DOI: 10.1002/JLB.5HI0720-466R

JLB JOURNAL OF LEUKOCYTE BIOLOGY

HIGHLIGHTED ARTICLE

Genome-wide DNA methylation profiling of peripheral blood reveals an epigenetic signature associated with severe COVID-19

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Abstract

The global pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly pathogenic RNA virus causing coronavirus disease 2019 (COVID-19) in humans. Although most patients with COVID-19 have mild illness and may be asymptomatic, some will develop severe pneumonia, acute respiratory distress syndrome, multi-organ failure, and death. RNA viruses such as SARS-CoV-2 are capable of hijacking the epigenetic landscape of host immune cells to evade antiviral defense. Yet, there remain considerable gaps in our understanding of immune cell epigenetic changes associated with severe SARS-CoV-2 infection pathology. Here, we examined genome-wide DNA methylation (DNAm) profiles of peripheral blood mononuclear cells from 9 terminally-ill, critical COVID-19 patients with confirmed SARS-CoV-2 plasma viremia compared with uninfected, hospitalized influenza, untreated primary HIV infection, and mild/moderate COVID-19 HIV coinfected individuals. Cell-type deconvolution analyses confirmed lymphopenia in severe COVID-19 and revealed a high percentage of estimated neutrophils suggesting perturbations to DNAm associated with granulopoiesis. We observed a distinct DNAm signature of severe COVID-19 characterized by hypermethylation of IFN-related genes and hypomethylation of inflammatory genes, reinforcing observations in infection models and single-cell transcriptional studies of severe COVID-19. Epigenetic clock analyses revealed severe COVID-19 was associated with an increased DNAm age and elevated mortality risk according to GrimAge, further validating the epigenetic clock as a predictor of disease and mortality risk. Our epigenetic results reveal a discovery DNAm signature of severe COVID-19 in blood potentially useful for corroborating clinical assessments, informing pathogenic mechanisms, and revealing new therapeutic targets against SARS-CoV-2.

KEYWORDS

epigenetics, COVID-19, DNA methylation, IFN, SARS-CoV-2

Received: 24 July 2020 | Revised: 17 November 2020 | Accepted: 29 December 2020

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RNA viruses such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are capable of inducing immune dysfunction through hijacking host immune cell epigenomes and altering transcriptional programs to evade immune defenses.[1] Epigenetics offers a window into understanding host-pathogen interactions decoding the biologic dialogue between host and pathogen and understanding pathogen-related disease outcomes.[2–5] Increasing evidence has shown that various components of the host immune system are dramatically altered during SARS-CoV-2 infection and the extent of immune dysregulation relates to severe COVID-19 disease and mortality.[6] Yet, our knowledge of the epigenetic landscape of immune cells during severe SARS-CoV-2 infection remains limited.

Previous work showed that the host cell epigenetic landscape of DNA methylation (DNAm) is altered[7] modulating immune antigen presentation during coronavirus infection.[8] A comparative transcriptomic study of influenza viruses and coronaviruses revealed coronavirus manipulation of host antiviral IFN responses.[9] Notably, initial transcriptional studies of SARS-CoV-2 infection of target host cells revealed a unique transcriptional signature compared with other respiratory viruses characterized by suppressed antiviral IFN gene induction and elevated chemokine/cytokine gene expression patterns supporting initial observations of coronavirus suppression of innate antiviral responses.[10] Single-cell RNA-Seq studies of peripheral blood mononuclear cells from patients infected with SARS-CoV-2 suggest a distinct peripheral immune transcriptional signature of severe COVID-19 consisting of perturbations to IFN-stimulated genes, antigen presentation genes, and proinflammatory genes.[11,12] Yet, whether aberrant DNAm patterns of host immune cells is present in severe COVID-19 remains unknown.

