An Immune Cell Atlas Reveals Dynamic COVID-19 Specific Neutrophil Programming Amendable to Dexamethasone Therapy

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Summary

SARS-CoV-2 is a novel coronavirus that causes acute respiratory distress syndrome (ARDS), death and long-term sequelae. Innate immune cells are critical for host defense but are also the primary drivers of ARDS. The relationships between innate cellular responses in ARDS resulting from COVID-19 compared to other causes of ARDS, such as bacterial sepsis is unclear. Moreover, the beneficial effects of dexamethasone therapy during severe COVID-19 remain speculative but understanding the mechanistic effects could improve evidence-based therapeutic interventions. To interrogate these relationships, we developed an scRNAseq atlas that is freely accessible (biernaskielab.ca/COVID_neutrophil). We discovered that compared to bacterial ARDS, COVID-19 was associated with distinct neutrophil polarization characterized by either interferon (IFN) or prostaglandin (PG) active states. Neutrophils from bacterial ARDS had higher expression of antibacterial molecules such as PLAC8 and CD83. Dexamethasone therapy in COVID patients rapidly altered the IFN^{active} state, downregulated interferon responsive genes, and activated the $IL1R2^{+ve}$ neutrophils. Dexamethasone also induced the emergence of immature neutrophils expressing immunosuppressive molecules ARG1 and ANXA1, which were not present in healthy controls. Moreover, dexamethasone remodeled global cellular interactions by changing neutrophils from information receivers into information providers. Importantly, male patients had higher proportions of IFN^{active} neutrophils and a greater degree of steroid-induced immature neutrophil expansion. Indeed, the highest proportion of IFN^{active} neutrophils was associated with mortality. These results define neutrophil states unique to COVID-19 when contextualized to other life-threatening infections, thereby enhancing the relevance of our findings at the bedside. Furthermore, the molecular benefits of dexamethasone therapy are also defined. The identified molecular pathways can now be targeted to develop improved therapeutics.

COVID-19 ARDS host responses contextualized to bacterial ARDS.

A broad array of infections including SARS-CoV-2 and bacterial sepsis can induce acute respiratory distress syndrome (ARDS), respiratory failure and death[1-3]. Neutrophils are thought to be key drivers of both COVID-19 and bacterial ARDS[4-6], yet it is unclear if this is related to intrinsic and/or irreversible cellular responses. We studied critically ill patients admitted to intensive care units and compared COVID-19 ARDS to bacterial sepsis with ARDS (herein referred to as bacterial ARDS) (Extended Fig 1a-b). Patient cohorts had comparable ages, sex, days on life support and time in hospital, but COVID-19 patients had broader racial diversity (Extended Fig 1c, Extended Data Table 1). Bacterial ARDS induced significant neutrophilia, and relative thrombocytopenia compared to the near normal circulating neutrophil numbers in COVID-19, while both had similar degrees of lymphopenia (Extended Fig 1d). Both cohorts had comparable PaO2 / FiO2 (P/F) ratios, which is an indicator of the severity of ARDS[7], but bacterial ARDS patients had significantly more kidney injury demonstrated by higher levels of serum creatinine (Extended Fig 1e). We further compared families of soluble inflammatory markers used to distinguish prototypical states, including those identified during cytokine storm (Extended Fig 1f) and cytokine release syndrome (Extended Fig 1g)[8], which demonstrated significantly more IL-1α and IFN-γ in COVID-19, but similar levels of IL-6 and TNF-α to bacterial ARDS patients. Therefore, in the context of life-threatening bacterial ARDS, COVID-19 ARDS patients had normal neutrophil counts, comparable IL-6 levels, and less organ failure, all of which have been previously proposed as markers of COVID disease severity[9,10]. This prompted us to further investigate immune states and composition in response to COVID-19 compared to bacterial ARDS.

The online companion atlas (biernaskielab.ca/COVID_neutrophil) contains accessible scRNAseq data performed on freshly obtained whole blood at timepoint 1 (t1, <72h after ICU admission) and then at timepoint 2 (t2, 7 days after t1). Cellular identity was mapped to 30 immune cell types/states using a UMAP projection from 21 patients and 86,935 cells (Fig 1a, Extended Figure 2 a-b). Global magnitude of gene expression was directly compared between COVID-19 and bacterial ARDS patients (Extended Data

Table 2), which revealed a more globally altered distribution of differential expression at t1 than at t2. Altered regulation of genes was most pronounced in neutrophils at t1, with lower neutrophil gene expression in COVID-19 compared to bacterial ARDS (Fig 1b; Extended Fig 2c). At t2, the global alterations in gene expression when comparing COVID-19 to bacterial ARDS were most pronounced in plasmablasts (Fig 1c; Extended Fig 2d). We further compared and quantified the proportions of known peripheral blood cellular constituents, which highlighted significant differences in CD4 T cells, CD8 T cells and NK cells (Extended Fig 2e). These data highlight that significant global differences in immune cell gene expression exist between COVID-19 ARDS and bacterial ARDS.

COVID-19 drives specific neutrophil maturation states.

Neutrophils are a primary participant in the development of ARDS[11]; yet despite similar severity of ARDS between our bacterial and COVID-19 cohorts, the numbers of circulating neutrophils from clinical cell counts were significantly different (Extended Fig 1e). We hypothesized that neutrophil qualitative states may be important determinants of disease. Neutrophils were subjected to velocity analysis[12,13] to reconstruct maturation dynamics. Louvain clusters (Fig 1d), clinical cohort, individual patient, and velocity length were overlayed on velocity vector fields (Extended Fig 3a-d). The proportions of distinct neutrophil states were compared at t1 and this revealed a divergent expansion of IFN^{active} neutrophils (clusters 2, 4 and 5) in COVID-19, which became similar to bacterial ARDS at t7 (Fig 1e, f). Relative to healthy donors, the IFN^{active} population in both COVID-19 and bacterial ARDS patients were significantly higher (Extended Fig 4a-f), suggesting that pathogen challenge alters neutrophil dynamics and that comparing COVID-19 neutrophils to healthy neutrophils would mask COVID-19 specific responses. Hence, comparing neutrophils from COVID-19 to bacterial ARDS imparts nuanced understanding of the neutrophil response specific to COVID-19.

