

Cross-species comparison of amniote single-cell transcriptomes reveals evolutionary conservation and divergence in the chicken immune system

Corresponding Author: Professor Yu Jiang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have generated single cell RNA seq data from 1.57 million cells across 157 cell types from 36 tissues in the chicken. They also generated some data for turtles and used existing human data to perform comparisons. They focused primarily on immune cells and identify both similarities and differences between the three species. The observed variation in subtypes and proportions of $\gamma\delta$ T cells across the three species reflected the evolution of pathogen recognition and signaling mechanisms among amniotes. They support the chicken data with some data from duck. The analysis seems solid and the paper is well written.

There is no justification for why they focus only on the immune system. Are the other tissues/organs more similar or different or did they simply not look? I'm fine with the immune system being the main focus of the paper, but it would be good with a summary of what happens in the rest of the data.

Line 58: typo: "provides"

Reviewer #2

(Remarks to the Author)

This is a well written manuscript of international interest that provides a comprehensive molecular characterization of avian cells in comparison to distantly related humans and a reptile. It provides a reference atlas and spatial transcriptomic map of the chicken embryo including assessment of a range of cell types and 36 tissues. The evolutionary rates of selected immune cells were preferentially analysed demonstrating a rapid evolution of avian cells due to changes in gene regulatory networks. The analysis provides fresh insight into the role of embryonic follicular dendritic cells in the development of the bursa of Fabricius and how they promote B cell proliferation and migration within follicles.

A high degree of confidence in the data and interpretation is provided by the spatial transcriptomics heatmaps. The study set out to equally study immune system, specifically the bursa of Fabricius and gizzard as examples of where divergence may have been greatest between chickens and humans. A significant part of the Results and Discussion is committed to $\gamma\delta$ T-cells and dendritic cell lineages with very little attention provided to the gizzard or other differences in avian intestinal anatomy which were put forward as key drivers for the study. This should be addressed in the Results and Discussion. Similarly the discussion on mammalian adrenal cortical function could have further explored the ontogeny and evolutionary divergence of corticosterone and cortisol as the dominant adrenal cortical hormones in mature birds and mammals, respectively. For completeness it would be relevant to have included in the Methods the sexes of the adult animals that were used for the study since that may have influenced adrenal gene expression if only females were not included. The expression of genes relating to lipid and lipoprotein transport and heme scavenging in chicken plasmacytoid dendritic cells and their clustering with mononuclear phagocytes is an important finding that could explain differences in heme degradation pathways between birds, reptiles and mammals. It also provides a potential explanation for avian macrophage lipid metaplasia in xanthomatous lesions. Finally I was disappointed to not find an expected divergence in ARG1 gene expression between avian, reptile and human cells. Some explanation for this might be worth considering in the Discussion.

Reviewer #4

(Remarks to the Author)

The manuscript by Wang et al. presents an impressive and comprehensive single-cell atlas of chicken tissues, including cross-species comparisons with human, turtle, and duck. The dataset represents an excellent resource for the scientific community and will be of high value for studies of immune evolution. However, several aspects of the manuscript would benefit from clarification, restructuring, and stronger focus on the immune system, as suggested by the title.

Comments

1. Scope and Relevance

a The resource itself is excellent and of high community value.

b However, given the title emphasizes chicken immune systems, certain sections (e.g., ionocytes in the first results section, lines 190–221 and Fig. 2) appear only loosely related to immunity. Immune-relevant cells such as microglia and macrophages are shown in Fig. 2F but not discussed here. A stronger emphasis on immune subsets would better align with the stated focus.

2. Methods and Materials

The methods mention the use of two chicken breeds for scRNA-seq, but the manuscript does not specify which breed was used for each analysis. This should be clarified.

3. Definitions and Claims

a Line 182: The term “rapid evolution” is vague and should be explicitly defined, with criteria and examples.

b Lines 187–189: The arguments to conclude evolution to endothermy are unclear and this conclusion would be more appropriate in the discussion section.

4. Innate Lymphoid Cells (ILCs)

a Fig. 3A shows ILC2s in chicken but not in humans, which contradicts previous comparative studies (e.g., Vivier et al., Nat Immunol 2016, PMID 27328009). The discrepancy must be addressed in the discussion.

b Figs. 3B/C: Cell identities are not clearly labeled (chicken vs. human). Furthermore, comparative plots should avoid including cell types detected in only one species, as this prevents meaningful comparison.

5. Figure 4

a Large and small B cells are not defined—please provide criteria.

b Fig 4I; This is an interpretation of the data and as such should be shown within the discussion section.

6. Follicular Dendritic Cells (FDCs)

This section is particularly interesting, especially the suggestion of a hematopoietic origin in chicken. However:

a Line 368: A reference for the claimed human FDC origin is missing

b In the discussion (lines 707–708) the references provided on the human FDC origin were mouse, not human.

c Line 720; there are other publications from well suited to refer to the formation and presence of the FDC within mouse and human, such as PMID 18424165

7. Data Interpretation and Clarity

a Fig. 6A: Selection criteria for cells/genes are unclear. CTSG should be defined (Cathepsin G), and justification provided for using it as a marker.

b Lines 534–535: The term “innovative gene expression profiles” is vague; please define.

c Line 542: “CoRCs of T cells” is not explained earlier—please clarify.

d Lines 553–554: The comparison between human PBMCs and chicken tissues is not valid; this should be acknowledged as a limitation.

e Lines 585–591: The relationship of the 6.7% proportion to other comparisons is unclear.

f Line 590: Define “substantive.”

g. Spatial and Ligand-Receptor Analyses

h Lines 613–615 & Fig. 7D–E: The link between ligand-receptor pairs and cell identity is not convincingly demonstrated.

i Fig. 7D; Histological validation, as provided for other tissues in other figures, would be helpful to interpret the data shown.

j Interpretations in Fig. 7E are overstated.

k Lines 618–619: Statements appear assumptive without clear argument.

l Lines 637–638: The concept of “gradual refinement” of cells needs mechanistic clarification—does this occur in situ, postnatally?

m Lines 643–649 & Fig. 7I: Conclusions based on expression of only two chemokine receptors are overreaching.

9. Terminology

a Line 701: Please clarify what is meant by “low evolutionary conservation.”

b Chicken gene names should be annotated similarly as mouse, so first capital letter followed by small and at the gene in italics.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed the questions raised by the reviewers.

Reviewer #2

(Remarks to the Author)

The revised manuscript satisfies my previous comments.

Reviewer #4

(Remarks to the Author)

I would like to thank the authors for the considerable effort they have made to address my comments. The majority of my points have been handled satisfactory. However, there remains one important issue concerning the overall focus of the manuscript that, in my view, has not yet been fully resolved. This concern is similar to the first comment raised by Reviewer #1, who also questioned the central focus of the study. Although the authors have responded to both comments, the revisions do not sufficiently clarify or adjust the manuscript's focus, and therefore this issue persists.

Specifically, it remains unclear why certain cell types are highlighted while others are not. For example, in Supplementary Figure 7, several cell types are presented, yet many additional populations appear equally interesting and relevant. I am missing a clear rationale for emphasizing PBMCs, smooth muscle cells, fibroblasts, ionocytes, adrenal cells, and others, while excluding, for instance, tuft cells or GABAergic neurons.

In addition, Supplementary Figure 7 shows notable differences among immune cell populations (e.g. plasmablasts), which are largely not discussed in the main text. As also noted by Reviewer #1, I do not object to the manuscript focusing either on immune or non-immune cell types. However, it is important that the authors clearly articulate the criteria used to select the highlighted cell populations. Are these cells chosen because they are the most divergent, the most conserved, the most biologically informative, or because they serve as quality controls for cross-species comparison? Without such clarification, the selection appears somewhat arbitrary.

Clarifying this rationale would substantially strengthen the coherence and interpretability of the manuscript.

A last, minor point, is that I would like to suggest to also add the classification of the large and small Bcells based on size measured by flowcytometry within the manuscript text, as the authors pointed out in the rebuttal letter.

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Reviewer #1:

The authors have generated single cell RNA seq data from 1.57 million cells across 157 cell types from 36 tissues in the chicken. They also generated some data for turtles and used existing human data to perform comparisons. They focused primarily on immune cells and identify both similarities and differences between the three species. The observed variation in subtypes and proportions of $\gamma\delta$ T cells across the three species reflected the evolution of pathogen recognition and signaling mechanisms among amniotes. They support the chicken data with some data from duck. The analysis seems solid and the paper is well written.

