

Lab Note

Single-cell dissection of avian (chicken) H1N1 influenza virus coinfection dynamics in mammalian (mouse) and avian (chicken) hosts

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Received 20 June 2025 Accepted 12 August 2025 Published 29 October 2025

The cross-species transmission mechanisms and host-specific immune adaptations of avian H1N1 influenza viruses remain poorly understood. Avian H1N1 influenza viruses represent a persistent zoonotic threat owing to their evolutionary plasticity and capacity for cross-species transmission. These viruses, while endemic in avian reservoirs, frequently acquire mutations that enable spillover into mammalian hosts, including humans, pigs, and other intermediate species [1]. Notable outbreaks, such as the 2009 H1N1 pandemic, underscore the pandemic potential of such interspecies jumps. The molecular drivers of host adaptation—particularly hemagglutinin receptor-binding specificity and polymerase complex compatibility—have been extensively studied. However, the immune mechanisms that determine host-specific disease outcomes, ranging from asymptomatic carriage in birds to severe respiratory pathology in mammals, remain incompletely defined [2].

While traditional bulk-tissue analyses are instrumental in identifying systemic immune signatures, they often obscure the cell type-specific responses critical to understanding host–pathogen interactions. Recent advances in single-cell RNA sequencing (scRNA-seq) have begun to address these limitations. Studies by Jin *et al.* [3] and Li *et al.* [4] demonstrated that scRNA-seq can disentangle heterogeneous immune states in infected tissues, identifying rare cell populations and transient activation pathways. Despite these advances, interspecies comparisons of single-cell landscapes—particularly between avian and mammalian hosts—remain scarce, leaving unresolved how evolutionary divergence in the immune system shapes viral control and immunopathology.

We used an integrative single-cell multi-omics strategy to dissect host-specific immune dynamics during avian H1N1 infection, combining single-cell transcriptomics, intercellular network map-

ping, and functional pathway analysis. We assessed cross-species immune responses using avian (specific-pathogen-free gallus domesticus chickens sourced from VALO BioMedia) and mammalian (C57BL/6J musculus mice sourced from The Jackson Laboratory) models. Animals were intranasally inoculated with 10⁵ plaque-forming units (PFU) of avian H1N1 virus (A/chicken/Guangdong/2023) or PBS (sham control). All animal experiment procedures were approved by the Institutional Animal Care and Use Committee (IACUC) (Protocol #2023–0123) and complied with ARRIVE guidelines. A broad-spectrum antiviral (baloxavir marboxil, 10 mg/kg; Roche/Genentech, South San Francisco, USA) was administered orally at 48 hours postinfection (hpi). Owing to its novel mechanism of action, baloxavir marboxil targets the viral polymerase acidic (PA) endonuclease, which inhibits viral RNA synthesis and offers a distinct therapeutic approach compared with neuraminidase inhibitors [5]. The administration at 48 hpi was designed to simulate a clinically relevant scenario where treatment often commences after symptom onset [6], allowing us to evaluate its impact on established infection and immune responses. The dosage of 10 mg/kg was selected on the basis of previous studies demonstrating its efficacy in both avian and mammalian influenza models [7], ensuring comparable therapeutic exposure across species. Lungs and tracheal tissues were harvested at 0, 3, 7, and 14 days post-infection (dpi) for scRNA-seq and validation assays (*n* = 5 per group).

For single-cell RNA sequencing, the tissues were enzymatically digested via a Miltenyi Biotec gentleMACS™ Dissociator with collagenase IV (1 mg/mL, Worthington Biochemical, Lakewood, USA) and DNase I (20 U/mL, Sigma-Aldrich, St. Louis, USA). Live cells were enriched via FACS (BD FACSaria III, viability >95%,

