Translating known drivers of COVID-19 disease severity to design better SARS-CoV-2 vaccines

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The SARS-CoV-2 pandemic has highlighted how an emergent disease can spread globally and how vaccines are once again the most important public health policy to combat infectious disease. Despite promising initial protection, the rise of new viral variants calls into question how effective current SARS-CoV-2 vaccines will be moving forward. Improving on vaccine platforms represents an opportunity to stay ahead of SARS-CoV-2 and keep the human population protected. Many researchers focus on modifying delivery platforms or altering the antigen(s) presented to improve the efficacy of the vaccines. Identifying mechanisms of natural immunity that result in the control of infection and prevent poor clinical outcomes provides an alternative approach to the development of efficacious vaccines. Early and current evidence shows that SARS-CoV-2 infection is marked by potent lung inflammation and relatively diminished antiviral signaling which leads to impaired immune recognition and viral clearance, essentially making SARS-CoV-2 'too hot to handle'.

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Inflammation: good or bad for infections and vaccines?

The SARS-CoV-2 pandemic has resulted in an estimated 248 million cases worldwide with over 5 million deaths [1]. Though healthy adults and children generally clear infection with mild to no symptoms, they are able to spread the virus to more vulnerable populations (elderly, obese, diabetic, immunocompromised, etc.) who are more susceptible to developing severe COVID-19 disease. A wealth of literature has been published on SARS-CoV-2 infection and one common theme observed is that severe COVID-19 is a disease characterized by production of potent inflammatory mediators including IL-1b and IL-6, both in the lungs and systemically [2,3,4**,5,6]. While inflammation is a necessary component of protective immune responses to viral infection, too much inflammation and/or the wrong type of inflammation can lead to poor outcomes. Likewise in vaccination, inflammation is observed to be part of the signature of a good vaccine response [7–10]. However there are multiple types of inflammation (Interferon-driven, NF-κB driven, MAPK driven, Type 1 versus Type 2) and which types of inflammation are good versus bad at promoting protective immune responses is highly dependent on the nature of the pathogen. So how do we go about investigating what is good inflammation in SARS-CoV-2 vaccination and leveraging this to develop better vaccines? One approach is to leverage Systems Immunology to identify the immune signatures during vaccination which are associated with vaccine efficacy. Arunachalam *et al.* used this approach to identify the components of the immune response which modulate efficacy of the Pfizer-BioNTech BNT162b2 mRNA vaccine. They showed that boosting of this vaccine resulted in significant increases in polyfunctional CD4 and CD8 T cell responses, as well as a potent induction of interferon and inflammatory signaling in monocytes. Interestingly, this inflammation was only present after the boost and did not persist a week after boosting. Could the lack of sustained inflammation account for the waning immunity observed post-vaccination? How can we promote and sustain a proper inflammatory response to SARS-CoV-2 vaccination? Only detailed studies of the approved SARS-CoV-2 vaccines can answer these questions but such studies and data are not currently available.

Approved SARS-CoV-2 vaccine platforms

Six COVID-19 vaccines have been approved by the WHO which span four different platforms and have efficacies ranging from 50 to 95%. The four platforms are: 1) the Moderna and Pfizer mRNA vaccines [11,12]; 2) the Johnson & Johnson human Adenovirus (AD26.COV 2.S) [13]; 3) the AstraZeneca AZD1222 chimpanzee Adenovirus (ChAdOx1 vector) vaccine [14]; and 4) the Sinopharm and Sinovac-CoronaVac inactivated virus vaccines [15,16]. The first three platforms encode for the full-length prefusion stabilized spike protein. That efficacy varies among the platforms even though the antigen is

similar, with the mRNA platforms having the highest efficacy, supporting the idea that there are specific immune pathways induced by vaccination which promote protection and vary by platform.

The development of more broadly efficacious vaccines is critical for continuing efforts to fight the SARS-CoV-2 pandemic. The emergence of variants including the delta (B.1.617.2) and omicron (B.1.1.529) variants have already begun to raise questions about the efficacy rates of current COVID-19 vaccines [17••,18,19,20••]. It has been shown that the neutralizing activity of vaccine-induced antibodies is reduced against variants including the delta, beta (B.1.351), gamma (P.1), kappa (B.1.617.1) and mu (B.1.621) variants [21–25]. Compounding this scenario is the fact that herd immunity has not been, and may never be, reached by a combination of vaccination and natural infection due to a multitude of factors including limitations on vaccine availability around the globe, the fact that high vaccine efficacy rates are benchmarked against preventing severe disease not infection/transmission, vaccine hesitations/concerns and preexisting medical conditions. Herd immunity is a population state in which a large enough percentage of individuals are immune to infection such that it nearly abrogates disease transmission between individuals thus providing protection of the whole population to the spread of disease. Common examples of vaccine-induced herd immunity are measles, mumps, and smallpox for which infections do still occur but they are not able to spread. Higher rates of infection and transmission associated with SARS-CoV-2 variants further limits the potential for herd immunity. As a result, SARS-CoV-2 viruses continue to circulate, inevitably giving rise to new variants which will further impact the efficacy of approved vaccines. This may lead to a seasonal infection similar to Influenza and the other endemic β-Coronaviruses. So where do we go from here to develop a better vaccine?

