

Single-cell transcriptomics of bronchoalveolar lavage during PRRSV infection with different virulence

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Porcine reproductive and respiratory syndrome virus (PRRSV) causes significant economic losses in the global swine industry due to its high genetic diversity and different virulence levels, which complicate disease management and vaccine development. This study evaluated longitudinal changes in the immune cell composition of bronchoalveolar lavage fluid and the clinical outcomes across PRRSV strains with varying virulence, using techniques including single-cell transcriptomics. In highly virulent infection, faster viral replication results in an earlier peak lung-damage time point, marked by significant interstitial pneumonia, a significant decrease in macrophages, and an influx of lymphocytes. Viral tracking reveals less than 5% of macrophages are directly infected, and further analysis indicates bystander cell death, likely regulated by exosomal microRNAs as a significant factor. In contrast, the peak intermediate infection shows a delayed lung-damage time point with fewer cell population modifications. Furthermore, anti-inflammatory M2-like macrophages (SPPI-CXCL14^{high}) are identified and their counts increase during the peak lung-damage time point, likely contributing to local defense and lung recovery, which is not observed in high virulent infection. These findings provide a comprehensive description of the immune cellular landscape and differential PRRSV virulence mechanisms, which will help build new hypotheses to understand PRRSV pathogenesis and other respiratory infections.

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important viral diseases affecting the global swine industry as it can cause mild to severe reproductive disorder in sows and respiratory problems in infected newborn and growing pigs. The causative agent, porcine reproductive and respiratory syndrome virus (PRRSV), is an enveloped, single-stranded positive-sense RNA

virus belonging to the genus *Betaarterivirus*, family *Arteriviridae*, and order *Nidovirales*. The virus infects cells via the CD163 receptor, which is expressed exclusively in myeloid cells. PRRSV primarily infects subpopulations of tissue-resident macrophages in the lungs (porcine alveolar macrophages [PAMs]) and less efficiently in circulating blood monocytes and monocyte-derived cells^{1–3}. Infection

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results in viremia that lasts for 4–5 weeks; however, the virus can persist in lymphoid organs including tonsils and lymph nodes, thus establishing a life-long infection⁴.

The aberrant host immune responses, which are characterized by dysregulated cell-mediated immune response with impaired T-cell activation and delayed/dampened activation of adaptive immunity, are induced by PRRSV infection^{4–6}. Systemically, the virus induces limited levels of proinflammatory cytokines in the serum unlike other swine viruses, such as ASFV or influenza virus infection. Additionally, peripheral T cell response during PRRSV infection appears in a delayed form after three weeks post-infection^{7,8}. However, a local increase of proinflammatory cytokines/chemokines in the lung is known to correlate with the development of interstitial pneumonia along with the modulation of cell population, which is characterized by PAM loss and immune cell infiltration, within 7–14 days post-infection^{8–12}. Given the high genetic heterogeneity of PRRSV, which is the highest among RNA viruses^{13,14}, the more virulent viruses, such as Chinese high pathogenic PRRSV-2 (HP-PRRSV) or PRRSV1 subtype 3 Lena strain, induce higher viral loads and faster disease progression with earlier cell population modulation in the lungs^{11,15–17}.

Bronchoalveolar lavage fluid (BALF) has provided valuable insights into the microenvironment for a wide range of cell populations, including molecules that mediate cell communication such as cytokines, chemokines, and exosomes, cell membrane-derived vesicles that transfer materials, such as microRNAs (miRNAs) and viral proteins^{18–20}. For instance, single-cell RNA sequencing (scRNA-seq) on BALF cells from patients with COVID-19²¹ or from a ferret model²² has identified potential mechanisms underlying the pathogenesis and recovery of COVID-19. Analysis of exosomes isolated from the BALF of patients with coronavirus infection revealed that host molecules, as well as viral proteins, could be loaded into exosome, facilitating viral entry and immune escape^{23,24}.

BALF has also been widely utilized in studying PRRSV pathogenesis. Transcriptome studies using the total RNA of PRRSV-infected BALF cells have indicated the upregulation of multiple proinflammatory cytokines (IL-8 and TNF- α) and chemokines and their receptor genes (CXCL10, CCR5, CCL4, and CCL2)^{25–27}. However, immunohistochemistry of the lung sections⁹ or flow cytometry of BALF cells^{15,26} reveals that no more than 2% of the PAMs harbor viral antigens, which indicates that the transcriptome responses detected from whole BALF cells do not reflect the responses of cells directly infected with PRRSV but rather combined responses from both infected and bystander cells. A study using PRRSV expressing green fluorescent protein to discriminate between the infected and bystander cells showed that the upregulated negative immune regulators, including NF- κ B inhibitors and immune checkpoint molecules (PD-L1, PD-L2, IL-10, IDO1, and TGF β 2), were detected from PRRSV-infected PAMs compared to bystander cells²⁶. Given the complexity of cell-to-cell communication in the context of PRRSV infection, there is a growing need for single-cell-based transcriptome analysis^{28,29}.

This study employed three PRRSV-2 strains (JB15-N-M8-GN; NA8, JA142, and JB15-N-PJ10-GN; NA10) with varying levels of virulence^{10,30,31}. The results demonstrate the single-cell landscape in BALF for both the negative and PRRSV-infected pigs, and the data were compared across a time-series to quantitatively assess cell changes and identify differential immunological mechanisms (Fig. 1a). The time-series landscape for the immune microenvironment revealed significant shifts in the proportions and characteristics of immune cells over time. Subpopulations of myeloid (macrophages and DCs), lymphoid 1 (NK and T cells), and lymphoid 2 cells (B cells) were constructed and showed distinct gene expression patterns and viral tracking in the myeloid population. Additionally, the differential functions related to cell death signals using exosomes in BALF were validated.

Results

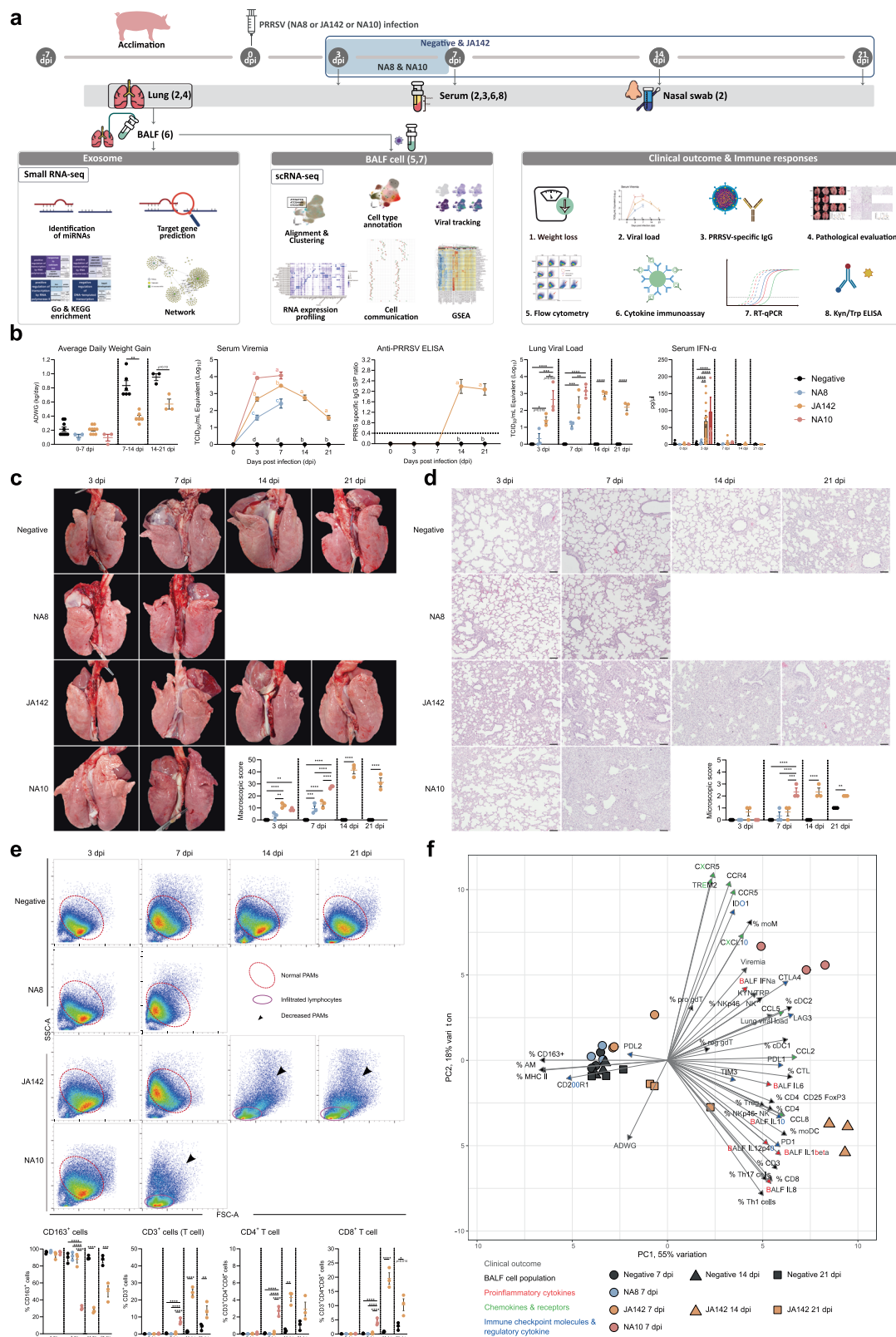
Clinical outcomes of PRRSV infections with different virulence

During the infection period, infected pigs displayed clinical outcomes in a virulence-dependent manner. This was demonstrated by the average daily weight gain (ADWG), viral loads, and lung pathology. The ADWG of both infected and negative pigs were found to be similar during 0–7 dpi period. JA142-infected pigs showed significantly lower ADWGs than the negative pigs during the 7–14 dpi period, but started to recover their weight gain during the 14–21 dpi period (Fig. 1b). The highest serum virus titers of all infected groups were detected at 7 dpi, with a virulence-dependent manner ($10^{2.41}$, $10^{3.47}$, and $10^{4.07}$ 50% tissue culture infectious dose [TCID₅₀/mL for the NA8, JA142, and NA10, respectively). Furthermore, at the initial viremia at 3 dpi ($10^{1.59}$, $10^{2.68}$, and $10^{3.92}$ TCID₅₀/mL for the NA8, JA142, and NA10, respectively). A similar trend was observed in the shedding of the virus measured through nasal swab. Following the peak viral load, the virus titer exhibited a gradual decrease in the JA142-infected group, as expected. Only serum IFN- α could be detected from the JA142- and NA10-infected groups in a virulence-dependent manner, while other cytokines were under the detection limits. The results from the lungs collected at 3, 7, 14, and 21 dpi showed that the initial lung viral load at 3 dpi was also dependent on virulence ($10^{0.33}$, $10^{1.42}$, and $10^{2.65}$ TCID₅₀/mL for NA8, JA142, and NA10, respectively), with the peak lung viral load for JA142-infected lungs occurring at 14 dpi, whereas that for NA10 occurred at 7 dpi. The macroscopic (Fig. 1c) and microscopic (Fig. 1d) lung lesions exhibited a similar trend. The structural differences between NA8, JA142, and NA10 are illustrated in Supplementary Fig. 1.

Immunological responses in BALF

A significant reduction in the number of CD163⁺ cells or normal PAMs, was observed in the BALF cells of JA142 and NA10 at 14 dpi and 7 dpi, respectively, in which the cell population reached only ~30% of the total cell population (Fig. 1e, red arrow). In contrast, all negative animals and the NA8 group stably maintained their respective populations (Fig. 1e, red circle). Given that the most significant modulation of cell population occurred concurrently with the highest lung viral load and histopathology, 14 dpi of JA142 and 7 dpi of NA10 were identified as the peak lung-damage time points. At the same peak lung-damage time point for each strain, a population compatible with a mixture of neutrophils, monocytes, and lymphocytes was observed to infiltrate the BALF (Fig. 1e, pink circle)¹⁵. A significant infiltration of CD3⁺ T cells was observed in the BALF, with a higher population of CD8⁺ T cells than CD4⁺ T cells in both infected groups. Further analysis was conducted on monocyte/DC subsets (AM ϕ , moM ϕ , moDC, cDC1, and cDC2), T cell subsets (CTL, Th1, Th17, T_{reg}, and CD4⁺CD25⁺FoxP3⁺ cells), and NK cells (NKp46[−] NK and NKp46⁺ NK cells) (Supplementary Fig. 2) exhibited a corresponding increase in number to the decrease in the CD163⁺ cells. All cell subsets of JA142 that increased at 14 dpi gradually decreased at 21 dpi.

In addition, significant increases in BALF for the proinflammatory cytokines (IFN- α , IL-1 β , IL-6, IL-8 and IL-12p40) and the regulatory cytokine IL-10 were detected in the BALF of JA142 14 dpi, whereas the levels of proinflammatory cytokines (IFN- α , IL-1 β , and IL-8) and IL-10 were significantly increased in the NA10 group at 7 dpi, but to a lesser extent than in the JA142 at 14 dpi (Supplementary Fig. 3). Significantly upregulated transcript-level expression of chemokines (CCL2, CCL5, CCL8, and CXCL10) and receptors (CCR4, CCR5, CXCR5, and TREM2) in the BALF cells was detected in the NA10 group at 7 dpi, whereas transcript-level expression of chemokines but not receptors was significantly upregulated at 14 dpi in the JA142 group (Supplementary Fig. 4). Transcript-level expression of the immune checkpoint molecules (PD-1, PD-L1, CTLA4, TIM3, LAG3, and IDO1) was also significantly upregulated at the peak lung-damage time point in both strains



(Supplementary Fig. 5). Consistent with the upregulation of IDO1, the ratio of serum kynurenine to tryptophan (Kyn/Trp) was significantly increased in the JA142 and NA10 groups and showed a negative correlation with ADWG (Supplementary Fig. 6). All measured variables were summarized using principal component analysis and are shown in Fig. 1f.

