INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has a spectrum of clinical outcomes, ranging from asymptomatic to fatal. There is a need to parse out the role of individual immune cell types and molecular pathways that contribute to effective control of viral infection in asymptomatic/mild disease and those leading to organ failure during severe coronavirus disease 2019 (COVID-19). Increased proinflammatory cytokines (1–3), deficient type I interferon (IFN) responses (4–6), and activation of inflammasomes (7), neutrophils (8–10), and monocytes/macrophages (3, 10–14) have all been associated with severe COVID-19. Coordinated activation of CD8+ and CD4+ T cells, T cell activation state, and antigen (Ag) specificity have all been linked to favorable outcomes of SARS-CoV-2 infection (15–20). Whereas neutralizing antibodies are clearly protective in immune hosts, T cell responses may also contribute to the protection provided by vaccination and natural infection (21–24).

Most studies of immune correlates of COVID-19 disease severity in humans have focused on sampling of peripheral blood. Nevertheless, some studies have observed infiltration of immune cells into the bronchoalveolar lavage (BAL) fluid, into postmortem lung tissue acquired from fatal COVID-19 cases, or from individuals undergoing medically necessary procedures (12, 14, 25, 26). On autopsy, several reports have observed an unexpected lack of immune cells infiltrating into extrapulmonary tissues despite the presence of high levels of virus (27–31). These data highlight the importance of understanding the early host response in pulmonary and extrapulmonary tissues in the first few days after SARS-CoV-2 infection.

Animal models can be used to obtain a detailed understanding of the host response in infected tissues. Studies in SARS-CoV-2–susceptible species provide insights into COVID-19 disease pathogenesis. For example, transgenic mouse strains expressing human angiotensin converting enzyme 2 (ACE2) (K18-hACE2), and mice induced to express hACE2 with viral vectors, are highly susceptible to SARS-CoV-2 infection (32–36). Syrian hamsters and ferrets are also moderately susceptible and shed infectious virus (37–41). On the other hand, species more resistant to SARS-CoV-2 induced disease are useful tools in examining mechanisms of efficient control of viral replication. Several nonhuman primate (NHP) species can be experimentally infected with SARS-CoV-2. Rhesus
macaques, cynomolgus macaques, and African green monkeys typically develop mild symptoms after SARS-CoV-2 infection (42–52). SARS-CoV-2–immune and SARS-CoV-2–vaccinated rhesus macaques are protected from reinfection primarily by neutralizing antibodies and, to a lesser extent, anamnestic T cell responses (53–64). Thus, current NHP models are suitable for the study of protective host immune responses associated with mild SARS-CoV-2 infection, but not the mechanisms of pathogenesis during severe disease.

In this study, we use the rhesus macaque model of mild COVID-19 to examine the (i) kinetics of lung inflammation using \(^{18}\)F-fluorodeoxyglucose (\(^{18}\)FDG) positron emission tomography/computed tomography (PET/CT) imaging, (ii) innate immune responses using single-cell RNA sequencing (scRNAseq), and (iii) the tissue distribution of SARS-CoV-2–specific T cell responses by flow cytometry. Our findings suggest that mild SARS-CoV-2 disease and efficient control of the infection are temporally correlated with activation of myeloid cells by type I IFN before the induction of Ag-specific T and B cell responses. Moreover, they reveal a strong propensity for Ag-specific T cell migration into the pulmonary compartment compared with other mucosal sites of infection.

### RESULTS

#### Radiologic and virological outcomes of SARS-CoV-2 infection in rhesus macaques

Six male rhesus macaques were infected with \(1 \times 10^6\) tissue culture infectious dose (TCID)\(_{50}\) intranasally (i.n.) and \(1 \times 10^5\) TCID\(_{50}\) intratracheally (i.t.), for a total dose of \(2 \times 10^6\) TCID\(_{50}\) of SARS-CoV-2/USA-WA1 (table S1). \(^{18}\)FDG-PET/CT imaging showed evidence of heterogeneous inflammatory foci with increased \(^{18}\)FDG uptake (Fig. 1, A and B) and lesion density (Fig. 1, A and C) in the lungs of five of six animals at day 3 after infection, which resolved by day 9. Total genomic Nucleocapsid (gN) and subgenomic Nucleocapsid (sgN) RNA levels from nasal and throat swabs peaked 1 to 2 days after infection and decreased to undetectable levels by days 7 to 10 (Fig. 1D). Viral RNA was also found in the BAL of all animals at day 4 after infection and was mostly cleared by days 7 to 10. It should be noted that day 4 after infection likely does not represent the peak of viremia in the BAL, and previous studies indicate that peak viral loads are reached at day 1 after infection in the BAL (42). Viral RNA was essentially absent from plasma at all time points, consistent with previous reports (42).

The animals were necropsied at day 10 after infection for tissue analysis. A three-dimensional (3D) reconstruction of the day 3 PET/CT images with conducting airways was used to locate and individually collect the previously PET hot lung regions and normal lung tissue separately. SARS-CoV-2 gN RNA was found on day 2 in all secondary lymphoid organs (SLOs) and nonlymphoid tissues (NLTs) tested, including the previously PET hot and normal lung tissue, nasal turbinates, salivary gland, and tonsils (Fig. 1E). sgN RNA was present at lower levels compared with gN RNA and was highest in lung tissue (Fig. 1F). The persistence of viral RNA at day 10 was confirmed with RNAscope immunohistochemical analysis (Fig. 1G). There was a correlation between genomic and subgenomic RNA levels in the mucosal swabs, BAL, and tissues with detectable RNA (Fig. S1, A to C). We did not observe a correlation between lung lesion severity at day 3 and viral RNA levels from nasal swabs and BAL at days 1 and 4, respectively (Fig. S1, D to G).

Consistent with previous reports in macaques, various forms of CD62P\(^-\) and fibrin\(^-\) microthrombi were still detectable in the lungs on day 10 after infection (Fig. 1H) (44). Thus, in rhesus macaques, SARS-CoV-2 viral loads peak in the upper airways ~1 to 2 days after exposure, and this results in mild and transient radiographic evidence of lung inflammation at ~3 days after infection, with residual viral RNA in tissues and microthrombi in the lungs at day 10.

#### Longitudinal scRNAseq analysis of BAL and PBMC

To compare cellular immune responses in circulation versus airways, scRNAseq was performed on cryopreserved peripheral blood mononuclear cells (PBMCs) and BAL samples obtained before infection and at days 4, 7, and 10 after infection. Uniform Manifold Approximation and Projection (UMAP) and nearest-neighbor clustering of PBMCs from all time points identified multiple myeloid and T/natural killer (NK) cell populations along with B cells, platelets, and a mixture of proliferating cells (Fig. 2A). Because of PBMC isolation and cryopreservation, granulocyte populations were not accounted for in this study. Myeloid and T/NK cell populations were selected for subsequent clustering. We identified nine distinct T/NK cell subsets in PBMCs across all time points (Fig. S2). Overall, we did not detect major alterations in the T/NK cell composition from PBMCs, but at day 4 after infection, we did observe a drop in naïve CD8\(^+\) T cells and an increase in central memory CD4\(^+\) T cells (PBMC T/NK subpopulations 3 and 1, respectively) (fig. S2). Further clustering of myeloid cells identified seven distinct myeloid subsets in PBMCs (Fig. 2, B and C). There were major changes to CD14\(^+\) monocytes after infection. At baseline, a subpopulation of CD14\(^+\) monocytes expressing PGTS2 (PBMC myeloid subpopulation 3) were predominant (Fig. 2, C and D). At day 4 after infection, there was a marked loss of the PGTS2\(^+\) monocytes with an accompanying increase in two inflammatory monocyte populations with IFN-responsive gene signatures (PBMC myeloid subpopulations 0 and 1) (Fig. 2, C to F). PBMC myeloid population 1 had a more prominent expression pattern of IFN-stimulated genes at day 4 as compared with PBMC myeloid population 0, i.e., MX1, MX2, IFI16, IFI16, IFI27, ISG15, and OAS2, although both populations showed evidence of response to IFN (Fig. 2, B, E, and F). In contrast to the major changes in CD14\(^+\) monocytes, CD16\(^+\) monocytes (PBMC myeloid population 5) did not increase in relative abundance after infection (Fig. 2, B to D).

