Limited TCR repertoire and ENTPD1 dysregulation mark late-stage COVID-19

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Highlights

Transcriptome profiling of COVID-19 autopic tissue and PBMC was carried out

There is limited TCR repertoire in lung, kidney and PBMC of severe COVID-19 cases

There are increased CD39 levels in PBMC of severe COVID-19 patients

High HIF-1α and STAT-3 and low CD39-antisense might be linked with CD39 increase

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Limited TCR repertoire and ENTPD1 dysregulation mark late-stage COVID-19

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SUMMARY
T cell exhaustion and dysfunction are hallmarks of severe COVID-19. To gain insights into the pathways underlying these alterations, we performed a comprehensive transcriptome analysis of peripheral-blood-mononuclear-cells (PBMCs), spleen, lung, kidney, liver, and heart obtained at autopsy from COVID-19 patients and matched controls, using the nCounter CAR-T-Characterization panel. We found substantial gene alterations in COVID-19-impacted organs, especially the lung where altered TCR repertoires are noted. Reduced TCR repertoires are also observed in PBMCs of severe COVID-19 patients. ENTPD1/CD39, an ectoenzyme deﬁning exhausted T-cells, is upregulated in the lung, liver, spleen, and PBMCs of severe COVID-19 patients where expression positively correlates with markers of vasculopathy. Heightened ENTPD1/CD39 is paralleled by elevations in STAT-3 and HIF-1α transcription factors; and by markedly reduced CD39-antisense-RNA, a long-noncoding-RNA negatively regulating ENTPD1/CD39 at the post-transcriptional level. Limited TCR repertoire and aberrant regulation of ENTPD1/CD39 could have permissive roles in COVID-19 progression and indicate potential therapeutic targets to reverse disease.

INTRODUCTION
Coronavirus disease 2019 (COVID-19) is a potentially fatal multisystem inﬂammatory disease caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). This serious illness has been associated with alterations in T cell immunity (Sette and Crotty, 2021), which vary according to the disease stage (Peng et al., 2020; Zhang et al., 2020). Aberrant features include CD8 T cell cytopenia (Laing et al., 2020), upregulation of HLA-DR and CD45RO activation markers and immune exhaustion (Kusnadi et al., 2021): a phenotype that becomes more pronounced with disease progression (Wang et al., 2020). Investigations at the transcriptional level have analyzed the single cell landscape of peripheral blood-derived lymphomononuclear cells in small patients’ cohorts. These earlier studies have indicated HLA-class II downregulation (Wilk et al., 2020), increases in Fas-induced T cell apoptosis (Zhu et al., 2020) and decreased expression of genes associated with NK and CD8 T cell cytotoxicity in moderate to severe COVID-19 cases (Yao et al., 2021).

To gain better insights into the mechanistic pathways and gene signatures linked with these alterations, we have performed a comprehensive transcriptome analysis of peripheral blood mononuclear cells (PBMCs), spleen, lung, kidney, liver and heart obtained at autopsy from COVID-19 patients and matched controls. We report that restrictions in T cell receptor (TCR) repertoire and aberrant regulation of the ENTPD1/CD39 ectoenzyme might underlie, at least in part, the T cell dysfunction and putative immune exhaustion observed in severe COVID-19.

RESULTS
Limited TCR repertoire in COVID-19
We used the nCounter CAR-T Characterization Panel (NanoString Technologies, Seattle, WA) (see Table S1) to proﬁle 770 genes of 43 T cell-related pathway annotations in PBMCs and autopic tissues, obtained from COVID-19 patients and controls. PBMCs were obtained from 45 COVID-19 patients, of whom 20 had...
Figure 1. Limited TCR repertoire in lung, kidney, and PBMCs of severe COVID-19 patients

(A) Ultrasound-guided biopsies of autopsic lung (n = 5), heart (n = 3), liver (n = 5), kidney (n = 3), and spleen (n = 3) were obtained from COVID-19 patients and non-COVID-19 controls (n = 2 for all organs). Tissue samples were placed in formalin and, 24 h later, processed and paraffin embedded. Total RNA was extracted and subjected to hybridization prior to NanoString processing and profiling.

(B–F) Heatmaps showing differentially expressed genes (DEGs) included in the nCounter CAR-T Characterization Panel in lung (B), heart (C), liver (D), kidney (E), and spleen (F) from COVID-19 patients versus controls. Upregulated and downregulated genes are shown in red and blue, respectively. DEGs were defined based on p value ≤ 10⁻⁴ (false discovery rate <0.05 in all cases).
Figure 1. Continued

(i) Moderate disease, 15 had severe disease and 10 were convalescents, and from 10 healthy controls. Autopsy tissues included biopsies from lung, heart, liver, kidney, and spleen of 5 COVID-19 patients and 2 controls, for a total of 29 samples. Demographic and clinical data of COVID-19 patients are summarized in Table S2.

