outcomes.

Declarations

Ethics approval and consent to participate

The study protocol and its amendments were approved by the NedMec Medical Research Ethics Committee (reference no. NL43279.041.13) or the Biobank Research Ethics Committee (TCBio) (reference no. 24-231). The Mini Donor Service received positive approval from the TCBio of the UMCU (reference no. 07-125/C). This study was conducted in accordance with the declaration of Helsinki. All included participants or their next of kin provided written informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The data supporting the findings of this study are not publicly available due to participant privacy considerations but are available from the corresponding author upon reasonable request.

Competing interests

The authors have no competing interests as defined by BMC, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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Authors' contributions

Conception and design: LVD, SHB, EJF, CWA, LPHL, KJPW, LK, NV, FH

Acquisition, analysis, or interpretation of data: LVD, SHB, EJF, CWA, LPHL, KJPW, LK, NV, FH

Drafting of the article: LVD, SHB, NV

Critical revision of article for intellectual content: LVD, SHB, EJF, CWA, LPHL, KJPW, LK, NV, FH

Statistical analysis: LVD, SHB

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Figures

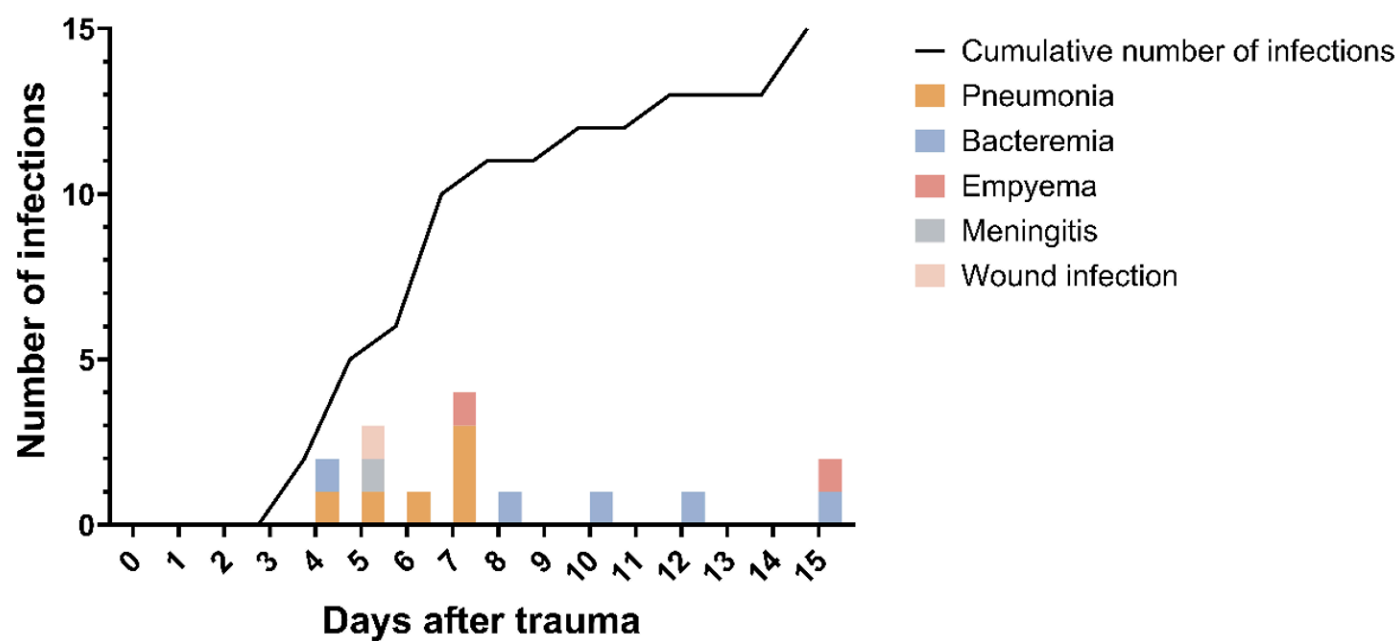


Figure 1

Cumulative number and type of infectious complications over time after severe traumatic injury. The onset of categorised infectious complications is shown per day in stacked bars. Multiple infectious complications could occur on the same day and/or in the same patient. Selective digestive decontamination (SDD) was administered according to institutional intensive care unit (ICU) protocols. The cumulative number of infections that occurred over time is shown as a black line.

