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Systems analysis of human innate immunity in COVID-19

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ABSTRACT

Recent developments in sequencing technologies, the computer and data sciences, as well as increasingly high-throughput immunological measurements have made it possible to derive holistic views on pathophysiological processes of disease and treatment effects directly in humans. We and others have illustrated that incredibly predictive data for immune cell function can be generated by single cell multi-omics (SCMO) technologies and that these technologies are perfectly suited to dissect pathophysiological processes in a new disease such as COVID-19, triggered by SARS-CoV-2 infection. Systems level interrogation not only revealed the different disease endotypes, highlighted the differential dynamics in context of disease severity, and pointed towards global immune deviation across the different arms of the immune system, but was already instrumental to better define long COVID phenotypes, suggest promising biomarkers for disease and therapy outcome predictions and explains treatment responses for the widely used corticosteroids. As we identified SCMO to be the most informative technologies in the vest to better understand COVID-19, we propose to routinely include such single cell level analysis in all future clinical trials and cohorts addressing diseases with an immunological component.

1. Introduction

The numerous waves of SARS-CoV-2 virus variants [1] have caused a rather broad spectrum of disease courses of COVID-19 [2–4]. The spectrum of symptoms induced by different SARS-CoV-2 strains varies [4], but for all virus variants disease courses range from mainly asymptomatic to very severe and critical courses in a smaller subset of patients [4,5]. It became obvious that the interaction between the human immune system and the virus is a major driver of viral evolution and in contrast to other coronaviruses SARS-CoV-2 is still evolving very fast [6]. Patients with immune deficiencies cannot clear the virus very efficiently, which can prolong viremia and accelerate viral evolution [7,8], clearly indicating that the functional status of the immune system including its innate arm is a major determinant of disease course and severity. Furthermore, comorbidities including obesity, diabetes and cardiovascular diseases associated with chronic inflammation are major risk factors for severe courses [9,10] further supporting the notion that the immune system is a decisive component when it comes to disease course and severity. Genetic studies have identified a small number of mutations related to autoimmune or inflammatory diseases [11]. Furthermore, patients with mutations in TLR7 are at elevated risk for severe disease courses [12,13]. Similarly, mutations in interferon (IFN) pathways downstream of pattern recognition receptor signaling also point towards an important role of the innate immune system in defining disease courses and severity [14].

The time course of COVID-19 is best described by an initial phase of viremia followed by the second inflammatory response phase, which turns into viral sepsis in severe and critical cases [15]. In case of recovery, the second phase is followed by a third phase of convalescence with a return to immune homeostasis. While the dynamics of the disease phases is varying widely between patients, sophisticated time course analysis of high-content data suggest that convalescence is associated with a universal cellular and molecular program [16]. A smaller percentage of patients are not recovering and either experience prolonged clinical symptoms or even develop new symptoms after a period without any disease symptoms [4,17,18]. This clinical syndrome also termed long COVID has been linked to chronic inflammation, but also to the development of functional autoantibodies and even prolonged viral titers have been suggested as pathophysiological mechanisms [19,20]. In contrast to acute COVID-19, the role of the immune system and its individual cellular components including innate immune cells in long COVID are still not sufficiently understood. However, we are convinced

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that systems immunology approaches as we and others applied them to better understand the role of the immune system in acute COVID-19 [21–25] will also be most instrumental to better understand the role of the immune system in long COVID.

Here, we focus on knowledge gained from studies interrogating the immune response to COVID-19 with technologies supporting systems level analysis and description of the dynamic processes occurring during the different phases of the infection. We focus on the surprisingly strong heterogeneity of activation of cellular components of the innate immune system not only at the entry site of the virus but also during systemic inflammation. Further, we summarize what is known so far about processes during convalescence and lay out further necessary steps to better understand the heterogeneity of the pathophysiology during long COVID. We will highlight some surprising innate immune functions of a subset of T cells during SARS-CoV-2 infections and the potential link to endothelial damage. Finally, we will give an outlook on how we envision to further develop systems immunology approaches into a response system for emerging infectious diseases.

2. Systems level interrogation of the human immune response to COVID-19

Prior to the pandemic, a large consortium of European scientists had worked for over two years to design a roadmap toward improving