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Response: Thank you for raising this valuable question.

Since immune cells are widely distributed in various tissues and organs, our single-cell atlas of multiple chicken tissues offers a unique opportunity to systematically characterize the global landscape of chicken immune cells and further provide insights into the immune evolution of amniotes. We thus focused on the immune system.

We examined the similarity of all shared cell types among chickens, humans and turtles. Overall, most cell types showed statistically significant correlations, including those of the immune, digestive, and circulatory systems, underscoring a broadly conserved gene expression at cellular levels across amniotes. Interestingly, some cell types associated with the endocrine and urinary systems, such as chromaffin cells, capsular cells, and distal convoluted tubule cells, did not exhibit significant correlations, indicating that gene expression profiles of these cell types exhibit considerable interspecies variation. This summary has now been incorporated into the Results as below (Line176-183):

“Overall, most cell types across species showed statistically significant correlations, including those of the immune, digestive, and circulatory systems, underscoring a broadly conserved gene expression at cellular levels across amniotes. Interestingly, however, certain cell types associated with the endocrine and urinary systems, such as chromaffin cells, capsular cells, and distal convoluted tubule cells, did not exhibit significant correlations (Fig. 2a and Supplementary Fig. 7a, b), indicating that gene expression profiles of these cell types exhibit considerable interspecies variation.”

Line 58: typo: “provides”

Response: Thanks. We have revised it.

Reviewer #2 :

This is a well written manuscript of international interest that provides a comprehensive molecular characterization of avian cells in comparison to distantly related humans and a reptile. It provides a reference atlas and spatial transcriptomic map of the chicken embryo including assessment of a range of cell types and 36 tissues. The evolutionary rates of selected immune cells were preferentially analysed demonstrating a rapid evolution of avian cells due to changes in gene regulatory networks. The analysis provides fresh insight into the role of embryonic follicular dendritic cells in the development of the bursa of Fabricius and how they promote B cell proliferation and migration within follicles. A high degree of confidence in the data and interpretation is provided by the spatial transcriptomics heatmaps.

Response: We sincerely appreciate your recognition.

The study set out to equally study immune system, specifically the bursa of Fabricius and gizzard as examples of where divergence may have been greatest between chickens and humans. A significant part of the Results and Discussion is committed to $\gamma\delta$ T-cells and dendritic cell lineages with very little attention provided to the gizzard or other differences in avian intestinal anatomy which were put forward as key drivers for the study. This should be addressed in the Results and Discussion. Similarly the discussion on mammalian adrenal cortical function could have further explored the ontogeny and evolutionary divergence of corticosterone and cortisol as the dominant adrenal cortical hormones in mature birds and mammals, respectively.

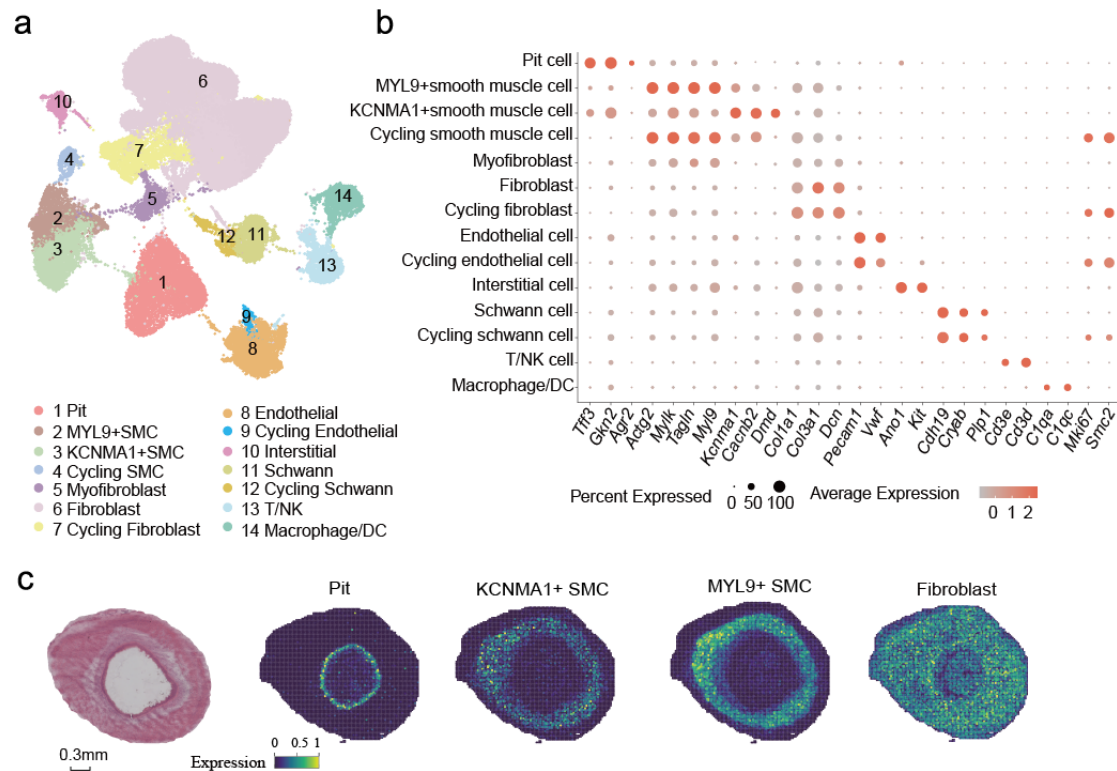
Response: Thank you. We highlighted both the bursa of Fabricius and the gizzard as key examples of evolutionary divergence in the introduction. For the gizzard, scRNA-seq analysis identified 14 major cell types (Supplementary Fig. 3a, b). Interestingly, two muscle cell types with distinct transcriptional signatures were observed. One cluster was enriched in genes encoding myosin (*Myh9*, *Myh11*), while the other cluster had abundant transcripts that enable muscle contraction (*Kcnma1*, *Cacnb2*, *Dmd*)(PMID: 30422117; 31427379; 11917091) (Supplementary Fig. 3b). The spatial localization of cell populations in the early gizzard showed that pit cells were localized to the center of gizzard, whereas the two types of smooth muscle cell and fibroblast consistently mapped to the periphery of the gizzard (Supplementary Fig. 3c). The muscle cell type that expresses genes crucial for robust muscular contraction may enhance its efficient grinding functionality. This adaptation compensates for the absence of teeth and provides a survival advantage by decoupling feeding from mastication, thereby reducing predation risk and improving foraging efficiency (PMID: 33986452). The adrenal cortex cells are responsible for steroid hormones and show relatively low correlations between chicken and human. And a fundamental divergence exists between humans and birds in their predominant glucocorticoid profiles: cortisol is the major glucocorticoid in humans, whereas corticosterone plays this role in birds (PMID: 18165137). This divergence may be attributed to several factors. Anatomically, the human adrenal cortex is organized into three distinct histological layers: the zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR)(PMID: 38718896). In contrast, the chicken adrenal cortex is

structurally simpler, comprising two principal functional zones: a subcapsular region analogous to the mammalian ZG and a larger inner zone that resembles the combined ZF and ZR(PMID: 18165137). Our single-cell data corroborate this simplified organization in chickens, identifying only two principal adrenocortical subpopulations: a ZG population and a second population analogous to the combined ZF/ZR. Second, the expression of key steroidogenic enzyme for glucocorticoid synthesis is divergent in chickens and humans. The key enzyme for cortisol synthesis, *Cyp17a1*, is scarcely expressed in chicken adrenal cells(Supplementary Fig. 7d), but is abundant in human adrenal cells(PMID: 38718896), fundamentally altering the glucocorticoid profiles between them. Furthermore, birds produce only minimal amounts of the primary mineralocorticoid, aldosterone(PMID: 18165137). Since corticosterone binds to the mineralocorticoid receptor with a higher affinity than cortisol (though corticosterone and cortisol can activate it)(PMID: 15947887), its elevated production in birds likely serves as a compensatory mechanism to maintain electrolyte and fluid balance.