When analyzing autopsy tissue (Figure 1A), the gene repertoires in COVID-19 lung, heart, liver, kidney, and spleen differed substantially from those of control tissues, as indicated in the heatmaps presented in Figures 1B–1F. Analysis of signature scores indicated that, compared with controls, organs from COVID-19 patients underwent most substantial changes (i.e. >50% genes, for which the p value was ≤10^{-4} when compared with healthy control samples) in the expression of genes included in metabolic pathways, namely oxidative phosphorylation (lung, heart, liver, and kidney), glycolysis (lung) and glutamine metabolism (kidney) (see Figures S1A–S1D and S2A–S2F). In addition to these metabolic alterations, likely resulting in the production of aberrantly high levels of ATP, we noted that COVID-19 lungs displayed upregulation of genes associated with Th9 cell immunity (see Figures S1A and S2G), which was found upregulated in peripheral blood samples of COVID-19 convalescents, even 2 months after recovery (Orologas-Stavrou et al., 2020); whereas in COVID-19 spleen genes related to memory cell markers were among those mostly upregulated (see Figure S1E).

Notably, genes associated with TCR repertoire (see Table S3) were among the most downregulated in COVID-19 lung and kidney (Figures 1G, 1H, S1A, and S1D). These changes mainly impacted the alpha, beta and, in part, the gamma and delta variable genes (Figures 1G and 1H). TRAV9-1, TRBV16, and TRBV11-3 in the lung and TRAV9-2, TRAV41, TRAV22, and TRBV29-1 in the kidney were among the genes most downregulated (Figures 1G and 1H). When analyzing TCR alpha, beta, gamma, and delta constant genes (see Table S3), only TRG1 was significantly downregulated in COVID-19 lung when compared with control (Figure 1G); no significant changes were observed in TCR constant genes in COVID-19 kidney (Figure 1H).

When analyzing PBMC samples (Figure 2A), we noted substantial changes in the gene expression makeup of moderate and severe COVID-19 cases, when compared with convalescents and healthy controls (Figure 2B). Signature score analysis indicated that, compared to healthy controls, PBMCs obtained from severe patients showed marked alterations (i.e. ≥50% genes, for which the p value was ≤10^{-4} when compared with healthy control samples) in memory cell markers and Myc targets (see Figure S3A). CCR7, which was previously reported being upregulated in CD4 T-cells from children with multisystem inflammatory syndrome associated with SARS-CoV-2 (Carter et al., 2020), and the apoptotic markers FASLG and TP53 were among the most significantly downregulated genes in these pathways (Figure 2C). Although at a rate lower than 50%, TCR alpha, beta, and, in part, gamma, and delta variable genes were downregulated in PBMC samples obtained from severe COVID-19 patients (Figure 2D). Signature score analysis on PBMCs obtained from moderate and convalescent cases indicated less marked changes in gene pathways in these two groups of subjects (see Figures S3B and S3C). When analyzing TCR alpha, beta, gamma, and delta constant genes, TRAC, TRBC1/2, and TRDC were significantly downregulated in PBMCs from severe COVID-19 patients when compared with control samples (Figure 2D).

The frequency of CD4 and CD8 cells positive for TCR V alpha 2 and TCR V beta 3.1 – two of the TCR variable genes that also underwent profound changes and for which detection using fluorochrome conjugated monoclonal antibodies was available – was determined by flow cytometry (Figures 2E, 2F, and S4A–S4D). There was a significant decrease in the frequency of CD4 cells positive for TCR V alpha 2 or TCR V beta 3.1 in severe COVID-19 patients, when compared with controls (Figures 2E, 2F, S4A, and S4C). The frequency of CD4 TCR V alpha 2+ cells was also decreased in moderate COVID-19 patients and convalescents when compared with controls (Figures 2E and S4A). When considering CD8 cells, we noted decreased CD8 TCR V alpha 2+ cell frequencies in severe COVID-19, when compared with moderate...
Figure 2. Limited TCR repertoire in PBMCs of severe COVID-19 patients

(A) Peripheral blood mononuclear cells (PBMCs) were obtained from severe (n = 6), moderate (n = 6) and convalescent (n = 6) COVID-19 patients and from healthy controls (n = 6). Total RNA was extracted and subjected to hybridization prior to NanoString processing and profiling.

(B) Heatmap showing differentially expressed genes (DEGs) included in the nCounter CAR-T Characterization Panel in PBMCs from moderate, severe and convalescent COVID-19 cases compared with controls. Upregulated and downregulated genes are shown in red and blue, respectively. DEGs were defined based on p value ≤ 10^{-3} (false discovery rate <0.05 in all cases).

(C) Volcano plot showing down- and upregulated genes in severe COVID-19 patients compared with controls. X and Y axes correspond to fold change and p value, respectively. Downregulated and upregulated genes are shown in red and blue. The most significantly downregulated genes are indicated.
COVID-19 cases, convalescents, and healthy individuals (Figures 2E and S4B). The frequency of CD8⁺ TCR V β 3.1⁺ cells in severe and moderate COVID-19 patients was lower than that in healthy controls (Figures 2F and S4D); this decrease trended to significance in both cases (Figure 2F).

