Unraveling new characteristics of γδ T cells using scRNA-seq in TCR KO chicken

Theresa von Heyl[1], Milena Brunner[1], Antonia Schmauser[1], Christine Wurmser[2], Simon Früh[3,4], Benjamin Schusser[1,5*]

[1] Reproductive Biotechnology, TUM School of Life Science, Technical University of Munich, 85354 Freising, Germany

[2] Chair of Animal Physiology and Immunology, TUM School of Life Sciences, Technical University of Munich, 85354 Freising, Germany

[3] Department of Veterinary Sciences, Ludwig-Maximilians-Universität München, 80539 Munich, Germany

[4] Virale Integration, Tumorigenesis and Virus evolution, Institute of Virology, Freie Universität Berlin, 14163 Berlin, Germany

[5] Center for Infection Prevention (ZIP), Technical University of Munich, 85354 Freising, Germany

*** Correspondence:**
Benjamin Schusser
benjamin.schusser@tum.de

Keywords: T cells, chicken, gamma/delta T cells, genetically modified animals, scRNA-seq

Abstract

The characterization of T cells in chickens has proven to be challenging, primarily due to the lack of specific markers to differentiate classical Th1, Th2, and Th17 subsets. Furthermore, chickens possess a notably high proportion of γδ T cells, making them a unique model for investigating the poorly understood role of these cells not only in chickens but also in mammals. To gain deeper insights into the functions and characteristics of the αβ and γδ T cell subsets in chickens, whole transcriptome analysis (WTA) on $CD3^+$ single cells isolated from TCR Cβ knockout (KO), TCR Cγ KO, and wild type (WT) chickens at embryonic day (ED) 18, day (d) 3, and d14 post-hatch was conducted. The results indicate that γδ T cells exhibit cytolytic activity in both TCR Cβ KO and WT chickens, a feature absent in TCR Cγ KO chickens. The severe phenotype observed in TCR Cβ KO chickens revealed a distinct cluster of γδ T cells expressing genes associated with the interferon pathway. Additionally, novel gene markers were identified that more precisely define the αβ and γδ T cell subsets. These findings provide new insights into chicken T cell biology and contribute to a better understanding of the mechanisms underlying the severe phenotype observed in TCR Cβ KO chickens.

1 Introduction

Chickens are an indispensable livestock species, as not only the chicken meat but also the eggs are principal protein sources worldwide. Compared to other livestock, chicken meat is healthier and cheaper (Farrell 2011, Salter 2018). Livestock diseases often pose significant threats, leading to great

economic losses. Moreover, diseases like avian influenza pose a considerable zoonotic risk to humans (Bodewes, Bestebroer et al. 2015, Rahman, Sobur et al. 2020, Abdelwhab and Mettenleiter 2023). Therefore, a detailed understanding of the chicken immune system is essential to ensure the health of chickens and humans.

T cells are key players in the adaptive immunity of chickens (Cooper and Alder 2006). They are categorized into αβ T cells and γδ T cells based on their T cell receptor chains (Chen, Sowder et al. 1989). The role of αβ T cells is defined through co-expression of CD4 and CD8 (Arstila, Vainio et al. 1994, Luhtala 1998). The $CD4^+$ αβ T cells are activated through antigens presented on MHC class II molecules, to produce cytokines that stimulate B cells to secrete immunoglobulins (Arstila, Vainio et al. 1994). The $CD8^+$ T cells can directly lyse pathogens by secreting cytotoxic cytokines upon activation through MHC class I molecules (Luhtala 1998). Compared to mammals, there is no clear classification into Th1, Th2 and Th17 cells of αβ Tcells in chickens, as the classification is based on the secretion of various interleukins, some of which have not yet been described (Mousset, Hobo et al. 2019).

The other major T cell subset, γδ T cells, remains poorly understood in both chickens and mammals. Chickens present a compelling model for studying the role of γδ T cells in greater detail, as they are a γδ-high species, with comprising up to 50% γδ T cells of circulating lymphocytes throughout their lifespan (Sowder, Chen et al. 1988). In contrast, mice and humans typically harbor only 1–10% γδ T cells (Sowder, Chen et al. 1988, Holderness, Hedges et al. 2013). To date, chicken γδ T cells are known to exhibit cytotoxic activity when stimulated *in vitro* (Fenzl, Gobel et al. 2017). They are abundantly present in peripheral epithelial tissues (Hayday, Roberts et al. 2000), playing a crucial role in the immune response to infections such as Salmonella (Berndt and Methner 2001). γδ T cell subsets have been previously characterized by Berndt et al. into three groups: $CD8^{high+}$, $CD8^{dim+}$, and $CD8^{neg-}$. These γδ T cell subsets respond differently to infections like salmonellosis (Berndt, Pieper et al. 2006).

To characterize T cell subsets in more detail, we generated knockout chickens that lack αβ or, respectively γδ T cells and analyzed them phenotypically. The phenotypical analysis of the TCR Cγ KO chicken showed a comparable phenotype to wild type (WT) chickens (von Heyl, Klinger et al. 2023). Additionally, infection of TCR Cγ KO chickens with MDV showed increased disease incident and tumor formation compared to their WT siblings, leading to the conclusion that γδ T cells play an important role in the pathogenesis and tumor formation (Sabsabi, Kheimar et al. 2024). Moreover, the TCR Cβ KO chickens exhibit a severe phenotype two weeks after hatch, with granuloma formation at the comb, beak, and legs. Additonally, inflammation of the stomach and spleen, as well as an impaired development of the lymphatic organs was observed. It remains elusive what agent causes the phenotype and how much the remaining γδ T cells contribute to this phenotype (von Heyl, Klinger et al. 2023).

Using scRNA-seq analysis for the first time in chickens, Maxwell et al. identified leukocyte populations such as B cells, eosinophils, basophils, monocytes, red blood cells, and thrombocytes. The description of T cell subtypes proved to be challenging in chickens, but led to new conclusions of cytotoxic characteristics of particular T cells (Maxwell, Soderlund et al. 2024).

Here, we used scRNA-seq to perform whole transcriptome analysis (WTA) of $CD3^+$ T cells sorted from thymus and PBMCs of TCR Cβ KO, TCR Cγ KO, and WT animals on embryonal day (ED18), day 3 (d3) and day 14 (d14) post hatch. Our data revealed two different cytotoxic γδ T cell clusters in the TCR Cβ KO at 14 days post hatch, which were absent in TCR Cγ KO and WT chickens. Moreover, useful gene markers to identify γδ and αβ T cell clusters were analyzed.

2 Material and Methods:

2.1 Animals:

White Leghorn (Lohmann selected White Leghorn (LSL), Lohmann-Tierzucht GmbH, Cuxhaven, Germany) chickens were used. Animal experiments were approved by the government of Upper Bavaria, Germany (ROB-55.2-2532.Vet_02-17-101 & 55.2-1-54-2532-104-2015). Experiments were performed according to the German Welfare Act and European Union Normative for Care and Use of Experimental Animals. All animals received a commercial standard diet and water *ad libitum*. Genetically modified animals were generated as previously described (von Heyl et al.).

2.2 Experimental setup:

For the sampling of the thymus of ED18 chicks, TCR CγKO and TCR CβKO eggs were incubated for 18 days in egg incubators (HEKA-Brutgeräte, Rietberg, Germany) at standard conditions (37,8°C. 55% relative humidity, six rotations/day). Infertile eggs or dead embryos were removed by candeling the eggs on day 7 after incubation. *In ovo* genotyping was performed on ED10. On ED18, eggs were opened, and embryos were decapitated to remove the thymus, for the other time points, TCR CβKO, TCR CγKO, and their WT sibling were hatched. Blood was drawn for genotyping on d1 post hatch. On d3 and d14 post hatch, chickens were euthanized by terminal bleeding. 5ml blood was collected for sorting, and the thymus was removed and strained through a 100µL corning cell strainer (Sigma Aldrich, Taufkirchen, Germany) for further processing. From all genotypes (TCR CβKO, TCR Cγ KO and WT) n=3 animals were used at each time point (ED18, d3, d14).

2.3 Genotyping assays:

For *in ovo* genotyping, allantois samples were collected on ED10. Briefly, a candling light was used to determine fertility of the egg, embryo viability, and to mark the areas for creating a hole with a lancet where no blood vessels were visible. The allantoic fluid was removed using an insulin syringe with a 30G cannula (BD Micro Fine™, Heidelberg, Germany). Allantoic fluid was directly used for genotyping without a gDNA isolation step.

For the hatched chicks, a drop of blood was collected one day after hatch from the *vena jugularis* using a syringe with a 27G cannula (B. Braun, Meisungen, Germany). gDNA was isolated from blood, by adding 1µL blood into 200µL STM buffer and centrifuging for 5min at 100g. After removing the supernatant, 400µL TEN buffer containing 100µg/mL Pronase E was added, and the mix was incubated for 1h at 37°C (250rpm). The Pronase E was subsequently inactivated at 65°C for 20min (Bailes, Devers et al. 2007).

