CORONAVIRUS

Host-microbe multiomic profiling reveals age-dependent immune dysregulation associated with COVID-19 immunopathology

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Age is a major risk factor for severe coronavirus disease 2019 (COVID-19), yet the mechanisms behind this relationship have remained incompletely understood. To address this, we evaluated the impact of aging on host immune response in the blood and the upper airway, as well as the nasal microbiome in a prospective, multicenter cohort of 1031 vaccine-naïve patients hospitalized for COVID-19 between 18 and 96 years old. We performed mass cytometry, serum protein profiling, anti–severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antibody assays, and blood and nasal transcriptomics. We found that older age correlated with increased SARS-CoV-2 viral abundance upon hospital admission, delayed viral clearance, and increased type I interferon gene expression in both the blood and upper airway. We also observed age-dependent up-regulation of innate immune signaling pathways and down-regulation of adaptive immune signaling pathways. Older adults had lower naïve T and B cell populations and higher monocyte populations. Over time, older adults demonstrated a sustained induction of pro-inflammatory genes and serum chemokines compared with younger individuals, suggesting an age-dependent impairment in inflammation resolution. Transcriptional and protein biomarkers of disease severity differed with age, with the oldest adults exhibiting greater expression of pro-inflammatory genes and proteins in severe disease. Together, our study finds that aging is associated with impaired viral clearance, dysregulated immune signaling, and persistent and potentially pathologic activation of pro-inflammatory genes and proteins.

INTRODUCTION

Age is a major risk factor for severe coronavirus disease 2019 (COVID-19), with older adults experiencing markedly greater rates of acute respiratory distress syndrome and death compared to younger individuals (*1*–*3*). Even with primary series vaccination rates above 90% (*4*), adults over 75 years of age are 140 times more likely to die if infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (*5*). Despite these epidemiological associations, the biological mechanisms underlying the impact of aging on COVID-19 remain incompletely understood.

Observational cohort studies of healthy adults (*6*, *7*) demonstrate that aging leads to baseline increases in plasma concentrations of pro-inflammatory cytokines (*6*, *8*), several of which, such as interleukin-6 (IL-6), are well-known biomarkers of COVID-19 severity, suggesting potential connections between the pathophysiology of human aging and COVID-19 (*8*). Juxtaposed against this state of aging-associated inflammation are functional impairments in innate and adaptive immune signaling observed during the vaccination of aged individuals (*9*–*12*). Furthermore, recent human in vitro data indicate that aging results in impaired production of type I interferons (IFNs) in monocytes and dendritic cells after Toll-like receptor (TLR) ligation, suggesting disrupted innate immunity (*13*–*17*).

Comparative upper respiratory tract transcriptional profiling has demonstrated that mild SARS-CoV-2 infection induces a more robust innate and adaptive immune response in children compared with adults (*18*, *19*). Paradoxically, among adults hospitalized for COVID-19, a more robust immune response underlies the pathogenesis of severe disease, suggesting more complicated relationships between aging and host defense for older individuals. Adding further complexity, and highlighting the need for additional investigation, is the association between increased age, development of anti-IFN autoantibodies, and disease severity (*2*, *20*, *21*).

The pathophysiology of COVID-19 involves a dynamic relationship between SARS-CoV-2 and the host immune response (*22*, *23*), yet studies of COVID-19 and aging have assessed each

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independently. Furthermore, heterogeneity in human physiology necessitates a large sample size to optimally study aging and host immunity. To address these gaps, we leveraged data from 1031 vaccine-naïve adults who were hospitalized for COVID-19 and enrolled in the IMPACC (IMmunoPhenotyping Assessment in a COVID-19 Cohort) cohort (*2*, *24*) and performed a multiomic, host-microbe systems immunoprofiling study of aging.

From 2523 longitudinally collected blood and nasal swab samples, we investigated the impact of aging on SARS-CoV-2 viral relative abundance, anti–SARS-CoV-2 antibody (Ab) titers, host gene expression, inflammatory protein expression, immune cell populations, and the nasal microbiome. Our study leverages a robust multicenter cohort to gain insights into aging and immunity by integrating both host and microbe data. This work builds on landmark clinical studies demonstrating that age is a major risk factor for COVID-19 severity (*1*–*3*), assesses the generalizability of early translational studies that included smaller numbers of study participants (*8*, *13*, *25*, *26*), and generates additional insights into the age-dependent aspect of COVID-19 pathophysiology.

RESULTS

Study cohort

We analyzed blood and nasal swab specimens from 1031 vaccine-naïve adults with COVID-19 enrolled in the IMPACC cohort from 20 hospitals across the United States (Fig. 1A and table S1) (*2*, *24*). Participants were grouped into age quintiles (18 to 46, 47 to 54, 55 to 62, 63 to 70, and 71 to 96 years old), ranging from 187 to 223 participants (median 206 participants) per age group (Fig. 1B). Samples were collected from each patient in up to six visits, of which visit 1 was at the time of hospital admission. We analyzed the age distributions across five previously defined COVID-19 disease trajectory groups (TGs) (*2*), ranging from mild disease with brief length of hospital stay (trajectory group 1, TG1) to severe disease and death (TG5). We found that advanced age was more common in those in the more severe disease TGs compared with the mild-moderate groups ($P < 1 \times 10^{-15}$; Fig. 1C) and in those who died compared with survivors ($P < 1 \times 10^{-15}$; Fig. 1D). To investigate host immune and microbial features associated with age, we used a wide range of assays at visit 1 baseline (within 72 hours of hospital admission) and longitudinally after hospital admission (Fig. 1A). These included transcriptional profiling of peripheral blood mononuclear cells (PBMCs) and nasal swabs, serum inflammatory protein profiling (Olink assay), whole blood mass cytometry (CyTOF), nasal metatranscriptomics, and anti–SARS-CoV-2 Ab assays.

Aging is associated with higher SARS-CoV-2 viral abundance, impaired viral clearance, and lower SARS-CoV-2 antibody titers

In our study, we measured the SARS-CoV-2 viral relative abundance using the SARS-CoV-2 reads per million (rpM) obtained from nasal swab metatranscriptomics. We verified that the metatranscriptomic viral abundance was highly correlated with the quantitative polymerase chain reaction viral load as measured by the cycle threshold ($P < 1 \times 10^{-15}$; fig. S1). Therefore, we solely used SARS-CoV-2 rpM in all of our analyses that involved viral abundance.

Older adults (63 to 96 years old) had a significantly higher SARS-CoV-2 viral abundance at visit 1 compared with the younger adults (18 to 62 years old) ($P = 0.0092$; Fig. 1E). This age-related increase in viral abundance was not explained by differences in time from symptom onset (fig. S2). Longitudinal analysis also revealed significant differences in viral abundance dynamics, with the oldest adults demonstrating delayed viral clearance compared with the youngest adults ($P = 0.0030$; Fig. 1F). We also assessed anti–SARS-CoV-2 receptor binding domain (RBD) immunoglobulin G (IgG) Ab titers across the five age groups and found that no difference in IgG titers was observed at visit 1 (fig. S3A), but the longitudinal dynamics of Ab titers varied with age (fig. S3B).

Age-dependent differences were observed in immune cell populations

We quantified differences in proportions of immune cell populations in the peripheral blood by CyTOF to assess whether aging altered cell type frequencies (*24*). At visit 1, we found 21 immune cell types significantly associated with age (adjusted $P < 0.05$; Fig. 2, A and B). Increased age correlated with higher proportions of classical monocytes ($CD14^+$ $CD16^-$), nonclassical monocytes ($CD14^-$ $CD16^+$), and intermediate monocytes ($CD14^+$ $CD16^+$). Terminally differentiated/exhausted natural killer (NK) cell ($CD56^{lo}$ $CD16^{hi}$ $CD57^{hi}$) proportions also increased with age, as did activated $CD4^+$ T cells and central memory (CM) $CD8^+$ T cells (Fig. 2, B and C). In contrast, older adults had lower proportions of naïve $CD8^+$ T cells, naïve B cells, gamma-delta (γδ) T cells, and plasmablasts (Fig. 2, B and C). Last, we found that the age-related differences in cell type frequencies were not affected by SARS-CoV-2 viral abundance (fig. S4).

Age-dependent changes in PBMC gene expression are apparent at the time of hospitalization

Next, we performed PBMC transcriptional profiling and identified 3763 genes significantly associated with age at hospital admission (visit 1), controlling for sex and disease severity (adjusted $P < 0.05$; Fig. 3A). Gene set enrichment analysis (GSEA) revealed the up-regulation of several innate immune-related pathways in older participants, including IFN-α/β, IFN-γ, and TLR signaling (Fig. 3B and data file S1). In contrast, several adaptive immunity-related pathways were down-regulated in older individuals, such as B cell receptor (BCR) signaling, T cell receptor (TCR) signaling, and programmed cell death protein 1 (PD1) signaling.

Prior studies have demonstrated a direct relationship between SARS-CoV-2 viral abundance and IFN-stimulated gene (ISG) expression (*18*, *27*). We, therefore, hypothesized that the strong positive correlation between age and viral abundance (Fig. 1E) might contribute to the up-regulation of innate immunity genes and pathways that we observed in older adults. To test this hypothesis, we repeated the differential expression and GSEA analyses while additionally controlling for SARS-CoV-2 viral abundance. Age-related increases in IFN-γ, TLR signaling, and neutrophil degranulation remained significant (adjusted $P < 0.05$; Fig. 3B). IFN-α/β, IL-2, and caspase activation signaling, however, lost statistical significance, suggesting that the enrichment of these pathways in older patients was due to the age-related increase in viral abundance (data file S2). Given that several comorbidities were associated with age, we also asked whether they might influence our findings. After controlling for the presence of the three most prevalent comorbidities (hypertension, diabetes, and chronic cardiac disease; table S1), we found that GSEA results remained largely unchanged (fig. S5).