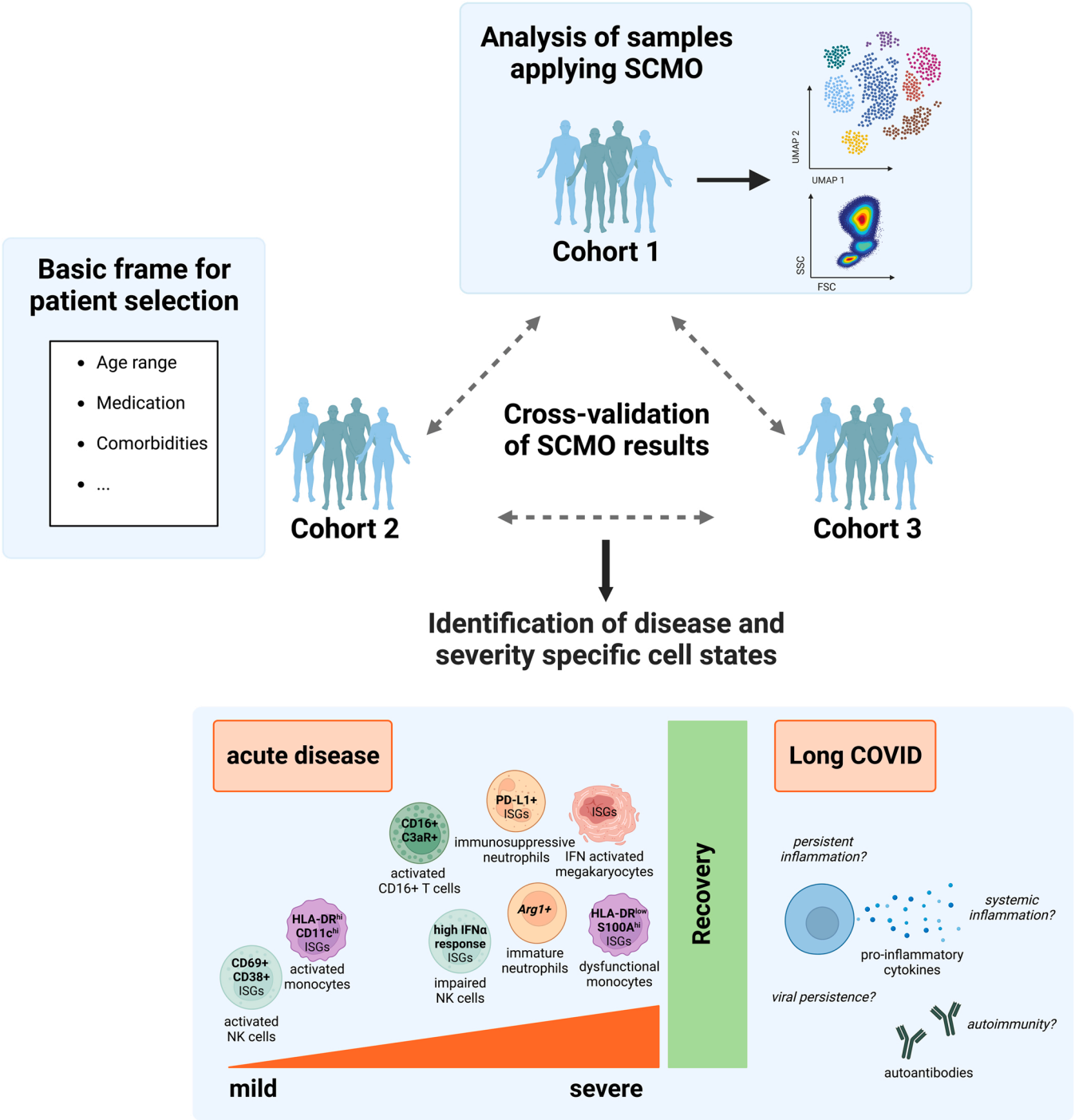

Fig. 1. General setup in clinical cohorts and validation cohorts to study COVID-19 and Long COVID by high-resolution single cell multi-omics technologies.

European healthcare through cell-based interceptive medicine [26]. This roadmap includes three major areas to be developed for clinical applications: 1. Single-cell multi-omics (SCMO), 2. Artificial intelligence / machine learning (AI/ML), and 3. Patient-derived experimental disease models. All three areas are major 'ingredients' of the big data-driven circle of systems immunology we previously suggested [27] and recently extended to SCMO applications [28]. At the beginning of the pandemic, we conceptualized how these technologies could be utilized to better understand the pathophysiology of the SARS-CoV-2 infection and to determine mechanisms that might explain the heterogeneous dynamics and disease trajectories of COVID-19 [15]. Looking at the major breakthroughs for a better understanding of COVID-19, it can be argued that genome-wide methods on the single cell level, in particular single cell RNA-seq technologies, have contributed most to a fast understanding of disease trajectories, cell types involved, key pathways altered and important genes dysregulated as a consequence of the infection [15,29]. However, it is also important to mention that scRNA-seq data very successfully guided follow-up studies including functional assessment of immune cells, phenotyping of immune deviations using targeted single cell technologies such as flow cytometry-based assays. Further, it became rather clear that systems level interrogation at scale requires clinical validation, which we introduced early on in the pandemic [21], illustrating that data integration - as it is currently being pursued for example in the Human Cell Atlas for generating healthy tissue maps - might be complemented or replaced by classical approaches from clinical research, namely clinical validation trials independently validating and confirming findings from early studies. Collectively, our experience during the first three years of the pandemic clearly point towards the enormous advantage of genome-wide single cell technologies in accelerating the identification of the major disease-specific and -associated changes in any given organ system and that targeted approaches are better suited for follow up or validation and confirmation studies.