Chickens were genotyped as described before (von Heyl et al.). Briefly, primers were used to detect TCR CβKO chickens: Forward: 5’ GGTTCGAAATGACCGACCAAGC 3’; Reverse: 5’ GGCTTGCACACTCAGCTCTATAG 3’. A second primer pair was used to detect the TCR Cβ WT allele: Forward: 5’ GGTTCGAAATGACCGACCAAGC 3’; Reverse: 5’ CACACCATTCACCTTCCAGAC 3’. FIREPol Multiplex DNA Polymerase Mastermix (Solis Biodyne, Tartu, Estonia) was used according to manufactures instructions with an annealing temperature of Tm 58°C. For the TCR CγKO chickens’ primers were used to detect the TCR CγKO: Forward: 5’ GCCATTCCTATTCCCATCCTAAGT 3’; Reverse: 5’ GGTTCGAAATGACCGACCAAGC 3’. A second primer pair was used to detect the WT constant region of the TCR γ chain: Forward: 5’ GAGCTCCACGCCATGAAACCATAG 3’; Reverse: 5’ GTTGTCACTGTCACTGGCTG 3’. FIREPol Multiplex DNA Polymerase Mastermix (Solis

Biodyne, Tartu, Estonia) was used according to manufactures instructions with an annealing temperature of Tm 60°C.

2.4 Cell sorting:

5mL blood was collected from hatched chickens. The thymus was removed and strained through a 100µm cell strainer into a falcon holding 5mL ice-cold PBS. Lymphocytes were isolated from blood and the strained thymus single-cell solution using histopaque density gradient centrifugation (Sigma, Taufkirchen, Germany). Cells were washed with 1% BSA in PBS and stained with CD3-PE (clone CT-3) (Biozol, Eching, Germany) for 20 min in the dark on ice. Subsequently, cells were again washed and cell sorting was performed using a CytoFLEX SRT Cell sorter (BD, Heidelberg, Germany). Data were analyzed with FlowJo 10.8.1 software (FlowJo, Ashland, USA).

2.5 Multiplexing:

The BD Flex Single-Cell Multiplexing Kit A, B, and C (BD, Heidelberg, Germany) were used according to the manufacturer's protocol. Briefly, cells were resuspended in 180µL PBS+ 1%FBS. 20µL of each sample tag was added and incubated. Samples were washed and pooled together.

2.6 Single-cell capture and cDNA Synthesis

The BD Rhapsody™ cDNA Kit and BD Rhapsody Enhanced Cartridge Reagent Kit (BD, Heidelberg, Germany) were used according to the manufacturer's protocol. The 8-Lane Cartridge was loaded with 70.000 cells.

2.7 mRNA Whole Transcriptome Analysis and Sample Tag

The BD Rhapsody™ WTA Reagent Kit (BD, Heidelberg, Germany) was used according to the manufacturers protocol (23-24119(02)). Briefly, random priming and extension (RPE) reactions of the Exonuclease I-treated beads containing the whole cellular transcriptome and cDNA was performed. After this step two parallel workflows were performed, one for the WTA libraries, using the RPE product from the supernatant and the other one for the Sample Tag libraries from the post-RPE beads. The RPE supernatant underwent WTA library preparation following the manufacturers' instructions from the BD Rhapsody™ System mRNA Whole Transcriptome Analysis (WTA) Library Preparation Protocol that included the steps of RPE cleanup, performing RPE PCR, RPE PCR cleanup, performing WTA Index PCR and cleanup. The Sample Tag products were amplified and purified through PCR. A Sample Tag Index PCR was performed to add full-length Illumina Sequencing adapters and indices.

WTA libraries and Sample Tag libraries were pooled and sequenced on a NovaSeqX 10B Sequencer by Novogene (Cambridge, UK). Libraries were sequenced as paired-end 2x150bp reads with an estimated depth of 1,250 M reads per cartridge lane.

2.8 Sequencing and analysis:

Fastq files from BD Rhapsody samples were processed using BD Rhapsody™ WTA Analysis Pipeline, v1.9.1 in the Seven Bridges Genomics cloud platform. WTA reads were aligned to the *gallus gallus* (chicken) GRCg7b reference genome (Accession Number GCF_016699485.2) and demultiplexed. The generated Seurat output files were analyzed using Seurat (Version 5.1.0) on Rstudio (Version 2024.04.2+764) for the Cluster analysis. Gene markers were classified using PANTHER (Version 19.0) for gene ontology analysis.

2.9 Immunohistochemistry:

Immunohistochemistry was performed as described before (von Heyl et al.). As primary antibody a mix of anti-αβ T cell Vb1 and Vb2 (clone TCR2 and TCR3) or anti-γδ T cell (clone TCR1) (Biozol, Eching, Germany) were used in a concentration of 5µg/mL.

3 Results:

3.1 No overlapping clusters between the TCR Cβ KO and TCR Cγ KO $CD3^+$ T cells

In this study, whole transcriptome analysis was conducted on sorted $CD3^+$ T cells isolated from TCR Cγ KO, TCR Cβ KO, and WT animals. Thymus samples were collected from ED18, d3, and d14 chickens, while PBMC samples were obtained from d3 and d14 chickens (Figure 1A). Due to the limited blood volume in ED18 embryos, it was not feasible to sort the needed 20,000 cells per embryo. Generally, the $CD3^+$ population in the thymus ranged from 15% to 49,4% at all time points. The $CD3^+$ population in blood increased from d3 to d14. At d3 the population ranged between 0,73% to 8,98% of all PBMCs with no differences between the genotypes. At d14 TCR Cβ KO chickens showed a smaller population of $CD3^+$ PBMCs (mean 2,57%), compare to TCR Cγ KO (mean 8,88%) and WT (mean 10,17%) animals (Supplement Figure 1). From each sample 20,000 cells were sorted, except for one TCR Cβ KO thymus sample at d14 where only 8,000 sorted cells were achieved. The sequencing quality control for each timepoint is summarized in Table 1. At all three timepoints over 90% of the reads were aligned to a putative cell. At d3 the highest mean with 14,650.78 reads per cell were found. Clustering of the cells was performed for different resolutions. Since TCR Cβ KO and TCR Cγ KO clusters have both either αβ or γδ T cell subpopulation missing, a smaller resolution was chosen for the KO chickens compared to the WT chickens. In general, the clusters of TCR Cβ KO and TCR Cγ KO showed no overlapping, and merging the clusters of both TCR KOs showed the same clusters as in WT animals. Only in the thymus samples on ED18 a few overlapping single cells were detected between TCR Cβ KO and TCR Cγ KO animals (Figure 2A, B).

3.2 TARP as a useful marker to identify γδ T cell clusters

TARP was identified for γδ T cells as a useful marker to annotate γδ T cell clusters since it is expressed in every cell in the TCR Cβ KO chickens. We found that *TARP* and *GATA3* are highly expressed in the γδ T cells, while *MAF* is only expressed in activated and mature T cells and is not exclusively expressed in γδ T cells but also αβ T cells. The genes *KK34* and *INPP5A* are only expressed in γδ T cells but showed a low expression. Alongside, the markers *PDE1A* and *CD82* are highly expressed in the γδ T cells. However, only *PDE1A* seems to be completely absent in TCR Cγ KO chickens, while *TARP* and *CD82* is also expressed in some cells of the TCR Cγ KO chickens. *IL20RA* showed low expression but is a selective marker for mature γδ T cells (Figure 3A, B). To identify a large population of αβ T cells *CD4* was used, and only few $CD4^+$ γδ T cells were detected. *CD5* and *CCR7* were used to identify undifferentiated αβ T cells. Equal clusters of CD8α and *CCR7* cells can be found in TCR Cγ KO chickens compared to TCR Cβ KO chickens (Figure 4).

3.3 Annotation of lineage defining markers revealed T cell subclusters

Clusters of $CD3^+$ T cells were manually annotated by assessing the expression of lineage-defining marker genes on ED18, d3 and d14. Markers that were used to identify each cluster are shown in

Tables 2-6 (supplementary Figure 5-19). In TCR Cβ KO and TCR Cγ KO chickens a few αβ or respectively γδ T cells were found in the thymus (Figure 5). This was also confirmed by staining for αβ (TCR2+3) or respectively γδ (TCR1) T cells in the thymus of TCR Cβ KO and WT chickens using Immunohistochemistry (Supplement Figure 2). In the PBMCs, no αβ T cells were detected in TCR Cβ KO chickens, and no γδ T cells were observed in TCR Cγ KO chickens. Both T cell subpopulations were seen in the WT animals (Figure 5, 6). Despite sorting for $CD3^+$ T cells, small clusters of thrombocytes and erythrocytes were still detectable in most samples, except on ED18, regardless of genotype, and in both thymus and PBMCs. Thrombocytes were identified by the expression of *GP1BB, GP9,* and *F13A1,* while erythrocytes were characterized by the expression of *HBE1, SLC4A1, EPB42, SPTB,* and *DMTN* (Figure 5, 6)

3.4 Subclustering of T cells on ED18 reveals memory T cells in TCR Cβ KO chickens

The T cell subclusters were annotated based on the expression of specific genes (Tab. 2, Supplementary Figure 5-7). By subclustering $CD3^+$ T cells from WT and TCR KO chickens, we identified gene signatures that characterize the functional state of the T cells. At ED18, TCR Cβ KO chickens exhibited a large subpopulation of mature γδ T cells in the thymus, along with smaller populations of activated and memory γδ T cells. Additionally, division-active precursor and proliferating γδ T cells were observed. In WT siblings, all these γδ T-cell subclusters were also present, except for memory γδ T cells. Notably, the division-active precursor T cells in WT and TCR Cγ KO chickens formed a distinct cluster compared to their TCR Cβ KO siblings. In TCR Cγ KO chickens, mature and differentiating αβ T cells constituted the largest subpopulations. Division-active precursor and proliferating T cells were also observed. Importantly, there were no differences in the αβ T cell clusters between TCR Cγ KO birds and their WT siblings (Figure 5).