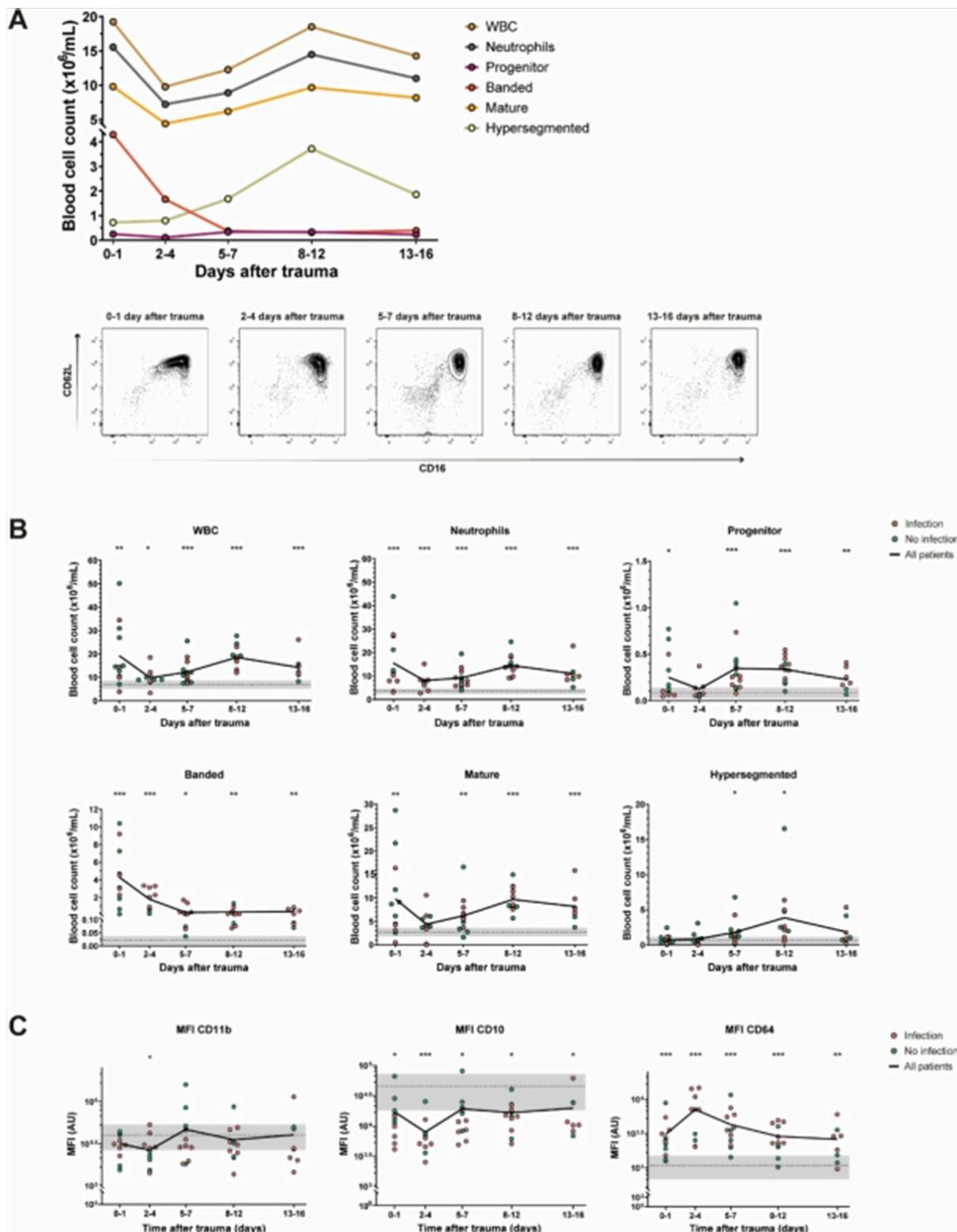


Figure 2

Blood cell counts and surface marker expression over time after severe traumatic injury. Overview of white blood cell counts (WBC) and neutrophil subsets (progenitor, CD16^{low} -banded, mature and $\text{CD62L}^{\text{low}}$ -hypersegmented) over time, shown as means per time point (**A**) or with individual data points, color-coded for infection status (**B**). A representative example of CD16 and CD62L plots over time in a trauma patient is also shown in panel A. Expression of surface markers CD11b , CD10 and CD64 on neutrophils with individual

