CHARACTERIZATION OF ANTIGEN-PRESENTING CELLS IN CHICKEN PEYER'S PATCHES BY IMMUNOHISTOCHEMICAL STAINING

A Thesis

by

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ABSTRACT

In the chicken, Peyer's patches (PPs) represent a crucial gut-associated lymphoid tissue (GALT) responsible for antigen sampling and activation of T-cells and B-cells. This involves antigen presentation in the context of major histocompatibility complex class II (MHC-II) by professional antigen-presenting cells (APCs). This study aims at elucidating the microanatomical organization of the APCs in the PPs in order to better understand their role in initiating the response to orally administered vaccines. PPs can be most readily identified in young birds (3-12 weeks of age) as an ovoid white patch about 1-cm in length on the antimesenteric side of the mucosa in the distal ileum between the ceca and cephalic to the cecal tonsils. The hallmarks that make the PPs different from the adjacent intestinal tissue include thickened villi, heavy lymphocyte infiltration, and isolated follicles deeply embedded in the muscularis mucosae/submucosa of the intestine. In this study, PPs and the adjacent tissue were rolled up like a Swiss-roll, snap frozen in liquid nitrogen (vapor phase), cryosectioned at 5μm and 10μm and fixed by cold acetone and 80% methanol prior to immunofluorescent visualization of CD205 (DCs), CD40 and/or MHC-II (APCs), IgM (B-cells) and CD3 (T-cells). In the center of the PP follicle, CD40 and surface IgM were abundantly expressed, whereas the expression of MHC-II and CD205 was relatively scarce. $CD3^+$ cells were predominantly distributed in the peripheral zone of the PP follicle, the lamina propria of the adjacent villi, and localized intraepithelially. MHC-II^+ APCs were packed subepithelially throughout the lamina propria, with some penetrating the follicle-associated epithelium (FAE) towards the lumen. $CD205^+$ DCs appeared as single cells near the crypts and were occasionally found inside the follicles. $CD40^+$ APCs were clustered both inside and outside the follicles. These

results show that, much like in mammalian PPs, naïve B-cells are the major cell type occupying the follicles of chicken PPs, while T-cells are found in the interfollicular areas.

DEDICATION

To my Mom and Dad

To my Grandparents

To the future of immunology

And to my beloved friend and brother, Song

To my boyfriend, James

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NOMENCLATURE

APC	Antigen-presenting cells
CD	Cluster of differentiation
DC	Dendritic cells
FAE	Follicle-associated epithelium
GALT	Gut-associated lymphoid tissues
GC	Germinal center
H&E	Hematoxylin and Eosin
IEL	Intraepithelial lymphocytes
IF	Immunofluorescent
IFR	Interfollicular region
IHC	Immunohistochemistry
LE	Lympho-epithelium
LF	Lymphoid follicle
MALT	Mucosa-associated lymphoid tissues
MHC-II	Major histocompatibility complex class II
PP	Peyer’s patches
SED	Subepithelial dome region
SER	Subepithelial region

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CHAPTER I

INTRODUCTION

The adaptive immune response that follows a vaccination initiates crosstalk between the professional antigen-presenting cells (APCs: dendritic cells, macrophages, and B-cells) and naïve lymphocytes in secondary immune organs. Antigen peptides are loaded onto major histocompatibility complex (MHC) molecules of class I or II and presented to naïve $CD8^+$ and $CD4^+$ lymphocytes, cytotoxic and T-helper cells, respectively. The initiation of an adaptive immune response in avian species depends largely on dendritic cells (DCs). Since poultry are perhaps the most heavily vaccinated among all livestock, research of chicken DCs is vital to discovering an efficient vaccination mechanism (Staines, Young and Butter, 2013). The focus on chicken APCs, especially DCs, has not been optimal in recent research. Additionally, there are limited histological and immunological data on chicken Peyer's patches (PPs), one of the classical lymphoid organs in the intestine (Oláh *et al.*, 2003). The development of an oral vaccine targeting CD40 expressed on the cell surface of chicken APCs (Chen *et al*., 2010; 2012) and the isolation of $CD205^+$ DCs from chicken peritoneal exudate cells (Vuong *et al.*, manuscript in preparation), stimulated our interest in visualization and microanatomical characterization of APCs in chicken PPs by immunohistochemistry and confocal microscopy. Monoclonal antibodies (mAb) against chicken CD40 (designated "2C5") and chicken CD205 (anti-cCD205 mAb) were prepared in Dr. Luc Berghman's lab at Texas A&M University (College Station, TX) based on previous work. Synthetic peptides complexed with 2C5 were previously used as an *in vivo* targeted immunogen for chicken APCs (Chen *et al*., 2010) and cCD205 mAb was utilized in the fundamental characterization of peritoneal exudate-derived DCs (Vuong *et al*., manuscript in preparation).

These mAbs represent a valuable tool for validation of the presence of chicken DCs and other APCs in different tissues, like the PPs. The use of immunohistochemistry and immunofluorescent staining of cryosections will demonstrate the specific binding affinity of new primary antibodies to cCD40 and cCD205 and reveal their distribution and expression levels on chicken APCs, and will enrich the current morphological and histological understanding of chicken PPs in the ileum via the modified Swiss-roll technique (Bialkowska *et al*., 2016; Moolenbeek and Ruitenberg, 1981).

CHAPTER II

LITERATURE REVIEW

Avian immune system

Although birds have evolved more simplified immune organs compared to those in mammals, the lymphoid organs are well-preserved and developed in the avian immune system. The primary lymphoid organs consist of 14-16 thymus lobes in the neck, and a bursa of Fabricius located dorsally to the cloaca (Kendall, 1980; Oláh, Nagy and Vervelde, 2014). The secondary lymphoid organs, such as the spleen, lymph nodes and mucosa-associated lymphoid tissues (MALT), are sites for lymphocyte maturation into effector T-cells and B-cells after their encounter with antigens (Cesta, 2006). Chickens lack encapsulated lymph nodes that are present in aquatic species like geese and ducks (Jeurissen *et al.*, 1989). Instead, they develop diffuse lymphoid tissues and an extensive MALT to ensure immune functions and a healthy internal environment (Jeurissen *et al.*, 1989). The cellular composition of the avian immune system (B-cells, T-cells, macrophages, DCs) is very similar to that in humans and mice, but heterophils are the avian analog to neutrophils in mammals (Hansell *et al*., 2007).

Mucosal-associated lymphoid tissue (MALT)

In both mammalian and avian species, the mucosa acts as a first line of defense against exogenous agents and consists of non-specific barriers such as epithelial cells, mucus, and extreme pH values (Jeurissen *et al*., 1989). Vertebrates possess sophisticated lymphoid tissues in the mucosa, which form the MALT to protect against pathogens and respond to antigenic stimulation (Jeurissen and Veldman, 2002). MALT can be found in all mucosal surfaces, including the

gastrointestinal (GI), respiratory, and urogenital tracts, where they encounter the external environment (Jeurissen *et al*., 1989). The MALT is primarily located in the GI tract where it is referred to as the gut-associated lymphoid tissue (GALT). Other well-documented lymphoid tissues include the eye-associated lymphoid tissue in the Harderian gland, nasopharynx-, bronchus-, conjunctiva- and genital-associated lymphoid tissues (Cesta, 2006; Oláh, Nagy and Vervelde, 2014). The organs/components of MALT share a similar morphology and function, *i.e.* the production of immunoglobulin A (IgA), described by Cesta (2006) as an "anatomically separated" but "functionally connected" common mucosal immune system. The antigen presentation and activation of B-cells at the inductive site (GALT) can cause a release of secretory IgA (sIgA) at other effector sites, such as the lamina propria of the intestine (Jeurissen *et al*., 1989; Kiyono and Fukuyama, 2004).

Gut-associated lymphoid tissue (GALT)

The GALT has been extensively studied in both mammals and avian species (Casteleyn *et al.*, 2010; Liebler-Tenorio and Pabst, 2006). It comprises defined organs lining the entire length of the digestive tract, including the pharyngeal tonsils, Meckel's diverticulum, cecal tonsils, bursa of Fabricius, and Peyer's patches (PPs). Scattered along the tract are numerous diffuse lymphoid aggregates in the form of small lymphoid accumulations, follicles, and solitary nodules (Oláh *et al*., 2003; Oláh, Nagy and Vervelde, 2014). The development of GALT begins at the lamina propria of the villi and may extend to the submucosa of the intestine (Casteleyn *et al.*, 2010; Oláh, Nagy and Vervelde, 2014). The lymphoid tissues feature a specialized follicle-associated epithelium (FAE), a term introduced by Bockman and Cooper (1973), or lympho-epithelium (LE) characterized by infiltration of lymphoid cells (Befus *et al.*, 1980). The FAE or LE contains

specialized enterocytes known for their microfold morphology; M-cells. The role of these cells includes surveillance and uptake of antigens followed by transport to the underlying lymphoid tissues (Gebert *et al.*, 2004; Owen, 1999). Vacuoles in the epithelial cells where active pinocytosis often occurs may indicate the presence of M-cells in the chicken GALT (Befus *et al*., 1980). The antigen sampling and processing by lympho-epithelial cells is critical to the initiation of immune responses in the avian GALT. Since many vaccines are administered orally, a better understanding of the GALT immune system could optimize the oral delivery of drugs and vaccines in the poultry industry (Casteleyn *et al.*, 2010).

Peyer's patches

PPs were described by Macro Severino in his publication on comparative anatomy as early as 1645 in Italy (Reynolds, 1985; Jung, Hugot and Barreau, 2010). However, they were named after the Swiss anatomist and pathologist Johann Conrad Peyer, who first studied this tissue in great detail in 1677 and described them as "elevated lymphoid nodules in the mucous membrane" of the human small intestines (Heel *et al.*, 1997). Based upon the initial concept of the mucus secretion of PPs, Bruecke in 1851 refined the definition and interpreted the "milky white area" as a follicle composed of lymphocytes that were similar to "vacuoles" in the lymph nodes (Reynolds, 1985). In 1885, Flemming noted high mitotic activity in areas of both the PPs and lymph nodes, thus coining the term "germinal center" for the site of lymphocyte proliferation (Reynolds, 1985).

Since the last century, scientists have been trying to unveil the mystery of PPs in the chicken gut and their immune function as putative antigen sampling sites (Befus *et al.*, 1980; Burns, 1982; Burns and Maxwell, 1986; Jeurissen *et al.*, 1989; Kajiwara *et al.*, 2003; Vaughn *et al.*, 2006). However, the collective scientific literature remains scarce in comparison to the extensive study

of human and mammalian PPs (Owen and Jones, 1974; Heel *et al.*, 1997). In rats and humans, PPs are aggregations of lymphoid follicles lining the antimesenteric border of the mucosa in the jejunum and ileum (Heel *et al.*, 1997). Most chicken PPs are scattered throughout the jejunum, but one of them can consistently be found in the distal ileum, in a position that is 5-10 cm cephalic to the ileocecal junction **(Fig. 1a)** (Vaughn *et al.*, 2006). Chicken PPs are perceived as a rough and swollen oval-shaped white patches with some redness in the texture and about 1-cm in length **(Fig. 1b)** (Vaughn *et al.*, 2006). Unlike mammalian PPs, which are evident nodules scattered most frequently in the ileum and the subdominant jejunum, chicken PPs are unpredictably scattered and are hard to distinguish from the serosal surface with the naked eye (Befus *et al.*, 1980; Jeurissen *et al.*, 1989; Heel *et al.*, 1997).

The embryonic development of chicken PPs starts on day 13 of embryogenesis, where two PPs can be identified near the ileocecal junction and Meckel's diverticulum (Kajiwara *et al.*, 2003). Macroscopically, PPs start to be visible at 10 days of age in neonatal birds, one with a consistent location as mentioned above, and up to six PPs are scattered in the lower intestine depending on the birds' growth and condition **(Fig. 1c)** (Casteleyn *et al.*, 2010). Their number and volume continue to grow until 13 weeks of age (Casteleyn *et al.*, 2010). After one year, with atrophy taking place in the ileum, PPs have regressed to a single site when the birds reached 52-58 weeks of age (Befus *et al.*, 1980).

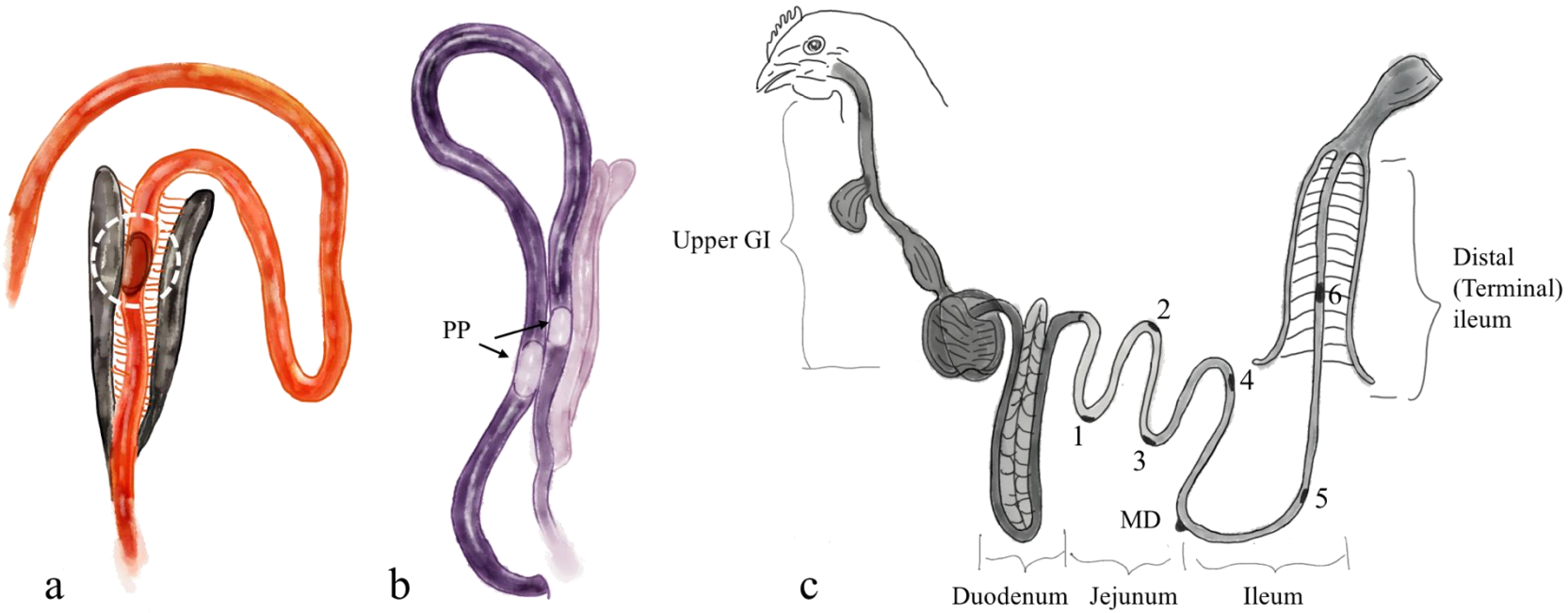

Figure 1. The location of Peyer's patches in the chicken gut by gross staining (a-b). a, Consistent PP site in the distal ileum cranial to the ileocecal junction (circle). **b,** Possible locations of PPs indicated by a pale pink-white focal area on the serosal surface. **c,** A simplified drawing of the chicken intestinal tract indicates the Peyer's patches **(1-6)** in the jejunum and the ileum (MD, Meckel's diverticulum). Illustrations **(a-b)** were modified after Vaughn *et al.* (2006) and **(c)** Casteleyn *et al.* (2010).