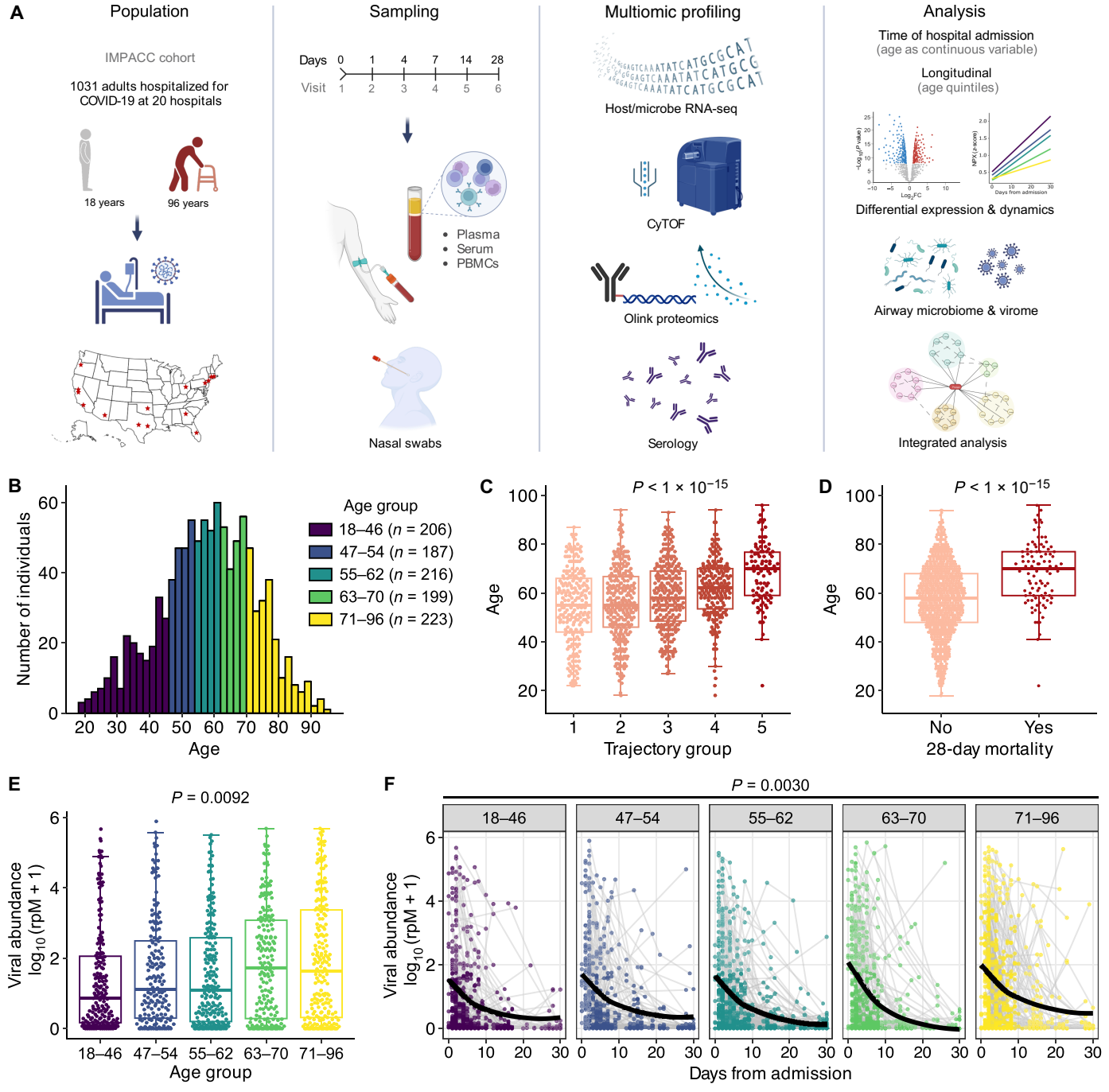

Fig. 1. Older patients have more severe COVID-19 and greater SARS-CoV-2 viral abundance. (**A**) Graphical study overview. This study evaluated 1031 patients with COVID-19 between the ages of 18 and 96 enrolled in the IMPACC cohort at 20 hospitals across the United States. (**B**) Age distribution of the participant cohort. (**C** and **D**) The box plot shows the relationship between patients' age and TG severity (C) or mortality (D). In (C), $n = 217$, 270, 251, 191, and 102 patients in TG1 to TG5, respectively. In (D), $n = 102$ and 929 patients for the dead and surviving groups, respectively. (**E**) Nasal swab SARS-CoV-2 viral abundance at visit 1 (reads per million, or rpM, as measured by metatranscriptomics) in each age group ($n = 191$, 175, 205, 190, 212 patients in youngest to oldest age groups). In (C) to (E), the boxes indicate the first and third quartiles of the distributions, the boxes' center lines indicate median values, and the whiskers indicate 1.5× the interquartile range below the first quartile and above the third quartile. (C to E) *P* values were calculated using the Kruskal-Wallis test to test whether the medians of all groups were the same. (**F**) Nasal swab SARS-CoV-2 viral abundance over time in each age group ($n = 448$, 451, 544, 507, 573 samples in youngest to oldest age groups). Each gray line connects longitudinal samples from the same patient. The black line indicates the generalized additive mixed-effects model fit in each age group. The *P* value was calculated for the interaction between days from admission and age groups with generalized additive mixed-effects modeling.

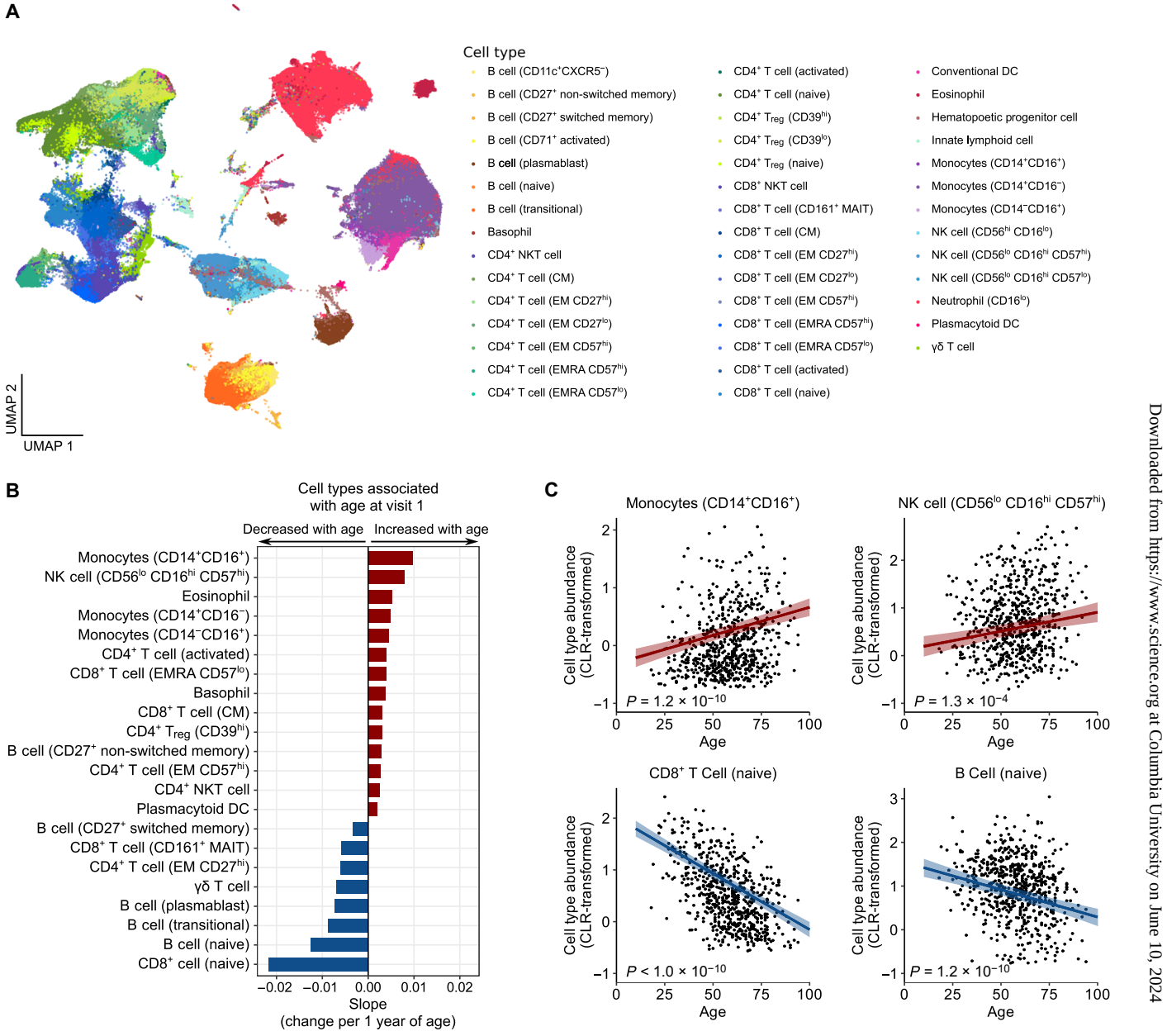

Fig. 2. Aging alters immune cell populations during COVID-19. (**A**) Uniform Manifold Approximation and Projection (UMAP) plot shows blood cell types analyzed by CyTOF (n = 643 patients). (**B**) Bar plot shows blood cell types that are increased (red) or decreased (blue) with age at visit 1. (**C**) Scatter plots depict centered log ratio (CLR)–transformed proportions of four example cell types against age. The red and blue lines indicate the linear fit, and the shaded regions indicate the 95% confidence intervals of the fits. *P* values were calculated using linear modeling with Benjamini-Hochberg correction.