As a consequence, we propose for any future threat by (re-)emerging infectious and also non-infectious diseases to apply SCMO - best combined with AI/ML applications - early on during the exploratory phase complemented by targeted validation studies (Fig. 1), which then will also allow to develop patient-derived experimental disease models, which could further accelerate the identification and development of therapeutic targets, an area that requires more attention in the future.

3. Acute innate immune response to COVID-19

Here, we summarize findings that have been mainly obtained by studies using systems immunology technologies, in particular single cell omics approaches.

3.1. Local immune response to SARS-CoV-2

The respiratory tract is the major entry route for SARS-CoV-2, where it binds to ACE2 receptors on nasal and oral epithelial cells, mainly goblet secretory cells [30–33]. A first line of defense against SARS-CoV-2 infection are IgA antibodies in sputum and saliva [34]. If local cells are productively infected by SARS-CoV-2, tissue-resident immune cells are activated in the upper respiratory tract, which in turn leads to activation and recruitment of circulating immune cells to the site of infection through inflammatory mediators [15]. This process is performed in close interaction of epithelial cells and tissue-resident immune cells, which sense the virus and initiate a defense response. By production of chemoattractants, innate and adaptive immune cells are recruited from the circulation to support the ongoing immune response. Following viral clearance, the resolution of the inflammatory response is initiated to inhibit hyperactivation of the immune system, which can induce exacerbated tissue damage.

Several single cell studies examining bronchoalveolar lavage fluid (BALF) and nasopharyngeal swabs from COVID-19 patients have yielded insight into the role of the innate immune response to SARS-CoV-2. A single cell study of nasopharyngeal swabs from COVID-19 patients identified increased frequencies of IFN-responsive ciliated epithelial cells in mild and moderate disease contributing to the antiviral response in the upper respiratory tract [33]. Another single cell study including samples from the upper (nasopharyngeal/pharyngeal swabs) and lower respiratory tract (BALF) during the first wave of the pandemic showed a stronger inflammatory profile for alveolar tissue-resident macrophages in the lower airways [35]. Furthermore, in critically ill patients, there were more ligand-receptor interactions predicted between epithelial cells and immune cells [35]. Together with the high pro-inflammatory profile of the interacting immune cells such as monocyte-derived macrophages and cytotoxic T cells, the prediction of a large number of ligand-receptor interactions between immune cells and epithelial cells is likely indicative for a higher number of highly activated infiltrating cells into the lung and more inflammatory tissue damage in critical compared to moderate disease [35]. Whether this difference is still valid for later SARS-CoV-2 variants such as omicron characterized by reduced involvement of the lower respiratory tract [36] requires further evaluation. An early finding was the identification of $ACE2^{-}$ tissue resident and monocyte-derived alveolar macrophages, which harbored SARS-CoV-2 transcripts likely from uptake of escaped virus particles through phagocytosis [37,38]. SARS-CoV-2 harboring myeloid cells showed a distinct transcriptional program including upregulation of cytokine and chemokine transcripts such as *CCL4, CCL20, CXCL10, CXCL11, IL1B* [37].

The release of chemoattractants by innate immune cells is likely responsible for the influx of additional immune cells to the lung, which is reflected by elevated levels of activated monocytes, monocyte-derived macrophages and neutrophils in BALF derived from COVID-19 patients [37,38]. In contrast, the number of tissue-resident alveolar macrophages is drastically reduced in severe disease [38,39]. While the remaining alveolar macrophage pool is altered in its functionality including reduced antigen presentation capacity [39], depending on disease severity and phase of the infection, the infiltrating immune cells have been described as hyperinflammatory [40]. Moreover, lung infiltrating monocyte-derived macrophages acquire a pro-fibrotic phenotype in severe disease [23].

In addition to BALF, local immune activation was also assessed by spatial single cell transcriptomics in post-mortem lung tissues. Among several studies, we highlight two of the larger studies [41,42]. In the first study, 116,314 nuclei from lungs of 19 COVID-19 infected lungs and 7 control lungs contained highly activated myeloid cells including monocytes, monocyte-derived macrophages and alveolar macrophages in the lungs of deceased COVID-19 patients [41]. In the second study, lung, heart, liver, and kidney tissues were studied by scRNA-seq [42]. Similar to early studies in BALF [37,38], SARS-CoV-2 RNAs were primarily detected in endothelial cells and myeloid phagocytes, which was associated with higher expression of viral response genes, chemokines, and cytokines [42]. Collectively, SARS-CoV-2 infection induces a profound local innate immune response, however, the magnitude and quality of this response in context of different viral strains, disease severity and phase, including prolonged immune deviations in long COVID are neither comprehensively characterized on the single cell level nor completely understood.

