Morphological and immunological characterization of primary cultured chicken caecal epithelial cells

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ABSTRACT

Cell cultures are models in biological and medical research to understand physiological and pathological processes. Cell lines are not always available depending on cell type and required species. In addition, the immortalization process often affects cell biology. Primary cells generally maintain a greater degree of similarity in short-term culture to the cells in tissue. Goal of this study was to verify the suitability of chicken primary epithelial caecal cells (PECCs) for in vitro investigations of host–pathogen interactions. Epithelial nature of PECCs was confirmed by detection of tight and adherens junctions and cobblestone-like cell morphology. Sialic acids distribution was similar to that in caecal cyrosections. To understand the capacity of PECCs to respond to microbial challenges, the Toll-like receptors (TLRs) repertoire was determined. Exposure of PECCs to polyinosinic-polycytidylic acid (poly(I:C)) or lipopolysaccharide (LPS) led to upregulation of type I and III interferon (IFN) as well as interleukin (IL-) 1β, IL-6 and IL-8 mRNA expression. Overall, the PECCs showed properties of polarized epithelial cells. The presence of TLRs, their differential expression, as well as pattern recognition receptor dependent immune responses enable PECCs to act as suitable in vitro model for host–pathogen interaction studies, which are difficult to conduct under in vivo conditions.

KEYWORDS

intestinal epithelial cells, chicken, characterization, TLR repertoire, interleukin, interferon

INTRODUCTION

As one of the major health problems in poultry production, gut infections have an enormous economic impact. For example, Blake et al. [1] estimated the global cost of coccidiosis in 2016 to be 10.4 billion GB£. Additionally, colonization of the chicken gut by zoonotic pathogens such as Salmonella Enteritidis or Campylobacter jejuni is of major public concern [2, 3].

Many studies on gut physiology, biochemical pathways, pharmaceutical reactions, and the pathophysiology of gut infections or diseases employ cultivated enterocytes [4, 5], including well-characterized human cell lines such as Caco2 or T84 [6–8]. Due to the lack of permanent enterocyte cell lines from a variety of animal species (including chickens), many investigations have relied upon extrapolation from studies with human cell lines, which have been limited to tissues of the species under study or to using epithelial cells from nonintestinal tissues such as primary chicken kidney cell cultures [9–11]. While many of these studies provide important information, it is important to note that many host pathogen responses differ according to the host species. Of particular relevance to the current manuscript, the chicken genome contains a different array of pattern recognition receptors than does the...
mammalian genome [12, 13]. For example, some Toll-like receptors (TLRs) are homologous to those found in humans, and some are distinct [12, 14–16]. Even when TLRs are shared between humans and chickens, it is clear that there are differences in the reactivity or response profiles of these TLRs [17, 18].

The isolation and culture of primary intestinal epithelial cells is challenging. However, in recent years, isolation and culture techniques for primary intestinal epithelial cells have been developed and used in various studies. These studies included the cultivation of embryonic intestinal cells [19, 20] as well as cells from older chickens [21]. Cultured enterocytes will continue to be useful in determining the biology of both the epithelial cells and infections that target these cells. For example, a range of infections in birds involve interactions with sialic acid residues on target cells [22–25]. Hence, it is important to develop a range of culture systems for chicken enteric epithelial cells to understand the biology of this important cell type and interactions with a wide range of avian opportunistic and facultative pathogens. Furthermore, primary intestinal epithelial cells form the basis for intestinal organoids [26, 27], which are excellent ex vivo models for investigating gut physiology and biology.

In this study, we verified the suitability of primary epithelial caecal cells (PECCs) of posthatch chicken as a model for investigating caecum epithelial cell–pathogen interactions. Here, we report the cultivation and characterization of PECCs in terms of morphology, the expression of E-cadherin, sialic acids and the expression of mRNAs encoding TLRs. We also characterized the response of PECCs after exposure to polyinosinic-polycytidylic acid encoding TLRs. Here, we report the cultivation and characterization of avian opportunistic and facultative pathogens. Further, this important cell type and interactions with a wide range of chicken enteric epithelial cells to understand the biology of these cells and infections that target these cells. For example, a range of infections in birds involve interactions with sialic acid residues on target cells [22–25]. Hence, it is important to develop a range of culture systems for chicken enteric epithelial cells to understand the biology of this important cell type and interactions with a wide range of avian opportunistic and facultative pathogens. Furthermore, primary intestinal epithelial cells form the basis for intestinal organoids [26, 27], which are excellent ex vivo models for investigating gut physiology and biology.