In this study, we investigated genome-wide DNAm profiles using the Infinium MethylationEPIC array[13] in peripheral blood mononuclear cells of 9 individuals with severe COVID-19 compared with 9 uninfected controls, 5 individuals hospitalized with influenza A or B, 9 individuals with primary HIV-1 infection (treatment naïve; mean CD4 count: 627 cells/mm^3), and 9 individuals coinfected with mild/moderate COVID-19 and HIV-1 (on antiretroviral therapy; median days from symptom onset to first visit: 34 days; mean CD4 count: 544 cells/mm^3) (Fig. 1A). Severe COVID-19 participants were receiving mechanical ventilation or supplemental oxygen, showed detectable plasma SARS-CoV-2 RNA by droplet digital PCR, and lymphopenia (0.1–1.1 lymphocytes/mcL). Clinical features of our severe COVID-19 participants support findings of SARS-CoV-2 RNA detected in blood termed RNAemia[14] and lymphopenia[15] associated with severe COVID-19. Severe COVID-19 participants had treatment histories including use of hydroxychloroquine, chloroquine, zithromycin, lopinavir/ritonavir, vancomycin, ceftriaxone, or piperacillin/tazobactam.

Abbreviations: SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; COVID-19, Coronavirus disease 2019; DNAm, DNA methylation; HIV, human immunodeficiency virus; IFN, Interferon; NK, natural killer; FDR, false discovery rate.

Based on accumulating evidence reporting dramatic change in the composition of immune cell types in severe COVID-19,[12,16–18] our initial analysis of our DNAm dataset investigated differences in estimated cell-type proportions between groups utilizing cell-type-specific differential methylation profiles.[16] We utilized the Epigenetic Dissection of Intra-Sample-Heterogeneity (EpiDISH) package[19,20] to infer fractions of 7 priori immune cell subtypes (B-cells, CD4+ T Cells, CD8+ T cells, NK cells, monocytes, neutrophils, and eosinophils) based on 3 methods: Robust Partial Correlations,[16] CIBERSORT,[21] and Constrained projection[22] methods. As expected, we observed a significant difference in CD4 [$F(4, 36 = 28.47$, $P < 0.0001$] and CD8 T cell percentage for all 5 groups [$F(4, 36 = 14.68$, $P < 0.0001$]. Post hoc comparisons using the Tukey's test indicated a significant loss in DNAm inferred CD4+ and CD8+ T cell percentages in severe COVID-19 compared with both uninfected controls, primary HIV samples, and influenza ($P < 0.05$) (Fig. 1B). Supporting findings in HIV, we observed a significant decrease in estimated CD4 T cell percentage ($P = 0.0001$) and increase in CD8 T cell percentage ($P = 0.006$) in primary HIV and HIV/COVID-19 coinfection compared with uninfected controls (Fig. 1B). We observed a significant decrease in NK cell proportions associated with severe COVID-19 compared with primary HIV and HIV/COVID-19 coinfection ($P < 0.05$). The most striking DNAm inferred cell-type composition change was a significant increased proportion of neutrophils observed in severe COVID-19 participants compared with uninfected controls, primary HIV infection, hospitalized influenza, and HIV/COVID-19 coinfection ($P < 0.0001$; Fig. 1B). We calculated a DNAm inferred neutrophil–lymphocyte ratio (NLR) by dividing the relative % inferred neutrophils by the relative % inferred lymphocytes (CD4+, CD8+ T cells, and B cells). Severe COVID-19 participants had a significantly increased NLR compared with uninfected controls, HIV infection, hospitalized influenza, and HIV/COVID-19 coinfection ($P < 0.0001$; Fig. 1C), supporting work showing that clinical laboratory testing-based NLR ratio predicts severe COVID-19 in the early stages of infection.[16] These findings also support recent single-cell transcriptomic research of peripheral blood highlighting a potential shift in cellular trajectory toward a neutrophil population in COVID-19,[12] a report of the low-density inflammatory neutrophils arising in severe COVID-19 related to coagulopathy,[23] and neutrophil lung infiltration from autopsied COVID-19 patients.[24] This large proportion of peripheral neutrophils associated with severe COVID-19 could be a source of excess neutrophil extracellular traps across body compartments resulting in permanent organ damage and potential death. To examine this hypothesis, we profiled DNAm patterns in postmortem lung tissues from 15 individuals with COVID-19 and 4 uninfected controls and observed a significant increase in neutrophils inferred by DNAm-based cell-type deconvolution analysis in COVID-19 postmortem lung compared with uninfected lung tissues (Fig. 1D).