Upregulation of ENTPD1/CD39 in severe COVID-19

Alterations of purinergic signaling were previously reported in the context of viral infections (Gupta et al., 2015; Ravimohan et al., 2017) and found to be associated with worse disease outcome (Ravimohan et al., 2017). One of the key mediators of the purinergic pathway is ENTPD1/CD39, an ectonucleotidase that scavenges pro-inflammatory ATP and ADP into AMP, subsequently converted into immunosuppressive adenosine by NT5E/CD73, the ectoenzyme that works in tandem with CD39. ENTPD1 was one of the most highly upregulated genes within the Treg pathway in the lung, liver, and spleen of COVID-19 patients, when compared with controls (Figures 3A and 3B). In these organs, CD39 was elevated not only at the RNA (Figure 3B) but also at the protein level, as indicated by immunohistochemistry staining (Figure 3C).

When analyzing PBMC samples, severe COVID-19 patients had higher levels of ENTPD1, when compared with moderate patients, convalescents, and healthy controls (Figure S5A); this finding being confirmed also by qPCR analysis, performed on PBMCs from a larger cohort of COVID-19 patients (Figure 3D). Importantly, when considering moderate and severe patients, ENTPD1 mRNA levels were positively correlated with markers of vasculopathy like D-Dimer and prothrombin time (Figures 3E and 3F) and with length of stay in hospital and intensive care unit (Figures 3G and 3H).

We next subdivided COVID-19 patients into low and high ENTPD1 expressing groups, based on the median value obtained after pooling the ENTPD1 gene expression levels of severe and convalescent cases (median ENTPD1 levels: 9.9, range: 9.43–10.77). We noted that high ENTPD1 was associated with low expression of TRGC1, CCR7, and CCR4 (Figures 4A and S6A), decreased levels of which have been also linked with impaired Treg suppression and exacerbation of inflammation in other infectious disease contexts (Bertolini et al., 2018); and with high levels of IL-6 (Figures 4A and S6A), a cytokine involved in CD39 induction via signal transducer and activator of transcription-3 (STAT-3) (Chalmin et al., 2012), IFNA1, and IL-33 (Figures 4A and S6A), known to be linked with poor COVID-19 outcome (Burke et al., 2020).

Gene set enrichment analysis (GSEA), where genes of the nCounter CAR-T Characterization panel were ranked based on ENTPD1 expression levels of severe and convalescent cases, indicated that the high ENTPD1-expressing group mainly displayed enrichment in genes associated with chemokine and interleukin signaling pathways (Figures 4B and S6B), whereas the low ENTPD1-expressing group was inversely associated with TCR repertoire and interactions with non-lymphoid cell pathway (Figures 4B and S6B). Further analysis conducted to detect potential interactions between ENTPD1/CD39 and other protein-coding genes included in the nCounter CAR-T panel and identified links with IL-6 and CCR7 that further corroborated the functional associations with these two molecules (Figure 4C).

Additional GSEA where the nCounter CAR-T genes were ranked based on the expression levels of IL-6 and IL-33 in the same groups of subjects, indicated that the high IL-6 and IL-33-expressing group (median IL-6: 4.8, range: 3.1–6.9; median IL-33: median 4.55, range: 3.6–6.1) displayed enrichment in genes associated with chemokine and interleukin signaling (Figures S7A and S7B); whereas the low IL-6 and IL-33-expressing group displayed an inverse association with genes related to the TCR repertoire (Figures S7A and S7B). nCAR-T gene ranking based on the expression levels of CCR7 (median: 8.8, range: 6.1–9.9) revealed that
A. Heatmaps representing the Treg gene set in lung, liver, and spleen of COVID-19 patients, compared with controls. Upregulated and downregulated genes are shown in red and blue.

B. Box plots showing the median ENTPD1 levels in biopsies from autopsic lung, heart, liver, kidney, and spleen of COVID-19 patients (lung n = 5, liver n = 5, spleen n = 3) and controls (n = 2 for all organs). Results were obtained by NanoString. *p ≤ 0.05; **p ≤ 0.01 using two-sided unpaired t test. Samples were run in triplicate and experiments performed independently three times.

C. Expression of CD39 was tested in paraffin embedded sections obtained from biopsies of autopsic COVID-19 and control lung, heart, liver, kidney, and spleen. Immunohistochemistry staining is shown from one COVID-19 patient and one control. Arrows indicate positively stained cells (magnification 20 x, scale bar 100 μM). A representative of three independent staining is shown.

D. ENTPD1 mRNA levels in PBMCs were determined by qPCR in moderate (n = 20), severe (n = 15), convalescent (n = 6) COVID-19 cases and in healthy controls (n = 6). Mean ± SEM is shown. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 using one-way ANOVA followed by Tukey’s multiple comparisons test. Samples were run in triplicate and experiments performed independently three times.

Figure 3. Increased ENTPD1 levels in lung, liver, spleen, and PBMCs from severe COVID-19 patients

(A) Heatmaps representing the Treg gene set in lung, liver, and spleen of COVID-19 patients, compared with controls.

