Research article

Immunohistochemistry for Detection of MHC Class II-Expressing Cells in Japanese Flounder *Paralichthys olivaceus*

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ABSTRACT—Antigen-presenting cells characterised by the expression of MHC class II molecules play a crucial role in differentiating and activating helper T cells at lymphoid organs. We attempted to establish an immunohistochemical staining technique for the detection of MHC class II-expressing cells in Japanese flounder to study the localisation of these cells in tissues. We produced a specific antibody against the α chain peptide of MHC class II. The antibody reacted to the recombinant protein of the $\alpha 2$ segment of the class II molecule, the native molecule of the class II α chain in soluble proteins from the spleen and in membrane proteins from intraperitoneal infiltrated cells. Immunohistochemical staining using the antibodies could be utilised for the detection of MHC class II-expressing cells in paraffin-embedded tissue specimens. MHC class II-expressing cells were found in various organs and tissues such as the spleen, kidney, thymus, intestine, stomach, liver, heart, gills, skin, olfactory organs, and brain. The immune-positive cells showed an elongated- or rounded-shape. Considering our observations of morphological features and tissue localisation along with previously published information, we suggest that the MHC class II-expressing cells are dendritic cells, macrophages, B cells, microglia, and epithelial cells that have been considered antigen-presenting cells.

Key words: antigen-presenting cells, lymphoid organs, anti-MHCII α chain antibody, immunostaining, histology, immunoblotting, spleen, teleost

Major histocompatibility complex (MHC) molecules are cell surface proteins associated with self- and non-self-recognition by T cell-mediated immunity (Abbas *et al.*, 2015). MHC molecules are divided into two groups; class I molecules are expressed on virtually all nucleated cells, whereas class II molecules are expressed only on dendritic cells, B cells, macrophages, and a few other cell types (Neefjes *et al.*, 2011). Class II molecules are composed of two polypeptide chains, an α chain and a β chain, and display self-peptides and foreign peptides accommodated in a peptide-binding groove formed by both polypeptide chains for recognition by the antigen receptor (TCR) of CD4-positive T cells (Guermonprez *et al.*, 2002; Neefjes *et al.*, 2011). The specialised cells that display peptide antigens with MHC class II molecules and activate CD4-positive T cells are called antigen-presenting cells (APCs) (Itano and Jenkins, 2003). APCs play an important role in the

maturation, differentiation, and activation of helper T (Th) cells that exhibit the CD4 phenotype, and they are responsible for controlling the cellular and humoral adaptive immune response (Abbas *et al.*, 2015).

In secondary lymphoid organs such as lymph nodes and the spleen, professional APCs drive naive Th cells to differentiate into effector Th cells by binding the interactions between foreign peptide-MHC class II complexes on the APCs and TCRs on the T cells, costimulatory molecules CD80/86 on the APCs, and costimulatory receptors CD28 on the T cells (Itano and Jenkins, 2003; Mildner and Jung, 2014). In the thymus, thymic APCs such as thymic epithelial cells and dendritic cells are essential for the maturation and selection of T cells because they bind the interaction between self-peptide-MHC molecule complexes on the APCs and TCRs on thymocytes (Alexandropoulos and Danzl, 2012; Takaba and Takayanagi, 2017).

Adaptive immunity and related immune cells such as T cells, B cells, and dendritic cells have also been found in teleost fish (Lugo-Villarino *et al.*, 2010; Bassity

