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Singular insights into the immunology of SARS-CoV-2 infection

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Abstract

Gaining a better understanding of the immune cell subsets and molecular factors associated with protective or pathological immunity against SARS-CoV-2 could aid the development of vaccines and therapeutics for coronavirus disease (COVID-19). Single-cell technologies, such as flow cytometry, mass cytometry, single-cell transcriptomics and single-cell multiomic profiling, offer considerable promise in dissecting the heterogeneity of immune responses among individual cells and uncovering the molecular mechanisms of COVID-19 pathogenesis. Particularly noteworthy single-cell immune profiling studies have identified innate and adaptive immune cell subsets that correlate with COVID-19 disease severity, as well as immunological factors/pathways of potential relevance to the development of vaccines and treatments for COVID-19. To facilitate integrative studies and meta-analyses, we provide to the scientific research community in download-ready, standardized form 21 published single-cell sequencing datasets (over 3.2 million cells in total), as well as an interactive visualization portal for data exploration.

High-throughput single-cell technologies such as flow cytometry and mass cytometry, which can measure features on millions of individual cells, and high-dimensional single-cell technologies such as single-cell RNA sequencing (scRNA-seq), which can measure potentially thousands of features in individual cells, are well-suited to support studies for the heterogeneity of immune responses and of how immune cells interact with other host cells and with pathogens. Specific applications of single-cell technologies in the field of immunology include identifying host immunological correlates of disease severity (potentially aiding the design of effective vaccines and therapeutics, as well as allowing for the monitoring of each person's response to those approaches), elucidating molecular

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Author contributions

Y.T., E.W.N., and R.G. conceived the project. Y.T. and H.E.R.M. collected, processed, and standardized the single-cell transcriptomics datasets. M.A.Z. built the visualization portal. Y.T., L.N.C., E.W.N., and R.G. wrote the manuscript. E.W.N. and R.G. supervised the project.

Competing interests

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mechanisms of disease and enabling the identification of predictive biomarkers of disease outcome.

The ongoing COVID-19 pandemic has been described as "an explosive pandemic of historic proportions"[1], with over 220 million confirmed cases and over 4.5 million confirmed deaths worldwide so far[2]. In addition to basic measures such as physical distancing and mask wearing, optimal management of the pandemic may involve a diverse armamentarium of scientific tools including effective and safe preventative vaccines[3], early therapeutic interventions that can blunt progression to severe disease[4,5], and anti-inflammatory treatments to counteract the harmful 'cytokine storm' in patients with severe disease[6–8]. Towards the development of these tools, there is an urgent need to understand severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) interactions with host cells and the host immune response[9].

Here we provide an overview of single-cell technologies that have been applied to COVID-19 studies and list the pertinent experimental aspects of each study (e.g., sample size, technology platform, and patient characteristics). We describe our efforts to organize and curate available single-cell sequencing datasets into an easily downloadable format, providing information on how to access these datasets. We also review key insights obtained from single-cell immune profiling and discuss opportunities and challenges of integrative analysis of publicly available datasets.

Single-cell technologies and available datasets

Single-cell technologies that have been used in COVID-19 studies to date are summarized in Table 1. They include 62 published and 2 preprint articles describing studies that applied one or more single-cell technologies in the context of COVID-19. Figure 1 displays the sample size and dimensionality of the studies; Supplementary Table 1 presents relevant experimental details and locations of publicly available data sets (raw fcs files are publicly available for 6 flow cytometry and 3 mass cytometry datasets, and 24 single-cell sequencing datasets are publicly available). Whereas flow cytometry is the most commonly used technology in these studies, scRNA-seq and single-cell multiomic profiling are also increasingly being used. The flow/mass cytometry studies analyzed data from up to ~300 individuals and up to 62 markers with one or multiple panels; 25 out of the 54 datasets include longitudinal data (Fig. 1a). Most single-cell sequencing studies analyzed >50,000 cells from fewer than 150 individuals; only a few of them included longitudinal data (Fig. 1b).

In the following text, we briefly summarize key conclusions from the 64 studies shown in Figure 1, focusing on the insights obtained via single-cell technologies, including studies still at the preprint stage or that have relatively small sample sizes. As the vast majority of studies have been performed in peripheral blood mononuclear cells (PBMCs), only limited conclusions can be drawn about the respiratory tract – the primary site of infection. Most studies focused on transcriptional (as opposed to protein or epigenetic) readouts. In the sections below, we summarize these finding in the context of innate immune cells, B cells

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and T cells, finally summarizing how these single-cell data may correlate with immune protection.

Innate immune responses

Most flow cytometry-based studies of COVID-19 reported to date that analyzed PBMCs from patients with COVID-19 report reduced frequencies or abundances of circulating basophils[10,11], monocytes[12–14] (especially CD14^{low}CD16^{high} non-classical monocytes[15]), dendritic cells (DCs)[10,13,14,16], and NK cells[13,14,16–18] when compared with healthy donors, with greater reductions in individuals with severe than with mild COVID-19[14–18]. Conversely, patients with COVID-19 have shown increased frequencies or abundances of circulating neutrophils, eosinophils, and monocytic myeloid-derived suppressive cells (M-MDSCs) compared with healthy donors, with greater increases in individuals with severe than with mild COVID-19[11,13,15,16,18]. The neutrophil-to-lymphocyte ratio has also been reported to be associated with severe COVID-19[16].

NK cells.

High-dimensional flow cytometry has enabled in-depth characterization of immune cell subsets. A report featuring a 28-color NK-cell-oriented panel described fewer circulating (yet more highly activated and proliferating) NK cells in COVID-19 patients compared to healthy controls[17]. Worse clinical outcomes were also correlated with increased levels of circulating adaptive NK cells (NKG2C$^+$CD57$^+$CD56^{dim}) and higher levels of perforin expression in CD56^{bright} NK cells[17], implicating adaptive and activated NK cells in COVID pathogenesis.

The above study is of particular notice because it incorporated an analysis of publicly available scRNA-seq data (NK cells in bronchoalveolar lavage (BAL) fluid from patients with COVID-19[19]). The data reveal high activation of NK cells in COVID-19 and corroborate results from flow cytometry[17].

Neutrophils.

Neutrophil extracellular traps (NETs) have been implicated in severe COVID-19[20,21]. A ‘developing neutrophil’ subpopulation, specifically increased in patients with acute respiratory distress syndrome, was identified through scRNA-seq and featured expression of neutrophil granule-related genes and a lack of expression of canonical neutrophil markers[14].

