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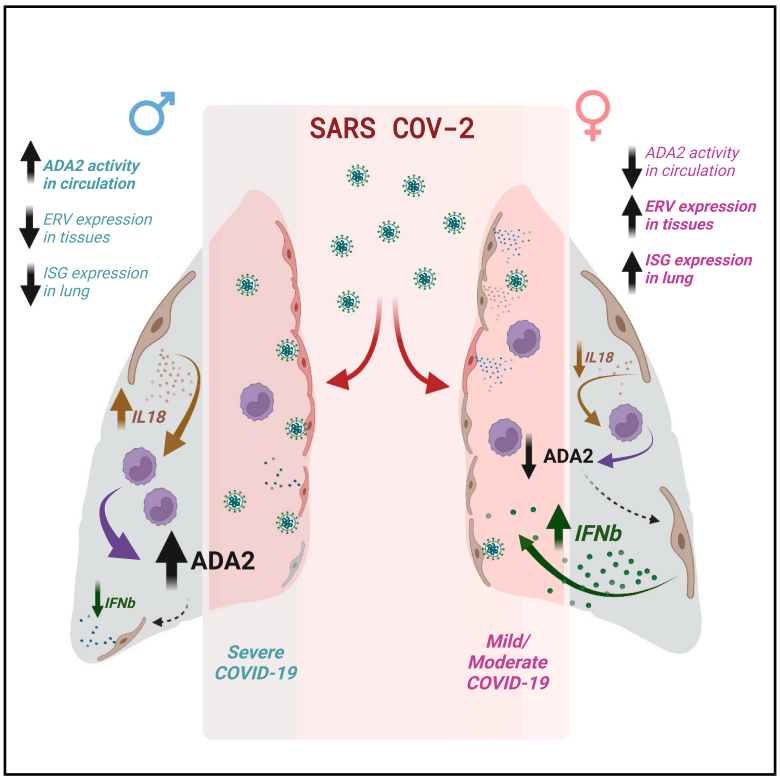
Sex differences in adenosine deaminase activity associate with disparities in SARS-CoV-2 innate immunity

Graphical abstract



Highlights

- ADA activity, a suppressor of the hERV-ISG response, is lower in females
- hERVs and ISGs are higher in the lungs of healthy and COVID-19 females
- Monocytes are the cellular source of sex-specific ADA expression
- Male lung epithelial cells produce higher IL-18, which correlates with ADA

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In brief

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Sex differences in adenosine deaminase activity associate with disparities in SARS-CoV-2 innate immunity

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SUMMARY

Females demonstrate elevated type-I interferon production and a stronger antiviral immune response; however, the mechanisms underlying sex-based differences in antiviral immunity are incompletely understood. We previously reported that low adenosine deaminase (ADA) activity perturbs the methylation-based transcriptional silencing of endogenous retroviral elements (hERV), which stimulates IFN-Stimulated Genes (ISG) and primes antiviral immunity. Here we demonstrate lower ADA activity in females compared to their male counterparts, which correlated with higher hERV and ISG expression in female lungs. Sex differences in ADA2 were linked to the number and expression profiles of blood and lung-derived monocyte populations. Single-cell RNA sequencing of respiratory cells from patients with COVID-19 showed a significant female bias in hERV-ISG signatures, and implicated IL-18 as a driver of sex-specific ADA2 expression. Observations in healthy and COVID-19 cohorts indicate that higher ADA activity is associated with suppressed antiviral innate immunity in the male respiratory tract, which may drive adverse COVID-19 outcomes.

INTRODUCTION

Evidence for sex as a biological variable in the prognosis of viral diseases is compelling.[1] For infections with Hepatitis C and Human Immunodeficiency Virus (HIV), males tend to exhibit higher viral loads compared to their female counterparts.[2,3] During the Middle East Respiratory Coronavirus (MERS-CoV) outbreak of 2012, fatality rates of 52% were observed in males compared to 23% in females.[4] Similarly, during the Severe Acute Respiratory Coronavirus 1 (SARS-CoV-1) outbreak in 2003, male mortality was recorded at 22% versus 13% in females. Across the globe, biological males are at a significantly higher risk of developing complications from SARS-CoV-2 infection and acute COVID-19 disease. Compared to female patients with COVID-19 , males are 46% more likely to be admitted to the ICU[5] and 45% more likely to die from COVID-19 complications.[6] A recent review of the impact of biological sex in COVID-19 vaccine response and efficacy reveals that females also display significantly higher vaccine-driven humoral responses.[7] At the cellular level, female sex is linked to more efficient intracellular viral recognition[8] and higher production of type-1 Interferon (Type I IFN).[9] While these observations support the existence of significant sex-based differences in antiviral innate immunity at the cellular and molecular levels, the mechanistic underpinnings are important for the understanding of COVID-19 pathogenesis and yet remain incompletely understood.

An early and robust innate immune response to virus infection is crucial for controlling viral load, limiting viral spread, and effectively triggering the adaptive immune response in order to fully eliminate the virus and virus infected cells.[10–12] Type I IFNs are crucial components of the innate immune system and act as the first line of defense against viral infections. These cytokines trigger antiviral responses in nearby cells, inhibiting viral replication and limiting viral spread.[13] They also recruit immune cells,

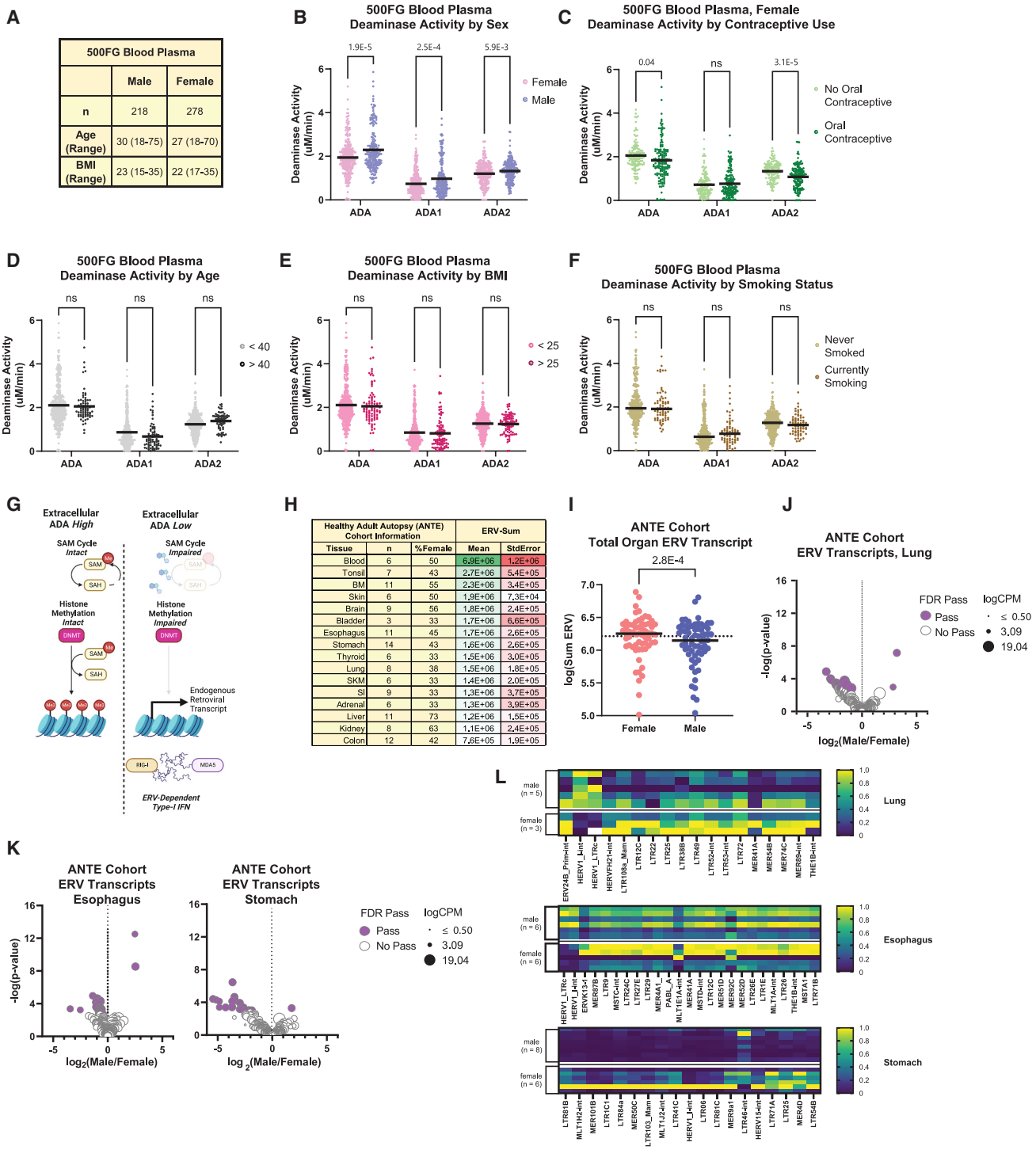

Figure 1. Healthy female individuals exhibit lower peripheral ADA and higher tissue hERV

(A) Demographic table of the 500 Functional Genomes (500FG) cohort.[34]

(B–F) Total, EHNA-sensitive (i.e., ADA1) or EHNA-resistant (i.e., ADA2) adenosine deaminase enzyme activity measured in peripheral blood from healthy individuals and stratified by (B) sex, (C) contraceptive use, (D) age, (E) BMI, and (F) smoking history. Data are represented as individual points with bar = mean.

(G) Schematic representation of the mechanism by which ADA suppresses hERV gene methylation and type-I IFN.

(H) Cohort description for bulk measurements of hERV transcripts from 134 human tissue samples from Oncobox Atlas of Normal Tissue Expression (ANTE, Suntsova et al. (2019) Sci Data).

(I) Total hERV expression in solid organs stratified by sex. Data are represented as individual points with bar = mean.

(legend continued on next page)

such as natural killer (NK) cells and macrophages, to help eliminate infected cells and orchestrate a broader immune response.[14] Type I IFNs are produced in direct response to the cellular sensing of foreign, ectopic, or aberrant nucleic acids and their by-products.[15,16] Following viral entry, aberrant nucleic acids are recognized by endosomal Toll-like receptors (TLR) such as TLR3 and TLR7/8[17] or cytoplasmic sensors such as retinoic acid–inducible gene-I (RIG-I),[18] melanoma differentiation–associated protein 5 (MDA5),[18] or cyclic guanosine monophosphate–adenosine monophosphate synthetase (cGAS).[19] Engagement of innate cytosolic sensors in turn activates key signaling adaptor proteins, including mitochondrial antiviral signaling (MAVS) or stimulator of IFN genes (STING), which drive IFNβ production downstream of the signaling proteins Tank-Binding Kinase-1 (TBK-1) and Interferon Regulatory Factor 3 (IRF-3).[20,21] IFNβ works in coordination[22] or competition[23] with NLRP3 inflammasome-derived signals to recruit peripheral leukocytes and subsequently activate a CD8-T/NK-cell mediated anti-viral immune program. Early IFN responses are essential for mitigating the severity of acute COVID-19 disease. Research has shown that individuals with impaired or delayed IFN responses tend to experience more prolonged viral replication and severe disease.[24] Conversely, a robust early IFN response is associated with better clinical outcomes.[24] Furthermore, age and sex differences influence the strength of the IFN response, with younger individuals and women generally exhibiting stronger antiviral immune responses, which may explain the lower severity of COVID-19 observed in these groups.[1] Thus, enhancing or preserving the early IFN response may be key in reducing COVID-19 severity, and understanding the molecular and cellular factors that modulate early IFN response could inform therapeutic strategies. In contrast to its protective role in acute disease, a delayed or exaggerated IFN response in severe adult COVID-19 cases can lead to an overproduction of ISG contributing to progressive tissue damage and impaired type I IFN activity, exacerbating inflammatory responses, driving hyperinflammation and lung damage and severe progression of COVID-19.[25] This dysregulated immune response contributes to complications such as acute respiratory distress syndrome (ARDS).[26,27] These findings underscore the importance of exploring more thoroughly the mechanisms that drive early vs. late IFN response. In this context, changes in ADA2 activity may play a clinically relevant role in suppressing Type I IFN-mediated immunity and inflammation during acute and chronic COVID-19.

We previously reported a mechanism by which the adenosine deaminase enzymes ADA1 and ADA2 function to inhibit cellular IFNβ production and the resulting IFN-stimulated gene (ISG) mediated cellular antiviral response.[15] The mechanism involves mainly ADA2-dependent deamination of the extracellular bioactive nucleoside deoxyadenosine, whose subsequent transport and accumulation inside cells destabilizes methionine metabolism and perturbs the *trans*-methylation of the DNA genome, driving the expression of dozens of immune-stimulatory human endogenous retroviral elements (hERV). hERVs are genomic-encoded, aberrant RNAs that engage both the TLR and cytosolic dsRNA sensing pathways to stimulate low levels of IFNβ and ISG production, which train or prime cell-intrinsic innate immune signaling pathways resulting in an enhanced cellular antiviral response to virus infection.[15,28,29] In this study, we report significant sex-specific differences in ADA2 activity and expression, and explore its relationship to the magnitude of the cellular innate immune response to SARS-CoV-2 and clinical outcomes of COVID-19. In multiple independent human cohorts, including both healthy individuals and patients with acute COVID-19, ADA enzyme activity was quantified in plasma using high-resolution liquid chromatography-mass spectrometry techniques.[30] Publicly available RNA sequencing databases and other resources were used to further examine the relationship between ADA and hERV or ISG expression in human blood and tissues. Our results demonstrate for the first time that circulating ADA2 activity is significantly lower in healthy females compared to their male counterparts, and tissue hERVs are overexpressed in female tissues, including the lung. In patients with SARS-CoV-2 infection, elevated hERV expression was directly linked to the extent of antiviral ISG signature induction, with female patients demonstrating significantly higher ISG expression levels. Respiratory tract sampling and single-cell transcriptomics showed that ADA2 expression in patients with acute COVID-19 is predominantly derived from myeloid cells, with the higher expression of ADA2 in specific male-derived monocyte populations. In respiratory epithelial cells from male patients with COVID-19, higher levels of the cytokine IL-18, which induces ADA2 in peripheral blood mononuclear cells,[31] correlated with higher ADA2 activity and expression. Together, our results suggest that in males, inflammatory cytokines such as IL-18 induce higher ADA2 expression and activity. Higher ADA2 activity is associated with lower hERV expression and suppressed ISG antiviral responses in males compared to their female counterparts, which may contribute to male sex being a risk factor for increased morbidity and mortality in severe COVID-19 disease.

RESULTS

Healthy females exhibit lower adenosine deaminase activity compared to their male counterparts

Differences in peripheral ADA activity in healthy individuals have been documented in pediatric populations compared to adults,[32] however little is known about the influence of sex, age and BMI on variance in ADA activity in adults. We measured natural variation in *de novo* adenosine deamination using mass spectrometry analysis of peripheral blood samples from the Human Functional Genomics Project[33] (500FG, $n = 496$, Figure 1A;

(J) Volcano plot shows the differential expression of individual hERV transcripts in the lung.
(K) Differential expression of hERV transcripts in the esophagus and stomach. Gray/purple delineate significance cutoff per legend.
(L) Heatmaps of differentially expressed hERV transcript in lung ($n = 5$ males, 3 females), esophagus ($n = 6$ males, 6 females), and stomach ($n = 8$ males, 6 females). Stats = with univariate linear regression, $n = 474$ (Figures 1B–1F), multivariate linear regression, covariates = individual, tissue, sex (Figure 1I), differential gene expression analysis with Mann-Whitney post-hoc analysis (Figures 1J and 1K). CPM = counts per million. See also Figure S1, Table S1.

Table S1), where higher ADA enzymatic activities specific to ADA1 and ADA2 were detected in the male samples compared to matched female counterparts (Figure 1B). Interestingly, lower serum ADA2 activity was observed in females taking estrogen and progestin-containing hormonal contraceptives (Figure 1C), accounting for the lower ADA2 activity observed in the younger female subset of the 500FG cohort (Figures S1A and S1B). In contrast, no significant correlation was observed between ADA activity and age, body mass index (BMI), or tobacco smoking (Figures 1D–1F). Together, these results are the first to demonstrate sex-specific and hormone-sensitive variations in peripheral ADA activity in healthy adults, where females demonstrate significantly lower ADA1 and ADA2 specific enzymatic activity compared to their male counterparts.

Increased human endogenous retroviral elements expression in female mucosal tissues

We previously reported that loss of extracellular ADA2 expression or enzymatic activity drives cellular uptake of extracellular deoxyadenosine, which, once transported inside cells destabilizes intracellular S-adenosyl-methionine metabolism and perturbs the *trans*-methylation of dozens of hERV loci encoded in the DNA genome[15] (Figure 1G). Methylation-sensitive transcription of immune-stimulatory hERV elements is an established immune-stimulatory mechanism for infection-naïve, IFNβ-driven priming or training of the cellular innate immune response,[15,35] resulting in enhanced immune-inflammatory responses to subsequent virus infection.[15] Given that significantly lower serum ADA2 activity was observed in females of the 500FG cohort, we reasoned that hERV expression may be selectively elevated in females compared to males. In 500FG, whole genome RNA sequencing of peripheral blood mononuclear cells (PBMC) revealed no significant sex bias in total hERV expression (Figure S2A). The previous study indicates that nucleoside accumulation in the extracellular environment is required for cellular hERV-IFNβ priming,[15] which would occur more efficiently in a local tissue microenvironment than systemic circulation. To investigate hERV expression in human tissues, we analyzed whole genome RNA sequencing data from The Oncobox Atlas of Normal Tissue Expression (ANTE), a community-acquired, postmortem solid tissue specimen collection[36] (Figure 1H). hERV expression, represented as a sum of all detected hERV transcripts, was significantly higher in female tissues when controlled for individual donor and tissue type (Figure 1I). Of note, when sub-classed into individual tissues, relative hERV overexpression in female tissues was detected in the lung (Figure 1J) and tissues of the upper gastrointestinal tract, including the esophagus and stomach (Figures 1K, 1L, S2B, and S2C). Together, these data demonstrate for the first time a selective female bias in hERV expression in mucosal epithelial tissues.

Sex-specific variations in ADA2 expression and activity in monocytes

Globally, male sex is an established risk factor for developing severe COVID-19 disease and COVID-19-related mortality,[37] although the immune-related mechanisms underlying this disparity are not fully understood. Early production of the antiviral cytokine IFNβ has been shown to significantly reduce COVID-19 severity in humans,[38] emphasizing the importance of cellular innate immunity and the IFNβ/ISG-driven antiviral response to SARS-CoV-2 in positive COVID-19 outcomes. To evaluate ADA activity in COVID-19 we analyzed serum samples from vaccination-naïve, patients with acute COVID-19 and controls, which were collected at Cedars Sinai Health System in the summer and fall of 2020 (Figure 2A; Table S2), as part of the Embarking Beyond Acquired Risks in Communities (EMBARC) longitudinal study cohort.[39] In the EMBARC cohort, male sex was a biological variable that significantly increased the likelihood for ICU admission due to COVID-19 (Figure 2B). Interestingly, age was not significantly associated with disease severity in these patients (Figure 2B); however, this likely reflects an underrepresentation of younger patients in this first-wave sampling of hospitalized patients with COVID-19. We observed that ADA activity was significantly increased in acute COVID-19 (Figure 2C), with a specific increase in ADA2 activity accounting for the variation. While increased ADA2 activity was observed in the patients with COVID-19, this variation was not related to the severity of COVID-19 as graded by ICU admission or intubation (Figure S3). When separated by sex, the female patients were the primary contributors of the increase in ADA2 activity in COVID-19, whereas male ADA2 activity was stable (Figure 2D). Taken together, these results demonstrate increased ADA2 activity in the setting of COVID-19, specific in female patients.

It is unknown how male sex influences ADA2 activity or expression. In healthy individuals, ADA2 is mainly produced by circulating monocytes,[31,41] and increased ADA2 enzymatic activity is a hallmark of monocyte activation. To examine ADA2 expression in cell populations of the lung, we analyzed single-cell sequencing data from bronchoalveolar lavage fluid (BALF) and sputum sampling of patients with vaccine-naïve acute COVID-19[40] (Figure 2E). In the setting of acute SARS-CoV-2 infection in the lung, monocytes and macrophages were the major sources of ADA2 transcripts in the BALF and sputum (Figure 2F). Stratifying samples based on sex revealed that the male-derived monocytes displayed significantly higher ADA2 transcript expression compared to female counterparts (Figure 2G). The sex-bias in ADA2 expression was observed in the most abundant monocyte sub-populations, including classical monocytes (Cluster 1 ($CD14^+$ $CCL3^+$), Cluster 2 ($CD14^+$, $HLA\text{-}DPB\text{-}1^+$), and Cluster 3 ($CD14^+$, $VCAN^+$)) and non-classical monocytes (Cluster 5 ($CD16^+$)) (Figure 2H). Male bias in ADA2 expression was absent in Cluster 4 ($CD14^+$ $CD16^+$), corresponding to intermediate monocytes. These observations support classical and non-classical lung monocytes as the primary cellular drivers of higher ADA2 expression in males with COVID-19. Likewise, we also found that the number of monocytes in blood positively correlated with ADA2 activity measured in the healthy individuals of the 500FG cohort, particularly in the male participants (Figure 2I). In healthy males, classical monocytes account for the majority of the correlation between monocyte numbers and ADA2 enzyme activity, while in healthy females, the association is observed mainly in intermediate monocytes and to a lesser extent nonclassical monocytes (Figure 2J). Together, these data show that sex-specific variation in ADA2 expression relates to differences in the number, type,

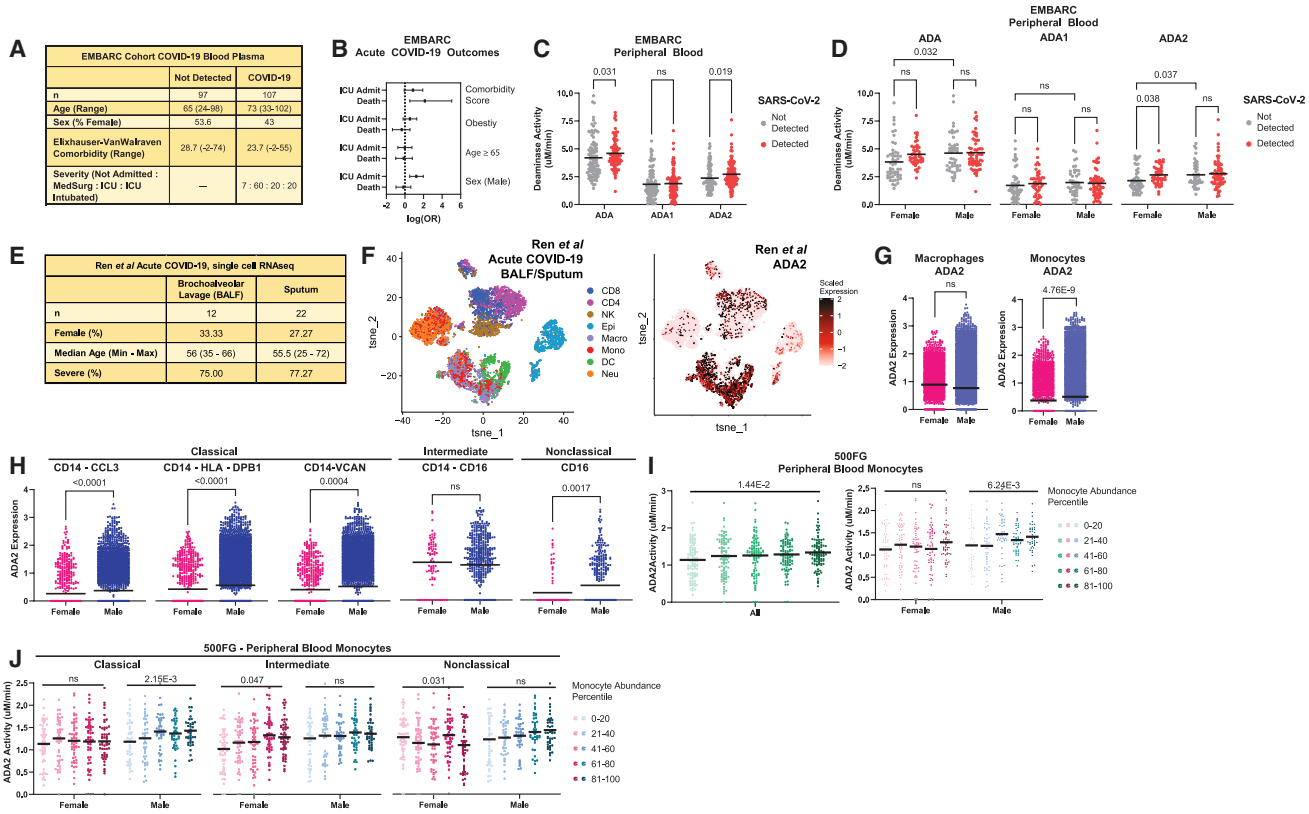

Figure 2. Sex differences in ADA2 activity and expression are linked to monocyte populations

(A) Demographics table for the EMBARC COVID-19 cohort.

(B) Forest plot of the odds ratio for ICU admission or mortality in patients with EMBARC COVID-19. Error = 95% CI, vertical rectangle = mean log odds ratio (C–D) Adenosine deaminase activity in patients stratified by (C) COVID-19 status or (D) COVID-19 status and sex. Data are represented as individual points with bar = mean.

(E) Demographic table of Ren et al. COVID-19 cohort.[40]

(F) Two-dimensional tSNE plot of single-cell RNAseq analysis of COVID-19 BALF and sputum (n = 24), stratified by cell type (left) and relative ADA2 mRNA expression (right).

(G) ADA2 mRNA expression in monocytes and macrophages from patients with COVID-19,[40] stratified by patient sex.

(H) ADA2 mRNA expression in patients with COVID-19,[40] stratified by monocyte clusters and patient sex.

(I and J) ADA2 activity measured in bulk populations of monocytes ($CD14^+$) (I) or monocyte subsets (J) by flow cytometry, (500FG, n = 474). Data are represented as individual points with bar = mean. Stats = univariate logistic regression (Figure 2B); multivariate logistic regression, covariates = age, sex, BMI (Figures 2C and 2I left), covariates = age, BMI (Figures 2D and 2I right; Figure 2J); covariates = patient (Figures 2G and 2H). See also Figure S2, Table S2.

and expression profiles of monocytes, which are the major producers of ADA2 in the blood and respiratory tract.

Higher human endogenous retroviral elements and IFN-stimulated genes expression in female lung tissue

We observed significant male bias in ADA2 enzymatic activity and expression, which correlated with reduced hERV expression in SARS-CoV-2 target tissues such as the lung. The inhibitory effects of higher ADA2 activity on hERV-IFNβ signaling may underlie the sex-specific discrepancies observed in acute COVID-19 pneumonia. While robust evidence supports IFNβ induction as a protective feature in SARS-CoV-2 infection and acute COVID-19[37], the relationship between hERV-mediated priming of cell-intrinsic viral sensing pathways and the type-I IFN response has not been described in this setting. Single-cell RNA-seq analysis across the myeloid, lymphoid, and epithelial populations in acute COVID-19 BALF and sputum[40] revealed a broad distribution of hERV transcript expression across all of these cell populations (Figure 3A). Type-I IFN gene signatures were enriched in certain respiratory cell populations in an infection-specific and sex-specific manner. In cells where SARS-CoV-2 transcripts were detected, ISG expression was higher relative to cells without detectable virus transcripts, particularly in the epithelial and myeloid cell subsets, which are the reported cell targets for SARS-CoV-2 infection (Figure S4A).[40] Interestingly, an unbiased analysis of gene ontology and biological pathway (GOBP) enrichment identified gene signatures specific to the cellular antiviral IFN/ISG program only in lung epithelial cells of the female donors (Figure 3B), nor was it observed in other cell types known to be ISG producers in this dataset (Figure S4B). We next examined the co-enrichment of the ISG signature with hERV in the epithelial cells. Indeed, hERV expression positively correlated with multiple ISGs in these cells (Figure 3C). When stratified by sex, we observed that the male patients displayed

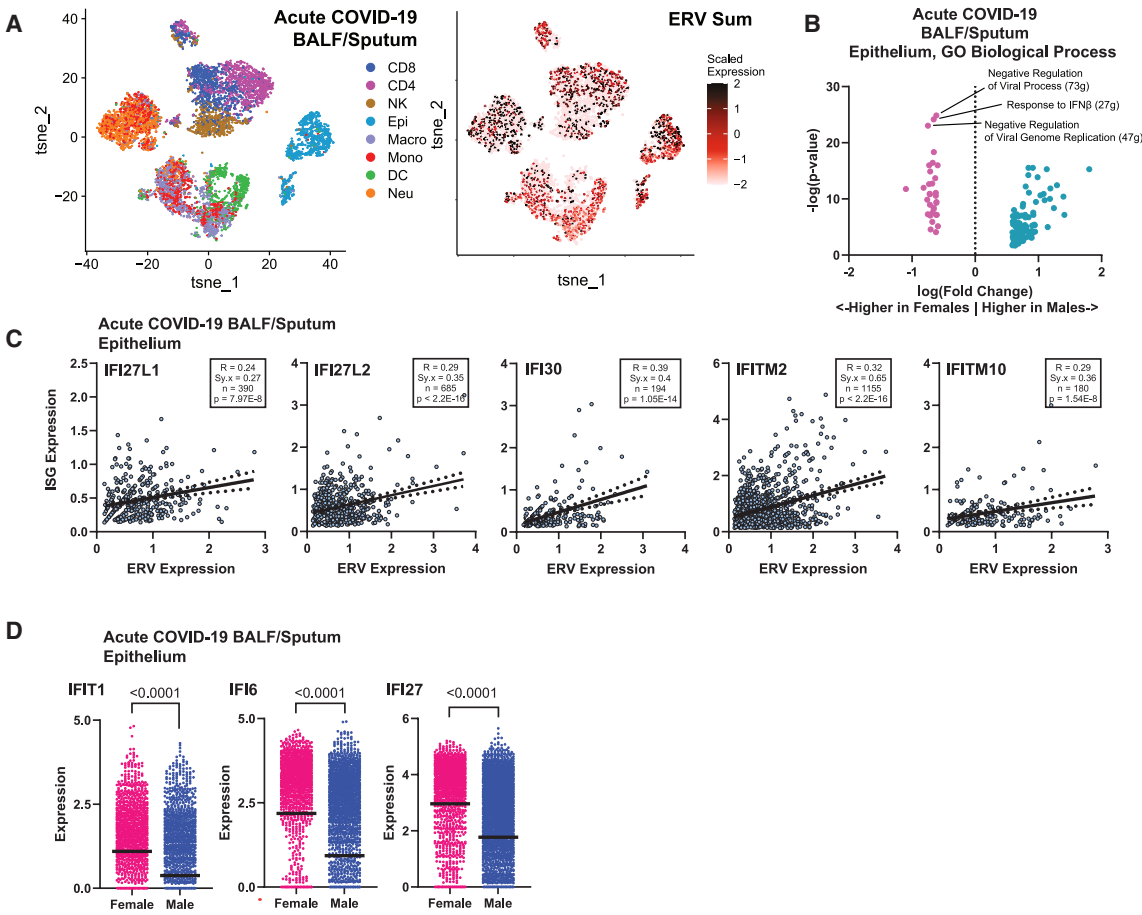

Figure 3. Expression of hERV and ISG signatures in the lungs of patients with COVID-19

(A) Two-dimensional tSNE representation of single-cell RNAseq analysis of BALF and sputum from patients with COVID-19, stratified by cell type (left) and relative hERV transcript levels (right).[40]

(B) Volcano plot of gene ontology and biological pathway (GOBP) terms differentially expressed between BALF/sputum-derived single epithelial cells from male and female patients with COVID-19.

(C) Scatterplots of hERV transcript levels relative to ISG transcript levels in epithelial cells without detectable COVID-19 transcripts. Linear regression with a 95% confidence interval is shown.

(D) ISG expression stratified by patient sex. Stats = Mann-Whitney nonparametric (Figure 3B), univariate linear regression (Figure 3C), multivariate linear regression, covariates = patient (Figure 3D).

See also Figures S3 and S4.

significantly lower magnitude of ISG expression (Figure 3D). Together, these data show that females with COVID-19 exhibit robustly detectable signatures of cell-intrinsic antiviral priming compared to their male counterparts, where higher hERV expression co-expresses with a stronger innate immune type-I IFN response.

Lung epithelial cell-derived IL-18 signals promote local ADA2 induction in monocytes

Increased ADA2 enzymatic activity has been suggested as a functional readout for myeloid cell activation by pro-inflammatory cytokines such as IL-18, which function in the pathogenesis of Macrophage Activation Syndrome (MAS), a life-threatening complication of systemic juvenile idiopathic arthritis.[31] Using measures of cytokine concentrations in the 500FG cohort, we observed a significant positive association between circulating ADA2 enzymatic activity and serum IL-18 concentrations (Figure 4A) and IL-1β concentrations (Figure 4B). By contrast, concentrations of the pro-inflammatory cytokine IL-6, which has been implicated in cross-regulation of IFN/ISG in older, convalescent COVID-19 males,[42] did not significantly associate with ADA2 activity (Figure S5). Notably, in contrast to IL-1β, which showed no significant difference between males and females, there was a significant male bias in blood IL-18 concentrations (Figure 4A). At the mRNA level, COVID-19 single cell RNA sequencing data showed that epithelial cells are the major producers of IL-18 in BALF (Figure 4C). Separating the epithelial cells by patient sex, male cells demonstrate significantly higher overall IL-18 expression compared to female cells (Figure 4D). These data support a model whereby an epithelial cell-derived cytokine IL-18 axis drives higher ADA2 expression and activity in males compared to females.

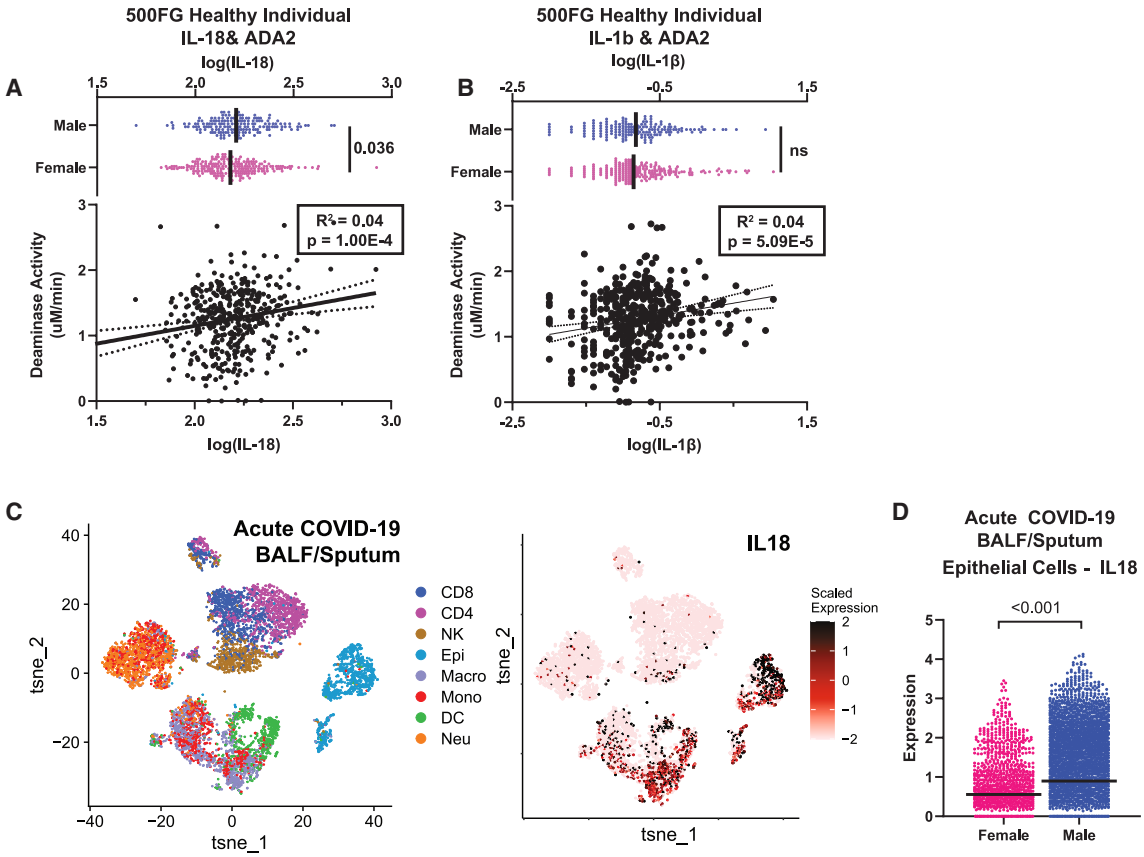

Figure 4. IL-18 expressed by lung epithelial promote ADA2 expression by monocytes

(A and B) Variation of (A) IL-18, and (B) IL-1b cytokine concentration by sex (top) or ADA2 activity (bottom), in blood of healthy individuals in the 500FG cohort (n = 408).

(C) Two-dimensional tSNE representation of single-cell RNAseq analysis of COVID-19 patient BALF/sputum, stratified by cell type (left) and relative IL-18 expression (right).

(D) IL-18 mRNA expression in BALF/sputum-derived lung epithelial cells, stratified by patient sex. Data are represented as individual points with bar = mean. Stats = multivariate linear regression, covariate = sex (Figures 4A and 4B); multivariate linear regression, covariates = patient (Figure 4D). See also Figure S5.

DISCUSSION

Bio-active purine nucleosides play critical roles in host-pathogen interactions and inflammatory diseases. As bona fide signaling molecules that modulate immune cells and inflammatory systems through binding to adenosine receptors,[43,44] purinergic signaling has been shown to skew immune cells such as T-cells, monocytes, macrophages, mast cells, and endothelial cells to an anti-inflammatory profile.[45] Purine nucleosides also exert bio-active effects inside stromal and myeloid cell populations after uptake from the extracellular environment, where they alter the homeostasis of cellular metabolic pathways and activate the innate immune antiviral pathway.[15] Purine metabolites also stimulate the Intracellular Pathogen Response (IPR) in the nematode C. elegans, which is involved in host defense to intracellular pathogens, suggesting that the immune-stimulatory function of purines in cell-intrinsic innate immunity is conserved.[46] Accordingly, it has been observed that lower adenosine levels in patient sera correlated with poor prognosis for COVID-19 infections.[47] The metabolic enzymes that control purine nucleoside metabolism likewise exert effects on immunity and inflammation in mammals[48–50] and invertebrates.[51] ADA1, which is mostly intracellular, and ADA2, which almost exclusively extracellular/in circulation, catalyze the irreversible deamination of adenosine or deoxyadenosine into inosine or deoxyinosine, respectively, though they differ significantly in localization, binding efficiency, and catalytic activity.[52,53] Approximately 15% of patients with Severe Combined Immune Deficiency (SCID), who suffer from severe impairment of both adaptive and innate immune cells, harbor loss-of-function mutations in the ADA1 gene.[54] In contrast, an increase in serum ADA2 activity is indicative of monocyte and/or macrophage activation[31] and loss-of-function mutations in the ADA2 gene cause DADA2, an autoinflammatory disorder with clinical features of fever, skin rashes, joint pain, and vasculitis.[55] The pathophysiology of severe COVID-19 also involves a combination of vascular inflammation[56] and macrophage activation.[57] Given the role of ADA in restraining these hallmark features of COVID-19, it emphasizes the importance of understanding the regulation of ADA activity during SARS-COV-2 infection both in circulation and in tissue. While

previous studies describe changes to ADA1 and ADA2 levels and activity in convalescent serum of patients with COVID-19 with mild disease,[58] this study is the first of its kind to delve into the sexual dimorphism of ADA expression and activity, and indicate an immune mechanism that renders biological males more vulnerable to viral pneumonias such as COVID-19 due to the innate immune-suppression of the ADA-regulated hERV-IFNβ axis.

A prior study has shown that children exhibit higher levels of ADA1 activity compared to adults; however, ADA activity does not appear to further change as a function of age in the adult population.[32] Biological variables such as smoking and high BMI, which are linked to systemic inflammation, had no discernible effect on ADA activity (Figure 1). Studies of ADA in metabolic syndrome report increased ADA2 activity associated with hyperlipidemia and T2DM without reporting significant variation by any particular demographic categorizations.[59] However, in these studies, variations in ADA enzymatic activity by sex or contraceptive use were either ineffectively powered or not accounted for. Prior to this study, the relationship between biological sex and circulating ADA activity was most explicitly examined in ischemic disease. In a large retrospective case-control study of coronary artery disease (CAD), diminished plasma ADA activity was observed in patients with CAD was most pronounced in the male patients,[60] though the specific ADA1 and ADA2 activities were not resolved. It is unclear if this observation is phenotypically linked to the small/medium vessel vasculitis observed in ADA2-deficient patients, which can uncommonly include early onset coronary artery calcification.[61,62] DADA2 classically presents with neurologic deficits secondary to lacunar ischemic stroke,[63] which may arise from post-vasculitis luminar narrowing. Conversely, ADA1 activity has been reported to be uniquely upregulated in females undergoing acute stroke,[64] which likely occurs secondary to cell and tissue damage. Here, we report higher peripheral ADA2 activity in biological males compared to female counterparts across multiple independent human patient cohorts. Our group previously demonstrated that lower ADA2 enzymatic activity allows for increased uptake of extracellular deoxyadenosine by cells, leading to a cascade of changes in cellular metabolism that culminate with the transcription of hERVs.[15] hERV overexpression triggers the production of Type I IFNβ and IFNβ signaling, which primes or trains the cellular innate immune response to virus infection. Interestingly, increased ADA2 activity in males did not translate into significant sex-based differences in hERV transcript expression in circulating leukocytes. However, hERV expression in tissues showed significant sex-based differences, including the lung, which is notably targeted by SARS-CoV-2 infection (Figure 1).

Monocytes and macrophages are the primary producers of ADA2. In this study, we found that biological males display a significant correlation between serum ADA2 activity and monocyte counts compared to females. Accordingly, healthy males exhibit increased numbers of total circulating monocytes compared to females;[65] however, this may vary by ethnicity. In spite of this, higher numbers of classical ($CD14^{++}$, $CD16^{-}$), intermediate ($CD14^{+}$, $CD16^{+}$), and non-classical ($CD14^{+}$, $CD16^{++}$) monocyte subsets are consistently reported in males, and a recent study indicated that older men develop a bias toward enhanced innate immunity, whereas older women toward adaptive immunity.[66] The epigenomic signature of aging involves a decrease in naive T cell counts and an increase in monocyte and cytotoxic cell activities, and these alterations are more pronounced in males.

In our current study, we observed that ADA2 activity in circulation increases significantly in patients with COVID-19, driven by the female demographic (Figures 2C and 2D), suggesting a potential sex-specific mechanism for the suppression of Type I IFN levels later in the infection cycle. Considering data obtained from both COVID-19 cohorts, the selective increase of ADA2 activity in the blood of female patients with COVID-19 occurs in parallel to lower relative ADA2 expression in female lung-infiltrated monocytes compared to their male counterparts. The difference likely relates to the orthogonal regulation of blood ADA2 expression, perhaps involving cell subsets other than monocytes, after SARS-CoV-2 infection. Increased blood ADA2 activity measured in the EMBARC COVID-19 cohort may reflect physiological processes absent from the lung/BALF samples in the Ren cohort, including the contribution of inflamed and/or activated endothelial cells which also express functional ADA2.[15] Female endothelial cells are often more responsive to inflammatory stimuli due to hormonal regulation and immune system differences,[67] and female endothelial cells or another cell type in circulation might express higher levels of ADA2 as part of a more active immune response to various stimuli, such as ongoing infection or inflammation. A notable limitation in our study is the use of two different cohorts to assess ADA2 in the lungs and blood of patients with COVID-19. While both cohorts correspond to symptomatic infection, differences in biological factors such as age, sex, comorbidities, or even the timing of sample collection might differentially influence ADA2 measurements. Variability in disease severity or treatment regimens could also contribute to discrepancies between tissue-level and circulating ADA2. These findings highlight the complex and sex-specific interplay of cytokines, immune cells, and tissue-specific responses during COVID-19 and warrant further investigation into the regulatory mechanisms underlying ADA2 activity using a controlled longitudinal cohort analysis.

We show here that males exhibit higher ADA2 activity and lower hERV expression compared to females at baseline, rendering males more vulnerable to early viral infection and poorer disease outcomes. The TLR7 gene escapes X chromosome inactivation,[68,69] thus females with two X chromosomes express higher levels of TLR7 compared to males, with only one X chromosome. Lower TLR7 expression in males has been linked to more severe disease outcomes in infectious diseases such as COVID-19.[70,71] Notably, studies have demonstrated that, aside from intracellular receptors such as RIG-I and MDA5, TLR3 and TLR8 in humans and TLR7 in mice are responsive to certain hERV species.[72] Given this, it is possible that higher TLR7 expression in females may contribute to enhanced hERV-dependent IFN production compared to males.

Ultimately, our study and others show that male sex correlates with the immune suppression of the early antiviral response to SARS-CoV-2, which is critical for controlling the viral load in affected organs and viral spread to new sites. While it remains to be determined how sex-based variation in ADA expression

and activity is regulated at the molecular level, our results in females taking hormonal contraceptives indicate that sex hormones play a role (Figure 1C), with ADA2 activity lower in women taking hormonal contraceptives. Estrogen generally exerts immune-stimulatory effects, which have been shown to enhance both cell-mediated and humoral immune responses to virus infection.[73,74] The effects of estradiol and estrogen receptor activity generally promote innate immune signaling pathways and the development of myeloid cells, which can vary significantly depending on dosage.[75,76] Thus, the administration of oral contraceptives appears to play a role in antiviral immunity, an intriguing concept that also warrants further study within the context of studying sex-based differences in the antiviral response to SARS-CoV-2.

Limitations of the study

We offer several limitations in our study and its conclusions. First, the observations derive from pre-existing cohorts or publicly available datasets of healthy individuals or patients with COVID-19 sampled at different time points, thereby limiting our ability to perform a longitudinal analysis of ADA, hERV, and IFN/ISG expression during SARS-CoV-2 infection. Second, we did not directly demonstrate that epithelial-derived IL-18 drives sex-specific ADA expression using an *in vitro* coculture system with male and female derived monocytes.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Sonia Sharma (soniasharma@lji.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This article analyzes existing, publicly available data, accessible under the NCBI Gene Expression Omnibus (GEO); accession numbers for each series are listed with the associated cohort subheading under Cohorts, when relevant, and here as follows: "Large-scale single-cell analysis reveals critical immune characteristics of COVID-19 patients" NCBI GEO: GSE158055; "RNA-Seq whole blood of Dutch 500FG cohort" NCBI GEO: GSE134080; "Atlas of RNA sequencing profiles of normal human tissues" NCBI GEO: GSE120795. All data supporting the findings of this study, including patient information and demographics, LC-MS feature quantitative measurement and raw LC-MS data, and gene expression data, are contained within this article, the referenced article, or referenced public database, or available within supplemental information. This article does not contain original code. Any additional information required to reanalyze the data reported in this article is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation and data collection were performed by Priyanka Saminathan, Ian Mathews, Ahmad Alibadi, and Kai Fung. Data analysis, interpretation, and figure design were performed by Priyanka Saminathan, Ian Mathews, Ahmad Alibadi, Kai Fung, and Kiyokazu Kakugawa. Material support and patient sampling were performed by Leo AB Joosten, Mihai G Netea, Mohit Jain, Susan Cheng, Catherine C Hedrick, and Sonia Sharma. The first draft of the article was written by Priyanka Saminathan, Ian Mathews, and Sonia Sharma, and all authors commented on previous versions of the article. All authors read and approved the final article.

DECLARATION OF INTERESTS

Authors Sharma S and Jain M have financial interests in Sapient Bioanalytics. The remaining authors have no financial or non-financial interests to disclose.

STAR★METHODS

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

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
2′-Deoxyinosine	Sigma-Aldrich	CAS# 890-38-0
2′-Deoxyadenosine ($^{13}C_{10}$, 98%; $^{15}N_5$, 96-98%)	Cambridge Isotope Libraries	CAS# 2483830-27-7
Hypoxanthine	Sigma-Aldrich	CAS# 68-94-0
Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA)	Sigma-Aldrich	CAS# 58337-38-5
Methanol (HPLC Grade)	Sigma-Aldrich	CAS# 67-56-1
Acetonitrile (HPLC Grade)	Sigma-Aldrich	CAS# 75-05-8
Water (HLPC Grade)	Sigma-Aldrich	CAS# 7732-18-5
SeQuant® ZIC®-pHILIC (5 μm) HPLC Columns	Sigma	UNSPSC# 41115709
Deposited data		
Large-scale single-cell analysis reveals critical immune characteristics of COVID-19 patients	NCBI Gene Expression Omnibus (GEO)	GSE158055
RNASeq whole blood of Dutch 500FG cohort	NCBI Gene Expression Omnibus (GEO)	GSE134080
Atlas of RNA sequencing profiles of normal human tissues	NCBI Gene Expression Omnibus (GEO)	GSE120795
Software and algorithms		
Seurat v5.0.3	R	RRID:SCR_007322
AUCell	R	RRID:SCR_021327
Bowtie2		RRID:SCR_016368
Graphpad Prism 10.4.0	Prism	RRID:SCR_002798

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cohorts

500 Functional Genomes (500FG)

500FG (NCBI GEO: GSE134080) is a community-acquired, healthy human population cohort (n = 496) with peripheral immune phenotypic measurements as a component of the Human Functional Genomics Project (Ethical Committee of Radboud University Nijmegen, the Netherlands, #42561.091.12). Participants were appropriately consented prior to participation and venous blood sampling. Covariates in linear modeling for regression are reported in figure legends and include patient age, reported sex, and BMI (www.humanfunctionalgenomics.org).

Oncobox Atlas of Normal Tissue Expression (ANTE)

ANTE (NCBI GEO: GSE120795) is a community-acquired post-mortem solid tissue specimen collection (n = 133 samples across 16 organs donated from 17 healthy individuals) as approved by the ethics committees of the Faculty of Medicine at Moscow State University and of the National Research Center for Hematology.[36] Informed consent was obtained by donor's legal representative, and tissue of origin was confirmed for each sample by a pathologist prior to analyses.

Bronchioalveolar lavage and sputum from acute COVID-19 patients

Bronchioalveolar lavage and sputum sampling were collected from a community-acquired, COVID-19 vaccination-naïve patient population (n = 34) hospitalized for acute COVID-19 in Guangzhou or Shenzhen, China (NCBI GEO: GSE158055) and processed for single-cell RNA sequencing.[40] While prior COVID-19 infections were not exclusion criteria in participant recruitment, the timing of sample acquisition 01/2021 makes prior COVID-19 infection highly unlikely and prior COVID-19 vaccinations impossible. The study was approved by the ethics committees of 19 participating institutions, and appropriate informed consents obtained from all participants prior to sample collection.

Embarking Beyond Acquired Risks in Communities (EMBARC)

The EMBARC study (embarc-study.org) includes a cohort of human blood plasma sampling prospectively collected from a community-acquired, COVID-19 vaccination-naïve population (n = 204) admitted to the Cedar Sinai Hospital in Los Angeles, USA,[39,77–81] allocated by SARS-CoV-2 nucleic acid amplification testing status to not detected or COVID-19 groups. While prior COVID-19 infections were not exclusion criteria in participant recruitment, the timing of sample acquisition 03/24/2020 to 05/10/2020 makes prior COVID-19 infection highly unlikely and prior COVID-19 vaccinations impossible. All study participants provided their written informed consent prior to participation in all protocols, and all protocols were approved by the CS institutional review board.

METHOD DETAILS

Adenosine deaminase activity assay

Measurement of adenosine deaminase activity was performed in freshly frozen blood plasma. Briefly, human blood plasma was diluted 1:10 in cold phosphate-buffered saline and incubated with 100μM ^{15}N-fully labeled 2′-deoxyadenosine (Cambridge Isotope Laboratories) for 30 and 120 minutes at 37°C. To determine enzyme-specific activity, 50μM erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was co-incubated to inhibit ADA1 enzymatic activity selectively. Reactions were quenched at a ratio of 80:20 ice-cold, HPLC-grade methanol:reaction volume, centrifuged at 14,000 rpm for 10min to remove insoluble material, and supernatant was isolated for LC-MS analysis (see "Metabolite Extraction and Liquid Chromatography Tandem Mass Spectrometry"). Enzymatic activity was determined via total, ENHA-resistant, and ENHA-sensitive reactions for each sample and represented as nanomoles of labeled deoxyinosine and hypoxanthine generated per liter of reaction volume per minute.

Metabolite Extraction and LC-MS/MS

Extraction of deoxyadenosine and deamination products for liquid chromatography tandem mass spectrometry has been described previously.[15,30] Fresh frozen blood plasma was thawed from −80°C storage overnight in light-free conditions at 4°C before enzymatic reactions were performed. Analytes were vacuum concentrated before resuspension in 40:40:20 UPLC-grade acetonitrile:methanol:water with isotopically labeled internal standards and transferred to glass LCMS inserts for analysis.

Liquid chromatography was performed using a Thermo Vanquish UPLC system coupled to a Thermo QExactive Orbitrap mass spectrometer. To measure deoxyadenosine, deoxyinosine, and hypoxanthine, 2μL of the sample were injected onto a ZIC-pHILIC HPLC column (5μm particle size, 100 x 2.1mm dimension) held at 45°C. The injection needle was washed with 50:25:25:0.1 water:acetonitrile:isopropanol:acetic acid following each sample draw, with the sample eluted on a gradient mobile phase of Mobile Phase A: 20mM ammonium bicarbonate in water, pH 9.6, and Mobile Phase B: acetonitrile. The gradient mobile phase was a constant 0.4mL/min following 90% B from -3.25 to 0.25 minutes, 90% to 55% B from 0.25 to 4.00 minutes, and 55% B from 4.00 to 6.00 minutes. Mass spectrometry utilized a HESI source for ionization in positive mode, +3.5kV spray voltage. Capillary and aux gas temperatures were 275°C and 350°C, respectively, with a sheath gas flow of 40, aux gas flow of 20, and sweep gas flow of 2. Profile data were collected in positive mode, with data quantification by peak height and with tandem mass spectra acquired in data-dependent acquisition as described previously. Protonated adducts of ^{15}N-labeled deoxyinosine and hypoxanthine were identified via in-house analytic standards.

QUANTIFICATION AND STATISTICAL ANALYSIS

hERV transcript annotation and expression analysis

hERV transcripts were annotated from a publicly available whole blood transcriptomics dataset, acquired from a subset of 500FG as described by Aguirre-Gamboa et al.[82] (NCBI GEO: GSE134080). Briefly, whole blood RNA isolation and globin transcript filtration from age- and sex-balanced individuals were performed. Sequencing was performed on the Illumina HiSeq 2000 platform with 2x 50-bp reads from TruSeq 2.0 library preparations. Quality control and alignment to the ensemble genome were performed using FastQC and STAR 2.3.0/SAMTools, respectively.

hERV transcripts were similarly annotated from publicly available tissue transcriptomics, acquired from a subset of the Oncobox Atlas of Normal Tissue Expression (ANTE) cohort of otherwise healthy individuals who were victims of lethal road accidents[36] (NCBI GEO: GSE120795). Samples include either freshly frozen or formalin-fixed paraffin-embedded blocks prior to RNA extraction. Peripheral blood and mononuclear cell isolates were excluded from the analysis. Ribosomal RNA depletion with library preparation was performed using KAPA Biosystem RNA Hyper with RiboErase, and sequencing was performed on an Illumina HiSeq 3000 system (50 bp read length, 30 million raw reads per sample). Quality control and alignment were performed using FASTQ and Start aligners, respectively.

hERV annotation was performed via mapping to the reference genome GRCh38.gencode v26 using Bowtie2 (version 2.1.0). The alignment process was performed with default parameters across all samples. Post-alignment, RepEnrich was employed to perform hERV mapping and annotation, specifically targeting the LTR of the hg38 human reference genome. This analysis enabled the comparative assessment of hERV expression between male and female samples.

Single-cell RNA analysis in COVID-19 BAL and sputum

Single-cell RNA sequencing data, including the expression matrix, metadata, and cell type annotations, were downloaded from the NCBI Gene Expression Omnibus database comprising bronchoalveolar lavage and sputum sampling from acute COVID-19 patients[40] (NCBI GEO: GSE158055) (n = 34, age range 25-72, age median 56, male-to-female ratio 24:10). Bronchoalveolar lavage (BALF) samples were collected from acute COVID-19 patients during endotracheal intubation and subsequently passed through 100μm nylon strainers into RPMI 1640. Sputum samples were collected from subjects following oral cavity wash and hypertonic saline induction by collection and oropharyngeal swab prior to agitation in Dulbecco's Phosphate-Buffered Saline for 15 minutes and 40μm strainer filtration. Cell isolates were processed for 10x single cell transcriptomics by 10x Chromium Single Cell 5′ library for barcoding and Chromium Single Cell 3′ v2, v3, and v5 reagent kits (10x Genomics), with a target of 300-14,000 single cells per reaction. Sequencing was performed on either DIPSEQ, BGISEQ, or Illumina platforms. For in-depth methodology concerning single-cell quality control, alignment against human and SARS-CoV-2 reference genomes, and cell subset annotation and clustering.[40]

The data was examined and downsampled to address the potential bias in the analysis due to the wide variation in cell numbers between patients, sexes, and cell types. First, based on the variation we observed, the data was downsampled to a maximum of 200 cells per patient. Subsequently, it was downsampled to a maximum of 500 cells per sex and cell type. Since the data had been previously cleaned and clustered, we skipped these stages, focusing primarily on dimensionality reduction, visualization, pathway, and DEG analysis. The data was processed using the Seurat v5.0.3 R package.[83]

To be consistent with the original paper, the top 1500 variable genes were calculated for each sample, and the top 1500 most common variable genes were used as the final list to compute the principal component (PC). The Harmony package[84] was utilized for batch correction of the data, using the top 20 principal components (PCs) and treating the sample and dataset as technical covariates, with theta values set to 2.5 and 1.5, respectively. The top 20 Harmony dimensions were then used to generate tSNE dimensionality reduction.

The AUCell R package version 1.24.0[85] was utilized to explore Gene Ontology (GO) Biological Processes at the cellular level. For the analysis, only gene sets containing at least 10 genes from our dataset were included. These gene sets were obtained from the GSEA database. To identify differentially expressed genes or pathways, the Mann-Whitney test using the 'FindMarkers' function from the Seurat package was employed with a ''logfc.threshold'' of 0.59 and a ''min.pct'' of 0.25. The ''pseudocount.use'' was set to 1 and 0.25 for gene and pathway, respectively.

Flow cytometry and cytokine quantification in 500FG

Peripheral blood flow cytometry and cytokine were performed as described by Aguirre-Gamboa et al. and Li et al., respectively.[34,86] Peripheral blood cells were performed 2-3 hours after venipuncture, stained, and analyzed on a 10-color Navios flow cytometer, with data analysis using Kaluza 1.3 software (Beckman Coulter). Monocytes were quantified as a $CD45^{+}$, $CD14^{+}$ population, with $CD14^{++}$, $CD16^{-}$ as classical monocytes, $CD14^{++}$, $CD16^{+}$ as intermediate monocytes, and $CD14^{+}$, $CD16^{+}$ as non-classical monocytes. Full gating strategy and reagents, including fluorochromes, were outlined.[34] Measurement of IL-1β, IL-6 and IL-18 cytokine commercials were performed from commercial ELISA kits (R&D Systems) and measured against commercially available standard (Protein Simple).

Statistical analysis

Multiple hypothesis correction, where performed, utilized the appropriate test as outlined in each assay's associated methods section and figure legends in either R or GraphPad Prism v10. Where employed, multivariate regression was performed in R using covariates known to produce variation at the population level in the associated dependent variable and were outlined in associated figure legends.