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CHAPTER ONE

Pulmonary macrophages and SARS-Cov2 infection

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Abstract

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to the largest global pandemic in living memory, with between 4.5 and 15M deaths globally from coronavirus disease 2019 (COVID-19). This has led to an unparalleled global, collaborative effort to understand the pathogenesis of this devastating disease using state-of-the-art technologies. A consistent feature of severe COVID-19 is dysregulation of pulmonary macrophages, cells that under normal physiological conditions play vital roles in maintaining lung homeostasis and immunity. In this article, we will discuss a selection of the pivotal findings examining the role of monocytes and macrophages in SARS-CoV-2 infection and place this in context of recent advances made in understanding the fundamental immunobiology of these cells to try to understand how key homeostatic cells come to be a central pathogenic component of severe COVID-19 and key cells to target for therapeutic gain.

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1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in late 2019 and has to date (late summer 2021) led to over 200M infections and between 4.5 and 15M deaths globally from coronavirus disease 2019 (COVID-19), the clinical illness caused by SARS-CoV-2 infection (The Economist, 2021). Most people have a mild course of disease, but around 15% of cases manifest severe illness (defined by presence of hypoxia) and 5% become critically unwell with acute respiratory distress syndrome (ARDS), usually necessitating intubation and mechanical ventilation as supportive measures for respiratory failure (COVID-19 Treatment Guidelines, 2022). While several organs have the potential to be involved during COVID-19, clinical and pathophysiological abnormalities of the respiratory system, characterized by bilateral pulmonary infiltrates and progressive hypoxia, are a near universal manifestation of severe and critical disease. Principally transmitted by airborne spread, it is thought that SARS-CoV-2 gains access to the lower airways and alveoli via inhalation/aspiration from the upper airways. SARS-CoV-2 can enter host cells by the viral spike protein binding to ACE2 (Angiotensin converting enzyme 2) followed by cleavage by host proteases. In humans, ACE2 is highest expressed within nasal epithelial cells, followed by airway epithelium (particularly secretory and ciliated cells) with only low level expression in alveolar epithelium (<1% of alveolar epithelial cells (Sungnak et al., 2020)), despite the alveolus being the site of an extensive inflammatory reaction and epithelial injury in severe cases. This paradox of low level ACE2 expression within alveolar epithelium is most likely explained by the presence of additional attachment receptors at this site, with recent description of lectins fulfilling this role (Lempp et al., 2021).

Although severe respiratory disease secondary to pathogen infection is a well-recognized phenomenon and shared with many other pathogens (e.g., influenza, respiratory syncytial virus (RSV), bacterial pneumonia, etc.), some features of COVID-19 are sufficiently different to suggest that both shared and unique features were occurring in this disease. Unlike influenza, that mainly causes mortality in extremes of age (both young and old), the main risk factor for development of severe COVID-19 is increasing age, with younger age groups having a much lower (but not absent) risk of hospitalization and mortality. Several other host factors also influence the risk of severe disease, including male sex and the presence of co-morbidities such as obesity, hypertension and diabetes (Budinger et al., 2021). In addition, the duration from onset of symptoms to development of severe disease is

particularly long in COVID-19 infection being in the order of 10 days (nearly a week longer than the kinetics in influenza). These observations raised early suspicion that host factors were principally responsible for driving deleterious pulmonary inflammation and exacerbating organ damage, something that is now borne out by numerous studies of the host immune response as well as the reductions in mortality afforded by anti-inflammatory treatment with the glucocorticoid dexamethasone, as well as interleukin-6 receptor blockade (NEJM, 2021; The Lancet, 2021). There is now compelling evidence that dysregulated monocytes and macrophages are a key feature of severe COVID-19 (Velu et al., 2021). How and why these cells come to play such a deleterious role is incompletely understood. Here we will first review recent advances in our understanding of lung macrophage biology before discussing the role of monocytes and macrophages in SARS-CoV-2 infection. Due to the vast number of publications and pre-prints in this area, we focus on the pivotal findings that have informed our understanding of monocytes/macrophages in COVID-19.

2. Macrophages in the healthy lung

Before discussing how the macrophage compartment changes in context of SARS-CoV-2 infection, it is important to understand the composition of this compartment in health. The healthy lung harbors at least two populations of macrophages that occupy distinct anatomical niches. Alveolar (or airway) macrophages (AMs) are present in the bronchoalveolar space and are phenotypically, transcriptionally and functionally distinct from their counterparts in the lung interstitium (interstitial macrophage; IM). Importantly, neither lung macrophage population can be defined using the M1/M2 nomenclature that has become rooted in the macrophage biology field in the last 20 years. In the early 2000s, in an attempt to simplify the nomenclature of macrophage activation states, it was proposed that macrophages could be classified as M1 or M2, similar to the classification for helper T cells (Th1 vs. Th2) (Mills et al., 2000). M1 (or "classically" activated) macrophages were proposed to represent highly bactericidal, pro-inflammatory macrophages induced by IFNγ and/or endotoxin. In contrast, M2 (or "alternatively activated") macrophages were said to arise in response to IL-4 and adopt a "tissue repair" phenotype. As an extension of this, different flavors of M2 macrophages were proposed to differentiate in response to other cytokines, including IL-13, IL-10 or TGFβ (Mantovani et al., 2004). However, because macrophages are highly plastic and these "activation states" were mostly defined using *in vitro* culture systems

involving a single stimulus and often defined by a single cell surface marker, it is rather unsurprising that this binary classification system fails to capture the degree of macrophage diversity seen *in vivo*. Indeed, recent transcriptional profiling of macrophages across tissues shows that tissue resident macrophages can have both "M1" and "M2" features in the absence of any injury or insult (Gibbings et al., 2017; Lavin et al., 2014), again highlighting the inadequacy of this system. We agree with Nahrendorf and Swirski that the continued use of the M1/M2 nomenclature stifles discovery rather than promoting it and should be abandoned by the field (Nahrendorf and Swirski, 2016).