Another study featuring scRNA-seq, high-dimensional flow cytometry, and mass cytometry reported that severe COVID-19 was associated with a substantial increase in circulating immature neutrophils and the presence of a neutrophil cluster characterized by upregulated *S100A8* and *S100A9* (calprotectin) among other genes[15].

Dendritic cells.

A study by Arunachalam et al.[22] is notable due to its relatively large sample size (n=52 COVID-19 patients and n=62 healthy controls) and its use of a phospho-CyTOF panel to immunophenotype PBMCs in patients with COVID-19 along with CITE-seq to profile gene

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and protein expression in dendritic cell (DC)-enriched PBMC samples from patients with COVID-19.

One major conclusion from this study included a decrease in the frequency of plasmacytoid DCs (pDCs) in patients with COVID-19. Another key finding was reduced expression of mammalian target of rapamycin (mTOR) signaling proteins in these pDCs, suggesting that pDCs may have deficient interferon (IFN)-α signaling in patients with COVID-19.

Monocytes.

In another important work also distinguished by a relatively large sample size (n=53 COVID-19 patients, n=8 patients with flu-like illness, and n=48 controls, over two independent cohorts), researchers used single-cell transcriptomic (scRNA-seq) and proteomic (CyTOF) interrogation[23] to test if distinct innate immune responses are associated with the clinical course of COVID-19. The study revealed that, compared with healthy controls, patients with mild COVID-19 had increased levels of inflammatory huma leukocyte antigen (HLA)-$DR^{hi}CD11c^{hi}CD14^{+}$ monocytes. Conversely, patients with severe COVID-19 were distinguished not only by monocyte populations with low expression of HLA-DR (indicative of monocyte dysfunction[24]) and enhanced expression of genes related to anti-inflammatory macrophage functions (e.g. *CD62L*, *CD163*), but also an abundance of immature neutrophils (including pro- and pre-neutrophils) expressing markers indicative of immunosuppression and/or dysfunction. These findings identify a dysfunctional monocyte response as well as dysregulated myelopoiesis as potentially important processes underlying the development of severe disease.

As discussed below, immune-cell phenotyping via single-cell technologies has yielded potential insights into the immune pathways that may be dysregulated in severe COVID-19 (i.e., COVID-19-associated cytokine storm[25]) and in multisystem inflammatory syndrome (MIS-C) associated with SARS-CoV-2 infection. These insights could in turn inform ongoing and future development of anti-inflammatory treatment strategies of COVID-19 by targeting immunological factors with therapeutic potential. Evidence that such anti-inflammatory treatment strategies may be effective is provided by the encouraging preliminary results of the RECOVERY trial of dexamethasone in patients who were hospitalized with COVID-19[26] (which resulted in ongoing corticosteroid trials[27–29] to stop early based on the data and safety monitoring board (DSMB) recommendations), along with the results of meta-analyses of randomized clinical trials of corticosteroids in patients with severe COVID-19[30,31]. Moreover, flow cytometry, mass cytometry, and scRNA-seq have all demonstrated reduced HLA-DR and CD86 expression on monocytes in patients with severe COVID-19[15,22,32] as well as in children with MIS-C associated with SARS-CoV-2 infection[33], implying potentially impaired antigen presentation to T cells. The downregulation of HLA-DR on monocytes could potentially be driven by interleukin-6 (IL-6), which has been shown to be elevated in patients with severe COVID-19[10,18,34,35] as well as in pediatric patients with MIS-C[33,36], as the decreased HLA-DR expression can be partially restored by tocilizumab[32] (a humanized IgG1κ monoclonal antibody (mAb) that targets interleukin-6 receptor (IL-6R) and blocks IL-6 signaling[37]).

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Tocilizumab has no efficacy in preventing disease progression[38] or intubation or death in hospitalized patients with moderate COVID-19[39]; however, the latter study[39] had wide confidence intervals in the efficacy comparisons, and another report[40] suggested potential benefit of tocilizumab in reducing the need for ventilation and/or death. Thus, the question of whether IL-6R blockade can benefit patients with moderate or severe COVID-19 disease remains open[40], and other chemokine receptor blockers are also being tested in ongoing clinical trials[41,42]. Additional applications of single-cell technologies in this context yielded the identification of the chemokine receptor CCR1[11,43] as a potential therapeutic target based on scRNA-sequencing of nasopharyngeal samples from patients with critical or non-critical COVID-19[43] or of nasopharyngeal and bronchial samples from patients with moderate or critical COVID-19[11].

Macrophages.

These[11,43] and other[19,44] single-cell studies of airway and alveolar cells have also yielded particular insight into the role of macrophages in COVID-19, specifically, that a dysregulated macrophage response may drive pathological inflammation[45]. Patients with critical COVID-19 have manifested increased ligand–receptor interactions between epithelial cells and immune cells, upregulation of pro-inflammatory chemokine and cytokine genes in non-resident macrophages, and *CCR1* upregulation in neutrophils, macrophages and $CD8^+$ T cells[11]. These findings suggest the influence of cycles of recruitment of immune cells to the lung (monocytes that differentiate to inflammatory macrophages, further recruitment and activation of more immune cells) on the epithelial damage seen in severe COVID-19. scRNA-seq analysis of cells in bronchoalveolar lavage fluid (BALF) has revealed that the proportion of bronchoalveolar macrophages and the levels of inflammatory cytokine and chemokine receptors are positively associated with disease severity[19]. Macrophages in severe COVID-19 were also distinguished by high expression of *FCN1* (a member of the complement cascade) and *SPP1* (a proinflammatory cytokine), suggesting that alveolar macrophages may drive local inflammation in patients with severe COVID-19[19]. Finally, flow cytometry and scRNA-seq data of BALF cells from patients with pneumonia caused by SARS-CoV-2 infection suggest that high levels of monocytes, as well as $CD4^+$ and $CD8^+$ T cells, are found in the alveolar space.[44] Single-cell RNA-seq identified multiple clusters corresponding to tissue-resident alveolar macrophages and monocyte-derived alveolar macrophages, along with expression of *IFNG* in T cells from patients with pneumonia caused by SARS-CoV-2 infection. The identification of an interferon response signature by bulk sequencing of flow cytometry-sorted alveolar macrophages, and the finding of SARS-CoV-2 RNA in alveolar macrophages (which suggests that SARS-CoV-2 can replicate in alveolar macrophages), gives strength to the hypothesis that activated T cells in severe COVID-19 release IFN-γ. In turn, this IFN-γ drives an IFN response in alveolar macrophages that leads to the recruitment of monocyte-derived alveolar macrophages, completing an ‘inflammatory signaling loop’[44].

