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CHICKEN AIR SACS AND MESENTERY: A HISTOMORPHOMETRICAL AND IMMUNOLOGICAL STUDY

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Abstract: Fat-associated lymphoid clusters (FALCs) are novel lymphoid tissues that have been reported in the mesenteric and mediastinal fat tissue of mouse and human. It plays role in the progression of respiratory and intestinal inflammation and parasitic infestations. However, their occurrence in the chicken air sacs and mesenteric adipose tissue has not yet been identified. Here we investigated the occurrence and distribution of FALCs in the air sacs (cervical, clavicular, thoracic, and abdominal) and mesenteric adipose tissue of healthy chicken. The latter air sacs and mesentery were immediately harvested after anesthesia and cutting the chicken heads then fixed in 4% paraformaldehyde fixative solution for histopathological examination. The degree of FALCs development among different specimens was measured and statistically analyzed. Our results revealed lymphoid clusters associating with the adipose tissues in mesentery, and air sacs (clavicular, thoracic, and abdominal), but not the cervical one. Interestingly, the thoracic air sacs showed significant higher FALCs size in comparison to that of other air sac types and the mesentery. Our findings suggested other possible immunological role of the air sacs and mesentery that could have impact on the progression of air sacculitis and mesenteritis- associate diseases. However, further investigations are required for clarification of air sacs and mesenteric FALCs in the progressions of respiratory and digestive tract diseases.

Key words: air sacs; chicken; fat-associated lymphoid clusters; air sacculitis; mesenteritis

Introduction

Fat-associated lymphoid clusters (FALCs) are unique lymphoid clusters (LCs) that have been reported to be associated with the gonadal, mesenteric, pericardial and mediastinal adipose tissue of healthy mouse and human (1-3). It is consisted mainly of lymphocytes, some macrophages, and granulocytes that were in direct contact with the surrounding adipocytes. Furthermore, novel innate lymphoid cells "natural helper cells" were detected in such clusters. Unlike other immune organs including lymph nodes, and spleen, no fibrous capsule was observed around such clusters, and no zonation for B- and T- lymphocytes (1, 3).

Interestingly, it has been reported that inflammation induces the development of FALCs (4, 5). Moreover, we documented strain difference in the degree of mediastinal FALCs development among healthy mice strains with well and less developed clusters in Th1-biased C57BL/6N (B6), and Th2-biased DBA/2Cr (DBA) mice,

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respectively (3). Interestingly, the role of FALCs in the pathogenesis of various respiratory and digestive tract diseases in mammals has been recently clarified. FALCs have been reported to control local IgM secretion during pleural infection and lung inflammation (6). Furthermore, the role of FALCs in controlling the *Nippostrongylus brasiliensis* helminth infection in mice has been revealed via B-1 B cell proliferation and promotes goblet cell hyperplasia (1, 7). Furthermore, our previous research revealed that mice strain difference in the susceptibility to Mycoplasma pulmonis infection correlates with degree of FALCs development (8).

The respiratory system differs in mammals than that in birds due to the facts that the birds required more efficient system than that of mammals for transferring more oxygen. The birds lack diaphragm and have relatively smaller lung (9, 10), but they have a complex system of well-developed air sacs which represent a much larger percentage of the respiratory system (11). In chickens and ducks, the air sacs volume has been reported to larger than the lung volume by approximately 10× (12, 13).

The air sacs in birds consisted of thin-walled bubble-like pockets connected with the lung, usually eight or nine in number (11, 14). Air sacculitis is an inflammatory disease of the air sac in birds, that is frequently associated with infection, mostly bacteria (15). Our previous report revealed the occurrence of mediastinal FALCs in the mice mediastinal adipose tissue extending from diaphragm to the thymus. However, no reports concerning the occurrence of FALCs in the bird's air sacs and mesentery and its possible role in the development of various respiratory and digestive diseases.

Here we investigated the occurrence of a novel lymphoid clusters in the air sacs (cervical, clavicular, thoracic, and abdominal) and mesenteric adipose tissue of healthy chicken. Except for the cervical air sacs, our results revealed the presence of FALCs in other types of air sacs as well as in the mesenteric adipose tissue. Interestingly, dramatic differences in the development of FALCs among different types of air sacs and the mesentery were highest development in the thoracic air sacs. Our data suggested an additional immunological role of air sacs and mesentery in chicken. However, further investigations are required to clarify its significant contribution in inflammatory conditions of the respiratory and digestive tract.

