The influence of embryonic testosterone treatment on bursal mesenchymal environment

M.Sc. thesis

Molecular Genetics, Cell-and Developmental Biology specialization

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Contents

Abbreviations

1. TP	Testosterone propionate
2. BF	bursa of Fabricius
3. PFA	paraformaldehyde
4. PBS	phosphate-buffered saline
5. BSA	Bovine Serum Albumin
6. ECM	Extracellular matrix
7. SPF	Specific-pathogen-free
8. ABC	Avidin-biotin peroxidase complex
9. FAE	follicle-associated epithelium
10. E	embryonic
11. TB	tail bud
12. IFE	inter-follicular epithelium
13. SDC	secretory dendritic cell
14. VIF	vimentin-intermediate filaments
15. BSDC	bursal secretory dendritic cell
16. HG	hindgut
17. ED	embryonic day

I. Introduction

The immune system is a complex network of lympho-myeloid organs and cells that play crucial role to distinguish the „self" molecules from harmful „nonself" antigens and eliminate those that could make you ill. In contrast to the innate immunity, the adaptive immune system is able to provide long-lasting defense against different antigens maintained by B and T lymphocytes. Starting from early '60s it is widely accepted, that there are two types of adaptive immune responses: the humoral mediated responses involving antibodies and cell mediated responses. This dogma was strongly supported by immunological research conducted in avian experimental model system that contributed to the establishment of the main fundamental immunological concept: the antibody mediated immune response is carried out by bursa of Fabricius (B)-dependent lymphocytes (bone marrow is the bursa-equivalent organ in mammals), while in cell mediated response thymus (T)-dependent lymphocytes are involved. Since the discovery the role of chicken bursa of Fabricius as the primary lymphoid organ the avians become one of the most important model organisms for immunological studies.

In contrast to most of the tetrapods (mammals, amphibians and reptiles) where the bone marrow is the main site of B-lymphocyte development, in birds, the bursa of Fabricius is exclusively responsible for the development of B-lymphocytes. Inside the bursa of Fabricius, the maturation of B-lymphocytes takes place in lymphoepithelial follicles, the functional unit of the bursa, in close contact with the lining epithelium of the bursa lumen. Treatment of the avian embryo with testosterone propionate interferes with the normal ontogenesis of the bursal lymphoid tissue, resulting in functional bursectomy and immunosuppression. However, it is still not clear how the testosterone treatment influences the stromal compartment, namely the mesenchyme or the epithelial cells of the bursa of Fabricius. The proper humoral immune response of birds depends on the normal differentiation and function of bursal microenvironment; therefore, it is important to understand the complexity and reveal the development of the bursa specific microenvironment.

During the last two decades in the Laboratory of Stem Cells and Experimental Embryology at Semmelweis University, embryo manipulation methods combined with immunohistochemical techniques were extensively used to explore the normal and pathological development of the avian lymphoid organs. In the present study, I have examined the pattern of extracellular matrix differentiation in developing chicken bursa of Fabricius during the testosterone-induced hormonal bursectomy.

II. Overview of the literature

1. Structure of the bursa of Fabricius

The bursa of Fabricius (BF) – also known as bursa cloacalis – was first described by Hieronymus Fabricius ab Aquapendente in the 17th century, as a cloaca associated, blind ending sac, first thought to be responsible for storing sperm cells in birds. The main function of the organ was discovered centuries later, by Bruce Glick and Timothy Chang, who described the bursa as an essential site for antibody production and the generation of immunological response (Glick, 1956; Ribatti, 2010).

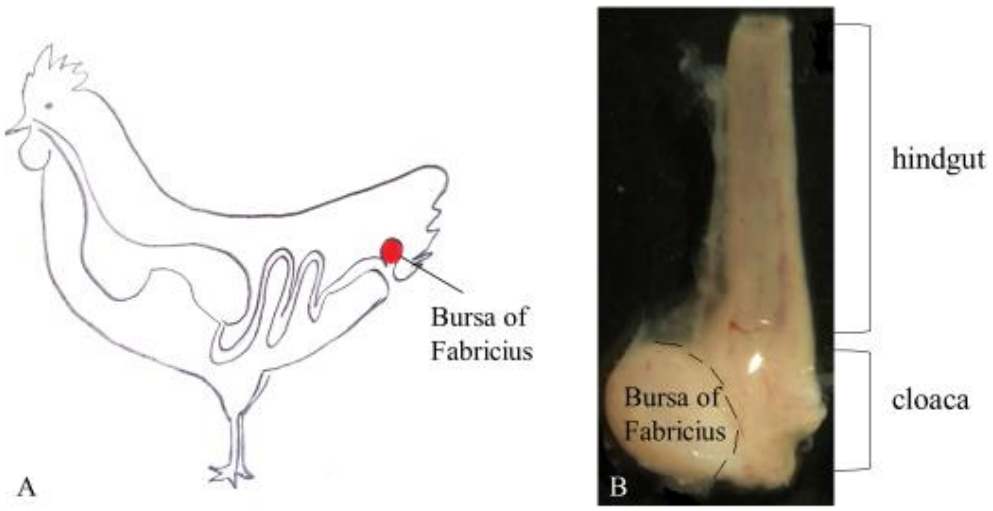

Figure 1. *A: Schematic drawing of the cloaca associated bursa of Fabricius in adult chicken. B: Anatomical location of the bursa of Fabricius in the 16-day-old embryo*

The BF is an avian specific, chestnut-sized organ that is located between the cloaca and the sacrum (Fig.1) (Nagy and Oláh, 2010; Balic et al., 2019) and is connected to the proctodeum via the bursal duct (Madej et al., 2013). As a central lymphoepithelial organ it is responsible for proliferation and differentiation of B-lymphocytes and is a primary site of antigen specific antibody production (Sari et al., 2015; Madej et al., 2013; Nagy and Oláh, 2010). In adult birds the bursal lumen is divided by 12-20 longitudinal folds, where a large number of lymphoid follicles are arranged next to each other (Oláh and Glick, 1978). These follicles are composed of a central medullary and an outer cortical region, which are separated by a basement membrane (Balic et al., 2019). The medulla is composed of cytokeratin+/E-cadherin+ epithelial reticular cells of ectodermal origin, while the cortex is of mesodermal origin and is composed of vimentin+/desmin+ mesenchymal reticular cells (Fig. 2A) (Balic et al., 2019).

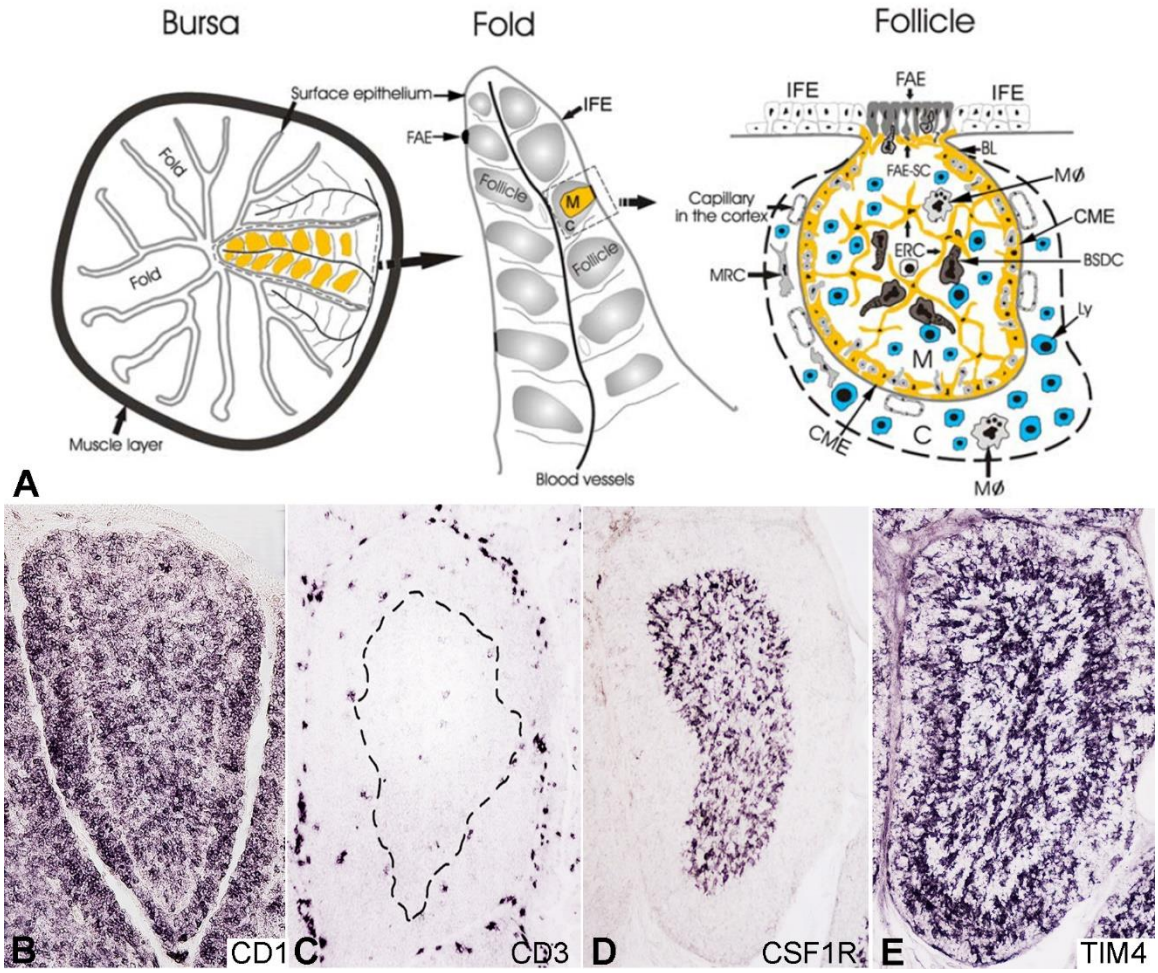

Figure 2. The structure of the bursa of Fabricius. *A: Schematic drawing of the bursa of Fabricius, highlighting the bursal folds, filled with lymphoid follicles and its main cellular components: M- medulla, C- cortex, FAE – follicle associated epithelium, FAE-SC- follicle associated epithelium supporting cell, IFE – interfollicular epithelium, MRC- mesenchymal reticular cell, ERC-epithelial reticular cell, BSDC- bursal secretory dendritic cell, MΦ – macrophage, Ly-lymphocyte, CMAFC- cortico-medullary arch forming cells, BL- basal laminaB: Hematopoietic cell populations in the bursal follicles: B-lymphocytes (B), T-lymphocytes (C), bursal secretory dendritic cells (D), macrophages (E).*
(Nagy et al., 2022, Structure of the avian lymphoid system. In Avian Immunology, ed. by Kaspers B, Schat K, Göbel T, Vervelde L, Academic Press)