**MATERIALS AND METHODS**

**Experimental procedure**

The investigations were divided into two parts and are overviewed in Table 1.

For each experimental part, PECCs were prepared from at least 2–3 chickens. Isolated crypts were randomly pooled and plated at a dilution of 3,000 crypts/well. Each experiment was conducted at least in triplicate per treatment and time point. The expression of selected cytokines and interferon (IFN) mRNAs was evaluated with 5–7 repeats/treatment and at a range of time points.

In Part A, morphological characteristics were determined microscopically every 8–12 h to describe PECCs cultures after preparation and to determine the lifespan of these cells and the time points at which the cultures underwent age-related microscopic changes. To investigate viability, the confluence of the PECCs after 24, 36 and 48 h of culture was estimated, and the percentage of live cells was determined via trypan blue staining (Experiment 1). The epithelial origin of the PECCs was confirmed by immunofluorescence staining for cytokeratin and E-cadherin. Chicken embryo fibroblasts (CEF) were used as a negative control (Experiment 2). In addition, transmission electron microscopy (TEM) of the PECCs was performed to screen for morphological markers of the intestinal epithelium (Experiment 3). Additionally, the cultures were investigated for possible contaminating macrophages by immunohistochemistry. After two days of culture, the PECCs were fixed in methanol, and together with acetone-fixed caecum cryosections of the same donor animals, they were stained with anti-chicken monocent antibodies (Experiment 4). The distribution of α2,3- and α2,6-linked sialic acids was investigated by lectin staining of methanol-fixed PECCs 24 h after seeding. Caecum cryosections from the same donor animals were used as a positive control (Experiment 5).

In Part B, selected immunological parameters of PECCs were determined. For investigations of the TLR repertoire, 30 wells of a 24-h PECCs culture were randomly assigned to 3 pools of 10 wells each. Real-time quantitative RT-PCR (qRT–PCR) was performed to detect TLR 1–5, 7, 15 and 21. The avian macrophage line HD11 was used as a control and is known to express these TLRs to different extents [28] (Experiment 6). In addition, we investigated the ability of the PECCs to react to stimuli through changes in the mRNA expression patterns of selected cytokines. Therefore, we stimulated cells with poly(I:C) to detect the expression of IFNα, IFNβ and IFNλ mRNA (Experiment 7) and with LPS from *Escherichia coli* to detect the expression of interleukin (IL-1β, IL-6 and chicken IL-8 homolog mRNA (Experiment 8) by qRT–PCR.

**Chickens**

Specific pathogen-free (SPF) chicken eggs were purchased from VALO BioMedia GmbH (VALO BioMedia GmbH, Osterholz-Scharmbeck, Germany) and incubated until
hatch. Chickens were raised in a cage-free aviary system with wood shavings under confined conditions in the facilities of the Clinic of Poultry, University of Veterinary Medicine Hannover. Birds had ad libitum access to water and feed (‘all-mash L’, Deutsche Tiernahrung Cremer GmbH & Co. KG, Düsseldorf, Germany). Between five and twelve weeks, posthatch chickens were humanely sacrificed according to the welfare regulations of Lower Saxony, Germany, to collect fresh ceca. The number of sacrificed chickens was announced to the animal welfare officer of the University of Veterinary Medicine (according to §4 Animal Protection Law, 11.11.2015) and subsequently reported to the authorities according to the German Decree on the Reporting of Laboratory Animals.