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and Clark, 2012; Secombes and Wang, 2012). However, the distribution and tissue structure of lymphoid organs in teleost fish are different from that of mammals. Teleost fish lack lymph nodes and Peyer's patches, which are known as secondary lymphoid organs in mammals (Zapata *et al.*, 1995). The spleen is also classified as a secondary lymphoid organ in teleost fish, but the white pulp is poorly developed and no germinal centres are present (Zapata *et al.*, 1995; Press and Evensen, 1998; Udrouiu and Sgura, 2017). The kidney of teleost fish is known as a primary haematopoietic organ (Kobayashi *et al.*, 2016). Yet Zwollo *et al.* (2005) reported that B cell activation occurred in the posterior kidney of rainbow trout *Oncorhynchus mykiss*, suggesting the presence of a secondary immune site like the spleen. Some peripheral lymphoid tissues have been found in mucosa of the intestine, skin, and olfactory organs of teleost fish (Salinas, 2015). The common histological feature of these tissues is the presence of diffuse immune cells and the absence of organised lymphoid structures. Salmonid fish have substantial intraepithelial aggregates dominated by lymphocytes located at the terminal portion of the interbranchial septum, termed interbranchial lymphoid tissue (ILT) (Koppang *et al.*, 2010). The epithelial cells in ILT of Atlantic salmon *Salmo salar* show gene expression of chemokine CCL19, which attracts naive T cells in mammals (Björge *et al.*, 2019). Spiny-rayed fish such as European perch *Perca fluviatilis* and witch flounder *Glyptocephalus cynoglossus* do not have typical ILT found in salmonid fish, but lymphoid aggregates within the branchial epithelium at the base of gill filaments have been observed (Rességuier *et al.*, 2020). Recently, some types of immune cells such as T cells and APCs have been found in milky spots of intraperitoneal adipose tissue in rainbow trout (Pignatelli *et al.*, 2014; Veenstra *et al.*, 2019). Veenstra *et al.* (2019) suggested that the visceral adipose mass likely plays an important role in vaccine antigen uptake and presentation by APCs, as well as subsequent T cell activation and differentiation.

Although various lymphoid organs and tissues that likely act as lymph nodes and Peyer's patches have been found in teleost fish, the interaction between APCs and T cells in these lymphoid sites, which is necessary for development of adaptive immunity, has not yet been directly observed. A lack of immunological tools for detecting each immune cell in teleost fish has hindered progress on the functional study of the lymphoid organs and tissues. While several specific antibodies are available for detecting immune cells in limited fish species, those needed for studying the lymphoid sites have not been sufficiently developed for marine fish species important to Japanese aquaculture. This study attempted to develop immunohistochemistry using a specific antibody for the MHC class II molecule of Japanese flounder, *Paralichthys olivaceus*, a major commercial species

in Japan.

Materials and Methods

Production of specific antibody against the α chain of MHC class II molecule (poMHCII α)

The peptide (VTEGTSLNVPYPNT) on the $\alpha 2$ segment of class II molecules was selected as an antigen for immunisation from predicted amino acid sequence data (accession number: BAD13364) of the α chain of MHC class II in Japanese flounder (Srisapoomee *et al.*, 2004) by Eurofins Genomics. Rabbit antiserum was prepared by immunisation with the synthetic peptides conjugated with a carrier protein, keyhole limpet hemocyanin (KLH) by Eurofins Genomics. Immunoglobulin G (IgG) antibodies were purified from the antiserum by affinity chromatography using protein A/G agarose resins (Thermo Fisher Scientific), and then antibodies against KLH were eliminated from the purified polyclonal IgG by absorption using acetone powder from KLH.

Preparation of protein samples

A piece of spleen tissue (approximately 50 mg) of Japanese flounder (approximately 20.5 g) was put in 500 μ L EzApply (ATTO) and homogenised at 4,300 rpm for 10 s with a zirconia bead (diameter: 5 mm) using Micro Smash MS-100 (Tomy Seiko). The supernatants were collected from the homogenised samples by centrifugation at 12,000 rpm for 15 min at 4°C and kept at -20°C until use. Infiltrated cells were harvested from the peritoneal cavity of Japanese flounder (approximately 23.7 g) 2 days after intraperitoneal injection of formalin-fixed *Edwardsiella piscicida* NJB0401 (1 mg). The cells (1×10^6 cells) were lysed by mixing with 100 μ L EzApply by pipetting and kept at -20°C until use. Biotinylation of cell membrane proteins was performed for intraperitoneally infiltrated cells prepared as mentioned above using a fish weighing 26.5 g. The cells (3×10^6 cells) were incubated with 0.1 mg EZ-Link NHS-LC-Biotin (Thermo Fisher Science) in 150 μ L PBS for 30 min at room temperature. After two washes, the cells were solubilised with 300 μ L lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA) for 30 min on ice. The supernatants were collected by centrifugation at 12,000 rpm for 15 min at 4°C and incubated with 50 μ L NeutrAvidin agarose resins (Thermo Fisher Scientific) for 2 h on ice. The mixture was occasionally agitated during the incubation periods. After three washes with PBS, the resins were mixed with 50 μ L EzApply and kept at -20°C until use.

