Full title: **Tracking inflammation resolution signatures in lungs after SARS-CoV-2 omicron BA.1 infection of K18-hACE2 mice**

Short title: Inflammation resolution in lungs of a mouse model of SARS-CoV-2 infection

Agnes Carolin[1#], Kexin Yan[1#], Cameron R. Bishop[1#], Bing Tang[1], Wilson Nguyen[1], Daniel J. Rawle[1†], Andreas Suhrbier[1,2†].

[1] QIMR Berghofer Medical Research Institute, Brisbane, Queensland, 4029, Australia.

[2] GVN Centre of Excellence, Australian Infectious Disease Research Centre, Brisbane, Queensland 4029 and 4072, Australia.

[#] should be considered joint first authors
[†] should be considered joint last authors

[*]Corresponding author. Email: Andreas. Suhrbier@qimrberghofer.edu.au or Daniel.Rawle@qimrberghofer.edu

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Abstract

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes Coronavirus Disease 2019 (COVID-19), which can result in severe disease often characterised by a 'cytokine storm' and the associated acute respiratory distress syndrome. However, many infections with SARS-CoV-2 are mild or asymptomatic throughout the course of infection. Although blood biomarkers of severe disease are well studied, less well understood are the inflammatory signatures in lung tissues associated with mild disease or silent infections, wherein infection and inflammation are rapidly resolved leading to sequelae-free recovery. Herein we described RNA-Seq and histological analyses of lungs over time in an omicron BA.1/K18-hACE2 mouse infection model, which displays these latter features. Although robust infection was evident at 2 days post infection (dpi), viral RNA was largely cleared by 10 dpi. Acute inflammatory signatures showed a slightly different pattern of cytokine signatures compared with severe infection models, but where much diminished 30 dpi and absent by 66 dpi. Cellular deconvolution identified significantly increased abundance scores for a number of anti-inflammatory pro-resolution cell types at 5/10 dpi. These included type II innate lymphoid cells, T regulatory cells, and interstitial macrophages. Genes whose expression trended downwards over 2 – 66 dpi included biomarkers of severe disease and were associated with 'cytokine storm' pathways. Genes whose expression trended upward during this period were associated with recovery of ciliated cells, AT2 to AT1 transition, reticular fibroblasts and innate lymphoid cells, indicating a return to homeostasis. Very few differentially expressed host genes were identified at 66 dpi, suggesting near complete recovery. The parallels between mild or subclinical infections in humans and those observed in this BA.1/K18-hACE2 mouse model are discussed.

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a global pandemic of Coronavirus Disease 2019 (COVID-19), with over 770 million cases and 7 million deaths [1, 2]. Severe acute disease is primarily characterised by infection of the respiratory tract and pathological hyper-inflammatory responses, often referred to as a "cytokine storm" that can lead to acute respiratory distress syndrome (ARDS) and ultimately mortality [3-5]. Post-acute and chronic sequelae of COVID-19 are now well described, with post-COVID conditions affecting 10-30% of COVID-19 patients [6] and long-COVID at least 10% of patients [7-9].

The inflammatory mediators that are associated with severe COVID-19, as distinct from mild disease, have been the focus of many studies, with such insights having the potential to identify therapeutic anti-disease interventions [3, 5, 10-17]. Elevated mRNA or protein levels of a range of cytokines have been identified in peripheral blood of severe COVID-19 patients, these include *inter alia* IL-6, IL-1, IL-2, IL-17, IL-18, TNF, IFNG and CSF2 (GM-CSF) (*ibid*). Elevated IL-10 in the peripheral blood has also been associated with severe disease, but may be useful later in the infection to suppress the hyper-cytokinemia associated with ARDS [18-20]. Elevated Th2 [10, 21-23] and Th17 responses [10, 24] have been associated with poor outcomes, with Th1/Th2 [25-27] and Th1/Th17 imbalances [28] also reported to be detrimental. T regulatory cells (Tregs) are viewed as providing protection against the cytokine storm [29], whereas neutrophils promote the latter and contribute to disease severity [10]. Monocytes and macrophages play key protective roles against SARS-CoV-2 infection, but depending on their differentiation and activation state, are also important contributors to over-production of inflammatory cytokines [30, 31]. Higher viral loads appear to positively associate with COVID-

19 severity [32, 33], although this may be associated with more prolonged high viral loads in patients with severe disease [34].

An estimated 25-30% of SARS-CoV-2 infections are asymptomatic throughout the course of infection [35, 36], with a constellation of factors potentially contributing [37, 38]. Although fatal disease is associated with higher viral loads, asymptomatic individuals often do not have lower acute viral loads than symptomatic patients [39-42]. Humans have also been shown to tolerate a certain level of lower respiratory tract infection (and associated radiographic abnormalities), without reporting symptoms [43]. Persistent asymptomatics do, nevertheless, appear able to clear the virus more rapidly [44, 45]. For instance, only minimal PCR-detectable viral RNA was present in saliva of a small cohort of such individuals by days 9-10 post first positive diagnosis (after daily saliva testing) [44]. Individuals with silent infections may have lower levels of circulating proinflammatory cytokines [45], although this is not a universal finding [46]. Silent or subclinical infections have been associated with powerful antiviral responses [47], early and robust innate immune responses [48], as well as specific gene expression patterns [49, 50], such as higher plasma levels of the anti-inflammatory cytokines IL-10, IL-1RA and IL-19 [46]. Although in humans there can be long-term consequences of asymptomatic SARS-CoV-2 infections, these are significantly more prevalent after symptomatic infections [51].

Herein we provide the transcriptional profile of lung infection over time in a model of SARS-CoV-2 omicron BA.1 infection of K18-hACE2 mice, wherein the majority of mice showed no significant overt signs or symptoms of disease [52]. The acute phase was characterised by a significant lung infection and a pattern of proinflammatory cytokine signatures differing only slightly from those reported previously [53]. By 10 days post infection (dpi), virus had been largely cleared and a series of cell types associated with damage control, suppression of inflammation, homeostasis and repair were identified by cellular deconvolution. Few

differentially expressed genes (DEGs) were identified in lungs by 66 dpi, arguing that the model describes near complete resolution of the infection, without long-term pulmonary sequelae. The term "protective inflammation" has been coined to describe the inflammatory processes that lead to subclinical or mild disease, resulting in sequelae-free outcomes [54]. Herein we provide insights into the transcriptional signatures in lungs that characterise resolution of inflammation and return to homeostasis, and also identify a series of parallels with human studies.

Materials and methods

Ethics statements and regulatory compliance

Collection of nasal swabs from consented COVID-19 patients was approved by the QIMR Berghofer Medical Research Institute Human Research Ethics Committee (P3600).

All mouse work was conducted in accordance with the Australian code for the care and use of animals for scientific purposes (National Health and Medical Research Council, Australia). Mouse work was approved by the QIMR Berghofer MRI Animal Ethics Committee (P3600). All infectious SARS-CoV-2 work was conducted in the BioSafety Level 3 (PC3) facility at the QIMR Berghofer MRI (Department of Agriculture, Fisheries and Forestry, certification Q2326 and Office of the Gene Technology Regulator certification 3445). Breeding and use of GM mice was approved under a Notifiable Low Risk Dealing (NLRD) Identifier: NLRD_Suhrbier_Oct2020: NLRD 1.1(a). Mice were euthanized using carbon dioxide.

The SARS-CoV-2 omicron BA.1 isolate

The BA.1 omicron isolate was obtained at QIMR Berghofer MRI from nasal swabs from a

consented COVID-19 patient by culture in Vero E6 cells (ATCC C1008). The omicron BA.1 isolate, SARS-CoV-2_{QIMR01} (SARS-CoV-2/human/AUS/QIMR01/2022), belongs to the BA.1.17 lineage (GenBank: ON819429 and GISAID EPI_ISL_13414183) [55, 56].

