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Primary nasal viral infection rewires the tissue-scale memory response

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ABSTRACT

The nasal mucosa is frequently the initial site of respiratory viral infection, replication, and transmission. Recent work has started to clarify the independent responses of epithelial, myeloid, and lymphoid cells to viral infection in the nasal mucosa, but their spatiotemporal coordination and relative importance remain unclear. Furthermore, understanding whether and how primary infection shapes tissue-scale memory responses to secondary challenge is critical for the rational design of nasal-targeting therapeutics and vaccines. Here, we generated a single-cell RNA-sequencing (scRNA-seq) atlas of the murine nasal mucosa sampling three distinct regions before and during primary and secondary influenza infection. Primary infection was largely restricted to respiratory mucosa and induced stepwise changes in cell type, subset, and state composition over time. Interferon (IFN)-responsive neutrophils appeared 2 days post infection (dpi) and preceded transient IFN-responsive/cycling epithelial cell responses 5 dpi, which coincided with broader antiviral monocyte and NK cell accumulation. By 8 dpi, monocyte-derived macrophages expressing *Cxcl9* and *Cxcl16* arose alongside effector cytotoxic CD8 and *Ifng*-expressing CD4 T cells. Following viral clearance (14 dpi), rare, previously undescribed *Meg3*+*MHC-II*+ epithelial cells and **K**rt13+ **n**asal **i**mmune-**i**nteracting **f**loor **e**pithelial (KNIIFE) cells expressing multiple genes with immune communication potential increased concurrently with tissue-resident memory T (T_{RM})-like cells and IgG+/IgA+ plasma cells. Proportionality analysis coupled with cell-cell communication inference underscored the CXCL16–CXCR6 signaling axis in effector CD8 T cell and T_{RM} cell formation in the nasal mucosa. Secondary influenza challenge administered 60 dpi induced an accelerated and coordinated myeloid and lymphoid response with reduced IFN-responsive epithelial activity, illustrating how tissue-scale memory to natural infection engages both myeloid and lymphoid cells without broad epithelial inflammation. Together, this atlas serves as a reference for viral infection in the upper respiratory tract and highlights the efficacy of local coordinated memory responses upon rechallenge.

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INTRODUCTION

As the primary passage to the lower airway, the nasal mucosa balances the complex roles of olfaction, filtration and conditioning of inhaled air, and host defense. To accomplish these diverse functions, the nose contains distinct anatomical structures, harbors a varied yet organized cellular composition, and secretes a multitude of proteins with distinct and complementary roles (Harkema et al., 2006). In the face of pathogens, the nasal mucosa is thought to mount a variety of incompletely understood defense mechanisms to protect against infection and limit spread to the lower respiratory tract (Bosch et al., 2013). Nevertheless, many respiratory pathogens manage to infect or colonize the upper airways and disseminate into the lungs, causing millions of cases of severe disease, hospitalizations, and deaths annually (Clark, 2020; Roth et al., 2018; Shinya et al., 2006).

There is a growing appreciation for how the inflammatory state of nasal tissue affects respiratory viral infection outcome. The COVID-19 pandemic has helped accelerate research to understand the roles of interferons (IFNs) in nasal protection and disease trajectory, with studies highlighting the importance of sample timing and location, viral burden, and strain (Bastard et al., 2022; Kim and Shin, 2021; Park and Iwasaki, 2020; Sposito et al., 2021). Study of the human nasopharynx during SARS-CoV-2 infection showed muted IFN-response in epithelial cells from individuals who went on to develop severe disease when compared with mild cases (Ziegler et al., 2021). Expression of specific IFN stimulated genes (ISGs) like *OAS1* associate with protection from severe COVID-19 and may even drive viral mutations to overcome host protection (Wickenhagen et al., 2021). More generally, evidence of a recent prior infection in children receiving a live-attenuated influenza vaccine was associated with enhanced ISG signaling and lower viral shedding (Costa-Martins et al., 2021). Collectively, this suggests that the present nasal state, cellular composition, and antiviral signaling capacity, as informed by the cumulative history of environmental exposures, may drive disease outcomes (Bastard et al., 2020; Habibi et al., 2020; Ordovas-Montanes et al., 2020; Weisberg et al., 2021; Zhang et al., 2020).

Following viral infection or intranasal (i.n.) vaccination, immune memory in the nasal mucosa can provide long-term protection both systemically and at the mucosal barrier, reducing pathology and infection burden in the lower airways and elsewhere (Johnson et al., 1986; Johnson Jr. et al., 1985; Rutigliano et al., 2010). Local protection is afforded by both cellular and humoral immune mechanisms. For example, CD8+ tissue-resident memory (T_{RM}) cells that form following upper respiratory tract influenza A virus (IAV) infection correlate with enhanced protection against heterologous IAV strain rechallenge (Pizzolla et al., 2017). Protective mucosal IgA producing plasma cells, and antibodies capable of neutralizing virus, can be generated in the nasal mucosa

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following IAV, vesicular stomatitis virus, respiratory syncytial virus, and SARS-CoV-2 infections (Johnson Jr. et al., 1985; Liew et al., 2023; Sterlin et al., 2021; Wellford et al., 2022; Weltzin et al., 1996). Even so, respiratory infections like IAV remain epidemic and kill up to 500,000 people each year (Iuliano et al., 2018).

To develop protective, durable, and efficacious vaccines for respiratory viruses, we must reach a deeper understanding of the establishment, timing, and cooperation of tissue-scale memory following natural infection (Morens et al., 2023). Immune responses to pathogens often occur in stepwise fashion: recognition of pathogen-associated molecular patterns by immune and/or epithelial cells leads to cytokine production that broadly activates innate immune cells that in turn recruit pathogen-specific effector lymphocytes, some of which will develop into circulating and tissue-resident memory cells (Iwasaki and Medzhitov, 2015). Prior infection or vaccination, however, has the capacity to re-order and even invert these circuits locally in the barrier tissues that re-encounter infection (Kadoki et al., 2017; Kaufmann et al., 2018; Ols et al., 2020; Ordovas-Montanes et al., 2020; Schenkel et al., 2014). During viral rechallenge, T_{RM} cells exhibit antiviral effector functions and can act as sentinels that send antigen-specific inflammatory “alarms” to local immune cells to activate multicellular anti-microbial responses at the site of infection (Ariotti et al., 2014; McMaster et al., 2015; Schenkel et al., 2014; Steinbach et al., 2016). In mucosal vaccination, IFN-γ produced by antigen-specific T cells is sufficient to induce increased inflammatory cytokine production by both distal (Bosch-Camós et al., 2022; Stary et al., 2015) and local (Yao et al., 2018) antigen presenting cells, suggesting that recruited and/or tissue-resident cells can contribute to rapid memory responses. Similarly, antibodies can directly neutralize virus and also orchestrate a variety of antiviral effector functions through antibody Fc-receptor mediated binding by NK cells, macrophages, and neutrophils (Boudreau and Alter, 2019). However, most of these studies to date have focused on the role of individual cell types or limited interactions during a memory response.

Here, we present a tissue-scale single-cell RNA-sequencing (scRNA-seq) atlas of the murine nasal mucosa before and during primary IAV infection and secondary rechallenge. By sampling multiple regions, timepoints, and cell lineages, we develop a compositional landscape of the tissue and reveal how the diversity of cell subsets and states dynamically changes in response to infection and during a memory response. Primary IAV infection induced reproducible stepwise shifts in cell composition starting with increased IFN-responsive neutrophil subsets followed by broader antiviral/IFN-stimulated responses in epithelial, myeloid, and lymphoid immune cells. Next, monocyte-derived macrophages (MDMs) accumulated along with effector CD8 and CD4 T cells. Following viral resolution, T_{RM} cells and plasma cells are established

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alongside increased frequencies of rare *Meg3+MHC-II+* epithelial cells and **K**rt13+ **n**asal **i**mmune-interacting **fl**oor **e**pithelial (KNIIFE) cells expressing genes for several ligands and receptors known to modulate immune cell activity. Learning cell cluster identity in samples generated in memory and during IAV rechallenge, we demonstrate the applicability of our atlas to inform newly generated data and show that the nasal memory response to IAV is accelerated and coordinated compared to primary infection. Collectively, our spatial and temporal datasets enumerate and characterize the diversity of cell types, states, and subsets in the murine nasal mucosa and highlight those recruited and local cell subsets that exhibit memory and respond to viral infection.

RESULTS

Nasal mucosa infection with influenza virus and tissue processing

We administered 10^4 plaque forming units (pfu) of IAV H1N1 strain PR8 i.n. to awake, naïve mice in a small volume (5 μl/nostril) to restrict infection to the upper respiratory tract (Klinkhammer et al., 2018; Pizzolla et al., 2017), and collected and processed nasal mucosa tissue (n=3 biological samples/timepoint) by scRNA-seq at 0, 2, 5, 8, and 14 days post infection (dpi; “primary”). Later, additional samples were collected on 60 dpi and immediately following IAV rechallenge with sample collection 2 and 5 days post rechallenge (dprc) during the memory response (n=3/timepoint) (**Figure 1A**).

Anatomically, the murine nasal mucosa can be divided into several distinct morphological, histological, and functional tissue regions (Harkema et al., 2006). We mapped the cellular and structural diversity in the naïve nasal mucosa by immunofluorescence imaging, observing broad heterogeneity in epithelial, immune, and neural distribution throughout the tissue (**Figure 1B**). Thus, to capture region-specific changes in cell composition and response following IAV infection, we micro-dissected the tissue and separated into three different regions: (1) respiratory mucosa (RM), inclusive of the nasal and maxillary turbinates, septum, and vomeronasal organ; (2) olfactory mucosa (OM), inclusive of the ethmoid turbinates; and (3) the lateral nasal gland (LNG), which sits underneath the RM and OM in the maxillary sinus (see **Methods**).

Single-cell spatiotemporal atlas of primary influenza infection in the nasal mucosa

Across all primary infection timepoints, regions, and replicates (n=45), we collected 156,572 single-cell transcriptomes after filtering low-quality cell barcodes and hash-annotated cell multiplets (**Methods**). Top-level clustering on the entire primary infection dataset captured dozens of clusters belonging to neural, epithelial, immune, and stromal (endothelial, fibroblast, and

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others) cell lineages demarcated by known lineage-restricted genes (**Figures 2A and S1A**). We captured variable numbers of each cell type, with neurons and epithelial cells making up more than half of the dataset (**Figure S1B**). Major cell types were found to be distributed differently across nasal regions, with enrichment of neurons, granulocytes, B cells, and hematopoietic stem cells (HSCs) in the OM and more epithelial cells, fibroblasts, myeloid cells, and T & NK cells in the RM (**Figures 2B and S1C**). Since we collected bone (and thus bone marrow) along with the mucosa, the relative enrichment of specific immune cell types — including HSCs and other immune progenitors — in the OM can likely be attributed to cells localized to bone marrow (see **Figure 1B**).

Sample replicates were called by demultiplexing oligo-hashtag count tables (Li et al., 2020) and did not exhibit strong batch effects (**Figure S1D**). While clustering and differential expression were performed on all singlets regardless of successful hash assignment, we counted only those cells with annotated sample replicates for downstream compositional analyses.

To delineate the diversity of cell subsets and states present in the nasal mucosa, we split the dataset by cell type and conducted new clustering analyses, yielding 9-to-28 subclusters per cell type for a total of 127 clusters across the dataset (**Figure S1F and Supplementary Table 1**). By counting the number of cells assigned to each cluster in each sample replicate and normalizing across samples, we calculated cell cluster abundances to interrogate the relationship between samples in cell compositional space (see **Methods**). Performing principal component analysis (PCA) on samples over cell cluster abundances, we saw strong separation by region (**Figure 2C**), reinforcing that the nasal mucosa contains distinct regions with specific functions. Examination of the PCA loadings revealed that LNG is defined by higher abundances of odorant binding protein (OBP)-expressing cells, serous cells, and capillary endothelial cells (**Figure S1G**). OM has relatively more Schwann cells, sustentacular cells (Brann et al., 2020), and olfactory sensory neurons, while RM is enriched for vomeronasal sensory neurons, chondrocytes, and resting basal cells. Collectively, this atlas represents a high-resolution, comprehensive view of the mouse nasal mucosa enabling characterization of the dynamic differences in cellular composition within and between nasal regions during infection.

Influenza infection is largely restricted to the RM and induces reproducible changes in cellular composition

While viral, immune cell, and epithelial cell dynamics following IAV infection of the lung have been partially mapped (Bouvier and Lowen, 2010; Boyd et al., 2020; Manicassamy et al., 2010; Matsuoka et al., 2009; Steuerman et al., 2018), responses in the nasal mucosa are less

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studied. Viral titers of entire nasal mucosa showed robust infection 2 dpi that waned through 8 dpi and was completely cleared by 14 dpi (**Figure 2D**), while data from lungs showed sporadic spread of virus from the nasal mucosa only occurring between 5 and 8 dpi (**Figure S2A**). Aligning to a joint IAV and mouse genome, we also captured viral transcripts by scRNA-seq and thus could identify which cells may have been infected or contained virus. Individual genes like NP and HA were detected most strongly in epithelial and myeloid cells (**Figure S2B**). We calculated a summative IAV unique molecular identifier (UMI) count for every single cell to assess "positivity" for IAV (**Figure 2E**). Infected cells producing large amounts of virus may not survive tissue dissociation and processing, leading to lower-than-expected numbers of IAV+ cells. Looking across time points and regions in epithelial and myeloid cells, we captured low, but reproducible numbers of IAV+ cells 2, 5, and 8 dpi in the RM, but no positive cells in the OM, and only at 5 dpi in the LNG (**Figures S2C-D**).

Staining for IAV NS2 and 5 dpi confirmed that infection was largely restricted to epithelial cells in the RM (**Figures 2F and S2E**) and is consistent with expression of binding receptors marked by α2,3-linked sialic acid in mucous producing cells (Ibricevic et al., 2006). We performed qPCR of total RM to validate an early response to infection and found robust upregulation of type I and III IFNs 2 dpi, with even higher expression 5 dpi despite relatively lower viral titers (**Figure S2G**). As expected, *Ifng* expression exhibited delayed kinetics peaking 8 dpi.

To understand how infection remodels each nasal region, we applied PCA to the sample replicates within each region. In OM, 5 and 14 dpi samples separated from each other and the other time points (**Figure S2H**). In LNG, only 14 dpi samples separated from the rest (**Figure S2I**). Comparatively, PCA of RM samples showed clear separation between all timepoints in chronological order across the first two PCs (**Figure 2G**) suggesting dynamic and linked responses occur over the course of infection in this region. PCA loadings from RM highlight a shift in composition from resting basal cells, fibroblasts, and ciliated cells in naïve mice and 2 dpi to diverse activated myeloid and lymphoid clusters 5 and 8 dpi that gave way to specific goblet/secretory cells, T_{RM}-like cells, and mature myeloid cells following viral resolution 14 dpi (**Figure 2H**). Even though virus had been cleared by 14 dpi, we note that all three nasal regions reached compositions distinct from naïve mice at this timepoint.

Cataloging the epithelial subsets and states present in the murine nasal mucosa at baseline and throughout infection

Cellular diversity of the nasal epithelium

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Having acquired high-level knowledge of how IAV infection broadly impacts the nasal mucosa, we next sought to understand the variety of epithelial cells present across the tissue and how they respond during infection as the main target of infection. Subclustering on all epithelial cells yielded 28 clusters encompassing diverse differentiation states and functions (**Figures 3A and S3A**). We categorized these clusters into broader subsets including basal (*Krt5, Krt14*), ciliated (*Foxj1, Dnah5*), serous (*Ltf*, *Ccl9*), glandular (*Bpifb9b, Odam*), goblet/secretory (*Reg3g, Selenom, Scgb1c1,* and mucin-encoding genes), ionocyte (*Cftr, Coch*), tuft (*Trpm5, Il25*), and sustentacular cells (*Sec14l3, Cyp2g1*) (**Figure 3B**). In addition to known subsets, we also identified unique clusters of epithelial cells potentially specific to the nasal mucosa and present in naïve mice and throughout primary infection that separated distinctly in UMAP space: *Scgb-b27+Cck+*, *Klk1+Fxyd2+*, *Meg3+MHC-II+*, and *Krt13+Il1a+* cells. Epithelial clusters were differentially distributed across regions (**Figure 3C**): recently described nasal tuft cells, ionocytes, and *Dclk1+* cells (Ualiyeva et al., 2022), as well as olfactory sensory neuron supportive sustentacular cells (Brann et al., 2020), were enriched in OM, whereas serous and glandular cells were specific to LNG.

Cycling and IFN-responsive epithelial cells arise in the RM during infection

To understand the impact of IAV infection on the nasal epithelial compartment, we first determined which clusters harbored viral reads. Looking at the distribution of IAV UMIs across all epithelial clusters, we found that IAV+ cells were most prevalent in the IFN-stimulated cluster followed by cycling basal and ciliated cells (**Figure S3B**). The IFN-stimulated cluster, largely restricted to the RM, exhibited high levels of *Krt5*, a basal cell marker, but also *Cxcl17*, which in our dataset is expressed at steady state in serous cells in the LNG and some goblet/secretory subsets. While infection was well-established at 2 dpi, we were surprised to see that IFN-stimulated epithelial cells only arose starting at 5 dpi and made up ~20% of all RM epithelial cells 5 and 8 dpi (**Figure 3D**). We cannot rule out that the lack of IFN-responsive cells 2 dpi may have been from loss of infected cells during processing, but host silencing mechanisms by IAV itself may have contributed as well (Kochs et al., 2007). Comparing cells within the IFN-stimulated cluster by presence of IAV transcripts, we found relatively higher levels of ISGs but lower expression of transcription factors *Atf3*, *Egr1*, and *Junb* in IAV+ cells (**Figure S3C**), suggesting dysregulation of epithelial cell state.

Cycling basal cells demonstrated a similar transient increase in abundance, peaking 5 dpi (**Figure 3E**). Interestingly, differential expression across timepoints revealed many ISGs were upregulated in these cells 5 dpi (**Figure S3D**). Given recent work demonstrating that the epithelial

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IFN-response can impede proliferation and tissue repair in the lower airway (Broggi et al., 2020; Major et al., 2020), we leveraged our single-cell resolution to assess if individual nasal basal cells co-express pathways for cell cycle and IFN-response. Gene set analysis confirmed significant enrichment for both cell cycle and IFN-response pathways in cycling basal cells. Additionally, a largely mutually exclusive apoptosis pathway was also significant (**Figures S3E-F**). Pathway module scoring showed that while the IFNα response score changed over time, the G2M Checkpoint score was equally distributed across time points and independent of IFNα response (**Figure 3F**). Thus, unlike their lower respiratory tract counterparts, nasal cycling basal cells proliferate during primary infection and may concurrently support ISG expression alongside non-proliferative IFN-stimulated basal cells.

Rare unique epithelial cell subsets with lymphoid and myeloid communication potential accumulate following viral clearance

Looking at the compositional PCA, RM samples from 14 dpi separated from other timepoints (**Figure 2F**). Among epithelial cells, two clusters, *Emp1+Ccdc3+* basal cells and *Gp2+Lyz2+* goblet/secretory cells, accumulated to 9-12% of all epithelial cells following viral clearance by 14 dpi (**Figure S3G**). In addition to *Gp2* and *Lyz2*, the goblet/secretory subset was also enriched for *Il33*, *Muc1*, *Isg20*, and *Cd14* suggesting a potential shift in the epithelium toward an antibacterial state. Additionally, two rare and transcriptionally distinct subsets of epithelial cells (*Meg3*+MHC-II+ and *Krt13+Il1a+*), each only making up ~1% of all RM epithelial cells prior to viral clearance, accumulated at 14 dpi (p=0.032 and p=0.067, respectively). The *Meg3*+MHC-II+ subset expressed high levels of maternally imprinted Meg genes (*Meg3*, *Rian*) alongside *Cd74*, H2 class II genes, *Wnt5a*, *Cxcl12*, and *Ccl25* (**Figure 3G**). They also uniquely expressed *Fezf2*, a transcription factor studied in the context of thymic self-antigen expression and immune tolerance induction by thymic epithelial cells (Takaba et al., 2015), but not *Aire,* which is necessary in the thymus for presentation of autoantigens. Whether these cells can promote tolerance in the upper respiratory tract, similar to their thymic counterparts, remains to be determined.

The *Krt13+Il1a+* subset uniquely expressed *Krt13* (93.8% expressing within cluster vs 0.6% expressing in other clusters), a keratin previously described on "hillock" cells in the trachea with undetermined functions (Montoro et al., 2018). These nasal cells exhibited several of the markers specific to tracheal hillock cells including *Lgals3*, *Ecm1,* and *Anxa1* but were not enriched for *Cldn3* or club cell marker *Scgb1a1*. Unlike hillock cells, this nasal subset expressed genes for several secreted and membrane bound immune cell regulatory factors including *Il1a*, *Tnf*, *Cd274* (PD-L1), *Ifngr2*, and *Cxcl16* as well as secretory proteins like *Defb1*, *Muc4*, and *Muc1* (**Figure**

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3H). Given the potential for these *Krt13*+ cells to communicate with immune cells, we stained the mucosa for Krt13 to understand their distribution throughout the nasal cavity. We found the strongest signal for Krt13 along the nasal floor in the anterior RM (**Figure 3I**) and more distally where the nasal mucosa meets the oral mucosa (**Figure S3H**). Comparing samples from naïve mice and 14 dpi, we saw increased Krt13 staining and colocalization with PD-L1 in the post-infection samples (**Figure 3J**). Thus, following resolution of IAV infection, rare subsets of nasal epithelial cells with immune communication potential accumulate in the RM.