data points, color-coded for infection status (C). Normal values derived from control blood are visualized as a horizontal dotted line (mean) and grey area (SD). Statistical significance was tested using Generalized Estimating Equations (GEE) to compare mean values of the total patient population to controls at each time point. Statistical significance is represented as *: $P < 0,05$; **: $P < 0,01$; ***: $P < 0,001$, only significant effects are shown.

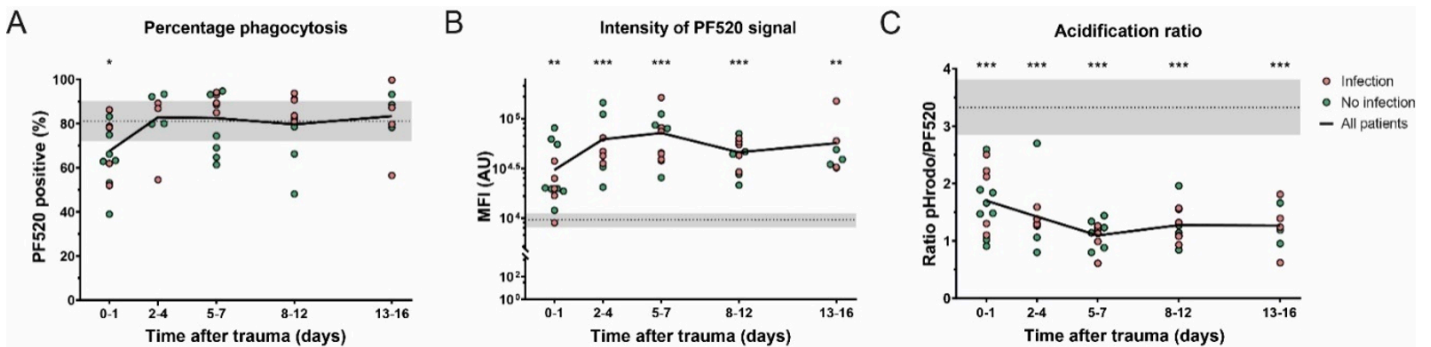


Figure 3

Phagocytosis and acidification capacity of neutrophils over time after severe traumatic injury. The percentage of neutrophils that phagocytosed *S. aureus* bioparticles (PF520+ neutrophils) (A). The intensity of the PF520 signal within phagocytosing neutrophils, used as a surrogate for the amount of ingested particles per cell (B). The ratio of the median fluorescent intensity (MFI) of pHrodo over the MFI of PF520 (to correct acidification signal for amount of phagocytosed particles) (C). Data are visualized as means per time point with individual data points, color coded for infection status. Normal values derived from control blood are visualized as a horizontal dotted line (mean) and grey area (SD). Statistical significance was tested using Generalized Estimating Equations (GEE) to compare mean values of the total patient population at each time point to controls. Statistical significance is represented as *: $P < 0,05$; **: $P < 0,01$; ***: $P < 0,001$, only significant effects are shown.

