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Et al.

Diverse Stimuli engage different Neutrophil Extracellular Trap pathways

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Abstract

Neutrophils release neutrophil extracellular traps (NETs) which ensnare pathogens and have pathogenic functions in diverse diseases. We examined the NETosis pathways induced by five stimuli; PMA, the calcium ionophore A23187, nigericin, *Candida albicans* and Group B Streptococcus. We studied NET production in neutrophils from healthy donors with inhibitors of molecules crucial to PMA induced NETs including protein kinase C, calcium, reactive oxygen species, the enzymes myeloperoxidase (MPO) and neutrophil elastase. Additionally, neutrophils from chronic granulomatous disease patients, carrying mutations in the NADPH oxidase complex or a MPO-deficient patient were examined. We show that PMA, *C. albicans* and GBS use a related pathway for NET induction whereas ionophores require an alternative pathway but that NETs produced by all stimuli are proteolytically active, kill bacteria and composed mainly of chromosomal DNA. Thus, we demonstrate that NETosis occurs through several signalling mechanisms, suggesting that extrusion of NETs is important in host defence.

Keywords

Neutrophil, Neutrophil Extracellular Traps, NETs, reactive oxygen species, myeloperoxidase, neutrophil elastase, myeloid cells, cell death, innate immune response, signal transduction.

Introduction

Neutrophils are the most abundant white blood cell in the circulation and serve as the first line of host defence against pathogen attack. They are terminally differentiated, short lived cells that emerge from the bone marrow ready to react to the presence of pathogens (Amulic et al., 2012, Kolaczkowska and Kubes, 2013). Once a foreign molecule or endogenous threat is identified the neutrophil has a battery of mechanisms it can deploy to insure optimum removal of the hazard. These include the ability to phagocytose, degranulate and produce reactive oxygen species (ROS). The neutrophil can also produce chemokines and cytokines to alert other cells in the vicinity to the danger and thus maximise the host's immune response (Scapini and Cassatella, 2014).

Another form of defence utilised by the neutrophil is the release of decondensed chromatin decorated with antimicrobial peptides that can capture the pathogen in a process termed neutrophil extracellular trap (NET) formation (Brinkmann et al., 2004). NETosis has been primarily examined in response to phorbol 12-myristate 13-acetate (PMA), a potent mitogen and a robust NET inducer. Neutrophils also initiate NETosis in response to microbial infections and, similarly to PMA, these activate protein kinase C (PKC), which in turn leads to calcium fluxes within the cell and activation of the NAPDH oxidase signalling cascade resulting in the production of reactive oxygen species (ROS) (Hakkim et al., 2011, Kaplan and Radic, 2012). The hydrogen peroxide (H_2O_2) produced is in turn consumed by myeloperoxidase (MPO) to produce hypochlorous acid as well as other oxidants (Papayannopoulos et al., 2010). The production of ROS is responsible for the activation of the azurosome, a protein complex composed of MPO, the serine protease neutrophil elastase (NE) and cathepsin G among other granular proteins. The generation of oxidants by MPO liberates NE from the azurosome, allowing it to translocate to the nucleus where it aids in the decondensation of the chromatin by proteolyzing histones (Metzler et al., 2014). Finally, the

cytoplasmic milieu mixes with the nuclear material as the nuclear and subsequently the plasma membrane break down, resulting in release of the NET.

This study describes the different pathways leading to NETs by comparing the induction of NETosis by several stimuli. Primary neutrophils from healthy donors were treated with five stimuli: (1) PMA, (2) the calcium ionophore A23187, (3) the bacterial toxin nigericin that acts as a potassium ionophore, (4) the dimorphic fungus *Candida albicans* and (5) the gram positive bacteria Group B Streptococcus (GBS) and examined for the production of NETs. We tested a range of inhibitors against proteins involved in NETosis to clarify the essential elements in NET induction.

To study the role of ROS in NETosis we tested neutrophils isolated from chronic granulomatous disease (CGD) patients. These patients have mutations in genes coding for subunits of the NADPH oxidase complex and as such their neutrophils cannot make ROS (Heyworth et al., 2003). Thus, these patients are highly susceptible to bacterial and fungal infections. We also tested neutrophils from a patient with a mutation in MPO.

Citrullination is a post-transcriptional modification resulting in the conversion of arginine to citrulline and is catalysed by a group of calcium-dependent proteins known as peptidylarginine deiminases (PADs) (Fuhrmann et al., 2015). Recent studies have shown that citrullination occurs during NETosis (Lewis et al., 2015). We therefore also investigated if histone H3 is citrullinated during the induction of NETosis in response to the different stimuli.

Finally, we showed that the NETs generated by the five stimuli have similar properties and that NETosis is a unique form of cell death, different from classical cell death pathways involving apoptosis and necroptosis.

Results

A wide range of stimuli induce Neutrophil Extracellular Traps (NETs)

We selected five representative and well described NET inducers that are effective over a 2.5–4 hour time period: (1) PMA, (2) the calcium ionophore A23187 which is produced during the growth of *Streptomyces chartreusensis*, (3) the potassium ionophore nigericin which is derived from the bacteria *Streptomyces hygroscopicus*, (4) *Candida albicans* hyphae and (5) *Streptococcus agalactiae* or Group B streptococcus (GBS) and examined NETosis (Figure 1A). We visualised and quantified NETs in samples that were fixed and stained with antibodies directed against a complex of histone 2A, histone 2B and chromatin (Losman et al., 1992) and against neutrophil elastase (NE). Finally, the DNA was stained with the DNA-intercalating dye Hoechst 33342. We used the DNA stain to count the total number of neutrophils and NETs were quantified based on the presence of extracellular chromatin and a size exclusion protocol previously described (Brinkmann et al., 2012). Activating neutrophils with each of the stimuli resulted in a similar NET structure containing extracellular DNA co-localised with NE and chromatin (Figure 1B-G).

Figure 1 – figure supplement 1 shows that PMA (B), A23187 (C) and nigericin (D) produced NETs with similar kinetics over a 3-4 hour time course. *C. albicans* and GBS induced a slower rate of NETosis and non-stimulated cells remained NET free for the duration of the experiment.

Supplemental videos 1-6 also visualise the induction of NETosis in response to the aforementioned stimuli over a 6 hour time course. All stimuli resulted in the release of extracellular DNA; however the temporal order of nucleus decondensation and plasma membrane rupture was varied.

PKC is required for PMA, *C. albicans* and GBS induced NETosis. Only PMA and nigericin require calcium to make NETs

PMA is a direct protein kinase C (PKC) activator which, in turn, leads to calcium fluxes within the cell and both of these processes are required for PMA induced NETosis (Gupta et al., 2014, Fuchs et al., 2007). As anticipated, PMA induced NET formation was blocked by the PKC inhibitor Gö6976 (Figure 2A) (Gray et al., 2013). *C. albicans* and GBS NET induction was also blocked by the PKC inhibitor, albeit to a lesser degree (Figure 2C). The two ionophores, conversely, did not require PKC (Figure 2B).

PMA (Figure 2D) and nigericin (Figure 2E), induced NETosis was impaired by the calcium chelator BAPTA-AM. This chelator reduced NET formation only slightly in response to A23187 (Figure 2E). Early work on neutrophil signalling revealed that ionomycin can induce a massive influx of calcium into the neutrophil, reaching a concentration greater than 1 μM (Gennaro et al., 1984). This abundance of intracellular calcium may have overwhelmed the ability of the BAPTA-AM to chelate the calcium at the concentration used. Pre-treatment of the neutrophils with higher concentrations of the calcium chelator resulted in spontaneous NET formation (data not shown) therefore making the A23187 calcium requirements difficult to assess. A previous study demonstrated a role for calcium in NETosis however, suggesting that A23187 does in fact require the calcium flux it induces to produce NETs (Gupta et al., 2014). Notably, calcium chelation did not impair NETosis in response to *C. albicans* or GBS (Figure 2F).

Finally, as previously shown for PMA and *C. albicans*, A23187, nigericin and GBS NET induction is independent of transcription (Figure 2G-I) (Sollberger et al., 2016).

Differential ROS requirements of NETs

Generation of reactive oxygen species (ROS) is a hallmark of PMA induced NETosis. Figure 3A confirms that PMA induced a ROS burst in primary human neutrophils, peaking after 20 minutes of stimulation. This ROS burst was largely abolished by pre-treating the neutrophils with the ROS scavenger pyrocatechol. A23187 (Figure 3B) also induced a ROS burst, although with slower kinetics than PMA, peaking around 80 minutes post stimulation. Pyrocatechol also prevented this ROS burst. In contrast, nigericin did not induce any ROS production (Figure 3B). Opsonized *C. albicans* generated ROS (Figure 3C) peaked, like PMA 20 minutes after activation. GBS induced ROS production to similar levels but with slower kinetics. ROS release by both microbes was abrogated by pyrocatechol. PMA, A23187, *C. albicans* and GBS induced ROS returned to basal levels 2 hours post-stimulation.

To test whether ROS were required for NETosis, we pre-incubated neutrophils with pyrocatechol before stimulation. As expected, ROS was absolutely required for PMA induced NETosis (Figure 3D). Conversely, pyrocatechol did not affect the level of NETosis in response to A23187 or nigericin (Figure 3E). Interestingly, *C. albicans* induced NETosis was impaired in the presence of the ROS scavenger, but GBS induced NETosis was not (Figure 3F).

To confirm the ROS requirements for NETosis we isolated neutrophils from 5 patients with chronic granulomatous disease (CGD, mutations outlined in Table 1). As expected, the neutrophils from these patients were deficient in ROS production (Figure 3 – figure supplement 1A). As previously described (Fuchs et al., 2007), CGD patient neutrophils did not undergo NETosis in response to PMA (Figure 3G) and were also significantly impaired in NET production in the presence of *S. aureus* (Figure 3 – figure supplement 1B).

Notably, and confirming our data with the ROS scavenger, neither A23187 nor nigericin required ROS to generate NETs (Figure 3H). Intriguingly, and in contrast to data obtained with the ROS scavenger, there was no significant difference in the levels of NETosis

178 comparing healthy vs. CGD patient neutrophils in response to *C. albicans*. This discrepancy
179 was explained by the fact that *C. albicans* can induce a ROS burst itself in the absence of
180 neutrophils and this was inhibited in the presence of the ROS scavenger pyrocatechol (Figure
181 3 – figure supplement 1C). Indeed, we confirmed that *C. albicans* produces sufficient ROS to
182 allow NETosis. We pre-incubated the fungus with pyrocatechol (Figure 3 - figure supplement
183 1D) and showed that scavenging *C. albicans* derived ROS also inhibited NET production.
184 Moreover, by inhibiting ROS in both the neutrophils and the fungus NETosis was inhibited to
185 a greater extent. Thus, the amount of ROS produced by the *C. albicans* was sufficient to allow
186 NETs induction in CGD neutrophils. The amount of NETs were, however, decreased in CGD
187 neutrophils infected with GBS (Figure 3I) when compared with cells isolated from healthy
188 donors.
189 Overall these data show that ROS generated by the NADPH oxidase, while absolutely
190 essential for PMA induced NETosis, is not necessary for NET production in response to both
191 ionophores and only partially required for *C. albicans* and GBS induced NETosis.
192
193 **Differential myeloperoxidase requirements of NETs**
194 We explored whether myeloperoxidase (MPO) is also differentially required by the different
195 stimuli. Aminobenzoic acid hydrazide (ABAH) is a potent and irreversible small molecule
196 inhibitor of MPO. Pre-treatment of neutrophils with ABAH did not induce spontaneous
197 NETosis and, as anticipated, significantly decreased PMA induced NET formation (Figure
198 4A). This was confirmed with neutrophils isolated from a MPO-deficient patient stimulated
199 with PMA (Figure 4D). Similar to the lack of ROS requirement in NETosis in response to
200 ionophores, the MPO inhibitor did not affect NET production by A23187 or nigericin (Figure
201 4B) and neutrophils from a MPO-deficient individual underwent NETosis in response to both
202 stimuli (Figure 4E). Interestingly, and contrary to the subtle role of ROS in NETosis

induction, NETs induced by *C. albicans* or GBS stimulation required MPO (Figure 4C and F).

Downstream of ROS and MPO, neutrophil elastase is differentially required for NETosis

Pre-treatment of healthy neutrophils with a highly specific small molecule NE inhibitor (Macdonald et al., 2001) did not result in spontaneous NETosis and significantly impaired PMA, *C. albicans* and GBS induced NETs (Figure 5A and C). NETosis in response to A23187 and nigericin did not require NE (Figure 5B). These data show that ionophores do not require the molecules relevant in other forms of NET induction such as ROS, MPO and NE.

Histone citrullination occurs but is not required for NET induction

Stimulation of neutrophils with A23187, nigericin, *C. albicans* and GBS, but not PMA, resulted in Histone 3 citrullination (cit-H3) within 90 min as shown by western blot analysis, (Figure 6A) confirming previous publications for both PMA and the calcium ionophore (Neeli and Radic, 2013). The citrullination data was confirmed by quantifying NETosis and the number of cit-H3 positive cells by immunofluorescence. Very few of the PMA induced NETs (Figure 6B) stained positively for cit-H3. Each of the other stimuli induced both NETosis and citrullination to varying levels (Figure 6C and D), confirming the data seen by western blot analysis.

We next explored if citrullination was required for NET formation. Neutrophils were pre-treated with three inhibitors of PAD proteins: Cl-amidine and BB-Cl-amidine, both of which inhibit PAD2 and PAD4, and Thr-Asp-F-amidine (TDFA), a potent specific PAD4 inhibitor. Treating neutrophils with Cl-amidine, BB-Cl-amidine or TDFA did not induce NETosis spontaneously (Figure 6E). PMA induced NETosis was not affected by these inhibitors, consistent with the data obtained with GSK199, another PAD4-selective inhibitor (Figure 6E)

(Lewis et al., 2015). Importantly, in response to A23187 or nigericin stimulation, NETosis remained intact after incubation with the three inhibitors (Figure 6F). Similarly, these inhibitors did not affect *C. albicans* or GBS induced NETosis (Figure 6G). These data are the combination of 10 independent experiments with different donors. Each individual experiment is shown in Figure 6 – figure supplement 2. The three inhibitors reduced citrullination in response to A23187, *C. albicans* and GBS, confirming that these inhibitors were active (Figure 6 – figure supplement 1A-C).

These data show that histone H3 citrullination occurs during the course of NETosis in response to A23187, nigericin, *C. albicans* and GBS but not PMA induced NETs. Moreover, the inhibitor assays demonstrate that PAD2 and PAD4 are not required for NETosis.

All stimuli induce NETs that are proteolytically active, kill bacteria and are composed primarily of nuclear DNA

We next examined the properties of the NETs generated by the different stimuli. We began by examining the proteolytic activity of the NETs. As previously shown for PMA (Papayannopoulos et al., 2010), the induction of NETosis with all five stimuli resulted in the degradation of histone H3 at both 90 and 180 minutes (Figure 7A). Stimulation with PMA and nigericin resulted in strong degradation at the 90 minute time point and a total loss of the full length histone H3 at 180 minutes. Conversely, A23187, *C. albicans* and GBS stimulation led to less degradation overall. This was further confirmed by assaying the degradation of FITC-labelled casein in the presence of NETs isolated from healthy neutrophils treated with the five stimuli (Figure 7B). NETs from all five stimuli were capable of degrading FITC-labelling casein to a similar level indicating they are proteolytically active. The supernatant of non-stimulated neutrophils was used to determine the background level of degradation and a known concentration of trypsin was added as a positive control.

Next we tested the ability of the NETs to kill *E.coli*. Healthy neutrophils were treated for 4 hours with the stimuli to induce NETosis, phagocytosis was blocked with the addition of Cytochalasin D and *E.coli* were added for 1 hour in the presence or absence of DNase 1 (to degrade the NETs). NETs produced by all five stimuli were capable of limited killing that was blocked in the presence of DNase 1 (Figure 7C). The NETs were sonicated post *E.coli* incubation to release bacteria potentially trapped in clumps and skewing the killing counts. This did not affect the bacterial counts indicating that the NETs were in fact killing the bacteria.

We also examined the NETs for the presence of mitochondrial DNA as this is seen in response to autoimmune stimuli such as ribonucleoprotein immune complexes (Lood et al., 2016). NETs produced in response to PMA, nigericin, *C. albicans* and GBS contained around 10% mitochondrial DNA and A23187 NETs contained around 25% (Figure 7D). This is in line with previous work demonstrating that NETs are mainly generated from chromosomal DNA (Lood et al., 2016).

Taken together this data revealed that NETs produced in response to all stimuli tested can degrade proteins, kill bacteria and mostly contain nuclear DNA.

NETosis is distinct from other forms of cell death

To conclude, we compared NETosis, apoptosis, necrosis and necroptosis in neutrophils. We treated neutrophils with a caspase-3 inhibitor to block apoptosis or necrostatin to prevent necroptosis and measured NET formation revealing that NETosis is not affected by either of these inhibitors (Figure 8A-C).

Importantly, the apoptosis inducer staurosporine did not induce NET formation even after 6 hours incubation (Figure 8D). As a control, we showed that staurosporine induced apoptosis in neutrophils as demonstrated by the presence of cleaved caspase-3. This cleavage was blocked by a caspase-3 inhibitor (Figure 8 – figure supplement 1A). These data were

confirmed with the pan-caspase inhibitor Z-VAD-FMK (data not shown). As expected, staurosporine did not induce LDH release (Figure 8 – figure supplement 1B).

Finally, we induced necrosis with α-hemolysin from *Staphylococcus aureus* or necroptosis with a cocktail of TNF-α, Z-VAD-FMK and a SMAC mimetic or cycloheximide (CHX) and measured NETosis. Neither necrosis nor necroptosis activation induced NETosis beyond the level seen in the non-stimulated cells over a 6 hour time course (Figure 8E). LDH assays confirmed that α-hemolysin and the necroptosis cocktail induced cell death (Figure 8 – figure supplement 1C and D). As a control, we verified that necrostatin blocked cell death due to necroptosis (Figure 8 – figure supplement 1D).

Lastly we investigated whether LDH release occurs during NETosis. Figure 8 – figure supplement 1E showed that PMA, nigericin, *C. albicans* and GBS stimulation resulted in LDH release greater than non-stimulated cells at 4 hours. A23187 treatment also resulted in LDH release but to a lesser extent.

In conclusion these data show that NETosis is a unique form of cell death that does not utilise components of the pathways associated with apoptosis, necrosis or necroptosis.

Discussion

Recent work focusing on the various stimuli that lead to NETosis has yielded contradictory results in response to the same stimuli. These discrepancies may arise from technical issues such as differences in neutrophil isolation protocols, the culture of neutrophils in different types of cell culture media and the concentration of stimuli used. With this in mind we aimed here to confirm that our stimuli of interest generate NETs by use of the benchmark for NETosis analysis: quantifiable analysis along with fixed cell imaging and live cell videos.

Using these methods, we demonstrate here that NETs can be robustly induced by a broad range of stimuli, including PMA, the ionophores A23187 and nigericin and the more physiologically relevant stimuli *C. albicans* and GBS. Using neutrophils isolated from healthy donors and patients as well as small molecule inhibitors, we show that NET formation occurs through different signalling pathways. The NETs generated by all five stimuli were proteolytically active, kill bacteria and are composed mainly of chromosomal DNA. We also show that NETosis is distinct from other cell death pathways such as apoptosis, necrosis and necroptosis.

The data shown here demonstrates that PMA induced NETs requires PKC, calcium, ROS, MPO and NE. Conversely, Histone 3 is not citrullinated upon stimulation with PMA and transcription plays no role as has been previously shown (Sollberger et al., 2016).

Clarifying the mechanisms of NET formation in response to *C. albicans* and GBS proved nevertheless to be challenging, perhaps due to the need to culture fresh microbes for each experiment. Similar to PMA both microbes require PKC, MPO and NE and do not require transcriptional activation for NET formation. However, both microbes induce the citrullination of histone 3. Despite this PAD4 activity is not required for NETosis in response to the microbes.

The role of ROS is less conclusive as there is a discrepancy between comparing healthy neutrophils treated with ROS scavengers and cells isolated from CGD patients. Indeed, *C.*

albicans induces significantly less NETs when ROS were pharmacologically abrogated in neutrophils from healthy donors. In contrast, neutrophils from CGD patients produced similar amounts of NETs as those isolated from healthy controls. This suggests that the ROS used by *C. albicans* do not originate from the NADPH oxidase complex. We show here that indeed *C. albicans* itself can produce ROS thus circumventing the ROS requirements of the neutrophil by producing sufficient levels of ROS to allow CGD neutrophils to form NETs. These results are very much in line with the clinical phenotype in which patients with CGD suffer more frequently from invasive infections with *A. fumigatus* than with *C. albicans* (Henriet et al., 2012). Recently a study demonstrated that CGD patient neutrophils produce significantly less NETs in response to *A. fumigatus* than healthy neutrophils further adding to our evidence that neutrophils from CGD patients react diversely to fungal infections (Gazendam et al., 2016).

The role of ROS in GBS induced NETosis is confounded by the ability of healthy neutrophils to produce normal levels of NETs in the presence of the ROS scavenger but a significantly reduced level of NETosis in the CGD patient neutrophils. The CGD patient data suggests a requirement for NAPDH oxidase-dependent ROS for GBS induced NETs that is perhaps not revealed by the ROS scavenger. As seen in Figure 3C, there was some residual ROS production in response to GBS in the presence of the ROS scavenger. This level of ROS may be sufficient for the GBS to induce NETosis in a manner similar to *C. albicans* in which the amount of ROS produced by the fungus allows NETosis to occur in the CGD patient neutrophils. It must also be noted that although the ability of GBS to induce NETosis was significantly reduced in the CGD patients, a high level of NETosis still occurred in these cells. Taken together these data suggest that while PMA absolutely relies on NADPH oxidase derived ROS for NETosis *C. albicans* and GBS can circumvent this need to some degree. This could be due to the ability of the microbes to generate ROS themselves that is then hijacked by the neutrophil to generate NETs in the absence of a self-source of ROS.

This is in contrast with NETosis induction by *S. aureus* which was dependent on the ability of the neutrophils to generate ROS as outlined in the original study on NETosis in CGD patient neutrophils (Fuchs et al., 2007). This suggests that NETosis induced by physiologically relevant stimuli is also very diverse in the signalling pathways utilised and hence challenging to clarify. Indeed, *Leishmania amazonensis* can induce NETosis in the absence of ROS production (Rochael et al., 2015, DeSouza-Vieira et al., 2016).

Indeed the role of ROS production in the production of NETs is further confounded by recent work demonstrating that the mitochondria can also be a source of ROS in NETosis in response to calcium ionophores (Douda et al., 2015) or ribonucleoprotein-containing immune complexes (RNP ICs) (Lood et al., 2016). These data demonstrate that outside of the NADPH-oxidase complex the neutrophil has other ROS sources that are sufficient to induce NETosis.

Conversely, activation with the ionophores A23187 and nigericin does not require PKC, ROS, MPO or NE or transcriptional activation and calcium only has a limited role. Histone 3 is citrullinated upon activation by these stimuli; however, pre-treatment with PAD inhibitors does not affect the ability of the neutrophils to make NETs. This suggests that citrullination (of histone 3 at least) is a consequence of NETosis, and that PAD4 is not required for NET formation. Ionophore and PMA induced NETosis appears to be distinct, at least in the few components of the signal transduction cascade already described.

A23187 is a calcium ionophore that causes a massive influx of calcium and nigericin stimulates potassium effluxes in cells which also results in the influx of calcium demonstrating the similarity between the ionophores in their mechanism of NET induction (Yaron et al., 2015). This flooding of the neutrophil with calcium ions could thus result in perturbation of the membrane potential and cell death that ultimately releases NETs. While the method to initiate NET induction by the ionophores is very different to that seen in response to PMA, the end product appears to be similar.

Auto-antibodies directed against citrullinated proteins are commonly found in the serum of rheumatoid arthritis patients (van Venrooij et al., 2004) and as such elucidating the origin of these modified proteins is of great therapeutic interest. Recent research suggests that PAD enzymes, in particular PAD4, are associated with the induction of NETosis. Consistent with this PAD4-deficient mice do not generate NETs in response to a calcium ionophore which is in direct contrast to the data presented here (Martinod et al., 2013). However, the readout for NETosis used in this study was the presence of extracellular DNA decorated with citrullinated histone 3. Since deficiency in PAD4 results in no citrullination of histones this study lacks a readout in the PAD4-deficient cells that would confirm the presence or absence of NETs such as staining with antibodies against NE or MPO on the extracellular DNA. It is also important to note that these experiments were carried out using murine neutrophils which may not behave similarly to human cells (Bardoel et al., 2014). Importantly, studies examining the requirements of the PAD enzymes in human NETosis, using the same PAD inhibitors also demonstrate a very limited inhibition of NETosis in response to a calcium ionophore and *S. aureus* (Hosseinzadeh et al., 2016, Lewis et al., 2015*)*. Thus, it appears that while PAD enzymes might be important for murine neutrophils to generate NETs, this effect is not seen in human neutrophils.

One additional discrepancy between our data and published reports is the observation that PAD inhibitors (both selective and pan-PAD) show efficacy in multiple mouse models of SLE and RA (Knight et al., 2015, Ghari et al., 2016, Kawalkowska et al., 2016). These studies suggest that citrullination is important in disease pathogenesis and as such could affect NET function. We do not understand how NETs function as antimicrobials, immune cell activators or in coagulation. It is possible that these NET functions are altered by the citrullination of NETs components. Indeed, the phenotype observed in the PAD4-deficient mice could potentially be attributed to the effectiveness of NETs and not necessarily NET formation.

A recent review highlights the wide range of proteins and pathways required for NETosis in response to a variety of stimuli with emphasis on the questions surrounding the role of citrullination in NETosis (Konig and Andrade, 2016). It states that ionophores and bacterial pore-forming toxins induce a pathway within neutrophils that results in the citrullination of histones but that is distinct from NETosis. They term this form of neutrophil cell death leukotoxic hypercitrullination (LTH) and suggest it is not antimicrobial but a bacterial strategy to kill neutrophils. The data shown here demonstrates that in the presence of the calcium ionophore or nigericin nuclear decondensation occurs and results in the extrusion of DNA, chromatin and antimicrobial peptides from neutrophils, albeit in a different manner to that utilised by PMA, *C. albicans* and GBS. Whether these extruded DNA and proteins are antimicrobial, however, requires further investigation.

The different mechanisms of neutrophil cell death have been studied in detail and as such the data presented here can be included in the body of evidence that NETosis is in fact a distinct cell death mechanism utilised to aid in pathogen killing by neutrophils (Fuchs et al., 2007, Remijsen et al., 2011b, Remijsen et al., 2011a). However, two recent studies on the role of necroptosis in NETs induction present contrasting evidence for and against the requirements of necroptosis (Amini et al., 2016, Desai et al., 2016). Our data strengthens the argument that necroptosis is a separate cell death signalling cascade that is not required by neutrophils to induce NETosis.

Our observations show that there are different paths to NETosis in human cells. The elucidation of these pathways is of importance due to the ancient nature of chromatin release as a form of host defence as has been identified in both the animal and plant kingdoms. Therefore, it is unsurprising that NETosis is induced through a wide range of pathways (Tran et al., 2016).

Consequently, the clarification of these different pathways to NETosis has definite therapeutic relevance. There is a genuine need to identify NET inhibitors to alleviate or prevent many

diseases including cystic fibrosis, thrombosis, malaria and sepsis (Kaplan and Radic, 2012, Brinkmann and Zychlinsky, 2012). NETs are present in the sputum of cystic fibrosis (CF) patients and contribute to the viscosity of the sputum (Manzenreiter et al., 2012). NETs are evident in the thrombus in deep vein thrombosis (DVT) and disease activity is reflective of NET markers in the plasma (Fuchs et al., 2012). Many autoimmune diseases such as Systemic lupus erythematosus (SLE) and vasculitis also show a very strong NET phenotype with regards the presence of autoantibodies against proteins readily released from neutrophils in the process of NETosis such as anti-dsDNA and anti-neutrophil cytoplasmic autoantibodies (Hakkim et al., 2010, Kessenbrock et al., 2009).

This study will aid in the development of tools to help combat the detrimental effects of NETosis while balancing this with the need for the neutrophils to fulfil their purpose in the presence of a pathogen and induce their unique cell death program.

Materials and Methods

Inhibitors

Gö6976 (PKC, Biozol), BAPTA-AM (Thermo Fisher Scientific), Actinomycin D, GW311616A (NE) and pyrocatechol (Sigma-Aldrich), 4-Aminobenzoic acid hydrazide (ABAH, Cayman chemical), Cl-amidine (Causey and Thompson, 2008) , BB-Cl-amidine (Knight et al., 2015), TDFA (Jones et al., 2012), caspase-3 inhibitor and necrostatin (Merck-Millipore).

Donor consent

Blood samples were collected according to the Declaration of Helsinki with study participants providing written informed consent. All samples were collected with approval from the ethics committee–Charité –Universitätsmedizin Berlin. Healthy neutrophils were isolated from blood donated anonymously at the Charité Hospital Berlin.

Strains and media

Candida albicans clinical isolate SC5314 was cultured overnight at 30ºC in YPD media. GBS was growth on a 6% sheep blood agar plate overnight at 37ºC, sub-cultured in Todd-Hewitt broth (Sigma-Aldrich) for 2-3 hours until the OD_{600nm} reached 0.5. *Staphylococcus aureus* was prepared as previously described (Fuchs et al., 2007). *E. coli* XL1-Blue (Stratagene) was cultured overnight at 37ºC in LB plus tetracycline. The *C. albicans*, GBS and *S.aureus* were opsonised for 30 minutes at 37ºC with 10 % human serum before addition to the neutrophils. This ensured hyphal growth of the *C. albicans*.

Neutrophil isolation and NET induction

Human neutrophils were isolated by centrifuging heparinized venous blood over Histopaque 1119 (Sigma-Aldrich) and subsequently over a discontinuous Percoll (Amersham

Biosciences) gradient as previously described (Fuchs et al., 2007). Experiments were performed in RPMI-1640 (w/o phenol red) supplemented with 10 mM HEPES and 0.05% human serum albumin. Cells were seeded at 10^5/well (24 well plate) for NET experiments and stimulated with PMA, staurosporine, cycloheximide (Sigma-Aldrich), A23187 (Santa Cruz Biotechnology Inc.), Nigericin (InvivoGen), *Candida albicans* SC5314 hyphae, GBS, α-hemolysin (generated as previously described (Virreira Winter et al., 2016)), *Staphylococcus aureus* (prepared as previously described (Fuchs et al., 2007)), TNF-α (Thermo Fisher Scientific), z-VAD-FMK (Enzo) or SMAC mimetic (Birinapant, ChemieTek) for 2 – 6 hrs. Where applicable, cells were pre-treated inhibitors for 30 min before stimulation.

NET staining and quantification

Neutrophils seeded on glass coverslips were stained and quantified as previously described (Brinkmann et al., 2012). Briefly, cells were fixed in 2% paraformaldehyde (PFA) post NET induction, permeabilized on 0.5% Triton-X100, blocked for 30 min in blocking buffer. Neutrophils were then stained with the following primary antibodies: anti-neutrophil elastase (Calbiochem: 481001, RRID:AB_212213) and antibodies directed against the subnucleosomal complex of Histone 2A, Histone 2B, and chromatin ((Losman et al., 1992), generated in house). The secondary antibodies donkey anti-mouse Cy3 (Jackson ImmunoResearch Labs: 715-175-150, RRID:AB_2340819) and donkey anti- rabbit Alexa Fluor488 (Life Technologies: A11008, RRID:AB_143165) were used. Finally the samples were stained with Hoechst 33342 (Immunochemistry: 639, RRID:AB_2651135) and mounted with Mowiol. Image acquisition was done using a Leica DMR upright fluorescence microscope equipped with a Jenoptic B/W digital microscope camera and analysed using ImageJ/FIJI software.

Confocal Microscopy

Primary human neutrophils (10^6) were washed once by centrifugation (300g, 10min, RT) in imaging medium (20mM HEPES, 2.5mM KCl, 1.8mM CaCl2, 1mM MgCl2, 0.1% Human Serum Albumin, pH 7.4) (Sigma Aldrich) and then resuspended in 4ml imaging medium containing 2µM draq5 (Biostatus) and 0.5µM Sytox Green (Thermo Fischer Scientific). Each well of an 8 well ibidi treat dish (ibidi) was filled with 200µl of that suspension and cells were allowed to settle down for 30 min at imaging temperature. NETs were induced by PMA, A23187, nigericin, *C. albicans* or GBS at the concentrations outlined above. Imaging was performed with a Leica SP8 AOBS confocal microscope equipped with a motorized stage and temperature-controlled chamber at 36°C. Images (2048*2048 pixels) were acquired at 0.5% maximal laser intensities every 2min for each well for a total duration of 6hrs.

ROS assay

Neutrophils were seeded at concentration of $1x10^5$ cells per well in 200µl RPMI (w/o phenol red) supplemented with 10 mM HEPES, 0.05% human serum albumin, 50 µM luminol and 1.2 units/ml horseradish peroxidase and pre-treated with pyrocatechol for 30min at 37°C. The cells were then stimulated for 2 hr with the indicated stimuli and luminescence was measured over time in a VICTOR Light luminescence counter from Perkin Elmer.

Western blot

Neutrophil lysates were generated from $5x10^6$ cells 90, 180 minutes (cit-H3 and H3) or 3 hr (caspase-3) post stimulation by lysis in RIPA buffer (50mM Tris-HCl Ph 8.0, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10mM sodium fluoride, 25mM sodium pyrophosphate) supplemented with protease inhibitor cocktail (Sigma-Aldrich), 20 µM neutrophil elastase V inhibitor and 20 µM cathepsin G inhibitor (219372, both Calbiochem). Protein lysates were quantified by bicinchoninic acid assay (BCA assay,

Pierce) according to manufacturer's instructions. Protein lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by analysis via Western immunoblotting using an anti-citrullinated Histone H3 primary antibody (abcam: ab5103, RRID:AB_304752), an anti-histone H3 antibody (Active Motif: 39164, discontinued) an anti-cleaved Caspase-3 antibody (9661, RRID:AB_2341188), anti-β-actin (5057S, RRID:AB_10694076) or anti-GAPDH (all Cell Signaling Technology: 5014S RRID:AB_10693448) and anti-rabbit HRP (Jackson ImmunoResearch Labs: 111-035-144, RRID:AB_2307391).

Protease activity assay

NETs were generated as described above using $1.5x10^6$ cells/point. The NETs were isolated as previously described (Barrientos et al., 2013). Briefly the samples were treated with 4 U/ml *Alu*I, the NETs were collected, their DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fischer Scientific) and the protease activity of 200ng/ml of NET DNA was quantified using the Pierce Fluorescent Protease Assay kit according to the manufacturer's instructions. 100 µl of non-stimulated neutrophil supernatant was used to calculate background protease activity and 125 ng/ml trypsin was sued as a positive control.

Bacterial Killing assay

NETs were generated as described above using $1x10^6$cells/point and stimulated for 4 hrs. Bacterial killing was assayed as previously described (Ermert et al., 2009). Briefly, once NETosis was induced (visualised by light microscopy) the cells were treated with 10 µg/ml Cytochalasin D (Sigma-Aldrich) for 15 minutes to block phagocytosis. A subset of samples were treated with DNase 1 at 50 U/ml to degrade the NETs prior to killing. A tetracycline resistant *E. coli* strain was added to the neutrophils at an MOI of 1 and incubated at 37ºC for 1 hr. The cells and *E. coli* were collected, a subset of samples were sonicated to release any

563 trapped bacteria, serially diluted, plated on tetracycline treated agar plates and incubated at
564 37ºC for 24hr. Finally CFUs were counted.
565
566 **Quantitative real-time PCR of mitochondrial and nuclear content of NETs**
567 NETs were generated as described above using $1.5x10^6$cells/point for 4 hours. NETs were
568 released, DNA was isolated and analysed for nuclear (*S18)* versus mitochondrial (*S16*)
569 content by Q-PCR as previously described (Lood et al., 2016).
570
571 **LDH assay**
572 Neutrophils were seeded at $1x10^5$ cells/well in a 96 well plate and treated for 21hr with the
573 indicated stimuli. LDH release was quantified from the supernatants using Cytotox 96 Non-
574 Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions.
575
576 **Statistics**
577 Data are presented as means ± SEM unless otherwise noted and were analysed using a two-
578 sided Student t test. All analyses were performed using GraphPad Prism software (Version
579 6.04). Results were considered significant at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
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Competing interests

P.R.T. is a consultant to Bristol-Myers Squibb. The remaining authors declare no competing financial interests.

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Donor	Age	Nucleotide change	Mutation	Amino acid change	Residual activity
1	24	CYBB c.742dupA	insertion	p.Ile248AsnfsX36	No
2	25	CYBB c.868C > T	nonsense	p.Arg290X	No
3	18	CYBB c.1421T > G	missense	p.Leu474Arg	No
4	26	CYBB c.868C > T	nonsense	p.Arg290X	No
5	29	CYBA c.371C > T	missense	p.Ala124Val	Yes

Table 1. CGD patient donors

Nomenclature for genotypes is according to den Dunnen and Antonarakis (den Dunnen and Antonarakis, 2001).

Figure Legends

Figure 1. NETosis induction by a range of stimuli

Primary human neutrophils were stimulated for the indicated times with 50 nM PMA, 5 μM A23187, 15 μM nigericin, MOI 5 opsonized *C. albicans* or MOI 10 opsonized group B streptococcus (GBS), fixed with 2% PFA and incubated with a DNA stain (Hoechst) and immunolabeled with antibodies directed against Neutrophil Elastase (NE) and chromatin (A-G). (A) NETosis rate was quantified by immunofluorescence. Graph shows mean ± SEM from independent experiments with 3 different donors. (B-G) Representative confocal microscopy images of (B) non-stimulated neutrophils (-) or NETs induced by (C) PMA (D) A23187, (E) nigericin (F) *C. albicans* or (G) GBS and stained with Hoechst (blue) and immunolabeled for NE (green) and chromatin (red). Scale bars, 50 μm.

Figure 1 – figure supplement 1. NET induction over time with the five stimuli of interest

Primary human neutrophils were stimulated for the indicated times with 50 nM PMA (B), 5 μM A23187 (C), 15 μM nigericin (D), MOI 5 *C. albicans* (E) or MOI 10 GBS (F), fixed with 2% PFA and incubated with a DNA stain (Hoechst) and immunolabeled with antibodies directed against Neutrophil Elastase (NE) and chromatin. NETosis rate was quantified by immunofluorescence. Graph shows mean ± SEM from independent experiments with 3 different donors.

Figure 2. Differential requirements for PKC and calcium and a lack of requirement of transcription for NET induction by the stimuli of interest

(A-C) NETosis rate in PKC inhibited neutrophils. Primary neutrophils were pre-treated with the PKC inhibitor Gö6976 (1 μM) for 30 mins and stimulated with (A) PMA, (B) A23187 or nigericin, and (C) *C. albicans* or GBS for 2.5 – 4 hrs and analysed for NET production by immunofluorescence. (D-F) NETosis rate in neutrophils pre-treated with the calcium chelator

BAPTA-AM (10 μM) for 30 mins and stimulated with (D) PMA, (E) A23187 or nigericin and (F) *C. albicans* or GBS for 2.5 – 4 hrs and analysed for NET production by immunofluorescence. (G-I) NETosis rate in neutrophils pre-treated with actinomycin D (1 μg/ml) for 30 mins and stimulated with (G) PMA, (H) A23187 or nigericin and (I) *C. albicans* or GBS for 2.5 – 4 hrs and then analysed for NET production by immunofluorescence. Graphs show mean ± SEM from 3 independent experiments. *$P < 0.05$, NS = not significant.

Figure 3. Diverse stimuli have different ROS requirements for NETosis

ROS production by neutrophils (A-C). ROS production was measured over a 2 hour time course in the presence or absence of the ROS scavenger pyrocatechol (pyro, 30 μM) in response to (A) PMA, (B) A23187 or nigericin and (C) *C. albicans* or GBS stimulation. Shown is a representative of three independent experiments. (D-F) NETosis rate of neutrophils pre-treated for 30 mins with pyrocatechol or (G-I) NETosis rate of healthy control neutrophils and CGD patients stimulated with (D & G) PMA, (E & H) A23187 or nigericin and (F & I) *C. albicans* or GBS. (A-C) Graphs show mean ± SD from a representative of three independent experiments. (D-F) Graphs shows mean ± SEM from 3 independent experiments. (G-I) Graphs show mean ± SEM from 5-7 independent experiments using neutrophils from 5 independent GCD patients (each represented by a red circle). *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = not significant.

Figure 3 - figure supplement 1. No ROS production in CGD patient neutrophils, S. aureus requires ROS for NET production & *C. albicans* produces ROS

(A) ROS production by CGD neutrophils. Neutrophils from a healthy control donor and (B) a CGD patient were examined for the production of ROS in response to PMA stimulation over a 2 hr time course. Graph shows mean ± SD from a representative of 5 independent ROS

assays carried out with CGD patient neutrophils. (B) CGD patient neutrophils are impaired for *S. aureus* induced NETosis. Healthy and CGD patient neutrophils were stimulated with *S. aureus* at a MOI of 20 for 4hr and NETosis was examined as previously described. Graph shows mean ± SEM from 3 independent experiments. (C) *C. albicans* produces ROS. *C. albicans* induced ROS was measured over a 3 hr time course in the presence or absence of either neutrophils (PMN) or pyrocatechol (pyro). Graph shows mean ± SD from a representative of three independent experiments. (D) NETosis in response to *C. albicans* utilises ROS generated from *C. albicans*. Healthy neutrophils or *C. albicans* were pre-treated with pyrocatechol for 30 mins. Cells were then stimulated with *C. albicans* at a MOI of 5 for 3hr and NETosis was examined as previously described. Graph shows mean ± SEM from 3 independent experiments.

Figure 4. Myeloperoxidase is essential for PMA, *C. albicans* and GBS induced NETosis but not for A23187 and nigericin NET formation

(A-F) NETosis rate in response to (A & D) PMA, (B & E) A23187 or nigericin and (C & F) *C. albicans* or GBS. (A-C) Primary neutrophils were pre-treated for 30mins with 500 μM ABAH or a DMSO control, stimulated as indicated for 2.5 – 4 hrs and analysed for NET production by immunofluorescence. Graphs show mean ± SEM from 3 independent experiments. (D-F) healthy control neutrophils and neutrophils from a MPO-deficient patient were stimulated as outlined above. Graphs show mean ± SD from a representative of two independent experiments from a single MPO-deficient donor. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = not significant.

Figure 5. Neutrophil elastase is required for PMA, *C. albicans* and GBS induced NETosis but not for A23187 or nigericin NET production

(A-C) NETosis rate of neutrophils during NE inhibition. Primary neutrophils were pre-treated for 30 mins with a neutrophil elastase inhibitor (GW311616A, 20 μM) or a DMSO control and stimulated for 2.5-4 hrs with (A) PMA, (B) A23187 or nigericin and (C) *C. albicans* or GBS and analysed for NET production by immunofluorescence. Graphs show mean ± SEM from 3 independent experiments. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = not significant.

Figure 6. Citrullination of histone H3 occurs during NETosis but is not required for NET induction

(A-D) Histone H3 was citrullinated during NETosis in response to all stimuli bar PMA. (A) Primary neutrophils were stimulated for 90 mins with PMA, A23187, nigericin, *C. albicans* or GBS, lysed and assayed for the presence of citrullinated histone H3 and GAPDH by SDS-PAGE electrophoresis and Western immunoblotting. (B-D) NETosis rate and percentage of citrullinated cells in response to (B) PMA, (C) A23187 or nigericin and (D) *C. albicans* or GBS. Graphs show mean ± SD from a representative of two independent experiments. (E-G) NETosis rate in neutrophils pre-treated with the PAD inhibitor Cl-amidine at 200 μM, BB-Cl-amidine at 10 μM, TDFA at 200 μM, or DMSO as control and stimulated with (E) PMA, (F) A23187 or nigericin and (G) *C. albicans* or GBS and analysed for NET production by immunofluorescence. Graphs show mean ± SEM from 10 independent experiments. *$P <$ 0.05, NS = not significant.

Figure 6 - figure supplement 1. PAD inhibitors reduce histone H3 citrullination

Neutrophils were pre-treated with (A) 200 μM Cl-amidine, (B) 10 μM BB-Cl-amidine or (C) 200 μM TDFA, stimulated with A23187, C. albicans or GBS for 3-4hrs, fixed, stained with an antibody against citrullinated histone H3 and hoechst and analysed for percentage of cells

912 citrullinated on histone H3 by immunofluorescence. Graphs show mean ± SEM from 3
913 independent experiments.
914
915 **Figure 6 – figure supplement 2. PAD inhibitors do not prevent NETosis**
916 (A – J) Neutrophils were pre-treated with Cl-amidine at 200 μM (light grey bars), BB-Cl-
917 amidine at 10 μM (dark grey bars), TDFA (black bars) at 200 μM, or DMSO as control (white
918 bars), stimulated with PMA, A23187, nigericin, *C. albicans* or GBS for 3-4hrs and analysed
919 for NET production by immunofluorescence. Graphs show mean ± SD from 10 independent
920 experiments.
921
922 **Figure 7. NETs are proteolytically active, kill bacteria and are mainly composed of**
923 **chromosomal DNA**
924 (A) NETosis leads to histone H3 degradation. Primary neutrophils were stimulated for 90 and
925 180 mins with PMA, A23187, nigericin, *C. albicans* or GBS, lysed and assayed for the
926 presence of histone H3 and GAPDH by SDS-PAGE electrophoresis and Western
927 immunoblotting. Shown is a representative of 3 independent experiments. (B) Isolated NETs
928 are proteolytically active. NETosis was induced for 4 hr, NETs were isolated after treatment
929 with *Alu*I for 20min, the DNA content was determined and 200ng/ml DNA was tested for its
930 proteolytic active using the Pierce Fluorescent Protease Assay Kit according the
931 manufacturer's instructions. 100 μl of non-stimulated neutrophil supernatant was used to
932 determine the background activity and 125 ng/ml trypsin was added as a positive control. (C)
933 NETs can kill *E. coli*. Neutrophils were stimulated to produce NETs for 4 hr. Phagocytosis
934 was inhibited by the addition of Cytochalasin D and *E. coli* at an MOI of 1 was added in the
935 presence or absence of 50U/ml DNase 1. After 1 hr the cells, NETs and *E. coli* were collected
936 (selected samples were sonicated), serially diluted, plated on tetracycline resistant agar plates
937 and incubated for 24hr at 37ºC followed by CFU counts to determine killing. (D) NETs are

938 primarily composed of chromosomal DNA. 4hr post NET induction the NETs were isolated
939 by MNase treatment, followed by proteinase K treatment. NET DNA was isolated by phenol-
940 chloroform extraction and the ratio of *S18* to *S16* DNA was analysed by real-time PCR.
941 Graphs show mean ± SEM from 3 independent experiments.
942
943 **Figure 8. NETosis is a unique form of cell death different from apoptosis, necrosis and**
944 **necroptosis**
945 (A-C) NETosis occurs in the presence of apoptosis and necroptosis inhibitors. Primary human
946 neutrophils were pre-treated for 30 mins with 20 μM caspase-3 inhibitor or 30 μM necrostatin
947 or a DMSO control and stimulated with (A) PMA, (B) A23187 or nigericin and (C) *C.*
948 *albicans* or GBS for 2.5 – 4 hrs and analysed for NET production by immunofluorescence.
949 Graphs show mean ± SEM from 3 independent experiments. (D) NETosis rate in the presence
950 of the apoptosis inducer staurosporine. Primary neutrophils were stimulated for 2 – 6 hrs with
951 staurosporine (500 nM) or PMA and analysed for NET induction by immunofluorescence.
952 Graphs show mean ± SEM from 3 independent experiments. (E) NETosis rate in response to
953 necrosis or necroptosis inducers. Primary neutrophils were stimulated with α-hemolysin (25
954 μg/ml) to induce necrosis or with TNF-α (50 ng/ml), Z-VAD-FMK (50 μM) and a SMAC
955 mimetic (100 nM) or cycloheximide (25 μg/ml) to induce necroptosis for 6 hrs and analysed
956 for NET production by immunofluorescence. Graphs show mean ± SEM from 3 independent
957 experiments. NS = not significant.
958
959 **Figure 8 - figure supplement 1. Apoptosis, necrosis and necroptosis can be induced in**
960 **primary neutrophils, NETosis results in LDH release**
961 (A) Staurosporine induced caspase-3 cleavage in neutrophils. Primary neutrophils were pre-
962 treated with a caspase-3 inhibitor for 30 mins, stimulated with staurosporine for 3 hrs; cell
963 lysates were generated and assayed for the presence of cleaved caspase-3 and β-actin by SDS-

PAGE electrophoresis and Western immunoblotting. Data shown is a representative of 3 independent experiments. (B) Staurosporine does not induce LDH release. Neutrophils were stimulated with staurosporine for 21 hrs and LDH release was measured as per the manufacturer's instructions. (C) Neutrophils were stimulated for 21 hrs with α-hemolysin and LDH release was measured. (D) Neutrophils were pre-treated with a necrostatin inhibitor for 30 mins and stimulated with TNF-α, Z-VAD-FMK and a SMAC mimetic or cycloheximide (CHX) for 21 hrs and LDH release was measured. (C-D) Graphs show mean ± SD from a representative of two independent experiments. (E) LDH is released in NETosis. Neutrophils were treated with the indicated stimuli for 4 hrs and LDH release was measured. Graph shows mean ± SEM from 3 independent experiments. Treatment of neutrophils with triton-X100 was used to normalise the data with triton treatment set to 100% LDH release.

Supplementary figure legends

Video 1. No NETosis in non-stimulated primary neutrophils

Primary neutrophils were stained with Draq5 (blue) and cell impermeable Sytox Green (green) and imaged for 6 hours using a Leica SP8 AOBS confocal microscope. Video is representative of three independent experiments.

Video 2. PMA induced NETosis in primary neutrophils

Primary neutrophils were stained with Draq5 (blue) and cell impermeable Sytox Green (green), stimulated with 50nM PMA and imaged for 6 hours using a Leica SP8 AOBS confocal microscope. The appearance of the green colour indicated NETosis. Video is representative of three independent experiments.

Video 3. A23187 induced NETosis in primary neutrophils

Primary neutrophils were stained with Draq5 (blue) and cell impermeable Sytox Green (green), stimulated with 5 μM A23187 and imaged for 6 hours using a Leica SP8 AOBS confocal microscope. The appearance of the green colour indicated NETosis. Video is representative of three independent experiments.

Video 4. Nigericin induced NETosis in primary neutrophils

Primary neutrophils were stained with Draq5 (blue) and cell impermeable Sytox Green (green), stimulated with 15 μM nigericin and imaged for 6 hours using a Leica SP8 AOBS confocal microscope. The appearance of the green colour indicated NETosis. Video is representative of three independent experiments.

Video 5. *C. albicans* induced NETosis in primary neutrophils

Primary neutrophils were stained with Draq5 (blue) and cell impermeable Sytox Green (green), stimulated with MOI 5 *C. albicans* and imaged for 6 hours using a Leica SP8 AOBS confocal microscope. The appearance of the green colour indicated NETosis. Video is representative of three independent experiments.

Video 6. GBS induced NETosis in primary neutrophils

Primary neutrophils were stained with Draq5 (blue) and cell impermeable Sytox Green (green), stimulated with MOI 10 GBS and imaged for 6 hours using a Leica SP8 AOBS confocal microscope. The appearance of the green colour indicated NETosis. Video is representative of three independent experiments.

Supplementary File 1. Graphical abstract: The diverse mechanisms of NETosis

In this study we investigated whether NETosis occurs through a single signalling pathway or is induced by the five stimuli of interest in a diverse mechanism. As demonstrated, NETosis in response to C. albicans and GBS requires ROS, MPO and NE and induces histone H3 citrullination. This is in comparison to the NETosis seen in response to A23187 and nigericin during which none of the molecules highlighted above are required but citrullination of histone H3 does occur. Finally we re-confirm that PMA induced NETosis requires ROS, MPO and NE but does not result in the citrullination of histone H3.

Titles and figure legends for source data.

Figure 1 - source data 1

This data is the mean values of three independent NETosis assays in response to the five stimuli of interest and was used to generate the histogram in figure 1A.

Figure 2 - source data 1

This data is the mean values of three independent NETosis assays in response to the five stimuli of interest in the presence of the PKC inhibitor Gö6976 (figure 2A-C), the calcium chelator BAPTA-AM (figure 2 D-F) and actinomycin D (figure 2 G-I) and was used to generate the histograms in figure 2.

Figure 3 - source data 1

This data is the mean values of three independent NETosis assays in response to the five stimuli of interest in the presence of the ROS scavenger pyrocatechol and was used to calculate the histograms in figure 3 D-F. This data also shows the means from 7 independent experiments with CGD patient neutrophils and was used to generate the histograms in figure 3 G-I.

Figure 4 - source data 1

This data is the mean of three independent NETosis assays in response to the five stimuli of interest in the presence of the MPO inhibitor ABAH and was used to generate the histograms in figure 4 A-C. This data also shows the raw data used to calculate the mean of a representative experiment using MPO-deficient neutrophils used to generate the histograms in figure 4 D-F.

Figure 5 - source data 1

This data is the mean of three independent NETosis assays in response to the five stimuli of interest in the presence of a NE inhibitor and was used to generate the histograms in figure 5 A-C.

Figure 6 - source data 1

This data is the mean of ten independent NETosis assays in response to the five stimuli of interest in the presence of the PAD inhibitors and was used to generate the histograms in figure 6.

Figure 8 - source data 1

This data is the mean of three independent NETosis assays in response to the five stimuli of interest in the presence of necrostatin or caspase 3 inhibitor and was used to generate the histograms in figure 8 A-C. This data also shows the mean of three independent NETosis experiments in response to staurosporine (figure 8 D), hemolysin (to induce necrosis), TNF-α, Z-VAD-FMK and a SMAC mimetic or cycloheximide (to induce necroptosis) and was used to generate figure 8E.

Figure 1

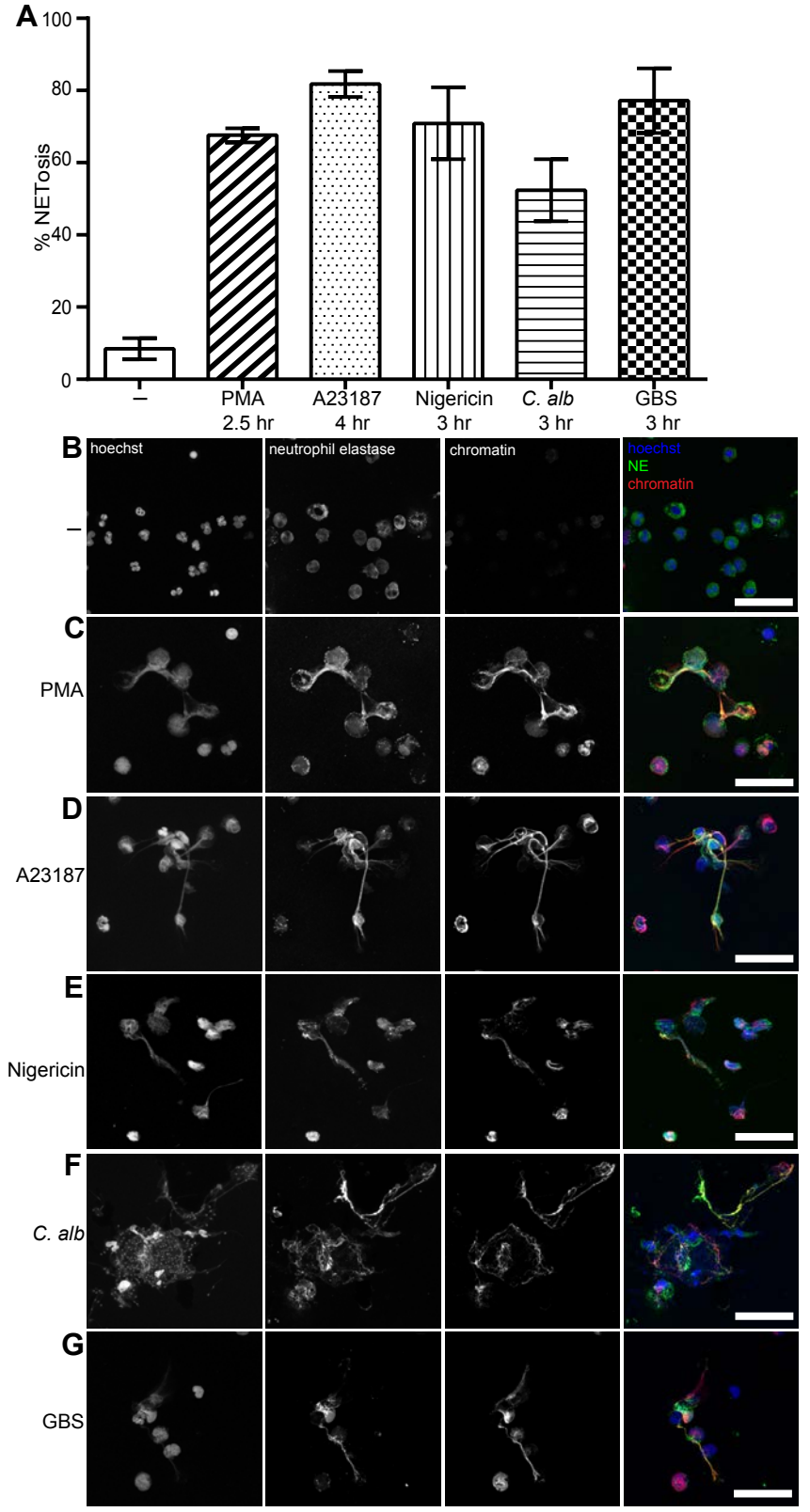
A
100
80
60
40
20
0
% NETosis
–
PMA
2.5 hr
A23187
4 hr
Nigericin
3 hr
C. alb
3 hr
GBS
3 hr
B
hoechst
neutrophil elastase
chromatin
hoechst
NE
chromatin
–
C
PMA
D
A23187
E
Nigericin
F
C. alb
G
GBS

Figure 1 - figure supplement 1

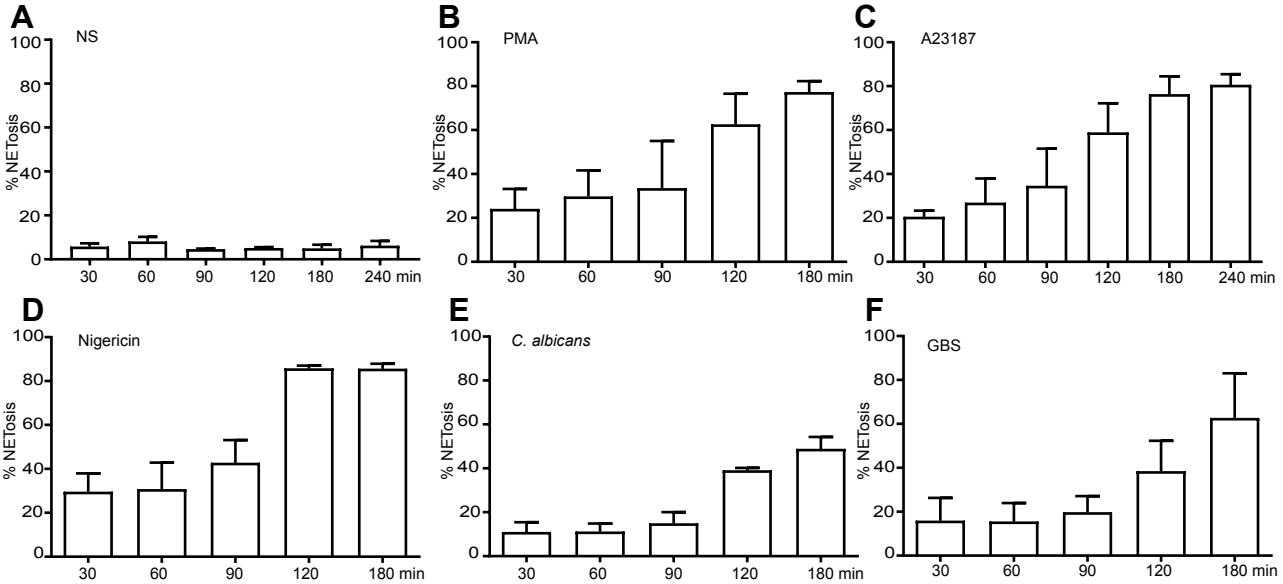

Figure 2

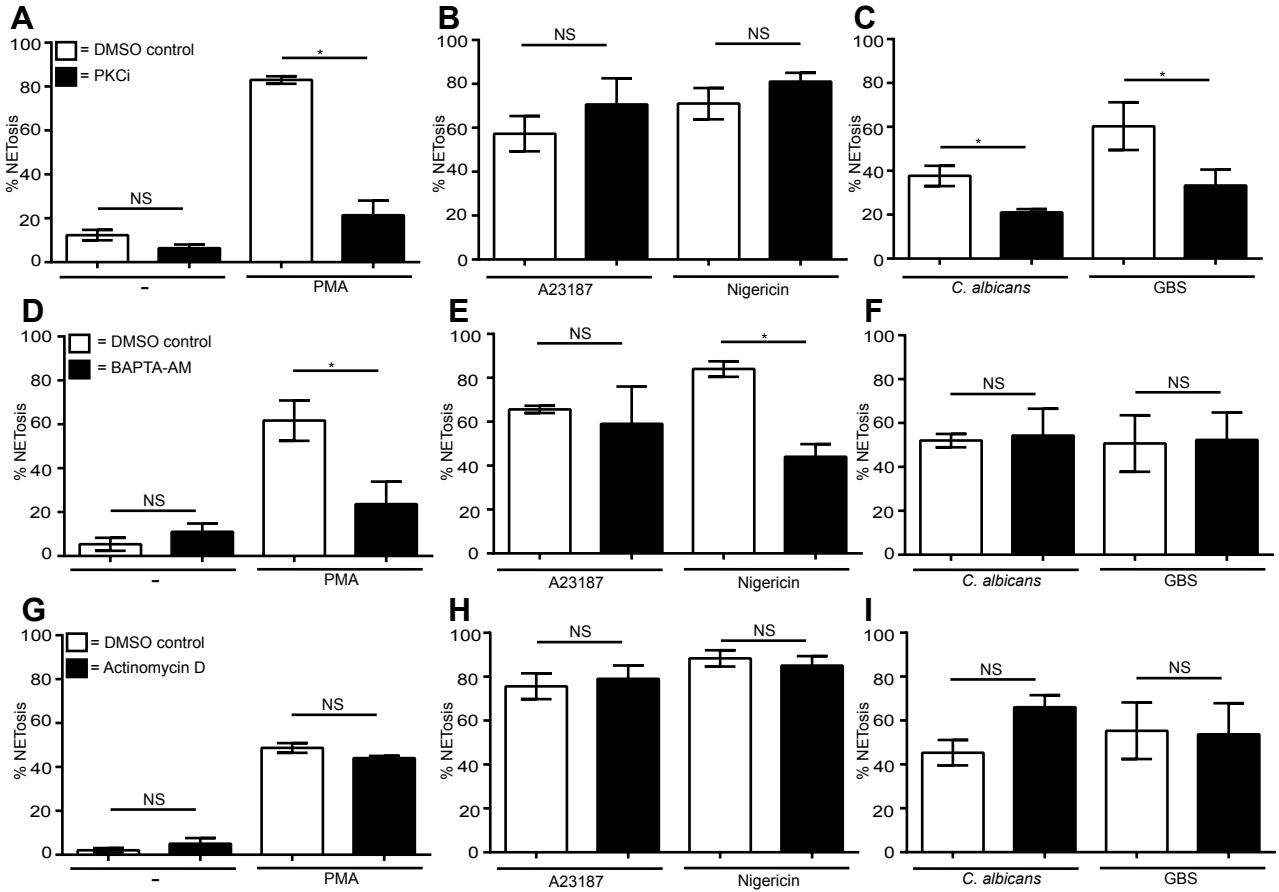
A
= DMSO control
= PKCi
% NETosis
NS
*
-
PMA
B
NS
NS
A23187
Nigericin
C
*
*
C. albicans
GBS
D
= DMSO control
= BAPTA-AM
E
F
G
= DMSO control
= Actinomycin D
H
I

Figure 3

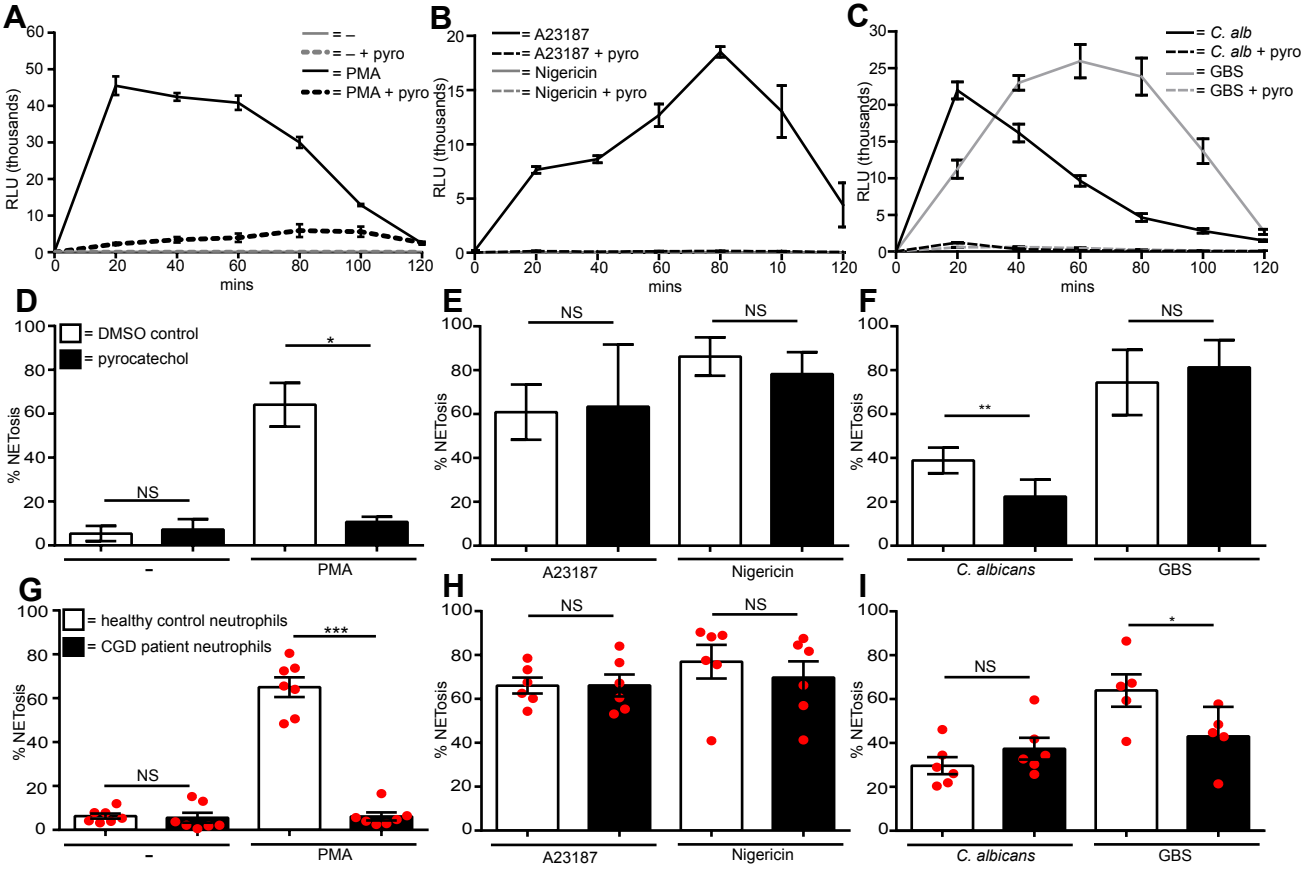
A
RLU (thousands)
mins
= –
= – + pyro
= PMA
= PMA + pyro
B
RLU (thousands)
mins
= A23187
= A23187 + pyro
= Nigericin
= Nigericin + pyro
C
RLU (thousands)
mins
= C. alb
= C. alb + pyro
= GBS
= GBS + pyro
D
% NETosis
= DMSO control
= pyrocatechol
NS
*
–
PMA
E
% NETosis
NS
NS
A23187
Nigericin
F
% NETosis
**
NS
C. albicans
GBS
G
% NETosis
= healthy control neutrophils
= CGD patient neutrophils
NS
***
–
PMA
H
% NETosis
NS
NS
A23187
Nigericin
I
% NETosis
NS
*
C. albicans
GBS

Figure 3 - figure supplement 1

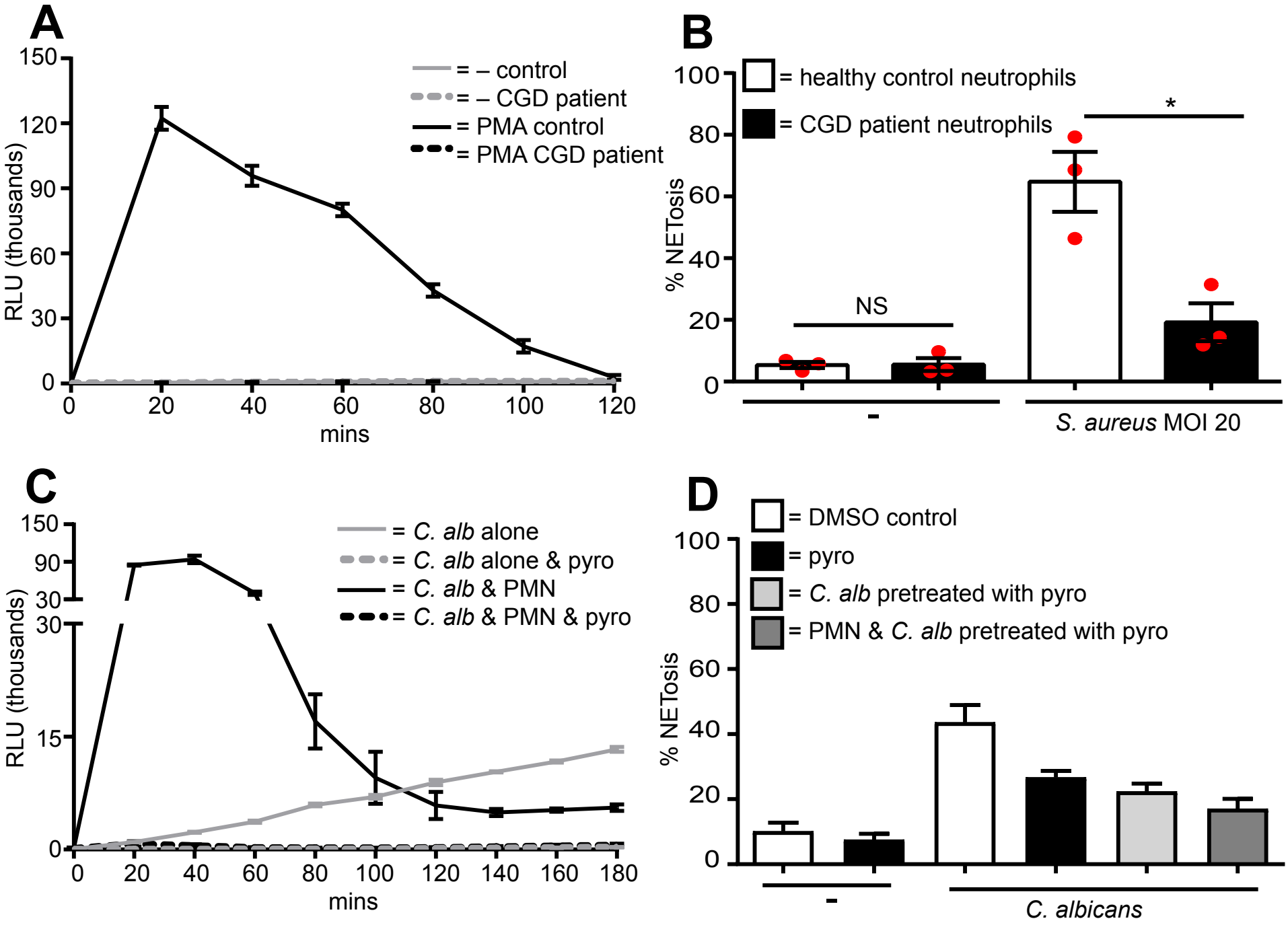

Figure 4

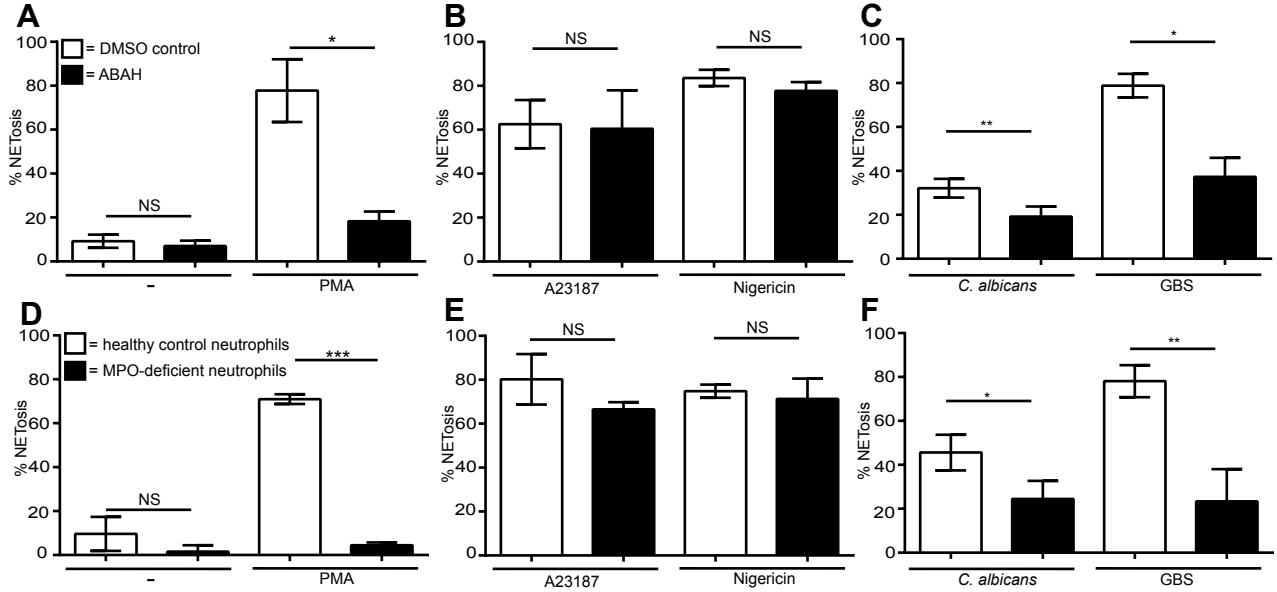
A
= DMSO control
= ABAH
% NETosis
NS
*
-
PMA
B
NS
NS
A23187
Nigericin
C
**
*
C. albicans
GBS
D
= healthy control neutrophils
= MPO-deficient neutrophils
***
E
F

Figure 5

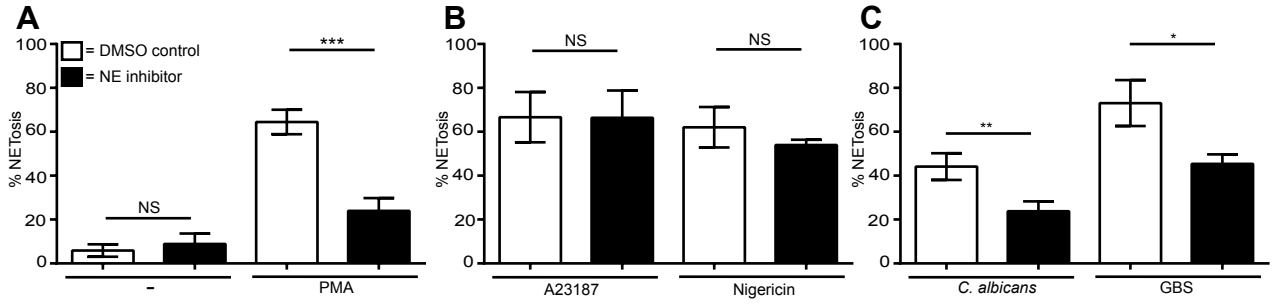
A
= DMSO control
= NE inhibitor
% NETosis
100
80
60
40
20
0
NS
***
-
PMA
B
% NETosis
NS
NS
A23187
Nigericin
C
% NETosis
**
*
C. albicans
GBS

Figure 6

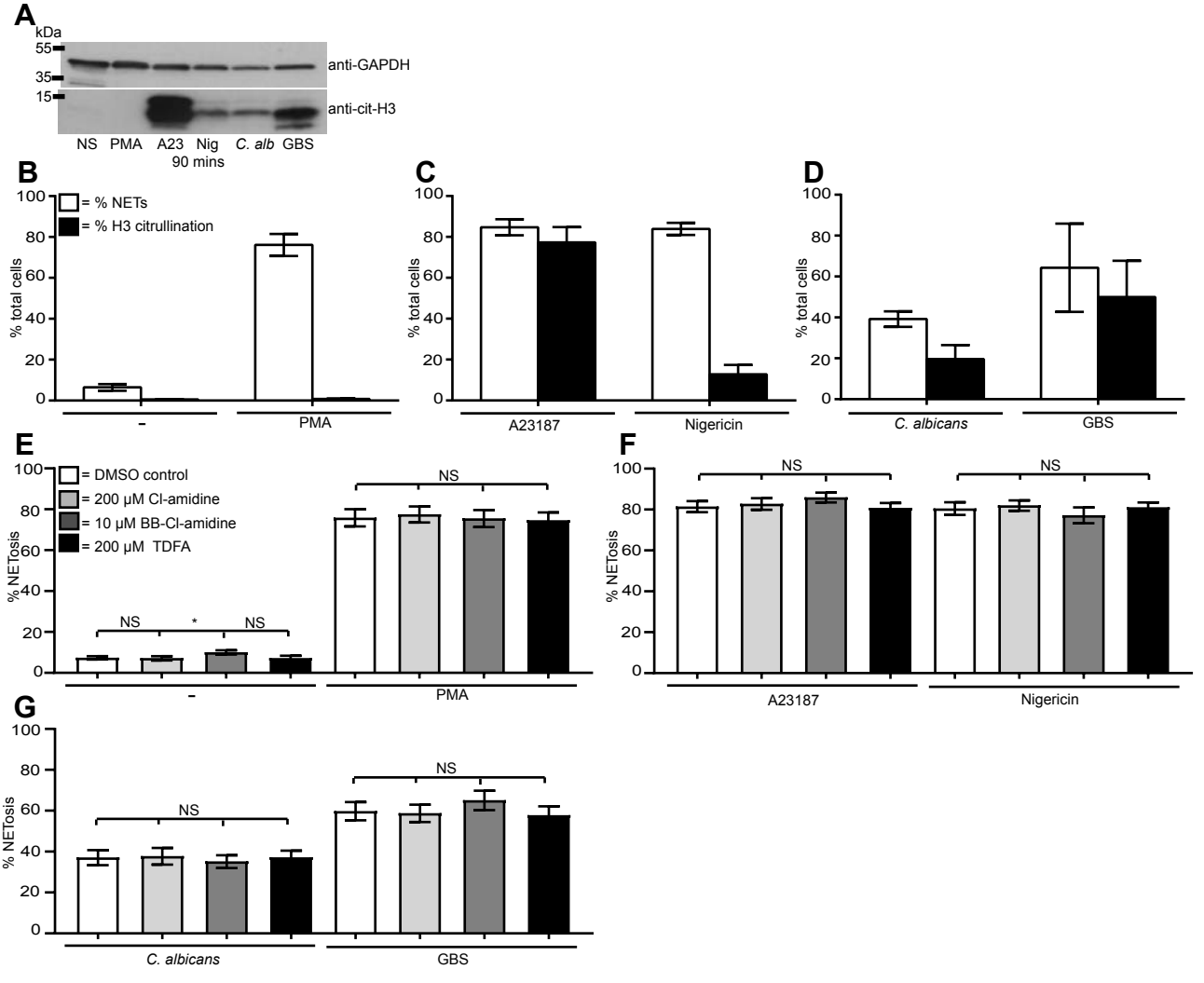
A
kDa
55
35
15
anti-GAPDH
anti-cit-H3
NS PMA A23 Nig C. alb GBS
90 mins
B
= % NETs
= % H3 citrullination
% total cells
PMA
C
A23187
Nigericin
D
C. albicans
GBS
E
= DMSO control
= 200 µM Cl-amidine
= 10 µM BB-Cl-amidine
= 200 µM TDFA
% NETosis
NS
*
F
G

Figure 6 - figure supplement 1

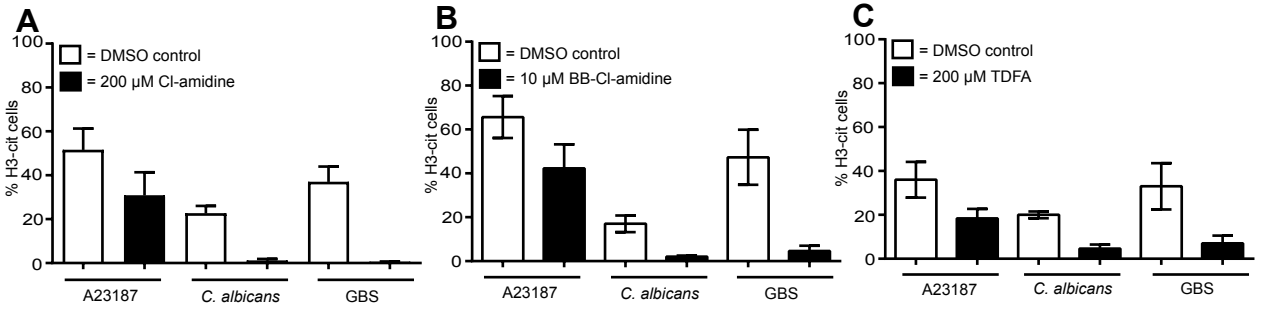

Figure 6 - figure supplement 2

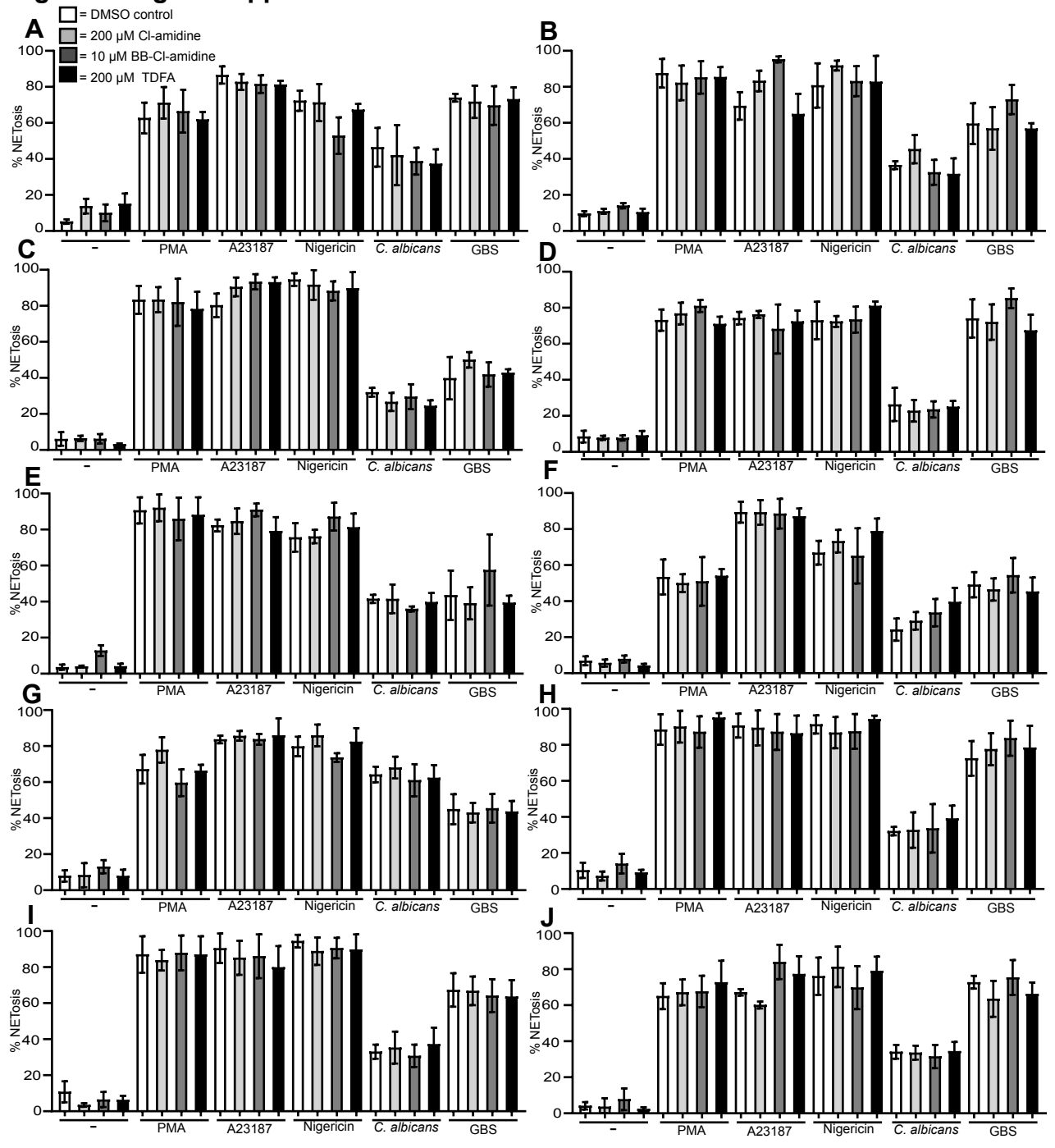

Figure 7

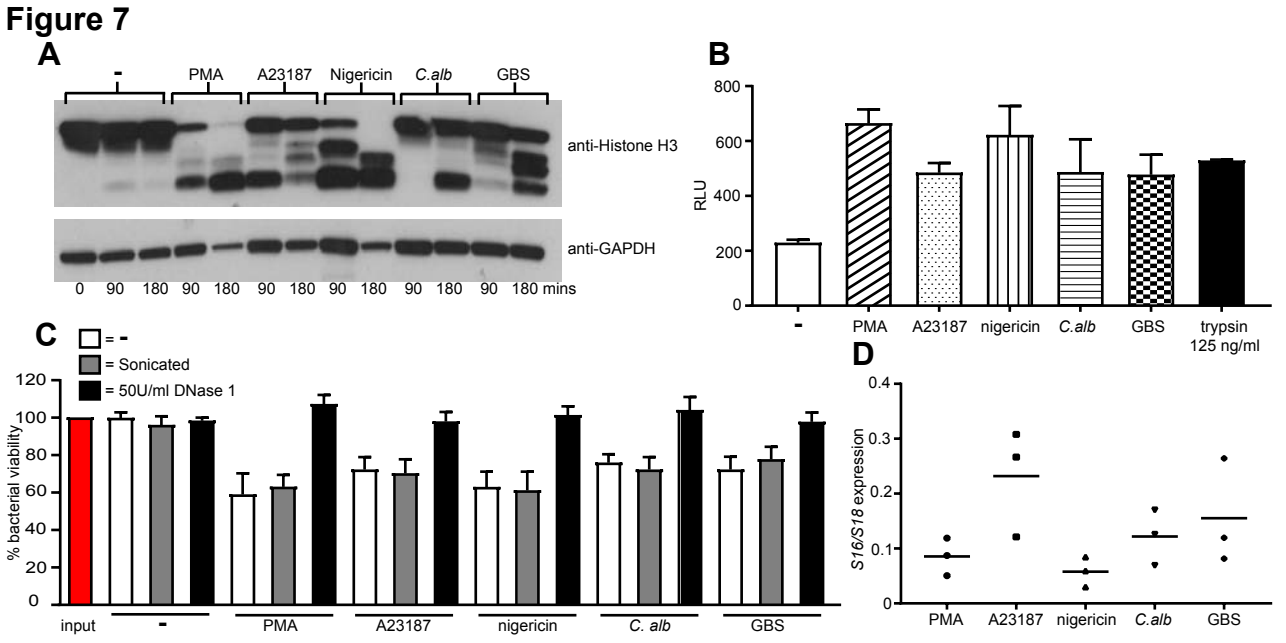
A
-
PMA
A23187
Nigericin
C.alb
GBS
anti-Histone H3
anti-GAPDH
0 90 180 90 180 90 180 90 180 90 180 90 180 mins
B
RLU
800
600
400
200
0
-
PMA
A23187
nigericin
C.alb
GBS
trypsin
125 ng/ml
C
= -
= Sonicated
= 50U/ml DNase 1
% bacterial viability
120
100
80
60
40
20
0
input
-
PMA
A23187
nigericin
C. alb
GBS
D
S16/S18 expression
0.4
0.3
0.2
0.1
0
PMA
A23187
nigericin
C.alb
GBS

Figure 8

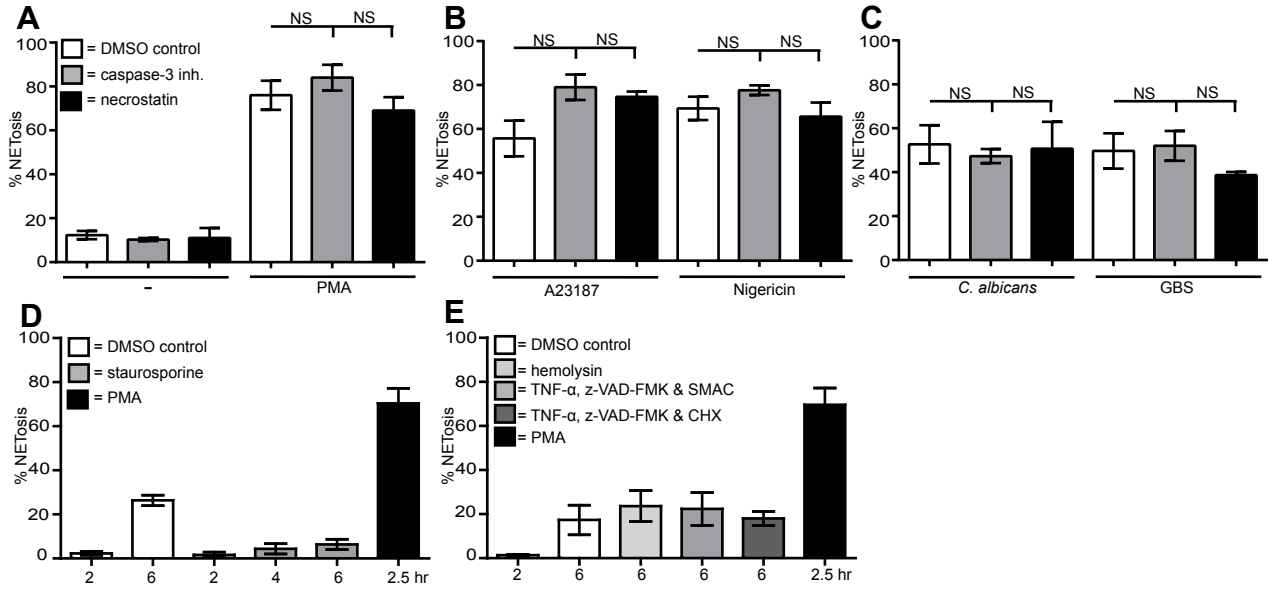
A
= DMSO control
= caspase-3 inh.
= necrostatin
% NETosis
NS
NS
-
PMA
B
A23187
Nigericin
C
C. albicans
GBS
D
= staurosporine
= PMA
2
6
2
4
6
2.5 hr
E
= hemolysin
= TNF-α, z-VAD-FMK & SMAC
= TNF-α, z-VAD-FMK & CHX

Figure 8 - figure supplement 1