(B) Box plots showing the median ENTPD1 levels in biopsies from autopsic lung, heart, liver, kidney, and spleen of COVID-19 patients (lung n = 5, liver n = 5, spleen n = 3) and controls (n = 2 for all organs). Results were obtained by NanoString. *p ≤ 0.05; **p ≤ 0.01 using two-sided unpaired t test. Samples were run in triplicate and experiments performed independently three times.

(C) Expression of CD39 was tested in paraffin embedded sections obtained from biopsies of autopsic COVID-19 and control lung, heart, liver, kidney, and spleen. Immunohistochemistry staining is shown from one COVID-19 patient and one control. Arrows indicate positively stained cells (magnification 20 x, scale bar 100 μM). A representative of three independent staining is shown.

(D) ENTPD1 mRNA levels in PBMCs were determined by qPCR in moderate (n = 20), severe (n = 15), convalescent (n = 6) COVID-19 cases and in healthy controls (n = 6). Mean ± SEM is shown. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 using one-way ANOVA followed by Tukey’s multiple comparisons test. Samples were run in triplicate and experiments performed independently three times.
the high CCR7-expressing group was enriched in genes related to the TCR repertoire and interactions with non-lymphoid cell pathway (Figure S7C), whereas the low CCR7-expressing group showed an inverse association with genes of the chemokine and interleukin signaling pathways (Figure S7C).

Aberrant ENTPD1/CD39 regulation in late-stage COVID-19

Others and we have previously shown that ENTPD1/CD39 has a pivotal role in the maintenance of immune homeostasis, being regulated at the genetic level by polymorphisms in the promoter region that are associated with low mRNA levels (Friedman et al., 2009; Rissiek et al., 2015; Timperi et al., 2016; Adhikary et al., 2020). ENTPD1/CD39 can be also regulated at the transcriptional level by transcription factors such as the aryl hydrocarbon receptor (AHR), a toxin mediator that modulates adaptive immunity (Quintana et al., 2008; Veldhoen et al., 2008), which has been recently involved in SARS-CoV-2 pathology (Giovannoni et al., 2021); STAT-3 that induces CD39 in Th17 cells (Chalmin et al., 2012) and indirectly by hypoxia-inducible-factor-1-alpha (HIF-1α) via Sp1 (Eltzschig et al., 2009). In PBMCs, we detected an increase in the levels of both STAT-3 and HIF-1α, in severe COVID-19 patients, when compared with healthy controls and convalescents (Figures 4D and 4E), while no differences were noted in the levels of AHR among different groups of patients (Figure 4F).

We have recently shown that ENTPD1/CD39 can be also regulated by an endogenous antisense RNA, which is present in multiple splice variants in antisense position to ENTPD1 at 10q24.1 (Harshe et al., 2020). qPCR analysis indicated downregulation in the levels of CD39-AS RNA in severe COVID-19 patients, when compared with convalescents and controls (Figure 4G). Levels of CD39-AS RNA in moderate COVID-19 patients were also lower than in controls (Figure 4G).

DISCUSSION

Our data show that in severe COVID-19 patients there are alterations in the expression of TCR variable genes along with aberrant regulation of ENTPD1/CD39, the latter resulting in the upregulation of this ectoenzyme at both systemic and organ levels. A marked decrease in the expression of alpha and beta variable genes was noted when analyzing samples obtained from lung, kidney, and PBMCs of severe COVID-19 patients, as compared with other COVID-19 subgroups and controls. Changes in the TCR constant genes were less evident, suggesting a more prominent impact on the TCR variable region. Although derived from a relatively small number of samples, the flow cytometry data showing decrease in the frequency of CD4 and CD8 cells positive for TCR V alpha 2 and of CD4 cells positive for TCR V beta 3.1 in severe COVID-19 patients corroborate the findings obtained at the gene level. Our data indicate that alterations in TCR repertoire are present in both CD4 and CD8 cell subsets, although studies on larger patients’ cohorts are needed to confirm these data.

Decreases in TCR repertoire have been previously linked to immune exhaustion, as in severe HIV and AIDS (Hernandez et al., 2018), early septic shock (Tomino et al., 2017), and tuberculosis (Luo et al., 2012). These changes may lead to inadequate adaptive immune function as a result of limited capability of antigen recognition by T lymphocytes. Further studies at the single-cell level would aid determining which specific T helper/cytotoxic cell subset undergoes downregulation of TCR-related genes in different stages of COVID-19 and whether these subsets are phenotypically and functionally exhausted.