Isolation of primary epithelial caecal cells (PECCs)

The isolation of PECCs was performed as described earlier [29], with some minor modifications. Briefly, SPF chickens aged 5–12 weeks were sacrificed, and the caeca were aseptically removed, washed in Hank’s Balanced Salt Solution (Biochrom GmbH, Berlin, Germany), minced and digested enzymatically in digestion medium (Dulbecco’s Modified Eagle’s medium (DMEM)/Ham’s F12 [1:1; Biochrom GmbH, Berlin, Germany], 1% fetal bovine serum [FBS; Biochrom GmbH, Berlin, Germany], 50 μg/mL gentamicin [Sigma–Aldrich, St. Louis, Missouri, USA], 100 U/mL penicillin, 100 μg/mL streptomycin [Biochrom GmbH, Berlin, Germany], 1 U/mL dispase II [Sigma–Aldrich, St. Louis, Missouri, USA] and 75 U/mL collagenase [Biochrom GmbH, Berlin, Germany]) for 2 h. Single cells and bacteria were removed from the tissue suspension by using repeated sorbitol gradient centrifugation (DMEM/Ham’s F12 [1:1], 2% d-sorbit [Carl Roth GmbH, Karlsruhe, Germany], 2.5% FBS, 50 μg/mL gentamicin) at 100×g for 3 min at 37 °C until the supernatant was clear. The remaining pellet of crypts was resuspended in growth medium (DMEM/Ham’s F12 [1:1], 2.5% FBS, 10 μg/mL insulin [Sigma–Aldrich, St. Louis, Missouri, USA], 1.4 μg/mL hydrocortisone [Sigma–Aldrich, St. Louis, Missouri, USA], 5 μg/mL transferrin [Sigma–Aldrich, St. Louis, Missouri, USA], 1 μg/mL fibronectin [Biochrom GmbH, Berlin, Germany], 100 μg/mL penicillin, 100 μg/mL streptomycin; 50 μg/mL gentamicin). The crypts in the final suspension were counted microscopically and adjusted by dilution to a concentration of 6,000 crypts/mL. Crypts were seeded on collagen-coated 24-well cell culture plates (500 μL/well; Greiner Bio-One GmbH, Frickenhausen, Germany) and incubated at 37 °C in a 5% CO₂ atmosphere. After 24 h, the medium was replaced with fresh growth medium (500 μL/well).

Isolation of chicken embryo fibroblasts

Isolation of CEFs was performed as described previously [30]. Cells were harvested from SPF chicken eggs at embryonation day 10 and seeded at a density of 7.5 × 10⁵/well in 24-well cell culture plates (Sarstedt AG & Co., Nümbrecht, Germany) in L-15 Leibovitz medium/McCoy’s 5A modified medium (1:1; Biochrom GmbH, Berlin, Germany), 10% FBS, 1% L-Glutamine (200 mM; Biochrom GmbH, Berlin, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were incubated at 37 °C in a 5% CO₂ atmosphere for two days and subsequently used as controls in Experiment 2.

Viability of the PECCs (experiment 1)

After the growth of PECCs for 24, 36 or 48 h, the cells were washed two times with prewarmed phosphate-buffered saline (PBS), after which the confluence of the wells was estimated via microscopic examination. Subsequently, the cells were treated with 150 μL of trypsin/EDTA (Biochrom GmbH, Berlin, Germany). Immediately after detachment, 150 μL of a 0.4% trypsin blue solution (Amresco, Solon, OH, USA) was added, and the cells were counted in a Neubauer counting chamber to differentiate live cells from dead cells.

Immunofluorescence staining for epithelial markers (experiment 2)

Methanol-fixed PECCs and CEFs were rehydrated and blocked with 0.5% bovine serum albumin (BSA; Carl Roth GmbH, Karlsruhe, Germany) in PBS. The cells were incubated with the following primary antibodies in PBS supplemented with 0.5% BSA for 1 h at 37 °C: polyclonal rabbit-anti-cytokeratin (1:250; Dako North America, Inc., Carpinteria, CA, USA) for the detection of cytokeratin and monoclonal mouse-anti-L-CAM (1:45; Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) for the detection of E-cadherin [31]. After three washes with 0.5% BSA in PBS, the cells were incubated with secondary anti-rabbit or anti-mouse fluoroscent labelled antibodies (1:1000, Alexa Fluor 568 and Alexa Fluor 488, Life Technologies, Grand Island, New York, USA) for 1 h at 37 °C. The cells were subsequently washed two times with PBS and one time with distilled water. Finally, the cells were mounted with ProLong Gold Antifade with DAPI (Life Technologies, Carlsbad, CA, USA) for the detection of tight junctions and microvilli. The stained cells were examined by using an inverted fluorescence microscope (Nikon Eclipse Ti, Tokyo, Japan). CEFs cultures were included as negative controls.

