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Temporal, Phenotypic, and Quantitative Characterization of Thyroid Infiltrating Mononuclear Cells During Development of Spontaneous Autoimmune Thyroiditis in Obese Strain Chickens

Katelyn M. Clark
University of Arkansas, Fayetteville

Chrysta N. Beck
University of Arkansas, Fayetteville

Gisela F. Erf
University of Arkansas, Fayetteville

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Meet the Student-Author

Katelyn Clark

I am a Summa Cum Laude May 2023 graduate with a B.S. in Poultry Science and B.A. in Spanish. Prior to attending the University of Arkansas, I went to Fayetteville High School. I am currently interning with Cobb Vantress in their lab and have previously worked at a veterinary clinic in high school and college. In high school, I was also part of the Veterinary Science team for FFA, participated in Beef Quiz Bowl, and took several animal science courses. These activities ultimately inspired me to major in Poultry Science. I was also part of the Health Occupations Students of America Veterinary Science team and competed at their International Leadership Conference before serving as a mentor for the veterinary science team at Fayetteville High School while in college the next year. Having a keen interest in animal health and welfare, I was very interested in immunology, which drew me to my honors thesis topic. I have enjoyed my time at the University of Arkansas and within the honors college and am extremely grateful for the opportunities that both have afforded me. I would like to thank Dr. Gisela F. Erf and Chrysta Beck for their continued guidance and my committee members, Drs. Sara K. Orlowski and Adnan Alrubaye, for their assistance. I would also like to thank Jossie Santamaria for his assistance in the lab and my friends and family who have supported me throughout this chapter of my life.

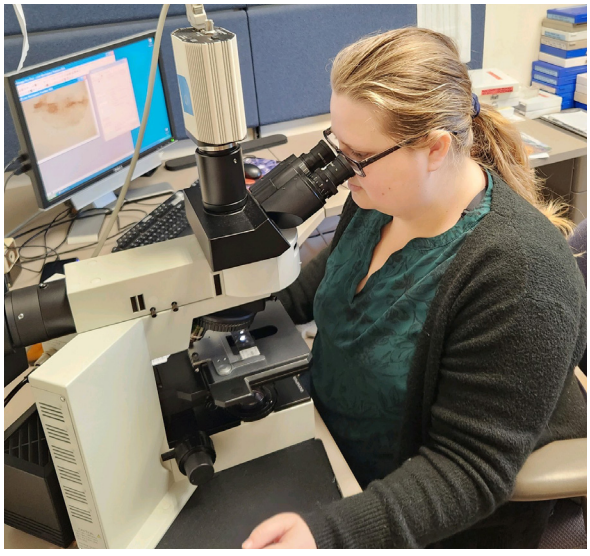

Photo credit: Russell Cothren

Katelyn examining the thyroid tissues under a microscope to evaluate the relative amounts of various immune cells present based on brown staining.

Research at a Glance

- Thyroids were collected from Obese strain chickens prone to spontaneously develop an autoimmune disease affecting the thyroid glands mimicking Hashimoto's thyroiditis in humans.
- Thyroid samples were immunochemically stained to identify different types of mononuclear immune cells infiltrating the thyroid. The samples could then be viewed under a microscope to estimate the proportion of the gland tissue occupied by each type of immune cell type.
- Immune cell infiltration was first observed at 7 days of age, and from 3 weeks onwards, infiltration was nearly complete across most samples. T cells and B cells were the most numerous immune cells present.

Temporal, Phenotypic, and Quantitative Characterization of Thyroid Infiltrating Mononuclear Cells During Development of Spontaneous Autoimmune Thyroiditis in Obese Strain Chickens

