Avian pathogenic *Escherichia coli* infection of a chicken lung epithelial cell line

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A B S T R A C T

Virulent strains of *Escherichia coli* (Avian Pathogenic *E. Coli*: APEC) can cause initial infection of the respiratory tract in chickens potentially leading to systemic infection called colibacillosis, which remains a major cause of economic losses in the poultry industry. The role of epithelial lung cells as first targets of APEC and in initiating the innate immune response is unclear and was investigated in this study. APEC was able to adhere and subsequently invade cells from the chicken lung epithelial CLEC213 cell line exhibiting pneumocyte type II-like characteristics. Invasion was confirmed using confocal microscopy after infection with GFP-labelled APEC. Moreover, the infection resulted in a significant increase in IL-8 gene expression, a chemo-attractant of macrophages and heterophils. Gene expression of interferon α and β were not significantly upregulated and chicken Surfactant Protein A, also did not show a significant upregulation on either gene or protein level. The immune response of CLEC213 cells towards APEC was shown to be similar to stimulation with *E. coli* LPS. These results establish CLEC213 cells as a novel model system for studying bacterial infection of the lung epithelium and show that these cells may play a role in the initial innate response towards bacterial pathogens.

1. Introduction

Escherichia coli (*E. coli*) is a natural inhabitant of the chicken's intestinal tract and to a lesser extent also of the trachea. However, virulent strains of *E. coli* (Avian Pathogenic *E. coli*: APEC) can cause initial infection of the respiratory tract potentially leading to systemic infection and disease in chicken (Dziva and Stevens, 2008). Colibacillosis is currently a major cause of economic losses in the poultry industry, due to decreased hatching rates, egg production, growth and increased mortality (Zhuang et al., 2014). There is no highly effective vaccine available to protect against APEC mainly due to the diversity of APEC strains in the field, and therefore often antibiotic-based treatment is required.

It is still unclear what makes an *E. coli* strain virulent in chickens. APEC strains from more than 6 serotypes have been identified often displaying multiple antibiotic resistance genes, but no clear systematic association with the APEC phenotype can be found (Guabiraba and Schouler, 2015). However the presence of 4 specific genes located on a the large colV virulence plasmid is found in approximately 70% of APEC strains (Schouler et al., 2012). Nevertheless, a study comparing virulent and non-virulent *E. coli* could actually not find significant differences in lung histology of infected chickens nor in induction of apoptotic activity in lung cells, indicating again the lack of a thorough understanding of what makes certain *E.* coli strains pathogenic in chicken (Horn et al., 2012).

APEC pathogenesis has been studied mainly through the use of experimental infection models (Antao et al., 2008; Matthijs et al., 2009, 2017). Upon infection, heterophils and macrophages are attracted to the site of infection where they interact with bacteria. Heterophils are the fastest responders to an *E. coli* infection appearing within 6 h post infection contributing to bacterial clearance by degranulation, and the release of antibacterial compounds (Pourbakhsh et al., 1997). Phagocytosis of *E. coli* by macrophages has been observed *in vivo* and some studies actually correlates virulence genes of *E. coli* to resistance towards phagocytosis (Pourbakhsh et al., 1997; Mellata et al., 2003). In a recent study from our group it was shown that chicken macrophages (HD11 cells) *in vitro* are capable of providing an immune response towards APEC comparable to non-pathogenic *E.* coli (Peng et al., 2018a).

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Besides macrophages and heterophils, other leukocytes such as NK cells could also contribute significantly to the innate response towards *E. coli*, but not many studies are present on this subject.

The exact role of lung epithelial cells as first line cellular target initiating innate immune responses towards invading respiratory APEC has not been studied in the chicken. A few studies have described adhesion characteristics of APEC to primary cell cultures of type II pneumocytes derived from 14 day-old chicken embryos (Zhang et al., 2014; Peng et al., 2018b), but more extensive studies on immune responses are hampered by the relative difficulty to isolate epithelial cells from tissues. However, recently a new chicken lung epithelial cell line (CLEC213 cells) was described that showed many characteristics of type II pneumocytes, including the presence of cilia, alkaline phosphatase activity, and importantly the presence of pulmonary Surfactant Protein A (SP-A) mRNA (a protein abundantly expressed by mammalian lung epithelial type II cells) (Esnault et al., 2011). This cell line can be permissive to various chicken pathogens and is capable of developing a pro-inflammatory immune response, as was shown towards Influenza A viral infection and upon LPS stimulation (Esnault et al., 2011; Meyer et al., 2017). In addition, CLEC213 cells were described as a novel chicken epithelial model system to study gametogony of *Eimeria Tenella* (Bussiere et al., 2018). However, except for a single study where the cell line was mainly used as a tool to determine the importance of the Salmonella T3SS secretion system (Rossignol et al., 2014), no bacterial infection studies with these cells have been performed. In this study we determined the interaction of APEC with these chicken epithelial cells. Invasion characteristics of APEC were determined, and the innate immune response of epithelial cells was measured and compared to stimulation of these cells by *E. coli* LPS.

