Developmental and Comparative Immunology 61 (2016) 48–59

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Localization and developmental expression of two chicken host defense peptides: cathelicidin-2 and avian β-defensin 9

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ARTICLE INFO

Article history:
Received 19 January 2016
Received in revised form
9 March 2016
Accepted 9 March 2016
Available online 10 March 2016

Keywords:
Chicken
Host defense peptides
Development
Innate immunity
Cathelicidin
Defensin

ABSTRACT

In the first weeks of life young chickens are highly susceptible to infectious diseases due to immaturity of the immune system. Little is known about the expression of host defense peptides (HDPs) during this period. In this study we examined the expression pattern of two chicken HDPs, the cathelicidin CATH-2 and the β-defensin AvBD9 by immunohistochemistry in a set of organs from embryonic day 12 until four weeks posthatch. AvBD9 was predominantly found in enteroendocrine cells throughout the intestine, the first report of in vivo HDP expression in this cell type, and showed stable expression levels during development. CATH-2 was exclusively found in heterophils which decreased after hatch in most of the examined organs including spleen, bursa and small intestine. In the lung CATH-2 expression was biphasic and peaked at the first day posthatch. In short, CATH-2 and AvBD9 appear to be expressed in cell types strategically located to respond to infectious stimuli, suggesting these peptides play a role in embryonic and early posthatch defense.

1. Introduction

During the neonatal period chickens are highly susceptible to infectious diseases that cause relatively few problems later in the life of the animals. Chickens younger than 2 weeks often develop clinically apparent disease and severe symptoms when infected with *Escherichia coli* or *Salmonella* (Gast and Beard, 1989; Goren, 1978; Johnson et al., 2001). This susceptibility is reflected in high antibiotic usage in broilers during the first week of life (GD Animal Health, 2014). When chicks hatch, their immune system is immature and marked development occurs over the first weeks of life. During this period partial protection is offered by maternal antibodies, transferred to chicks via the yolk. Most of these antibodies are depleted by 10 days posthatch (Gharaibeh and Mahmoud, 2013). Though neonatal and even embryonic chicks are able to mount humoral responses of their own, these are generally very low. Antibody titers against orally or intramuscularly administered BSA were higher in vaccinated animals older than 10 days compared to animals vaccinated in the first week posthatch (Bar-Shira et al., 2003; Mast and Goddeeris, 1999). Additionally, at hatch the gut-associated lymphoid tissue is only poorly developed. The numbers of lymphocytes in the intestinal wall are low at hatch, but increase rapidly during the first week (Bar-Shira et al., 2003). Germinal centers in the cecal tonsils are not identified until two weeks posthatch (Gomez Del Moral et al., 1998). Development of the innate immune system in neonatal chicks is less well described, though heterophils of 1 day old chicks show lower phagocytic and bactericidal capacities compared to cells of animals of 4 and 7 days old (Wells et al., 1998).

Host defense peptides (HDPs) are important multifunctional effector molecules of the innate immune system. Two of the main classes of HDPs are cathelicidins and defensins. In mammals, these peptides are believed to be very important in neonatal defense (Kai-Larsen et al., 2014). Expression of the human cathelicidin LL-37 and its murine counterpart CRAMP is higher in neonatal compared to adult skin (Dorschner et al., 2003). In contrast, the expression of intestinal α-defensins is lower in newborns, possibly predisposing neonates to intestinal infections such as necrotizing enterocolitis (Mallow et al., 1996). In the chicken, four cathelicidins and 14 β-defensins have been described. Expression analysis of chicken HDPs showed clear differences over the course of development. Cathelicidin-1, -2 and -3 showed a strongly increased expression in

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http://dx.doi.org/10.1016/j.dci.2016.03.008

the cecum and tonsils at 4 weeks posthatch (Achanta et al., 2012). Intestinal expression of the β-defensins 1, 2, 4 and 6 decreased in the first week posthatch, but increased again in the second week (Bar-Shira and Friedman, 2006; Crhanova et al., 2011). However, this research was solely based on mRNA levels and did not take into account the cell types expressing these peptides and possible changes herein during development.

In this work we aimed to elucidate the cellular localization and developmental expression pattern of two chicken HDPs, cathelicidin-2 (CATH-2) and β-defensin 9 (AvBD9, previously also named GAL6) at the protein level, hereby increasing the knowledge about the role of HDPs during chicken development.

2. Materials and methods

2.1. Animals and tissue sampling

This animal experiment was conducted in accordance with a protocol approved by the Dutch Animal Experimental Licensing Committee (DEC). Twelve-day incubated Ross 308 broiler eggs were obtained from a commercial hatchery (Lagerwey, Lunteren, The Netherlands) and further incubated at the research facility of the Department of Farm Animal Health (Utrecht University). After hatch, chickens were housed in a ground stable under controlled hygienic conditions. Chickens were fed a commercial broiler diet without antibiotics or coccidostats and were given access to water and food *ad libitum*. At multiple embryonic (ed12, 14, 16, 18, 20) and posthatch (d1, 4, 7, 14, 21, 28) timepoints, eggs/chickens (n = 4/5) were chosen at random for sample collection. Animals were euthanized by cervical dislocation until posthatch day 7 and by electrocution and bleeding at later timepoints.

Organs in contact with the external environment (intestine, lung, skin) and a selection of lymphoid organs (spleen, bursa) were chosen for analysis. Additional organs (yolk, pancreas, thymus, kidney) were also sampled, but only perfunctory analysis on the absence or presence of CATH-2 and AvBD9 expression was performed for this study. During the experiment no symptoms of disease were noticed in live birds or at post-mortem. Birds were free of infectious bronchitis virus based on serology at d28.

2.2. Antibodies

Rabbit polyclonal antibodies against CATH-2 and AvBD9 were generated at Biogenes (Berlin, Germany). CATH-2 antibody production was previously described (van Dijk et al., 2009a). For the production of AvBD9 antibody, two short peptides derived from the AvBD9 sequence (CSFVASRAPSVD and LASRQSHGSC) were conjugated to *Limulus polyphemus* hemocyanin (LPH) at the cysteine residue and the mixture of peptides was used to immunize two rabbits. Antiserum was collected from the immunized animals and purified by affinity chromatography on CNBr-Sepharose columns coated with the peptides. Monospecific IgG was eluted from the columns with 0.2 M Glycine-HCl buffer (250 mM NaCl, pH 2.2), neutralized with 1 M Tris-HCl (pH 7.5) and centrifuged to remove remaining debris. Antibodies were aliquoted and stored at −80 °C. For the staining of enteroendocrine cell (EEC) products, commercial rabbit antibodies were used which had previously been shown to work on chicken tissue samples: anti-glucagon-like peptide 1 (GLP-1, GA1176, Enzo, Farmingdale, USA), anti-gastric inhibitory peptide (GIP, T-4340, Peninsula Laboratories, San Carlos, USA) and anti-serotonin (5-HT, 20080, Immunostar, Hudson, USA). Antibodies were used in the following dilutions: 1:50 for anti-AvBD9, 1:100 for anti-CATH-2 and anti-5-HT, 1:200 for anti-GLP-1 and 1:500 for anti-GIP.

2.3. Tissue processing and sectioning

Tissue samples were fixed in 4% paraformaldehyde (w/v) in phosphate buffer (pH 7.2) for 24 h and subsequently paraffin-embedded. Sections (5 μm for single or sequential staining or 2 μm for serial sections) were mounted on glass slides, deparaffinized and rehydrated.

