Establishing an RNA-sequencing approach to analyse the migration of B cells in the chicken embryo

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B lymphocytes have unquestionable importance in the immune system. In chickens, the B cell precursors initiate their development in the spleen and later migrate into the bursa of Fabricius via the blood, to become fully mature and diversified. However, the mechanisms and signals regulating such migration remain mostly unknown, as the methodology to isolate B cells in such early stages is challenging. In this work, a FACS-based methodology and RNA-sequencing analysis were established to isolate B cells from the crucial time points and locations of their development, starting at embryonic day 12 and from the spleen, bursa and blood. Previous studies identified the CXCR4/CXCL12 mechanism as involved in B cell migration into the bursa, yet it was considered not necessary. RNA-sequencing analysis performed in this study revealed seven mechanisms possibly involved in B cell migration. Two of these are implicated in negative regulation, and five in positive regulation of cell migration. Overall, the CXCR5 receptor and its ligand CXCL13 are proposed here as a necessary and sufficient mechanism for B cell migration, with their genes being highly expressed in the ED14 and ED16 bursal B cells. Further pathway enrichment analysis disclosed that the most prevalent pathways involved in B cell migration into the bursa were the cytokine-cytokine receptor interaction, MAPK and axon guidance. Overall, in this work, a protocol for B cell isolation and a pipeline for obtaining good results with further RNA-sequencing analysis were established.

Keywords: B lymphocytes, CXCR5/CXCL13, CXCR4/CXCL12, Chicken, Bursa, Migration, Spleen, Chemokines, RNA-sequencing, Transcriptomics

Introduction

The development of B cells in the chicken comprises different locations and mechanisms that differ from those in humans. It initiates at embryonic day (ED) 6 to 7 with the colonisation of the spleen by hematopoietic stem cells (HSC) from the intraembryonic mesenchyme [1]. Some HSCs undergo the first step of the V(D)J recombination processes and can become committed to the B cell lineage [2]. From ED8 until ED14, the B cell precursors exit the spleen and migrate via the blood circulation towards the bursa [3]. In this location, the lymphocytes finalise the V(D)J recombination events and undergo the process of gene conversion, becoming fully mature and diversified B cells. Later, the functional lymphocytes start to emigrate from the bursa to secondary lymphoid organs to exert their function [4].

Such intricate pathways of B cell migration from the spleen into the bursa require highly regulated processes. These have been studied to identify mechanisms necessary and sufficient for the B cells to migrate from the spleen into the bursa. So far, it is known that the expression of a B cell receptor (BCR) is not solely required for B cells to migrate into the bursa, as BCR^{neg} cells were seen inside the bursa undergoing gene conversion [5]. Besides, as in humans, the mechanism involving the receptor CXCR4 and the ligand CXCL12 are relevant for B cell migration, it was proposed that it could also have a crucial role in chickens [6]. Nevertheless, it was recently found that this process is BCR-dependent, so it is insufficient for cell translocation into the bursa [6,7]. BCR^{neg} B cells, which lack the CXCR4/CXCL12 mechanism, were still able to migrate into the bursa. Therefore, two other mechanisms were hypothesised as necessary, namely the CXCR5/CXCL13 and CCR6/ CCL20, as they were deeply involved in B cell migration in mice and humans [7]. However, no further studies have been made in this regard. Consequently, the signals that are both necessary and sufficient for B cell entrance into the bursa remain unknown.

To answer this concern, this work proposes a methodology to sort B cells and analyse them by RNA-sequencing. In more detail, a Fluorescence associated cell sorting (FACS) based methodology was established for isolating B cells in the early time points and locations of development, namely ED12, ED14 and ED16, from the spleen, bursa and blood. Next, the RNA-sequencing analysis was performed, and signals involved in cell migration were assessed. Seven mechanisms that probably participate in B cell migration were identified.

Overall in this study, a methodology to isolate B cells in the early stages of development was established, and an RNA-sequencing analysis was performed to evaluate the differentially expressed genes (DEGs) between the samples. The RNA-sequencing analysis revealed that the CXCR5/CXCL13 mechanism may be necessary and sufficient for B cell migration towards the bursa.

