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Heidi B.T. Huttenhuis · Nicla Romano ·
Carolien N. Van Oosterhoud · Anja J. Taverne-Thiele ·
Lucia Mastrolia · Willem B. Van Muiswinkel ·
Jan H.W.M. Rombout

The ontogeny of mucosal immune cells in common carp (*Cyprinus carpio* L.)

Abstract The ontogeny of carp (*Cyprinus carpio* L.) immune cells was studied in mucosal organs (intestine, gills and skin) using the monoclonal antibodies WCL38 (intraepithelial lymphocytes), WCL15 (monocytes/macrophages) and WCI12 (B cells). In addition, recombination activating gene 1 expression was examined in the intestine with real time quantitative PCR and in situ hybridization to investigate extrathymic generation of lymphocytes. WCL38$^+$ intraepithelial lymphocytes (putative T cells) appeared in the intestine at 3 days post-fertilization (dpf), which is shortly after hatching but before feeding, implying an important function at early age. These lymphoid cells appear in the intestine before the observation of the first thymocytes at 3–4 dpf, and together with the expression of recombination activating gene 1 in the intestine, suggests that similar to mammals at least part of these cells are generated in the intestine. WCL15$^+$ monocytes/macrophages appeared in the lamina propria of the intestine at 7 dpf, but considerably later in the epithelium, while WCI12$^+$ (B) cells appeared in intestine and gills at 6–7 weeks. From these results it can be concluded that putative T cells occur much earlier than B cells, and that B cells appear much later in the mucosae than in other internal lymphoid organs (2 wpf).

Keywords Growth and development · Lymphocytes · Mucosal immunity · Fishes macrophages

H. B.T. Huttenhuis · C. N. Van Oosterhoud ·
A. J. Taverne-Thiele · W. B. Van Muiswinkel ·
J. H.W.M. Rombout (✉)
Cell Biology and Immunology Group, Wageningen Institute
of Animal Sciences, Wageningen University, Marijkeweg 40,
6709 PG, Wageningen, The Netherlands
E-mail: jan.rombout@wur.nl
Tel.: +31-31-7482729
Fax: +31-31-7483955

N. Romano · L. Mastrolia
Department of Environmental Sciences,
Functional Anatomy and Developmental Biology,
Tuscia University, Viterbo, Italy

Introduction

Over the past 15 years, production of farmed fish more than doubled, of which carp species are quantitatively the most important (Vannuccini 2004). A major problem accompanying this vast increase is the occurrence of infectious disease, which causes high losses in cultured fish, especially larvae and juveniles. Vaccination or immuno-stimulation can restrict this problem, although the latter method is rather unprecedented. Vaccines are often administrated via injection, but administration via mucosal surfaces is less stressful and may be a cost-effective and labour-extensive alternative (Lamers 1985; Collado et al. 2000). When applying antigens or immuno-stimulants to mucosal surfaces however, knowledge of the ontogeny of the mucosal immune system is imperative, because fish become immuno-tolerant when vaccinated via the intestine at a very early age (Joosten et al. 1995).

Carp (*Cyprinus carpio* L.) embryos hatch at 2 days post-fertilization (dpf) and start feeding at 5 dpf. Cells (Romano et al. 1998) and humoral factors (Huttenhuis et al. 2005a) of the innate immune system are already present at 2 dpf, while the thymus (the first organ of the adaptive immune system) starts developing from 3 to 4 dpf (Romano et al. 1999; Huttenhuis et al. 2005). Systemic antibody responses appear considerably later at 1–2 months, while juvenile tolerance is evoked before this age (Mughal and Manning 1985; Van Muiswinkel et al. 1985; Mughal et al. 1986). Knowledge on the ontogeny of the mucosal immune system in carp however is limited.

In adult fish, mucosal immune cells are diffusely distributed (Rombout et al. 1989a, b, 1993a, b), and do not form localized accumulations of immune cells (e.g. Peyers Patches) like in mammals. The carp intestine contains many immunoglobulin positive (Ig $^+$) lymphoid cells [recognized with the mAb WCI12 (Koumans-Van Diepen et al. 1995)] and granulocytes in the lamina propria, but also (Ig $^+$) macrophages [recognized with the mAb WCL15 (Weyts et al. 1997; Romano et al. 1998)] and even more Ig$^-$ lymphoid cells in the epithelium (Rombout et al. 1993a, b). Macrophages (Joosten et al. 1995) have been implicated in the uptake of macromolecules in the second segment of the intestine (Rombout et al. 1985). A considerable amount of data indicates that the mucosal immune system is separate from the systemic immune system (Rombout et al. 1986, 1989a, b, 1998). It has been established that mucosal antibodies in adult carp differ from serum antibodies in composition (Rombout et al. 1993a, b) and response after change of route of administration and antigenic composition (Rombout et al. 1989a, b).

The monoclonal antibody (mAb) WCL38 is reactive with an Ig$^-$ subpopulation of carp intraepithelial lymphocytes (IEL), which are present in large numbers in the epithelium of intestine, gills and skin, but not in thymus, head kidney (which is together with the trunk kidney homologue to the bone marrow of mammals regarding haematopoietic function), or peripheral blood leucocytes (PBL) (Rombout et al. 1998). Rainbow trout (*Oncorhynchus mykiss*) IEL exhibited non-specific cytotoxic activity (McMillan and Secombes 1997), but further information on fish IEL is extremely limited. Mammalian IEL are almost exclusively T cells of the T-cell receptor (TCR)$\gamma\delta^+$ or TCR$\alpha\beta^+$ category (Hayday et al. 2001; Cheroutre 2004).

Intraepithelial lymphocytes of tetrapods can originate from the gut epithelium itself instead of from the thymus (Lefrancois and Puddington 1995; Matsunaga and Rahman 2001), although inefficient and mostly skewed to the TCR$\gamma\delta^+$ population (Cheroutre 2004). Since fish appeared before other vertebrates, a considerable number of T cells may generate in the gut, although a thymus is present. Mature T- or B cells have experienced V(D)J recombination of their B cell receptor (BCR) or T cell receptor (TCR) genes. It is convincingly established that this recombination coincides with the expression of recombination activating gene-1 (*rag-1*) and *rag-2* (Schatz et al. 1989; van Gent et al. 1995), and can be performed exclusively by these proteins (McBlane et al. 1995; Hiom and Gellert 1997). Recently, *rag-1* was sequenced in carp (Huttenhuis et al. 2005), enabling us to investigate *rag-1* expression in the carp intestine.

In this paper, the appearance of WCL38$^+$ (IEL), WCI12$^+$ (B cells) and WCL15$^+$ cells (monocytes/macrophages) was studied with flow cytometry, immunohistochemistry and (immuno)electron-microscopy to provide an overview of cell kinetics in the ontogeny of carp mucosal organs. In addition, *rag-1* expression in the intestine was studied using real time quantitative PCR (RQ-PCR) and in situ hybridization, and correlated with the appearance of lymphoid cells.