BA.1 viral stocks were propagated in Vero E6 cells as described [57]. UV inactivation of the BA.1 virus was undertaken using the UVC 500 Ultraviolet Crosslinker (Hoefer) (dose 7650 J/m^2 UVC) as described [57]. Virus stocks were titered using $CCID_{50}$ assays [58].

K18-hACE2 mice, infection and monitoring

Heterozygous K18-hACE2 mice (strain B6.Cg-Tg(K18-ACE2)2Prlmn/J, JAX Stock No: 034860) were purchased from The Jackson Laboratory, USA, and were maintained in-house as heterozygotes by backcrossing to C57BL/6J mice (Animal Resources Center, Canning Vale WA, Australia). Heterozygotes were inter-crossed to generate homozygous K18-hACE2 mice (on a C57BL/6J background [53, 59]) as described [52, 53]. Mice were held under standard animal house conditions (for details see [55]) and homozygous female mice received intrapulmonary infections delivered via the intranasal route with 5×10^4 $CCID_{50}$ of virus in 50 μl RPMI 1640 whilst under light anesthesia as described [60]. For each group for each time point, a comparable mean age and age distribution of mice was used (mean age 26. 3 $\pm$ SD 1.1. weeks). Mice were weighed and monitored as described [60]. Mice were euthanized using CO_2, lungs were removed, with the left lung fixed in formalin for histology, the right lung inferior lobe placed in RNAlater for RNA-Seq and the remaining lobes used for tissue titers determination by $CCID_{50}$ assays using Vero E6 cells as described [57, 60].

$CCID_{50}$ assays

Tissue titers were determined as described [57]. Briefly, 5 fold serial dilutions of clarified tissue homogenates were applied in duplicates to Vero E6 cells in 96 well plates. After 6 days cytopathic effects were observed by inverted light microscope. The virus titer was determined by the method of Spearman and Karber [61].

Histology

Lungs were fixed in 10% formalin, embedded in paraffin, and sections stained with H&E (Sigma Aldrich). Slides were scanned using Aperio AT Turbo (Aperio, Vista, CA, USA). Areas with overt leukocyte infiltrates (with high focal densities of dark purple staining nuclei) were measured manually using the "Pen Tool" in the Aperio ImageScope software v10 (Leica Biosystems, Mt Waverley, Australia). White space analysis was undertaken using QuPath v0.2.3 [62].

Immunohistochemistry

Immunohistochemistry was undertaken as described using the anti-SARS-CoV-2 spike monoclonal antibody, SCV2-1E8 [56], except that the monoclonal (IgG2a) was purified using Protein A affinity chromatography and applied to sections at 4 µg/ml for 1 hr.

RNA-Seq and bioinformatics

RNA-Seq was undertaken as described using Illumina Nextseq 2000 platform generating 75 bp paired end reads [53]. Mean quality scores were above Q20 for all samples. Mouse RNA-Seq

reads were aligned to a combined mouse (GRCm39, version M27) and SARS-CoV-2 BA.5 reference genome [52] using STAR aligner. Viral read counts were generated using Samtools v1.16. Read counts for host genes were generated using RSEM v1.3.1. Low count genes were identified and removed separately for each time point using DESeq2 v1.40.2. Differentially expressed genes were identified using DESeq2 using a FDR cut-off of 0.05. Pathway analysis was performed with host DEGs using Ingenuity Pathway Analysis (IPA, v84978992) (QIAGEN) using the Canonical pathways, Up-Stream Regulators (USR) and Diseases or Functions features as described [59]. Gene Set Enrichment Analyses (GSEAs) were undertaken using GSEA v4.1.0 with gene sets provided in MSigDB (≈ 45,000 gene sets) and in the Blood Transcription Modules [63], and gene lists generated using DESeq2 ranked by $\log_2$ fold-change. Relative abundances of cell types were estimated in R v4.1.0 from RSEM 'expected counts' using SpatialDecon v1.4.3 [64] and cell-type expression matrices obtained from the "Mouse Cell Atlas Lung Cell expression matrix" and the "NanoString Immune Cell Family expression matrix"

Statistics

The t-test (with Welch's correction) was used if the difference in variances was <4 fold, skewness was > - 2 and kurtosis was <2. The t test significance and variance were determined using Microsoft Excel. Skewness and kurtosis were determined using IBM SPSS Statistics for Windows v19.0 (IBM Corp., Armonk, NY, USA). Otherwise, the non-parametric Kolmogorov-Smirnov exact test was performed using GraphPad Prism 10.

Results

SARS-CoV-2 omicron BA.1 provided robust lung infection in K18-hACE2 mice that was cleared by 10 dpi

K18-hACE2 mice were given an intrapulmonary inoculum via the intranasal route of SARS-CoV-2 BA.1 ($5x10^4$ $CCID_{50}$) or the same inoculum of UV-inactivated BA.1 virus. Lungs were harvested at 2, 5, 10, 30 and 66 days post infection (dpi) and tissue titers (Fig. 1a), RNA-Seq (Fig. 1b and see below) and histology (see below) undertaken. Lung viral titers reached a mean titer of 7.86 $\pm$ SD 0.49 $log_{10}CCID_{50}$/g on 2 days post infection (dpi) (Fig. 1a). Using the same assay system, infection with an original strain isolate in K18-hACE2 mice provided a comparable titer at this time (7.19 $\pm$ SD 0.44 $log_{10}CCID_{50}$/g) [53]. However, at 5 dpi the mean titers in BA.1 infected lungs was 4.04 $\pm$ SD 0.8 $log_{10}CCID_{50}$/g (Fig. 1a), 1.8 logs lower than those seen after infection with an original strain isolate (5.8 $\pm$ SD 0.9 $log_{10}CCID_{50}$/g) [53]. By 10 dpi, 3 of 4 BA.1 infected mice showed no detectable virus titers (Fig. 1a).

Fig 1. Viral loads and immunohistochemistry of viral antigen in lungs of BA.1 infected K18-hACE2 mice. **a** Lung tissue titers at the indicated days post infection with BA.1 (limit of detection ≈ 2 $log_{10}CCID_{50}$/g). **b** Viral read counts per million reads, determined by RNA-Seq for K18-hACE2 mice infected with BA.1 or inoculated with UV-inactivated BA.1 virus. Statistics by Kolmogorov Smirnov Exact tests. **c** Viral read counts per million reads, determined by RNA-Seq for K18-hACE2 mice infected with original strain isolates; regraphed from Bishop et al. 2022 [53]. **d** Immunohistochemistry of BA.1-infected lungs at 2 dpi, stained with an anti-spike monoclonal antibody. Staining of bronchial epithelia cells (dark brown) can be seen top left and

bottom right (left image), with enlargements of these two areas shown in the right 2 images. **e** As for d showing staining of cells surrounding alveoli; morphology consistent with that of pneumocytes (alveolar epithelial cells). * alveolar air sacs.

Viral read counts of infected mice obtained from RNA-Seq data showed a similar trend (Fig. 1b) to viral titers. No significant read counts were obtained from mice inoculated with UV-inactivated virus (Fig. 1b). Previously reported RNA-Seq viral read count data for infection of K18-hACE2 mice by original strain isolates is provided as a comparison (Fig. 1c). This data is taken from three independent studies [53, 65, 66] and has been presented previously [53], but is regraphed herein in a slightly different format to facilitate comparison (Fig. 1c). Mean lung viral RNA levels on 2 dpi with an original strain isolate were ≈ 17 fold higher than those seen after BA.1 infection, and they did not substantially decline thereafter (Fig. 1c). Overall the reduced lung viral loads in BA.1-infected mice is consistent with previous reports [67, 68].

As described previously [52, 67, 68], most mice in the BA.1/K18-hACE2 model do not develop a brain infection. Brain infection is associated with >20% weight loss and an ensuing ethical requirement for euthanasia (S1a Fig). After infection of K18-hACE2 mice with original strain isolates, mice succumb to brain infections between 4 and 7 dpi [52, 60]. This BA.1 model thus provides a significant lung infection, and permits analysis of long term responses to SARS-CoV-2.