Katelyn M. Clark, Chrysta N. Beck,† and Gisela F. Erf§*

Abstract

The Obese strain (OS) of chickens spontaneously develops autoimmune thyroiditis (SAT) and is a well-established biomedical model for Hashimoto's thyroiditis in humans. Both conditions are characterized by the infiltration of thyroid glands with mononuclear immune cells resulting in the destruction of thyroid tissue and impairment of the thyroid's endocrinological functions. Past studies described immune cell infiltration in thyroids of the OS chickens, but the time-course, cell composition, and relative amounts of the various immune cells infiltrating the thyroids have not been well defined. In this project, frozen and stored thyroid glands that were previously collected at 1, 4, 7, 14, 21, 28, 35, and 42 days of age (n = 4 to 5 OS birds/age) were used. Frozen thyroid sections (8-µm thick) were prepared and used in an indirect immunohistochemical staining procedure to identify macrophages, B cells, T cells, T helper cells, cytotoxic T cells, γδ T cells, and MHC II-expressing cells. Stained sections were evaluated by microscopy, and the percentage of tissue area occupied by various cell types was determined. Thyroid infiltration was first observed at 7 days of age, and immune cells occupied the entire tissue in most samples from 3 weeks onwards. Macrophages were the first cells to infiltrate, but T cells dominated the response. MHC II expression reached very high levels by 14 days and remained at nearly 100% thereafter. This study provided new insights regarding the participating immune cells and the chronological order of their infiltration into thyroid glands during SAT development in OS chickens.

* Katelyn Clark is a May 2023 honors program graduate from the Department of Poultry Science.
† Chrysta Beck, Graduate Assistant, Department of Poultry Science.
§ Gisela Erf, the faculty mentor, is a professor in the Department of Poultry Science.

Introduction

Autoimmune diseases have a significant impact on quality of life as they often require long-term management. Hashimoto's thyroiditis is one of the most common autoimmune diseases in the United States, affecting one to two percent of the population (MedlinePlus, 2020). Hashimoto's thyroiditis is characterized by the infiltration of the thyroid with a variety of immune cells that results in damage and destruction of functional thyroid tissue and consequently hypothyroidism. Infiltrating T cells were shown to have cytotoxicity towards thyroid antigens resulting in apoptosis of thyroid follicular cells, while B cells produce specific autoantibodies to thyroid-associated molecules (Ajjan and Weetman, 2015).

High levels of thyroid-stimulating hormone (TSH), low levels of thyroxine (T4), and the presence of anti-thyroid peroxidase and anti-thyroglobulin antibodies are the most critical diagnostic criteria, but Hashimoto's thyroiditis is often not diagnosed until there is considerable damage to the thyroid (Mincer and Jialal, 2022). Once the thyroid is damaged, a broad range of symptoms can occur, such as weight gain, cold intolerance, goiter, depression, joint pain, memory lapses, and fatigue (Mayo Clinic, 2020). Because Hashimoto's thyroiditis goes undiagnosed until late into the disease progression, studying the progression of the autoimmune disease in humans is difficult. As a result, biomedical animal models can play an important role in research focused on the development and progression of autoimmune destruction in the thyroid.

The Obese strain (OS) of chicken housed at the University of Arkansas System Division of Agriculture in Fayetteville, Arkansas, develops spontaneous autoimmune thyroiditis (SAT). The OS line was developed by Randall Cole at Cornell University after he noticed odd physical characteristics in a few of the pullets in the Cornell strain (CS) in 1955 that are now associated with OS chickens: the smaller stature, long and silky feathers, increased subcutaneous fat accumulation, and diminished reproductive development (Cole, 1966). The SAT in OS chickens mimics Hashimoto's thyroiditis and provides an excellent opportunity for investigation into the development of this organ-specific autoimmune disease, especially as SAT develops rapidly with mononuclear immune cell infiltration of thyroid glands beginning within the first week of life (Dietrich et al., 1997). This early, spontaneous, and predictable onset of SAT makes this animal model particularly suitable to study the immunopathology before onset and during the development and progression of the disease (Erf, 2021).

Autoimmune diseases are derived from a loss of self-tolerance of T- and/or B-lymphocytes that results in a specific immune response to self-antigens. In organ-specific autoimmunity, like SAT, other mononuclear cells, such as macrophages, may also play a role in tissue destruction (Janeway et al., 2001). Characterizing the infiltrating mononuclear cells (lymphocytes and macrophages) present both before and during thyroid destruction in OS chickens could provide information on how Hashimoto's disease is initiated and progresses. This study aims to characterize and quantify the mononuclear cells, specifically B cells, various T cell subpopulations, and macrophages, which infiltrate thyroid glands in OS chickens, as well as establish a timeline of mononuclear cell infiltration within the first six weeks of the OS chicken's life.