Materials and Methods

Animals

Wild-type white leghorn chickens were bred and maintained under specific-pathogen-free (SPF) conditions in the animal facility at the Technical University of Munich (School of Life Sciences, Weihenstephan, Freising, Germany). Chickens were kept and provided free water access and a standard chicken diet *ad libitum*. Fertilised eggs were incubated at 37.8 ºC and 55 % humidity and rocked three times per day.

Isolation of embryonic spleen and bursa

The eggs were opened at ED12, ED14 and ED16, and the embryos were euthanised by decapitation. Spleen and bursa were harvested, and per every 5 embryos, organs were disaggregated through a 40 µm nylon cell strainer in a Petri dish and then pooled in one single cell suspension.

In ovo Blood Collection

Blood collection was performed on ED12, ED14 and ED16. The maximum volume possible to be retrieved from a single embryo varies with age. The anticoagulant was prepared by diluting Heparin 1:100 in RPMI, and for every 50 embryos, 1 mL of this solution was divided to coat

all syringes. Blood was extracted resorting to 1 mL syringes (MarMed, I010033) with 27G needles (MarMed, I023001), and was placed inside EDTA tubes (NC9990563, Fisher Scientific) to avoid coagulation. The blood was pooled in a 15 mL Falcon® tube and diluted 1:1 with ice-cold PBS.

Mononuclear Leucocytes Isolation

A density gradient centrifugation with a 1:1 volume of Histopaque® 1077 Hybrid-Max medium (H8889-100 mL, Sigma-Aldrich®) was performed on the spleen, bursa and blood cell suspensions. The cell solution was layered on top and centrifuged at 650 *x g* for 12 min at 4 ºC. The (white) cell layer was collected from the interface and transferred into a FACS tube with 1 mL PBS+BSA. The cells were washed 222 *x g* for 5 min at 4 ºC, and 2 mL of Fluo-Buffer (500 mL PBS, 50 mg NaN_3 (#A1430, AppliChem), 5 g Albumin Fraction V (#A1391, AppliChem)) was added.

Cell Counting

Cell counting was performed using a Neubauer chamber (0.1 mm, 0.0025 m^3, Paul Marienfeld GmbH & Co.KG) and an inverted light microscope with phase contrast (Labovert FS/Biomed, Leica Microsystems). Live cells were counted in a 1:10 dilution of Trypan Blue and concentrated to $5x10^6$ cells/mL with Fluo-Buffer.

MACS and FACS analysis

The magnetic associated cell sorting (MACS) procedure followed the Miltenyi Biotec for Streptavidin MicroBeads cell separation protocol [8]. Cell suspensions of 1×10^6 cells each were plated and stained with the following antibodies for MACS: Mouse Anti-Chicken Monoclonal Bu-1-Biotin-Antibody (1:200) (#MA5-28700, Thermo Fisher Scientific) or Mouse Anti-Chicken Monoclonal Bu-1-FITC-Antibody (1:200) (#MA5-28701, Thermo Fisher Scientific) or Mouse Anti-Chicken Monoclonal Bu-1-AF647-Antibody (1:200)(#8395-1, Southern Biotech). For the isolation of the antibodies, the following microbeads were used: Streptavidin MicroBeads (1:100) (#130-048-102, Miltenyi Biotec), Anti-FITC MicroBeads (1:100) (#130-048-701, Miltenyi Biotec), Anti-Cy5/Anti-Alexa Fluor 647 MicroBeads (1:100) (#130-091-395, Miltenyi Biotec). For both MACS and FACS, the cell suspensions were centrifuged at 700 *x g* for 1 min at 4 °C. and live/dead staining was performed with the Fixable Viability Dye eFluor™ 780-staining (1:1000 dilution) (#65-0865-14, Invitrogen™). The cells stained with the Bu-1-Biotin-Antibody and the samples for FACS were additionally stained with Bu-1-FITC-Antibody (1:200 dilution) (#MA5-28701, Thermo Fisher Scientific). Flow Cytometry analysis of MACS sorted B cells were performed with the Invitrogen Attune NxT Flow Cytometer. FACS cell sorting of B cells was performed with the CytoFLEX SRT Cell Sorter, and cells were directly sorted in Lysis Buffer (ReliaPrep™ RNA Cell Miniprep kit (#Z6010, Promega Corporation) for RNA extraction. Data analysis was performed using the FlowJo™ v10.8 Software (BD Life Sciences) [9].