While the morphology of the avian PPs is distinct from that of mammals, there are also a lot of similarities. In contrast to the adjacent normal villi, avian PPs have thickened villi, heavy lymphocyte infiltration and aggregated follicles covered by a distinct LE or FAE **(Fig. 2a)** (Befus *et al*., 1980). The shape of the lymphoid aggregates varies from diffuse lymphoid tissues to follicles with germinal centers in the submucosa (Befus *et al*., 1980; Burns and Maxwell, 1986). The FAE is devoid of goblet cells, and the brush border of microvilli is sometimes interrupted by M-cells, in contrast to the adjacent intestinal epithelium **(Fig. 2b)** (Befus *et al*., 1980). A few reports have documented M-cells in the avian PPs **(Fig. 2c)** (Befus *et al*., 1980; Burns and Maxwell, 1986) in many ways, they are similar to mammalian PPs (Owen and Jones, 1974) and chicken GALTs, including Meckel's diverticulum and cecal tonsils (Jeurissen, Wagenaar and Janse, 1999). The follicular region of mammalian PPs consists of naïve B-cells which form germinal centers and are in intertwined with follicular DCs and macrophages and flanked by T-cells in the interfollicular space **(Fig. 3)** (Neutra, Mantis and Kraehenbuhl, 2001). T-lymphocytes are present in the lamina propria and reside among epithelial cells characterized as intraepithelial leukocytes (IEL) (Heel *et al.*, 1997). High endothelial venules are present in mammalian PPs where they associated with the transport of naïve lymphocytes, but their equivalent has not been identified in avian species (Befus *et al.*, 1980).

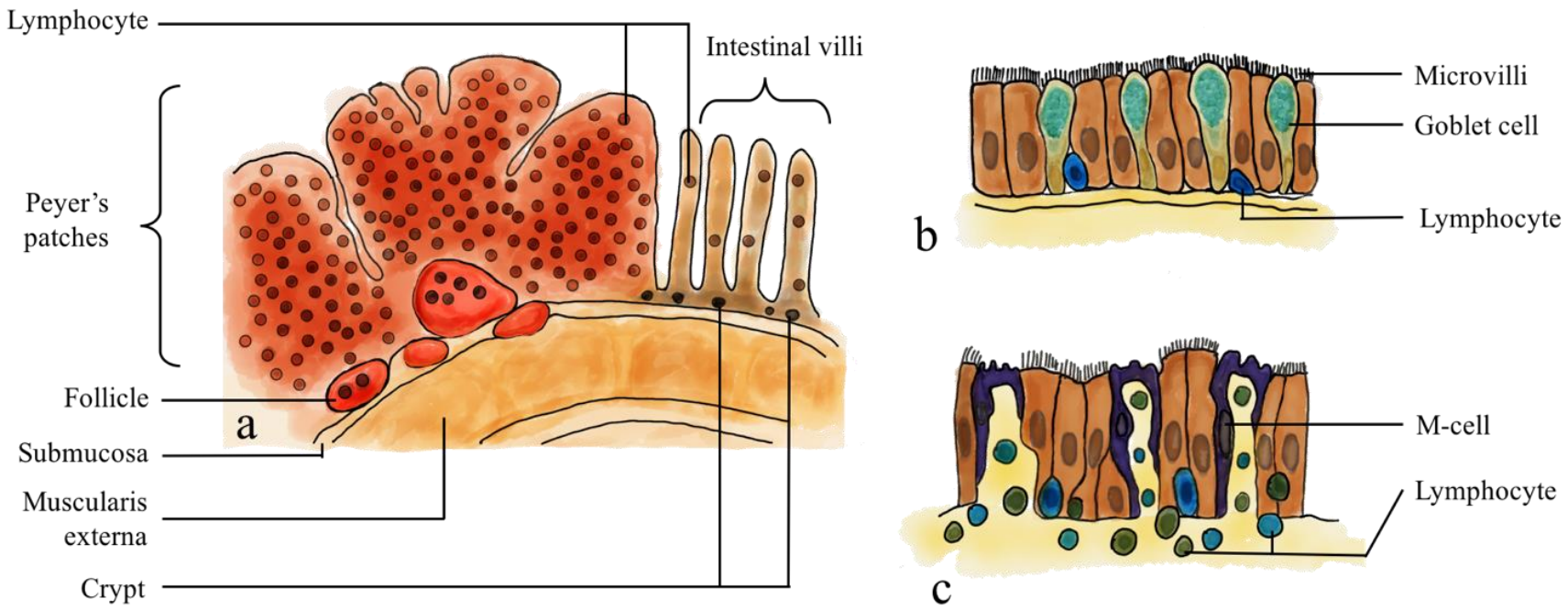

Figure 2. The morphology of the avian Peyer's patches. a, The chicken PP is characterized by massive lymphoid infiltration in contrast to the adjacent normal villi to the right. **b,** Goblet cells and microvilli are present in the normal intestinal epithelium, **(c)** but are missing from the lympho-epithelium of the chicken PP, which are infiltrated with lymphocytes and M-cells. Illustrations were modified after Befus *et al.* (1980).

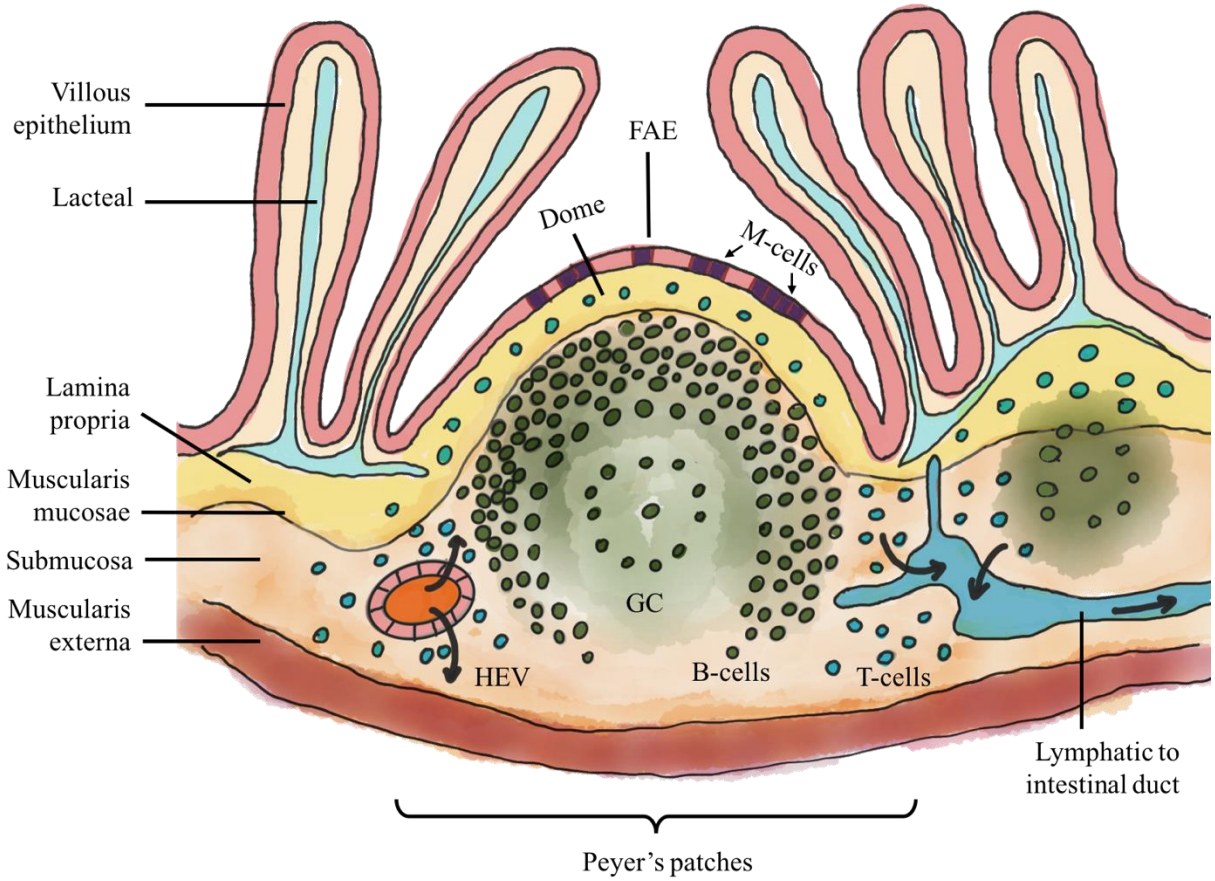

Figure 3. A drawing scheme of the mammalian Peyer's patches. The primary cellular component of the PPs includes follicles of the B-cells with a formation of germinal center (GC) and the interfollicular region of T-cells. The follicle-associated epithelium (FAE) covers the follicle that forms into a dome. Diffuse lymphoid cells and follicles are also present in the lamina propria. High endothelial venules are present in mammalian PPs. The illustration was modified after Owen *et al.* (2013).

The Swiss-roll technique

The Swiss-roll was first reported when it was used to study the human gastric mucosa (Magnus, 1937). Later on, it was widely applied for the study of rodent gastrointestinal tissues by light microscopic examination (Bialkowska *et al.*, 2016; Moolenbeek and Ruitenberg, 1981; Park *et al.*, 1987). In this procedure, the intestine is cut into strips, slit open longitudinally, and rolled up like a Swiss roll, to then proceed to paraffin-embedded sectioning (Moolenbeek and Ruitenberg, 1981). In this way, a significant portion of the intestine is included in one section allowing histological visualization of the intestine lengthwise (Park *et al.*, 1987). Bialkowska *et al.* (2016) improved the Swiss-rolling technique and used it for immunohistochemical and immunofluorescent analyses, and this study formed the basis for our studies. Apart from its application in humans and rodents, this method has not been well-described in chickens. Furthermore, the use of histological H&E staining alternated with immunofluorescent staining on adjacent cryosections is a fairly innovative approach for a micro-anatomical study. In this study, we performed a Swiss-roll specimen using OCT-embedded cryosections and optimized the fixation method for immunofluorescent staining.

The function of Peyer's patches

PPs are the first sites of T-cell priming and proliferation following oral administration of antigens in the gut (Jung, Hugot and Barreau, 2010). The function of PPs was unexplained until 1922 when Kenzaburo Kumagai observed the uptake of *Mycobacterium tuberculosis* in the epithelial dome of PPs (Owen, 1999). However, Kumagai characterized it as non-specific since he observed that sheep erythrocytes and heat-killed bacteria were also absorbed (Jung, Hugot and Barreau, 2010; Owen, 1999). Later in 1972, ultrastructural studies made it possible to distinguish

the M-cells from enterocytes and lymphocytes in the FAE of PPs (Owen and Jones, 1974). Transcytosis through the M-cells in the intestinal lymphoid tissues thus provided the morphological basis for further functional studies of the PPs (Owen, 1999).

M-cells internalize luminal particles within their reach by pinocytosis; particles are transported in vacuoles and released into the subepithelial space of M-cell pockets to engage with professional APCs (memory B-cells, DCs, and macrophages) and $CD4^+$ T-cells **(Fig. 4)** (Neutra, Mantis and Kraehenbuhl, 2001; Owen, 1999). Most immature DCs are localized in the subepithelial dome (SED) region of the PPs, where they capture antigens resulting from M-cell transcytosis and initiate the adaptive mucosal immune response (Neutra, Mantis and Kraehenbuhl, 2001; Sato and Iwasaki, 2005). The mechanisms that DCs utilize for the uptake of antigens vary between biological materials: viruses are taken up by endocytosis in the vesicles of M-cells, contrary to bacteria and large particles, which are internalized via phagocytosis by macrophages and DCs (Jung, Hugot and Barreau, 2010). Following this, APCs migrate to the lymphoid follicles of the PPs and present antigenic peptides in the context of MHC-II and -I to naïve $CD4^+$ or $CD8^+$ T-lymphocytes in the interfollicular T-cell region, respectively (Neutra, Mantis and Kraehenbuhl, 2001; Owen, 1999). These processes generate antigen-specific B-cells in the local germinal centers under the cytokine control of $CD4^+$ T helper cells. Primed B-cells then migrate out of the inductive sites of the PPs to the effector sites of the intestinal mucosa via the bloodstream (Madej and Bednarczyk, 2016). The immune cells are navigated by surface integrins and extravasated by cell adhesion molecules on the vascular endothelium in the intestinal lamina propria (Brandtzaeg, Farstad and Haraldsen, 1999). Finally, B-cells proliferate and differentiate into plasma cells and secrete sIgA on the epithelial surface of the intestine (Madej and Bednarczyk, 2016). Thus, PPs

are the inductive sites in the gut for antigen presentation and elicit the mucosal immune response following oral immunization (Madej and Bednarczyk, 2016).

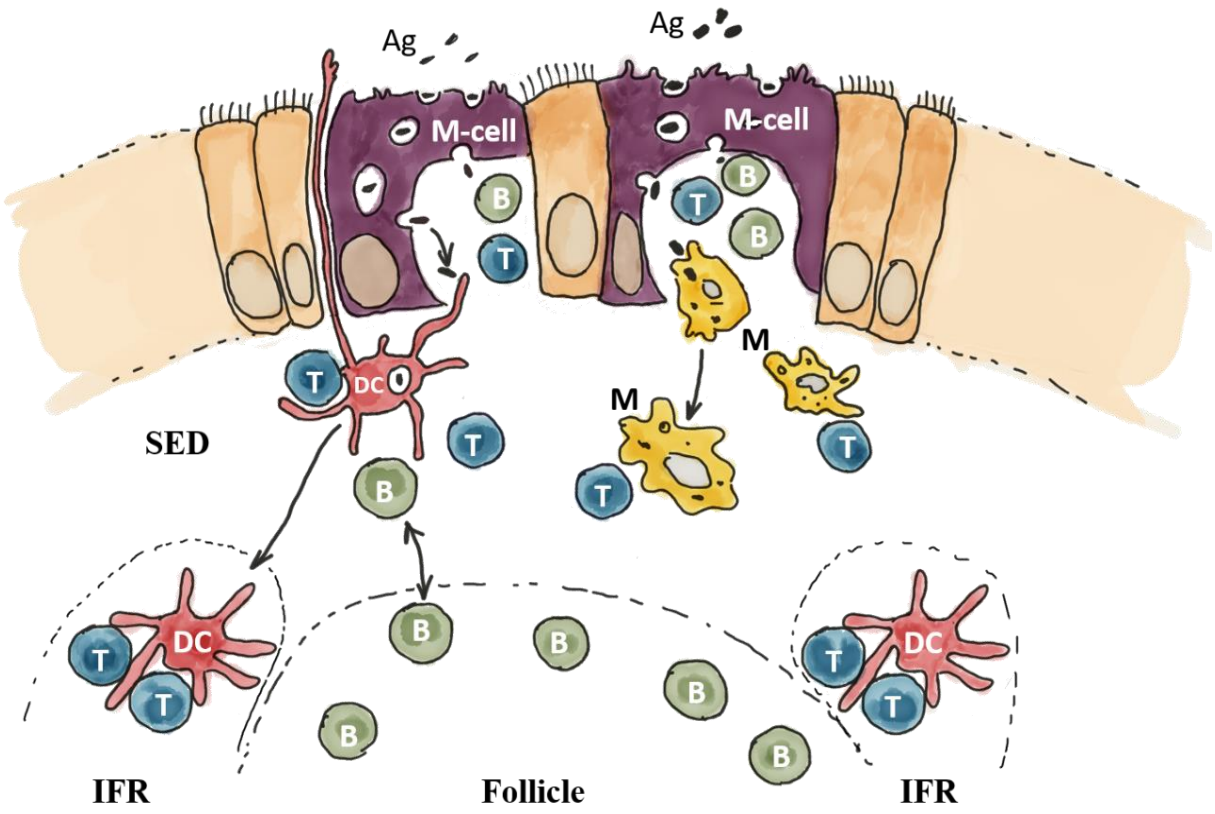

Figure 4. The assumed pathways of pathogenic antigens after M-cell transcytosis. Antigens are released at the M-cell pocket and may be captured by DCs and macrophages (M) in the subepithelial dome region (SED). Then, DCs may present antigens to the local T-cells or migrate to the interfollicular region (IFR). Memory B-cells may be also function as APCs by capturing antigens in the M-cell pocket and present them to T-cells. The illustration was modified after Neutra, Mantis and Kraehenbuhl (2001).