Transmission electron microscopy (experiment 3)

For the detection of tight junctions and microvilli in PECCs, standard procedures for TEM (Zeiss EM 10 C) were performed at the Institute for Pathology, University of Veterinary Medicine Hannover [32]. Thin sections (70–90 nm) of a two-day-old PECCs culture were evaluated for tight junctions, adherens junctions and microvilli (10,000× and 20,000× magnification).

Immunohistochemical detection of macrophage contamination (experiment 4)

After two days of culture, the PECCs were fixed in methanol and together with acetone-fixed 5 μm caecum cryosections stained as described previously [33]. The cells and sections
were stained with monoclonal mouse anti-chicken monocyte/macrophage antibodies (KUL01; 1:500; Southern Biotech, Birmingham, AL, USA). Biotinylated secondary horse anti-mouse IgG antibodies and ABC reagent were used according to the manufacturer’s instructions (Vector Laboratories, New York, CA, USA). Peroxidase activity was investigated microscopically, and positive cells were identified by 3,3′-diaminobenzidine staining.

Lectin staining for the detection of sialic acids (experiment 5)

Methanol-fixed PECCs and acetone-fixed caecal cryosections (5 μm) were washed thrice with PBS. Afterwards, the cells and sections were blocked with streptavidin and biotin solution (Vector Laboratories, New York, CA, USA) for 15 min each and with Carbo-free blocking solution (Vector Laboratories, New York, CA, USA) for 30 min at room temperature (RT) with a single wash in PBS, as suggested by the manufacturer. After washing three times with PBS, the PECCs and sections were incubated with biotinylated Maackia Amurensis lectin II (MAL; specific for α2,3-linked sialic acids; Vector Laboratories, New York, CA, USA) or Sambucus nigra lectin (SNA; specific for α2,6-linked sialic acids; Vector Laboratories, New York, CA, USA) in PBS for 40 min at RT. Technical controls were incubated with PBS without lectin. The cells and sections were washed thrice with PBS and incubated with the AB reagent from the VECTASTAIN Elite ABC HRP Kit (Vector Laboratories, New York, CA, USA). Peroxidase activity was investigated microscopically as described previously [34].

Detection of Toll-like receptor mRNA expression by qRT–PCR (experiment 6)

After two days of culture, the PECCs were harvested with trypsin/EDTA (Biochrom GmbH, Berlin, Germany). The samples were stored in RNA Later (Sigma–Aldrich, St. Louis, Missouri, USA) until further investigation. Culture of HD11 cells was performed for comparison with that of PECCs with respect to TLR expression as described previously [35]. The mRNA expression of TLR was determined for both cell types following previously published procedures [33].

Detection of the mRNA expression of selected cytokines by qRT–PCR (experiments 7 + 8)

PECCs were stimulated with 2.5 μg/mL LPS from E. coli 0127:B8 (Sigma–Aldrich, St. Louis, Missouri, USA) or 50 μg/mL poly(I:C) (InvivoGen, San Diego, CA, USA) in conservation medium (DMEM/Ham’s F12 [1:1], 2.5% FBS, 10 μg/mL insulin) or left untreated in conservation medium for controls. Four and eight or 12 and 24 h after stimulation with LPS or poly(I:C), respectively, cells were washed and detached with 250 μL of trypsin/EDTA (Biochrom GmbH, Berlin, Germany). After detachment, the cells were stored in trypsin/EDTA solution at −80 °C until RNA isolation.