Single-cell transcriptomes of BALF cells and viral tracking according to PRRSV virulence

A total of 306,052 BALF cells were obtained from 36 pigs after filtering out low-quality cells. They were classified into seven cell types (DC, NK cells, T cells, B cells, alveolar epithelial type I [ATI], and alveolar epithelial type II [ATII]), excluding unknown cells (Fig. 2a). These cells

Fig. 1 | Study design, clinical outcomes, and immune responses in bronchoalveolar lavage fluid (BALF) with porcine reproductive and respiratory syndrome virus (PRRSV) infections of varying virulence. **a** Schematic representation of the study design. **b** Clinical outcomes including average daily weight gain (ADWG), serum viremia, S/P ratio of PRRSV-specific IgG, lung viral load, and serum IFN- α protein levels of each pig from negative control ($n = 12$) and PRRSV-infected groups including NA8 ($n = 6$), JA142 ($n = 12$) and NA10 ($n = 6$). Circles indicate a single biological replication and the data are presented as mean values $\pm$ SEM. **c** Representative gross and histopathological appearances of the lungs in each group. Gross lung lesion scores were calculated as the average of the five lobes measured on a range of 0–50. Circles indicate a single biological replication and the data are presented as mean values $\pm$ SEM. **d** Representative histopathologic appearance of the lung in each group. The microscopic score for lung lesions measured the extent of interstitial pneumonia in the five lobes of the lung, ranging from 0 to 3 and then averaged. The scale bar size indicates 100 μ m. Circles indicate

a single biological replication and the data are presented as mean values $\pm$ SEM. **e** Representative flow cytometry dot plot of BALF cells and proportion of each cell type (CD163 $^{+}$ cells, CD3 $^{+}$ cells, CD4 $^{+}$ T cells and CD8 $^{+}$ T cells). Red circles indicate normal PAMs, pink circles indicate lymphocytes, and red arrows indicate decreased PAMs. In the scatter plot, circles indicate a single biological replication and the data are presented as mean values $\pm$ SEM. **f** Principal Components Analysis (PCA) of clinical outcomes and immune responses measured in this study. Statistical analysis was performed using two-way ANOVA analysis with Tukey's multiple comparisons test for four groups (0, 3, and 7 dpi) or Sidak's multiple comparison test for two groups (14 and 21 dpi). For ADWG, one-way Kruskal–Wallis test (0–7 dpi) and two-tailed Mann–Whitney test (7–14 dpi and 14–21 dpi) was performed. Statistically significant differences between groups have been marked with asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$) or letters ($p < 0.05$) for serum viremia and PRRSV-specific IgG. Source data are provided as a Source Data file.

were further classified into three categories (myeloid, lymphoid, and epithelial) (Fig. 2b). The myeloid category accounted for the majority (92.7%; $n = 283,735$), lymphoid accounted for a minority (6.5%; $n = 19,998$), and epithelial cells were rare (0.1%; $n = 221$). Macrophages ($n = 282,069$) and DCs ($n = 1,666$) were identified in myeloid cells. NK ($n = 1,937$), T ($n = 11,835$), and B ($n = 6,226$) cell types were identified in the lymphoid tissue. Both ATI ($n = 179$) and ATII ($n = 42$) cells were detected in the epithelium. The representative gene expression levels are shown in Fig. 2c and the levels in each virulence are shown in Supplementary Fig. 7. Among the cell populations in each of the four groups (negative, NA8, JA142, and NA10; Supplementary Fig. 8a), the negative ($n = 117,039$) and NA8 ($n = 61,915$) groups were found to have over 99% myeloid cells, whereas the JA142 ($n = 83,808$) and NA10 ($n = 43,290$) groups were found to have 12% and 21% lymphoid and myeloid cells, respectively (Supplementary Fig. 8b).

The time-series changes in the BALF cells for each strain were also identified (Fig. 2d), and the cell numbers in each group and time point are shown in Fig. 2e. In accordance with the flow cytometry results (Fig. 1e) total cell numbers at 14 and 21 dpi for the JA142 and 7 dpi for the NA10 were significantly lower than the other tested time points and groups (Fig. 2e, Supplementary Fig. 9a), and high proportions of the lymphoid cells were observed at 14 and 21 dpi for the JA142 (46% and 20%, respectively) and 7 dpi for the NA10 (77%) (Fig. 2g). Viral infected cells and their densities were identified based on the open reading frame (ORF)7 viral transcripts (>10 transcripts) in each group with the exclusion of a small proportion of lymphoid cells showing low expression levels (<10 transcripts) (Supplementary Fig. 8c, d), and PRRSV-infected cells in the myeloid were identified in the NA8 ($n = 13$, 0.02%), JA142 ($n = 1,507$, 2.08%), and NA10 groups ($n = 481$, 1.32%) (Supplementary Data 1). The time-series distribution of the infected cells was identified for each group (Fig. 2f, h) in accordance with the lung viral load (Fig. 1b). The JA142 and NA10 groups had a very low number of infected cells, whereas the NA8 group had even lower numbers of infected cells ($\sim 5\%$) (Fig. 2h). No significant differences were observed in the proportions of the infected cells identified among 7 dpi for the NA8, three-time points (7, 14, and 21 dpi) for the JA142, and two-time points (3 and 7 dpi) for the NA10 (Supplementary Fig. 9b).

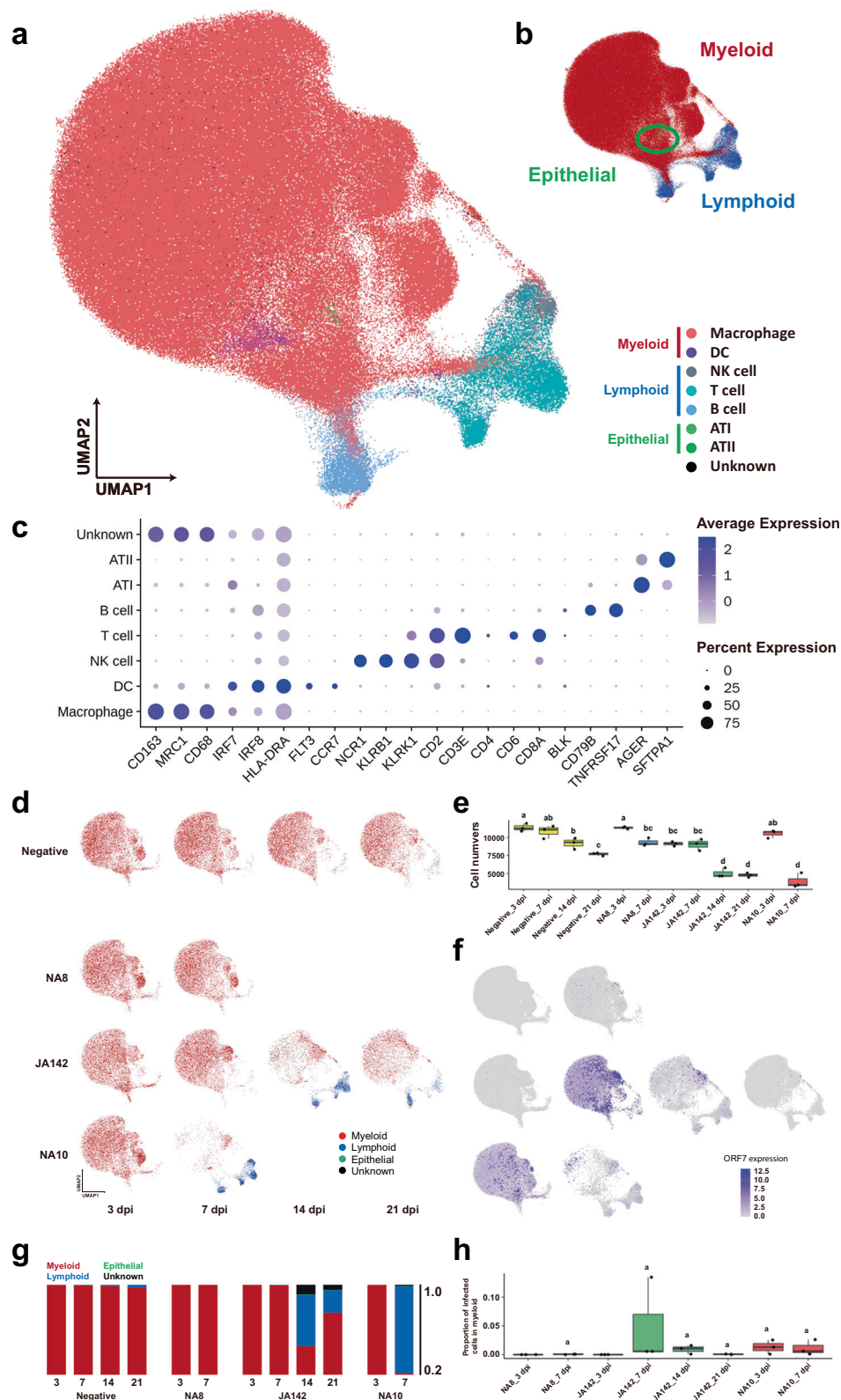
Time-series changes in macrophage and DC subpopulation

Macrophage and DC (myeloid) subpopulations ($n = 283,735$) were independently focused on and scrutinized to identify detailed sub-cell types. This subpopulation consisted of 23 clusters based on the described specific features, excluding unknown features (Fig. 3a). Most cells were evenly distributed; some sub-cell types were primarily identified in clusters of SPPI-CXCL14 high macrophages, proliferating SPPI high macrophages, and PDCDILG2 high macrophages (Fig. 3a, Supplementary Fig. 10a). The distribution of infected cells was confirmed in macrophage and DC subpopulations (Fig. 3b, c). MRC1-COX2 high

macrophages showed high expression levels for the viral transcripts in both JA142 and NA10 infections; however, their densities were not consistent in some regions. Although high densities of infected cells in the MRC1-COX2 high macrophages are commonly identified, ISAG high macrophages in JA142 and MT1A high macrophages in the NA10 also showed high densities. However, only a few infected cells were observed in the MT1A high macrophages because of the low expression of viral transcripts (Supplementary Fig. 10b). A table of representative gene expression is shown in Fig. 3d and the levels in each virulence are shown in Supplementary Fig. 11. The IFN signaling-associated gene (ISAG) high macrophages and proliferating ISAG high macrophages showed high expression levels of *ISG15*, *MX1*, *MX2*, and *OAS1*. SPPI-expressing sub-cell types (SPPI-CXCL14 high macrophages, SPPI-CCL2 high macrophages, and proliferating SPPI high macrophages) also expressed *TREM2*, *MS4A7*, *SPON2*, and *ACP5*. Time-series changes in the proportion of sub-cell types were identified and compared among the strains (Fig. 3e, Supplementary Fig. 12). The macrophage and DC sub-cell types in the negative and NA8 groups exhibited no quantitative changes across different time points. However, certain time points for specific sub-cell types showed high variability, such as MRC1-NREP high macrophages at 21 dpi in the negative group and MT1A high macrophages at 7 dpi in the NA8 group. Most sub-cell types in the JA142 group were maintained up to 7 dpi, then decreased at 14 dpi, and recovered at 21 dpi. The sub-cell types in the NA10 group were maintained up to 3 dpi and rapidly decreased at 7 dpi. In contrast to the decreasing number of macrophages, SPPI-expressing sub-cell types (SPPI-CXCL14 high macrophages, SPPI-CCL2 high macrophages, and proliferating SPPI high macrophages) and DCs (cDC and pDC) increased at the same time points. Only MT1A high macrophages showed a different pattern, as the JA142 group had low proportions at all dpi.

Time-series changes in NK and T cell subpopulation

NK and T cell (lymphoid 1) subpopulations ($n = 13,772$) were dissected to identify sub-cell types, resulting in 18 clusters (Fig. 4a). The JA142 and NA10 groups were the predominant groups, showing even cell distribution (Fig. 4b). A table of representative gene expression is shown in Fig. 4c and the levels in each virulence are shown in Supplementary Fig. 13. NK and T cells are largely separated based on the expression of *NCR1*, *KLRB1*, and *CD3E*. Activated NK cells were defined using the expression of *IL2RA*. In the T sub-cell types, most ISAG high -expressing cells (ISAG high CD4 $^{+}$ T cells, ISAG high CD8 $^{+}$ T cells, and proliferating ISAG high CD8 $^{+}$ T cells) showed high *ISG15*, *MX1*, and *MX2* expression. In addition, some regulatory T cells (regulatory [exhausted] CD8 $^{+}$ T and T $_{reg}$ cells) indicated specific features, i.e., high *PDCD1*, *IL10*, *IRF8*, *ICOS*, and *CTLA4* expression. In this subpopulation, time-series changes in the proportion of sub-cell types were identified and compared among the strains (Fig. 4d, Supplementary Fig. 14). The negative group showed a few numbers in some NK and T cell sub-cell



types (mature NK cells, ZNF683⁺ [Tissue-resident] CD8⁺ T cells, proliferating CD8⁺ T cells, CD2⁺ gamma-delta T cells, and CD2⁺ gamma-delta T cells) at 14 and 21 dpi. Although the NA8 group showed no quantitative changes after infection, the JA142 and NA10 groups showed an increasing trend. The NK and T sub-cell types in the NA10 group rapidly increased at 7 dpi; however, the number of cells increased relatively less in the JA142 group at 14 dpi and then decreased at 21 dpi.

