S3.3c

Alveolar macrophage-mediated host resistance against *Aspergillus fumigatus*

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S3.3 Innate immune responses to pathogenic fungi, September 21, 2022, 4:45 PM - 6:15 PM

Alveolar macrophages (AlvMφ) reside on the luminal surface of the airways serving as the primary phagocyte within the airways of the lungs where they act as immune sentinel cells sensing and responding to microbial and environmental exposures. In this role, AlvMφ must be able to respond in a manner that is appropriate to the threat posed which has been hypothesized to occur through sensing microbial vitality and/or patterns of pathogenesis. It is well-established that AlvMφ interact with phagocytose and respond to *A. fumigatus*, but their role in host resistance against *A. fumigatus* is currently controversial. Here I will discuss the role of AlvMφ play in orchestrating a robust and effective antifungal innate immune response to mediate *A. fumigatus* clearance. AlvMφ orchestrate the protective innate immune response against *A. fumigatus* by sensing live fungal conidia using the cytosolic RNA-sensing MDA5 receptor to initiate the host protective type I and type III interferon response in both mice and humans. The activation of MDA5/MAVS signaling appears to be mediated by both fungal dsRNA-dependent and fungal dsRNA-independent mechanisms. Thus, AlvMφ serve as a central hub for regulating and tuning the antifungal immune response within the respiratory tract.

S3.3d

Influenza versus COVID-19-associated pulmonary aspergillosis: Profiling lower respiratory tract epithelial and myeloid innate immunity in patient samples

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Objectives: Up to 20% and 15% of critically ill influenza and coronavirus disease 2019 (COVID-19) patients are affected by influenza- and COVID-19-associated pulmonary aspergillosis (IAPA and CAPA) respectively. These viral-fungal coinfections are difficult to diagnose and are associated with increased mortality. Mechanistic insights into the development of IAPA and CAPA are a prerequisite for the development of new biomarkers and novel immunomodulatory therapeutic targets. However, data on the pathophysiology are scarce. With this study, we aimed at expanding our knowledge of IAPA and CAPA pathophysiology in an explorative way, resorting to lower respiratory tract samples and focusing on the epithelial and myeloid innate immunity components of the antifungal host response.

Methods: We performed nCounter gene expression analyses of 755 genes linked to innate immunity, and determined protein levels of 47 cytokines, chemokines, growth factors, and other inflammatory mediators on bronchoalveolar lavage (BAL) fluid samples from 166 ICU-admitted influenza and COVID-19-patients with or without aspergillosis. Additionally, we performed spatial transcriptomics and RNAscope on *in vivo* tracheobronchial biopsies from four IAPA and CAPA patients.

Results: Several genes encoding proteins with important effector functions in antifungal immunity are downregulated in BAL fluid of IAPA and CAPA patients compared with influenza-only or COVID-19-only patients. Cellular deconvolution of the gene expression data reveals a significantly lower BAL neutrophil fraction in CAPA patients compared to COVID-19-only patients.

IAPA and CAPA patients have high BAL fluid levels of pro-inflammatory cytokines, but these are not significantly different from the levels seen in influenza-only and COVID-19-only patients. By integrating the BAL fluid cytokine levels with their respective transcriptional responses, we show that IAPA patients, and to a lesser extent CAPA patients, have an aberrant transcriptional response to pro-inflammatory cytokines as well as type I and type II interferons, which may result in poor cellular effector functions (Fig. 1a). Interferon-gamma signaling is abrogated in both IAPA and CAPA patients when compared with influenza-only and COVID-19-only patients.

We observe significantly higher levels of growth factors associated with lung fibrosis in both IAPA and CAPA BAL fluid, which may contribute to the higher mortality seen in these coinfections (Fig. 1b).

Using spatial transcriptomics, we show that different epithelial defense mechanisms are at play in IAPA and CAPA (Fig. 2a).

Finally, using RNAscope ultrasensitive single-molecule RNA *in situ* hybridization, we visualize fungal and viral co-localization in CAPA tracheobronchial tissue, proving that virus-induced epithelial barrier disruption paves the way for tissue-invasive aspergillosis (Fig. 2b).

Conclusion: Using state-of-the-art techniques in lower respiratory tract samples obtained from a large representative patient cohort, we provide arguments for a three-level breach in antifungal immunity in IAPA and CAPA. A hampered ability to phagocytize and kill fungal spores enables *Aspergillus* germination and growth, leading to hyphae that are not contained because of restrained extracellular defense mechanisms. These hyphae may easily become tissue-invasive through an epithelium that is weakened by the viral infection, causing detrimental damage to the respiratory system. Functional studies will be necessary to further unravel the pathophysiology of IAPA and CAPA.

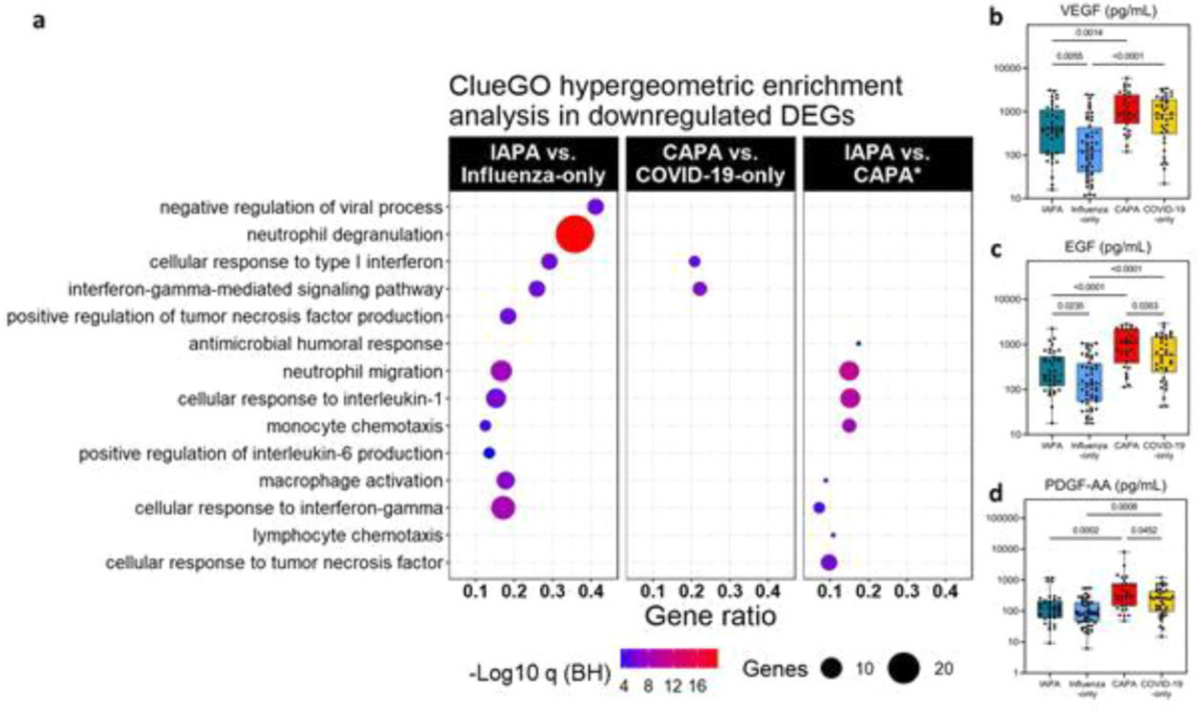

Figure 1. BAL fluid analyses reveal downregulation of genes associated with pro-inflammatory pathways in IAPA, and type I & II interferon signaling in IAPA and CAPA, while levels of fibrosis-related growth factors are elevated in IAPA and CAPA compared to influenza-only and COVID-19-only.
Panel (**a**): dot plot showing pathway analyses based on the downregulated differentially expressed genes (DEG) in BAL fluid for disease comparisons IAPA vs. influenza-only, CAPA vs. COVID-19-only and IAPA vs. CAPA. For the comparison IAPA vs. CAPA, DEGs with q-value <0.20 were included to generate the analyses, while DEGs with q-value< 0.05 were used for the other comparisons.
Panels (**b-d**): levels of fibrosis-related growth factors per disease are displayed as box plots with whiskers set from minimum to maximum. Asterisks represent significant differences between groups (Kruskall-Wallis with follow-up Benjamini-Krieger-Yekutieli; only statistics for comparisons IAPA vs. influenza-only, CAPA vs. COVID-19-only, IAPA vs. CAPA and influenza-only vs. COVID-19-only are shown).