Both medullary and cortical compartments are filled with CD1+ B-lymphocytes (Fig. 2B), which proliferate and differentiate in the follicular microenvironment after seeding the organ during early embryonic development (Madej et al., 2013). Microenvironmental factors in the bursal follicles are essential for the generation of the antigen specific B-lymphocyte population via the mechanism of gene conversion, a phenomenon which in birds is restricted to the bursal follicles and occurs in a restricted period of time before the involution of the organ upon sexual maturation (Reynaud et al., 1994). A major cell type in the follicular medulla are bursal

secretory dendritic cells (BSDC) (Fig. 2D), which have been described to play a crucial role in B-lymphocyte maturation (Nagy et al., 2004; Oláh and Nagy, 2013). Chicken dendritic cells express many of the surface markers described in mammals, such as CD11c, CSF1R, CD83 (Nagy et al., 2016; Balic et al., 2019). In contrast, in the follicular cortex the major myeloid cell populations are cortical macrophages (Fig.2E), only a few BSDCs can be detected scattered between the mesenchymal reticular cells. The cortex reaches maturity after hatching, mature B-lymphocytes leave the bursa through the cortical capillaries localized at the cortico-medullary border and seed the peripheral lymphoid organs (Igyártó et al., 2008).

2. Embryonic development of the bursa of Fabricius

Embryonic development of the BF can be divided into several well characterized stages: appearance of the pre-programmed bursal epithelial anlage, colonization by hematopoietic cells that results in lymphoid follicle formation, follicle-associated epithelium differentiation and appearance of the follicular cortex (Nagy and Oláh, 2010).

First stage: appearance of the bursal epithelial anlage in the tail bud mesenchyme.

The epithelio-mesenchymal rudiment of the BF appears around E4-E5 of development, when the epithelial anlage of the BF starts growing into the tail bud mesenchyme (Nagy et al., 2004; Meyer et al., 1959). Quail-chicken chimera experiments demonstrate that the bursal epithelium is of ectodermal origin (Nagy and Oláh, 2010). The proliferation and vacuolization of the epithelium results in the formation of the bursal lumen, which connects the developing lymphoid organ with the proctodeal region of the avian cloaca.

Second stage: colonization by hematopoietic cells and follicle bud formation.

Hematopoietic cells colonize the bursa in a short window of time, between E9-E15 of development (Fig. 3A) (Madej et al., 2013), which induces further structural development and bursal lymphoid follicle formation (Nagy et al., 2004). On the 10th day of embryonic development, two well-defined cells can be distinguished in the mesenchyme: light and dark cells (Nagy et al., 2001; Oláh et al., 1979). Dark cells were named "dark" based on their highly basophilic staining with toluidine blue (Nagy et al., 2001). The dark cells migrate into the surface epithelium and induce follicle bud formation, while the light cells remain in the mesenchyme and differentiate to cortical mesenchymal reticular cells (Nagy et al., 2001; Lupetti et al., 1990). The first cell type to reach the surface epithelium has been later characterized as a dendritic cell precursor, which express CD45, CSF1R, MHCII, vimentin antigens (Nagy et al., 2004). Colonization of the epithelium by dendritic cell precursors results

in the formation of a specialized dendro-epithelial tissue, which is considered the initiation step of bursal lymphoid follicle formation (Fig. 3B) (Nagy et al., 2004; Ackerman and Knouff, 1991). In the second wave, chB6+ B-lymphocyte precursors appear in large numbers in the bursal mesenchyme and colonize the follicle buds starting from E12 onwards (Fig. 3C). Each follicle bud is colonized by 2-3 B-lymphocyte precursors, which upon colonization of the epithelium express surface IgM. Proliferation and differentiation of the B-lymphocytes in the follicular microenvironment give rise to the lymphoid cell population of the follicular medulla (Fig. 3D).

Third stage: differentiation of the follicle-associated epithelium (FAE).

The bursal surface epithelium will differentiate into a follicle-associated epithelium at E18, while between the follicles a simple inter-follicular epithelium (IFE) layer forms (Romano et al., 1996). The exact origin of the FAE is still unclear, but according to the current scientific knowledge, the FAE develops from the undifferentiated surface epithelium of the bursal folds, although it has also been suggested to have mesenchymal origin (Dolfi et al., 1988; Nagy et al., 2001; Oláh et al., 1986). This specialized epithelium does not have a basement membrane, FAE-bordering cells surround and support the FAE cells within the lymphoid follicles, and link the FAE and IFE (Romano et al., 1996). Similar to microfold cells of the gastrointestinal tract, the FAE is believed to facilitate antigen transfer between the bursal lumen and the follicular medulla (Fig. 3E, F).

Fourth stage: development of the follicular cortex.

The follicular cortex starts developing, when B-lymphocytes migrate outside of the basement membrane of the epithelial follicle buds (Fig. H). The first cortical cells appear around hatching and the cortex is fully developed 2 weeks later (Oláh and Vervelde, 2008). In contrast to the medulla, the cortex is highly vascularized, capillaries are localized near the cortico-medullary border.

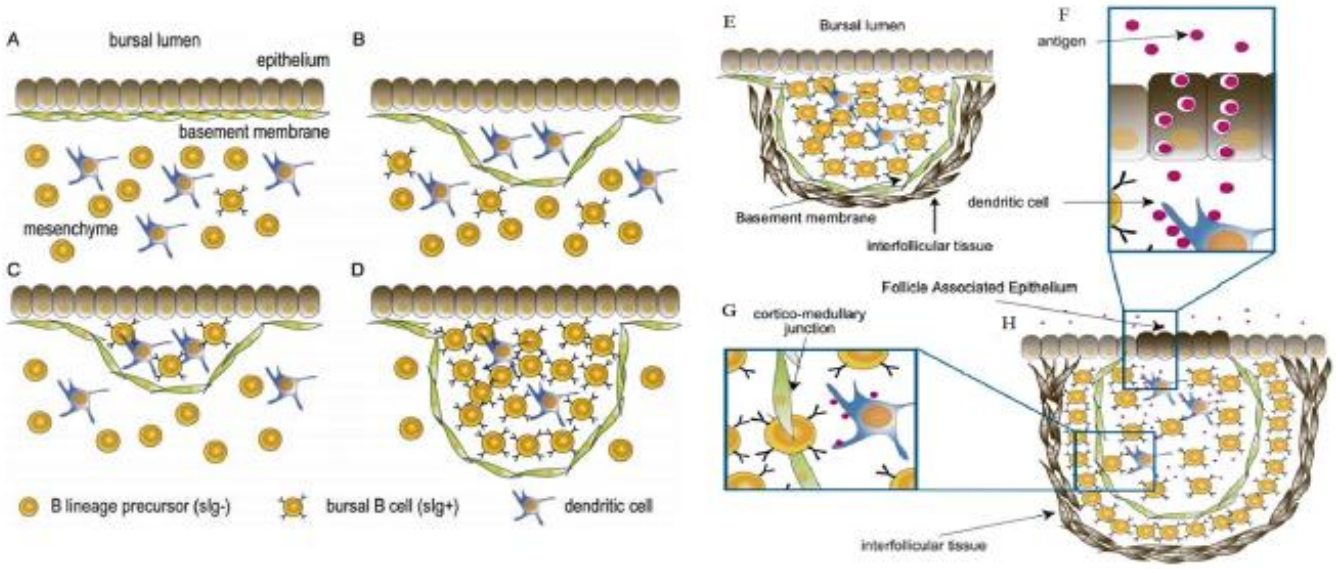

Figure 3. Left side: Hematopoietic colonization of the bursal surface epithelium. ***A:*** *Starting from E10 the bursal mesenchyme is colonized by large basophilic dendritic cells and lymphoid precursors.* ***B:*** *The first cell type to colonize the surface epithelium and induce follicle bud foramtion are dendritic cell precursors.* ***C:*** *The epithelial buds are colonized by two to three surface immunoglobulin expressing B lineage precursors.* ***D:*** *Surface immunoglobulin expressing B cells undergo rapid proliferative expansion within the epithelial buds.*
Right side: Development of the bursal follicles after hatching. ***E:*** *At the time of hatching, epithelial buds are filled with B cells.* ***F:*** *Shortly after hatching, cells of the follicle associated epithelium develop and actively transport antigens from the bursal lumen into the follicular medulla.* ***G:*** *Some bursal lymphocytes migrate across the basement membrane, which develops into a complex cortico-medullary junction.* ***H:*** *Structure of a mature bursal follicle post-hatching (*Ratcliffe and Hartle, 2022. B cells, the bursa of Fabricius, and the generation of antibody repertoires).