RNA Extraction

RNA was extracted with the ReliaPrep™ RNA Cell Miniprep kit (#Z6010, Promega Corporation). The total RNA was analysed using the Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Pico Kit. Only RNA samples with a minimum RNA integrity number (RIN) of 8.0 were used for further Next-Generation sequencing (NGS) analysis.

cDNA Synthesis, Library Preparation and Illumina Next-Generation Sequencing

The cDNA synthesis and library preparation were performed by Christine Wurmser (TU München, Freising, Germany), and the Illumina Next-Generation Sequencing by the IMGM laboratories (Martinsried, Germany). The cDNA synthesis from total RNA and the library preparation were performed according to the SMART-Seq® v4 PLUS Kit from Takara Bio USA, Inc., following the manufacturer's instructions. cDNA quality and quantity were determined with the Agilent 2100 Bioanalyzer and Agilent's High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). The library pool was quantified with the highly sensitive fluorescent dye-based Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific) and the NEBNext® Library Quant Kit for Illumina (New England Biolabs). A quantitative PCR (qPCR) was used to determine the concentration of the library on the ViiA7™ Real-Time PCR System (Thermo Fisher Scientific). All quantification steps were carried out according to the manufacturer´s instructions. The High Sensitivity DNA Kit (Agilent Technologies) 2100 Bioanalyzer (Agilent Technologies) was employed for the quality. Library sequencing was performed on the Illumina NovaSeq® 6000 SP Next-Generation Sequencing system in XP mode in 100-cycle run, with single-read chemistry, a read length of 1 x 100 bp, 25 M reads per sample and 400 M reads in total [10]. Raw data were quality-controlled, demultiplexed and transferred without further processing. Technical quality parameters were evaluated with the SAV software, namely the percentage of Phred score (Q), where Illumina classifies this sequencing run type as good quality when Q30 bases ≥ 85 % [10].

RNA-Sequencing Analysis

RNA-sequencing analyses were run with the default settings of the DNASTAR Lasergene Genomics® [11] package. In the SeqMan NGen® [11] software, the assembly was performed against the annotated chicken genome GRCg6a (GCA_000002315.5) downloaded from NCBI [12]. The normalisation method utilised was Reads Per Kilobase Million (RPKM), as in the experimental setup no samples could be defined as control. The assembly results were subsequently analysed in the ArrayStar® [11] software. Replicates were evaluated by performing a Pearson correlation test within the software, where R^2 values were calculated for each replicate comparison, and a heat map was constructed with GraphPad Prism [13] to visualise these results. In the differentially DEGs analysis, gene lists were created based on a gene expression fold change of 10, up and down, between samples. The DEGs were grouped based on their gene ontology (GO) terms, and the "Locomotion" term was selected and new gene sets of interest were created. This term was selected to include the genes involved in the migration of the B cells, in line with the scope of this project. To visualise the data, heat maps in ArrayStar® [11] and protein-protein interaction (PPI) network analysis with the STRING® [14] tool were performed, with default settings and selecting *gallus gallus* as the target organism. Additionally, with the STRING® [14] tool, a pathway enrichment analysis was performed.

Results and Discussion

MACS vs FACS for B cell isolation

To evaluate if the MACS technique to isolate B cells was successful, three pairs of antibodies and beads were tested. However, none resulted in an enrichment of B cells in both the spleen and bursa, and most of the cells died (data now shown). FACS based cell sorting was then tested, and it yielded much better results in sorting cells. One representative example of these assays is in **Figure 1.** The percentage of Bu-1$^+$ cells in the spleen was 88.10 %, and in the bursa, 74.00 % after sorting. The value of live cells after FACS in the splenic sample was 55.60 % and in the bursal cells was 18.90 %. Henceforth, FACS was the sorting method selected to isolate B cells from all the locations and time points.