Materials and methods

Animals

Adult healthy Rhode Island red (RIR) chickens were used in the current experiment at age of one years old. All experiment procedures were approved in accordance with the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University, Japan (approval number 20–0171).

Experimental design

Four RIR chickens of both sexes were employed at 12 months of age. The chickens were anaesthetized by intravenous injection of pentobarbital sodium (25 mg/kg) through the ulnar cutaneous wing vein. After confirming the disappearance of immobilization, pain sensation, and corneal reflex, the chickens were euthanized by exsanguination and cutting the head. Then the chest and abdomen were opened, and both air sacs (cervical, clavicular, thoracic, and abdominal) and mesentery were immediately harvested and fixed in 4% paraformaldehyde fixative solution at 4°C. After overnight fixation, the air sacs and mesentery were washed in distilled water, then subjected to histopathological procedures.

Tissue preparation for histopathological observations

Following washing of the fixed specimens, the air sacs and mesentery were subjected to gradual dehydration in ascending graded alcohol (70%, 80%, 90%, absolute alcohol I, II, and III), cleared in xylene (I, II, and III, 30 min/ each), embedded in paraffin, paraffin blocking, and 3 μm paraffin sections were prepared and were used for both routine histopathological observations following staining with hematoxylin and eosin (H&E) staining.

Histomorphometrical measurements

Morphometrical analysis of the degree of FALCs development among different studied air sacs and mesentery in H&E- stained sections according to our previous report (16). Briefly, the H&E- stained sections of various air sac types and mesentery were converted to virtual slides using Nano Zoomer 2.0 RS (Hamamatsu Photonics Co.; Hamamatsu, Japan). Then, we measured the area of both LCs, and total adipose tissue (AT) by the NDP. view2 (Hamamatsu Photonics Co., Ltd.) software, and the percentage of area ratio for LCs /total AT was calculated and compared among different studied air sac types and mesentery.

Statistical analysis

The values were presented as mean± standard error (SE). Kruskal-Wallis test was conducted to assess the significant differences among studied groups, followed by Scheffé's test for multiple comparisons among the studied groups when significant differences (*P*-values < 0.05) observed among different groups.

Results

Grossly, the chicken had nine air sacs, one of which was single (interclavicular "saccus clavicularis" air sac) and four were paired (cervical "Sacuus cervicalis", cranial thoracic "Saccus thoracicus cranialis", caudal thoracic "Saccus thoracicus caudalis", and abdominal "Saccus abdominalis" air sacs). The air sacs have direct connection with the lung secondary bronchi and indirectly with parabronchi except the abdominal air sac which connected with the primary bronchi. The medullary cavities of pneumatic bones in fowl skeleton are included and aerated by diverticula of the air sacs (Figures 1A, 1B).

Histologically, the air sacs lined by simple squamous to cuboidal cells, supported by connective tissue containing numerous white adipose tissues. The adipose tissue associating with the cervical air sacs lacked lymphoid clusters, however, scattered lymphoid cells could be observed among the adipocytes (Figures 1C, 1D).

