DEVELOPMENT OF THE CECAL TONSIL IN CHICKENS

By OLAITAN IJITIMEHIN
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Scientific Advisor: Nandor Nagy PhD.

SEMMELWEIS UNIVERSITY BUDAPEST
Faculty of Medicine
Department of Human Morphology and Developmental Biology

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INTRODUCTION

- Mucosal immunity

The mucosal surfaces of the respiratory, reproductive and digestive tracts are by far the most extensive surface in contact with the external environment. They take part in a variety of physiological functions which include digestion, respiration and reproduction and in addition to these they have a protective function via diverse mechanisms which include barrier functions and specialized, highly developed lymphoid tissues such as the bronchial associated lymphoid tissues (BALT) and the gut associated lymphoid tissues (GALT) (Davidson et al., 2008). The vast majority of human pathogens initiate infections at mucosal surfaces, thus making the gastrointestinal, urogenital and respiratory tracts major routes of entry into the body. This is especially the case in the gastrointestinal tract where organisms reside as part of the normal flora and with the constant influx of pathogens from the external milieu result in an even higher pathogen load which necessitates the need for a highly effective immune capability within it. The lymphoid tissue network sentinels the body from infection. The GALT as a major immunologic system is estimated to contain more immune cells than any other lymphoid tissue (Kasahara et al., 1993) such as the lymph nodes and the spleen (Schat and Myers, 1991). Mucosal immunity has been largely investigated in the gut associated lymphoid tissues such as the tonsils, Peyer's patches and appendix of mammals or bursa of Fabricius and cecal tonsil of birds. In spite of many immunological and morphological studies performed, the precise origin and development of the GALT is less understood.

Chickens are known to be a suitable model for the study of the immunity, morphology and embryology of vertebrate lymphomyeloid organs. Avians do not have lymph nodes as found in mammals; instead they have well developed gut associated lymphoid tissues (Matsumoto and Hashimoto, 2000). Their mucosal associated lymphoid tissues (MALT) are similar to those found in humans. They are also easy to maintain in laboratory settings, its major advantage is the easy accessibility throughout all developmental stages, permitting embryologic manipulations.

- Gut associated lymphoid tissues (GALT)

The GALT of the chicken is comprised of the following (del Cacho et al., 1993; Kajiwara et al., 2003).

a. Esophageal tonsil- The first part of the GALT, located at the junction between the proventriculus and the esophagus (Olah et al., 2003). It is comprised of up to 8 isolated tonsillar units at the base of each esophageal fold (Nagy et al., 2005), therefore the number of tonsillar units is identical to the number of esophageal folds. Each of the tonsillar units is composed of a crypt lined by lymphoepithelium and the surrounding dense lymphoid tissue. Its anatomical location proximal to the stomach allows it to be exposed to undigested environmental antigens.

b. Pyloric tonsil- which consists of 15-20 connective tissue encapsulated tonsillar units forming a ring at the beginning of the duodenum (Nagy and Olah, 2007).

c. Peyer's patches (PP) - are present at the anti-mesenteric side of the jejunum as well as another located in the ileum 5-10cm proximal to the ileo-cecal transition (Befus et al., 1980; Burns and Maxwell, 1986). It consists of lymphoid follicles and inter-follicular lymphoid tissue with overlying lymphoepithelium.

d. Meckel's diverticulum- a remnant of the yolk sac located at the junction between the duodenum and the jejunum (Olah et al., 1984). It is visible as a vermiform protrusion at the anti-mesenteric side of the jejunal wall.

e. Bursa of Fabricius- is a primary lymphoid organ. It is a dorsal outgrowth of the proctodeum restricted to avian species (Cortes et al., 1995). It is diffusely infiltrated with lymphoid cells located in the proximity of the cloacal duct (Odend'hal and Breazile, 1979) and is responsible for the development of B cells (Ratcliffe, 2006).

g. Additional GALT have been described in the region of the cloaca where they are present as diffuse follicles and lymphocytes within the sub-epithelium (Bryant et al., 1973).

f. Cecal Tonsil (CT) - located at the entry to the paired cecal pouches. It is visible macroscopically as an enlargement of the proximal portion of the paired ceca.

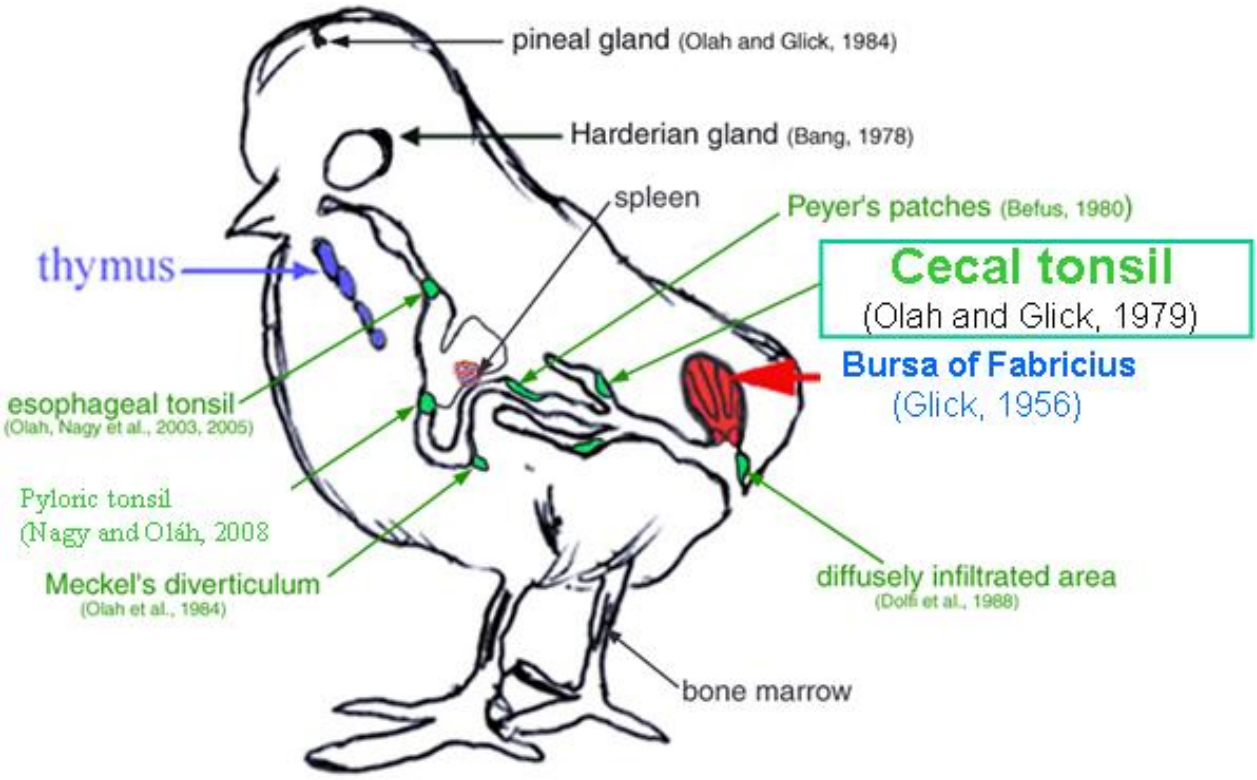

Figure 1: A schematic illustration of the chicken intestinal tract indicating the locations of GALT. The cecal tonsils are located at the base of the paired ceca.

In the adult chicken, the cecal tonsils represent one of the major components of the GALT; it has an important role in antigen sampling (Sorvari and Sorvari, 1977) and is involved in antigen-specific humoral response (Jankovic and Mitrovic, 1967). It is also known to be the site of Eimeria tenella infection which may either elicit no lesion (Whitlock et al., 1975) or cause slight damage (Bemrick and Hammer, 1978) of the cecal tonsil. Newcastle disease is an acute viral infection of great economic significance to the poultry industry worldwide. The viral infection produces hemorrhagic and necrotic lesions within the cecal tonsils. Concerning these immunological and pathologic points of view, the normal morphology and development is of very important.

