HHS Public Access

Author manuscript

Science. Author manuscript; available in PMC 2019 September 19.

Published in final edited form as:

Science. 2018 June 22; 360(6395): . doi:10.1126/science.aas9457.

Response to Comment on "Sterilizing immunity in the lung relies on targeting fungal apoptosis-like programmed cell death"

Neta Shlezinger[1], **Henriette Irmer**[2], **Sourabh Dhingra**[3], **Sarah R. Beattie**[3], **Robert A. Cramer**[3], **Gerhard H. Braus**[2], **Amir Sharon**[4,*], **Tobias M. Hohl**[1,5,*]

[1]Infectious Disease Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY 10075, USA

[2]Department of Molecular Microbiology and Genetics, Institute for Microbiology and Genetics, and Göttingen Center for Molecular Biosciences, University of Göttingen, D-37077 Göttingen, Germany

[3]Department of Microbiology and Immunology, Geisel School of Medicine, Dartmouth College, Hanover, NH 03755, USA

[4]Department of Molecular Biology and Ecology of Plants, Tel Aviv University, Tel Aviv 69978, Israel

[5]Immunology Program, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, NY 10075, USA

Abstract

Aouacheria *et al.* question the interpretation of contemporary assays to monitor programmed cell death with apoptosis-like features (A-PCD) in *Aspergillus fumigatus.* Although our study focuses on fungal A-PCD for host immune surveillance and infectious outcomes, the experimental approach incorporates multiple independent A-PCD markers and genetic manipulations based on fungal rather than mammalian orthologs to circumvent the limitations associated with any single approach.

Our manuscript reports that human and mouse leukocytes trigger A-PCD in inhaled mold conidia as a mechanism of mucosal barrier immune surveillance (1). We distinguish fungal A-PCD from metazoan apoptosis and from accidental cell death; the latter process is defined as "virtually immediate and...insensitive to pharmacologic or genetic interventions of any kind" (2). According to a recently published 2018 guideline (3), the A-PCD process observed in conidia during cellular interactions with innate immune cells is best understood as a regulated cell death subroutine with apoptosis-like features. Our study relied on a combination of assays to monitor markers of conidial A-PCD (i.e., nuclear condensation and histone degradation, fungal caspase-like activities, DNA double-strand breaks, and viability by clonogenic assay), in accordance with accepted practice (4–7).

*Corresponding author. hohlt@mskcc.org (T.M.H.); amirsh@tauex.tau.ac.il (A.S.).

The Comment by Aouacheria *et al.* (8) highlights differences between animal apoptosis and fungal A-PCD, and points to longstanding knowledge gaps in the study of regulated cell death in fungi. In particular, the fungal factors responsible for TUNEL reactivity or caspase-like activity, or those that trigger fungal cell death in mammalian cell death assays, remain undefined (9). Notably, the authors' assertion regarding the low specificity of individual assays holds true for mammalian cells as well. Thus, although we fully agree with the authors that improvements in methodologies will advance the study of fungal cell death, our study did not aim to answer this general question. In addition, Aouacheria *et al.* raise several issues that require clarification.

First, at the outset, the authors state that "Shlezinger *et al.* reported the existence of an 'apoptosis-like' programmed death pathway in the opportunistic pathogen *Aspergillus fumigatus,* a multicellular fungus responsible for life-threatening infections." A-PCD in fungi, and specifically in *A. fumigatus,* is well known and broadly supported by numerous studies conducted over the past 20 years [reviewed in (7)]. Among filamentous molds, the best-understood examples of regulated cell death processes include heterokaryon incompatibility (10, 11), appressorium morphogenesis (12), senescence (13, 14), and the development of reproductive structures and infectious propagules (15). We make no such claim of discovery; in fact, the existence of A-PCD in fungi was the basis for our research hypothesis.