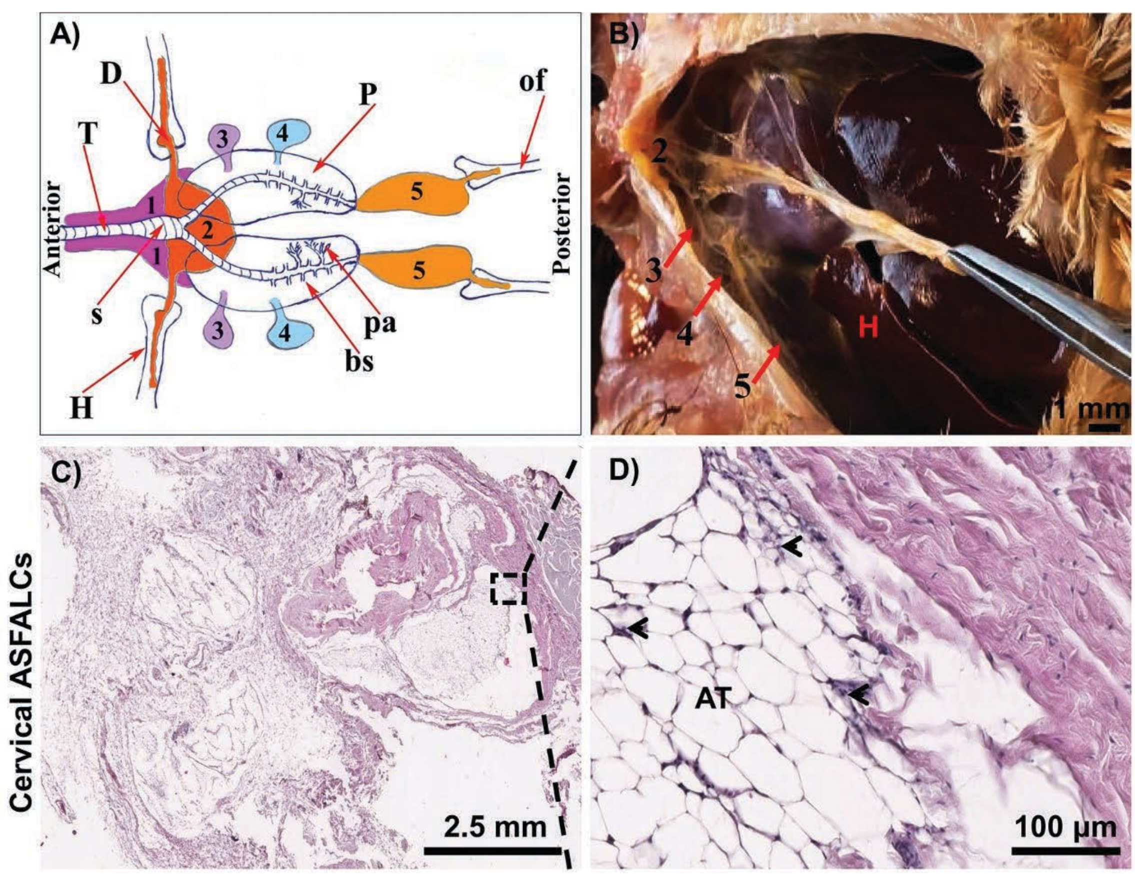

Figure 1: A schematic diagram of fowl air sacs (A), Macroscopic localization of air sacs after opening the chest and abdominal cavities of chicken (B). Notice Trachea (T), Syrinx (S), Pulmo (P), Bronchi secundarii (bs), Parabronchi (pa), Humerus (H), Os femoris (Of), Sacuus cervicalis (1), Saccus clavicularis (2), Saccus thoracicus cranialis (3), Saccus thoracicus caudalis (4), Saccus abdominalis (5), Divericula of air sac (D) and hepar (h). (C, D) Light micrographs of H&E-stained section of the cervical air sac. Notice scattered lymphoid cells (arrow heads), adipose tissue (AT)