3.5 Subclustering on d3 shows activated γδ T cells in Thymus and PBMCs of TCR Cβ KO chickens

The T cell subclusters on d3 were annotated based on the expression of specific genes (Tab. 3+4, Supplementary Figure 8-13), with analyses conducted in both the thymus and PBMCs samples. In WT chickens, only mature and differentiating γδ T cells were identified in the thymus. However, in the thymus of TCR Cβ KO birds, an additional cluster of activated γδ T cells was observed. In d3 PBMCs of TCR Cβ KO birds, a large population of activated and differentiating γδ T cells was present, with only a small population of mature γδ T cells. In contrast, WT chickens exhibited a larger population of mature γδ T cells a few activated and no differentiating γδ T cells. By comparing the thymus of TCR Cγ KO and WT chickens on d3, TCR Cγ KO birds displayed an increased population of mature αβ T cells and a reduced population of memory T cells, while the opposite was seen in WT chickens. Interestingly, the differentiating and proliferating αβ T cell subpopulations were similar between both genotypes. The PBMCs on d3 in TCR Cγ KO chickens exhibited fewer memory T cells and lacked differentiating αβ T cells compared to WT chickens. Other subclusters, including mature, proliferating, and activated αβ T cells, were comparable between TCR Cγ KO and WT birds (Figure 5, 6).

3.6 Cluster annotation reveals two different cytotoxic γδ T cell clusters on d14

The T cell subclusters on d14 were annotated based on the expression of specific genes (Tab. 5+6, Supplementary Figure 14-19), The cluster annotation of $CD3^+$ T cells in the thymus of chickens at d14, revealed no differences in the γδ T cell clusters between TCR Cβ KO and WT chickens. Comparing TCR Cγ KO and WT T cell clusters in the chicken's thymus on d14 the clusters of

mature αβ T cells, differentiating αβ T cells and memory T cells were similar. Only in TCR Cγ KO chickens a proliferating αβ T cell cluster was found in the thymus at d14 (Figure 5). Both, TCR Cβ KO and WT chickens, have equal clusters of mature γδ T cells, memory T cells and cytotoxic $CD8^+$ T cells on d14 in the PBMCs. In the TCR Cβ KO chickens we found an additional cluster expressing genes involved in the interferon pathway, such as *MX1*, *STAT1* and *IFNGR1*. In the WT chickens, no interferon-stimulated γδ T cells were found (Figure 6). The comparison of TCR Cγ KO and WT chicken's PBMCs on d14 revealed similar clusters of differentiating and memory T cells.

3.7 Interferon pathway highly activated in TCR Cβ KO chickens

Comparing genes which are significantly upregulated in TCR Cβ KO compared to WT chickens at d14 after hatch, revealed genes which are involved in the interferon pathway. These genes include *STAT1, MX1, IFNGR1* and *IKZF2* (Figure 7A). A heatmap analysis of genes that are involved in the interferon pathway (*STAT1, IFI6, MX1, USP18, IFNGR1, OASL, RSAD2, IFIT5)* showed upregulation of these genes in the thymus and PBMCs TCR Cβ KO chickens on d14. While there are no cell clusters in WT chickens on d14 that express genes involved in the interferon pathway. Interestingly in the Thymus and PBMCs of TCR Cγ KO chickens on d14 also some cell clusters showed expression of interferon-stimulated genes (Figure 7B). On ED18 and d3 no upregulation of the interferon pathway was visible (Supplement Figure 3 A, B). The string networks show the close relation of the genes involved into interferon pathway, such as *STAT1, Mx1* and *OASL* which were found significantly upregulated in TCR Cβ KO chickens compared to WT at d14 (Figure 7C).

3.8 Cytolytic CD8+ γδ T cells secrete Granulysin and Granzyme A to induce apoptosis

The analysis of cytolytic genes on d14 in the T cells showed, that TCR Cβ KO chickens express cytokines that are related to lysis and apoptosis. High expression of Granulysin (*GNLY*), Granzyme A (*GZMA*), Granzyme M (*GZMM*) and *IL2RB* in the PBMCs of TCR Cβ KO chickens on d14 was detected, while this was not observed in the thymus of these chickens (Figure 8A). Also, on ED18 and d3 there is no such upregulation of cytotoxic γδ T cells visible in any genotype (Supplement Figure 4 A, B). The string networks show the close relation of the cytokines and chemokines involved in cytotoxicity (Figure 8B).

4 Discussion:

Single cell RNA-sequencing is a novel tool to understand gene expression patterns in cells to define clusters (Tang, Barbacioru et al. 2009, Eschke, Moore et al. 2023). In this study, we used WTA to define T cell clusters in TCR KO chickens (TCR Cβ KO and TCR Cγ KO) (von Heyl, Klinger et al. 2023) compared to WT birds from embryo (ED18) until two weeks post hatch (d14). Sorted $CD3^+$ T cells from PBMCs and thymocytes were used for single cell RNA-sequencing WTA. The Sequencing quality was overall good since over 96% of the reads passed the quality filter and over 90% of the reads were assigned to a putative cell (Table 1).

On ED18, it was not possible to sort 20,000 $CD3^+$ T cells from the blood, therefore, $CD3^+$ T cell sorting was limited to the thymus at this time point (data not shown). The first T cells that move into the thymus are $TCR1^+$ cells which occur first on ED12 and are first detectable in the spleen by ED15. The second wave of T cells colonize the thymus at ED15 and move towards spleen on ED19 (Chen, Sowder et al. 1989, Coltey, Bucy et al. 1989, Chen, Gobel et al. 1994). Even though there should be some $CD3^+$ T cells in the blood of ED18, we were not able to sort enough cells for scRNA-seq.

In the thymus of TCR Cβ KO chickens, not only γδ T cells were observed, but also αβ T cells. Similarly, in the thymus of TCR Cγ KO chickens, alongside αβ T cells, a small population of γδ T

cells was present. In contrast, neither αβ T cells in TCR Cβ KO nor γδ T cells in TCR Cγ KO were detected in the PBMCs on days 3 or 14. Previously, it was demonstrated via flow cytometry that neither αβ nor γδ T cells could be detected in the blood of TCR Cβ KO or TCR Cγ KO chickens (von Heyl, Klinger et al. 2023). Based on these findings, we speculate that progenitor T cells migrate into the thymus of TCR KO chickens. However, due to the incomplete TCR, these T cells are unable to exit the thymus and enter the bloodstream. No such a population has been reported in the thymus of mice with a knockout in the TCR before, since neither staining nor sequencing of the thymus has been done (Roberts, Smith et al. 1996, Dianda, Hanby et al. 1997).

Through the use of TCR KO chicken, it was possible to detect different genes that are specific markers for γδ or αβ T cells. Before, *TARP, MAF, GATA3, KK34* and *INPP5A* were described as γδ T cell markers (Maxwell, Soderlund et al. 2024). Likewise, in this study we also found that *TARP* and *GATA3* are highly expressed in the γδ T cells, while *MAF* is only expressed in activated and mature T cells and is not exclusively expressed in γδ T cells but also αβ T cells. The genes *KK34* and *INPP5A* show a low expression but are exclusively expressed in γδ T cells. Alongside we found that the markers *PDE1A* and *CD82* are highly expressed in the γδ T cells. However, only *PDE1A* seems to be absent in TCR Cγ KO chickens, while *TARP* and *CD82* is also expressed in some cells of the TCR Cγ KO chickens. *IL20RA* showed low expression but is a selective marker for mature γδ T cells. *IL20RA* is known to be a subunit of the IL20 receptor and is involved in the regulation of inflammatory mediators by regulating the *JAK-STAT* pathway (Lamichhane, Mo et al. 2021). *CD82* (also known as *KAI1* or *R2*) is an inducible membrane marker that was initially identified from a T cell activation study (Gaugitsch, Hofer et al. 1991). Here, we highlight for the first time, that *CD82* is not exclusively expressed in γδ T cells, but comparing TCR Cβ KO with TCR Cγ KO and WT chickens, we see that most of γδ T cells express *CD82* while only few αβ T cells express *CD82*. Until now there is no literature on γδ T cells expressing the calmodulin-dependent phosphodiesterase 1A (*PDE1A*) exclusively. This study reveals for the first time the absence of *PDE1A* in TCR Cγ KO chickens and shows that only a specific γδ T cell cluster express *PDE1A*.

Before, scRNA-seq in chicken was performed in peripheral blood leukocytes (Maxwell, Soderlund et al. 2024). Here, the T cell clusters were subdivided in CD4, T_{regs}, CD8, γδ T cells and cytolytic cells. However, in our study we subdivided the T cells further due to their differentiation status into proliferation, differentiation, memory T cells and mature T cells (Figure 5). Likewise, in comparison to Maxwell et al. we found cytolytic T cells, that express Granulysin and Granzyme A and M in TCR Cβ KO and wildtype animals 14 days after hatch (Maxwell, Soderlund et al. 2024). Other than shown before, here we can proof that this cytolytic T cells are γδ T cells, since they are absent in TCR Cγ KO chickens, while present in TCR Cβ KO and WT birds. Interestingly, these cytolytic γδ T cells do neither express perforin nor Granzyme B.