Materials and Methods

The animal experiment and tissue collection for this study were previously conducted by Dr. G.F. Erf, Department of Poultry Science, University of Arkansas System Division of Agriculture, and her team. A total of 36 birds from the OS line were used for the study. The birds were raised in floor pens under conventional husbandry practices at the Poultry Health Lab. All procedures involving the experimental animals were approved by the University of Arkansas System Division of Agriculture, Institutional Animal Care and Use Committee (IACUC; protocol 21077).

At 0, 4, 7, and 14 days of age, 5 birds were euthanized via CO_2 inhalation, and their thyroid glands were collected. After 14 days, 4 birds were euthanized for thyroid collection each week until the birds were 6 weeks of age. The thyroid glands collected at each time point were placed in plastic histology molds, covered in OCT freezing medium, snap frozen in liquid nitrogen, and stored at -80 °C, with the thyroid glands from the left side being in one mold and the thyroid glands from the right side in another mold.

For this project, an indirect immunochemical staining procedure was used to identify various immune cells and molecules in the thyroid glands, following the procedures described by Sorrick et al. (2022). Specifically, the thyroid glands were cut with a cryostat at -24 °C into 8-µm thick sections, individual sections placed on poly-L-lysine coated glass microscope slides, and sections fixed in acetone for 5 minutes. To prevent non-specific binding of the reagents used for the immunostaining to the cells, the sections were incubated overnight with 10% horse serum in phosphate-buffered saline (PBS) in a humidifying chamber. After incubation and each subsequent incubation step (Table 1), sections were washed with PBS. Next, the sections were incubated for 30 minutes with primary mouse anti-chicken (mac) monoclonal antibodies, which included antibodies specific to the cell surface molecules: CD3 (pan T cell marker), CD4 (T helper cells), CD8 (cytotoxic T cells) and γδ T cell receptor (TCR), Bu-1 (B cells), KUL-01 (macrophages), and MHC II (appears on

a variety of cells and functions as an antigen-presenting molecule). All primary mac-monoclonal antibodies were IgG_1. Hence, to check for the presence of non-specific binding of the reagents used for the immunostaining of the cells, a mouse IgG_1 monoclonal antibody with irrelevant specificity (isotype control) was used instead of the primary specific antibody on a thyroid section. All primary antibodies and the isotype control were purchased from Southern Biotech, Birmingham, Alabama.

To detect binding of the primary antibodies, sections were then incubated for 30 minutes with the secondary antibody, a biotinylated horse anti-mouse (ham) IgG antibody that binds to the primary mac-antibodies (Vector Laboratories, Inc., Burlington, California). The tissue sections were then incubated with ABC reagent, consisting of avidin, which was preincubated with biotin conjugated with horse radish peroxidase (HRP) enzyme following manufacturer instructions (Vector Laboratories). Following the 30-minute incubation with ABC reagent, diamino-benzidine tetrahydrochloride (DAB) that was charged with peroxide was then added to the sections to serve as a substrate for the HRP enzyme. As a result of the enzyme-substrate reaction, a brown product was formed that precipitated at the site of formation; the brown precipitate visually identified the cell with the cell surface molecule the specific primary antibodies bound to, hence identifying a cell based on its unique cell-surface molecule. When the brown precipitate developed, the sections had a final wash with PBS and were counterstained with Methyl Green nuclear stain. The sections were then sealed with a glass coverslip and VectaMount mounting medium (Vector Laboratories).

Tissues were viewed at 40x magnification using a bright field Olympus BX50 microscope to determine the location and proportion of the types of immune cells/cell surface molecules identified by immunohistochemical staining. Tissue images were captured via a cool SNAP camera connected to a computer with Image-Pro Plus software. Images of each section were visually evaluated for the extent of brown precipitation for each cell-type/marker. The areas of brown precipitation and, in the case of overall infiltration, the dense areas of green nuclear staining were assessed subjectively as the portion (%) of the thyroid section with brown or green stain, respectively, by the same evaluator. Proportions were averaged for tissues requiring evaluation of more than one image. One-way analysis of variance was used for statistical analysis to determine the effect of age for each cell type/marker. In the case of a significant age effect ($P \leq 0.05$), Fisher's least significant difference multiple means comparisons tests were conducted to determine differences ($P \leq 0.05$) between means at each age.