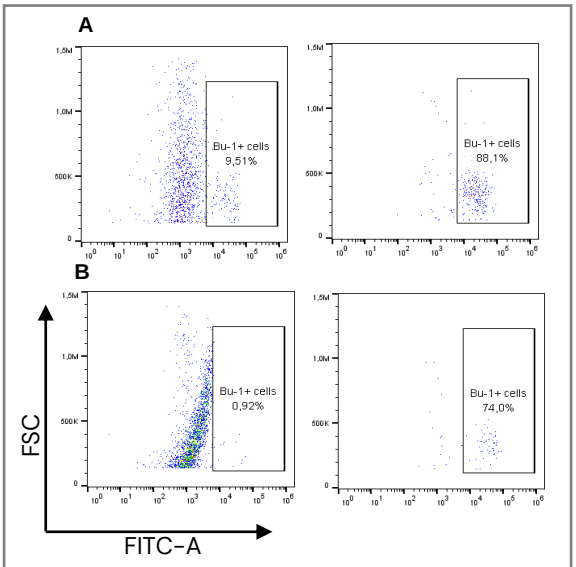

Figure 1. FACS data of Bu-1$^+$ cells from an ED13 splenic (A) and bursal sample (B). Organs from 15 embryos in ED13 were utilised. The B cells were stained with Bu-1-FITC and Fixable Viability Dye eFluor™ 780. **A.** Percentage of Bu-1$^+$ cells in the spleen before (9.5 %) and after FACS (88.1 %). **B.** Percentage of Bu-1$^+$ cells in the bursa before (0.9 %) and after FACS (74.0 %). The Figures were obtained with FlowJo™ v10.8 Software (BD Life Sciences) [9].

Migration of embryonic B cells may occur at later time points

The splenic, bursal and blood B cells in ED12, ED14 and ED16, were stained with Bu-1-FITC antibody and detected in the CytoFLEX SRT Cell Sorter. Regarding ED12, the percentage of Bu-1$^+$ cells was highest in the bursa (8.16 % ± 1.12) when compared to the spleen (9.35 % ± 1.87) and blood (0.73 % ± 0.28) **(Figure 2)**. In ED14, the percentage of Bu-1$^+$ cells increased in the spleen (11.61 % ± 3.21), bursa (15.9 % ± 5.96) and blood (1.28 % ± 0.46), being the highest still in the bursa. Lastly, in ED16, the percentage of Bu-1$^+$ cells decreased in the spleen (3.81 % ± 2.70) and blood (0.68 % ± 0.06) and was higher in the bursa (37.03 % ± 7.41). In previous studies, it was seen a decrease in the percentage of B cells in the spleen from ED12, detecting that cell migration towards the bursa occurred between ED8-14 [3,6,7]. However, the results obtained in this project indicate that the migration of the lymphocytes from the spleen towards the bursa occurs later than ED12, as the percentage of Bu-1$^+$ cells increased from ED12 to ED14 in the spleen. Additionally, the Bu-1$^+$ cell percentage decreased heavily in the spleen after ED14 and parallelly increased intensely in the bursa **(Figure 2)**. Overall, these results suggest that the migration of B cells to the bursa occurs until in a later time point, and more intensely after ED14.

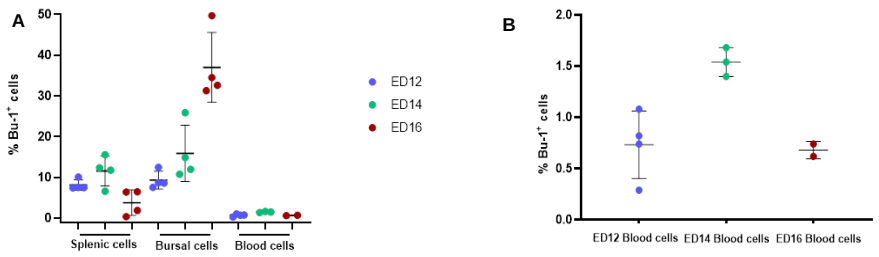

Figure 2. Summary of the migration of B cells in their developmental stages. A. Percentage of Bu-1^{+} cells in the spleen, bursa and blood for the time points ED12 (blue), ED14 (green) and ED16 (red). **B.** Amplification of the data from the blood represented in A. Each dot corresponds to one experiment, for each location and time point at least three replicates were obtained. The data was plotted in a scatter plot with the mean, the error bars correspond to the standard deviation (SD). This figure was designed with GraphPad Prism [13].

DEGs analysis with PPI networks and pathways enrichment

Replicate similarities evaluated with the Pearson correlation test revealed that all replicates clustered among the expected group, except for one from ED16 bursal B cells, thus was excluded. From the PPI networks and pathway enrichment analysis performed with STRING® [14], three most pathways were seen as the most prevalent, namely the cytokine-cytokine receptor interactions, MAPK signalling and axon guidance **(Figure 3)**. These pathways were present in all the PPI networks in the multiple comparisons performed (data not shown).