3.2. Heterogeneous deviations in the blood myeloid cell compartment

Analysis of the peripheral blood gives insight into the systemic effects of SARS-CoV-2 infection. Several studies examining peripheral blood from COVID-19 patients using SCO revealed complex immune response regulations during SARS-CoV-2 infection [43]. Despite different sampling times, study design (cross sectional versus longitudinal), SCO technologies used and heterogeneity of important clinical metadata (e.g. sex, age, comorbidities, ongoing medication including immunosuppressive drugs such as corticosteroids), several immune

deviations were reported independently by several studies strongly supporting these changes to be hallmarks of COVID-19 [44]. For example, concerning cell type frequencies it was shown that circulating non-classical CD16^{+} monocytes were reduced in moderate and even more so in severe disease [21,45–47]. At the same time, SCO revealed additional transcriptional states within the myeloid cell compartment, which differed between disease phase and severity. While monocyte states identified in mild to moderate disease were characterized by expression of genes related to a productive antiviral immune response such as CD83 or IFN-stimulated genes (ISGs), dysregulated molecular phenotypes were revealed in patients with severe disease, for example monocyte states characterized by reduced expression of *HLA-DR* genes [21,46–48]. *HLA-DR* genes encoding for MHC molecules are crucial for antigen presentation and the induction of an adaptive immune response. A recent study combining murine data from a murine virus infection and single cell RNA seq data of human PBMCs from COVID-19 patients, found strong differences in the functional capacity of monocytes and dendritic cells to productively present antigens to CD8 + T cells depending on the disease severity. A transcriptional program, taking place in antigen-presenting cells induced by type-1 IFNs and CD4 + T cell help, was found to be required to effectively activate anti-viral CD8 + T cells. Importantly, DCs and monocytes from mild but not severe COVID-19 patients have high chromatin accessibility and transcription of these genes, which correlates with the CD8 + T cell response in those patients [49]. Focusing on monocytes in severe COVID-19, cell states could be further subdivided into HLA-DR^{low} monocytes appearing early during infection, characterized by expression of *CD163* and alarmins *S100A4/8/9/12*, while later during severe infection elevated *CD163* expression was lost [21,46]. Despite some early discrepancies concerning the role of the interferon system in COVID-19 [50], in monocytes expression levels of ISGs are highest at early time points, which is even more prominent in severe COVID-19, and levels consistently decrease over time, linking the IFN response inversely to disease severity and phase [21,51]. This transient expression of ISGs correlated with plasma IFN-α levels [48]. However, the regulation of the IFN response is even more complex as in a subset of critically ill COVID-19 patients type-1 IFN-specific autoantibodies were found, which are associated with an impaired type-1 IFN response in myeloid populations including monocytes [52]. An interesting observation was that those monocytes displaying the highest type-1 IFN response also expressed ligands and receptors for interaction with platelets [25]. When directly comparing to monocytes in severe influenza infection, a monocyte cell state in severe COVID-19 patients was associated with higher TNF and IL-1b driven responses together with an enrichment of a type-1 IFN gene signature [53], which was often related to hyperinflammation. Since these patients were also characterized by harboring HLA-DR^{low} tolerizing or suppressed monocytes, we would suggest that the myeloid compartment in severe COVID-19 is better described as being a major part of a viral sepsis phenotype with COVID-19 related immune deviations.

