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T Lymphocyte Selection is Indispensible for the Development of Goose Bursa of Fabricius

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ABSTRACT

The process of T Lymphocyte involvement in the development of avian bursa of fabricius remains unclear. In order to clarify the feature of this process, the fetal goose bursas of fabricius from E17 (17-day-embryo) to 18 d (18-day after hatching) were adopted to study the morphological indices, 4 and CD8 co-localization in bursas of fabricius were determined by fluorescence microscopy. Cell apoptosis rate were analyzed the apoptosis with software Image Pro Plus 6.0. Like an appendix, the goose bursa of fabricius was located on the back of cloaca. The lymphatic follicle increased during the prenatal stage and became degenerated after hatching. For the first time, CD4 and CD8 co-expression (double positive) was found in the connective tissue and cortex and co-expression level increased from E17 to E26, then decreased from E28 to 7d. It was significantly different ($p<0.01$) among E17, E24, E26, E28, 18 and 3d. The apoptosis rate decreased from E17 to 3d, then dramatically increased after hatching and was extremely different ($p<0.01$) among E17, E24, E26, E28, 18 and 3d. The coordination between co-expression level and apoptosis rate in bursa of fabricius is significant. It is deduced that T lymphocyte selection in bursa of fabricius sharing the same feature as the T lymphocyte selection in thymus is indispensible for the development of goose bursa of fabricius.

Key words: Bursa of fabricius, goose, CD4/CD8 co-expression, development, T-lymphocyte selection

INTRODUCTION

Bursa of fabricius is one of central lymphoid organs in birds (Rodriguez-Mendez *et al*., 2010). In bursal development, it undergoes striking changes including the rapid development from the late embryogenesis to post-hatching and degeneration after sexual maturity (Luna *et al*., 2008). It contains many lymphatic follicles and is known as the primary lymphoid organ which dominates proliferation and diversification of B cell (Ackerman and Knouff, 1959; Cooper *et al*., 1969; Olah and Glick, 1978; Reynaud *et al*., 1991). It plays a central role in avian B-cell development and participates in the B-cell proliferation and immunoglobulin V gene diversification (Brown *et al*., 2004).

Previous studies revealed that there was a Diffusely Infiltrated Area (DIA) of lymphoid cells just dorsal to the chicken bursal duct opening (Odend'hal and Breazile, 1980; Dolfi *et al.*, 1988) and DIA was previously described as a T-dependent bursal area (Cortes *et al.*, 1995). DIA cells are almost T lymphocytes including $CD4^+$ cells, $CD8^+$ cells and TCR lymphocytes (Cortes *et al.*, 1995) can activate B-cell (Larosa and Orange, 2008).

Avian T-cell populations can be divided into different subsets based on their different cell-surficial antigens such as CD4, CD8 and TCR (Erf *et al.*, 1998). The CD4 and CD8 receptors are made of single polypeptides. Most $CD4^+$ cells are helper T-cells responding to exogenous antigen in association with major histocompatibility complex Class II (MHC II) molecules; while $CD8^+$ cells serve as cytotoxic T-cells responding to endogenous antigen in association with MHC I molecules. T lymphocyte subpopulations plays an important role in the innate immune response against intracellular pathogens (Liu *et al.*, 2011).

The CD4 and CD8 expressions can be detected in the thymus and spleen of 2-7 week-old broilers (Erf *et al.*, 1998). At early embyo phase, the thymocytes express neither CD4 nor CD8. Then CD4 and CD8 were co-expressed in immature thymocyte at middle embyo phase. The double-positive cells react with self antigens and induce apoptosis in the thymus (negative selection), thus negative selection results in the occurance of $CD4^+CD8^-$ or $CD4^-CD8^+$ T-cells (Oguma *et al.*, 2009). CD4 and CD8 co-expression is considered as a marker of immature cell. $CD4^+$ or $CD8^+$ single-positive T-cells are mature cells which enter secondary immune organs and travel in the circulatory lymphatic systems (Erf *et al.*, 1998).

All in all, whether T-dependent bursal area participates in B cell proliferation in bursa of fabricius remains unclear (Yamamoto *et al.*, 1996), especially in goose. Up to now, little is known about T or B lymphocyte selection in lymphoid organs. Unveiling the role of T lymphocyte selection in bursa of fabricius will contribute to understand their roles in immune response in goose. Therefore, we marked T cells with CD4 and CD8 expression in bursa of fabricius during bursal development. The objective of study was demonstrated that whether T lymphocyte selection in bursa of fabricius is similar to T cell development in the thymus and is indispensible for bursal development or not.

MATERIALS AND METHODS

Materials and reagents: Goose embryos [17-day (E17), 24-day (E24), 26-day (E26) and 28-day (E28) in appendix embryos] and young geese [3-day (3d), 7-day (7d) and 18-day (18d)](supplement material Fig. 1A-D in appendix) were collected from Sanyuan Breeding Limited-liability Company (Wuwei, Anhui Province, China).

Polyclonal rabbit anti-rat/mouse CD4 antibody and goat anti-rabbit IgG-FITC were bought from Beijing Biosynthesis Biotechnology Co Ltd (Beijing, China). Monoclonal mouse anti-human CD8 antibody, goat anti-mouse IgG-TRITC (tetraethyl rhodamine isothiocyanate), APES (aminopropyl-triethoxysilane), BSA (bovine serum albumin) and PBS (phosphate buffered saline) were bought from Wuhan Boster Biology Technology Ltd. (Wuhan, China).

Histology development of goose bursa of fabricius: The bursa of fabricius collecting from E17 to 18d samples (n = 5 at each developmental stage) were fixed in paraformaldehyde for 24 h at room temperature, dehydrated in ethanol and embedded in paraffin wax. Tissue sections (5 μm thick) were cut with a microtome and mounted onto APES-coated glass slides. Serial sections were cleared in dimethylbenzene, rehydrated in a graded series of ethanol and then incubated for Hematoxylin-Eosin (HE) staining.