Adjuvants as a means to the end of better vaccine efficacy

Altering the antigenic component of a vaccine is not the only way to improve the efficacy of the response. The components of the vaccine platform themselves, including delivery methods, formulation, and adjuvant, can directly impact how the immune system responds to the vaccination and modulate whether or not protective responses are induced. Recent results from Arunachalam et al. showed that modifying the adjuvant included with a RBD protein subunit can modulate immune responses including Ab magnitude and breadth of variant neutralization, T cell responses and protection from viral challenge supporting the idea that inclusion of adjuvants which drive the proper protective immune responses is critical for enhancing efficacy [26••]. Similar differences in vaccine efficacy based on adjuvant inclusion were shown, specifically, for Alum and MF59 in the context

of SIV/HIV [27,28•] as well as TB and Influenza [29]. This work, done by our group and others, demonstrates that Alum and MF59 induce distinct inflammatory immune profiles during vaccination which directly modulates the efficacy of vaccine responses; whether this modulation promotes or reduces vaccine efficacy is platform and pathogen dependent. That distinct adjuvants induced a different magnitude and quality of the SARS-CoV-2 (and others) vaccine response isn't surprising, yet the expedited nature of SARS-CoV-2 vaccine development meant no testing was performed to study the impact of adjuvants. Understanding the inflammatory pathways which drive protective COVID-19 vaccine responses and how these pathways are differentially modulated by available vaccine platforms and adjuvants is critical to the development of next generation COVID-19 vaccines. Unfortunately, the data does not currently exist to comprehensively compare the protective immune responses across the currently approved COVID-19 vaccine platforms. In the absence of such data, how can we begin to understand the key inflammatory cells and pathways important for vaccine responses to SARS-CoV-2?

Informative and relevant knowledge could be garnered from studies of an efficient natural immune response triggered upon SARS-CoV-2 infection and which would lead to the protection from dissemination of infection and from the development of poor clinical outcomes in infected subjects. Understanding the dysregulation of natural immunity during infection will provide insights into immune pathways which contribute to pathology and thus might not be effective at preventing infection. At the same time, immune pathways which are suppressed in severe cases compared to mild or asymptomatic cases could represent key targets to boost the immune response and improve vaccine efficacy. We will now discuss a critical immune mechanism associated with severe disease that can help inform on developing more efficacious vaccines.

Inflammation is a hallmark of COVID-19 disease progression

COVID-19 is characterized by progressive inflammation, lung damage, and lymphopenia which culminate in severe respiratory distress and cytokine storm in patients with severe COVID-19 disease [2,3,4••,5,6]. It has become evident that the SARS-CoV-2 virus utilizes multiple subversions of the human immune system including driving inflammation and the inhibition of the antiviral innate interferon responses [30,31]; this promotes viral persistence, mainly in individuals with pre-existing comorbidities (age, high BMI, diabetes, etc.) or who are immunocompromised, by driving an aberrant immune response that is not capable of controlling the SARS-CoV-2 virus within the first 1–2 weeks of infection, as is normally the case for most infected individuals. Dampened interferon (IFN) responses during acute infection *in*

vitro and *in vivo* distinguish SARS-CoV-2 from other respiratory infections including Respiratory Syncytial virus (RSV) and Influenza A virus (IAV) [32••,33]. Select human clinical trials using IFN treatment have shown efficacy in reducing disease severity, disease longevity, recovery from symptoms and mortality, indicating that interferons may be critical for limiting COVID-19 disease [34–36]. This early IFN signaling is responsible for priming cells towards an anti-viral state that allows the control of viral dissemination. In the absence of a potent induction of antiviral interferons stimulated genes (ISGs), SARS-CoV-2 virus can persist in the upper and lower airway epithelium leading to infectious spread to the circulation and progressive COVID-19 disease. It is known that inflammasomes and IFNs regulate each other; inflammasomes antagonize IFN responses by cleaving cGAS to limit IFN production downstream of cGAS/STING; IFN signaling has been shown to suppress inflammasome activation in response to multiple stimuli including the adjuvant Alum [37–39]. Thus, scales tipped too far in favor of inflammation over IFN signaling could be a major contributing factor to the severity of acute infection, the subsequent down regulation of effective innate and adaptive immune responses, and disease progression. So then, what is the source of this deleterious inflammation?