2.1 Alveolar macrophages

Much of what is known about the biology of lung macrophages has come from studies using mouse models. There is consensus that AMs in the healthy mouse lung can be defined by their high expression of CD11c, SiglecF and CD169, along with CD64 (high affinity Fcγr1) and Mer-tyrosine kinase (MerTK), a receptor that allows recognition and uptake of apoptotic cells (Gautier et al., 2012a; Mowat et al., 2017). In humans, AMs are defined as HLA-DR$^+$CD11b$^+$CD64$^+$CD169$^+$CD206$^+$ cells (Bharat et al., 2016). Despite the limited overlap of surface marker expression between mouse and human AMs, recent cross-species transcriptomic analyses have shown that there is conservation of large parts of the transcriptome between mouse and man. In particular, genes involved the lipid catabolism (e.g., *PPARG, FABP4, FFAR4, CES1*) are prominent features of this conserved program (Leach et al., 2020; McQuattie-Pimentel et al., 2021). This is consistent with the homeostatic role of AMs in the regulation of pulmonary surfactant, the protein-lipid rich complex produced by alveolar type II epithelial (AT2) cells to lubricate the luminal surface of the lung. AMs are indispensable for this function, as evidenced by accumulation of surfactant and the development of pulmonary alveolar proteinosis (PAP) in humans and mice in whom AM function is defective (Dranoff et al., 1994; Martinez-Moczygemba et al., 2008; Stanley et al., 1994; Suzuki et al., 2014; Willinger et al., 2011). Similarly, AMs are key for the removal of effete cells and particulate matter from the airways (Felton et al., 2018; Poon et al., 2014). AMs are in constant communication with the alveolar epithelium, dialogue that is mediated by a series of receptor ligand interactions (Fig. 1). These include CD200 (Snelgrove et al., 2008), CD172a (Janssen et al., 2008) and IL-10 (Fernandez et al., 2004) expression

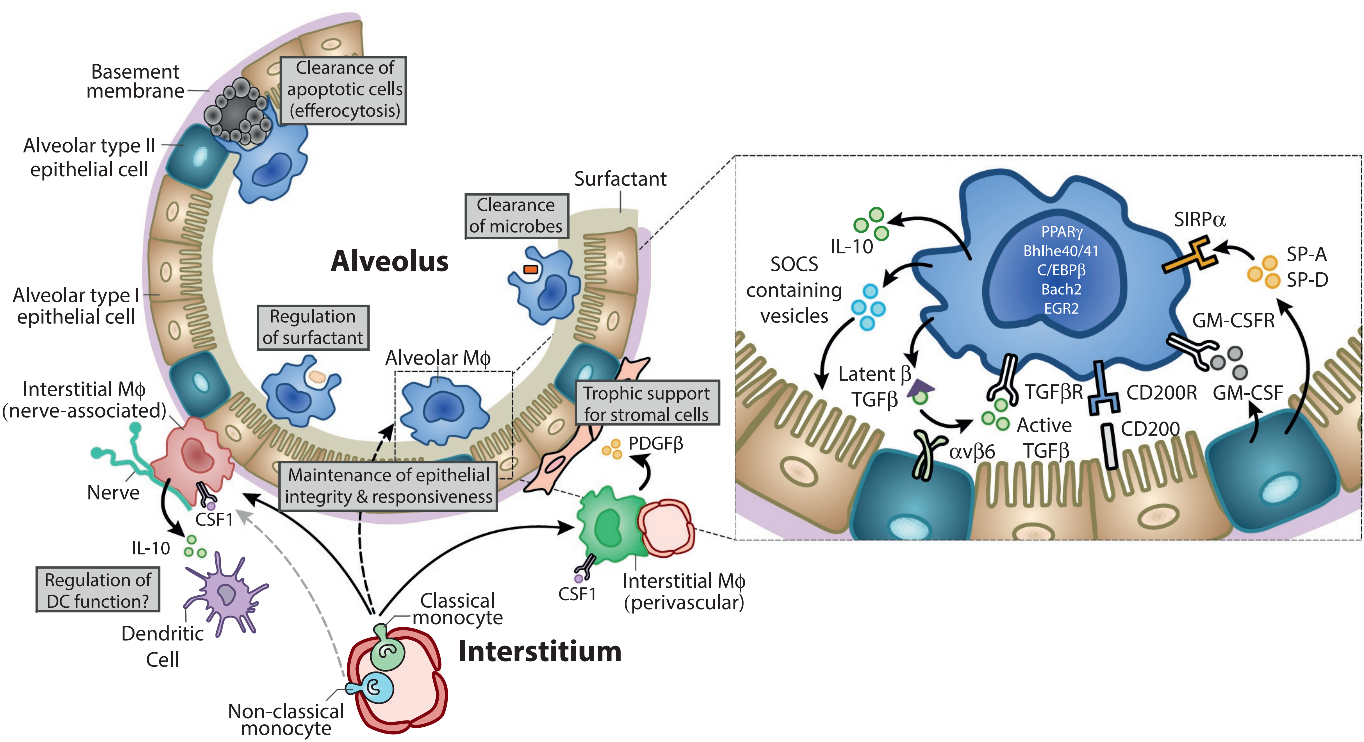

Fig. 1 See figure legend on next page.

