IMMUNITY TO FUNGAL INFECTIONS: INSIGHTS FROM THE INNATE IMMUNE RECOGNITION AND ANTIFUNGAL EFFECTOR MECHANISMS

EDITED BY: Rodrigo Tinoco Figueiredo, Allan J. Guimaraes and David L. Moyes
PUBLISHED IN: Frontiers in Microbiology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88971-325-7
DOI 10.3389/978-2-88971-325-7

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

IMMUNITY TO FUNGAL INFECTIONS: INSIGHTS FROM THE INNATE IMMUNE RECOGNITION AND ANTIFUNGAL EFFECTOR MECHANISMS

Topic Editors:
Rodrigo Tinoco Figueiredo, Federal University of Rio de Janeiro, Brazil
Allan J. Guimaraes, Fluminense Federal University, Brazil
David L. Moyes, King's College London, United Kingdom

Citation: Figueiredo, R. T., Guimaraes, A. J., Moyes, D. L., eds. (2021). Immunity to Fungal Infections: Insights From the Innate Immune Recognition and Antifungal Effector Mechanisms. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88971-325-7

Table of Contents

frontiers in Microbiology

EDITORIAL
published: 15 July 2021
doi: 10.3389/fmicb.2021.714013

Editorial: Immunity to Fungal Infections: Insights From the Innate Immune Recognition and Antifungal Effector Mechanisms

David L. Moyes[1]*, *Allan J. Guimarães*[2]* and *Rodrigo T. Figueiredo*[3]*

[1] Centre for Host-Microbiome Interactions, Faculty of Dentistry, Oral and Craniofacial Sciences, King's College London, London, United Kingdom, [2] Department of Microbiology and Parasitology, Biomedical Institute, Fluminense Federal University, Niterói, Brazil, [3] Campus Duque de Caxias, Federal University of Rio de Janeiro, Duque de Caxias, Brazil

Keywords: mycoses, innate immunity, leukocytes, lymphocytes, cytokines

Edited by:
Karl Kuchler,
Medical University of Vienna, Austria

Reviewed by:
Oliver Kurzai,
Julius Maximilian University of Würzburg, Germany
Bruce Klein,
University of Wisconsin-Madison, United States

****Correspondence:***
David L. Moyes
david.moyes@kcl.ac.uk
Allan J. Guimarães
allanguimaraes@id.uff.br
Rodrigo T. Figueiredo
figueiredo.rt@xerem.ufrj.br

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *24 May 2021*
Accepted: *23 June 2021*
Published: *15 July 2021*

Citation:
Moyes DL, Guimarães AJ and Figueiredo RT (2021) Editorial: Immunity to Fungal Infections: Insights From the Innate Immune Recognition and Antifungal Effector Mechanisms. Front. Microbiol. 12:714013. doi: 10.3389/fmicb.2021.714013

Editorial on the Research Topic

Immunity to Fungal Infections: Insights From the Innate Immune Recognition and Antifungal Effector Mechanisms

Fungal infections represent a major health concern which, according estimations, account for nearly 1.6 million deaths annually (Brown et al., 2012). Epidemiological reports have demonstrated a growth in the incidence of invasive mycoses which are associated with conditions associated with immunossupression, such as diabetes, immunosupressive therapy for solid organ transplantation or autoimmune diseases, cancer and neutropenia (Bitar et al., 2014; Rayens et al., 2021). Risk factors for invasive mycosis represent medical advances, including the use of intravenous catheters, cancer chemotherapy and immunosupressive therapies which have created a growing population of vulnerable patients. Invasive mycoses are associated with unacceptable high lethality rates (Suleyman and Alangaden, 2016). Although antifungal drug resistance is not a major problem in most cases of invasive mycoses, it is an additional threat (Fairlamb et al., 2016). Recent outbreaks of infections caused by *Candida auris*, a multidrug-resistant fungus, as well as associations of fungal infections with influenza and COVID-19 cases, as observed for aspergillosis and mucormycosis, have created new challenges for the managements of patients in critical care units (Spivak and Hanson, 2018; Dewi et al., 2021; Gandra et al., 2021).

Integration of innate immune recognition and adaptive immunity is critical for the control of invasive mycoses. The relevance of the basic knowledge in the immunopathogenesis of mycoses can be observed by the growth in clinical immunomodulation studies (Williams et al., 2020). Thus, comprehension of the immunity to fungal pathogens offers new possibilities for the treatment of invasive mycoses. This topic discusses aspects of the innate immunity and leukocyte activation to fungal pathogens, the repertoire of B and T cell antigen receptors in experimental models of pneumocystosis, as well as a discussion about the control of the expression of fungal toxins involved in the modulation of host responses.

Here, Thompson et al. investigate the role of Dectin-2 and Mincle in recognizing different *Candida* species—notably *C. albicans, C. parapsilosis, C. tropicalis*, and *C. glabrata*. They compare the cell wall composition of the different species, looking at cell wall thickness and phosphomannan content, and discuss the importance of Mincle and Dectin-2 recognition in systemic murine infection models varies with the *Candida* species, indicating the varied nature of fungal detection in the innate immune response.

Neutrophil DNA extracellular traps (NETs) are formed in response to fungal pathogens and show fungicidal/fungistatic activity (Urban and Nett, 2019). Eosinophils ETs (EETs) have been observed in human samples from bronchopulmonary aspergillosis cases and human eosinophils release ETs in response to *A. fumigatus* conidia (Muniz et al., 2018). In this topic, Silva et al. present a review discussing the mechanisms involved in the release of NETs and EETs, in response to fungal pathogens, as well as the role of ETs in the immunity and pathogenesis of fungal infections. In this issue, Barroso et al. describe the signaling pathways involved in the EET formation in response to *A. fumigatus*. *A. fumigatus*-induced EET release requires the activity of Src kinases, class IA phosphatidylinositol 3-kinase δ (PI3KIδ), Akt and p38 kinase. Intracellular calcium mobilization is also required for EET formation in response to *A. fumigatus*, while PAD4-mediated histone citrullination is dispensable. EET release does not require *A. fumigatus* viability, indicating that recognition of *A. fumigatus* molecular patterns is able to trigger EETosis, while fungal viability and expression of virulence factors are dispensable for the eosinophil activation culminating in the ET release.

Fungal infections are a particular problem in HIV-infected individuals. Indeed, one of the defining features of AIDS is respiratory *Pneumocystis* infection. Normally, these infections are prevented by robust B and T cell responses (Thomas and Limper, 2007). Although gross examination of the different subsets of T cells during these infections has been identified, only recently the tools have been developed to increase the resolution of the T cell immunophenotypes. In this issue, Yang et al. use a combination single cell TCR-Seq and single cell RNA-Seq to identify the subsets of T cells that respond to *Pneumocystis* infection. In doing so, they reveal the composition and characteristics of clonally expanding T cells, along with their TCR repertoire.

In a similar approach, Sun et al. integrated single-cell RNA and BCR sequencing of immune cells from mouse lungs to detail the dynamic nature of B cell responses during Pneumocystis infection, with ongoing increased plasma cells elevated ratio of (IgA+ IgG) to (IgD+ IgM) after infection. Despite the clonal expansion post-infection, BCR repertoire diversity decreased, with B cell transcriptional changes including a biased usage of V(D)J genes and higher frequency of somatic hypermutation in comparison to naïve B cells, offering valuable information and tools for the development of immunotherapeutic targets and diagnostic biomarkers.

The modulation of inflammatory responses during fungal infections, particularly in the context of how each IL-1 family (IL-1, IL-18, and IL-36) member, despite of their clearly defined roles, could act together in determining the disease outcome is reviewed by Griffiths et al.. The authors address their mechanism of induction, the main cellular types responsible for their expression and processing and the immunological and other functional roles of each subfamily member. The authors tackle deeply how several endemically important fungal pathogens could differentially induce each of these IL-1 subfamily cytokines. Their therapeutic potential is further described as modulation of receptors or proteins regulating the IL-1 subfamily might enhance protective anti-fungal immunity or resolve excessive damaging immune responses.

Host and fungus interactions trigger responses in a two-way signaling that leads to fungal adaptation to the host environment, for example the hyphal growth in *Candida* species. Notably, these interactions can lead to the secretion of different mycotoxins, including ochratoxin, which can mediate different aspects of the pathology associated with infection. In this issue, Gao et al. review the role of G protein coupled receptors (GCPR) in fungi, and their role in regulating the synthesis of mycotoxins. Accordingly, the authors explore the current limited state of knowledge of fungal GPCR biology and how these circuits regulate fungal behavior and mycotoxin synthesis. The role of GPCR as potential targets in modulating the synthesis of secondary fungal metabolites and regulating fungal behavior is also discussed.

AUTHOR CONTRIBUTIONS

All authors contributed to manuscript revision and draft, read, and approved the submitted version.

FUNDING

RF was supported by Brazilian Agency Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, E-26/203.200/2017). AG was supported by FAPERJ (E-26/202.696/2018) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant 311470/2018-1). DM was supported by the Biotechnology and Biological Sciences Research Council, UKRI (BB/S016899/1) and Unilever.

REFERENCES

Bitar, D., Lortholary, O., Le Strat, Y., Nicolau, J., Coignard, B., Tattevin, P., et al. (2014). Population-based analysis of invasive fungal infections, France, 2001–2010. *Emerg. Infect. Dis.* 20, 1149–1155. doi: 10.3201/eid2007.140087

Brown, G. D., Denning, D. W., Gow, N. A., Levitz, S. M., Netea, M. G., and White, T. C. (2012). Hidden killers: human fungal infections. *Sci. Transl. Med.* 4:165rv113. doi: 10.1126/scitranslmed.3004404

Dewi, I. M., Janssen, N. A., Rosati, D., Bruno, M., Netea, M. G., Brüggemann, R. J., et al. (2021). Invasive pulmonary aspergillosis associated with viral pneumonitis. *Curr. Opin. Microbiol.* 62, 21–27. doi: 10.1016/j.mib.2021.04.006

Fairlamb, A. H., Gow, N. A., Matthews, K. R., and Waters, A. P. (2016). Drug resistance in eukaryotic microorganisms. *Nat. Microbiol.* 1:16092. doi: 10.1038/nmicrobiol.2016.92

Gandra, S., Ram, S., and Levitz, S. M. (2021). The "Black Fungus" in India: the emerging syndemic of COVID-19-associated mucormycosis. *Ann. Intern. Med.* 2021:2354. doi: 10.7326/M21-2354

Muniz, V. S., Silva, J. C., Braga, Y.a.V., Melo, R. C. N., Ueki, S., Takeda, M., et al. (2018). Eosinophils release extracellular DNA traps in response to *Aspergillus fumigatus*. *J. Allergy Clin. Immunol.* 141, 571–585.e577. doi: 10.1016/j.jaci.2017.07.048

Rayens, E., Norris, K. A., and Cordero, J. F. (2021). Mortality trends in risk conditions and invasive mycotic disease in the United States, 1999–2018. *Clin. Infect. Dis*. 2021:ciab336. doi: 10.1093/cid/ciab336

Spivak, E. S., and Hanson, K. E. (2018). *Candida auris*: an emerging fungal pathogen. *J. Clin. Microbiol.* 56:17. doi: 10.1128/JCM.01588-17

Suleyman, G., and Alangaden, G. J. (2016). Nosocomial fungal infections: epidemiology, infection control, and prevention. *Infect. Dis. Clin. North Am.* 30, 1023–1052. doi: 10.1016/j.idc.2016.07.008

Thomas, C. F., and Limper, A. H. (2007). Current insights into the biology and pathogenesis of *Pneumocystis pneumonia*. *Nat. Rev. Microbiol.* 5, 298–308. doi: 10.1038/nrmicro1621

Urban, C. F., and Nett, J. E. (2019). Neutrophil extracellular traps in fungal infection. *Semin. Cell Dev. Biol.* 89, 47–57. doi: 10.1016/j.semcdb.2018.03.020

Williams, T. J., Harvey, S., and Armstrong-James, D. (2020). Immunotherapeutic approaches for fungal infections. *Curr. Opin. Microbiol.* 58, 130–137. doi: 10.1016/j.mib.2020.09.007

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 10 February 2021
doi: 10.3389/fmicb.2021.633047

Role for IL-1 Family Cytokines in Fungal Infections

James S. Griffiths, Giorgio Camilli, Natalia K. Kotowicz, Jemima Ho, Jonathan P. Richardson and Julian R. Naglik*

Centre for Host-Microbiome Interactions, Faculty of Dentistry, Oral and Craniofacial Sciences, King's College London, London, United Kingdom

Fungal pathogens kill approximately 1.5 million individuals per year and represent a severe disease burden worldwide. It is estimated over 150 million people have serious fungal disease such as recurrent mucosal infections or life-threatening systemic infections. Disease can ensue from commensal fungi or new infection and involves different fungal morphologies and the expression of virulence factors. Therefore, anti-fungal immunity is complex and requires coordination between multiple facets of the immune system. IL-1 family cytokines are associated with acute and chronic inflammation and are essential for the innate response to infection. Recent research indicates IL-1 cytokines play a key role mediating immunity against different fungal infections. During mucosal disease, IL-1R and IL-36R are required for neutrophil recruitment and protective Th17 responses, but function through different mechanisms. During systemic disease, IL-18 drives protective Th1 responses, while IL-33 promotes Th2 and suppresses Th1 immunity. The IL-1 family represents an attractive anti-fungal immunotherapy target. There is a need for novel anti-fungal therapeutics, as current therapies are ineffective, toxic and encounter resistance, and no anti-fungal vaccine exists. Furthering our understanding of the IL-1 family cytokines and their complex role during fungal infection may aid the development of novel therapies. As such, this review will discuss the role for IL-1 family cytokines in fungal infections.

Keywords: fungi, fungal immunology, IL-1, *Candida*, *Aspergillus*

OPEN ACCESS

Edited by:
Allan J. Guimaraes, Fluminense Federal University, Brazil

Reviewed by:
Flavio Vieira Loures, Federal University of São Paulo, Brazil
Teresa Zelante, University of Perugia, Italy
Agostinho Carvalho, University of Minho, Portugal

****Correspondence:***
James S. Griffiths
james.griffiths@kcl.ac.uk

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *24 November 2020*
Accepted: *15 January 2021*
Published: *10 February 2021*

Citation:
Griffiths JS, Camilli G, Kotowicz NK, Ho J, Richardson JP and Naglik JR (2021) Role for IL-1 Family Cytokines in Fungal Infections. Front. Microbiol. 12:633047. doi: 10.3389/fmicb.2021.633047

INTRODUCTION

Fungal pathogens represent an increasingly severe disease burden and are responsible for ∼1.5 million deaths per year. Patients who are immunocompromised, have undergone invasive clinical procedures or suffered trauma are particularly susceptible to fungal infection. Fungi can be frequently encountered, such as *Aspergillus* through inhalation or *Candida*, which colonizes mucosal barriers (Brown et al., 2012). Anti-fungal responses must strike a careful balance to provide protection and maintain homeostasis. Regularly encountered fungal pathogens must be cleared with minimal effect on the host, while commensal fungi must be maintained without reducing barrier integrity. The majority of serious fungal disease arises from poorly cleared infection or disrupted barrier integrity (Rautemaa-Richardson and Richardson, 2017). Here, the IL-1 family play a crucial role mediating both barrier and systemic anti-fungal immunity. As such, modulating IL-1 family cytokines to enhance anti-fungal immunity may provide valuable therapeutic strategies that overcome current therapeutic inadequacies.

The IL-1 family possess numerous potent biological activities and mediate a wide range of immunological responses (Garlanda et al., 2013). IL-1 was identified in the 1980's but had been

investigated for many years under various aliases. The discovery of IL-1 was initially met with skepticism that a molecule at such low concentration could have potent, systemic effect. Since then, our understanding of the IL-1 family has grown and now comprises four sub-families containing eleven signaling members, five primary receptors and six co-receptors (**Figure 1**). Of the eleven signaling members, seven are pro-inflammatory and four are anti-inflammatory (**Table 1**). The signaling members of the IL-1 family share a highly conserved gene sequence and structure and (except for IL-18 and IL-33) are clustered on human chromosome 2 (Sims and Smith, 2010). Due to these similarities, the genomic identification of IL-1 family members largely preceded the discovery of their function. Aside from IL-1 receptor antagonist (IL-1Ra), all other IL-1 family cytokines lack a secretion signal peptide and either require cleaving and activation or are active in their precursor form. Signaling typically occurs when cytokines bind their primary receptor and recruit a co-receptor, which induces signaling through the Toll/interleukin 1 receptor (TIR) domain resulting in mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) activation (Fields et al., 2019). In this review, we describe each IL-1 subfamily (IL-1, IL-18, and IL-36) and investigate the mechanism of induction and functional role of each subfamily member within the context of fungal disease.

THE IL-1 SUBFAMILY

Interleukin-1 was the first interleukin to be identified and represented a huge leap forward in immunology. The IL-1 gene cluster encodes the pro-inflammatory cytokines IL-1α and IL-1β, and anti-inflammatory receptor antagonist IL-1Ra (Garlanda et al., 2013). IL-1α/β bind their receptor IL-1R1, which recruits IL-1RAcP and permits signaling through both receptor's TIR domain (Fields et al., 2019). This domain is well conserved throughout IL-1 and Toll-like receptor (TLR) signaling where it drives inflammation (Heguy et al., 1992). IL-1α/β are recognized as major inflammatory cytokines that mediate innate and adaptive immunity, and also general health. IL-33, a recent addition to the IL-1 subfamily, was discovered in 2005 and signals through its receptor IL-1R4 (formerly ST2) in complex with IL-1RAcP. IL-33 has many functional capabilities which influence barrier integrity and inflammation (Mehraj et al., 2016). IL-33 signaling has been implicated in numerous allergic-type diseases and, along with IL-1α/β, has recently been shown as a mediator of adaptive immunity (Kamijo et al., 2013).

IL-1 Subfamily Expression and Processing

Although IL-1α and IL-1β signal through the same receptor and have similar biological activities, these two pro-inflammatory cytokines differ in several aspects. IL-1α is constitutively expressed in epithelial and mesenchymal cell types and expression increases in response to growth factors, inflammation or stress-associated stimuli (Di Paolo and Shayakhmetov, 2016). Here, IL-1α is released from cells during damage or necrosis (Chen et al., 2007). IL-1α also possesses a nuclear localization signal and can interact with histone acetyltransferase complexes to mediate transcription of cytokines including IL-6 and IL-8 (Werman et al., 2004). However, nuclear translocation of IL-1α during apoptosis inactivates IL-1α signaling, likely sequestering it and blocking inflammatory effects (Cohen et al., 2010). IL-1α also functions as an active membrane bound precursor promoting inflammation through IL-1R1 binding. Membrane-associated IL-1α is present on the surface of numerous immune cells including macrophages (Kurt-Jones et al., 1985), monocytes, and B lymphocytes (Zola et al., 1993).

Interleukin-1β is mainly produced by mononuclear phagocytes as an inactive precursor and activated via a two-step process. During the initial priming step, pro-IL-1β is induced by the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRR) (Takeuchi and Akira, 2010). Activation requires pro-IL-1β cleavage by the intracellular cysteine protease caspase-1 and is regulated by inflammasomes (Franchi et al., 2009). Although caspase-1 is the main protease responsible for pro-IL-1β activation, Fas stimulation is also able to induce secretion of biologically active IL-1β from caspase-1-deficient murine macrophages (Bossaller et al., 2012). Once activated, IL-1β is secreted through one or more non-conventional secretory pathways (Lopez-Castejon and Brough, 2011). Here, another role for caspase-1 has been described in that it can cleave the pore forming toxin gasdermin D, which induces pore formation in the plasma membrane, and pyroptosis to enhance IL-1β release (Heilig et al., 2018). Importantly, the release of pro-IL-1β upon membrane disruption has functional consequences. Multiple proteases, mainly derived from neutrophils and mast cells, can cleave and activate pro-IL-1β in the extracellular environment which drives inflammation (Stehlik, 2009).

Interleukin-33 is constitutively expressed in multiple cell types but is mainly found in fibroblasts, epithelial and endothelial cells (Moussion et al., 2008), with expression further increasing during inflammation (Liew et al., 2016). In macrophages, IL-33 induction was dependent on glutaredoxin-1/TRAF6 and NF-κB signaling (Weinberg et al., 2019). Similar to IL-1α, IL-33 has dual functions acting as a transcriptional repressor of NF-κB following nuclear localization (Ali et al., 2011) and enhancing inflammation after being released from damaged or necrotizing cells (Moussion et al., 2008). Again like IL-1α, IL-33 is also sequestered during apoptosis (Bessa et al., 2014). Interestingly, caspase-1 IL-33 cleavage attenuated inflammation (Luthi et al., 2009), while neutrophil proteases enhanced biological activity (Lefrancais et al., 2012). Following the release of IL-1α and IL-33, and the release and activation of IL-1β, the cytokines drive potent immunological functions.

The Immunological Function of the IL-1 Subfamily

The first discovered IL-1 cytokine was named hemopoietin-1 after its signaling drove myeloid "emergency" responses (Pietras et al., 2016). Since then, the sub-family has expanded and IL-1α/β are now known to promote myelopoiesis and inflammation, lead to the release of antimicrobial compounds,

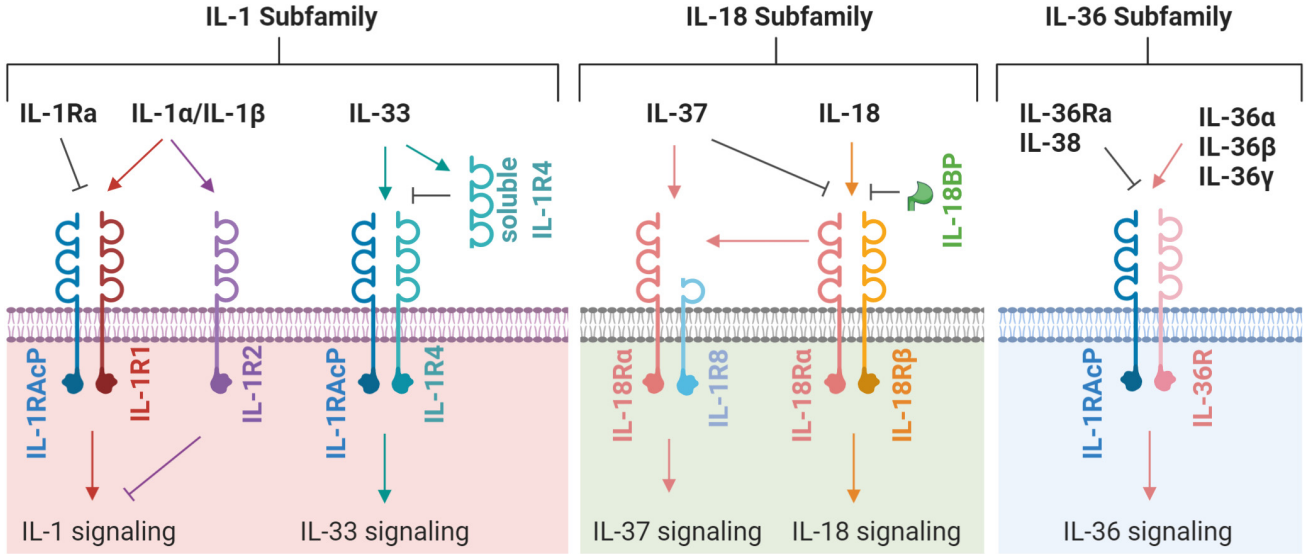

FIGURE 1 | A schematic representation of IL-1 family receptors, co-receptors, and ligands. Each ligand, receptor and co-receptor are separated into their appropriate IL-1 subfamily. An arrow indicates the induction of signaling; a flat line indicates the blocking of signaling. The latest nomenclature is used, for previous/alternative nomenclature see **Table 1**.

TABLE 1 | Nomenclature and function of IL-1 family members.

Cytokine		Primary receptor		Co-receptor		Effect
Name	**Alternate name(s)**	**Name**	**Alternate name(s)**	**Name**	**Alternate name(s)**	
IL-1α	IL-1F1	IL-1R1/IL-1R2 (Decoy)		IL-1RAcP	IL-1R3	Pro-inflammatory
IL-1β	IL-1F2	IL-1R1/IL-1R2 (Decoy)		IL-1RAcP	IL-1R3	Pro-inflammatory
IL-1Ra	IL-1F3	IL-1R1				Anti-inflammatory
IL-33	IL-1F11	IL-1R4	ST2	IL-1RAcP	IL-1R3	Pro-inflammatory
IL-18	IL-1F4	IL-18Rα	IL-1R5	IL-18Rβ	IL-1R7	Pro-inflammatory
IL-37	IL-1F7	IL-18Rα	IL-1R5	IL-1R8	SIGIRR	Anti-inflammatory
IL-36α	IL-1F6	IL-36R	IL-1Rrp2/IL-1R6	IL-1RAcP	IL-1R3	Pro-inflammatory
IL-36β	IL-1F7	IL-36R	IL-1Rrp2/IL-1R6	IL-1RAcP	IL-1R3	Pro-inflammatory
IL-36γ	IL-1F8	IL-36R	IL-1Rrp2/IL-1R6	IL-1RAcP	IL-1R3	Pro-inflammatory
IL-36Ra	IL-1F5	IL-36R	IL-1Rrp2/IL-1R6			Anti-inflammatory
IL-38	IL-1F10	IL-36R	IL-1Rrp2/IL-1R6			Anti-inflammatory

and mediate immunity (Dinarello, 2018). Following the release of IL-1α and active IL-1β, the cytokines bind their receptor IL-1R1 and drive MAPK and NF-κB signaling through the myeloid differentiation primary response (MyD88) adaptor protein (Cohen, 2014). Two mechanisms antagonize the effects of IL-1α/β. Firstly, IL-1Ra binds IL-1R1 to prevent IL-1α/β-IL-1R1 interactions. The importance of IL-1Ra is highlighted in IL-1Ra deficient mice, which spontaneously develop Th17-associated rheumatoid arthritis (Koenders et al., 2008). Secondly, IL-1R2 acts as a decoy receptor and lacks a TIR domain, therefore blocking downstream signaling. Overexpressing IL-1R2 in mice reduced inflammation in numerous IL-1-induced inflammatory diseases (Peters et al., 2013).

While IL-1α/β have central roles in driving inflammation and mediating immune responses, each cytokine possesses distinct function. IL-1α acts as an alarmin and is the principal trigger of inflammation following cell membrane disruption or cellular necrosis. In this context, constitutively expressed IL-1α is released from cells and drives neutrophil recruitment and local inflammation (Chen et al., 2007; Eigenbrod et al., 2008). IL-1β signaling promotes the recruitment of monocytes, macrophages, and neutrophils; enhanced phagocytosis and killing; increased reactive oxygen species/nitrogen oxide synthase (ROS/NOS) production; and Th1 and Th17 immunity (Garlanda et al., 2013; Altmeier et al., 2016; Dinarello, 2018). Mice deficient in caspase-1 were protected from Th1 and Th17-associated experimental autoimmune encephalomyelitis (Gris et al., 2010) and mice deficient in IL-1Ra developed spontaneous Th17-associated autoimmunity (Horai et al., 2000). IL-1 is also associated with numerous autoinflammatory diseases, with blockade of IL-1 signaling rapidly improving symptoms (Dinarello et al., 2012). Interestingly, even when the autoimmune disease is primarily

tumor necrosis factor (TNF)/IL-6 driven (as in rheumatoid arthritis), blocking IL-1β signaling reduced disease severity (Dinarello, 2011). This highlights the central role of IL-1α/β mediating immunity and health.

Interleukin-33 acts in a similar fashion to IL-1α, being released from barrier cells and functioning as a damage-associated molecular pattern (DAMP). Here, IL-33 activates T-cells directly through PRR/IL-1R4 binding or indirectly through local inflammatory responses. Immune cells such as mast cells, basophils, dendritic cells, macrophages, natural killer cells, and Th2 cells are receptive to IL-33 and express IL-1R4 (Martin and Martin, 2016). The soluble form of IL-1R4 sequesters "off-target" IL-33 and antagonizes IL-33 activity (Kakkar and Lee, 2008). IL-33 was originally identified as driving allergic and anti-helminthic immunity but is also a key mediator of adaptive immunity. During acute infection, IL-33 promotes tissue remodeling and drives Th1, Th2, and T regulatory (Treg) responses. In contrast, during chronic infection, IL-33 enhanced local inflammation, tissue damage and fibrosis (Li et al., 2014). IL-33 signaling has been implicated in numerous diseases associated with exacerbated inflammation including allergic-asthma (Salter et al., 2016), Crohn's disease (Pastorelli et al., 2010), and rheumatoid arthritis (Tang et al., 2013). While the broad and potent consequences of IL-1α/β and IL-33 signaling are known, the induction of these cytokines during fungal infection is less clear.

Fungal Induction of the IL-1 Subfamily

Numerous fungal pathogens infect mucosal barrier sites and systemic disease often results from a loss in barrier integrity. It is therefore unsurprising that IL-1α/β and IL-33 have been implicated in anti-fungal immunity. All three cytokines are induced following *Aspergillus fumigatus* infection, a pathogen that typically infects mucosal surfaces. IL-1α expression in the lung is positively correlated with *A. fumigatus* strain virulence (Caffrey-Carr et al., 2017). This is likely a result of conidial germination and subsequent damage to mucosal barriers. IL-1β was rapidly induced and sustained for 2 days in an *A. fumigatus* keratitis model. Here, IL-1β induction was dependent on Dectin-1 signaling and c-Jun N-terminal kinase (JNK) phosphorylation (Yuan et al., 2017). Numerous PRRs that induce IL-1β signaling, including C-type lectin-like receptors (CLRs) and TLRs, are involved during *A. fumigatus* infection (Steele et al., 2005). The requirement of CLRs for IL-33 induction appears more complex. During acute *A. fumigatus* infection the induction of IL-33 occurred independently of Dectin-1 (Garth et al., 2017), while induction during chronic allergic-type infection required Dectin-1 (Lilly et al., 2012).

Candida albicans infection also results in IL-1α/β and IL-33 induction. IL-1α induction has been described in oral and vaginal epithelial cells (Steele and Fidel, 2002) and required membrane disruption and Ca^{2+} influx (Gross et al., 2012). Here, the peptide toxin candidalysin, secreted when *C. albicans* forms hyphae, disrupts host membranes resulting in IL-1α release (Moyes et al., 2016). IL-33 is also induced during *C. albicans* infection at barrier sites (Le et al., 2012) and likely requires candidalysin expression in a similar manner to IL-1α. The induction of IL-1β in monocytes was only achieved with live *Candida* and does not require CLR involvement (Castro et al., 1996). In contrast, dendritic cells (that require two signals for IL-1β induction and activation) were dependent on spleen tyrosine kinase (Syk) signaling for IL-1β induction during *C. albicans* infection (Gross et al., 2009). In macrophages, IL-1β is induced by *C. albicans, C. tropicalis,* and *C. krusei,* although the requirement for CLRs is disputed (Joly et al., 2009; Kasper et al., 2018). As such, there are several mechanisms by which *Candida* induce inflammasome activation and IL-1β release (Camilli et al., 2020). One particularly interesting mechanism involves the non-tyrosine kinase Tec which was found to activate non-canonical caspase-8 exclusively following fungal challenge (Zwolanek et al., 2014). IL-1α/β induction during mucosal *C. albicans* infection occurs through NF-κB and a biphasic MAPK response. Activation of NF-κB and the first MAPK c-Jun phase was dependent on fungal PAMP recognition, while the second MAPK MKP1/c-Fos phase was dependent on hyphae formation and fungal burden. This identifies an interesting mechanism by which the host may detect the switch from commensalism to pathogenicity (Moyes et al., 2010). A similar biphasic recognition mechanism has not yet been described for other fungal infections, although such a mechanism may also differentiate between *A. fumigatus* conidia and hyphae during infection.

Interleukin-1 subfamily members are also induced by *Cryptococcus neoformans*, *Paracoccidioides brasiliensis,* and *Paracoccidioides lutzii* [the causative agents of paracoccidioidomycosis (PCM)], and *Sporothrix schenckii* (the causative agent of sporotrichosis). IL-1α/β and IL-33 are induced in both mucosal and systemic compartments during *C. neoformans* infection (Flaczyk et al., 2013; Alvarez et al., 2019). During *P. brasiliensis* challenge, IL-1β was induced in human monocytes, macrophages and plasmacytoid dendritic cells (pDC). As was found with *Candida*, CLRs and Syk signaling were required for pDC IL-1β induction (Kurokawa et al., 2007; Preite et al., 2018). IL-1β release from macrophages was caspase-1 dependent; however, a non-canonical IL-1β processing pathway requiring Dectin-1 and caspase-8 activity has been described (Ketelut-Carneiro et al., 2018). *P. brasiliensis* challenge also induced IL-1α release through a macrophage-derived IFN-β/procaspase-11 pathway which enhanced pore-mediated cell lysis (Ketelut-Carneiro et al., 2019). Patients with severe PCM possessed high levels of IL-1β, IL-33, and IL-1R4 in serum which decreased following anti-fungal therapy (Silva et al., 1995; Alves et al., 2018). Recently, IL-1β has been implicated in *S. schenckii* infection. Here, IL-1β was induced in a caspase-1 dependent manner with increased IL-1β correlating with higher fungal burden (Goncalves et al., 2015). While it is clear the IL-1 subfamily is induced by fungal pathogens and contributes to fungal immunity, the sequence and mechanism of IL-1 subfamily induction during fungal challenge is still unclear.

The Role of the IL-1 Subfamily in Fungal Immunology

Interleukin-1α/β drive crucial immune mechanisms and are central mediators of immunity. Following their induction during

fungal infection, both IL-1α/β play a key role orchestrating the immune response. While IL-33 is induced during fungal disease, its functional role is not yet fully delineated. Much of our understanding of the IL-1 subfamily during fungal disease has been revealed using mice deficient in IL-1 components. Mice lacking IL-1R1 exhibit reduced neutrophil recruitment and emergency granulopoieses following oropharyngeal candidiasis (OPC) challenge. Here, both IL-1β from hematopoietic cells and IL-1α from non-hematopoietic cells promoted neutrophil recruitment (Altmeier et al., 2016). However, neutrophil recruitment is not always protective, since in vulvovaginal candidiasis (VVC) IL-1α promotes damaging inflammation and neutrophil recruitment (Barousse et al., 2004). In agreement, mutations increasing Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome activity are associated with VVC in patients (Jaeger et al., 2016; Roselletti et al., 2017).

Mice deficient in IL-1α or IL-1β were more susceptible to systemic *C. albicans* infection, with mice lacking both IL-1α/β possessing the highest fungal burdens and lowest survival. Interestingly, IL-1α/β deficient mice continued to possess higher fungal burdens in a neutropenic model of systemic *C. albicans* infection, suggesting the role of IL-1α/β is not just in neutrophil recruitment. Instead, IL-1α was found to activate macrophages and IL-1β enhanced neutrophil killing, and both IL-1α and IL-1β were involved in the development of IFN-γ Th1 and IL-10 Th2 responses (Vonk et al., 2006). A role for epithelial and hematopoietic IL-1α/β has also been described to promote essential Th17 immunity during mucosal *Candida* infection (Bishu et al., 2014; Verma et al., 2017, 2018). In patients, mutations in caspase recruitment domain-containing protein 9 (CARD9) or TLR1 (pathways that lead to IL-1β induction) resulted in defective Th17 responses and increased *Candida* susceptibility (Glocker et al., 2009; Plantinga et al., 2012). Th17 immunity is also vital during *Coccidioides* infection. Here, Th17 responses were dependent on CARD9 and IL-1R1, with IL-1R1 deficient mice lacking Th17 immunity and being highly susceptible to *C. immitis* infection (Hung et al., 2014; Viriyakosol et al., 2018). IL-1R1 deficient mice are highly susceptible to *C. neoformans* infection; however, susceptibility was associated with the induction of harmful Th2 immunity (Shourian et al., 2017).

Both IL-1α and IL-1β drive protective immunity during PCM. *In vivo* models of *P. brasiliensis* infection determined mice with impaired IL-1β responses had higher fungal burden and a dysregulated inflammatory response. IL-1α promoted local inflammatory responses, nitric oxide production and Th17 immunity (Ketelut-Carneiro et al., 2018, 2019). Whilst signaling through IL-1R1 is required for protective immunity against pulmonary *A. fumigatus* infection; unlike *Candida* and *P. brasiliensis* infection, only IL-1α was required. Here, IL-1α promoted neutrophil and macrophage recruitment through monocyte-induced CXCL1 signaling. Administration of CXCL1 partially restored neutrophil recruitment in IL-1R1 deficient mice (Caffrey et al., 2015), while mice lacking inflammasome components, and thus IL-1β activation, cleared *A. fumigatus* infection (Caffrey et al., 2015). However, IL-1β may play a key role during systemically disseminated *Aspergillus* infection, as was described with systemic *Candida* infection (Vonk et al., 2006). The Dectin-1 Y238X polymorphism, which results in diminished Dectin-1 activity and reduced IL-1β responses, significantly enhanced patient's *Aspergillus* and *Candida* susceptibility (Plantinga et al., 2009; Cunha et al., 2010).

While the potent effects of IL-1α/β are clear during fungal disease, the effect of IL-1Ra and IL-1R2 are less well explored. Patients with severe fungal-sensitized asthma have higher levels of IL-1α/β and IL-1Ra, indicating the antagonist is involved. In an animal model of fungal-sensitized asthma, a lack of IL-1Ra enhanced Th1 and Th17 immunity, increased neutrophil recruitment and exacerbated disease. Treatment with recombinant IL-1Ra (Anakinra) reduced these responses and resolved disease. In the same model, IL-1R1 deficient mice displayed improved lung function, suggesting IL-1α/β enhance chronic disease (Godwin et al., 2019). IL-1Ra also has a protective role in VVC by restraining activation of damage-enhancing NLRP3 through an IL-22/NLR family CARD domain-containing protein 4 (NLRC4)/IL-1Ra pathway. Here, mice deficient in IL-1Ra displayed enhanced disease that could be rescued with the administration of Anakinra (Borghi et al., 2015). While the role of IL-1Ra and IL-1R2 in balancing IL-1 agonist activity during fungal disease requires further investigation, early indications suggest IL-1Ra and IL-1R2 play important roles in mediating and resolving potentially damaging inflammation during acute and chronic disease.

The role of IL-33 signaling is now being explored in the context of fungal infection. During *C. neoformans* infection, IL-1α/β drives protective Th17 immunity and reduces Treg responses, while IL-33 promotes the suppressive function of Treg cells (Alvarez et al., 2019) and enhances IL-5 and IL-13-derived Th2 immunity. This IL-33-Th2 response resulted in increased fungal burdens and reduced survival in murine models (Flaczyk et al., 2013). A deleterious role for IL-33 has also been described during acute and chronic *A. fumigatus* disease. Mice deficient in IL-33 produced more IL-17A and IL-22 and displayed enhanced fungal clearance when challenged with acute *A. fumigatus* infection. Furthermore, blocking IL-1R4 in a model of *Aspergillus*-sensitized asthma improved airway hyperresponsiveness and fibrosis (Ramaprakash et al., 2011). In contrast, pre-treatment of mice with IL-33 prior to peritoneal and systemic *C. albicans* infection resulted in fungal clearance and improved survival. Here, IL-33 enhanced the recruitment of neutrophils and increased their killing capability while also mediating T-cell tolerance (Le et al., 2012; Tran et al., 2015).

Therapeutic Potential of the IL-1 Subfamily

The activity of IL-1α, IL-1β, and IL-33 must be carefully regulated. Protection from pathogen-derived and autoinflammatory diseases likely involves a balance of IL-1 subfamily agonist and antagonist activity. Excessive IL-1/Th17 signaling results in numerous diseases including asthma, chronic obstructive pulmonary disease (Gurczynski and Moore, 2018), inflammatory bowel disease (IBD), and psoriasis (Beringer et al., 2016). As such, the IL-1/Th17 pathway has been therapeutically

targeted. Anakinra blocks IL-1α/β activity and is used as a therapy in multiple inflammatory diseases (Dinarello et al., 2012). Similarly, targeting IL-1 signaling components is known to reduce inflammation and pathology in fungal-induced respiratory disease (Gresnigt et al., 2016; Griffiths et al., 2018; Godwin et al., 2019). Alongside Anakinra, numerous other IL-1 therapeutic strategies have been investigated. Examples include EBI-005, an IL-1R1 antagonist (Hou et al., 2013), Rilonacept (trade name Arcalyst), a decoy IL-1R1 (Economides et al., 2003), SL1067, a DNA aptamer that disrupts IL-1α (Ren et al., 2017), AF10847, a peptide inhibitor of IL-1R1 (Vigers et al., 2000), and AMG108, an antibody inhibitor of IL-1R1 (Cohen et al., 2011). IL-33 also has therapeutic potential and was effective in supporting immune defenses prior to *C. albicans* infection (Tran et al., 2015). The exogenous administration of IL-1 sub-family agonists or antagonists has potential to enhance protective anti-fungal immunity or resolve excessive, damaging immune responses. Another therapeutic route may involve the modulation of receptors or proteins that regulate IL-1 sub-family signaling. A clear link between CLRs and IL-1α/β signaling exists, as evidenced by CARD9-deficient patient's reduced IL-1α/β expression and susceptibility to fungal infection (Drummond et al., 2015). Furthermore, Dectin-1-induced IL-1β protected against mucosal *Candida* infections becoming systemic and lethal (Hise et al., 2009). In summary, modulating mediators of IL-1 sub-family induction, translation and processing may provide novel therapeutic targets in the fight against fungal infections.

THE IL-18 SUBFAMILY

Interleukin-18 was first described in 1989 as IFN-γ inducing factor before it was re-named when its function was described as pro-inflammatory (Dinarello, 2018). IL-18 and IL-1β share similar structures and signaling pathways, and are produced as inactive precursors requiring caspase-1 activation; however, the two cytokines are functionally distinct (Dinarello, 2019). IL-18 forms a low affinity signaling complex by binding IL-18Rα, the ligand for mature IL-18. A high affinity signaling complex is formed in cells that also express the co-receptor IL-18Rβ (Kaplanski, 2018). Similar to IL-1α/β, signal transduction requires the TIR domain and drives NF-κB and MAPK signaling (Hoshino et al., 1999; Wyman et al., 2002). IL-18 is regulated by IL-18 binding protein (IL-18BP), a soluble protein with high affinity for IL-18 (Krumm et al., 2014), and IL-37 which binds IL-18Rα and inhibits recruitment of IL-18Rβ. Moreover, IL-37 binding of IL-18Rα induces recruitment of IL-1R8 (also called SIGIRR), which induces anti-inflammatory signaling (Nakanishi, 2018).

IL-18 Subfamily Expression and Processing

Interleukin-18 is a pleiotropic cytokine that plays a central role in immunity (Novick et al., 2013). Like IL-1β, IL-18 is produced as an inactive precursor and must be processed by the inflammasome/caspase-1 complex in order to be activated and secreted (Franchi et al., 2009). However, unlike IL-1β, IL-18 is constitutively expressed by several hemopoietic and non-hemopoietic cells, including macrophages, dendritic cells, Kupffer cells, keratinocytes, osteoblasts, adrenal cortex cells, intestinal epithelial cells, microglial cells, and synovial fibroblasts (Gracie et al., 2003). Fas stimulation was able to induce secretion of biologically active IL-18 in caspase-1-deficient murine macrophages (Bossaller et al., 2012). In addition, proteases secreted by the inflammatory cell infiltrate contribute to inflammation via activation of IL-18 (Omoto et al., 2006, 2010). IL-18 can be secreted from monocytes and macrophages in its active form (Fantuzzi et al., 1999), or released from dying endothelial and epithelial cells in its precursor form before being activated outside the cell (Sugawara et al., 2001). IL-37 acts as an IL-18 antagonist and is found in many human tissues (Dinarello et al., 2016), although more specific roles for IL-37 reducing inflammation in the lung, spleen, plasma and in dendritic cells have been described (Nold et al., 2010). IL-37 is similar to IL-1α and IL-33, functioning as a dual-action cytokine that does not require activation (Li et al., 2015), however, caspase-1 processing was required for IL-37 to translocate to the nucleus to modulate transcription. IL-37 can be released in a processed or precursor form, resulting from exogenous administration of ATP (Bulau et al., 2014). IL-18BP also acts as an IL-18 antagonist and contains a signal peptide that directs secretion into the extracellular environment (Novick et al., 2001).

The Immunological Function of the IL-18 Subfamily

Following induction and activation, IL-18 signaling drives inflammation and promotes innate and adaptive immunity. Accordingly, IL-18 signaling can direct robust anti-pathogen immunity but is also associated with numerous autoinflammatory diseases. A clear role for IL-18 promoting Th1 immunity through IFN-γ induction has been described during models of viral and bacterial infection. In addition, IL-18BP is targeted by viruses to reduce host-protective IL-18 mediated immunity (Born et al., 2000; Reading and Smith, 2003). Mice deficient in IL-18 components were highly susceptible to viral and bacterial challenge (Mastroeni et al., 1999; Kinjo et al., 2002; Freeman et al., 2015). However, IL-18 can only induce IFN-γ and Th1 immunity in combination with IL-12 or IL-15 signaling; IL-18 signaling alone enhances Th2 immunity (Nakanishi et al., 2001), which is typically detrimental for fungal clearance. An IL-18 synergy primarily with IL-12 but also IL-15 exists with each cytokine modulating the others transcription, expression, and receptor expression (Fantuzzi et al., 1999). Recently, IL-18 has also been shown to drive IL-17 production from γδ T-cells and promote Th17 responses (Sutton et al., 2012), modulate adhesion molecules in endothelium (Carrascal et al., 2003), promote nitric oxide (NO) synthesis (critical for viral and bacterial killing) and enhance production of numerous chemokines (Kaplanski, 2018). IL-18 contributes to autoimmune disease including Type-1 diabetes, psoriasis, IBD, asthma and numerous myocardial and kidney diseases (Nakanishi et al., 2001; Garlanda et al., 2013). Here, increased expression of IL-18 and IL-18BP has been

observed suggesting that poor agonist/antagonist balance may result in disease. In agreement, the majority of IL-18 associated autoimmune diseases result from excess Th2 immune responses (Monteleone et al., 1999; Tanaka et al., 2001; Gerdes et al., 2002), potentially from IL-18 activity without IL-12 or IL-15.

Interleukin-37 has a broad anti-inflammatory effect limiting IL-18 responses. Reducing IL-37 in human cells increased the production of cytokines (IL-1β/TNF/IL-6) induced by IL-1 and TLRs (Nold et al., 2010). IL-37 is the only member of the IL-1 family not to have a mouse homolog, as such *in vivo* work is only possible with transgenic IL-37 models. Here, the anti-inflammatory effects of IL-37 have been demonstrated to reduce colitis, metabolic syndrome, acute lung injury, myocardial infarction, and asthma (Wu et al., 2014; Li et al., 2015). IL-18BP also acts to reduce IL-18 agonist activity and an imbalance of IL-18 and IL-18BP has been described in Wegener's granulomatosis and systemic lupus erythematosus (Novick et al., 2009, 2010). Administration of IL-18BP reduced inflammation in a model of rheumatoid arthritis; however, at high concentrations IL-18BP also bound IL-37 and the anti-inflammatory effect was lost (Banda et al., 2003). The functional implications of IL-18 signaling driving inflammation, innate and adaptive immune responses in autoimmune and anti-pathogen disease are clear. However, the induction, function, and balance of the IL-18 subfamily during fungal disease is less well explored.

Fungal Induction of the IL-18 Subfamily

Interleukin-18 is a crucial cytokine that mediates innate and adaptive immunity and likely plays a key role during fungal infection. The cytokine is expressed in cells of mesenchymal origin and hematopoietic cells and therefore may share functions with both IL-1α and IL-1β. In agreement, the induction of IL-18 has been observed during systemic and mucosal fungal infections. IL-18 was induced following acute *Aspergillus* challenge and during a chronic model of fungal-sensitized asthma (Cenci et al., 1997, 1998). A recent study revealed IL-18 expression increased rapidly following *Aspergillus* challenge peaking at 24 h before resolving over the next 48 h (Cheng et al., 2020). However, the signaling events leading to IL-18 induction in these models has not been defined. *Aspergillus* conidia frequently interact with barrier surfaces and systemic disease arises from germination in this setting. Understanding the induction of IL-18 as *Aspergillus* conidia persist, germinate, and promote disease may lead to important findings.

Interleukin-18 is also induced during *Candida* infection. Oral epithelial cells constitutively expressed IL-18 mRNA and precursor IL-18. During *C. albicans* challenge expression of IL-18 mRNA and pre-IL-18 was reduced while active IL-18 was released in a caspase-1 dependent manner (Rouabhia et al., 2002). A similar effect was seen in a model of human oral mucosa where *C. albicans* challenge resulted in active IL-18 expression. In agreement, patients with oral candidiasis possess increased levels of active IL-18 in saliva samples (Tardif et al., 2004). There is limited evidence describing the signaling events required for IL-18 induction in fungal disease. Recent investigation determined that Dectin-1 signaling and activation of the non-canonical NF-κB subunit RelB resulted in IL-18 induction (Shen et al., 2020); however, little else is known. Investigating the mechanism of IL-18 induction during mucosal and systemic *Candida* disease may provide insight into protective anti-*Candida* immunity. Aside from the induction of IL-18 during *Aspergillus* and *Candida* disease, IL-18 has been functionally implicated in *P. brasiliensis* infection (Panagio et al., 2008; Alves et al., 2018), *S. schenckii* infection (Goncalves et al., 2015), and *C. neoformans* infection (Kawakami et al., 2000b; Wang et al., 2011), suggesting at the very least a general role in host immune response. Whether IL-37 and IL-18BP are induced during fungal disease currently unclear; however, increased serum IL-37 was identified in PCM patients with severe disease (Alves et al., 2018).

The Role of the IL-18 Subfamily in Fungal Immunology

While the induction of IL-18 requires further investigation, the functional consequences of IL-18 signaling during fungal disease highlight the importance of this IL-1 subfamily member. During acute *Aspergillus* lung infection, IL-18 promoted protective immunity and enhanced Th1 immunity and neutrophil recruitment in concert with IL-12 and IFN-γ (Blease et al., 2001). Skewing toward Th2 immunity during acute *Aspergillus* infection is non-protective and promotes chronic disease (Cenci et al., 1998). A study of acute *Aspergillus* infection determined 72 h after infection IL-18 mediates protection independently of IFN-γ, suggesting that the IL-18/IFN-γ axis occurs rapidly and IL-18 continues to mediate immunity independently of IFN-γ if the infection persists (Brieland et al., 2001). However, in immunocompromised models, IFN-γ was essential throughout infection and exogenous administration of IFN-γ was consistently protective (Nagai et al., 1995; Cenci et al., 1997, 1998). In an *Aspergillus*-sensitized asthma model, IL-18 was again found to be protective and acted without IL-12 or IFN-γ to enhance *Aspergillus* clearance. Depleting IL-18 in this model increased fungal burden and resulted in persistent airway hyperactivity and fibrosis (Blease et al., 2001). These results suggest IL-18 is protective during both acute and chronic *Aspergillus* disease but provides protection through distinct mechanisms.

Interleukin-18 is also vital for protection during *Candida* infection. Similar to *Aspergillus* infection, IL-18 induced protective Th1 immunity against *Candida*. Mice deficient in caspase-1 displayed reduced Th1 responses and were susceptible to *Candida* challenge. Here, the exogenous administration of IL-18, without IL-1β, restored Th1 responses and protection (Mencacci et al., 2000). In addition, the exogenous administration of IL-18BP reduced IFN-γ-derived Th1 immunity in human whole blood cultures (Netea et al., 2002) and in mice (Fantuzzi et al., 1999). In agreement, administration of anti-IL-18 antibodies prior to systemic *Candida* infection depleted IFN-γ responses and enhanced fungal disease (Stuyt et al., 2002), whereas increasing IL-18 enhanced IFN-γ and Th1 responses which ultimately promoted protection (Stuyt et al., 2004). Interestingly, the role for IL-18 mediating neutrophil recruitment is unclear with one study suggesting IL-18 is uncoupled from neutrophil recruitment (Netea et al., 2003).

This aligns with the protective role of IL-18 during VVC, where neutrophil recruitment enhances disease. VVC disease results from unrestrained NLRP3 activation/continuous IL-1β stimulation and is regulated by the IL-22/NLR4C axis. In this setting, IL-18 acts in a cross-circuit with IL-22 with both cytokines regulating each other and reducing NLRP3 activity (Borghi et al., 2019). IL-18 appears to be broadly protective during *Candida* infection, although, as was found with *Aspergillus*, IL-18 acts through many distinct mechanisms to promote immunity.

Interleukin-18 signaling has also been described during *C. neoformans* and *P. brasiliensis* infection. IL-18 deficient mice exhibit increased *C. neoformans* fungal burdens and reduced IFN-γ/IL-12 responses (Kawakami et al., 2000a,b). In addition, IL-18R deficient mice were more susceptible to *C neoformans* than IL-1R deficient mice, suggesting IL-18 and not IL-1α/β signaling mediates *C. neoformans* immunity (Wang et al., 2011). While IFN-γ and Th1 immunity are vital during PCM, the role of IL-18 is controversial. IL-18 deficient mice on a BALB/c background were protected from PCM challenge and displayed increased survival and reduced fungal burden (Panagio et al., 2008). In contrast, IL-18 deficient mice on a C57BL/6 background were susceptible to PCM and displayed enhanced fungal burdens (Ketelut-Carneiro et al., 2015). In patients with PCM, increased IL-18 in serum correlated with more severe forms of disease (Corvino et al., 2007), suggesting the C57BL/6 mouse model may be more appropriate.

A role for IL-37 has been described in a murine model of pulmonary aspergillosis. Here, administration of IL-37 decreased NLRP3 activity and IL-1β expression through the SIGIRR signaling pathway and resulted in reduced inflammatory cell recruitment. This reduced tissue damage during acute *Aspergillus* infection, and dampened adaptive responses in chronic *Aspergillus* infections (Moretti et al., 2014). SIGIRR signaling has been described to prevent lethal dysregulated IL-1 dependent Th17 responses in fungal disease (Warris et al., 2005). The induction and function of IL-18BP and IL-37 during fungal disease requires investigation but these antagonists may provide important functions mediating IL-18 signaling, enhancing immune responses, and resolving inflammatory effect. Numerous viruses target IL-18BP as an immune evasion strategy; whether fungi can do the same would be interesting to determine.

Therapeutic Potential of the IL-18 Subfamily

The exogenous administration of IL-18 enhances immunity in systemic models of *C. albicans* infection. This strategy may also provide protection during *Aspergillus* and *Cryptococcus* infection where IL-18 responses also confer protection. IL-18 therapy may be targeted at barrier sites or systemically once any difference in IL-18 induction and function at these two sites is determined. Although the exogenous administration of IL-18BP and anti-IL-18 antibodies enhanced acute fungal susceptibility, both these IL-18 depleting strategies promote the resolution of autoinflammatory disease and improve chronic autoimmune disease (Kanai et al., 2001; Sivakumar et al., 2002). Although no IL-18 therapeutics are currently licensed, IL-18BP therapy was examined in rheumatoid arthritis and psoriasis patients with positive tolerance and safety profiles (Tak et al., 2006). IL-37 has clear therapeutic potential as the administration of IL-37 during *Aspergillus* infection promoted beneficial inflammatory resolution in both acute and chronic disease (Moretti et al., 2014).

THE IL-36 SUBFAMILY

Interleukin-36 is a recent addition to the IL-1 superfamily that was discovered and characterized 20 years ago. Initially, IL-36 was thought to be similar to IL-1 as the two members of the IL-1 family shared similar gene sequences, exon-intron arrangements and predicted protein structure (Smith et al., 2000). Intriguingly, however, these new IL-36 cytokines were unable to bind IL-1R or any known orphan receptors in the IL-1 superfamily (O'Neill and Dinarello, 2000). Shortly after, two studies determined that IL-36 cytokines signal through a complex of IL-36R and IL-1RAcP leading to NF-κB and MAPK activation, and IL-6 and IL-8 production (Debets et al., 2001; Towne et al., 2004). We now know the IL-36 subfamily is comprised of four IL-36 isoforms, three agonists IL-36α, IL-36β, and IL-36γ driving proinflammatory functions (Towne et al., 2004), and the IL-36Ra antagonist mediating inflammation (Debets et al., 2001). It is worth noting that although the IL-36 subfamily was renamed in 2010, the previous nomenclature is still frequently encountered. IL-36α, IL-36β, IL-36γ, and IL-36Ra were known as IL-1F6, IL-1F8, IL-1F9, and IL-1F5, respectively, and IL-36R was named IL-1Rrp2 (Dinarello et al., 2010). IL-38 also belongs to the IL-36 sub-family, signals through IL-36R and functions as a receptor antagonist similar to IL-36Ra (van de Veerdonk et al., 2012).

IL-36 Subfamily Expression and Processing

The IL-36 subfamily plays a key role driving immune responses at mucosal barriers in the skin, respiratory tract and intestine and are the only cytokines constitutively expressed in epithelium (D'Erme et al., 2015). This location-specific expression is not found with IL-1 or IL-18 (Gresnigt and van de Veerdonk, 2013). IL-36 agonist expression is predominantly restricted to epithelial cells (Towne et al., 2004; Blumberg et al., 2007), although expression has also been observed in macrophages, dendritic cells and monocytes (Smith et al., 2000; Vigne et al., 2011; Mutamba et al., 2012; Boutet et al., 2016). IL-36R transcripts are highly prevalent in keratinocytes and epithelial cell types (Kumar et al., 2000; Towne et al., 2004), and have been found in naïve CD4 + T-cell subsets (Vigne et al., 2012), monocytes and dendritic cells (Vigne et al., 2011). IL-36 agonists bind the same receptor complex and are expressed in similar cells; adding each IL-36 agonist to keratinocytes resulted in similar immunological outcomes (Swindell et al., 2018). However, certain isoforms have been described in specific disease settings such as IL-36α in arthritis (Frey et al., 2013) and IL-36γ in psoriasis (D'Erme et al., 2015), with the blocking of IL-36γ achieving a reduction in psoriasis-associated inflammation in a

3D skin model (Todorovic et al., 2019). Whether there is isoform redundancy or whether each isoform has its own role, potentially associated with location or stimulus, remains unclear.

Interleukin-36 cytokines, like all members of the IL-1 family, are expressed without a signal peptide and are not secreted via the classical secretory pathway (Rubartelli et al., 1990). Unlike IL-1 and IL-18, IL-36 cytokines do not possess a leading peptide sequence required for caspase-1 cleavage and are therefore regulated independently of the inflammasome (Barton et al., 2000). However, a role for IL-36 cytokines facilitating activation of the NLRP3 inflammasome has been described (Chi et al., 2017). IL-36 cytokines are produced as inactive, full-length proteins that must undergo N-terminal truncation 9 amino acid residues upstream of a conserved A-X-D motif for biological activity. This precise cleavage of IL-36 cytokines increased their receptor affinity over 10,000-fold (Towne et al., 2011). It is thought that IL-36 expression is regulated by epidermal growth factor receptor (EGFR) signaling (Satoh et al., 2020) and positive feedback loops associated with Th17 cytokines (Carrier et al., 2011). There has been a recent focus on proteases that can cleave and activate IL-36 cytokines. Interestingly, blocking these proteases in inflammatory disease may have therapeutic potential. The neutrophil-derived proteases elastase and proteinase 3 appear to cleave IL-36 agonist cytokines, although their non-specific protease activity rarely activated IL-36. While neutrophils are not abundantly resident at mucosal barrier surfaces, neutrophil elastase was able to specifically activate IL-36Ra (Macleod et al., 2016), suggesting neutrophils may mediate inflammation once recruited. A clear role for the cysteine protease cathepsin S activating IL-36 agonists has also been described. Crucially, cathepsin S is found in fibroblasts and keratinocytes and its activity was increased in psoriatic lesions (Ainscough et al., 2017). IL-38 lacks a signal peptide and caspase-1 cleavage site, suggesting that, unlike IL-36Ra, activation is not required. IL-38 is expressed mostly in the skin but has also been found in B-cells (Lin et al., 2001).

The Immunological Function of the IL-36 Subfamily

Following their induction and activation, IL-36 signaling has potent effects on barrier immunity and can lead to protective responses against pathogens or drive autoinflammatory disease. IL-36 agonist signaling leads to activation of MAPK and NF-κB pathways (He et al., 2013). Downstream this results in anti-microbial peptide release from keratinocytes (Nguyen et al., 2012), increased recruitment and maturation of myeloid cells (Foster et al., 2014), increased macrophage phagocytosis and microbial killing (Tao et al., 2017), and the robust production of IL-6, IL-8, TNF, CCL3, CCL4, CCL5, CCL20, CXCL1, CXCL2, and CXCL8 (Carrier et al., 2011; Ramadas et al., 2012; Foster et al., 2014; Dietrich et al., 2016). It is likely a finely tuned balance of IL-36 agonist and antagonist activity promotes protective immunity. This is evidenced in generalized pustular psoriasis (the most severe form of psoriasis) where patients lack IL-36Ra due to a missense mutation in the IL-36Ra gene. Furthermore, IL-36Ra deficiency is associated with systemic inflammation, suggesting that uncontrolled IL-36 agonist signaling has systemic effect (Marrakchi et al., 2011).

The IL-36 subfamily also bridges innate and adaptive immunity. IL-36α/γ secreted from immune and epithelial cells directly acts on $CD4^+$ T-cells and results in the release of IL-36β, which through a feedback loop promoted IL-2 secretion, T-cell expansion, and Th1 differentiation (Vigne et al., 2012). More recently, IL-36 gene expression has been associated with Th17 immunity. Here, IL-36 agonist cytokines regulated their own expression and drove the expression and function of Th17 cytokines and immunity (Carrier et al., 2011). While our knowledge of IL-36 is increasing, little information exists about the function of IL-38. The induction of IL-38 occurs in apoptotic cells to limit inflammation (Mora et al., 2016). However, as yet no induction of IL-38 in disease settings has been described apart from the inhibition of *Candida*-induced Th17 immunity in a similar manner to IL-36Ra (van de Veerdonk et al., 2012).

Fungal Induction of the IL-36 Subfamily

Although there is good evidence describing the function of the IL-36 subfamily, there is limited evidence for the induction and function of IL-36 during fungal infection. Given the important barrier function of IL-36, these cytokines likely mediate interactions with both commensal and pathogenic fungi. *C. albicans* induced IL-36γ expression in human keratinocytes (Braegelmann et al., 2018), IL-36α/γ in TR146 cells (a human epithelial cell line), and all IL-36 agonists during an *in vivo* OPC model (Verma et al., 2018). IL-36α/γ expression was significantly increased within 24 h of OPC challenge, while IL-36β increased at 48 h. The induction of IL-36 following *Candida* challenge was dependent on candidalysin, with a candidalysin-null *Candida* strain inducing drastically reduced IL-36 expression (Verma et al., 2018). The induction of IL-36 during systemic *Candida* infection has yet to be demonstrated. However, systemic clinical infections typically arise from disrupted barrier integrity. Replicating this *in vivo* is challenging and systemic infections are achieved through intravenous injection.

The signaling events that lead to IL-36 induction following *Candida* infection have been investigated using the OPC model. Multiple signaling pathways are activated during OPC including MAPK, PI3K, and NF-κB (Moyes et al., 2010, 2014; Verma et al., 2018) and blocking p38-MAPK but not JNK-MAPK or ERK1/2 MAPK impaired IL-36α/γ expression (Verma et al., 2018). Further investigation revealed that blocking c-Fos impaired IL-36α/γ expression, reducing c-Jun increased IL-36α expression without effecting IL-36γ. In addition, when the MAPK phosphatase MKP1 (which negatively regulates p38-MAPK and JNK-MAPK) was knocked down, IL-36α/γ expression increased (Verma et al., 2018). While these results suggest p38 MAPK induces IL-36 expression and this is negatively regulated by MKP1, MAPK pathways did not fully account for IL-36 expression. Here, while NF-κB was able to mediate some IL-36α/γ expression, blocking PI3K reduced IL-36α/γ to resting levels. These data suggest significant roles for p38-MAPK, NF-KB and PI3K in inducing IL-36 expression, with PI3K playing the most prominent role (Verma et al., 2018).

Aspergillus fumigatus infection also resulted in IL-36 induction, interestingly in a morphology and-time dependent manner. IL-36γ was induced in human peripheral blood mononuclear cells (PBMCs) following incubation with live conidia and heat-killed hyphae, while IL-36Ra was induced following incubation with live conidia, heat-killed conidia and live hyphae. As expected, IL-36α was not induced in PBMCs (Gresnigt et al., 2013). The induction of IL-36γ during *Aspergillus* challenge was dependent on Dectin-1 Syk signaling and TLR4 (Gresnigt et al., 2013). Dectin-1 and TLR4 signaling results in NF-κB activation which, as with *Candida*, controlled IL-36α/γ expression. The CLR Dectin-2 signals through PI3K and has an important role driving Th17 immunity against *Candida* (Saijo et al., 2010; Lee et al., 2016). Aside from *Candida* and *Aspergillus*, only *Trichophyton mentagrophytes* has been shown to induce IL-36 expression (Braegelmann et al., 2018). Although not extensively investigated, the induction of IL-36 cytokines appears to be cell-type, fungal morphology and time-dependent.

The Role of the IL-36 Subfamily in Fungal Immunology

Following their induction, the role of IL-36 cytokines during fungal infection is poorly understood. IL-36 can be solely responsible for inflammatory disease and clearly has potent immunological effects. Therefore, following fungal induction of IL-36, the cytokines likely mediate important immunological responses. In agreement with this, IL-36 is protective during mucosal *Candida* disease. IL-36R deficient mice when challenged with OPC displayed increased fungal burden and reduced IL-23 expression (Verma et al., 2018). IL-36 also induces IL-23 in macrophages isolated from psoriasis patients, suggesting a consistent link between the two cytokines (Bridgewood et al., 2018). IL-23 drives the proliferation and survival of Th17 cells vital for anti-*Candida* immunity. Mice deficient in IL-23 (IL-23p19-/-) experienced severe OPC disease associated with a lack of neutrophil recruitment and anti-microbial peptides (Conti et al., 2009). It was thought IL-1 and IL-36 worked in tandem to enhance protective Th17 immunity (Verma et al., 2017); however, IL-36R deficient mice had normal IL-17 gene expression suggesting a distinct, unconnected role for each (Verma et al., 2018). As such, the IL-36/IL-23 axis may complement the IL-1/Th17 response through an uncoupled mechanism (Verma et al., 2018). This contrasts with *Aspergillus* infection which showed blockade of IL-36R with IL-36Ra reduced IL-17 and IFN-γ responses (Gresnigt et al., 2013).

The role of IL-36 during *Candida* infection appears to be tightly linked with candidalysin activity, which was required for IL-36 induction (Verma et al., 2018). Commensal (yeast) *Candida* does not produce candidalysin and subsequently does not initiate inflammation. Instead, candidalysin is expressed when *Candida* becomes invasive (through hypha formation) and results in inflammation and the loss of barrier integrity (Moyes et al., 2016). Here, IL-36 signaling may facilitate host discrimination between commensal and pathogenic *Candida*. Furthermore, excessive IL-36 signaling is damaging and leads to inflammatory disease. Thus, *Candida* may induce IL-36 to promote inflammation, disrupt barrier integrity and enhance disease. This may potentially explain why psoriasis patients are particularly susceptible to *Candida* infection (Pietrzak et al., 2018).

The function of IL-36 during *Aspergillus* infection has been less well explored. IL-36γ is induced following *A. fumigatus* infection in a Dectin-1 Syk dependent manner. In support of this, Dectin-1 deficient mice produced defective Th17 immune responses (LeibundGut-Landmann et al., 2007) and are highly susceptible to *Aspergillus* lung infection (Werner et al., 2009). Many systemic fungal infections are acquired across mucosal surfaces where IL-36 induction has potent effects. Understanding the function of IL-36 signaling and the balance of protective and excessive responses may provide valuable therapeutic targets to mediate barrier inflammation and integrity.

Therapeutic Potential of the IL-36 Subfamily

Modulating IL-36 signaling has been investigated with some early success. Recently, a small molecule inhibitor of IL-36γ successfully attenuated IL-36γ induced responses (Todorovic et al., 2019). A phase 1 study in generalized pustular psoriasis patients has been completed showing a monoclonal antibody against IL-36R rapidly reduced patient pustules and psoriasis severity score (Bachelez et al., 2019). Anti-IL-23 antibodies have also been trialed with success in psoriasis patients (Reich et al., 2011). While therapeutically inhibiting IL-36 signaling may produce rapid disease improvement in autoimmune settings, IL-36 has a protective role during infectious disease and a careful balance of IL-36 signaling must be achieved.

DISCUSSION

The IL-1 family of cytokines are central to immunity and health. It is no surprise that this family plays a crucial role during fungal infection and in determining fungal disease outcomes. Lacking IL-1 agonist activity during acute fungal disease is often severely detrimental for the immunocompromised host, resulting in fungal growth and dissemination. However, excessive IL-1 family agonist activity, either through over expression/activation or through a lack of antagonist activity, can be equally destructive. Excessive IL-1 family signaling is associated with numerous inflammatory disorders and in the context of fungal infection, can exacerbate chronic disease and lead to barrier disruption and fungal dissemination. Therefore, potent IL-1 signaling must be carefully regulated through balancing levels of protease activation and antagonist activity, to successfully promote protection and immunity.

Understanding of the functional role, mechanism of induction and downstream regulators of each IL-1 family member would greatly improve our knowledge of anti-fungal immunity. Here, we have reviewed the effects of different IL-1 family members that provide action at various locations and in response to multiple stimuli. We graphically summarize our current understanding of this topic in **Figure 2**. It is also important that our investigations

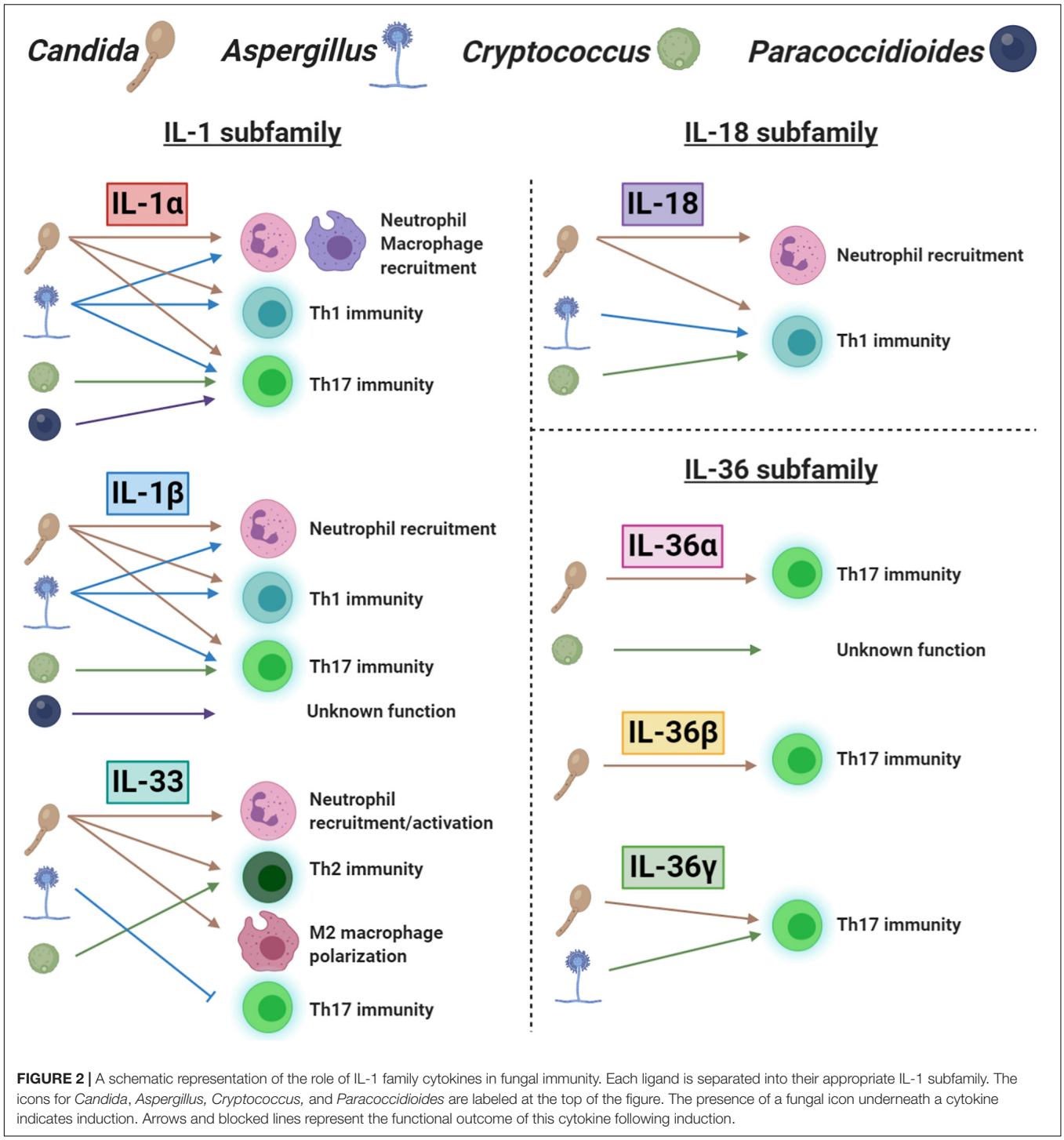

FIGURE 2 | A schematic representation of the role of IL-1 family cytokines in fungal immunity. Each ligand is separated into their appropriate IL-1 subfamily. The icons for *Candida*, *Aspergillus*, *Cryptococcus*, and *Paracoccidioides* are labeled at the top of the figure. The presence of a fungal icon underneath a cytokine indicates induction. Arrows and blocked lines represent the functional outcome of this cytokine following induction.

of IL-1 family members consider collaboration and redundancy that occurs throughout the IL-1 family. Although individual IL-1 family members appear to have clearly defined roles in specific locations and disease settings, the interaction between IL-1 family signaling likely contributes to the overall immune response and disease outcome. While enhancing individual IL-1 family agonists or antagonists to promote disease resolution is therapeutically effective, targeting multiple members at once may provide the best outcome. Furthermore, while the exogenous administration of IL-1 family members is being investigated with some success, targeting the receptors or enzymes that drive IL-1 family induction and activation is another strategy that may provide therapeutic benefit.

As fungal diseases become an increasingly severe worldwide burden contributing to millions of deaths per year, the extensive use of immune-modulating therapies also continues

to increase. Current therapies are inadequate, toxic, highly drug interactive, and frequently encounter resistance. In addition, there is no current fungal vaccine available for use. Therefore, immunotherapies that enhance anti-fungal immunity will be a vital component of future anti-fungal therapies.

AUTHOR CONTRIBUTIONS

JG conceptualized and wrote the manuscript. GC contributed sections and produced figures. NK and JH contributed sections. JR and JN edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Wellcome Trust (214229_Z_18_Z), Biotechnology and Biological Sciences Research Council (BB/N014677/1), National Institutes of Health (R37-DE022550), and the NIH Research at Guys and St. Thomas's NHS Foundation Trust and the King's College London Biomedical Research Centre (IS-BRC-1215-20006).

ACKNOWLEDGMENTS

The figures were created using BioRender.

REFERENCES

Ainscough, J. S., Macleod, T., McGonagle, D., Brakefield, R., Baron, J. M., Alase, A., et al. (2017). Cathepsin S is the major activator of the psoriasis-associated proinflammatory cytokine IL-36γ. *Proc. Natl. Acad. Sci. U S A.* 114, E2748–E2757. doi: 10.1073/pnas.1620954114

Ali, S., Mohs, A., Thomas, M., Klare, J., Ross, R., Schmitz, M. L., et al. (2011). The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription. *J. Immunol.* 187, 1609–1616. doi: 10.4049/jimmunol.1003080

Altmeier, S., Toska, A., Sparber, F., Teijeira, A., Halin, C., and LeibundGut-Landmann, S. (2016). IL-1 Coordinates the neutrophil response to C. albicans in the Oral Mucosa. *PLoS Pathog.* 12:e1005882. doi: 10.1371/journal.ppat.1005882

Alvarez, F., Istomine, R., Shourian, M., Pavey, N., Al-Aubodah, T. A., Qureshi, S., et al. (2019). The alarmins IL-1 and IL-33 differentially regulate the functional specialisation of Foxp3(+) regulatory T cells during mucosal inflammation. *Mucosal Immunol.* 12, 746–760. doi: 10.1038/s41385-019-0153-155

Alves, A., David, M. A., de Castro, L. F., da Silva, R. M., Longhi, L. N. A., Blotta, M., et al. (2018). Differential production of interleukin-1 family cytokines (IL-1beta, IL-18, IL-33 and IL-37) in patients with paracoccidioidomycosis: correlation with clinical form and antifungal therapy. *Med. Mycol.* 56, 332–343. doi: 10.1093/mmy/myx050

Bachelez, H., Choon, S. E., Marrakchi, S., Burden, A. D., Tsai, T. F., Morita, A., et al. (2019). Inhibition of the interleukin-36 pathway for the treatment of generalized pustular psoriasis. *N. Engl. J. Med.* 380, 981–983. doi: 10.1056/NEJMc1811317

Banda, N. K., Vondracek, A., Kraus, D., Dinarello, C. A., Kim, S. H., Bendele, A., et al. (2003). Mechanisms of inhibition of collagen-induced arthritis by murine IL-18 binding protein. *J. Immunol.* 170, 2100–2105. doi: 10.4049/jimmunol.170.4.2100

Barousse, M. M., Van Der Pol, B. J., Fortenberry, D., Orr, D., et al. (2004). Vaginal yeast colonisation, prevalence of vaginitis, and associated local immunity in adolescents. *Sex Transm. Infect.* 80, 48–53. doi: 10.1136/sti.2002.003855

Barton, J. L., Herbst, R., Bosisio, D., Higgins, L., and Nicklin, M. J. (2000). A tissue specific IL-1 receptor antagonist homolog from the IL-1 cluster lacks IL-1, IL-1ra, IL-18 and IL-18 antagonist activities. *Eur. J. Immunol.* 30, 3299–3308. doi: 10.1002/1521-4141(200011)30:11<3299::aid-immu3299>3.0.co;2-s

Beringer, A., Noack, M., and Miossec, P. (2016). IL-17 in chronic inflammation: from discovery to targeting. *Trends Mol. Med.* 22, 230–241. doi: 10.1016/j.molmed.2016.01.001

Bessa, J., Meyer, C. A., de Vera Mudry, M. C., Schlicht, S., Smith, S. H., Iglesias, A., et al. (2014). Altered subcellular localization of IL-33 leads to non-resolving lethal inflammation. *J. Autoimmun.* 55, 33–41. doi: 10.1016/j.jaut.2014.02.012

Bishu, S., Hernández-Santos, N., Simpson-Abelson, M. R., Huppler, A. R., Conti, H. R., Ghilardi, N., et al. (2014). The adaptor CARD9 is required for adaptive but not innate immunity to oral mucosal *Candida albicans* infections. *Infect. Immun.* 82, 1173–1180. doi: 10.1128/IAI.01335-1313

Blease, K., Kunkel, S. L., and Hogaboam, C. M. (2001). IL-18 modulates chronic fungal asthma in a murine model; putative involvement of Toll-like receptor-2. *Inflamm. Res.* 50, 552–560. doi: 10.1007/PL00000233

Blumberg, H., Dinh, H., Trueblood, E. S., Pretorius, J., Kugler, D., Weng, N., et al. (2007). Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation. *J. Exp. Med.* 204, 2603–2614. doi: 10.1084/jem.20070157

Borghi, M., De Luca, A., Puccetti, M., Jaeger, M., Mencacci, A., Oikonomou, V., et al. (2015). Pathogenic NLRP3 inflammasome activity during candida infection is negatively regulated by IL-22 via activation of NLRC4 and IL-1Ra. *Cell Host Microbe.* 18, 198–209. doi: 10.1016/j.chom.2015.07.004

Borghi, M., Pariano, M., Solito, V., Puccetti, M., Bellet, M. M., Stincardini, C., et al. (2019). Targeting the Aryl hydrocarbon receptor with Indole-3-Aldehyde protects from vulvovaginal candidiasis via the IL-22-IL-18 cross-Talk. *Front. Immunol.* 10:2364. doi: 10.3389/fimmu.2019.02364

Born, T. L., Morrison, L. A., Esteban, D. J., VandenBos, T., Thebeau, L. G., Chen, N., et al. (2000). A poxvirus protein that binds to and inactivates IL-18, and inhibits NK cell response. *J. Immunol.* 164, 3246–3254. doi: 10.4049/jimmunol.164.6.3246

Bossaller, L., Chiang, P. I., Schmidt-Lauber, C., Ganesan, S., Kaiser, W. J., Rathinam, V. A., et al. (2012). Cutting edge: FAS (CD95) mediates noncanonical IL-1beta and IL-18 maturation via caspase-8 in an RIP3-independent manner. *J. Immunol.* 189, 5508–5512. doi: 10.4049/jimmunol.1202121

Boutet, M. A., Bart, G., Penhoat, M., Amiaud, J., Brulin, B., Charrier, C., et al. (2016). Distinct expression of interleukin (IL)-36alpha, beta and gamma, their antagonist IL-36Ra and IL-38 in psoriasis, rheumatoid arthritis and Crohn's disease. *Clin. Exp. Immunol.* 184, 159–173. doi: 10.1111/cei.12761

Braegelmann, J., Braegelmann, C., Bieber, T., and Wenzel, J. (2018). Candida induces the expression of IL-36gamma in human keratinocytes: implications for a pathogen-driven exacerbation of psoriasis? *J. Eur. Acad. Dermatol. Venereol.* 32, e403–e406. doi: 10.1111/jdv.14994

Bridgewood, C., Fearnley, G. W., Berekmeri, A., Laws, P., Macleod, T., Ponnambalam, S., et al. (2018). IL-36γ is a strong inducer of IL-23 in psoriatic cells and activates angiogenesis. *Front. Immunol.* 9:200. doi: 10.3389/fimmu.2018.00200

Brieland, J. K., Jackson, C., Menzel, F., Loebenberg, D., Cacciapuoti, A., Halpern, J., et al. (2001). Cytokine networking in lungs of immunocompetent mice in response to inhaled *Aspergillus fumigatus*. *Infect. Immun.* 69, 1554–1560. doi: 10.1128/IAI.69.3.1554-1560.2001

Brown, G. D., Denning, D. W., Gow, N. A., Levitz, S. M., Netea, M. G., and White, T. C. (2012). Hidden killers: human fungal infections. *Sci. Transl. Med.* 4:165rv13. doi: 10.1126/scitranslmed.3004404

Bulau, A. M., Nold, M. F., Li, S., Nold-Petry, C. A., Fink, M., Mansell, A., et al. (2014). Role of caspase-1 in nuclear translocation of IL-37, release of the cytokine, and IL-37 inhibition of innate immune responses. *Proc. Natl. Acad. Sci. U S A.* 111, 2650–2655. doi: 10.1073/pnas.1324140111

Caffrey, A. K., Lehmann, M. M., Zickovich, J. M., Espinosa, V., Shepardson, K. M., Watschke, C. P., et al. (2015). IL-1alpha signaling is critical for leukocyte recruitment after pulmonary *Aspergillus fumigatus* challenge. *PLoS Pathog.* 11:e1004625. doi: 10.1371/journal.ppat.1004625

Caffrey-Carr, A. K., Kowalski, C. H., Beattie, S. R., Blaseg, N. A., Upshaw, C. R., Thammahong, A., et al. (2017). Interleukin 1alpha is critical for resistance

against highly virulent aspergillus fumigatus isolates. *Infect. Immun.* 85:e00661-17. doi: 10.1128/IAI.00661-617

Camilli, G., Griffiths, J. S., Ho, J., Richardson, J. P., and Naglik, J. R. (2020). Some like it hot: *Candida activation* of inflammasomes. *PLoS Pathog.* 16:e1008975. doi: 10.1371/journal.ppat.1008975

Carrascal, M. T., Mendoza, L., Valcarcel, M., Salado, C., Egilegor, E., Telleria, N., et al. (2003). Interleukin-18 binding protein reduces b16 melanoma hepatic metastasis by neutralizing adhesiveness and growth factors of sinusoidal endothelium. *Cancer Res.* 63, 491–497.

