Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens

Nora Branzk1, Aleksandra Lubojemska1, Sarah E Hardison2, Qian Wang1, Maximiliano G Gutierrez3, Gordon D Brown2 & Venizelos Papayannopoulos1

Neutrophils are critical for antifungal defense, but the mechanisms that clear hyphae and other pathogens that are too large to be phagocytosed remain unknown. We found that neutrophils sensed microbe size and selectively released neutrophil extracellular traps (NETs) in response to large pathogens, such as Candida albicans hyphae and extracellular aggregates of Mycobacterium bovis, but not in response to small yeast or single bacteria. NETs were fundamental in controlling fungal clearance when they were small enough to be phagocytosed.

The immune system controls microbes of varying size, ranging from small viruses and bacteria to dimorphic fungi and large multicellular parasites. Large microbes and parasites evade phagocytosis and can prove difficult to clear. It is unknown how the immune system senses microbe size and clears large pathogens. There has been considerable progress in the understanding of how receptors of the immune system distinguish between soluble microbial ligands and particle-associated microbial ligands. However, little is known about how cells of the immune system distinguish between microbial particles of varying size and whether they can respond selectively to combat microbes of varying size.

Fungi present a considerable challenge to the immune system, as they grow not only in a small yeast form that can be countered by phagocytosis but also in a filamentous form that is too large to be phagocytosed. Both small forms and large forms are thought to be critical for fungal virulence. Due to its large size, the filamentous form is resistant to the immune system, while the small yeast or spores are required for dissemination. Macrophages release higher concentrations of the cytokine interleukin 1β in response to hyphae than in response to yeast, but the mechanism that drives this difference in response and, most notably, the relevance of this hypersensitivity for fungal clearance are unclear.

Neutrophils are critical in controlling fungal pathogens that cause potentially lethal infections in neutropenic and immunosuppressed people. While neutrophils kill yeast by phagocytosis and release factors that indirectly starve the fungus of important metal ions, how neutrophils directly kill filamentous hyphae is poorly understood. Neutrophils were long thought to undertake a single antimicrobial program involving phagocytosis and degranulation. The discovery of neutrophil extracellular traps (NETs) raised the question of whether neutrophils can deploy their antimicrobial strategies selectively to generate an efficient immune response that is tailored to pathogens with different attributes.

NETs are large, extracellular, web-like structures composed of decondensed chromatin and neutrophil antimicrobial factors. NETs trap and kill a variety of microbes, but their contribution to innate immune defense is unclear. NETs are released mainly via the cell-death program known as ‘NETosis’ that requires reactive oxygen species and the granule proteins myeloperoxidase (MPO) and neutrophil elastase (NE). Upon activation of neutrophils, NE is released from azurophilic granules into the cytosol and translocates to the nucleus, where it cleaves histones to decondense chromatin. Myeloperoxidase consumes H2O2 to generate HOCl and other oxidants and is required for translocation of NE to the nucleus during NETosis. Fungi trigger NETosis and are killed by NETs. Furthermore, NET deficiency is associated with susceptibility to fungal infection in patients with chronic granulomatous disease who do not generate ROS and in MPO-deficient patients. A case study of humans has hinted that NETs may serve a critical antifungal role by controlling the large filamentous form of fungi, but due to the requirement for ROS and MPO in phagocytic killing, the importance of NETs in antifungal immunity has been difficult to delineate.

The aberrant release and defective clearance of NETs has also been associated with various human pathologies, such as autoimmune
systemic lupus erythematosus and vasculitis associated with anti-neutrophil cytoplasmic antibodies. Although their interaction is poorly understood, fungal infection and aberrant NETosis have been linked to inflammatory and autoimmune diseases\(^{19,20}\). Therefore, NETosis must be tightly regulated, but the mechanisms that control the deployment of NETs in different scenarios remain unknown. Here we identify a cell-autonomous mechanism that allows cells of the immune system, such as neutrophils, to sense microbe size. We found that by regulating the size-dependent release of NETs, this mechanism allowed selective implementation of neutrophil antimicrobial strategies to clear fungal infections while minimizing tissue damage.

**RESULTS**

**NETosis depends on microbe size**

To address how neutrophils respond to microbes of varying size, we assessed the ability of several microbes to induce NETosis in isolated human peripheral neutrophils using the cell-impermeable SYTOX dye to assess cell viability and stain extracellular DNA. Fungal pathogens are good inducers of NETosis. Notably, NETosis in response to *Candida albicans* required plasma (Fig. 1a). Because plasma drove the formation of hyphae by *C. albicans* (Fig. 1a), we investigated whether fungal morphology has a role in NET formation. We found that large preformed hyphae grown in RPMI medium induced NETosis in the absence of plasma (Fig. 1b and Supplementary Fig. 1a). In contrast, a mutant of *C. albicans* that is locked in the yeast form ('yeast-locked') and is unable to form hyphae due to a deficiency in the gene encoding the hypha-specific cyclin hgc1 (\(hgc1\))\(^{21}\) failed to induce NETosis in the presence of plasma or RPMI medium (Fig. 1b and Supplementary Fig. 1b). Finally, hyphae triggered histone degradation in neutrophils, a key step in driving chromatin decondensation during NETosis\(^{14}\), but yeast did not (Fig. 1c). These data suggested that NET release was selective and was dependent on fungal morphology.

Beyond their difference in size, *C. albicans* yeast and hyphae also vary in metabolic activity and surface molecule expression\(^{22,23}\). Notably, selective NETosis was not dependent on microbial enzymatic activity, since heat-inactivated hyphae induced the release of NETs as efficiently as live hyphae did (Supplementary Fig. 1c,d). We next investigated whether selective NETosis was driven by the expression of different fungal surface molecules or was driven solely by a difference in microbe size. For this, we presented small yeast particles to neutrophils over a modified Transwell insert that allowed direct contact with the microbes but prevented phagocytosis. We removed the rim of the Transwell insert to enable the insert to sink to the bottom of the well and bring neutrophils in contact with the membrane. The membrane pore was small enough to prevent neutrophil transmigration and phagocytosis but large enough to allow neutrophils to extend filopodia and contact the microbes on the other side. To ensure that neutrophils were coming in contact with the microbes, we removed the

![Image](image-url)