by the alveolar epithelium that acts on corresponding receptors on AMs to regulate their responsiveness to exogenous stimulation to prevent unwanted and potentially damaging responses to innocuous material. Regulation is reciprocal with AMs releasing suppressor of cytokine signalling (SOCS)-containing vesicles which dampen the responsiveness of alveolar epithelial cells (Bourdonnay et al., 2015; Draijer et al., 2020). AMs may also help maintain the integrity of the epithelial barrier. For instance, under normal physiological conditions, AMs in both mouse and man produce fibronectin, which can act as a pro-survival and proliferative factor for airway epithelium (Han and Roman, 2006). Another conserved feature is the expression of key scavenger receptors, such as macrophage receptor with collagenous structure (MARCO) which is crucial for the recognition and uptake of a variety of respiratory pathogens, including *Streptococcus pneumoniae, Cryptococcus neoformans* and *Mycobacterium tuberculosis* (Arredouani et al., 2004; Dorrington et al., 2013; Xu et al., 2017). Genetic ablation of key apparatus for pathogen recognition or selective depletion of AMs in mouse models renders the host

Fig. 1 Composition, homeostatic functions and regulation of pulmonary macrophages. Alveolar macrophages (AMs) in the bronchoalveolar space of the lung are crucial for maintaining patency of the alveolar space, where they regulate surfactant levels and phagocytose inhaled microbes and other particulate materials. AMs are intimately associated with the alveolar epithelium and control the renewal and integrity of the barrier, as well as remove dead or senescent cells. AMs release suppressor of cytokines (SOCS)-containing vesicles that act to control the responsiveness of alveolar epithelial cells to exogenous stimulation. In turn, alveolar type II (AT2) epithelial cells produce GM-CSF to control the development and maintenance of AMs. Together with autocrine TGFβ, which is activated by epithelial αvβ6 integrin, GM-CSF induces expression of the transcription factor PPARγ, which in turn induces EGR2 and C/EBPβ, which drive particular aspects of alveolar macrophage differentiation. Bhlhe40 and Bhlhe41 control the identity and the self-renewal capacity of alveolar macrophages. The transcription factor Bach2 is also implicated in AM development. Epithelial-derived surfactant can also alter alveolar macrophage function by, e.g., altering phagocytosis. The interstitial macrophage (IM) compartment is heterogeneous with nerve-associated and blood vessel-associated (perivascular) macrophages, both of which appear to depend on CSF1, although the source of CSF1 is unclear. The homeostatic functions of interstitial macrophages are poorly understood, although they constitutively produce high levels of IL-10 which may alter the behavior of conventional dendritic cells (cDC). They also produce growth factors such as PDGFβ which may support fibroblasts. The transcription factors controlling IM differentiation and function remain largely unexplored. While originally seeded by embryonic progenitors, the IMs are replaced by circulating monocytes progressively with age. The contribution of monocytes to the steady state maintenance of alveolar macrophages remains controversial and to be determined with certainty.

more susceptible to a variety of infectious agents, demonstrating the key role these cells play in protective immunity.

AMs develop from fetal liver-derived monocytes in the first few days of life in parallel with alveolarization of the lung (Bharat et al., 2016; Guilliams et al., 2013; Tan and Krasnow, 2016), in a process highly dependent on granulocyte-macrophage colony stimulating factor (GM-CSF, also known as CSF-2) from AT2 cells (Gschwend et al., 2021; Schneider et al., 2014a). As such, mice or humans in whom the GM-CSF-GM-CSFR axis has been disrupted have considerably fewer mature alveolar macrophages and those present are dysfunctional, resulting in PAP development (Dranoff et al., 1994; Robb et al., 1995; Stanley et al., 1994; Suzuki et al., 2008, 2014). Autocrine transforming growth factor beta (TGFβ) also plays a crucial role in AM development (McCowan et al., 2021; Yu et al., 2017). GM-CSFR and TGFβR signalling induces expression of key transcription factors, including PPARγ (Baker et al., 2010; Gautier et al., 2012b; Malur et al., 2011; Schneider et al., 2014a), PU.1 (Shibata et al., 2001), C/EBPβ (Cain et al., 2013), Bhlhe40/41 (Rauschmeier et al., 2019) and Bach2 (Nakamura et al., 2013) to drive the niche-specific identity and function of AMs. Moreover, we have recently shown the transcription factor early growth response 2 (EGR2) to be indispensable for the identity and immune protective features of AMs (McCowan et al., 2021).

As discussed elsewhere in this review series, the ontogeny of tissue macrophages has received considerable attention in recent years with the realization that some tissue macrophages can derive from yolk sac mesenchyme and persist for long periods of time with little or no replenishment from blood monocytes. This has led to a revision of the long-accepted linear model of the mononuclear phagocyte system in which tissue macrophages were thought to be maintained exclusively through replenishment by blood monocytes, although it is important to note that some early studies reported the long-lived nature of tissue macrophages, including lung alveolar macrophages, and proposed that they may maintain themselves autonomously (Tarling et al., 1987). Indeed, alveolar macrophages can and do proliferate under normal physiological conditions (Hashimoto et al., 2013). However, whether *in situ* self-renewal is sufficient to sustain the alveolar macrophage compartment remains somewhat controversial. Studies using monocytopenic *Ccr2*$^{-/-}$ mice and parabiotic mice support the idea that alveolar macrophages maintain themselves independent of blood monocytes throughout adulthood (Hashimoto et al., 2013), findings supported by tracing of alveolar macrophage origin in human sex mismatched lung transplant

studies (Eguíluz-Gracia et al., 2016). However, this is at odds with longitudinal fate mapping using *Flt3*^{Cre}- and *Ms4a3*^{Cre}-based reporter mice, which allow tracing of hematopoietic stem cell (HSC)-derived and granulocyte-monocyte progenitors (GMPs)-derived cells, respectively (Gomez Perdiguero et al., 2015; Liu et al., 2019), as well as fate mapping using tissue protected bone marrow chimeric mice (Bain et al., 2016). Moreover, a more recent study using single cell RNA sequencing (scRNA-seq) to examine AM repopulation dynamics in sex mismatched lung transplant tissue has shown that AMs are largely replaced by recipient monocyte-derived cells (Byrne et al., 2020). Thus, more work is needed to understand the parameters that control alveolar macrophage maintenance even in the absence of inflammation, infection or injury.

