Lung Hybrid Neutrophils and Extracellular Traps Are Protective in COVID-19-Associated Pulmonary Aspergillosis

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Background

COVID-19-associated pulmonary aspergillosis (CAPA)

- *Aspergillus* superinfection in critically ill COVID-19 patients
- Increased mortality, diagnosis is difficult
- Pathophysiological insight is lacking.

Methods

Single-cell RNA sequencing

- 37 BAL samples of 37 critically ill COVID-19 patients
  - 22 COVID-19-only patients (no aspergillosis)
  - 6 early CAPA patients (sampling <5 days after diagnosis)
  - 9 late CAPA patients (sampling 5-11 days after diagnosis)

Neutrophil extracellular trap (NET) levels

- 57 BAL samples of 57 critically ill COVID-19 patients
  - 33 COVID-19-only patients
  - 24 early CAPA patients

1A

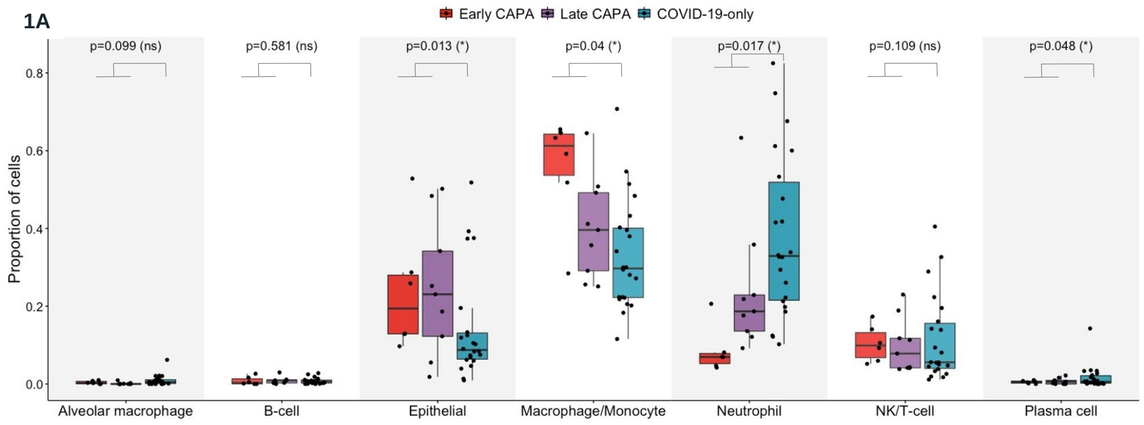


Figure 1 panel (**A**): BALF neutrophil proportions as analyzed by single-cell RNA sequencing using the Seurat R package are significantly lower in CAPA patients compared to COVID-19-only patients. Patients with early CAPA have significantly lower BALF neutrophil proportions than patients with late CAPA. Macrophage/monocyte and epithelial cell proportions are reciprocally increased in CAPA patients compared to COVID-19-only patients. P-values shown for differences between the pooled CAPA patients and the COVID-19-only patients. P-values were calculated using a generalized linear model correcting for age, Charlson Comorbidity Index at hospital admission, and administration of corticosteroids (prednisone equivalent dose 20 mg or higher) within 48 hours of BALF sampling.

1B

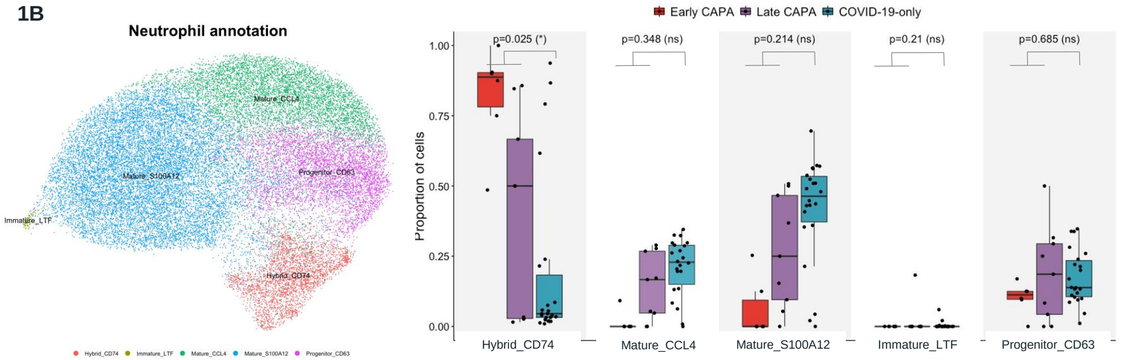


Figure 1 panel (**B**): UMAP of the neutrophil fraction, and box plots depicting the proportions of neutrophil subtypes in BAL fluid. P-values shown for differences between the pooled CAPA patients and the COVID-19-only patients. P-values were calculated using a generalized linear model correcting for age, Charlson Comorbidity Index at hospital admission, and administration of corticosteroids (prednisone equivalent dose 20 mg or higher) within 48 hours of BALF sampling.

Results

scRNA-seq

- 69008 cells passed quality filtering
- Lower neutrophil proportions in CAPA vs. COVID-19-only (Fig. 1A)
- Neutrophil subclustering (Fig. 1B)
  - Immature & mature clusters
  - "Hybrid" neutrophil cluster
    - Genes ~ antigen-presenting functions
    - Dominant in CAPA

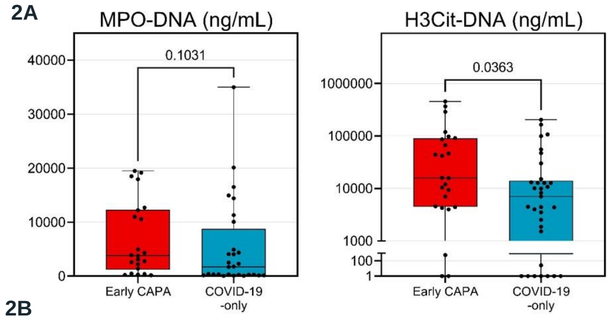
NETosis

- Significantly higher citrullinated histone H3 DNA complexes levels (H3Cit-DNA) in CAPA patients (Fig. 2A)
  → Explains low neutrophil proportions
- Significantly lower survival in CAPA patients with the lowest H3Cit-DNA levels (Fig. 2B).

Conclusion

- Extremely high NET levels in CAPA lower respiratory tract, which are likely to be protective in CAPA patients specifically
- Hybrid neutrophil formation in CAPA, probably upon encountering the fungus

2A



2B

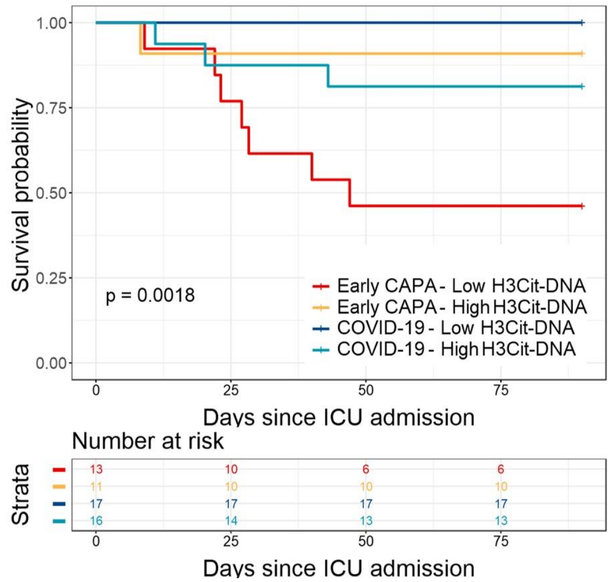


Figure 2 panel (**A**): Myeloperoxidase (MPO) DNA complexes were analyzed as biomarkers for general NET-formation, while citrullinated histone H3 DNA (H3Cit-DNA) levels were analyzed for PAD4-dependent NET-formation, in BALF samples from early CAPA and COVID-19-only patients. Similar MPO-DNA levels were found in early CAPA patients, while H3Cit-DNA levels were significantly higher in early CAPA compared to COVID-19-only patients. P-values calculated using Mann-Whitney *U* test.
Figure 2 panel (**B**): Kaplan-Meier analysis of patients with NETosis analyses, divided in early CAPA and COVID-19-only patients and subdivided according to H3Cit-DNA levels (cut-off at 20000 ng/mL for early CAPA and at 8000 ng/mL for COVID-19-only). Log-rank test was used to compare survival distributions. For the comparison early CAPA (low H3Cit-DNA) versus early CAPA (high H3Cit-DNA), the p-value was 0.033.

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