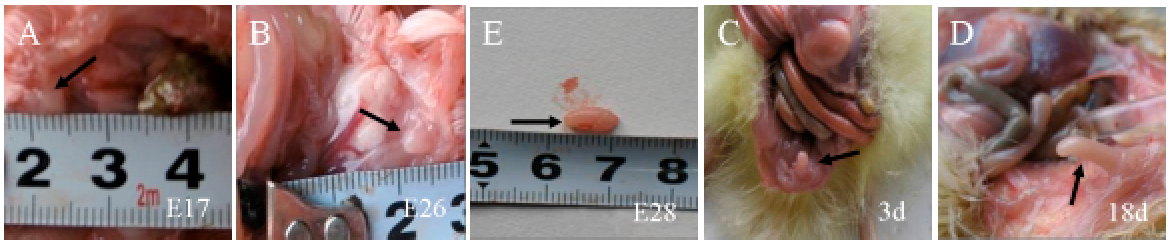

Fig. 1 (A-D): The anatomical photos of goose bursa of fabricius at different stages

Immunofluorescence analysis, CD4 CD8 expression and T lymphocyte localization: Tissue sections were incubated with polyclonal rabbit anti-rat/mouse CD4 antibody (at a dilution of 1:100) and mouse monoclonal anti-CD8 antibody (at a dilution of 1:100) in 1% PBS at 4°C overnight. After being washed (3×10 min) with PBS, sections were incubated at 37°C 1h with the secondary antibodies: Goat anti-rabbit IgG-FITC (1:32) and goat anti-mouse IgG-TRITC (1:32). After being rinsed (3×10 min) with PBS, sections were sealed with glycerin. CD4 and CD8 co-localization were observed via fluorescence microscopy with lasers at excitation wavelengths of 488 nm (FITC), 514 nm (TRITC), 350 nm (Hoeschst33342), respectively. OLYMPUS BX61 fluorescence microscopy observation analysis were performed to determine CD4 and CD8 co-localization in bursa of fabricius.

Apoptosis analysis of bursal development: Sections were also sealed with 1% BSA, then stained with 1% Hoeschst33342 in PBS at 37°C for 15 min, rinsed (3×10 min) with PBS and coated with glycerin, analyzed the apoptosis rate with software Image Pro Plus 6.0.

Statistical analysis: The data were expressed as Mean±SE and analyzed using one-way ANOVA. Data were transformed to ensure homogeneity of variance. LSD's multiple comparisons were applied to identify differences with homogeneity of variance. Tamhane's multiple comparisons were adopted to check non-homogeneity of variance. Probability values were considered to be significant at the 5% between groups. Statistical analysis was performed using SPSS13.0 package version by a two-tailed test.

RESULTS

Anatomical profile of goose bursa of fabricius at different stages: The goose bursa of fabricius was connecting with cloacal back wall via the bursal duct (black arrows, Fig. 1A-D). It increased in length and diameter from E17 (0.4 cm) to 18d (1.6 cm) and reached maximum at 18d (1.6 cm). The bursa of fabricius was like a sac, linking up with cloaca via ostioles. The bursal cystic wall was similar to digestive canal, consisted of mucous membrane, the submucosa and muscularis and outer membrane.

The bursal histologic changes at different stages: The morphology of goose embryonic bursa of fabricius:

E17: The mucosal epithelium of bursa of fabricius was composed by stratified epithelium; the submucosa was consisted of loose connective tissue. The outer membrane was the serosa which comprised mesothelium and connective tissue (Fig. 2A)

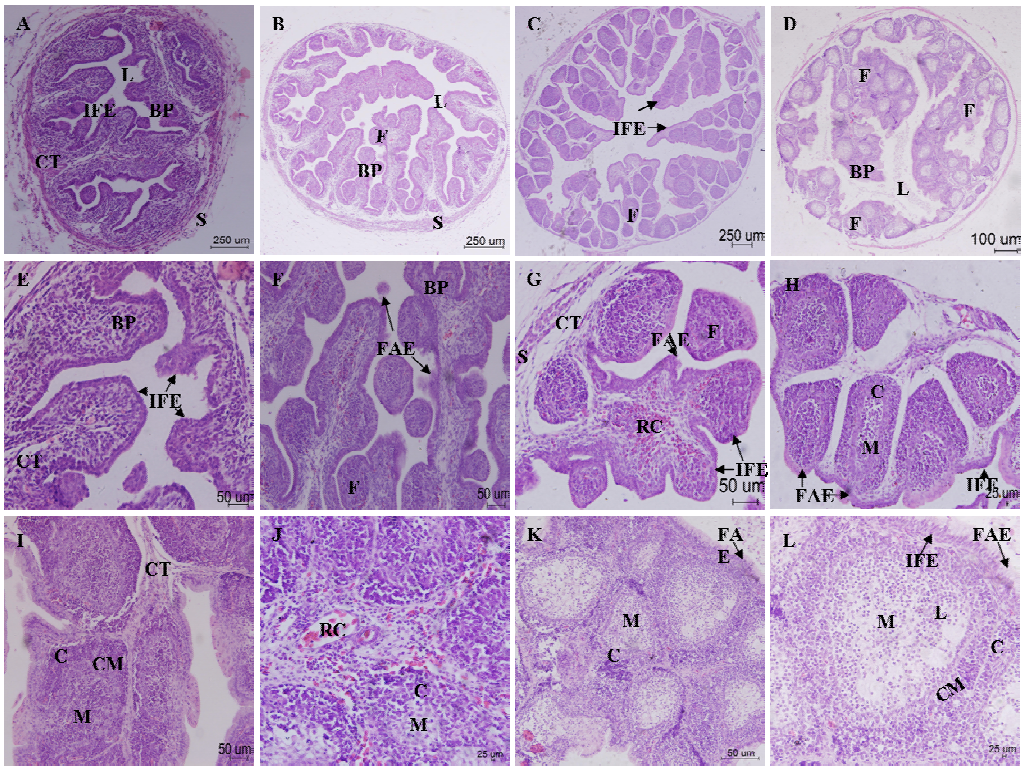

Fig. 2 (A-L): Histological changes of bursal follicle and epithelium at different stages Goose bursal sections were stained with Hematoxylin-eosin (HE). A-D: Structure of goose bursa of fabricius at E17, E24, E28, 18d, Magnification in A-D: x40, bars 250 μm. E-K: Structure at E17, E24, E26, E28, 3d, 7d, 18d; Magnification in (E, F, G, I, K): x200, bars 50 μm; (H, J): x400, bars 25 μm. L: Structure of lymphoid follicles, Magnification in: x400, bars 25 μm. BP: Bursal plicae; F: Bursal follicles; L: Lumen; CT: Connective tissue; M: Medulla; C: Cortex; CM: Corticomedullary; IFE: Interfollicular epithelium; FAE: Follicle-associated epithelium; RC: Red cell