MAPK signalling molecules activate kinases (JNK, p38 and ERK) involved in cell migration

The MAPK includes three kinases relevant to cell migration, namely Jun N-terminus kinase (JNK), p38 and Extracellular signal-regulated kinases (ERK), and each one regulates cell migration by different mechanisms [15]. The most expressed genes in the three time points were considered more pertinent for B cell migration and were selected. These were FTL1, FGF10, PDGFRA, VEGFC, KIT, EFNA5 PGF, TGFB and IL-1β. Additionally, most were not expressed in the ED16 bursa, which could indicate that they were needed only for the migration until this location. Henceforth, these molecules might pose new signals for B cell migration.

The growth factors (TNF, EGF, PDGF, TGF-β, PDGFRA, PGF and VEGFC) are part of the stimuli that activate the kinase JNK, which is

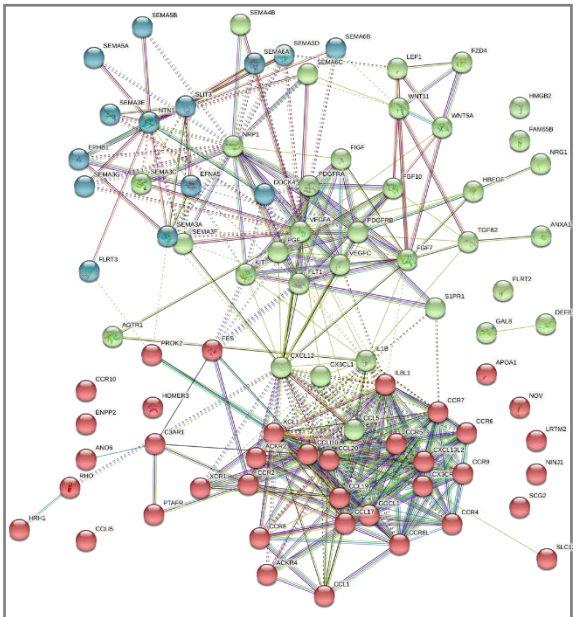

Figure 3. Protein-Protein Interaction Network of the DEGs from in all comparisons. The list of DEGs in all comparisons was merged and subjected to a PPI analysis with STRING®. The cytokine receptor interactions (red), MAPK signalling (green) and axon guidance (blue) were the most prevalent pathways. The edges represent the protein-protein associations, the known interactions (light blue and purple), the predicted interactions (green, orange, blue) and others (yellow, black, violet). A kmeans clustering was performed with the creation of 3 clusters shown by the dotted line between the proteins.

involved in increased cell migration, as its targets are mostly proteins engaged in cell migration [15,16]. Another kinase involved in cell motility, the p38, is activated by the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), FGF10 and IL-1β, PDGF and TNF. Finally, regarding KIT and EFNA5, these do not appear as activators of the JNK or p38 kinases. KIT codifies a receptor tyrosine kinase involved in the intracellular signalling of neural stem cell migration, and its activation leads to the stimulation of the ERK cascade, which engages in cell migration [17]. The EFNA5 gene encodes for the ephrin-A5 ligand, which binds to an ephrin receptor tyrosine kinase resulting in MAPK pathway activation, thus, is involved in cell mobility [18]. Hence the genes involved in the MAPK mostly activate kinases (JNK, p38 and ERK) related to cell migration.

Axon guidance genes probably regulate chemokine signalling of B cell homing

This group of molecules was analysed as they pose an exciting opportunity to identify novel mechanisms important in the migration of other cell types, such as axons and neural cells [7]. The SEMA5A, SEMA3F, SEMA6B and SLIT3 were the genes with the highest expression in the crucial time points for cell migration (ED12, ED14 spleen and ED14, ED16 bursa). SEMA5A, SEMA3F, and SEMA6B codify for semaphorins and are enrolled in regulating neuronal migration, immune responses, tumour growth and angiogenesis [19]. In more detail, SEMA5A was seen to elicit endothelial cell proliferation and reduce apoptosis [20]. SEMA3F was shown to inhibit the migration of the thymocytes towards the chemokine CXCL12 [21]. SEMA6B has a role in cell viability, migration and invasiveness in cancer cell lines, via the Notch signalling pathway [22]. Concerning SLIT3, it has been reported to inhibit the chemoattractant-induced migration of various cell types, such as leucocytes. Nevertheless, a more recent study in monocytes showed that SLIT3 stimulated cell migration via chemoattractants, such as CXCL12 [23]. Hence, molecules involved in the axon guidance signalling expressed in the early stages of B cell development mostly regulate their migration by stimulating or inhibiting chemokine signalling.

