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Editorial

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Does autonomous macrophage-driven inflammation promote alveolar damage in COVID-19?

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SARS-CoV-2 has caused devastating effects with over 550 million infections by July 2022 and approximately 6.4 million deaths [1]. Societal and economic impacts will reverberate for years, with continuous evolution of SARS-CoV-2 as it persistently spreads through the human population as exemplified by reduced activity of vaccines and monoclonals against Omicron BA.4 or BA.5 subvariants [2]. A greater understanding of pathogenesis and more tailored therapeutic approaches are therefore essential.

Central to pathogenesis has been the spread of disease from the upper airway to the lower airway, due to the lack of upper respiratory tract mucosal immunity controlling viral translocation. In severe cases this results in hypoxemia and respiratory failure. Pathogenesis resembles acute respiratory distress syndrome (ARDS), but also shows some differences. Post-mortem studies have demonstrated key features of ARDS such as diffuse alveolar damage, thrombosis and bronchopneumonia were frequent but variable findings [3]. However, expansion of interstitial macrophages in the lung parenchyma occurs to a greater extent in COVID-19, compared to influenza or other causes of ARDS [4]; and the burden of microthrombus formation is greater in COVID-19 compared to influenza [5]. Induction of robust and persistent pulmonary inflammation by SARS-CoV-2 is associated with life-threatening disease. Indeed, clinical trial platforms demonstrated improved survival with anti-inflammatory therapies (e.g. glucocorticoids and interleukin-6 receptor blockers) in hospitalised patients who are hypoxemic[6, 7]. In post-mortem studies SARS-CoV-2 spike (S) protein was found in the respiratory epithelium, but pulmonary inflammation did not map well to areas of viral persistence lung, suggesting the existence of autonomous inflammatory circuits at the time of death from respiratory failure [3].

Establishing the extent of lower airway viral replication, the cells and receptors that support productive viral infection and the extent to which direct viral replication as opposed to autonomous inflammation drives lung injury have been key questions. Establishing functional models of viral replication has been a major requirement to supplement inference based on expression data and associative studies. Many studies have identified low-level and restricted expression of angiotensin converting enzyme 2 (*ACE2*), the major receptor implicated in SARS-CoV-2 cell binding, in the alveolar space, but reports have varied widely in terms of the exact levels of expression. As highlighted by Ziegler and co-workers, inferring expression from single-cell RNA sequencing (scRNA-seq) data for low-copy transcripts such as those for ACE2 and the protease transmembrane serine protease 2 (*TMPRSS2*) implicated in processing the viral S protein that interacts with ACE2 is problematic, since low sensitivity of detection may result in an underestimate of real expression and protein levels may not correlate with transcription [8]. Multiple stimuli induce *ACE2* expression in the human lung, including type 1 interferons (IFN-1s) [8] or cigarette smoke [9]. In addition, other receptors (e.g. Basignin (BSG), Asialoglycoprotein receptor 1 (ASGR1), Kingle-containing transmembrane protein 1 (KREMEN 1) and proteases (e.g. Cathepsin L, Furin) may allow SARS-CoV-2 cell entry on the basis of their interactions with related coronaviruses, from *in vitro* studies of SARS-CoV-2 or in some cases from *in vivo* mouse studies of COVID-19 [10, 11].

Hönzke and co-workers used sc or single-nuclear (sn) RNA-seq to show that ACE2 was only expressed at low levels in approximately 1.5/1000 alveolar epithelial type (AT) 2 but not

other cells in alveoli [12]. This contrasted with higher level expression of *TMPRSS2*, *Furin*, *BSG*, *ASGR1*, and *KREMEN1* in a broader range of cell-types. Importantly, they validated protein expression in tissue explants, COVID-19 post-mortem lung tissue, and adult human stem cell-derived bronchial organoids. IFN-β (an IFN-1 subtype) failed to upregulate ACE2 at the protein level in contrast to induction of mRNA. The findings align with Wang and co-workers, who found similarly low levels of ACE2 by snRNA-seq in healthy lung tissue, primarily in AT2 cells, and identified a low number of chromatin sites co-accessible with the ACE2 promoter [13]. This suggested limited sites with open chromatin, denoting a hallmark of *cis*-regulatory elements that bind combinations of transcription factors to regulate spatiotemporal patterns of gene expression limit expression [14].