E24: The bursa of fabricius became thicker. There were some elongated mucosal folds on the mucosal surface. The number of nodular lymphocyte cell masses gradually increased. The loose connective tissue in the submucosa was forming large trabecular meshwork. The muscularis layer was hypoplasia. A few follicle-associated epithelium were located on the mucosal surface and played a role in phagocytosis (Fig. 2B)

E26: Bursal mucosal epithelium became thicker increasingly and formed many leafy mucosal folds before lymphoid follicle emergence. The lymphoid follicles included cortex and medulla, whereas their boundary was not evident. There are several small lymphocytes, B lymphocytes, T lymphocytes and many macrophages existing in the follicles (Fig. 2C)

E28: The number and volume of lymphoid follicles increased significantly. The cell density in the cortex was much more than that in the medulla. Some heterophilic leukocytes and lymphocytes-like cells dispersed in the cortex. The number of follicular absorptive epithelium in the lymphoid follicle increased and follicular secretory epithelium appeared (Fig. 2D)

The morphology of goose bursa of fabricius after hatching:

3d: The bursa of fabricius developed well. The shape of most lymphoid follicles was irregular. The cortex and medulla can be easily distinguished in the submucosa. The number of follicular absorptive epithelium and follicular secretory epithelium increased (Fig. 2E)

7d: The shape of lymphoid follicles became oval. A large number of the reticular cell and reticular fiber were present in lymphoid follicles; lots of heterophilic leukocytes around follicles; many blood cell in the trabecular meshwork (Fig. 2F)

18d: The lymphoid follicles degenerated gradually. The boundary between cortex and medulla was still clear but there were only few lymphocytes in the medulla. Vacuolated structure was relatively obvious (Fig. 2G)

The comparison of the follicular number at different stages: There were approximately 3 bursal follicles at E17, 10 at E24, 17 at E26, 100 at E28, 128 at 3d, 120 at 7d and 78 at 18d (Table 1). It was clear that the volume and number of the lymphatic follicle increased from E17 to 3d and decreased after 3d (Fig. 3). The vacuolated structure was seen in lymphoid follicle after hatching.

The CD4 and CD8 expression in goose bursa of fabricius

The T lymphocyte distribution and CD4 CD8 co-expression during bursal development: The single positive CD4 ($CD4^+$) were mainly expressed in the connective tissue around the mucosal folds and cortex in the lymphoid follicles. The single positive CD8 ($CD8^+$) were also expressed in the connective tissue adjacent to the serosa and cortex in the follicles.

The CD4 and CD8 co-expression was predominantly in the connective tissue and rarely in the follicle at E17, also widely discovered in the connective tissue near the serosa at E24. The co-expression level increased and reached the peak in the connective tissue at E26. Although the co-expression in connective tissue gradually reduced at E28, it increased in the cortex and corticomedullary of follicles at 3d. Co-expression was more evident in the cortex at 7d while it is almost absent in the connective tissue. Then it gradually increased in the cortex and connective tissue near the junction of follicle at 18d. It is indicated that CD4 and CD8 co-expression were simultaneously distributed but more in the connective tissue and cortex during development (Fig. 4).

In summary, the co-expression clearly rised from E17 to E26, then reduced from E28 to 7d and increased at 18d. It was almost present in bursal plicae, follicle-associated epithelium, follicular secretory epithelium and lumen which were described as T-independent bursal areas.

Statistical analysis of double positive area percentage: The statistical results of $CD4^+CD8^+$ expression (double positive) in goose bursal areas during different periods were analyzed and shown in Table 2. The double positive area percentage relationship between different periods was analysed using software SSPS13.0. The results showed that it were significant differences between

Table 1: The statistics of the bursal follicular number during different periods

Days	E17	E24	E26	E28	3d	7d	18d
The follicular number	3	10	17	100	128	120	78

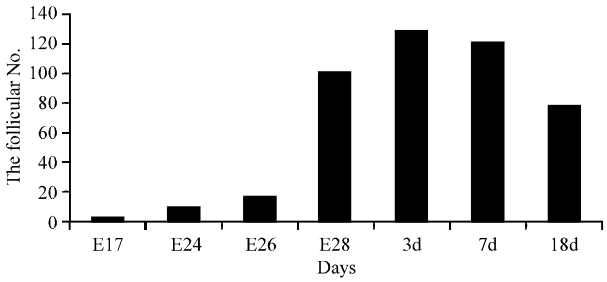

Fig. 3: The comparison on the lymphoid follicular number during different periods

Table 2: Statistical analysis showing double positive area percentage results in the goose bursa of fabricius during different periods

Days	E17	E24	E26	E28	3d	7d	18d
Area percentage (%)	3.950±0.130	4.253±0.233	7.527±0.190**	2.748±0.036	1.683±0.040	1.546±0.034	4.701±0.042**

Data are expressed as Mean±SE. **Indicate that difference is extremely notable compared with 3d after hatching ($p<0.01$)

Table 3: The comparative analysis on goose bursal double positive area percentage during different periods

Days	E17	E24	E26	E28	3d	7d
18d	0.004	0.857	0.000	0.000	0.000	0.000
7d	0.000	0.000	0.000	0.000	0.310	-
3d	0.000	0.000	0.000	0.000	-	-
E28	0.000	0.002	0.000	-	-	-
E26	0.000	0.000	-	-	-	-
E24	0.999	-	-	-	-	-

The mean difference is significant at the 0.05 level

group ($F(6,63) = 264.212$, $p<0.01$ ($p = 0.000$)) using one-way ANOVA. It come to conclusion that it did not have the homogeneity of variance ($p<0.05$ ($p = 0.000$)) through test of homogeneity of variance (supplemental Table S1, S2, S3 in appendix). The multiple comparative analysis on goose bursal double positive area percentage during different periods was performed difference using Tamhane with non-homogeneity of variance (Table 3). The lowest was 0.000 and the highest was 0.999. The stem-and-leaf diagram showed that had significant differences during different periods (Fig. 5).