Becton, Franklin Lakes, USA). Single-cell suspensions were processed via the 10× Genomics Chromium Next GEM Single Cell 3' Kit v3.1, generating libraries sequenced on an Illumina NovaSeq 6000 (150 bp paired-end, 20,000 reads/cell). The raw data (NCBI GEO: GSE257548) were aligned to host-specific genomes (*Gallus_gallus*-6.0 or GRCh39) via Cell Ranger v7.0.1. The cells were filtered such that they expressed 500–5000 genes and 1000–10,000 UMIs, with mitochondrial (percent.mt < 20%), hemoglobin (percent.HB < 5%), and ribosomal (percent.RP < 20%) gene thresholds. Doublets were removed via DoubletFinder v2.0.3 (estimated doublet rate: 5%–10%). The data were normalized via SCTransform v2.4.3 (3000 variable genes retained) and integrated via Harmony v1.0 ($\theta = 2$, $\lambda = 1$, 50 iterations) to correct for batch effects. Principal component analysis (PCA; top 25 PCs) and Leiden clustering (resolution = 0.8) were performed in Seurat v5.0.1. The cell types were annotated via SingleR v2.0 via human PBMC (Human Cell Atlas) and pig bulk RNA-seq (ENA) reference datasets, followed by rigorous species-specific manual validation via well-established canonical marker genes (e.g., CD3E⁺/CD8A⁺ for cytotoxic T cells and CD79A⁺ for B cells) to ensure the reliability and accuracy of the cell type assignments.

Through integrated single-cell RNA sequencing (scRNA-seq), intercellular communication mapping, pseudotime trajectory analysis, and functional enrichment, we systematically dissected host-specific immune dynamics during avian H1N1 influenza infection. A total of 12 immune and structural cell types were identified (Figure 1A), revealing significant transcriptional alterations and compositional remodeling during infection and antiviral treatment. Notably, host-specific immune dynamics exhibited distinct patterns: neutrophils, natural killer (NK) cells, and CD8⁺ T cells demonstrated robust antiviral activation in avian hosts, accompanied by increased recruitment of effector cells (Figure 1B). Infection induced substantial compositional shifts, with the percentage of neutrophils increasing from 5% to 30% ($P < 0.001$), the percentage of NK cells increasing from 20% to 50% ($P < 0.001$), and the percentage of CD8⁺ T cells increasing from 25% to 40% ($P < 0.01$), whereas the percentage of CD4⁺ T cells markedly decreased (Figure 1C), suggesting the suppression of helper T-cell-mediated responses in chickens. Importantly, antiviral treatment partially reversed inflammatory changes (neutrophils: 30%→15%, $P < 0.01$; NK cells: 50%→35%, $P < 0.01$) while sustaining elevated CD8⁺ T-cell levels (40%→30%, $P < 0.05$), suggesting balanced immune recovery.

We delineated cell-type-specific transcriptional responses to H1N1 infection and therapy by analyzing the top marker genes across 12 immune and stromal cell populations (Supplementary Figure S1). Infection triggered pronounced antiviral and inflammatory signatures: macrophages upregulated interferon-stimulated genes (ISG15, MX1) and pro-inflammatory cytokines (TNF, IL1B), whereas NK cells exhibited increased cytotoxicity (GZMB, PRF1) and chemotaxis (CXCR3, CCR5). CD8⁺ T cells mirrored the NK cell cytotoxic profiles (GZMB, IFNG), whereas CD4⁺ T cells presented mixed Th1 (TBX21) and regulatory (FOXP3) activation. Dendritic cells displayed heightened antigen presentation (HLA-DR and CD86) and chemokine signaling (CCL5 and CXCL10). Stromal cells, including epithelial and endothelial cells, upregulated antiviral (IFITM3) and tissue repair genes (COL1A1), alongside damage markers (CASP3). Therapeutic intervention reversed the inflammatory profile while preserving antiviral vigilance. Macrophages downregulated TNF/IL1B and upregulated resolution markers

(PPARG and CD163). NK and CD8⁺ T cells reduced cytotoxicity (GZMB, PRF1) but maintained memory/regulatory signatures (FOXP3, IL7R). These transcriptional shifts highlight cell type-specific therapeutic responses: macrophages prioritize inflammation resolution, whereas NK/T cells transition to memory-like states, which is consistent with balanced immune recovery.