Although the basic histology and frequency of immunocompetent cells within the cecal tonsil have been studied, the development and cellular differentiation of dendritic cells is currently unknown. Therefore, my research has been carried out to understand the embryonic development of the cecal tonsils of chickens.

- The Cecal Tonsil (CT)

The ceca are 2 blind-ended elongated sacs extending forward from the point of origin at the junction between the hindgut and the foregut; each has three parts that is a base/ proximal part, a middle part and an apex/ distal part. The proximal part has a thickened wall and a relatively narrow lumen, in this region there are large masses of diffuse and nodular lymphatic tissue within the lamina propria and in the submucosa. The cecal tonsils are secondary lymphomyeloid organs occupying up to 18mm at the base of each of the paired ceca and are the most extensively studied structure of the avian GALT. They consist of large aggregates of lymphatic tissue with M-cell rich epithelium that overlies a lamina propria which is highly populated by multiple follicles and a range of leukocytes. It is the largest lymphoid organ of the GALT (Gomez Del Moral et al., 1998). It is a vestigial appendage (Looper, 1929) similar to the human vermiform appendix. Macroscopically the cecal tonsil can be seen as an enlargement in the proximal region of each of the ceca (Muthmann, 1913; Calhoun, 1932; Glick et al., 1978).

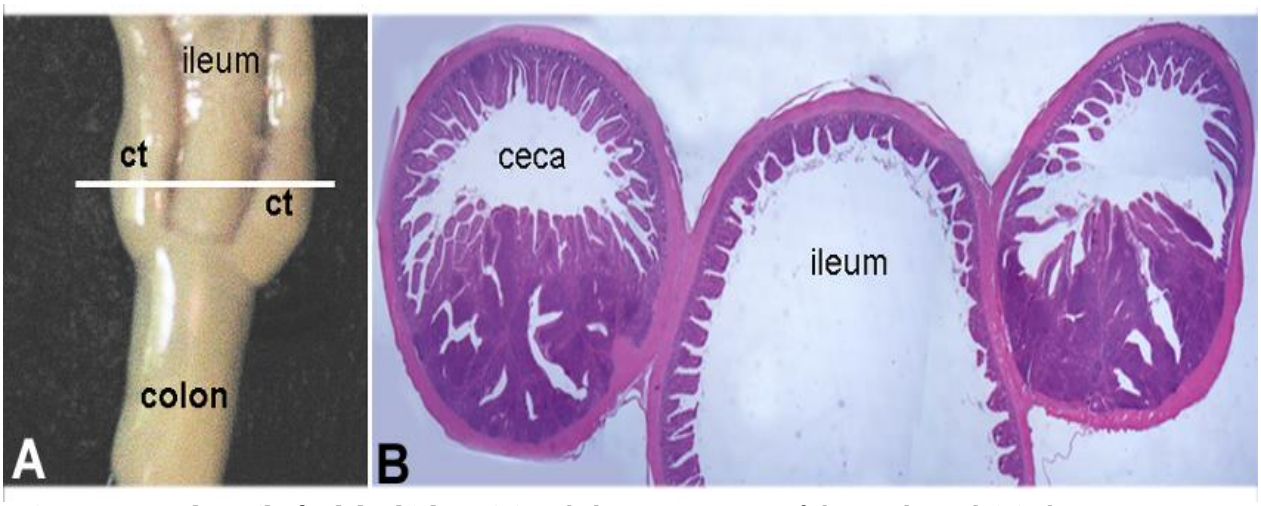

Figure 2: Cecal tonsil of adult chicken. (A). Whole mount picture of the cecal tonsil (ct) showing its gross morphology. (B) Hematoxylin-eosin stained cross section of the adult chicken cecal tonsil showing the asymmetric accumulation of lymphoid tissue.

The luminal view shows spatula-type villi (Calhoun, 1932; Wilkins and Lee, 1974; Glick et al., 1978) among which are mounts of lymphoid aggregates covered by overlying lymphoepithelium.

Histologically the CT is recognized as an aggregate of lymphoid tissue which displaces the muscularis mucosae and tunica muscularis (Kitagawa et al., 1998) at the anti-mesenteric border of the proximal region of the ceca. It consists of a specialized lymphoepithelium, a sub-epithelial region, germinal follicles and inter-follicular areas. The lymphoepithelium contains columnar epithelial cells and is M-cell rich (Kitagawa et al., 1998; Kitagawa et al., 2000); this epithelium is different from that of neighboring tissues (Jeurissen et al., 1999). The epithelium overlies a lamina propria which is highly populated with multiple follicles and a range of different leukocytes e.g. dendritic cells, granulocytes and macrophages. The aggregates contain B and T lymphocytes (Albini and Wick, 1974) and leukocytes. Within the lymphoid aggregates are 2 morphologically distinct types of germinal centers; a) encapsulated- that is encircled by reticular cells and b) partially encapsulated- that is incompletely surrounded by reticular cells (Olah and Glick, 1979). The germinal centers are composed of primitive reticular cells, small lymphocytes and large lymphocytes, lymphoblasts, immature and mature plasmacytic cells. The germinal centers consist of IgG and IgM positive cells (Jeurissen et al., 1989) as well as T lymphocytes, within the germinal centers there is also a secretory cell with yet unidentified function, large central lymphoblasts and small peripheral lymphoblasts (Glick et al., 1981). The plasma cells are located outside the germinal centers deep within the tunica propria and in the sub-epithelial zones (Hoshi and Mori, 1973; Olah and Glick, 1979).

The basic unit of the cecal tonsil consists of a central crypt which has several branches, germinal centers and interspersed T cell regions (Casteleyn et al., 2010); this unit is structurally similar to the mammalian palatine and lingual tonsils. The crypt is surrounded by diffuse lymphoid tissue and several germinal centers and its epithelium is invaded by small lymphocytes. The follicles with the epithelial cells above them are classified as a tonsillar unit; tonsillar units are separated from neighboring units by connective tissue. There are calculated to be approximately 400 basic units in the polycryptic cecal tonsil (Glick and Olah, 1981).