Animals, materials and methods

Animals

Common carp (*Cyprinus carpio* L.) of the R3R8 strain were bred and kept in the facilities of 'de Haar Vissen', Department of Animal Sciences, Wageningen University (The Netherlands). They were kept at 25°C for the first 5–6 weeks and subsequently at 23°C in circulating, filtered, UV-treated water. The embryos hatch at 2–3 dpf and were fed with *Artemia salina* nauplii starting at 4–5 dpf for 3 weeks followed by Trouvit K30 pellets (Trouw & Co., Putten, The Netherlands). The principles of laboratory animal care (NIH no. 86-23) and the Dutch law on experiments on animals were followed. Fish were anaesthetized with 0.03% tricaine methane sulphonate (Crescent Research Chemicals, Phoenix, AZ, USA) buffered with 0.06% sodium bicarbonate to pH 7.2 in aquarium water.

Monoclonal antibodies

WCI12 (mouse IgG$_1$) is reactive with the IgM heavy chain and subsequently B cells of carp (Koumans-Van Diepen et al. 1995). WCL38 (mouse IgM) reacts with intraepithelial lymphocytes in mucosal organs (putative T cells) (Rombout et al. 1998). WCL15 (mouse IgM) reacts (on tissues) with the cytoplasm of macrophages and monocytes (Weyts et al. 1997; Romano et al. 1998).

Flow cytometry

All steps were performed at 4°C and all washing steps consisted of centrifuging twice at 1,800 rpm (rotations per minute) for 10 min. Between 1 and 10 dpf, whole fish were sampled. From 10 dpf onwards it was possible to separately sample blood, thymus, gills, gut, spleen and head kidney. Blood was collected in RPMI medium after adding 10% water (c-RPMI) with heparin (50 IU/ml) upon cutting the tail until 6 weeks post-fertilization (wpf). In older animals it was sampled from the caudal vein using a syringe containing c-RPMI with heparin. Leucocytes from blood and organs from 3 to 50 individuals (number decreasing from 50 to 3 with age) were pooled in order to obtain enough cells for analysis. The procedure was performed on two offspring. Cell suspensions were prepared in c-RPMI containing 0.1% sodium azide (c-RPMI$^+$) by teasing the tissues through a nylon gauze filter (50 μm mesh). Cell suspensions and blood were washed once in c-RPMI$^+$, layered over a discontinuous gradient of Percoll (Pharmacia AB, Uppsala, Sweden), and diluted in c-RPMI$^+$ to yield densities of 1.060 and 1.070 g/ml. Skin cells (that come

of during the sampling procedure) with a density between 1.020 and 1.060 g/ml mainly contain WCL38$^+$ skin epithelial cells, whereas skin cells with a density between 1.060 and 1.070 g/ml predominantly contain lymphoid cells (Rombout et al. 1998). After centrifugation (30 min at 2,000 rpm) both interfaces were removed and washed once in c-RPMI$^+$ with 1% bovine serum albumin (c-RPMI^{++}). Leucocytes were incubated with WCL38 (1:50) or WCI12 (1:100) for 1 h, washed and incubated with fluorescein-conjugated goat–anti-mouse Ig (GAM-FITC: Dako, Glostrup, Denmark) diluted 1:100 in c-RPMI^{++}. After washing, 10^4 cells were measured with a flow cytometer (FACStar, Becton Dickinson Immunocytometry System, Mountain View, USA) and analysed using the DataMate analysis package. Until 6 wpf peripheral blood leucocytes (PBL) of adult fish were used as positive controls. The percentage of mAb positive cells was calculated by subtracting the percentage of cells labelled with only the secondary antibody (GAM-FITC).

Immunohistochemistry

Immunohistochemistry was performed as described before (Huttenhuis et al. 2005). Note that animals were bled completely before the procedure. Sections to be labelled with WCI12 (1:100) were fixed in 4% paraformaldehyde (PFA) in pH 7.5 phosphate-buffered saline (PBS), whereas sections to be labelled with WCL38 (1:50) or WCL15 (1:50) were fixed in ice-cold acetone for 5 min. Sections labelled with WCI12 were incubated with GAM-FITC (Dako, Glostrup, Denmark), and sections labelled with WCL38 or WCL15 with horseradish peroxydase-labelled goat–anti-mouse immunoglobulins (GAM-HRP, Bio-Rad Laboratories, Hercules, CA, USA). Afterwards, sections labelled with WCI12 were washed twice in PBS, embedded in Vecta-shield mounting medium with propidium-iodide (Vector Laboratories, Burlingame, CA, USA) and examined with a laser-scanning microscope (Carl Zeiss laser scanning system LSM 510). Sections labelled with WCL38 or WCL15 were washed in 0.05 M, pH 5 sodium acetate buffer. 3-Amino-9ethyl-carbazole (AEC: Sigma-Aldrich, St. Louis, MO, USA) in sodium acetate buffer with 0.03% H_2O_2 was added and after staining (2–6 min), rinsed thrice with double distilled water. Sections were stained in haematoxylin (BDH Laboratory Supplies, Poole, England) diluted 1:1 with distilled water for 20 s, rinsed in running tap water, embedded in Kaiser's glycerin gelatin (Merck, Darmstadt, Germany) and examined by light microscopy. Sections treated without first antibody showed no staining.

Whole mount immunohistochemistry

Carp aged 2–10 dpf were fixed in 4% PFA in PBS for 12 h and stored in 1% PFA in PBS at 4°C. Samples were washed thrice in PBS containing 15% sucrose, and incubated in PBS with 0.2% Triton X-100 and 10% foetal calf serum for 8 h at room temperature. After three rinses (10 min) the embryos/larvae were incubated with WCL15 (1:10) for 72 h at 4°C. After three washes, samples were incubated with GAM-FITC (1:50: DAKO) for 16 h at 4°C, rinsed in water and mounted on slides with Vecta-Shield Mounting Medium with propidium-iodide (Vector Laboratories, Burlingame, CA, USA). Samples were examined with a laser-scanning microscope (LSM 510, Carl Zeiss, Germany). Samples treated without first antibody showed no staining.

Electron microscopy

Cell suspensions of 2 and 3 dpf animals were obtained from the 1.060 to 1.070 g/ml interface and labelled with WCL38 as described before (*Flow cytometry*). WCL38-labelled cells were washed in c-RPMI^{++} and incubated (30 min at 4°C) with 25-nm gold-conjugated goat–anti-mouse Ig (Aurion, Wageningen, The Netherlands) diluted 1:5. The cells were washed, suspended in 1 ml of c-RPMI^{++} and centrifuged (1,000g) to yield a compact pellet. The pellet and 3–9 dpf carp larvae were fixed in 1% (w/v) $K_2Cr_2O_7$, 2% (v/v) glutaraldehyde, 1% (w/v) OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h at 4°C, washed in double-distilled water, dehydrated in ethanol and propylene oxide, and embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, PA, USA). Ultra-thin sections were cut on a Reichert Ultracut S (Leica, Rijswijk, The Netherlands), and stained with uranyl acetate and lead citrate. Sections were examined with a Philips 208 electron microscope (Philips, Eindhoven, The Netherlands).