Second, Aouacheria *et al.* criticize the use of the fluorescein isothiocyanate (FITC)–conjugated tripeptide Val-Ala-Asp fused to fluoromethylketone (FITC-VAD-FMK) as a marker of fungal A-PCD. Fungal genomes encode metacaspases, cysteine-dependent proteases that share structural properties with metazoan caspases and hydrolyze proteins after Arg or Lys rather than Asp residues (16). Deletion of the two *A. fumigatus* metacaspases, CasA and CasB, does not render cells sensitive to oxidative stress and does not attenuate a FITC-VAD-FMK signal under these conditions (17); the dependency of *Aspergillus* cell death routines on metacaspase activity varies according to the specific stimulus (18, 19). Nonetheless, a substantial body of work demonstrates that the pan-caspase inhibitor Z-VAD-FMK effectively inhibits the activation of apoptosis-like cell death in fungal cells (4, 20–22) and in particular in *A. fumigatus* (18). On the basis of these studies, FITC-VAD-FMK has been used extensively for detection of caspase-like activity in yeast and filamentous fungi (23–26). In addition, numerous studies reported that activation of fungal apoptotic cell death correlated with the appearance of caspase-like activities with a substrate specificity similar to that of initiator caspases [with Val-Glu-Ile-Asp (VEID)- and Ile-Glu-Thr-Asp (IETD)-hydrolyzing activities] that could be abolished by Z-VAD-FMK (15, 24, 27, 28). Together with our findings, the above studies support a model in which unidentified caspase-like protease(s) act in fungal A-PCD downstream of BIR1-dependent regulation. Thus, the identification and functional characterization of putative *A. fumigatus* cysteine proteases in A-PCD represents an important direction of future research.

Our results do not support the notion that FITC-VAD-FMK "can also nonspecifically label living nonpermeabilized yeast cells." We detected a fluorescent signal in live *A. fumigatus* conidia that were engulfed by innate immune cells in vivo. If the probe were incorporated

nonspecifically by live conidia in the murine lung, we would predict detection of the same signal in free conidia found in infected airways of the same animal. This was not the case.

Third, the authors state that “Shlezinger *et al.* took a different approach to address a causal role for fungal caspase-like activities, leading to the identification of a virulence mechanism” and that the manuscript assumes “that AfBIR1 behaves similarly to the human inhibitor of apoptosis (IAP) protein survivin, based on the assumption that survivin inhibits apoptotic caspases.” It is critical to note that AfBIR1 belongs to a conserved family of proteins in fungi (and animals) that encode a BIR domain (4, 16), the hallmark of IAP proteins. Because all other characterized fungal proteins that contain a BIR domain exert anti-PCD activity (17–21), we functionally analyzed AfBIR1 to establish a direct relationship among AfBIR1-dependent regulation of conidial A-PCD, fungal virulence, innate immune surveillance in the lung, and infectious outcomes. Although we monitored fungal caspase-like activity during A-PCD and its dependency on AfBIR1 expression levels, the precise mechanism by which this regulation occurs represents a focus for follow-up studies. Thus, the premise for our experiments and conclusions rests on a body of work conducted in fungal rather than mammalian systems that collectively strongly supports a role for fungal BIR1 orthologs in regulated cell death processes.

Fourth, the authors criticize the use of S12 as an AfBIR1 inhibitor. Because a genetic loss-of-function approach was not possible, we used a complementary pharmacologic approach to target the fungal BIR domain. The impact of S12 on oxidative stress-induced A-PCD correlated with AfBIR1 expression levels in different strains. In vivo, administration of S12 accelerated fungal clearance, in contrast to the delayed clearance and invasive disease caused by an increase in AfBIR1 expression. Thus, the results of the S12 experiments are in line with and extend complementary genetic gain-of-function experiments. However, further work is necessary to demonstrate direct binding of S12 with AfBIR1.

Our results support a model in which the respiratory innate immune system triggers NADPH oxidase-dependent A-PCD in inhaled mold conidia and advance the concept that a higher eukaryote can exploit a regulated cell death process in a lower eukaryote to maintain barrier immunity. Although the Comment by Aouacheria *et al.* does not detract from these conclusions, it enlightens longstanding conceptual and technical questions related to fungal A-PCD, highlights important knowledge gaps for future research and advancement of the field as a whole, and engenders a lively conversation about these fascinating topics.