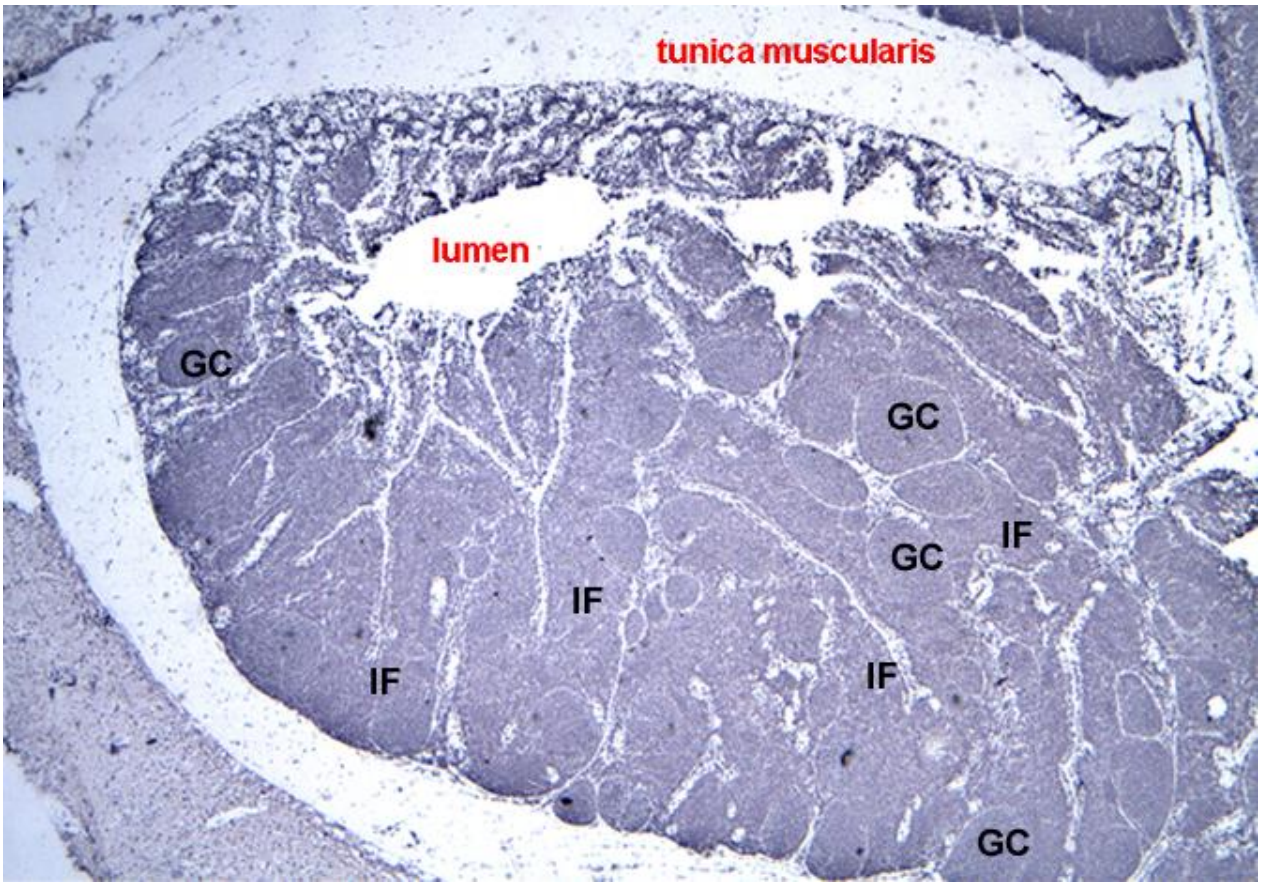

Figure 3: A typical cecal tonsil of the chicken. Frozen section stained with anti-CD45 (common leukocyte antigen) antibody shows two tonsillar units composed of germinal centers (GC) and inter-follicular regions (IF).

Bacteria (streptobacilli) are attached to the surface villi but do not penetrate the cells (Fuller and Turvey, 1971). The bacterial presence is influenced by the environment and plays a role in regulating the intestinal flora. These bacteria adhere to the proximal end and not to the distal end of the ceca; this is thought to reflect a difference in the function of the surface epithelium of these regions. The epithelium in this proximal region is highly reactive to stimulation by antigens as noted by an increase in pyroninophilia after antigen challenge.

The development of the germinal centers within the cecal tonsil relies on the function of the bursa of fabricius and the thymus (Jankovic et al., 1966), removal or elimination of these influences either by resection or irradiation results in a markedly reduced number of germinal centers and an altered cellular makeup within the cecal tonsil (Jankovic et al., 1966; Hoshi and Mori, 1973). It appears in the late embryonic period but its main development occurs after hatching. Lymphoid nodules appear approximately a week after hatching but diffuse lymphoid tissue may be seen earlier, the initial cells are the small and large lymphocytes, later

monocytes and granulocytes appear which are thought to arise from the embryonal mesenchymal layer. The number of germinal centers increases with age and reaches a maximum after approximately 8 weeks of life. The stimulus for its development remains unknown, the colonization of the gut by flora or the presence of food was thought to be the initiating event but other studies showed that the lymphocyte homing in the cecal tonsil may occur in the absence of these stimuli (Kajiwara et al 2003). It has been reported that the precursors of dendritic cells of the primary lymphoid organs, which express CD45 (common leukocyte antigen) and MHC II, migrate into the bursal epithelium and form the lymphoid bud of the follicles. These cells provide a dendro-epithelial environment for the differentiation of lymphocytes (Nagy et al 2009; Nagy and Olah 2010). These findings suggest that the dendritic precursor cells work as the inducer cells for the lymphoid organogenesis. However the role of such inducer cells in the cecal tonsil or other GALT is not currently understood.

AIMS

1) The immunocytochemical characterization of the cellular components of the cecal tonsil.
2) To follow the embryonic formation of the cecal tonsil using hematopoietic cell specific antibodies (T lymphocytes, B lymphocytes, dendritic cells and macrophages).

MATERIALS AND METHODS

Animals:

Fertilized White Leghorn Chicken (Gallus gallus) specific-pathogen-free (SPF) eggs were obtained from BiOvo Ltd. (Mohács, Hungary). Eggs were incubated to desired stages at 38°C and 60%-os humidity in a humidifier incubator. Embryos were staged according to the number of days of incubation (ED). Adult animals were staged according to their age after hatching (W: week; M: month). Experimental design and conditions were approved by the Animal Ethical Committee of Semmelweis University, Budapest, Hungary (Leg. No.779-000-2005). The animals were killed by decapitation after anesthesia with a ketamine - diazepam combination, for up to one week post-hatching cervical dislocation was sometimes used.

Clone name	**Antigen**	**Species reactivity (in this study)**	**Host species**	**Isotype**	**Dilution**	**Manufacturer**
HIS C7	CD45	Ch	M	IgG_{2a}	1:200	Prionics (formerly known as CEDI Diagnostics)
BoA1	Bu1a+b	Ch Q	M	IgG_1	undiluted supernatant	developed in our laboratory*
5-11G2	Bu1b	Ch*	M	IgG_1	1:200	SouthernBiotech
Tap1	MHC class II	Ch	M	IgG_{2a}	1:100	Developmental Studies Hybridoma Bank
CVI.Ch.74.2	unknown (present on chicken macrophages)	Ch	M	IgG_1	1:200	Prionics (formerly known as CEDI Diagnostics)
Rabbit Anti-Human CD3	CD3	Ch Q	R	polyclonal	1:200	DakoCytomation
CVI.Ch.74.3	unknown (present on chicken dendritic	Ch	M	I gG_1	1:200	Prionics (formerly known as CEDI Diagnostics)

	cells)					
Amf-17b	Vimentin	Ch Q	M	IgG_1	undiluted supernatant	Developmental Studies Hybridoma Bank
Lu-5	Cytokeratin intermediate filament	Ch	M	IgG1	1:100	BMA, Biomedicals AG Augst, Switzerland
1A4	Alpha-smooth muscle actin	Ch	M	IgG_{2a}	1:200	DAKO
3H11	laminin	Ch	M	IgG1	1:5	Developmental Studies Hybridoma Bank

Table 1. Primary antibodies.

Embryo dissection and tissue preparation

Sample collection:

Midgut-hindgut junctions with cecal primordia were isolated using fine forceps and iris scissors. Samples were collected in PBS to avoid drying.

Gelatin-sucrose embedding: For embryonic organs

In order to avoid organ distortion guts were stabilized with insect pins in silicon coated petri dishes. Tissues were fixed in 4% paraformaldehyde (PFA) for 1 hour at room temperature, then washed in PBS three times for 5 minutes and infiltrated with 15% sucrose (Reanal) in PBS overnight at 4°C. The solution was changed to 7.5% gelatin (Fluka; 04055) with 15% sucrose at 37°C for 1–2 h. Specimens were placed into plastic plates containing warm gelatin-sucrose solution. After setting the proper position the plates were left at room temperature for 5 minutes to allow the gelatin solidify. Tissue blocks were cut out and fixed on labeled carton papers with Shandon Cryomatrix (Anatomical Pathology Ind., 67690006), they were rapidly frozen at −60°C in isopentane (Fluka, 59075). Blocks were stored at -80°C till use.