An alternative RNA-seq analysis of PBMCs from four healthy donors, five influenza patients, and eight COVID-19 patients reported that classical monocytes in patients with severe COVID-19 are distinguished by an IFN type I (IFN-I)-related transcriptional signature and an IL-1β–related inflammatory transcriptional signature[46]. This finding

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generated the hypothesis that the IFN-I response contributes to detrimental inflammation in severe COVID-19. In a much larger sample size (including 130 patients with COVID-19 from three different centers in the UK), scRNA-seq and quantification of 188 cell surface proteins[47] revealed a positive association between the frequency of proliferating monocytes and *MKI67*- and *TOP2A*-expressing dendritic cells with COVID-19 disease severity. The same study also describes how platelet expansion was associated with severe COVID-19, along with enhanced interactions of platelets with C1QA/B/C+CD16+ monocytes in COVID-19. These findings lend support to the role of both platelets and monocytes in the tissue thrombosis that has been reported in COVID-19[48].

B cell responses

Neutralizing antibodies (nAbs) have been heavily implicated in protection against SARS-CoV-2 infection and COVID-19 disease[49–53] and thus intense interest has focused on identifying potent nAbs against SARS-CoV-2. Such analysis requires a single-cell approach, owing to the extensive VDJ recombination and somatic hypermutation in B cells[54].

Immunoglobulin sequencing.

A general strategy for identifying relevant antibody sequences has been to sort and process via scRNA-seq individual antigen-specific memory B cells (for example, specific to the receptor-binding domain (RBD) in the spike glycoprotein[55] or the spike trimer[14,56–58]) from convalescent COVID-19 patients. VDJ sequencing performed at the single-cell level (scVDJ-seq[33]) has led to identification of nAbs with prophylactic and/or therapeutic efficacy against SARS-CoV-2, feeding into the robust pipeline of nAb clinical trials[59].

In a study that employed high-throughput scRNA/VDJ-seq, about 9,000 RBD-binding B-cell clonotypes were identified, yielding 14 potent nAbs, one of which was shown to protect against SARS-CoV-2 in a mouse model[55]. The coupled scRNA-seq data allowed the identification of naïve and memory B cell subsets and helped improve the efficiency of nAb selection by filtering out clonotypes enriched in naïve and exhausted B cells[55]. A different study used a similar strategy to identify 19 potent nAbs, including RBD-binding and non-RBD binding nAbs, one of which was shown to protect against SARS-CoV-2 in a hamster model[60]. The integration of two parallel workflows, both of which featured scRNA-seq and one of which incorporated single-cell functional assays along with scVDJ-seq, has led to identification of five major classes of nAbs with different reactivities to the spike glycoprotein and cross-reactivity with SARS-CoV[53]. Unterman et al.[62] performed a multi-omic integration of single-cell analysis, including scRNA-seq and CITE-seq, along with B-cell receptor (BCR) and T cell receptor (TCR) sequencing, on PBMCs from patients with ‘stable’ COVID-19 (who were hospitalized and ultimately discharged) and patients with ‘progressive’ COVID-19 (who were treated in ICU and ultimately succumbed to the disease)[61]. Their results supported a complex B-cell response in COVID-19, including a high proportion of unmutated immunoglobulin gamma heavy chain (IGHG) B-cell clones present alongside multiple mutated B-cell clones that did not appear to increase levels of somatic hypermutation over time. The latter could potentially be explained by memory B

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cell cross-reactivity with other coronaviruses or failed formation of robust germinal center reactions[58].

B cell markers.

High-dimensional flow cytometry has been used to characterize and compare B-cell responses in patients with different severities of COVID-19 disease. A 24-marker B-cell focused panel designed to identify B cell populations, evaluate their activation status, and assess homing potential[62] allowed the identification of a correlation between overactivation of extrafollicular B cells and COVID-19 disease severity, along with greater expansion of antibody-secreting cells in severe versus milder disease. Patients with severe disease had high serum titers of RBD-targeting SARS-CoV-2 nAbs, raising the question of whether this distinct B-cell response in patients with severe COVID-19 is ineffective or potentially even pathogenic. Other studies have described how the humoral immune response (as assessed by BCR clonal expansion and B cell activation) could be correlated with disease severity[63]; however, it remains an open question whether these positive correlations are simply due to increased initial viral load; this is a particularly complex question, with studies having reported direct[64–71], inverse[72,73], or no[74] correlation. These discrepant findings may be the result of differences in sampling compartment[67] (e.g., saliva, blood, or anal), timing of sample collection, and/or population differences. In any case, it is clear that future studies are warranted to define the cellular and molecular determinants that dictate protective versus non-protective humoral responses in infected individuals. The potential of antibody-dependent enhancement of COVID-19 disease must also be considered[75].

Convergent antibody clusters — antibodies with highly similar VDJs shared by multiple patients, which generally comprise only a small proportion of the virus-specific B-cell response[76] — have also been identified[77], with the suggestion that the majority of COVID-19 patients have convergent immunoglobulin heavy chains against the RBD, which may bode well for spike- or RBD-based vaccines.

B-cell phenotypes.

In a study looking at longitudinal samples from individuals who had recovered from mildly symptomatic COVID-19, Rodda et al.[78] used RBD tetramer enrichment and flow cytometry to phenotype the rare population of RBD-specific B cells. RBD-specific memory B cells increased from one to three months post-symptom onset and were substantially higher in COVID-19 samples than in healthy controls. Moreover, memory B cells displaying a $Tbet^{low}IgG^{+}CD21^{+}CD27^{+}$ phenotype (that is, a ‘classical’ memory B cell phenotype) also increased from one to three months post symptom onset, whereas levels of $Tbet^{hi}$ memory B cells, which are typically found in chronic infections, remained low. The question of whether SARS-CoV-2 specific memory B cells can produce nAbs after reactivation by secondary infection has also been addressed: sorting and sequencing of RBD-specific single B cells and their BCRs suggested that memory B cells may help protect from secondary infections.

Human tissue-imaging platforms coupled with multi-color immunofluorescence and multispectral imaging via quantitative automated scanning microscopy have been used to study, at the single cell level, thoracic lymph nodes and spleens obtained via from autopsy of

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patients who succumbed to COVID-19[58]. Compared with single-cell approaches requiring dissociation of tissue, an advantage of this approach is that tissue architecture could be largely preserved, enabling study of cell–cell interactions. A loss of germinal centers (GCs) in the lymph nodes of these patients, accompanied by substantial reductions in GC B cells and follicular helper T (Tfh) cells in the lymph nodes and spleens, was reported using this approach[58]. As optimal germinal center reactions are essential for the production of high-affinity antibodies[79], these results suggest that the increased proportions of plasmablasts observed in patients with COVID-19 (particularly severe COVID-19)[10,14,16,58] are a correlate of suboptimal antiviral humoral immunity and disease rather than protection. Other emerging single-cell imaging-based techniques, such as single-cell spatial transcriptomics, may be particularly relevant to the emerging field of pathological studies in organs from deceased COVID-19 patients[80,81].