Cytokine receptor interaction analysis showed that the CXCR5/CXCL13 mechanism might be critical for B cell migration to the bursa

For this pathway, all the genes involved in chemotaxis signalling were selected, not only the DEGs, to perform a broader comparison. From this analysis, seven mechanisms were up regulated and perhaps relevant in the early stages of B cell development **(Figure 4)**. Four were classified as involved in the positive regulation of B cell movement, yet probably not necessary. It is the case for the CXCR4/CXCL12, CCR6/CCL20, CCR7/CCL21/CCL19 and CCR9/CCL25, as deletions of the ligands or receptors were reported to still allow the lymphocytes to enter the lymph nodes in mice, or human [6,24–26]. Concerning, CXCR4 and CXCL12, where the receptor is highly expressed throughout all stages, while the ligand is mainly expressed in the spleen **(Figure 4)**. Recent studies showed that $CXCR4^{pos}$ B cells migrate towards the $CXCL12^{pos}$ bursa, yet $CXCR4^{neg}$ cells can still migrate inside the bursa [6]. The expression of CXCL12 mostly in the spleen instead of the bursa can occur as the CXCL12-producing bursal stromal cells were not sorted [7]. Hence the expression of the ligand was only

visible in the spleen, which probably arises from B cells or macrophages. Besides this, the signal concerning CCR6/CCL20, which was hypothesised in previous studies as necessary for B cell migration, is not highly expressed in the time points assessed [7]. Additionally, studies indicate that it is mostly involved in the migration of cells inside the lymph nodes, and in the secondary immune responses [24].

On the other hand, two mechanisms were classified as probably involved in the negative regulation of B cell locomotion, namely the CCR2/CCL2 and the CCR5/CCL5/CCL4/CCL3 mechanisms [27,28].

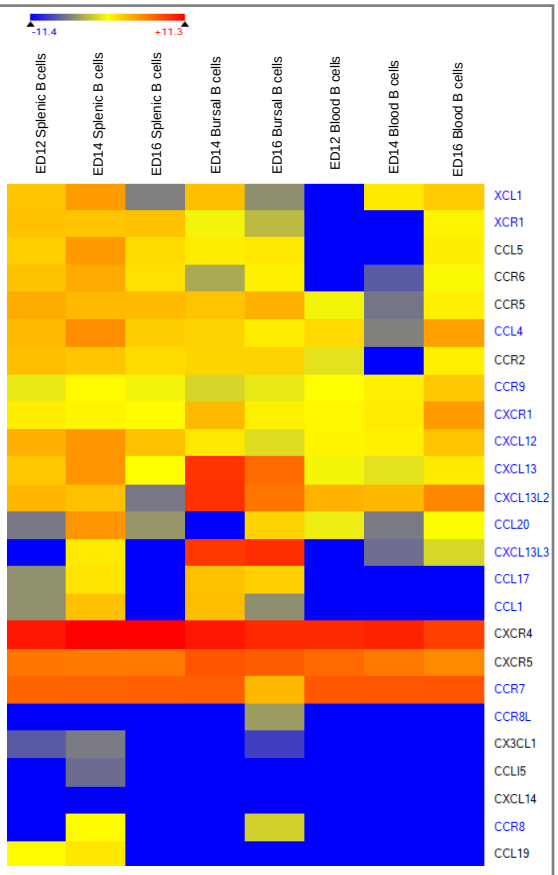

Figure 4. Heat map of the genes involved in cytokine-cytokine interaction. The genes involved in the cytokine pathway were selected and plotted in a heat map to compare the expression between the samples. In blue are the low expressed genes, in yellow the medium expressed and in red the highly expressed. This figure was obtained with ArrayStar® [11].