Progression to severe COVID-19 is highlighted by a local and systemic inflammatory response and the subsequent degeneration of respiratory function. Evidence from SARS-CoV-2 infected patients highlight inflammasomes and IL-1β as potential critical mediators of this inflammation [5]. Inflammasomes are cytosolic, multi-protein complexes which mediate activation of inflammatory caspases and the eventual processing of pro-IL-1β and pro-IL-18 into their biologically active forms. Inflammasomes can be activated in response to a wide range of stimuli, including bacteria [40–44], fungi [45–47] and host DAMPs [48,49] as well as, to a lesser extent, viruses [50–52]. These cytokines are potent inflammatory regulators which drive leukocyte chemotaxis, activation, and differentiation. IL-1β in tissue activates potent neutrophil and macrophage responses to combat extracellular infection and cellular dysfunction caused by tissue damage [46,53–56]. In line with this, altered neutrophil and macrophage responses are hallmarks of COVID-19 lung damage and disease progression [57,58,59••,60–62]. Evidence of inflammasome activation is also present in the blood of patients with severe COVID-19 [63•,64].

Inflammasome activation also routinely induces a lytic form of cell death called pyroptosis which is mediated by cleavage of Gasdermins, canonically Gasdermin-D, and their insertion as multimeric pores into cellular membranes [65,66]. The resulting perturbation in ion homeostasis (K+ efflux and Ca2+ influx/mobilization) leads to cellular rupture and release of inflammatory components normally sequestered within the cytosol of the cell. One of these components, Lactate Dehydrogenase or LDH, is a marker for COVID-19 disease severity and progression supporting that inflammasome induced cell death is a driver of COVID-19 pathology [67,68•,69]. Heightened cell death induced by inflammasomes combined with the capacity of IL-1β to promote chemokine production and extravasation of cells into tissues could explain the acute and persistent lymphopenia observed in COVID-19 patients [70–72]. Systemically, IL-1β mediates its effects through direct binding to the cell as well as through inducing production of secondary inflammatory mediators including IL-6 and CRP [73–75], both of which have been identified as critical determinants of disease progression and severity [74,76–79]. With this mounting evidence for inflammasome signaling being a central player in COVID-19 disease progression, the next step would be to identify potential cellular sources of SARS-CoV-2-dependent inflammasome activation.

Lung inflammation spreads from epithelial cells to infiltrating cells

Given that the upper airway and lungs are the primary target of SARS-CoV-2 infection, it is logical to hypothesize that cells resident to and/or infiltrating into the lungs would be the initial source of this inflammasome activation. *In vitro* infection of human monocytes with SARS-CoV-2 induced inflammasome activation and cell death [63•,80]. Yet, airway epithelial cells are the main targets of natural SARS-CoV-2 infection, not monocytes.

To understand how IL-1 and inflammasome signaling is activated and spreads among the first line of immune defense, we probed publicly available datasets of single cell RNA sequencing of bronchioalveolar lavage fluid (BALF) [81••]. Liao *et al.* found that in severe COVID-19 disease there is heightened infiltration of macrophages and neutrophils into the BALF. To confirm these findings, we mined transcriptional profiles from the Human Cell Atlas and Human Protein Atlas to encapsulate a wider set of cells and visualize clusters using UMAP. Visual comparison of cluster frequency between healthy, mild, and severe disease shows accumulation of epithelial cells, neutrophils, and a shift in the monocyte population (Figure 1a). Quantitative assessment of frequencies of cell clusters shows significant increase in the frequency of epithelial cells within BALF from patients with severe disease compared to healthy controls and mild disease (Figure 1b). Gene Set Enrichment Analysis (GSEA) was performed to identify if IL-1 and inflammasome related pathways were being differentially modulated in the epithelial cells from severe COVID-19 as compared to mild COVID-19 and healthy controls. Indeed, we observed heightened expression of key pathways of IL-1 signaling and NF-κB inflammation in epithelial cells from severe COVID-19 compared to mild disease or healthy controls (Figure 1c). Multiple key inflammatory