3. <u>Microenvironment of B-cells in the bursa of Fabricius</u>

B-lymphocytes are an essential branch of adaptive immunity, as they recognize and eliminate foreign antigens and form immunological memory (Marsman et al., 2022). The microenvironmental factors that drive precursor cell proliferation, somatic gene recombination and gene conversion, B-cell maturation and terminal differentiation into plasma cell or memory cell fates are precisely regulated in designated primary and secondary lymphoid organs. These lymphoid organs are the bone marrow in mammals (Abdou and Abdou, 1972), ileal Peyer's patches in ruminants (Reynolds and Morris, 1984), the appendix in rabbits (Pinheiro et al., 2011), which have all been described as bursa-equivalent organs after the initial discovery of the BF as an avian specific primary lymphoid organ, which is specifically responsible for the generation of avian B-lymphocytes. In birds, the BF is the sole region for B-cell maturation, the bursal follicular microenvironment supports development isolated from other lymphoid, myeloid and erythroid lineages (Nagy and Oláh, 2010).

Chicken B-cell development can be broken down into various stages: pre-bursal, bursal and post-bursal phases (Nagy et al., 2020). In the pre-bursal stage, committed chB6+ B-cell progenitors originating from extra-bursal hematopoietic sites colonize the BF mesenchyme (Reynaud et al., 1985). In the bursal stage B-cells migrate to the developing lymphoid follicles and start expressing surface IgM molecules within the follicular microenvironment (Vainio et al., 1996). In post-hatch animals non-lymphoid cells of the bursal lymphoid follicles are epithelial reticular cells, bursal secretory dendritic cells, macrophages and mesenchymal reticular cells (Nagy et al., 2022). Epithelial reticular cells form a three-dimensional cellular network in the medulla, where there is no detectable extracellular matrix. At the cortico-medullary border epithelial cells form epithelial arches, which enclose CD45+ lymphoblast-like cells. The cortico-medullary arch forming cells are sitting on a basement membrane, that separates the medullary and cortical compartments. Embedded in the medullary epithelial network are bursal secretory dendritic cells, which are highly elongated and contain large cytoplasmic granules, which are recognized by the 74.3 monoclonal antibody. During embryonic development BSDCs transiently produce the CXCL12 chemokine, and guide migration of the CXCR4+ B-cells that colonize the lymphoid follicles. Post-hatch CXCL12 expression is restricted to the cortex, while BSDCs are localized in the follicular medulla (Nagy et al., 2020). Highly phagocytotic macrophages are present in both medullary and cortical compartments and express classical macrophage markers such as MHCII, Lep100, KUL01, 68.2, TIM4. In contrast with the epithelial reticular cells in the medulla, mesenchymal cells in the cortex produce a large variety of extracellular matrix proteins, such as collagen-I, collagen-III, perlecan, tenascin-C, which form a gradient along the cortico-medullary border, indicating extensive cell migration through the cortex. In the post-bursal stage, B-lymphocytes migrate to the periphery through the capillaries of the cortex and colonize the secondary lymphoid organs. It has been described that cortical B-lymphocyte populations are heterogeneous: all cortical B-cells express the chB6 antigen, however the CXCR4 receptor is present only on a subpopulation of lymphocytes along the cortico-medullary border. It is suggested that cortical B-cells leave the CXCL12 chemokine rich cortical microenvironment, through downregulation of the CXCR4 chemokine receptor, a mechanism which is essential for secondary lymphoid organ colonization (Nagy et al., 2020).

4. Methods to influence normal bursal development and antibody production

Following the initial discovery of the bursa derived antibody producing B-lymphocytes and the thymus derived lymphocytes, different methods to interfere with the development of early hematopoietic cells have been implemented to understand the function of these two lymphoid lineages. Neonatal removal of the thymus resulted in delayed homograft rejection, but did not affect antibody production, while bursectomized chicken did not produce antibodies and the average time to reject skin homografts was similar in control and bursaless chicken. Surgical removal of these primary lymphoid organs, combined with irradiation resulted in the dissociation of the two branches of adaptive immunity, with the division of bursa derived antibody producing B-lymphocytes and thymus-derived lymphocytes that mediate graft versus host reaction.

4.1. Surgical removal of bursa of Fabricius

Characterization of the immunological role of the bursa began with the discovery that surgical removal of the organ in post-hatch chicken results in the absence of normal antibody response. Surgical methods to eliminate B-lymphocytes and antigen specific antibody responses to induce immunosuppression include *in ovo* embryonic bursectomy and neonatal bursectomy, which is often combined with sub-lethal X-irradiation to eliminate the germinal centers in the spleen. The surgical removal of the BF results in severe immunosupression and complete loss of antigen specific antibody production, which is most effective when performed at hatching and is followed by irradiation within 24 hours (Glick and Oláh, 1984). *In ovo* surgical bursectomy is performed between days 17-19 of embryonic development, with a 70% approximate survival rate. Compared to neonatally bursectomized birds, experiments that implemented *in ovo* surgical bursectomy greatly increased the number of birds without germinal centers and secondary immune responses. It was observed that 60 hours port-bursectomy, these *in ovo* bursectomized birds are incapable of antibody production against nine different antigens (Mansikka et al., 1990). Furthermore, isotype switching and generation of IgG producing B-lymphocytes in case of *in ovo* bursectomized birds was absent, which raised the role of the bursal microenvironment in IgM to IgG transformation (van Alten et al., 1968). Germinal center formation, plasma cell and immunoglobulin synthesis can be restored in bursectomized-irradiated chicks by injection of control bursal lymphocytes (van Alten et al., 1968).

4.2. Hormonal treatment induced bursectomy

It was observed that androgen injections in newborn chicken cause severe immunosuppression and lack of antigen specific antibody production, similar to surgically bursectomized birds. It was later demonstrated that testosterone treatment results in immunosuppression through inhibiting bursal development and causing bursal involution (Glick and Oláh, 1984). 19-nortestosterone dissolved in corn oil injected into the fertilized eggs on the 5th day of incubation (Meyer et al., 1959) results in impaired bursal development, embryos and post-hatch animals lack a cloaca associated bursa of Fabricius (Meyer et al., 1959). Histological observations confirmed the failure of follicle bud formation on the 10th embryonic day of BF development (Meyer et al., 1959). A later study revealed that more than 80% of testosterone treated chicken lacked a visible bursa between the 13th and 21st embryonic days (Glick, 1991) and the injection of testosterone into the allantoic cavity prevents the appearance of lymphoid tissue in the BF (Glick and Oláh, 1984).

Hormonal bursectomy can also be achieved by dipping eggs in steroid solutions, which equally affects bursal development (Glick and Oláh, 1984). One study examined several androgen-like hormones and their effects on bursal development (Aspinall et al., 1961) and found that the following androgen-like hormones had a strong inhibitory effect on lymphoepithelial tissue formation: dihydrotestosterone; androsterone, androstane-3,17-dione; 19-nortestosterone, methylandrostenediol, testosterone-cyclo-pentyl-propionate; testosterone (Aspinall et al., 1961). Compounds are listed from most effective to less effective in their capacity to induce hormonal bursectomy (Aspinall et al., 1961).

The molecular and cellular mechanisms by which TP treatment abrogates bursal development is not fully characterized. Quail-chick tissue recombination experiments show that recombination of testosterone treated epithelium with control mesenchyme results in no follicle formation. In contrast, association of testosterone treated mesenchyme with non-treated BF epithelium results in normal bursal development, hematopoietic colonization and follicle formation (Le Douarin et al., 1980). It has also been reported that testosterone treatment abrogated the development of the dark cells in the bursal mesenchyme, which might be responsible for the absence of follicle bud formation (Oláh et al., 1986). Whether testosterone treatment induces hormonal bursectomy via affecting development of the epithelium, mesenchyme or the immigrating hematopoietic cells deserves further investigation.

III. Aims

The androgens, such as the testosterone propionate treatment of chicken embryo results in bursectomy. Previous developmental studies raised the possibility that testosterone affect the mesenchymal compartment of the bursa of Fabricius and during bursectomy primary impairment occurs in the mesenchyme. The general aim of this project is to understand the developmental changes of the mesenchymal microenvironment of the bursa of Fabricius during testosterone treatment.

This will be split in two specific aims:

- Compare the expression pattern of extracellular matrix proteins during development of normal and testosterone treated bursa of Fabricius.
- Determine the role of the different glycoproteins (tenascin and fibronectin) on B-cell migration.

IV. Materials and methods

1. Experimental animals

Fertilized White Leghorn chickens (*Gallus gallus domesticus*) were used as experimental animals. Eggs were obtained from commercial breeders (Ceva-Phylaxia Zrt. Hungary). Eggs were incubated at 37,7°C in a humidified incubator (HekaBrutgerate, TS-7000C). The age of the embryos was determined by embryonic day (ED). We have used ED 10, 12 and 14 chicken embryos for our experiments.

2. Testosterone propionate treatment

Testosterone treatment was performed as previously described (Oláh et al., 1986). After 24 hours of incubation, the eggs were dipped in 2,5% testosterone propionate (Sigma-Aldrich, cat. no.: #57-85-2) (Fig.4) dissolved in absolute ethanol (Molar Chemicals Kft., cat. no.: #02910-101-340) for 5 seconds. The treated eggs were returned to the incubator, and the embryos were further incubated up to 10, 12, 14th days.

