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Aspergillus fumigatus corneal infection is regulated by chitin synthases and by neutrophil–derived acidic mammalian chitinase

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Abstract

Aspergillus fumigatus is an important cause of pulmonary and systemic infections in immune compromised individuals, and of corneal ulcers and blindness in immune competent patients. To examine the role of chitin synthases in *Aspergillus* corneal infection, we analyzed *Aspergillus* mutants of chitin synthase family 1 and family 2, and found that compared with the parent strain, the quadruple mutants from both families were more readily killed by neutrophils in vitro, and that both also exhibited impaired hyphal growth in the cornea. Further, inhibition of chitin synthases using Nikkomycin Z enhanced neutrophil killing in vitro and in vivo in a murine model of *A. fumigatus* corneal infection. Acidic mammalian chitinase (AMCase) is mostly produced by macrophages in asthmatic lungs; however, we now demonstrate that neutrophils are a major source of AMCase, which inhibits hyphal growth. In *A. fumigatus* corneal infection, neutrophils are the major source of AMCase, and addition of AMCase inhibitors or adoptive transfer of neutrophils from AMCase$^{-/-}$ mice resulted in impaired hyphal killing. Together, these findings identify chitin synthases as important fungal virulence factors and neutrophil-derived AMCase as an essential mediator of host defense.

Keywords

Acidic mammalian chitinase; *Aspergillus fumigatus*; Corneal infection; Chitin synthase; Keratitis; Neutrophil

Introduction

The incidence of fungal infections has been increasing worldwide, especially those caused by *Candida* yeast and by filamentous fungi including *Aspergillus fumigatus*, which is ubiquitous in the environment [1, 2]. *A. fumigatus* is an important cause of systemic and pulmonary disease, especially in immunosuppressed individuals; however, *Aspergillus* and *Fusarium* molds also other cause blinding corneal infections in immune competent individuals worldwide [3]. The major risk factor is ocular trauma caused by airborne particles with attached conidia (spores) or conidiophores, which penetrate the tight junctions of the corneal epithelium and enter the corneal stroma. Once in the stroma, conidia germinate and form hyphae, which can migrate throughout the stroma and into the anterior chamber and posterior eye. Hyphae activate resident macrophages to produce CXC chemokines that mediate recruitment of neutrophils from peripheral, limbal capillaries. This results in loss of corneal clarity, opacification, and visual impairment, and in severe cases, blindness [4].

We reported that neutrophils are the predominant cells in patients with corneal ulcers caused by *Aspergillus* or *Fusarium* [5], and that neutrophils are the first cells recruited to corneas in murine models of *Aspergillus* and *Fusarium* infected mice [6, 7]. Neutrophils play an essential role in regulating hyphal growth in the cornea by oxidative and nonoxidative mechanisms, including limiting iron and zinc availability to hyphae. Inhibition of growth by nutrient deprivation is also termed nutritional immunity [8–10].

Another potential target on pathogenic fungi is the cell wall, and we showed a role for β-1,3 glucan and α-mannose, which activate the c-type lectins Dectin-1 and Dectin-2, respectively [11]. In that study, we also showed that in the absence of the RodA hydrophobin protein on conidia, the host response to cell wall components was more rapid, leading to clearance of the organisms.

In fungi, chitin forms the inner, rigid layer of the cell wall, and chitin fibrils covalently attach to β (1, 3)-glucans [12, 13]. Chitin is a polymer of β-(1-4)-*N*-acetyl-D-glucosamine, which is synthesized from UDP *N*-acetylglucosamine by transmembrane chitin synthases. In addition to fungi, chitin is also present in the cyst walls of amoebae, the exoskeleton of crustaceans and insects, and in nematodes, making it the most abundant polysaccharide in nature after cellulose [14–16]. However, although mammals do not have chitin, mammals can produce enzymatically active Acidic mammalian chitinase (AMCase, *Chia1*) and chitotriosidase (*CHIT1*), which can hydrolyze chitin in the environment [17, 18]. In humans, AMCase is produced by macrophages, eosinophils, and epithelial cells in asthmatic lungs, where it is thought to contribute to disease severity [19–22].

In the current study, we show that human and murine neutrophils are an important source of AMCase, Using a well-established murine model of *A. fumigatus* corneal infection [6, 9, 23] we show that neutrophil AMCase and chitin synthases play an important role in limiting fungal growth during *A. fumigatus* infection.

Results

Nikkomycin Z inhibition of chitin synthase activity

Transmembrane chitin synthases (*CHS*) convert UDP-*N*-acetylglucosamine to chitin. As a first approach to examine the role of chitin synthases in *A. fumigatus* killing by neutrophils, hyphae were incubated with Nikkomycin Z, which is a specific inhibitor of chitin synthase enzymatic activity [24]. Human peripheral blood neutrophils from healthy volunteers were incubated with the *A. fumigatus* RFP-expressing strain Af293-RFP in the presence of Nikkomycin Z, and fungal mass was quantified as total RFP.

As shown in Fig. 1A, the fungal mass was significantly lower when incubated with neutrophils compared with hyphae incubated in RPMI alone; however, when Nikkomycin Z was added to the culture with neutrophils, hyphal growth was significantly inhibited compared with neutrophils alone. There was no effect of 1 μM Nikkomycin Z on hyphal growth in the absence of neutrophils. To examine the effect of Nikkomycin Z in vivo, we used a well-characterized model of *A. fumigatus* corneal infection [6, 8, 11]. Corneas of C57BL/6 mice were infected with Af293-RFP, and after 6 h, mice were injected intrastromally with either Nikkomycin Z or with vehicle alone. As shown in Fig. 1B–D, there was significantly less RFP hyphae in infected corneas given Nikkomycin Z compared with those given vehicle alone. Consistent with this finding, there was also lower viability of Nikkomycin Z - treated corneas as determined by CFU.

