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Distribution and spatiotemporal development of organised lymphoid tissues in the chicken intestinal tract

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ABSTRACT

Chickens exhibit a distinct immune architecture characterised by the absence of draining lymph nodes and the presence of a well-developed mucosa-associated lymphoid tissue. The structure and spatiotemporal development of chicken lymphoid tissues in the intestine are poorly documented. The macroscopically indistinct structure of chicken Peyer's patches has impeded studies into their development. The generation of *CSF1R*-eGFP reporter transgenic chickens enables visualisation of the development, organisation and extent of chicken lymphoid tissues by unique macroscopic views. *CSF1R*-eGFP reporter transgenic chickens were used to investigate the distribution and spatiotemporal development of PP and caecal tonsils in embryonic day 18 to 8-week-old chickens. Peyer's patch anlagen are present at ED18 with a similar frequency and distribution pattern observed in 2- and 8-week-old chickens. These findings can support *in ovo* and post-hatch mucosal vaccination strategies and the development of vaccine delivery systems targeted to the specialized epithelium overlying the Peyer's patches.

1. Introduction

The most common methods of mass vaccination in the poultry industry are via spray/aerosol, drinking water, and *in ovo* routes, which are expected to induce an effective local immune response at mucosal surfaces in the young chick. To improve the induction of robust vaccine responses at the mucosal surfaces, a better understanding of the development of the mucosal immune system and the efficient uptake of the vaccine is required.

The chicken gut-associated lymphoid tissue (GALT) is a collective name for the lymphoid tissues associated with the intestinal tract and disseminated in different anatomical regions. GALT includes organized lymphoid tissues, such as pharyngeal, oesophageal, and pyloric tonsils, Peyer's patches (PP), Meckel's diverticulum (MD), and paired caecal tonsils (CT), in addition to the diffuse lymphoid follicles and aggregations, found in the cervical and thoracic regions of the oesophagus, proventriculus, caecum, rectum, and proctodaeum (Arai et al., 1988; Befus et al., 1980; Casteleyn et al., 2010; Del Moral et al., 1998; Nagy and Oláh, 2007; Oláh et al., 1984, 2003; Vervelde and Jeurissen, 1993). Moreover, all immune cells scattered in the lamina propria and epithelium are considered in chicken GALT (Nagy et al., 2022). Classical structure of PP is compartmentalized into B; Oláh et al., 1984, 2003 cell follicles, interfollicular T cell regions, subepithelial dome regions, and follicle-associated epithelium (FAE) overlaying the lymphoid follicles. FAE of chicken PP has a different cellular composition compared to the villous epithelium and is composed of enterocytes, few goblet cells, and antigen-sampling M cells (Befus et al., 1980; Burns and Maxwell, 1986). While goblet cells are easily distinguishable due to their shape and secretary mucin granules, detection of intestinal M cells is hampered due to the difficulty in finding chicken PP and the lack of appropriate M cell markers. Species-specific differences in GALT structure have been observed in pigs, cows, sheep, goats, and dogs, which develop two types of PP in the small intestine, in the jejunum multiple discrete PP exist while the there is a continuous PP on the ileum. Both are structurally and functionally different from each other (Haley, 2003).

In mammals, the development of PP begins during embryonic days (ED) and continues postnatally in an antigen-independent manner while its full maturation is antigen-dependent. In chicken, PP development begins pre-hatch when accumulations of $MHCII^+$ cells, and $chB6^+$, IgM^+ cells emerge in two sites, one after MD and the other near the ileocaecal junction at ED13, in parallel with the emergence of similar aggregations in the CT region (Jeurissen et al., 1988; Kajiwara et al., 2003). Although chicken PP anlagen appear pre-hatch, macroscopically PP are not visible at the time of hatching. By 10 days post-hatch, one or two PP become

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prominent. Apart from the ileal PP anterior to the ileocaecal junction, other PP are described as randomly distributed in the small intestine and disappear in birds older than one year due to age-dependent involution (Befus et al., 1980; Burns and Maxwell, 1986). Owing to the paucity of information on the precise locations of chicken PP in the literature, most PP studies have been limited to two sites situated in the jejunum (caudal of the MD) and in the ileum (Vaughn et al., 2006).

The generation of colony-stimulating factor 1 receptor (*CSF1R*)-reporter transgenic chickens has enabled us to visualise the chicken lymphoid tissues by fluorescence microscopy (Balic et al., 2014). In these birds, lymphoid follicles are detectable as aggregations of *CSF1R*-transgene$^+$ dendritic cells, follicular dendritic cells, and macrophages in the lymphoid follicles and inter-follicular T cell regions, respectively (Balic et al., 2014). In this study, we used *CSF1R*-eGFP reporter transgenic chickens to investigate the distribution and spatiotemporal development of PP and CT across age groups including ED18, 1-day, 2-week, and 8-week-old chickens, which correspond to crucial points in vaccination schedules for both broilers and layers. The results showed that *CSF1R*-eGFP$^+$ PP anlagen are present in the ED18 intestine with a similar frequency and distribution pattern to PP in 2- and 8-week-old birds.

