



## Mitochondria in human neutrophils mediate killing of *Staphylococcus aureus*

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### ABSTRACT

**Background:** Neutrophils play a role in innate immunity and are critical for clearance of *Staphylococcus aureus*. Current understanding of neutrophil bactericidal effects is that NADPH oxidase produces reactive oxygen species (ROS), mediating bacterial killing. Neutrophils also contain numerous mitochondria; since these organelles lack oxidative metabolism, their function is unclear. We hypothesize that mitochondria in human neutrophils contribute to the bactericidal capacity of *S. aureus*.

**Methods:** and Findings: Using human neutrophils isolated from healthy volunteers (n = 13; 7 females, 6 males), we show that mitochondria are critical in the immune response to *S. aureus*. Using live-cell and fixed confocal, and transmission electron microscopy, we show mitochondrial tagging of bacteria prior to ingestion and surrounding of phagocytosed bacteria immediately upon engulfment. Further, we demonstrate that mitochondria are ejected from intact neutrophils and engage bacteria during *vital NETosis*. Inhibition of the mitochondrial electron transport chain at Complex III, but not Complex I, attenuates *S. aureus* killing by 50 ± 7%, comparable to the NADPH oxidase inhibitor apocynin. Similarly, mitochondrial ROS scavenging using MitoTEMPO attenuates bacterial killing 112 ± 60% versus vehicle control. Antimycin A treatment also reduces mitochondrial ROS production by 50 ± 12% and NETosis by 53 ± 5%.

**Conclusions:** We identify a previously unrecognized role for mitochondria in human neutrophils in the killing of *S. aureus*. Inhibition of electron transport chain Complex III significantly impairs antimicrobial activity. This is the first demonstration that *vital NETosis*, an early event in the antimicrobial response, occurring within 5 min of bacterial exposure, depends on the function of mitochondrial Complex III. Mitochondria join NADPH oxidase as bactericidal ROS generators that mediate the bactericidal activities of human neutrophils.

### 1. Introduction

Neutrophils are the most abundant white blood cell, comprising up to 70% of all leukocytes. Neutrophils are the main cellular defense against bacterial infections and are exceptionally important for combating *Staphylococcus aureus* (*S. aureus*), the most common cause of skin infections in North America [1] and a leading cause of lethal systemic infections, like sepsis. The incidence of Staphylococcal bacteremia is 20–50 cases/100,000 population per year and studies indicate a high mortality rate (10%–30%) in infected patients [2]. More recently, the annual number of US deaths due to *S. aureus* sepsis was reported to be 20,000 people [3]. The importance of neutrophils in combatting this

pathogen is highlighted by the observation that patients with deficiencies in neutrophil function, or with reduced neutrophil numbers due to chemotherapy for cancer, are particularly susceptible to chronic and recurrent *S. aureus* infections [4,5]. In healthy individuals, neutrophils rapidly migrate out of the vasculature and are the first immune cell to arrive at the local nidus of infection. Guided by chemotactic factors derived from both pathogens and infected host tissue, neutrophils attempt to eradicate the pathogen through an armamentarium of defenses, including intracellular production of reactive oxygen species (ROS), antimicrobial peptides/proteins and the production of Neutrophil Extracellular Traps (NETs) [6].

Early literature suggested that neutrophils initially phagocytose

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bacteria and subsequently use intracellular ROS and proteases to kill bacteria in the phagolysosome [7]. The production of ROS by neutrophils has classically been attributed to the function of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [8]. Subsequently, it was recognized that as a last resort to stop the spread of the pathogen, neutrophils deploy NETs [9]. In this process, neutrophils lyse and eject their genetic material forming extracellular DNA-rich net-like structures, that are decorated with histones and granular proteins capable of entrapping exogenous bacteria, a process dubbed 'lytic NETosis'. A defining component of lytic NETosis is the requirement for rupture of the neutrophil's plasma membrane and neutrophil death. However, *Staphylococcus* bacteria also secrete numerous toxins capable of killing human neutrophils, including haemolysins, leukotoxins and phenol-soluble modulins [10], causing pathogen-induced immune cell lysis. The similarities between lytic NETosis and pathogen-induced immune cell lysis make these two phenomena difficult to distinguish and have led to controversy as to whether lytic NETosis is an active antimicrobial defense mechanism or just a manifestation of neutrophil death, caused by *Staphylococcus*-secreted toxins [11]. Recent evidence suggests that NETosis may also occur in the absence of neutrophil lysis, which would favor it serving an active antimicrobial role. This form of NETosis is termed 'vital NETosis' and it allows persistence of neutrophil function after NET formation [12–14].

The potential role(s) of neutrophil mitochondria in combatting bacterial infections has received little attention. This is likely due to the limited oxidative metabolic activity exhibited by neutrophil mitochondria [15], which mistakenly suggests that these organelles are relatively inactive or unimportant. A potential role for the neutrophil mitochondria in the immune response was described by Yousefi et al. They demonstrated the presence of mitochondrial DNA in NET-like structures released from human neutrophils [14] and human eosinophils [16]. However, this group also identified major knowledge gaps, including uncertainty as to where energy for NETosis is generated. Mitochondrial DNA replication has also been implicated in the neutrophil's immune response. Zhou et al. report that knockout of DNA polymerase gamma, POLG, (the mitochondria's DNA polymerase) drastically decreased neutrophil motility, and showed that neutrophil motility is directly correlated with mitochondrial ROS production in zebrafish [17]. These observations in neutrophils and other leukocytes indicate that the mitochondria may be participants in the innate immune response.

Here, we study the role of mitochondria in neutrophils, isolated from healthy human volunteers, on phagocytosis, ROS production, ejection of NETs, and bacterial killing. To explore the functions of neutrophil mitochondria, we employ a live-cell interrogation strategy to visualize neutrophils and their mitochondria while simultaneously imaging *S. aureus* expressing green fluorescent protein (GFP). We report, for the first time, that in healthy human neutrophils, functional neutrophil mitochondria contribute importantly to the antimicrobial defense against *S. aureus* infection. This study focused on replication of physiologic mechanisms of neutrophil activation and thus we did not stimulate neutrophils with non-physiologic agents like phorbol myristate acetate (PMA) or a calcium ionophore. We found that while inhibition of neutrophil mitochondria at various mitochondrial targets increased the number of engulfed *S. aureus*, only inhibition of electron transport chain Complex III impaired infection-induced mitochondrial ROS formation, NET formation (both vital and lytic NETosis), and bacterial killing. While identifying a key role for mitochondria in killing *S. aureus*, several noncanonical mitochondrial functions were identified, including expulsion of mitochondria from the neutrophil and preparatory tagging of bacteria, which appears to be a prelude to phagocytosis.

## 2. Materials & methods

Detailed methods are provided in the Supplementary Information. All experiments were approved by Queen's University HSREB (protocols #6025141 and #6029805), or University of Calgary (protocol REB15-

1943) and informed consent was obtained from all study participants. All reagents were purchased from Sigma (Oakville, Ontario, Canada) unless otherwise specified.

### 2.1. Study volunteers

Healthy volunteers were sought from the Queen's University (or University of Calgary) communities. Volunteers were provided with a consent form outlining procedures and relevant exclusion criteria, namely donor age of <45 years [18]. Volunteers who were included in this study remained anonymous to all members of the study team, except the consent form administrator and phlebotomist. Only age and sex of donors was reported to study team. The volunteer population for studies conducted at Queen's University is outlined in Table 1.