3.3. Elevation and transcriptional reprogramming of neutrophils in COVID-19

Many single cell studies of peripheral blood are based on the isolation of peripheral blood mononuclear cells (PBMC), which misses out the large majority of granulocytes including neutrophils. However, in diseases such as COVID-19, where neutrophils have been shown to be significantly elevated and potential drivers of the disease pathology [21,50,54] these studies might have missed important pathophysiology of the disease. Bulk RNA sequencing of whole blood samples already indicated that the blood neutrophil compartment in severe COVID-19 is characterized by signatures reminiscent of pre-/immature neutrophils and simultaneous inflammatory and suppressive features, arguing for a complex dysregulation best captured by SCO technologies [55]. Indeed, studying the complete white blood cell compartment in COVID-19 patients by scRNA-seq led to the discovery of an enrichment of neutrophil precursors and immature neutrophils pointing towards emergency myelopoiesis, which was not present in patients with flu-like illness or healthy controls [21]. Generation of single cell transcriptomes of the bone marrow compartment from patients with severe COVID-19 validated this hypothesis illustrating accumulation of granulocyte-monocyte precursors (GMPs) and an upregulation of transcription factors determining the granulocyte lineage [56]. Accumulation of immature *Arginase 1* (*ARG1*$^{+}$) neutrophils in peripheral blood was also confirmed for critically ill COVID-19 patients and bacterial induced acute respiratory distress syndrome (ARDS) patients that were admitted to intensive care units but not in healthy individuals [57]. Not only the presence of immature neutrophils but also significant alterations in the mature neutrophil pool characterized COVID-19. In severe COVID-19 a peculiar neutrophil state occurs, which simultaneously expresses interferon response signature genes and genes associated with immunosuppression, e.g. *CD274* (PD-L1) or *ARG1* [21,45,47,57]. Immunosuppressive neutrophils have been previously reported in the context of systemic inflammation, e.g. in septic shock patients and after *in* vivo stimulation with LPS, to inhibit lymphocyte proliferation [58,59]. In severe COVID-19 a similar mechanism might take place [54]. However, he precise effect of these dysregulated neutrophil states on cell-cell interaction and the disease outcome requires further investigation [60]. Additionally, neutrophils with high expression of alarmins *S100A8* and *S100A9* were detected in the peripheral blood and BALF from severe COVID-19 patients [21,47]. CyTOF analysis confirmed the suppressive phenotype showing increased expression of PD-L1 and CD62L downregulation on mature and immature neutrophils from severe COVID-19 patients [21].

Taken together, neutrophils are not only increased in frequencies in COVID-19, but show dramatic transcriptional alterations leading to novel cell states (activated/ immunosuppressive) but also reprogramming of known differentiation-associated neutrophil states, particular precursor and immature neutrophils, reminiscent of emergency hematopoiesis. The assessment of the neutrophil compartment by SCO technologies is probably a prime example of the power of the application of high-resolution technologies to complete tissues including blood, when it comes to a fast, comprehensive understanding of pathophysiological processes. This then also allows to prioritize downstream analysis and potentially target identification for biomarker and therapies.

3.4. Transcriptional alterations in the megakaryocyte compartment

Recent findings strongly support innate-like functions of megakaryocytes and platelets [61,62]. A large enough longitudinal single cell multi-omics study identified an increase of megakaryocytes with an activated phenotype and a strong type-1 IFN signature in the peripheral blood of COVID-19 patients [63], which was later confirmed and extended to the identification of megakaryocyte progenitors [25]. The expression of megakaryocyte genes in the early hematopoietic stem cell-derived progenitor cells in peripheral blood and bone marrow from COVID-19 points towards emergency megakaryopoiesis, a phenomena also seen in other infections to replenish the peripheral platelet consumption during acute infection, both in patients and murine models [25,56,64]. Megakaryocytes expanded in COVID-19 patients have increased expression of *PKM2*, encoding a pyruvate kinase, which points towards higher pyruvate metabolism and glycolysis in this cell compartment during COVID-19 [63]. There is some indication that platelets, the product of megakaryocytes, promote thromboinflammation in SARS-CoV-2 pneumonia [65], but much more work is necessary to fully appreciate the role of molecular reprogramming of the megakaryocyte pool in COVID-19.

3.5. Persistently dysfunctional NK cells in severe COVID-19

For effective control of many viral infections cytokine production

and cell-mediated cytotoxicity by NK cells is essential [66]. Under physiological conditions, cytokine-producing $CD56^{bright}$ and cytotoxic $CD56^{dim}$ NK cells are the most prevalent subsets [67]. During acute COVID-19, there is a decrease in circulating NK cells, especially in severe COVID-19 patients [22,68]. NK cells present in the circulation have clear signs of activation as assessed by the surface expression of CD69 and CD38, which is increased in moderate and severe COVID-19 patients [45]. Longitudinal characterization of NK cells from acute COVID-19 patients by sc-RNA-seq identified high expression of IFN stimulated genes and a prolonged expression of genes involved in IFNa signaling in severe COVID-19, while TNF-related genes were upregulated in early moderate disease [22]. scRNA-seq data also predicted functional impairment (virus control, cytokine production and cell-mediated cytotoxicity) in severe COVID-19, which was supported by functional flow cytometry assays [22]. A subsequent study confirmed NK cell dysfunctionality and linked these to an unbalanced TGF-b response during the early phases of the infection, which altered the expression of genes related to cell-mediated cytotoxicity, granule exocytosis and cell-cell adhesion [68]. How more recent single cell transcriptome studies, describing hyper-activated NK cells and NK cell-platelet aggregates [69] or the appearance of a memory-like NK cell subset [70], are related to earlier studies requires further investigation.