Hönzke and co-workers also found that SARS-CoV-2 replicated with only moderate efficiency in lung tissues and with less efficiency than influenza A virus (IAV) or Middle East respiratory syndrome coronavirus (MERS-CoV). SARS-CoV-2 replication was only observed in bronchial organoids (which lack AT2-like cells with constitutive *ACE2* expression) after genetic manipulation to induce *ACE2* expression. Similarly, in alveolar organoids which contained AT2-like cells expressing *ACE2* in low number, low-level viral replication was confined to $ACE2^{+}$ cells. A strong cytosolic staining pattern for virus was specific to $ACE2^{+}$ cells in both $ACE2^{+}$ bronchial and alveolar organoids. In contrast, alveolar macrophages (AM) had only punctate staining for viral protein or RNA, which the authors ascribed to phagocytosis/efferocytosis of AT2 cells or endocytosis of virus. Conversely, adenoviral transfection of *ACE2* in lung explants broadened cell tropism. In post-mortem COVID-19 lung specimens, evidence of virus was infrequent with viral replication in rare AT2 cells and low-level viral staining in macrophages in keeping with viral uptake rather than productive infection. In summary, the findings suggest ACE2 is the receptor for productive infection, which is confined to occasional AT2 cells, while lung macrophages take up virus but do not support viral replication. However, some other studies have suggested lung macrophages can support viral replication. Sefik and co-workers found evidence of markers of viral replication (subgenomic RNA, double-stranded RNA and viral RNA-dependant RNA polymerase (RdRp)) in lung macrophages in humanized mice infected with SARS-CoV-2, validating RdRp findings in post-mortem human lungs [15]. Grant and co-workers identified anti-sense viral RNA in AM from bronchoalveolar lavage (BAL) of intubated patients with severe COVID-19 [16]. The reasons for the varying findings are not clear. However, Sefik and co-workers found that ACE2 blockade dramatically reduced viral uptake by macrophages, while Hönzke found that if lung macrophages had forced expression of *ACE2*, they became productively infected, suggesting that the absence of *ACE2* expression in lung macrophages in the models studied underpin Hönzke's findings. Sefik also identified potential uptake of virus via CD16 and antibody-dependent uptake, which can contribute to uptake of viral particles in macrophages. Sefik and co-workers also suggest induction of pyroptosis limits viral replication in infected macrophages [15]. Therefore, it remains plausible that replicative infection in lung macrophages is rare if it occurs, and that the major drivers of macrophage activation are detection of S protein, low fucosylation of the Fc region of anti-S IgG antibody, viral RNA in ingested virus and indirect cellular stress induced by the virus, as sensed by detection of DNA by the cGMP-AMP synthase-stimulator of interferon genes pathway or other inflammatory pathways [17-19].

These intriguing results suggest that alveolar viral replication is not the major driver of alveolar injury. Hönzke and co-workers mined single-cell and bulk transcriptional data from their infected lung explants and post-mortem COVID-19 lung tissue to illustrate lung macrophages demonstrated viral uptake. *In vitro*, SARS-CoV-2, along with MERS-CoV and SARS-CoV, induced distinct AM transcriptional responses from IAV, with prominent induction of IFN-stimulated genes (ISG) and inflammatory responses in cells with viral uptake. There was a loss of AM and their replacement by inflammatory macrophages in SARS-CoV-2 infected explants and post-mortem lungs, consistent with findings in humanised mice [20]. There were also clusters of activated macrophages and a population of MERTK^{+} monocyte-derived macrophages. Viral endocytosis was seen in inflammatory macrophages, the population of macrophages most associated with ISG expression. There was also evidence for early induction of IL-1β and chemokines (e.g. CCL3, CCL20, CXCL5, CXCL8) in inflammatory macrophages. It was suggested that activated macrophages may have ingested infected AT2 cells by efferocytosis, and cells with viral uptake were associated with enhanced *NF-κB, TNF* and *IL-1* expression. There was induction of the inflammasome activator P2RX7 which would also aid processing of IL-1β. These data therefore strongly support an early pro-inflammatory macrophage response in response to viral products. Although viral replication in alveoli may not be a direct driver of injury, viral load is associated with systemic inflammation and clinical outcome, supporting the concept that this macrophage response is primed proportional to viral load [21].

This important study raises many questions for future investigation. While the functional consequences of ACE2 were explored with genetic approaches, the direct role of macrophages and specific inflammatory pathways in driving alveolar injury will require confirmation by manipulation in relevant models. In a recently published humanized mouse model of COVID-19, it was confirmed that SARS-CoV-2 induced weight loss, lung pathology, ISG induction in association with expansion of inflammatory macrophages and monocyte-derived macrophages, and reduction in CD206^{+} AM [20]. Both dexamethasone and early therapeutic administration of SARS-CoV-2 mAbs reduced viral-associated perturbations in macrophage populations in association with improved outcomes. The exact identity of the activated and inflammatory macrophage populations will require further characterisation. Rendeiro and co-workers found that interstitial macrophages of monocyte origin were the major population expanded in the COVID-19 lung [4] while monocyte-derived macrophages predominated in BAL in severe COVID-19 [22], features that appear distinct from severe IAV infection. What are the relative contributions of macrophages derived from recently recruited monocytes or of interstitial macrophages replicating in the lung to the expanded interstitial macrophage population? Is there evidence of replication and reprogramming of resident AM in the inflammatory niche of the COVID-19 lung to the inflammatory and activated macrophage phenotypes responding to virus? These questions could benefit from lineage-tracing approaches and measurement of *in situ* replication. To what extent does differential ontogeny or changes in environment altering macrophage plasticity underpin COVID-19-related alveolar macrophage phenotype [23, 24]? Mechanisms of AM loss require clarification. Apoptosis pathways were upregulated in Hönzke's study, but is this the primary mechanism of loss of AM? Sefik and co-workers have demonstrated NLRP3 inflammasome activation and pyroptosis of lung macrophages containing virus, which may be an additional mechanism of cell loss [15]. Genetic variation in *DPP9*, a negative regulator of the inflammasome, is associated with risk of critical illness with COVID-19 [25]. The