Lastly, one mechanism stood out from the remaining. The receptor CXCR5 and the ligand CXCL13 were suggested to be both necessary and sufficient for B cell migration. The receptor was highly expressed throughout all stages, and the ligand in the ED14 and ED16 bursal B cells, which is concordant with the crucial time points of B cell migration **(Figure 4)**. In mice, $CXCR5^{pos}$ B cells migrated towards CXCL13, and in $CXCR5^{neg}$ animals, most B cells failed to enter the lymph nodes and Peyer's patches. Moreover, these animals had barely any lymph nodes [29–31]. Henceforth, these results suggest that CXCR5/CXCL13 may be a necessary and sufficient mechanism for B cell migration into the bursa, even if in murine it was not sufficient, as still some B cells migrated.

Conclusion

Until now, it was challenging to investigate how B cells migrate, as no methodology to isolate the cells in the early stages of development was defined. In this study, a FACS based cell sorting methodology was established to sort B cells in ages as early as ED12 from the spleen, bursa, and blood. So far, no study combined these three samples (spleen, bursa and blood) from the same embryo. Additionally, RNA-sequencing analysis was performed to assess which molecules may be relevant for cell movement into the bursa. The mechanisms CXCR4/CXCL12, CCR6/CCL20, CCR7/CCL21/CCL19 and CCR9/CCL25 were highlighted in this work as probably involved in the B cell migration, yet these are most likely not sufficient. However, the receptor CXCR5 expressed by B cells, and the ligand CXCL13 present in the bursa, were identified as a mechanism that may be both necessary and sufficient for cells to migrate.

Additionally, multiple pathways deeply involved in the regulation of the migration were underlined, and the cytokine receptor interaction, MAPK pathway and axon guidance stood out. To fully evaluate and confirm if the mechanisms proposed in this work are involved, and if the CXCR5/ CXCL13 is sufficient for the B cell migration, chemotaxis essays would need to be performed. It could be by either knocking out the cytokine receptors or treating the cells with a receptor antagonist and assessing if cells still migrate towards the bursa. In conclusion, this project has contributed with a defined protocol to isolate B cells and, besides, allowed to enlarge the knowledge regarding the early stages of the immune system development in chickens, and the RNA-sequencing data obtained in this work facilitate further studies in avian B cell development.

References

1. Lassila O, Eskola J, Toivanen P. Prebursal Stem Cells in the Intraembryonic Mesenchyme of the Chick Embryo at 7 Days of Incubation. *The Journal of Immunology*. 1979;123(5).
2. Reynaud CA, Imhof BA, Anquez V, Weill JC. Emergence of committed B lymphoid progenitors in the developing chicken embryo. *EMBO J*. 1992;11(12):4349-4358. doi:10.1002/J.1460-2075.1992.TB05534.X
3. Kaspers B, Schat KA (Karel A, Göbel TW, Vervelde L. *Avian Immunology*. 3rd ed.; 2022.
4. Mastellera EL, Pharra GT, Funk' PE, Thompson CB. *Avian B Cell Development*. Vol 15.; 1997.
5. Schusser B, Collarini EJ, Yi H, et al. Immunoglobulin knockout chickens via efficient homologous recombination in primordial germ cells. *Proc Natl Acad Sci U S A*. 2013;110(50):20170-20175. doi:10.1073/PNAS.1317106110
6. Laparidou M, Schlickenrieder A, Thoma T, Lengyel K, Schusser B. Blocking of the CXCR4-CXCL12 Interaction Inhibits the Migration of Chicken B Cells Into the Bursa of Fabricius. *Front Immunol*. 2020;10:3057. doi:10.3389/FIMMU.2019.03057/BIBTEX
7. Nagy N, Busalt F, Halasy V, et al. In and Out of the Bursa—The Role of CXCR4 in Chicken B Cell Development. *Front Immunol*. 2020;11:1468. doi:10.3389/FIMMU.2020.01468/BIBTEX
8. *Streptavidin MicroBeads*. Accessed January 7, 2022. www.miltenyibiotec.com/
9. FlowJo™ Software (for Windows) Version 10.8. Becton, Dickinson and Company; 2021. .
10. Illumina. NovaSeq 6000 Sequencing System. Accessed October 12, 2022. www.illumina.com.
11. Lasergene Genomics®. Version 17.3.3. DNASTAR. Madison, WI.
12. National Center for Biotechnology Information (NCBI)[Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [1988] – [cited 2022 Jun-Sep]. Available from: https://www.ncbi.nlm.nih.gov/.
13. GraphPad Prism version 9.3.0 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.
14. Szklarczyk D, Gable AL, Nastou KC, et al. The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Res*. 2021;49(D1):D605-D612. doi:10.1093/NAR/GKAA1074
15. Huang C, Jacobson K, Schaller MD. MAP kinases and cell migration. *J Cell Sci*. 2004;117(Pt 20):4619-4628. doi:10.1242/JCS.01481
16. Yu J, Moon A, Kim HRC. Both platelet-derived growth factor receptor (PDGFR)-α and PDGFR-β promote murine fibroblast cell migration. *Biochem Biophys Res Commun*. 2001;282(3):697-700. doi:10.1006/BBRC.2001.4622
17. Lam LPY, Chow RYK, Berger SA. A transforming mutation enhances the activity of