Depending on the expression of specific cytokines, helper T cells are subdivided in Th1, Th2, Th17 and T_{regs} in humans and mammals (Raphael, Nalawade et al. 2015). Still, it was not possible to define these T cell subsets for the helper T cells in chickens due to missing annotations of some cytokines. In this analysis, the T cells were subdivided into different clusters depending on their activation status. However, we did not find individual clusters for different helper T cells subclass, since in this study the sorted $CD3^+$ T cells in the wildtype showed no expression of IFN-γ (Th1), IL-4 (Th2), IL-17 (Th17) or IL-10 (T_{reg}). Even though these markers are already annotated in the chicken genome.

Additionally, small clusters of Thrombocytes, expressing the marker *GP1BB*, *GP9* and *F13A1*, and Erythrocytes, expressing *HBE1*, *SLC4A1* and *EPB42* (Figure 2+5) were found even though the samples were sorted for $CD3^+$ T cells. These cluster are distinct from the T cell clusters and do not interfere with the analysis.

In this study, we identified for the first time a subset of γδ T cells that are present exclusively in TCR Cβ KO chickens but absent in both TCR Cγ KO and WT chickens. These cells exhibit the

expression of genes associated with the interferon pathway, including *MX1*, *STAT1*, and *OASL* (Mountford, Gheyas et al. 2022). TCR Cβ KO chickens display a severe phenotype as early as 14 days post hatch, characterized by inflammation of the gut, spleen, and skin. This condition is driven by an imbalance between humoral and cytotoxic responses within the adaptive immune system (von Heyl, Klinger et al. 2023). The interferon-activated γδ T cells described here appear to play a defining role in the severity of the phenotype observed in TCR Cβ KO chickens. Further studies are needed to determine whether these T cells are also present in other inflammatory diseases in chickens and to assess if their differentiation is triggered by the activation of dysregulated monocytes.

This study revealed that cytolytic T cells, characterized by the expression of Granulysin and Granzymes, are predominantly γδ T cells. Additionally, we identified interferon-activated γδ T cells, which are likely contributors to the severe phenotype observed in TCR Cβ KO chickens, as described previously. The precise trigger for this phenotype remains unclear, necessitating further sequencing analysis of monocytes and B cells in future investigations.

Overall, this study provides new insights into chicken T cell biology, including T cell markers for specific subsets such as αβ T cells and γδ T cells, as well as their functional states, such as proliferation and differentiation. Notably, the use of TCR Cβ KO chickens has uncovered novel characteristics of γδ T cells, offering valuable perspectives for further research.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

TvH performed and analyzed the experiments and wrote the paper. BS supervised the work and wrote the paper. MB and SPF helped with the analysis of the data. AS and CW performed experiments. All authors approved the submitted version.

7 Funding

This project was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) in the framework of the Research Unit ImmunoChick (FOR5130) project SCHU 2446/6-1 (awarded to BS) and an Emmy-Noether research fellowship (DFG Schu2446/3-1 awarded to BS).

8 Acknowledgments:

ChatGPT (https://chat.openai.com/) was used to improve conciseness of language and coding, with no contribution to the scientific content.

9 Data Availability Statement

Raw data for this study can be found on SRA under the accession number PRJNA1226579.

10 Literature:

Abdelwhab, E. M. and T. C. Mettenleiter (2023). "Zoonotic Animal Influenza Virus and Potential Mixing Vessel Hosts." Viruses **15**(4).

Arstila, T. P., O. Vainio and O. Lassila (1994). "Central role of CD4+ T cells in avian immune response." Poult Sci **73**(7): 1019-1026.
Bailes, S. M., J. J. Devers, J. D. Kirby and D. D. Rhoads (2007). "An inexpensive, simple protocol for DNA isolation from blood for high-throughput genotyping by polymerase chain reaction or restriction endonuclease digestion." Poult Sci **86**(1): 102-106.
Berndt, A. and U. Methner (2001). "Gamma/delta T cell response of chickens after oral administration of attenuated and non-attenuated Salmonella typhimurium strains." Vet Immunol Immunopathol **78**(2): 143-161.
Berndt, A., J. Pieper and U. Methner (2006). "Circulating gamma delta T cells in response to Salmonella enterica serovar enteritidis exposure in chickens." Infect Immun **74**(7): 3967-3978.
Bodewes, R., T. M. Bestebroer, E. van der Vries, J. H. Verhagen, S. Herfst, M. P. Koopmans, R. A. Fouchier, V. M. Pfankuche, P. Wohlsein, U. Siebert, W. Baumgartner and A. D. Osterhaus (2015). "Avian Influenza A(H10N7) virus-associated mass deaths among harbor seals." Emerg Infect Dis **21**(4): 720-722.
Chen, C. H., T. W. Gobel, T. Kubota and M. D. Cooper (1994). "T cell development in the chicken." Poult Sci **73**(7): 1012-1018.
Chen, C. H., J. T. Sowder, J. M. Lahti, J. Cihak, U. Losch and M. D. Cooper (1989). "TCR3: a third T-cell receptor in the chicken." Proc Natl Acad Sci U S A **86**(7): 2351-2355.
Coltey, M., R. P. Bucy, C. H. Chen, J. Cihak, U. Losch, D. Char, N. M. Le Douarin and M. D. Cooper (1989). "Analysis of the first two waves of thymus homing stem cells and their T cell progeny in chick-quail chimeras." J Exp Med **170**(2): 543-557.
Cooper, M. D. and M. N. Alder (2006). "The evolution of adaptive immune systems." Cell **124**(4): 815-822.
Dianda, L., A. M. Hanby, N. A. Wright, A. Sebesteny, A. C. Hayday and M. J. Owen (1997). "T cell receptor-alpha beta-deficient mice fail to develop colitis in the absence of a microbial environment." Am J Pathol **150**(1): 91-97.
Eschke, M., P. F. Moore, H. Chang, G. Alber and S. M. Keller (2023). "Canine peripheral blood TCRalphabeta T cell atlas: Identification of diverse subsets including CD8A(+) MAIT-like cells by combined single-cell transcriptome and V(D)J repertoire analysis." Front Immunol **14**: 1123366.
Farrell, D. (2011). "The role of poultry in human nutrition." FAO Poultry Production.
Fenzl, L., T. W. Gobel and M. L. Neulen (2017). "gammadelta T cells represent a major spontaneously cytotoxic cell population in the chicken." Dev Comp Immunol **73**: 175-183.
Gaugitsch, H. W., E. Hofer, N. E. Huber, E. Schnabl and T. Baumruker (1991). "A new superfamily of lymphoid and melanoma cell proteins with extensive homology to Schistosoma mansoni antigen Sm23." Eur J Immunol **21**(2): 377-383.
Hayday, A. C., S. Roberts and E. Ramsburg (2000). "gammadelta cells and the regulation of mucosal immune responses." Am J Respir Crit Care Med **162**(4 Pt 2): S161-163.
Holderness, J., J. F. Hedges, A. Ramstead and M. A. Jutila (2013). "Comparative biology of gammadelta T cell function in humans, mice, and domestic animals." Annu Rev Anim Biosci **1**: 99-124.
Lamichhane, S., J. S. Mo, G. Sharma, T. Y. Choi and S. C. Chae (2021). "MicroRNA 452 regulates IL20RA-mediated JAK1/STAT3 pathway in inflammatory colitis and colorectal cancer." Inflamm Res **70**(8): 903-914.
Luhtala, M. (1998). "Chicken CD4, CD8alphabeta, and CD8alphaalpha T cell co-receptor molecules." Poult Sci **77**(12): 1858-1873.
Maxwell, M., R. Soderlund, S. Hartle and E. Wattrang (2024). "Single-cell RNA-seq mapping of chicken peripheral blood leukocytes." BMC Genomics **25**(1): 124.

Mountford, J., A. Gheyas, L. Vervelde and J. Smith (2022). "Genetic variation in chicken interferon signalling pathway genes in research lines showing differential viral resistance." Anim Genet **53**(5): 640-656.
Mousset, C. M., W. Hobo, R. Woestenenk, F. Preijers, H. Dolstra and A. B. van der Waart (2019). "Comprehensive Phenotyping of T Cells Using Flow Cytometry." Cytometry A **95**(6): 647-654.
Rahman, M. T., M. A. Sobur, M. S. Islam, S. Ievy, M. J. Hossain, M. E. El Zowalaty, A. T. Rahman and H. M. Ashour (2020). "Zoonotic Diseases: Etiology, Impact, and Control." Microorganisms **8**(9).
Raphael, I., S. Nalawade, T. N. Eagar and T. G. Forsthuber (2015). "T cell subsets and their signature cytokines in autoimmune and inflammatory diseases." Cytokine **74**(1): 5-17.
Roberts, S. J., A. L. Smith, A. B. West, L. Wen, R. C. Findly, M. J. Owen and A. C. Hayday (1996). "T-cell alpha beta + and gamma delta + deficient mice display abnormal but distinct phenotypes toward a natural, widespread infection of the intestinal epithelium." Proc Natl Acad Sci U S A **93**(21): 11774-11779.
Sabsabi, M. A., A. Kheimar, Y. You, D. von La Roche, S. Hartle, T. W. Gobel, T. von Heyl, B. Schusser and B. B. Kaufer (2024). "Unraveling the role of gammadelta T cells in the pathogenesis of an oncogenic avian herpesvirus." mBio: e0031524.
Salter, A. M. (2018). "The effects of meat consumption on global helth." Revue Scientifique et Technique **37**: 47-55.
Sowder, J. T., C. L. Chen, L. L. Ager, M. M. Chan and M. D. Cooper (1988). "A large subpopulation of avian T cells express a homologue of the mammalian T gamma/delta receptor." J Exp Med **167**(2): 315-322.
Tang, F., C. Barbacioru, Y. Wang, E. Nordman, C. Lee, N. Xu, X. Wang, J. Bodeau, B. B. Tuch, A. Siddiqui, K. Lao and M. A. Surani (2009). "mRNA-Seq whole-transcriptome analysis of a single cell." Nat Methods **6**(5): 377-382.
von Heyl, T., R. Klinger, D. Aumann, C. Zenner, M. Alhussien, A. Schlickenrieder, K. Lengyel, H. K. Vikkula, T. Mittermair, H. Sid and B. Schusser (2023). "Loss of alphabeta but not gammadelta T cells in chickens causes a severe phenotype." Eur J Immunol **53**(12): e2350503.