Recombinant proteins for the $\alpha 2$ segment of class II molecules were also prepared. The $\alpha 2$ segment gene was amplified using a primer pair designed for using In-Fusion cloning (5'-TACCCTCGAGGGATCGGAGCCTCCCTCCAGTCCGATG-3', 5'-CGACAAGCTTGAATT

CCCAGATCTTGGTCAGTGATTG-3') and PrimeSTAR MAX DNA polymerase (Takara Bio). The PCR products were ligated into pCold I vector digested with BamHI and EcoRI using the In-Fusion HD cloning kit (Takara Bio). The constructs were transformed into *Escherichia coli* JM109 and replicated. The recombinant plasmids were purified using NucleoSpin Plasmid EasyPure (Takara Bio) and retransformed into *E. coli* BL21. After growth at 37°C for 7 h in LB broth containing ampicillin, the recombinant proteins were induced by adding 0.1 mM isopropyl thiogalactoside to the cell suspension and culturing at 15°C for 24 h. The cells were lysed using BugBuster Protein Extraction Reagent (Merck) supplemented with 0.1 mg lysozyme and 25 U DNase I (Takara Bio). Consequently, the recombinant protein formed inclusion bodies.

Western blotting

Protein samples were electrophoresed on e-Pagel precast gel (E-T15L, ATTO) and then transferred to Clear Blot Membrane-P plus (ATTO). The membrane was blocked by 4% Block Ace (DS-Pharma). Immunoblotting was performed with rabbit polyclonal antibodies against poMHCII α (0.3 mg/mL in TBS-T containing 0.1% Tween 20, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl), followed by a secondary antibody conjugated using alkaline phosphatase (Thermo Fisher Scientific). Primary antibodies such as 6x-His Tag monoclonal antibody (Thermo

Fisher Scientific) and Purified Rabbit Polyclonal Isotype Ctrl (BioLegend) were used as controls instead of anti-poMHCII α antibodies. The immune reaction was visualised by NBT/BCIP solution (Roche). The membrane to which spleen proteins were transferred was stained by CBB solution (0.25% Coomassie Brilliant Blue R250, 7.5% acetic acid, 5% methanol).

The specificity of anti-poMHCII α antibodies was confirmed by an inhibition test using the antigen peptide that was used in the production of the specific antibody. Briefly, the antibodies reacted with the antigen peptide on the $\alpha 2$ segment for 30 min were used as a primary antibody. Another synthetic peptide (SPPWKVENNTQVSH) was prepared as a negative control.

Immunohistochemistry

Japanese flounder (20–300 g) were fixed with 10% neutral buffered formalin for 2 days. Internal organs were washed with water and kept in 50% isopropanol. Head parts were decalcified with 27 mM EDTA, 7.4 mM sodium acetate, and 4% formalin adjusted to pH 7.4 using sodium hydroxide for 7 days. After washing with water, the heads were kept in 50% isopropanol. All organs and tissues were embedded in paraffin and tissue sections (3–5 μ m in thickness) were prepared. The deparaffinised specimens were soaked in 20% H₂O₂ for 20 h to bleach and eliminate endogenous melanin and peroxidase activity, respectively. After blocking of the