**Figure 1**  Hyphae selectively induce NETosis.  
(a) Release of NETs by human peripheral neutrophils left unstimulated (US) or stimulated with wild-type (WT) *C. albicans* (at a multiplicity of infection (MOI) of 10), in the presence (bottom) or absence (top) of 3% plasma, followed by staining of extracellular DNA with SYTOX at 4 h after stimulation (left and middle), and phase-contrast microscopy of cells as at left (right). (b) Quantification of NET release by human peripheral neutrophils left unstimulated or stimulated with *hgc1* (US). (c) NET release by human peripheral neutrophils left unstimulated or stimulated with wild-type *C. albicans* hyphae (Hyphae (WT)), without plasma, presented as SYTOX + events relative to total neutrophils (top). Below, formation of NETs (below the zero line) by nuclei (left) and phase-contrast microscopy of cells above (bottom). (d) Experimental setup (top left) and quantification of results from f (below), presented as in b. Scale bars (a,d,f), 50 \(\mu\)m. *\(P < 0.01\), **\(P < 0.001\) and ***\(P < 0.0001\) (one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison post-test). Data are representative of at least three independent experiments.
modified Transwell insert after 1 h of incubation, rinsed the bottom and imaged the cells with the membrane-permeable DNA-binding stain DAPI. We found considerable attachment of neutrophils to the bottom of the modified Transwell insert (Supplementary Fig. 1e). In the presence of the Transwell, small yeast particles triggered NETosis only when the Transwell insert was in direct contact with neutrophils (Fig. 1d) but not when the insert was suspended in the well to prevent direct contact between neutrophils and microbes (Supplementary Fig. 1f). Hence, neutrophils were in physical contact with yeast in the modified Transwell; otherwise, neutrophils in the suspended Transwell would also have yielded NETs. Therefore, yeast triggered NETosis only when they were presented as larger particles. In contrast, heat-inactivated hyphae that were fragmented into particles small enough to be phagocytosed (Supplementary Fig. 2) lost their ability to induce NETosis (Fig. 1f.g). Therefore, selective NETosis was independent of the expression of molecules on the surface of fungi or the enzymatic activity of fungi and was regulated only by differences in microbe size. Likewise, neutrophils released NETs in response to Aspergillus fumigatus hyphae or large aggregated A. fumigatus conidia but failed to form NETs in response to small single conidia (Supplementary Fig. 3).

Although bacteria are reported to induce the release of NETs11,13,24,25, our data suggested that bacteria that were small enough to be phagocytosed would not induce NETosis. Consistent with that, neutrophils did not release NETs in response to Escherichia coli or Klebsiella pneumoniae (Fig. 2a.b) but NETosis was induced when bacteria were presented by the Transwell system (Fig. 2a.b). Many pathogenic bacteria are known to circumvent phagocytosis through virulence strategies that include the formation of large aggregates36–38. Clumping of Mycobacterium species is thought to prevent phagocytosis and contribute to pathogenesis39. We incubated neutrophils with a mixture of single bacteria and small and large aggregates of Mycobacterium bovis bacillus Calmette-Guérin (BCG) conjugated to the red fluorescent protein DsRed (BCG-DsRed) and found that after 4 h, some neutrophils released NETs (Fig. 2c.d, group ii), whereas others underwent necrosis with small nuclei that did not decondense (Fig. 2c.d, group i). In these endpoint experiments, NETs seemed to be released by neutrophils that were associated with large bacterial aggregates. However, the accumulation of small, phagocytosed bacteria in neutrophils made it difficult to determine the original size of the bacterial particles that triggered these responses. Therefore, we used time-lapse video microscopy to observe neutrophils infected with BCG-DsRed (Fig. 2e). While single bacteria underwent phagocytosis without triggering NETosis, neutrophils failed to phagocytose large BCG-DsRed aggregates and eventually released NETs (Fig. 2e). Therefore, the size dependence of the release of NETs applied to fungi and bacteria and might apply to other large pathogens.

**NETs control hyphae in vivo**

Our findings suggested that perhaps NETs evolved as a specific mechanism for countering large hyphae extracellularly, as they cannot be phagocytosed. To address this point, we first investigated whether NETs were released selectively in vivo by detecting DNA, MPO and citrullinated histone H3 (a marker specific for NETs). Notably, only wild-type C. albicans that was able to form both yeast and hyphae induced the release of NETs in the lungs of wild-type mice (Fig. 3a). In contrast, the yeast-locked hgc1Δ mutant failed to induce NETosis (Fig. 3a), which indicated that neutrophils selectively released NETs in response to the size of C. albicans both in vitro and in vivo. To address whether selective NETosis is critical for controlling the hyphal form, we infected MPO-deficient mice (which do not form NETs) and wild-type control mice with a low dose of wild-type or yeast-locked hgc1Δ C. albicans. Wild-type mice that were able to both phagocytose and form NETs cleared both wild-type and yeast-locked hgc1Δ C. albicans strains and fully recovered from the infection (Fig. 3b). Notably, MPO-deficient mice, which were able to kill only via phagocytosis, cleared the yeast-locked hgc1Δ strain that does not form hyphae (Fig. 3b), which indicated that NETs were not required for clearance of yeast. In contrast, the MPO-deficient mice succumbed to infection with the wild-type fungus that forms both yeast and hyphae (Fig. 3b), which indicated that while MPO-deficient mice were able to control yeast in the absence of NETs, they were unable to control hyphae. The wild-type fungus established infection in the lungs of MPO-deficient mice and disseminated to the spleen (Fig. 3c). Therefore, NETs were required specifically for control of the hyphal form. To investigate whether these differences were due to the possibility that the hgc1Δ mutant is a
Phagocytosis inhibits NETosis by sequestering NE

To investigate the mechanism by which size-dependent NETosis is regulated, we first assessed the activation of Syk and Erk, two kinases involved downstream of fungal recognition and linked to the NETosis pathway by triggering ROS production. We found only subtle differences in the dynamics of the phosphorylation of Syk upon induction with yeast or hyphae (Supplementary Fig. 5a). Erk appeared to be more strongly induced by hyphae. To determine if these differences in kinase activation were triggering differences in ROS activation, we compared the neutrophil ROS burst in response to yeast or hyphae. We found two major classes of neutrophils in populations from healthy human donors: for half of the donors (n = 6) there was no major difference in ROS dynamics, while for the other half, neutrophils exhibited a delayed response to yeast but yielded much higher ROS concentrations (Supplementary Fig. 5b). Notably, NETs were selectively released only in response to hyphae regardless of these variations in ROS dynamics among different human donors, which indicated that that while signaling via these kinases that regulate the ROS burst may have been required for NETosis, it did not regulate the ‘decision’ of whether to make NETs in response to microbe size.