2. Materials and methods

2.1. Animals

Experiments were conducted using male and female Hy-line 1-day- to 8-week-old *CSF1R*-eGFP reporter transgenic chickens (*Gallus gallus*) and ED18 embryos, provided by the National Avian Research Facility, The Roslin Institute, Edinburgh, UK (Balic et al., 2014). Birds received food and water *ad libitum* and were non-vaccinated. Animal experiments were approved by The Roslin Institute Animal Welfare and Ethical Review Body. Experiments were carried out in accordance with the UK Home Office project licences (PPL 70/8940 and PE263A4FA) and subject to the UK Home Office Animals Act 1986 (Scientific Procedures).

2.2. Tissue preparation and whole-mount imaging

The spatiotemporal development of lymphoid aggregations in the small and large intestines was investigated in ED18, 1-day-old, 2- and 8-week-old chickens (3 birds per each age group) based on the accumulation of eGFP$^+$ cells. For the whole-mount fluorescence imaging, the intestine was dissected from the ventriculus to the cloaca of *CSF1R*-eGFP reporter transgenic chickens and placed in a Petri dish. The mesenteric membranes were removed and the presence of the lymphoid tissues based on eGFP expression was investigated using Axio Zoom.V16 fluorescence stereo zoom microscope without prior fixation or immunostaining. Images were analysed by ZEN software (Carl Zeiss Zen pro-2012 software). For the purpose of the quantification, a PP anlage and PP were defined as any *CSF1R*-eGFP$^+$ aggregate in ED18 and 1-day-old birds and *CSF1R*-eGFP$^+$ aggregations greater than 1 mm in 2- and 8-week-old birds, respectively. After whole-mount fluorescence imaging, the length of each segment was measured. The different segments of the small intestine, including duodenum, jejunum, and ileum, were defined as shown in Fig. 1. Both caeca and the rectum were considered as the large intestine.

2.3. Histology

To study the developmental changes in PP structure over time, the duodenal PP, jejunal PP situated caudal to MD, ileal PP, and CT were selected. Small intestinal PP and CT were collected immediately after fluorescence imaging and fixed in 10% neutral buffered formalin (NBF, Cell Path) for 24 h at room temperature. Trimmed tissue pieces were embedded in paraffin and sectioned at 8 μm, mounted on SuperFrost Plus slides (Thermo Fisher Scientific) and incubated at 50°C overnight. Formalin-fixed paraffin-embedded (FFPE) sections were dewaxed and stained with Haematoxylin (Harris modified, Sigma-Aldrich) and Eosin. FFPE sections were taken from ED18, 2-, and 8-week-old chickens. In ED18 embryos, an aggregation was considered a PP anlage if it had thickened villi or elevated lamina propria, whereas, in older birds, an aggregation was considered structurally matured PP if it had multiple B cell follicles and the FAE with a reduced number of goblet cells compared to the villous region. Images were captured on a Nikon Bright-field microscope and analysed by ZEN software (Carl Zeiss, blue edition).

3. Results

3.1. Anatomical localisation and distribution of CSF1R-eGFP$^+$ lymphoid aggregations in the intestine

The whole-mount microscopy of *CSF1R*-eGFP$^+$ aggregates in ED18 embryos indicated a pattern of 1 duodenal, 3–4 jejunal, and 1 ileal PP from the serosa of the small intestine (Figs. 1A and 2A). To confirm the presence of the aggregations from the mucosal surface, the intestine was opened longitudinally along the mesenteric border and each aggregation was imaged from the luminal aspect as shown in red boxes (Fig. 1A). Duodenal PP anlage appeared as an elongated (Fig. 1A) and flat aggregation that occurred on the mesenteric aspect or between mesenteric and anti-mesenteric sides of the duodenum oriented towards the pancreas (Fig. 2B). In the jejunum, 3–4 PP anlagen were located on the anti-mesenteric or between mesenteric and anti-mesenteric sides (Fig. 2B). Except for the nearly compact anlage always emerging caudal to the MD, other jejunal PP anlagen were either continuous or discontinuous elongated patches (Fig. 1A). The ileal PP anlage was found as a compact *CSF1R*-eGFP$^+$ aggregation at the anti-mesenteric side located at the proximal end of the ileum (Fig. 2B). In the caeca, apart from the caecal tonsil anlage, 1–3 caecal patches were observed at the curve of mid-caecum (Fig. 1A, image number 10). In contrast to the small intestine, the large intestine contained multiple isolated lymphoid follicles (ILF) in the ED18 gut (Fig. 1A, image numbers 9 and 10). None of the reported aggregations in the intestine were macroscopically detectable from the serosa.

In 1-day-old chickens, a pattern of 1 duodenal, 2–3 jejunal, and 1 ileal PP with a similar phenotype and distribution to ED18 anlagen were observed (Figs. 1B and 2A). The noticeable differences with the intestines of ED18 embryos were the emergence of *CSF1R*-eGFP$^+$ aggregations at the base of the MD and a reduction in scattered ILF in the large intestine. Duodenal PP anlagen were flat and elongated (Fig. 1B) and at the mesenteric side (Fig. 2B). In jejunum, PP located proximal to the MD were generally flat and elongated (Fig. 1B) and located on all sides (Fig. 2B) whereas the jejunal PP which was located at the anti-mesenteric side of distal MD (Fig. 1B). Ileal PP exhibited flat and compact (Fig. 1B) morphology and positioned at the anti-mesenteric side (Fig. 2B).