2.4. Immunohistochemistry

For antigen retrieval, sections were boiled in citrate buffer (pH 6.0) for 10 min (AvBD9, GLP-1 and GIP staining). Endogenous peroxidase activity was blocked by incubating the sections for 30 min in 1% H_2O_2 in methanol. Subsequently, sections were blocked with 10% normal goat serum and 2.5% BSA for 1 h before incubation with the primary antibody (see section 2.2). Incubation times of the primary antibodies were as follows: CATH-2, 1 h; AvBD9, GLP-1, GIP and 5-HT overnight (16–20 h). Immunostaining with rabbit serum or in absence of the primary antibody served as the negative control. Sections for CATH-2 staining were then incubated with the Horse Radish Peroxidase labelled anti-rabbit polymer from the EnVision + system (Dako, Glosstrup, Denmark). All other sections were incubated with a biotinylated goat-anti-rabbit antibody (1:250, Vector Laboratories, Burlingame, USA) for 30 min followed by incubation with ABC reagent (Vector Laboratories). Staining was visualized by incubating the sections with diaminobenzidine (DAB) for 5–10 min. Finally, sections were counterstained with hematoxylin, dehydrated and mounted with Pertex.

2.5. Sequential stain with HE or Giemsa

Sections were sequentially stained with hematoxylin and eosin (HE) followed by anti-AvBD9 or Giemsa followed by anti-CATH-2 to elucidate morphology of AvBD9 and CATH-2 expressing cells. Routine HE staining was performed in a Leica Autostainer XL (Son, The Netherlands). For Giemsa stain, tissue sections were incubated for 1 h in 20% Giemsa's Azure Eosin Methylene Blue solution (Merck, Darmstadt, Germany) and differentiated in diluted acetic acid for 15 min. Subsequently, differentiation was stopped by dipping sections in ethanol 96% followed by incubation in aceton. Photographs of distinctive cells were taken and subsequently sections were incubated overnight in fresh xylene to remove the cover slips. Sections were then destained by incubation in acidified ethanol (27 ml HCl 37% and 973 ml ethanol 70%) for 5 min. After rehydration, sections were stained with antibodies against CATH-2 or AvBD9 as described above (section 2.4).

2.6. Silver staining

Fontana-Masson silver stain was performed on gastro-intestinal tissue sections as previously described (Grimelius, 2004). Briefly, sections were incubated for 1 h in prewarmed ammoniacal silver solution (5% $AgNO_3$ with NH_4OH) and rinsed in Milli-Q water. Sections were counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, USA), dehydrated and mounted with Vecta-Mount (Vector Laboratories). Silver stained sections were compared with serially cut sections stained with antibody against AvBD9.

2.7. Quantitative analysis

Five to ten random photographs were made of each tissue section using an Olympus BX51 microscope with movable table at 400x magnification (Field size 0.09 μm^2). Images were analyzed

using cellSens Dimension software (Olympus, Tokyo, Japan) to obtain stained area percentages and stained cell numbers. To account for the variation in empty space in the field of view, a region including tissue only was manually created. Cell numbers

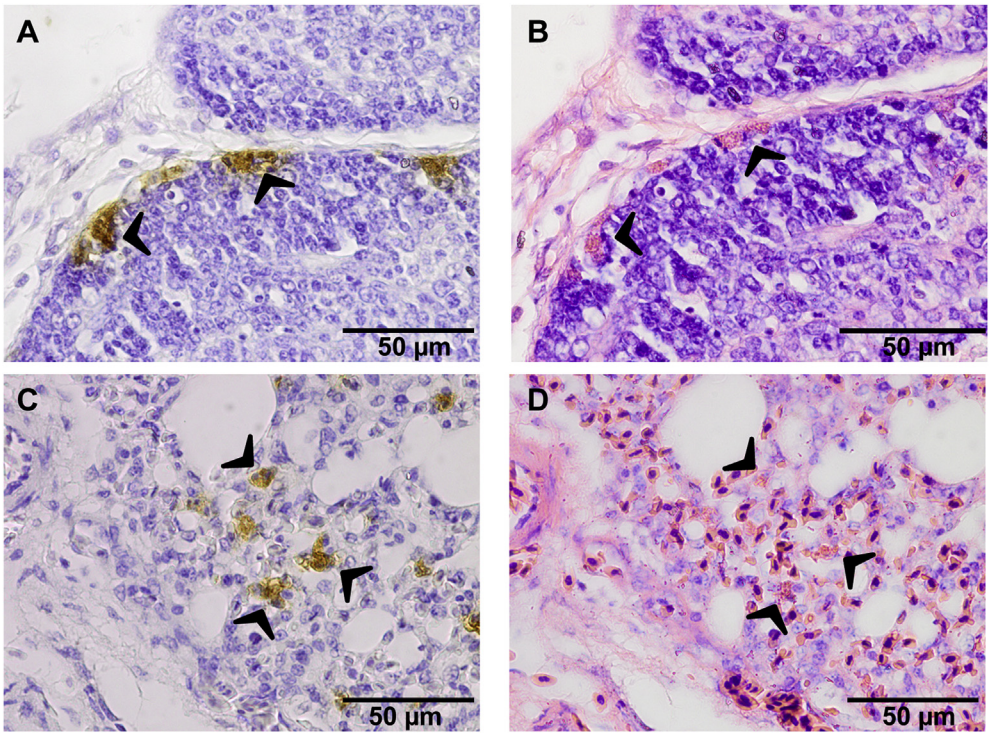

Fig. 1. Identification of CATH-2 expressing cells as heterophils via sequential stain of sections with CATH-2 antibody (A,C) and Giemsa (B,D). Lung (A,B) and bursa (C,D) at d28. Arrows denote examples of CATH-2 expressing cells overlapping with cells identified as heterophils.

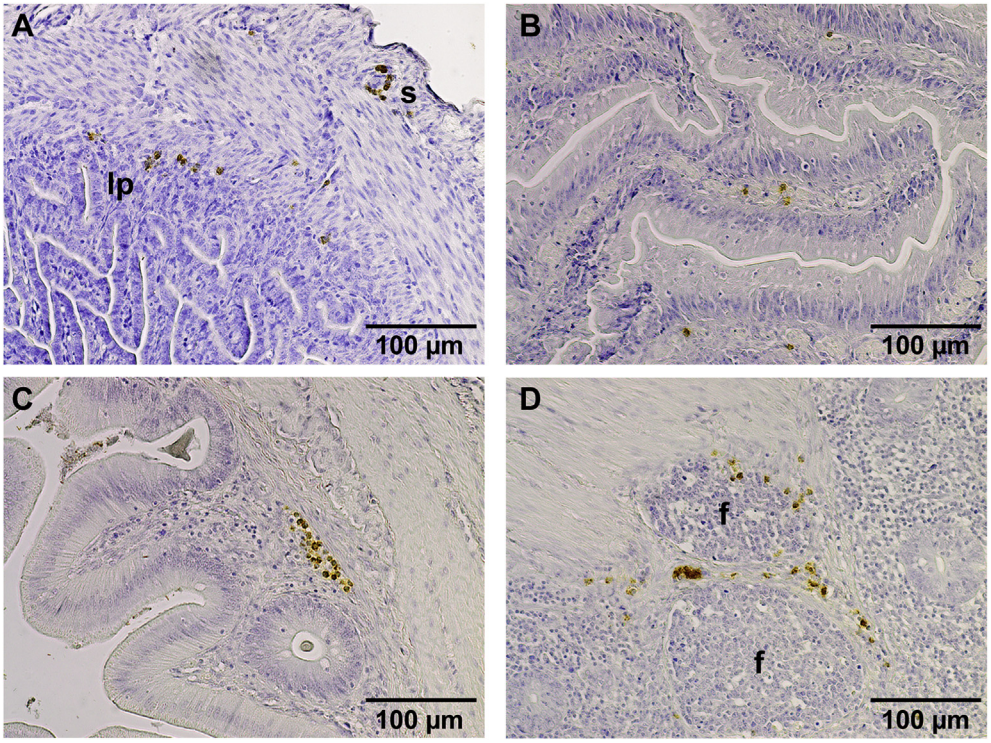

Fig. 2. Localization of CATH-2 expressing heterophils in the gastro-intestinal tract during development. A: duodenum, ed18, B: duodenum, d28, C: cecum, d28, D: tonsil, d28. s: serosa, lp: lamina propria, f: lymphoid follicle.

could not be obtained for CATH-2 stained sections because of the clustering of cells. The result of stained area percentage or stained cell numbers for each tissue sample was calculated by taking the average of the individual 5–10 image values.