Dendritic cells

DCs were first identified in the skin as Langerhans cells and later isolated from the mice spleen (Wu *et al*., 2010). DCs vary in size and are characterized by their hallmark stellate morphology with numerous protrusions and loose attachments to each other (Quéré *et al*., 2013). Their morphology is sometimes hard to distinguish from macrophages (Quéré *et al*., 2013). DCs are the true sentinel cells of the immune system due to their migratory properties. Also, specialized DC subsets orchestrate the innate and adaptive immune responses, which are immature non-activated $CD209^+$ DCs, immature activated $CD205^+$ DCs regulating T-cell tolerance, and mature activated $CD83^+$ DCs stimulating T-cell immune response (Faure-André *et al*., 2008; Schwede, Alfer and von Rango, 2014; Summerfield and McCullough, 2009). The majority of DCs are immature with a low expression of costimulatory receptors, MHC-II and adhesion molecules (Chen *et al*., 2016). These cells efficiently sample the environment while internalizing antigens, although they are poor at antigen presentation and activating T-cells due to the relative scarcity of essential surface molecules (Caminschi, Lahoud and Shortman, 2009; Chen *et al*., 2016). The endocytosis of immature DC consists of three mechanisms: firstly, by 'specific' phagocytosis of exogenous antigens; secondly, by the receptor-mediated endocytosis pathway following up-regulation of the CD205 (DEC205) receptor, and lastly, via 'non-specific' macropinocytosis of large volumes of soluble antigens (Chen *et al*., 2016; Guermonprez *et al*., 2002; Platt *et al*., 2010). Following this, antigenic cargo is internalized into endosomes, degraded into peptides and loaded onto MHC-I or -II molecules (Faure-André *et al*., 2008). The outcome of antigen presentation depends on the type of antigen. Self-antigens do not send 'danger' signals to DCs and result in tolerance to limit auto-reactivity, whereas microbial intruders alert the DCs to undergo a maturation program and to establish adaptive immunity (Caminschi, Lahoud and Shortman, 2009).

Upon antigenic stimulation, their capacity to migrate enables immature DCs to home to peripheral lymphoid organs where they mature and present antigens to naïve T-cells as peptide-MHC complexes (Faure-André *et al*., 2008; Takahashi *et al*., 2001).

Immature DCs undergo dramatic changes in terms of functionality and morphology, including acidification of their lysosomal compartment (to enhance antigen processing), up-regulation of co-stimulatory receptors, and reformation of surface MHC-II from the late endosomal compartment, aimed at T-cell recognition (Platt *et al*., 2010). Part of this transformation is the synthesis of chemokine receptor CCR7 (Summerfield and McCullough, 2009) and upregulation of C-type lectin receptor DEC205 (CD205) for endocytosis and antigen presentation (Jiang *et al*., 1995). Mature DCs shut down the antigen acquisition program, but are optimized for antigen presentation and T-cell priming, including cytokine secretion and proliferation (Caminschi, Lahoud and Shortman, 2009; Faure-André *et al*., 2008). Interdigitating DCs represent a subset of mature DCs that form a network of MHC-II rich processes and dominate the T-cell area of secondary lymphoid organs such as the PPs, the spleen and the lymph nodes (Witmer and Steinman, 1984).

In addition, DCs appear in the B-cell follicles as germinal center DCs and are different from stromal follicular DCs. FDCs are not considered typical APCs that present antigens in the context of MHC molecules; rather, they present immune complexes and present to B-cells (Wu and Kaiser, 2011). Follicular DCs are distributed in the outer aspect of the germinal center and are identified in the chicken PPs, cecal tonsils, and spleen (Witmer and Steinman, 1984; Wu and Kaiser, 2011). Apart from the central lymphoid organs, DCs reside in the peripheral tissues, such as in the skin (as Langerhans cells), the airways, the bloodstream and the afferent lymphatics where they surveil the environment while facilitating antigen internalization and trafficking to lymphoid

tissue (Wu and Kaiser, 2011). Chicken DCs were first observed in the cecal tonsils, and later in various tissues, such as the epidermis, the spleen and the bursa (del Cacho *et al.*, 2009; Gallego *et al.*, 1997; Igyarto *et al*., 2006; Oláh and Glick, 1995). Although the relative paucity of specific immune reagents initially made it difficult to confirm the existence of chicken DCs, Wu *et al*. (2010) successfully cultured bone marrow cells in the presence of recombinant chicken GM-CSF and IL-4. Wu *et al.* (2010) also observed dendritic-like cells with high levels of MHC-II, moderate CD40, low CD86 and no CD205. Upon stimulation with lipopolysaccharide and CD40L, these cells showed increased expression of surface antigens (CD40, CD205, CD83 and CD86) along with impaired endocytic activity and a switch from gene expression of the immature marker CCR6 to the mature marker CCR7 (Wu and Kaiser, 2011; Yasmin *et al*., 2015).

The lack of surface markers and poor cross-reactivity of heterologous mAbs or polyclonal Abs have hampered the study of DC identification and characterization (Hansell *et al*., 2007). Vuong *et al.* (manuscript in preparation) developed mAb against the chicken DCs surface marker CD205 (anti-chCD205 mAb), facilitating future research into chicken DCs residing in various tissues, including, but not limited to the PP, the spleen, the bursa, and the thymus.

Co-stimulatory receptor

Along with high levels of constitutively expressed MHC-II molecules, costimulatory receptors are expressed on the surface of APCs and bind to their cognate ligand on the surface of T-cells, for example. The ligation between a receptor and its ligand initiates a cross-talk between APCs and naïve lymphocytes, resulting in bidirectional signals to induce or regulate long-lasting adaptive immune responses (Hashem *et al*., 2014). Activation of naïve T-cells is comprised of three signaling stages: first, a primary signal from the binding of a ($CD4^+$) T-cell receptor to the antigenic peptide in the context of MHC-II on an APC; second, costimulatory signals mediate the ligation of ligands and receptors on T-cells and APCs; finally, bidirectional intercellular cytokines stimulate both cell types and induce naive T-cells to become effector T-cells (Curtsinger *et al*., 1999).

CD40

CD40 is a 48 kDa transmembrane glycoprotein receptor, belonging to the tumor necrosis factor (TNF) superfamily. Among the various surface receptors, mammalian CD40 is a crucial regulator expressed constitutively on all professional APCs, monocytes, thrombocytes, and also on non-hematopoietic cells such as fibroblasts, endothelial and epithelial cells (Hashem *et al.*, 2014; van Kooten and Banchereau, 2000). Chicken CD40 is predominantly expressed on B-cells, macrophages, monocytes; it is moderately expressed on bone marrow-derived DC, and also, to a lesser extent, on thrombocytes (Ratcliffe and Härtle, 2014; Tregaskes *et al.*, 2005; Wu *et al.*, 2010). The ligand of CD40, CD154 a.k.a. CD40L with a mass of 34-39 kDa, is transiently expressed on the cell membrane of chicken activated $CD4^+$ T-cells, and mammalian activated T-cells of both subsets ($CD4^+$ and $CD8^+$) (Hashem *et al.*, 2014; Quezada *et al.*, 2004). Other peripheral cells such

as monocytes, thrombocytes, and smooth muscle cells also express CD40L following inflammatory stimuli (Quezada *et al.*, 2004; Tregaskes *et al.*, 2005). The gene for CD154 is located on chicken chromosome 4 and on the human X chromosome (Tregaskes *et al.*, 2005). The putative chicken CD154 is the ortholog of mammalian CD154 with 44-49% similarity of the amino acid sequence. The evolutionary conservation of mammalian and avian CD154 is reflected in their functional conservation, *i.e.* positive and negative regulation of B-cell survival and Ig production (Miyashita *et al.*, 1997; Tregaskes *et al.*, 2005).

The CD40-CD40L ligation alters the phenotype of APCs and stimulates the survival and expansion of APCs, T-cells, and B-cells (Hashem *et al.*, 2014). The ligation of DCs and $CD4^+$ T-cells promotes the differentiation of DCs into fully competent APCs with cytokine secretion, MHC upregulation, and more importantly, the expression of co-stimulatory receptors such as CD40, CD80, and CD86 (Hashem *et al.*, 2014).

B-cells act as both APCs and antibody-secreting cells in immune responses (Hashem *et al.*, 2014). The CD40 signaling on B-cells promotes antigen presentation and B-cell activation and differentiation (Hashem *et al.*, 2014). Chicken B-cells differentiate into plasmablast-like phenotypes under CD40L stimulation, resulting in reduced surface IgM expression and increased cytoplasmic Ig production (Ratcliffe and Härtle, 2014). In general, CD40 signaling results in B-cell clonal expansion, the rescue from apoptosis, cytokine secretion, Ig isotype switching and antibody production, formation of a germinal center, and memory B-cell maturation. Finally, B-cells develop into plasma cells and memory cells posterior to survival in the germinal center (Grewal and Flavell, 1998; Grammer and Lipsky, 2001; Kothlow *et al.*, 2008).

Monoclonal antibodies against CD40 (anti-CD40 mAb) have been used to substitute the role of CD40L in activating B-cells of T-cell dependent humoral immunity (Hollenbaugh,

Grosmaire and Kullas, 1992). Chicken agonistic anti-chicken CD40 mAbs (2C5) were first developed by Chen *et al*. (2010) to mimic the activity of chicken CD40L, *i.e*. to activate macrophages (HD11) and immature B-cells (DT40), suggesting the potential to use this mAb as an immunological adjuvant in chickens (Chen *et al.*, 2012). Chen *et al.* (2012) showed that *in vivo* CD40-targeting a synthetic peptide to APCs, specifically B-cells, can enhance the isotype-switched antigen-specific IgG response in the chicken. Later, Chou *et al.*, (2016) substantiated that a single application of 2C5-peptide complex via the mucosal route was able to induce a sIgA immune response in chickens, strongly suggesting that the activation of APCs in the mucosal inductive sites was a result of CD40 targeting. Hence, the visualization of CD40 distribution and expression in chicken PPs will serve as the morphological basis for future CD40-targeting studies in the chicken GALT.

CD205

CD205 (Ly75 or more commonly DEC205) is possibly the most unfamiliar member of the macrophage mannose receptor family of C-type multilectins, situated in the coated pits at the plasma membrane (Butler *et al*., 2007; Mahnke *et al*., 2000). It harbors 10 calcium-dependent recognition domains, a transmembrane domain of cytoplasmic tail, and a coated pit sequence for endocytosis in the cytoplasmic domain of the membrane-proximal region (Jiang *et al*., 1995; Mahnke *et al*., 2000). CD205 was first cloned in mice and named for its molecular weight (205kDa) and the predominant distribution on DCs and thymic epithelial cells in both chickens and mammals (Butler *et al*., 2007; Nagy, Bódi and Oláh, 2016). The gene structure and sequence of chicken CD205 are highly conserved with its ortholog in humans. Sequence identity with human is 51% and with mouse 48% (Staines, Young and Butter, 2013). CD205 is observed on different

phenotypes of DCs: bone-marrow derived DCs (chicken), interdigitating DCs (mice) and Langerhans cells of the skin (mice) (Kraal *et al*., 1986; Wu *et al*., 2010). CD205 has a low-level expression on other cell types (macrophages, B- and T-cells) and in non-lymphoid tissues (cardiac muscle and endothelium of the vascular) (Staines, Young and Butter, 2013).

In the receptor-mediated endocytosis, extracellular proteins that bind to receptors, *i.e*. CD205 are rapidly internalized using coated vesicles and processed in intracellular compartments (Goldstein, Anderson and Brown, 1979). As such, CD205 is an endocytic receptor capable of antigen uptake and contributes to antigen presentation by delivering antigens to the endosomal compartments of MHC molecules loading pathways (Butler *et al*., 2007). It also enhances ligand uptake, induces T-cell tolerance or immunity, and clears apoptotic and necrotic thymocytes by thymic epithelial cells (Mahnke *et al*., 2000; Staines, Young and Butter, 2013). CD205 on immature DCs is massively upregulated upon maturation to enhance antigen presentation while losing its endocytic activity probably due to a large extent of cytoskeletal changes (Butler *et al*., 2007). The upregulation is a consequence of both *de novo* synthesis and redistribution from intracellular compartments of immature DCs to the extracellular spaces of mature DCs, which closely resembles the translocation of MHC-II molecules upon maturation (Butler *et al*., 2007). Another function of CD205 in addition to endocytosis and antigen internalization is to interact with cells in the secondary lymphoid organs where most mature DCs are. The upregulation of CD205 on mature DCs can reflect the time span for DCs to migrate to the local lymph nodes (Butler *et al.*, 2007).

There are limited mAbs to characterize DC phenotypically (Wu and Kaiser, 2011) and studies are still in scarcity to chicken CD205. However, truncated chicken CD205 was recently cloned by Vuong *et al*. (manuscript in preparation) and used to raise monoclonal antibodies. Those

mAbs were then used to isolate $CD205^+$ DC-like cells from chicken peritoneal exudate cells and made the morphological study of avian DCs in this study possible.

CHAPTER III

MATERIALS AND METHODS

Antibodies

Mouse anti-chCD40 (2C5) (Chen *et al*., 2010) and anti-CD205 mAbs (Vuong *et al*., to be published) were generated and biotinylated in Dr. Luc Berghman's lab at Texas A&M University (College Station, TX). Mouse Anti-Chicken L-CAM mAb (7D6, E-cadherin) (DSHB Cat# 7d6, RRID: AB_528115) was purchased from Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA). Mouse Anti-Chicken IgM-FITC (Cat. No.8310-02), Mouse Anti-Chicken CD3-Alexa Fluor® 647 (Cat. No.8200-31), Mouse Anti-Chicken MHC Class II-UNLB (Cat. No.8350-01), and Mouse Anti-Chicken MHC Class II-FITC (Cat. No.8350-02) were purchased from SouthernBiotech (Birmingham, AL). Rhodamine Red™-X (RRX) AffiniPure Goat Anti-Mouse IgG (H+L) (Code: 115-295-146), Fluorescein (FITC) AffiniPure Sheep Anti-Mouse IgG (H+L) (Code: 515-095-062), Biotin-SP AffiniPure Goat Anti-Mouse IgG (H+L) (Code: 155-065-062), Alexa Fluor® 488 Streptavidin (Code: 016-540-084), and Rhodamine Red™-X (RRX) Streptavidin (Code: 016-290-084) were purchased from Jackson ImmunoResearch (West Grove, PA). Mouse Anti-Chicken MHC Class II-UNLB was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Cat. No. 21327) according to the commercialized protocol by Thermo Scientific (Rockford, IL). Antibodies used in this project were summarized in **Table 1**.

Table 1. The surface receptors and molecules to be studied in this research.