An increase in certain subsets of dendritic cells (DC2) have been associated with moderate/severe disease in patients with COVID-19 (65, 66). At day 4 after infection, we observed an increase in CD1c\(^+\) conventional DC2s [cDC2s; (67)] (PBMC myeloid subpopulation 4), which contracted by day 10. cDC1s (XCR1\(^+\) and BATF3\(^-\)—expressing PBMC myeloid population 6) were less abundant than cDC2s and changed relatively little in abundance during infection. The major alterations in the CD14\(^+\) monocytes substantially declined by day 7 after infection and returned to baseline levels by day 10 (Fig. 2, C and D). The PGTS2-expressing monocytes that were lost at day 4 returned by day 10 after infection and did not show major changes in gene expression (Fig. 2, C, D, and G).

In the BAL, multiple distinct T cell and myeloid populations were identified, along with proliferating cells, B cells, plasmacytoid DCs (pDCs), mast cells, and epithelial cells (Fig. 3A). Further clustering of BAL T cells identified five populations of T cells (fig. S2D). The largest change was the appearance on day 4 of a population with a mixture of CD8\(^+\) and CD4\(^+\) T cells that had a prominent
Fig. 1. Mild disease and rapid viral clearance in rhesus macaques infected with SARS-CoV-2. Six rhesus macaques infected with $2 \times 10^6$ TCID$_{50}$ of SARS-CoV-2/USA-WA1 i.n. (1 $\times 10^6$) and i.t. (1 $\times 10^6$). (A) 3D rendering of lung $^{18}$FDG-PET/CT images before infection and at days 3 and 9 after infection. (B) Quantification of the metabolic activity [mean $^{18}$FDG SUV body weight (bw)] and volume of tissue with $>$–300 HU (size of dot) from individual lesions based on VOIs defined at day 3 after infection. (C) Quantification of density (mean HU) and volume of tissue with $>$–300 HU (size of dot) from individual lesions based on VOI defined at day 3 after infection. DGCX did not have any detectable lung lesions. DGRX and DG4i did not have PET/CT imaging done at day 9 after infection. (D) Quantification of viral genomic RNA (left column) and subgenomic RNA (right column) of the N gene from nasal swabs, throat swabs, BAL, and plasma in copies per milliliter by RT-qPCR. Cutoff for positivity for genomic RNA is 3000 copies/ml, and cutoff for subgenomic RNA is 2500 copies/ml (nasal/throat) or 3000 copies/ml (BAL/plasma). (E and F) Quantification of viral genomic RNA (E) and subgenomic RNA (F) of the N gene from tissues at day 10 after infection in copies per gram of tissue by RT-qPCR with individual samples and median. Cutoff for genomic RNA is 1000 copies/g of tissue, and cutoff for subgenomic RNA is 1000 copies/g of tissue. (G) Representative images of staining for SARS-CoV-2 genomic RNA by RNAscope from the lung and nasopharynx at day 10 after infection. Red is viral RNA. Scale bars, 50 $\mu$m. gRNA, guide RNA. (H) Representative images of clotting patterns in the lung at day 10 after infection. Red is platelet staining for CD62P, brown is for fibrin, and blue is for nuclei. Scale bars, 100 $\mu$m.

IFN-stimulated gene signature (BAL T cell subpopulation 3) (fig. S2, D to F). These IFN-activated T cells were no longer detectable by day 7 after infection. Further clustering of BAL myeloid cells revealed 10 distinct populations of myeloid cells (Fig. 3, B and C). At baseline, BAL cells were mostly composed of multiple MRC1$^+$MARCO$^+$ myeloid subsets (BAL myeloid subpopulations 0, 2, and 3), which are likely alveolar macrophages (Fig. 3C). At day 4 after infection, there were major increases in populations of IFN-activated monocytes and macrophages in the BAL (BAL myeloid subpopulations 1 and 6), which declined by day 7 and returned to baseline levels by...
Platelets also responded to infection by up-regulating type I IFN–responsive subpopulation (Fig. 3C). The cDC2 in the BAL had a pattern of I IFN–responsive genes up-regulated at day 4. If log fold change is ≥0.5 and adjusted value is <0.01. Biological processes associated with the genes are indicated on the side, and the blue box highlights type P of the heatmap, and the first and second color bars distinguish the time point and animal, respectively. Genes were considered differentially expressed between time points if log fold change is >0.5 and adjusted P value is <0.01. Biological processes associated with the genes are indicated on the side, and the blue box highlights type I IFN–responsive genes at day 4 after infection (Fig. 3E). By day 10, the cDC2 had down-regulated the type I IFN genes and up-regulated genes associated with responses to lipopolysaccharide, including additional chemokines and IL1B, as well as the macrophage markers MRC1 and MARCO.

Fig. 2. Rapid and transient alterations in CD14+ monocytes in PBMCs after SARS-CoV-2 infection. (A) UMAP plot representing the clustering pattern of cells from scRNAseq data of PBMCs from four animals (DGCX, DG3V, DHGF, and DHKM) (left). Each dot denotes a cell and is colored on the basis of the automated cluster identification. Clusters of cells belonging to a certain cell type are demarcated and indicated on the plot. Expression levels of cell type defining markers are shown as a dot plot (right).

(B) UMAP2 plot of the myeloid cells from (A). Clusters were annotated with cell types based on gene expression patterns as shown on the dot plot and are identified with color intensity and dot size represent the level of expression and percent of cells in that cluster expressing the gene as defined in the key.

(C) UMAP plots separated by time depict the kinetic of the myeloid cells characterized in (B) before infection and at days 4, 7, and 10 after infection. (D) Fraction of cells that comprise each myeloid cell type for each of the four time points shown in (C) is summarized. (E to G) Heatmap represents the hierarchical clustering of normalized expression levels of differentially expressed genes for each cell for three myeloid clusters. The cluster names are indicated on top.