Whole mount in situ hybridization

Intestines of 1 and 2 wpf carp were fixed overnight in 4% PFA in PBS and stored at 4°C in 1% PFA in PBS. Unfortunately, it was not possible to examine intestines of older fish with in situ hybridization, because the background reaction was too high then (Huttenhuis et al. 2005). Whole mount in situ hybridization was performed as described previously with a carp *rag-1* probe (Huttenhuis et al. 2005). In short, samples were incubated with a pre-heated DIG-labelled *rag-1* probe in hybridization buffer at a concentration of 0.05–0.5 ng/μl overnight at 70°C. Subsequently, a number of stringent washing steps were applied with decreasing concentrations of formamide, sodium chloride and sodium citrate containing Tween at 70°C. During the last washing step samples were allowed to cool to room temperature. Then, samples were blocked with 1% blocking reagent and incubated with anti-DIG/alkaline phosphatase (Roche Applied Science, Basel, Switzerland). For the staining reaction, samples were incubated 3 h at room temperature and then overnight at 4°C in the dark with

nitrobluetetrazolium (NBT; 0.09 µl/ml; Roche Applied Science) and 5′-bromo-4′-chloro-3′-indolyl phosphatase (BCIP; 0.035 µl/ml; Roche Applied Science) according to the manufacturers' protocol. Samples were fixed in 4% PFA, washed, frozen, sectioned, embedded in aqua mount improved (BDH Laboratory Supplies, Amsterdam, The Netherlands) and examined (see Immunohistochemistry).

RNA extraction and cDNA synthesis

Intestines from 1 to 30 animals (dependent on size) from 4 dpf up to 0.5 year were pooled (10–1 dependent on age), snap frozen in liquid nitrogen and stored at −80°C. RNA was isolated and cDNA was synthesized as described previously (Huttenhuis et al. 2005). The stages 4–7 dpf and 4–26 wpf were obtained from different offspring.

Real time quantitative polymerase chain reaction (RQ-PCR)

Real time quantitative polymerase chain reaction was performed as described previously (Huttenhuis et al. 2005). In short, cDNA was added to QuantiTect SYBR Green PCR kit (Qiagen, Leusden, The Netherlands), forward and reverse primers and additional water. The PCR protocol was run on a Rotorgene (Corbett, Sydney, Australia) according to the manufacturer. *β-actin* and *40S* were used as house keeping genes and *rag-1* as gene of interest. *R*-values were calculated with Pfaffl's method:

$$E^{\mathrm{GeneOfInterest}^{\hat{}}}\left(\mathrm{Ct}^{\mathrm{reference}} - \mathrm{Ct}^{\mathrm{sample}}\right)/$$
$$E^{\mathrm{HouseKeepingGene}^{\cdot}}\left(\mathrm{Ct}^{\mathrm{reference}} - \mathrm{Ct}^{\mathrm{sample}}\right)$$

where *E* is the efficiency of the primer combination, the Ct value depicts the cycle number when product exceeds a pre-determined threshold, using a sample with insignificant *rag-1* expression as a reference. *R*-values calculated with either *β-actin* or *40S* as house keeping genes were comparable if not identical, consequently, only values calculated with *β-actin* are shown. Non-template controls and non-RT controls were always included and consistently presented high (> 30) Ct values.

Results

Ontogeny of WCL38$^+$ cells

Figure 1a and c show the gate (that was shown previously to contain lymphoid cells; Verburg-Van Kemenade et al. 1994) used to calculate the relative percentage of WCL38$^+$ cells. In whole fish macerates of 4 dpf (panel a), the percentage of gated lymphoid cells was 42.6%. At the same age, a considerable number of WCL38$^+$ cells (panel b; not gated) displayed charac-

teristics of lymphoid cells on the FSC/SSC (forward scatter/sideward scatter) dot blot, while others clearly did not. At 8 wpf however (panel c), the intestine contained a higher percentage of gated lymphoid cells (87.2%). The majority of the WCL38$^+$ cells in the intestine at the same age (panel d; not gated) presented lymphoid characteristics on the FSC/SSC dot blot.

WCL38$^+$ cells first appeared in whole fish macerates at 4–5 dpf using flow cytometry (Fig. 1e). From 10 dpf onwards, WCL38$^+$ cells mainly resided in gut and gills. Positive cells were also present in thymus, spleen, head kidney and PBL, though at a considerably lower frequency.

Using immunohistochemistry (Fig. 2), WCL38$^+$ cells first appeared at 3 dpf in the epithelium of the intestine (panel a), although they were rare and only weakly reactive. From 5 to 6 dpf the number of WCL38$^+$ cells in the intestine (predominantly the epithelium) increased substantially (results not shown), until they were numerous by 10 dpf (panel c). From 7 dpf, WCL38$^+$ were also present in the epithelium of the gills (panel b) and the skin (panel d). Positive cells were rare in other tissues, such as muscle, liver and neural tissue.

Using immuno electron microscopy, lymphoid WCL38$^+$ cells were detected in whole fish macerate cell suspensions already at 3 dpf (Fig. 3). In addition, putative intraepithelial lymphoid cells (based on the almost straight cut from base to apex of the surrounding epithelial cells, and the fact that intestinal epithelial cells divide from the apex and not from the basal layer) were located near the basal layer in the intestinal epithelium at 5 dpf (Fig. 4). IEL sometimes contained a high cytoplasm/nucleus ratio.

Ontogeny of WCL15$^+$ cells in mucosal organs

WCL15$^+$ cells appeared in the lamina propria of the intestine at 7 dpf (results not shown), and were more numerous at 9 dpf (Fig. 5a, b). Numerous WCL15$^+$ cells were also present dorsally to the intestine, in the haematopoietic tissue along the vena cardinalis (Huttenhuis et al. 2005b). Intestinal intraepithelial WCL15$^+$ cells were not present at least up to 2 wpf. WCL15$^+$ cells appeared in the dermis of the skin at 9–10 dpf (Fig. 5c, d), with numerous cells with extensions present at 14 dpf (Fig. 5e).

Ontogeny of WCI12$^+$ cells (B cells)

Using flow cytometry, WCI12$^+$ cells first appeared in head kidney and spleen from 3 wpf, and in PBL from 5 wpf (Fig. 6). WCI12$^+$ cells were present in mucosal organs (gut and gills) from 6 to 7 wpf, and in thymus at 8 wpf. WCI12$^+$ cells were never abundant in thymus and intestine.