Neutrophils mature and activate in the RM immediately following IAV infection

Neutrophil accumulation in IAV infected lung has largely been associated with severe disease and poor prognosis (Brandes et al., 2013; Johansson and Kirsebom, 2021; Tang et al., 2019), but their role in the nasal viral infection is unknown. Given recent work showing a relationship between neutrophil activation in the nose prior to RSV infection and symptom occurrence (Habibi et al., 2020), and intrigued by the large number of neutrophils and mast cells captured across the nasal mucosa (n=7,987), we investigated the transcriptional programs and change in frequency of granulocytes in the nasal mucosa. Subclustering and UMAP embedding revealed a continuum of granulocyte development starting with granulocyte-myeloid precursor like cells (*Elane, Mpo)*, differentiating through immature states (*Camp, Mmp8, Retnlg*), and ending with several clusters of mature neutrophils (*Il1b, H2-D1, Siglecf*) (**Figures 4A and S4A**). Mast cells expressing *Il6*, *Gata2*, and *Il4* were also detected in small numbers. Most neutrophils originated from OM samples (**Figures 2C and S4B**) and are likely present in high numbers in the bone marrow. Pseudotime analysis across both the OM and RM recapitulated known maturation gene expression patterns in the blood (Grieshaber-Bouyer et al., 2021) and systemically (Xie et al., 2020) (**Figures 4B and S4C**), suggesting that precursors and immature neutrophils in OM bone marrow may give rise to activated and mature subsets in the RM.

By 2 dpi, neutrophil composition in the RM transitioned into mature IFN-stimulated and MHC-I-Hi states alongside an antimicrobial immature subset near the end of the pseudotime development trajectory (**Figure 4C**). The accumulation of these neutrophil clusters is one of the earliest changes in the RM following infection and may make up some of the earliest responses to local IFN, with epithelial IFN-Stim responses in either infected or uninfected cells not arising until 5 dpi. Interestingly, the OM exhibited increased frequencies of mast cells, progenitors, and cycling immature granulocytes in 2-of-3 mice at 5 dpi, likely within bone marrow, indicating IAV infection may induce changes to nearby hematopoiesis (**Figure S4D**). Thus, neutrophil activation

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and maturation mark the earliest detectable responses using our sampling strategy in the nasal mucosa to IAV infection.

Recruited monocytes and monocyte-derived macrophages constitute the myeloid effector response to IAV

Next, we explored heterogeneity among non-granulocyte myeloid cells (**Figure 4D and S5A**) — i.e., macrophages, monocytes, and dendritic cells (DCs). We captured a spectrum of macrophage (*Cd74, C1qb, Ccl4*) clusters including a putative tissue-repair subset expressing *Trem2, Fcrls,* and *Il1a*, an innate immune recruiting subset expressing *Ccl7, Ccl8,* and *Pf4* (CXCL4), and a small cluster of osteoclasts (*Ctsk, Mmp9*). Monocytes clustered into classical (*Ly6c2, Ccr2, Chil3*) and non-classical (*Ace*, *Ear2, Itgal*) subsets (Jung et al., 2022) alongside IFN-stimulated monocytes and monocyte-derived macrophages (MDMs). DCs separated into distinct clusters including Langerhans-like (*Epcam, Ccl17, Ccl22*), intraepithelial (*Cd103, Xcr1, Tlr3*), migratory (*Ccr7, Ccl22, Cd274)*, and a subset uniquely expressing *Cd209a* (DC-SIGN), *Tnfsf9* (4-1BB), and *Klrd1*. We also captured plasmacytoid DCs (*Siglech, Irf8*; pDCs). Most myeloid clusters were present in all nasal regions with some exceptions like the IFN-stimulated monocytes and MDMs, which were restricted to the RM (**Figure S5B**).

While non-existent at baseline, upward of 30-40% of all myeloid cells belonged to these antiviral monocyte and MDM clusters 5 and 8 dpi, respectively (**Figure 4E**). The appearance and accumulation of monocytes and MDMs is concordant with lower frequencies of several tissue macrophage clusters, likely reflecting an overall increase in the total number of myeloid cells in the tissue as monocytes infiltrate from circulation. To understand the difference between the IFN-stimulated monocytes and MDMs, we performed differential expression analysis between clusters (**Figure 4F**). While the monocyte cluster had higher ISG expression than the MDM cluster, the MDMs still had relatively high ISG expression when compared with resting tissue macrophages. Notably, IFN-stimulated MDMs expressed higher levels of *Cxcl9* and *Cxcl16*, whose receptors (CXCR3 and CXCR6, respectively) have been implicated in T_{RM} cell development in the lung (Slütter et al., 2013; Wein et al., 2019). This cluster also had the largest number of IAV+ cells of all myeloid cells (**Figure S5C**). Bystander analysis showed higher ISG expression in IAV+ myeloid cells, like IAV+ epithelial cells (**Figure S5D**). However, IAV+ MDMs also had lower expression of MHC-II genes and inflammatory cytokine genes *Ccl6* and *Cxcl9* suggesting reduced antigen presentation and inflammatory signaling capacity, consistent with prior research in the lung showing IAV suppresses myeloid cell activation and maturation (Moriyama et al., 2016; Zhang et al., 2022a).

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The rapid shift in the myeloid compartment from IFN-stimulated monocytes at 5 dpi to a predominance of IFN-stimulated MDMs 3 days later (**Figure 4E**) suggested that recruited monocytes differentiated into MDMs within the RM during this interval. To test this idea, we treated mice daily with an anti-CCR2 antibody (Mack et al., 2001) to deplete circulating monocytes from 3 to 5 dpi (**Figure S5E**). We stained for differentiating monocytes in the nasal mucosa by flow cytometry using MHC-II, CD11c, F4/80, CD64, and an intravascular CD45 stain to separate cells that had extravasated into the tissue from those in circulation (**Figure S5F**). Ly6C+ and Ly6C++ cells represent recently recruited monocytes with and without patrolling function; expression of MHC-II, in addition to F4/80 and CD64, by monocytes reflects differentiation into MDMs. Treatment with anti-CCR2 antibody reduced frequencies of Ly6C+ monocytes overall. Moreover, the proportion and number of differentiated Ly6C++ and Ly6C+ cells (i.e., MDMs) were considerably lower, suggesting monocytes recruited after 7 dpi had not yet differentiated (**Figures 4G and S5G**). Together, these data show that IAV infection induces a large recruitment of antiviral monocytes 5 dpi that differentiate into MDMs by 8 dpi in the RM.

Antiviral NK cell responses precede transient effector T cells that are replaced by durable T_{RM} cells following viral clearance

Following the accumulation of inflammatory and chemokine secreting monocytes and MDMs at 5 and 8 dpi, we anticipated a strong lymphocyte response during IAV infection. Thus, we next further investigated NK and T cells, the latter of which have been shown to be essential in clearing IAV infection in the lungs (Hufford et al., 2015) and nasal mucosa (Pizzolla et al., 2017). Subclustering revealed NK cell subsets (*Klrb1c, Ncr1*), type 2 (*Areg, Il13*) and type 3 (*Il22, Rorc*) innate lymphoid cells (ILC), γδT cells (*Trdc, Cd163l1*), and a spectrum of αβT cell subsets and states including naïve/central memory CD8 (*Ccr7, Dapl1*), effector CD8 (*Gzmb, Gzmk*), T_{RM}-like CD8 cells (*Itgae* [CD103]), resting CD4 (*Cd4, Tnfrsf4* [OX40]), Th1 CD4 (*Ifng, Cd200*), Th17 (*Cd40lg, Il17a*), and a cluster of Helios (*Ikzf2*) expressing cells (**Figures 5A and S6A**). Most T and NK cell clusters were enriched or restricted to the RM, but *Ccr7*+ CD8 T cells, ILCs, and γδT cells were also found in the OM and LNG (**Figure S6B**).

Looking at T and NK cell frequencies, we found that IFN-stimulated T and NK cells accumulated 5 dpi alongside cytotoxic NK cells that remained elevated through 8 dpi (**Figure S6C**). By 8 dpi, the T and NK cell compartment completely shifted towards effector antiviral T cell subsets, with high abundances of *Gzmk*+ CD8, Th1-like *Ifng*+*Cd200*+ CD4, cycling, and Helios+ T cell clusters (**Figure 5B**). These effector responses were short-lived, however, and were followed by increased frequencies of T_{RM} cells and resting CD4 T cells (**Figure 5C**). Notably, while

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detectable infection was largely restricted to the RM, T_{RM} cells also increased in OM and LNG 14 dpi (**Figure S6D**), supporting the notion that even low levels of infection can result in T_{RM} accumulation and development (Jiang et al., 2012).

To contextualize the T_{RM}-like cells that arise following IAV clearance, we examined two recently published signatures separating resident memory from central/circulating memory. A universal T_{RM} gene score (Milner et al., 2017) reasonably separated our T_{RM} cluster from effector and naïve/memory CD8 T cells, but IFN-stimulated T cells also scored highly (**Figure 5D**). Conversely, the T_{RM}-like cells also scored low for the associated circulating memory gene score, while naïve/memory CD8 T cells scored highest (**Figure S6E**). Closer inspection of known resident memory and central memory markers and transcription factors (Crowl et al., 2022) confirmed restriction of *CD103* (*Itgae*) expression to our T_{RM} cluster, but *Cd69* was only highly expressed in IFN-stimulated cells. *Runx3* was also most highly expressed in T_{RM}-like cells but also at lower levels in effector CD8 T cells. The *Cd103*+ CD8 cluster lacked the known naïve/central memory transcription factors *Klf2* and *Tcf7* (**Figure 5E**). In summary, effector T cell responses in the RM 8 dpi are replaced by T_{RM}-like cells across all nasal mucosa regions following viral clearance.

Plasma cells populate throughout the NM following viral clearance

Following IAV infection, local mucosal plasma cells and activated B cells produce and secrete neutralizing soluble IgA into the airways (Rossen et al., 1970; Wellford et al., 2022; Woof and Mestecky, 2005). In the lungs, resident memory B cells form after primary infection and can be recruited upon secondary challenge to produce additional antibodies (MacLean et al., 2022), suggesting infection can lead to long term changes in both local and distal B cell subsets. Clustering of B cells in the nasal mucosa (**Figures S7A-B**) revealed mature subsets (*Ighd, Cd74*), plasma cells (*Mki67, Ighg2b, Igha*), lambda-chain-high expressing cells (*Iglc1, Iglc2*), nucleoside diphosphate kinase (NME) expressing cells (*Nme1*, *Nme2*) and, primarily in OM, developing subsets including pro-B (*Dntt, Vpreb1, Rag1*), pre-B (*Bub1b, Mki67, Sox4*), and immature B cells (*Ms4a1, Ifi30*). The preponderance of precursor and developing B cell subsets in OM likely reflects bone marrow cells, whereas IgG+/IgA+ plasma cells were found at highest frequency within LNG tissue, but class-switched B cells were also detectable in RM and OM (**Figure S7C**).

Looking at changes in cluster frequency over the course of IAV infection, we found that pro-B and pre-B cells collectively comprised up to 80-90% of all B cells in the OM 5 dpi increasing from 5-15% at baseline, suggesting that IAV infection may induce local B cell proliferation and differentiation in the bone marrow following infection and/or egress of mature B cells from this

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region (**Figure S7D**). This increase in B cell precursor frequency in OM 5 dpi paralleled that of granulocyte precursors (**Figure S4D**), supporting the notion of the activation of nasal bone marrow. By 14 dpi, IgG+/IgA+ plasma cells were reliably detected in the RM in all three replicate samples. However, plasma cell recovery was more variable in OM and LNG samples, which could be due to biological variability and/or inconsistent cell capture. Indeed, imaging of the RM and LNG at 30 dpi confirmed increased IgA+ cells in both regions when compared with naïve mice, with the largest number of IgA-expressing cells localizing to the LNG (**Figure S7E**). Thus, B cells may undergo proliferative development during acute IAV infection and plasma cells accumulate in the RM and LNG following clearance.

Proportionality guided cell-cell communication analysis highlights the CXCL16-CXCR6 signaling axis in effector and memory T cell responses

To understand how compositional changes in the tissue during primary infection may be coordinated across multiple cell subsets, we next characterized relationships between pairs of cell clusters over time using our compositional data. We employed proportionality analysis (Lovell et al., 2015), an alternative to correlation that avoids intra-sample abundance dependence present in compositional data (Quinn et al., 2018), to find cell clusters with significantly similar abundance trajectories (see **Methods**). Given that the RM was the major site of infection and showed temporally structured changes in cell composition over time (**Figure 2G and H)**, we applied our proportionality analysis to all samples from this region. We discovered a highly structured proportionality landscape with 101 significantly proportional cluster pairs (FDR < 0.05) (**Figures 6A and S8A**). To understand coordination among larger groups of cell clusters, we built a network of all significantly proportional cluster pairs (**Figure S8B**). The network revealed larger groups of proportional responses made up of clusters from several different cell types and smaller and single-pair groups with 1-2 contributing cell types. Next, to further characterize the coordination among immune cells and between immune and epithelial cells, we investigated subsets of clusters with high proportionalities by cell-cell communication analysis.

IFN-stimulated MDMs – Gzmk+ CD8 T cells – Ifng+Cd200+ CD4 T cells

The strongest proportionality was observed among highly networked IFN-stimulated clusters and effector T cell clusters (**Figure 6B**). Given the strong myeloid and T cell responses 8 dpi and the possibility that activated MDMs may function as APCs within the nasal mucosa, we focused on the relationship between the IFN-stimulated MDM, *Gzmk*+ CD8 T cell, and *Ifng*+*Cd200*+ CD4 T cell clusters. Plotting abundance values confirmed overlapping trajectories

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of these three clusters with transient accumulation starting 5 dpi, peaking at 8 dpi, and waning by 14 dpi (**Figure 6C**). We next assessed cell-cell communication potential using NICHES, an approach that finds single-cell pairs with multiplicative high expression of known interacting ligands and receptors (Raredon et al., 2023). Differential ligand-receptor expression between groups of cell-pairs was then used to identify interactions specific to pairs of clusters (see **Methods and Supplementary Table 2**). Applied to cells from these three clusters at 8 dpi, we found several predicted interactions between the MDM cluster and both effector T cell clusters including *Cd274–Pdcd1* (PD-L1–PD-1), *Cd86–Ctla4*, and *Cxcl16–Cxcr6* (**Figure 6D**).

Cd103+ DCs – Dusp2+Icam1+ mature neutrophils – Gp2+Lyz2+ goblet/secretory cells

The second largest networked group included various myeloid, granulocyte, and epithelial cell clusters. Intrigued by the inclusion of the late arising Gp2+Lyz2+ goblet/secretory cell cluster (**Figure S3G**), we took a closer look at this cluster and the two clusters most proportional with it: *Cd103*+ DCs and *Dusp2*+*Icam1*+ mature neutrophils. Plotting cluster abundances confirmed that all three clusters peaked following viral clearance 14 dpi (**Figure S8C**). Closer inspection of differentially expressed ligand-receptor pairs revealed potential pro-inflammatory signaling by Gp2+Lyz2+ Gob/Sec cells to mature neutrophils via *Sftpd–Sirpa*, which blocks Cd47 binding (Gardai et al., 2003), *Cirbp–Trem1* which has been shown to occur in sepsis (Denning et al., 2020), and *Tgfb2–Tgfbr1*. DC–neutrophil interactions included *Ccl2–Ccr1* and *Il18–Il18rap* suggesting mutual immune recruitment/homeostasis (**Figure S8D**). These interactions suggest that late arising *Gp2+Lyz2+* goblet/secretory cells may recruit and regulate *Cd103*+ DCs and mature neutrophils in the RM following viral clearance.

Krt13+Il1a+ epithelial cells express Cxcl16 and increase when T_{RM}-like cells accumulate

Like IFN-stim MDMs producing *Cxcl16* 8 dpi in concert with abundant *Cxcr6* expressing effector CD8 and CD4 T cells, the late arising *Krt13+Il1a*+ epithelial cell subset expressed *Cxcl16* alongside increasing frequencies of *Cxcr6*+ T_{RM} and CD4 T cells 14 dpi (**Figures 6E-F**). Although not significantly proportional over all time points (ρ = 0.53, 0.32, 0.77), we applied cell-cell communication analysis to cells from 14 dpi in these three clusters given the role of CXCL16–CXCR6 signaling in T_{RM} localization in the lower airways (Morgan et al., 2008; Wein et al., 2019). In addition to discovering the *Cxcl16-Cxcr6* interaction, the analysis also captured additional interactions like *Cd274–Pdcd1*, *Timp2–Itgb1*, *Il18–Cd48*, *Alcam–Cd6*, and *Il16–Cd9* (**Figure 6G**). RNAscope of the nasal floor 14 dpi confirmed expression of *Cd274* (PD-L1) and *Cxcl16* by *Krt13*+ cells in the vicinity of cells expressing *Cxcr6* (**Figures S8E and 6H**). Considering the

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transcriptional programming and localization of these cells, we propose the name ***K**rt13*+ **n**asal **i**mmune-**i**nteracting **f**loor **e**pithelial (KNIIFE) cells. Notably, KNIIFE cells are a fraction of many cells on the nasal floor expressing *Cxcl16* at 14 dpi, suggesting that this region of the nasal mucosa may be important in instructing T_{RM} cells following viral clearance and/or tissue damage.

In summary, proportionality analysis coupled with cell-cell communication approaches reveal temporally synced cell cluster abundance changes over the course of primary infection and highlight potential cell-cell interactions contributing to T cell function and residual inflammation following viral clearance.

Learning the cellular composition of new scRNA-seq datasets of the nasal mucosa

To assess the ability of our primary IAV infection atlas to contextualize and analyze new scRNA-seq data generated from murine nasal mucosa, we utilized the replicate structure of our dataset to test label transfer methods. Separating one RM replicate from each timepoint as a query set, we compared Seurat's built-in weighted nearest neighbors method (Hao et al., 2021) to the generative model approach used in single-cell Annotation using Variational Inference (scANVI) (Xu et al., 2021). First trying cluster annotation on the entire RM query dataset, we found poor accuracy in assigning correct cluster identity in both methods and several clusters were completely lost in the predicted annotations (**Figure S9A**). Since the primary infection atlas clusters were found following multiple rounds of clustering, we next applied the same stepwise approach for label transfer: assign a cell type label and then split into cell types for cluster label predictions (**Figure 7A**). Using this stepwise approach, we correctly labeled 99.66% cell types using Seurat and 99.27% using scANVI (**Figure S9B**). Cluster identity calling, however, was more accurate in Seurat with 89.11-95.19% of cells correctly annotated across cell types, whereas scANVI had 69.33-92.31% properly labeled (**Figure S9C**). Moving forward with Seurat given its superior predictions in this use case, we next validated the output by calculating cell cluster abundances using the predicted cell cluster labels and projecting these "query" replicates into the primary infection RM compositional PCA. Remarkably, the query replicates aligned very closely to their real sample replicate counterparts in compositional space (**Figure S9D**). Thus, we validated a label transfer approach to learn cell cluster identities and cellular composition of new scRNA-seq data using our primary infection atlas as a reference.

IAV rechallenge is characterized by accelerated and concurrent myeloid and lymphocyte memory responses

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Having developed a tissue-scale response timeline of acute IAV infection in the nasal mucosa, we next asked how a memory response differs from primary infection on cluster and compositional levels. Thus, we applied our label transfer approach — using the primary infection data as a reference — to new RM samples harvested from mice 60 dpi and 2- and 5-days post rechallenge (dprc) with the same strain of IAV (**Figures 1A and 7A**). All cell types present in the primary infection dataset were captured in the rechallenge samples (**Figure 7B**) and UMAP projection showed strong overlap between the datasets (**Figure S10A**). Plaque assays detected infectious virus in 1-of-5 mice in nasal mucosa, and in 0-of-6 mice in lung upon rechallenge, suggesting immediate control of infection or baseline resistance (**Figure S10B**). If protection was completely neutralizing antibody mediated, however, we would not expect to see large changes in tissue composition. Surprisingly, despite this viral rechallenge paradigm with a matched strain leading to barely any infectious viral shedding, we measured increased proportions of granulocytes, T & NK cells, and B cells following rechallenge that were concordant with relatively reduced proportions of epithelial, endothelial, and stromal cells, indicating immune cell accumulation in the tissue (**Figures 7C and S10C**).

Following annotation, we compared changes in cluster abundance over time between the primary and secondary responses to IAV infection (**Figures 7D and S10D**). Like primary infection, IFN-Stim and MHC-I-Hi neutrophil subsets accumulated immediately and maintained elevated levels through 5 dprc. Interestingly, IFN-stimulated MDMs rapidly accumulated while monocytes only slightly increased. Given the total proportion of myeloid cells in the dataset was similar between 60 dpi, 2 dprc, and 5 dprc, these data suggest that MDMs already inside the RM prior to rechallenge quickly responded. Effector Th1 CD4 T cells were also elevated 2 dprc, but effector CD8 T and T_{RM} cells were slower to accumulate. Interestingly, plasma cell abundances, though variable, remained at similar levels between 60 dpi and 2 dprc; however, these abundances decreased 5 dprc, concurrent with an overall reduction in B cell proportion. Cycling basal cells showed no change in abundance following rechallenge, and the increase in IFN-responsive epithelial cells was stunted. Notably, both the *Meg3*+*MHC-II*+ subset and KNIIFE cells that arose following viral clearance in primary infection remained at low levels throughout rechallenge. While we do not yet understand whether their roles may be restricted to resolving primary infection, there may be a necessary inflammation threshold for their expansion, or they may take longer to increase in frequency than the relatively early sampling timepoints post re-challenge.

To understand if the quality of antiviral effector responses changed between primary and secondary infection, we performed differential expression analysis within cell clusters (**Figure 7E and Supplementary Table 3**). Compared to 8 dpi, *Gzmk*+ CD8 T cells at 5dprc exhibited reduced

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expression of cytotoxic and activation genes, but higher levels of cell survival genes *Birc5* and *Selenoh*, and histone *H2afv*, suggesting induction of epigenetic modifications. T_{RM} cells had lost some activation surface markers by 60 dpi; however, following rechallenge they showed similar expression profiles to those that appeared 14 dpi. Interestingly, expression of *Cd103* and *Gzma* were highest 5 dprc. *Ifng*+*Cd200*+ CD4 T cells may become more prone to cell death during a memory response, with loss of *Mapkapk2*, *Bcl2l1*, and *Cd200*. However, they expressed higher levels of *Itga1*, which has been shown to mark a subset of CD4 T cells that rapidly secrete IFN-γ in the airways following IAV infection (Chapman and Topham, 2010). IFN-stimulated MDMs at 2 dprc compared to 8 dpi had lower expression of ISGs, *Ccl4*, and *Fcgr1*, but higher levels of *Tnfaip2*, *Atp5k*, and ribosomal protein coding genes.

While changes in abundance or gene expression on an individual cell subset/state level highlight specific differences between primary and secondary responses, we sought to understand how the collective RM tissue-scale response differs. To contextualize on the compositional level, we projected the memory and rechallenge sample replicates into the previously derived compositional PC space for RM in primary infection (**Figure 7F**). The RM 60 dpi samples were separated from naïve samples and most resembled 2 dpi, suggesting that even though IAV was cleared by 14 dpi, the nasal mucosa sustained significant changes in composition. The first memory timepoint 2 dprc recapitulated the variance described by PC1, but there was no significant shift along PC2 unlike in primary infection, suggesting increases in effector immune responses but not broad antiviral activation across all cell types like those seen 5 dpi (i.e., IFN-Stim cluster) (**Figure 2G**). Notably, by 5 dprc, the tissue had almost returned to “memory baseline” at 60 dpi in PC-space, indicating that responses had already largely resolved. In case projection obscured important variation between the primary and secondary samples, we also re-ran PCA with abundances from all timepoints and found that the first two PCs were remarkably similar (**Figure S10E**). PC3 captured the differences between datasets, with specific epithelial cell subsets higher in primary infection and chondrocytes, plasma cells, and T_{RM} cells enriched in samples during and after rechallenge (**Figure S10F**).

To quantify the overall difference between timepoints, we calculated compositional distances between all pairs of sample replicates (**Figure S10G**). In primary infection, RM increasingly separated from the naïve state up through 8 dpi but then became closer as infection is resolved (**Figure 7G**). Corroborating the PCA, the nasal mucosa 60 dpi was still distinct from its naïve state. Upon rechallenge, RM also separated from 60 dpi; however, the extent of that difference (MD=3.50) was less than between naïve and 5 dpi (MD=5.37) and 8 dpi (MD=6.67) indicating that the memory response was more succinct (**Figure 7H**). Comparing primary infection

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timepoints with peak memory response, each primary infection timepoint was similarly distinct from 2 dprc, suggesting that prior infection rewired the RM response to IAV infection (**Figure 7I**). Summarizing the primary and secondary responses to infection described here, we present a timeline of the key immune and epithelial cell responses during IAV infection and rechallenge illustrating that many of the stepwise changes seen in primary infection occur in a more coordinated and accelerated fashion (**Figure 7J**).

DISCUSSION

Here, we present a longitudinal, multi-region, scRNA-seq atlas of the murine nasal mucosa during primary and secondary IAV infection. Cataloguing the distribution and temporal dynamics of the diverse cell types, subsets, and states present, we develop and apply a compositional framework to understand tissue-scale changes occurring throughout primary and memory responses to viral infection. Neutrophil activation responses following infection precede broader IFN-stimulated responses in epithelial, myeloid, and lymphoid cells. By 8 dpi, effector CD8 and CD4 T cell subsets accumulate alongside recently differentiated MDMs. Following viral clearance at 14 dpi, T_{RM}-like cells and IgG+/IgA+ plasma cells appear in the nasal mucosa, which has achieved distinct cellular composition from the naïve state with these adaptive immune subsets being sustained until 60 dpi. Careful investigation of the epithelial cell compartment also revealed rare, previously undescribed subsets, including KNIIFE cells. We validate and localize the presence of these cells, provide evidence for their interaction with *Cxcr6*-expressing lymphocytes, and show co-expression of *Krt13*, *Cd274* (PD-L1), and *Cxcl16* on the nasal floor following viral clearance. Even using the same viral strain, which was effectively controlled to limit almost any detectable viral replication, our use case of the primary infection atlas to understand secondary infection shows that rechallenge induces surprisingly widespread yet coordinated and accelerated changes to cellular composition. In particular, we identify accelerated neutrophil, macrophage, and T cell responses in memory with a reduced burden on epithelial cells to express the joint interferon and proliferative response programs of primary infection.

Comprehensively understanding airway mucosal immunity has become an urgent unmet need in the face of emerging and recurring respiratory pathogens (Lavelle and Ward, 2022; Morens et al., 2023; Roth et al., 2018; Russell et al., 2020). In particular, the nasal mucosa is at the forefront of mammalian host responses to airborne pathogens and functions as both an entry site and the primary barrier for infections of the respiratory tract. Consequently, the nasal mucosa is thought to be the site of initial engagement of respiratory viruses to generate both local T cell memory (Pizzolla et al., 2017) and neutralizing antibodies (Liew et al., 2023; Sterlin et al., 2021;

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Wellford et al., 2022; Weltzin et al., 1996). Determining how these responses occur following primary infection, and how immune and non-immune cells in the nasal mucosa contribute to viral clearance and subsequent memory, is critical to inform the design of next-generation nasal vaccines and therapeutics.

Although live-attenuated IAV mucosal vaccines work in children (Costa-Martins et al., 2021; Johnson Jr. et al., 1985), recent attempts to administer new vaccine formulations i.n. in both children and adults have had mixed results (Madhavan et al., 2022; Nouën et al., 2022; Vaca et al., 2023). New approaches utilizing “prime-and-pull” strategies (Mao et al., 2022) and mucosa-targeting delivery platforms (Hartwell et al., 2022) support the promise of i.n. vaccines. A better understanding of natural mucosal immune memory may inform a more comprehensive assessment of the efficacy of these and other immunization approaches and potentially reveal avenues for further improvement. The present atlas of murine nasal mucosa comprehensively catalogues the tissue-scale response following primary and secondary IAV infection, highlighting the dynamic and cooperative cell subsets and states contributing to viral resolution and memory recall.

One focus of the present work is the multi-faceted epithelial makeup of the nasal mucosa and its response to IAV infection. Basal cells in the nasal mucosa give rise to epithelial cells reminiscent of pseudostratified epithelium found in the trachea (Davis and Wypych, 2021), but we and others have also captured several additional epithelial cell subsets not found in other parts of the airway. Our atlas validates recent work in mouse describing sustentacular cells, ionocytes, nasal tuft cells, and serous cells (Brann et al., 2020; Ualiyeva et al., 2022). Moreover, we describe for the first time several clusters of epithelial cells with undetermined function: (1) *Scgb-b27*+*Cck*+, (2) *Meg3*+MHC-II+, (3) *Klk1*+*Fxyd2*+, and (4) KNIIFE cells. Except for the *Scgb-b27*+*Cck*+ cluster, the remaining previously undescribed clusters all exist at low frequencies (< 1% of all epithelial cells) in naïve mice and may have been missed in experiments without sufficient cell numbers or utilized cell sorting.

The viral signaling and proliferative capacities of epithelial cells associate with COVID-19 disease trajectory (Sposito et al., 2021; Ziegler et al., 2021). Our data confirm that transient IFN-responsive epithelial cell subsets, including cycling basal cells, arise in the nasal mucosa during IAV infection. During the peak response, nasal basal cells co-expressed cell cycle and IFN-response programs, which have been previously described as non-compatible in lungs (Broggi et al., 2020; Major et al., 2020). Given the diverse roles of nasal epithelial cells and the need to protect olfactory sensory neurons (Dumm et al., 2020), nasal basal cells may be more tolerant of IFN-response signaling during proliferation than basal cells in the lower airways. Notably,

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epithelial IFN-induced responses were significantly reduced upon rechallenge. This difference could reflect several non-exclusive mechanisms during recall, including an overall reduction or shortening in IFN production or signaling, lower viral load, or a potential tolerized basal cell state. Airway basal cells can develop transcriptional memory in vitro (Adamson et al., 2022), but whether primary infection can confer durable memory to viral immunity, as has been seen for allergic inflammation (Ordovas-Montanes et al., 2018), requires further investigation.

In addition to epithelial responses, IAV infection also precipitated a highly dynamic, stepwise response by immune cells that was initially dominated by myeloid subsets. Specifically, we observed a substantial early influx of neutrophils followed by monocytes that then differentiated into MDMs concurrent with the arrival of effector T cells. The evidence for both resident (Yao et al., 2018) and recruited (Aegerter et al., 2020) macrophages in the lungs to engage in memory responses suggests that a similar phenomenon may occur in the nasal mucosa. After depleting circulating monocytes during acute infection, MDM formation was markedly reduced, indicating that the majority of nasal MDMs at 8 dpi differentiated from newly recruited monocytes. Interestingly, during a memory response MDMs, but not monocytes, increased in abundance 2 dprc even though overall myeloid frequencies remained unchanged, indicating that either recruited MDMs replaced local myeloid cells or MDMs already present in the tissue expanded to exert antiviral effector functions. If the latter, understanding the mechanisms by which enhanced myeloid function is maintained and recalled in the nasal mucosa could yield a new avenue for designing improved mucosal vaccines.

The role of adaptive immune responses to IAV infection have been well described in the lower respiratory tract (Chapman and Topham, 2010; Krammer, 2019; McMaster et al., 2015; Onodera et al., 2012; Slütter et al., 2013; Wein et al., 2019), but their dynamics and quality in the nasal mucosa are less understood. Antibody-mediated immunity following primary and secondary IAV infection has been described (Chen et al., 2018) and falls outside the scope of the present study. Capturing substantial bone marrow resident cells in the OM samples during primary infection, we noted a marked increase in this region of granulocyte and B cell progenitors 5 dpi. Systemic and circulating cytokine responses are documented to induce hematopoiesis during infection (Zaretsky et al., 2014), so it follows that local type I inflammation in the nasal mucosa alongside high levels of IFNs could induce rapid granulo- and lymphopoiesis. Antibody-producing B cell response dynamics, however, were variable in our model with sizeable frequencies of plasma cells detected in 1- or 2-of-3 mice at 14 dpi, 60 dpi, and 2 dprc. Wellford et al., recently showed in an influenza B model that the OM requires mucosa resident plasma cells to prevent transmission to the brain; the RM, alternatively, can receive neutralizing antibodies from both

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serum and local plasma cells (Wellford et al., 2022). To what extent local nasal plasma cell derived IgG and IgA play roles in stymying infection during rechallenge, and whether non-neutralizing antibody functions help activate other immune subsets (e.g., Fc receptor mediated signaling), must be further explored.

IAV infection of the nasal mucosa resulted in classical T cell responses with both antiviral effector CD8 T cells expressing cytotoxic genes and Th1 CD4 T cells expressing *Ifng* and *Tnfrsf4* (OX40) arising at 8 dpi. Proportionality analysis revealed their coordination with an influx of IFN-Stim MDMs expressing *Cxcl9* and *Cxcl16*, and NICHES predicted several modes of communication between all three clusters, suggesting MDMs may provide activation signals for T cells in the nasal mucosa. While infectious titers waned between 2 and 8 dpi, effector T cell responses likely played a critical role in completely extinguishing IAV infection by 14 dpi. Finding upward of ~50% of all T and NK cells by 14 dpi belonged to the T_{RM}-like cluster in the RM tissue, we validate their presence and phenotype in the nasal cavity following IAV infection (Pizzolla et al., 2017; Wiley et al., 2001). Unlike in the lung where T_{RM} cells quickly wane following infection (Slütter et al., 2017), our data demonstrate robust frequencies out to 60 dpi that are further amplified during rechallenge; moreover, in addition to the RM, we find T_{RM} in OM and LNG tissue, where virus is only detected at low levels or not at all. While T_{RM} contribute to an effective memory response upon rechallenge (Ariotti et al., 2014; McMaster et al., 2015; Schenkel et al., 2014; Steinbach et al., 2016), differential expression across timepoints of the *Cd103*+ CD8 T cell cluster in our dataset did show strong gene expression differences between these cells at 2 dprc and following primary infection; moreover, their relative proportion only increased by ~2x over levels at 60 dpi. Given recent work highlighting the importance of T_{RM} in mitigating nasal viral infections (Mao et al., 2022; Pizzolla et al., 2017), understanding which cell subsets and signals establish and maintain the T_{RM} niche could help guide mucosal vaccine strategies with heterotypic protection.

Following viral clearance, several unique subsets of epithelial cells with potential immune signaling and inflammatory regulation capacity substantially increased in abundance. In addition to a large cluster of goblet/secretory cells with predicted DC/neutrophil communication ability, we also discovered a subset of epithelial cells uniquely expressing *Krt13* and *Krt6a* in the nasal mucosa, hereto named ***K**rt13*+ **n**asal **i**mmune-**i**nteracting **f**loor **e**pithelial (KNIIFE) cells. Phenotypically, KNIIFE cells were reminiscent of the recently described “hillock” cells in the trachea expressing *Krt13, Ecm1*, and *Lgals3* (Montoro et al., 2018); however, KNIIFE cells additionally expressed several genes often found in macrophages including *Cd274* (PD-L1), *Il1a*, *Tnf*, and *Cxcl16*. This cluster was present at low levels throughout infection until expanding 14

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dpi and remained stable during rechallenge. At 14 dpi, we measured *Krt13* and *Cxcl16* co-expression in situ nearby *Cxcr6* expressing cells, especially along the nasal floor. These results raise the possibility that KNIIFE cells, by providing a source for CXCL16 beyond that expressed by myeloid cells, may contribute to the establishment of the resident memory T cell pool in the nasal mucosa, as has been suggested for this chemokine pathway in other tissues (Clark et al., 2006; Morgan et al., 2008; Tse et al., 2014; Wein et al., 2019). The enrichment of KNIIFE cells along nasal floor and below the vomeronasal organ prompts the question of whether these cells interact with particles or irritants just entering or settling in the nose and play a regulatory role in tissue tolerance and/or immunity.

Compositional scRNA-seq analyses are becoming more common to discern differences between disease trajectories (Ordovas-Montanes et al., 2018; Smillie et al., 2019; Zheng et al., 2021), treatment groups (Darrah et al., 2020; Zhang et al., 2022b), and/or species (Chen et al., 2022; Li et al., 2022). Current tools focus on identifying specific clusters or gene programs that are compositionally distinct between groups (Büttner et al., 2021; Cao et al., 2019; Dann et al., 2022). However, the power of compositional scRNA-seq data lies in its structure; namely, singular changes in composition cannot be independent and must correspond with mutual changes in other clusters/programs. Leveraging the biological replicates and multiple timepoints present in our atlas, we utilized straightforward tools for compositional analysis adapted from microbiome research (Gloor et al., 2017; Lin and Peddada, 2020; Quinn et al., 2018) to understand tissue-scale changes within the nasal mucosa throughout IAV infection. PCA of cell cluster abundances across sample replicates separated nasal regions and depicted structured stepwise changes in epithelial and immune cell subsets throughout infection trajectory. Proportionality analysis, which avoids the spurious associations present in Pearson correlation applied to compositional data (Lovell et al., 2015), revealed pairs and groups of clusters with significantly similar compositional trajectories (e.g., IFN-stimulated MDMs and effector CD4 and CD8 T cells) and can be readily applied to discover similarities across various metadata. Finally, metrics like Aitchison distance (Aitchison et al., 2000) capture holistic changes in tissue-scale cellular composition and support standard tests for differences between group means (e.g., Welch's ANOVA) to assess global similarity and compositional distance traveled by a tissue. Applied to our datasets, the RM "travels" less during the memory response to IAV than during primary infection, suggesting prior infection induces a coordination of responses that were previously unsynchronized. We propose that these approaches for analyzing scRNA-seq data constitute a new framework for understanding and summarizing whole tissue- and biopsy-scale changes in cellular composition at high resolution in health, disease, and/or under perturbation.

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Collectively, our murine nasal mucosa atlas of primary IAV infection longitudinally catalogues the cell types, subsets, and states present throughout distinct nasal regions. We demonstrate the utility of our dataset to serve as an annotation reference for newly generated scRNA-seq datasets and apply it to understand how the response to infection in the RM differs during a memory recall following IAV rechallenge. These findings will help contextualize studies of the nose in humans and highlight key immune and epithelial cell responses to recapitulate in future nasal vaccines and therapeutics to drive increased synchronicity in nasal memory responses.

Limitations of the study

First, we acknowledge that cluster abundance-based compositional analyses are inherently dependent on how clustering was performed, and thus implicitly incorporates, to some degree, operator bias. While we believe our approach to be as impartial as possible through use of iterative clustering, it will be imperative to implement robust, reproducible clustering analyses (Hu et al., 2019; Patterson-Cross et al., 2021; Zheng et al., 2021) prior to compositional analysis moving forward. Partial labeling of cells by hashing antibodies may also have obscured changes in composition over time. Second, detection of IAV transcripts by scRNA-seq was limited. Other studies have included spike-in primers to facilitate additional capture of viral nucleic acids (Ratnasiri et al., 2023); it is possible that we were not sufficiently sensitive to IAV transcripts without these spike-in primers. Also, cells productively infected with virus may not be sufficiently viable through our tissue processing pipeline, leading to artificially low numbers of cells containing IAV reads. Third, to increase the relative proportion of non-epithelial cells in our scRNA-seq dataset, we performed a partial EpCAM depletion using magnetic beads. This decision was made following preliminary experiments comparing this approach to un-depleted RM tissue in naïve mice and 2 dpi; we found that depletion similarly reduced all epithelial cell clusters without bias and did not result in the loss of IAV+ cells. Thus, the cellular compositions throughout the study represent the nasal mucosa tissue after both dissociation and epithelial cell depletion and, therefore, do not reflect the true frequencies of cell types within intact nasal mucosa. Nevertheless, our atlas can still be used to assign cell cluster labels to new datasets where epithelial cells have not been depleted and could inform spatial transcriptomics approaches to derive more accurate cell abundances in vivo. Indeed, spatial transcriptomics and/or multiplexed immunofluorescence approaches will help validate the spatial organization and quantification of cell clusters defined here; however, given the complexity of the nasal mucosa and difficulty in sectioning through the nasal bone, further work will need to be done to validate, adapt and refine

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imaging protocols for this unique tissue. Additional experiments to test how various influenza strains, other respiratory pathogens, and vaccination strategies impact the composition and timing of responses in the nasal mucosa to IAV challenge are warranted (Rutigliano et al., 2010).

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Author contributions

Conceptualization, S.W.K., C.M., U.H.vA., and J.O-M,;
Methodology, S.W.K. and C.M.;
Software, S.W.K., E.M.L., and T.J.L.;

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Writing – Review & Editing, S.W.K., C.M., E.M.L., U.H.vA., and J.O-M.;
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Declaration of Interests

S.W.K. reports compensation for consulting services with Monopteros Therapeutics, Flagship Pioneering, and Radera Biosciences. J.O.M. reports compensation for consulting services with Cellarity, Tessel Biosciences, and Radera Biotherapeutics. U.H.v.A. is a paid consultant with financial interests in Avenge Bio, Beam Therapeutics, Bluesphere Bio, Curon, DNAlite, Gate Biosciences, Gentibio, Intergalactic, intrECate Biotherapeutics, Interon, Mallinckrodt Pharmaceuticals, Moderna, Monopteros Biotherapeutics, Morphic Therapeutics, Rubius, Selecta and SQZ.

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METHODS

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Jose Ordovas-Montanes (jose.ordovas-montanes@childrens.harvard.edu).

Material availability

All the mouse lines used in this study are available from Jackson Laboratories. The anti-CCR2 antibody MC-21 was provided as a gift by Prof. Matthias Mack. This study did not generate new unique reagents.

Data and code availability

All sequencing data reported in this paper will be available in FASTQ read format and cellbender corrected gene expression matrix format at Gene Expression Omnibus upon publication as we plan to submit this work for pre-print. The annotated data can also be explored at the Broad Institute Single Cell Portal under study numbers SCP2216 and SCP2221. All the code generated and used to analyze the data reported in this paper will be available on GitHub in the jo-m-lab/IAV-nasal-sc-atlas repository.

Experimental model and subject details

Mice

All experiments were approved by the Harvard University Institutional Animal Care and Use Committee and run following NIH guidelines. C57BL/6J (B6) mice 6 to 8 weeks old were purchased from The Jackson Laboratory and experiments commenced 1 to 3 weeks following their arrival. Mice were infected with 10^4pfu PR8 in a 10μL volume that was administered by pipette dropwise to the nares to allow each drop to be inhaled. Mice were restrained during this administration but not anesthetized, to maintain the virus in the upper respiratory tract. All mice were housed in a BSL-2+ facility with specific pathogen free conditions.

Method details

Virus growth, quantification, and mouse infections

IAV strain A/Puerto Rico/8/1934 (PR8) and Madin-Darby canine kidney (MDCK) cells were generously provided by Dr. Daniel Lingwood and Dr. Maya Sangesland of the Ragon Institute of

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Mass General, MIT, and Harvard. Virus was propagated and quantified in MDCK cells. MDCK cells were grown at 37°C with 5% CO_2 in cell growth media: Dulbecco's modified eagle's medium (DMEM) (Corning, #10-017-CV), 10% fetal bovine serum (FBS; Gemini #100-106), 1X Penicillin:Streptomycin (Gemini, 100X stock: 400109). PR8 was grown in MDCK cells in influenza growth media: Iscove's DMEM (Corning, # 10-016-CV), 0.2% bovine serum albumin (BSA; EMD Millipore, EM-2960), 1Xm Penicillin:Streptomycin, and 2μg/mL TPCK treated Trypsin (Sigma, T1426).

For viral load quantification experiments, mice were sacrificed in CO_2 and lungs and heads were separated. For the nasal cavity, fur and skin were removed and the lower jaws cut off. The entire nasal cavity or lungs were collected into 1mL PBS with 2.3mm Zirconia/Silica beads (Biospec Products, 11079125z) and stored on ice. The tissue was homogenized in an OMNI Bead Ruptor Elite at 3m/s for 30 seconds twice, centrifuged 500g for 5 minutes, and supernatant was collected and stored at -80°C until thawed for plaque assays. Virus titers were measured by plaque assays in confluent MDCK cells in 6-well plates. MDCK cells were grown in cell growth media, washed with sterile phosphate-buffered saline (PBS), then washed with influenza growth media. Media was removed and serial dilutions of viral supernatant in influenza growth media were added to each well in a 400μL volume, incubated for 1 hour at 37°C, then overlayed with 0.3% agarose in influenza growth media. Infected cells were incubated for three days at 37°C, fixed with 4% paraformaldehyde, stained with crystal violet, washed, and plaques were counted.

Tissue harvesting, single-cell suspension preparation, and hashtag labeling

Three separate regions of the nasal tissue were harvested independently: (1) the respiratory mucosa (RM), (2) the olfactory mucosa (OM), and (3) the lateral nasal gland (LNG). The nasal tissue was collected by removing the skin and connective tissue from around the head, cutting off the lower jaw, and opening the nasal cavity by peeling away the nasal bone from the rest of the skull. Tissue separation and collection was performed using a dissection scope with a 4x objective. All nasal tissue surrounding the nasoturbinates, maxillary turbinates, and septum, including the mucosa that runs along the nasal lateral walls between the nasoturbinates and maxillary turbinate, and the mucosal tissue under the nasal bone that connects the nasoturbinates and septum, were collected together and constitute the RM. After removal of the RM the ethmoid turbinates were collected including both the mucosal tissue and the bone and cartilage of the turbinates, but not the surrounding skull, constituting the OM. After removal of the OM, the LNG was exposed and could be collected without any bone or cartilage. The nasal-associated lymphoid tissue (NALT) was not collected in any of the three regions.

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Each nasal tissue region was collected into 750µL Wash Media (RPMI 1640, 2% FBS, 10 mM HEPES, and 100U/ml penicillin G, 100µg/ml streptomycin) and stored on ice. Tissues were chopped with scissors then 750µL Digestion Media (Wash Media with 100µg/mL Liberase (Sigma, #5401127001) and 100µg/mL of DNAse I (Roche, #10104159001)) was added. Tissues were incubated at 37°C with end-over-end rotation, 30 minutes for RM and OM, 20 minutes for LNG. 13.3µL EDTA (0.5M) was added to each sample and then cells were washed with HBSS Media (HBSS (Ca, Mg Free, 500 mL), 10mM EDTA, 10mM HEPES, 2% FBS) and filtered through a 70µm nylon cell strainer. Cells were pelleted by centrifugation 500g for 10 minutes, resuspended with ACK (Ammonium-Chloride-Potassium) lysis buffer for 1 minute on ice, and then diluted with 9mL HBSS Media and centrifuged 500g for 5 minutes twice. Cells were then resuspended in 1mL Isolation Buffer (PBS, 0.1% BSA, 2mM EDTA) pre-mixed with 25µL anti-EpCAM-biotin-Dynabeads (anti-EpCAM-biotin antibody (G8.8, Biolegend) bound to Dynabeads Biotin Binder (ThermoFisher)) for a light epithelial cell depletion, incubated for 15 minutes on ice, washed with Isolation Buffer and placed on a Dynamag for 2 minutes. Supernatants were collected, centrifuged 500g for 5 minutes, resuspended in 100µL Staining Buffer (PBS, 1% BSA, 0.01% Tween) and 10µL Fc block, and incubated on ice for 10 minutes. Next, 0.5µg Biolegend TotalSeq Hashing antibodies B0301, B0302, or B0303 were added so that each mouse had all three nasal regions (RM, OM, and LNG) stained with one of the three antibodies, and incubated on ice for 20 minutes. Cells were then washed extensively to remove excess antibody with 10mL Staining Buffer and centrifugation at 500g for 5 minutes twice. Cells were resuspended in Loading Buffer (PBS and 0.04% BSA), counted, and pooled equally (13,500 cells/sample) between three mice for each region. Finally, each set of pooled cells were centrifuged 500g for 5 minutes and resuspended in 42µl Loading Buffer for downstream scRNA-seq processing.

Single-cell RNA-seq

Pooled samples from each nasal region (RM, OM, and LNG) were processed using the 10x Genomics Chromium Next GEM Single Cell 3' Kit v3.1 and Feature Barcoding Kit with dual indices per the manufacturer's instructions. Approximately 40,000 cells per pooled reaction were loaded on the 10x Genomics Chromium Controller. Library quality was evaluated using the Agilent TapeStation 4200 (Agilent). Prior to sequencing, the gene expression and hashtag libraries were pooled 20:1. Sequencing was performed on either the NovaSeq 6000 or NextSeq 2000 (Illumina) with an average RNA read depth of 16,000 reads/cell and hashtag read depth of 500 reads/cell.

Immunofluorescence Microscopy

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Mice were euthanized in CO_2 and their heads following skin, fur, and lower jaw removal were placed in 4% paraformaldehyde for 1-4 hours on ice for fixation. Heads were transferred to 0.5M EDTA for 2-3 days at 4°C for bone decalcification. Heads were transferred to 30% sucrose in PBS for cryoprotection for 2 days at 4°C then rapidly frozen in NEG-50 using dry ice. Frozen heads were stored at -20°C until cryostat sectioning. Mouse nasal tissues were cut into 50-100μm sections, permeabilized with 0.3% Tween in PBS (PBST) for 1 hour, then incubated overnight at 4°C with antibodies, DAPI, and Fc block at a 1:200 dilution in PBST. Antibodies used: anti-Influenza A virus NS1 (PA5-32243, ThermoFisher), anti-acetyl-α-tubulin (Ly640, D20G3, Cell Signaling Technology), anti-CD45 (30-F11, Biologend), anti-EpCAM (G8.8, Biolegend), anti-Krt13 (EPR3671, Abcam), anti-PD-L1 (10F.9G2, Biolegend), and anti-IgA (mA-6E1, ThermoFisher). Samples were then washed 3 times with PBST in 15-minute intervals at room temperature, mounted on glass slides with Prolong Gold, and visualized with an Olympus FLUOVIEW FV3000 confocal laser scanning microscope.

qPCR

RM tissue was collected as described above from mice and lysed in Buffer RLT (Qiagen) + 1% beta-mercaptoethanol (Sigma) via gentleMACS Octo Dissociator in M-Tubes (Miltenyi Biotec). RNA was extracted from tissue lysate by RNAEasy Mini column purification (Qiagen) following the manufacturer’s instructions. cDNA was then generated following the SmartSeq II protocol as previously described (Trombetta et al., 2014). qPCR was performed using TaqMan reagents and probes (ThermoFisher) on a CFX384 Real-Time PCR System.

Antibody-based depletion

Naïve or PR8 infected B6 mice were administered daily 20μg anti-CCR2 depleting antibodies (MC-21 generously provided by Prof. Matthias Mack, Universität Regensburg) or rat IgG2b,κ isotype control (Biolegend, #400644) intraperitoneally (i.p.). 24h following one administration, blood was collected from naïve mice by tail vein bleed into FACS buffer (PBS, 0.5% BSA, 2mM EDTA) and stored on ice before processing for flow cytometry.

PR8 infected mice were administered antibodies 3, 4, and 5 dpi in 24h intervals. For this experiment, mice were euthanized at 8 dpi.

Flow cytometry

Blood was processed for flow cytometry by pelleting cells by centrifugation and resuspending with ACK lysis buffer to remove RBCs. Cells were then washed with FACS buffer

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and stained in 50 µL for flow cytometry using the following antibodies: anti-CCR2 (475301, R&D Systems), anti-CD11b (M1/70, Biolegend), anti-CD19 (6D5, Biolegend), anti-CD3e (145-2C11, Biolegend), anti-CD45 (30-F11, Biologend), anti-Ly6C (HK1.4, Biolegend), anti-Ly6G (1A8, Biolegend), and anti-NK1.1 (PK136, Biolegend). Cells were analyzed using the Beckman Coulter CytoFLEX.

For RM tissue, mice were anesthetized i.p. with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) prior to euthanasia and administered 1µg anti-CD45 antibody (30-F11, Biologend) by retroorbital intravascular injection to label CD45+ cells in circulation. Mice were then euthanized 3 minutes later in CO_2. RM tissue was processed as described above for tissue harvesting and single-cell suspension preparation through ACK lysis and dilution. Cells were then centrifuged and resuspended in 100 µL FACS buffer LIVE/DEAD Fixable Aqua Dead Cell Stain (ThermoFisher #L34966) per manufacturer's instructions. Cells were then washed and stained for 30min at 4°C in the dark with Fc block diluted 1:200 and the following antibodies: anti-CD11b (M1/70, Biolegend), anti-CD11c (HL3, BD Biosciences), anti-CD19 (6D5, Biolegend), anti-CD3e (145-2C11, Biolegend), anti-CD45 (30-F11, Biologend), anti-CD64 (X54-5/7.1, Biolegend), anti-Ly6C (HK1.4, Biolegend), anti-Ly6G (1A8, Biolegend), anti-F4/80 (BM8, Biolegend), anti-MHC-II (M5/114.15.2, Biolegend), and anti-NK1.1 (PK136, Biolegend). Following staining, cells were washed in FACS buffer, and analyzed. To determine cell counts, AccuCheck Counting Beads (ThermoFisher #PCB100) were added to every sample.

RNAscope Microscopy

RNA *in situ* hybridization was performed according to manufacturer's instructions for the RNAscope Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics ACD, 323270) on 20 µm thin sections of fixed-frozen murine nasal mucosa tissue collected 14 dpi. We implemented the following modifications to preserve tissue integrity: 1) 5 min PBS wash preceding initial baking of slides was removed; 2) slides were baked for 30 min at 60°C following EtOH dehydration; 3) target retrieval time was reduced to 5 min; 4) slides were baked for 60 min at 60°C following target retrieval; and 5) tissue sections were incubated in Protease Plus instead of Protease III for milder protease digestion. Probes used included Mm-Krt13 (ACD, 575341), Mm-Cxcr6-C2 (ACD, 871991-C2), Mm-Cxcl16-C3 (ACD, 466681-C3), and Mm-Cd274-C3 (ACD, 420501-C3). Following signal amplification, Opal 520 (Akoya Biosciences, FP1487001KT), Opal 570 (Akoya Biosciences, FP1488001KT), and Opal 690 (Akoya Biosciences, FP1497001KT) dyes were used, diluted 1:1000 in TSA buffer (ACD, 322809). Nuclei were stained with DAPI and slides were

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mounted with VECTASHIELD PLUS (Vector Laboratories, H-1900). Confocal images were collected using an Olympus FLUOVIEW FV3000 confocal laser scanning microscope.

Quantification and statistical analysis

Single-cell RNA-seq alignment, cleanup, and pre-processing

To detect reads originating from IAV, we built a combined genome of mm10 (GRCm39) and the sequences for PR8 (NCBI Taxonomy ID #211044). The eight PR8 genomic viral segment sequences (NC_002023.1, NC_002022.1, NC_002021.1, NC_002020.1, NC_00219.1, NC_2018.1, NC_002017.1, and NC_002016.1) and associated IAV gene annotations were added to the GRCm39 FASTA and GTF files and processed using the CellRanger's built in "mkref" function. Sequences were then aligned and quantified using this combined genome with the CellRanger toolkit (v6.0.1) via Cumulus tools (Li et al., 2020) (https://cumulus.readthedocs.io/en/stable/). Cell sample identity was assigned from the measurement of TotalSeqB aligned counts using the cumulus demultiplexing tool for feature barcoding, calling identity for any cell with at least 100 barcodes. To correct for transcript spill-over, cellbender (Fleming et al., 2022) was applied to the raw output UMI matrices from CellRanger with the following parameters: *expected_cells=30000*, *fpr=0.01, total_droplets_included=50000*. Cellbender corrected cells were then filtered based on Unique Molecular Identifiers (UMI) count (>750 & <10000), number of detected genes (>500), and percentage of mitochondrial genes (<15%). Finally, cells labeled as doublets by demultiplexing were removed.

Iterative clustering, cell cluster annotation, and IAV+ cell calling

Downstream analysis was performed using Seurat (v.4.2.1) (Hao et al., 2021). Briefly, the entire primary infection dataset underwent normalization using the *scTransform* function followed by principal component analysis (PCA), shared nearest neighbors (SNN) graph generation, Louvain clustering, and UMAP embedding. Clustering was performed at multiple resolutions to help annotate similar and dissimilar clusters. Using clustering *resolution = 0.6,* cluster specific/enriched markers were calculated. Each cluster was labeled by major cell type based on the expression of known lineage markers (e.g., *Omp*, *Epcam*, *Ptprc, Flt1,* etc.). Doublet clusters were also annotated based on the lack of unique markers and/or the presence of multiple mutually exclusive lineage markers (e.g., *Omp+Ptprc+* cells). Following annotation, doublet clusters were removed, and the normalization/clustering/doublet removal process was repeated twice more (total of three times) until no doublet clusters were discernable.

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The dataset was then divided into separate objects by cell type label for further subclustering. Following the same routine applied to the full dataset, clusters for each cell type were annotated with subset/state labels based on prior knowledge and previously published scRNAseq datasets of the nasal mucosa (Brann et al., 2020; Ualiyeva et al., 2022; Ziegler et al., 2021). After the first set of annotations in every cell type object, it was apparent that there were still intra-sample doublets present: mostly contaminating cell types, but also within cell type doublets (e.g., ionocyte/sustentacular doublets). These clusters were iteratively removed like in the analysis of the full dataset, for a total of three rounds in each cell type, yielding a total of 127 clusters across the whole dataset. To visualize these clusters' relationships and distribution across nasal regions, we built a cell cluster "phylogenetic tree" using ARBOL (Zheng et al., 2021), where the first tier encodes major cell type, the second tier encodes defined subtypes, and the third tier encodes cluster identity (**Figure S1F**).

Since IAV transcript capture was sparse, we classified IAV+ cells as any cell with 2 or more UMIs aligned to any IAV PR8 gene.

Compositional analyses

After removing multiplets, immune (>97.5%) and endothelial cells (89%) had nearly all cells assigned a sample replicate while neurons (30.7%), epithelial cells (65.9%), fibroblasts (52.4%), and other stromal cells (56.4%) had lower sample annotation rates. We note that cells without a sample replicate assignment were excluded from all compositional analyses. Within cell type frequencies were calculated on a per replicate basis by counting the number of cells within each cluster label and dividing by the total number of cells for that cell type captured in that replicate. For tissue- and region-level compositional analyses, cell cluster abundances were calculated by deriving cell cluster frequencies over all labeled cells in each sample replicate, scaling to 3,000 cells per replicate, and log transforming. Subsequent PCAs were calculated from these log-transformed cell cluster abundances.

Proportionality analysis was performed using the propr package (v4.2.6) (Quinn et al., 2017) on non-log-transformed cell cluster abundances across all RM sample replicates. We compared the proportionality statistic **ρ** to standard Pearson correlation across all pairwise comparisons and found **ρ** to be more stringent for significance cutoffs (FDR<0.05) generated by permutating testing (**Figure S8A**). We built a network using Cytoscape (v3.9.1) comprised of all significantly proportional cell cluster pairs to assess groups of cell clusters with similar cell abundance trajectories throughout the infection time course.

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RM sample replicate distances were calculated using the Aitchison distance (Aitchison et al., 2000). Cell cluster abundances were first normalized using the center-log-ratio (clr). Euclidean distance was then calculated between all pairs of RM sample replicates, yielding three distances within a timepoint and nine distances between two timepoints. Statistics on Aitchison distances were performed using a one-sided non-parametric Welch's ANOVA and Dunnett's T3 test for multiple comparisons in Prism.

Neutrophil pseudotime analysis

The neutrophil pseudotime analysis was performed using diffusion mapping as previously described (Grieshaber-Bouyer et al., 2021). A principal component analysis was run on all cells assigned to granulocyte clusters, excluding mast cells. The first 20 principal components were used to compute a cell-to-cell distance matrix using *1 – Pearson correlation coefficient* as the distance metric. Using the destiny package in R (Angerer et al., 2016), we computed a diffusion map with standard parameters with density normalization and rotate enabled. We manually selected "Progenitor" cells as the root of the trajectory and used the DPT function to calculate the pseudotime values, manually scaling the values from 0 to 1.

Cell-cell signaling analysis

Three cell networks (IFN-stimulated MDMs : *Gzmk*+ CD8 T cells : *Ifng+Cd200*+ CD4 T cells; *Cd103*+ DCs : *Dusp2+Icam1*+ mature neutrophils : *Gp2+Lyz2*+ goblet/secretory cells*;* *Krt13+Il1a*+ epithelial cells : *Cd103*+ CD8 T cells : CD4 T cells) were selected based on high proportionality throughout the primary infection time-course or specific biological interest to the authors. Data for the clusters of interest in each network were then subset to RM and timepoint of interest to best capture individual cells with sufficient spatial and temporal proximity to plausibly interact, and re-normalized with Seurat's *NormalizeData* function. Since NICHES calculates the multiplicative expression of ligand-receptor pairs from a random sampling of cells from each cell type to predict cell-cell communication, Adaptively thresholded Low-Rank Approximation (ALRA) imputation was applied to each cell network to reduce the impact of technical zeros due to potential dropout events (Linderman et al., 2022). NICHES was then run on each cell network individually, drawing from the OmniPath database of ligand-receptor pairs (Türei et al., 2016) to generate a cell interaction object whereby rows are ligand-receptor pairs and columns are cell type pairs (Raredon et al., 2023). These objects were then scaled and passed through principal component analysis and UMAP dimensionality reduction to generate low-dimensional embeddings of cell interactions. Differentially expressed interactions were identified using the

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Seurat *FindAllMarkers* function, and highly differentially expressed interactions of interest were selected for display as heatmaps.

Cluster annotation in memory samples

To assign cluster labels to new scRNA-seq datasets generated from the nasal mucosa, we leveraged the structure of our data to test the label transfer methods provided in Seurat (Hao et al., 2021) and scANVI (Xu et al., 2021). We separated the cells from one RM replicate from each time point as a query dataset, using the remaining cells as the reference. With Seurat, we implemented the FindTransferAnchors function on the scTransformed data, using the first 40 PCs from the PCA as the reference. Labels were then assigned with the TransferData function using either cell cluster or cell type identities. With scANVI, we first built a scVI model on the reference data, and then a scANVI model using either cell cluster or cell type identities as labels. The reference scANVI model was then used to train a model on the query data. We calculated the percentage of correctly called cell labels using each method for both sets of labels and found calling to be superior on the cell type level. Thus, we next repeated each procedure within each cell type to learn cell cluster labels and found Seurat to perform better across all cell types (**Figure S9C**). To further validate, we calculated cell cluster abundances using the predicted cell cluster labels for the query replicates and projected into the PCA calculated across the RM samples.

We next applied the two-step label transfer approach using Seurat to new data generated from RM 60 dpi and 2 and 5 dprc. Before performing label transfer, we removed all hashtag annotated cell doublets from the new dataset and applied the same filtering criteria as above. We next performed *scTransform* and PCA on the new dataset. We then predicted cell type labels using all cells from RM samples in the primary infection dataset for reference. We next removed any cells from subsequent analysis that had a maximum assignment score < 0.8 (i.e., 80% is the greatest confidence in label prediction), making up 5% of the total dataset. Given our loading strategy and number of intrasample doublets found in the primary infection dataset, we chose a more stringent cutoff following cell type label prediction. Separating into each cell type and using the processed data from the matching cell type in the primary infection dataset as reference, we performed the same procedure. Here, we were more liberal, keeping all cells with a maximum assignment score ≥ 0.4 since very similar clusters within cell types could receive almost equal prediction probability (e.g., Resting Basal and Abi3bp Resting Basal). With cell cluster labels assigned, we then calculated cell cluster abundances as above and performed downstream differential expression analysis.

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MAIN TEXT FIGURES

Figure 1

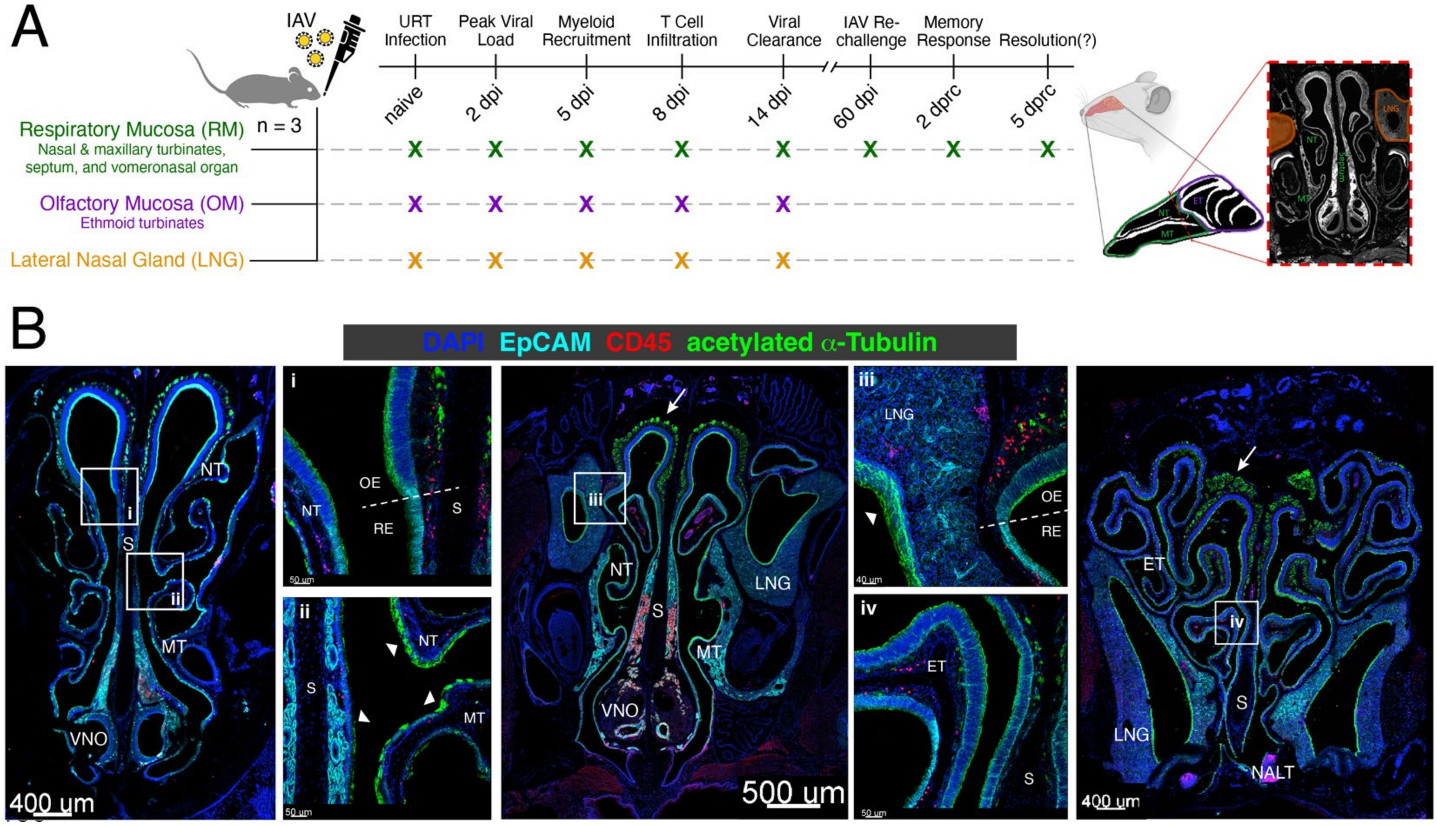

Figure 1: Experimental design and the structure of the murine nasal mucosa

(A) Schema depicting the sampling scheme and three tissue regions collected and processed for scRNA-seq: respiratory mucosa (RM), olfactory mucosa (OM), and lateral nasal gland (LNG). For both primary infection and rechallenge, 10^4 pfu Influenza A Virus (IAV) PR8 was administered intranasally (5µl/nostril). dpi = days post infection; dprc = days post rechallenge.

(B) Representative immunofluorescence images of coronal slices of the nasal mucosa from a naïve mouse moving from anterior (left) to dorsal (right) staining for epithelial cells (EpCAM, teal), immune cells (CD45, red), and ciliated cells/neurons (α-acetylated tubulin, green). Distinct regions of the mucosa are labeled. Labeled white boxes outline higher resolution images below. White arrows point to olfactory sensory nerve bundles; white arrowheads point to cilia. The olfactory epithelium (OE) and respiratory epithelium (RE) both reside within the collected RM tissue and are differentiated by morphology and the presence of olfactory sensory neurons. NT = nasoturbinate; S = septum; MT = maxillary turbinate; VNO = vomeronasal organ; LNG = lateral nasal gland; ET = ethmoid turbinate; NALT = nasal-associated lymphoid tissue.

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Figure 2

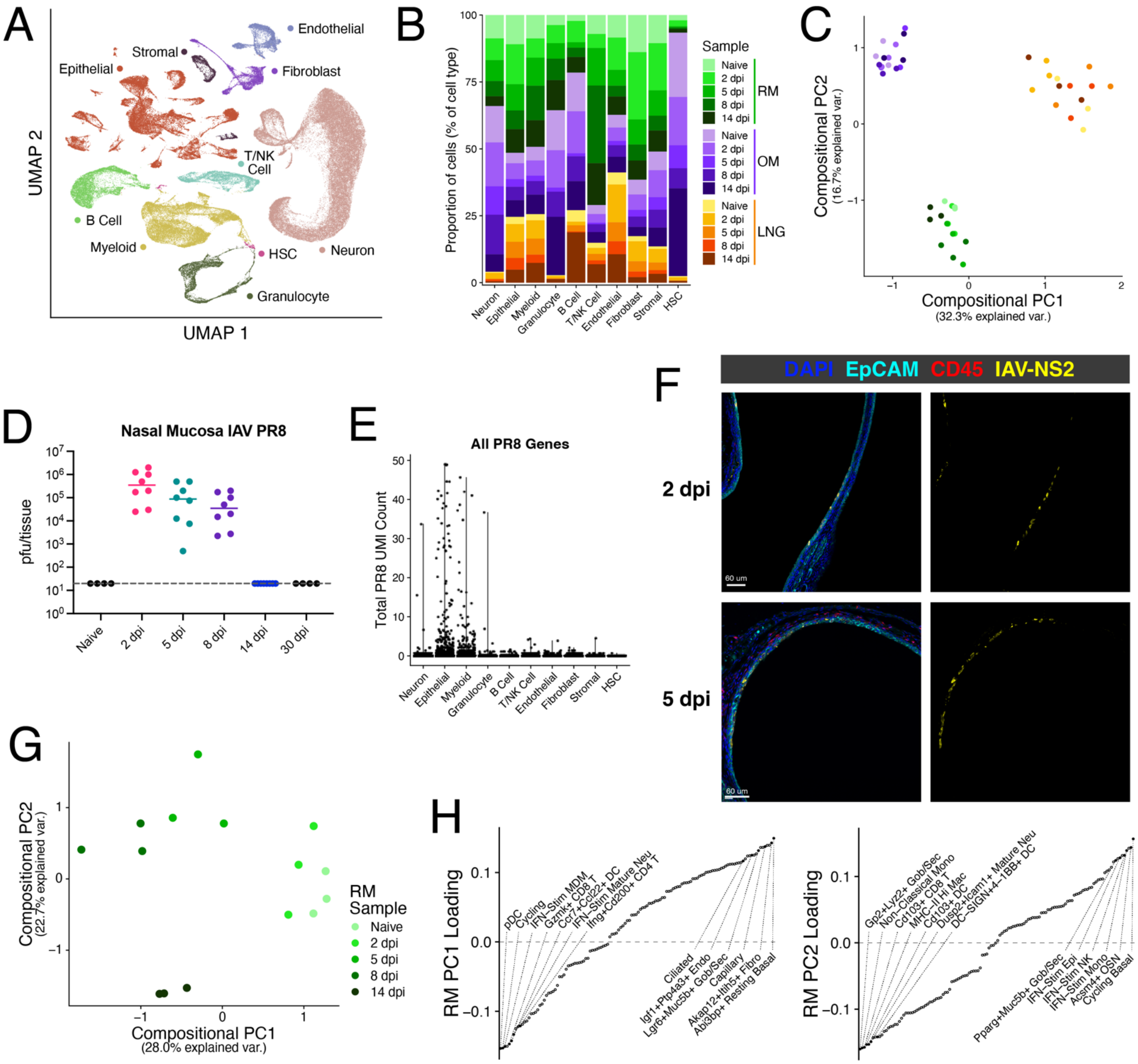

Figure 2: Single-cell atlas of the nasal mucosa during primary IAV infection

(A) UMAP embedding of 156,572 nasal cells across three tissue regions and five time points (n=3 per region/timepoint) colored by cell type. HSC = hematopoietic stem cell.

(B) Stacked bar chart depicting the relative proportions of cells annotated for each cell type by region and time point.

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(C) Compositional principal component analysis (PCA) of all acute infection sample replicates. Each point represents a sample replicate and distance reflects variation in cell cluster abundance (scaled cell counts). Dots are colored by region and time point as in **B**.

(D) Infectious IAV PR8 quantification in plaque forming units (pfu) of the entire nasal mucosa.

(E) Summative scTransform-corrected UMI counts per cell across all IAV PR8 genes split by cell type.

(F) Representative images of IAV infection in RM taken from mice 2 dpi (top) and 5 dpi (bottom). Staining for EpCAM (teal), CD45 (red), and IAV-NS2 (yellow). Images on the right depict only the signal in the IAV-NS2 channel.

(G) Compositional PCA of all cell clusters from only RM samples.

(H) Cell cluster abundance loadings for PC1 (left) and PC2 (right) from (G). Cell cluster names for several of the most negative and most positive weights for each PC are depicted.

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Figure 3

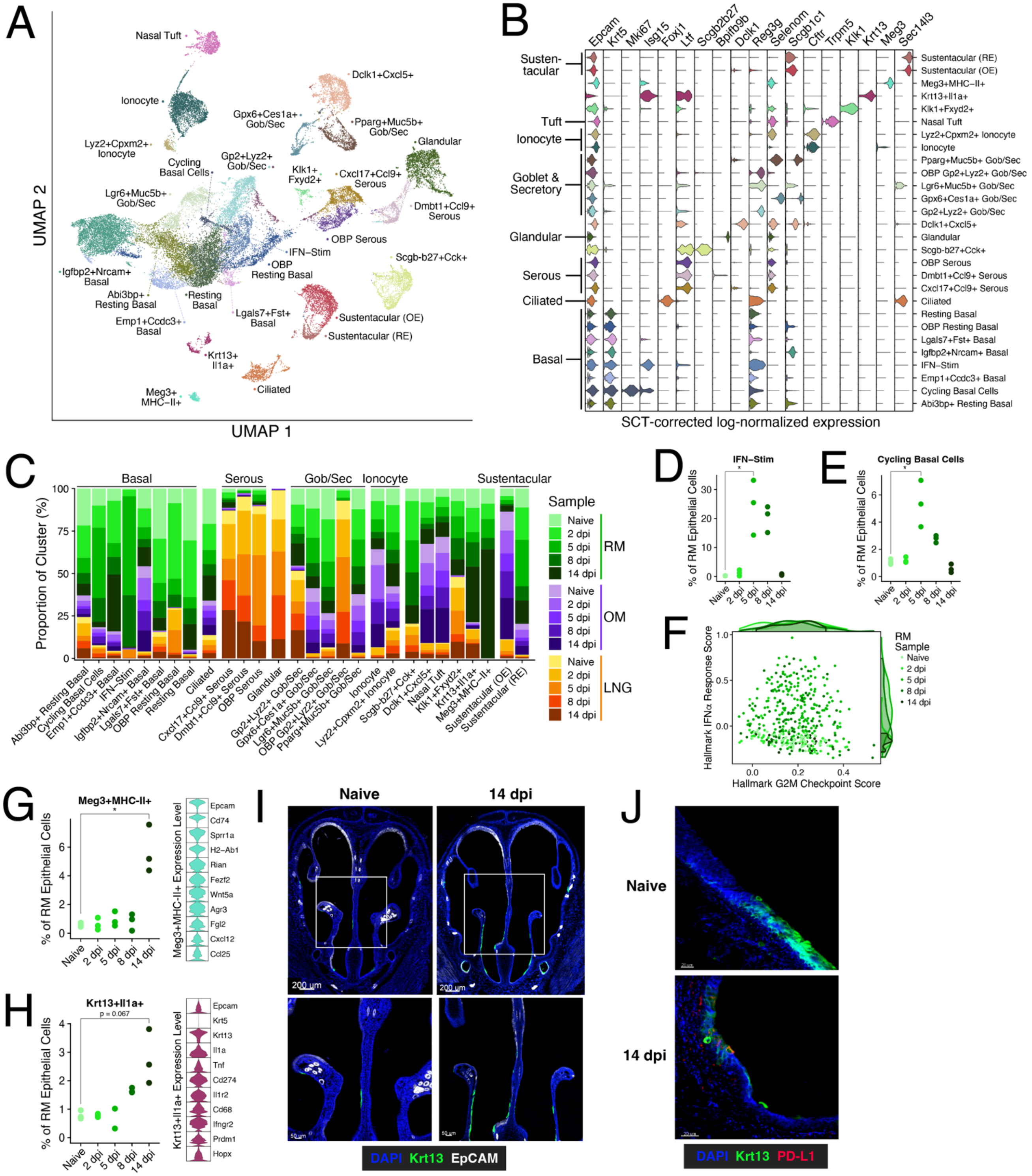

Figure 3: Epithelial cell subclustering reveals diverse subsets including transient IFN-responsive/cycling cells and rare cells with immune-interaction potential arising 14 dpi

(A) UMAP embedding of 38,367 epithelial cells across 27 clusters.

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(B) Violin plot depicting epithelial lineage and subset marker expression levels (scTransform-corrected log-normalized UMI counts) across all discovered clusters (see **Supplementary Table 1**).

(C) Stacked bar chart depicting the relative proportions of cells annotated for each cluster by region and time point.

(D & E) Relative frequencies of cells clustered as IFN-Stim (D) and Cycling Basal (E) as a proportion of all epithelial cells per replicate RM sample. Only cells with assigned hash calls are included. Welch's t test, $^*p < 0.05$.

(F) Scatter plot of gene module scores for the Hallmark IFNα Response and Hallmark G2M Checkpoint gene lists (MsigDB v7.5.1) in cycling basal cells. Density plots represent the scatter plot data.

(G & H) Relative frequency plots of *Meg3*+MHC-II+ (G) and *Krt13*+*Il1a*+ (KNIIFE cells) (H) clusters (left) as a proportion of all epithelial cells per replicate RM sample. Violin plots of select cluster specific/enriched genes, except for *Krt5* (FDR corrected p-values $\leq 10^{-242}$ by 1-vs-rest Wilcoxon Rank Sum Test) (right).

(I) Representative immunofluorescence images of the very anterior nasal mucosa in naïve mice (left) and 14 dpi (right) staining for Krt13 (green) and EpCAM (white).

(J) Representative images within the region shown in (I) in naïve mice (top) and 14 dpi (bottom) staining for Krt13 (green) and PD-L1 (red). Welch's t test, $^*p < 0.05$.

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Figure 4

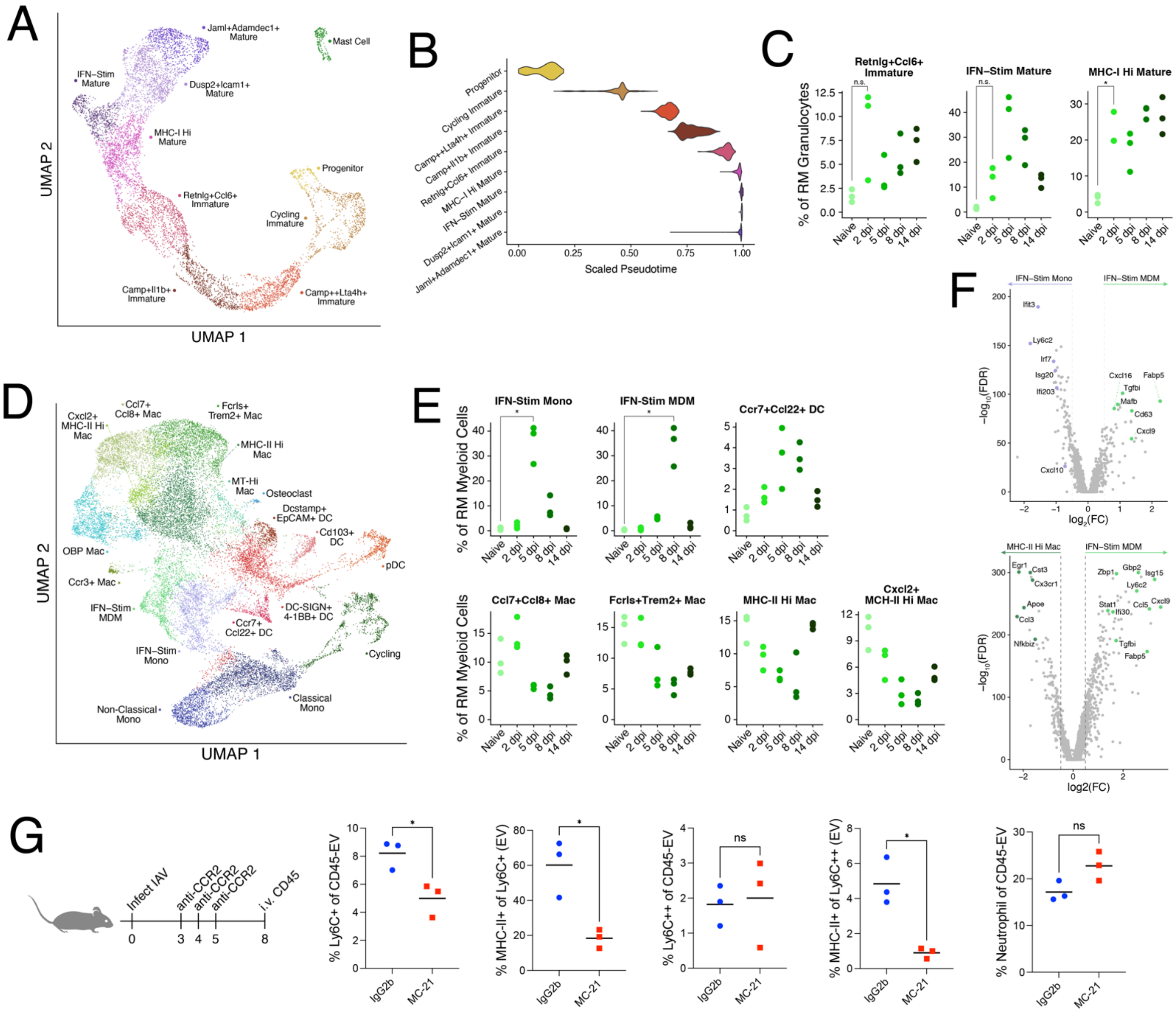

Figure 4: Immediate neutrophil responses are bolstered by recruited antiviral monocytes that differentiate into antiviral monocyte-derived macrophages

(A) UMAP embedding of 7,987 granulocytes across 10 clusters.

(B) Violin plot of assigned pseudotime values to all granulocytes (except mast cells) split by cluster identity.

(C) Relative frequencies of various neutrophil clusters as a proportion of all granulocytes per replicate RM sample. Only cells with assigned hash calls are included. Welch's t test, $^*p < 0.05$.

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(D) UMAP embedding of 22,654 macrophages, monocytes, and dendritic cells (DCs) across 17 clusters.

(E) Relative frequencies of various myeloid cell clusters as a proportion of all macrophages, monocytes, and DCs per replicate RM sample. Welch's t test, $^*p < 0.05$.

(F) Volcano plots depicting differentially expressed genes ($|\log2FC| \geq 0.5$; $FDR < 0.01$) between IFN-Stim MDMs and IFN-Stim Mono (top) and between IFN-Stim MDMs and MHC-II-Hi Macs (bottom). Only cells from RM were used in the differential expression analysis. Genes of interest are labeled.

(G) Mice were infected with 10^4 pfu IAV PR8 and then treated 3, 4, and 5 dpi with either MC-21 (anti-CCR2; n=3) or IgG2b (isotype control; n=3). At 8 dpi mice received anti-CD45 intravascularly immediately prior to euthanasia to distinguish cells in the tissue (extravascular, EV) from those in circulation (IV). Flow cytometry statistics are gated on CD45-EV+ cells; Welch's t test, $^*p < 0.05$.

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Figure 5

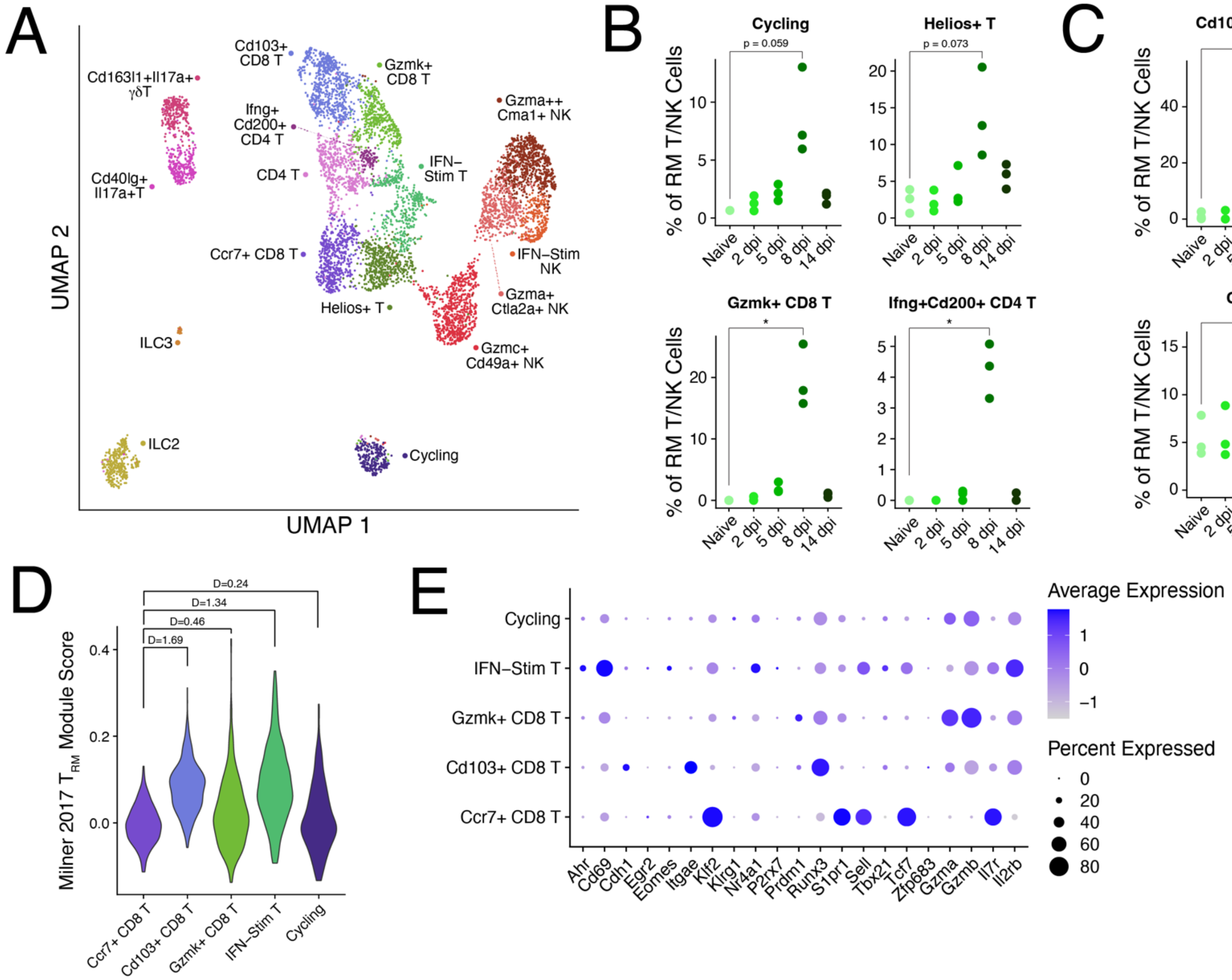

Figure 5: Effector CD4 and CD8 T cells 8 dpi are replaced by T_{RM} cells following viral clearance

(A) UMAP embedding of 6,573 T, NK, and innate lymphoid cells across 16 clusters.

(B & C) Relative frequencies of various T cell clusters as a proportion of all T and NK cells per replicate RM sample. Only cells with assigned hash calls are included.

(D) Violin plot depicting a gene module score derived from the universal T_{RM} signature as published in (Milner et al., 2017) across all CD8 T cell clusters for cells collected from RM. Cohen's D for effect size is reported between *Ccr7*+ CD8 T cells and each other cluster. Welch's t test, $^*p < 0.05$, $^{**}p < 0.01$.

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(E) Dot plot of genes encoding for canonical surface markers, proteases, and transcription factors enriched or absent in T_{RM} cells from circulating memory and naïve CD8 T cells. Gene list derived from (Crowl et al., 2022).

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Figure 6

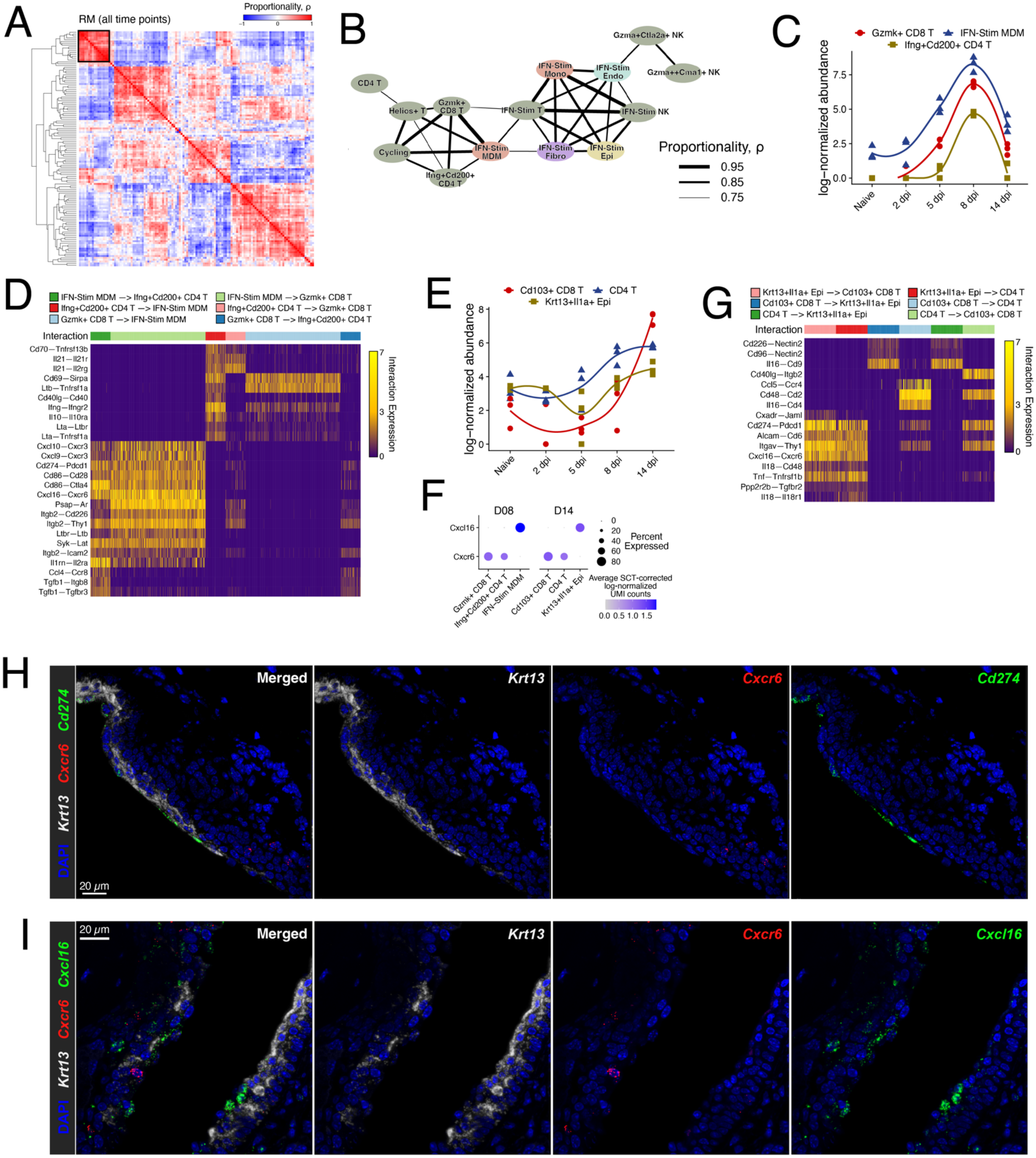

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Figure 6: Proportionality and cell-cell communication analyses highlight the CXCL16–CXCR6 signaling pathway in T cell:MDM and T cell:KNIIFE cell interactions

(A) Hierarchically clustered similarity heatmap of sample replicate proportionality calculated across all RM primary infection time points. Black box surrounds the proportionality results for the cell clusters depicted in (B).

(B) Network of significantly proportional (FDR<0.01) cell clusters as in (A). Nodes are colored by cell type and edge weight is representative of proportionality.

(C) Abundance plot of *Gzmk*+ CD8 T cells, IFN-Stim MDMs, and *Ifng*+*Cd200*+ CD4 T cells in replicate RM samples. Smoothed lines are calculated using local polynomial regression fitting.

(D) Heatmap depicting a subset of differentially expressed receptor-ligand interaction pairs between single-cell pairs identified by NICHES (Raredon et al., 2023) for the clusters depicted in (C); see **Supplementary Table 2**. Interaction expression is the multiplicative expression of receptor and ligand gene expression for each member of a single-cell pair. See for all receptor-ligand interaction pairs.

(E) Abundance plot of *Cd103*+ CD8 T cells, CD4 T cells, and *Krt13*+*Il1a*+ epithelial (KNIIFE) cells in replicate RM samples.

(F) Dot plot of scTransform-corrected log-normalized *Cxcl16* and *Cxcr6* expression 8 dpi (left) in the clusters depicted in (C) and 14 dpi (right) in the clusters depicted in (E).

(G) Heatmap like (D) for the clusters shown in (E).

(H) Representative RNAscope in situ staining for *Krt13* (gray)*, Cxcr6* (red)*, and Cd274* (i.e., PD-L1; green) of the nasal floor 14 dpi. Images depict a maximal intensity projection across 5 µm (10 slices) in the z-plane.

(I) RNAscope as in (H) staining with *Krt13* (gray)*, Cxcl6* (red)*, and Cxcl16* (green). Here, maximal intensity projection is across 3 µm (6 slices) in the z-plane.

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Figure 7

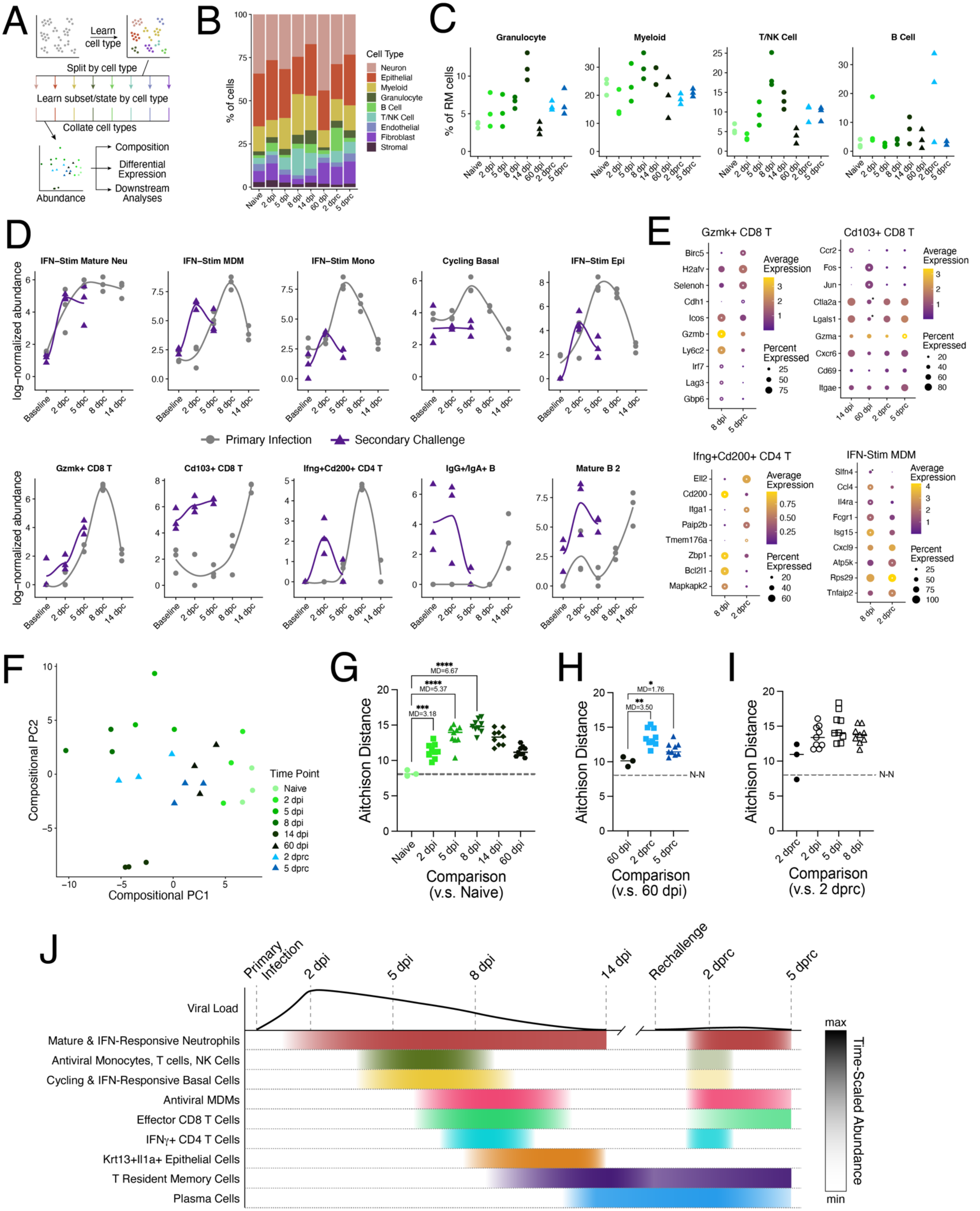

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Figure 7: IAV rechallenge induces accelerated and coordinated memory immune responses

(A) Analysis scheme applied to RM samples collected 60 dpi and 2 and 5 days post IAV rechallenge (dprc) to learn cell cluster identity.

(B) Stacked bar chart depicting the relative proportions of cells annotated for each cell type by time point. Cells from RM samples only.

(C) Relative frequencies of immune cell types as a proportion of all sequenced cells per RM replicate sample in primary infection and following rechallenge.

(D) Abundance plots of various clusters showing overlaid primary infection (gray) and rechallenge (purple) responses. Baseline refers to samples from naïve mice in primary infection and to samples from 60 dpi in rechallenge. dpc = days post challenge. Smoothed lines are calculated using local polynomial regression fitting.

(E) Dot plots depicting select cluster-specific and differentially expressed genes between primary infection and rechallenge time points in *Gzmk*+ CD8 T cells (top left), *Cd103*+ CD8 T cells (top right), *Ifng*+*Cd200*+ CD4 T cells (bottom left), and IFN-Stim MDMs (bottom right). Time points were chosen from peak responses in each challenge. Significantly enriched (white star) or decreased (black star) genes are labeled at each timepoint (FDR<0.01). See **Supplementary Table 3** for all differentially expressed genes in each comparison.

(F) Compositional PCA of both primary infection and secondary challenge RM sample replicates. Here, secondary challenge samples were projected into the PC space calculated across only primary infection samples (see **Figure 2F**).

(G, H, & I) Euclidean distances calculated between center log ratio transformed abundance values (Aitchison distance). The farther the Aitchison distance between two sample replicates, the less similar their compositions. (G) All pairs of naïve replicates and primary infection + 60 dpi replicates. (H) All pairs of 60 dpi replicates and rechallenge replicates. (I) Pairs of replicates between 2 dprc and 2, 5, and 8 dpi. Dotted line plotted at the median naïve-naïve Aitchison distance (N-N). P values reported for multiple hypothesis corrected Welch's ANOVA. MD = mean difference; *p<0.05; **p<0.01; ***p<0.001, ****p< 0.0001.

(K) Timeline schematic of primary IAV infection and rechallenge depicting viral load trajectory and immune and epithelial cell cluster response timing and duration.

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SUPPLEMENTARY MATERIAL

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Figure S1

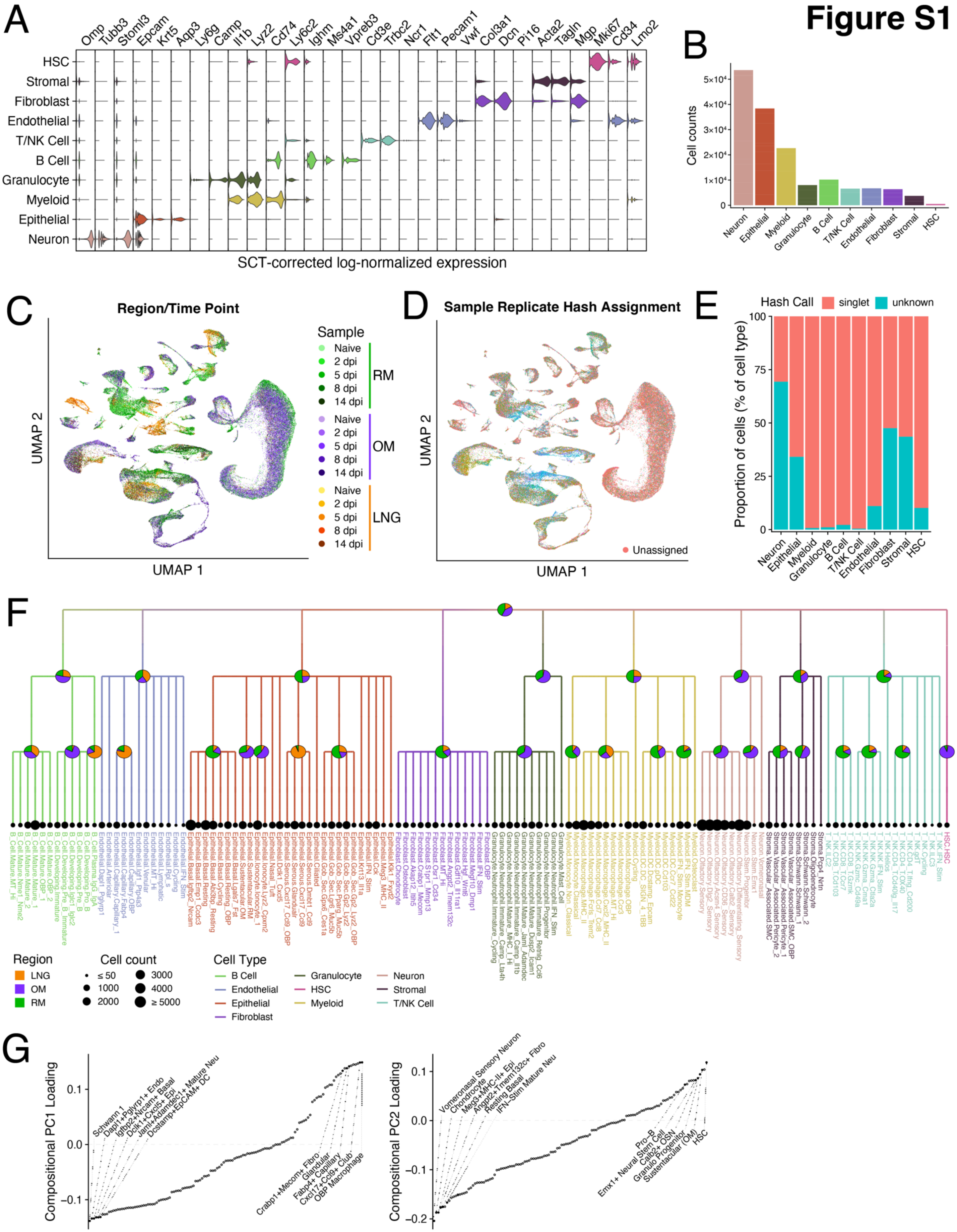

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Figure S1: Clustering and sample replicate assignment across nasal mucosa regions and cell types

(A) Violin plots of representative genes used to assign cell type identity to clusters.

(B) Numbers of cells classified as each cell type across all samples, including cells that did not receive a hash call.

(C) UMAP embedding as in **Figure 2A** colored by region and time point.

(D) UMAP as in (D) colored by hash call assignment. There are 45 sample replicates across the dataset in addition to cells without definitive hash identities (“Unassigned”).

(E) Stacked bar chart depicting the relative proportion of cells with assigned sample replicate identity (i.e., hash call) by cell type. Singlet = single sample replicate call; unknown = too few barcodes measured to assign a sample replicate identity.

(F) Cell lineage tree generated with ARBOL (Zheng et al., 2021) depicting all 127 clusters found in the dataset through cell type subclustering. Branches are colored by cell type. Pie charts at each branching point depict the relative proportion of cells from each nasal mucosa region. Dot size at each end node is proportional to the number of cells assigned to that cluster. See **Supplementary Table 1** for all differentially expressed markers across clusters within each cell type.

(G) Cell cluster abundance loadings from the PCA shown in **Figure 2C** for PC1 (left) and PC2 (right) from (F). Cell cluster names for several of the most negative and most positive weights for each PC are depicted.

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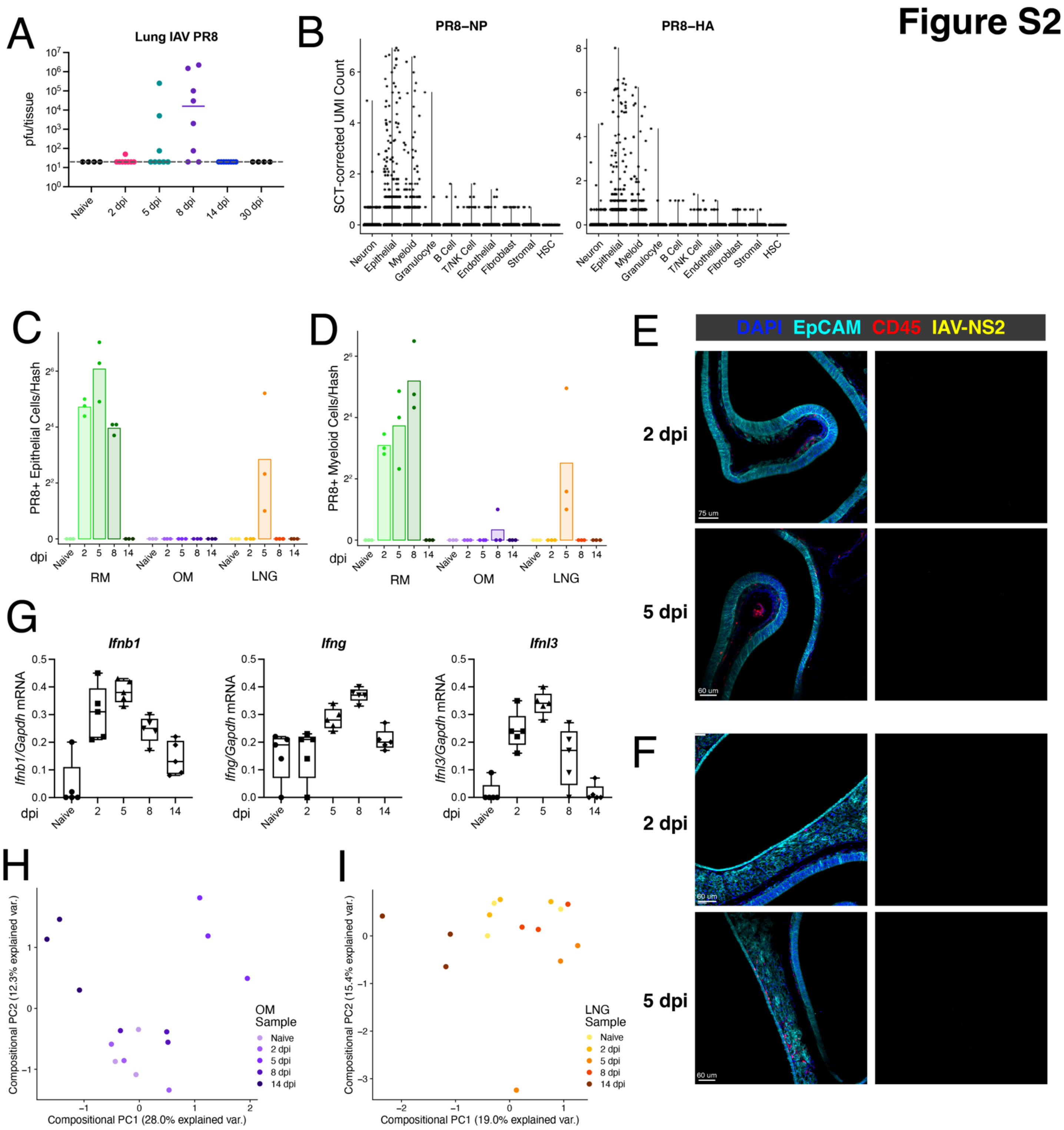

Figure S2: Viral transcript capture, global antiviral responses, and changes in OM and LNG composition

(A) Infectious IAV PR8 quantification in plaque forming units (pfu) of the entire lung.

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(B) scTransform-corrected UMI counts for the IAV PR8 genes encoding NP (left) and HA (right) by cell type.

(C & D) Number of PR8+ epithelial cells (D) and myeloid cells (E) by time point and region. PR8+ cells are classified by having at least 2 UMI aligning to PR8 genes.

(E & F) Representative images of IAV infection in OM (E) and LNG (F) taken from mice 2 dpi (top) and 5 dpi (bottom). Staining for EpCAM (teal), CD45 (red), and IAV-NS2 (yellow). Images on the right depict only the signal in the IAV-NS2 channel.

(G) qPCR of *Ifnb1* (left), *Ifng* (center), and *Ifnl3* from RNA extracted from RM tissue. C_q ratios are normalized by *Gapdh* C_q. n = 5 per time point.

(H & I) Compositional PCA of only OM samples (H) and only LNG samples (I).

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Figure S3

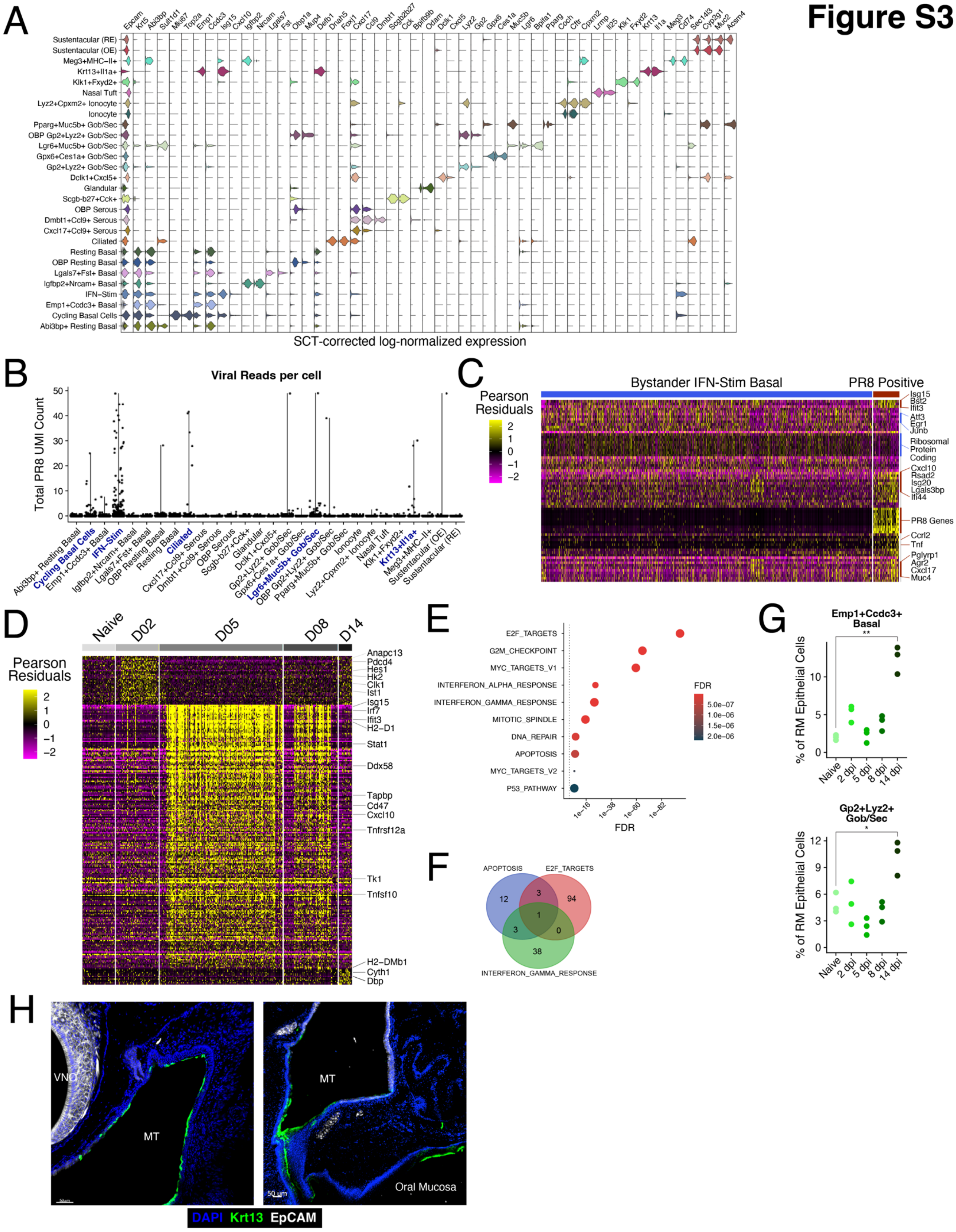

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Figure S3: Epithelial cell heterogeneity and response cluster dynamics

(A) Violin plots depicting differentially expressed marker genes (FDR<0.01) across all 28 epithelial clusters (see **Supplementary Table 1**).

(B) Summative scTransform-corrected UMI counts across all 8 IAV genes by epithelial cell cluster. Clusters with ≥5 cells with more than 2 PR8 UMIs have their cluster names bolded in blue.

(C) Heatmap depicting all differentially expressed genes between PR8 positive (≥2 PR8 UMIs) and bystander IFN-Stim epithelial cells from RM 5 and 8 dpi. Scaled Pearson residuals from scTransform are plotted.

(D) Heatmap depicting all differentially expressed genes (FDR<0.01) in Cycling Basal cells from RM between timepoints. Scaled Pearson residuals from scTransform are plotted.

(E) Gene set analysis (hypergeometric test) of all differentially enriched genes in Cycling Basal cells compared to all other epithelial cell clusters (FDR<0.01). The Hallmark pathways from MsigDB (v7.5.1) were used.

(F) Venn diagram showing the number genes used in (E) that are within the Hallmark Apoptosis, E2F Targets, and Interferon Gamma Response pathways.

(G) Relative frequencies of *Emp1*+*Ccdc3*+ basal cells (top) and *Gp2*+*Lyz2*+ Gob/Sec cells (bottom) as a proportion of all epithelial cells per replicate RM sample. Only cells with assigned hash calls are included. Welch's t test, *p < 0.05, **p < 0.01.

(H) Representative immunofluorescence images of the nasal mucosa 14 dpi taken more posterior than **Figure 3H** where the nasal mucosa connects to the oral cavity.

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Figure S4

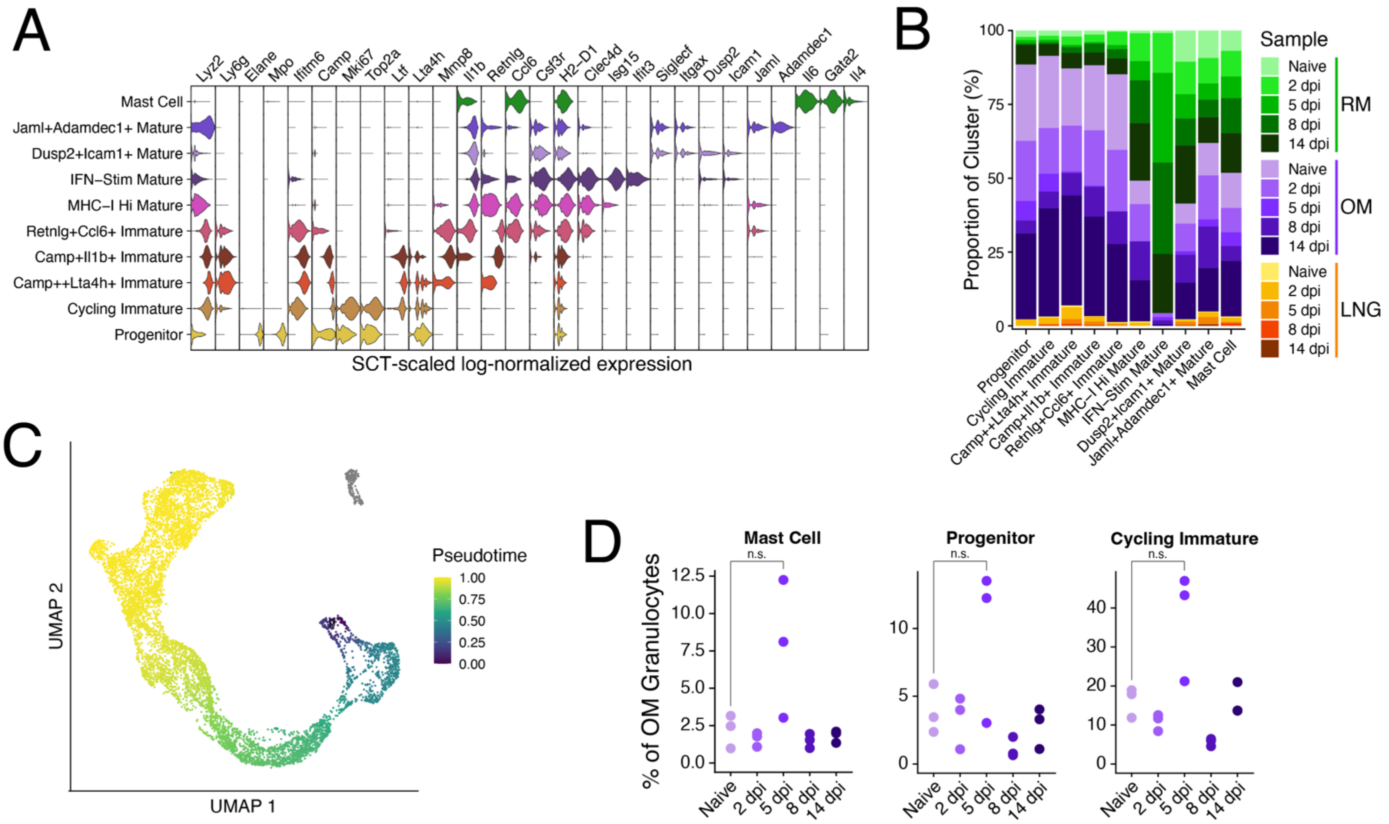

Figure S4: Granulocyte heterogeneity

(A) Violin plots depicting differentially expressed marker genes (FDR<0.01) across all 10 granulocyte clusters (see **Supplementary Table 1**).

(B) Stacked bar chart depicting the relative proportions of cells annotated for each granulocyte cluster by region and time point.

(C) UMAP of granulocytes colored by pseudotime. Mast Cells were not included in the pseudotime analysis and are colored gray.

(D) Relative frequencies of Mast cells (left), progenitors (center), and cycling immature (right) as a proportion of all granulocytes per replicate OM sample.

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Figure S5

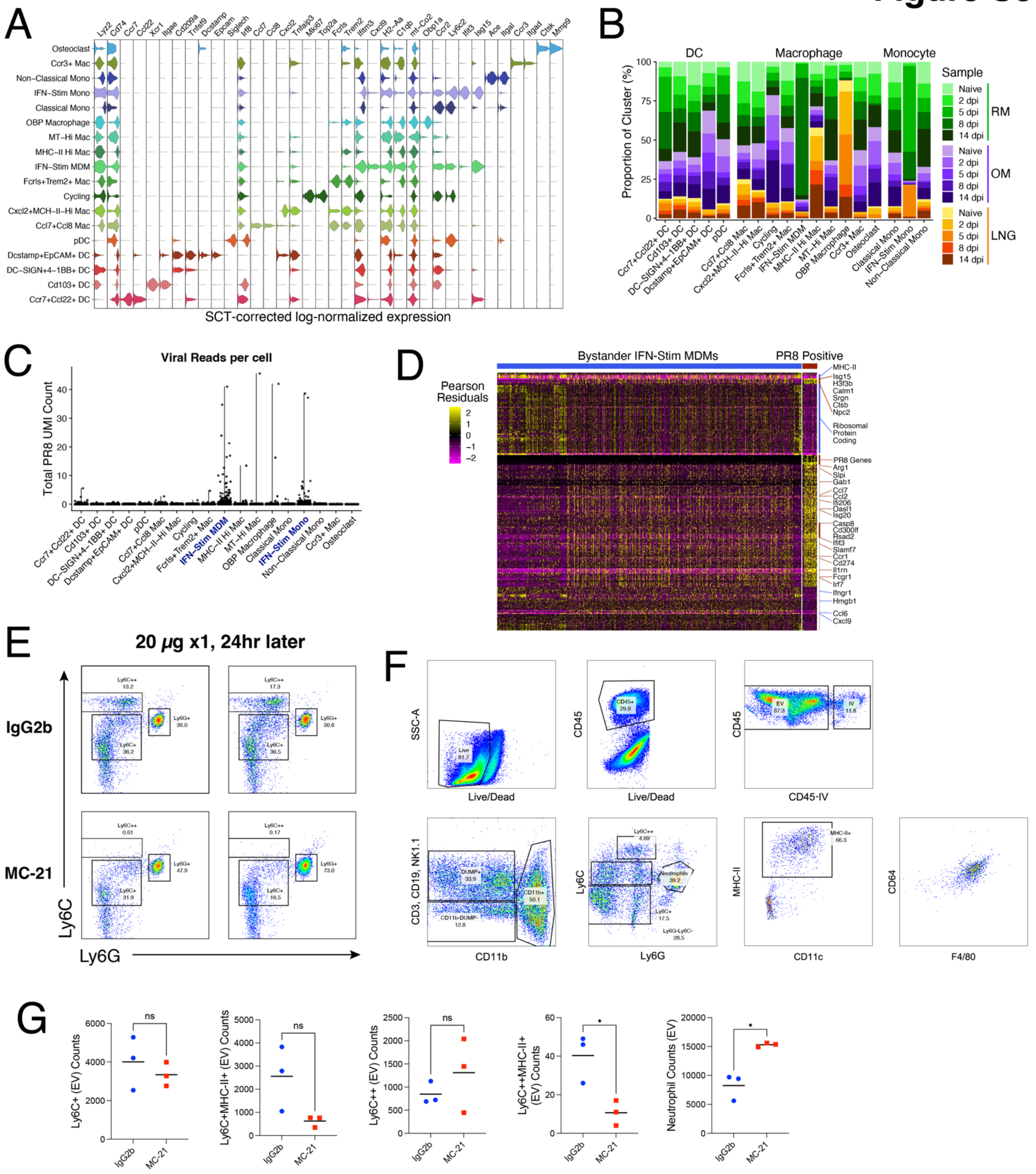

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Figure S5: Myeloid heterogeneity, viral+ cells, and monocyte depletion

(A) Violin plots depicting differentially expressed marker genes (FDR<0.01) across all 18 macrophage, monocyte, and DC clusters (see **Supplementary Table 1**).

(B) Stacked bar chart depicting the relative proportions of cells annotated for each myeloid cluster by region and time point.

(C) Summative scTransform-corrected UMI counts across all 8 IAV genes by myeloid cell cluster. Clusters with ≥5 cells with more than 2 PR8 UMIs have their cluster names bolded in blue.

(D) Heatmap depicting all differentially expressed genes between PR8 positive (≥2 PR8 UMIs) and bystander IFN-Stim MDMs from RM 8 dpi. Scaled Pearson residuals from scTransform are plotted.

(E) Mice (n = 4) were treated i.p. with control antibody (top) or anti-CCR2 antibody (bottom) and blood was collected 24 hours later for flow cytometry. Pre-gated on Dead–CD45+CD3–CD19–CD11b+.

(F) Representative gating scheme for Ly6C+ and Ly6C++ monocytes in the nasal mucosa.

(G) Cell counts for the experiment performed in **Figure 4G**; Welch’s t test, $^*p < 0.05$.

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Figure S6

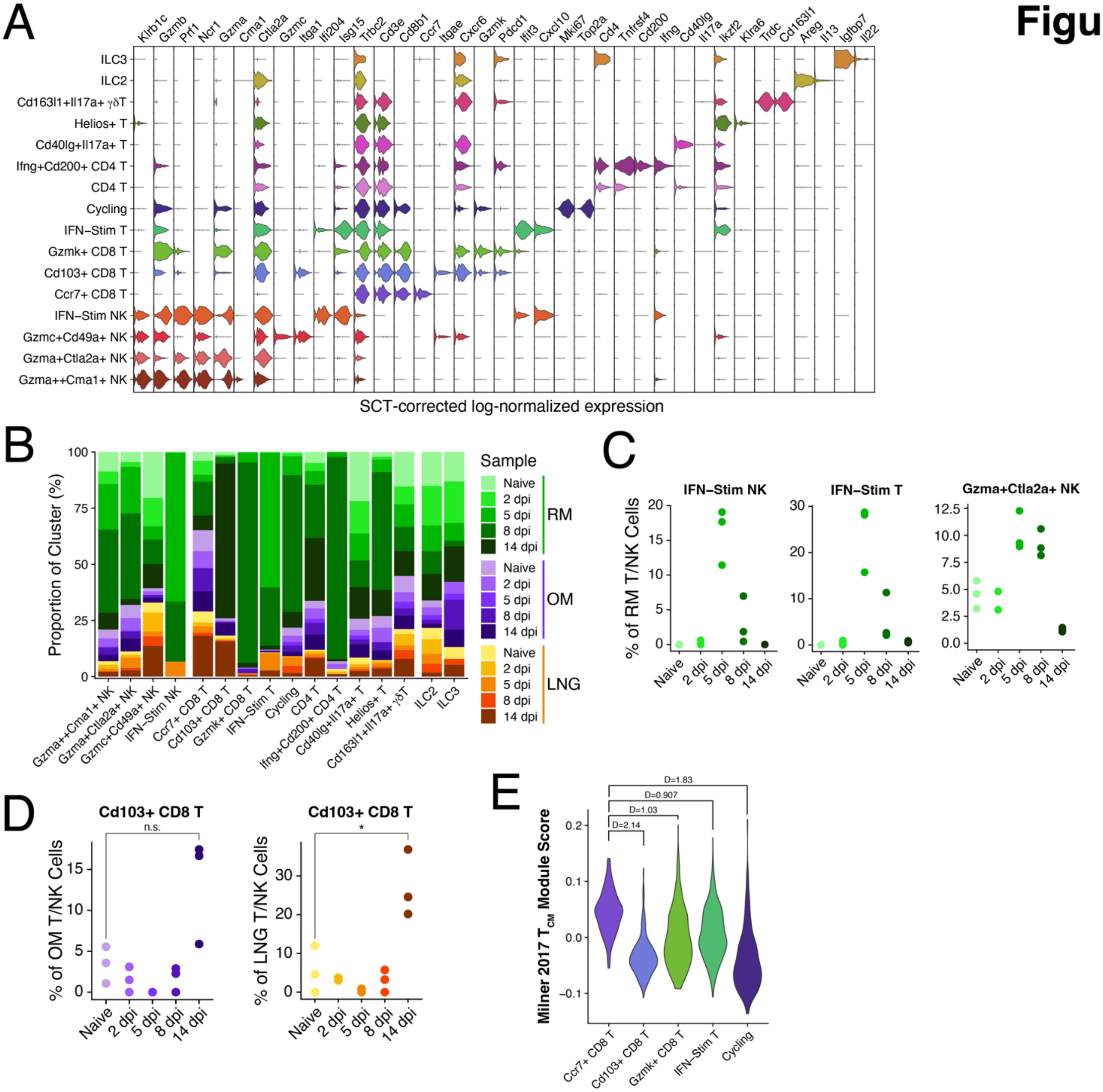

Figure S6: T cell, NK cell, and innate lymphocyte heterogeneity and T_{RM} responses

(A) Violin plots depicting differentially expressed marker genes (FDR<0.01) across all 16 T cell, NK cell, and innate lymphocyte cell clusters (see **Supplementary Table 1**).

(B) Stacked bar chart depicting the relative proportions of cells annotated for each myeloid cluster by region and time point.

(C) Relative frequencies of IFN-Stim NK cells (left), IFN-Stim T cells (middle), and *Gzma+Ctla2a+* NK cells (right) as a proportion of all T cells, NK cells, and innate lymphocytes per RM replicate sample.

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(D) Relative frequencies of *Cd103*+ CD8 T cells as a proportion of all T cells, NK cells, and innate lymphocytes per OM replicate sample (left) and LNG replicate sample (right).

(E) Violin plot depicting a gene module score derived from the universal T circulating memory (T_{CM}) cell signature as published in (Milner et al., 2017) across all CD8 T cell clusters. Cohen's D for effect size is reported between *Ccr7*+ CD8 T cells and each other cluster.

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Figure S7

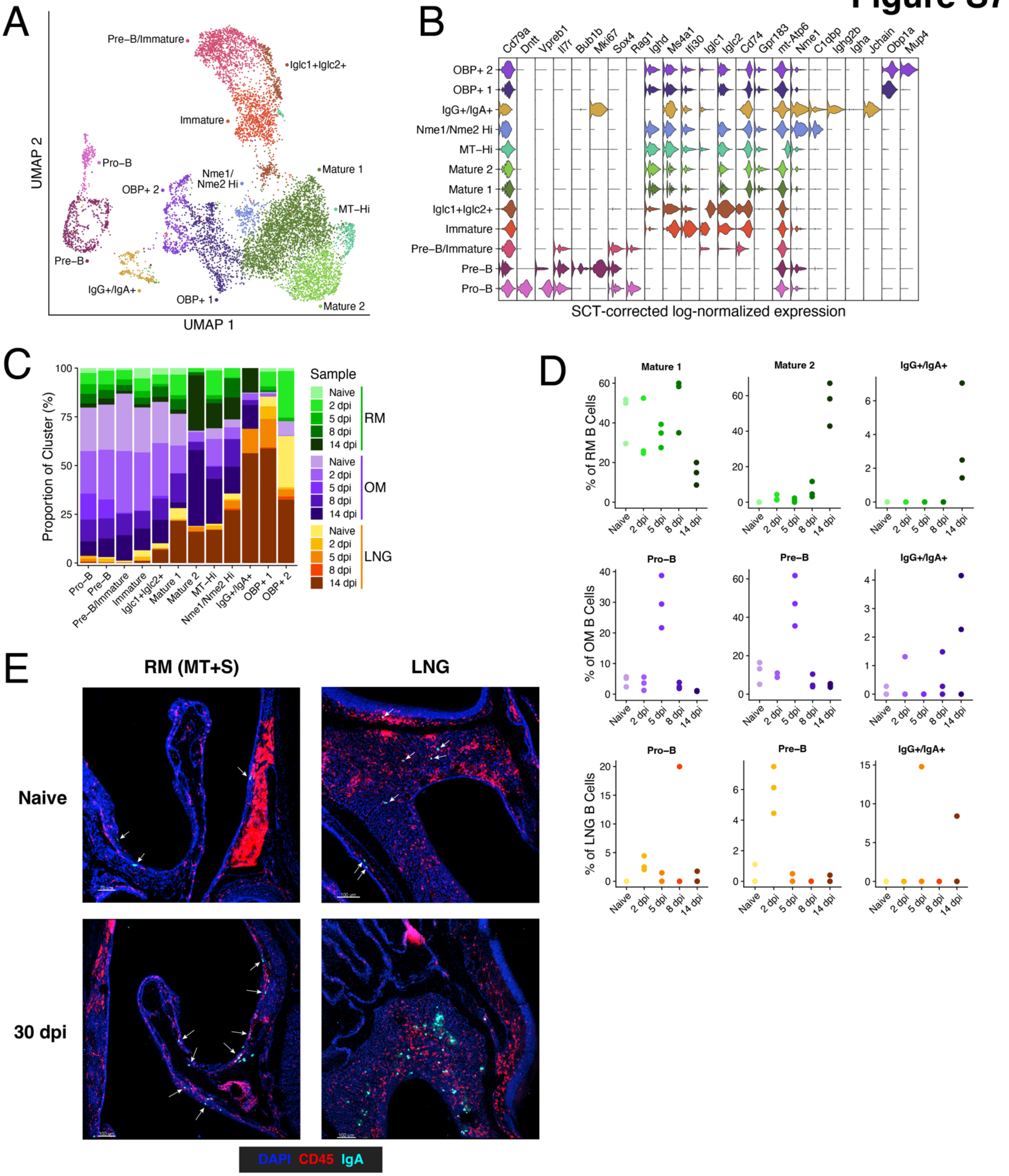

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Figure S7: B cell heterogeneity and cluster dynamics

(A) UMAP embedding of 10,167 B cells across 12 clusters.

(B) Violin plots depicting differentially expressed marker genes (FDR<0.01) across all 12 B cell clusters (see **Supplementary Table 1**).

(C) Stacked bar chart depicting the relative proportions of cells annotated for each B cell cluster by region and time point.

(D) Relative frequencies of several B cell clusters as proportions of all B cells per RM replicate sample (top), OM replicate sample (middle), and LNG replicate sample (bottom).

(E) Representative immunofluorescence images staining for IgA producing cells in the RM (left) and LNG (right) in naïve mice (top) and 30 dpi (bottom). White arrows point to IgA+ cells in the sparser images.

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Figure S8

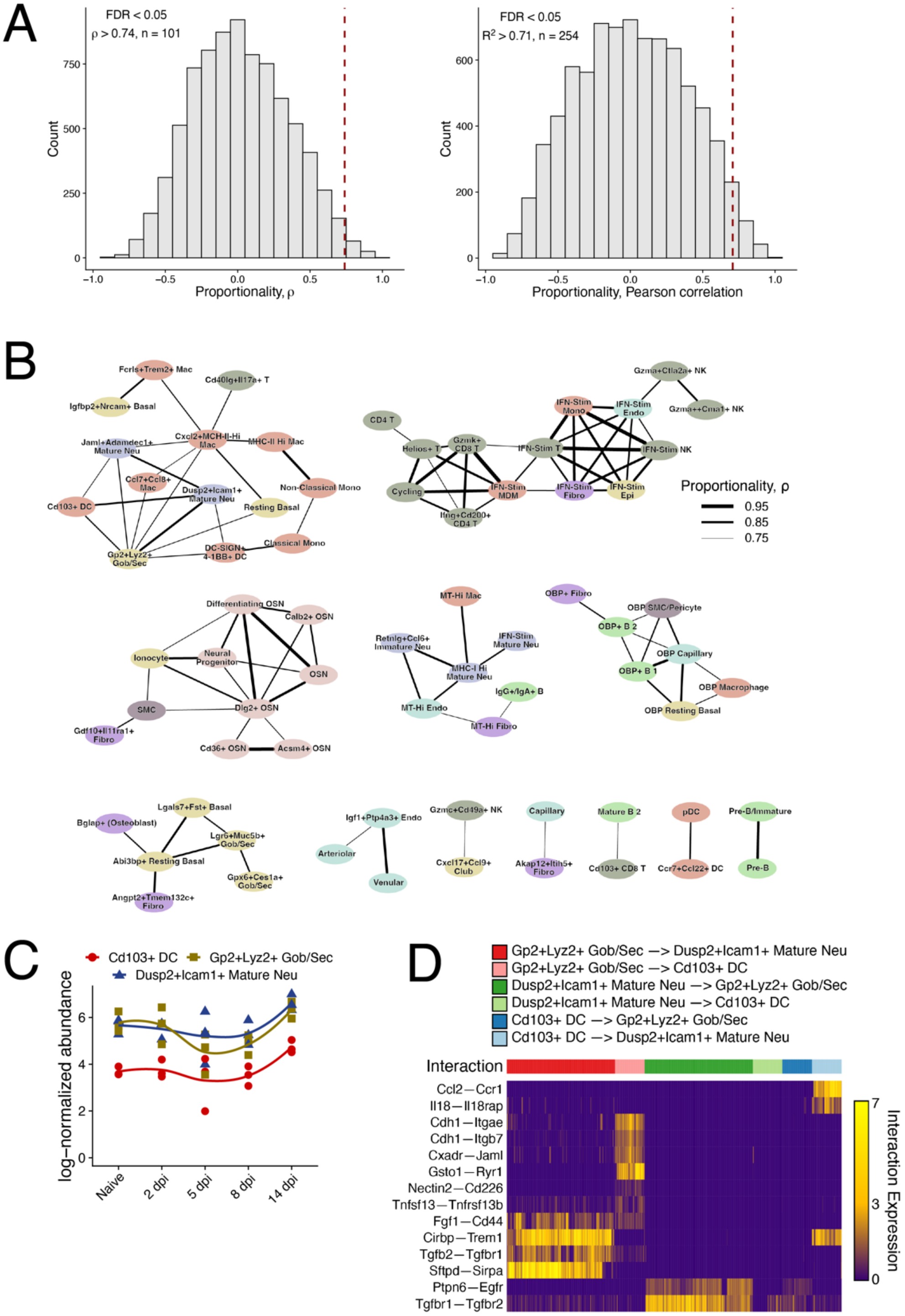

Figure S8: Proportionality and cell-cell communication analysis

(A) Histograms of calculated proportionality (left) and Pearson correlation (right) statistics across all RM sample replicates. The significance cutoff (FDR<0.05) for each statistic is marked by the red dashed line and was calculated from a background of 1000 permutations of the data.

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(B) Network of all significantly proportional (FDR<0.01) cell clusters across all RM replicate samples. Nodes are colored by cell type and edge weight is representative of proportionality.

(C) Abundance plot of *Cd103*+ DCs, *Gp2*+*Lyz2*+ Gob/Sec cells, and *Dusp2*+*Icam1*+ Mature Neutrophils in replicate RM samples. Smoothed lines are calculated using local polynomial regression fitting.

(D) Heatmap depicting a subset of differentially expressed receptor-ligand interaction pairs between single-cell pairs identified by NICHES (Raredon et al., 2023) for the clusters depicted in (C); ; see **Supplementary Table 2**. Interaction expression is the multiplicative expression of receptor and ligand gene expression for each member of a single-cell pair.

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Figure S9

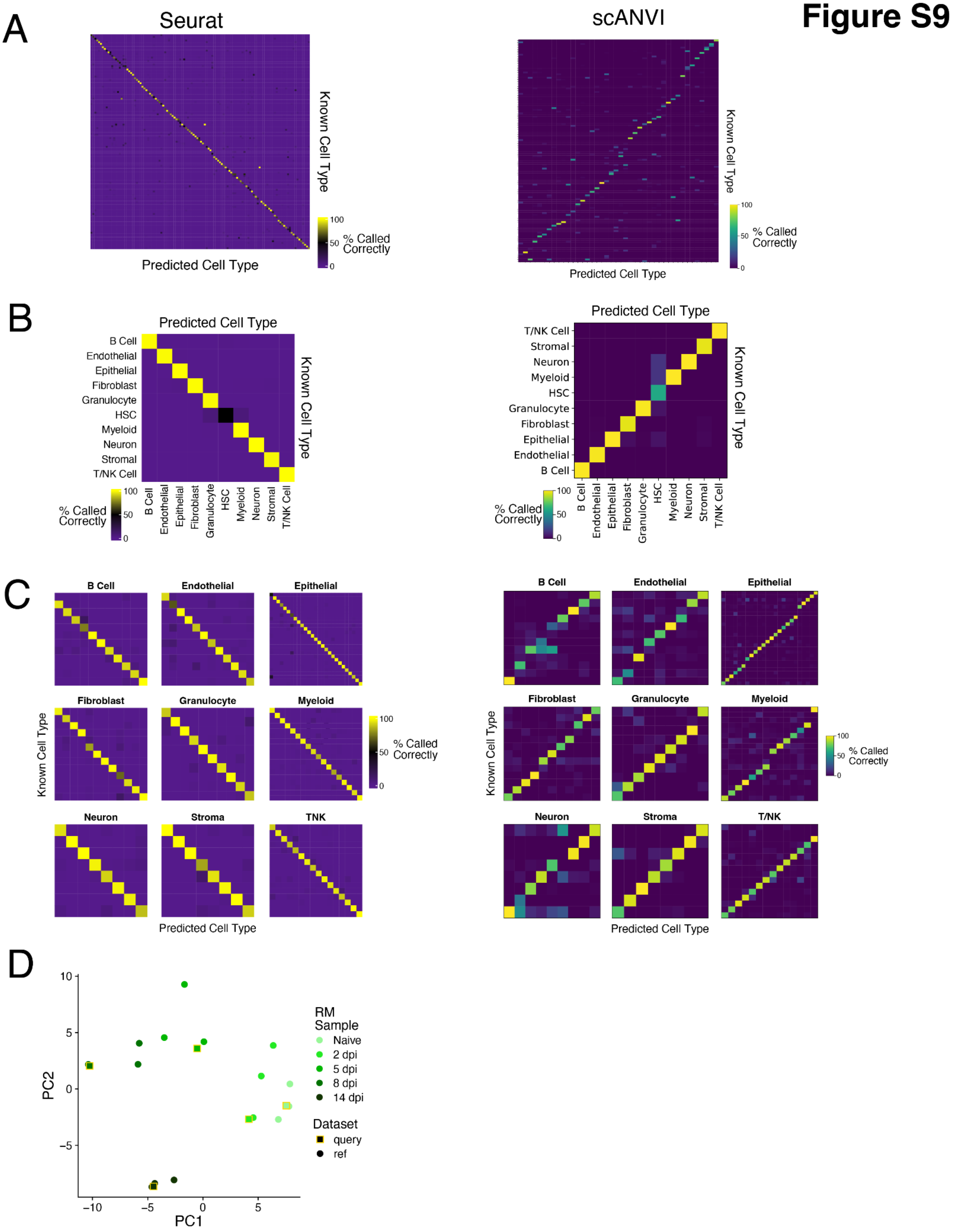

Figure S9: Validating label transfer methods to assign cell cluster identities to new nasal mucosa samples

One sample replicate per timepoint was separated from the RM dataset to be used as a query trained using a reference made from the remaining sample replicates and unclassified cells. The left and right side depict results from Seurat and scANVI respectively.

(A) Heatmaps depicting the per-cluster on-target prediction frequency when calculated across all 127 cluster labels.

(B) Heatmaps depicting the per-cell-type on-target prediction frequency when calculated across the 9 cell type labels.

(C) After predicting cell type labels, new query and reference pairs were generated within each cell type and label transfer was performed within each. Heatmaps depicting the per-cluster on-target prediction frequency when calculated within all clusters within each respective cell type.

(D) Compositional PCA from **Figure 2F** where the query sample replicates were projected using the predicted cell cluster labels.

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Figure S10

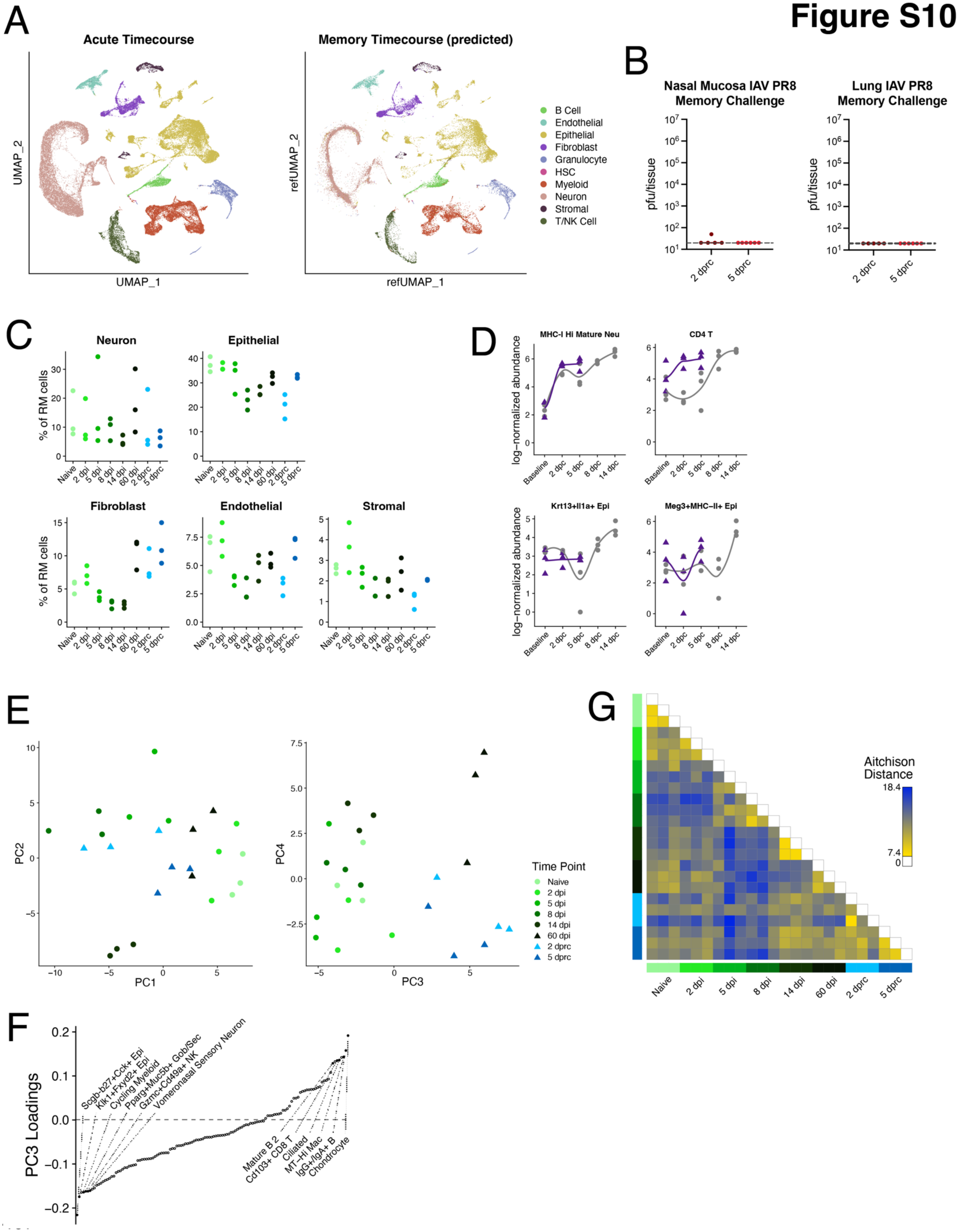

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Figure S10: Changes in RM composition following secondary challenge

(A) UMAP of all RM cells from the primary infection dataset (left) and the projected UMAP of all cells in the rechallenge dataset (right) colored by cell type.

(B) Infectious IAV PR8 quantification in pfu of the entire nasal mucosa (left) and lung (right) during IAV rechallenge.

(C) Relative frequencies of non-immune cell types as a proportion of all sequenced cells per RM replicate sample in primary infection and following rechallenge.

(D) Abundance plots of various clusters showing overlaid primary infection (gray) and rechallenge (purple) responses. Baseline refers to samples from naïve mice in primary infection and to samples from 60 dpi in rechallenge. dpc = days post challenge. Smoothed lines are calculated using local polynomial regression fitting.

(E) Compositional PCA recalculated using both primary infection and secondary challenge RM sample replicates. PCs 1-2 (left) and 3-4 (right).

(F) Cell cluster abundance loadings for PC3 (E). Cell cluster names for several of the most negative and most positive weights for each PC are depicted.

(G) Heatmap depicting all pairwise Aitchison distances between all RM sample replicates.

Supplementary Table 1: Differentially expressed genes across clusters within each cell type. Differential expression analysis was performed across cells from all samples within each cell type using the Wilcoxon Rank Sum test.

Supplementary Table 2: Differentially expressed receptor-ligand pairs identified by NICHES. Within each NICHES analysis, differential expression was performed across all cell-pairs at the specific timepoint of interest using the ROC test built into Seurat. See **Figure 6**.

Supplementary Table 3: Differentially expressed genes between primary infection and secondary challenge. Differential expression analysis ("bidomal" test) was performed between timepoints using cells from RM only within the specified clusters in **Figure 7E**.

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