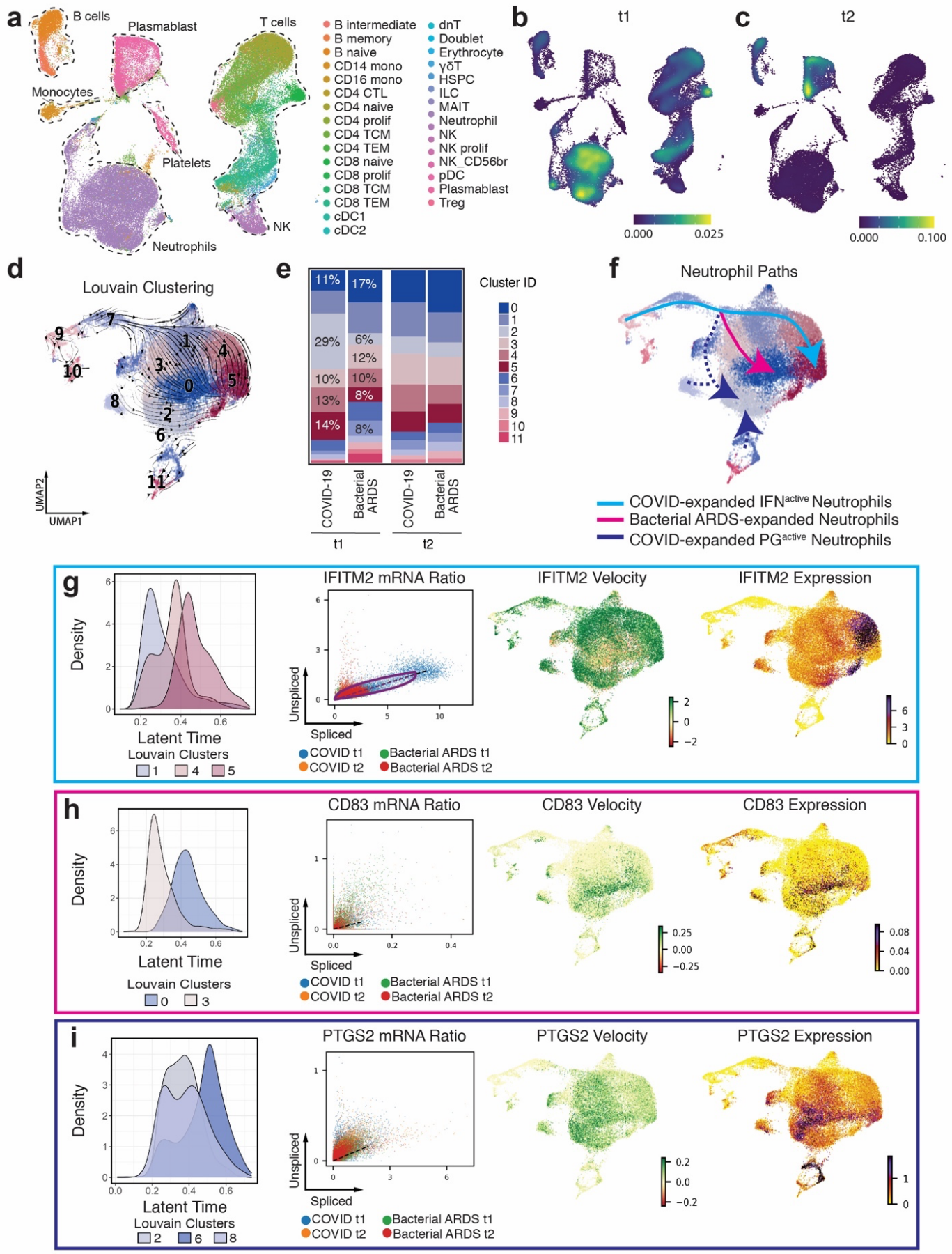
a
B cells
Plasmablast
T cells
Monocytes
Platelets
NK
Neutrophils
B intermediate
B memory
B naive
CD14 mono
CD16 mono
CD4 CTL
CD4 naive
CD4 prolif
CD4 TCM
CD4 TEM
CD8 naive
CD8 prolif
CD8 TCM
CD8 TEM
cDC1
cDC2
dnT
Doublet
Erythrocyte
γδT
HSPC
ILC
MAIT
Neutrophil
NK
NK prolif
NK_CD56br
pDC
Plasmablast
Treg
b
t1
0.000
0.025
c
t2
0.000
0.100
d
Louvain Clustering
UMAP2
UMAP1
e
Cluster ID
COVID-19
Bacterial ARDS
t1
t2
f
Neutrophil Paths
COVID-expanded IFNactive Neutrophils
Bacterial ARDS-expanded Neutrophils
COVID-expanded PGactive Neutrophils
g
Density
Latent Time
Louvain Clusters
IFITM2 mRNA Ratio
Unspliced
Spliced
COVID t1
COVID t2
Bacterial ARDS t1
Bacterial ARDS t2
IFITM2 Velocity
IFITM2 Expression
h
CD83 mRNA Ratio
CD83 Velocity
CD83 Expression
i
PTGS2 mRNA Ratio
PTGS2 Velocity
PTGS2 Expression

Figure 1 – COVID-19 alters neutrophil maturation. a. UMAP projection of 86,935 whole blood cells from 21 patient samples, coloured by Azimuth reference-mapped immune cell states. **b-c.** Kernel density estimates depicting magnitude of molecular response elicited by immune cell subsets during COVID-19 compared to Bacterial ARDS at t1 (b) and t2 (c) calculated by summing DEG fold changes for each cell state shown in Panel a. **d.** UMAP plotting RNA velocity analysis of 29,653 subclustered neutrophils undergoing state transitions, coloured by cluster ID. **e.** Stacked bar plot depicting cluster composition of clinical cohorts examined. **f.** UMAP coloured by neutrophil clusters and overlaid with summary path curves based on vector fields and neutrophil state compositions in Panel d and e, respectively to determine neutrophil states. **g-i.** Transcriptional kinetics driving expansion of IFN^{active} (g), Bacterial ARDS-enriched (h), and PG^{active} (j) neutrophils. Latent time distribution of trajectory-associated louvain clusters (left), phase portraits with equilibrium slopes of spliced–unspliced ratios (center), and RNA velocity and gene expression (right) of selected genes driving divergent maturation trajectories. Phase portraits are coloured by clinical cohort.

Classically, peripheral neutrophils are considered terminally differentiated and non-dividing, however the increase in velocity length suggested the ability to alter phenotypic states once in circulation along specific paths or 'lineages'. COVID-19 neutrophils followed unique maturation paths compared to bacterial ARDS, culminating in three distinct terminal states: Interferon active (IFN^{active}), prostaglandin active (PG^{active}) or bacterial ARDS enriched (Fig 1d-f; Extended Fig 3e-k). Interestingly, the apex of this trajectory was marked by high velocity lengths, characteristic of cells undergoing differentiation (Extended Fig 3c, d). COVID-19 neutrophils preferentially transitioned from the apex of the trajectory, which was an immature state (TOP2A expressing; Extended Fig 3f) to an IFN responsive state characterized by IFITM1, IFITM2 and IFI6 expression (Cluster 1 to 4 and 5; Fig 1g; Extended Fig 3e, g, i, l). This is clearly illustrated in Extended Video 1. This immature state was not present in healthy controls, though it is present in both comparator groups, suggesting these states are liberated into circulation upon pathogen exposure (Extended Fig 4e-f). The lineage relationship was less clear for COVID-19 enriched PG^{active} clusters defined by prostaglandin responsive genes (clusters 2, 6 and 8), with notable increases in PTGER4 and PTGS2 (or COX2), a proposed therapeutic target in COVID-19[14] (Fig 1i; Extended Fig 3e, h, k, l). The dominant conventional bacterial ARDS state was characterized by antibacterial proteins CD83[15] and PLAC8[16] (cluster 3 to 0; Fig 1h; Extended Fig 3e, j, l). Taken together, this data demonstrated that peripheral neutrophils have dynamic programming abilities which result in COVID-19 specific neutrophil polarization defined by the emergence of IFN^{active} and PG^{active} neutrophil states.

Unique transcriptional regulatory pathways drive neutrophil maturation in COVID-19.