Antibody	Antigen	Specificity	Reference	Catalog number	Dilution recommendation
7D6	E-cadherin	Epithelial cells	Gallin *et al.*, 1983. DSHB	Cat# 7d6, RRID: AB_528115	4µg/ml
Anti-ch MHC-II-UNLB Anti-ch MHC-II-FITC	MHC-II	APC	SouthernBiotech	Cat. No.8350-01 Cat. No.8350-02	3µg/ml
2C5	CD40	APC	Chen *et al.*, 2010. Dr. Berghman Lab, TAMU, College Station, TX		5µg/ml
anti-chCD205	CD205	Dendritic cells	Vuong *et al.*, manuscript in preparation. Dr. Berghman Lab, TAMU, College Station, TX		5µg/ml
anti-chIgM-FITC	IgM	Subsets of B-cells	SouthernBiotech	Cat. No.8310-02	3µg/ml
anti-chCD3-AF 647	CD3	T-cells	SouthernBiotech	Cat. No.8200-31	5µg/ml

Animals and tissues

All tissues used in this study were collected post-mortem from animals that were used in other projects. Peyer's patches and spleen were collected from post-mortem broilers at 42 days offered by Dr. Morgan Farnell lab (TAMU, TX, AUP IACUC 2016-0270), and post-mortem layers at 35 days and 52+ week-old hens offered by Dr. Luc Berghman lab (TAMU, TX, AUP IACUC 2015-0387).

Post-mortem tissue collection

All tissues used in this study were collected post-mortem from animals that were used in other projects. Peyer's patches and spleen were collected from post-mortem broilers at 42 days and post-mortem layers at 35 days and spent hens of more than one year. The post-mortem birds and tissues were offered by Dr. Morgan Farnell lab at Poultry Science Department, TAMU, TX, AUP IACUC 2016-0270, and by Dr. Luc Berghman lab, AUP IACUC 2015-0387.

Chicken spleen and PP of the consistent location of the distal ileum were collected for investigation. Mesenteric attachments were removed when the intestine was extended, and the ceca lengthwise to measure the distal ileum, from the ileocecal junction to the blind end of the ceca. The distal ileum was excised and placed in the petri dish, and flushed by a 10cc syringe, 25 gauge 1-in, needle filled with PBS to remove the ingesta. After removing the mesenteries, the ileum was opened from the mesenteric border to reveal the putative PP site. The PP was excised and proceed to cryopreservation.

Swiss-roll technique

A modified tissue processing method, *i.e.* “Swiss-roll” technique, was performed on the intestine of 35d layers and spent hens in order to locate the PP based on the method described by Bialkowska *et al*. (2016) and Moolenbeek and Ruitenberg (1981). The small intestine from the Meckel's diverticulum to the ileocecal junction was removed from the abdomen and rearranged as displayed in **Fig. 6a**. The distal ileum as defined above was flushed with PBS and cut longitudinally from the mesenteric side to expose the mucosa **(see Fig. 5)**. The segment with the putative PP was spread out in a clean petri dish with the luminal side up. Then, the caudal end was held in place with the forceps and rolled up using a wooden toothpick. The roll had to be tight with the mucosa facing inward, taking care that the epithelium lining remained intact throughout the procedure. Then the roll was immersed in OCT with the spiral side up in a metal cryo-mold and further processed for cryosectioning.

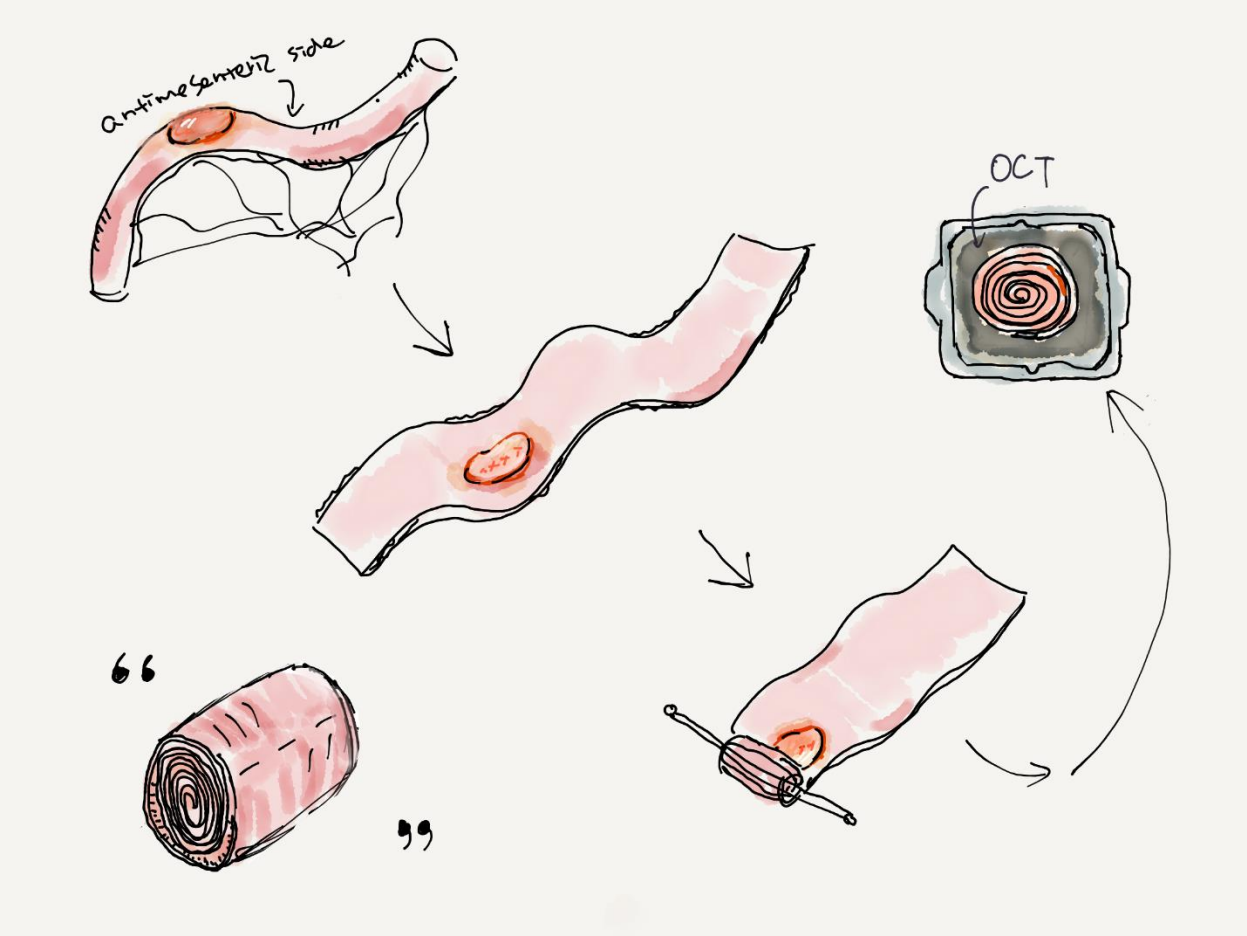

Figure 5. Schematic drawing of the "Swiss-roll" technique illustrating the rolling of the intestine with the PP embedded in it. The gut was slit open from the mesenteric side to reveal the mucosa. Half a portion of the terminal ileum with the PP was rolled up from one end using a wooden toothpick and the luminal side facing upward. The standing roll was embedded in the OCT medium and proceeded with snap-freezing and frozen section procedure. The technique was made and modified after Bialkowska *et al.* (2016) and Moolenbeek and Ruitenberg (1980).

Cryosections

Fresh tissues were embedded into the OCT compound medium (TissueTek®; Sakura Finetek, Torrance, CA) in cryo-molds and fast-frozen in liquid phase nitrogen and stored at -80˚C until further processing. Cryostat (Leica CM1900) sections of 5μm and 10μm thicknesses were picked up on slides for Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) staining, respectively. Then, the sections were air-dried for 20-30 minutes at room temperature (RT) (or stored at -80˚C) and proceeded to fixation with successive -20˚C acetone for 3 minutes and 4˚C 80% methanol for 5 minutes. Following this, samples were gently rinsed in PBS in the up-straight Coplin jar to get rid of extra OCT and fixatives. The sections were ready to proceed with H&E staining and IHC staining following the protocols tailored to this experiment.

H&E staining of cryopreserved sections

H&E staining procedures were as follows: basifying slides in tap water before the hematoxylin stain (Grill No.2, Sigma-Aldrich, Saint Louis, MO) for 4 minutes. Differentiation in a hydrochloric acid alcohol solution (formulated by 1% HCl in 70% Ethanol) for 30 seconds and bluing in Scott's tap water (formulated by magnesium sulfate 10.0g, sodium bicarbonate 0.67g in 1L tap water) for 30 seconds. Next, counterstaining with eosin-Y (formulated by 5g eosin and 2.5ml acetic acid in 1L 70% Ethanol) for 2 minutes. Between each of the steps detailed above, the sections were washed three times with PBS. Then, going through 1-minute decolorizing with 95% ethanol, three changes of 1-minute dehydration with absolute alcohol, and finally three changes of 3-minute clearing with xylene. Sections were mounted with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI) and observed and photographed under the microscope (Olympus IX71) and the scanner (Leica, Aperio CS2).

Direct immunofluorescence staining

Nonspecific binding sites were blocked by either 1% (w/v) bovine serum albumin (BSA) (Rockland Immunochemicals Inc., Gilbertsville, PA) in PBS, pH 7.4 for an hour or 10% goat serum in PBS, pH 7.4 for 30 minutes. Antibodies and reagents were diluted in blocking buffers. All rinses were performed in PBS for three minutes of three times. All procedures were carried out in the dark chamber at room temperature (RT) to avoid bleaching and coverslipped with Vectashield Mounting Medium with DAPI (Vector Laboratories Inc. Burlingame, CA) to stain the nuclei.

Single detection of B-cell surface IgM

Cryosections of PP were blocked with 1% BSA/PBS and incubated with mouse anti-chicken IgM-FITC (3µg/ml) for an hour.

Double detection of B-cell surface IgM and T-cell marker CD3

Swiss-roll sections of 35d layers were blocked and incubated with anti-chicken IgM-FITC as described above. Then, the sections were rinsed in PBS to proceed with another primary antibody anti-chicken CD3-Alexa 647 (5µg/ml) for an hour.

Indirect immunofluorescence staining

Single staining of CD40 and CD205

The sections of broiler PP and spleen were blocked with 10% goat serum and stained with anti-chicken CD205 (clone 3B6, purified) and anti-chicken CD40 (2C5) (clone 2C5, purified). CD205 and 2C5 ascites were diluted at 1/500 in 10% goat serum/PBS with overnight incubation

and further detected by biotinylated goat anti-mouse IgG (1/600-) for 45 minutes. CD205 was labeled by streptavidin-Alexa 488 (4µg/ml) and CD40 by streptavidin-Rhodamine Red (4µg/ml) for 20 minutes.

Single staining of E-cadherin

The Swiss-roll sections of 35d layer ileum were blocked as described above, and then incubated with the primary antibody 7D6 (3µg/ml) overnight and the secondary antibody goat anti-mouse IgG-Rhodamine Red (1/600) for an hour.

Dual staining of E-cadherin and MHC-II

The Swiss-roll sections of 35d layers were blocked as described above, incubated with the primary antibody 7D6 overnight, and detected by the secondary antibody goat anti-mouse IgG-Rhodamine Red for an hour. Then, slides were rinsed and incubated with biotinylated anti-MHC-II (3µg/ml) for 45 minutes and finally labeled by streptavidin-Alexa 488 (4µg/ml) for 20 minutes.

Dual staining of CD40 and MHC-II

Biotinylated 2C5 (5µg/ml) was applied to slides for 45 minutes following with streptavidin Rhodamine Red (4µg/ml) for 20 minutes. Slides were rinsed well to proceed with FITC conjugated anti-MHC-II (3µg/ml) for an hour.

Confocal microscopy

The fluorescence specimens were studied by a confocal microscope (Leica DMi8) using an image software (Leica Application Suite X).

CHAPTER IV

RESULTS

Gross and microscopic observations of Peyer's patches

Based on our findings, the optimal location to identify a PP was the terminal ileum, from the ileocecal junction to the length of a cecum, on the antimesenteric border of the intestine **(Fig. 6)**. Regardless of birds' sex, this area featured a consistent PP, which was readily visible in birds of young age (4 to 8 weeks) and virtually absent from aged hens of 52+ weeks old. The morphological structure of the ileum and the PP was similar during each time point in all post-mortem samples (with ages ranging from 35d to 42d, and both in broilers and laying hens), with no apparent deviations. In most healthy birds, the ileal PP was barely recognizable from the serosal surface **(Fig. 6a)**, but was much more accessible from the luminal side after cutting along the mesenteric border **(Fig. 6b)**. The serosal look of PP resembled an oval-shaped bump with a pale to reddish color that was similar to the smooth surface of the intestine, whereas the mucosal surface of PP was clearly distinguishable from its surroundings. This tissue lacked a connective tissue capsule and hence, was directly exposed to the luminal contents of the intestine. The PP was perceived as an elevated bump with a granular texture and a white appearance and was oval about 1/4 to 1/2 inch, in length. Some PPs appeared to have a reddish color due to blood infiltration. In birds with infections, the PP was swollen and more evident with hemorrhagic lesions that were visible from the serosal surface.

The light microscopic observation of H&E sections from the longitudinal sections of excised broiler tissues provided the first glance of the PP **(Fig. 7)**. The hallmarks of these tissues were massive sub-epithelial lymphocyte infiltration in the lamina propria, lymphoid follicles in the

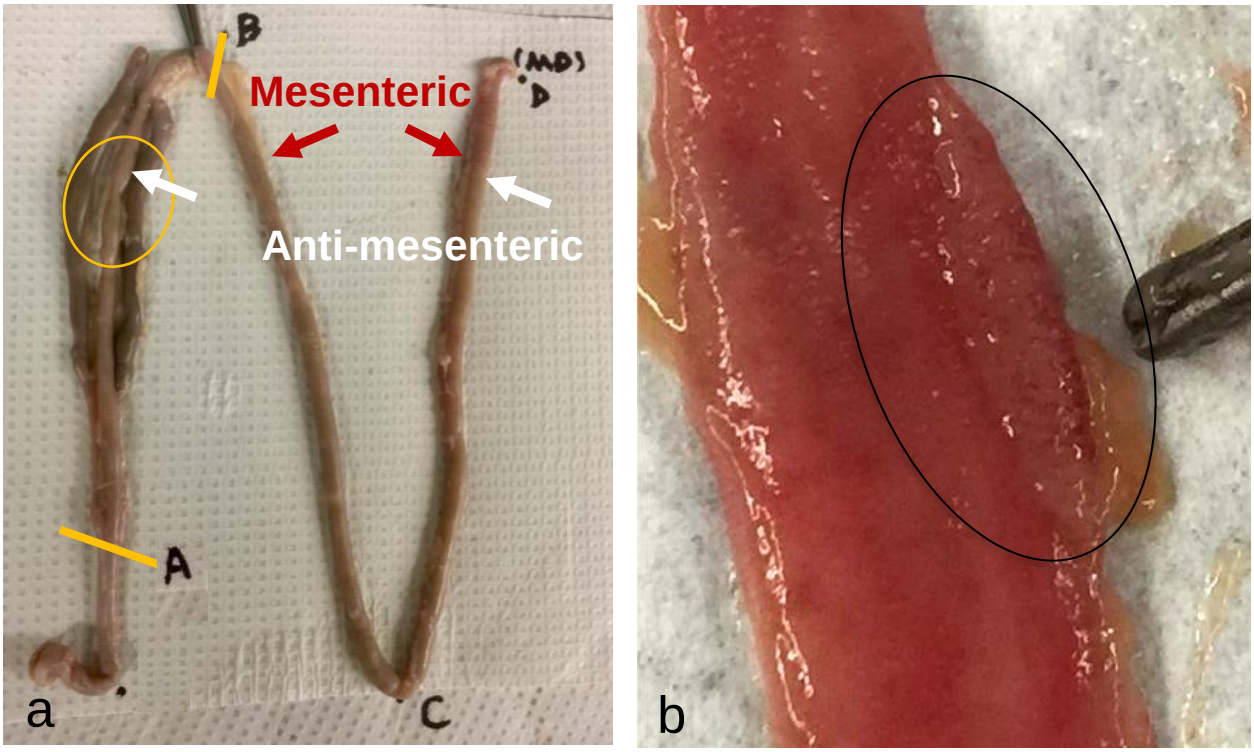

Figure 6. The macroanatomy and the overall location of the PP. Images show the general location and appearance of the ileal PP in a 35d-layer **(a)** and a 3-week old broiler **(b)**. The ileal segments of A-B (as indicated in **Fig. 1a**) were slit open to locate the PP spanning a longitudinal trajectory of approx. 10 mm. A portion of the terminal ileum containing the PP was rolled up by the Swiss-roll technique to make cross-sections, and broiler PPs were excised from the intestine to make longitudinal sections. All tissues were cryosectioned at 5µm and 10µm for H&E histological staining and immunofluorescent single or double staining. **a,** Segment A-D represents the entire ileum. Segment A-B represents the terminal ileum from the ileocecal junction to the tips of the ceca. The relative position of the ileal PP on the antimesenteric border of the intestine is indicated by the circle. This PP is not visible on the serosal surface. **b,** This photograph shows one half of a PP in the mucosa of the terminal ileum. The tissue is elevated from the mucosal surface with a granular texture and a lighter appearance. Some PPs show a meshwork of blood capillaries.

muscularis mucosae, and diffuse lymphoid tissues in the interfollicular regions. These findings were entirely in line with those described by Vaughn *et al.* (2006) and Befus *et al.* (1982) in broiler PPs. Numerous small lymphoid nodules were evident in the lamina propria characterized by intense lymphoid cell infiltration **(Fig. 7b)**. However, the longitudinal sections of a single patch that were made preceding the Swiss-roll sections were ultimately not suitable to illustrate the overall morphology of the PP.