2. Methods

2.1. Bacterial strains

Avian pathogenic *Escherichia coli* 506 (O78, K80) isolated from chicken (Cuperus et al., 2016) was used in this study. Bacteria were cultured in Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) at 37 °C. For preparation of the green fluorescent protein (GFP) – expression APEC strain, the plasmid pWM1007 was transformed into APEC 506 (O78, K80) by electroporation using an Electro Cell Manipulator according to the manufacturer's instructions. GFP-expression APEC was cultured in the same condition as APEC (Peng et al., 2018a).

2.2. Chicken lung epithelial cells

The chicken lung epithelial cell line CLEC213 (Esnault et al., 2011) was maintained in a humidified 41 °C incubator with 5% CO_2 and cultured in advanced DMEM supplemented with 4% Fetal calf serum, GlutaMax (Thermo Fisher Scientific) and antibiotics (100 U penicillin/mL, 100 µg streptomycin/mL). Aliquots of cell suspension were seeded into 12 well plates at 2×10^5 cells/well and cultured overnight to reach 100% confluence at about 4×10^5 cells/well before being used for assays described below.

2.3. Bacterial adhesion and invasion assays

Before CLEC213 cells were incubated with APEC, culture medium was removed, and cells were washed twice with PBS. APEC was grown from a 1/1000 dilution of an o/n culture to log-phase in 3 h in TSB. Bacteria were pelleted by centrifugation for 10 min at 2500 x g, washed 1x in PBS and resuspended in cell culture medium (see paragraph 2.2) without antibiotics. Aliquots of 1 mL of bacterial suspension ($10^6 – 10^8$ CFU/mL) were added to each well. For association assays, the CLEC213 cells were incubated for 1, 2 and 3 h with APEC, washed three times with advanced DMEM medium (without supplements) and lyzed in 1% Triton X-100 in PBS at room temperature for 5 min to release the

associated bacteria. The suspensions were serially diluted and 100 µl of each dilution was plated on Trypticase Soy Agar (TSA, Oxoid Limited). From this, total cell-associated viable bacteria (both cell-adherent and intracellular) were calculated. For invasion assays, 1 ml of colistin at 250 µg/ml per well in advanced DMEM supplemented with Glutamax and 4% FCS was added to the CLEC213 cultures for 1 h to kill the remaining extracellular bacteria. Then the cells were washed, treated with Triton X-100 and plated out as described above to enumerate the number of invaded, intracellular bacteria. The number of adhered bacteria was calculated as : number of cell associated – number of intracellular bacteria. Experiments were performed in at least three independent experiments in duplicate.

2.4. Metabolic activity

Metabolic activity of CLEC213 cells was determined by the WST-1 assay according to the manufacturer's instructions (Roche, Basel, Switzerland). Absorbance was measured after 30 min at 450 nm with a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany) and was corrected for absorbance at 630 nm. Non-infected control cells were defined as 100% mitochondrial activity.

2.5. Gene expression

CLEC213 cells were infected with 1 ml of 1×10^7 CFU/ml APEC (MOI = 25) at 41 °C for 3 h and subsequently treated with 250 µg/ml colistin as described above, or were stimulated with several doses of LPS ranging from 0.1 to 50 mg/ml (LPS EB: from E. coli O111:B4, Invivogen, Toulouse, France). After 4 and 24 h of culture total RNA was extracted by Trizol reagent (Ambion, Carlsbad, CA) according to manufacturer's instructions. RNA (500 ng) was reverse transcribed using the iScript cDNA synthesis kit according to the manufacturer's instructions. Quantitative real time PCR was performed on a CFX Connect qPCR with CFX Manager 3.0 (Bio-Rad). Reactions were performed as follows: 3 min at 95 °C; 40 cycles: 10 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Relative gene expression levels were normalized against the expression levels of the house keeping genes GAPDH and 28S. Primer and probe sequences of the genes determined are depicted in Table 1.