T-cell responses

Multiple studies using flow cytometry of PBMCs from patients with COVID-19 have reported T lymphopenia: compared with the lower limit of normal or with levels observed in healthy controls, substantially reduced T-cell[13,16,18,82,83], $CD4^+$ T-cell[16,56,57,82,83], $CD8^+$ T-cell[10,16,56,57,82,83], $CD8^+$ mucosal-associated invariant T (MAIT) cells[16], γδ T-cell[10], αβ T-cell (some subsets)[10] and regulatory T-cell[83] counts have been observed in patients with mild or severe COVID-19. Similar T-cell lymphopenia has been observed in patients with COVID-19-associated MIS-C[33,36,84,85]. Whereas Mudd et al.[12] reported that the reductions in $CD4^+$ and $CD8^+$ T cells were comparable between patients with COVID-19 and patients with influenza, Giamarellos-Bourboulis et al.[32] have observed that the reduction in $CD4^+$ T cells is stronger in patients with COVID-19 than in patients with influenza. Decreased T-cell populations have also been associated with disease severity, as both $CD4^+$[56,83] and $CD8^+$[56] T cells were shown to be substantially reduced in patients with severe versus moderate or mild COVID-19, or in intensive care unit (ICU) versus non-ICU cases[82]. An increased $CD4^+/CD8^+$ T-cell ratio has also been reported in patients with COVID-19[18,57], suggesting that SARS-CoV-2 infection might preferentially impact $CD8^+$ T cells.

T-cell phenotype.

Multiparametric flow cytometry has also revealed phenotypic and functional alterations in T cells of patients with COVID-19, with a general theme emerging of hyperactivation of T cells in COVID-19, as demonstrated by elevated subpopulations expressing activation, proliferation or exhaustion markers. Levels of activated (HLA-DR^+CD38^+[10,13,16,57], $CD38^+$[86], HLA-DR^+[87], or $CD25^+CD4^+/CD8^+$[10]), proliferating ($Ki67^+CD8^+$[57,86] or $Ki67^+CD4^+$[86]), and exhausted (PD-$1^+CD8^+/CD4^+$[82,86], $TIGIT^+$[87], or $NKG2A^+$[88]) T cells have been shown to dramatically increase in patients with COVID-19 or in patients with severe COVID-19 compared with healthy controls, and most activated ($CD38^+$ PD-1^+) $CD8^+$ T cells in patients with acute COVID-19 have been shown to be specific for SARS-CoV-2[86]. However, perhaps due to differences in the timing of sampling, healthy controls, patients with influenza, and patients with COVID-19 with similarly low abundances of HLA-DR^+CD38^+ activated $CD8^+$ T cells have been described. Notably, scRNA-seq and scTCR-seq analysis of $CD8^+$ T cells in BALF samples identified signatures of tissue-

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resident memory T cells and increased levels of clonal expansion associated with mild disease, whereas elevated proliferative capacity was correlated with severe disease[19]. Single-cell RNA-seq analysis of cerebrospinal fluid leukocytes isolated from COVID-19 patients with neurological sequelae ('Neuro-COVID') has uncovered high levels of CD4$^+$ T cells expressing exhaustion markers (e.g., *ICOS*, *HAVCR2/TIM3 and CD226*) compared with those in control patients.

Flow cytometry and scRNA-seq have also identified altered T-cell differentiation and cytotoxicity in COVID-19. Patients with severe COVID-19 had higher proportions of circulating cytotoxic CD8$^+$ T cells compared with healthy controls[16], and CD8$^+$ T cells in nasopharyngeal and bronchial samples from patients with severe COVID-19 have increased expression of cytotoxic molecules[11]. This increased cytotoxicity has been proposed to contribute to epithelial damage[11]. Regarding differentiation, a decrease in peripheral naïve and central memory CD8$^+$ T cells and an increase in senescent and effector memory CD45RA$^+$ CD8$^+$ T cells in patients with COVID-19[18] have been reported, suggesting a skew towards terminal differentiation. In another study[58], CD4$^+$ Bcl-6$^+$ germinal center type T follicular helper cells were substantially decreased in thoracic lymph node and spleen autopsy tissue from patients with COVID-19, accompanied by high levels of tumor necrosis factor (TNF)-α at the follicle and increased numbers of T helper type 1 cells (T_{H1}) cells. These findings suggest that COVID-19 impairs T follicular helper cell differentiation, which may explain the lack of germinal centers mentioned above.

T-cell specificity.

SARS-CoV-2-specific T cells recognizing both spike- and non-spike epitopes have been identified in patients with acute COVID-19 and in convalescent patients through various flow cytometry-based techniques such as intracellular cytokine staining (ICS), activation induced marker (AIM) assays (including antigen-reactive T-cell enrichment (ARTE)), and peptide–MHC multimers (Supplementary Table 1), demonstrating the formation of virus-specific memory T cells after infection[78,86,89–96]. Although patients with severe COVID-19 showed overall higher breadth and magnitude of SARS-CoV-2-specific T-cell responses than patients with mild COVID-19, higher proportions of SARS-CoV-2 specific CD8$^+$ T cells were observed in mild versus severe cases[95]. SARS-CoV-2-specific CD4$^+$ T cells are predominantly Th1 cells and largely display a central memory T phenotype, whereas SARS-CoV-2-specific CD8$^+$ T cells are more enriched in effector memory and terminally differentiated effector subsets[93,96,97]. scRNA-seq and scTCR-seq analysis of SARS-CoV-2-reactive CD4$^+$ T cells revealed an association between increased SARS-CoV-2 specific cytotoxic CD4$^+$ T cells and cytotoxic Tfh cells with disease severity, and an inverse association of regulatory T cells with disease severity[98]. SARS-CoV-2-reactive CD8$^+$ T cells were shown to have enhanced expression of cytotoxic and inflammatory genes, with higher levels of TCR clonal expansion, in patients with severe disease, supporting an association of overactivated antigen-specific T cells with COVID-19 pathogenesis[99]. As *in vitro* stimulation can radically alter the gene expression profiles of reactive T cells, further studies using peptide–MHC multimers together with single-cell sequencing should provide complementary and additional insights into the phenotype and function of SARS-CoV-2-specific T cells. In addition, single-cell multiomic technologies, such as scATAC-seq, could

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be applied to investigate whether SARS-CoV-2-specific T cells retain epigenetic fingerprints that may dictate their recall responses.