2.2 Interstitial macrophages

Interstitial macrophages (IMs) are embedded in the lung parenchyma between the alveoli and capillary beds. Their high phagocytic and bactericidal capacity suggests they may represent an additional layer of innate protection below the basement membrane (Gibbings et al., 2017; Schyns et al., 2019). Furthermore, they may provide trophic support for fibroblasts, endothelia and epithelia through production of mediators such as platelet-derived growth factor beta (PDGFβ) and amphiregulin (Brody et al., 1992; Schyns et al., 2019). While the AM compartment appears to be relatively homogenous in health, the IM compartment is more heterogeneous. Consensus is yet to reached on the best approach to define discrete subsets of these cells, but it is clear that, in mice, they all express high levels of CD11b, CD64 and MerTK, with at least two independent populations defined by their expression of CD206 (mannose receptor). CD206^{-} IMs express high levels of MHCII and associate with nerve fibers. In contrast, CD206^{+} IMs generally lack MHCII expression, but express CD36 and Lyve-1, and are enriched in the perivascular regions of the lung parenchyma (Chakarov et al., 2019; Gibbings et al., 2017). The phenotype and function of human lung IMs is less well-defined owing to difficulty in obtain healthy lung tissue for these analyses. Nevertheless, a limited number of studies have shown that these cells are defined by their HLA-DR^{+}CD68^{+}CD11b^{+}CD206^{+} profile (Bharat et al., 2016; Leach et al., 2020), consistent with recent work by the Willinger group using so-called 'MISTRG' humanized mice (Evren et al., 2021). Moreover, discrete IM subsets may exist in the human lung defined by their levels of HLA-DR and CD36 expression and there appears to be a

degree of conservation of gene expression between mouse and human (Leach et al., 2020). Whether these subsets occupy distinct anatomical niches in the lung interstitium is not yet known. Functionally, studies in mice have shown that $CD206^{-}$ IMs are more capable of supporting the differentiation of regulatory T cells, which is consistent with their expression of MHCII and likely aided by their constitutive production of IL-10 (Sabatel et al., 2017; Schyns et al., 2019).

In contrast to AMs, IMs originally develop from yolk sac-derived progenitors (Tan and Krasnow, 2016) but these are displaced, and by adulthood all interstitial macrophages derive from HSC in the adult mouse (Gomez Perdiguero et al., 2015). When combined with the fact that there is only low-level replenishment of interstitial macrophages in *Cx3cr1*$^{\text{Cre-ERT2}}$. *Rosa26* reporter mice (Schyns et al., 2019), in the context of parabiosis (Tan and Krasnow, 2016) or tissue protected bone marrow chimeric mice (Gibbings et al., 2017; Hawley et al., 2018), this suggests HSC-derived macrophages seed the lung in late embryonic development or early post-natal development and become relatively long-lived. Notably, despite the heterogeneity in surface phenotype, transcriptional profile and anatomical positioning of interstitial macrophage subsets, they appear to have similar replenishment kinetics (Chakarov et al., 2019; Gibbings et al., 2017; Schyns et al., 2019).

Finally, in addition to AMs and IMs, the mammalian lung also appears to have a population of intravascular macrophages, although notably these are not present in rodents (Brain et al., 1999; Dehring and Wismar, 1989; Evren et al., 2021; Longworth, 1997). Transcriptional profiling of these macrophages from humanized mice has shown them to express molecules involved in iron recycling (e.g., *CD163, SLC40A1, SLC48A1*) and the transcription factor *SPIC* (Evren et al., 2021), which controls the differentiation program of red pulp macrophages in the spleen. Thus, intravascular macrophages may allow filtering of the blood in the lung, similar to that occurring in the spleen and liver. Whether they play additional roles in homeostasis, and indeed disease, remains to be investigated.

3. Inflammation as a driver of COVID-19 severity

Early during the pandemic it was appreciated that the development of severe COVID-19 did not relate solely to an increased viral presence within the airways (Merad and Martin, 2020; Rubin et al., 2021). Indeed, a report from our own group, analyzing autopsy collected tissue from decedents that

had succumbed to severe disease, explored SARS-CoV-2 organotropism by multiplex PCR, viral sequencing and *in situ* viral S protein immunohistochemistry. This revealed that the duration of illness (from symptom onset to death) was not correlated with the number of organs with viral RNA present, and within lung tissue viral presence was not directly linked to the presence and severity of pulmonary inflammation (Dorward et al., 2021). This evidence of virus-independent immunopathology, rather than direct pathogen-mediated organ inflammation and injury, as a primary driver of severe COVID-19 was swiftly followed by the discovery that immunosuppression with corticosteroids (dexamethasone) limits death in severe cases with established hypoxic respiratory failure (NEJM, 2021). Interestingly, that report demonstrated that patients with a longer duration of symptoms (who were more likely to have had established respiratory failure) and those receiving invasive mechanical ventilation had the greatest benefit, while in patients not on oxygen there was a trend towards worse outcomes with dexamethasone treatment. Overall, these data are consistent with a requirement for early robust inflammation in response to infection, but in later stage disease inflammation directly contributing to disease severity (Velu et al., 2021). Understanding what "type" of inflammation drives disease pathogenesis remains critical to understanding how best to intervene with therapeutics in clinical trials and has been a major focus of COVID-19 research.