Collectively, these observations demonstrate that loss of chitin integrity in the cell wall results in increased *A. fumigatus* susceptibility to neutrophil-mediated killing in vitro and in infected corneas.

Chitin synthases are required for resistance to neutrophil killing

A. fumigatus has eight *CHS* genes, which belong to two families based on sequence data—family 1, *CHSA*, *B*, and *C*, and family 2 (*CSMA*, *CSMB*, *CHSF*, and *CHSD*), which work cooperatively to synthesize chitin [25, 26]. Therefore, as a second approach to examine the importance of chitin integrity on *A. fumigatus* resistance to neutrophil killing, we incubated human neutrophils with chitin synthase mutants *ChsA/B/C/G* (family 1) or with *CSMA/CSMB/D/F* (family 2), and measured the metabolic activity of hyphae using the cell viability dye XTT. As shown in Fig. 2A, viability of Ku80 hyphae was impaired in the presence of neutrophils compared with RPMI alone; however, chitin synthase mutants exhibited significantly less viability of the following incubation with neutrophils compared to the parent Ku80 strain.

To examine the role of *A. fumigatus* chitin synthases during infection, *chsA/B/C/G* and *CSMA/CSMB/D/F* conidia were injected into C57BL/6 mouse corneas, and after 48 h, eyes were homogenized and fungal survival was quantified by CFU. We found significantly lower CFU in the chitin synthase mutants compared with the parent Ku80 strain (Fig. 2B), indicating that normal chitin synthesis is required for *A. fumigatus* hyphal growth in the cornea. Consistent with lower CFU, we observed significantly less corneal disease (measured by corneal opacity) caused by *CSMA/CSMB/D/F* strain compared with Ku80, although there was no difference between the *chsA/B/C/G* and Ku80 (Fig. 2C–E). These

findings indicate a more important role for family 1 chitin synthases during corneal infection. We also found no difference in virulence between Ku80 and single chitin synthase mutants (data not shown), indicating functional redundancy among these chitin synthases.

Neutrophil AMCase inhibits growth of *Aspergillus* hyphae in vitro

To determine if AMCase has a direct role in the ability of neutrophils to inhibit *A. fumigatus* hyphal growth, human peripheral blood neutrophils were incubated with *A. fumigatus* hyphae in the presence of the chitinase inhibitors Bisdionin C, which has broad activity for chitinases, or Bisdionin F, which is specific for AMCase [27]. Neutrophils were incubated with live *A. fumigatus* hyphae for 16 h, and fungal mass was measured using XTT [28].

We found significantly less hyphal mass when neutrophils were incubated with growing hyphae, (Fig. 3A); However, in the presence of Bisdionin F or Bisdionin C, fungal mass was significantly higher, indicating impaired neutrophil killing, thereby indicating that neutrophil AMCase has an important role in killing *A. fumigatus* hyphae.

As a second approach to examine the role of neutrophil AMCase in hyphal killing, we isolated neutrophils from the bone marrow of AMCase$^{-/-}$ (*Chit1*$^{-/-}$) mice, which were generated by Dr. Lori Fitz [29], and incubated them with growing hyphae. As shown in Fig. 3B, hyphal growth was significantly lower in the presence of bone marrow neutrophils from C57BL/6 mice compared with neutrophils from AMCase$^{-/-}$ mice. These combined studies demonstrate an important role for AMCase in neutrophil killing of *A. fumigatus* hyphae.

Neutrophils are the predominant source of AMCase in *A. fumigatus* infected corneas

To determine the source of AMCase in *A. fumigatus* corneal infection, corneas of C57BL/6 mice were injected with 4×10^4 *A. fumigatus* conidia in 2 μL PBS. After 24 h, infected corneas were dissected and homogenized, and AMCase was detected either by Western blot analysis, or by immunohistochemistry (IHC) to identify AMCase and Ly6G+ neutrophils. In addition, bone marrow neutrophils from naïve C57BL/6 mice were stimulated in vitro with AspHE, and AMCase protein was detected by Western blot.

As shown in Fig. 4A, AMCase was detected in bone marrow neutrophils from C57BL/6 mice, and increased following incubation with *Aspergillus* extracts. AMCase was not detected in corneas from uninfected (naïve) mice (Fig. 4B), which have resident macrophages, but no neutrophils; however, AMCase is clearly present in infected corneas after neutrophils have been recruited 24 h postinfection. Although AMCase is produced by macrophages in the lungs [17], we reported that neutrophils are the predominant cell type recruited to infected corneas at early time points [6, 8, 11].

To determine if neutrophils are the major source of AMCase in infected corneas, 5 μm corneal sections were immunostained with antibodies to Ly6G and AMCase. Figure 4C and D show that AMCase colocalized with Ly6G+ neutrophils in the corneal stroma of infected mice, whereas naïve mice, which do not have neutrophils, were negative for AMCase. Consistent with this finding, we also examined infiltrating CD45+ cells by flow cytometry, and found that 80–90% of the recruited CD45+ cells were Ly6G+ neutrophils and 10–20% were Ly6C+ monocytes (Fig. 4E, F), with <3% F4/80+ macrophages (Supporting

Information Fig. 1). Also, there was no difference in the total number of Ly6G+ neutrophils recruited to the corneas of AMCase$^{-/-}$ compared with C57BL/6 mice (Fig. 4G), indicating that AMCase has no role in recruiting neutrophils to infected corneas.