The increases in ENTPD1/CD39 noted in the lung, liver, and spleen, and PBMCs of severe COVID-19 cases indicate likewise associations with disease progression and severity. Such linkages have been previously reported in the context of HIV infection, where high levels of CD39 in CD8 T-cells were associated with immune exhaustion (Gupta et al., 2015) and early mortality (Ravimohan et al., 2017). Increases in the expression of CD39 in peripheral blood-derived CD4 and CD8 cells were reported earlier in hospitalized COVID-19 patients, and further associated with increases in CD38, PD-1, and HLA-DR levels, as determined by high-dimensional flow cytometry analysis (Mathew et al., 2020). However, whether these cellular changes were present in end-organs (lung, kidney) was not tested in earlier studies. Our data indicating a positive correlation between ENTPD1 mRNA levels...
Figure 4. ENTPD1 is aberrantly regulated in severe COVID-19 patients

(A) Volcano plot showing down- and upregulated genes within the high ENTPD1 expressing group. High and low ENTPD1 expression was defined based on the median value (median ENTPD1 levels: 9.9, range: 9.43–10.77 obtained after pooling the ENTPD1 gene expression levels of severe and convalescent cases. nCounter CAR-T genes were then subdivided based on ENTPD1 levels and subsequently associated with high or low ENTPD1 expression. X and Y axes correspond to fold change and p value respectively. Downregulated and upregulated genes are shown in blue and red. The most significantly downregulated and upregulated genes are indicated.

(B) Gene set enrichment analysis (GSEA) showing the gene pathways associated with high and low ENTPD1 expression levels (p value < 0.05 and false discovery rate q value < 0.05). High and low ENTPD1 expression was defined based on the median value obtained after pooling the ENTPD1 gene expression levels of severe and convalescent cases. nCounter CAR-T genes were then subdivided based on ENTPD1 levels and associated with high or low ENTPD1 expression. Lines

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| Figure 4 | ENTPD1 is aberrantly regulated in severe COVID-19 patients |
|----------|----------------------------------------------------------|
| A        | Volcano plot showing down- and upregulated genes within the high ENTPD1 expressing group. High and low ENTPD1 expression was defined based on the median value (median ENTPD1 levels: 9.9, range: 9.43–10.77 obtained after pooling the ENTPD1 gene expression levels of severe and convalescent cases. nCounter CAR-T genes were then subdivided based on ENTPD1 levels and subsequently associated with high or low ENTPD1 expression. X and Y axes correspond to fold change and p value respectively. Downregulated and upregulated genes are shown in blue and red. The most significantly downregulated and upregulated genes are indicated. |
| B        | Gene set enrichment analysis (GSEA) showing the gene pathways associated with high and low ENTPD1 expression levels (p value < 0.05 and false discovery rate q value < 0.05). High and low ENTPD1 expression was defined based on the median value obtained after pooling the ENTPD1 gene expression levels of severe and convalescent cases. nCounter CAR-T genes were then subdivided based on ENTPD1 levels and associated with high or low ENTPD1 expression. Lines |
with D-dimer, prothrombin time, and other clinical parameters like the length of stay in hospital or intensive care unit suggest a role for CD39 as a prognostic marker for disease severity.

Our GSEA results showing a negative association between high CD39 levels and limited TCR repertoire might suggest that the overly suppressive microenvironment, derived from high CD39 and adenosine levels, might predispose to further inflammatory insults and immune compromise. Future studies should be directed at testing whether increase in CD39 expression is indeed associated with increased ectoenzymatic activity and, consequently, heightened adenosine generation.

Our data suggest important functional associations between ENTPD1/CD39 with IL-6, IL-33, and CCR7. In this regard, GSEA studies show that high (or low) expression of these molecules was associated with the chemokine and interleukin signaling gene sets and the TCR repertoire, postulating a role for CD39, IL-6, IL-33, and CCR7 in the modulation of these pathways in the setting of COVID-19.

As shown by our findings, upregulation of ENTPD1/CD39 in late-stage COVID-19 could be associated with aberrant regulation of this ectoenzyme, as indicated by heightened levels of STAT-3 and HIF-1α, which contribute to CD39 modulation at the transcriptional level (Chalmin et al., 2012; Xie et al., 2018; Mascarenhas et al., 2015). Aberrant CD39 regulation in COVID-19 might also derive from decreased levels of CD39-AS RNA, as we have recently reported (Harshe et al., 2020). This endogenous antisense RNA regulates ENTPD1 expression and was previously found elevated in the peripheral blood and lamina propria-derived Tregs and Th17 cells of Crohn’s disease patients (Harshe et al., 2020). It remains to be determined whether high levels of ENTPD1 in severe COVID-19 subjects are also associated with the possession of GG alleles at rs10748643, as others and we have previously shown (Friedman et al., 2009; Rissiek et al., 2015; Timperi et al., 2016; Adhikary et al., 2020). Further investigations in larger cohorts of COVID-19 patients and controls are needed to address this important point.

It is unclear whether T cells with specificity for viral epitopes do preferentially express high levels of CD39. Previous work in the context of cancer has shown that tumor-infiltrating, non-specific “bystander” CD8 T-cells preferentially recognize viral epitopes (e.g. influenza and cytomegalovirus) instead of tumor antigens and lack CD39 expression (Simoni et al., 2018). Based on this earlier study, it is unlikely that T cells recognizing COVID-19 epitopes are those expressing high levels of CD39, although additional work at the functional and/or single-cell level might help elucidating this.