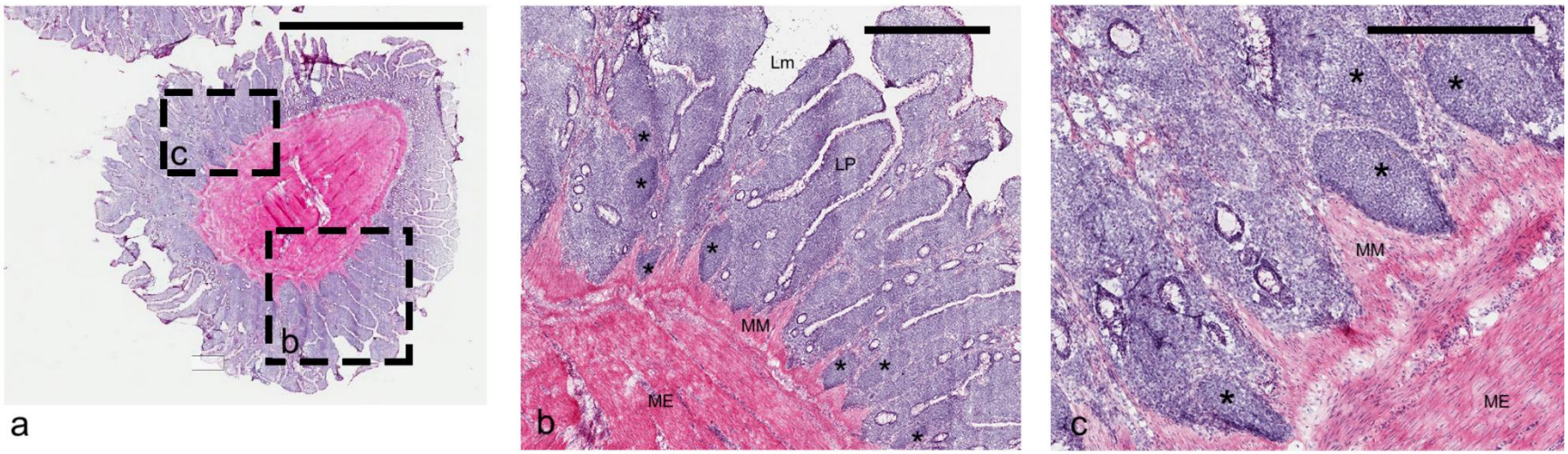

Figure 7. A longitudinal section of excised 42d-old broiler PP tissue stained by Hematoxylin and Eosin. a, A scanned preview of the tissue, which is enlarged to see details. Scale bar, 3mm. **b,** The predominant feature of the PP is the numerous lymphatic follicles (indicated by asterisk) at the base of the villi projections. The lamina propria has an intense infiltration of lymphatic cells. Scale bar, 600µm. **c,** The image is further enlarged to see the follicles embedded in the muscularis mucosae. Scale bar, 300µm (Lm: lumen, LP: lamina propria, MM: muscularis mucosae, ME: muscularis externa).

The “Swiss roll” processing revealed substantial lymphoid aggregations in the putative PP area **(Fig. 8; Fig. 9)**. In the Swiss-roll sections, we were able to observe that the morphology of the PP was more organized and markedly distinct from the surrounding intestinal tissues **(Fig. 8a; Fig. 9a)**. In contrast to the surrounding areas with regular intestinal villi, the PP was covered by thickened villi, no goblet cells were observed, and neither was the brush border of microvilli **(Fig. 8b)**. M-cells were not identifiable with the H&E stain, but intraepithelial lymphocytes (IEL) were visible in the lympho-epithelium (LE) **(Fig. 9b)**. Lymphocytes in the villi were organized into characteristic egg-shaped or cylindrical lymphoid follicles that resided at the base of lamina propria **(Fig. 8b&c)** and in some cases, germinal centers were observed inside these follicles **(Fig. 9c)**. Interestingly, our sections of ileal PP demonstrated a typical subepithelial dome (SED) structure covering the follicle that has been described in mice and humans (Jung, Hugot & Barreau, 2010; Makala, Suzuki & Nagasawa, 2003) **(Fig. 8b)**. However, the lymphoid tissues of chicken PPs did not further penetrate into the submucosa as in mammals but settled in the muscularis mucosae to generate follicles **(Fig. 8b&c)**. However, other lymphoid aggregates scattered in the ileum occasionally had a dome-shape and an epithelium that resembled lympho-epithelium **(Fig. 8d)**. These dome structures with diffuse lymphoid tissue were also observed in the 52+ week-old hen that lacked ileal PPs **(Fig. 10)**.

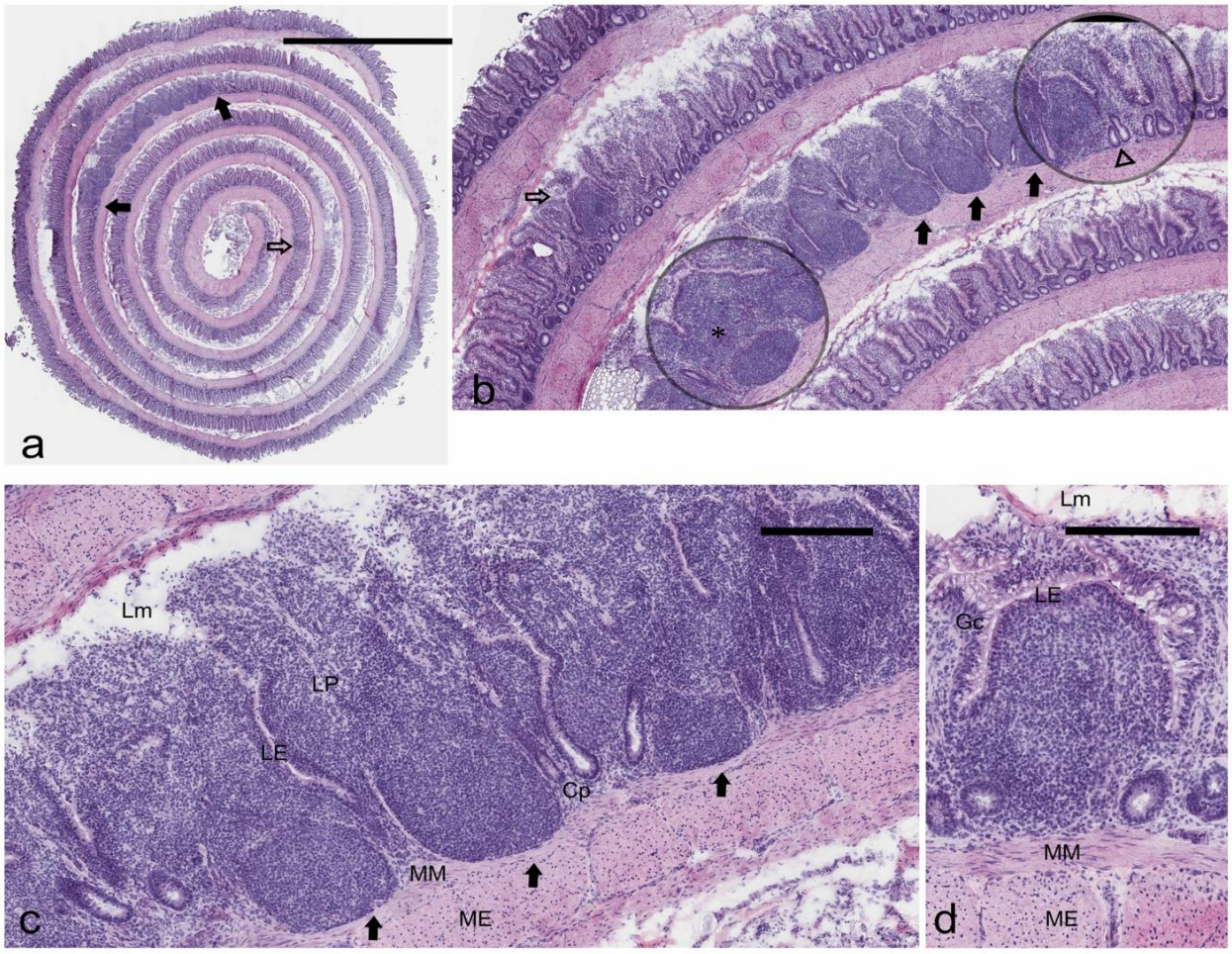

Figure 8. The detailed morphology of the 35d-old layer PP and diffuse lymphoid tissues in the terminal ileum made from the Swiss-roll cross-sections. a, The PP spans a 10mm longitudinal trajectory in the ileum and a 4mm transverse trajectory in the Swiss-roll section. The solid arrow marks the boundary of PP and the open arrow denotes a diffuse lymphoid tissue in the distal ileum. Scale bar, 4mm. **b,** Chicken PP may have the alike subepithelial dome structure on top of a follicle as of those in the mammals (indicated by the asterisk). An abrupt change from the normal intestine to the PP is on the right side (indicated by the open triangle). The open arrow indicates a dome-shaped diffuse lymphoid tissue in the intestine that is not in the PP. Scale bar, 600μm. **c,** The image is further enlarged to see the details. The chicken PP consists of massive lymphoid tissues in the mucosa of the distal ileum on the anti-mesenteric side. The lymphoid tissue is exposed to the lumen without a capsule. The PP tissue varies into thickened villi, lymphocytes infiltrated lamina propria, cylindrical to egg-shaped follicles, and a differentiated lympho-epithelium devoid of goblet cells. The follicles localize at the base of the lamina propria in adjacent to the crypt. Scale bar, 200μm. **d,** Goblet cells of villous epithelium were visible in the intestine, but absent in lympho-epithelium of the PP and the dome epithelium of the diffuse lymphoid tissue. Scale bar, 160μm (Gc: goblet cells, Cp: Crypt, LE: lympho-epithelium).

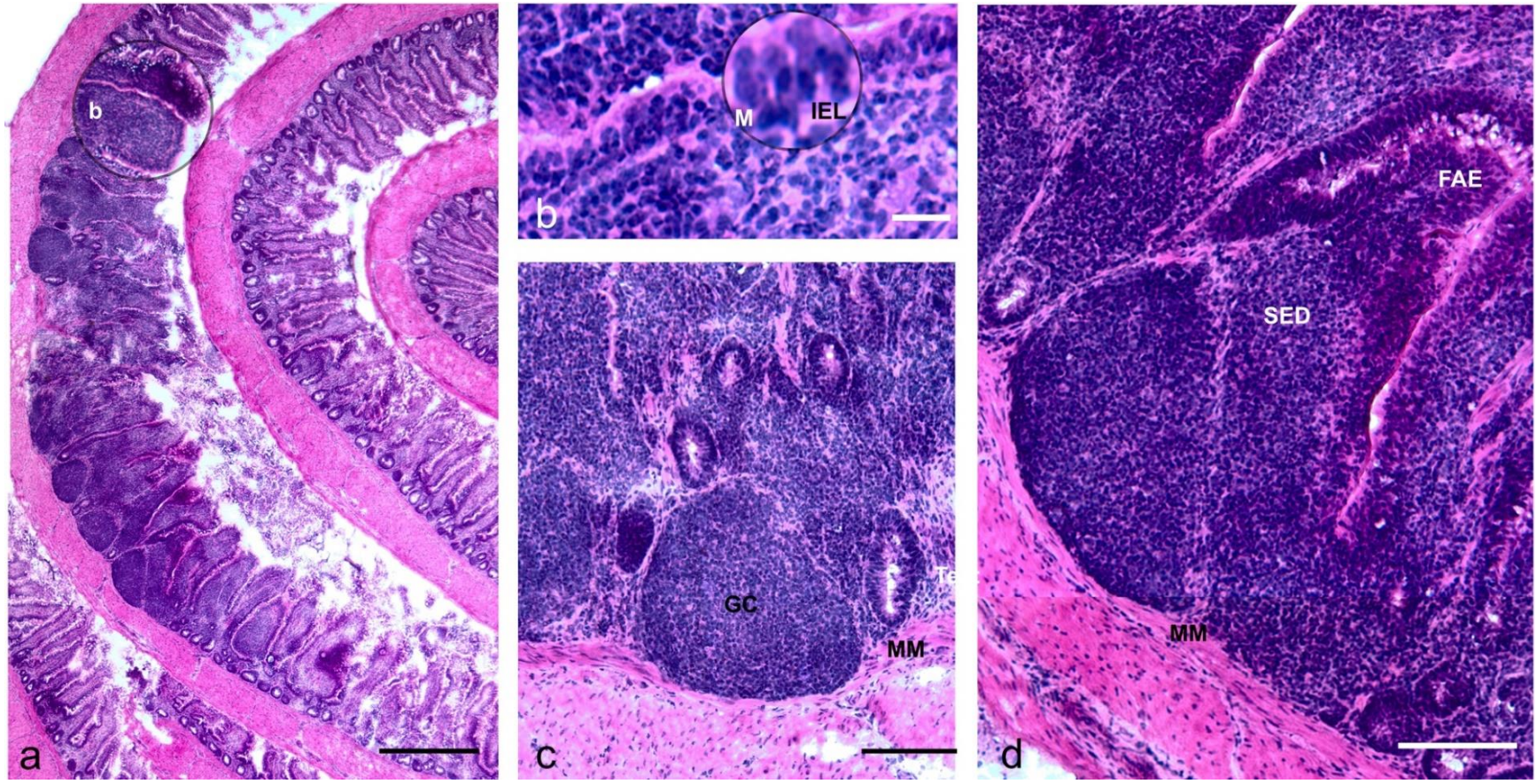

Figure 9. The follicular and structural morphology of the ileal PP from the 35d-old layer Swiss-roll cross-sections. a, An overview of the PP in the terminal ileum. The magnifier indicates the lymphoid tissue organized into a dome and the transition from goblet-cell-rich intestinal epithelium to the lympho-epithelium of the PP; numerous follicles are arranged in the same layer. Scale bar, 500µm. **b,** The circle magnifies a macrophage and intraepithelial lymphocytes in the lympho-epithelium. Scale bar, 20µm. **c,** The germinal center is present in the lymphoid follicle with a dark outer cortex and a light inner medulla. The follicle disrupts the muscularis mucosae. Scale bar, 100µm. **d,** The follicle-associated epithelium and the subepithelial dome region covers the basal follicle. Scale bar, 100µm (M: macrophage, IEL: intraepithelial lymphocytes, GC: germinal center, SED: subepithelial dome region).