We summarized results of the above analysis in our revised manuscript as below:

Section: Result of chicken gizzard (Line 135-143)

*“We focused on chicken gizzard combining scRNA-seq and spatial transcriptomics. ScRNA-seq analysis identified 14 major cell types(Supplementary Fig. 3a, b). Interestingly, two muscle cell types with distinct transcriptional signatures were observed. One cluster was enriched in genes encoding myosin (*Myl9*, *Myh11*), while the other cluster had abundant transcripts that enable muscle contraction (*Kcnma1*, *Cacnb2*, *Dmd*)^{1, 2, 3}(Supplementary Fig. 3b). The spatial localization of cell populations in the early gizzard showed that pit cells were localized to the center of gizzard, whereas the two types of smooth muscle cell and fibroblast consistently mapped to the periphery of the gizzard (Supplementary Fig. 3c)⁴. ”*



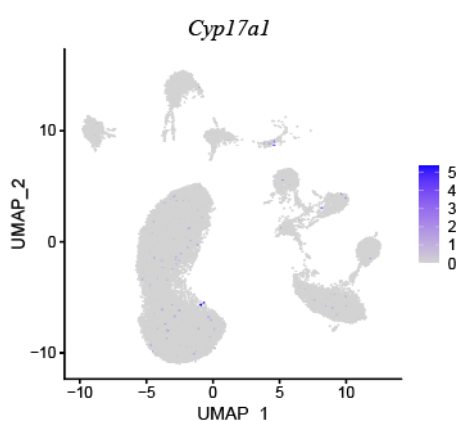
Supplementary Fig. 3. ScRNA-seq profiling and spatial visualization of cell clusters in the gizzard of the chicken. **a** UMAP visualization of all clusters colored by major cell types. **b** Dot plot for expression of marker genes of the identified cell populations in chicken gizzard. **c** Spatial visualization of cells in gizzard of the chicken embryo.

Section: Discussion about the evolutionary aspects of the gizzard (Line 691-697) and adrenal gland (Line 715-735).

“The gizzard represents a specialized digestive organ unique to birds, functioning in the mechanical grinding of food. Our analysis identified two distinct muscle cell subtypes, including one enriched in genes critical for strong muscular contraction, which may support its efficient grinding function. This adaptation compensates for the absence of teeth and provides a survival advantage by decoupling feeding from mastication, thereby reducing predation risk and improving foraging efficiency⁴.”

“The adrenal cortex cells are responsible for steroid hormones and show relatively low correlations between chicken and human. And a fundamental divergence exists between humans and birds in their predominant glucocorticoid profiles: cortisol is the major glucocorticoid in humans, whereas corticosterone plays this role in birds⁵. This divergence may be attributed to several factors. Anatomically, the human adrenal cortex is organized into three distinct histological layers: the zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR)⁶. In contrast, the chicken adrenal cortex is structurally simpler, comprising two principal functional zones: a subcapsular region analogous to the mammalian ZG and a larger inner zone that resembles the combined ZF and ZR⁵. Our single-cell data corroborate this simplified

organization in chickens, identifying only two principal adrenocortical subpopulations: a ZG population and a second population analogous to the combined ZF/ZR. Second, the expression levels of key steroidogenic enzyme for glucocorticoid synthesis is different between chickens and humans. The key enzyme for cortisol synthesis, *Cyp17a1*, is scarcely expressed in chicken adrenal cells (Supplementary Fig. 7d), whereas it is abundant in human adrenal cells⁶, fundamentally altering the glucocorticoid profiles between them. Furthermore, birds produce only minimal amounts of the primary mineralocorticoid, aldosterone⁵. Since corticosterone binds to the mineralocorticoid receptor with a higher affinity than cortisol (though both can activate it)⁷, its elevated production in birds likely serves as a compensatory mechanism to maintain electrolyte and fluid balance.”



Supplementary Fig. 7d. UMAP visualization of *Cyp17a1* expression in chicken adrenal cells.

For completeness it would be relevant to have included in the Methods the sexes of the adult animals that were used for the study since that may have influenced adrenal gene expression if only females were not included.

Response: Thanks for your kind reminder. For Xiangjia Black Phoenix chickens, two males and one female were used for sample collection. For Leghorn chickens, turtles, and ducks, gender information was not recorded. The single-cell data of chicken adrenal glands was obtained from Xiangjia Black Phoenix chickens, including samples from both male and female chickens. We have supplemented the sex-related details in the Materials and Methods section as below (Line 794-795):

“For Xiangjia Black Phoenix chickens, two males and one female were used for sample collection. For Leghorn chickens, turtles, and ducks, gender information was not recorded.”

The expression of genes relating to lipid and lipoprotein transport and heme scavenging in chicken plasmacytoid dendritic cells and their clustering with mononuclear phagocytes is an important finding that could explain differences in heme degradation pathways between birds, reptiles and mammals. It also provides a potential explanation for avian macrophage lipid metaplasia in xanthomatous

lesions. Finally I was disappointed to not find an expected divergence in ARG1 gene expression between avian, reptile and human cells. Some explanation for this might be worth considering in the Discussion.

Response: Thank you for your suggestions. In our cross-species analysis, we focused on 12,546 one-to-one orthologous genes. Due to the absence of an orthologous gene for ARG1 in chickens, it was excluded from our cross-species expression comparison.

We checked and confirmed the absence of ARG1 in the chicken genome. we aligned the turtle ARG1 protein sequence using tBLASTn to the two chicken reference genomes (7b and GGswu1, GGswu1 is a T2T genome released by a recent study (PMID: 36780517)). The chicken ARG2 gene exhibited the highest sequence similarity and alignment coverage (57% identity) (Response Fig. 1). In parallel, we conducted a BLASTp search of the human ARG1 protein sequence against the chicken protein sequences, which predominantly aligned to chicken ARG2 (arginase-2) and AGMAT (agmatinase), with sequence identities of 59% and 26%, respectively (Response Fig. 2). These analyses consistently indicate that ARG1 is absent in the chicken genome.

In humans, ARG1 encodes arginase, an enzyme that catalyzes the hydrolysis of arginine to ornithine and urea (PMID: 39952693). In birds, however, ammonia derived from amino acid catabolism is primarily converted to uric acid (PMID: 12670782). This metabolic divergence may have driven the loss of the ARG1 gene in the chicken.

Score = 404 bits (1037), Expect = 2e-140, Method: Compositional matrix adjust.
Identities = 184/324 (57%), Positives = 248/324 (77%), Gaps = 2/324 (1%)

```
Query 31 RMSAKPKTSAGIIWAPFSKQAREGTEEGPAVVKEAGLIDKLTAAQGCDDVKDYGALQFDDI 90|
      R+S + S ++ APFS+GQ R G + GPA ++ AGL+++L GC + D+G L F +
Sbjct 20 RLSRRAH-SVALVGAPFSRGQKRRGVDHGPATLRAAGLVERLAGLGCQLYDFGDLNFTQV 78

Query 91 PDDRPCHNA-KNPRSVGRANEKLASAVAIEVKKSGRTCLVVGGDHSLGIGSISGHAKIHPD 149
      P+D +N PRSVG A++ LA AV+ +G +C+ +GGDHSL +GS+SGHA+ P
Sbjct 79 PNDELNNLIYYPRSVGLASQVLADAVSRAVAAGHSCVTIGGDHSLALGSVSGHARQCPH 138

Query 150 LGVIWFDAHTDINTPLTSLSGNMHGQPVSFLLKELKDKMPDVPGFWSWITPCLSAEDIVYI 209
      LGVIW DAH DINTPLT+ SGN+HGQP+SFL+EL+DK+P +PGFSW+ PCLSA DIVYI
Sbjct 139 LGVIWVDAHADINTPLTTQSGNLHGQPLSFLRELQDKVPQLPGFSWLKPCLSASDIVYI 198

Query 210 GLRDVDNAEHCILKSLGIKYYSMTIDQLGIRKVMEEETLCYLLANKKRPIHLSFDVDGLD 269
      GLRDVD AE+ ILK+ I+Y+SM ++D+LGI+KVMET L+ ++RPIHLSFD+D D
Sbjct 199 GLRDVDPAEYYILKNYDIQYFSMRDVRDLGIQKVMERTFEQLMGRRRQRPIHLSFDIDAFD 258

Query 270 PSLAPATGTPVPGGVTLREGIYVAEELYRTGLLSAVDIMEINPALGKTQEEVNNTTVNTAV 329
      PSLAPATGTPV GG+T REG+Y+AE++ TG+LSAVD++E+NP LG +QEEVN T + AV
Sbjct 259 PSLAPATGTPVLGGLTYREGMYIAEIIHNTGMLSAVDMVEVNPLL GASQEEVNATASLAV 318

Query 330 SIILSCFGKEREGSHSSSYRIPKP 353
      +I +CFG+ REG+H++ +P P
Sbjct 319 DVIATCFGQTREGAHTAFDELPTP 342
```

