Title: Lung structural cell dynamics are altered by influenza virus infection experience leading to rapid immune protection following viral re-challenge

Authors: Julie C. Worrell[1†*], Kerrie E. Hargrave[1], George E. Finney[1], Chris Hansell[1], John Cole[1], Jagtar Singh Nijjar[2], Fraser Morton[1], Marieke Pingen[1], Tom Purnell[1] Kathleen Mitchelson[1†], Euan Brennan[1], Jay Allan[3], Georgios Ilia[3], Vanessa Herder[3], Claire Kennedy Dietrich[4], Yoana Doncheva[4], Nigel B. Jamieson[4], Megan K.L MacLeod[1*]

Affiliation(s):

1.School of Infection and Immunity, University of Glasgow, Glasgow, UK.

Current Affiliation: † Conway Institute and School of Medicine, University College Dublin, Ireland.

2. Weatherden Ltd, London, UK

3. MRC-University of Glasgow Centre for Virus Research, Glasgow, UK

4. Institute of Cancer Sciences, University of Glasgow, Wolfson Wohl Cancer Research Center, University of Glasgow, UK

***Corresponding Authors:** Julie C. Worrell and Megan K.L. MacLeod

Correspondence: School of Infection and Immunity, University of Glasgow, B527, SGDB, 120 University Place, Glasgow, G12 8TA, UK.

Email: Julie.worrell@ucd.ie Megan.MacLeod@glasgow.ac.uk

Abstract

Lung structural cells, including epithelial cells and fibroblasts, form barriers against pathogens and trigger immune responses following infections such as influenza A virus. This response leads to the recruitment of innate and adaptive immune cells required for viral clearance. Some of these recruited cells remain within the lung following infection and contribute to enhanced viral control following subsequent infections. There is growing evidence that structural cells can also display long-term changes following infection or insults. Here we investigate long-term changes to mouse lung epithelial cells, fibroblasts, and endothelial cells following influenza virus infection and find that all three cell types maintain an imprint of the infection, particularly in genes associated with communication with T cells. Lung epithelial cells from IAV-infected mice display functional changes by more rapidly controlling influenza virus than cells from naïve animals. This rapid anti-viral response and increased expression of molecules required to communicate with T cells demonstrates sustained and enhanced functions following infection. These data suggest lung structural cells could be effective targets for vaccines to boost durable protective immunity.

Keywords: lung; epithelial cells; fibroblasts; T cells; influenza virus; SpiB, antigen presentation

Introduction

The lung is constantly exposed to microbes that can trigger immune responses. Respiratory viruses, including influenza A virus (IAV), are leading causes of disease worldwide[1,2]. The World Health Organization estimates annual epidemics result in approximately 3 to 5 million cases of severe illness and about 290,000 to 650,000 deaths.

Many different cell types are required to control the pathogens that enter the lung. These include structural cells, epithelial cells and stromal cells (fibroblast and endothelial cells); and immune cells that can be resident or recruited following the infection. The early responses to IAV by lung structural cells have been well characterized[3-8]. Epithelial cells are the primary cell type infected *in vivo* and are the cells most likely to produce new virus[9,10]. They, and nearby fibroblasts, trigger an early wave of inflammatory cytokines and chemokines that can help control the infection until dedicated immune cells migrate to the infection site[3-6]. Blood endothelial cells (BECs) play a key role in alerting circulating immune cells and recruiting them to the lung via chemokines[7,11].

IAV can be cleared within 7 to 10 days, but the consequences of the infection are more sustained. While epithelial cells and fibroblasts are involved in tissue repair, damage can persist for many months in mouse infection models[12-14]. Lung immune cells are also altered by infection: adaptive T and B cells can take up long-term residence in tissues as Tissue resident memory (Trm) cells and circulating monocytes can differentiate into alveolar macrophages. These cells can provide both pathogen specific and non-specific protection to future challenges and are considered arms of adaptive and innate memory[15-22].

Recently, evidence is growing that structural cells can also display long-term changes to infections[23,24]. In the context of IAV, surfactant producing club epithelial cells that survive IAV infection can produce a rapid anti-viral response following a second challenge which may contribute to tissue damage[25,26]. Other epithelial cells can also survive IAV infection by reducing communication with lung CD8 T cells[27]. However, an in-depth analysis of long-term changes by IAV to lung epithelial cells is lacking. Moreover, little is known about the long-term changes to other lung structural cells following influenza virus infection.

To address these questions, we used transcriptomic and flow cytometry analysis to investigate long-term changes to lung structural cells following primary infection and re-challenge of mice with

IAV. Our data reveal that, in addition to epithelial cells, fibroblasts and BECs maintain an imprint of the IAV infection. Our analysis demonstrates that antigen processing and presentation is a shared pathway upregulated in all three cell types for at least 40 days following infection and implicates the Ets family transcription factor, SpiB[27], in regulating these changes.

More rapid control of IAV is expected in re-challenge infections, even when different IAV strains are used in a heterosubtypic infection to avoid neutralizing antibodies[18,21,28,29]. We find that lung epithelial cells rapidly upregulate the expression of molecules required for communication with T cells and present antigen to IAV-specific CD4 and CD8 T cells *in vitro*. Despite this ability to communicate with lung T cells, *in vivo* rapid virus control within the first two days of a re-infection is independent of T cells. Instead, our data show that epithelial cells from previously infected mice can more rapidly control IAV *in vitro* independently of immune cells. Together, our data suggest that lung structural cells maintain an imprint of IAV infection that can enhance their ability to communicate with T cells and for epithelial cells, also bolsters their own immunity against subsequent infections.

Results

IAV infection causes prolonged up-regulation of immune-related genes in lung structural cells

To advance our understanding of the long-term consequences of IAV infection on lung structural cells, we used fluorescence activated cell sorting (FACS) to isolate three major cell types in the lung: epithelium, endothelium, and fibroblasts. We sorted the cells based on the expression of EpCAM1 (epithelial cells), CD31 (blood endothelial cells, BECs) and CD140a (fibroblasts) from mice at day 10 (up to viral clearance) and 40 post-infection (long after viral clearance) and compared their gene expression to cells sorted from naïve animals. The gating strategy, sort purities and Principal Component Analysis (PCA) are shown in Supp Fig 1. PCA demonstrated substantial changes at day 10 post infection for all cell types compared to naïve controls. We identified 996 differentially expressed genes (DEG) in epithelial cells (764 up, 232 down), 3128 DEG in fibroblasts (1772 up, 1356 down) and 1292 DEG in endothelial cells (968 up, 324 down) between day 10 IAV and naïve groups (Fig1A-C). Over representation analysis (ORA) detected enrichment in cell cycle in all three subsets and defense response to virus in epithelial cells and fibroblasts, Fig 1A-C.

At day 40 post infection, all three cell types continued to show differential gene expression compared to cells from naïve mice, although the number of DEG were reduced compared to day 10: epithelial cells: 144 up, 61 down; fibroblasts 159 up, 43 down; BECs: 58 up and 21 down (Fig 2A,B). Closer

inspection of overlapping upregulated DEG from our RNAseq datasets at day 10 and 40 post-infection, revealed that several genes remained persistently elevated with 80, 131 and 43 DEG in epithelial cells, fibroblasts, and BECs respectively (Fig 2C). BECs had the lowest number of genes that remained upregulated (43 genes), suggesting these cells may be more likely to return to homeostasis than epithelial cells or fibroblasts. Taken together, these data reveal sustained transcriptional alterations in immune related genes in lung epithelial cells and fibroblasts long after viral clearance.

We next performed a gene set enrichment network analysis on all structural cell types at both day 10 and 40 post infection (Fig 3A-C). This was performed as ORA can enrich for highly similar genes and may only represent a portion of the enriched biology. Network analysis revealed conserved upregulation of gene networks governing shared biological themes in all three cell types, including antigen processing and presentation at day 40 post IAV infection. These findings indicate that IAV infection leads to long-term changes in the expression of immune-related genes in lung structural cells.

Expression of SpiB in airway epithelial cells found near immune cell clusters is dependent on viral replication

Analysis of the upstream regulators of the DEG identified at day 10 and 40 in epithelial cells and fibroblasts indicated a number of potential upstream regulators including ETS1, NFκB1 and CITED2 (Supp Fig 2). These molecules are downstream of Interferon (IFN)γ, CD40L and IFNα which can be produced by multiple cell types during IAV infection including epithelial cells, T cells and NK cells[30]. Interestingly, both epithelial cells and fibroblasts had significantly elevated gene expression levels of the ETS transcription family member *SpiB* at day 40 post infection and we confirmed increased expression by qPCR (Supp Figure 2B). SpiB can co-ordinate differentiation and chemokine production by splenic fibroblasts following viral insult (LCMV)[31].

To investigate the potential contribution of SpiB to the transcriptional changes in lung structural cells following IAV infection, we compared the persistently upregulated DEG identified in our RNAseq analysis with genes identified as SpiB targets using a mouse ChIP-seq data set[32]. In epithelial cells, 25% of persistently upregulated DEG following IAV infection were SpiB target genes (20/80), in fibroblasts SpiB target genes (22/131) accounted for 17% of DEG (Fig 4A). 27% of the SpiB targets induced by IAV infection were conserved between lung epithelial cells and fibroblasts (9/33): *H2-Q4*, *H2-Q5*, *H2-Q7, Psmb8*, *H2-K1*, *H2-Q6*, *SpiB*, *H2-Ab1*, *Psmb9* (Fig 4B). These data indicate that structural cells share an imprint of previous infection and SpiB may be a marker of this infection experience.

To determine the location of transcriptionally altered epithelial cells and fibroblasts, we performed RNAscope on paraffin embedded lung tissue from naïve animals or mice infected 10 or 40 days previously with IAV. We found clusters of B220+ B cells that were SpiB+ cells in the parenchyma, as described by ourselves and others (Supp Fig3)[13,17,33-36]. SpiB+ epithelial cells were rare in the airway epithelium of naïve animals but were increased in SpiB+ structural cells in infected animals, but only present in areas of the lung close to clusters of immune cells (Fig4C).

This link in location between infection altered epithelial cells and immune clusters could indicate sites of viral replication during the initial infection. To ask if viral replication was required for SpiB expression, we infected mice with a modified IAV virus, S-FLU, which, due to a mutation in the vRNA encoding HA, can only undergo one round of replication in host cells[37]. Despite this reduced replication, S-FLU does induce systemic and local adaptive immune responses[37,38]. *SpiB* expression was not detected in lung epithelial cells (or fibroblasts) in animals infected with S-FLU 30 days previously (Fig4D). These mice did not display overt clinical signs of infection, with no reduction in total body weight following infection with S-FLU as previously reported[37] (Supp Fig 4A). At 30 days post infection lungs from mice infected with S-FLU had reduced levels of inflammatory infiltrate compared to those infected with the WT-IAV strain (Supp Fig 4B) shown by H&E staining (Supp Fig 4C). Viral replication was also necessary for the formation of immune cell clusters. These mice lacked B220+ and CD4+ cell containing clusters, compared to IAV-X31 infected mice (Supp Fig 4D). Taken together, our findings indicate that viral replication is necessary for SpiB expression by lung epithelial cells.

IAV infection alters the number and MHCII expression of epithelial cell subsets

The lung contains multiple subsets of epithelial cells that are specialized for different roles and that respond distinctly to IAV infection[3,26,39]. We used multi-parameter flow cytometry to investigate the consequences of IAV on lung epithelial cell subsets. To explore which populations express SpiB we generated SpiB reporter mice. In these transgenic (Tg) animals the sequence for a 2A peptide (T2A[40]) and the fluorescent molecule, mCherry were inserted into the *SpiB* locus. As expected B cells[41] and plasmacytoid DCs[42] were positive for the reporter (Supp Fig 5A).

In naïve mice, SpiB was expressed by multiple cell types including those of the upper airway: ciliated (CD24hiSca1hi), club (CD24+Sca1+), progenitor cells (CD24+Sca1neg), Sca1+ progenitor cells (Sca1+CD24neg), and alveolar epithelial cells (ATI: CD24negSca1negPdpn+ and ATII: CD24negSca1negPdpnnegMHCII+) from the distal lung (Fig 5A). Gating strategy is shown in Supp Fig5B and based on previous studies[43,44]. In comparison to SpiB negative EpCAM1+ cells, the SpiB+ population was more likely to contain ATII cells but fewer ATI, club cells and CD24+ and Sca1+ progenitor cells (Fig5A).

As SpiB is known to regulate molecules involved in MHCII antigen processing and presentation[32] we examined the expression of MHCII on the epithelial cell populations, excluding ATI cells that are defined as MHCII negative. For all the populations, SpiB+ cells were more likely to be MHCII+ than SpiB negative populations (Fig5B).

IAV infection led to a decline of CD24 progenitor cells and SpiB+ ATII cells at day 10 (Fig5C). In contrast, Sca1+ SpiB+ progenitors more than doubled at day 10 post-infection. This increase may reflect that Sca1+ progenitor cells can give rise to both bronchiolar and alveolar cells to repair the damaged epithelium[45]. All populations had returned to naïve levels by day 30 apart from a small increase in the number of SpiB+ club cells.

Infection led to an increased proportion of SpiB negative and positive ciliated cells that were MHCII+ and this was sustained at day 30, although at a lower level than at day 10 post-infection (Fig 5D). The amount of MHCII expressed by MHCII+ SpiB+ epithelial cells subsets was measured *via* MHCII geometric MFI on each cell population (Supp Fig 6). SpiB negative and positive cells that were MHCII+ expressed broadly similar levels of MHCII as measured by MFI. SpiB negative and positive Sca1+ progenitor cells expressed higher levels of MHCII at day 10 but not day 30 post-infection compared to cells from naïve mice. In contrast, SpiB+ club cells expressed increased levels of MHCII at day 10 and these levels were still increased at day 30 post-infection. We also found small but significant increases of MHCII on CD24+ progenitor and ATII populations.

Together, these data show that SpiB+ epithelial cells form a large proportion of the upper and distal airway epithelium, are generally more likely to express MHCII than SpiB negative cells, and that multiple epithelial cell populations alter their expression of MHCII following IAV infection.

Epithelial cells from IAV re-challenged mice have less virus than those from primary infected animals

We next investigated the consequences of a second IAV infection on lung epithelial cells and fibroblasts. To reduce the impact of neutralizing antibody responses, we infected mice with two different strains of IAV, WSN in the first infection and X31 in the re-challenge. We compared the response in the re-challenged mice with a primary X31 infection in age-matched naïve animals.

To identify infected epithelial cells and fibroblasts by flow cytometry we used a fluorescently labeled anti-IAV nucleoprotein (NP) antibody (Fig 6A). Mice infected 30 days earlier with WSN had reduced percentages and numbers of IAV-NP+ epithelial cells two days after infection with IAV-X31 compared to infected naïve mice (Fig 6A).

To examine this difference in more detail, we gated on the epithelial cell subsets and examined the percentages of these cells that were IAV-NP+ (Fig 6B). In primary infected animals, ciliated and Sca1+ progenitor cells were the most likely to be infected, followed by club cells. Very few CD24+ progenitor and ATII cells were infected and too few ATI cells were detected to examine. Prior infection led to reduced IAV-NP in ciliated, club, and a small decline in ATII cells but no differences in the progenitor cells.

Few fibroblasts (CD140a+) were IAV-NP+ after IAV infection and there were too few cells detected to examine this further. Interferon-responsive[3] fibroblasts were reduced in re-infected mice compared to primary infection (Figure 6C-D). These data highlight that while fibroblasts are much less likely to be infected, they are susceptible to local microenvironmental changes.

To determine if the reduction in IAV-NP levels by lung structural cells could be explained by enhanced/rapid phagocytosis of the virus by immune cells, IAV-NP levels were measured in lung myeloid and B cells. We found no evidence for this, all cells contained equivalent levels of NP apart from conventional dendritic cells 1 (cDC1) which contained less NP in the re-infected compared to the primary infected animals, Supp Fig 7.

Re-challenge with IAV leads to rapid up regulation of genes involved in communication with T cells by lung epithelial cells

To investigate the consequences of re-infection in more depth, we compared gene expression in FACS sorted EpCAM1+ cells 2 days after a primary or re-challenge infection. EpCAM1+ cells from re-infected mice had increased expression of molecules in class I and II antigen presentation pathways (*B2m*, *H2-K1, H2-DMB2*, *Ciita*) and virus detection (*Nlrc5*) (Figure 7A-B). Detection of IAV-NP+ cells require fixation making it difficult to investigate gene expression in FACS sorted cells. Therefore, we investigated whether gene expression is different in infected cells in primary infected and re-infected animals by combining RNAscope with GeoMx spatial transcriptomics. First, we identified IAV-infected airways in primary and re-infected animals using a probe for IAV-X31 (Figure 7C). We examined gene expression in virus+ airway areas, and virus negative areas either in the same airway as the infected cells or from adjacent airways. We also included airways from sections of the lungs which did not contain any virus taken from primary and re-infected animals.

As expected, in primary infected mice, a comparison of gene expression in virus+ areas versus airways from non-infected sections demonstrated an upregulation of anti-viral genes including *Cxcl10*, *Isg15* and *Irf7* and upregulation of pathways related to 'defense to viruses' and 'type 1 IFN', Supp Fig 8A. An

anti-viral response was also evident in virus negative areas within infected airways and adjacent virus negative airways.

Only three of the five re-infected mice that we analyzed contained virus+ areas and we found fewer virus+ airways in these three mice compared to the primary infected mice. However, similar to the primary infected mice, in re-infected animals, genes within 'type 1 IFN' and 'response to virus pathways' were upregulated compared to airway cells from non-infected sections from the same mice, Supp Fig 8B. To compare the responses between primary and re-infected animals, we performed two types of analysis. First, we counted the number of upregulated genes within the Gene Ontogeny (GO) terms 'Inflammation' and 'Type 1 IFN' identified in virus+, virus negative areas within virus+ airways (close), and virus negative adjacent airways (further) and calculated these as a percentage of the total DEG within each area (Fig 7E).

All three areas had genes within both GO terms in primary infected animals, although 'further' areas had the lowest number. In contrast, in re-infected animals while genes within GO term 'Inflammation' were upregulated in virus+ and 'close' airways, no genes in this term were found in 'further' areas and no genes in GO term 'Type 1 IFN' were found in 'close' or 'further' areas in re-infected animals. These data suggest that while IAV may induce inflammatory type 1 IFN responses in infected cells primary and re-infected mice, the response is much more contained in re-infected animals.

Second, we compared gene expression between virus+ airways taken from primary or re-infected animals. We found increased expression of genes in the GO term 'Cellular Response to IFNγ' including *Ccl5* and *Cxcl9* (Fig 7E). These data suggest that IFN-γ producing immune cells, including CD4 and CD8 T cells[46] act on infected cells that in turn upregulate molecules that enhance communication between immune and infected structural cells.