The results showed that: significant difference ($p<0.01$) were observed among E17 ($p = 0.000$), E24 ($p = 0.000$), E26 ($p = 0.000$), E28 ($p = 0.000$), 18d ($p = 0.000$) and 3d.

The apoptosis analysis on goose bursa of fabricius

Hoeschst33342 stained nuclei analysis: The small blue dot was apoptotic body of nuclei (the white arrows in Fig. 6h). At E17, nuclei were sparse in connective tissue and serosa, while they are intensive in the interfollicular epithelium and large numbers of apoptotic cells were seen in bursal follicle. At E24, nuclei arranged neatly and closely in the interfollicular epithelium and bursal follicle whereas loosely in the connective tissue connecting with serosa. At E26, E28 and 3d, the nuclei were compacted to each other but sparse in connective tissue and aligned closely in the cortex, while they scattered in the medulla at 7d and 18d.

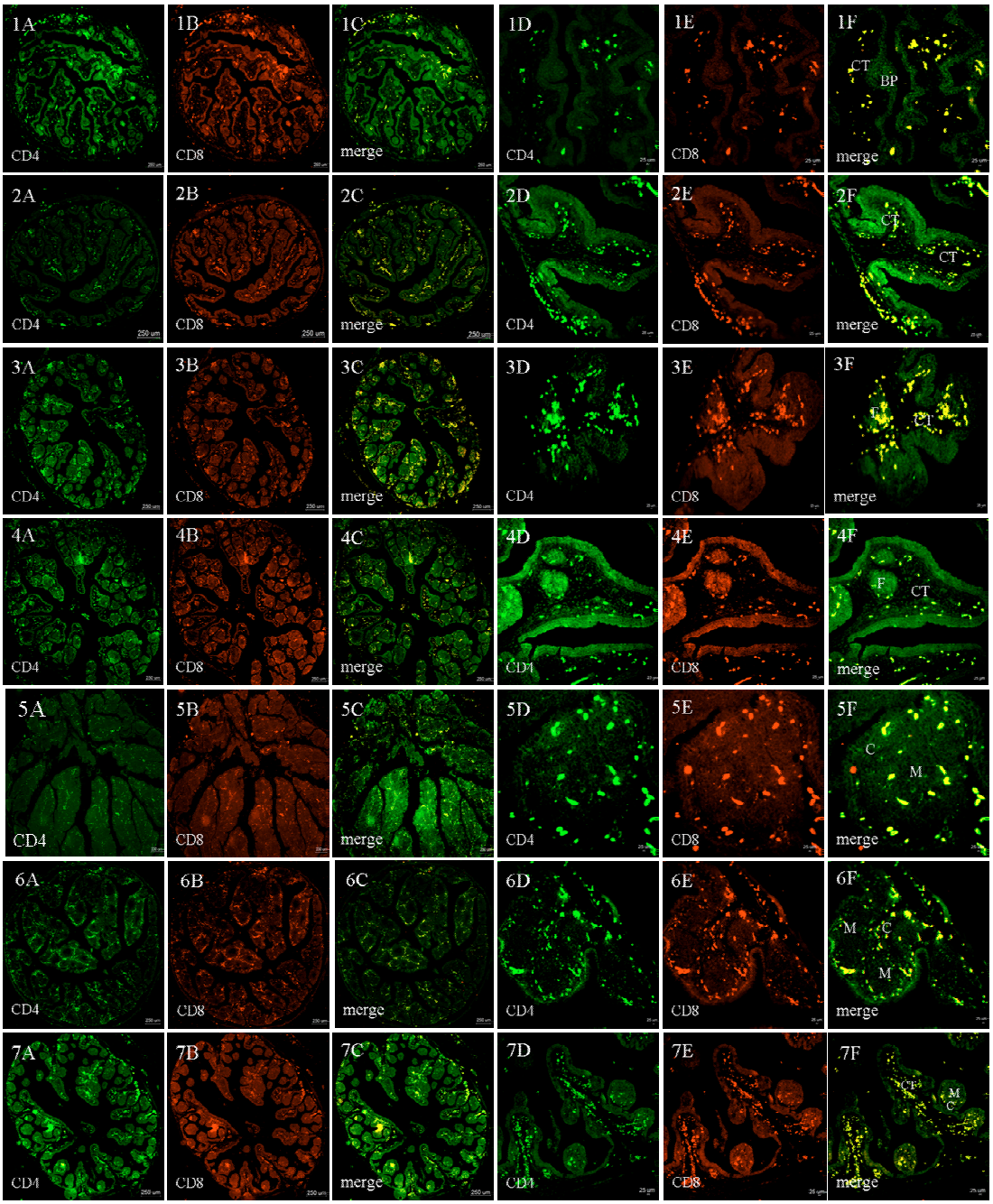

Fig. 4: CD4 and CD8 immunoreactivity in goose bursa of Fabricius during different periods 1-7 represents E17, E24, E26, E28, 3d, 7d, 18d, respectively. A, D: CD4 expression (green, 1st Ab: polyclonal rabbit anti-rat/mouse CD4 IgG 1:100; 2nd Ab: goat anti-rabbit IgG-FITC, 1:32); B,E: CD8 expression (red, 1st Ab: mouse monoclonal anti-CD8 IgG 1:100; 2nd Ab: goat anti-mouse IgG-TRITC 1:32) in bursal follicles. C, F is shown in yellow after merging images. Magnification in A-C: 40x, bars 250 μm; in (D-F): 400x, bars 25 μm. BP: Bursal plicae; F: Bursal follicles; CT: Connective tissue; M: Medulla; C: Cortex