2.8. Statistics

Statistical analysis was performed using SPSS 22 software (IBM, Armonk, USA). Differences between timepoints were evaluated

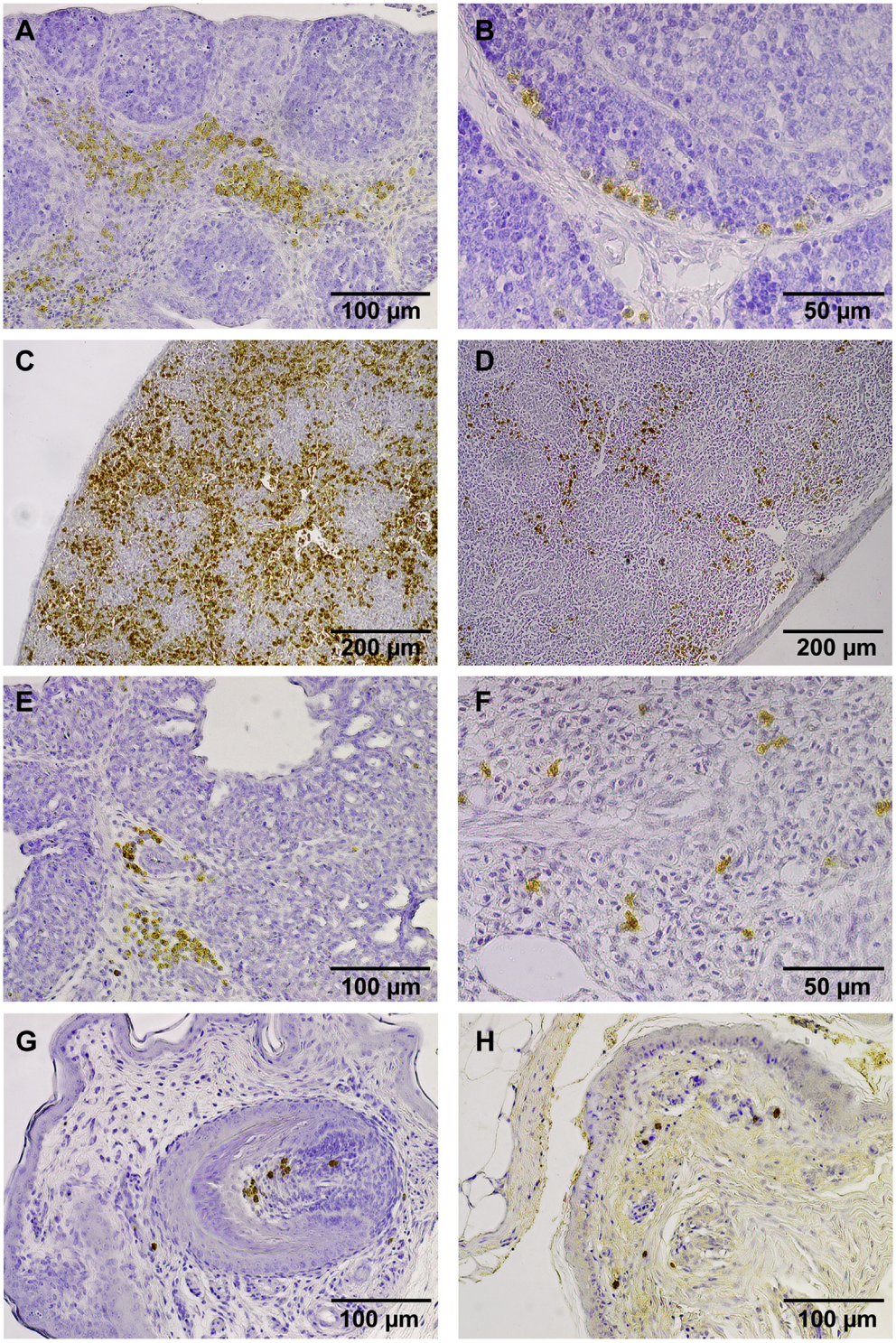

Fig. 3. Localization of CATH-2 expressing heterophils in multiple organs during development. Bursa (A: ed18, B: d21), spleen (C: ed18, D: d21), lung (E: ed16, F: d21), and skin (G: ed18, H: d21).

using a one-way analysis of variance (ANOVA) or non-parametric Kruskal-Wallis test when data were non-normally distributed. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Localization and identity of CATH-2 expressing cells and developmental stage of heterophils

CATH-2 expressing cells were identified as heterophils based on sequential staining with Giemsa (Examples of sequential staining in bursa and lung shown in Fig. 1). All heterophils (mature and developing) observed in this study expressed CATH-2. In all analyzed tissues and in blood smears CATH-2 was never seen in other cell types. CATH-2 expressing cells were observed from the earliest timepoint (ed12) onwards in every organ investigated with considerable variation in the numbers of CATH-2 expressing cells between different organs.

In the gastro-intestinal tract, CATH-2 expressing heterophils were sparsely present as single cells in the lamina propria of the intestinal villi or in small groups at the base of the villi (Fig. 2A–C), but hardly ever in the epithelial layer. In embryonic (Fig. 2A) or neonatal samples (d1, d4, data not shown), CATH-2 expressing cells were also found in the serosa. In the lymphoid tissue of the cecal tonsils, CATH-2 expressing heterophils were quite numerous around the developing lymphoid follicles (Fig. 2D).

In the bursa and spleen, CATH-2 expressing cells were very numerous during embryonic and neonatal (d1) timepoints. Clusters of these cells filled the bursal mesenchyme between the developing lymphoid follicles (Fig. 3A) and the red pulp of the spleen (Fig. 3C). From 1 week posthatch onwards, very few CATH-2 expressing cells remained in the bursa and were seen only at the edge of some follicles in small groups, but never in the follicular medulla (Fig. 3B). In the spleen, CATH-2 expressing heterophils remained present in relatively large numbers until the last timepoint (d28) scattered throughout the red pulp (Fig. 3D). Throughout embryonic development, clusters of CATH-2 expressing heterophils similar to those observed in the bursa and spleen were also seen in the yolk,