Carrier, Y., Ma, H. L., Ramon, H. E., Napierata, L., Small, C., O'Toole, M., et al. (2011). Inter-regulation of Th17 cytokines and the IL-36 cytokines in vitro and in vivo: implications in psoriasis pathogenesis. *J. Invest. Dermatol.* 131, 2428–2437. doi: 10.1038/jid.2011.234

Castro, M., Bjoraker, J. A., Rohrbach, M. S., and Limper, A. H. (1996). Candida albicans induces the release of inflammatory mediators from human peripheral blood monocytes. *Inflammation* 20, 107–122. doi: 10.1007/BF01487749

Cenci, E., Mencacci, A., Fe, d'Ostiani, C., Del Sero, G., Mosci, P., et al. (1998). Cytokine- and T helper-dependent lung mucosal immunity in mice with invasive pulmonary aspergillosis. *J. Infect. Dis.* 178, 1750–1760. doi: 10.1086/314493

Cenci, E., Perito, S., Enssle, K. H., Mosci, P., Latge, J. P., Romani, L., et al. (1997). Th1 and Th2 cytokines in mice with invasive aspergillosis. *Infect. Immun.* 65, 564–570. doi: 10.1128/IAI.65.2.564-570.1997

Chen, C. J., Kono, H., Golenbock, D., Reed, G., Akira, S., and Rock, K. L. (2007). Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat. Med.* 13, 851–856. doi: 10.1038/nm1603

Cheng, Z., Li, M., Wang, Y., Chai, T., Cai, Y., and Li, N. (2020). Pathogenicity and immune responses of *Aspergillus fumigatus* infection in chickens. *Front. Vet. Sci.* 7:143. doi: 10.3389/fvets.2020.00143

Chi, H. H., Hua, K. F., Lin, Y. C., Chu, C. L., Hsieh, C. Y., Hsu, Y. J., et al. (2017). IL-36 signaling facilitates activation of the NLRP3 inflammasome and IL-23/IL-17 axis in renal inflammation and fibrosis. *J. Am. Soc. Nephrol.* 28, 2022–2037. doi: 10.1681/ASN.2016080840

Cohen, I., Rider, P., Carmi, Y., Braiman, A., Dotan, S., White, M. R., et al. (2010). Differential release of chromatin-bound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. *Proc. Natl. Acad. Sci. U S A.* 107, 2574–2579. doi: 10.1073/pnas.0915018107

Cohen, P. (2014). The TLR and IL-1 signalling network at a glance. *J. Cell Sci.* 127:2383. doi: 10.1242/jcs.149831

Cohen, S. B., Proudman, S., Kivitz, A. J., Burch, F. X., Donohue, J. P., Burstein, D., et al. (2011). A randomized, double-blind study of AMG 108 (a fully human monoclonal antibody to IL-1R1) in patients with osteoarthritis of the knee. *Arthritis Res. Ther.* 13:R125. doi: 10.1186/ar3430

Conti, H. R., Shen, F., Nayyar, N., Stocum, E., Sun, J. N., Lindemann, M. J., et al. (2009). Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J. Exp. Med.* 206, 299–311. doi: 10.1084/jem.20081463

Corvino, C. L., Mamoni, R. L., Fagundes, G. Z., and Blotta, M. H. (2007). Serum interleukin-18 and soluble tumour necrosis factor receptor 2 are associated with disease severity in patients with paracoccidioidomycosis. *Clin. Exp. Immunol.* 147, 483–490. doi: 10.1111/j.1365-2249.2006.03308.x

Cunha, C., Di Ianni, M., Bozza, S., Giovannini, G., Zagarella, S., Zelante, T., et al. (2010). Dectin-1 Y238X polymorphism associates with susceptibility to invasive aspergillosis in hematopoietic transplantation through impairment of both recipient- and donor-dependent mechanisms of antifungal immunity. *Blood* 116, 5394–5402. doi: 10.1182/blood-2010-04-279307

Debets, R., Timans, J. C., Homey, B., Zurawski, S., Sana, T. R., Lo, S., et al. (2001). Two novel IL-1 family members, IL-1 delta and IL-1 epsilon, function as an antagonist and agonist of NF-kappa B activation through the orphan IL-1 receptor-related protein 2. *J. Immunol.* 167, 1440–1446. doi: 10.4049/jimmunol.167.3.1440

D'Erme, A. M., Wilsmann-Theis, D., Wagenpfeil, J., Holzel, M., Ferring-Schmitt, S., Sternberg, S., et al. (2015). IL-36gamma (IL-1F9) is a biomarker for psoriasis skin lesions. *J. Invest. Dermatol.* 135, 1025–1032. doi: 10.1038/jid.2014.532

Di Paolo, N. C., and Shayakhmetov, D. M. (2016). Interleukin 1alpha and the inflammatory process. *Nat. Immunol.* 17, 906–913. doi: 10.1038/ni.3503

Dietrich, D., Martin, P., Flacher, V., Sun, Y., Jarrossay, D., Brembilla, N., et al. (2016). Interleukin-36 potently stimulates human M2 macrophages, langerhans cells and keratinocytes to produce pro-inflammatory cytokines. *Cytokine* 84, 88–98. doi: 10.1016/j.cyto.2016.05.012

Dinarello, C., Arend, W., Sims, J., Smith, D., Blumberg, H., O'Neill, L., et al. (2010). IL-1 family nomenclature. *Nat. Immunol.* 11:973. doi: 10.1038/ni1110-973

Dinarello, C. A. (2011). Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood* 117, 3720–3732. doi: 10.1182/blood-2010-07-273417

Dinarello, C. A. (2018). Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol. Rev.* 281, 8–27. doi: 10.1111/imr.12621

Dinarello, C. A. (2019). The IL-1 family of cytokines and receptors in rheumatic diseases. *Nat. Rev. Rheumatol.* 15, 612–632. doi: 10.1038/s41584-019-0277-278

Dinarello, C. A., Nold-Petry, C., Nold, M., Fujita, M., Li, S., Kim, S., et al. (2016). Suppression of innate inflammation and immunity by interleukin-37. *Eur. J. Immunol.* 46, 1067–1081. doi: 10.1002/eji.201545828

Dinarello, C. A., Simon, A., and van der Meer, J. W. (2012). Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat. Rev. Drug Discov.* 11, 633–652. doi: 10.1038/nrd3800

Drummond, R. A., Collar, A. L., Swamydas, M., Rodriguez, C. A., Lim, J. K., Mendez, L. M., et al. (2015). CARD9-Dependent neutrophil recruitment protects against fungal invasion of the central nervous system. *PLoS Pathog.* 11:e1005293. doi: 10.1371/journal.ppat.1005293

Economides, A. N., Carpenter, L. R., Rudge, J. S., Wong, V., Koehler-Stec, E. M., Hartnett, C., et al. (2003). Cytokine traps: multi-component, high-affinity blockers of cytokine action. *Nat. Med.* 9, 47–52. doi: 10.1038/nm811

Eigenbrod, T., Park, J. H., Harder, J., Iwakura, Y., and Nunez, G. (2008). Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. *J. Immunol.* 181, 8194–8198. doi: 10.4049/jimmunol.181.12.8194

Fantuzzi, G., Reed, D. A., and Dinarello, C. A. (1999). IL-12-induced IFN-gamma is dependent on caspase-1 processing of the IL-18 precursor. *J. Clin. Invest.* 104, 761–767. doi: 10.1172/JCI7501

Fields, J. K., Günther, S., and Sundberg, E. J. (2019). Structural basis of IL-1 family cytokine signaling. *Front. Immunol.* 10:1412. doi: 10.3389/fimmu.2019.01412

Flaczyk, A., Duerr, C. U., Shourian, M., Lafferty, E. I., Fritz, J. H., and Qureshi, S. T. (2013). IL-33 signaling regulates innate and adaptive immunity to *Cryptococcus neoformans*. *J. Immunol.* 191, 2503–2513. doi: 10.4049/jimmunol.1300426

Foster, A. M., Baliwag, J., Chen, C. S., Guzman, A. M., Stoll, S. W., Gudjonsson, J. E., et al. (2014). IL-36 promotes myeloid cell infiltration, activation, and inflammatory activity in skin. *J. Immunol.* 192, 6053–6061. doi: 10.4049/jimmunol.1301481

Franchi, L., Eigenbrod, T., Munoz-Planillo, R., and Nunez, G. (2009). The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat. Immunol.* 10, 241–247. doi: 10.1038/ni.1703

Freeman, B. E., Raue, H. P., Hill, A. B., and Slifka, M. K. (2015). Cytokine-Mediated activation of NK cells during viral infection. *J. Virol.* 89, 7922–7931. doi: 10.1128/JVI.00199-115

Frey, S., Derer, A., Messbacher, M. E., Baeten, D. L., Bugatti, S., Montecucco, C., et al. (2013). The novel cytokine interleukin-36alpha is expressed in psoriatic and rheumatoid arthritis synovium. *Ann. Rheum. Dis.* 72, 1569–1574. doi: 10.1136/annrheumdis-2012-202264

Garlanda, C., Dinarello, C. A., and Mantovani, A. (2013). The interleukin-1 family: back to the future. *Immunity* 39, 1003–1018. doi: 10.1016/j.immuni.2013.11.010

Garth, J. M., Reeder, K. M., Godwin, M. S., Mackel, J. J., Dunaway, C. W., Blackburn, J. P., et al. (2017). IL-33 signaling regulates innate IL-17A and IL-22 production via suppression of prostaglandin E2 during lung fungal infection. *J. Immunol.* 199, 2140–2148. doi: 10.4049/jimmunol.1602186

Gerdes, N., Sukhova, G. K., Libby, P., Reynolds, R. S., Young, J. L., and Schonbeck, U. (2002). Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis. *J. Exp. Med.* 195, 245–257. doi: 10.1084/jem.20011022

Glocker, E. O., Hennigs, A., Nabavi, M., Schaffer, A. A., Woellner, C., Salzer, U., et al. (2009). A homozygous CARD9 mutation in a family with susceptibility to fungal infections. *N. Engl. J. Med.* 361, 1727–1735. doi: 10.1056/NEJMoa0810719

Godwin, M. S., Reeder, K. M., Garth, J. M., Blackburn, J. P., Jones, M., Yu, Z., et al. (2019). IL-1RA regulates immunopathogenesis during fungal-associated allergic airway inflammation. *JCI Insight* 4:e129055. doi: 10.1172/jci.insight.129055

Goncalves, A. C., Maia, D. C., Ferreira, L. S., Monnazzi, L. G., Alegranci, P., Placeres, M. C., et al. (2015). Involvement of major components from Sporothrix schenckii cell wall in the caspase-1 activation, nitric oxide and cytokines production during experimental sporotrichosis. *Mycopathologia* 179, 21–30. doi: 10.1007/s11046-014-9810-9810

Gracie, J. A., Robertson, S. E., and McInnes, I. B. (2003). Interleukin-18. *J. Leukoc Biol.* 73, 213–224. doi: 10.1189/jlb.0602313

Gresnigt, M. S., Rekiki, A., Rasid, O., Savers, A., Jouvion, G., Dannaoui, E., et al. (2016). Reducing hypoxia and inflammation during invasive pulmonary *Aspergillosis* by targeting the interleukin-1 receptor. *Sci. Rep.* 6:26490. doi: 10.1038/srep26490

Gresnigt, M. S., Rosler, B., Jacobs, C. W., Becker, K. L., Joosten, L. A., van der Meer, J. W., et al. (2013). The IL-36 receptor pathway regulates Aspergillus fumigatus-induced Th1 and Th17 responses. *Eur. J. Immunol.* 43, 416–426. doi: 10.1002/eji.201242711

Gresnigt, M. S., and van de Veerdonk, F. L. (2013). Biology of IL-36 cytokines and their role in disease. *Semin. Immunol.* 25, 458–465. doi: 10.1016/j.smim.2013.11.003

Griffiths, J. S., Thompson, A., Stott, M., Benny, A., Lewis, N. A., Taylor, P. R., et al. (2018). Differential susceptibility of Dectin-1 isoforms to functional inactivation by neutrophil and fungal proteases. *FASEB J.* 32, 3385–3397. doi: 10.1096/fj.201701145R

Gris, D., Ye, Z., Iocca, H. A., Wen, H., Craven, R. R., Gris, P., et al. (2010). NLRP3 plays a critical role in the development of experimental autoimmune encephalomyelitis by mediating Th1 and Th17 responses. *J. Immunol.* 185, 974–981. doi: 10.4049/jimmunol.0904145

Gross, O., Poeck, H., Bscheider, M., Dostert, C., Hannesschlager, N., Endres, S., et al. (2009). Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* 459, 433–436. doi: 10.1038/nature07965

Gross, O., Yazdi, A. S., Thomas, C. J., Masin, M., Heinz, L. X., Guarda, G., et al. (2012). Inflammasome activators induce interleukin-1alpha secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity* 36, 388–400. doi: 10.1016/j.immuni.2012.01.018

Gurczynski, S. J., and Moore, B. B. (2018). IL-17 in the lung: the good, the bad, and the ugly. *Am. J. Physiol. Lung. Cell Mol. Physiol.* 314, L6–L16. doi: 10.1152/ajplung.00344.2017

He, Q., Chen, H. X., Li, W., Wu, Y., Chen, S. J., Yue, Q., et al. (2013). IL-36 cytokine expression and its relationship with p38 MAPK and NF-kappaB pathways in psoriasis vulgaris skin lesions. *J. Huazhong Univ. Sci. Technol. Med. Sci.* 33, 594–599. doi: 10.1007/s11596-013-1164-1161

Heguy, A., Baldari, C. T., Macchia, G., Telford, J. L., and Melli, M. (1992). Amino acids conserved in interleukin-1 receptors (IL-1Rs) and the Drosophila toll protein are essential for IL-1R signal transduction. *J. Biol. Chem.* 267, 2605–2609. doi: 10.1016/s0021-9258(18)45924-8

Heilig, R., Dick, M. S., Sborgi, L., Meunier, E., Hiller, S., and Broz, P. (2018). The Gasdermin-D pore acts as a conduit for IL-1beta secretion in mice. *Eur. J. Immunol.* 48, 584–592. doi: 10.1002/eji.201747404

Hise, A. G., Tomalka, J., Ganesan, S., Patel, K., Hall, B. A., Brown, G. D., et al. (2009). An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen Candida albicans. *Cell Host Microbe* 5, 487–497. doi: 10.1016/j.chom.2009.05.002

Horai, R., Saijo, S., Tanioka, H., Nakae, S., Sudo, K., Okahara, A., et al. (2000). Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. *J. Exp. Med.* 191, 313–320. doi: 10.1084/jem.191.2.313

Hoshino, K., Tsutsui, H., Kawai, T., Takeda, K., Nakanishi, K., Takeda, Y., et al. (1999). Cutting edge: generation of IL-18 receptor-deficient mice: evidence for IL-1 receptor-related protein as an essential IL-18 binding receptor. *J. Immunol.* 162, 5041–5044.

Hou, J., Townson, S. A., Kovalchin, J. T., Masci, A., Kiner, O., Shu, Y., et al. (2013). Design of a superior cytokine antagonist for topical ophthalmic use. *Proc. Natl. Acad. Sci. U S A.* 110, 3913–3918. doi: 10.1073/pnas.1217996110

Hung, C. Y., Jimenez-Alzate Mdel, P., Gonzalez, A., Wuthrich, M., Klein, B. S., and Cole, G. T. (2014). Interleukin-1 receptor but not Toll-like receptor 2 is essential for MyD88-dependent Th17 immunity to Coccidioides infection. *Infect. Immun.* 82, 2106–2114. doi: 10.1128/IAI.01579-1513

Jaeger, M., Carvalho, A., Cunha, C., Plantinga, T. S., van de Veerdonk, F., Puccetti, M., et al. (2016). Association of a variable number tandem repeat in the NLRP3 gene in women with susceptibility to RVVC. *Eur. J. Clin. Microbiol. Infect. Dis.* 35, 797–801. doi: 10.1007/s10096-016-2600-2605

Joly, S., Ma, N., Sadler, J. J., Soll, D. R., Cassel, S. L., and Sutterwala, F. S. (2009). Cutting edge: *Candida albicans* hyphae formation triggers activation of the Nlrp3 inflammasome. *J. Immunol.* 183, 3578–3581. doi: 10.4049/jimmunol.0901323

Kakkar, R., and Lee, R. T. (2008). The IL-33/ST2 pathway: therapeutic target and novel biomarker. *Nat. Rev. Drug Discov.* 7, 827–840. doi: 10.1038/nrd2660

Kamijo, S., Takeda, H., Tokura, T., Suzuki, M., Inui, K., Hara, M., et al. (2013). IL-33-mediated innate response and adaptive immune cells contribute to maximum responses of protease allergen-induced allergic airway inflammation. *J. Immunol.* 190, 4489–4499. doi: 10.4049/jimmunol.1201212

Kanai, T., Watanabe, M., Okazawa, A., Sato, T., Yamazaki, M., Okamoto, S., et al. (2001). Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease. *Gastroenterology* 121, 875–888. doi: 10.1053/gast.2001.28021

Kaplanski, G. (2018). Interleukin-18: biological properties and role in disease pathogenesis. *Immunol. Rev.* 281, 138–153. doi: 10.1111/imr.12616

Kasper, L., Konig, A., Koenig, P. A., Gresnigt, M. S., Westman, J., Drummond, R. A., et al. (2018). The fungal peptide toxin candidalysin activates the NLRP3 inflammasome and causes cytolysis in mononuclear phagocytes. *Nat. Commun.* 9, 4260. doi: 10.1038/s41467-018-06607-6601

Kawakami, K., Koguchi, Y., Qureshi, M. H., Kinjo, Y., Yara, S., Miyazato, A., et al. (2000a). Reduced host resistance and Th1 response to *Cryptococcus neoformans* in interleukin-18 deficient mice. *FEMS Microbiol. Lett.* 186, 121–126. doi: 10.1111/j.1574-6968.2000.tb09092.x

Kawakami, K., Koguchi, Y., Qureshi, M. H., Miyazato, A., Yara, S., Kinjo, Y., et al. (2000b). IL-18 contributes to host resistance against infection with Cryptococcus neoformans in mice with defective IL-12 synthesis through induction of IFN-gamma production by NK cells. *J. Immunol.* 165, 941–947. doi: 10.4049/jimmunol.165.2.941

Ketelut-Carneiro, N., Ghosh, S., Levitz, S. M., Fitzgerald, K. A., and da Silva, J. S. (2018). A Dectin-1-Caspase-8 pathway licenses canonical caspase-1 inflammasome activation and interleukin-1beta release in response to a pathogenic fungus. *J. Infect. Dis.* 217, 329–339. doi: 10.1093/infdis/jix568

Ketelut-Carneiro, N., Silva, G. K., Rocha, F. A., Milanezi, C. M., Cavalcanti-Neto, F. F., Zamboni, D. S., et al. (2015). IL-18 triggered by the Nlrp3 inflammasome induces host innate resistance in a pulmonary model of fungal infection. *J. Immunol.* 194, 4507–4517. doi: 10.4049/jimmunol.1402321

Ketelut-Carneiro, N., Souza, C. O. S., Benevides, L., Gardinassi, L. G., Silva, M. C., Tavares, L. A., et al. (2019). Caspase-11-dependent IL-1alpha release boosts Th17 immunity against *Paracoccidioides brasiliensis*. *PLoS Pathog.* 15:e1007990. doi: 10.1371/journal.ppat.1007990

Kinjo, Y., Kawakami, K., Uezu, K., Yara, S., Miyagi, K., Koguchi, Y., et al. (2002). Contribution of IL-18 to Th1 response and host defense against infection by *Mycobacterium tuberculosis*: a comparative study with IL-12p40. *J. Immunol.* 169, 323–329. doi: 10.4049/jimmunol.169.1.323

Koenders, M. I., Devesa, I., Marijnissen, R. J., Abdollahi-Roodsaz, S., Boots, A. M., Walgreen, B., et al. (2008). Interleukin-1 drives pathogenic Th17 cells during spontaneous arthritis in interleukin-1 receptor antagonist-deficient mice. *Arthritis Rheum.* 58, 3461–3470. doi: 10.1002/art.23957

Krumm, B., Xiang, Y., and Deng, J. (2014). Structural biology of the IL-1 superfamily: key cytokines in the regulation of immune and inflammatory responses. *Protein Sci.* 23, 526–538. doi: 10.1002/pro.2441

Kumar, S., McDonnell, P. C., Lehr, R., Tierney, L., Tzimas, M. N., Griswold, D. E., et al. (2000). Identification and initial characterization of four novel members of the interleukin-1 family. *J. Biol. Chem.* 275, 10308–10314. doi: 10.1074/jbc.275.14.10308

Kurokawa, C. S., Araujo, J. P. Jr., Soares, A. M., Sugizaki, M. F., and Peracoli, M. T. (2007). Pro- and anti-inflammatory cytokines produced by human monocytes challenged in vitro with *Paracoccidioides brasiliensis*. *Microbiol. Immunol.* 51, 421–428. doi: 10.1111/j.1348-0421.2007.tb03929.x

Kurt-Jones, E. A., Beller, D. I., Mizel, S. B., and Unanue, E. R. (1985). Identification of a membrane-associated interleukin 1 in macrophages. *Proc. Natl. Acad. Sci. U S A.* 82, 1204–1208. doi: 10.1073/pnas.82.4.1204

Le, H. T., Tran, V. G., Kim, W., Kim, J., Cho, H. R., and Kwon, B. (2012). IL-33 priming regulates multiple steps of the neutrophil-mediated anti-Candida albicans response by modulating TLR and dectin-1 signals. *J. Immunol.* 189, 287–295. doi: 10.4049/jimmunol.1103564

Lee, M. J., Yoshimoto, E., Saijo, S., Iwakura, Y., Lin, X., Katz, H. R., et al. (2016). Phosphoinositide 3-Kinase delta regulates Dectin-2 signaling and the generation of Th2 and Th17 Immunity. *J. Immunol.* 197, 278–287. doi: 10.4049/jimmunol.1502485

Lefrancais, E., Roga, S., Gautier, V., Gonzalez-de-Peredo, A., Monsarrat, B., Girard, J. P., et al. (2012). IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G. *Proc. Natl. Acad. Sci. U S A.* 109, 1673–1678. doi: 10.1073/pnas.1115884109

LeibundGut-Landmann, S., Gross, O., Robinson, M. J., Osorio, F., Slack, E. C., Tsoni, S. V., et al. (2007). Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat. Immunol.* 8, 630–638. doi: 10.1038/ni1460

Li, D., Guabiraba, R., Besnard, A. G., Komai-Koma, M., Jabir, M. S., Zhang, L., et al. (2014). IL-33 promotes ST2-dependent lung fibrosis by the induction of alternatively activated macrophages and innate lymphoid cells in mice. *J. Allergy Clin Immunol.* 134, 1422-1432.e11. doi: 10.1016/j.jaci.2014.05.011

Li, S., Neff, C. P., Barber, K., Hong, J., Luo, Y., Azam, T., et al. (2015). Extracellular forms of IL-37 inhibit innate inflammation in vitro and in vivo but require the IL-1 family decoy receptor IL-1R8. *Proc. Natl. Acad. Sci. U S A.* 112, 2497–2502. doi: 10.1073/pnas.1424626112

Liew, F. Y., Girard, J. P., and Turnquist, H. R. (2016). Interleukin-33 in health and disease. *Nat. Rev. Immunol.* 16, 676–689. doi: 10.1038/nri.2016.95

Lilly, L. M., Gessner, M. A., Dunaway, C. W., Metz, A. E., Schwiebert, L., Weaver, C. T., et al. (2012). The beta-glucan receptor dectin-1 promotes lung immunopathology during fungal allergy via IL-22. *J. Immunol.* 189, 3653–3660. doi: 10.4049/jimmunol.1201797

Lin, H., Ho, A. S., Haley-Vicente, D., Zhang, J., Bernal-Fussell, J., Pace, A. M., et al. (2001). Cloning and characterization of IL-1HY2, a novel interleukin-1 family member. *J. Biol. Chem.* 276, 20597–20602. doi: 10.1074/jbc.M010095200

Lopez-Castejon, G., and Brough, D. (2011). Understanding the mechanism of IL-1beta secretion. *Cytokine Growth Factor Rev.* 22, 189–195. doi: 10.1016/j.cytogfr.2011.10.001

Luthi, A. U., Cullen, S. P., McNeela, E. A., Duriez, P. J., Afonina, I. S., Sheridan, C., et al. (2009). Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* 31, 84–98. doi: 10.1016/j.immuni.2009.05.007

Macleod, T., Doble, R., McGonagle, D., Wasson, C. W., Alase, A., Stacey, M., et al. (2016). Neutrophil elastase-mediated proteolysis activates the anti-inflammatory cytokine IL-36 receptor antagonist. *Sci. Rep.* 6:24880. doi: 10.1038/srep24880

Marrakchi, S., Guigue, P., Renshaw, B. R., Puel, A., Pei, X. Y., Fraitag, S., et al. (2011). Interleukin-36-receptor antagonist deficiency and generalized pustular psoriasis. *N. Engl. J. Med.* 365, 620–628. doi: 10.1056/NEJMoa1013068

Martin, N. T., and Martin, M. U. (2016). Interleukin 33 is a guardian of barriers and a local alarmin. *Nat. Immunol.* 17, 122–131. doi: 10.1038/ni.3370

Mastroeni, P., Clare, S., Khan, S., Harrison, J. A., Hormaeche, C. E., Okamura, H., et al. (1999). Interleukin 18 contributes to host resistance and gamma interferon production in mice infected with virulent *Salmonella* typhimurium. *Infect. Immun.* 67, 478–483. doi: 10.1128/IAI.67.2.478-483.1999

Mehraj, V., Ponte, R., and Routy, J. P. (2016). The dynamic role of the IL-33/ST2 axis in chronic viral-infections: alarming and adjuvanting the immune response. *EBioMedicine* 9, 37–44. doi: 10.1016/j.ebiom.2016.06.047

Mencacci, A., Bacci, A., Cenci, E., Montagnoli, C., Fiorucci, S., Casagrande, A., et al. (2000). Interleukin 18 restores defective Th1 immunity to *Candida albicans* in caspase 1-deficient mice. *Infect. Immun.* 68, 5126–5131. doi: 10.1128/iai.68.9.5126-5131.2000

Monteleone, G., Trapasso, F., Parrello, T., Biancone, L., Stella, A., Iuliano, R., et al. (1999). Bioactive IL-18 expression is up-regulated in Crohn's disease. *J. Immunol.* 163, 143–147.

Mora, J., Schlemmer, A., Wittig, I., Richter, F., Putyrski, M., Frank, A. C., et al. (2016). Interleukin-38 is released from apoptotic cells to limit inflammatory macrophage responses. *J. Mol. Cell Biol.* 8, 426–438. doi: 10.1093/jmcb/mjw006

Moretti, S., Bozza, S., Oikonomou, V., Renga, G., Casagrande, A., Iannitti, R. G., et al. (2014). IL-37 inhibits inflammasome activation and disease severity in murine aspergillosis. *PLoS Pathog.* 10:e1004462. doi: 10.1371/journal.ppat.1004462

Moussion, C., Ortega, N., and Girard, J. P. (2008). The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS One.* 3:e3331. doi: 10.1371/journal.pone.0003331

Moyes, D. L., Runglall, M., Murciano, C., Shen, C., Nayar, D., Thavaraj, S., et al. (2010). A biphasic innate immune MAPK response discriminates between the yeast and hyphal forms of *Candida albicans* in epithelial cells. *Cell Host Microbe* 8, 225–235. doi: 10.1016/j.chom.2010.08.002

Moyes, D. L., Shen, C., Murciano, C., Runglall, M., Richardson, J. P., Arno, M., et al. (2014). Protection against epithelial damage during *Candida albicans* infection is mediated by PI3K/Akt and mammalian target of rapamycin signaling. *J. Infect. Dis.* 209, 1816–1826. doi: 10.1093/infdis/jit824

Moyes, D. L., Wilson, D., Richardson, J. P., Mogavero, S., Tang, S. X., Wernecke, J., et al. (2016). Candidalysin is a fungal peptide toxin critical for mucosal infection. *Nature* 532, 64–68. doi: 10.1038/nature17625

Mutamba, S., Allison, A., Mahida, Y., Barrow, P., and Foster, N. (2012). Expression of IL-1Rrp2 by human myelomonocytic cells is unique to DCs and facilitates DC maturation by IL-1F8 and IL-1F9. *Eur. J. Immunol.* 42, 607–617. doi: 10.1002/eji.201142035

Nagai, H., Guo, J., Choi, H., and Kurup, V. (1995). Interferon-gamma and tumor necrosis factor-alpha protect mice from invasive aspergillosis. *J. Infect. Dis.* 172, 1554–1560. doi: 10.1093/infdis/172.6.1554

Nakanishi, K. (2018). Unique action of interleukin-18 on T Cells and other immune cells. *Front. Immunol.* 9:763. doi: 10.3389/fimmu.2018.00763

Nakanishi, K., Yoshimoto, T., Tsutsui, H., and Okamura, H. (2001). Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev.* 12, 53–72. doi: 10.1016/s1359-6101(00)00015-10

Netea, M. G., Stuyt, R. J., Kim, S. H., Van der Meer, J. W., Kullberg, B. J., and Dinarello, C. A. (2002). The role of endogenous interleukin (IL)-18, IL-12, IL-1beta, and tumor necrosis factor-alpha in the production of interferon-gamma induced by *Candida albicans* in human whole-blood cultures. *J. Infect. Dis.* 185, 963–970. doi: 10.1086/339410

Netea, M. G., Vonk, A. G., van den Hoven, M., Verschueren, I., Joosten, L. A., van Krieken, J. H., et al. (2003). Differential role of IL-18 and IL-12 in the host defense against disseminated *Candida albicans* infection. *Eur. J. Immunol.* 33, 3409–3417. doi: 10.1002/eji.200323737

Nguyen, T. T., Niyonsaba, F., Ushio, H., Akiyama, T., Kiatsurayanon, C., Smithrithee, R., et al. (2012). Interleukin-36 cytokines enhance the production of host defense peptides psoriasin and LL-37 by human keratinocytes through activation of MAPKs and NF-kappaB. *J. Dermatol. Sci.* 68, 63–66. doi: 10.1016/j.jdermsci.2012.07.010

Nold, M. F., Nold-Petry, C. A., Zepp, J. A., Palmer, B. E., Bufler, P., and Dinarello, C. A. (2010). IL-37 is a fundamental inhibitor of innate immunity. *Nat. Immunol.* 11, 1014–1022. doi: 10.1038/ni.1944

Novick, D., Elbirt, D., Dinarello, C. A., Rubinstein, M., and Sthoeger, Z. M. (2009). Interleukin-18 binding protein in the sera of patients with Wegener's granulomatosis. *J. Clin. Immunol.* 29, 38–45. doi: 10.1007/s10875-008-9217-9210

Novick, D., Elbirt, D., Miller, G., Dinarello, C. A., Rubinstein, M., and Sthoeger, Z. M. (2010). High circulating levels of free interleukin-18 in patients with active SLE in the presence of elevated levels of interleukin-18 binding protein. *J. Autoimmun* 34, 121–126. doi: 10.1016/j.jaut.2009.08.002

Novick, D., Kim, S., Kaplanski, G., and Dinarello, C. A. (2013). Interleukin-18, more than a Th1 cytokine. *Semin Immunol.* 25, 439–448. doi: 10.1016/j.smim.2013.10.014

Novick, D., Schwartsburd, B., Pinkus, R., Suissa, D., Belzer, I., Sthoeger, Z., et al. (2001). A novel IL-18BP ELISA shows elevated serum IL-18BP in sepsis and extensive decrease of free IL-18. *Cytokine* 14, 334–342. doi: 10.1006/cyto.2001.0914

Omoto, Y., Tokime, K., Yamanaka, K., Habe, K., Morioka, T., Kurokawa, I., et al. (2006). Human mast cell chymase cleaves pro-IL-18 and generates a novel and biologically active IL-18 fragment. *J. Immunol.* 177, 8315–8319. doi: 10.4049/jimmunol.177.12.8315

Omoto, Y., Yamanaka, K., Tokime, K., Kitano, S., Kakeda, M., Akeda, T., et al. (2010). Granzyme B is a novel interleukin-18 converting enzyme. *J. Dermatol. Sci.* 59, 129–135. doi: 10.1016/j.jdermsci.2010.05.004

O'Neill, L. A., and Dinarello, C. A. (2000). The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defense. *Immunol. Today* 21, 206–209. doi: 10.1016/s0167-5699(00)01611-x

Panagio, L. A., Tristao, F. S., Moreira, A. P., Pereira, M. S., Cavassani, K. A., Milanezi, C. M., et al. (2008). Role of interleukin (IL)-18 in experimental paracoccidioidomycosis. *Med. Mycol.* 46, 435–442. doi: 10.1080/13693780801895444

Pastorelli, L., Garg, R. R., Hoang, S. B., Spina, L., Mattioli, B., Scarpa, M., et al. (2010). Epithelial-derived IL-33 and its receptor ST2 are dysregulated in ulcerative colitis and in experimental Th1/Th2 driven enteritis. *Proc. Natl. Acad. Sci. U S A.* 107, 8017–8022. doi: 10.1073/pnas.0912678107

Peters, V. A., Joesting, J. J., and Freund, G. G. (2013). IL-1 receptor 2 (IL-1R2) and its role in immune regulation. *Brain Behav. Immun.* 32, 1–8. doi: 10.1016/j.bbi.2012.11.006

Pietras, E. M., Mirantes-Barbeito, C., Fong, S., Loeffler, D., Kovtonyuk, L. V., Zhang, S., et al. (2016). Chronic interleukin-1 exposure drives haematopoietic stem cells towards precocious myeloid differentiation at the expense of self-renewal. *Nat. Cell Biol.* 18, 607–618. doi: 10.1038/ncb3346

Pietrzak, A., Grywalska, E., Socha, M., Rolinski, J., Franciszkiewicz-Pietrzak, K., Rudnicka, L., et al. (2018). Prevalence and possible role of candida species in patients with psoriasis: a systematic review and meta-analysis. *Med. Inflamm.* 2018:9602362. doi: 10.1155/2018/9602362

Plantinga, T. S., Johnson, M. D., Scott, W. K., van de Vosse, E., Velez Edwards, D. R., Smith, P. B., et al. (2012). Toll-like receptor 1 polymorphisms increase susceptibility to candidemia. *J. Infect. Dis.* 205, 934–943. doi: 10.1093/infdis/jir867

Plantinga, T. S., van der Velden, W. J., Ferwerda, B., van Spriel, A. B., Adema, G., Feuth, T., et al. (2009). Early stop polymorphism in human DECTIN-1 is associated with increased candida colonization in hematopoietic stem cell transplant recipients. *Clin. Infect. Dis.* 49, 724–732. doi: 10.1086/604714

Preite, N. W., Feriotti, C., Souza, de Lima, D., da Silva, B. B., Condino-Neto, A., et al. (2018). The syk-coupled C-type lectin receptors Dectin-2 and Dectin-3 are involved in *Paracoccidioides brasiliensis* recognition by human plasmacytoid dendritic cells. *Front. Immunol.* 9:464. doi: 10.3389/fimmu.2018.00464

Ramadas, R. A., Ewart, S. L., Iwakura, Y., Medoff, B. D., and LeVine, A. M. (2012). IL-36alpha exerts pro-inflammatory effects in the lungs of mice. *PLoS One* 7:e45784. doi: 10.1371/journal.pone.0045784

Ramaprakash, H., Shibata, T., Duffy, K. E., Ismailoglu, U. B., Bredernitz, R. M., Moreira, A. P., et al. (2011). Targeting ST2L potentiates CpG-mediated therapeutic effects in a chronic fungal asthma model. *Am. J. Pathol.* 179, 104–115. doi: 10.1016/j.ajpath.2011.03.032

Rautemaa-Richardson, R., and Richardson, M. D. (2017). Systemic fungal infections. *Medicine* 45, 757–762. doi: 10.1016/j.mpmed.2017.09.007

Reading, P. C., and Smith, G. L. (2003). Vaccinia virus interleukin-18-binding protein promotes virulence by reducing gamma interferon production and natural killer and T-cell activity. *J. Virol.* 77, 9960–9968. doi: 10.1128/jvi.77.18.9960-9968.2003

Reich, K., Langley, R. G., Papp, K. A., Ortonne, J. P., Unnebrink, K., Kaul, M., et al. (2011). A 52-week trial comparing briakinumab with methotrexate in patients with psoriasis. *N. Engl. J. Med.* 365, 1586–1596. doi: 10.1056/NEJMoa1010858

Ren, X., Gelinas, A. D., von Carlowitz, I., Janjic, N., and Pyle, A. M. (2017). Structural basis for IL-1alpha recognition by a modified DNA aptamer that specifically inhibits IL-1alpha signaling. *Nat. Commun.* 8:810. doi: 10.1038/s41467-017-00864-862

Roselletti, E., Perito, S., Gabrielli, E., Mencacci, A., Pericolini, E., Sabbatini, S., et al. (2017). NLRP3 inflammasome is a key player in human vulvovaginal disease caused by Candida albicans. *Sci. Rep.* 7:17877. doi: 10.1038/s41598-017-17649-17648

Rouabhia, M., Ross, G., Page, N., and Chakir, J. (2002). Interleukin-18 and gamma interferon production by oral epithelial cells in response to exposure to Candida albicans or lipopolysaccharide stimulation. *Infect. Immun.* 70, 7073–7080. doi: 10.1128/iai.70.12.7073-7080.2002

Rubartelli, A., Cozzolino, F., Talio, M., and Sitia, R. (1990). A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *Embo J.* 9, 1503–1510. doi: 10.1002/j.1460-2075.1990.tb08268.x

Saijo, S., Ikeda, S., Yamabe, K., Kakuta, S., Ishigame, H., Akitsu, A., et al. (2010). Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against Candida albicans. *Immunity* 32, 681–691. doi: 10.1016/j.immuni.2010.05.001

Salter, B. M., Oliveria, J. P., Nusca, G., Smith, S. G., Tworek, D., Mitchell, P. D., et al. (2016). IL-25 and IL-33 induce Type 2 inflammation in basophils from subjects with allergic asthma. *Respir Res.* 17:5. doi: 10.1186/s12931-016-0321-z

Satoh, T. K., Mellett, M., Meier-Schiesser, B., Fenini, G., Otsuka, A., Beer, H. D., et al. (2020). IL-36γ drives skin toxicity induced by EGFR/MEK inhibition and commensal *Cutibacterium acnes*. *J. Clin. Invest.* 130, 1417–1430. doi: 10.1172/jci128678

Shen, H., Yu, Y., Chen, S. M., Sun, J. J., Fang, W., Guo, S. Y., et al. (2020). Dectin-1 Facilitates IL-18 production for the generation of protective antibodies against *Candida albicans*. *Front. Microbiol.* 11:1648. doi: 10.3389/fmicb.2020.01648

Shourian, M., Ralph, B., Angers, I., Sheppard, D. C., and Qureshi, S. T. (2017). Contribution of IL-1RI signaling to protection against cryptococcus neoformans 52D in a mouse model of infection. *Front. Immunol.* 8:1987. doi: 10.3389/fimmu.2017.01987

Silva, C. L., Silva, M. F., Faccioli, L. H., Pietro, R. C., Cortez, S. A., and Foss, N. T. (1995). Differential correlation between interleukin patterns in disseminated and chronic human paracoccidioidomycosis. *Clin. Exp. Immunol.* 101, 314–320. doi: 10.1111/j.1365-2249.1995.tb08357.x

Sims, J. E., and Smith, D. E. (2010). The IL-1 family: regulators of immunity. *Nat. Rev. Immunol.* 10, 89–102. doi: 10.1038/nri2691

Sivakumar, P. V., Westrich, G. M., Kanaly, S., Garka, K., Born, T. L., Derry, J. M., et al. (2002). Interleukin 18 is a primary mediator of the inflammation associated with dextran sulphate sodium induced colitis: blocking interleukin 18 attenuates intestinal damage. *Gut* 50, 812–820. doi: 10.1136/gut.50.6.812

Smith, D. E., Renshaw, B. R., Ketchem, R. R., Kubin, M., Garka, K. E., and Sims, J. E. (2000). Four new members expand the interleukin-1 superfamily. *J. Biol. Chem.* 275, 1169–1175. doi: 10.1074/jbc.275.2.1169

Steele, C., Fidel, P. L., and Jr. (2002). Cytokine and chemokine production by human oral and vaginal epithelial cells in response to Candida albicans. *Infect. Immun.* 70, 577–583. doi: 10.1128/iai.70.2.577-583.2002

Steele, C., Rapaka, R. R., Metz, A., Pop, S. M., Williams, D. L., Gordon, S., et al. (2005). The beta-glucan receptor dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*. *PLoS Pathogens* 1:323–334. doi: 10.1371/journal.ppat.0010042

Stehlik, C. (2009). Multiple interleukin-1beta-converting enzymes contribute to inflammatory arthritis. *Arthritis Rheum.* 60, 3524–3530. doi: 10.1002/art.24961

Stuyt, R. J., Netea, M. G., van Krieken, J. H., van der Meer, J. W., and Kullberg, B. J. (2004). Recombinant interleukin-18 protects against disseminated Candida albicans infection in mice. *J. Infect. Dis.* 189, 1524–1527. doi: 10.1086/382955

Stuyt, R. J., Netea, M. G., Verschueren, I., Fantuzzi, G., Dinarello, C. A., Van Der Meer, J. W., et al. (2002). Role of interleukin-18 in host defense against disseminated *Candida albicans* infection. *Infect. Immun.* 70, 3284–3286. doi: 10.1128/iai.70.6.3284-3286.2002

Sugawara, S., Uehara, A., Nochi, T., Yamaguchi, T., Ueda, H., Sugiyama, A., et al. (2001). Neutrophil proteinase 3-mediated induction of bioactive IL-18 secretion by human oral epithelial cells. *J. Immunol.* 167, 6568–6575. doi: 10.4049/jimmunol.167.11.6568

Sutton, C. E., Mielke, L. A., and Mills, K. H. (2012). IL-17-producing gammadelta T cells and innate lymphoid cells. *Eur. J. Immunol.* 42, 2221–2231. doi: 10.1002/eji.201242569

Swindell, W. R., Beamer, M. A., Sarkar, M. K., Loftus, S., Fullmer, J., Xing, X., et al. (2018). RNA-Seq analysis of IL-1B and IL-36 responses in epidermal keratinocytes identifies a shared MyD88-Dependent gene signature. *Front. Immunol.* 9:80. doi: 10.3389/fimmu.2018.00080

Tak, P. P., Bacchi, M., and Bertolino, M. (2006). Pharmacokinetics of IL-18 binding protein in healthy volunteers and subjects with rheumatoid arthritis or plaque psoriasis. *Eur. J. Drug Metab. Pharmacokinet.* 31, 109–116. doi: 10.1007/BF03191127

Takeuchi, O., and Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell* 140, 805–820. doi: 10.1016/j.cell.2010.01.022

Tanaka, H., Miyazaki, N., Oashi, K., Teramoto, S., Shiratori, M., Hashimoto, M., et al. (2001). IL-18 might reflect disease activity in mild and moderate asthma exacerbation. *J. Allergy Clin. Immunol.* 107, 331–336. doi: 10.1067/mai.2001.112275

Tang, S., Huang, H., Hu, F., Zhou, W., Guo, J., Jiang, H., et al. (2013). Increased IL-33 in synovial fluid and paired serum is associated with disease activity and autoantibodies in rheumatoid arthritis. *Clin. Dev. Immunol.* 2013:985301. doi: 10.1155/2013/985301

Tao, X., Song, Z., Wang, C., Luo, H., Luo, Q., Lin, X., et al. (2017). Interleukin 36alpha attenuates sepsis by enhancing antibacterial functions of macrophages. *J. Infect. Dis.* 215, 321–332. doi: 10.1093/infdis/jiw535

Tardif, F., Goulet, J. P., Zakrazewski, A., Chauvin, P., and Rouabhia, M. (2004). Involvement of interleukin-18 in the inflammatory response against oropharyngeal candidiasis. *Med. Sci. Monit.* 10, BR239–BR249.

Todorovic, V., Su, Z., Putman, C. B., Kakavas, S. J., Salte, K. M., McDonald, H. A., et al. (2019). Small molecule IL-36gamma antagonist as a novel therapeutic approach for plaque psoriasis. *Sci. Rep.* 9:9089. doi: 10.1038/s41598-019-45626-w

Towne, J. E., Garka, K. E., Renshaw, B. R., Virca, G. D., and Sims, J. E. (2004). Interleukin (IL)-1F6, IL-1F8, and IL-1F9 signal through IL-1Rrp2 and IL-1RAcP to activate the pathway leading to NF-kappaB and MAPKs. *J. Biol. Chem.* 279, 13677–13688. doi: 10.1074/jbc.M400117200

Towne, J. E., Renshaw, B. R., Douangpanya, J., Lipsky, B. P., Shen, M., Gabel, C. A., et al. (2011). Interleukin-36 (IL-36) ligands require processing for full agonist (IL-36α, IL-36β, and IL-36γ) or antagonist (IL-36Ra) activity. *J. Biol. Chem.* 286, 42594–42602. doi: 10.1074/jbc.M111.267922

Tran, V. G., Kim, H. J., Kim, J., Kang, S. W., Moon, U. J., Cho, H. R., et al. (2015). IL-33 enhances host tolerance to *Candida albicans* kidney infections through induction of IL-13 production by CD4+ T Cells. *J. Immunol.* 194, 4871–4879. doi: 10.4049/jimmunol.1402986

van de Veerdonk, F. L., Stoeckman, A. K., Wu, G., Boeckermann, A. N., Azam, T., Netea, M. G., et al. (2012). IL-38 binds to the IL-36 receptor and has biological effects on immune cells similar to IL-36 receptor antagonist. *Proc. Natl. Acad. Sci. U S A.* 109, 3001–3005. doi: 10.1073/pnas.1121534109

Verma, A. H., Richardson, J. P., Zhou, C., Coleman, B. M., Moyes, D. L., Ho, J., et al. (2017). Oral epithelial cells orchestrate innate type 17 responses to Candida albicans through the virulence factor candidalysin. *Sci. Immunol.* 2:eaam8834. doi: 10.1126/sciimmunol.aam8834

Verma, A. H., Zafar, H., Ponde, N. O., Hepworth, O. W., Sihra, D., Aggor, F. E. Y., et al. (2018). IL-36 and IL-1/IL-17 drive immunity to oral candidiasis via parallel mechanisms. *J. Immunol.* 201, 627–634. doi: 10.4049/jimmunol.1800515

Vigers, G. P., Dripps, D. J., Edwards, C. K. III, and Brandhuber, B. J. (2000). X-ray crystal structure of a small antagonist peptide bound to interleukin-1 receptor type 1. *J. Biol. Chem.* 275, 36927–36933. doi: 10.1074/jbc.M006071200

Vigne, S., Palmer, G., Lamacchia, C., Martin, P., Talabot-Ayer, D., Rodriguez, E., et al. (2011). IL-36R ligands are potent regulators of dendritic and T cells. *Blood* 118, 5813–5823. doi: 10.1182/blood-2011-05-356873

Vigne, S., Palmer, G., Martin, P., Lamacchia, C., Strebel, D., Rodriguez, E., et al. (2012). IL-36 signaling amplifies Th1 responses by enhancing proliferation and Th1 polarization of naive CD4+ T cells. *Blood* 120, 3478–3487. doi: 10.1182/blood-2012-06-439026

Viriyakosol, S., Walls, L., Okamoto, S., Raz, E., Williams, D. L., and Fierer, J. (2018). Myeloid differentiation factor 88 and interleukin-1R1 signaling contribute to resistance to Coccidioides immitis. *Infect. Immun.* 86:e00028-18. doi: 10.1128/IAI.00028-18

Vonk, A. G., Netea, M. G., van Krieken, J. H., Iwakura, Y., van der Meer, J. W., and Kullberg, B. J. (2006). Endogenous interleukin (IL)-1 alpha and IL-1 beta are crucial for host defense against disseminated candidiasis. *J. Infect. Dis.* 193, 1419–1426. doi: 10.1086/503363

Wang, J. P., Lee, C. K., Akalin, A., Finberg, R. W., and Levitz, S. M. (2011). Contributions of the MyD88-dependent receptors IL-18R, IL-1R, and TLR9 to host defenses following pulmonary challenge with *Cryptococcus neoformans*. *PLoS One* 6:e26232. doi: 10.1371/journal.pone.0026232

Warris, A., Netea, M. G., Verweij, P. E., Gaustad, P., Kullberg, B. J., Weemaes, C. M., et al. (2005). Cytokine responses and regulation of interferon-gamma release by human mononuclear cells to *Aspergillus fumigatus* and other filamentous fungi. *Med. Mycol.* 43, 613–621. doi: 10.1080/13693780500088333

Weinberg, E. O., Ferran, B., Tsukahara, Y., Hatch, M. M. S., Han, J., Murdoch, C. E., et al. (2019). IL-33 induction and signaling are controlled by glutaredoxin-1 in mouse macrophages. *PLoS One* 14:e0210827. doi: 10.1371/journal.pone.0210827

Werman, A., Werman-Venkert, R., White, R., Lee, J. K., Werman, B., Krelin, Y., et al. (2004). The precursor form of IL-1alpha is an intracrine proinflammatory activator of transcription. *Proc. Natl. Acad. Sci. U S A.* 101, 2434–2439. doi: 10.1073/pnas.0308705101

Werner, J. L., Metz, A. E., Horn, D., Schoeb, T. R., Hewitt, M. M., Schwiebert, L. M., et al. (2009). Requisite role for the dectin-1 beta-glucan receptor in pulmonary defense against *Aspergillus fumigatus*. *J. Immunol.* 182, 4938–4946. doi: 10.4049/jimmunol.0804250

Wu, B., Meng, K., Ji, Q., Cheng, M., Yu, K., Zhao, X., et al. (2014). Interleukin-37 ameliorates myocardial ischaemia/reperfusion injury in mice. *Clin. Exp. Immunol.* 176, 438–451. doi: 10.1111/cei.12284

Wyman, T. H., Dinarello, C. A., Banerjee, A., Gamboni-Robertson, F., Hiester, A. A., England, K. M., et al. (2002). Physiological levels of interleukin-18 stimulate multiple neutrophil functions through p38 MAP kinase activation. *J. Leukoc Biol.* 72, 401–409.

Yuan, K., Zhao, G., Che, C., Li, C., Lin, J., Zhu, G., et al. (2017). Dectin-1 is essential for IL-1beta production through JNK activation and apoptosis in *Aspergillus fumigatus* keratitis. *Int. Immunopharmacol.* 52, 168–175. doi: 10.1016/j.intimp.2017.09.008

Zola, H., Flego, L., Wong, Y. T., Macardle, P. J., and Kenney, J. S. (1993). Direct demonstration of membrane IL-1 alpha on the surface of circulating B lymphocytes and monocytes. *J. Immunol.* 150, 1755–1762.

Zwolanek, F., Riedelberger, M., Stolz, V., Jenull, S., Istel, F., Koprulu, A. D., et al. (2014). The non-receptor tyrosine kinase Tec controls assembly and activity of the noncanonical caspase-8 inflammasome. *PLoS Pathog* 10:e1004525. doi: 10.1371/journal.ppat.1004525

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 12 February 2021
doi: 10.3389/fmicb.2021.631392

Fungal G-Protein-Coupled Receptors: A Promising Mediator of the Impact of Extracellular Signals on Biosynthesis of Ochratoxin A

*Jing Gao[1], Xinge Xu[2], Kunlun Huang[2] and Zhihong Liang[1,2]**

[1] Beijing Laboratory for Food Quality and Safety, Beijing, China, [2] College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China

G-protein-coupled receptors (GPCRs) are transmembrane receptors involved in transducing signals from the external environment inside the cell, which enables fungi to coordinate cell transport, metabolism, and growth to promote their survival, reproduction, and virulence. There are 14 classes of GPCRs in fungi involved in sensing various ligands. In this paper, the synthesis of mycotoxins that are GPCR-mediated is discussed with respect to ligands, environmental stimuli, and intra-/interspecific communication. Despite their apparent importance in fungal biology, very little is known about the role of ochratoxin A (OTA) biosynthesis by *Aspergillus ochraceus* and the ligands that are involved. Fortunately, increasing evidence shows that the GPCR that involves the AF/ST (sterigmatocystin) pathway in fungi belongs to the same genus. Therefore, we speculate that GPCRs play an important role in a variety of environmental signals and downstream pathways in OTA biosynthesis. The verification of this inference will result in a more controllable GPCR target for control of fungal contamination in the future.

Keywords: G protein-coupled receptors, ochratoxin A, quorum sensing, oxylipin, trans-kingdom, transcription factor

OPEN ACCESS

Edited by:
David L. Moyes, King's College London, United Kingdom

Reviewed by:
Xuehua Xu, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), United States
Massimo Reverberi, Sapienza University of Rome, Italy

***Correspondence:**
Zhihong Liang
lzh105@cau.edu.cn

Specialty section:
This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology

Received: *20 November 2020*
Accepted: *21 January 2021*
Published: *12 February 2021*

Citation:
Gao J, Xu X, Huang K and Liang Z (2021) Fungal G-Protein-Coupled Receptors: A Promising Mediator of the Impact of Extracellular Signals on Biosynthesis of Ochratoxin A. Front. Microbiol. 12:631392. doi: 10.3389/fmicb.2021.631392

INTRODUCTION

Ochratoxin A (OTA), which is a type of mycotoxin produced mainly by various *Aspergillus* and *Penicillium* species (Frisvad et al., 2019), was classified as a group IIB carcinogen by the International Agency for Research on Cancer (IARC) (International Agency For Research On Cancer, 1993). The latest *in vitro* toxicology experiment showed that alteration of DNA methylation occurred? in OTA-induced G0/G1 phase arrest (Zhang et al., 2019). Numerous cell and animal experiments have reported that OTA exposure can cause many toxicological effects, which include teratogenicity, carcinogenicity, mutagenicity, hepatotoxicity, and was a main causative agent of human Balkan endemic nephropathy (Pfohl-Leszkowicz and Manderville, 2012; Zheng et al., 2013; Qi et al., 2014; Zhang et al., 2014; Zhao et al., 2017; Hou et al., 2020). OTA widely contaminates feed and food commodities, and it is accumulated through the food chain in animals (Wenying et al., 2018). Global warming promotes the poleward movement of toxigenic fungi, causes contamination in previously unsuitable geographic regions, and exacerbates this threat (Bebber et al., 2013). OTA pollution occurs at various stages of agricultural production (i.e., plant growth, harvest, processing,

transportation, and storage), and the occurrence of toxigenic fungi and the biosynthesis of OTA was influenced significantly at different stages of synthesis by external environmental factors, signaling molecules, and trans-kingdom communication.

Sensing and responding to environmental fluctuations are crucial to the survival of microorganisms. GPCRs are ubiquitous and the largest family of transmembrane receptors in both prokaryotes and eukaryotes. They contain seven transmembrane domains (TMDs) and are connected by alternating intracellular coils (IC1-IC3) and extracellular loops (EC1-EC3) (Baldwin, 1993). The extracellular amino acid? fragments sense the environment by interacting with a diverse array of ligands, then transmit this interaction through protein folding modifications to the intracellular carboxy-terminal end that recognizes the cognate heterotrimeric G proteins (Gαβγ) (Ballesteros et al., 2001; Shapiro et al., 2002). Sensitization of a GPCR by ligands results in the exchange of GTP for GDP on the Gα subunit, which leads to the dissociation of Gβγ; both GTP-Gα and Gβγ dimers can interact with respective effectors to activate or inhibit specific downstream pathways (Khan et al., 2013; Brown et al., 2018). In fungi, GPCR-regulated signaling pathways that include the cAMP-activated Protein Kinase A (PKA) pathway (Xue et al., 2006), mitogen-activated protein kinases (MAPK) cascades pathway (Chen and Thorner, 2007; Atoui et al., 2008; Hamel et al., 2012; Ma and Li, 2013), and the phospholipase C (PLC) pathway (Ansari et al., 1999) influence gene expression to regulate cell growth, morphogenesis, mating, stress responses, and metabolism in a complex and intersecting way (Rispail et al., 2009).

The regulator of G protein signaling (RGS) is a GTPase activating protein (GAP), which accelerates the decomposition of GTP-binging Gα by enhancing Gα subunit GTPase activity that negatively regulates G protein signal transduction (Kasahara et al., 2000; McPherson et al., 2018). Another G protein signal-regulating protein, Phosducin-like protein (PhLP), is involved in the G protein signal transduction pathway by positively regulating Gβγ subunits (Lukov et al., 2014). Significant differences exist in the abundance and diversity of these receptors in fungi and the potential ligands that they detect. A variety of GPCRs relates to OTA biosynthesis, which involves a wide range of environmental signals and downstream pathways (**Figure 1**). Because of their cell surface location and central mediating role, GPCRs are specific targets to control the biosynthesis of fungal toxins and to intervene in fungal disease and mycotoxin contamination.

The well-researched GPCR-mediated, G-protein signaling pathway, which regulates fungal behavior in response to a variety of signals, is highly conserved in animals, plants, and microorganisms. This phenomenon and the mechanism of model strains have been studied thoroughly enough to provide the guiding ideology and practical basis for research on GPCRs in fungi. The bovine rhodopsin and human beta2-adrenergic GPCRs were crystallized and based on homology and structural similarity, which served as models for the structure of other members of the GPCR family (Stenkamp et al., 2002; Rasmussen et al., 2007). Although GPCR signaling is essential for fungal biology, the identified interactions of GPCR–G-protein, the studies of receptor binding ligands, and the resolved GPCR crystal structures are only the beginning of what we need to know. Perfecting the above information will prove vital in understanding the upstream ligand–receptor relationship of the OTA synthesis regulatory pathways and the development of novel fungal GPCR-targeting of mycotoxin control. This paper reviews the current understanding of fungal GPCR-mediated signaling pathways that regulate fungal behavior and OTA synthesis; this has important theoretical significance for improving our knowledge about the function of GPCRs and the synthesis of secondary metabolites. It is expected that we will eventually use GPCR as targets to prevent and control the harm caused by fungal toxins.

GPCRS SENSE NUTRIENTS AND PHEROMONES

Fungal adaptation to distinct microenvironments, which include hosts, natural habitats, and culture media, is essential for their success (Brown and Goldman, 2016). G-protein signaling pathways play a vital role in sensing external ligands, which include nutrients, hormones, proteins, and peptides (including pheromones), ions, hydrophobic surfaces, and light (Kochman, 2014). This enable fungi to coordinate function, metabolism, and development that, in turn, promotes their survival, propagation, and virulence (Van Dijck et al., 2017). At present, based on structural similarity and homology, fungal GPCRs are classified into 14 categories, which include six original classes and eight novel classes (**Supplementary Table 1**; Lafon et al., 2006; Li et al., 2007; Zheng et al., 2010; Grice et al., 2013; Cabrera et al., 2015; Brown et al., 2018; Nadarajah et al., 2018). The model filamentous ascomycete fungus *A. nidulans* possesses 86 putative GPCRs, which are classified into 16 receptors, named GprA∼GprP and NopA, constitute nine categories of GPCRs; only a few have been characterized functionally (Lafon et al., 2006; Krishnan et al., 2012; Brown et al., 2015).

Many studies have found that GPCR-mediated perception of signaling molecules, especially pheromones, nutritions and oxylipins, are closely related to fungal reproduction and the production of secondary metabolites, which seems to imply that there are cross-effects of different GPCRs. This complex regulatory network provides numerous targets for controlling OTA biosynthesis.

Perception of Nutrients

G-protein-coupled receptors sense nutrients, and then they influence fungal development and metabolism. An enormous amount of basic insights into nutrient utilization by fungi has been established. The interaction of GPCR protein Gpr1 with Gα protein Gpa2 is necessary for the stimulation of cAMP synthesis by sugars. In the model organism *Saccharomyces cerevisiae*, Gpr1 is a high-affinity sucrose and a low-affinity glucose sensor. The addition of glucose to a glucose starvation culture triggers a rapid and transient increase in the cAMP level, which sets off a PKA-mediated protein phosphorylation cascade. Deletion of Gpr1 and/or Gpa2 affected a variety of physiological events

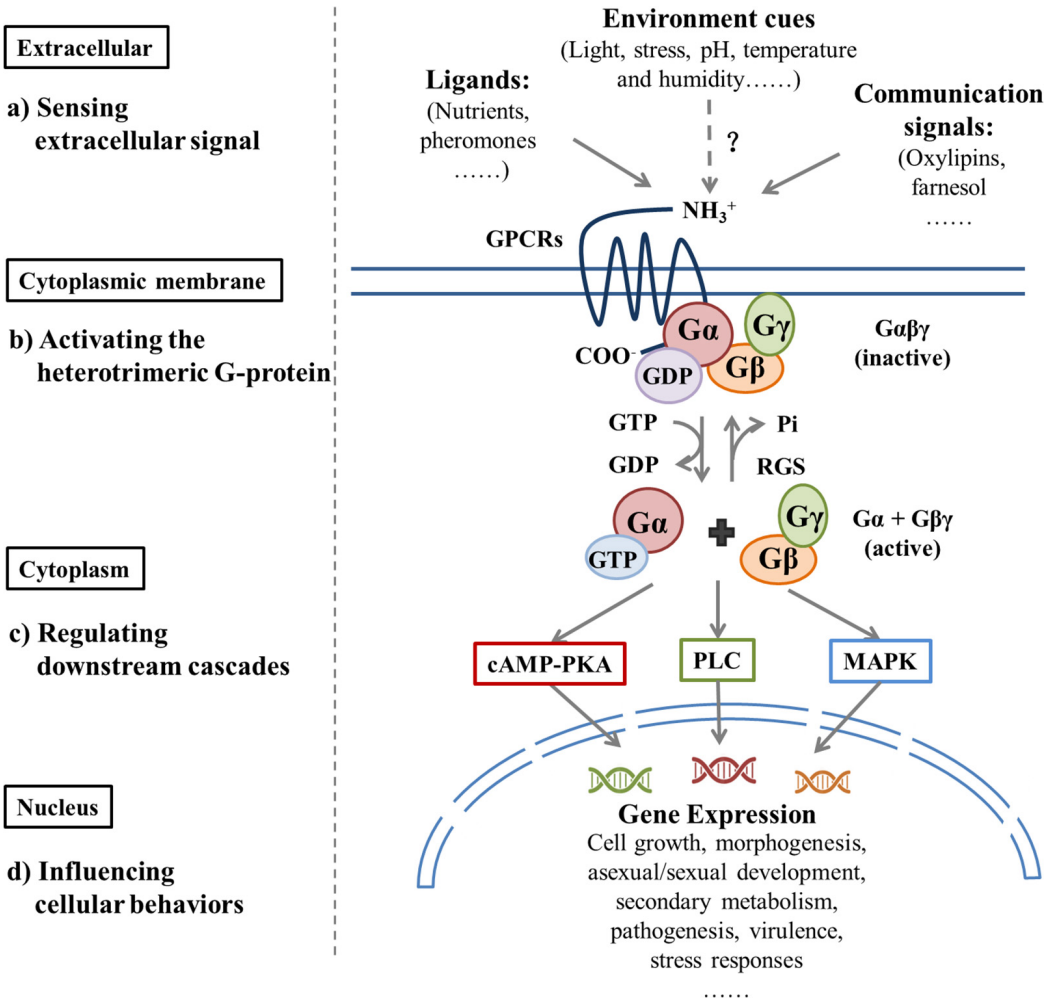

FIGURE 1 | Extracellular signals regulate downstream pathways by activating G protein signaling and, thus, influence cellular behavior.| Different classes of GPCRs sense various extracellular factors, which include ligands, environmental cues, and communication signals that bind to the GPCR; this causes GDP-GTP exchange on the Gα protein and dissociation of Gα and Gβγ. Both the Gα-GTP and Gβγ dimers may trigger respective downstream signal cascades, which include the cAMP-activated Protein Kinase A (PKA) pathway, mitogen-activated protein kinase (MAPK) cascades pathway, and the phospholipase C (PLC) pathway. This process influences cellular growth, reproduction, metabolism, virulence, and stress responses. GTP hydrolysis by the Gα subunit results in the reassociation of Gα-GDP with the Gβγ dimer and GPCR, which completes the G protein cycle. The Regulator of G-protein Signaling (RGS) proteins can inactivate G protein signaling rapidly by increasing intrinsic GTPase activity of GTP-bound Gα subunits. In this figure, dashed arrows with the question mark (?) indicates hypothetical interactions.

controlled by cAMP-dependent protein kinases (cAPKs), such as mobilization of trehalose and glycogen and a rapid loss of stress resistance (Rolland et al., 2000; Lemaire et al., 2004). The absence of Gpr1 can be rescued by the constitutively activated $Gpa2^{Val-132}$ allele, which supports the interaction of GPCR and its cognate G protein in regulation (Kraakman et al., 2010). GprC and GprD (Class III) in *Aspergilli* are analogous to Gpr1 in *S. cerevisiae*, which is involved in the sensing of sugar and oxylipin (De et al., 2013). Deletion of the *gprC* or *gprD* gene in *A. fumigatus* affected the ability of strains to produce several toxic secondary metabolites and then affected the growth and pathogenicity in model murine infections (Gehrke et al., 2010). In *A. flavus*, deletion of genes *gprC* and/or *gprD* resulted in alteration of quorum sensing (QS), sporulation, sclerotia formation, and biosynthesis of aflatoxin (AF) (Affeldt et al., 2012). GprK (ClassVI) in *A. fumigatus* was involved in sensing external carbon sources, which is a more complex sensor protein that contains a RGS domain in addition to the 7-TM domains. Overexpression of *gprK* significantly activated toxin in the biosynthesis-related cAMP-PKA pathway, then activated the MAPK signaling pathway, which partially regulates the expression of genes that code for catalase and superoxide dismutase and, thus, indirectly affects secondary metabolism.

Even in the absence of carbon sources, Δ*gprK* mutants showed a severe morphological alteration, a change in asexual reproduction, and a reduction in tolerance to different stressing factors, which included oxidative stress and destruction of the ability to produce gliotoxin (Jung et al., 2016). Both GprM (ClassVII, homologous to rat growth hormone-releasing factor

receptors) and GprJ (Class IV, nitrogen source sensors) were involved in regulating the production of DHN-melanin in *A. fumigatus*, and this regulation partially occurred through the activation of MpkA (Manfiolli et al., 2019; Filho et al., 2020). GprH (ClassV), a common glucose and tryptophan sensor, has been found in many filamentous fungi, which regulates hyphal growth and sexual reproduction during carbon starvation in *A. nidulans*. In cultures supplemented with glucose and tryptophan, the deletion of *gprH* caused a decrease in cAMP-PKA activity under stress conditions (Brown et al., 2015), which suggested that a single GPCR interacted with multiple ligands. In *Cryptococcus neoforme*, Gpr4 was similar to Gpr1 in *S. cerevisiae* and to GprH in *A. nidulans*, which induced the cAMP–PKA pathway. Glucose, but not methionine, acted on Δ*gpr4* mutants to cause a change in the cAMP level, and the deficiencies in capsule formation and mating defects were reverted by cAMP supplementation. Therefore, Gpr4 protein has been proposed to be a sensor of amino acids, particularly for methionine (Xue et al., 2006).

There are also examples of other types of nutrient sources that are sensed and regulated by fungal GPCR. Four cAMP-type GPCR genes (*gpr1* to *gpr4*) in *Trichoderma atroviride* belong to class V, and expression of all four *gpr* genes increased after carbon starvation. Gpr1 protein is essential to conidium germination and mycelial growth. Expression of *gpr3* and *gpr4* responded to exogenous cAMP, and the addition of hyphal fragments and cellulosic material also increased the expression of *gpr4* (Brunner et al., 2008). The Pth11 type GPCR of *Neurospora crassa* was involved in the detection of cellulose materials, plant cell walls, or their degradation products, which triggered a response that favored fungal infection of plant hosts (Cabrera et al., 2015; Thieme et al., 2017).

The above results showed that fungi can sense a variety of nutrients through GPCRs, and they can regulate cell growth, development, immune evasion, invasiveness, mycotoxin production, chemotropism, and virulence. Disrupted nutrient-sensing GPCRs could be targeted to reduce fungal virulence and mycotoxin contamination.

Perception of Pheromones

Pheromones are another type of typical GPCR ligand that influences fungal reproduction and virulence. The benefits of rapid evolution that is driven by sexual reproduction of virulence and resistance are essential for promoting genetic diversity and evolutionary competition with hosts. Pheromones secreted by one type of mating cell and recognized by the opposite type of mating cell trigger downstream signal transduction cascades that lead to cell mating, and they also play a potential role in the production of secondary metabolites (Karlson and Lüscher, 1959; Herskowitz, 1995; Xue et al., 2008; Kim and Borkovich, 2010). In *S. cerevisiae*, the GPCR sex pheromone sensor proteins Ste2 and Ste3 detect the a and α sex peptide pheromones, respectively, which can activate the MAPK cascade to result in cell cycle arrest and cell fusion with the opposite mating type (Burkholder and Hartwell, 1985; Kuchler et al., 1989; Herskowitz, 1995; Chen and Thorner, 2007). PcPRE1 and PcPRE2, which are homologs of Ste2 and Ste3 pheromone receptors involved in mating, have been found in *P. notatum*, *P. chrysogenum,* and *Acremonium chrysogenum;* previously, it was thought that these fungi were incapable of sexual reproduction (Paoletti et al., 2005; Poggeler et al., 2008; Bohm et al., 2013; Terfehr et al., 2014).

The mating-type loci *MAT1-1-1* and *MAT1-1-2* were found in *A. chrysogenum*, and the mating-type genes of the recombinant strains control conidia formation, hyphal differentiation, and penicillin production. In the Δ*mat1* mutant, the expression of biosynthetic genes in penicillin decreased because the pellet size and structure were affected by hyphal differentiation (Poggeler et al., 2008). In *A. nidulans*, GprA and GprB are required for sexual development (without vegetative growth), which include formation of self-fertilized fruiting bodies, asexual development, Hülle cell production, and heterothallic sexual development (Han et al., 2004). Single Δ*gprA* and Δ*gprB* mutants and double Δ*gprA*Δ*gprB* mutants were defective in sexual reproduction. Interestingly, GprD-mediated carbon sensing also acted as a repressor of sexual development through the PKA pathway; when grown in the presence of glucose, GprD promoted hyphal growth and conidial germination. The sexual developmental activator NsdD functioned downstream of GprA or GprB, whereas GprD-mediated attenuation of sexual development functioned upstream of GprA and GprB (Han et al., 2004; Seo et al., 2004). On the other hand, GprB and GprD affected QS, spore and sclerotia formation, mycotoxin production, and other metabolic pathways (De et al., 2013). The putative carbon and amino acid receptor GprH worked upstream of the cAMP-PKA pathway, which promoted glucose uptake and hyphal growth and repressed sexual development during carbon starvation.

Cross-Talk and Interaction Among GPCR-Mediated Pathways

Ligands, GPCRs, and downstream regulatory pathways are not strictly corresponding; this cross-over internal relationship increases the possibility of GPCR as targets for regulating cell behavior. Nutritional state and the perception of sexual partners regulate sexual developmentbecause nutrient limitation reduces pheromone signaling and, in turn, mating efficiency. Nutrient and pheromone GPCR pathways significantly influence the next step of cell growth or reproduction as cell cycle arrest by integrating environmental and internal signals.

In *C. albicans*, the carbon source sensing protein *Ca*Gpa2 not only regulated the cAMP level, but it also inhibited pheromone-mediated cell cycle arrest; the absence of *Ca*Gpa2 caused pheromone hypersensitivity and mating (Dignard et al., 2008). *Ca*Gpa2 was also important for activating the mating MAPK pathway, which showed a link between the nutrient-sensing pathway and the pheromone-responsive pathway (Bennett and Johnson, 2010). The *A. nidulans An*GprD protein regulates hyphal growth and conidial germination and represses sexual development during growth on glucose. In *A. fumigatus*, although GrpC and GprD are similar to the *S. cerevisiae* glucose receptor Gpr1, experiments showed that they were not involved in glucose and cAMP sensing. *Afu*GprC and *Afu*GprD proteins regulated growth, morphogenesis, reactive oxygen species (ROS), and temperature tolerance, and virulence

in a murine model of pulmonary aspergillosis, although it had an opposing influence on the transcriptional regulation of primary and secondary metabolism (Gehrke et al., 2010). In *A. flavus*, inactivating each of 15 GPCR proteins separately showed mixed effects on the response to carbon sources, nitrogen sources, lipid molecules, environment stress signals, cell growth, conidiation, production of secondary metabolites, and virulence; two or more these responses were altered in several null mutants (Cabrera et al., 2015). It seems that GPCR-mediated signal transduction pathways cross each other in downstream cascades, which resulted in different outcomes? that were expected for a single GPCR target (Martín et al., 2019). Therefore, signals generated by distinct GPCR-mediated nutrient and pheromone-sensing pathways are potentially integrated into one biological outcome through downstream, dual-function signaling elements. In addition, GPCRs are adapted to detect multiple environmental cues and to bind multiple ligands to induce different signaling pathways. Thus, the interlinked signaling pathways are modulated differentially and fine-tuned to regulate multiple aspects of fungal development, metabolism, and virulence.

POTENTIAL ROLE OF GPCRS IN A FUNGAL-SENSING ENVIRONMENT

The secondary metabolism biosynthetic genes in the fungal genome are clustered on the chromosome, which contain several key structural genes that encode multimodular enzymes that belong to the polyketide synthases (PKSs) or non-ribosomal peptide synthetases (NRPSs) families. These enzymes facilitate the construction of the main scaffold structure of many secondary metabolites. Additional enzymes introduced various modifications to the original structure (Alkhayyat and Yu, 2014). This complex process requires one or more cluster-specific transcription factors (TFs) to regulate, which operates downstream of G-protein signaling. The regulation of global TFs related to environmental signals is also at an upper level of cluster-specific regulation.

For example, the most studied pathway-specific TF, AflR, which regulates the AF/sterigmatocystin (ST) biosynthetic gene cluster, was affected by CreA (carbon source), AreA (nitrogen source), Velvet (light), PacC (pH), and other global TFs (Macheleidt et al., 2016; Caceres et al., 2020; Gil-Serna et al., 2020). Other environmental factors, such as temperature, humidity, and osmotic pressure, also have a comprehensive influence on microbial behavior. High-similarity, specific gene clusters were found in several common OTA-produced *Aspergillus* and *Penicillium* species, which contained four highly conserved biosynthetic genes that encoded polyketide synthase (PKS), non-ribosomal peptide synthetase (NRPS), halogenate or chloroperoxidase (HAL/CHL), cytochrome P450 (P450), and a bZIP transcription factor (Antonia et al., 2016; Ferrara et al., 2016; Gil-Serna et al., 2018; Yan et al., 2018). bZIP transcription factor is supposed to be a specific regulator for secondary metabolite biosynthesis and the hub that accommodates various inputs that lead to a single output, and it altered the expression of all four biosynthetic genes (*pks, nrps, p450, hal*) (**Figure 2**; Reverberi et al., 2008; Nadia, 2015; Yan et al., 2018). Based on a similar mechanism of secondary metabolite production, we speculate that environmental signals may regulate OTA biosynthetic gene clusters in a cluster-specific, transcription factor-dependent manner; complex and integrated GPCRs likely play an important transfer role in this process.

Carbon/Nitrogen Sources

Fungi can utilize a diverse array of nutrient sources, which include hexose, pentose, and complex saccharides. This nutritional plasticity may be reflected, therefore, in the dramatic expansion of putative GPCRs (Wang et al., 2020). A wider evaluation of the function of all 15 classical GPCRs in *A. flavus* revealed multiple GPCRs that were potentially involved in carbon sensing because the individual absence of the receptors GprA, GprC, GprJ, GprK, or GprR impaired growth on several carbon sources (Dierking and Pita, 2020). In addition to the GPCR mentioned above (Section " Perception of Nutrients"), nutrients also regulate life activities through global transcription factors. In the presence of favored carbon sources that can be metabolized rapidly to provide quick energy, fungi will repress the utilization of alternative, less preferred carbon sources, which is known as carbon catabolite repression (CCR) (Ruijter and Visser, 1997). The CCR mechanism is regulated in part by the C_2H_2 zinc-finger global transcription factor CreA, which represses the expression of genes required for catabolism of less preferable carbon sources, gluconeogenic genes, and nutrient acquisition genes in response to carbon starvation (Adnan et al., 2018). CreA up-regulated AF/ST biosynthesis by affecting the regulatory factor AflR (Hicks et al., 2001). Notably, loss of CreA in *A. nidulans*, *Fusarium oxysporum*, *P. chrysogenum,* and *Colletotrichum gloeosporoides* was fatal, but it did not affect the activity of *N. crassa*, *T. reesei,* and *A. fumigatus* (Ries et al., 2018). Recent studies revealed that Gα (GanB) and PKA (PkaA) participated in CreA-independent CCR, then regulated mycotoxin synthesis at the transcriptional level in *A. nidulans* (Kunitake et al., 2019; **Figure 2**). This study indicated that the G-protein signaling pathway and the global regulator pathway partially overlapped in their perception of a carbon source.

Nitrogen is another important nutrient that affects mycotoxin synthesis. In *A. carbonarius*, inorganic nitrogen and ammonium chloride strongly reduced the OTA level, but organic nitrogen promoted OTA yield (Tudzynski, 2014). Similarly, there was a nitrogen catabolite repression (NCR) for nitrogen sources, which was affected by the C_2H_2 zinc-finger, global transcription factor AreA (Wong et al., 2008). However, AreA contributed to, but was not essential for, virulence of *A. fumigatus* (Kmetzsch et al., 2011).

Light

Photon is a type of ligand of rhodopsin-like GPCRs, which is a special animal neuronal photoreceptor that converts light signals into electrical signals. After photons are absorbed by rhodopsin, the voltage of the photoreceptor cell membrane is changed, thereby regulating life activities, and G protein plays a role in the conduction and amplification of this photoelectric

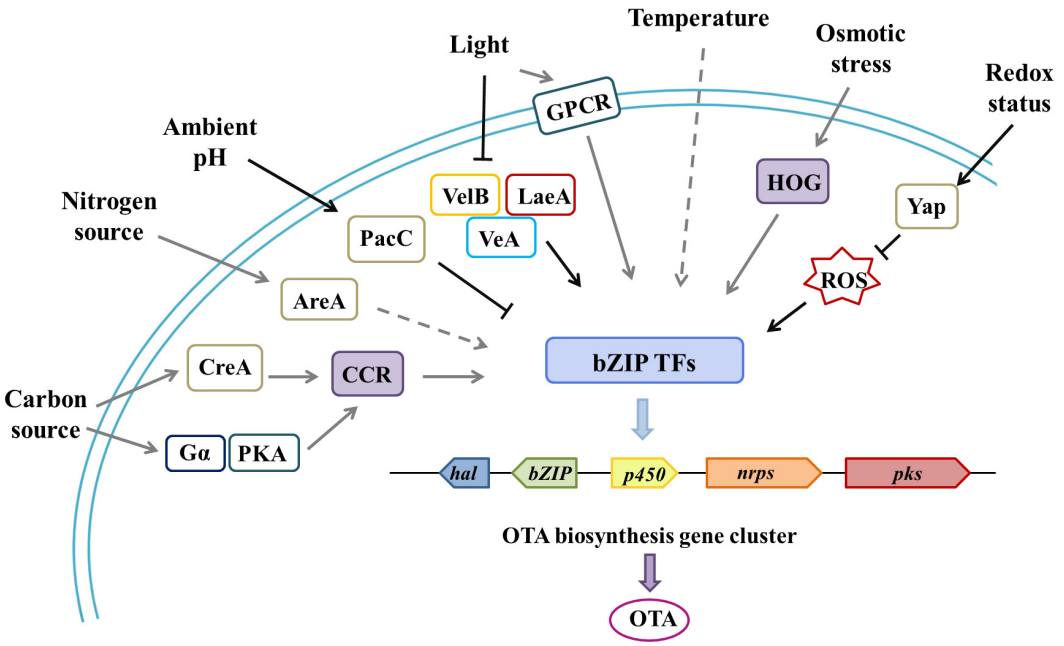

FIGURE 2 | Environmental factors and associated regulatory elements that affect OTA biosynthesis through cluster-specific transcription factors.| Through various global transcription factors or specific pathways, related environmental cues affect the regulation of OTA gene clusters, which contain four highly conserved biosynthetic genes (*pks, nrps, hal/chl, p450*) and a cluster-specific, transcription factor gene (*bZIP*). bZIP plays a pivotal role in processing various environmental signals for a carbon source (CreA transcription factor and the carbon catabolite repression pathway), a nitrogen source (AreA transcription factor and the nitrogen catabolite repression pathway), light (Velvet complex proteins, VelB-VeA-LaeA, and the global regulator VosA), temperature and humidity, redox status (Yap transcription factor and ROS), and osmotic stress (the high osmolarity glycerol system). At the same time, there are also some examples where G protein pathways are partly involved in the perception of environmental cues, such as Gα(GanB) and PKA (PkaA) in *A. nidulans* and GPCR (NopA) in *A. fumigatus*. In this figure, the black solid arrows indicate connections verified in OTA-producing fungi; the gray solid arrows indicate connections that have been verified in other toxin-producing fungi, but not verified in OTA-producing fungi; and the gray dashed arrows indicate possible, yet unproven, connections.

signal (Nagy, 1991). In microorganisms, the bacterial rhodopsins bind retinal and act as light-driven proton pumps that pump protons from intracellular to extracellular regions? areas? to form a proton gradient, which is used by ATPase to synthesize ATP (Ignatov and Mosin, 2014). One class of fungal GPCR that is similar to bacterial rhodopsin has been identified. In *Neurospora crassa*, the NOP-1 receptor that is homologous to the bacterial opsin was verified and classified as a class IX GPCR. Results from Northern analysis supported light-based regulation of *nop-1* gene expression, and the NOP-1 protein functions as rhodopsin in *N. crassa* photobiology; (Bieszke et al., 1999) a unique homolog of NOP-1 was also identified in *Aspergillus* species (**Supplementary Table 1**).