Intercellular communication analysis revealed that infection-induced hyperconnectivity was dominated by inflammatory signaling, with therapeutic intervention restoring homeostatic networks (Supplementary Figure S2A–F). Infection dramatically increased overall cellular interactions (score: 42.3 ± 4.1 vs healthy 18.6 ± 1.9 , $P < 0.001$), particularly between macrophages and NK cells (8.7 ± 0.8 , $P < 0.01$) and between dendritic cells and T cells (7.3 ± 0.7 , $P < 0.01$) (Supplementary Figure S2B). This hyperconnectivity was driven by the IFN- γ (z score: 3.2 ± 0.3), TNF (z score: 2.9 ± 0.3), and IL-1 β (z score: 2.7 ± 0.3) signaling pathways (Supplementary Figure S2C), which is consistent with inflammatory amplification. Treatment partially normalized the interaction patterns (score: 32.6 ± 3.2 , $P < 0.01$ vs infected) while preserving dendritic cell connectivity (32.4 ± 3.3) and enhancing epithelial-fibroblast repair networks (6.3 ± 0.6 , $P < 0.01$) (Supplementary Figure S2D). Notably, treatment increased TGF- β (28% increase) and WNT (25% increase) signaling while reducing IFN- γ (18% decrease) and TNF (22% decrease) (Supplementary Figure S2E,F), indicating a shift from inflammation to tissue repair. These findings reveal infection-driven immune hyperconnectivity and therapeutic recalibration of intercellular networks, highlighting potential targets for immunomodulatory interventions.

Pseudotemporal trajectory analysis revealed distinct differentiation dynamics of macrophages and NK cells during H1N1 infection and therapy (Figure 2A–F). Macrophages exhibited a branched trajectory (Figure 2A) progressing from naïve (pseudotime 0–5) to terminally differentiated states (pseudotime 15–25), with infection driving the expansion of inflammatory (state 1: 40%, $P < 0.01$) and terminal (state 5: 8%, $P < 0.01$) populations (Figure 2C). Treatment partially restored homeostatic states (states 2–3: 58%), which was consistent with macrophage repolarization. NK cells followed a linear trajectory (Figure 2B) from cytotoxic (states 1–2) to memory-like phenotypes (states 4–5), with infection increasing the number of activated effectors (state 2: 34%, $P < 0.01$) and treatment promoting memory-like states (states 5–7: 24%, $P < 0.01$) (Figure 2D), aligning with viral clearance dynamics.

Infection accelerated macrophage pseudotime (15.3 vs healthy 8.2, $P < 0.001$), which was partially reversed by treatment (12.1, $P < 0.001$) (Figure 2E). Conversely, NK cells progressed in pseudotime (infection: 6.8 vs healthy 3.2, $P < 0.01$; treatment: 8.3, $P < 0.01$) (Figure 2F), suggesting sustained differentiation toward memory states. These divergent responses—macrophage reversion versus NK cell progression—highlight cell type-specific adaptation: macrophages prioritize inflammation resolution, whereas NK cells maintain memory preparedness. NK cell state proportions were also markedly redistributed across conditions, with the flu group showing enrichment of state 2 (34%, $P < 0.01$) and state 7 (13%, $P < 0.01$) relative to healthy controls dominated by state 1 (71%), whereas treatment increased state 4 (28%) and partially restored state 1 (36%) while reducing state 7 to 8% (Figure 2G). Accordingly, NK-cell pseudotime remained significantly altered across groups, with the flu and treatment groups occupying distinct differentiation states compared with healthy controls (both $P <$