### 2.2. Statistics

Data are expressed as mean  $\pm$  SEM, relative to vehicle control where stated. Statistical analyses of data include descriptive statistics to determine if standard deviations are equal, identification of outliers using the ROUT method. Tests for statistical significance include Shapiro-Wilk normality test, unpaired *t*-test, and mixed effects ANOVA adjusted for multiple comparisons, as appropriate. All analyses were performed using Graphpad Prism Version 9.1.12 (San Diego, California, USA). A *p* value < 0.05 is considered statistically significant. *P* values are reported directly in figure panels.

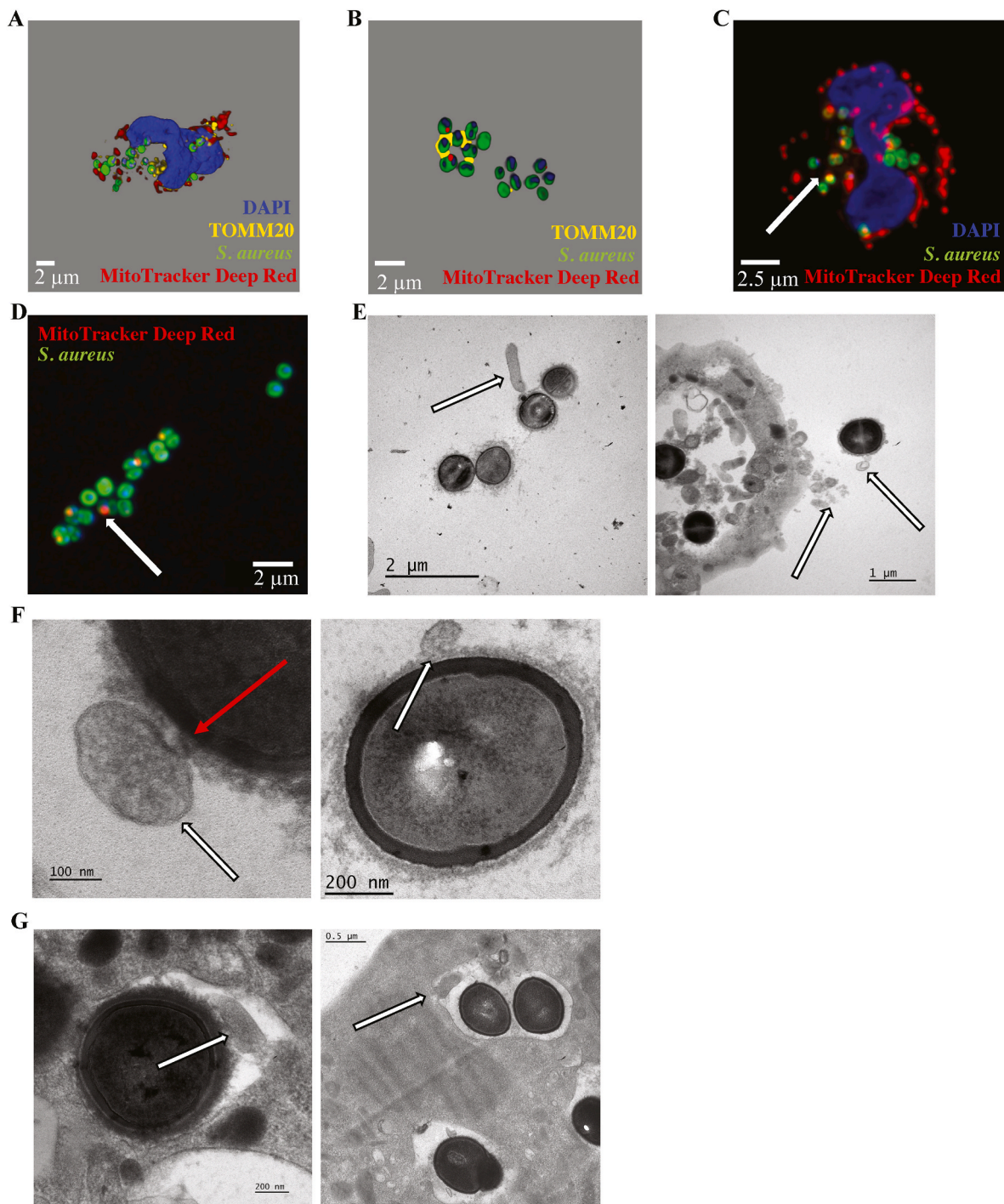
## 3. Results & discussion

### 3.1. Neutrophil mitochondria 'tag' *S. aureus* for phagocytosis

To investigate the role of mitochondria in bacterial sensing, human neutrophils were imaged live prior to and after inoculation with *S. aureus* (S1A Fig.). Mitochondria were observed to interact with extracellular bacteria even before engulfment (S1–S3 Videos) and in many cases, the neutrophil 'tagged' the extracellular bacteria with a small portion of a mitochondrion. Our conclusion that these tags were indeed a mitochondrion, or portion of a mitochondrion, was demonstrated by three lines of evidence. First, the composition of the mitochondrial tag included a mitochondrial-specific protein, translocase of the outer mitochondrial membrane (TOMM20). Second, the tag took up MitoTracker Deep Red (MTDR), a membrane-potentiometric dye that fills the mitochondrial matrix in active, polarized mitochondria. TOMM20- and MTDR-containing tags were seen both on engulfed bacteria inside neutrophils (Fig. 1A and C) and on extracellular bacteria (Fig. 1B and D). Third, transmission electron microscopy of neutrophils inoculated with bacteria confirmed the presence of mitochondria adherent to extracellular *S. aureus*, as well as mitochondrial adherence to *Staphylococcus* within phagosomes (Fig. 1E–1G). We confirmed that

**Table 1**  
Demographics of study participants.

	Total (n = 13)
Age	
<b>N</b>	13
<b>Mean (SD)</b>	31.4 (7)
<b>Median</b>	32
<b>Range</b>	23, 42
Sex, n (%)	
<b>Female</b>	7 (53.8%)
<b>Male</b>	6 (46.2%)
Race, n (%)	
<b>Asian</b>	1 (7.7%)
<b>Caucasian</b>	8 (61.5%)
<b>Middle Eastern</b>	2 (15.4%)
<b>South Asian</b>	1 (7.7%)
<b>Latinx</b>	1 (7.7%)