Infection experienced structural cells more effectively control IAV than cells from naïve mice

The potentially increased communication between infected airway cells and T cells indicated that at this early timepoint following infection, the enhanced protection provided by a prior IAV-infection will be dependent on T cells. To test this, we infected two cohorts of C57BL/6 mice with IAV-WSN and treated one cohort with IgG and the second with depleting CD4 and CD8 antibodies (Fig 8A). Mice were treated 2 days prior and 2 days following a second infection with X31; control experiments demonstrated a loss of CD4 and CD8 T cells in lymphoid organs and the lung at day 2 post-re-infection (Fig 8B). In data consistent with Epstein *et al*[28]., we found that mice treated with IgG and T cell depleting antibodies had less virus in their lungs compared to primary infected animals (Fig 8C). Moreover, there was no difference in levels of virus between the two re-infected groups. These data

suggest that T cells are not required for enhanced protection at day 2 post-infection and that, potentially, enhanced viral control responses are from the infected cells themselves.

We chose to use a reductionist *in vitro* model to investigate this finding further, reducing the complexity of a rapidly changing response *in vivo* (Fig 9A). First, we asked whether the prior infection altered the ability of the structural cells to present antigen to CD4 and CD8 T cells. To achieve this, we isolated CD45 negative cells from naïve and day 30 IAV-WSN infected animals and rested these overnight before infecting them with IAV-X31 *in vitro*. The following day, T cells were isolated from the spleens of mice infected with IAV (X31; day 9/10 post infection) to generate a pool of IAV specific CD4 and CD8 T cells. These cells were co-cultured with the infected CD45negative cells and expression of T cell activation markers, CD25 and CD69, was analyzed by flow cytometry after a further 24 hours.

CD4 and CD8 T cells co-cultured with *in vitro* IAV-infected CD45negative cells from either naïve mice or previously infected mice expressed higher levels of CD25 and CD69 than T cells co-cultured with control CD45 negative cells that did not receive IAV *in vitro*. This demonstrates that the structural cells could process and present IAV antigens to CD4 and CD8 T cells (Figure 9B and Supp Fig 9).

We expected that CD45negative cells from previously infected animals might present antigens more effectively to T cells given their increased expression of molecules involved in these processes. Expression of CD25 and CD69 were equivalent between CD4 T cells cultured with IAV-infected structural cells taken from naïve or IAV-infected mice. There was a slight but significantly lower expression of CD25 and CD69 on CD8 T cells cultured with IAV-X31 infected structural cells from previously infected animals compared to X31-infected structural cells from naïve mice.

Potentially, prior infection enabled the CD45negative cells to control the virus more effectively and thus these cells might contain lower levels of antigen. To test this, we harvested a separate plate of structural cells 24 hours after *in vitro* infection and examined the levels of IAV-NP in epithelial cells, fibroblasts and BECs by flow cytometry. Few BECs were infected with IAV, in contrast, clear populations of fibroblasts and epithelial cells were IAV-NP+ (Fig 9C). Despite the low percentage of IAV-NP+ cells in the BECs, cells from previously infected animals were less likely to be IAV-NP+ than cells from naïve animals. This distinction was not found in fibroblasts, but epithelial cells from previously infected mice had significantly less virus than cells from naïve animals. These data suggest a prior IAV infection enhances the ability of epithelial cells to control a subsequent challenge.

To ask whether this reduced infection led to a lower inflammatory response, we examined culture supernatants for the presence of cytokines and chemokines 24h after the *in vitro* infection using a multiplex array. The supernatants from CD45negative cells isolated from previously infected mice,

contained higher levels of IL-1α, IL-12 p40 and p70, IL-13, IL-15, and CXCL9 (Fig 9D). Type I and III IFN were not detectable by ELISA (below the limit of detection; 31.3 pg/ml). In conclusion, these data suggest that the rapid control of virus by the structural cells is accompanied by an increased inflammatory cytokine response, perhaps to warn and attract immune cells to control any virus not contained by the epithelial cells themselves.

Discussion

Our functional and geographical analysis of the post-IAV lung demonstrates substantial and sustained changes to the three main structural lung cell types, epithelial cells, fibroblasts, and endothelial cells. All three cell types maintain upregulated expression of genes involved in antigen processing and presentation, suggesting that they may have an enhanced ability to communicate with T cells. While interactions between T cells and lung structural cells may be important[47,48], we found that lung epithelial cells from mice previously infected with IAV can themselves control IAV more efficiently than cells from naïve mice. These data demonstrate enhanced protective responses by epithelial cells indicating these cells display protective immune memory.

Evidence for innate or trained immunity in multiple cell types has been growing over the last ten years[8,24,49]. Skin epithelial cells previously exposed to inflammatory stimuli respond more rapidly through an inflammasome-dependent mechanism[50] and lung epithelial cells that survive an IAV infection have an altered gene expression and inflammatory response following re-infection[25-27]. Fibroblasts are known to play important roles in lung anti-viral responses and in other organs, including synovial fibroblasts in joints and gingival fibroblasts, can display evidence of memory to previous insults[49,51-54]. Most of the evidence for sustained changes in BECs come from the field of cardiovascular disease where chronic inflammation may sustain altered responses[49,55-57]. Our data extended the field by examining all three cell types within one organ, demonstrating a shared sustained response in all cell types suggestive of coordinated tissue memory.

Epithelial cells, most notably ATII cells can express MHCII[47,48,58]. Antigen presentation by lung epithelial cells is required for normal responses to *Streptococcus pneumoniae* and Sendai virus infections in mice[47,48,58]. We found that IAV infection led to upregulation of MHCII either by the proportion of cells that were MHCII+ or increased expression on upper (ciliated, club), progenitor (CD24+, Sca1+), and lower airway (ATII) epithelial cells at day 10 post-infection. Some of these changes, including more ciliated cells expressing MHCII, persisted at day 30 post-infection. As ciliated cells can live for months[59], this suggests these may persist from the infection, while other changes may be sustained by the progenitor epithelial cells.

We bioinformatically identified the transcription factor, SpiB, as a potential upstream regulator of the altered MHCII expression and upregulation of gene expression in multiple molecules involved in antigen processing and presentation[32]. In addition to B cells, SpiB is expressed by plasmacytoid dendritic cells[60], Micro-fold cells[60], tuft cells, secondary lymphoid organs[61], and some thymic epithelial cells[62]. Our data suggest that it is also expressed by multiple populations of lung epithelial cells and that SpiB+ positive cells express higher levels of MHCII. Recently, two studies identified SpiB+ epithelial cells in distinct anatomical locations. Surve *et al*., performed re-analysis of publicly available single -cell RNA sequencing data and detected rare M cells in the homeostatic mouse trachea [63]. While Barr *et al*., used single-nucleus RNAseq to detect lung M cells expressing *Tnfaip2*, *Sox8*, *Spib*, *Ccl9*, *Ccl20*, and *Tnfrsf11a* [64].

We showed that following IAV infection, SpiB+ upper airway epithelial cells were likely to be close to persistent cluster of immune cells. These observations are consistent with the study by Barr *et al*., where SpiB+ cells in the IAV infected lung persisted from day 5 to 56 post infection[64]. However, our study provides additional insight by validating this at the protein level and by examining MHCII expressing in naïve and infected animals. We and others have characterized these clusters which predominately contain B and T cells[13,17,33-36]. We showed that following IAV, SpiB+ upper airway epithelial cells were likely to be close to persistent clusters of immune cells. The close proximity of these immune cells suggests that they may release molecules to sustain the altered epithelial cells, and/or their proximity evidences the past location of viral replication.

A classic characteristic of immune memory is the ability to respond rapidly to re-infection[65]. By measuring gene expression and developing an *in vitro* infection model, we demonstrated that lung epithelial cells and fibroblasts display an enhanced response to re-infection and that, for epithelial cells, this leads to more rapid control of IAV. This enhanced functional response was accompanied by increased production of cytokines. Krausgruber *et al.,* found that even in naïve animals, structural cells are poised, ready to produce molecules more often associated with immune cells[66]. Our data extend this finding, suggesting that exposure to pathogens boosts this homeostatic immune gene expression making structural cells an even more important component to protective immunity. Our study also provides rationale for attempting to target structural protective immune potential as part of vaccine design, for example, by adjuvants. This concept was investigated in a recent study by Denton *et al*., who found that targeting TLR4 using adjuvants improved the MAdCAM-1 + stromal cell response to immunization and facilitated longer term protection post vaccination[67].

Our data show that early protection to re-infection was independent of T cells, but using plaque assays and RNAscope, virus is still present at day 2 following the re-challenge infection. Both CD4 and CD8 T cells protect mice and humans from IAV re-infection, especially when priming and re-challenge strains express different hemagglutinin and neuraminidase proteins[18,20,21,28,29,68,69]. The sustained expression of inflammatory chemokines and molecules involved in the processing and presentation of antigen following infection suggests that lung structural cells will have an enhanced ability to attract and communicate with local T cells. In summary, therefore, our data suggests that lung epithelial cells may play both early T cell independent and then later T cell dependent roles in accelerating viral clearance following re-infection.

Materials and Methods

Animals

SpiB-mCherry reporter mice were generated at MRC Harwell and bred at the university of Glasgow. The sequence for a 2A peptide (T2A[40]) with a GSG linker and the fluorescent molecule, mCherry, was inserted into the SpiB locus. Ensembl Version http://www.ensembl.org/Mus_musculus/Gene/Summary?db=core;g=ENSMUSG00000008193;r=7:44175417-44181495. The knock in was inserted at the end of the coding sequence. SpiB reporter mice were viable, fertile and born at expected Mendelian ratios.

Ten-week-old female C57BL/6 mice were purchased from Envigo (United Kingdom) and male and female SpiB reporter mice were bred at the University of Glasgow. SpiB and C57BL/6 mice were maintained at the University of Glasgow under specific pathogen-free conditions in accordance with UK home office regulations (Project Licenses P2F28B003 and PP1902420) and approved by the local ethics committee. No formal randomization was performed, and sample size was based on previous studies.

IAV infections

SpiB or C57BL/6 mice were briefly anesthetized using inhaled isoflurane and infected with 100–200 plaque-forming units (PFU) of IAV WSN strain or 200PFU IAV-X31 strain in 20 µL of phosphate-buffered saline (PBS) i.n. depending on their age, weight, and sex. IAV was prepared and titered in Madin-Darby Canine Kidney cells (MDCK). Infected mice were weighed daily from day 4 post-infection. Any animals that lost more than 20% of their starting weight were humanely euthanized. Cages with mice that lost weight were given soft diet until all mice returned to their starting weight or above.

S FLU virus

S-FLU virus[37] was obtained from the laboratory of Alain Townsend at the university of Oxford. C57BL/6 were infected with 5 HAU (hemagglutinating units) either the parental strain of the virus IAV-WT X31 or the S-FLU-X31.

T cell depletion experiment

Two cohorts of C57BL/6 mice were infected with IAV-WSN and one cohort injected with 400µg of rat IgG (LTF-2) and the second with depleting 200µg CD4 (GK1.5) and 200µg CD8 (2.43) antibodies. Mice were treated intraperitoneally with anti-CD4/CD8 2 days prior to re-challenge with IAV-X31 and 2 days following infection. Mice were culled 2 days post re-challenge. Control IgG and anti-CD4/CD8

treatments were given to mice within the same cages to prevent cage-specific effects acting as confounders.

Plaque assay on mouse lung homogenates

Once euthanized, mouse lungs were extracted and frozen at −80 °C. Lungs were thawed, weighed and homogenized with 1 ml of DMEM. Homogenates were cleared by centrifugation (3,000 r.p.m., 4 °C) and titrated by plaque assay. Confluent monolayers of MDCK cells (1×10^6 cells seeded the day before infection in six-well plates,) were washed once with PBS, infected with serial dilutions of virus and incubated for 1 h at 37 °C to allow virus adsorption to the cells. After virus removal, cells were overlaid with MEM with oxoid agar and incubated for 2 days at 37°C, 5% CO2. After removing the overlay, cells were fixed with PBS:4% formaldehyde and stained with a 0.08% Crystal Violet solution in 6.25% methanol solution for at least 1 h. Staining solution was rinsed under tap water, plates were air-dried, plaques were then counted.

Tissue preparation

Mice were euthanized by cervical dislocation (RNA-sequencing, FACS sorting and flow cytometry analysis) or alternatively with a rising concentration of carbon dioxide with PBS-5mM EDTA to remove blood cells from the lungs (lung histology and imaging). Single cell suspensions of lungs were prepared by enzymatic digestion with a final concentration of 1.6 mg/mL Dispase, 0.2 mg/mL collagenase P (Roche, UK) and 0.1 mg/mL DNase (Sigma, UK) for 40 minutes at 37°C in a shaking incubator and tissues disrupted by passing through a 100µm filter. Red blood cells were lysed with lysis buffer (ThermoFisher).

For analysis of CD4 and CD8 T cells by flow cytometry prior to euthanasia by cervical dislocation, mice were injected intravenously with 1µg AF488-conjugated anti-CD45 (clone, 30F11, ThermoFisher) and organs harvested after 3 minutes. Single-cell suspensions of lungs were prepared by digestion of snipped lung tissue with 1 mg/mL collagenase and 30µg/mL DNase (Sigma) for 40 minutes at 37 °C in a shaking incubator and tissues disrupted by passing through a 100µm filter. Spleens and lymph nodes were processed by mechanical disruption. Red blood cells were lysed from spleen and lungs with lysis buffer (ThermoFisher). Cells were counted using a hemocytometer with dead cells excluded using Trypan Blue.

Lung digestion and isolation of mouse lung structural cells by Fluorescence-Activated Cell sorting

For fluorescence activated cell sorting, single cell suspensions were generated as follows: enzymatic digestion with a final concentration of 0.4mg/mL Collagenase P, 0.2mg/mL Dnase-1 and 3.2mg/mL

Dispase-II. Lung samples were incubated at 37°C for 20 minutes. Red blood cells were lysed using RBC buffer (ThermoFisher). Cells were simultaneously stained with CD45 microbeads (Miltenyi Biotec) and antibodies: anti-CD45 FITC (Biolegend, clone:30-F11), anti-CD31 PE (BioLegend, MEC13.3), CD326/Epcam-1 PerCP Cy5.5 (Biolegend, G8.8), CD140a/Pdgfra APC (Biolegend, APA5). Hematopoietic cells were depleted using the LS MACS (Miltenyi Biotec) column as per manufacturer's instructions. CD45 depleted lung cells were stained with eFlour 780 viability stain (Thermofisher), and cells sorted on BD FACS Aria IIu sorter. Cells were either sorted directly into RLT or first sorted into 50% FCS/RPMI, washed with PBS and then lysed in RLT buffer.

RNA extraction and qPCR

RLT suspensions were centrifuged through Qiagen QIA-Shredders and RNA was extracted from sorted cells using the RNeasy mini kit (Qiagen,UK) with the following changes to manufacturer's instruction: input to the RNeasy column was at a ratio of 100µL sorted cells/MACS buffer: 350µL RLT: 200µL 100% EtOH. Following extraction samples were DNase-1 treated and purified using an RNA Concentration and Clean-Up kit as per manufacturer's instruction (Zymo, Cambridge Biosciences, R1013). The integrity and quantity of total RNA were determined using Agilent 2100 Bioanalyzer (Agilent Technologies), samples with a RIN value of ≥8 were included for downstream analysis. For qPCR analysis, briefly, the first strand cDNA synthesis was carried out by using a Reverse Transcription System kit according to the instructions of the manufacturer (Promega, Madison, WI) and analyzed by qPCR (SYBR Green FastMix (Quanta Bioscience). Spib and Gapdh standards were generated from spleen cells from IAV-infected mice (Standard Primers: SpiB Forward: TTGCTCTGGAGGCTGCA; Reverse: GGTGAGTTTGCGTTTGACCT; Gapdh Forward: AAGGGCATCTTGGGCTACAC; Reverse: TAAAGTTCGCTGCACCCACA) and purified by gel extraction (Quick Gel Extraction kit, Invitrogen). qPCR was performed on a QuantStudio 7 flex and expression was calculated using standard curves and results normalized to gapdh expression (qPCR Primers: Spib Forward: GATGGCCCACACTTAAGCTG; Reverse: AGCAGGATCGAAGGCTTCAT; Gapdh Forward: AACTCCCACTCTTCCACCTTC; Reverse: AAGGAGTAAGAAACCCTGGACC).

RNA sequencing

Samples were pooled from 6-8 mice in each group. Cells were sorted into 50% FCS/RMPI, centrifuged and washed x1 with PBS. Supernatant removed and 350µl of RLT added. Samples were centrifuged through a Qiashredder and samples were stored at -80C. RNA was extracted using mini or micro-RNA kits as per manufacturer's instruction. RNA was purified from total RNA (100ng) using poly-T oligo-attached magnetic beads (Life Technologies, CA, USA). Sequencing libraries were generated using NEB Next Ultra Directional RNA Library Prep Kit for Illumina (NEB) in accordance with the

manufacturer's recommendations. Products were purified and quantified using the Agilent high sensitivity DNA assay on the Agilent Bioanalyzer 2100 system. After the clustering of the index-coded samples, each library preparation was sequenced on an Illumina NextSeq™ 500 platform with 2x75bp paired end sequencing. An average sequencing depth of 20M reads per sample was achieved. Reads were aligned to the mouse genome with STAR version 2.4.2a (/doi.org/10.1093/bioinformatics/bts635) in two-pass mode, and on the second pass reads mapping to genes were quantified. Both the genomic DNA sequence (Mus_musculus. GRCm38 primary assembly) and gene annotations (GTF file GRCm38.86) were downloaded from Ensembl (Zerbino et al. doi:10.1093/nar/gkx1098). Quality control of the samples was carried out with FASTQC (bioinformatics.babraham.ac.uk/projects/fastqc) and differential expression analysis and further sample quality control were performed in R with SearchLight2[70] .

NanoString nCounter analysis of sorted epithelial cells

Briefly, RNA from sorted lung epithelial cells of either naïve or IAV infected mice (day 30) was analyzed using the nCounter Mouse Immunology Panel (catalog # 115000052 XT_PGX_MmV1_immunology). This panel includes 547 genes covering core pathways and processes of the immune response, and 14 internal reference genes, a custom code set of 30 genes was used in conjunction with this panel. Each sample consisted of RNA from sorted epithelial cells from one individual mouse. Assays were performed using 50ng input RNA and quantified on the nCounter FLEX system, sample preparation station, and digital analyzer (NanoString Technologies) according to the manufacturer's instructions. Briefly, reporter and capture probes were hybridized to target analytes for 20 h at 65°C as per[68.] After hybridization, samples were washed to remove excess probes. Purified target-probe complexes were aligned and immobilized onto the nCounter cartridge, and the transcripts were counted via detection of the fluorescent barcodes within the reporter probe. Raw gene expression data were analyzed using NanoString nSolver analysis software, version 4.0 Background subtraction was performed using the included negative controls included using the default threshold settings. Data normalization was performed using 6 house-keeping genes (*Gapdh, GusB, Hprt, Oaz1, Polr2 and Sdha*), these genes had been selected based on stability of expression levels from previous experiments. Ratios of transcript count data were generated for primary infected epithelial cells (day 2) versus IAV-rechallenged epithelial cells (day 30+2) . Genes with an FDR value <0.05 were considered statistically significant.