Apart from the organized lymphoid aggregations in the MD, a pattern of 1 duodenal, 3–5 jejunal and 1 ileal PP was observed in the small intestine of 2-week-old chickens (Figs. 1C and 2A). Compared to the intestinal tract of 1-day-old chicks, various-sized patches were considerably extended throughout the intestine. ILF were occasionally seen in the duodenum, jejunum, and ileum (Supplementary Fig. 1). Duodenal PP followed the same morphological features as that of in ED18 embryos and 1-day-old chickens and appeared as a flat and elongated patch (Fig. 1C) at the mesenteric side of the intestine (Fig. 2B). Jejunal PP which were located cranial to the MD displayed an elongated

structure (Fig. 1C) majority of which were flat whereas the jejunal PP located caudal to the MD was relatively compact and macroscopically bulged. While the jejunal PP cranial of the MD were found at anti-mesenteric side or between anti-mesenteric and mesenteric aspect, jejunal PP caudal MD were largely observed at the anti-mesenteric side (Fig. 2B). The ileal PP was located at the anti-mesenteric side (Fig. 2B) and was mostly compact and slightly bulged. Caecal tonsils were distinctively enlarged and 2–6 caecal patches were detected at the caecal curve (Fig. 1C, image number 10). ILF, duplets, and smaller patches were present at the caecum (Fig. 1C, image number 11). In the rectum, ILF and duplets were scattered along the intestinal wall (Fig. 1C).

In 8-week-old chickens, a pattern of 1 duodenal, 3–4 jejunal, and 1 ileal PP was observed in the small intestine (Figs. 1D and 2A). In the duodenum, ILF were scattered in the descending part of the duodenum (Supplementary Fig. 1) and a well-developed duodenal PP was detected in a similar position and with a similar morphology to duodenal PP in younger birds (Fig. 2B). In the jejunum, apart from PP, multiple ILF, duplets, and small patches were present (Supplementary Fig. 1). Jejunal PP located proximal to the MD were mostly flat and showed both elongated (Fig. 1D) and compact structures with variable lengths. In contrast, jejunal PP distal to the MD appeared elliptical and compact and bulged out on the anti-mesenteric side of the intestinal wall (Fig. 2B). A large number of ILF, duplets, and triplets were found in the distal part of the jejunum and this pattern continued to the proximal part of the ileum (Supplementary Fig. 1). The ileal PP macroscopically appeared as a milky pink patch, likely due to higher vascularisation, with a distinctive bulge structure at the proximal part and anti-mesenteric side of the ileum (Fig. 2B). In the caeca, large caecal tonsils are macroscopically apparent and a group of expanded patches were located at the caecal curve while disc-like patches were spread at the caecal tips (Fig. 1D, image numbers 9 and 10, respectively). In the rectum, a clear pattern of ILF were observed that were in 2–3 rows (Fig. 1D). In general, the size of the PP and number of ILF increased with age.

The overall distribution of the PP in the small intestine relative to the length of the small intestine at each age is given in Fig. 2A. The graph illustrates a consistent spatiotemporal distribution of PP within and across age groups. The duodenal PP was always found at the ascending part of the duodenal loop (Fig. 2B). Jejunal PP were mostly concentrated at the proximal jejunum whereas the jejunal PP caudally located to the MD was found at the distal jejunum (Fig. 2B). The location of the PP was highly consistent in the ileum where the ileal PP occurred at the proximal ileum and anti-mesenteric side of the intestinal wall. Nonetheless, PP are not all anti-mesenteric; while the duodenal PP is either located on the mesenteric side or in between, the jejunal PP are largely located in anti-mesenteric or between mesentery and anti-mesentery.