REFERENCES

1. Shlezinger N et al., Science 357, 1037–1041 (2017). [PubMed: 28883073]
2. Galluzzi L et al., Cell Death Differ. 22, 58–73 (2015). [PubMed: 25236395]
3. Carmona-Gutierrez D et al., Microb. Cell 5, 4–31 (2018). [PubMed: 29354647]
4. Sharon A, Finkelstein A, Shlezinger N, Hatam I, FEMS Microbiol. Rev 33, 833–854 (2009). [PubMed: 19416362]
5. Semighini CP, Harris SD, Methods Mol. Biol 638, 269–279 (2010). [PubMed: 20238276]
6. Carmona-Gutierrez D et al., Cell Death Differ. 17, 763–773 (2010). [PubMed: 20075938]
7. Gonçalves AP, Heller J, Daskalov A, Videira A, Glass NL, Front. Microbiol 8, 1837 (2017). [PubMed: 28983298]

8. Aouacheria A et al., Science 360, eaar6910 (2018). [PubMed: 29930109]
9. Hardwick JM, Cheng WC, Dev. Cell 7, 630–632 (2004). [PubMed: 15525523]
10. Daskalov A et al., PLOS Biol. 13, e1002059 (2015). [PubMed: 25671553]
11. Daskalov A et al., Proc. Natl. Acad. Sci. U.S.A 113, 2720–2725 (2016). [PubMed: 26903619]
12. Veneault-Fourrey C, Barooah M, Egan M, Wakley G, Talbot NJ, Science 312, 580–583 (2006). [PubMed: 16645096]
13. Hamann A, Brust D, Osiewacz HD, Mol. Microbiol 65, 948–958 (2007). [PubMed: 17627766]
14. Knuppertz L, Warnsmann V, Hamann A, Grimm C, Osiewacz HD, Autophagy 13, 1037–1052 (2017). [PubMed: 28368682]
15. Thrane C, Kaufmann U, Stummann BM, Olsson S, Fungal Genet. Biol 41, 361–368 (2004). [PubMed: 14761796]
16. Tsiatsiani L et al., Cell Death Differ. 18, 1279–1288 (2011). [PubMed: 21597462]
17. Richie DL et al., Mol. Microbiol 63, 591–604 (2007). [PubMed: 17176258]
18. Mousavi SA, Robson GD, Fungal Genet. Biol 39, 221–229 (2003). [PubMed: 12892635]
19. Mousavi SA, Robson GD, Microbiology 150, 1937–1945 (2004). [PubMed: 15184579]
20. Al-Dhaheri RS, Douglas LJ, J. Med. Microbiol 59, 149–157 (2010). [PubMed: 19892857]
21. Wang X, Wang Y, Zhou Y, Wei X, Mycologia 106, 881–888 (2014). [PubMed: 24895430]
22. Shirazi F et al., Antimicrob. Agents Chemother 57, 4360–4368 (2013). [PubMed: 23817366]
23. Herker E et al., J. Cell Biol 164, 501–507 (2004). [PubMed: 14970189]
24. Hauptmann P et al., Mol. Microbiol 59, 765–778 (2006). [PubMed: 16420350]
25. Shirazi F, Lewis RE, Kontoyiannis DP, Microb. Cell 2, 445–450 (2015). [PubMed: 28357269]
26. Johansson M, Chen X, Milanova S, Santos C, Petranovic D, FEMS Yeast Res. 16, fow007 (2016). [PubMed: 26833421]
27. Madeo F et al., Curr. Genet 41, 208–216 (2002). [PubMed: 12172961]
28. Guaragnella N, Bobba A, Passarella S, Marra E, Giannattasio S, FEBS Lett. 584, 224–228 (2010). [PubMed: 19941863]