Flow Cytometry Staining

Cells were incubated for 10 mins with Fc block (homemade containing 24G2 supernatant and mouse serum) surface stained with anti-CD45 FITC (Biolegend, 30-F11) or anti-CD45-PE (BD, 30-F.11) or anti-CD45 eflour450, anti-CD31 PE (Biolegend, MEC13.3) or anti-CD31-FITC (BioLegend, MEC13.3),

CD326/EpCAM1-1 PerCP-Cy5.5 (Biolegend, G8.8) or CD326/EpCAM1-1 BV711 (Biolegend, G8.8),anti-CD140a/Pdgfra APC (Biolegend, APA5) or anti-CD140a/Pdgfra BV605 (Biolegend, APA5), Gp38/podoplanin PeCy7 (Biolegend, 8.8.1), anti-Sca1 APC-Cy7 (BioLegend, W18174A), anti-CD24 BV421 (Biolegend, M1/69), anti-MHCII BUV395 (BD: 2G9), anti-Siglec F-APC (BioLegend, S17007L), anti-CD11b-PeCy7 (BioLegend, M1/70), anti-Ly6G-BV785 (BioLegend, 1A8), anti-Ly6C-PerCP-Cy5.5 (ThermoFisher, HK1.4), anti-CD64-BV711 (BioLegend, X54-5/7.1), anti-CD11c-eFluor780 (ThermoFisher, N418), anti-B220-eFluor 450 (RA3-6B2, ThermoFisher), anti-CD103-Qdot 605 (BioLegend, 2E7). Cells were stained with a fixable viability dye eFluor 780 or eFluor 506 (both ThermoFisher) as per the manufacturer's recommendations. Cells were fixed with cytofix/cytoperm (BD Bioscience) for 20 min at 4°C and stained in permwash buffer with anti-IAV-NP FITC (Invitrogen, D67J) for 1 h at room temperature.

For T cells, anti-CD4 APC-Alexa647 (RM4-5, ThermoFisher), anti-CD8 BUV805 (BD 53-6.7), anti-CD44 BUV395 (BD, IM7), anti-CD25 BV711 (Biolegend, PC61), anti-CD69 PE (BD, H1.2F3) anti-B220 eFluor 450 (RA3-6B2), anti-MHCII eFluor 450 (M5114) and anti- F480 eFluor 450 (BM8) all ThermoFisher. Anti-B220, MHCII and F480 were used as a 'dump' gate. Following washing with permwash buffer, samples were acquired on a BD Fortessa and analyzed using FlowJo (version 10 BD Bioscience, USA).

Analysis of DEG

Quality control of the samples was carried out with FASTQC and then SearchLight[70]. FDR with a p-value of less than 0.05 was considered statistically significant.

Over Representation Analysis

Over Representation Analysis (ORA). In ORA significantly different genes were compared to a database (e.g. GO terms, KEGG) of pre-defined lists of genes (gene-sets). A hyper-geometric test was then used to determine whether each gene-set is enriched or not for the significantly differential genes. We examined the 10 most enriched gene-sets when using all significantly differential genes, significantly upregulated genes, and significantly downregulated genes. The false discovery rate (FDR) was calculated to correct the p value. The significant ORA terms were defined an FDR < 0.05.

RNAscope *in situ* hybridization

Naïve wildtype and IAV infected mice were euthanized by cervical dislocation and lungs were removed. Briefly, lung tissues were fixed with a 10% neutral buffered formalin solution (Sigma-Aldrich, UK) for 24-36 hours and subsequently embedded in paraffin. Lung tissue was cut with a microtome (Leica) at 6µm per section and mounted on Superfrost slides (Fisher Scientific). Slides were stored at

R/T until use, then baked at 60°C for one hour prior to commencing the RNA scope protocol. Pre-treatment of tissue sections and RNAscope ISH was carried out according to manufacturer protocols (Advanced Cell Diagnostics, Inc) with protease for 30 mins, followed by heat induced target retrieval, using the RNAscope Red 2.5 kit (ACD-Biotechne; Cat# 322350) with Mm-SpiB (408789), DapB (Cat# 310043) and Mm-Ubc (Cat# 310771). For X31-IAV RNAscope, probe 1226621-C1 (ACD) was used, slides were baked at 37°C overnight and pre-treated, as above, combined with boiling for target retrieval.

Spatial transcriptomics using Nanostring GeoMx Digital Spatial Profiler (DSP)

X31-IAV RNAscope was used to select virus positive and negative areas from FFPE lungs of each mouse and these cores combined to generate a TMA. Two serial 5mm thick slices were cut, one slide tested to confirm virus presence/absence by RNAscope and the second slide used for GeoMX DSP. The GeoMx DSP experiment consisted of slide preparation, with the nuclei stained with Syto13, tissue hybridization with UV-photocleavable probes (Mouse Whole Transcriptome Atlas panel of probes corresponding to 19,963 genes, Nanostring), slide scanning, region of interest (ROI) selection, probe collection, library preparation, sequencing, data processing and analysis. Detailed slide preparation has been previously described[71].

Region of interest and area of illumination selection and probe retrieval: Slides were scanned and imaged at ×20 magnification using the GeoMx DSP with the integrated software suite. Virus positive and negative ROIs were selected using the polygon segmentation tool with reference to the RNAscope, 161 ROIs were selected in total. Images were then used to identify multiple ROIs on which the instrument focuses UV light (385 nm) to cleave the UV-sensitive probes with the subsequent release of the hydridized barcodes with the subsequent aspirate collected into a 96-well DSP collection plate.

Library preparation, sequencing and data analysis: Libraries were prepared using GeoMx Seq Code primers (NanoString) and 1× PCR Master Mix (NanoString) and AMPure XP purification. Library quality was checked using an Agilent Bioanalyzer. The libraries were run on an Illumina NovaSeq sequencing system (GeneWiz/Azenta). The FASTQ files from sequenced samples were converted into Digital Count Conversion (DCC) files using the GeoMx NGS pipeline on NanoString's DND platform. The DCC files were uploaded onto the GeoMx DSP analysis suite (NanoString), where they underwent quality control, filtering, and Q3 normalization[71]. Normalised GeoMx data was analysed using R base functions and packages including Searchlight2[70].

Immunohistochemistry

Samples were prepared for immunohistochemistry as described above. De-paraffinized and rehydrated sections were incubated in 2% hydrogen peroxide/methanol for 15 minutes. Antigen retrieval (pH 7 or 9) was performed using Vector antigen unmasking (low pH) solution (Vector cat #H-3300) and pH 9 (#H-3301) at 100°C for 20 minutes. Sections were blocked with an avidin/biotin kit (SP-2001; Vector Laboratories, Burlingame, CA) for 20 minutes, then with 10% normal goat serum (NGS, Invitrogen) in PBS for 20 minutes. Slides were incubated overnight at 4°C with primary antibodies at 1:200 directed against B220 (Invitrogen 14-0452-82, clone; RA36A3). Slides were washed with PBS and then incubated with biotinylated goat anti-rat IgG (ThermoFisher, 31830) for 1.5 hours. Amplification of antigen was achieved using an R.T.U. Vectastain kit (Vector, PK-7100), and positive cells were visualized by 3,3- diaminobenzidine tetrahydrochloride (Vector, SK-4100). Hematoxylin and Eosin staining was performed as previously described[17] **.**

Immunofluorescence

Mice were euthanized by rising concentrations of carbon dioxide (CO_2) and the vena cava cut. Lungs were perfused with PBS-5 mM ethylenediaminetetraacetic acid to remove red blood cells and 1% paraformaldehyde was used to fix the lungs. Lung inflation was achieved using 1–3 mL 1% warm UtraPure low melt agarose administrated via the trachea. Lungs with solidified agarose were incubated in 1% paraformaldehyde overnight at 4 °C followed by incubation in 30% sucrose for a further 2–5 days at 4 °C. Lung lobes were frozen in Optimal Cutting Temperature (OCT) (Tissue-Tek 4583, UK) and stored at −80 °C. Lungs were sectioned into 10 µm slices on a Shandon Cryotome FE (Thermo Scientific 12087159, UK) and mounted onto Super Frost slides (Thermo Scientific). Slides were fixed in 100% cold acetone and stored at −20 °C.

Slides were rehydrated in PBS containing 0.5% bovine serum albumin (BSA) for 5 minutes and incubated with Fc block (24G2) for 30–60 minutes at room temperature. The sections were stained with antibodies at 4 °C overnight: anti-CD4 AlexaFluor647 (BD UK, RM4-5), anti-MHCII eFluor 450 (M5114, ThermoFisher, UK), anti-B220-Phycoerythrin (ThermoFisher, RA3-6B2), Slides were washed in PBS-BSA and mounted with VectorSheild (Vector, UK). Images were collected on an LSM880 (Zeiss, Germany) confocal microscope at 20× magnification.

Digital slide scanning

Excised lung tissues for histological analysis were fixed in 10% neutral buffered formalin for 24 hours and paraffin-embedded in a tissue processor (UK, Shandon Pathcentre Tissue Processor). Formalin-fixed and paraffin-embedded tissues were sectioned (6µm) and processed for subsequent staining

with H&E using standard protocols. Stained slides were scanned on a Leica Aperio VERSA 8 bright-field slide scanner (Leica Biosystems, UK) at 10X or 20X magnification and were visualized/analyzed using Aperio ImageScope version 12.1.0.5029 (Aperio Technologies Inc., Vista, USA.

***In vitro* stromal infection and T cell interaction assay**

CD45 negative cells were isolated from naïve mice or those that had been challenged with IAV-WSN 30 days previously, as per flow cytometry tissue preparation above. Cells were seeded at $1x10^6$ cells per well of a 48 well plate and allowed to adhere overnight. Approximately 18 hours later cells were infected with IAV-X31 (Multiplicity of Infection (MOI) 1). T cells were isolated from the spleens of mice that had been infected with IAV-X31 9 days previously to generate a pool of IAV-specific T cells using a T cell isolation kit (Stem cell, as per manufacturer's instructions). T cells were cultured with stromal cells for 24h. Flow cytometry analysis was performed as described above.

Luminex assay

The culture supernatants from the *in vitro* infected stromal cells were harvested, centrifuged to remove cellular debris, and stored at −80 °C until assayed by Luminex (Merck). Each experimental condition was carried out in duplicate. Chemokine/cytokine levels in plasma were analyzed by quantification with the Milliplex MCYTOMAG-70K assay; IL1a, IL1b, IL10, RANTES/ CCL5, MIG/ CXCL9, IP10/CXCL10, IL6, IL15, IL13, IL17A, IL12p70, IL12p40, GRO/CXCL1 and Milliplex MTH17MAG-47K-03 assay; IL33, IL17F,IL22 (EMD Millipore) according to the manufacturer's instructions.

Statistical Analysis

Data were analysed using GraphPad Prism version 10.01 for Windows (GraphPad, San Diego, California, USA). Data were tested for normality using the Shapiro-Wilk test with (α=0.05). Data are presented as mean ± SEM and p values were calculated as indicated the in the figure legends. P values < 0.05 were considered significant.

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Figure 1: IAV infection induces conserved transcriptional changes in cell cycle and anti-viral response genes in lung structural cells at day 10 post infection

RNA sequencing was performed on sorted lung (A) epithelial cells, (B), fibroblasts, and (C) endothelial cells from naïve and C57BL/6 mice infected with IAV 10 days previously. Differentially expressed genes (DEG) with a 2-fold change and an FDR q-value < 0.05 in lung structural cells were considered statistically significant. 7-8 mice were combined to make 4 samples per time point. Volcano plot showing the relationship between the fold change and the associated p−value with the top 10 DEG by fold change named (A) epithelial cells, (B) fibroblasts and (C) endothelial cells at 10 post IAV infection compared to naïve mice. Over Representation Analysis (ORA) bar charts showing ten most enriched gene-sets when using significantly upregulated genes with numbers next to each bar showing the number of genes in the pathway differentially expressed. To correct for multisampling a Benjamini-Hochberg correction was applied. Gene sets with an adjusted p-value of 0.05 and an absolute log2fold enrichment above 1 were considered significant.

Figure 2: IAV infection induces prolonged transcriptional changes in immune related genes in lung structural cells at day 40 post infection

RNA sequencing was performed on sorted lung (A) epithelial cells, fibroblasts, and blood endothelial cells from naïve and C57BL/6 mice infected with IAV 40 days previously. Differentially expressed genes (DEG) with a 2-fold change and an FDR q-value < 0.05 in lung epithelial cells and fibroblasts were considered statistically significant. 7-8 mice were combined to make 4 samples per time point. (B) Volcano plot showing the relationship between the fold change and the associated p−value in epithelial cells and fibroblasts at 40 days post IAV infection compared to naïve mice with the top 10 DEG by fold changed. (C) Venn diagrams showing overlap between differentially expressed upregulated genes at day 10 and 40 post IAV infection.

Figure 3: Network analysis shows conserved upregulation of genes involved in antigen processing and presentation in lung structural cells at day 40 post infection

Networks of all significantly enriched genes sets in (A) epithelial cells, (B) fibroblasts and (C) endothelial cells, highlighting significance and gene set interconnectivity. Networks are given for enrichment amongst all significantly differentially expressed genes (p.adj < 0.05, absolute log2 fold > 0.5) between day 10 and naïve and day 40 and naïve. Enrichment analysis was performed using Hypergeometric Gene Set Enrichment on the gene set databases STRING11.5. For each network nodes represent gene sets and edges represent two gene sets with a Szymkiewicz-Simpson coefficient of at least 0.5. Node colour represents -log10 enrichment p-value, top 5 nodes for each timepoint are listed.

Fig 4: SpiB is upregulated in lung epithelial cells adjacent to areas of inflammation and this is dependent on viral replication

DEG from the experiments described in Fig1 and 2 were compared with target genes of the transcription factor, SpiB, identified using a publicly available ChiPseq dataset (Solomon[32] *et al*.) (A) Heatmaps show SpiB target genes in epithelial cells and fibroblasts, respectively. Expression across each gene (or row) is scaled, with a Z-score computed for all DEG. Targets specific to epithelial cells (blue boxes), targets specific to fibroblasts (pink boxes) and those that overlap between cell types (purple boxes). Samples with relatively high expression of a given gene are marked in red and samples with relatively low expression are marked in blue. (B) Venn diagram shows overlap between 'SpiB target genes' and differentially expressed genes (DEG) in lung epithelial cells (blue) and fibroblasts (pink) post IAV infection. (C) Lungs were taken from naïve or C57BL/6 mice infected with IAV 30-40 days previously. Infected lungs with no inflammation have *SpiB* negative airways while *SpiB* (black arrows) is localized in areas of inflammation (dark red color, indicated by white star) within the lung and in airways, close to areas of inflammation. Data are (n=4-6) mice per experimental group, combined from 2 independent experiments. Each symbol represents a mouse and bars show mean with SEM. Data analysis using Kruskal-Wallis with Dunn's post hoc comparison test. **: $p < 0.01$ and ***: $p < 0.001$. (D) C57BL/6 mice were infected intranasally with IAV (either wild type or single cycle (S-FLU), Airways (Aw), blood vessels (Bv) inflammatory foci/immune cell clusters (labelled with white stars) and *SpiB* positive airway epithelial cells (black arrows). All scale bars are 200µm.

Figure 5: IAV infection results in upregulated MHCII expression by SpiB+ ciliated and progenitor cells at day 10

SpiB-mCherry reporter mice were infected with IAV (100-200PFU) and lungs taken at day 0, 10 or 30 post infection. EpCAM1+ epithelial cell populations were identified by gating on lineage negative (CD45, CD31 and CD140a neg), SpiB negative or SpiB+ populations. (A) Frequency of SpiB positive or negative lung epithelial cells by cell subset in naive mice. (B) Frequency of MHCII+ cells by each epithelial cell subset in naïve mice. (C) Numbers of SpiB negative or positive populations at the different timepoints. (D). Percentage of each population expressing MHCII at day 0, 10 and 30 post infection. In all experiments, data are combined with N=11 naïve, N=7 primary and N=10 memory from 2 independent experiments. In (A) data show mean with SEM and differences tested by ANOVA and Šidák's multiple comparison test. In B-D: each symbol represents a mouse and the bars show means with SEM error bars. In (B) data for Sca1+ progenitor cells are not normally distrusted. In (C) numbers of ciliated, club, CD24+ progenitor, and ATI cells are also not normally distributed and in (D) Sca1 progenitor data are not normally distributed. All other data are normal and tested by either

paired T-tests (B) or (C-D) ANOVA with Šidák's multiple comparison test comparing day 0 and infected groups within SpiB negative/positive populations. Data not normally distributed tested by (A) paired Wilcoxon test or (C-D) ANOVA and a Dunn's multiple comparison test comparing day 0 with the infected groups. *:p <0.05, **:p<0.01,*** :p<0.001, ****: p<0.0001.

Figure 6: Epithelial cells from IAV re-challenged mice have less virus than those from primary infected animals

C57BL/6 mice that were either naïve or infected 30 days earlier with IAV-WSN, were infected with IAV-X31, their lungs harvested 2 days later, and epithelial cells and fibroblasts examined by flow cytometry. (A) The percentages and number or EpCAM1+ cells positive for IAV-NP were examined by flow cytometry. (B) The percentages of each epithelial cell type that were IAV-NP+. (C) Representative FACS plots of IFN-responsive fibroblasts indicating positive populations and (D) the percentages and numbers of IFN-responsive fibroblasts. Data are not normally distributed (mean +/- SD). Primary: n=8 mice, and Re-challenge: n=9 mice. Each symbol represents a mouse and the bars are mean with SEM error bars. Data are from two independent experiments combined. For two group comparisons (A,B,D: Mann-Whitney U test), *: p <0.05, ***: p<0.001.

Figure 7: Spatial analysis of gene expression in primary and re-infected animals show altered inflammatory and type I IFN response and increased IFNγ response in re-infected animals

C57BL/6 mice that were either naïve or infected 30 days earlier with IAV-WSN, were infected with IAV-X31, their lungs harvested 2 days later, and their epithelial cells sorted by FACS and gene expression analyzed by Nanostring nCounter or lungs fixed and embedded in paraffin. (A-B) Heatmap and example boxplots showing DEG from primary or re-infected animals in FACS sorted epithelial cells. DEG with a 2-fold change and an FDR q value < 0.05 in lung epithelial were considered statistically significant. Primary group (n=4) and re-challenge group (n=7), data are from two independent experiments combined and each dot represents a mouse. (C) RNAscope analysis for X31-IAV was performed on FFPE lung tissue, representative X31-IAV+ areas are shown in red and labelled with black arrows to highlight virus + bronchial epithelium. Bars, 200 micrometres. Virus positive and negative airways from 6 primary and 3 re-infected mice were analysed by GeoMX. (D) Heatmap showing DEG between X31-IAV positive airways in primary versus re-challenge groups, DEG with a 2-fold change and an FDR q-value < 0.05 in lung epithelial cells were considered statistically significant. (E) DEG between normal tissue and 3 areas: virus+ airway cells; virus negative cells within the same airway (Close); and adjacent virus negative airways (Further) were classified based on presence within MGI GO terms 'Inflammation' or 'Type 1 IFN'. (F) Enriched pathways found in DEG identified between viral+ airways in day 2 primary or re-infected C57BL/6 mice analysed by GeoMx. The x-axis represents the

−log10 of the P value given during the analysis; (FDR < 0.05) with the number of DEGs listed from each pathway.