Day: 0: before 4: 7: 10: Animal: DG3V: DGCX: DHGF: DHKM
Fig. 3. Myeloid cell activation in the airways after SARS-CoV-2 infection. (A) UMAP plot of scRNAseq data from BAL of four rhesus macaques (DGX, DG3V, DHGF, and DHKM) (top). Cell clusters are annotated on the basis of broad cell types and are circled and indicated on the plot. Each dot represents a cell and is colored by cluster. dot plot displays the expression level of markers used to identify the cell types (bottom). Color intensity and dot size represent the level of expression and percent of cells in that cluster expressing the gene marker as defined in the key. (B) UMAP plot (left) of the subclustering of the myeloid cells from (A). Clusters were annotated with cell types based on gene expression patterns as shown on the dot plot and are identified with different numbers and colors on the plots (right). (C) UMAP plot (left) of the subclustering of the myeloid cells from (A). Clusters were annotated on the basis of broad cell types and are circled and indicated on the plot. Each dot represents a cell and is colored by cluster. dot plot displays the expression level of markers used to identify the cell types (bottom). Color intensity and dot size represent the level of expression and percent of cells in that cluster expressing the gene marker as defined in the key. (D and E) Normalized gene expression from cells of two BAL myeloid clusters is visualized as a hierarchically clustered heatmap. The time points and animals are indicated as colored bars above the heatmap and are defined in the color key. Only genes that were differentially expressed between time points (log fold change ≥ 0.5 and adjusted P value < 0.01) are shown. Biological processes associated with the genes are indicated on the side, and the blue box highlights type I IFN–responsive genes up-regulated at day 4. (F) Spearman’s correlation matrix was based on the kinetics of viral loads, and the fraction of cells from BAL and PBMC myeloid and lymphoid clusters was calculated and visualized as a correlation network. Each circle represents a parameter with the different colors indicating a viral, BAL, or PBMC cluster parameter. The size of the circle is proportional to the number of significant correlations (adjusted P value < 0.05). A connecting line between two parameters indicates a significant correlation, with green and pink lines signifying a positive and negative correlation, respectively. (G) Average expression of IFN and IFN-stimulated genes from all BAL cells separated by time is clustered and represented as a heatmap. Genes that show a significant difference (adjusted P value < 0.05) in expression over time are indicated with asterisks.

Correlation analysis revealed strong positive correlations between viral RNA levels in the BAL, nasal swabs, and throat swabs (Fig. 3F). Viral RNA from BAL and nasal swabs was positively correlated with IFN-activated monocytes, macrophages, and T cells in the BAL. In contrast, PGT52+ monocytes and naive CD8+ T cells from PBMCs negatively correlated with viral RNA from nasal swabs and BAL. To ask whether type I, II, or III IFN was the stimulus for the IFN gene signature observed in many cell subsets, we analyzed IFNB1, IFNG, and IFNL1 gene expression across all cell types (Fig. 3G). We found that IFNB1 was up-regulated at day 4 after infection, the time point when IFN-activated immune cells were highest. IFNG and IFNL1 showed a relative increase at day 10, when viral RNA had already
Early B cell responses to SARS-CoV-2 infection

We measured multiple B cell subsets in PBMCs and BAL by flow cytometry, including resting naïve B cells (CD20^+ IgD^+ CD95^-), activated naïve B cells (CD20^+ IgD^- CD95^+), germinal center B cells (CD20^+ IgD^- BCL6^- Ki67^-), plasmablasts (CD20^+ IgD^- BCL-6^- CD38^hi CD27^-), and activated memory B cells (CD20^+ IgD^- BCL-6^- CD95^+)(fig. S3, A and B). Activated memory B cells were further subdivided into immunoglobulin M positive (IgM^+), IgG^+, IgA^+, and isotype undefined. After infection, we observed a decrease in total B cells in PBMCs (fig. S3C), an increase of 2 to 3% in the proportion of activated naïve B cells from PBMC at days 4 and 7 (fig. S3D), and a decrease in the overall proportion of activated B cells in PBMCs that are isotype undefined (fig. S3E). At necropsy, the frequency of B cells varied across tissues. Whereas the spleen had the largest fraction of B cells, the BAL and lung had the highest proportion of activated memory B cells (fig. S3, D to H). Anti-spike (S) IgM and IgG were first detectable in the plasma and BAL at ~day 10 after infection in most animals, although levels were low compared with positive controls (~2- to 5-fold above background) (fig. S4). Low levels of neutralizing antibodies against SARS-CoV-2/USA-WA1 were also detected in the plasma on day 10. Overall, there were very few changes in B cell populations over the first 10 days of infection, and antibody responses were just developing on day 10 after infection.

Kinetics of SARS-CoV-2–specific effector CD8^+ and CD4^+ T cell responses in the BAL and PBMC

We next performed a flow cytometric analysis of the Ag-specific T cell response to SARS-CoV-2. We observed only minor changes in the activation of bulk T cell responses in the PBMC after infection, with more dynamic changes in the BAL after infection (fig. S5, A and B). To examine SARS-CoV-2–specific T cell responses, we performed intracellular cytokine staining after ex vivo restimulation with peptide pools from the viral S, N, and membrane (M) proteins, as well as peptide pools (megapools) derived from multiple SARS-CoV-2 Ags found to be immunogenic in humans (68, 69). As expected, Ag-specific T cell responses were not detected at day 4 after infection in PBMCs or BAL (fig. 4A to D). CD4^+ T cell responses to S, N, and megapool each reached ~4 to 6%, on average, by day 7, whereas Ag-specific CD8^+ T cells were ~1% at this time point in the BAL (fig. 4, B and D). Consistent with a slightly delayed response, Ag-specific CD8^+ T cells in the BAL continued to expand in frequency and maintained Ki67 expression between days 7 and 10 after infection, whereas Ag-specific CD4^+ T cells peaked in frequency at day 7 and decreased Ki67 expression between days 7 and 10 (Fig. 4, D and E). frequencies of Ag-specific T cells were ~10- to 20-fold higher in the BAL versus PBMC. Moreover, CD8^+ and CD4^+ T cell responses against S and N were consistently immunodominant in comparison to M-specific T cells.

In addition to producing IFNγ and tumor necrosis factor (TNF) after peptide stimulation, most of the Ag-specific CD8^+ T cells in the BAL also expressed granzyme B and degranulated after restimulation, as indicated by CD107a/b surface staining (Fig. 4F). About 25 to 60% of Ag-specific CD4^+ T cells in the BAL also made interleukin-2 (IL-2). Furthermore, both CD8^+ and CD4^+ Ag-specific T cells in the BAL up-regulated markers of tissue residence, CD69 and CD103, between days 7 and 10 after infection (fig. S5, C and D). Thus, SARS-CoV-2–specific CD8^+ and CD4^+ T cells in the airways displayed typical effector functions associated with cytotoxic T lymphocyte (CTL) and T helper 1 (Th1) cells, respectively.

Distribution of SARS-CoV-2–specific CD8^+ and CD4^+ T cell responses in mucosal tissues

At the day 10 necropsy, we examined SLO and NLT from the upper and lower respiratory tract for bulk and Ag-specific T cells. Tissue-resident memory (Trm) CD8^+ and CD4^+ T cells (CD95^- CD69^- CD103^-) were detected in all NLTs measured, including the lung, nasal turbinates, salivary glands, and tonsils (Fig. 5, A and B). CD103^- Trm cells were more abundant among CD8^+ compared with CD4^+ T cells in the BAL, salivary glands, and lymph nodes (LNs), which has been shown in other model systems (70, 71). Using intravenous antibody staining to distinguish between tissue parenchymal and intravascular cells (72–74), we confirmed that most cells in the BAL, nasal turbinates, salivary gland, tonsils, and LNs were from the tissue parenchyma (fig. S6). As expected for such a highly vascularized tissue, most cells from the lung were intravascular stain positive, but a small population of CD69^iv^- cells were detectable in the lungs, confirming that tissue-resident cells were also detected in pulmonary tissue. We next quantified the magnitude of SARS-CoV-2–specific T cells in each of these tissues. S-, N-, and megapool-specific CD8^+ and CD4^+ T cells were detected in the BAL, previously PET hot lung lesions, pulmonary LNs, peripheral LNs, spleen, and PBMC (i.e., the frequency of IFNγ^+ and/or TNF^+ cells after peptide restimulation was statistically significantly higher than the unstimulated samples). Unexpectedly, Ag-specific CD8^+ and CD4^+ T cell responses could not be detected in most of the nasal turbinates, salivary gland, and tonsils (Fig. 5C). The absence of Ag-specific T cells cannot be accounted for by poor T cell isolation from tissues (Fig. 5, A and B) or lack of virus replication at these sites (Fig. 1, D to F). Thus, the early clonal burst of SARS-CoV-2–specific T cells is highly focused on the BAL and unexpectedly undetectable in the nasal and oral mucosa.