In conclusion, we have shown that perturbations in T cell immunity in COVID-19 can be linked to limitations in the TCR repertoire, systemically with blood cells and locally, at the organ level. We also note aberrant elevations of ENTPD1/CD39, possibly associated with enhanced expression of CD39-related transcription factors along with decreases in the levels of CD39 endogenous antisense. These changes might contribute to a purinergic imbalance that perpetuates immunosuppression, resulting in metabolic changes and T cell dysfunction. Strategies that interfere with these purinergic alterations as in the case of antibodies to CD39 might be beneficial in preventing COVID-19 progression or in stabilizing those patients with severe disease.

**Limitations of the study**

We acknowledge that the analysis of a small number of biopsies, obtained at autopsy from non-COVID-19 controls, is a limitation of our study and that a larger number of such biopsies is needed for a complete

**Figure 4. Continued**

represent individual gene sets. Upregulated genes are located on the left, while downregulated genes are located on the right of the plot.

(C) PPI network between ENTPD1/CD39 and other protein-coding genes included in the nCounter CAR-T characterization panel was derived by STRING (threshold value of median confidence of 0.4).

(D–F) Box plots showing the median (D) STAT-3, (E) HIF-1α, and (F) AHR mRNA levels in PBMCs from moderate (n = 6), severe (n = 6) and convalescent (n = 6) COVID-19 patients and healthy controls (n = 6). Data were obtained by NanoString analysis. **p ≤ 0.01 and ***p ≤ 0.001 using one-way ANOVA followed by Tukey’s multiple comparisons test. Samples were run in triplicate and experiments performed independently three times.

(G) Levels of ENTPD1-AS RNA were tested by qPCR in moderate (n = 20), severe (n = 15), convalescent (n = 6) COVID-19 cases and in healthy controls (n = 6). Mean ± SEM ENTPD1-AS RNA is shown. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 using one-way ANOVA followed by Tukey’s multiple comparisons test. Samples were run in triplicate and experiments performed independently three times. See also Figures S6 and S7.
validation of our findings. However, due to the clear time constraints related to the ongoing COVID-19 pandemic and the clinical and logistical limitations in obtaining additional controls at autopsy, we have preferred making these earlier investigations available to the scientific community now.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and software availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHODS DETAILS
  - Sample preparation
  - RNA extraction and NanoString transcriptomic profiling
  - qPCR
  - Immunohistochemistry
  - Flow cytometry
- QUANTIFICATION AND STATISTICAL ANALYSIS

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103205.

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**AUTHOR CONTRIBUTIONS**

Acquisition of data, analysis and interpretation of data, and drafting of the manuscript, N.W., M.V., A.K.; acquisition and analysis of data, J.J.G.; acquisition and interpretation of data, C.D., E.M., and K.G.; critical revision of the manuscript, J.L.H., S.S., S.C.R., and M.S.L.; writing of the manuscript, M.S.L.; obtained funding, M.S.L. and S.C.R.

**DECLARATION OF INTERESTS**

C.D. and K.G. are employees and shareholders of NanoString Technologies, Inc. E.M.M. was an employee and shareholder of NanoString Technologies, Inc at the time of study.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-human CD39 | Sigma Aldrich | Cat. # HPA014067; RRID:AB_1848178 |
| Goat anti-rabbit IgG antibody | Vector Laboratories | Cat. # BA-1000; RRID:AB_2313606 |
| TCR V alpha 2 monoclonal antibody | Invitrogen | Cat. # TCR2663; RRID:AB_417093 |
| TCR V beta 3.1 monoclonal antibody | Invitrogen | Cat. # TCR2740; RRID:AB_223625 |
| PerCP/Cyanine 5.5 anti-human CD4 | Biolegend | Cat. # 317428; RRID:AB_1186122 |
| PE anti-human CD8 | Biolegend | Cat. # 344706; RRID:AB_1953244 |
| Brilliant Violet 785 anti-human CD3 | Biolegend | Cat. # 317330; RRID:AB_2563507 |
| **Biological samples** |        |            |
| COVID-19 PBMC samples | Massachusetts General Hospital and Brigham’s and Women’s Hospital | https://www.massgeneral.org; https://www.brighamandwomens.org |
| COVID-19 and non-COVID-19 FFPE samples | Beth Israel Deaconess Medical Center | https://www.bidmc.org |
| Healthy controls PBMC samples | Blood Donor Center at Boston Children’s Hospital | https://www.childrenshospital.org/ways-to-help/donate-blood |
| **Critical commercial assays** |        |            |
| RNeasy Mini kit (50) | Qiagen | Cat. # 74104 |
| AllPrep DNA/RNA FFPE kit | Qiagen | Cat. # 80234 |
| nCounter CAR-T Characterization Panel | NanoString | Cat. # XT-CSO-CART1-12 |
| iScript cDNA synthesis kit | Bio-Rad Laboratories | Cat. # 1708891BUN |
| **Oligonucleotides** |        |            |
| Human CD39/ENTPD1 forward | Harshe et al. (2020) | NA |
| 5’- AGTGCCCATATGGCTGGATTAC-3’ |        |            |
| Human CD39/ENTPD1 reverse | Harshe et al. (2020) | NA |
| 5’- CCAAGGCTCAAGGTITCC-3’ |        |            |
| Human CD39-AS forward | Harshe et al. (2020) | NA |
| 5’- AATACGGCAGCGACCACCAGG-3’ |        |            |
| Human CD39-AS reverse | Harshe et al. (2020) | NA |
| 5’- TGAATCCACTCTGCTCCTGCA-3’ |        |            |
| Human β-actin forward | This paper | NA |
| 5’- CTCTCCAGCCTCCCTTCT-3’ |        |            |
| Human β-actin reverse | This paper | NA |
| 5’- AGCAGCTGTGTGGCGTACAG-3’ |        |            |
| **Software and algorithms** |        |            |
| nSolver™ Analysis Software, 4.0 | NanoString Technologies | https://www.nanostring.com/products/analysis-solutions/ncounter-advanced-analysis-software/ |
| R software, 4.0.2 | R | https://cran.r-project.org |
| pheatmap package | R environment | https://CRAN.R-project.org/package=pheatmap |
| limma package | Ritchie et al. (2015) | https://bioconductor.org/packages/release/bioc/html/limma.html |
| clusterProfiler package | Yu et al. (2012) | http://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Maria Serena Longhi (mlonghi@bidmc.harvard.edu).