Liver embedding: For adult tissues

Lymphoid organs (cecal tonsil, spleen, thymus, bursa of fabricius) were collected in PBS and placed on pieces of carton paper. Specimens were covered by liver pieces in order to avoid drying and for cryoprotection. Liver embedded blocks were frozen in liquid nitrogen and stored at -80°C until sectioning.

Solutions:

- Phosphate buffered solution (PBS): for 1liter: NaCl 8g, KCl 0,2g, Na_2HPO_4 x $2H_2O$ 1,42g, KH_2PO4 0,2g, 1000ml distilled water.
- 4%-os paraformaldehyde (PFA): 40g paraformaldehyde powder were added to 700ml distilled water at 60°C and placed on onto a magnetic stirrer. Precipitation was dissolved by adding 20% (5M) NaOH. After filtering the solution 250ml 0,24M (10x concentrated) PBS were added and pH was adjusted to 7.2, then the solution's volume was filled up to 1000ml. The fixative was stored in aliquots at -20°C.
- PBS-BSA: 1g Bovine Serum Albumin (Sigma A9647) was dissolved in PBS. Sodium-Acid (NaN_3) was added for 0.1% final concentration as preservative. Stored at 4°C.
- Chloronapthol - chromogen for immunohistochemistry: In preparing a stock solution, 500mg of 4-chloro-1-naphtol (Sigma, C8890) was dissolved in 2ml of absolute ethanol, and then was stored at -20°C. For use in procedures, the stock solution was dissolved in PBS to get a 25mg/100ml final concentration.

Sectioning:

Frozen sections were made by using a Shandon Cryotome. Gelatin blocks were sectioned at -24°C. In case of liver embedded specimens working temperature was set to -19°C. 8-12 μm thick sections were made and collected onto poly-L-lysine (Sigma, P8920) coated slides. Slides were stored at -20°C.

Immunohistochemistry and immunofluorescence:

Immunohistochemistry:

The slides were put into 37°C PBS for 10mins in order to rehydrate the sections and to dissolve the gelatin. Sections were incubated with primary antibodies. (See Table 1) (40-80µl / section) for 45minutes at room temperature within a humid chamber. Then the slides were washed in PBS for (3x 5minutes).

Biotinylated secondary antibodies were diluted 1:200 in PBS-BSA and were chosen regarding to the host species and isotypes of the primary antibodies (anti-mouse IgG Vector Laboratories BA-2000). Sections were incubated with secondary antibodies for 60 minutes followed by a PBS washing step (3x5 minutes). Endogenous peroxidase activity was quenched by 1% H_2O_2 diluted in PBS (Sigma, H1009) for 8-10 minutes. Another washing step in PBS was carried out (3x5 minutes). Secondary antibodies were detected by avidine-biotin-peroxidase complex (ABC; Vectastain Elite PK-6100; Vector Laboratories). ABC components were diluted 1:100 in PBS. Sections were incubated with ABC for 30 minutes and washed in PBS for (3x5 minutes). Visualization of the immunoreactions was carried out by 25 mg 4-chloro-1-naphtol (Sigma; C8890) dissolved in 100 ml PBS in the presence of 500 µl 3% H_2O_2. The sections were immersed in the filtered chromogen for 30 minutes. The reaction was stopped by washing in PBS (3x5 minutes). Slides were covered with water soluble mounting medium (Poly-Aqua Mount, Polyscience Inc., Warrington PA, USA; 18606) and glass coverslips. Slides were stored in slideboxes at 4°C.

Immunofluorescence:

- Single immunofluorescence:

Rehydration and primary antibody steps were the same as written above followed by adding 40-80µl fluorescein coupled secondary antibodies (Table 2) with suitable species and isotype reactivity. Secondary antibodies were used in 1:100 dilutions in PBS. 20 minutes incubation with DAPI solution (4,6 diamidino-2-phenylindole-dihydrochloride, Invitrogen, D1306) was used for cell nuclei visualization. Slides were mounted with water based mounting medium. All steps were carried out protected from light.

- Double immunofluorescence:

Double immunofluorescence staining was carried out by using two single immunofluorescence solutions done one after the other. During the second immuno-labeling, fluorescein conjugated secondary antibodies were chosen having distinct isotype and emission spectrum from the former labeled antibody.

		Peak excitation wavelength	
reactivity	**dilution**	**488nm**	**594nm**
Anti-Mouse IgG	1:100	A-11001	A-11005
Anti-Mouse IgG_1	1:100	A-21121	A-21125
Anti-Mouse IgG_{2a}	1:100	A-21131	A-21135

Table 2- Fluorescein coupled secondary antibodies obtained from Invitrogen (Life Technologies)

Image processing:

Sections were photographed with Zeiss Axiophot microscope equipped with a Zeiss AxioCam HRC digital camera. Image post-processing was done by using Adobe Photoshop 7.01 software.

RESULTS

In order to follow and characterize the development of the cecal tonsil in the chicken embryo, immunohistochemical staining was performed.

- **The development of the cecal tonsil in embryonic life**

Gross anatomical examination: The cecal swelling can be discerned morphologically by the fourth embryonic day, by the 5^{TH}-6^{TH} embryonic day the cecal buds can be recognized as two blind diverticula extending ventrolaterally from the junction between the ileum and the colon.

NINTH EMBRYONIC DAY: At the 9^{TH} day of embryogenesis, the cecal tonsil was not macroscopically discernable. Histological examination of the ileo-cecal transition was made. The layers of the wall were present (mucosa, submucosa, muscular layer and the serosa), the muscular layer was well developed (Fig 4c), the lumen of the intestine was patent and devoid of content except for trace amounts of a mucus-like material. The luminal wall was lined with columnar epithelial cells. There is an infiltration of the intestine by sparse amounts of CD45 expressing cells (figure 4b), the cells are randomly scattered throughout the tissue without any discernable aggregation. These cells were located within the lamina propria and submucosa, they exhibit a round morphology.

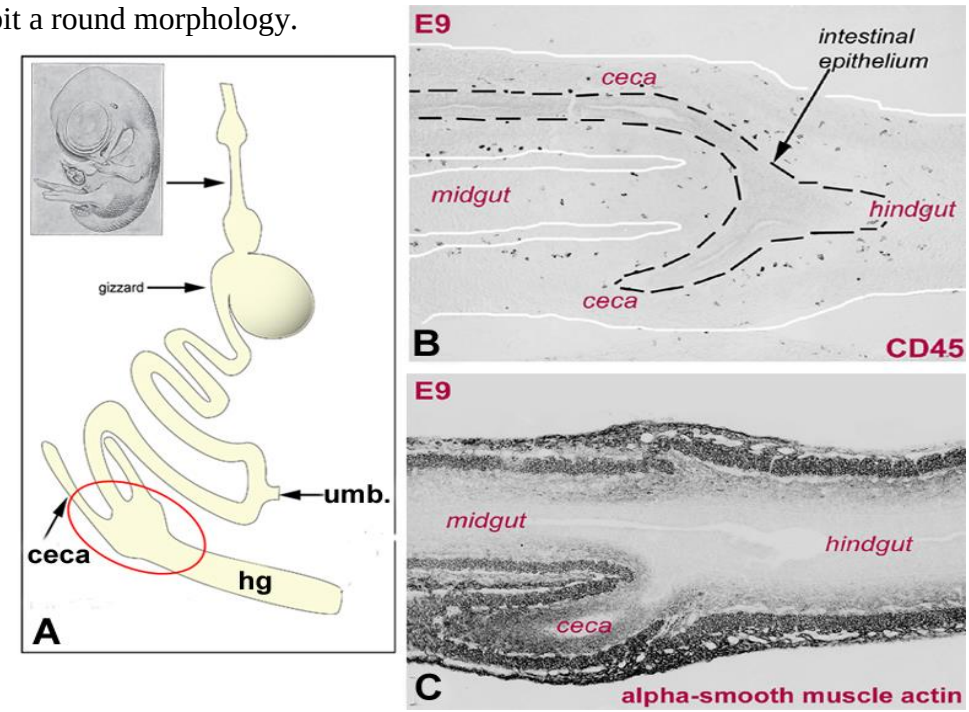

Figure -4: Development of the chicken cecal tonsil in the 9 day old chicken embryo. (A) Schematic diagram shows the region of the subsequent sections (hg-hindgut; umb-umbilicus). Longitudinal section of the ileo-cecal transition stained immunohistochemically with CD45. (B) and alpha-smooth muscle actin (C)