1 Figures

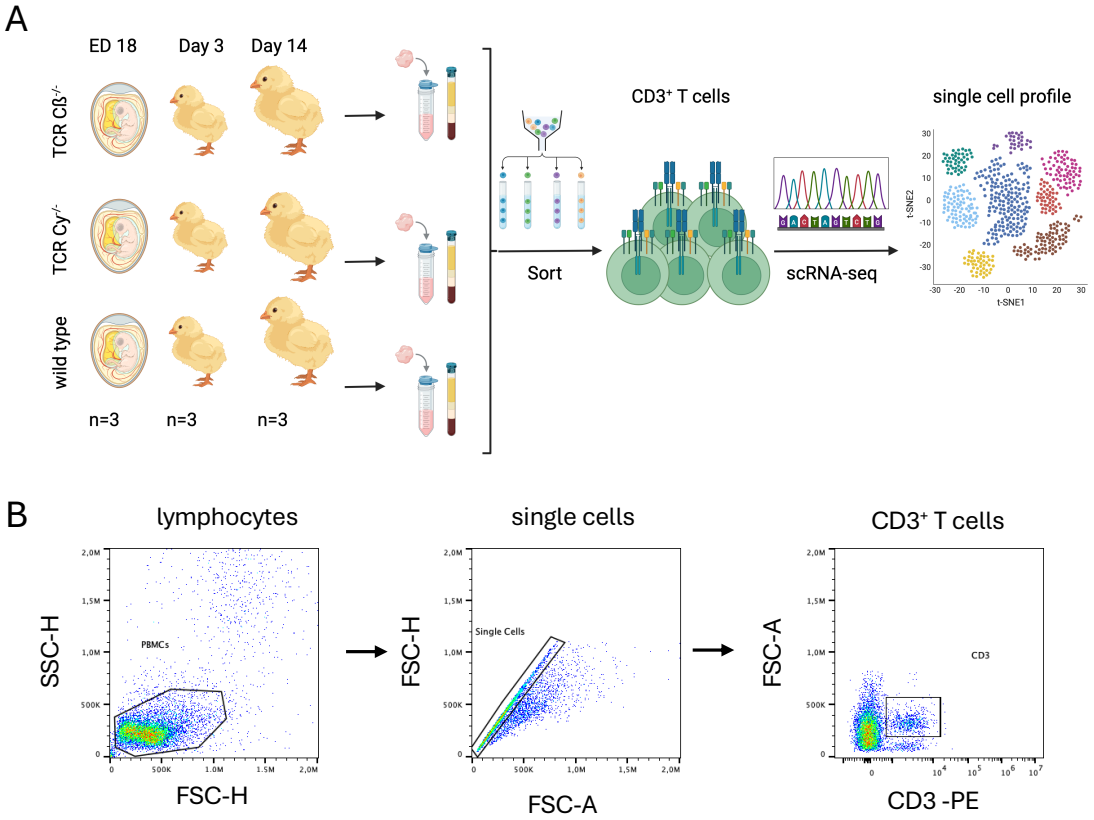

Figure 1. Experimental setup for the single cell analysis of chicken CD3^{+} T cells. (A) PBMC and thymocytes of three chickens from each genotype TCR Cβ KO, TCR Cγ KO and WT were used. CD3^{+}T cells were isolated by fluorescence-activated cell sorting (FACS) and applied to 5' single-cell RNA sequencing (5' scRNA-seq) for whole transcriptome analysis (WTA) (created with BioRender). (B) The gating strategy for isolation of CD3^{+} T cells by FACS.

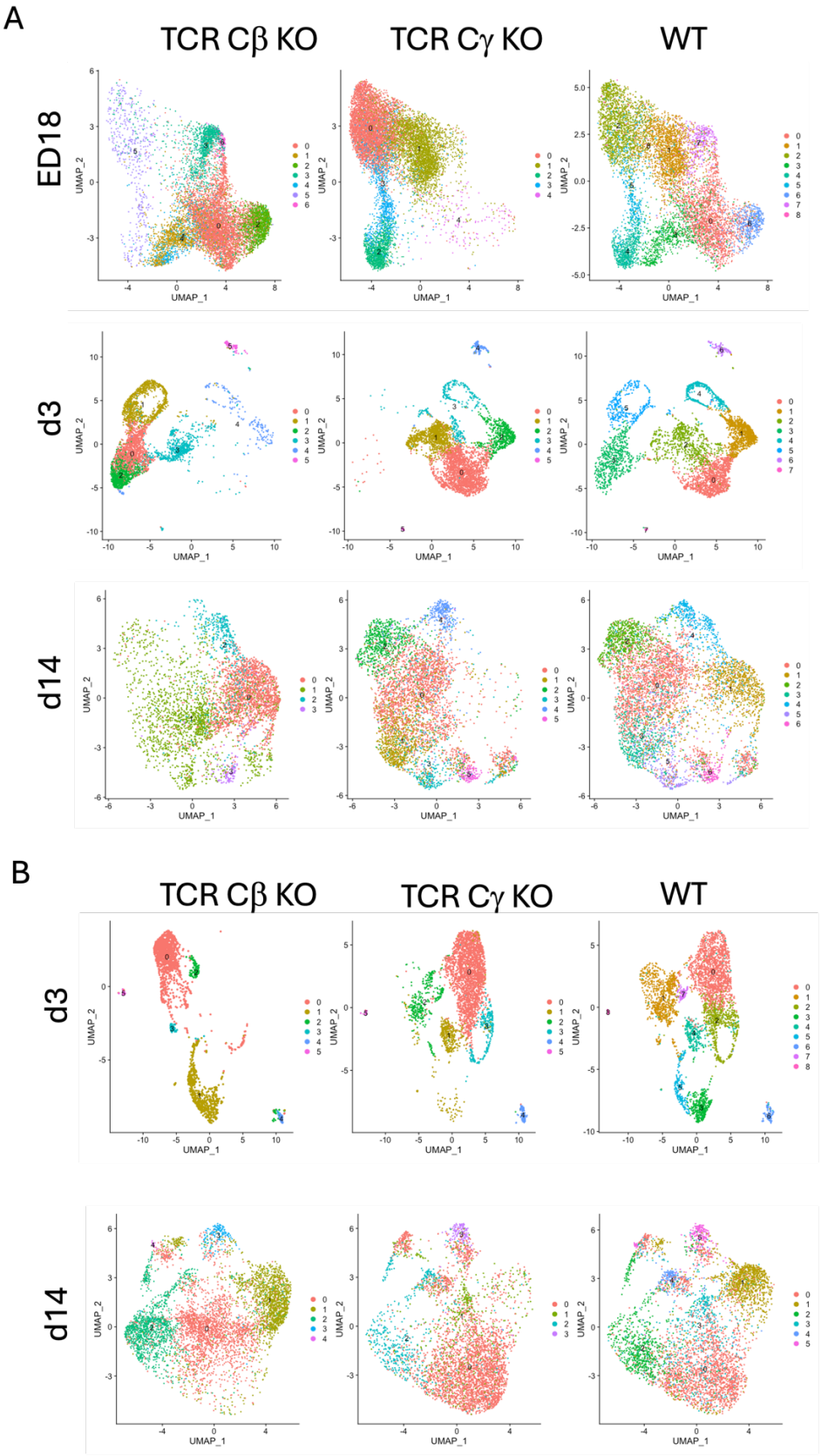

Figure 2. Single-cell RNA sequencing of CD3^{+} T cells isolated from the (A) thymus and (B) PBMCs of TCR Cβ KO, TCR Cγ KO, and WT chickens at ED18, d3, and d14 post hatch revealed distinct and contradictory gene clusters in the UMAP plots. The T cell clusters from TCR Cβ KO and TCR Cγ KO chickens collectively form a UMAP distribution comparable to WT chickens.