RNA extraction

Total RNA was extracted from cell samples using the MasterPure RNA Purification Kit (Epicentre, Madison, WI, USA) according to the manufacturer’s instructions. The isolated RNA was stored at −80 °C until qRT–PCR analysis.

qRT–PCR

qRT–PCR was performed using a Stratagene MX 3005P RT–qPCR cycler (Stratagene, La Jolla, CA, USA), a Step-One Plus qPCR machine (Applied Biosystems, Waltham, MA, USA) and an AgPath-ID One-Step RT–PCR kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer’s instructions [33, 34, 36]. The sequences of the primers and probes used for the detection of the mRNA expression of IL-1β, IL-6 and the chicken IL-8 homolog, IFNα, IFNβ, IFNλ, and TLR targets as well as the housekeeping gene 28S were previously published [33–35, 37, 38]. Three microliters of diluted total RNA in 25 μL of reaction mix was used with the following cycle: one cycle at 45 °C for 10 min and 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 57 °C for 45 s. The cycle threshold (Ct) values of the expressed mRNAs of the investigated genes were normalized against those of the expressed housekeeping gene 28S rRNA of the same sample (ΔCt) as described by Powell et al. [39]. The overall 28S rRNA expression was comparable between samples independent of the treatment. The ΔCt values of the samples are presented as fold changes and were related to the ΔCt values from negative control groups at the same sampling time point.

Statistical analysis

Statistical analyses were performed using Statistix version 10.0 (Analytical software, Tallahassee, FL, USA). One-way analysis of variance with Tukey HSD All-Pairwise Comparisons Test was used for comparison of confluence levels in the viability assay. Two-sample t-test as well as the Wilcoxon Rank Sum Test were used for assessing the differences in cytokine and TLR mRNA expression levels. P < 0.05 was considered to indicate statistical significance.

Ethics statement

The German animal welfare guidelines were met. The birds were sacrificed at the age of 5–12 weeks posthatch, at which points fresh caeca were collected. The number of sacrificed chickens was reported to the authorities of the University of Veterinary Medicine Hannover and Lower Saxony State Office for Consumer Protection and Food Safety according to German welfare regulations.

RESULTS

Morphology and lifespan

The PECCs proliferated for one to three days after seeding. After the attachment of the crypts to the culture plates, the
cells proliferated and migrated across the culture area, reaching at least 75–80% confluence after 24 h (occasionally up to 95% confluence was reached). The cells exhibited a typical cobblestone-like, epithelial morphology. The PECCs cultures were stable for two to five days after seeding if the growth medium was carefully replaced every 24–48 h. When vigorous washing was applied, the monolayer coverage decreased more rapidly, as single cells detached from the outside margins of the monolayers. Often, the confluence of the cell monolayer decreased slightly over time (rarely >60%), but the cell viability remained high (Fig. 1).

To confirm the epithelial origin of the isolated cells, immunofluorescence staining of the cytoskeletal epithelial protein cytokeratin and the epithelial-specific cell–cell adhesion glycoprotein E-cadherin [40] was performed (Experiment 2). Chicken embryo fibroblasts served as a negative control. PECCs were positive for cytokeratin and E-cadherin, while chicken embryo fibroblasts were negative for both (Fig. 2). Moreover, the morphology of the PECCs determined by TEM confirmed the epithelial origin of these cultured cells, as indicated by the presence of tight junctions (Fig. 3). The cultures did not contain contaminating macrophages, as judged by a lack of staining with the KUL01 antibody, which detects chicken monocytes and macrophages, while parallel stained caecum cryosections showed KUL01-positive cells in the subepithelial structures (data not shown). Microscopy revealed low numbers of fibroblasts in the PECCs cultures at 24 and 48 h post seeding, but the number of fibroblasts increased significantly after five to seven days of culture. Therefore, PECCs cultures were routinely used after 24 h of culture and up to three days after seeding to avoid complications associated with fibroblast outgrowth.

Epithelial cells can be characterized by the nature of the sialic acids on their surfaces, and many pathogens exploit these sialic acids for recognition of target cells. The PECCs cultures showed a low surface staining intensity for the MAL...
lectin, which recognizes α2,3-linked sialic acids, whereas there was strong staining with the SNA lectin, indicating high levels of α2,6-linked sialic acids. Cryosections collected from the caecum of the same animals were used as positive controls and showed the same staining pattern (Fig. 4).