Fig. 1. Immunoblot analysis of the α chain protein of MHC class II in spleen and intraperitoneally infiltrated cells of Japanese flounder. (A) The recombinant protein of poMHCII α . Lane 1: anti-poMHCII α . Lane 2: anti-6xHis. (B) Spleen. Lane 1: CBB. Lane 2: anti-poMHCII α . Lane 3: anti-poMHCII α pretreated with antigen peptide. Lane 4: anti-poMHCII α pretreated with control peptide. (C) Intraperitoneally infiltrated cells. Lane 1: the soluble proteins reacted with anti-poMHCII α . Lane 2: the membrane proteins reacted with anti-poMHCII α .

specimens using 1% Block Ace, immunostaining was performed with anti-poMHCII α antibodies (0.3 mg/mL in 0.4% Block Ace/PBS), followed by TaKaRa POD Conjugate Anti Rabbit (Takara Bio). Purified Rabbit Polyclonal Isotype Ctrl (BioLegend) was used as a negative control instead of anti-poMHCII α antibodies. The immune reactive cells were visualised by development with 3,3'-diaminobenzidine, followed by counterstaining with haematoxylin and eosin.

The specificity of anti-poMHCII α antibodies was confirmed by an inhibition test using the antigen peptide. Briefly, the antibodies reacted with the antigen peptide on the $\alpha 2$ segment for 30 min were used as a primary antibody. The control peptides mentioned above were also used instead of the antigen peptides in a negative control.

Results

Specificity of rabbit polyclonal antibodies against poMHCII α molecules

The antibodies reacted to recombinant proteins for the $\alpha 2$ segment of class II molecules (Fig. 1A). A single protein was detected in the lysates of spleen and intraperitoneally infiltrated cells by immunoblotting using anti-poMHCII α antibodies (Fig. 1B, 1C). However, no immune reaction was observed using the isotype control (data not shown).

The molecular weight of this protein was estimated to be approximately 28 kDa relative to standard molecular markers. A biotinylated protein from cell membrane of intraperitoneally infiltrated cells was also detected at the position of approximately 28 kDa by immunoblotting using anti-poMHCII α antibodies (Fig. 1C).

The antigen peptides inhibited the binding of anti-poMHCII α antibodies to the target protein (Fig. 1B). However, control peptides failed to inhibit the immune binding between anti-poMHCII α antibodies and the target protein (Fig. 1B).

Tissue distribution of MHC class II-expressing cells

Immunohistochemistry using anti-poMHCII α antibodies detected positive cells in various organs and tissues of Japanese flounder. Inhibition of immune binding when using the antigen peptide, but not when using the control peptide, concealed the positive cells (Fig. 2E, 2F). Immune-positive cells were not observed in the specimens by immunohistochemistry using the isotype control (Fig. 2D, 3–13B).

The immunohistochemical features observed in each organ and tissue are described below based on results from at least three fishes.

Spleen: Positive cells were detected in ellipsoids, splenic cords, around the melanomacrophage centres (MMCs), and a part of the area around central arterioles (Fig. 2A, 2B, 2C). A few cells with a positive

reaction were rarely found in MMCs. The positive cells had elongated or round shapes.

Kidney: Positive cells were detected in the renal interstitium, tubular epithelium, and sinusoids surrounding tubules (Fig. 3A). Some melanomacrophages were positive in MMCs (Fig. 3A). The positive cells had elongated or round shapes.

Thymus: Positive cells were largely detected in the medulla, rather than in the cortex. Many positive cells with elongated or round shapes showed reticular spread in the medulla (Fig. 4A). In the cortex, positive elongated cells were observed surrounding the aggregation of thymocytes. Large cells with round shapes were also found as positive cells in the cortex. Thymocytes showed a negative reaction for anti-poMHCII α antibodies (Fig. 4A).

Intestine: Positive cells were detected in the mucosal epithelium and lamina propria (Fig. 5A). In particular, the positive cells were abundant in a basal part of the mucosal epithelium. These cells had elongated or round shapes.