Using immunohistochemistry (Fig. 7), WCI12$^+$ appeared in the intestine from 5 wpf onwards (6 wpf is
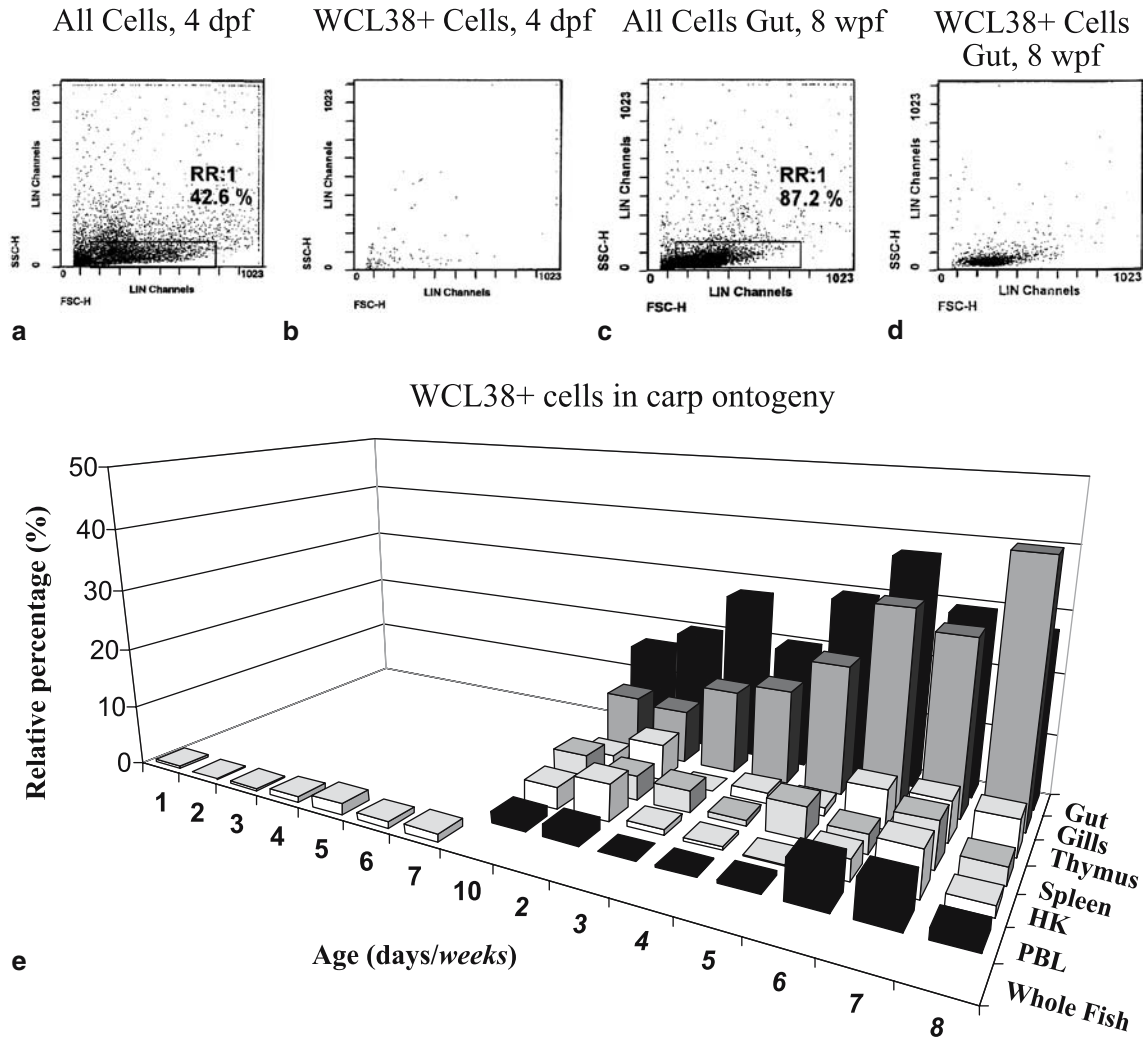
Fig. 1 Forward scatter/sideward scatter (*FSC/SSC*) profiles and relative percentages of WCL38$^+$ cells in distinct organs during carp development determined with flow cytometry. Panels **a**–**d** represent FSC/SSC profiles from the following fractions: whole fish macerate at 4 dpf (**a**, **b**) and intestine at 8 wpf (**c**, **d**) showing all cells (**a**, **c**) and WCL38$^+$ cells only (**b**, **d**). Panels **a** and **c** display the gate used shown in Fig. 7a), in the gills from 6 wpf (Fig. 7b), and in the skin at 3 months (Fig. 7c: at 2 months positive cells were not present). WCI12$^+$ cells in the intestine were located in the lamina propria. Large WCI12$^+$ cells in the epithelium of the second segment, representing Ig$^+$ intraepithelial macrophages, appeared from 6 wpf (results not shown). In the skin, however, positive cells were not present in the dermis but in the epidermis (epithelium), while the dermis reacted diffusely with WCI12.

Rag-1 expression in the intestine

Rag-1 was expressed in carp intestine (Fig. 8). Using RQ-PCR (panel a), expression increased from 4 to 5 dpf in both offspring tested. In juvenile and adult carp

(*RR:1*) with the percentage of gated cells. The percentage of WCL38$^+$ cells relative to all gated lymphoid cells with a density between 1.06 and 1.07 g/ml was measured by flow cytometry in whole fish macerate (*whole fish*), peripheral blood leucocytes (*PBL*), head kidney (*HK*), spleen, thymus, gills and gut of carp (*panel **e***)

(4 and 26 wpf), *rag-1* expression was lower than in larvae from 5 and 7 dpf.

Using in situ hybridization (panel b), *rag-1*$^+$ cells were present in the intestine at 1 and 2 wpf. Positive cells were located in the lamina propria (panel b), but it cannot be excluded that some positive cells were present in the epithelium (results not shown). The number of positive cells was very low (approximately 1 cell per 20 sections).

Discussion

This study for the first time describes the development of the mucosal immune system of fish. WCL38$^+$ IEL first appeared in the intestine at 3 dpf, which is after hatching but before feeding, while their numbers increased

Fig. 2 Immunohistochemistry using WCL38 on carp tissue sections. WCL38$^+$ cells first appeared in the intestine at 3 dpf (**a**), and in the gills (**b**) and skin (**d**) at 7 dpf (*arrows*). The number of positive cells in the intestine had increased considerably by 10 dpf (*panel* **c**). The cells with the intensely black granules (*panels* **a** *and* **b**) are melanocytes. *Ca* cartilage of large gill lamella, *Fi* small gill filament. *Arrows* point to WCL38$^+$ cells. *Bar* is 20 μm
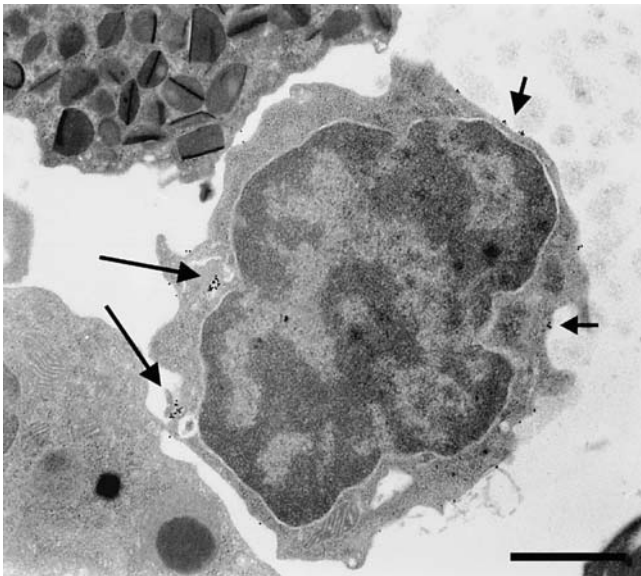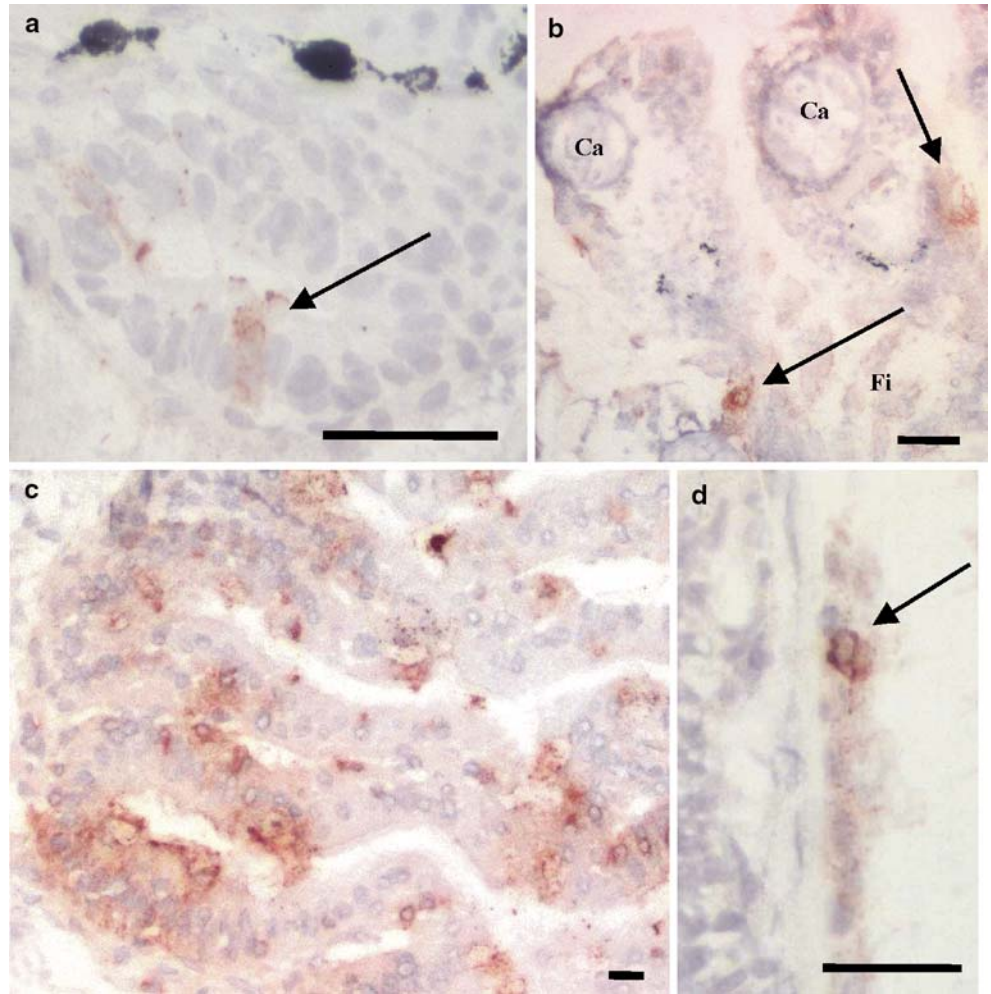

Fig. 3 Electron microscopy picture of a WCL38$^+$ lymphoid cell from a 3-dpf whole fish macerate cell suspension. *Arrows* point to gold particles representing WCL38 immunoreactive molecules. Note the WCL38$^-$ neutrophilic granulocyte (*top*) and basophilic granulocyte (*left bottom*). *Bar* is 1 μm

substantially at the time of first food ingestion (5 dpf). The distribution and abundance of these cells in carp larvae is very similar to that in adult animals (Rombout et al. 1998), implying that WCL38$^+$ IEL play a significant role in mucosal organs already early in development. According to a previous study (Rombout et al. 1998), WCL38$^+$ cells are not B cells or natural killer cells. Knowledge on the IEL function in teleost fish is unfortunately very limited, although non-specific cytotoxic activity was observed in rainbow trout (McMillan and Secombes 1997). In carp, WCL38$^+$ cells exhibit no specific or non-specific cytotoxic responses (Rombout et al., unpublished results 2005). Mammalian IEL are T cells that are implicated in the conventional role of mounting specific immune responses, but also in the preservation of the mucosal epithelial layer (Hayday et al. 2001), gut metabolism (Fahrer et al. 2001), and the prevention of undesirable immune responses (Cheroutre 2004). TCR$\alpha\beta^+$ IEL bear the hallmarks of conventional T cells, whereas TCR$\gamma\delta^+$ IEL and TCR$\alpha\beta^+$CD8$\alpha\alpha$ IEL also have characteristics of innate immune cells: they respond to antigens not restricted by the conventional major histocompatibility complex (Hayday et al. 2001) and recognize conserved antigens (Boismenu and
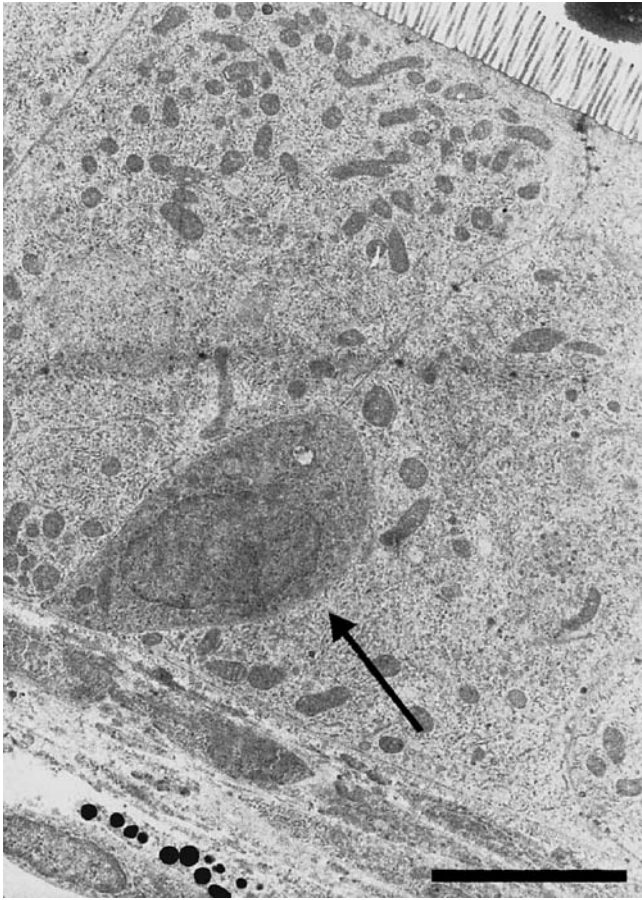
Fig. 4 Electron microscopy picture of a putative intraepithelial lymphocyte (*arrow*) in the intestine at 5 dpf. *Bar* is 5 μm

Havran 1997; Matsunaga 1998). Consequently, the early appearance of WCL38$^+$ cells, which are similar to mammalian IEL regarding location and morphology, suggests the presence of T cells with at this age most probably innate characteristics, although their function remains to be investigated.

WCL38$^+$ cells appeared before the development of a cortex and medulla in the thymus (Huttenhuis et al. 2005; Romano et al. 1999), and also before the onset of *rag-1* expression in the thymus (Huttenhuis et al. 2005). Assuming WCL38$^+$ IEL are T cells, these data strongly suggest that at least part of these cells originate from the intestine itself. This is corroborated by the expression of *rag-1* in the intestine around 1 wpf, when the number of WCL38$^+$ cells is increasing considerably. However, the level of expression and the number of *rag-1* positive cells is very low compared to the thymus or even kidney (Huttenhuis et al. 2005), and the number of WCL38$^+$ cells only increases substantially after the appearance of the thymus, suggesting the thymus is still the main provider of these cells. This is consistent with data obtained in mammals, in which *rag-1* expression in IEL suspensions was considerably lower than in thymic cells (Guy-Grand et al. 1992), and a large amount of evidence shows a thymic origin of all or most IEL (Lefrancois and Puddington 1995; Cheroutre 2004). The limited *rag-1*

expression in carp can also be explained by the fact that part of the WCL38$^+$ IEL originate from the thymus but via a short partway without negative selection as is suggested for mammals (Cheroutre 2004), or by clonal expansion of a limited number of differentiated IEL.

IEL that are generated in the mammalian gut are mostly the TCR$\gamma\delta^+$ population (Cheroutre 2004), and this subpopulation is also the most abundant in young mammals (Hayday 2000). With respect to these data it is interesting that γ and δ TCR genes were recently identified in teleost fish (Nam et al. 2003). TCR$\gamma\delta^+$ cells are suggested to have evolved before $\alpha\beta$TCR$^+$ cells and to represent the first step in the development of adaptive immunity (Matsunaga 1998).

Rag-1 expression was located in the lamina propria epithelium of the intestine, but it cannot be excluded that *rag-1* positive cells were also present in the epithelium. The first location is consistent with data in mammals where cryptopatches in the lamina propria are associated with the generation of IEL (Matsunaga 1998; Cheroutre 2004). In contrast to our data, *rag-1* was abundantly expressed in both intestinal epithelium and lamina propria in zebrafish (cyprinidae) (Danilova and Steiner 2002), suggesting that the extent of extrathymic T cell production might differ among fish species.

As WCL38$^+$ cells are present in the intestine before the appearance of the thymus, they possibly are the progeny of precursors from tissue homologue to foetal liver or bone marrow in mammals (Hayday et al. 2001). Progenitors of WCL38$^+$ cells tentatively originate from the intermediate cell mass, a tissue described in zebrafish (Al-Adhami and Kunz 1977; Willett et al. 1999) and carp embryos (Huttenhuis et al. 2005b), which expresses many genes implicated in haematopoiesis including *i-karos* (Willett et al. 2001), a transcription factor specifically of the lymphoid lineage. The intermediate cell mass transforms into the 'dorsal aorta' in zebrafish (Willett et al. 1999), which is the equivalent of the aorta–gonad–mesonephros region, the first site of definitive haematopoiesis in tetrapods.

WCL15$^+$ cells (monocytes/macrophages) first appeared in the lamina propria of the intestine at 7 dpf, which is about the same age as in other studies (Romano et al. 1998), while intraepithelial macrophages (IEM) appear considerably later around 4 wpf (Joosten et al. 1995). These intraepithelial macrophages have been shown to ingest antigens, and are proposed to have a role in antigen presentation (Rombout et al. 1989a, b; Joosten et al. 1995). Ig$^+$ IEM are first detectable at 6 wpf, which corresponds to the appearance of plasma-cells at 4 wpf (Koumans-Van Diepen et al. 1994). The delayed appearance of IEM indicates an immature mucosal immune system in juvenile carp. WCL15$^+$ cells were also present in the dermis of the skin. The long extensions and network formation are similar to mammalian Langerhans cells (antigen presenting cells), although these cells are present in the epidermis of the skin. Whether these cells also function as Langerhans cells remains to be investigated.
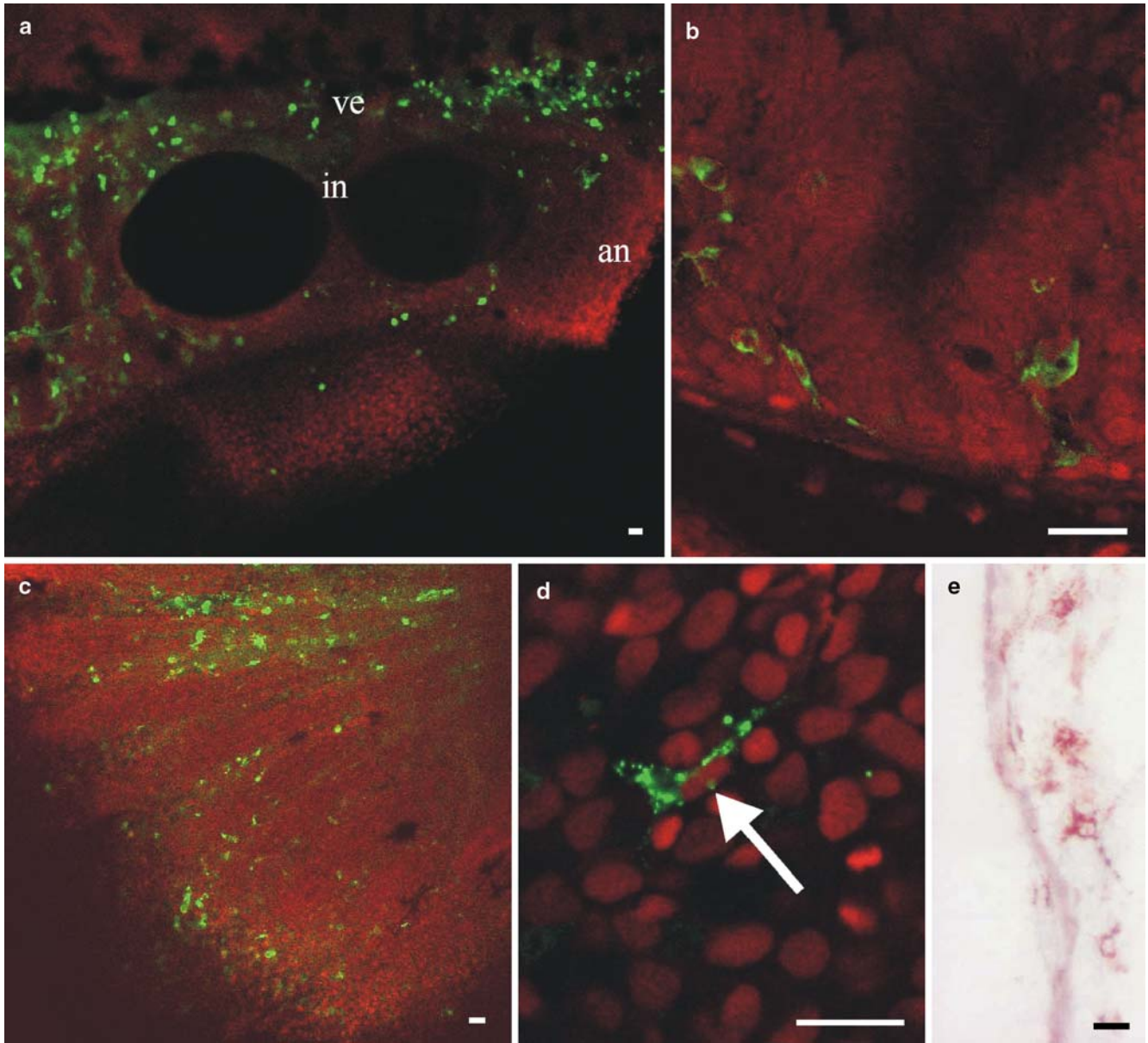
Fig. 5 Monocytes/macrophages (WCL15$^+$ cells) detected with immunohistochemistry in mucosal organs during the first 2 weeks of carp development. WCL15$^+$ cells are *green fluorescent* and tissue is *red fluorescent* after treatment with propidium-iodide (*panels* **a–d**), or WCL15$^+$ cells are *reddish-brown* and tissue is *purple* after counter staining with diluted haemalum (*panel* **e**). WCL15$^+$ cells were present in intestine (**a–b**), and skin from 9 dpf. Panels **c–d** and **e** represent the skin at 10 and 14 dpf ,respectively. In panel **a** *Artemia* eggshells are present as non-fluorescent circles. Panel **c** represents the skin in the tail of the larva, showing the abundance of WCL15$^+$ cells. The *arrow* in panel **d** points to the nucleus of the WCL15$^+$ cell. *Ve* blood vessel, *in* intestine, *an* anus. *Bar* is 20 μm

WCI12$^+$ cells (B cells) first appeared in head kidney and spleen and then in PBL, which is consistent with earlier studies (Koumans-Van Diepen et al. 1994; Romano et al. 1997), and suggests that B cells, like in mammals home to the spleen for maturation. Subsequently, B cells were situated in intestine and gills at 5–6 wpf, suggesting B cells are now mature because this is also the first appearance of B cells outside kidney and spleen. The 'lag' period between appearance of B cells in head kidney and spleen compared to mucosal organs corresponds with data obtained for sea bass (*Dicentrarchus labrax*; Picchietti et al. 1997)

and spotted wolffish (*Anarhichas minor*; Grontvedt and Espelid 2003). The appearance of 'mature' B cells in peripheral organs (intestine and gills) at 5–6 wpf corresponds to the appearance of plasma cells (Koumans-Van Diepen et al. 1994), and the mounting of antibody responses against *Aeromonas salmonicida* (Mughal and Manning 1985). B cells appeared in the skin considerably later than in intestine, which is consistent with data in spotted wolffish (Grontvedt and Espelid 2003). In contrast to intestine, B cells are situated in the epithelium (epidermis), similar to spotted wolffish (Grontvedt and Espelid 2003), with

Fig. 6 Relative percentages of WCI12$^+$ cells in distinct organs during carp development determined with flow cytometry. The percentage of WCI12$^+$ cells relative to all gated lymphoid cells with a density between 1.06 and 1.07 g/ml was measured by flow cytometry in whole fish macerate (*whole fish*), thymus, gut, gills, peripheral blood leucocytes (*PBL*), spleen and head kidney (*HK*) of carp. The gate used is shown in Fig. 1 (RR:1)
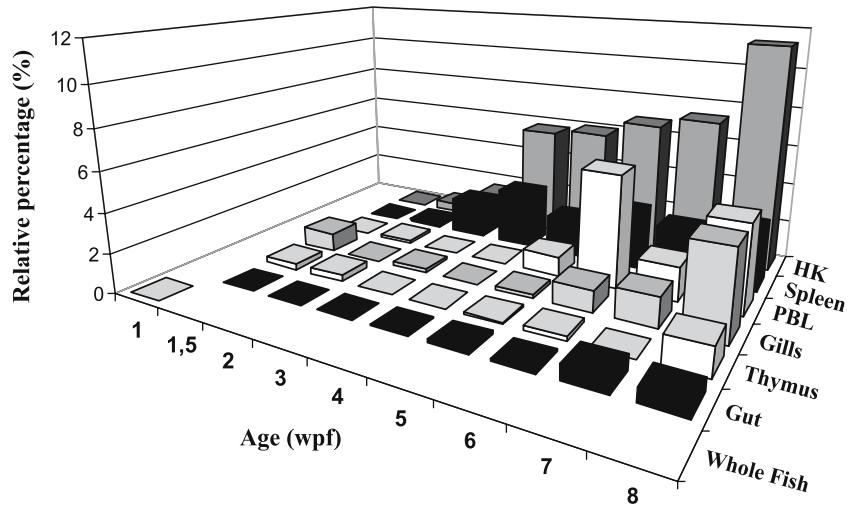
the dermis reacting diffusely with WCI12 like in adult carp (Rombout et al. 1998).

The induction of tolerance in juvenile carp after oral vaccination with *Vibrio anguillarum* up to 4 wpf and the development of an increased antibody response at 2 months (Joosten et al. 1995), corresponds to the appearance of Ig$^+$ intraepithelial macrophages and B cells, but not to the earlier presence of WCL38$^+$ IEL or Ig$^-$ intraepithelial macrophages (Joosten et al. 1995). Consequently, the ability to mount a systemic antibody response against oral vaccines is correlated to the presence of Ig$^+$ IEM and B cells but not to WCL38$^+$ IEL.