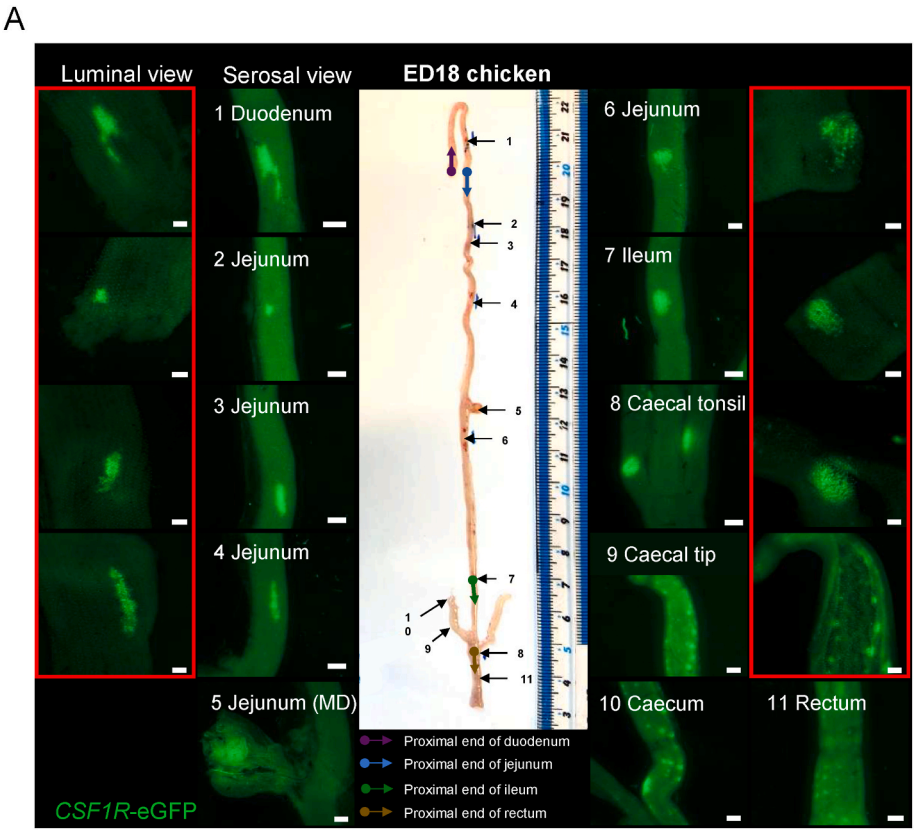

Fig. 1. Visualisation of lymphoid aggregations in the small and large intestine of *CSF1R*-eGFP reporter transgenic embryos and chickens. Whole-mount fluorescence images from the serosal surface indicate *CSF1R*-eGFP^{+} lymphoid aggregations in (A) ED18 embryos, (B) 1-day-old chicks, (C) 2-week-old chickens and (D) 8-week-old chickens. Red boxes illustrate images of the *CSF1R*-eGFP^{+} aggregations from the luminal surface in higher magnification to ensure the presence of the aggregations. The coloured arrows illustrate the proximal end of each intestinal segment. Three individual embryos or chickens were used for imaging at each age. Scale bar is 200 μm in A and B and 1000 μm in C and D. MD: Meckel's diverticulum.