To assess whether our observations were specific to COVID-19 or reflected the general effects of aging, we compared our GSEA results against a meta-analysis of public data from 14,983 healthy adults (*6*). First, we compared the directionality of age-related transcriptional changes on an individual gene level between patients with COVID-19 and healthy controls (fig. S6A). We identified 120 age-associated genes with opposing expression patterns based on COVID-19 status (data file S3). For instance, *TREML1*, which protects against acute lung injury and moderate inflammation in a murine model of sepsis (*28*), was down-regulated with age in COVID-19 but up-regulated in healthy controls. Conversely, the expression of *CD93*, which supports plasma cell longevity leading to elevated Ab titers (*29*) and may function in the clearance of apoptotic cells (*30*), increased with age in patients with COVID-19 but decreased with age in healthy controls.

Next, we compared our data to the healthy control data on a pathway level. Some pathways were up-regulated with age in both data sets (e.g., IFN-γ and TLR signaling), whereas others were uniquely up-regulated in the context of COVID-19, including caspase activation and tumor necrosis factor (TNF) receptor–associated factor

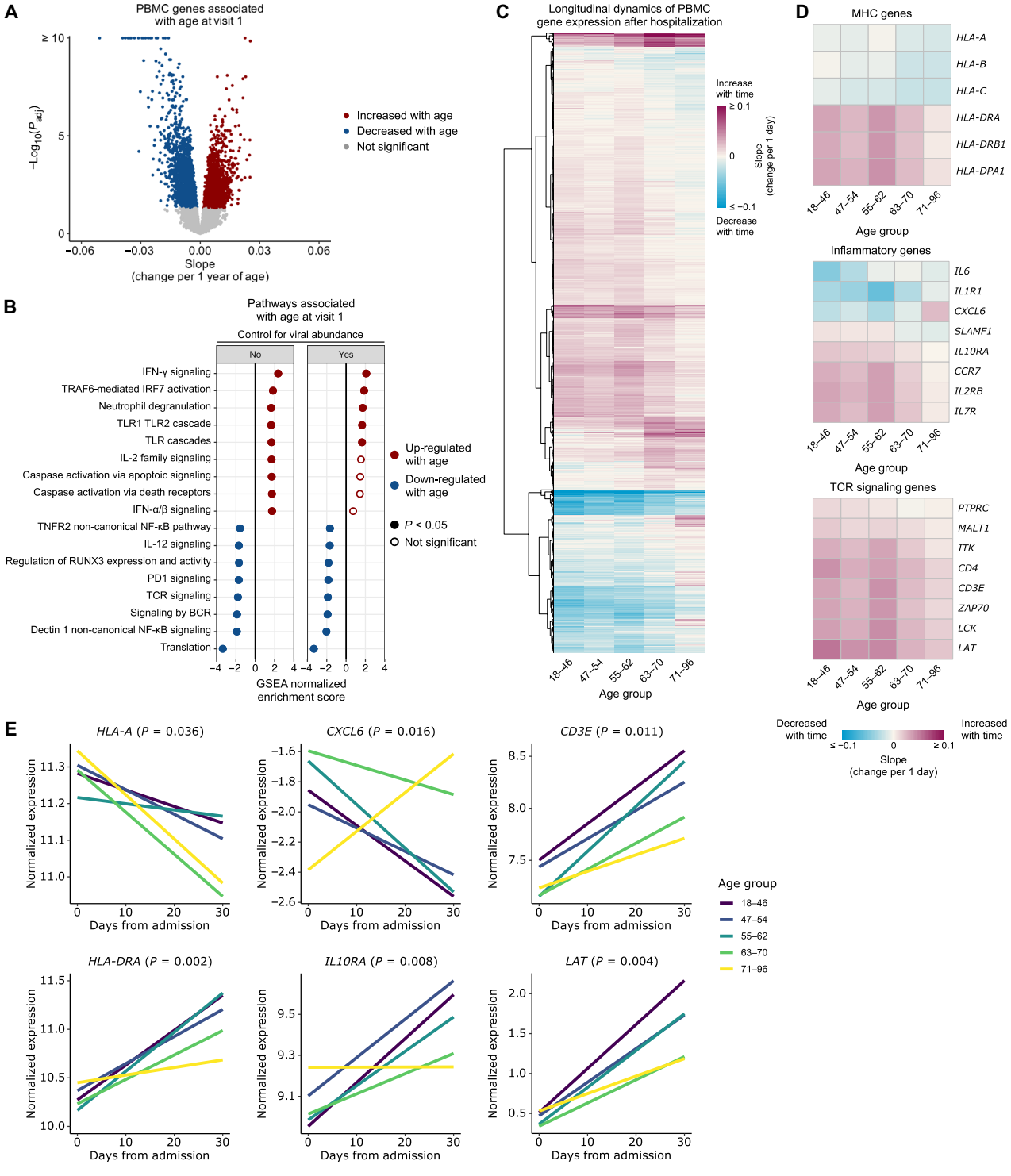

Fig. 3. Aging leads to changes in PBMC gene expression during COVID-19. (**A**) Volcano plot highlights genes associated with age at visit 1 in PBMC RNA-seq data (n = 833 patients). (**B**) The dot plot demonstrates select Reactome pathways associated with age at visit 1, with (right) and without (left) controlling for SARS-CoV-2 viral abundance, in PBMC samples (data files S1 and S2). P values in (A) and (B) were calculated with the limma linear model and Benjamini-Hochberg correction. NF-κB, nuclear factor κB; RUNX3, run-related transcription factor 3. (**C**) Heatmap shows the longitudinal slopes (change in gene expression per day) of 2737 genes that significantly differ longitudinally between the five age groups (adjusted $P < 0.05$). n = 379, 384, 469, 449, and 485 samples in youngest to oldest groups. (**D**) Heatmaps show the longitudinal slopes of select MHC, inflammatory, and TCR signaling genes from (C). (**E**) Plots display the longitudinal dynamics of six example genes from (D). P values in (C) to (E) were calculated for the interaction term between days from admission and age groups using linear mixed-effects modeling and Benjamini-Hochberg correction. (Full longitudinal dynamics plots with confidence intervals are provided in fig. S7.)

6–mediated interferon regulatory factor 7 activation (fig. S6B and data file S4). Similarly, age-related down-regulation of TCR and BCR signaling, as well as several other pathways, was unique to COVID-19 (fig. S6C).

Age-dependent differences were observed in PBMC gene expression dynamics

We next performed a longitudinal analysis of PBMC transcriptomics data over approximately 28 days after hospital admission to identify genes that exhibited age-dependent differences in longitudinal dynamics while controlling for sex and severity TG. Using linear mixed-effects modeling, we identified 2737 genes that had different longitudinal dynamics across age quintiles (Fig. 3C and data file S5). For example, the expression of major histocompatibility complex (MHC) class II genes (e.g., *HLA-DRA*) increased over time after hospitalization in all age groups, but the rate of increase was greater in younger participants (Fig. 3, D and E, and fig. S7). In contrast, the expression of MHC class I genes (e.g., *HLA-A*) decreased over time across all five age groups, but the rate of decrease was greater in older participants.

The longitudinal dynamics of several inflammatory genes also differed between age groups (fig. S7). For instance, the expression of the gene C-X-C motif chemokine ligand 6 (*CXCL6*) increased over the course of hospitalization in the oldest age group, whereas in younger participants, its expression decreased markedly. In contrast, expression of the anti-inflammatory gene *IL10RA* increased over time in the youngest participants while remaining stable in the oldest participants. TCR signaling genes (e.g., *CD3E* and *LAT*) were globally up-regulated over time; however, their induction was attenuated in the oldest age quintile compared with the youngest. Together, our results suggested both greater activation and impaired attenuation of immune signaling with advanced age.

Age-dependent differences were observed in cytokine and chemokine expression upon hospitalization and over time

The impact of aging on immune signaling in COVID-19 was also evident at the protein level. We identified 43 serum proteins that were significantly correlated with age at the time of hospital admission (adjusted $P < 0.05$; Fig. 4, A and B, and fig. S8A). Of these, 31 increased with age, and the protein with the greatest change was C-X-C motif chemokine ligand 9 (CXCL9), a T cell chemoattractant induced by IFN-γ and produced by neutrophils and macrophages (*7*). Twelve proteins significantly decreased with age (adjusted $P < 0.05$), including TNF superfamily member 11 (TNFSF11), which is involved in the regulation of T cell–dependent immune responses and group 2 innate lymphoid cell–mediated type 2 immunity (*31*), and sirtuin 2 (SIRT2), which may attenuate aging-associated inflammation through de-acetylation of the nucleotide-binding domain and leucine-rich repeat-containing (NLR) family, pyrin domain-containing protein 3 (NLRP3) inflammasome (*32*).