Materials availability
This study did not generate new unique reagents.

Data and software availability
NanoString bulk transcriptomics data are provided in Table S4 and are available from Mendeley Data at https://doi.org/10.17632/mkmv3mgz86.1.

qPCR and all other data reported in this paper are available from the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS
PBMC samples were obtained from a total of 45 COVID-19 patients recruited from Massachusetts General Hospital (n = 30) and Brigham and Women’s Hospital (n = 15) as part of the Massachusetts Consortium on Pathogen Readiness initiative. All patients had laboratory confirmed COVID-19 (detected by nasopharyngeal or oropharyngeal swabs). Of these 45 patients, 20 had moderate disease while 15 had severe disease and 10 were convalescents. As per World Health Organization definition, severe cases were defined as those with clinical signs of pneumonia in association with one of the following signs: respiratory rate higher than 30 breaths/min, severe respiratory distress and SpO2<93% on room air. In moderate and severe cases, PBMC samples were collected at the time of hospital admission. In the case of convalescents, PBMCs were obtained during recovery, 4–12 weeks after the onset of symptoms. PBMCs were also obtained from 10 healthy controls (6 males and 4 females; median age 39 years, range 26–55).

Ultrasound guided biopsies of autopic lung, heart, kidney, liver and spleen were obtained from 5 COVID-19 patients (1 moderate, 4 severe) and 2 non-COVID-19 controls (one male, 65 year-old; one female 59 year-old). These were carried out in the Pathology Department, Beth Israel Deaconess Medical Center (BIDMC), Boston. In COVID-19 cases, death occurred as a result of COVID-19 related multi-organ failure; whereas in the non-COVID-19 controls death was due to brain fungal infection in one case and shock in the setting of acute on chronic liver failure from established, non-infectious liver cirrhosis, in the other. The histopathological findings in the organs of COVID-19 patients were representative of SARS-CoV-2-related injury and consistent with previous reports (Simoni et al., 2018). Demographic and clinical data of all COVID-19 patients recruited in the study are summarized in Table S2. The study received IRB approval (protocol # 2020P000675) by the Committee of Clinical Investigations, BIDMC. Written informed consent was obtained from all study participants prior to inclusion in the study.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| enrichplot package  | Yu et al. (2012) | https://yulab-smu.top/biomedical-knowledge-mining-book/ |
| Cytoscape software, 3.8.2 | Shannon et al. (2003) | https://cytoscape.org/ |
| FlowJo, 10          | FlowJo | https://www.flowjo.com |
| QuantStudio Design & Analysis software, 1.5.1 | Applied Biosystems | https://www.thermofisher.com/us/en/home/global/forms/life-science/quantstudio-3-5-software.html |
METHODS DETAILS

Sample preparation
Peripheral blood samples were collected in ethylenediaminetetraacetic acid-anticoagulated tubes. PBMCs were obtained by density gradient centrifugation on Ficoll-Paque (GE Healthcare Life Sciences, Pittsburgh, PA), resuspended in 90% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) and 10% dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO) and stored at −140°C until use. Mononuclear cell viability, determined by Trypan Blue exclusion, exceeded 98%.

Autopsic tissue samples were placed in formalin immediately after collection; after 24 hours, samples were processed and paraffin embedded. Formalin fixed paraffin embedded (FFPE) tissue samples were kept at 4°C until RNA was extracted.

RNA extraction and NanoString transcriptomic profiling
Total RNA was obtained from PBMC and FFPE samples using the RNeasy Mini kit (Qiagen, Germantown, MD) and the AllPrep DNA/RNA FFPE kit according to the manufacturer’s instructions. RNA quantification was carried out using Nanodrop whereas RNA integrity was verified using Agilent Bioanalyzer. Samples with DN200 higher than 70% were used for subsequent steps. NanoString profiling was performed using the nCounter Human CAR-T characterization panel. 100 ng of total RNA was used as input into hybridization reactions containing reporter and capture probes, according to the manufacturer’s instructions. RNA samples were hybridized at 65°C for 24 hours and subsequently processed and each analyzed in a single nCounter cartridge lane. Post hybridization processing was carried out in an nCounter MAX System.

RCC files were compiled and analyzed using nSolver analysis software (version 4.0), as per instructions of the manufacturer. The internal reference genes included in the probe set were used for normalization. All PBMC and FFPE samples subjected to NanoString analysis were run in triplicate and experiments repeated independently three times.

qPCR
Expression of human CD39 mRNA and CD39-AS RNA was determined by qRT-PCR. Total RNA was obtained from PBMC and FFPE samples, as indicated above. mRNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. ENTPD1/CD39 and antisense primer sequences are as follows:

CD39/ENTPD1
Forward 5’ AGGTGCCTATGGCTGGATTAC 3’
Reverse 5’ CCAAAGCTCCAAAGGTTTCCT 3’

CD39-AS
Forward 5’ AATACTGTGACACTGACCACCAGG 3’
Reverse 5’ TGAATCCACTCTGTCTCCTGCA 3’

Samples were run in triplicate on a QuantStudio 3 (Applied Biosystems, Foster City, CA). Experiments were repeated independently three times. Results were analyzed by matched software and expressed as relative quantification. Relative gene expression was determined after normalization to human β-actin.

Immunohistochemistry
Paraffin embedded lung, heart, liver, kidney, and spleen tissue sections were subjected to antigen retrieval (Liberal et al., 2016). Sections were subsequently incubated overnight at 4°C with rabbit polyclonal anti-human CD39 (cat. # HPA014067, Sigma Aldrich) at 1/100 (Vuerich et al., 2021). Following endogenous peroxidase blocking with 3% H2O2, sections were incubated with 1/1,000 goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) for one hour at room temperature. After treatment with Vectastain Elite ABC kit (Vector Laboratories), ImmPACT DAB (Vector Laboratories) was applied and sections examined by light microscopy.
Flow cytometry

PBMCs were stained with PerCP-Cyanine 5.5 anti-human CD4 (clone # OKT4, Biolegend, San Diego, CA), PE anti-human CD8 (clone # SK1, Biolegend), FITC anti-human TCR V alpha 2 (clone # F1, Invitrogen, Waltham, MA) and FITC anti-human TCR V beta 3.1 (clone # 8F10, Invitrogen). Cells were acquired on a CytoFLEX LX flow cytometer (Beckman Coulter, Pasadena, CA) and analyzed using FlowJo 2 software (version 10, TreeStar, Ashland, OR). Fluorescence compensation was adjusted based on fluorescence-minus-one method.

QUANTIFICATION AND STATISTICAL ANALYSIS

R software (version 4.0.2) was used for further data analysis of normalized data, differentially expressed gene (DEG) results and gene set scores obtained from nSolver Advanced Analysis software. Heatmaps of DEGs were obtained using heatmap package. DEGs where defined based on P value ≤ 10^{-4} when analyzing samples from organs; and P value ≤ 10^{-3} when analyzing PBMC samples. Two different threshold for P values were adopted because the changes in gene expression were more substantial in the organs than PBMC samples. False-discovery-rate (FDR) was controlled by the Benjamini-Yekutiel method. All DEGs had FDR consistently lower than 0.05.

When subdividing the genes of the nCounter CAR-T Characterization Panel based on high and low ENTPD1 expression levels (high and low ENTPD1 expression was based on the median expression levels in severe and convalescent COVID-19 patients), limma package was used (Ritchie et al., 2015). For this set of analysis, genes with a P value < 0.05 and a |Log fold change| > 0.585 were defined as DEGs.

To identify enriched pathways, GSEA was carried out on the pathway annotations included in the nCounter CAR-T Characterization Panel and visualized using clusterProfiler package (Yu et al., 2012). Gene sets with nominal P value < 0.05 and FDR q value < 0.05 were considered significant. Pathway enrichment results were also visualized using enrichplot package (Yu et al., 2012).

Protein-protein interaction (PPI) network was retrieved from the STRING database using a threshold value of median confidence of 0.4. Results were inputted and analyzed using Cytoscape software (version 3.8.2) (Shannon et al., 2003). Cytohubba plugin in Cytoscape was used to visualize the relationships within the PPI network, based on the degree ranking in Cytoscape.

qPCR and flow cytometry data are presented as Mean ± SEM. Normality of variable distribution was assessed by Kolmogorov-Smirnov goodness-of-fit test. Comparisons were performed using unpaired Student’s t test. One-way ANOVA test, followed by Tukey’s multiple comparison test, were used when comparing more than two sets of data. Correlation analysis was performed using Pearson’s correlation coefficient. P ≤ 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism, version 7.0a (GraphPad Software, San Diego, CA).