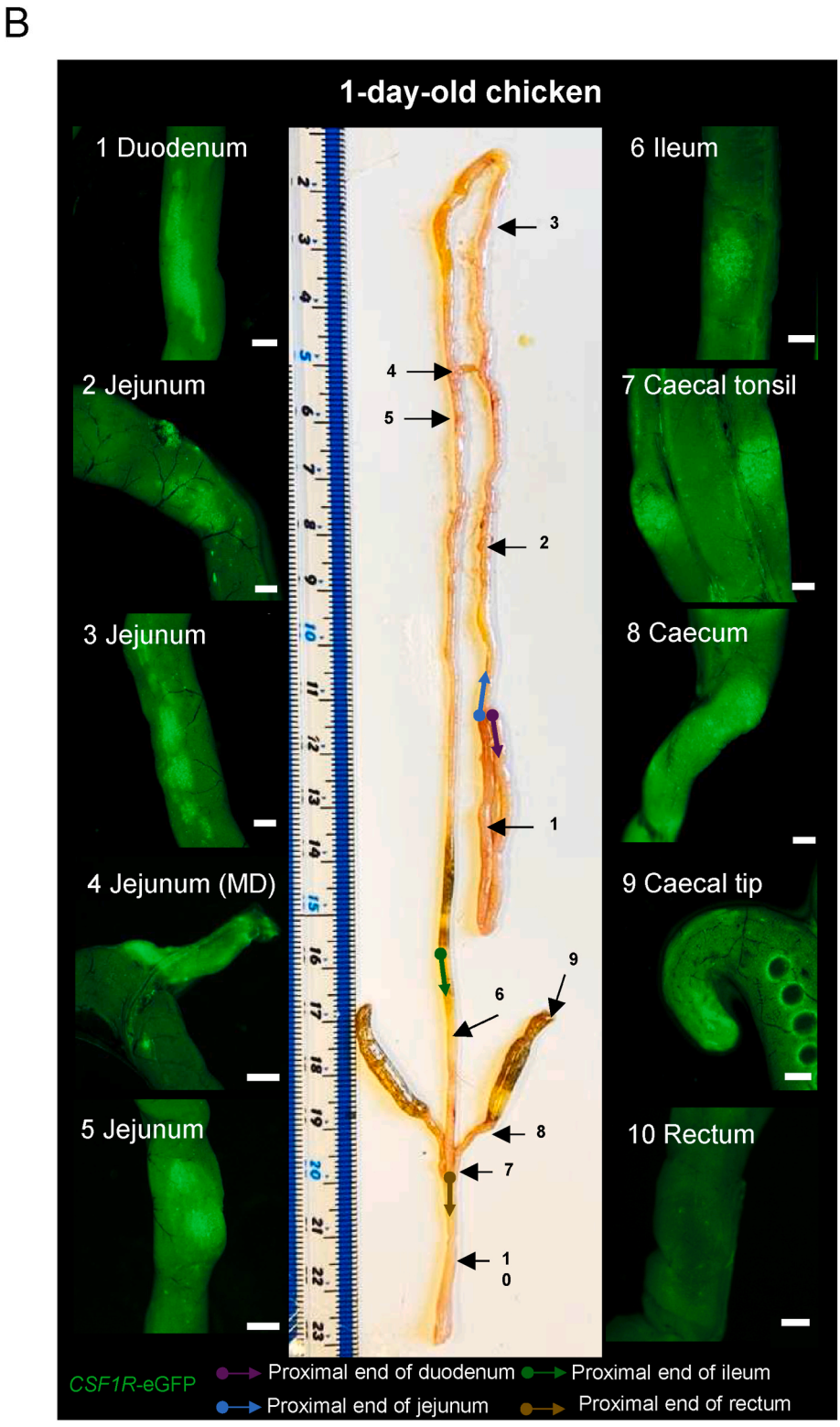

Fig. 1. (*continued*).

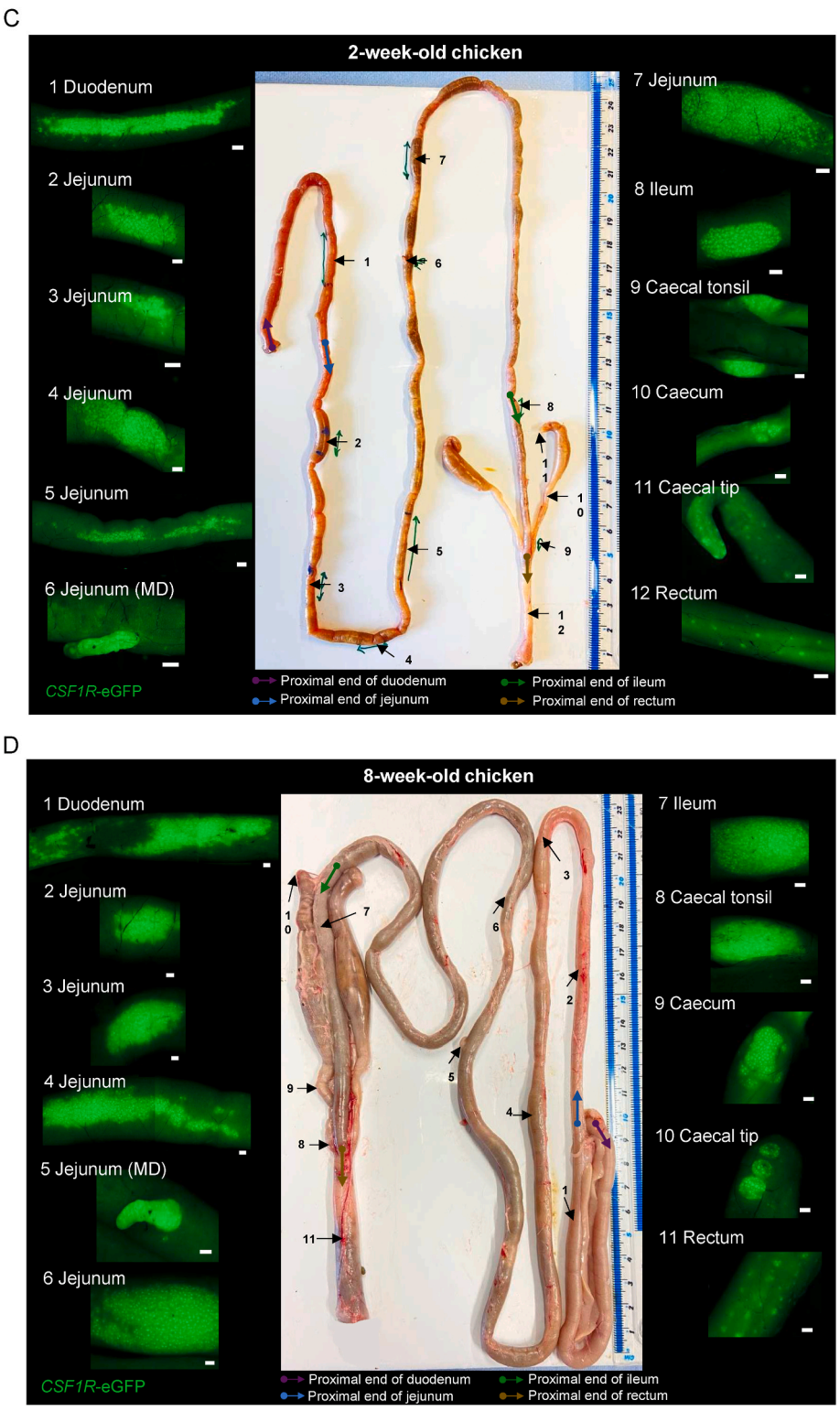

Fig. 1. (*continued*).