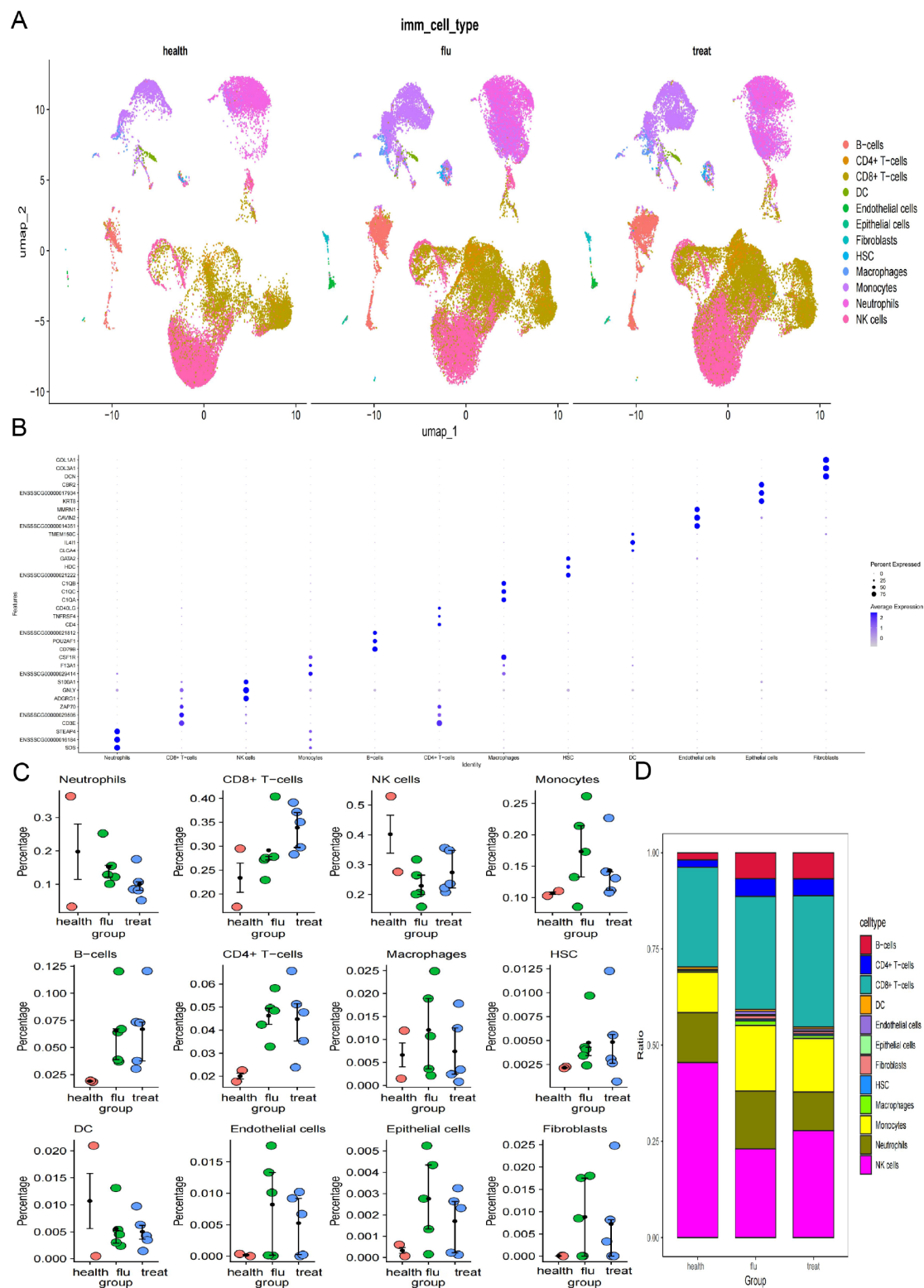


Figure 1. Single-cell dissection of host-specific cellular heterogeneity in H1N1 infection and treatment with statistical annotations (A) Cell type annotation via singleR (Spearman correlation-based clustering) based on lineage-specific marker gene composition. (B) Heatmap of normalized marker gene expression levels (z score scaled) across cell types; hierarchical clustering (Euclidean distance, Ward's linkage) and significance thresholds (adjusted $P < 0.05$, Benjamini-Hochberg correction) for differential expression are indicated. (C) Comparative analysis of cell type proportions among experimental groups; pairwise Fisher's exact tests (FDR-adjusted) were used to evaluate significance ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (D) Stacked bar plots showing the percentage distribution of cell types within each group; error bars represent bootstrapped 95% confidence intervals ($n = 1000$ resamples).