Taken together, these findings demonstrate that neutrophils are the major source of AMCase in *A. fumigatus* corneal infection.

Neutrophil AMCase regulates *A. fumigatus* hyphal growth in infected corneas

To ascertain if AMCase has a role in regulating *A. fumigatus* hyphal growth in infected corneas, corneas of C57BL/6 and AMCase$^{-/-}$ mice were infected with dsRed expressing Af293, and after 48 h, hyphal mass and fungal viability were quantified. We found no significant difference in corneal opacification between C57BL/6 and AMCase$^{-/-}$ mice; however, there was significantly higher RFP hyphae and CFU in infected AMCase$^{-/-}$ mice compared with C57BL/6 mice (Fig. 5A–C, Supporting Information Fig. 2).

To examine more directly the role for neutrophil AMCase in infection, we used CD18$^{-/-}$ mice as recipients, as circulating CD18$^{-/-}$ neutrophils are unable to extravasate to inflamed tissues due to their inability to form CD11a/CD18 or CD11b/CD18 heterodimeric integrins (LFA1, MAC1). Bone marrow neutrophils were isolated from C57BL/6 and AMCase$^{-/-}$ mice by negative bead selection, and 4×10^6 cells were injected intravenously in 150 μL 4 h after corneal infection with Af293-RFP. Corneas were examined 24 h later, and CFU were quantified. As shown in Fig. 5D, CFU in corneas of recipient mice given AMCase$^{-/-}$ neutrophils was significantly higher than those given C57BL/6 neutrophils.

Finally, as AMCase targets chitin, and the chitin synthase quadruple mutants have less chitin [26], we predicted that the chitin synthase mutants would grow to the same extent as the parent strain in the absence of AMCase. We therefore infected corneas of AMCase$^{-/-}$ mice with either the *ChsE/Eb/D/F* quadruple mutant that induced less disease in C57BL/6 mice (Fig. 2) or with the parent KU80 strain, and examined CFU 48 h postinfection. We found that as anticipated, there was no difference in CFU between the *ChsE/Eb/D/F* and KU80 in AMCase$^{-/-}$ mice (Fig. 5E).

Taken together, these observations indicate that neutrophil derived AMCase regulates hyphal growth in *A. fumigatus* corneal infection by targeting cell wall chitin.

Discussion

The results of our study clearly demonstrate an essential role for chitin synthases and for neutrophil derived AMCase in the pathogenesis of *A. fumigatus* infection. The requirement for chitin synthases is supported by the findings that pharmacological inhibition or genetic deletion of chitin synthases results in reduction of *A. fumigatus* survival in the presence of neutrophils, and a loss of virulence in an animal model of corneal infection. Consistent with the role for chitin, we show that the enzymatically active chitinase AMCase has a nonredundant role in the keratitis model where neutrophils are the major players, and in an in vitro neutrophil hyphal killing assay.

Latge and coworkers demonstrated that *A. fumigatus* cell wall chitin is synthesized by eight chitin synthases (*Chs*) belonging to two families, *Chs* A, B, C, and G in family 1 and *Chs* F, D, CSMA, and CSMB in family 2. The differences between these *Chs* families is based on organization of the enzymatic region in relation to the transmembrane regions of the proteins [25, 30], and mutants deleted in *Chs* genes of family 1 (*chsA/B/C/G)*, or family 2 (*chsE/Eb/D/F)* had impaired chitin synthase activity that correlates with a restructuring of the cell wall [12, 25, 26]. In the pulmonary aspergillosis model, where mice are immunosuppressed, there was no difference between family 1 and family 2 Chs mutants compared with the Ku80 parent strain of *A. fumigatus* [30]. However, those findings are in contrast to the current study, where family 1 and family 2 *Chs* mutants were more susceptible than the parent Ku80 to neutrophil killing in vitro, and were less virulent in infected corneas. Interestingly, the *chsE/Eb/D/F* family 2 mutants induced less corneal disease than the *chsA/B/C/G* family 1 mutants despite there being no difference in CFU recovered from the cornea.

In support of this observation, we also showed that *A. fumigatus* hyphae grown in the presence of Nikkomycin Z were more susceptible to neutrophil killing, and in vivo injection of Nikkomycin Z at the time of infection resulted in impaired fungal growth. Nikkomycin Z is a potent inhibitor of chitin synthases [13], where it impairs growth by inhibiting the chitin synthases Chs1 and Chs3 as shown for *Candida albIcans* [24, 31]. *A. fumigatus* is also sensitive to Nikkomycin Z, especially when used together with other anti-fungal agents [32]. We show here that inhibition of chitin synthesis augments susceptibility to the neutrophil anti-fungal response.

Although we have yet to determine the underlying reason for the increased susceptibility of those mutants to neutrophils, it may relate to differences in the size of the chitin microfibrils, as *chsE/Eb/D/F* mutants have short-chitin microfibrils compared with longer chitin microfibrils in the *chsA/B/C/G* mutants [30]. It may also relate to the relative levels of chitin and the deacetylated form of chitin (chitosan) in the cell wall. Smaller chitin fragments were reported by Elias et al. to induce IL-10 production by macrophages compared with larger fragments that induce proinflammatory responses in macrophages and in murine models of pulmonary inflammation [14, 33–36]; however, Levitz and coworkers showed that chitosan rather than chitin activates the NLRP3 inflammasome to secrete IL-1β in LPS–-stimulated macrophages when given as a second signal, and that smaller rather than larger chitosan particles are more proinflammatory [37]. In a more recent study, van de Veerdonk et al. showed that chitin must be bound to IgG to induce either an anti-inflammatory IL-1Ra response through FCRγRII/Syk activation, or to enhance IL-1β production in the presence of LPS or other TLR ligands [38].