Contribution of T cells to COVID-19 immunity.

Many questions remain regarding how prior exposure to endemic coronaviruses and cross-reactive memory T cell immunity shape the immune response to SARS-CoV-2.[100,101] Intracellular cytokine staining/flow cytometry, ELISpot, and FluoroSpot assays have been key single-cell technologies in detecting cross-reactive memory $CD4^+$ T cells in SARS-CoV-2-unexposed individuals (ranging from ~20 to 50% of individuals tested, across geographically diverse cohorts), whereas cross-reactive memory $CD8^+$ T cells are much less common[86,89–93,102]. It has been postulated that cross-reactive memory $CD4^+$ T cells may provide protective immunity against SARS-CoV-2 infection and reduce disease severity by promoting B-cell and antibody responses and/or mediating rapid local antiviral immunity at sites of infection, including the lung and/or upper respiratory tract[101]. However, Bacher et al.[104] reported that pre-existing cross-reactive memory $CD4^+$ T cells may not only have low TCR avidity with reduced clonal expansion, but also exacerbate inflammation and disease severity, especially in the elderly[103]. Thus, the roles of pre-existing cross-reactive memory T cells in SARS-CoV-2 infection and vaccination in the general population warrant further investigation.

Correlates of immune protection at the single-cell level

With the rapid growth of single-cell datasets on the immunology of COVID-19, one open question is how to best leverage current and emerging public datasets. Ongoing efficacy trials of candidate COVID-19 vaccines are generating single-cell immune-profiling datasets that could be applied to identifying correlates of risk and correlates of protection against primary and secondary endpoints in these trials, for instance as we have done previously for the RV144 HIV vaccine efficacy trial[104]. Because of limited specimen volumes in COVID-19, vaccine efficacy trials, and the large number of immunological biomarkers that could potentially be evaluated as correlates, it will be critical to perform pilot studies to optimize and identify assays and associated immune signatures with favorable statistical properties for correlates analyses. Important factors in determining the best correlates include high reproducibility, large dynamic range in vaccine recipients, and low response range (low false-positive rate) at baseline in vaccine recipients and in placebo recipients post-placebo. To help expedite correlates analyses, existing single-cell datasets could be explored to optimize and identify highly reproducible single-cell immunological signatures, which will be useful in variable down-selection.

Another major application of these collated datasets is the potential for integrative analyses and meta-analyses, such as the recent meta-analysis of 107 lung single-cell RNA-seq studies that identified additional proteases that may potentially be involved in SARS-CoV-2 infection[105].

Box 1 (together with Figs 2 and 3) illustrates one way to organize publicly available single-cell data for use in integrative analysis. Readers should note, however, that a search

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for biomarkers and signatures associated with COVID-19 disease severity or progression using such published data faces several challenges.

One challenge is whether and how to account for trial-participant demographics, as ethnic[106–109], sex[108,110], and age[111–115] differences in clinical presentation, immune responses, and outcomes of SARS-CoV-2 infection have been identified, even when adjusting for potential confounders.

A second major challenge is the substantial heterogeneity thus far in the ordinal scales that have been used for assessing coronavirus disease severity[116]: out of the 44 studies that categorized disease severity, 7 used WHO scores, 5 used National Health Commission of China guidelines, 3 used NIH scores, 1 used German Robert Koch Institute symptom classification, 1 used National Early Warning Score, 4 did not provide relevant information on how disease severity was defined, and 23 used custom scoring (Supplementary Table 1). Moreover, for studies that have obtained samples at ‘early’ and ‘late’ disease stages, there is substantial heterogeneity in how the times post-symptom onset are defined. The incorporation of standardized definitions of disease severity into future single-cell immune profiling studies would facilitate such integrative and meta-analyses. As one approach to overcome this problem, Zheng et al.[117] have defined seven disease severity categories and manually assigned standardized categories in an integrated analysis of 4,780 PBMC transcriptomic samples from patients infected with a different virus (16 in total, across 26 datasets) — an approach that may help mitigate the problem caused by non-standardized definitions of disease severity. Integrative analysis of bulk sequencing data has supported the hypothesis that there is a conserved pan-viral response associated with disease severity, Zheng et al.[118] also have performed an integrative analysis of three single-cell data sets (two CITE-seq[22,118] and one scRNA-seq[14]; 264,224 cells from 71 PBMC samples overall) from three independent cohorts including healthy controls[14,22,118], patients with SARS-CoV-2 infection[14,22,118], and patients with influenza or RSV infection[22], reporting that a ‘Meta-Virus Signature’ score in single myeloid cells is positively correlated with viral infection severity across different virus strains.

The lack of standardized experimental protocols and analysis pipelines is an additional challenge in integrating single-cell datasets. As a result, published results and datasets can be difficult to compare and integrate due to experimental and computational variation on how the cells and datasets were processed. Fortunately, the research community has worked hard to provide computational approaches that can be used to reduce technical variation and produce standardized cell annotations that be compared, visualized and modeled across datasets[119,120]. These approaches have already been used in many of the COVID single-cell studies published to date to correct for batch effects[118,118]. The Human Cell Atlas[121] initiative has also been working on standardization of the different aspects of single-cell sequencing, and the Human Cell Atlas Data Portal provides publicly available data sets processed by standardized pipelines[122].

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Conclusions

Single-cell analyses have held up to their promise of overcoming certain limitations of bulk methods and enabling a deep dive into the cellular heterogeneity of antiviral immune responses. Multiple single-cell immune profiling studies of COVID-19 patients have identified distinct cell subsets of the innate and adaptive immune systems that correlate with disease severity; this body of evidence supports the hypothesis that such subsets may have important functions in blunting (or even enhancing) COVID-19 disease severity. There is also evidence to suggest that targeting certain immunological factors, such as cytokine/chemokine receptors (e.g. IL-6R and CCR1), might curb pathogenic responses and/or improve protective immunity. In the near-term future, studies applying single-cell multiomics technologies such as CITE-seq and single-cell BCR-seq/TCR-seq with peptide–MHC multimers are needed to further characterize the phenotypes and functions of immune cell subsets implicated in COVID-19 protection and of those implicated in progression. Application of multiomic technologies such as scATAC-seq to understand the epigenetic changes associated with SARS-CoV-2 infection in immune cells, especially in antigen-specific T and B cells, may help identify new avenues to pursue for COVID-19 therapies. Moreover, additional multiomic spatial immune profiling studies are needed to further dissect local immune responses against SARS-CoV-2 in tissues such as lung. In the longer-term, single-cell immune profiling studies with sufficiently large sample sizes and participant diversity will be valuable for helping investigate potential sex-related and age-related differences in COVID-19, e.g. whether the immune cell subsets of interest described above vary in frequency or in function in males vs females. Application of single-cell immune profiling to better understand the mechanisms driving the range of post-COVID conditions (e.g. long COVID) is also a relatively underexplored area with many unanswered questions.