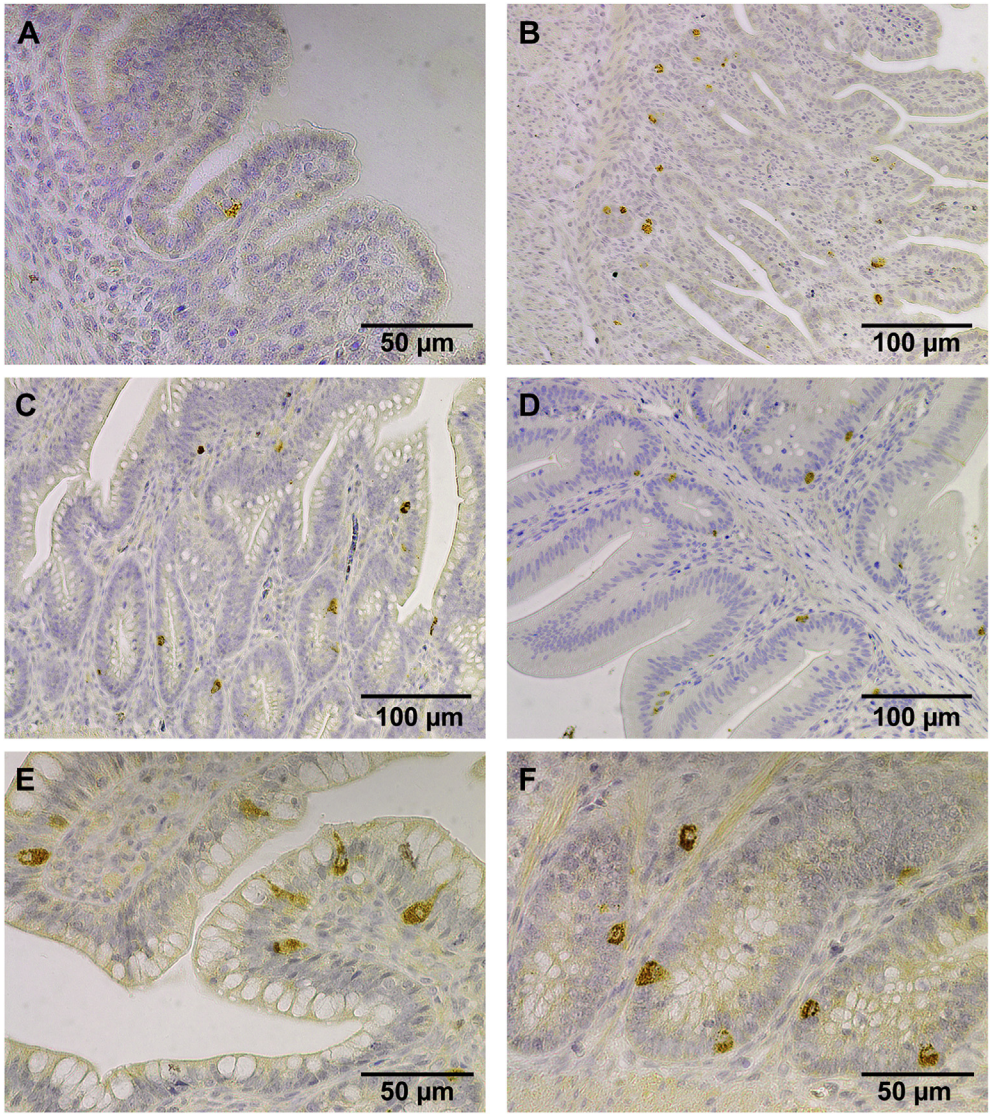

Fig. 4. Overview of AvBD9 expressing cells in the gastro-intestinal tract. Expression was first seen in rare cells at ed14 (A), expressing cell numbers grew much more numerous in later stages (B, ed18). AvBD9 expressing cells were seen in all parts of the intestine (jejunum, C and cecum, D). Typical shapes of AvBD9 expressing cells were spindle shaped in villi (E, ileum) and round or pyramidal in crypts (F, duodenum).

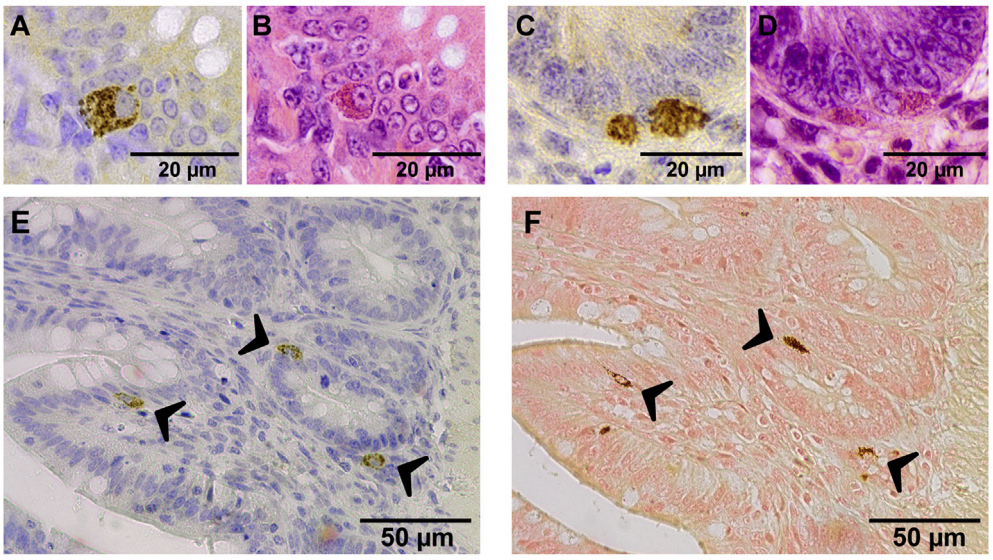

Fig. 5. Identity of the AvBD9 expressing cells in the intestine. A-D: Sequential stain with AvBD9 antibody (A,C) and HE (B,D) of jejunum (A,B) and cecum (C,D). Serial stain of ileum with AvBD9 antibody (E) and argentaffin Fontana-Masson silver stain (F). Arrows indicate overlap between AvBD9 expressing cells and silver stain.