3.2. Visualising the spatiotemporal development of lymphoid aggregations in the chicken intestine through light microscopy

In our spatiotemporal study on chicken PP, we aimed to explore the developmental dynamics at different ages that are related to the time points at which vaccination may occur in the field. However, the developmental changes of PP anlagen occurring between ED18 and 1 day of age are relatively subtle and therefore we excluded 1-day-old chickens from further histological studies. Bright-field microscopic analysis of the duodenal PP anlage at ED18 revealed an occasional enlarged villus or enlarged lamina propria at the villus base (Fig. 3 and Supplementary Fig. 2). In contrast, in 2-week-old chickens, the duodenal PP occupied a substantial portion of the tissue, encompassing up to half of the lumen, and exhibited the presence of 10 lymphoid follicles per cross section (Fig. 3). These follicles were partially or fully encapsulated (Supplementary Fig. 2). The FAE regions overlying the PP had a reduced

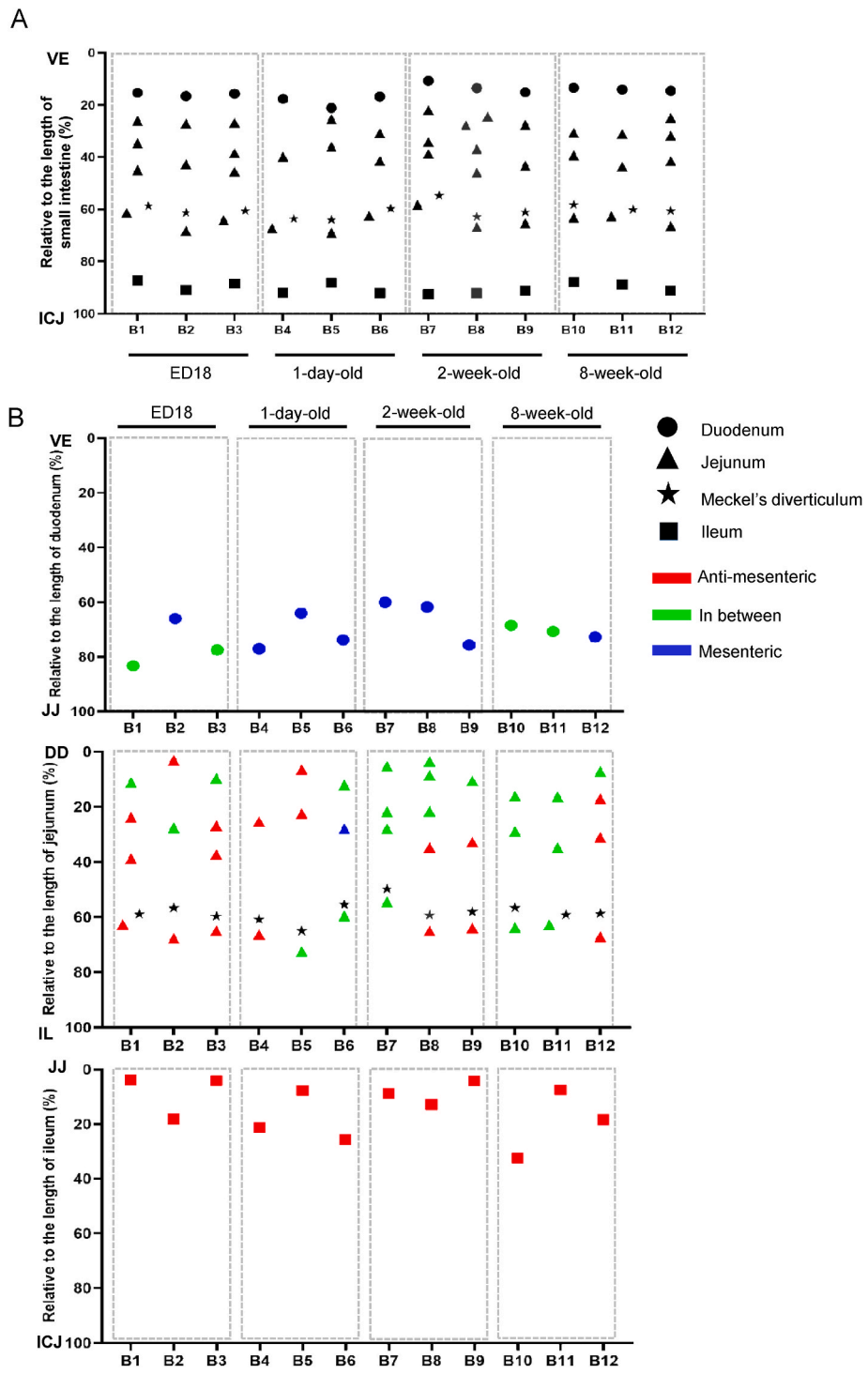

Fig. 2. Distribution of Peyer's patches in the small intestine of ED18 to 8-week-old birds. (A) Relative location of PP or PP anlagen was determined across the small intestine in 4 ages based on *CSF1R*-eGFP^{+} aggregations. (B) Relative location of each Peyer's patch to the length of each segment in 4 age groups. The orientation of PP with regard to the mesentery is colour-coded. Data are derived from 3 embryos or chickens in each age group (B1 to B12). VE: Ventriculus, DD: Duodenum, JJ: Jejunum, IL: Ileum, ICJ: Ileocaecal junction.

number of goblet cells compared to the adjacent villi. In 8-week-old chickens, a noticeable expansion was evident in both the length and width of the duodenal PP compared to the duodenum in 2-week-old birds (Fig. 3). Cross-sectional views revealed a varied count of 18–34 lymphoid follicles, with the majority exhibiting partial or complete encapsulation (Supplementary Fig. 2). These follicles were found throughout the lamina propria from the base to the tip of villus. The size and encapsulation of the follicles was not related to their location, suggesting that maturation of the germinal centers occurred throughout the villus.

