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## **Comment on “Sterilizing immunity in the lung relies on targeting fungal apoptosis-like programmed cell death”**

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**Shlezinger et al. (Reports, 8 September 2017, p. 1037) report that the common fungus *Aspergillus fumigatus*, a cause of aspergillosis, undergoes caspase-dependent apoptosis-like cell death triggered by lung neutrophils. However, evidence for fungal cells dying via a protease signaling cascade that is thwarted by a fungal caspase inhibitor homologous to human Survivin is built on compromised assumptions about the technologies.**

Shlezinger et al. (1) reported the discovery of an 'apoptosis-like' programmed cell death pathway in the opportunistic pathogen *Aspergillus fumigatus*, a multicellular fungus responsible for life-threatening infections. However, this conclusion is compromised by several technical problems with the methods leading to unsupported conclusions. The technical problems arise from the use of mammalian apoptosis assays designed to detect biochemical events that are not present nor molecularly defined in fungi. A related problem stems from the assumption that fungal proteins with limited sequence homology to mammalian apoptosis regulators will function analogously in fungal cells. In this study, a chemical sensor and a small molecule inhibitor of mammalian apoptosis regulators (caspases and IAP proteins, respectively) were applied to fungal cells with the expectation of binding to distant fungal homologs but without direct evidence for such. Furthermore, the proposed fungal target proteins (metacaspases and BIR1) appear to lack the corresponding binding sites for these chemical reagents. Thus, the evolutionary divide between fungi and mammals appears to be too great for what might seem like a safe extrapolation regarding apoptosis – generally defined in mammals as the classic biochemical and morphological consequences of caspase-3 or -7 activation.

Using a transgenic strain of *Aspergillus fumigatus* with a dual fluorescent probe to distinguish dying cells from dead cells, Shlezinger *et al.* (1) report that mold conidia exposed to oxidative stress *in vitro*, or engulfed by neutrophils *in vivo*, exhibit features of apoptotic mammalian cells prior to loss of viability. The authors measured fungal caspase activity by flow cytometry or fluorescence microscopy using the fluorescent FITC-conjugated tripeptide Val–Ala–Asp (VAD) fused to fluoromethylketone (FMK). FITC-VAD-FMK is a mammalian caspase reporter that mimics the required Asp cleavage site found in natural substrates of mammalian caspases. The FMK moiety results in tight binding to the caspase active site cysteine, potently inhibiting caspase activity. By binding to active caspases, the fluorescent FITC moiety is retained in animal cells for easy detection.

However, despite careful scrutiny, sequence-based bioinformatics approaches have failed to detect caspase homologs neither in fungal genomes, nor more generally in non-metazoan organisms (2). *A. fumigatus* encodes two metacaspases (CasA and CasB) (3),

which are also cysteine proteases related to animal caspases. However, metacaspases cleave their substrates after Arg and Lys, rather than Asp (D), the key residue in FITC-VAD-FMK. This raises a critical question – what enzymatic activities are responsible for the FITC-VAD-FMK caspase reporter activity in dying fungi? One possibility is that peptide-FMK reporters are promiscuous. They have been reported to bind/inhibit other classes of cysteine proteases in mammalian cells including cathepsins (4), and for these reasons have fallen from favor for use in mammalian models (5). Similarly, FITC-VAD-FMK could potentially monitor vacuolar cathepsins in fungi, but it can also nonspecifically label living non-permeabilized yeast cells (6). Based on its promiscuity, FITC-VAD-FMK could theoretically bind fungal metacaspases. However, although a double knockout of both *A. fumigatus* metacaspases CasA and CasB exhibited cell growth abnormalities it was not resistant to cell death induced by oxidative stress or other stimuli tested (3).