**TLR expression and responses of PECCs to poly(I:C) and LPS**

The expression of TLR mRNA in PECCs was assessed by qRT–PCR and compared with that in the well-studied avian macrophage line HD11 [35]. Both PECCs and HD11 cells expressed mRNA from all ten avian TLRs. Interestingly, the mRNA expression levels of TLR1.1, TLR3 and TLR5 were significantly higher in PECCs than in HD11 cells (P < 0.05). TLR2.1 and TLR2.2 mRNA levels were similar in PECCs and HD11 cells. In contrast, HD11 cells expressed significantly greater amounts of the TLR 1.2, TLR4, TLR7, TLR15 and TLR21 mRNAs than did PECCs (P < 0.05; Fig. 5). Within the PECCs, the most highly expressed TLRs were 1.1, 3 and 4 (40-Ct values: 9.25–10.19). The TLR 2.2, 5, 7 and 15 were expressed to a distinct lower extent (40-Ct values: 11.18–13.2). The TLR 1.2, 2.1 and TLR 21 (40-Ct values: 10.19). Hence, PECCs may differentially recognize certain TLR agonists, which could affect their participation in enteric immune responses.

To test the capacity of the PECCs to participate in immune responses via TLR-dependent pathways, the cells were exposed to the well-defined agonists, poly(I:C) (which stimulates TLR3 to induce type I IFN responses) and LPS (which stimulates TLR4-induced upregulation of inflammatory cytokines and chemokines such as IL-8 and CXCL8) [35]. Transcriptional regulation after the activation of pattern recognition receptors such as TLR3 is known for avians, there are differences in anatomy, morphology and human cells [41, 42]. Increased levels of IFNα, β and λ mRNA were readily detected after 12 and/or 24 h of exposure to poly(I:C). The upregulation of IFNα and IFNβ mRNA occurred later and was markedly lower than that detected for IFNα. IFNα and β upregulation of approximately threefold (P < 0.05) was detected only at 24 h post exposure, and high levels of background IFNα and β mRNA were detected in nonstimulated control cells (data not shown). In contrast, IFN-λ mRNA was strongly upregulated by poly(I:C) treatment at 12 and 24 h, with 2564-fold and 437-fold upregulation, respectively, compared to that in the nonstimulated controls (Fig. 6). In the case of IFNα, very low signals were detected in nonstimulated controls.

Exposure of PECCs to LPS led to the upregulation of IL-1β and IL-6 mRNA (10- to 15-fold) expression at 8 h post exposure compared to that in the nonstimulated control group. The mRNA levels of the IL-8 (CXCL8) homolog were significantly upregulated at four hours post stimulation compared to those in the nonstimulated control group but not at eight hours post stimulation (Fig. 7).

**DISCUSSION**

The majority of investigations characterizing the biological activities of intestinal cells and their interactions with stimulants or pathogens have been performed with human-derived gut cells, mainly cell lines [43]. However, not all findings can be generalized to other species. Due to the relatively great evolutionary distance between humans and avians, there are differences in anatomy, morphology and...
physiology [44]. No caecum-derived cell lines from post-
hatch chickens are available. Therefore, we established a
crypt-derived primary gut cell culture, confirmed the
polarized epithelial characteristics of these cells and inves-
tigated their biological characteristics to determine their
potential use as an in vitro model for the chicken gut and for
investigating pathogen-gut cell interactions. We were spe-
cifically interested in the distribution of \( \alpha_{2,3} \) and
\( \alpha_{2,6} \) sialic acids, which are known to serve as receptors for various
pathogens [22–25], and their capacity to participate in
innate immune responses.

Within 24–48 h, the cells proliferated and reached a
confluence of 75–80%. We observed variations in prolifer-
ation and viability between the experimental days. These
variations seemed to depend on unknown factors, and we
hypothesized that they were associated with the donor ani-
mals, suggesting the influence of age or the microbiota of the
host. Cells isolated from animals from the same rearing
group exhibited similar trends in growth characteristics,
including growth dynamics and extent of confluence. We
observed microscopically that different batches varied in
growth with slow-growing cell cultures showing longer
viability than fast-growing cell cultures and vice versa (data
not shown).