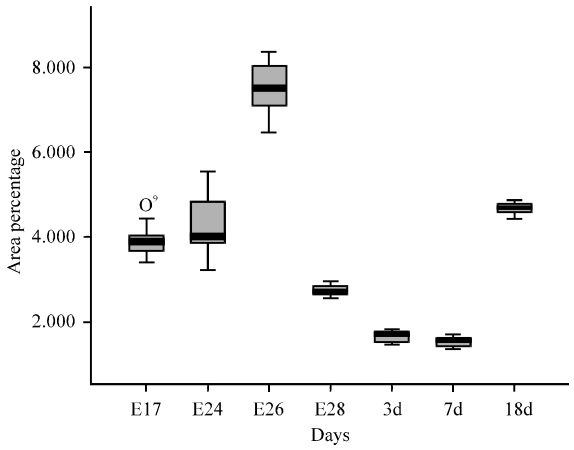

Fig. 5: The stem-and-leaf diagram about double positive area percentage during bursal development. Rectangular box is the subject of the diagram, it shows the value of 25-75%. The thick black lines represent average value. o represents outliers; It shows 1 outliers in 70 numerical values

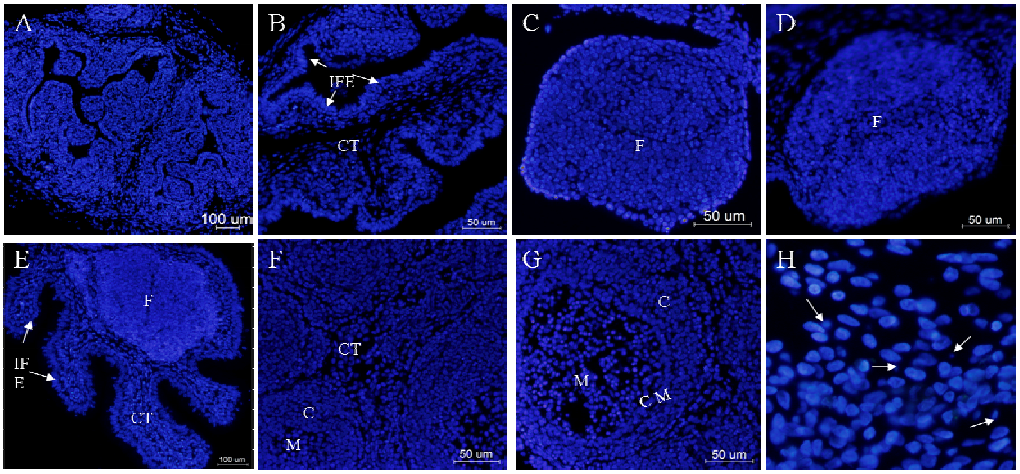

Fig. 6 (A-H): Hoeschst33342 stained nuclei in goose bursa of fabricius during different periods A-G: separately represents E17, E24, E26, E28, 3d, 7d, 18d. Magnification in (A, E): x100, bars 100 μm; (B, C, D, F, G): x200, bars 50 μm. H: showing the apoptotic bodies of goose bursal nuclei, the white arrows pointed to apoptotic bodies. F: Bursal follicles; CT: Connective tissue; M: Medulla; C: Cortex; IFE: Interfollicular epithelium; CM: Corticomedullary

Statistical analysis on apoptosis rate: Statistical analysis were carried on goose bursal apoptosis rate at different periods (Table 4). The stem-and-leaf diagram showed that there were significant differences among 3d and E17, E24, E28, 18d (Fig. 7).

There were significant differences between groups ($F_{(6,63)} = 5.811$, $p<0.01$ ($p = 0.000$)) using one-way ANOVA and it had the homogeneity of variance ($p>0.05$ ($p = 0.068$)) through test of homogeneity of variance (supplemental Table S4, S5, S6 in appendix). The multiple comparison

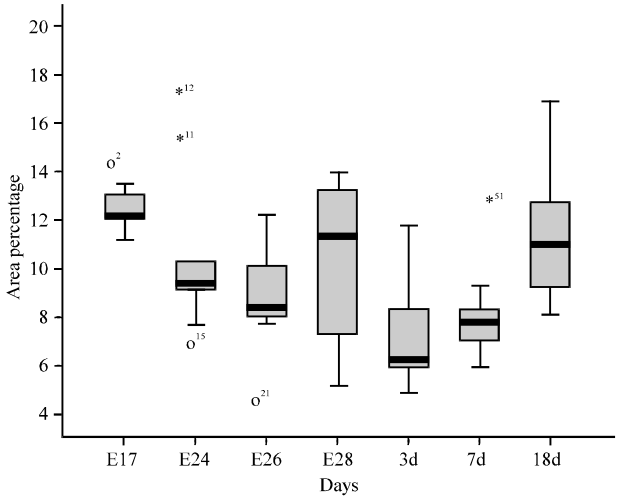

Fig. 7: The stem-and-leaf diagram on apoptosis rate at different periods. Rectangular box is the subject of the diagram, it shows the value of 25-75%. The thick black lines represent average value. o represents outliers; * represents extreme value. It shows 3 outliers and 3 extreme values in 70 numerical values

Table 4: The apoptosis rate in goose bursa of fabricius at different periods ($\overline{X} \pm S$)

Days	E17	E24	E26	E28	3d	7d	18d
Apoptosis rate (%)	12.485±0.355**	10.552±1.121**	8.802±0.671	10.397±1.008**	7.058±0.659	8.089±0.603	11.293±0.855**

Data are expressed as Mean±SE. **Indicate that difference is extremely notable compared with 3d after hatching ($p<0.01$)

Table 5: The multiple comparison on the apoptosis rate the difference during different periods

Days	E17	E24	E26	E28	3d	7d
18d	0.291	0.509	0.029	0.426	0.000	0.006
7d	0.000	0.031	0.526	0.043	0.360	-
3d	0.000	0.003	0.124	0.004	-	-
E28	0.066	0.890	0.159	-	-	-
E26	0.002	0.123	-	-	-	-
E24	0.089	-	-	-	-	-

The mean difference is significant at the 0.05 level

on goose bursal apoptosis rates among different periods was performed difference using LSD with homogeneity of variance (Table 5). It was testified that extremely significant difference ($p<0.01$) were observed among E17 ($p = 0.000$), E24 ($p = 0.003$), E28 ($p = 0.004$), 18d ($p = 0.000$) and 3d.