Response Fig1. Turtle ARG1 protein sequence maps to chicken ARG2 (arginase-2).

arginase-2, mitochondrial [Gallus gallus]
Sequence ID: [NP_001186633.2](#) Length: **356** Number of Matches: **1**

Range 1: 21 to 343 [GenPept](#) [Graphics](#) ▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Method	Identities	Positives	Gaps
413 bits(1062)	3e-145	Compositional matrix adjust.	192/323(59%)	239/323(73%)	1/323(0%)
Query 1	MSAKSRTIGIIGAPFSKGQPRGGVEEGPTVLRKAGLLEKLKEQECQDKYDGLPFADIPN	60			
	+S ++ ++ ++GAPFS+GQ R GV+ GP LR AGL+E+L C + D+GDL F +PN				
Sbjct 21	LSRAHSVALVGAPFSRGQKRRGVDHGPTLRAAGLVERLAGLGCQLYDFGDLNFTQVPI	80			
Query 61	DSPFQ-IVKNPRSVGKASEQLAGKVAEVKKNGRISLVLGGDHS LAIGSISGHARVHPDLG	119			
	D + ++ PRSVG AS+ LA V+ G + +GGDHS LA+GS+SGHAR P LG				
Sbjct 81	DELYNNLIYYPRSVGLASQVLADAVSRAVAAGHSCVTIGGDHSLALGSVSGHARQCPhLG	140			
Query 120	VIWDAHTDINTPLTTTSGNLHGQPVSFLLKELKGKIPDVPGFSNVTPCISAKDIVYIGL	179			
	VIWDAH DINTPLTT SGNLHGQ+SFLL+EL+ K+P +PGFSW+ PC+SA DIVYIGL				
Sbjct 141	VIWDAHADINTPLTTQSGNLHGQPLSFLLRELQDKVPQLPGFSWLKPCLASADIVYIGL	200			
Query 180	RDVDPGEHYILKTLGIKYFSMTEVDRLGIGKVMETLSYLLGRKKRPIHLSFDVDGLDP	239			
	RDVDP E+YILK I+YFSH +VDRLGI KVME T L+GR++RPIHLSFD+D DPS				
Sbjct 201	RDVDPAEYYILKNYDIQYFSHRDVDRLGIQKVMERTFEQLMRRQRPIHLSFDIDAPS	260			
Query 240	FTPATGTPVVGGLTYREGLYITEEYKTLGLSLGDI MEVNP SLGKTP EEVTRTNTAVAI	299			
	PATGTPV+GGLTYREG+YI EEI+ TG+LS +D++EVNP LG + EEV T + AV +				
Sbjct 261	LAPATGTPVLGGLTYREGMYIAEEIHNTGMSAVDMVEVNP LLGASQEEVNATASLAVDV	320			
Query 300	TLACFLAREGNHKPIDYLNPPK 322				
	CFG REG H D L P				
Sbjct 321	IATCFGQTREGAHTAFDELTPS 343				

Related Information
[Gene](#) - associated gene details
[AlphaFold Structure](#) - 3D structure displays
[Genome Data Viewer](#) - aligned genomic context

agmatinase, mitochondrial precursor [Gallus gallus]
Sequence ID: [NP_989474.2](#) Length: **340** Number of Matches: **1**

Range 1: 55 to 311 [GenPept](#) [Graphics](#) ▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Method	Identities	Positives	Gaps
80.9 bits(198)	5e-17	Compositional matrix adjust.	74/282(26%)	114/282(40%)	28/282(9%)
Query 2	SAKSRTIGIIGAPFSKGQPRGGVEEGPTVLRKAGLLEKLKEQECQDKYDGLPFADIPN	60			
	SA+ +G P G R G GP +R ++ + +L AD+				
Sbjct 55	SAEGLDAAFVGVPDGTGTSNRPGARFGPQQIRAESVMVRRYNASTGAAPFDSLLVADV-G	113			
Query 61	DSPFQIVKNPRSVGKASEQLAGKVAEVKKNGRISLVLGGDHS LAIGSISGHARVHPDLGV	120			
	D + + P S + E VA +G + L LGGDHS+ + A H +G+				
Sbjct 114	DVNNLYNLPDSCRRIRESYQKIVA----SGCVPLTLGGDHSITYPILQAVAEKHGPVGL	169			
Query 121	INWDAHTDINTPLTTTSGNLHGQPVSFLLKELKGKIPDVPGFSNVTPCISAKDIVYIGLR	180			
	+ VDAHTD + + HG P + E G + + +V IG+R				
Sbjct 170	VHVAHTD-TSDMALGEKIYHGTFFRRCVDE-----GL-----LDCSRVVQIGIR	213			
Query 181	--DVPGEHYILKTLGIKYFSMTEVDRLGIGKVMETLSYLLGRKKRPIHLSFDVDGLDP	238			
	P + + G + E + +M E + P+++SFD+DGLDP				
Sbjct 214	GSSYAPNPYKYCWDQGFRRVPAEECWKSLVPLMGEVRQQM---GDGPVVISFDIDGLDP	270			
Query 239	SFTPATGTPVVGGLTYREGLYITEEYKTLGLSLGDI MEVNP 280				
	++ P TGTP + GLT + L I ++ G D++EV P				
Sbjct 271	AYAPGTGTPEIAGLTPMQALEIIRGCKGLNIV-GCDLVEVAP 311				

Related Information
[Gene](#) - associated gene details
[AlphaFold Structure](#) - 3D structure displays
[Genome Data Viewer](#) - aligned genomic context

Response Fig2. Human ARG1 protein sequence maps to chicken ARG2 (arginase-2) and AGMAT (agmatinase).

Reviewer #4:

The manuscript by Wang et al. presents an impressive and comprehensive single-cell atlas of chicken tissues, including cross-species comparisons with human, turtle, and duck. The dataset represents an excellent resource for the scientific community and will be of high value for studies of immune evolution. However, several aspects of the manuscript would benefit from clarification, restructuring, and stronger focus on the immune system, as suggested by the title.

Response: We sincerely appreciate your valuable feedback.

Comments**1. Scope and Relevance**

a The resource itself is excellent and of high community value.

b However, given the title emphasizes chicken immune systems, certain sections (e.g., ionocytes in the first results section, lines 190–221 and Fig. 2) appear only loosely related to immunity. Immune-relevant cells such as microglia and macrophages are shown in Fig. 2F but not discussed here. A stronger emphasis on immune subsets would better align with the stated focus.

Response: Thank you for your suggestion. For these sections (lines 190–221) with only loose relevance to immune systems, we have simplified the results and included figures in the Supplementary Fig. 7c. At the same time, we compared the differences in gene transcription patterns between chicken and human microglial cells. Gene expression analysis revealed 367 genes with species-biased expression patterns, of which 145 and 222 were upregulated and downregulated in chicken microglia, respectively (Fig. 2b). These species-biased genes were enriched in regulation of small GTPase mediated signal transduction, cytokine-mediated signaling pathway and negative regulation of neuron apoptotic process (Fig. 2c). For instance, well-characterized microglial genes *CX3CR1* and *C3*, which are critical for microglial function (PMID: 34492237; 22632727), were downregulated in chicken (Fig. 2d). Disruption of their signaling leads to dysregulation of host-defense pathways, resulting in abnormal neuroinflammatory response and neuronal damage (PMID: 34492237; 22632727). This finding suggested that microglia might exhibit species-specific differences in host defense and neuroprotective responses between chickens and humans. We added it to the Results as below (Line186-198):

*“Given the relatively low similarity between human and chicken microglia, we further explore the differences in gene expression patterns of chicken microglia with human microglia. Gene expression analysis revealed 367 genes with species-biased expression patterns, of which 145 and 222 were upregulated were downregulated in chicken microglia, respectively (Fig. 2b). These species-biased genes were enriched in regulation of small GTPase mediated signal transduction, cytokine-mediated signaling pathway and negative regulation of neuron apoptotic process (Fig. 2). For instance, well-characterized microglial genes *CX3CR1* and *C3*, which are critical for microglial function^{8,9}, were downregulated in chicken (Fig. 2d). Disruption of their signaling leads to dysregulation of host-defense pathways, resulting in abnormal neuroinflammatory response and neuronal damage^{8,9}. This finding suggests that*

microglia might exhibit species-specific differences in host defense and neuroprotective responses between chickens and humans.”