BA.1 infected bronchial epithelial cells and pneumocytes in K18-hACE2 mouse lungs

Immunohistochemistry (IHC) of lungs taken 2 dpi (peak viral load), using an anti-spike

monoclonal antibody [56], illustrated clear staining of bronchial epithelial cells (Fig. 1d) and cells surrounding alveoli that have a morphology consistent with pneumocytes (Fig. 1e). The BA.1/K18-hACE2 model thus recapitulates the main cell tropisms seen in human lung tissues, with infection of bronchial and alveolar epithelial cells by omicron variants well described [69, 70].

BA.1 induced histopathological lesions in lungs that resolved by 66 dpi

H&E stained sections of lung tissues from BA.1 infected K18-hACE2 mice illustrated a series of histopathological features (Fig. 2, S2 Fig) previously identified in SARS-CoV-2 mouse models [71], although they appeared less severe than those seen after infection of K18-hACE2 mice with original strain isolates, consistent with previous reports [67, 68]. For instance, in the latter, pronounced sloughing of the bronchial epithelium and fulminant occlusions of bronchi (with serum and red blood cells) were observed [66, 72].

Fig 2. Histology of lungs after BA.1 infection of K18-hACE2 mice. **a** H&E staining of lung tissue sections at the indicated days after infection/inoculation. Green dotted lines encircle overt foci of cellular infiltrates. **b** Quantitation of cellular infiltrates. All cellular infiltrates were marked as in “a” and the summed areas as a percentage of the total lung section area was determined for each mouse. Statistics by Kolmogorov Smirnov Exact tests. **c** Whole H&E stained lung sections from day 5 after infection/inoculation, illustrating reduced white space in lungs from infected mice. **d** QuPath digital analysis of white space in H&E stained whole lung sections such as those shown in “c”. Statistics by t test.

In BA.1 infected mice (as in other models [55, 71]), focal areas of leucocyte infiltration were clearly evident, characterized by dense clusters of dark-purple staining leukocyte nuclei; although the infiltrates were more diffuse at 2 dpi (Fig. 2a, green dashed lines). These areas were measured manually using the Aperio ImageScope "Pen Tool" (Fig. 2a, green dashed lines), providing quantitation of leukocyte infiltration. Significantly higher levels of infiltration were seen for BA.1-infected versus UV-inactivated BA.1-inoculated mice at all time-points except 66 dpi, with infiltrates also substantially reduced by 30 dpi (Fig. 2b) (Aperio Positive Pixel Count assessment of leukocyte infiltrates also showed significantly higher infiltrate levels at 5 dpi; S1b Fig). Lung consolidation with resulting loss of white space, primarily as a result of diminished alveolar air sack volumes, was also clearly seen at 5 dpi (Fig. 2c). Significant loss of white space at 5 dpi was confirmed by image analysis of scanned lung sections using QuPath (Fig. 2d). Minor intrusion of red blood cells and serum into bronchi, and some alveolar edema were also occasionally observed in BA.1-infected mice (S2 Fig). H&E images from PBS and naïve control lungs are shown in S3 Fig.

In summary, lungs of BA.1 infected K18-hACE2 mice displayed some typical acute histopathological features described previously for SARS-CoV-2 infections, although less severe than after infection with original strain isolates. At 30 dpi BA.1 infection-associated histopathology was clearly resolving, and by 66 dpi had largely resolved.

Cell types associated with resolution of inflammation were evident on 5 and 10 dpi

BA.1 infected lungs and UV BA.1 inoculated lungs were compared at each time point (2, 5, 10,

30 and 66 dpi) by RNA-Seq, with DESeq2 [73]. Full gene lists and bioinformatic analyses are provided for 2 dpi (S1 Table), 5 dpi (S2 Table), 10 dpi (S3 Table), 30 dpi (S4 Table), and 66 dpi (S5 Table).

Ingenuity Pathway analysis (IPA) Diseases or Functions annotations for Activation of leukocytes, Chemotaxis of leukocytes, Leukocyte migration and Quantity of leukocytes showed a progressive decline over time, with a slight elevation 10 dpi (Fig. 3a), consistent with Fig. 2b. To gain insights into the type of cellular infiltrates present in the lungs over time post infection with BA.1, cellular deconvolution (SpacialDecon) analysis using full gene sets (S1 – S5 Tables) were used together with the gene expression matrices from the "Mouse Cell Atlas (MCA) Lung Cell expression matrix" and the "NanoString (NS) Immune Cell Family expression matrix". As might be expected [74, 75], a series of innate mononuclear leukocyte populations were identified at 5 dpi, which included macrophages, monocytes, dendritic cells, and NK cells, as well as gamma delta T cells [76] (Fig. 3b). Increased abundance of nuocytes, ILC2 cells, was also identified on both 5 and 10 dpi (Fig. 3b,c, nuocytes). These innate effector leukocytes mediate type-2 immunity [77] and are involved in the initiation of inflammation, but also have key roles in damage control, homeostasis and repair [78, 79]. Lower frequencies of ILC2 cells have been associated with severe COVID-19 [80]. An increase abundance score for pericytes was also identified in infected lungs at 5 dpi (but not other times) (S4 Fig). Pericytes express ACE2 and binding of spike protein has been shown *in vitro* to induce profound transcriptional changes in these cells [81, 82], which may explain this result.

Fig 3. Cellular infiltrates and types identified by RNA-Seq of lungs for 2 – 30 dpi. **a** Z scores for the indicated annotations from Ingenuity Pathway analysis (IPA) Diseases or Function analysis plotted against dpi. (N.I. not identified; for 66 dpi there were insufficient DEGs for IPA

analysis). **b** Using cellular deconvolution (SpatialDecon) and the gene expression matrices from the "Mouse Cell Atlas (MCA) Lung Cell expression matrix" and the "NanoString (NS) Immune Cell Family expression matrix", cell types whose abundance scores were significantly different on day 5 were identified (q values provided). Blue text indicates cell type associated with inflammation resolution. **c** As for b but for day 10. Blue text indicates cell type associated with inflammation resolution. We assume "colon" T reg are well annotated in the NS matrices (and are thus readily identified), and represent tissue T regs, rather than implying that colonic T regs have migrated to the lungs.

At 10 dpi, in addition to nuocytes, significantly increased abundance of three more cell types associated with repair and return to homeostasis was evident (Fig. 3c, blue text). Inducible T regulatory cells (T regs) characterised by low neurophilin expression (Nrp-1^{lo}) [83] were more abundant in infected lungs. T regs secrete *inter alia* IL-10 and are generally protective [84], not just by dampening proinflammatory responses, but also via prevention of fibrosis and maintenance of tissue homeostasis [85]. Interstitial macrophages were also elevated in infected lungs, with these cells having an inherently anti-inflammatory phenotype, secreting *inter alia* IL-10 (USR z score 1.69, q=1.39E-29, S3 Table) and supporting T regs in the lung [86]. An Ear2+ alveolar macrophage population was also identified, with Nra4a1+, Ear2+ macrophages recently found to have a novel reparative phenotype [87]. Although perhaps proinflammatory, CD8 T cells were also more abundant at 10 dpi (Fig. 3c), with CD8 T cells recently identified as key to protection against SARS-CoV-2 [88].