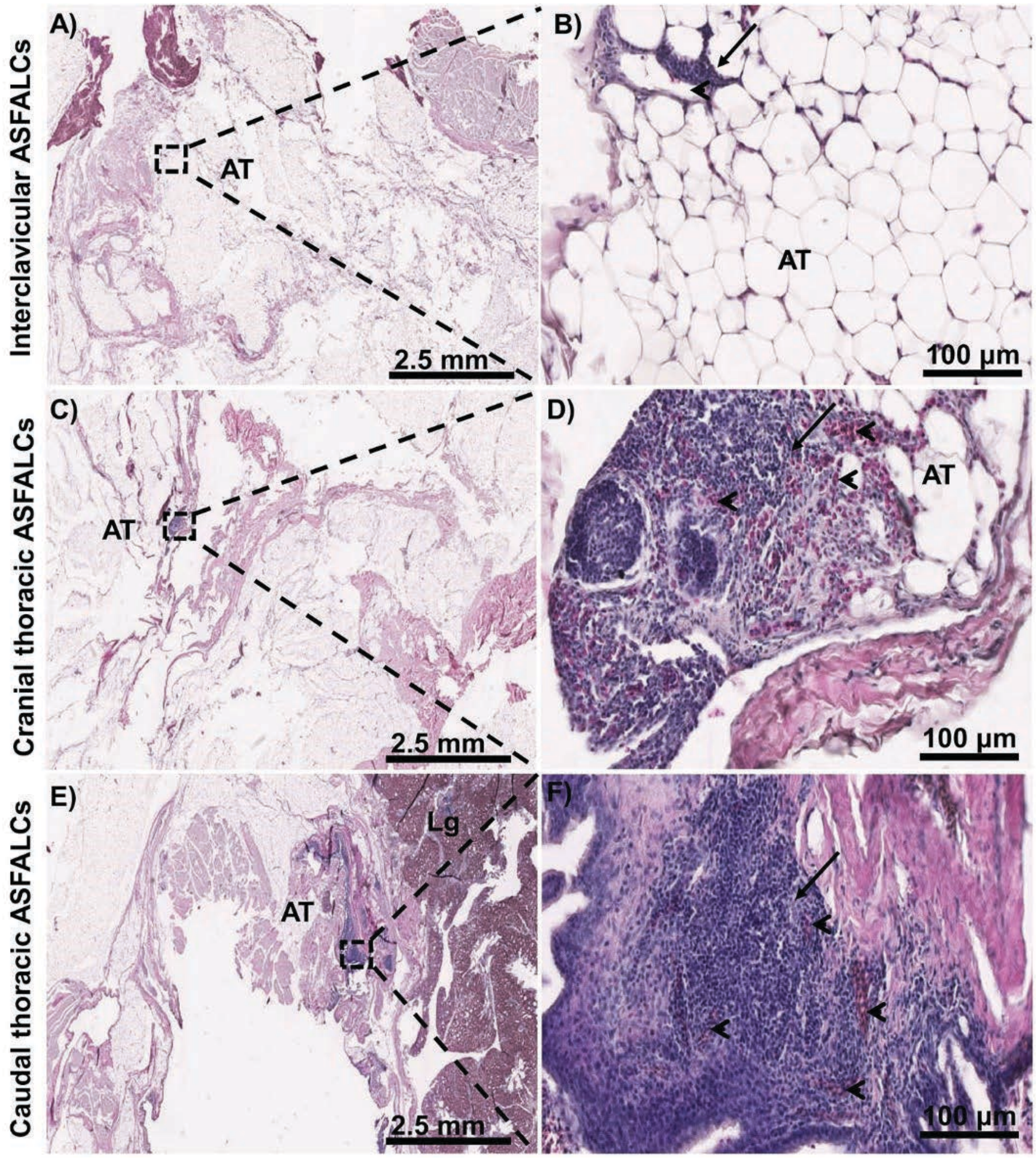

Figure 2: Light micrographs of H&E-stained section of the interclavicular (A), cranial thoracic (C), and caudal thoracic (E) air sacs. (B, D, F) higher magnification to the boxed area in (A, C, E), respectively. Notice blood vessels (arrow heads), lung (Lg), less- and well-developed lymphoid clusters (arrows) in the adipose tissue (AT) of interclavicular and thoracic air sacs, respectively

As shown in Figure 2, the interclavicular air sac showed small sized lymphoid clusters among the adipose tissue and we named it as interclavicular air sac FALCs "interclavicular ASFALCs" (Figures 2A, 2B). On the other hand, well developed lymphoid clusters were observed in the adipose tissue of both cranial and caudal thoracic air sacs especially in the adipose tissue close to the lung tissue and we termed such clusters as thoracic ASFALCs (Figures 2C, 2F).

Similar to the mammalian FALCs, the ASFALCs and mesenteric FALCs in chicken were highly vascularized and lack surrounding connective tissue capsule and their immune cells showed direct contact with the surrounding adipocytes. Interestingly, numerous blood vessels were observed in both cranial and caudal thoracic ASFALCs than that of the abdominal one (Figures 2B, 2D, 2F). Furthermore, the caudal thoracic ASFALCs were frequently observed in numerous number the nearby to the lung tissue (Figure 2E).

As shown in Figure 3, less developed lymphoid clusters were observed in the adipose tissue of abdominal air sacs especially close to the liver tissue and we termed such clusters as abdominal ASFALCs (Figures 3A, 3B). Interestingly, moderate sized lymphoid clusters were noticed to be associated with the adipose tissue of the mesentery and we termed it as mesenteric FALCs (Figures 3C, 3D).