Stomach: Positive cells with elongated shapes were detected in the mucosal epithelium and a space between gastric glands in the lamina propria (Fig. 6A), similar in location to positive cells in the intestine.

Liver: Positive cells were found embedded among hepatocytes and located adjacent to the hepatic sinusoids, but not in the sinusoids (Fig. 7A). These cells had elongated shapes.

Heart: Positive cells were detected in a space between cardiac myofibrils of the ventricle (Fig. 8A). This space consists of connective tissues (Sanchez-Quintana *et al.*, 1996). The positive cells had elongated or round shapes. Some positive cells were also found in a space between myofibrils of the atrium (data not shown), similar in location to positive cells in the ventricle.

Gills: Positive cells were detected in the gill epithelium and a basal part of secondary lamellae (Fig. 9A). No ILT-like structure could be identified in the specimens of Japanese flounder. The positive cells had elongated shapes.

Skin: The surface of the lower jaw was observed. Positive cells were largely detected in the epidermis, rather than the dermis (Fig. 10A). The positive cells had elongated or round shapes.

Olfactory organs: Positive cells were detected in the mucosal epithelium and lamina propria, and they were prominent in a basal part of the epithelium (Fig. 11A). The positive cells had elongated or round shapes.

Brain: Positive cells with elongated shapes were detected both in the molecular and granular layers of the grey matter (Fig. 12A, 13A). A positive reaction was not found in the brain from one of the three fish examined in this study.

Figs. 2–13. MHC class II-expressing cells in various organs and tissues of Japanese flounder. Immunoreactive cells are brown in colour. Melanin was bleached by pre-treatment with 20% H_2O_2 . Scale bar: 50 μm . **Fig. 2.** Spleen. EP: ellipsoid. SC: splenic cord. MMC: melanomacrophage centre. (A and B) Anti-poMHCII α . Ellipsoid is circled with a broken line. Arrowheads indicate a central arteriole. (C) Anti-poMHCII α . Ellipsoid in high magnification. Arrow indicates sheathed artery. Scale bar: 20 μm . (D) Isotype control. (E) Inhibitory effect of the antigen peptide on immune binding. Haematoxylin counterstain. (F) Result using the control peptide. Haematoxylin counterstain. **Fig. 3.** Kidney. RI: renal interstitium. RT: renal tubule. MMC: melanomacrophage centre. Arrows indicate a sinusoid surrounding tubule. (A) Anti-poMHCII α . (B) Isotype control. **Fig. 4.** Thymus. TM: thymic medulla. TC: thymic cortex. (A) Anti-poMHCII α . (B) Isotype control. **Fig. 5.** Intestine. ME: mucosal epithelium. LP: lamina propria. (A) Anti-poMHCII α . (B) Isotype control. **Fig. 6.** Stomach. ME: mucosal epithelium. LP: lamina propria. (A) Anti-poMHCII α . (B) Isotype control. **Fig. 7.** Liver. Arrows indicate a hepatic sinusoid. (A) Anti-poMHCII α . (B) Isotype control. **Fig. 8.** Ventricle. Arrowheads indicate the cardiac myofibril. (A) Anti-poMHCII α . (B) Isotype control. **Fig. 9.** Gill. Arrows indicate the secondary lamella. (A) Anti-poMHCII α . (B) Isotype control. **Fig. 10.** Skin. Asterisks indicate epidermis. (A) Anti-poMHCII α . (B) Isotype control. **Fig. 11.** Olfactory organ. ME: mucosal epithelium. LP: lamina propria. (A) Anti-poMHCII α . (B) Isotype control. **Fig. 12.** A molecular layer of the grey matter in brain. (A) Anti-poMHCII α . (B) Isotype control. **Fig. 13.** A granular layer of the grey matter in brain. (A) Anti-poMHCII α . (B) Isotype control.