mechanism though of rapid large-scale loss of resident AM within a couple of days of infection [20], a finding we also observed in K18 *ACE2* transgenic mice (McHugh and Dockrell unpublished data), suggests loss of the original long-lived yolk-sac originating fetal liver-derived population of AM. Which viral recognition pathways mediate the inflammatory responses in macrophages? And what is the role of altered oxidative stress and mitochondrial dysfunction? Recently, mitochondrial fission, altered mitochondrial oxidative phosphorylation, reactive oxygen species generation and hypoxia inducible factor-1α expression have been identified in lung macrophages during COVID-19 [26].

In addition to these questions, it is important to consider how does the change in macrophage populations in the alveolar space, and the ongoing inflammatory environment, imprint future responses in macrophages. Is there loss of plasticity? Are there consequences for future infections, and do alterations in macrophage phenotype influence the resolution of inflammation and tissue repair with consequences for the development of lung fibrosis? In IAV infection in mice, the monocyte-derived macrophages that are recruited into the alveolar space persist over time, but their phenotype reverts gradually to one resembling the original resident AM, but long after resolution of infection [27]. Are the kinetics of this transition prolonged in COVID-19? In addition, Wendisch and co-workers identified accumulation of $CD163^+$ monocyte-derived macrophages with profibrotic transcriptional phenotype that resemble profibrotic macrophages in idiopathic pulmonary fibrosis. The extent to which this phenotype which expresses MERTK arises because of direct uptake of virus or from altered macrophage ontogeny is an open question as recently debated [28]. Irrespective of all the questions simulated by Hönzke's manuscript, this study suggests perturbations in AM populations offset the tight homeostatic control exerted by resident AM in the healthy lung and promote circuits of autonomous inflammation that may explain the alveolar damage in severe COVID-19 independent of direct viral induced cytopathology (Figure 1).

Figure 1: Perturbed macrophage populations in the distal lung in pathogenesis of alveolar injury in Covid-19
AT1: alveolar epithelial type 1 cells; AT2: alveolar epithelial type 2 cells; AM: alveolar macrophage; MDM: monocyte-derived macrophage; IM: interstitial macrophage.

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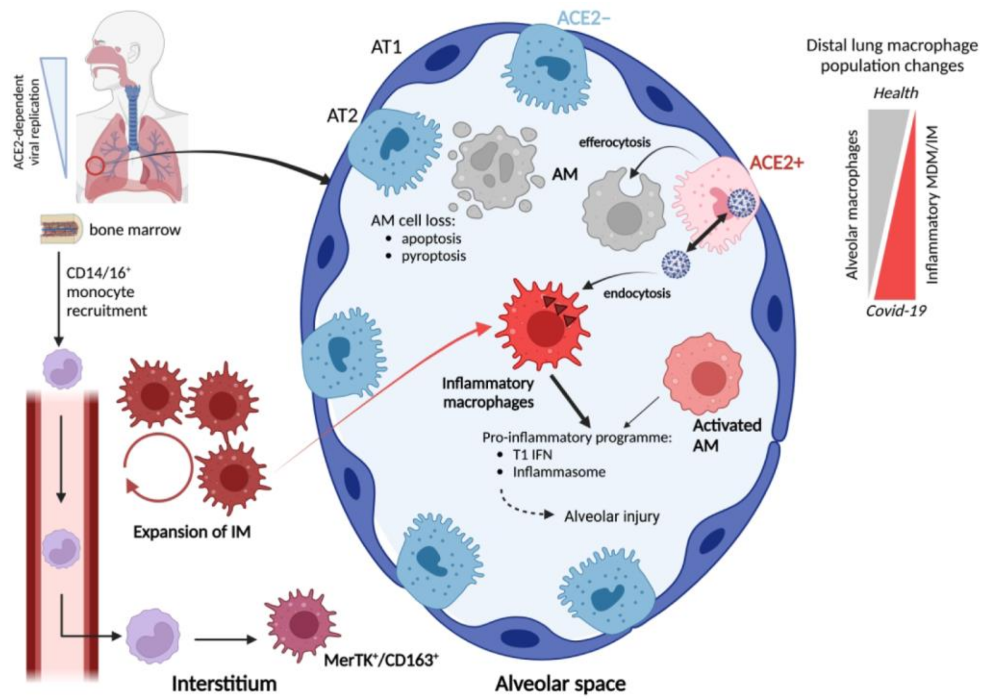

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Figure 1: AT1: alveolar epithelial type 1 cells; AT2: alveolar epithelial type 2 cells; AM: alveolar macrophage; MDM: monocyte-derived macrophage; IM: interstitial macrophage.