To analyze the degree of development of the FALCs observed in the adipose tissue in different types of air sacs and mesentery, we calculated the ratios of total lymphoid clusters areas to that of the total adipose tissue areas and compared such values among studied air sacs and mesenteric adipose tissue. Interestingly, the statistical analysis revealed significant higher value for the thoracic ASFALCs than other ASFALCs (Figure 3E).

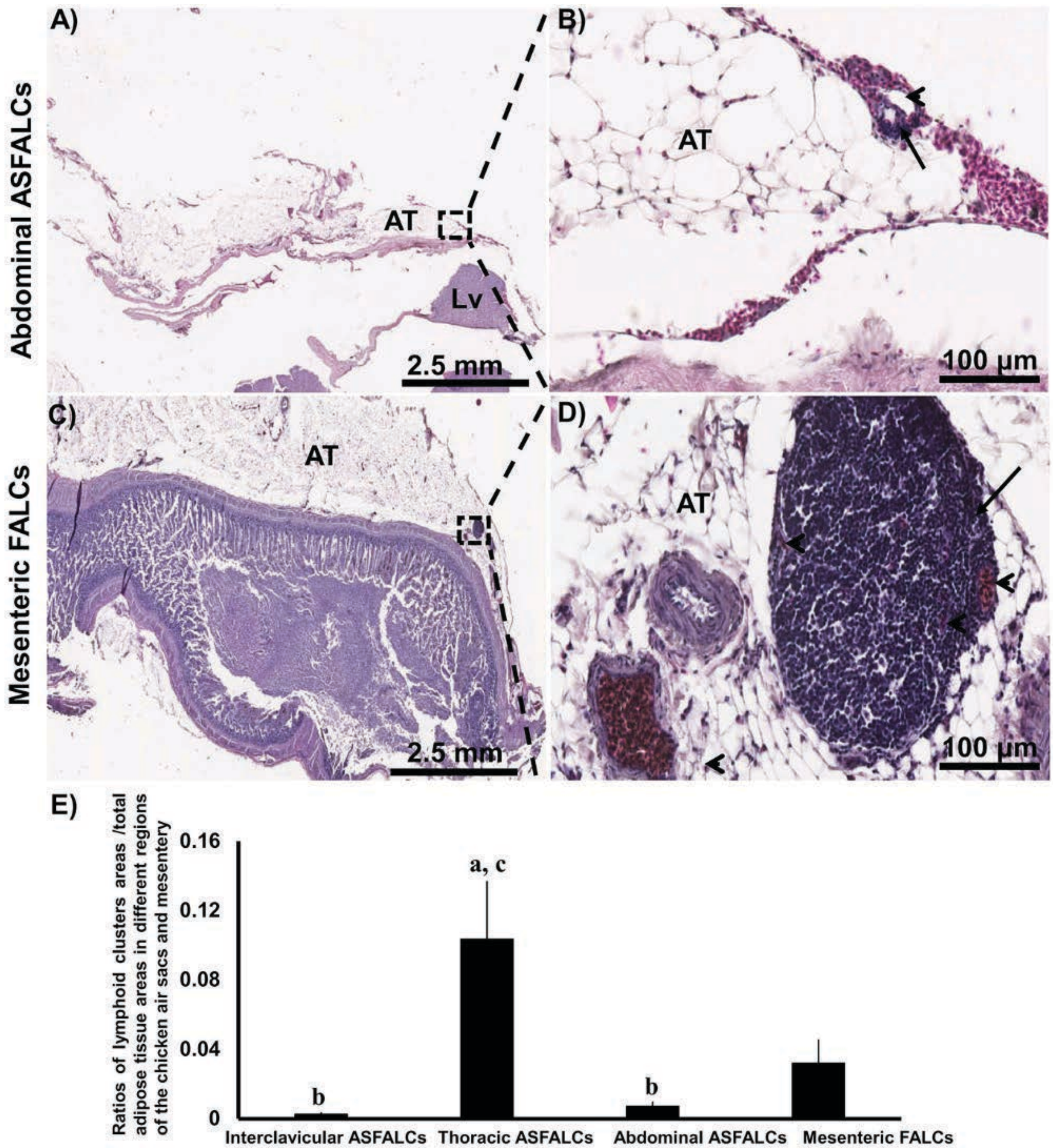

Figure 3: Light micrographs of H&E-stained section of the abdominal air sacs (A), and mesentery (C). (B, D) higher magnification to the boxed area in (A, C), respectively. Notice blood vessels (arrow heads), liver (Lv), lymphoid clusters (arrows) in the adipose tissue (AT) of abdominal air sacs (A, B), and mesentery (C, D). Graph showing the morphometrical data of the ratios of the lymphoid clusters area/ total adipose tissue area in the chicken air sacs and mesentery (E). The letters a, b, c, indicate significant differences between interclavicular air sacs (a), thoracic air sacs (b), and abdominal air sacs (c), with $P < 0.05$, n= 4/experimental tissue group, analyzed by the Kruskal-Wallis test, followed by Scheffé's method. Data are presented as mean values ± SEM

Discussion

The functional activity of the respiratory system in mammals varied extensively from that in birds due to structural variation. The lung in mammals is homogenously partitioned and performs both ventilation and gas exchange simultaneously via its common structures including the respiratory bronchioles, alveolar ducts and alveoli. On the other hand, the respiratory system in birds is heterogeneously partitioned and therefore the functions of ventilation and gas exchange were completely separated (17, 18). The birds characterized by having a unique structure, the air sacs, that serve as bellows to store extra air and allows continuous stream of air to pass through the lungs in one direction (19, 20). Furthermore, other function of the avian air sacs is to remove excess heat as the bird breaths through its connection with the pneumatic bones (21, 22). In birds, it has been reported that the lung is relatively smaller than that of mammals and comprised 10% of the total respiratory system and consisted of parabronchi rather than alveoli. On the other hand, the remaining 90% is occupied by the air sacs (18).