Time-serial changes in B cell subpopulation

The B cell (lymphoid 2) subpopulation ($n = 6,226$) was divided into 17 sub-cell types (Fig. 5a). The JA142 and NA10 groups were predominant, showing an even cell distribution in the B cells and a JA142 majority in the plasma cells (Fig. 5b). A table of representative gene expression is shown in Fig. 5c and the levels in each virulence are shown in Supplementary Fig. 15. Among the sub-cell types, more B cells expressed *BLK* and *CD79B* than the plasma cells. In addition, plasma cells showed

Fig. 2 | Single-cell transcriptomes of bronchoalveolar lavage fluid (BALF) cells from negative and porcine reproductive and respiratory syndrome virus (PRRSV)-infected pigs. **a** Uniform Manifold Approximation and Projection (UMAP) of 306,052 BALF cells from negative ($n = 12$), NA8-infected ($n = 6$), JA142-infected ($n = 12$), and NA10-infected ($n = 6$) pigs. The colors indicate annotated cell types. **b** UMAP showing three cell categories (myeloid, lymphoid, and epithelial). **c** Specific marker gene expression levels for seven basic cell types. Average expression levels were calculated using log-normalized values. **d** Time-serial UMAP projection at 3 ($n = 34,040$), 7 ($n = 32,433$), 14 ($n = 27,531$), and 21 ($n = 23,035$) dpi for the negative; 3 ($n = 33,989$) and 7 ($n = 27,926$) dpi for the NA8; 3 ($n = 27,361$), 7 ($n = 27,011$), 14 ($n = 15,114$), and 21 ($n = 14,322$) dpi for the JA142; and 3 ($n = 31,525$) and 7 ($n = 11,765$) dpi for the NA10 groups with annotations for the cell categories

(myeloid, lymphoid, and epithelial). **e** Average cell numbers for each time point and group. **f** Time-series viral tracking based on the log-normalized ORF7 expression levels in each virulence group (NA8, JA142, and NA10). **g** Time-series proportions for the cell categories (myeloid, lymphoid, and epithelial). **h** Average proportion of infected myeloid cells at each time point and for each virulence group. **e, h** Each group is depicted using a box-and-whisker plot, illustrating three biological replications. The lower boundary of the box corresponds to the first quartile (Q1), the middle line marks the median, and the upper boundary represents the third quartile (Q3). The whiskers extend to indicate the minimum and maximum values within the dataset, respectively. Different lowercase letters above box indicate significant differences ($p < 0.05$) determined using one-way ANOVA analysis with Tukey's multiple comparison test. Source data are provided as a Source Data file.

relatively higher expression levels for the *JCHAIN* and *MZB1* than the B cells. Time-series changes were identified for the proportions of B cells and plasma sub-cell types (Fig. 5d, Supplementary Fig. 16). In both the JA142 and NA10 groups, the proportions of B and plasma sub-cell types increased at 14 and 7 dpi, respectively. Subsequently, most proportions of B sub-cell types in the JA142 group decreased at 21 dpi, but those of plasma sub-cell types increased. In addition, the proportion of HSPA6^{high} plasma cells increased at 14 and 21 dpi after the JA142 infection but did not increase in NA10.

Time-serial changes in epithelial cells

Epithelial cells ($n = 221$) consisted of the ATI and ATII sub-cell types, and time-series changes in their proportions were identified (Supplementary Fig. 17). In both the JA142 and NA10 groups, the proportions of the ATI sub-cell type increased at 14 and 7 dpi, respectively, and decreased at 21 dpi in the JA142 group. ATII sub-cell type also indicated a similar pattern to ATI, but the 14 dpi in the JA142 group showed high variability.

Characterization of cytokine, chemokine, and their receptors

The expression of cytokines, chemokines, and their receptors was analyzed using the time-series data for each strain. In the negative group, the expression of *CXCL2*, *PPBP* (*CXCL7*), ENSSSCG00000017920 (*CXCL16*), *CCL14*, and ENSSSCG00000035736 (*CCL24*) in the macrophages was common at all dpi (Supplementary Fig. 18a, Supplementary Fig. 19a, Supplementary Fig. 20a, Supplementary Fig. 21a). In addition, CXCL2^{high}, SPPI-CCL2^{high}, and proliferating SPPI^{high} macrophages showed higher expression of *TNF*, *IL1A*, *CXCL8*, *CCL3L1*, *CCL4*, *CCL5*, and *CCL8*. SPPI-CCL2^{high}- and ISAG-expressing macrophages also showed higher expression of *CXCL10*. The NA8 group showed cytokine and chemokine expression comparable to that observed in the negative group (Supplementary Fig. 22a, Supplementary Fig. 23a). Similar expression patterns were confirmed in macrophages at 3 and 7 dpi for the JA142 (Supplementary Fig. 24a, Supplementary Fig. 25a); however, the expression of *IL18*, *IL27*, *CXCL2*, *CXCL10*, *CXCL14*, *CCL2*, *CCL4*, *CCL5*, and *CCL8* increased at 14 dpi (Supplementary Fig. 26a), and the expression of *CXCL2*, *CXCL10*, *CCL2*, *CCL4*, *CCL5*, and *CCL8* decreased at 21 dpi (Supplementary Fig. 27a). At 3 dpi, the NA10 group had comparable cytokine and chemokine expression patterns to the negative group in terms of their macrophages (Supplementary Fig. 28a), and at 7 dpi showed patterns similar to those observed at 14 dpi for JA142 (Supplementary Fig. 29a). However, unlike macrophages at 14 dpi in JA142, 7 dpi in NA10 showed higher expression of ENSSSCG00000039214 (*IL1B*) and lower expression of *CXCL14* and ENSSSCG00000035736 (*CCL24*). To identify the characteristics of the other cell types (DC, NK, T, and B cells), 14 dpi in JA142 and 7 dpi in NA10 were focused on, as they have abundant cell numbers. DC and NK cells showed high expression levels for *IL18* and *XCL1* in both JA142 and NA10 cells, respectively (Supplementary Fig. 26a, Supplementary Fig. 29a). Approximately 50% of the NK and T cells exhibited high *IL16* expression, and T_{reg} cells showed high *IL10*

expression. In addition, CD8-expressing T cells showed higher expression of *CCL5* when compared with other T cells, and regulatory (exhausted) CD8⁺ T cells showed higher expression of *IFNG*, *CCL3L1*, and *CCL4*.

In the negative control group, *IFNAR1*, *IFNGR1*, *IFNGR2*, ENSSSCG00000012397 (*IL2RG*), *CSF2RB* (*IL5RB*), *NCK1* (*IL2ORB*), *ACKR4*, and *CCR1* were expressed at all dpi analyzed (Supplementary Fig. 18b, Supplementary Fig. 19b, Supplementary Fig. 20b, Supplementary Fig. 21b). SPPI-expressing macrophages showed relatively higher expression of *TNFRSF1A*, *TNFRSF1B*, *IL6R*, *IL7R*, *IL10RA*, *CCR2*, and *CCR5* than other macrophages, but lower *ACKR4* expression. The NA8 group had receptor expression patterns similar to those in the negative group (Supplementary Fig. 22b, Supplementary Fig. 23b). Comparable expression patterns with negative expression were confirmed in macrophages at 3 and 7 dpi for JA142 (Supplementary Fig. 24b, Supplementary Fig. 25b); the expression of *IL10RA*, ENSSSCG00000021815 (*IL15RA*), *CXCR4*, and *CCR5* increased, and *ACKR4* expression decreased at 14 dpi (Supplementary Fig. 26b). The expression of *IL10RA*, ENSSSCG00000021815 (*IL15RA*), and *CCR5* decreased, and the expression of *ACKR4* increased at 21 dpi; however, the expression of *CXCR4* was still high (Supplementary Fig. 27b). At 3 dpi, the NA10 group showed expression patterns for their receptors similar to the negative group for macrophages (Supplementary Fig. 28b); however, at 7 dpi, the NA10 group showed similar expression to the JA142 group at 14 dpi (Supplementary Fig. 29b). NK cells showed high expression levels of ENSSSCG00000029668 (*IL2RB*) and *CXCR4* in both the JA142 and NA10 groups (Supplementary Fig. 26b, Supplementary Fig. 29b). T cells showed high expression of *TNFRSF1B*, ENSSSCG00000012397 (*IL2RG*), ENSSSCG00000029668 (*IL2RB*), *CXCR3*, *CXCR4*, *CXCR6*, and *CCR5*, whereas B cells showed high expression of *IL6R*, *IL21R*, *CCR2*, and *CCR10*. In the NA10 group, the B cells had higher expression of *IL6R*, *IL21R*, *CCR2*, and *CCR10* than JA142 cells, with more B cells with *IFNAR1*, *IFNAR2*, and *IFNGR2* expression also observed.

Investigations were conducted on macrophage populations ISAG^{high} and SPPI-CXCL14^{high} macrophages, both of which may play important roles in the immune response during infection. Significant differences were identified in the expression levels of chemokines (ligands) and receptors, distinguishing between responses to high (NA10) and intermediate (JA142) virulence (Supplementary Fig. 30). In the JA142 group, ISAG^{high} macrophages exhibited significantly elevated CXCL14 expression despite a marked reduction in cell numbers at 14 and 21 dpi; however, no changes were observed at 7 dpi in the NA10 group. Additionally, the expression levels of ENSSSCG00000035736 (*CCL24*) and *ACKR4* were significantly reduced at 14 dpi in the JA142 group and at 7 dpi in the NA10 group. In the JA142 group, SPPI-CXCL14^{high} macrophages displayed a rapid increase in cell numbers at 14 and 21 dpi and exhibited relatively high levels of CXCL14 expression, along with low levels of ENSSSCG00000035736 (*CCL24*) and *ACKR4* expression, though these changes were not statistically significant. Similarly, at 7 dpi in the NA10 group, SPPI-CXCL14^{high} macrophages

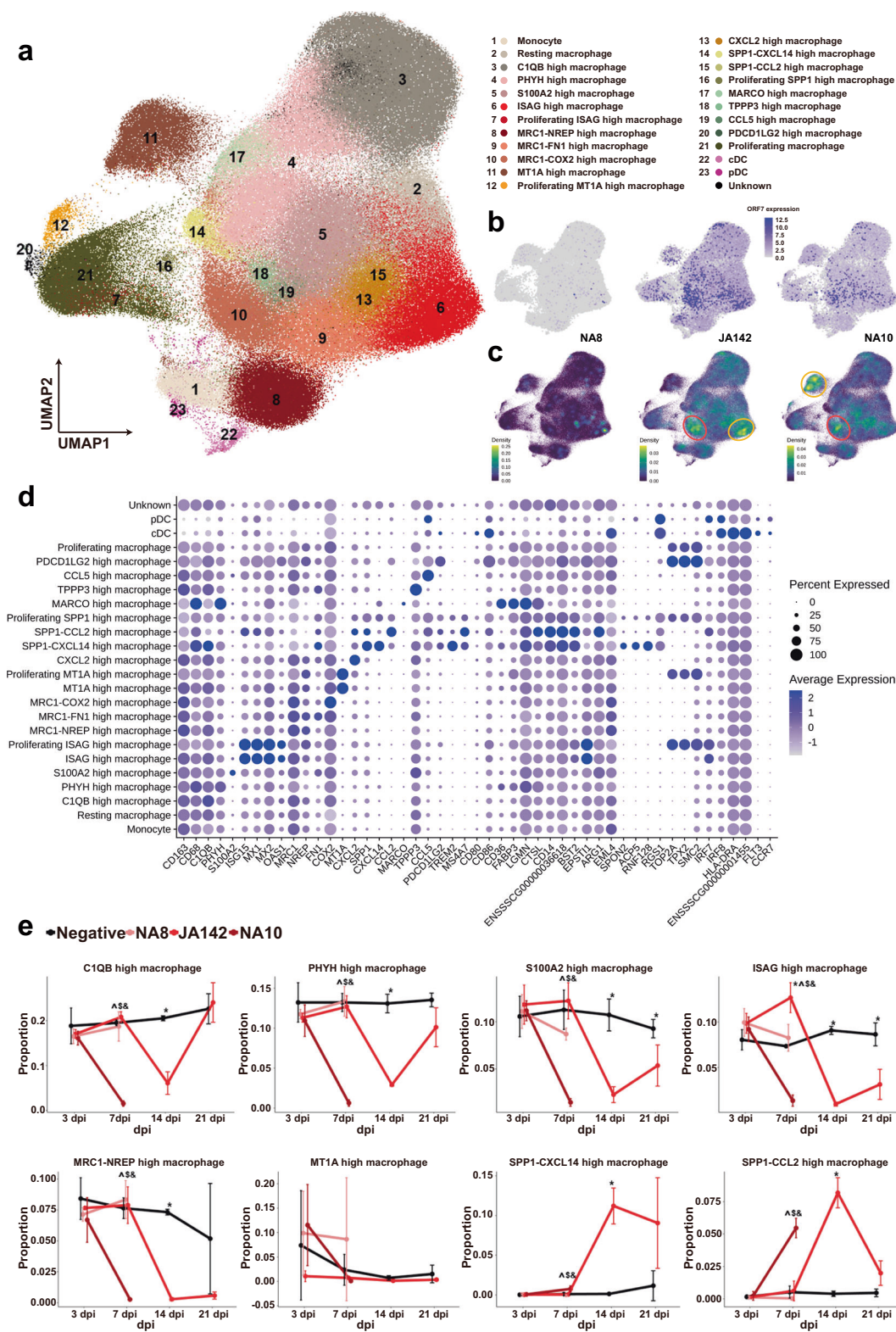


Fig. 3 | Subpopulation analysis of the macrophages and dendritic cells (DCs). **a** UMAP of the macrophage and DC subpopulation ($n = 283,735$), comprising 23 sub-cell types, in all groups. Colors and numbers indicate sub-cell types of the macrophages and DCs. Viral tracking of the macrophages and DC subpopulation based on the **b** log-normalized ORF7 expression levels in each virulence group (NA8, JA142, and NA10) and **c** log-normalized ORF7 expression density. Red circles indicate common high-density regions in JA142 and NA10. Yellow circles indicate different high-density regions in JA142 and NA10. **d** Specific marker gene expression levels for 23 sub-cell types. Average expression levels were calculated using log-

normalized values. **e** Time-series proportions of the representative sub-cell types in each group (negative; black, NA8; light red, JA142; red, and NA10; dark red). Dots indicate three biological replications and the data are presented as mean values $\pm$ SD. Statistically significant differences between groups are marked with exclamation (!, NA8-negative), asterisk (*, JA142-negative), circumflex (^, NA10-negative), ampersand (&, NA10-JA142), hash (#, JA142-NA8), and dollar (\$, NA10-NA8) signs. Significant differences ($p < 0.05$) determined using one-way ANOVA analysis with Tukey's multiple comparison test. Source data are provided as a Source Data file.