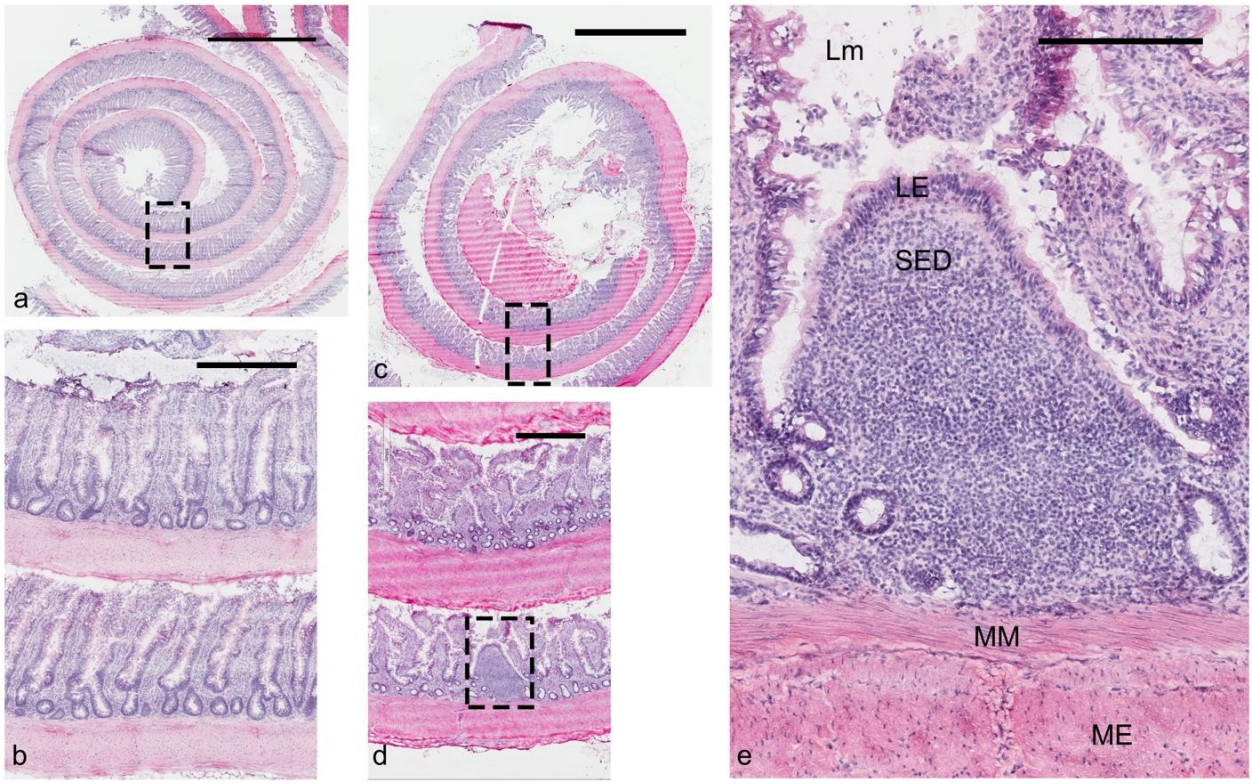

Figure 10. The ileal morphology and the regression of PP in 52+ week-old hen. a-d, The ileal segment of the spent hen does not contain a consistent PP in the ileum as described before, except for some diffuse lymphoid tissues in the ileum. Scale bars from a-d: 3mm, 400µm, 3mm, 500µm. **e,** This structure has a goblet-cell-free lympho-epithelium and a dome-shaped diffuse lymphoid tissue as of those identified in the 35d layers. Scale bar, 200µm.

Expression of MHC-II and E-cadherin in the Swiss-roll sections of layers

To visualize enterocytes in the intestinal epithelium, we performed a single IF staining of E-cadherin. Later on, we combined this staining with the labeling of MHC-II in dual staining because the dual staining of E-cadherin and MHC-II yielded an excellent picture of the morphology of ileal PPs. The E-cadherin staining by the 7D6 antibody was intense, and clearly delineated the outline of the intestine from the crypt base to the villus **(Fig. 11a)** and depicted the tight junction of the epithelial cells **(Fig. 11b)**. MHC-II$^+$ APCs were abundant both in the PP and in the intestinal lamina propria **(Fig. 12a)**. The expression of MHC-II was especially intense in the subepithelial region, and APCs were occasionally penetrating the lympho-epithelium and protruding into the lumen **(Fig. 12b-c)**. A small number of MHC-II$^+$ APCs was present in the follicles, but MHC-II$^+$ APCs were a lot more numerous in the interfollicular and subepithelial regions **(Fig. 13)**.

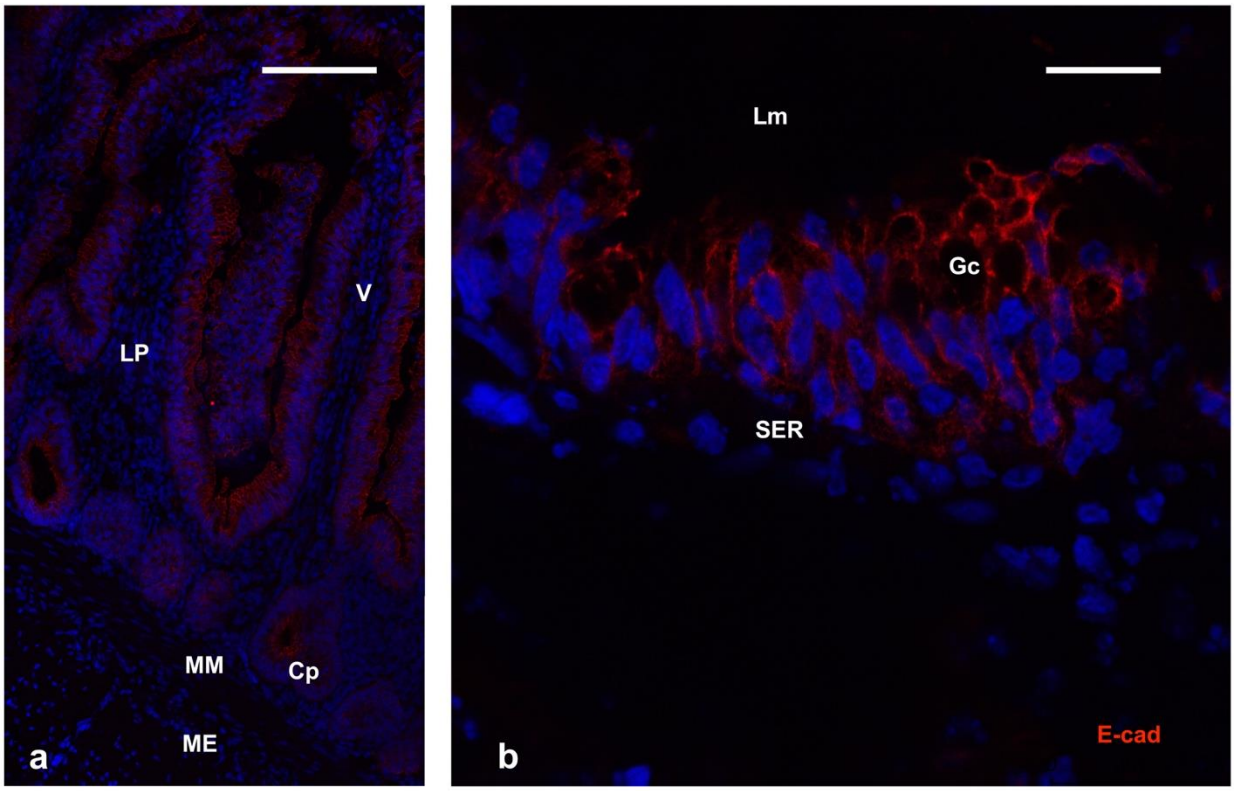

Figure 11. The expression of E-cadherin outlining the intestine and showing the structure of the epithelial cells. a, The uniform expression traced along the intestinal epithelium and outlined the entire intestinal villi and the crypts. Scale bar, 100µm. **b,** The higher magnification reflected the tight junction of epithelial cells. The vacuoles may indicate goblet cells. Scale bar, 50µm. (V: villi, SER: subepithelial region, Gc: goblet cell).

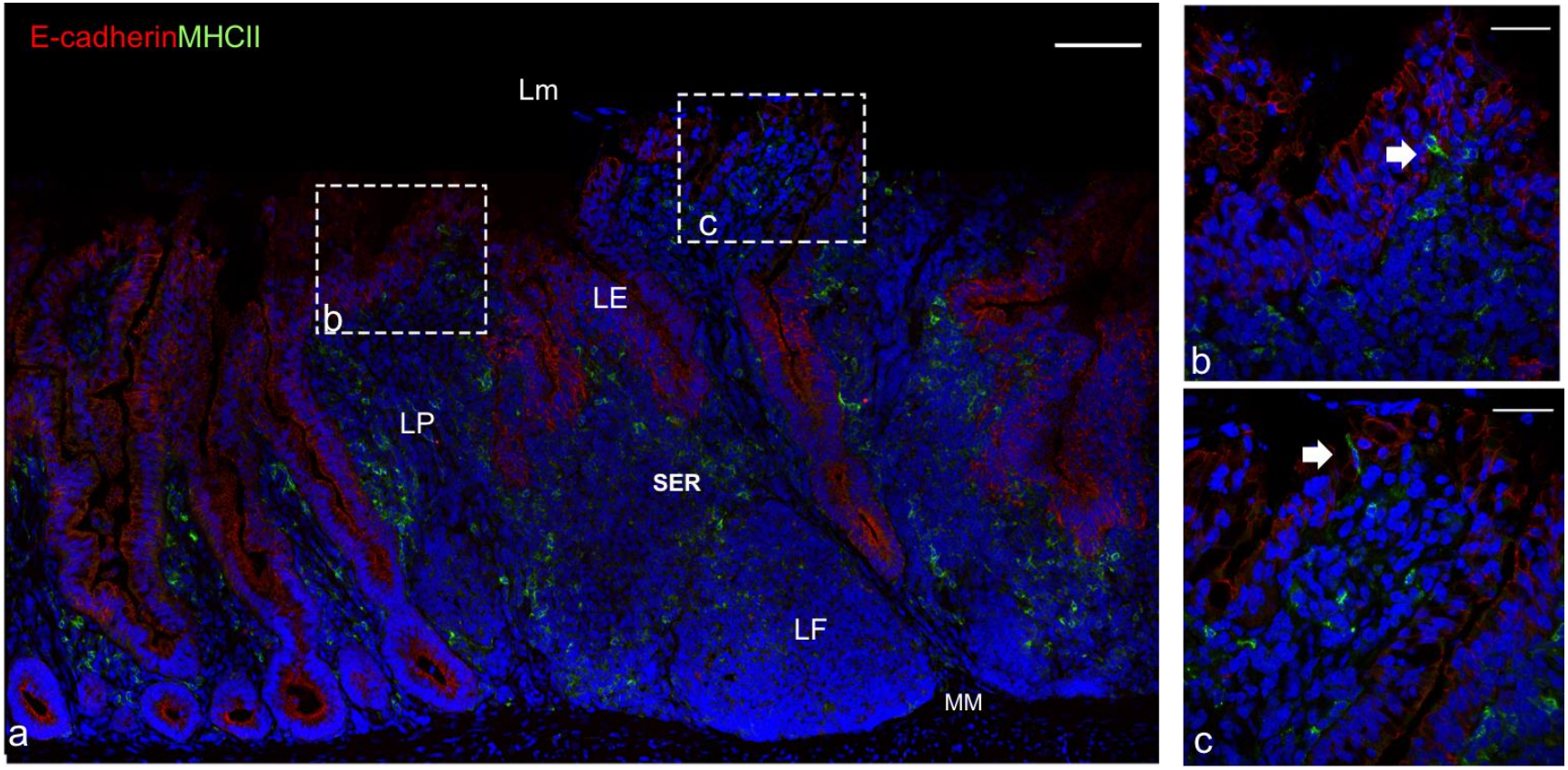

Figure 12. The dual staining of MHC-II and E-cadherin in the Swiss-roll processed tissue illustrating the morphology of ileal PP in the 35d-old layer. The lumen is always facing upward, and the connective tissue of smooth muscle layers is at the bottom. **a,** Part of the PP in the distal ileum with the adjacent intestinal villi. The staining of enterocytes outlines the epithelium. Thickened villi are present in the PP with a dilated lamina propria, in contrary to the normal intestinal villi to the left. The expression of APC is abundant in the lamina propria of the intestine and the subepithelial lamina propria of the PP. The interfollicular region has more MHC-II expression than the lymphoid follicle. Scale bar, 75µm. **b-c,** APCs are penetrating the epithelium. Scale bars, 25µm. **c,** An intraepithelial APC that depicts a dendritic-cell like cell with an elongated cytoplasm. (LF: lymphoid follicle, IFR: interfollicular region).

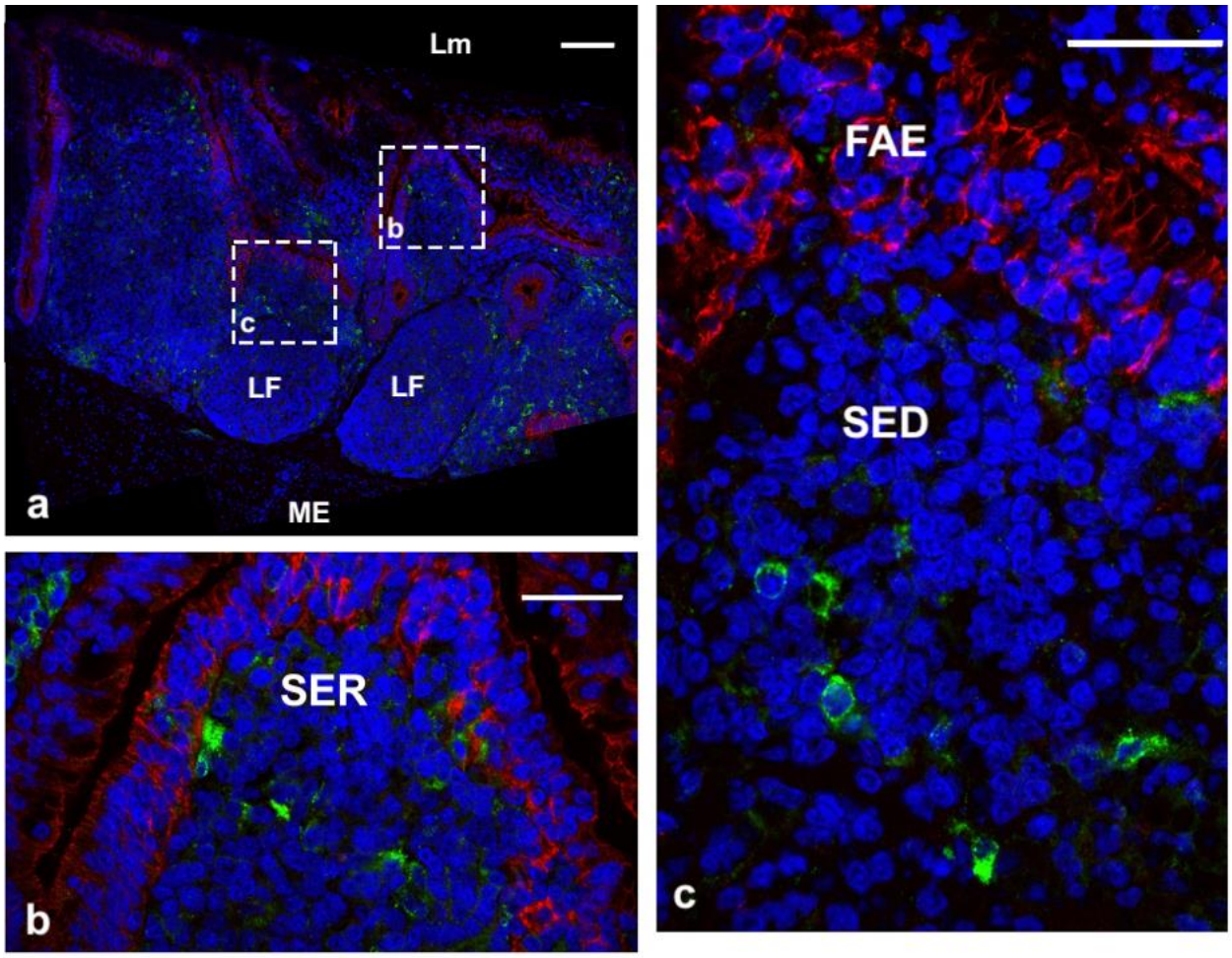

Figure 13. The predominant distribution of MHC-II$^+$ APCs in the subepithelial zones of the PP in the 35d-old layer. a, The MHC-II expression is mainly detected below the epithelium. Scale bar, 75µm. **b-c,** MHC-II$^+$ APCs in the subepithelial region and the subepithelial dome region underneath the follicle-associated epithelium. Scale bars, 25µm (FAE: follicle-associated epithelium, SER: subepithelial region, SED: subepithelial dome).