3.6. Innate immune function of T cells during COVID-19

While the importance of T cells in antigen-specific immune responses required for viral control and clearance is unquestioned [71], single cell transcriptomics was critical in identifying innate immune functionality of T cells in COVID-19 [24]. Here, a subset of $CD4^+$ T cells, $CD8^+$ T cells as well as gamma-delta T cells all expressed CD16 on their surface. While natural killer T (NKT) cells, an unconventional T cell population recognizing antigen in context of CD1d molecules, are also known to express CD16 [72], they make out only a small proportion of the activated CD16 + T cells in COVID-19 patients [24]. These $CD16^+$ T cells were significantly elevated in severe COVID-19 in the circulation and the lung, but absent in influenza infection, and exerted high TCR-independent cytotoxic potential by immune-complex-mediated degranulation. CD16 expression on T cells was induced by the complement protein C3a and both elevated plasma levels of C3a and higher frequencies of $CD16^+$ T cells were associated with fatal outcome. The expression of CD16 on T cells was confirmed in a recent study also demonstrating that $CD16^+$ cells are activated via soluble multimeric immune complexes, which are present in ~80% of severe and critically ill COVID-19 patients [73].

Collectively, acute COVID-19 is characterized by reprogramming of all major innate immune compartments even inducing innate immune-like functionality in T cells and certain immune deviations within the innate immune system are directly linked to more severe or even fatal outcome of COVID-19, further underlining the importance to not only understand the adaptive arm of the anti-viral immune response, but also changes in the innate immune system.

Table 1
Definitions of Long COVID-19.

Name	Symptoms/ clinical manifestation	Time after COVID-19	COVID-19 severity	Reference
Post COVID-19 condition/ syndrome	fatigue, shortness of breath, cognitive dysfunction or other symptoms affecting everyday functioning	> 3 months, lasting at least 2 months	mild-severe	WHO, 2021 https://www.who.int/publications/i/item/WHO-2019-nCoV-Post_COVID-19_condition-Clinical_case_definition-2021.1
	fatigue, exertion intolerance, partly meeting the criteria for ME/CFS	4–15 months	mild-moderate	[91]
	fatigue, exertion intolerance, partly meeting the criteria for ME/CFS	7–19 months	mild-moderate	[86]
	respiratory symptoms, pulmonary abnormalities	3–6 months	severe	[94]
	Mainly cognitive and neurological symptoms	> 3 months	na	[84]
Post-acute sequelae of COVID-19 (PASC)	one or more of the following: constitutional, respiratory, cardiopulmonary, gastrointestinal, genitourinary, reologic, rash, musculoskeletal symptoms, trouble sleeping	> 3 months	mild-severe	[78]
	respiratory, gastrointestinal, neurological symptoms, anosmia/dysgeusia	2–3 months	mild-severe	[20]
	pulmonary and extrapulmonary symptoms	2–3 months	severe	[96]
	fatigue, respiratory, digestive, neurological, skin and mucous membrane, circulation symptoms	> 1 months	mild	[76]
	different symptoms including fatigue, dyspnea, respiratory, body aches	> 1 months	mainly mild-moderate	[79]
	respiratory symptoms, interstitial lung changes	3–12 months	mild-severe	[95]
	pulmonary and extrapulmonary symptoms	2–3 months	severe	[96]
	According to WHO case definition	> 6 months	mild-severe (mainly hospitalized)	[97]
Long COVID	fatigue, dyspena, chest pain	> 4 months	mild-severe	[77]
	breathing difficulties/breathlessness, fatigue/malaise, chest/throat pain, anxiety/depression, headache, myalgia, other pain, cognitive, abdominal symptoms	3–6 months	mild-severe	[71]
	33 symptoms based on WHO case definition for post COVID-19 condition	> 4 months	mild	[18]
	with and without cognitive symptoms (e.g. "brain fog")	na	mainly mild	[93]
	Physical symptoms (e.g. arthralgia/myalgia, fatigue, cough), exertion intolerance	12 months	severe	[80]
	e.g. fatigue, neurological, cardiopulmonary, gastrointestinal symptoms, trouble sleeping	~4 months	mild-severe	Peluso et al., 2022
	Fatigue, cough, shortness of breath	3–12 months	mild-severe	[85]
	One or more symptoms of the following e.g. pulmonary, systemic, neurological or psychiatric symptoms	12 months	mild and moderate/severe	[83]
Post-acute COVID-19 syndrome	different symptoms including fatigue, memory issues, loss of smell, abdominal pain	~7 months	mainly mild	[81]