**Fig. 1.** Neutrophil mitochondria 'tag' *S. aureus* prior to phagocytosis. After PMN exposure to GFP-labelled *S. aureus*, extracellular bacteria are 'tagged' by neutrophil mitochondria. **A)** Vehicle-treated PMN after 30-min exposure to *S. aureus* showing engulfed bacteria with yellow 'tags' (mitochondria fragments). **B)** Extracellular *S. aureus* exhibiting mitochondrial tagging by mitochondrial TOMM20 (yellow). Bacteria (green) and nuclei (blue) are also evident. **C)** MitoTracker Deep Red (MTDR)-loaded PMN after 30-min bacterial exposure showing engulfment of MTDR-tagged bacteria (mitochondrial tag indicated by white arrow). **D)** Extracellular *S. aureus* with a MTDR mitochondrial tag. For immunofluorescence: **A, B** - mitochondrial TOMM20 (yellow), mitochondrial matrix (MitoTracker Deep Red-red), bacteria (green), nuclei (blue); **C, D** - mitochondrial matrix (MitoTracker Deep Red-red), bacteria (green), nuclei (blue). **E)** TEM image series demonstrating mitochondrial tagging of *S. aureus* (macro view, mitochondrial fragment indicated by white arrow). **F)** TEM image series of extracellular *S. aureus* (bacterial edge indicated by red arrow, left) with a mitochondrial fragment serving as a 'tag' (mitochondrial fragment indicated by white arrows). **G)** TEM image series demonstrating association of mitochondria with engulfed bacteria (mitochondria indicated by white arrows). Representative TEM images selected from  $n = 4$  patients. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

bacterial tagging by mitochondria does not reflect off-target staphylococcal staining by the immunofluorescence TOMM20 antibody or MTDR. The possibility of nonspecific IF staining by TOMM20 antibody or secondary antibodies was eliminated because bacteria were incubated with human plasma for all experiments, blocking all free protein A and other staphylococcal proteins that are known to bind the Fc tail region of antibodies. Our method of MTDR immunofluorescent labelling protocol excluded nonspecific dye adherence to bacteria. Specifically, neutrophils were loaded with MTDR and rigorously washed prior to inoculation with bacteria, removing any extramitochondrial dye. Thus, the only source of MTDR was from mitochondria within neutrophils.

### 3.2. Neutrophil mitochondria actively participate in phagocytosis and NETosis

To examine a possible role for neutrophil mitochondria after initial bacterial sensing, we conducted live-cell, time-course imaging of neutrophils before and after inoculation with GFP-*S. aureus* (**S1A and S1B Figs.**). These neutrophils were loaded with MTDR and subsequently washed to remove any extracellular dye prior to addition of *S. aureus* and initiation of live-cell imaging. Neutrophil mitochondria were present at the leading edge of the neutrophil, subjacent to the site of imminent phagocytosis, as is seen in time-course confocal immunofluorescence imaging (**Fig. 2A**). In many cases, the mitochondria formed lasso-like structures that participated in bacterial engulfment, with the labelled mitochondrial membrane enveloping the bacteria that were being phagocytosed (**Fig. 2A, S1 Video**). The neutrophil response to bacterial inoculation was rapid. Neutrophils engaged with and engulfed  $10 \pm 2.3$  bacteria/neutrophil within 30 min of exposure (**Fig. 2B–2D**).

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.redox.2021.102225>.

In addition to actively participating in phagocytosis, mitochondria were also observed in the material expelled during both vital and lytic NETosis (**Fig. 3**). Vital NETosis was observed in inoculated neutrophils beginning within 5 min of bacterial exposure. Vital NETosis occurred without stimulation with PMA or other known NETosis-inducing agents (**Fig. 3A and 3B**). Vital NETosis was also imaged via TEM, revealing mitochondria within these vital NETs (**Fig. 3C**), and demonstrating that the NETs surrounded bacteria (**Fig. 3D**). By 20 min post-inoculation, a dramatic increase in staphylococcal-induced neutrophil lysis and lytic NETosis was observed (**Fig. 3E and 3F**). NET formation was quantified over 30 min via immunofluorescence (**Fig. 4A**) demonstrating a time-dependent increase in NET formation (**Fig. 4B**). Serial measurements revealed an initial rapid deployment of vital NETs, within 5 min of inoculation with *S. aureus*, followed by increasing numbers of lytic NETs by 20 min, which was accompanied by PMN lysis.

### 3.3. Inhibition of the ETC complex III, but not complex I, increases bacterial engulfment

To further demonstrate the requirement of healthy, active, mitochondria for neutrophil function, isolated neutrophils were treated with either Antimycin A (inhibitor of the  $Q_i$  site of Complex III [19]), myxothiazol (inhibitor of the  $Q_o$  site of Complex III [20]), S3QEL (a suppressor of electron leak from the  $Q_o$  site of Complex III [21]), rotenone, (inhibitor of the ubiquinone binding site of Complex I [22]), or apocynin (an NADPH oxidase [NOX] inhibitor and traditional inhibitor of neutrophil function [23]). These drugs were given 60 min prior to inoculation with bacteria (**S1C Fig.**). The mitochondrial metabolic inhibitors are also classically used to interrogate organelle health and metabolism, and to identify the site of production of mitochondrial reactive oxygen species (ROS), namely superoxide anion ( $O_2^{\bullet-}$ ), within the ETC [24–28]. Mitochondria have not classically been considered the primary source of ROS production in neutrophils [29]; rather this function is attributed to NADPH oxidase isoform 2 (NOX2), the dominant NOX isoform in neutrophils [8]. NOX2 catalyzes the redox-coupled

oxidation of NADPH and reduction of molecular oxygen ( $O_2$ ), resulting in generation of  $O_2^{\bullet-}$ . NOX2-generated ROS participate in the oxidative burst that neutrophils generate to kill engulfed bacteria [8,30]. Apocynin prevents this bacteria-killing oxidative burst and increases bacterial survival.

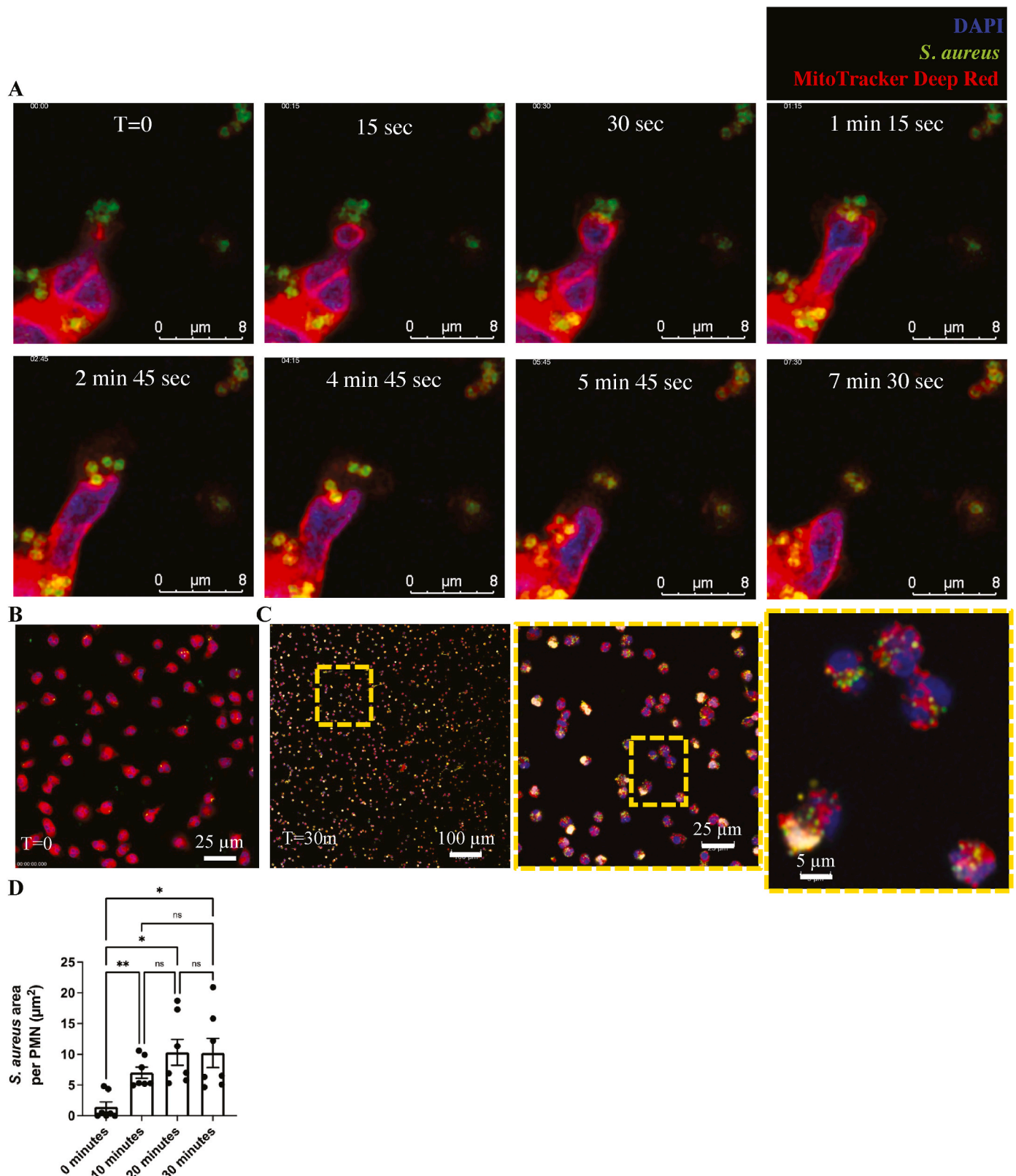
To determine the effect of mitochondrial metabolic inhibition on phagocytosis, engulfment of *S. aureus* was quantified 30 min post-inoculation (**Fig. 5A and 5B**). Interestingly, pre-treatment of PMN with all inhibitors increased the amount of engulfed *S. aureus*, with significant increases observed in myxothiazol- and apocynin-treated PMN (**Fig. 5B**). While there was significant variation between patients, there was a visible trend towards increased phagocytosis, which we hypothesize could be due to an impairment of effective bacterial killing mechanisms within the neutrophil, which allow accumulation of bacteria.

### 3.4. ETC inhibition alters NET production and mitochondrial ROS formation

NETosis was also quantified in neutrophils that were pre-treated with NOX2 and ETC inhibitors, 30 min post-inoculation with *S. aureus* (**S1C Fig.**). NETosis was visualized by measuring dsDNA immunofluorescence in non-permeabilized cell preparations (so that all DNA signal was extracellular, **Fig. 6A and 6B**). Antimycin A, rotenone and apocynin pre-treatment all significantly reduced the volume of NETs (**Fig. 6B**). To investigate the role of mitochondrial ROS formation in neutrophil function,  $O_2^{\bullet-}$  was quantified in neutrophils 60 min post-inoculation with *S. aureus*. Inhibition of Complex III with Antimycin A significantly impaired mitochondrial  $O_2^{\bullet-}$  production, while Complex I inhibition with rotenone resulted in a modest, and statistically insignificant reduction in mitochondrial ROS (**Fig. 6C**). Inhibitors of different mitochondrial ETC complexes decreased ROS production to varying degrees. Complexes I and III are the primary sources of mitochondria-derived ROS [31], particularly from reverse electron transport in more highly oxidative cell types [32].

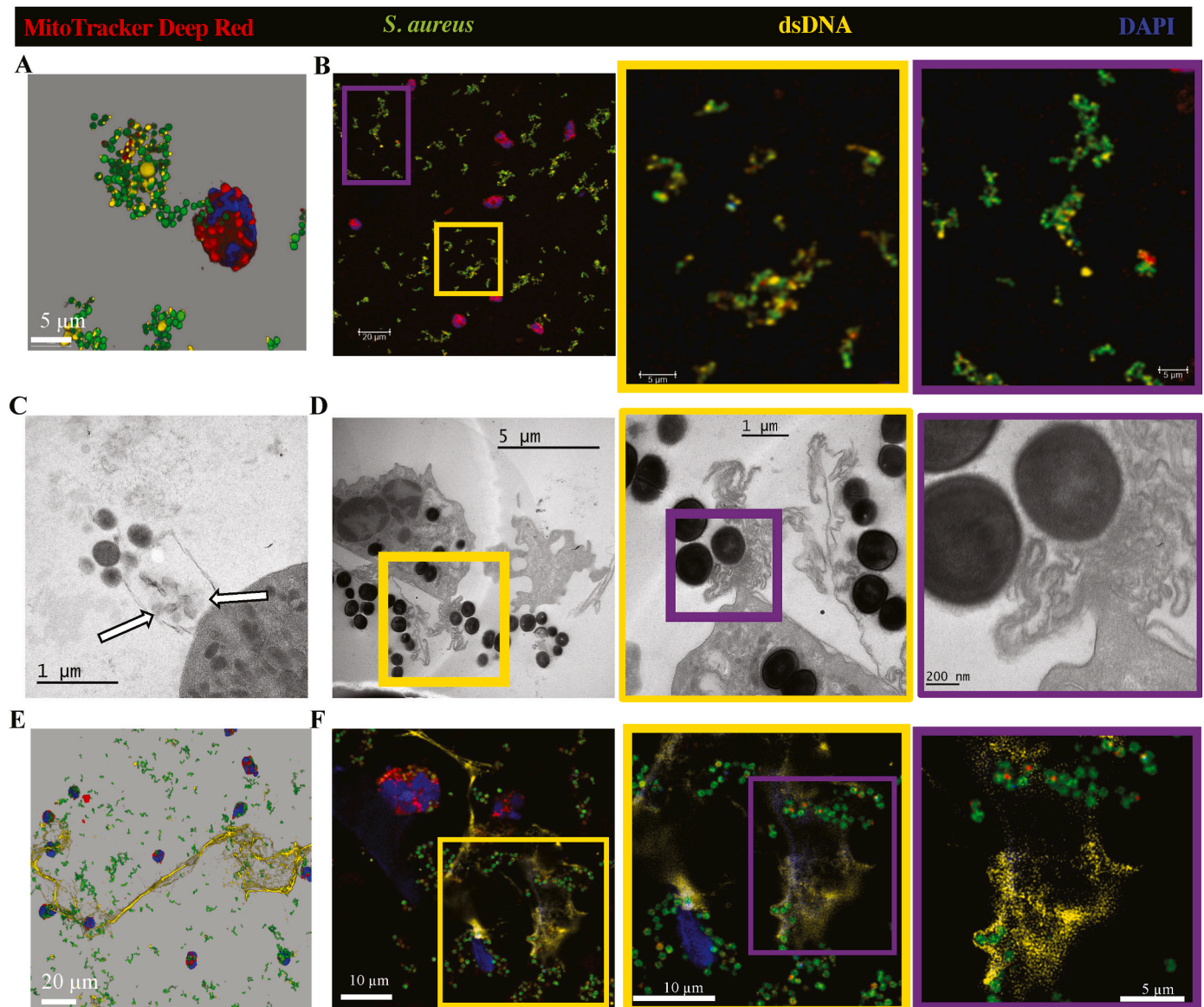
### 3.5. Inhibition of ETC complex III impairs neutrophil bactericidal activity

Because ETC Complex III-inhibited PMNs had increased accumulation of bacteria, reduced NET formation and, with Antimycin A also manifested impaired *S. aureus*-induced mitochondrial ROS formation, we sought to examine the role of mitochondria in bacterial killing, the terminal step of innate immunity. Neutrophils were again pre-treated with mitochondrial ETC or NOX2 inhibitors and then incubated with *S. aureus* (**S1C Fig.**). While ETC and NOX2 inhibitors have no direct effects on the growth of *S. aureus* (**Fig. 7A**), apocynin treatment significantly reduced bacterial killing, evidenced by a dramatically higher surviving colony count compared to vehicle-treated neutrophils (**Fig. 7B**). Antimycin A inhibited neutrophil killing of *S. aureus* to a magnitude comparable to apocynin whereas rotenone had no effect on bacterial survival (suggesting a selective role for Complex III in bacterial killing). A similar result was also observed in the myxothiazol-treated PMN (although myxothiazol-induced inhibition of *S. aureus* killing did not reach statistical significance;  $p = 0.056$ ). The inhibitory effect of Antimycin A on bacterial killing was also confirmed in a second cohort of patients, demonstrating reproducibility of results (**Fig. 7C**). To determine if ROS generated by Complex III are the regulator of mitochondria-mediated killing of *S. aureus*, PMN were pre-treated with 50  $\mu$ M MitoTEMPO [33] prior to bacterial exposure. MitoTEMPO is a manganese superoxide dismutase (MnSOD, SOD2) mimetic that converts superoxide to hydrogen peroxide, and is a combination of the antioxidant piperidine nitroxide (“TEMPO”) and triphenylphosphonium, a lipophilic cation that permits TEMPO to cross mitochondrial membranes and accumulate within mitochondria. MitoTEMPO has been previously demonstrated to quench mitochondrial ROS in neutrophils exposed to *Leishmania* parasites [33]. Indeed, pre-incubation with MitoTEMPO significantly attenuated killing of *S. aureus*, and as



**Fig. 2.** Neutrophil mitochondria actively participate in phagocytosis of *S. aureus*

After inoculation with GFP-labelled *S. aureus*, PMN mitochondria are present at the leading edge of activated cells. **A)** Representative 3D image series captured from Supplementary Video 1. **B)** Representative 2D image captured immediately after inoculation of MitoTracker Deep-Red-loaded PMN with GFP-*S. aureus*, captured at 25 μm scale. **C)** Representative 2D image series illustrating a 25-frame tile-scan image, captured at 100 μm scale (left), 25 μm scale (center), and 5 μm scale (right). Phagocytosis was quantified throughout time-course by generating an image ‘mask’ using the red mitochondrial signal and verifying PMN morphology using brightfield imaging. The total area of GFP-*S. aureus* contained within the image mask was measured in 10-min intervals for 30 min. **D)** Quantitation of total *S. aureus* area per PMN. Data are mean ± SEM of n = 7 patients; RM one-way ANOVA with Tukey post-test; \*p < 0.05, \*\*p < 0.01. For immunofluorescence: mitochondria (red), *S. aureus* (green), nuclei (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



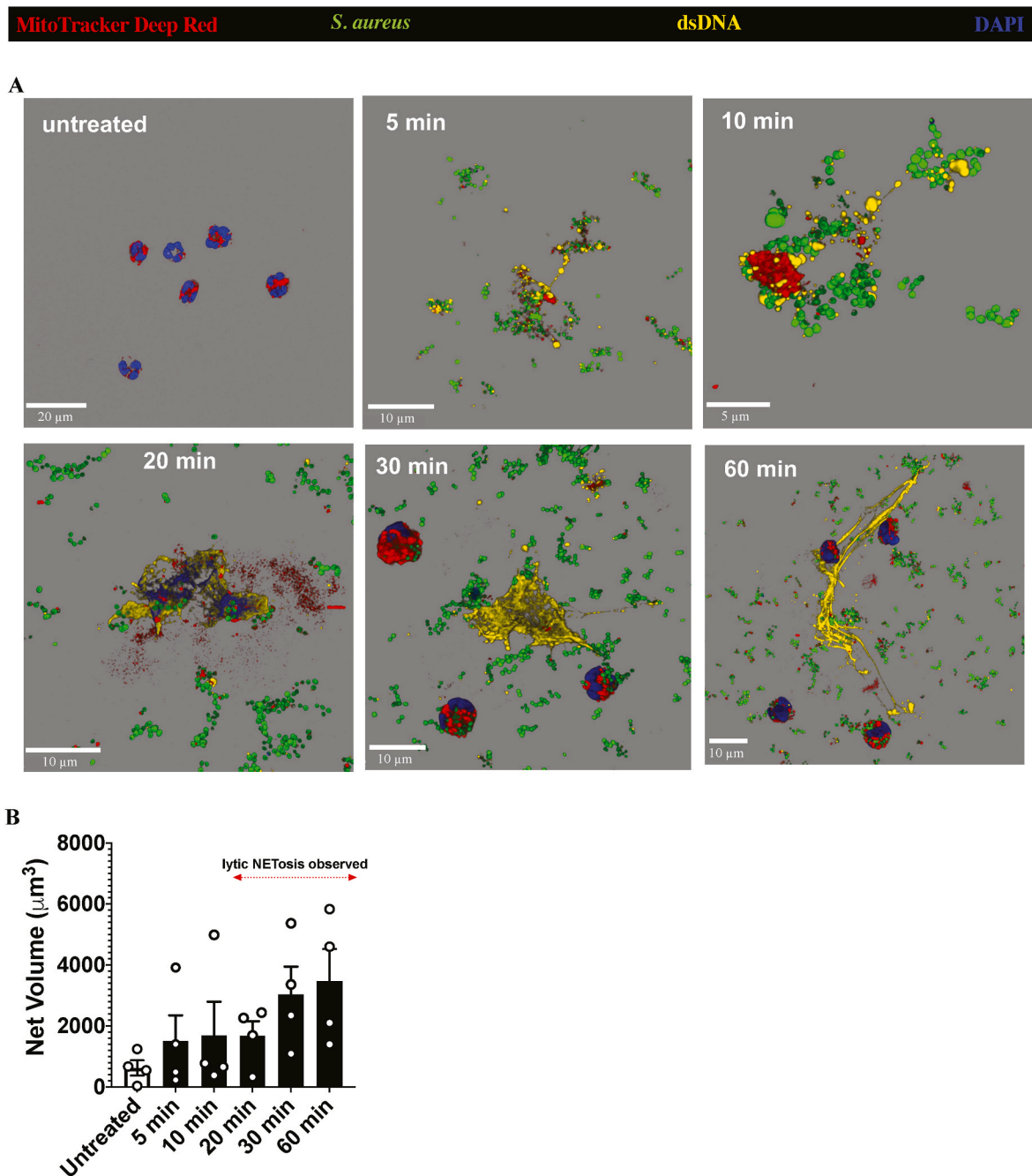
**Fig. 3.** Neutrophil mitochondria actively participate in *S. aureus*-induced NETosis

After inoculation with GFP-labelled *S. aureus*, PMN mitochondria are present in vital and lytic NETs. **A)** Representative 3D image of vital NETosis showing that mitochondria are expelled with genetic material as early as 5 min post-bacterial infection. **B)** Representative 2D image series of vital NETosis with two insets showing expulsion of vital NETs 5 min post-inoculation with GFP-*S. aureus*. **C)** Representative TEM image of vital NETosis showing that mitochondria (white arrows) are expelled with genetic material whilst the neutrophils are otherwise intact. **D)** Representative TEM image series of vital NETosis with increasing magnification (left to right) revealing the expulsion of genetic material from an intact PMN. **E)** Representative 3D image of lytic NETosis showing that mitochondria are expelled with genetic material during cell lysis. **F)** Representative 2D confocal images (from different patients) of lytic NETosis showing expulsion of mitochondria within the NETs.

expected, this effect was not enhanced by co-incubation of PMN with MitoTEMPO and Antimycin A in combination (Fig. 7D and 7E), reflecting the fact that the ROS are originating from Complex III. These data demonstrate that healthy mitochondria, specifically with active Complex III, are required for bacterial killing and constitute an important part of the innate immune response, along with NOX2.

The role of mitochondria in neutrophils has historically been restricted to apoptosis rather than canonical ATP production [34] because neutrophils exhibit remarkably low mitochondrial oxygen consumption [15]. Chacko et al. [15] conducted comprehensive bioenergetic profiling of platelets and white blood cells and found that neutrophils are effectively non-respiratory (with respect to classic oxidative metabolism), reflecting that neutrophils do not use the ETC to generate ATP via oxidative metabolism in the manner that occurs in other cells. Chacko et al. only report a detectable oxygen consumption

signal in neutrophils stimulated with PMA. It was further noted that this PMA-induced oxygen consumption was inhibited in the presence of DPI, which the authors correctly attribute as oxygen consumption by NOX2 for the oxidative burst, and in doing so, present a viable method for oxidative burst quantification. These findings are reinforced by van Raam et al. who report that neutrophils derive their ATP largely via glycolysis [35]. This group also reports that, unlike mitochondria in other leukocytes, neutrophil mitochondria do not form ETC super-complexes, and do not maintain mitochondrial membrane potential ( $\Delta\Psi_m$ ) by means of classical hydrogen pumping which occurs in tandem with reduction of molecular oxygen to water by the ETC [35]. While neutrophils have enzyme activity in ETC Complexes II and V (ATP synthase) comparable to other leukocytes, activity at Complex IV (where terminal electron acceptor oxygen is reduced to water) is virtually absent [35]. Instead, it is the activity of the glycerol-3-phosphate shuttle,



**Fig. 4.** Neutrophils release NETs within 5 min of inoculation with *S. aureus*.

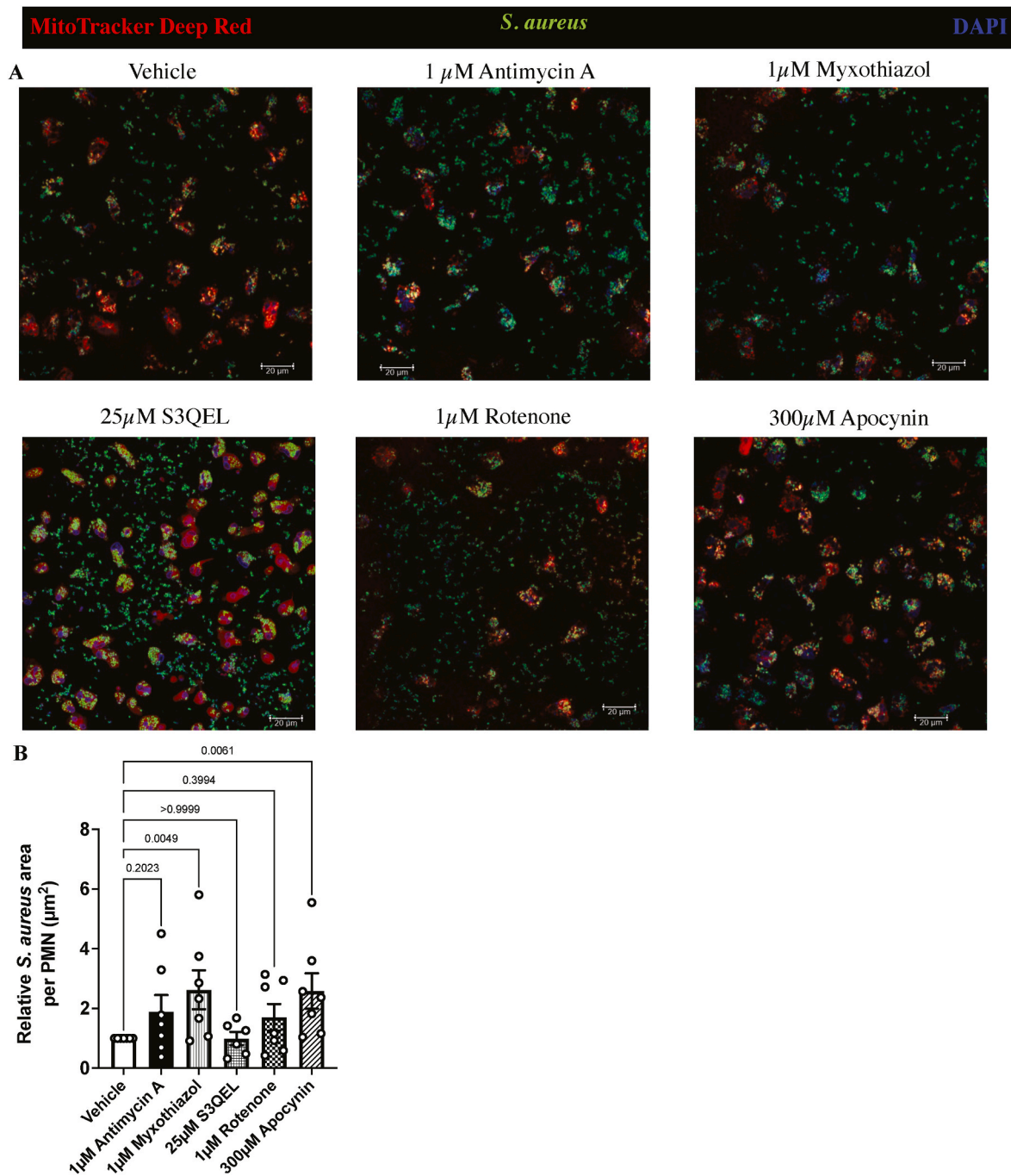
**A)** Representative 3-dimensional time-course image series of uninhibited neutrophils inoculated with GFP-labelled *S. aureus*. Vital NETosis is observed at < 20 min, followed by increasing incidence of lytic NETosis at later time points (>20 min post-inoculation with *S. aureus*). Samples were fixed in 4% PFA at the stated time post-inoculation and subjected to non-permeabilizing immunofluorescence staining. While all samples were fixed with DAPI-containing fixative, some representative images do not have intact PMN in frame, and as such, there is no DAPI signal. **B)** Time-course quantitation of NET formation in neutrophils with annotation for vital versus lytic NETosis. For immunofluorescence: mitochondria (red), *S. aureus* (green), nuclei (blue), double-stranded DNA (yellow). For TEM, mitochondria are indicated by white arrows. Data are mean  $\pm$  SEM of  $n = 4$ ; Friedman test in H  $p = 0.0435$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

which translocates electrons generated via glycolysis directly to ETC Complex III (coenzyme Q – cytochrome c reductase) that creates  $\Delta\Psi_m$  in neutrophil mitochondria. This is consistent with our observation that complex III is a plausible mediator of neutrophil killing of *S. aureus*. Also, since  $\Delta\Psi_m$  determines ROS production, and is established by Complex III, this supports our observation that inhibition of Complex III reduces mitochondrial ROS generation. ROS inhibition is part of the explanation for antimycin's inhibitory effect on the neutrophil's antibacterial capacity. Complex III is demonstrated herein to be the primary

source of mitochondrial ROS in neutrophils, an observation supported by van Raam's studies of neutrophil metabolism and membrane potential which identified Complex III as having paramount importance in neutrophil metabolism [35].