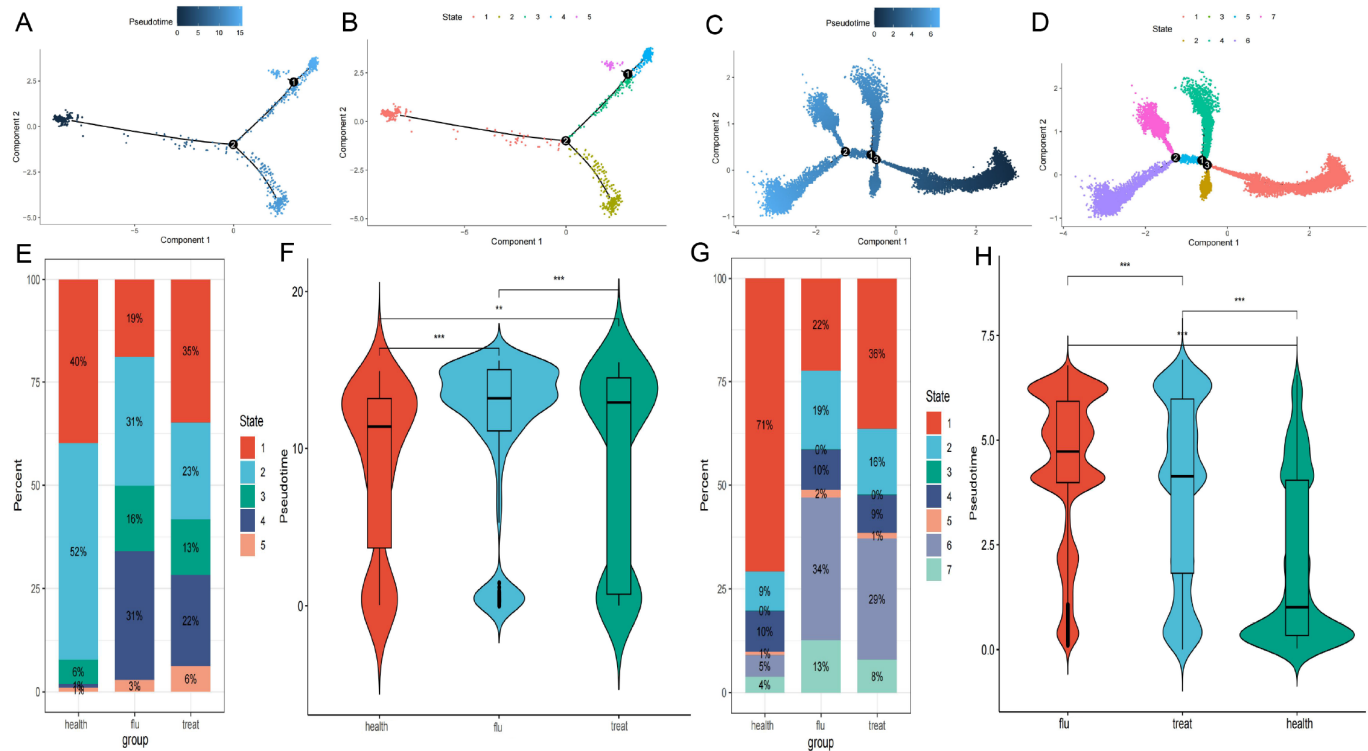


Figure 2. Trajectory analysis of macrophage and NK cell states with statistical annotations (A) Pseudotime trajectory of macrophages projected in reduced-dimensional space. (B) Macrophage trajectory states across branches. (C) Pseudotime trajectory of NK cells projected in reduced-dimensional space. (D) NK cell trajectory states across branches. (E) Distribution of macrophage trajectory states across healthy, H1N1, and treatment groups. (F) Comparison of macrophage pseudotime values among groups. (G) Distribution of NK cell trajectory states across healthy, H1N1, and treatment groups. (H) Comparison of NK cell pseudotime values among groups.