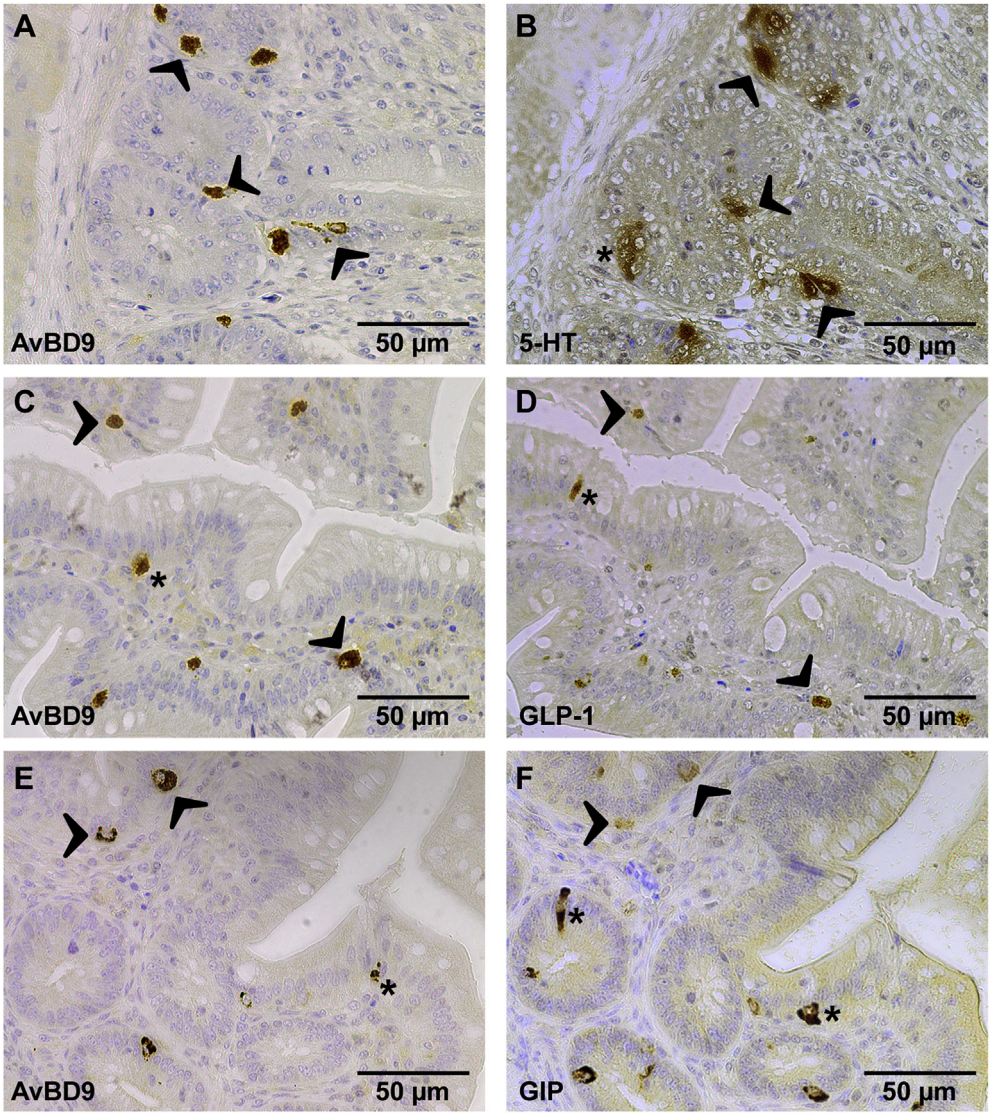

Fig. 6. Identity of the AvBD9 expressing cells in the intestine. Serial intestinal sections stained with AvBD9 (A,C,E) and enteroendocrine products 5-HT (B, cecum), GLP-1 (D, jejunum) and GIP (F, jejunum). Arrows denote examples of overlapping cells, asterisks indicate non-overlapping cells.

pancreas, thymus and kidney (data not shown).

In the embryonic lung at ed12 and ed14, only few dispersed heterophils were found (data not shown). At ed16, clusters of CATH-2 expressing cells were seen surrounding the blood vessels (Fig. 3E). At all posthatch timepoints, CATH-2 expressing heterophils were observed throughout the entire lung parenchyme as single cells or in small groups (Fig. 3F).

In the skin at all studied timepoints, small numbers of CATH-2 expressing cells were seen scattered throughout the dermis as single cells, but in embryonic and neonatal (d1, d4) samples the cells also occurred as small clusters (Fig. 3H). Only from ed14 to ed20 heterophils could also be observed in the pulp of the feather follicles (Fig. 3G).

The developmental stage of heterophils (based on descriptions in (Lucas and Jamroz, 1961)) in spleen, bursa and cecum was defined by assessment of the morphology of heterophils in Giemsa stained sections at ed18 and d28. At ed18, the majority of heterophils seen in spleen, bursa and cecum were identified as immature cells, mainly meso- and metamyelocytes with round granules and a large nucleus (Fig. S1A–C). A small number of promyelocytes with pale granules were also present in the spleen, but blast forms were not observed. Mature heterophils, characterized by rod-shaped granules and a bi-lobed nucleus were only occasionally seen. Four weeks posthatch, most of the heterophils in the spleen and the cecum were compact mature cells, though a minority of mesomyelocytes with round granules was still present (Fig. S1B,F). In contrast, in the bursa at d28, among the small number of heterophils present, the majority were of an immature phenotype (Fig. S1D).

3.2. Localization and identity of AvBD9 expressing cells

AvBD9 expression was mainly found in the intestine. Cells expressing AvBD9 were observed in all examined parts of the intestine, from the duodenum to the colon. Rare AvBD9 expressing cells were first seen at ed14, numerous stained cells were observed from ed16 onwards (Fig. 4A–B). Single AvBD9 expressing cells were spread throughout the villi (more frequently in the lower half) and crypts (Fig. 4C,D), always located basally in the epithelial layer. Two different cell morphologies of AvBD9 expressing cells were observed. Bottle-shaped or elongated cells were seen in the villi of the small intestine, often with a slender apical process reaching the luminal surface (Fig. 4E). Round or pyramid shaped AvBD9 expressing cells were found in the cecum and in crypts throughout the intestine (Fig. 4F). AvBD9 staining was cytoplasmic with a granular pattern and a clearly non-staining nucleus.

By staining intestinal sections sequentially with HE followed by AvBD9 antibody, we assessed the morphology of AvBD9 expressing cells (Fig. 5A–D). In HE stained sections, AvBD9 expressing cells showed a basal concentration of bright pink staining granules (Fig. 5B,D). These morphological characteristics gave reason to believe that AvBD9 expressing cells were enteroendocrine cells (EECs). To confirm this, intestinal sections were stained with the argentaffin Fontana-Masson silver stain, traditionally used to detect EECs. When comparing serial sections stained with Fontana-Masson and AvBD9, silver-stained cells were shown to overlap with AvBD9 expressing cells in all parts of the intestine (Fig. 5E,F).

To further characterize the identity of this AvBD9 expressing cell type, serial intestinal sections were stained pairwise with AvBD9 antibody and antibodies against three EEC products: serotonin (5-HT), gastric-inhibitory peptide (GIP), and glucagon-like peptide (GLP-1). AvBD9 expressing cells overlapped strongly with cells expressing 5-HT in all studied parts of the intestine, though cells expressing only 5-HT and no AvBD9 were also observed (Fig. 6A,B). AvBD9 expression displayed only partial overlap with GLP-1 and GIP expressing cells. Overlap of AvBD9 and GLP-1 expressing cells was most pronounced in the cecum, with only little overlap seen in the small intestine (as shown for jejunum in Fig. 6C,D). In the case

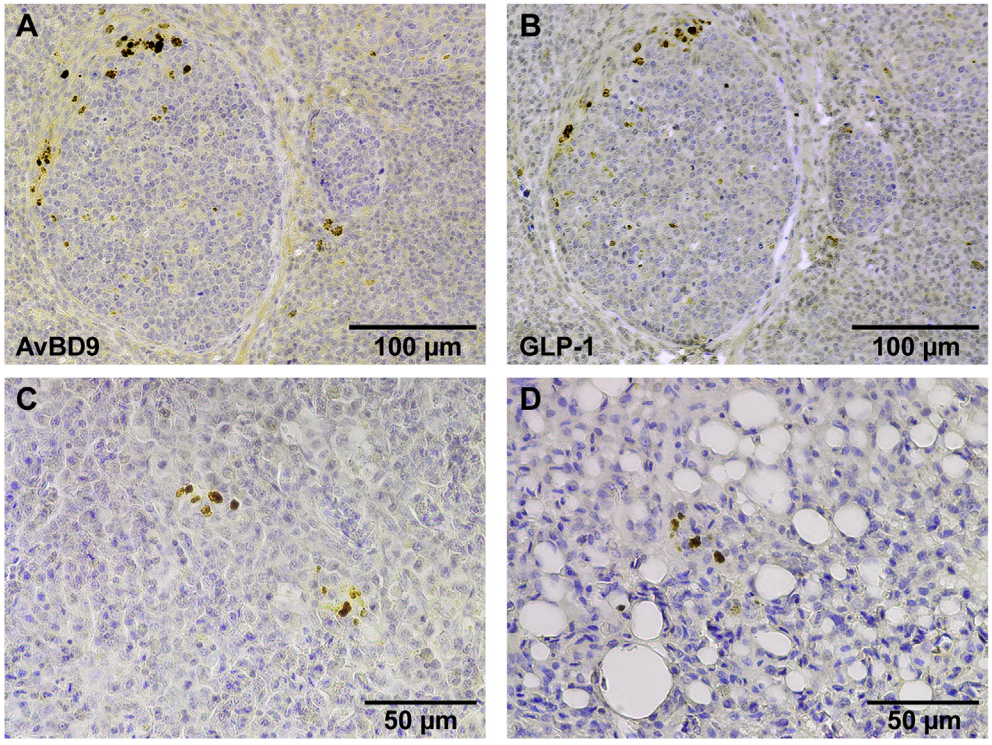

Fig. 7. AvBD9 expression in cecal tonsils, lung and spleen. A-B: serial stain of cecal tonsil with AvBD9 antibody (A) and GLP-1 antibody (B). Representative examples of AvBD9 expression in spleen (C) and lung (D) at d28.

of GIP, AvBD9 expressing cells overlapped in many cases with cells displaying a weak GIP signal, while cells with a strong GIP signal did not overlap with AvBD9 expressing cells (Fig. 6E,F).