The jejunal PP anlage, located after the MD in ED18 embryos, exhibited a slightly thickened lamina propria (Fig. 3). Jejunal PP in 2-week-old birds expanded across half of the lumen and adopted a dome-like structure (Supplementary Fig. 3). Within a cross-sectional view, up to 7 lymphoid follicles were discernible and all follicles were positioned above the muscularis mucosae. The jejunal PP in 8-week-old

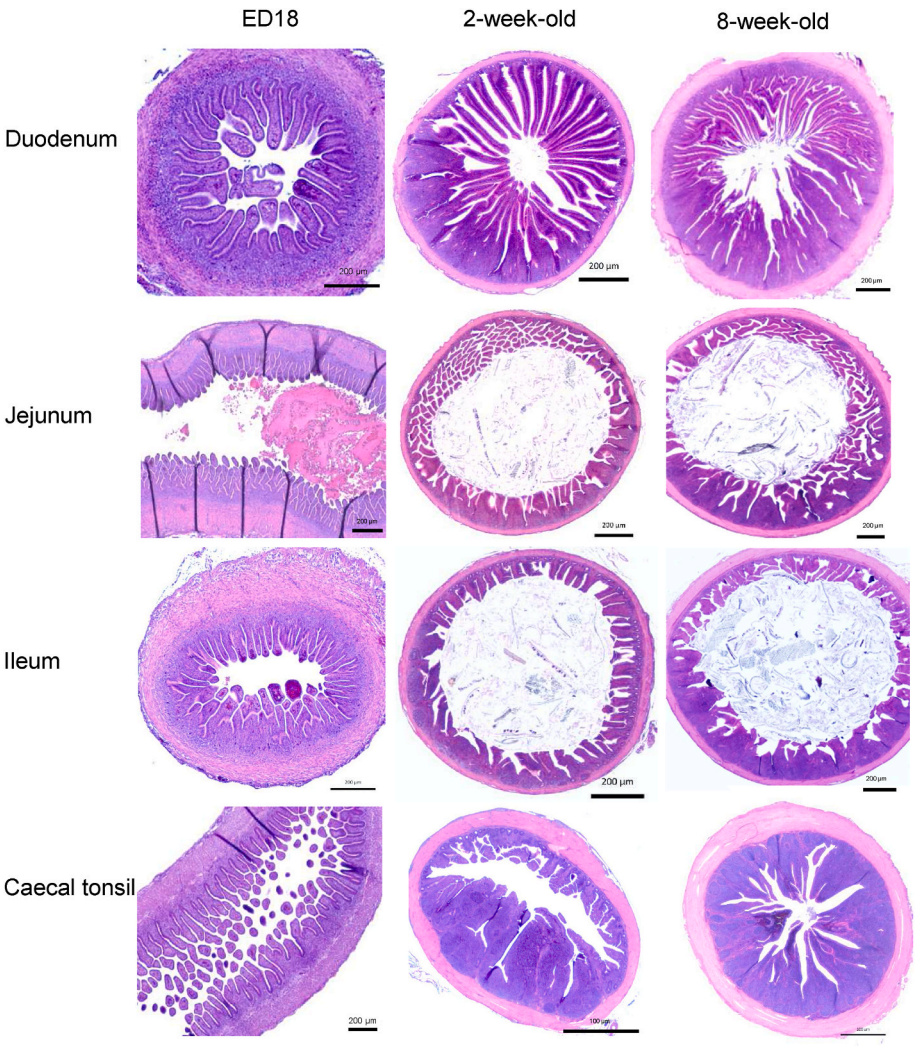

Fig. 3. Spatiotemporal development of intestinal Peyer's patches and caecal tonsil. FFPE sections of selected PP of ED18, 2-week-old and 8-week-old duodenum, jejunum, ileum and caecal tonsil (CT) were stained for H&E. Scale bar is 200 μm except in CT scale bar is 100 μm. Image enlargements of Peyer's patches, CT, and follicle associated epithelium are shown in Supplementary Figs. 2–5. Data are derived from 3 embryos or chickens in each age group.

chickens resembled that of 2-week-old birds and occupied half of the tissue but its structure was further expanded, with 27–31 lymphoid follicles located above the muscularis mucosae (Supplementary Fig. 3).