Figure 1

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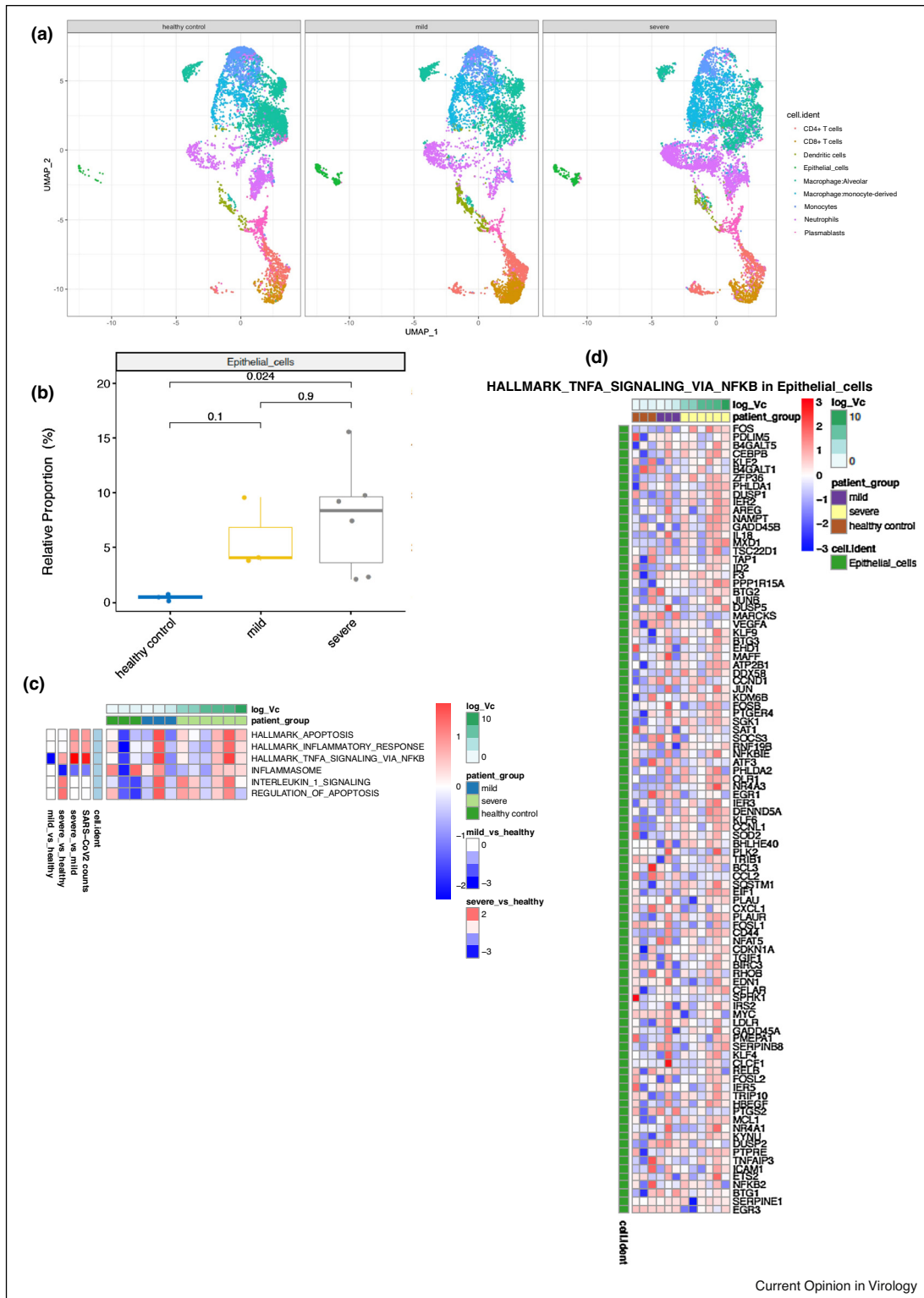
(Figure 1 Legend) Epithelial cells in the BALF of severe COVID-19 patients are elevated in frequency and have heightened inflammatory signaling. Single cell sequencing of the BALF from three healthy controls and three mild and six severe COVID-19 patients was analyzed. Cells were clustered using UMAP and cell identify annotated using the Human Cell Atlas and Human Protein Atlas. **(a)** UMAP visualization of cells/clusters split into healthy control, mild and severe COVID-19. **(b)** The frequency of epithelial cells in the BALF is significantly enhanced in severe disease compared to healthy controls, with a similar trend seen in mild versus healthy controls. **(c)** Gene Set Enrichment Analysis (GSEA) was performed to identify how pathways of inflammation and IL-1 signaling compared between severe disease and mild disease or healthy controls. Inflammatory signaling including by NF-kB is significantly increased in severe disease. **(d)** Heatmap of expression of the leading edge genes from NF-kB signaling which are enhanced in severe disease. Comparison of cluster frequencies was performed using paired-Wilcoxon sum rank test.

mediators (including IL-18, CXCL1, CCL2) and transcriptional regulators (including KLF4, FOS, JUN, MYC, NFKB2, NFKBIE) were among the leading-edge genes from the Hallmark NF-κB pathway (Figure 1d). Interestingly, expression of genes in the INFLAMMASOME pathway, including CASP1 which encodes for Caspase-1, are reduced in severe disease. The simultaneous heightened IL-1/inflammatory signaling and diminished inflammasome expression in epithelial cells, normally upregulated by inflammatory signaling, suggests that epithelial cells in severe disease which express inflammasomes die; the resulting inflammatory signaling then spreads to the remaining neighboring epithelial cells.