The heterotrimeric Velvet complexes (VelB-VeA-LaeA) is a light-dependent regulator in fungi, which contain transcription factors (i.e., VelB, VelC, and VosA), and the global regulator (VeA) interacts with methyltransferase (LaeA) to regulate fungal development and secondary metabolism (Dasgupta et al., 2016; Zhang et al., 2018; Gil-Serna et al., 2020). VeA regulates the synthesis of ST in *A. nidulans* and AF in *A. flavus* (Eom et al., 2018); the Δ*veA* and Δ*velB* mutations impair ST production in *A. nidulans* (Kim et al., 2020) and OTA production in *A. carbonarius* (Crespo-Sempere et al., 2013) and *A. ochraceus* (Wang et al., 2019). The LaeA protein acts as a positive regulator on conidia production, OTA biosynthesis, and oxidative stress tolerance in *A. niger* (Ruitao et al., 2019). VeA is not a light sensor, but shows light-dependent mobility. Under light exposure, it occurs abundantly in the cytoplasm and is associated with filamentous bodies, but in the dark it is transferred into the nuclei by integrins. There, it is functionally active and interacts in concert with other proteins to form the velvet complex; it supports sexual reproduction and secondary metabolism in the dark and sporulation under light conditions (Calvo et al., 2016). Studies in *A. nidulans* linked the light-regulated Velvet pathway and GPCR, in which VeA regulators act as the bond. GprH,GprI (Class V), and GprM (Class VII) receptors collectively represent a carbon starvation-induced, nutrient sensing mechanism, which sensed glucose and propagated signals through the light-responsive VeA pathways and cAMP-PKA pathways to promote vegetative growth. GprH coordinated sexual development by regulating VeA nuclear localization and activity and then it repressed sexual development and ST production (Dos Reis et al., 2019).

Interestingly, different wavelengths are sensed by different light-receptors and have almost the opposite effect on mycotoxin production. This similar phenomenon was found in *Cherax quadricarinatus* because the activation of the Gq protein was related to the wavelength for light stimulation (Yan et al., 2003). Major OTA-produced *Aspergillus* and *Penicillium* spp. exposed to white light consistently reduced OTA production, whereas

Fusarium spp. produced more fumonisin under light conditions (Fanelli et al., 2016). In *A. ochraceus*, *A. niger,* and *Penicillium* spp., red and blue wavelengths reduced OTA biosynthesis by modulating the level of expression of ochratoxin polyketide synthase. However, the red light for *A. carbonarius* and the yellow and green light for *A. steynii* caused increased OTA production (Fanelli et al., 2016). Different wavelengths of light are known to be accepted by different protein receptors, but the association with GPCRs needs to be explored further.

Stress

Fungi have sophisticated signaling cascades to sense and to respond to different stressors, which include UV irradiation, temperature, osmotic shock, high salt, oxidative or nitrosative damage, and exposure to antifungal drugs (Arroyo et al., 2009). Mainly involved is the high osmolarity glycerol (HOG) system and its activated MAPK pathway. In addition to stress control, the fungal HOG pathway regulates cell-cycle progression, sexual development, and morphological differentiation. *Cryptoccus neoformans* has developed a specialized HOG pathway. Hog1 of *C. neoformans* is constitutively phosphorylated under normal conditions and represses sexual reproduction and the synthesis of capsule and melanin. In *P. verrucosum, P. nordicum*, and *A. carbonarius*, osmotic stress is associated with severe changes in OTA biosynthesis through the HOG pathway (De Assis et al., 2020). Metabolic reactions are triggered in part by cells in response to oxidative stress (redox state). In *A. parasiticus*, oxidative stress represented by intracellular ROS accumulation, enhanced AF production, but the application of antioxidants, such as butanol, reduced AF production (Jayashree and Subramanyam, 2000; Reverberi et al., 2005).The transcription factor Yap1 in *S. cerevisiae* controlled toxins produced in response to oxidative stress. Deletion of the *Yap-1* homologous gene *Aoyap1* in *A. ochraceus* damaged the activity of superoxide dismutases and catalases and, therefore, did not effectively scavenge high-level ROS to maintain the redox balance, which triggered the biosynthesis of OTA (Reverberi et al., 2012). Yap1 orthologs are coregulators of oxidative stress response with secondary metabolism in other aspergilli, such as *napA* in *A. nidulans*, *Apyap1* in *A. parasiticus*, and *Afap1* in *A. flavus* (Reverberi et al., 2007, 2008, 2012; Yin et al., 2013; Guan et al., 2019; Mendoza-Martinez et al., 2020). On the other hand, ROS catalyzed the production of oxylipin non-enzymatically, which is the ligand of receptors GprC and GprD, and they affected cellular activity that included toxin synthesis (described below). However, evidence for a strong link between the redox state and GPCR is still lacking.

pH

G-protein-coupled receptors are mainly located on the surface of cell membranes and are regularly exposed to a wide range of dynamic pH microenvironments. Human OGR1 and GPR4 are typical proton-sensing, G-protein coupled receptors involved in pH homeostasis. GPR4, GPR65, and GPR68 receptors can be activated directly by pH and elicit cAMP formation (Rowe et al., 2020). However, no fungal proton-sensing GPCRs have been reported. In fungi, the perception of pH is closely related to the PacC pH adaptation signaling pathway. The dedicated proteins PalH, PalI, PalF, PalC, PalA, and PalB transmit environmental pH changes to transcription factor PacC (Cornet and Gaillardin, 2014). PacC effectively inhibits the expression of the known acid-expressed genes and undergoes pH-dependent proteolytic cleavage under alkaline ambient pH conditions, and then it regulates neutral-alkaline sensing in filamentous fungi (Tilburn et al., 1995; Penalva et al., 2008; Bignell, 2012). PacC is involved in the synthesis of secondary metabolites in fungi that activates penicillin biosynthesis at an alkaline pH (Brakhage, 1998), but it inhibits the biosynthesis of AF in *A. parasiticus*, ST in *A. nidulans*, OTA in *A. ochraceus*, fumonisin in *F. verticillioides* (Keller et al., 1997; O'callaghan et al., 2006), and gliotoxin in *A. fumigatus* (Kapetanakou et al., 2009). The regulation of pH in OTA synthesis is related to *pks* genes in the OTA biosynthesis gene cluster. Expression of the *otapksPN* gene in *P. nordicum* has been reported to be lower under acidic conditions below pH 5 (Geisen, 2004). Currently, there is no evidence that the pH sensor interacts with GPCRs in fungi. However, the structure of sensing external signals contained in pH sensors is similar to GPCR, which contains 7TMDs and a cytoplasmic C-terminus (Herranz et al., 2005; Hervas-Aguilar et al., 2010), and both regulate OTA synthesis at the transcriptional level. Therefore, we speculate that there is a certain connection between potential GPCRs and pH sensing in fungi, just like human pH-sensing GPCRs.

Temperature and Humidity

Temperature and humidity both are key factors in the environmental regulation of fungal growth and secondary metabolites (MarÍN et al., 1998; Tai et al., 2020; Wang et al., 2020). The minimum temperature for fungal growth increases with the enhanced inhibition of other environmental factors on growth, and mycotoxin production increases with the humidity increasing at the same temperature. However, there was not always a positive correlation between water activity and mycotoxin production in different cultures. The temperature range for mycotoxin production is stricter than for fungal growth. For example, suboptimal growth conditions that enhanced mycotoxin production is the optimum temperature for the biosynthesis of AF (i.e., 30°C), but the optimum temperature for *Aspergillus* growth is 37°C (Marc et al., 2002). The RNA-seq results of Yu et al. (year?) showed that most of the genes involved in AF synthesis, which included the cluster-specific regulatory genes *aflR* and *aflS*, were highly upregulated at low temperatures (Yu et al., 2011). Affeldt et al. (year?) annotated 15 GPCR genes in the *A. flavus* genome. The expression of *gprC*, *gprD*, *gprF*, *gprG*, and *gprO* were significantly different at three temperatures (20°C, 28°C, and 37°C). The deletion of either *gprC* or *gprD* resulted in a severe, temperature-dependent growth defect that was independent of the carbon source in *A. fumigatus* (Gehrke et al., 2010; Han et al., 2019). Knockout of *gprD* at a low temperature (20°C) increased the yield of AF, but there was no significant difference between wild-type and mutant under 28°C and 37°C (Han et al., 2019); this may have been due to the existence of alternative pathways to maintain normal biological functions.

The type of GPCR receptor and their ligands are being explored continuously, and the crossover phenomenon suggests that we cannot completely separate the G-protein signaling pathway and environmental factors perception for the regulation of OTA synthesis. Macroscopically, the dominant influence of temperature and humidity on OTA yield has been observed, and oxidative stress also plays a significant role; common stimuli, such as light and pH, also play a role. Indeed, studies have linked this regulation with GPCR. Although a large number of environmental factors influence the phenotypic phenomena associated with OTA biosynthesis, further confirmation of these is lacking. GPCRs play a critical role in perceiving these environmental stimuli and regulating the appropriate signaling pathways. However, the importance of GPCRs and the functional connections in regulating secondary metabolism are unknown.

GPCRS MEDIATE TRANS-KINGDOM COMMUNICATION

The natural interactions of fungi with bacteria, and cross-talks between infecting fungi and their infected hosts, induce many response signals that are sensed by fungi. Fungal G-protein signaling pathways that regulate cell behavior are also influenced by signaling molecules derived within and between fungal species (Fischer and Keller, 2016; Van Dijck et al., 2017), which implies that communication between species is partly mediated through GPCRs. This suggests that further study of these communication events is necessary to find targets to control mycotoxin biosynthesis.

Fungal Quorum Sensing

Fungal QS is one of the main mechanisms for intra- and interspecific communication. Hormone-like small molecular compounds known as quorum-sensing molecules (QSMs) are secreted by fungi and enter into other cells through specific transporters and activate the expression of corresponding genes in a density-dependent manner within cells?. The QS mechanism plays a role in monitoring population density and regulating the physiology of fungal cells, that affect growth, sexual/asexual reproduction, apoptosis, secondary metabolism, and pathogenesis. This mechanism causes fungi to coordinate their actions and to enhance their survival, host immune evasion, and infection ability to adapt to environmental changes. Currently, verified fungal QSMs include alcohols, oxylipins, small molecule peptide pheromones, and certain volatile substances (Wongsuk et al., 2016; Barriuso et al., 2018; Padder et al., 2018). The perception of pheromones and their effects on fungal biology are mediated by GPCRs, and oxylipin is also a class of ligand for GPCRs.

Oxylipins, which is a large family of enzymatic or non-enzymatic oxidation products of fatty acids, are common signaling molecules in animals, plants, and microorganisms (Wasternack and Feussner, 2018). Treatment of *A. nidulans* wild-type with exogenous oxylipins resulted in cAMP accumulation, but this could be prevented in the absence of the *gprD* gene (De et al., 2013). Indeed, studies on both the GprD protein of *A. nidulans* and GprC and/or GprD proteins of *A. fumigatus* revealed that oxylipins were likely to be a type of ligand of these fungal GPCRs and to activate the cAMP pathway (Affeldt et al., 2012).

9S-Hydroxyoctadecadienoic acid (9-HODE), 13S-Hydroxyoctadecadienoic acid (13-HODE), and their derivatives that are derived from linoleic acid, act as crucial signals and elicitors of secondary metabolites in fungi and plants. 9(S)-HODE inhibited *A. ochraceus* sporulation and promoted the production of OTA, but 13(S)-HODE promoted the sporulation and inhibited the production of OTA (Reverberi et al., 2010). 13(S)-HPODE inhibited the expression of mycotoxin synthesis genes of *A. parasiticus* (*ver-1*) and *A. nidulans* (*stcU*) and significantly reduced the production of AFB1 and ST, although the inhibition effect at the same concentration of 9(S)-HPODE was not obvious (Burow et al., 1997).

Precocious sexual inducer (PSI) factors are mixed oxylipin signals produced by PpoA-C oxygenases, which are the main oxidases that regulate oxylipin synthesis in *Aspergillus* species, and the ratio of psiA-C determines if fungi enter sexual or asexual development (Fischer and Keller, 2016). *A. fumigatus* and *A. flavus* can produce the secreted PpoA oxylipin 5,8-dihydroxyoctadecadienoic acid (5,8-diHODE), which use a model of an autocrine-like mechanism to regulate hyphal branching. Also, the rice blast pathogen *Magnaporthe grisea* produces the branching-inducing oxylipin 7,8-diHODE. When exposed to exogenous 5,8-diHODE, *M. grisea* germlings differentiated predominantly into appressoria, the infectious structure required for plant penetration (Niu et al., 2020).On the other hand, *M. grisea* can produce the autocrine signal 7,8-diHODE to induce branching, and it is proposed that there is a cross-genera recognition of fungal dihydroxyl oxylipins between *Aspergillus* and *M. grisea*, where the 5,8-diHODE acts as a paracrine signaling molecule between cells of some fungal species.

Knocking out the *ppo* genes in *A. nidulans* reduced the production of oxylipins and then it disrupted the balance of sexual/asexual sporulation, although the double Δ*ppoA*Δ*ppoC* mutants and triple Δ*ppoA–C* mutants lost their ability to produce the mycotoxin ST, but showed an overproduction of the antibiotic penicillin. It also weakened the fungal ability to colonize in peanuts and maize hosts (Tsitsigiannis and Keller, 2010). These phenotypes were similar to the constitutively activated Gα, *An*Fad^{AG42R}, which suppressed the ST inducer gene *AnAflR*, but enhanced the penicillin biosynthetic gene *AnIpnA* that was mediated through the cAMP-PKA pathway (Tag et al., 2010). Disruption of *Ppo* orthologs also reduced T2 production in *Fusarium sporotrichioides* (Mcdonald et al., 2003). After knocking out all five dioxygenase genes (*ppoA-D* and *lox*), *A. flavus* lost its density-dependent regulation of sporulation and AF production; both the Δ*ppoC* and Δ*lox* mutant strains produced high levels of AF at any population density (Brown et al., 2009). Oxylipins added at different concentrations exogenously affected the normal morphological development of AF. In addition, when fungi were exposed to oxidative stress, such as an increased ROS level, this led to the synthesis of oxylipins and induced toxin production.

Therefore, the QS mechanism represented by fungal oxylipins can impact fungal sporulation, mycotoxin production, and virulence in a density-dependent regulation that is partly mediated by GPCRs.

Interspecies Fungal Communication

Fungi communicate with other fungi and bacteria to produce signaling molecules that also regulate their growth form and virulence through G-protein signaling pathways. For example, in co-cultivation of *C. albicans* and *A. nidulans*, farnesol produced by the former affected the latter by inhibiting its growth and/or inducing apoptosis (Semighini et al., 2006). Exposure of *A. nidulans* to farnesol did not influence the emergence of the germ-tube, but relied on G-protein signaling to affect mitochondrial function and ROS production; what was dependent? dependent on autophagy and PKC signaling, which caused cell apoptosis. Experiments have shown that the Δ*flbA* mutation affected activity of the Gα protein FadA that caused a significant increase in the sensitivity of farnesol, which revealed the signal transduction role of the FadA-G protein complex in this fungal interspecies communication process (Semighini, 2006; Savoldi et al., 2010). Conversely, another QSM pantothenic acid has positive impacts on fungal growth. Both *C. neoformans*-conditioned media, which contained *C. neoformans* autocrine pantothenic acid, and exogenous pantothenic acid increased the growth of *C. albicans* and *S. cerevisiae* (Albuquerque et al., 2014).

Fungi and bacteria can interact through signaling molecules. *Pseudomonas aeruginosa* is often found with *C. albicans* in mixed mammalian infections; the former can grow on and damage filamentous hyphae, but not budding yeast cells, and the bacterial QSM homoseryl lactone inhibits filamentation (Hogan et al., 2004; Cugini et al., 2010). Recent studies have shown that in mixed *C. albicans* and *S. aureus* biofilm, farnesol produced by the former induced ROS in the latter bacterium, which resulted in the up-regulation of drug efflux pumps that protected bacterial cells from antibiotic damage (Kong et al., 2017).

Host–Pathogen Communication

There is a complex GPCR-mediated communication between fungi and their hosts, in which oxylipins may play a completely different, but crucial role (**Figure 3**). For example, Gpr1 of

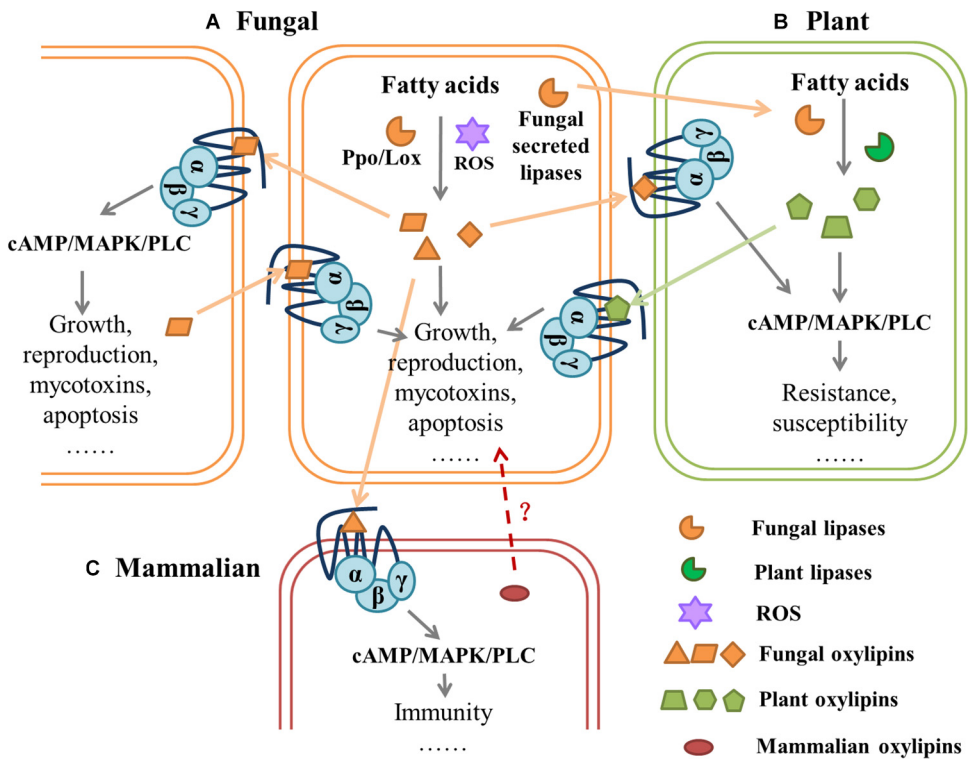

FIGURE 3 | Hypothetical model of mediated communication of G-protein-coupled receptors between fungi and their hosts.| **(A)** Fungal lipoxygenase (Lox) and psi-producing oxygenases (Ppo) or reactive oxygen species (ROS) can catalyze the oxidation of fatty acids (FAs) to oxylipins, which affect fungal behavior through downstream pathways. Different species of fungi can sense oxylipins secreted *in vitro* by GPCRs, which regulate cell growth, reproduction, mycotoxins, and apoptosis. **(B)** Fungal lipases are secreted into plant cells where fatty acid substrates are cleaved and processed by fungi- secreted lipoxygenases and/or plant lipoxygenases for oxylipin production. Plant-produced oxylipins are perceived and exploited by fungi to regulate GPCR-mediated behavior. **(C)** In mammalian cells, different signals from microbial-derived oxylipids, mediate inflammatory responses, and fungi can affect host physiology through GPCRs. It is also possible that mammalian oxylipins affect fungal activity. All fungal lipases and oxylipins are orange, all plant lipases and oxylipins are green, mammalian oxylipins are red. Dashed arrows with the question mark (?) indicates hypothetical interactions, and solid arrows represent proven interactions.

S. cerevisiae showed structural and functional homology with mammalian GPCRs, and 9(S)-HODE and other oxidized free fatty acids bound to the mammalian G2A (oxylipins receptor), which inhibited cellular cAMP accumulation and MAP kinase activation (Obinata et al., 2005). Further, different eicosanoids mediated inflammatory responses through GPCRs in mammals (Dennis and Norris, 2015). *Ca*Gpr1 in *C. albicans* detected l-lactate released by the gut microorganism *Lactobacillus reuteri,* which promoted fungal β-glucan masking and evaded the mammalian immune system (Ballou et al., 2016). *C. albicans* can secrete QSMs tyrosol and farnesol; the former hindered the killing of mammalian host neutrophils by inhibiting ROS production (Josef et al., 2010), and the latter induced macrophage apoptosis (Abe et al., 2010). In the entomopathogenic *Metarhizium* species, deletion of the GPCR *Mr*Gpr8 (Class XIV Pth11-like GPCR) substantially impaired the nucleus translocation in MAPK, which resulted in the failure of appressorium to form on different substrates and the loss of virulence during topical infection of insects. The Δ*MrGpr8* mutants could not be rescued with the addition of cAMP for appressorium formation (Shang et al., 2020). This model recognizes that the G protein signaling pathway can integrate intra- and interspecific signal transmission and cellular activity. In humans and mice, it is well-established that GPCRs detect microbial-derived signals and that the microbiota can affect host physiology through GPCRs (Dierking and Pita, 2020).

Plant hosts secrete a variety of elicitors that induce defense responses against the infecting fungi, but fungal signal molecules also modulate host immunity and damage host cells (Ariyo et al., 1997; Liu et al., 2001; Benz et al., 2014). The plant oxylipin jasmonate inhibited fungal reproduction and secondary metabolism as a defense against necrotrophic fungal pathogens (Calvo et al., 1999), and it can also promote *F. oxysporum* infection (Thatcher et al., 2009). Plant oxylipins seem to have a completely different effect on fungi. Linoleic acid and 9S-HPODE promoted mycotoxin synthesis in *Aspergillus*, whereas 13S-HPODE inhibited it (Burow et al., 1997). *A. ochraceus* prefers to infect crops that contain more fatty acid and produce more OTA (Caiyan et al., 2017), which is speculated to be related to the oxidation of plant fatty acids to produce oxylipins. Who proposed? proposed a hypothetical model that the host-derived oxylipins can bind with fungal GPCR to regulate growth, sporulation, and synthesis of mycotoxin; on the other hand, these oxylipins may stimulate biosynthesis of fungal oxylipins. The psiB factor in *Aspergillus* is also derived from linoleic acid.

The complementation of Δ*ppoA*Δ*ppoC* mutants with the maize *ZmLOX3* gene restored conidiation and, thus, the ability of plant oxylipins to mimic or to interfere with fungal signaling may be due to structural similarities (Brodhagen et al., 2010). Destroying the *ZmLOX3* gene of maize caused a deficiency in 9-LOX derivatives, which compromised conidiation, pathogenicity, and mycotoxin production of maize pathogenic *Fusarium verticillioides*, and this promoted resistance to other fungal pathogens (Wilson et al., 2001). On the contrary, maize that lacked the *lox3* gene were more susceptible to *Aspergillus* infection, and AF contamination was more serious, which indicated that host oxylipins promoted pathogenesis in addition to resisting pathogenic (Reverberi et al., 2010; Yan et al., 2015).

Another mechanism proposed in *F. graminearum* is that *Aspergillus* can secrete lipases and LOX in host cells to regulate host lipid metabolism by cleaving off free fatty acids and oxidizing them to produce oxylipins. Fungi can then use host-derived oxylipins to facilitate invasive growth, spores, and mycotoxin production (Voigt et al., 2010). Similarly, host-derived 3-hydroxyoxylipin promoted the ability of *C. albicans* to grow and become more virulent within mammalian cells, whereas salicylic acid treatment inhibited fungal development and biofilm formation (Tsitsigiannis and Keller, 2007).

In summary, QS plays an important role in intra- and interspecies communication, which impact fungal development, mycotoxin regulation, and disease. The key role of oxylipins and their status as GPCR ligands suggest that these mechanisms are related to the G-protein signaling pathway, but the receptors capable of sensing these QSMs and the specific mechanisms remain to be discovered.

CONCLUSION

Biological detoxification methods have greater safety, availability, and cost-effectiveness than physical and chemical detoxification methods. Currently, research advances in OTA biodetoxification has been made in degradation, adsorption, or enzymatic degradation[9]. With the development of bioinformatics and molecular biology, researchers have turned to studies of molecular biological mechanisms to crack the code of fungal physiology. Fungal GPCRs have been proposed as targets for controlling mycotoxins. The importance of the GPCR signaling pathway to fungal biology and virulence is underexplored, and only a limited number of receptors have been shown to regulate OTA synthesis directly. However, there are a large number of unclassified orphan GPCRs. The ligands of these orphan GPCRs are unidentified, and their physiological role is yet to be determined, which implies a huge number of possible targets link the perception of extracellular signals with mycotoxin synthesis, such as pH-sensing GPCRs. Another area worthy of further study is that of quorum sensing inhibition through the blockage of signal production (i.e., Quorum Quenching), which limits fungal growth and mycotoxin production by affecting intercellular communication. This can be achieved partly by inhibiting the GPCR's reception of QSMs such as oxylipins (Turan and Engin, 2018). Therefore, fungal-specific GPCRs represent promising and unexplored targets to potentially intervene or to reduce the impact of mycotoxin contamination and fungal diseases. Deeper understanding of fungal GPCRs will enhance our ability to develop novel strategies in agricultural and clinical settings to promote human, animal, plant, and even ecosystem health.

AUTHOR CONTRIBUTIONS

JG contributed to conception and design of the study, and wrote the first draft of the manuscript. XX wrote sections of the manuscript. KH final approval of the version to be published. ZL wrote sections of the manuscript and final approval of the version to be published. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 31671947).

ACKNOWLEDGMENTS

We would like to thank Thomas A. Gavin, Professor Emeritus, Cornell University, for help with editing this paper.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.631392/full#supplementary-material

Supplementary Table 1 | Classification of G-protein-coupled receptors in fungi (Martín et al., 2019).

REFERENCES

Abe, S., Tsunashima, R., Iijima, R., Yamada, T., and Maruyama, N. (2010). Suppression of anti-*Candida* activity of macrophages by a quorum-sensing molecule, farnesol, through induction of oxidative stress [J]. *Microbiol. Immunol.* 53, 323–330. doi: 10.1111/j.1348-0421.2009.00128.x

Adnan, M., Zheng, W. H., Islam, W., Arif, M., Abubakar, Y. S., Wang, Z. H., et al. (2018). Carbon catabolite repression in filamentous fungi [J]. *Int. J. Mol. Sci.* 19:48. doi: 10.3390/ijms19010048

Affeldt, K. J., Brodhagen, M., and Keller, N. P. (2012). *Aspergillus oxylipin* signaling and quorum sensing pathways depend on g protein-coupled receptors [J]. *Toxins* 4, 695–717. doi: 10.3390/toxins4090695

Albuquerque, P., Nicola, A. M., Nieves, E., Paes, H. C., Williamson, P. R., Silva-Pereira, I., et al. (2014). Quorum sensing-mediated, cell density-dependent regulation of growth and virulence in *Cryptococcus neoformans* [J]. *mBio* 5:e0986-13.

Alkhayyat, F., and Yu, J. H. (2014). Upstream regulation of mycotoxin biosynthesis [J]. *Adv. Appl. Microbiol.* 86, 251–278. doi: 10.1016/b978-0-12-800262-9.00005-6

Ansari, K., Martin, S., Farkasovsky, M., Ehbrecht, I. M., and Kuntzel, H. (1999). Phospholipase C binds to the receptor-like GPR1PROTEIN and controls pseudohyphal differentiation in *Saccharomyces cerevisiae* [J]. *J. Biol. Chem.* 274:30052. doi: 10.1074/jbc.274.42.30052

Antonia, S., Proctor, R. H., Massimiliano, M., Miriam, H., Antonia, G., Logrieco, A. F., et al. (2016). Variation in fumonisin and ochratoxin production associated with differences in biosynthetic gene content in *Aspergillus niger* and *A. welwitschiae* isolates from multiple crop and geographic origins [J]. *Front. Microbiol.* 7:1412. doi: 10.3389/fmicb.2016.01412

Ariyo, B. T., And, C. B., and Keshaverz, T. (1997). Alginate oligosaccharides as enhancers of penicillin production in cultures of penicillium chrysogenum [J]. *Biotechnol. Bioeng.* 53, 17–20. doi: 10.1002/(sici)1097-0290(19970105)53:1<17::aid-bit3>3.0.co;2-1

Arroyo, J., Bermejo, C., Garcia, R., and Rodriguez-Pena, J. M. (2009). Genomics in the detection of damage in microbial systems: cell wall stress in yeast [J]. *Clin. Microbiol. Infect.* 15, 44–46. doi: 10.1111/j.1469-0691.2008.02676.x

Atoui, A., Bao, D. P., Kaur, N., Grayburn, W. S., and Calvo, A. M. (2008). *Aspergillus nidulans* natural product biosynthesis is regulated by mpkB, a putative pheromone response mitogen-activated protein kinase [J]. *Appl. Environ. Microbiol.* 74, 3596–3600. doi: 10.1128/aem.02842-07

Baldwin, J. M. (1993). The probable arrangement of the helices in G protein-coupled receptors [J]. *EMBO J.* 12, 1693–1703. doi: 10.1002/j.1460-2075.1993.tb05814.x

Ballesteros, J. A., Jensen, A. D., Liapakis, G., Rasmussen, S. G. F., and Javitch, J. A. (2001). Activation of the β2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6 [J]. *J. Biol. Chem.* 276:29171. doi: 10.1074/jbc.m103747200

Ballou, E. R., Avelar, G. M., Childers, D. S., Mackie, J., Bain, J. M., Wagener, J., et al. (2016). Lactate signalling regulates fungal β-glucan masking and immune evasion [J]. *Nat. Microbiol.* 2:16238.

Barriuso, J., Hogan, D. A., Keshavarz, T., and Martinez, M. J. (2018). Role of quorum sensing and chemical communication in fungal biotechnology and pathogenesis [J]. *FEMS Microbiol. Rev.* 42, 627–638. doi: 10.1093/femsre/fuy022

Bebber, D. P., Ramotowski, M. A. T., and Gurr, S. J. (2013). Crop pests and pathogens move polewards in a warming world [J]. *Nat. Clim. Change* 3, 985–988. doi: 10.1038/nclimate1990

Bennett, R. J., and Johnson, A. D. (2010). The role of nutrient regulation and the Gpa2 protein in the mating pheromone response of *C. albicans* [J]. *Mol. Microbiol.* 62, 100–119. doi: 10.1111/j.1365-2958.2006.05367.x

Benz, J. P., Chau, B. H., Zheng, D., Bauer, S., Glass, N. L., and Somerville, C. R. A. (2014). comparative systems analysis of polysaccharide-elicited responses in Neurospora crassa reveals carbon source-specific cellular adaptations [J]. *Mol. Microbiol.* 91, 275–299. doi: 10.1111/mmi.12459

Bieszke, J. A., Braun, E. L., Bean, L. E., Kang, S. C., Natvig, D. O., and Borkovich, K. A. (1999). The nop-1 gene of Neurospora crassa encodes a seven transmembrane helix retinal-binding protein homologous to archaeal rhodopsins [J]. *Proc. Natl. Acad. Sci. U.S.A.* 96, 8034–8039. doi: 10.1073/pnas.96.14.8034

Bignell, E. (2012). The molecular basis of pH sensing, signaling, and homeostasis in fungi [J]. *Adv. Appl. Microbiol.* 79, 1–18. doi: 10.1016/b978-0-12-394318-7.00001-2

Bohm, J., Hoff, B., O'gorman, C. M., Wolfers, S., Klix, V., Binger, D., et al. (2013). Sexual reproduction and mating-type-mediated strain development in the penicillin-producing fungus *Penicillium chrysogenum* [J]. *Proc. Natl. Acad. Sci. U.S.A.* 110, 1476–1481. doi: 10.1073/pnas.1217943110

Brakhage, A. A. (1998). Molecular regulation of beta-lactam biosynthesis in filamentous fungi [J]. *Microbiol. Mol. Biol. Rev.* 62, 547–585. doi: 10.1128/mmbr.62.3.547-585.1998

Brodhagen, M., Tsitsigiannis, D. I., Hornung, E., Goebel, C., and Keller, N. P. (2010). Reciprocal oxylipin-mediated cross-talk in the *Aspergillus*-seed pathosystem [J]. *Mol. Microbiol.* 67, 378–391. doi: 10.1111/j.1365-2958.2007.06045.x

Brown, N. A., Dos, Reis TF, Ries, L. N., Caldana, C., Mah, J. H., Yu, J. H., et al. (2015). G-protein coupled receptor-mediated nutrient sensing and developmental control in *Aspergillus nidulans* [J]. *Mol. Microbiol.* 98, 420–439. doi: 10.1111/mmi.13135

Brown, N. A., and Goldman, G. H. (2016). The contribution of *Aspergillus fumigatus* stress responses to virulence and antifungal resistance [J]. *J. Microbiol.* 54, 243–253. doi: 10.1007/s12275-016-5510-4

Brown, N. A., Schrevens, S., Dijck, P. V., and Goldman, G. H. (2018). Fungal G-protein-coupled receptors: mediators of pathogenesis and targets for disease control [J]. *Nat. Microbiol.* 3, 402–414. doi: 10.1038/s41564-018-0127-5

Brown, S. H., Scott, J. B., Bhaheetharan, J., Sharpee, W. C., and Keller, N. P. (2009). Oxygenase coordination is required for morphological transition and the host-fungus interaction of *Aspergillus flavus* [J]. *Mol. Plant Microbe Interact.* 22:882. doi: 10.1094/mpmi-22-7-0882

Brunner, K., Omann, M., Pucher, M. E., Delic, M., Lehner, S. M., Domnanich, P., et al. (2008). Trichoderma G protein-coupled receptors: functional characterisation of a camp receptor-like protein from *Trichoderma atroviride* [J]. *Curr. Genet.* 54, 283–299. doi: 10.1007/s00294-008-0217-7

Burkholder, A. C., and Hartwell, L. H. (1985). The yeast alpha-factor receptor: structural properties deduced from the sequence of the STE2 gene [J]. *Nuclie Acids Res.* 13, 8463–8475. doi: 10.1093/nar/13.23.8463

Burow, G. B., Nesbitt, T. C., Dunlap, J., and Keller, N. P. (1997). Seed lipoxygenase products modulate *Aspergillus* mycotoxin biosynthesis [J]. *Mol. Plant Microbe Interact.* 10, 380–387. doi: 10.1094/mpmi.1997.10.3.380

Cabrera, I. E., Pacentine, I. V., Lim, A., Guerrero, N., Krystofova, S., Li, L., et al. (2015). Global analysis of predicted G protein coupled receptor genes in the filamentous fungus, *Neurospora crassa* [J]. *G3 Genes Genomes Genet.* 5, 2729–2743. doi: 10.1534/g3.115.020974

Caceres, I., Al Khoury, A., El Khoury, R., Lorber, S., Oswald, I. P., El Khoury, A., et al. (2020). Aflatoxin biosynthesis and genetic regulation: a review [J]. *Toxins* 12:150. doi: 10.3390/toxins12030150

Caiyan, L., Yanmin, S., Lu, X., Kunlun, H., and Zhihong, L. (2017). Initial spore density has an influence on ochratoxin a content in *Aspergillus ochraceus* CGMCC 3.4412 in PDB and its interaction with seeds [J]. *Toxins* 9:146. doi: 10.3390/toxins9040146

Calvo, A. M., Hinze, L. L., Gardner, H. W., and Keller, N. P. (1999). Sporogenic effect of polyunsaturated fatty acids on development of *Aspergillus* spp [J]. *Applenvironmicrobiol* 65, 3668–3673. doi: 10.1128/aem.65.8.3668-3673.1999

Calvo, A. M., Lohmar, J. M., Ibarra, B., and Satterlee, T. (2016). *18 Velvet Regulation of Fungal Development [M]*. Berlin: Springer International Publishing.

Chen, R. E., and Thorner, J. (2007). Function and regulation in MAPK signaling pathways: lessons learned from the yeast *Saccharomyces cerevisiae* [J]. *Biochim. Biophys. Acta* 1773:1311. doi: 10.1016/j.bbamcr.2007.05.003

Cornet, M., and Gaillardin, C. (2014). pH signaling in human fungal pathogens: a new target for antifungal strategies (vol 13, pg 342, 2014) [J]. *Eukaryotic Cell* 13:691. doi: 10.1128/ec.00073-14

Crespo-Sempere, A., Marin, S., Sanchis, V., and Ramos, A. J. (2013). VeA and LaeA transcriptional factors regulate ochratoxin A biosynthesis in *Aspergillus carbonarius* [J]. *Int. J. Food Microbiol.* 166, 479–486. doi: 10.1016/j.ijfoodmicro.2013.07.027

Cugini, C., Calfee, M. W., Farrow, J. M., Morales, D. K., and Hogan, D. A. (2010). Farnesol, a common sesquiterpene, inhibits PQS production in *Pseudomonas aeruginosa* [J]. *Mol. Microbiol.* 65, 896–906. doi: 10.1111/j.1365-2958.2007.05840.x

Dasgupta, A., Fuller, K. K., Dunlap, J. C., and Loros, J. J. (2016). Seeing the world differently: variability in the photosensory mechanisms of two model fungi [J]. *Environ. Microbiol.* 18, 5–20. doi: 10.1111/1462-2920.13055

De, S. W. R., Rezende, M. E., Graciele, K. N., Marcela, S., Goldman, M. H. S., Fernando, R., et al. (2013). Identification of metabolic pathways influenced by the G-protein coupled receptors GprB and GprD in *Aspergillus nidulans* [J]. *PLoS One* 8:e62088. doi: 10.1371/journal.pone.0062088

De Assis, L. J., Silva, L. P., Liu, L., Schmitt, K., Valerius, O., Braus, G. H., et al. (2020). The high osmolarity glycerol mitogen-activated protein kinase regulates glucose catabolite repression in filamentous fungi [J]. *PLos Genet.* 16:e1008996. doi: 10.1371/journal.pgen.1008996

Dennis, E. A., and Norris, P. C. (2015). Eicosanoid storm in infection and inflammation (vol 15, pg 511, 2015) [J]. *Nat. Rev. Immunol.* 15:511. doi: 10.1038/nri3859

Dierking, K., and Pita, L. (2020). Receptors mediating host-microbiota communication in the metaorganism: the invertebrate perspective [J]. *Front. Immunol.* 11:1251. doi: 10.3389/fimmu.2020.01251

Dignard, D., Andre, D., and Whiteway, M. (2008). Heterotrimeric G-protein subunit function in *Candida albicans*: both the alpha and beta subunits of the pheromone response G protein are required for mating [J]. *Eukaryotic Cell* 7, 1591–1599. doi: 10.1128/ec.00077-08

Dos Reis, T. F., Mellado, L., Lohmar, J. M., Silva, L. P., Zhou, J. J., Calvo, A. M., et al. (2019). GPCR-mediated glucose sensing system regulates light-dependent fungal development and mycotoxin production [J]. *PLoS Genet.* 15:e1008419. doi: 10.1371/journal.pgen.1008419

Eom, T. J., Moon, H., Yu, J. H., and Park, H. S. (2018). Characterization of the velvet regulators in *Aspergillus flavus* [J]. *J. Microbiol.* 56, 893–901. doi: 10.1007/s12275-018-8417-4

Fanelli, F., Geisen, R., Schmidt-Heydt, M., Logrieco, A. F., and Mule, G. (2016). Light regulation of mycotoxin biosynthesis: new perspectives for food safety [J]. *World Mycotoxin J.* 9, 129–145. doi: 10.3920/wmj2014.1860

Ferrara, M., Perrone, G., Gambacorta, L., Epifani, F., Solfrizzo, M., and Gallo, A. (2016). Identification of a halogenase involved in the biosynthesis of ochratoxin a in *Aspergillus carbonarius* [J]. *Appl. Environ. Microbiol.* 82, 5631–5641. doi: 10.1128/aem.01209-16

Filho, A. P. D. C., Brancini, G. T. P., De Castro, P. A., Valero, C., Ferreira Filho, J. A., Silva, L. P., et al. (2020). *Aspergillus fumigatus* G-protein coupled receptors GprM and GprJ are important for the regulation of the cell wall integrity pathway, secondary metabolite production, and virulence [J]. *mBio* 11:e02458-20.

Fischer, G. J., and Keller, N. P. (2016). Production of cross-kingdom oxylipins by pathogenic fungi: An update on their role in development and pathogenicity [J]. *J. Microbiol.* 54, 254–264. doi: 10.1007/s12275-016-5620-z

Frisvad, J. C., Hubka, V., Ezekiel, C. N., Hong, S. B., Novakova, A., Chen, A. J., et al. (2019). Taxonomy of *Aspergillus* section Flavi and their production of aflatoxins, ochratoxins and other mycotoxins [J]. *Stud. Mycol.* 93, 1–63. doi: 10.1016/j.simyco.2018.06.001

Gehrke, A., Heinekamp, T., Jacobsen, I. D., and Brakhage, A. A. (2010). Heptahelical receptors GprC and GprD of *Aspergillus fumigatus* are essential regulators of colony growth, hyphal morphogenesis, and virulence [J]. *Appl. Environ. Microbiol.* 76, 3989–3998. doi: 10.1128/aem.00052-10

Geisen, R. (2004). Molecular monitoring of environmental conditions influencing the induction of ochratoxin A biosynthesis genes in *Penicillium nordicum* [J]. *Mol. Nutr. Food Res.* 48, 532–540. doi: 10.1002/mnfr.200400036

Gil-Serna, J., GarcíA-Díaz, M., González-JaéN, M. T., Vázquez, C., and Pati, O. B. (2018). Description of an orthologous cluster of ochratoxin A biosynthetic genes in *Aspergillus* and *Penicillium* species. A comparative analysis [J]. *Int. J. Food Microbiol.* 268, 35–43. doi: 10.1016/j.ijfoodmicro.2017.12.028

Gil-Serna, J., Vazquez, C., and Patino, B. (2020). Genetic regulation of aflatoxin, ochratoxin A, trichothecene, and fumonisin biosynthesis: A review [J]. *Int. Microbiol.* 23, 89–96. doi: 10.1007/s10123-019-00084-2

Gomar-Alba, M., Jiménez-Martí, E., and Del Olmo, M. (2012). The *Saccharomyces cerevisiae* Hot1p regulated gene YHR087W (HGI1) has a role in translation upon high glucose concentration stress [J]. *BMC Mol. Biol.* 13:19. doi: 10.1186/1471-2199-13-19

Grice, C. M., Bertuzzi, M., and Bignell, E. M. (2013). Receptor-mediated signaling in *Aspergillus fumigatus* [J]. *Front. Microbiol.* 4:26. doi: 10.3389/fmicb.2013.00026

Guan, X., Zhao, Y., Liu, X., Shang, B., Xing, F., Zhou, L., et al. (2019). The bZIP transcription factor Afap1 mediates the oxidative stress response and aflatoxin biosynthesis in *Aspergillus flavus* [J]. *Rev. Argentina Microbiol.* 51, 292–301. doi: 10.1016/j.ram.2018.07.003

Hamel, L. P., Nicole, M. C., Duplessis, S., and Ellis, B. E. (2012). Mitogen-activated protein kinase signaling in plant-interacting fungi: distinct messages from conserved messengers [J]. *Plant Cell* 24, 1327–1351. doi: 10.1105/tpc.112.096156

Han, G., Zhao, K., Yan, X., Xiang, F., and Tao, F. (2019). Differential regulation of mycelial growth and aflatoxin biosynthesis by *Aspergillus flavus* under different temperatures as revealed by strand-specific Rna-Seq [J]. *MicrobiologyOpen* 8:e897.

Han, K. H., Seo, J. A., and Yu, J. H. A. (2004). putative G protein-coupled receptor negatively controls sexual development in *Aspergillus nidulans* [J]. *Mol. Microbiol.* 51, 1333–1345. doi: 10.1111/j.1365-2958.2003.03940.x

Herranz, S., Rodriguez, J. M., Bussink, H. J., Sanchez-Ferrero, J. C., Arst, H. N. Jr., Penalva, M. A., et al. (2005). Arrestin-related proteins mediate pH signaling in fungi [J]. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12141–12146. doi: 10.1073/pnas.0504776102

Herskowitz, I. (1995). MAP kinase pathways in yeast: for mating and more [J]. *Cell* 80, 187–197. doi: 10.1016/0092-8674(95)90402-6

Hervas-Aguilar, A., Galindo, A., and Penalva, M. A. (2010). Receptor-independent Ambient pH signaling by ubiquitin attachment to fungal arrestin-like PalF [J]. *J. Biol. Chem.* 285, 18095–18102. doi: 10.1074/jbc.m110.114371

Hicks, J., Lockington, R. A., Strauss, J., Dieringer, D., Kubicek, C. P., Kelly, J., et al. (2001). RcoA has pleiotropic effects on *Aspergillus nidulans* cellular development [J]. *Mol. Microbiol.* 39, 1482–1493.

Hogan, D. A., Vik, A., and Kolter, R. A. (2004). *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology [J]. *Mol. Microbiol.* 54, 1212–1223.

Hou, L. L., Le, G. N., Lin, Z. M., Qian, G., Gan, F., Gu, C., et al. (2020). Nontoxic concentration of ochratoxin A decreases the dosage of cyclosporine A to induce chronic nephropathy model via autophagy mediated by toll-like receptor 4 [J]. *Cell Death Dis.* 11:153.

Ignatov, I., and Mosin, O. (2014). Visual perception and electromagnetic conception for the eyesight. rhodopsin and bacteriorhodopsin in nano- and biotechnologies [J]. *J. Health Med. Nurs.* 4:14947.

Inadome, H., Noda, Y., Adachi, H., and Yoda, K. (2005). Immunoisolaton of the yeast golgi subcompartments and characterization of a novel membrane protein, Svp26, discovered in the Sed5-containing compartments [J]. *Mol. Cell. Biol.* 25, 7696–7710.

International Agency For Research On Cancer (1993). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 56. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins [M]*. Lyon: IARC.

Jayashree, T., and Subramanyam, C. (2000). Oxidative stress as a prerequisite for aflatoxin production by *Aspergillus parasiticus* [J]. *Free Radic. Biol. Med.* 29, 981–985.

Josef, C., Vassilia, V., and Ilja, B. (2010). 2,4-(Hydroxyphenyl)-ethanol, an antioxidative agent produced by *Candida* spp., impairs neutrophilic yeast killing in vitro [J]. *FEMS Microbiol. Lett.* 2, 319–325.

Jung, M. G., Kim, S. S., Yu, J. H., and Shin, K. S. (2016). Characterization of gprK encoding a putative hybrid G-protein-coupled receptor in *Aspergillus fumigatus* [J]. *PLoS One* 11:e0161312. doi: 10.1371/journal.pone.0161312

Kapetanakou, E., Panagou, E. Z., Gialitaki, M., Drosinos, E. H., and Skandamis, P. N. (2009). Evaluating the combined effect of water activity, pH and temperature on ochratoxin A production by *Aspergillus ochraceus* and *Aspergillus carbonarius* on culture medium and *Corinth raisins* [J]. *Food Control* 20, 725–732.

Karlson, P., and Lüscher, M. (1959). Pheromones': a new term for a class of biologically active substances [J]. *Nature* 183, 55–56.

Kasahara, S., Wang, P., and Nuss, D. L. (2000). Identification of bdm-1, a gene involved in G protein β-subunit function and α-subunit accumulation [J]. *Proc. Natl. Acad. Sci. U.S.A.* 97, 412–417.

Keller, N. P., Nesbitt, C., Sarr, B., Phillips, T. D., and Burow, G. B. (1997). pH regulation of sterigmatocystin and aflatoxin biosynthesis in *Aspergillus* spp [J]. *Phytopathology* 87, 643–648.

Khan, S. M., Sleno, R., Gora, S., Zylbergold, P., Laverdure, J.-P., Labbe, J.-C., et al. (2013). The expanding roles of Gβγ subunits in G protein–coupled receptor signaling and drug action [J]. *Pharmacol. Rev.* 65, 545–577.

Kim, H., and Borkovich, K. A. A. (2010). pheromone receptor gene, pre-1, is essential for mating type-specific directional growth and fusion of trichogynes and female fertility in *Neurospora crassa* [J]. *Mol. Microbiol.* 52, 1781–1798.

Kim, H., Wright, S. J., Park, G., Ouyang, S., Krystofova, S., and Borkovich, K. A. (2012). Roles for receptors, pheromones, G proteins, and mating type genes during sexual reproduction in *Neurospora crassa* [J]. *Genetics* 190, 1389–1404.

Kim, J., Bortz, E., Zhong, H., Leeuw, T., Leberer, E., Vershon, A. K., et al. (2000). Localization and signaling of G(beta) subunit Ste4p are controlled by a-factor receptor and the a-specific protein Asg7p [J]. *Mol. Cell. Biol.* 20:8826.

Kim, M. J., Lee, M. K., Pham, H. Q., Gu, M. J., Zhu, B. H., Son, S. H., et al. (2020). The velvet regulator VosA governs survival and secondary metabolism of sexual spores in *Aspergillus nidulans* [J]. *Genes* 11:103.

Kmetzsch, L., Staats, C. C., Simon, E., Fonseca, F. L., Oliveira, D. L., Joffe, L. S., et al. (2011). The Gata-type transcriptional activator Gat1 regulates nitrogen uptake and metabolism in the human pathogen *Cryptococcus neoformans* [J]. *Fungal Genet. Biol.* 48, 192–199.

Kochman, K. (2014). Superfamily of G-protein coupled receptors (GPCRS) - extraordinary and outstanding success of evolution [J]. *Postepy Higieny I Medycyny Doswiadczalnej* 68, 1225–1237.

Kong, E. F., Tsui, C., Kucharíková, S., Dijck, P. V., and Jabra-Rizk, M. A. (2017). Modulation of *Staphylococcus aureus* response to antimicrobials by the *Candida albicans* quorum sensing molecule farnesol [J]. *Antimicrob. Agents Chemother.* 61:e01573-17.

Kraakman, L., Lemaire, K., Ma, P., Teunissen, A. W., and Thevelein, J. M. (2010). A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the camp pathway during the transition to growth on glucose [J]. *Mol. Microbiol.* 32, 1002–1012.

Krishnan, A., Almen, M. S., Fredriksson, R., and Schioth, H. B. (2012). The origin of Gpcrs: identification of mammalian like rhodopsin, adhesion, glutamate and frizzled GPCRs in fungi [J]. *PLoS One* 7:e29817. doi: 10.1371/journal.pone.0029817

Kuchler, K., Sterne, R. E., and Thorner, J. (1989). *Saccharomyces cerevisiae* STE6 gene product: a novel pathway for protein export in eukaryotic cells [J]. *EMBO J.* 8, 3973–3984.

Kunitake, E., Li, Y., Uchida, R., Nohara, T., Asano, K., Hattori, A., et al. (2019). CreA-independent carbon catabolite repression of cellulase genes by trimeric G-protein and protein kinase A in *Aspergillus nidulans* [J]. *Curr. Genet.* 65, 941–952.

Lafon, A., Han, K.-H., Seo, J.-A., Yu, J.-H. Y., and D'enfert, C. G. - (2006). protein and CAMP-mediated signaling in aspergilli: A genomic perspective [J]. *Fungal Genet. Biol.* 43, 490–502.

Lemaire, K., De Velde, S. V., Van Dijck, P., and Thevelein, J. M. (2004). Glucose and sucrose act as agonist and mannose as antagonist ligands of the G protein-coupled receptor Gpr1 in the yeast *Saccharomyces cerevisiae* [J]. *Mol. Cell* 16, 293–299.

Li, L., and Borkovich, K. A. G. P. R. - (2006). 4 is a predicted G-protein-coupled receptor required for carbon source-dependent asexual growth and development in *Neurospora crassa* [J]. *Eukaryotic Cell* 5, 1287–1300.

Li, L., Wright, S. J., Krystofova, S., Park, G., and Borkovich, K. A. (2007). Heterotrimeric G protein signaling in filamentous fungi [J]. *Annu. Rev. Microbiol.* 61:423.

Liu, G., Casqueiro, J., Gutiérrez, S., Kosalková, K., and MartíN, J. F. (2001). Elicitation of penicillin biosynthesis by alginate in *Penicillium chrysogenum*, Exerted on pcbAB, pcbC, and penDE genes at the transcriptional level [J]. *J. Microbiol. Biotechnol.* 11, 812–818.

Lorenz, M. C., and Pan, X. (2000). The G protein-coupled receptor Gpr1 is a nutrient sensor that regulates pseudohypal [J]. *Genetics* 154, 609–622.

Lukov, G. L., Hu, T., Mclaughlin, J. N., Hamm, H. E., and Willardson, B. M. (2014). Phosducin-like protein acts as a molecular chaperone for G protein βγ dimer assembly [J]. *EMBO J.* 24, 1965–1975.

Ma, D., and Li, R. (2013). Current Understanding of HOG-MAPK Pathway in *Aspergillus fumigatus* [J]. *Mycopathologia* 175, 13–23.

Macheleidt, J., Mattern, D. J., Fischer, J., Netzker, T., Weber, J., Schroeckh, V., et al. (2016). Regulation and role of fungal secondary metabolites [J]. *Annu. Rev. Genet.* 50, 371–392.

Manfiolli, A. O., Siqueira, A. F. S., Reis, A. T. F. D., Dijck, A. P. V., and Schrevens, C. S. (2019). Mitogen-activated protein kinase cross-talk interaction modulates the production of melanins in *Aspergillus fumigatus* [J]. *mBio* 10:e00215-19.

Marc, Y. L., Huchet, V., Bourgeois, C. M., Guyonnet, J. P., Mafart, P., and Thuault, D. (2002). Modelling the growth kinetics of Listeria as a function of temperature, pH and organic acid concentration [J]. *Int. J. Food Microbiol.* 73, 219–237.

MaríN, S., Companys, E., Sanchis, V., Ramos, A. J., and Magan, N. (1998). Effect of water activity and temperature on competing abilities of common maize fungi [J]. *Mycol. Res.* 102, 959–964.

Martín, J. F., van den Berg, M. A., Ver Loren van Themaat, E., and Liras, P. (2019). Sensing and transduction of nutritional and chemical signals in filamentous fungi: Impact on cell development and secondary metabolites biosynthesis [J]. *Biotechnol. Adv.* 37:107392.

Mcdonald, T., Devi, T., Shimizu, K., Sim, S., and Keller, N. (2003). Signaling events connecting mycotoxin biosynthesis and sporulation in *Aspergillus* and *Fusarium* spp [J]. *JSM Mycotoxins* 2003(Suppl.3), 139–147.

McPherson, K. B., Leff, E. R., Li, M. H., Meurice, C., Tai, S., Traynor, J. R., et al. (2018). Regulators of G-protein signaling (RGS) proteins promote receptor coupling to G-protein-coupled inwardly rectifying potassium (GIRK) channels [J]. *J. Neurosci. Off. J. Soc. Neurosci.* 38, 8737–8744.

Mendoza-Martinez, A. E., Cano-Dominguez, N., and Aguirre, J. (2020). Yap1 homologs mediate more than the redox regulation of the antioxidant response in filamentous fungi [J]. *Fungal Biol.* 124, 253–262.

Nadarajah, K., Kumar, I. S., Sangapillai, V., and Omar, N. S. (2018). Recent advances in understanding the fungal G-protein-coupled receptors [J]. *Malaysian J. Microbiol.* 14, 611–623. doi: 10.21161/mjm.1461823

Nadia, P. (2015). Mycotoxins are a component of Fusarium graminearum stress-response system [J]. *Front. Microbiol.* 6:1234. doi: 10.3389/fmicb.2015.01234

Nagy, K. (1991). Biophysical processes in invertebrate photoreceptors - recent progress and a critical overview based on limulus photoreceptors [J]. *Q. Rev. Biophys.* 24, 165–226. doi: 10.1017/S0033583500003401

Niu, M., Steffan, B. N., Fischer, G. J., Venkatesh, N., Raffa, N. L., Wettstein, M. A., et al. (2020). Fungal oxylipins direct programmed developmental switches in filamentous fungi [J]. *Nat. Commun.* 11:5158. doi: 10.1038/s41467-020-18999-0

Obinata, H., Hattori, T., Nakane, S., Tatei, K., and Izumi, T. (2005). Identification of 9-hydroxyoctadecadienoic acid and other oxidized free fatty acids as ligands of the G protein-coupled receptor G2A [J]. *J. Biol. Chem.* 280, 40676–40683. doi: 10.1074/jbc.M507787200

O'callaghan, J., Stapleton, P. C., and Dobson, A. D. (2006). Ochratoxin A biosynthetic genes in *Aspergillus ochraceus* are differentially regulated by pH and nutritional stimuli [J]. *Fungal Genet. Biol.* 43, 213–221. doi: 10.1016/j.fgb.2005.11.005

Padder, S. A., Prasad, R., and Shah, A. H. (2018). Quorum sensing: a less known mode of communication among fungi [J]. *Microbiol. Res.* 210:51. doi: 10.1016/j.micres.2018.03.007

Paoletti, M., Rydholm, C., Schwier, E. U., Anderson, M. J., Szakacs, G., Lutzoni, F., et al. (2005). Evidence for sexuality in the opportunistic fungal pathogen *Aspergillus fumigatus* [J]. *Curr. Biol.* 15, 1242–1248. doi: 10.1016/j.cub.2005.05.045

Penalva, M. A., Tilburn, J., Bignell, E., and Arst, H. N. (2008). Ambient pH gene regulation in fungi: making connections [J]. *Trends Microbiol.* 16, 291–300. doi: 10.1016/j.tim.2008.03.006

Pfohl-Leszkowicz, A., and Manderville, R. A. (2012). An update on direct genotoxicity as a molecular mechanism of ochratoxin a carcinogenicity [J]. *Chem. Res. Toxicol.* 25, 252–262. doi: 10.1021/tx200430f

Poggeler, S., Hoff, B., and Kuck, U. (2008). Asexual cephalosporin C producer *Acremonium chrysogenum* carries a functional mating type locus [J]. *Appl. Environ. Microbiol.* 74, 6006–6016. doi: 10.1128/AEM.01188-08

Qi, X., Yang, X., Chen, S., He, X., Dweep, H., Guo, M., et al. (2014). Ochratoxin A induced early hepatotoxicity: new mechanistic insights from microRNA, mRNA and proteomic profiling studies [J]. *Sci. Rep.* 4:5163. doi: 10.1038/srep05163

Raisley, B., Zhang, M., Hereld, D., and Hadwiger, J. A. A. (2004). camp receptor-like G protein-coupled receptor with roles in growth regulation and development [J]. *Dev. Biol.* 265, 433–445. doi: 10.1016/j.ydbio.2003.09.035

Rasmussen, S. G., Choi, H. J., Rosenbaum, D. M., Kobilka, T. S., Thian, F. S., Edwards, P. C., et al. (2007). Crystal structure of the human beta2 adrenergic G-protein-coupled receptor [J]. *Nature* 450, 383–387. doi: 10.1038/nature06325

Reverberi, M., Fabbri, A. A., Zjalic, S., Ricelli, A., Punelli, F., and Fanelli, C. (2005). Antioxidant enzymes stimulation in *Aspergillus parasiticus* by *Lentinula edodes* inhibits aflatoxin production [J]. *Appl. Microbiol. Biotechnol.* 69, 207–215. doi: 10.1007/s00253-005-1979-1

Reverberi, M., Gazzetti, K., Punelli, F., Scarpari, M., Zjalic, S., Ricelli, A., et al. (2012). Aoyap1 regulates OTA synthesis by controlling cell redox balance in *Aspergillus ochraceus* [J]. *Appl. Microbiol. Biotechnol.* 95, 1293–1304. doi: 10.1007/s00253-012-3985-4

Reverberi, M., Punelli, F., Scarpari, M., Camera, E., Zjalic, S., Ricelli, A., et al. (2010). Lipoperoxidation affects ochratoxin A biosynthesis in *Aspergillus ochraceus* and its interaction with wheat seeds [J]. *Appl. Microbiol. Biotechnol.* 85, 1935–1946. doi: 10.1007/s00253-009-2220-4

Reverberi, M., Zjalic, S., Punelli, F., Ricelli, A., Fabbri, A. A., and Fanelli, C. (2007). Apyap1 affects aflatoxin biosynthesis during *Aspergillus parasiticus* growth in maize seeds [J]. *Food Addit. Contaminants A Chem. Anal. Control Exposure Risk Assess.* 24, 1070–1075. doi: 10.1080/02652030701553244

Reverberi, M., Zjalic, S., Ricelli, A., Punelli, F., and Camera, E. (2008). Modulation of antioxidant defense in *Aspergillus parasiticus* is involved in aflatoxin biosynthesis: a role for the ApyapA gene [J]. *Eukaryotic Cell* 7, 988–1000. doi: 10.1128/EC.00228-07

Ries, L. N. A., Beattie, S., Cramer, R. A., and Goldman, G. H. (2018). Overview of carbon and nitrogen catabolite metabolism in the virulence of human pathogenic fungi [J]. *Mol. Microbiol.* 107, 277–297. doi: 10.1111/mmi.13887

Rispail, N., Soanes, D. M., Ant, C., Czajkowski, R., Grünler, A., Huguet, R., et al. (2009). Comparative genomics of MAP kinase and calcium-calcineurin signalling components in plant and human pathogenic fungi [J]. *Fungal Genet. Biol. Fg B* 46, 287–298. doi: 10.1016/j.fgb.2009.01.002

Rolland, F., De Winde, J. H., Lemaire, K., Boles, E., Thevelein, J. M., and Winderickx, J. (2000). Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process [J]. *Mol. Microbiol.* 38, 348–358. doi: 10.1046/j.1365-2958.2000.02125.x

Rowe, J., Kapolka, N., Taghon, G., Morgan, W., and Isom, D. (2020). Mechanistic Insights into pH-sensing GPCRs [J]. *FASEB J.* 34:1. doi: 10.1096/fasebj.2020.34.s1.06154

Ruijter, G. J. G., and Visser, J. (1997). Carbon repression in aspergilli [J]. *FEMS Microbiol. Lett.* 151, 103–114. doi: 10.1111/j.1574-6968.1997.tb12557.x

Ruitao, X., Haoyu, C., Meirong, C., Mengwei, Z., and Jian, Z. (2019). LaeA regulates the morphological development, ochratoxin synthesis and oxidation tolerance of *Aspergillus niger* [J]. *Food Res. Dev.* 40, 189–195.

Savoldi, M., Malavazi, I., Soriani, F. M., Capellaro, J. L., Kitamoto, K., Ferreira, M. E. D. S., et al. (2010). Farnesol induces the transcriptional accumulation of the *Aspergillus nidulans* Apoptosis-Inducing Factor (Aif)-like mitochondrial oxidoreductase [J]. *Mol. Microbiol.* 70, 44–59. doi: 10.1111/j.1365-2958.2008.06385.x

Sekito, T., Nakamura, K., Manabe, K., Tone, J., Sato, Y., Murao, N., et al. (2014). Loss of ATP-dependent lysine uptake in the vacuolar membrane vesicles of *Saccharomyces cerevisiae* ypq1 mutant [J]. *Biosci. Biotechnol. Biochem.* 78, 1199–1202. doi: 10.1080/09168451.2014.918489

Semighini, C. P. (2006). Functional characterization of the putative *Aspergillus nidulans* Poly(ADP-Ribose) polymerase homolog PrpA [J]. *Prog. Pharm. Sci.* 173, 87–98. doi: 10.1534/genetics.105.053199

Semighini, C. P., Hornby, J. M., Dumitru, R., Nickerson, K. W., and Harris, S. D. (2006). Farnesol-induced apoptosis in *Aspergillus nidulans* reveals a possible mechanism for antagonistic interactions between fungi [J]. *Mol. Microbiol.* 59, 753–764. doi: 10.1111/j.1365-2958.2005.04976.x

Seo, J. A., Han, K. H., and Yu, J. H. (2004). The gprA and gprB genes encode putative G protein-coupled receptors required for self-fertilization in *Aspergillus nidulans* [J]. *Mol. Microbiol.* 53, 1611–1623. doi: 10.1111/j.1365-2958.2004.04232.x

Shang, J. M., Shang, Y. F., Tang, G. R., and Wang, C. S. (2020). Identification of a key G-protein coupled receptor in mediating appressorium formation and fungal virulence against insects [J]. *Sci. China Life Sci.* [Epub ahead of print]. doi: 10.1007/s11427-020-1763-1

Shapiro, D. A., Kristiansen, K., Weiner, D. M., Kroeze, W. K., and Roth, B. L. (2002). Evidence for a model of agonist-induced activation of 5-Hydroxytryptamine 2A serotonin receptors that involves the disruption of a strong ionic interaction between helices 3 and 6 [J]. *J. Biol. Chem.* 277, 11441–11449. doi: 10.1074/jbc.M111675200

Stenkamp, R. E., Teller, D. C., and Palczewski, K. (2002). Crystal structure of rhodopsin: a G-protein-coupled receptor [J]. *Chembiochem* 3, 963–967. doi: 10.1002/1439-7633(20021004)3:10<963::AID-CBIC963>3.0.CO;2-9

Tag, A., Hicks, J., Garifullina, G., Ake, C., and Keller, N. G. (2010). -protein signalling mediates differential production of toxic secondary metabolites [J]. *Mol. Microbiol.* 38, 658–665. doi: 10.1046/j.1365-2958.2000.02166.x

Tai, B. W., Chang, J. H., Liu, Y., and Xing, F. G. (2020). Recent progress of the effect of environmental factors on *Aspergillus flavus* growth and aflatoxins production on foods [J]. *Food Quality Saf.* 4, 21–28. doi: 10.1093/fqsafe/fyz040

Terfehr, D., Dahlmann, T. A., Specht, T., Zadra, I., Kurnsteiner, H., and Kuck, U. (2014). Genome sequence and annotation of *Acremonium chrysogenum*, producer of the β-Lactam antibiotic cephalosporin C [J]. *Genome Announc.* 2:e00948-14. doi: 10.1128/genomeA.00948-14

Thatcher, L. F., Manners, J. M., and Kazan, K. (2009). Fusarium oxysporum hijacks COI1-mediated jasmonate signaling to promote disease development in Arabidopsis [J]. *Plant J.* 58, 927–939. doi: 10.1111/j.1365-313X.2009.03831.x

Thieme, N., Wu, V. W., Dietschmann, A., Salamov, A. A., Wang, M., Johnson, J., et al. (2017). The transcription factor PDR-1 is a multi-functional regulator and

key component of pectin deconstruction and catabolism in *Neurospora crassa* [J]. *Biotechnol. Biofuels* 10:149. doi: 10.1186/s13068-017-0807-z

Tilburn, J., Sarkar, S., Widdick, D. A., Espeso, E. A., Orejas, M., Mungroo, J., et al. (1995). The *Aspergillus* Pacc zinc-finger transcription factor mediates regulation of both acid-expressed and alkaline-expressed genes by ambient Ph [J]. *EMBO J.* 14, 779–790. doi: 10.1002/j.1460-2075.1995.tb07056.x

Tsitsigiannis, D. I, and Keller, N. P. (2010). Oxylipins act as determinants of natural product biosynthesis and seed colonization in *Aspergillus nidulans* [J]. *Mol. Microbiol.* 59, 882–892. doi: 10.1111/j.1365-2958.2005.05000.x

Tsitsigiannis, D. I., and Keller, N. P. (2007). Oxylipins as developmental and host-fungal communication signals [J]. *Trends Microbiol.* 15, 109–118. doi: 10.1016/j.tim.2007.01.005

Tudzynski, B. (2014). Nitrogen regulation of fungal secondary metabolism in fungi [J]. *Front. Microbiol.* 5:656. doi: 10.3389/fmicb.2014.00656

Turan, N. B., and Engin, G. Ö (2018). Quorum quenching [J]. *Comprehensive Anal. Chem.* 81, 117–149. doi: 10.1016/bs.coac.2018.02.003

Van Dijck, P., Brown, N. A., Goldman, G. H., Rutherford, J., Xue, C. Y., and Van Zeebroeck, G. (2017). Nutrient sensing at the plasma membrane of fungal cells [J]. *Microbiol. Spectr.* 5:31. doi: 10.1128/9781555819583.ch19

Villa, N. Y., Moussatche, P., Chamberlin, S. G., Kumar, A., and Lyons, T. J. (2011). Phylogenetic and preliminary phenotypic analysis of yeast PAQR receptors: potential antifungal targets [J]. *J. Mol. Evol.* 73, 134–152. doi: 10.1007/s00239-011-9462-3

Voigt, C. A., Schäfer, W., and Salomon, S. A. (2010). secreted lipase of *Fusarium graminearum* is a virulence factor required for infection of cereals [J]. *Plant J.* 42, 364–375. doi: 10.1111/j.1365-313X.2005.02377.x

Wang, G., Wang, Y., Zhang, H., Zhang, C., Yang, B., Huang, S., et al. (2020). Factors that affect the formation of mycotoxins: a literature review [J]. *Mygosystema* 39, 477–491.

Wang, G., Zhang, H. Y., Wang, Y. L., Liu, F., Li, E. F., Ma, J. N., et al. (2019). Requirement of LaeA, VeA, and VelB on asexual development, ochratoxin a biosynthesis, and fungal virulence in *Aspergillus ochraceus* [J]. *Front. Microbiol.* 10:2759. doi: 10.3389/fmicb.2019.02759

Wasternack, C., and Feussner, I. (2018). The oxylipin pathways: biochemistry and function [J]. *Annu. Rev. Plant Biol.* 69, 363–386. doi: 10.1146/annurev-arplant-042817-040440

Wenying, C., Chen, L., Boyang, Z., Zheng, Z., Yingbin, S., Xin, L., et al. (2018). Advances in biodetoxification of ochratoxin A-a review of the past five decades [J]. *Front. Microbiol.* 9:1386. doi: 10.3389/fmicb.2018.01386

Wilson, R. A., Gardner, H. W., and Keller, N. P. (2001). Cultivar-dependent expression of a maize lipoxygenase responsive to seed infesting fungi [J]. *Mol. Plant Microbe Interact.* 14:980. doi: 10.1094/MPMI.2001.14.8.980

Wong, K. H., Hynes, M. J., and Davis, M. A. (2008). Recent advances in nitrogen regulation: a comparison between *Saccharomyces cerevisiae* and filamentous fungi [J]. *Eukaryotic Cell* 7, 917–925. doi: 10.1128/EC.00076-08

Wongsuk, T., Pumeesat, P., and Luplertlop, N. (2016). Fungal quorum sensing molecules: role in fungal morphogenesis and pathogenicity [J]. *J. Basic Microbiol.* 56, 440–447. doi: 10.1002/jobm.201500759

Xue, C., Bahn, Y. S., Cox, G. M., and Heitman, J. G. (2006). protein-coupled receptor Gpr4 senses amino acids and activates the cAMP-PKA pathway in *Cryptococcus neoformans* [J]. *Mol. Biol. Cell* 17:667. doi: 10.1091/mbc.e05-07-0699

Xue, C. Y., Hsueh, Y. P., and Heitman, J. (2008). Magnificent seven: roles of G protein-coupled receptors in extracellular sensing in fungi [J]. *FEMS Microbiol. Rev.* 32, 1010–1032. doi: 10.1111/j.1574-6976.2008.00131.x

Yan, S. J., Liang, Y. T., Zhang, J. D., Chen, Z., and Liu, C. M. (2015). Autoxidated linolenic acid inhibits aflatoxin biosynthesis in *Aspergillus flavus* via oxylipin species [J]. *Fungal Genet. Biol.* 81, 229–237. doi: 10.1016/j.fgb.2014.11.005

Yan, W., Liuqing, W., Fan, W., Fei, L., Qi, W., Xiaoling, Z., et al. (2018). A consensus ochratoxin a biosynthetic pathway: insights from the genome sequence of *Aspergillus ochraceus* and a comparative genomic analysis [J]. *Appl. Environ. Microbiol.* 84:e01009-18. doi: 10.1128/AEM.01009-18

Yan, X. U., Wei-Jia, Y., Yun-Long, Z., and Jing, X. U. (2003). Identification of the Gq-protein in cherax quadricar-inatus photoreceptor and the influence of wave- length Light on the Content of Gqα [J]. *Zool. Res.* 24, 602–606.

Yin, W. B., Reinke, A. W., Szilagyi, M., Emri, T., Chiang, Y. M., Keating, A. E., et al. (2013). bZIP transcription factors affecting secondary metabolism, sexual development and stress responses in *Aspergillus nidulans* [J]. *Microbiol. Sgm* 159, 77–88. doi: 10.1099/mic.0.063370-0

Yu, J., Fedorova, N. D., Montalbano, B. G., Deepak, B., Cleveland, T. E., Bennett, J. W., et al. (2011). Tight control of mycotoxin biosynthesis gene expression in *Aspergillus flavus* by temperature as revealed by RNA-Seq [J]. *FEMS Microbiol. Lett.* 2, 145–149. doi: 10.1111/j.1574-6968.2011.02345.x

Zhang, B., Shen, X. L., Liang, R., Li, Y., Huang, K., Zhao, C., et al. (2014). Protective role of the mitochondrial Lon protease 1 in ochratoxin A-induced cytotoxicity in HEK293 cells [J]. *J. Proteom.* 101, 154–168. doi: 10.1016/j.jprot.2014.02.017

Zhang, B., Zhu, L., Dai, Y., Li, H., and Xu, W. (2019). An in vitro attempt at precision toxicology reveals the involvement of DNA methylation alteration in ochratoxin A-induced G0/G1 phase arrest [J]. *Epigenetics* 15, 199–214. doi: 10.1080/15592294.2019.1644878

Zhang, J., Chen, H. Y., Sumarah, M. W., Gao, Q., Wang, D. P., and Zhang, Y. (2018). veA gene acts as a positive regulator of conidia production, ochratoxin A biosynthesis, and oxidative stress tolerance in *Aspergillus niger* [J]. *J. Agric. Food Chem.* 66, 13199–13208. doi: 10.1021/acs.jafc.8b04523

Zhao, T., Shen, X. L., Chen, W., Liao, X., Yang, J., Wang, Y., et al. (2017). Advances in research of nephrotoxicity and toxic antagonism of ochratoxin A [J]. *Toxin Rev.* 36, 39–44. doi: 10.1080/15569543.2016.1243560

Zheng, H., Zhou, L., Dou, T., Han, X., Cai, Y., Zhan, X., et al. (2010). Genome-wide prediction of G protein-coupled receptors in *Verticillium* spp [J]. *Fungal Biol.* 114, 359–368. doi: 10.1016/j.funbio.2010.02.008

Zheng, J., Zhang, Y., Xu, W., Luo, Y., Mao, J., Shen, X. L., et al. (2013). Zinc protects HepG2 cells against the oxidative damage and DNA damage induced by ochratoxin A [J]. *Toxicol. Appl. Pharmacol.* 268, 123–131. doi: 10.1016/j.taap.2013.01.021

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 18 February 2021
doi: 10.3389/fmicb.2021.634043

Neutrophil and Eosinophil DNA Extracellular Trap Formation: Lessons From Pathogenic Fungi

*Juliana da Costa Silva[1], Glaucia de Azevedo Thompson-Souza[2], Marina Valente Barroso[1,2], Josiane Sabbadini Neves[2] and Rodrigo Tinoco Figueiredo[3]**

[1] Institute of Microbiology Paulo de Góes, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [2] Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [3] Campus Duque de Caxias, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Fungal infections represent a worldwide health problem. Fungal pathogens are responsible for a variety of conditions, including superficial diseases, allergic pathologies and potentially lethal invasive infections. Neutrophils and eosinophils have been implicated as effector cells in several pathologies. Neutrophils are major effector cells involved in the control of fungal infections and exhibit a plethora of antifungal mechanisms, such as phagocytosis, reactive oxygen species production, degranulation, extracellular vesicle formation, and DNA extracellular trap (ET) release. Eosinophils are polymorphonuclear cells classically implicated as effector cells in the pathogenesis of allergic diseases and helminthic infections, although their roles as immunomodulatory players in both innate and adaptive immunity are currently recognized. Eosinophils are also endowed with antifungal activities and are abundantly found in allergic conditions associated with fungal colonization and sensitization. Neutrophils and eosinophils have been demonstrated to release their nuclear and mitochondrial DNA in response to many pathogens and pro-inflammatory stimuli. ETs have been implicated in the killing and control of many pathogens, as well as in promoting inflammation and tissue damage. The formation of ETs by neutrophils and eosinophils has been described in response to pathogenic fungi. Here, we provide an overview of the mechanisms involved in the release of neutrophil and eosinophil ETs in response to fungal pathogens. General implications for understanding the formation of ETs and the roles of ETs in fungal infections are discussed.

Keywords: neutrophil extracellular traps, eosinophil extracellular traps, pathogenic fungi, neutrophils, eosinophils

OPEN ACCESS

Edited by:
Constantin Felix Urban, Umeå University, Sweden

Reviewed by:
Jeniel E. Nett, University of Wisconsin-Madison, United States
Olaf Kniemeyer, Leibniz Institute for Natural Product Research and Infection Biology, Germany

***Correspondence:**
Rodrigo Tinoco Figueiredo
figueiredo.rt@xerem.ufrj.br

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *26 November 2020*
Accepted: *01 February 2021*
Published: *18 February 2021*

Citation:
Silva JC, Thompson-Souza GA, Barroso MV, Neves JS and Figueiredo RT (2021) Neutrophil and Eosinophil DNA Extracellular Trap Formation: Lessons From Pathogenic Fungi. Front. Microbiol. 12:634043. doi: 10.3389/fmicb.2021.634043

INTRODUCTION

Fungi represent major human pathogens. Estimates indicate that approximately 300 different fungal species are pathogenic to humans (Taylor et al., 2001; Wardeh et al., 2015), with fungal infections contributing to nearly 1.5–1.6 million deaths annually (Brown et al., 2012). The number of individuals affected by fungal diseases is increasing worldwide. Risk factors for the development of severe invasive mycoses include immunosuppression, cancer, bone marrow or solid organ transplantation, and the aging (Suleyman and Alangaden, 2016). Although fungal infections

contribute greatly to human morbidity and mortality, the true magnitude of these diseases in humans is unknown due to underreporting or misdiagnosis (Vallabhaneni et al., 2016). In cases of invasive fungal infections, the mortality rate of patients can exceed 50% (Brown et al., 2012; Vallabhaneni et al., 2016). In addition to acting as causative agents of infections, fungi are also implicated in allergic pathologies. Exposure to environmental fungi and their antigens promotes allergic pathologies such as asthma and rhinitis (Knutsen et al., 2012), and fungal colonization is a common complication of asthma resulting in allergic bronchopulmonary mycoses (ABPMs) (Agarwal and Chakrabarti, 2013; Ishiguro et al., 2014).

Neutrophils and eosinophils have been implicated as effector cells in infections caused by fungal pathogens and in diseases associated with allergic sensitization to fungi (Yoon et al., 2008; Lilly et al., 2014; Gazendam et al., 2016a; Figueiredo and Neves, 2018). Conditions resulting in neutropenia or deficiencies in neutrophil responses are major risk factors for the development of severe systemic mycoses (Lilic, 2012; Gazendam et al., 2016a). Neutrophils and eosinophils are promptly recruited to inflammatory settings where their activation contributes to tissue damage and immunopathology through the release of toxic components of their granules, the generation of reactive oxygen species (ROS), the production of inflammatory mediators and the formation of DNA extracellular traps (ETs) (Amulic et al., 2012; Fulkerson and Rothenberg, 2013).

Neutrophils are peripheral blood cells of the myeloid lineage and the most abundant leukocytes in human blood, constituting approximately 70% of the leukocytes in human peripheral blood (and approximately 30% of the leukocytes in mouse blood). Neutrophils can be distinguished from other granulocytes by the absence of granule staining upon exposure to acidic or basic dyes (neutral property for which these cells were named), and in addition, the presence of multilobed nuclei allows their identification as polymorphonuclear leukocytes (PMNs) (Amulic et al., 2012). Neutrophils are essential for the clearance of fungal pathogens. Neutrophils kill fungi by a variety of mechanisms, including degranulation, phagocytosis, oxidative burst, the release of extracellular vesicles, and the formation of neutrophil DNA extracellular traps (NETs) (Urban et al., 2006; Gazendam et al., 2016a; Shopova et al., 2020). Neutrophils express pattern recognition receptors (PRRs) involved in the recognition of fungi, such as Toll-like receptor (TLR) 2 and TLR4, C-type lectin receptors (CLRs), such as Dectin-1, Dectin-2, and Mincle, the β_2-integrin Mac-1 (macrophage-1 antigen, also known as complement receptor 3/CR3, $\alpha_M\beta_2$ integrin or CD11b/CD18) (Futosi et al., 2013; Patin et al., 2019). In summary, these receptors trigger signaling pathways that coordinate neutrophil responses involved in the killing of fungal pathogens (Gazendam et al., 2016a; Lehman and Segal, 2020).