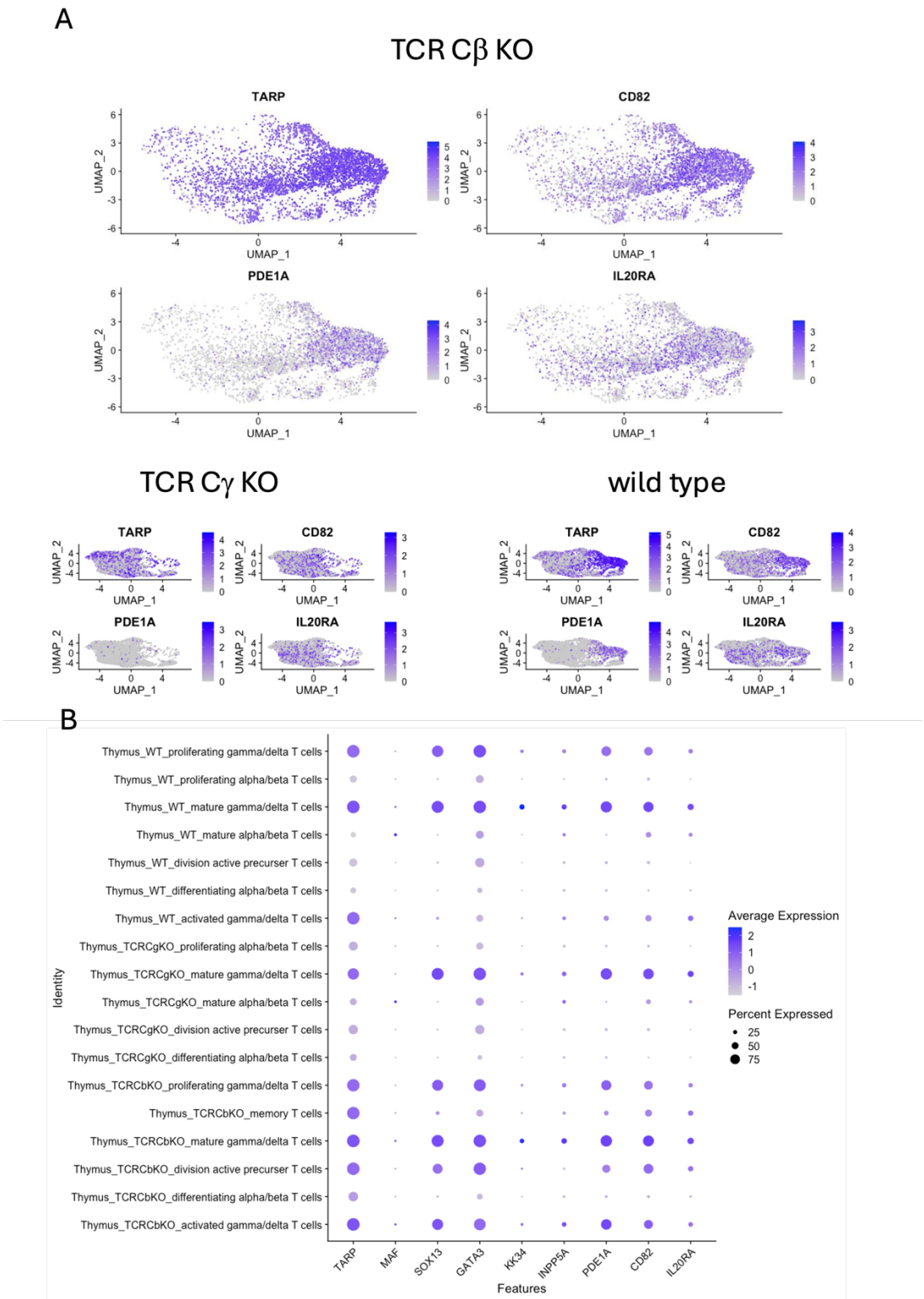

Figure 3. Identification of markers for γδ T cell subsets among chicken CD3^{+} cells. (A) Feature plot of potential γδ T cell markers in TCR Cβ KO, TCR Cγ KO and WT chickens on d14 post hatch. The color intensity corresponds to scaled expression values. (B) Dot plot representing the expression of a selection of γδ T cell associated genes in CD3^{+} clusters. The radius of the dot corresponds to the percentage of cells in each cluster expressing the gene, and color intensity corresponds to scaled expression values (average_log2 fold change).

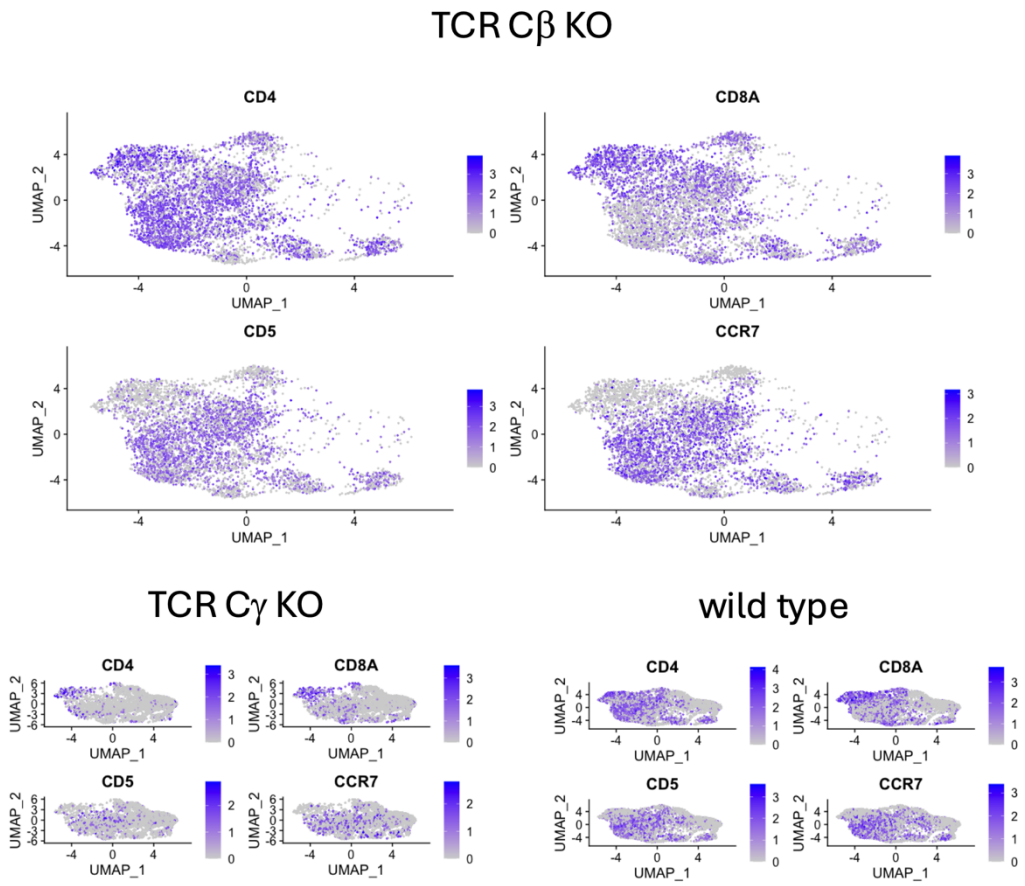

Figure 4. Identification of markers for αβ T cell subsets among chicken $CD3^+$ cells from PBMCs. Feature plot of potential αβ T cell markers in TCR Cβ KO, TCR Cγ KO and WT chickens on d14 post hatch. The color represents the expression level (average_log2 fold change).

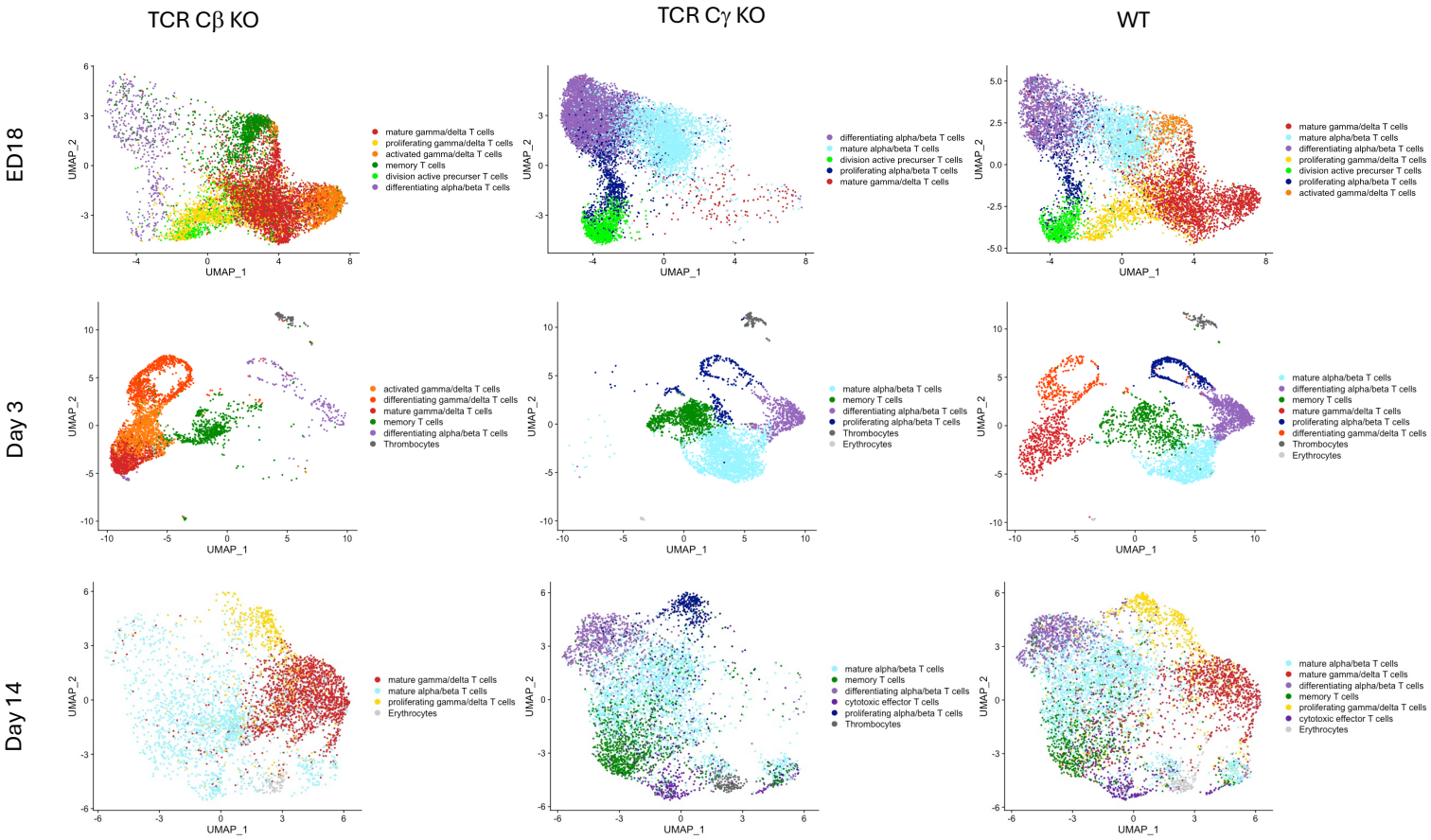

Figure 5. Identification of T cell subsets among chicken CD3$^+$ clusters on ED18, d3 and d14 post hatch from TCR Cβ KO, TCR Cγ KO and WT chickens from thymus. UMAPs showing CD3$^+$ T cell clusters from thymocytes were annotated according to specific gene markers.

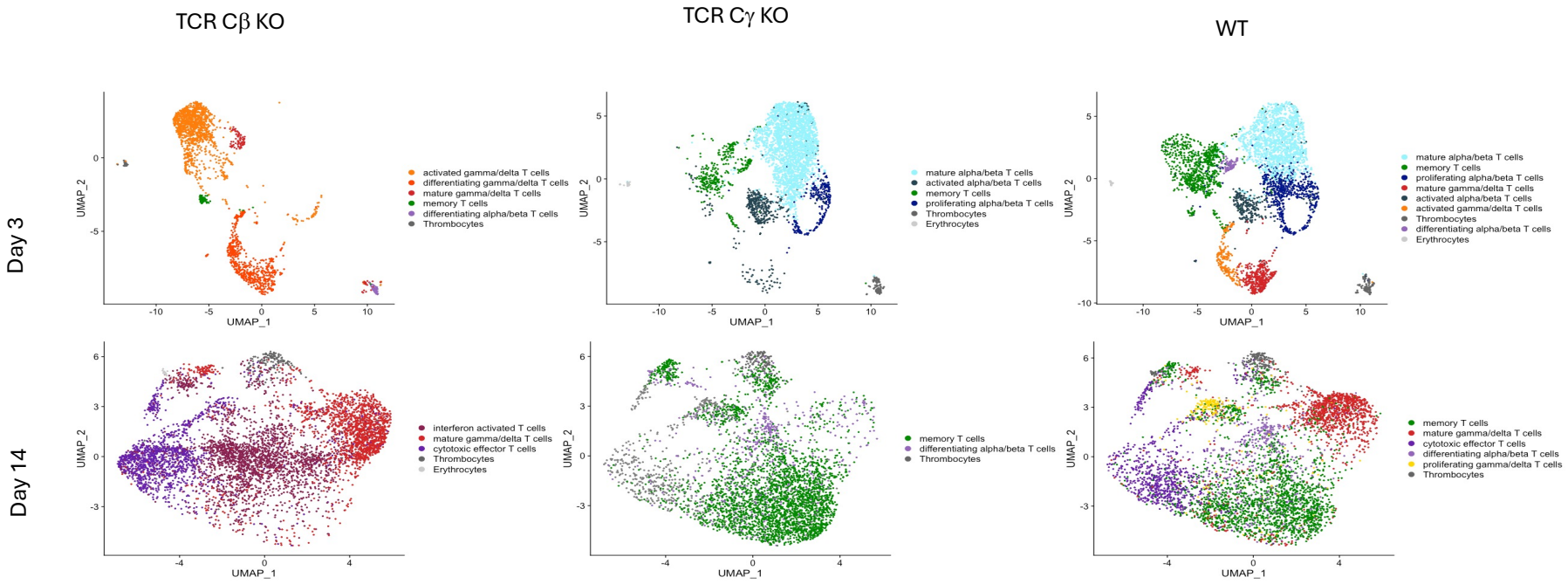

Figure 6. Identification of T cell subsets among chicken CD3+ clusters on ED18, d3 and d14 post hatch from TCR Cβ KO, TCR Cγ KO and WT chickens from PBMCs. UMAPs showing $CD3^{+}$ T cell clusters from PBMCs were annotated according to specific gene markers seen in Table 2-6.

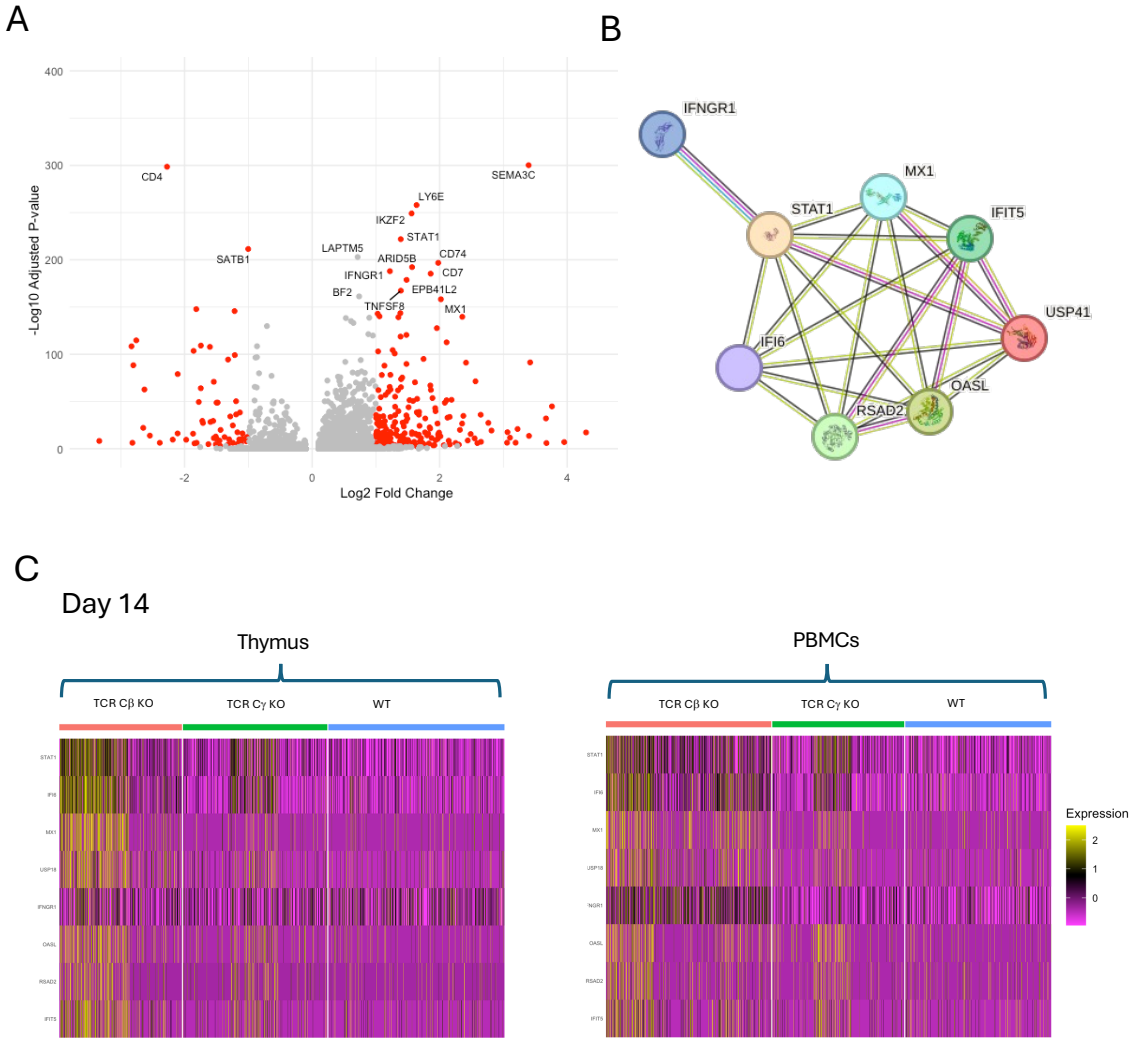

Figure 7. Interferon-activated γδ T cell clusters. A) Volcano plot of differentially expressed genes (DEGs) between TCR Cβ KO and WT chickens at d14 post hatch. B) String Network of DEGs from TCR Cβ KO chickens involved in interferon pathway. C) Heatmap of clusters from thymus and PBMCs of TCR Cβ KO, TCR Cγ KO and WT chickens at d14 showing genes involved in the interferon pathway. The color represents the gene expression level (average_log2 fold change).

