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Avian pathogenic *Escherichia coli*-induced activation of chicken macrophage HD11 cells

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) can cause severe respiratory diseases in poultry. The initial interaction between APEC and chicken macrophages has not been characterized well and it is unclear how effective chicken macrophages are in neutralizing APEC. Therefore, the effect of APEC on activation of chicken macrophage HD11 cells was studied. Firstly, the effect of temperature (37 vs 41 °C) on phagocytosis of APEC by HD11 cells was determined. The results showed that APEC was more susceptible to being phagocytosed by HD11 cells at 41 °C than 37 °C. Subsequently, the capacity of HD11 cells to kill APEC was shown. In addition, HD11 cells produced nitric oxide (NO) at 18 h post-infection and a strong increase in the mRNA expression of IL-8, IL-6, IL-1β and IL-10 was detected, while IFN-β gene expression remained unaffected. Finally, it was shown that the response of HD11 was partially dependent on viability of APEC since stimulation of HD11 cells with heat-killed APEC resulted in a reduced expression level of these cytokines. In conclusion, APEC induces an effector response in chicken macrophages by enhanced NO production and cytokines gene expression.

1. Introduction

Avian pathogenic *Escherichia coli* strains (APEC) can cause severe infections in poultry, such as omphalitis, salpingitis, cellulitis and respiratory tract infections (Dho-Moulin and Fairbrother, 1990; Guabiraba and Schouler, 2015). In all of these infections, the bacteria can enter the bloodstream and become systemic resulting in colibacillosis (Guabiraba and Schouler, 2015; Horn et al., 2012). Nowadays, avian colibacillosis is one of main causes leading to mortality and morbidity in poultry resulting in huge economic losses in the poultry industry (Zhuang et al., 2014). So far, there is no highly effective vaccination to protect against APEC strains mainly due to the diversity of APEC strains in the field. Treatment of APEC infection mainly relies on antibiotics, but the increasing emergence of drug resistance makes treatment less successful.

An APEC infection originally starts in the respiratory tract, crosses to the blood stream and can subsequently infect internal organs causing septicemia (Dziva and Stevens, 2008). In the last few years, APEC pathogenesis to the host has been studied through the use of experimental infection models (Antao et al., 2008; Matthijs et al. 2009, 2017) and identification of virulence genes (Dozois et al., 2000; Lymberopoulos et al., 2006; Ma et al., 2014; Caza et al., 2011). Furthermore, APEC superinfection with infectious bronchitis virus infection leads to severe pathogenesis in the respiratory tracts as virus damages the respiratory mucosa and facilitates APEC colonization (Guabiraba and Schouler, 2015). Despite the importance of APEC pathogenesis, the knowledge about APEC invasion and interactions with host cells in chicken is limited and poorly understood.

In order to respond to a microbial infection, host innate immune cells are activated by interaction with (parts of) the pathogen. Activation of these immune cells subsequently leads to activation of intracellular signalling pathways resulting in production of cytokines and microbial killing (Kogut et al., 2012). Macrophages are one of the first responder innate cells upon a new infection, as seen in infection models where APEC infection causes a quick increase in the number of macrophages (Ariaans et al., 2008). They can phagocytize bacteria and subsequently produce multifunctional compounds including reactive oxygen species (ROS), nitric oxide (NO) and cytokines to kill the infectious microorganisms (Li et al., 2011; Okamura et al., 2005; Withanage et al., 2005) and signal to other immune cells to establish an appropriate response to the infection.

A number of *in vitro* studies have shown phagocytosis and immune responses of chicken macrophages upon challenge with different bacterial strains, using primary macrophages, or chicken macrophage cell lines (Wisner et al., 2011; He et al., 2012b; Jarvis et al., 2017; Hashimoto et al., 2017). Although most bacteria tested are

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phagocytized by macrophages, some bacteria such as *Staphylococcus aureus*, can still escape from macrophages through the activation of caspase-3 followed by macrophage cell death (Flannagan et al., 2016). However, the role of macrophages in controlling APEC infection and the interaction between APEC and macrophages in chickens is less studied and remains mostly unclear.

In this study, we investigated the phagocytic capacity of HD11 macrophages towards APEC and the response towards infection as a first step towards understanding role of chicken macrophages in APEC infection.

