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Primordial germ cell-mediated *RAG1* knockout in chickens uncovers RAG1-independent B cells and conserved NK subsets

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Introduction

Chickens are powerful models for comparative immunology, combining mammalian-like adaptive mechanisms with distinctive avian features. Unlike mammals, chicken B cells develop in the bursa of Fabricius and use a single functional V and J gene per chain, diversifying their antibody repertoire by post-rearrangement gene conversion from upstream pseudogenes (Reynaud *et al*., 1985; Reynaud *et al*., 1989). Embryonic B cells can persist without a functional B cell receptor (BCR), but post-hatch B cell development normally fails in its absence, as shown in J_H-knockout birds (Schusser *et al*., 2013). However, post-hatch bursal B cells have recently been described in *RAG1*-deficient chickens lacking functional BCRs, revealing a previously unrecognized RAG1-independent pathway (Lee *et al*., 2022).

The innate compartment also diverges from mammals, particularly in natural killer (NK) cell biology. T cell transcriptional programs are relatively conserved across species, whereas NK cell programs are more divergent (Carmona *et al*., 2017). Human and mouse NK cells segregate into immature, weakly cytotoxic and mature, strongly cytotoxic subsets, while chicken NK cells remain poorly defined because their transcriptional profiles overlap extensively with T cells (Chiossone *et al*., 2009; Ferlazzo *et al*., 2004; Narni-Mancinelli *et al*., 2011).

Detailed analysis of chicken immunity has further been constrained by limited pan-markers and suitable models. However, primordial germ cells (PGCs), which transiently circulate in the blood at Hamburger–Hamilton (HH) stages 14–16, provide an accessible, long-term-culturable germline progenitor that can be precisely edited with platforms such as CRISPR/Cas9 (Whyte *et al*., 2015). It enabled creation of diverse immune gene-targeted chicken models (Lee *et al*., 2022; Schusser *et al*., 2013).

In this study, we discovered *RAG1*-independent post-hatch B cell pathway and delineation of NK cell subsets without confounding T cell populations using *RAG1*-deficient chickens generated by introducing *RAG1*-disrupted PGCs. Collectively, these PGC-based genome-editing strategies establish the chicken as a genetically tractable system for dissecting avian lymphocyte development and for refined comparative studies across vertebrate immune systems.

Materials & Methods

Generation of *RAG1*-deficient chicken model. *RAG1* knockout chickens were generated as described previously (Lee *et al*., 2022). Briefly, White Leghorn (WL) primordial germ cells (PGCs) were transfected with 2 µg each of CRISPR/Cas9 and donor plasmids in 1 ml Opti-MEM (Thermo Fisher Scientific) using Lipofectamine 2000. Transfected PGCs (1×10^5) were selected with G418 (300 µg/ml) for up to 7 days, and over 3,000 edited cells were microinjected into the dorsal aorta of Korean Ogye (KO) embryos at HH stages 14–17. After window closure with paraffin film, eggs were incubated to hatching. Hatched chicks were reared to maturity, and KO roosters carrying WL-specific PCR signals in sperm were crossed with wild-type WL hens to produce homozygous *RAG1*-deficient offspring.

Single-cell RNA sequencing (scRNA-seq) analysis. Single-cell suspensions were prepared from spleens of 1-week-old wild-type and *RAG1*-deficient chickens as previously described (Woo *et al*., 2025). Single-cell libraries were generated and scRNA seq raw data were processed by Macrogen Inc. (Seoul, Korea) using their in-house pipeline. For cross-species integration, published human and murine NK cell scRNA-seq datasets from GEO were analyzed (Crinier *et al*., 2018). After quality control, 1:1:1 orthologous genes across the three species were identified using Ensembl BioMart, and batch effects were corrected with Harmony v1.2.1.