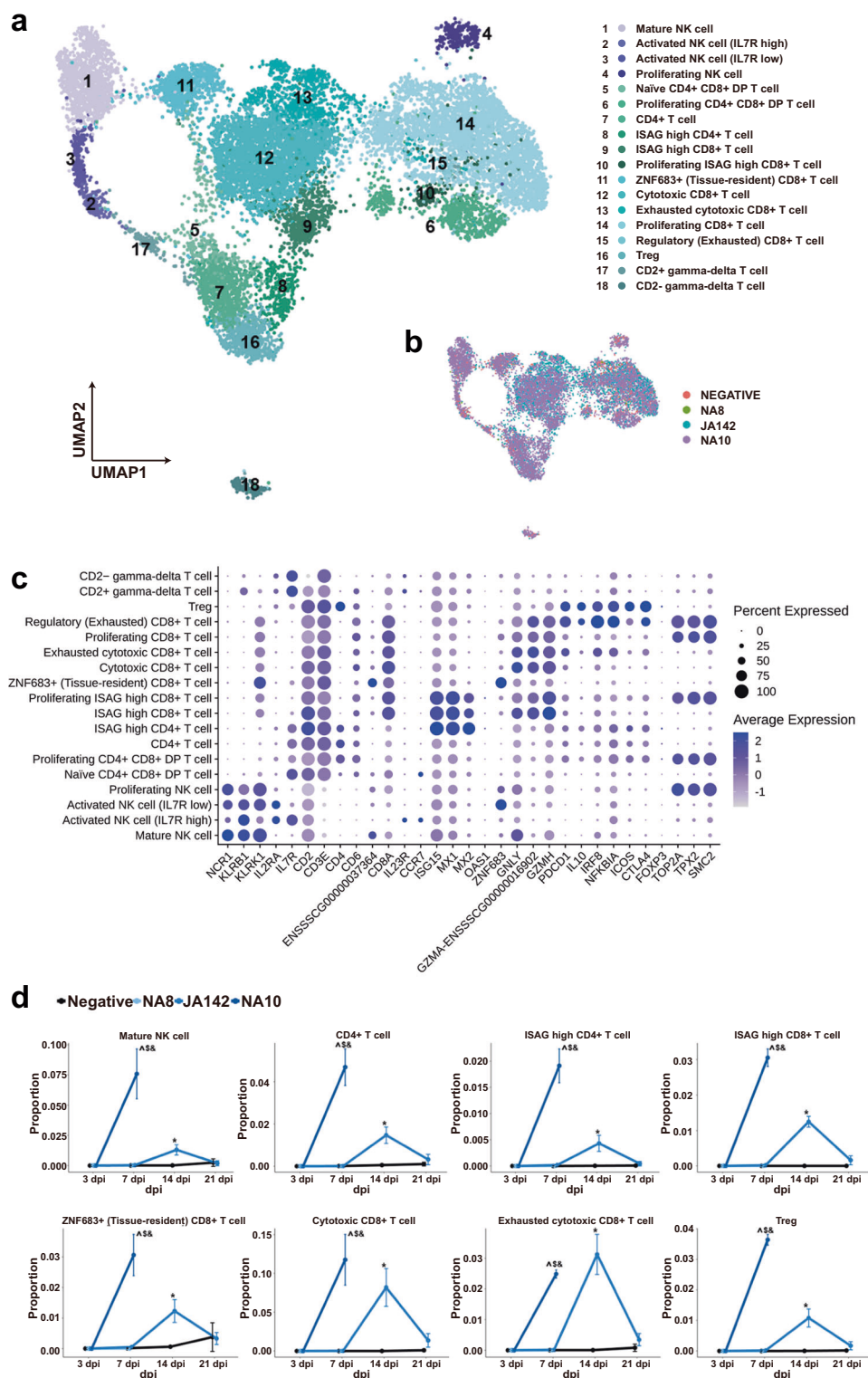


Fig. 4 | Subpopulation analysis of natural killer (NK) and T cells. a Uniform Manifold Approximation and Projection (UMAP) of the NK and T cell subpopulation ($n = 13,772$), consisting of 18 sub-cell types, in all groups. Colors and numbers indicate sub-cell types for the NK and T cells. **b** UMAP of the NK and T cell subpopulations with group annotations (negative, NA8, JA142, and NA10). **c** Specific marker gene expression levels for 18 sub-cell types. Average expression levels were calculated using log-normalized values. **d** Time-series proportions for representative sub-cell types in each group (negative; black, NA8; light blue, JA142; blue, and

NA10; dark blue). Dots indicate three biological replications and the data are presented as mean values $\pm$ SD. Error bars indicate standard deviations. Statistically significant differences between groups are marked with exclamation (!, NA8-negative), asterisk (*, JA142-negative), circumflex (^, NA10-negative), ampersand (&, NA10-JA142), hash (#, JA142-NA8), and dollar (\$, NA10-NA8) signs. Significant differences ($p < 0.05$) were determined using one-way ANOVA analysis with Tukey's multiple comparison test. Source data are provided as a Source Data file.

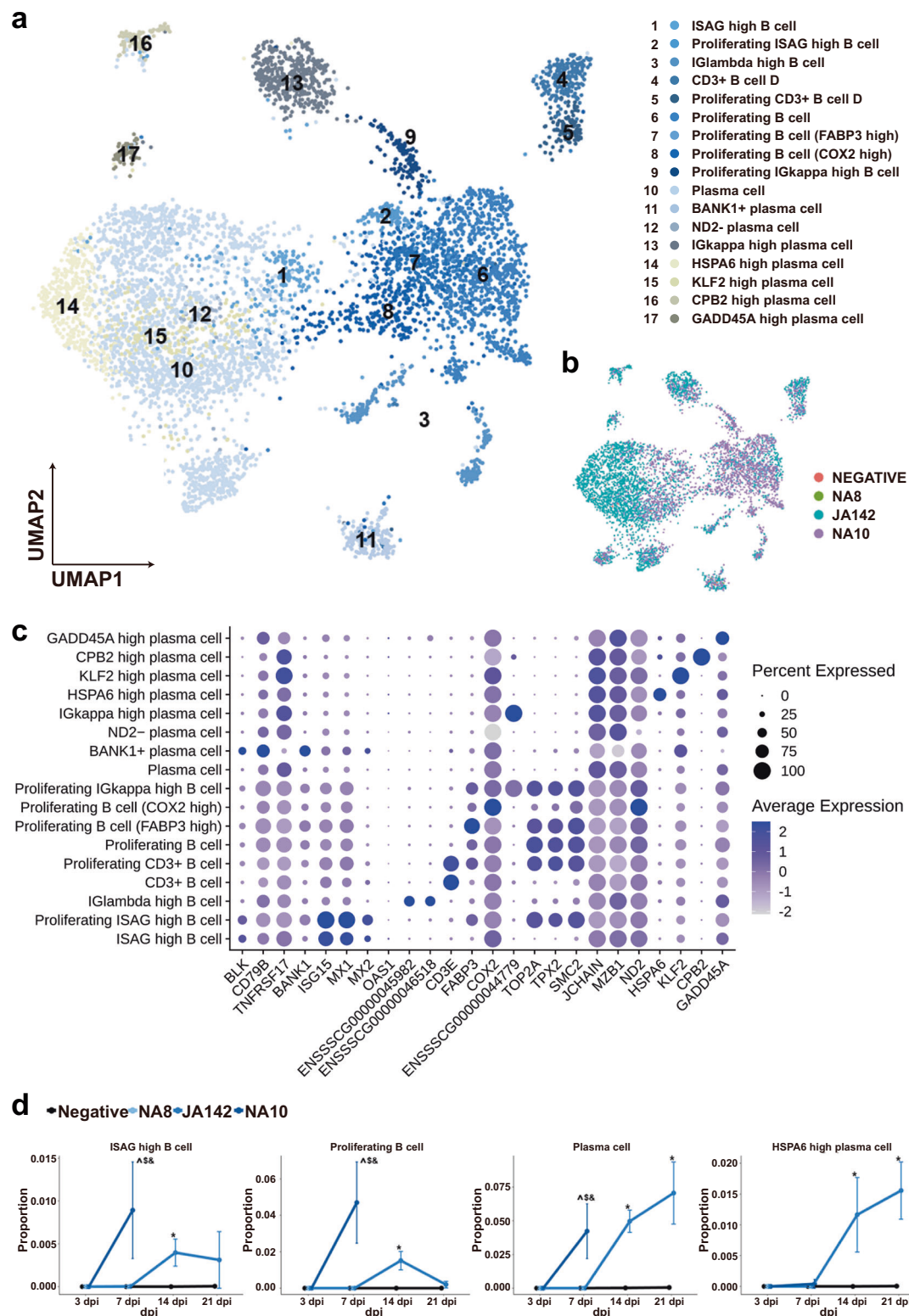


Fig. 5 | Subpopulation analysis of B cells. a Uniform Manifold Approximation and Projection (UMAP) of the B cell subpopulation ($n = 6,226$), consisting of 17 sub-cell types, in all groups. Colors and numbers indicate sub-cell types for the B cells. The superscript D indicates doublet possibility. **b** UMAP of the B cell subpopulation with group annotation (negative, NA8, JA142, and NA10). **c** Specific marker gene expression levels for 17 sub-cell types. Average expression levels were calculated using log-normalized values. **d** Time-serial proportions of representative sub-cell types in each group (negative; black, NA8; light blue, JA142; blue, and NA10; dark

blue). Dots indicate three biological replications and the data are presented as mean values $\pm$ SD. Error bars indicate standard deviations. Statistically significant differences between groups are marked with exclamation (!, NA8-negative), asterisk (*, JA142-negative), circumflex (^, NA10-negative), ampersand (&, NA10-JA142), hash (#, JA142-NA8), and dollar (\$, NA10-NA8) signs. Significant differences ($p < 0.05$) determined using one-way ANOVA analysis with Tukey's multiple comparison test. Source data are provided as a Source Data file.

exhibited very low levels of ENSSCG00000035736 (CCL24) and *ACKR4* expression.

Intercellular communication

Immune cells in the lungs exhibit distinct patterns of communication and circulation that vary according to specific physiological and pathological conditions³². Intercellular cross-talk in the BALF environment of pigs after PRRSV infection is largely unclear. Ligand-receptor interactions were found to be substantially elevated at peak lung-damage time points (JA142 14 dpi and NA10 7 dpi) (Supplementary Fig. 31a). We focused on JA142 14 dpi and NA10 7 dpi, both of which exhibited a substantial number of interactions. In both groups, macrophage sub-cell types showed dominant interactions. Interactions in JA142 14 dpi indicated a slightly higher number of interactions (including both outgoing and incoming signals) compared to NA10 7 dpi (Supplementary Fig. 31b). Upon examining the differences in signaling interactions between JA142 14 dpi and NA10 7 dpi, it was confirmed that various differences, including those related to immune responses, were observed (Supplementary Fig. 32a). Considering the dominant number of interactions in macrophages, we identified ligand-receptor pairs involved in SPP1, MHC-II, chemokine, and cytokine signaling within the SPP1-CXCL14^{high} macrophage sub-cell type, which showed the largest proportional difference between JA142 and NA10 infections (Supplementary Fig. 32b). More SPP1-(ITGAV + ITGB5), SPP1-(ITGAV + ITGB1), and CCL8-ACKR4 pairs in macrophage sub-cell types and CCL5-CCR5 pairs in CD8⁺ T cell sub-cell types were present in JA142 at 14 dpi (compared to NA10 at 7 dpi).

Differential functions according to PRRSV virulence

The functional differences between infected and bystander cells in the same macrophage sub-cell types were compared (Fig. 6a). Infected macrophages were primarily identified at 14 dpi and 7 dpi in the JA142 and NA10 groups, respectively. Infected macrophages generally showed lower enrichment of metabolic (adipogenesis, oxidative phosphorylation, reactive oxygen species pathway, fatty acid metabolism, and glycolysis), immune (IFN- α response, IFN- β response, IL2-STAT5 signaling, and complement), and cell signaling (apoptosis, p53 pathway, angiogenesis, Notch signaling, and TGF β signaling) than the bystander cells in both JA142 and NA10. In addition, NA10-infected and bystander macrophages showed lower enrichment scores for these functional terms than JA142-infected macrophages. High enrichments of immune (IL2-STAT5 signaling, complement, coagulation, IL6-JAK-STAT3 signaling, and inflammatory response) and cell signaling (apoptosis, p53 pathway, angiogenesis, Notch signaling, and TGF β signaling) were also confirmed in the SPP1-CXCL14^{high} macrophages. Proliferating macrophages showed high enrichment levels for genes associated with DNA replication (mitotic spindle, E2F targets, and the G₂-M checkpoint). Key genes were selected based on the results of differential expression gene analyses among negative and virulence groups (NA8, JA142, and NA10), and represented key genes were visualized in Fig. 6b. Following PRRSV infection, *ISG15* expression significantly increased universally, whereas *SIOA6* and *ISG12(A)* expression were significantly elevated only in the JA142 and NA10 groups. Additionally, *PONI* expression was significantly lower in the JA142 and NA10 groups, whereas *SPP1* and *CXCL14* expression rapidly increased only at 14 dpi in the JA142 group. *DPYS* and *MT1A* expression were peculiarly increased in the early stages (3 dpi) of the NA8 and NA10 groups.

All the bystander macrophage sub-cell types were enriched for Hallmark functions (Supplementary Fig. 33a). The negative and NA8 groups had lower enrichment levels at all dpi; however, the later the time point, the higher the score. Similar enrichment patterns were observed at 7 dpi and 14 dpi for the NA10 and JA142 groups, respectively, which generally showed higher enrichment levels. NA10 specifically, had a higher enrichment level for immune signaling

(IFN- α response, IFN- β response, inflammatory response, TNF- α signaling via NF- κ B, and IL6-JAK-STAT3 signaling) and a lower enrichment level for metabolic signaling (protein secretion, reactive oxygen species pathway, fatty acid metabolism, oxidative phosphorylation, and peroxisome) than JA142. Commonly, SPP1-CXCL14^{high} macrophages are highly enriched in epithelial-mesenchymal transition, whereas MARCO^{high} macrophages are highly enriched in myogenesis. In addition, proliferating macrophages showed a high level of enrichment in relation to DNA replication (mitotic spindle, E2F targets, and G₂-M checkpoints). Additionally, there were high levels of enrichment for the cell death signature at 7 dpi and 14 dpi in the NA10 and JA142 groups, respectively (Supplementary Fig. 33b). In particular, bystander macrophages in NA10 showed a higher enrichment for necroptosis and pyroptosis signatures than the JA142 group.