Results and Discussion

There was no brown precipitation on the thyroid sections stained with the isotype control, and hence no non-specific binding of any of the reagents used. As only green counterstain was visible on these sections, they were used to evaluate total mononuclear cell infiltration. There was an effect of age for infiltration ($P < 0.001$; Figs. 1–3). Although there was variation in the severity of infiltration within each time point, some general trends were observed from the day of hatch to 42 days of age (Fig. 1). There was no noticeable infiltration until 7 days, when there was mild infiltration at 13 ± 7.8%. The incidence and severity of infiltration increased until 28 days, when nearly the entire thyroid section consisted of mononuclear cells instead of

Table 1. Reagents for immunohistochemistry.

Slide	PBS/10% HS[a]	1° Ab[b]	2° Ab (hamIG^{biotin})[c]	ABC[d]	DAB[e]
1	+	Isotype Control[f]	+	+	+
2	+	mac-KUL-01	+	+	+
3	+	mac-CD3	+	+	+
4	+	mac-Bu-1	+	+	+
5	+	mac-CD4	+	+	+
6	+	mac-CD8	+	+	+
7	+	mac-γδ	+	+	+
8	+	mac-MHC II	+	+	+

[a] PBS = phosphate buffered saline; HS = horse serum; Ab = antibody.

[b] All primary antibodies were mouse anti-chicken (mac) IgG_1 isotype.

[c] ham = horse anti-mouse IgG antibody, conjugated with biotin.

[d] ABC = avidin-biotin complex, a pre-reacted mixture of avidin and peroxidase labeled biotin.

[e] DAB = 3,3'-Diaminobenzidine, substrate for the peroxidase enzyme on ABC that results in formation of a brown product that precipitates at the site of formation.

[f] Isotype control = non-specific binding control, mouse IgG_1 antibody with irrelevant specificity.