We next assessed changes in genome-wide DNAm associated with severe COVID-19 by comparing with uninfected controls and identified 40,904 differentially methylated loci associated with severe COVID-19 at ($\Delta\beta$-value $> |0.20|$ and significant at FDR adjusted $P < 0.05$). Comparing genome-wide DNAm associated with severe

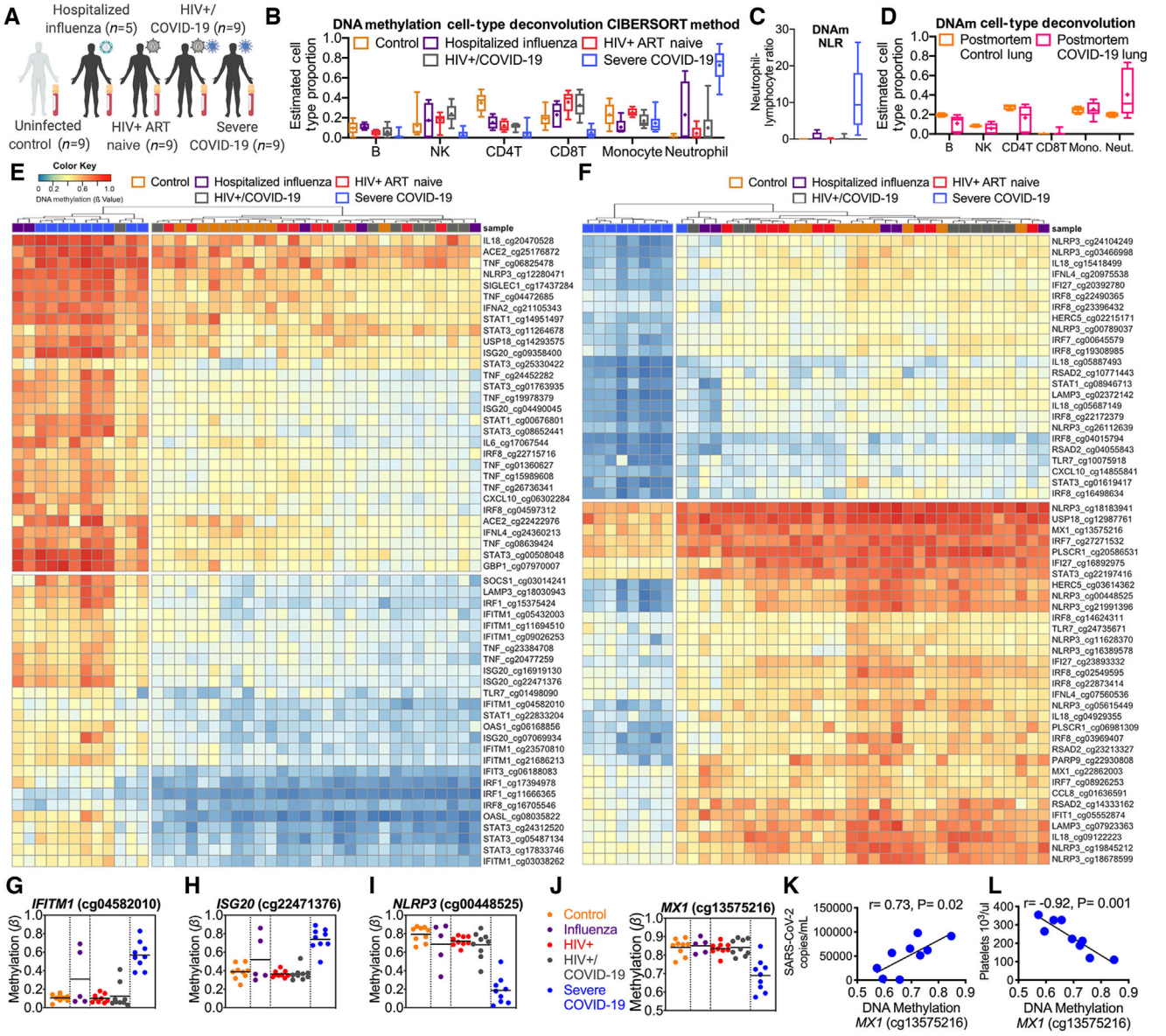

FIGURE 1 **DNA methylation signature associated with severe COVID-19**. (A) Overview of experimental design for comparative DNA methylation profiling. (B) Box and whisker plots of DNA methylation cell type deconvolution by the CIBERSORT method[21] showing estimated cell type proportions of B cells, NK cells, CD4 T cells, CD8 T cells, monocytes, and neutrophils for uninfected control (orange), hospitalized influenza (purple), primary HIV+ ART naïve (red), coinfection HIV+/COVID-19 (gray), and severe COVID-19 (blue) PBMCs. (C) DNA methylation (DNAm)-based neutrophil–lymphocyte ratio (NLR). (D) Box and whisker plots of DNA methylation cell-type deconvolution showing estimated cell-type proportions of B cells, NK cells, CD4 T cells, CD8 T cells, monocytes, and neutrophils in postmortem lung tissues from COVID-19 and uninfected controls. (E) Heatmap of hypermethylated CpGs in uninfected control (orange), hospitalized influenza (purple), primary HIV+ ART naïve (red), coinfection HIV+/COVID-19 (gray), and severe COVID-19 (blue) PBMCs; each participant indicated at top of row. GeneID associated with each CpG displayed for each row. Unsupervised hierarchic clustering above columns identified 2 main clusters. Methylation values displayed as ranging from low methylation (0; blue) to high methylation (1, red). (F) Heatmap of hypomethylated CpGs in uninfected control (orange), hospitalized influenza (purple), primary HIV+ ART naïve (red), coinfection HIV+/COVID-19 (gray), and severe COVID-19 (blue) PBMCs. GeneID associated with each CpG displayed for each row. Unsupervised hierarchic clustering above columns identified 2 main clusters. Methylation values displayed as ranging from low methylation (0; blue) to high methylation (1, red). (G–J) Dot plots of DNA methylation levels at CpGs related to *IFITM1*, *ISG20*, *NLRP3*, and *MX1* genes. (K and L) Correlation plots of SARS-CoV-2 plasma viral copies/ml and platelets count with DNA methylation levels of CpG at the *MX1* genes

COVID-19 against influenza identified 26,733 differentially methylation loci at ($\Delta\beta$-value > |0.20| and significant at FDR adjusted $P < 0.05$) and an RNA viral infection associated with lymphopenia and immunosuppression, primary HIV infection identified 51,728 differentially methylation loci at ($\Delta\beta$-value > |0.20| and significant at FDR adjusted $P < 0.05$). Notably, among the differentially methylated loci associated with severe COVID-19, we observed significant hypermethylation in regulatory regions of genes involved in the type I IFN response associated with severe COVID-19 (Fig. 1E) including first line antiviral defense genes such as *IFITM1* and *ISG20* (Fig. 1G and H) supporting the notion of SARS-CoV-2 suppressing host IFN responses.[10] We also observed aberrant levels of DNAm associated with severe COVID-19 related to the SARS-CoV-2 viral host receptor *ACE2* gene supporting work suggesting up-regulation of *ACE2* during SARS-CoV-2 infection.[25] In contrast, we observed significant hypomethylation in regulatory regions of genes involved in immune inflammation and cytokine genes associated with severe COVID-19 (Fig. 1F) including loci in a regulatory region of the *NLRP3* inflammasome and antiviral *MX1* genes (Fig. 1I and J). DNAm in severe COVID-19 at antiviral gene *MX1* significantly associated with plasma SARS-CoV-2 viral load and platelet count (Fig. 1K and L). These findings suggest that DNAm patterns in immune cells may provide a signature of severe COVID-19 represented by an imbalanced epigenetic orchestration of inflammatory and IFN gene transcriptional programs reported by various gene expression studies of SARS-CoV-2.[10–12] These data support the hypothesis that SARS-CoV-2 alters the immune cell epigenome in a 2-hit wave by (1) imprinting a shutoff of IFN transcriptional programs and (2) embedding an unfettered inflammatory cytokine-trained immune response leading to severe COVID-19.

We also examined which immune cell types may be driving the methylation signal associated with severe COVID-19. We used the eFORGE 2.0 tool[26] to identify a cell-type-specific signal for the top 1,000 hypo- and hyper-methylated differentially methylated sites associated with COVID-19 based on detecting enrichment of overlap with histone marks from the Roadmap Epigenomics project. We found that the top 1,000 hypomethylated CpGs were significantly enriched in enhancer regions of primary neutrophils from peripheral blood (E030) using the 15 chromatin states model from the Roadmap Epigenomics project[27] ($P = 7.11e{-}315$, Q value $= 1.1e{-}310$). In contrast, we found that the top 1,000 hypermethylated CpGs typically associated with transcriptional repression were significantly enriched in transcription start site regions of (E033) primary T cells from cord blood ($P = 1.2e{-}285$, Q value $= 1.85e{-}281$), (E041) primary T helper cells ($P = 1.08e{-}264$, Q value $= 8.34e{-}261$), and (E044) primary T regulatory cells ($P = 8.48e{-}239$, Q value $= 4.38e{-}235$). These findings confirm dysregulation of neutrophils and T cells as disease-relevant cell types for severe COVID-19 and suggest an epigenomic signature of COVID-19 based on aberrant DNAm at cell-type-specific regulatory regions of the host genome.

Genome-wide DNAm data have been used to develop epigenetic clocks to accurately estimate chronologic age and a predictor of lifespan in immune cells and tissues.[28–31] Infectious diseases such as HIV accelerate the epigenetic clock[32,33] suggestive of damaging effects of an RNA pathogen on the host immune cell epigenome. To study epigenetic age in severe COVID-19, we examined Horvath's measure of epigenetic age acceleration according to the PhenoAge epigenetic clock[29] and assessed DNAm inferred mortality risk according to GrimAge.[30] Individuals with severe COVID-19 were estimated to have a significantly increased epigenetic age acceleration compared with uninfected controls and influenza ($P < 0.05$; Fig. 2A). Moreover, we also observed a significant increase in mortality risk in severe COVID-19 compared with uninfected controls, primary HIV, and HIV/COVID-19 coinfection ($P < 0.05$; Fig. 2B). Interestingly, we did not observe significant decreases in DNAm-based telomere length in severe COVID-19 compared with uninfected controls (P value $= 0.22$; Fig. 2C). However, validating previous reports in HIV, we found that individuals coinfected with HIV and mild/moderate COVID-19 had an estimated significantly shorter telomere length compared with uninfected controls, influenza, and severe COVID-19 ($P < 0.05$; Fig. 2C). We also utilized our DNAm dataset to infer previously validated DNAm-based biomarker estimates for mortality including renal function biomarker cystatin C and fibrosis marker TIMP metallopeptidase inhibitor 1 (TIMP-1).[30] Estimated levels of biomarkers cystatin and TIMP-were significantly increased in severe COVID-19 compared with uninfected controls ($P < 0.05$) and influenza ($P < 0.05$) (Fig. 2D and E). Additionally, cystatin and TIMP-1 were significantly increased in individuals coinfected with HIV and mild/moderate COVID-19 compared with primary HIV ($P < 0.05$). Overall, these findings suggest severe COVID-19 is detrimental to host immune cell epigenomes and perturb the epigenetic clock.

Altogether, this epigenetic DNAm profiling study provides the first evidence for a distinct methylome signature in peripheral blood obtained from severe COVID-19 participants characterized by dramatic cell-type composition changes, hypomethylation of inflammatory genes, hypermethylation of IFN-related genes, and perturbations to the epigenetic clock and epigenetic inferred mortality risk. Our findings support the notion that SARS-CoV-2 dramatically reshapes peripheral blood and lung tissue host immune cell landscapes and may hijack the host epigenome by modifying cellular DNAm states. This may occur through viral RNA shed from dying cells in tissues and circulating viral protein components such as the ORF3a,[34] ORF6,[35] spike, membrane, and nucleocapsid[36] that have been shown to hamper the host immune response. SARS-CoV-2 likely alters other epigenetic mechanisms such as histone modifications and noncoding RNA in a cell-type and context-dependent fashion. Future research will need to need to replicate these findings in additional COVID-19 cohorts, examine whether DNAm differences are apparent in mild/moderate cases of COVID-19 that progress to severe disease, and whether an indelible epigenetic imprint of COVID-19 persist after recovery in convalescent patients.

AUTHORSHIP

M.J.C., A.P.P., and L.C.N. designed and carried out experiments. M.J.C. and L.C.N. drafted the manuscript. T.P. and J.B.S. analyzed and

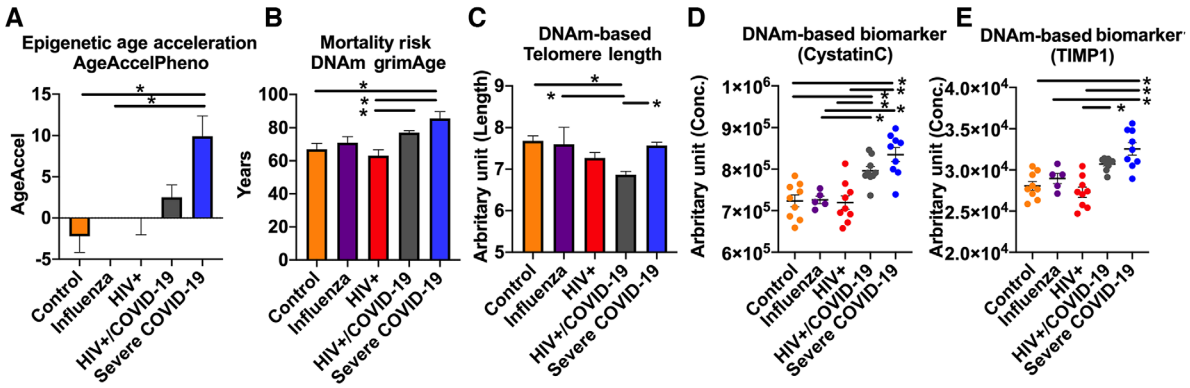

FIGURE 2 **Comparative epigenetic clock analyses of uninfected controls, influenza infection, primary HIV infection, HIV+/COVID-19 coinfection, and severe COVID-19.** (A) Bar graph displaying significant elevation in estimated epigenetic age acceleration in severe COVID-19 participants compared to uninfected controls and influenza. (B) Bar graph displaying significant elevation in estimated mortality risk according to DNAm GrimAge[30] in severe COVID-19 participants compared with uninfected controls and primary HIV. (C) Bar graph displaying significant decrease in DNAm-based telomere length in primary HIV compared with uninfected control. (D and E) Scatter plot showing severe COVID-19 participants significant elevations in DNAm-based biomarker estimates of cystatin C and TIMP1 compared with uninfected control, influenza, HIV+, and coinfection HIV+/COVID-19. One-way ANOVA was utilized to identify significant group differences. Post hoc multiple comparisons testing utilized Tukey test. Significance was set at $P = 0.05$

interpreted data. P.M., B.P., H.S., Y.B., V.C., M.P., A.B., R.E.S., T.H., K.D., H.S., and S.G.D. recruited study subjects and provided samples. All authors reviewed and edited manuscript.

ACKNOWLEDGMENTS

We gratefully acknowledge the patients and study participants who made this work possible. This work was supported by the following grants: NIH/NHBLI (K01 HL140271, M.J.C.), NIH/NIAID (3R01AI141003-03S1, T.J.H.).

DISCLOSURES

None of the authors have any relevant conflict of interest to disclose with the exception of L.C.N. who served on a Scientific Advisory Board for Abbvie.

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SUPPORTING INFORMATION

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How to cite this article: Corley MJ, Pang AP, Dody K, et al. Genome-wide DNA methylation profiling of peripheral blood reveals an epigenetic signature associated with severe COVID-19. *J Leukoc Biol*. 2021;110:21–26. https://doi.org/10.1002/JLB.5HI0720-466R