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ORIGINAL ARTICLE

Chicken endothelial cells are highly responsive to viral innate immune stimuli and are susceptible to infections with various avian pathogens

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ABSTRACT

It is well established that the endothelium plays a prominent role in the pathogenesis of various infectious diseases in mammals. However, little is known about the role of endothelial cells (EC) as targets for avian pathogens and their contribution to the pathogenesis of infectious diseases in galliform birds. First, we explored the innate immune response of primary chicken aortic endothelial cells (pchAEC), obtained from 18-day-old embryos, to stimulation with pathogen-associated molecular patterns or recombinant chicken interferons (type I, II and III IFNs). In spite of the abundant expression of a number of innate immune receptors, marked cytokine responses to stimulation with pathogen-associated molecular patterns were only seen in pchAEC treated with the TLR3 agonist polyI:C (pI:C) and the MDA5 agonist liposome-complexed polyI:C (L-pI:C), as was assessed by quantitative PCR and luciferase-based IFN-I/NFκB reporter assays. Treatments of pchAEC with IFN-α, IFN-γ and IFN-λ resulted in STAT1-phosphorylation/activation, as was revealed by immunoblotting. Next, we demonstrated that pchAEC are susceptible to infection with a variety of poultry pathogens, including Marek's disease virus (MDV), infectious bursal disease virus (IBDV), avian pathogenic *Escherichia coli* (APEC) and *Eimeria tenella*. Our data highlight that chicken EC are potential targets for viral, bacterial and protozoan pathogens in gallinaceous poultry and may partake in the inflammatory and antimicrobial response. The pchAEC infection model used herein will allow further studies interrogating avian pathogen interactions with vascular EC.

RESEARCH HIGHLIGHTS

- Use of a well-defined primary chicken aortic endothelial cell (pchAEC) culture model for studying avian host–pathogen interactions.
- pchAEC are responsive to innate immune stimulation with viral pathogen-associated molecular patterns and chicken type I, II and III interferons.
- pchAEC are susceptible to infections with economically important poultry pathogens, including MDV, IBDV, APEC and *Eimeria tenella.*

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Chicken; endothelial cells; innate immunity; inflammation; immune receptors; avian pathogens

Introduction

The vascular endothelium lines the interior surface of blood vessels and encompasses a massive amount of endothelial cells (EC) within the vertebrate body. However, EC not only form the vascular barrier, they also possess a wide array of distinct physiological functions, ranging from the control of the vasomotor tone, blood coagulation and vascular permeability. Furthermore, it is well established that EC are potent immune regulator cells controlling the trafficking of various leukocyte populations and promoting or impeding immune reactions (Young, 2012; Rodrigues & Granger, 2015; Yau *et al.*, 2015).

Thus, it is not surprising that endothelial cell dysfunction (ECD) plays a central role in the pathogenesis of a large variety of pathological conditions, including various infectious diseases. In general, microbial pathogens can cause ECD, either by directly targeting and damaging endothelial cells, or by inducing an exacerbated cytokine response that leads to uncontrolled EC activation. The possible outcomes of pathogen-induced ECD are multifold and may include endothelial barrier breakdown, vascular leakage, oedema, disseminated intravascular coagulation, haemorrhages and excessive inflammation. Intriguingly, these ECD-associated pathological patterns are hallmarks of

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some of the most fatal human microbial diseases including viral haemorrhagic fevers, influenza A virus-induced acute respiratory distress syndrome, *Plasmodium falciparum* malaria and bacterial sepsis (Steinberg *et al.*, 2012; Miller *et al.*, 2013; Khakpour *et al.*, 2015).

EC-mediated inflammatory responses to pathogens are generally confined to the infected tissue compartment, but, especially in the case of highly pathogenic infectious diseases, may become systemic. These responses are initiated through activation of membrane and intracellular pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs) and RIG-like receptors (RLRs), by pathogen-associated molecular patterns (PAMPs) or damaged tissue debris (Salvador *et al.*, 2016). EC activation results in the production of inflammatory mediators (including chemokines and cytokines) and the upregulation of surface adhesion molecules (e.g. selectin E/P and VCAM-1), thereby promoting the early recruitment of innate immune cells, such as neutrophils and monocytes, into the damaged/infected tissue (Langer & Chavakis, 2009; McEver, 2015; Silva *et al.*, 2018).

Collectively, these observations emphasize the major role for EC, or more precisely ECD, in the pathogenesis of various fatal infectious diseases in mammals. As discussed later in this paper, various lines of evidence indicate that EC infection and/or ECD may also play a central role in the pathogenesis of some of the most important infectious diseases in gallinaceous poultry, including Marek's disease (MD) and avian colibacillosis. Recently, we have reported on the use of a chicken EC culture model to study the mechanisms underlying the distinctive endotheliotropism of highly pathogenic avian influenza (HPAI) viruses in gallinaceous birds (Lion *et al.*, 2018). In the present study, we applied a well-established protocol for the preparation of macrovascular EC from aortic explants (McGuire & Orkin, 1987) to isolate and culture primary EC from the aortas of 18-day-old chicken embryos. The primary chicken aortic endothelial cells (pchAEC) were used to assess, for the first time, (i) the innate immune responses of chicken EC stimulated with various pathogen-associated molecular patterns (PAMPs) or type I, II and III interferons (IFN-α, IFN-γ and IFN-λ); and (ii) the susceptibility of chicken EC to infections with a panel of poultry pathogens, including Marek's disease virus (MDV), infectious bursal disease virus (IBDV), avian pathogenic *Escherichia coli* (APEC), and the protozoan *Eimeria tenella (E. tenella)*. Our data identify chicken EC as a biologically relevant cell type that may partake in the disease development induced by all the avian pathogens tested. The well-defined pchAEC infection model presented here is expected to spur further studies on the interactions of microbial pathogens with EC of galliform host species.

Materials and methods

Cell culture

Primary chicken aortic endothelial cells (pchAEC) were prepared from 18-day-old embryos by applying a tissue/cell culture protocol for obtaining EC from aorta explants, exactly as described previously (Lion *et al.*, 2018). Chicken embryos were obtained from outbred White Leghorn PA12 chickens (Vu Manh *et al.*, 2014) raised under specific-pathogen-free (SPF) conditions at the PFIE animal experimental platform (INRA Centre Val de Loire, 37380 Nouzilly, France). Localization of the ascending aorta in the embryonic chicken heart, outgrowth of primary cells from cultured aorta explants and pchAEC monolayer formation (passage 2) are shown in Figure 1(a,b). For use in the immune stimulation assays and infection experiments described below, pchAEC (passage 2) were distributed in either 24-well ($1–5 \times 10^5$ cells per well) or 6-well culture plates (10^6 cells per well) and cultured until approx. 80% confluence for 24–48 h.

LMH chicken hepatocellular carcinoma cells (ATCC, CRL-2117) and CLEC213 chicken lung epithelial cells (Esnault *et al.*, 2011) were cultured at 40°C (5% CO_2) in DMEM/F12 medium (Gibco) supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and 1% penicillin–streptomycin (Sigma), in gelatin-coated and not-coated plastic Petri dishes respectively. Chicken embryo fibroblasts (CEF) were prepared from 10-day-old embryos of PA12 chickens raised under SPF conditions at the PFIE animal experimental platform, or of White Leghorn chickens at the ANSES poultry facility (ANSES Ploufragan-Plouzané Laboratory, 22440 Ploufragan, France) using a standard protocol (Xing & Schat, 2000). CEF were cultured at 40°C (5% CO_2) in DMEM (Lonza) supplemented with 7.5% heat-inactivated foetal bovine serum and 1% penicillin–streptomycin. For use in the infection experiments and assays described below, LMH, CLEC213 and CEF were distributed in either 24-well ($1–5 \times 10^5$ cells per well) or 6-well culture plates (10^6 cells per well) and cultured until approx. 80% confluence for 24 h.