Results & Discussion

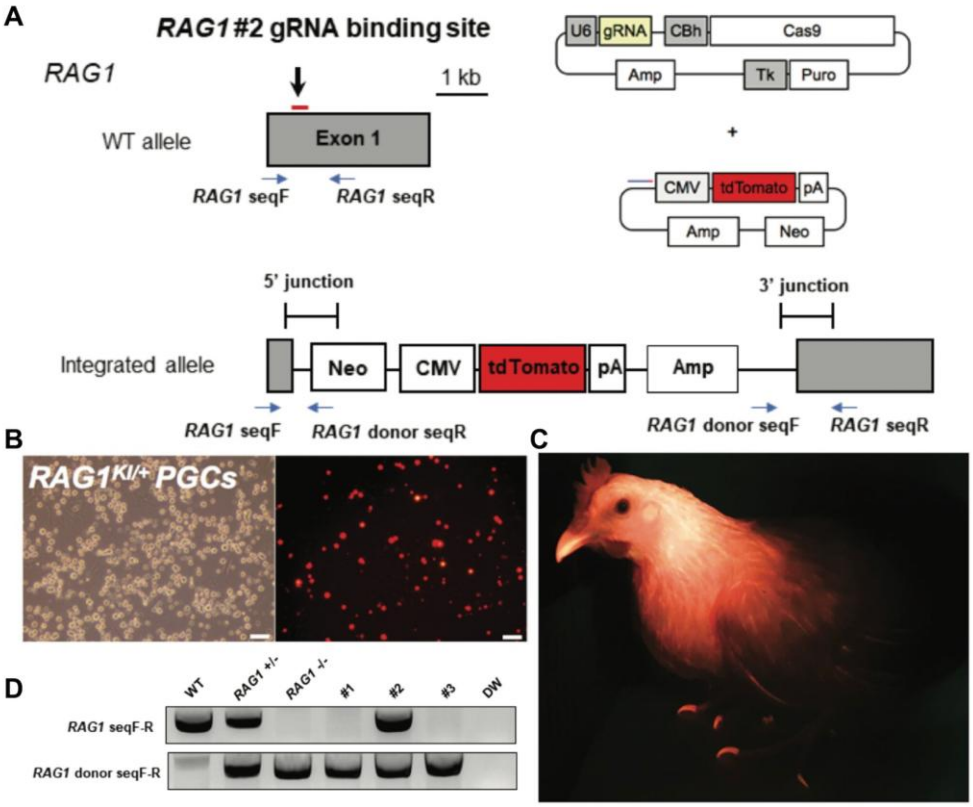

Figure 1. Generation of *RAG1*-deficient chicken. (A) Design of *RAG1* targeting in chicken PGCs through introducing tdTomato red fluorescence gene. (B) *RAG1*-disrupted PGCs emitting red fluorescence. (C) *RAG1*-deficient chicken model generated by injecting *RAG1*-disrupted PGCs. (D) Genotyping of *RAG1*-deficient chicken progenies through junctional PCR. Modified from (Lee *et al*., 2022).

A CRISPR/Cas9 construct targeting the region upstream of chicken *RAG1* exon 1 was designed, and a donor plasmid carrying a tdTomato cassette was co-transfected to introduce a premature stop codon in *RAG1* (Fig. 1A). *RAG1*-disrupted PGCs were identified by red fluorescence after transfection (Fig. 1B) and injected into HH14–17 embryonic blood vessels to generate germline chimeras, which were then crossbred with wild-type hens to obtain red fluorescence *RAG1*-deficient offspring (Fig. 1C). *RAG1* genotypes were analyzed by junctional PCR using primer sets specific for the endogenous *RAG1* locus and the integrated donor sequence (Fig. 1D).

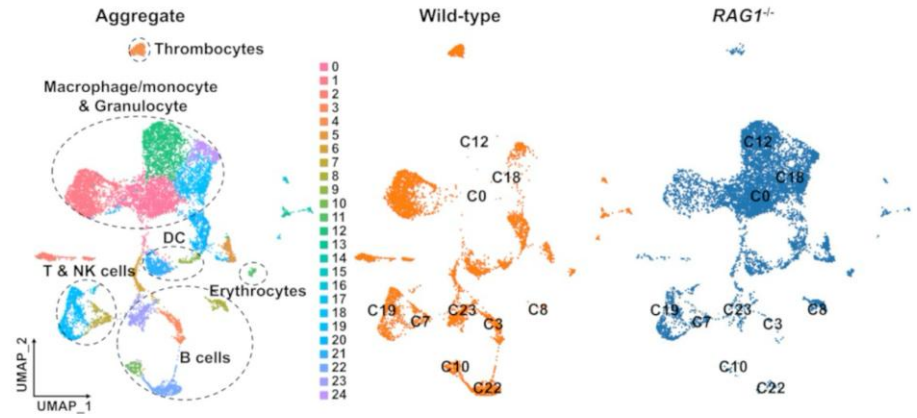

Figure 2. scRNA-seq of chicken leukocytes. UMAP showing 25 clusters between wild-type and *RAG1*$^{-/-}$ 1-week-old chicken spleen. Chicken leukocytes were annotated based on gene marker expression. Modified from (Woo *et al*., 2025)