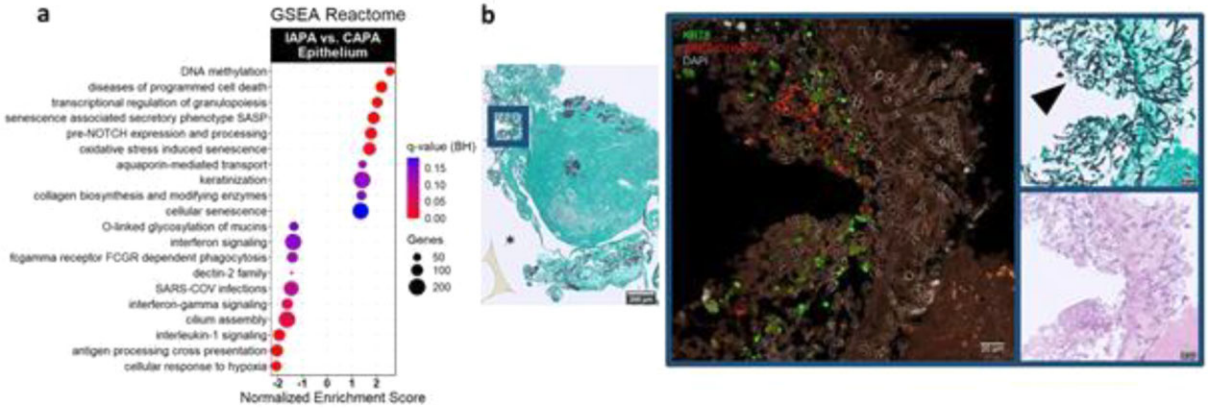

Figure 2. Epithelial transcriptional responses differ in IAPA and CAPA, and SARS-CoV-2-induced epithelial barrier damage facilitates tissue invasion by Aspergillus hyphae.

Panel (**a**) shows gene set enrichment analysis (GSEA) based on the gene expression of epithelial regions of interest in IAPA vs. CAPA *in vivo* tracheobronchial biopsies.

Panel (**b**) shows an *in vivo* tracheobronchial biopsy of a CAPA patient with invasive *Aspergillus* tracheobronchitis. The image on the left shows a slide stained with Grocott-Gomori's methenamine silver (GMS), which makes fungi appear black (examples indicated by black arrowheads) and the background tissue green. The asterisk shows the localization of the lumen of the respiratory tract. The middle image shows an RNAscope image of an adjacent slide of the magnified area (blue square in left image) in which the red puncta reflect *SARS-CoV-2-N* RNA. The two images on the right show consecutive H&E and GMS stainings of the magnified area.

S3.4a

The repurposing approach identifies pitavastatin (calcium) making fluconazole fungicidal by inhibiting ergosterol synthesis

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S3.4 Free oral paper session, September 21, 2022, 4:45 PM - 6:15 PM

Objectives: Making fluconazole (FLC) fungicidal in combination with adjuvants is a promising strategy to avoid the emergence of FLC resistance and eliminate the persistence and recurrence of fungal infections. To address this question, we combined *in vitro* screening of a library of FDA-approved drugs to identify compounds for making FLC fungicidal.

Methods: We performed a high-throughput screen of an FDA-approved compound library (HY-L022, MCE®), which contains 2372 drugs, to identify potentially novel FLC synergistic lethal adjuvants using broth microdilution and dose-matrix titration assays. The abilities of candidate drugs to turn FLC from fungistatic to fungicidal were further investigated by FLC disk diffusion assays carried out by four tested strains with different FLC tolerance levels (SC5314, SN152, cmp1-aidΔ/cmp1-aidΔ, and ADH1p-UPC2 strains). We determined the median lethal dose (LD50) of Candidate compounds by the Up-and-Down procedure (UDP) (OECD 425, 2008) via the intraperitoneal route in adult mice and used cyclosporine A and geldanamycin as control drugs to screen FLC synergistic lethal adjuvants with lower toxicity. Finally, we constructed heterozygous deletion mutants for ergosterol synthesis-related genes to identify the mechanism of action of the synergistic lethality of pitavastatin (calcium) (PIT) and FLC (Fig. 1a).

Results: We found that 200 compounds (≤100 μM) could make FLC (4 μg/ml) fungicidal and further confirmed that 30 compounds turned FLC (4 μg/ml) from fungistatic to fungicidal at a concentration lower than 12.5 μM by broth microdilution assays (Fig. 1b). We further identified that 12/30 compounds (≤3.125 μM) can make FLC fungicidal (≤4 μg/ml) using dose-matrix titration assays. Among these compounds, PIT can make FLC fungicidal at as low as 0.78 μM (Fig. 1c). In the FLC disk diffusion assay, we identified 8 compounds (5 μM) that were superior to or equivalent to the abilities of the control drugs to eliminate the FLC tolerance of four tested strains. It was worth noting that PIT could make FLC fungicidal against all four tested strains (Fig. 1d). The LD50 value of PIT is 103.6 mg/kg and the highest of the tested compounds. Spot assay results showed feeding exogenous 100 μM ergosterol counteracted the antifungal activity of PIT (16 μM) (Fig. 2a), but did not restore the growth defect of Tet-HMG1/hmg1Δ mutant, in which the HMG1 gene expression would be inhibited by tetracycline