The scientific community has mobilized in unprecedented fashion in response to the ongoing COVID-19 pandemic. Large collaborative efforts such as the US National Institutes of Health (NIH)-funded Immunophenotyping Assessment in a COVID-19 Cohort (IMPACC) study (NCT04378777) and the COVID-19 Cell Atlas[123] (Wellcome Sanger Institute/Chan Zuckerberg Initiative), to name a few, are generating freely available, open access datasets at the single-cell level and work is being done on standardizing protocols and metadata as well. These datasets have been derived from patients with COVID-19 of varying severity and include different tissues, cohort features, and time points. Looking ahead, we anticipate that the amount and complexity of single-cell datasets will rapidly grow, including in important populations, such as pediatric patients (for whom little single-cell data currently exists), and that re-analyses and meta-analyses will become more common as standards become available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1.

Organization and curation of single-cell sequencing datasets

With the aim of facilitating integrative analyses and meta-analyses, we have organized, curated and standardized a set of publicly available single-cell transcriptomics datasets. Of the 22 scRNA-seq and 4 CITE-seq studies in Table 1, openly accessible and downloadable FASTQ, count matrix, Seurat object, or H5AD data are available for 23. Of these 23, we excluded ref.[12], as only raw sequence reads were available, and ref.[125], as only four cell populations (monocytes, B cells, megakaryocytes, and cell precursors) were available. Thus, our analysis processed 21 datasets. We first downloaded the datasets from such repositories as Gene Expression Omnibus (GEO), ArrayExpress, and the Genome Sequence Archive. The vast majority of scRNA-seq and CITE-seq studies (22 out of 26) used the Seurat package[124,126] for data analysis, whereas three studies used Scanpy[127] and one study used Pagoda2[128]. Thus, for simplicity, we prepared the datasets as Seurat objects in R and performed quality control, normalization, dimensionality reduction, clustering, and cell-type annotation using Seurat v4. Seurat v4 multimodal CITE-seq reference mapping enables not only more accurate resolution of granular cell types that are difficult to distinguish by transcriptomic data alone, but also imputation of the expression of over two hundred surface proteins[124]. We collected single-cell sequencing data of over 3.2 million single cells across 21 datasets[11,14,15,19,22,23,43,44,46,47,98,99,103,118,129–135] and mapped each cell to the human PBMC CITE-seq reference[124].

As shown in Figure 2a, substantial diversity was observed with respect to age, and there were more male patients with moderate or severe COVID-19 than female patients. Additionally, the vast majority of the samples were blood as opposed to another tissue type, such as from the airways (Fig. 2b). As only cell types in the Seurat v4 multimodal CITE-seq PBMC reference set can be correctly mapped, for visualization purposes we took only the PBMCs and whole blood datasets (16 in total, the latter minus neutrophils and basophils) and created a ‘merged dataset’ consisting of >2.5 million cells. (We note that as additional references and/or integration methods become available, other tissues could potentially be integrated as well.) Figure 2c presents a visual representation of all cells in the merged dataset in the same Uniform Manifold Approximation and Projection (UMAP) space, along with standardized cell type annotations at three levels of granularity.

We also include standardized cell metadata (e.g. disease severity and time points) from the original publication when available. Briefly, the standardized disease severity categories are based on information provided in datasets and supplementary materials and defined as mild: not hospitalized, moderate: hospitalized (no ICU), and severe: ICU. Standardized time points are defined as the number of days between symptom onset and the sampling time if provided and were estimated for patients where it was not provided (See more details in the Supplementary Text).

To illustrate the usage of the merged dataset, Figure 2d shows the frequencies of $CD4^+$ T cells, $CD8^+$ T cells, mucosal-associated invariant T (MAIT) cells, $CD56^{bright}$ NK

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cells, CD16$^+$ monocytes, and CD14$^+$ monocytes in samples across 12 datasets grouped by disease severity and time points for COVID-19 samples based on the standardized metadata. Consistent with published results[16,32,56,82,83,88,136,137], reductions in CD4$^+$ T cells, CD8$^+$ T cells, MAIT cells, and CD56^{bright} NK cells were each associated with COVID-19 severity. When sampled at an early or intermediate stage of their clinical course, we also observed decreased CD16$^+$ monocytes in patients with severe vs mild COVID-19 (Fig. 2d), which is consistent with published results[15,23]. Conversely, an increase in CD14$^+$ monocytes was observed in patients with severe versus mild COVID-19, regardless of when in the clinical course the samples were obtained (Fig. 2d). To facilitate standardization and sharing of future datasets by the community, the code used to process the datasets is available at github (https://github.com/RGLab/covid19_sc).

Furthermore, we provide interactive visualization of these datasets on a visualization portal (https://atlas.fredhutch.org/fredhutch/covid). Figure 3 shows an example screenshot of the visualization portal. The processed Seurat objects can be easily downloaded from the visualization portal; to ensure that our resource remains maximally relevant to the research community, we encourage users to request datasets to be added to the portal as new studies emerge. We plan to update the portal as needed to ensure that it includes highly requested studies. We hope that the standardized datasets we present here will facilitate re-analyses and meta-analyses and accelerate much-needed translational discoveries to help stem the COVID-19 crisis.

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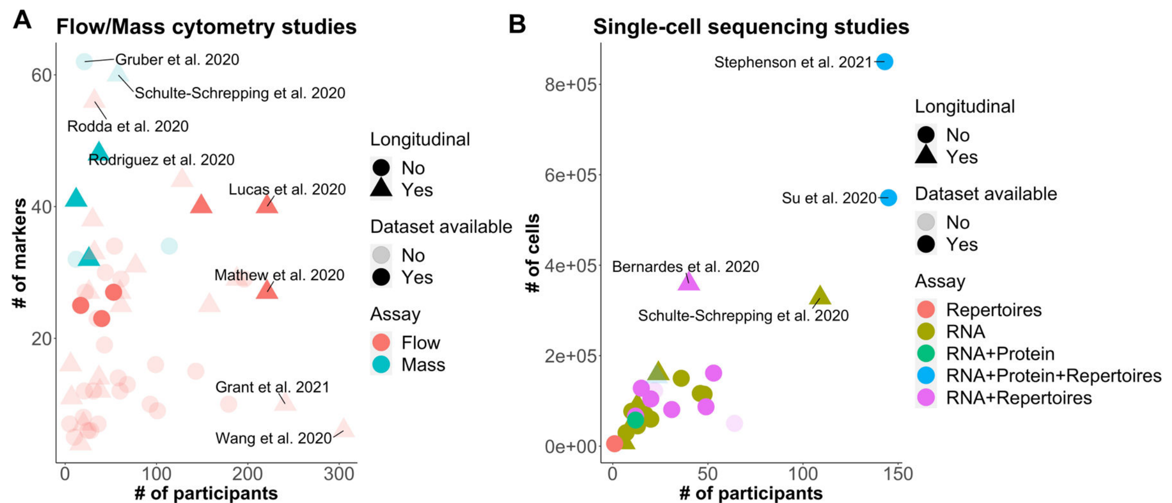

Figure 1.