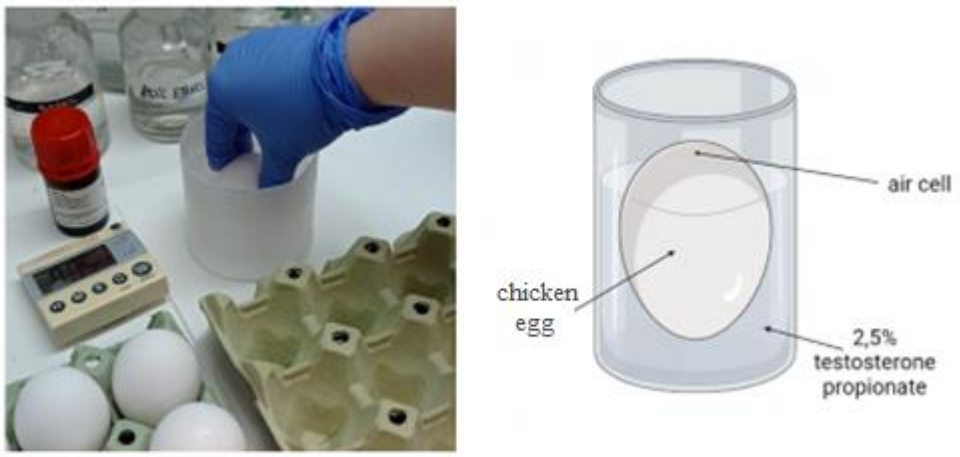

Figure 4: *Chemical bursectomy of chicken embryo with testosterone.*

3. Histological analysis of the samples

3.1. Gelatine embedding of histological samples

The bursa of Fabricius (BF) has been isolated, and the tissue was fixed by 4% PFA (Renal Labor, cat. no.: #25550-0-13-38) for 1 hour then infiltrated with 15% sucrose (Reanal, 0714000838) overnight at 4°C. 24 hours later the sucrose solution was replaced by 7,5% gelatin (Sigma, cat. No: #25550-0-13-38) for 2 hours at 37°C. The samples were placed onto the gelatin layer, which was made on a plastic mold surface during the sample incubation time. The samples were covered with a second gelatin layer. After solidification of the gelatin at room temperature the organs, we cut out with a surgical blade. Gelatin blocks were snap frozen in isopentane (2 methylbutane, Sigma, cat. no #M32631) at -60°C with liquid nitrogen for 1 min. We stored the frozen tissue samples at -20°C or for long-term at -80°C.

3.2. Cryosectioning of the embedded blocks

Cryosectioning of the gelatin blocks was performed with a Shandon cryotome at -25-30°C. The 12 μm thick sections were applied to slides treated by 0,01% poly-L-Lysine (Sigma, cat. no: #P8920) and stored at -20°C until use.

3.3. Immunohistochemistry

The frozen sections were rehydrated in 37°C 1x PBS for 10 minutes. After rehydration we incubated the sections with the primary antibodies (Table 1) for 45 minutes (or overnight at 4°C) at room temperature in a humid chamber. The primary antibodies were diluted in 1% PBS-BSA (Sigma; cat. no.: #A9647). After multiple washing steps (1x PBS 3 times for 5 minutes), we have incubated the sections with the biotinylated secondary antibody (Table 2) of the isotype and host specificity corresponding to the primary antibody at RT for 45 min. The secondary antibodies were diluted 1:200 in 1% PBS-BSA. After PBS wash, the samples were placed in 30% H_2O_2 (AnalaR NORMAPUR, analytical reagent, cat. no.: #23619.297) solution for 10 minutes to quench the endogenous peroxidase activity. After removing the hydrogen peroxide, 1:100 diluted in 1x PBS avidin-biotin peroxidase complex solution (ABC Peroxidase Staining Kit Standard, Life Technologies, cat. no.: #32020) was added to the samples and incubated for 30 minutes at RT. During this time, we prepared the chloronaphtol solution (Chloronaphthol

Substrate Powder, 4-chloro-1-naphthol, ThermoScientific, cat. no.: #34010): 50 µl chloronaphtol was added to 50 ml 1x PBS and stirred in a dark for 30 minutes then added 250 µl 3% H_2O_2 (as the substrate of the horseradish peroxidase enzyme). After the ABC incubation, the samples were washed 3x5 minutes in 1x PBS. As the final step, the chromogen reaction was developed for 30 minutes in dark at RT: the conjugated peroxidase enzyme produced purple precipitate from the choloronaphtol on the binding site of the primary antibody, thus marking the individual cell types within the tissue. Finally, we covered the sections with aqueous mounting solution (Mowiol 4-88, Sigma Aldrich, cat. no.: #81381-25G).

Table 1. Primary antibodies used in this study.

Primary antibody (clone)	**Host, Isotype**	**Dilution in PBS-BSA**	**Binding structures**	**Supplier**
CD45 (HISC7)	mouse, IgG2A	1:200	hematopoietic cell	Prionics Co.
EIVE-12	mouse, IgG1	1:5	chicken B-cell	Dr. Todd Pharr (USA)
Bu-1b/ChB6	mouse, IgG1	supernatant	chicken B-cell	Southern Biotech
anti-tenascin-C/M1-B4	mouse, IgG1	supernatant	extracellular matrix	DSHB
anti-collagen I (SPID8)	mouse, IgG1	1:2	extracellular matrix	DSHB
CSF1R	mouse, IgG1	1:80	chicken dendritic cell	Dr. Adam Balic Roslin Institute Edinburgh
SMA (1A4)	mouse, IgG1	1:200	smooth muscle cell	DAKO
Laminin (3H11)	mouse, IgG1	1:2	extracellular matrix	DSHB
anti-fibronectin (B3D6)	mouse, IgG1	1:3	extracellular matrix	DSHB
anti-cytokeratin (Lu-5)	mouse, IgG1	1:100	basal cell cytokeratin	Millipore

Table 2. Secondary antibodies used in this study.

Secondary antibody	Organism of origin	Supplier and catalogue number
Biotinylated anti-mouse IgG (H+L)	Donkey	Vector Laboratories; BA-9200
Biotinylated anti-mouse IgM (H+L)	Goat	Vector Laboratories; BA-2020

4. *In vitro* migration assay of B-cells

For the *in vitro* migration assay, 13 ED chicken BF was cultured on laminin, fibronectin or tenascin-coated Petri dish (Orange Scientific, cat. no.: # 5550200) with a diameter of 6 cm. Before the experiment, plastic Petri dishes were covered with 20 µg/ml laminin (Sigma), 20 µg/ml fibronectin (FN, Thermo Fisher Scientific, cat. no.: # 33010018) or 20 µg/ml tenascin (Sigma) for 30 minutes at 37°C. The embryonic organs were placed on the scratches made in the middle of the Petri dishes with forceps in order to ensure the adherence of the tissues to the surface. The matrix solutions were dissolved in DMEM (Dulbecco's Modified Eagle Medium/Nutrient Mixture; Sigma-Aldrich, cat. no.: # D5429) solution containing 100 µg/ml Penicillin-Streptomycin (Sigma Aldrich, cat. no.: # P0781). We stimulated the B-cell migration with addition 150 ng/ml SDF1 (CXCL12, 100 ng/ml; R&D Systems, cat. no.: # 6448SD025) in an Eppendorf incubator for 24 hours at 37°C with 5% CO_2 level. At the end of the experiment the organ culture was fixed in 4% PFA for 20 minutes and processed for immunofluorescence staining.

5. Evaluation of the samples

Images were recorded using a Nikon Eclipse E800 microscope and whole-mount images were recorded using a Nikon SMZ25 (with Prior L200/E unit) stereomicroscope and following software: Adobe Photoshop CS 7.01 and ImageJ (http://rsb.info.nih.gov/ij/) for image processing and analysis.

Table 3. Solutions used in this study

Solution	Ingredients	Supplier	Catalogue number
10x PBS	1,42 g $Na_2HPO_4x2\ H_2O$, 8 g NaCl, 0,2 g KH_2PO_4, 0,2 g KCl, distilled water	prepared in our lab	-
1x PBS	1/10 of 10x PBS mixed with 9 parts of distilled water and stored at room temperature.	prepared in our lab	-
15% sucrose	15 g D (+) sucrose dissolved in 100 ml 1x PBS.	Renal	07140-0-08-38
7,5 % gelatin	75 g D (+) sucrose and 37,5 g gelatin dissolved in 500 ml warm 1x PBS on a magnetic stirrer.	Sigma	G2500
4% PFA (buffered paraformaldehyde)	20 g of paraformaldehyde dissolved in 400 ml 80°C distilled water on a magnetic stirrer. 100 µl 1N NaOH was added to help dissolve any remaining PFA precipitate. The solution was filtered, and 50 ml of 10x PBS was added. To reach the final volume (500 ml) it was completed with distilled water.	Renal Labor	25550-0-13-38
1% PBS-BSA	1 g of BSA dissolved in 100 ml 1x PBS. After filtration 0,1 % Na-azid (NaN_3) was added.	Sigma	A9647
2,5 % Testosterone propionate	5 g testosterone propionate were dissolved in 200 ml absolute ethanol.	Sigma-Aldrich	57-85-2
Chloronaphtol	500 mg 4-chloro-1-naphthol dissolved in absolute ethanol, stored at -20°C.	Sigma	C8890

V. Results

1. Testosterone treatment disrupts hematopoietic colonization of bursa of Fabricius.

Testosterone propionate treatment of early chick embryos prevent bursal development and lead to severe bursal involution (bursectomy), thereby antigen specific antibody productions were not detectable in posthached birds (Szenberg and Warner, 1962). It is widely accepted that embryonic testosterone treatment affects the differentiation of the bursal stromal cells, (either the epithelium or the mesenchymal compartment), thereby, leading to bursectomy, preventing bursa-dependent B-cell development and subsequently causing immunosuppression.