In live organisms, it is also possible that disruption of the chitin cell wall results in increased penetrability of ROS and neutrophil antimicrobial products. We reported that neutrophils regulate growth of *Aspergillus* hyphae in vitro and in infected corneas by oxidative and also by nonoxidative mechanisms, including sequestering iron and zinc binding proteins; however, neutrophil extra-cellular traps were not required for hyphal killing in this model [8, 9, 23, 39]. Results of the current study identify AMCase as an additional regulator of hyphal growth by neutrophils, as inhibition of AMCase using Bisdionin F or Bisdionin C results in

impaired fungal killing by neutrophils in vitro. Further, AMCase$^{-/-}$ neutrophils were impaired in their ability to inhibit hyphal growth in vitro and during corneal infection. It is possible that short-chain chitins are more readily degraded by AMCase than long- chain chitins, thereby facilitating the penetration of neutrophil oxidative and nonoxidative mediators to the fungal cell membrane and into the cytoplasm.

Despite the impaired fungal killing in corneas of AMCase$^{-/-}$ mice, we found no significant difference in corneal opacification between these and C57BL/6 mice. This is likely because the number neutrophils recruited infected corneas was the same as C57BL/6 mice, and the absence of AMCase has no effect on neutrophil production of ROS or proteolytic enzymes that are associated with tissue damage.

In contrast to our findings, Steele and coworkers recently reported that AMCase$^{-/-}$ mice were more resistant to acute pulmonary aspergillosis than control mice, although AMCase $^{-/-}$ mice also showed less airway hyper-responsiveness compared with control mice in a model of chronic fungal asthma [40]. Those authors indicated that the increased resistance in the acute model was due to elevated production of proinflammatory cytokines that mediate fungal killing, which we did not detect in our studies.

An important difference between that study and ours is the site of infection and the critical role for neutrophils in the cornea compared with the lungs. We recently reported that neutrophil calprotectin (S100A8/A9) was required to control *Aspergillus* hyphae in infected corneas, but not in the lungs, as we showed that S100A9$^{-/-}$ mice were more susceptible to corneal, but not pulmonary *Aspergillus* infection [8]. In that report, we speculated that the difference was at least in part due to the large neutrophil infiltrate in acute corneal infection, whereas the lung infiltrate at the time examined also included macrophages and eosinophils. Given the current findings with corneal infection, and the nonredundant role for AMCase in the ability of neutrophils to kill *A. fumigatus* hyphae in vitro, it is highly likely that the difference between our studies and those of Steele et al. relates to the major role that neutrophils play in corneal compared with pulmonary disease.

The role for AMCase in pulmonary disease is somewhat controversial. Elias and coworkers, and others reported that AMCase has an important role in the pathology of asthma by inducing neutrophil and eosinophil infiltration and airway hyper-responsiveness [20, 41]. AMCase is highly expressed in OVA—sensitized mice that develop airway hyper—responsiveness and in lung tissue from asthmatic patients [22]. Moreover, chemical inhibition of AMCase ameliorated allergic inflammation by decreasing eosinophil and neutrophil recruitment to the lungs of OVA-sensitized mice [20]. In contrast, Fitz and colleagues reported that despite the absence of chitinase activity in models of pulmonary inflammation and allergic airway disease, the absence of AMCase had no significant effect on cellularity or airway responses [29]. Wynn et al. also found no role for AMCase in airway disease induced by house dust mite allergens, although AMCase was essential for developing a protective type II immune response to intestinal nematodes [42]. Locksley and coworkers showed that older mice spontaneously develop pulmonary fibrosis and die earlier than WT controls, at least in part due to failure to digest environmental chitin fragments that

accumulate in the lungs [43]. These investigators concluded that AMCase production by specific lung epithelial cells is important for chitin degradation in the airways.

Most of those studies identified macrophages as the primary source of AMCase, whereas our findings identified murine and human neutrophils as a major source of this enzyme. To the best of our knowledge, there is only one other paper showing this, where AMCase production by neutrophils from patients with type-2 diabetes was elevated compared with healthy individuals [44].

In summary, the current study identified a novel role for neutrophil derived AMCase in targeting *A. fumigatus* cell wall chitin in a clinically relevant model of *A. fumigatus* corneal infection. These data also indicate that inhibiting chitin synthases or enhancing host chitinase activity are potential targets for therapeutic intervention for fungal infections.

Materials and methods

Reagents

Neutrophils were detected using NIMPR14 rat monoclonal antibody to Ly6G (Abcam ab2557), and rabbit polyclonal antibodies to AMCase were obtained from Proteintech (cat no 143631-AP). Bisdionin C and Nikkomycin Z were purchased from Sigma; Bisdionin F was purchased from EMD Millipore.

Source of mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and AMCase−/− mice were obtained from Dr. Lori Fitz through a material transfer agreement between UC Irvine and Pfizer. Mice were bred under specific pathogen-free conditions and maintained according to institutional guidelines. All animals were treated in accordance with the guidelines provided in the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by Case Western Reserve University and UC Irvine IACUC committees.

Isolation of murine bone marrow neutrophils

To isolate bone marrow neutrophils, mice were euthanized by CO_2 asphyxiation, and femurs and tibias were dissected. Cells were flushed out by placing the tip of a needle containing sterile PBS in one end of the bones and releasing the PBS. Cells were centrifuged at 1300 rpm for 5 min at 4°C. Red blood cells (RBCs) were lysed in 5 mL of RBC lysis buffer (eBioscience), and neutrophils were isolated with a MojoSort mouse neutrophil isolation kit using negative selection (Biolegend). This approach typically yields a >95% neutrophil population.