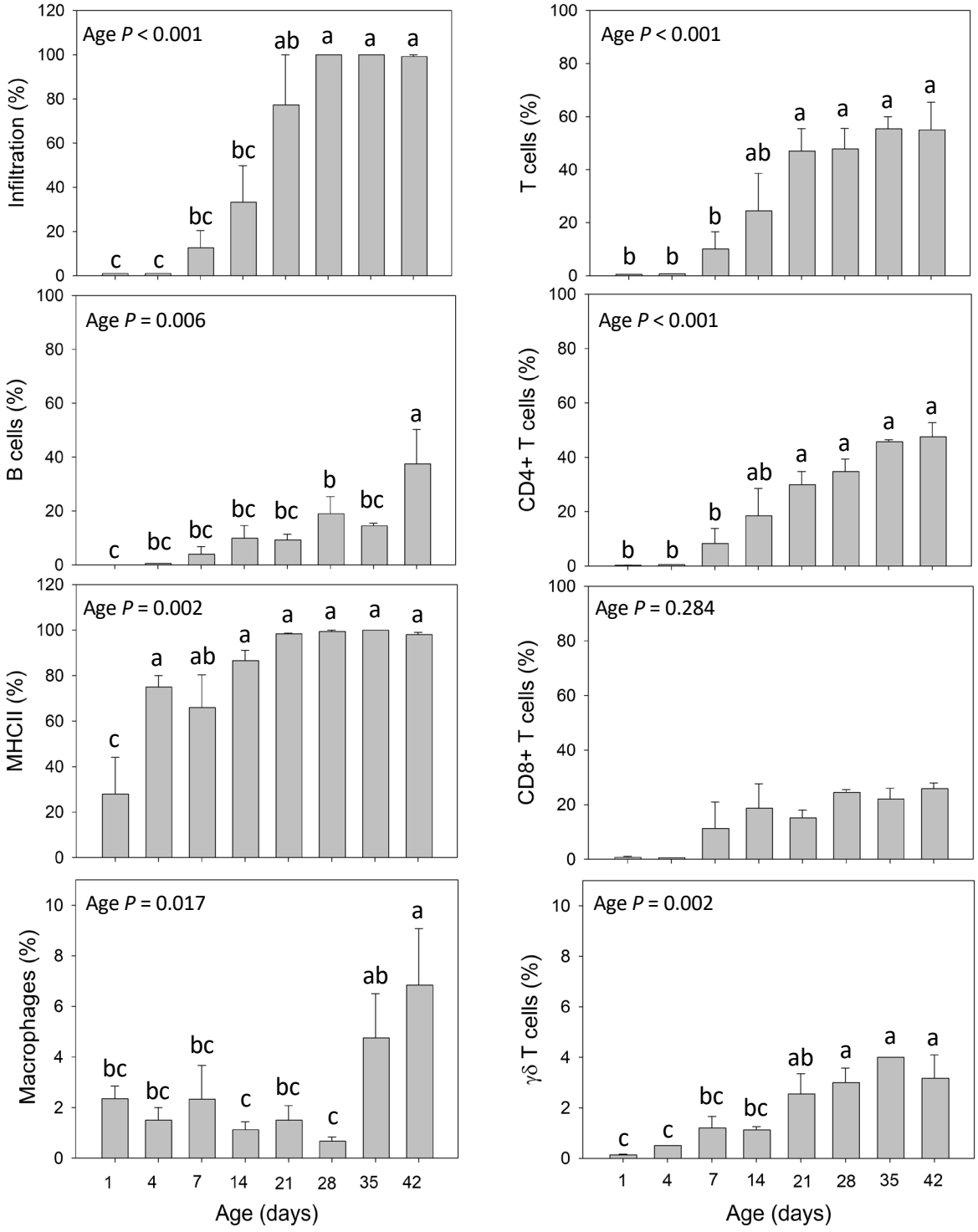

Fig. 1. Thyroid infiltrating immune cells in thyroids from Obese strain chickens during the development of spontaneous autoimmune thyroiditis. Frozen sections of thyroids collected at various ages from day of hatch until 42 days of age were stained via indirect immunohistochemistry using mouse anti-chicken primary antibodies specific for T cells (CD3), B cells (Bu-1), T cell subsets (CD4, CD8, and γδ T cell receptor), macrophages (KUL01) and MHC II expression. Data are mean ± SEM of the estimated percentage of thyroid tissue occupied by various cell types. n = 3 to 5 thyroids per time point; a-c: means without a common letter are different at $P \leq 0.05$.

thyroid follicles. Similar observations of nearly complete replacement of thyroid tissue by infiltrating mononuclear cells were made in thyroids collected at 35 and 42 days (Fig. 1). At 14 days, the first observation of nearly complete infiltration was made (Figs. 1 and 2), but other tissues had much lower severity with a third or less of the tissue occupied by mononuclear cells (Fig. 3).

T lymphocytes were the most prominent leukocyte within the thyroid tissue, and there was an effect of age ($P = 0.004$). T cell proportions in the thyroids followed a similar trend to overall infiltration, with the first noticeable appearance at 7 days with an average of 10 ± 6.5%, but the proportion of T lymphocytes stabilized around 21 days at 47.0 ± 8.5% reaching maximal levels at 35 and 42 days of 55 ± 4.6% and 55 ± 10.4%, respectively (Fig. 1). Like T lymphocytes, B lymphocytes also first appeared on day 7, but occupied only 4% of the thyroid section area (Figs. 1 and 3). B cell levels then gradually increased, reaching their highest proportion at 38 ± 12.8% at 42 days (Fig. 1).

The CD4+ and CD8+ T cells were present in relatively equal proportions up to 14 days of age (Fig. 1). The CD4+ to CD8+ ratio within this two-week period ranged from 0.76 to 1. From 7 days to 42 days, the levels of CD4+ T cells ranged from 8.3 ± 5.5% up to 48% ± 5.2% at 42 days (age, $P < 0.001$), whereas the CD8+ T cell population fluctuated between 11 ± 9.7% and 26 ± 2.1% with no differences (age, $P = 0.284$), resulting in a CD4+ to CD8+ T cell ratio (CD4:CD8 ratio) of 1.89 ± 0.25 at 42 days. While the average proportion of γδ T cells never exceeded 5% of the area of the thyroid tissue, the proportions of this cell type also increased with time (age, $P = 0.002$) (Fig. 1).