In order to characterize the early stages of bursal development we have examined by immunocytochemistry 10, 12 and 14-day-old bursa of Fabricius in control and testosterone treated chicken embryos. Previously it has been reported that testosterone propionate treatment impairs bursal development, however the mechanisms and microenvironmental factors that results in chemical bursectomy are still not fully characterized (Oláh et al., 1986). We have observed after testosterone treatment, that the survival rate of embryos significantly changed; less than half of the embryos survived until E16. 80% of embryos survived at 10 ED. At this early developmental stage, the bursa primordia was already rudimentary, its lumen was barely visible.

Consecutive histological sections of 10, 12 and 14 ED chicken embryo tail bud and dissected bursa of Fabricius were examined by monoclonal antibodies specific to hematopoietic cells (anti-CD45, common leukocyte antigen) and anti-chB6 specific monoclonal antibody specific for B-cells and its precursors, which colonize the bursal rudiment starting from 10 ED and 12ED, respectively. At 10 ED control embryos CD45+ hematopoietic cells characterized by round and ramified morphology are uniformly scattered in the bursal mesenchyme, with a few ramified cells localized in small groups at the level of the surface epithelium (Fig. 5A). At this early developmental stage chB6 immunoreactive B-lymphocytes are absent from the bursal rudiment (Fig. 5B). In contrast to this, hematopoietic colonization delayed in the testosterone treated bursa: CD45 immunoreactive cells are scattered in the tail bud mesenchyme, but clustering of the cells in the bursal rudiment is not visible (Fig. 5C).

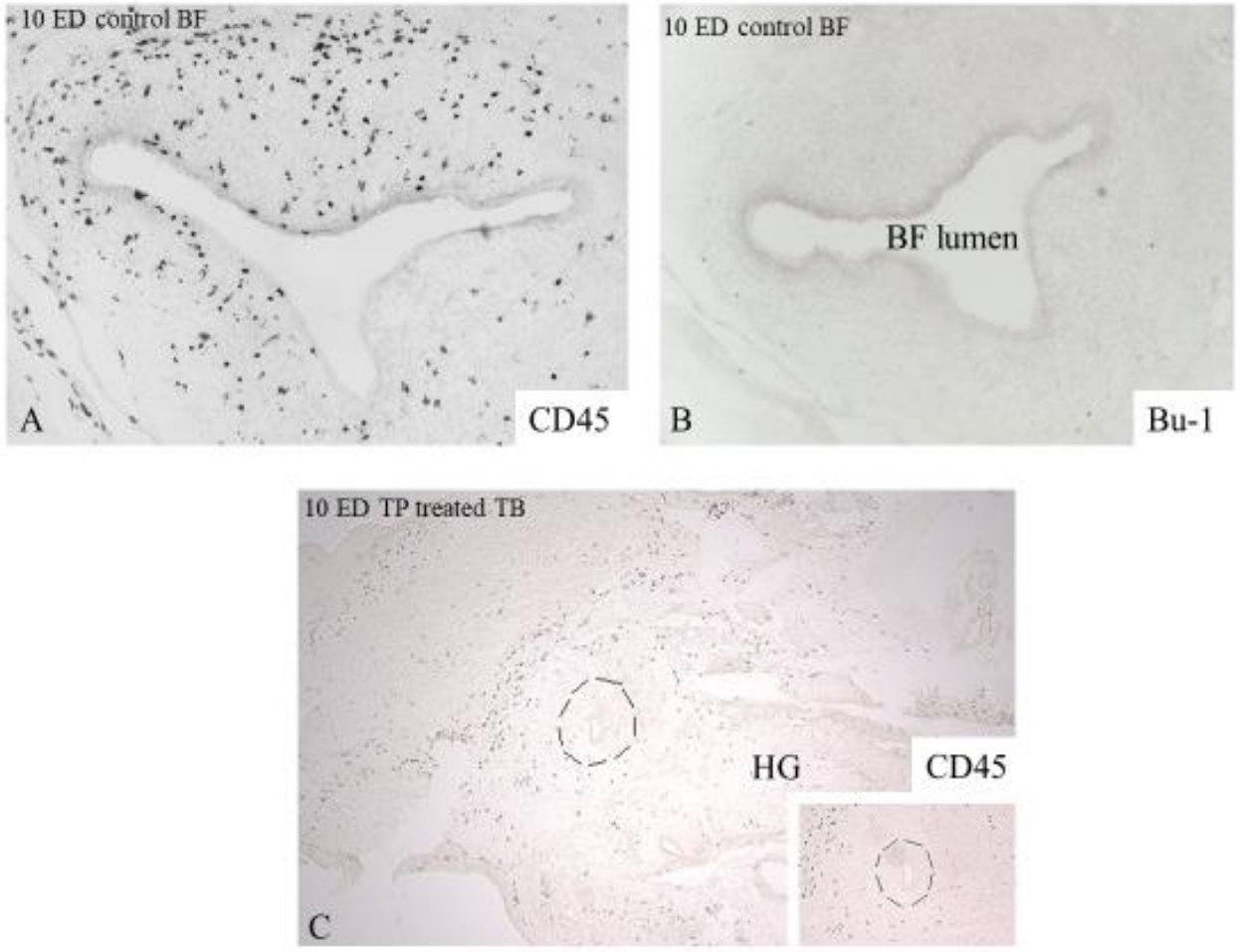

Figure 5. Serial sections of 10 ED control (A, B) and testosterone treated embryo tail bud (C). *__A:__ hematopoietic cells marked with CD45 in control BF. __B:__ B cells marked with Bu-1 specific monoclonal antibody. __C:__ immunostaining of testosterone treated embryo tail bud with anti-CD45 monoclonal antibody.*

Lymphoid follicle formation in the bursa of Fabricius is a sequential process, which occurs between days 10-15 of embryonic development and it is initiated by the colonization of the surface epithelium with dendritic cell precursors, macrophages, and B-lymphocytes (Nagy et al, 2004). To study the bursal folliculogenesis, histological sections of E12 and E14 control and testosterone treated bursa of Fabricius were immunolabelled with antibodies specific for lympho-myeloid cell lineages, such as anti-CD45, EIVE-12, CSF1R and anti-chB6 (Fig. 6, Fig. 7). All hematopoietic cells are labelled by CD45 antibody (Fig. 6A, B). The anti-EIVE-12 (Fig. 6 C, D) antigen is present on a subpopulation of colonizing hematopoietic cells, which are recognized as a precursor of dendritic cells in the early bursa (Nagy et al., 2004; Nagy et al.,

2016). EIVE-12 antibody is not a cell line-specific marker, since later colonizing B-cells also express this antigen (Pharr et al., 1995; Felfoldi et al., 2015). In contrast, the chB6 antigen (Fig. 6 G, H) is a B-cell specific marker, which labels only bursal lymphocytes.

In the control and testosterone treated 12 ED bursa of Fabricius clear differences can be observed in the number and localization of immigrating hematopoietic cells. Compared to the control bursa, where the proliferation and differentiation of the mesenchyme induce the formation of bursal folds, testosterone treated bursa of Fabricius show little differentiation, with a small central lumen surrounded by undifferentiated mesenchyme and no detectable follicle buds at the level of the surface epithelium. In the testosterone treated embryos significantly fewer CD45+ and EIVE-12+ cells were present in the 12-day-old embryos. Immigrating hematopoietic cells are usually localized in the distal part of the developing lymphoid organ (Fig. 6B).

Immigration of the chB6+ B-cells to the primordium of the bursa of Fabricius occurs at later stages of the hematopoietic colonization, where the colonization of the B-cell precursors starts from 12 ED of development (Nagy et al., 2004; 2022). In control bursa of Fabricius chB6+ cells appear in the axis of the bursal folds. chB6+ B-cells are completely absent from the bursal mesenchyme following testosterone treatment. CSF1R+ (Fig. 6E, F) dendritic precursor cells are present in both control and testosterone treated bursa of Fabricius, but their number is significantly decreased in the treated bursa of Fabricius compared to the control.

More characteristic differences were observed 48hours later, where in control bursa of Fabricius the mesenchyme of the bursal folds and the developing follicle buds were filled with CD45+ hematopoietic cells (Fig.7A). EIVE-12+ cells are localized at the level of the epithelium and inside the developing epithelial buds (Fig. 7B), while the majority of immigrating chB6 immunoreactive B-lymphocytes occur in the mesenchyme, with only a few B-cells present in the follicle buds (Fig. 7C). In testosterone treated embryos, the bursa of Fabricius shows little progression in morphological development, the epithelio-mesenchymal primordium shows no organization in bursal fold-like structures, colonization of the epithelium by CD45+ cells cannot be observed (Fig. 7B). At this stage, chB6+ B-cells (Fig. 7 E, F) also occurred in the mesenchyme of the bursa, but their number was lower than in the untreated bursa of Fabricius.