the c-Kit soluble tyrosine kinase domain. *Biochemical Journal*. 1999;338(Pt 1):131. doi:10.1042/0264-6021:3380131

18. Davy A, Robbins SM. Ephrin-A5 modulates cell adhesion and morphology in an integrin-dependent manner. *EMBO J*. 2000;19(20):5396. doi:10.1093/EMBOJ/19.20.5396

19. Ko PH, Lenka G, Chen YA, et al. Semaphorin 5A suppresses the proliferation and migration of lung adenocarcinoma cells. *Int J Oncol*. 2020;56(1):165. doi:10.3892/IJO.2019.4932

20. Saxena S, Hayashi Y, Wu L, et al. Pathological and functional significance of Semaphorin-5A in pancreatic cancer progression and metastasis. *Oncotarget*. 2018;9(5):5931-5943. doi:10.18632/ONCOTARGET.23644

21. Mendes-da-Cruz DA, Brignier AC, Asnafi V, et al. Semaphorin 3F and Neuropilin-2 Control the Migration of Human T-Cell Precursors. *PLoS One*. 2014;9(7):103405. doi:10.1371/JOURNAL.PONE.0103405

22. Lv XJ, Chen X, Wang Y, Yu S, Pang L, Huang C. Aberrant expression of semaphorin 6B affects cell phenotypes in thyroid carcinoma by activating the Notch signalling pathway. *Endokrynol Pol*. 2021;72(1):29-36. doi:10.5603/EP.A2020.0072

23. Geutskens SB, Hordijk PL, Hennik PB van. The Chemorepellent Slit3 Promotes Monocyte Migration. *The Journal of Immunology*. 2010;185(12):7691-7698. doi:10.4049/JIMMUNOL.0903898

24. Noelle Samuel RO, Hess H, Lord GM, et al. CCR6-Dependent Positioning of Memory B Cells Is Essentialfor Their Ability To Mount a Recall Response to Antigen. Published online 2022. doi:10.4049/jimmunol.1401553

25. Pedro Pereira J, Kelly LM, Cyster JG. Finding the right niche: B-cell migration in the early phases of T-dependent antibody responses. *Int Immunol*. 22(6):413-419. doi:10.1093/intimm/dxq047

26. Wurbel MA, Malissen M, Guy-Grand D, et al. Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor γδ+ gut intraepithelial lymphocytes. *Blood*. 2001;98(9):2626-2632. doi:10.1182/BLOOD.V98.9.2626

27. Flaishon L, Becker-Herman S, Hart G, Levo Y, Kuziel WA, Shachar I. Expression of the chemokine receptor CCR2 on immature B cells negatively regulates their cytoskeletal rearrangement and migration. *Blood*. 2004;104(4):933-941. doi:10.1182/BLOOD-2003-11-4013

28. Honczarenko M, Le Y, Glodek AM, et al. CCR5-binding chemokines modulate CXCL12 (SDF-1)-induced responses of progenitor B cells in human bone marrow through heterologous desensitization of the CXCR4 chemokine receptor. *Blood*. 2002;100(7):2321-2329. doi:10.1182/BLOOD-2002-01-0248

29. Ansel KM, Ngo VN, Hyman PL, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature*. 2000;406(6793):309-314. doi:10.1038/35018581

30. Förster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell*. 1996;87(6):1037-1047. doi:10.1016/S0092-8674(00)81798-5

31. Müller G, Lipp M. Shaping up adaptive immunity: The impact of CCR7 and CXCR5 on lymphocyte trafficking. *Microcirculation*. 2003;10(3-4):325-334. doi:10.1038/SJ.MN.7800197