The isolation method did not fully exclude the possibility
that other cell types in addition to PECCs may be isolated
and contaminate the culture. We detected low contamina-
tion with fibroblasts in the PECCs after isolation. These fi-
broblasts contribute to the short duration of PECCs culture,
as they replicate and outgrow the PECCs after approxi-
mately five to seven days. Unfortunately, fibroblast
contamination could not be fully avoided despite different
isolation attempts or modifications of culture conditions
(data not shown). Therefore, PECCs should be used only
for up to 3 days post plating to avoid the influence of fibroblasts.

Sialic acids are important attachment targets for various
pathogens, such as Avian Influenza Virus, Newcastle Disease
Virus, Infectious Bronchitis Virus or Salmonella ssp.
[22–25]. PECCs exhibited intense SNA lectin staining, which
indicates a high density of \( \alpha_{2,6} \)-linked sialic acids. Staining
by the \( \alpha_{2,3} \)-linked sialic acid-specific MAL was less intense.
These observations are interesting because they contrast

Fig. 6. Upregulation of interferon (IFN) mRNA expression in PECCs after stimulation with polyninosinic-polycytidylic acid (poly(I:C)) (Exp. 7). PECCs were stimulated with 25 μg/mL poly(I:C). Cells were collected 12 and 24 h post stimulation (hps), after which IFNα (a), IFNβ (b) and IFNλ (c) mRNA expression was investigated. The results are presented as the fold change in expression compared to that in nonstimulated cultures. Relative quantification was performed by qRT–PCR, and the values were normalized to those of 28S rRNA. Error bars indicate the standard error of the mean (SEM). Asterisks indicate significant differences compared to the nonstimulated controls at the investigated time points (n = 5–6 wells/treatment and time point; P < 0.05; Two-Sample T Test, Wilcoxon Rank Sum Test)

Fig. 7. Upregulation of interleukin (IL-)1ß, IL-6 and IL-8 mRNA expression in PECCs after stimulation with lipopolysaccharide (LPS) (Exp. 8). PECCs were stimulated with 2.5 μg/mL LPS. Cells were collected four and eight hours post stimulation (hps), and IL-1ß (a), IL-6 (b) and IL-8 (c) mRNA expression was investigated via qRT–PCR. The results are presented as the fold change in expression compared to that in nonstimulated cultures. Relative quantification was performed by qRT–PCR, after which the data were normalized to 28S rRNA. Error bars indicate the standard error of the mean (SEM). Asterisks indicate significant differences compared to the nonstimulated controls at the investigated time points (n = 5–7/treatment and time point; P < 0.05; Two-Sample T Test)
with previous findings in the chicken intestine, in which a greater density of α2,3-linked sialic acids was found in the small intestine [22, 45] and in which they were equally distributed in the caecum [45], which may be due to variations in sialic acid distribution according to breed, location or age. Age-related changes in sialic acid distribution and density have been described in the rat intestine, for example [46]. The genotype of the chicken may also play a role in the distribution of α2,3-linked and α2,6-linked sialic acids. For example, comparisons of MAL- and SNA-positive cells in different organs of Silky Fowl and White Leghorn chickens revealed significant differences in caecal tissue [47]. However, staining of caeca cryosections from the donor animals matched the staining pattern of the PECCs.