Fungal strains

Aspergillus fumigatus strains used in this work are Af293 expressing red fluorescent protein as described [6], chitin synthase Family 1 mutants Δ*ChsA/B/C/G*, and Family 2 mutants Δ*ChsE/Eb/D/F* that were derived from the parent Ku80 as described [30]. Strains were grown at 37°C in either Aspergillus minimal medium [45] containing 1% glucose + 5 mM

ammonium tartrate, YG (1% glucose, 0.5% yeast extract), Sabouraud (2% glucose, 1% neopep-tone) (Difco), RPMI (Sigma Aldrich) or 2% malt agar (Cristomalt). Conidia were collected from agar media after 10 days of growth at RT, using 0.05% Tween 80 solution.

Human peripheral blood neutrophils

Healthy donors between ages 18 and 65 years provided human peripheral blood in accordance with the Declaration of Helsinki guidelines, and with the approval of the Institutional Review Board of University Hospitals of Cleveland. Informed consent was obtained in writing from each volunteer.

We used 3% dextran (Sigma-Aldrich, St. Louis, MO) in PBS to separate RBCs from whole blood, and neutrophils were purified from the remaining cells by overlay on a Ficoll density gradient (GE Healthcare) following centrifugation for 25 min at $500 \times g$. The remaining RBCs were lysed, and neutrophils were resuspended in RPMI 1640 medium. This process resulted in >95% purity.

Hyphal growth inhibition assay

Isolated conidia were prepared as a stock of 10 000 conidia/200 μL SDA and cultured in black 96-well plates with an optical clear bottom (CoStar 3720) for 6 h at 37°C to obtain germ tubes. Super-natants were removed and cells were washed ×3 with ddH$_2$O. Cells were then incubated with RPMI alone or RPMI + human neutrophils (200 000 neutrophils/well) and incubated at 37°C for 16 h. For drug studies, neutrophils were incubated with the drug for 30 min at 37°C prior to the stimulation and then added to the plates. Next day, cells were washed ×3 with ddH$_2$O and fungal viability was measured by incubating hyphae with the XTT dye as previously described [28].

Murine model of *A. fumigatus* keratitis

Male and female mice aged 6–8 weeks were infected as previously described [9, 11]. *A. fumigatus* strains were cultured in VMM agar in 25 cm^2 tissue culture flasks. Dormant conidia were recovered with a bacterial L-loop and harvested in 5 mL PBS. Pure coni-dial suspensions were obtained by passing the culture suspension through PBS-soaked sterile gauges placed at the tip of a 10 mL syringe. Conidia were quantified and a stock was made at a final concentration of 2.5×10^4 conidia/μL in PBS. Mice were anaesthetized and the corneal epithelium was abraded with a 30 gauge needle to allow insertion of a 33-gauge Hamilton needle through which 2 μL PBS containing 4×10^4 conidia were injected into the corneal stroma. Nikkomycin Z was suspended in cyclodextran, and 2 μL 10 mg/mL stock (20 ng) were injected into the corneal stroma at 6 h postinfection.

Mice were euthanized by CO$_2$ asphyxiation and positioned in a three-point stereotactic mouse restrainer. Corneal opacity (brightfield) and corneal fungal growth (fluorescence) were photographed using a high-resolution stereo fluorescence MZFLIII microscope (Leica Micro-systems) and Spot RT Slider KE camera (Diagnostics Instruments). Images were captured using SpotCam software (RT Slider KE; Diagnostics Instruments). MetaMorph imaging software was used to quantify the percent area of opacity and the integrated corneal opacity as described by Leal et al. [23].

Adoptive transfer model of *A. fumigatus* keratitis

To examine a specific role for neutrophils in vivo, we used $CD18^{-/-}$ mice, as circulating neutrophils are unable to extravasate to inflamed tissues. Bone marrow neutrophils were isolated from C57BL/6 and $AMCase^{-/-}$ mice, and 4×10^6 cells were injected intravenously in 150 μL 4 h after corneal infection with *Aspergillus*. After 24 h, corneas were examined as described above. We reported that this experimental approach results in neutrophil recruitment to infected corneas [23].

Quantification of CFUs

For assessment of fungal viability, whole eyes were homogenized under sterile conditions in 1 mL PBS, using the Mixer Mill MM300 (Retsch, Qiagen, Valencia, CA) at 33 Hz for 4 min. Subsequently, serial log dilutions were performed and plated onto Sabouraud dextrose agar plates (Becton Dickenson). Following incubation for 24 h at 37°C, CFUs were quantified by direct counting.

Flow cytometric analysis

Infected corneas were incubated in 500 μL collagenase type I (Sigma-Aldrich) at 82 U/cornea for 1–2 h at 37°C. Cells were resuspended in 200 μL FACS buffer containing Fc block anti-mouse CD16/CD32 (clone 93, 16-0161-86; eBioscience), and then incubated with anti-mouse neutrophil antibody (Ly6G, NIMP-R14-PE, ab125259; Abcam, Cambridge, MA, USA), anti-CD11b-APC FAB1124A (R&D Systems, Minneapolis, MN, USA), CD45-FITC (30-F11), Ly6C APC-Cy7 (HK1.4), and Ep-CAM-PECy7 (G8.8). All antibodies were purchased from BioLegend. Total cells were examined in an ACEA Novocyte™ flow cytometer and analyzed as we described, and in accordance with guidelines for flow cytometry [39, 46, 47].