We reasoned that size-dependent ‘decision-making’ might be regulated downstream of ROS. Because phagocytosis proceeds faster than NETosis, we hypothesized that the uptake of yeast could potentially sequester NE to the phagosome and prevent the translocation of NE to the nucleus. Hence, we monitored the localization of NE after stimulation with yeast or hyphae. We stained neutrophils for NE, MPO and CD63. NE and MPO localize together in the same azurophilic granules in resting neutrophils, but NE selectively translocates to the phagosome during NET formation, leaving MPO behind.

The results presented above suggested that fusion of the phagosome to azurophilic granules containing NE was the critical step in the regulation of selective NETosis. To investigate whether sequestration of NE to the phagosome suppresses NET release in response to yeast, we blocked the fusion of granules to phagosomes by inhibiting acidification with bafilomycin A1 or by inhibiting microtubule polymerization with nocodazole. Each treatment increased NET formation in response to hgc1Δ C. albicans yeast (Fig. 5a), indicative of a simple competition mechanism whereby phagocytosis inhibits NETosis. Since ROS are important upstream effectors in

**Figure 3** Selective NETosis is critical for clearance of hyphae in vivo. (a) NET release (white arrows) in the lungs of wild-type (C57BL/6) mice 24 h after intratracheal infection with 1 × 10⁶ colony-forming units (CFU) of wild-type or hgc1Δ C. albicans, assessed by immunofluorescence microscopy of citrullinated histone H3 (Cit-H3; red), MPO (green) and DAPI-stained DNA (blue). Bottom row, enlargement of area outlined in top left image. Scale bars, 20 µm. (b) Weight of wild-type (C57BL/6) mice (WT; n = 6) and MPO-deficient mice (ΔMPO; n = 5) infected with 1 × 10⁶ CFU of wild-type or hgc1Δ C. albicans, presented relative to starting weight (at day 0), set as 100%. Each symbol represents an individual mouse (n = 3 mice per group); small horizontal lines indicate the mean. *P < 0.01 and **P < 0.0001 (two-way ANOVA, followed by Sidak’s (b) or Tukey’s (c) multiple-comparison post-test). Data are representative of two independent experiments (error bars (b), s.d.).
NETosis, we also investigated whether these compounds elicited NETosis by increasing the generation of ROS. However, in the presence of bafilomycin A1 or nocodazole, ROS concentrations were lower (Fig. 5b), as assembly of the NADPH oxidase involves microtubule-enhanced fusion of specific granules to the phagosome membrane. Notably, the remaining concentration of ROS was sufficient to trigger NETosis in response to yeast (Fig. 5a). Furthermore, preincubation of neutrophils with yeast to induce phagocytosis before the induction of NETosis with hyphae decreased the efficiency of NET release after restimulation with hyphae, in a dose-dependent manner (Fig. 5c); this highlighted the importance of NE availability in driving NET formation. NETosis was similarly inhibited by feeding of neutrophils with opsonized polystyrene beads of a size similar to that of yeast (Fig. 5d), which indicated that the formation of phagosomes was sufficient to downregulate NETosis without a requirement for additional stimulation by microbial factors in yeast. Therefore, phagocytosis negatively regulated NETosis by depleting NE before it was able to translocate to the nucleus. Such a mechanism would prevent unnecessary release of NETs when the pathogen is small enough to be eliminated intracellularly by phagocytosis and might help to ‘fine tune’ the immune response.

Dectin-1 negatively regulates NETosis

Next we investigated whether the regulation of NETosis by phagocytosis is important in ‘fine-tuning’ the immune response to reduce immunopathology. We reasoned that a deficiency in phagocytic receptor activity might disrupt the size-dependent selectivity of NET release due to inefficient phagocytosis. Dectin-1 is one of the main antifungal phagocytic receptors34,35, and we found that human primary neutrophils treated with a blocking antibody to dectin-1 (anti-dectin-1) exhibited lower rates of phagocytosis than those of untreated neutrophils and took up less than half the number of yeast taken up by untreated neutrophils (Fig. 6a). That residual phagocytosis was probably driven by other phagocytic receptors that quickly became saturated in the absence of active dectin-1. The reduction in phagocytosis was accompanied by considerable upregulation of NETosis in response to yeast-locked Δhgc1 C. albicans (Fig. 6b). In line with published reports36, in the absence of the blocking antibody, phagocytosing human neutrophils died after engulfing microbes to their maximal capacity but failed to decondense their nuclei (Fig. 6c and Supplementary Movie 1), consistent with the lack of translocation of NE to the nucleus (Fig. 4a,b). In contrast, when phagocytosis was diminished in the presence of anti-dectin-1, neutrophils readily decondensed their nuclei and made NETs in response to yeast (Fig. 6c and Supplementary Movie 2). Immunofluorescence microscopy showed that the amount of NE localized to the phagosome in neutrophils treated with anti-dectin-1 and incubated for 1 h with yeast (Fig. 6d) was similar to that of untreated control neutrophils (Fig. 4c), and that NE localized together with CD63 (Fig. 6d), indicative of delivery of NE via fusion of azurophilic granules with the phagosome. In addition, in contrast to results obtained for untreated neutrophils, in neutrophils treated with anti-dectin-1, NE also translocated to the nucleus and away from CD63 (Fig. 6d), which is characteristic of the translocation of NE during NETosis that is independent of membrane-fusion events14,15. Therefore, the lower number of phagosomes formed per neutrophil upon inhibition of dectin-1 resulted in reduced sequestration of NE, which left enough NE to translocate to the nucleus and drive NETosis.
Figure 5 Phagocytosis inhibits NETosis via sequestration of NE. (a) NET release by human peripheral neutrophils left untreated (UT) or treated with 1 μM bafilomycin A1 (Baf) or 2.5 μM nocodazole (Noc) and left unstimulated (−) or stimulated (+) with hgc1Δ C. albicans at an MOI of 10 (presented as in Fig. 1b). (b) Production of reactive oxygen species (ROS) by human peripheral neutrophils left untreated or treated with bafilomycin A1 or nocodazole and stimulated with hgc1Δ C. albicans at an MOI of 10. LU, luminescence units. (c) NET release by neutrophils preincubated for 1 h with hgc1Δ C. albicans at an MOI of 0, 20, 40, or 80 and then left unstimulated (−) or stimulated (+) with C. albicans hyphae at an MOI of 10 (presented as in Fig. 1b). (d) NET release by neutrophils preincubated for 1 h with 0.1-μm-polystyrene beads and then stimulated with C. albicans hyphae at an MOI of 10 (presented as in Fig. 1b). *P < 0.01 and **P < 0.0001 (two-way ANOVA followed by Sidak’s multiple-comparison post-test). Data are representative of at least three independent experiments.