Eosinophils constitute a minor leukocyte population in the bloodstream, comprising from 1 to 5% of circulating cells, and a sudden increase in eosinophil blood counts in certain pathological conditions indicates that eosinophils are linked to the onset and maintenance of inflammatory processes (Hogan et al., 2008; Fulkerson and Rothenberg, 2013). Eosinophils are characterized by their numerous cytoplasmic granules, including crystalloid granules, primary granules, and secretory vesicles. Among these, crystalloid granules are the largest, and unique to eosinophils, they contain a variety of preformed granule-stored proteins, including cationic proteins, such as major basic protein (MBP), which is the most abundant granular protein in eosinophils; eosinophilic peroxidase (EPO); eosinophil cationic protein (ECP); and eosinophil-derived neurotoxin (EDN) (Muniz et al., 2012; Acharya and Ackerman, 2014). The abundance of cationic proteins makes eosinophils stainable by acidic dyes, such as eosin, which can be used to distinguish them from other polymorphonuclear leukocytes. Classically, eosinophils are considered effector cells in allergic pathologies and helminthic infections and have been implicated in tissue damage in allergic inflammatory pathologies. However, evolving knowledge in the field has revealed that eosinophils exhibit inflammatory and immunomodulatory functions and play roles in tissue remodeling (Lee et al., 2004; Jacobsen et al., 2008; Fulkerson and Rothenberg, 2013).

The increase in eosinophil blood counts, as well as tissue eosinophilia in allergic diseases associated with fungal sensitization/colonization, has been widely recognized (Knutsen et al., 2012; Figueiredo and Neves, 2018). Exposure and sensitization to fungal allergens is an important factor in patients with respiratory allergies, and in this context, fungi play important roles in the development, severity and persistence of allergic lung diseases, especially asthma (Knutsen et al., 2012). ABPMs are characterized by robust inflammation due to fungal colonization of the airways, particularly in patients with asthma or cystic fibrosis (Knutsen et al., 2012). One of the main characteristics indicating a diagnosis of ABPM is eosinophilia, in addition to increased serum IgE levels and colonization of the airways by fungi (Asano et al., 2020). Although ABPMs have a profile of a Th2 inflammatory response with eosinophilic infiltrates, the nature of the interactions between eosinophils and fungi is unclear, and the participation of eosinophils in fungal infections continues to be extensively discussed, as eosinophils played no role in certain aspects of the pulmonary pathology in the experimental ABPM induced by *Aspergillus fumigatus* exposure (Dietschmann et al., 2020). However, whether these findings are relevant clinically or are limited to the experimental model utilized remains to be elucidated. In the last decade, new data have shown an important antifungal role of eosinophils against several species of fungi, such as *Alternaria alternata, Cryptococcus neoformans,* and *A. fumigatus*, the latter being the main cause of ABPMs (Yoon et al., 2008; Garro et al., 2011; Lilly et al., 2014).

Extracellular traps are produced by neutrophils and eosinophils in response to fungal pathogens and are involved in the killing and/or entrapment of fungi (Muniz et al., 2018; Urban and Nett, 2019). Considerable effort has been dedicated to the elucidation of the mechanisms of neutrophil and eosinophil ET formation. While this work has focused on the formation of DNA extracellular traps in response to fungi, more detailed information on the basic aspects of NET and eosinophil extracellular trap (EET) formation can be found in recent publications (Mukherjee et al., 2018; Papayannopoulos, 2018). Here, we review the mechanisms by which ETs are released from

neutrophils and eosinophils in response to fungal pathogens and the role of these ETs in pathologies caused by fungal infections or hypersensitivity responses to fungi and their antigens.

NEUTROPHIL EXTRACELLULAR TRAPS

Takei et al. (1996) characterized a previously undiscovered process of cell death in human neutrophils stimulated with phorbol-12-myristate-13-acetate (PMA). Neutrophil death induced by PMA is characterized by morphological changes in the shape of the nucleus, chromatin decondensation, and further leakage of the nuclear envelope without markers of apoptosis or necrosis (Takei et al., 1996). Later, it was demonstrated that the PMA-induced neutrophil death represents a novel effector mechanism that culminates with the release of DNA fibers involved in the killing gram-negative and gram-positive bacteria (Brinkmann et al., 2004). These DNA web-like structures released by neutrophils are called NETs and contain histones and granular proteins such as neutrophil elastase (NE) and myeloperoxidase (MPO) (Brinkmann et al., 2004). The process involved in the NET release represents a novel type of cell death, called NETosis, characterized by the loss of the lobulated nuclear structure, fragmentation of the nuclear envelope, interaction of granular proteins with decondensed chromatin and the subsequent extrusion of nuclear content into the extracellular compartment (Fuchs et al., 2007).

However, further investigations demonstrated that NET release can also occur without membrane rupture and can occur independent of neutrophil death (Yousefi et al., 2009; Yipp et al., 2012). The origin of the DNA released has also been investigated. Neutrophils primed with granulocyte/macrophage colony-stimulating factor (GM-CSF) and then further stimulated with lipopolysaccharide (LPS) or complement factor 5a (C5a) release mitochondria-derived DNA by a process that maintains the integrity of the nuclear membrane and neutrophil viability. Mitochondrial NETs do not present nuclear components, such as lamin B or nuclear DNA; however, these structures contain mitochondrial DNA in association with granular proteins, such as NE and MPO. The release of mitochondrial NETs does not involve cell death, membrane rupture or nuclear disruption and is faster than the NET formation involving the nuclear DNA release, with mitochondrial NETs released within 15 min of stimulation in a mechanism that requires the generation of ROS by NADPH oxidase (Yousefi et al., 2009). The general contribution of mitochondrial NETs, however, is still unclear since the NETs formed in response to several relevant stimuli and pathogens are composed basically of nuclear DNA (Pilsczek et al., 2010; Kenny et al., 2017). Furthermore, in many experimental models and clinical samples, histones are associated with NETs, indicating that nuclear chromatin is the source of these NETs (Brinkmann et al., 2004; Guimarães-Costa et al., 2009; Röhm et al., 2014).

Neutrophils are terminally differentiated cells unable to initiate mitosis; however, signaling pathways involved in the mitotic program are involved in NETosis. Induction of NETosis leads to the expression of the mitotic marker Ki-67 and the phosphorylation of the retinoblastoma protein pRb, lamin A/C and serine 10 in histone 3, modifications characteristic of cell cycle entry. NET inducers, however, do not promote neutrophil DNA replication or cell division. Neutrophils express CDK4 and 6, and CDK6 is required for NET formation (Amulic et al., 2017). Thus, pathways in NETosis induction and mitosis are shared, with the activation of pre-mitotic machinery inducing a neutrophil-specific cell death program that leads to nuclear disruption and chromatin release into the extracellular environment.

Autophagy has been described as a process required for NETosis based on the observation of autophagic vesicles and the inhibition of NET formation by class III phosphatidylinositol 3-kinase (PI3K) inhibitors, such as wortmannin and 3-methyladenine (3-MA) (Mitroulis et al., 2011; Remijsen et al., 2011). The role of autophagy in the NET release, however, has not been confirmed in ATG5 mice conditionally deficient in neutrophils and eosinophils or by the pharmacological inhibition of autophagosome acidification (Germic et al., 2017). Thus, it seems that the effects of inhibitors of class III PI3Ks, such as wortmannin, reflect other pharmacological targets, such as class I PI3Ks, the activity of which is required for NETosis induced by *Leishmania amazonensis* and *A. fumigatus* (DeSouza-Vieira et al., 2016; Silva et al., 2020). Alternatively, since Germic et al. investigated the formation of ETs by a rapid mechanism demonstrated for stimuli that induce the formation of mitochondrial ETs (Germic et al., 2017), they could not exclude the participation of autophagic mechanisms in the ETosis in response to inducers that promote nuclear fragmentation and release of chromatin, by a process that takes hours to occur, resulting in cell death (Fuchs et al., 2007). Thus, it remains to be established whether autophagy is a consequence of neutrophil activation and occurs in parallel but is not involved in the mechanisms triggering ETosis, or whether autophagy is necessary under some conditions leading to ETosis, particularly ET formation originating from nuclear chromatin (Fuchs et al., 2007; Thiam et al., 2020).

Several inflammatory stimuli have been described as inducers of NET formation, including calcium ionophores, PMA, a protein kinase C activator, bacteria, fungi, protozoans, viruses, and immune complexes (Brinkmann et al., 2004; Urban et al., 2006; Bruns et al., 2010; McCormick et al., 2010; Saitoh et al., 2012; Neeli and Radic, 2013; Muraro et al., 2018; Veras et al., 2020). NETosis induced by PMA, a protein kinase C (PKC) activator, bacteria, fungi, or immune complexes, requires the activity of NADPH oxidase in a mechanism that relies on the generation of ROS (Fuchs et al., 2007; Ermert et al., 2009; Behnen et al., 2014; Kenny et al., 2017). A role for oxidants generated by the NADPH oxidase complex has been demonstrated by the impairment in NET formation by neutrophils obtained from individuals suffering from chronic granulomatous disease (CGD), which show impaired NADPH oxidase activity (Fuchs et al., 2007). The role of ROS generation downstream of NADPH oxidase activity has been supported by considerable evidence: (1) exogenous H_2O_2 or the extracellular generation of H_2O_2 by the incubation of neutrophils with glucose oxidase induces NETosis in the absence of NADPH oxidase activity, and (2) antioxidants are effective inhibitors of NET formation

in response to many neutrophil activators (Fuchs et al., 2007; Kenny et al., 2017). In addition, NADPH oxidase-independent mechanisms for NET formation have been described in response to a toxin-producing *Staphylococcus aureus* strain (Pilsczek et al., 2010). NET formation by ROS-independent mechanisms has also been observed for a calcium ionophore and nigericin (Kenny et al., 2017).

In addition to the roles of ROS generated by NADPH oxidase, NETosis requires the activity of NE and MPO. Genetic deficiency of MPO results in impairment of NET release in response to PMA and other NET inducers (Metzler et al., 2011). During NETosis, NE and MPO are released from azurophilic granules into the cytosol (Papayannopoulos et al., 2010; Metzler et al., 2014). NE is then translocated to the nucleus, where it degrades histones. This process is amplified by MPO, which also shows nuclear localization upon the induction of NETosis (Papayannopoulos et al., 2010). The inhibition of NE results in impaired NET formation, and NE-deficient mice do not exhibit NETs in the lungs in an experimental model of infection by *Klebsiella pneumoniae* (Papayannopoulos et al., 2010). The cellular compartmentalization of NE during neutrophil activation is a determinant of the induction of NETosis. Recognition of pathogens under conditions favorable to phagocytosis does not result in NETosis, while fungal morphotypes, such as hyphae, that are not phagocytosed by neutrophils induce NET formation (Branzk et al., 2014). The trigger for NET release has been attributed to the release of NE from azurophilic granules to the cytosol in response to the recognition of pathogens in the absence of internalization by neutrophils. In contrast, phagocytosis results in the targeting of elastase to phagosomes, which turns off the NETosis program due to the reduction in the amount of cytosolic NE required for nuclear translocation and histone degradation (Branzk et al., 2014).

Histone citrullination by peptidyl arginine deiminase 4 (PAD4) has also been implicated in the induction of NETosis (Wang et al., 2009; Li et al., 2010). Peptidyl arginine deiminases constitute a group of enzymes that modify arginine residues in polypeptide chains by deimination or demethylimination, resulting in the formation of citrulline (Wang and Wang, 2013). PAD4 is highly expressed in neutrophils, and stimuli, such as calcium ionophores, TNF and LPS, promote histone citrullination (Neeli et al., 2008; Wang et al., 2009). The role of PAD4 in NET formation was first revealed by the observation that HL-60 leukemia cells when differentiated to a neutrophil-like phenotype undergo histone citrullination in response to a calcium ionophore that is associated with ET formation (Wang et al., 2009). The release of ETs by the HL-60 cell line requires the activity of PAD4 since its inhibition results in the absence of ET formation and histone citrullination. The role of PAD4 in the HL-60 model was attributed to chromatin decompaction by histone citrullination and was also observed when HL-60 cells were primed with CXCL8 followed by stimulation with *Shigella flexneri* (Wang et al., 2009). Following these observations, the involvement of PAD4 in the process of NET formation has been described in many experimental settings (Li et al., 2010; Lewis et al., 2015). A recent study demonstrated that NETosis proceeds through actin cytoskeleton dismantling, nuclear envelope rupture and chromatin decondensation that precede DNA release, and nuclear rupture requires PAD4 nuclear localization and activity (Thiam et al., 2020). The relevance of PAD4-mediated histone citrullination for NET formation has been demonstrated in PAD4-KO mice (Li et al., 2010). PAD4-deficient neutrophils do not show histone citrullination or NET release when stimulated with calcium ionophores, LPS, PMA, H_2O_2, or *S. flexneri*, and PAD4-KO mice show an increased susceptibility to a DNAse-deficient *Streptococcus pyogenes* strain (Li et al., 2010). A role for PAD4 in the formation of NETs in experimental models of inflammatory pathologies and infections has also been demonstrated (Hemmers et al., 2011; Martinod et al., 2013, 2017).

However, the role of PAD4-mediated histone citrullination as a universal promoter of NETosis has been questioned. PMA does not promote histone citrullination in human neutrophils under conditions in which NETs are formed (Neeli and Radic, 2013). Furthermore, PMA inhibits the histone citrullination induced by calcium ionophores without interfering with the NET release promoted by these stimuli (Neeli and Radic, 2013). NETosis in response to many pathogens, such as group B streptococci, *K. pneumoniae* and fungi, does not require PAD4-mediated histone citrullination (Kenny et al., 2017; Claushuis et al., 2018; Guiducci et al., 2018; Silva et al., 2020; Thompson-Souza et al., 2020). PAD4-dependent histone citrullination has been suggested to be an essential process for the induction of NETosis in response to calcium ionophores (Li et al., 2010; Lewis et al., 2015), but this supposition has been questioned (Kenny et al., 2017). The reasons for the discrepancies in the roles of PAD4 in NETosis are not clear. Although the data seem to indicate that PAD4-mediated histone citrullination is the mechanism responsible for the induction of NETosis, PAD4 can mediate other signaling mechanisms, possibly through the citrullination of non-histone proteins (Loos et al., 2008, 2009; Proost et al., 2008; Jang et al., 2015; Sun et al., 2017). Thus, other roles for PAD4 that do not involve chromatin decompaction by histone citrullination may be involved indirectly in the induction of NETosis *in vitro* and *in vivo*. Alternatively, the involvement of PAD4-mediated histone citrullination in the NETosis must not reflect a common process for NET formation but must be dispensable depending on the NET inducer.

While NADPH oxidase-induced ROS generation and PAD4 histone citrullination have been identified as downstream mediators of NET formation, the receptors and upstream signaling pathways critical for triggering NETosis are less understood. PMA has been used as a prototypical NET inducer requiring ROS generation by the NADPH oxidase complex (Fuchs et al., 2007). PMA-induced NETosis involves the activity of conventional PKCβ isoforms (Neeli and Radic, 2013). In contrast, calcium ionophores require the atypical PKCζ isoform for the induction of histone citrullination and NETosis (Neeli and Radic, 2013). Interestingly, PMA not only fails to promote histone citrullination but also inhibits the histone citrullination induced by a calcium ionophore by means of PKCα/β activity (Neeli and Radic, 2013). Thus, divergent programs of NETosis can be initiated through the activity of distinct PKC isoforms. A large screening of small-molecule compounds revealed that a selective inhibitor of c-Raf impaired PMA-induced NETosis at

an early stage of nuclear disruption and chromatin expansion. Furthermore, PMA-induced NETosis involves PKC activation of the MEK/ERK pathway, with the c-Raf/MEK/ERK pathway upstream of NADPH oxidase activation (Hakkim et al., 2011).

EOSINOPHIL EXTRACELLULAR TRAPS

The release of DNA extracellular traps by eosinophils (named EETs) was first described by Yousefi et al. (2008), 4 years after the characterization of ET release from neutrophils. In the first study, the authors observed multiple extracellular DNA fibers associated with MBP and ECP in colon biopsy samples taken from patients with Crohn's disease, schistosomiasis, or intestinal spirochetosis (Yousefi et al., 2008). *In vitro*, it was observed that human eosinophils primed with IFN-γ or IL-5 and activated with LPS, C5a, or eotaxin/CCL11 released EETs through a ROS-dependent mechanism. Interestingly, the origin of the DNA in the EETs was characterized as mitochondrial, and the phenomenon did not involve cell death (Yousefi et al., 2008). Yet in this same study, the authors showed that human eosinophils released EETs in response to opsonized *Escherichia coli,* and these EETs presented bactericidal activity. A few years later, a subsequent study showed that the stimulation of human eosinophils with thymic stromal lymphopoietin (TSLP), a cytokine secreted by epithelial cells and known to contribute to the promotion of Th2 responses, also induced the release of EETs of mitochondrial origin in a mechanism independent of cell death but dependent on the activation of NADPH oxidase and a β_2-integrin (Morshed et al., 2012). Non-cytolytic mitochondrial EET formation occurred independently of autophagy in eosinophils primed with GM-CSF and stimulated with C5a or LPS or with low concentrations of PMA without cell priming (Germic et al., 2017).

Afterward, the process of EET release involving cell death was described, introducing the concept of EETosis (similar to the mechanism observed for neutrophils), where eosinophils undergo a cytolytic process with nuclear disruption, DNA mixing with intact granules and release of chromatin and associated granules into the extracellular medium in an NADPH oxidase-dependent mechanism (Ueki et al., 2013). In this study, the EETs released by human eosinophils were observed *in vitro* after stimulation with immobilized immunoglobulins (IgG and IgA), PAF, calcium ionophore or PMA. The DNA that constituted the EETs was characterized as having nuclear origin, and histones were found to be associated with these EET structures. Thus, EETs can be either released from live eosinophils, after mitochondrial DNA mobilization, associated with ECP and EPO (Yousefi et al., 2008), or as part of a slower process that results in the death of eosinophils by cytolysis and extrusion of histone-enriched nuclear DNA associated with clusters of intact granules (Ueki et al., 2013).

Eosinophil extracellular traps are formed in either an oxidative NADPH oxidase-dependent or oxidative-independent manner. NADPH oxidase-dependent mechanisms are required for mitochondrial EET formation in response to IFN-γ/IL-5 cell priming followed by stimulation with LPS, C5a or eotaxin/CCL11 (Yousefi et al., 2008). The release of nuclear-derived EETs in response to PAF, IgG/IgA immune complexes or PMA also relied on ROS generation by NADPH oxidase as did EET formation induced by TSLP (Yousefi et al., 2008; Morshed et al., 2012; Ueki et al., 2013). However, lysophosphatidylserine (LysoPS) induces EET formation by a ROS-independent mechanism (Kim et al., 2020). As observed for neutrophils (Asaga et al., 2001; Nakashima et al., 2002), human eosinophils also express the enzyme PAD4 (Asaga et al., 2001), and PAD4-mediated histone citrullination is necessary for LysoPS-induced EET formation (Kim et al., 2020). However, the role of PAD4-mediated histone citrullination in EET formation is unknown for several ETT inducers, such as PMA, immune complexes, PAF and monosodium urate crystals (Schorn et al., 2012; Ueki et al., 2013).

EETs and/or EETosis have been implicated in various eosinophil-associated allergic diseases, including rhinosinusitis with nasal polyps, eosinophilic esophagitis, allergic asthma, eosinophilic bronchopulmonary aspergillosis, eosinophilic otitis, and chronic obstructive pulmonary disease (Simon et al., 2015; Ueki et al., 2016; Uribe Echevarría et al., 2017; Muniz et al., 2018; Hwang et al., 2019; Persson et al., 2019). EETs have also been characterized as pro-inflammatory entities in non-allergic processes, such as atherosclerotic plaque formation and thrombosis (Marx et al., 2019), sepsis and colitis (Yousefi et al., 2008).

NETs AND PATHOGENIC FUNGI

In the context of pathogenic fungi, the NET release phenomenon has been described for *Candida albicans* and other *Candida* spp. *A. fumigatus, Paracoccidioides brasiliensis, Scedosporium apiospermum*, and *Histoplasma capsulatum* (Urban et al., 2006; Bruns et al., 2010; McCormick et al., 2010; Svobodová et al., 2012; Mejía et al., 2015; Rocha et al., 2015; Campos-Garcia et al., 2019; Luna-Rodríguez et al., 2020; Negoro et al., 2020; Thompson-Souza et al., 2020). Fungi present great diversity in terms of interactions with host cells and tissue colonization, forms of development and immune evasion strategies. Neutrophils and other immune cells must deal with small fungal structures, such as conidia and yeast cells, which can be phagocytosed, as well as large multicellular forms, such as hyphae, that require non-phagocytic effector mechanisms. Most of the current knowledge on the induction of NETosis and EETosis is based on investigations using non-physiological inducers, such as PMA or calcium ionophores, which are useful for the elucidation of the basic mechanisms of NET formation, but do not account for the true complexity of pathogens. In this sense, fungal pathogens have provided valuable information about the mechanisms of ET formation and the roles of ETs in immunity and pathology.

Candida albicans-INDUCED NET FORMATION

The dimorphic fungus *C. albicans* is a component of the human microbiota colonizing tissues such as skin, the genitourinary tract, gastrointestinal tract and oral mucosa. *C. albicans* is

the major causative agent of mucosal fungal infections in healthy individuals and a relevant pathogen causing life-threatening disseminated infections in immunocompromised patients worldwide, particularly neutropenic patients (Poulain, 2015). *C. albicans* hyphae and yeast cells induce NET release in human neutrophils, and these extracellular traps ensnare and kill both morphotypes (Urban et al., 2006). While *C. albicans* hyphae are efficient inducers of NETosis, in the absence of the morphological transition to hyphae, yeast cells are unable to induce NET release (Branzk et al., 2014; Guiducci et al., 2018; Wu et al., 2019). The inability of *C. albicans* yeast cells to induce NETosis is a consequence of yeast phagocytosis, which reroutes NE from azurophilic granules to phagosomes, while hyphae that are not internalized trigger NET formation, with the release of NE to the cytosol and its subsequent nuclear translocation, leading to the NE-mediated degradation of histones (Branzk et al., 2014). NETs contain associated calprotectin, a cytosolic protein released during NETosis but not during neutrophil degranulation (Urban et al., 2009). NET-associated calprotectin chelates zinc, which inhibits *C. albicans* growth, and immunodepletion of calprotectin abolishes the fungicidal effect of NETs on *C. albicans*. Corroborating the role of calprotectin *in vitro*, calprotectin-deficient mice show increased susceptibility in experimental models of *C. albicans* infection (Urban et al., 2009).

Different mechanisms have been proposed for *C. albicans*-induced NET formation. NETosis induced by *C. albicans* in mouse neutrophils requires an oxidative burst by NAPDH oxidase, as demonstrated by the impaired NET release from *gp91*$^{-/-}$ neutrophils in response to *C. albicans* hyphae (Ermert et al., 2009). *C. albicans*-induced NETosis by human neutrophils requires the generation of ROS; however, neutrophils obtained from CGD patients show similar NET formation. The requirement for a ROS-dependent mechanism for *C. albicans*-induced NETosis was attributed to ROS generation by opsonized *C. albicans*, as the preincubation of *C. albicans* hyphae with a ROS scavenger abolished NET release; thus, ROS generation by *C. albicans* cells can supply the oxidant environment necessary for the induction of NETosis (Kenny et al., 2017). NADPH oxidase-independent NETosis has been described for *C. albicans* in the absence of serum opsonization by a mechanism involving Dectin-2 recognition, Syk, PKCδ, calcium mobilization and PAD4-mediated histone citrullination (Wu et al., 2019). Adding more complexity to the role of NADPH oxidase-dependent ROS generation in *C. albicans*-induced NETosis, a faster process (30–50 min) for NET formation has been described for neutrophils adhered to fibronectin in response to β-glucans or *C. albicans* (Byrd et al., 2013). Crosstalk involving fibronectin-derived signals and β-glucan recognition by Mac-1/CR3 results in NET formation via a ROS-independent pathway in response to β-glucans and *C. albicans* (Byrd et al., 2013). Thus, neutrophils exhibit flexible mechanisms for NET formation. In the absence of serum opsonization, Dectin-2 must recognize α-mannans on the surface of *C. albicans* hyphae (Saijo et al., 2010); in contrast, in the presence of serum opsonins, *C. albicans* recognition by Mac-1 leads to NADPH oxidase-dependent NETosis (Wu et al., 2019). The opsonins involved in the NETosis in response to *C. albicans* are unknown. Possible candidates for *C. albicans* opsonization for NET formation include iC3b or fibrinogen, well-established ligands for Mac-1 (Diamond et al., 1993). Furthermore, in the context of extracellular matrix adhesion upon tissue migration, signaling by fibronectin recognition must be associated to the direct recognition of fungal β-glucans by Mac-1 to trigger NET release in the absence of an oxidative burst.

The contribution of the distinct mechanisms for NETosis in the context of *C. albicans* infection remains a point of discussion. In an experimental model of *C. albicans* peritonitis, pharmacological inhibition of PAD4 abolished NET formation *in vivo*, leading to *C. albicans* dissemination to the kidneys (Wu et al., 2019). In contrast, in an intravenous model of *C. albicans* infection, PAD4-KO mice showed a slight increase in *C. albicans* load in the kidneys without increased kidney pathology or increased fungal burden at later timepoints, indicating that PAD4 is dispensable for immunity in a model of systemic candidiasis (Guiducci et al., 2018). Moreover, PAD4-deficient neutrophils show similar fungicidal activity and NETosis in response to *C. albicans* (Guiducci et al., 2018). The mechanistic differences in *C. albicans*-induced NETosis *in vivo* are not clear, but it is possible that, in the context of a local infection, in the absence of *C. albicans* opsonization, the Dectin-2/PAD4-dependent pathway is critical for NETosis (Wu et al., 2019), while a PAD4-independent mechanism leads to NET formation in systemic infection in which *C. albicans* must be exposed to serum opsonins (Guiducci et al., 2018). **Figure 1** summarizes the mechanisms described for *C. albicans*-induced NET release.

Aspergillus fumigatus-INDUCED NETosis

Aspergillus spp. are saprophytic mold fungi with a worldwide distribution. *Aspergillus* species produce small spores (2–3 μm), conidia, which are critical for the fungal dispersion. Under adequate conditions, conidia germinate, giving rise to the filamentous multicellular morphotype, the hyphae. *Aspergillus* spp. conidia are easily dispersed in the air which results in continuous human exposure to the inhalation of these conidia (Latgé and Chamilos, 2019). *A. fumigatus* is an environmental fungus and is also a common opportunistic agent causing invasive human respiratory diseases, such as allergic bronchopulmonary aspergillosis (ABPA) and a severe pulmonary infection, invasive aspergillosis (IA) (Kosmidis and Denning, 2015). The major predisposing factors for the development of invasive aspergillosis are immunosuppression associated with neutropenia or deficiencies in neutrophil effector functions (Brown et al., 2012; Gazendam et al., 2016a).

Aspergillus fumigatus resting conidia, swollen conidia, germ tubes and hyphae induce the formation of NETs (Bruns et al., 2010; McCormick et al., 2010). Web-like DNA structures are also formed in the lungs of *A. fumigatus*-infected mice and are absent in neutrophil-depleted mice, indicating that they are a consequence of NETosis *in vivo* and not a result of cell damage during infection (Bruns et al., 2010). In contrast to the NET formed against *C. albicans*, the NETs are not

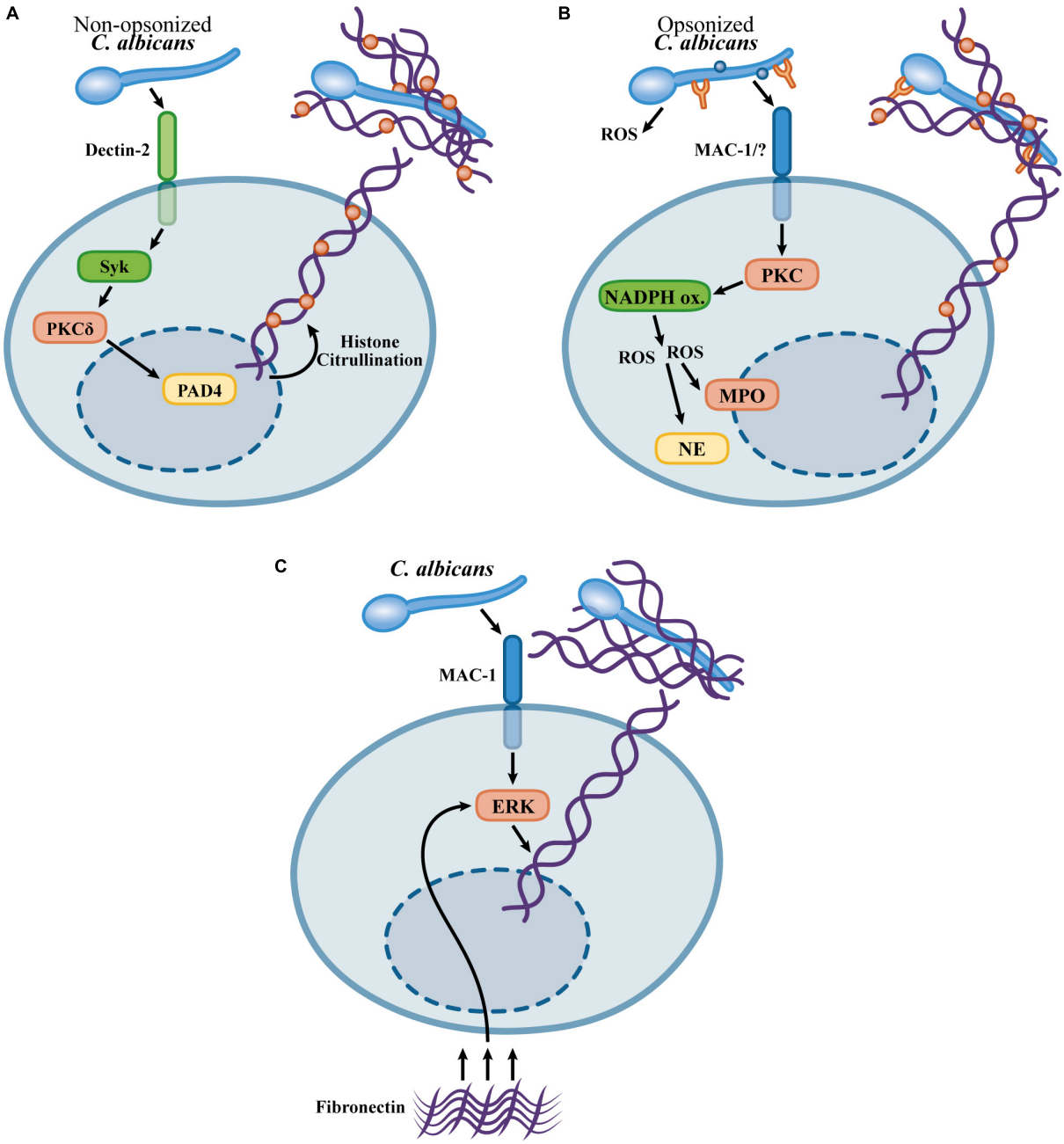

FIGURE 1 | Mechanisms proposed for NET formation in response to *Candida albicans*. **(A)** Dectin-2 mediates NETosis in response to non-opsonized *C. albicans* via a pathway involving Syk, PKCδ, and the PAD4-mediated histone citrullination (Wu et al., 2019). **(B)** Opsonized *C. albicans* triggers NETosis by a ROS-dependent pathway involving NADPH oxidase activity (mouse neutrophils) (Ermert et al., 2009) or ROS generation by *C. albicans* (human neutrophils) (Kenny et al., 2017). PAD4-mediated citrullination is not required for NETosis in response to opsonized *C. albicans*, however, histone citrullination occurs during NETosis (Kenny et al., 2017; Guiducci et al., 2018). **(C)** Fast NETosis induced by the crosstalk upon the adhesion to fibronectin and recognition of *C. albicans* β-glucans by Mac-1 (Byrd et al., 2013).

fungicidal for *A. fumigatus* or *A. nidulans* conidia; instead, these NETs exert fungistatic effects that have been attributed to zinc chelation by calprotectin (McCormick et al., 2010; Bianchi et al., 2011). Neutrophils cause a slight decrease in the metabolic rate of *A. fumigatus* hyphae, and DNAse I addition restores hyphal metabolic activity (Bruns et al., 2010). Thus, it seems that although they do not play roles in the killing of *A. fumigatus*, NETs must inhibit fungal growth and germination. Furthermore, NETs capture *A. fumigatus* conidia and hyphae, which prevents tissue dissemination. Interestingly, two-photon confocal analyses reveal neutrophils carrying swollen conidia and small hyphae in proximity to other neutrophils, which

results in the entrapment of fungal structures, a process that must maximize neutrophil effector activity, such as NETosis and oxidative killing (Bruns et al., 2010).

NETosis in response to *A. fumigatus* requires superoxide generation by the NADPH oxidase complex. In an experimental model of *A. fumigatus* pulmonary infection, NADPH oxidase-deficient (*Ncf1* KO, p47-deficient) mice did not form NETs in lung tissue, and p47-deficient neutrophils were unable to release NETs in response to *A. fumigatus* hyphae (Röhm et al., 2014). *In vitro* results confirmed the role of NADPH oxidase in NETosis in response to *A. fumigatus* conidia (Bruns et al., 2010; Silva et al., 2020). The role of NADPH oxidase in NETosis in response to *A. fumigatus* hyphae seems to differ according to the experimental conditions. Opsonization of *A. fumigatus* hyphae with human serum induces NET formation in a NADPH oxidase-independent way (Gazendam et al., 2016b), while in other experimental settings without human serum, NETosis in response to hyphae requires NADPH oxidase activity (Bruns et al., 2010).

TLR2, TLR4, Dectin-1, and the β_2-integrin Mac-1 are involved in the immune recognition of *A. fumigatus* (Mambula et al., 2002; Meier et al., 2003; Hohl et al., 2005; Steele et al., 2005; Gersuk et al., 2006; Gazendam et al., 2016b). While, TLR2, TLR4, and Dectin-1 are involved in cytokine production by macrophages in response to *A. fumigatus*, these receptors are dispensable for NETosis induced by *A. fumigatus* (Clark et al., 2018; Silva et al., 2020). Mac-1 recognizes fungal β-glucans through a lectin domain distinct from the so-called I-domain involved in the recognition of iC3b, fibrinogen and ICAM-1 (Thornton et al., 1996; Xia and Ross, 1999). NETosis in response to the *A. fumigatus* hyphal extracts, and curdlan, a particulate preparation of β-glucans, requires the recognition mediated by Mac-1 through its lectin domain, as evaluated by the blockade with a monoclonal antibody that targets this domain (Clark et al., 2018). This mechanism differs from the mechanisms of NET release and ROS generation induced by *A. fumigatus* live conidia, which are mediated by Mac-1 through the I-domain without the participation of the lectin domain (Silva et al., 2020). The I-domain is the region in the α_M chain critical for binding to fibrinogen, ICAM-1 and iC3b (Diamond et al., 1993). Recognition of β-glucans by the lectin domain in Mac-1 promotes an active conformational change in the Mac-1 complex that shows increased binding through the I-domain. Priming of Mac-1 by the lectin domain also induces the exposure of an activation epitope in the I-domain that is the binding site of the monoclonal antibody CBRM1/5 (O'Brien et al., 2012). Thus, it seems possible that distinct mechanisms function during fungal recognition mediated by Mac-1: (1) a priming by the β-glucan recognition through the lectin domain that must promote the I-domain recognition of *A. fumigatus* and (2) direct recognition of *A. fumigatus* molecules by the I-domain in living conidia.

NETosis and neutrophil ROS generation induced by *A. fumigatus* conidia require the activity of Src kinases, Syk, and class I PI3Kδ (Silva et al., 2020). Mac-1 signaling involves the immunoreceptor tyrosine-based activation motif (ITAM)-containing membrane proteins DAP-12 and the FcRγ chain (Mócsai et al., 2006). Src kinases promote the phosphorylation of the ITAM motifs in DAP12 and the FcRγ chain, which recruits Syk, resulting in Syk phosphorylation (Mócsai et al., 2006). This signaling module is required for ROS generation and adhesion resulting from the β_2-integrin adhesion (Lowell et al., 1996; Mócsai et al., 1999, 2006). Interestingly, neither Src kinases, Syk, DAP12 nor the FcRγ chain are required for chemotaxis and neutrophil migration *in vitro* and *in vivo* (Mócsai et al., 2002, 2006; Kovács et al., 2014), indicating that Src kinases and Syk are involved in specific neutrophil responses, such as ROS generation, adhesion and NETosis. Interestingly, Syk is required for NETosis in response to immune complexes and *S. aureus*, indicating that Syk is a convergent signaling molecule for NETosis induction by several stimuli (Van Ziffle and Lowell, 2009; Behnen et al., 2014).

Class I PI3Ks are involved in neutrophil responses, including oxidative burst, adhesion and chemotaxis (Hirsch et al., 2000; Sasaki et al., 2000; Sadhu et al., 2003; Boyle et al., 2011). Class IB PI3Kγ is activated downstream of G coupled-protein receptors (GPCRs), while class IA PI3 kinases α, β, and δ are activated by tyrosine kinase pathways (Luo and Mondal, 2015). Selective inhibition of class IA PI3Kδ abolishes ROS generation and NETosis in response to *A. fumigatus* conidia. In contrast, class I PI3Kγ inhibition exerts only partial effects on ROS generation and does not affect *A. fumigatus*-induced NET release at concentrations in which the selective effects of class I PI3Kγ are observed (Silva et al., 2020). NADPH oxidase activation in response to *A. fumigatus* hyphae requires the associated activity of class I PI3Kδ and β; however, the role of class I PI3Kβ in *A. fumigatus*-induced NETosis has not been established; thus, it remains to be investigated whether class IA PI3Kδ cooperates with another class IA PI3K to provide signaling for NETosis induction by *A. fumigatus* (Boyle et al., 2011). How class I PI3Ks trigger NETosis and ROS generation in response to *A. fumigatus* remain unclear. Class I PI3Ks phosphorylate membrane phosphoinositides (PIs) at the 3rd position of the inositol moiety, generating PtdIns(3,4,5)P_3 (Luo and Mondal, 2015). Akt/PKB serine-threonine kinases are activated upon the translocation to the cell membrane and their PH domain interacts with PtdIns(3,4,5)P_3 produced by the activity of class I PI3Ks (Luo and Mondal, 2015). In neutrophils, Akt2 phosphorylates and activates the NADPH oxidase subunit p47 in response to C5a and fMLP, which is required for the assembly of NADPH oxidase and its subsequent activity (Chen et al., 2010). In contrast, opsonized zymosan induces ROS generation in the absence of Akt activity, indicating that Akt activation must not be a general mechanism for the NADPH oxidase activation (Chen et al., 2010). Thus, it remains to be established whether Akt isoforms are downstream effectors of class I PI3Ks for NETosis and ROS generation in response to *A. fumigatus* and other fungi.

PAD4-mediated histone citrullination occurs during neutrophil activation with curdlan, a preparation of particulate β-glucans, *A. fumigatus* hyphae and conidia (Clark et al., 2018; Silva et al., 2020). NET formation in response to curdlan is partially dependent on histone citrullination mediated by PAD4, and in an experimental model of *A. fumigatus* corneal infection, histone citrullination was abolished in PAD4-KO mice (Clark et al., 2018). NET formation in response to *A. fumigatus*

conidia, however, does not require PAD4 activity (Silva et al., 2020). The reason for the discrepant role of PAD4 in β-glucan- and *A. fumigatus*-induced NETosis is unknown. Since PAD4 histone citrullination makes only a partial contribution to β-glucan-induced NETosis, another pathway contributes to NET release (Clark et al., 2018). Furthermore, while curdlan is a particulate β-glucan preparation, *A. fumigatus* conidia express a variety of different molecules on their surface, which must trigger NETosis through a mechanism that overcomes a possible requirement for PAD4 activity. **Figures 2A–C** summarizes the mechanisms described for *A. fumigatus*-induced NET formation.

NET RELEASE IN RESPONSE TO THE DIMORPHIC ENDEMIC FUNGI *H. capsulatum* var. *capsulatum* AND *P. brasiliensis*

Histoplasmosis is an endemic disease whose etiologic agent is the fungus *H. capsulatum*, a thermally dimorphic fungus in the Americas and Africa that can affect both immunocompromised and immunocompetent individuals (Wheat et al., 2016; Oladele et al., 2018). *H. capsulatum* lives in the soil as a filamentous fungus. *H. capsulatum* mycelia produce sporulated structures, macroconidia and microconidia (8–14 and 2–5 μm diameter, respectively). Inhalation of *H. capsulatum* conidia and mycelial fragments results in pulmonary infections that can, in some cases, become disseminated, particularly in individuals presenting deficiencies in T-cell-mediated immune responses. Once in the host tissues at 37°C, *H. capsulatum* undergoes a morphological transition, giving rise to yeast cells that reside in an intracellular niche when phagocytosed by macrophages (Woods, 2016).

The interaction of neutrophils with *H. capsulatum* var *capsulatum* yeast cells results in phagocytosis and NET extrusion (Schnur and Newman, 1990; Newman et al., 1993, 2000; Thompson-Souza et al., 2020). Although neutrophils are capable of phagocytosing *H. capsulatum* cells, phagocytosis does not seem to underlie their antifungal activities (Brummer et al., 1991; Kurita et al., 1991; Newman et al., 1993). It was previously shown that *H. capsulatum* yeast cell phagocytosis by neutrophils triggers an oxidative burst process that is interestingly unable to contribute to fungal killing (Schnur and Newman, 1990; Kurita et al., 1991). The capacity of *H. capsulatum* to neutralize the fungicidal effect of the oxidative burst is attributed to its efficient system of antioxidative enzymes (Youseff et al., 2012; Holbrook et al., 2013). Another observation that corroborates this finding is that neutrophils obtained from patients with CGD show fungistatic activity similar to that of neutrophils that generate an oxidative burst (Newman et al., 1993). Neutrophils can bind opsonized *H. capsulatum* through Mac-1 (CD11b/CD18), CR1, and FcγRIII in a cooperative manner (Newman et al., 1993). As mentioned, ROS do not seem to be a relevant mechanism for *H. capsulatum* killing by neutrophils; however, components of primary granules such as cathepsin G, bactericidal/permeability-increasing protein (BPI) and defensins seem to play fungistatic roles (Newman et al., 1993, 2000). Interestingly, this fungistatic effect did not require uptake of yeast cells (Newman et al., 1993). Thus, phagocytosis does not explain the fact that neutrophils have antifungal capabilities against *H. capsulatum* yeast cells.

Thompson-Souza et al. described that *H. capsulatum* yeast cells induce NET release by human neutrophils, and the extracellular killing of *H. capsulatum* requires NET formation (Thompson-Souza et al., 2020). This process occurred through a signaling pathway mediated by NADPH oxidase-dependent ROS generation, β_2-integrin-mediated recognition, Src kinases and Syk, and culminated in the loss of neutrophil viability. Neutrophil ROS production in response to *H. capsulatum* required Src kinase and Syk activity, demonstrating a role for NADPH oxidase-dependent oxidative burst downstream of the signaling cascade promoting NETosis (Thompson-Souza et al., 2020). In addition, NETs formed in response to *H. capsulatum* show bona fide NET markers, such as associated NE and citrullinated histones. However, extrusion of *H. capsulatum*-induced NETs occurs independently of PAD4-mediated histone citrullination (Thompson-Souza et al., 2020).

The fact that NETs exhibit fungicidal activity against *H. capsulatum* may explain the apparent contradiction found by Newman et al. (1993) who showed that the uptake of yeast cells was not required for the antifungal activities of neutrophils. While previous works have evaluated total killing through neutrophil lysis and *H. capsulatum* CFU counts after relatively short periods of incubation, Thompson-Souza et al. (2020) investigated extracellular *H. capsulatum* killing by propidium iodide entry into yeast cells after 6 h. Thus, it seems that *H. capsulatum* yeast cells are able to survive the intracellular microbicidal activity of neutrophils, while the extracellular yeasts trapped by NETs are vulnerable to the toxic components present in the NETs. The presence of NETs, as well as the role of these structures, in infections caused by *H. capsulatum* have not been investigated; therefore, it remains to be established whether NETs contribute to the control of *H. capsulatum* in the context of infection or even whether NETs contribute to the immunopathogenesis during histoplasmosis. **Figure 3A** shows the mechanisms involved in *H. capsulatum*-induced NETosis.

Paracoccidioides brasiliensis is a pathogenic dimorphic fungus that causes paracoccidioidomycosis, a systemic disease prevalent in Latin America (Colombo et al., 2011). Infections caused by *P. brasiliensis* seem to be established upon the inhalation of conidia that subsequent differentiate in the host environment to establish intracellular yeast parasitism in macrophages (Cezar-Dos-Santos et al., 2020). Human neutrophils form NETs in response to *P. brasiliensis* conidia and yeast cells, and NETs are found in the lesions obtained from patients suffering from paracoccidioidomycosis (Della Coletta et al., 2015; Mejía et al., 2015). NETosis induced by *P. brasiliensis* yeast cells requires ROS generation by the NADPH oxidase complex, while conidia trigger NET release by an NADPH oxidase-independent mechanism. In any case, neutrophils are unable to kill *P. brasiliensis* yeast cells. Consistent with the lack of fungicidal activity of neutrophils, NET degradation by DNAse I, inhibition of NADPH oxidase or phagocytosis do not affect the viability of this fungus during the interaction with neutrophils (Mejía et al., 2015). NETosis in response

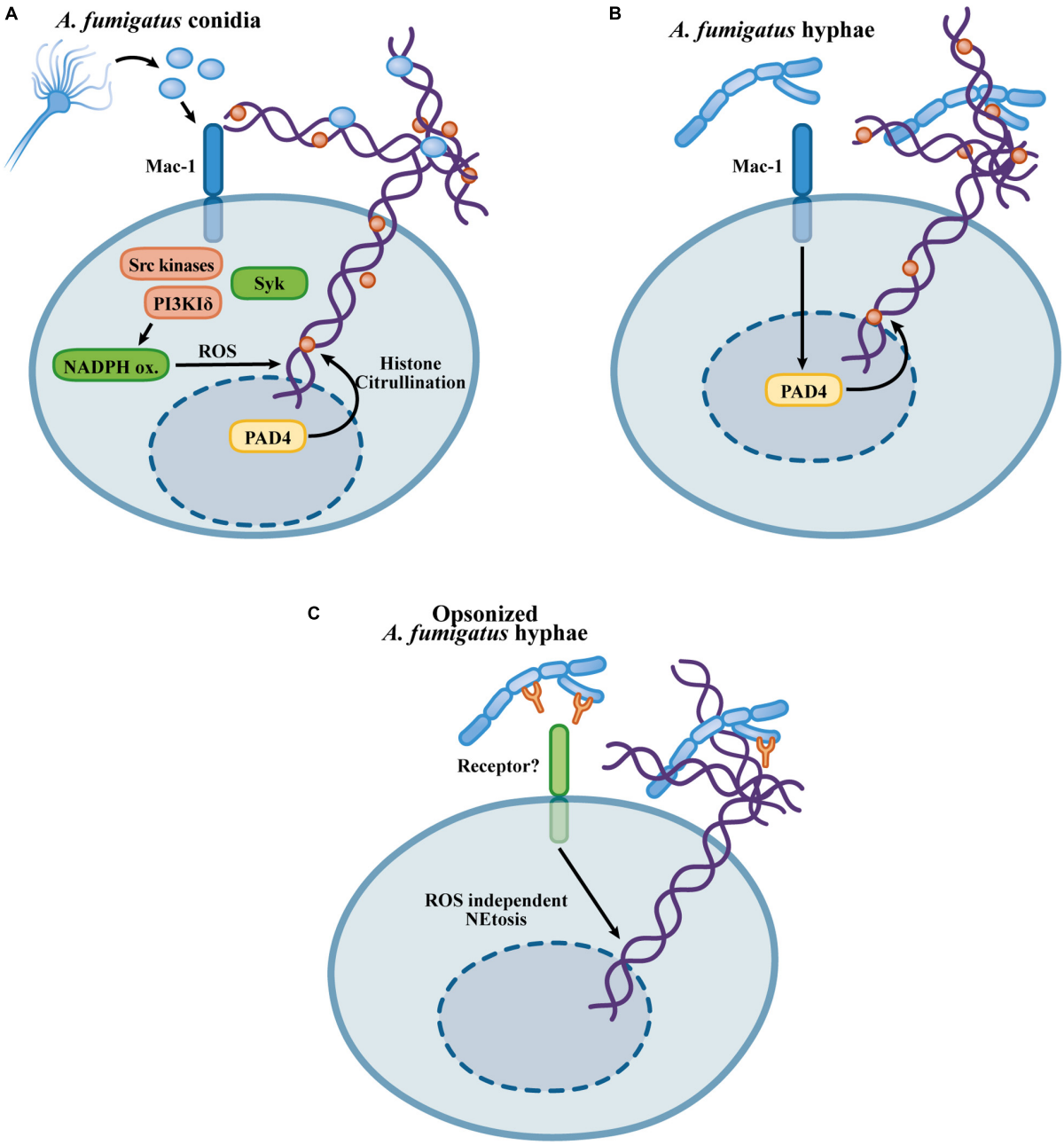

FIGURE 2 | Mechanisms proposed for the NETosis in response to *Aspergillus fumigatus*. **(A)** Recognition of *A. fumigatus* triggers NETosis by a Mac-1/Src kinase/Syk and class I PI3Kδ mechanism, inducing a NET-releasing pathway that requires ROS generation by the NADPH oxidase but occurs independently of the PAD4-mediated histone citrullination (Silva et al., 2020). **(B)** *A. fumigatus* extracts, as well as β-glucans, induce NETosis through a Mac-1 and PAD4-dependent mechanism (Clark et al., 2018). **(C)** *A. fumigatus* opsonized hyphae promote NETosis through a NADPH oxidase-independent pathway (Gazendam et al., 2016b).

to *P. brasiliensis* is partially dependent on Dectin-1, while TLR2 and TLR4 are dispensable (Bachiega et al., 2016). Although human neutrophils exhibit reduced fungicidal activity against *P. brasiliensis*, neutrophil primming with TNF, GM-CSF, or IFN-γ results in *P. brasiliensis* killing by a mechanism requiring NET formation (Bachiega et al., 2016). Thus, although *P. brasiliensis* evades neutrophil killing, their ability to escape from neutrophil fungicidal mechanisms can be overcome by neutrophil activation induced by pro-inflammatory cytokines, leading to NET-associated fungicidal activity. It would be interesting to evaluate whether pro-inflammatory cytokines lead to a different NET composition or whether cooperation of NETs with other microbicidal mechanisms can be induced by neutrophil-activating cytokines, thus enabling fungal killing. **Figures 3B,C** illustrates the findings concerning the NET formation in response to *P. brasiliensis*.

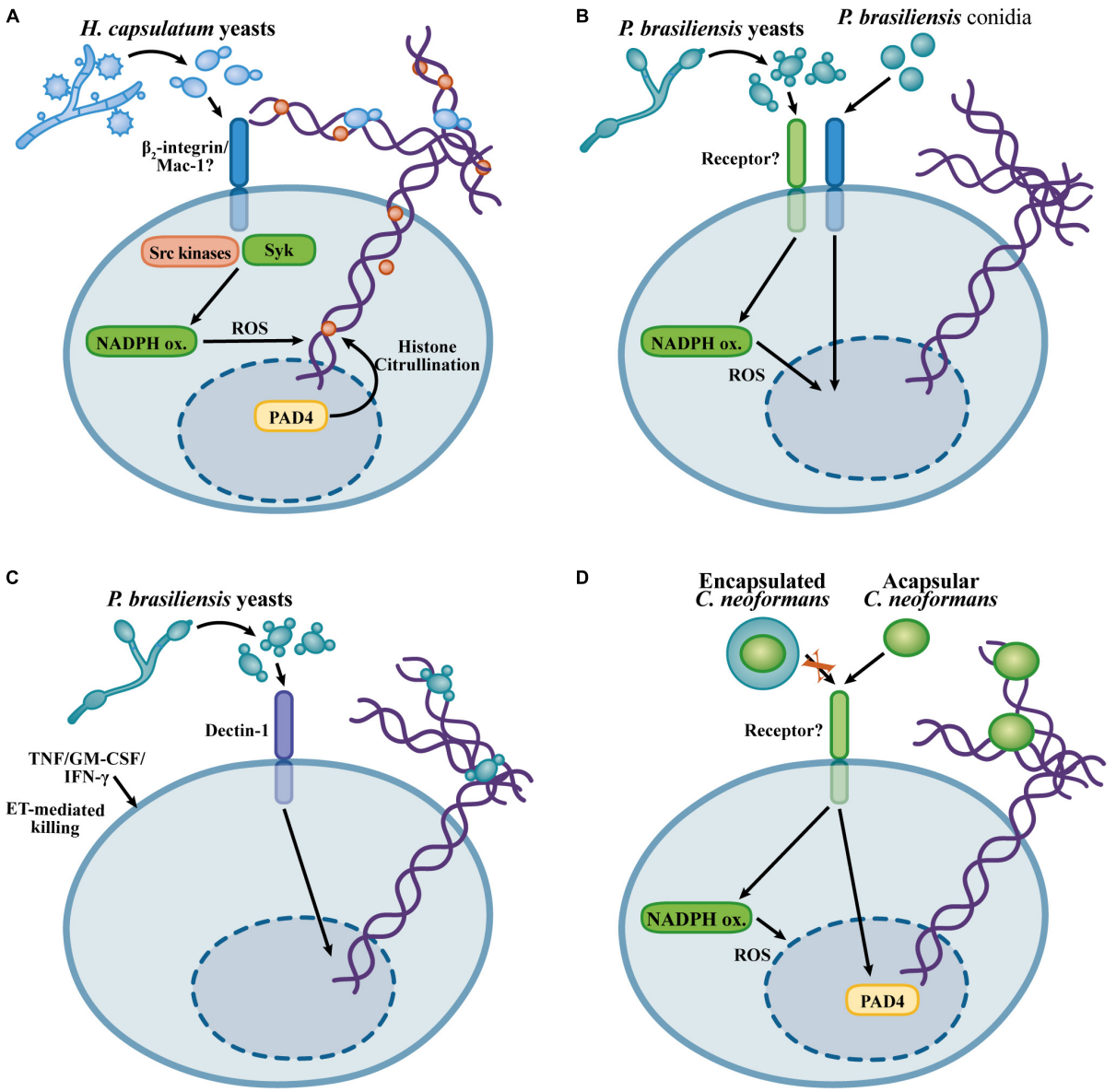

FIGURE 3 | Overview of the mechanisms described for the NET induction in response to *Histoplasma capsulatum*, *Cryptococcus neoformans,* and *Paracoccidioides brasiliensis*. **(A)** *H. capsulatum* NETosis relies on β_2-integrin recognition, Src and Syk kinase, via a NADPH oxidase-dependent process, but in a histone citrullination-independent manner (Thompson-Souza et al., 2020). **(B)** NET formation in response to *P. brasiliensis* yeast cells and conidia occurs through NADPH oxidase-dependent and NADPH oxidase -independent mechanisms, respectively (Mejía et al., 2015; Bachiega et al., 2016). **(C)** Dectin-1 mediated recognition of *P. brasiliensis* yeast cells promotes NET formation. Although *P. brasiliensis* yeast cells are resistant to the neutrophil killing, neutrophil-activating cytokines promote fungicidal activity against *P. brasiliensis* through NET-mediated fungal damage (Bachiega et al., 2016). **(D)** Mechanisms of NETosis in response to *C. neoformans*. Encapsulated *C. neoformans* evades NETosis, while acapsular yeast cells induce NET formation by NADPH oxidase- and PAD4-dependent mechanisms (Rocha et al., 2015).

Cryptococcus gattii AND *Cryptococcus neoformans*

Cryptococcus gattii and *C. neoformans* are environmental fungi able to grow as encapsulated yeast cells. Infections caused by *C. neoformans* and *C. gattii* are initiated by the inhalation of environmental yeast cells or spores, which establishes a pulmonary infection and subsequent dissemination to the central nervous system leading to meningitis. While *C. gattii* has been identified as a primary pathogen causing infections in immunocompetent individuals, *C. neoformans* infections are mostly associated with deficiencies in T cell-mediated immunity, especially in HIV-infected patients (May et al., 2016). Human neutrophils exhibit fungicidal activity against *C. neoformans* by either oxidative or non-oxidative mechanisms (Mambula et al., 2000). Through the fractionation of human

neutrophils, in cytosolic and granular preparations, the antifungal components calprotectin and defensins (human neutrophil proteins 1 and 3, HNP-1 and HNP-3), have been identified as anticryptococcal molecules present in the cytosol and primary granules, respectively (Mambula et al., 2000). Killing of *C. gattii* by neutrophils requires serum opsonization, phagocytosis and serine protease activity, but the NADPH oxidative burst is dispensable (Ueno et al., 2019).

Cryptococcus gattii yeast cells growing on plant-derived material produce extracellular fibrils. The formation of extracellular fibrils results in increased resistance of *C. gattii* yeast cells to killing by neutrophils. Paradoxically, the production of extracellular fibrils by *C. gattii* results in increased NET formation, indicating that although extracellular fibrils induce neutrophil activation, these structures confer resistance to neutrophil effector mechanisms (Springer et al., 2010). NETs show fungicidal activity against *C. neoformans* yeast cells due to the microbicidal effects of MPO, elastase, histones and collagenase (Urban et al., 2009; Rocha et al., 2015). While encapsulated *C. neoformans* yeast cells do not induce NET formation, a capsule-deficient strain leads to NETosis via an NADPH oxidase- and PAD4-dependent mechanism (Rocha et al., 2015). Glucuronoxylomannan (GXM) is the major component of the *C. neoformans* capsule (O'Meara and Alspaugh, 2012). Purified GXM inhibits NET formation and neutrophil ROS generation, and incubation of a non-encapsulated *C. neoformans* strain with GXM results in inhibition of NETosis, thus indicating that GXM is the component in the *C. neoformans* capsule critical for the inhibition of NETosis. Interestingly, glucuronoxylomannogalactan (GXMGal), a minor component of the *C. neoformans* capsule, is an inducer of NET formation through a ROS-independent mechanism (Rocha et al., 2015). Thus, the secretion of extracellular polysaccharides by *Cryptococcus* spp. represents a major evasion mechanism for the fungicidal activity of NETs. **Figure 3D** illustrates the knowledge about NET formation in response to *C. neoformans*.

EETs AND FUNGI

Different studies have characterized eosinophils as capable of recognizing fungi, as well as fungal molecular patterns, which promote eosinophil activation and antifungal responses (Inoue et al., 2005; Yoon et al., 2008; Garro et al., 2011; Lilly et al., 2014). Human eosinophils respond to *A. alternata* hyphae by releasing their granular eosinophilic content, which reduces fungal viability (Yoon et al., 2008). Eosinophil degranulation was induced by β-glucans, but not chitin, through recognition mediated by the β_2-integrin Mac-1 (Yoon et al., 2008). In addition to the recognition of β-glucans, eosinophils also recognize proteases secreted by *A. alternata* in a mechanism that involves protease-activated receptor-2 (PAR-2) (Matsuwaki et al., 2009, 2011). Eosinophils are required for the clearance of *A. fumigatus* in an experimental model of pulmonary infection (Lilly et al., 2014). In addition, *in vitro* murine bone marrow-differentiated eosinophils present fungicidal properties against *A. fumigatus* conidia in a mechanism that does not depend on contact (Lilly et al., 2014). Thus, despite different studies exploring the mechanisms of fungal recognition by eosinophils, there is still a shortage of evidence describing how eosinophils contribute to fungal infections by releasing EETs.

Bronchial secretions of patients with ABPA show EETs in association with large numbers of eosinophils with clear nuclear characteristics of EETosis. EETs in the mucus obtained from ABPA individuals show citrullinated histone 3 (Muniz et al., 2018). Human eosinophils form EETs in response to *A. fumigatus* conidia through a cytolytic process. EETs show labeling for MBP, a granular protein; however, in contrast to NETs that exhibit an association with free granule proteins, such as NE and MPO, EETs exhibit large punctuated immunostaining for cationic proteins, suggesting that these proteins are not freely attached to these structures (Muniz et al., 2018). The presence of clusters of intact free eosinophil granules associated with EETs has been described for other stimuli, such as a calcium ionophore and LysoPS (Ueki et al., 2013; Kim et al., 2020). Recent evidence indicates that intact granules are associated with *A. fumigatus*-induced EETs (Muniz et al., 2018), which must reflect a general mechanism by which many stimuli can lead to the cytolytic process in EET formation. In contrast, the presence of clusters of eosinophil granules attached to EETs was not observed in studies reporting mitochondrial-derived EET release (Yousefi et al., 2008; Morshed et al., 2012). In addition, there is no evidence showing that faster non-cytolytic mitochondrial EETs are released in response to fungal exposure. However, this is an interesting possibility that cannot be discarded.

In neutrophils, NE and MPO are directed to the nucleus and have roles in the chromatin decondensation that precedes nuclear membrane rupture, mixture of nuclear content and NET extrusion, as previously mentioned (Fuchs et al., 2007; Papayannopoulos et al., 2010). However, for eosinophils, it is uncertain whether granular proteins have roles in the chromatin decompaction and nuclear rupture that precede EETosis, or which molecules are involved in this process. Thus, the mechanisms that underlie EET extrusion considering the roles of granular proteins and possible mediators of EETosis remain to be elucidated.

Reactive oxygen species generation has traditionally been suggested as an important mechanism of host defense against pathogens and a downstream signal for the induction of NETosis (Fuchs et al., 2007; Winterbourn et al., 2016). However, pharmacological inhibition of NADPH oxidase or mitochondrial ROS generation does not interfere with *A. fumigatus*-induced EET formation, indicating that EETosis occurs independently of the major systems critical for ROS production in leukocytes (Muniz et al., 2018). Furthermore, eosinophils do not exhibit ROS production in response to *A. fumigatus*, while PMA is able to induce an oxidative burst in eosinophils in an NADPH oxidase-dependent manner. This difference is interesting because the release of EETs differs from NETs formed by neutrophils responding to *A. fumigatus*, and also from other neutrophil responses in which ROS involvement is clearly important (Bruns et al., 2010; Röhm et al., 2014; Winterbourn et al., 2016; Muniz et al., 2018; Silva et al., 2020).

Considerable discussion is reported regarding the receptors and proteins that may be involved in the recognition of the pathogens and signaling pathways that trigger the NET formation. In eosinophils, Mac-1 mediates the process of *A. fumigatus*-induced EET release (Muniz et al., 2018). Interestingly, Mac-1 emerges as a pattern recognition receptor (PRR) involved in neutrophil and eosinophil responses to fungal pathogens; however, some aspects remain uncharacterized, for example, (i) does Mac-1 directly recognize *A. fumigatus* molecules, and if it does, what is the identity of these molecules? (ii) Is there cooperation between Mac-1 and other PRR(s) during *A. fumigatus* recognition by neutrophils and eosinophils? Interestingly, peripheral human eosinophils do not express Dectin-1 (Yoon et al., 2008; Muniz et al., 2018), and neutralization of Dectin-1 does not impact the EET release induced by *A. fumigatus*, findings consistent with the inability of Dectin-1 to induce NET formation (Branzk et al., 2014; Muniz et al., 2018). However, it remains to be established whether there are roles for other well-established PRRs involved in fungal recognition, such as TLRs or other C-type lectin receptors (CLRs), in EETosis and NETosis.

Similarly, as observed for neutrophils, human eosinophils stimulated with *A. fumigatus* also release EETs in a Syk-dependent manner (Muniz et al., 2018; Silva et al., 2020). Investigations regarding the participation of other signaling components in the *A. fumigatus* stimulation of eosinophils are still needed. The roles of PAD4 and histone citrullination in EET release in response to *A. fumigatus* are also unknown. Furthermore, more investigations regarding the capacity of eosinophils to release EETs in response to other fungi, particularly those involved in ABPMs (such as *A. alternata* and *C. albicans*), are necessary. **Figure 4** summarizes the findings on the mechanisms of *A. fumigatus*-induced EET formation.

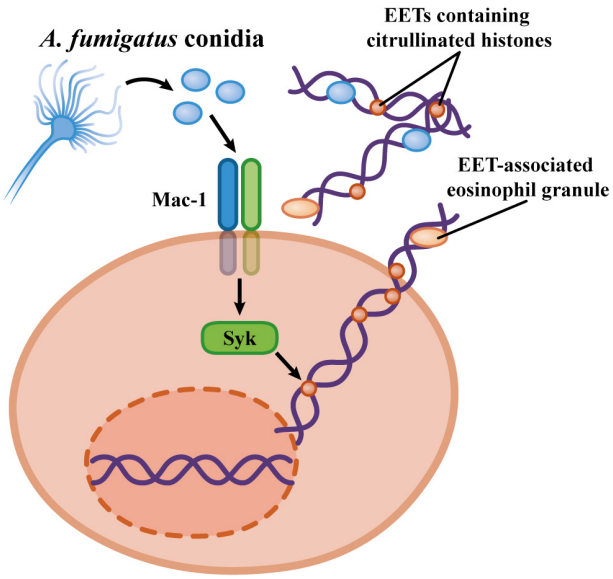

FIGURE 4 | Mechanisms proposed by EET formation in response to *Aspergillus fumigatus*. Human eosinophils release ETs by a cytolytic process via the Mac-1 and Syk activation in a ROS-independent mechanism (Muniz et al., 2018).

MECHANISMS OF EVASION OF THE FUNGICIDAL ACTIVITY OF ETs

Neutrophil extracellular trap formation is a relevant microbicidal mechanism of neutrophils, and many pathogenic fungi present mechanisms that prevent the induction of NETosis or attenuate the toxicity of NETs. Growth in a biofilm inhibits the killing of *C. albicans* hyphae by neutrophils (Johnson et al., 2016). *C. albicans* biofilms impair *C. albicans*-induced NETosis and ROS generation by neutrophils, as well as NET formation and ROS generation in response to PMA. The inhibitory activity of the *C. albicans* biofilm on neutrophil effector responses, such as NETosis and ROS generation, requires intact mannosylation of *C. albicans*. The mutant *C. albicans* strain $PMR1^{\Delta/\Delta}$ lacks a Ca^{+2}-ATPase necessary for the activity of mannosyltransferases in the Golgi complex, which results in the formation of truncated mannan chains. Neutrophils show increased NET formation in response to $PMR1^{\Delta/\Delta}$ *C. albicans* compared with wild-type *C. albicans*, and this phenotype is reversed by the restoration of mannosylation upon *PMR1* expression. Thus, the *C. albicans* biofilm seems to down modulate neutrophil signaling pathways possibly downstream of PKC activation, thus reducing the impact of NET toxicity (Johnson et al., 2016).

Preventing NET formation has also been observed in an encapsulated *C. neoformans* strain that does not induce NET release, in contrast with a non-encapsulated *C. neoformans* strain that promotes extensive NETosis and ROS generation. Interestingly, purified GXM inhibits PMA-induced NET formation and renders an acapsular *C. neoformans* strain unable to induce NET formation, thus indicating that, similar to the *C. albicans* biofilm, GXM acts as a secreted fungal molecule that inhibits neutrophil responses (Rocha et al., 2015). The expression of an external layer of hydrophobic proteins on *A. fumigatus* conidia conceals the ligands for PRRs, which decreases the release of NETs (Bruns et al., 2010) and the activation of PRRs, such as TLRs and Dectin-1 (Aimanianda et al., 2009). Thus, fungal pathogens evade the toxic effects of neutrophils by the secretion or surface expression of components able to inhibit NETosis induction.

The exopolysaccharide galactosaminogalactan (GAG) is found in the *A. fumigatus* cell wall, and this polysaccharide is composed of galactose and N-acetylgalactosamine (GalNAc) (Fontaine et al., 2011). The expression of GAG by *A. fumigatus* mediates its adherence and is required for full virulence and biofilm formation (Fontaine et al., 2011; Gravelat et al., 2013). *Aspergillus* species with lower pathogenicity, such as *A. nidulans*, present decreased expression of GalNAc in their GAGs, while *A. fumigatus* naturally produces GAG with higher amounts of GalNAc (Lee et al., 2015). The generation of *A. nidulans* strains overexpressing the enzymes involved in the synthesis of GalNAc results in increased resistance to neutrophil killing. This reduced susceptibility caused by the increased content of GalNAc in the

A. nidulans-derived GAGs is a consequence of the resistance to the toxic effects of NETs, as indicated by DNAse treatment resulting in similar susceptibility of wild-type *A. nidulans* (Lee et al., 2015). The overexpression of GalNAc in *A. nidulans* strains resulted in increased virulence in murine experimental models of aspergillosis, which was similar to the virulence of *A. fumigatus* under the same experimental conditions (Lee et al., 2015). Thus, the expression of GAGs with a high content of GalNAc seems to be a determinant for the virulence of *A. fumigatus*. A recent paper demonstrated the activation of the NLRP3 inflammasome by *A. fumigatus* GAGs. The activation of the NLRP3 inflammasome by GAG was required for the control of infection in experimental models of aspergillosis, with an *A. fumigatus* strain deficient in GAG production showing increased virulence. Interestingly, the activation of the NLRP3 inflammasome by GAGs depended on the degree of N-acetylation, with acetylated GAGs unable to promote inflammasome activation (Briard et al., 2020). Thus, recognition of *A. fumigatus* GAGs by the NLRP3 inflammasome may reflect a mechanism for detecting a virulence factor produced by *A. fumigatus*; in contrast, increases in the degree of acetylation of GAGs may contribute to the evasion from the toxic effects of NETs and inflammasome activation. Therefore, it would be interesting to evaluate whether differences in GAG acetylation in clinical isolates of *A. fumigatus* are correlated with virulence.

The production of nucleases is a mechanism used by bacterial pathogens (Buchanan et al., 2006; Berends et al., 2010) and *Leishmania infantum* (Guimarães-Costa et al., 2014) to degrade NETs, thus avoiding neutrophil-mediated killing. *C. albicans* strains produce DNAse activity during interactions with neutrophils, and there is a correlation between the amount of DNAse produced and the susceptibility of different *C. albicans* strains to killing by neutrophils (Zhang et al., 2017). This work, however, did not identify the putative *C. albicans* DNAse(s) or offer formal proof using *C. albicans* mutants with deficient DNAse activity. Therefore, it remains to be established whether the production of DNase enzymes is a mechanism employed by pathogenic fungi to evade NET-induced toxicity.

ROLES OF DNA EXTRACELLULAR TRAPS IN FUNGAL INFECTIONS

Neutrophil extracellular traps are mediators of the antimicrobial arsenal of neutrophils and have been implicated in the elimination and control of many pathogens (Brinkmann and Zychlinsky, 2012). In addition, many investigations have demonstrated a role for NETs in the pathogenesis in many experimental models and human diseases. NET-induced pathology has been observed in autoimmune diseases (Hakkim et al., 2010), atherosclerosis (Warnatsch et al., 2015), and thrombosis (Fuchs et al., 2010). Thus, the roles of NETs in fungal infections must involve both antifungal immunity and immunopathology. While NETs have been demonstrated to contribute to the antifungal activity of neutrophils *in vitro*, the roles of NETs in fungal infections are still poorly characterized. NETs have been described in experimental models of aspergillosis, scedosporiosis and candidiasis (Urban et al., 2009; Bruns et al., 2010; Wu et al., 2019; Luna-Rodríguez et al., 2020).

Using different experimental models of *C. albicans* infection, Urban et al. (2009) revealed that NETs are formed in infected tissues and restrict *C. albicans* spread. Calprotectin-deficient mice show increased susceptibility in experimental models of cutaneous, pulmonary, and systemic *C. albicans* infection, which is manifested by increased fungal loads (pulmonary infection), lethality (pulmonary and systemic infections), and formation of skin abscesses (Urban et al., 2009). The increased susceptibility of calprotectin-deficient mice does not result from inefficient neutrophil tissue recruitment or NET formation, indicating that the effect of calprotectin is a consequence of its intrinsic antifungal activity (Urban et al., 2009). Since calprotectin is released in association with NETs and is also required for NET antifungal activity, the role of calprotectin in the immune response to *C. albicans* must reflect the fungicidal activity of NET-associated calprotectin. However, Urban et al. (2009) did not evaluate the formal roles of NETs *in vivo,* for example, by NET removal by DNAse *in vivo* treatment; therefore, it remains possible that calprotectin acts in a NET-independent way, as a fungicidal molecule. In an experimental model of intraperitoneal infection by *C. albicans*, NETs were formed, and the degradation of these NETs by the intraperitoneal administration of micrococcal nuclease (MNase) resulted in *C. albicans* dissemination to the kidneys in association with reduced *C. albicans* loads in the peritoneal cavity, which indicates that the peritoneal entrapment of *C. albicans* by NETs prevented the systemic spread of infection (Wu et al., 2019).

Although NETs are formed in the lung parenchyma in a murine model of experimental aspergillosis (Bruns et al., 2010; Röhm et al., 2014), their roles in immunopathogenesis remain unclear. In a murine model of pulmonary aspergillosis, PAD4-KO mice showed decreased fungal loads and alveolar edema; however, neutrophil recruitment and pulmonary pathology were not affected by PAD4 deficiency (Alflen et al., 2020). The results obtained with PAD4-KO mice have been interpreted as evidence of roles for NETs in experimental pulmonary aspergillosis. These conclusions, however, are based on the premise that PAD4 is required for *A. fumigatus*-induced NETosis, which has not been observed (Silva et al., 2020). Moreover, Alflen et al. did not formally evaluate the roles of NETs in the aspergillosis by determining whether (1) NETs were evident in the lung tissues of wild-type or PAD4-KO mice, (2) NETs were formed by wild-type or PAD4-KO neutrophils or whether (3) DNAse treatment had an effect in the outcome of infection (Alflen et al., 2020). NADPH oxidase deficiency resulted in the absence of *A. fumigatus*-induced NET release by murine neutrophils, and *Ncf1*$^{-/-}$ mice showed the absence of NETs in lung tissues in an experimental model of pulmonary aspergillosis (Röhm et al., 2014). NADPH oxidase-deficient mice are extremely susceptible to pulmonary aspergillosis; however, the role of NADPH oxidase in aspergillosis is complex and must involve oxidative killing of *A. fumigatus*, as well as the control of lung inflammation, and probably does not reflect merely the absence of NETosis (Morgenstern et al., 1997; Winterbourn et al., 2016). A case study of gene therapy for CGD revealed that restoring NADPH oxidase activity in

hematopoietic cells resulted in the recovery of disseminated infection by *A. nidulans*, which was associated with restored NETosis and the NET-mediated inhibition of *A. nidulans* germination (Bianchi et al., 2009). However, considering the complexity of this clinical study and the evident technical limitations, it is not possible to conclude a specific role for NETosis in CGD gene therapy, especially considering all the roles of NADPH oxidase in immunity. Interestingly, therapy with DNAse is an approach for the treatment of cystic fibrosis. *A. fumigatus* pulmonary infections are common conditions in individuals suffering from cystic fibrosis. A clinical study investigating risk factors for the development of pulmonary aspergillosis pointed to therapy with recombinant DNAse as a factor associated with the incidence of pulmonary aspergillosis in pediatric patients with cystic fibrosis (Jubin et al., 2010). Therefore, it seems possible that the continuous formation of NETs prevents *A. fumigatus* colonization of the airways in cystic fibrosis and other pulmonary conditions where mucus accumulation is present. Alternatively, NETs must promote tissue pathology, contributing to the severity of pulmonary aspergillosis. Thus, more detailed investigations of the role of NETs in experimental models of aspergillosis are necessary.

The roles of EETs in fungal infections are even more elusive. The bronchiolar mucus of patients with ABPA presents extracellular EETs and abundant infiltrates of eosinophils with clear nuclear characteristics of EETosis. Human eosinophils, however, do not show fungicidal/fungistatic effects on *A. fumigatus* conidia under experimental conditions in which EETosis occurs (Muniz et al., 2018). Thus, it is possible that EETs ensnare viable *A. fumigatus* conidia in the bronchial tree, which must cause chronic colonization of *A. fumigatus* and perpetuation of allergic inflammation. In a mouse model of ABPM induced by intranasal challenge with *A. fumigatus* conidia, eosinophils were dispensable for pathological alterations, such as mucus production and pulmonary edema. Although the absence of eosinophils did not interfere with IL-5 and IL-13 production, eosinophils were required for IL-4, IL-17, and IL-23 production (Dietschmann et al., 2020). Thus, eosinophils play immunomodulatory roles in the experimental ABPM, although some aspects of the pulmonary pathology occur independently of eosinophil activity. However, this work did not evaluate parameters such as fungal load, respiratory mechanics, airway remodeling, collagen deposition and formation of EETs. Thus, more investigations are necessary to establish the roles of EETs and eosinophils in ABPMs.

Signaling pathways involved in the formation of NETs and EETs are also implicated in other neutrophil and eosinophil responses, such as the modulation of apoptosis, microbicidal killing and production of inflammatory mediators. For example, NADPH oxidase is critical for microbial oxidative killing, as well as the modulation of the inflammatory response and neutrophil apoptosis (Winterbourn et al., 2016; Warnatsch et al., 2017). Thus, the use of NADPH oxidase-deficient mice would fail to reveal specific roles of NETs in experimental models of fungal infection. The same situation must occur in the context of PAD4, NE, and MPO deficiency. Therefore, complementary approaches must be used to establish the role of NETs in fungal infections, such as the use of DNAse *in vivo* treatment, use of experimentally induced neutrophil/eosinophil selective deficiency of signaling molecules or receptors, neutrophil/eosinophil transfer experiments, and the detection of NETs/EETs *in vivo*.

CONCLUSION

Neutrophils are critical effectors of antifungal immunity, but they also contribute to the immunopathology associated with infections. Although the roles of eosinophils in fungal diseases are still poorly understood, growing evidence indicates that these leukocytes show antifungal activity and immunomodulatory mechanisms in fungal-associated pathologies. Neutrophils and eosinophils release ETs, structures that exhibit microbicidal actions but also promote tissue injury. Although considerable information has been obtained through *in vitro* experiments, NETosis and EETosis are still poorly characterized in experimental models of mycoses. Thus, the comprehension of the mechanisms involved in NETosis/EETosis in response to fungal pathogens must contribute for the design of new therapeutic strategies, including new pharmacological targets.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the following programs from the Brazilian Agency, Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ): (1) Jovem Cientista do Estado do Rio de Janeiro (granted to RF), (2) Cientista do Estado do Rio de Janeiro (granted to JN), and (3) Apoio a Grupos Emergentes do Estado do Rio de Janeiro (JN and RF). JS, MB, and GT-S were supported by fellowships from the Brazilian Federal Agency Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). JN was also supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil).

REFERENCES

Acharya, K. R., and Ackerman, S. J. (2014). Eosinophil granule proteins: form and function. *J. Biol. Chem.* 289, 17406–17415. doi: 10.1074/jbc.R113.546218

Agarwal, R., and Chakrabarti, A. (2013). Allergic bronchopulmonary aspergillosis in asthma: epidemiological, clinical and therapeutic issues. *Future Microbiol.* 8, 1463–1474. doi: 10.2217/fmb.13.116

Aimanianda, V., Bayry, J., Bozza, S., Kniemeyer, O., Perruccio, K., Elluru, S. R., et al. (2009). Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature* 460, 1117–1121. doi: 10.1038/nature08264

Alflen, A., Aranda Lopez, P., Hartmann, A. K., Maxeiner, J., Bosmann, M., Sharma, A., et al. (2020). Neutrophil extracellular traps impair fungal clearance in a mouse model of invasive pulmonary aspergillosis. *Immunobiology* 225:151867. doi: 10.1016/j.imbio.2019.11.002

Amulic, B., Cazalet, C., Hayes, G. L., Metzler, K. D., and Zychlinsky, A. (2012). Neutrophil function: from mechanisms to disease. *Annu. Rev. Immunol.* 30, 459–489. doi: 10.1146/annurev-immunol-020711-074942

Amulic, B., Knackstedt, S. L., Abu Abed, U., Deigendesch, N., Harbort, C. J., Caffrey, B. E., et al. (2017). Cell-cycle proteins control production of neutrophil extracellular traps. *Dev. Cell* 43, 449.e5–462.e5. doi: 10.1016/j.devcel.2017.10.013

Asaga, H., Nakashima, K., Senshu, T., Ishigami, A., and Yamada, M. (2001). Immunocytochemical localization of peptidylarginine deiminase in human eosinophils and neutrophils. *J. Leukoc Biol.* 70, 46–51.