Air sacculitis is common inflammatory condition of the air sacs in birds that is often observed in many infectious diseases including Mycoplasma (23), Newcastle disease (24), Escherichia coli (25), and infectious laryngotracheitis , avian influenza (26, 27). Previously, we reported novel lymphoid clusters associating with the mediastinal fat tissue of healthy mice strains (3). Furthermore, we revealed their role in the development of numerous respiratory diseases including septic condition such as bleomycin induced pneumonitis (5), as well as aseptic infection with Mycoplasma pulmonis (8). Interestingly, in birds more close contact of air carrying infectious agent could lead to the development of air sacculitis due to its functional attribution in allowing continuous flow of the air in one way direction. However, no reports concerning the immunological role of air sacs. Therefore, we histologically examined different types of chicken air sacs for the occurrence of ASFALCs that could be similar structure to that of mammals.

The present investigation revealed less developed ASFALCs in the adipose tissue associated with interclavicular and abdominal air sacs. But well developed ASFALCs were observed in that of both cranial and caudal thoracic air sacs suggesting their possible role in the development of lung associated diseases.

Interestingly, the air sacs of broiler chickens experimentally infected with E.coli revealed the presence of numerous aggregated inflammatory cells replacing the normal air sacs structure (28). This could suggest that the inflammation induce the development of ASFALCs similar to that observed in mammalian FALCs (4, 5). Previous reports indicated that the air sacs communicate with the lung tissues via five perforations on the lung ventral surface of each side (29). Our results revealed well developed thoracic ASFALCs in the vicinity of lung tissue, suggesting their possible role in inflammatory lung progression.

Additionally to the latter, It is known that all air sacs give aeration to specific pneumatic bone of bird except cranial and caudal thoracic air sacs which never aerate any bone and not have any diverticula (30), that may explained the immunological role of thoracic air sacs by presence of higher percentage ASFALCs than other air sacs which have role in ventilation.

Additionally, our results revealed the occurrence of FALCs in chicken mesentery. Similar lymphoid clusters were reported in the mesentery of both human and mice (1). The chicken ASFALCs and mesenteric FALCs showed similar characteristic features to that reported in human and mice where a close contact of immune cells with the surrounding adipocytes and well-developed vasculature were observed (1, 3).

Inconclusions, the current investigations suggest an immunological role of the air sacs and mesenteric adipose tissue in chicken. However, further investigations are required to examine the role of ASFALCs and mesenteric FALCs in the pathogenesis of various avian respiratory and digestive tract diseases.

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