Discussion

We were successful in producing a specific antibody against the α chain of MHC class II molecules of Japanese flounder. The antibody reacted to a protein with a molecular weight of approximately 28 kDa in the soluble proteins from the spleen and intraperitoneally infiltrated cells. The size of the target molecule was slightly larger than the deduced size from an amino acid sequence (BAD13364) of poMHCII α registered in the database. Glycosylation probably affects the molecular size, because Srisapoome *et al.* (2004) suggested the presence of a N-linked glycosylation site in poMHCII α .

The peptide that is an epitope for anti-poMHCII α antibodies blocked the antibodies from binding to the target protein, but another peptide used as a control did not. This result ensured the specificity of the antibody to poMHCII α .

The antibody recognised a membrane protein on the intraperitoneally infiltrated cells in Japanese flounder. MHC class II molecules are generally known as a membrane protein that enables APCs to display peptide antigens (Neefjes *et al.*, 2011). The fact that poMHCII α is a membrane protein raises the possibility that the MHCII in Japanese flounder functions as a molecule responsible for antigen presentation. Functional

analysis of the MHCII molecules is necessary to confirm its role in antigen presentation.

Immune-positive cells were found in various organs and tissues of Japanese flounder by immunohistochemistry using the anti-pMHCII α antibody. Inhibition of the immunoreaction by the antigen peptide was also confirmed in the tissue sections. These observations demonstrated that immunohistochemistry was useful for specifically detecting MHCII-expressing cells in tissues of Japanese flounder. Koppang *et al.* (2003) reported tissue distribution of MHCII-expressing cells in Atlantic salmon by immunohistochemistry using the specific antibody for MHCII β molecules. Positive cells were detected in the reticular matrix of the ellipsoidal sheath and in the white pulp surrounding the ellipsoids of the spleen, the renal interstitium, the thymus medulla and cortex, mucosal epithelium of the stomach and the intestine, interhepatocytes, the primary and secondary lamellae of the gills, and epidermis (Koppang *et al.*, 2003). However, these authors did not include observations on the heart, olfactory organs, and central nervous system. Wittammer *et al.* (2011) generated a zebrafish reporter line expressing green fluorescent protein (GFP) driven by the MHCII β promoter gene. The presence of zebrafish MHCII-expressing cells was indicated in the spleen, the kidney, the thymus, the intestine, the skin, and the brain by analysis of the GFP-reporter line (Wittammer *et al.*, 2011; Ferrero *et al.*, 2018). Tissue distribution of MHCII-expressing cells were equivalent among these fish species including Japanese flounder.

Information on MHCII-expressing cells in the heart and olfactory organs of teleost fish has been unavailable until now. This study revealed that MHCII-expressing cells are distributed in a space between the cardiac myofibrils and mucosal epithelium of olfactory organs in Japanese flounder. Hart and Fabre (1981) reported that MHCII-expressing cells appeared in interstitial connective tissues of the heart in the brown rat *Rattus norvegicus*. These observations suggest that MHCII-expressing cells are present in the heart, and this may be a common feature among animal species. The olfactory organ (olfactory rosette) is located at the bottom of the nasal cavity (Kasumyan, 2004), which consists of ciliated columnar epithelium cells (Menke *et al.*, 2011). LaParta *et al.* (2015) reported that vaccination by the nasal route was effective in rainbow trout and that the immune response, including blood vessel formation and leukocyte infiltration, was elicited in lamina propria of the olfactory organ after vaccination. Tacchi *et al.* (2014) reported that most immune-relevant genes of rainbow trout were expressed at similar levels in the olfactory organ and the head kidney, which is a major lymphoid organ. These reports suggest that the olfactory organ in teleost fish plays a similar role to secondary lymphoid organs in the nasal mucosa of mammals. In mammals, MHCII-expressing cells are distributed in

mucosal epithelium and the upper parts of the lamina propria, and they are responsible for controlling mucosal immunity (Jahnsen *et al.*, 2004; Lee *et al.*, 2015).