The correlation analysis on co-expression area percentage and apoptosis: The statistical analysis between double positive area percentage and apoptosis rate also showed that there were significant differences among 3d and E17, E24, E28, 18d. Spearman's rho was adopted to analyze the correlation between double positive area percentage and apoptosis rate. The results showed that: their correlation coefficient is 0.242, $p = 0.043$ ($p<0.05$). It is indicated that the correlation had significant difference between them (Table 6). It is suggested that there is cooperative relationship between them in bursal development.

Table 6: The correlations analysis between double positive area percentage and apoptosis rate

O levels	Double positive area percentage	Apoptosis rate
Double positive area percentage		
Correlation coefficient	1.000	0.242 (*)
Sig. (2-tailed)		0.043
N	70	70
Apoptosis rate		
Correlation coefficient	0.242(*)	1.000
Sig. (2-tailed)	0.043	
N	70	70

*Correlation is significant at the 0.05 level (2-tailed)

DISCUSSION

The bursa of fabricius is responsible for B cell maturation and antigen specific IgM-IgG switch (Nagy and Olah, 2009; Nagy and Olah, 2010; Ricci *et al*., 1996) or peptides (Garcia-Espinosa *et al*., 2008). The goose bursal development in this study was similar to chicken bursa of fabricius (Glick *et al*., 1956; Nagy and Olah, 2009). Except for different incubation period, the size and developing pattern were consistent to those of the chicken during embryogenesis and early weeks after hatching (Luna *et al*., 2005). It is reported that the morphology of human intestinal Peyer patches, rabbit appendices, Sheep Ileal Peyer Patches (SIPP) and the avian bursa of fabricius are similar (Dasso *et al*., 2000). In mammals and birds, B-cell differentiation starts from VDJ recombination in central lymphoid organs (Butler, 1997; Griebel and Hein, 1996). Historically, there is Germinal Center (GC) structure in the rabbit appendix, follicular structure in the avian bursa of fabricius and SIPP (Hodges, 1974; Reynolds and Morris, 1983). It was also be revealed that the avian bursal follicle can be divided into cortex and medulla. In present study, the vacuolated structure appeared after hatching, however, SIPP not were found. It was shown that there were structural differences in avian bursa of fabricius, Peyer patches and appendix in mammals. It is known that continuing T-cell can activated B cells and initiate the GC reaction, or differentiate into short-lived plasma cells (Larosa and Orange, 2008). Our viewpoints are in agreement with previous reports (Odend'hal and Breazile, 1980; Dolfi *et al*., 1988) that goose had T-dependent area in the bursa of fabricius. We come to conclusion that connective tissue and cortex were T-dependent bursal areas in the goose. This research provides theoretical basis and evidences for avian embryology and immunology researches.

T cells in both the human and rabbit appendix are fewer than B cells. T cells in the rabbit appendix range from 7 to 40% (Dasso *et al*., 2000; Hanaoka *et al*., 1977; Bast *et al*., 1979) and in human from 19 to 50% (Kawanishi, 1987; Mizumoto, 1976; Neiburger *et al*., 1976; Alexopoulos *et al*., 1976). The majority of T cells lie in T-cell rich interfollicular region and a few are also found in B-cell rich follicular region. But peripheral $CD4^+CD8^+$ T-cells are reported in partially inbred and MHC-homozygous H.B15 chickens by Luhtala *et al*. (1997). Cell-mediated immunity plays a useful role in laying hens (Babu *et al*., 2005). In this study for the first time it was proved that CD4 or CD8 expression in goose bursa of fabricius is consistent with process in chicken thymus: co-negative $CD4^-CD8^-$ firstly emergence, then co-positive $CD4^+CD8^+$ formation, single-positive $CD4^+$ or $CD8^+$ appearance at last (Oguma *et al*., 2009; Parel and Chizzolini, 2004). The most interesting thing of all is T Lymphocyte selection in goose bursa of fabricius was unexpectedly similar to T cell development in thymus.

The results are approximately in accordance with that bursal $CD4^+$, $CD8^+$ T cells and macrophages were observed in the MDV-infected and controlled chicken. In addition, MDV-infected

cells up-regulate MHC II molecules in bursa of fabricius (Niikura *et al*., 2007) and down-regulate MHC I after MDV infection (Gimeno *et al*., 2001; Hunt *et al*., 2001; Levy *et al*., 2003). Therefore, it can be concluded that T lymphocytes existed in avian bursa of fabricius and exerted an important role in B cell proliferation and immune system maintenance.

In this study, it was demonstrated that T Lymphocyte completely involved in the development of goose bursa of fabricius. It is well known that T cell function can be divided into three categories according to TCR$\alpha\beta$: T helper (Th1, Th2) and T suppressor (Ts); cytotoxic T lymphocyte (Tc). Th2 cells specialize in facilitating B-cell antibody responses, produce IL-4(drive B-cell proliferation), IL-5 and IL-13, while IL-4 and IL-5 enable IgE production (Larosa and Orange, 2008). It is well known that the surface receptor (CD28, CD40L and MHC etc.) on T lymphocytes is also surface antigen on B cell. The CD28 family ligand is B7-1 (CD80) and B7-2 (CD86). Previous studies showed that combination between IL-4 and CD40L:CD40 sustained B-cell activation and differentiation, promoted the memory B cell's production and immunoglobulin class switch recombination (Larosa and Orange, 2008; Lafrenz and Feldbush, 1981; Miller and Sprent, 1971). Under these conditions the present study demonstrate that T lymphocytes is essential for B cell activation differentiation and maturation in the goose bursa of fabricius. Understanding the process of T lymphocyte selection in bursa of fabricius will greatly help study the immune response in goose.