Statistical analysis

Statistical analysis was performed using either an unpaired *t* test or ANOVA with Tukey posttest (Prism GraphPad). A *p* value of <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

Abbreviations:

CHS	chitin synthases
IHC	immunohistochemistry

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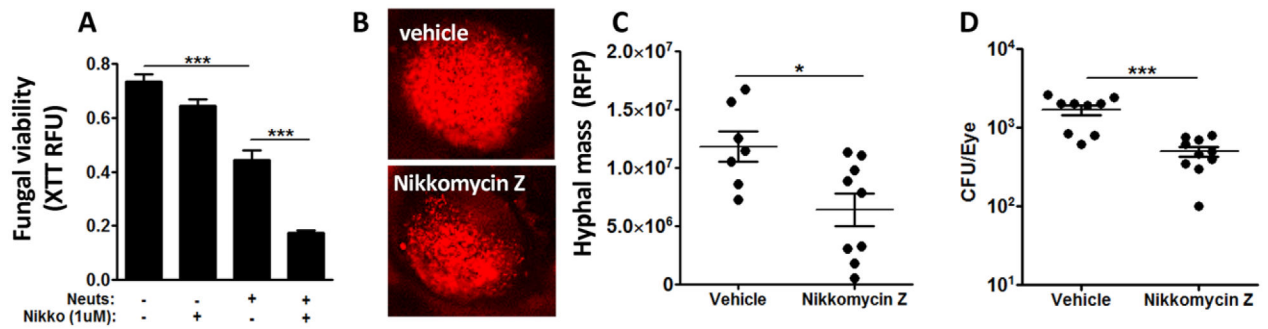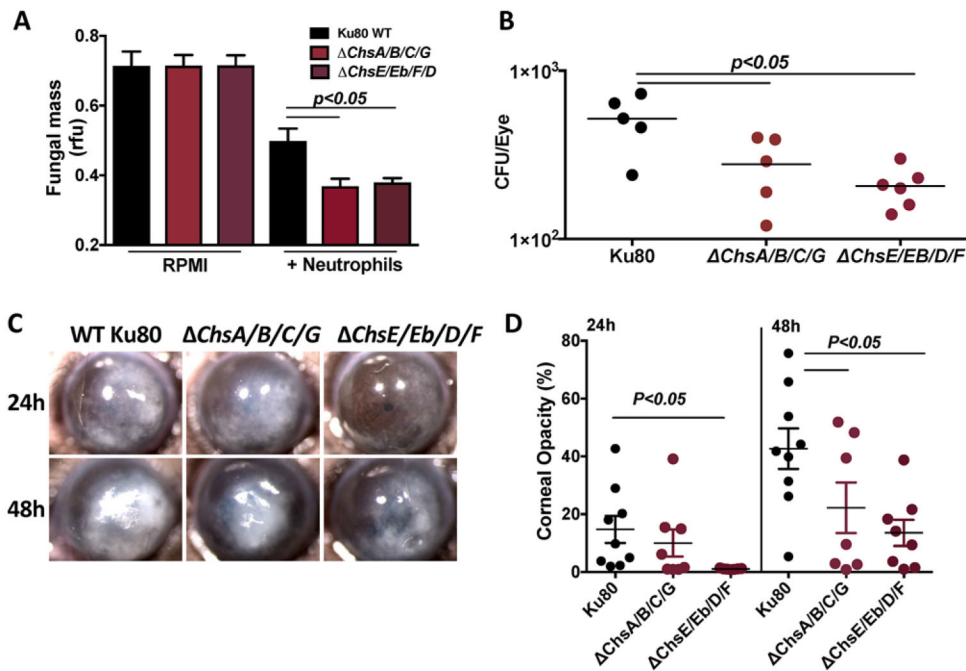
Figure 1.
Nikkomycin Z inhibition of *A. fumigatus* chitin synthesis. (A) Human neutrophils were incubated with *A. fumigatus* for 16 h in the presence or absence of neutrophils and Nikkomycin Z (Nikko). Fungal viability was detected by XTT and quantified by fluorimetry and represented as fungal mass (three biological replicates representing three repeat experiments). (B) Representative corneas showing hyphal growth of RFP expressing *A. fumigatus* 48 h infected C57BL/6 mice that were given systemic Nikkomycin Z or vehicle control (original magnification is ×20). Quantification of *A. fumigatus* RFP fluorescence (C) and CFU (D) of 48 h infected eyes. A: mean ± SD of neutrophils from a single donor (four technical replicates), which was repeated using a second donor. (C, D) Data points represent individual infected corneas from 6–10 mice per group; experiments were repeated twice with similar results. *p* values were determined following ANOVA analyses and a Tukey posttest (A) or t test to compare two groups (C, D). *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

Figure 2.