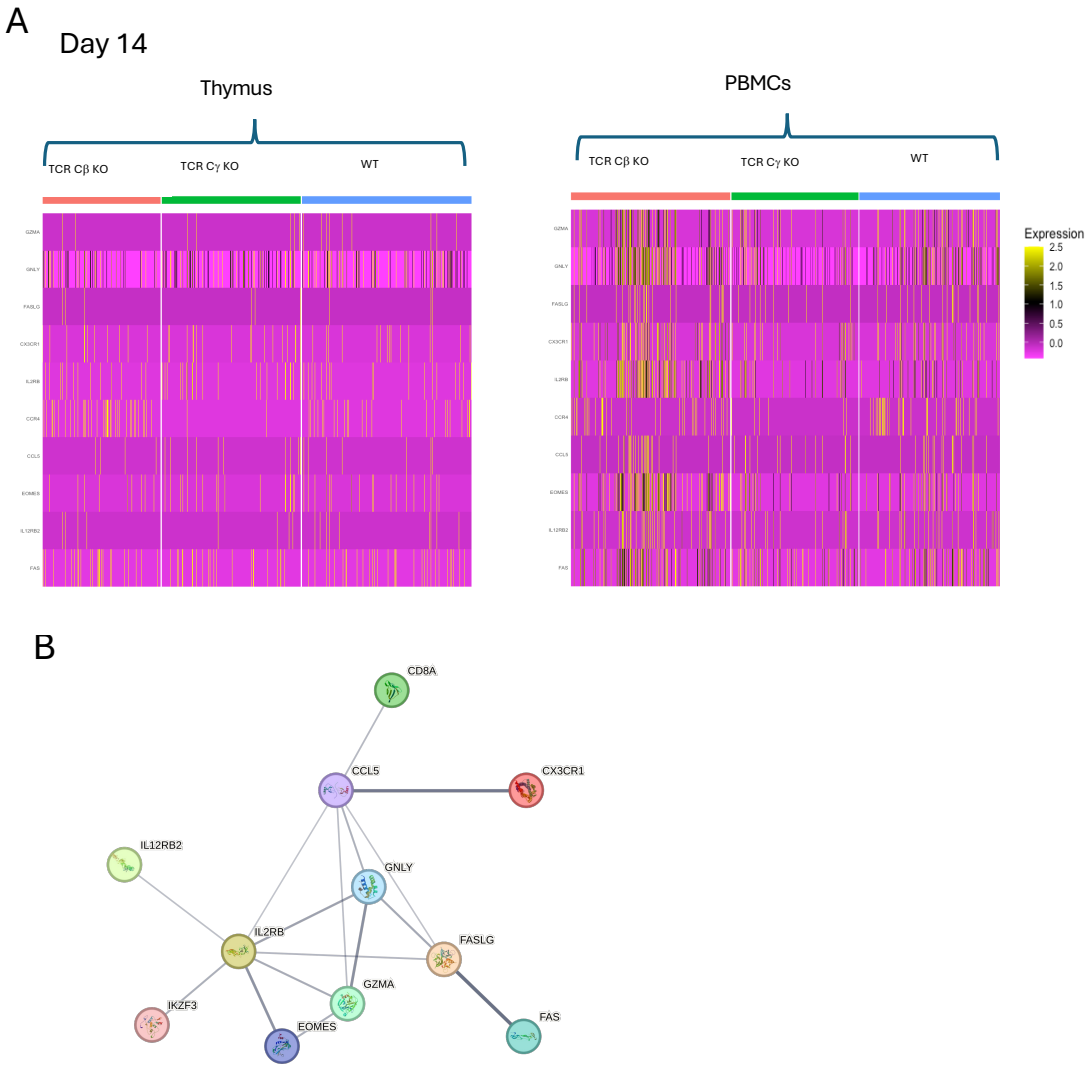

Figure 8. Cytolytic γδ T cell clusters. A) Heatmap of Clusters from thymus and PBMCs of TCR Cβ KO, TCR Cγ KO and WT chickens 14 days after hatch showing genes involved in cytolytic activity. The color represents the gene expression level (average_log2 fold change). B) String Network of DEGs involved in cytolytic activity.

2 Tables

Table 1. Sequencing quality of cells and sample multiplexing:

	Cells				Sample Multiplexing	
Day	Putative Cells	Reads Passing Quality Filter	Reads from putative cells	Mean Reads per cell	Valid Reads aligned to Sample Tags	Reads from putative cells
ED18	41,998	97,33%	93,87%	13,258.31	5,611,637	65,20%
d3	37,975	96,98%	90,83%	14,650.78	7,944,922	68,93%
d14	54,683	96,83%	92,96%	12,617.67	6,633,932	71,24%

Table 2. Cluster annotation ED18

Markers	Genes	TCR Cβ KO	TCR Cγ KO	Wild type
TARP, CD82, PDE1A, IL20RA	mature γδ T cell	0	4	0
CCR7, CD5, THEMIS	mature αβ T cells		1	1
RAG1, RAG2, CD4, CD8A, FOXP1	differentiating αβ T cells	5	0	2
TARP, CDCA7, MCM2,3, 4,5,6,	proliferating γδ T cells	1		3
CDK1, TOP2A, CCNBI, BUB1B, CCNA2, CDC20, PLK1 CENPF, ASPM, KIF11, KIFF20A, TACC	division active precursor T-cells	4	2	4
CDCA7, MCM2,4,5,6, TOP2A, CDK1	proliferating αβ T cells		3	5
CHAC1, ASNS, MTHFD2, SLC1A4, PSAT, CD28, CD44, TARP	activated γδ T cells	2		6
FOXO1, KLF2, IL7R, P2RY8, CD38, RORA	memory T cells	3		
IL2RA (=CD25), IL2RB, IL1R1, IL18R1	activated γδ T cells	6		

Table 3. Cluster annotation d3 from Thymus

Markers	Genes	TCR Cβ KO	TCR Cg KO	Wild Type
CCR7, CD5, THEMIS	mature αβ T cells		0	0
RAG1, RAG2, CD4, CD8A, FOXP1	differentiating αβ T cells	4	2	1
FOXO1, KLF2, IL7R, P2RY8, CD38, RORA	memory T cells	3	1	2
TARP, CD82, PDE1A, IL20RA,	mature γδ T cell	2		3
CDCA7, MCM2,4,5,6, TOP2A, CDK1	proliferating αβ T cells	4	3	4
TARP, CDCA7, MCM2,4,5,6	proliferating γδ T cells	1		5
GP1BB, GP9, F13A1	thrombocytes	5	4	6
HBE1, SLC4A1, EPB42, SPTB, DMTN	erythrocytes		5	7
IL2RA (=CD25), IL2RB, IL1R1, IL18R1	activated γδ T cells	0		

Table 4. Cluster annotation d3 from PBMCs

Markers	Genes	TCR Cβ KO	TCR Cγ KO	Will Type
CCR7, CD5, THEMIS	mature αβ T cells		0	0
FOXO1, KLF2, IL7R	memory T cells	0	2	1
CDCA7, MCM2,4,5,6	proliferating αβ T cells		3	2
TARP, CD82, IL20RA, PDE1A	mature γδ T cell	1		3
IL2RA, CD4, IL18R1	activated αβ T cells		1	4
IL2RA (=CD25), IL2RB, IL1R1, IL18R1	activated γδ T cells	1		5
GP1BB, GP9, F13A1	thrombocytes	4	4	6
RAG1, RAG2	differentiating αβ T cells	2	2	7
HBE1, SLC4A1, EPB42, SPTB, DMTN	erythrocytes	5	5	8
GNLY, GZMM, CCL5, IL2RB, EOMES, XCL1,	CD8+ effector cells	3		

Table 5. Cluster annotation d14 from Thymus

Genes	Cluster ID	TCR Cβ KO	TCR Cγ KO	Wild Type
CCR7, CD5, THEMIS	mature αβ T cells	1	0	0
TARP, CD82, IL20RA, PDE1A	mature γδ T cell	0		1
RAG1, RAG2	differentiating αβ T cells		2	2
FOXO1, KLF2, CCR7	memory T cells		1	3
TARP, CDCA7, MCM2,4,5,6	proliferating γδ T cells	2		4
GNLY, GZMM, CCL5, IL2RB, EOMES, XCL1,	cytotoxic effector T cells		3	5
HBE1, SLC4A1, EPB42, SPTB, DMTN	erythrocytes	3	5	6
CDCA7, MCM2,4,5,6, TOP2A, CDK1	proliferating αβ T cells		4	

Table 6. Cluster annotation d14 from PBMCs

Genes	Cluster ID	TCR Cβ KO	TCR Cγ KO	Wild Type
FOXO1, KLF2, CCR7	memory T cells		0	0
TARP, CD82, IL20RA, PDE1A	mature γδ T cell	1		1
GNLY, GZMM, CCL5, IL2RB, EOMES, XCL1,	cytotoxic effector T cells	2	2	2
RAG1, RAG2, CD4, CD8A, FOXP1	differentiating αβ T cells		1	3
TARP, CDCA7, MCM2,4,5,6	proliferating γδ T cells			4
GP1BB, GP9, F13A1	thrombocytes	3	3	5
CD8A, CD28, STAT1, BACH2, IFNGR1, IL7R,	interferon-stimulated γδ T cells	0		
HBE1, SLC4A1, EPB42, SPTB, DMTN	erythrocytes	4		