

Attenuation of neutrophil driven inflammation and fungal growth by pilocarpine hydrochloride

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Research Article

Keywords: Candida albicans, pilocarpine, neutrophils, fungal infection, anti-inflammatory

Posted Date: October 12th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10766704/v1>

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Additional Declarations: Competing interest reported. AGR serves as the co-Editor-in-Chief of the Journal of Inflammation. To ensure impartiality, the peer-review of this manuscript was handled by an independent member of the editorial board, and the submission underwent the standard review process applied to all manuscripts.

Abstract

Background

An increasing incidence of fungal infections and antifungal resistance highlight the urgent need for novel treatment approaches. Current antifungal treatment primarily targets the pathogen and does not address modulation of the host immune response. However, infections trigger a rapid and intense recruitment of immune cells, particularly neutrophil granulocytes. Although these first responders play a critical role in pathogen clearance, their activation can concurrently cause damage to surrounding tissues. This is especially detrimental in tissues like the cornea, where scarring and opacification induced by inflammation can lead to permanent visual impairment. A drug with simultaneous effects on fungal growth and immune reaction could reduce immune-mediated tissue damage while improving antifungal efficacy. Pilocarpine hydrochloride is a muscarinic receptor agonist previously shown to inhibit *C. albicans* hyphae formation and reduce granulocyte-mediated inflammation in *Galleria mellonella* larvae, which could be repurposed for the localised treatment of fungal infection.

Results

We investigated the effect of pilocarpine hydrochloride on fungal growth and on neutrophil responses *in vitro*. Growth of not only *C. albicans*, but also filamentous fungi *A. flavus* and *F. keratoplasticum* was reduced upon treatment. In neutrophils, pilocarpine induced apoptosis in a concentration-dependent manner. It further downregulated neutrophil activation as measured by CD11b and CD66b receptor expression, independent of calcium flux. Shape changes and chemotaxis in response to the chemoattractant N-formylmethionine-leucyl-phenylalanine (fMLF) were reduced by pilocarpine treatment. The drug further influenced neutrophil-mediated antimicrobial activities against *C. albicans*, including killing, reactive oxygen species (ROS) production, degranulation and phagocytosis. While pilocarpine itself did not induce NETs, it was able to downregulate ROS-dependent NETosis. We further investigated NET-release in response to heat-inactivated yeast, dead hyphae, and live *C. albicans* during its transition from yeast to hyphae under pilocarpine treatment. In a novel three-dimensional *in vitro* cornea infection model, neutrophil chemotaxis was reduced by pilocarpine, while fungal growth into the model was inhibited.

Conclusions

In summary, pilocarpine inhibits fungal metabolism while downregulating neutrophil activities and inducing apoptosis. Hence, pilocarpine could serve as a therapeutic option for local fungal infections, as it provides a dual benefit by targeting fungi and immune regulation.

1. Background

Incidences of fungal infections are increasing worldwide, and additionally pose a growing challenge due to rising antifungal drug resistance (1, 2). The most common human fungal pathogen, *Candida albicans*,

is associated with a broad spectrum of infections, ranging from invasive systemic to localised conditions. Localised infections are seen in mucocutaneous areas, such as skin, oropharyngeal or vaginal region, in the gastrointestinal tract, and on the eye surface (3, 4). One of the key virulence factors of *C. albicans* is its ability to transition from yeast to hyphal growth (5), enabling biofilm formation. Biofilms result in less efficient drug response, as their extracellular matrix protects the fungus from both antimicrobial agents and the host immune system (6, 7).

Among the first immune cells to respond to an infection are neutrophil granulocytes, which migrate to the site of infection to prevent the pathogen from spreading. Here, they phagocytose small pathogens, such as bacteria or yeast forms of fungi. In contrast, fungal hyphae are primarily targeted by extracellular defence mechanisms due to their size, such as the release of neutrophil extracellular traps (NETs) (8). Additional key mechanisms of neutrophil defence are the release of granule components and reactive oxygen species (ROS), both of which are involved in pathogen killing. Following infection resolution, the remaining short-lived neutrophils can undergo apoptosis, a non-inflammatory form of cell death (9). While the immune response against fungal infections aims to eliminate the invading pathogen, it simultaneously results in destruction of surrounding tissue.

Current treatment for localised fungal infections does not focus on modulating the host immune response (10, 11). However, a therapeutic agent that promotes the resolution of inflammation while exerting antifungal activity could accelerate healing and mitigate further tissue damage. This reinforces the need for novel antifungal strategies, which effectively inhibit fungal growth while avoiding activation of the immune response.

One such drug candidate is pilocarpine hydrochloride. While pilocarpine is an FDA approved drug for oral and topical ocular administration for non-infectious conditions, it has recently been shown to inhibit hyphae formation of *Candida albicans* (12). Its effect on filamentation appears to result primarily from a reduction of fungal metabolism rather than from direct fungicidal effects, and is accompanied by diminished biofilm formation. This drug is a muscarinic receptor agonist with a higher affinity for M1 over M3 receptors (13). *Candida* expresses an Rrp9 protein with similarity to the human M1 receptor (14), and the muscarinic receptor antagonist scopolamine was able to revert the effects of pilocarpine (12). Mechanistically, exposure to pilocarpine hydrochloride induces alterations of lipid, sphingolipid and protein content in *C. albicans* (15).

In contrast, data regarding the effect of pilocarpine on the immune response remain limited. Using pilocarpine treatment on moth (*Galleria mellonella*) larvae, the drug alone did not induce hemocyte recruitment or aggregation, and no activation of the insect granulocytes was observed at a 10.5 mM concentration. Furthermore, pilocarpine treatment of *C. albicans*-infected moth larvae resulted in reduced granulocyte numbers in the circulation, and decreased inflammation. It was therefore suggested to promote clearance of the fungal infection while limiting bystander tissue damage (12).

In this study, we investigate the effect of pilocarpine on human neutrophil responses during *C. albicans* fungal infection. Uncovering the drug's impact on the immune response, and the associated neutrophil-

driven tissue damage, is important to determine whether pilocarpine could serve as a therapeutic option that provides a dual benefit by targeting fungi and immune regulation in fungal infections.

2. Material and methods

2.1. Pilocarpine hydrochloride preparation

Pilocarpine hydrochloride (Tocris Bioscience 0694/100) was prepared in 1x lipopolysaccharide (LPS)-free phosphate-buffered saline (10x PBS (Sigma P5493) diluted in endotoxin-free ultra-pure water (Merck TMS-011-A)) to a stock concentration of 100 mM on the day of each experiment.

2.2. Fungal strains and culture

All fungal strains were sourced from in-house strain collections or American Type Culture Collection (ATCC). The fungal strains used in this study are *C. albicans* ATCCMYA2876, *C. albicans* CAF2-dTomato (16), *A. flavus* ATCC200026 and *F. keratoplasticum* ATCC36031. *C. albicans* in yeast form was prepared from a single colony picked from a Potato Dextrose agar (PD, Millipore 70139) plate and inoculated into PD broth (Millipore P6685) to grow overnight at 30°C under motion. The overnight culture was adjusted to OD₅₉₅ 0.1 and further incubated until mid-log phase (OD₅₉₅ 0.4–0.8). Yeast cells were washed three times with 0.9% sterile saline, and concentrations adjusted in RPMI medium (Gibco 11835063) by haemocytometer counting. *Aspergillus flavus* and *Fusarium keratoplasticum* spores were prepared by harvesting from three to five days (30°C) PD agar growth plates with PBS with 0.025% Tween 80 (Sigma P1754). Harvested spores were washed and the concentration was calculated by haemocytometer counting.

2.3. Fungal growth quantification (XTT and crystal violet)

C. albicans ATCCMYA2876 yeast and *F. keratoplasticum* spores were diluted to 10^4 mL^{-1} , whilst *A. flavus* was diluted to $10^5 \text{ spores mL}^{-1}$. They were washed and resuspended in pilocarpine (0, 1, 3, 5, 8, 10, and 50 mM) or in 70% ethanol, in RPMI for yeast and PD broth for filamentous fungi. 200 µL of each yeast/spore solution was plated in 96-well plates in triplicates. All pathogens were incubated for 18 hours at 30°C for hyphae formation. Each well was imaged using a Nikon Eclipse TSR2 microscope. For metabolic activity, XTT assay was performed as per manufacturer's instruction (Thermo Fisher Scientific X12223). Absorbance was read after three hours XTT reagent exposure at 450 nm. For biomass measurements, each well was washed in distilled water followed by incubation in 0.1% crystal violet (Merck V5265) diluted in water at room temperature for 20 minutes. Wells were washed four times in distilled water and left to air dry. Bound dye was eluted in 70% ethanol and absorbance read at 595nm.

2.4. Neutrophil isolation

Blood was collected from healthy human volunteers following informed consent and ethical approval granted by the Lothian Research Ethics Committee (EMREC Reference: 21-EMREC-041 and 25-EMREC-023) at the University of Edinburgh. Neutrophils were isolated from whole blood by PolymorphPrep

(Progen 1114683) isolation as published (17). For this purpose, whole blood was layered at a 1:1 ratio onto the sodium diatrizoate and dextran solution and neutrophils were separated by density gradient centrifugation at 470 xg at room temperature, 30 minutes, without using a centrifuge brake. The polymorphonuclear cell layer was obtained and washed once using endotoxin-free PBS (Sigma-Aldrich P5493-1L, 10× PBS diluted in endotoxin-free water to 1× concentrated). Remaining erythrocytes were lysed using sterile water for 15 seconds. The isolated neutrophils were resuspended in RPMI with or without autologous serum depending on the assay.

2.5. Neutrophil viability and apoptosis

Neutrophils were incubated for three and six hours with pilocarpine at a range of concentrations (0, 1, 3, 5, 8, 10, and 50 mM) in RPMI with 5% autologous serum. Pan-caspase inhibitor Q-VD (R&D systems OPH001, 10 μM), cyclin-dependent kinase inhibitor roscovitine (Merck 557360, 10 μM) and 50% dimethyl sulfoxide (DMSO, Sigma D2438) were used as controls. Viability, apoptosis and necrosis were assessed using Pacific Blue Annexin V (BioLegend 640918) and Draq7 (Abcam ab109202, 0.3 μM final concentration) by flow cytometry. Pacific Blue Annexin V was diluted 1:500 in binding buffer (BioLegend 433301), and 200 μL were added to 50 μL neutrophils. After 10 minutes incubation on ice, Draq7 was added to the samples immediately before analysis on a NovoCyte Penton flow cytometer.

2.6. Cytocentrifuge preparations

Neutrophils were incubated for six hours with and without pilocarpine at 50 mM in RPMI with 5% autologous serum. Roscovitine (Merck 557360, 10 μM) was used as control for apoptosis induction. 10^5 neutrophils were spun against a glass slide at 300 rpm for three minutes using medium acceleration (Shandon Cytospin 4). After air drying, the samples were fixed in 100% methanol for ten minutes, then stained using Diff-Kwik solutions 2 and 3 (Thermo Scientific). The slides were washed with water, and imaged using a Zeiss Observer widefield microscope (63x objective with oil immersion).