Expression of IgM and CD3 in the Peyer's patches

To further confirm the cellular composition of lymphoid follicles, we used FITC-labeled B-cell marker IgM and Alexa 647-labeled anti-CD3 on various tissue sections. The results revealed that IgM-bearing cells form clusters within all follicles **(Fig. 14a, e-g)** and their distribution was especially abundant in lamina propria of the intestine of young birds **(Fig. 14c)**. The expression was much weaker in the old laying hens where only a few cells were spotted along the lamina propria **(Fig. 14h-i)**. Signals of IgM can also be recognized in the bloodstream of the muscularis externa. $CD3^+$ T-cells were mainly distributed in the interfollicular area, in the lamina propria of the subepithelial region, and penetrating the epithelium of the intestine as intraepithelial lymphocytes (IEL) **(Fig. 14c-d)**.

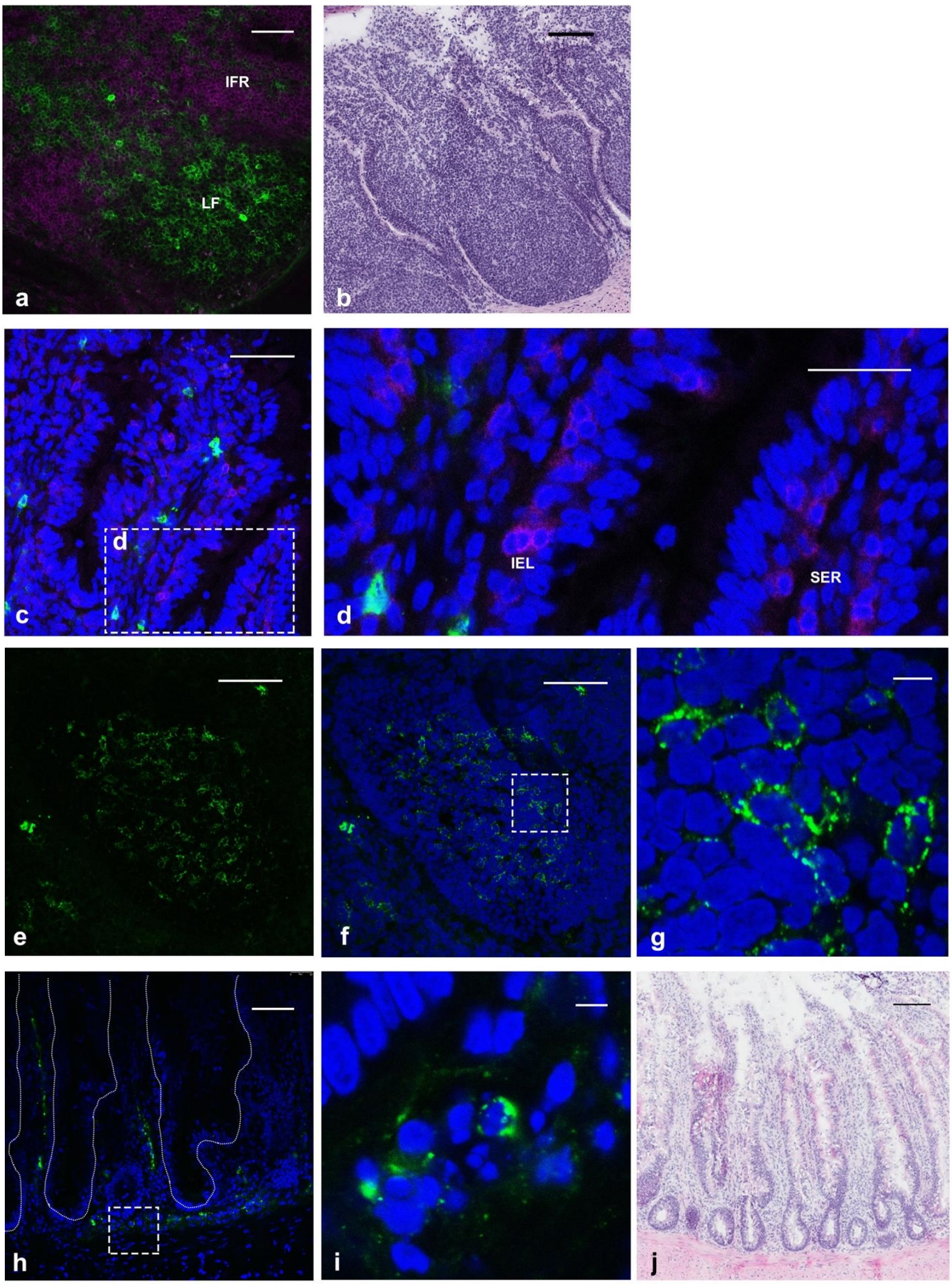

Figure 14. The follicular composition of chicken PPs and the distribution of lymphocytes in the distal ileum by a dual staining of IgM and CD3. a, In 35d-old layers, the follicle contained a large number of B-cells which formed in a germinal center. T-cells were in the interfollicular region. Scale bar, 50μm. **b,** H&E staining of the follicle. Scale bar, 100μm. **c,** IgM^+ cells scattered from the crypts to the villi tips in the intestine. Scale bar, 50μm. **d,** The subepithelial region and the mucosal epithelium and contained intraepithelial lymphocytes, many of which were T-cells. Scale bar, 25μm. **e-g,** In 42d-old broilers, follicular B-cells expressed high IgM. Scale bars: 50μm, 50μm, 5μm. **h-i,** In 52+ week-old hen, the expression of IgM was low and B-cells were scarce in the lamina propria. Scale bars: 50μm, 5μm. **j,** The H&E staining of the terminal ileum illustrated the morphology of the aged hen. Scale bar, 100μm (IFR: interfollicular region, SER: subepithelial region).

Expression of CD40 in the Peyer's patches

Besides the follicular expression of IgM and MHC-II, we also identified the CD40 expression in the follicles by a single IF staining of 2C5. We found clusters of $CD40^+$ cells in the follicle and a high expression in the interfollicular region. To further confirm the cell types of $CD40^+$, we performed a dual staining with MHC-II to identify the APCs.

Immunofluorescent staining of broiler PPs using 2C5 anti-CD40 mAbs revealed a fair level of CD40 expression in the interfollicular area and a moderate to high expression in the follicles **(Fig. 15a-d)**. The dual staining of MHC-II and CD40 revealed clusters of cells expressing CD40 near the crypts of the Lieberkühn **(Fig. 15e-f)** and the presence of APCs in the epithelium **(Fig. 15g)**. We also detected a high CD40 expression in lamina propria MHC-II^+ cells, where the distribution of CD40 seemed to match that of MHC-II **(Fig. 15h)**. However, no yellow fluorescence was observed, indicating the absence of dual labeling at the molecular level.

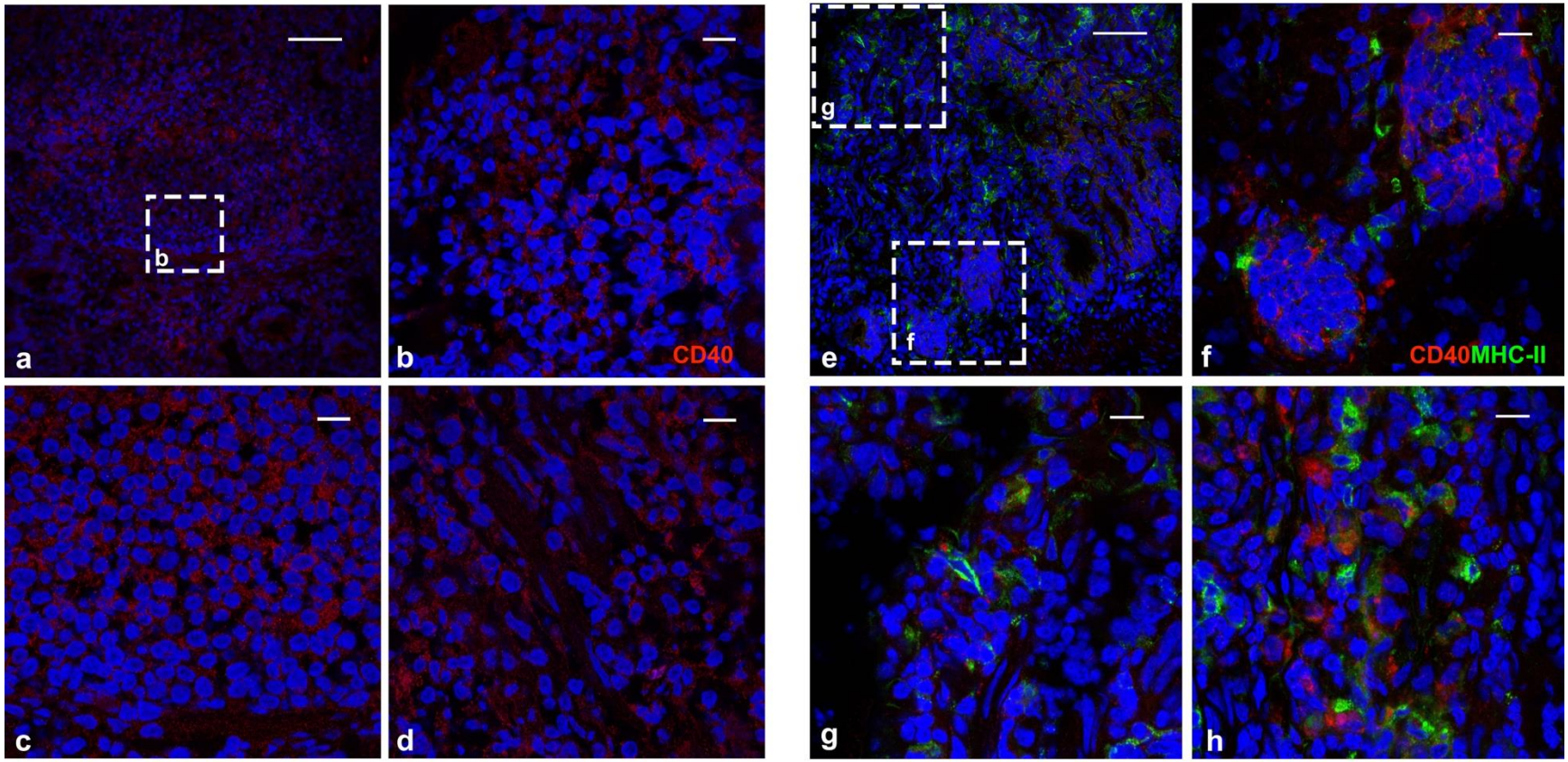

Figure 15. The follicular and lamina propria expression of CD40 (left) and the co-expression of CD40 on MHC-II$^+$ APCs (right) in the 42d-old broiler PPs. a-d, A moderate to high-level expression of CD40 in the lymphoid follicles of PPs. **d,** Some CD40 expression in the lamina propria. Scale bars, a: 50µm, b-d: 10µm. **e-h,** A dual staining of CD40 and MHC-II showing MHC-II$^+$ APCs and CD40$^+$ cells. Scale bars, e: 50µm, f-h: 10µm. **f,** Clusters of CD40$^+$ next to the crypts at the base of the lamina propria. **g,** An intraepithelial APC. **h,** Co-expression of CD40 and MHC-II on APCs in the lamina propria. The loci of CD40 are present in MHC-II molecules.

Expression of CD205 in the Peyer's patches and the spleen as observed by immunofluorescent staining of cryosections

We first investigated the immunofluorescent expression of CD205 on putative DCs in broiler PPs and spleen using monoclonal mouse anti-cCD205 (diluted ascites fluid) in combination with a FITC-labeled secondary mouse IgG. $CD205^+$ cells (putative immature DCs) were scarce in the lymphoid follicles of the PP **(Fig. 16a-b)** and mostly appeared near the crypts **(Fig. 16c-d)**. DC-like cells were scattered single cells that were loosely attached to each other in the tissue **(Fig. 16e-n)**. In contrast, $CD205^+$ DCs were abundant in the spleen, compared to their relative scarcity in the PP **(Fig. 16k-n)**.

Labeling experiments with anti-chicken CD205 mAb revealed multiple maturation stages of $CD205^+$ cells. One of the expression patterns was characterized by an abundance of fine $CD205^+$ granules surrounding the cell nucleus **(Fig. 16e)**, in combination with an elongated cytoplasm **(Fig. 16f)**. Another characteristic consisted of distinct granular particles on the cell surface, resembling vesicles or intracellular organelles **(Fig. 16g-i)**. The intensity of $CD205^+$ labeling was variable between cells, ranging from relatively dim, diffuse labeling to condensed, bright fluorescent spots in the cell **(Fig. 16e-j)**. The phenotype characterized by fine granules was more frequently observed in splenic $CD205^+$ DCs **(Fig. 16o)**.