4. Macrophage as mediators of organ damage in COVID-19?

4.1 Defining the macrophage compartment in COVID-19

While many groups rapidly delineated the cellular and soluble mediator profile of circulating blood from COVID-19 patients, it is clear that these peripheral abnormalities are not always reflective of the changes occurring within inflamed lung tissue (Dress and Ginhoux, 2021). As described above, whole lung tissue is exceedingly challenging to acquire (especially out with autopsy sampling of fatal cases), whereas sampling of the airways via acquisition of bronchoalveolar lavage fluid (BALF) allows for analysis of luminal cells, with repeated sampling also possible. An early report detailing scRNA-seq of BALF cells from three patients with moderate COVID-19 and six patients with severe disease demonstrated significant abnormalities within the macrophage compartment (Liao et al., 2020). Most notably, tissue resident (*FABP4*$^+$, *MARCO*$^+$) AMs were depleted during COVID-19,

with severe disease typified by a marked expansion of elicited macrophages (Liao et al., 2020). These were divided into three clusters based on transcriptional profiles; group 1 expressed typical monocyte markers (e.g., *S100A8/MRP8, FCN1* and *CD14*), group 2 had high level expression of chemokines (e.g., *CCL2, CCL3, CXCL10*) and group 3 expressed immunoregulatory and pro-fibrotic genes (e.g., *SPP1, TREM2*). Analysis of whole lung tissue from autopsy cases of fatal COVID-19 has also demonstrated marked changes in macrophage compartment of non-luminal pulmonary tissue. Specifically, there is an accumulation of monocytes and macrophages frequently expressing MRP8/S100A8 (a subunit of the calcium binding protein calprotectin) consistent with monocytic origin and an IM rather than AM phenotype (Dorward et al., 2021). In addition, a pulmonary arteritis (inflammation of blood vessel walls) was also observed in around half of analyzed cases, with mononuclear cells being the predominant infiltrating cell type. Whether this contributes to the endothelial dysfunction and high rate of thrombosis seen in COVID-19 (Bing et al., 2021; Dolby et al., 2021), or is merely a secondary response to endothelial injury, remains to be clarified. In keeping with a key role for monocytes and macrophages in severe COVID-19, an elegant study analyzing host genetic variants associated with critical illness identified that high genotype-inferred expression of the monocyte–macrophage chemotactic receptor CCR2 is associated with severe COVID-19 (Pairo-Castineira, 2020). Since these early reports, numerous studies have implicated macrophages and their products in the pathogenesis of severe SARS-CoV-2 infection, and some of the more notable studies will now be discussed (Fig. 2).

A longitudinal analysis of immune cells and soluble factors in blood and BALF fluid, investigating different severities of COVID-19, found that disease severity was associated with increased abundance of chemokines involved in myeloid/monocyte recruitment including CCL4, CCL2 and CXCL9 and also soluble complement factor C5a generation (Carvelli et al., 2020). The C5a receptor (C5aR1/CD88) was highly expressed on both blood and BALF neutrophils and monocytes and multiplex IHC of lung tissue from fatal COVID-19 showed expansion of macrophages ($CD68^+$/$CD163^+$ cells), many of which also expressed C5aR1. In addition, arteritis was also observed in this study and was associated with infiltration of these $C5aR1^+$ macrophages around the arteries and within thrombus. The potential therapeutic benefits of targeting the C5a–C5aR1 axis was also tested in a pre-clinical model of lung injury with the monoclonal antibody avdoralimab that prevents C5a binding to C5aR1. Although experimental lung injury was induced in