Analysis of infiltrating leukocyte populations revealed that there were significantly elevated neutrophils in severe disease compared to mild or healthy controls (Figure 2a). Increased monocyte derived macrophages and decreased alveolar macrophages are a feature of both mild and severe disease, with severe disease showing more significant modulations (Figure 2b). Pathway analysis for inflammatory and IL-1 pathways reveals that inflammatory pathways are significantly elevated in severe versus mild disease for all subsets of monocytes/macrophages probed and neutrophils. Thus, not only are there more neutrophils and monocyte derived macrophages in severe disease but these cells are also in a heightened inflammatory status. IL-1 signaling pathway genes upregulated in neutrophils during severe disease include IL-1B itself, suggesting a potential additional source of IL-1β *in vivo*, early targets (IL1RN, HMGB1) and regulators of IL-1/NF-kB signaling (IL1R1, IRAK1/3, RELA, NFKB1/2, NFKBIA). In this way, severe COVID-19 disease is marked by the infiltration of circulating neutrophils and monocytes, which become macrophages, that sense the inflammation driven by IL-1 signaling in epithelial cells and become primed for inflammatory responses. These potently activated neutrophils and macrophages can then mediate tissue damage through the production of proteases (MMPs, Elastase, etc.) which degrade extracellular components, cytokine production and cell death. Cell death releases inflammatory mediators which further exacerbate cell activation and tissue damage. Inflammation generated in the lungs can then spread to the periphery, as evidenced by previous discussion of systemic markers of inflammasome and inflammation, where a cytokine storm drives progressive dysfunction and disease severity. Inflammasome

activation that is localized to the lungs and limited in the circulating blood could explain why the cytokine storm in COVID-19 does not lead to the rapid and high mortality traditionally associated with microbial sepsis. Microbial sepsis [82] is a disease where inflammasome activation and inflammation within the blood drives rapid multi-system organ failure and death in contrast to the prolonged disease course associated with the cytokine storm seen in severe COVID-19. Figure 3 provides a model of initial inflammatory signaling that is activated in the lungs in response to SARS-CoV-2, and potentially mucosal microbial dysbiosis, and spreads to the periphery, driving disease severity and eventually mortality. Thus, vaccines which include adjuvants, or vectors, that augment or exacerbate tissue inflammation as is seen in SARS-CoV-2 infection may not produce robust or effective immune responses following vaccination.

Implications for vaccine development
Avoiding the bad inflammation
So what does this mean for the development of the next generation of COVID-19 vaccines? If inflammasome and IL-1 activation do not induce protective immunity to natural infection, we may consider avoiding using vaccine components which can potently activate the inflammasomes. Though inflammasome/IL-1 activation is often thought of only as a modulator of innate immunity, it also modulates adaptive immune function. It has been shown that inflammasome activation in vaccines can enhance CD4 polyfunctionality, T follicular helper responses and the magnitude of Ab titers [83,84]. In yellow fever virus (YFV), vaccination with YFV17D is associated with inflammasome activation which, combined with complement and interferons, leads to a broad and polyfunctional T cell and B cell response post-vaccination [85]. IL-1 and IL-18 are associated with T helper subset skewing of Th1 and Th17 responses [83,86–88]. IL-18 produced by DCs has also been shown to induce antigen-independent production of IFN-gamma from effector CD8+ T cells [89].

The licensed adjuvant Alum [90,91] is known to activate the NLRP3 inflammasome. Likewise, certain DNA viral vectors including Adenoviruses [92,93] and modified vaccinia virus Ankara (19543380) lead to activation of inflammasomes including AIM2 [94], IFI16 and NLRP3. The widespread use of Alum as an adjuvant suggests that inflammasome activation is important for vaccine