The expression pattern of TLRs in human intestinal epithelial cells has been well described [48–50], but the knowledge about the TLRs repertoire of chicken gut epithelial cells is insufficient. For the first time, we investigated in this study the TLR mRNA expression in isolated caecal epithelial cells from chickens, while others had just described it in caecal tissue [51, 52]. There was no correlation between the level of mRNA expression of the single TLR according to our results for isolated caecal cells and the results of these investigations. In both other studies, whole-tissue samples of the caecum were analysed. In contrast to our investigations, this should include tissue from the Lamina propria mucosae, Lamina muscularis mucosae, Tunica muscularis, Tunica serosa, immune cells and blood cells, which could explain the differences in TLR expression in the analysed samples. The different genetic backgrounds of broiler and SPF-layer-type chickens may also be a possible explanation for the different results [53]. It is known that during embryological development, TLR expression in tissues undergoes fluctuations [54], and in the first days of life, chicken enterocytes and the microbiome undergo maturation [55]. Differences in the age of the donor chickens and maturation of tissues could also explain the differences in TLR expression patterns. Another study analysing the avian TLR repertoire in different tissues, including the caecum of eight-week-old White Leghorn chickens as well as in selected chicken cell lines including HD11 cells, supported our findings. The ratio of TLR mRNA expression in caecal tissue to that in HD11 cells showed the same trend for all investigated TLR in PECCs and HD11 cells [56].

The use of the PECCs in virus–host interaction studies requires data on the baseline expression of immune parameters. In an in vitro study with chicken duodenal epithelial cells, infection with low pathogenic Avian Influenza Virus A and velogenic Newcastle Disease virus induced the upregulation of type I and III IFN [21]. Therefore, PECCs were also investigated for their ability to express type I and III IFN after TLR3 stimulation by the synthetic dsRNA poly(I:C) [17].

The low expression levels of Type I IFN mRNA and high upregulation of Type III IFN mRNA after poly(I:C) stimulation correspond to in vitro viral infection studies on avian small intestinal epithelial cultures [21] and respiratory [34] and oviduct tissues of chickens [57]. Furthermore, the expression of IFNλ in chickens is similar to the expression of mammalian IFNλ [58, 59], which is induced predominantly in mucosal epithelial cells and plays a critical role in the epithelial antiviral immune response [60].

Many bacterial infections are associated with the upregulation of proinflammatory cytokines [37, 38, 61]. The upregulation of IL-1β, IL-6 and IL-8 mRNA expression in adult PECCs exposed to LPS is in accordance with the findings of studies in avian primary embryo intestinal cells [19, 62] after bacterial infection or stimulation with LPS. Although the expression of the IL-8 mRNA was significantly upregulated at 4h post stimulation (P < 0.05) compared to that in the unstimulated controls, only low expression levels of the IL-8 mRNA were detected. Comparison of IL-8 secretion in IFNγ-primed and unprimed human intestinal epithelial HT-29 cells after stimulation with Vibrio cholae LPS revealed a distinct, dose-dependent upregulation of IL-8 secretion in IFNγ-primed cells but no upregulation in unprimed HT-29 cells. This finding suggested the necessity of co-factors for intestinal epithelial cells to produce IL-8 [63]. However, adult chicken PECCs upregulated IL-8 mRNA expression without priming with IFNγ.

In conclusion, our study revealed that PECCs from adult chickens represent a useful tool for investigating chicken-pathogen interactions at the intestinal epithelial level. Despite its limited lifetime, PECCs differentiated and showed epithelial properties. The presence of binding sites as well as pattern recognition receptors and a tissue-specific immunologic response enable PECCs to act as suitable in vitro models for host–pathogen interaction studies, which are difficult to conduct under in vivo conditions.

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Authors’ contributions: TW, AK and SR planned the experiments; TW and AK performed the experiments except the experiments; XH and AS investigated the TLR repertoire; TW and SR wrote the manuscript, which was reviewed by all the authors.

Conflict of interest: The authors declare no conflicts of interest.

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LIST OF ABBREVIATIONS

BSA bovine serum albumin
CEFs chicken embryo fibroblasts
DAPI 4',6-diamidino-2-phenylindole
DMEM Dulbecco’s Modified Eagle’s medium
FBS fetal bovine serum
hps hours post stimulation
IL interleukin
LPS lipopolysaccharide
MAL Maackia Amurensis
PBS phosphate-buffered saline
PECCs primary epithelial caecal cells
poly(I:C) polyinosinic-polycytidylic acid
qRT-PCR real-time quantitative RT-PCR
RT room temperature
SD standard deviation
SEM standard error of the mean
SNA Sambucus nigra
SPF specific pathogen-free
TEM transmission electron microscopy
TLR Toll-like receptor
TLRs Toll-like receptors

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