On the basis of our prior work (*18*), we hypothesized that aging might affect the relationship between protein expression and viral abundance. Consistent with this idea, we identified eight cytokines and chemokines whose expression correlated with SARS-CoV-2 viral abundance (Fig. 4C) and observed differences in this relationship between the oldest and youngest age groups. For instance, the expression of IL-10, a key anti-inflammatory cytokine, increased more strongly in response to viral abundance in younger patients. C-X3-C motif chemokine ligand 1 (CX3CL1), a chemoattractant of T cells and monocytes, exhibited a similar relationship (Fig. 4C).

We next evaluated the longitudinal dynamics of protein expression in the serum after hospitalization (Fig. 4D). The expression of several cytokines, such as TNFSF11, increased over time in younger adults but lagged in the oldest adults (Fig. 4E and fig. S8B). Conversely, the expression of several pro-inflammatory cytokines and chemokines, such as CXCL8, CXCL9, and IL-6, decreased rapidly over time in younger adults, whereas, in the oldest adults, their expression increased over time (CXCL8 and CXCL9) or declined more slowly (IL-6) (Fig. 4E).

Age-dependent changes occurred in the upper airway gene expression and microbiome during COVID-19

We next asked whether aging was associated with changes in host gene expression and the upper airway microbiome (including the virome), which has been found to correlate with outcomes in patients with COVID-19 (*33*–*35*), using nasal swab metatranscriptomics. We identified 913 host genes that were significantly associated with age (adjusted $P < 0.05$; Fig. 5A), representing several key immune signaling pathways (Fig. 5B). TLR signaling, which plays an important role in microbial recognition, was up-regulated with age, as were genes related to IFN-α/β, IL-1, IL-4, IL-13, IL-10, and IL-17 signaling. In contrast, T cell–related pathways (TCR signaling, co-stimulation by the CD28 family, and PD1 signaling) were down-regulated with age, similar to our observations in the peripheral blood. In silico prediction of upstream cytokine activation demonstrated age-related activation of the cytokines TNF, IL-6, IFN-γ, IL-1α/β, IL-22, and CSF1 (Fig. 5C).

Our study design enabled the assessment of the extent to which gene expression in the blood and the upper respiratory tract was coordinated. To this end, we calculated Pearson's correlation coefficients of gene expression between matched PBMC and nasal samples in the youngest and oldest age groups separately. We found 52 genes that had relatively high correlation coefficients in both groups (Pearson's correlation coefficient, >0.5), in particular those related to type I IFN signaling (e.g., *IFI6*, *IFI44*, and *IFIT3*) and antigen presentation [human leukocyte antigen (HLA) genes] (Fig. 5D).

Because TLR pathways in the upper airway were strongly up-regulated with age, we asked whether this could be due to differences in SARS-CoV-2 viral abundance or the nasal microbiome. We found that at visit 1, the bacterial relative abundance correlated with both ISG and TLR gene expression, whereas SARS-CoV-2 viral abundance only correlated with ISG expression (Fig. 5E). We thus considered whether variations in bacterial abundance across the age groups could explain the observed age-related TLR signaling differences; however, no variation was found (fig. S9).

We also considered whether age-related differences in specific taxa within the upper airway microbiome might contribute to the aforementioned differences in TLR signaling. Metatranscriptomic analysis identified only one significantly different genus, *Lawsonella*, whose relative abundance decreased with higher age ($P = 8.7 \times 10^{-8}$; Fig. 5F). *Lawsonella* relative abundance positively correlated with TLR gene expression across all age groups, however, demonstrating that it did not account for the age-related up-regulation in TLR signaling (Fig. 5G). Last, we evaluated the upper respiratory tract virome and observed reactivation of herpes simplex virus (HSV) and cytomegalovirus (CMV) over the course of hospitalization in the

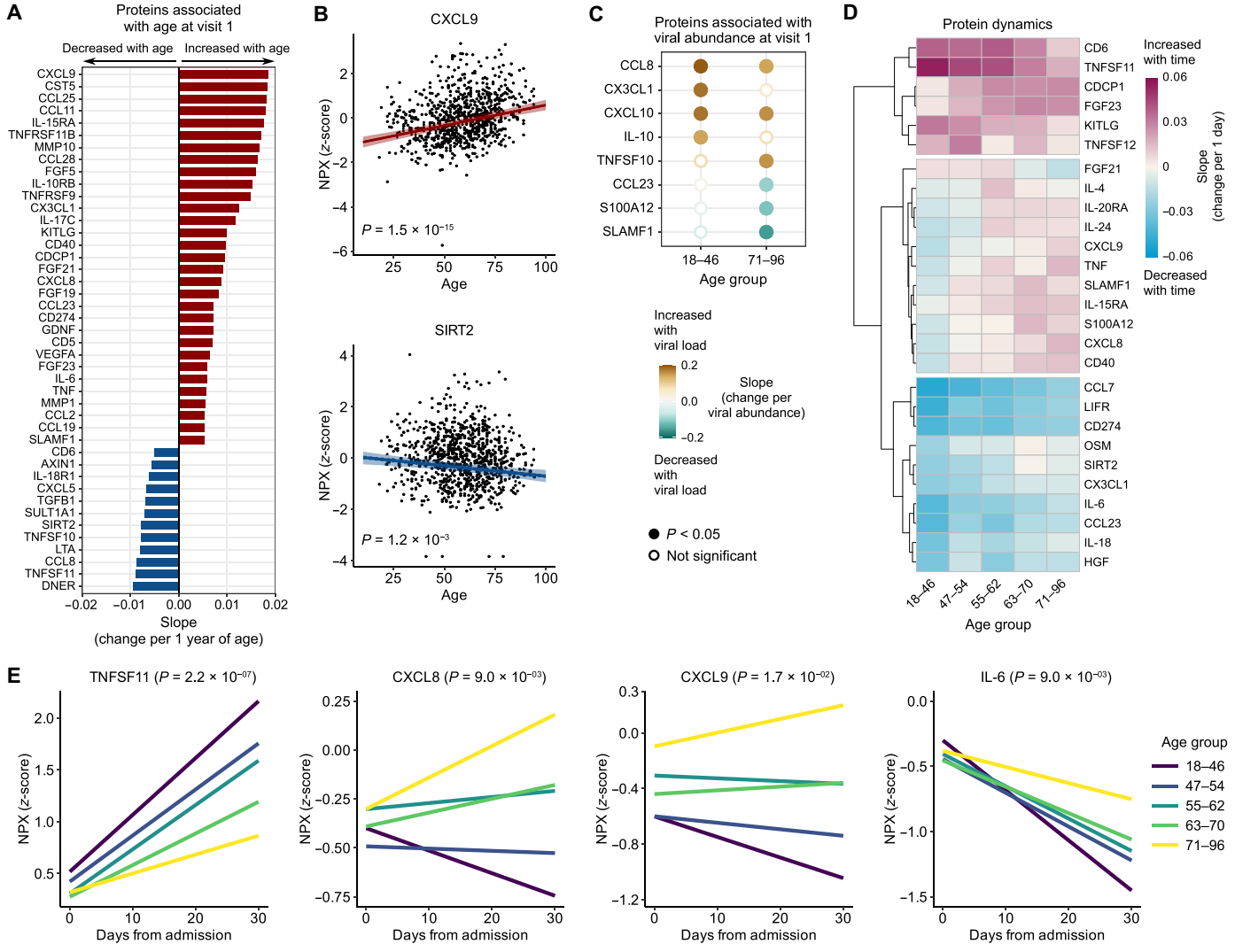

Fig. 4. Aging is associated with differences in serum inflammatory proteins during COVID-19. (**A**) The bar plot highlights proteins that are significantly up-regulated (red) or down-regulated (blue) with age at visit 1 (adjusted $P < 0.05$). $n = 895$ patients. (**B**) Scatter plots show the normalized protein expression (NPX) of two representative proteins, CXCL9 and SIRT2, as a function of age. The red and blue lines indicate the linear fit, and the shaded regions indicate the 95% confidence intervals of the fits. (**C**) The dot plot represents the slope of cytokine expression versus viral abundance in the youngest and oldest age quintiles, 18 to 46 ($n = 179$ patients) and 71 to 96 ($n = 198$ patients), respectively. In (A) to (C), P values were calculated using linear regression and Benjamini-Hochberg correction. (**D**) Heatmap displays longitudinal slopes (change in protein expression per day) of all cytokines that display significant age-dependent longitudinal dynamics (adjusted $P < 0.05$). $n = 412$, 414, 456, 466, and 525 samples in the youngest to oldest age groups. (**E**) Plots show the longitudinal dynamics of four example cytokines from (D). P values in (D) and (E) were calculated for the interaction between days from admission and age groups using linear mixed-effects modeling and Benjamini-Hochberg correction.

oldest age quintile, but not in younger participants (Fig. 5H and table S2).

Relationships were observed between aging, immune response, and COVID-19 severity

Previous studies have established that severe COVID-19 involves a dysregulated host response characterized by inappropriate activation of inflammatory and immunoregulatory pathways (*36*–*38*). We, therefore, sought to examine the intersection of aging, COVID-19 severity, and host immune responses by assessing PBMC gene expression differences at visit 1 between participants with mild/moderate COVID-19 [baseline National Institute of Allergy and Infectious Diseases Ordinal Scale (NIAID-OS) (*2*) of COVID-19 severity between 3 and 4] and severe COVID-19 (baseline NIAID-OS between 5 and 6) within the youngest and oldest age groups. Several immune signaling pathways were associated with disease severity in an age-dependent manner. For example, only in the oldest age quintile was severe COVID-19 associated with up-regulation of the IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor signaling pathway; the IL-4 and IL-13 signaling pathway; TLR signaling pathways; and the NRLP3 inflammasome signaling pathway in the upper airway (Fig. 6A). In the blood, the IL-1 signaling pathway was only up-regulated in severe COVID-19 in the oldest adults. We also identified several pathways that were associated with COVID-19 severity independent of age. For example, in the blood of both the youngest and oldest patients, severe disease was associated with up-regulation

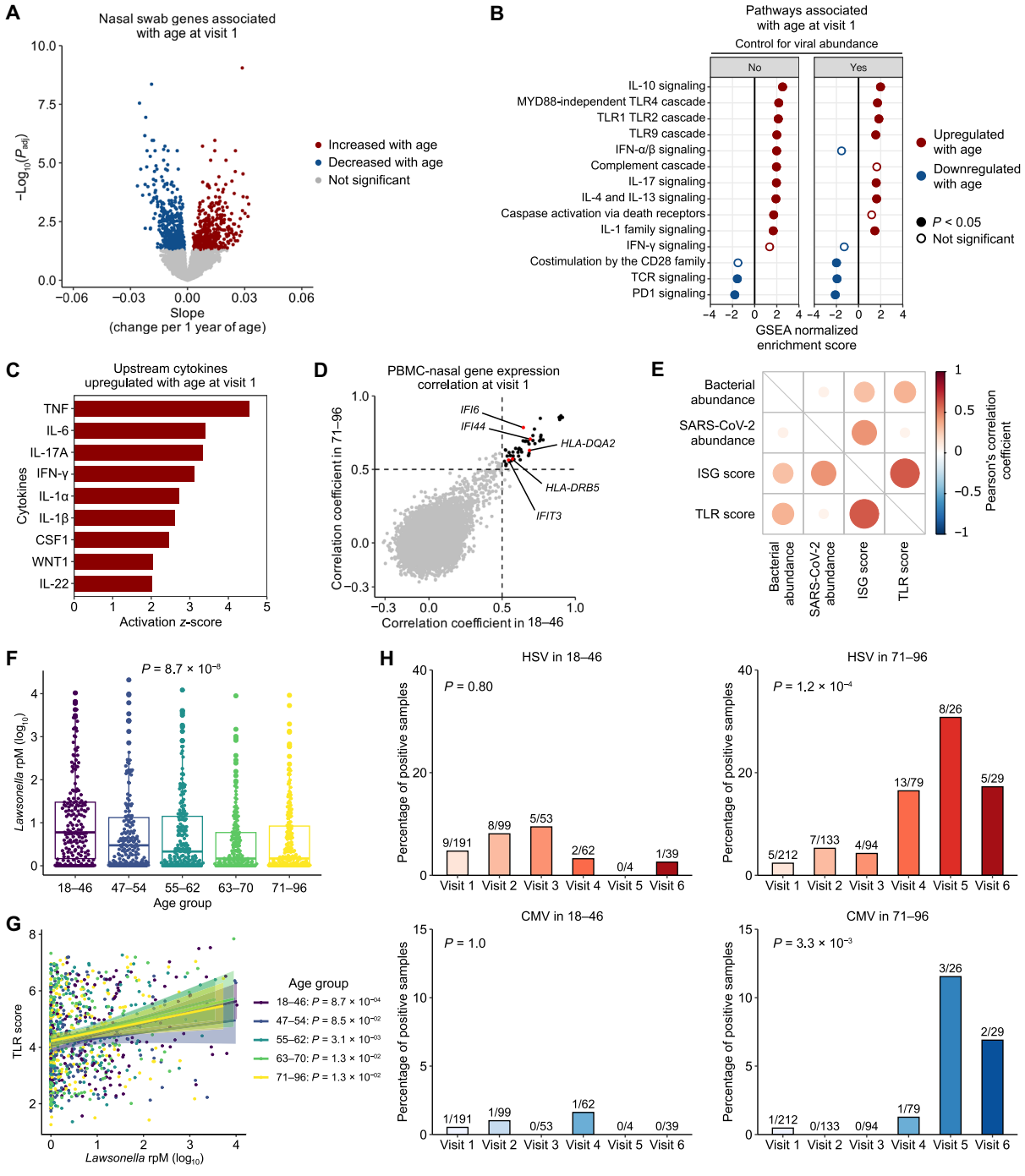

Fig. 5. Aging is associated with upper airway gene expression and changes to the nasal microbiome in COVID-19. (**A**) Volcano plot shows genes associated with age at visit 1 in nasal samples ($n = 915$ patients). (**B**) Dot plots show select Reactome pathways associated with age at visit 1, with (right) and without (left) controlling for SARS-CoV-2 viral abundance. MYD88, myeloid differentiation primary response protein 88. (**C**) Bar plot displays cytokines predicted from nasal gene expression to be upregulated with age by Ingenuity Pathway Analysis. (**D**) The scatter plot displays the Pearson's correlation coefficient between PBMC expression and nasal expression of a gene in the youngest (*x* axis) and oldest (*y* axis) age quintiles. $n = 155$ and 170 patients in the youngest and oldest age groups, respectively. Fifty-two genes (black dots) have a strong correlation in both age groups (correlation coefficient, >0.5). (**E**) The dot plot shows correlations between SARS-CoV-2 and total bacterial relative abundance, ISG expression score, and TLR gene expression score. (**F**) The box plot shows *Lawsonella* relative abundance across the age quintiles. $n = 198$, 185, 215, 203, and 221 patients in the youngest to oldest age group. *P* values were calculated with a one-way analysis of variance (ANOVA) test and Benjamini-Hochberg correction. (**G**) Correlation between *Lawsonella* relative abundance and TLR gene expression across the age quintiles. *P* values were calculated using the test of association with Pearson's correlation coefficient and Benjamini-Hochberg correction. (**H**) Percentages of cases with detected HSV or CMV transcripts in the youngest and oldest age quintiles. The number above each bar indicates the number of positive cases over the number of total samples. *P* values were calculated by Fisher's exact test to compare the distribution of positive cases across six visits between the young and old age groups for HSV and separately for CMV.

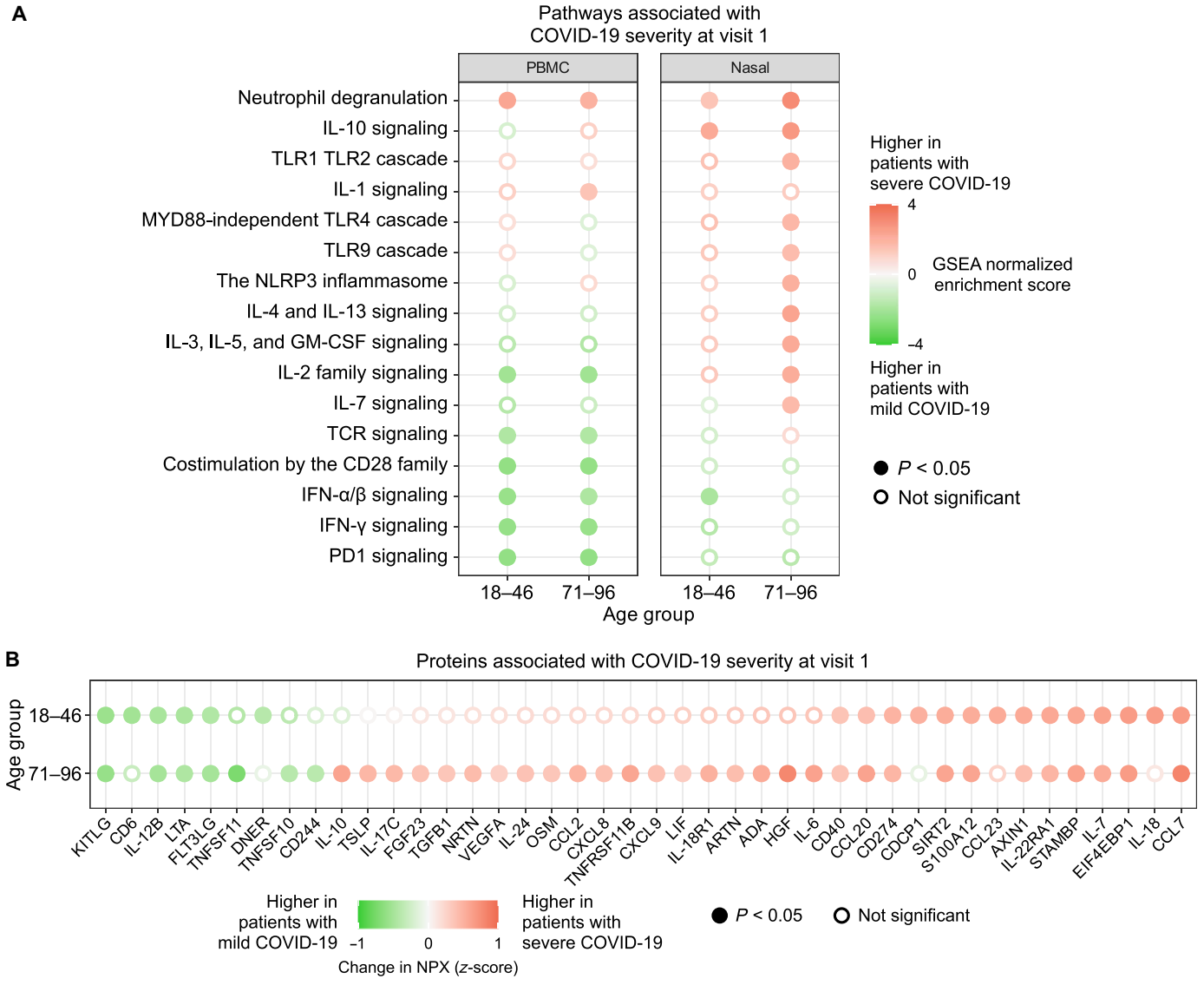

Fig. 6. Aging is associated with COVID-19 severity. (**A** and **B**) The dot plots highlight select Reactome pathways in PBMC or nasal swab RNA-seq data (A) and serum proteins (Olink) (B) that were up-regulated in participants with severe COVID-19 (baseline respiratory severity ordinal scale 5 to 6) compared with mild/moderate COVID-19 (ordinal scale 3 to 4) at visit 1, stratified by age group (youngest or oldest). In (A), $n = 165$ and 182 patients with PBMC samples in the 18 to 46 and 71 to 96 age groups, respectively; $n = 181$ and 199 patients with nasal swab samples in the 18 to 46 and 71 to 96 age groups, respectively. In (B), $n = 179$ and 198 patients in the 18 to 46 and 71 to 96 age groups, respectively. *P* values in (A) and (B) were calculated with linear modeling and Benjamini-Hochberg correction.

of neutrophil degranulation genes and down-regulation of pathways related to TCR, IFN-α/β, IFN-γ, IL-2, and PD1 signaling.

Assessment at the protein level provided further insights regarding the immunological intersection of aging and COVID-19 severity (Fig. 6B). We found that the higher abundance of several pro-inflammatory cytokines and chemokines, such as IL-6, oncostatin M (OSM), CXCL8, and CXCL9, was uniquely associated with more severe disease in the oldest adults. Increased expression of the anti-inflammatory cytokines TGF-β1 and IL-10 in individuals with severe disease was also specific to the oldest age quintile. Serum concentrations of several other proteins increased in severe disease independent of age, including CCL7, a leukocyte chemoattractant (*39*); S100A12, a neutrophil-derived cytosolic pro-inflammatory protein (*40*); and CD274 (also known as PDL1), a ligand for the immune inhibitory receptor PD1. Similarly, we found that severity was associated with reduced expression of several cytokines regardless of age, including IL-12B and LTA (also known as TNF-β). Differences in SARS-CoV-2 viral abundance did not influence the results (fig. S10).

Integrated analyses of aging in COVID-19 revealed multilayered immune cross-talk in the blood and airway

Last, we sought to integrate findings across genes and proteins, as well as between the blood and upper airway compartments. Integrated network analysis of statistically significant age-associated genes and age-associated proteins from the blood (adjusted $P < 0.05$;

Figs. 3A and 4A) identified three prominent nodes related to chemokine ligand (CCL) signaling, T cell signaling, and the cell cycle (Fig. 7A). In addition, analysis of the 10 proteins most significantly associated with age (by largest magnitude of age-associated slope) and their immediate downstream genes illuminated the complex cross-talk between several key immune mediators. For example, CXCL9, which interacts with the receptors CXCR3 and CXCR5 in an activating manner (fig. S11), was positively associated with age. Conversely, the genes encoding the receptors, *CXCR3* and *CXCR5*, were negatively correlated with age.

To investigate how aging could potentially affect ligand-receptor interactions in both the blood and upper airway, we focused on serum ligands that were significantly associated with age at visit 1 (adjusted $P < 0.05$) and examined the expression of the genes that

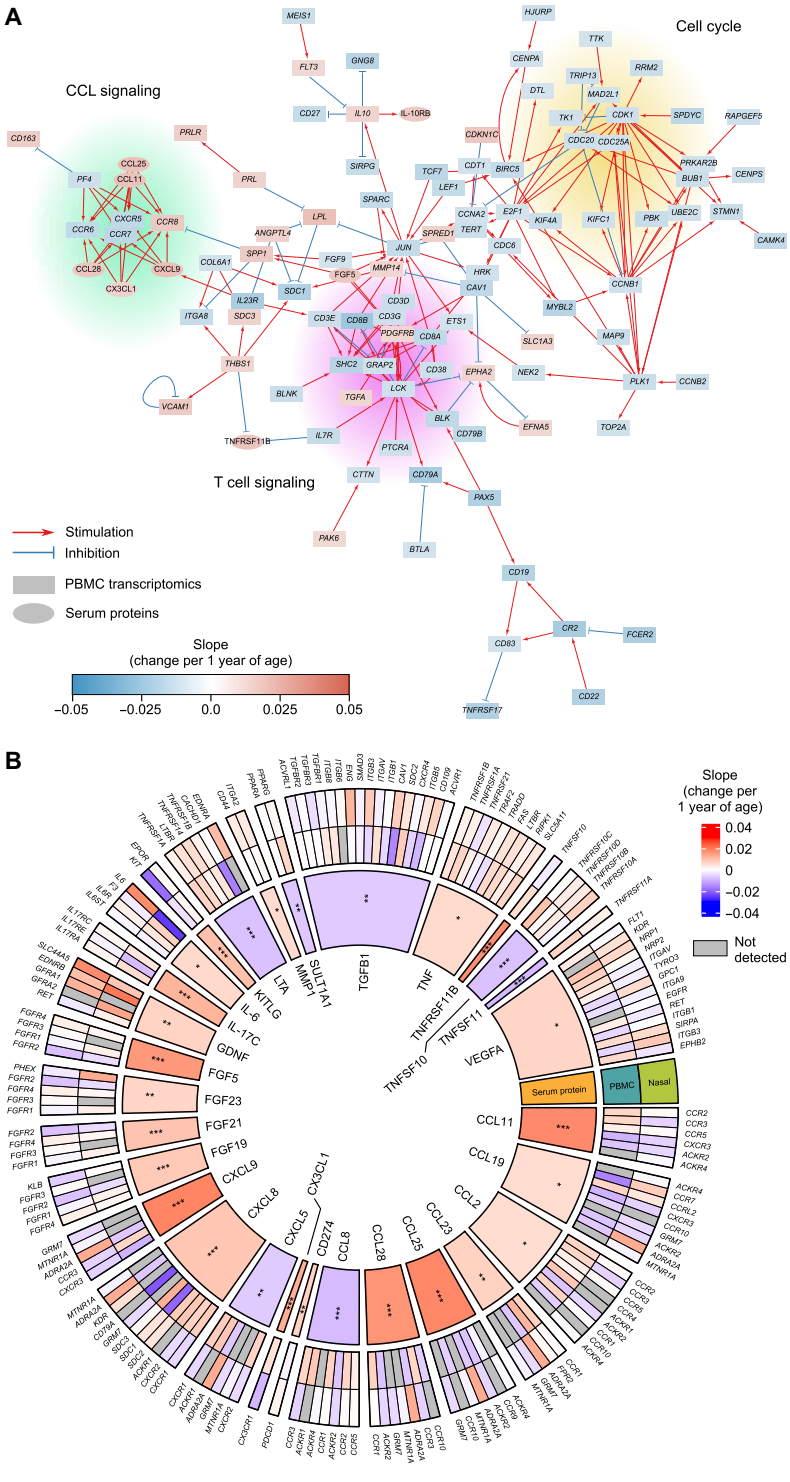

Fig. 7. Integrated analyses of aging in COVID-19 revealed multilayered immune cross-talk in the blood and airway. (**A**) Network analysis was performed on serum cytokines and chemokines and PBMC genes significantly associated with age at visit 1 using protein-protein interactions reported in OmniPath. (**B**) Analysis of ligand-receptor interactions from cytokine protein data, PBMC RNA-seq data, and nasal RNA-seq data. The innermost ring shows the significant cytokines and chemokines from visit 1 analysis (adjusted $P < 0.05$) and the magnitude of their average change per 1 year of age. The two outer rings illustrated genes that encode known receptors for each cytokine and their associated change per one year of age. *P* values for the cytokines were calculated using linear models and Benjamini-Hochberg correction. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

encoded their cognate receptors. This analysis further highlighted transcriptomic/proteomic concordance and discordance (Fig. 7B). For example, aging was associated with increased expression of serum CXCL9 and CCL11 but decreased expression of *CXCR3*, which encodes a receptor for these ligands. In contrast, the expression of TNF and many of its receptors (e.g., *TNFRSF1A*, *TNFRSF1B*, and *LTBR*) were positively associated with age.

Several recent studies have highlighted the role of different monocyte subsets in aging-related chronic inflammatory diseases (*13*, *41*, *42*); thus, we further examined the role of monocytes in the context of COVID-19 and aging. From the PBMC transcriptomics data, we found that many genes and pathways were significantly correlated with proportions of classical ($CD14^+CD16^-$), nonclassical ($CD14^-CD16^+$), and intermediate ($CD14^+CD16^+$) monocyte subsets (adjusted $P < 0.05$; fig. S12). For instance, in the youngest age quintile, an increasing classical monocyte population correlated with the up-regulation of the IL-10 signaling pathway, an anti-inflammatory signaling pathway. In contrast, an increasing nonclassical monocyte population in the oldest quintile was associated with the down-regulation of several innate and adaptive immune signaling pathways, suggesting that nonclassical monocytes were associated with an anti-inflammatory response in the oldest patients.

We then asked whether our multiomic dataset could reveal latent molecular subgroups in the oldest age quintile. To this end, we integrated PBMC RNA sequencing (RNA-seq) data, nasal RNA-seq data, and serum protein data using principal components analysis, controlling for the patient's sex and disease severity. We found that the first and second principal components (PC1 and PC2) explained 19.83 and 18.19% of the variance in the data, respectively (fig. S13, A and B). On the basis of whether the PC1 value was negative or positive, we divided the patients into two subgroups: $PC1^+$ and $PC1^-$ (fig. S13A). The two subgroups had similar distributions of age, sex, and TG, but the $PC1^-$ subgroup was enriched for patients with severe disease, based on the NIAID-OS (fig. S13, C to F).

These subgroups exhibited substantial differences in the nasal compartment but not in the blood (fig. S14, A to C). The upper respiratory tract of $PC1^-$ patients demonstrated up-regulation of many innate immune signaling pathways, including type I IFN signaling, TLR signaling, and interleukin signaling (adjusted $P < 0.05$; fig. S14D), as well as significantly higher bacterial abundance ($P = 3.9 \times 10^{-10}$; fig. S14, E to G). The difference in bacterial abundance was strongly driven by the Pseudomonadota phylum (fig. S14H). Although there were no differentially expressed PBMC genes (fig. S14B), GSEA suggested that TCR signaling, BCR signaling, and the complement cascade pathways were up-regulated in the blood compartment of $PC1^+$ patients (fig. S14D). Together, these findings suggest the existence of two molecular subgroups within the oldest patients, which are characterized by differences in many innate immune pathways and the microbiome in the upper respiratory tract.

DISCUSSION

Effective host response to viral infection depends on potent early innate immune activation, engagement of adaptive immune effectors, and then, upon effective viral clearance, attenuation of this inflammatory signaling to prevent excessive tissue damage and pathologic consequences (*43*). We observed age-dependent dysregulation of this program at the transcriptional, protein, and cellular levels, manifesting in an imbalance of inflammatory responses over the course of hospitalization. Our results identify discrete innate and adaptive immune signaling pathways that are altered with age, suggesting potential targets for age-dependent therapeutic intervention.

The role of type I IFN signaling in age-related immune dysregulation during COVID-19 has remained unclear, with some reports suggesting impaired induction of ISGs in older adults (*8*, *16*, *25*) and others demonstrating the contrary (*13*, *26*). We found that older age was associated with increased type I IFN signaling in both the blood and respiratory tract but that the relationship was principally driven by aging-related differences in SARS-CoV-2 viral relative abundance. In contrast, IFN-γ signaling, which is associated with poor prognosis in COVID-19 participants (*44*), was up-regulated with age independent of viral abundance. Several factors likely contribute to higher SARS-CoV-2 viral abundance in older adults, including impaired T and B cell immunity and impaired MHC antigen presentation, each of which was observed at the transcriptional, protein, and cellular levels in our dataset. Delayed viral clearance due to these age-related factors could facilitate the evolution of SARS-CoV-2 variants (*45*, *46*).

Older adults had lower proportions of naïve $CD8^+$ T cells and γδ T cells, which contribute to the effective clearance of viral pathogens (*25*, *47*, *48*). Terminally differentiated/exhausted NK cells, which are associated with severe COVID-19 (*49*), were more prevalent in older adults, as previously observed (*13*), as were central memory $CD8^+$ T cells. Impaired T cell signaling in older adults was also observed at the transcriptional level in both the blood and the upper respiratory tract upon hospitalization. Longitudinal analyses demonstrated attenuated expression dynamics of several TCR signaling-related genes in the older participants' blood samples. We also observed differences in the relationship between SARS-CoV-2 viral abundance and cytokines important for T cell recruitment in the oldest versus youngest adults, such as the chemokine CX3CL1.

Younger adults exhibited a much more robust induction of MHC class II gene expression over the course of hospitalization. This is consistent with a previous study that reported *HLA-DR* expression increases over time after symptom onset in younger patients with COVID-19 but not in older patients (*13*). We also found that expression of MHC class I genes decreased more rapidly after hospitalization in the oldest versus the youngest adults. Because SARS-CoV-2 can subvert immune responses by reducing MHC class I surface expression in infected cells (*50*), our observations suggest that older participants may be even more vulnerable to this viral immune evasion mechanism.

Evidence of impaired B cell immunity was also observed in the older participants, consistent with prior studies (*13*, *25*). Age was associated with reduced expression of BCR signaling–related genes at the time of hospitalization. Furthermore, we observed lower proportions of naïve B cells and plasmablasts in the oldest adults. This could partly explain the lower anti–SARS-CoV-2 RBD Ab titers in the oldest patients upon hospitalization.

Effective modulation of inflammatory responses is critical for the restoration of immune homeostasis and mitigation of excessive tissue damage. We found consistent evidence of prolonged, potentially pathologic inflammatory responses in the oldest adults from transcriptomics and protein analyses. For instance, upon hospitalization, pro-inflammatory cytokines and chemokines such as TNF, IL-6, CXCL8, and CXCL9 were higher in the blood of older

participants and continued to increase over time. In contrast, these cytokines decreased over time in the blood of younger participants. Our results suggest that age-related changes may exacerbate inflammatory signaling in severe COVID-19, supporting an early hypothesis (*13*, *25*).

The oldest adults in our study had evidence of HSV and CMV reactivation in the upper airway over the first 28 days after hospitalization. This may reflect impaired antiviral immune defenses in older adults exacerbated in the context of SARS-CoV-2 infection. Furthermore, reactivation of latent Herpesviridae may contribute to excessive inflammatory responses observed in older adults, as has been described in studies of participants with HIV infection (*51*).

Advanced age was also associated with up-regulation of TLR signaling genes in both the upper airway and the blood, independent of SARS-CoV-2 viral abundance. We found that upper airway relative bacterial abundance correlated with TLR expression independent of age, and compositional differences in the microbiome across age groups did not explain this association, suggesting that age-related increases in TLR gene expression were caused by microbe-independent factors. Consistent with this hypothesis, studies have demonstrated that the up-regulation of innate immune receptors, including TLRs, could be an intrinsic feature of inflammaging (*25*, *52*).

Severe COVID-19 is characterized by dysregulated, pathologic inflammatory responses (*36*, *53*, *54*). We found that aging was associated with higher expression of several signaling pathways previously implicated in this pathologic immune dysregulation. For instance, in the oldest adults, severe COVID-19 was disproportionately associated with the hyperactivation of canonical proinflammatory cytokines, such as IL-6, OSM, CXCL8, and CXCL9. In the upper airway, severe COVID-19 in the oldest age group led to greater evidence of NLRP3 inflammasome and TLR activation compared with the youngest group. These differences raise the possibility that older adults with severe COVID-19 may respond differently, and perhaps more favorably, to immunomodulatory therapies directed at certain inflammatory cytokines.

Limitations of our study include the lack of a concurrently enrolled SARS-CoV-2-negative control group, the lack of a nonhospitalized COVID-19 group, and the lack of lower respiratory tract samples. To partially address the first limitation, we analyzed publicly available gene expression datasets to incorporate findings from unrelated, healthy cohorts. Although we found that adjusting for the three most prevalent age-associated comorbidities in our PBMC transcriptional analysis did not strongly influence the results, our study was not designed to specifically evaluate the impact of comorbidities on host immune responses in COVID-19, which can be explored in future dedicated studies. Moreover, participants in our current study were enrolled before the introduction of SARS-CoV-2 vaccines, and patient enrollment ended during the emergence of the B.1.1.7 alpha variant. As a result, age-related differences in host immune responses may differ from a contemporary cohort because of variations in both vaccination status and the circulating SARS-CoV-2 variants. Although this aspect limits the extrapolation of our findings to currently immunized older adults, the naïve state of our study population was also a strength, because our results were not confounded by prior vaccination or infection, providing a window into age-related differences in immune response to an emerging viral respiratory pathogen.

In summary, we found that aging has marked impacts on host immune and viral dynamics in patients hospitalized for COVID-19. Older adults exhibited impaired viral clearance, dysregulated immune signaling, and persistent and potentially pathologic activation of pro-inflammatory genes and cytokines. Together, these findings will help to inform therapeutic strategies for this at-risk population.

MATERIALS AND METHODS

Study design

This study leveraged data from 1031 participants in the IMPACC observational cohort study (*2*, *24*), which enrolled from 20 hospitals across 15 medical centers in the United States between 5 May 2020 and 19 March 2021. The end of this enrollment period represented the beginning of the emergence of the B.1.1.7 alpha variant; hence, SARS-CoV-2 variants of concern were not widely represented in the cohort. Eligible participants were those hospitalized with SARS-CoV-2 infection confirmed by reverse transcription polymerase chain reaction and symptoms or signs consistent with COVID-19. The detailed study design and schedule for clinical data and biologic sample collection and shared core platform assessments were previously described (*24*, *37*). Detailed clinical assessments and sampling of blood and upper respiratory tract were performed within approximately 72 hours of hospitalization (visit 1) and on approximately days 4, 7, 14, 21, and 28 after hospital admission (which corresponded to visits 2 to 6). As previously described (*24*), biological sample collection and processing followed a standard protocol used by every participating academic institution.

Ethics

NIAID staff conferred with the Department of Health and Human Services Office for Human Research Protections (OHRP) regarding the potential applicability of the public health surveillance exception [45CFR46.102(l)(2)] to the IMPACC study protocol. OHRP concurred that the study satisfied the criteria for the public health surveillance exception, and the IMPACC study team sent the study protocol, and participant information sheet for review, and assessment to institutional review boards (IRBs) at participating institutions. Twelve institutions elected to conduct the study as public health surveillance, and three sites with prior IRB-approved biobanking protocols elected to integrate and conduct IMPACC under their institutional protocols (University of Texas at Austin, IRB 2020-04-0117; University of California San Francisco, IRB 20-30497; Case Western Reserve University, IRB STUDY20200573) with informed consent requirements. Participants enrolled under the public health surveillance exclusion were provided information sheets describing the study, samples to be collected, and plans for data deidentification and use. Those who requested not to participate after reviewing the information sheet were not enrolled. In addition, participants did not receive compensation for study participation while inpatient and subsequently were offered compensation during outpatient follow-ups. The International Committee of Medical Journal Editors recommendations were followed for this study.

Statistical analysis

All clinical and immunophenotyping data were obtained from the IMPACC study (clinical data version 2021-11-11-frozen) and are publicly available under restricted access (*2*, *24*). Quality control and assessments and raw data processing to generate computable

matrices were previously performed by the IMPACC study (*2*, *37*, *55*). All data analyses were done in R (v4.0.2). For each data type, we investigated the behavior of features both at visit 1 (within 72 hours of hospital admission for most of the participants) and longitudinally for scheduled visits (visits 1 to 6, up to 30 days after hospital admission, both inpatient and outpatient samples). No formal sample size calculations were carried out for this study. Severity was assessed using a previously described seven-point severity OS based on the degree of respiratory illness at the time of sampling (*2*). For comparisons between two or more groups that did not require controlling for covariates, we used the Kruskal-Wallis test or analysis of variance (ANOVA) test as indicated in the figure captions. For visit 1 analysis of RNA-seq data, protein data, and mass cytometry data, we used linear modeling with age as a continuous variable and controlled for sex and baseline respiratory severity. *P* values were adjusted with Benjamini-Hochberg correction.

In the longitudinal analyses, we used age quintiles (18 to 46, 47 to 54, 55 to 62, 63 to 70, and 71 to 96) and controlled for sex and disease severity clinical TG, a previously defined metric of COVID-19 severity over time (*2*). For longitudinal analysis of SARS-CoV-2 nasal viral abundance and serum anti–SARS-CoV-2 RBD IgG, we used generalized additive models with mixed effects from the package gamm4 (v0.2.6) to evaluate the effects of age while controlling for sex and TG. Generalized additive modeling was preferred for these features because of their nonlinear trajectories. For all other data types, we used linear mixed-effects models from the package lme4 (v1.1.25). *P* values in all analyses were adjusted with Benjamini-Hochberg correction. An adjusted $P < 0.05$ was considered statistically significant.

Supplementary Materials

The PDF file includes:
Materials and Methods
Figs. S1 to S14
Tables S1 and S2
Legends for data files S1 to S5
References (*56*–*63*)

Other Supplementary Material for this manuscript includes the following:
Data files S1 to S5
MDAR Reproducibility Checklist

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Acknowledgments: We would like to thank S. Thomas, M. Cooney, S. Rao, S. Vignolo, and E. Morrocchi from the Clinical and Data Coordinating Center; A. Naeim, M. Bernardo, S. Sanchez, S. Intluxay, C. Magyar, J. Brook, E. Ramires-Sanchez, M. Llamas, C. Perdomo, C. E. Magyar, J. A. Fulcher, and the University of California Los Angeles (UCLA) Center for Pathology Research Services and the Pathology Research Portal from the David Geffen School of Medicine at UCLA; and M. C. Muenker, D. Duvilaire, M. Kuang, W. Ruff, K. Raddassi, D. Shepherd, H. Wang, O. Chaudhary, S. Salahuddin, J. Fournier, M. Rainone, and M. Kuang from Yale School of Medicine. **Funding:** The study was funded by the US NIH through the following grants: 5R01AI135803-03 (to F.K.), 5U19AI118608-04 (to O.L., H.S., and J.D.-A.), 5U19AI128910-04 (to C.B.C. and E.K.H.), 5U19AI089992 (to R.R.M.), 4U19AI090023-11 (to B.P., N.R., and S.B.), 4U19AI118610-06 (to F.K. and S.K.-S.), 5U19AI057229-17 (to H.T.M.), 5U19AI128913-03 (to J.S.), 3U19AI077439-13 (to D. J. Erle, C.S.C., C.R.L., and W.E.), 3U19AI089992-09 (to R.R.M.), 3U19AI128913-03 (to M.C.A.), 3U19AI1289130 (to E.R.), U19AI128913-04S1 (to E.R.), and 5R01HL155418 (to C.R.L.) and by the Chan Zuckerberg Biohub (to C.R.L.). **Author contributions:** H.V.P. and C.R.L. conceived the idea for the project. H.V.P., A.T., C.P.M., and B.L. analyzed the data. W.E. and S.B. generated the PBMC transcriptomic data. M.C.A. and N.D.J. generated the nasal transcriptomic data. E.K.H., P.M.B., S.K.-S., J.C., A.H., H.P., P.v.Z., M.C.A., A.D.A., C.S.C., S.B., C.B.C., W.E., L.G., N.D.J., S.H.K., F.K., H.T.M., A.O., B.P., N.R., R.R.M., E.R., J.S., H.S., O.L., and J.D.-A. provided input on analyses and findings. H.V.P., A.T., and C.R.L. wrote the manuscript. All authors reviewed and edited the manuscript. **Competing interests:** F.K. has the following financial interests: The Icahn School of Medicine at Mount Sinai has filed patent applications relating to SARS-CoV-2 serological assays, NDV-based SARS-CoV-2 vaccines, influenza virus vaccines, and influenza virus therapeutics that list F.K. as coinventor ["Influenza Virus Vaccines and Uses Thereof (Chimeric HA 2)," 9,371,366; "Influenza Virus Vaccines and Uses Thereof (Chimeric HA 1)," 10,131,695; "Influenza Virus Vaccines and Uses Thereof (Chimeric HA 2)," 2934581; "Influenza Virus Vaccines and Uses Thereof (Chimeric HA 2)," 9,968,670; "Influenza Virus Vaccines and Uses Thereof (Chimeric HA2)," 10,137,189; "Influenza Virus Vaccines and Uses Thereof (Chimeric HA2)," 10,583,188; "Influenza Virus Vaccines and Uses Thereof (Chimeric HA 1)," EP2758075; "Influenza Virus Vaccination Regimens (Neuraminidase)," 10,736,956; "Anti-Influenza B Virus Neuraminidase Antibodies and Uses Thereof," 11254733; and "Influenza Virus Hemagluttinin Proteins and Uses Thereof (Mosaic)," 7237344]. Mount Sinai has spun out a company, Kantaro, to market serological tests for SARS-CoV-2 and another company, Castlevax, to develop SARS-CoV-2 vaccines. F.K. is a cofounder and a scientific advisory board member of Castlevax. F.K. has consulted for Merck, Curevac, Seqirus, and Pfizer and is currently consulting for Third Rock Ventures, GSK, Gritstone, and Avimex. The Krammer laboratory is also collaborating with Dynavax on influenza vaccine development. S.K. receives consulting fees from Peraton. C.B.C. has received funding from the Bill & Melinda Gates Foundation for COVID-19 work paid to their institution; has received consulting fees from bioMerieux on clinical biomarkers; serves as DSMB, advisory board for Convalescent Plasma COVID-19 study for the National Heart, Lung and Blood Institute (NHLBI); and is acting leadership as president board of directors for the National Foundation of Emergency Medicine (NFEM), a nonprofit supporting emergency medicine research and researchers. C.S.C. has received grants from Bayer, Roche-Genentech, and Quantum Leap Healthcare Collaborative and has received consulting fees from Vasomune, Gen1e Life Sciences, Cellenkos, Arrowhead, Calcimedica, NGMBio, and Janssen. C.S.C. and C.R.L. are coinventors on a provisional patent filed by Regents of the University of California and the Chan Zuckerberg BioHub titled "Integrated Host-Microbe Metagenomics of Cell-Free Nucleic Acid for Sepsis Diagnosis," application number 63/342,528. R.R.M. has a leadership counselor role from 2018 to 2021 for the Society of Leukocyte Biology. O.L. has received support as a speaker for a presentation regarding the coronavirus pandemic from the Mid-Size Bank Coalition of America (MBCA) and Moody's Analytics. N.R. has research grants from Pfizer, Merck, Sanofi, Quidel, Immorna, Vaccine Company, and Lilly; serves on safety committees for ICON and EMMES and the advisory boards of Moderna, Seqirus, Pfizer, and Sanofi; and is a paid safety consultant for ICON, CyanVac, and EMMES. The other authors declare that they have no competing interests. **Data and materials availability:** All data associated with this study are in the paper or the Supplementary Materials. Patient-level data are available under restricted access to comply with the NIH Public data sharing policy for IRB-exempted public surveillance studies. Access can be obtained via AccessClinicalData@NIAID (https://accessclinicaldata.niaid.nih.gov/study-viewer/clinical_trials). More information can also be found at https://docs.immport.org/home/impaccslides/. Data files were previously generated (*2*, *37*, *55*) and are available at ImmPort under accession number SDY1760 and dbGAP accession number phs002686.v1.p1 (clinical data version 2021-11-11-frozen). The healthy control aging transcriptomic dataset was obtained from a supplementary data file from Peters *et al.* (*6*). All analysis code has been deposited on Zenodo at https://doi.org/10.5281/zenodo.10729751.

In addition to the authors, the following additional IMPACC Consortium members contributed to patient enrollment, cohort development, biospecimen collection, clinical data collection, biological assay data generation, biospecimen quality control, data quality control, or study oversight:

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Submitted 30 June 2023
Accepted 15 March 2024
Published 17 April 2024
10.1126/scitranslmed.adj5154