ELEVENTH EMBRYONIC DAY: a cluster of CD45 expressing cells were present near the ileo-cecal junction (Fig 5a) which is considered to be the presumptive site of the cecal tonsil. The cells were localized within the tunica propria of the mucosal layer (Fig 5b). The cells exhibit a similar round morphology as the earlier stage. Among these cells, serial sections of the same area revealed the presence of 1-2 Bu1 expressing cells (Fig 5c) and a few MHC II expressing cells (Fig 5d). At this stage is the first discernable aggregation of cells within the lamina propria denoting the site of the future cecal tonsil, the appearance of these cells before hatching implies that this site is genetically determined and not reliant on environmental antigens as previously suggested. The cell subpopulations also suggest that the MHC II cell population is present in the CT prior to the Bu1 expressing cell population.

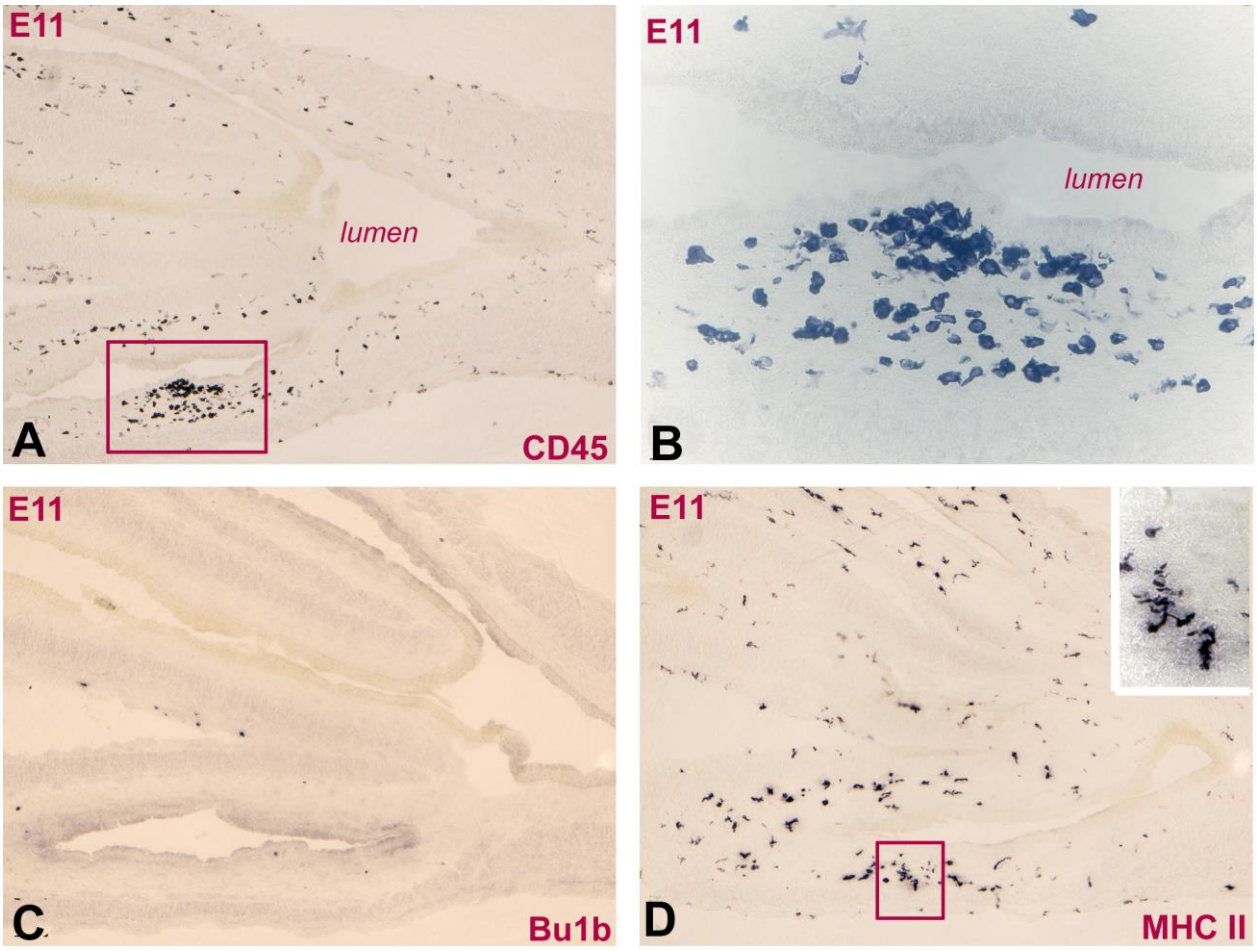

Figure -5: Development of the chicken cecal tonsil in the 11th day old chicken embryo. Serial sections of the cecal tonsil taken at E11 (A) accumulation of CD45 expressing cells beneath the epithelium of the cecal tonsil. (B) a magnified view of the area denoted by the box in A (C) Bu1b stained section shows 1-2 positive cells among this population (D) MHC II stained section shows a higher population of positive cells, the magnified view of the area delineated by the red box (right upper corner of picture).

THIRTHEENTH EMBRYONIC DAY: The muscular wall of the intestine is better developed (Fig 6a). The cluster of CD45 expressing cells expanded in the lamina propria of the CT (Fig 6b) as well as the population of cells infiltrating the intestine. Of the cell cluster, two distinct morphologies could be discerned a) round cells and b) ramified cells (Fig 6c). The population of MHC II expressing cells in this area also increased as observed in serial sections (Fig 6d). These MHC II expressing cells showed a similar ramified morphology. Serial section of the same region reveals an increase in the population of Bu1 expressing cells (Fig 6e); these cells were noted to be of round morphology.