2.6. Griess assay

To determine NO production, CLEC213 cells were incubated with APEC as described above for qPCR analysis. Subsequently nitrite, a stable metabolite of NO, was measured by the Griess assay in the cell culture supernatant as described before (Peng et al., 2018a).

Table 1
Primer and probe sequences for qPCR.

Gene		5'→3'sequence
GAPDH	Forward	GCCGTCCTCTCTGGCAAAG
	Reverse	GTAAACCATGTAGTTCAGATCGATGA
	probe	AGTGGTGGCCATCAATGATCCC
IL-8	Forward	GCCCTCCTCCTGGTTTCA
	Reverse	CGCAGCTCATTCCCCATCT
	probe	TGCTCTGTCGCAAGGTAGGACGCTG
cSP-A	Forward	GGAATGACAGAAGGTGCAATCAG
	Reverse	GCAATGTTGAGTTTATTAGCTACAAATG
	probe	CCGGCTTGTTGTCTGCCAGTTTTAGTGG
IFNα	Forward	GACAGCCAACGCCAAAGC
	Reverse	GTCGCTGCTGTCCAAGCATT
	probe	CCGGCTTGTTGTCTGCCAGTTTTAGTGG
IFNβ	Forward	CCTCCAACACCTCTTCAACATG
	Reverse	TGGCGTGTGCGGTCAAT
	probe	TCC-ACCGCTACACCCAGCACCTCG

2.7. Confocal microscopy

CLEC 213 cells were seeded on a 12 mm coverslip in 24-well plate and incubated overnight at 41 °C to reach confluence. Cells were subsequently infected with GFP-APEC (MOI = 25) for 3 h at 41 °C. After three wash steps with plain advanced DMEM, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature (RT). Subsequently, cells were incubated with 50 mM NH$_4$Cl in PBS for 10 min at RT and blocked with 5% normal goat serum in PBS for 1 h to block non-specific antibody staining. Then, cells were stained with *E. coli* antiserum (1:500) (Dwars et al., 2009) for 1 h. After the wash steps, cells were incubated with Donkey anti-Rabbit Alexa 647 (1:100) (Jackson ImmunoReasearch,West Grove, PA, USA) for 1 h. Finally, cells were washed with PBS or water and mounted in FluoroSave. Slides were observed on a Leica SPE-II DMI4000 microscope with LAS-AF software (Leica, Wetzlar, Germany) using a 63 × HCX PLAN APO OIL CS objective.

2.8. Western blotting

The presence of cSP-A in the CLEC 213 protein fraction was measured by Western Blot using mouse anti-cSP-A antibodies as described before (Zhang et al., 2016a). In short, CLEC213 cells were infected with APEC or stimulated with LPS as described above, after which CLEC213 cell proteins and secreted proteins in the supernatant, were dissolved in denaturing SDS sample buffer and separated on a 10% SDS-PAGE gel. Subsequently, proteins were blotted on nitrocellulose (Protran BA83, Whatmann, Sigma-Aldrich). cSP-A was detected using monoclonal mouse anti-cSP-A antibodies as 1st antibody and horse radish peroxidase labelled Goat anti Mouse antibody (Sigma-Aldrich) as 2nd antibody.

2.9. Statistical analyses

Results are presented as the mean $\pm$ standard error of the mean (SEM) of at least three independent experiments. Statistical significance was assessed with Two-way ANOVA followed by the Tukey Post-Hoc test in Prism software, version 6.02 (Graphpad, La Jolla, CA, USA). Differences were considered statistically significant at p < 0.05.

3. Results and discussion

3.1. Adhesion and invasion characteristics of APEC

Initial adherence studies were performed for 1 h with different densities of APEC (1 ml 10^6 – 10^8 CFU/ml; corresponding to an MOI of 2.5, 25 and 250, respectively) as shown in Fig. 1A. Increased inoculum density resulted in an increased APEC adherence. If the adherence was expressed as percentage of the inoculum, the values correspond to approximately 7.5% for MOI 2.5 and 25, and 2.5% for the highest bacterial density (MOI 250). Based on this, an inoculum density of 10^7 CFU/ml (MOI 25) was chosen for further studies.