The bursa of fabricius is one of the most important lymphoid system during cell apoptosis and its apoptosis rate is several fold higher than that of the thymus (Luna *et al*., 2005; Paramithiotis *et al*., 1995). This study uncovered that the distribution of the nucleus at different periods. A significant finding in the study was that goose bursal apoptosis decreased between E17 and 3d and increased after hatching. The apoptosis rate is very low while $CD4^+CD8^+$ co-expression level became higher. It was coincided with the fact that co-positive thymocytes were more sensitive than single-positive thymocytes via various reagents to induce apoptosis (Oguma *et al*., 2009). The apoptotic cells were also increased by virus inoculation (Wang *et al*., 2011). B-lymphocytes in the bursa of fabricius undergo proliferation, differentiation and apoptosis (Garcia-Espinosa *et al*., 2003). It has been estimated that only about 5% of these juvenile B-cells emigrate successfully, while the rest of the cells die in situ via apoptosis (Lassila, 1989). T-lymphocytes subsets may play a role in cellular immune response of colon cancer (Attallah *et al*., 2006). It was found that lymphocyte accompanied by extensive apoptotic cell death during avian development. The results indicated that T lymphocyte selection and apoptosis were correlated with B-cell development in goose bursa of fabricius, the concrete function is yet unclear and further study is needed to clarify the underlying sense.

CONCLUSION

It was proved that T-dependent area also existed in goose bursa of fabricius, involved B cell differentiation and maturation and shamed the same feature as the T lymphocyte selection in thymus. T lymphocytes are indispensible for the development of goose bursa of fabricius.

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APPENDIX

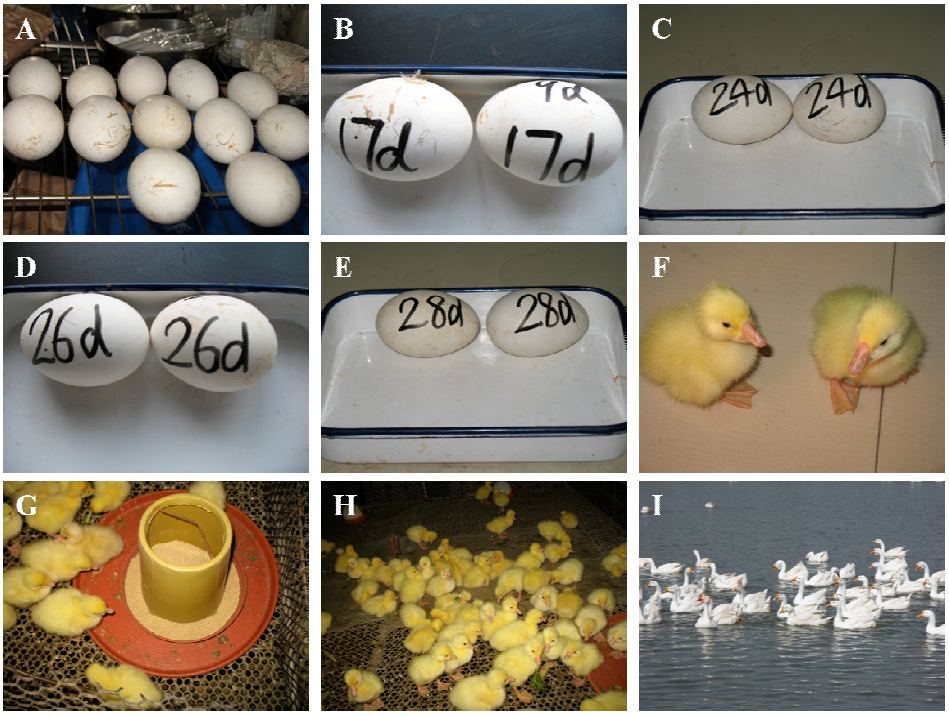

Fig. S1: B-H represents E17, E24, E26, E28, 3d, 7d, 18d, respectively. A represents goose incubation in oven at 37°C; I represent mature White goose

Table S1: The descriptions on double positive area percentage of geese bursa

Days	Mean (%)	N	SD	SEM	Minimum	Maximum	Variance
E17	3.950	10	0.411	0.130	3.401	4.789	0.169
E24	4.253	10	0.736	0.233	3.218	5.575	0.542
E26	7.527	10	0.601	0.190	6.465	8.377	0.361
E28	2.748	10	0.114	0.036	2.557	2.960	0.013
3d	1.683	10	0.125	0.039	1.491	1.830	0.016
7d	1.546	10	0.107	0.034	1.384	1.715	0.011
18d	4.701	10	0.133	0.042	4.450	4.886	0.018

Table S2: The result of one-way ANOVA on double positive area percentage of geese bursa

SOV	Sum of squares	df	Mean square	F	Sig.
Between groups	255.948	6	42.658	264.212	0.000
Within groups	10.172	63	0.161		
Total	266.119	69			

Table S3: The multiple comparisons on double positive area percentage of geese bursa with LSD

					95% confidence interval	
Days (I)	Days (J)	Mean difference (I-J)	SE	Sig.	Lower bound	Upper bound
E17	E24	-0.303	0.180	0.097	-0.662	0.056
	E26	-3.578 (*)	0.180	0.000	-3.937	-3.218