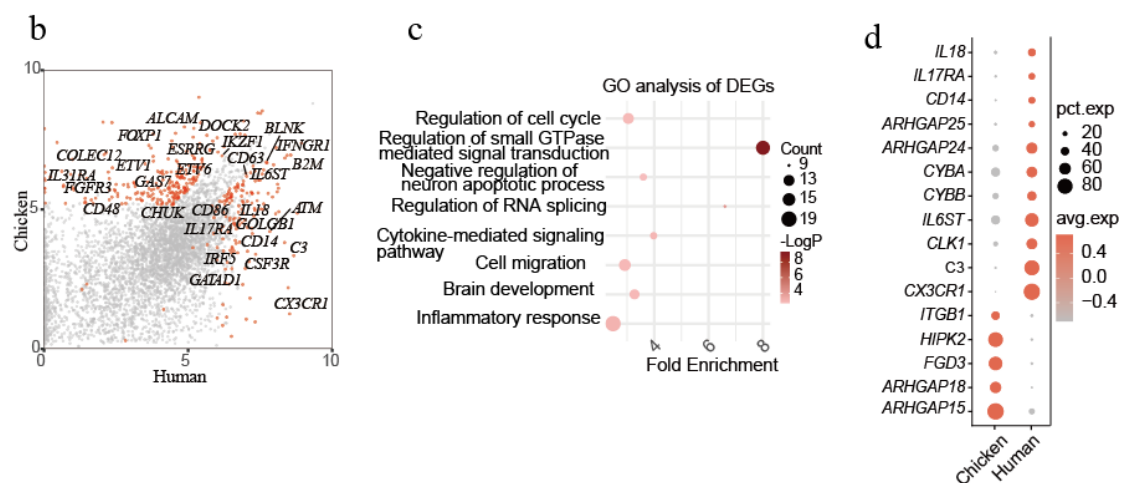


Fig. 2 b Scatter plot comparing average expression levels of individual genes (dots) in microglia between humans and chickens. Red dots denote immune genes with $p < 0.05$ (MAST differential gene expression test). Scale: $\log(\text{CPM}+1)$. **c** GO analysis of different expression genes between chicken and human. **d** Dot plot for expression of different expression genes.

2. Methods and Materials

The methods mention the use of two chicken breeds for scRNA-seq, but the manuscript does not specify which breed was used for each analysis. This should be clarified.

Response: For the construction of the chicken single-cell atlas (Figure 1b, Supplementary Fig1a, b), we used data from two breeds to capture a broader spectrum of cell types in chickens. We have included the breed origins corresponding to the single-cell data for each tissue in Supplementary Table 1. We prioritized White Leghorn chicken data for cross-species comparisons of common cell type similarity to minimize potential platform-specific biases (Fig.2a and the Supplementary Fig7a, b). If a specific cell type was absent in the White Leghorn dataset due to the lack of single-cell data in the corresponding tissue, data from the Xiangjia Black Phoenix chickens were used as a substitute. We have included the breed origin for each cell type in Fig.2a and the Supplementary Fig7a, b. For all other results, we exclusively utilized data from White Leghorn chickens. We added it to the Methods as below (Line 795-802):

“For the construction of the chicken single-cell atlas (Fig. 1b, Supplementary Fig.1a, b), we used data from two breeds to capture a broader spectrum of cell types in chickens. We prioritized White Leghorn chicken data for cross-species comparisons of common cell type similarity to minimize potential platform-specific biases (Fig.2a and the Supplementary Fig7a, b). If a specific cell type was absent in the White Leghorn dataset due to the lack of single-cell data in the corresponding tissue, data from the Xiangjia Black Phoenix chickens were used as a substitute. For all other results, we

exclusively utilized data from White Leghorn chickens.”

3. Definitions and Claims

a Line 182: The term “rapid evolution” is vague and should be explicitly defined, with criteria and examples.

Response: Thank you for your comments.

Among the 31 cell types conserved across chickens, humans, and turtles, we first computed the mean expression profile for each cell type, normalized as $\ln(\text{CPM}+1)$. Pairwise Spearman correlation coefficients were then calculated between chicken and human, as well as between chicken and turtle. For each cell type, the two correlation values (chicken-human and chicken-turtle) were averaged. These average similarity scores were subsequently ranked in order. The results indicated that erythrocytes exhibited the second-lowest cross-species expression similarity. We further investigated the differences in transcriptional patterns of erythrocytes. For instance, we identified 591 differentially expressed genes between chicken and human erythrocytes (Fig. 2f). The upregulated genes in chicken were enriched for transcription, translation, and cellular responses to viruses(Fig. 2g). For chicken and turtle erythrocytes, 976 genes showed differential expression (Fig. 2h). Genes upregulated in chicken were again enriched in transcription, translation, and cellular responses to viruses, consistent with results from the chicken-human comparison above (Fig. 2i). For example, genes related to interferon signaling and antigen presentation exhibited high expression in chicken erythrocytes (Fig. 2j), whereas they were expressed at low levels or were absent in human and turtle erythrocytes. We added to the Results as below (Line201-213):

“Some cell types, such as erythrocytes, showed lower similarity across amniotes, with the mean Spearman correlation coefficients between chicken vs. human and chicken vs. turtle ranking the second lowest among all conserved cell types. For instance, we identified 591 differentially expressed genes between chicken and human erythrocytes (Fig. 2f). The upregulated genes in chicken were enriched for transcription, translation, and cellular responses to viruses(Fig. 2g). For chicken and turtle erythrocytes, 976 genes showed differential expression (Fig. 2h). Genes upregulated in chicken were again enriched in transcription, translation, and cellular responses to viruses, consistent with results from the chicken-human comparison above (Fig. 2i). For example, genes related to interferon signaling and antigen presentation exhibited high expression in chicken erythrocytes (Fig. 2j), whereas they were expressed at low levels or were absent in human and turtle erythrocytes.”

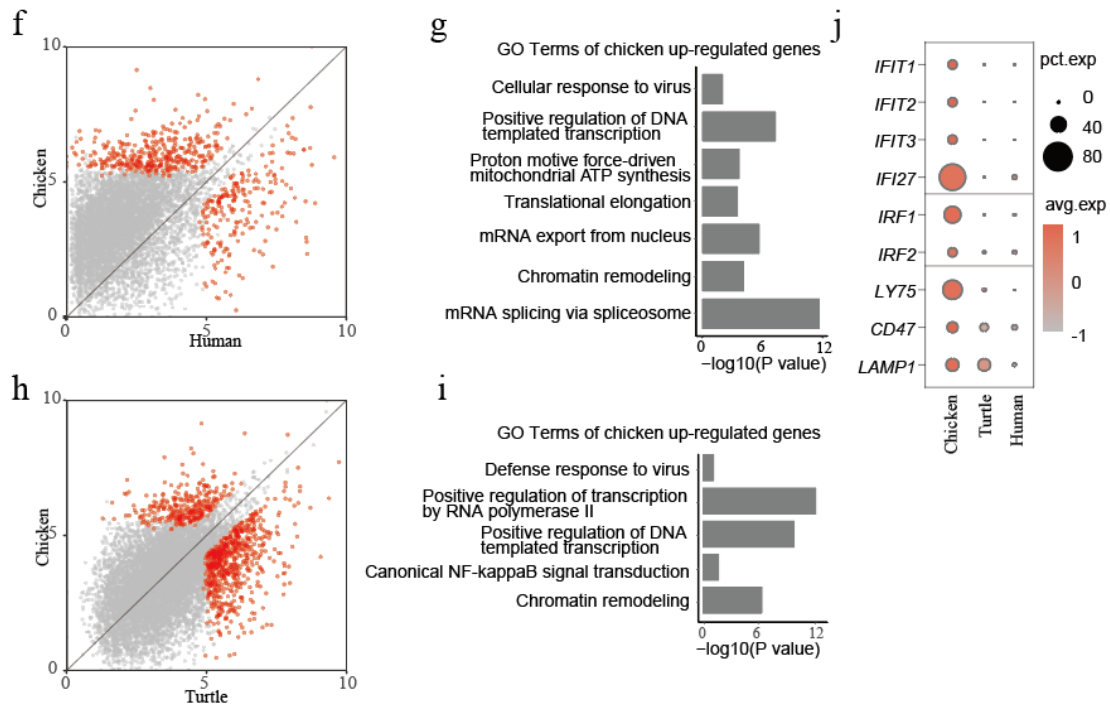


Fig. 2 f, h Scatter plot showing DEGs of erythrocytes between chicken and human(f) or turtle(h). **g, i** Gene ontology analysis of up-regulated genes in chicken erythrocytes compared to those of human(b) and turtle(d). **j** Dot plot showing the expression of interferon and antigen presentation-related genes in turtle, chicken and human erythrocytes.

b Lines 187–189: The arguments to conclude evolution to endothermy are unclear and this conclusion would be more appropriate in the discussion section.