The DC, NK, T, and B sub-cell types were enriched in Hallmark functions (Supplementary Fig. 33c). Most sub-cell types were involved at 7 dpi and 14 dpi in the NA10 and JA142 groups, respectively. The DCs in particular showed high enrichment levels for metabolic (adipogenesis, oxidative phosphorylation, reactive oxygen species pathway, fatty acid metabolism, and glycolysis), immune (IL2-STAT5 signaling, complement, coagulation, IL6-JAK-STAT3 signaling, inflammatory response, and TNF- α signaling via NF- κ B), and cell signaling (apoptosis, p53 pathway, and Notch signaling), and ISAG^{high} T cells indicated high enrichment levels for immune signaling (IFN- α and IFN- β response). Overall, the DC, NK, T, and B sub-cell types were more enriched in all functions for the NA10 group than for the JA142 group.

Comparison of differentially expressed (DE) exosomal miRNAs according to PRRSV virulence

Exosome size and exosome markers were measured in purified exosomes, and the sizes ranged from 118.74 to 356.20 nm (Supplementary Fig. 34a). The markers, CD63 and CD9, were detected, whereas the Grp94 marker for the endoplasmic reticulum was not detected (Supplementary Fig. 34b).

Time-serial DE exosomal miRNAs were identified in each strain based on the negative group, and the target genes were predicted (Supplementary Fig. 35). At 3 dpi, the NA8 group had one and three DE miRNAs with upregulated and downregulated expressions, respectively, and predicted 0 and 119 target genes, respectively. Details of the DE exosomal miRNAs and target genes are described in Supplementary Data 2 and 3.

At 7 and 14 dpi for NA10 and JA142, respectively, JA142 had relatively high levels of DE miRNAs and target genes, the functional enrichment of whom was evaluated. DE exosomal miRNAs between NA10 and JA142 indicated 18% overlap (13 miRNAs), which is a low proportion, and the predicted target genes only showed 13% overlap (184 genes) (Fig. 7a). In both the NA10 and JA142 groups, the predicted target genes for the miRNAs with upregulated and downregulated expressions were highly enriched in terms associated with the regulation of transcription and cell signaling, including cell death pathways (Fig. 7b, Supplementary Data 4). NA10 and JA142 at 7 and 14 dpi, respectively, were compared to assess their exosomal miRNAs, and the results identified 12 and four DE miRNAs with upregulated and downregulated expressions, respectively (Supplementary Data 2). The DE miRNAs predicted 282 and 61 target genes (Supplementary Data 3), and a functional network based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was constructed (Fig. 7c, Supplementary Data 4). Target genes for miRNAs with upregulated expressions (miR-124-3p, miR-92b-3p, and miR-423-5p) were associated with the terms metabolic (metabolic pathway, various types of N-glycan biosynthesis, N-glycan biosynthesis, fatty acid degradation, glutathione metabolism, carbon metabolism, galactose metabolism, and biosynthesis of amino acids) and cell signaling (FoxO and PI3K-Akt signaling pathways), whereas those for miRNAs with downregulated expressions (miR-145-5p and miR-335-5p) were associated with the terms cell

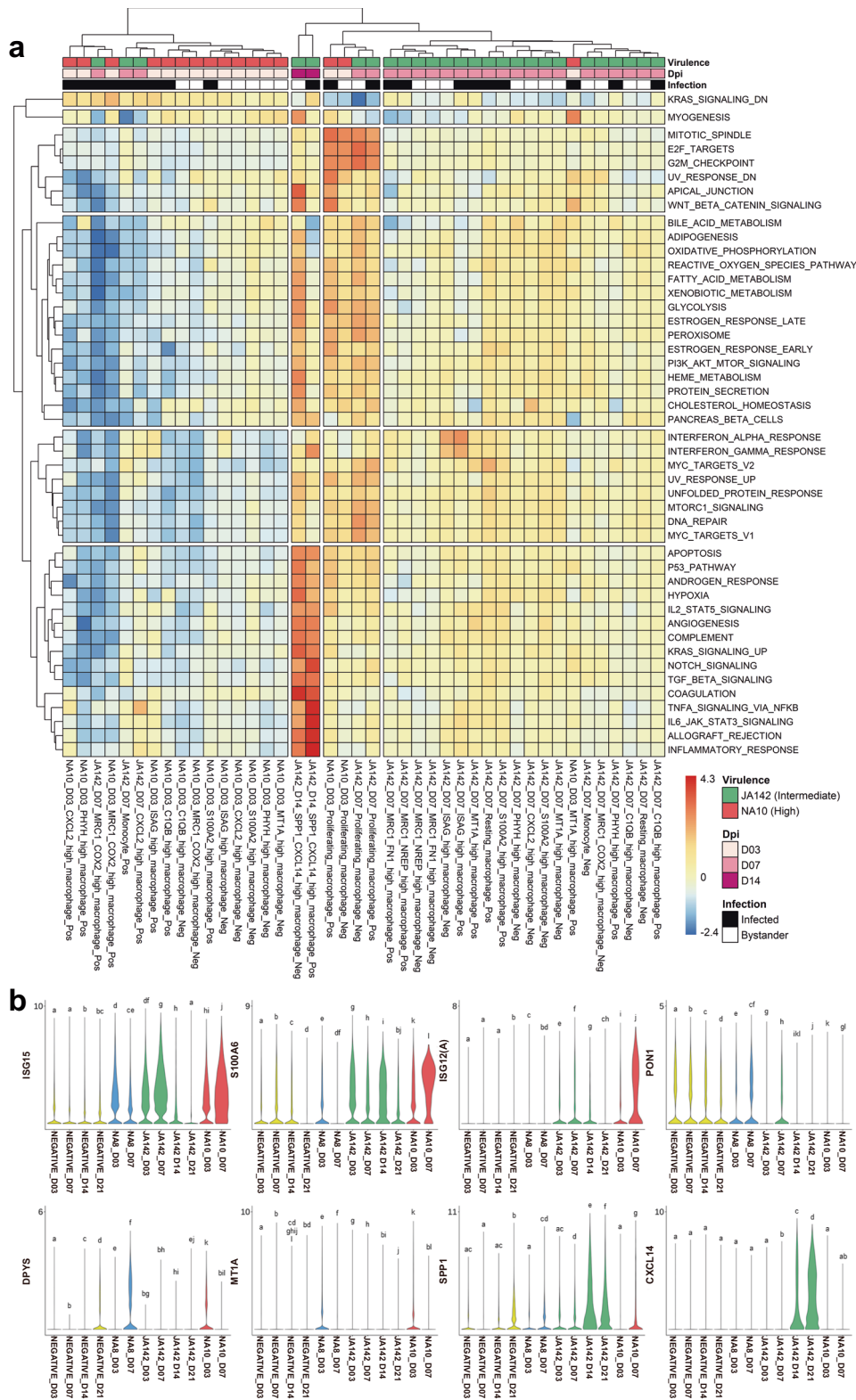


Fig. 6 | Gene set enrichment analysis of PRRSV-infected and bystander cells for each time point and virulence group (JA142 and NA10). a The heatmap was generated with more than 20 cell counts using z-score standardized enrichment scores. Significant differences ($p < 0.05$) determined using one-way ANOVA analysis with Tukey's multiple comparison test. At least one significant term among group

comparisons was used. **b** Key genes based on the differential expression gene analyses. Different lowercase letters above violin indicate significant differences ($p < 0.05$) determined using one-way ANOVA analysis with Tukey's multiple comparison test. Source data are provided as a Source Data file.

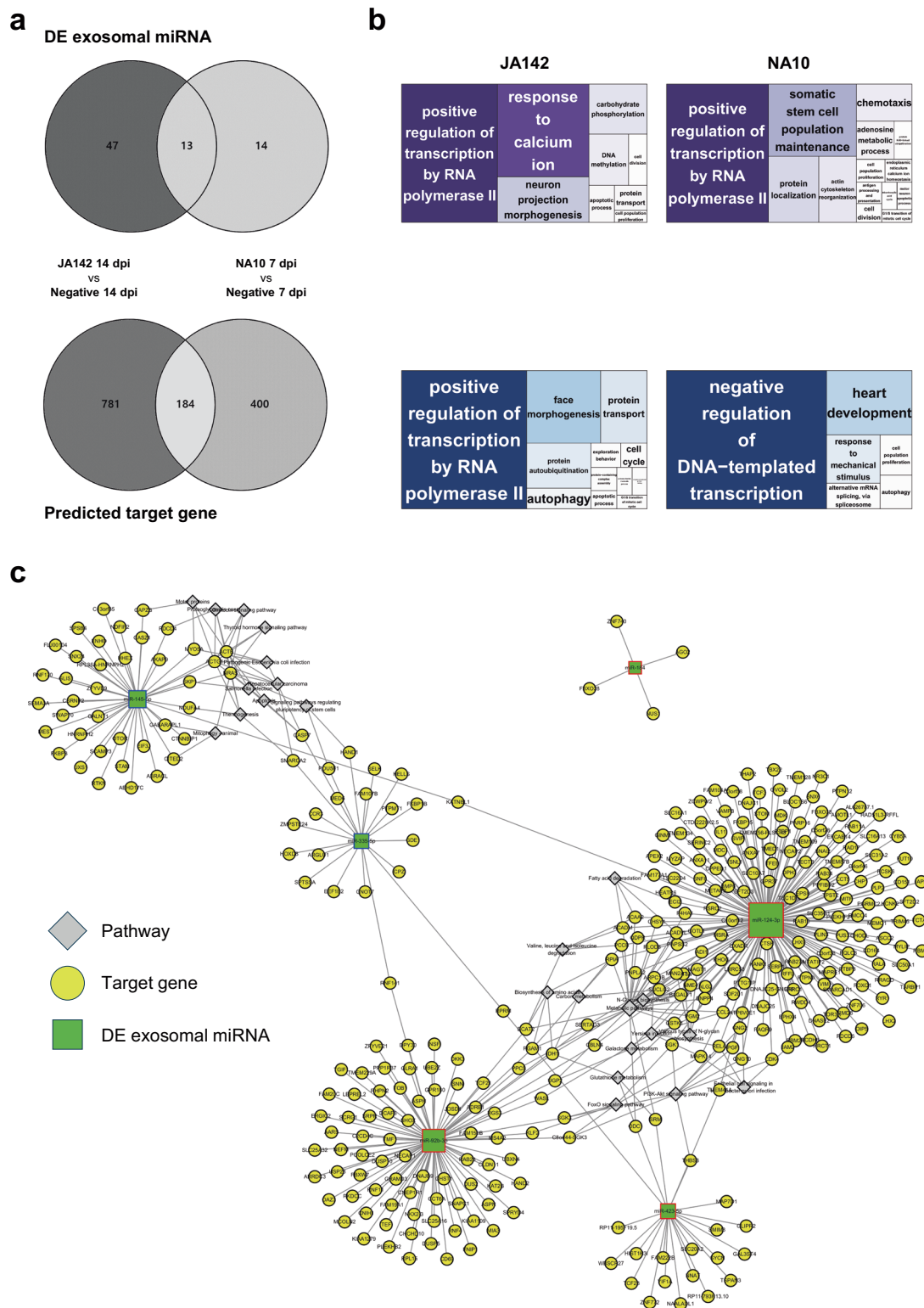


Fig. 7 | Functional comparisons of the exosomal microRNAs (miRNAs) in the JA142 and NA10 groups. **a** Venn diagram showing differentially expressed (DE) exosomal miRNAs and their predicted target genes at specific time points in the JA142 (14 dpi) and NA10 (7 dpi) groups based on the negative group. **b** Functional enrichment visualized using treemaps based on gene ontology (GO) analysis for the predicted target genes of the JA142 (14 dpi) and NA10 (7 dpi) groups. Upper and

lower parts indicate the results when using target genes for the miRNAs with upregulated and downregulated expressions, respectively. **c** Functional network using significant KEGG pathways with DE exosomal miRNAs and their target genes, obtained from comparisons of the NA10 (7 dpi) and JA142 (14 dpi) groups. Node size increases with the number of interactions (edges).

(thermogenesis, apoptosis, hepatocellular carcinoma, mitophagy–animal, and signaling pathways regulating pluripotency of stem cells) and infection signaling (pathogenic *Escherichia coli* and *Salmonella* infections).

Discussion

Although PRRSV has a high level of genetic heterogeneity and the genetic differences that alter clinical outcomes and host immune responses have not yet been fully elucidated, certain genetic markers such as mutations in ORF5 or insertion/deletion patterns in non-structural protein 2 have been suggested^{16,33}. The term virulence of a virus refers to its clinical phenotypes, including the occurrence of lesions in organs, which are accompanied by inflammatory cytokine cascades, elevated viral loads, and an increased ability to induce regulated cell death in the lungs or lymphoid tissues¹⁶. In order to characterize the immunological landscape based on different virulence levels, as was the goal of this study, the three PRRSV-2 strains, NA8 (low virulence), JA142 (intermediate virulence), and NA10 (high virulence), were selected with great care. One of the most widely tested PRRSV strains, JA142, induces moderate virulence with typical PRRSV clinical signs but low mortality, and the immunopathogenesis of this strain has been previously investigated in animal experiments¹⁰. The virulence of Korean wild-type PRRSV strains NA8 (modified live vaccine variant) and NA10 was also investigated in a previous animal study³⁰. While NA8 exhibited low virulence, NA10 induced near-100% mortality within 10–12 dpi with significantly higher virus replication, exhibiting characteristics of high virulence comparable to other highly virulent PRRSV strains, such as Chinese HP-PRRSV or PRRSV1 Lena strain^{11,15–17}. Consequently, despite the limited resources available, the current study was designed based on predetermined *in vivo* virulence, the observed viral replication patterns and lung lesions reflected the expected virulence levels (Fig. 1b, c).