Macrophages also were present in small amounts. The most notable time points in terms of the presence of macrophages occurred at 35 and 42 days (age, $P = 0.017$), but even

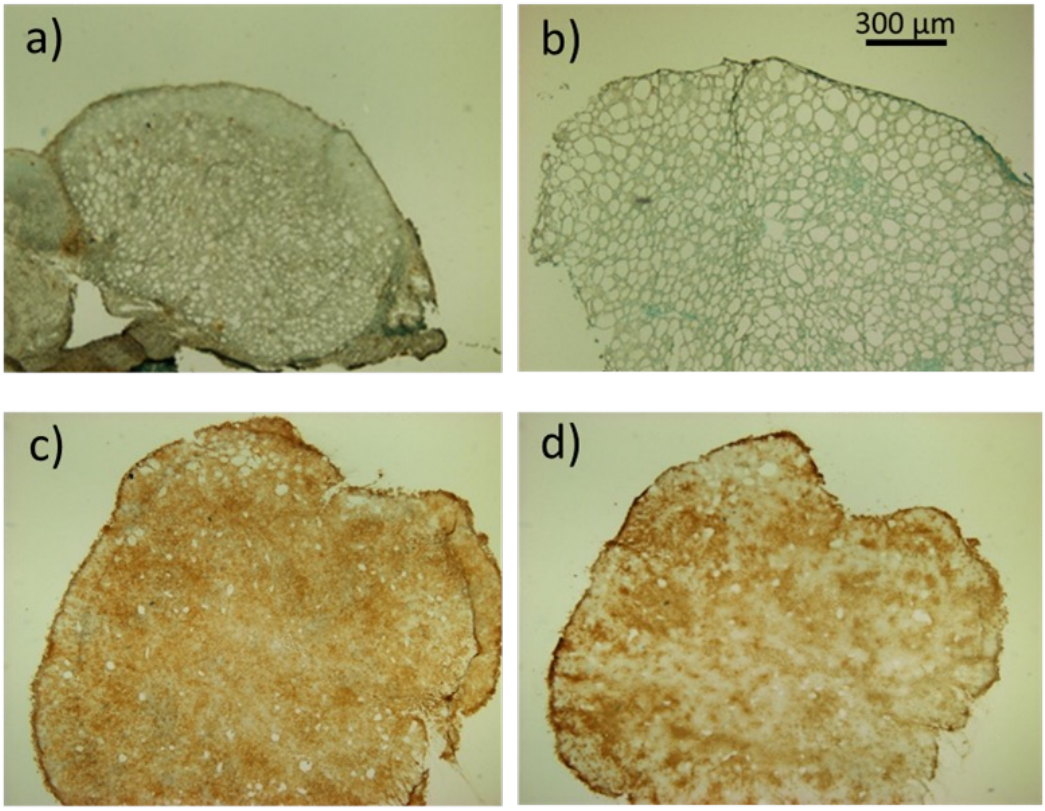

Fig. 2. Chicken thyroid sections with and without immune cell infiltration. a-b) Methyl Green-stained frozen sections of normal thyroids from a) a 4-day-old autoimmune thyroiditis-prone Obese Strain (OS) chick and b) a 6-week-old White Leghorn control chicken showing normal thyroid tissue without immune cell infiltration. c-d) Extensive lymphocyte infiltration of a thyroid gland from a 14-day-old OS chicken; T cells (c) and B cells (d) were identified by indirect immunohistochemical staining using chicken-CD3 and Bu-1 specific primary mouse monoclonal antibodies, respectively. Note: nearly all the thyroid tissue is occupied by T and B cells. Pictures were taken at 40x magnification on a bright field Olympus BX50 microscope equipped with a coolSNAP™ camera.

at the maximal point at 42 days, macrophages were only present at 6.8 ± 2.2%. It should be noted, however, that macrophages were present at a small proportion (1 to 2%) as early as the day of hatch and 4 days, unlike other leukocytes examined (Fig. 1).