Overall, the kinetics of SARS-CoV-2 replication and innate/adaptive immune response in rhesus macaques appears typical of an acute viral infection (Fig. 5D). SARS-CoV-2 replication peaks within 1 to 2 days after infection and rapidly decreases thereafter. IFN-γ-responsive myeloid responses are rapidly detected in the PBMC and BAL at day 4 after infection. IFN-driven innate immune responses and lung inflammation then decline by day 7 after infection, followed by an increase in inflammatory DCs and Ag-specific T cells at day 10.

DISCUSSION

We show here that during mild COVID-19 in rhesus macaques, SARS-CoV-2 replication is largely suppressed before the induction of virus-specific T cell responses. PET/CT imaging showed regions of ground glass opacity and consolidation with elevated 18F-FDG uptake in the lungs on day 3 after SARS-CoV-2 infection, which completely resolved by day 9. A longitudinal scRNAseq analysis identified early type I IFN–responsive monocyte, macrophage, and
Fig. 4. Kinetics of SARS-COV-2–specific CD8⁺ and CD4⁺ T cell responses in the airways. (A to D) Ag-specific CD8⁺ and CD4⁺ T cell responses in the blood and BAL enumerated by production of cytokines (IFNγ and/or TNF) after ex vivo peptide stimulation with peptide pools to S, N, M, and an optimized SARS-CoV-2 peptide megapool (see Materials and Methods for description of peptides). Representative flow cytometry plots of Ag-specific CD8⁺ and CD4⁺ T cells from DG4i at day 10 after infection from unstimulated, megapool, S, N, and M peptides from blood (A) and BAL (B) gated on activated T cells, i.e., CD8⁺CD95⁺ or CD4⁺CD95⁺. Quantification of Ag-specific T cells from all animals over time in blood (C) and BAL (D), calculated by subtracting the frequency of IFNγ⁺ and/or TNF⁺ in the unstimulated samples from the frequency in the stimulated samples. Bottom row of graphs is an overlay of the mean CD8⁺ and CD4⁺ Ag-specific responses with SE and a two-way ANOVA with a Sidak’s multiple comparison test of CD4 versus CD8 responses at each time point. DGCX and DG3V do not have quantification of S, N, and M responses from BAL at day 4 and are only represented by megapool at day 4. (E) Representative flow cytometry plots of Ki67 expression by Ag-specific CD8⁺ and CD4⁺ T cells from the BAL after S peptide stimulation at days 7 and 10 after infection from DG4i. Graphs indicate the percent Ki67⁺ of Ag-specific CD8⁺ and CD4⁺ T cells responding to megapool, S, and N peptides from BAL at days 7 and 10 after infection. Only samples with >35 data points were included. Paired t test of day 7 versus day 10 for CD8 and CD4 separately and CD8 day 10 versus CD4 day 10. Ki67 staining was not done for DGCX and DG3V. (F) Representative flow cytometry plots of granzyme B, CD107a/b, and IL-2 expression by Ag-specific CD8⁺ and CD4⁺ T cells from the BAL after S peptide stimulation at days 7 and 10 after infection from DG4i. Graphs indicate the percent granzyme B⁺, CD107a/b⁺, or IL-2⁺ of Ag-specific CD8⁺ and CD4⁺ T cells responding to megapool, S, and N peptides from BAL at days 7 and 10 after infection. Only samples with >35 data points were included. Paired t test of day 7 versus day 10 for CD8 and CD4 separately. For all statistical analysis, P < 0.05 for the given test is considered significant. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
**Fig. 5. Distribution of SARS-CoV-2–specific effector CD8+ and CD4+ T cells in mucosal tissues.** (A and B) CD69 and CD103 and Ag-specific T cell responses after ex vivo peptide stimulation from SLOs and NLTs at day 10 after infection. (A) Representative flow cytometry plots of CD69 and CD103 expression on CD8+CD95+ or CD4+CD95+ from the BAL, previously hot lung sections, normal lung sections, nasal turbinates, salivary gland (parotid), tonsil, previously hot pulmonary LN, normal pulmonary LN, peripheral LN (axillary), spleen, and blood from DHGF in unstimulated samples at day 10 after infection. (B) Enumeration of percent CD69+ and CD103+ of CD8+CD95+ or CD4+CD95+ in unstimulated samples. Peripheral LN includes axillary, inguinal, and cervical LNs. Previously hot lung sections not done for DGCX and DG3V. Sidak’s multiple comparison test for values on CD8+ versus CD4+ T cells for each tissue. (C) Tissue distribution diagram and quantification of the frequency of Ag-specific (IFN-γ+ and/or TNF+ in CD4+CD95+ (left graph) or CD8+CD95+ (right graph) in each tissue against peptide pools for S, N, M, and an optimized SARS-CoV-2 peptide megapools for CD4+ and CD8+ T cells (see Materials and Methods for description of peptides). Frequency calculated by subtracting the frequency of IFN-γ+ and/or TNF+ in the unstimulated samples from the frequency in the stimulated samples. Statistics are paired t tests of stimulated versus unstimulated for each condition. Ag-specific T cells from BAL at day 10 were shown in Fig. 4, with addition of CD8 megapool here. Tissue graphic was created with BioRender.com. (D) Representative summary graphs of the immune response to SARS-CoV-2 infection in rhesus macaques. Median genomic and subgenomic viral RNA levels from nasal swabs on a log scale, as in Fig. 1. Mean frequency of myeloid subpopulations 1 and 6 in BAL, as in Fig. 3. Mean frequency of cDC2 (BAL myeloid subpopulation 4) in BAL, as in Fig. 3. Mean frequency of the sum of Ag-specific CD4+ and CD8+ T cells (S + N + M peptide pools) in BAL, as in Fig. 4. For all statistical analysis, \( P < 0.05 \) for the given test is considered significant. \( * P < 0.05 \), \( ** P < 0.01 \), \( *** P < 0.001 \), and \( **** P < 0.0001 \). ns, not significant; MΦ/Mono, macrophage/monocyte.
DCs in PBMC and BAL that mostly dissipated before the arrival of virus-specific CD8+ and CD4+ T cells. SARS-CoV-2–specific effector T cells were abundant in the pulmonary compartment but undetectable in nasal turbinates, tonsils, and salivary glands, highlighting major differences in the localization of Ag-specific T cells in pulmonary and extrapulmonary mucosal tissues during SARS-CoV-2 infection.

Type I IFN is emerging as a critical mediator of control of SARS-CoV-2 infection (4–6). Type I IFN likely acts directly on infected epithelial cells to suppress viral replication, but it may also be important in driving the activation of antiviral responses by myeloid cells. On day 4 after infection, there was a notable increase in two populations of IFN-activated myeloid cells in the BAL, one having monocyte-like features and the other more closely resembling alveolar macrophages, indicating that recruited monocytes and lung-resident macrophages are key early responders to type I IFN after SARS-CoV-2 infection. In our study, the abundance of type I IFN–activated myeloid cells in the BAL positively correlated with viral loads in the nasal swabs and BAL. These results are consistent with the data from African green monkeys showing a strong type I IFN gene signature in macrophages from lung tissue 3 days after SARS-CoV-2 infection (48). However, several scRNAseq studies from patients with COVID-19 have found that inflammatory monocytes/macrophage populations are increased with disease severity (3, 10, 12, 14), suggesting that early type I IFN responses are host protective, but prolonged activation of this pathway may be detrimental. Consistent with this hypothesis, in this model of mild disease, we observed a rapid resolution of IFN-activated phagocytes in both the blood and BAL by day 7 after infection.

On day 10 after infection, we observed a major reduction in the frequency of alveolar macrophages, which has also been documented in individuals with severe COVID-19 (12, 75). Our data indicate that loss of alveolar macrophages can also occur after mild SARS-CoV-2 infection, so their loss may not be directly related to disease outcome. We also observed a major increase in the proportion of CD1c+ DCs in the BAL on day 10 after infection, a time when Ag-specific T cells were abundant in the airways. CD1c+ DCs displayed a distinct gene expression profile on day 10 with a notable increase in IL-1β and multiple proinflammatory chemokines. These cells may correspond to a population of inflammatory cDC2 that have been shown to appear in the lung after viral infection in mice (76). These cells were found to have an enhanced ability to stimulate both virus-specific CD4+ and CD8+ T cells. Collectively, our data indicate that there may be two distinct phases of cellular innate immune responses in the BAL after SARS-CoV-2 infection. The first is characterized by an early wave of type I IFN–activated phagocytes, which may play an important role in the initial suppression of viral replication. The second is characterized by an abundance of proinflammatory DCs, which may be key stimulators of the Ag-specific T cells that simultaneously arrive in the airways.

Ag-specific T cell responses were substantially greater in the BAL versus PBMCs, with the average sum of S+, N+, and M-specific T cells reaching ~12% of CD4+ T cells and ~ 7% of CD8+ T cells in the BAL compared with ~1 and ~ 0.2% in PBMC, respectively. In the BAL, virus-specific T cell responses preceded CTL responses. The CD8+ T cell clonal burst likely had not yet peaked, evidenced by their maintained expression of Ki67 at day 10 after infection. The lack of virus-specific T cells in the nasal turbinates, salivary glands, and tonsils, despite virus infection and subsequent clearance from these tissues, was unexpected. The mechanisms underlying the lack of Ag-specific effector T cells in the infected nasal and oral mucosa are not clear. It remains possible that SARS-CoV-2–specific T cell responses were not detected in these sites because they produce molecules other than IFNγ, TNF, IL-2, granzyme B, or degranulation markers CD107a/b after ex vivo peptide stimulation (i.e., Tia2 cytokines, which were not measured here). Alternative techniques for functionally agnostic detection of SARS-CoV-2–specific T cell responses, such as the activation-induced marker assay, should be tested in future studies (17, 69, 77). Nevertheless, it is unlikely that T cells in these tissues specific to Ags other than the ones tested here, although this too seems unlikely, because the peptide pools used contain numerous immunogenic peptides from across the entire viral genome (68).

Last, it is possible that T cells accumulate in these tissues after day 10, and further studies will be needed to determine the longevity and breadth of SARS-CoV-2–specific T cell responses in tissues at later time points. The paucity of SARS-CoV-2–specific T cells in the nasal and oral mucosa may at least partly explain the observation of individuals previously infected with SARS-CoV-2 becoming reinfected (78–80). Our findings support the hypothesis that control of primary SARS-CoV-2 infection in these tissues is largely T cell independent, which is consistent with a recent report showing that rhesus macaques depleted of CD4+ and/or CD8α+ cells before SARS-CoV-2 infection controlled the virus in the upper and lower respiratory tract, albeit perhaps with a slight delay (64). Another study also found that CD8 depletion in cynomolgus macaques had no impact on control of SARS-CoV-2 infection (81). It is important to point out, however, that our data do not rule out a critical role for T cells in other settings of SARS-CoV-2 infection. For example, T cells likely play a role when SARS-CoV-2 infection does not resolve quickly, such as during moderate and severe COVID-19. T cells have been implicated in control of SARS-CoV-2 in other susceptible animal models, like the hACE2-expressing mouse lines and Syrian hamsters (82–84). Furthermore, N-specific CD8α+ T cells are correlated with less severe disease in patients (20). In addition, preclinical studies suggest that depletion of CD8α+ T cells from vaccinated monkeys before SARS-CoV-2 challenge significantly impairs control of virus replication (59). T cells may also play a major role in vaccine-elicited protection, and T cell–targeted peptide vaccines are currently being developed (20, 85, 86). Vaccine-elicited T cells may prove critical in protection against SARS-CoV-2 variants of concern that are able to evade neutralizing antibodies, because T cell epitopes are thought to be more conserved across isolates (87–92). It should also be noted that we cannot rule out the possibility that T cells may have played a role in clearance of any residual virus–infected cells remaining in the lungs after the first week when T cells arrived in the tissue.

Together, these data show that mild SARS-CoV-2 infection is associated with effective innate immune-mediated control. Future studies are needed to determine the importance of individual innate and adaptive immune cell types in suppression of SARS-CoV-2 replication.

MATERIALS AND METHODS

Study design
The study was designed with the goal of assessing the differential kinetics of lung inflammation, viral replication, and innate and
adaptive cellular immune responses against SARS-CoV-2 infection in a model of mild disease. The study had a predetermined end point of day 10 after infection. The number of animals included in the study was based on previous experience in detecting immune responses in NHP infection studies and practical limitations. The first four animals to be infected were selected for subsequent scRNAseq analysis.

**Animals and infection**

Six male rhesus macaques aged 2.5 to 6 years, weighing 3 to 10 kg, were infected with SARS-CoV-2/USA-WA1 (table S1). For infection, animals were anesthetized as described below and administered \( 2 \times 10^5 \) TCID\(_{50}\) total of SARS-CoV-2/USA-WA1: \( 1 \times 10^6 \) TCID\(_{50}\) in 3 ml i.t. with a plastic gavage tube attached to a 5-ml syringe and \( 5 \times 10^5 \) TCID\(_{50}\) in 0.5 ml i.n. in each nostril. The animals were examined daily with a health scoring sheet, as previously described (42). Animals were anesthetized with ketamine and dexmedetomidine before infection (days −4 to −55) and days 0, 1, 2, 3, 4, 7, and 10 (necropsy) for exams, PET/CT scans, viral swabs, blood and BAL fluid draws, and analysis of complete blood count and C-reactive protein. During anesthesia, animals were weighed and monitored for heart rate, respiratory rate, body temperature, and oxygen saturation. Glycopyrrolate and atipamezole were given for recovery from anesthesia. For each animal, the preinfection/baseline PET/CT scans are as follows: DGCX and DG3V, day −55; DHGF and DHKM, day −17; and DGRX and DG4i, day −26. The preinfection time points for swabs, blood, and BAL are as follows: DGCX and DG3V, day −4; DHGF and DHKM, day −14; and DGRX and DG4i, day −5. Graphs, including data plotted longitudinally, of any pre-infection time points were represented as day 0.

All animal experiments were approved by the Animal Care and Use Committee (ACUC), and all methods were performed under animal safety protocol LPD-25E at the National Institutes of Health. Experiments were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited animal biosafety level 3 (BSL-3) vivarium facility. Animals were singly housed in vented air cages with a 12-hour light/12-hour dark cycle. The animals were monitored twice daily, with a detailed physical exam once per day during the study. The Institutional Biosafety Committee approved all work with SARS-CoV-2 in the BSL-3 level facility and approved any inactivation methods used.

\(^{18}\text{FDG}-\text{PET/CT acquisition and data analysis}\)

Rhesus were sedated and imaged by PET/CT during mechanical ventilation (Hallowell Ventilator Model 2002) at baseline and on days 3 and 9 after infection. To reveal metabolic hyperactivity consistent with inflammation, a \(^{18}\text{F}\)-FDG dose of 0.5 mCi/kg was given intravenously 1 hour before PET imaging. During the uptake time, a high-resolution CT scan of the lungs was acquired with a breath hold on a LFER 150 PET/CT scanner (Mediso Inc., Budapest, Hungary) as previously described (93). The raw CT and PET data were reconstructed using the Nucline software (Mediso Inc., Budapest, Hungary) to create individual DICOM files that were coregistered using MIM Maestro (version 7.0, MIM Software Inc., Cleveland, OH).

By aligning the baseline PET/CT-fused images and those taken at days 3 and 9 in MIM Maestro, specific lung regions with abnormal density \([\sim \sim 550 \text{ Hounsfield units (HU)}]\) or metabolic activity \([\sim 1.5 \text{ standardized uptake value (SUV)}]\) were identified as volumes of interest (VOIs) or lesions similar to methods used previously, rather than using whole-lung VOI (94). For each animal, the lesion VOIs (day 3 in this study) were transferred to the aligned PET/CT images acquired at baseline and the day 9 time point, adjusting for position variations but keeping the same volume. Disease volume was estimated by using two density thresholds: tissues denser than −550 HU or denser than −300 HU for evaluating change over time. Regarding metabolic activity, PET parameters were estimated using a threshold of >2 SUV. Similar reference VOIs were used to identify metabolically activated tissues (SUV > 2) within pericarinal LNs. LN \(^{18}\text{F}\)-FDG uptake was measured in activated regions of the hilar and subcarinal LNs of each animal. Our analysis also included calculations of total lesion glycolysis. Two readers independently performed image analysis for each animal using consistent lesion labeling determined by a third reviewer. 3D projections of \(^{18}\text{FDG}\) uptake in the lung regions were generated using OsiriX version 5.9 software (Pixmeo, Geneva, Switzerland) as previously described (95).

**Blood and BAL collection**

Blood and BAL collection procedures followed ACUC-approved standard operating procedures and limits. Blood was collected in EDTA tubes and centrifuged at 2000 rpm for 10 min at 22°C to isolate the plasma. After plasma removal, the remaining blood was diluted 1:1 with 1× phosphate-buffered saline (PBS). Fifteen milliliters of 90% Ficoll-Paque density gradient (Cytiva, catalog no. 17144002), diluted with 10× PBS, was added to SepMate PBMC Isolation Tubes (STEMCELL Technologies, catalog no. 85450) and centrifuged at 1000 g for 1 min at 22°C to collect Ficoll below the separation filter. Blood and PBS mix was added to the SepMate tube with Ficoll-Paque and centrifuged at 1200g for 10 min at 22°C. The upper layer was poured into a 50-ml conical tube and brought to 50 ml with PBS + 1% fetal bovine serum (FBS) and then centrifuged at 1600 rpm for 5 min at 4°C. The cell pellet was resuspended at \(2 \times 10^7\) cells/ml in X-VIVO 15 media + 10% FBS for subsequent analysis. BAL was collected after intubation by instillation of 50 ml of warm pharmaceutical-grade PBS, 10 ml at a time. For cellular analysis, BAL was filtered through a 100-µm filter into a 50-ml conical tube and centrifuged at 1600 rpm for 15 min at 4°C. The cell pellet was resuspended at \(2 \times 10^7\) cells/ml in X-VIVO 15 media + 10% FBS for subsequent analysis.

**Necropsy**

Intravenous antibody was administered before euthanasia, as previously described (73). In brief, before necropsy, 10 ml of blood was drawn as a negative control and anti–CD45-biotin (100 µg/kg; clone: ITS_rhCD45 developed by M. Roederer’s laboratory) was infused. The infusion was circulated for 10 min before terminal exsanguination and necropsy. The BAL and another ~60 ml of blood were collected during exsanguination. After prossection of the lung and airways, specific lung regions observed to have abnormal HU density or FDG uptake in the day 3 images were scan-matched and collected separately as follows. The PET/CT scan images from day 3 after infection were used to locate the airway vascular bundle adjacent to the abnormality. At necropsy, the lung parenchyma was resected back to reveal the bronchus upstream of the abnormality, which was followed to the target region to be resected. We have used a similar technique for PET/CT scan matching at necropsy after *Mycobacterium tuberculosis* infection (96). LNs identified as having regions of SUV > 2.5 were collected separately from those.
with lower SUV on day 3. Normal lung sections were taken from each lobe. The resected lung sections, nasal turbinates, salivary gland, tonsils, spleen, and LNs along with were then divided for RNA isolation, histology, and flow cytometry analysis.

**Viral RNA quantification**

RNA from the nose and throat was collected by swabbing each nostril or back of the throat, respectively, with a sterile swab for 10 s. Swabs were placed in 1 ml of viral transport media [1× Hanks’ balanced salt solution, 2% FBS, gentamicin (100 µg/ml), and amphotericin B (0.5 µg/ml)] and stored on ice until RNA extraction. Swabs were vortexed in swab media before removing the swab tip. For RNA extraction, 140 µl of sample (plasma, first BAL wash, or swab media) was processed using a Viral RNA mini kit (QIAGEN, catalog no. 52906) and eluted in 50 µl of ribonuclease (RNase)–free water. For RNA isolation from tissues, tissue pieces were weighed before placing in 1 ml of RNAlater media (Sigma-Aldrich, catalog no. R0901) and stored at 4°C overnight and then stored at −80°C long term. Tissues were then thawed and processed in the RNeasy Plus Mini Kit (QIAGEN, catalog no. 74136) and eluted in 50 µl of RNase-free water. Eluted RNA was stored at −80°C long term.

Extracted RNA was used in a reverse transcription quantitative polymerase chain reaction (RT-qPCR) for detection of total or subgenomic RNA from the N gene of SARS-CoV-2. Total RNA reactions amplify both genomic viral RNA and subgenomic RNA from the N gene of SARS-CoV-2. Total RNA polymerase chain reaction (RT-qPCR) for detection of total or subgenomic RNA cutoff, CT < 37 was used. For genomic RNA, this was also limited to CT < 35 based on the manufacturer’s instructions. For subgenomic RNA cutoff, CT < 37 was used.

**Tissue digestion**

Tissues were processed for single-cell suspension before flow cytometry or peptide stimulation as follows. The spleen (about 0.5 inch by 0.5 inch portion) and LNs were placed in 5 ml of PBS + 1% FBS in a gentleMACS C tube (Miltenyi, catalog no. 13006334) and run on gentleMACS Octo Dissociator (Miltenyi), with n;spleen_02_01 setting, then filtered through a 100-µm filter into a 50-ml conical tube, and centrifuged at 1600 rpm for 5 min at 4°C. Salivary gland was gentleMACS dissociated as above, and after centrifugation, cells were resuspended in 7 ml of 44% Percoll (Sigma-Aldrich, catalog no. P1644) with 1× PBS and centrifuged at 2000 rpm for 20 min at 22°C without brake. The tonsil and lung were gentleMACS dissociated in 5 ml of digestion buffer [RPMI + deoxyribonuclease I (50 U/ml) + hyaluronidase (1 mg/ml) + collagenase D (1 mg/ml) (Roche)] and then agitated on a shaker at 220 rpm for 45 min at 37°C. Digestion reaction was stopped with equal parts PBS + 20% FBS and centrifuged at 1600 rpm for 5 min at 22°C. The cell pellet was resuspended in Percoll gradient, as above for salivary gland. After processing, the spleen and lung were cleared of red blood cells by resuspending cell pellet in 2 ml of ACK lysing buffer (Quality Biologicals, catalog no. 118-156-101) for 2 min at room temperature and then stopping the reaction with 10 to 20 ml of PBS + 1% FBS. Cells were resuspended at 2 × 10^7 cells/ml in X-VIVO 15 media + 10% FBS for further analysis.

**Peptide stimulation assay**

Single-cell suspensions were plated at 2 × 10^7 cells/ml in 200 µl in 96-well plates with X-VIVO 15 media + 10% FBS, Brefeldin (1000×; Invitrogen, catalog no. 00-4506-51) and monensin (1000×; Invitrogen, catalog no. 00-4505-51), CD107a allophycocyanin (APC; 1:50), CD107b APC (1:50), and peptide pools at 1 µg/ml. If cell counts from individual lung sections were below 5 × 10^5 total cells, then sections of similar kind were pooled together to reach the concentration for stimulation. Cells were then stimulated for 6 hours at 37°C + 5% CO_2 before surface staining. S peptide pool consisted of PepTivator SARS-CoV-2 Prot_S1 (Miltenyi, catalog no. 130-127-048) and PepTivator SARS-CoV-2 Prot_S (Miltenyi, catalog no. 130-127-953). N peptide pool consisted of PepTivator SARS-CoV-2 Prot_N (Miltenyi, catalog no. 130-126-699). M peptide pool consisted of PepTivator SARS-CoV-2 Prot_M (Miltenyi, catalog no. 130-126-703). CD4 megapool consisted of CD4_S_MP and CD4_R_MP, and CD8 megapool consisted of CD8_MP_A and CD8_MP_B, as described (69). After stimulation, cells were centrifuged at 1600 rpm for 5 min at 4°C and proceeded with surface staining. For enumeration of the frequency of Ag-specific populations responding each peptide pool, the frequency of IFNγ+ or TNF+ CD4+ or CD8+ T cells in each condition was adjusted for background staining in unstimulated wells (% stimulated − % unstimulated). For Fig. 4 (C and D), statistical analysis was done on frequencies of Ag-specific CD4+ and CD8+ T cells after background subtraction. In Fig. 5C, the statistical analysis was a paired t test of frequencies of Ag-specific CD4+ or CD8+ T cells between stimulated and unstimulated samples for reporting of the detection of Ag-specific populations above background in each tissue.

**Flow cytometry and antibody staining**

Cells were resuspended in 50 µl of surface stain antibodies diluted in PBS + 1% FBS and incubated for 20 min at 4°C. Cells were washed three times with PBS + 1% FBS before fixation with eBioscience...
Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific, catalog no. 88-8824-00) for 16 hours at 4°C. After fixation, cells were centrifuged at 2200 rpm for 5 min at 4°C without brake and washed once with eBioscience Permeabilization Buffer. Cells were resuspended in 50 μl of intracellular stains diluted in eBioscience Permeabilization Buffer and stained for 30 min at 4°C. After staining, cells were washed with eBioscience Permeabilization Buffer two times and resuspended in PBS + 1% FBS + 0.05% sodium azide for running on the BD Symphony platform.

B cells were resuspended in 50 μl of human Fc block (BD, catalog no. 564220) diluted to 1:500 in PBS + 1% FBS and incubated for 30 min at 4°C before washing and surface staining. Antibodies used can be found in table S2. The biotinylated intravenous antibody was detected with streptavidin conjugated to the indicated fluorophore included in the surface stain. The intravenous stain–positive cells are localized to the tissue vasculature, whereas intravenous stain–negative cells are localized to the tissue parenchyma. For phenotypic analysis of Ag-specific T populations, a cutoff of >35 peptide-specific T cells per sample was used for subsequent analysis to prevent the introduction of error from quantifying too few events.

Fluorescent enzyme-linked immunosorbent assay

High-binding polystyrene half-area 96-well plates (Greiner Bio-One, catalog no. 675077) were coated with recombinant SARS-CoV-2 spike trimer (Miltenyi, catalog no. 130-127-683) at 2 μg/ml and 25 μl per well for at least 16 hours at 4°C. Plates were washed with wash buffer (PBS + 0.05% Tween 20) using an automatic plate washer (five times with 180 μl). Plates were blocked with 25 μl per well of blocking buffer (PBS + 1% bovine serum albumin + 0.01% Tween 20) for at least 16 hours at 4°C. Plates were washed again as above. Plasma and BAL fluid were initially diluted 1:10 with blocking buffer and threefold serial diluted for a total of 12 dilutions per sample. The dilutions were plated at 25 μl per well and incubated for 1 hour at 37°C. Plates were washed again as above. Goat anti-monkey IgG–horseradish peroxidase (HRP) (Invitrogen, catalog no. PAI-84631) and goat anti-monkey IgM–HRP (Alpha Diagnostics, catalog no. 70031) were diluted with blocking buffer (1:3000 and 1:2000, respectively). Secondary antibodies were plated at 25 μl per well and incubated 30 min at 37°C. Plates were washed again as above. Plates were developed with the QuantaRed Enhanced Chemiflourescent HRP Substrate Kit (Thermo Fisher Scientific, catalog no. 15159), following the manufacturer’s instructions for reagent preparation, and incubated for 15 min for IgG and 30 min for IgM, and the reaction was stopped with the manufacturer-provided stop solution. Plates were immediately read on a SpectraMax Gemini fluorescent microplate reader (Molecular Devices) with an excitation of 570 nm and an emission of 585 nm.

Live virus neutralization assay

Vero-E6 cells stably expressing human TMPRSS2 (Vero-E6T2) (97), a gift from the laboratory of J. Yewdell (National Institute of Allergy and Infectious Diseases), were maintained at 37°C and 5% CO₂ in D10 + medium [Dulbecco’s modified Eagle medium (DMEM) + 10% FBS, 1X GlutaMAX, 1X antibiotic-antimycotic (Gibco), and hygromycin B (250 μg/ml; InvivoGen)]. One day before the assay, cells were plated in 12-well plates (Falcon, catalog no. 353043) at a density of 0.4 million cells per well in 2 ml of D10 + medium. At the day of the assay, 12-well plates were washed twice with D2 medium (DMEM + 2% FBS and 1X GlutaMAX).

Rhesus macaque plasma samples, collected at days 0 and 10 after infection with SARS-CoV-2, were heat-inactivated at 56°C for 30 min. After an initial 1:5 dilution, plasma samples were further twofold serially diluted with D2 medium, and 100 μl of each dilution was incubated for 1 hour at 37°C with an equivalent volume of D2 medium containing 40 to 50 plaque-forming units of SARS-CoV-2/USA-WA1/2020 virus. After the incubation, 100 μl of plasma/virus mixture was added to Vero-E6T2 in duplicate wells containing 300 μl of D2 medium. The virus was allowed to absorb onto the cells for 1 hour at 37°C and 5% CO₂ with occasional mixing. At the end of the incubation, 1.5 ml of DMEM supplemented with 0.6% of methyl cellulose was added per well, and the plates were maintained for another 66 to 72 hours at 37°C and 5% CO₂.

At the end of the incubation, the medium was removed and replaced with 1 ml of crystal violet solution containing 5% ethanol and 3% neutral-buffered formamide. The wells were stained for 20 min at room temperature, then washed briefly with deionized water, and air-dried in the biosafety cabinet. Once dry, the plates were scanned at 300 dots per inch, and plaques were counted from the images. The percentage of inhibition was calculated on the basis of the number of plaques observed for each dilution compared with controls. The IC₅₀ (median inhibitory concentration) of the percent inhibition for the dilution series was calculated on the basis of nonlinear regression. Each experiment included the following controls: uninfected cells as negative control, virus in the absence of rhesus macaque serum, and virus incubated with a dilution of a known positive serum (provided by SAB Biotherapeutics).

Immunohistochemistry and RNAscope

Tissues for histology were collected in 10% neutral-buffered formalin and stored at room temperature for 16 hours. Fixed tissues were transferred to 70% ethanol and stored at room temperature until processing. Slides were cut 10 μm thick using standard RNAse precautions. Immunohistochemical slides were deparaffinized and treated with AR6 buffer (Akoya Biosciences, USA) for 20 min at 100°C. Tissues were then permeabilized using 0.2% Triton X-100 (MilliporeSigma, USA) for 10 min. After blocking, slides were incubated with primary antibodies against CD62P (clone EPR22850-190, Abcam, USA) and fibrin (clone 59D8, MilliporeSigma, USA) at a 1:500 and 1:200 concentration, respectively. After washing, slides were stained according to the protocol for the ImmPRESS Duet Double Staining Polymer Kit (Vector Laboratories, USA) and counterstained with hematoxylin. Slides used for in situ hybridization were deparaffinized and treated with RNAscope epitope retrieval buffer (ACD Bio-Techne, USA) for 20 min at 100°C. Endogenous peroxidases were then blocked with hydrogen peroxide, and tissue was permeabilized with a diluted RNAscope Protease Plus for 20 min at 40°C. Probes for SARS-CoV-2, containing 20 pairs of probes spanning S gene (category no. 848561, ACD Bio-Teche, USA), were incubated for 2 hours at 40°C. Slides were then processed according to RNAscope 2.5 HD Assay-RED (ACD Bio-Teche, USA) protocol and counterstained with hematoxylin. Slides stained immunohistochemically or by in situ hybridization were imaged using Aperio VERSA (Leica Microsystems, USA) and analyzed using QuPath, an open-source software developed by the University of Edinburgh. SARS-CoV-2 puncta were confirmed using both positive and negative controls to ensure accurate staining.
Cells from the BAL fluid and PBMCs from blood were obtained as described above and cryopreserved in 1 ml of RPMI + 40% FBS + 15% dimethyl sulfoxide. PBMC and BAL samples from days −7, 4, 7, and 10 for monkeys DHGF, DG3V, and DHKM and both sample types from days −7, 4, and 10 for monkey DGCGX were processed for scRNAseq using the 10X Genomics Chromium Single Cell 3’ Kit (v3.1). In brief, cryopreserved samples were quickly thawed using a water bath set to 37°C and washed twice using 10% FBS in RPMI. Samples were then stained with unique TotalSeq-A hashtag antibodies [hash tag oligonucleotide (HTO)] as per the manufacturer’s (BioLegend) protocol. Equal number of cells from each sample was pooled and super-loaded on a 10X Genomics NextGEM chip, and single-cell GEMs were generated on a 10X Chromium Controller as previously described (98). Subsequent steps to generate complementary DNA and HTO libraries were performed following the 10X Genomics and BioLegend’s protocol, respectively. Libraries were pooled and sequenced on Illumina NovaSeq S1 and S2 flow controllers as previously described (99). Subsequent steps to generate complementary DNA and HTO libraries were performed following the 10X Genomics and BioLegend’s protocol, respectively. Libraries were pooled and sequenced on Illumina NovaSeq S1 and S2 flow controllers as previously described (99). Subsequent steps to generate complementary DNA and HTO libraries were performed following the 10X Genomics and BioLegend’s protocol, respectively. Libraries were pooled and sequenced on Illumina NovaSeq S1 and S2 flow controllers as previously described (99). Subsequent steps to generate complementary DNA and HTO libraries were performed following the 10X Genomics and BioLegend’s protocol, respectively.

The sequenced data were processed using Cell Ranger (version 5.0) to demultiplex the libraries. The reads were aligned to Macaca mulatta mmul_10mm to generate count tables. The count tables were then further processed and analyzed using the Seurat (version 4.0) in R (version 4.1.0). Samples from different PBMC and BAL libraries were integrated using IntegrateData function to account for possible batch effects and to generate one integrated dataset for each tissue type. Cells were then filtered for less than 15% mitochondrial contamination, and only singlets as determined by the HTOs were included, resulting in 16,769 PBMCs and 7274 BAL cells for downstream analysis. Data were normalized and scaled, and FindVariableFeatures function was used to identify variable genes to subset and integrate the data to correct for animal bias. Principal components analysis was performed to find neighbors and clusters, and UMAP reduction was performed with 20 dimensions. FindAllMarkers with a filter of log fold change ≥ 0.25 and percent of cells expressing the marker ≥ 0.25 was used to identify gene markers that distinguish the cell clusters, and the clusters were manually assigned cell types on the basis of identified canonical markers. Myeloid and T (and NK in case of PBMC) cell clusters were further subclustered, and clusters were again manually annotated on the basis of gene markers determined by the FindAllMarkers function.

Differentially expressed genes between time points of a particular cluster were identified by running FindMarkers function with MAST and comparing one time point to all other time points or one time point to another in a pairwise manner. Genes with a log fold change ≥ 0.5, percent of cells expressing the marker ≥ 0.25, and adjusted P value ≤ 0.01 were considered significant, and these genes were hierarchically clustered and displayed as a heatmap using the ComplexHeatmap function in R. Gene Ontology enrichment analysis of genes up-regulated at a particular time point was performed using clusterProfiler to identify biological processes (adjusted P value ≤ 0.05). The AverageExpression function was used to calculate average gene expression of IFN and IFN-stimulated genes across all cells over time and was visualized using heatmap.

Spearman’s correlation test was performed between viral loads from various sites and fraction of cells in a particular cluster at all available time points and filtered for adjusted P value < 0.05. Correlations were visualized using a network diagram generated using igraph in R. Hashtag antibodies used can be found table S2.
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