In rainbow trout, the expression of MHCII molecules in dendritic cells, macrophages, and B cells has been demonstrated by flow cytometry (Bassity and Clark, 2012; Granja *et al.*, 2015; Castro *et al.*, 2017). In zebrafish, B cells have been identified as MHCII-expressing cells by immunofluorescence staining (Zhu *et al.*, 2014). Another approach using the zebrafish reporter line expressing GFP driven by the MHCII β promoter gene has indicated that dendritic cells, macrophages, B cells, and eosinophils express MHCII molecules (Wittammer *et al.*, 2011). We also need to identify cell types that are expressing MHCII molecules in Japanese flounder, but trials are difficult to conduct at present. Multicolour immunostaining using anti-pMHCII α antibodies cannot be applied because the antibodies fail to react to live cells used for flow cytometry, even though other specific antibodies useful for identifying Japanese flounder leukocytes are available (Matsuyama *et al.*, 2009).

Morphological observations revealed that MHCII-expressing cells in Japanese flounder were elongated and round. Koppang *et al.* (2003) attempted to identify cell types by morphological observation in a single-colour immunohistochemistry and suggested that MHCII-expressing cells may be lymphocytes, macrophages, dendritic cells, and epithelial cells in Atlantic salmon. Because our observations on cell shapes and tissue distribution are similar to those on Atlantic salmon, we suggest that MHCII-expressing cells in Japanese flounder are also similar: elongated cells may be dendritic cells, and round cells may be macrophages or lymphocytes. The positive cells that were abundant in the thymus medulla appear to be thymic epithelial cells. These cells may be responsible for the deletion of autoreactive T cells. The positive cells with elongated shapes detected in the gill epithelium seem to be lamellar epithelial cells. Kato *et al.* (2008) have also found a cell population expressing MHCII molecules in the lectin UEA-1⁺ gill epithelial cells of rainbow trout. These cells were mainly distributed in the epithelium of secondary lamellar. The positive cells that reacted with anti-pMHCII α antibodies were also observed in the brain of Japanese flounder. The cells with elongated shapes are regarded as microglia. Microglia are known as mononuclear phagocyte lineage in the central nervous system (Gehrmann *et al.*, 1995). The microglia expressing MHCII molecules were also found in the brain of zebrafish (Ferrero *et al.*, 2018). Although microglia can express MHCII molecules, the state of expression changes according to physiological and pathological conditions (Sedgwick *et al.*, 1991). In fact, the appearance of MHCII-expressing cells in the brain of Japanese flounder depended on the individual in this

study. In future studies, the identification of MHCII-expressing cells in Japanese flounder will be accomplished through analyses using other specific probes for each cell type and observations of structural cellular components via electron microscopy.

In this study, we did not find any remarkable positive reaction with anti-pMHCII α antibodies in component cells of splenic MMCs. Koppang *et al.* (2003) also indicated the presence of unstained melanomacrophages by immunohistochemistry in Atlantic salmon. Teleost MMCs have been known as sites of antigen retention and are hypothesised to play a role in the humoral adaptive immune response (Steinel and Bolnick, 2017). Vigliano *et al.* (2006) supposed that melanomacrophages are the phylogenetic precursors of follicular dendritic cells (FDCs), which can stimulate B cells in lymphoid follicles from a common profile in a cell surface marker. FDCs also lack the expression of MHCII molecules (Gray *et al.*, 1991) like teleost melanomacrophages. It remains unclear whether teleost melanomacrophages can activate B cells via direct interaction with these cells.

In conclusion, we developed an immunohistochemical method for detecting MHC class II-expressing cells in Japanese flounder. Some of the MHCII-expressing cells must be involved with antigen presentation. Studies on the interaction between APCs and Th cells are now possible by combining detection methods such as immunostaining and *in situ* hybridisation of T cells. This technique will be useful for finding secondary lymphoid organs or tissues that take the place of lymph nodes and Peyer's patches.

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