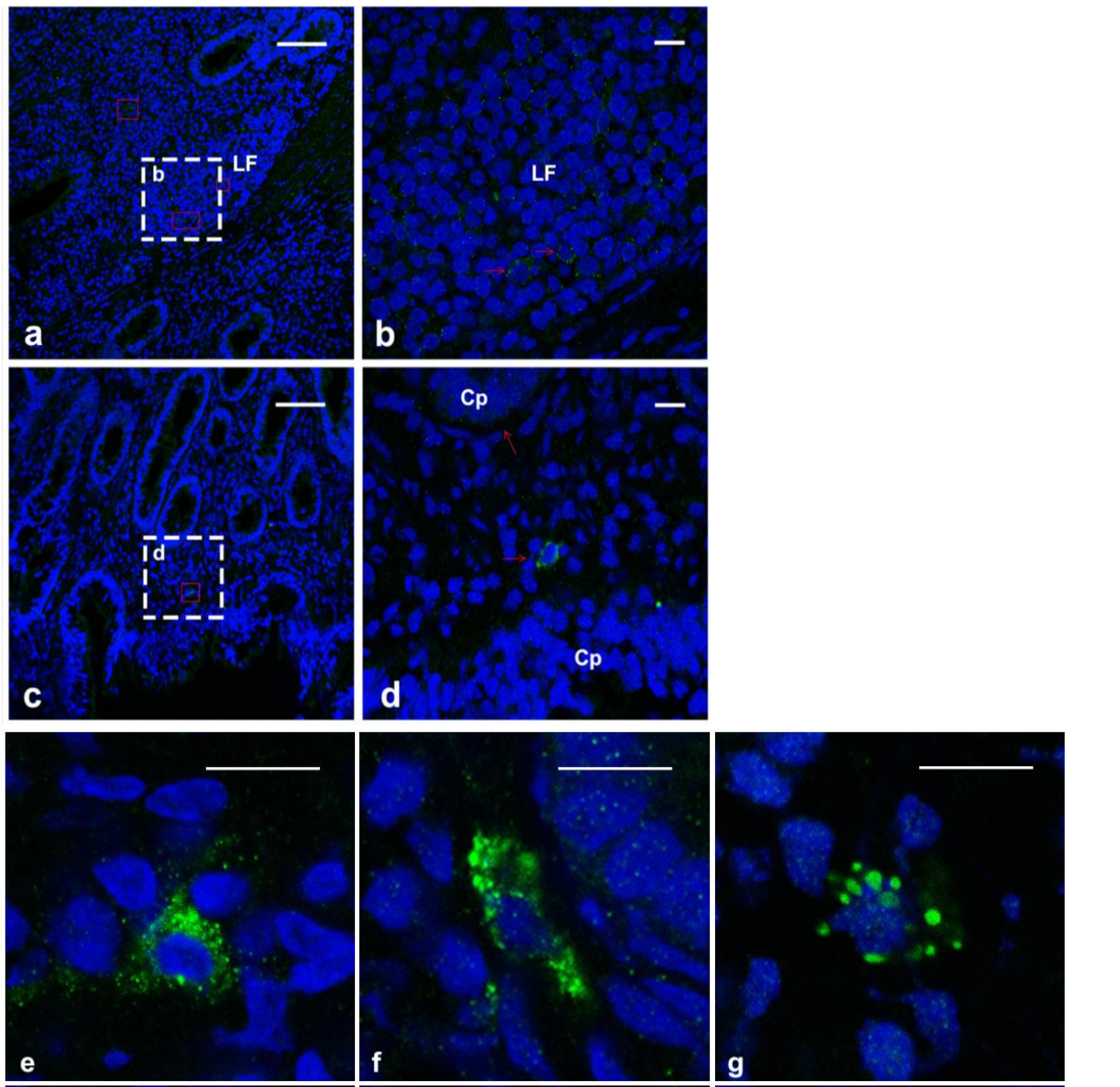

Figure 16. The expression and distribution of CD205: DC-like cells in the PP (a-j) and in the spleen (k-o) of 42d-old broilers, suggesting a variety of maturation stages. Peyer's patches. a-b, Relatively few CD205^{+} DCs were observed in the broiler PPs with a very low expression in the follicles. Scale bars: 50µm, 10µm. **c-d,** Most DCs were identified around the crypts and appeared as single cells. Scale bars: 50µm, 5µm. **e-j,** Phenotypically different CD205^{+} DCs were identified in the PP. CD205-labeling in the form of abundant fine cytoplasmic granules **(e)**, with some cells displaying an elongated cytoplasm **(f)**. Scale bars: 10µm. **g-i,** Distinct granular particles that are typical to CD205^{+} DCs. **j,** Degraded fluorescent spots in the cell. The expression levels range from bright to dim. Such CD205 expression can be brilliant or diminished and condensed into bright spots on the cell surface. **Spleen. k-n,** The overall appearance of splenic CD205 expression in fine granules. Scale bars, k-m: 25µm, l-n: 10µm. **o,** A splenic CD205^{+} DC showing intracellular granules and an elongated cytoplasm. Scale bars: 8µm.

CHAPTER V

DISCUSSION AND CONCLUSIONS

The immune system of the chicken is in many respects less sophisticated than its mammalian counterparts, but it nevertheless clearly maintains the necessary functionality. The contents of the chicken ileum represent a complicated environment of digesta, microbiota and pathogenic antigens, thus making it a crucial site for complex immune interactions. The ileal GALT consists of a variety of immune organs, among which the Peyer's patches have the most pronounced and intricate micro-anatomical organization (Simon *et al.,* 2014).

In this study, we have identified the PP in the terminal ileum of the chicken **(Fig. 6)** based on previous observations (Befus *et al*., 1980; Vaughn *et al*., 2006). We also revealed the in-depth longitudinal morphology of the PPs by the use of the Swiss-roll technique (Bialkowska *et al*., 2016; Moolenbeek and Ruitenberg, 1981). Moreover, we observed the PPs of *Eimeria*-infected broilers (post-mortem tissues offered by Dr. Farnell) **(Fig. 6)**. We found that in birds with coccidiosis, the PPs were extremely conspicuous and easy to locate from both intestinal surfaces (serosal and mucosal). In these birds, the PPs were swollen and reddish, indicating the vasodilation (data not shown), whereas the PPs of healthy birds were unremarkable and smaller in size **(Fig. 6)**. These observations are in line with reports about poorly developed PPs in mice from "antigen-free" environment, and abnormally developed lymphopoietic zones in isolated PPs of the mouse, rabbit, and sheep (Reynolds, 1987).

Based on our macroanatomy observations, PPs were non-encapsulated and exposing the lymphatic tissue to the lumen of the intestine, which is similar to the lymphatic nodules of chicken tonsils (Casteleyn *et al.*, 2010). As in previous studies (Befus *et al.*, 1980; Vaughn *et al.*, 2006),

our findings confirmed the consistent location of the distal. The ileal PP, flanked by the ceca, was cranial to the ileocecal junction and in proximity to the ceca blind end. However, other locations as described in Casteleyn *et al.* (2010) and Vaughn *et al.* (2006) were failed to detect in this research. PPs were macroscopically visible in young broilers of 3-week-old **(Fig. 6b)** and were more distinguishable in birds from 5 (35d layer) to 6 (42d broiler) weeks of age. The aged hen of more than 52 weeks did not possess a PP in the distal ileum **(Fig. 10)**. These findings were correlated with previous studies that PPs are further developed from 10-day-old neonatal birds and increase in size and quantity as birds reach 13 weeks of age (Casteleyn *et al.*, 2010). Finally, PPs are regressed in birds of 52-58 weeks of age (Befus *et al.*, 1980). Although PPs were not present at the time we collected the tissue, 52+ week-old hen remained a single dome-shaped diffuse lymphoid tissue in the distal ileum **(Fig. 10e)** as of those recognized in young layers **(Fig. 8)**. The nature of this structure is rarely known and has not been described in chickens before. However, from the research in humans, the dome might indicate a primordial PP site and the arched appearance is caused by the germinal center formation (Jung, Hugot and Barreau, 2010).

Most of our findings regarding the morphology of PP were consistent with previous descriptions (Befus *et al.*, 1980; Casteleyn *et al.*, 2010; Jeurissen *et al*., 1989; Vaughn *et al*., 2006). The ileal PP had the characteristics of broad villi, dilated lamina propria with lymphoid cell infiltration, numerous egg-shaped follicles at the base of the mucosa and a lympho-epithelium containing intraepithelial lymphocytes but no goblet cells **(Fig. 9)**. M-cells were not identifiable by the light microscopy though their presence was confirmed by Befus *et al*. (1980), nor were the high endothelial venules found in PPs of mammals (Jung, Hugot and Barreau, 2010). In our 35d layer specimens, most follicles did not have a distinct germinal center **(Fig. 9c)** which may imply the incomplete maturation of follicles according to Jeurissen *et al*. (1989), that fully matured

follicles with a germinal center are observed in birds at 12 weeks of age. We also noted significant variations from mammals that the submucosa was not identifiable in the chicken ileum and follicles resided at the base of the lamina propria in the same level, and in some circumstances, follicles might interrupt the muscularis mucosae **(Fig. 7-9)**. Unlike in mammals, PPs are transmucosal with multiple layers of follicles in the submucosa, and the lymphoid tissue is organized in dome-shaped structures in the mucosa (Makala, Suzuki and Nagasawa, 2003).

The knowledge of the cellular composition of PPs in the chicken is relatively superficial, compared to the situation in mammals. To address this knowledge gap, we immunostained the tissue using various cell surface markers on thin (5µm) cryosections to preserve the antigenicity in combination with biotinylated antibodies and fluorescently labeled streptavidin to amplify the signal and also to overcome the limitations of primary antibodies produced in the same host (mouse) in the dual staining experiments. Moreover, we optimized a post-sectioning fixation method consisting of -20˚C cold acetone followed by -4˚C 80% methanol.

PPs are widely scattered in the intestinal wall and only take up a small portion of the ileum. Therefore, it is impossible to visualize them all at the same a time. Also, the intestine is too long to permit systematic analysis of every millimeter along its entire trajectory. Producing a countless number of transverse sections along the entire length of the ileum is not achievable, and risks overlooking essential details without creating a meaningful overview. To compensate for this, we performed transverse sections of "Swiss rolls" made from the lengthwise opened ileal segments according to Bialkowska *et al.* (2016), who used it for paraffin sectioning of the mouse intestine, and proved that this method also produces excellent results when used for cryosectioning and immunofluorescent analysis of the chicken ileum. The Swiss-roll technique provided a longitudinal view of the PP, as each Swiss roll section depicted a longitudinal view of 5-10 mm of

the distal ileum **(Fig. 8-10)**. The epithelium lining was better preserved in the Swiss-roll tissue **(Fig. 8-10)** when compared to sections of the excised tissue **(Fig. 7)**.

E-cadherin is one of the primary cell adhesion molecules in the intestinal epithelium that is closely in association with cell-cell interactions. The chicken homolog of E-cadherin (L-CAM) has been used to identify the architecture of the avian intestine (Escaffit *et al.*, 2005; Gallin, Edelman and Cunningham, 1983). Therefore, we decided to use dual fluorescent labeling of both MHC-II (a constitutively expressed APC marker) and E-cadherin, in combination with the DNA stain DAPI as nuclear staining method. This method was most helpful in guiding us through the basic morphology of the intestine, specifically to ascertain the microscopic location of the PP as well as to map the detailed morphology of the terminal ileum **(Fig. 11-13).** This strategy permitted thus to provide an additional level of information, complementing the basic histology initially obtained by H&E staining.

APCs were predominantly observed along the crypt-villus axis in the lamina propria of the intestine. In PPs, APCs were especially abundant in the subepithelial zone, but they were also occasionally observed in intraepithelial locations. The expression of MHC-II was much higher in the subepithelial dome region than in the follicles, which correlates with previous studies in mammals (Neutra, Mantis and Kraehenbuhl, 2001) that DCs, macrophages, and B-cells capture antigens—either directly or through M-cell transcytosis, in the subepithelial region. The organization in the chicken also implies that the primary sites for antigen presentation are in the subepithelial zone, which is characterized by a high MHC-II expression, whereas the low MHC-II expression in the follicles indicates that fewer APCs are actively participating in antigen presentation.

From the IgM and CD3 expression in the ileum, we identified that the ileal PP contained separate B- and T-lymphocyte areas and IgM-positive germinal centers as expected from previous studies (Jeurissen *et al.*, 1989). It was similar to mammalian PPs and chicken GALTs that the follicle has a germinal center of B-lymphocytes flanking by T-lymphocytes in the interfollicular region (Casteleyn *et al.*, 2010; Neutra, Mantis and Kraehenbuhl, 2001). Besides, T-lymphocytes were diffusely scattered under the epithelium and penetrated the intestinal epithelium regarded as intraepithelial lymphocytes as described by Heel *et al*. (1997).

To investigate the CD40 expression in the PPs, we initially conducted a single immunofluorescent staining (using monoclonal antibody 2C5 against chicken CD40), and then followed with additional dual staining for MHC-II to identify the APCs. We found clusters of $CD40^+$ cells in the follicle and high expression in the interfollicular region **(Fig. 15)**. We also observed the expression of CD40 along with MHC-II in the epithelium and a predominant CD40 expression in the bottom cell clusters that seemed to be located near the crypt base **(Fig. 15)**. This is in accordance with previous studies, reporting the expression of CD40 on the cell surface of all APCs, but mainly on macrophages and B-cells in chickens where it functions as the primary co-stimulatory receptor assisting in antigen presentation (Hashem *et al*., 2014; Ratcliffe and Härtle, 2014; Tregaskes *et al*., 2005). Confocal microscopy also revealed that CD40 was present on the surface of $MHC\text{-}II^+$ APCs but in a lower amount compared to the expression of MHC-II. The high power magnification images suggested that both molecules were expressed on the same cell surface and that the respective primary antibodies were not cross-reacting. Molecules were not dual labeled as indicated by the green and red fluorescence on the cell surface, and the absence of any yellow fluorescence **(Fig. 15h)**. These findings, suggesting that APCs in the lamina propria are mainly macrophages expressing both CD40 and MHC-II, are in line with the report by Carvallo

et al. (2011) that macrophages are predominant in chicken lamina propria. Mammalian species, including mice and humans, also express CD40 on endothelial as well as epithelial cells (van Kooten and Banchereau, 2000), which is in line with our findings of CD40 expression on the epithelium of the ileal crypts in the chicken.

Immunofluorescent staining for CD205 revealed that DCs were sparse in the PP compared to DC clusters observed in the spleen. Based on our observations, the intracellular and extracellular CD205 protein appeared as fine granules around the nucleus, or in the form of discrete particles on the plasma membrane, as well as small fluorescent spots in the cell cytoplasm **(Fig. 16)**. As a marker of immature DCs, CD205 is representative of the stage of endocytosis and initial maturation of DCs. In the initial stages of this differentiation process, CD205 is gradually translocated from the intracellular compartments onto the cell surface (Butler *et al*., 2007). The CD205 receptor then binds antigens, internalizes them in coated vesicles and delivers them via endocytosis to the MHC-II synthetic pathway supporting antigen presentation (Butler *et al.*, 2007; Goldstein, Anderson and Brown, 1979). Since immature DCs are capable of endocytosis and mature DCs are specialized in antigen presentation (Caminschi, Lahoud and Shortman, 2009), the expression levels and patterns of CD205 reflect the activity of DCs. Cells with abundant CD205 in diffuse granules appear to indicate the early formation of CD205 as can be seen in the spleen, where most of the splenic DCs are in the immature state. The vesicular appearance of CD205 suggests active endocytosis of DCs when antigens are bound to CD205 on the cell surface and internalized in coated vesicles invaginating from the plasma membrane (Goldstein, Anderson and Brown, 1979), and then CD205 finally appears in the cytoplasm as granules in the intracellular compartment. The presence of degraded CD205 spots on the cell surface may explain the recycling

process of CD205 back to the cell surface after endocytosis (Mahnke *et al*., 2000) and may represent a more matured DC cell type.

In conclusion, our immunohistochemical and microanatomical characterization of the ileal PPs in the chicken confirm the presence of all necessary functional elements permit an essential role of the PPs as inductive sites in the chicken GALT, including antigen capture and presentation, followed by formation of germinal centers reflecting the clonal expansion of activated B-cells. Based on the abundant expression of CD40, which is in sharp contrast with the relatively sparse expression of CD250 in and on DCs, our studies provide a rationale for the continued use and further optimization of CD40-targeted oral vaccination strategies. While CD205 has been reported as a target for DC-mediated internalization of antigens, our observations do not warrant major efforts in pursuing CD205-targeted oral vaccination strategies.

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