Using a live immune cell suspension for BALF, which may provide more unbiased information for the targeted lung tissue, a high number of CD163⁺ macrophage cell deaths accompanied by immune cell infiltrations, elevated proinflammatory cytokines, chemokines with upregulated expressions, and immune checkpoint molecules were identified in JA142 and NA10 infections (Fig. 1e, f). It is noteworthy that, the time points at which such modulations occurred and the trajectory of the immune responses differed between JA142 and NA10. Therefore, a comparison of the lung microenvironments and immune landscapes for the two PRRSV-2 infection models may facilitate of an understanding of the immunopathogenesis of PRRSV. This study represents the assessment of single-cell transcriptome changes in porcine BALF samples with different types of PRRSV infections, including a negative sample. A substantial quantity of porcine BALF single-cell data ($n = 306,052$) and classified sub-cell type data (23 myeloid, 35 lymphoid, and two epithelial cells), were generated, including data pertaining to PRRSV-related cell types. Additionally, exosomal miRNAs that might contribute to recovery related to anti-inflammation and bystander cell death were also identified and combined with the single-cell data, revealing the differential host mechanisms of the PRRSV infections according to their virulence.

In PRRSV infection, *in vivo* studies have demonstrated that the virus induces the death of both infected and non-infected bystander cells^{34,35}, in which cell death pathways of apoptosis, necrosis, necroptosis, and pyroptosis have been proposed^{34–37}. In consistent to previous studies that PRRSV directly infects only small proportion of PAMs^{16,33}, viral tracking showed that <5% of cells were directly infected by PRRSV, in which the highest number of infected cells were detected at the time points (JA142 at 7 dpi and NA10 at 3 dpi) prior to peak lung-damage time points (JA142 at 14 dpi and NA10 at 7 dpi) and none of the viral transcripts were observed during the recovery-phase of the JA142 infection (Fig. 2f, Supplementary Data 1). The infection was commonly favorable to MRC1-COX2^{high} macrophages (Fig. 3b, Supplementary

Data 1), and the infected macrophages showed markedly reduced functions, including metabolic, immune, and cell signaling when compared with the bystander cells, suggesting that infected or zombie cells are functionally useless³⁸ (Fig. 6a). Notably, bystander cells of the NA10 at 3 dpi and JA142 at 7 dpi, when the highest viral transcripts were detected through viral tracking (Fig. 2f), showed high enrichment of IFN signaling pathways in similar levels (Supplementary Fig. 33a). However, the pathway was more enriched at the peak damage time point of NA10 (7 dpi) compared to JA142 (14 dpi) (Supplementary Fig. 33a). Phenotypically, higher concentrations of serum and BALF IFN- α were detected for the NA10-infected group when compared with the JA142 infected group at an earlier time point (Fig. 1b, Supplementary Fig. 3), and it is generally known that increased levels of IFN- α is correlated with the development of interstitial pneumonia in PRRSV infection^{8,39}. Furthermore, in bulk RNA level, NA10 at 3 dpi showed significantly higher expression of cell death pathway-related genes associated with apoptosis (caspase-7) and necroptosis (RIPK1, RIPK3, and MLKL) than in other groups, whereas JA142 only showed significant upregulation of caspase-1, which is related to pyroptosis, at 7 dpi (Supplementary Fig. 36).

Given that somewhat excessive immune responses and cell deaths were observed at the peak lung-damage time point, while only a small proportion (<5%) of cells were directly infected with PRRSV, it is highly plausible that certain cell signaling mechanisms from infected or zombie cells to bystander cells could be involved in these responses, in accordance with the differences in virulence. Among possible mechanisms, we focused on exosome as it is involved in various immunological and pathological processes in viral infections such as HIV and SARS-CoV2²⁴, in which exosome release are significantly influenced by cell death pathways^{40,41}. In viral tracking, some T and B cells, which are non-permissive cells of PRRSV, also had a low number of viral transcripts at the peak lung-damage time point. Therefore, it was speculated that those viral transcripts were resulted from the direct transfer of PRRSV-infected cell-derived exosomes in the BALF environment, as previous studies demonstrated that those exosomes could contain viral genomic RNAs, partial viral proteins (GP5, M, and N)⁴², and miRNAs that regulate programmed cell death, such as apoptosis, autophagy, necroptosis, and pyroptosis^{43,44}. Subsequently, the DE exosomal miRNAs in the three PRRSV strains (NA8, JA142, and NA10) were based on the negative groups at all time points; the highest numbers of DE miRNAs were detected at the peak lung-damage time point for JA142 and NA10 (Supplementary Data 2, Supplementary Fig. 35), and the predicted target genes of the miRNAs for both groups were functionally involved in transcription and cell signaling (including apoptotic process and autophagy) (Fig. 7a, b). However, relatively few miRNAs and target genes were common between the JA142 group at 14 dpi and the NA10 group at 7 dpi (Fig. 7a, b, Supplementary Data 3). Thus, these groups could be involved in differentially upregulated cell death pathways in bystander cells, especially with the increased enrichment of necroptosis and pyroptosis in the NA10 group compared to that in the infected and negative cells (Fig. 6a, Supplementary Fig. 33b). When DE exosomal miRNAs of NA10 were compared with those of JA142, three upregulated miRNAs (miR-124-3p, miR-92b-3p, and miR-423-5p) enriched in metabolic and cell death pathways, and two downregulated miRNAs (miR-145-5p and miR-335-5p) enriched in cell death and infection pathways were identified (Fig. 7c). Strikingly, the miR-124-3p and miR-423-5p are known to target NLRP3 and play important roles in inflammasomes, promoting cell pyroptosis^{45,46}. Collectively, we hypothesize that exosomes and exosomal miRNAs derived from PRRSV-infected cells (<5% of total BALF cells) may commonly participate in cell deaths in non-infected bystander cells in the early stage of PRRSV infection. In virulence-dependent manner, severe cell death could be accelerated by highly virulent PRRSV (NA10 in this study) through higher level of inflammatory pyroptosis induced by certain exosomal miRNAs (miR-

124-3p and miR-423-5p) and more exosomes released by robust cell deaths, in which exosomes from pyroptotic cells are known to induce immune cell death and amplify inflammation⁴⁰.

At the peak lung-damage time points for JA142 and NA10, most macrophage populations tended to decrease, except for that of a high proportion of SPPI-expressing macrophages which increased (SPPI-CXCL14^{high} macrophages, SPPI-CCL2^{high} macrophages, and proliferating SPPI^{high} macrophages) (Fig. 3e, Supplementary Fig. 12), and the overall upregulation of *SPPI* was confirmed via RT-qPCR at the total RNA level (Supplementary Fig. 36). Notably, the population of SPPI-CXCL14^{high} macrophages increased only in JA142 infection, but not in the NA10 infection. The population expressed M2 macrophage markers (*SPPI*, *TREM2*, and *CXCL14*) (Fig. 3d)^{47–49} which were enriched in epithelial-mesenchymal transition, a process that can be induced by M2 macrophages contributes to tissue repair (Supplementary Fig. 33a)^{50,51}. However, in contrast to the commonly identified M2 macrophages, which show high expression levels for *CD163*, SPPI-CXCL14^{high} macrophages demonstrated slightly lower expression levels for *CD163* and *MRC1*. The increase in the population of macrophages expressing lower levels of *CD163*, which is the major target receptor of PRRSV, at the peak lung-damage time point may explain the subsequent gradual decrease in the viral load. The specific cell population should be further investigated in the future.

Within the expression pattern for the cytokines, chemokines, and their receptors at the single-cell level, high expression of *CXCL14*, which has been reported to inhibit PRRSV replication via the down-regulation of the expression of the overexpressed *CEBPB* transcription factor⁵², was detected in most macrophage subsets at 14 dpi with the JA142 infection (Supplementary Fig. 26); but was almost none at 7 dpi with the NA10 infection (Supplementary Fig. 29). The expression of *CCL24* (ENSSSCG00000035736), an important factor in the inhibition of pulmonary inflammation⁵³, was high in all macrophages at all time points in the negative, NA8, and JA142 groups; however, the expression in the NA10 group rapidly decreased in all macrophages at 7 dpi, except for the C1QB^{high} macrophages (Supplementary Fig. 26, Supplementary Fig. 10). The reduced level of immune responses, including the inflammatory response, in the JA142 group compared to the NA10 group appeared to be influenced by recovery mechanisms via M2-like macrophages that have roles in anti-inflammatory responses (Supplementary Fig. 33a). Taken together, host recovery from PRRSV infection might be abrogated by CXCL14 activation in M2-like macrophages (SPPI-CXCL14^{high} macrophages) and CCL24 maintenance in the macrophages. The aforementioned miR-145-5p, whose expression was upregulated in the JA142 groups (Fig. 7c), could be involved as miR-145-5p has been reported to contribute to anti-inflammation associated with immunosuppression by targeting the immunoreceptor⁵⁴ and could lead to M2-like polarization in PRRSV infections⁵⁵ or colorectal cancer via cancer-derived extracellular vesicles⁵⁶. Moreover, another upregulated miRNA in the JA142 infection group, miR-335-5p, may also be involved, as it is known to inhibit the inflammatory and innate immune responses of macrophages^{57,58}.

Lymphoid cell populations, including NK and T cells (Fig. 4) and B cells (Fig. 5), infiltrated into the BALF at the peak lung-damage time points, in accordance with the decrease or death of myeloid populations, as shown by both flow cytometry (Fig. 1e) and single-cell analysis (Fig. 2). Although the variety of NK and T cell subsets was almost identical for both NA10 and JA142 infection, the proportion of NK cell subsets and several T cell subsets, including ISAG^{high} CD4⁺, CD8⁺ T, and T_{reg} cells, was significantly higher at 7 dpi with the NA10 infection than that at 14 dpi with the JA142 infection (Fig. 4d, Supplementary Fig. 14). It was postulated that early and strong induction of the IFN signaling and cell death pathways (Supplementary Fig. 33a), induced by the highly virulent NA10, could initiate strong infiltration of NK and ISAG^{high} T cells, which are at the frontline of the defense system against viruses, including PRRSV. At the same time, for immunological

homeostasis, excessive regulatory processes were evidenced by the significantly increased expression of strong immunoregulatory receptors, including *CCR4* and *CCR5* (Supplementary Fig. 4) and T cell exhaustion markers, including *CTLA4* and *IDO1* (Supplementary Fig. 5) at the bulk RNA level (Fig. 1f). Collectively, the evidence suggests that excessive pro- and anti-inflammatory immune responses without sufficient levels of tissue-healing M2-like macrophages (SPPI-CXCL14^{high} macrophages), may be a cause for mortality induced by highly virulent PRRSV infections.

In summary, this study represents a significant contribution to the field, as it provides data on the immunological and single-cell transcriptomic landscapes under three different virulent PRRSV infections, including a negative control group, in porcine BALF. The potential mechanisms for recovery and bystander cell death in PRRSV infection according to virulence, are illustrated in Fig. 8. The results offer a comprehensive understanding of the immunological dynamics according to PRRSV virulence, which will facilitate the formulation of new hypotheses to elucidate PRRSV pathogenesis and other respiratory infections.

Methods

Viruses and cells

Korean PRRSV-2 strains JB15-N-M8-GN (GenBank: MZ287322) and JB15-N-PJ10-GN (GenBank: MZ287321) were selected for viral infection in this study based on their genetic characteristics, pathogenicity, and the severity of clinical outcomes reported in previous studies^{10,31,59}. In brief, the lineage 5-modified live virus vaccine variant strain JB15-N-M8-GN showed mild pathogenicity, and the Korean-specific lineage KOR B strain JB15-N-PJ10-GN exhibited high pathogenicity with a high viral load in the serum and lungs, which induces high mortality in piglets within 10–12 dpi. In this study, the JB15-N-M8-GN and JB15-N-PJ10-GN strains were designated NA8 and NA10, respectively. The PRRSV-2 reference strain JA142 (GenBank: AY424271) from lineage 8, which has been widely used in previous studies^{10,26,60}, was also selected for viral infection as it is known to induce typical PRRS symptoms with high viremia but has low mortality¹⁰. Collectively, PRRSV-2 isolates NA8, JA142, and NA10 were designated as representative strains with low, intermediate, and high virulence, respectively.

PAMs were used for viral propagation and assays. These cells were collected from the lungs of 4-week-old PRRSV-negative piglets and maintained in the laboratory. The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco® RPMI-1640, Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies), 2 mM L-glutamine, and an antibiotic–antimycotic cocktail (Anti-Anti, Life Technologies) containing 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL Fungizone® [amphotericin B] in a humidified chamber with 5% CO₂ at 37 °C. The PRRSV-2 isolates used in this study were propagated and titrated in PAMs following a previously described protocol; PAM isolates at the appropriate dilutions were then used as the pig inoculum.