Asano, K., Hebisawa, A., Ishiguro, T., Takayanagi, N., Nakamura, Y., Suzuki, J., et al. (2020). New clinical diagnostic criteria for allergic bronchopulmonary aspergillosis/mycosis and its validation. *J. Allergy Clin. Immunol.* S0091-6749, 31240–31249. doi: 10.1016/j.jaci.2020.08.029

Bachiega, T. F., Dias-Melicio, L. A., Fernandes, R. K., De Almeida Balderramas, H., Rodrigues, D. R., Ximenes, V. F., et al. (2016). Participation of dectin-1 receptor on NETs release against *Paracoccidioides brasiliensis*: role on extracellular killing. *Immunobiology* 221, 228–235. doi: 10.1016/j.imbio.2015.09.003

Behnen, M., Leschczyk, C., Möller, S., Batel, T., Klinger, M., Solbach, W., et al. (2014). Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcγRIIIB and Mac-1. *J. Immunol.* 193, 1954–1965. doi: 10.4049/jimmunol.1400478

Berends, E. T., Horswill, A. R., Haste, N. M., Monestier, M., Nizet, V., and Von Köckritz-Blickwede, M. (2010). Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps. *J. Innate Immun.* 2, 576–586. doi: 10.1159/000319909

Bianchi, M., Hakkim, A., Brinkmann, V., Siler, U., Seger, R. A., Zychlinsky, A., et al. (2009). Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood* 114, 2619–2622. doi: 10.1182/blood-2009-05-221606

Bianchi, M., Niemiec, M. J., Siler, U., Urban, C. F., and Reichenbach, J. (2011). Restoration of anti-*Aspergillus* defense by neutrophil extracellular traps in human chronic granulomatous disease after gene therapy is calprotectin-dependent. *J. Allergy Clin. Immunol.* 127, 1243.e7–1252.e7. doi: 10.1016/j.jaci.2011.01.021

Boyle, K. B., Gyori, D., Sindrilaru, A., Scharffetter-Kochanek, K., Taylor, P. R., Mócsai, A., et al. (2011). Class IA phosphoinositide 3-kinase β and δ regulate neutrophil oxidase activation in response to *Aspergillus fumigatus* hyphae. *J. Immunol.* 186, 2978–2989. doi: 10.4049/jimmunol.1002268

Branzk, N., Lubojemska, A., Hardison, S. E., Wang, Q., Gutierrez, M. G., Brown, G. D., et al. (2014). Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat. Immunol.* 15, 1017–1025. doi: 10.1038/ni.2987

Briard, B., Fontaine, T., Samir, P., Place, D. E., Muszkieta, L., Malireddi, R. K. S., et al. (2020). Galactosaminogalactan activates the inflammasome to provide host protection. *Nature* 588, 688–692. doi: 10.1038/s41586-020-2996-z

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., et al. (2004). Neutrophil extracellular traps kill bacteria. *Science* 303, 1532–1535. doi: 10.1126/science.1092385

Brinkmann, V., and Zychlinsky, A. (2012). Neutrophil extracellular traps: is immunity the second function of chromatin? *J. Cell Biol.* 198, 773–783. doi: 10.1083/jcb.201203170

Brown, G. D., Denning, D. W., Gow, N. A., Levitz, S. M., Netea, M. G., and White, T. C. (2012). Hidden killers: human fungal infections. *Sci. Transl. Med.* 4:165rv113. doi: 10.1126/scitranslmed.3004404

Brummer, E., Kurita, N., Yosihida, S., Nishimura, K., and Miyaji, M. (1991). Fungistatic activity of human neutrophils against *Histoplasma capsulatum*: correlation with phagocytosis. *J. Infect. Dis.* 164, 158–162. doi: 10.1093/infdis/164.1.158

Bruns, S., Kniemeyer, O., Hasenberg, M., Aimanianda, V., Nietzsche, S., Thywissen, A., et al. (2010). Production of extracellular traps against *Aspergillus fumigatus* in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. *PLoS Pathog.* 6:e1000873. doi: 10.1371/journal.ppat.1000873

Buchanan, J. T., Simpson, A. J., Aziz, R. K., Liu, G. Y., Kristian, S. A., Kotb, M., et al. (2006). DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. *Curr. Biol.* 16, 396–400. doi: 10.1016/j.cub.2005.12.039

Byrd, A. S., O'brien, X. M., Johnson, C. M., Lavigne, L. M., and Reichner, J. S. (2013). An extracellular matrix-based mechanism of rapid neutrophil extracellular trap formation in response to *Candida albicans*. *J. Immunol.* 190, 4136–4148. doi: 10.4049/jimmunol.1202671

Campos-Garcia, L., Jimenez-Valdes, R. J., Hernandez-Bello, R., Palma-Nicolas, J., Gonzalez, G. M., and Sanchez-Gonzalez, A. (2019). *Candida albicans* and non-*albicans* isolates from bloodstream have different capacities to induce neutrophil extracellular traps. *J. Fungi* 5:28. doi: 10.3390/jof5020028

Cezar-Dos-Santos, F., Assolini, J. P., Okuyama, N. C. M., Viana, K. F., De Oliveira, K. B., and Itano, E. N. (2020). Unraveling the susceptibility of paracoccidioidomycosis: insights towards the pathogen-immune interplay and immunogenetics. *Infect. Genet. Evol.* 86:104586. doi: 10.1016/j.meegid.2020.104586

Chen, J., Tang, H., Hay, N., Xu, J., and Ye, R. D. (2010). Akt isoforms differentially regulate neutrophil functions. *Blood* 115, 4237–4246. doi: 10.1182/blood-2009-11-255323

Clark, H. L., Abbondante, S., Minns, M. S., Greenberg, E. N., Sun, Y., and Pearlman, E. (2018). Protein deiminase 4 and CR3 regulate *Aspergillus fumigatus* and β-glucan-induced neutrophil extracellular trap formation, but hyphal killing is dependent only on CR3. *Front. Immunol.* 9:1182. doi: 10.3389/fimmu.2018.01182

Claushuis, T. A. M., Van Der Donk, L. E. H., Luitse, A. L., Van Veen, H. A., Van Der Wel, N. N., Van Vught, L. A., et al. (2018). Role of peptidylarginine deiminase 4 in neutrophil extracellular trap formation and host defense during *Klebsiella pneumoniae*-Induced pneumonia-derived sepsis. *J. Immunol.* 201, 1241–1252. doi: 10.4049/jimmunol.1800314

Colombo, A. L., Tobón, A., Restrepo, A., Queiroz-Telles, F., and Nucci, M. (2011). Epidemiology of endemic systemic fungal infections in Latin America. *Med. Mycol.* 49, 785–798. doi: 10.3109/13693786.2011.577821

Della Coletta, A. M., Bachiega, T. F., De Quaglia e Silva, J. C., Soares, Â, De Faveri, J., Marques, S. A., et al. (2015). Neutrophil extracellular traps identification in tegumentary lesions of patients with paracoccidioidomycosis and different patterns of NETs generation in vitro. *PLoS Negl. Trop. Dis.* 9:e0004037. doi: 10.1371/journal.pntd.0004037

DeSouza-Vieira, T., Guimarães-Costa, A., Rochael, N. C., Lira, M. N., Nascimento, M. T., Lima-Gomez, P. S., et al. (2016). Neutrophil extracellular traps release induced by *Leishmania*: role of PI3Kγ, ERK, PI3Kσ, PKC, and [Ca2+]. *J. Leukoc Biol.* 100, 801–810. doi: 10.1189/jlb.4A0615-261RR

Diamond, M. S., Garcia-Aguilar, J., Bickford, J. K., Corbi, A. L., and Springer, T. A. (1993). The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. *J. Cell Biol.* 120, 1031–1043.

Dietschmann, A., Schruefer, S., Krappmann, S., and Voehringer, D. (2020). Th2 cells promote eosinophil-independent pathology in a murine model of allergic bronchopulmonary aspergillosis. *Eur. J. Immunol.* 50, 1044–1056. doi: 10.1002/eji.201948411

Ermert, D., Urban, C. F., Laube, B., Goosmann, C., Zychlinsky, A., and Brinkmann, V. (2009). Mouse neutrophil extracellular traps in microbial infections. *J. Innate Immun.* 1, 181–193. doi: 10.1159/000205281

Figueiredo, R. T., and Neves, J. S. (2018). Eosinophils in fungal diseases: an overview. *J. Leukoc Biol.* 104, 49–60. doi: 10.1002/JLB.4MR1117-473R

Fontaine, T., Delangle, A., Simenel, C., Coddeville, B., Van Vliet, S. J., Van Kooyk, Y., et al. (2011). Galactosaminogalactan, a new immunosuppressive polysaccharide of *Aspergillus fumigatus*. *PLoS Pathog.* 7:e1002372. doi: 10.1371/journal.ppat.1002372

Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., et al. (2007). Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* 176, 231–241. doi: 10.1083/jcb.200606027

Fuchs, T. A., Brill, A., Duerschmied, D., Schatzberg, D., Monestier, M., Myers, D. D., et al. (2010). Extracellular DNA traps promote thrombosis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15880–15885. doi: 10.1073/pnas.1005743107

Fulkerson, P. C., and Rothenberg, M. E. (2013). Targeting eosinophils in allergy, inflammation and beyond. *Nat. Rev. Drug Discov.* 12, 117–129. doi: 10.1038/nrd3838

Futosi, K., Fodor, S., and Mócsai, A. (2013). Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int. Immunopharmacol.* 17, 638–650. doi: 10.1016/j.intimp.2013.06.034

Garro, A. P., Chiapello, L. S., Baronetti, J. L., and Masih, D. T. (2011). Rat eosinophils stimulate the expansion of *Cryptococcus neoformans*-specific CD4(+) and CD8(+) T cells with a T-helper 1 profile. *Immunology* 132, 174–187. doi: 10.1111/j.1365-2567.2010.03351.x

Gazendam, R. P., Van De Geer, A., Roos, D., Van Den Berg, T. K., and Kuijpers, T. W. (2016a). How neutrophils kill fungi. *Immunol. Rev.* 273, 299–311. doi: 10.1111/imr.12454

Gazendam, R. P., Van Hamme, J. L., Tool, A. T., Hoogenboezem, M., Van Den Berg, J. M., Prins, J. M., et al. (2016b). human neutrophils use different mechanisms to kill *Aspergillus fumigatus* conidia and hyphae: evidence from phagocyte defects. *J. Immunol.* 196, 1272–1283. doi: 10.4049/jimmunol.1501811

Germic, N., Stojkov, D., Oberson, K., Yousefi, S., and Simon, H. U. (2017). Neither eosinophils nor neutrophils require ATG5-dependent autophagy for extracellular DNA trap formation. *Immunology* 152, 517–525. doi: 10.1111/imm.12790

Gersuk, G. M., Underhill, D. M., Zhu, L., and Marr, K. A. (2006). Dectin-1 and TLRs permit macrophages to distinguish between different *Aspergillus fumigatus* cellular states. *J. Immunol.* 176, 3717–3724. doi: 10.4049/jimmunol.176.6.3717

Gravelat, F. N., Beauvais, A., Liu, H., Lee, M. J., Snarr, B. D., Chen, D., et al. (2013). *Aspergillus* galactosaminogalactan mediates adherence to host constituents and conceals hyphal β-glucan from the immune system. *PLoS Pathog.* 9:e1003575. doi: 10.1371/journal.ppat.1003575

Guiducci, E., Lemberg, C., Küng, N., Schraner, E., Theocharides, A. P. A., and Leibundgut-Landmann, S. (2018). *Candida albicans*-induced NETosis is independent of peptidylarginine deiminase 4. *Front. Immunol.* 9:1573. doi: 10.3389/fimmu.2018.01573

Guimarães-Costa, A. B., Desouza-Vieira, T. S., Paletta-Silva, R., Freitas-Mesquita, A. L., Meyer-Fernandes, J. R., and Saraiva, E. M. (2014). 3'-nucleotidase/nuclease activity allows *Leishmania* parasites to escape killing by neutrophil extracellular traps. *Infect. Immun.* 82, 1732–1740. doi: 10.1128/IAI.01232-13

Guimarães-Costa, A. B., Nascimento, M. T., Froment, G. S., Soares, R. P., Morgado, F. N., Conceição-Silva, F., et al. (2009). *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6748–6753. doi: 10.1073/pnas.0900226106

Hakkim, A., Fuchs, T. A., Martinez, N. E., Hess, S., Prinz, H., Zychlinsky, A., et al. (2011). Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nat. Chem. Biol.* 7, 75–77. doi: 10.1038/nchembio.496

Hakkim, A., Fürnrohr, B. G., Amann, K., Laube, B., Abed, U. A., Brinkmann, V., et al. (2010). Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9813–9818. doi: 10.1073/pnas.0909927107

Hemmers, S., Teijaro, J. R., Arandjelovic, S., and Mowen, K. A. (2011). PAD4-mediated neutrophil extracellular trap formation is not required for immunity against influenza infection. *PLoS One* 6:e22043. doi: 10.1371/journal.pone.0022043

Hirsch, E., Katanaev, V. L., Garlanda, C., Azzolino, O., Pirola, L., Silengo, L., et al. (2000). Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* 287, 1049–1053. doi: 10.1126/science.287.5455.1049

Hogan, S. P., Rosenberg, H. F., Moqbel, R., Phipps, S., Foster, P. S., Lacy, P., et al. (2008). Eosinophils: biological properties and role in health and disease. *Clin. Exp. Allergy* 38, 709–750. doi: 10.1111/j.1365-2222.2008.02958.x

Hohl, T. M., Van Epps, H. L., Rivera, A., Morgan, L. A., Chen, P. L., Feldmesser, M., et al. (2005). *Aspergillus fumigatus* triggers inflammatory responses by stage-specific beta-glucan display. *PLoS Pathog.* 1:e30. doi: 10.1371/journal.ppat.0010030

Holbrook, E. D., Smolnycki, K. A., Youseff, B. H., and Rappleye, C. A. (2013). Redundant catalases detoxify phagocyte reactive oxygen and facilitate *Histoplasma capsulatum* pathogenesis. *Infect. Immun.* 81, 2334–2346. doi: 10.1128/IAI.00173-13

Hwang, C. S., Park, S. C., Cho, H. J., Park, D. J., Yoon, J. H., and Kim, C. H. (2019). Eosinophil extracellular trap formation is closely associated with disease severity in chronic rhinosinusitis regardless of nasal polyp status. *Sci. Rep.* 9:8061. doi: 10.1038/s41598-019-44627-z

Inoue, Y., Matsuwaki, Y., Shin, S. H., Ponikau, J. U., and Kita, H. (2005). Nonpathogenic, environmental fungi induce activation and degranulation of human eosinophils. *J. Immunol.* 175, 5439–5447. doi: 10.4049/jimmunol.175.8.5439

Ishiguro, T., Takayanagi, N., Kagiyama, N., Shimizu, Y., Yanagisawa, T., and Sugita, Y. (2014). Clinical characteristics of biopsy-proven allergic bronchopulmonary mycosis: variety in causative fungi and laboratory findings. *Intern. Med.* 53, 1407–1411. doi: 10.2169/internalmedicine.53.2230

Jacobsen, E. A., Ochkur, S. I., Pero, R. S., Taranova, A. G., Protheroe, C. A., Colbert, D. C., et al. (2008). Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells. *J. Exp. Med.* 205, 699–710. doi: 10.1084/jem.20071840

Jang, B., Kim, H. W., Kim, J. S., Kim, W. S., Lee, B. R., Kim, S., et al. (2015). Peptidylarginine deiminase inhibition impairs Toll-like receptor agonist-induced functional maturation of dendritic cells, resulting in the loss of T cell-proliferative capacity: a partial mechanism with therapeutic potential in inflammatory settings. *J. Leukoc Biol.* 97, 351–362. doi: 10.1189/jlb.3A0314-142RR

Johnson, C. J., Cabezas-Olcoz, J., Kernien, J. F., Wang, S. X., Beebe, D. J., Huttenlocher, A., et al. (2016). The extracellular matrix of *Candida albicans* biofilms impairs formation of neutrophil extracellular traps. *PLoS Pathog.* 12:e1005884. doi: 10.1371/journal.ppat.1005884

Jubin, V., Ranque, S., Stremler Le Bel, N., Sarles, J., and Dubus, J. C. (2010). Risk factors for Aspergillus colonization and allergic bronchopulmonary aspergillosis in children with cystic fibrosis. *Pediatr. Pulmonol.* 45, 764–771. doi: 10.1002/ppul.21240

Kenny, E. F., Herzig, A., Krüger, R., Muth, A., Mondal, S., Thompson, P. R., et al. (2017). Diverse stimuli engage different neutrophil extracellular trap pathways. *eLife* 6:e24437. doi: 10.7554/eLife.24437

Kim, H. J., Sim, M. S., Lee, D. H., Kim, C., Choi, Y., Park, H. S., et al. (2020). Lysophosphatidylserine induces eosinophil extracellular trap formation and degranulation: Implications in severe asthma. *Allergy* 75, 3159–3170. doi: 10.1111/all.14450

Knutsen, A. P., Bush, R. K., Demain, J. G., Denning, D. W., Dixit, A., Fairs, A., et al. (2012). Fungi and allergic lower respiratory tract diseases. *J. Allergy Clin. Immunol.* 129, 280–291. doi: 10.1016/j.jaci.2011.12.970 quiz 292-283,

Kosmidis, C., and Denning, D. W. (2015). The clinical spectrum of pulmonary aspergillosis. *Thorax* 70, 270–277. doi: 10.1136/thoraxjnl-2014-206291

Kovács, M., Németh, T., Jakus, Z., Sitaru, C., Simon, E., Futosi, K., et al. (2014). The Src family kinases Hck, Fgr, and Lyn are critical for the generation of the in vivo inflammatory environment without a direct role in leukocyte recruitment. *J. Exp. Med.* 211, 1993–2011. doi: 10.1084/jem.20132496

Kurita, N., Terao, K., Brummer, E., Ito, E., Nishimura, K., and Miyaji, M. (1991). Resistance of *Histoplasma capsulatum* to killing by human neutrophils. Evasion of oxidative burst and lysosomal-fusion products. *Mycopathologia* 115, 207–213. doi: 10.1007/BF00462229

Latgé, J. P., and Chamilos, G. (2019). *Aspergillus fumigatus* and Aspergillosis in 2019. *Clin. Microbiol. Rev.* 33:e00140-18. doi: 10.1128/CMR.00140-18

Lee, J. J., Dimina, D., Macias, M. P., Ochkur, S. I., Mcgarry, M. P., O'neill, K. R., et al. (2004). Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 305, 1773–1776. doi: 10.1126/science.1099472

Lee, M. J., Liu, H., Barker, B. M., Snarr, B. D., Gravelat, F. N., Al Abdallah, Q., et al. (2015). The fungal exopolysaccharide galactosaminogalactan mediates virulence by enhancing resistance to neutrophil extracellular traps. *PLoS Pathog.* 11:e1005187. doi: 10.1371/journal.ppat.1005187

Lehman, H. K., and Segal, B. H. (2020). The role of neutrophils in host defense and disease. *J. Allergy Clin. Immunol.* 145, 1535–1544. doi: 10.1016/j.jaci.2020.02.038

Lewis, H. D., Liddle, J., Coote, J. E., Atkinson, S. J., Barker, M. D., Bax, B. D., et al. (2015). Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. *Nat. Chem. Biol.* 11, 189–191. doi: 10.1038/nchembio.1735

Li, P., Li, M., Lindberg, M. R., Kennett, M. J., Xiong, N., and Wang, Y. (2010). PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J. Exp. Med.* 207, 1853–1862. doi: 10.1084/jem.20100239

Lilic, D. (2012). Unravelling fungal immunity through primary immune deficiencies. *Curr. Opin. Microbiol.* 15, 420–426. doi: 10.1016/j.mib.2012.06.003

Lilly, L. M., Scopel, M., Nelson, M. P., Burg, A. R., Dunaway, C. W., and Steele, C. (2014). Eosinophil deficiency compromises lung defense against *Aspergillus fumigatus*. *Infect. Immun.* 82, 1315–1325. doi: 10.1128/IAI.01172-13

Loos, T., Mortier, A., Gouwy, M., Ronsse, I., Put, W., Lenaerts, J. P., et al. (2008). Citrullination of CXCL10 and CXCL11 by peptidylarginine deiminase: a naturally occurring posttranslational modification of chemokines and new dimension of immunoregulation. *Blood* 112, 2648–2656. doi: 10.1182/blood-2008-04-149039

Loos, T., Opdenakker, G., Van Damme, J., and Proost, P. (2009). Citrullination of CXCL8 increases this chemokine's ability to mobilize neutrophils into the blood circulation. *Haematologica* 94, 1346–1353. doi: 10.3324/haematol.2009.006973

Lowell, C. A., Fumagalli, L., and Berton, G. (1996). Deficiency of Src family kinases p59/61hck and p58c-fgr results in defective adhesion-dependent neutrophil functions. *J. Cell Biol.* 133, 895–910. doi: 10.1083/jcb.133.4.895

Luna-Rodríguez, C. E., González, G. M., Montoya, A. M., Treviño-Rangel, R. J., and Sánchez-González, A. (2020). Production of neutrophil extracellular traps (NETs) in response to *Scedosporium apiospermum* in a murine model of pulmonary infection. *Microb. Pathog.* 149:104349. doi: 10.1016/j.micpath.2020.104349

Luo, H. R., and Mondal, S. (2015). Molecular control of PtdIns(3,4,5)P3 signaling in neutrophils. *EMBO Rep.* 16, 149–163. doi: 10.15252/embr.201439466

Mambula, S. S., Sau, K., Henneke, P., Golenbock, D. T., and Levitz, S. M. (2002). Toll-like receptor (TLR) signaling in response to *Aspergillus fumigatus*. *J. Biol. Chem.* 277, 39320–39326. doi: 10.1074/jbc.M201683200

Mambula, S. S., Simons, E. R., Hastey, R., Selsted, M. E., and Levitz, S. M. (2000). Human neutrophil-mediated nonoxidative antifungal activity against *Cryptococcus neoformans*. *Infect. Immun.* 68, 6257–6264. doi: 10.1128/iai.68.11.6257-6264.2000

Martinod, K., Demers, M., Fuchs, T. A., Wong, S. L., Brill, A., Gallant, M., et al. (2013). Neutrophil histone modification by peptidylarginine deiminase 4 is critical for deep vein thrombosis in mice. *Proc. Natl. Acad. Sci. U.S.A.* 110, 8674–8679. doi: 10.1073/pnas.1301059110

Martinod, K., Witsch, T., Erpenbeck, L., Savchenko, A., Hayashi, H., Cherpokova, D., et al. (2017). Peptidylarginine deiminase 4 promotes age-related organ fibrosis. *J. Exp. Med.* 214, 439–458. doi: 10.1084/jem.20160530

Marx, C., Novotny, J., Salbeck, D., Zellner, K. R., Nicolai, L., Pekayvaz, K., et al. (2019). Eosinophil-platelet interactions promote atherosclerosis and stabilize thrombosis with eosinophil extracellular traps. *Blood* 134, 1859–1872. doi: 10.1182/blood.2019000518

Matsuwaki, Y., Wada, K., Moriyama, H., and Kita, H. (2011). Human eosinophil innate response to *Alternaria* fungus through protease-activated receptor-2. *Int. Arch. Allergy Immunol.* 155(Suppl. 1), 123–128. doi: 10.1159/000327498

Matsuwaki, Y., Wada, K., White, T. A., Benson, L. M., Charlesworth, M. C., Checkel, J. L., et al. (2009). Recognition of fungal protease activities induces cellular activation and eosinophil-derived neurotoxin release in human eosinophils. *J. Immunol.* 183, 6708–6716. doi: 10.4049/jimmunol.0901220

May, R. C., Stone, N. R., Wiesner, D. L., Bicanic, T., and Nielsen, K. (2016). *Cryptococcus*: from environmental saprophyte to global pathogen. *Nat. Rev. Microbiol.* 14, 106–117. doi: 10.1038/nrmicro.2015.6

McCormick, A., Heesemann, L., Wagener, J., Marcos, V., Hartl, D., Loeffler, J., et al. (2010). NETs formed by human neutrophils inhibit growth of the pathogenic mold *Aspergillus fumigatus*. *Microbes Infect.* 12, 928–936. doi: 10.1016/j.micinf.2010.06.009

Meier, A., Kirschning, C. J., Nikolaus, T., Wagner, H., Heesemann, J., and Ebel, F. (2003). Toll-like receptor (TLR) 2 and TLR4 are essential for *Aspergillus*-induced activation of murine macrophages. *Cell Microbiol.* 5, 561–570. doi: 10.1046/j.1462-5822.2003.00301.x

Mejía, S. P., Cano, L. E., López, J. A., Hernandez, O., and González, Á (2015). Human neutrophils produce extracellular traps against *Paracoccidioides brasiliensis*. *Microbiology* 161, 1008–1017. doi: 10.1099/mic.0.000059

Metzler, K. D., Fuchs, T. A., Nauseef, W. M., Reumaux, D., Roesler, J., Schulze, I., et al. (2011). Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood* 117, 953–959. doi: 10.1182/blood-2010-06-290171

Metzler, K. D., Goosmann, C., Lubojemska, A., Zychlinsky, A., and Papayannopoulos, V. (2014). A myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis. *Cell Rep.* 8, 883–896. doi: 10.1016/j.celrep.2014.06.044

Mitroulis, I., Kambas, K., Chrysanthopoulou, A., Skendros, P., Apostolidou, E., Kourtzelis, I., et al. (2011). Neutrophil extracellular trap formation is associated with IL-1β and autophagy-related signaling in gout. *PLoS One* 6:e29318. doi: 10.1371/journal.pone.0029318

Mócsai, A., Abram, C. L., Jakus, Z., Hu, Y., Lanier, L. L., and Lowell, C. A. (2006). Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat. Immunol.* 7, 1326–1333. doi: 10.1038/ni1407

Mócsai, A., Ligeti, E., Lowell, C. A., and Berton, G. (1999). Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. *J. Immunol* 162, 1120–1126.

Mócsai, A., Zhou, M., Meng, F., Tybulewicz, V. L., and Lowell, C. A. (2002). Syk is required for integrin signaling in neutrophils. *Immunity* 16, 547–558. doi: 10.1016/S1074-7613(02)00303-5

Morgenstern, D. E., Gifford, M. A., Li, L. L., Doerschuk, C. M., and Dinauer, M. C. (1997). Absence of respiratory burst in X-linked chronic granulomatous disease mice leads to abnormalities in both host defense and inflammatory response to *Aspergillus fumigatus*. *J. Exp. Med.* 185, 207–218. doi: 10.1084/jem.185.2.207

Morshed, M., Yousefi, S., Stöckle, C., Simon, H. U., and Simon, D. (2012). Thymic stromal lymphopoietin stimulates the formation of eosinophil extracellular traps. *Allergy* 67, 1127–1137. doi: 10.1111/j.1398-9995.2012.02868.x

Mukherjee, M., Lacy, P., and Ueki, S. (2018). Eosinophil extracellular traps and inflammatory pathologies-untangling the web! *Front. Immunol.* 9:2763. doi: 10.3389/fimmu.2018.02763

Muniz, V. S., Silva, J. C., Braga, Y. A. V., Melo, R. C. N., Ueki, S., Takeda, M., et al. (2018). Eosinophils release extracellular DNA traps in response to *Aspergillus fumigatus*. *J. Allergy Clin. Immunol.* 141, 571.e7–585.e7. doi: 10.1016/j.jaci.2017.07.048

Muniz, V. S., Weller, P. F., and Neves, J. S. (2012). Eosinophil crystalloid granules: structure, function, and beyond. *J. Leukoc Biol.* 92, 281–288. doi: 10.1189/jlb.0212067

Muraro, S. P., De Souza, G. F., Gallo, S. W., Da Silva, B. K., De Oliveira, S. D., Vinolo, M. A. R., et al. (2018). Respiratory syncytial virus induces the classical ROS-dependent NETosis through PAD-4 and necroptosis pathways activation. *Sci. Rep.* 8:14166. doi: 10.1038/s41598-018-32576-y

Nakashima, K., Hagiwara, T., and Yamada, M. (2002). Nuclear localization of peptidylarginine deiminase V and histone deimination in granulocytes. *J. Biol. Chem.* 277, 49562–49568. doi: 10.1074/jbc.M208795200

Neeli, I., Khan, S. N., and Radic, M. (2008). Histone deimination as a response to inflammatory stimuli in neutrophils. *J. Immunol.* 180, 1895–1902. doi: 10.4049/jimmunol.180.3.1895

Neeli, I., and Radic, M. (2013). Opposition between PKC isoforms regulates histone deimination and neutrophil extracellular chromatin release. *Front. Immunol.* 4:38. doi: 10.3389/fimmu.2013.00038

Negoro, P. E., Xu, S., Dagher, Z., Hopke, A., Reedy, J. L., Feldman, M. B., et al. (2020). Spleen tyrosine kinase is a critical regulator of neutrophil responses to *Candida* species. *mBio* 11:e02043-19. doi: 10.1128/mBio.02043-19

Newman, S. L., Gootee, L., and Gabay, J. E. (1993). Human neutrophil-mediated fungistasis against *Histoplasma capsulatum*. Localization of fungistatic activity to the azurophil granules. *J. Clin. Invest.* 92, 624–631. doi: 10.1172/JCI116630

Newman, S. L., Gootee, L., Gabay, J. E., and Selsted, M. E. (2000). Identification of constituents of human neutrophil azurophil granules that mediate fungistasis against *Histoplasma capsulatum*. *Infect. Immun.* 68, 5668–5672. doi: 10.1128/iai.68.10.5668-5672.2000

O'Brien, X. M., Heflin, K. E., Lavigne, L. M., Yu, K., Kim, M., Salomon, A. R., et al. (2012). Lectin site ligation of CR3 induces conformational changes and signaling. *J. Biol. Chem.* 287, 3337–3348. doi: 10.1074/jbc.M111.298307

Oladele, R. O., Ayanlowo, O. O., Richardson, M. D., and Denning, D. W. (2018). Histoplasmosis in Africa: an emerging or a neglected disease? *PLoS Negl. Trop. Dis.* 12:e0006046. doi: 10.1371/journal.pntd.0006046

O'Meara, T. R., and Alspaugh, J. A. (2012). The *Cryptococcus neoformans* capsule: a sword and a shield. *Clin. Microbiol. Rev.* 25, 387–408. doi: 10.1128/CMR.00001-12

Papayannopoulos, V. (2018). Neutrophil extracellular traps in immunity and disease. *Nat. Rev. Immunol.* 18, 134–147. doi: 10.1038/nri.2017.105

Papayannopoulos, V., Metzler, K. D., Hakkim, A., and Zychlinsky, A. (2010). Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J. Cell Biol.* 191, 677–691. doi: 10.1083/jcb.201006052

Patin, E. C., Thompson, A., and Orr, S. J. (2019). Pattern recognition receptors in fungal immunity. *Semin. Cell Dev. Biol.* 89, 24–33. doi: 10.1016/j.semcdb.2018.03.003

Persson, E. K., Verstraete, K., Heyndrickx, I., Gevaert, E., Aegerter, H., Percier, J. M., et al. (2019). Protein crystallization promotes type 2 immunity and is reversible by antibody treatment. *Science* 364:eaaw4295. doi: 10.1126/science.aaw4295

Pilsczek, F. H., Salina, D., Poon, K. K., Fahey, C., Yipp, B. G., Sibley, C. D., et al. (2010). A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J. Immunol.* 185, 7413–7425. doi: 10.4049/jimmunol.1000675

Poulain, D. (2015). *Candida albicans*, plasticity and pathogenesis. *Crit. Rev. Microbiol.* 41, 208–217. doi: 10.3109/1040841X.2013.813904

Proost, P., Loos, T., Mortier, A., Schutyser, E., Gouwy, M., Noppen, S., et al. (2008). Citrullination of CXCL8 by peptidylarginine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation. *J. Exp. Med.* 205, 2085–2097. doi: 10.1084/jem.20080305

Remijsen, Q., Vanden Berghe, T., Wirawan, E., Asselbergh, B., Parthoens, E., De Rycke, R., et al. (2011). Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res.* 21, 290–304. doi: 10.1038/cr.2010.150

Rocha, J. D., Nascimento, M. T., Decote-Ricardo, D., Côrte-Real, S., Morrot, A., Heise, N., et al. (2015). Capsular polysaccharides from *Cryptococcus neoformans* modulate production of neutrophil extracellular traps (NETs) by human neutrophils. *Sci. Rep.* 5:8008. doi: 10.1038/srep08008

Röhm, M., Grimm, M. J., D'auria, A. C., Almyroudis, N. G., Segal, B. H., and Urban, C. F. (2014). NADPH oxidase promotes neutrophil extracellular trap formation in pulmonary aspergillosis. *Infect. Immun.* 82, 1766–1777. doi: 10.1128/IAI.00096-14

Sadhu, C., Masinovsky, B., Dick, K., Sowell, C. G., and Staunton, D. E. (2003). Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. *J. Immunol.* 170, 2647–2654. doi: 10.4049/jimmunol.170.5.2647

Saijo, S., Ikeda, S., Yamabe, K., Kakuta, S., Ishigame, H., Akitsu, A., et al. (2010). Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. *Immunity* 32, 681–691. doi: 10.1016/j.immuni.2010.05.001

Saitoh, T., Komano, J., Saitoh, Y., Misawa, T., Takahama, M., Kozaki, T., et al. (2012). Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. *Cell Host Microbe* 12, 109–116. doi: 10.1016/j.chom.2012.05.015

Sasaki, T., Irie-Sasaki, J., Jones, R. G., Oliveira-Dos-Santos, A. J., Stanford, W. L., Bolon, B., et al. (2000). Function of PI3Kgamma in thymocyte development. T cell activation, and neutrophil migration. *Science* 287, 1040–1046. doi: 10.1126/science.287.5455.1040

Schnur, R. A., and Newman, S. L. (1990). The respiratory burst response to *Histoplasma capsulatum* by human neutrophils. Evidence for intracellular trapping of superoxide anion. *J. Immunol.* 144, 4765–4772.

Schorn, C., Janko, C., Latzko, M., Chaurio, R., Schett, G., and Herrmann, M. (2012). Monosodium urate crystals induce extracellular DNA traps in neutrophils, eosinophils, and basophils but not in mononuclear cells. *Front. Immunol.* 3:277. doi: 10.3389/fimmu.2012.00277

Shopova, I. A., Belyaev, I., Dasari, P., Jahreis, S., Stroe, M. C., Cseresnyés, Z., et al. (2020). Human neutrophils produce antifungal extracellular vesicles against *Aspergillus fumigatus*. *mBio* 11:e00596-20. doi: 10.1128/mBio.00596-20

Silva, J. C., Rodrigues, N. C., Thompson-Souza, G. A., Muniz, V. S., Neves, J. S., and Figueiredo, R. T. (2020). Mac-1 triggers neutrophil DNA extracellular trap formation to *Aspergillus fumigatus* independently of PAD4 histone citrullination. *J. Leukoc Biol.* 107, 69–83. doi: 10.1002/JLB.4A0119-009RR

Simon, D., Radonjic-Hösli, S., Straumann, A., Yousefi, S., and Simon, H. U. (2015). Active eosinophilic esophagitis is characterized by epithelial barrier defects and eosinophil extracellular trap formation. *Allergy* 70, 443–452. doi: 10.1111/all.12570

Springer, D. J., Ren, P., Raina, R., Dong, Y., Behr, M. J., Mcewen, B. F., et al. (2010). Extracellular fibrils of pathogenic yeast *Cryptococcus gattii* are important for ecological niche, murine virulence and human neutrophil interactions. *PLoS One* 5:e10978. doi: 10.1371/journal.pone.0010978

Steele, C., Rapaka, R. R., Metz, A., Pop, S. M., Williams, D. L., Gordon, S., et al. (2005). The beta-glucan receptor dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*. *PLoS Pathog.* 1:e42. doi: 10.1371/journal.ppat.0010042

Suleyman, G., and Alangaden, G. J. (2016). Nosocomial fungal infections: epidemiology, infection control, and prevention. *Infect. Dis. Clin. North Am.* 30, 1023–1052. doi: 10.1016/j.idc.2016.07.008

Sun, B., Dwivedi, N., Bechtel, T. J., Paulsen, J. L., Muth, A., Bawadekar, M., et al. (2017). Citrullination of NF-κB p65 promotes its nuclear localization and TLR-induced expression of IL-1β and TNFα. *Sci. Immunol.* 2:eaal3062. doi: 10.1126/sciimmunol.aal3062

Svobodová, E., Staib, P., Losse, J., Hennicke, F., Barz, D., and Józsi, M. (2012). Differential interaction of the two related fungal species *Candida albicans* and *Candida dubliniensis* with human neutrophils. *J. Immunol.* 189, 2502–2511. doi: 10.4049/jimmunol.1200185

Takei, H., Araki, A., Watanabe, H., Ichinose, A., and Sendo, F. (1996). Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA) accompanied by changes different from typical apoptosis or necrosis. *J. Leukoc Biol.* 59, 229–240. doi: 10.1002/jlb.59.2.229

Taylor, L. H., Latham, S. M., and Woolhouse, M. E. (2001). Risk factors for human disease emergence. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 356, 983–989. doi: 10.1098/rstb.2001.0888

Thiam, H. R., Wong, S. L., Qiu, R., Kittisopikul, M., Vahabikashi, A., Goldman, A. E., et al. (2020). NETosis proceeds by cytoskeleton and endomembrane disassembly and PAD4-mediated chromatin decondensation and nuclear envelope rupture. *Proc. Natl. Acad. Sci. U.S.A.* 117, 7326–7337. doi: 10.1073/pnas.1909546117

Thompson-Souza, G. A., Santos, G. M. P., Silva, J. C., Muniz, V. S., Braga, Y. A. V., Figueiredo, R. T., et al. (2020). *Histoplasma capsulatum*-induced extracellular DNA trap release in human neutrophils. *Cell Microbiol.* 22:e13195. doi: 10.1111/cmi.13195

Thornton, B. P., Vetvicka, V., Pitman, M., Goldman, R. C., and Ross, G. D. (1996). Analysis of the sugar specificity and molecular location of the beta-glucan-binding lectin site of complement receptor type 3 (CD11b/CD18). *J. Immunol.* 156, 1235–1246.

Ueki, S., Konno, Y., Takeda, M., Moritoki, Y., Hirokawa, M., Matsuwaki, Y., et al. (2016). Eosinophil extracellular trap cell death-derived DNA traps: Their presence in secretions and functional attributes. *J. Allergy Clin. Immunol.* 137, 258–267. doi: 10.1016/j.jaci.2015.04.041

Ueki, S., Melo, R. C., Ghiran, I., Spencer, L. A., Dvorak, A. M., and Weller, P. F. (2013). Eosinophil extracellular DNA trap cell death mediates lytic release of free secretion-competent eosinophil granules in humans. *Blood* 121, 2074–2083. doi: 10.1182/blood-2012-05-432088

Ueno, K., Yanagihara, N., Otani, Y., Shimizu, K., Kinjo, Y., and Miyazaki, Y. (2019). Neutrophil-mediated antifungal activity against highly virulent *Cryptococcus gattii* strain R265. *Med. Mycol.* 57, 1046–1054. doi: 10.1093/mmy/myy153

Urban, C. F., Ermert, D., Schmid, M., Abu-Abed, U., Goosmann, C., Nacken, W., et al. (2009). Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. *PLoS Pathog.* 5:e1000639. doi: 10.1371/journal.ppat.1000639

Urban, C. F., and Nett, J. E. (2019). Neutrophil extracellular traps in fungal infection. *Semin. Cell Dev. Biol.* 89, 47–57. doi: 10.1016/j.semcdb.2018.03.020

Urban, C. F., Reichard, U., Brinkmann, V., and Zychlinsky, A. (2006). Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cell Microbiol.* 8, 668–676. doi: 10.1111/j.1462-5822.2005.00659.x

Uribe Echevarría, L., Leimgruber, C., García González, J., Nevado, A., Álvarez, R., García, L. N., et al. (2017). Evidence of eosinophil extracellular trap cell death in COPD: does it represent the trigger that switches on the disease? *Int. J. Chron Obstruct Pulmon. Dis.* 12, 885–896. doi: 10.2147/COPD.S115969

Vallabhaneni, S., Mody, R. K., Walker, T., and Chiller, T. (2016). The Global Burden of Fungal Diseases. *Infect. Dis. Clin. North Am.* 30, 1–11. doi: 10.1016/j.idc.2015.10.004

Van Ziffle, J. A., and Lowell, C. A. (2009). Neutrophil-specific deletion of Syk kinase results in reduced host defense to bacterial infection. *Blood* 114, 4871–4882. doi: 10.1182/blood-2009-05-220806

Veras, F. P., Pontelli, M. C., Silva, C. M., Toller-Kawahisa, J. E., De Lima, M., Nascimento, D. C., et al. (2020). SARS-CoV-2-triggered neutrophil extracellular traps mediate COVID-19 pathology. *J. Exp. Med.* 217:e20201129. doi: 10.1084/jem.20201129

Wang, S., and Wang, Y. (2013). Peptidylarginine deiminases in citrullination, gene regulation, health and pathogenesis. *Biochim. Biophys. Acta* 1829, 1126–1135. doi: 10.1016/j.bbagrm.2013.07.003

Wang, Y., Li, M., Stadler, S., Correll, S., Li, P., Wang, D., et al. (2009). Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J. Cell Biol.* 184, 205–213. doi: 10.1083/jcb.200806072

Wardeh, M., Risley, C., Mcintyre, M. K., Setzkorn, C., and Baylis, M. (2015). Database of host-pathogen and related species interactions, and their global distribution. *Sci. Data* 2:150049. doi: 10.1038/sdata.2015.49

Warnatsch, A., Ioannou, M., Wang, Q., and Papayannopoulos, V. (2015). Inflammation. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. *Science* 349, 316–320. doi: 10.1126/science.aaa8064

Warnatsch, A., Tsourouktsoglou, T. D., Branzk, N., Wang, Q., Reincke, S., Herbst, S., et al. (2017). Reactive oxygen species localization programs inflammation to clear microbes of different size. *Immunity* 46, 421–432. doi: 10.1016/j.immuni.2017.02.013

Wheat, L. J., Azar, M. M., Bahr, N. C., Spec, A., Relich, R. F., and Hage, C. (2016). Histoplasmosis. *Infect. Dis. Clin. North Am.* 30, 207–227. doi: 10.1016/j.idc.2015.10.009

Winterbourn, C. C., Kettle, A. J., and Hampton, M. B. (2016). Reactive oxygen species and neutrophil function. *Annu. Rev. Biochem.* 85, 765–792. doi: 10.1146/annurev-biochem-060815-014442

Woods, J. P. (2016). Revisiting old friends: developments in understanding *Histoplasma capsulatum* pathogenesis. *J. Microbiol/* 54, 265–276. doi: 10.1007/s12275-016-6044-5

Wu, S. Y., Weng, C. L., Jheng, M. J., Kan, H. W., Hsieh, S. T., Liu, F. T., et al. (2019). *Candida albicans* triggers NADPH oxidase-independent neutrophil extracellular traps through dectin-2. *PLoS Pathog.* 15:e1008096. doi: 10.1371/journal.ppat.1008096

Xia, Y., and Ross, G. D. (1999). Generation of recombinant fragments of CD11b expressing the functional beta-glucan-binding lectin site of CR3 (CD11b/CD18). *J. Immunol.* 162, 7285–7293.

Yipp, B. G., Petri, B., Salina, D., Jenne, C. N., Scott, B. N., Zbytnuik, L. D., et al. (2012). Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat. Med.* 18, 1386–1393. doi: 10.1038/nm.2847

Yoon, J., Ponikau, J. U., Lawrence, C. B., and Kita, H. (2008). Innate antifungal immunity of human eosinophils mediated by a beta 2 integrin. CD11b. *J. Immunol.* 181, 2907–2915. doi: 10.4049/jimmunol.181.4.2907

Youseff, B. H., Holbrook, E. D., Smolnycki, K. A., and Rappleye, C. A. (2012). Extracellular superoxide dismutase protects *Histoplasma* yeast cells from host-derived oxidative stress. *PLoS Pathog.* 8:e1002713. doi: 10.1371/journal.ppat.1002713

Yousefi, S., Gold, J. A., Andina, N., Lee, J. J., Kelly, A. M., Kozlowski, E., et al. (2008). Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nat. Med.* 14, 949–953. doi: 10.1038/nm.1855

Yousefi, S., Mihalache, C., Kozlowski, E., Schmid, I., and Simon, H. U. (2009). Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ.* 16, 1438–1444. doi: 10.1038/cdd.2009.96

Zhang, X., Zhao, S., Sun, L., Li, W., Wei, Q., Ashman, R. B., et al. (2017). Different virulence of *Candida albicans* is attributed to the ability of escape from neutrophil extracellular traps by secretion of DNAse. *Am. J. Transl. Res.* 9, 50–62.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 18 February 2021
doi: 10.3389/fmicb.2021.633696

Structural and Signaling Events Driving *Aspergillus fumigatus*-Induced Human Eosinophil Extracellular Trap Release

Marina Valente Barroso[1,2†], Isabella Gropillo[1†], Marcella A. A. Detoni[1], Glaucia A. Thompson-Souza[2], Valdirene S. Muniz[2], Claudia Regina Isaías Vasconcelos[2], Rodrigo T. Figueiredo[3], Rossana C. N. Melo[4] and Josiane S. Neves[2]*

[1] Institute of Microbiology Paulo de Góes, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [2] Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [3] Institute of Biomedical Sciences/Campus of Duque de Caxias, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [4] Laboratory of Cellular Biology, Department of Biology, Institute of Biological Sciences, Federal University of Juiz de Fora, Juiz de Fora, Brazil

OPEN ACCESS

Edited by:
Frank Ebel,
Ludwig Maximilian University of Munich, Germany

Reviewed by:
Sven Krappmann,
University of Erlangen-Nuremberg, Germany
Anurag Singh,
Tübingen University Hospital, Germany

***Correspondence:**
Josiane S. Neves
jneves@icb.ufrj.br

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: 25 November 2020
Accepted: 27 January 2021
Published: 18 February 2021

Citation:
Barroso MV, Gropillo I, Detoni MAA, Thompson-Souza GA, Muniz VS, Vasconcelos CRI, Figueiredo RT, Melo RCN and Neves JS (2021) Structural and Signaling Events Driving *Aspergillus fumigatus*-Induced Human Eosinophil Extracellular Trap Release. Front. Microbiol. 12:633696. doi: 10.3389/fmicb.2021.633696

Eosinophils are granulocytes classically involved in allergic diseases and in the host immune responses to helminths, fungi, bacteria and viruses. The release of extracellular DNA traps by leukocytes is an important mechanism of the innate immune response to pathogens in various infectious conditions, including fungal infections. *Aspergillus fumigatus* is an opportunistic fungus responsible for allergic bronchopulmonary aspergillosis (ABPA), a pulmonary disease marked by prominent eosinophilic inflammation. Previously, we demonstrated that isolated human eosinophils release extracellular DNA traps (eosinophil extracellular traps; EETs) when stimulated by *A. fumigatus in vitro*. This release occurs through a lytic non-oxidative mechanism that involves CD11b and Syk tyrosine kinase. In this work, we unraveled different intracellular mechanisms that drive the release of extracellular DNA traps by *A. fumigatus*-stimulated eosinophils. Ultrastructurally, we originally observed that *A. fumigatus*-stimulated eosinophils present typical signs of extracellular DNA trap cell death (ETosis) with the nuclei losing both their shape (delobulation) and the euchromatin/heterochromatin distinction, followed by rupture of the nuclear envelope and EETs release. We also found that by targeting class I PI3K, and more specifically PI3Kδ, the release of extracellular DNA traps induced by *A. fumigatus* is inhibited. We also demonstrated that *A. fumigatus*-induced EETs release depends on the Src family, Akt, calcium and p38 MAPK signaling pathways in a process in which fungal viability is dispensable. Interestingly, we showed that *A. fumigatus*-induced EETs release occurs in a mechanism independent of PAD4 histone citrullination. These findings may contribute to a better understanding of the mechanisms that underlie EETs release in response to *A. fumigatus*, which may lead to better knowledge of ABPA pathophysiology and treatment.

Keywords: eosinophils, *A. fumigatus*, extracellular DNA traps, allergic bronchopulmonary aspergillosis, ABPA

INTRODUCTION

Eosinophils are bone marrow-derived granulocytes that are typically abundant in inflammatory infiltrates in the defense against helminthic parasites and in allergic diseases. However, various immunoregulatory actions and functions have been also described for eosinophils, such as lymphocyte recruitment, tissue repair, organ development, antigen presentation, antimicrobial and antifungal activities, among other functions (Strandmark et al., 2016). Two of the main eosinophil activation mechanisms are degranulation and, more rarely, phagocytosis (Shamri et al., 2011). Recently, another cell effector mechanism has been described for eosinophils—the release of extracellular DNA traps (Yousefi et al., 2008; Ueki et al., 2013). The release of extracellular DNA traps (ETs) by leukocytes has been considered an important mechanism of the immune response in different inflammatory conditions. Although most ET knowledge is based on neutrophil studies (Brinkmann et al., 2004; Fuchs et al., 2007; Parker et al., 2012), it is well known that other leukocytes are also able to release ETs, including eosinophils (Yousefi et al., 2008), mast cells (von Kockritz-Blickwede et al., 2008), monocytes/macrophages (Chow et al., 2010), dendritic cells (Ramirez-Ortiz et al., 2011; Loures et al., 2015) and basophils (Schorn et al., 2012; Morshed et al., 2014). Various stimuli are capable of inducing the release of ETs, which essentially are structures of disrupted chromatin filaments coated with granular and cytosolic proteins, histones, and proteases (Brinkmann et al., 2004; Fuchs et al., 2007; Guimaraes-Costa et al., 2009; Parker et al., 2012). ET release involves non-lytic (Yousefi et al., 2008, 2009; Yipp et al., 2012) or lytic processes (named ETosis) that in general require cell chromatin decondensation (Brinkmann et al., 2004; Fuchs et al., 2007; Guimaraes-Costa et al., 2009; Parker et al., 2012; Ueki et al., 2013). Some studies indicate that this process is due to histone hypercitrullination by the enzyme PAD4 (peptidylarginine deiminase 4), in which arginine residues in histones are converted to citrulline, allowing DNA fibers to unfold (Wang et al., 2009; Li et al., 2010; Lewis et al., 2015; Van Avondt and Hartl, 2018). However, controversy remains about the relative importance of PAD4 for ETs release (Neeli and Radic, 2013; Kenny et al., 2017; Claushuis et al., 2018; Guiducci et al., 2018; Silva et al., 2019; Thompson-Souza et al., 2020). Interestingly, some studies have indicated that mitochondria are the source of the DNA that composes ETs (Yousefi et al., 2008, 2009). ETs help leukocytes immobilize bacteria, fungi and viruses, creating a microenvironment that favors a more efficient elimination of pathogens (Brinkmann et al., 2004; Fuchs et al., 2007; Papayannopoulos et al., 2010; Parker et al., 2012). However, recent evidence has emerged suggesting that ETs also have a role in non-infectious sterile inflammation (Jorch and Kubes, 2017). In eosinophils, the process by which they produce and release eosinophil extracellular DNA traps (EETs) can result in cell death (named EETosis in eosinophils) (Ueki et al., 2013); or in a non-lytic process by which eosinophils rapidly produce EETs but do not lose their viability (Yousefi et al., 2008, 2009).

Exposure and sensitization to fungal allergens is an important factor in patients with respiratory allergies; in this context, fungi play an important role in the development, severity and persistence of allergic lung diseases (Knutsen et al., 2012; Denning et al., 2014). Allergic bronchopulmonary mycoses (ABPMs) are characterized by fungal colonization and are known to worsen lung function, which is commonly associated with the development of severe asthma (Denning et al., 2013, 2014; Chowdhary et al., 2014). *Aspergillus fumigatus* is the most common cause of ABPMs; to a lesser extent, *C. albicans* and *Alternaria* species are also related to the development of these diseases (Shah and Panjabi, 2002; Chowdhary et al., 2014). ABPA, a form of non-invasive eosinophilic pulmonary aspergillosis, is a multifaceted pulmonary disorder caused by immunological reactions in response to repeated antigen exposure and/or colonization by *A. fumigatus* (Agarwal et al., 2013; Ueki et al., 2018). Susceptibility is related to the pathophysiology of comorbidities such as asthma, sinusitis, cystic fibrosis and alveolitis (Kousha et al., 2011; Denning et al., 2014). We recently described the presence of EETs in sputum samples from ABPA patients and found that human eosinophils are capable of releasing EETs *in vitro* in response to *A. fumigatus* conidia in a lytic non-oxidative process that involves CD11b and the Syk signaling pathway (Muniz et al., 2018). However, the ultrastructural features and signaling events that characterize and drive *A. fumigatus*-induced EETosis in human eosinophils are not completely understood. Thus, the purpose of this study was to unravel the intracellular mechanisms that direct the process of extracellular DNA trap release by *A. fumigatus*-stimulated eosinophils.

METHODOLOGY

Study Approval

All protocols and experimental procedures that involved human blood-isolated eosinophils were approved by the Committee on Human Research at Clementino Fraga Filho Hospital (Federal University of Rio de Janeiro). Written informed consent was obtained under institutional review board approved protocols (license number CAAE 31968020.9.0000.5257).

Fungal Culture and Conidial Preparation

A. fumigatus conidia ATCC 46645 (strain NCPF 2109) cryopreserved in liquid nitrogen and maintained in medium that contained 0.9% saline, 0.01% Tween, and 30% glycerol was thawed and spread onto solid potato dextrose agar medium (Neogen, MI, United States). The culture was incubated for 5–7 days at 37°C. Plates that contained mycelium of *A. fumigatus* were scraped with sterile PBS containing 0.05% Tween-20 (Bio-Rad, CA, United States), and the conidia were collected via filtration through a sterile nylon mesh with a porosity of 40 μm (BD Biosciences, NJ, United States). The conidia were pelleted by centrifugation (3150 *g*, 25°C, 15 min), resuspended in RPMI 1640 (phenol red-free) (Sigma, MO, United States), and counted using a Neubauer chamber and an optical microscope (Leica Microsystems, Wetzlar, Germany) at 40× magnification. The cell concentration was adjusted for the subsequent stimulation experiments. For a specific set of experiments, *A. fumigatus* conidia were fixed in 4% paraformaldehyde (PFO) for 30 min

at room temperature, extensively washed with PBS (2 ml, three times), centrifuged (3160 *g*, 15 min) and resuspended in RPMI.

Eosinophil Purification

Eosinophils were isolated from the blood of healthy donors using negative selection as previously described (Muniz et al., 2018). The viability and purity of the freshly isolated eosinophils were more than 99%, as analyzed by trypan blue exclusion and panoptic kit staining, respectively.

Fluorimetric Assay for EET Quantification

Purified human eosinophils (2×10^5/200 μL) were resuspended in RPMI 1640 (phenol red-free) supplemented with 0.1% heat-inactivated fetal calf serum (Life Technologies, CA, United States), 1% L-glutamine (Life Technologies) and antibiotics (penicillin and streptomycin). The eosinophils were stimulated in 96-well tissue culture plates with *A. fumigatus* conidia at a cell:fungus ratio of 1:10 for 6 h at 37°C, conditions that were previously determined in previous studies (Muniz et al., 2018). Ten minutes before the end of the incubation time, Sytox Green (5 μM, Life Technologies), an extracellular DNA probe impermeable to viable cells, was added to the wells. The samples were analyzed in a FlexStation plate reader (Molecular Devices, CA, United States) with a wavelength combination of excitation at 485 nm and emission at 538 nm. The values are expressed in relative fluorescence units. To evaluate the participation of different signaling pathways in *A. fumigatus*–induced release of EETs, the eosinophils were pretreated for 30 min before fungal stimulation with the following inhibitors: PP2 (10 μM, Caymann, MI, United States), a pharmacological inhibitor for Src kinases; wortmannin (100 ηM, Sigma), a PI3K pan-inhibitor; Akt inhibitor VIII (2.6 μM, Cayman), an Akt inhibitor; SB202190 (10 μM, Calbiochem, CA, United States), a p38 MAPK inhibitor; AS605240 (Cayman), at 10 μM, a selective inhibitor of the class I PI3K family; IC-87114 (Cayman), at 1 μM, a selective PI3K δ inhibitor; GSK484 (10 μM, GlaxoSmithKline, Brentford, United Kingdom), a PAD4 inhibitor; and BAPTA-AM (10 μM, Sigma), a calcium chelating agent. In all conditions, the inhibitor vehicle, dimethyl sulfoxide (DMSO), was tested at the corresponding dilution. 0

Confocal Microscopy

Purified human eosinophils (2×10^5/1000 μL) were placed in a 24-well plate that contained coverslips pretreated with poly-L-lysine (0.001%) (Sigma). Before fungal stimulation, the eosinophils were pretreated for 30 min with the pharmacological inhibitors described above. The cells were subsequently stimulated with *A. fumigatus* conidia at a cell: fungus ratio of 1:10 and were maintained at 37°C with 5% CO_2 for 6 h. At the end of the incubation time, the culture medium was removed, and the adhered cells were fixed with 4% PFO for 15 min at room temperature (RT). The cells were washed three times with sterile PBS. Sytox Green (5 μM) or Hoechst (1:1000, Life Technologies) was subsequently added and incubated for 10 min for DNA labeling. For histone labeling, the samples were fixed with 4% PFO and permeabilized with PBS buffer containing 1% Triton X-100 and 2% NP40. They were then labeled with a mouse anti-human citrullinated histone H3 antibody (0.8 μg/mL, Abcam, Cambridge, United Kingdom) diluted in PBS containing 0.05% Tween-20, 2% BSA, and 5% human serum. Following an overnight incubation at 4°C, the cells were washed and incubated for 1 h with a rabbit anti-mouse IgG fluorescein isothiocyanate–conjugated antibody (1:1000, Jackson ImmunoResearch, ME, United States). The wells were then washed 3 times, followed by the addition of the mounting medium Aqua-Poly/Mount. Images were acquired using a fluorescence confocal microscope (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany) and were analyzed with the ImageJ program (Fiji).

Immunoblotting

Purified human eosinophils (1×10^6/200 μL) were placed in a 24-well plate and pretreated for 30 min with the PAD4 inhibitor GSK484 (10 μM, GlaxoSmithKline) or its vehicle and then stimulated with *A. fumigatus* conidia (cell: fungus ratio of 1:10, 37°C, 6 h). After incubation, the cells were lysed in sample buffer (Tris HCl 62 mM, 10% glycerol, 5% β-mercaptoethanol, 2% SDS). The samples were boiled (100°C for 5 min), centrifuged to remove insoluble debris, run on a polyacrylamide gel (12%), and then transferred to a nitrocellulose membrane using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad). The membranes were blocked with TBS-T (Tris-buffered saline + 0.05% Tween) containing 5% BSA and then incubated overnight with a primary rabbit anti-human citrullinated histone H3 polyclonal antibody (0.8 μg/mL, Abcam) or a rabbit anti-human histone H3 monoclonal antibody (clone D1H2, Cell Signaling, Massachusetts, United States). The membranes were subsequently incubated for 1 h with a secondary goat anti-rabbit IgG peroxidase-conjugated antibody (Sigma) and were revealed by chemiluminescence (ECL, Thermo Fisher Scientific, MA, United States).

Transmission Electron Microscopy

Blood eosinophils were added to chamber slides (2.5×10^5/chamber), stimulated or not with A. fumigatus conidia (cell: fungus ratio of 1:10) for 6 h, and immediately fixed in a mixture of freshly prepared aldehydes (2.5% paraformaldehyde, 2.5% glutaraldehyde) in 1 M sodium cacodylate buffer, pH 7.4, for 30 min at room temperature. To allow optimal cell morphology and observation of extracellular trap formation in situ, all electron microscopy procedures were performed at RT directly on the slide surface as previously described (Ueki et al., 2013). The cells were post-fixed in 1% osmium tetroxide and processed for transmission electron microscopy (TEM) according to previous studies (Melo et al., 2009). Resin embedding was performed by inverting resin-filled plastic capsules over the slide-attached cells. After polymerization at 60°C for 16 h, thin sections were cut using a diamond knife on an ultramicrotome (Leica, Bannockburn, IL, United States). Sections were mounted on uncoated 200-mesh copper grids (Ted Pella, California, United States) before staining with lead citrate and then viewed with a transmission electron microscope (Tecnai G2 Spirit, FEI/Thermo Fisher Scientific) at 80 kV. A total

of 77 randomly acquired electron micrographs were analyzed at different magnifications.

Scanning Electron Microscopy (SEM)

Purified human eosinophils (2×10^5/200 μL) were placed in a 24-well plate that contained coverslips pretreated with poly-L-lysine (0.001%) (Sigma). *A. fumigatus* conidia were added at a cell: fungus ratio of 1:10, and the samples were maintained at 37°C with 5% CO_2. After 6 h of incubation, the culture medium was removed, and cells that adhered to the coverslip were fixed with 2.5% glutaraldehyde, 4% formaldehyde (0.1 M) in sodium cacodylate buffer for 2 h at RT. After three washes in sodium cacodylate buffer (0.1 mol/L), the samples were post-fixed with 1% osmium tetroxide and 0.8% potassium ferrocyanide in sodium cacodylate buffer for 30 min. The samples were again washed three times in sodium cacodylate buffer, followed by dehydration in graded ethanol: 30, 50, 70, 90, and 100% for 15 min each. The critical point technique with CO_2 was subsequently performed, followed by mounting on a metallic support with carbon tape. The samples were then covered with a thin layer of 20 nm gold (metallization), followed by examination under a conventional QUANTA 250 FEI scanning electron microscope (ThermoFisher Scientific).

Statistical Analysis

The results were analyzed with GraphPad Prism 8 using ANOVA with repeated measures and the Newman-Keuls post-test, with statistically significant differences defined as $p < 0.05$.

RESULTS

A. fumigatus-Induced EETosis Is Characterized by Marked Nuclear Alterations

To investigate the intracellular structural events associated with the formation of EETs by human eosinophils in response to *A. fumigatus*, cells incubated with this pathogen in chamber slides were fixed and processed for TEM directly on the slide surface, without any additional procedures that could interfere with the cell morphology. This ultrastructural analysis revealed for the first time that eosinophils undergo marked EETosis-associated nuclear alterations upon interaction with *A. fumigatus*. The typically bilobed nuclei with well-defined euchromatin/heterochromatin areas as seen in control cells (**Figure 1A**) lost their shape in parallel with chromatin decondensation/expansion, and the distinction between euchromatin/heterochromatin disappeared (**Figure 1B**). Rupture of the nuclear envelope and plasma membrane (**Figure 1C**) allowed the release of chromatin-formed EETs (arrowheads in **Figure 1Ci**) from cytolytic eosinophils and free extracellular granules (FEGs) (**Figure 1C**). Accordingly, DNA-citrullinated histone EETs released in response to *A. fumigatus* were consistently immunolabeled (**Figures 1D,Di**), as previously demonstrated (Muniz et al., 2018).

Analyses at high resolution by both SEM (**Figures 2A,Ai,C**) and TEM (**Figures 2B,D**) revealed the ultrastructure of EETs being released from cytolytic eosinophils. In three dimensions (3D), EETs appeared as typical elongated DNA fibers (**Figures 2A,Ai**), while *in situ*, TEM images of adhered cells showed the two-dimensional appearance of the extruded EETs, which covered large areas outside the cells (**Figures 2B** colored in purple, **2D**). *A. fumigatus* conidia entrapped by EETs were observed in 3D (**Figures 2A,Ai,C** colored in green) and in thin sections by TEM, which revealed that the fungus cell wall was completely involved in the DNA ETs (**Figures 2B,D**). Free extracellular granules (FEGs) with preserved limiting membranes and typical morphology represented by an electron-dense core surrounded by an electron-lucent matrix were frequently observed in association with cytolytic eosinophils, thus demonstrating that the EETosis triggered by *A. fumigatus* leads to the release of nearly intact granules (**Figures 2Ai** in yellow, **2D**). Quantitative TEM of 54 randomly acquired cell sections demonstrated that most eosinophils (87.2%) in interaction with *A. fumigatus* were cytolytic, with morphological changes typical of EETosis.

Release of EETs in Response to *A. fumigatus* Involves Src Kinase Family Activation

We previously showed that *A. fumigatus*-induced EETs release is dependent on integrin CD11b and Syk kinases (Muniz et al., 2018). Syk and Src kinases have been shown to mediate cell signaling via different classes of receptors involved in fungal recognition, including integrins (Mocsai et al., 2002, 2006). Thus, the role of Src kinases in *A. fumigatus*-induced EETs release was investigated. In the presence of PP2 (10 μM), which is known to inhibit a broad spectrum of Src kinases, the release of extracellular DNA traps by eosinophils was abolished (**Figure 3A**). Confocal fluorescence microscopy confirmed these results (**Figure 3B**).

Release of EETs in Response to *A. fumigatus* Requires the PI3 Kinase, Akt and p38-MAPK Signaling Pathways

PI3K has been described as involved in the process of extracellular DNA trap release in neutrophils and eosinophils in response to different stimuli (Behnen et al., 2014; DeSouza-Vieira et al., 2016; Germic et al., 2017; Silva et al., 2019). Using fluorimetry (**Figure 4A**) and confocal fluorescence microscopy (**Figure 4B**), we observed that in the presence of 100 ηM wortmannin, a pan-PI3K inhibitor, EETs release in response to *A. fumigatus* was abolished. Class IA PI3K has been described as important for neutrophil extracellular DNA trap (NETs) (DeSouza-Vieira et al., 2016; Silva et al., 2019) and has been implicated as critical in different eosinophil responses (Kang et al., 2012; Saito et al., 2014). More specifically, class I PI3K δ has been described as critical for neutrophil extracellular trap release in response to *A. fumigatus* conidia (Silva et al., 2019) and *Leishmania* (DeSouza-Vieira et al., 2016), as well as for eosinophil trafficking, migration and morphology (Kang et al., 2012). Thus, the role of the class I PI3K family and the class I PI3K δ isoform in EETs release in response to *A. fumigatus* was assessed. As wortmannin does not distinguish among PI3K classes, we subsequently used

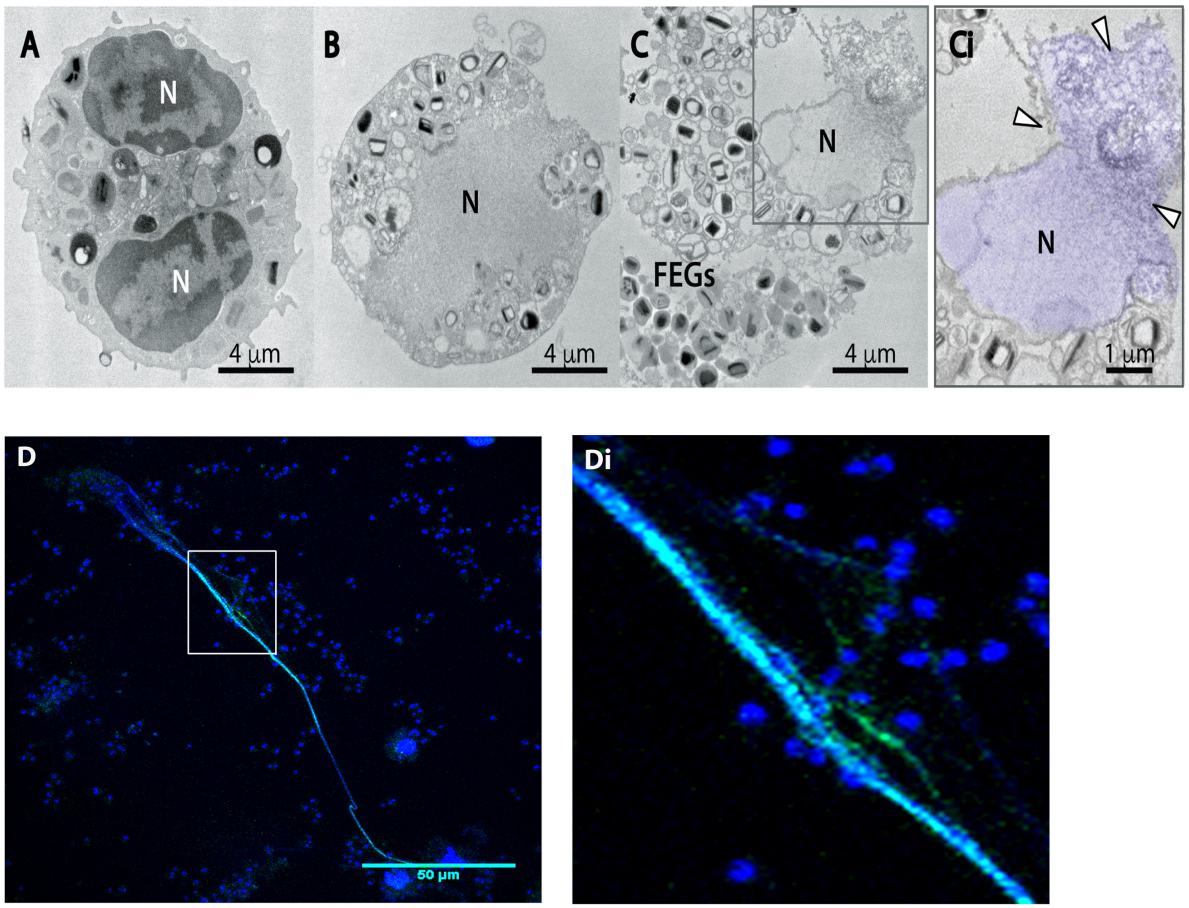

FIGURE 1 | Human eosinophils exposed to *A. fumigatus* show typical EETosis-associated nuclear changes and release chromatin-citrullinated histone EETs. Human eosinophils were stimulated with *A. fumigatus* conidia (cell:fungus ratio of 1:10) for 6 h **(A)** A representative unstimulated eosinophil with a typical bilobulated nucleus (N) and well-defined euchromatin and heterochromatin. **(B,C,Ci)** After interaction with the fungal conidia, eosinophil nuclei undergo delobulation, disintegration of the nuclear envelope, chromatin decondensation/expansion (colored in purple) and release of extracellular traps [arrowheads in panel **(Ci)**]. Most free extracellular granules (FEGs) maintain their morphology. **(D)** EETs and citrullinated histone colocalization are shown by confocal fluorescence microscopy after staining for DNA (Hoechst, blue) and anti-citrullinated H3 histone antibodies (green). Panel **(Di)** is the boxed area at a higher magnification. Representative images of 3 independent experiments (n = 3).

the compound AS605240, which at 10 μM is an inhibitor of class I PI3K. We verified that 10 μM AS605240 completely inhibited EETs release (**Figure 4C**, left panel). Confocal fluorescence microscopy studies in which the samples were stained for DNA (Sytox Green, green) confirmed these findings (**Figure 4D**). In the presence of a selective PI3Kδ inhibitor (1 μM IC87114), we observed that the release of EETs in *A. fumigatus*-stimulated human eosinophils was inhibited (**Figures 4C**, right panel and **4D**), suggesting that the process is dependent on the PI3K p110δ subunit.

Akt kinase is commonly observed as a downstream activation molecule in the class I PI3K pathway (Hawkins et al., 2010). In addition, the participation of Akt has been demonstrated in the context of NETs release following immunocomplex recognition and signaling via Mac-1 (Behnen et al., 2014). Thus, we aimed to investigate the role of Akt in EETs release in response to *A. fumigatus*. As observed by fluorimetry (**Figure 5A**) and confocal fluorescence microscopy (**Figure 5C**), when we blocked the Akt signaling pathway via its inhibitor (iAkt VIII, 2.6 μM), the eosinophils did not release EETs.

p38-MAPK is known to be involved in the release of NETs in response to immune complexes (Behnen et al., 2014), calcium ionophores and PMA (Douda et al., 2015). Therefore, we investigated whether p38-MAPK was involved in *A. fumigatus*-induced EETs release. We found that compound SB202190 (10 μM) inhibited the process of EETs release (**Figure 5B**). Confocal fluorescence microscopy confirmed the fluorimetric findings (**Figure 5C**).

Release of EETs in Response to *A. fumigatus* Requires Calcium

Calcium increase in leukocytes is intimately associated with the pro-inflammatory functions of these cells (Dixit and Simon, 2012). Therefore, we tested the impact of the calcium chelator BAPTA-AM on the process of *A. fumigatus*-induced EETs release.

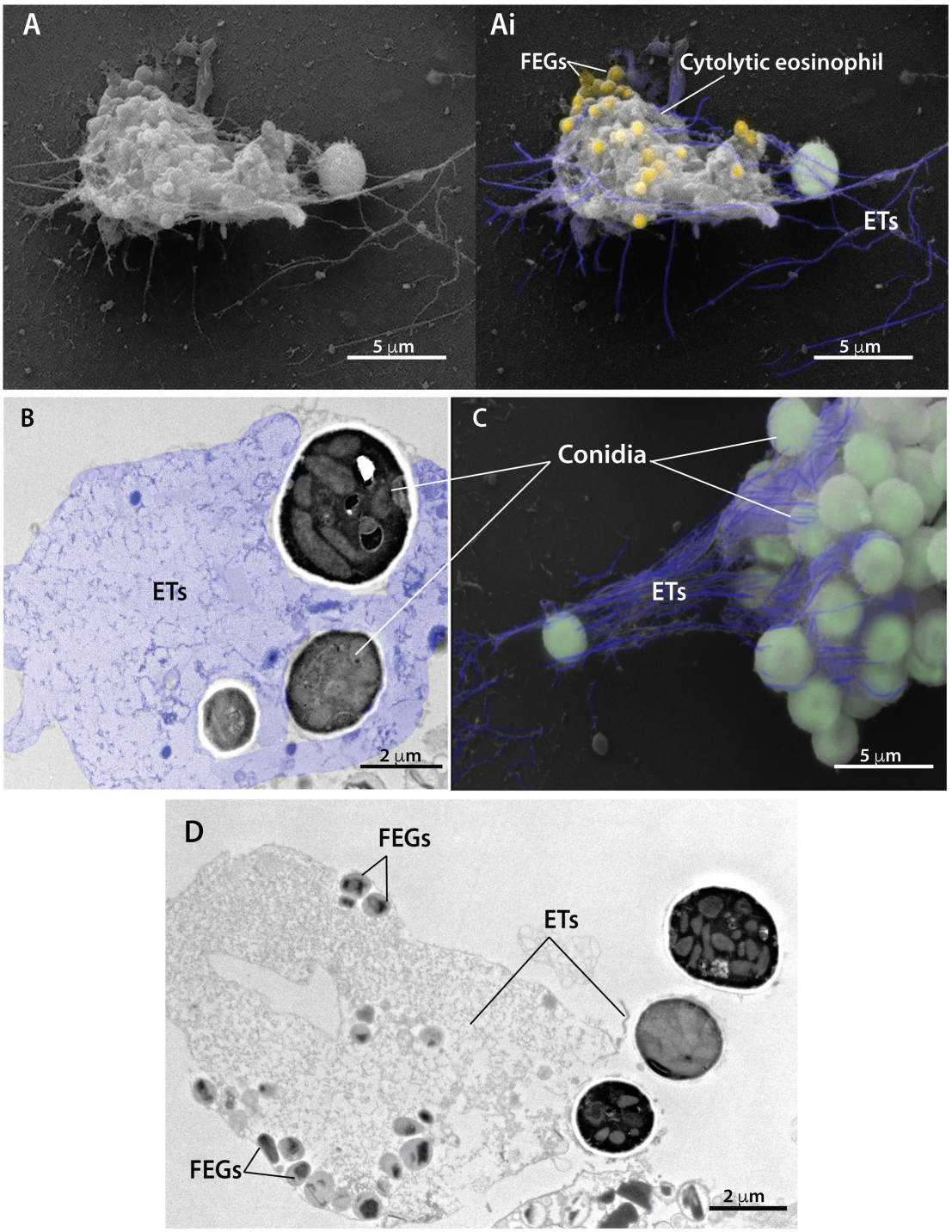

FIGURE 2 | Extracellular traps released from cytolytic eosinophils entrap conidia from *A. fumigatus*. Human eosinophils were stimulated with *A. fumigatus* conidia (cell: fungus ratio of 1:10) for 6 h and processed for conventional TEM and SEM. **(A,Ai,C)** SEM and **(B,D)** TEM revealed eosinophil extracellular traps (ETs) composed of fibers of DNA (highlighted in purple) emerging from cytolytic human eosinophils and entrapping conidia (colored in green). Note in a thin section (**B**) that the fungal cell wall is completely surrounded by eosinophil ETs. Eosinophil ETs are decorated with free extracellular granules (FEGs) seen in both 3D [Panels **(A,Ai)** in yellow] and two dimensions **(D)**. The typical eosinophil granule ultrastructure with an electron-dense core and less dense matrix is observed (**D**).

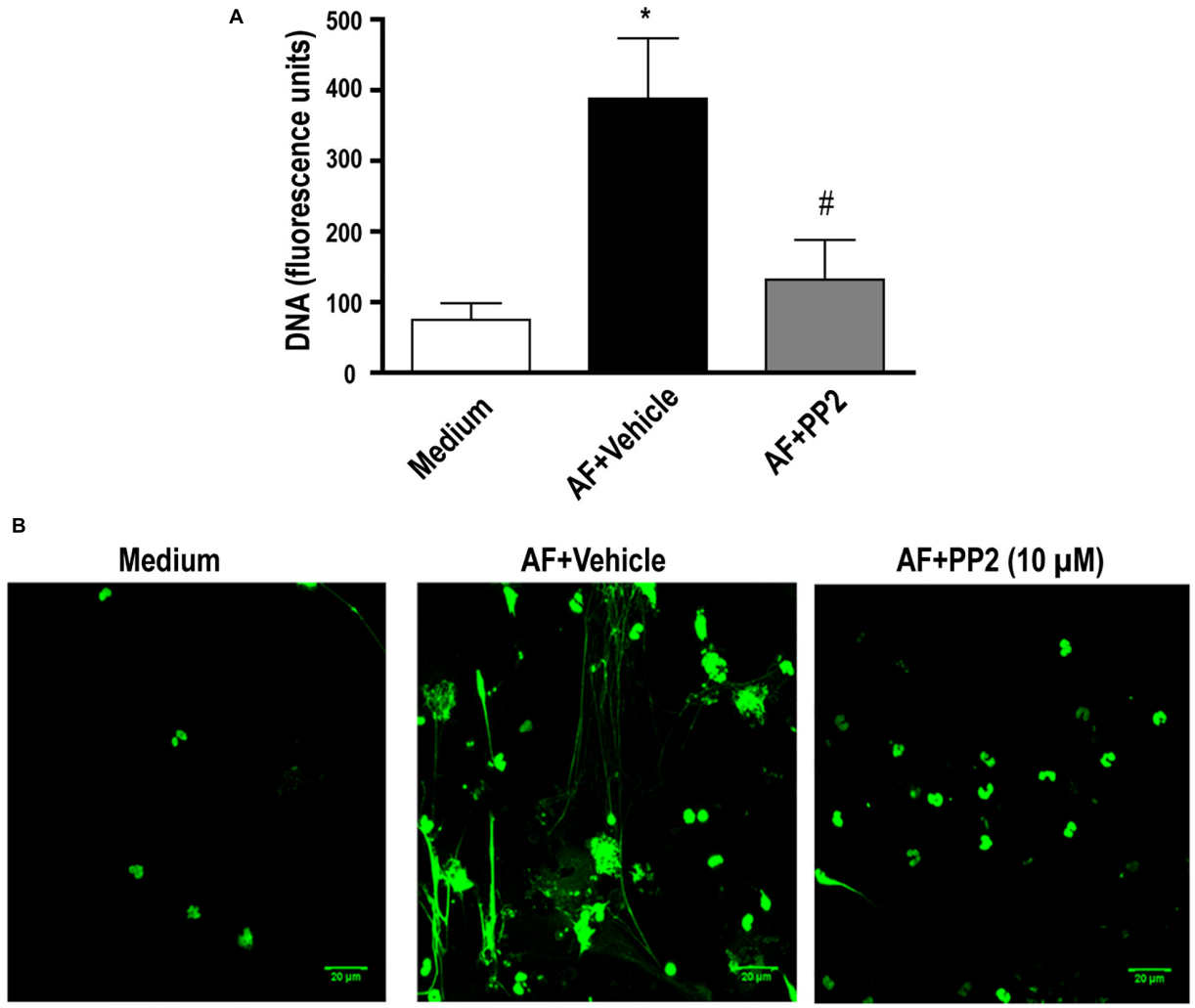

FIGURE 3 | *A. fumigatus*-induced EETs release depends on the Src kinase family. Human eosinophils were pretreated with a Src kinase family inhibitor (PP2) (10 μM), or its vehicle (DMSO) for 30 min and were subsequently stimulated with *A. fumigatus* conidia (cell: fungus ratio of 1:10) for 6 h. **(A)** EETs were quantified in the samples using a fluorescence microplate reader after Sytox Green addition (5 μM). The graph represents the mean ± SEM of 4 independent experiments from different donors. *$P < 0.05$ compared to the non-stimulated sample (medium); #$P < 0.05$ compared to AF + vehicle; one-way ANOVA followed by the Newman-Keuls test. **(B)** Confocal fluorescence microscopy of human eosinophils pretreated with PP2 (10 μM) or its vehicle (DMSO) for 30 min and subsequently stimulated with *A. fumigatus* conidia (cell: fungus ratio of 1:10) for 6 h after staining for DNA (Sytox Green, green). Representative images of 4 independent experiments ($n = 4$). AF = *Aspergillus fumigatus*.

We observed a complete inhibition of EETs release (**Figure 6A**). Accordingly, fluorescence microscopy confirmed the prevention of fungal-induced EETs extrusion (**Figure 6B**).

PAD4-Mediated Histone Citrullination Is Dispensable for *A. fumigatus*-Induced EETs Release

In this study (**Figure 1D**) and in a previous study (Muniz et al., 2018), we showed that EETs released in response to *A. fumigatus* were associated with citrullinated histone H3. Histone citrullination is considered to play an essential role in the nuclear-derived EET formation mediated by the action of PAD4 (Li et al., 2010; Rohrbach et al., 2012; Lewis et al., 2015; Kim et al., 2020), although some controversy remains about the relative importance of PAD4 for ETs release (Neeli and Radic, 2013; Kenny et al., 2017; Claushuis et al., 2018; Guiducci et al., 2018; Silva et al., 2019; Thompson-Souza et al., 2020). Thus, using fluorimetry (**Figure 7A**) and confocal fluorescence microscopy (**Figure 7B**), we observed that in the presence of GSK484 (10 μM), a selective PAD4 inhibitor, EETs release in response to *A. fumigatus* was not inhibited (cell: fungal ratio of 1:10, 6 h). Citrullinated histone H3 expression was increased in the presence of *A. fumigatus* and negatively modulated in the presence of GSK484 (10 μM), as assessed by immunoblotting (**Figure 7C**). Interestingly, similar EET-like structures were observed in eosinophils pretreated with the PAD4 inhibitor and further stimulated with *A. fumigatus*; however,

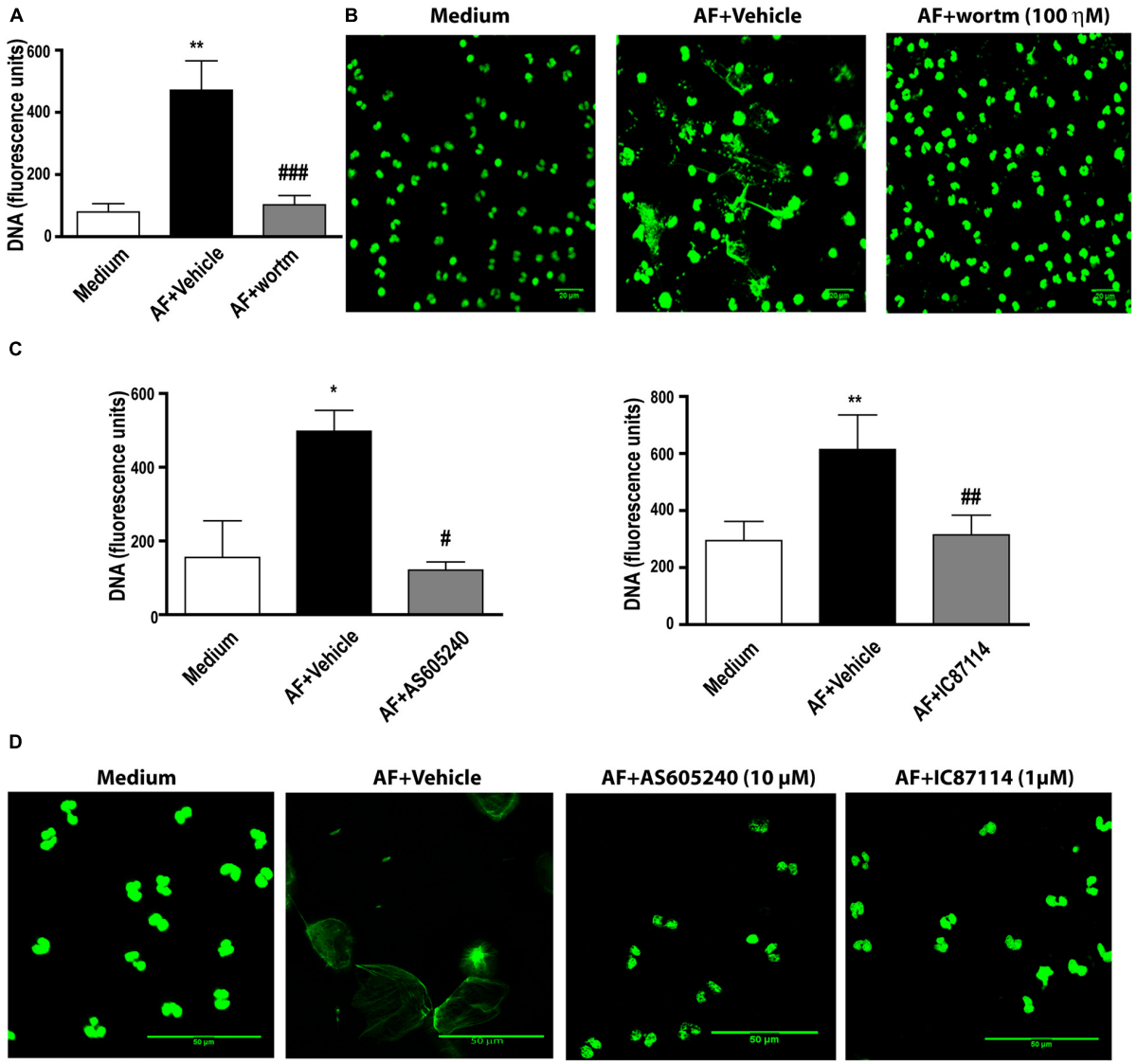

FIGURE 4 | *A. fumigatus*-induced EETs release requires PI3 kinase activation. Human eosinophils were pretreated with **(A,B)** the pan-PI3K inhibitor wortmannin (wortm – 100 ηM), **(C,D)** a class I PI3K inhibitor (AS605240 – 10 μM) and a PI3Kδ inhibitor (IC87114 – 1 μM), or their vehicle (DMSO), for 30 min and were subsequently stimulated with *A. fumigatus* conidia (cell: fungus ratio of 1:10) for 6 h. **(A,C)** EETs were quantified in the samples using a fluorescence microplate reader after Sytox Green addition (5 μM). The graphs represents the mean ± SEM of 3 (wortm), 3 (AS605240) and 7 (IC87114) independent experiments from different donors. *$P < 0.05$ and **$P < 0.01$ compared to the non-stimulated sample (medium); # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to AF + vehicle condition; one-way ANOVA followed by the Newman-Keuls test. **(B,D)** Confocal fluorescence microscopy of human eosinophils pretreated with **(B)** 100 ηM wortmannin, **(D)** 10 μM AS605240 and 1 μM IC87114 or their respective vehicle dilutions (DMSO) for 30 min and subsequently stimulated with *A. fumigatus* conidia (cell: fungus ratio of 1:10) for 6 h after staining for DNA (Sytox Green, green). Representative images of 3 (wortm), 3 (AS605240) and 7 (IC87114) independent experiments (n = 3, 3 and 7). AF = *Aspergillus fumigatus*.

citrullinated histone H3 sites were not detectable (**Figure 7D**, EETs stained by Hoechst in blue and by anti-citrullinated histone H3 antibodies in green).

Fungus Viability Is Dispensable for *A. fumigatus*-Induced EETs Release

To evaluate whether *A. fumigatus* conidia viability is crucial for the process of EETs release, we stimulated eosinophils with both viable and PFO-fixed *A. fumigatus* conidia. As shown in **Figure 8**, eosinophils responded by releasing EETs when co-cultured with either live or fixed *A. fumigatus* conidia (cell: fungal ratio of 1:10, 6 h).

DISCUSSION

Eosinophils are the major leukocytes involved in ABPA, which is the most prevalent fungal allergic manifestation among

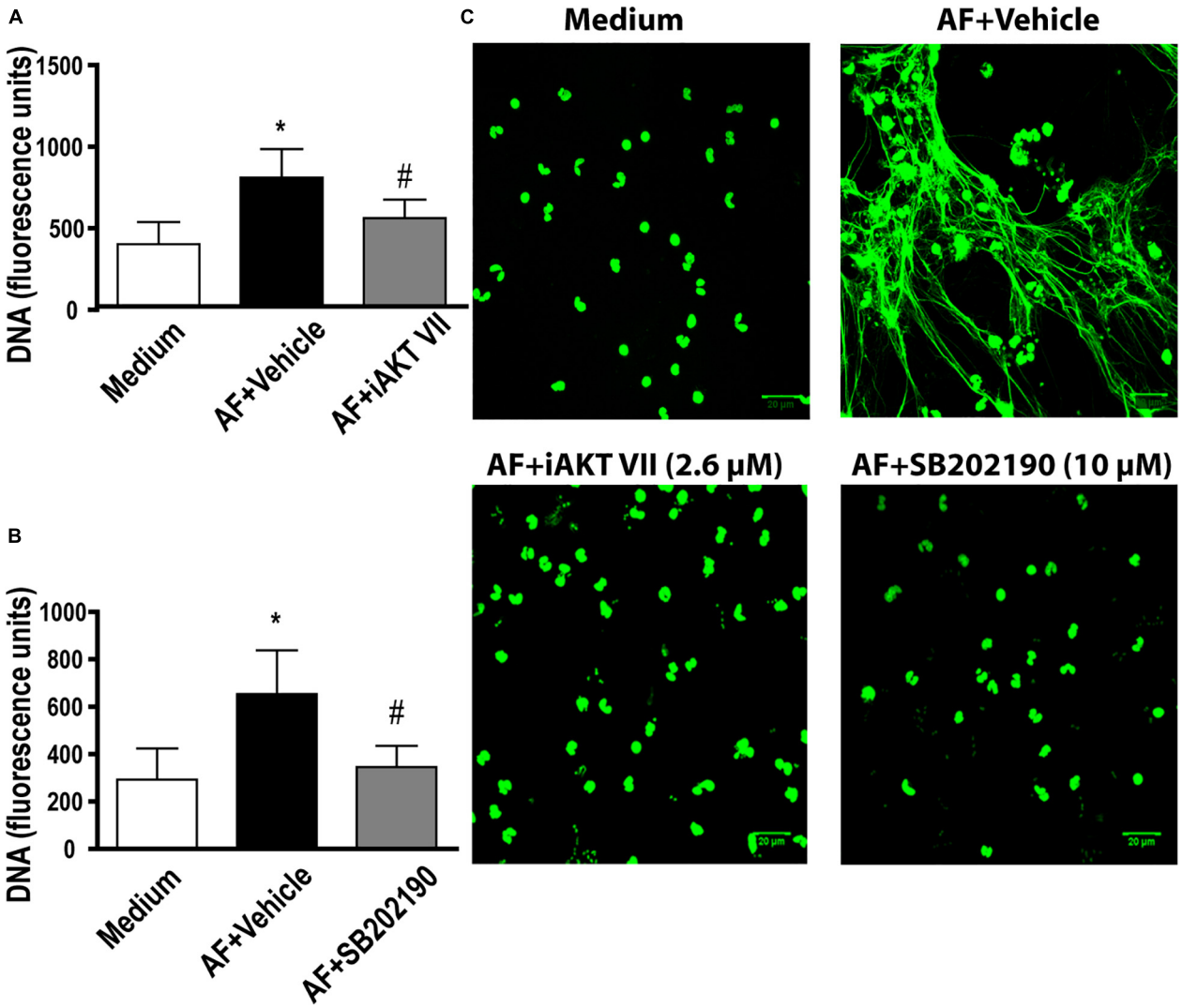

FIGURE 5 | *A. fumigatus*-induced EETs release involves Akt and p38-MAPK signaling pathways. Human eosinophils were pretreated with **(A,C)** an Akt inhibitor (iAkt VIII – 2.6 μM) and **(B,C)** a p38-MAPK inhibitor (SB202190 – 10 μM) or their vehicles (DMSO) for 30 min and were subsequently stimulated with *A. fumigatus* conidia (cell: fungus ratio of 1:10) for 6 h. **(A,B)** EETs were quantified in the samples using a fluorescence microplate reader after Sytox Green addition (5 μM). The graphs represent the mean ± SEM of 3 **(A)** and 4 **(B)** independent experiments from different donors. *$P < 0.05$ compared to the non-stimulated sample (medium); # $P < 0.05$ compared to AF + vehicle; one-way ANOVA followed by the Newman-Keuls test. Samples were analyzed by **(C)** confocal fluorescence microscopy after staining for DNA (Sytox Green, green). Representative images of 3 (iAkt) and 4 (SB202190) independent experiments ($n = 3$ and $n = 4$). AF = *Aspergillus fumigatus*.

ABPMs (Shah and Panjabi, 2002; Chowdhary et al., 2014). Thus, studies that contribute to a better understanding of ABPA pathology and can elucidate possible therapeutic targets for patient treatment are extremely relevant. Previous studies have shown that EETs are present in the mucus plugs of ABPA patients and that human eosinophils release EETs in response to *A. fumigatus* conidia *in vitro* (Muniz et al., 2018; Ueki et al., 2018). Nevertheless, efforts to understand eosinophil-*A. fumigatus* recognition and the mechanisms that drive this interaction are still in progress. Here, we demonstrated by both TEM and SEM that eosinophils responding to *A. fumigatus* stimulation present prominent morphological alterations typical of EETosis. Moreover, *A. fumigatus*-induced EETs release depends on the calcium, Src, PI3K, p38 MAPK and Akt signaling pathways. Interestingly, we determined that human eosinophils release EETs in response to *A. fumigatus* independently of PAD4 and histone citrullination through a process in which fungus viability is dispensable (**Figure 9**).

EETosis with a cytolytic profile and extrusion of extracellular traps has increasingly been identified in several eosinophilic diseases (Ueki et al., 2016a,b, 2018). This process of cell death associated to ETs release is morphologically distinct from other classic cell death processes, such as apoptosis and necrosis (Fuchs et al., 2007; Brinkmann and Zychlinsky, 2012), and has been associated with different leukocytes, especially neutrophils (Brinkmann et al., 2004), mast cells

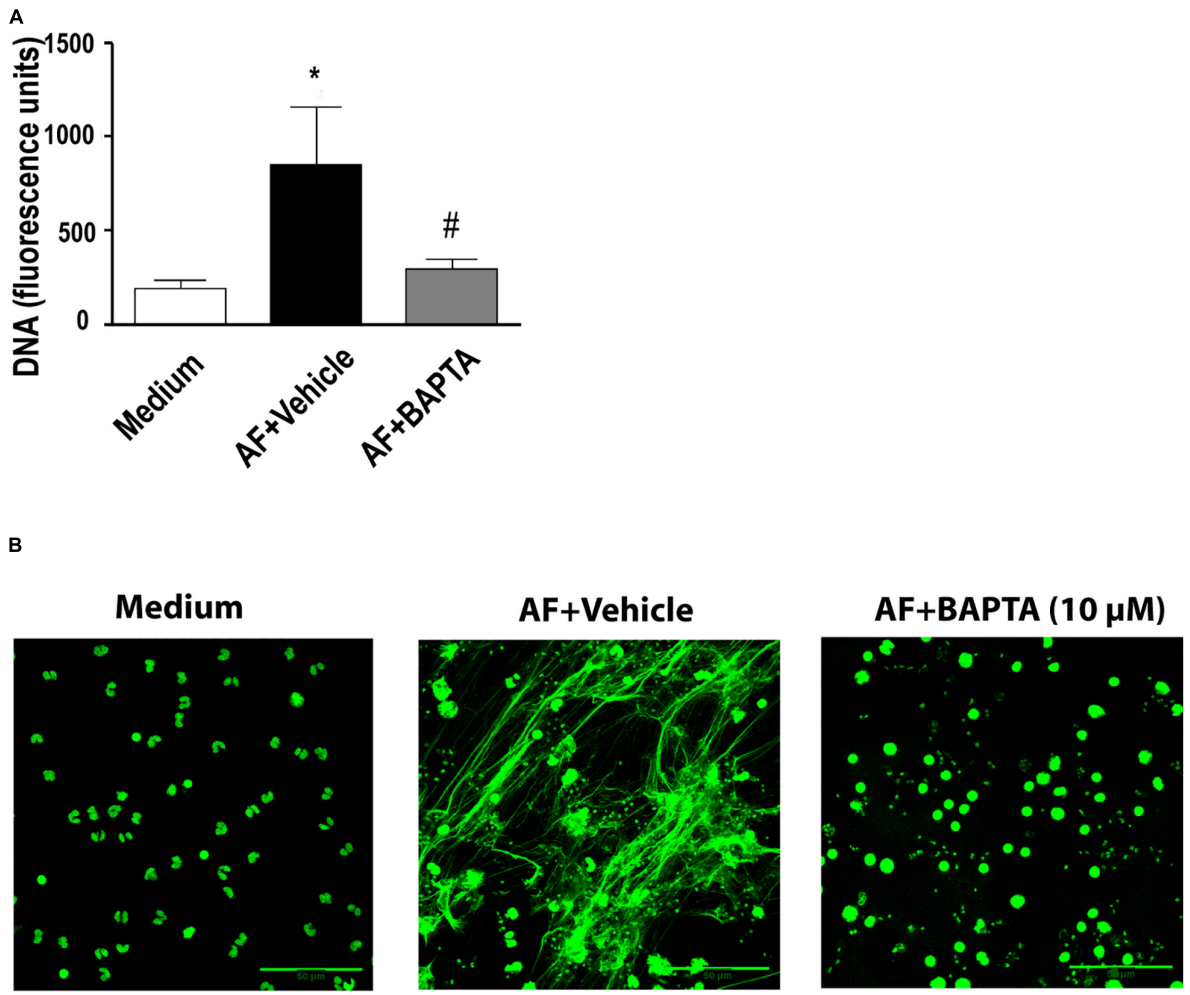

FIGURE 6 | *A. fumigatus*-induced release of EETs depends on calcium. Human eosinophils were pretreated with the calcium chelator BAPTA-AM (10 µM) or its vehicle (DMSO) for 30 min and were subsequently stimulated with *A. fumigatus* conidia (cell: fungus ratio of 1:10) for 6 h. **(A)** EETs were quantified in the samples using a fluorescence microplate reader after Sytox Green addition (5 µM). The graph represents the mean ± SEM of 3 independent experiments from different donors. *$P < 0.05$ compared to the non-stimulated sample (medium); #$P < 0.05$ compared to AF + vehicle; one-way ANOVA followed by the Newman-Keuls test. **(B)** Confocal fluorescence microscopy of human eosinophils pretreated with BAPTA-AM (10 µM) or its vehicle (DMSO) for 30 min and subsequently stimulated with *A. fumigatus* conidia (cell: fungus ratio of 1:10) for 6 h after staining for DNA (Sytox Green, green). Representative images of 4 independent experiments ($n = 4$). AF = *Aspergillus fumigatus*.

(von Kockritz-Blickwede et al., 2008) and eosinophils (Ueki et al., 2013). Here, we used high-resolution TEM to characterize intracellular events associated with EETosis. Our TEM findings revealed that *A. fumigatus*-stimulated eosinophils elicited nuclear changes typical of EETosis (decondensation, delobulation/rounding, disruption of nuclear envelope, chromatin expansion in the cytoplasm and further release of EETs) (**Figure 1**). These processes are well characterized in neutrophils (Fuchs et al., 2007), but are poorly understood in human eosinophils. In contrast to the process of ETosis in neutrophils (termed NETosis), which leads to the release of granule contents mixed with released chromatin (Brinkmann et al., 2004; Fuchs et al., 2007), EETosis enables the release of clusters of FEGs, with preserved morphology and contents, together with EETs. Our ultrastructural results showing FEGs by both TEM (**Figures 1C**, **2D**) and SEM (**Figures 2A,Ai**) are in accordance with previous works demonstrating that EETosis induced by different stimuli, including the lipid mediator lysophosphatidylserine and *A. fumigatus* conidia, occurs in the presence of punctual MBP labeling (indicative of intact granules) and in the absence of linear immunostaining for MBP or ECP (indicative of disrupted granules) (Ueki et al., 2013; Muniz et al., 2018; Kim et al., 2020). This means that distinct mechanisms are operating in neutrophils and eosinophils in the process of granule product release in association with DNA traps during ETosis. For eosinophils, FEGs are secretory-competent organelles acting as "cluster bombs" that selectively release proteins under specific stimuli (Neves et al., 2008; Ueki et al., 2013). However, the consequences of these secretory entities associated with EETs still require clarification.

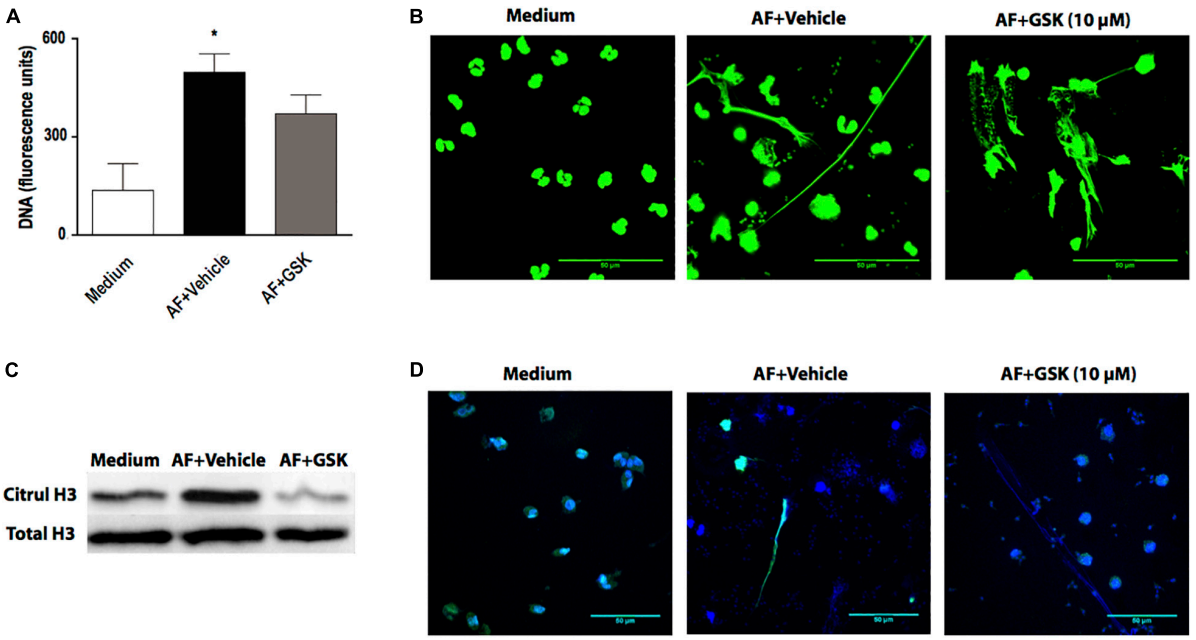

FIGURE 7 | Histone citrullination mediated by PAD4 is dispensable for *A. fumigatus*-induced EETs release. Human eosinophils were pretreated with the PAD4 selective inhibitor GSK484 (10 μM) or its vehicle (DMSO) for 30 min and were subsequently stimulated with *A. fumigatus* conidia (cell: fungus ratio of 1:10) for 6 h. **(A)** EETs were quantified in the samples using a fluorescence microplate reader after Sytox Green addition (5 μM). The graph represents the mean ± SEM of 3 independent experiments from different donors. *$P < 0.05$ compared to the non-stimulated sample (medium); one-way ANOVA followed by the Newman-Keuls test. **(B)** Samples were analyzed by confocal fluorescence microscopy after staining for DNA (Sytox Green, green). Representative images of 3 independent experiments ($n = 3$). Histone citrullination was evaluated by **(C)** immunoblotting and **(D)** confocal fluorescence microscopy after staining for DNA (Hoechst, blue) and anti-citrullinated H3 histone antibodies (green). Representative immunoblotting images of 3 independent experiments ($n = 3$). AF = *Aspergillus fumigatus*.

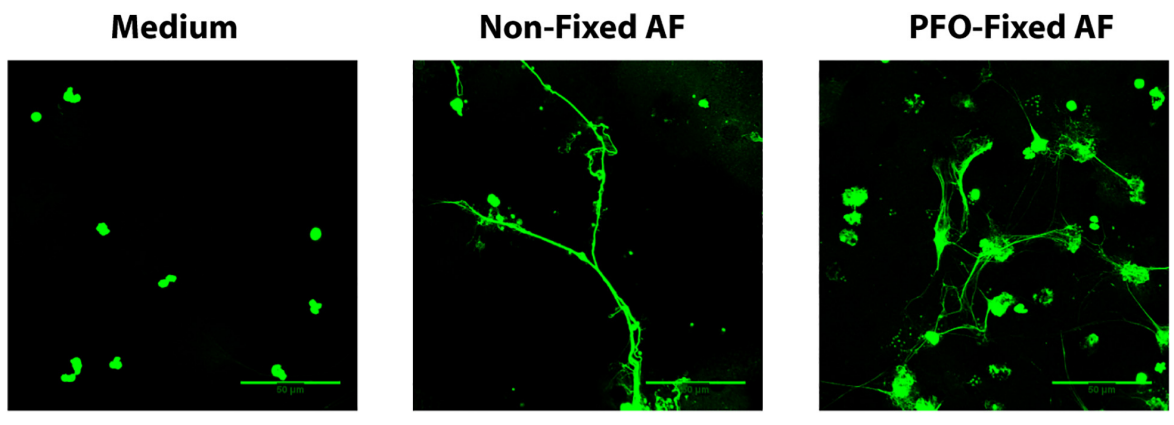

FIGURE 8 | Fungus viability is dispensable for *A. fumigatus*-induced EETs release. Human eosinophils were stimulated with both viable and paraformaldehyde (PFO)-fixed *A. fumigatus* conidia (cell: fungus ratio of 1:10, 6 h). Samples were analyzed by confocal fluorescence microscopy after staining for DNA (Sytox Green, green). Representative images of 4 independent experiments ($n = 4$). AF = *Aspergillus fumigatus*.

The capacity of chromatin to actively participate in NETosis is now recognized (Neubert et al., 2018), in addition to its classical role in regulating gene expression. During NETosis, after certain signaling events and enzymatic reactions (including histone citrullination and phosphorylation events), the chromatin expands, which drives the rupture of the nuclear envelope, determining the point of no return. Here, we noted the same event for human eosinophils undergoing EETosis. Although signaling molecules are usually the focus of studies on cell activation processes, recent works have demonstrated that chromatin changes are crucial in determining cell fate (Neubert et al., 2018). Thus, our ultrastructural observations bring a new appreciation of the importance of linking intracellular signaling pathways to nuclear entities and structural protein modifications to better delineate a stimulus-specific cell activation process.

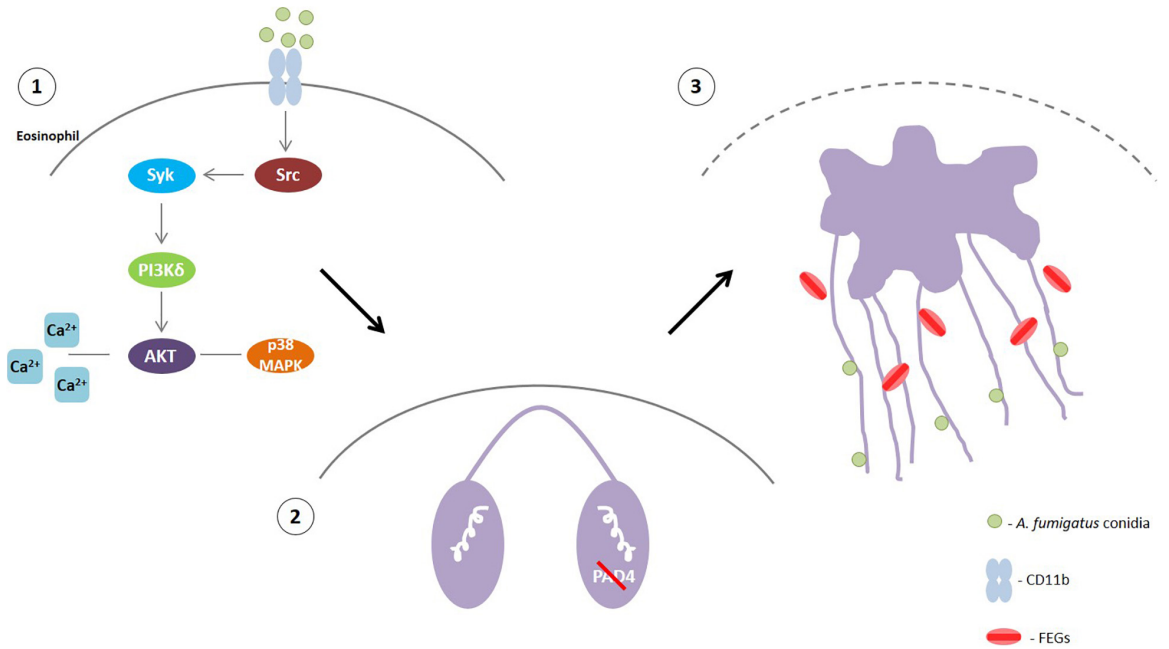

FIGURE 9 | (1) *A. fumigatus* conidia-human eosinophil interaction triggers the activation of Src that will phosphorylate Syk tyrosine kinases. Then, PI3δK will be activated followed by Akt, probably with the participation of calcium. p38 MAPK will also likely be activated downstream the PI3K signaling **(2)** This signaling leads to histone citrullination mediated by PAD4 and chromatin decondensation. However, PAD4 and H3 citrullination are not essential for EETs extrusion. **(3)** Rupture of the nuclear envelope and plasma membrane allowed the release of chromatin-formed EETs that entrap *A. fumigatus* conidia and free extracellular granules (FEGs).

We previously demonstrated that intracellular signaling involved in the release of EETs in response to *A. fumigatus* is Syk- and CD11b β2-integrin-dependent (Muniz et al., 2018). Src and Syk kinases have been implicated in the cell signaling of various classes of receptors involved in fungal molecule recognition, including integrins (Mocsai et al., 2002, 2006; Jakus et al., 2007) and C-type lectins (Rogers et al., 2005; Kerrigan and Brown, 2010). Src-family kinases are involved in β_2 integrin-mediated responses in which the Src kinases are implicated in Syk phosphorylation in neutrophils (Mocsai et al., 2006). In line with these findings, our results reveal that *A. fumigatus*-induced EETs release is blocked in the presence of a Src inhibitor. Additionally, in agreement with our findings, neutrophils respond to *A. fumigatus* conidia by releasing NETs in a Syk-Src-dependent signaling pathway (Silva et al., 2019).

PI3Ks are enzymes that catalyze the phosphorylation of one or more inositol phospholipids in the 3-position of the inositol ring. PI3Ks can be divided into classes I, II, and III. Class IA PI3Ks, which include PI3K α, β, and δ, are activated by stimulation of tyrosine kinase-based receptors. PI3Kγ, the only member of class IB, is activated by stimulation of GPCR subunits (Vanhaesebroeck et al., 2010). We found that both a non-selective (wortmannin) and a class I-selective (AS605240, 10 μM) PI3K inhibitor blocked *A. fumigatus*-induced EET release. Because there have been many studies of wortmannin as a non-selective inhibitor of the different PI3K classes and of other possible targets (Makni-Maalej et al., 2013; Germic et al., 2017), we additionally used the compound AS605240. AS605240 at 10 μM has been reported as an inhibitor of class I PIKs but is unable at this concentration to distinguish between class A (PI3K α, β and δ) and B (PI3Kγ) (Sadhu et al., 2003; Silva et al., 2019). In mammals, class I PI3Ks are present in all cell types, including eosinophils, with PI3Kδ and γ highly enriched in leukocytes (Kok et al., 2009). As mentioned, we have previously described the dependence of *A. fumigatus*-induced EET release on Syk signaling (Muniz et al., 2018). Syk is a tyrosine kinase that is crucial in the signaling pathways mediated by Dectin-1 and the β_2 integrin CD11b/CD18 (Mocsai et al., 2002; Rogers et al., 2005). Taking in account that receptors utilizing protein tyrosine kinase activation are known to be more related to the activation of class IA PI3Ks (PI3K α, β, and δ), we investigated the role of class I PI3K δ in *A. fumigatus*-induced EET release. We observed that compound IC-87114 at 1 μM (concentration known to be selective for the class I δ isoform) (Sadhu et al., 2003; Silva et al., 2019) completely inhibited EETs release. Indeed, the importance of PI3K δ in eosinophils and in experimental models of allergic inflammation has been previously reported (Lee et al., 2006; Nashed et al., 2007; Kang et al., 2012). Kang and colleagues demonstrated that treatment with ICI87114 reduced murine bone marrow-derived eosinophil adhesion and Mac-1 expression and had inhibitory effects on eotaxin-1-induced chemotaxis and shape change (Kang et al., 2012). However, the authors used 10 μM ICI87114. In our studies, we used 1 μM ICI87114. Some studies suggest that 10 μM ICI87114 might also have prominent effects on PI3Kγ (Sadhu et al., 2003; Silva et al., 2019). Regarding the PI3Kγ isoform, some important effects have also been described in human eosinophils but are related to the activation of GPCRs (Saito et al., 2014). Saito and colleagues described the

effects of specific inhibition of the PI3Kγ isoform on human eosinophil chemotaxis, adherence and degranulation induced by eotaxin (Saito et al., 2014). Since PI3Kγ signaling is associated with GPCRs (such as CCR3), whereas PI3K α, β, and δ are activated by receptor tyrosine kinase, it is possible that PI3Kγ and δ isoforms might have distinct roles in eosinophil intracellular signaling and function depending on the stimuli and the cognate receptor involved. In fact, PI3K activation has been implicated as crucial for ROS generation-induced DNA trap release in eosinophils and neutrophils stimulated with GM−CSF and C5a or with low concentrations of PMA (Germic et al., 2017); and in neutrophils stimulated with immobilized immune complexes (Behnen et al., 2014). In contrast, other studies have pointed that ROS and PI3K is dispensable for lysophosphatidylserine-induced EETs extrusion (Kim et al., 2020). The PI3K δ isoform specifically has been described as critically involved in NETs release in response to *Leishmania amazonensis* (DeSouza-Vieira et al., 2016) and *A. fumigatus* (Silva et al., 2019) in a signaling pathway upstream of ROS production. In contrast, the PI3Kγ isoform is not involved in *A. fumigatus*-induced NETs release (Silva et al., 2019).

The activation of Akt has been reported to be the downstream target of PI3K in various cells, including eosinophils (Alessi et al., 1997; Machida et al., 2005; Hawkins et al., 2010). The participation of Akt has been demonstrated in the context of NETs release following immunocomplex recognition and signaling via Mac-1 (Behnen et al., 2014). Moreover, it has been demonstrated that PMA-induced NETs formation is dependent on Akt activation, which suppresses apoptosis via the inhibition of caspases (Douda et al., 2014). In agreement with these findings, we found that the release of EETs in response to *A. fumigatus* requires the PI3 kinase and Akt signaling pathways. Behnen and colleagues showed that CR3 (CD11b/CD18) activation through the recognition of immunocomplexes by the FcγRIIIB receptor induces NETs release via the Src/Syk, PI3K/Akt, p38 MAPK, and ERK1/2 signaling pathways (Behnen et al., 2014). The involvement of p38 MAPK in NETs release induced by different stimuli, such as bacteria or PMA, was previously reported (Keshari et al., 2013; Behnen et al., 2014; Douda et al., 2015; Ma et al., 2018). In agreement with these studies, we found that the release of EETs was inhibited when eosinophils were pretreated with SB202190, suggesting the involvement of p38-MAPK signaling in *A. fumigatus*-induced EETs release.

As we observed that *A. fumigatus*-induced EETs release triggered the Src/Syk, PI3K/Akt and p38 MAPK signaling pathways, we also examined calcium. Changes in leukocyte calcium levels have been consistently related to several leukocyte functions, including cell adhesion, chemotaxis and degranulation, among others (Dixit and Simon, 2012; Hann et al., 2020). The relationship between calcium and ETosis has been supported by different studies in eosinophils, but mostly by studies in neutrophils (Parker et al., 2012; Gupta et al., 2014; Ueki et al., 2016a; Kenny et al., 2017; de Bont et al., 2018). Moreover, the activation of Akt has been found to be regulated by an elevation of calcium during the NETosis process (Douda et al., 2014). Thus, our finding that the calcium chelator BAPTA-AM inhibited EETs release induced by *A. fumigatus* corroborates these previous observations. Ueki and colleagues found that EDTA inhibited the EETs extrusion induced by A23187, PMA, or immobilized IgG in human eosinophils (Ueki et al., 2016a). In neutrophils, Kenny and colleagues demonstrated the relevance of intracellular calcium for PMA-induced NET production by showing a strong inhibition of NETosis after treating neutrophils with BAPTA-AM (Kenny et al., 2017). In agreement with this observation, another work also provided evidence that external calcium is dispensable for NETs extrusion induced by PMA (Douda et al., 2015). In contrast, a different study showed that IL-8-mediated NET formation requires calcium fluxes from both intracellular and extracellular pools, while only extracellular calcium appeared to be important for PMA-mediated NET generation (Gupta et al., 2014). Accordingly, ionomycin and other calcium ionophores have been widely used as known inducers of NETs and EETs (Douda et al., 2015; Ueki et al., 2016a). In this context, it is clear that calcium plays a crucial role in *A. fumigatus*-induced ETTs extrusion. However, whether calcium is important for Akt activation during this EETosis process or which is the major source of calcium (intracellular or extracellular or both) are questions still to be answered.

Although several studies have implicated a role for PAD4 in histone citrullination and chromatin decondensation in the process of ETosis (Wang et al., 2009; Li et al., 2010; Lewis et al., 2015; Van Avondt and Hartl, 2018; Kim et al., 2020), others have suggested that PAD4 might not be essential for this process even when H3 citrullination is reduced (Neeli and Radic, 2013; Kenny et al., 2017; Claushuis et al., 2018; Guiducci et al., 2018; Silva et al., 2019; Thompson-Souza et al., 2020). According to most ET studies, the citrullination of histone 3 by the enzyme PAD4, expressed in the nuclei of eosinophils and other granulocytes (Asaga et al., 2001; Nakashima et al., 2002; Kim et al., 2020), results in weakened DNA–histone binding, thereby facilitating the release of DNA from the nucleus and out of the cell (Van Avondt and Hartl, 2018). However, other studies have shown that histone citrullination-independent mechanisms occur in the process of NETs release in response to *Candida albicans* (Guiducci et al., 2018), bacteria (*Streptococcus* and *Klebsiella pneumoniae*) (Kenny et al., 2017; Claushuis et al., 2018), *Histoplasma capsulatum* (Thompson-Souza et al., 2020) and *A. fumigatus* (Silva et al., 2019). Under our conditions, we observed that EETs generated in response to *A. fumigatus* exhibited histone citrullination that was PAD4-dependent; however, PAD4 and H3 citrullination are not essential for EETs extrusion. In fact, the existence of NET release pathways independent of histone citrullination has been described for PMA, one of the most recognized promoters of NET release (Neeli and Radic, 2013; Kenny et al., 2017). In this context, the dependency of ETs extrusion on PAD4 and histone citrullination is questionable and might depend on the stimuli and cell type involved. One question that remains in eosinophils is which molecule is responsible for chromatin decondensation, since it can occur independently of PAD4 and histone citrullination. In neutrophils, elastase and myeloperoxidase have been described as important for this process (Papayannopoulos et al., 2010), but in eosinophils, this is a point for future investigations. Moreover, the consequences of the release of non-citrullinated

EETs for the host immune response, for the fungus, and for ABPA development remain unknown. We previously showed that EETs do not contribute to the killing and impairment of *A. fumigatus* conidia (Muniz et al., 2018). Thus, the impact of non-citrullinated EETs on fungal viability is potentially not critical. However, other consequences of these non-citrullinated EETs regarding ABPA development and the host immune response cannot be discarded. In a study of *Klebsiella pneumoniae*–induced pneumonia, NETs formed in the absence of PAD4 and histone citrullination did not affect bacterial growth or lung inflammation (Claushuis et al., 2018). Whether the same is valid for eosinophils or eosinophilic lung inflammation in the context of ABPA remains to be addressed. Our findings also demonstrated that *A. fumigatus* is capable of inducing EETs release independent of fungal viability. In previous works, dead *A. fumigatus* conidia were also capable of inducing neutrophils to release extracellular DNA traps (Bruns et al., 2010; McCormick et al., 2010). Thus, fungal cell metabolism seems to play no active role in this interaction, both in EETs and NETs formation.

In this study, we identified several components that drive EETs release in response to the fungus *A. fumigatus*. Based on this and previous studies (Muniz et al., 2018), we believe that the excessive release of EETs may contribute to the formation of sticky mucus, which in turn contributes to airway obstruction and lung function impairment. Furthermore, because EETs lack killing activity against *A. fumigatus* (Muniz et al., 2018), their association with clusters of functional FEGs may potentiate the pro-inflammatory roles of these structures. Thus, therapeutic interventions to avoid or degrade these DNA traps may represent an interesting approach to minimize inflammatory lung damage without major impacts on fungal clearance. In addition to clarifying mechanisms that underlie the interaction between *A. fumigatus* and eosinophils, these findings may help improve our understanding of ABPA pathogenesis and treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Committee on Human Research at Clementino Fraga Filho Hospital (Federal University of Rio de Janeiro). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MVB, IG, MAAD, GAT-S, VSM, and CRIV conducted the experiments and acquired and analyzed the data. MVB, RCNM, RTF, and JSN designed the research studies, analyzed the data and provided reagents. MVB and JSN wrote the manuscript. RCNM and RTF critically revised the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Brazilian agencies Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) (#E-26/203.312/2017 granted to JSN) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (#409057/2018-5 and #309216/2017-6 granted to JSN; #309734/2018-5 and 434914/2018-5 granted to RCNM). MVB (post-doc scholarship) and IG (MSc scholarship) especially thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support.

ACKNOWLEDGMENTS

We thank Grasiela Ventura for technical assistance in image acquisition in the confocal microscopy facility of the Biomedical Sciences Institute (ICB, UFRJ, Brazil) and Gustavo Rocha for technical assistance in image acquisition in the scanning electron microscopy facility at Centro Nacional de Biologia Estrutural e Bioimagens (CENABIO, UFRJ, Brazil). We also thank Katiane Oliveira Vieira for technical assistance with the various experiments performed in this study.

REFERENCES

Agarwal, R., Chakrabarti, A., Shah, A., Gupta, D., Meis, J. F., Guleria, R., et al. (2013). Allergic bronchopulmonary aspergillosis: review of literature and proposal of new diagnostic and classification criteria. *Clin. Exp. Allergy* 43, 850–873. doi: 10.1111/cea.12141

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., et al. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr. Biol.* 7, 261–269. doi: 10.1016/s0960-9822(06)00122-9

Asaga, H., Nakashima, K., Senshu, T., Ishigami, A., and Yamada, M. (2001). Immunocytochemical localization of peptidylarginine deiminase in human eosinophils and neutrophils. *J. Leukoc. Biol.* 70, 46–51.

Behnen, M., Leschczyk, C., Moller, S., Batel, T., Klinger, M., Solbach, W., et al. (2014). Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcgammaRIIIB and Mac-1. *J. Immunol.* 193, 1954–1965. doi: 10.4049/jimmunol.1400478

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., et al. (2004). Neutrophil extracellular traps kill bacteria. *Science* 303, 1532–1535. doi: 10.1126/science.1092385

Brinkmann, V., and Zychlinsky, A. (2012). Neutrophil extracellular traps: is immunity the second function of chromatin? *J. Cell Biol.* 198, 773–783. doi: 10.1083/jcb.201203170

Bruns, S., Kniemeyer, O., Hasenberg, M., Aimanianda, V., Nietzsche, S., Thywissen, A., et al. (2010). Production of extracellular traps against *Aspergillus fumigatus* in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. *PLoS Pathog.* 6:e1000873. doi: 10.1371/journal.ppat.1000873

Chow, O. A., von Kockritz-Blickwede, M., Bright, A. T., Hensler, M. E., Zinkernagel, A. S., Cogen, A. L., et al. (2010). Statins enhance formation of

phagocyte extracellular traps. *Cell Host Microbe* 8, 445–454. doi: 10.1016/j.chom.2010.10.005

Chowdhary, A., Agarwal, K., Kathuria, S., Gaur, S. N., Randhawa, H. S., and Meis, J. F. (2014). Allergic bronchopulmonary mycosis due to fungi other than *Aspergillus*: a global overview. *Crit. Rev. Microbiol.* 40, 30–48. doi: 10.3109/1040841X.2012.754401

Claushuis, T. A. M., van der Donk, L. E. H., Luitse, A. L., van Veen, H. A., van der Wel, N. N., van Vught, L. A., et al. (2018). Role of peptidylarginine deiminase 4 in neutrophil extracellular trap formation and host defense during *Klebsiella pneumoniae*-induced pneumonia-derived sepsis. *J. Immunol.* 201, 1241–1252. doi: 10.4049/jimmunol.1800314

de Bont, C. M., Koopman, W. J. H., Boelens, W. C., and Pruijn, G. J. M. (2018). Stimulus-dependent chromatin dynamics, citrullination, calcium signalling and ROS production during NET formation. *Biochim. Biophys. Acta Mol. Cell. Res.* 1865(11 Pt A), 1621–1629. doi: 10.1016/j.bbamcr.2018.08.014

Denning, D. W., Pashley, C., Hartl, D., Wardlaw, A., Godet, C., Del Giacco, S., et al. (2014). Fungal allergy in asthma-state of the art and research needs. *Clin. Transl. Allergy* 4:14. doi: 10.1186/2045-7022-4-14

Denning, D. W., Pleuvry, A., and Cole, D. C. (2013). Global burden of allergic bronchopulmonary aspergillosis with asthma and its complication chronic pulmonary aspergillosis in adults. *Med. Mycol.* 51, 361–370. doi: 10.3109/13693786.2012.738312

DeSouza-Vieira, T., Guimaraes-Costa, A., Rochael, N. C., Lira, M. N., Nascimento, M. T., Lima-Gomez, P. S., et al. (2016). Neutrophil extracellular traps release induced by Leishmania: role of PI3Kgamma, ERK, PI3Ksigma, PKC, and [Ca2+]. *J. Leukoc. Biol.* 100, 801–810. doi: 10.1189/jlb.4A0615-261RR

Dixit, N., and Simon, S. I. (2012). Chemokines, selectins and intracellular calcium flux: temporal and spatial cues for leukocyte arrest. *Front. Immunol.* 3:188. doi: 10.3389/fimmu.2012.00188

Douda, D. N., Khan, M. A., Grasemann, H., and Palaniyar, N. (2015). SK3 channel and mitochondrial ROS mediate NADPH oxidase-independent NETosis induced by calcium influx. *Proc. Natl. Acad. Sci. U.S.A.* 112, 2817–2822. doi: 10.1073/pnas.1414055112

Douda, D. N., Yip, L., Khan, M. A., Grasemann, H., and Palaniyar, N. (2014). Akt is essential to induce NADPH-dependent NETosis and to switch the neutrophil death to apoptosis. *Blood* 123, 597–600. doi: 10.1182/blood-2013-09-526707

Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., et al. (2007). Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* 176, 231–241. doi: 10.1083/jcb.200606027

Germic, N., Stojkov, D., Oberson, K., Yousefi, S., and Simon, H. U. (2017). Neither eosinophils nor neutrophils require ATG5-dependent autophagy for extracellular DNA trap formation. *Immunology* 152, 517–525. doi: 10.1111/imm.12790

Guiducci, E., Lemberg, C., Kung, N., Schraner, E., Theocharides, A. P. A., and LeibundGut-Landmann, S. (2018). *Candida albicans*-induced NETosis is independent of peptidylarginine deiminase 4. *Front. Immunol.* 9:1573. doi: 10.3389/fimmu.2018.01573

Guimaraes-Costa, A. B., Nascimento, M. T., Froment, G. S., Soares, R. P., Morgado, F. N., Conceicao-Silva, F., et al. (2009). Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6748–6753. doi: 10.1073/pnas.0900226106

Gupta, A. K., Giaglis, S., Hasler, P., and Hahn, S. (2014). Efficient neutrophil extracellular trap induction requires mobilization of both intracellular and extracellular calcium pools and is modulated by cyclosporine A. *PLoS One* 9:e97088. doi: 10.1371/journal.pone.0097088

Hann, J., Bueb, J. L., Tolle, F., and Bréchard, S. (2020). Calcium signaling and regulation of neutrophil functions: still a long way to go. *J. Leukoc. Biol.* 107, 285–297. doi: 10.1002/jlb.3ru0719-241r

Hawkins, P. T., Stephens, L. R., Suire, S., and Wilson, M. (2010). PI3K signaling in neutrophils. *Curr. Top. Microbiol. Immunol.* 346, 183–202. doi: 10.1007/82_2010_40

Jakus, Z., Fodor, S., Abram, C. L., Lowell, C. A., and Mocsai, A. (2007). Immunoreceptor-like signaling by beta 2 and beta 3 integrins. *Trends Cell Biol.* 17, 493–501. doi: 10.1016/j.tcb.2007.09.001

Jorch, S. K., and Kubes, P. (2017). An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat. Med.* 23, 279–287. doi: 10.1038/nm.4294

Kang, B. N., Ha, S. G., Ge, X. N., Reza Hosseinkhani, M., Bahaie, N. S., Greenberg, Y., et al. (2012). The p110delta subunit of PI3K regulates bone marrow-derived eosinophil trafficking and airway eosinophilia in allergen-challenged mice. *Am. J. Physiol. Lung. Cell Mol. Physiol.* 302, L1179–L1191. doi: 10.1152/ajplung.00005.2012

Kenny, E. F., Herzig, A., Kruger, R., Muth, A., Mondal, S., Thompson, P. R., et al. (2017). Diverse stimuli engage different neutrophil extracellular trap pathways. *eLife* 6:e24437. doi: 10.7554/eLife.24437

Kerrigan, A. M., and Brown, G. D. (2010). Syk-coupled C-type lectin receptors that mediate cellular activation via single tyrosine based activation motifs. *Immunol. Rev.* 234, 335–352. doi: 10.1111/j.0105-2896.2009.00882.x

Keshari, R. S., Verma, A., Barthwal, M. K., and Dikshit, M. (2013). Reactive oxygen species-induced activation of ERK and p38 MAPK mediates PMA-induced NETs release from human neutrophils. *J. Cell. Biochem.* 114, 532–540. doi: 10.1002/jcb.24391

Kim, H. J., Sim, M. S., Lee, D. H., Kim, C., Choi, Y., Park, H. S., et al. (2020). Lysophosphatidylserine induces eosinophil extracellular trap formation and degranulation: implications in severe asthma. *Allergy* 75, 3159–3170. doi: 10.1111/all.14450

Knutsen, A. P., Bush, R. K., Demain, J. G., Denning, D. W., Dixit, A., Fairs, A., et al. (2012). Fungi and allergic lower respiratory tract diseases. *J. Allergy Clin. Immunol.* 129, 280–291. doi: 10.1016/j.jaci.2011.12.970

Kok, K., Geering, B., and Vanhaesebroeck, B. (2009). Regulation of phosphoinositide 3-kinase expression in health and disease. *Trends Biochem. Sci.* 34, 115–127. doi: 10.1016/j.tibs.2009.01.003

Kousha, M., Tadi, R., and Soubani, A. O. (2011). Pulmonary aspergillosis: a clinical review. *Eur. Respir. Rev.* 20, 156–174. doi: 10.1183/09059180.00001011

Lee, K. S., Lee, H. K., Hayflick, J. S., Lee, Y. C., and Puri, K. D. (2006). Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *FASEB J.* 20, 455–465. doi: 10.1096/fj.05-5045com

Lewis, H. D., Liddle, J., Coote, J. E., Atkinson, S. J., Barker, M. D., Bax, B. D., et al. (2015). Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. *Nat. Chem. Biol.* 11, 189–191. doi: 10.1038/nchembio.1735

Li, P., Li, M., Lindberg, M. R., Kennett, M. J., Xiong, N., and Wang, Y. (2010). PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J. Exp. Med.* 207, 1853–1862. doi: 10.1084/jem.20100239

Loures, F. V., Rohm, M., Lee, C. K., Santos, E., Wang, J. P., Specht, C. A., et al. (2015). Recognition of *Aspergillus fumigatus* hyphae by human plasmacytoid dendritic cells is mediated by dectin-2 and results in formation of extracellular traps. *PLoS Pathog.* 11:e1004643. doi: 10.1371/journal.ppat.1004643

Ma, F., Chang, X., Wang, G., Zhou, H., Ma, Z., Lin, H., et al. (2018). *Streptococcus suis* serotype 2 stimulates neutrophil extracellular traps formation via activation of p38 MAPK and ERK1/2. *Front. Immunol.* 9:2854. doi: 10.3389/fimmu.2018.02854

Machida, K., Inoue, H., Matsumoto, K., Tsuda, M., Fukuyama, S., Koto, H., et al. (2005). Activation of PI3K-Akt pathway mediates antiapoptotic effects of beta-adrenergic agonist in airway eosinophils. *Am. J. Physiol. Lung. Cell Mol. Physiol.* 288, L860–L867. doi: 10.1152/ajplung.00131.2004

Makni-Maalej, K., Chiandotto, M., Hurtado-Nedelec, M., Bedouhene, S., Gougerot-Pocidalo, M. A., Dang, P. M., et al. (2013). Zymosan induces NADPH oxidase activation in human neutrophils by inducing the phosphorylation of p47phox and the activation of Rac2: involvement of protein tyrosine kinases, PI3Kinase, PKC, ERK1/2 and p38MAPkinase. *Biochem. Pharmacol.* 85, 92–100. doi: 10.1016/j.bcp.2012.10.010

McCormick, A., Heesemann, L., Wagener, J., Marcos, V., Hartl, D., Loeffler, J., et al. (2010). NETs formed by human neutrophils inhibit growth of the pathogenic mold Aspergillus fumigatus. *Microbes Infect.* 12, 928–936. doi: 10.1016/j.micinf.2010.06.009

Melo, R. C. N., Spencer, L. A., Perez, S. A., Neves, J. S., Bafford, S. P., Morgan, E. S., et al. (2009). Vesicle-mediated secretion of human eosinophil granule-derived major basic protein. *Lab. Invest.* 89, 769–781. doi: 10.1038/labinvest.2009.40

Mocsai, A., Abram, C. L., Jakus, Z., Hu, Y., Lanier, L. L., and Lowell, C. A. (2006). Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat. Immunol.* 7, 1326–1333. doi: 10.1038/ni1407

Mocsai, A., Zhou, M., Meng, F., Tybulewicz, V. L., and Lowell, C. A. (2002). Syk is required for integrin signaling in neutrophils. *Immunity* 16, 547–558.

Morshed, M., Hlushchuk, R., Simon, D., Walls, A. F., Obata-Ninomiya, K., Karasuyama, H., et al. (2014). NADPH oxidase-independent formation of

extracellular DNA traps by basophils. *J. Immunol.* 192, 5314–5323. doi: 10.4049/jimmunol.1303418

Muniz, V. S., Silva, J. C., Braga, Y. A. V., Melo, R. C. N., Ueki, S., Takeda, M., et al. (2018). Eosinophils release extracellular DNA traps in response to *Aspergillus fumigatus*. *J. Allergy Clin. Immunol.* 141, 571.e7–585.e7. doi: 10.1016/j.jaci.2017.07.048

Nakashima, K., Hagiwara, T., and Yamada, M. (2002). Nuclear localization of peptidylarginine deiminase V and histone deimination in granulocytes. *J. Biol. Chem.* 277, 49562–49568. doi: 10.1074/jbc.M208795200

Nashed, B. F., Zhang, T., Al-Alwan, M., Srinivasan, G., Halayko, A. J., Okkenhaug, K., et al. (2007). Role of the phosphoinositide 3-kinase p110delta in generation of type 2 cytokine responses and allergic airway inflammation. *Eur. J. Immunol.* 37, 416–424. doi: 10.1002/eji.200636401

Neeli, I., and Radic, M. (2013). Opposition between PKC isoforms regulates histone deimination and neutrophil extracellular chromatin release. *Front. Immunol.* 4:38. doi: 10.3389/fimmu.2013.00038

Neubert, E., Meyer, D., Rocca, F., Günay, G., Kwaczala-Tessmann, A., Grandke, J., et al. (2018). Chromatin swelling drives neutrophil extracellular trap release. *Nat. Commun.* 9:3767. doi: 10.1038/s41467-018-06263-5

Neves, J. S., Perez, S. A., Spencer, L. A., Melo, R. C.N., Reynolds, L., Ghiran, I., et al. (2008). Eosinophil granules function extracellularly as receptor-mediated secretory organelles. *Proc. Natl. Acad. Sci. U.S.A.* 105, 18478–18483. doi: 10.1073/pnas.0804547105

Papayannopoulos, V., Metzler, K. D., Hakkim, A., and Zychlinsky, A. (2010). Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J. Cell Biol.* 191, 677–691. doi: 10.1083/jcb.201006052

Parker, H., Dragunow, M., Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (2012). Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *J. Leukoc. Biol.* 92, 841–849. doi: 10.1189/jlb.1211601

Ramirez-Ortiz, Z. G., Lee, C. K., Wang, J. P., Boon, L., Specht, C. A., and Levitz, S. M. (2011). A nonredundant role for plasmacytoid dendritic cells in host defense against the human fungal pathogen *Aspergillus fumigatus*. *Cell Host Microbe* 9, 415–424. doi: 10.1016/j.chom.2011.04.007

Rogers, N. C., Slack, E. C., Edwards, A. D., Nolte, M. A., Schulz, O., Schweighoffer, E., et al. (2005). Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* 22, 507–517. doi: 10.1016/j.immuni.2005.03.004

Rohrbach, A. S., Slade, D. J., Thompson, P. R., and Mowen, K. A. (2012). Activation of PAD4 in NET formation. *Front. Immunol.* 3:360. doi: 10.3389/fimmu.2012.00360

Sadhu, C., Masinovsky, B., Dick, K., Sowell, C. G., and Staunton, D. E. (2003). Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. *J. Immunol.* 170, 2647–2654. doi: 10.4049/jimmunol.170.5.2647

Saito, Y., Takeda, M., Nishikawa, J., Konno, Y., Tamaki, M., Itoga, M., et al. (2014). The effect of pharmacological PI3Kgamma inhibitor on eotaxin-induced human eosinophil functions. *Pulm. Pharmacol. Ther.* 27, 164–169. doi: 10.1016/j.pupt.2013.11.006

Schorn, C., Janko, C., Latzko, M., Chaurio, R., Schett, G., and Herrmann, M. (2012). Monosodium urate crystals induce extracellular DNA traps in neutrophils, eosinophils, and basophils but not in mononuclear cells. *Front. Immunol.* 3:277. doi: 10.3389/fimmu.2012.00277

Shah, A., and Panjabi, C. (2002). Allergic bronchopulmonary aspergillosis: a review of a disease with a worldwide distribution. *J. Asthma* 39, 273–289.

Shamri, R., Xenakis, J. J., and Spencer, L. A. (2011). Eosinophils in innate immunity: an evolving story. *Cell Tissue Res.* 343, 57–83. doi: 10.1007/s00441-010-1049-6

Silva, J. C., Rodrigues, N. C., Thompson-Souza, G. A., Muniz, V. S., Neves, J. S., and Figueiredo, R. T. (2019). Mac-1 triggers neutrophil DNA extracellular trap formation to *Aspergillus fumigatus* independently of PAD4 histone citrullination. *J. Leukoc Biol.* 10, 69–83. doi: 10.1002/JLB.4A0119-009RR

Strandmark, J., Rausch, S., and Hartmann, S. (2016). Eosinophils in homeostasis and their contrasting roles during inflammation and helminth infections. *Crit. Rev. Immunol.* 36, 193–238. doi: 10.1615/CritRevImmunol.2016018726

Thompson-Souza, G. A., Santos, G. M. P., Silva, J. C., Muniz, V. S., Braga, Y. A. V., Figueiredo, R. T., et al. (2020). Histoplasma capsulatum-induced extracellular DNA trap release in human neutrophils. *Cell Microbiol.* 22:e13195. doi: 10.1111/cmi.13195

Ueki, S., Hebisawa, A., Kitani, M., Asano, K., and Neves, J. S. (2018). Allergic bronchopulmonary aspergillosis-A luminal hypereosinophilic disease with extracellular trap cell death. *Front. Immunol.* 9:2346. doi: 10.3389/fimmu.2018.02346

Ueki, S., Konno, Y., Takeda, M., Moritoki, Y., Hirokawa, M., Matsuwaki, Y., et al. (2016a). Eosinophil extracellular trap cell death-derived DNA traps: their presence in secretions and functional attributes. *J. Allergy Clin. Immunol.* 137, 258–267. doi: 10.1016/j.jaci.2015.04.041

Ueki, S., Melo, R. C. N., Ghiran, I., Spencer, L. A., Dvorak, A. M., and Weller, P. F. (2013). Eosinophil extracellular DNA trap cell death mediates lytic release of free secretion-competent eosinophil granules in humans. *Blood* 121, 2074–2083. doi: 10.1182/blood-2012-05-432088

Ueki, S., Tokunaga, T., Fujieda, S., Honda, K., Hirokawa, M., Spencer, L. A., et al. (2016b). Eosinophil ETosis and DNA traps: a new look at eosinophilic inflammation. *Curr. Allergy Asthma Rep.* 16:54. doi: 10.1007/s11882-016-0634-5

Van Avondt, K., and Hartl, D. (2018). Mechanisms and disease relevance of neutrophil extracellular trap formation. *Eur. J. Clin. Invest.* 48(Suppl. 2), e12919. doi: 10.1111/eci.12919

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., and Bilanges, B. (2010). The emerging mechanisms of isoform-specific PI3K signalling. *Nat. Rev. Mol. Cell Biol.* 11, 329–341. doi: 10.1038/nrm2882

von Kockritz-Blickwede, M., Goldmann, O., Thulin, P., Heinemann, K., Norrby-Teglund, A., Rohde, M., et al. (2008). Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood* 111, 3070–3080. doi: 10.1182/blood-2007-07-104018

Wang, Y., Li, M., Stadler, S., Correll, S., Li, P., Wang, D., et al. (2009). Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J. Cell Biol.* 184, 205–213. doi: 10.1083/jcb.200806072

Yipp, B. G., Petri, B., Salina, D., Jenne, C. N., Scott, B. N., Zbytnuik, L. D., et al. (2012). Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat. Med.* 18, 1386–1393. doi: 10.1038/nm.2847

Yousefi, S., Gold, J. A., Andina, N., Lee, J. J., Kelly, A. M., Kozlowski, E., et al. (2008). Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nat. Med.* 14, 949–953. doi: 10.1038/nm.1855

Yousefi, S., Mihalache, C., Kozlowski, E., Schmid, I., and Simon, H. U. (2009). Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Diff.* 16, 1438–1444. doi: 10.1038/cdd.2009.96

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 25 February 2021
doi: 10.3389/fmicb.2021.633229

Dependence on Mincle and Dectin-2 Varies With Multiple *Candida* Species During Systemic Infection

*Aiysha Thompson[1,2], Diogo M. da Fonseca[1,3], Louise Walker[4], James S. Griffiths[1,5], Philip R. Taylor[1,2], Neil A. R. Gow[4,6] and Selinda J. Orr[1,3]**

[1] Division of Infection and Immunity, Systems Immunity Research Institute, Cardiff University School of Medicine, Cardiff, United Kingdom, [2] UK Dementia Research Institute, Cardiff, United Kingdom, [3] School of Medicine, Dentistry and Biomedical Science, Wellcome Wolfson Institute for Experimental Medicine, Queen's University Belfast, Belfast, United Kingdom, [4] Aberdeen Fungal Group, University of Aberdeen, Aberdeen, United Kingdom, [5] Faculty of Dentistry, Oral & Craniofacial Sciences, Centre for Host-Microbiome Interactions, King's College London, London, United Kingdom, [6] Medical Research Council Centre for Medical Mycology, University of Exeter, Exeter, United Kingdom

More than 95% of invasive *Candida* infections are caused by four *Candida* spp. (*C. albicans, C. glabrata, C. tropicalis, C. parapsilosis*). C-type lectin-like receptors (CLRs), such as Dectin-1, Dectin-2, and Mincle mediate immune responses to *C. albicans*. Dectin-1 promotes clearance of *C. albicans, C. glabrata, C. tropicalis*, and *C. parapsilosis*, however, dependence on Dectin-1 for specific immune responses varies with the different *Candida* spp. Dectin-2 is important for host immunity to *C. albicans* and *C. glabrata*, and Mincle is important for the immune response to *C. albicans.* However, whether Dectin-2 drives host immunity to *C. tropicalis* or *C. parapsilosis,* and whether Mincle mediates host immunity to *C. glabrata, C. tropicalis* or *C. parapsilosis* is unknown. Therefore, we compared the roles of Dectin-2 and Mincle in response to these four *Candida* spp. We demonstrate that these four *Candida* spp. cell walls have differential mannan contents. Mincle and Dectin-2 play a key role in regulating cytokine production in response to these four *Candida* spp. and Dectin-2 is also important for clearance of all four *Candida* spp. during systemic infection. However, Mincle was only important for clearance of *C. tropicalis* during systemic infection. Our data indicate that multiple *Candida* spp. have different mannan contents, and dependence on the mannan-detecting CLRs, Mincle, and Dectin-2 varies between different *Candida* spp. during systemic infection.

Keywords: Mincle, Dectin-2, *Candida*, CLR, fungal

OPEN ACCESS

Edited by:
David L. Moyes, King's College London, United Kingdom

Reviewed by:
Todd B. Reynolds, The University of Tennessee, Knoxville, United States
Teresa Zelante, University of Perugia, Italy

***Correspondence:**
Selinda J. Orr
S.Orr@qub.ac.uk

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *24 November 2020*
Accepted: *08 February 2021*
Published: *25 February 2021*

Citation:
Thompson A, da Fonseca DM, Walker L, Griffiths JS, Taylor PR, Gow NAR and Orr SJ (2021) Dependence on Mincle and Dectin-2 Varies With Multiple Candida Species During Systemic Infection. Front. Microbiol. 12:633229. doi: 10.3389/fmicb.2021.633229

INTRODUCTION

Candida spp. are the second most common agents of human fungal infection after dermatophytes (Brown et al., 2012). The vast majority of *Candida* infections (candidiasis) are superficial and pose no serious threat to immunocompetent individuals, however, recurrent mucosal infections impart a significant and widespread morbidity affecting more than 100 million women each year (Kullberg and Arendrup, 2015; Denning et al., 2018). Although invasive candidiasis is much less frequent

than other superficial mycoses, it is associated with high mortality rates (Yapar, 2014; Mccarty and Pappas, 2016; Pappas et al., 2018). Indeed, invasive candidiasis is a worrying health concern as it is estimated to affect 250,000 people every year with associated mortality ranging from 46 to 75% (Brown et al., 2012; Bongomin et al., 2017), This high mortality stems mainly from the fact that current antifungal therapies for invasive candidiasis are limited, often administered late in the course of infection, and because the patients are often suffering from one or more predisposing factors (Zaoutis et al., 2005; Lockhart, 2014; Pappas et al., 2018; Sam et al., 2018). Immunotherapy as a potential treatment for invasive candidiasis has yet to be developed, however, this would require an in-depth understanding of the anti-fungal immune response (Segal et al., 2006; van de Veerdonk et al., 2010; Sam et al., 2018).

C. albicans is the most virulent and frequently isolated pathogen from patients with invasive candidiasis, accounting for 50–70% of all infections (Pfaller and Diekema, 2007; Guinea, 2014), however, invasive infections caused by other non-*albicans Candida* spp., such as *C. parapsilosis*, *C. glabrata*, and *C. tropicalis* and recently *C. auris* have been rising over the past decades (Arendrup et al., 2002; Yapar, 2014; Lamoth et al., 2018), and pose an emerging health concern. Nevertheless, most research tends to focus on *C. albicans* as a model pathogen of candidiasis, with much less research on other clinically relevant *Candida* spp.

Innate immune cells deploy several molecular mechanisms to control *Candida* proliferation and dissemination into host tissues, and to initiate adaptive immunity in order to ultimately curtail the infection. Innate phagocytes, such as macrophages and dendritic cells (DCs) recognize *Candida* spp. via an array of pattern-recognition receptors (PRRs), such as C-type lectin-like receptors (CLRs) and Toll-like receptors (TLRs) (Patin et al., 2019). These receptors sense pathogen-associated molecular patterns (PAMPs) mainly present in the fungal cell wall, the outermost structure of the *Candida* cell. The CLR, Dectin-1 was long identified as the main fungal β-(1,3)-glucan-binding PRR (Brown et al., 2002; Taylor et al., 2007), and was shown to be involved in the activation of protective immune mechanisms against *C. albicans* and *C. glabrata* via phagocytosis, the respiratory burst, neutrophil extracellular traps release, cytokine/chemokine secretion, recruitment of inflammatory cells and T cell response activation (Taylor et al., 2007; Hardison and Brown, 2012; Branzk et al., 2014; Chen et al., 2017). Dectin-1 has been shown to contribute to host resistance against multiple forms of *C. albicans*-driven candidiasis (Ferwerda et al., 2009; Plantinga et al., 2009, 2010), however, we recently showed that the requirement for Dectin-1 for specific immune responses to *Candida* is species-dependent. We found that Dectin-1 abrogation significantly increases mortality in mice during *C. albicans*-induced systemic candidiasis but does not affect survival following infection with *C. glabrata*, *C. tropicalis* or *C. parapsilosis* (Thompson et al., 2019). However, fungal burden is increased in Dectin-1 KO mice following infection with all four *Candida* spp. (Thompson et al., 2019).

The outer cell wall of *Candida* is dominated by a layer of highly mannosylated cell wall proteins for which the mannan component determines the majority of the molecular mass. Mannans represent a shield that hides the inner glucan layer from recognition by Dectin-1, whilst innate cells recognize a range of mannans in the outer cell wall via a number of CLRs (Gow et al., 2011; Yadav et al., 2020). Mannans can be linked to cell wall proteins via amide linkages (*N*-mannan) or ether linkages [*O*-mannan or via phosphodiester linkages to *N*- or *O*-mannan (phosphomannan)] (Netea et al., 2006; Hall and Gow, 2013). Therefore, host cells rely on various mannan-binding receptors for their recognition including the mannose receptor, DC-SIGN, Galectin-3, Dectin-2, and Mincle. The ligands for these mannan-binding CLRs can either be superficial or buried within the inner cell wall (Vendele et al., 2020). Dectin-2 was shown to bind mannose-rich structures present in the *Candida* cell wall (McGreal et al., 2006; Sato et al., 2006), and to contribute to host protection during systemic infections with *C. albicans* and *C. glabrata* (Ifrim et al., 2014, 2016; Haider et al., 2019; Thompson et al., 2019). In *C. albicans* infections, Dectin-2 was shown to contribute toward the generation of protective Th1 and Th17 responses mainly by promoting production of IL-12p40, IL-1β, and IL-23 by DCs (Robinson et al., 2009; Saijo et al., 2010). Mincle can bind α-mannose residues present in fungal cell wall mannans (Taylor et al., 2005) and also acts as a death sensor by recognizing SAP130, an alarmin released by dying host cells that induces neutrophil infiltration into damaged tissues (Yamasaki et al., 2008). Although it was shown to have a redundant role in *C. albicans* phagocytosis by macrophages, Mincle is recruited to macrophage phagocytic synapse sites, and was shown to be involved in TNF production (Wells et al., 2008). Dependence on Mincle for resistance against *C. albicans*-driven systemic candidiasis appears to be strain-specific, as Mincle was reported to be necessary for controlling *C. albicans* 3630 clearance in the kidneys (Wells et al., 2008), but we recently showed that it plays a redundant role in conferring resistance toward *C. albicans* SC5314 (Thompson et al., 2019). Indeed, other authors have shown that Mincle does not bind multiple *Candida* spp., including *C. albicans* (Yamasaki et al., 2008), making it difficult to ascertain its importance for host protection during candidiasis.

Here we compare the cell wall composition of the four main *Candida* spp. responsible for the vast majority of invasive candidiasis *(C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*) and determine the role of Mincle and Dectin-2 in response to these different pathogens in a systemic model of infection.

MATERIALS AND METHODS

Mice

C57BL/6J (WT), Mincle KO (*Clec4e*$^{-/-}$), and Dectin-2 KO (*Clec4n*$^{-/-}$) mice were maintained and handled according to institutional and U.K. Home Office guidelines. Animals used were age- and gender-matched. In accordance with the 3Rs to reduce mouse numbers, wherever possible the same WT mice were used as controls for multiple KO mice strains in concurrent experiments. Where the same controls have been used for different figures, this is highlighted in the figure legend.

Ethics Statement

All *in vivo* experimental procedures were approved by and performed in strict accordance to both Cardiff University's Animal Welfare and Ethical Review Body and the U.K. Home Office. Animal care and use adhered to the Animals (Scientific Procedures) Act 1986.

Reagents

IFN-γ and IL-17 ELISAs were obtained from R&D. IL-1β, IL-6, IL-10, IL-12p40, and TNF ELISAs were obtained from Life Technologies. M-CSF and GM-CSF used for bone marrow cell differentiation were obtained from Peprotech. Greiss reagent was purchased from Sigma.

Preparation of *Candida* Cultures

Candida albicans SC5314 reference strain was obtained from ATCC. The clinical isolates *Candida glabrata* SCS74761, *Candida tropicalis* SCS74663, *Candida parapsilosis* SCSB588, were kindly donated by Dr. Donna MacCallum (University of Aberdeen). *Candida* spp. were propagated on YPD agar plates overnight at 30°C, then grown in YPD broth for ∼16 h at 30°C in a shaking incubator. *Candida* spp. were washed three times with PBS with centrifugation at 350 × g, for 5 min, and then resuspended at the appropriate concentration for experimental assays.

Candida Preparation for TEM, Cell Wall Thickness, Mannan Fibril Length, and Alcian Blue Assay

High-pressure freezing–freeze substitution transmission electron microscopy (TEM) of *Candida* spp. was performed as previously described (Walker et al., 2018). Briefly, cells were snap-frozen in liquid nitrogen at high pressure using a Leica Empact high-pressure freezer (Leica, Milton Keynes, United Kingdom) and fixed using a Leica AFS freeze substitution system. Samples were then processed in a Lynx tissue processor and embedded in TAAB812 (TAAB Laboratories, Aldermaston, United Kingdom) epoxy resin. One-hundred-nanometer sections were cut with a Leica Ultracut E microtome and stained with uranyl acetate and lead citrate. Samples were imaged with a JEOL 1400 plus transmission microscope (JEOL UK Ltd., Hertfordshire, United Kingdom) and imaging with an AMT UltraVUE camera (Deben, Suffolk, United Kingdom). Five measurements for cell wall thickness and mannan fibril length were taken per cell and 15 cells were measured for each *Candida* spp. Alcian Blue binding assay was used to determine the phosphomannan content of each *Candida* spp., as previously described (Hobson et al., 2004). Briefly, exponentially grown cells from each *Candida* spp. were collected, washed twice with milliQ water and diluted 1 in 10. Cell pellets were resuspended in 1 ml of 30 μg/ml Alcian Blue and incubated at room temperature for 10 min. The cell pellet was then collected and the OD 620_{nm} of the supernatant was measured to determine the content of Alcian Blue. The concentration of Alcian Blue bound to cells of each *Candida* spp. was then calculated.

Cell Culture

Femurs and tibiae of mice were flushed with PBS to harvest bone marrow. Bone marrow cells were cultured for 6 days in DMEM supplemented with 10% heat inactivated fetal bovine serum, 5% heat inactivated horse serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 10 mM HEPES and 10 ng/ml M-CSF to generate bone marrow-derived macrophages (BMDMs) (Patin et al., 2016). Bone marrow cells were cultured for 8 days in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 10 mM HEPES, 1% NEAA, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol and 10 ng/ml GM-CSF to generate bone marrow-derived dendritic cells (BMDCs).

Cell Stimulations and Cytokine Assays

Differentiated BMDMs were harvested using 8 mg/ml lidocaine, washed and resuspended in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum and 100 U/ml penicillin/streptomycin. BMDMs/BMDCs were plated in 96-well plates at a density of 1×10^5 cells/well and incubated overnight at 37°C. Media was removed the following day, and cells were stimulated with 1×10^5 *Candida* CFUs/well for 24 h in a final volume of 200 μl fresh media. 2.5 μg/ml amphotericin B was added 2 h after stimulation. Cell culture supernatants were harvested and cytokine levels were measured by ELISA assays, according to the manufacturers' protocols. Nitric oxide production was detected using the Griess test (Sun et al., 2003). Briefly, following *in vitro* stimulation of host cells with *Candida* spp., cell culture supernatants were harvested and incubated with equal volumes of Griess reagent for 10 min. Absorbance was read at 540_{nm} using a Multiskan Spectrum plate reader (Thermo Fisher Scientific) and nitrite levels in samples were calculated from a standard curve generated using a serially diluted sodium nitrite solution.

In vivo Candida spp. Infections

Candida spp. was suspended in PBS and intravenously injected in mice in a volume of 100 μl. The *Candida* spp. dose administered varied between different experiments (outlined in figure legends). Mice were weighed daily and examined twice a day by using a predefined scoring system, with 20% body weight loss set as an additional humane endpoint. Mice were culled by CO_2 asphyxiation and death was confirmed by posterior cervical dislocation. At the end of each experiment blood was collected by cardiac puncture and kidneys, brains and spleens were harvested as previously described (Patin et al., 2016). Serum was extracted from blood by centrifugation at 10,000 rpm for 10 min at 4°C in serum tubes and used in ELISA assays for cytokine detection. The left kidney and right brain were homogenized in 1 mL PBS, homogenates were serially diluted and spotted on petri dishes containing 50 μg/ml chloramphenicol in YPD and incubated for 24–48 h at 30°C. Colonies were counted and fungal burden was calculated as CFUs/g of organ. The spleen was homogenized and erythrocytes were lysed using ACK lysis buffer. Splenocytes were washed by centrifugation and resuspended in IMDM supplemented with 10% heat inactivated fetal bovine

serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 50 μM β-mercaptoethanol. Cells were plated at 1×10^6 cells/well and left unstimulated or stimulated with 2×10^6 CFUs/well *Candida* spp. for 48 h at 37°C. 2.5 μg/ml amphotericin B was added 2 h after stimulation. After 48 h, supernatants were collected and used for IFN-γ and IL-17 ELISA assays.

Statistical Methods

Data were analyzed using GraphPad Prism. For statistical analysis of two groups, Student's *t* test was performed, and for three or more groups, One-way ANOVA with Bonferonni's post-test or Two-way ANOVA with Bonferonni's post-test was used. Datasets were transformed by Y = sqrt(Y + 0.5) when data did not follow a Gaussian distribution and analyzed by either ANOVA or Student's *t*-test, or non-parametric tests if normality was not achieved after transformation. One sample *t*-test was used if all the samples were zero in one group. Statistical significance was considered to be achieved when *p*-values were less than 0.05: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. All data are presented as means ± SEM.

RESULTS

Candida spp. Display Differences in Their Cell Wall

We compared the cell wall structure of *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* by TEM of high-pressure frozen *Candida*. We observed significant differences in cell wall thickness of the inner wall in the following descending order: *C. albicans, C. parapsilosis, C. glabrata, C. tropicalis* (**Figures 1A,B**). Mannosylated protein fibrils extend out from the inner layer in the TEM images. The *Candida* spp. displayed decreasing mannan fibril length in the following order: *C. albicans, C. parapsilosis = C. tropicalis, C. glabrata* (**Figures 1A,C**). We then analyzed the cell wall phosphomannan content using Alcian Blue binding. The *Candida* spp. displayed decreasing phosphomannan content in the following order: *C. albicans, C. tropicalis = C. parapsilosis, C. glabrata* (**Figure 1D**). The significant differences in the *Candida* cell walls particularly in length/type of mannan structures present may affect the immune response mediated by CLRs, such as Dectin-2 and Mincle in response to these different clinically relevant *Candida* spp.

Mincle and Dectin-2 Regulate Cytokine Responses to Multiple *Candida* spp. in BMDCs

Mincle has been shown to mediate TNF production from BMDMs in response to *C. albicans* (Wells et al., 2008) while Dectin-2 has been shown to drive cytokine production from BMDCs in response to *C. albicans* mannans (Saijo et al., 2010) and from macrophages during systemic infection with *C. glabrata* (Ifrim et al., 2014, 2016). Here, we investigated the role of Mincle and Dectin-2 during cytokine production from BMDMs and BMDCs in response to four *Candida* spp. To investigate this, BMDMs and BMDCs from WT and Mincle KO or WT and Dectin-2 KO mice were stimulated with *C. albicans* SC5314, *C. glabrata* SCS74761, *C. tropicalis* SCS74663, or *C. parapsilosis* SCSB5882. We did not observe any major roles for Mincle (**Supplementary Figures 1A,C**) or Dectin-2 (**Supplementary Figures 1B,C**) in regulating cytokine production or nitric oxide production in response to these four *Candida* spp. This is largely unsurprising as BMDMs do not basally express either Mincle or Dectin-2 (**Supplementary Figure 2**). However, both Mincle and Dectin-2 regulate cytokine production in response to multiple *Candida* spp. in BMDCs. Mincle mediates IL-12p40 production and inhibits IL-1β production in response to multiple *Candida* spp. (**Figure 2A**). While Dectin-2 promotes IL-12p40 and IL-10 production in response to multiple *Candida* spp. (**Figure 2B**). Neither CLR is required for nitric oxide production from BMDCs (**Figure 2C**). Therefore, Mincle and Dectin-2 regulate cytokine production from BMDCs in response to multiple *Candida* spp. but they are largely uninvolved in cytokine production from BMDMs.

In vivo Clearance of *C. tropicalis* Is Partially Dependent on Mincle

As Mincle regulates cytokine production in response to multiple *Candida* spp. we next sought to determine whether Mincle plays any roles during systemic infection with these different *Candida* spp. To this end, WT and Mincle KO mice were systemically infected with *C. albicans*, *C. glabrata*, *C. tropicalis*, or *C. parapsilosis*.

The infection doses used were previously determined (Thompson et al., 2019). One-week post-infection, Mincle KO mice showed reduced presence of *C. glabrata* within the brain and decreased clearance of *C. tropicalis* from the brain compared to WT mice (**Figure 3A**). The WT and Mincle KO mice did not display any other major differences in the early clearance of these four *Candida* spp. from the main target organs. Mincle KO mice also displayed a subtle elevation in serum IL-6 levels 1-week post infection with *C. tropicalis* likely due to the increased fungal burden in these mice (**Figure 3B**). We did not observe any other major differences in serum cytokine levels between WT and Mincle KO mice following infection with these *Candida* spp. In addition, we tested the ability of WT and Mincle KO splenocytes to produce IFN-γ and IL-17 upon antigen restimulation with live *Candida* spp. in the presence of amphotericin B. We did not observe any major differences in the ability of these splenocytes to produce IFN-γ or IL-17. Finally, we examined the requirement for Mincle for controlling systemic infection with these *Candida* spp. over time. Mincle was largely unimportant for controlling and/or clearing systemic infection with these different *Candida* spp. However, similar to the early clearance of *C. tropicalis*, Mincle once again played a role in the clearance of *C. tropicalis* over time as the kidneys in Mincle KO mice displayed increased fungal burden compared to WT mice after 21 days (**Figures 4A,B**). Mincle is therefore relatively unimportant for controlling systemic *Candida* infections with the specific strains used in this study, however, it plays a role during systemic infection with *C. tropicalis*.

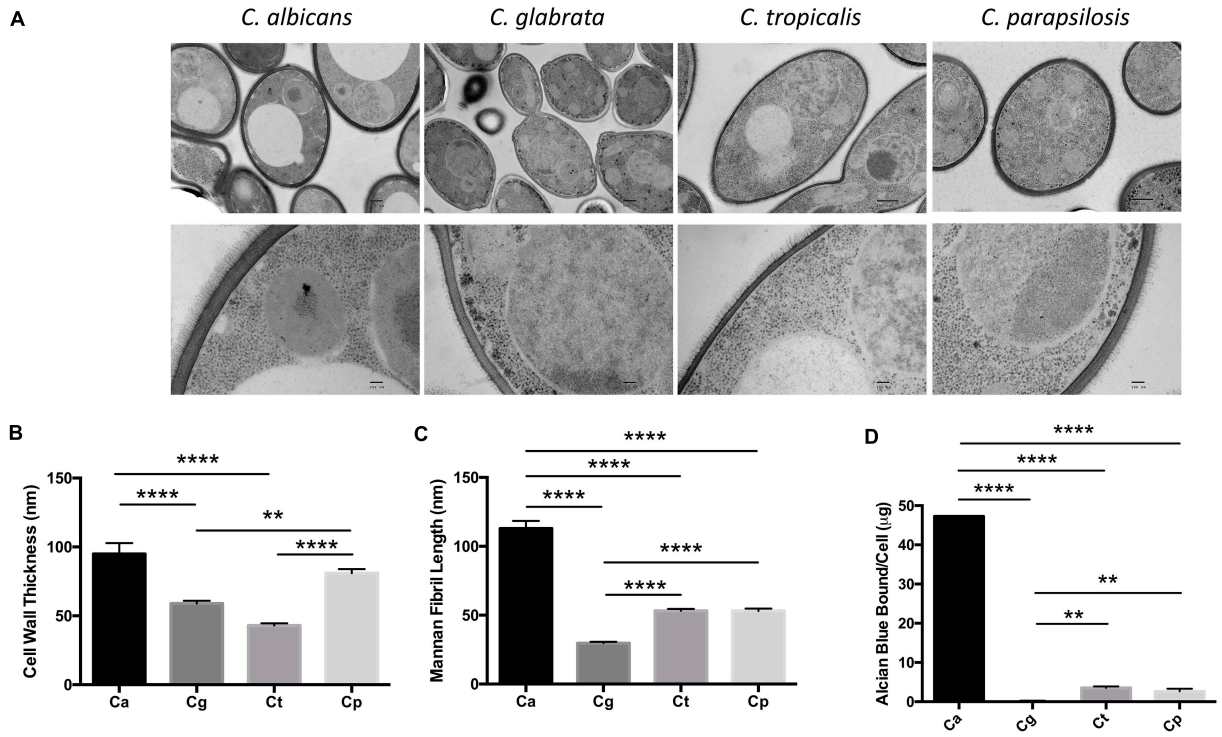

FIGURE 1 | *Candida* spp. yeast display differences in their cell wall. **(A)** Electron micrograph images showing the ultrastructure of *Candida* cells taken at 4,000× (upper panel) and 30,000× (lower panel). Scale bars are 500 nm (upper panel) and 100 nm (lower panel). Images are representative of three separate preparations. **(B,C)** Cell wall thickness **(B)** and mannan fibril length **(C)** were measured from the TEM images. Graphs show the mean ± SEM from 15 *Candida* cells (1-way ANOVA, Bonferroni's post-test). **(D)** *Candida* spp. were incubated in 30 μg/ml Alcian Blue for 10 min and the level of dye associated with the cell wall was measured by absorbance. Graph displays the mean amount of dye bound per cell ± SEM from three independent experiments (One-way ANOVA with Bonferroni's post-test). $^{**}p < 0.01$, $^{****}p < 0.0001$.

Dectin-2 Is Important for *in vivo* Clearance of Multiple *Candida* spp.

As Dectin-2 mediates cytokine production in response to multiple *Candida* spp. and it has been shown to play a role during infections with *C. albicans* (Saijo et al., 2010) and *C. glabrata* (Ifrim et al., 2014, 2016), we next analyzed the role for Dectin-2 at different timepoints during systemic infection with these four clinically relevant *Candida* spp. To this end, WT and Dectin-2 KO mice were systemically infected with *C. albicans*, *C. glabrata*, *C. tropicalis*, or *C. parapsilosis*. One-week post-infection, Dectin-2 KO mice showed decreased clearance of *C. parapsilosis* from the brain compared to WT mice (**Figure 5A**). The WT and Dectin-2 KO mice did not display any other major differences in the early clearance of these four *Candida* spp. from the main target organs. We did not observe any major differences in serum cytokine levels between WT and Dectin-2 KO mice following infection with these *Candida* spp., however, Dectin-2 KO mice displayed a subtle elevation in serum IL-6 levels 1-week post infection with *C. albicans* or *C. tropicalis* (**Figure 5B**). Dectin-2 KO splenocytes produced more IFN-γ upon antigen restimulation with live *C. albicans* and *C. glabrata* and increased IL-17 production following restimulation with *C. albicans* (**Figure 5C**). Finally, we examined the requirement for Dectin-2 for controlling systemic infection with these *Candida* spp. over time. Dectin-2 was important for clearance of all four *Candida* spp. (**Figures 6A,B**). Thus, Dectin-2 is important during early infection with *C. parapsilosis* and at later timepoints for controlling systemic *Candida* infections with all four *Candida* spp.

DISCUSSION

In this study, we demonstrated that the cell wall thickness and mannan composition varied between *C. albicans* and three other clinically relevant, non-*albicans* species. We found that Mincle and Dectin-2 are involved in regulating cytokine production in response to all four *Candida* spp. However, in an *in vivo* model of systemic infection, we showed that Mincle is involved in clearance of *C. tropicalis* while Dectin-2 is involved in the clearance of all four *Candida* spp. Overall, we found that both Mincle and Dectin-2 play roles in response to different *Candida* spp. however this is host cell-, species- and likely strain- and site of infection-dependent.

As Mincle and Dectin-2 bind mannose/mannans in fungal cell walls, we hypothesized that differences in the cell wall mannan content between these four clinically relevant *Candida* spp. would determine whether Mincle or Dectin-2 are involved in the immune response to the different *Candida* spp. Mincle

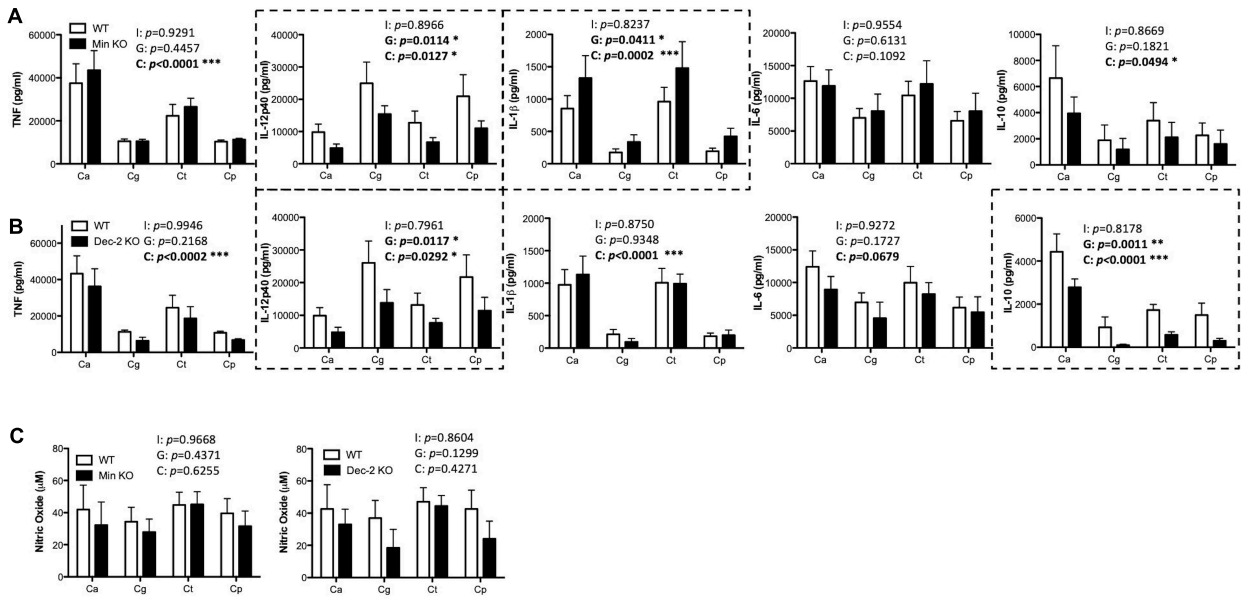

FIGURE 2 | Mincle and Dectin-2 regulate cytokine responses to multiple *Candida* spp. in BMDCs. **(A–C)** BMDCs from WT and Mincle KO mice **(A,C)** or from WT and Dectin-2 KO mice **(B,C)** were stimulated with *C. albicans* SC5314 (Ca), *C. glabrata* SCS74761 (Cg), *C. tropicalis* SCS74663 (Ct), and *C. parapsilosis* SCSB5882 (Cp) at a multiplicity of infection (MOI) of 1:1 (*Candida* sp.: BMDCs). Cytokine levels **(A,B)** and nitric oxide levels **(C)** in the supernatants were measured after 24 h incubation. Results are presented as means ± SEM of 4 **(A)**, 3–4 **(B)**, or 3 **(C)** independent experiments (Two-way ANOVA with Bonferroni's post-test). I = Interaction between "Genotype" and "*Candida*" variables, G (Genotype) = impact of BMDC genotype (WT vs. Mincle KO; WT vs. Dectin-2 KO), C (*Candida*) = impact of different *Candida* spp. tested (*albicans, glabrata, tropicalis, parapsilosis*). Graphs where Genotype is significant have been surrounded by a dashed box. **(B)** WT data is the same as in **(A)** for three out of three replicates for TNF, three out of four replicates for IL-12p40, IL-1β, and IL-6, and two out of three replicates for IL-10. **(C)** WT data in the right graph is the same as in the left graph for two out of three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

has been shown to bind mannose (Lee et al., 2011) and Dectin-2 has been shown to bind high mannose structures (e.g., $Man_9GlcNAc_2$) (McGreal et al., 2006). Therefore, we compared the cell wall mannan content of these four clinically relevant *Candida* spp. Interestingly, we found large differences in the mannan fibril length and phosphomannan content. This would indicate significant differences in the N-glycan structures on the surface of these four *Candida* spp. In agreement with our data, Nguyen et al. (2018) found that the molecular weights of *Candida* mannans decreased in the following order: *C. albicans, C. tropicalis, C. glabrata*. The predicted structures of these mannans differed substantially with much larger and more complex *C. albicans* mannans compared to the other *Candida* spp. (Nguyen et al., 2018). In addition, Shibata et al. (2007) showed that the mannan structure changed as *C. albicans* yeast transformed to hyphae. Moreover, several factors like different morphogenetic and morphological stages of *Candida* spp., the physicochemical properties of the substrate, such as the carbon source, pH, oxygen levels, metal ion micronutrients and temperature, and also certain environmental stresses, such as the presence of different antifungal drugs were shown to alter carbohydrate synthesis and orientation in the fungal cell wall (Ballou et al., 2016; Pradhan et al., 2019; Vendele et al., 2020). Therefore, various factors will likely determine whether specific PRRs, such as Mincle and Dectin-2 are important for immune responses to these different *Candida* spp. These include: differences in the mannan content of the four *Candida* spp. when grown under the same conditions; the complexity of the mannans on the fungal cell wall at yeast, and likely hyphal stages; and the fact that some of these species do not form hyphae.

In this study, we next assessed whether Mincle or Dectin-2 influenced cytokine release and nitric oxide production in response to these four *Candida* spp. In BMDCs, we found that Mincle mediates IL-12p40 production and regulates IL-1β production, while Dectin-2 drives production of IL-12p40 and IL-10 upon *Candida* spp. stimulation. Wells et al. (2008) reported that Mincle was involved in TNF production from macrophages following stimulation with *C. albicans* 3630. We did not observe any defect in TNF production in Mincle KO BMDMs or BMDCs. The difference between our results and those previously published by Wells et al. (2008) could be due to the timepoints (1–2 h vs. 24 h) or the strains of *C. albicans* (3630 vs. SC5314) used in the experiments. Dectin-2 was previously shown to be important for production of IL-12p40, TNF, IL-1β, and IL-10 from BMDCs in response to *C. albicans* mannans (Saijo et al., 2010). In addition, naïve Dectin-2 KO peritoneal macrophages generally produced lower levels of TNF, IL-6, KC, IL-1α, and IL-1β compared to WT controls in response to *C. albicans* (Ifrim et al., 2016). However, cytokine production from naïve Dectin-2 KO peritoneal macrophages was similar to WT cells in response to *C. glabrata* (Ifrim et al., 2014). The different results could be due to the use of different cell types and whole *C. albicans* vs. *C. albicans* mannans. The altered IL-12p40 and IL-1β levels that we observed in Mincle KO BMDCs and the attenuated IL-12p40

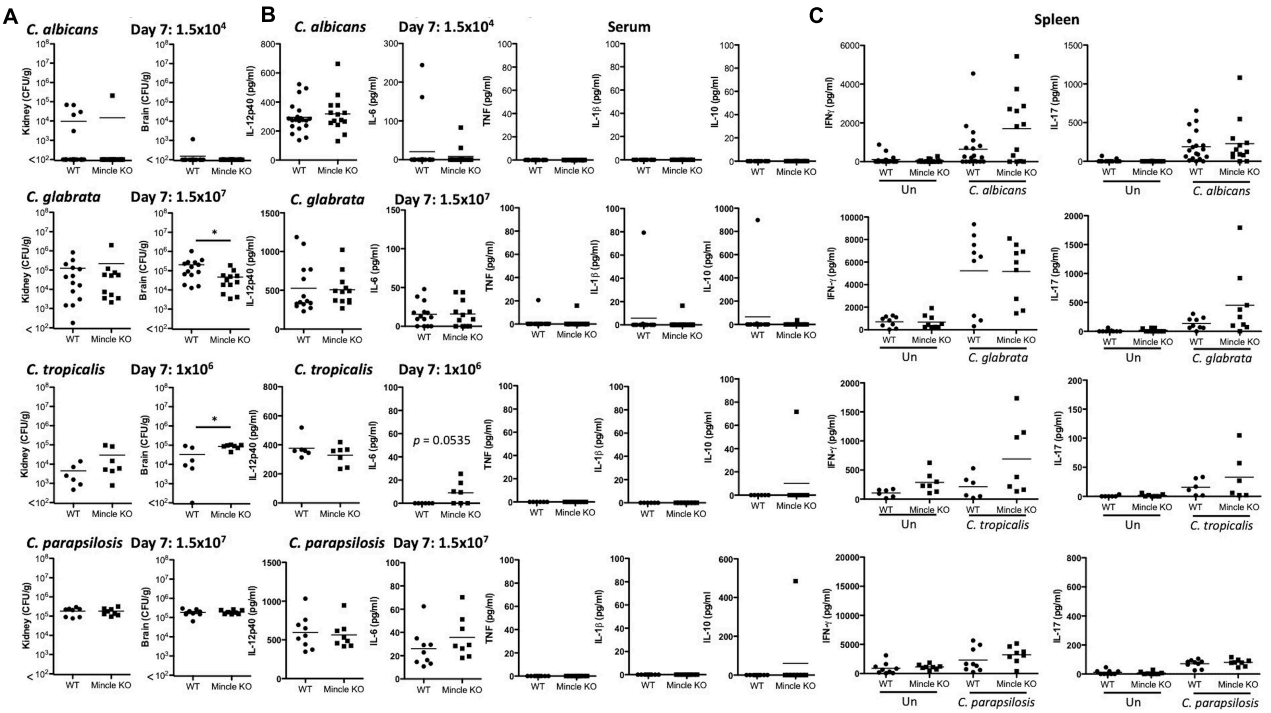

FIGURE 3 | Early clearance of *C. tropicalis* is partially dependent on Mincle. **(A–C)** WT and Mincle KO mice were intravenously infected with the indicated doses of *C. albicans*, *C. glabrata*, *C. tropicalis*, or *C. parapsilosis* for 7 days. **(A)** CFU in the kidneys and brains of mice at 7 days post-infection were determined. Mann-Whitney test (*C. albicans* kidney on transformed data; *C. glabrata* kidney and brain), One sample *t*-test (*C. albicans* brain), Student's *t*-test (*C. tropicalis* kidney; *C. tropicalis* brain on transformed data; *C. parapsilosis* kidney and brain). **(B)** Cytokine levels in the serum of mice at 7 days post-infection. Student's *t*-test (*C. albicans* IL-12p40 on transformed data; *C. glabrata* IL-12p40 and IL-6 on transformed data; *C. tropicalis* IL-12p40; C. *parapsilosis* IL-12p40 and IL-6 on transformed data), Mann-Whitney test (*C. albicans* IL-6 on transformed data; *C. glabrata* TNF, IL-1β, and IL-10 on transformed data), One sample *t*-test (*C. tropicalis* IL-6 and IL-10; C. *parapsilosis* IL-10). **(C)** Splenocytes from infected mice were unstimulated or restimulated with live *Candida* spp. for 48 h and IFN-γ and IL-17 levels were measured by ELISA. Kruskal Wallis with Dunn's post-test (*C. albicans* IFN-γ and IL-17 on transformed data; *C. glabrata* IL-17 on transformed data; *C. parapsilosis* IFN-γ; *C. parapsilosis* IL-17 on transformed data), One-way ANOVA with Bonferroni's post-test (*C. glabrata* IFN-γ; *C. tropicalis* IFN-γ; *C. tropicalis* IL-17 on transformed data). **(A–C)** Graphs are the cumulative result of 2–4 independent experiments. Each symbol represents an individual mouse. Fifteen out of twenty WT mice in the *C. albicans* graphs, and five out of nine WT mice in the *C. parapsilosis* graphs are the same as **Figure 4** in a previous publication from our group (Thompson et al., 2019). $^*p < 0.05$.

and IL-10 levels in Dectin-2 KO BMDCs would suggest that despite significant differences in the mannan levels/structures on the different *Candida* spp. that ligands for both these CLRs are present on *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*. While we observed roles for Mincle and Dectin-2 in mediating/regulating cytokine production from BMDCs, this may differ for myeloid cell populations in target organs, such as the kidney or spleen.

As Mincle regulated cytokine production *in vitro* in response to these four *Candida* spp., we then assessed whether Mincle had any impact during systemic infection. We did not observe any major effect of Mincle deficiency on the clearance of *C. albicans* or *C. parapsilosis* either at early or late timepoints. This is in agreement with our previous findings (Thompson et al., 2019), while Wells et al. (2008) observed increased fungal burden in Mincle KO mice 5 days after systemic infection with *C. albicans*. The *C. albicans* strain, time point and dose differed between our studies and that by Wells et al. (2008). In our study, Mincle KO mice displayed slightly enhanced early clearance of *C. glabrata* from the brain, however, fungal burden was similar to WT at later timepoints and cytokine production was unaltered suggesting a minimal role. Interestingly, we found that although Mincle does not significantly influence survival, it plays a role in the clearance of *C. tropicalis* from the brain and kidneys of systemically infected mice at early and late timepoints. In previous studies with *C. albicans*, we have observed elevated IL-6 levels in the serum of mice with higher fungal burdens, and our data with Mincle KO mice suggest a similar trend during infection with *C. tropicalis* (Orr et al., 2013; Thompson et al., 2019). While we identified a role for Mincle in regulating cytokine production from all four *Candida* spp. *in vitro*, we only observed a major role for Mincle in regulating the clearance of *C. tropicalis in vivo*. Based on our findings we cannot rule out other roles for Mincle *in vivo* in response to these four *Candida* spp., however, our data indicate that while a ligand for Mincle may be present on all four *Candida* spp. tested, Mincle is only important for the clearance of *C. tropicalis* during systemic infection.

Similar to our experiments with Mincle, we then assessed whether Dectin-2 had any impact during systemic infection with all four *Candida* spp. We found that Dectin-2 is

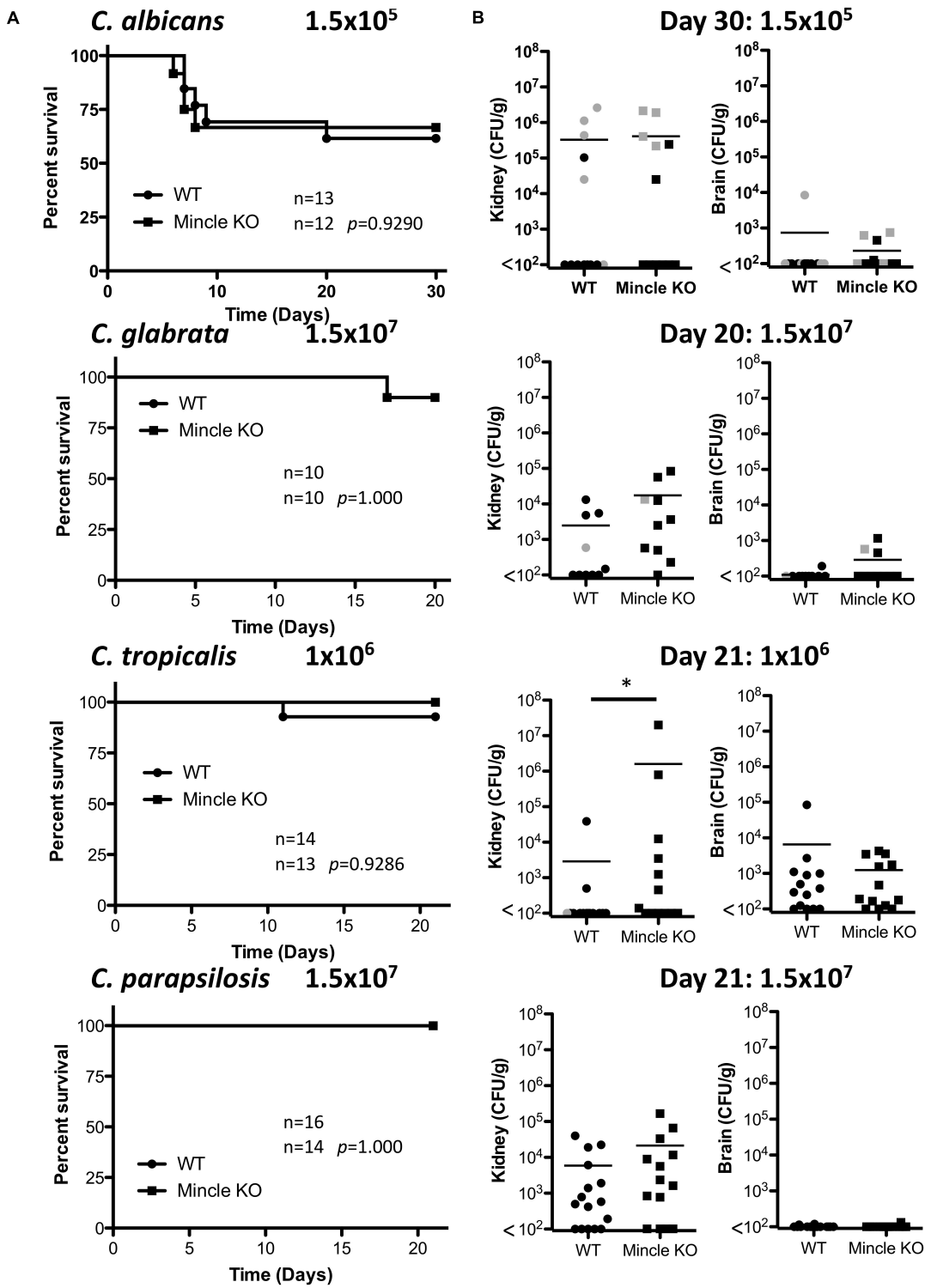

FIGURE 4 | Clearance of *C. tropicalis* is partially dependent on Mincle. **(A)** WT and Mincle KO mice were intravenously infected with the indicated doses of *C. albicans* for 30 days, *C. glabrata* for 20 days, *C. tropicalis* for 21 days, or *C. parapsilosis* for 21 days. Survival curves based on humane end-point of infected WT (filled circles) and Mincle KO mice (filled squares). Graphs are the cumulative result of 2–3 independent experiments. Log-rank test. **(B)** CFU in the kidneys of WT and Mincle KO mice at 20–30 days after infection (black symbols) or at time of death by humane end point (gray symbols). Graphs are the cumulative result of 2–3 independent experiments. Each symbol represents an individual mouse. Mann-Whitney test on transformed data (*C. albicans* kidney and brain; *C. glabrata* brain; *C. tropicalis* kidney and brain; *C. parapsilosis* kidney and brain), Student's *t*-test on transformed data (*C. glabrata* kidney). Four out of fourteen WT mice in the *C. tropicalis* graphs are the same as **Figure 3** of a previous publication from our group (Thompson et al., 2019). $^*p < 0.05$.

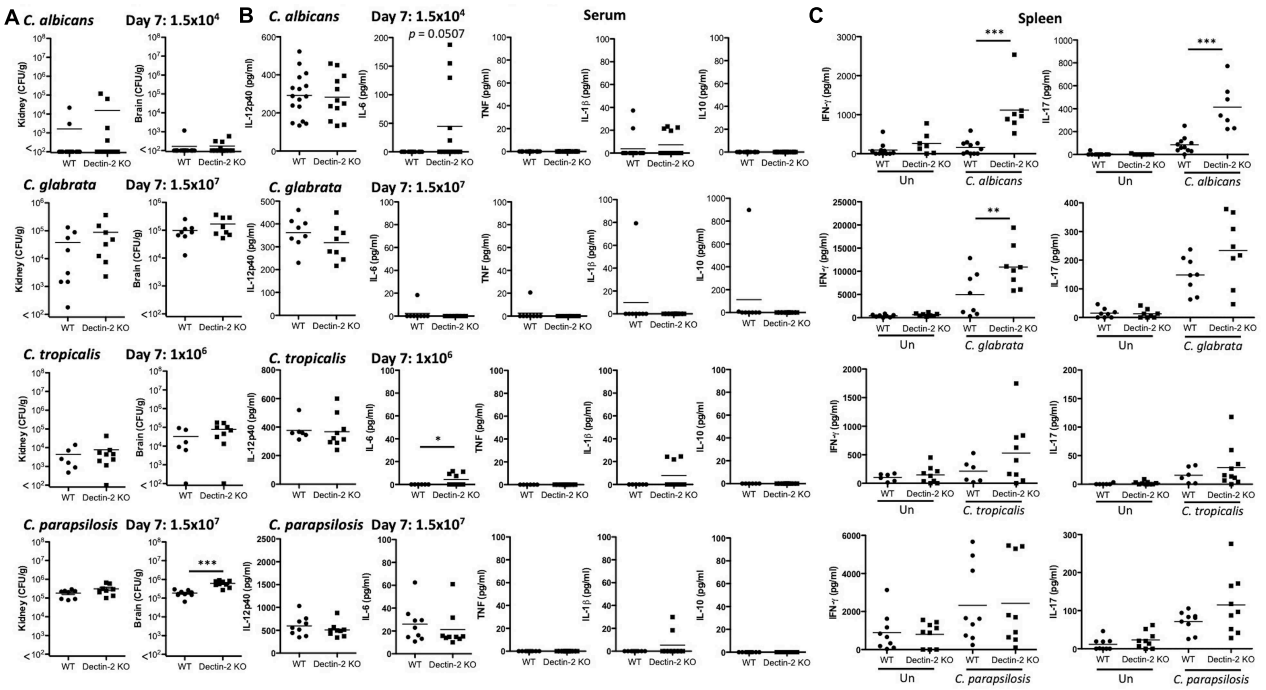

FIGURE 5 | Early clearance of *C. parapsilosis* is partially dependent on Dectin-2. **(A–C)** WT and Dectin-2 KO mice were infected intravenously with the indicated doses of *C. albicans*, *C. glabrata*, *C. tropicalis*, or *C. parapsilosis* for 7 days. **(A)** CFU in the kidneys and brains of mice at 7 days post-infection were determined. Mann-Whitney test (*C. albicans* kidney and brain on transformed data), Student's *t*-test (*C. glabrata* kidney and brain on transformed data; *C. tropicalis* kidney and brain on transformed data; *C. parapsilosis* kidney and brain). **(B)** Cytokine levels in the serum of mice at 7 days post-infection. Student's *t*-test (*C. albicans* IL-12p40; *C. albicans* IL-1β on transformed data; *C. glabrata* IL-12p40; *C. tropicalis* IL-12p40), Mann-Whitney test (C. *parapsilosis* IL-12p40 and IL-6), One sample *t*-test (*C. albicans* IL-6; *C. glabrata* IL-6, TNF, IL-1β, and IL-10; *C. tropicalis* IL-6 and IL-1β; C. *parapsilosis* IL-1β). **(C)** Splenocytes from infected mice were unstimulated or restimulated with live *Candida* spp. for 48 h and IFN-γ and IL-17 levels were measured by ELISA. One-way ANOVA with Bonferroni's post-test (*C. albicans* IFN-γ and IL-17 on transformed data; *C. glabrata* IFN-γ; *C. glabrata* IL-17 on transformed data; *C. tropicalis* IFN-γ and IL-17 on transformed data; *C. parapsilosis* IFN-γ and IL-17 on transformed data). **(A–C)** Graphs are the cumulative result of 2–4 independent experiments. Each symbol represents an individual mouse. Seven out of sixteen WT mice in the *C. albicans* graphs **(A,B)** and 7 out of 11 WT mice in the *C. albicans* graphs **(C)** are the same as **Figure 3**. Four out of eight WT mice in the *C. glabrata* graphs are the same as **Figure 4** in a previous publication from our group (Thompson et al., 2019). Four out of eight WT mice in the *C. glabrata* graphs, six out of six WT mice in the *C. tropicalis* graphs, and nine out of nine WT mice in the *C. parapsilosis* graphs are the same as **Figure 3** in a previous publication from our group (Thompson et al., 2019). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

important for clearance of all *Candida* spp. tested, and that it significantly contributes to survival during *C. albicans* infection. The *C. albicans* and *C. glabrata* data is in agreement with previous findings (Saijo et al., 2010; Ifrim et al., 2014, 2016; Thompson et al., 2019). Serum IL-6 was also elevated to a significant or near significant level in Dectin-2 KO mice following infection with *C. albicans* and *C. tropicalis* similar to our previous observations (Orr et al., 2013; Thompson et al., 2019). Splenocytes from *C. albicans*- or *C. glabrata*-infected Dectin-2 KO mice displayed enhanced IL-17 and/or IFN-γ production following restimulation with the same *Candida* sp. Ifrim et al. (2016) also showed similar enhanced T-cell associated cytokine production from Dectin-2 KO splenocytes following infection with *C. albicans* and restimulation with heat-killed *C. albicans*, although the time points differed from our study. However, in contrast to our findings, Ifrim et al. (2014) showed reduced T-cell associated cytokine production following infection with *C. glabrata* and restimulation with heat-killed *C. glabrata.* In addition, IL-17 production by splenocytes following infection with *C. albicans* was decreased with the addition of anti-Dectin-2 (Robinson et al., 2009). The experimental setup for each of these findings differ considerably, which could potentially explain some of the discrepancies. However, we believe that the enhanced IL-17 and/or IFN-γ production from the Dectin-2 KO splenocytes in our study is likely associated with enhanced T cell exposure to the *Candida* spp. *in vivo* due to the inability of these mice to clear/control these infections. Saijo et al. (2010) showed reduced T-cell associated cytokine production from purified naïve Dectin-2 KO $CD4^{+}$ T cells following stimulation with supernatants from BMDCs cultured with *C. albicans* indicating an inability of Dectin-2 KO T cells to appropriately respond to *C. albicans*. Therefore, our data indicate that Dectin-2 is important for the clearance of all four *Candida* spp., similar to our *in vitro* cytokine findings, however, roles for Dectin-2 in specific responses *in vivo* during systemic infection varies with *Candida* spp. Our data does not rule out additional roles for Dectin-2 in mediating production of other cytokines in response to these four *Candida* species or at different sites of infection.

At the start of this study, we postulated that differences in host immune responses and consequent *in vivo* fungal

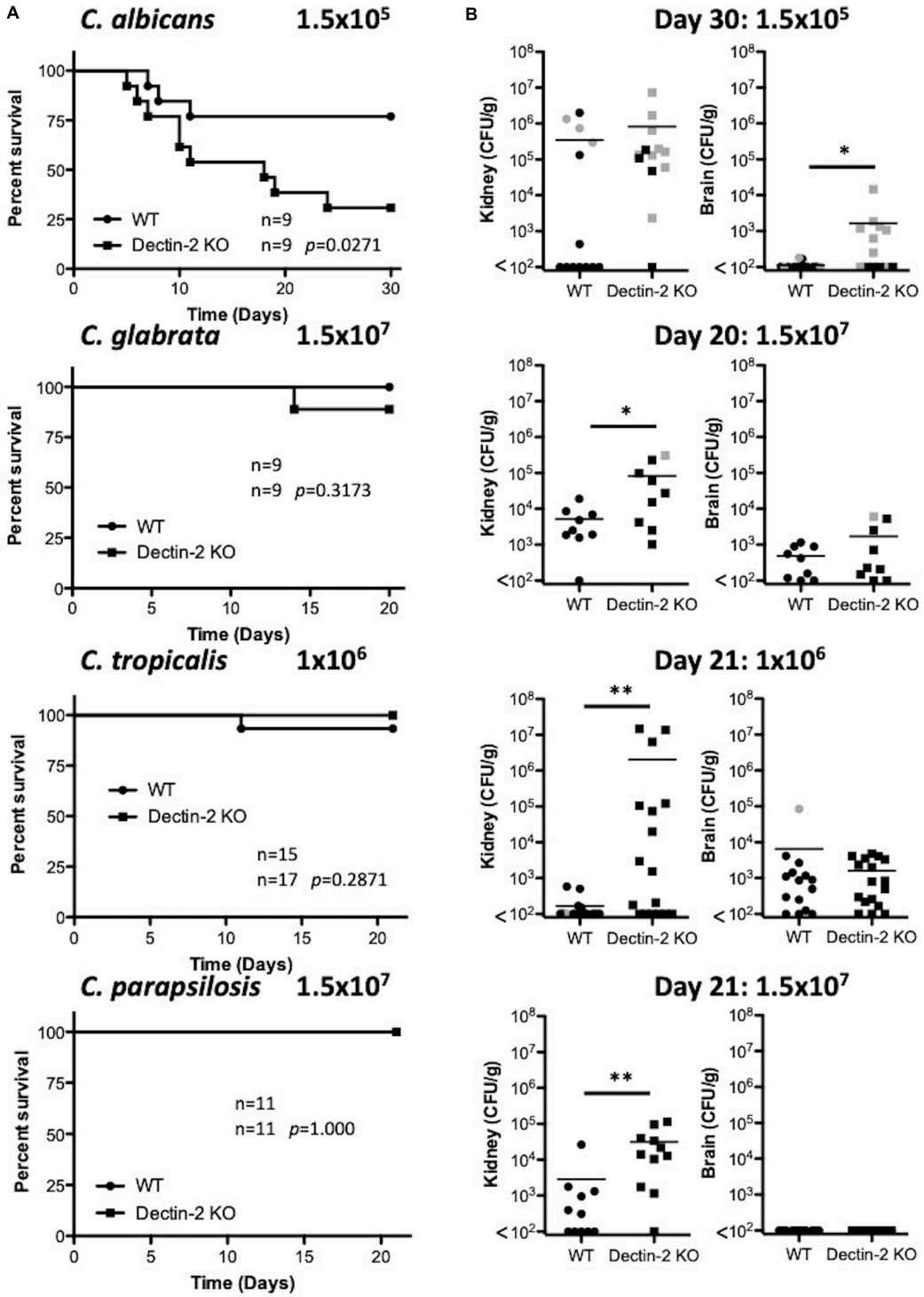

FIGURE 6 | Dectin-2 is important for clearance of multiple *Candida* spp. **(A)** WT and Dectin-2 KO mice were infected intravenously with the indicated doses of *C. albicans* for 30 days, *C. glabrata* for 20 days, *C. tropicalis* for 21 days, or *C. parapsilosis* for 21 days. Survival curves based on humane end-point of infected WT (filled circles) and Dectin-2 KO mice (filled squares). Graphs are the cumulative result of 2–3 independent experiments. Log-rank test. **(B)** CFU in the kidneys of WT and Mincle KO mice at 20–30 days after infection (black symbols) or at time of death by humane end point (gray symbols). Graphs are the cumulative result of 2–3 independent experiments. Each symbol represents an individual mouse. Mann-Whitney test on transformed data (*C. albicans* kidney and brain; *C. tropicalis* kidney and brain; *C. parapsilosis* kidney), Student's *t*-test on transformed data (*C. glabrata* kidney and brain), One sample *t*-test (*C. parapsilosis* brain). Nine out of nine WT mice in the *C. glabrata* graphs are the same as **Figure 3** and 10 out of 14 WT mice in the *C. tropicalis* graphs are the same as **Figure 4** of a previous publication from our group (Thompson et al., 2019). $^{*}p < 0.05$, $^{**}p < 0.01$.

clearance of four clinically relevant *Candida* spp. could be due to different mannan levels/structures on the cell wall. While we found significant differences in the mannan fibril length and phosphomannan in the four *Candida* spp., our results do not suggest a consistent relationship between type/amount of mannan and Mincle or Dectin-2 involvement in cytokine production or fungal clearance. Spatial orientation and exposure of different mannan ligands on the *Candida* cell wall was recently shown to greatly influence recognition by mannan-binding CLRs, including Dectin-2 (Vendele et al., 2020). It is possible that differences in cell wall architecture of the distinct *Candida* spp. tested here, could translate to different exposure of these ligands and greatly influence the ability of immune cells to recognize them via Mincle and Dectin-2. An in-depth analysis of the cell wall and the mannan structure on these *Candida* spp. may help to identify a pattern that could connect a specific mannan structure/type that is associated with each CLR, however, from our data, this pattern is not currently evident. Overall in this study, we have shown that Dectin-2 in particular, but also Mincle are important for *Candida*-mediated immune responses during systemic infection, although the level of CLR importance varies with species and likely strain of *Candida*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Cardiff University's Animal Welfare and Ethical Review Body.

AUTHOR CONTRIBUTIONS

AT designed and performed the experiments. DF performed the experiments and wrote the manuscript. LW and JG performed the experiments. PT and NG guided the research. SO conceptualized and guided the research, designed and performed the experiments, and wrote the manuscript. All authors contributed to the manuscript revision, and read and approved the submitted version.

FUNDING

SO was supported by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (Grant No. 099953/Z/12/Z) and by a Wellcome Trust Cross-Disciplinary Award. NG acknowledges Wellcome Trust support of a Senior Investigator (101873/Z/13/Z), Collaborative (200208/A/15/Z) and Strategic Awards (097377/Z11/Z) and the MRC Centre for Medical Mycology (MR/N006364/2). PT was funded by a Wellcome Trust Investigator Award (107964/Z/15/Z) and the UK Dementia Research Institute.

ACKNOWLEDGMENTS

We wish to acknowledge the NIH-sponsored Mutant Mouse Regional Resource Center (MMRRC) National System as the source of genetically altered mice (C57BL/6-*Clec4e*$^{tm1.1Cfg}$/Mmucd 031936-UCD) for use in this study. The mice were produced and deposited to the MMRRC by the Consortium for Functional Glycomics supported by the National Institute of General Medical Sciences (GM62116). We also thank the Microscopy and Histology Core Facility at the University of Aberdeen for expert assistance with TEM.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.633229/full#supplementary-material

Supplementary Figure 1 | *Candida*-induced responses in BMDMs are largely independent of Mincle and Dectin-2. **(A–C)** BMDMs from WT and Mincle KO mice **(A,C)** or from WT and Dectin-2 KO mice **(B,C)** were stimulated with *C. albicans* SC5314 (Ca), *C. glabrata* SCS74761 (Cg), *C. tropicalis* SCS74663 (Ct), and *C. parapsilosis* SCSB5882 (Cp) at a MOI of 1:1 (*Candida* sp.: BMDMs). Cytokine levels **(A,B)** and nitric oxide levels **(C)** in the supernatants were measured after 24 h incubation. Results are presented as means ± SEM of 4–7 **(A)**, 3 **(B)**, or 3 **(C)** independent experiments (Two-way ANOVA with Bonferroni's post-test). I = Interaction between "Genotype" and "*Candida*" variables, G (Genotype) = impact of BMDM genotype, C (*Candida*) = impact of different *Candida* spp. tested. **(A)** WT data is the same as in[26], for 2 out of 4–7 replicates for TNF, IL-12p40, IL-1β, IL-6, and IL-10. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Supplementary Figure 2 | CLR Expression on BMDMs and BMDCs. Representative flow cytometry histograms showing basal Mincle and Dectin-2 surface expression and Mincle expression following 4 h LPS stimulation on BMDMs and BMDCs. Plots are representative of 2–3 independent experiments. Dashed line = isotype control, solid line = CLR.

REFERENCES

Arendrup, M., Horn, T., and Frimodt-Møller, N. (2002). In vivo pathogenicity of eight medically relevant candida species in an animal model. *Infection* 30, 286–291. doi: 10.1007/s15010-002-2131-0

Ballou, E. R., Avelar, G. M., Childers, D. S., Mackie, J., Bain, J. M., Wagener, J., et al. (2016). Lactate signalling regulates fungal β-glucan masking and immune evasion. *Nat. Microbiol.* 2:16238.

Bongomin, F., Gago, S., Oladele, R., and Denning, D. (2017). Global and multi-national prevalence of fungal diseases—estimate precision. *J. Fungi* 3:57. doi: 10.3390/jof3040057

Branzk, N., Lubojemska, A., Hardison, S. E., Wang, Q., Gutierrez, M. G., Brown, G. D., et al. (2014). Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat. Immunol.* 15, 1017–1025. doi: 10.1038/ni.2987

Brown, G. D., Denning, D. W., Gow, N. A., Levitz, S. M., Netea, M. G., White, T. C., et al. (2012). Hidden killers: human fungal infections. *Sci. Transl. Med.* 4:165rv13. doi: 10.1126/scitranslmed.3004404

Brown, G. D., Taylor, P. R., Reid, D. M., Willment, J. A., Williams, D. L., Martinez-Pomares, L., et al. (2002). Dectin-1 is a major β-glucan receptor on macrophages. *J. Exp. Med.* 196, 407–412. doi: 10.1084/jem.20020470

Chen, S. M., Shen, H., Zhang, T., Huang, X., Liu, X. Q., Guo, S. Y., et al. (2017). Dectin-1 plays an important role in host defense against systemic *Candida glabrata* infection. *Virulence* 8, 1643–1656. doi: 10.1080/21505594.2017.1346756

Denning, D. W., Kneale, M., Sobel, J. D., and Rautemaa-Richardson, R. (2018). Global burden of recurrent vulvovaginal candidiasis: a systematic review. *Lancet Infect. Dis.* 18, e339–e347.

Ferwerda, B., Ferwerda, G., Plantinga, T. S., Willment, J. A., van Spriel, A. B., Venselaar, H., et al. (2009). Human dectin-1 deficiency and mucocutaneous fungal infections. *N. Engl. J. Med.* 361, 1760–1767.

Gow, N. A. R., van de Veerdonk, F. L., Brown, A. J. P., and Netea, M. G. (2011). *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. *Nat. Rev. Microbiol.* 10, 112–122. doi: 10.1038/nrmicro2711

Guinea, J. (2014). Global trends in the distribution of *Candida* species causing candidemia. *Clin. Microbiol. Infect.* 20, 5–10. doi: 10.1111/1469-0691.12539

Haider, M., Dambuza, I. M., Asamaphan, P., Stappers, M., Reid, D., Yamasaki, S., et al. (2019). The pattern recognition receptors dectin-2, mincle, and FcRγ impact the dynamics of phagocytosis of *Candida*, *Saccharomyces*, *Malassezia*, and *Mucor* species. *PLoS One* 14:e0220867. doi: 10.1371/journal.pone.0220867

Hall, R. A., and Gow, N. A. R. (2013). Mannosylation in *Candida albicans*: role in cell wall function and immune recognition. *Mol. Microbiol.* 90, 1147–1161. doi: 10.1111/mmi.12426

Hardison, S. E., and Brown, G. D. (2012). C-type lectin receptors orchestrate antifungal immunity. *Nat. Immunol.* 13, 817–822. doi: 10.1038/ni.2369

Hobson, R. P., Munro, C. A., Bates, S., MacCallum, D. M., Cutler, J. E., Heinsbroek, S. E., et al. (2004). Loss of cell wall mannosylphosphate in *Candida albicans* does not influence macrophage recognition. *J. Biol. Chem.* 279, 39628–39635. doi: 10.1074/jbc.m405003200

Ifrim, D. C., Bain, J. M., Reid, D. M., Oosting, M., Verschueren, I., Gow, N. A., et al. (2014). Role of dectin-2 for host defense against systemic infection with *Candida glabrata*. *Infect. Immun.* 82, 1064–1073. doi: 10.1128/iai.01189-13

Ifrim, D. C., Quintin, J., Courjol, F., Verschueren, I., van Krieken, J. H., Koentgen, F., et al. (2016). The role of dectin-2 for host defense against disseminated candidiasis. *J. Interf. Cytokine Res.* 36, 267–276. doi: 10.1089/jir.2015.0040

Kullberg, B. J., and Arendrup, M. C. (2015). Invasive candidiasis. *N. Engl. J. Med.* 373, 1445–1456.

Lamoth, F., Lockhart, S. R., Berkow, E. L., and Calandra, T. (2018). Changes in the epidemiological landscape of invasive candidiasis. *J. Antimicrob. Chemother.* 73, i4–i13.

Lee, R. T., Hsu, T. L., Huang, S. K., Hsieh, S. L., Wong, C. H., Lee, Y. C., et al. (2011). Survey of immune-related, mannose/fucose-binding C-type lectin receptors reveals widely divergent sugar-binding specificities. *Glycobiology* 21, 512–520. doi: 10.1093/glycob/cwq193

Lockhart, S. R. (2014). Current epidemiology of *Candida* Infection. *Clin. Microbiol. Newsl.* 36, 131–136. doi: 10.1016/j.clinmicnews.2014.08.001

Mccarty, T. P., and Pappas, P. G. (2016). Invasive candidiasis. *Infect. Dis. Clin. NA* 30, 103–124.

McGreal, E. P., Rosas, M., Brown, G. D., Zamze, S., Wong, S. Y., Gordon, S., et al. (2006). The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for high mannose. *Glycobiology* 16, 422–430. doi: 10.1093/glycob/cwj077

Netea, M. G., Gow, N. A., Munro, C. A., Bates, S., Collins, C., Ferwerda, G., et al. (2006). Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest.* 116, 1642–1650. doi: 10.1172/jci27114

Nguyen, T. N. Y., Padungros, P., Wongsrisupphakul, P., Sa-Ard-Iam, N., Mahanonda, R., Matangkasombut, O., et al. (2018). Cell wall mannan of *Candida krusei* mediates dendritic cell apoptosis and orchestrates Th17 polarization pathway via TLR-2/MyD88-dependent pathway. *Nat. Sci. Rep.* 8:17123.

Orr, S. J., Burg, A. R., Chan, T., Quigley, L., Jones, G. W., Ford, J. W., et al. (2013). LAB/NTAL facilitates fungal/PAMP-induced IL-12 and IFN-γ production by repressing β-catenin activation in dendritic cells. *PLoS Pathog.* 9:e1003357. doi: 10.1371/journal.ppat.1003357

Pappas, P. G., Lionakis, M. S., Arendrup, M. C., Ostrosky-zeichner, L., and Kullberg, B. J. (2018). Invasive candidiasis. *Nat. Rev. Diease Prim.* 4, 1–20.

Patin, E. C., Jones, A. V., Thompson, A., Clement, M., Liao, C. T., Griffiths, J. S., et al. (2016). IL-27 induced by select candida spp. via TLR7/NOD2 signaling and IFN-β production inhibits fungal clearance. *J. Immunol.* 197, 208–221. doi: 10.4049/jimmunol.1501204

Patin, E. C., Thompson, A., and Orr, S. J. (2019). Pattern recognition receptors in fungal immunity. *Semin. Cell Dev. Biol.* 89, 24–33. doi: 10.1016/j.semcdb.2018.03.003

Pfaller, M. A., and Diekema, D. J. (2007). Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* 20, 133–163. doi: 10.1128/cmr.00029-06

Plantinga, T. S., Hamza, O. J., Willment, J. A., Ferwerda, B., van de Geer, N. M., Verweij, P. E., et al. (2010). Genetic variation of innate immune genes in HIV-infected african patients with or without oropharyngeal candidiasis. *J. Acquir. Immune Defic. Syndr.* 55, 87–94. doi: 10.1097/qai.0b013e3181e53c64

Plantinga, T. S., van der Velden, W. J., Ferwerda, B., van Spriel, A. B., Adema, G., Feuth, T., et al. (2009). Early stop polymorphism in human DECTIN-1 is associated with increased *Candida* colonization in hematopoietic stem cell transplant recipients. *Clin. Infect. Dis.* 49, 724–732. doi: 10.1086/604714

Pradhan, A., Avelar, G. M., Bain, J. M., Childers, D., Pelletier, C., Larcombe, D. E., et al. (2019). Non-canonical signalling mediates changes in fungal cell wall PAMPs that drive immune evasion. *Nat. Commun.* 10, 1–14.

Robinson, M. J., Osorio, F., Rosas, M., Freitas, R. P., Schweighoffer, E., Gross, O., et al. (2009). Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J. Exp. Med.* 206, 2037–2051. doi: 10.1084/jem.20082818

Saijo, S., Ikeda, S., Yamabe, K., Kakuta, S., Ishigame, H., Akitsu, A., et al. (2010). Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. *Immunity* 32, 681–691. doi: 10.1016/j.immuni.2010.05.001

Sam, Q. H., Yew, W. S., Seneviratne, C. J., Chang, M. W., and Chai, L. Y. A. (2018). Immunomodulation as therapy for fungal infection: are we closer? *Front. Microbiol.* 9:1612. doi: 10.3389/fmicb.2018.01612

Sato, K., Yang, X. L., Yudate, T., Chung, J. S., Wu, J., Luby-Phelps, K., et al. (2006). Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor γ chain to induce innate immune responses. *J. Biol. Chem.* 281, 38854–38866. doi: 10.1074/jbc.m606542200

Segal, B. H., Kwon-Chung, J., Walsh, T. J., Klein, B. S., Battiwalla, M., Almyroudis, N. G., et al. (2006). Immunotherapy for fungal infections. *Clin. Infect. Dis.* 42, 507–515.

Shibata, N., Suzuki, A., Kobayashi, H., and Okawa, Y. (2007). Chemical structure of the cell-wall mannan of *Candida albicans* serotype A and its difference in yeast and hyphal forms. *Biochem. J.* 404, 365–372. doi: 10.1042/bj20070081

Sun, J., Zhang, X., Broderick, M., and Fein, H. (2003). Measurement of nitric oxide production in biological systems by using griess reaction assay. *Sensors* 3, 276–284. doi: 10.3390/s30800276

Taylor, P. R., Gordon, S., and Martinez-pomares, L. (2005). The mannose receptor: linking homeostasis and immunity through sugar recognition. *Trends Immunol.* 26, 104–110. doi: 10.1016/j.it.2004.12.001

Taylor, P. R., Tsoni, S. V., Willment, J. A., Dennehy, K. M., Rosas, M., Findon, H., et al. (2007). Dectin-1 is required for β-glucan recognition and control of fungal infection. *Nat. Immunol.* 8, 31–38. doi: 10.1038/ni1408

Thompson, A., Davies, L. C., Liao, C. T., da Fonseca, D. M., Griffiths, J. S., Andrews, R., et al. (2019). The protective effect of inflammatory monocytes during systemic *C. albicans* infection is dependent on collaboration between C-type lectin-like receptors. *PLoS Pathog.* 15:e1007850. doi: 10.1371/journal.ppat.1007850

Thompson, A., Griffiths, J. S., Walker, L., da Fonseca, D. M., Lee, K. K., Taylor, P. R., et al. (2019). Dependence on dectin-1 varies with multiple *candida* species. *Front. Microbiol.* 10:1800. doi: 10.3389/fmicb.2019.01800

van de Veerdonk, F. L., Netea, M. G., Joosten, L. A., van der Meer, J. W., and Kullberg, B. J. (2010). Novel strategies for the prevention and treatment of *Candida* infections: the potential of immunotherapy. *FEMS Microbiol. Rev.* 34, 1063–1075.

Vendele, I., Willment, J. A., Silva, L. M., Palma, A. S., Chai, W., Liu, Y., et al. (2020). Mannan detecting C-type lectin receptor probes recognise immune epitopes with diverse chemical, spatial and phylogenetic heterogeneity in fungal cell walls. *PLoS Pathog.* 16:1007927. doi: 10.1371/journal.ppat.1007927

Walker, L., Sood, P., Lenardon, M. D., Milne, G., Olson, J., Jensen, G., et al. (2018). The viscoelastic properties of the fungal cell wall allow traffic of ambisome as intact liposome vesicles. *mBio* 9, 1–15.

Wells, C. A., Salvage-Jones, J. A., Li, X., Hitchens, K., Butcher, S., Murray, R. Z., et al. (2008). The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to *Candida albicans*. *J. Immunol.* 180, 7404–7413. doi: 10.4049/jimmunol.180.11.7404

Yadav, B., Mora-Montes, H. M., Wagener, J., Cunningham, I., West, L., Haynes, K., et al. (2020). Differences in fungal immune recognition by monocytes and macrophages: N-mannan can be a shield or activator of immune recognition. *Cell Surf.* 6:100042. doi: 10.1016/j.tcsw.2020.100042

Yamasaki, S., Ishikawa, E., Sakuma, M., Hara, H., Ogata, K., Saito, T., et al. (2008). Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat. Immunol.* 9, 1179–1188. doi: 10.1038/ni.1651

Yamasaki, S., Matsumoto, M., Takeuchi, O., Matsuzawa, T., Ishikawa, E., Sakuma, M., et al. (2008). C-type lectin mincle is an activating receptor for pathogenic fungus, *Malassezia*. *Proc. Natl. Acad. Sci. U S A.* 106, 1897–1902. doi: 10.1073/pnas.0805177106

Yapar, N. (2014). Epidemiology and risk factors for invasive candidiasis. *Ther. Clin. Risk Manag.* 10, 95–105. doi: 10.2147/tcrm.s40160

Zaoutis, T. E., Argon, J., Chu, J., Berlin, J. A., Walsh, T. J., Feudtner, C., et al. (2005). The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the united states: a propensity analysis. *Clin. Infect. Dis.* 41, 1232–1239. doi: 10.1086/496922

Conflict of Interest: The handling editor declared a shared affiliation with one of the authors JG at time of review.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 20 April 2021
doi: 10.3389/fmicb.2021.637500

Single-Cell TCR Sequencing Reveals the Dynamics of T Cell Repertoire Profiling During *Pneumocystis* Infection

Hu-Qin Yang, Yi-Shan Wang, Kan Zhai and Zhao-Hui Tong**

Department of Respiratory and Critical Care Medicine, Beijing Institute of Respiratory Medicine, Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China

OPEN ACCESS

Edited by:
David L. Moyes, King's College London, United Kingdom

Reviewed by:
Fabien P. Blanchet, Institut National de la Santé et de la Recherche Médicale (INSERM), France
Prasanta Kumar Dash, University of Nebraska Medical Center, United States

***Correspondence:**
Kan Zhai
zhaikan@ccmu.edu.cn
Zhao-Hui Tong
tongzhaohuicy@sina.com

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *03 December 2020*
Accepted: *29 March 2021*
Published: *20 April 2021*

Citation:
Yang H-Q, Wang Y-S, Zhai K and Tong Z-H (2021) Single-Cell TCR Sequencing Reveals the Dynamics of T Cell Repertoire Profiling During Pneumocystis Infection. Front. Microbiol. 12:637500. doi: 10.3389/fmicb.2021.637500

T cell responses play critical roles in host adaptive immunity against *Pneumocystis*. However, the dynamics and diversity of the T cell immune repertoire in human immunodeficiency virus (HIV)-negative *Pneumocystis* pneumonia (PCP) remains unclear. In this study, single-cell RNA and single-cell T cell receptor (TCR) sequencing were applied to cells sorted from lung tissues of mice infected with *Pneumocystis*. Our findings demonstrated the clonal cells were mainly composed of $CD4^+$ T cells in response to *Pneumocystis*, which were marked by highly expressed genes associated with T cell activation. Mice infected with *Pneumocystis* showed reduced TCR diversity in $CD4^+$ T cells and increased diversity in $CD8^+$ T cells compared with uninfected controls. Furthermore, Th17 cells were mostly clonal $CD4^+$ T cells, which exhibited the phenotype of tissue-resident memory-like Th17 cells. In addition, *Pneumocystis*-infected mice showed biased usage of TCRβ VDJ genes. Taken together, we characterized the transcriptome and TCR immune repertoires profiles of expanded T cell clones, which demonstrate a skewed TCR repertoire after *Pneumocystis* infection.

Keywords: *Pneumocystis* pneumonia, T cell receptors, single-cell TCR sequencing, single-cell RNA sequencing, T cell immune repertoire

INTRODUCTION

Pneumocystis pneumonia (PCP) is a life-threatening complication in human immunodeficiency virus (HIV)-negative immunocompromised patients with more abrupt clinical manifestation, worse prognosis, and higher mortality than HIV patients (Roblot et al., 2002; Roux et al., 2014). The mortality of HIV-negative PCP patients is estimated to range from 20 to 80%, which is higher than in HIV patients (5–15%) (Rilinger et al., 2019; Shoji et al., 2020). The incidence of PCP in HIV-negative patients has continued to increase in recent years (Cilloniz et al., 2019; Lee et al., 2019), highlighting the need to better understand the immune mechanism against *Pneumocystis* to identify new therapeutic targets.

Adaptive immune responses, especially $CD4^+$T cells, play a central role in clearing *Pneumocystis* infection and directly influence clinical prognosis (Otieno-Odhiambo et al., 2019). Thus, various

studies attempted to elucidate the specific function of $CD4^+$ T cells subsets and found that diverse $CD4^+$ T cells subsets participate in the immune response against *Pneumocystis* such as Th1 (Garvy et al., 1997), Th17 (Rudner et al., 2007), Treg (McKinley et al., 2006), and Th9 cells (Li et al., 2018). However, those studies were based on whole tissues or bulk populations of cells, which could not account for T-cell heterogeneity at a high resolution, and thus may obscure some important mechanistic mediators.

Recently, the advent of single-cell RNA sequencing (scRNA-seq) has facilitated in dissecting the heterogeneity of immune cells. Furthermore, single-cell T cell receptor (TCR) sequencing (scTCR-seq) can effectively identify clonal cells and characterize their relationships. In infectious diseases, the combination of scTCR-seq and scRNA-seq allows for simultaneous analysis of paired TCR sequences and the transcriptome to track T cell clones (Azizi et al., 2018; Yost et al., 2019). Waickman et al. (2019) characterized the activated and clonally expanded T cells in response to the vaccine of dengue virus and assessed the T cell immune response following vaccination. Wang et al. (2021) delineated the TCR repertoires of coronavirus disease 2019 (COVID-19) patients and observed distinct T cell clonal expansion and skewed VDJ gene usage in COVID-19. Liao et al. (2020) detected the transcriptome and TCR profile of the bronchoalveolar lavage fluid of patients with COVID-19 and found highly clonally expanded $CD8^+$ T cells in patients with mild COVID-19.

Li Pira et al. (2001) investigated the characteristics of the TCR repertoire of *Pneumocystis*-specific $CD4^+$ T cells in HIV patients using spectratyping; however, changes in TCR repertoires and clonal expansion in multiple T cell subsets in PCP remains unclear.

In this study, we quantitatively tracked the expanded T cell clones and revealed their phenotype during *Pneumocystis* infection using scRNA-seq coupled with scTCR-seq. We comprehensively analyzed the composition and characteristics of clonal T cells, diversity of TCR repertoire, distribution of CDR3 length, and usage patterns of TCRβ VDJ gene segments and combination at different time points post-infection. Our study characterized the dynamics and diversity of TCR profiling across different stages of *Pneumocystis* infection, which contribute to elucidating host adaptive immunity and discovering novel therapeutic targets.

MATERIALS AND METHODS

Mice and Pneumocystis Infection

Adult male C57BL/6J mice and SCID mice aged 6–8 weeks (Vital River Lab Animal Co., Ltd., Beijing, China) were housed under specific pathogen-free conditions at the Beijing Institute of Respiratory Medicine (Beijing, China). C.B-17 SCID mice were inoculated with *P. murina* (American Type Culture Collection, Manassas, VA, United States) as we previously reported (Zhang et al., 2020). For the *Pneumocystis* infection, 1×10^6 *Pneumocystis* cysts contained in 100 μL PBS (Solarbio, Ca^{2+}/Mg^{2+}-free) were injected through the trachea of each mouse. Control mice were transtracheally injected with 100 μL PBS. To determine the *Pneumocystis* burden, real-time PCR was conducted to determine the copy number of *Pneumocystis* rRNA from right lung lobes by TaqMan assays. The primers were 5′-AGGTGAAAAGTCGAAAGGGAAAC-3′ and 5′-AAAACCTCTTTTCTTTCACTCAGTAACA-3′. The sequence of probe was 5′-FAM-CCCAGAATAATGAATAAAG-MGBNFQ-3′. Li et al. (2018) and Rong et al. (2019) demonstrated that the *Pneumocystis* burden would increase until the third week, and then decrease in the fourth week after generation of the PCP model. Based on the previously reported *Pneumocystis* burden and our results (**Supplementary Figure 1A**), we designed this study using mice infected with *Pneumocystis* from 1 to 4 weeks. Thus, research on the mice infected with *Pneumocystis* from 0 to 4 weeks could reflect the full dynamic immune response to *Pneumocystis*.

Lung Tissue Processing and Cell Sorting

Single cell suspensions were isolated from mouse lungs by collagenase digestion following recently published protocols (Reyfman et al., 2019). Briefly, mice were anesthetized with an overdose of 0.5% pentobarbital, then the lungs were perfused with 1 mL complete 1,640 medium (Solarbio) with 10% FCS (Hyclone) containing collagenase IV (Solarbio) and Dnase I (Sigma) through the trachea, chopped with scissors, and subsequently incubated for 20 min at 37°C with mild agitation. The resulting lung homogenate was passed through a 40-μm filter and resuspended in ACK buffer (BD) for 15 min on ice. Then, the cells were centrifuged at 400 *g* for 6 min and incubated with Percp-Cy5.5 anti-mouse CD45 antibody (BD) and Ghost Dye™ Red 780 (Tonbo) for 15 min on ice. BD FACS ARIA II cell sorter was applied to sort $CD45^+$ cells from lung cell suspensions pooled from three mice.

scRNA-Seq and Data Processing

Single-cell 5′ RNA-Seq libraries were generated using Single Cell 5′ Library and Gel Bead Kit and Chromium Controller (10 × Genomics) following automated cell counting and a sample quality test (Countess® II Automated Cell Counter with AO/PI reagent). After assessing the quality of libraries by Agilent 4200, sequencing libraries were loaded onto an Illumina Novaseq platform. Cell Ranger (v.3.0.2) was applied to demultiplex align reads (mouse GRCm38/mm10 as reference genome) and produce gene-barcode matrices. The Cell Ranger aggr pipeline was used to combine data from all five samples.

Cell Clustering and Annotation

Quality control and cell clustering were performed using Seurat (version 3.1.2) (Butler et al., 2018). If the genes were less than 200 or more than 4,000 and the mitochondrial genes were more than 20% of total unique molecular identifiers (UMIs), then the cells were excluded from analysis. After performing quality control, gene expression levels were normalized *via* the NormalizeData function and the top 2,000 variable genes were calculated using the FindVariableFeatures function. To reduce the dimensions, the scaled data were used to conduct principal component analysis. Based on the first 20 principal components, the subclusters of T

cells were recognized utilizing uniform manifold approximation and projection (UMAP) and t-distributed stochastic neighbor embedding (t-SNE) projections. Clusters were annotated based on the Seurat-based cluster-specific marker gene list and the expression of canonical marker genes, including T cells (Cd3d, Cd3e, and Cd3g), naïve $CD4^+$ T cells (Cd4, Sell), effector $CD4^+$ T cells (Cd4 and Cd44), naïve $CD8^+$ T cells (Cd8a, Sell), effector $CD8^+$ T cells (Cd8a and Cd44), γδT cells (Tcrg-C1), double-positive T cells (DPT, Cd4, and Cd8a), NKT cells (Nkg7 and Klrc1), and cycling cells (Mki67 and Stmn1).

We performed Scrublet to exclude cell doublets (Wolock et al., 2019). We calculated the doublet score for each single cell using default parameters and used a cluster-level approach to remove clusters that contained more than 20% doublet cells.

scTCR-Seq and Analysis

Full-length TCR VDJ segments were generated using the Chromium Single-Cell V(D)J Enrichment kit following the manufacturer's protocol. The Cell Ranger vdj pipeline was applied to assemble the TCR sequences and identify the CDR3 sequence and TCR genes. Then, the cells were filtered according to the following steps: (1) Cells annotated as T cell clusters in scRNA-seq were kept and (2) Cells that possessed productive TCR α and β chains were incorporated into the analysis. If more than one α or β chain were detected in a cell, then we retained the chain with the highest UMIs (Zheng et al., 2017). We defined the expanded clonal cells as a pair of TCR α and β chains that appeared in at least three cells.

V-J gene combinations were visualized *via* chord diagrams using the circlize package (version 0.4.8). To explore the common and unique V-J and V-D-J gene combinations, we obtained a list of specific V-J and V-D-J gene combinations for all samples, then utilized the Venn Diagram package (version 1.6.20) in R software (version 3.6.1) to display the relationship of those combinations in each sample.

Differential Expression and Pathway Analyses

The FindMarkers function was used to identify the differentially expressed genes among cell clusters. Genes were considered differentially expressed when the logFC > 0.25, expressed in $>25\%$ of cells in the cluster and the adjusted P value was < 0.05 based on the Wilcoxon rank sum test. Gene pathway analyses were implemented using the DAVID website[1].

Analysis of $CD4^+$ T Cells' Residency and Migration Scores

Genes of $CD4^+$ T cells' residency and migration were obtained from previous studies (Zhao et al., 2021), then were converted into mouse genes using the Mouse Genome Informatics Database (**Supplementary Table 1**; Blake et al., 2017). The AddModuleScore function in Seurat was used to calculate the $CD4^+$ T cells' residency and migration scores.

[1]https://david.ncifcrf.gov

RESULTS

scRNA-seq and scTCR-seq Profiling of Lung T Cells

To investigate the dynamics and diversity of the T cell repertoire during PCP, we first performed scRNA-seq and scTCR-seq on lung $CD45^+$ cells from mice infected with *Pneumocystis* for 0–4 weeks (**Figure 1A**). *Pneumocystis* burden continued to increase until 3 weeks post-infection (**Supplementary Figure 1A**), which was concordant to the previous results of our team. Transcriptional and TCR profiles of single cells were obtained using the 10 × Chromium platform. The sequencing information is shown in **Supplementary Table 2**. A total of 15,780 T cells were captured in our scTCR-seq analysis from five lung samples, and 61.4% (9,685/15,780) of the T cells detected had full TCR sequences. To simultaneously analyze the transcriptome and immune repertoire of T cells, we only retained the barcodes detected in both scRNA-seq and scTCR-seq. In addition, we excluded the non-productive TCR chains and only kept 2,870 cells with paired α and β chain for subsequent analysis. Finally, unique and productive α chains constituted 47.5% (1,363/2,870) and productive β chains constituted 20.4% of the cells (585/2,870).

Expansion of Clonal $CD4^+$ T Cells After *Pneumocystis* Infection

T cells were annotated as eight clusters, including naive $CD4^+$ T cells (C1), naive $CD8^+$ T cells (C2), effector $CD4^+$ T cells (C3), effector $CD8^+$ T cells (C4), double-positive T cells (C5), NKT cells (C6), cycling T cells (C7), and γδT cells (C8) (**Figure 1B**). All five samples were well distributed across eight clusters (**Figure 1C**). The expression of selected marker genes is shown in **Figure 1D**. We next investigated the dynamic changes of the proportion of each T cell subset during *Pneumocystis* infection. Among these, naive $CD4^+$ and $CD8^+$ T cells gradually decreased, while effector $CD4^+$ T cells significantly increased during *Pneumocystis* infection (**Figure 1E**). Effector $CD4^+$ T cells accounted for only 7.5% of the T cells in the uninfected mice; however, the proportion of effector $CD4^+$ T cells significantly increased to 36.8% in the fourth week after *Pneumocystis* infection, supporting previous reports that the role of $CD4^+$ T cells in *Pneumocystis* infection was fundamental (Otieno-Odhiambo et al., 2019).

In addition to the T cell frequency, we further analyzed the composition of clonal cells. According to the UMAP projections, the clonal cells were mainly manifested as effector CD4 cells, effector CD8 cells, and dividing cells, while naïve T cells showed minimal clonal expansion (**Figure 1F**). Among the clonally expanded T cells, 44.8% were $CD4^+$ T cells, 36.6% were $CD8^+$ T cells, and 18.6% were cycling T cells (**Figure 1G**). Collectively, these results suggested that clonal $CD4^+$ T cells expanded in lung tissues after *Pneumocystis* infection.

We next focused on the potential changes in the transcriptome of clonal T cell populations in PCP. Compared with the unexpanded cells, clonally expanded $CD4^+$ T cells were mainly enriched with cytokines, chemokine signaling, and the cell

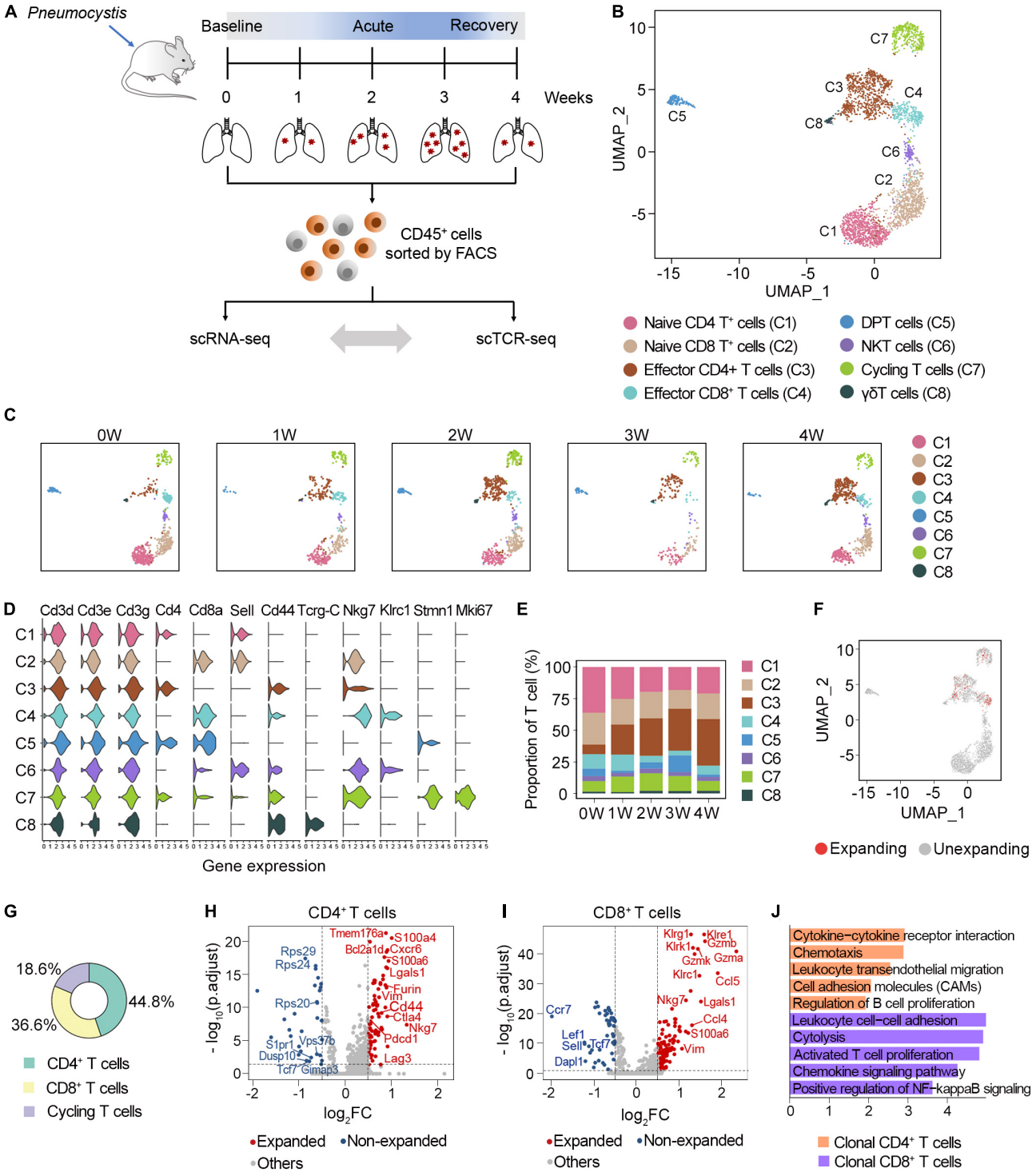

FIGURE 1 | Characterization of clonal T cells by scRNA-seq and scTCR-seq. **(A)** Schematic representation of the experimental design. Immune single cells were isolated from various timepoints during PCP. **(B)** UMAP visualization of T cells. Approximately 2,870 T cells from all samples clustered by UMAP. **(C)** UMAP of T cell clusters from each sample. **(D)** Vlnplot exhibiting the expression of selected markers in each cluster. **(E)** The proportion of T cell subsets at different timepoints. **(F)** UMAP displayed the distribution of clonal T cells. **(G)** The composition of clonal T cells. **(H,I)** Volcano plot showing differentially expressed genes between the expanded and non-expanded cells in $CD4^+$ T cells **(H)** and $CD8^+$ T cells **(I)**. Each dot indicates one gene; red dots represent a $\log_2FC > 0.5$, and blue dots depict a $\log_2FC < -0.5$. Significant genes were selected with adjusted p-value < 0.05 based on Wilcoxon rank sum test. **(J)** Gene set enrichment analyses of clonal $CD4^+$ T cells and $CD8^+$ T cells using DAVID website.

adhesion pathway. Furthermore, clonal $CD4^+$ T cells showed the upregulation of genes associated with inhibitory receptors, including Pdcd1, Lag3, and Ctla4 (**Figures 1H,J**). The increasing expression level of Nkg7, Gzma, Gzmb, and Gzmk in clonal $CD8^+$ T cells was related to cytotoxicity (**Figures 1I,J**). Together, these results indicated the activated, cytotoxic function of clonal T cells in the lung of PCP mice.

Distinct TCR Immune Repertoire After *Pneumocystis* Infection

To evaluate the distribution of clonal TCR, most cells contained unique TCR, while $CD4^+$ T cells showed the maximum level of alterations after *Pneumocystis* infection. We found 1.26–10% of the $CD4^+$ T cells in the *Pneumocystis* infected lung tissues harbored clonal TCR, which was much higher than the 0.98% clonal TCR in the control mice (**Figure 2A** and **Supplementary Figure 1B**). By comparison, the frequency of clonal cells was lower in the $CD8^+$ subset and did not markedly change in the total T cells after the *Pneumocystis* infection (**Figure 2A** and **Supplementary Figures 1C,D**).

Then, we attempted to characterize the diversity of the TCR repertoire by calculating the D50 value (Kuo et al., 2019). If the value of D50 is closer to 50, then the sample is more diverse. For the $CD4^+$ T cells, the D50 of the uninfected sample was 50.7, whereas the values for the *Pneumocystis* infection from weeks 1, 2, 3, and 4 were 51.3, 54.4, 52.2, and 56.5, respectively. For $CD8^+$ T cells, the D50 of uninfected samples was 58.2, whereas those of *Pneumocystis* infection from 1 to 4 weeks were 55.0, 50.8, 50.9, and 53.3, respectively (**Figure 2B**). Furthermore, Gini coefficient was also used to evaluate the diversity and revealed similar results (**Figure 2B**). Taken together, these results indicated the reduced diversity of $CD4^+$ T cells and increased diversity of $CD8^+$ T cells in PCP.

TCR β chains are generated by the rearrangement of the V, D, and J genes, which is indicative of a greater diversity than the TCR α chains, and the complementarity determining region 3 (CDR3) is the most variable region of the TCR (Nikolich-Zugich et al., 2004). Therefore, we next analyzed the length of the CDR3 aa in the TCRβ chain. Regardless of the cluster of total T cells, $CD4^+$ T cells, or $CD8^+$ T cells, the average length of CDR3 aa was 27 bp (range: 16–40 bp), and the length distributions of TCRβ CDR3 were nearly identical in both infected and uninfected tissues (**Figure 2C**).

Expansion of Clonal Tissue-Resident Memory-Like Th17 Cells

Because $CD4^+$ T cells were the most expanded clonal cells after *Pneumocystis* infection, we performed sub-clustering analysis of $CD4^+$ T cells from all five samples and identified six subclusters (**Figure 3A**). According to the gene expression program, two clusters were annotated as naïve CD4 cells, which expressed high levels of Ccr7, Sell, Lef1, and Tcf7. Furthermore, Th1 cell (Tbx21, Il2, Ifng, Cxcr3, and Ccl5), Treg cell (Il2ra, Foxp3, and Ikzf2), and Th17 cell (Rora, Il17a, Il17f, Il23r, and Ccr6) associated gene signatures were identified. Of note, a mixed effector CD4 cluster was observed, which showed upregulated expression of Th1, Th2, and Treg-related genes (**Figure 3B**). Quantification of cell subsets from scRNA-seq data demonstrated decreased fraction of naïve CD4 cells, while Th1, Th17, and Treg significantly increased after *Pneumocystis* infection. Specifically, the proportion of Th1 cells increased from 14.0% of $CD4^+$ T cells at baseline to 26.1% at 2 weeks post-infection. Th17 cells continued to increase from 2.3% of $CD4^+$ T cells at baseline to 16.5% at 4 weeks post-infection. Treg cells accounted for only 0.7% of $CD4^+$ T cells in the uninfected group, but increased to 12.3% at 3 weeks post-infection, and began to decrease at 4 weeks post-infection (**Figure 3C**).

To further investigate the composition of clonal $CD4^+$ T cells, we compared the clonal expansion of each $CD4^+$ T cell subcluster. Interestingly, only Th17 and mixed cells underwent clonal expansion, which accounted for 53.4 and 46.6% of the total clonal $CD4^+$ T cells, respectively (**Figures 3D–F**). Recent studies have demonstrated that Th17 cells could acquire a tissue-resident phenotype in the lungs (Amezcua Vesely et al., 2019). Based on the gene sets obtained from a previous research (Zhao et al., 2021), we calculated the residency and migratory scores of Th17 cells and found that the Th17 cells in our study have a relatively higher residency score (**Figure 3G**), suggesting that Th17 cells might exhibit the phenotype of tissue-resident memory-like Th17 cells (Trm-like 17 cells).

The Altered Usage Patterns of TCRβ VDJ Gene Segments and Combination After *Pneumocystis* Infection

To gain insights into the preference of TCRβ VDJ gene segments, a total of 21 V gene and 12 J gene segments were detected in TCRβ from five samples. As shown in the heat map, the usage patterns of the V and J gene segments were similar between the different time points of the PCP and healthy control groups (**Figures 4A–C** and **Supplementary Table 3**). For the total T cells, the most frequent V gene segments in *Pneumocystis* infected in the 0–4 W groups were TRBV13-2 (14.3%), TRBV13-2 (9.8%), TRBV3 (11.9%), TRBV1 (11.1%), and TRBV3 (10.7%), respectively (**Supplementary Figure 1E**). For CD4 cells, the most frequent V gene segments in the 0–4 W groups were TRBV13-2 (10.4%), TRBV13-2 (10.4%), TRBV3 (14.1%), TRBV3/TRBV1 (13.6%), and TRBV3 (12.4%), respectively (**Supplementary Figure 1F**). For CD8 cells, the most frequent V gene segments in the 0–4 W groups were TRBV13-2 (19.5%), TRBV19 (9.3%), TRBV13-2 (11.6%), TRBV13-2/TRBV13-3/TRBV4 (14%), and TRBV13-3 (9.1%), respectively (**Supplementary Figure 1G**). TRBJ2-7 accounted for the largest number of TRBJ in the total T cells and CD8 cells for each sample, except for TRBJ1-1 in CD4 cells at 3 W after infection (**Supplementary Figures 1E–G**). TRBD gene usage was also examined, and TRBD1 genes were used most frequently across all samples. Therefore, alterations in the TCRβ VDJ gene segments mainly occurred in the V gene in PCP.

We also examined the make-up of V-J and V-D-J gene combinations for TCRβ and identified 245 distinct V-J gene combinations and 385 distinct V-D-J gene combinations (**Supplementary Tables 4**, **5**). To identify the disease-specific

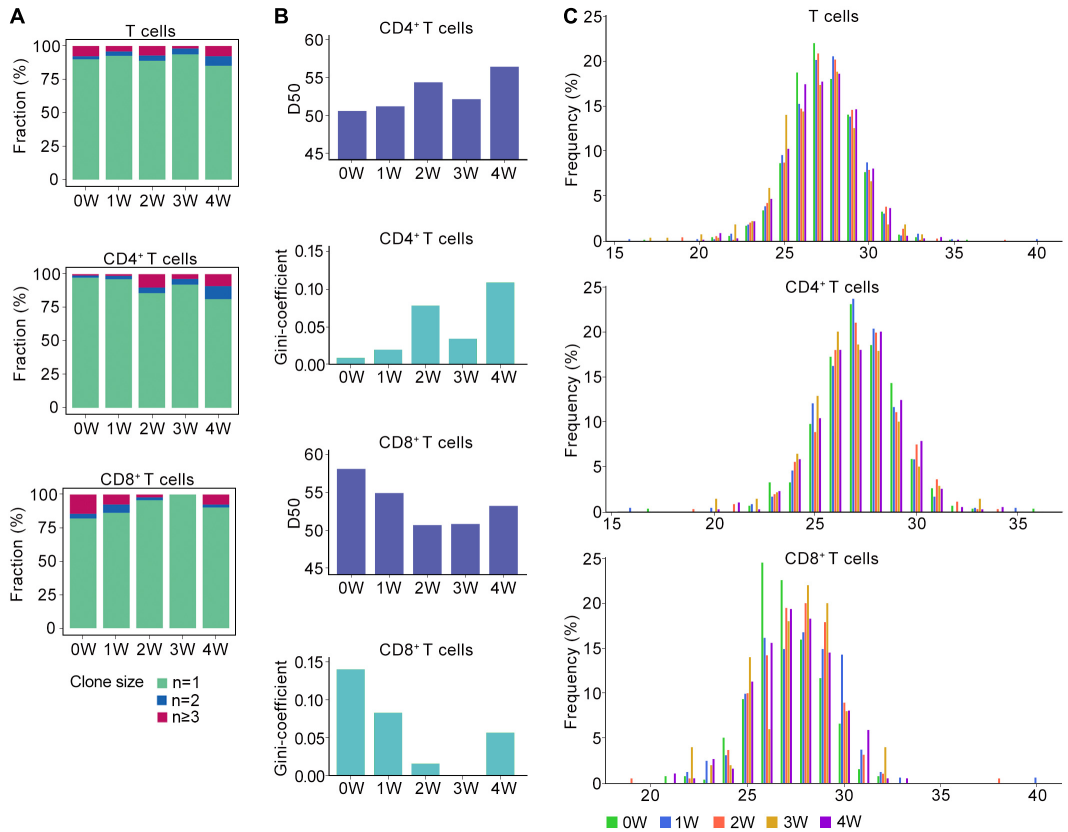

FIGURE 2 | Distinct TCR immune repertoire after *Pneumocystis* infection. **(A)** The proportion of unique and non-unique TCRs expression in T cells (upper), $CD4^+$ T cells (middle), and $CD8^+$ T cells (lower) across five samples. Unique TCRs ($n = 1$), duplicated TCRs ($n = 2$), and the clonal TCRs ($n \geq 3$) were labeled with different colors. **(B)** The value of D50 and Gini-coefficient of $CD4^+$ and $CD8^+$ T cells across samples. **(C)** Distributions of the TCRB CDR3 aa lengths in T cells (upper), $CD4^+$ T cells (middle), and $CD8^+$ T cells (lower) across all samples.

TRB V-J and V-D-J gene combinations, we quantified the common and unique V-J and V-D-J gene combinations between infected and uninfected samples (**Supplementary Table 6**). The results showed that of all the 241 V-J combinations in *Pneumocystis*-infected mice, 189 (78.4%) were shared with uninfected controls, and 52 (21.6%) were unique (**Figure 4D**). The common and unique V-J combinations between each sample is shown in **Figure 4E**, which suggest that unique V-J combinations existed at every time point. Greater variation was observed in the V-D-J gene combinations, of all the 369 V-D-J combinations in *Pneumocystis*-infected mice, 234 (63.4%) were shared with uninfected controls and 135 (36.6%) were unique (**Figure 4F**). Unique V-D-J combinations were also obtained in all samples, ranging from eight to 28 (**Figure 4G**). The frequency of all V-J and V-D-J combinations in T, $CD4^+$ T and $CD8^+$ T cells were visualized *via* circos plots and heat maps, respectively (**Supplementary Figures 2**, **3**). The most frequently used V-J and V-D-J combinations are listed in **Supplementary Table 7**. In addition, we constructed heat maps of top 10 V-D-J gene combinations that were most frequently used in T, $CD4^+$ T, and $CD8^+$ T cells, which showed distinct patterns of V-D-J gene combinations for TCRβ at each time point (**Figures 4H–J**). These results demonstrated that the distributions of the V-J and V-D-J combinations were significantly shaped by *Pneumocystis* infection.

DISCUSSION

An increasing number of studies have attempted to elucidate the mechanism of T cells in controlling *Pneumocystis* infection, which indicate that *Pneumocystis* infection activates helper T cells and promotes cytokine production (Otieno-Odhiambo et al., 2019). However, the modulation of TCR immune repertoires in PCP remains unclear. Concordant with previous studies, we revealed that *Pneumocystis* induced the enrichment of effector $CD4^+$ T cells. By quantifying the clonal cells, we found that the highly expanded clones were mainly composed of $CD4^+$ T cells. In addition, *Pneumocystis* infection induced the proliferation of clonal $CD4^+$ T cells, and the expansion degree of clonal $CD4^+$ T cells was greater than that of $CD8^+$ T cells. In combination with single-cell transcriptomics, we discovered that the clonal $CD4^+$ T cells expressed genes were associated with cytokine, chemokine signaling, and cell adhesion pathway, whereas CD8 cells mainly expressed cytotoxic effector genes. The clonally expanded $CD4^+$ T cells highly expressed immune checkpoint

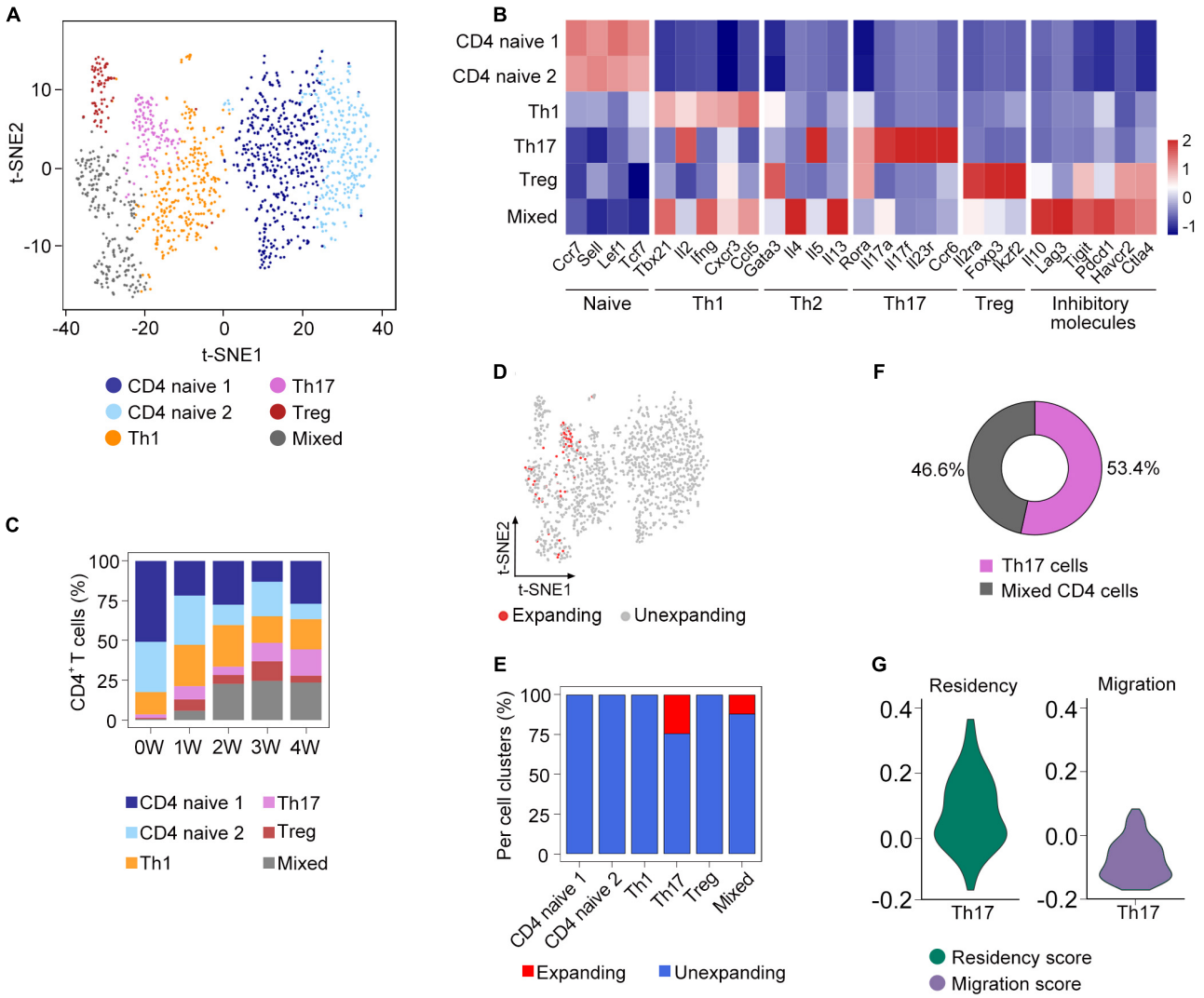

FIGURE 3 | Sub-clustering analysis of clonal CD4$^+$ T cells. **(A)** t-SNE plot of the subsets of CD4$^+$ T cells from all samples. **(B)** The expression of selected genes in the subsets of CD4$^+$ T cells. **(C)** Fraction of each cell subset across five samples. **(D)** Distribution of clonally expanded CD4$^+$ T cells on the t-SNE plot. **(E)** Proportion of clonally expanded cells in each CD4$^+$ T cells subsets. **(F)** The composition of clonally expanded CD4$^+$ T cells. **(G)** Vlnplot showing CD4$^+$ T cells' migration and tissue residency score of all Th17 cells from five samples.

molecules, including Pdcd1, Lag3, and Ctla4, which confirmed our recent findings of elevated expression of PD-1/PD-L1 in PCP. Zhang et al. (2020) also found anti-PD-1 antibody could promote the clearance of *Pneumocystis*, which might be due to the inhibition of expansion in these clonal CD4 cells with highly expressed inhibitory checkpoints.

In addition, we discovered the decreased diversity of CD4$^+$ T cells and increased diversity of CD8$^+$ T cells in PCP, which suggested differences in the roles of CD4$^+$ and CD8$^+$ T cells in response to *Pneumocystis*. Prior studies have shown that CD4$^+$ T cell-mediated response is absolutely pivotal in controlling *Pneumocystis* infection (Kelly and Shellito, 2010), as we observed clonal expansion and decreased diversity of CD4$^+$ T cells. However, the role of CD8$^+$ T cells in PCP remains controversial, with researchers describing both protective and detrimental functions (McAllister et al., 2004; Gigliotti et al., 2006; Zhang et al., 2019). In our study, as shown in **Figure 2A**, little clonal expansion was observed in CD8$^+$ T cells after the *Pneumocystis* challenge.

The sub-clustering analysis of CD4$^+$ T cells revealed that *Pneumocystis* induced the accumulation of Th17 cells, and clonally CD4$^+$ T cells were mostly represented as Th17 cells. Concordant to our results, previous studies have demonstrated that neutralizing IL-17A would lead to a significant increase in *Pneumocystis* burden in the lungs (Rudner et al., 2007), suggesting that Th17 response play a critical role in *Pneumocystis* clearance. In addition, we observed that Th17 cells might resemble the phenotype of Trm17 cells. Recent studies have shown that Trm17 cells could be induced in the lungs following bacterial or fungi infection. Trm17 cells were thought to play a

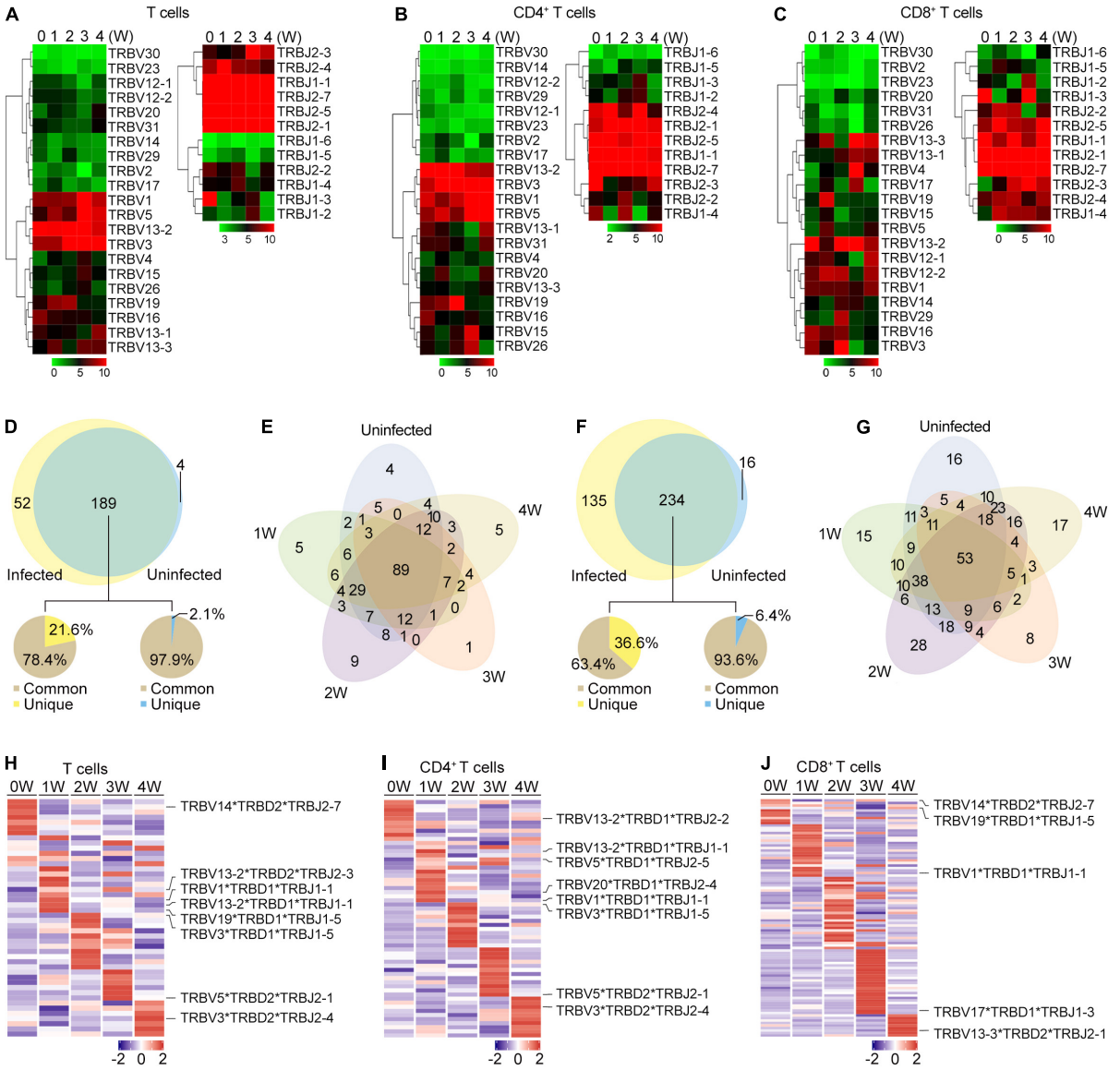

FIGURE 4 | TRB gene usage in *Pneumocystis* infected and uninfected mice. **(A–C)** Heat maps of V and J gene usage for TRB in T cells **(A)**, CD4$^+$ T cells **(B)**, and CD8$^+$ T cell **(C)** at different timepoints. **(D)** Venn diagram showing the common and unique V-J gene pairs of *Pneumocystis*-infected mice and uninfected controls. Pie charts showing the proportion of cells that harbored common and unique V-J pairs in *Pneumocystis*-infected mice (left) and uninfected controls (right). **(E)** Venn diagram showing the common and unique V-J gene pairs of mice infected with *Pneumocystis* from 0 to 4 weeks. **(F)** Common and unique V-D-J gene pairs of *Pneumocystis*-infected mice and uninfected controls. Pie charts showing the proportion of cells that harbored common and unique V-D-J pairs in *Pneumocystis*-infected mice (left) and uninfected controls (right). **(G)** Venn diagram showing the common and unique V-D-J gene pairs of mice infected with *Pneumocystis* from 0 to 4 weeks. **(H–J)** Heat maps showing the top 10 V-D-J gene combinations that were most frequently used in T cells **(H)**, CD4$^+$ T cells **(I)**, and CD8$^+$ T cells **(J)**, of which the most frequently used combinations are shown in labels.

crucial role in protecting from *Klebsiella* pneumonia infection (Amezcua Vesely et al., 2019). These results indicated the potential role of Trm17 cells in *Pneumocystis* clearance; however, its specific function in PCP requires further investigation.

Although the distribution of the CDR3 aa lengths remained similar after *Pneumocystis* infection, we found that the frequencies of some V and J gene segments significantly increased at different time points upon *Pneumocystis* infection, as well as the preferential usage of V-J and V-D-J gene combinations. Furthermore, through investigating the common and unique V-J and V-D-J combinations between all samples, we found that the unique combinations significantly increased in PCP compared to the control group. However, the function of these unique V-J and V-D-J combinations remains unclear.

While this work comprehensively delineates changes in clonal T cells and V(D)J gene usage for PCP, several key matters require validation. First, we only obtained data from mice infected with *Pneumocystis* at different timepoints, which is not sufficient

to reflect the pathogenesis of human PCP. Second, readily visible clonal expansion was observed in Th17 cells that had a phenotype resembling Trm17 cells after *Pneumocystis* infection. The number and function for Trm17 cells in PCP need to be further explored. V and J gene segments and combinations that significantly increased in PCP in this study also need validation. Finally, the limited sample size of our study is another limitation, therefore, the results of this study require validation using a larger PCP patient cohort.

In conclusion, investigations on T cell immunity have always been an important subject in PCP. Due to the lack of studies on the TCR repertoire of PCP, we performed scTCR-seq and scRNA-seq to comprehensively reveal the reconstructed TCR repertoire during *Pneumocystis* infection at the single-cell resolution and to track the clonal cells uniting TCR clonotype and transcriptome phenotype, thereby providing novel insights to improve our understanding of adaptive immune response in PCP.

DATA AVAILABILITY STATEMENT

The sequence data in this study were deposited in the Gene Expression Omnibus (GEO). The accession number of scTCR-seq data is GSE156843 and that of scRNA-seq is GSE157627.

ETHICS STATEMENT

The animal study was reviewed and approved by Capital Medical University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

H-QY conducted the experiments and drafted the manuscript. H-QY and Y-SW performed the data analysis. KZ and Z-HT designed the experiments, supervised the study, and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The National Natural Science Foundation of China (Grant no. 81870004) supported this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.637500/full#supplementary-material

Supplementary Figure 1 | *Pneumocystis* burden, TCR clones, and V/J gene segments usage at each time point. **(A)** Changes in *Pneumocystis* burden in mice 0–4 weeks post-infection. **(B–D)** The proportion of clonal cells ($n \geq 3$) in $CD4^+$ T cells **(B)**, T cells **(C)**, and $CD8^+$ T cells **(D)** at each time point. **(E–G)** The fraction of V and J gene frequency for TCRβ in T cells **(E)**, $CD4^+$ T cells **(F)**, and $CD8^+$ T cells **(G)** visualized using bar plots.

Supplementary Figure 2 | TRB V-J gene combinations in five samples. V-J gene combinations were labeled when the frequency was more than 1%.

Supplementary Figure 3 | Usage frequency of V-D-J gene combinations at each time point. **(A–C)** Heat maps showing usage frequency of V-D-J gene combinations in T cells **(A)**, $CD4^+$ T cells **(B)**, and $CD8^+$ T cells **(C)**.

Supplementary Table 1 | Gene list. This table provides the gene information of residency and migratory scores of $CD4^+$ T cells.

Supplementary Table 2 | Sequencing information of scTCR-seq. This table provides the information about scTCR-seq of all five samples, which were summarized by CellRanger.

Supplementary Table 3 | Frequency of V, D, and J gene segments in T, $CD4^+$ T, and $CD8^+$ T cells in each sample.

Supplementary Table 4 | Frequency of TRB V-J pairs for T, $CD4^+$ T, and $CD8^+$ T cells in each sample.

Supplementary Table 5 | Frequency of TRB V-D-J combinations in T, $CD4^+$ T, and $CD8^+$ T cells in each sample.

Supplementary Table 6 | A list of common and unique V-J combinations in **Figure 4E** and V-D-J combinations in **Figure 4G**.

Supplementary Table 7 | The most frequently used V-J and V-D-J combinations for T, $CD4^+$ T and $CD8^+$ T cells across all samples.

REFERENCES

Amezcua Vesely, M. C., Pallis, P., Bielecki, P., Low, J. S., Zhao, J., Harman, C. C. D., et al. (2019). Effector TH17 Cells Give Rise to Long-Lived TRM cells that are essential for an immediate response against bacterial infection. *Cell* 178, 1176.e15–1188.e15. doi: 10.1016/j.cell.2019.07.032

Azizi, E., Carr, A. J., Plitas, G., Cornish, A. E., Konopacki, C., Prabhakaran, S., et al. (2018). Single-cell map of diverse immune phenotypes in the breast tumor microenvironment. *Cell* 174, 1293.e36–1308.e36. doi: 10.1016/j.cell.2018.05.060

Blake, J. A., Eppig, J. T., Kadin, J. A., Richardson, J. E., Smith, C. L., Bult, C. J., et al. (2017). Mouse Genome Database (MGD)-2017: community knowledge resource for the laboratory mouse. *Nucleic Acids Res.* 45, D723–D729. doi: 10.1093/nar/gkw1040

Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018). Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* 36, 411–420. doi: 10.1038/nbt.4096

Cilloniz, C., Dominedo, C., Alvarez-Martinez, M. J., Moreno, A., Garcia, F., Torres, A., et al. (2019). Pneumocystis pneumonia in the twenty-first century: HIV-infected versus HIV-uninfected patients. *Expert Rev. Anti. Infect. Ther.* 17, 787–801. doi: 10.1080/14787210.2019.1671823

Garvy, B. A., Wiley, J. A., Gigliotti, F., and Harmsen, A. G. (1997). Protection against Pneumocystis carinii pneumonia by antibodies generated from either T helper 1 or T helper 2 responses. *Infect. Immun.* 65, 5052–5056. doi: 10.1128/IAI.65.12.5052-5056.1997

Gigliotti, F., Crow, E. L., Bhagwat, S. P., and Wright, T. W. (2006). Sensitized CD8+ T cells fail to control organism burden but accelerate the onset of lung injury during *Pneumocystis carinii* pneumonia. *Infect. Immun.* 74, 6310–6316. doi: 10.1128/IAI.00668-06

Kelly, M. N., and Shellito, J. E. (2010). Current understanding of pneumocystis immunology. *Future Microbiol.* 5, 43–65. doi: 10.2217/fmb.09.116

Kuo, H. C., Pan, C. T., Huang, Y. H., Huang, F. C., Lin, Y. S., Li, S. C., et al. (2019). Global investigation of immune repertoire suggests kawasaki disease has infectious cause. *Circ. J.* 83, 2070–2078. doi: 10.1253/circj.CJ-19-0206

Lee, H. Y., Choi, S. H., Kim, T., Chang, J., Kim, S. H., Lee, S. O., et al. (2019). Epidemiologic trends and clinical features of pneumocystis jirovecii pneumonia

in non-HIV patients in a tertiary-care hospital in Korea over a 15-year-period. *Jpn. J. Infect. Dis.* 72, 270–273. doi: 10.7883/yoken.JJID.2018.400

Li, T., Rong, H. M., Zhang, C., Zhai, K., and Tong, Z. H. (2018). IL-9 deficiency promotes pulmonary Th17 response in murine model of pneumocystis infection. *Front. Immunol.* 9:1118. doi: 10.3389/fimmu.2018.01118

Li Pira, G., Bottone, L., Fenoglio, D., Terranova, P., Pontali, E., Ivaldi, F., et al. (2001). Analysis of the antigen specific T cell repertoires in HIV infection. *Immunol. Lett.* 79, 85–91. doi: 10.1016/s0165-2478(01)00269-3

Liao, M., Liu, Y., Yuan, J., Wen, Y., Xu, G., Zhao, J., et al. (2020). Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19. *Nat. Med.* 26, 842–844. doi: 10.1038/s41591-020-0901-9

McAllister, F., Steele, C., Zheng, M., Young, E., Shellito, J. E., Marrero, L., et al. (2004). T cytotoxic-1 CD8+ T cells are effector cells against pneumocystis in mice. *J. Immunol.* 172, 1132–1138. doi: 10.4049/jimmunol.172.2.1132

McKinley, L., Logar, A. J., McAllister, F., Zheng, M., Steele, C., and Kolls, J. K. (2006). Regulatory T cells dampen pulmonary inflammation and lung injury in an animal model of pneumocystis pneumonia. *J. Immunol.* 177, 6215–6226. doi: 10.4049/jimmunol.177.9.6215

Nikolich-Zugich, J., Slifka, M. K., and Messaoudi, I. (2004). The many important facets of T-cell repertoire diversity. *Nat. Rev. Immunol.* 4, 123–132. doi: 10.1038/nri1292

Otieno-Odhiambo, P., Wasserman, S., and Hoving, J. C. (2019). The contribution of host cells to pneumocystis immunity: an update. *Pathogens* 8:52. doi: 10.3390/pathogens8020052

Reyfman, P. A., Walter, J. M., Joshi, N., Anekalla, K. R., McQuattie-Pimentel, A. C., Chiu, S., et al. (2019). Single-cell transcriptomic analysis of human lung provides insights into the pathobiology of pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* 199, 1517–1536. doi: 10.1164/rccm.201712-2410OC

Rilinger, J., Staudacher, D. L., Rieg, S., Duerschmied, D., Bode, C., and Wengenmayer, T. (2019). Extracorporeal membrane oxygenation in *Pneumocystis jirovecii* pneumonia: outcome in HIV and non-HIV patients. *Crit. Care* 23:356. doi: 10.1186/s13054-019-2661-9

Roblot, F., Godet, C., Le Moal, G., Garo, B., Faouzi Souala, M., Dary, M., et al. (2002). Analysis of underlying diseases and prognosis factors associated with *Pneumocystis carinii* pneumonia in immunocompromised HIV-negative patients. *Eur. J. Clin. Microbiol. Infect. Dis.* 21, 523–531. doi: 10.1007/s10096-002-0758-5

Rong, H. M., Li, T., Zhang, C., Wang, D., Hu, Y., Zhai, K., et al. (2019). IL-10-producing B cells regulate Th1/Th17-cell immune responses in *Pneumocystis pneumonia*. *Am. J. Physiol. Lung Cell Mol. Physiol.* 316, L291–L301. doi: 10.1152/ajplung.00210.2018

Roux, A., Canet, E., Valade, S., Gangneux-Robert, F., Hamane, S., Lafabrie, A., et al. (2014). Pneumocystis jirovecii pneumonia in patients with or without AIDS, France. *Emerg. Infect. Dis.* 20, 1490–1497. doi: 10.3201/eid2009.131668

Rudner, X. L., Happel, K. I., Young, E. A., and Shellito, J. E. (2007). Interleukin-23 (IL-23)-IL-17 cytokine axis in murine *Pneumocystis carinii* infection. *Infect. Immun.* 75, 3055–3061. doi: 10.1128/IAI.01329-06

Shoji, K., Michihata, N., Miyairi, I., Matsui, H., Fushimi, K., and Yasunaga, H. (2020). Recent epidemiology of *Pneumocystis pneumonia* in Japan. *J. Infect. Chemother.* 26, 1260–1264. doi: 10.1016/j.jiac.2020.07.006

Waickman, A. T., Victor, K., Li, T., Hatch, K., Rutvisuttinunt, W., Medin, C., et al. (2019). Dissecting the heterogeneity of DENV vaccine-elicited cellular immunity using single-cell RNA sequencing and metabolic profiling. *Nat. Commun.* 10:3666. doi: 10.1038/s41467-019-11634-7

Wang, P., Jin, X., Zhou, W., Luo, M., Xu, Z., Xu, C., et al. (2021). Comprehensive analysis of TCR repertoire in COVID-19 using single cell sequencing. *Genomics* 113, 456–462. doi: 10.1016/j.ygeno.2020.12.036

Wolock, S. L., Lopez, R., and Klein, A. M. (2019). Scrublet: computational identification of cell doublets in single-cell transcriptomic data. *Cell Syst.* 8, 281.e9–291.e9. doi: 10.1016/j.cels.2018.11.005

Yost, K. E., Satpathy, A. T., Wells, D. K., Qi, Y., Wang, C., Kageyama, R., et al. (2019). Clonal replacement of tumor-specific T cells following PD-1 blockade. *Nat. Med.* 25, 1251–1259. doi: 10.1038/s41591-019-0522-3

Zhang, C., Rong, H. M., Li, T., Zhai, K., and Tong, Z. H. (2020). PD-1 deficiency promotes macrophage activation and T-helper cell type 1/T-Helper Cell Type 17 response in pneumocystis pneumonia. *Am. J. Respir. Cell Mol. Biol.* 62, 767–782. doi: 10.1165/rcmb.2019-0234OC

Zhang, N. N., Huang, X., Feng, H. Y., Huang, L. N., Xia, J. G., Wang, Y., et al. (2019). Circulating and pulmonary T-cell populations driving the immune response in non-HIV immunocompromised patients with *Pneumocystis jirovecii* pneumonia. *Int. J. Med. Sci.* 16, 1221–1230. doi: 10.7150/ijms.34512

Zhao, Y., Kilian, C., Turner, J. E., Bosurgi, L., Roedl, K., Bartsch, P., et al. (2021). Clonal expansion and activation of tissue-resident memory-like Th17 cells expressing GM-CSF in the lungs of severe COVID-19 patients. *Sci. Immunol.* 6:eabf6692. doi: 10.1126/sciimmunol.abf6692

Zheng, C., Zheng, L., Yoo, J. K., Guo, H., Zhang, Y., Guo, X., et al. (2017). Landscape of infiltrating t cells in liver cancer revealed by single-cell sequencing. *Cell* 169, 1342.e16–1356.e16. doi: 10.1016/j.cell.2017.05.035

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 May 2021
doi: 10.3389/fmicb.2021.636250

Signatures of B Cell Receptor Repertoire Following *Pneumocystis* Infection

Han Sun, Hu-Qin Yang, Kan Zhai and Zhao-Hui Tong**

Department of Respiratory and Critical Care Medicine, Beijing Institute of Respiratory Medicine, Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China

B cells play vital roles in host defense against *Pneumocystis* infection. However, the features of the B cell receptor (BCR) repertoire in disease progression remain unclear. Here, we integrated single-cell RNA sequencing and single-cell BCR sequencing of immune cells from mouse lungs in an uninfected state and 1–4 weeks post-infection in order to illustrate the dynamic nature of B cell responses during *Pneumocystis* infection. We identified continuously increased plasma cells and an elevated ratio of (IgA + IgG) to (IgD + IgM) after infection. Moreover, *Pneumocystis* infection was associated with an increasing naïve B subset characterized by elevated expression of the transcription factor *ATF3*. The proportion of clonal expanded cells progressively increased, while BCR diversity decreased. Plasma cells exhibited higher levels of somatic hypermutation than naïve B cells. Biased usage of V(D)J genes was observed, and the usage frequency of *IGHV9-3* rose. Overall, these results present a detailed atlas of B cell transcriptional changes and BCR repertoire features in the context of *Pneumocystis* infection, which provides valuable information for finding diagnostic biomarkers and developing potential immunotherapeutic targets.

Keywords: *Pneumocystis* infection, B cell receptor, single-cell BCR sequencing, single-cell RNA sequencing, somatic hypermutation

OPEN ACCESS

Edited by:
Allan J. Guimaraes, Fluminense Federal University, Brazil

Reviewed by:
Raz Somech, Sheba Cancer Center, Sheba Medical Center, Israel
Xiao Liu, Tsinghua University, China

****Correspondence:***
Kan Zhai
zhaikan@ccmu.edu.cn
Zhao-Hui Tong
tongzhaohuicy@sina.com

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *01 December 2020*
Accepted: *01 April 2021*
Published: *31 May 2021*

Citation:
Sun H, Yang H-Q, Zhai K and Tong Z-H (2021) Signatures of B Cell Receptor Repertoire Following Pneumocystis Infection. Front. Microbiol. 12:636250. doi: 10.3389/fmicb.2021.636250

INTRODUCTION

Pneumocystis jirovecii pneumonia (PJP) is a severe opportunistic infectious disease found in immunocompromised patients (Schmidt et al., 2018). The incidence of PJP has been increasing in patients without human immunodeficiency virus (HIV) infection due to the widespread use of immunosuppressive medications (Bienvenu et al., 2016). Compared to HIV cases, the symptoms observed in HIV-negative PJP patients are more severe and abrupt, and the clinical outcome is worse (Guo et al., 2014), which indicates the significance of understanding the cellular and molecular basis of disease progression and developing better treatment strategies.

B cells have been demonstrated to play a vital role in host defense against *Pneumocystis* infection by antigen presentation and antibody production (Kolls, 2017; Otieno-Odhiambo et al., 2019). The B cell receptor (BCR), a B cell surface membrane immunoglobulin, possesses the

Abbreviations: ATF3, activating transcription factor-3; BCR, B cell receptor; CDR3, complementarity-determining region 3; DEGs, differentially expressed genes; HIV, human immunodeficiency virus; IGH, immunoglobulin heavy chains; IGK, immunoglobulin light chains kappa; IGL, immunoglobulin light chains lambda; PJP, *Pneumocystis jirovecii* pneumonia; SHM, somatic hypermutation; scRNA-seq, single-cell RNA sequencing; UMAP, uniform manifold approximation and projection.

ability to specifically recognize and bind antigens, with the complementarity-determining region 3 (CDR3) of the heavy chain as the major determinant of antibody specificity (Xu and Davis, 2000). The diversity of BCR repertoires in several infectious diseases has been explored, such as in coronavirus disease 2019 (Wen et al., 2020), dengue (Parameswaran et al., 2013), and chronic hepatitis C virus infection (Tucci et al., 2018). These results show that encountering a specific antigen could elicit clonal B-cell proliferation, thus altering the selective distribution of the BCR spectrum. *Pneumocystis*-specific BCR was reported to be required for adequate priming of T cells against *Pneumocystis*, rather than the mere presence of B cells (Opata et al., 2015), indicating the indispensable role of antigen-specific BCR in *Pneumocystis* infection. However, the specific characteristics of the BCR repertoire after *Pneumocystis* infection remains unknown.

The combination of single-cell RNA sequencing (scRNA-seq) and single-cell BCR sequencing enables us to simultaneously investigate the heterogeneity of the transcriptome and analyze the BCR features of B cell clones, which may lead to new insights into the host immune response (Zheng et al., 2017; Goldstein et al., 2019). Here, we integrated the transcriptome data and single-cell paired BCR analysis of B cells from mice lungs at different timepoints during *Pneumocystis* infection, aiming to elucidate the *Pneumocystis*-specific shuffling of the BCR repertoire and phenotypic characteristics of B cell clones.

MATERIALS AND METHODS

Mice and *Pneumocystis* Lung Infection

C57BL/6 mice and severe combined immunodeficient mice (6–8 weeks of age) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Animals were housed in specific pathogen-free conditions. *Pneumocystis murina* (ATCC, Manassas, VA, United States) was maintained in severe combined immunodeficient mice as previously described (Hu et al., 2017). Mouse models of *Pneumocystis* infection were prepared by intratracheally inoculating with 1×10^6 cysts diluted in 100 μl phosphate-buffered saline under anesthesia, while non-infected mice were inoculated with 100 μl phosphate-buffered saline (Rong et al., 2019a). The animal studies were approved by the Capital Medical University Animal Care and Use Committee.

Preparation of Single-Cell Suspensions and Cell Sorting

At specific time points, mice were sacrificed by exsanguination under deep anesthesia. Lungs were cut into pieces and transferred to a digestion medium containing DNAse I (Sigma), collagenase IV (Solarbio), and 10% fetal bovine serum in RPMI 1640 medium. After incubation for 20 min at 37°C with manual shaking every 5 min, samples were filtered with 40-μm nylon mesh (Biologix) and centrifuged at 400 × g for 6 min. Red blood cells were removed by lysing buffer (BD). Following surface-staining with Ghost Dye Red 780 (TONBO) and anti-mouse CD45 PerCP-Cyanine5.5 (BD) according to the operations manual, single-cell suspensions of the lung tissue from three mice were mixed together. Afterward, cell sorting was performed on a FACS Aria II (BD Biosciences) and $CD45^+$ living cells were collected.

scRNA-Seq and Preprocessing

Using Single Cell 5′ Library and Gel Bead kit (10x Genomics) and Chromium Single Cell A Chip kit (10x Genomics), cell suspensions were loaded onto a Chromium single cell controller (10x Genomics) to generate single-cell gel beads in the emulsion. The RNA of captured cells was released and barcoded through reverse transcription, and then the complementary DNA was amplified to establish the 5′ gene expression libraries. An Agilent 4200 system was used for quality assessment and after that, the libraries were sequenced using an Illumina Novaseq sequencer. Raw gene expression matrices were generated by Cell Ranger count pipeline with default parameters and mouse GRCm38/mm10 as the reference genome. The dataset integration of all five samples was achieved by Cell Ranger AGGR.

Single-Cell Transcriptome Data Analysis

R software (v.4.0.2) with the Seurat (Butler et al., 2018) package (v.3.2.2) was applied for quality control. Low-quality cells were discarded if they met the following criteria: (1) the number of genes expressed were <200 or more than 4,000, or (2) > 25% unique molecular identifiers were derived from the mitochondrial genome. After normalizing the gene expression matrices by log normalization, the top 2,000 highly variable genes were calculated via the FindVariableFeatures function. The RunPCA function was conducted for linear dimensionality reduction, and non-linear dimensional reduction was performed with the RunUMAP function. Finally, cells were clustered together according to common features. The corresponding cell types of cell clusters were annotated based on the expressions of canonical markers.

Differential Gene Expression Analysis and Functional Enrichment

Differential gene expression analysis was performed using the FindMarkers function in Seurat by a two-sided Wilcoxon test with Bonferroni correction. Genes with a maximum adjusted *p*-value of 0.01 and an absolute value of $\log_2$(fold change) > 0.5 were considered to be differentially expressed genes (DEGs) (Zhang J.Y. et al., 2020). Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses of DEGs were conducted using the Metascape (Zhou et al., 2019) webtool[1].

Single Cell Trajectory Analysis

We used the Monocle 2 (Qiu et al., 2017) package (version 2.16.0) to conduct pseudotime analysis. Ordering genes were calculated by the "differentialGeneTest" function ($q < 0.01$). To generate a two-dimensional projection of the underlying developmental trajectory, the "DDRTree" reduction method was used with default parameters. The "orderCells" function was applied to obtain cell ordering (pseudotime) along the lineage trajectory and

[1]www.metascape.org

the visualization function "plot_cell_trajectory" was used to plot the minimum spanning tree.

Single-Cell BCR V(D)J Sequencing and Analysis

Full-length BCR V(D)J segments were enriched from amplified cDNA from 5′ libraries using a Chromium Single-Cell V(D)J Enrichment kit according to the manufacturer's protocol. BCR sequences for each single B cell were assembled by Cell Ranger vdj pipeline (v.3.0.2). Only cells with both productive immunoglobulin heavy chains (IGH) and productive immunoglobulin light chains kappa (IGK) or lambda (IGL) were kept. If more than one heavy chain or light chain were detected in one cell, the chain with the highest amount of unique molecular identifiers was retained (Zheng et al., 2017).

A clonotype was defined as a unique combination of an IGH-IGK/IGL pair. A cell was considered to be clonally expanded if its clonotype was shared by at least two cells. The clonality of a clonotype could be indicated by the number of cells with the same clonotype. Using barcode information, B cells with prevalent BCR clonotypes were projected on a uniform manifold approximation and projection (UMAP) plot. The somatic hypermutation (SHM) rate of each B cell was defined as the percentage of mismatching nucleotides in the VH portion compared with the most similar germline gene (Kuri-Cervantes et al., 2020). Lineage analysis was performed on the protein sequences of the CDR3 region in both heavy and light chains. BCR sequences with the same V(D)J assignment were grouped together into a lineage if their CDR3 sequences differed by no more than one amino acid (Jiang et al., 2013). Plots of the lineage structures were generated with Cytoscape (v.3.5.1).

RESULTS

Study Design and Single-Cell Profiling of B Cells

To assess the signatures of B-cell receptor diversity in B lymphocytes following *Pneumocystis* infection, we performed scRNA-seq and single-cell BCR sequencing on $CD45^+$ immune cells from the lung tissue of uninfected mice (0 w) and mouse models at 1–4 weeks post-infection (1 w, 2 w, 3 w, and 4 w). Each sample contained $CD45^+$ cells from three mice. Based on the single-cell transcriptome data, a total of 11,062 B cells were obtained after filtering. We integrated the scRNA-seq and single-cell paired BCR analysis for each subject, and only cells with full-length productive paired IGH-IGK/IGL chains were retained. Finally, 3,280 B lymphocytes were included for subsequent analysis.

Features of B Cell Subsets After *Pneumocystis* Infection

UMAP clustering analyses were performed on B lymphocytes, yielding four cell clusters (**Figure 1A**). According to the differential expression of canonical genes, B cells were categorized into naïve B (clusters 1, 2, and 3) and plasma cells (cluster 4). Naïve B cells expressed high levels of *IGHD* and *MS4A1*, while plasma cells were identified on the basis of high expression of *XBP1* and *SDC1* (**Figure 1B**).

We then explored the gene expression pattern of each B cell subset (**Figure 1C**) and the relative frequencies of the four clusters among each sample (**Figure 1D**). Cluster 1 accounted for the largest portion of B cells in each sample. *IGHD* and homing marker *SELL* were present at high levels in cluster 1, suggesting that cluster 1 was retained in a relatively naïve state. Genes related to B cell activation (*CD79B*, *MS4A1*, and *CD24A*) were markedly expressed in cluster 2 (**Figure 1C** and **Supplementary Figure 1A**). DEGs preferentially expressed in cluster 2 were mainly enriched in B cell activation and B cell differentiation (**Supplementary Figure 1B**), indicating that cluster 2 was in a relatively activated state. The proportion of cluster 3 in total B cells presented an increasing trend over time, from 5.7% in the uninfected sample to 16.0% in the 4 w sample. Activating transcription factor-3 (ATF3), a key regulator of inflammatory responses, was significantly upregulated in cluster 3 (**Figure 1E**). In addition, the high level of *TAGLN2* expression, which was reported to be a potential marker of activated B cells (Kiso et al., 2017), implied that cluster 3 was also activated to some extent. DEGs significantly enriched in cluster 3 were associated with regulation of transmembrane transport, cell adhesion, and Th17 cell differentiation (**Figure 1F**), indicating the effector functions of cluster 3. Compared with that in the uninfected mice, the percentage of plasma cells rose continuously during the infection process, suggesting a key role for plasma cells in the control of *Pneumocystis* infection and the development of adaptive immunity by inducing specific antibodies.

In order to discover the cell-state transitions, we performed a pseudotime analysis using Monocle 2, an algorithm for the lineage reconstruction of biological processes based on transcriptional similarity (**Figure 1G**). A trajectory of B cell populations was ordered from a starting point of cluster 1 and bifurcated into two diverse branches, with clusters 2 and 3 in one terminal, while plasma cells were in the other terminal, representing two major cell lineages in the process of B cell differentiation after *Pneumocystis* infection. The cell trajectory analysis supported the above speculation that cluster 1 is in a more primitive stage, which could transit to relatively activated naïve B subsets (clusters 2 and 3) and plasma cells (cluster 4) in response to *Pneumocystis* antigenic stimulation.

Clonal Expansion and IgH Class Switching of B Cells After *Pneumocystis* Infection

To quantitatively assess the clonal diversity of B cells, the D50 value (Kuo et al., 2019) was calculated. The D50 value of the 0 w sample was 49.9, which was higher than the infected samples (49.8, 48.3, 48.0, and 47.3 for the 1 w, 2 w, 3 w, and 4 w samples, respectively). The D50 value analysis showed that B-cell diversity decreased after *Pneumocystis* infection.

We then evaluated the distribution of clonal expanded B cells. As seen in the UMAP projections, clonal expanded B cells mainly belonged to plasma cells (79.2%), while naïve B cells exhibited

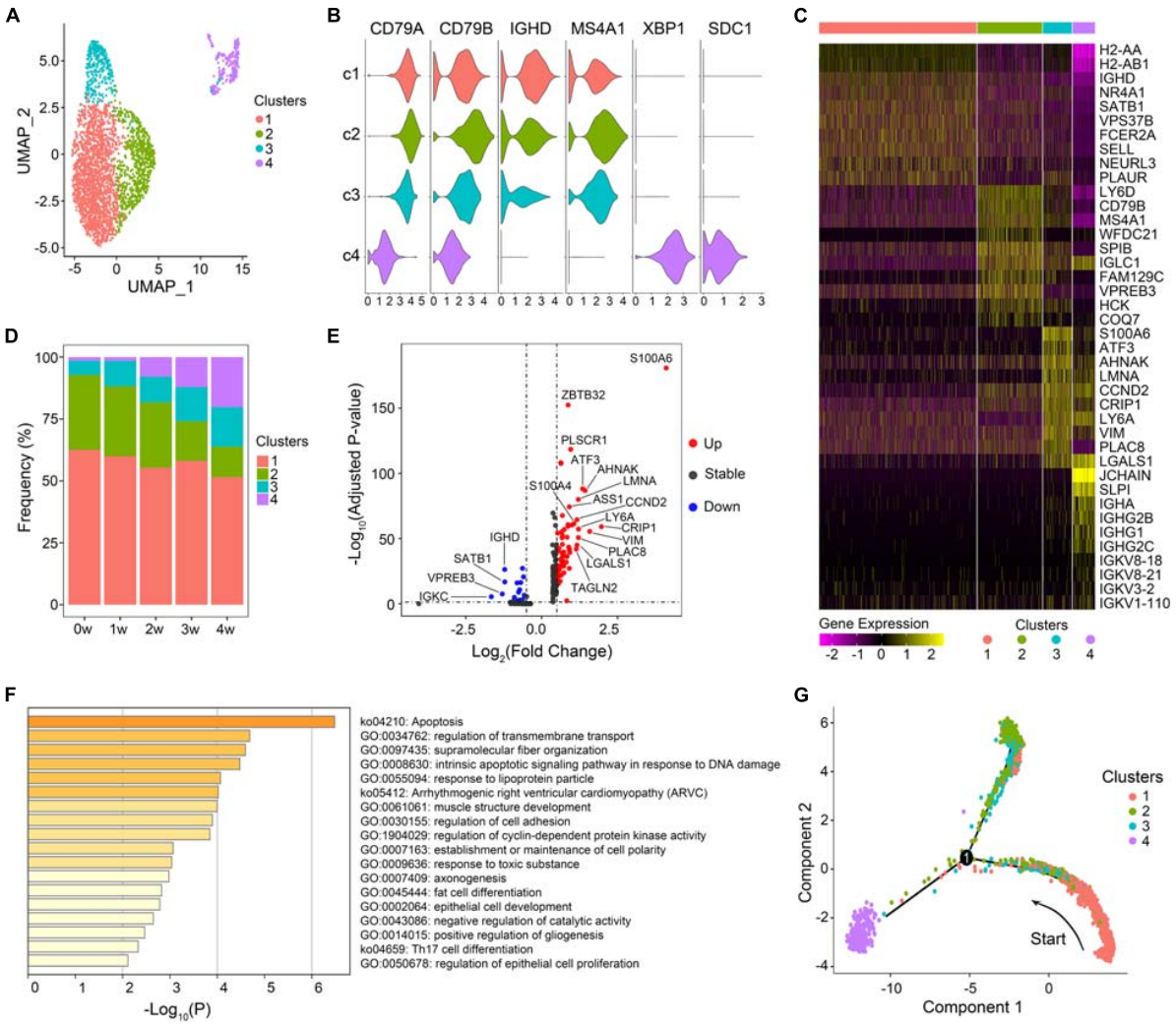

FIGURE 1 | Features of B cell subsets. **(A)** Uniform manifold approximation and projection (UMAP) projection of B cells from all five samples. Each dot corresponds to a single cell, colored according to cell clusters. **(B)** Violin plots show expression distribution of canonical cell markers in B cell subsets. **(C)** The expression of the top 10 marker genes of each cluster. **(D)** Proportion of B cell clusters in each sample. **(E)** Differentially expressed genes of cluster 3 in comparison with all other cells. Genes with a maximum adjusted *p*-value of 0.01 and an absolute value of $\log_2$(fold change) > 0.5 were considered to be differentially expressed genes. **(F)** Gene enrichment analyses of upregulated differentially expressed genes of cluster 3. **(G)** Trajectory reconstruction of all B cells through pseudotime analysis.

little clonality (**Figure 2A**). The percentage of clonal expanded cells was 4.6% in ATF3$^+$ naïve B cells (cluster 3), which is higher than those in the other 2 subclusters of naïve B cells, both 0.4%. According to the bar plot (**Figure 2B**), the proportion of clonal cells progressively increased from 0.6% (0 w) to 7.5% (4 w) after *Pneumocystis* infection. Among the B cell clusters, 93.1% of naïve B cells owned a unique BCR clonotype, while 36.9% of plasma cells showed clonal expansions (**Figure 2C**).

IgH class switching occurs after responding to antigens and confers distinct functional characteristics to B cells (Stavnezer and Schrader, 2014). The delineation of isotypes is therefore essential for the comprehensive analysis of the BCR repertoire. To further investigate the dynamic changes of IgH class switching after *Pneumocystis* infection, we evaluated the distribution of IgA, IgD, IgG, and IgM, as IgE was not detected (**Figure 2D**). A total of 64.7% of naïve B cells expressed the IgM isotype, followed by IgD (32.2%), IgG (1.6%), and IgA (0.7%). Plasma cells preferentially expressed the IgG isotype (64.9%), and IgA made up a relatively small proportion (25.4%; **Figure 2E**). IgM remained the predominant immunoglobulin, whereas the abundance of IgG apparently increased with the disease progression (**Figure 2F**). Compared to the uninfected state, the ratio of (IgA + IgG) to (IgD + IgM) increased after infection, suggesting that *Pneumocystis* induced an intensive antibody response.

SHM in the immunoglobulin variable gene allows for the generation of high-affinity antibodies (Wrammert et al., 2008). We assessed the SHM of VH portion in every unique sequence. The mutation rate was divided into four groups (0, 0–1%, 1–10%, and > 10%), which are shown in the UMAP plot (**Figure 2G**). Less than 10% of B cells exhibited SHM in sample 0 w and

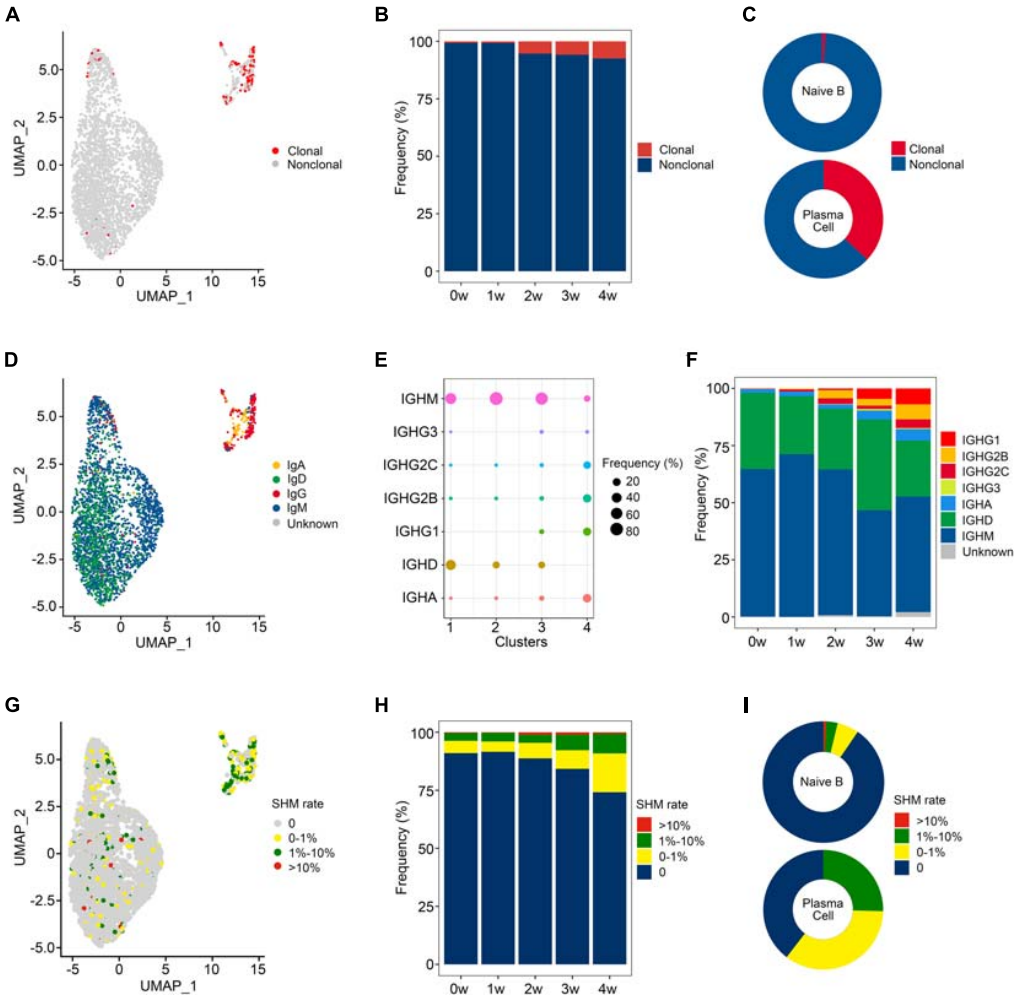

FIGURE 2 | Clonal expansion and immunoglobulin class switching of B cells after *Pneumocystis* infection. **(A)** Uniform manifold approximation and projection (UMAP) plot shows the distribution of clonal expanded B cells. **(B)** The proportion of clonal expanded B cells in each sample. **(C)** The pie plot shows the proportion of clonal expanded B cells in naïve B and plasma cells. **(D)** UMAP plot of B cells colored by immunoglobulin heavy chain expression. **(E)** The proportion of each immunoglobulin isotype in B cell subsets. **(F)** The proportion of different immunoglobulin isotype in each sample. **(G)** UMAP plot of B cells colored by somatic hypermutation (SHM) rate. **(H)** The proportion of B cells with SHM in each sample. **(I)** The pie plot shows the proportion of B cells with SHM in naïve B and plasma cells.

sample 1 w, while an increasing proportion of cells experienced SHM in the 2 w, 3 w, and 4 w samples (11.2%, 15.7%, and 25.8%, respectively, **Figure 2H**). A total of 90.5% of naïve B cells were unmutated, while 60.4% of plasma cells experienced SHM (**Figure 2I**).

CDR3 Length and Specific Rearrangements of V(D)J Genes After *Pneumocystis* Infection

We then calculated the CDR3 length of the BCR heavy chain, since the length of the CDR3 region affects the three-dimensional structure of the CDR3 ring, thus influencing antigen-binding specificity. The length of CDR3 in total B cells ranged from 6 to 25 amino acids (aa), with an average of 14 aa for each sample (**Figure 3A**). There was no significant difference in the CDR3 length between naïve B and plasma cells, and their average CDR3 aa lengths were both 14 aa. For naïve B cells, the length of CDR3 varied from 6 to 25 aa, and for plasma cells, the CDR3 length varied from 7 to 25 aa (**Figure 3B**).

To study biased V(D)J rearrangements of the BCR heavy chain, we determined differences in the usage frequency of the V, D, and J gene segments across all five samples. According to the sequencing results, a total of 96 IGHV gene segments, 13 IGHD gene segments, and 4 IGHJ gene segments were identified in the whole B cells.

We generated a distribution histogram of IGHJ genes usage frequency for total B cells and discovered that *IGHJ2* was most frequently used in the first four samples, while the usage of *IGHJ4* gradually increased from sample 1 w to sample 4 w, and became the most frequently used IGHJ gene segment in the 4 w sample. *IGHJ1* shared the lowest utilization (**Figure 3C**).

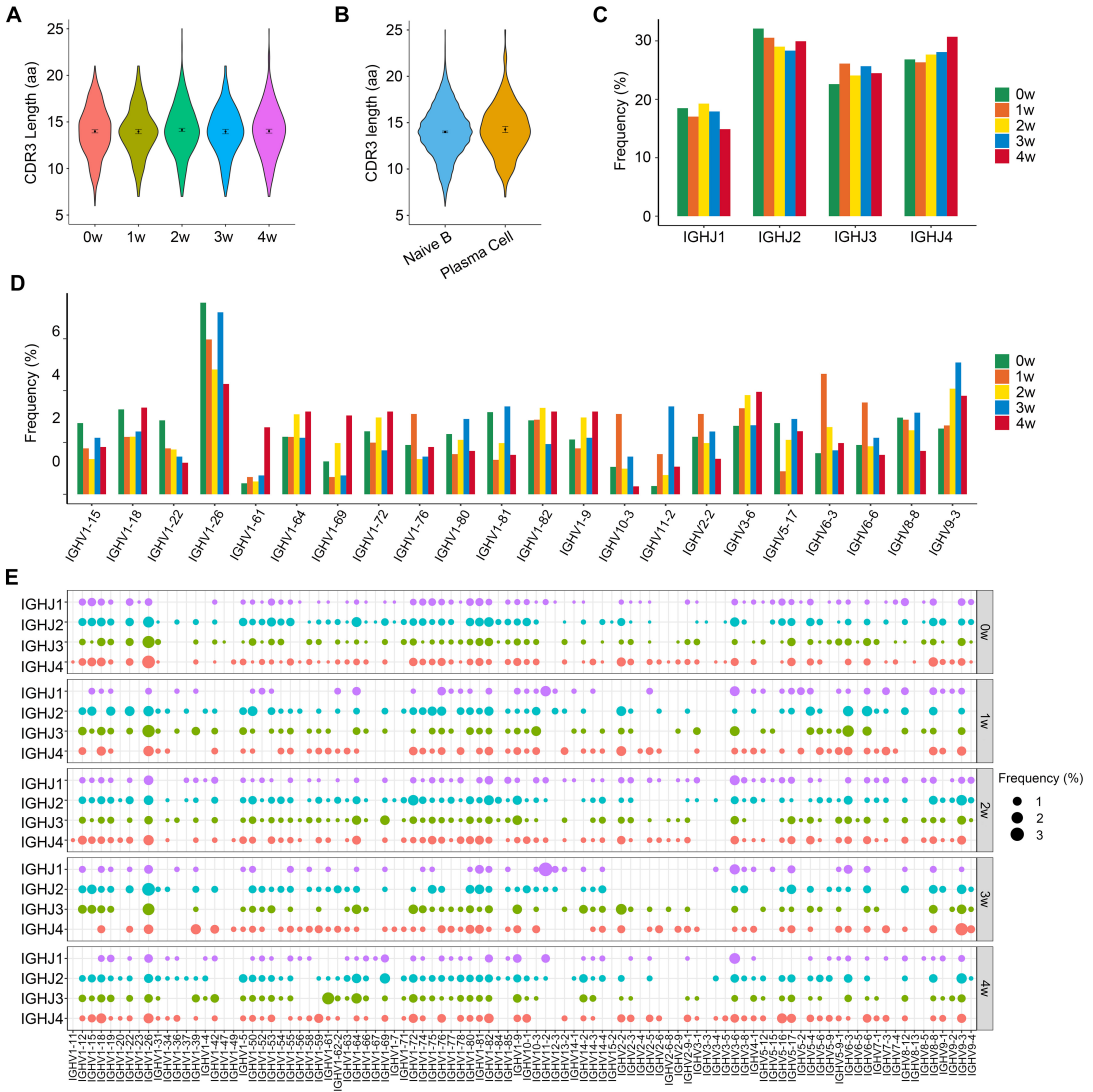

FIGURE 3 | The distribution of immunoglobin heavy chains (IGH) CDR3 length and selective usage of V and J genes. **(A)** Violin plots show the distribution of IGH CDR3 amino acid length in each sample. **(B)** Distribution of IGH CDR3 amino acid length in naïve B and plasma cells. **(C)** Selective usage of J gene segments in each sample. **(D)** Usage of some V gene segments in each sample. **(E)** Bubble chart shows the usage frequency of V–J gene combination in each sample.

The selective usage of IGHV genes was analyzed (**Figure 3D** and **Supplementary Figures 2A,B**). For total B cells, we detected an over-representation of the *IGHV1* family, especially *IGHV1-26*, which occupied the most frequent IGHV gene segment in each sample, with frequencies of 7.4%, 6.0%, 4.8%, 7.1%, and 4.3% for the 0 w, 1 w, 2 w, 3 w, and 4 w samples, respectively. It is remarkable that *IGHV9-3* was gradually enriched following the infectious process and peaked at 3 w post-infection (5.1%). In addition, we observed the over-representation of *IGHV6-3* in the 1 w sample (4.6%) and *IGHV 1-61* in the 4 w sample (2.6%).

A total of 332 unique V–J combinations were identified in the whole B cells (**Figure 3E**). The top paired V–J frequency in the uninfected sample was *IGHV1-26*/*IGHJ4* (2.6%), while those in the 1 w, 2 w, 3 w, and 4 w samples were *IGHV1-26*/*IGHJ3* (2.4%), *IGHV1-72*/*IGHJ2* (1.6%), *IGHV11-2*/*IGHJ1* (3.1%), and *IGHV1-61*/*IGHJ3* (2.3%), respectively. A total of 1,386 IGHV-IGHD-IGHJ rearrangements were found in all B cells. The most preferred rearrangements in the 0 w, 1 w, 2 w, 3 w, and 4 w samples were *IGHV1-26*/*IGHD1-1*/*IGHJ2* (0.7%), *IGHV1-76*/*IGHD1-1*/*IGHJ2* (1.0%), *IGHV3-6*/*IGHD1-1*/*IGHJ1* (1.1%), *IGHV11-2*/*IGHD2-6*/*IGHJ1* (2.7%), and *IGHV1-61*/*IGHD1-1*/*IGHJ3* (2.5%), respectively.

We also compared the CDR3 length and the usage of V and J genes in BCR light chains among each sample. Light chain analysis demonstrated preferential IGK use with an IGK/IGL ratio of 12.2:1. The average CDR3 lengths of BCR light chains were 11 aa in all five samples (**Figure 4A**). The usage pattern of J gene segments was displayed (**Figure 4B**), and shuffling of the V gene usage preference and V–J combinations in

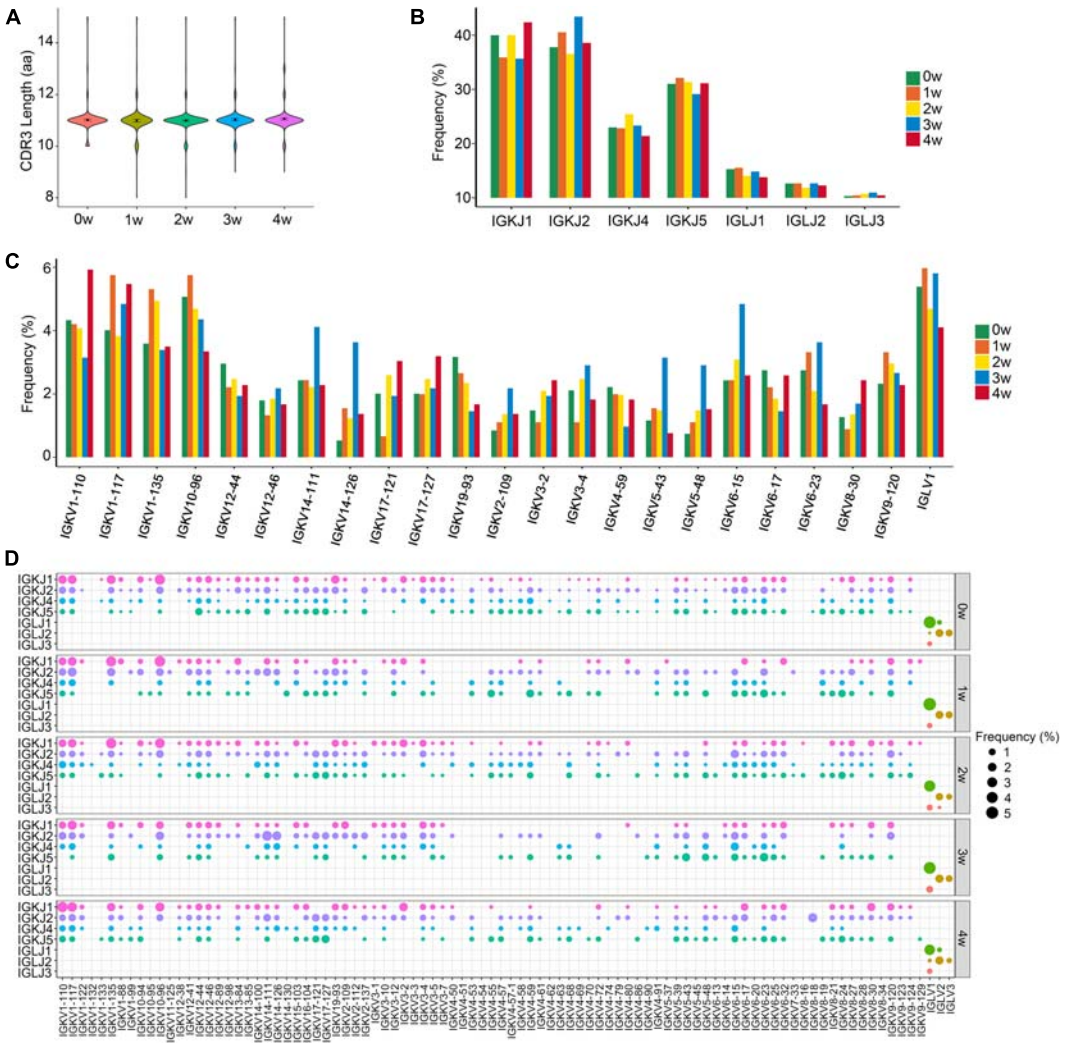

FIGURE 4 | The distribution of immunoglobin light chains kappa (IGK)/immunoglobin light chains lambda (IGL) CDR3 length and selective usage of V and J genes. **(A)** Distribution of IGK/IGL CDR3 amino acid length in total B cells. **(B)** Selective usage of J gene segments in total B cells. **(C)** Selective usage of some IGK/L V gene segments in each sample. **(D)** Bubble chart shows the usage frequency of V–J gene combination in each sample.

light chains was observed (**Figures 4C,D** and **Supplementary Figures 3A,B**).

In order to explore *Pneumocystis*-specific BCRs, we performed lineage analysis on all B cells. Two sequences were clustered into a lineage if they shared the same V(D)J combination and their CDR3 region differed by no more than one amino acid. The lineage structures of each sample are shown in **Supplementary Figures 4–8**. Lineages which contained only one cell remained dominant in all five samples and few sequences from different samples could be clustered into the same lineage. A total of 11.3% of plasma cells in the 4 w sample were grouped together into a lineage, with a SHM rate of 0–1.4%. These IgG1-expressing B cells shared the IGH-IGK pair of *IGHV1-61*/*IGHD1-1*/*IGHJ3*–*IGKV8-18*/*IGKJ2*. The protein sequences of the CDR3 region in the heavy chain and light chain were ARNGYYGSSRFAYW and CQHNHGS(T)FLPYTF, respectively.

DISCUSSION

PJP is a fatal disease in the non-HIV infected immunosuppressed population. Host immune response against *Pneumocystis* infection not only plays an antifungal role, but it also leads to simultaneous pathogenic injury of the tissue, which determines the disease severity, progression, and outcome. B cells represent a major component of the humoral immune system. In this study, we explored the B cell transcriptome profiles and BCR repertoires in mouse lungs before and after *Pneumocystis* infection to investigate the immune mechanisms underlying the responsiveness to the pathogen. Our integrated single-cell transcriptomic and BCR sequencing analysis resulted in several insights into the immunobiology of PJP.

In our study, *Pneumocystis* infection caused the elevated proportion of a subcluster of naïve B cells, with the high

expression of the *ATF3* gene. ATF3 is a fundamental transcription factor in the endoplasmic reticulum-oxidative stress pathway, and it has a significant function in the cellular adaptive-response network. In mouse models, ATF3 could be superinduced by reactive oxygen species and protect against endotoxic shock by inhibiting innate cytokines (Hoetzenecker et al., 2011). In addition, ATF3 facilitates the pathogen clearance in *S. pneumoniae* infection by promoting IL-17A production in γδ T cells (Lee et al., 2018), and it inhibits the secretion of inflammatory cytokines induced by *Mycoplasma pneumonia in vitro* and *in vivo* (Wang et al., 2017). As evidenced by our analysis, the $ATF3^{+}$ B cells highly expressed genes associated with regulation of transmembrane transport, cell adhesion, and Th17 cell differentiation. The immunologic function of ATF3 in PJP remains in need of further exploration.

B cell responses to antigens are characterized by the activation of reactive B cell clones. Based on our results, the percentage of clonal expanded B cells continuously increased after *Pneumocystis* infection, while the BCR diversity exhibited a downward tendency. The BCR is an essential functional receptor, allowing B cells to specifically recognize antigens. Once the antigen is recognized, B cells proliferate and undergo affinity maturation by accumulation of somatic mutations (Roy et al., 2017). The clonal expansion of B cells was caused by chronic stimulation of foreign antigens, and the reduction of BCR diversity reflected the selection of B cells that were adapted to recognize *Pneumocystis*-specific antigens. Over 60% of plasma cells exhibited SHM, suggesting enhanced BCR affinity and specificity.

As the most variable region of the BCR sequence, the CDR3 region directly determines the specificity of antigen binding to the BCR (Bashford-Rogers et al., 2018). The length of CDR3 was reported to be associated with antibody polyreactivity and autoimmunity (Meffre et al., 2001). The average CDR3 length in patients with systemic lupus erythematosus or systemic sclerosis was significantly shorter than that in the healthy controls (Odendahl et al., 2000; Shi et al., 2020), and B cell clones from patients with rheumatoid arthritis were enriched for longer heavy chain CDR3 sequences (Doorenspleet et al., 2014). Moreover, longer CDR3 length was demonstrated to be preferentially selected during persistent infection (Breden et al., 2011). In this study, we measured the CDR3 aa lengths of BCR heavy chains and their distribution among each sample. The average CDR3 aa lengths in the five samples were all 14 aa. Although a higher percentage of cells with CDR3 aa lengths longer than 20 aa was detected in plasma cells, there was no significant difference in the CDR3 aa lengths between naïve B and plasma cells.

The differential use of IGHV genes contributes to the diversity of the BCR repertoire. Compared with the uninfected control sample, significant changes in IGHV gene segment usage were observed in samples with *Pneumocystis* infection. In addition to *IGHV1-26*, which remained the most frequently used IGHV gene segment in each sample, the usage frequency of *IGHV9-3* elevated after *Pneumocystis* infection, and it became the second most frequently used IGHV gene segment in the 2 and 3 w samples. In terms of the previous research of our group, the fungal burden of *Pneumocystis* peaked 3 weeks post-infection and then gradually cleared (Li et al., 2018; Rong et al., 2019b; Zhang C. et al., 2020). The increased usage of *IGHV9-3* might provide a direction for further study of finding prognostic or diagnostic biomarkers for *Pneumocystis* pneumonia.

The single-cell resolution data acquired from scRNA-seq and single-cell BCR V(D)J sequencing enabled us to carry out lineage analysis based on the similarity of protein sequences in CDR3 regions. Due to the relative shallow sampling of immune cells from each mouse, most of the BCR lineages did not show any enrichment, and contained only a single cell. A lineage of plasma cells expressed the protein sequence of ARNGYYGSSRFAYW and CQHNHGS(T)FLPYTF in the heavy chain and light chain CDR3 region, constituting 11.3% of plasma cells in the 4 w sample. However, no naïve B cells in the 4 w sample expressed this sequence and no BCRs from other samples could be clustered into the same lineage. It remains to be confirmed that whether this CDR3 sequence was induced by antigen stimulation, and its affinity with *Pneumocystis*-specific antigens needs to be detected.

Taken together, our work integrated the transcriptomic data and single-cell paired BCR profiles, revealing the dynamic change of the BCR repertoire during *Pneumocystis* infection. This study identified the alterations in B cell subtypes and BCR clonal expansion, laying the foundation for further understanding of host immune mechanisms against *Pneumocystis* infection. It is likely to provide novel insight into finding diagnostic biomarkers and developing effective immunotherapies for PJP.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/ and GSE157627; https://www.ncbi.nlm.nih.gov/, GSE162533.

ETHICS STATEMENT

The animal study was reviewed and approved by the Capital Medical University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

Z-HT and KZ: conception and design. HS and H-QY: collection and assembly of data. HS: data analysis and interpretation. All authors: manuscript writing, final approval of manuscript.

FUNDING

This work was supported by National Natural Science Foundation of China (Grant no: 81870004).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.636250/full#supplementary-material

Supplementary Figure 1 | Gene enrichment analyses of differentially expressed genes in cluster 2. **(A)** Differentially expressed genes of cluster 2 in comparison with all other cells. Genes with a maximum adjusted *p*-value of 0.01 and an absolute value of $\log_2$(fold change) > 0.5 were considered to be differentially expressed genes. **(B)** Gene enrichment analyses of upregulated differentially expressed genes of cluster 2.

Supplementary Figure 2 | Selective usage of immunoglobin heavy chains (IGH) V genes. **(A)** Selective usage of V gene segments in each sample. **(B)** Heatmaps of V gene segment usage.

Supplementary Figure 3 | Selective usage of immunoglobin light chains kappa/lambda (IGK/L) V genes. **(A)** Selective usage of V gene segments in each sample. **(B)** Heatmaps of V gene segment usage.

Supplementary Figure 4 | Lineage analysis of all B cells in sample 0 w. Each dot represents a unique CDR3 protein sequence. The area of a dot is proportional to the number of cells with identical CDR3 protein sequences. Two dots are linked if they differ by one amino acid in the CDR3 region.

Supplementary Figure 5 | Lineage analysis of all B cells in sample 1 w. Each dot represents a unique CDR3 protein sequence. The area of a dot is proportional to the number of cells with identical CDR3 protein sequences. Two dots are linked if they differ by one amino acid in the CDR3 region.

Supplementary Figure 6 | Lineage analysis of all B cells in sample 2 w. Each dot represents a unique CDR3 protein sequence. The area of a dot is proportional to the number of cells with identical CDR3 protein sequences. Two dots are linked if they differ by one amino acid in the CDR3 region.

Supplementary Figure 7 | Lineage analysis of all B cells in sample 3 w. Each dot represents a unique CDR3 protein sequence. The area of a dot is proportional to the number of cells with identical CDR3 protein sequences. Two dots are linked if they differ by one amino acid in the CDR3 region.

Supplementary Figure 8 | Lineage analysis of all B cells in sample 4 w. Each dot represents a unique CDR3 protein sequence. The area of a dot is proportional to the number of cells with identical CDR3 protein sequences. Two dots are linked if they differ by one amino acid in the CDR3 region. The lineage with a red border showed enrichment, with CDR3 protein sequences in the heavy chain and light chain of ARNGYYGSSRFAYW and CQHNHGS(T)FLPYTF, respectively.

REFERENCES

Bashford-Rogers, R. J. M., Smith, K. G. C., and Thomas, D. C. (2018). Antibody repertoire analysis in polygenic autoimmune diseases. *Immunology* 155, 3–17. doi: 10.1111/imm.12927

Bienvenu, A. L., Traore, K., Plekhanova, I., Bouchrik, M., Bossard, C., and Picot, S. (2016). Pneumocystis pneumonia suspected cases in 604 non-HIV and HIV patients. *Int. J. Infect Dis.* 46, 11–17. doi: 10.1016/j.ijid.2016.03.018

Breden, F., Lepik, C., Longo, N. S., Montero, M., Lipsky, P. E., and Scott, J. K. (2011). Comparison of antibody repertoires produced by HIV-1 infection, other chronic and acute infections, and systemic autoimmune disease. *PLoS One* 6:e16857. doi: 10.1371/journal.pone.0016857

Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018). Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* 36, 411–420. doi: 10.1038/nbt.4096

Doorenspleet, M. E., Klarenbeek, P. L., de Hair, M. J., van Schaik, B. D., Esveldt, R. E., van Kampen, A. H., et al. (2014). Rheumatoid arthritis synovial tissue harbours dominant B-cell and plasma-cell clones associated with autoreactivity. *Ann. Rheum. Dis.* 73, 756–762. doi: 10.1136/annrheumdis-2012-202861

Goldstein, L. D., Chen, Y. J., Wu, J., Chaudhuri, S., Hsiao, Y. C., Schneider, K., et al. (2019). Massively parallel single-cell B-cell receptor sequencing enables rapid discovery of diverse antigen-reactive antibodies. *Commun. Biol.* 2:304. doi: 10.1038/s42003-019-0551-y

Guo, F., Chen, Y., Yang, S. L., Xia, H., Li, X. W., and Tong, Z. H. (2014). Pneumocystis pneumonia in HIV-infected and immunocompromised non-HIV infected patients: a retrospective study of two centers in China. *PLoS One* 9:e101943. doi: 10.1371/journal.pone.0101943

Hoetzenecker, W., Echtenacher, B., Guenova, E., Hoetzenecker, K., Woelbing, F., Bruck, J., et al. (2011). ROS-induced ATF3 causes susceptibility to secondary infections during sepsis-associated immunosuppression. *Nat. Med.* 18, 128–134. doi: 10.1038/nm.2557

Hu, Y., Wang, D., Zhai, K., and Tong, Z. (2017). Transcriptomic analysis reveals significant B lymphocyte suppression in corticosteroid-treated hosts with pneumocystis pneumonia. *Am. J. Respir. Cell Mol. Biol.* 56, 322–331. doi: 10.1165/rcmb.2015-0356OC

Jiang, N., He, J., Weinstein, J. A., Penland, L., Sasaki, S., He, X. S., et al. (2013). Lineage structure of the human antibody repertoire in response to influenza vaccination. *Sci. Transl. Med.* 5:171ra119. doi: 10.1126/scitranslmed.3004794

Kiso, K., Yoshifuji, H., Oku, T., Hikida, M., Kitagori, K., Hirayama, Y., et al. (2017). Transgelin-2 is upregulated on activated B-cells and expressed in hyperplastic follicles in lupus erythematosus patients. *PLoS One* 12:e0184738. doi: 10.1371/journal.pone.0184738

Kolls, J. K. (2017). An emerging role of B cell immunity in susceptibility to pneumocystis pneumonia. *Am. J. Respir. Cell Mol. Biol.* 56, 279–280. doi: 10.1165/rcmb.2016-0360ED

Kuo, H. C., Pan, C. T., Huang, Y. H., Huang, F. C., Lin, Y. S., Li, S. C., et al. (2019). Global investigation of immune repertoire suggests kawasaki disease has infectious cause. *Circ. J.* 83, 2070–2078. doi: 10.1253/circj.CJ-19-0206

Kuri-Cervantes, L., Pampena, M. B., Meng, W., Rosenfeld, A. M., Ittner, C. A. G., Weisman, A. R., et al. (2020). Comprehensive mapping of immune perturbations associated with severe COVID-19. *Sci. Immunol.* 5:eabd7114. doi: 10.1126/sciimmunol.abd7114

Lee, S., Kim, G. L., Kim, N. Y., Kim, S. J., Ghosh, P., and Rhee, D. K. (2018). ATF3 stimulates IL-17A by regulating intracellular Ca(2+)/ROS-dependent IL-1beta activation during streptococcus pneumoniae infection. *Front. Immunol.* 9:1954. doi: 10.3389/fimmu.2018.01954

Li, T., Rong, H. M., Zhang, C., Zhai, K., and Tong, Z. H. (2018). IL-9 deficiency promotes pulmonary Th17 response in murine model of pneumocystis infection. *Front. Immunol.* 9:1118. doi: 10.3389/fimmu.2018.01118

Meffre, E., Milili, M., Blanco-Betancourt, C., Antunes, H., Nussenzweig, M. C., and Schiff, C. (2001). Immunoglobulin heavy chain expression shapes the B cell receptor repertoire in human B cell development. *J. Clin. Invest.* 108, 879–886. doi: 10.1172/JCI13051

Odendahl, M., Jacobi, A., Hansen, A., Feist, E., Hiepe, F., Burmester, G. R., et al. (2000). Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. *J. Immunol.* 165, 5970–5979. doi: 10.4049/jimmunol.165.10.5970

Opata, M. M., Hollifield, M. L., Lund, F. E., Randall, T. D., Dunn, R., Garvy, B. A., et al. (2015). B lymphocytes are required during the early priming of CD4+ T cells for clearance of pneumocystis infection in mice. *J. Immunol.* 195, 611–620. doi: 10.4049/jimmunol.1500112

Otieno-Odhiambo, P., Wasserman, S., and Hoving, J. C. (2019). The contribution of host cells to pneumocystis immunity: an update. *Pathogens* 8:52. doi: 10.3390/pathogens8020052

Parameswaran, P., Liu, Y., Roskin, K. M., Jackson, K. K., Dixit, V. P., Lee, J. Y., et al. (2013). Convergent antibody signatures in human dengue. *Cell Host Microbe* 13, 691–700. doi: 10.1016/j.chom.2013.05.008

Qiu, X., Mao, Q., Tang, Y., Wang, L., Chawla, R., Pliner, H. A., et al. (2017). Reversed graph embedding resolves complex single-cell trajectories. *Nat. Methods* 14, 979–982. doi: 10.1038/nmeth.4402

Rong, H. M., Li, T., Zhang, C., Wang, D., Hu, Y., Zhai, K., et al. (2019a). IL-10-producing B cells regulate Th1/Th17-cell immune responses in pneumocystis pneumonia. *Am. J. Physiol. Lung Cell Mol. Physiol.* 316, L291–L301. doi: 10.1152/ajplung.00210.2018

Rong, H. M., Qian, X. J., Zhang, C., Li, T., and Tong, Z. H. (2019b). IL-17 inversely correlated with IL-10 via the STAT3 gene in pneumocystis-infected mice. *Mediators Inflamm.* 2019:6750861. doi: 10.1155/2019/6750861

Roy, B., Neumann, R. S., Snir, O., Iversen, R., Sandve, G. K., Lundin, K. E. A., et al. (2017). High-throughput single-cell analysis of B cell receptor usage among autoantigen-specific plasma cells in celiac disease. *J. Immunol.* 199, 782–791. doi: 10.4049/jimmunol.1700169

Schmidt, J. J., Lueck, C., Ziesing, S., Stoll, M., Haller, H., Gottlieb, J., et al. (2018). Clinical course, treatment and outcome of pneumocystis pneumonia in immunocompromised adults: a retrospective analysis over 17 years. *Crit. Care* 22:307. doi: 10.1186/s13054-018-2221-8

Shi, X., Shao, T., Huo, F., Zheng, C., Li, W., and Jiang, Z. (2020). An analysis of abnormalities in the B cell receptor repertoire in patients with systemic sclerosis using high-throughput sequencing. *PeerJ* 8:e8370. doi: 10.7717/peerj.8370

Stavnezer, J., and Schrader, C. E. (2014). IgH chain class switch recombination: mechanism and regulation. *J. Immunol.* 193, 5370–5378. doi: 10.4049/jimmunol.1401849

Tucci, F. A., Kitanovski, S., Johansson, P., Klein-Hitpass, L., Kahraman, A., Durig, J., et al. (2018). Biased IGH VDJ gene repertoire and clonal expansions in B cells of chronically hepatitis C virus-infected individuals. *Blood* 131, 546–557. doi: 10.1182/blood-2017-09-805762

Wang, J., Cheng, W., Wang, Z., Xin, L., and Zhang, W. (2017). ATF3 inhibits the inflammation induced by mycoplasma pneumonia in vitro and in vivo. *Pediatr. Pulmonol.* 52, 1163–1170. doi: 10.1002/ppul.23705

Wen, W., Su, W., Tang, H., Le, W., Zhang, X., Zheng, Y., et al. (2020). Immune cell profiling of COVID-19 patients in the recovery stage by single-cell sequencing. *Cell Discov.* 6:31. doi: 10.1038/s41421-020-0168-9

Wrammert, J., Smith, K., Miller, J., Langley, W. A., Kokko, K., Larsen, C., et al. (2008). Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. *Nature* 453, 667–671. doi: 10.1038/nature06890

Xu, J. L., and Davis, M. M. (2000). Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* 13, 37–45. doi: 10.1016/s1074-7613(00)00006-6

Zhang, C., Rong, H. M., Li, T., Zhai, K., and Tong, Z. H. (2020). PD-1 deficiency promotes macrophage activation and T-helper cell type 1/T-helper cell type 17 response in pneumocystis pneumonia. *Am. J. Respir. Cell Mol. Biol.* 62, 767–782. doi: 10.1165/rcmb.2019-0234OC

Zhang, J. Y., Wang, X. M., Xing, X., Xu, Z., Zhang, C., Song, J. W., et al. (2020). Single-cell landscape of immunological responses in patients with COVID-19. *Nat. Immunol.* 21, 1107–1118. doi: 10.1038/s41590-020-0762-x

Zheng, C., Zheng, L., Yoo, J. K., Guo, H., Zhang, Y., Guo, X., et al. (2017). Landscape of infiltrating T cells in liver cancer revealed by single-cell sequencing. *Cell* 169, 1342–1356.e16. doi: 10.1016/j.cell.2017.05.035

Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A. H., Tanaseichuk, O., et al. (2019). Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat. Commun.* 10:1523. doi: 10.1038/s41467-019-09234-6

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership