Supplementary Materials for

Immune signatures underlying post-acute COVID-19 lung sequelae

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The PDF file includes:

Figs. S1 to S16

Other Supplementary Material for this manuscript includes the following:

Data files S1 to S4
Figure S1

A

|     | Sex | Age | Vaccine |
|-----|-----|-----|---------|
| CON-1 | M   | 65  |         |
| CON-2 | M   | 66  |         |
| CON-3 | F   | 77  |         |
| CON-4 | M   | 73  |         |
| CON-5 | M   | 73  | Y       |
| CVD-1 | M   | 64  |         |
| CVD-2 | M   | 83  |         |
| CVD-3 | M   | 63  |         |
| CVD-4 | M   | 65  |         |
| CVD-5 | M   | 74  |         |
| CVD-6 | F   | 66  |         |
| CVD-7 | M   | 62  |         |
| CVD-8 | F   | 76  | Y       |
| CVD-9 | F   | 63  |         |
| CVD-10 | M  | 68  |         |

Days post diagnosis with COVID 19

B

Relative expression to HPRT

C

|     | FEV1  | FVC  | FEV1/FVC | DLCO |
|-----|-------|------|----------|------|
|     | % of reference | % of reference | % of ratio | % of reference |
| CON CVD |       |       |          |      |
| CVD |       |       |          |      |

D

Original CT image

Original CT image

CALIPER analysis

CON

CVD

Recovered

CVD

Moderate

CVD

Severe

E

|     | % of total volume | % of total volume | % of total volume | % of total volume | % of total volume |
|-----|-------------------|-------------------|-------------------|-------------------|-------------------|
| CON CVD |       |       |          |      |      |
| CVD |       |       |          |      |      |

Mild

P = 0.0193*

Spared

P = 0.0553

GGO

P = 0.0280*

Reticulate

P = 0.0193*

Vessel

P = 0.0127*
**Fig. S1. Functional and pathological characterization of lung sequelae in aged COVID-19 convalescents.**

**A.** Cohort information of aged healthy control and COVID-19 convalescents.  

**B.** RT-PCR of SARS-CoV-2 N1 gene in the BAL or PBMC of COVID-19 convalescents. (-), negative control of uninfected human alveolar macrophages (AMs). (+), positive control of SARS-CoV-2 infected AMs.  

**C.** Pulmonary function parameters of control or COVID-19 convalescents.  

**D.** Representative lung CT image and CALIPER analysis (Green/Dark Green, “spared/relatively spared”; Yellow, “GGO”; Orange, “Reticular/Consolidation”).  

**E.** Lung pathological parameters of control or COVID-19 convalescents. **C and E.** Statistical significance was calculated using Mann-Whitney test. * p < 0.05.
Fig. S2. Inflammatory cytokine levels in the blood and BAL of COVID-19 convalescents. A. Cytokines were detected by multiplex cytokine assay in plasma. B. Cytokines were detected by multiplex assay in concentrated BAL. Statistical significance was calculated using Mann-Whitney test.
Figure S3

A  BAL

B  Blood
Fig. S3. **Flow cytometric analysis and gating strategy of innate cells.** **A.** Gating strategy of innate cells in human BAL. Flow cytometry was performed on BAL cells. **B.** Gating strategy of innate cells in human blood.
Figure S4

A  BAL

B  Blood
Fig. S4. Flow cytometric analysis and gating strategy of lymphocytes. A. Gating strategy of adaptive cells in human BAL. Flow cytometry was performed on BAL cells. B. Gating strategy of adaptive cells in human PBMC.
Figure S5

A

B

C

D

BAL-N  BAL-RBD  BAL-S1  Plasma-N  Plasma-RBD  Plasma-S1

IgG

IgA

IgM

Dilution

Vacc.  Vacc.  Vacc.  Vacc.

N-IgG  N-IgA  RBD-IgG  RBD-IgA  S1-IgG  IFNγ-CDF8+  CD89+CD103+CD4+

CD89+CD103+CD4+  Naive CD8+

T_{em} CD8+  T_{em} CD4+  T_{efw} CD8+  T_{efw} CD4+

RBD*F  RBD*F

R value

0.8  0.6  0.4  0.2  0.0  -0.2  -0.4  -0.6  -0.8  -1
Fig. S5. Characterization of respiratory or blood humoral responses at the convalescent stage. **A.** SARS-CoV-2 (N, RBD or S1) specific IgG (top row), IgA (bottom row) responses in BAL or plasma. **B.** SARS-CoV-2 (N, RBD or S1) specific IgM responses in BAL or plasma. Blue arrow indicates vaccinated control subject and red arrow indicates vaccinate COVID-19 convalescent. **C, D.** Correlation matrix between viral specific antibodies and lymphocyte subsets in BAL (**C**) or blood (**D**). **C, D.** Pearson correlation, $r$ values are indicated by color and circle size. Significant correlations were indicated by white asterisks.
Figure S6

A) Live CD8⁰ T cells

B) UMAP

C) Heatmap

D) PBMC-CD8⁰ T cells

E) BAL-CD8⁰ T cells

F) BAL-CD8⁰ T cells in CVD

G) BAL, PBMC

H) IFNγ, TNFα, IL-2

I) MHC-cytolytic
Fig. S6. Phenotypes of CD8$^+$ T cell in control and COVID-19 convalescents. A. UMAP analysis of the different sub-populations of CD8$^+$ T cells based on BAL control subjects (CON), BAL COVID-19 convalescents (CVD), PBMC CON and PBMC CVD groups. B. Surface marker expression of merged CD8$^+$ T cells. C. Heat map of different marker expression in CD8$^+$ T cell subsets. D. % of indicated populations in PBMC CD8$^+$ T cells. E. Cell numbers of BAL (left) and PBMC (right) CD8$^+$ T cell subtypes. F. Expression of indicated molecules on BAL CD8$^+$ T$_{RM}$s including (CD103$^+$ and CD103$^-$) in CVD groups. G. Representative flow cytometry plots on cytokine expression by CD8$^+$ cells stimulated by SARS-CoV-2 peptide pools for 6 hrs in CVD groups. H. Expression of cytokines on BAL CD8$^+$ T$_{RM}$s stimulated by SARS-CoV-2 peptide pools. I. Pie chart graph on % of single, double or triple cytokine$^+$ CD8$^+$ T cells in the BAL or PBMC Statistical significance was calculated using Mann-Whitney test (D and E) or paired t-test (F and H). * < 0.05, ** < 0.01 and *** < 0.001.
Fig. S7. Phenotypes of CD4+ T cell in control and COVID-19 convalescents. A. UMAP analysis of the different sub-populations of CD4+ T cells based on BAL CON, BAL CVD, PBMC CON and PBMC CVD groups. B. Heat map of different marker expression in CD4+ T cell subsets. C, D. Frequencies (C) or cell numbers (D) of indicated CD4+ T cells in BAL or PBMC. E. Representative flow cytometry plots on cytokine expression by CD4+ cells stimulated by SARS-CoV-2 peptide pools for 6 in CVD groups. F. Expression of indicated molecules in BAL CD4+ T_{RM} including (CD103+ and CD103-) in CVD groups. G. Pie chart graph on % of single, double or triple cytokine+ CD4+ T cells in the BAL or PBMC. Statistical significance was calculated using Mann-Whitney test (C and D) or paired t-test (F). * < 0.05 and ** < 0.01.
Figure S8

Correlation between lung function parameters with BAL CD8\(^+\) T cell subsets.

Correlation of DLCO and FEV1 with % of total CD8\(^+\), CD69\(^+\) CD8\(^+\), CD69\(^+\)CD103\(^-\) CD8\(^+\) or CD69\(^+\)CD103\(^+\) CD8\(^+\) T cells in the BAL of COVID-19 convalescents.
Fig. S9. Correlation between lung function parameters with BAL lymphocytes in non-vaccinated subjects. A. Correlation of DLCO and FEV1 with % of total CD8\(^+\), CD69\(^+\) CD8\(^+\), CD69\(^+\)CD103\(^-\) CD8\(^+\) or CD69\(^+\)CD103\(^+\) CD8\(^+\) T cells in the BAL of non-vaccinated control and COVID-19 convalescents. M: male, F: female. B. Correlation of DLCO, FEV1, Reticular and GGO with % of total CD8\(^+\), CD69\(^+\) CD8\(^+\), CD69\(^+\)CD103\(^-\) CD8\(^+\) or CD69\(^+\)CD103\(^+\) CD8\(^+\) T cells in the BAL of non-vaccinated male control and COVID-19 convalescents.
Fig. S10. CD8+ T cells are a driver of chronic lung impairment following viral pneumonia during aging. Aged mice were infected or not (Naive) as in Figure 3. 3 weeks later, mice were treated with weekly with CD8-depleting antibody (αCD8) or isotype control (Rat IgG) until 50 days post-infection. Lung function was measured via forced oscillation technique. Parameters from the constant-phase model (Rn left column, Gti middle column, Zrs right column) were measured prior (top row) to and after inflation (middle row) of the lungs to total lung capacity. The bottom row is normalized data from the top and middle rows indicating % of the total lung capacity used at baseline for each parameter by each mouse. 3 independent pooled experiments * p < 0.05 and ** p < 0.01 for each indicated multiple comparison by one-way (left and middle columns) or two-way (right column) ANOVA following Fisher’s LSD test.
**Fig. S11. scRNA-seq of respiratory and circulating T cells.** CD3⁺ T cells from BAL or PBMC were enriched with magnetic beads and analyzed with scRNA-seq.  

A. UMAP plot visualization of T cell clusters from BAL and PBMC of COVID-19 convalescents (n=6).  

B. UMAP representation from (A) colored based on T cells source.  

C. UMAP plot visualization of BAL and PBMC CD4⁺ T cell clusters (n=6).  

D. UMAP representation from (C) colored based on T cells source.  

E. Volcano plot showing the differentially expressed genes between BAL and PBMC CD4⁺ T cells.  

F. Expression of the indicated gene signature is elevated between BAL and PBMC CD4⁺ T cells.  

G. UMAP plot showing BAL CD4⁺ T cell clusters.  

H. Cell frequency of each cluster in total BAL CD4⁺ T cells.  

I. The percentage of each cluster in total BAL CD4⁺ T cells divided by individual donor.  

J. Heatmap shows the relative expression of the 15 most differentially expressed genes for each cluster of BAL CD4⁺ T cells.  