Rapid and robust IFN responses protect against COVID-19 severe disease, while delayed responses could exacerbate systemic and pulmonary inflammation[17,18]. Moreover, neutrophil IFN responses are not traditionally considered during infections and neutrophils are generally considered to be homogenous, with a uniform proinflammatory capacity. Global neutrophil expression aligned with neutrophil state specific markers, such as interferon response genes (IFITM1, RSAD2, IFI6, and ISG10), being more highly expressed in COVID-19 neutrophils (Fig 2a; Extended Fig 3i). The inverse was the case for anti-bacterial proteins like PLAC8 (Fig 2a; Extended Fig 3f, g, i). However, the discovery of differential neutrophil states prompted further exploration of the factors driving neutrophil state polarization. Gene regulatory network reconstruction using SCENIC analysis[19] revealed differentially activated transcription factors STAT1, IRF1 and PRDM1 in COVID-19 (Fig 2b), while bacterial ARDS neutrophils had increased prototypical granulocyte transcription factors such as CEBPA, CEBPB, STAT5B and less defined factors such as NFE2 (Fig 2b, Extended Fig 5a). PRDM1 activation was most pronounced in the IFN^{active} neutrophil population and was likely responsible for driving expression of interferon response elements (IFIT1, ISG15, IFI6) and antiviral signaling, such as RSAD2 and STAT1 (Fig 2c; Extended Fig 5b-h). A hallmark of PG^{active} neutrophil polarization was the activation of an E2F4 pathway (Fig 2d), while neutrophil programming during bacterial ARDS included STAT5B (Fig 2e). To summarize, in response to COVID-19, neutrophils were polarized by unique transcriptional regulation towards one of two main populations, either an IFN^{active} population or a PG^{active} population (Fig 2f).

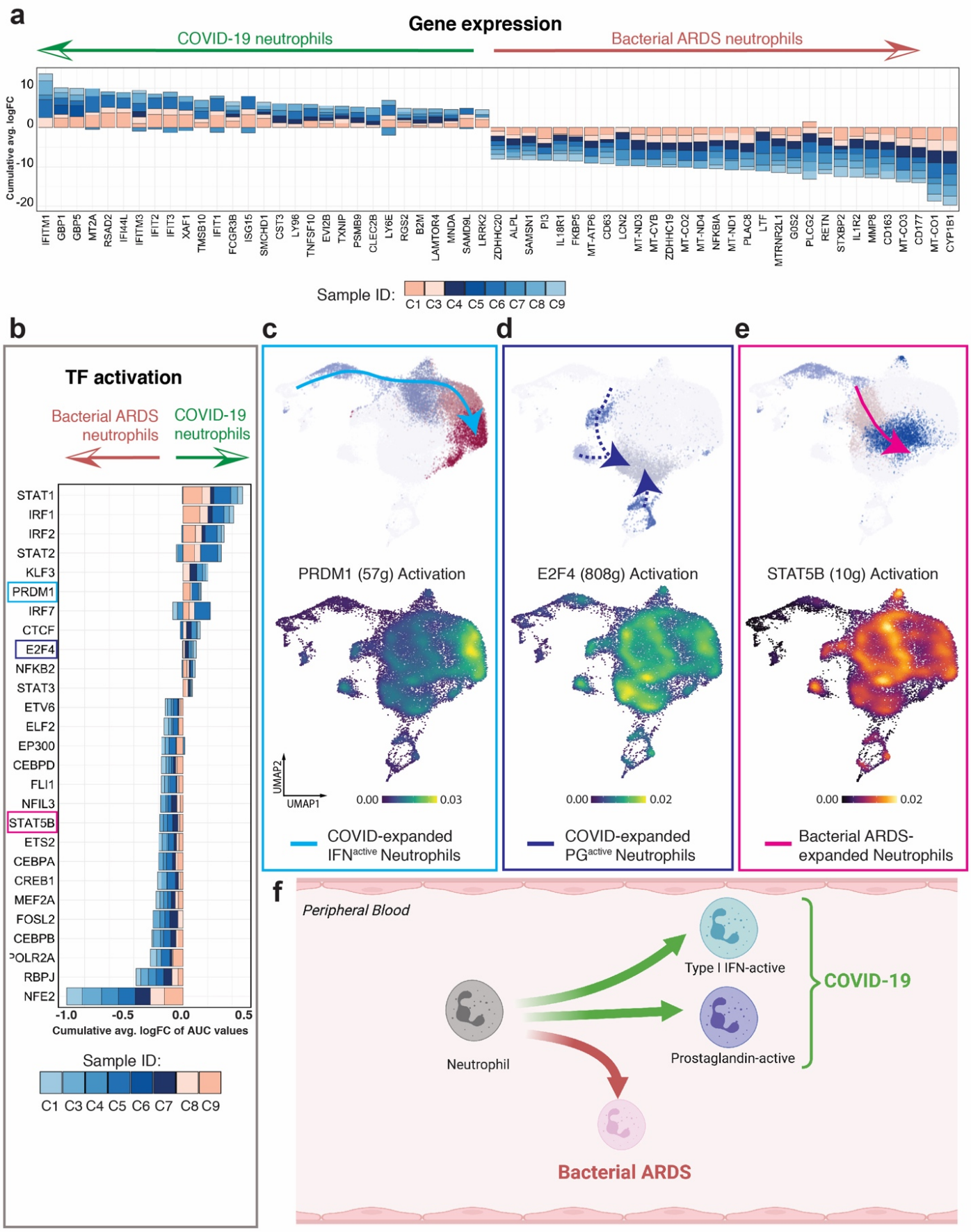
a
Gene expression
COVID-19 neutrophils
Bacterial ARDS neutrophils
Cumulative avg. logFC
10
0
-10
-20
IFITM1 GBP1 GBP5 MT2A RSAD2 IFI44L IFITM3 IFIT2 IFIT3 XAF1 TMSB10 IFIT1 FCGR3B ISG15 SMCHD1 CST3 LY96 TNFSF10 EVI2B TXNIP PSMB9 CLEC2B LY6E RGS2 B2M LAMTOR4 MNDA SAMD9L LRRK2 ZDHHC20 ALPL SAMSN1 PI3 IL18R1 FKBP5 MT-ATP6 CD63 LCN2 MT-ND3 MT-CYB ZDHHC19 MT-CO2 MT-ND4 NFKBIA MT-ND1 PLAC8 LTF MTRNR2L1 G0S2 PLCG2 RETN STXBP2 IL1R2 MMP8 CD163 MT-CO3 CD177 MT-CO1 CYP1B1
Sample ID:
C1 C3 C4 C5 C6 C7 C8 C9
b
TF activation
Bacterial ARDS neutrophils
COVID-19 neutrophils
STAT1
IRF1
IRF2
STAT2
KLF3
PRDM1
IRF7
CTCF
E2F4
NFKB2
STAT3
ETV6
ELF2
EP300
CEBPD
FLI1
NFIL3
STAT5B
ETS2
CEBPA
CREB1
MEF2A
FOSL2
CEBPB
POLR2A
RBPJ
NFE2
-1.0 -0.5 0.0 0.5
Cumulative avg. logFC of AUC values
Sample ID:
C1 C3 C4 C5 C6 C7 C8 C9
c
PRDM1 (57g) Activation
UMAP2
UMAP1
0.00 0.03
COVID-expanded IFNactive Neutrophils
d
E2F4 (808g) Activation
0.00 0.02
COVID-expanded PGactive Neutrophils
e
STAT5B (10g) Activation
0.00 0.02
Bacterial ARDS-expanded Neutrophils
f
Peripheral Blood
Neutrophil
Type I IFN-active
Prostaglandin-active
COVID-19
Bacterial ARDS

Figure 2 – Distinct regulatory programs drive divergent neutrophil maturation. a. Consensus neutrophil DEGs upregulated (positive FC) or suppressed (negative FC) during COVID-19 in at least 3 of 8 patients at t1 relative to Bacterial ARDS. **b.** Differentially activated consensus transcription factors (TFs) in neutrophils from patients with COVID-19 relative to bacterial ARDS at t1. Stacked bars depict logFC contributions of each COVID-19 patient. **c-e.** Gene-regulatory networks preferentially driving IFN^{active} (PRDM1, c), PG^{active} (E2F4, d), and bacterial ARDS-enriched (STAT5B, e) neutrophil states. Scale bars depict kernel density estimates approximating magnitude of TF activation inferred by SCENIC-calculated AUCell scores. **f.** Schematic summarizing neutrophil fates favoured during COVID-19 versus bacterial ARDS.