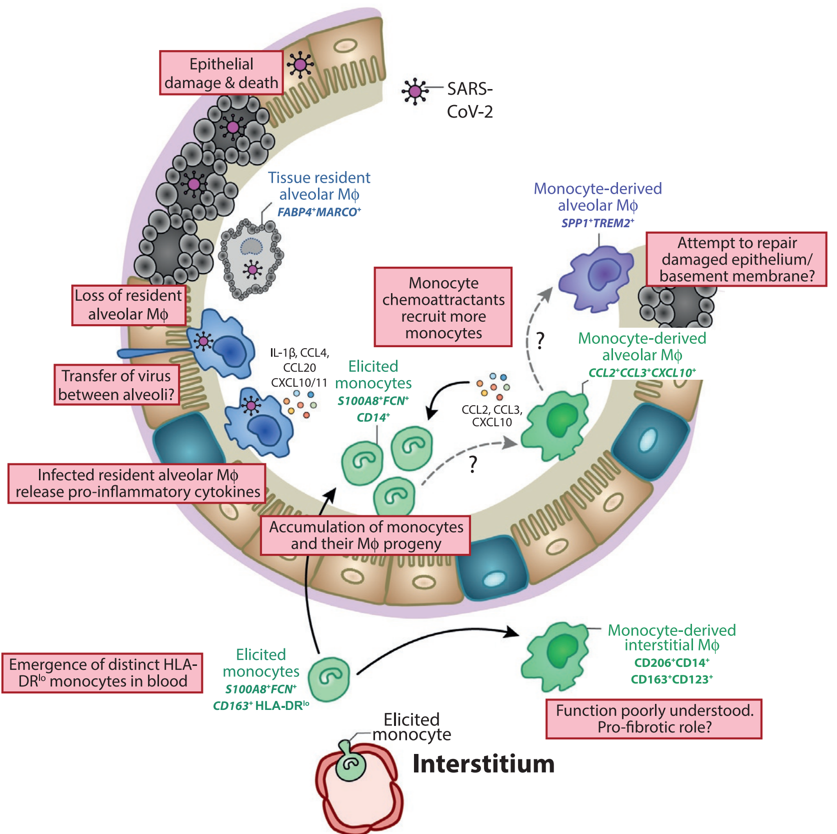

Fig. 2 Composition of pulmonary macrophages during severe COVID-19. In severe COVID-19, tissue resident alveolar macrophages (AMs), defined by expression of *FABP4* and *MARCO*, are diminished in number. Whether this occurs in response to direct infection or due to the hyperinflammatory environment is unclear. Alternatively, because there is epithelial cell damage and death, this could reflect a loss of survival signals for resident AMs. Infected tissue resident AMs express higher levels of *IL1B, CCL4, CCL20, CXCL10* and *CXCL11* than their uninfected counterparts, which leads to monocyte (and T cell; not shown) recruitment. Given that AMs have been shown to be able to transit between alveoli, it has been suggested that AMs may facilitate the spread of SARS-Cov-2 within the lung. Monocytes accumulate in vast numbers in the bronchoalveolar space, as do transcriptionally distinct macrophage subsets. Current evidence supports the idea that these macrophages are derived from recruited monocytes, although the development relationship, if any, between macrophages displaying pro-inflammatory features (*CCL2, CCL3, CXCL10*) and pro-fibrotic features (*SPP1, TREM2*) is unclear. Elevated chemokine production creates a positive feedback loop to recruit more monocytes, thereby maintaining the inflammatory response. The significance of *SPP1*$^{+}$*TREM2*$^{+}$ macrophages is unclear, although they could represent an attempted response to repair the damaged barrier. Monocytes and their progeny also accumulate in the lung parenchyma, although their role in COVID-19 pathogenesis and disease outcome is unclear. Importantly, circulating monocytes in severe COVID-19 are phenotypically and transcriptionally distinct from their counterparts in health, suggestion changes in monocyte output at the level of hematopoiesis in the bone marrow.

human C5aR1 knock-in mice (as avdoralimab is human specific) and in response to local delivery of recombinant C5a (and thus limiting the generalizability of the findings), several parameters of lung injury were attenuated with avdoralimab treatment (Carvelli et al., 2020). In an elegantly planned and executed study, Szabo and colleagues performed longitudinal profiling of paired peripheral blood and BALF cells from patients with severe COVID-19 (average 6–7 paired samples per patient) and complemented this with analysis of autopsy lung tissue (Szabo et al., 2021). Spectral flow cytometry based phenotyping alongside transcriptomic and cytokine profiling revealed that aberrant $CD163^{+}$ and $HLA\text{-}DR^{lo}$ monocytes predominated in COVID-19 peripheral blood, with these populations expressing CCR2. In health, myeloid populations in blood and airways were phenotypically distinct, whereas COVID-19 airway myeloid cells displayed a significant phenotypic overlap with circulating blood myeloid subsets, suggesting myeloid cell infiltration from blood to airways. Consistent with this, CCL2, CCL3 and CCL4 (among other cytokines and chemokines) were elevated in airways, with CCL2 and CCL3 undetectable in blood. Furthermore, airway myeloid cells themselves expressed high levels of CCL2, CCL3, and CCL4 transcripts and airway monocytes and macrophages (along with airway T cells) exhibited hyperinflammatory transcriptional signatures. Analysis of whole lung tissue confirmed a significant increase in numbers of $CD163^{+}$ monocytes and macrophages, but airway transcriptomics did not demonstrate high expression of genes associated with proliferation (e.g., Ki67). Overall these data supported active myeloid recruitment from blood to lung as a major mechanism that may directly exacerbate inflammation and organ injury in severe COVID-19 (Szabo et al., 2021). These findings have been independently replicated by another group, whereby BALF macrophages had increased transcriptional similarity to circulating monocytes in COVID-19, again suggesting a differential origin of alveolar macrophages (monocyte derived) in SARS-CoV-2 infection (Stephenson et al., 2021). Similarly, targeted proteomics of autopsy lung and splenic tissue from our own group has demonstrated elevated abundance of pulmonary CCL7 (MCP-3), along with enhanced splenic CSF-1 in COVID-19, consistent with monocyte/macrophage production at extrapulmonary sites followed by recruitment into lung tissue (Russell et al., 2022).

One of the major outstanding questions in COVID-19 is how much of the observed alterations in immune responses is a response to widespread epithelial injury, and therefore likely to be shared between other causes of severe lung injury/inflammation (e.g., influenza, sepsis, bacterial pneumonia), and how much is truly specific and unique to SARS-CoV-2