In addition to EECs, intestinal AvBD9 expression was also seen in the lymphoid tissue of the cecal tonsils. AvBD9 expressing cells here were mostly found at the edges of the lymphoid follicles in small clusters (Fig. 7A). Morphologically, these cells appeared large and round with much cytoplasm and occasionally vacuoles. These AvBD9 expressing cells also demonstrated faint Fontana-Masson staining (data not shown) and overlapped with GIP (data not shown) and GLP-1 stain (Fig. 7A–B).

Outside of the intestine, AvBD9 expression was only found in rare instances in the spleen and lungs of 3 and 4 week old chickens as single expressing cells or small clusters (Fig. 7C,D). In appearance, these were small round cells. No clearly defined anatomical location for this AvBD9 expression was apparent. AvBD9 expressing cells were not found in the kidney, trachea, skin, bursa or in blood smears.

3.3. Quantitative expression of CATH-2 during development

The developmental expression pattern of CATH-2 was different between the studied organs. In gastro-intestinal tissues, numbers of CATH-2 expressing heterophils were low in general, but a pattern of higher expression in embryos and neonatal animals compared to older animals was seen in the small intestine (Fig. 8A–C). In the duodenum, expression at d7 was lower compared to all previous measured days (Fig. 8A). In the ileum and jejunum, expression at d7 and 14 respectively was significantly lower compared to d1 (Fig. 8B,C). In cecum and tonsils, no significant differences in CATH-2 expression were seen during development (Fig. 8D,E).

In the spleen and bursa, CATH-2 expression was highest at ed18 (Fig. 9A,B). In the spleen, CATH-2 expressing heterophils covered more than 20% of the measured area at this timepoint. As development proceeds, a steady decline in expression was seen until d4 in the bursa and d7 in the spleen. After this, CATH-2 expression remained relatively low and no further changes were seen.

In the lung, expression did not exceed 1% of the measured area (Fig. 9C). CATH-2 expression was biphasic with an increase from ed16 to d1, after which a decrease was seen until d14. Expression at d1 is the highest and significantly higher than expression at ed16, d4, 7 and 14. In the skin, CATH-2 expression was low at all measured timepoints and no significant differences in expression were seen (Fig. 9D).

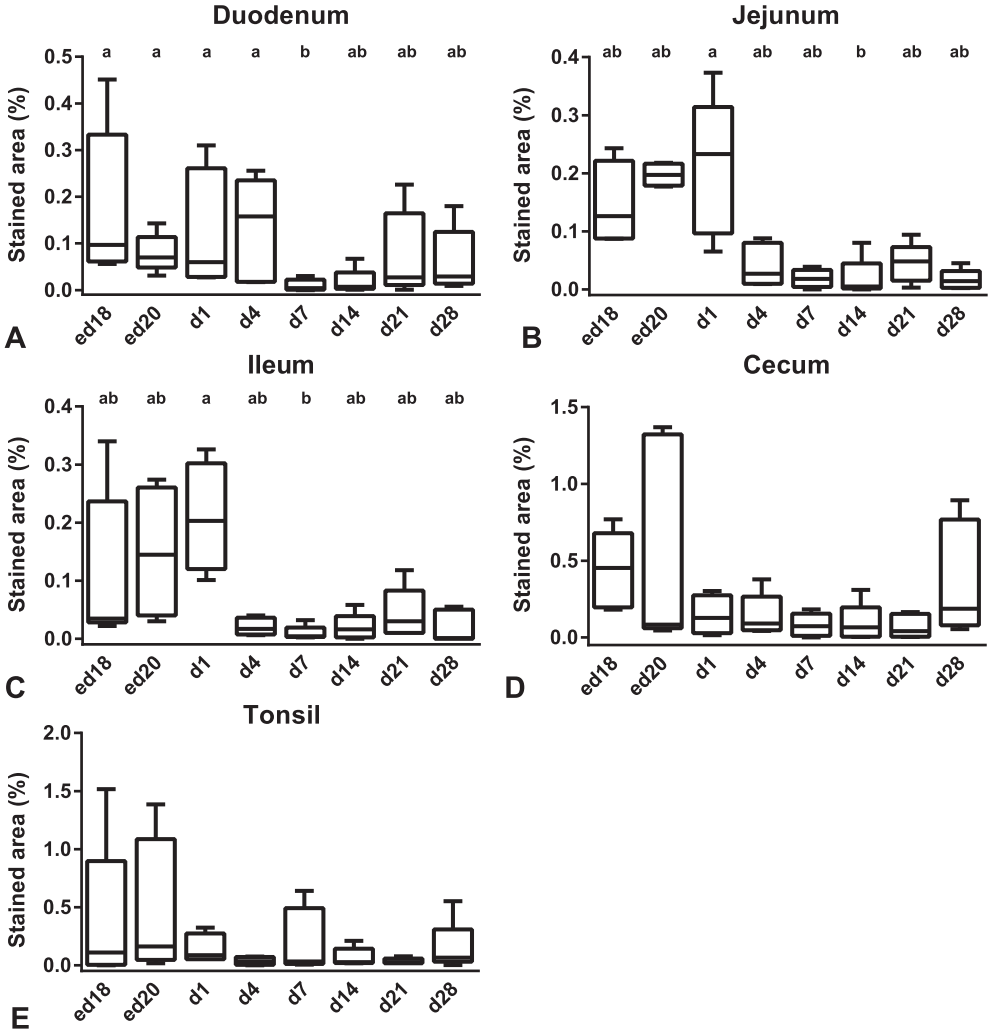

Fig. 8. Quantitative expression of CATH-2 in the gastro-intestinal tract during development: duodenum (A), jejunum (B), ileum (C), cecum (D) and cecal tonsil (E) (n = 3–5). Expression is presented as percentage stained area, as an average of 10 fields (400x magnification, 0.09 μm^2) per sample. Different characters at the top of the graphs indicate statistically significant differences between developmental stages ($p < 0.05$). Data for lung, spleen and skin were analyzed by one-way ANOVA, data for bursa were analyzed using a Kruskal-Wallis test.