Indirect immunofluorescence assay (IFA)

Primary chAEC were grown on 13 mm glass coverslips until confluence, fixed with 4% paraformaldehyde (30 min), permeabilized with 1% Triton X-100 (15 min), and overlaid overnight at 4°C with an IFA blocking solution containing 2% BSA in PBS 0.5% Triton X-100. Abundance of endothelial cell-specific antigens was tested using a rabbit polyclonal antibody anti-human von Willebrand Factor (anti-vWF, Dako, Agilent, Santa Clara, CA) at a dilution of 1:500, and a rabbit polyclonal antibody anti-human endoglin/CD105 (anti-CD105, Interchim, Montluçon, France) at a

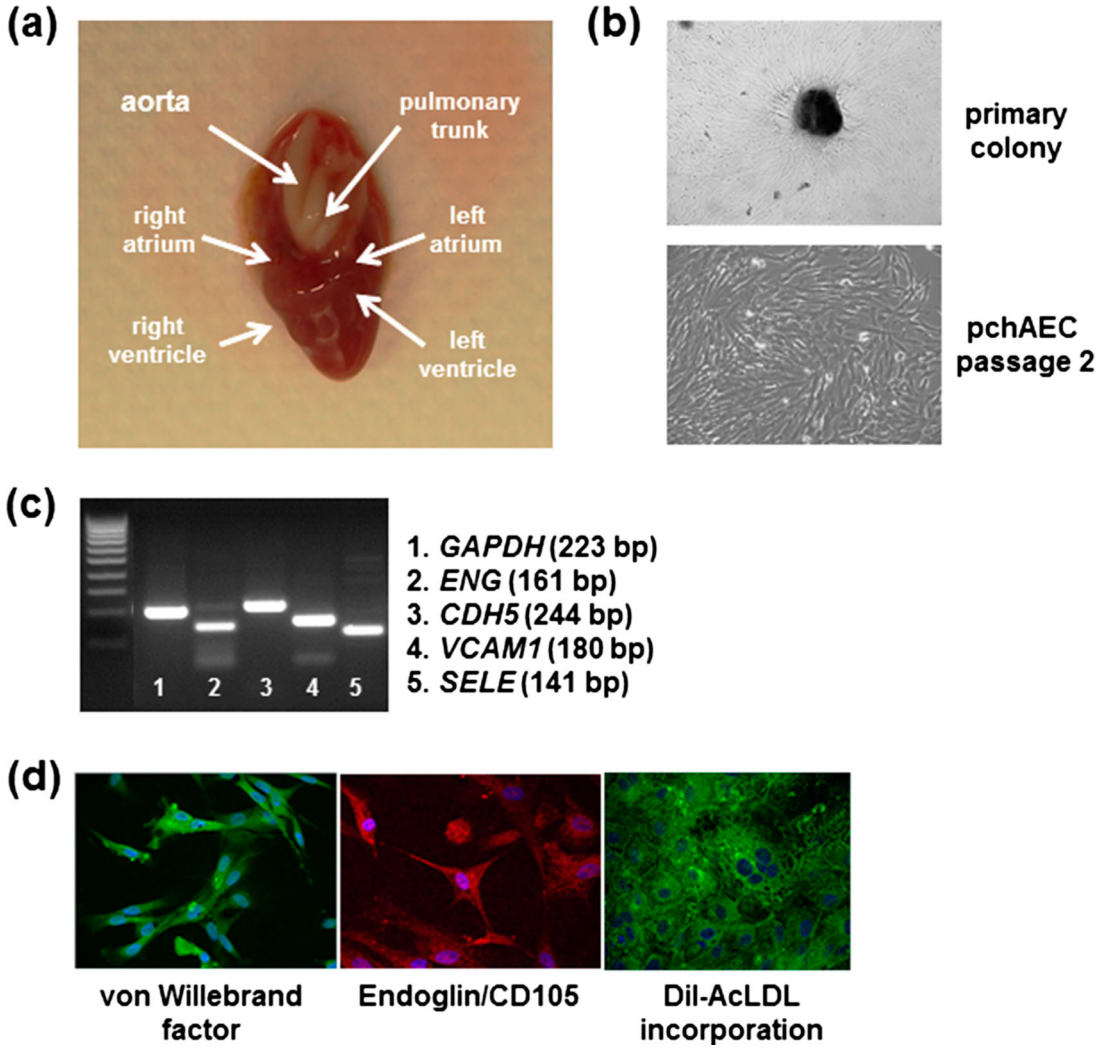

Figure 1. Primary chicken aortic endothelial cell (pchAEC) preparation, morphology and phenotyping. (a) 18-day-old embryo heart and aorta. (b) pchAEC colony formation and cell morphology at passage 2. (c) Transcription of endoglin (*ENG*), VE-cadherin (*CDH5*), VCAM (*VCAM1*) and E-selectin (*SELE*) genes as assessed by PCR. (d) Fluorescence microscopy images showing that pchAEC score positive for the presence of von Willebrand Factor (green) and endoglin (red). Cells actively incorporate Dil-acetylated-LDL (Dil-AcLDL) (green). Colour images are provided in the online Open Access version of this article (https://doi.org/10.1080/03079457.2018.1556386). At least three different cell batches were tested.

dilution of 1:20. Non-specific binding of rabbit immunoglobulins was tested using a rabbit polyclonal antibody directed against influenza virus non-structural protein 1 (Lion *et al.*, 2018) at a dilution of 1:500 as a negative control. Primary antibodies diluted in the IFA blocking solution were spread on the glass coverslips containing fixed cell monolayers for 1 h at room temperature (RT). Following washes with PBS, expression of vWF and CD105 was revealed by staining for 1 h at RT with an Alexa® 488- or Alexa® 594-conjugated secondary goat anti-rabbit IgG polyclonal antibody (Molecular Probes, Thermo Fisher Scientific, Waltham, MA) at a dilution of 1:2000.

Dil-Acetyl-LDL uptake assay

Primary chAEC were grown on 13 mm glass coverslips, and active incorporation of acetylated low-density lipoproteins (AcLDL) was tested by incubating the cells for 4 h at 37°C and 5% CO_2 with a 1:200 dilution of Alexa® 488-conjugated AcLDL (Molecular Probes) in complete cell culture medium.

Fluorescence imaging

Glass coverslips with antibody- or Alexa® 488-AcLDL-stained cells were mounted by dispersing 25 µl of the VECTASHIELD® H-1200 mounting reagent containing DAPI (Vector Laboratories, Burlingame, CA) on the coverslips. Mounted coverslips were imaged using a Zeiss Axiovert 200M inverted epifluorescence microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Gene expression analysis

RNA extraction and reverse transcription

Total RNA was extracted from pchAEC grown in 6-well or 24-well culture plates using the Nucleospin® RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Concentration and purity of

the RNA preparations were assessed using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA). For cDNA synthesis, 1 µg of RNA was DNAse-treated (RQ1 RNase-Free DNase, Promega, Madison, WI). After thermal denaturation for 5 min at 70°C, reverse transcription was carried out at 42°C for 1 h in a final volume of 25 µl Tris-HCL buffer, adding 0.5 µg Oligo(dT)15 primer, 25 units ribonuclease inhibitor RNAsin (Promega), 200 units M-MLV Reverse Transcriptase (Promega) and 5 µl dNTP (10 mM).

Polymerase chain reaction (PCR)

PCR amplification was carried out in a 50 µl reaction mixture containing 100 ng template DNA, 0.5 µl DNA polymerase Go Taq DNA Flexi (Promega), 5 pMol of each primer (Eurogentec, Liege, Belgium), 0.5 µl dNTP (10 mM), 5 µl $MgCl_2$ (25 mM), and 10 µl Go Taq Flexi buffer (5×) (Promega). Amplification was performed for 30 cycles (95°C for 45 s, optimum annealing temperature (54–56°C) for 30 s, 72°C for 30 s) in a thermal cycler (GeneAmp®PCR system 2700, Applied Biosystems, Foster City, CA). Sequences of specific primers for PCR amplification of *GAPDH* and the EC marker genes endoglin/CD105 (*ENG*), VE-cadherin (*CDH5*), *VCAM1* and E-selectin (*SELE*), as well as the NCBI accession numbers of the corresponding *Gallus gallus* mRNAs, are given in Table 1. PCR products were separated by electrophoresis in 1.5% agarose gels and visualized under UV light after staining with ethidium bromide.

Quantitative PCR (qPCR)

Baseline transcriptional expression levels of some selected pathogen recognition receptors (TLR3, TLR4, TLR5, TLR7 and MDA5) and the IFN I, II and III receptor subunits IFNAR1, IFNGR1 and IL28RA were assessed by SYBR Green qPCR for pchAEC, CLEC213 and CEF. Cells were grown in 6-well culture plates. Total RNA was extracted using the Nucleospin® RNA kit and reverse-transcribed cDNAs were synthesized as described above. For each cell type, 3–6 biological samples were analysed. PCR quantification was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). PCR amplification was carried out in 15 µl reaction mixtures containing 50 ng template DNA, 0.75 µl of forward and reverse primers (10 pmol/µl), and 7.5 µl iQ SYBR® Green PCR Master Mix (Bio-Rad). The amplification protocol was as follows: 95°C for 10 min; 40 cycles at 95°C for 10 s and at 62–68°C for 30 s. Sequences of specific primer pairs are given in Table 1. Cycle threshold (Ct) values and specificity of amplification products were analysed using the CFX Manager software (Bio-Rad). Determination of the Ct values for a serially-diluted standard plasmid (pGEM®-T Easy cloning vector, Promega) harbouring the corresponding amplicon sequence allowed calculation of the mRNA copy numbers (per 1 µg total RNA) for each target.

To explore immune gene expression dynamics in stimulated cells, pchAEC grown in 24-well culture plates (6 wells per condition) were treated for 6 h or 18 h with the following PAMPs diluted in endothelial growth medium: lipopolysaccharide (LPS from *E. coli* O55:B5, Sigma-Aldrich, St. Louis, MO) at a conc. of 10 µg/ml; HMW polyI:C (pI:C, InvivoGen, San Diego, CA) at a conc. of 10 µg/ml; LyoVec® (liposome)-complexed HMW polyI:C (L-I:C, InvivoGen) at a conc. of 1 µg/ml; and flagellin (from *Salmonella typhimurium*, InvivoGen) at a conc. of 100 ng/ml. Non-treated pchAEC (6 wells) served as controls Expression levels of *IFNB*, *MX1*, *IL1B*, *IL6*, *IL8*, *TNFSF15*, *CX3CL1* and *SELE* were assessed by applying the SYBR Green qPCR protocol described above. Sequences of the primer pairs used for qPCR-based gene expression analysis and the NCBI accession numbers of the corresponding *Gallus gallus* mRNAs are given in Table 1. Relative expression (fold change relative to control) of the targeted immune genes in response to treatments was calculated using the $2^{-\Delta\Delta CT}$ method with *GAPDH* serving as the reference gene for normalization.

IFN-I/NF-κB reporter assays

The effects of an immunostimulation of pchAEC on IFN-I signalling and NF-κB activation were measured by dual luciferase assays employing the Firefly reporter plasmid pGL3-chMx-Luc (kindly provided by Dr Nicolas Ruggli, IVI Mittelhäusern, Switzerland (Liniger *et al.*, 2012)) or pNF-kB-Luc (Agilent Technologies, Santa Clara, CA), and the Renilla normalization plasmid pRL-TK (Promega). Transfection mixtures containing 100 ng of pGL3-chMx-Luc or pNF-kB-Luc, 10 ng pRL-TK, 1 µl Lipofectamine® 2000 (Thermo Fisher Scientific) and 100 µl Opti-MEM (Thermo Fisher Scientific) were deposited into each well of a 24-well culture plate with pchAEC covered with endothelial growth medium (400 µl per well). Twenty-four hours post-transfection, the cells were washed with PBS and treated with 10 µg/ml LPS, 10 µg/ml pI:C, or 1 µg/ml L-pI:C. Non-treated pchAEC served as controls. Following 6 h or 18 h of treatment/culture, pchAEC were lysed with 200 µl 1x Passive Lysis Buffer (Promega), and Firefly/Renilla luciferase activity in the lysates was assessed using the Dual-Luciferase® reporter assay system (Promega) and a GloMax®-Multi luminescence plate reader (Promega).

STAT1 activation assay – western blot analysis

The responsiveness of pchAEC to treatments with IFN I, II and III was tested by a western blotting (WB) protocol for the detection of phosphorylated (activated) STAT1. Primary chAEC grown in 6-well culture plates

Table 1. PCR and real-time quantitative PCR primers.

Chicken gene name	GenBank accession number	Forward	Reverse
GAPDH	NM_204305.1	GTCCTCTCTGGCAAAGTCCAAG	CCACAACATACTCAGCACCTGC
ENG	NM_001080887.1	TCCTGATGCTGAACAACTGC	GTAGGAGGCGATGATGCTGT
CDH5	NM_204227.1	CCAAAGAAGCCCCTGGACTTCGA	CGTCGGGGTCATGTGCCCAA
VCAM1	XM_004936551.1	AGCTTGATGTCAAAGTTCCTCCT	AGGTTCCATTGACTGCTGGT
SELE	XM_422246.4	AATGCAAAGCTGTGACCTGC	GCGTGGATTGTCCTGTCAGA
TLR3	NM_001011691.3	GAAAGAGTTTCACACAGGATG	ACTGTGAGGTTTGTTCCTTG
TLR4	NM_001030693.1	TGCACAGGACAGAACATCTC	ATGTGGCACCTTGAAAGA
TLR5	NM_001024586.1	ACGAGATTTCTTGCCTGGGG	CTGAAGTACCTGCTCTGGGC
TLR7	NM_001011688.2	CACCGGAAAATGGTACATC	GGTTAATAGTCAGGGTCAGGT
MDA5	NM_001193638.1	CGCGACCCCGGATGGTTCAC	GTCTCAATCCCAACCAGGTCCTCC
IFNAR1	NM_204859.1	GATCTGGCACCCTCGACTTT	TGTTTACCGCCAGCTGTTCC
IFNGR1	NM_001130387.1	AACCTGAGCATCCCAGTTCC	TTGTGTACTCCAAGCCTGCG
IL28RA	XM_004947909.1	GGCATCACATTCTCACAGTTCC	CTCACTTCCAGGCAGTCGTT
IFNB	NM_001024836.1	GCTTCGTAAACCAAGGCACG	GAGCTCGACTTTTCATCCATTG
MX1	NM_204609.1	ACGTCCCAGACCTGACACTA	TTTAGTGAGGACCCCAAGCG
IL1B	NM_204524.1	TGGGCATCAAGGGCTACAAG	CCAGGCGGTAGAAGATGAAG
IL6	NM_204628.1	TTCGCCTTTCAGACCTACCT	TGGTGATTTTCTCTATCCAGTCC
IL8L2	NM_205498.1	CTGCGGTGCCAGTGCATTAG	AGCACACCTCTCTTCCATCC
TNFSF15	NM_001024578.1	TCTGAAGCAGCGAGCAGTAG	CAGGTATCACCAGTGCGTTG
CX3CL1	NM_001077232.1	TTCTCTCCAGATCCCGTTTG	GTTCAGTCTCGCGGTAGCTC

were treated for 30 min or 120 min with the following three chicken interferon preparations: (i) 2500 U/ml (final concentration) IFN-α produced in *E. coli* (Schultz *et al.*, 1995) and purchased from Bio-Rad; (ii) 100 ng/ml IFN-γ (final concentration) produced in *E. coli* (Weining *et al.*, 1996) and kindly provided by Prof Bernd Kaspers, LMU Munich, Germany; and (iii) a 1:50 dilution (final concentration) of a cell culture supernatant from HEK-293 cells overexpressing IFN-λ (Reuter *et al.*, 2014), kindly provided by Prof Peter Stäheli, University Freiburg, Germany. Non-treated cells served as controls. Treated and non-treated pchAEC were lysed using 2x Laemmli buffer and cell lysates were heated for 5 min at 95°C. Proteins were then separated on a 7.5% polyacrylamide gel in Tris-Glycin-SDS buffer and transferred on to a Porablot® nitrocellulose membrane (Macherey-Nagel). Following overnight immersion at 4°C in a WB blocking solution (3% non-fat milk powder in Tris-NaCl-Tween), the membranes were washed and incubated for 1 h at RT with either of the following primary antibodies diluted in the WB blocking buffer: mouse monoclonal antibody (mAb) anti-GAPDH (MAB374, 1:500, Merck-Millipore, Burlington, MA), mouse mAb anti-STAT1 (1:250, BD Biosciences, Franklin Lakes, NJ) and rabbit pAb anti-Phospho(Tyr701)-STAT1 (1:1000, Thermo Fisher Scientific). Membranes were washed and incubated for 1 h at RT with a mouse or rabbit IgG-specific secondary antibody coupled to horseradish peroxidase (1:10,000, Sigma). Finally, after washing, the membranes were overlaid with the WesternBright® ECL peroxidase substrate (Advansta, San Jose, CA) and chemiluminescence was visualized using a Fusion FX imaging platform (Vilber Lourmat, Collégien, France).

MDV infection

Primary chAEC seeded in 6-well culture plates were infected by co-culture with 50,000 chicken embryonic skin cells (CESC) infected with the BAC20-derived recEGFPVP22 virus encoding the MDV-VP22 tegument protein fused at its 5′ end to the *EGFP* gene (Blondeau *et al.*, 2008). BAC20 is an avirulent MDV cloned as an infectious bacterial artificial chromosome (Schumacher *et al.*, 2000). At 2, 3 and 6 days post infection (dpi), EGFP fluorescence was imaged using a Zeiss Axiovert 200M inverted epifluorescence microscope. Images of green-fluorescent plaques were captured using a CCD Axiocam MRm camera and the Axiovision software (Zeiss).

IBDV infection

CEF or pchAEC were seeded on 13 mm glass coverslips in 6-well culture plates. The following day, at confluence, cells were infected at an MOI of 1 with the following IBDV strains: Ct (serotype 1, attenuated vaccine strain adapted to CEF cell culture), 89163 (serotype 1, very virulent strain, unable to replicate in CEF) or TY89 (serotype 2, avirulent strain able to replicate in CEF). Twenty-four hours post-infection, cells were fixed with an ice-cold 1:1 mix of ethanol and acetone and an IFA protocol was carried out using a mouse monoclonal antibody anti-VP3 (mAb18) as the primary antibody. Nuclear counterstaining was performed with Hoechst 33342 (Molecular Probes).

APEC association/invasion assay

APEC strain BEN2908 (serotype O2:K1:H5) was used for cellular association/invasion assays as described previously (Chanteloup *et al.*, 2011). Briefly, exponentially growing bacteria, at a concentration corresponding to an MOI of 10, were added to pchAEC (as well as LMH and CLEC213 cells used for comparison) grown in 24-well culture plates and incubated with the cells for 2 h. For invasion assays, the cell monolayers were washed with PBS, incubated for 90 min with cell culture media containing gentamicin (100 µg/ml) to kill

extracellular bacteria, and again washed with PBS. Cells were then hypotonically lysed with water for 20 min at 4°C. For association assays, the gentamicin treatment step was omitted. Serial dilutions of the inoculum and cell lysates were plated onto LB agar to enumerate viable bacteria. Association and invasion were respectively calculated as the number of associated (adherent and intracellular) or intracellular bacteria, divided by the number of bacteria in the inoculum, and expressed as a percentage.

E. tenella *invasion/replication assay*

Eimeria invasion and replication assays were performed with sporozoites of the Wis YFP+ strain of *E. tenella* (Gras *et al.*, 2014). Harvesting of unsporulated oocysts from the caeca of experimentally infected White Leghorn chickens and protocols for the recovery of freshly excysted sporozoites were carried out exactly as previously described (Gras *et al.*, 2014). Wis YFP+ sporozoites were used to infect pchAEC grown on 13 mm glass coverslips at an MOI of 1. From 48 to 92 hours post infection (hpi), cultures were observed to follow the development of the parasite. Cells were washed and fixed with 2.7% paraformaldehyde. Nuclei were counterstained with DAPI (Vectashield). Intracellular YFP-positive developmental stages of *E. tenella* were imaged using a Zeiss Axiovert 200M fluorescence microscope and the Axiovision software (Zeiss).

Results

Primary cells prepared from chicken embryonic aortas (pchAEC) display an EC phenotype

Phenotyping approaches were used to ascertain that the pchAEC preparation protocol (McGuire & Orkin, 1987) allows reliable recovery of primary cells with an EC phenotype. Primary chAEC colonies were developed from explants obtained from embryonic aortas (Figure 1(a)) after 48 h of culture (Figure 1(b)) and amplified after trypsin treatment. Morphology of confluent EC layers from passage 2 is shown in Figure 1(b). All pchAEC preparations displayed an EC-specific gene expression profile as was assessed by RT–PCR targeting *ENG* (endoglin/CD105), *CADH5* (VE-cadherin), *VCAM1* and *SELE* (E-selectin) (Figure 1(c)). This EC phenotype was further confirmed by the protein expression level by immunofluorescence staining of von Willebrand Factor (vWF) and endoglin/CD105 (Figure 1(d)). In addition, the prepared cells had the ability to actively incorporate Dil-Ac-LDL (Figure 1(d)). Together, these data confirmed the successful preparation of pchAEC displaying the expected EC phenotype. Collectively, these data showed that pchAEC preparations display a *bona fide* endothelial cell phenotype.

Expression profile of PRRs and IFNα-, γ- and λ-receptor subunits in pchAEC, CLEC213 and CEF

Gene expression levels of the main chicken PRRs involved in viral/bacterial pathogen sensing (TLR3, TLR4, TLR5, TLR7 and MDA5), as well as the IFN-α/β, γ and λ-receptor subunits IFNAR1, IFNGR1 and IL28RA, were assessed by qPCR in pchAEC, as well as in CLEC213 and CEF which were used as cellular controls (Figure 2). Compared to CEF, pchAEC exhibit minor differences in mRNA copy numbers of the PRR and the IFNR genes tested, with the exception of markedly reduced transcriptional levels of *TLR4* and *IFNAR1* and a significantly higher transcriptional level of *TLR7*. Compared to CLEC213, pchAEC express significantly higher transcriptional levels of *TLR4*, *TLR7* and *MDA5*, but lower levels of *TLR3* and *IFNGR1*.

Signalling response of pchAEC to stimulation with PAMPs

The innate immune response pattern in pchAEC that were treated for 6 h or 18 h with the PAMPs LPS, flagellin, pI:C and L-pI:C was assessed by qPCR protocols targeting *IFNB*, *MX1*, *IL1B*, *IL6*, *IL8*, *TNFSF15*, *CX3CL1* and *SELE*. Overall, by far the strongest stimulatory effects were observed after treatment with the TLR3 agonist pI:C and with the MDA5 agonist L-pI:C (Figure 3(a)). Stimulation with the TLR4 and TLR5 agonists LPS and flagellin, respectively, had no appreciable effect on the transcriptional expression of any of the targeted innate immune genes (Figure 3(a)). L-pI:C stimulation resulted in highly elevated *IFNB* expression levels at 6 h (approx. 2500-fold) and 18 h (approx. 6000-fold) post-treatment. This L-pI:C-induced IFNβ response was paralleled by a stark increase of the expression levels of the IFN-stimulated gene (ISG) *MX1* (approx. 200 and 500-fold at 6 and 18 h, respectively). Although pI:C stimulation had virtually no effect on the transcriptional expression of *IFNB*, we observed markedly increased expression levels of *MX1* at 6 h (approx. 40-fold) and 18 h post-treatment (approx. 20-fold). Transcriptional expression of all cytokine genes tested (i.e. *IL1B*, *IL6*, *IL8L2*, *TNFSF15* and *CX3CL1*) generally peaked at 18 h post-treatment with both L-pI:C and pI:C (Figure 3(a)). At this time-point, L-pI:C induced a strong increase in the gene expression levels of the pro-inflammatory cytokines IL1β (approx. 70-fold), IL6 (approx. 600-fold), IL8 (approx. 2000-fold) and the chemokine CX3CL1/Fractalkine (approx. 100-fold). For the neovascularization inhibitor TNFSF15 and the endothelial-leukocyte adhesion factor E-selectin (*SELE*), transcriptional expression also peaked at 18 h post-treatment with L-pI:C (approx. 650-fold and 14-fold increase respectively). Treatment with the TLR3 agonist pI:C generally induced modest changes in the expression of *IL1B*, *IL6*, *IL8L2*, *TNFSF15*, *CX3CL1*,

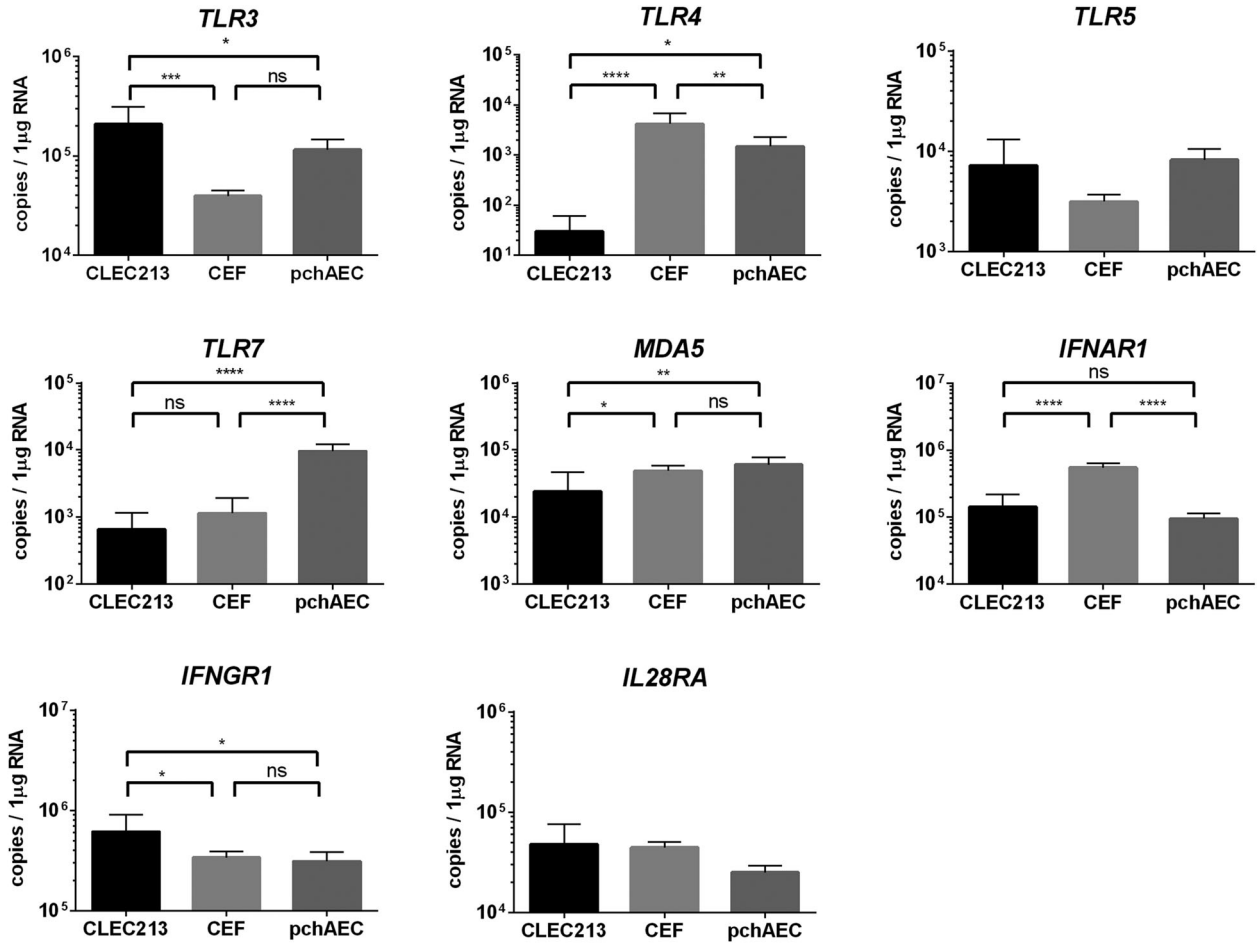

Figure 2. pchAEC express PRRs and interferon receptor genes. Expression levels for *TLR3*, *TLR4*, *TLR5*, *TLR7*, *MDA5*, *IFNAR1*, *IFNGR1* and *IL28RA* in chicken lung epithelial cells (CLEC213), chicken embryo fibroblasts (CEF) and pchAEC, as assessed by qPCR. The most distinctive features of pchAEC are their lower levels of *IFNAR1* and *TLR4* expression, along with a higher level of *TLR7* expression compared with CEF, and their higher levels of *TLR4*, *TLR7* and *MDA5* expression compared with CLEC213. Results are expressed as cDNA copy numbers obtained from 1 µg RNA after reverse transcription (mean ± SD from 3 to 6 biological samples for each cell type). **** $P < 10^{-4}$; *** $P < 10^{-3}$; ** $P < 0.01$; * $P < 0.05$ in ANOVA followed by Dunnett multiple pairwise comparisons.

and *SELE*, as compared to those induced by the cytoplasmically-delivered, MDA5 agonist L-pI:C. Nonetheless, pI:C stimulation resulted in considerably elevated expression levels of *IL1B* (approx. 25-fold at 6 h and 50-fold at 18 h post-treatment) and *CX3CL1* (approx. 20-fold at 6 and 18 h post-treatment). In summary, the data from the qPCR analysis showed that pchAEC barely respond to stimulation with the bacterial PAMPs flagellin and LPS, but mount a robust innate immune response to L-pI:C and pI:C that mimic viral genomic RNA or replication intermediates. Specifically, the gene expression pattern observed in viral PAMPs-stimulated pchAEC indicates the onset of an IFN-I and pro-inflammatory cytokine response and EC activation. Interestingly, our supplementary data on *IFNB*, *MX1*, *IL1B*, *IL8L2* and *TNFSF15* expression kinetics in CEF and CLEC213 treated with L-pI:C point to a relatively higher IFN-I response and a reduced pro-inflammatory cytokine response of these cells to viral PAMPs, in comparison to pchAEC (Supplementary Figure 1).

To corroborate the findings from the qPCR analysis of the innate immune gene expression profile in PAMPs-stimulated pchAEC, we performed luciferase-based IFN-I/NF-κB-reporter assays on pchAEC that were treated for 6 h or 18 h with LPS, pI:C and L-pI:C. As is shown in Figure 3(b), the data from the IFN-I-reporter (chicken Mx promoter activation) assay are largely in line with the findings presented above: Treatment of pchAEC with pI:C and L-pI:C induced activation of the Mx-promoter, whereas LPS-treatment had no such effect. Specifically, pI:C-stimulation led to an early onset activation of the Mx-promoter (approx. 30-fold at 6 h post-treatment) which diminished over time, down to approx. 20-fold at 18 h post-treatment. L-pI:C-stimulation, in contrast, had a delayed effect on Mx-promoter activation, as is demonstrated by an approx. 5-fold change at 6 h post-treatment and an approx. 25-fold change at 18 h post-treatment. Overall however, the luciferase-based Mx-promoter activation values obtained for pI:C and L-pI:C showed only limited correlation with the magnitude of changes in the transcriptional expression of *MX1* (and *IFNB*) assessed by qRT-PCR. The data from the NF-κB-reporter assay revealed an even more pronounced discrepancy between reporter-activation values and transcriptional

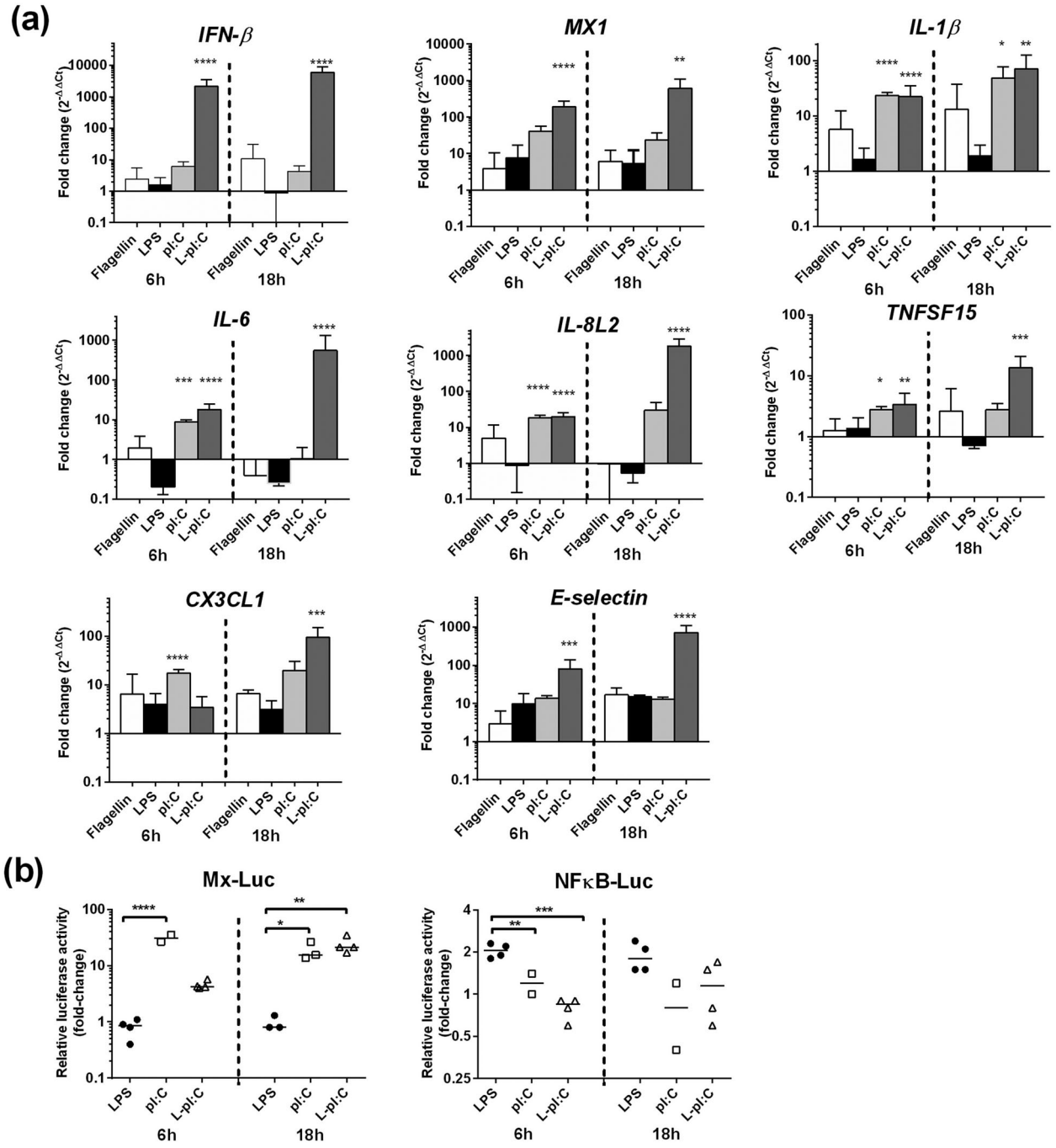

Figure 3. pchAEC respond to PRR agonists. (a) Innate immune gene expression responses to flagellin (100 ng/ml), LPS (10 µg/ml), polyI:C (pI:C, 10 µg/ml) and LyoVec-polyI:C (L-pI:C, 1 µg/ml) as assessed by qPCR. Data are expressed as fold-change in gene expression relative to non-stimulated controls (mean ± SD from 6 replicates); **** $P < 10^{-4}$; *** $P < 10^{-3}$; ** $P < 0.01$; * $P < 0.05$ in ANOVA followed by Dunnett pairwise comparisons with the control. (b) Inducible Mx/NF-κB promoter activities after PRR agonist treatment. Results for dual luciferase assays are expressed as fold-change in luciferase activity relative to non-stimulated controls (data combined from four independent experiments). L-pI:C and pI:C were the most efficient stimuli in inducing luciferase expression under the control of the chicken Mx promoter at 6 and 18 h post-treatment**;** **** $p < 10^{-4}$; *** $P < 10^{-3}$; ** $P < 0.01$; * $P < 0.05$ in Dunnett multiple pairwise comparisons (ANOVA) between pI:C or L-pI:C *versus* LPS.

expression changes (Figure 3(b)): pI:C-stimulation resulted in a slight, 1.2-fold activation of NF-κB that was only observed 6 h post-treatment and returned to the basal level (0.8) at 18 h. For L-pI:C, the NF-κB activation pattern was inverted, with fold-changes of 0.8 and 1.2 at 6 h and 18 h post-treatment, respectively. In contrast to our qRT-PCR data demonstrating steady transcriptional expression levels in LPS-treated pchAEC, LPS-stimulation resulted in sustained activation of NF-κB (2-fold change and 1.9-fold change at 6 h and 18 h, respectively). From these data, we concluded that luciferase-based IFN-I reporter assays provide a suitable means to assess the onset of an antiviral IFN-I response in pchAEC. The NF-κB-reporter assay, however, showed poor sensitivity and/or incongruent results and seems not to be suitable for testing for the onset of inflammatory cytokine response in these primary cells.

Primary chAEC respond to stimulation with type I, II and III IFNs

In view of our finding that pchAEC expressed substantial levels of *IFNAR1*, *IFNGR1* and *IL28RA*, we were interested to see whether the cells are responsive to treatment with type I, II and III IFNs. As shown in Figure 4, at 30 min post-treatment with recombinant chicken IFN-α or IFN-γ, phosphorylation of the signal transducer and activator of transcription 1 (STAT1) was readily detected by western blotting, illustrating the rapid onset of the IFN-I/II-induced STAT1 activation/signalling in pchAEC. This STAT1 activation decreased over time but was still detected at 120 min post-treatment. Following treatment with recombinant chicken IFN-λ, we detected faint, but clearly visible bands of phosphorylated STAT1 at 30 min and 120 min post-treatment (Figure 4), indicating that pchAEC are responsive to IFN-III stimulation. This IFN-III-induced STAT1 activation pattern in pchAEC is reminiscent of our data showing that IFN-λ-treatment induces STAT1 activation in CLEC213 (A. Lion, unpublished data), which were previously shown to mount an efficient antiviral response upon treatment with recombinant chicken IFN-λ (Reuter *et al.*, 2014).

Primary chAEC are susceptible to infection with various poultry pathogens

MDV

After infection of pchAEC with the recombinant, BAC-derived MDV-EGFPVP22, we monitored the replication and propagation of the virus at 2, 3 and 6 dpi by visualizing the expression of the EGFP-fused tegument protein VP22 (Figure 5(a)). We observed the formation of typical MDV plaques starting from 2 dpi and a significant increase of average plaque size over time (2-6 dpi) with 4.6% of EGFP-positive cells (i.e. infected cells) being detected by flow cytometry at 6 dpi (data not shown). These observations demonstrate that pchAEC are susceptible and fully permissive to MDV infection, suggesting that EC may present a relevant target cell type for MDV with a potential role in MD pathogenesis.

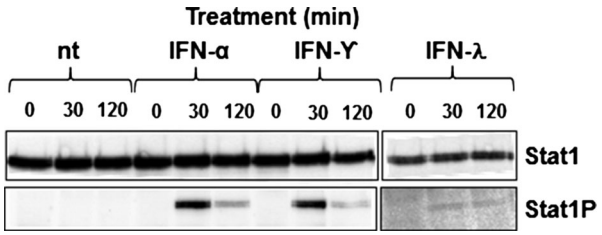

Figure 4. pchAEC respond to type I, II and III IFNs. Inducible Stat1 phosphorylation was analysed by western blotting after treatment (30 min) with recombinant chicken IFN-α (type I), IFN-γ (type II) and IFN-λ (type III). nt = protein extract from non-treated control cells.

IBDV

We next analysed the replication of the lytic IBDV in pchAEC through immunofluorescence at 24 hpi, using an IBDV-specific (anti-VP3) monoclonal antibody. Although non-infected control cells showed no or very faint fluorescent signals, bright cytoplasmic fluorescence was observed in numerous cells from pchAEC and CEF cultures that were inoculated with either the Ct or TY89 attenuated vaccine strains of IBDV (Figure 5(b)). In contrast, no fluorescence was observed for either cell type upon infection with the very virulent strain 89163. These observations suggest that, similar to the situation seen in CEF, the susceptibility of pchAEC to IBDV infection is essentially dependent on the viral strain and serotype.

APEC

The APEC strain BEN2908 showed similar capacities to adhere to pchAEC (471.2% association) and the chicken epithelial cell lines CLEC213 (530.4% association) and LMH (389.2% association) (Table 2). The