Kite et al. (1969) reported a predominance of large mononuclear cells based on conventional histology. While the time course and extent of mononuclear cell infiltration of OS thyroids in the present study agreed with Kite et al. (1969), specific identification of macrophages based on the expression of macrophage cell surface molecule KUL-01 revealed a bimodal pattern of macrophage infiltration. In the current study, we found low but early presence of macrophages, with relative proportions dropping during the major lymphocyte infiltration phase and increasing again when tissue destruction was extensive. Our observations regarding macrophages also agree with Hala et al. (1996), who concluded that macrophages were one of the first cells to infiltrate the thyroid, followed shortly thereafter by B- and T-lymphocytes. Together these observations point towards the role of the macrophages in the initiation of the mononuclear cell infiltration, as well as in the later removal of dying and dead cells during the autoimmune destruction of the thyroid tissue (Abbas et al., 2018).

As described by Hala et al. (1996) and observed in the current study, macrophage infiltration was followed

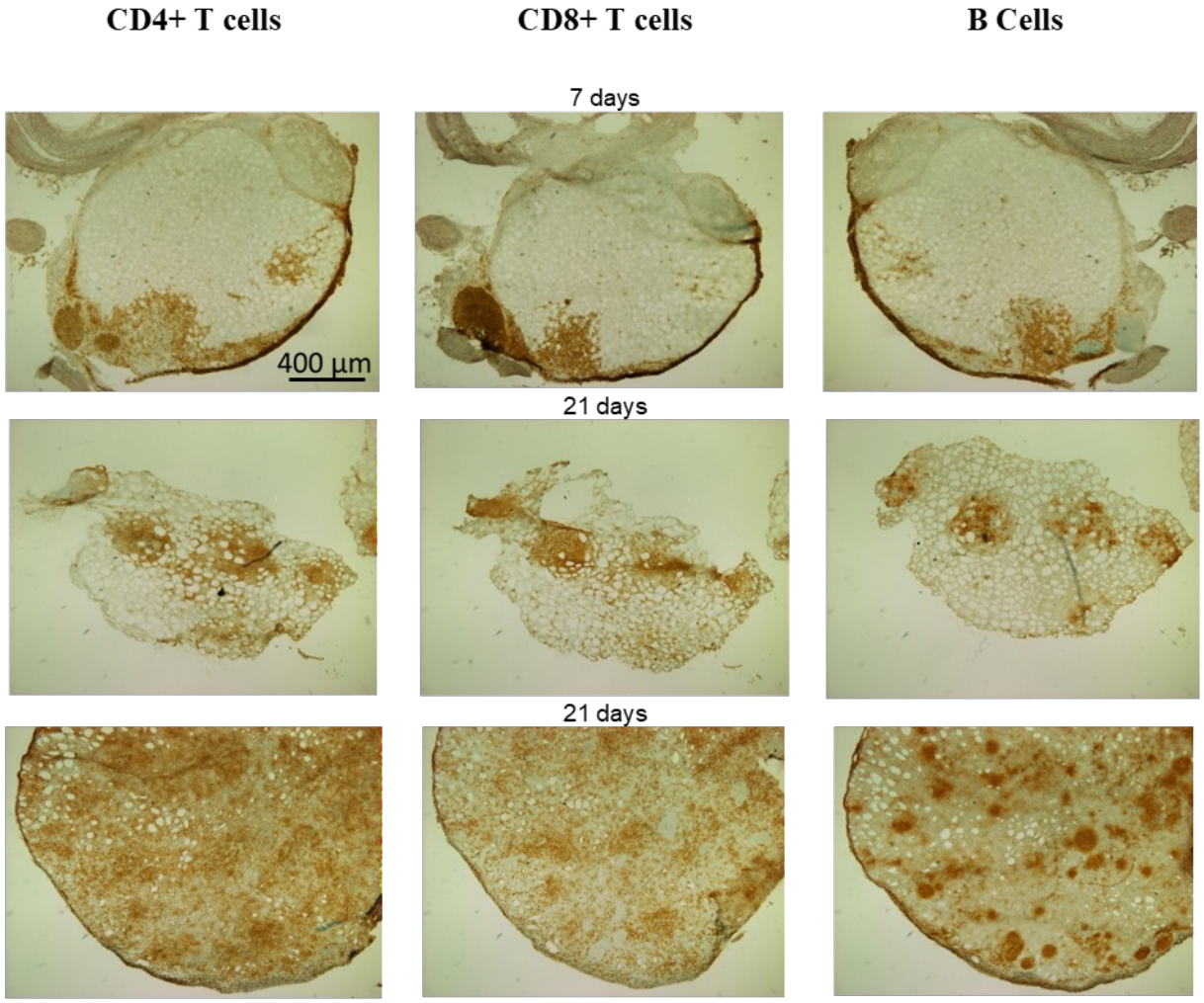

Fig. 3. Images of CD4+ T cell-, CD8+ T cell-, and B cell-infiltration in thyroids during the development of spontaneous autoimmune thyroiditis in Obese Strain chickens at 7- and 21-days of age. Frozen sections of thyroids collected from Obese Strain chickens at various ages from the day of hatch until 6 weeks of age were stained using an indirect immunohistochemical staining procedure utilizing a mouse anti-chicken primary antibody specific for CD4, CD8, and Bu-1 to identify CD4+ T cells, CD8+ T cells and B cells, respectively. Pictures were taken at 40x magnification on a bright field Olympus BX50 microscope equipped with a coolSNAP™ camera.

shortly thereafter by B- and T-lymphocytes, and these small mononuclear cells appeared to be the primary constituents of the thyroid infiltrating cells, with more than 2-fold higher proportions of T cells than B cells at specific time points. As expected, B cells were organized into tightly packed follicles, surrounded by T cells, which also were often observed as aggregates. B cell follicles, described as germinal centers by Hala et al. (1996) and Wick et al. (2006), contain B cells at various stages of activation and differentiation, including proliferating cells, B cells with different isotypes of B cell receptors, and antibody-producing B cells, aka plasma