Shlezinger *et al.* (1) took a different approach to address a causal role for fungal caspase-like activities, leading to the identification of a virulence mechanism. They found that fungal strains overexpressing *A. fumigatus* BIR1 (AfBIR1) are resistant to oxidative stress (H<sub>2</sub>O<sub>2</sub>) in vitro and are more virulent in mice. AfBIR1 shares amino acid sequence similarity to a family of mammalian inhibitor of apoptosis (IAP) proteins that, like AfBIR1, contain baculovirus IAP repeat (BIR) domains. The authors' proposed model suggests that AfBIR1 behaves similarly to the human IAP protein Survivin, based on the assumption that Survivin inhibits apoptotic caspases. However, Survivin is not a direct caspase inhibitor and it lacks the sequences found in the BIR region of mammalian XIAP that interact with and directly inhibit caspases (7, 8). To provide evidence that endogenous AfBIR1 regulates caspase-like activity while overcoming the lethality of *AfBIR1* deletions, Shlezinger *et al.* (1) sought to inhibit AfBIR1 by treating fungal conidia and mice with S12, a small molecule reported to inhibit Survivin (9). S12 was originally identified through chemical and computational screens for compounds that target a cavity located near the Survivin dimerization interface. However, residues critical for S12 binding to Survivin are not well conserved in AfBIR1 (Fig.1). Thus, studies to verify direct binding of S12 to AfBIR1 are needed, as their interaction cannot be inferred solely based on multiple sequence alignments.

The other hallmark of mammalian apoptosis used throughout the Shlezinger *et al.* study (1) is nuclear DNA fragmentation, which is quantified using TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assays. The TUNEL assay is useful for labeling the new ends of nuclear DNA following caspase-dependent activation of the mammalian DNase CAD (caspase-activated DNase) (10). However, the TUNEL assay is not specific to apoptosis and detects DNA breaks resulting from non-apoptotic cell death (11). TUNEL and many other features of dying mammalian cells have been observed in a diverse range of non-metazoan taxa, including protists, microalgae, yeast, bacteria and plants.

However, the genes directly responsible are largely unknown. Thus, the central unanswered question is whether the fungal factors responsible for TUNEL reactivity, caspase-like activity or other mammalian assays play an active and causal role in fungal cell death, or only serve to mark dead and dying cells with degrading DNA, vacuolar permeability and depletion of ATP.

Shlezinger *et al.* (1) provide convincing genetic arguments that host phagocyte NADPH oxidase (NOX) mediates the demise of ingested fungal conidia. The possibility that NOX-derived ROS induces a FITC-VAD-FMK-traceable protease signaling modulated by AfBIR is also intriguing, but more evidence will be required to distinguish this model from other possibilities, including direct killing of the engulfed pathogens by neutrophils without pro-death contributions from fungal “caspase-like activities”. Some fungal species have orthologs of mammalian MLKL that mediate necroptosis and fungal homologs of DNFA5/gasdermin that mediate mammalian pyroptosis (12, 13). Even if these pro-necrotic death factors do not promote regulated death pathways in fungi, this does not deny the existence of genetically controlled cell death in fungi (14).

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## Figure legend

Fig. 1. Residues in human Survivin required for binding S12 are not well conserved in AfBIR1. Amino acid sequence of the single BIR domain region of human Survivin is aligned using ClustalW with each of the two BIR domains in BIR1 of *Aspergillus fumigatus*. Disruption of either Phe86 or Val89 (red frames) in Survivin inhibits S12 binding (9). Asterisks mark residues forming the interface between Survivin dimers (9).

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A. Fumigatus (XP_752777) BIR(I) 9  MSFDLVLFERRRTSSAISKPI S WPHSR - - PSFAELAHAGFYNYFYETNPDNITTO LGCALIGWEPEDNPI TEHLKHSKGGAL I MDI EQHSNPAE I EDP 110
SURVIVIN (NP_001159) BIR 1  -MGAPTLPPAVQPFLKDR I STFKMVFLECCAC I PER I LAAGFI HCPITENEPLACG I CFKLELGMEPDDPI EEHKNHSSGAE I LSVKKGFEEL I LGEFL I KLD 105
* *
A. Fumigatus (XP_752777) BIR(II) 99 - - QHSNPAE I EDTSER I AQARCAT I G I QMPHDG I RGM I VEGG I YFCPTESND I ASGAY I G I KLS I I G WEP I KDP I FEEL I YR I SSSDGS I F I V I AQPPGK I K I KSSRS I K I KS 203
SURVIVIN (NP_001159) BIR 1  M E A P I L P P A V Q P F L K D R I S T F K M V F L E G C A C T P E R - - L A E A G F I H C P T E N E P L A C G I C F K L E L G W E P D D P I E E H K N H S S G A E I L S V K K G F E E L I L G E F L I K L D 105
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