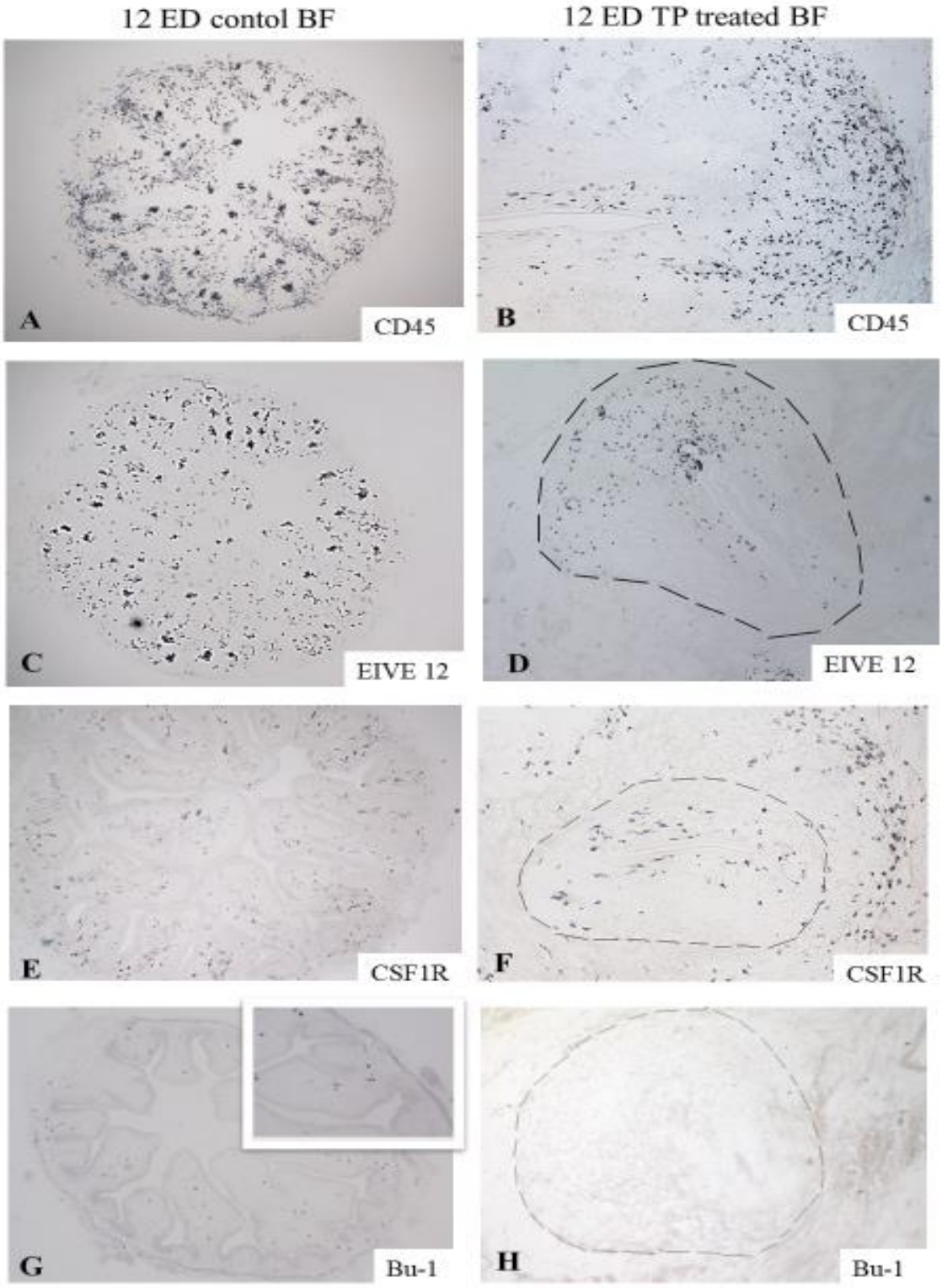

Figure 6. Distribution of lympho-myeloid cells in 12 ED control and testosterone propionate treated bursa of Fabricius.

Comparison of hematopoietic cell colonization of control (A, C, E, G) and testosterone treated (B, D, F, H) bursa of Fabricius at 12 ED. Hemopoietic (CD45; A and B); myeloid and B-cell precursors (EIV-E12; C and F); dendritic cells (CSF1R; E and F); B-cells (Bu-1); G and H) markers have been used to demonstrate the different cell populations. In contrast to testosterone treated bursa (B) CD45+ cells heavily infiltrate the control bursa mesenchyme(A) and colonize the follicle buds.

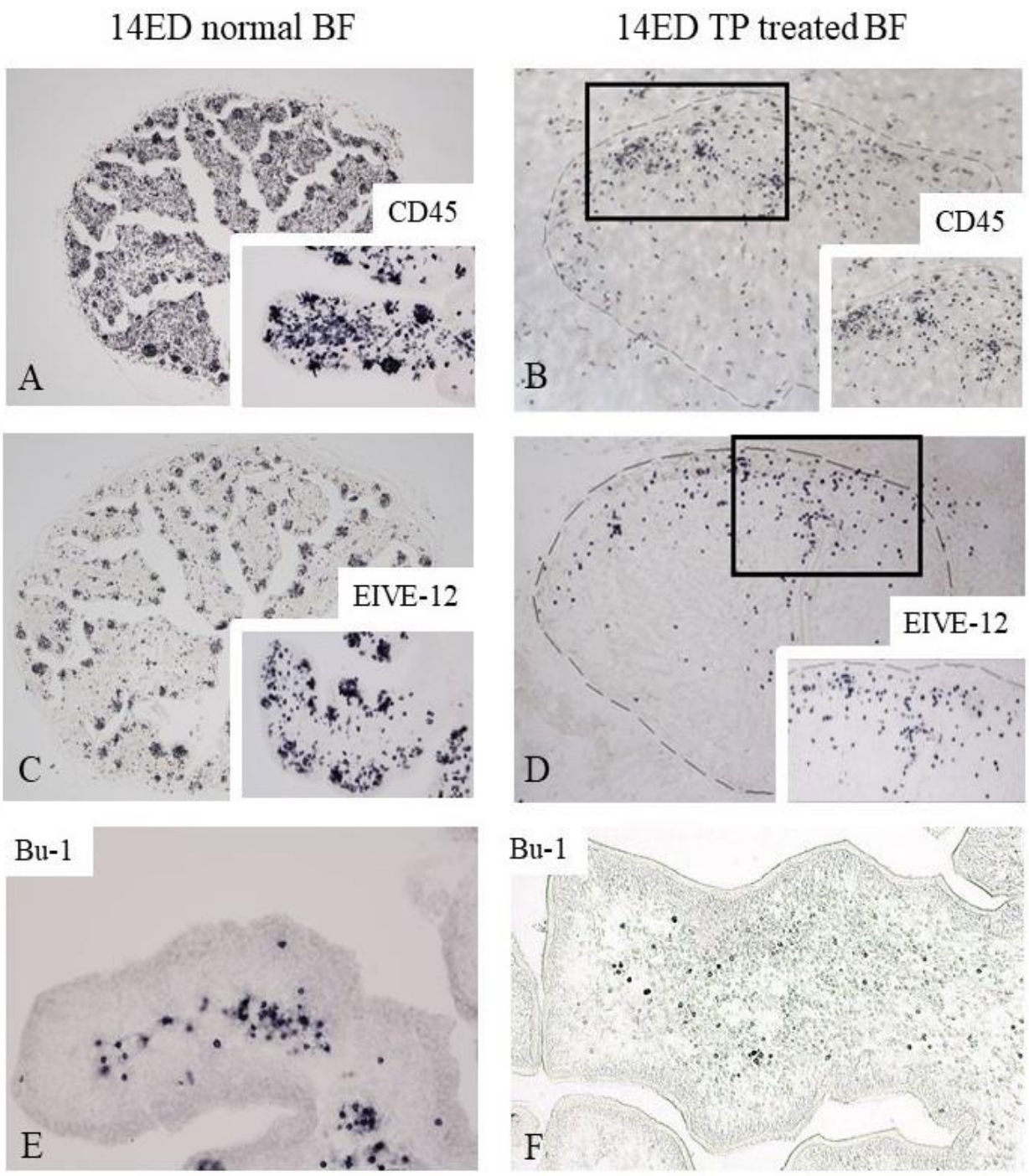

Figure 7. Distribution of lympho-myeloid cells in 14 ED control and testosterone propionate treated bursa of Fabricius.

Comparison of hematopoietic cell colonization of control (A, C, E) and testosterone treated (B, D, F) bursa of Fabricius at 14 ED. Inset: magnified view. Hemopoietic (CD45; A and B); myeloid and B cell precursors (EIV-E12; C and D); and B cells (Bu-1); E and F) markers have been used again to compare the distribution of the different lympho-myeloid cell populations. In control bursa Bu-1+ B-lymphocytes already settle down in the bursal follicle. In testosterone treated bursa no sign of follicle formation using anti-CD45 and Bu-1 specific monoclonal antibodies (B) CD45+ cells heavily infiltrate the control bursa mesenchyme(A) and colonize the follicle buds.

2. Extracellular matrix protein expressed during bursa of Fabricius development

Mesenchymal reticular cells in the bursal rudiment produce a complex array of extracellular matrix proteins, which are expressed during the primordium formation and the hematopoietic colonization of the bursa. The effect of testosterone treatment on the extracellular matrix composition of the developing bursal mesenchyme has been compared in consecutive histological sections of 11 ED, 12 ED and 14 ED (Figs. 8, 9), using mouse monoclonal antibodies specific for tenascin-C, laminin, fibronectin, and collagen type-I respectively. We observed that the expression pattern of fibronectin and collagen-I was similar in control animals (data not shown). Fibronectin is uniformly present in the tail bud and bursal mesenchyme (Fig. 8A, C). Compared to fibronectin, tenascin-C is absent from the bursal mesenchyme (Fig. 9B, E, F, G) at 11 ED, 12 ED and 14 ED. Laminin immunoreactivity was specific for basement membrane of surface epithelium and blood vessel endothelial cells (Fig. 8D). In testosterone treated bursa high level of tenascin-C expression. Tenascin-C expression in the bursa follicles appears first at the 16th day of embryogenesis when B cells complete the colonization of the developing follicle buds (data not shown).