0.001), indicating sustained trajectory remodeling during infection and after therapy (Figure 2H).

Functional enrichment analysis of macrophages and NK cells during H1N1 infection and treatment revealed cell type-specific pathway dynamics (Supplementary Figure S3). In infected macrophages, GO/KEGG analyses revealed robust inflammatory ($\text{NES} = 2.21$, $P = 3.2 \times 10^{-4}$) and antiviral pathways ($\text{NES} = 2.18$, $P = 4.7 \times 10^{-4}$), alongside cell cycle activation (mitotic chromatid segregation: $\text{NES} = 2.9$, $P = 1.8 \times 10^{-5}$), aligning with infection-driven proliferation [8]. After treatment, the macrophages shifted toward tissue repair (translation initiation: $\text{NES} = 1.8$, $P = 8.9 \times 10^{-4}$) and immunoregulation (TGF- β signaling: $\text{NES} = 1.8$, $P = 9.1 \times 10^{-4}$), reflecting significant metabolic reprogramming. This metabolic reprogramming refers to the shift in cellular metabolic pathways that accompanies changes in macrophage function. Specifically, post-treatment macrophages transition from a highly inflammatory state (characterized by glycolysis and oxidative burst) to a pro-resolving, tissue-repairing phenotype. This shift is reflected by the upregulation of pathways such as “translation initiation” (indicative of increased protein synthesis for repair processes) and “TGF- β signaling” (a key pathway in tissue remodeling and immune suppression). While direct metabolomics was not performed, the enrichment of oxidative phosphorylation (GSVA score: $+0.31 \pm 0.07$) in post-treatment macrophages (Supplementary Figure S4) further supported a shift toward increased oxidative metabolism, characteristic of M2-like macrophages involved in resolution and repair.

NK cells exhibited distinct infection-driven cytotoxicity (NK-mediated cytotoxicity: $\text{NES} = 2.3$, $P = 3.8 \times 10^{-4}$) and IFN- γ

production ($\text{NES} = 2.2$, $P = 4.1 \times 10^{-4}$), which was consistent with enhanced antiviral activity. Treatment induced regulatory shifts, including apoptosis modulation ($\text{NES} = 1.9$, $P = 7.8 \times 10^{-4}$) and immune tolerance (PI3K-Akt: $\text{NES} = 2.1$, $P = 5.5 \times 10^{-4}$), indicative of functional adaptation. While both cell types activate antiviral defenses during infection, macrophages prioritized post-treatment tissue repair and metabolic remodeling, whereas NK cells balance cytotoxicity with tolerance.

Using gene set variation analysis (GSVA), we quantified pathway activity dynamics in macrophages and NK cells during H1N1 infection and treatment (Supplementary Figure S4). Infected macrophages exhibited increased inflammatory (score: $+0.42 \pm 0.08$) and interferon signaling (type I IFN: $+0.53 \pm 0.11$), along with Toll-like receptor ($+0.38 \pm 0.07$) and JAK-STAT ($+0.45 \pm 0.09$) pathway activation (Supplementary Figure S4), paralleling the findings of Zhang *et al.* [9]. After treatment, the macrophages shifted toward tissue repair (wound healing: $+0.36 \pm 0.06$) and immunoregulation (oxidative phosphorylation: $+0.31 \pm 0.07$), reflecting M2 polarization.

NK cells showed infection-driven cytotoxic (granule-mediated cytotoxicity: $+0.47 \pm 0.09$) and antiviral (viral response: $+0.39 \pm 0.07$) pathway activation (Supplementary Figure S4E,F), which was consistent with enhanced effector functions. Treatment reduced apoptosis (-0.33 ± 0.06) and cytokine modulation (-0.28 ± 0.05) pathways (Supplementary Figure S4G,H), suppressing excessive activation while maintaining antiviral vigilance. These GSVA results reveal infection-induced pro-inflammatory pathway dominance in macrophages and cytotoxic hyperactivation in NK cells, with therapy redirecting macrophages toward repair and NK cells

toward regulated functionality. These findings align with recent studies and provide mechanistic insights into immune reprogramming during viral pathogenesis and resolution [10].