Visual representation of characteristics of the 64 published articles or publicly posted preprints on COVID-19 that have used one or more single-cell technologies (March 2020 – March 2021). (**a,b**) Scatter plots showing the number of participants versus (a) the number of flow cytometry markers or (b) the number of cells for which data are available in each study. Each symbol represents a dataset using one of the single-cell technologies from a single article/preprint. Opacity indicates dataset availability to the public (light, no; dark, yes), shape indicates whether the dataset has longitudinal data (circle, no; triangle, yes), and color indicates assay type [(a) red, flow cytometry; cyan, mass cytometry; (b) red, repertoires; gold, RNA; green, RNA plus protein; blue, RNA plus protein plus +repertoires; and magenta, RNA plus repertoires].

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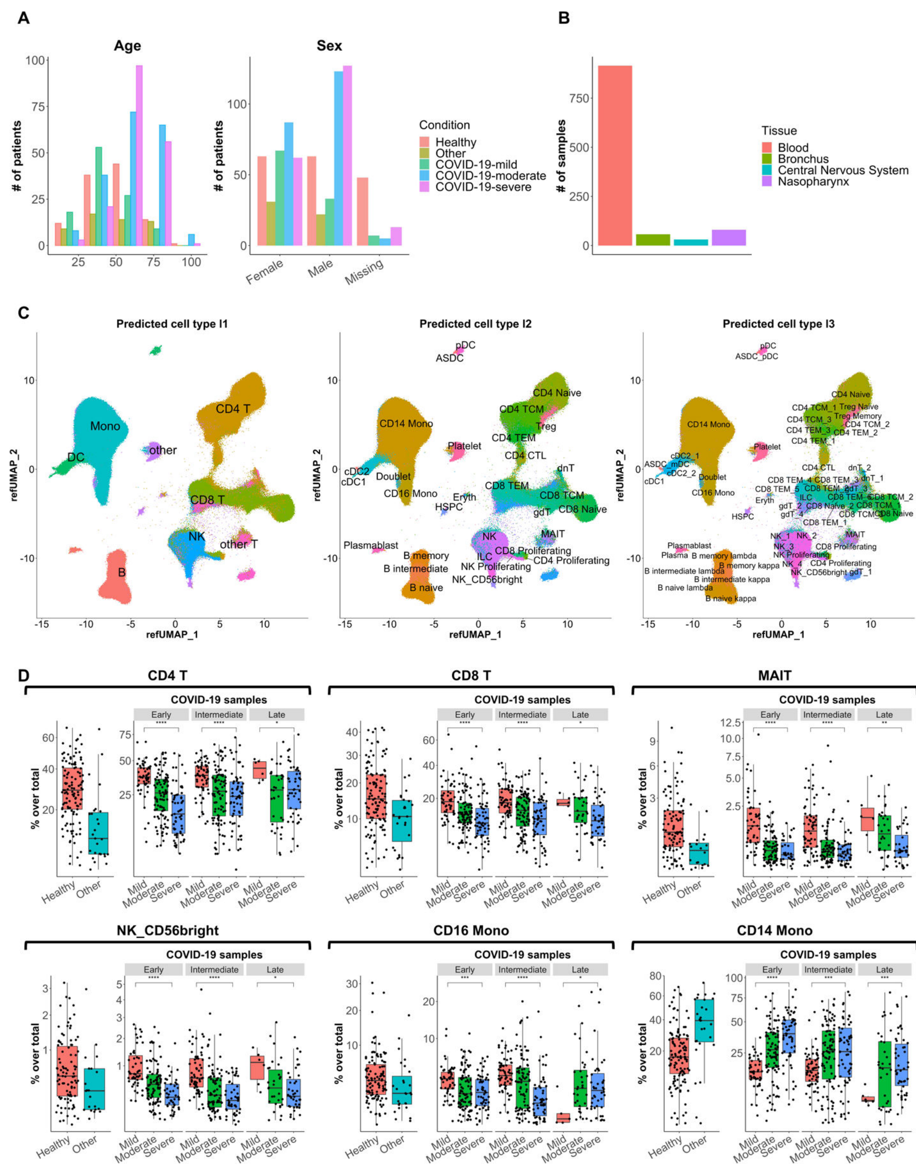

Figure 2.

Visual representation of single-cell transcriptomics data. (a) Plots showing the distribution of age (left panel) and sex (right panel) among individuals included in the collected 21 datasets. (b) Bar plot showing the number of samples per tissue type among the collected 21 datasets. (c) Uniform manifold approximation and projection (UMAP) plots showing the projection of over 2.5 million single cells from 16 PBMC and whole blood datasets (the latter minus neutrophils and basophils) mapped to the Seurat CITE-seq reference, colored and labeled by reference-defined cell type annotations at level 1 (left panel), level 2 (middle panel), and level 3 (right panel) granularity. (D) Box plots showing the frequencies of $CD4^+$ T cells, $CD8^+$ T cells, mucosal-associated invariant T (MAIT) cells, $CD56^{bright}$ NK cells, $CD16^+$ monocytes, and $CD14^+$ monocytes among samples grouped by disease severity and time points for COVID-19 samples. Samples from Arunachalam et al.[22] (enriched for DCs), Meckiff et al.[98] (enriched for antigen-specific $CD4^+$ T cells), Kusnadi et al.[99] (enriched for

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antigen-specific $CD8^+$ T cells), and Bacher et al.[103] (enriched for antigen-specific $CD4^+$ T cells) were excluded. Early, <= 8 days post symptom onset; intermediate, > 8 and <= 15 days post symptom onset; late, > 15 days post symptom onset. Statistical significance between mild and severe was determined by Wilcoxon test. *: p <= 0.05, **: p <= 0.01, ***: p <= 0.001, ****: p <= 0.0001.