Response: We have moved it to the discussion section and revised it as below (Line706-715):

“Erythrocytes are the most prevalent cells in blood. We observed that chicken erythrocytes, which are nucleated, express high levels of genes related to interferon signaling and antigen presentation. Studies showed that the maintenance of warmer core temperatures supports the rapid proliferation of potentially infectious organisms^{10 11}. The higher expression of these genes in the erythrocytes of endothermic chickens, compared to those of ectothermic turtles, might enhance their capacity for a swift immune response. In contrast to chickens, human mature erythrocytes lack a nucleus and organelles¹², thereby abolishing their transcriptional activity and likely precluding immune-related gene expression.”

4. Innate Lymphoid Cells (ILCs)

a Fig. 3A shows ILC2s in chicken but not in humans, which contradicts previous comparative studies (e.g., Vivier et al., Nat Immunol 2016, PMID 27328009). The discrepancy must be addressed in the discussion.

Response: We addressed in the discussion as below (Line681-689):

“Notably, certain cell type, such as ILC2, were absent from the human single-cell

dataset we downloaded, precluding their inclusion in our analysis. The absence of ILC2s in this dataset likely stems from their relatively low abundance in tissues, which leads to failed capture during scRNA-seq. Consistent with this, previous scRNA-seq studies have also failed to capture and identify ILC2s in human gut tissue and other immune tissues^{13, 14}. For such relatively rare cell types, strategies including the analysis of substantially larger cell numbers or flow cytometry-based sorting prior to sequencing may help mitigate these technical constraints.”

b Figs. 3B/C: Cell identities are not clearly labeled (chicken vs. human). Furthermore, comparative plots should avoid including cell types detected in only one species, as this prevents meaningful comparison.

Response: We have added labels in Fig. 3b/c. As suggested, we retained cell types shared in chicken and human for re-analysis. The results are presented below. Consistent with previous results, genes related to lipid and lipoprotein transport, and those involved in scavenging free heme for iron recycling, were expressed in chicken pDCs, whereas in humans these genes were primarily expressed in macrophages (Fig. 3b, c). Additionally, genes related to leukocyte migration and adhesion were expressed in chicken pDCs, while in humans their expression was predominantly observed in DC1 (Fig. 3b, c). We revised it in the Results.

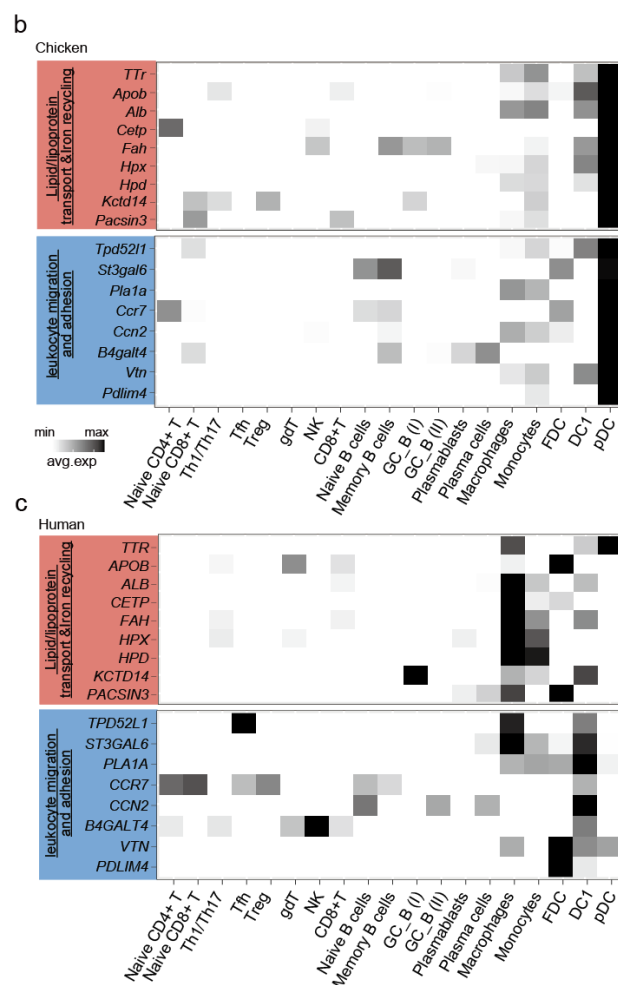


Fig. 3 **b, c** Heatmaps showing different expressions of genes involved in cell metabolism and migration in chicken and human immune cell types, respectively.

5. Figure 4

a Large and small B cells are not defined—please provide criteria.

Response: In previous research (PMID: 30442912), B cells isolated from the embryonic bursa were classified into two subsets via flow cytometry based on size: small B cells and large B cells. Large B cells exhibited greater proliferative activity than small B cells. Furthermore, compared to small B cells, large B cells showed higher mRNA expression of both proliferation-associated gene *Mki67* and the gene conversion-related gene *Aicda*. We annotated these B cell populations based on the distinct transcriptional profile characteristics of large and small B cells. We added it to the Result and further added *Mki67* and *Aicda* to the Fig4b as below (Line308-311):

“Here we analyzed 34,861 single cells from the embryonic bursa of Fabricius. A total of 11 cell types were identified(Fig. 4a). Large and small B cells were characterized by differential expressions of gene conversion-related gene Aicda and proliferation-associated gene Mki67 based on previous research(Fig. 4b)¹⁵.”

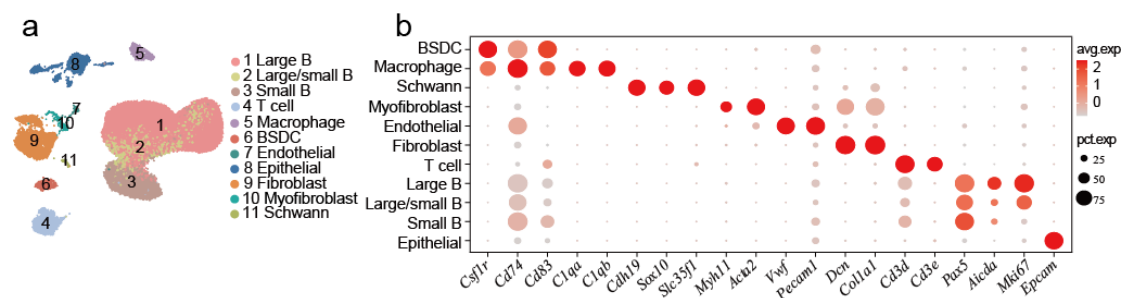


Fig. 4 **a** UMAP visualization of major cell types in the embryonic bursa of Fabricius in chicken. **b** Dot plot for expression of marker genes of the identified cell populations in the embryonic bursa of Fabricius in chicken.

b Fig 4I; This is an interpretation of the data and as such should be shown within the discussion section.

Response: We have moved it to the Discussion as below (Line749-754):

“However, avian FDCs were of hematopoietic origin, enabling their presence during embryonic hematopoiesis without mature B cells support. This early presence may offer two primary advantages for birds. First, it can assist B cell progenitors in migrating to the bursa of Fabricius. Second, it may enhance the efficiency of germinal center formation and antibody production in birds (Supplementary Fig. 16).”

6. Follicular Dendritic Cells (FDCs)

This section is particularly interesting, especially the suggestion of a hematopoietic origin in chicken. However:

a Line 368: A reference for the claimed human FDC origin is missing

Response: Thanks for your kind reminder. Our statement is inaccurate. In humans,

relevant investigations have indicated a close lineage relationship between fibroblasts and FDCs. For instance, FDCs share multiple markers with fibroblasts (PMID: 7797422). Additionally, fibroblast-like synoviocytes display intrinsic FDC-like properties, such as stable interactions with germinal center (GC) B cells and suppression of apoptosis (PMID: 10229832). These observations suggest a close lineage relationship between FDC and fibroblast. In mice, FDCs have been shown to differentiate from precursors of stromal cells (PMID: 22770220). The accessibility of genetic editing in mice facilitates more straightforward tracing of FDC origins. We incorporated additional references addressing human FDC origins into the Results as below (Line363-367):

“Human FDCs share multiple markers with fibroblasts¹⁶, and fibroblast-like synoviocytes display FDC-like properties, such as stable interactions with germinal center (GC) B cells and suppression of apoptosis¹⁷. These observations suggest a close lineage relationship between FDC and fibroblast. In mice, FDCs have been shown to differentiated from precursors of stromal cells¹⁸”

b In the discussion (lines 707-708) the references provided on the human FDC origin were mouse, not human.