The time-dependent adhesion and invasion of APEC to the CLEC213 cells is shown in Fig. 1B and C. Adherence of APEC at the initial MOI 25 significantly increased over time from 1 h to 3 h of incubation, roughly corresponding to 6 bacteria adhering to one CLEC213 cell after 3 h (2.5 × 10^6 CFU/ well vs 4 × 10^5 CLEC213 cells/well) (Fig. 1B). Invasion showed a similar trend towards higher number of invading bacteria with 4.5 × 10^4 CFU/well after 3 h (Fig. 1C). This indicates that after 3 h less than 2% of adhered bacteria was able to invade CLEC213 cells. In addition, after removal or killing of extracellular bacteria, the number of invaded bacteria was followed over time. As shown in Fig. 1D, the viability of invaded bacteria actually decreased over time: 1 log reduction in viability (10% survival) was observed within the first 6 h leading to a complete loss of detection of viable bacteria after 24 h. Additional time points would have aided in a more precise

determination of the kinetics of viability loss, but from the data it is clear that invasion did not lead to multiplication of APEC intracellularly but actually led to killing of APEC. It is unclear if this could be a bacterial strain specific effect or whether CLEC213 cells can potentiate an efficient intracellular immune response against a broader range of (invaded) bacteria.

Studies with isolated cultured chicken type II pneumocytes have shown similar high adherence of APEC, causing cell damage and the loss of microvilli (Zhang et al., 2014, 2016b), but invasion into type II cells was not determined. APEC was also able to adhere to chicken breast and human colorectal adenocarcinoma cells (HCT-8) cells indicating that the adhesion is not specific for pneumocytes (LeStrange et al., 2017). With respect to invasion characteristics of APEC, one study tested this on a chicken hepatocyte cell line and found it to be relatively high (8% of the total adhered bacteria) but this number was much lower (0.2%) when tested on human type II cells (Chanteloup et al., 2011). Overall this is the first study quantitively determining the time and density dependency of APEC adhesion and invasion of chicken lung epithelial cells.

Next, confocal microscopy was used to confirm the presence of intracellular bacteria after APEC infection in CLEC213 cells. GFP-producing APEC was used to infect CLEC213 cells for 3 h after which an antibody derived against *E. coli* was used to detect the remaining adherent bacteria. Since the CLEC213 cells were not permeabilized, only extracellular APEC was detected by the antibody. As shown in Fig. 2, extracellular and intracellular bacteria can clearly be distinguished. Double labelled (yellow) are available for the anti- *E. coli* antibody and thus are extracellular, while intracellular APEC are shielded from the antibody and only show the green GFP signal. Control experiments to validate the model without infection or with permeabilized cells showed no staining or only double labelled bacteria, respectively, (data not shown). These results confirm that APEC can invade CLEC213 cells and shows that the more indirect results obtained by the adhesion/invasion assay was not caused by, for example, incomplete killing of extracellular bacteria. In addition, a similar experiment was performed with APEC treated with gentamycin. No intracellular localization of these non-viable bacteria was observed, showing that invasion is an active process. Our results correspond well to an earlier study where intracellular APEC in cultured chicken type II pneumocytes were detected using transmission electron microscopy (Zhang et al., 2016b), although this technique requires several fixation and staining steps, unlike our current confocal imaging set-up.

3.2. Immune response upon APEC infection

CLEC213 cells were infected for 3 h with APEC after which extracellular bacteria were killed by colistin. At 4 and 24 hpi, gene expression of several immune genes were determined. The largest effect was observed for IL-8 which showed a 6-fold increase in gene expression at 4 hpi. Interestingly, IL-8 is known for its chemotactic activity for macrophages and heterophils in chicken (Poh et al., 2008), indicating that the observed increase in macrophages in APEC infections *in vivo* could be partially explained by the initial epithelial response after the first interaction with the respiratory epithelia. Surfactant protein A and IFN-α showed a tendency towards upregulation at 4 hpi but this difference did not reach statistical significance (Fig. 3A). Gene expression was also determined after 24 h, but no significant upregulation could be observed anymore indicating a relatively short immune response for at least the genes studied. Metabolic activity (WST-1 assay) or viability (cell count, tryphan blue exclusion) of CLEC213 cells was not affected by bacterial infection after 4 and 24 h (data not shown).