Furthermore, we observed much greater induction of NETs in the lungs of dectin-1-deficient mice infected with the lung pathogen A. fumigatus (Fig. 6e) or wild-type C. albicans (Supplementary Fig. 6a) than in the lungs of wild-type control mice infected similarly. Staining of these lung sections with an antibody to the neutrophil-specific marker MPO suggested that the difference in NET formation was not due to the recruitment of a lower number of neutrophils to the lungs of wild-type mice (Fig. 6e). Our in vitro data suggested that aberrant NETosis in dectin-1-deficient mice was the result of deregulated suppression of NETosis in response to yeast. Indeed, yeast-locked hgc1Δ C. albicans induced the release of NETs in the lungs of dectin-1-deficient mice but failed to induce NETosis in wild-type mice (Fig. 7a). Moreover, there was no significant difference in the burden of yeast-locked hgc1Δ C. albicans in the lungs of dectin-1-deficient mice versus those of wild-type mice (Supplementary Fig. 6b), which ruled out the possibility that the differences in NETosis were due to higher fungal load. Therefore, genetic disruption of phagocytosis in dectin-1-deficient mice led to deregulation of NETosis and resulted in the release of NETs from neutrophils in response to both yeast and hyphae.

Dectin-1 deficiency promotes NET-mediated pathology

Finally, we sought to address whether the mechanism noted above for the suppression of NETosis in response to small microbes such as yeast is important in protection against potential damage by excessive NET release. Aberrant NETosis has been linked to a range of inflammatory and autoimmune diseases. NETs are toxic to endothelial cells in culture, but the destructive role of NETs to tissues has not been addressed in vivo. We hypothesized that dysregulation of selective NETosis in dectin-1-deficient mice might lead to pathology. We infected wild-type and dectin-1-deficient mice with a high dose of yeast-locked hgc1Δ C. albicans and monitored their survival.

Figure 6 The phagocytic receptor dectin-1 negatively regulates NETosis. (a) Phagocytosis of hgc1Δ C. albicans (MOI, 40) by neutrophils (n = 10 per condition) left untreated (left) or treated with blocking antibody to dectin-1 (right); phagocytosed yeast particles per cell were quantified over 2 h by live microscopy (diagonal line: trend line fitted to data). (b) NET release by peripheral human neutrophils left untreated (left) or treated with blocking antibody to dectin-1 (right) and left unstimulated or stimulated with wild-type C. albicans hyphae or hgc1Δ C. albicans (MOI, 10), assessed 4 h after stimulation (presented as in Fig. 1b). *P < 0.0001 (one-way ANOVA followed by Sidak’s multiple-comparison post-test). (c) Time-lapse microscopy of live human peripheral neutrophils left untreated (top) or treated with blocking antibody to dectin-1 (bottom) and stimulated with heat-inactivated hgc1Δ C. albicans (MOI, 40); arrowheads indicate incomplete decondensation; arrows indicate NET release. Confocal images were obtained every 30 s. (d) Localization of NE to the phagosome (arrows) or nucleus (asterisk) in human peripheral neutrophils stimulated for 1 h with hgc1Δ C. albicans and then stained for NE (red) and CD63 (green) and DNA (DAPI; blue). (e) NET release in the lungs of dectin-1-deficient mice (Dectin-1-KO) and wild-type (C57BL/6) control mice (WT) infected intratracheally with 1 × 10^6 CFU of A. fumigatus, assessed 48 h later by immunofluorescence staining of citrullinated histone H3, MPO and DNA (DAPI). Scale bars, 20 μm (c,e) or 5 μm (d). Data are representative of two independent experiments.
Figure 7  Deregulation of NET release leads to pathology. (a) NET release in lungs of wild-type and dectin-1-deficient mice left untreated (left and middle) or treated with the NE inhibitor (+ NEI) (right) and then infected with 3 × 10⁶ CFU hgc1Δ C. albicans, assessed 24 h later by immunofluorescence confocal microscopy of lung sections stained for DNA (DAPI; blue), citrullinated histone H3 (red) and MPO (green). Scale bars, 50 μm. (b) Survival of wild-type (C57BL/6) mice and dectin-1-deficient mice (n = 4 per group) left untreated or treated with the NE inhibitor (NEi) or amphiregulin (AREG) and then infected intratracheally with 1 × 10⁷ CFU of hgc1Δ C. albicans. (c) Quantification of fibrin deposition (top two plots) and bleeding (bottom two plots) in lungs of wild-type (C57BL/6) mice and dectin-1-deficient mice infected with 3 × 10⁶ CFU of hgc1Δ C. albicans, assessed 36 h later and presented as average score for each mouse (top and third plots) or frequency of images from all mice with each score (second and bottom plots). Each symbol (top and third plots) represents an individual mouse; small horizontal lines indicate the mean. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (log-rank (Mantel-Cox) test (b) or two-way ANOVA followed by Tukey’s multiple-comparison post-test (c)). Data are representative of two independent experiments (error bars (c), s.d.).

We also treated these mice with either a specific inhibitor of NE that blocks NETosis or the tissue growth factor amphiregulin, which promotes tissue repair. Published studies have shown that dectin-1-deficient mice exhibit impaired cytokine responses and are better protected against cytokine-induced shock. Dectin-1-mediated activation drives the upregulation of proinflammatory cytokines such as interleukin 6, interleukin 12 and tumor-necrosis factor that can promote mortality during infection. Consistent with that, wild-type mice succumbed to infection with a high dose of yeast-locked hgc1Δ C. albicans and were not ‘rescued’ by the NE inhibitor (Fig. 7b), which suggested that NETs were not involved in the pathology in wild-type mice. In addition, treatment with amphiregulin did not increase the survival of wild-type mice (Fig. 7b), which indicated that tissue damage was not influencing mortality. Instead, the lungs of wild-type mice had substantially higher concentrations of tumor-necrosis factor than did dectin-1-deficient lungs (Supplementary Fig. 6c), which suggested that wild-type mice succumbed due to cytokine-induced shock.

Dectin-1-deficient mice also succumbed to infection, although their neutrophils were able to phagocytose yeast, albeit with lower capacity, and responded with significantly greater NETosis than that of wild-type mice (Fig. 7a). Microbial load was similar in wild-type and dectin-1-deficient mice (Supplementary Fig. 6b), which indicated that mortality of the mutant mice was not due to higher microbial load that resulted from insufficient fungal clearance. Instead, infected dectin-1-deficient mice exhibited enhanced survival not only when NETosis was blocked with the NE inhibitor (Fig. 7a) but also after treatment with amphiregulin (which promotes tissue repair) (Fig. 7b), which suggested that NETs not only were irrelevant in protecting these mice against the yeast-locked hgc1Δ C. albicans strain but also were detrimental to the host when present in large amounts. The yeast-locked hgc1Δ mutant elicited substantial infiltration of neutrophils into the lungs of both wild-type mice and dectin-1-deficient mice (Fig. 7a and Supplementary Fig. 6d), which indicated that their differences in survival were not due to differences in the recruitment of neutrophils but instead were due differences in subsequent neutrophil responses. The similar fungal load in both groups of mice suggested that neutrophils were exposed to similar amounts of microbial stimuli during infection.