Animals and experimental design

Experiments were performed on 4-week-old piglets ($n = 36$), composed of 24 female and 12 castrated male piglets, obtained from a PRRSV-negative farm. They were separated into negative ($n = 12$), NA8 ($n = 6$), JA142 ($n = 12$), and NA10 ($n = 6$) groups, with male and female animals equally distributed. After 7 d of acclimation, each of the 24 pigs (NA8, JA142, and NA10 groups) were intramuscularly inoculated with 2 mL of PRRSV-2 (1×10^3 TCID₅₀/mL) diluted in sterile phosphate-buffered saline (PBS). All pigs were provided with feed and water *ad libitum*. In the negative and JA142 groups, three pigs were humanely euthanized 3, 7, 14, and 21 dpi. In the NA8 and NA10 groups, three pigs were humanely euthanized at 3 and 7 dpi. A schematic of the experimental design is shown in Fig. 1A. Blood was collected from all pigs at

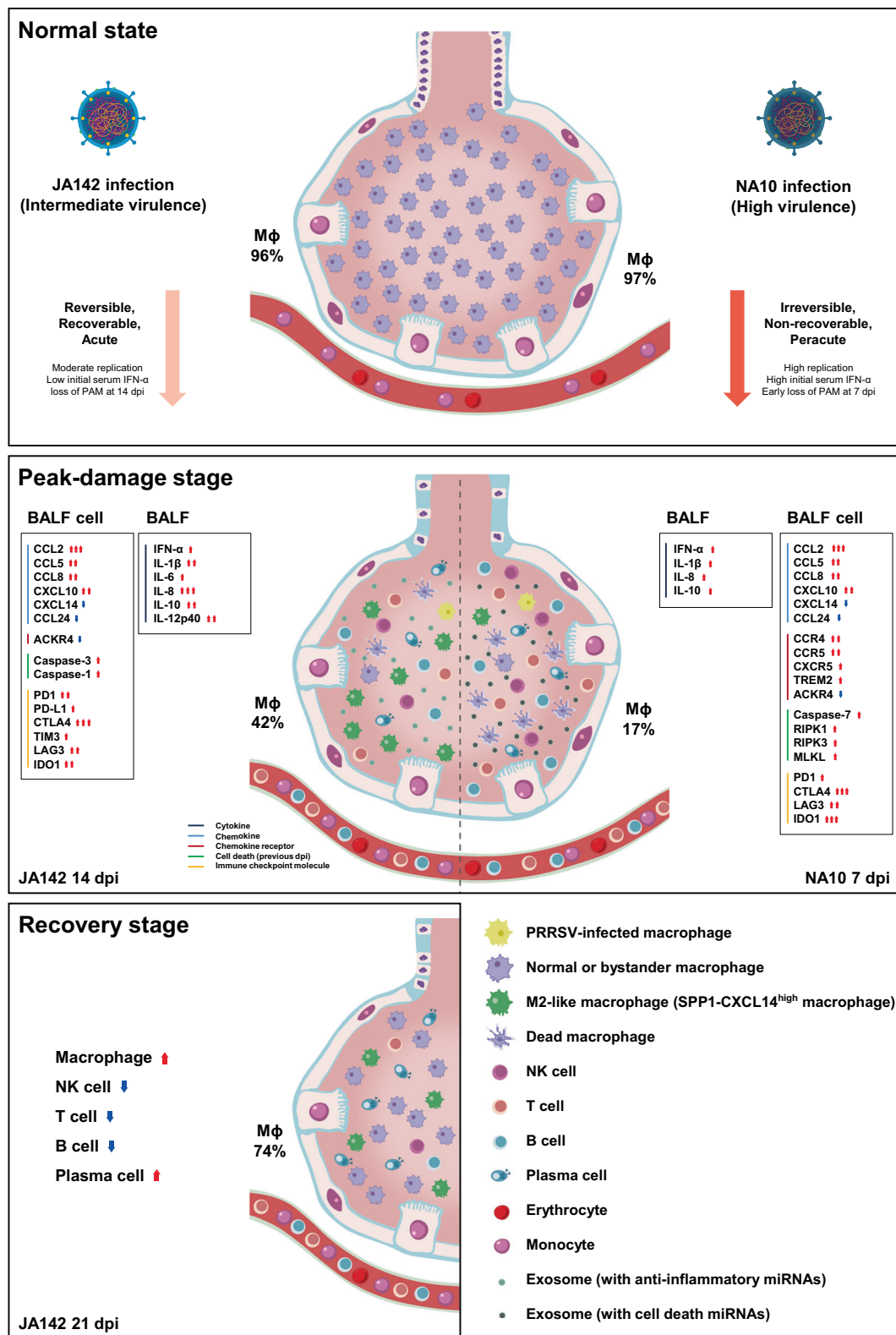


Fig. 8 | Illustration of chronological order of lung recovery and bystander cell death according to the virulence of PRRSV observed in this study. Lung microenvironment illustration showing cellular changes in the normal state, peak-damage stage, and recovery stage. Peak-damage stage is separated to show the JA142 (14 dpi; left) and NA10 (7 dpi; right) groups separately. Recovery stage is

shown for the JA142 (21 dpi; left) group. Cytokine and RNA expression changes in the BALF cells are shown in the peak-damage stage. In the recovery stage, the proportion of macrophages is partially recovered, and the cytokine and RNA expression levels are returned to a normal state, similar to that of the negative control.

0, 3, 7, 14, and 21 dpi, and serum was separated. The body weights of all pigs were measured at 0, 3, 7, 14, and 21 dpi. All animal experiments were approved by the Jeonbuk National University (JbNU) Institutional Animal Care and Use Committee, Republic of Korea (approval number JBNU 2021-0172).

Measurement of viral load

Serum viremia was measured 0, 3, 7, 14, and 21 dpi, and the viral load in the lungs of the euthanized pigs was also quantified 3, 7, 14, and 21 dpi. Lung tissues (approximately 0.1 g) were minced, diluted (1:10) in PBS, and then mechanically homogenized using a sterile Beadbeater TissueLyser II (Qiagen, Hilden, Germany). Viral RNA was extracted from the serum and lung homogenates using a nucleic acid extraction kit for viral DNA/RNA with a Tianlong automatic nucleic acid extractor (NP968, Xi'an Tianlong Science & Technology Co., Xi'an, China), according to the manufacturer's instructions. RT-qPCR was performed using the Prime-Q PCV2/PRRSV Detection Kit (Genet Bio, Daejeon, Republic of Korea) to quantify serum viremia and lung viral load, according to the manufacturer's instructions. To calculate the amount of PRRSV in each sample, the Cq values were converted to virus titers (TCID₅₀/mL) by generating a standard curve using the titration of the PRRSV-2 strain JA142 (Supplementary Fig. 37).

Measurement of anti-PRRSV-specific antibody

Serum samples were tested for anti-PRRSV antibodies (IgG) using a commercially available ELISA kit (PRRS Ab ELISA 4.0; BioNote Inc., Hwaseong, Republic of Korea), according to the manufacturer's instructions. Samples with a sample/positive (S/P) ratio (the ratio of the net optical density of the test samples to the net optical density of the positive controls) ≥ 0.4 were considered positive for the PRRSV antibody.

Collection of lung samples and isolation of BALF cells

After euthanasia, the lungs were aseptically extracted by clamping the tracheal tube and gross lesions were measured. The left mainstream bronchus was clamped, and tissue samples were collected from the clamped left lung lobes for RNA extraction and histopathological examination. The right lung was lavaged with 15 mL sterile PBS, containing 100 µg/mL ampicillin (USB Corporation Cleveland, OH, USA) and Anti-Anti, to retrieve the first lavage fluid. The second lavage was conducted using 75 mL PBS. The collected lavage fluids were then centrifuged at $1,000 \times g$ at room temperature for 10 min to separate the BALF and BALF cells. The resulting supernatant or BALF from the first lavage was used to quantify the cytokine and chemokine levels. Cell pellets or BALF cells from both the first and second lavages were then merged and used for flow cytometry, quantification of mRNA expression levels, and single-cell RNA sequencing. BALF cells were washed three times with PBS after red blood cell lysis. The tissues, BALF, and BALF cells were all collected in tubes, snap-frozen in liquid nitrogen, and stored immediately at -80°C until further analysis.

Pathological evaluation of lungs

The lungs of necropsied pigs were subjected to pathological evaluations on each day of necropsy, as previously described¹⁰. In brief, microscopic lung lesions were scored on a scale ranging from 0 to 3, indicating no lesions, mild interstitial pneumonia, moderate multifocal interstitial pneumonia, and severe interstitial pneumonia, respectively. Microscopic lesions were examined from five different lobes of the lungs and the average value was used for scoring.

Flow cytometry of BALF cells

The collected BALF cells were monitored using flow cytometry for phenotypic changes and infiltration of T cell subsets into the lungs. BALF cells (1×10^6 cells) were transferred to microtiter plates and washed with flow cytometry staining buffer (3% FBS in PBS and

0.02% sodium azide) for 5 min at 1500 rpm. The cells were then triple- or quadruple-stained for swine membrane markers and/or inter-nuclear and intranuclear markers, as previously described¹⁰. According to the gating strategies suggested in former studies^{10,61–63} with modifications, the BALF cells were phenotypically classified into sub-populations as follows: monocyte/DC panel including alveolar macrophages (AMφs and MHCII⁺CD172α⁺CD163^{high}), monocyte-derived macrophages (moMφs and MHCII⁺CD172α⁺CD163^{int}), moDC (MHCII⁺CD172α⁺CD163^{low}), cDC2 (MHCII⁺CD172α⁺CD163⁺), and cDC1 (MHCII⁺CD172α⁺CD163⁺) stained with porcine MHCII (myositis-specific antibody 3, cat.: WS0589S-100, Kingfisher Biotech), porcine CD163 (2A10/11, cat.: MCA2311GA, Bio-Rad), CD172α (Sirpα, 74-22-15, cat.: MCA6100PE, Bio-Rad), the Zombie Green live-dead fixable dye (cat.: 423112, BioLegend), rat adenomatous polyposis coli (APC) anti-mouse IgG1 (cat.: 406610, BioLegend), rat peridinin-chlorophyll-protein/cyanine 5.5 anti-mouse IgG2a (cat.: 407112, BioLegend); T cell panel including CD3⁺ T (CD3⁺), CD4⁺ T (CD3⁺CD4α⁺CD8α⁺), and CD8⁺ T (CD3⁺CD4α⁺CD8α⁺) cells stained with porcine CD3 (PPT3, cat.: MCA5951F, Bio-Rad), porcine CD4α (74-12-4, cat.: 561474, BD), and porcine CD8α (76-2-11, cat.: 559584, BD); T_{reg} (CD4α⁺CD8α⁺CD25⁺FoxP3⁺) cell panel stained with porcine CD4α, porcine CD8α, porcine CD25 (K231.3B2, cat.: MCA1736GA, Bio-Rad), FoxP3 (FJK-16s, cat.: 11-5773-82, eBioscience), and rat APC anti-mouse IgG1; NK cells panel including NKp46⁺ NK (CD3⁺CD8α⁺NKp46⁺) and NKp46⁺ NK (CD3⁺CD8α⁺NKp46⁺) cells stained with porcine CD3, porcine CD8α, and porcine CD335 or NKp46 (VIM-KM1, cat.: MCA5972GA, Bio-Rad); Th1/Th17 cells panel including Th1 (CD4α⁺CD8α⁺IFN-γ⁺) and Th17 (CD4α⁺CD8α⁺IL17⁺) cells stained with porcine CD4α, porcine CD8α, porcine IFN-γ (P2G10, cat.: 561481, BD), and IL17 (eBio64DEC17, cat.: 17-7179-42, eBioscience); CTL/porcine gamma-delta T cells (CTL/γδT) panel including CTLs (CD3⁺ T cell receptor (TCR)γδ-CD8α⁺IFN-γ⁺), regulatory γδT cells (CD3⁺TCRγδ⁺CD8α⁺), and proinflammatory γδT cells (CD3⁺TCRγδ⁺CD8α⁺) stained with porcine CD3, porcine CD8α, porcine IFN-γ, porcine TCR1 δ (PGBL22A, cat.: WS0621S-100, Kingfisher Biotech), and rat APC anti-mouse IgG1.

Cytokine immunoassay and ELISA for serum kynurenine and tryptophan

The cytokine levels (IFN-α, IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40, and TNF-α) in the sera and BALF were measured using a ProcartaPlex™ Porcine Cytokine & Chemokine Panel 1 9-Plex Immunoassay (ThermoFisher Scientific, Vienna, Austria), according to the manufacturer's instructions. Magnetic microsphere technology, based on porcine cytokine/chemokine antibody-immobilized magnetic beads, has been employed in immunoassays for cytokine quantification^{10,64}. The concentration of each cytokine was measured using a Luminex 200 system (Luminex Corporation, Austin, TX, USA). Appropriate standards provided with the kit were used to determine the concentration of each cytokine. The machine was verified and calibrated using a Luminex® 100/200™ verification kit and a Luminex® 100/200™ calibration kit (Luminex Corporation, Austin, TX, USA) prior to use. Serum kynurenine/tryptophan levels were measured using a commercial kynurenine/tryptophan ratio ELISA pack (ImmuSmol, France), according to the manufacturer's instructions.

Quantification of mRNA expression of chemokines and immune molecules in BALF cells

Total cellular RNA was extracted from 5×10^6 BAL cells using 0.5 mL of NucleoZOL® reagent and the NucleoSpin® RNA Set for NucleoZOL (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The extracted RNA was then confirmed using a NanoDrop spectrophotometer (Biospec-nano, Shimadzu Scientific Instruments, Kyoto, Japan), followed by Qubit 2.0 fluorometer using a Qubit RNA Broad Range assay kit for quantification. Next, 5 µg of RNA was used for complementary DNA (cDNA) synthesis using a WizScript™ cDNA

Synthesis Kit (Wizbiosolutions, Seongnam, Republic of Korea), according to the manufacturer's instructions. Real-time PCR was performed on a 7500 Fast Real-time PCR system (Applied Biosystems, Carlsbad, USA) with a Power SYBR[™] Green PCR master mix kit (Applied Biosystems, Carlsbad, USA). Target genes, such as chemokines and receptors (CCL2, CCL5, CCL8, CXCL10, CCR4, CCR5, CXCR5, and TREM2) and immune checkpoint molecules (PD-1, PD-L1, PD-L2, CTLA4, CD200R1, LAG3, TIM3, and IDO1) induced by PRRSV infection were chosen based on previous studies^{11,26,65,66}. The comparative cycle threshold (Ct) method ($2^{-\Delta\Delta C_t}$) was used for relative quantification. The quantification cycle (Cq) values of the target genes were normalized to those of the housekeeping gene porcine *RPS24*⁶². All qPCR results were confirmed by melt curve analysis. The primer set sequences for the target and housekeeping genes are provided in Supplementary Data 5^{62,65,67,68}.

Single-cell capture, library construction, and sequencing

For single-cell RNA sequencing (scRNA-seq) library construction, BALF cells were washed with cold Ca²⁺ and Mg²⁺ free 0.04% BSA/PBS at 300 g for 5 min at 4 °C. Samples were gently resuspended in cold Ca²⁺ and Mg²⁺ free 0.04% BSA/PBS, and then counted with a LUNA-FX7[™] Automated Fluorescence Cell Counter (Logos Biosystems, Anyang, Gyeonggi-do, Republic of Korea) using acridine orange and propidium iodide staining. scRNA-seq libraries were constructed with the Chromium Single Cell 3' v3.1 assay (10x Genomics, Pleasanton, CA, USA) using 36 BALF cells. According to the protocol, libraries were prepared using the 10x Chromium Controller and Next GEM Single cell 3' Reagent v3.1 kits. Briefly, the cell suspension (target recovery 10,000 cells) was mixed with RT master mix and loaded with Single Cell 3' Gel Beads and Partitioning Oil into a Single Cell G Chip to generate single-cell Gel Bead-in-emulsion (GEM). Polyadenylated mRNA from single cells was uniquely barcoded and reverse-transcribed within the GEM. After the barcoded full-length cDNA was generated from the mRNA by GEM-RT incubation, the barcoded cDNA molecules were amplified by PCR. To construct the 3' Gene Expression Library, the enriched cDNA was sequentially subjected to enzymatic fragmentation, end-repair, A-tailing, adaptor ligation, and index PCR. Purified libraries were quantified with qPCR, according to the qPCR Quantification Protocol Guide, and then qualified using an Agilent Technologies 4200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). The libraries were then sequenced using the NovaSeq 6000 System (Illumina, Inc., San Diego, CA, USA), and 150 bp paired-end reads were generated. The sequencing depth of 3' Gene Expression Library was approximately 20,000 read pairs per cell.