Role of chitin synthases in *A. fumigatus* survival in corneal infection. (A) Human neutrophils incubated 16 h with either WT Ku80, chitin synthase Family 1 mutants *ChsA/B/C/G*, or Family 2 mutants *ChsE/Eb/D/F*. Fungal viability was measured using the XTT viability dye. (B) The 48 h infected eyes were dissected and plated onto SDA agar plates overnight to determine CFU (corneas are 3 mm diameter). (C) Representative images of 24 h and 48 h infected corneas from C57BL/6 mice with Ku80, *ChsA/B/C/G*, or *ChsE/Eb/D/F* strains. (D) Quantification of percentage corneal opacity in 24 h and 48 h infected corneas. *p* values were determined following ANOVA analyses and a Tukey posttest. * = <0.05, ** = <0.001, *** = <0.0001. (A) Data are representative of two donors; (B, D) one representative experiment of three repeat experiments using 5–10 mice per group in each experiment where each data point represents an individual infected cornea.
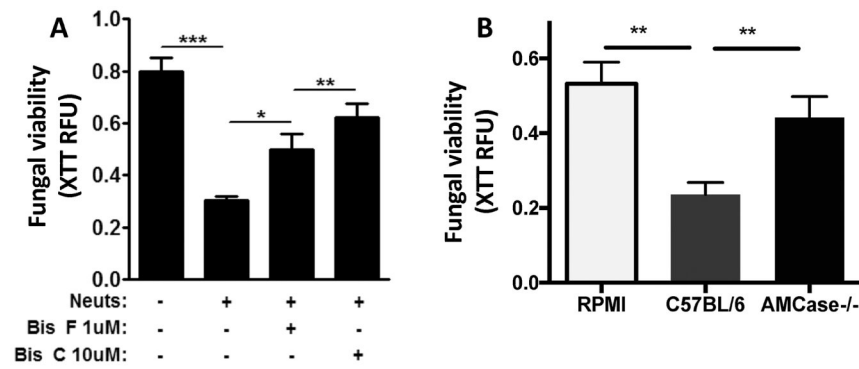
Figure 3.
AMCase dependent *A. fumigatus* hyphal killing in vitro. (A) Peripheral blood human neutrophils incubated 16 h with hyphae in the presence of the AMCase inhibitor Bisdionin F (BisF) or the general chitinase inhibitor Bisdionin C (BisC). (B) Bone marrow neutrophils from C57BL/6 or AMCase$^{-/-}$ mice incubated 16h with *A. fumigatus* hyphae. Viable hyphae were quantified using the XTT viability stain (A), and total chitin content was quantified using Calcofluor white (B). (A) Data are mean ± SD from a single donor; similar results were found using a second donor; (B), mean ± SD of neutrophils pooled from 3–5 mice per group. Similar results were obtained in three independent experiments. *p* values were derived by ANOVA analyses with a Tukey posttest. *<0.05, **<0.001, ***<0.0001.
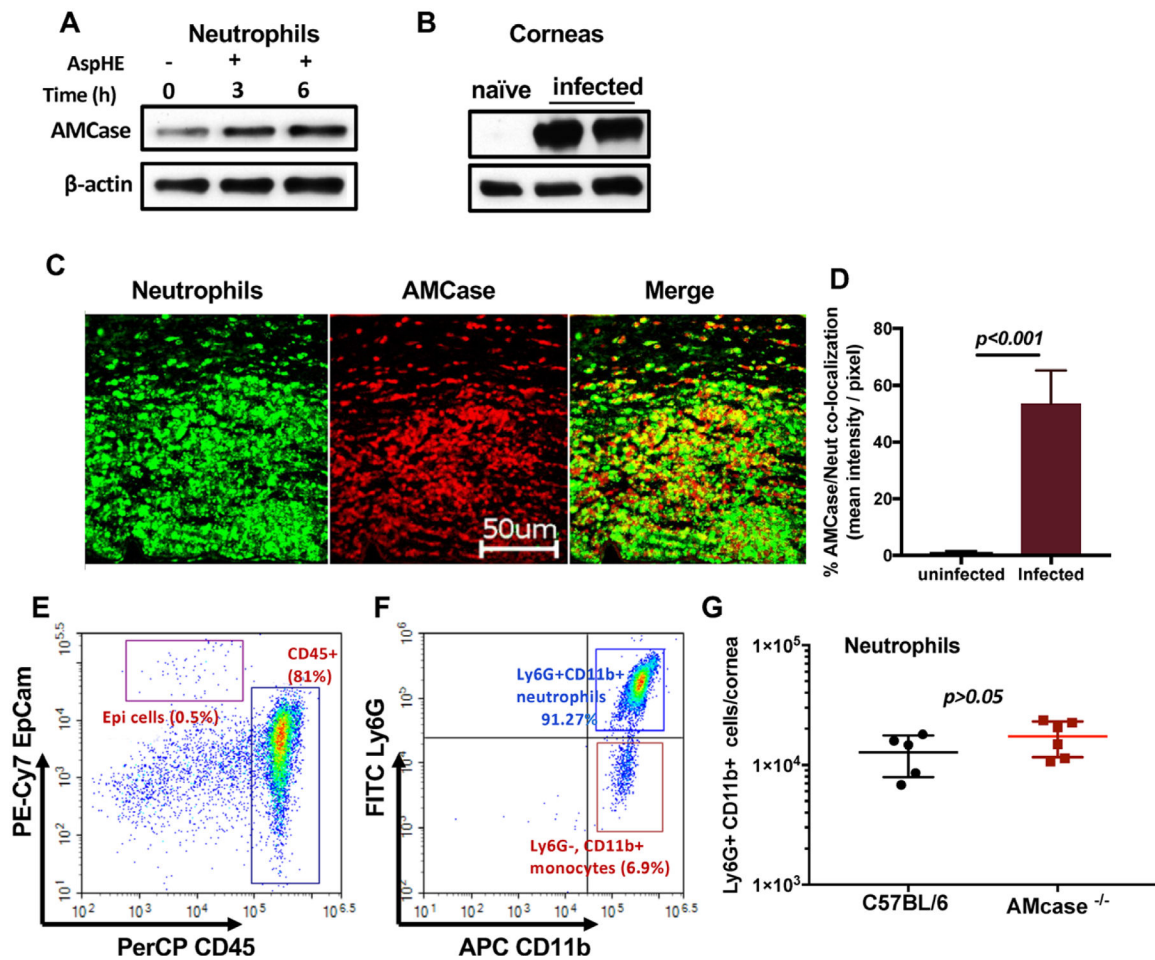
Figure 4.