2.7. Neutrophil activation

Neutrophils were resuspended at 10^7 mL^{-1} and treated with the following pilocarpine concentrations: 0 mM, 10 mM and 50 mM, each with or without 100 nM N-formylmethionine-leucyl-phenylalanine (fMLF; Sigma F3506). Cells were incubated for 30 minutes at 37°C. Treatments were stopped by pelleting and resuspending in BD® OMICS-Guard Sample Preservation Buffer (BD, 570911) overnight at 4°C. Neutrophils were then stained with 1:10,000 LIVE/DEAD™ Fixable Near IR Viability Kit (Thermo Fisher Scientific, L34992) for 15 minutes at 4°C. Following a wash in PBS, neutrophils were stained with a multi-antibody panel including Singlec-8 PE (BioLegend, # 347103), CD3 AF700 (#100215), MHC II = HLA-DR BV650 (#307649), CD15 BV605 (#323031), CD66b APC (#305117), and CD11b AF488/PerCP-cy5 (#301307) for 30 minutes at 4°C. Fluorescence Minus One (FMO) controls containing the antibody panels minus one antibody lacking either CD3, MHC2, CD15, CD66b, or CD11b were also included. Each sample was fixed in 4% paraformaldehyde (PFA, Thermo Scientific nJ19943-K2) for 20 minutes. Samples were analysed on a Cytex Aurora flow cytometer. Geometric mean for activation markers CD11b and CD66b were measured.

2.8. Calcium signalling

Neutrophils were incubated at 37°C with or without 1 mM EGTA for 15 minutes. Following pelleting neutrophils, cells underwent incubation in 4 µM Fluo-4 AM (Thermo Fisher Scientific F14217) at 37°C for 30 minutes. Following a wash in PBS, cells were seeded as 10^5 cells/well in 100 µL in a Greiner black 96-well plate with clear µClear bottom (SLS G655090). Fluorescence was read using Clariostar microplate reader (BMG Labtech) at Ex₄₉₀ /Em₅₂₀ every ten seconds for eight minutes. After 1-minute readings, 100 µL stimulating treatments were added using automatic injectors, in triplicates, and fluorescence read for the further seven minutes. Injected treatments were as followed: PBS only, 100 nM fMLF, 100 nM fMLF + 10mM pilocarpine, 100 nM fMLF + 50 mM pilocarpine, 10 mM pilocarpine, and 50 mM pilocarpine. Calcium flux changes were expressed as maximum fluorescence post-injection/average fluorescence pre-injection.

2.9. Shape changes and imaging flow cytometry

Neutrophil shape change was analysed after 30 minutes incubation with pilocarpine +/- 100 nM fMLF at 37°C, 300 rpm. Samples were fixed in 2% PFA and analysed for changes in standard deviation of forward scatter (FSC-A) using a BD FACS Aria II flow cytometer. Further, imaging flow cytometry was performed using a Thermo Attune Cytpix system. Single cell high-resolution images were analysed for neutrophil circularity, cell area, perimeter, and eccentricity, using Attune cytometric software.

2.10. Transwell migration assay

Neutrophil chemotaxis after pilocarpine treatment was analysed by neutrophil migration across transwell filter inserts (ThinCerts-inserts, Gibco 662631) with 3 µm pore size. First, neutrophils were stained using Syto 9 green fluorescent nucleic acid stain (Invitrogen S34854, 2.5 µM final) for ten minutes. They were then treated with pilocarpine in 0.5% fatty acid-free BSA (Merck A8806) in RPMI for 30 minutes at 37°C, 5% CO₂, before being transferred to the upper compartment of transwell filters. 10 nM fMLF in 0.5% fatty acid-free BSA-RPMI was used as chemoattractant in the lower compartment. After 90 minutes of incubation at 37°C, 5% CO₂, transmigrated neutrophils were collected from the lower compartment. Ultra-low attachment plates (Corning 3473) were used to facilitate neutrophil retrieval. Remaining cells were gently lifted using TrypLE Express (Gibco 12605-010). Fluorescent neutrophils were quantified using NovoCyte Penton flow cytometer.

2.11. Neutrophil killing assay

Opsonised *C. albicans* ATCCMYA2876 yeast were treated with pilocarpine at 10 mM and 50 mM final concentrations in the presence of 10% autologous serum. To half of the samples, 2.5×10^6 neutrophils were added, reaching a multiplicity of infection (MOI) of 0.01 for *C. albicans*. Samples were incubated with slow end over end incubation at 37°C, 5% CO₂ for two hours. Then, neutrophils were lysed by adding saponin (Sigma 84510, 0.05% in dH₂O) and potato dextrose broth, incubated for five minutes on ice, vortexed for two minutes and incubated for another five minutes on ice. Afterwards, samples were

serially diluted in saline and plated on PD agar in triplicate for quantification of colony forming units (CFUs) after overnight incubation at 37°C, 5% CO₂.

2.12. Phagocytosis

Phagocytosis of *C. albicans* CAF2-dTomato yeast by neutrophils in presence and absence of pilocarpine was quantified using flow cytometry. For this purpose, 5×10^5 neutrophils in RPMI and 10% autologous serum were incubated with pilocarpine and opsonised *C. albicans* CAF2-dTomato yeast (MOI 5). Phagocytosis was inhibited in some samples by pre-incubating the neutrophils for 20 minutes with Cytochalasin D (Sigma C2618, 10 µg/mL final conc.). After 20 minutes incubation of all samples at 37°C, 5% CO₂ under slow rotation, they were transferred to ice. Samples were stained for 30 minutes using APC Mouse anti-human CD15 (BD Pharmingen 561716, 23 ng/mL) to identify neutrophils, and with an Alexa Fluor 488 goat anti-human IgG (H + L) antibody (Molecular Probes A11013, 10 µg/mL) to stain non-phagocytosed *C. albicans* yeast. After a wash, all samples were fixed in 4% PFA. Flow cytometry controls included unstained neutrophils, single-stained neutrophils (CD15), *C. albicans* CAF2-dTomato yeast, and non-fluorescent *C. albicans* ATCCMYA2876 yeast opsonised and stained using secondary anti-human IgG 488 antibody. Samples were analysed using NovoCytte Pentec flow cytometer.

2.13. Reactive oxygen species (ROS)

Intracellular ROS was quantified by flow cytometric measurement of Dihydrorhodamine 123 (DHR; Sigma D1054) fluorescence intensity. For this, neutrophils were stained with 2 µM DHR for five minutes at 37°C before stimulation. Then, 2×10^5 neutrophils per condition were incubated with pilocarpine at final concentrations of 0, 10 and 50 mM, and stimulated with either RPMI as control, phorbol 12-myristate 13-acetate (PMA, 20 nM) or *C. albicans* ATCCMYA2876 yeast (MOI 2) for 30 minutes at 37°C in a heat block (300 rpm). Immediately after, samples were fixed with 4% PFA and analysed using NovoCytte Pentec flow cytometer.

For quantification of total ROS, a luminol-based chemiluminescence assay was used. White 96 well plates were blocked using 2% skimmed milk (Marvel, dissolved in dH₂O), before stimuli were added. These included pilocarpine (10 mM and 50 mM final conc.), phorbol 12-myristate 13-acetate (PMA; Sigma P8139, 20 nM final conc.), and *C. albicans* ATCCMYA2876 yeast (MOI 2). 5×10^5 neutrophil were added per well, as well as luminol (Sigma 8511, final conc. 156 µM) and horseradish peroxidase (HRP; Sigma P8375, 25 mU/µL final conc.). Luminescence reading was immediately started with readings every minute over a one-hour period at 37°C.

2.14. Matrix metalloproteinase-9 (MMP-9) release

Pilocarpine-treated neutrophils were stimulated with fMLF (100 nM), *C. albicans* ATCCMYA2876 yeast (MOI 10, opsonised with 10% autologous serum for 15 minutes) or RPMI for 30 minutes. Supernatants were collected, centrifuged at 500 xg for ten minutes, and stored at -20°C until further analysis. An MMP-9 ELISA (Proteintech KE00164) was used for quantification in sample supernatants following the manufacturer instructions. Duplicate samples of six blood donors were analysed at a 1:100 dilution.

2.15. Neutrophil extracellular trap assessment

Freshly isolated neutrophils were seeded at a concentration of 2×10^5 per well in a poly-L-lysine (Sigma P4707) coated 96 well μ -plate (ibidi, 89621). Pilocarpine was added to final concentrations of 0, 10 and 50 mM. Further stimuli include PMA (25 nM final concentration), heat-inactivated yeast (*C. albicans* ATCCMYA2876; MOI 2), 70% ethanol-killed hyphae (*C. albicans* ATCCMYA2876; MOI 2, grown for three hours in RPMI medium prior to fixation), and live *C. albicans* ATCCMYA2876 yeast at an MOI of 2. Samples were incubated for four hours at 37°C, 5% CO₂ and then fixed using 4% PFA.

For immunofluorescence staining, all samples were permeabilised for five minutes with 0.5% Triton X-100 (Sigma X100) in PBS, after which they were blocked for 20 minutes with 3% normal donkey serum (Sigma D9663), 3% gelatine from cold water fish skin (Sigma G7041), 1% bovine serum albumin (BSA, Sigma A4503), and 0.05% Tween20 (Merck P2287) in PBS. The samples were then incubated for one hour with a monoclonal mouse anti-DNA/histone-1 antibody (Sigma MAB3864, 0.5 μ g/mL final conc.) and a polyclonal rabbit anti-human myeloperoxidase antibody (Dako A0398, 10 μ g/mL final conc.) diluted in blocking buffer. Isotype controls were incubated with mouse IgG2a from murine myeloma (Merck M5409) and rabbit IgG control (R&D systems AB-105-C). After washing, secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (H + L) (Invitrogen A11001, 1 μ g/mL final conc.) and Alexa Fluor 633 goat anti-rabbit IgG (H + L) (Invitrogen A21070, 4 μ g/mL final conc.) in blocking buffer were applied for an hour. After further washes, samples were stained using Hoechst 33342 (molecular probes H1399, 20 μ g/mL in dH₂O) for ten minutes. The samples were kept in PBS until imaging.

Microscopy was performed on a Leica SP8 confocal laser scanning microscope (HC PL APO CS2, 40x oil immersion objective). Per sample, three randomly selected images were taken and used for quantification of NET-positive cells. Neutrophils were counted as NET negative when small, round or lobulated nuclei with a sharp rim were present. NET-positive cells either released DNA/histone-1 and myeloperoxidase positive extracellular off-shoots, were directly adjacent to an off-shoot, and/or had lost nucleus shape and density while increased in size. For NET experiments in presence of live *C. albicans*, the area per image covered by NETs was quantified from maximum projections of z-stack images. Images were processed using ImageJ software (version v1.54k).

2.16. 3D *in vitro* cornea infection model

The corneal fibroblast cell line hTK (18) was routinely grown using serum-free Keratinocyte Growth Medium (Lonza CC-3111).

For the cornea model, μ -slide Chemotaxis (ibidi 80326) was repurposed. First, fibroblasts were seeded into a collagen matrix in the central observation area: 6 μ L hTK cells at 5×10^5 /mL in a collagen matrix composed of 5 mg/mL Cultrex 3-D culture matrix rat collagen I (R&D systems, 344702001), 1 M HEPES buffer (Gibco 15630049), 37 mg/mL NaHCO₃ at pH 9.5, endotoxin-free ultra-pure water (Merck Millipore TMS-011-A) in a 3:1:1:5 ratio. The slides were incubated at 37°C, 5% CO₂ for 25 minutes to allow matrix

polymerisation, before cell culture medium (without antifungal) was added to both adjacent reservoirs, and cells were allowed to stretch out overnight. The next day, medium was removed from one reservoir, and 15 μL liquid containing 10^2 *C. albicans* ATCCMYA2876 yeast in PBS (untreated) or pilocarpine at 10 and 50 mM were added. Chambers were then incubated overnight at a 90° angle to let the *C. albicans* yeast sink onto the side of the collagen matrix, and allow for hyphae formation and growth into the central channel. The next day, medium was removed from the uninfected reservoir, and neutrophils at $5 \times 10^5/\text{mL}$ in RPMI + 10% autologous serum were gently added. Chambers were then incubated horizontally at 37°C , 5% CO_2 for two hours, and imaged using a widefield microscope to quantify the number of neutrophils migrated into the central observation area containing fibroblasts in collagen matrix.

2.17. Statistical analysis

Flow cytometry data was analysed using FCS Express 7 Research Edition and FlowJo (v10.10.0). Data was analysed using GraphPad Prism 9.5.1 (GraphPad Software, San Diego, CA, USA). Statistical differences were determined via repeated measures (RM)- or one-way ANOVA with subsequent comparisons as indicated in figure legends. Independent experiments were performed at least three times.

3. Results

Pilocarpine treatment reduces yeast and filamentous fungal biomass and metabolism

The fungistatic effect of pilocarpine has previously been demonstrated against *Candida albicans* yeast (12), however, its effect on filamentous fungi remained unknown. Filamentous fungi are important causes of a range of infections, therefore inhibition of their growth by pilocarpine hydrochloride could provide a new treatment option. *Aspergillus* spp. and *Fusarium* spp., in addition to *C. albicans* are important fungal pathogens causing corneal infections (19), and would be amenable to topical application of pilocarpine hydrochloride ophthalmic solutions. We therefore selected *Aspergillus fumigatus* and *Fusarium keratoplasticum* as exemplar filamentous fungi to measure fungistatic effects of pilocarpine. Along with *C. albicans*, these strains were grown in the presence of increasing concentrations of pilocarpine, and biomass and metabolic activity were measured. Both biomass and metabolic activity decreased in a dose dependent manner for all three fungal strains, and the required concentration of pilocarpine was comparable across the fungal species to achieve the same effect. Doses between 5–8 mM pilocarpine caused a significant reduction in biomass (Fig. 1a), and 8–10 mM resulted in a significant reduction in metabolic activity (Fig. 1b). A visible reduction in hyphae length, up to complete suppression of hyphae formation was observed for all three fungi when treated with pilocarpine at these concentrations (Fig. 1c).

Pilocarpine induces apoptosis of neutrophils in a concentration-dependent manner

Neutrophils act as the first responders to fungal infections. As a first step to characterise the effect of pilocarpine on human neutrophil viability and the mechanism of potential cytotoxic effects, neutrophils were incubated with freshly prepared pilocarpine at a range of concentrations previously evaluated against the fungi (0–50 mM). An induction of early apoptosis, measured by externalised phosphatidylserine (via Annexin V), was observed after three hours of treatment with 50 mM pilocarpine (Fig. 2a). A concentration-dependent but not statistically significant trend of increased apoptosis at lower concentrations was observed after six hours (Fig. 2a). Notably, the induction of apoptosis by 50 mM pilocarpine after six hours could be completely inhibited by pan-caspase inhibitor Q-VD-OPh (QVD) (Fig. 2a).

Secondary necrosis (measured by Draq7) significantly increased at three hours only for the 50 mM concentration, and after six hours starting from 10 mM pilocarpine (Fig. 2b). Morphologic changes of nuclear shape consistent with apoptosis were confirmed at six hours (Fig. 2c).

Consequently, concentrations of 10 mM and 50 mM pilocarpine were chosen for all subsequent experiments for several reasons. Firstly, these were the concentrations showing effects on both fungal growth and neutrophil apoptosis/secondary necrosis in our analysis. Secondly, 10.5 mM pilocarpine was used to analyse the effects of pilocarpine on *C. albicans* infection in *G. mellonella* larvae, indicating an influence on immune cells (12). Finally, 50 mM is a concentration of pilocarpine commonly used for clinical treatment of non-infectious eye conditions (clinical formulations range 1–4% w/v, equalling 40.87–163.4 mM pilocarpine hydrochloride).

Neutrophil activation is inhibited by pilocarpine via influence on receptor expression but not calcium signalling

After confirming that 10–50 mM pilocarpine induces neutrophil apoptosis over 3–6h, we next examined earlier effects of pilocarpine on neutrophil activation. A significant reduction of CD66b expression was observed in neutrophils treated with both 10 mM and 50 mM pilocarpine during N-formylmethionine-leucyl-phenylalanine (fMLF) stimulation (Fig. 3a). Likewise, the induction of CD11b expression by fMLF was markedly reduced in pilocarpine-treated neutrophils (Fig. 3b).

Neutrophil activation was determined by quantifying **(a)** CD66b and **(b)** CD11b expression by untreated and N-formylmethionine-leucyl-phenylalanine (fMLF, 100 nM) stimulated neutrophils, treated with or without pilocarpine (pilo., 10 mM and 50 mM). Relative geometric mean fluorescence intensity (gMFI) expressed as % of untreated neutrophils, $\pm$ SD ($n = 4$, individual data points are shown). The gating strategy is reported in supplementary figure S2. A one-way ANOVA followed by Tukey's multiple comparisons test displaying comparisons between untreated cells to respective treatments was conducted. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Calcium flux was measured using Fluo-4 AM. **(c)** representative calcium trace, **(d)** quantification of calcium flux, maximum relative fluorescence (RFU)/minimum RFU. Data represent mean $\pm$ SD ($n = 5$, individual data points are shown). A one-way ANOVA followed by Šídák's multiple comparisons test was performed, comparing pilocarpine treatment to the respective control, as well as between non-pilocarpine treated conditions. * $p < 0.05$, ** $p < 0.01$.

Calcium flux is a hallmark of neutrophil activation processes. We measured the influence of pilocarpine treatment on calcium flux of unstimulated and concurrently fMLF-stimulated neutrophils. fMLF at 100 nM induced a significant amount of intracellular calcium. Meanwhile, pilocarpine did not significantly influence the calcium flux in unstimulated or fMLF-stimulated neutrophils. However, a concentration-dependent trend in reduction of fMLF-induced calcium flux was observed (Fig. 3c, d).

Shape change, morphologic changes, and chemotaxis in response to inflammatory mediator N-formylmethionine-leucyl-phenylalanine (fMLF) are reduced by pilocarpine

Neutrophil activation goes in hand with a shape change of the cell, to allow for migration towards the stimulating signal. This morphological change was examined in response to fMLF using forward scatter area (FCS-A) measurement of unstained neutrophils in the presence and absence of pilocarpine (Fig. 4a, b). While pilocarpine alone did not affect neutrophil shape, the widening of FSC-A induced by fMLF was reverted back to baseline upon pilocarpine treatment at 50 mM. This influence on shape change was further confirmed by imaging flow cytometry and subsequent analysis of neutrophil area, perimeter, circularity, and eccentricity (Fig. 4c, d). Neutrophil area was the only measure significantly influenced by pilocarpine treatment (50 mM) in absence of additional stimulation, hinting at cell shrinking without affecting roundness. Meanwhile, fMLF-induced morphologic changes were concentration-dependently suppressed by pilocarpine. Functionally, the inhibition of shape change by 50 mM pilocarpine treatment is reflected in complete inhibition of migration over transwell filters towards fMLF as chemoattractant (Fig. 4e).

Pilocarpine influences neutrophil-mediated antimicrobial activity against *C. albicans*

While pilocarpine itself is fungistatic, not fungicidal (Fig. 5), we next sought to investigate how pilocarpine may influence the antimicrobial activity of neutrophils. Neutrophils were exposed to *C. albicans* yeast during treatment with pilocarpine at 10 and 50 mM for 2 hours, followed by neutrophil lysis (to enable quantification of both extracellular and intracellular viable pathogens). *C. albicans* viability was measured by assessment of colony-forming units (CFU). Killing of *C. albicans* by neutrophils was significantly reduced upon pilocarpine treatment (Fig. 5).

Neutrophil defence mechanisms phagocytosis, release of reactive oxygen species (ROS), degranulation, and neutrophil extracellular traps (NETs) are downregulated by pilocarpine

To investigate the reason for reduced antimicrobial activity against *C. albicans* during treatment with pilocarpine, neutrophil mechanisms to combat pathogens were examined. This included phagocytosis to investigate if uptake of the pathogen was influenced, reactive oxygen species (ROS) generation and neutrophil degranulation. Pilocarpine treatment at 50 mM significantly reduced phagocytosis of *C. albicans* yeast (Fig. 6a).

Further, the generation of intracellular ROS and total ROS (encompassing extracellular released and intracellular ROS) were investigated. Intracellular ROS (Fig. 6b) measured by DHR fluorescence intensity

resulted in a significant increase within 30 min using 20 nM phorbol 12-myristate 13-acetate (PMA) as positive control, but not with *C. albicans* yeast at the tested MOI of 2. This induction of intracellular ROS by PMA was significantly reduced by pilocarpine treatment at 10 mM and 50 mM concentrations. Conversely, measurement of total ROS (Fig. 6c) revealed a significant increase of total ROS levels triggered by *C. albicans* yeast (also MOI: 2), which were completely inhibited by simultaneous pilocarpine treatment at both tested concentrations.

In addition, the influence of pilocarpine on degranulation of antimicrobial contents was examined by ELISA, using matrix metalloproteinase-9 (MMP-9) release as an example (Fig. 6d). A significant increase of released MMP-9 was induced by *C. albicans* yeast (MOI: 10). Pilocarpine treatment resulted in reduced levels of MMP-9 after both fMLF and *C. albicans* yeast stimulation.

Next, the influence of pilocarpine at 10 and 50 mM on neutrophil extracellular trap (NET) release was assessed by immunofluorescence staining using DNA/histone-1-complexes and myeloperoxidase as NET markers (Fig. 7). Pilocarpine itself did not induce NET release in otherwise unstimulated neutrophils (Fig. 7a). In addition, many neutrophils lost their polymorphonuclear shape and displayed a small, round nuclear shape indicative of apoptosis at 50 mM pilocarpine. However, there was a strong inhibition of NADPH oxidase-dependent NETs triggered by PMA with the higher concentration of pilocarpine (Fig. 7b), feeding into the data previously displayed for inhibition of ROS by pilocarpine. To examine the effect of *C. albicans* yeast and hyphae on pilocarpine treated neutrophils, independently of the direct effects that pilocarpine has on live fungi, NETs were analysed in the presence of dead yeast (Fig. 7c) and dead hyphae (Fig. 7d). Heat-inactivated yeast alone induced a small number of NETs, but concurrent treatment with pilocarpine did not significantly affect the induction of NET-release. While extracellular yeast was almost completely cleared in the untreated and 10 mM pilocarpine conditions, more extracellular yeast can be seen in the brightfield image at 50 mM pilocarpine. This confirms the reduction in phagocytosis of yeast that we previously observed using flow cytometry. Ethanol-inactivated *C. albicans* hyphae alone induced NETs, which pilocarpine treatment did not affect significantly. Finally, in a set-up where untreated live *C. albicans* yeast develop into hyphae throughout the course of the experiment, an effect of 10 mM but not 50 mM pilocarpine on NET release was seen (Fig. 7e).

Pilocarpine treatment influences fungal growth, viability of cornea stromal cells, and migration of immune cells in a three-dimensional cell culture model of the infected cornea

Finally, we demonstrate a potential clinical application of pilocarpine treatment in a novel model of microbial keratitis, an infection of the cornea (Fig. 8). The three-dimensional *in vitro* model of fungal keratitis caused by *C. albicans* was established repurposing μ -slide chemotaxis chambers (Fig. 8a). In this system, corneal fibroblasts are grown inside a collagen I matrix in the middle channel, emulating the corneal stroma composition. Next, an infection with *C. albicans* is established with or without pilocarpine treatment from one side, and neutrophils are added to the opposite side after the fungi formed hyphae. This system allows neutrophils to migrate into the model towards the site of fungal infection, while mimicking topical eye-drop mediated treatment with pilocarpine. The time course of the model set-up is

highlighted in Fig. 8b. Pilocarpine-treated models showed reduced hyphal growth into the stromal compartment upon pilocarpine treatment (Fig. 8c). This resulted in reduced migration of neutrophils into the collagen matrix (corneal stroma) (Fig. 8d). In addition, morphological changes of fibroblasts were observed depending on pilocarpine concentration. While untreated fibroblasts remain elongated, the cells in the 50 mM-treated model rounded up in proximity to the site of drug application, and displayed cytoplasmic vacuolation further away (highlighted in Fig. 8c-i, ii).

4. Discussion

There is a great need for novel and improved antifungal treatment strategies for localised and systemic fungal infections, especially in the light of rising antifungal resistance. In this study, we have demonstrated a dual effect of pilocarpine hydrochloride on fungal growth and immune responses mediated by human neutrophils. While decreased filamentation and metabolic activity of the yeast *Candida albicans* had been demonstrated previously (12), we observed similar effects on filamentous fungi *Aspergillus flavus* and *Fusarium keratoplasticum* (Fig. 1). This suggests that pilocarpine may be useful for localised treatment of fungal infections beyond *Candida* species. *Aspergillus* spp., *Fusarium* spp., and *Candida* spp. are all on the World Health Organisation (WHO) fungal priority pathogens list (20), hence an effect by pilocarpine on their growth and biofilm formation targets some of the most critical fungal pathogens.

Since neutrophil granulocytes are the fast-responding, predominant immune cell type migrating towards the site of fungal infection, we further investigated the effect of pilocarpine on human blood-derived neutrophils. Pilocarpine has previously been shown to behave as muscarinic receptor M3 agonist or antagonist depending on the cell type and expression level of the receptor (21), but no data for neutrophils has been published to our knowledge. Pilocarpine could potentially interact with human neutrophils via muscarinic receptors, as these cells express M3 receptors (22). Neutrophil M3 receptor stimulation mediated by other drugs (e.g., levamisole) enhanced ROS and NET formation (23), whereas an M3 antagonist (4-DAMP) diminished neutrophil infiltration in lipopolysaccharide-induced lung injury (24). This shows that M3 interaction can result in a detrimental boost of immune-mediated activity, or dim it down.

We observed a significant induction of apoptosis (Fig. 2), a non-phlogistic type of cell death which promotes clearance of immune cells from the tissue and thereby contributes to resolution of inflammation. The selective induction of neutrophil apoptosis by other drugs, such as cyclin-dependent kinase inhibitors (25), resolvin E1 (26), or TNF-related apoptosis-inducing ligand (27), has proven beneficial in various types of neutrophilic inflammation. While it seems counterintuitive to suppress the antimicrobial response of neutrophils in an acute infection by localised treatment, the additional suppressive effect of pilocarpine on fungal growth might balance the lack of neutrophil response at the site of infection, and further enhance treatment susceptibility to antifungals by preventing biofilm formation. A major benefit of increased neutrophil apoptosis, and therefore decreased antimicrobial

activity, is the reduction of bystander tissue damage through release of histotoxic substances (28, 29). Moreover, apoptotic neutrophils themselves are an anti-inflammatory stimulus to other cell types (29).

We have further demonstrated that pilocarpine has a direct inhibitory effect on neutrophil activation as quantified by CD11b and CD66b receptor expression (Fig. 3). This downregulation of integrin CD11b was observed in the presence and absence of further stimulation by fMLF, while an increase of the driver of adhesion CD66b was inhibited by pilocarpine. Interestingly, pilocarpine did not induce calcium flux in neutrophils, nor did it significantly alter the calcium flux triggered by fMLF. This is in contrast to observations made in other cell types including skin fibroblasts and ciliary muscle cells, where pilocarpine drives cellular apoptosis through an increase of intracellular calcium (30, 31). However, no effect of pilocarpine on calcium mobilisation has been reported for human embryonic kidney cells and mouse insulinoma cells (21). Altogether, this suggests a cell-type dependent influence of pilocarpine on calcium signalling.

A further indicator for neutrophil activation is the measurement of shape change by forward scatter in label-free flow cytometry, and imaging flow cytometry reveals changes in cell size and morphology at a single cell level (32). Pilocarpine not only suppressed neutrophil activation and subsequent shape change induced by fMLF (Fig. 4), but also reduced cell size (neutrophil area) at 50 mM. As cell polarisation is required for extravasation and migration through tissues (33), we also observed reduced migration of neutrophils over transwell filters upon treatment with pilocarpine. This reduced migration of neutrophils is in agreement with a study investigating moth-larvae hemocyte responses to *Candida* infection (12). Here, granulocyte numbers were reduced in response to *Candida* infection upon pilocarpine treatment. This effect might be enhanced by a reduced release of chemokines IL-6 and IL-8 when treating fungal infection with pilocarpine, as observed on oral epithelial cells (34). In addition, a reduction of IL-6 release by immune cells (microglia, the tissue-resident macrophages of the central nervous system) through pilocarpine treatment has been shown (35). The same study also found an influence on shape change, reporting a recovery of morphological changes induced in microglia by lipopolysaccharide stimulation (35).

When evaluating the effect of pilocarpine on the interaction of neutrophils with fungi, we observed reduced killing of *C. albicans* (Fig. 5). There are two pathways for killing of *Candida* by neutrophils: Complement receptor 3 (CD11b) dependent killing of unopsonised *C. albicans*, and an Fcγ receptor dependent mechanism involving protein kinase C and ROS production for antibody-opsonised *C. albicans* (36). We assessed the latter pathway in our killing assay using antibody-opsonised *C. albicans*. However, the complement receptor 3 dependent pathway would likely be reduced as well under pilocarpine influence, due to the observed reduction in CD11b expression.

To further assess if neutrophil defence functions are directly affected by pilocarpine, or if their activity is merely reduced due to less fungal metabolic activity and sensing of the pathogen, we also examined ROS production (Fig. 6b, c), degranulation (Fig. 6d), and NETosis (Fig. 7) in response to both chemical stimuli and fungal pathogen (*C. albicans*). All of these functions, as well as phagocytosis of *C. albicans*

yeast (Fig. 6a), were reduced by pilocarpine treatment. This goes against previous findings in other cell types, where pilocarpine induced ROS/NO in resident host cell types (30, 31) as well as in *C. albicans* after 24 hours treatment (15). However, it does agree with findings made in microglia, where reduced phagocytosis of latex beads was observed under pilocarpine influence (35). This reduced phagocytosis might explain why amounts of total ROS are reduced upon pilocarpine treatment, since phagocytosis triggers intracellular ROS production (37).

A particular picture was observed for NET induction in response to different stimuli (Fig. 7), as NADPH oxidase-dependent NETs (induced by PMA) were downregulated by pilocarpine, whereas NET release triggered by dead *C. albicans* yeast and hyphae was unchanged by pilocarpine. The response of neutrophils to dead fungal forms was examined to investigate their influence on NETs without the additional influence of pilocarpine on fungal metabolism and yeast-to-hyphae transition. This is applicable, since NETosis is not dependent on microbial enzymatic activity, and heat-inactivated hyphae induced NET release as efficiently as live hyphae (8). The unchanged induction of NETs despite downregulation of NADPH-dependent NETosis can be explained by the multiple ways *Candida* induces NETs. Beyond ROS-dependent NET induction, unopsonised *C. albicans* triggers NADPH oxidase-independent NETs through dectin-2, mediated by calcium signalling (38), a signalling pathway we found to be unchanged in neutrophils by pilocarpine. Moreover, Candidalysin induces NETs over both NADPH oxidase activation and calcium influx (39). Ultimately, using live yeast developing into hyphae throughout the course of the assay, a significant reduction of NETs by 10 mM but not 50 mM pilocarpine was detected. This might be due to the finding that phagocytosis of yeast suppresses NET induction, however neutrophils revert to NETosis if phagocytosis is blocked (8). While phagocytosis was mostly unchanged at 10 mM pilocarpine (Fig. 6d), it is significantly inhibited at 50 mM pilocarpine. In addition, *C. albicans* hyphae remain smaller at 10 mM compared to untreated conditions, potentially resulting in improved phagocytosis of the smaller yeast forms. Overall, NET release in response to *C. albicans* growth forms seem largely unchanged by pilocarpine treatment.

The modifications in neutrophil mechanisms we observed are contradictory to expected muscarinic agonist effects. M3 receptor (M3R) subtypes are widely accepted to be G_q -coupled receptors, mediating an increase in intracellular calcium as downstream effect (40). One would therefore expect to see neutrophil activation. However, other G protein families can also interact with M3R, with G_s inducing an increase of the second messenger cyclic adenosine monophosphate (cAMP), and G_i a decrease of the latter (40). cAMP induction in neutrophils promotes resolution of inflammation by reducing pro-inflammatory cytokine release, migration, phagocytosis, ROS production (41), and NETs (42, 43), and by inducing granulocyte apoptosis (41). It has been suggested that non- G_q G proteins could be the primary effector of M3R in certain cell types or tissues (40).

Another explanation could be the fact that pilocarpine has previously been shown to act as partial agonist or antagonist of the M3 receptor in some cell types, depending on the level of M3R (21). For instance, pilocarpine does not induce calcium mobilisation via endogenous M3R in human embryonic kidney cell line 293T or mouse insulinoma cells, but rather antagonises other M3R agonists

(oxotremorine-M and carbachol) (21). It was suggested that pilocarpine does not activate but rather inhibits G_q -dependent signalling of M3R, and acts as partial agonist biased toward the β -arrestin-Src pathway in these cells (21). β -arrestin 2-biased activation by pilocarpine was also reported for microglial cells, resulting in suppression of their inflammatory response (35). This study observed similar effects in microglia as we have seen here in neutrophils, regarding suppression of phagocytosis and morphologic changes. It further proved the pathway by blocking pilocarpine-induced effects in microglial cells through β -arrestin 2 knockdown, and by demonstrating that an activation of β -arrestin but not G_q or G_i inhibits the inflammatory response (35). However, they did not investigate G_s activation.

While the effect of pilocarpine on the β -arrestin pathway in neutrophils had not been investigated so far, it is known that β -arrestin 1 and 2 are important regulators of neutrophil responses. They affect cytokine production, recruitment, and granule release (44). For example, β -arrestin 2 deficient mice have pro-inflammatory neutrophils, showing enhanced recruitment and increased expression of CD18 and CD62L *in vivo* (45) and enhanced calcium mobilisation and superoxide anion production *in vitro* (46). It was therefore suggested that β -arrestin 2 may downregulate neutrophil cytokine production and adhesion receptor expression upon inflammation (47). Ultimately, it has been suggested that β -arrestin expression is differently regulated in immune cells and tissues in inflammation (47), and that M3R plays a proinflammatory role on structural cells but not on immune cells in neutrophilic inflammation (48).

Finally, we assessed a potential application of pilocarpine treatment for fungal infection using a novel three-dimensional cell culture model of microbial keratitis, caused by *C. albicans* infection (Fig. 8). Pilocarpine is widely used in ophthalmology to treat non-infectious and non-inflammatory conditions such as glaucoma and presbyopia (age-related farsightedness). Using this cornea infection model, we confirmed the inhibitory effects of pilocarpine on both fungal growth and neutrophil migration. However, we also observed induction of cell death in corneal fibroblasts within the cornea model. This is in agreement with a previous study, where morphological abnormalities and apoptosis were caused in human corneal stromal cells *in vitro* by pilocarpine above 2.55 mM (49). Similarly, pilocarpine is toxic towards other ocular cell types *in vitro*, namely meibomian gland epithelial cells, at clinically relevant doses (4% w/v (163.45 mM)) and at a tenth of this concentration (50). Likewise, prolonged exposure (daily use over three months) lead to corneal epithelial and endothelial damage in rabbits (51). However, no adverse corneal effects, measured by corneal thickness and endothelial cell counts, were observed in a study examining human glaucoma patients undergoing 4% pilocarpine treatment over 18–78 months (52). In addition, keratocytes in the adjacent corneal stroma become apoptotic upon injury and in microbial keratitis, independent of pathogen type and treatment (53). This process results in an acellular stromal area, and is suggested to have protective effects by preventing the release of tissue-damaging intracellular contents and by inhibiting spread of the pathogen (53, 54). Keratocyte apoptosis is further involved in the initiation and modulation of corneal wound healing (54), and therefore the apoptosis that we observed in our model may be beneficial, does not necessarily preclude pilocarpine as a potential therapy for corneal infection or inflammation. However, pilocarpine-induced pupillary constriction (55) would be an unwanted effect in this context, and requires further investigation to determine whether the

benefits of reduced fungal growth and attenuation of the inflammatory response outweigh the associated mitotic effect for this application.

5. Conclusions

The majority of fungal infections worldwide are superficial (56), and rising antifungal resistance enhances the need for novel treatment approaches. We have demonstrated a dual effect of pilocarpine hydrochloride, a drug interacting with muscarinic receptors, on both fungi and immune response. Pilocarpine treatment dims down the neutrophil response, which could help to resolve host tissue damage. The observed induction of apoptosis might further promote quiescent removal by efferocytosis. This is particularly important in conditions where tissue inflammation goes along with long term detrimental effects, such as microbial keratitis, in which corneal proteolysis results in visual impairment and corneal perforation. Further research on tissue compatibility and use as an adjunct treatment to conventional antifungals is needed, however, we demonstrate here that pilocarpine hydrochloride might be of broad interest as anti-inflammatory drug in further neutrophil-driven inflammatory conditions.

Declarations

Ethics approval and consent to participate: Blood was collected through the CIR Blood Resource from healthy human volunteers following informed consent and ethical approval at the University of Edinburgh (21-EMREC-041 and 25-EMREC-023).

Consent for publication: Not applicable

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. Schematics created with BioRender are available at the following URLs: *(to be provided after manuscript acceptance)*.

Competing interests: AGR serves as the co-Editor-in-Chief of the Journal of Inflammation. To ensure impartiality, the peer-review of this manuscript was handled by an independent member of the editorial board, and the submission underwent the standard review process applied to all manuscripts.

Funding: This work was funded by a UKRI Future Leaders Fellowship (No. MR/V026097/1). A.G.R. was funded by a Medical Research Council (MRC) UK Programme Grant (MR/K013386/1). JG was supported by The Baillie Gifford Pandemic Science Hub. For the purpose of open access, the authors have applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising from this submission.

Authors' contribution: LF: Conceptualisation, Investigation, Data curation, Formal analysis, Methodology, Writing – original draft; FB and JG: Investigation, Data curation, Formal analysis, Methodology; SV:

Methodology, Editing; AGR: Methodology, Supervision, Editing; BM: Conceptualisation, Supervision, Funding acquisition, Editing. All authors read and approved the final manuscript.

Acknowledgements: Microscopy was carried out in the IRR Imaging facility at the University of Edinburgh. Flow cytometry was supported by the IRR Flow cytometry facility at the University of Edinburgh. The *C. albicans* CAF2-dTomato strain was gifted from the Munro group, the University of Aberdeen. The corneal fibroblast cell line hTK was gifted by the Stanton group, the University of Edinburgh.

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Figures

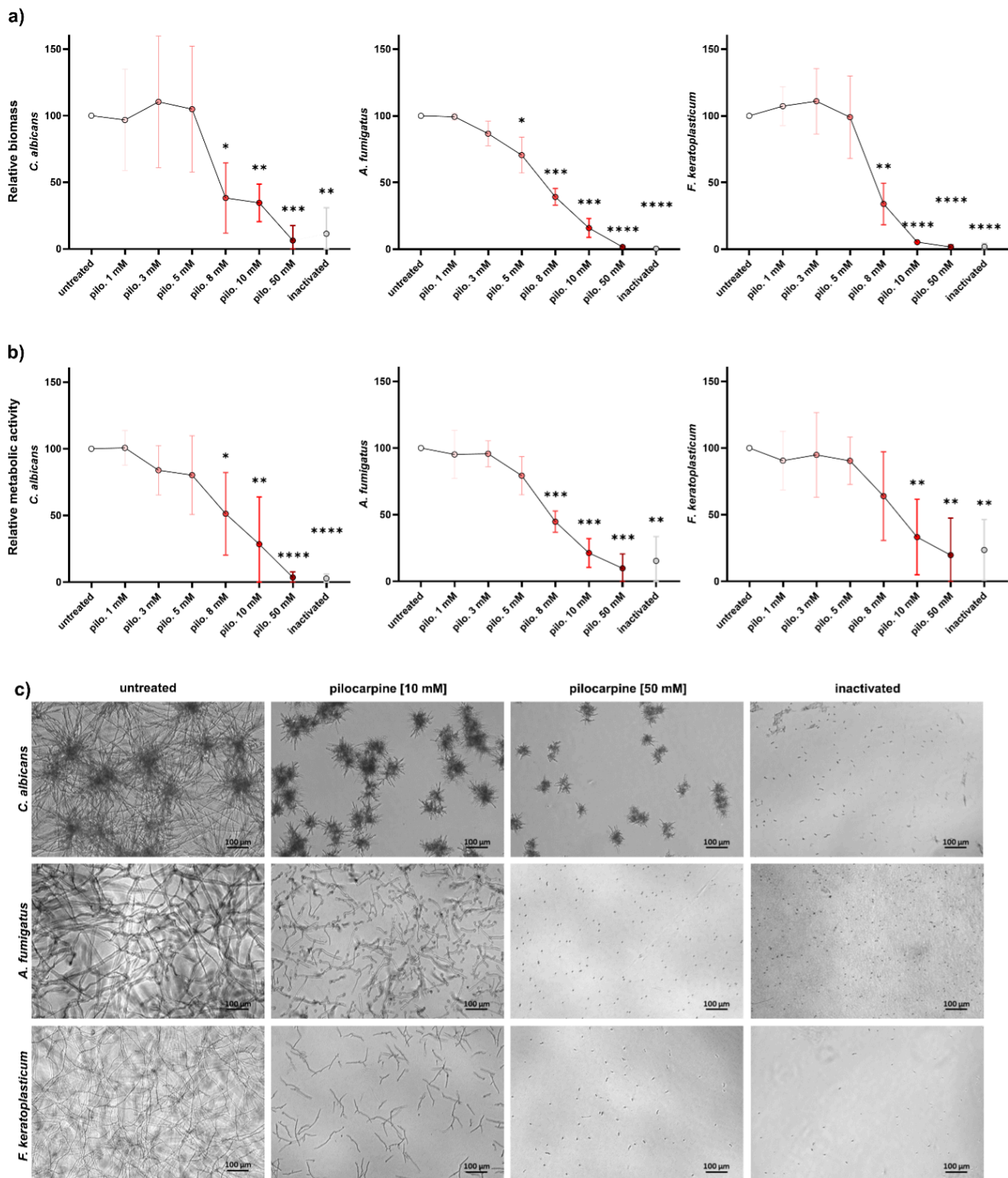


Figure 1

Pilocarpine inhibits biomass, metabolism, and hyphae formation in yeast and filamentous fungi.

Candida albicans, *Aspergillus fumigatus*, and *Fusarium keratoplasticum* yeast/spores were treated with pilocarpine (pilo. 1-50 mM) for 18 h. Control groups consisted of untreated and inactivated (70% ethanol) fungi. After treatment, **(a)** biomass was measured using crystal violet, **(b)** metabolism using XTT, and **(c)**

representative brightfield images showing fungal morphology were taken. Data represent mean $\pm$ SD (n=4-6). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by two-tailed one sample t-test.

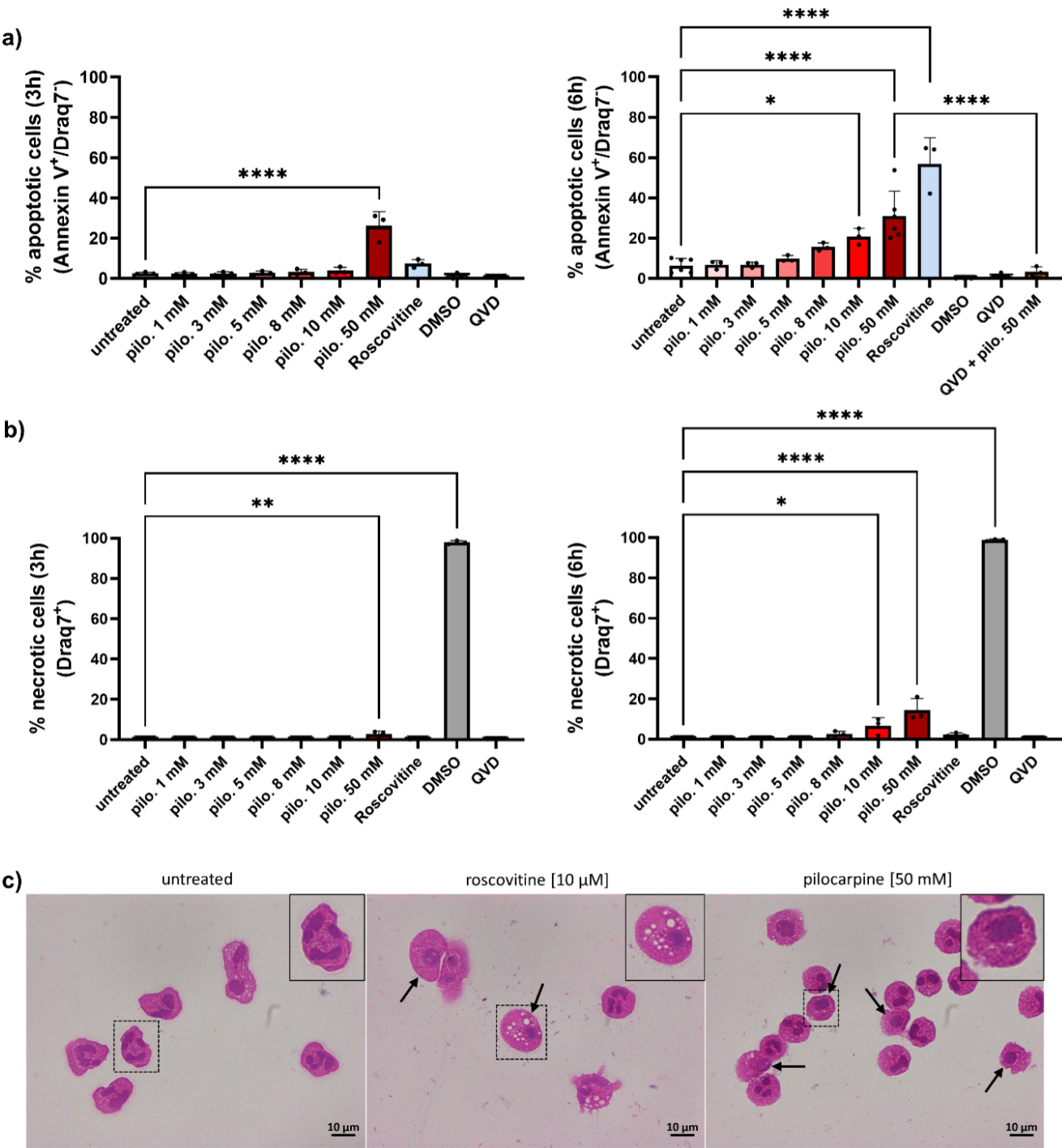


Figure 2

Cytotoxicity of pilocarpine towards human neutrophils. Concentration (1-50 mM) and time dependent (3h and 6h) induction of neutrophil **(a)** early apoptosis (Annexin V⁺/Draq7⁻ cells) and **(b)** necrosis (Draq7⁺

cells) by pilocarpine. Controls included roscovitine (10 μ M) for apoptosis induction, dimethyl sulfoxide (DMSO, 50% v/v) for necrosis induction and Q-VD-OPh (QVD; 10 μ M) for suppression of both. Bars show mean $\pm$ SD % of neutrophils with indicated staining pattern (n=3-6, indicated by individual data points). The gating strategy is displayed in Fig. S1. An ordinary one-way ANOVA followed by Dunnett's multiple comparisons test comparing untreated cells to respective treatments was conducted. *p<0.05, **p<0.01, ****<0.0001. **(c)** Cellular morphology was confirmed by cytocentrifuge of untreated, roscovitine (10 μ M), and pilocarpine (50 mM) treated neutrophils at 6h. Arrows indicate loss of nuclear lobulation typical for apoptotic cells. Inserts show magnified exemplary cells. Scale bar 10 μ m.

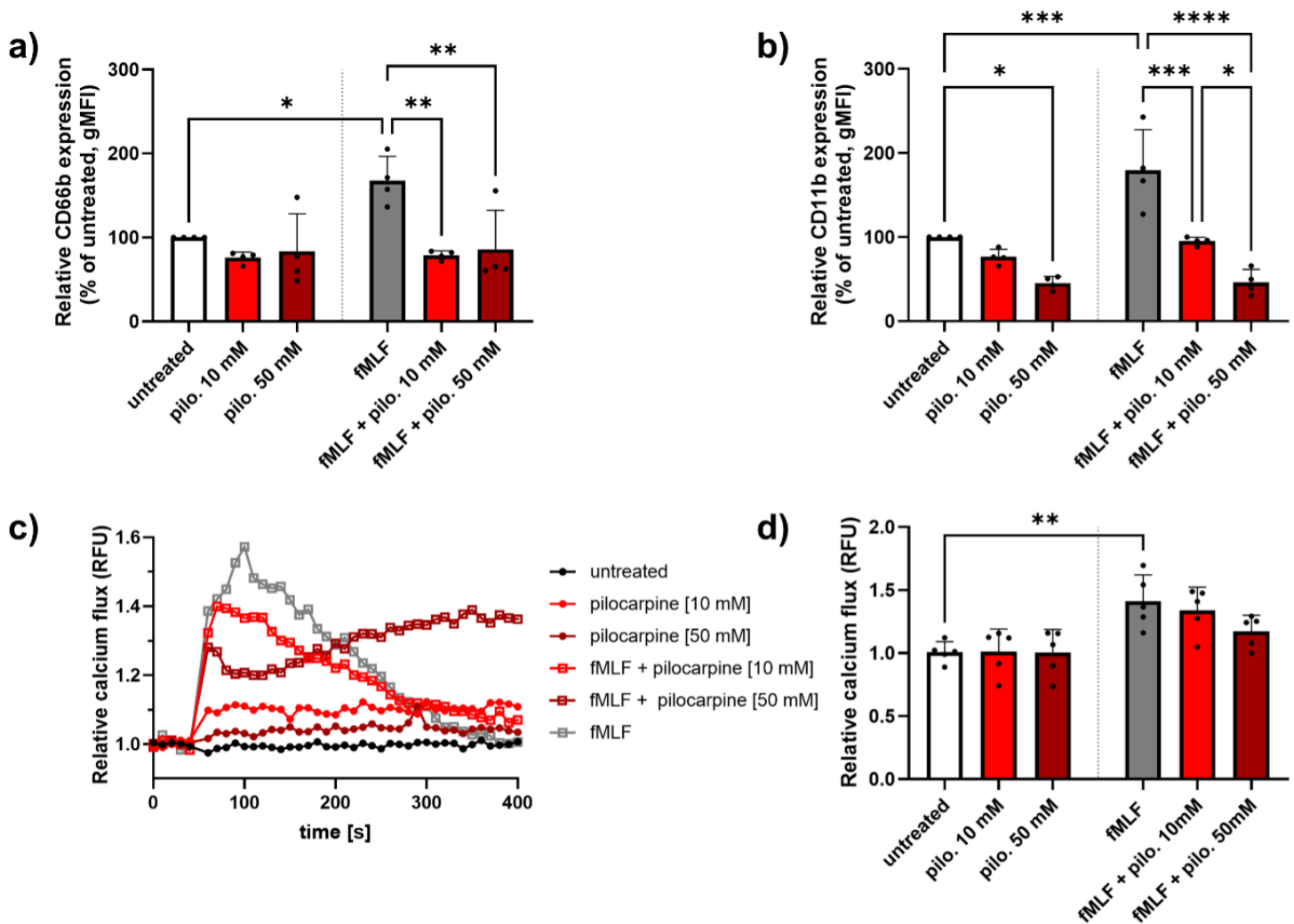


Figure 3

Influence of pilocarpine on neutrophil activation marker expression and calcium signalling.

Neutrophil activation was determined by quantifying **(a)** CD66b and **(b)** CD11b expression by untreated and N-formylmethionine-leucyl-phenylalanine (fMLF, 100 nM) stimulated neutrophils, treated with or without pilocarpine (pilo., 10 mM and 50 mM). Relative geometric mean fluorescence intensity (gMFI) expressed as % of untreated neutrophils, $\pm$ SD (n=4, individual data points are shown). The gating strategy is reported in supplementary figure S2. A one-way ANOVA followed by Tukey's multiple

comparisons test displaying comparisons between untreated cells to respective treatments was conducted. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Calcium flux was measured using Fluo-4 AM. **(c)** representative calcium trace, **(d)** quantification of calcium flux, maximum relative fluorescence (RFU)/minimum RFU. Data represent mean $\pm$ SD ($n=5$, individual data points are shown). A one-way ANOVA followed by Šídák's multiple comparisons test was performed, comparing pilocarpine treatment to the respective control, as well as between non-pilocarpine treated conditions. * $p<0.05$, ** $p<0.01$.

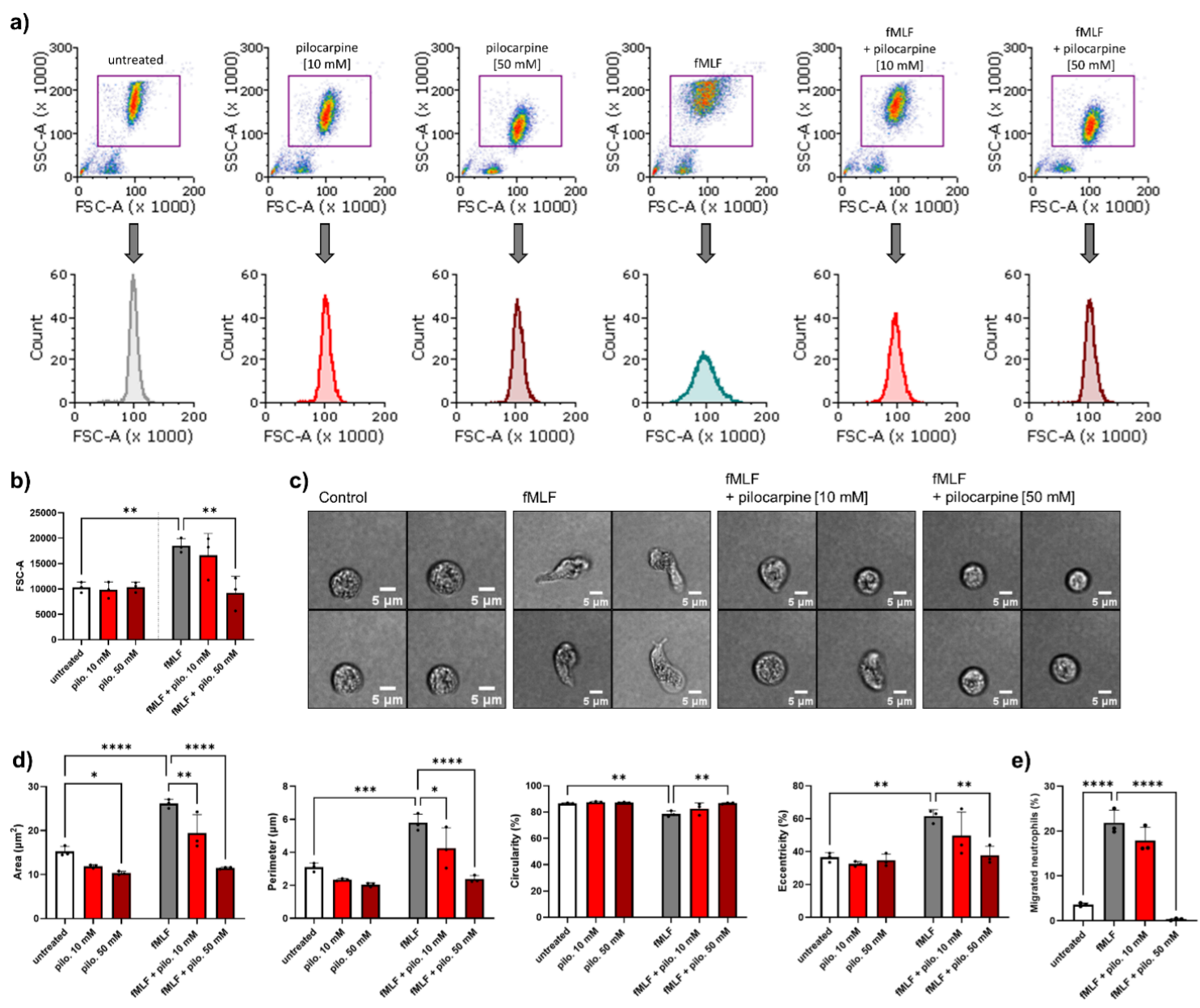


Figure 4

Influence of pilocarpine on neutrophil shape change and migration. Neutrophil shape change was determined by flow cytometry following 30 min stimulation with or without N-formylmethionine-leucyl-phenylalanine (fMLF, 100 nM) and treatment with pilocarpine (10 mM or 50 mM). **(a)** Representative flow cytometry forward scatter area (FSC-A) histograms, **(b)** FSC-A standard deviation quantification. Data represent mean $\pm$ SD ($n=3$, individual data points shown), the gating strategy is depicted in