2. Materials and methods

2.1. Bacterial strains

Avian pathogenic *Escherichia coli* (APEC) was isolated from chicken (Cuperus et al., 2016). *Salmonella enteritidis* (strain, ATCC 13368) was cultured in Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) at 37 °C. Heat-killed bacteria were prepared by incubating the bacterial suspension at 75 °C for 15 min; viability was checked by plating out heat treated bacteria on TSA plates. For preparation of the green fluorescent protein (GFP) – expressing APEC strain, the plasmid PWM1007 was transformed into APEC by electroporation using an Electro Cell Manipulator according to the manufacturer's instructions. GFP-expressing APEC was cultured in the same condition as APEC.

2.2. Chicken macrophages, HD11 cells

The chicken macrophage-like cell line (Beug et al., 1979), HD11, was maintained in a humidified 41 °C incubator with 5% CO_2 and cultured in RPMI 1640-glutaMAX supplemented with 10% FCS and antibiotics (100 U penicillin/mL and 100 µg streptomycin/mL). Aliquots of cell suspension were seeded into each well at 2.5×10^5 cells/well for a 24-well plate and 5.0×10^5 cells/well for 12-well plates and cultured overnight before being used for assays described below.

2.3. Effects of temperature on the growth of APEC

Log-phase bacteria were diluted to 1×10^5 CFU/mL in RPMI 1640-glutaMAX supplemented with 10% FCS. Aliquots of this bacterial suspension were added to 2 new tubes and incubated at 37 °C and 41 °C, respectively. The OD_{620} was measured at 0–6 h to determine kinetics of bacterial growth.

2.4. Effects of temperature on HD11 phagocytic capacity and cell viability

Before HD11 cells were incubated with APEC, culture medium was removed and cells were washed once with RPMI 1640-glutaMAX. Aliquots of 1 mL of bacterial suspension (10^6 CFU/mL) were added to each well with four replicate wells for 24-well plates, at a multiplicity of infection (MOI) of 2. Cells were incubated for 1, 2, 3 and 4 h at 37 °C and 41 °C. After incubation, the bacterial suspension was removed and HD11 cells were washed three times with RPMI 1640-glutaMAX. Then, RPMI 1640-glutaMAX containing 500 µg/mL gentamicin was added to each well in order to kill all extracellular, non-phagocytosed bacteria and the plates were placed back at 37 °C and 41 °C for 1 h. At each appropriate time point (2, 3, 4 and 5 h, after gentamicin treatment), infected cells in three wells were washed three times with RPMI 1640-glutaMAX and lysed by 1 mL 0.5% Triton X-100. After lysis, dilution series of cells were plated on TSA plates and incubated at 37 °C for 24 h to quantify viable bacteria. Cells in the fourth well received 0.1 mL 0.01% trypsin-EDTA and were stained with Trypan blue to quantify cell viability.

2.5. HD11 killing activity

Before incubation with APEC, HD11 cells were washed once with RPMI 1640-glutaMAX. Aliquots of 1 mL of bacterial suspensions (1×10^6 CFU/mL) were added to each well, with four replicate wells for 24-well plates at a multiplicity of infection (MOI) of 2 and incubated for 3 h at 41 °C. At 3 h, the bacterial suspension was removed and cells were washed three times with RPMI 1640-glutaMAX and replaced with RPMI 1640-glutaMAX containing 500 µg/mL gentamicin for 1 h. After this high-gentamicin treatment, medium was replaced by cell medium containing 62.5 µg/mL gentamicin and cells were incubated back at 41 °C. At each time point (4, 5, 6, 7 and 8 h), cells in three wells were washed three times with RPMI 1640-glutaMAX and lysed by 1 mL 0.5% Triton X-100. Then, suspensions were serially diluted, plated on TSA plates and incubated at 37 °C for 24 h to quantify viable intracellular bacteria. Cells in the fourth well received 0.1 mL 0.01% trypsin-EDTA and were stained with Trypan blue to quantify cell viability.

2.6. NO production assay

Nitrite, a stable metabolite of NO, produced by activated macrophages was measured by the Griess assay (Green et al., 1982). HD11 cells were incubated with live or heat-killed bacteria at 41 °C for 3 h and treated with 500 µg/mL gentamicin for 15 h. After 18 h incubation, aliquots of 50 µL supernatant were transferred to the wells of a 96-well flat bottom plate. