Cell Reports
Resource

Tracing Conidial Fate and Measuring Host Cell Antifungal Activity Using a Reporter of Microbial Viability in the Lung

Anupam Jhingran,[1] Katrina B. Mar,[1] Debra K. Kumasaka,[1] Sue E. Knoblaugh,[2] Lisa Y. Ngo,[1] Brahm H. Segal,[3] Yoichiro Iwakura,[4] Clifford A. Lowell,[5] Jessica A. Hamerman,[6] Xin Lin,[7] and Tobias M. Hohl[1,*]
[1]Vaccine and Infectious Diseases Division
[2]Comparative Medicine Shared Resources
Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA
[3]Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA
[4]Research Institute for Biomedical Sciences, Tokyo University of Science, Noda, Chiba 278-0022, Japan
[5]Immunology Program and the Department of Laboratory Medicine, University of California at San Francisco, San Francisco, CA 94143, USA
[6]Immunology Program, Benaroya Research Institute, Seattle, WA 98101, USA
[7]Department of Molecular and Cellular Oncology, University of Texas, MD Anderson Cancer Center, Houston, TX 77030, USA
*Correspondence: thohl@fhcrc.org
http://dx.doi.org/10.1016/j.celrep.2012.10.026

SUMMARY

Fluorescence can be harnessed to monitor microbial fate and to investigate functional outcomes of individual microbial cell-host cell encounters at portals of entry in native tissue environments. We illustrate this concept by introducing fluorescent *Aspergillus* reporter (FLARE) conidia that simultaneously report phagocytic uptake and fungal viability during cellular interactions with the murine respiratory innate immune system. Our studies using FLARE conidia reveal stepwise and cell-type-specific requirements for CARD9 and Syk, transducers of C-type lectin receptor and integrin signals, in neutrophil recruitment, conidial uptake, and conidial killing in the lung. By achieving single-event resolution in defined leukocyte populations, the FLARE method enables host cell profiling on the basis of pathogen uptake and killing and may be extended to other pathogens in diverse model host organisms to query molecular, cellular, and pharmacologic mechanisms that shape host-microbe interactions.

INTRODUCTION

The sequestration and killing of pathogenic microbes at portals of entry are fundamental properties of the immune system. Despite the importance of these processes, studies in microbial pathogenesis lack robust methods to quantify microbial uptake and killing by leukocytes in native tissue environments with single-encounter resolution. Studies with purified immune cells do not model the complexity and context of intact tissues, whereas in vivo studies of host-pathogen encounters often rely on surrogate endpoints, e.g., microbial tissue burden or host survival, that measure aggregate processes rather than individual cellular encounters.

Fluorescent proteins have long served as sensors of cellular environments, exemplified by genetically encodable probes that sense changes in ambient pH (Miesenböck et al., 1998). In pathogenesis studies, researchers have harnessed microbial fluorescent protein expression to report microbial localization, tissue burden, and host cell associations (Coombes and Robey, 2010). To measure host microbicidal activity with single-cell resolution, quantitation of both live and killed microbial cells represents a formidable obstacle, particularly for fluorescent microbes that are rapidly inactivated to nonfluorescent and, for many experimental purposes, invisible particles. Engineering microbes to emit a fluorescent signal coupled to loss of viability provides a potential solution to this obstacle.

Humans inhale mold conidia (asexual spores) daily. These infectious propagules do not divide into discrete cellular units but rather form tissue-invasive filaments when their growth is not checked by the respiratory innate immune system. The action of neutrophils and alveolar macrophages (AMs) prevents this transition, with most infections resolving in an asymptomatic fashion. However, defects in neutrophil trafficking (Bonnett et al., 2006; Mehrad et al., 1999), number (Gerson et al., 1984; Mircescu et al., 2009), or function (Park and Mehrad, 2009) predispose to invasive disease, commonly caused by *Aspergillus fumigatus*. Although the oxidative burst, degranulation, nutrient sequestration, and antimicrobial peptides contribute to neutrophil antifungal activity (Brown, 2011), understanding the mechanisms that control neutrophil conidiacidal activity in vivo has been difficult to achieve.

Neutrophil activation by *A. fumigatus* conidia is attributed to stage-specific exposure of C-type lectin receptor (CLR) and Toll-like receptor (TLR) ligands. The CLR Dectin-1 binds β-(1,3)-glucan on germinating conidia (Gersuk et al., 2006; Hohl et al., 2005; Steele et al., 2005) and transduces signals via an intracellular immunoreceptor tyrosine-based activation motif (ITAM)-like domain to spleen tyrosine kinase (Syk) and to caspase recruitment domain adaptor protein 9 (CARD9) (Mócsai et al., 2010). Syk- and CARD9-mediated antifungal immunity is principally ascribed to defects in cytokine regulation by

macrophages and dendritic cells (DCs) (Gross et al., 2006; Hara et al., 2007; Hsu et al., 2007), resulting in defects in the formation of T helper 17 (Th17) cells (LeibundGut-Landmann et al., 2007) and in susceptibility to mucosal candidiasis in humans (Glocker et al., 2009). Other studies link Dectin-1 (Li et al., 2011; Werner et al., 2009), CARD9 (Wu et al., 2009), integrins, and Syk to the activation of antimicrobial effectors, the latter exemplified by β2-integrin (CD18) and Syk-dependent neutrophil NADPH oxidase activity against sterile fungal hyphae (Boyle et al., 2011; Leal et al., 2012). In vitro, $Syk^{(-/-)}$ neutrophils are defective in the uptake of opsonized bacteria and in degranulation, resulting in delayed staphylococcal clearance (Van Ziffle and Lowell, 2009). Thus, despite increasing evidence that links CARD9 and Syk to cytokine regulation and to antimicrobial effector functions, the role and timing of CARD9 and Syk in neutrophil defense against inhaled mold conidia remain poorly defined.

Although fluorescent *A. fumigatus* strains have been generated for in vitro and in vivo applications (Balajee and Marr, 2002; Hohl et al., 2009; Leal et al., 2010; Wasylnka and Moore, 2002), tracing the outcome of fungal-host cell interactions remains elusive. In this study, we harness fluorescence to trace the outcome of conidia-host cell interactions in murine lungs. Herein, we introduce a *fl*uorescent *Aspergillus re*porter (FLARE) strain that emits two fluorescent signals—one to report conidial viability, the second to trace conidia independent of viability—to visualize and quantify conidial uptake and killing and to resolve sequential steps of neutrophil function during respiratory *A. fumigatus* challenge. Monitoring conidial fate with the FLARE method demonstrates that CARD9 is essential for lung neutrophil recruitment and for optimal phagocytic and conidiacidal responses, whereas Syk is indispensable for conidial uptake and killing. These studies indicate sequential and cell-type-specific roles for CARD9 and Syk in neutrophil-mediated clearance of inhaled mold spores. Our results illustrate the utility of a microbial reporter of viability to facilitate functional analysis of microbial cell-host cell encounters in native tissue environments.

RESULTS

A Fluorescent Reporter Distinguishes Live and Killed Conidia

The FLARE strain was developed to trace conidial fate and enable functional analysis of leukocyte conidiacidal activity in the lung. We reasoned that conidial expression of a red fluorescent protein variant, dsRed, could act as a viability indicator because dsRed has an ~45 min half-life in phagolysosomes (Maselli et al., 2002), the compartment implicated in conidial killing (Ibrahim-Granet et al., 2003). Given that killed conidia form crescent-shaped ghosts within phagocytes (Philippe et al., 2003), we attached a fluorescent tracer dye (Alexa Fluor 633; AF633) to the surface of live $dsRed^+$ conidia (Figure 1A) with the aim of detecting a signal from the tracer dye after conidial cell death. The modifications introduced into the *A. fumigatus* 293 parental strain did not alter bone marrow macrophage (BMM) cytokine responses to conidia (Figure 1B), suggesting that conidial dsRed expression and chemical labeling with AF633 do not interfere with or enhance macrophage responsiveness to conidia.

For measuring dsRed and AF633 fluorescence under conidiacidal conditions in vitro, $dsRed^+AF633^+$ conidia were exposed to increasing concentrations of H_2O_2, analyzed by flow cytometry, and plated for colony-forming units (cfu). $dsRed^+AF633^+$ conidia lost dsRed but retained AF633 fluorescence at conidiacidal H_2O_2 concentrations (Figure 1C). Extinction of dsRed fluorescence and the shift to a single fluorescent emission correlated with conidial killing, as measured by cfu analysis. Similar results were obtained when $dsRed^+AF633^+$ conidia were treated with 30 μM HOCl (data not shown). $dsRed^+AF633^+$ conidia are referred to as FLARE conidia hereafter.

FLARE Conidia Report Conidial Fate In Vivo

To assess dsRed as a reporter of conidial viability in vivo, C57BL/6 mice were infected with 3×10^7 FLARE conidia via the intratracheal (i.t.) route, and single-cell lung suspensions were analyzed for dsRed and AF633 fluorescence (Figure 1D). For $CD45^+CD11b^+Ly6G^+$ lung neutrophils, flow cytometric analysis gave rise to $dsRed^-AF633^-$ (Figure 1E, tan gate), $dsRed^-AF633^+$ (Figure 1E, blue gate), and $dsRed^+AF633^+$ (Figure 1E, red gate) cell populations. Similar results were observed for lung monocytes, macrophages, and $CD11b^+$ DCs (data not shown). Control mice infected with singly labeled conidia (AF633 or dsRed) gave rise to expected fluorophore-positive and fluorophore-negative neutrophil populations (Figures 1F and 1G).

To determine whether $dsRed^+AF633^+$ leukocytes contain live conidia, $dsRed^+AF633^+$ lung neutrophils, macrophages, and $CD11b^+$ DCs were sorted and plated for cfu. $dsRed^+AF633^+$ lung leukocytes harbored live conidia because these cells contain ~0.5–0.75 cfu per sorted event (Figure 1H, red bars). Dually fluorescent conidia were visible in $dsRed^+AF633^+$ neutrophils by fluorescence microscopy (Figure 1I) or imaging cytometry (Figure 1L). In contrast, $dsRed^-AF633^+$ lung leukocytes harbored killed conidia by cfu analysis, with <0.005 cfu per sorted event (Figure 1H, blue bars), despite clear visualization of $AF633^+$ conidia in neutrophils (Figures 1J and 1M). $dsRed^-AF633^-$ neutrophils (Figure 1E, tan gate) did not contain conidia by imaging cytometry (Figure 1K) and thus represent bystander cells. In sum, these data indicate that FLARE-dependent signals can distinguish bystander and fungus-engaged leukocytes and report the viability status of engulfed conidia.

To examine whether conidial killing is associated with leukocyte uptake, the frequency of live and killed conidia was compared in $CD45^-$ and in $CD45^+$ lung cell populations. Conidial viability was 72% at 12 hr postinfection (p.i.) and 61% at 36 hr p.i. in the $CD45^-$ gate (Figures 2A and 2B). The corresponding values were 18% and 7% in the $CD45^+$ gate (Figures 2A and 2B), indicating that leukocyte uptake generally precedes conidial killing. Neutrophils represent the predominant leukocyte subset that engulfs and kills FLARE conidia in infected lungs (Figures 2C–2E).

FLARE Conidia Detect Defects in Neutrophil Conidiacidal Activity

The molecular defect in chronic granulomatous disease (CGD) results in NADPH oxidase deficiency and is associated with an ~40% lifetime risk of invasive aspergillosis (IA). To validate the FLARE strain, we generated and infected mice that harbor