3.3. LPS stimulation of CLEC 213 cells

In the next set of experiments, CLEC213 cells were stimulated with the potent immune stimulant LPS derived from *E. coli* (Fig. 3B). At the
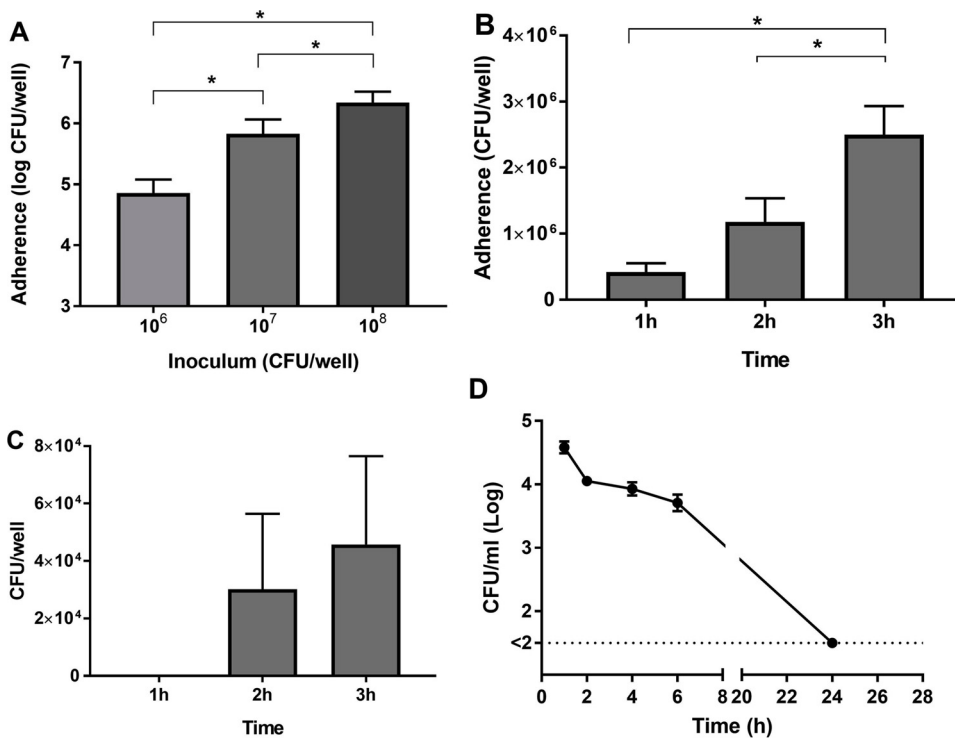
Fig. 1. Adherence and invasion characteristics of APEC.

A) CLEC213 cells were incubated for 1 h with different densities of APEC. After washing of excess bacteria, associated bacteria were plated out and counted. B) CLEC213 cells were incubated with 10^7 CFU/ml for 1–3 h, associated bacteria were determined as in A. C) CLEC213 cells were incubated with 10^7 CFU/ml for 1–3 h, after which extracellular bacteria were killed with colistin. Intracellular bacteria were determined by colony counting. D) CLEC213 cells were incubated with 10^7 CFU/ml for 3 h, after which extracellular bacteria were killed with colistin. Intracellular survival of APEC was followed in time, determined by colony counting. Data are shown as mean ± SEM of at least 3 independent experiments for each group (in duplicate). * indicates significant difference (P ≤ 0.05).
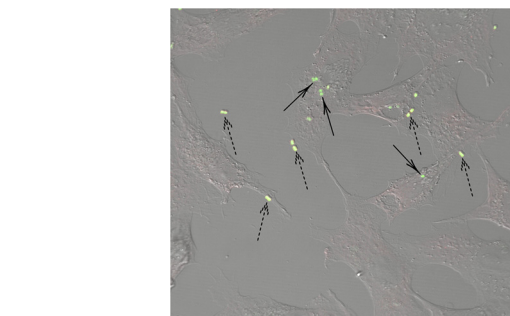

Fig. 2. APEC is partially located intracellularly in CLEC213 cells.

CLEC213 cells were incubated with GFP-APEC for 3 h. Subsequently, cells were washed and fixed, but not permeabilized. Extracellular APEC were labeled with rabbit anti E. coli antibody and Donkey anti-Rabbit Alexa 647 (red). CLEC213 cells appeared grey under the DIC channel.

Intracellular bacteria appear green (solid arrows) while extracellular bacteria appear as red/yellow (dotted arrows).

highest concentration of LPS a similar response as seen for APEC infection was observed and comparable to earlier studies using this cell line using LPS (Esnault et al., 2011) or influenza virus infection (Meyer et al., 2017). IL-8 was significantly upregulated, while cSP-A showed a non-significant tendency towards an increased expression. The large variation in cSP-A expression observed in both the LPS and APEC stimulation/infection of CLEC213 cells could be partially explained by the low absolute level of cSP-A gene expression. The levels of cSP-A mRNA that were measured in these experiments were close to or just beyond the detection limit where a linear concentration – response correlation was observed, likely causing lower reproducibility of the data. However, since this protein is highly expressed in type II cells (in mammals) it was still valuable to show. Besides gene expression, the presence of cSP-A was also tested on a protein level by western blot using cSP-A specific antibodies. No cSP-A could be detected in either bacterially infected, LPS-stimulated or non-stimulated CLEC213 cells or their supernatants (data not shown). This apparent lack of detectable cSP-A in CLEC213 cells could indicate that they are a different cell type than
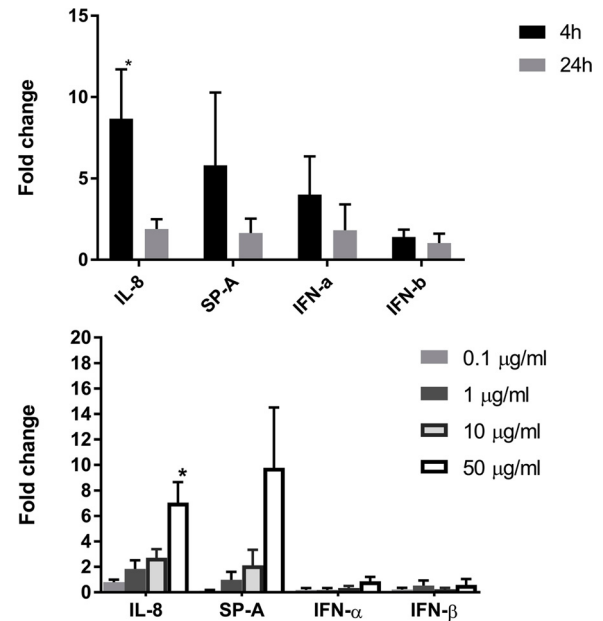

Fig. 3. Immune response of CLEC213 cells.

A) CLEC213 cells were incubated with 10^7 CFU/ml for 3 h, after which non-associated bacteria were washed away. At 4 and 24 h post infection mRNA was isolated and quantitative PCR was used to determine gene expression of selected genes. B) CLEC213 cells were incubated with *E. coli* LPS at the indicated concentrations for 4 h. Gene expression was determined using qPCR. Data are shown ± SEM of at least three independent experiments for each group (in duplicate). * indicates significant difference (P ≤ 0.05).

surfactant producing cells in the chicken lung, or that these cells require a different stimulus *in vitro* to produce cSP-A. Expression of cSP-A was expected in CLEC213 cells, based on the presence of lamellar bodies which have been detected in long-term cultures (Esnault et al., 2011), however the number remains much lower compared to what can be observed in the chicken lung by electron microscopy (Bodi et al., 2016).

In addition, lamellar bodies are not necessarily related to pulmonary surfactant synthesis, since they represent a general storage form of secretory lipids in multiple cell types (Schmitz and Muller, 1991).

In birds, there is not a clear distinction into only two types of alveolar epithelial cells as found in mammals. In the latter, alveoli contain elongated type I cells that are involved in gas exchange, while cuboidal type II cells contain secretory vesicles in which pulmonary surfactant is stored as surfactant-protein rich lamellar bodies. On the contrary, in birds besides granular secretory cells also squamous atrial and squamous respiratory cells (and squamous intermediate cells) are observed. Secretion of surfactant like material is not limited to the granular cells since also the squamous cells secrete a trilaminar substance that covers the epithelium (Bodi et al., 2016; Scheuermann et al., 1997). Although immunohistochemistry has shown the presence of cSP-A in specific atrial cells (Zhang et al., 2016a), while also another antibody CVI-ChNL 74-3 was described to recognize secretory type II cells (Kocsis et al., 2012), it should possibly be concluded that the simple classification in epithelial type I and type II cells is convenient, but oversimplified when used for chicken lungs.

Finally, besides gene expression, also the NO production by CLEC213 cells after LPS stimulation was measured. Although NO production is a common feature for (stimulated) macrophages, the current lack of knowledge on the exact lung epithelial immune defence prompted us to check this. As expected, NO levels were low irrespective of LPS concentration (1–50 mg/ml) or duration of stimulation (4–24 h) (data not shown).

Overall, this study indicates that CLEC213 cells are a valuable tool to determine host pathogen interaction in the chicken lung, and can help in understanding the host response towards bacterial infections.

Competing interest statement

The authors declare to have no competing interests.

Acknowledgements

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