RAG1 homozygous knockout (*RAG1*$^{-/-}$) chickens were used to characterize the reprogramming of adaptive and innate leukocytes in the absence of *RAG1*. Spleens from 1-week-old wild-type and *RAG1*$^{-/-}$ chickens were analyzed by scRNA-seq, identifying 25 distinct clusters that were annotated based on marker gene expression (Fig. 2).

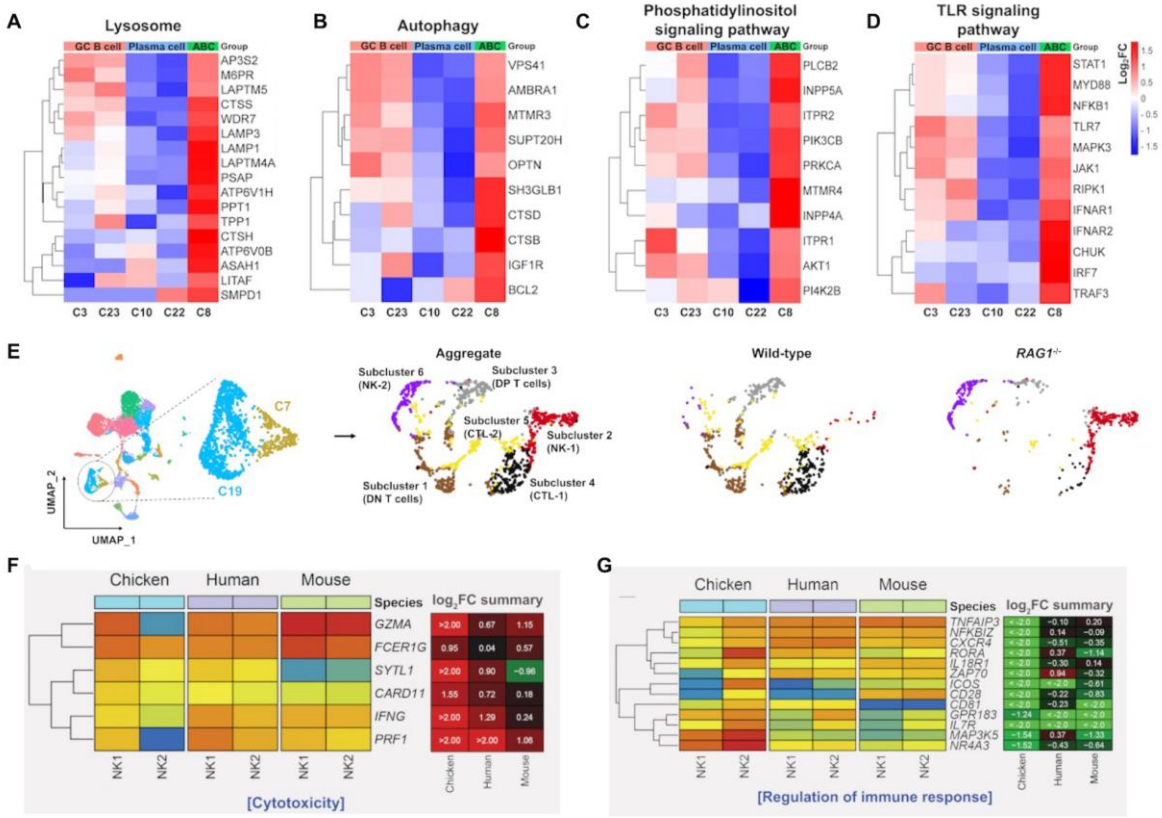

Figure 3. Identification of reprogrammed chicken leukocytes without *RAG1*. Analysis of differentially expressed genes (DEGs) related to (A) lysosome, (B) autophagy, (C) phosphatidylinositol, (D) TLR signalling pathway in c8 (atypical B cells; ABCs). (E) Identification of NK cell subsets in *RAG1*$^{-/-}$ chicken. Comparison of (F) mature chicken NK cell (NK-1) and (G) immature NK cell (NK-2) with humans and mice. Modified from (Woo *et al*., 2025).