K. BAL CD4⁺ T cell subclusters from control and COVID-19 convalescent donors revealed by scRNA-seq.  

L and M, Average (L) and individually (M) relative abundance of BAL CD4⁺ T cell subclusters from control or COVID-19 convalescent donors.
**Fig. S12.** scTCR-seq of respiratory and circulating CD4⁺ T cells in aged COVID-19 convalescents. CD3⁺ T cells from BAL or PBMC were enriched with magnificent beads, and analyzed with scTCR-seq. **A.** UMAP plot visualization of TCR clone expansion level from BAL and PBMC CD4⁺ T cells (n=6). **B.** The percentage of each level of TCR clone expansion in total CD4⁺ T cells from both PBMC and BAL divided by individual donor. **C.** UMAP plot visualization of TCR clone expansion level from BAL CD4⁺ conventional T cells (Tcon cells). **D.** The level of TCR clone expansion percentage in individual clusters of BAL CD4⁺ Tcon cells. **E.** The level of TCR clone expansion percentage in BAL CD4⁺ Tcon cells divided by individual donor. **F.** Volcano plot showing the differentially expressed genes between TCR clone high expanded (clone > 5) and low expanded (clone ≤ 5) BAL CD4⁺ Tcon cells. **G.** UMAP plot visualization of TCR clone expansion level from PBMC CD4⁺ Tcon cells. **H.** The level of TCR clone expansion percentage in PBMC CD4⁺ Tcon cells divided by the individual donor. **I.** Volcano plot showing the differentially expressed genes between TCR clone high expanded (clone > 5) and low expanded (clone ≤ 5) PBMC CD4⁺ Tcon cells.
**Fig. S13. scTCR-seq of respiratory and circulating CD8⁺ T cells in aged COVID-19 convalescents.**

**A.** Blood and BAL CD8⁺ T cell subsets in COVID-19 convalescents revealed by scRNA-seq. T_{eff}, effector T cells; Taa, aged-associated T cells; T_{PM}, peripheral memory T cells.

**B.** UMAP plot visualization of TCR clone expansion level from BAL and PBMC CD8⁺ T cells.

**C.** The percentage of each level of TCR clone expansion in total CD8⁺ T cells from both PBMC and BAL divided by the individual donor. **D.** Chord diagram of shared TCR sequences among BAL CD8⁺ T cell subclusters and PBMC CD8⁺ T cells in individual subject. **E.** The percentage of each level of TCR clone expansion in proliferating CD8⁺ T cells. **F.** Volcano plot showing the differentially expressed genes between TCR clone high expanded (clone > 5) and low expanded (clone ≤ 5) BAL total CD8⁺ T cells. **G.** Volcano plot showing the differentially expressed genes between TCR clone high expanded (clone > 5) and low expanded (clone ≤ 5) PBMC total CD8⁺ T cells.
Fig. S14. scRNA-seq of respiratory and circulating CD8$^+$ T cells in aged COVID-19 convalescents. A. UMAP of CD8$^+$ T cells grouped by the location of the cell source. B. Volcano plot showing the differentially expressed genes between BAL and PBMC CD8$^+$ T cells. C. Expression of the indicated gene signature is elevated between BAL and PBMC CD8$^+$ T cells. D. Cell number of each cluster in total tissue-resident CD8$^+$ T cells. E. Percentage of each cluster in total BAL CD8$^+$ T cells divided by individual donor. F. Heatmap showing the relative expression of the 20 most differentially expressed genes for each cluster of BAL CD8$^+$ T cells. G. Pseudotime analysis of BAL CD8$^+$ T cell development. H. Heatmap showing relative expression of genes associated with circulation in PBMC and BAL CD8$^+$ T cell clusters. I. TCR signaling gene set enrichment between cluster 0 and cluster 1 of respiratory CD8$^+$ T cells. J. Expression of the auto-aggressive CXCR6$^+$ CD8$^+$ T cell gene signature (24) was elevated in their respective subclusters of BAL CD8$^+$ T cells. K. BAL CD8$^+$ T cell subclusters (left) and relative abundance (right) from age-matched control (2), age-unmatched control from GSM4593888 (33) and COVID-19 convalescent donors (6) revealed by scRNA-seq.
Fig. S15. Shared TCR usage between BAL and PBMC CD8$^+$ T cells in aged COVID-19 convalescents. A. UMAP plot visualization of TCR clone expansion level from BAL CD8$^+$ T cells. B. The level of TCR clone expansion percentage in individual clusters of BAL CD8$^+$ T cells. C. Percentages of TCR clones shared with PBMC CD8$^+$ T cells within the individual BAL CD8$^+$ T cell clusters. Paired T test.
Fig. S16. Model on the potential roles of different respiratory CD8 T cell subsets in the development of chronic tissue sequelae.