Response: As mentioned above, in humans, previous studies have suggested a close relationship between fibroblasts and FDCs. We have corrected the reference in the revised manuscript (Lin748-749).

c Line 720; there are other publications from well suited to refer to the formation and presence of the FDC within mouse and human, such as PMID 18424165.

Response: We have updated the references in the manuscript as below (Line761-763):

“In humans, germinal centers appear in the first postnatal week to months¹⁹. In mice, germinal centers emerge in the submandibular and mesenteric lymph nodes at postnatal day 28²⁰.”

7. Data Interpretation and Clarity

a Fig. 6A: Selection criteria for cells/genes are unclear. CTSG should be defined (Cathepsin G), and justification provided for using it as a marker.

Response: According to the previous literature (PMID: 31730855), an undefined cluster was named with the genes highly expressed in this cluster. Given that the cell cluster highly expresses CTSG (the top-ranked highly expressed gene), we named it CTSG+ immune cell by referencing this method. CTSG has been defined. These were added to the Results in the manuscript as below (Line501-506):

“One cell cluster highly expressed the lymphocyte marker genes such as Cd3d and Cd4, as well as genes preferentially expressed in mammalian granulocytes or mast cells, such as Ctsg (Cathepsin G, the top-ranked highly expressed gene) (Fig. 6b). According to the previous literature²¹, an undefined cluster was named with the genes highly expressed in this cluster. Therefore, we named it CTSG+ immune cells (CTSG used as a marker).”

b Lines 534–535: The term “innovative gene expression profiles” is vague; please define.

Response: Thank you for pointing out our oversight. We have revised it to similar or different gene expression profiles in the manuscript as below (Line530-531):

“To examine T cells and innate lymphocytes with similar or different gene expression profiles in adult turtles, chickens, and humans, we integrated 16,998, 29,800, and 28,258 cells in turtles, chickens and humans, respectively.”

c Line 542: “CoRCs of T cells” is not explained earlier—please clarify.

Response: CoRCs means Core regulatory complexes. We have added an explanation where it first appears (Line 382).

d Lines 553–554: The comparison between human PBMCs and chicken tissues is not valid; this should be acknowledged as a limitation.

Response: Thank you for pointing that out. In our previous manuscript, we used the proportion of $\gamma\delta$ T cells in PBMCs to infer the proportion of IL-17A-producing $\gamma\delta$ T cells across human tissues. We updated our references with a study that analyzed ~360,000 human immune cells across multiple tissues and characterised $\gamma\delta$ T cell populations(PMID: 35549406). Notably, IL-17A was not expressed in the $\gamma\delta$ T cell populations, indicating that IL-17A-producing $\gamma\delta$ T cells represent a minimal subset in humans. We revised it on the Results as below (Line549-553):

“Although IL-17A-producing $\gamma\delta$ T cells exist in humans²², a cross-tissue scRNA-seq analysis of immune cells (~360,000 cells) revealed no expression of IL-17A in characterized $\gamma\delta$ T cell populations²³. These findings suggest that such cells represent a rare subset within the T cell compartment, whose low frequency may not be well represented in the human data we used.”

e Lines 585–591: The relationship of the 6.7% proportion to other comparisons is unclear.

Response: We apologize for the confusion. In the previous analysis, T cells derived from all tissues were pooled to determine $\gamma\delta$ T cell proportions relative to total T cells in chickens and humans, regardless of interspecies tissue conservation. This initial comparison demonstrated a 6.7% higher $\gamma\delta$ T cell proportion in chickens compared to humans. To reduce potential bias arising from tissues that are not shared between species, we subsequently restricted analyses to common tissues of both species. This refined analysis revealed that the proportion of $\gamma\delta$ T cells was 3.8% higher in chickens than in humans (15.4% vs. 11.6%). We have revised it in Results and Fig. 7a as below (Line583-584):

“Based on our data, the proportion of $\gamma\delta$ T cells was approximately 3.8% higher in chickens than in humans across the shared tissues(Fig. 7a).”

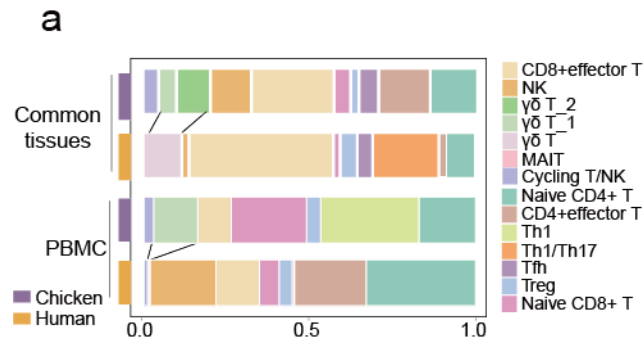


Fig. 7a Percentage frequency of T cell and innate lymphoid cell populations in adult chicken and human.

f Line 590: Define “substantive.”

Response: We have revised it in the manuscript as follows (Line589-590):

“This difference in the number of $\gamma\delta$ T cells and the effector genes they express between chickens and humans prompted us to explore the function and ontogeny of avian $\gamma\delta$ T cells.”

g. Spatial and Ligand-Receptor Analyses

h Lines 613–615 & Fig. 7D–E: The link between ligand-receptor pairs and cell identity is not convincingly demonstrated.

Response: In the single-cell data of cecal tonsils, $\gamma\delta$ T cells expressed IL17A, IL17F, ICOS, and TNFSF8(Fig. 7d); epithelial cells expressed IL17RA; and B cells expressed ICOSLG and TNFRSF8. We added it to the Results as below (Line609-613):

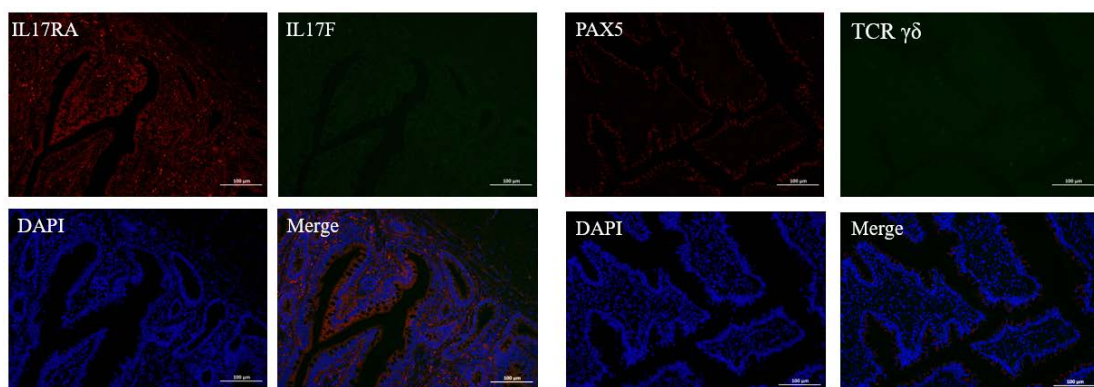
“To gain insight into the potential function of $\gamma\delta$ T cells, we examined their transcriptional profile and spatial distribution in chicken cecal tonsils, where immune cells accumulate. ScRNA-seq analysis revealed that $\gamma\delta$ T cells expressed IL17A, IL17F, ICOS, and TNFSF; epithelial cells expressed IL17RA, the receptor for IL17A and IL17F; and B cells expressed ICOSLG(ligand of ICOS) and TNFRSF8(ligand of TNFSF)(Fig. 7d).”



Fig. 7d Dot plot showing the expression of effector genes in cecal tonsil cells.

i Fig. 7D; Histological validation, as provided for other tissues in other figures, would be helpful to interpret the data shown.

Response: Thanks. To verify the interaction between $\gamma\delta$ T cells and B cells, as well as $\gamma\delta$ T cells and epithelial cells in the cecal tonsil, we attempted to perform immunofluorescence staining to confirm the interaction. More specifically, we purchased commercially available antibodies against $\gamma\delta$ T cell markers (e.g., IL17F, TCR gamma/delta) and epithelial cell-related markers (e.g., IL7RA), and B cell-related markers (e.g., PAX5). However, Chicken-specific antibodies are extremely limited. Researchers often rely on antibodies raised against human or mouse antigens. Among these, only a limited number are applicable for immunofluorescence. In our attempts, TCR $\gamma\delta$ and IL17F antibodies were unsuitable for immunofluorescence, and IL7RA antibodies produced nonspecific staining (Response Fig. below).