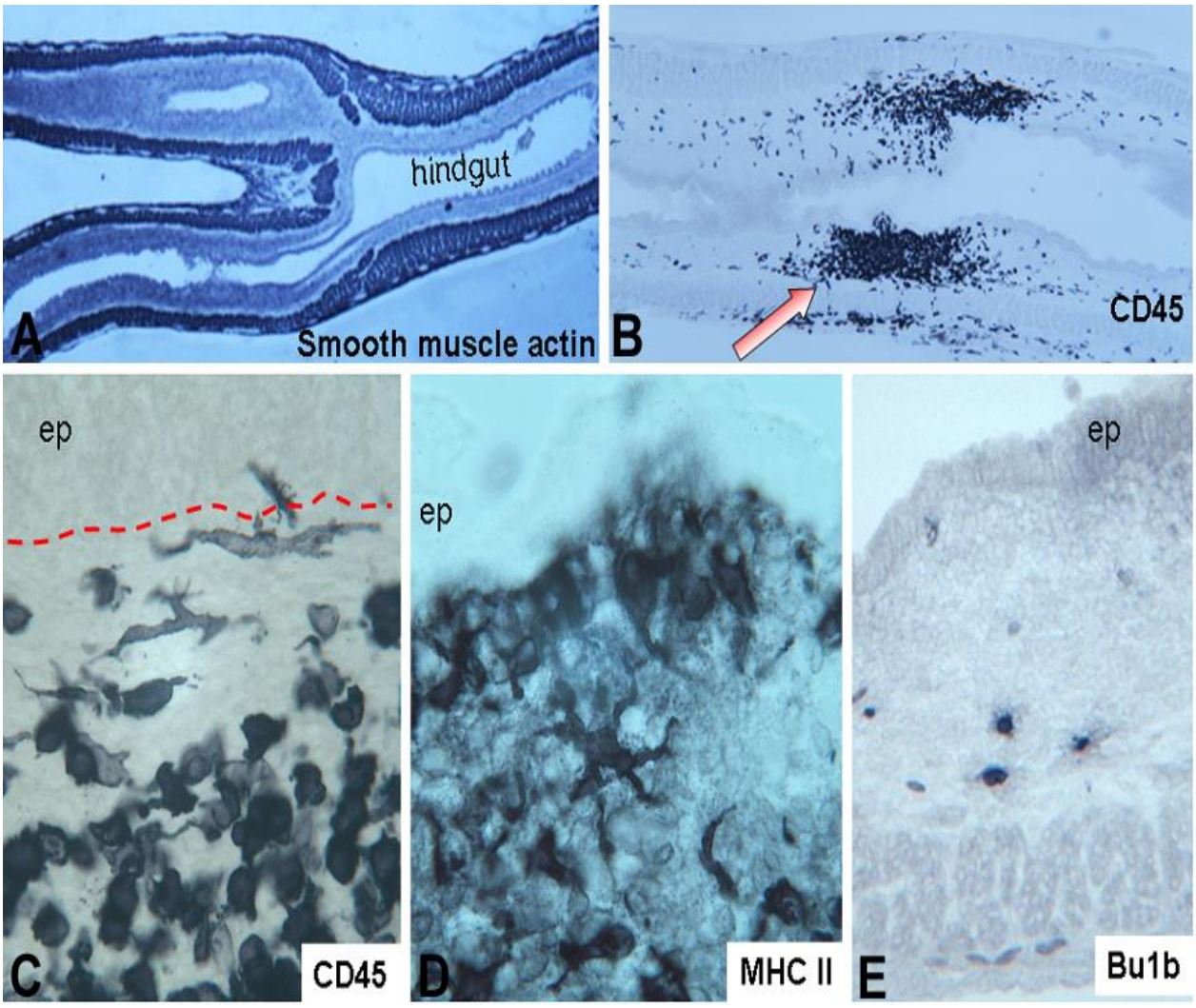

Figure -6: Development of the chicken cecal tonsil in the 13th day old chicken embryo. Immunohistochemical staining of serial sections of 13th embryonic day old chicken cecal tonsil. (A) Smooth muscle actin (B-C) CD45 (D) MHC II- shows the ramified structure of these cells (E) Bu1b.

FIFTHTEENTH EMBRYONIC DAY: At the 15TH day of embryogenesis the mucosal wall has numerous folds that protrude into the lumen of the gut. The cell populations aggregated underneath the epithelium of the cecal tonsil increase (Fig 7a). Serial sections of the same area show an increase in the Bu1 expressing (Fig 7b), 74.3 expressing (Fig 7c) and MHC II expressing cell populations (Fig 7e). These cells remain underneath the epithelium.

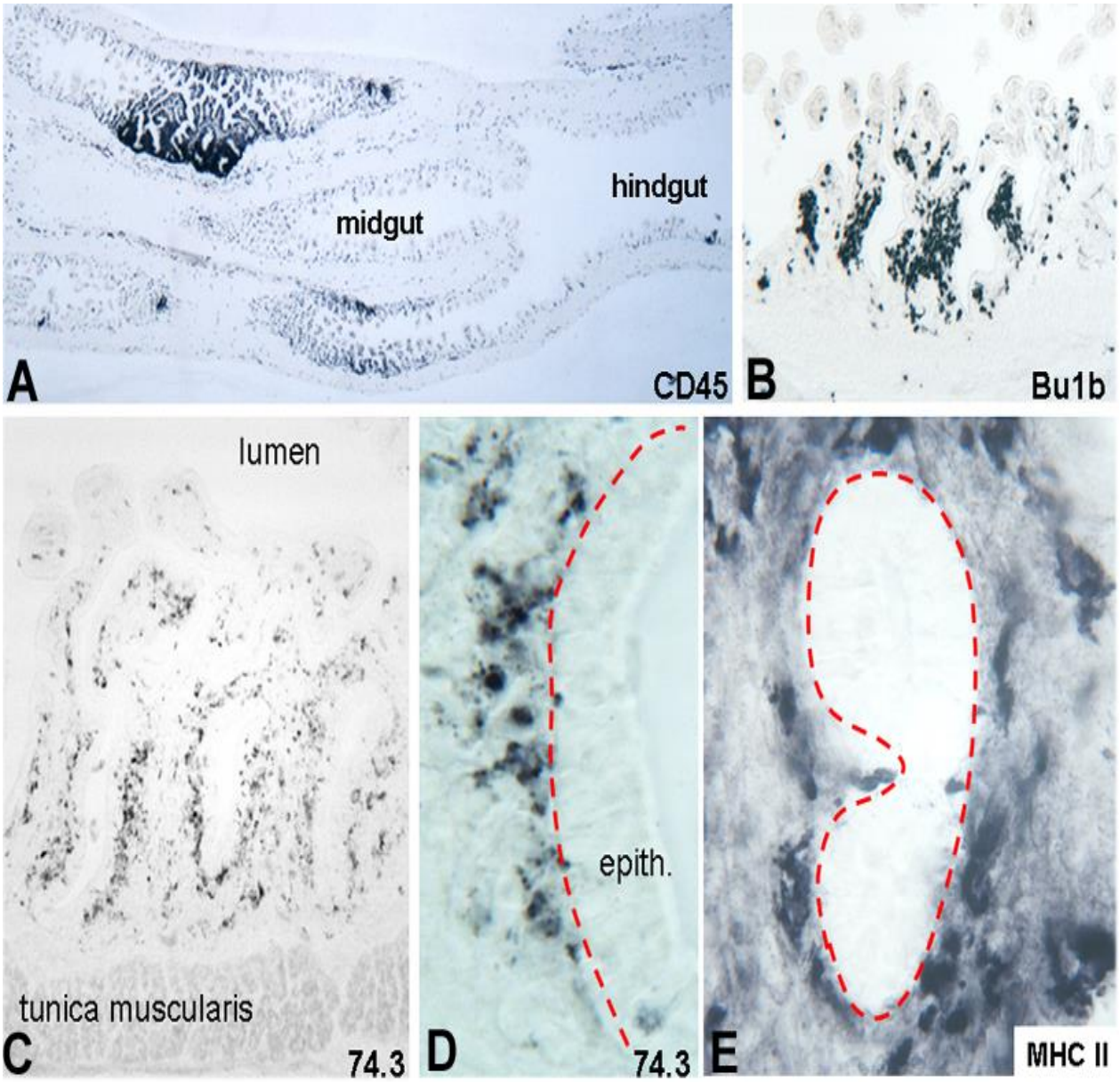

Figure - 7: Development of the chicken cecal tonsil in the 15 day old chicken embryo. Immunohistochemical staining of serial sections with (A) CD45 (B) Bu1b (C) 74.3 (D) a magnified view of C {74.3}, (E) MHC II.

SEVENTEETH EMBRYONIC DAY: As expected there was an increment in CD45 expressing cell population within the CT (Fig 8a). Some of these cells infiltrate the overlying epithelium (Fig 8b) to induce the formation of lymphoepithelium.