#### 4. Conclusion

We identify for the first time (to our knowledge) the critical role of neutrophil mitochondria in the innate immune response to *S. aureus*.



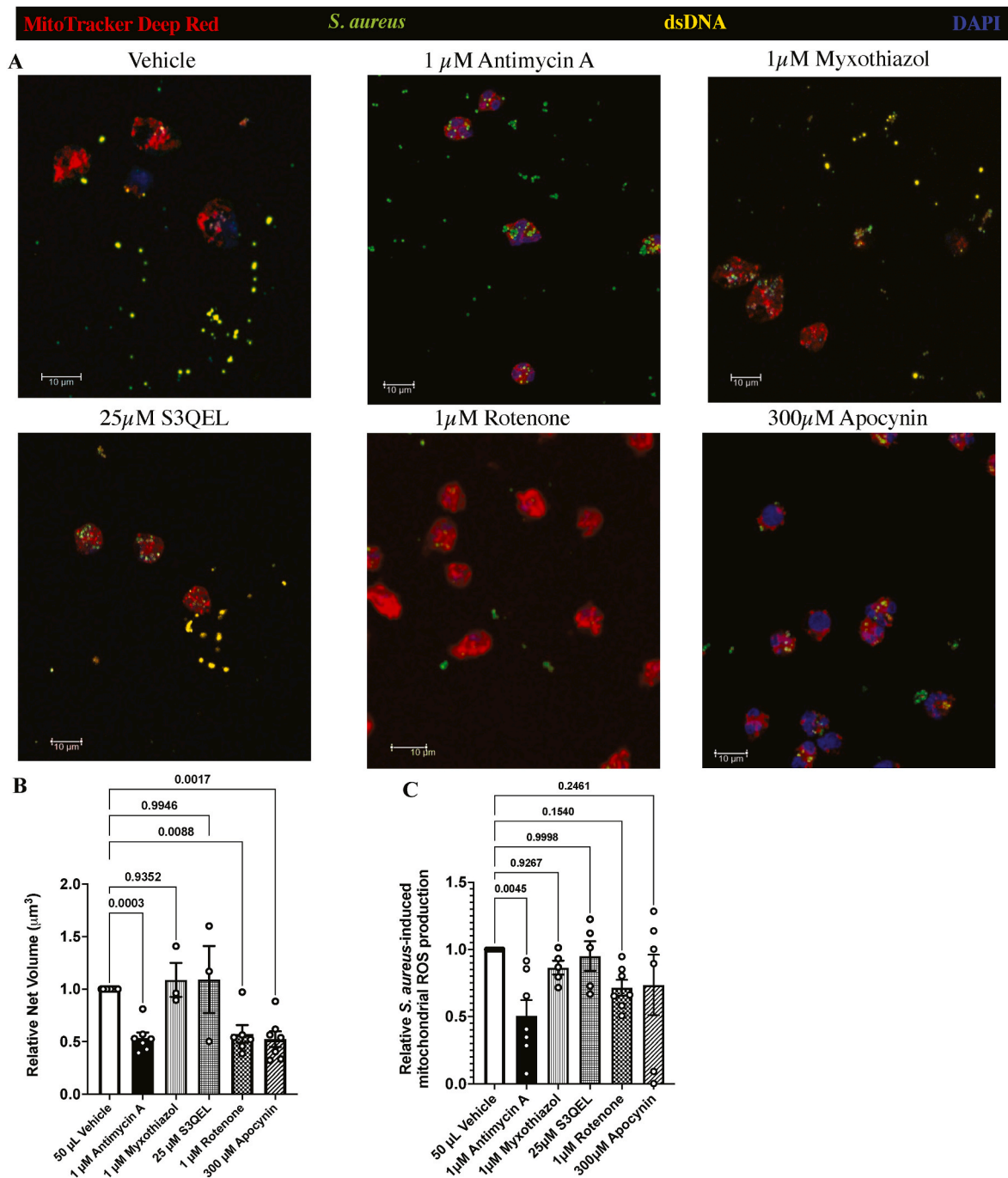
**Fig. 5.** Inhibition of the ETC Complex III, but not Complex I, increases the number of engulfed *S. aureus*.

After PMN exposure to *S. aureus*, neutrophil phagocytosis activities were assessed in neutrophils previously treated with mitochondrial inhibitors Antimycin A (1  $\mu$ M, Complex III), myxothiazol (1  $\mu$ M, Complex III), S3QEL (25  $\mu$ M, Complex III), rotenone (1  $\mu$ M, Complex I), an NADPH oxidase inhibitor apocynin (300  $\mu$ M), or vehicle (DMSO). **A**) Representative image series of vehicle- or inhibitor-treated neutrophils exposed to GFP-labelled *S. aureus*. Samples were imaged for 30 min post-inoculation with *S. aureus*, and the area of GFP-*S. aureus* per neutrophil were quantified. One tile from a 5  $\times$  5 image is shown for each condition. Immunofluorescence labelling: Mitochondria (MitoTracker Deep Red-red), bacteria (green), nuclei (blue); Magnification – 63X; Zoom – 2X; Scale bar – 15  $\mu$ m. **B**) Quantification of *S. aureus* per neutrophil, expressed as relative area of GFP signal detected inside intact PMN. Data are mean  $\pm$  SEM of n = 6–7 patients; p values indicated; mixed effects analysis with Dunnett post-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Previously assumed to be largely vestigial [34], we show that a portfolio of canonical neutrophil functions, including ROS production, vital NETosis, and importantly, bacterial killing, all require intact mitochondria with active ETC Complex III. We demonstrate that mitochondria contribute to bacterial killing with an effectiveness comparable to NOX2. Since mitochondria are thought to have arisen in eukaryotic cells endosymbiotically from uptake of prokaryotic progenitor bacteria [36,

37], this ability of mitochondria to kill bacteria is not only practical for mammals (and amphibians and fish) [38], but also interesting from an evolutionary perspective. We propose a novel role for neutrophil mitochondria, ‘aiding and abetting’ canonical NOX2-mediated bacterial killing. Neutrophil mitochondria may be a novel target for antimicrobial therapeutic agents.