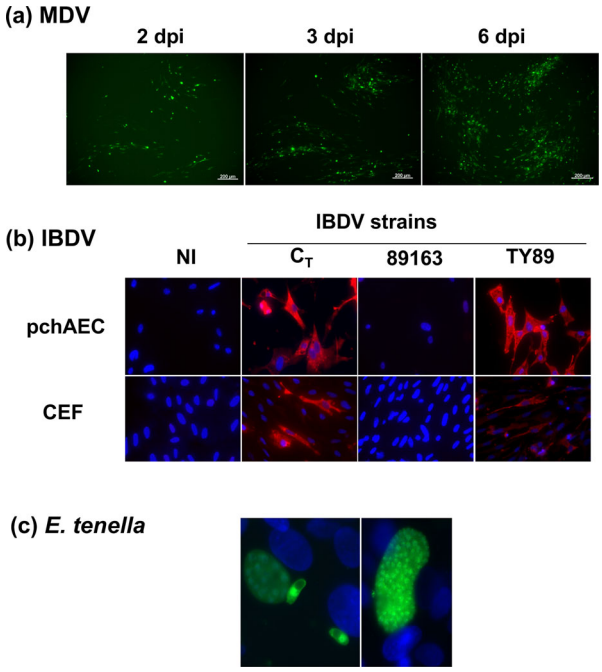

Figure 5. pchAEC are susceptible and/or permissive to a variety of avian pathogens. (a) Marek disease virus (MDV): fluorescent MDV-EGFPVP22 plaques (green) are detected from 2 days post-infection (dpi) with a significant size increase until 6 dpi (scale bar = 200 µm). (b) Infectious bursal disease virus (IBDV): immunostaining with an anti-VP3 mouse monoclonal antibody (mAb 18, red) reveals the presence or absence of IBDV replication in pchAEC and CEF at 24 hours post-infection (hpi) with the cell culture-adapted serotype 1 and 2 strains Ct and Ty89 (VP3-positive) and the very virulent serotype 1 strain 89163 (VP3-negative) at an MOI of 1. (c) *Eimeria*: Wis YFP recombinant *E. tenella* sporozoites are able to infect pchAEC and to undergo the first schizogony as shown by fluorescence microscopy capture of immature and mature schizonts at 48 hpi at an MOI of 1(green). Colour images are provided in the online Open Access version of this article (https://doi.org/10.1080/03079457.2018.1556386).

invasion rate of BEN2908 was lower in pchAEC (1.1% invasion) than in CLEC213 cells (2.7% invasion), but the difference between the two cell lines/types was not significant. In contrast, this same APEC strain was more invasive ($p < 0.05$) for LMH (23.1% invasion) than for pchAEC or CLEC213. These data demonstrate that pchAEC permit a low level of bacterial invasion by cell-invasive APEC strains such as BEN2908.

E. tenella

As is shown in Figure 5(c), immature and mature schizonts were detectable at 48 hpi of pchAEC with sporozoites from the recombinant YFP-expressing *E. tenella* strain Wis YFP+. Merozoites were observed in the cell supernatant at 72 hpi, but they were unable to re-enter pchAEC to initiate the second schizogony. These observations indicate that pchAEC are susceptible to infection with *E. tenella* but that they do not support the entire protozoan life cycle up to its completion.

Discussion

Primary cell (pchAEC) cultures were prepared from the aortas of 18-day-old chicken embryos by adapting an established protocol for the isolation and culture of mammalian macrovascular EC (McGuire & Orkin, 1987) as described previously (Lion *et al.*, 2018). All pchAEC preparations scored positive for the presence of a set of EC markers including von Willebrand Factor, endoglin, VE-cadherin, E-selectin and VCAM1. In addition, pchAEC actively incorporated acetylated low density lipoproteins (AcLDL), as a typical functional feature of EC (McGuire & Orkin, 1987). Thus, pchAEC exhibit the same phenotypic characteristics of cells that were previously described to be precursors of chicken EC isolated from whole blood (Bi *et al.*, 2014) and bone-marrow (Bai *et al.*, 2012; Davis *et al.*, 2018). However, the pchAEC preparation protocol provides the advantage of allowing the production of a large quantity of primary chicken EC in an easy tissue/cell culture procedure, thus permitting their widespread use in studies addressing avian host–pathogen interactions and/or the role of chicken EC in immune homeostasis and pathogenesis.

Table 2. Association/invasion of APEC BEN2908 in primary chAEC compared to the avian epithelial cell lines, CLEC213 and LMH.

	% association[a]	% invasion
CLEC213	530.44 +/− 69.13 [b]	2.68 +/− 0.53
pchAEC	471.18 +/− 63.18	1.05 +/− 0.02[c]
LMH	398.22+/− 62.32	23.10 +/− 3.87

[a]Percentage of inoculum associated with cells (association) or surviving to gentamicin treatment (invasion).
[b]Data are the means +/− standard error of 4 (CLEC213), 2 (pchAEC) and 5 (LMH) independent experiments performed in triplicate.
[c]Data of association and invasion assays were analysed using Kruskal–Wallis and Dunn tests. No difference between cell lines for association with BEN2908. No difference between pchAEC and CLEC213 for invasion. Significant difference between pchAEC and LMH for invasion ($P < 0.05$).

The majority of known TLRs were found to be expressed in all types of macro- and microvascular EC in mammals, but differences may be observed depending on the origin of the vascular bed and on the species (Pryshchep *et al.*, 2008; Erridge, 2009; Khakpour *et al.*, 2015). We showed here that pchAEC also express abundant mRNA levels of various PRRs, including TLR3, TLR4, TLR5 and MDA5. Genes encoding these PRRs genes are also abundantly expressed in CEF and CLEC213, although some differences in gene expression levels can be observed among the three chicken cell types. One striking difference concerns the markedly higher expression of *TLR7* in pchAEC compared to CEF and CLEC213. To explore which of the principal PRRs detecting viral and bacterial PAMPs in the chicken may be active in pchAEC, we analysed the cytokine responses to a panel of PRR agonists. In general, the MDA5 agonist L-pI:C, which delivers the synthetic double-stranded RNA analogue pI:C (mimicking viral RNA) directly into the cytoplasm, elicited the strongest stimulating effects on the transcription of *IFNB* and the ISG *MX1* next to the genes encoding pro-inflammatory cytokines/chemokines, including *IL1B*, *IL6*, *IL8L2*, *TNFSF15* and *CX3CL1*. Similar, yet more pronounced (*IFNB*, *MX1*) or less distinct (*IL1B*, *IL8L2*, *TNFSF15*) innate immune gene expression changes were seen in L-pI:C-treated CEF and CLEC213. Moreover, L-pI:C-treated pchAEC upregulated the EC activation marker E-selectin (*SELE*). Endothelial selectins, including P- and E-selectins and CX3CL1 (fractalkine) mediate the tethering and rolling of leukocytes in inflamed vascular beds and are responsible for early recruitment of neutrophils and monocytes at the site of pathogen injury (McEver, 2015; Lee *et al.*, 2018; Silva *et al.*, 2018). A robust innate immune response was also induced after stimulation of TLR3 with the non-complexed pI:C. In contrast, the overall immune response to PAMPs from bacterial wall components (LPS, flagellin) known to efficiently stimulate chicken leukocytes (St Paul *et al.*, 2013) was only poor despite an appreciable NF-κB activation detected by the respective luciferase-reporter assay. Yet, the chicken probably lacks the TRAM/TRIF signalling pathway (Keestra & van Putten, 2008), which is known to promote an accessory activation of NF-κB and IRF3 in response to LPS, thereby transmitting a robust LPS-induced IFN-I and inflammatory cytokine response in mammals (Kawai & Akira, 2010). In agreement with this notion, we could not detect any LPS-induced *IFNB* transcription at any time-point tested. Therefore, the pro-inflammatory cytokine response to LPS stimulation is likely to be low in the chicken, explaining the relative resistance of chickens to septic shock (Keestra & van Putten, 2008). However, for a better understanding of how the

endothelium of galliform birds responds to TLR/PRR-stimulation, we still have to explore the responsiveness of chicken EC derived from blood vessels other than macrovascular aortas, notably capillary-derived microvascular EC that are directly exposed to and involved in the triggering of inflammatory responses and leukocyte migration in infected/damaged tissues. Unfortunately, there are currently no protocols available for the isolation of microvascular chicken EC (or relevant chicken cell lines) that would allow comparing the data presented here. The current literature points to differences in TLR expression in mammalian EC and in TLR agonist-induced immune responses, that not only depend on the EC, but also on the species-origin (Erridge *et al.*, 2008; Khakpour *et al.*, 2015). This is notably the case for TLR4, the expression of which may or may not be upregulated by LPS stimulation, thereby explaining the phenomenon of high or low responsiveness of arterial EC from different species-origins (Shi *et al.*, 2010). In addition, we can only speculate about a potential TLR crosstalk effect in EC, entailing a robust and specific innate immune response to bacteria in the chicken as was previously seen in mammals (Tan *et al.*, 2014).