Response Fig. Immunofluorescence analysis of $\gamma\delta$ T cell, B cell and epithelial cell-related marker expression in chicken cecal tonsil.

j Interpretations in Fig. 7E are overstated.

Response: We have deleted this proposed model of $\gamma\delta$ T cell development.

k Lines 618–619: Statements appear assumptive without clear argument.

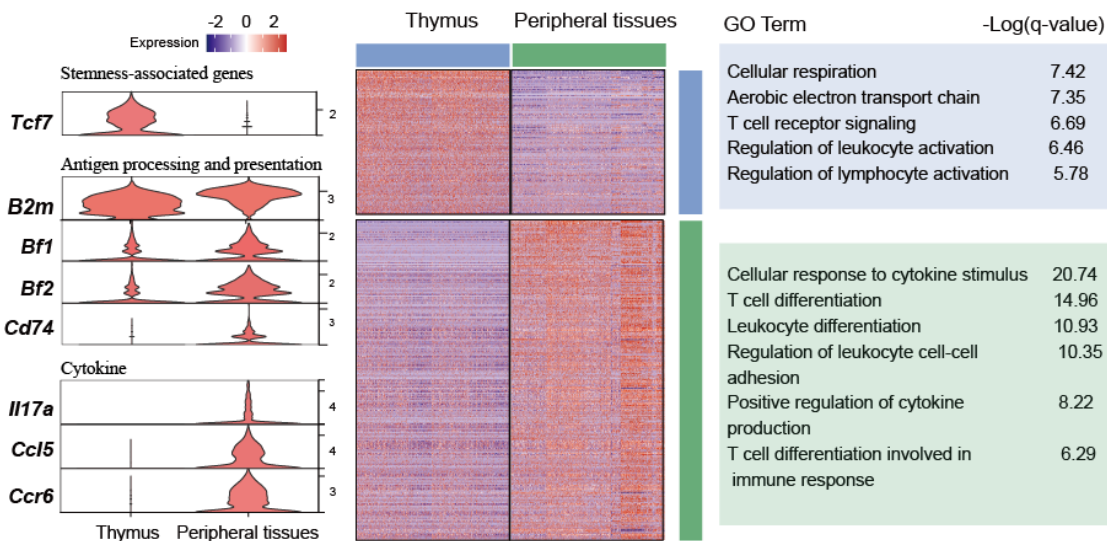
Response: Thank you for pointing out our inappropriate assumption. We deleted the following sentences:

“In mammals, IL17-expressing Th17 cells can function as B-cell helpers by not only triggering B cell proliferation but also promoting class-switch recombination. Interestingly, Th1/Th17 were approximately 23% lower in chicken intestine compared with those in human. Therefore, $\gamma\delta$ T cells express in IL-17 function similarly to Th17 cells to exert immune regulatory effects in chickens.”

l Lines 637–638: The concept of “gradual refinement” of cells needs mechanistic clarification—does this occur in situ, postnatally?

Response: Thank you for raising this valuable question. To investigate if the gradual refinement of immune function in $\gamma\delta$ T cells occurs in the periphery or the thymus, we compared the transcriptional profiles of thymic $\gamma\delta$ T cells and peripheral $\gamma\delta$ T₂ cells. Thymic $\gamma\delta$ T cells were enriched in stemness-associated genes, whereas their peripheral $\gamma\delta$ T₂ counterparts showed upregulation of genes related to antigen processing and presentation, as well as cytokine production (Supplementary Fig.15d). This pattern suggests that the adaptive immune features of $\gamma\delta$ T cells are largely acquired after they emigrate from the thymus. We added it to the Results as follows (Line635-637):

“Furthermore, genes associated with antigen processing and presentation and cytokine production were highly expressed in adult peripheral $\gamma\delta$ T₂ cells but not in thymic $\gamma\delta$ T(Supplementary Fig.15d). This indicated that the adaptive immune function of adult $\gamma\delta$ T cells was gradually refined after they emigrate from the thymus.”



Supplementary Fig.15d Genes highly expressed in $\gamma\delta$ T cells in the peripheral tissues and thymus and their enriched signalling pathways.

m Lines 643–649 & Fig. 7I: Conclusions based on expression of only two chemokine receptors are overreaching.

Response: We revised it as below (Line647-650):

“These findings suggested that the maturation and activation of $\gamma\delta$ T₁ cells accompany their egress from the thymus to seed peripheral tissues, where the local microenvironment shapes them into populations with distinct gene expression patterns.”

9. Terminology

a Line 701: Please clarify what is meant by “low evolutionary conservation.”

Response: Apologies for the confusion. It means that compared with adaptive immune cells, gene expression patterns of innate immune cells display low similarity during evolution. We revised it in the Discussion as below (Line740-741):

“Interestingly, compared with adaptive immune cells, gene expression patterns of innate immune cells display low similarity during evolution.”

b Chicken gene names should be annotated similarly as mouse, so first capital letter followed by small and at the gene in italics.

Response: Revised.

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REVIEWERS' COMMENTS

Reviewer #4 (Remarks to the Author):

I would like to thank the authors for the considerable effort they have made to address my comments. The majority of my points have been handled satisfactory. However, there remains one important issue concerning the overall focus of the manuscript that, in my view, has not yet been fully resolved. This concern is similar to the first comment raised by Reviewer #1, who also questioned the central focus of the study. Although the authors have responded to both comments, the revisions do not sufficiently clarify or adjust the manuscript's focus, and therefore this issue persists.

Specifically, it remains unclear why certain cell types are highlighted while others are not. For example, in Supplementary Figure 7, several cell types are presented, yet many additional populations appear equally interesting and relevant. I am missing a clear rationale for emphasizing PBMCs, smooth muscle cells, fibroblasts, ionocytes, adrenal cells, and others, while excluding, for instance, tuft cells or GABAergic neurons.

In addition, Supplementary Figure 7 shows notable differences among immune cell populations (e.g. plasmablasts), which are largely not discussed in the main text. As also noted by Reviewer #1, I do not object to the manuscript focusing either on immune or non-immune cell types. However, it is important that the authors clearly articulate the criteria used to select the highlighted cell populations. Are these cells chosen because they are the most divergent, the most conserved, the most biologically informative, or because they serve as quality controls for cross-species comparison? Without such clarification, the selection appears somewhat arbitrary. Clarifying this rationale would substantially strengthen the coherence and interpretability of the manuscript.

Response: We sincerely thank the reviewer for their valuable feedback and for recognizing the efforts made in our revisions.

About the focus of the manuscript on the immune system

Previous studies have identified substantial differences between the immune cells of chickens and humans, including the presence of bursal secretory dendritic cells unique to chickens and a higher proportion of $\gamma\delta$ T cells in chicken peripheral blood. These distinctions highlight the critical need to systematically characterize chicken immune cell types and their properties. Accordingly, we herein performed a systematic characterization of chicken immune cells, alongside a direct comparison with their human and turtle counterparts. We have added it to the Manuscript as below (Line 208):

“Previous studies have identified substantial differences between the immune cells of chickens and humans, including the presence of BSDCs unique to chickens and a higher proportion of $\gamma\delta$ T cells in chicken peripheral blood^{7,8}. These distinctions highlight the critical need to systematically characterize chicken immune cell types and their properties. Accordingly, we herein performed a systematic characterization of chicken immune cells, alongside a direct comparison with their human counterparts.”

About selection criteria for highlighted cell types in Supplementary Figure 7

Supplementary Figure 7 shows the similarity ranking of shared cell types between chicken and human, sorted by increasing similarity. Adrenal chromaffin cells exhibited

the lowest similarity among all shared cell types, which prompted us to focus on this cell type in the main text (Lines 165). In contrast, most other shared cell types, including plasmablasts, displayed significant similarity and thus were not further discussed in the main text.

A last, minor point, is that I would like to suggest to also add the classification of the large and small Bcells based on size measured by flowcytometry within the manuscript text, as the authors pointed out in the rebuttal letter.

Response: We added it to the Result as below(Line 274)

“Large and small B cells were designated according to previous studies³⁰, where they were classified by flow cytometry based on cell size. These two subsets were characterized by differential expressions of gene conversion-related gene Aicda and proliferation-associated gene Mki67.”