supplementary figure S3. A one-way ANOVA followed by Šídák's multiple comparisons test was performed, comparing pilocarpine treatment to the respective control, as well as between non-pilocarpine treated conditions. $**p<0.01$. **(c)** Representative images of neutrophil morphology after treatment, obtained by imaging flow cytometry. **(d)** Quantification of morphological changes. Data represent mean $\pm$ SD ($n=3$, individual data points shown). The gating strategy is depicted in supplementary figure S4. A one-way ANOVA followed by Šídák's multiple comparisons test was performed, comparing pilocarpine treatment to the respective control, as well as between non-pilocarpine treated conditions. $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$. **(e)** Neutrophil chemotaxis towards fMLF (10 nM) across a transwell filter with or without prior pilocarpine treatment (10 mM or 50 mM, 30 min) was quantified by cell count of transmigrated neutrophils after 90 min. Data represent mean $\pm$ SD ($n=3$, individual data points shown). A one-way ANOVA followed by Dunnett's multiple comparisons test comparing treatments to positive control (fMLF) was conducted. $****<0.0001$.

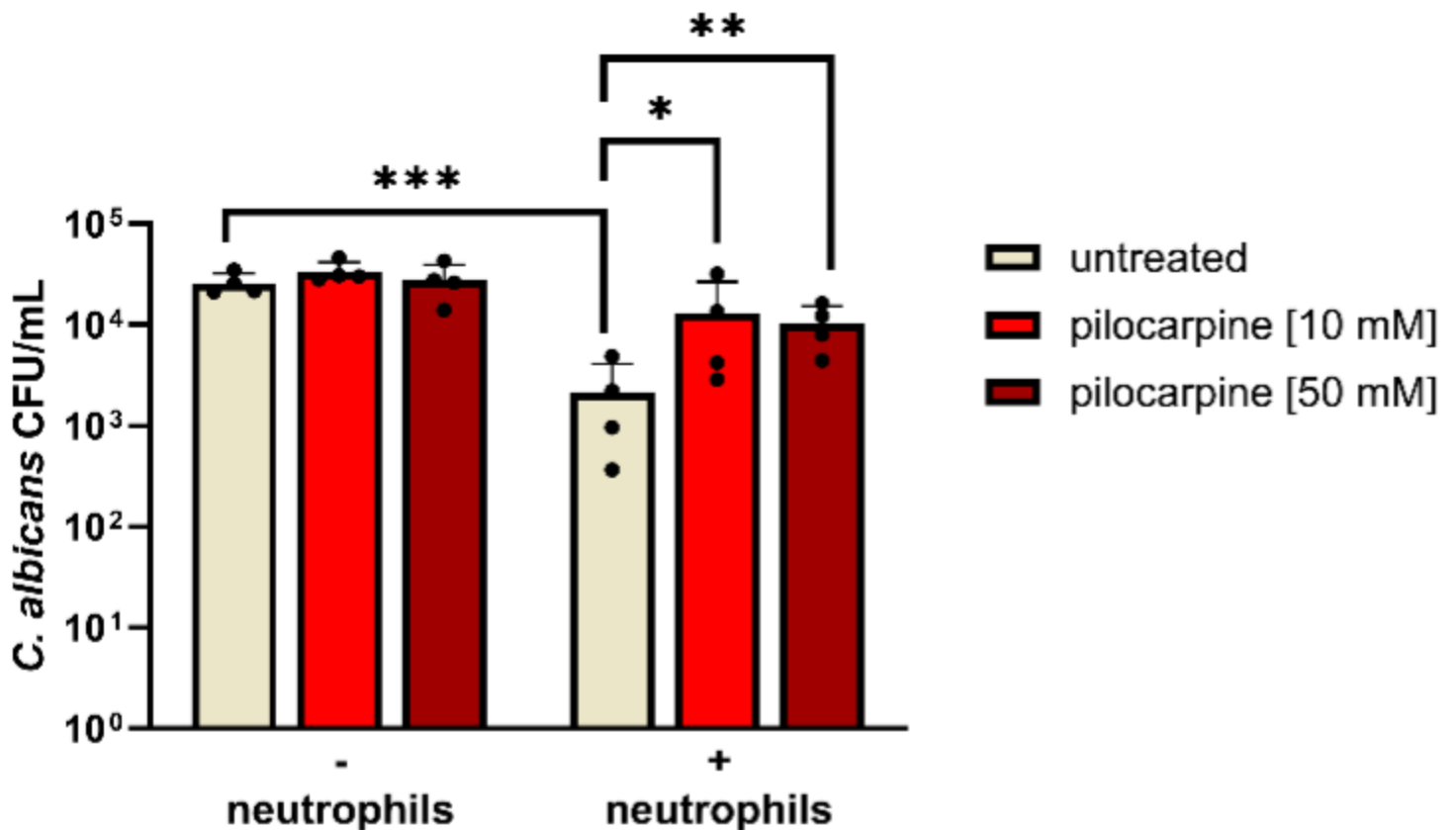


Figure 5

Killing of *C. albicans* by neutrophils during pilocarpine treatment. Inactivation of *C. albicans* yeast by neutrophils was quantified by counting colony-forming units (CFU), plated after 2h incubation of yeast with neutrophils, with or without pilocarpine (10 mM or 50 mM). Multiplicity of infection (MOI): 0.01. Data represent mean $\pm$ SD ($n=4$, individual data points are shown). An ordinary one-way ANOVA followed by Šídák's multiple comparisons test was performed on log-transformed CFU data, comparing treatment to respective controls, as well as between controls (yeast with or without neutrophils). $*p<0.05$, $**p<0.01$, $***p<0.001$.

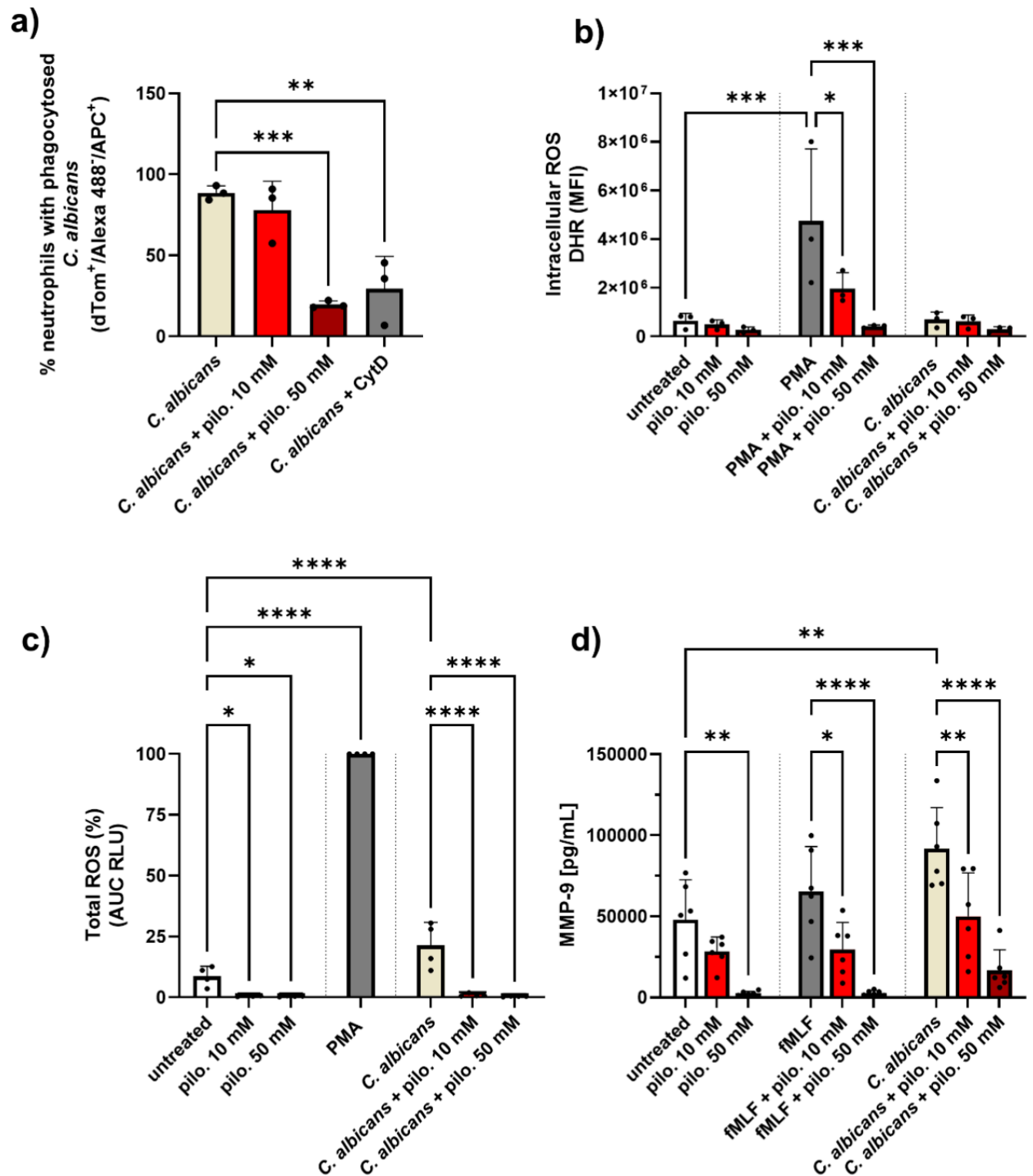


Figure 6

Influence of pilocarpine on neutrophil defence mechanisms. (a) Phagocytosis of fluorescent *C. albicans* yeast (dTomato⁺) by neutrophils (APC⁺ labelling CD15) was quantified using flow cytometry. Phagocytosis was measured with and without pilocarpine (pilo. 10 mM and 50 mM). *C. albicans* multiplicity of infection (MOI): 5. Cytochalasin D (CytD, 10 µg/mL) served as negative control (inhibitor) for phagocytosis. Neutrophils with adherent but not phagocytosed yeast (dTom⁺/Alexa488⁺) were

excluded from analysis. The gating strategy is shown in supplementary figure S5. The effect of pilocarpine (10 mM or 50 mM) on **(b)** intracellular and **(c)** total reactive oxygen species (ROS) production by neutrophils was quantified, with or without stimulation by phorbol 12-myristate 13-acetate (PMA, 20 nM), or *C. albicans* yeast (MOI: 2). In (b), intracellular ROS was quantified using Dihydrorhodamine 123 (DHR) mean fluorescence intensity (MFI). (c) Total ROS, combining intracellular ROS levels and extracellular released ROS was quantified by luminol-based chemiluminescence measurement. **(d)** Neutrophil degranulation was determined through quantification of the granule component matrix metalloproteinase-9 (MMP-9) into the supernatant. Neutrophils were stimulated with N-formylmethionine-leucyl-phenylalanine (fMLF, 100 nM) or *C. albicans* (MOI: 10), and treated with pilocarpine (pilo. 10 mM or 50 mM). Data represent mean $\pm$ SD of (a, b) n=3, (c) n=4, (d) n=6 independent repeats, individual data points shown. An ordinary one-way ANOVA followed by (a) Dunnett's or (b-d) Šídák's multiple comparisons test was performed, comparing pilocarpine treatment to the respective controls, as well as between non-pilocarpine treated conditions. *p<0.05, **p<0.01, ****p<0.0001, ***p<0.001.