Alignment and cell filtration

Reference genome sequences and gene information were downloaded from the Ensembl database (*Sus scrofa* 11.1.106, GCA_000003025.6). The PRRSV sequences (NA8, JA142, and NA10) were downloaded from National Center for Biotechnology Information GenBank (MZ287322.1, AY424271.1, and MZ287321.1). The pig reference genome and three pig-virus combined reference genomes (pig-NA8, pig-JA142, and pig-NA10) were utilized in this study and pre-processed for single-cell data using mkref (Cell Ranger; 10x Genomics). The sequence data for each sample were produced using a demultiplexing process (Cell Ranger). Sequence reads for each sample of the negative control group were aligned to the pig reference genome, and the sequence reads of the NA8, JA142, and NA10 groups were aligned to the pig-virus combined reference genomes corresponding to each group. A gene-by-cell unique molecular identifier (UMI) count matrices were created for each condition and subsequently aggregated into a single-count matrix. Empty droplets were identified and filtered out using the DropletUtils (v1.14.2) R package⁶⁹ with a false discovery rate (FDR) ≤ 0.05. Upon examining the outliers in the principal component (PC) analysis plot for the quality control metrics using the scatter (v1.22.0) R package⁷⁰, low-quality cells

with less than 600 UMIs, less than 100 detected genes, and greater than 30% of UMIs mapped to mitochondrial genes were eliminated. Before normalization, all samples were combined.

Dimensionality reduction and clustering

Normalization of the raw count matrix was performed to estimate the cell-specific size factors using the *scran* R package⁷¹, and then the normalized value was log₂-transformed with a pseudo-count of 1. The identification of highly variable genes across all cells was conducted using the same package with an FDR ≤ 0.05, except for viral genes. A Uniform Manifold Approximation and Projection (UMAP) plot for all cells was generated using the *Seurat* (v4.3.0) R package⁷² with batch-corrected for 15 PCs using the *harmony* (v0.1.1) R package⁷³ and a resolution of 1. To annotate cell types, 21 features (*CD163*, *MRC1*, *CD68*, *IRF7*, *IRF8*, *HLA-DRA*, *FLT3*, *CCR7*, *NCRI*, *KLRB1*, *KLRK1*, *CD2*, *CD3E*, *CD4*, *CD6*, *CD8A*, *BLK*, *CD79B*, *TNFRSF17*, and *SFTPA1*) were identified. Adjacent clusters, presumed to belong to the same cell type (feature), were integrated. The cell type anticipated to be a doublet is classified as unknown due to the simultaneous expression of markers specific to different cell types. This classification was meticulously conducted following re-integration.

Re-integration

Myeloid (macrophages and DC), lymphoid 1 (NK and T cells), and lymphoid 2 (B cells) subpopulations were re-integrated and re-clustered using the method described above. The UMAP plots for each subpopulation were visualized using batch-corrected 20 PCs at a resolution of 3. Features were selected based on the results (Supplementary Data 6) of the differential expression analysis (FindAllMarkers function) using *MAST*⁷⁴ in the *Seurat* (v4.3.0) R package⁷² and previously reported information. In the myeloid, 47 features (*CD163*, *CD68*⁷⁵, *CIQB*, *PHYH*, *S100A2*, *ISG15*, *MX1*, *MX2*, *OAS1*, *MRC1*⁷⁶, *NREP*⁷⁷, *FN1*, *COX2*, *MT1A*⁷⁸, *CXCL2*, *SPP1*, *CXCL14*⁷⁹, *CCL2*⁸⁰, *MARCO*, *TPPP3*, *CCL5*⁸¹, *PDCD1LG2*, *TREM2*⁸², *MS4A7*⁸³, *CD80*, *CD86*, *CD36*⁸⁴, *FABP3*⁸⁴, *LGMN*⁸⁵, *CTSL*⁸⁶, *CD14*⁸⁷, *ENSSSCG00000036618* (*FCGR3A*)⁸⁸, *BST2*, *EPSTI1*⁸⁹, *ARG1*⁹⁰, *EML4*⁹¹, *SPON2*⁹², *ACPS*⁹³, *RNF128*⁹⁴, *RGS1*⁹⁵, *TOP2A*, *TPX2*, *SMC2*, *IRF7*⁹⁶, *IRF8*, *HLA-DRA*, *ENSSSCG00000001455* (*SLA-DRB1*), *FLT3*, and *CCR7*⁹⁷) were used. In the lymphoid 1, 31 features (*NCRI*, *KLRB1*, *KLRK1*, *IL2RA*, *IL7R*, *CD2*, *CD3E*, *CD4*, *CD6*⁹⁸, *ENSSSCG000000037364* (*CD7*)⁹⁹, *CD8A*, *IL23R*¹⁰⁰, *CCR7*, *ISG15*, *MX1*, *MX2*, *OAS1*, *ZNF683*, *GZMA* (*ENSSSCG000000016902*), *GZMH*, *PDCD1*, *IL10*, *IRF8*, *NFKBIA*¹⁰², *ICOS*, *CTLA4*, *FOXP3*, *TOP2A*, *TPX2*, and *SMC2*) were used. Twenty-four lymphoid 2 features (*BLK*¹⁰³, *CD79B*, *TNFRSF17*, *BANK1*, *ISG15*, *MX1*, *MX2*, *OAS1*, *ENSSSCG000000045982*, *ENSSSCG000000046518*, *CD3E*, *FABP3*, *COX2*, *ENSSSCG000000044779*, *TOP2A*, *TPX2*, *SMC2*, *JCHAIN*¹⁰⁴, *MZB1*, *ND2*, *HSPA6*, *KLF2*, *CPB2*, and *GADD45A*) were analyzed. Adjacent clusters, presumed to belong to the same cell type (feature), were integrated. The cell type anticipated to be a doublet is classified as unknown due to the simultaneous expression of markers specific to different cell types. All unknown clusters were merged into a single unknown cell type. Key features distinguishing sub-cell types are summarized in Supplementary Data 7.

Differential gene expression analysis

Differential gene expression analysis was conducted using the *MAST* method with the 'FindAllMarkers' function of the *Seurat* (v4.3.0) R package. Comparisons were made among the negative group and the virulence groups (NA8, JA142, and NA10), focusing primarily on macrophages. Significance was defined as FDR < 0.05.

Viral tracking

The PRRSV sequences can be divided into nine main viral transcripts⁴. Based on the characteristics of PRRSV transcription, the ORF7 transcript was used to identify virus-infected cells. Furthermore, cells with

at least 10 viral transcripts (ORF7) were identified as productive infected¹⁰⁵. Three strains (NA8, JA142, and NA10) were used for viral tracking. Visualization of expression and density were performed using the Seurat (v4.3.0)⁷² and Nebulosa (v1.4.0)¹⁰⁶ R packages.

Cell-cell interaction analysis

Ligand-receptor interactions from scRNA-seq data were confirmed based on each time point of each group using the CellChat (v1.6.1)¹⁰⁷ according to the standard procedures. Cells with insufficient numbers (<20) were excluded from the analysis. The overall cell-cell communication probability and discovering significant interactions was calculated using default parameters.

Exosome isolation and characterization

Exosomes were isolated from 36 BALF samples using an Exo2d™-EV isolation kit (Exosome Plus, Suwon, Gyeonggi-do, Republic of Korea), according to the manufacturer's instructions. The purified exosomes were measured using a nanoparticle analyzer (SZ-100; HORIBA, Kyoto, Japan). Protein extracts were prepared in cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and protease inhibitor 20E [Millipore Sigma, Billerica, MA, USA]). Western blotting was performed using anti-CD63 (1:1,000; Santa Cruz Biotechnology, Dallas, TX, USA), anti-CD9 (1:1,000; Proteintech, Rosemont, IL, USA), and anti-Grp94 (1:1,000; Cell Signaling Technology). Chemiluminescent signals were detected using FluorChem HD2 (ProteinSimple, San Jose, CA, USA).

Exosomal small RNA sequencing

Small RNAs were extracted from the exosomes using a miRNeasy Serum/Plasma Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Small RNA concentrations were calculated using Quant-IT RiboGreen (Invitrogen, Waltham, MA, USA) with the Victor Nivo (PerkinElmer, Waltham, MA, USA), and integrity was calculated using the RNA 6000 Pico & Small RNA Kit (Agilent Technologies) with a Bioanalyzer2100 (Agilent Technologies). Only high-quality RNA preparations were used for RNA library construction. Small RNAs isolated from each BALF sample were used to construct sequencing libraries with the SMARTer smRNA-Seq Kit (Illumina), according to the manufacturer's protocol. Briefly, the input RNA was polyadenylated to provide a priming sequence for the oligo-(dT) primer. The cDNA synthesis was then primed by the 3' smRNA dT Primer, which incorporates an adapter sequence at the 5' end of each first-strand cDNA molecule. When the murine leukemia virus-derived PrimeScript™ Reverse Transcriptase reached the 5' end of each RNA template, nontemplated nucleotides were added, which were bound by SMART smRNA Oligos enhanced with locked nucleic acid technology for greater sensitivity. In the template-switching step, PrimeScript RT uses the SMART smRNA Oligos as a template for the addition of a second adapter sequence to the 3' end of each first-strand cDNA molecule. In the following step, full-length Illumina adapters (including index sequences for sample multiplexing) are added during PCR amplification. The forward PCR primer binds to the sequence added by the SMART smRNA Oligo, whereas the reverse PCR primer binds to the sequences added by the 3' smRNA dT Primer. The resulting cDNA library included the sequences required for clustering in an Illumina flow cell. The libraries were validated by checking their size, purity, and concentration using an Agilent Bioanalyzer (Agilent Technologies). The libraries were pooled in equimolar amounts and sequenced on an Illumina HiSeq 2500 instrument to generate 51 base reads. Image decomposition and quality value calculations were performed using Illumina pipeline modules.

Exosomal miRNA preprocessing and DE miRNA analysis

Total raw miRNA sequence reads were checked for quality using FAST quality control (QC) (v0.11.4)¹⁰⁸. The adaptor sequences and low-

quality sequences were trimmed using Trimmomatic (v0.39)¹⁰⁹ and the trimmed reads (≥15 nt) were used for further analysis. The trimmed reads were aligned to the porcine reference genome (*Sus scrofa* 11.1.106, GCA_000003025.6) using Bowtie1 (v1.3.1)¹¹⁰. The trimmed reads in FASTA format were recorded, and those >15 nt in length were aligned to the miRNAs in miRBase 22.1¹¹¹ with mapped FASTA data using the mapper.pl code in miRDeep2¹¹². Detailed information on trimming and mapping is provided in Supplementary Data 8. To identify DE miRNAs in each group, the DEseq2 (v1.40.2) R package¹¹³ was performed. DE miRNAs were considered significant when there was an absolute log₂ fold change ≥ 1 and $p < 0.01$.

Target gene prediction for DE exosomal miRNAs

To further explore the potential biological functions and processes of DE miRNAs in swine BALF, their potential target genes were predicted using the TargetScan (v8.0) tool¹¹⁴. Target genes with a context score percentile of >99 were selected for further analysis.

Functional annotation

For the scRNA-seq data, gene set enrichment analysis was performed using the escape package in R (v1.5.1)¹¹⁵. The Hallmark database¹¹⁶ and cell death signatures¹¹⁷ (apoptosis, necroptosis, autophagy, ferroptosis, and pyroptosis) were used to calculate signature scores, and the values were averaged among each cell type. For predicted target genes of DE exosomal miRNAs, the Biological Processes from Gene Ontology (GO) and KEGG databases were used to annotate functions using the Database for Annotation, Visualization, and Integrated Discovery (DAVID)¹¹⁸ with the default option. GO tree maps were generated using the REVIGO tool¹¹⁹ and enriched KEGG terms were visualized as a network with DE exosomal miRNAs and their predicted target genes.

qPCR validation

The expression of four chemokine and receptor genes (encoding CXCL14, CCL24, ACKR4, and SPP1) and 10 cell death signal-associated genes (encoding caspase-3, -7, -9, NLRP3, RIPK1, RIPK3, MLKL, caspase-1, -4, and GSDMD) in BALF cells was measured using RT-qPCR, as previously described. The primer set sequences for the target and housekeeping genes are provided in Supplementary Data 5^{62,65,67,68}.

Statistical analysis

The mean and standard deviation of the scRNA-seq data were calculated using R software (v4.1.3). Differences were analyzed using one-way analysis of variance, followed by Tukey's multiple comparison test. Significant mean differences were identified by $p < 0.05$. For other experimental measurements, the means and standard error means were calculated.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All raw and processed data for scRNA-seq of BALF cells are available from the Gene Expression Omnibus under super series accession GSE247257. All raw data for small RNA-seq of BALF exosomes are available from the Sequence Read Archive under the accession PRJNA640269. Source data are provided with this paper.

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Author contributions

K.-T.L., W.-I.K., and J.-M.K. supervised the study and designed the experiments. B.L. and S.-C.K. interpreted the data and wrote the manuscript. S.-C.K. and H.-J.K. performed the animal experiments and flow cytometry analysis. B.L. performed scRNA-seq analysis. B.L. and Y.-J.S. performed exosomal small RNA-seq analysis. D.-H.L. and K.P.

performed exosome experiments. S.-C.K. and H.-J.K. performed pathological analysis. J.-H.K., Y.-J.S., C.L., Y.P., S.S., and D.K. assisted the experiments and data analysis. All authors edited and approved the final paper.

Competing interests

The authors declare no competing interests.

Additional information

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