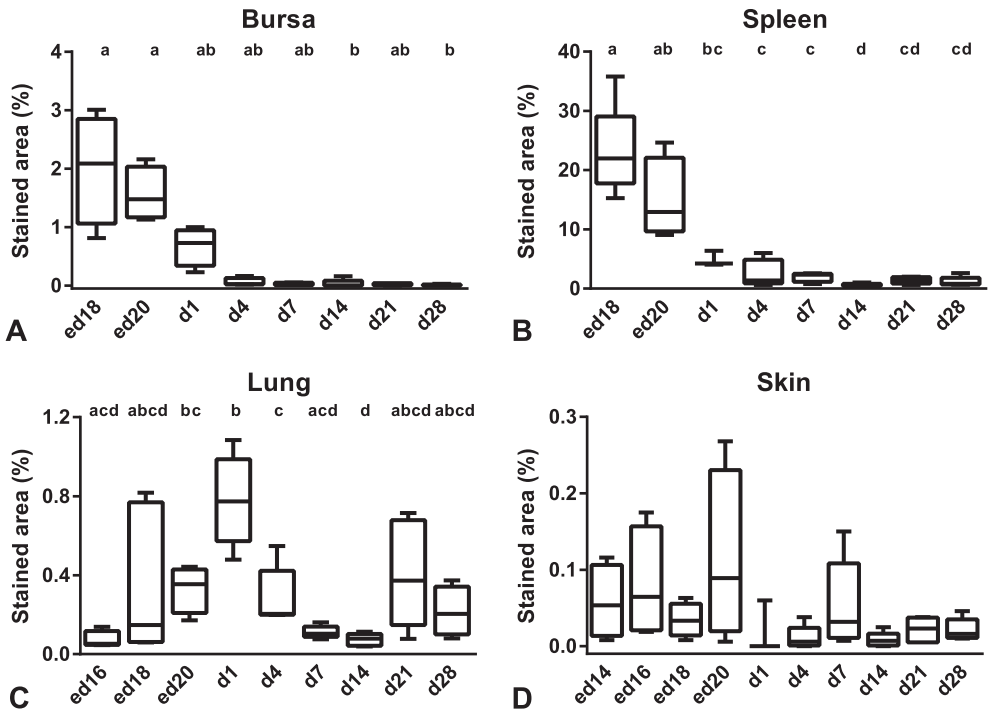

Fig. 9. Quantitative expression of CATH-2 in bursa (A), spleen (B), lung (C) and skin (D) during development (n = 3–5). Expression is presented as percentage stained area, as an average of 10 fields (400x magnification, 0.09 μm^2) per sample. Different characters at the top of the graphs indicate statistically significant differences between developmental stages ($p < 0.05$). Data for duodenum and cecum were analyzed by one-way ANOVA, data for jejunum, ileum and tonsil were analyzed using a Kruskal-Wallis test.

3.4. Quantitative expression of AvBD9 during development

AvBD9 expression was measured quantitatively only in the intestine. In general, about 5-15 AvBD9 expressing cells were seen per field (Fig. S2). Very little difference in expression was seen over the course of development, both in percentage of measured area and cell numbers. In the jejunum, the percentage expression of measured area was significantly lower at d14 compared to ed20, while in the duodenum and ileum, an apparent lower expression was seen at ed18 compared to later timepoints (Fig. 10).

4. Discussion

In this work, we report the expression patterns of two chicken HDPs: CATH-2 and AvBD9, during embryonic and posthatch development. Our group previously reported broad antimicrobial activity for both CATH-2 and AvBD9, and for CATH-2 immunomodulatory activities were also described, in line with the mammalian counterparts of these peptides (Coorens et al., 2015; van Dijk et al., 2007; van Dijk et al., 2009a; van Dijk et al., 2009b). In the present work, our earlier findings on CATH-2 localization (van Dijk et al., 2009a) are extended by examining a large set of tissues including intestinal, respiratory and lymphoid organs of numerous developmental stages, only finding CATH-2 expressed in heterophils, the avian counterpart of the mammalian neutrophil. Cathelicidins of multiple animal species were first isolated from neutrophils (Cowland et al., 1995; Larrick et al., 1991), but were later found to be expressed in multiple cell types. In addition to neutrophils, the human cathelicidin LL-37 is expressed amongst others in monocytes, lymphocytes and epithelia of the skin and gut (Agerberth et al., 2000; Dorschner et al., 2003; Hase et al., 2002). A similar expression profile has been reported for the mouse cathelicidin CRAMP, and the porcine cathelicidin PR-39 was shown to be expressed in multiple cell types of the lung (Hennig-Pauka et al., 2012). To our knowledge, a cathelicidin with exclusive protein expression in granulocytes has not been described to date. CATH-2 mRNA expression has a very broad tissue distribution, which corresponds well with expression in the ubiquitous heterophils (van Dijk et al., 2005). There are indications however that mRNA expression of CATH-2 may extend beyond heterophils (Sunkara et al., 2011). The chickens used in the present work were healthy animals, so the question remains whether CATH-2 expression can be induced in other cell types during infections. In young broilers infected with *Salmonella enteritidis* or *Campylobacter jejuni* CATH-2 expression in cecum and jejunum was only seen in heterophils (van Dijk et al., 2009a; van Dijk et al., 2012). If this exclusive heterophil expression remains true in other infectious states, CATH-2 may be considered as a heterophil marker, which could be very useful for infection and immune related research in poultry.

Based on CATH-2 staining, we were able to elucidate developmental distribution patterns of heterophils in multiple organs. In many of the organs studied a decrease in CATH-2 expressing heterophil numbers is seen shortly after hatch, most strongly in the bursa and the spleen. Bursa and spleen, but also kidney, pancreas and thymus are known sites of extramedullary hematopoiesis in the chicken embryo (Lucas and Jamroz, 1961; Romanoff, 1960; Shiojiri and Takahashi, 1991) and small-scale granulopoiesis in the intestine has also been reported (Bar-Shira and Friedman, 2006). This corresponds well with the presence of developing heterophils in bursa, spleen and cecum in embryonic samples. Posthatch, hematopoiesis in these organs largely seizes and is taken over by the bone marrow. In chicks younger than 1 week of age, very high heterophil numbers are seen in the blood, believed to be caused by the efflux of heterophils from the spleen (Lucas and Jamroz, 1961). These increased blood heterophil numbers may compensate for the relative immaturity of the adaptive immune system in this period. Interestingly, while in the spleen and cecum at day 28 we saw mostly mature heterophils, the bursa still