Figure 2
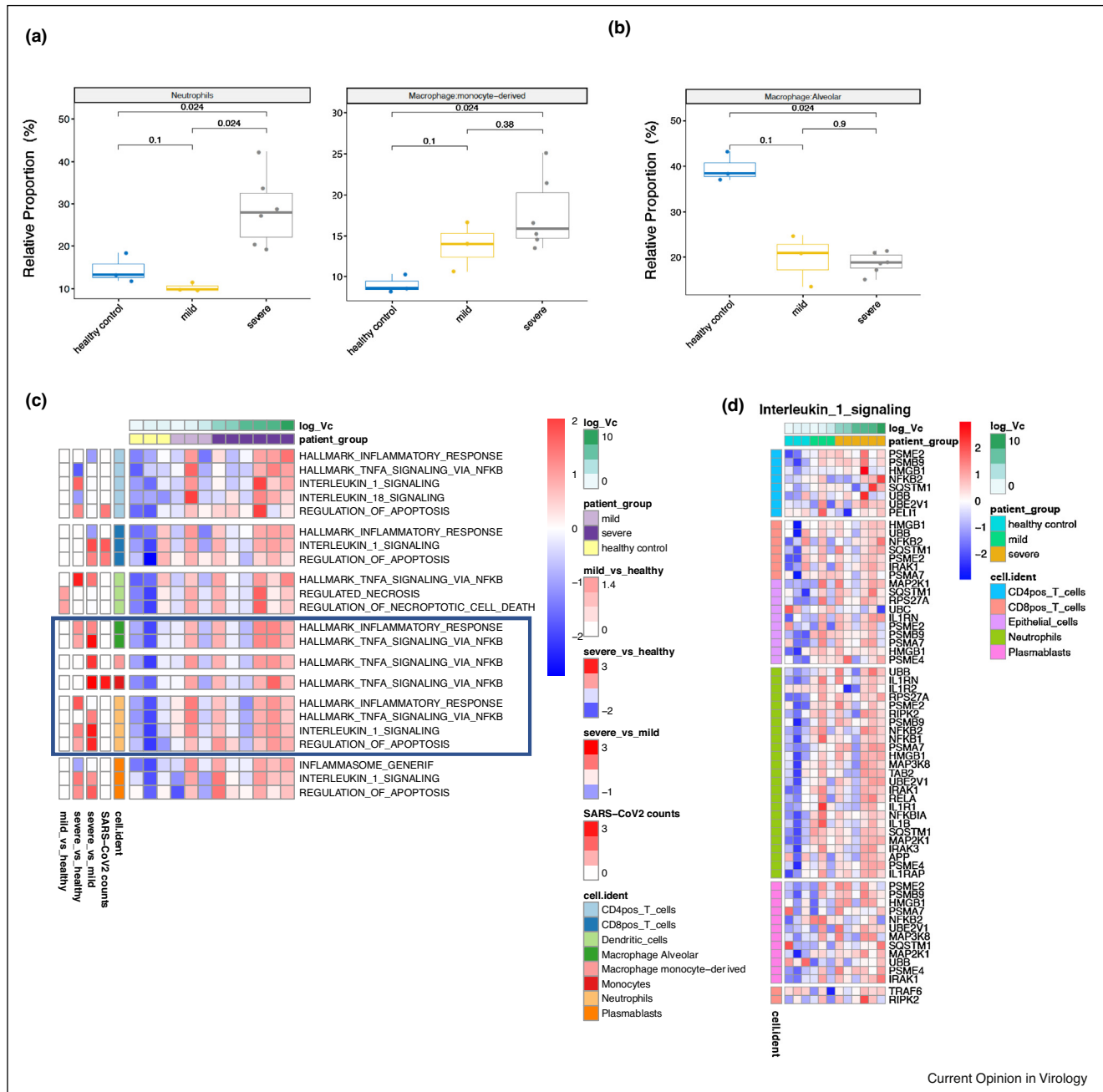
Infiltrating neutrophils and monocytes into the lung acquire an inflammatory phenotype and dominate the local immune response to SARS-CoV-2. Using the same data as in Figure 1, we analyzed the frequency and gene expression status of immune cells in the BALF. **(a)** Neutrophil frequencies were significantly increases in the BALF of severe disease versus mild disease and healthy controls. Monocyte-derived macrophages were also significantly increased in severe disease versus healthy controls, with an increase also observed in mild disease. **(b)** We observe a concomitant loss of resident alveolar macrophages in severe and mild disease. These data suggest that infiltrating neutrophils/monocytes begin to dominate the local lung immune response during severe COVID-19. **(c)** GSEA analysis per cell cluster reveals a consistent signature of upregulated inflammation in immune subsets including in monocytes/macrophages, alveolar macrophages and neutrophils. **(d)** Leading edge genes from the IL-1 signaling pathway reveal key regulators of IL-1β/NF-κB are upregulated in infiltrating immune cells in severe COVID-19 disease. Comparison of cluster frequencies was performed using paired-Wilcoxon sum rank test.

Figure 3
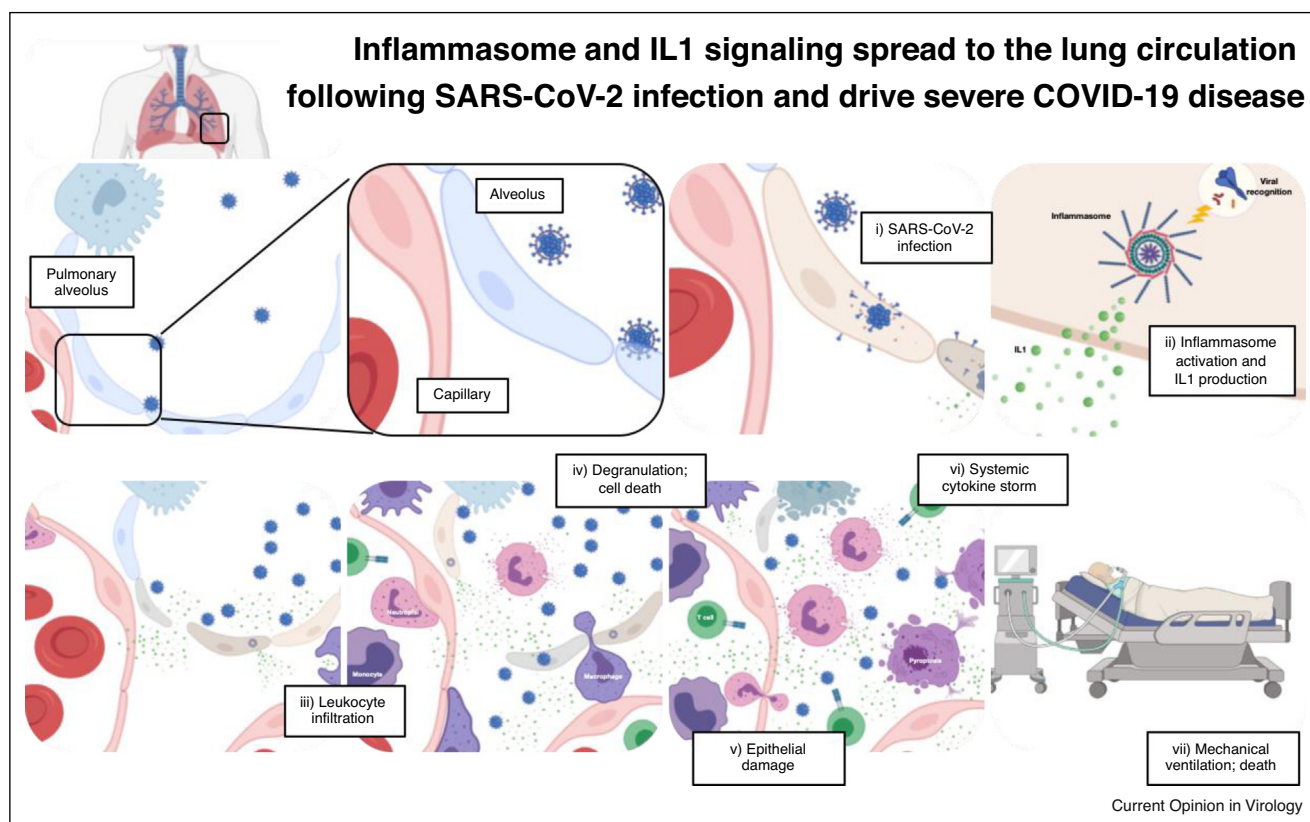
Model of the induction and spread of inflammasome/IL-1β driven inflammation from the lung to the periphery in COVID-19.
In **Step i**, SARS-CoV-2 virus infected epithelial cells and leads to intrinsic inflammasome activation and inflammatory signaling from infected epithelial cells in **Step ii**. This local inflammatory response promotes chemokine production and leads to the infiltration of circulating leukocytes including innate and adaptive immune cells to the lungs in **Step iii**. The rampant inflammatory response activates these infiltrating cells eventually leading to **Step iv** involving degranulation and/or cell death of immune cells further exacerbating inflammation; this process culminates in further damage to the lung tissue including epithelial cells in **Step v**. This inflammatory response does not remain local and spreads to the periphery causing (**Step vi**) cytokine storm and eventually the need for oxygen, mechanical ventilation and death in severe cases.

responses, however there is controversy concerning this subject. There are multiple publications which have published data showing that the NLRP3 inflammasome is dispensable for the adjuvant effect of Alum [95–97]. Similarly, while flagellin has been shown to be an adjuvant [98,99], it has been shown that the adjuvant activity of flagellin can be independent of NLRC4 [100,101], the inflammasome known to be activated by flagellin [100]. So while Alum is a potent adjuvant, it is far from clear how dependent this effect is on inflammasome activity. Inflammatory caspases are not the only mechanisms of activating IL-1 cytokines. There are multiple sources of proteases including neutrophils which can mediate cleavage and activation of IL-1 (reviewed in Ref. [102]).

Inflammasome activity in the context of other vaccines is also ambiguous. In influenza, it was recently shown that heightened inflammasome activation, resulting from antibiotic treatment, was associated with lower H1N1-specific neutralizing and binding IgG and IgA [103]. However in HIV/SIV, it has been shown that the canarypox vector ALVAC, a known activator of the inflammasome via AIM2 [104], promotes vaccine efficacy by inflammasome activation in monocytes NHP [105]. We have recently published that ALVAC induced CREB1 activation, a critical modulator of reduced HIV-1 acquisition in humans and protection from SIV challenge in NHP, was associated with IL-18 production which regulated pathways of immune activation in DCs [27].

In the context of SARS-CoV-2, the two approved inactivated virus vaccines, Sinopharm and Sinovac-CoronaVac, both use Alum as adjuvant and have reported lower efficacy rates than the mRNA-based vaccines from Moderna and Pfizer. A recent study used Systems Vaccinology to investigate the immune responses induced by the

Pfizer vaccine. Of pertinence, they did not observe potent or persistent inflammation post-vaccination, however they did not compare this to any other SARS-CoV-2 vaccine [106[••]]. It is prudent and necessary to better understand how inflammasome activation and inflammation during SARS-CoV-2 vaccination modulates vaccine efficacy and long-lived immunity. Controlled clinical studies are needed to compare Alum with other adjuvants (MF59, TLR ligands) to clearly understand the innate immune responses needed during vaccination to promote vaccine efficacy. We can learn lessons from what does and does not work for the immune response during natural infection to continue to inform vaccine development.

Augmenting the good inflammation

By identifying dampened Interferon antiviral responses as a hallmark of SARS-CoV-2 infection and disease, we can now envision designing vaccine regimens which potently induce IFN responses to augment the generation of cells which can combat SARS-CoV-2 infection. A recent study of Influenza vaccination identified epigenetic changes which persisted after vaccination in monocytes and DCs which were correlated with protection from subsequent viral challenge, both to homologous (same) and heterologous (different; Zika and Dengue) viruses [107[••]]. Another study, which used a Systems Immunology approach to dissect the immune responses generated in human to the Pfizer-BioNtech SARS-CoV-2 vaccine, showed that antiviral and IFN signaling pathways were more potently induced in monocytes and DCs after the boost (2nd dose) compared to the prime (1st dose) [106[••]]. Both of these studies indicate the acquisition of Trained Immunity, a newly described process whereby exposure of innate immune cells to pathogenic stimuli induces epigenetic changes which augment future innate immune responses to challenge [108–111]. This has tremendous implications for vaccine development as these heightened innate immune responses could help limit breakthrough infections in vaccinated individuals. This includes infections with viral variants as innate immune responses are antigen-independent and unlikely to be substantially impacted by mutations observed in viral variants.

Adjuvants are currently in development and pre-clinical/clinical testing which may potently induce interferon signaling including TLR agonists [112–114] and small molecule STING agonists [115–117]. We know from a recent study that inclusion of 5 distinct adjuvants in a protein subunit COVID-19 vaccine results in differential modulation of vaccine response. Specifically, 4 adjuvants targeting antiviral/interferon signaling showed efficacy while an oil-in-water formulation did not [26[••]]. Within the 4 successful adjuvants, there was variance in protection with all adjuvants inducing no detectable virus in the BAL but in the nares, there was no detectable viral RNA in four of five monkeys for AS03 and CpG-Alum compared to only three of five monkeys for AS37 and Alum. Different nucleic acid composition of vaccine vectors may be differentially sensed by TLRs and cytosolic sensors of nucleic acids leading to downstream differences in interferon signaling.

What we learn about the pathogenesis of SARS-CoV-2 infection can inform how the research community approaches profiling and identifying the immune correlates of protection during COVID-19 vaccination, across multiple platforms. A targeted approach to optimize the platform(s) ultimately chosen for future COVID-19 vaccines represents an opportunity to improve on the overall efficacy of vaccines, irrespective of antigen inclusion. While inflammation is a necessary component of vaccine responses, too much vaccine induced inflammation may not yield protective responses in COVID-19 vaccines. Identifying what constitutes good inflammation versus bad inflammation for SARS-CoV-2 vaccination requires controlled studies which access samples within days of vaccination and link these early signatures to long-term protection; a difficult task made even more difficult by the increasingly lower number of individuals naïve to SARS-CoV-2 infection and/or vaccination. An ideal adjuvant may be one that is capable of potently inducing interferon/antiviral responses while maintaining enough inflammation to drive chemotaxis and jump start the immune response. Identifying potential pathways ahead of time provides the opportunity to stay ahead of SARS-CoV-2 by not waiting potentially years for detailed immune analyses of current vaccine(s) to inform on vaccine design. Using available data on natural SARS-CoV-2 infection and COVID-19 disease allows us to be more proactive in designing the future of COVID-19 vaccines.

Methods
Single-cell transcriptomics

Raw count matrices for BALF single-cell RNA-seq data were extracted from SRA Archive GSE145926 and imported into the R package Seurat for preprocessing.

Cells with high mitochondrial content (>0.1 of reads) and low number of reads (<200) were filtered out for Quality Control. Expression was integrated across patients using the SCTransform approach from Seurat using 3000 genes as anchors on the basis of their high variance across cells, while an additional 4000 genes were also integrated using those same anchors. Principal component was performed on normalized integrated data, and the optimal number of components was inferred (25) using the Elbow method for clustering and UMAP dimension reduction.

SingleR was used to infer cell identity of individual clusters on the basis of the expression of their top 100 most differentially expressed genes (FindAllMarkers function, Seurat) in comparison with the Human Cell Atlas (https://

www.humancellatlas.org/) and Monaco reference profiles from the Human Protein Atlas (https://www.proteinatlas.org/). Clustered inferred with the same cell type were merged together to facilitate downstream analysis.

MAST was used to perform differential expression across severity groups, or versus viral counts/cell frequencies, by using raw counts and taking into account cellular detection rate and sample provenance as covariates.

Gene Set Enrichment Analysis (GSEA) was performed using the z score from the MAST analysis as ranking variable, and was tested against inflammasome/apoptosis genesets.

Median expression per gene/cell type/sample was computed and used for visualization purposes in heatmap format. The combined leading edge genes across contrasts was extracted on significant GSEA enrichments, and was leveraged to generate row-normalized heatmaps of pathways across cells and patients.

Authors' contribution
ANP performed bioinformatic analysis, interpretation of results and edited the manuscript. RPS aided in interpretation of results and preparation/editing of manuscript. JAT devised the conceptual basis of the manuscript, planned bioinformatic analysis, interpreted results and drafted the manuscript.

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Conflict of interest statement
Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- •• of outstanding interest

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highlights the point that one size does not fit all when it comes to vaccines and adjuvants.

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