The structure and morphology of ileal PP were comparable to those in jejunal PP of the same ages. Enlargement of the lamina propria was seen in the ileal PP anlage of ED18 embryos, whereas the ileal PP with a dome-like structure was extended up to half of the lumen in 2-week-old birds (Fig. 3). The ileal PP contained 7–9 lymphoid follicles and they were partially or fully encapsulated (Supplementary Fig. 4) and the FAE was devoid of goblet cells. In 8-week-old chickens, ileal PP appeared more expanded compared to 2-week-old birds and protruded towards the lumen (Supplementary Fig. 4). The PP lost its dome-like shape to a more continuous uniform patch. Around 30 follicles were found in a cross section of ileal PP, mostly encapsulated by a layer of reticular cells (Supplementary Fig. 4).

The structure and developmental changes of the CT were slightly different from the small intestinal PP. At ED18, the thickened villi and elevated lamina propria were already clearly apparent (Fig. 3). In 2-week-old birds, the CT occupied more than half of the lumen and the length (Fig. 3). Up to 9 follicles were scattered in CT at different heights from the base to the tip, the majority of which were surrounded by a thin layer of reticular cells (Supplementary Fig. 5). A lower number of goblet cells was observed in the epithelium lining the CT invaginations whereas the epithelium covering the tip of the CT still contained a considerable number of goblet cells similar to villi regions (Supplementary Fig. 5). In 8-week-old birds, CT was considerably expanded and only occasional villi were found next to the CT structures with typical morphology of villous lamina propria and epithelium (Fig. 3). The number of follicles reached 56 in cross sections and mostly were encapsulated (Supplementary Fig. 5).

4. Discussion

While organized GALT, such as PP and CT, could be the primary targets for widely administered oral and *in ovo* vaccines in the poultry industry, there is an extreme paucity of information on the ontogeny, location, and morphological features of PP in chickens. In this study, the development and distribution of chicken PP were studied based on *CSF1R*-eGFP^{+} cell aggregations. Our findings show that the development of all PP initiates during embryogenesis at pre-determined locations, whereas its compartmentalisation and structural maturation take place post-hatch.

The gross morphology of chicken PP and the lack of a systematic methodology to locate them have substantially limited the progress of studies into vaccine targeting and antigen uptake. In this study, PP with variable sizes were found on the mesenteric side, as well as anti-

mesenteric side and in between. This is contrary to the literature that only describes chicken PP at the anti-mesenteric side of the intestinal wall (Casteleyn et al., 2010). The jejunal and ileal PP always appeared in the caudal MD and cranial region of the ileocaecal junction, respectively, consistent with the literature (Befus et al., 1980; Burns and Maxwell, 1986; Kajiwara et al., 2003; Vaughn et al., 2006). Moreover, we identified another PP at the distal segment of the duodenum which was constantly present across different age groups. Morphologically, duodenal PP and the jejunal PP, located in cranial MD, formed elongated patches whereas jejunal PP located in caudal MD and ileal PP appeared more compact which likely contributes to the bulging structures in older birds. In addition, bulging could occur in villi regions devoid of PP. The consideration of other macroscopic features, such as rich blood supply, colour variation, shape, and overall morphological divergence between PP and adjacent villi, could offer corroborative cues for confirming PP presence (Befus et al., 1980; Burns, 1982; Vaughn et al., 2006).

While we did not conduct multicolour staining to specifically characterize *CSF1R*-eGFP^{+} cells in embryos and chickens, previous studies indicated that the distribution of these cells within the PP aggregates closely resembles that of follicular dendritic cells found in germinal centers (Balic et al., 2014; Eikelenboom et al., 1983; Jeurissen, 1993). In the process of embryonic development, at ED13, MHCII^{+} cells aggregate in the lamina propria. Subsequently, by ED15, these clusters expand, concurrent with an elevated presence of chB6^{+} (Bu-1) and IgM^{+} cells in this region (Kajiwara et al., 2003).

The chicken PP are distributed in a conserved pattern in the intestine compared to the human PP (Cornes, 1965; Reynolds et al., 1985). The majority of chicken PP were localized in the anterior part of the small intestine. The anatomical localisation of the jejunal PP caudal MD and ileal PP have been documented in other avian species, such as duck and turkey, implying the potential applicability of our data in other avian species (McGarry and Bourns, 1980; Talab et al., 2022). Up to six PP anlagen were detected along the small intestine of ED18 embryos with a relatively similar number and distribution in older birds. These findings are not comparable with those of Kajiwara et al. (2003) and Befus et al. (1980) who indicated that only two PP anlagen are formed during embryonic stages and this number increases up to 6 PP by 12–16 weeks post-hatch (Befus et al., 1980; Kajiwara et al., 2003). In fact, our data suggest the potential stability of PP numbers post-hatch similar to mice and contrary to human PP whose number increases after birth (Cornes, 1965; De Jesus et al., 2013), although further studies are required to clarify the potential alterations of PP abundance in birds older than 8 weeks.

In summary, this study indicated that the variation in size, shape, number, architecture, and location of chicken PP is greater than appreciated in the literature. The presence of all PP anlagen at ED18 with a similar frequency and distribution to 8-week-old chickens supports the hypothesis that the development of major PP is preprogramed at the embryonic stage but structurally-matured PP appear post-hatch. These findings can be implemented into the development of *in ovo* and post-hatch mucosal vaccination strategies and vaccine delivery systems by offering time windows for immunisation.

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Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2023.105096.

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