In conclusion, this study provides the first description of IEL ontogeny in carp, and suggests that these cells are partly produced in the intestine besides the thymus because of their early appearance (before the thymus) and concurrent *rag-1* expression in the intestine. Furthermore, monocytes/macrophages and especially B cells appeared later in development, suggesting particularly acquired mucosal immunity is not functional up to

Fig. 7 B cells (WCI12$^+$ cells) detected with immunohistochemistry in mucosal organs during the first weeks of carp development. WCI12$^+$ cells are green fluorescent and tissue is red fluorescent after treatment with propidium-iodide. Positive cells were present in the lamina propria of the intestine (**a**) and in gills (**b**) at 6 wpf, and in the epithelium of the skin at 3 months (**c**). *De* dermis, *Ed* epidermis, *ep* epithelium, *Fi* small gill filament, *Lf* large gill filament, *lp* lamina propria. *Arrows* point to positive cells. *Bar* is 100 μm
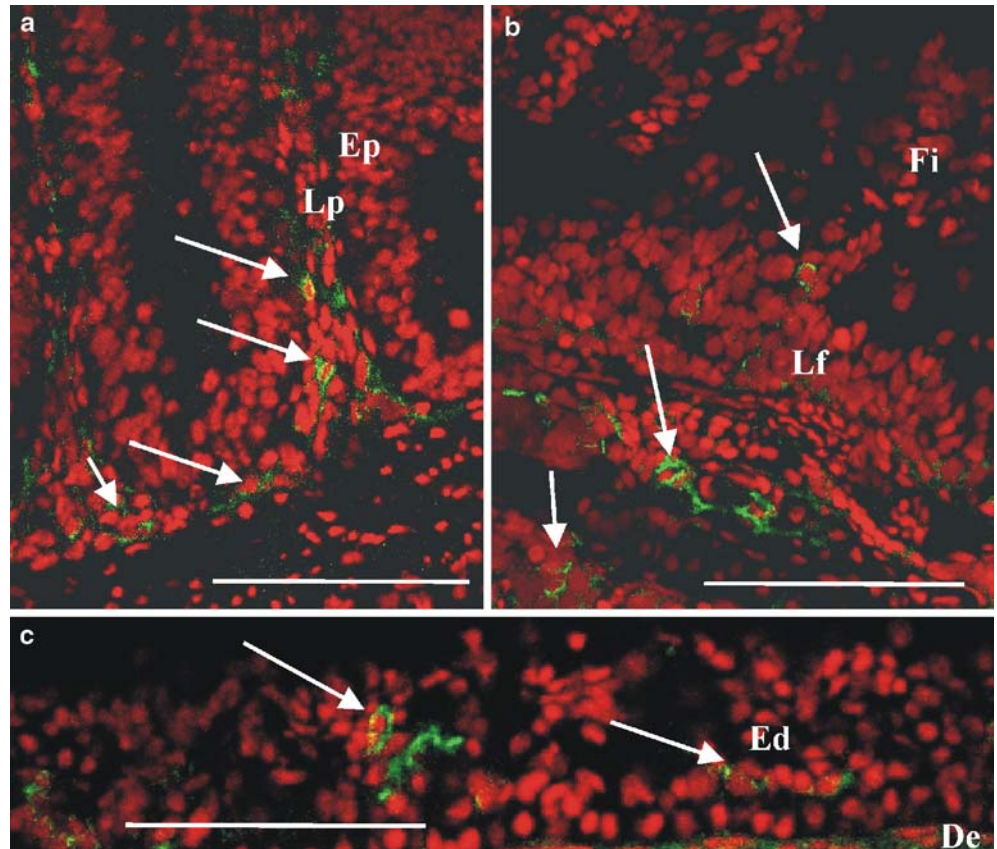
Fig. 8 *Rag-1* expression in carp intestine. Panel **a** displays *rag-1* expression detected with RQ-PCR in two different offspring (depicted with *white and black bars*) from 4 dpf up to 26 weeks. All data were normalized against *β-actin* levels and compared to a sample with insignificant *rag-1* expression (muscle of adult carp) with Pfaffl's method: *R*-values are displayed on the vertical axis. On the horizontal axis age is displayed in dpf (*d*) or wpf (*w*). Panel **b** shows *rag-1* positive cells (closed *arrows*; with in situ hybridization) in the intestine at 1 wpf. The *open arrow* points to a pigment cell. *Bar* is 50 μm
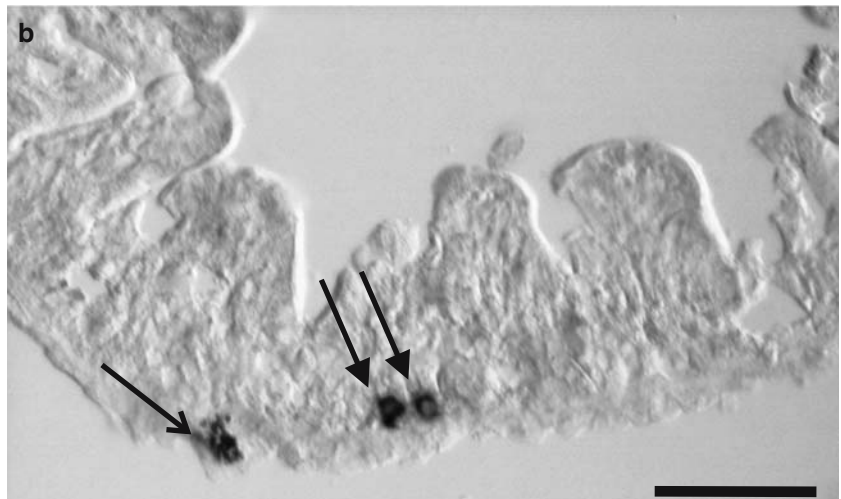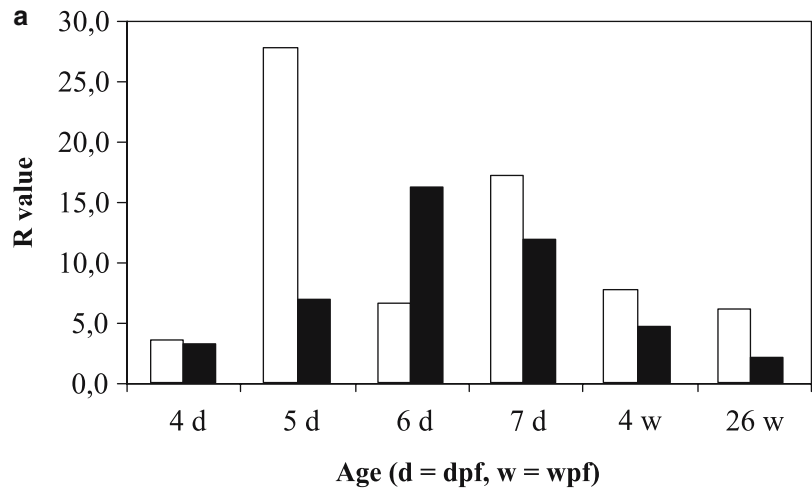
6–7 wpf, which is much later than the development of the systemic humoral immune system (2–3 wpf).

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