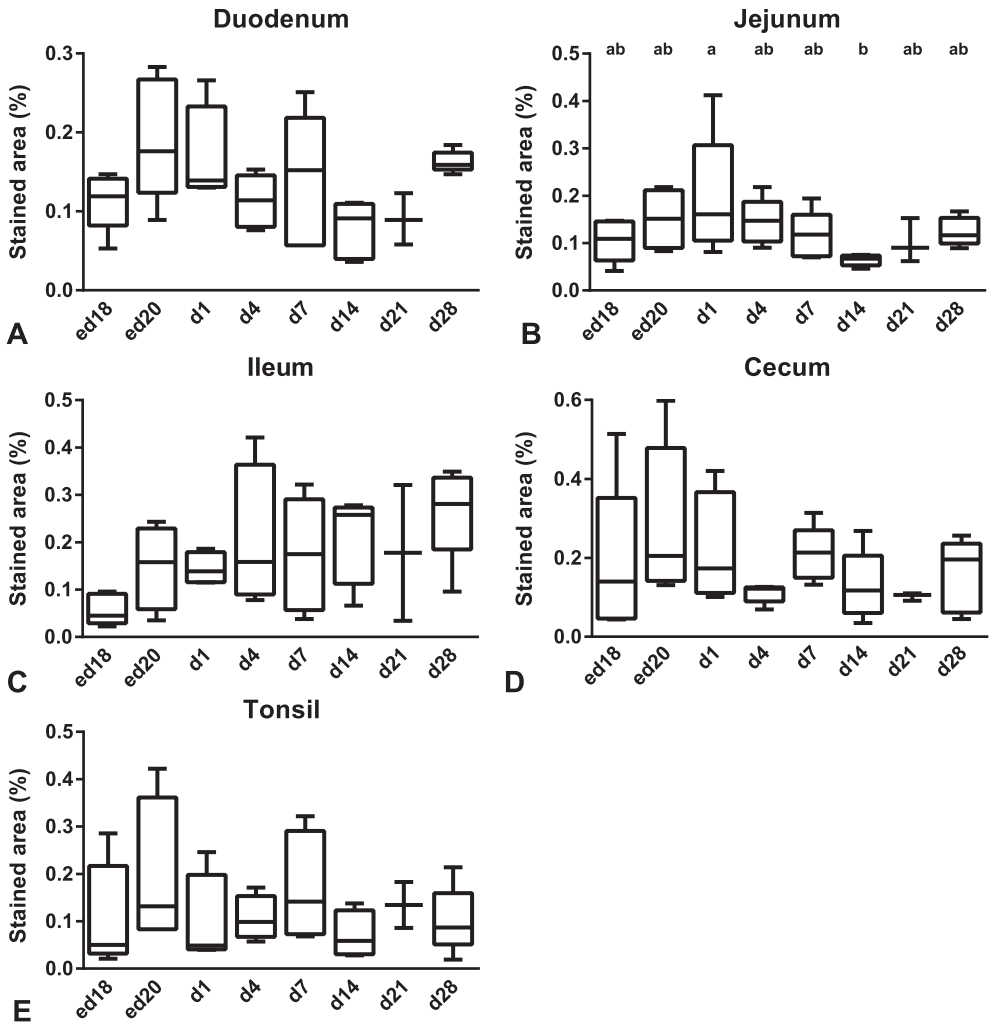

Fig. 10. Quantitative expression of AvBD9 in the gastro-intestinal tract during development (n = 3–5): duodenum (A), jejunum (B), ileum (C), cecum (D) and tonsil (E). Expression is presented as percentage stained area, as an average of 10 fields (400x magnification, 0.09 μm^2) per sample. Different characters at the top of the graphs indicate statistically significant differences between developmental stages ($p < 0.05$). Data were analyzed by one-way ANOVA.

contained developing cells at this timepoint. The presence of granulocytes around lymphoid follicles was previously described for the bursa (Aita and Minella, 1983) and was also seen in the cecal tonsils in our study. Heterophils might thus be involved in regulating B and T cell functions as is known for mammalian neutrophils (Mocsai, 2013). A biphasic expression pattern was found for CATH-2 expression in the lung. Possibly, the high CATH-2 expression at day 1 posthatch is due to heterophil recruitment resulting from the first exposure of this organ to the external environment upon hatching. The CATH-2 developmental expression reported here deviates from a previously reported analysis based on mRNA (Achanta et al., 2012) where an increase in the CATH-2 mRNA expression in the bursa, cecum and cecal tonsils was seen in the period from 2 to 28 days posthatch. As suggested above, mRNA expression of CATH-2 might be broader than the heterophil specific protein expression and therefore show a different developmental pattern.

The majority of AvBD9 expressing cells in the intestine could be identified as EECs based on cell morphology, overlap with argentaffin silver stain and co-localization with EEC products. Among the approximately 12 EEC cell types are enterochromaffin cells expressing serotonin (Furness et al., 2013; Rindi et al., 2004), which were found to be strongly co-localizing with AvBD9. As enterochromaffin cells are the most widely distributed of the EEC types, this co-localization corresponds well with the wide distribution of AvBD9 expressing cells in all parts of the chicken intestine. Some AvBD9 expression was also found in EECs expressing GIP and GLP-1, in mammals called K and L cells, respectively. Previously, mRNA expression of AvBD9 was reported to be low in the gastro-intestinal tract and high in the liver, kidney and bursa (van Dijk et al., 2007; Xiao et al., 2004).

Here we report for the first time in vivo protein expression of a defensin in EECs. Previously, expression of murine β-defensin 2 was reported in the EEC cell line STC-1, but expression was not confirmed in mouse tissues (Palazzo et al., 2007). EECs are well described for their role in regulation of digestion, intestinal motility and appetite, but a growing number of publications also ascribes to them a role in host defense. EECs were reported to express TLRs and respond to TLR ligands by secretion of both hormonal products and innate immune molecules (Bogunovic et al., 2007; Palazzo et al., 2007; Selleri et al., 2008). In addition, roles in defense have been described for some EEC derived hormones. For example, GLP-2 and cholecystokinin were shown to have anti-inflammatory effects in in vivo models and somatostatin was able to inhibit IL-1β and IL-8 secretion from intestinal epithelial cells (Chowers et al., 2000; Meng et al., 2002; Sigalet et al., 2007). In chickens, both somatostatin and 5-HT were shown to influence immune responses to

infectious challenges (Donato et al., 2015; Yang et al., 2007). Interestingly, deletion of the GLP-2 receptor in mice reduced Paneth cell α-defensin expression, showing another link between intestinal defensin expression and EECs (Lee et al., 2012).

EECs secrete their products basolaterally into the lamina propria, enabling interactions between these secreted compounds with residing immune cells. Human and murine enteric defensins have been shown to be chemotactic, to induce dendritic cell differentiation and maturation and to increase T cell cytokine production (Meisch et al., 2013; Presicce et al., 2009; Rohrl et al., 2010; Scudiero et al., 2010; Yang et al., 1999). It is interesting to speculate that a similar role may exist for AvBD9 released by chicken EECs.

In contrast to CATH-2, very little difference in AvBD9 expression was seen over the course of development from ed18 onwards. Though quantitative analysis was not possible before ed18, qualitatively a large increase in expression was seen after the first observation of AvBD9 expressing cells at ed14. Expression of many EEC products is also first seen around ed14, indicating functional maturation of EECs at this timepoint (Rawdon and Andrew, 1999). Previously, gene expression of avian intestinal defensins (AvBD1, -2, -4 and -6) was measured in the gut of developing chickens, showing clear developmental regulation with a decrease in the first week posthatch followed by an increase in the second week (Bar-Shira and Friedman, 2006; Crhanova et al., 2011). The expression of human β-defensin 1 is constitutive and already present in fetal intestines (O'Neil et al., 1999), it appears from our findings this is also true for AvBD9.

Data about the expression of AvBD9 in infected animals are rare, though infection with *Salmonella enteritidis* in geese increased AvBD9 mRNA expression in cecal tonsils, but not in small intestine (Ma et al., 2012). Even in the healthy chickens in our study, AvBD9 expression is not limited to EECs, as we also report expression in the lung, the spleen and the lymphoid tissue of the cecal tonsils. The AvBD9 mRNA expression has an even broader tissue distribution (Lynn et al., 2004; van Dijk et al., 2007; Xiao et al., 2004), suggesting that, even though protein expression was only found in a few organs in this study, expression might very well be induced in more organs and cell types under certain conditions.

In short, this study describes protein expression of two chicken host defense peptides during embryonic and posthatch development and reports two very distinct patterns of expression. Both peptides are constitutively expressed in specific cells, CATH-2 in heterophils and AvBD9 in EECs, cell types strategically located to quickly mount a response to infectious stimuli. CATH-2 and AvBD9 thus appear to be essential in host defense in both embryos and neonates, as well as in older animals.

Additional information

This work was supported by the Immuno Valley ALTernatives to ANTibiotics (ALTANT) Animal Specific Immuno-modulatory Antimicrobials (ASIA) 2 program of the Dutch Ministry of Economic Affairs.

Acknowledgments

We would like to thank Monique Tersteeg-Zijderveld of the Division Veterinary Public Health, Faculty of Veterinary Medicine, Utrecht University and José Nieuwenhuisen-van Wilgen of GD Animal Health for all their technical help and input. Erik Weerts of the Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University is thanked for his histological insights. We are grateful to Susanne Kirchhoff of the Department of Animals in Science and Society, Faculty of Veterinary Medicine, Utrecht University for the use of the Olympus BX51 microscope and Naomi de Bruijn of GD Animal Health for the use of the cellSens Dimension software.

Appendix A. Supplemental figures

Supplemental figures related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.03.008.

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