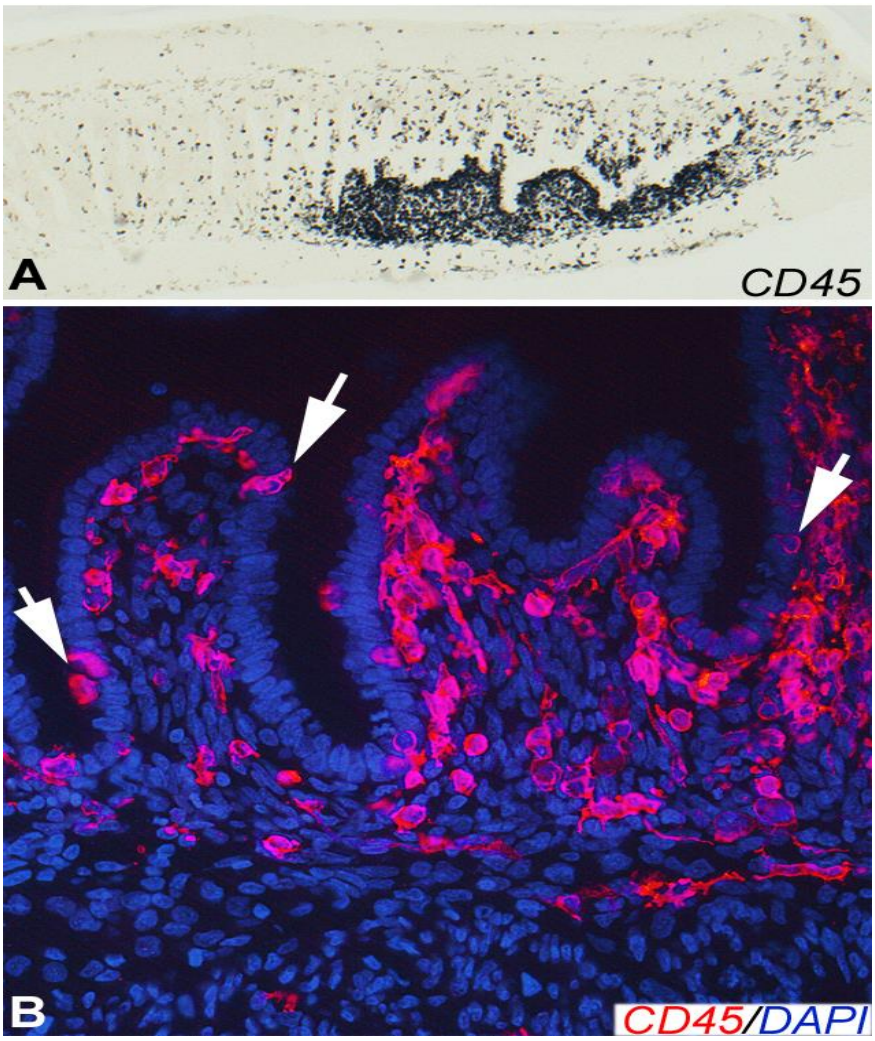

Figure -8: Development of the chicken cecal tonsil in the 17 day old chicken embryo. Longitudinal serial sections. (A) Immunohistochemical CD45 staining of the cecal tonsil. (B) Immunofluorescence staining of the cecal tonsil (DAPI-all cell nuclei; CD45-hematopoietic cells). Arrows: cells infiltrating the epithelium.

EIGTHTEENTH EMBRYONIC DAY: There is a further expansion of the CD45 cell population within the lamina propria (Fig 9a). Among these cells, serial sections of the same area shows the presence of 74.3 expressing cells (Fig9b), these 74.3 positive cells accumulated underneath the epithelium while other CD45 positive cells infiltrated the epithelium (Fig 9c-9d)

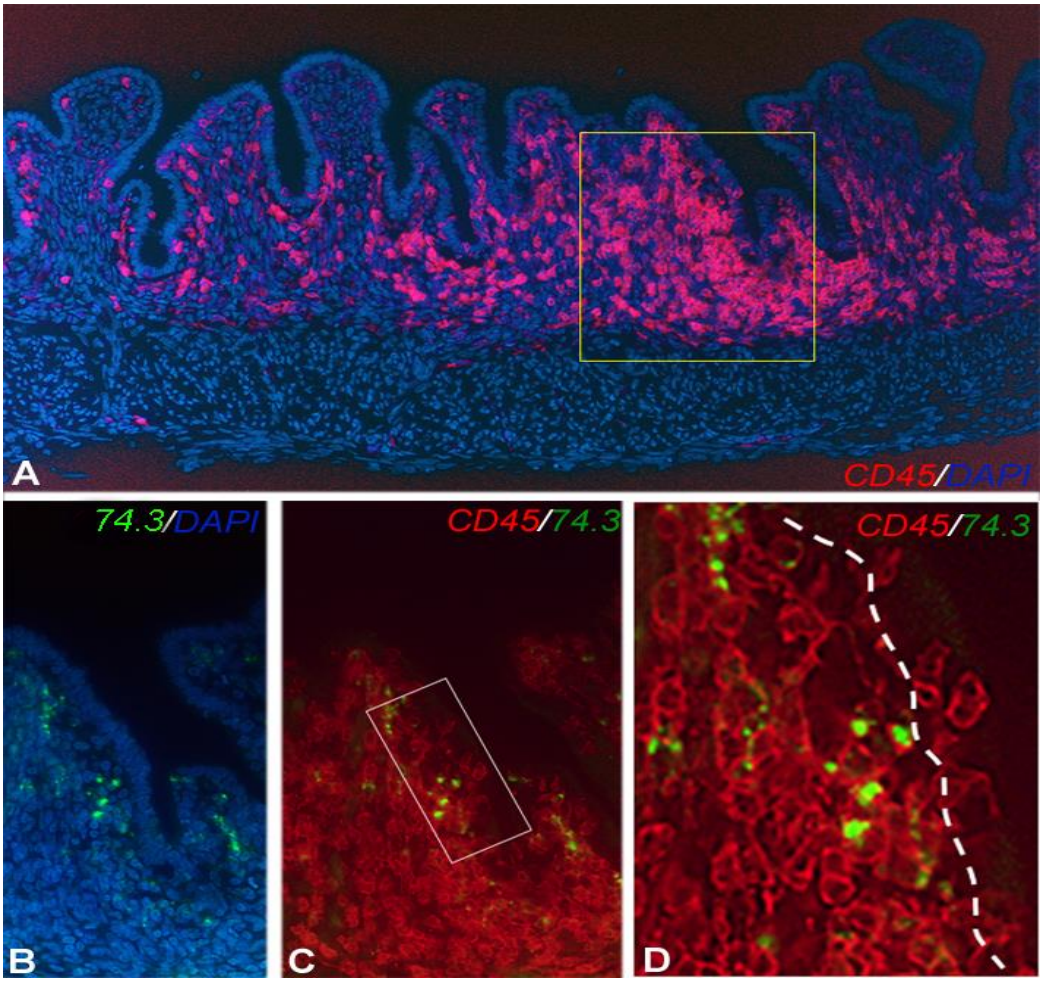

Figure - 9: Development of the chicken cecal tonsil in the 18 day old chicken embryo. Double immunofluorescence staining of serial sections (a) DAPI-all cell nuclei /CD45. (B) 74. 3/DAPI, (C) CD45/ 74.3 (D) magnified view of the area within the rectangle in C.

- **<u>The development of the cecal tonsil in post-embryonic life.</u>**

The cecal tonsil of the adult chicken is discernable morphologically as an enlargement of the proximal region of the paired ceca. There is an asymmetric accumulation of lymphoid tissue in the anti-mesenteric wall of the cecum. The mucosa overlying this medial wall is thrown into more extensive folds with fossulae and crypts than the adjacent mucosa. The lumen is lined with tall columnar cells interspersed with numerous goblet cells and is infiltrated by leukocytes. Within the lamina propria is a dense lymphoid tissue which displaces the muscularis mucosae. The submucosa contains follicles extending from the tunica propria. The muscular layer is composed of smooth muscle cells arranged in a thicker inner circular layer and a thinner outer longitudinal layer and is surrounded by a serous layer.