The effectiveness of treatment with the NE inhibitor or amphiregulin in ‘rescuing’ dectin-1-deficient mice suggested that their mortality was driven by tissue damage caused by aberrant NET release. Indeed, the lungs of dectin-1-deficient mice exhibited more tissue damage than those of wild-type mice, and this was associated with fibrin deposition and substantial bleeding in response to yeast-locked hgc1Δ C. albicans (Fig. 7c and Supplementary Fig. 7a). These symptoms were alleviated by treatment with the NE inhibitor (Fig. 7c and Supplementary Fig. 7a), which suggested that NETs were driving tissue damage in the lungs of dectin-1-deficient mice. NE can also cause acute tissue damage directly. Wild-type mice treated with the NE inhibitor exhibited slightly less lung damage than did untreated wild-type mice (Fig. 7c), and since NETs were absent from wild-type mice, some damage could have arisen through NET-independent mechanisms. However, because NETs formed only in dectin-1-deficient, but not in wild-type mice, the inability of the NE inhibitor to ‘rescue’ wild-type mice indicated that the benefit effect of the NE inhibitor and amphiregulin in dectin-1-deficient mice was mediated through suppression of NET-driven damage and not NET-independent mechanisms. Otherwise, lung damage would have been similar in wild-type mice and dectin-1-deficient mice. Therefore, while dectin-1 was important for potent cytokine induction, it was also critical in regulating neutrophil antimicrobial strategies (Supplementary Fig. 7b). By promoting the phagocytosis of smaller microbes, this phagocytic receptor downregulated NETosis in a microbe...
Our findings have identified a microbe size–sensing mechanism that allows neutrophils to selectively tailor their antimicrobial responses to pathogens on the basis of microbe size. The ‘decision’ that regulates NETosis relies on the competition of two cellular processes for NE. Phagocytosis is a rapid process, and neutrophils engulf a maximal number of yeast particles within 30–40 min. In contrast, NETosis is a slow process that requires approximately 4 h for completion. Phagocytosis drives the rapid fusion of azurophilic granules to the phagosome and the delivery of NE to its microbial contents to sequester NE away from the nucleus and prevent the proteolytic processing of histones that promotes chromatin decondensation during NETosis. When neutrophils encounter a microbe that is too large to be phagocytosed, the absence of phagosomes allows NE to be slowly released into the cytosol via an alternative pathway that does not involve membrane fusion. NE is then free to translocate to the nucleus and drive chromatin decondensation. This simple but effective mechanism allows phagocytes to distinguish between small pathogens and large pathogens. In contrast, the distinction of particle-associated cell-wall components, such as β-glucan, from their soluble form is driven by the localized exclusion of suppressive phosphatase activity from receptor clusters that selectively trigger a signaling cascade to initiate phagocytosis. This mechanism does not seem to have a role in the ability of neutrophils to distinguish between smaller particles and larger particles, since Syk, Erk and presumably other downstream kinases are sufficiently activated by both small yeast and large hyphae. Whether the mechanism we have presented here can serve as a paradigm to explain differences in the release of cytokines by macrophages in response to these different fungal forms remains to be addressed.

Notably, neutrophils that had completed phagocytosis died with kinetics similar to those of neutrophils entering NETosis, as indicated by entry of the cell-impermeable SYTOX dye. This suggested that neutrophils responding to yeast and hyphae entered a necrotic program that lysed the plasma membrane. However, chromatin remained condensed in response to yeast but decondensed in response to hyphae. These observations provide additional evidence to support a model in which the two processes share the same upstream regulatory signaling pathway and the formation of phagosomes acts downstream to dictate the divergence of the NE signal toward phagocytosis or NETosis.

Moreover, during the phagocytosis of smaller microbes and other particles, granules are thought to begin fusing with the plasma membrane before membrane closure, which results in leakage of some azurophilic granule contents to the extracellular space during phagocytosis. Our immunofluorescence studies showed that despite engagement of neutrophils with hyphae that lasted for several hours before the release of NETs, most of the NE remained inside the neutrophils and was not secreted. This suggests that azurophilic granules do not fuse with the plasma membrane during this lengthy engagement.

Our findings have defined the criteria that determine whether a microbe will trigger NETosis. Small microbes that are taken up in a mature phagolysosome are not good stimuli for NETosis. This mechanism ensures that phagocytosis acts as a checkpoint that determines whether a neutrophil will deploy NETs. Here we demonstrated that microbe size was a critical factor that regulated NETosis in response to fungi and bacteria. Like large C. albicans hyphae, aggregates of M. bovis BCG also triggered NETosis, while other single bacteria failed to induce the response. Our Transwell experiments indicated that the upstream pathways that initiate NETosis are probably similar for most microbes and that phagocytosis is the critical event that dictates the ‘decision’ to form NETs. Furthermore, NETosis may provide an immune response to pathogenic bacteria that circumvent phagocytosis through virulence strategies that include the formation of large aggregates or the adoption of bacterial filamentous forms that delay phagocytosis. But microbe size may not be the only virulence mechanism that triggers NETosis. Some bacteria are phagocytosed but can prevent the fusion of late endocytic organelles to the phagosome or even rupture the phagosome. We propose that these virulence mechanisms may have a critical role in the induction of NETosis in response to bacterial stimuli. Notably, M. tuberculosis and M. bovis BCG are known to inhibit phagosome maturation. Our analysis suggested that for M. bovis BCG, NETosis appeared to be dependent on microbe size. However, the possibility that bacteria–dependent manipulation of phagosome maturation may enhance the release of NETs cannot be excluded, and further work is needed to address this. Macrophages also release extracellular traps (‘METs’) in response to M. tuberculosis in a process that also depends on macrophage proteases, which suggests that NETs and METs may have important roles during infection. Notably, viruses such as human immunodeficiency virus trigger the release of NETs. On the basis of our model we predict that viruses may be good NET stimuli, since they may be too small to form sufficiently large endocytic organelles to result in substantial depletion of NE.

Notably, the ability to release NETs selectively is critical for minimizing unnecessary immunopathology associated with aberrant NETosis. Our data have demonstrated that interfering with phagocytosis through defects in dectin-1 disrupted the ability of neutrophils to make this important ‘decision’ and released NETs indiscriminately, which drove tissue damage and host mortality that was ‘rescued’ by pharmacological inhibition of NET formation. Published studies have reported that low expression of dectin-1 and mutations in genes encoding phagocytic receptors are associated with autoimmune pathologies. Our findings suggest that such deficiencies may disrupt the regulation of selective release of NETs and trigger or exacerbate the symptoms of autoimmune diseases.

Neutrophils have long been viewed as pathogenic effectors in a large number of inflammatory diseases. The mere presence of neutrophils at inflammatory sites is often associated with pathology. However, our findings suggest that neutrophils do not undertake a single antimicrobial program. Instead, after being recruited to the site of inflammation, neutrophils are able to make important ‘decisions’ that define the antimicrobial strategies they will implement to efficiently clear pathogens and minimize host damage.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
N.B. performed all experiments, except as noted below; S.E.H. and G.D.B. infected mice with A. fumigatus; N.B., A.L., Q.W. and V.P. did deoxyribonucleic acid and neutrophil immunofluorescence microscopy; M.G.G. provided advice for and contributed to the M. bovis BCG experiments; V.P. devised and directed the study; and N.B. and V.P. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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1. Scanianamico, S. et al. Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by Leishmania donovani promastigotes. Cell. Microbiol. 1, 19–32 (1999).

2. Gantner, B.N., Simmons, R.M. & Underhill, D.M. Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments. EMBO J. 24, 1277–1286 (2005).

3. Goodridge, H.S. et al. Activation of the innate immune receptor Dectin-1 upon formation of a ‘phagocytic synapse’. Nature 472, 471–475 (2011).

4. Gow, N.A., van de Veerdonk, F.L., Brown, A.J. & Netea, M.G. Neutrophil immunity. Nat. Rev. Immunol. 12, 579–590 (2012).

5. Metzler, K.D., Goosmann, C., Lubojemska, A., Zychlinsky, A. & Papayannopoulos, V. A myeloperoxidase-containing complex regulates neutrophil elastase release and neutrophil extracellular trap formation: implications for innate immunity. EMBO J. 20, 75–77 (2011).

6. Bianchi, M. Neutrophil extracellular traps kill bacteria. Sci. Transl. Med. 3, 102ra20 (2011).

7. Metzler, K.D. et al. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. Blood 117, 953–959 (2011).

8. Metzler, K.D. et al. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. J. Cell Biol. 176, 231–241 (2007).

9. Papayannopoulos, V., Metzler, K.D., Hakkim, A. & Zychlinsky, A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J. Cell Biol. 191, 627–635 (2010).

10. Metzler, K.D., Goosmann, C., Lubojemska, A., Zychlinsky, A. & Papayannopoulos, V. A myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis. Cell Rep. 8, 883–896 (2014).

11. Bianchi, M. et al. Restoration of NET formation by gene therapy in CGD controls aspergillosis. Blood 114, 2619–2622 (2009).

12. Lehrer, R.I. & Cline, M.J. Leukocyte myeloperoxidase deficiency and disseminated candidiasis - role of myeloperoxidase in resistance to Candida infection. J. Clin. Invest. 48, 1478–1488 (1969).

13. Parry, M.F. et al. Myeloperoxidase deficiency - prevalence and clinical significance. Ann. Intern. Med. 95, 293–301 (1981).

14. Banzk, N. & Papayannopoulos, V. Molecular mechanisms regulating NETosis in infection and disease. Semin. Immunopathol. 35, 513–530 (2013).

15. Caza, T., Oaks, Z. & Perl, A. Interplay of infections, autoimmunity, and immunosuppression in systemic lupus erythematosus. Int. Rev. Immunol. 33, 330–363 (2014).

16. Zheng, X., Wang, Y. & Wang, Y. Hgp1, a novel hypha-specific G1 cyclin-related protein regulates Candida albicans hyphal morphogenesis. EMBO J. 23, 1845–1856 (2004).

17. Martinez-Gomariz, M. et al. Proteomic analysis of cytoplasmic and surface proteins from yeast cells, hyphae, and biofilms of Candida albicans. Proteomics 9, 2230–2252 (2009).

18. Marthchenko, M., Alarco, A.M., Harsoua, D. & Whiteley, M. Superoxide dismutases in Candida albicans: transcriptional regulation and functional characterization of the hyphal-induced SODs gene. Mol. Biol. Cell 15, 456–467 (2004).

19. Grinberg, N., Elazar, S., Rosenshine, I. & Shpigel, N.Y. β-hydroxybutyrate regulates formation of bovine neutrophil extracellular traps and bactericidal activity against mammalian pathogenic Escherichia coli. Infect. Immun. 76, 2802–2807 (2008).

20. Morsi, Y. et al. Enolase of Streptococcus pneumoniae induces formation of neutrophil extracellular traps. J. Biol. Chem. 287, 10472–10481 (2012).

21. Domenech, M., Ramos-Sevillano, E., Garcia, E., Moscoso, M. & Yuste, J. Bioluminescence avoids complement immunity and phagocytosis of Streptococcus pneumoniae. Infect. Immun. 81, 2066–20615 (2013).

22. Walker, J.N. et al. The Staphylococcus aureus Arl8F two-component system is a novel regulator of agglutination and pathogenesis. PLoS Pathog. 9, e1003819 (2013).

23. McDow, M. et al. Preventing Staphylococcus aureus sepsis through the inhibition of its agglutination in blood. PLoS Pathog. 7, e1002307 (2011).

24. Bernut, A. et al. Mycobacterium abscessus cordis prevents phagocytosis and promotes abscess formation. Proc. Natl. Acad. Sci. USA 111, E943–E952 (2014).

25. Futa, A.K. & et al. Activation of the Ral-MEK-ERK pathway is required for neutrophil extracellular trap formation. Nat. Chem. Biol. 7, 75–77 (2011).

26. Nordenfelt, P. & Tagger, H. Phagosome dynamics during phagocytosis by neutrophils. J. Leukoc. Biol. 90, 271–284 (2011).

27. Villanueva, E. et al. Neutrophil neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. J. Immunol. 187, 538–552 (2011).

28. Cerna-Rivera, C., Zhao, W., Valayarthi, S. & Kaplan, M.J. Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix metalloproteinase-2. Ann. Rheum. Dis. doi:10.1136/ annrheumdis-2013-204837 (25 February 2014).

29. Taylor, P.R. et al. Dectin-1 is required for β-glucan recognition and control of fungal infection. Nat. Immunol. 8, 31–38 (2007).

30. Robinson, M.J. et al. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. J. Exp. Med. 206, 2037–2051 (2009).

31. Fujie, K. et al. Release of neutrophil elastase and its role in tissue injury in acute inflammation: effect of the elastase inhibitor, FR134043. Eur. J. Pharmacol. 374, 117–125 (1999).

32. Ishii, T. Neutrophil elastase contributes to acute lung injury induced by bilateral nephrectomy. Am. J. Pathol. 177, 1665–1673 (2010).

33. Kawabata, K., Hagio, T. & Matsuoka, S. The role of neutrophil elastase in acute lung injury. Eur. J. Pharmacol. 451, 1–10 (2002).

34. Prashar, A. et al. Filamentous morphology of bacteria delays the timing of phagosome morphogenesis in macrophages. J. Cell Biol. 203, 1081–1097 (2013).

35. Kusner, D.J. Mechanisms of mycobacterial persistence in tuberculosis. Clin. Immunol. 114, 239–247 (2005).

36. Deretic, V. Autophagy, an immunologic magic bullet: Mycobacterium tuberculosis phagosome maturation block and how to bypass it. Future Microbiol. 3, 517–524 (2008).

37. Simeone, R. et al. Phagosomal rupture by Mycobacterium tuberculosis results in toxicity and host cell death. PLoS Pathog. 8, e1002507 (2012).

38. Wang, K.W. & Jacobs, W.R. Jr. Mycobacterium tuberculosis exploits human interferon γ to stimulate macrophage extracellular trap formation and necrosis. J. Infect. Dis. 208, 109–119 (2013).

39. Saiotha, T. et al. Neutrophil extracellular traps mediate a host defense response against human immunodeficiency virus-1. Cell Host Microbe 12, 109–116 (2012).

40. Fossati-Jimack, L. et al. Phagocytosis is the main CR3-mediated function affected by the lupus-associated variant of CD11b in human myeloid cells. PLoS ONE 8, e57082 (2013).

41. Salazar-Altelefone, C. et al. Expression and function of dectin-1 is defective in monocytes from patients with systemic lupus erythematosus and rheumatoid arthritis. J. Clin. Immunol. 33, 368–377 (2013).
ONLINE METHODS

Human peripheral neutrophils and NET-release assay. Peripheral blood was collected from healthy adult volunteers (whose identity was masked) according to approved protocols of the ethics board of the National Institute for Medical Research. Neutrophils were freshly isolated over a Histopaque layer, followed by a discontinuous Percoll gradient as described. Neutrophils were plated 24-wells plates at a density of 5 x 10^6 cells per well in Hank’s balanced-salt solution plus Ca^{2+} and Mg^{2+} with 3% human plasma. If not stated otherwise, neutrophils were stimulated with with C. albicans yeast or preformed hyphae at an MOI of 10. 4 h later, SYTOX (S702; Invitrogen) was added and NETs were visualized in at least six random images (20x magnification) per sample. NET release was quantified with ImageJ software (US National Institutes of Health) as described, and results were plotted by the frequency function in Microsoft Excel as the area of distribution of SYTOX+ events relative to total cells counted by phase-contrast microscopy.

Fungal culture and hyphal preparation. Wild-type C. albicans (clinical isolate SC5314), yeast-locked hgc1A C. albicans and wild-type A. fumigatus were cultured overnight at 37 °C in yeast extract peptone dextrose medium. For all experiments, hgc1A C. albicans and A. fumigatus were subcultured in yeast extract peptone dextrose medium and wild-type C. albicans was subcultured for 4 h in RPMI medium to induce hyphal growth. Heat inactivation was achieved by incubation of C. albicans for 1 h at 90 °C. Inactivated hyphae were fragmented over an EmulsiFlex-C5 high-pressure homogenizer (Avestin).

Bacterial culture. E. coli and K. pneumoniae were cultured overnight at 37 °C in Luria-Bertani medium and were subcultured to an absorbance of 1.0 at 600 nm.

M. bovis BCG-DsRed preparation. BCG-DsRed was grown at 37 °C, with shaking at 100 r.p.m., to an absorbance of 0.8 at 600 nm in Middlebrook 7H9 medium supplemented with 10% oleic acid, albumin, dextrose and catalase (OADC supplement), 0.05% Tween-80, 0.4% glycerol and 50 µg/ml hygromycin. Bacterial cultures were centrifuged and supernatants were repeatedly passed through a syringe for the removal of large aggregates, which yielded single cells and small aggregates used for experiments.

Transwell assay. The rims of conventional 24-well Transwell inserts (pore size,0.4 µm; membrane thickness, 10 µm; Corning) were cut off to enable the insert to sit directly on the bottom of the culture dish, which allowed direct contact with the neutrophils plated in the well. For stimulation, C. albicans or bacteria were seeded in the Transwell insert. 4 h later, the release of NETs from neutrophils was analyzed as described above.

Confocal microscopy of fixed cells and tissue. 5 x 10^4 human peripheral neutrophils were plated on glass coverslips 1 h before stimulation. After stimulation, cells were fixed for 20 min at 37 °C with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline, then nonspecific binding was blocked with 2% bovine serum albumin and 2% donkey serum in phosphate-buffered saline and cells were incubated with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; Life Technologies) and primary antibodies (anti-NE (ab21595; Abcam), antibody to human and/or mouse MPO (AF3667 (R&D Systems) or A0398 (DAKO)), antibody to C. albicans (BP1006; Acris), anti-p40 (1.22; Millipore), anti-p47 (sc-17845; Santa Cruz) and anti-CD63 (RFAC4; Millipore)), which were detected with the following secondary antibodies (all from Invitrogen): Alexa Fluor 488–conjugated donkey anti-goat (A11055), Alexa Fluor 568–conjugated donkey anti-goat (A10037), Alexa Fluor 488–conjugated donkey anti-goat (A21206) and Alexa Fluor 568–conjugated donkey anti-goat (A10042). For lung histology, dissected lungs of infected mice were fixed overnight in 2% paraformaldehyde and were embedded in wax for sectioning. Sections were baked for 1 h at 60 °C, then were rehydrated in NeoClear (1.09843.5000; VWR) and series of ethanol solutions of decreasing concentration (100% to 50%) and then were incubated for 45 min at 95 °C in 10 mM sodium carbonate buffer for antigen retrieval. Then, nonspecific binding was blocked and sections were permeabilized as described above, followed by incubation with DAPI and the following primary antibodies: antibody to histone H3 (citrulline R2 + R8 + R17; ab5103; Abcam) and anti-MPO (A0598; DAKO). Stained cells and tissues were mounted in ProLong Gold (P36934; Molecular Probes) and examined by confocal microscopy. Images were analyzed with ImageJ software.

Immunofluorescence light microscopy of live cells and M. bovis. 6 x 10^5 human peripheral neutrophils were plated on glass-bottomed Petri dishes (MatTek); SYTOX was added for detection of NET release and neutrophils were stimulated with a mixture of single cells and aggregates of M. bovis. NET release was monitored by time-lapse video microscopy over a period of 4–12 h at 37 °C with 5% CO₂.

Confocal microscopy of live cells and phagocytosis rate. 3 x 10^5 human peripheral neutrophils were plated on glass-bottom Petri dishes (MatTEK), followed by incubation for 1 h in the presence of blocking antibody to dectin-1 (DD6; AbD Serotec) before stimulation. SYTOX was added for detection of NET release. Neutrophils were stimulated with C. albicans yeast and NET release was monitored over 4 h. Phagocytosed particles were quantified in time-lapse images by recording of the number of phagocytosed particles per cell for each frame.

Immunoblot analysis. 1 x 10^6 human peripheral neutrophils were plated in 12-well plates. Neutrophils were stimulated with C. albicans yeast or preformed hyphae. At the appropriate times, cells were lysed in SDS sample buffer and stored at −80 °C. Samples were resolved by SDS-PAGE and transferred to PVDF membranes, then nonspecific binding was blocked with 5% skim milk (Fluka Analytical) and membranes were probed with the following antibodies: antibody to histone H3 (07-690; Millipore), anti-Syk (N-19; Santa Cruz), antibody to Zap70 phosphorylated at Tyr319 or Tyr352 (2701s; Cell Signaling), antibody to Erk1 (p44) and Erk2 (p42) (9102S; Cell Signaling) and antibody to Erk1 and Erk2 phosphorylated at Tyr185 or Tyr187 (44680G; Life Technologies). Those primary antibodies were detected with the following horseradish peroxidase–conjugated secondary antibodies: rabbit anti-mouse (31455), rabbit anti-goat (31403) and goat anti-rabbit (31463; all from Thermo Scientific).

Quantification of NE localization. For analysis of NE distribution, confocal z-series micrographs (obtained every 0.8 µm) covering the entire neutrophil volume were analyzed by quantification of total NE and NE localized together with DNA through the use of a mask created with the corresponding DAPI (DNA) channel for each section. Results from individual sections were added to yield the total nuclear fraction of NE for each cell; 15–20 neutrophils per condition were processed.

Inhibition of vesicle fusion. Neutrophils were plated on the bottom of 24-well plates at a density of 5 x 10^4 cells per well and were preincubated for 1 h at 37 °C in the presence of 1 µM bafilomycin (Sigma) or 2.5 µM nocodazole (Sigma). Subsequently neutrophils were stimulated with hgc1A C. albicans (MOI = 10). NET release was assessed 4 h later as described above.

Phagocytosis of yeast or beads to inhibit NETosis. Small polystyrene beads (3 µm in diameter; Krisker Botech) were opsonized for 30 min at 37 °C with 100% plasma before stimulation. Neutrophils were plated on the bottom of 24-well plates at a density of 5 x 10^4 cells per well and were preincubated for 30 min at 37 °C with hgc1A C. albicans or polystyrene beads to induce phagocytosis. Subsequently, neutrophils were stimulated with preformed C. albicans hyphae (MOI = 10). NET release was assessed 4 h later as described above.

Mouse infection and quantification of fungal load. All mouse experiments conformed to the guidelines of the UK Home Office under an approved project license. Anesthetized mice were infected intratracheally with the appropriate dose(s) of hgc1A C. albicans or wild-type C. albicans (preformed hyphae only where appropriate). Weight and survival of mice were monitored daily. For analysis of fungal load, mouse organs (lungs and spleens) were dissected and homogenized in sterile saline and serial dilutions of the homogenates were spread onto sabouraud dextrose agar plates. Colonies were counted.
after incubation of plates at 37 °C. The NE inhibitor (GW611313A; Sigma) and amphiregulin (989-AR; R&D Systems) were administered 4 h before infection and subsequently every 24 h.

**Microbial strains.** The following microbes were used: wild-type *C. albicans* (clinical isolate SC5314) and hgcΔ*C. albicans*24, *A. fumigatus* (isolate 13073; American Type Culture Collection), *E. coli* (strain DH5α), *K. pneumoniae* (strain Kp52.145)57 and *M. bovis* BCG-DsRed56.

**Histological scores for tissue damage.** Dissected lungs of infected mice were fixed overnight in 2% paraformaldehyde and were embedded in wax for sectioning. The sections were rehydrated as described above, then were stained with hematoxilin and eosin and imaged with a VS120 virtual slide-scanning system (Olympus). 15 images of each section were randomly assigned for analysis of fibrin deposition and bleeding in the tissue according to the following scores: fibrin score, 0 (no fibrin deposition in airspace), 1 (few fibrin fibers), 2 (<50% of airspace filled with fibrin deposition) or 3 (>50% of airspace filled with fibrin deposition); and bleeding, 0 (none), 1 (mild: fewer than five red blood cells in at least 5–10% of alveoli ), 2 (moderate: five to ten red blood cells) or 3 (severe: over ten red blood cells in >10% of alveoli).

**Immunohistochemistry.** Lung sections treated as described above were stained with antibody to tumor-necrosis factor (ab6671; Abcam) followed by a biotinylated secondary antibody (BA-1000; Vector Laboratories) amplified with Streptavidin ABC (PK-6100; Vector Laboratories), then were treated with DAB (3,3-diaminobenzidine tetrahydrochloride; SK-4100; Vector Laboratories) and imaged by light microscopy. DAB reactions were performed in parallel and at the same time for all samples.

**Statistical analysis.** *P* values were calculated by one-way ANOVA followed by Tukey's or Sidak's multiple-comparison post-test. *P* values of 0.05 or less were considered significant.

55. Aga, E. et al. Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite *Leishmania major*. *J. Immunol.* 169, 898–905 (2002).
56. Kasnapour, B., Gronow, A., Bleck, C.K., Hong, W. & Gutierrez, M.G. Size-dependent mechanism of cargo sorting during lysosome-phagosome fusion is controlled by Rab34. *Proc. Natl. Acad. Sci. USA* 109, 20485–20490 (2012).
57. Benghezal, M. et al. Inhibitors of bacterial virulence identified in a surrogate host model. *Cell. Microbiol.* 9, 1336–1342 (2007).