4. Innate immune responses during convalescence and long COVID-19

Systems immunology approaches have also started to be applied to better understand recovery from acute COVID-19 and the development of long COVID [4,17–20], a heterogeneous clinical syndrome, which comes with different definitions and names and is still not well-defined (for more information see Table 1). For simplicity we use the term long COVID from here on. Gaining insight into the kinetics of recovery from COVID-19 is important to better understand deviations from the recovery process as seen in long COVID. The dynamics of recovery are rather patient specific and difficult to grasp when using chronological time. Applying computational modeling on longitudinally sampled blood transcriptomes from severe COVID-19 patients revealed common disease regression dynamics with reduction in neutrophils being the best predictor for convalescence, followed by an early rise in T cell activation and differentiation as well as the rebalancing between NF-κB and IFN signaling [16]. Normalization of cell numbers as assessed by SCMO was reported for circulating monocytes by an increase in non-classical monocytes and a reduction in classical monocyte frequency during convalescence [74,75]. While decreased HLA-DR expression is a hallmark of monocytes in severe COVID-19, in convalescent individuals *HLA-DR*^{lo}*S100A*^{hi} monocytes are absent and replaced by a monocyte subset with high antigen presentation capacity (high expression of *HLA-DQA* and *HLA-DPA*) [75]. However, in convalescent omicron patients, circulating immune cell type frequencies (e.g. activated monocyte subsets and megakaryocytes) as well as plasma cytokines and chemokines (e.g. CXCL10, CCL4, IL-9), remained altered around 42 days post-acute infection [76]. Patients fully clinically recovered from mild-to-moderate COVID-19 showed immune alterations even four months post-acute COVID-19, e.g. elevated pro-inflammatory plasma cytokines (IFN-β, IFN-λ1, IFN-γ, CXCL9, CXCL10, IL-8, soluble TIM-3) [77].

In contrast, in patients experiencing long COVID, IFN-β and IFN-λ1 remained elevated even 8 months after acute infection. Other studies described other sets of plasma cytokines (TNF, IFNγ, IL-10, IL-1β, IL-6, IL-12, IL-17) to be increased in patients with long COVID [78–80] further supporting the notion that long COVID is a rather heterogeneous syndrome with different endotypes or heterogeneous causes including persistent inflammation, viral persistence, autoimmune phenomena or a combination thereof [19]. Support for persistent inflammation as a cause of long COVID comes from studies describing elevated frequencies of circulating activated monocytes and pDCs [77]. Interestingly, a small observational study in SARS-CoV-2 infected patients with intestinal bowel disease (IBD) suggested viral persistence in gut mucosa derived from long COVID patients [81], a finding that certainly requires further investigation. An early single cell multi-omics study providing longitudinal data from acute infection until 2–3 months after infection suggested that risk factors for the development of long COVID include high level SARS-CoV-2 viremia, type 2 diabetes, Epstein-Barr virus reactivation during acute disease and the presence of autoantibodies [20]. Further validation of these intriguing findings are required to better understand whether the described patient subgroups are indeed endotypes of this heterogeneous syndrome. Another recent study links Epstein-Barr virus reactivation with a higher risk for the development of long COVID and especially fatigue and neurological symptoms [82]. Additionally, multiple studies have reported the association of autoantibodies with long COVID symptoms [83–85].

Major long COVID-associated symptoms resemble those of myalgic encephalomyelitis/ chronic fatigue syndrome (ME/CFS) [86]. ME/CFS is a complex, chronic disease, which is characterized by cognitive impairment, fatigue, post-exertional malaise but can also include chronic muscle pain and cardiovascular complaints [87,88]. The development of the syndrome is implicated with a preceded infection similar to long COVID [89,90]. Since precise biomarkers are lacking, the diagnosis of ME/CFS is difficult, mostly based on the clinical phenotype and the exclusion of other diseases. The molecular and cellular mechanisms underlying ME/CFS are not well understood. Signs of endothelial dysfunction were reported in long COVID patients with a ME/CFS like clinical phenotype, for example demonstrated by higher endothelin-1 plasma concentrations [91]. Given the broad overlap of symptoms between ME/CFS and long COVID similar pathophysiological mechanisms might occur, which could be identified by high-resolution technologies such as SCMO.

Other important clinical manifestations of long COVID are neurological alterations such as cognitive impairment and concentration deficits. Neurological symptoms have been described for both, acute COVID-19 and long COVID [92]. From a murine SARS-CoV-2 infection model inducing mild to moderate respiratory COVID-19 disease, we know that microglia/macrophage activation, depletion of oligodendrocytes, decreased myelination, and signs of impaired hippocampal neurogenesis are hallmarks of a prolonged disease phenotype persisting for more than 7 weeks post-acute infection [93]. At the same time, elevated cytokine levels including CCL11 in cerebrospinal fluid and plasma were associated with reduced neurogenesis and cognitive impairments. Elevated plasma levels of CCL11 were also identified in patients with lasting neurological symptoms, suggesting that a similar mechanism takes place in humans and mice [93]. Persistent neurological inflammation might be one mechanism explaining neurological symptoms in long COVID. A major challenge of future research will be the identification of risk factors predicting neurological symptoms, which occur only in a subset of long COVID patients. Worldwide efforts including the European consortium NeuroCOV (https://www.neurocov.eu/) are set up to define those molecular drivers of SARS-CoV-2-induced neuropathology that cause the manifestation of neurological symptoms and complications.