cells. Unfortunately, we did not have markers to identify the different populations of B cells. However, proliferating B cells and plasma cells would morphologically be described as large mononuclear cells, as would proliferating T cells, which may be present in the T cell aggregates. These lymphocyte populations could have been part of the large mononuclear cells described in Kite et al. (1969).

MHC II is found on a variety of cells, and many of the tissues were populated quite densely with MHC II positive cells. By 21 days (age, $P = 0.002$), almost the entire tissue was covered with brown precipitate indicating MHC II expression, which remained the case until 42 days of age (Fig. 1).

Considering that T helper cells need antigen-presentation in association with MHC II on antigen-presenting cells, the extensive expression of MHC II in affected thyroids may be important in their inflammatory activities. However, the extent of MHC II expression cannot be explained by the presence of antigen-presenting cells (macrophages, dendritic cells, and B cells alone) and is likely a reflection of interferon-γ production by infiltrating CD4+ T cells, which in chronic inflammation causes expression of MHC II on non-immune cells as well. Moreover, activated chicken T cells, like activated human T cells (but not mouse T cells), may also express MHC II (Abbas et al., 2018). Overall, the extensive expression of MHC II with increasing mononuclear cell infiltration is likely a reflection of the chronic inflammatory activity taking place in this thyroid-specific autoimmune response.

Conclusions

The current study revealed the time course and profiles of participating immune cells during the development of SAT in the Obese strain chicken model. Immune cell infiltration of the thyroid started within two weeks of life and progressed to complete tissue infiltration by 6 weeks; however, little is known regarding the functional activities of the thyroid infiltrating immune cells (e.g., cytokine production, cytotoxicity). Moreover, it is not clear what is happening to hormone production in the thyroid during this same time period and at what point the destruction of the thyroid begins to impact thyroid hormone concentrations. However, the current study laid the foundation to address these questions in future studies to elucidate what causes the onset and drives the progression of thyroid infiltration and tissue destruction and consequently, the development of hypothyroidism.

Acknowledgments

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