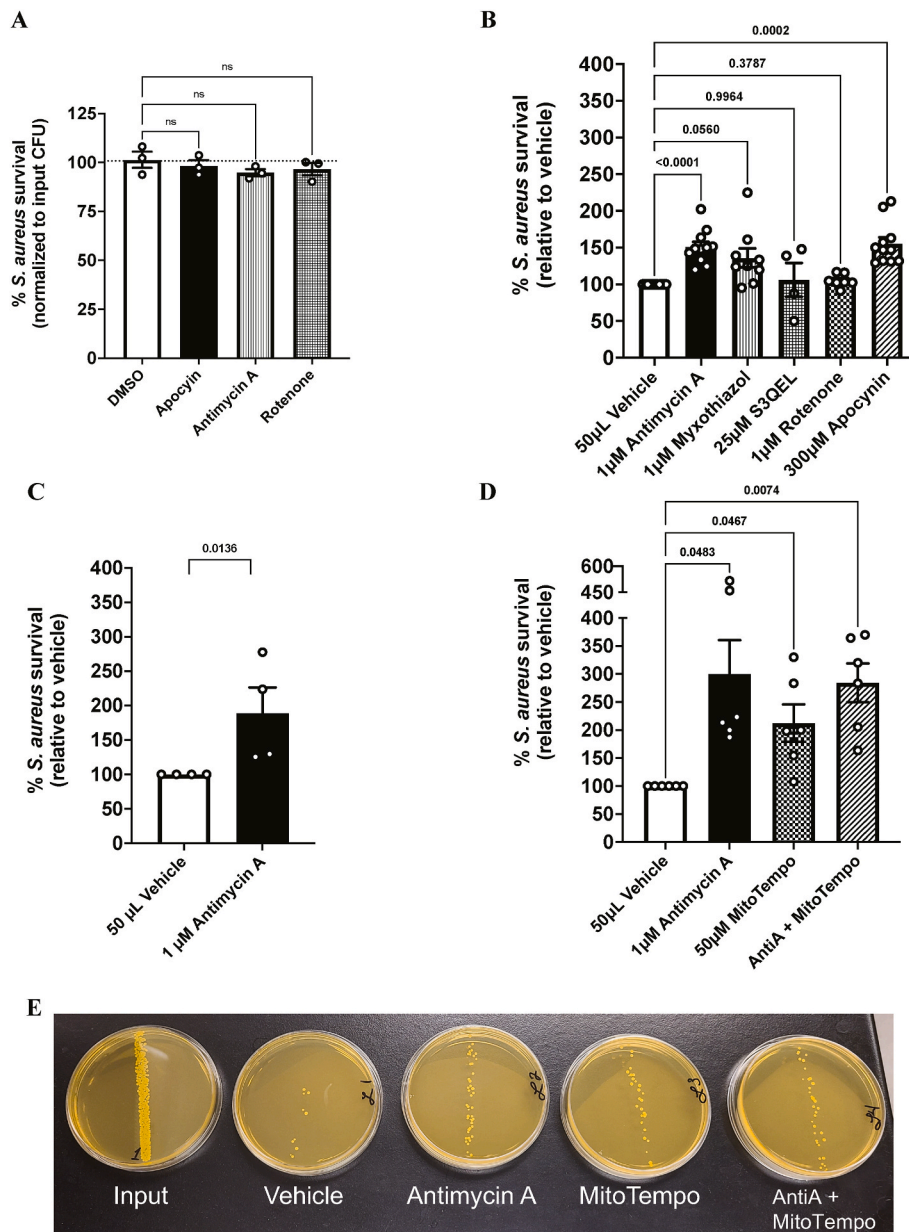


**Fig. 6.** Inhibition of the ETC alters production of NETs, and inhibition of Complex III, but not Complex I, reduces *S. aureus*-induced mitochondrial ROS formation. **A)** Representative image series of vehicle- or inhibitor-treated neutrophils exposed to GFP-labelled *S. aureus*. Samples were fixed after 45 min with 4% PFA and subjected to non-permeabilizing immunofluorescence. NET formation was quantified by measuring total area of extracellular double-stranded DNA. One tile from a  $5 \times 5$  image is shown for each condition. Immunofluorescence labelling: Mitochondria (MitoTracker Deep Red-red), bacteria (green), nuclei (blue), extracellular double-stranded DNA (yellow); Magnification – 63X; Zoom – 2X; Scale bar – 15  $\mu\text{m}$ . **B)** Quantification of extracellular double-stranded DNA, a marker of NET formation, expressed as relative area. Data are mean  $\pm$  SEM of  $n = 3-7$  patients; p values indicated; mixed effects analysis with Dunnett post-test. **C)** Quantitation of relative mitochondrial ROS formation in vehicle- and inhibitor-treated PMN during *S. aureus* exposure. Data are mean  $\pm$  SEM of  $n = 5-7$  patients; p values indicated; mixed effects analysis with Dunnett post-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

## 5. Future directions & limitations

Numerous questions surrounding the complex mechanism of mitochondria-mediated bacterial killing remain. Notably, our data indicate that, while healthy mitochondria are indeed required for all key aspects of bactericidal activity (i.e., phagocytosis, NETosis,

mitochondrial ROS production, and bacterial killing), the mechanism of mitochondria-mediated NETosis is likely disconnected from that of phagocytosis and killing. It is also possible that different electron transport chain components regulate various parts of this process, which may explain why Complex I inhibition with rotenone inhibited NETosis, but not phagocytosis or killing. While we demonstrate a definitive role



**Fig. 7.** Inhibition of ETC Complex III, but not Complex I, impairs bactericidal activity of neutrophils. **A)** Bacterial survival in ETC or NOX2 inhibitor-treated *S. aureus* determined by colony count after 3-h incubation with inhibitors and subsequent plating and overnight growth on BHI agar. Data are mean  $\pm$  SEM of  $n = 3$  replicates per treatment. Statistical analysis was a RM one-way ANOVA with Dunnett post-test. **B)** Bacterial survival determined by colony count after 3-h incubation with PMN and subsequent neutrophil lysis. Data are mean  $\pm$  SEM of  $n = 4$ –11 patients. Statistical analysis was a mixed effects analysis with Dunnett post-test. **C)** Analysis of bacterial survival of an additional small cohort of patients at a second experimental site (University of Calgary). Bacterial survival determined by colony count after 3-h incubation with PMN and subsequent neutrophil lysis. Data are mean  $\pm$  SEM of  $n = 4$  patients;  $p$  value reflects two-tailed  $t$ -test. **D)** Analysis of bacterial survival (determined via colony count) after pre-treatment with Antimycin A, MitoTEMPO, or combination. Data are mean  $\pm$  SEM of  $n = 6$  patients. Statistical analysis was a RM one-way ANOVA with Dunnett post-test. **E)** Representative image series displaying bacterial survival after PMN pre-treatment, 3-h incubation with *S. aureus* and subsequent PMN lysis + dilution series prior to plating.

for expelling mitochondrial DNA and production of mitochondrial ROS in *S. aureus* killing, further investigation is required to determine if the mechanism is similar to that described by Lood et al. in systemic lupus erythematosus, whereby mitochondrial ROS and release of oxidized mitochondrial DNA drive NETosis and may be a key player driving autoimmunity [39].

#### Author contributions

KDS, BS, JM, PK and SA conceived the study. KDS, BS, JM, OJ, RQ, AYM, RB, and PDAL performed all experiments. KDS, BS, and JM analyzed all data. KDS and SA wrote the manuscript. KDS, BS, PK, and SA edited the final version.

#### Data availability

The authors declare that all data are available in the manuscript (and its Supplementary Data and Information files). The authors note that the confocal microscopy raw data are extremely large files (>1 TB) and are

available from the corresponding author upon reasonable request.

#### Declaration of competing interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2021.102225>.

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