Cytokine signatures in BA.1 infected lungs and their resolution by 30-66 dpi

Differentially expressed genes (DEGs, q<0.05) (S1 –S5 Tables) were analyzed by IPA, with cytokine up-stream regulator (USR) z scores illustrated by a heat map (Fig. 4a). The acute cytokine responses were similar to those published previously for K18-hACE2 mouse models of SARS-CoV-2 infection [53]. When cytokine USR z scores at 2 dpi from BA.1 infection (Fig. 4a) were compared with those from original isolate infection [53], small differences in z scores (between ≈1 and -1) emerged (Fig. 4b). A series of USRs associated with less severe disease had slightly higher z score in BA.1 infected mice; these included EPO (erythropoietin) [89], IL-15 [90], and increased type I interferon responses [17, 48] (Fig. 4b). The z score was also higher for IL-10, with IL-10 elevated in severe COVID-19, but also able to suppress hyper-cyotokinemia [18-20]. Elevated IL-4, IL-9 and IL-13 have been associated with more severe disease [21, 22, 91], although IL-9, IL-13 (and IL-5) are produced by type 2 innate lymphoid cells (ILC2 cells) [92] (see below), and increased IL-4 and IL-13 signatures may (in this context) reflect a relatively more balanced Th1/Th2 response [25, 26]. Elevated OSM and IL-6 are also associated with more severe disease [17, 93]. Another group of cytokines/chemokines associated with increased disease severity (IL-2, IFNG, IL-17, CSF2, CCL5, CXCL8, CCL2 [10, 94, 95] showed slightly lower z scores for BA.1 infected lungs (Fig. 4b). IL-33 may drive fibrosis [96] and also showed a lower USR z score (Fig. 4b).

Fig 4. Inflammatory pathways identified by RNA-Seq of lungs for 2 – 30 dpi. a Heat map of cytokine UpStream Regulator (USR) z scores determined by IPA. White boxes means no cytokine USR annotation was returned for 30 dpi by IPA. RNA-Seq identified too few DEGs on

66 dpi for IPA analysis. **b** IPA cytokine USRs from BA.1 infected K18-hACE2 mice were compared with those previously reported for the more severe infection of K18-hACE2 mice by an original strain isolate. Differences in cytokine z scores for 2 dpi (with a cutoff filter of >0.2 and < -0.2) are plotted. BA.1 infected lungs show slightly increased z scores for some cytokine signatures associated with reduced disease severity (green) and slightly reduced z scores for some cytokine signatures associated with increased disease severity (red).

Importantly, the cytokine response signatures had substantially abated by 30 dpi (Fig. 4a), with the small number of DEGs on day 66 insufficient for meaningful pathway analysis. In addition, few, if any, upregulated inflammatory DEGs were identified at 66 dpi (see below).

Transcriptional responses 66 dpi reflected adaptive immunity and resolution

The RNA-Seq data sets and bioinformatic analyses comparing lungs from BA.1 infected mice 66 dpi with mice 66 days after inoculation with UV-inactivated virus (S5 Table) are summarized in Fig. 5. Only 29 DEGs were identified, of which 14 were immunoglobulin genes. This represents an insufficient number of DEGs for meaningful pathway analyses. DEGs were thus clustered into several groupings based on function (Fig. 5, described below). A plot of the first two principle components of normalized Variance Stabilizing Transformation (VST) counts from all experimental groups indicated that the relatively small number of DEGs for day 66 was due to limited separation between BA.5 infected and UV control groups (S5 Fig).

Fig 5. DEGs identified for 66 dpi. RNA-Seq data for day 66 dpi, with DEGs clustered by broad functions. The whole gene list was also interrogated by GSEAs using gene sets from MSigDB and Blood transcription modules.

Most of the upregulated DEGs in lungs at 66 dpi were immunoglobulin genes (Fig. 5) consistent with a more rigorous development of an adaptive immune response in BA.1 infected mice compared with mice inoculated with UV-inactivated virus. GSEAs also illustrate the development of T cell responses, with down-regulation of Nr4a family members potentially suggesting avoidance of T cell exhaustion [97]. However, the three Nr4a genes have a number of roles in T cell biology [98], and also have a diverse set of other activities in *inter alia* macrophages [87, 99] and epithelial cells [100].

The three upregulated, non-Ig genes, can be associated with wound healing/tissue repair; Grb7 (Growth factor receptor-bound protein 7) [101], Arfgap3 (ADP-ribosylation factor GTPase-activating protein 3) [102, 103], and Cap1 (cyclase-associated protein 1) [104]. In addition, Dusp8 (Dual specificity phosphatase 8) was down-regulated, with Dusp8 protein involved in negative regulation of MAP kinase superfamily members; thus Dusp down-regulation would promote cellular proliferation and differentiation [105].

Many of the remaining down-regulated genes have roles in various immune activities (Fig. 5): Prkd1 (Serine/threonine-protein kinase D1), involved in *inter alia* promoting neutrophil infiltration [106]; S1pr1 (Sphingosine-1-phosphate receptor 1), essential for immune cell trafficking [107]; Coq10b, required for coenzyme Q function [108, 109]; Kcne4 (Potassium voltage-gated channel subfamily E member 4), an immune regulator via modulation of Kv1.3 [110]; Atf3, a stress-inducible transcription factor [111]; Marco, a scavenger receptor [112]; and Thbs1 (thrombospondin), a matricellular protein involved in lung inflammation resolution and

repair [113]. Two stress-inducible genes, Atf3 and Hspa1b (a heat shock protein 70 member) were down-regulated, with Atf3 and Hsp70 upregulated in a large range of stress responses [100, 111].

In summary, at 66 dpi there were very few DEGs, with no indication of ongoing inflammation or tissue dysregulation, with identification of only a small number of upregulated genes that could be associated with wound healing processes.

Genes whose expression positively correlated with dpi were associated with ciliated epithelial cells and protein synthesis

Using the complete gene sets (S1 – S5 Tables), genes were identified whose expression showed significant positive correlation with dpi (Pearson correlations, FDR corrected p, $q<0.05$). Genes whose expression also showed significant positive correlation with days post inoculation of UV-inactivated virus were excluded from the analysis, thereby retaining only genes associated with active infection. Immunoglobulin genes were also removed as the development of immune responses is understood and established. The process identified 2786 genes (S6 Table).

Enrichr and IPA analyses of the 2786 genes indicated a significant association with ciliated epithelial cells and protein synthesis, with 59 of the 2786 representing ribosomal protein genes (Fig. 6a; S6 Table). Ciliated epithelial cells are a known target of SARS-CoV-2 infection [60, 114], with omicron variants showing a higher propensity to infect these cells [69]. The increase in ciliated epithelial cell genes thus indicates growth/recovery and differentiation of these cells over the 2-66 days of the experiment. The increase in signatures associated with protein translation during this period is consistent with the development of adaptive immune responses and ongoing tissue repair processes.

Fig 6. Genes whose expression correlated with dpi. **a** Using the whole gene lists, genes whose expression showed a statically significant positive correlation with dpi were identified. After removing genes that also positively correlated with dpi in the UV-inactivated BA.1 inoculated control groups, 2786 genes remained. The 2786 genes were analyzed by Enrichr and IPA (for the latter the r value was entered in the log2 expression column). Significant results clustered into two categories 'Ciliated epithelial cell' and 'Protein synthesis'. Of the 2786 genes, 59 were RPL (ribosomal protein) genes. **b** Using the whole gene lists, genes whose expression showed a statically significant negative correlation with dpi were identified. After removing genes that also negatively correlated with dpi in the UV control groups, 1996 genes remained. The 1996 genes were analyzed by IPA as above, with some top annotations shown. **c** Normalized expression data (VST - Variance Stabilizing Transformation) for five of the 1996 genes are plotted against dpi, with linear regression lines and 95% confidence intervals. Pearson correlation coefficients (r) and corrected significances, FDR (q), are provided for each gene. **d** Using cellular deconvolution (as in Fig. 3b,c), cell types whose abundance scores significantly positive correlated with dpi were identified. Linear regression lines and 95% confidence intervals, and Pearson correlation coefficients (r) with corrected significances, FDR (q), are provided. (Full data sets for this figure are provided in S6 Table).

Genes whose expression negatively correlated with dpi indicated inflammation resolution

Using the complete gene sets, genes were identified whose expression showed significant negative correlation with dpi (Pearson correlations, $q<0.05$). Genes whose expression also

showed significant negative correlation with days post inoculation of UV-inactivated virus were excluded from the analysis, thereby retaining only genes associated with active infection. The process identified 1996 genes (S6 Table).

IPA analyses of the 1996 genes identified reductions in cell movement/migration (Fig. 6b), consistent with Fig. 2 and 3a. A series of cytokine USRs were also down-regulated (S6 Table), with the top cytokine USRs (by negative z score) representing cytokines previously identified as being robustly associated with COVID-19 disease severity [10]. The latter was supported by identification of "Pathogen Induced Cytokine Storm Signaling Pathway" as a top IPA Canonical pathway annotation with a negative z score (Fig. 6b). Also informative were decreasing neutrophil degranulation and fibrosis signatures (Fig. 6b); both phenomena have been associated with long COVID [115, 116].

Negative correlations between expression and dpi are plotted for five of the 1996 genes (Fig. 6c). These genes were selected as their expression levels have been positively correlated with COVID-19 disease severity and/or they have been proposed as potential targets for prognosis or treatment; SerpinE1 [117], Cxcl10 [118], Nfkb1 [119], IL6 [120], and Hspa1b (a Hsp70 family member) [121, 122].

Cell types whose abundance scores increased with dpi indicated lung regeneration

Cell types whose abundance scores (determined by cellular deconvolution) showed a significant positive correlation (Pearsons $q<0.05$) with dpi were identified. The top scoring cell type was ciliated cells (Fig. 6d); consistent with Fig. 6a. The analysis also identified significant increases in AT1 cells, which likely reflects the AT2 to AT1 transition during alveolar epithelial cell

regeneration seen after SARS-CoV-2 alveolar damage in mouse models [123]. Increasing abundance scores for reticular fibroblasts, are also consistent with successful lung repair [49, 124], with no indication of significant fibrosis. Increasing numbers of innate lymphoid cells (ILC) likely also reflect the process of returning the tissue to homeostasis [125, 126].

Discussion

We describe herein the transcriptional profile of BA.1 omicron lung infections in K18-hACE2 mice, and the post-infection recovery up to 66 dpi. The expression of hACE2 via the keratin 18 promoter in the K18-hACE2 mice did not lead to overtly aberrant cell tropism in the lungs, with infection of bronchial and alveolar epithelia cells by omicron variants described previously [69, 70]. Compared to infection with original strain isolates, BA.1 viral loads in lungs were lower and were rapidly cleared, a feature associated in COVID-19 patients with less severe disease [34] or subclinical infections [44, 45]. By 66 dpi only a small number of DEGs were identified, arguing that the infection had been resolved and the tissue had largely returned to normal. UV-inactivated virus was used as a control to exclude responses to viral inoculation and restrict the analyses to effects arising from replicating virus. Features often associated with post-acute or long-COVID were not identified, for instance, there were no indications of prolonged infection [127], persistent inflammation [128], impaired lung regeneration [129], fibrosis [116], prolonged T cell activation [130], Th17 bias [131], chronic neutrophil activation [115] or clotting abnormalities [7].

The concept of “protective inflammation” was recently described for COVID-19 [54] and clearly applies to many other viral infections where subclinical or mild infections are also well documented [132]. Deleterious or pathological inflammatory disease processes tend to be the

focus of research, with perhaps less attention paid to characterisation of inflammatory responses that are resolved and lead to subclinical or mild disease and sequelae-free outcomes. Pathological versus protective responses are likely to have distinct gene expression profiles, patterns, magnitudes and sequence of events [133, 134], with the current study describing the responses associated with resolution of lung infection and inflammation. Importantly, at 10 dpi a series of cell types associated with damage control, suppression of inflammation, homeostasis and repair showed increased abundance scores (determined using transcription data from lung tissues and cellular deconvolution). Specifically these cells were, ILC2 cells [78, 79] (also seen at 5 dpi), inducible T regs [84, 85], interstitial macrophages [86] and Ear2+ macrophages [87]. Restoration of ciliated cells, regeneration of alveolar epithelial cell with AT2 to AT1 transition [123], and increases in fibroblast activity without fibrosis [49], also appear to characterize lung recovery in this model. Clearly, prompt clearance of virus [44, 48] (rather than viral persistence [127, 135]) and development of protective adaptive immune responses [47] are also likely to be key to such outcomes. The latter has recently been shown to include CD8 T cells [88], which may be represented by a population of CD8b1^{high} cells which reached significance by 10 dpi in the BA.1/K18-hACE2 model.

Defining key aspects of “protective inflammation” remains a challenge for human populations, as individuals with subclinical or mild disease are generally difficult to identify and recruit promptly. A better understanding of what characterizes rapid, sequelae-free resolution of inflammation, would clearly facilitate development of interventions for COVID-19 associated ARDS, with animal models providing ready access to lung tissues to help unravel the complex mechanisms that underpin such processes.

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Supplemental information

The paper is supported by supplementary figures (S1 – S5 Fig) and Tables (S1 – S6 Table).

S1 Fig. Overview of change in body weight overtime and ratio of purple over red staining in lungs at each timepoint.

S2 Fig. Histopathological features of viral infection in lungs of BA.1 infected mice in comparison to UV-inactivated BA.1 inoculated mice.

S3 Fig. H&E staining of control lungs from K18-hACE2 mice.

S4 Fig. Increased pericytes abundance score at 5 dpi.

S5 Fig. First two principle components of normalized VST-transformed counts from all experimental groups.

S1 Table Differential expression and pathway analysis for 2 dpi

S2 Table Differential expression and pathway analysis for 5 dpi

S3 Table Differential expression and pathway analysis for 10 dpi

S4 Table Differential expression and pathway analysis for 30 dpi

S5 Table Differential expression and pathway analysis for 66 dpi

S6 Table Gene expression vs dpi

Author Contributions

Conceptualization: Andreas Suhrbier, Daniel J. Rawle, Cameron R. Bishop.

Data curation; Cameron R. Bishop, Andreas Suhrbier.

Funding acquisition: Andreas Suhrbier, Daniel J. Rawle.

Investigation: Agnes Carolin, Kexin Yan, Bing Tang, Wilson Nguyen.

Methodology: Daniel J. Rawle, Cameron R. Bishop, Andreas Suhrbier.

Supervision: Daniel J. Rawle, Cameron R. Bishop, Wilson Nguyen, Andreas Suhrbier.

Visualization: Andreas Suhrbier, Cameron R. Bishop, Kexin Yan.

Writing –original draft: Andreas Suhrbier.

Writing – review & editing: Agnes Carolin, Cameron R. Bishop, Daniel J. Rawle.

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Figure 1

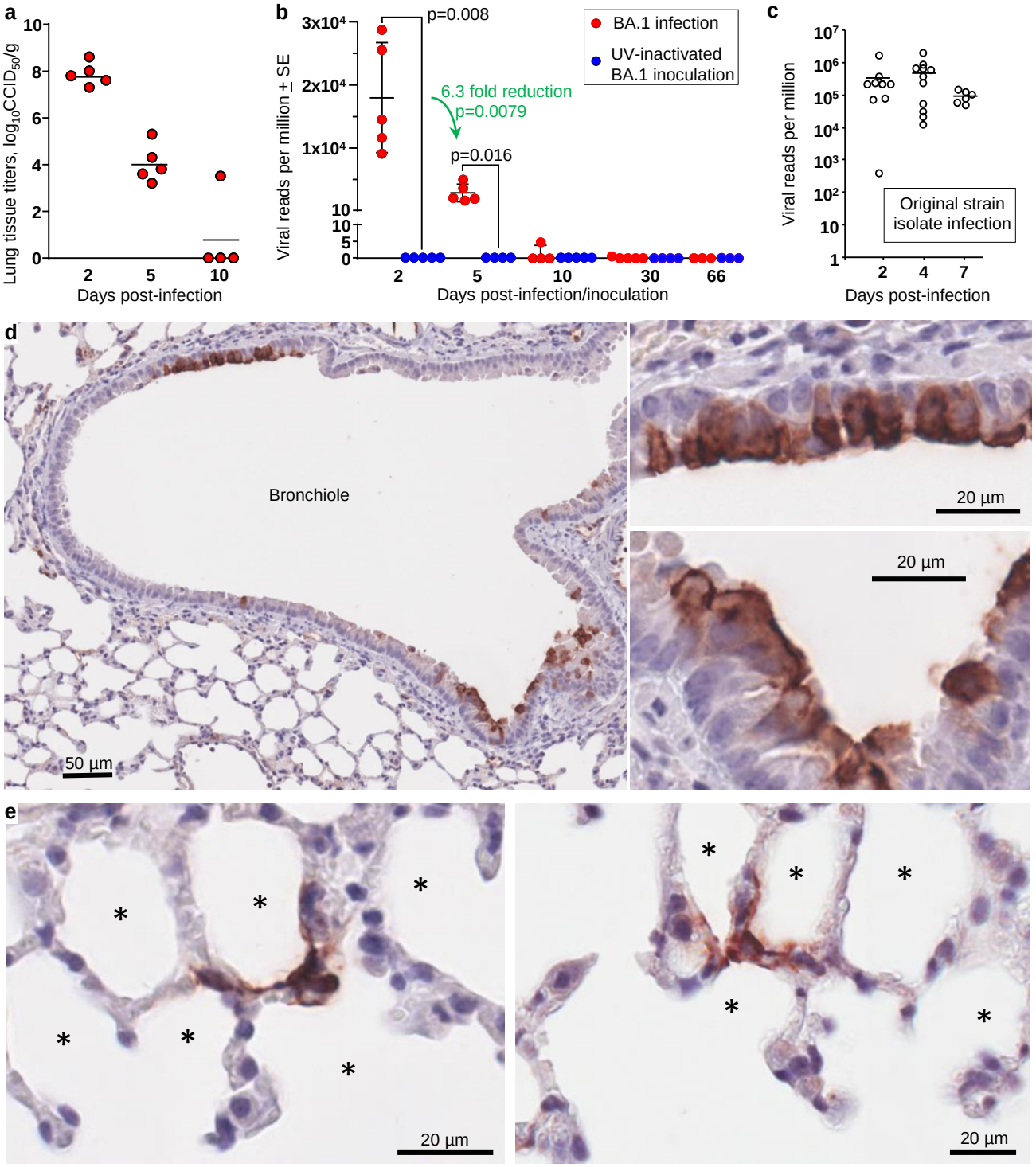

a
Lung tissue titers, log10CCID50/g
Days post-infection
b
Viral reads per million ± SE
p=0.008
6.3 fold reduction
p=0.0079
p=0.016
BA.1 infection
UV-inactivated BA.1 inoculation
Days post-infection/inoculation
c
Viral reads per million
Original strain isolate infection
Days post-infection
d
Bronchiole
50 µm
20 µm
20 µm
e
20 µm
20 µm

Figure 2

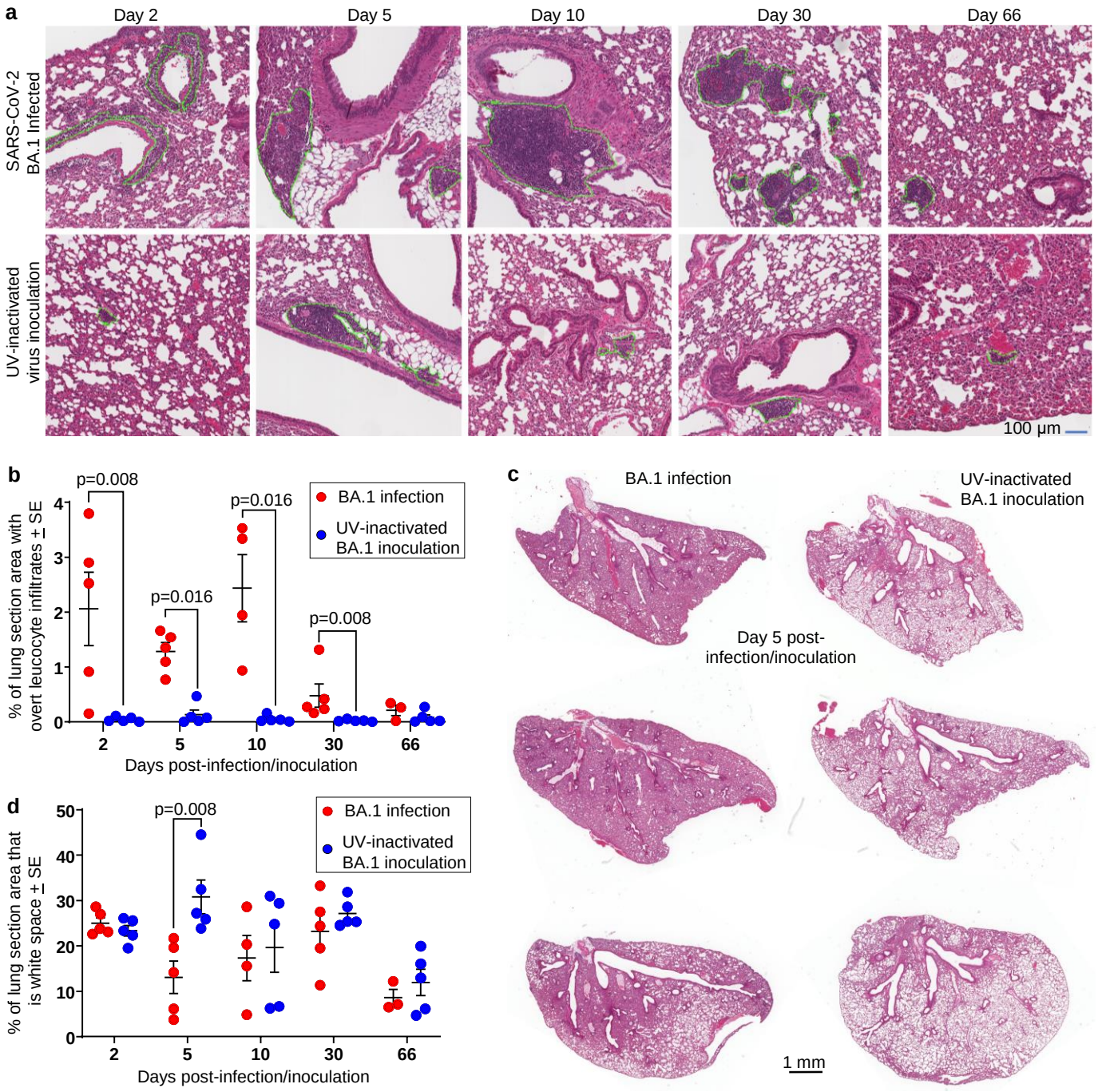

a
Day 2
Day 5
Day 10
Day 30
Day 66
SARS-CoV-2 BA.1 Infected
UV-inactivated virus inoculation
100 µm
b
p=0.008
p=0.016
p=0.016
p=0.008
BA.1 infection
UV-inactivated BA.1 inoculation
% of lung section area with overt leucocyte infiltrates ± SE
Days post-infection/inoculation
c
BA.1 infection
UV-inactivated BA.1 inoculation
Day 5 post-infection/inoculation
1 mm
d
p=0.008
BA.1 infection
UV-inactivated BA.1 inoculation
% of lung section area that is white space ± SE
Days post-infection/inoculation

Figure 3

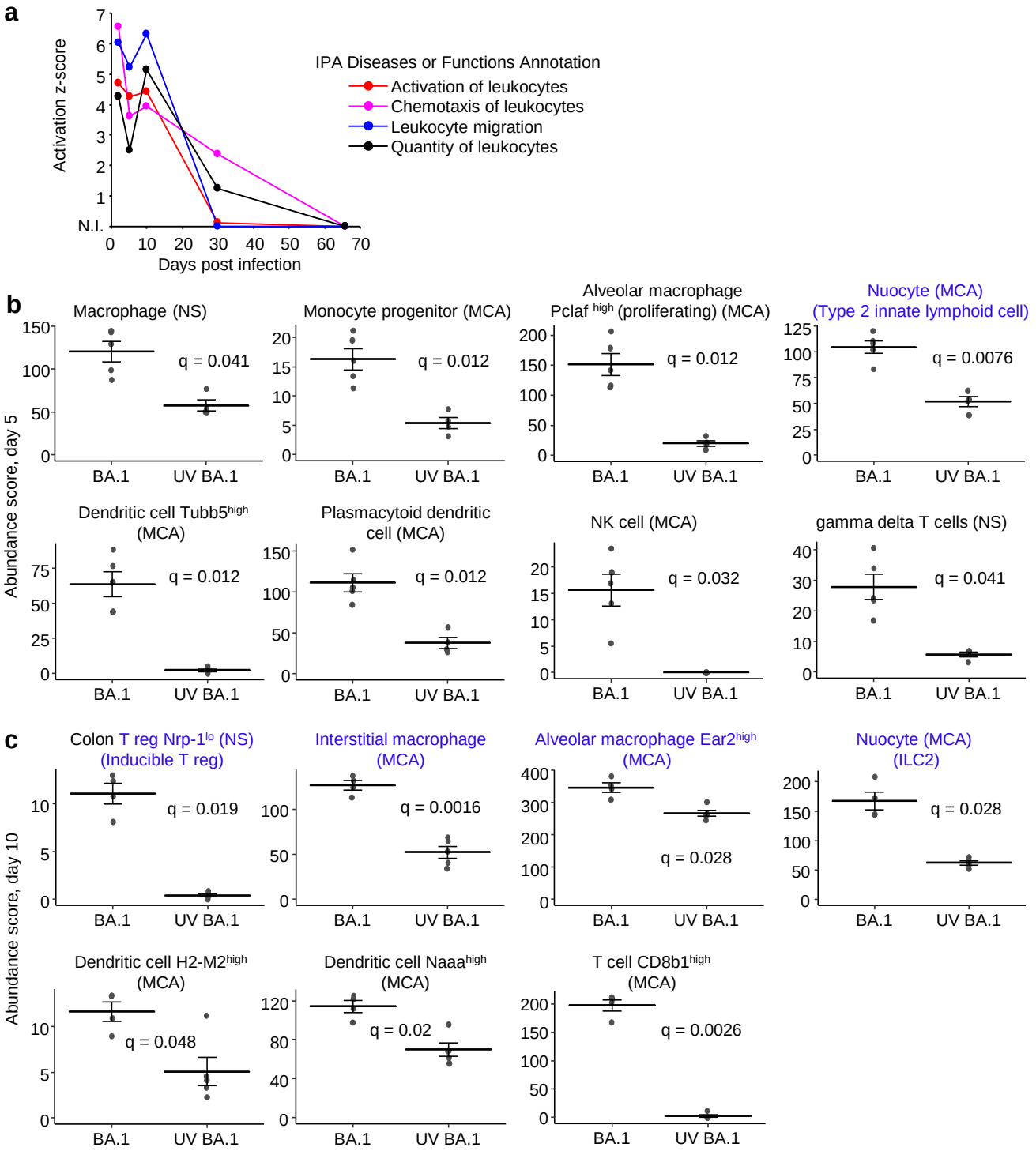

a
Activation z-score
Days post infection
IPA Diseases or Functions Annotation
Activation of leukocytes
Chemotaxis of leukocytes
Leukocyte migration
Quantity of leukocytes
b
Abundance score, day 5
Macrophage (NS)
q = 0.041
Monocyte progenitor (MCA)
q = 0.012
Alveolar macrophage Pclaf high (proliferating) (MCA)
q = 0.012
Nuocyte (MCA) (Type 2 innate lymphoid cell)
q = 0.0076
Dendritic cell Tubb5high (MCA)
q = 0.012
Plasmacytoid dendritic cell (MCA)
q = 0.012
NK cell (MCA)
q = 0.032
gamma delta T cells (NS)
q = 0.041
c
Abundance score, day 10
Colon T reg Nrp-1lo (NS) (Inducible T reg)
q = 0.019
Interstitial macrophage (MCA)
q = 0.0016
Alveolar macrophage Ear2high (MCA)
q = 0.028
Nuocyte (MCA) (ILC2)
q = 0.028
Dendritic cell H2-M2high (MCA)
q = 0.048
Dendritic cell Naaahigh (MCA)
q = 0.02
T cell CD8b1high (MCA)
q = 0.0026
BA.1
UV BA.1

Figure 4

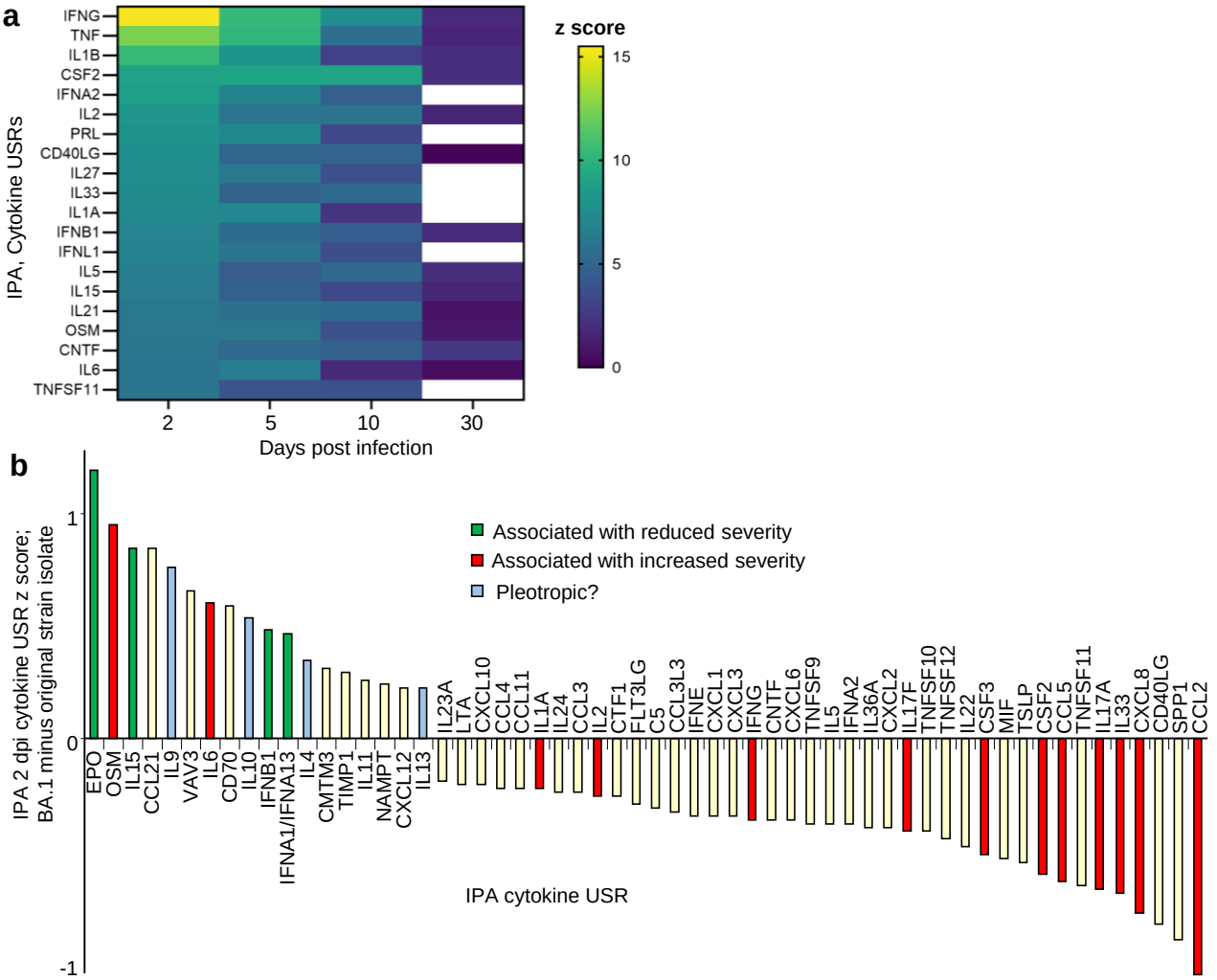

a
IPA, Cytokine USRs
IFNG
TNF
IL1B
CSF2
IFNA2
IL2
PRL
CD40LG
IL27
IL33
IL1A
IFNB1
IFNL1
IL5
IL15
IL21
OSM
CNTF
IL6
TNFSF11
2
5
10
30
Days post infection
z score
15
10
5
0
b
IPA 2 dpi cytokine USR z score;
BA.1 minus original strain isolate
1
0
-1
Associated with reduced severity
Associated with increased severity
Pleotropic?
EPO
OSM
IL15
CCL21
IL9
VAV3
IL6
CD70
IL10
IFNB1
IFNA1/IFNA13
IL4
CMTM3
TIMP1
IL11
NAMPT
CXCL12
IL13
IL23A
LTA
CXCL10
CCL4
CCL11
IL1A
IL24
CCL3
IL2
CTF1
FLT3LG
C5
CCL3L3
IFNE
CXCL1
CXCL3
IFNG
CNTF
CXCL6
TNFSF9
IL5
IFNA2
IL36A
CXCL2
IL17F
TNFSF10
TNFSF12
IL22
CSF3
MIF
TSLP
CSF2
CCL5
TNFSF11
IL17A
IL33
CXCL8
CD40LG
SPP1
CCL2
IPA cytokine USR

Figure 5

Lungs, BA.1 vs. UV-BA.1, day 66 (29 DEGs, q<0.05)

↑ Plasma cell development

DEG	log_2FC
Igkv3-10	6.89
Ighg2c	6.39
Igkv1-88	5.92
Ighv8-12	5.64
Igkv3-2	5.42
Igkv4-91	5.02
Igkv6-17	4.86
Ighv1-7	4.76
Igkv12-46	4.56
Igkv8-28	4.50
Igkv10-94	3.67
Igkv6-15	3.54
Ighv1-26	3.02
Igkc	2.40

Plasma cells & B cells, immunoglobulins (M156.0)
BTM GSEA
NES= 2.1 q=0.026

MM-GOBP_ Immunoglobulin production
MSigBD GSEA
NES= 3.31 q<0.001

↑ T cell responses

T cell activation (M7.3)
BTM GSEA
NES= 2.06 q=0.028

↓ T cell exhaustion?

DEG	log_2FC
Nr4a1	-3.12
(Nr4a2	-1.7*)
Nr4a3	-2.75

↓ DEGs associated with immune functions

DEG	log_2FC	Function
Prkd1	-0.60	Promotes neutrophil infiltration
S1pr1	-0.62	Regulates immune reponses
Coq10b	-1.03	Aerobic respiration/antioxidant
Kcne4	-1.41	Immune regulator
Atf3	-1.81	Stress-inducible transcription factor
Marco	-1.83	Scavenger receptor
Thbs1	-1.96	Matricellular protein, damage control

↓ Stress response DEGs

DEG	log_2FC
ATF3	-1.81
Hspa1b	-3.12

↑ DEGs associated with tissue repair

DEG	log_2FC	Function
Grb7	0.56	Cell migration
Arfgap3	0.34	Vesicle Transport
Cap1	0.28	cAMP proliferation
Dusp8	-1.00	MAPK inhibitor

GSEAs
MSigDB
Blood transcription modules

Figure 6

a

Genes whose expression levels positively correlated with dpi (Pearson correlations, q<0.05) n= 2786

Enrichr and IPA analyses

Ciliated epithelial cell

Enrichr		
Descartes Cell Types and Tissue 2021	Ciliated epithelial cells in Lung	q=1.11e-19, Combined score 134
CellMarker Augmented 2021	Ciliated cell:Lung	q=1.08e-13, Combined score 115
IPA Diseases or Functions	Formation of Cilia	p=7.88e-11 z= 2.58

Protein synthesis

Enrichr
GO Biological Process 2023 Cytoplasmic Translation. q= 3.26e-11, Combined score 181

59 ribosomal protein genes (RPL)

b

Genes whose expression levels negatively correlated with dpi (Pearson correlations, q<0.05) n=1996

IPA analysis

Cell movement/migration

IPA Diseases or Functions (top annotations)

Cell movement	p=1.75e-85	z= -14.71
Migration of cells	p=3.11e-85	z= -14.16

Cytokine responses

IPA Cytokine USRs (top annotations)

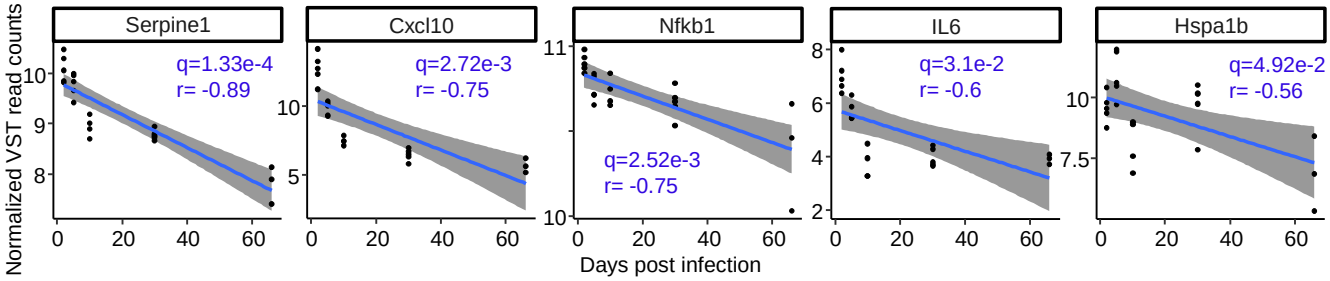
TNF	p=7.3e-73	z= -13.17
IFNG	p=1.4e-83	z= -12.42
IL1B	p=7.7e-54	z= -9.4
CSF2	p=2.5e-52	z= -9.33

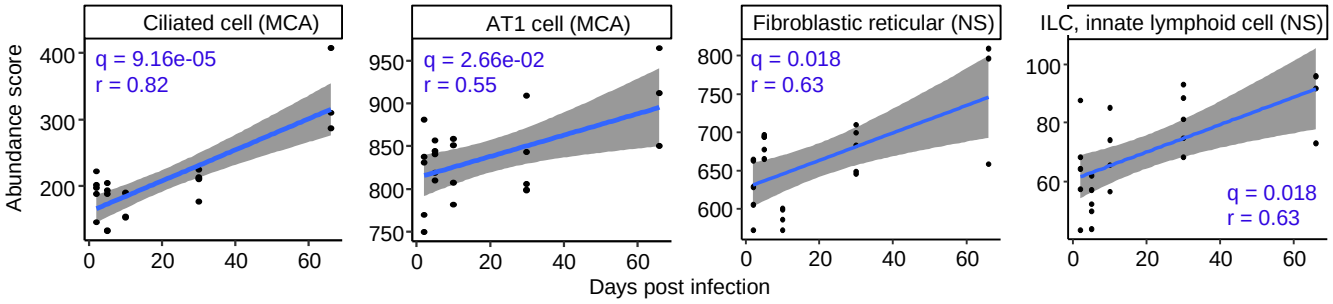
Cytokine storm, fibrosis and neutrophil degranulation

IPA Canonical (top examples)

Neutrophil degranulation	$-\log_{10}$p=19.3	z= -9.59
Pathogen Induced Cytokine Storm Signaling Pathway	$-\log_{10}$p=22.6	z= -8.79
Pulmonary Fibrosis Idiopathic Signaling pathway	$-\log_{10}$p=17.9	z= -8.3

c



d

Cell types whose abundance scores positively correlated with dpi (Pearson correlations, q<0.05)