We first examined B cell clusters (c3, c8, c10, c22, c23) and found that only c8 was markedly expanded in *RAG1*$^{-/-}$ spleen (Fig. 2), indicating a population of RAG1-independent B cells (hereafter atypical B cells; ABCs). The existence of ABCs therefore implies an alternative, RAG1-independent pathway of B cell development, challenging the paradigm that post-hatch chicken B cells cannot arise without a functional BCR. Transcriptomic profiling showed ABCs upregulate lysosomal autophagy, phosphatidylinositol and TLR signaling pathways (Fig. 3A–D), reminiscent of autoreactive B cells driven by phosphatidylinositol–TLR signaling in partially *RAG1*-deficient patients (Csomos et al., 2022).

Re-clustering of mixed T & NK cell clusters (c7 and c9) identified six subclusters. Subcluster 2, and 6 were expanded in *RAG1*$^{-/-}$ chicken and significantly expressed NK cell-related genes, thereby annotating subcluster 2 as NK-1 and subcluster 6 as NK-2 (Fig. 3E). Comparative analysis across human and mouse revealed functional conservation: NK-1 showed cytotoxic and effector gene signatures, while NK-2 exhibited immune-regulatory profiles similar to mammalian counterparts (Fig. 3F, G). These findings highlight evolutionary conservation of NK cell functions and support the chicken as a model for human translational immunology.

Conclusions

In conclusion, the genome edited PGC-mediated *RAG1*-deficient chicken provides a unique system to uncover alternative, RAG1-independent B lymphocyte pathways and to resolve NK cell heterogeneity without T cell interference. These findings broaden our understanding of avian immune organization and offer valuable insights to further analyse uncovered avian-adapted immune cells such as heterophils for gaining evolutionarily insight.

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References

Carmona S.J., Teichmann S.A., Ferreira L., *et al*. (2017) Genome Res 27(3):451-461. https://genome.cshlp.org/content/27/3/451

Chiossone L., Chaix J., Fuseri N., *et al*. (2009) Blood 113(22):5488-96. 10.1182/blood-2008-10-187179

Crinier A., Milpied P., Escalière B., *et al*. (2018) Immunity 49(5):971-986. https://www.sciencedirect.com/science/article/pii/S1074761318304242

Csomos K., Ujhazi B., Blazso P., *et al*. (2022) Nat Immunol 23(8):1256-1272. https://www.nature.com/articles/s41590-022-01271-6

Ferlazzo G., Thomas D., Lin S-L., *et al*. (2004) J Immunol 172(3):1455-62. 10.4049/jimmunol.172.3.1455

Lee K.Y., Choi H.J., Park K.J., *et al*. (2022) Front Immunol 13:892476. https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2022.892476/full

Narni-Mancinelli E., Vivier E., and Kerdiles Y.M. (2011) Int Immunol 23(7):427-31. https://academic.oup.com/intimm/article/23/7/427/675088?guestAccessKey=

Reynaud C.A., Anquez V., Dahan A., *et al*. (1985) Cell 40(2):283-91. https://www.sciencedirect.com/science/article/pii/0092867485901424?via=ihub

Reynaud C.A., Dahan A., Anquez V., *et al*. (1989) Cell 59(1):171-83. https://www.sciencedirect.com/science/article/pii/0092867489908799

Schusser B., Collarini E.J., Yi H., *et al*. (2013) Proc Natl Acad Sci U S A 110(50):20170-5. https://www.pnas.org/doi/10.1073/pnas.1317106110

Whyte J., Glover J.D., Woodcock M., *et al*. (2015) Stem Cell Reports 5(6):1171-1182. https://www.sciencedirect.com/science/article/pii/S2213671115003057

Woo S.J., Kim J., Lee H.J., *et al*. (2025) iScience 28(8):113144. https://www.sciencedirect.com/science/article/pii/S2589004225014051