Figure 8: T cell depletion does not alter the ability to control IAV infection in vivo at day 2 post re-challenge

(A) Schematic illustrating T cell depletion experimental timeline, C57BL/6 mice were infected with IAV-WSN on day 0 and treated with 400μg isotype control or 200μg each of anti-CD4 (GK1.5) and anti-CD8 (2.43) on days 28 and day 30 when mice were (re)-infected with IAV-X31. (B) Representative FACS plots showing CD4 and CD8 T cell depletion in the lung day 2 post infection, gated on live, single, CD45 iv negative lymphocytes, that are TCRβ+ and numbers of CD4 and CD8 T cells that are TCRβ+ in isotype control (IgG) and T cell deleted groups at day 32. Cell numbers from the lung, draining lymph node and spleen in IgG treated group compared to anti-CD4 and CD8 treated groups and compared by Student's t test. (C) Plaque assay showing viral titers as PFU per mg of lung tissue. Data are normally distributed. Primary (n=9) mice, recall IgG (n=9), recall aCD4aCD8 (n=11). Data are from two independent experiments combined. ONE-WAY ANOVA with Šidák's multiple comparison test. *:p<0.05, **: p <0.01,***: p<0.001, ****: p<0.0001. In all graphs, each symbol represents a mouse, the horizontal line shows the mean and error bars are SEM.

Figure 9: Infection experienced stromal cells display enhanced viral control and can reactivate T cells

(A) Schematic showing experimental design of IAV infection and *in vitro* re-challenge and co-culture. Lung CD45 negative cells were isolated from naïve or C57BL/6 mice infected 30 days earlier with IAV-WSN. After 24hours, the cells were infected with IAV-X31 and the cells examined by flow cytometry after a further 24hours or co-cultured with T cells isolated from the spleens of mice infected with IAV-X31 9 days earlier. (B) The percentage of CD44hi CD4 or CD8+ T cells that were CD25 or CD69 positive, normalized to the mean of the naïve- no infection control within each of the 2 experiments. CD4 data not normally distributed and tested *via* a ONE-WAY ANOVA and a Dunn's multiple comparison test. CD8 data are normally distributed and tested via ONE-WAY ANOVA with Šidák's multiple comparison test. Data are from two experiments with 4 mice within each naïve and IAV-memory group per experiment. (C) Representative FACS plots for IAV-NP for each cell type are shown for each group. Cells were gated based on live, single, CD45 negative populations that were CD31+ (endothelial cells), CD140+ (fibroblasts) and EpCAM1+ (epithelial cells). Numbers in plots indicate the percentages of cells that are IAV-NP+. Data are normally distributed and are from two combined experiments, with n=8

samples per timepoint. ONE-WAY ANOVA with Šidák's multiple comparison test. (D) Cytokine levels were measured by Luminex assay in the supernatants of *in vitro* IAV infected and re-challenged groups taken 24hours after the *in vitro* infection. Data were normally distributed, are mean +/- SEM and analyzed by T test. Data combined from the same two experiments. In all graphs, each symbol represents a mouse, the horizontal line shows the mean and error bars are SEM and *:$p<0.05$,**: $p<0.001$,***: $p <0.001$, ****: $p<0.0001$.

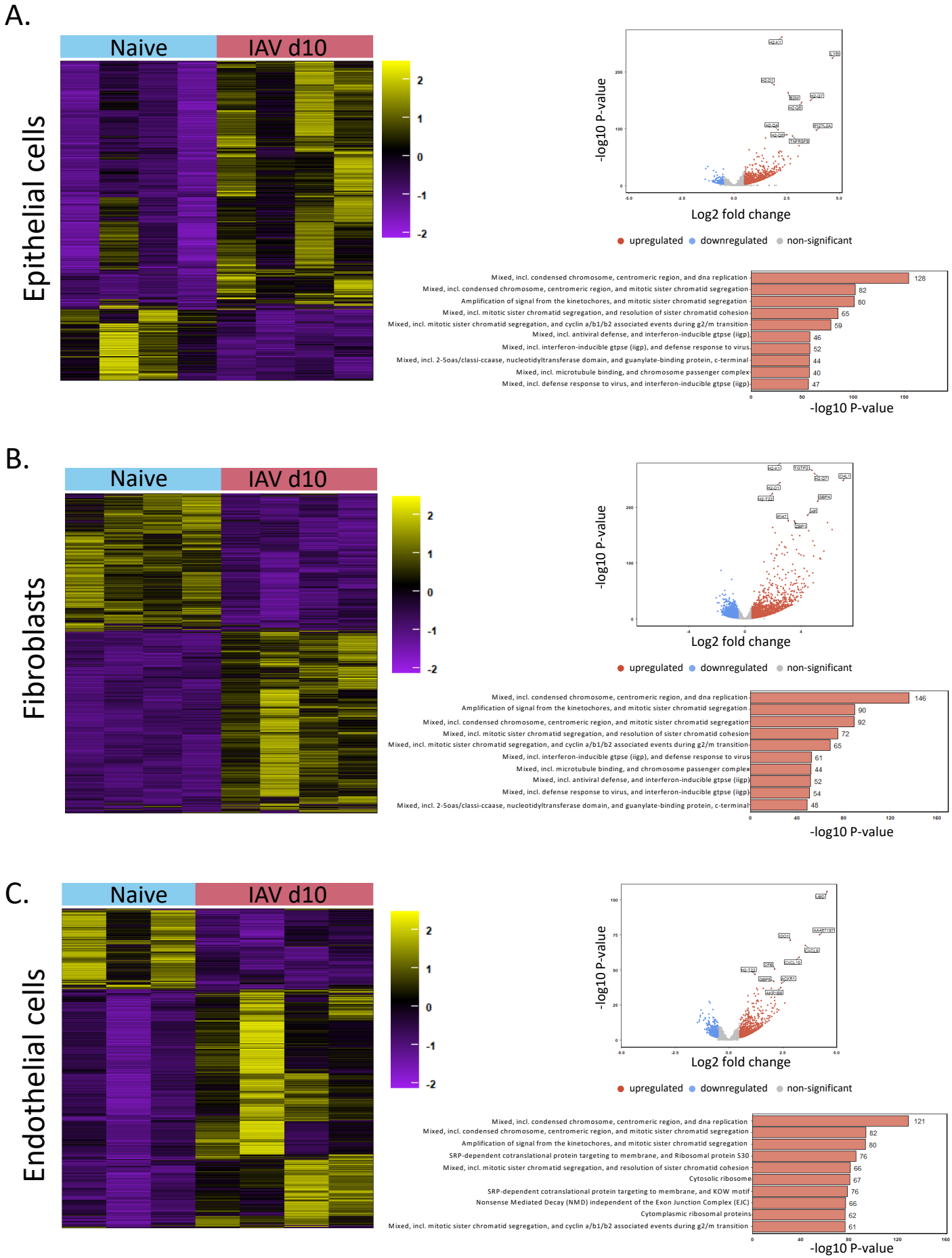


Figure 1: IAV infection induces conserved transcriptional changes in cell cycle and anti-viral response genes in lung structural cells at day 10 post infection

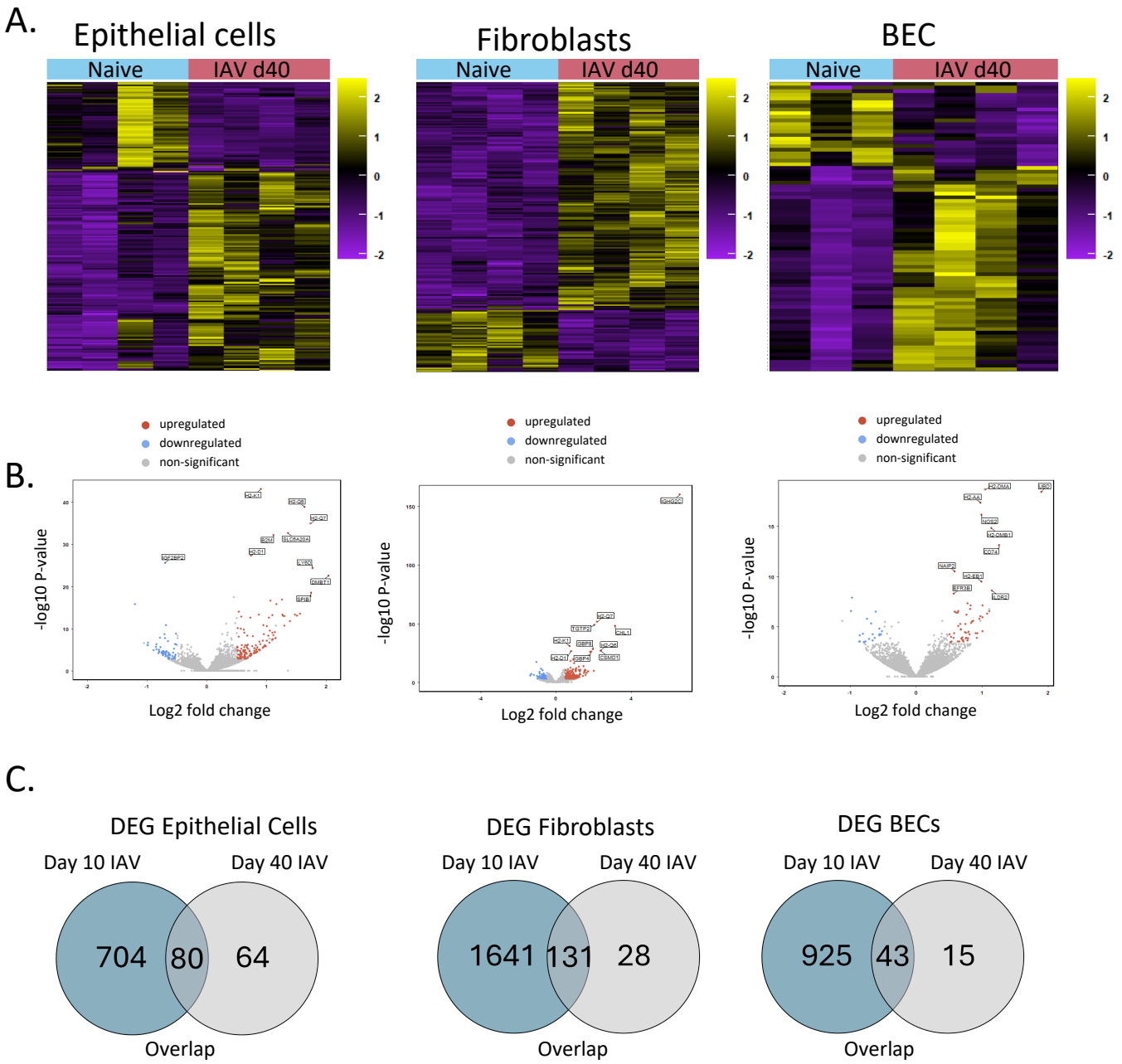
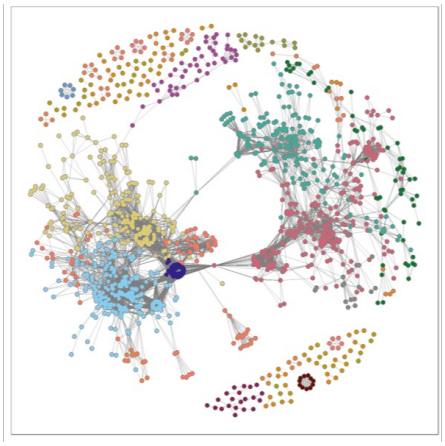


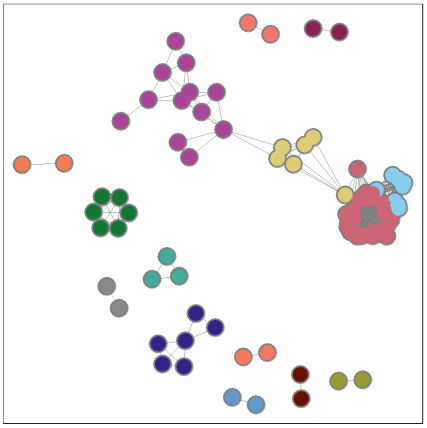
Figure 2: IAV infection induces prolonged transcriptional changes in immune related genes in lung epithelial cells and fibroblasts at day 40 post infection

A. Epithelial cells

Upregulated pathways at day 10



Upregulated pathways at day 40



Day 10

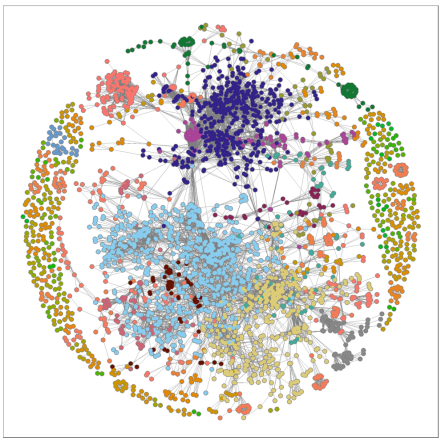
- Cell cycle regulation
- Regulation of immune response
- DNA replication repair
- Interferon response
- Regulation of adaptive immune response

Day 40

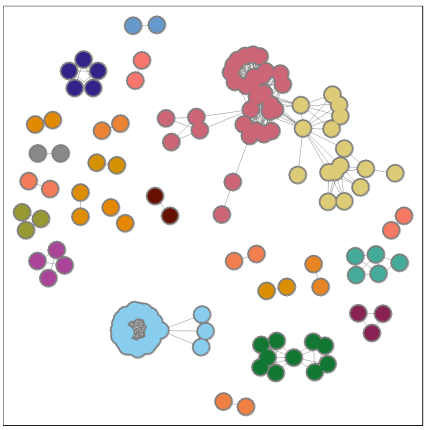
- Antigen processing and presentation
- Endosomal vascular pathway
- T lymphocytes
- Regulation of adaptive immune response
- Molting cycle process

B. Fibroblasts

Upregulated pathways at day 10



Upregulated pathways at day 40



Day 10

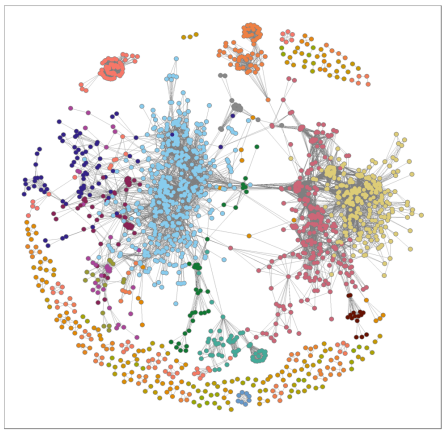
- Regulation of immune response
- Cell cycle and chromosome organisation
- Morphogenesis and chemotaxis
- Metabolic processes
- Regulation of kinases and cell cycle

Day 40

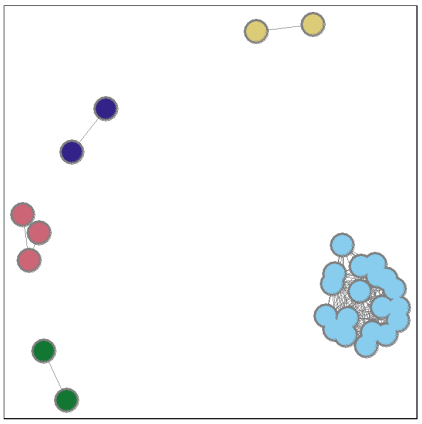
- Antigen processing and presentation
- Chemokine/chemotaxis
- Leukocyte regulation
- Interferon/virus defence
- Decreased type IV hypersensitivity

C. Blood Endothelial Cells

Upregulated pathways at day 10



Upregulated pathways at day 40



Day 10

- Regulation of immune cells
- Regulation of cell
- DNA repair and apoptosis
- Response to infection
- Ribosomes/translation

Day 40

- Antigen processing and presentation
- Regulation of immune cell proliferation
- Abnormal blood clotting
- Coagulation
- Negative regulation of cell activation/adhesion

Figure 3: Network analysis showing conserved upregulation of genes involved in antigen processing and presentation in lung structural cells at day 40 post infection

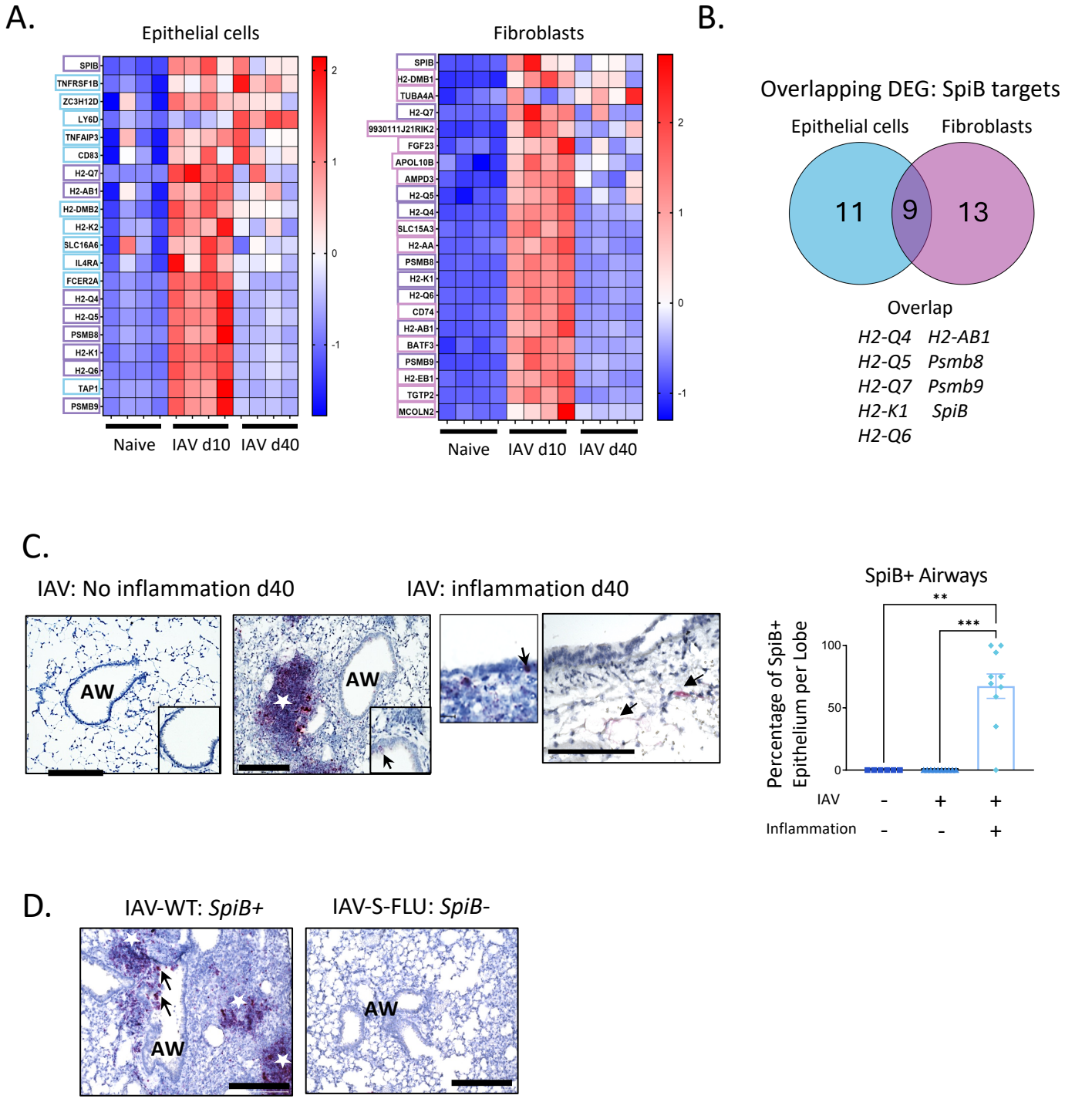


Figure 4: SpiB is upregulated in lung epithelial cells adjacent to areas of inflammation and this is dependent on viral replication

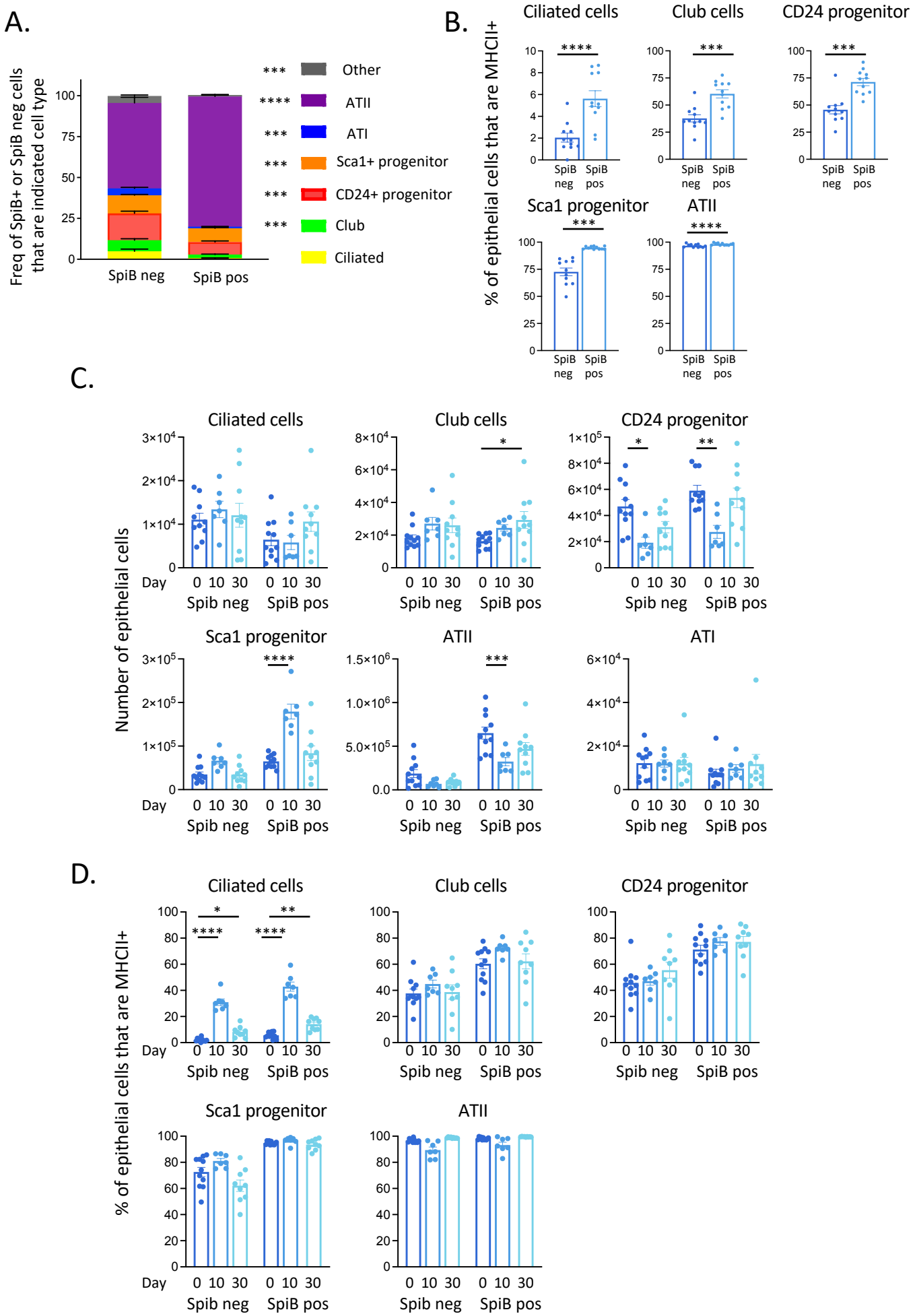


Figure 5: IAV infection results in upregulated MHCII expression by SpiB+ ciliated and progenitor cells at day 10

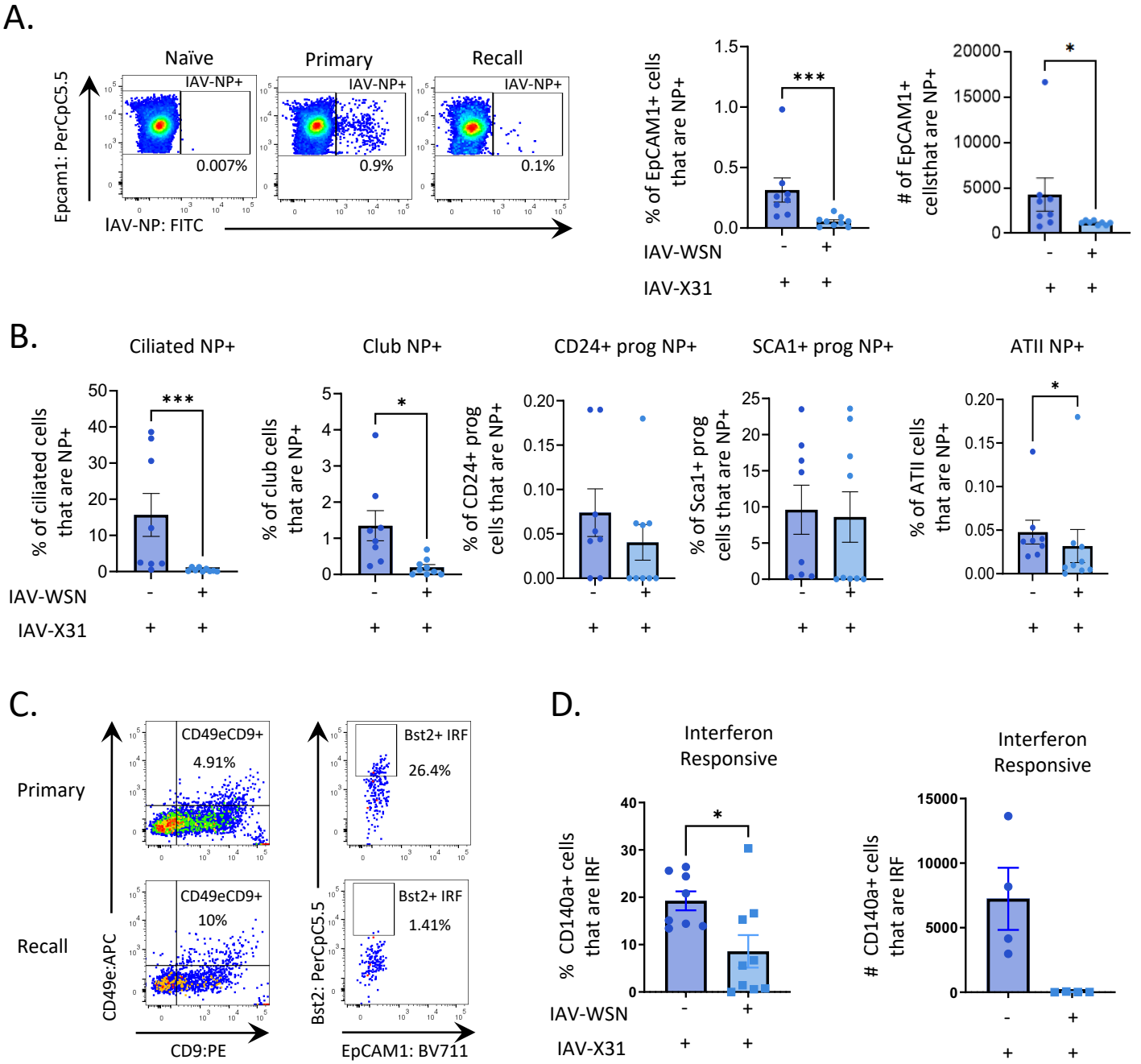


Figure 6: Epithelial cells from IAV re-challenged mice have less virus than those from primary infected animals

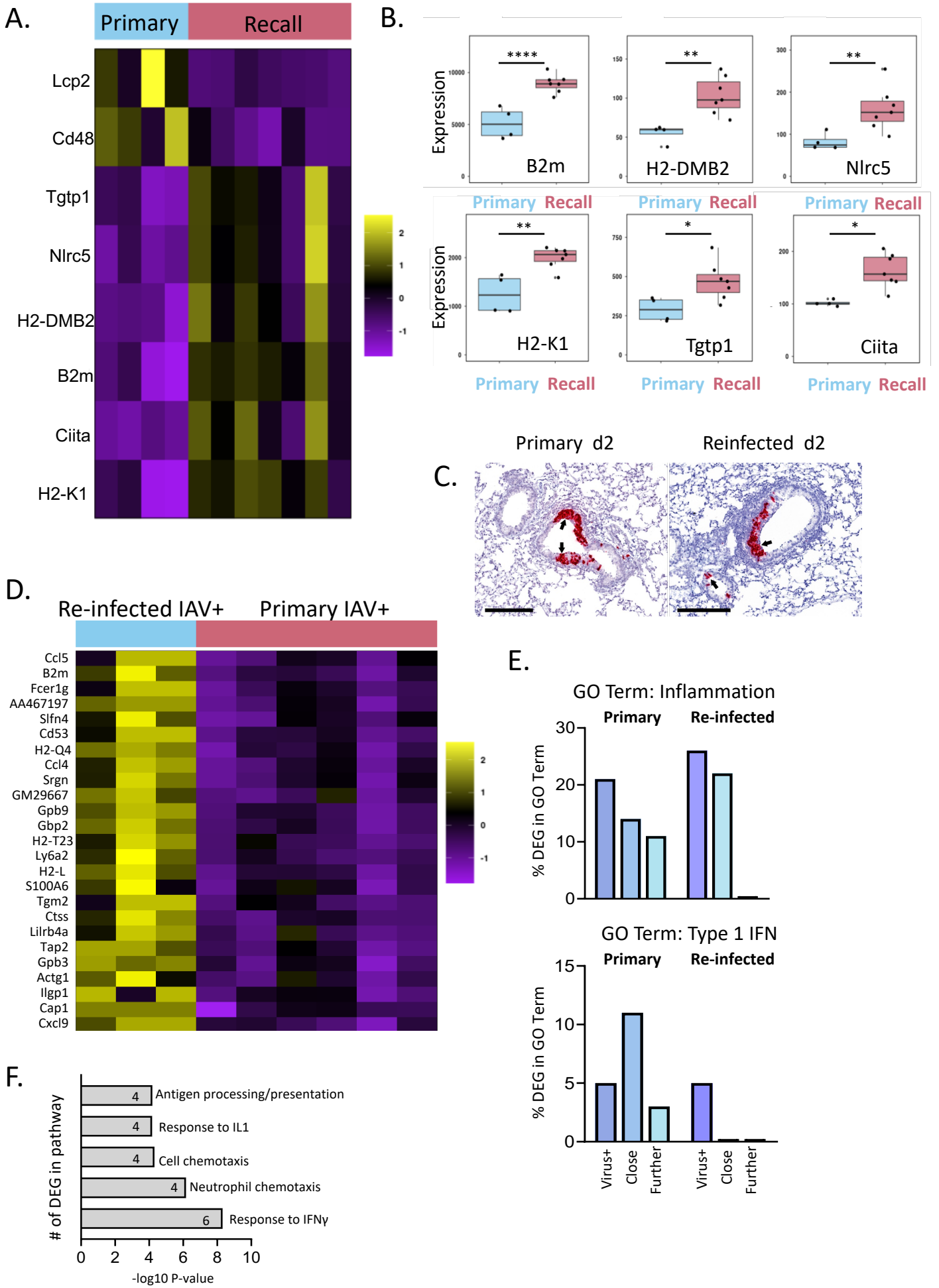
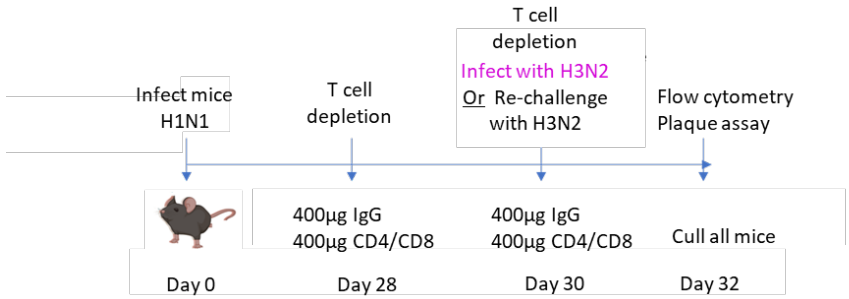


Fig7: Spatial analysis of gene expression in primary and re-infected animals show altered inflammatory and type I IFN response and increased IFNγ response in re-infected animals

A.



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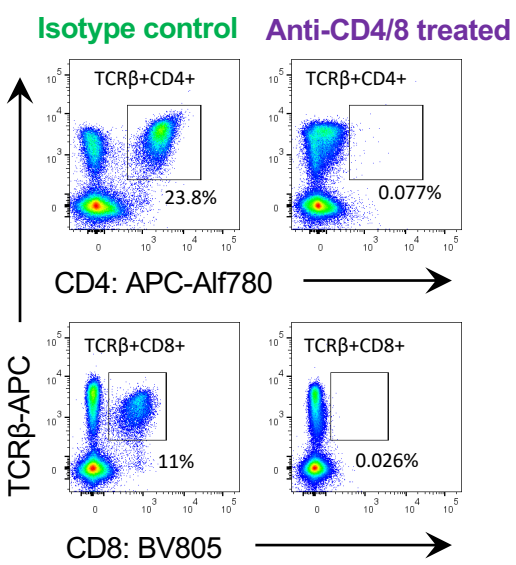
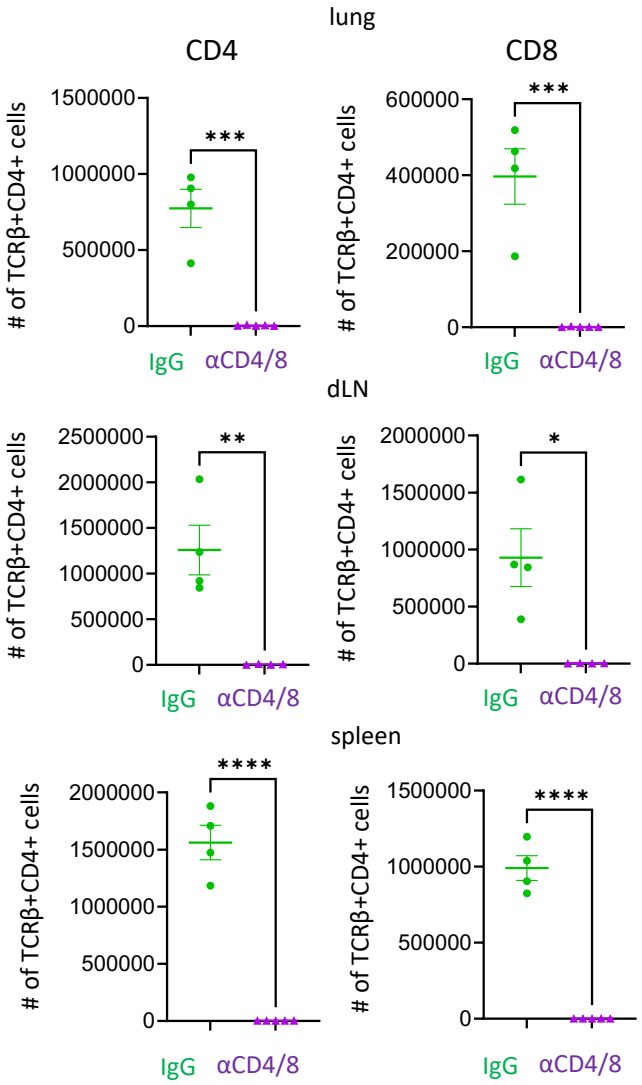




C.

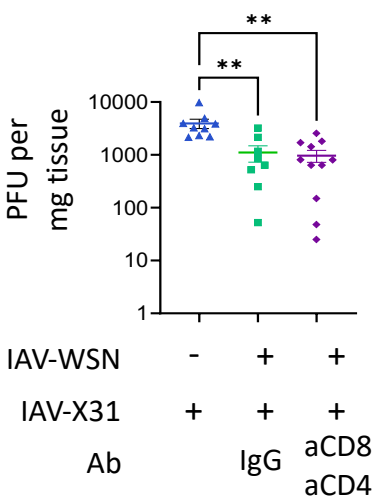


Fig 8: T cell depletion does not alter the ability to control IAV infection *in vivo* at day 2 post re-challenge

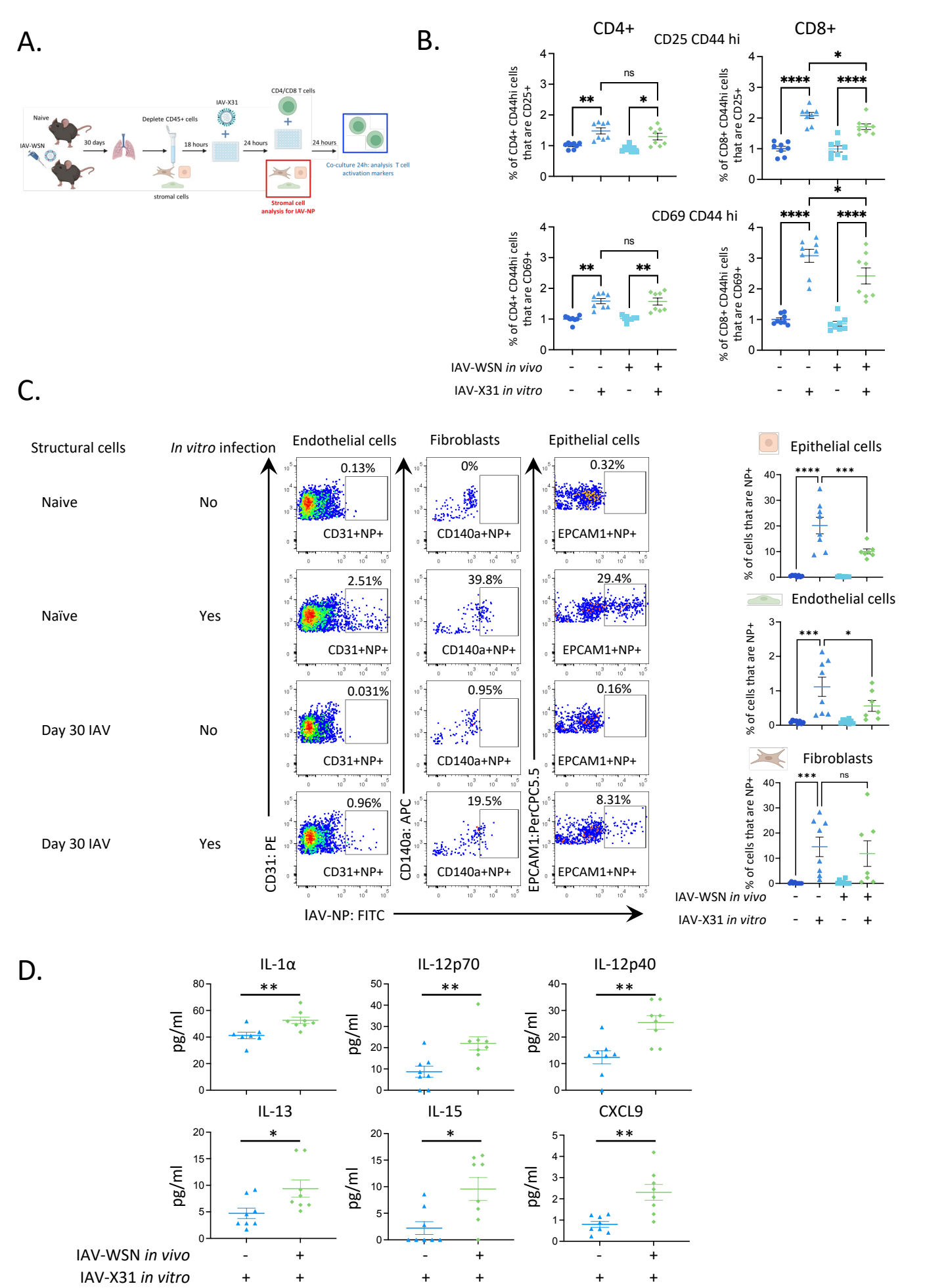
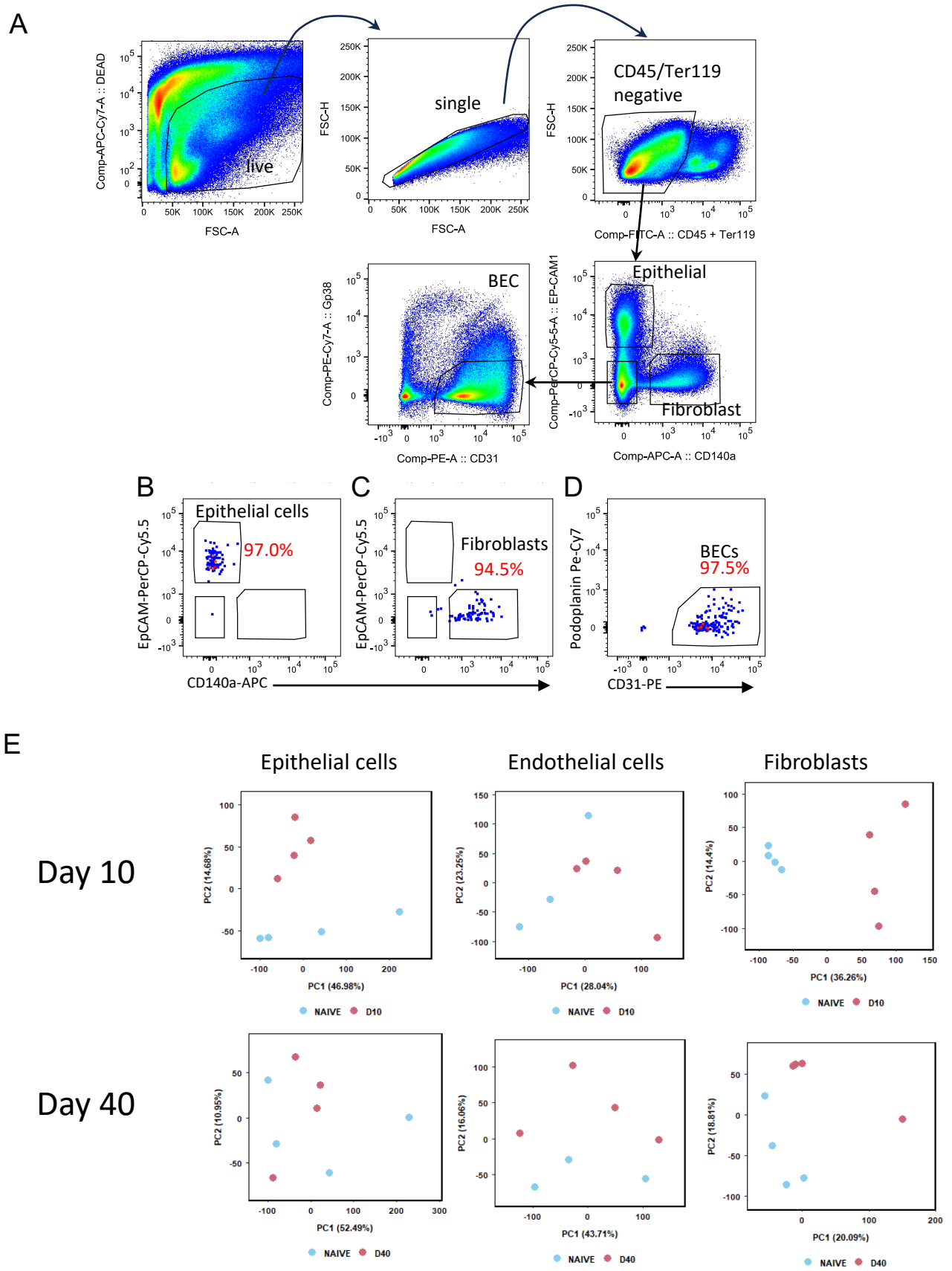


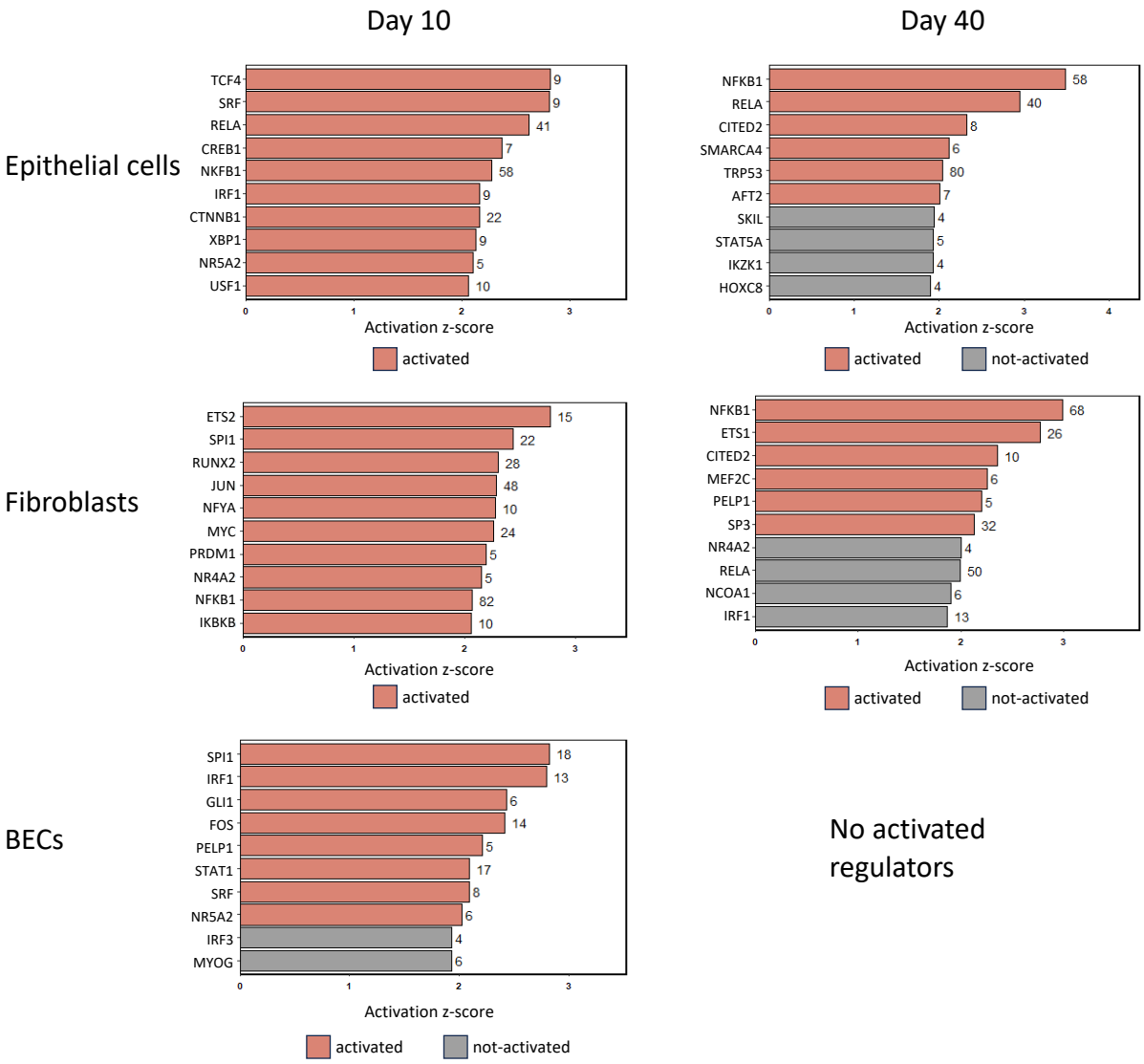
Figure 9: Infection experienced stromal cells display enhanced viral control and can reactivate T cells



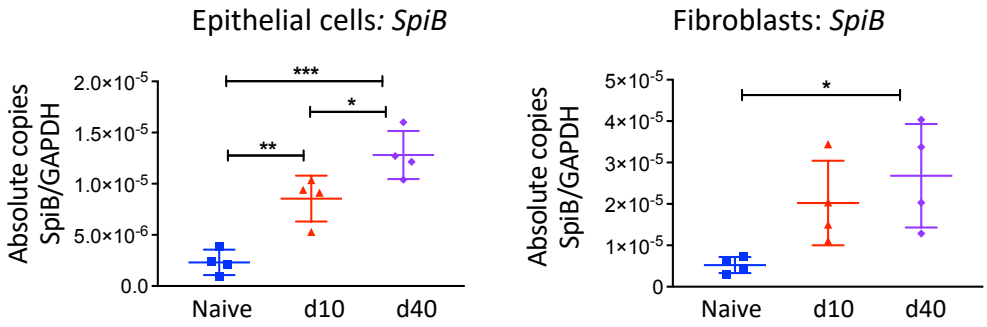
Supp Figure 1: Sorted stromal gating strategy, sort purity for RNA sequencing and PCA plots for day 10 and 40 post-infection

Stromal cells were identified by flow cytometry. (A) Lung structural cells gated as indicated. (B) Post sort epithelial cells (C) post sort fibroblasts (D) post sort BECs. (E) Principal Components Analysis (PCA) for day 10 and day 40. Each component describes a proportion of the total underlying variation between genes and samples (ordered by the % of the underlying variation that they describe).

A

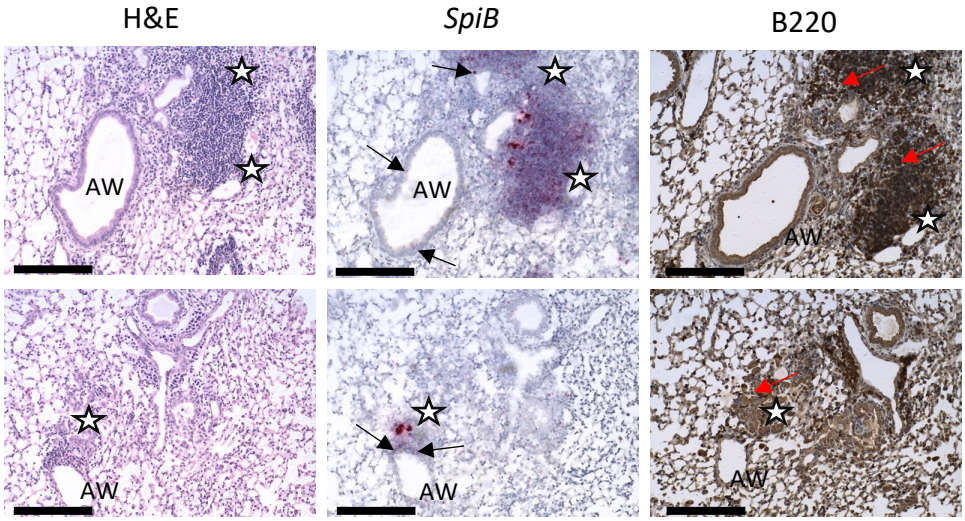


B



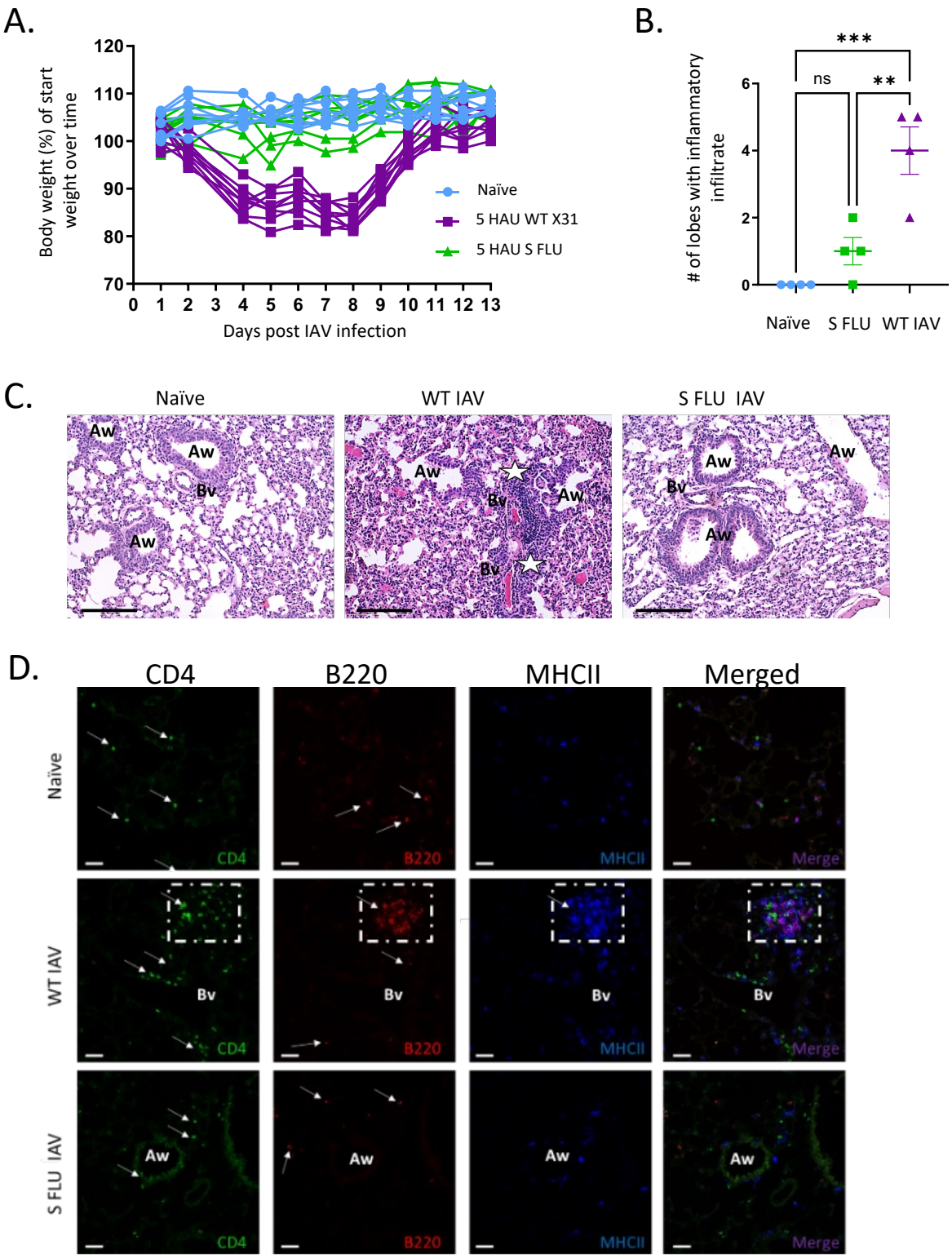
Supp Fig 2: Potential upstream regulators of DEG in lung structural cells post-IAV

A. RNA sequencing was performed on sorted lung epithelial cells, fibroblasts, and endothelial cells from naïve and C57BL/6 mice infected with IAV 10 and 40 days previously and potential upstream regulators identified. B. qPCR data on sorted cells showing absolute copy numbers of SpiB normalized to GAPDH in lung epithelial cells and fibroblasts. *P <0.05 ** P <0.01 and **P <0.001. Kruskal-Wallis with Dunn's post hoc test.



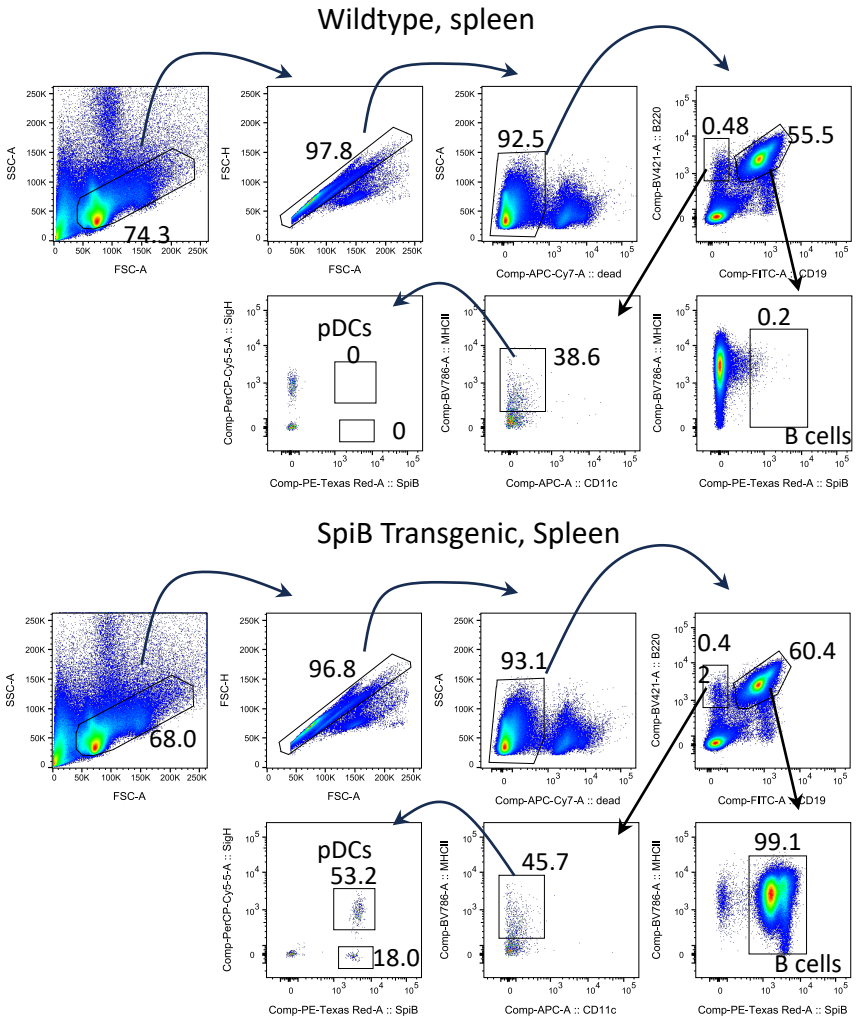
Supp Fig 3: SpiB+ immune cells located in clusters are also B220 positive

Immunohistochemistry and RNA-scope showing SpiB+ airway epithelial cells are located near B220+ immune cell clusters in the IAV infected lung at day 40 post iAV infection. Images taken at 20x magnification, scale bar 200µm. Airways (Aw), SpiB+ epithelial cells (black arrows), inflammatory foci (labelled with stars) and B220+ cells (red arrows).



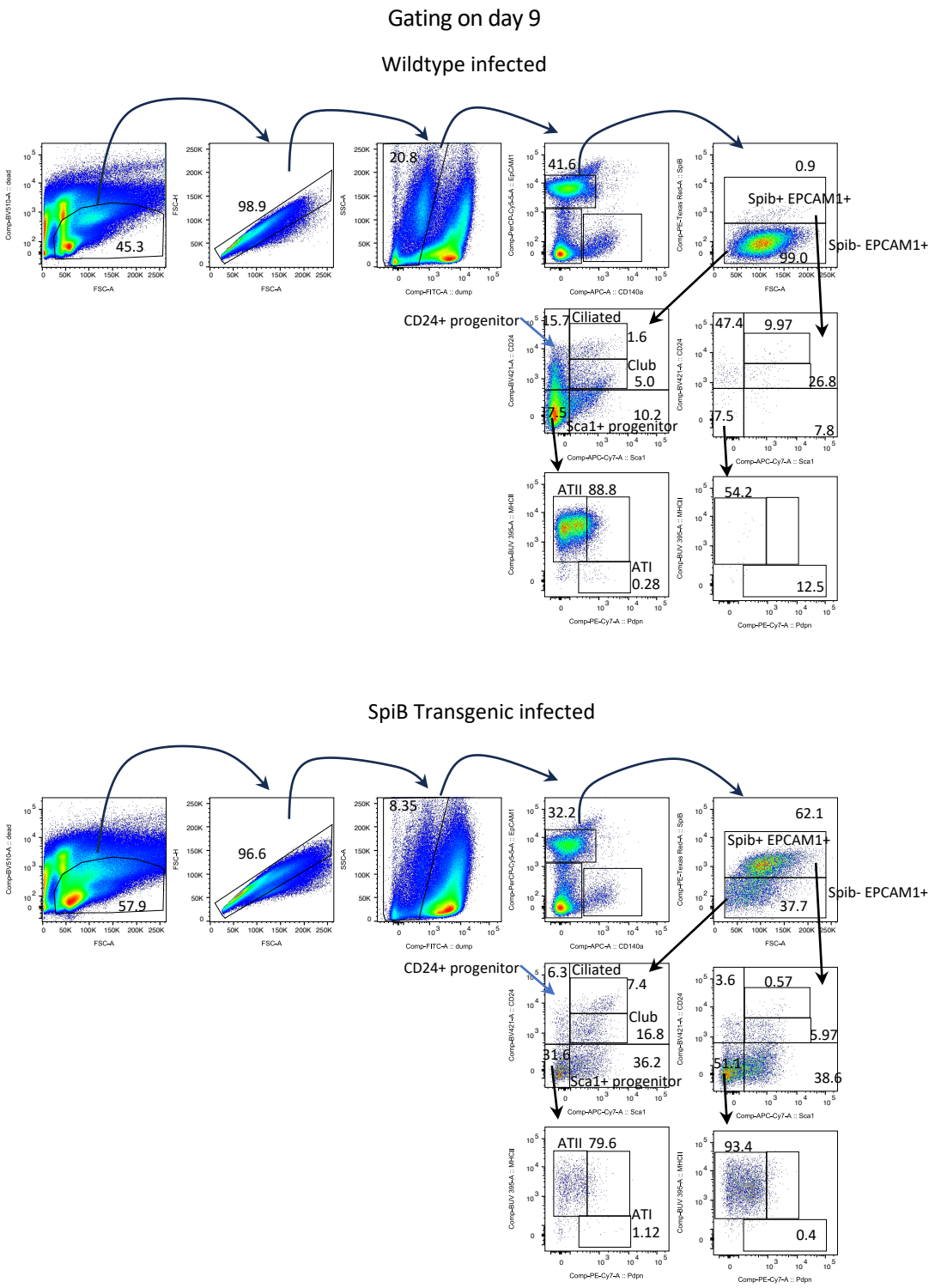
Supp Figure 4: Immune cell clusters do not form following infection with a replication deficient IAV virus (S-FLU)

C57BL/6 mice were infected i.n with IAV (X31 strain, either wild type or single cycle (S-FLU)) for 30 days. (A) Weight loss graph showing percentage of total body weight over time post infection (n=9) mice per group (naïve, WT-IAV and S-FLU). Data are combined from two independent experiments. (B) Histological analysis of inflammation in naïve, WT-IAV and S-FLU infected mice at day 30. Data are normally distributed and shown as mean +/- SEM. One way-ANOVA with Šidák's multiple comparison, **P <0.01, ***P <0.001. (C) H&E staining showing airways (Aw), blood vessels (Bv) inflammatory foci/immune cell clusters (labelled with white stars) in naïve, WT-IAV and S-FLU infected mice at day 30. All images taken at 200x magnification, scale bar 200µm. Immunofluorescent staining shows the localization of CD4 T cells (green), B cells (B220+, red) and antigen presenting cells (MHCII, blue) in lung sections from naïve and IAV infected mice culled at day 30 post infection. All images taken at 200x magnification, scale bar 200µm. Airways (Aw), blood vessels (Bv) inflammatory foci/immune cell clusters (labelled with dashed white box). Positive cells are indicated by white arrows.



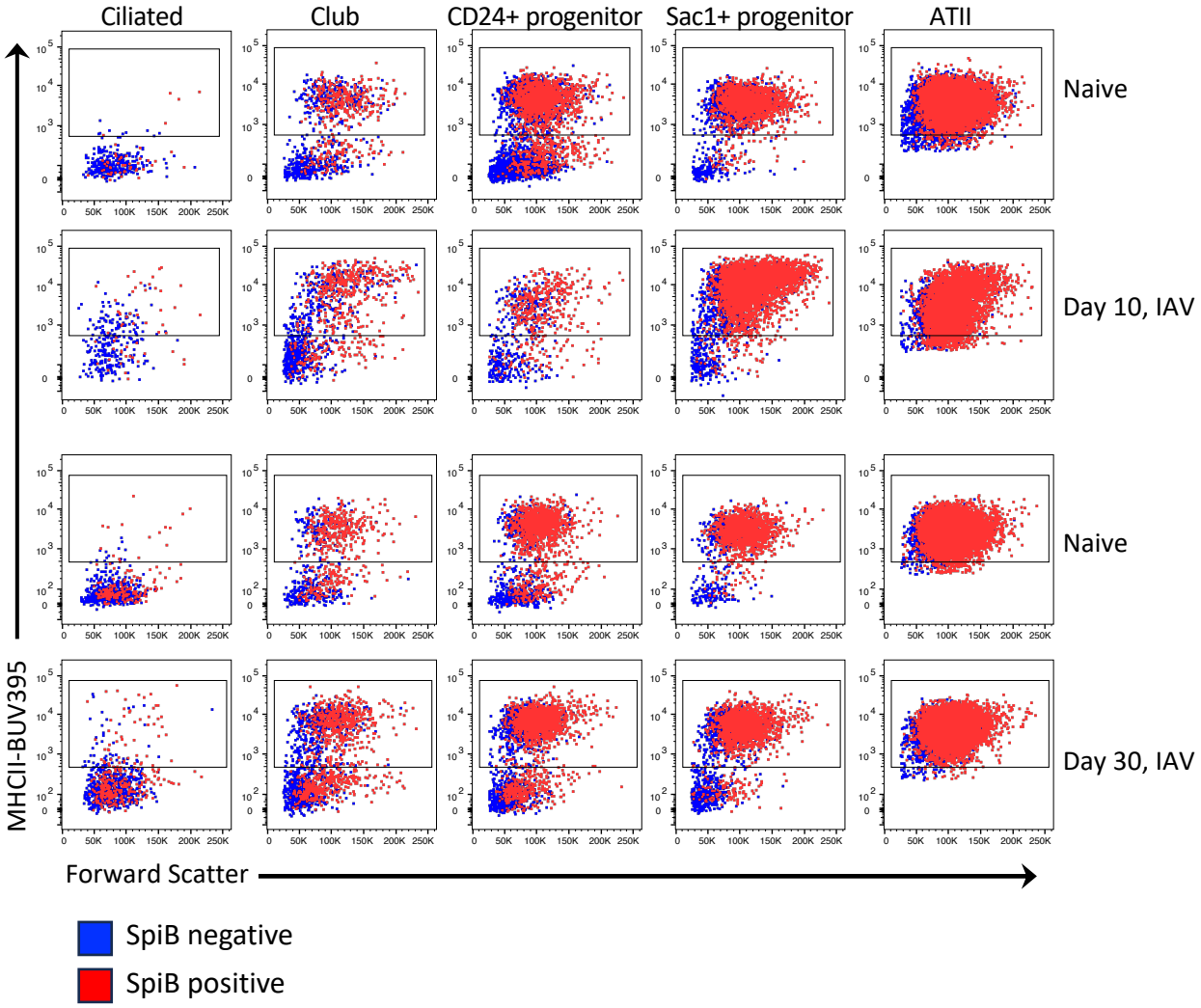
Supp Figure 5A: B cells and plasmacytoid DCs express the mCherry reporter molecule.

B.



Supp Figure 5B: SpiB reporter validation and gating strategy for lung epithelial cell subsets

C.

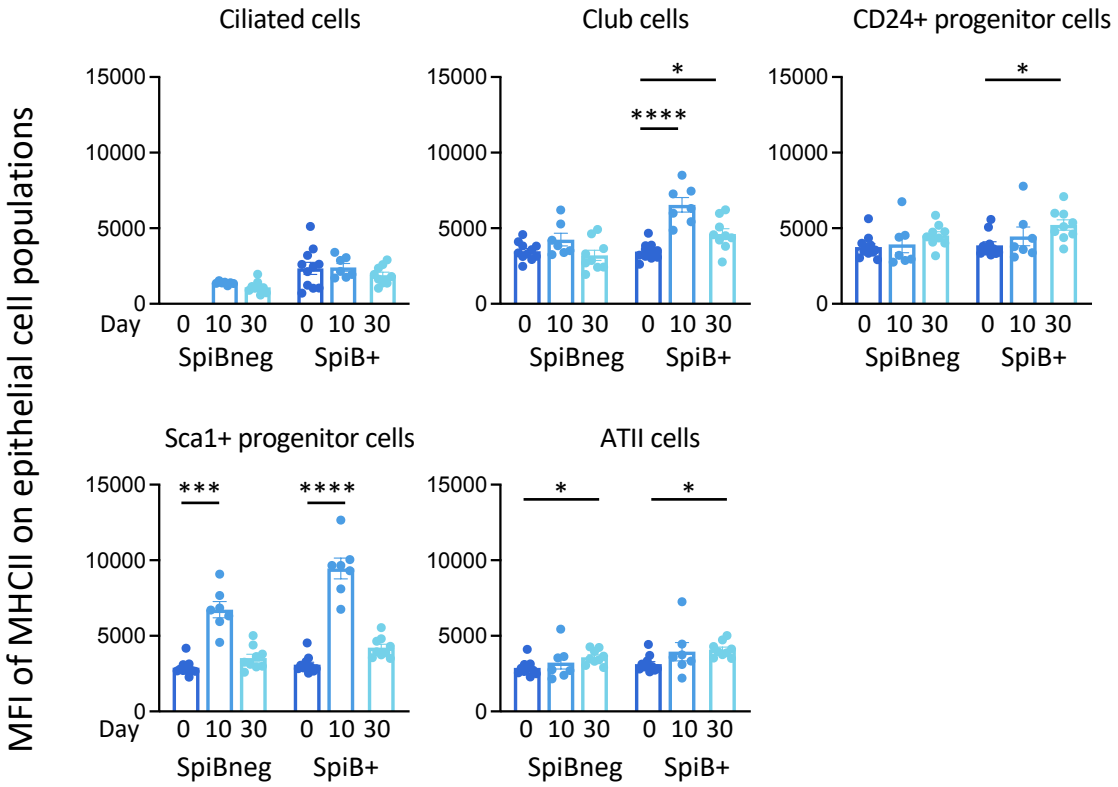


Supp Figure 5: SpiB reporter validation and gating strategy for lung epithelial cell subsets

(A): Spleen cells from naïve SpiB-mCherry reporter mice and wildtype C57BL/6 mice were analysed by flow cytometry and B cells and plasmacytoid DCs identified. Numbers indicate percentages within the indicated gates.

(B): SpiB-mCherry reporter mice and transgenic negative (Tg neg) littermates were infected i.n. with IAV (WSN)) on day 0 and culled at day 9 post infection. Lung cells were gated on live, single, lineage negative (CD45/31, CD1140a), EpCAM1+ epithelial cells that were either mCherry (SpiB) positive or mCherry (SpiB) negative. Subsets were then further identified: Ciliated cells (CD24hiSca1hi), club cells (CD24+Sca1+), CD24+ progenitors (CD24+Sca1-) Sca1+ progenitors (Sca1+CD24-), AT1 (CD24-Sca1- PDPN+) and ATII (CD24-Sca1-PDPN-MHCII+). Representative FACS plots are shown for (A) Tg negative littermates and (B) SpiB reporter mice. Numbers indicate the percentage within each gate.

(C): Representative plots of the SpiB-mCherry negative (blue) and positive (red) epithelial cell populations showing MHCII expression, percentages MHCII positive cells graphed in Figure 5.

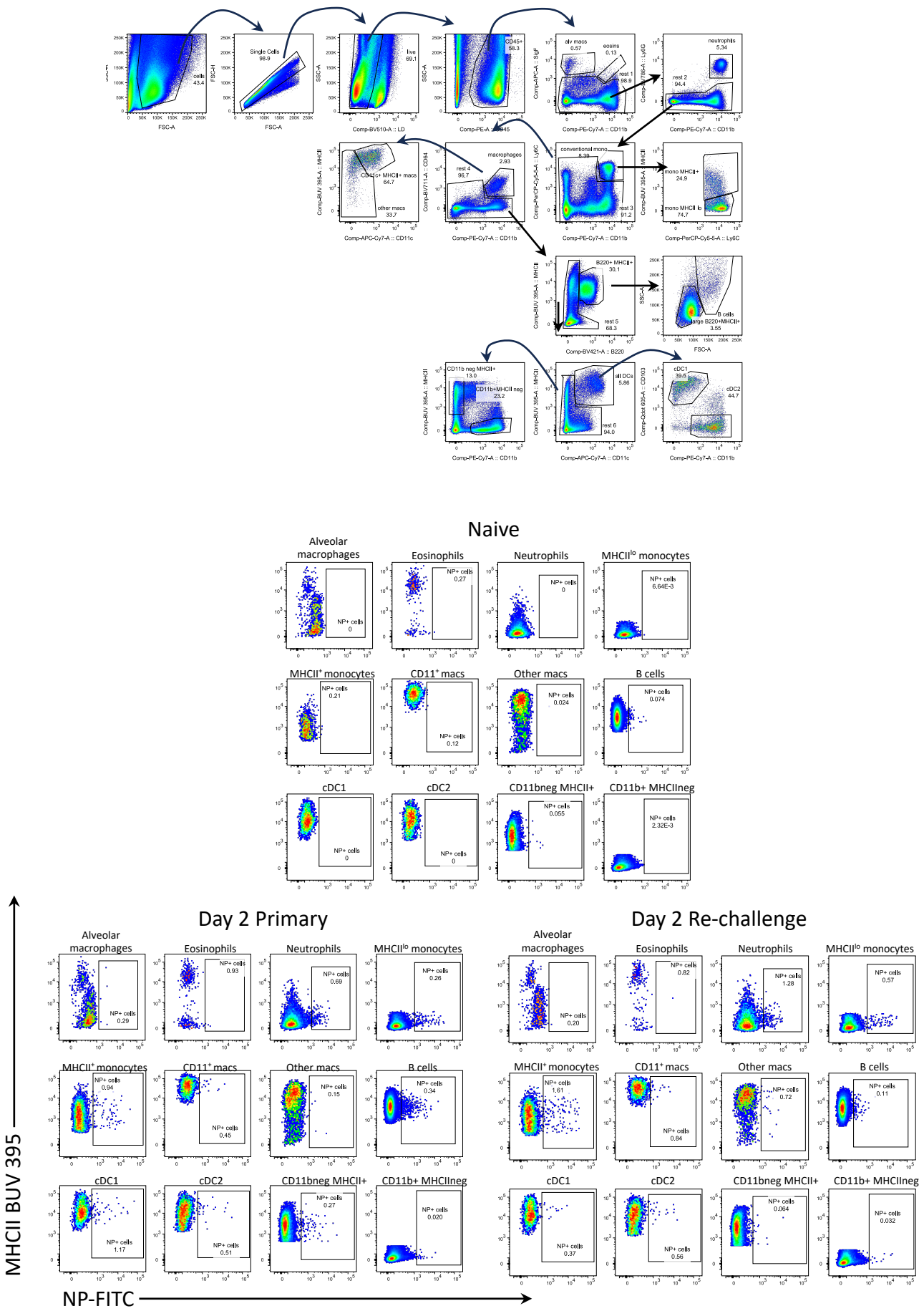


Supp Figure 6: MFI of MHCII on epithelial cell populations in SpiB negative and positive populations following infection

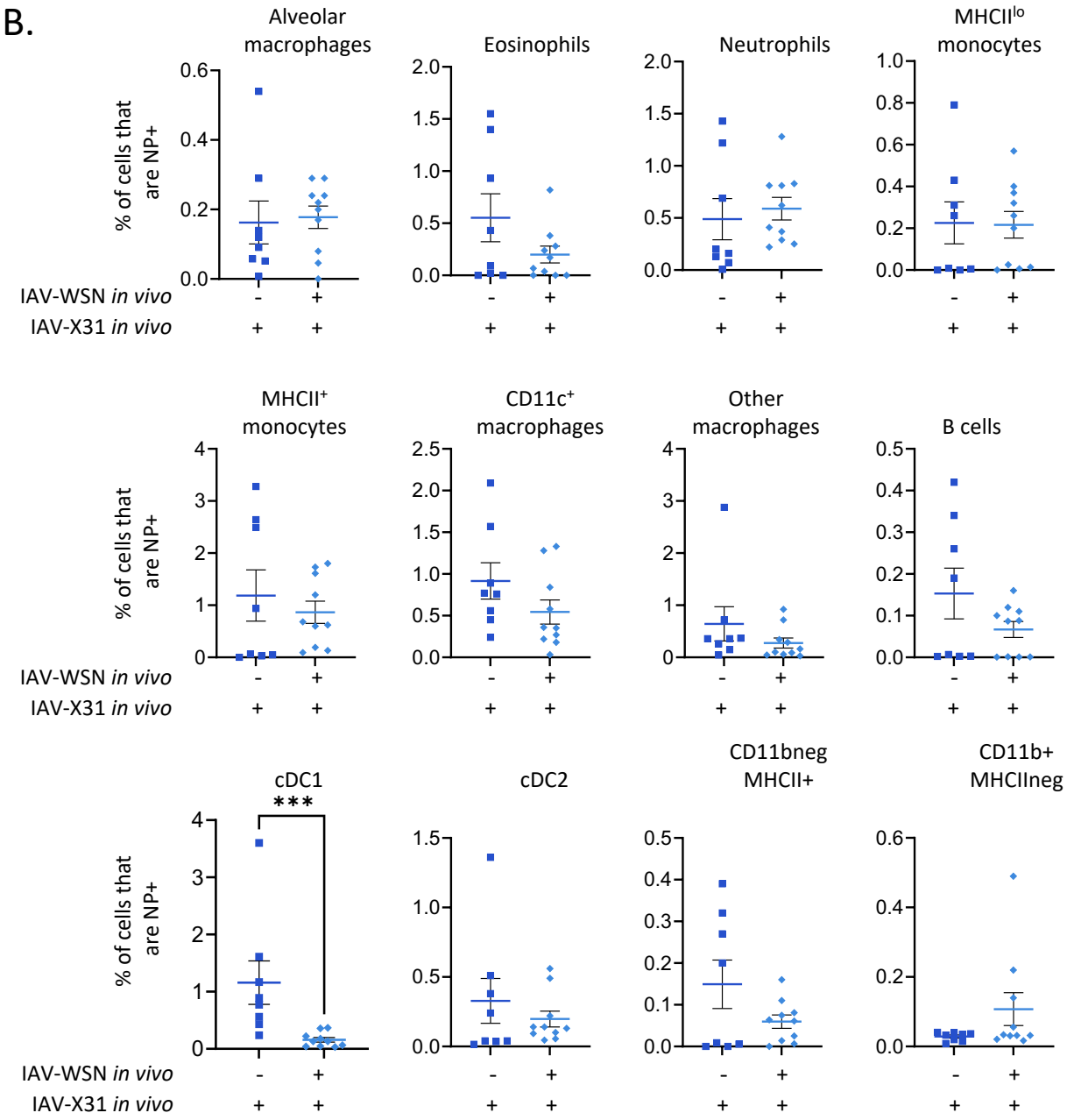
SpiB-mCherry reporter mice were infected with IAV (100-200PFU) and lungs taken at day 0, 10 or 30 post infection and epithelial cells examined by flow cytometry. The different epithelial cell subsets were identified as ciliated (CD24hiSca1hi), club (CD24+Sca1+), progenitor cells (CD24+Sca1neg), Sca1+ progenitor cells (Sca1+CD24neg), and alveolar epithelial cells (ATII: CD24negSca1negPdpnnegMHCII+). The MFI on the MHCII+ cells within each population was measured. Data are combined from two experiments with N=11 naïve, N=7 primary and N=10 memory with each mouse represented by a symbol and the bar showing the mean of the group with SEM error bars.

Data for ciliated and club cells are normally distributed, and are normally distributed for the other populations. No Spib negative cells are graphed for ciliated cells as too few MHCII+ cells were detected. Expression differences between cells at day 0 within each SpiB negative or positive subset were tested by ANOVA followed by Šidák's (normally distributed) or Dunn's (not normally distributed data) multiple comparison tests.

A.



Supp Fig 7A: Gating to identify IAV-NP+ CD45+ lung cells at day 2 post-infection



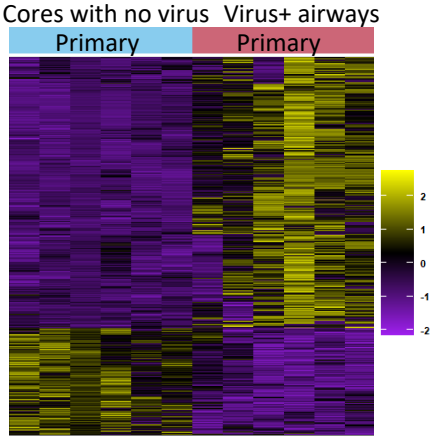
Supp Fig 7: Reduction in viral titers is not due to increased phagocytosis of IAV-NP antigen by APC after IAV re-challenge

C57BL/6 mice that were either naïve or infected 30 days earlier with IAV-WSN, were infected with IAV-X31, their lungs harvested 2 days later, and the presence of IAV-NP within the indicated CD45+ populations examined by flow cytometry. Cells are gated as shown in (A). Data are from two experiments with a combined number of (n=8) for X31 primary and (n=9) for re-infected groups with each symbol representing a mouse and the horizontal line showing the mean of the group, error bars are SEM. Data were not normally distributed, and differences tested by Mann Whitney, $p<0.001$=***.

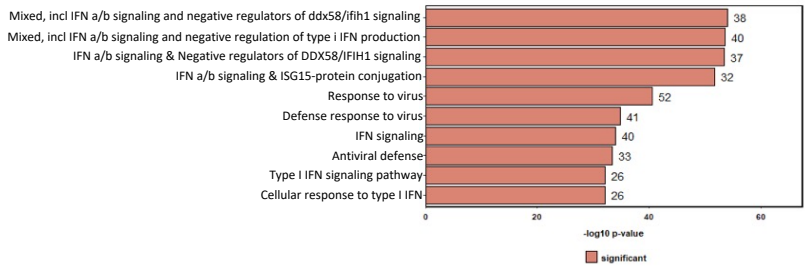
A.

Primary infected animals

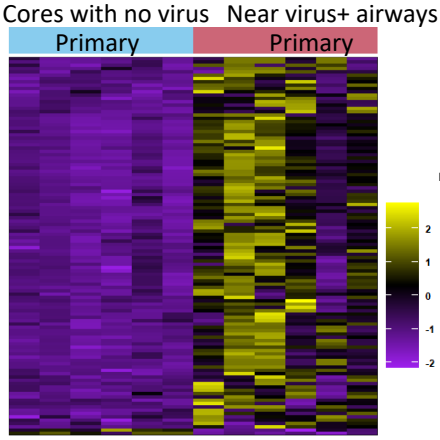
Cores with no virus Virus+ airways

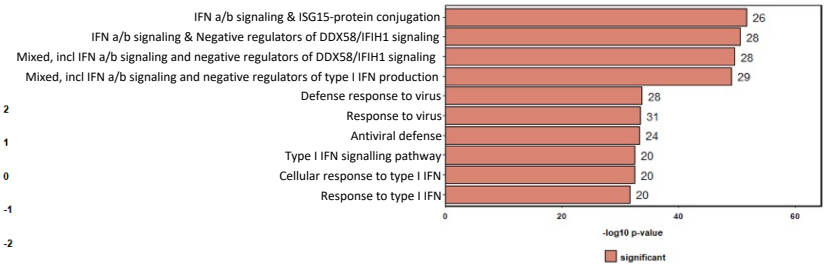


Over-representation Analysis

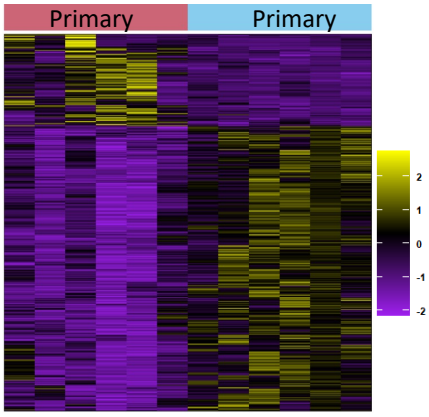


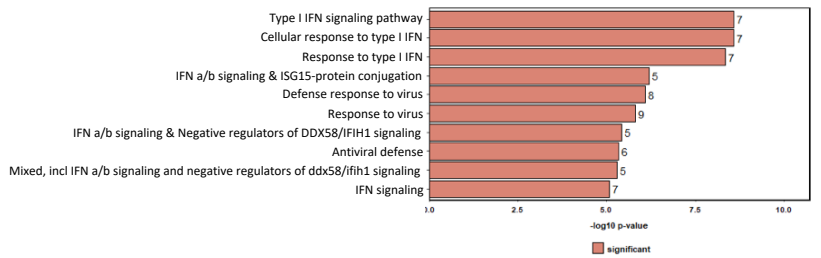
Cores with no virus Near virus+ airways





Cores with no virus Further from virus+ airways



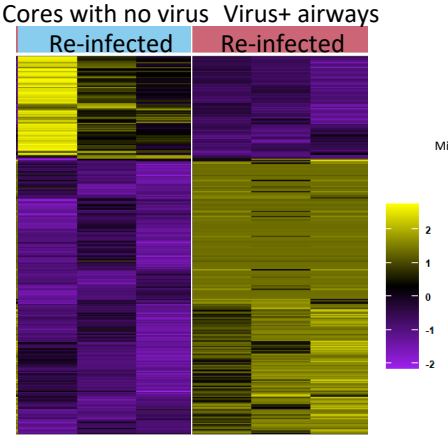


Supp Fig 8A: Primary and re-infected mice have a type I IFN response signature in virus+ airways

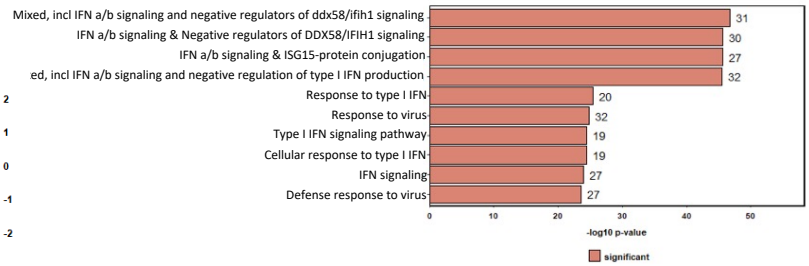
B.

Re-infected animals

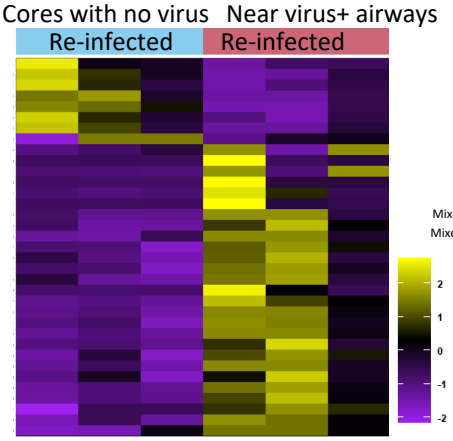
Cores with no virus Virus+ airways

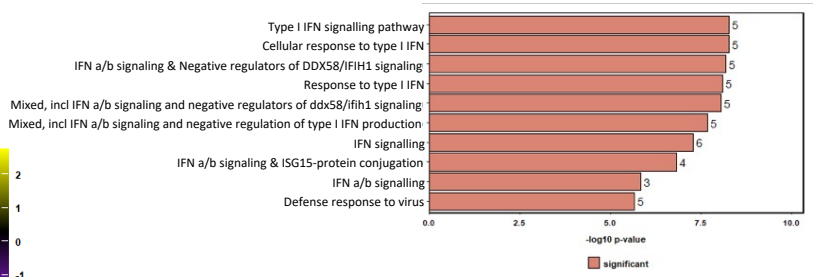


Over-representation Analysis

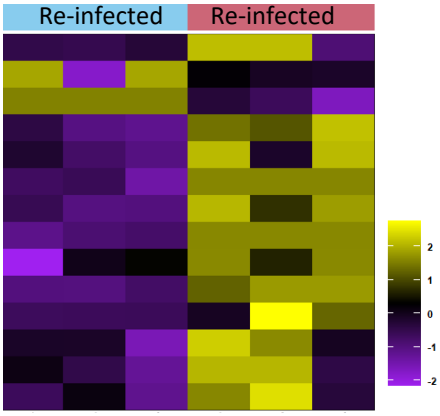


Cores with no virus Near virus+ airways





Cores with no virus Further from virus+ airways

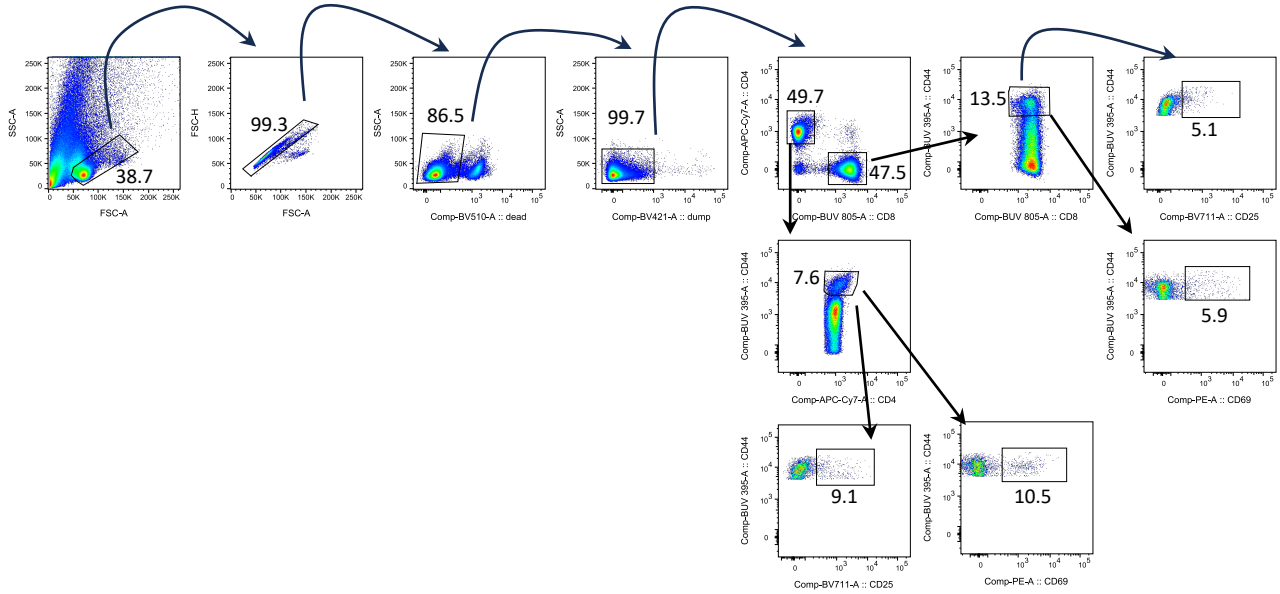


No over-enriched pathways

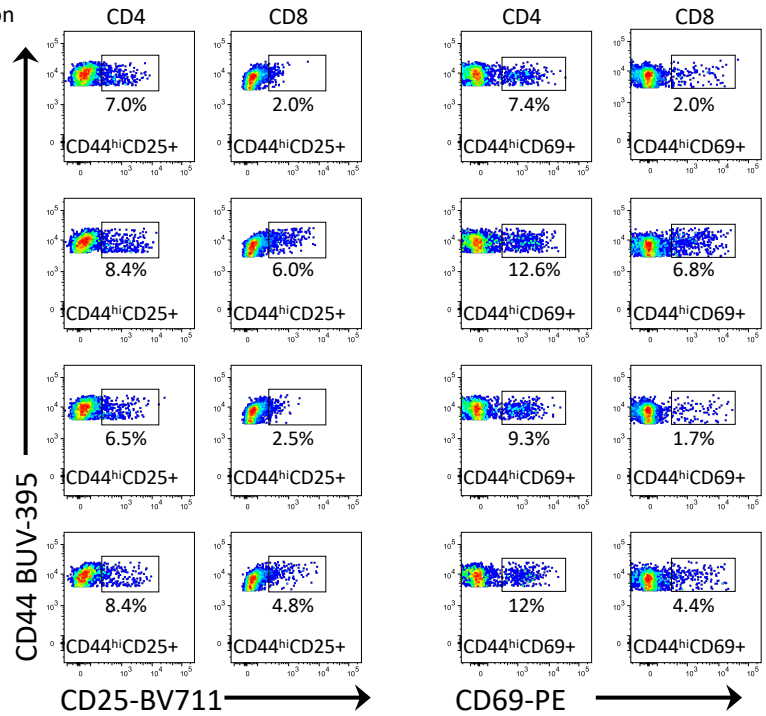
Supp Fig 8: Primary and re-infected mice have a type I IFN response signature in virus+ airways

C57BL/6 mice that were either naïve or infected 30 days earlier with IAV-WSN, were infected with IAV-X31, their lungs harvested 2 days later and embedded in paraffin. Virus positive and negative airways from 6 primary and 3 re-infected mice were analysed by GeoMX and heatmaps show DEG and enriched pathways in comparisons within primary or re-infected animals between airways from virus negative cores and either: virus+ airways; virus negative areas within the same airway as virus+ cells (Close); or airways adjacent to virus+ airways (Further).

A.



B.



Supp Fig 9: Lung structural cells can present IAV antigens to CD4 and CD8 T cells

Lung CD45 negative cells were isolated from naïve or C57BL/6 mice infected 30 days earlier with IAV-WSN. After 24 hours, the cells were infected with IAV-X31 and co-cultured with T cells isolated from the spleens of mice infected with IAV-X31 9 days earlier. Activated CD4 and CD8 T cells were gated as shown in A and representative FACS plots from each group are shown. Data are representative of two experiments with 4 mice within each naïve and Day 30 IAV group per experiment.