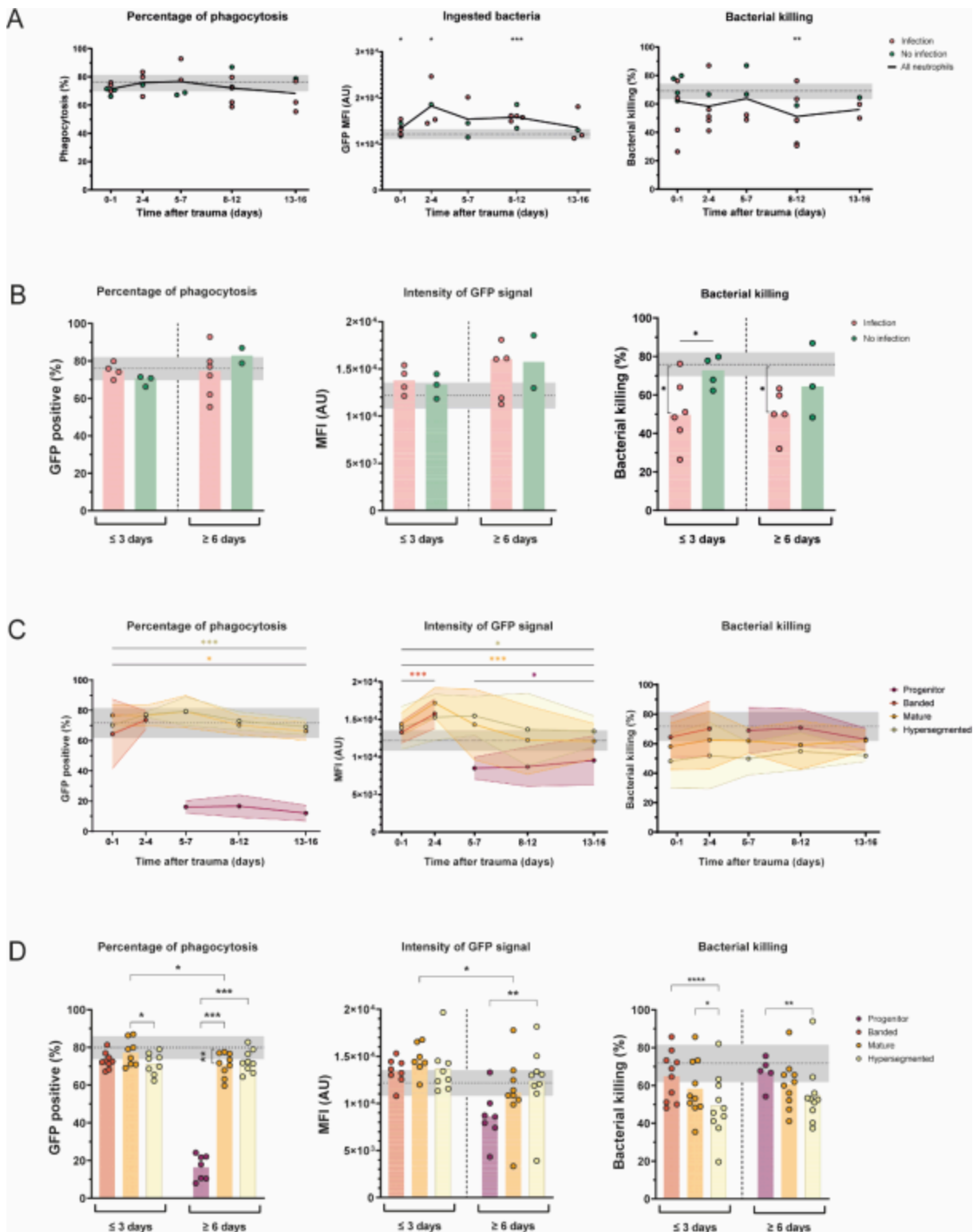


Figure 4

Capacity of neutrophils to phagocytose and kill live *S. aureus* after severe traumatic injury. Three functional parameters were assessed: the percentage of neutrophils that phagocytosed one or more bacteria, the intensity of the GFP signal after phagocytosis (a surrogate for the amount of ingested bacteria per cell) and the percentage of neutrophils that effectively killed phagocytosed bacteria. Data are visualized as means per timepoint with individual data points, color-coded for infection status. Statistical significance

was tested using Generalized Estimating Equations (GEE) to compare values at each timepoint to control values in the total patient population **(A)**. Kruskal-Wallis with Dunn's test, comparing infection and no-infection groups to each other and to control at ≤ 3 and ≥ 6 days post-trauma **(B)**. GEE to test the overall effect of time per subset **(C)**. A mixed-effects model (REML) with Fisher's LSD tests to compare neutrophil subsets within and between ≤ 3 and ≥ 6 days and with control values **(D)**. Data are visualized as medians with individual data points (B), means $\pm$ SD per subset (C), or means with individual data points (D). Normal values for the total neutrophil population (A, B) or mature neutrophils (C, D) derived from control blood are visualized as a horizontal grey area. Statistical significance is represented as *: $P < 0,05$; **: $P < 0,01$; ***: $P < 0,001$, only significant effects are shown.

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