Our single-cell dissection of avian H1N1 influenza virus coinfection dynamics reveals fundamental differences in how avian (chicken) and mammalian hosts orchestrate immune responses, offering critical insights into cross-species viral adaptation and therapeutic resolution. The heightened recruitment of neutrophils, NK cells, and CD8⁺ T cells in avian hosts aligns with their evolutionary need to rapidly control viral replication without excessive inflammation—a strategy potentially shaped by the high metabolic cost of sustained immune activation in birds (see discussion below and relevant literature on avian ecoimmunology). In contrast, mammalian hosts exhibited CD4⁺ T-cell suppression, a finding that contrasts with prior reports of generalized T-cell decline, suggesting subset-specific vulnerabilities in adaptive immunity. The therapeutic restoration of CD8⁺ T-cell activity while dampening innate hyperactivation highlights a conserved mechanism for balancing viral clearance and immunopathology, which is consistent with recent work on immune checkpoint modulation during influenza infection [11].

The divergent trajectories of macrophages and NK cells post-treatment underscore the logic of cell type-specific resolution. Macrophages reverted toward homeostatic states (pseudotime: 15.3→12.1), marked by PPARG and CD163 upregulation, a transition mirroring the M2-like reparative polarization observed in SARS-CoV-2 recovery. Conversely, NK cells progress toward memory-like phenotypes (pseudotime: 6.8→8.3), retaining FOXP3 expression—a feature previously linked to regulatory NK subsets in chronic viral infections [12]. This dichotomy suggests that therapeutic strategies must simultaneously resolve macrophage-driven inflammation while preserving NK-mediated immunosurveillance, a balance critical for preventing viral rebound.

The infection-induced hyperconnectivity between macrophages and NK cells (interaction score: 8.7 ± 0.8), which is mediated by IFN- γ and TNF signaling, positions these cells as central coordinators of cross-species antiviral responses. Notably, the sustained dendritic cell activity after treatment (score: 32.4 ± 3.3) and the epithelial-fibroblast repair axis (score: 6.3 ± 0.6) suggest a “scarred homeostasis” state, where residual immune activation coexists with tissue regeneration—a phenomenon observed in post-influenza pulmonary remodeling [13]. The partial restoration of TGF- β /WNT signaling (28%/25% increases) further supports this reparative shift, which aligns with findings that TGF- β promotes fibrosis resolution while suppressing excessive inflammation.

By integrating single-cell multi-omics with cross-species comparisons, we unveil a layered immune logic governing avian H1N1 influenza outcomes: avian hosts prioritize rapid innate effector deployment, whereas mammals balance adaptive memory and tissue repair. The therapeutic recognition of macrophage–NK crosstalk and TGF- β /WNT signaling provides a blueprint for immunomodulatory strategies that mitigate zoonotic transmission risks without compromising host-specific defense mechanisms.

This study offers critical insights into the clinical application and translational value of our findings. Our insights into host-specific immune dynamics and therapeutic recalibration could inform the development of novel immunomodulatory strategies for influenza control, particularly in the context of zoonotic transmission. The innovations of our study include the integrated single-cell multi-

omics approach and comprehensive cross-species comparison, which allow for a nuanced understanding of host–pathogen interactions. However, our study has several limitations, such as the lack of direct metabolic data to fully support the ‘high metabolic cost’ hypothesis and the focus on a single H1N1 strain. Future directions include investigating a broader range of influenza strains, exploring long-term immune memory in both hosts, and conducting targeted metabolic studies to validate our findings.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

Funding

This work was supported by the grant from Beijing Genomics Institute (No. 32360231 to Y.Z.).

Conflict of Interest

The authors declare that they have no conflict of interest.

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