We evidenced the expression of type I, II and III IFN receptor genes in pchAEC, as well as the responsiveness of the cells to stimulation with chicken IFN-α, IFN-γ and IFN-λ, as was revealed by STAT1 phosphorylation/activation in the stimulated pchAEC. Expression of the IFN-I/II cognate receptors IFNAR1 and IFNGR1 has been shown in mammalian EC and was demonstrated to permit IFN-I/II-induced antiviral signalling responses through STAT1 activation (da Silva *et al.*, 2002; Tissari *et al.*, 2005; Sikorski *et al.*, 2011). Our observations that pchAEC expressed the IFN-III receptor gene *IFN28R* and responded to treatment with chicken IFN-λ by STAT1 activation were somewhat unexpected. To our knowledge, expression of the IFN-III receptors in mammalian EC has never been reported. However, it has previously been demonstrated that microvascular EC from mouse brain are responsive to treatment with IFN-λ by modulating tight junctions and hampering the blood–brain barrier transit of West Nile virus (Lazear *et al.*, 2015). Therefore, the responsiveness of chicken EC to IFN-λ may play an as yet overlooked role in the maintenance of endothelial barriers at mucosal surfaces and in the central nervous system (CNS). Collectively, we concluded from our data that chicken EC, responding to PAMPS and to locally produced cytokines, may be important effector cells in the innate immune response, partaking in the initiation and/or amplification of the inflammatory process triggered by pathogen invasion.

In contrast to the situation in mammals, virtually nothing is known about the roles that the endothelium may play in the pathogenesis of infectious diseases in the chicken. Highly pathogenic avian influenza (HPAI) viruses present a notable exception, since they were frequently described to display a distinct tropism for the endothelium of chickens in various organs, such as brain, lung and heart (Perkins & Swayne, 2001; Nakatani *et al.*, 2005; Schat *et al.*, 2012; Hagag *et al.*, 2015). Using both the pchAEC culture model described in the present paper and cells that were serially passaged, we have recently discovered that productive infection of chicken EC by avian influenza viruses is not only determined by the presence of an HPAI virus-specific multibasic haemagglutinin cleavage site, but also depends on efficient viral innate immune escape (Lion *et al.*, 2018). Interestingly, we observed here that MDV, an alphaherpesvirus causing various clinical syndromes (including peripheral neuritis, T-cell lymphoma, immunosuppression, acute transient paralysis and brain oedema) in chickens (Osterrieder *et al.*, 2006), is pable to replicate in pchAEC following co-culture with infected chicken embryonic skin cells. MDV is characterized by being strictly cell-associated and by targeting a limited set of host cell types, B and T lymphocytes and keratinocytes, all of which play a critical role in the different phases of MDV infection in the chicken host (Osterrieder *et al.*, 2006; Couteaudier *et al.*, 2016). Various lines of evidence suggest that endothelial infection may also be involved in the pathogenesis of Marek's disease: Acute transient paralysis seen in chickens infected with very virulent (vv) or very virulent plus (vv+) strains of MDV has previously been attributed to vasogenic brain oedema, vasculitis and mononuclear perivascular cuffing together with a robust pro-inflammatory cytokine response in the brain (Xu *et al.*, 2012). Similar observations were made by Gimeno *et al.* and led the authors to speculate that directly infected and/or activated EC may play a central role in the pathogenesis of vv+ MDV infection in chickens (Gimeno *et al.*, 2001). By comparison, other herpesviruses, e.g. equine herpes virus type 1 (EHV-1) and human cytomegalovirus (HCMV), that preferentially infect macrophages and/or T-lymphocytes, are also able to infect endothelial cells thereby causing various potentially fatal conditions, including encephalitis (Jarvis & Nelson, 2007; Spiesschaert *et al.*, 2015). Brain vasculopathies characterized by inflammatory infiltrates and endothelial swelling are also observed in CNS manifestations of human infections with varicella zoster virus, an alphaherpesvirus sharing various biological features with MDV (Kleinschmidt-DeMasters *et al.*, 1996). Chicken primary aortic endothelial cells could thus constitute a new cellular model of infection to identify the importance they may have in MDV pathogenesis.

Infectious bursal disease (IBD) is caused by classical and very virulent serotype 1 strains of IBDV (Mahgoub *et al.*, 2012), with actively dividing IgM+ bursa cells being the major target cells *in vivo* for both serotypes.

We observed that both classical serotype 1 IBDV and avirulent serotype 2 IBDV, the target cells of which have not yet been identified *in vivo*, replicated efficiently in pchAEC. By contrast, these cells were not permissive to a very virulent serotype 1 IBDV, which also does not replicate in chicken embryo fibroblasts. However, very virulent IBDV strains are known to induce oedema and haemorrhagic lesions in the bursa of infected chickens that are accompanied by a systemic coagulopathy syndrome involving ecchymotic haemorrhages in the muscle and mucosa of the proventriculus (Singh *et al.*, 2015). Because very virulent IBDV are pable to efficiently infect macrophages, in addition to bursa B lymphocytes, one might speculate that an excessive innate immune response of infected macrophages could activate EC locally and/or throughout the body through pro-inflammatory cytokine the production, ultimately leading to perturbation of the coagulation pathway. This speculation is supported by *in vivo* observations by Lima *et al.* (2005). To address this issue, one straightforward approach would be to analyse the effect of co-culturing IBDV-infected macrophages with EC and to affirm the absence/presence of endothelial cell permissiveness of EC to IBDV according to the pathotype.

A wide range of avian pathogenic *E. coli* (APEC) strains induce either local or systemic types (distinguished by septicaemia and multiple organ inflammation) of colibacillosis in broilers and in laying hens (Collingwood *et al.*, 2014). The pathophysiology of colibacillosis is far from being fully elucidated (Guabiraba & Schouler, 2015). We demonstrated that the highly adhesive/invasive strain BEN2908 (also known as MT78) was able to adhere to and to be internalized by chicken primary aortic endothelial cells. However, this adhesion/invasiveness capacity is higher in chicken lung epithelial cells and even greater in chicken LMH hepatocytes. By comparison, BEN2908 is clearly able to invade human brain microvascular endothelial cells, in particular due to the presence of the *Ibe*A virulence factor, as in human meningitis-associated *E. coli* (Germon *et al.*, 2005). However, in regard to chicken colibacillosis, even if sometimes observed, meningitis remains a rare manifestation during the disease. Yet, interactions of APEC with EC might be of major importance in shaping the pathogenesis of colibacillosis, by inducing/enhancing inflammation that might participate in damaging the epithelial-endothelial barriers, as for example during pulmonary infection, or that might result in systemic coagulation disorders leading finally to fibrin deposition and multiple organ failure in septicaemia. Further studies are needed to test this disease model.

Finally, *Eimeria* are major avian parasites from the genus *Apicomplexa* causing great economic losses in poultry production. Although intestinal epithelial cells are the major first target cells of infection, data are still lacking on the multiple cell interactions that might take place during the developmental cycle of *E. tenella* across the intestinal mucosa and inside the intestinal villosity (Del Cacho *et al.*, 2014). We demonstrated here that chicken EC are able to support only the asexual multiplication of the parasite. However, even though infection of EC with avian *Eimeria spp.* might be a rare event *in vivo* that remains yet to be demonstrated, it is conceivable that the interplay between EC and some cycle steps of the parasite development may contribute to either exacerbate inflammation in order to reduce the parasite infection or, alternatively, favour parasite local dissemination. In support of this notion, other apicomplexan parasites have been shown to exhibit a complex relationship with the host endothelia, that can be modelled using appropriate cell culture systems. Sporozoites of *Eimeria* spp. infecting ruminants, such as *E. bovis*, *E. ninakohlyakimovae*, *E. arloingi*, *E. christenseni* and *E. bakuensis*, display a distinct tropism for the host endothelium, the site where protracted macromeront formation occurs, strikingly in the absence of a marked inflammatory response (Hermosilla *et al.*, 2012). By contrast, the crossing of endothelial barriers, including the blood–brain and blood-retinal barriers, by *Toxoplasma gondii* results in an excessive, disease-aggravating inflammation. Transmigration across endothelial cell layers has been demonstrated for free infectious tachyzoites pable to directly target endothelial cells as well as tachyzoite-infected mononuclear cells adhering to and traversing endothelial cells (Knight *et al.*, 2005; Lachenmaier *et al.*, 2011; Furtado, Bharadwaj, Ashander *et al.*, 2012; Furtado, Bharadwaj, Chipps *et al.*, 2012).

In summary, we show here that chicken EC are highly responsive to viral innate immune stimuli and may partake in the inflammatory and antimicrobial responses to various avian pathogens including MDV, IBDV, APEC and *E. tenella*. The development of infection models using easily accessible and well-characterized primary chicken aortic EC will pave the way for further elaborated studies on the interactions of infectious agents with the endothelium of galliform hosts.

Disclosure statement

No potential conflict of interest was reported by the authors.

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