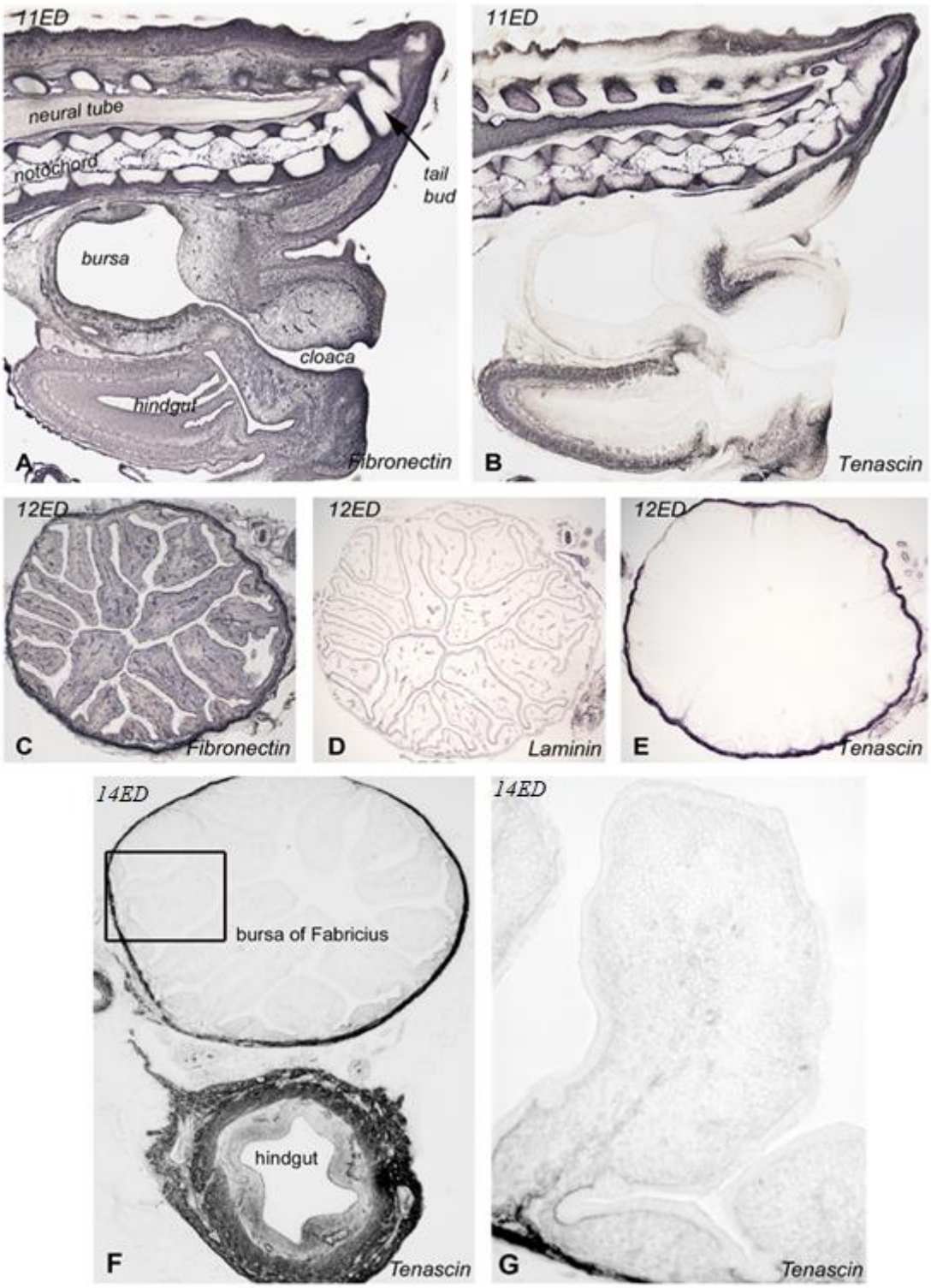

Figure 8. Glycoproteins are differentially expressed in developing chicken bursa of Fabricius. *Serial sagittal sections of 11ED tail bud and transverse sections of 12 ED and 14 ED chick bursa stained for fibronectin (A, C), tenascin-C (B, E, F, G), and laminin (D). Tenascin-C (B, E, F) is not expressed by the bursa mesenchyme. G) Inset is magnified views of a bursal fold.*

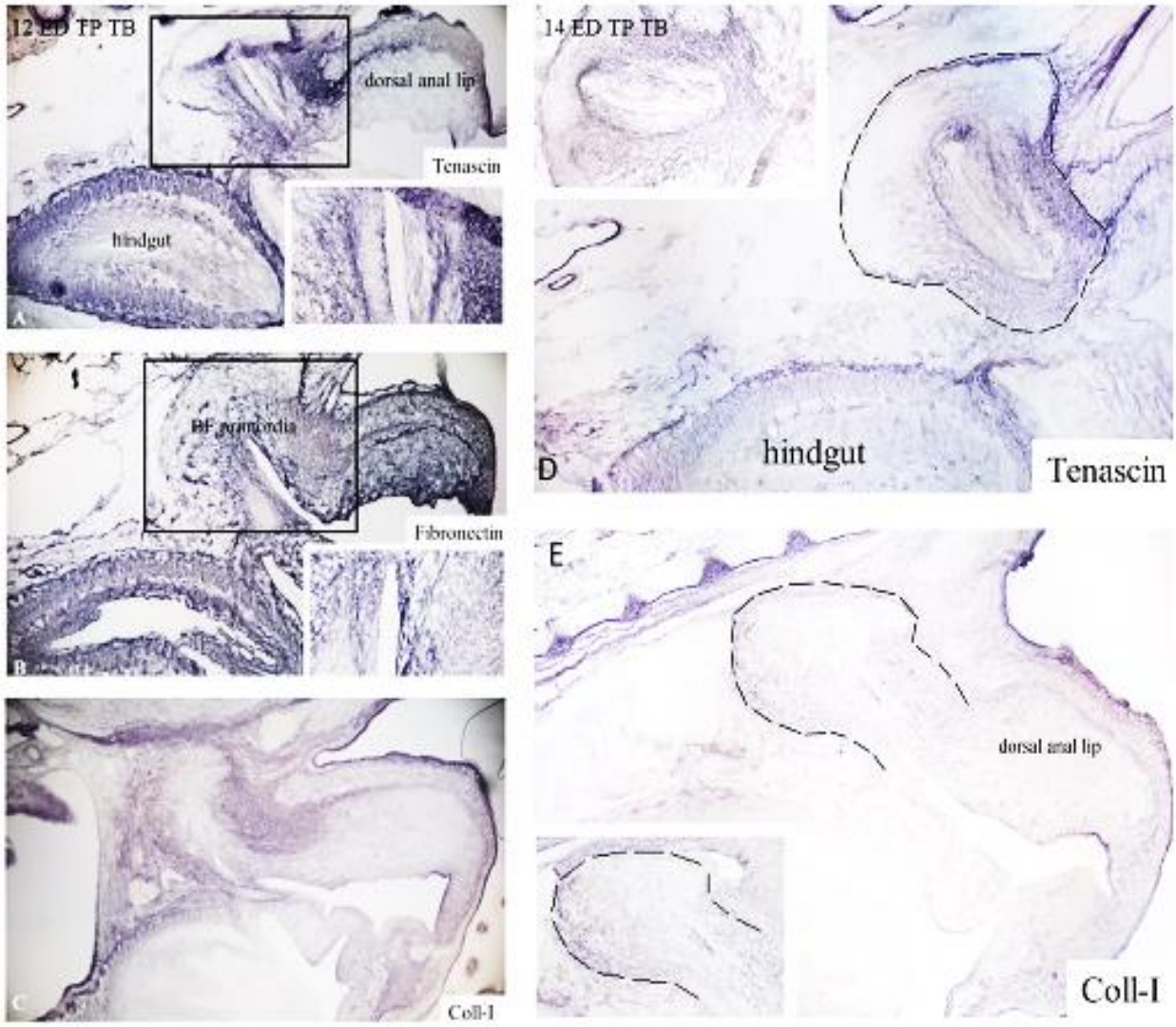

Figure 9. Testosterone treatment disrupts ECM development. *Serial sagittal sections of 12ED (A, B, C and 14 ED (D, E) tail bud stained for tenascin-C (A, D), fibronectin (B), and collagen type-I (C, E). Tenascin-C expression (B, E, F) is strongly induced in the bursal mesenchyme. G) Insets are magnified view of the rudimentary bursal primordium.*

3. Role of extracellular matrix proteins in avian B-cell precursor migration.

According to the previous reports, fibronectin play an important role in the avian B cell migration (Polajoki et al., 1993). Based on our immunohistohemical observations the bursa mesenchyme produces a large variety of ECM proteins during the hematopoietic colonization period. To assess the effect of different ECM proteins on avian B-lymphocyte migration, we applied an *in vitro* migration assay, where we explanted 13ED bursal folds on Petri-dishes

coated with tenascin-C (Fig. 10 B) or fibronectin (Fig. 10 C) proteins and cultured for 24 hours. In uncoated surface only a few B-lymphocytes migrated out from the explant in presence of the CXCL12 chemokine, which has been demonstrated to act as a chemoattractant agent for CXCR4+ bursal B-lymphocytes (Nagy et al., 2020). Fibronectin coated surface induced a robust cell migration. Migration was abolished, when embryonic bursa explants were cultured on the surface coated with 20 µg/ml tenascin-C, B-lymphocytes migrated out in large numbers from the explant, however cells could not spread as seen in fibronectin treated cultures.