Neutrophil production of AMCase in *A. fumigatus* corneal infection. (A) Production of AMCase in bone marrow neutrophils from naïve C57BL/6 that were incubated with *A. fumigatus* Af293 hyphal extract (AspHE). (B) Western blot of isolated corneas from naïve C57BL/6 mice and of corneas dissected 24 h postinfection with 4×10^4 *A. fumigatus* Af293 (two separate corneas). (C) Corneal sections (5 μm) 48 h postinfection incubated with antibodies to Ly6G+ (NIMP-R14) to identify neutrophils, AMCase and a merged image (original magnification is x400). (D) Colocalization of NIMP-R14 and AMCase staining in uninfected (naïve) and infected mice, and quantified using Image J software. Data are mean ± SD of three infected mice. (E, F) Representative flow cytometry plot of total cells from infected corneas (C57Bl/6 mice) showing total cells (E) and CD45+ cells (F).(EpCam: epithelial cell marker). Flow cytometry showing F/480 macrophage staining is shown in Supporting Information Fig. 1. (G) Quantification of total neutrophils from infected corneas from C57Bl/6 and AMCase$^{-/-}$ mice. (D) Mean ± SD of 10 fields each of two corneas. (G) Data points represent total neutrophils from five or six individual corneas per group. This experiment was repeated twice with similar results. Statistical significance in D and F was determined by Students *t* test to compare two groups.
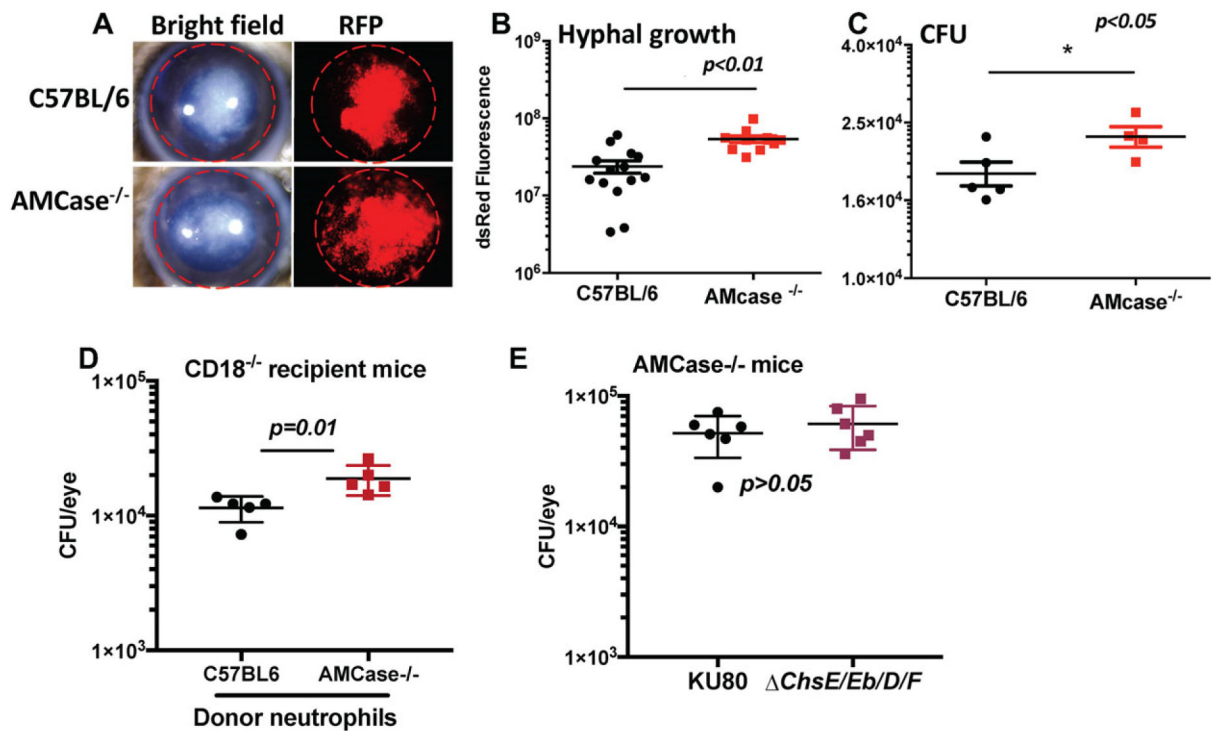
Figure 5.
The role of AMCase in *A. fumigatus* corneal infection. (A) Representative corneas from C57BL/6 and AMCase$^{-/-}$ mice 48 h after infection with RFP expressing Af293 (corneas are 3 mm diameter); quantification of RFP fluorescence (B) and CFU (C) in infected corneas. (D) Intravenous injection of bone marrow neutrophils from C57BL/6 or AMCase$^{-/-}$ mice into recipient CD18$^{-/-}$ mice. (E) CFU from AMCase–/– mice 48 h postinfection with the quadruple chitin synthase mutant *ChsE/Eb/D/F* compared or with the parent KU80 strain. (B–E) Each data point represents a single infected cornea with 5–6 mice per group in each experiment (RFP data were obtained from all 15 mice in a single experiment, whereas CFU were obtained from 5–6 mice per group. These experiments were repeated twice with similar results. Significance was determined by Students *t* test to compare two groups. *p* values are: *<0.05, ***<0.0001.