There was a numerical increase in the cell population within the cecal tonsil (Fig 10a and c). The population of Bu1 expressing cells is also increased as shown in serial sections (Fig 10b). The lymphoid tissue contains numerous germinal centers, within which there are mainly Bu1b and 74.3 expressing cells (Fig 10g-h) while the CD3 and 74.2 expressing cells were localized in the inter-follicular spaces (Fig 10f and 10i).

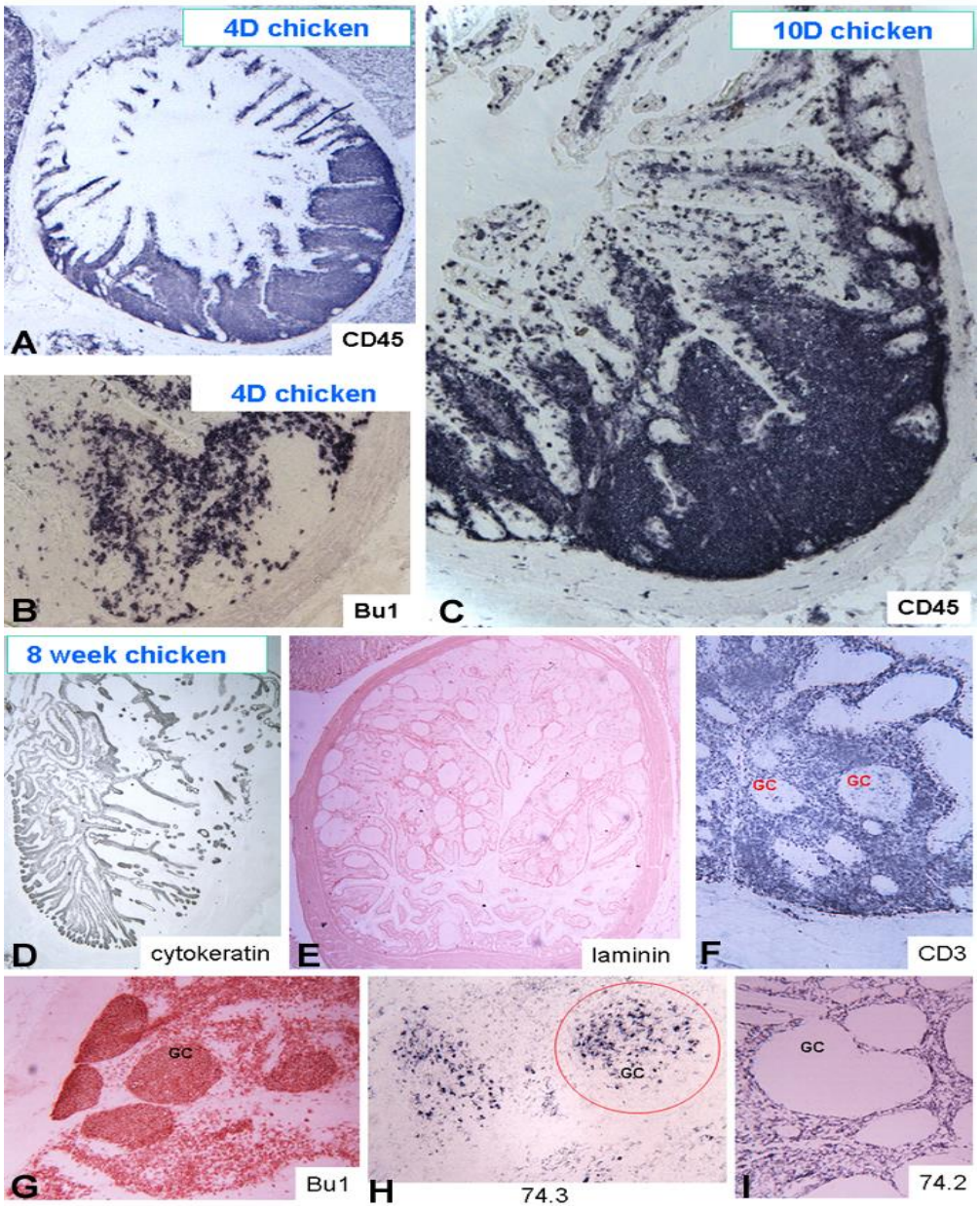

Figure 10: immunohistochemical staining of cross sections of the adult cecal tonsil. (A) Section taken from a 4 day old chicken showing the hematopoietic cells expressing CD45. (B) Section taken from a 4 day old chicken showing the B lymphocytes expressing Bu1. (C) cross section of the 10 day old chicken cecal tonsil stained with CD45 (hematopoietic cells),(D)-(I) serial sections of the cecal tonsil taken at the 8 week post-hatching, were stained immunohistochemically with cytokeratin (epithelial cells), laminin (basement membrane), CD3 (T lymphocytes), Bu1 (B lymphocytes), 74.3 (dendritic cells) and 74.2 (macrophages). gc- germinal center.

DISCUSSION:

The GALT of the chicken is made of solitary lymphoid cells and aggregates forming nodules or organs for example the cecal tonsil and bursa of fabricius. The cecal tonsil is the largest of these chicken gut associated lymphoid tissues.

Anatomically the cecal tonsil is located at the neck/proximal region of the paired blind-ended ceca. It starts as a ceca swelling as early as the 4th embryologic day, by the sixth day of embryogenesis the cecal buds can be recognized as two blind diverticula extending ventrolaterally at the ileo-cecal transition. The appearance of the cecal tonsil has been observed prior to hatching as lymphoid infiltration into the presumptive site. My research indicates that the infiltration of the gut by hematopoietic cells starts by the 9th day of embryogenesis although the definitive accumulation of cells within the cecal tonsil does not occur until the 11th day of embryogenesis. This accumulation is heralded by the appearance of CD45 expressing cells followed by MHC II and Bu1 cell populations. As embryonic development proceeds, there are continuous increments in the cell populations; by the 17th embryonic day some of these accumulated cells begin to infiltrate the epithelial layer overlying the aggregate to induce the formation of lymphoepithelium. These infiltrating cells were characterized via immunohistochemistry as CD45 positive but 74.3 negative cell populations, the 74.3 expressing cells remained underneath the epithelium unlike in the primary lymphoid organs such as the bursa of fabricius where the 74.3 expressing cells infiltrate the epithelium (Nagy et al, 2004. Nagy and Olah, 2010), instead in the cecal tonsil these cells remain in the sub-epithelium and induce mesenchymal development.

After hatching the cecal tonsil undergoes most of its growth as is noted by the rapid expansion of all cell populations via the proliferation of the resident cells as well as a massive migration of circulating lymphocytes to the region. The lymphoid tissue also undergoes organization into germinal centers (B lymphocyte and dendritic cell rich regions) and inter-follicular areas (T lymphocyte and macrophage rich regions). The cecal tonsil is further organized into units each made up of a central ramified crypt, germinal centers and their interspersed inter-follicular areas. The post-embryonic growth occurs rapidly, reaching adult conditions by the 4th day after hatching, the number of germinal centers continues to increase and reach a peak at week 8 after hatching.

In conclusion the results of my immunohistochemical characterization of the cecal tonsil suggests that the development of the cecal tonsil starts at the 11^{TH} day of embryogenesis and is hallmarked by the infiltration of the region by a CD45 expressing cell population, two days later (13^{TH} embryonic day) several of these cells express the MHCII antigen. The accumulation of these cells in the presumptive site of the cecal tonsil occurs without an antigen stimulus which suggests that the location and development of the cecal tonsil is genetically determined, the actual gene/genes responsible for this is yet to be characterized The cell population within the cecal tonsil gradually increase due to the migration and proliferation of cells. The appearance MHCII and 74.3 expressing dendritic cells under the epithelium supports the notion that the formation of the cecal tonsil is initiated by the accumulation of CD45+ve, MHC II+ve and 74.3+ve cells which go on to induce the formation of the lymphoepithelium.

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