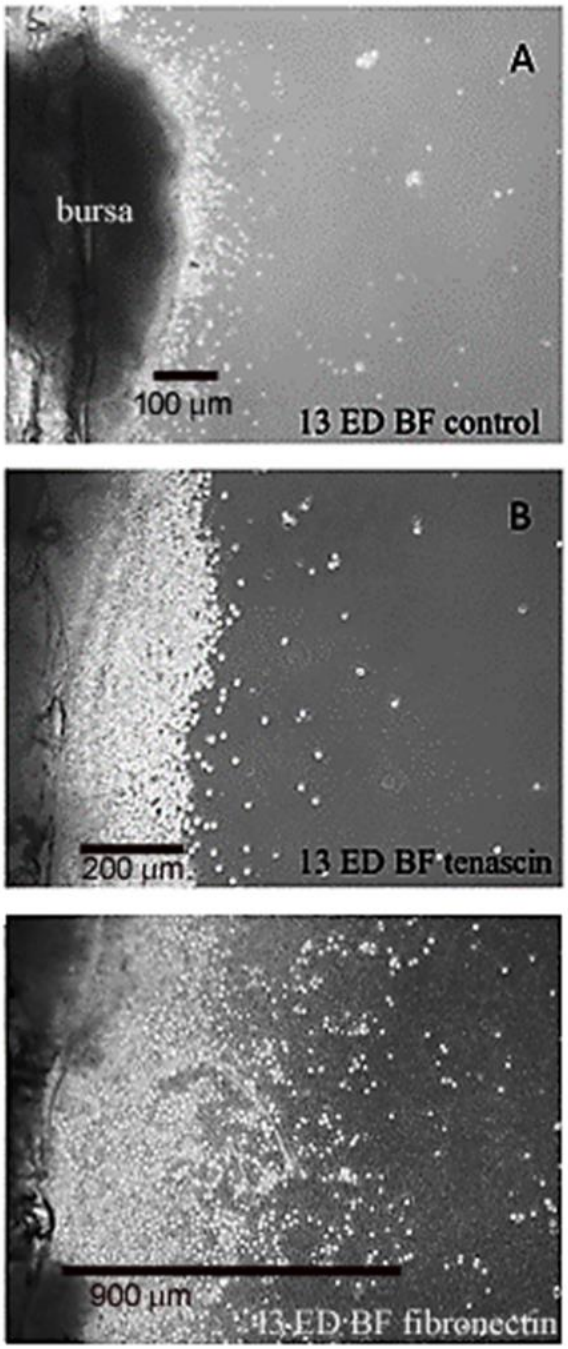

Figure 10. Tenascin-C is inhibitory to B-cell migration. *Control (A) 13-day-old chicken bursa of Fabricius cultured on the untreated surface of a plastic cell culture dish or treated either with tenascin-C (B) or fibronectin (C). The cell culture medium contained the migration-promoting chemokine CXCL12. B-cells migrate well on fibronectin as compared on surface coated with tenascin-C.*

VI. Discussion

My master thesis focused on the developmental changes of extracellular microenvironment of the bursa of Fabricius in testosterone treated and normal chicken embryos. The primary research aims were to characterize how the testosterone treatment influence hematopoietic cell colonization of the bursa of Fabricius and follow the developmental changes in extracellular components of the control and testosterone treated bursa using immunohistochemical methods. Employing *in vitro* organ culture methods, I also examined the role of tenascin-C and fibronectin on avian B cell migration.

Testosterone treatment produces damage in bursa primordium and ablate the immunocompetent B cells in chicken. Several studies have demonstrated that bursal epithelio-mesenchymal rudiment appears during E8-E15 day of chickens, the epithelio-mesenchymal rudiment of developing bursa is significantly smaller and only rudimentary follicles form (Udoumoh et al., 2022; Nagy et al., 2004; Oláh et al., 1986). The earliest marker of hematopoietic stem cells in chicken is CD45 molecule, which identifies the first cell colonizing the bursal rudiment at 8 ED (Dóra et al., 2017). We observed that after testosterone treatment migration of the CD45 immunoreactive hematopoietic stem cells show delayed colonization compared to the normal bursa. Furthermore, the first B cells occur at 12 ED in the normal bursa (Nagy et al., 2004; Dóra et al., 2027) and corresponding to CD45+ cells, the Bu-1+ (chB6) B cell precursors show reduced colonization level in testosterone treated embryonic bursa. These results indicate that delayed colonization of the bursa of Fabricius is either caused by incomplete lymphoid differentiation and inhibition of lymphoid cell proliferation or stromal microenvironment is altered following testosterone treatment. Testosterone treatment does not prevent the entry of CD45+/Bu-1+ B cells into the bursal mesenchyme but the rudimentary follicle buds are not colonized. Previous developmental studies raised the possibility that testosterone affect the mesenchymal compartment of the bursa of Fabricius and during bursectomy primary impairment occurs in the mesenchyme. Indeed, Olah et al (1986) reported that subepithelial mesenchymal cells become elongated and densely packed which may be the primary cause for inhibiting the migration of B cells from the mesenchyme into the epithelial follicle buds. In addition, in testosterone-treated chicken embryos mesenchymal alkaline phosphatase activity is almost completely inhibited, which may be responsible for the paused follicle bud colonization.

Therefore, during my student research studies I have also examined the distribution pattern of extracellular matrix molecules in testosterone treated bursa mesenchyme. Fibronectin is a glycoprotein that plays an important role in the growth and normal development of tissue structures (Palojoki et al., 1993). Expression of fibronectin in normal and testosterone treated bursa of Fabricius was similar at 12ED and 14ED. Collagen-I provides the essential structural framework for tissues (Kisling et al., 2019) and similar to fibronectin, distribution of collagen type-I was not influenced by testosterone treatment. Tenascin-C is an extracellular matrix protein that plays a crucial role in the migration of neural crest cells, primordial germ cells, hematopoietic cells, as well as, in progression of multiple tumor cell types (Orend and Chiquet-Ehrismann, 2006). My immunocytochemical studies revealed that the expression of tenascin-C was strongly induced in bursa mesenchyme of the testosterone treated embryos. Using cell migration experiments, I also demonstrated that addition of tenascin-C inhibits the migration of B cell precursors. This experiment supported the idea that stromal environment for immigrating B cells is not permissive, and ectopic expression of tenascin-C in testosterone treated embryo is inhibitory for the bursa colonization. Expression of tenascin-C can be induced by growth factors (Mackie, 1997). It has been published recently that during bursal hematopoietic cell colonization and lymphoid follicle formation the bursa mesenchyme produces the CXCL12 growth factor, responsible to guide migration of B-cells through the mesenchyme to the surface epithelium (Nagy et al., 2020). Further elucidation of the functional role of the CXCL12 during abnormal bursa development is needed.

In conclusion, my immunocytochemical and embryomanipulation studies demonstrate several novel roles for extracellular matrix in developing bursa of Fabricius and proposes a new hypothesis for tenascin-C produced in the developing bursa mesenchyme as inhibitory for the migration of B-cells. Given the complexity of matrix proteins that comprise the bursal stromal scaffold, a detailed transcriptomic approach would improve our understanding of normal bursa differentiation and early B cell development.

VII. Summary

Bursa of Fabricius is a peculiar primary lymphoid organ in birds. The avian bursa plays a significant role in antibody production, as it has been observed that its surgical removal leads to insufficient antibody production. It is widely accepted that embryonic development of the bursa of Fabricius is impaired under the influence of various androgens. Specifically, testosterone propionate treatment of early chicken embryo results in chemical bursectomy. Previous developmental studies raised the possibility that testosterone affect the mesenchymal compartment of the bursa of Fabricius and during bursectomy primary impairment occurs in the mesenchyme. The general aim of my MSc student research project was to understand the developmental changes of the mesenchymal microenvironment of the bursa of Fabricius during testosterone treatment. Monoclonal antibodies specific for lympho-myeloid cell lineages (anti-CD45, EIVE-12, CSF1R and anti-chB6) and extracellular matrix proteins (anti-laminin, -fibronectin, -collagen type I, and -tenascin-C) has been used to study the bursal folliculogenesis and differentiation of mesenchymal compartment. Testosterone propionate treatment disrupted the colonization of hematopoietic cells into the bursa epithelium. Here, we also identify glycoproteins, including fibronectin and tenascin-C, as important regulators of bursa colonization. In developing chicken bursa of Fabricius fibronectin is strongly expressed in the mesenchyme, while tenascin-C expression occurs later when the follicular cortex start to differentiate. Our *in vitro* migration studies demonstrate that fibronectin is permissive whereas tenascin-C is inhibitory to B cell migration. Increased tenascin-C immunoreactivity have been identified is the bursa mesenchyme followed by testosterone treatment and is absent in control bursa. These studies demonstrate several novel roles for extracellular matrix in developing bursa of Fabricius and proposes a new hypothesis for tenascin-C produced in the developing bursa mesenchyme as inhibitory for the migration of B-cells.

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