Table S3: Continued

Days (I)	Days (J)	Mean difference (I-J)	SE	Sig.	95% confidence interval	
					Lower bound	Upper bound
	E28	1.201 (*)	0.180	0.000	0.842	1.560
	3d	2.267 (*)	0.180	0.000	1.908	2.626
	7d	2.403 (*)	0.180	0.000	2.044	2.762
	18d	-0.752 (*)	0.180	0.000	-1.111	-0.393
E24	E17	0.303	0.180	0.097	-0.056	0.662
	E26	-3.275 (*)	0.180	0.000	-3.634	-2.916
	E28	1.504 (*)	0.180	0.000	1.145	1.863
	3d	2.570 (*)	0.180	0.000	2.211	2.929
	7d	2.7069*)	0.180	0.000	2.347	3.065
	18d	-0.449 (*)	0.180	0.015	-0.808	-0.090
E26	E17	3.578 (*)	0.180	0.000	3.218	3.937
	E24	3.275 (*)	0.180	0.000	2.916	3.634
	E28	4.779 (*)	0.180	0.000	4.420	5.138
	3d	5.845 (*)	0.180	0.000	5.486	6.204
	7d	5.981(*)	0.180	0.000	5.622	6.340
	18d	2.826 (*)	0.180	0.000	2.467	3.185
E28	E17	-1.201 (*)	0.180	0.000	-1.560	-0.842
	E24	-1.504 (*)	0.180	0.000	-1.863	-1.145
	E26	-4.779 (*)	0.180	0.000	-5.138	-4.420
	3d	1.066 (*)	0.180	0.000	0.707	1.425
	7d	1.202 (*)	0.180	0.000	0.843	1.561
	18d	-1.953 (*)	0.180	0.000	-2.312	-1.594
3d	E17	-2.267 (*)	0.180	0.000	-2.626	-1.908
	E24	-2.570 (*)	0.180	0.000	-2.929	-2.211
	E26	-5.845 (*)	0.180	0.000	-6.204	-5.485
	E28	-1.066 (*)	0.180	0.000	-1.425	-0.707
	7d	0.136	0.180	0.451	-0.223	0.495
	18d	-3.019 (*)	0.180	0.000	-3.378	-2.660
7d	E17	-2.403 (*)	0.180	0.000	-2.762	-2.044
	E24	-2.706 (*)	0.180	0.000	-3.065	-2.347
	E26	-5.981 (*)	0.180	0.000	-6.340	-5.622
	E28	-1.202 (*)	0.180	0.000	-1.561	-0.843
	3d	-0.136	0.180	0.451	-0.495	0.223
	18d	-3.155 (*)	0.180	0.000	-3.514	-2.796
18d	E17	0.752 (*)	0.180	0.000	0.393	1.111
	E24	0.449 (*)	0.180	0.015	0.090	0.808
	E26	-2.826 (*)	0.180	0.000	-3.185	-2.467
	E28	1.953 (*)	0.180	0.000	1.594	2.312
	3d	3.019 (*)	0.180	0.000	2.660	3.378
	7d	3.155 (*)	0.180	0.000	2.796	3.514

* The mean difference is significant at the 0.05 level

Table S4: The descriptions on apoptosis rate of geese bursa

Days	N	Mean (%)	SD	SE	95% confidence interval for mean		Minimum	Maximum
					Lower bound	Upper bound		
E17	10	12.485	1.123	0.355	11.682	13.289	11.163	15.012
E24	10	10.552	3.543	1.120	8.017	13.086	7.041	18.037

Table S4: Continued

	N	Mean (%)	SD	SE	95% confidence interval for mean		Minimum	Maximum
					Lower bound	Upper bound		
E26	10	8.801	2.122	0.671	7.283	10.320	4.584	12.235
E28	10	10.397	3.186	1.008	8.117	12.676	5.158	13.977
3d	10	7.059	2.085	0.660	5.567	8.550	4.860	11.799
7d	10	8.089	1.907	0.603	6.725	9.453	5.886	12.726
18d	10	11.294	2.705	0.855	9.359	13.229	8.125	16.886

Table S5: The result of one-way ANOVA on apoptosis rate of geese bursa

SOV	Sum of squares	df	Mean square	F	Sig.
Between groups	218.018	6	36.336	5.811	0.068
Within groups	393.928	63	6.253		
Total	611.946	69			

Table S6: The multiple Comparisons on apoptosis rate of geese bursa with LSD

Days (I)	Days (J)	Mean difference (I-J)	SE	Sig.	95% confidence interval	
					Lower bound	Upper bound
E17	E24	1.934	1.118	0.089	-0.301	4.168
	E26	3.684 (*)	1.118	0.002	1.449	5.919
	E28	2.0884	1.118	0.067	-0.147	4.323
	3d	5.427 (*)	1.118	0.000	3.192	7.661
	7d	4.396 (*)	1.118	0.000	2.161	6.631
	18d	1.192	1.118	0.291	-1.043	3.426
E24	E17	-1.934	1.118	0.089	-4.168	0.301
	E26	1.750	1.118	0.123	-0.485	3.984
	E28	0.155	1.118	0.891	-2.080	2.389
	3d	3.493 (*)	1.118	0.003	1.258	5.728
	7d	2.463 (*)	1.118	0.031	0.228	4.697
	18d	-0.742	1.118	0.509	-2.977	1.493
E26	E17	-3.684 (*)	1.118	0.002	-5.919	-1.449
	E24	-1.750	1.118	0.123	-3.985	0.485
	E28	-1.596	1.118	0.159	-3.830	0.639
	3d	1.743	1.118	0.124	-0.492	3.978
	7d	0.712	1.118	0.526	-1.522	2.947
	18d	-2.492 (*)	1.118	0.029	-4.727	-0.258
E28	E17	-2.088	1.118	0.067	-4.323	0.147
	E24	-0.155	1.118	0.891	-2.389	2.080
	E26	1.596	1.118	0.159	-0.639	3.830
	3d	3.338 (*)	1.118	0.004	1.104	5.573
	7d	2.308 (*)	1.118	0.043	0.0733	4.543
	18d	-0.897	1.118	0.426	-3.131	1.338
3d	E17	-5.427(*)	1.118	0.000	-7.661	-3.192
	E24	-3.493 (*)	1.118	0.003	-5.728	-1.258
	E26	-1.743	1.118	0.124	-3.978	0.492
	E28	-3.338 (*)	1.118	0.004	-5.573	-1.104
	7d	-1.030	1.118	0.360	-3.265	1.204
	18d	-4.235 (*)	1.118	0.000	-6.470	-2.000

Table S6: Continued

Days (I)	Days (J)	Mean difference (I-J)	SE	Sig.	95% confidence interval	
					Lower bound	Upper bound
7d	E17	-4.396 (*)	1.118	0.000	-6.631	-2.161
	E24	-2.463 (*)	1.118	0.031	-4.697	-0.228
	E26	-0.712	1.118	0.526	-2.947	1.522
	E28	-2.308 (*)	1.118	0.043	-4.543	-0.073
	3d	1.030	1.118	0.360	-1.204	3.265
	18d	-3.205 (*)	1.118	0.006	-5.439	-0.970
18d	E17	-1.192	1.118	0.291	-3.426	1.043
	E24	0.742	1.118	0.509	-1.493	2.977
	E26	2.492 (*)	1.118	0.029	0.258	4.727
	E28	0.897	1.118	0.426	-1.338	3.131
	3d	4.235 (*)	1.118	0.000	2.000	6.470
	7d	3.205 (*)	1.118	0.006	0.970	5.439

* The mean difference is significant at the 0.05 level

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