Dexamethasone therapy alters immune cell transcriptomics.

Conventional therapeutics have limited efficacy for COVID-19, and while dexamethasone offers a moderate benefit, the RECOVERY trial reported the benefit was greatest in the most severely affected patients[20]. However, the mechanisms underlying this benefit are unclear and not universal, so opportunity exists to optimize or better target this therapy. Global differences in transcription were apparent at t1 (within 72 hours of ICU admission) with clear upregulation of genes in neutrophils and some T cell subsets in COVID-19 patients treated with dexamethasone versus those that were not treated (Fig 3a-c, Extended Data Table 3). Dexamethasone globally downregulated genes at t1, including in naïve B cells, plasmablasts and some T cells (Extended Figure 6a-d). At t2 gene upregulation occurred in adaptive immune cells, including naïve and effector CD8 T cells, with limited alterations in the innate myeloid cell lineages including neutrophils. However, neutrophils demonstrated clear down regulation of genes at t2, as did CD4 naïve and central memory T cells (Extended Figure 6c, d). Proportionally, at t1, dexamethasone significantly increased the number of cytotoxic CD4 T cells, naïve B cells, plasmablasts, and decreased proliferating NK cells, and CD4 effector memory cells (Extended Fig 6e). By d7, dexamethasone therapy suppressed the proportions of neutrophils in circulation compared to untreated (13% vs 41%).

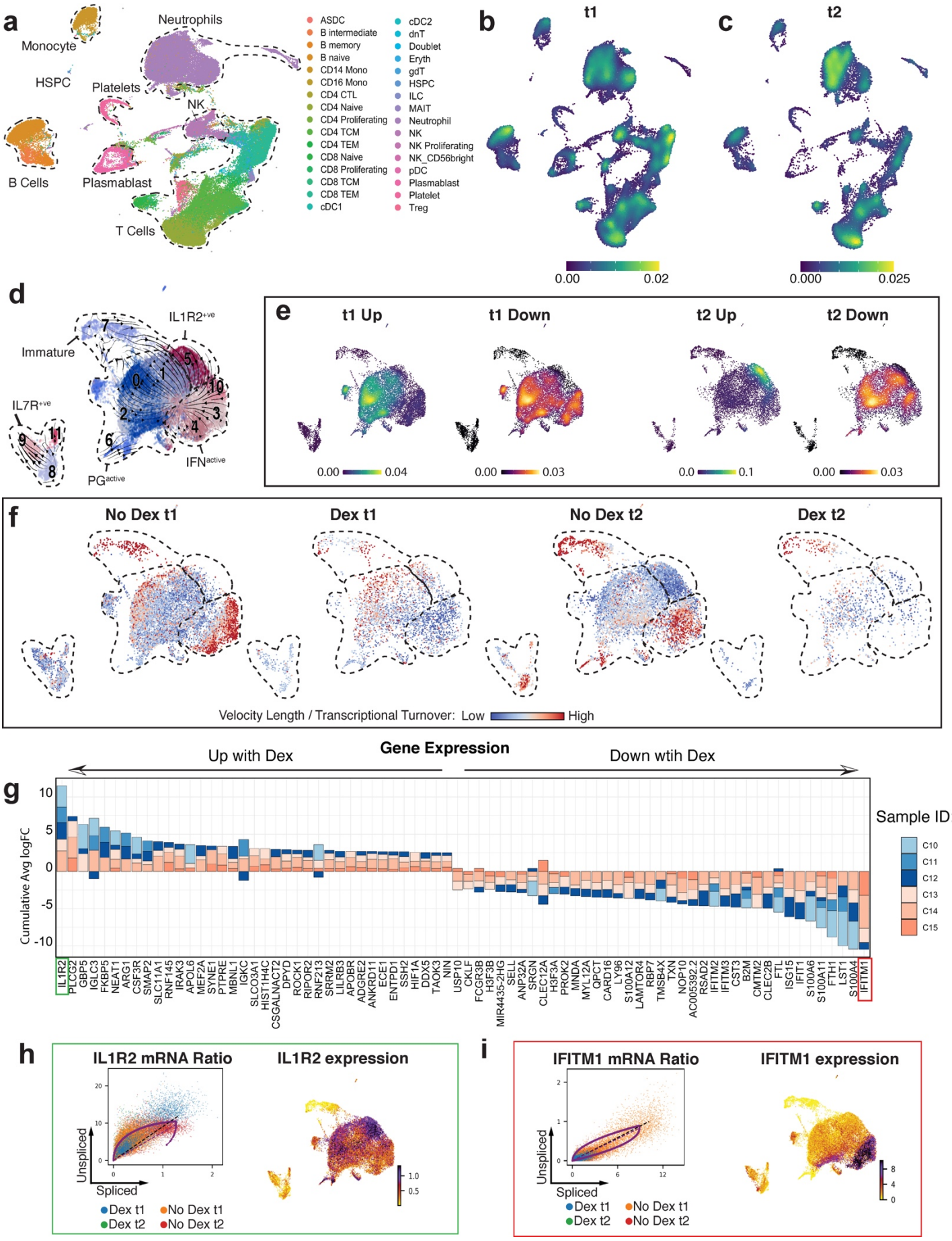
a
Monocyte
Neutrophils
HSPC
Platelets
NK
B Cells
Plasmablast
T Cells
ASDC
B intermediate
B memory
B naive
CD14 Mono
CD16 Mono
CD4 CTL
CD4 Naive
CD4 Proliferating
CD4 TCM
CD4 TEM
CD8 Naive
CD8 Proliferating
CD8 TCM
CD8 TEM
cDC1
cDC2
dnT
Doublet
Eryth
gdT
HSPC
ILC
MAIT
Neutrophil
NK
NK Proliferating
NK_CD56bright
pDC
Plasmablast
Platelet
Treg
b
t1
0.00
0.02
c
t2
0.000
0.025
d
IL1R2+ve
Immature
IL7R+ve
PGactive
IFNactive
e
t1 Up
t1 Down
t2 Up
t2 Down
0.00
0.04
0.00
0.03
0.0
0.1
0.00
0.03
f
No Dex t1
Dex t1
No Dex t2
Dex t2
Velocity Length / Transcriptional Turnover: Low
High
Gene Expression
Up with Dex
Down wtih Dex
g
Cumulative Avg logFC
Sample ID:
C10
C11
C12
C13
C14
C15
IL1R2
PLCG2
GBP5
IGLC3
FKBP5
NEAT1
ARG1
CSF3R
SMAP2
SLC11A1
RNF145
IRAK3
APOL6
MEF2A
SYNE1
PTPRE
MBNL1
IGKC
SLCO3A1
HIST1H4C
CSGALNACT2
DPYD
ROCK1
RIPOR2
RNF213
SRRM2
LILRB3
APOBR
ADGRE2
ANKRD11
ECE1
ENTPD1
SSH2
HIF1A
DDX5
TAOK3
NIN
USP10
CKLF
FCGR3B
H3F3B
MIR4435-2HG
SELL
ANP32A
SRGN
CLEC12A
H3F3A
PROK2
MNDA
MYL12A
QPCT
CARD16
LY96
S100A12
LAMTOR4
RBP7
TMSB4X
TXN
NOP10
AC005392.2
RSAD2
IFITM2
IFITM3
CST3
B2M
CMTM2
CLEC2B
FTL
ISG15
IFIT1
S100A6
S100A11
FTH1
LST1
S100A4
IFITM1
h
IL1R2 mRNA Ratio
IL1R2 expression
Unspliced
Spliced
Dex t1
No Dex t1
Dex t2
No Dex t2
i
IFITM1 mRNA Ratio
IFITM1 expression
Unspliced
Spliced
Dex t1
No Dex t1
Dex t2
No Dex t2

Figure 3 – Dexamethasone suppresses IFN programs and depletes IFN^{active} neutrophils in COVID-19. a. UMAP projection of 80,994 whole blood cells from 21 patient samples, coloured by Azimuth reference-mapped immune cell states. **b-c.** Kernel density estimates depicting magnitude of molecular response elicited by immune cell subsets following Dexamethasone treatment t1 (b) and t2 (c) calculated by summing DEG fold changes for each cell state shown in Panel A. **d.** Neutrophil states overlaid on a UMAP of 23,193 subclustered neutrophils from Dexamethasone- and non-Dexamethasone-treated COVID-19 patients, colored by cluster ID. **e.** Magnitude of molecular response elicited by each neutrophil state post-Dexamethasone treatment calculated by summing DEG fold changes for each cell state shown in Panel d. **f.** RNA velocity vector length (indicating rate of differentiation/state transition) in Dexamethasone- and non-Dexamethasone-treated neutrophils at t1 and t2. **g.** Consensus neutrophil DEGs upregulated (positive FC) or suppressed (negative FC) post-Dexamethasone in at least 3 of 6 COVID-19 patients at t1 relative to non-Dexamethasone COVID-19 controls. Stacked bars depict logFC contribution of each Dexamethasone-treated patient. **h-i.** Differential splicing kinetics drives activation of IL1R2 (h) and suppression of IFITM1 expression (i) post-Dexamethasone treatment. Phase portraits show equilibrium slopes of spliced–unspliced mRNA ratios. Green denotes most upregulated and red denotes most down regulated differentially expressed genes with COVID-19 (g-i).

Dexamethasone therapy restrains neutrophil IFN programs

Due to the early and sustained effects of dexamethasone on gene expression in neutrophils, the effects of dexamethasone therapy on neutrophil functional states were evaluated. Neutrophil reclustering again identified immature neutrophils at the apex of the maturation trajectory, accelerating and exhibiting maximal divergence prior to PG^{active} and IFN^{active} state commitments (Fig. 3 d, Extended Fig 7a-d). Interestingly, we also identified IL7R^{+ve} neutrophils (comprising roughly 8% of total neutrophils) whose trajectories remained completely separate (Fig. 3 d, Extended Fig 7h) suggesting an entirely distinct neutrophil state. Initially, dexamethasone therapy increased global transcription in PG^{active} neutrophils, while ongoing therapy resulted in the emergence of a PG^{active} neutrophils concomitant with high IL1R2 expression (IL1R2^{+ve}) (Fig. 3 e). Conversely, dexamethasone had a pronounced attenuation of global transcription of IFN^{active} neutrophils at t1 and t2 (Fig 3 d, e). Remarkably, dexamethasone administration at t1 halted dynamic state changes in IFN^{active} and IL7R^{+ve} neutrophils, followed by preferential depletion of IFN^{active} subsets (Fig 3 f). In the context of healthy controls, dexamethasone reduced IFN^{active} neutrophil proportions closer to that seen in healthy controls (Extended Fig 4).

Consensus DEG analysis highlighted that upregulation of IL1R2, a decoy receptor that sequesters IL-1, and downregulation of IFITM1 were the most prominent discriminating features of treatment with or

without steroids (Fig. 3g). Additionally, dexamethasone attenuated neutrophil expression of IFN pathways more broadly, highlighted by decreases in ISGs including IFITM1-3, IFIT1, ISG15 and RSAD2 (Fig 3g). Examination of unspliced pre-mRNA to mature spliced mRNA ratios supported the notion that induction of immunoregulatory systems (i.e., IL-1R2; Fig 3 h) and suppression of IFN (i.e., IFITM1; Fig 3 i) programs were driven by differential splicing kinetics.

Dexamethasone therapy intensifies neutrophil immunosuppressive function

Cortiocsteroid therapy shifted neutrophil state compositions. While IFN^{active} neutrophils were significantly depleted by seven days of therapy, there was >2-fold expansion in immature neutrophils relative to non-treated COVID-19 controls (Fig 4a), which were absent in the healthy controls. Albeit anecdotal, the dominance of IFN^{active} neutrophils at t1 in the patient who succumbed to COVID-19 in the non-dexamethasone cohort further supports depletion of IFN^{active} neutrophils as a mechanism by which dexamethasone is protective (Extended Fig 9 a-d). Assessment of gene regulatory networks demonstrated that IRF7 and MEF2A exhibited opposing activation patterns, with IRF7 being the most suppressed and MEF2A the most enhanced transcription factors identified with dexamethasone, which correlates with the emergence of PG^{active} and $IL1R2^{+ve}$ states and attenuation of the IFN^{active} neutrophil states (Fig 4b, Extended Fig 7e-k). Unexpectedly, steroids induced an increase in circulating immature neutrophils, which highly expressed TOP2A, and activated ATF4 and JDP2, transcription factors signatures seen in undifferentiated cells or those undergoing nuclear reprogramming (Extended Fig 7f, g). Interestingly, these immature neutrophils expressed high levels of ARG1, ANXA1 (Fig 4c), suggesting an immunomodulatory role[22,23,24-26] that was increased with dexamethasone treatment. Both ARG1 and ANXA1 express glucocorticoid response elements, supporting direct regulation post dexamethasone treatment[21,27].

To further understand the role of neutrophils during COVID-19 and the effects of dexamethasone, we investigated cellular connectomes. Cellular interactions between many cell types (including highly interactive neutrophils) were noted (Extended Fig 8a), and dexamethasone altered the globally predicted

interactions by suppressing intercellular signalling, in both number and strength of interactions (Extended Fig 7b, c). However, dexamethasone enhanced neutrophil specific signalling interactions (Fig 4d). Annexin family signalling, which was enhanced in the immature neutrophils and represent powerful immunomodulators, were augmented between neutrophils and the other circulating immune cells when patients received dexamethasone (Fig 4d). Of note is the direction of annexin family signaling, which switched from incoming toward neutrophils without dexamethasone treatment to being almost entirely outgoing from neutrophils toward B intermediate and memory cells and MAIT cells following dexamethasone (Fig 4e, f). Therefore, dexamethasone directly altered neutrophil functional states, by promoting expansion of an ARG1+/ANXA1+ immature state with immunosuppressive features and altered the global communication structure such that neutrophils became active instructors of some peripheral immune cells.

Neutrophil response to dexamethasone is sexually dimorphic

Given the apparent clinical benefit of dexamethasone is more evident in males[20], and since males are predisposed towards more severe COVID-19 presentations and outcomes[28] , we surmised that dexamethasone exhibits sexually dimorphic immunosuppressive effects. Interestingly, while neutrophils were depleted in both sexes post-dexamethasone, this was particularly pronounced in males (1.9 FC higher in males at t1 and 3.4 FC higher in males at t2, Extended Fig 9e). Of the two salient neutrophil state alterations, $ARG1^{+ve}$ immunosuppressive states were preferentially expanded in males (Extended Fig. 9e) whereas ISGs were preferentially suppressed (Extended Fig. 9f) and IFN^{active} states preferentially depleted in females (Extended Fig. 9g-h) at both t1 and t2. Sexually dimorphic effects of dexamethasone on neutrophil maturation kinetics in part explains these state alterations. Dynamo-reconstructed vector dynamics revealed that dexamethasone slowed IFN^{active} transitions (Extended Fig. 9i) whilst hastening immature ($ARG1^{+ve}$ immunosuppressive) neutrophil differentiation in females (Extended Fig. 9j) leading to a depleted immature neutrophil progenitor pool.

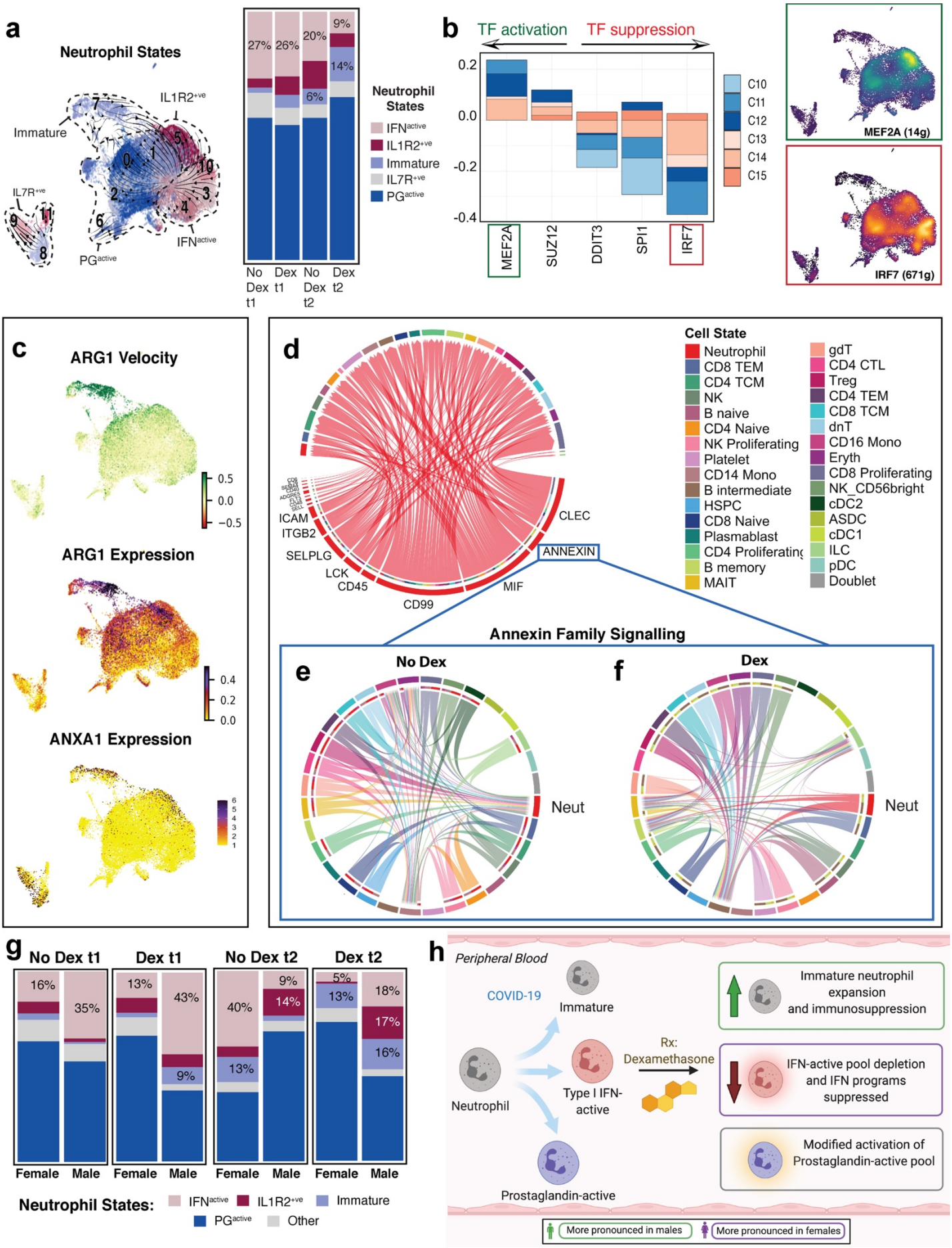
a
Neutrophil States
Immature
IL1R2+ve
IL7R+ve
PGactive
IFNactive
27% 26% 20% 9%
14%
6%
No Dex t1
Dex t1
No Dex t2
Dex t2
Neutrophil States
IFNactive
IL1R2+ve
Immature
IL7R+ve
PGactive
b
TF activation
TF suppression
0.2
0.0
-0.2
-0.4
MEF2A
SUZ12
DDIT3
SPI1
IRF7
C10
C11
C12
C13
C14
C15
MEF2A (14g)
IRF7 (671g)
c
ARG1 Velocity
0.5
0.0
−0.5
ARG1 Expression
0.4
0.2
0.0
ANXA1 Expression
d
Cell State
Neutrophil
CD8 TEM
CD4 TCM
NK
B naive
CD4 Naive
NK Proliferating
Platelet
CD14 Mono
B intermediate
HSPC
CD8 Naive
Plasmablast
CD4 Proliferating
B memory
MAIT
gdT
CD4 CTL
Treg
CD4 TEM
CD8 TCM
dnT
CD16 Mono
Eryth
CD8 Proliferating
NK_CD56bright
cDC2
ASDC
cDC1
ILC
pDC
Doublet
ICAM
ITGB2
SELPLG
LCK
CD45
CD99
MIF
ANNEXIN
CLEC
Annexin Family Signalling
e
No Dex
Neut
f
Dex
Neut
g
No Dex t1
Dex t1
No Dex t2
Dex t2
16% 35%
13% 43% 9%
40% 9% 14% 13%
5% 13% 18% 17% 16%
Female Male Female Male Female Male Female Male
Neutrophil States:
IFNactive
IL1R2+ve
Immature
PGactive
Other
h
Peripheral Blood
COVID-19
Immature
Neutrophil
Type I IFN-active
Prostaglandin-active
Rx:
Dexamethasone
Immature neutrophil expansion and immunosuppression
IFN-active pool depletion and IFN programs suppressed
Modified activation of Prostaglandin-active pool
More pronounced in males
More pronounced in females

Figure 4 – Dexamethasone expands immunosuppressive neutrophils and their interactions in COVID-19. a. Neutrophil states mapped onto Louvain-clustered UMAP, with comparison of neutrophil composition between Dexamethasone- and non-Dexamethasone-treated samples at t1 and t2. **b.** Differentially activated consensus TFs upregulated (positive logFC) or suppressed (negative logFC) post-Dexamethasone in at least 3 of 6 patients at t1. Green denotes most upregulated and red denotes most down regulated transcription factors with COVID-19. **c-d.** Predicted activity of MEF2A (c) and IRF7 (d), two of the most differentially regulated TFs post-dexamethasone. **c.** Immature and IL1R2^{+ve} neutrophil subsets express high levels of immunosuppressive neutrophil marker ARG1 and ANXA1. **d.** Neutrophil-driven signaling pathways induced post-Dexamethasone, identified using CellChat (MHC-I signalling filtered out). **e**, **f**. Topology of annexin signalling family without (e) and with dexamethasone (f) treatment (neutrophils are highlighted in red). **g.** Neutrophil state composition separated by sex and dexamethasone status at t1 and t2. **h.** Schematic summarizing the effects of dexamethasone on neutrophil fates and function in COVID-19 following dexamethasone treatment.

Conclusions

Surviving SARS-CoV-2 infection depends on striking a temporal balance between inciting viral clearance immune programs during the early stage and subsequently restraining those same programs at later stages to limit immunity-induced tissue damage. IFN signaling stands at the nexus between antiviral immunity and over active effector immune programs that inadvertently compromise tissue function and threaten survival[29]. Our work uncovered downstream IFN signalling as a signature of a stable neutrophil state that is selectively expanded during late stage COVID-19 infection from a common pool of immature progenitors. Given that inborn errors [18] and suppressed *early stage* [6] IFN signalling predicts COVID-19 severity, increased IFN^{active} neutrophils in females correlated with decreased mortality[30], and early initiation of IFN therapy has been suggested to mitigate disease severity [31,32], one may posit that IFN activity in neutrophils represents a concerted host antiviral program.

Interestingly, immunosuppression with dexamethasone, a corticosteroid known to improve mortality in hospitalized COVID-19 patients[20], suppressed COVID19-specific IFN regulatory networks and depleted COVID19-enriched IFN^{active} neutrophils in favour of expanding ARG1^{+} immunosuppressive neutrophils. These altered neutrophil states shared striking resemblances to bacterial ARDS, suggesting installation of generalized microbicidal programs ameliorate the overzealous neutrophil responses during COVID-19 (and perhaps during other viral infections). While neutrophil ISG activation may promote anti-viral

immunity during early stages of SARS-CoV-2 infection, sustained IFN activation during *late stages* (e.g., critically ill patients requiring intensive care) could drive immunopathology of COVID-19. Immunotherapies that support the innate antiviral immune response by decoupling IFN-exaggerated neutrophil response whilst reinforcing acquisition of suppressor states may limit the pathogenic potential of neutrophils and provide tremendous clinical benefit for treating severe COVID-19.

Methods

Patient enrolment. All patients were enrolled following admission to any of the four adult intensive care units at South Health Campus, Rockyview General Hospital, Foothills Medical Center or Peter Lougheed Center in Calgary, Alberta, Canada. Patient admission to the ICU was determined by the attending ICU physician based on the need for life sustaining interventions, monitoring and life-support. The research teams did not participate in clinical decisions. Study inclusion required a minimal age of 18, the ability to provide consent, or for most participants, the ability of a surrogate decision maker to provide regained capacity consent. All participants required an arterial catheter for blood draws, but the insertion of this catheter was at the discretion of the attending medical team. Participants required a positive clinical RNA COVID-19 test prior to enrolment and evidence of bilateral lung infiltrates and hypoxemia consistent with ARDS. The bacterial ARDS cohort required a negative COVID-19 test and a definitive microbiological diagnosis of bacterial pneumonia with chest imaging consistent with a diagnosis of ARDS. Patients were excluded if they were on immunosuppressive therapies as an outpatient, if they had established autoimmune disease, or active malignancy. Participants were required to have a definitive diagnosis and appropriate consent and samples collected within 72hrs of admission to the ICU in order to be included. Timepoint 1 (T1) refers to the first blood draw, while T2 was a repeat blood draw taken 7 days after T1, if the participant remained in the ICU, and had an arterial catheter. For each participant, whole blood was collected via the arterial catheter and immediately processed for analysis. This study was approved by the Conjoint Health Research Ethics Board (CHREB) at the University of Calgary (Ethics ID: REB20-0481) and is consistent with and the Declaration of Helsinki.

Serum cytokine assessment. Cytokines, chemokines and soluble cytokine receptors were quantitated on multiplex arrays that included a 65 MIlliPLEX cytokine/chemokine (6Ckine, BCA-1, CTACK, EGF, ENA-78, Eotaxin, Eotaxin-2, Eotaxin-3, FGF-2, Flt-3L, Fractalkine, G-CSF, GM-CSF, GRO, I-309, IFNα2, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-16, IL-17A, IL-18, IL-20, IL-21, IL-23, IL-28a, IL-33, IP-10, LIF, MCP-1, MCP-2, MCP-3, MCP-4, MDC, MIP-1α, MIP-1β, MIP-1d, PDGF-AA, PDGF-AB/BB, RANTES, SDF-1 a+b, sCD40L, SCF,TARC, TGFa, TNFa, TNFb, TPO, TRAIL, TSLP, VEGF) and a 14 MilliPLEX soluble cytokine (sCD30, sEGFR, sgp130, sIL-1RI, sIL-1RII, sIL-2Ra, sIL-4R, sIL-6R, sRAGE, sTNF RI, sTNF RII, sVEGF R1, sVEGF R2, sVEGF R3) arrays (Millipore Sigma, Oakville, ON, Canada) on a Luminex Model 200 Luminometer (Luminex Corporation, Austin, TX). EDTA-plasma samples were collected from each patient by venipuncture following a standard operating protocol (SOP) and stored at -80C until tested. Each run included a full range of calibrators.

Leukocyte and lymphocyte isolation. For lymphocyte isolation, whole blood (2mL) was collected in 5mL polystyrene round-bottom heparinized vacutubes. To isolate lymphocytes by immunomagnetic negative selection, 100μL of Isolation Cocktail and 100μL of Rapid Spheres (EasySep™ Direct Human Total Lymphocytes Isolation Kit: 19655, StemCell Technologies) were added to 2 mL of whole blood. After mixing and 5min incubation at RT, the sample volumes were topped up to 2.5mL with 0.04% bovine serum albumin (BSA) in PBS. The diluted sample was incubated in the magnet without lid for 5min, at RT and negatively selected lymphocytes were decanted into a new 5 mL polystyrene tube. Except the addition of Isolation Cocktail, all steps were repeated once. The final lymphocyte cell suspension was transferred to a 15 mL polypropylene tube and a volume of 5mL 0.04% BSA in PBS was added to the sample. Lymphocytes were precipitated by centrifugation for 5 min at 2000rpm, supernatant was discarded, and cells were resuspended in 5 mL of 0.04% BSA in PBS. This last step was repeated once, and cells were then resuspended in 100 μL of PBS+0.04% BSA. Cell density was quantified with a

hemacytometer, cell viability was assessed with Trypan Blue staining (T8154; Sigma Aldrich), and 7500 live lymphocytes were transferred to a sterile 1.5 mL microcentrifuge tube.

For leukocyte isolation, 1 mL of whole blood from heparin containing vacutubes was transferred to 5 mL polystyrene round-bottom tubes and 12µL of 0.5M EDTA was added. 2% FBS in PBS (1mL) and 50µL of EasySep RBC Depletion spheres (EasySep™ RBC Depletion Reagent: 18170, Stem Cell Technologies) were added to immunomagnetically deplete red blood cells. After 5 min of magnet incubation at RT, cell suspension containing leukocytes was decanted into a new 5mL polystyrene tube. To ensure complete removal of red blood cells, RBC depletion was repeated, and cell suspension containing leukocytes was decanted into a new 15mL polypropylene tube. Leukocytes were precipitated by centrifugation at 2000rpm for 5 min at 20ºC and resuspended in 5mL of 0.04% BSA in PBS. This last step was repeated once, and leukocytes were resuspended in 2 mL of 0.04% BSA in PBS. Cell viability and cell density were assessed, and 7500 live leukocytes were transferred to the microcentrifuge tube containing the lymphocyte cell suspension. The volume of the cell suspension containing 7500 lymphocytes and 7500 leukocytes in a total of 50 µL of 0.04% BSA in PBS.

Single-cell RNA-Seq library construction, alignment, and quality control. A total of 15,000 single cells (containing an equal proportion of leukocytes and lymphocytes) were loaded for partitioning using 10X Genomics NextGEM Gel Bead emulsions (Version 3.1). All samples were processed as per manufacturer's protocol (with both PCR amplification steps run 12X). Quality control of resulting libararies and quantification was performed using TapeStation D1000 ScreenTape assay (Agilent). Sequencing was performed using Illumina NovaSeq S2 and SP 100 cycle dual lane flow cells over multiple rounds to ensure each sample received approximately 32,000 reads per cell. Sequencing reads were aligned using CellRanger 3.1.0 pipeline[33] to the standard pre-built GRCh38 reference genome. Samples that passed alignment QC were aggregated into single datasets using CellRanger aggr with between-sample normalization to ensure each sample received an equal number of mapped reads per cell. Aggregated non-dexamethasone-treated COVID-19 (n = 12) and bacterial ARDS (n = 9) samples

recovered 1,872,659 cells that were sequenced to 38,410 post-normalization reads per cell. Likewise, aggregated COVID-19 samples with (n = 9) or without (n = 12) dexamethasone recovered 1,748,551 single cells sequenced to 51,415 post-normalization reads per cell. Aggregated healthy samples recovered 19,816 cells, including 1,912 post-QC neutrophils (n = 5).

Single-cell RNA-Seq computational analyses and workflows. Filtered feature-barcode HDF5 matrices from aggregated datasets were imported into the R package Seurat v.3.9 for normalization, scaling, integration, multi-modal reference mapping, louvain clustering, dimensionality reduction, differential expression analysis, and visualization [34]. Briefly, cells with abnormal transcriptional complexity (fewer than 500 UMIs, greater than 25,000 UMIs, or greater than 25% of mitochondrial reads) were considered artifacts and were removed from subsequent analysis. Since granulocytes have relatively low RNA content (due to high levels of RNases), QC thresholds were informed by [35] as they recently defined several rodent and human neutrophil subsets from scRNA-Seq samples. Cell identity was classified by mapping single cell profiles to the recently published PBMC single-cell joint RNA/CITE-Seq multi-omic reference [36]. Since no published reference automates granulocyte annotations, neutrophil clusters were manually annotated by querying known markers (i.e. CSF3R, S100A8, S100A9, MMP8, MMP9, ELANE, MPO) [37] and were corroborated using the R package SingleR [38]. To test whether cell composition was changed due to infection type (COVID-19 versus Bacterial ARDS) or treatment group (dexamethasone versus non-dexamethasone), a generalized linear mixed-effects model was employed where infection type and treatment group were considered fixed and individual patients were considered random effect. Fitting was done with Laplace approximation using the 'glmer' function in the 'lme4' R package [39] and p-values were calculated using the R package 'car'. Boxplots comparing cell type composition were generated using the ggplot2 package.

Differential cell-cell interaction networks were reconstructed using the Connectome R toolkit v0.2.2[40] and CellChat v1.0.0 [41]. Briefly, *DifferentialConnectome* queried Seurat R objects housing datasets integrated by infection type and dexamethasone status to define nodes and edges for downstream network analysis.

Total number of interactions and interaction strengths were calculated using CellChat's *compareInteractions* function. Differential edge list was passed through *CircosDiff* (a wrapper around the R package 'circlize') and CellChat's *netVisual_chord_gene* to filter receptor-ligand edges and generate Circos plots.

Consensus DEGs and perturbation scores. Differentially expressed genes (DEGs) were those with an average log fold change (FC) greater than 0.25 (p-adjusted < 0.05) as determined by Seurat's Wilcoxon rank-sum test. Consensus stacked bars showing cumulative log fold changes (colored by individual sample contributions) were generated using *constructConsensus* function [42] for genes exhibiting reproducible changes across patients (>3 for 72-hour comparisons, > 2 for 7-day comparisons). Gene Set Enrichment analyses of consensus DEGs were performed using gProfiler's g:GOSt (p-value cutoff <0.05). A cell state-specific 'perturbation score' was calculated to reflect the magnitude of response elicited by factoring in number and cumulative FC of consensus DEGs. Perturbation scores were visualized using Nebulosa-generated density plots [43].

Constructing cellular trajectories using RNA velocity. Analysis of neutrophil trajectories was performed by realigning CellRanger count-generated BAMs with RNA velocity command-line tool [13] using the *run10x* command and human (GRCh38) annotations. The output loom files containing spliced and unspliced counts were combined to compare neutrophils in COVID-19 with Bacterial ARDS controls and dexamethasone-treated with non-treated COVID-19 patients. For both analyses, combined looms were imported into Seurat v.3.9 using the *ReadVelocity* function in SeuratWrappers v.0.2.0, normalized using *SCTransform* v.0.3.2 [44], reduced and projected onto a UMAP, and exported as a .h5 file using the *SaveH5Seurat* function. Counts stored in H5 files were imported, filtered, and normalized as recommended in the scVelo v.0.2.1 workflow [12]. RNA velocities were estimated using stochastic and dynamical models. Since both models yielded comparable results, stochastic model was used as default for all subsequent analyses. Calculations stored in AnnData's metadata were exported as CSVs and kernel density lines depicting Velocity-inferred latent time distribution were plotted with ggplot2 v.3.1.1.

Gene Regulatory Network reconstruction. Single-cell regulatory network inference and clustering (SCENIC)[19] was employed to infer regulatory interactions between transcription factors (TFs) and their targetome by calculating and pruning co-expression modules. Briefly, neutrophils were subsetted from scVelo-realigned Seurat object and processed using default and recommended parameters specified in SCENIC's vignette (https://github.com/aertslab/SCENIC) using the hg19 RcisTarget reference. Regulon activity scores (in '3.4_regulonAUC.Rds', an output of the SCENIC workflow) were added to scVelo object (using *CreateAssayObject* function) to jointly project trajectory and TF activity onto the same UMAP embeddings. Consensus stacked bars showing cumulative logFC of AUCell scores for each TF (colored by individual sample contributions) were generated by modifying the *constructConsensus* function[42] for SCENIC assay. Targetome of TFs predicted as drivers of neutrophil states (stored in '2.6_regulons_asGeneSet.Rds') was profiled using g:Profiler's functional enrichment analysis and genes intersecting with the Interferon pathway were plotted using iRegulon (Cytoscape plugin)[45].

COVID Neutrophil Atlas. To enable intuitive exploration of single-cell datasets, a web portal (http://biernaskielab.ca/covid_neutrophil or http://biernaskielab.com/covid_neutrophil) was built using RShiny v1.1.0, shinyLP v.1.1.2, and shinythemes v.1.1.2 packages.

Data availability. Single cell RNA-Seq datasets and Velocyto-generated LOOM files can be provided upon request. Single-cell datasets can be further explored on our companion portal at http://biernaskielab.ca/COVID_neutrophil or http://biernaskielab.com/COVID_neutrophil.

Supplementary Information is available for this paper.

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