infection. In one of the few studies that has directly analyzed and compared whole lung tissue from different pulmonary insults, Rendeiro et al. performed high-parameter imaging mass cytometry to allow cellular and spatial profiling of pulmonary tissue at single cell resolution (Rendeiro et al., 2021). They compared a small cohort of 23 patients that died from either ARDS after influenza, ARDS after bacterial infection, acute bacterial pneumonia, early or late COVID-19 or non-pulmonary disease (control/normal lung tissue) to understand the cellular changes in the lungs during COVID-19 progression. Imaging mass cytometry was performed using a panel of 36 metal-labeled antibodies to delineate a spatial map of more than 660k cells at 1 μm resolution and was complemented by spatial transcriptomics. This revealed that immune infiltration in the COVID-19 lungs was quantitatively comparable to the other lung infections. However, qualitative differences were observed, with a significant increase in myeloid cells within COVID-19 lungs, with IMs (defined as $CD14^+/CD16^+/CD206^+/CD163^+/CD123^+$ cells) having the greatest expansion in COVID-19. Interestingly, neutrophil numbers were similar in early COVID-19 and the healthy lung with reduced neutrophil numbers in late COVID-19. How this reduction in neutrophils in late disease relates to ongoing inflammation and/or failed inflammation resolution and pulmonary repair requires further investigation. Additional immune-specific phenotyping showed increased levels of the calprotectin subunit S100A9/MRP14 in macrophages, neutrophils and alveolar epithelial cells, and complements the other studies discussed previously which reported increased calprotectin S100A8/MRP8 within monocytes and macrophages (Dorward et al., 2021; Liao et al., 2020). Out with immune lineage cells a reduction in endothelial cells was observed (consistent with the previously observed concurrent endothelial injury (NEJM, 2020)) and increased numbers of fibroblasts and collagen deposition noted in the COVID-19 groups, with obvious implications on recovery and pulmonary fibrosis from severe COVID-19.

4.2 The role of macrophage subsets in SARS-CoV-2 infection

Most of what is known about human pulmonary immunity in response to SARS-CoV-2 relates to severe or fatal disease, whereas most infections lead to mild disease. Thus, it remains incompletely understood if monocyte-macrophage expansion also occurs in mild disease and if these elicited macrophages have direct anti-viral effects and/or if they are responsible

for orchestrating epithelial damage that has occurred. However, limited analysis of "mild" COVID-19 shows that tissue resident AMs are lost, albeit to a lower extent than in severe COVID-19, and replaced by monocyte-derived macrophages. The loss of tissue resident macrophages in the setting of inflammation is a well described phenomenon, often referred to as the macrophage "disappearance reaction." Indeed, a common feature of murine models of lung bacterial, viral and fungal infection, as well as sterile injury, is the transient loss of AMs (Ghoneim et al., 2013; McCowan et al., 2021). In the context of SARS-CoV-2 infection, this could be an effect of infection of AM since viral S protein has been detected with macrophages and both S positive epithelial cells and S positive macrophages appear to display elevated levels of cleaved caspase-3 (Dorward et al., 2021; Grant et al., 2021; Rendeiro et al., 2021). Infection of AMs may occur following uptake of infected epithelial cells or through direct infection. Although *in vitro* studies suggest that macrophages do not support productive viral replication (Yang et al., 2020), recent scRNA-seq studies in which viral transcripts can be identified suggest AMs can and do become directly infected (Grant et al., 2021). While the loss of AM seems somewhat counterintuitive given that tissue resident macrophages are thought to be immune effector cells, apoptosis of AMs is required for efficient killing of certain bacterial pathogens (e.g., *Streptococcus pneumoniae, Haemophilus influenzae*) (Preston et al., 2019). Whether loss of tissue resident AMs in the context SARS-CoV-2 infection represents a killing mechanism is unknown, but something that warrants further investigation. Alternatively, loss of resident AMs may occur as a collateral result of hyper-inflammation, for instance in response to elevated IFNγ levels, as has been described in murine models of influenza A infection (Califano et al., 2018). Indeed, the presence of IFNγ-producing T cells is another defining feature of severe COVID-19. However, it is important to note that AMs are not bystanders in the anti-viral response. SARS-CoV-2-infected AMs produce higher levels of pro-inflammatory cytokines *IL1B* and monocyte and T cell chemoattractants, *CCL4* and *CXCL10,* than their uninfected counterparts (Grant et al., 2021). This is consistent with the role of resident AMs in other viral infections, such as influenza and RSV (Goritzka et al., 2015; Schneider et al., 2014b), where they are responsible for the recruitment of anti-viral monocytes through production of type I interferons and monocyte chemoattractants (Goritzka et al., 2015; (Kumagai et al., 2007)). Whether monocytes and their progeny are directly anti-viral in COVID-19 is also unclear. Observations from mouse models

may help to unravel this, with multiple mouse models of SARS-Cov-2 infection having been developed (Bao et al., 2020; Hassan et al., 2020; Israelow et al., 2020; Jiang et al., 2020; Sun et al., 2020). Importantly, monocyte/macrophage dynamics replicate observations in humans, with loss of tissue resident AMs and accumulation of monocytes and their progeny in response to elevated monocyte chemoattractants induced by type I interferons (Israelow et al., 2020). That monocytopenic *Ccr2*$^{-/-}$ mice have elevated viral burden when infected with mouse adapted SARS-CoV-2, suggests that elicited monocytes/macrophages may have a direct anti-viral role (Vanderheiden et al., 2021). However, this is complicated by the fact that monocytes and macrophages fail to accumulate in *Ifnar1*$^{-/-}$ mice yet viral clearance is unaffected. Instead, *Ifnar1*-deficiency is associated with significantly reduced inflammation, demonstrating the key role of type I interferons in driving the excessive inflammatory response through monocyte recruitment, consistent with earlier work in SARS-CoV-1 infection (Channappanavar et al., 2016). Continual recruitment of inflammatory monocytes may hinder the regeneration process. In support of this, previous studies had identified that ongoing inflammation, especially mediated by IL-1β, is capable of impairing alveolar epithelial regeneration by inhibiting the maturation of alveolar type I (AT1) cells and arresting them in in a damage-associated transient progenitor state (DATPs) (Choi et al., 2020; Kobayashi et al., 2020). Transcriptional profiling of alveolar epithelial cells during COVID-19 has revealed that alongside extensive epithelial destruction, initiation of regeneration also occurs. However, an incomplete transition of AT2 to AT1 cells is observed with increased numbers of epithelial cells with a transcriptional signature of DATPs (KRT8, CLDN4 and CDKN1A) in COVID-19 lungs (Melms et al., 2021). IL-1β has been noted to be highest expressed in pulmonary monocytes in "early" COVID-19 (up to 30 days from infection onset) (Rendeiro et al., 2021) and peripheral blood monocytes isolated from COVID-19 patients release mature IL-1β in response to viral S protein (alongside nigericin and after differentiation into macrophages by CSF-1) unlike healthy blood monocytes (Theobald et al., 2021). Thus, in early disease inflammatory monocytes and macrophages may directly worsen epithelial injury by liberation of inflammatory mediators such as IL-1β which subsequently impacts upon epithelial regeneration and exacerbates inflammation.