Most likely a different endophenotype of long COVID is characterized by prolonged pulmonary dysfunction, which seems to occur more frequently in patients with severe COVID-19, particularly after prolonged ICU treatment [94–96]. In these patients pulmonary symptoms persist for a prolonged period of time and are associated with alterations of the lung interstitium as assessed by computed tomography imaging [95]. Furthermore, plasma proteomics of these patients revealed cellular and molecular changes with enriched plasma IL17C levels and elevated numbers of neutrophils, which were characterized by increased expression of chemokines and neutrophil protease myeloperoxidase. Whether these changes are an effect of prolonged hospitalization and ICU treatment or specific for a protracted course of acute severe COVID-19 remains to be determined. There are indications that the risk to develop long COVID symptoms is linked to the severity of the acute disease. One study reported fewer mucosal CD8 + β7Integrin+ T cells and higher SARS-CoV-2 specific IgA levels in the circulation of long COVID patients with severe acute disease compared to milder acute disease [97]. Future studies need to further address, how other cellular and humoral characteristics of long COVID are connected to the severity of the acute disease.

Collectively, the heterogeneous syndrome long COVID following acute COVID-19 infections in a smaller subset of patients requires further research to better define patients at risk, determine endophenotypes, unravel different molecular mechanisms causing the myriad of clinical symptoms as a prerequisite for the development of patient-tailored therapy regimens. Large comparative therapy trials combined with biomarker platforms as they have been initiated for example in Germany (National Clinical Study Group for long COVID, https://long-coviddeutschland.org/nksg/) will be instrumental to reach these goals.

5. Conclusions and future perspectives

Systems level analysis of the human immune response to SARS-CoV-2 infection uncovered the major cells, pathways and genes involved in both, a productive immune response to the virus leading to protective immunity and even more so the immune deviations, particularly seen in

those patients with severe and critical disease trajectories. Systems approaches have the great advantage that all major components of the immune system are measured simultaneously, which allows to quickly determine a hierarchy of events at any given time of the infection and to prioritize mechanisms, pathways and genes for biomarker development but also to identify potential targets for treating immune deviation. While widespread clinical application of single-cell multi-omics was certainly in its infancies shortly prior to the start of the pandemic, this devastating world-wide event also triggered an accelerated use of these technologies and many hurdles that were anticipated to take years until solutions would be found, were overcome in much shorter time and leading to additional innovations. For us, one such innovation is the introduction of validation studies, when it comes to the clinical use of SCMO. Rather than integration of data from different sites, which is currently the preferred method for tissue atlasing, we are convinced that data obtained at different clinical sites should be treated as individual clinical trials or cohorts and used to validate findings from the other sites [21–24,63] (Fig. 1). One major result of SCMO-based systems approaches was the identification of disease stage- and severity-associated changes within the innate immune system in response to the SARS-CoV-2 infection [21]. Very surprising was the identification of innate functionality by cytotoxic $CD16^+$ T cells appearing within the major T cell compartments, namely $CD4^+$, $CD8^+$ and gamma-delta T cells [24]. Such findings are a particular strength of systems approaches since classical approaches, for example a classical gating strategy applied in flow cytometry, would probably have used CD16 expression to distinguish T cells from $CD16^+$ NK cells. In contrast, SCMO independently performed in two cohorts unequivocally established this T cell functionality. Further, functional validation experiments later confirmed the SCMO-predicted function.

Based on these exciting developments in clinical applications of SCMO-based technologies, two major directions require further attention. First, it needs to be determined if SCMO is only applicable during the exploratory phase and requires clinical validation of the initial findings via easier and more clinically applicable technologies. Or, if SCMO technologies can be further scaled and provided as much more cost-efficient technical solutions, with costs currently being one major hurdle for widespread clinical application. Second, even if these technologies would become widely clinically applicable, for a faster response towards (re-) emerging pandemic threats, we need to develop standardized and connected worldwide systems that would allow a fast and coordinated response when it comes to determine disease hallmarks and pathogen-specific immune responses and/or immune deviations. We have recently developed such a system, termed Swarm Learning [98] and are currently in the process to provide proof-of-principle that this AI- and block chain-based system could be used to build world-wide networks of hubs that are capable of measuring immune responses with highest resolution [99]. One big advantage of Swarm Learning is that there is no need to exchange data. Swarm Learning only exchanges insights, which makes working together across institutions and jurisdictions much easier.

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