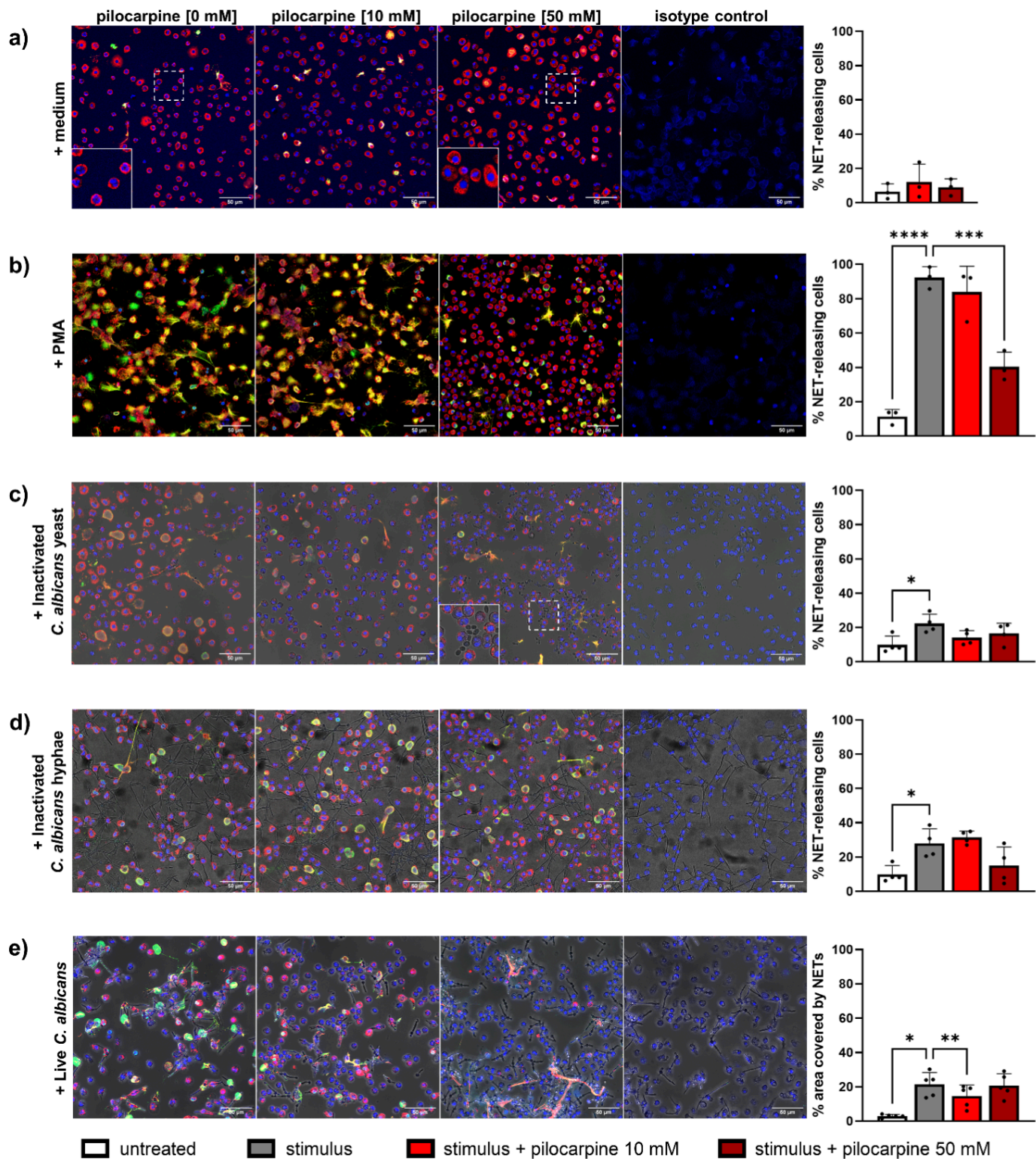


Figure 7

Influence of pilocarpine on neutrophil extracellular trap (NET) release. NET release by neutrophils was analysed after incubation (4h) with various stimulus. Representative images and quantification for each condition shown. All conditions included neutrophils, pilocarpine as indicated (or equivalent volume PBS in pilocarpine free conditions), and the following stimulus: **(a)** RPMI media (negative control); **(b)** phorbol 12-myristate 13-acetate (PMA, 25 nM); **(c)** heat-inactivated *C. albicans* yeast (MOI: 2); **(d)** ethanol-

inactivated *C. albicans* hyphae (MOI: 2); and **(e)** live *C. albicans* yeast (MOI: 2), which transformed into hyphae throughout the incubation period. NETs were quantified from immunofluorescence-stained microscopy images using DNA (Hoechst; blue), DNA/histone-1 complexes (green), and myeloperoxidase (red) as NET markers. Square inserts in (a) are exemplary cells magnified in the lower left corner, highlighting a loss of nuclear lobulation under pilocarpine treatment. The square insert in (c) highlights the presence of extracellular yeast in this condition. Data represent mean $\pm$ SD (n=3 for (a, b), n=4 for (c, d), n=5 for (e), individual data points are shown). A one-way ANOVA followed by Dunnett's multiple comparisons test was performed, comparing treatments to respective controls. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar 50 μ m.

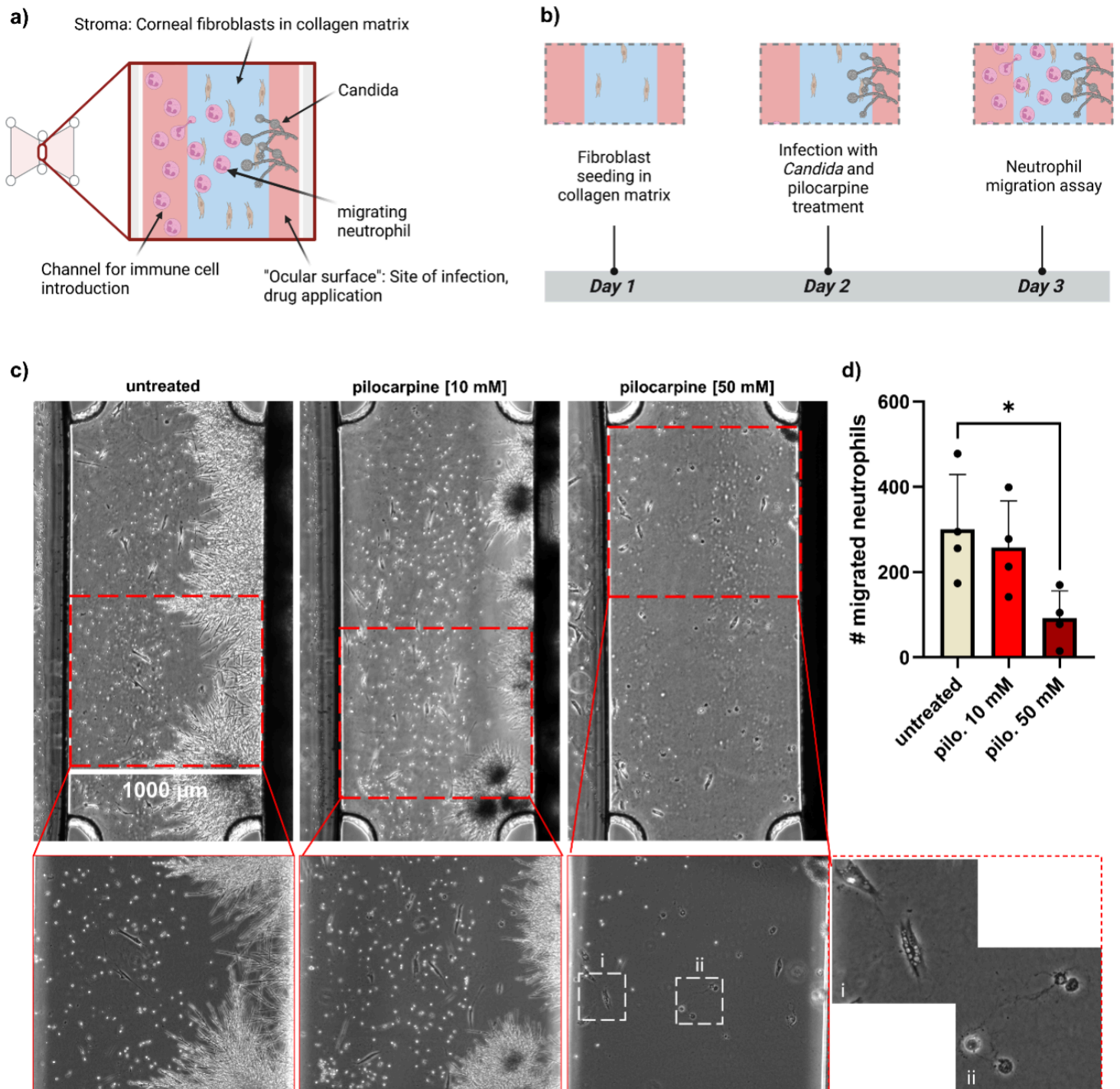


Figure 8

Influence of pilocarpine on *C. albicans* infection and neutrophil migration in model of cornea stroma. (a) Schematic showing mature *in vitro* corneal stromal infection model. Corneal fibroblasts were seeded into a collagen matrix in the middle channel of a chemotaxis chamber and infected with *C. albicans* from one side. Pilocarpine was applied from the *C. albicans* side. Neutrophil migration from the opposite side into the matrix-filled compartment towards the fungal hyphae was analysed. (b) Schematic of the chemotaxis assay. On day 1, corneal fibroblasts were seeded into a 3D collagen matrix, which was infected with *C. albicans* yeast from one side on the subsequent day, as well as treated with pilocarpine.

On the third day, blood-derived neutrophils were added to the uninfected side of the collagen matrix, and neutrophil migration within two hours was quantified. **(c)** Representative 10x brightfield images of the central channel, filled with elongated fibroblasts in collagen. In the untreated condition, *C. albicans* hyphae grow into the model from the infected side, triggering neutrophil response (white dot-like cells). The 20x magnification displays changes in fibroblast appearance upon pilocarpine treatment at 50 mM depending on proximity to the side of drug application, which are highlighted by square magnifications (c-i, ii). **(d)** Quantification of neutrophil migration into the stromal compartment of the corneal infection model. Data represent mean $\pm$ SD (n=4, individual data points are shown). A one-way ANOVA followed by Tukey's multiple comparisons test was performed. *p<0.05. Scale bar 1000 μ m.

Supplementary Files

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- [20260819SupplementaryInformation.pdf](#)
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