Until now, we have discussed the evidence that monocytes and their derivatives play a key role in lung inflammation and pathology in COVID-19. However, lessons from other pre-clinical models of lung

injury, including our own work, supports the notion that monocytes can adopt distinct functional profiles across a disease course. For instance, monocyte-derived AMs are proposed to be central to fibrogenesis following exposure to bleomycin, yet these same cells are indispensable for efficient lung repair in this model (Cui et al., 2020; Joshi et al., 2020; McCowan et al., 2021; Misharin et al., 2017). Indeed, the presence of transcriptionally distinct populations of monocyte-derived macrophages in the COVID-19 BALF suggests that monocytes can assume different effector profiles as they mature into macrophages. It could be that these cells occupy distinct niches, some of which support the differentiation of inflammatory macrophages, with others supporting a tissue repair phenotype. Indeed, as mentioned above, monocyte-derived macrophages with key pro-fibrotic characteristics, including *SPP1, TREM2, TGFB1* and *FGL2* expression have been identified (Liao et al., 2020; Wauters et al., 2021). Alternatively, these subsets of macrophages could reflect cells at different stages of a differentiation trajectory or cells that have arrived at distinct stages of the infection course. Whether *SPP1*$^{+}$ macrophages are involved in tissue repair or if they are indicative of an overactive fibrotic response following severe lung injury is not clear. However, given that pulmonary fibrosis appears to be a lasting effect of COVID-19 in a subset of individuals, the balance of these subsets may have a role in determining outcome from COVID-19.

5. Targeting the macrophage in severe COVID-19: Current and future therapeutics?

How does what we have learnt from studying macrophages in COVID-19 inform rationale choice of therapeutic agents for large scale clinical trials, and do the drugs that have already shown mortality benefits in severe COVID-19 act via effects on macrophages? While the broad spectrum anti-inflammatory glucocorticoid dexamethasone was the first pharmaceutical to show improved mortality in severe cases, the exact mechanism of action remains to be defined. We do know that dexamethasone can have several effects on macrophages including upregulation of the efferocytosis receptor MerTK which can enhance clearance of dead and dying cells (efferocytosis) generated during injury and can impact upon tissue repair (Bosurgi et al., 2017; Felton et al., 2018; Liu et al., 1999; McColl et al., 2009; Zagórska et al., 2014). In addition, dexamethasone can suppress pro-inflammatory cytokine production by macrophages including IL-6 and IL-1 (Heasman et al., 2004) by well investigated but as yet not completely

understood mechanisms (Barnes, 2017). Indeed, it has been proposed that glucocorticoid treatment of macrophages results in a unique pro-resolution type phenotype (McColl et al., 2007). Similarly, IL-6 receptor blockade reduces mortality in hospitalized COVID-19 patients with hypoxia and systemic inflammation; whether this acts on macrophages or macrophage-liberated IL-6 as a primary mechanism remains to be determined. But can more targeted therapeutics aiming to disrupt inflammatory macrophages be beneficial? This could take the approach of interfering with recruitment, their longevity within inflamed pulmonary tissue or blocking liberation of pro-inflammatory molecules identified in the above studies (Velu et al., 2021). Interestingly, both blocking (Temesgen et al., 2021) and augmenting (Bosteels et al., 2020) GM-CSF has been reported as having promise while preliminary studies on the tolerability of C5 blockade in human COVID-19 are beginning to appear (Zelek, 2020), but further studies to test therapeutic efficacy are awaited. Supporting the notion that blocking monocyte/macrophage pro-inflammatory molecules could be beneficial, a recent report evaluated the use of the IL-1α/β inhibitor anakinra (a recombinant version of the human interleukin 1 receptor antagonist protein) (Kyriazopoulou et al., 2021). By identifying patients at greatest risk of disease progression (using circulating soluble urokinase-type plasminogen activator receptor levels (suPAR)), and then intervening with anakinra (or placebo) alongside usual standard of care including dexamethasone, this demonstrated reduced mortality and length of hospital stay in the anakinra treated group.

It is likely that the approaches being taken to understand the basic biology of COVID-19 immunopathology will assist with prioritization and repurposing of existing therapeutics for ongoing clinical trials. The combined approaches of using human disease samples and pre-clinical models married to state-of-the-art technologies has led to an unprecedented pace at deciphering pulmonary immunity in SARS-CoV-2 infection. This serves as a paradigm to approach other infection-driven and pulmonary inflammatory diseases, especially when these continue to cause substantial global disability and premature mortality (WHO, n.d).

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