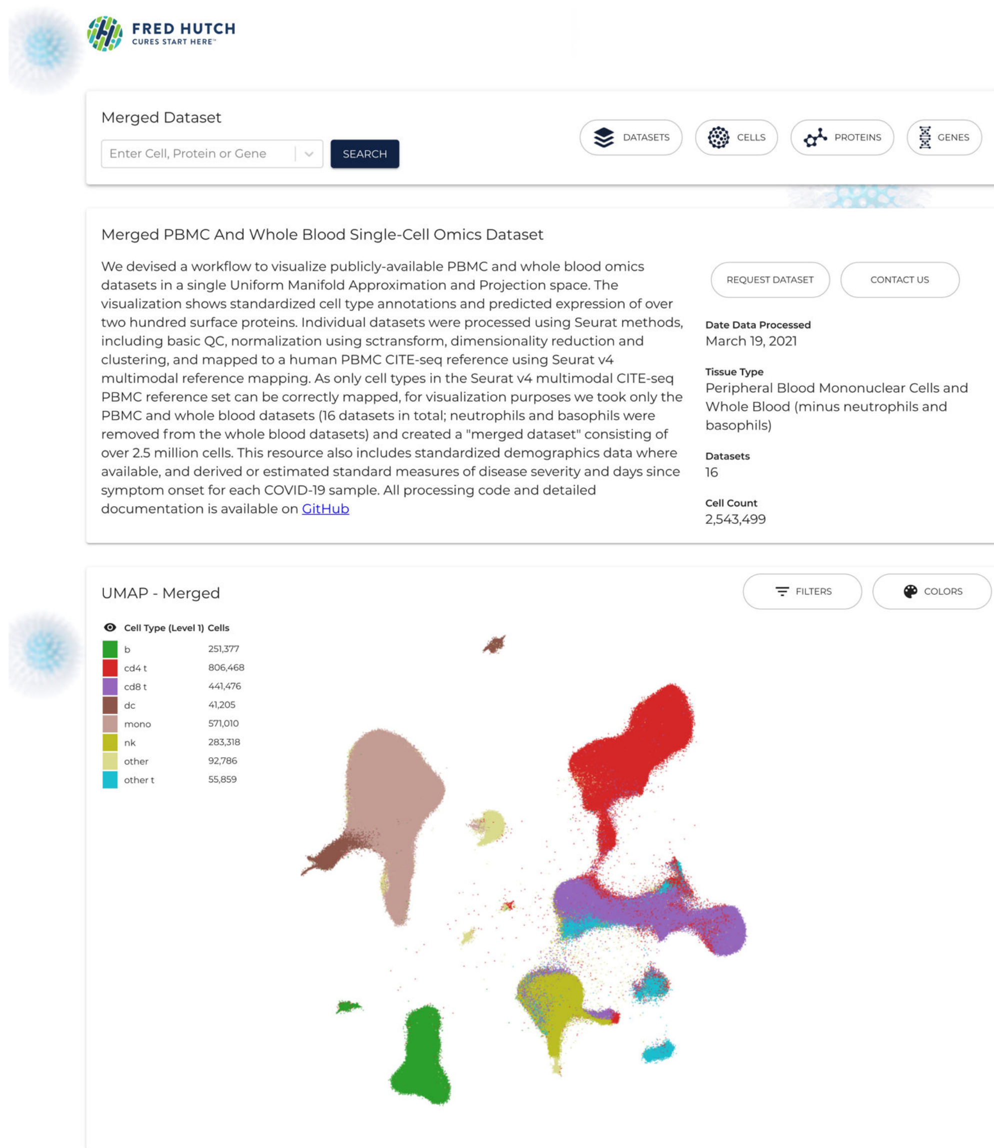

Figure 3.

An example screenshot of the visualization portal. The website provides visualization of 21 individual datasets and the merged dataset consisting of 16 datasets. The UMAP plot showing the merged dataset consisting of over 2.5 million cells mapped to the human PBMC CITE-seq reference[124]. The processed datasets can also be downloaded from the website at https://atlas.fredhutch.org/fredhutch/covid.

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Table 1.

Summary of single-cell technologies that have been applied to the study of COVID-19

Technology	Measurement	Methodology	Capacity	Pros and cons	Number of published or preprint COVID-19 articles*
Flow cytometry¶	Protein expression	Fluorophore-conjugated antibodies; cells are sorted into liquid droplets individually and flowed through a laser beam; the light emitted by each cell informs about marker expression	High throughput (millions of cells); up to ~30 markers	Pros: determination of immune cell subsets by well-defined surface markers and antibody panels; cells can be sorted for further analysis Cons: broad emission spectra of fluorophores	47
Mass cytometry (CyTOF)	Protein expression	Antibodies conjugated to heavy-metal isotopes; cells are nebulized and the metal-conjugated antibodies are ionized; signals are detected by a time-of-flight mass spectrometer	High throughput (up to millions of cells); many-dimensional (>40 cellular parameters/cell)	Pros: avoids spectral overlap between fluorophores Cons: slower flow rate than flow cytometry; expensive; destructive (not possible to sort cells for further analysis)	7
scRNA-seq	Gene expression	Single cells are isolated (e.g. through microfluidics, droplet-based methods, or flow cytometry-based sorting), lysed, and their transcripts captured. The subsequent workflow is similar to that of bulk RNA-seq	High dimensional (>10,000 features measured per cell)	Pros: comprehensive and unbiased sequencing	22
CITE-seq*	Simultaneous surface protein expression and gene expression	Barcoded, oligonucleotide-conjugated antibodies label single-cells that are analyzed by scRNA-seq	High dimensional (>100 proteins can be measured per cell in addition to (>10,000 genes)	Pros: gene expression integrated with multiomic profiling	4
scBCR/TCR-seq#	Immune antigen receptor repertoire	Single-cell V(D)J enriched libraries are generated utilizing microfluidics, 5′ molecular barcoding, and constant region–specific primers	Paired, full-length receptor sequences from T cells and/or B cells including isotypes.	Pros: comprehensive and unbiased sequencing; combination with multiomic profiling possible (gene and protein expression)	13

Abbreviations: CyTOF, cytometry by time of flight; CITE-seq, Cellular Indexing of Transcriptomes and Epitopes by Sequencing; scBCR/TCR seq, single-cell B-cell receptor/T-cell receptor sequencing; scRNA-seq, single-cell RNA sequencing.

*CITE-seq includes scRNA-seq as part of the workflow; to omit redundancy we did not count the CITE-seq studies in the scRNA-seq row.

¶Includes spectral flow cytometry (1 study), which is based on conventional flow cytometry but uses different optics and detectors.

#scBCR/TCR-seq can also be multiomic; here we include 12 multiomic studies that incorporated scBCR/TCR-seq and one scBCR-seq study.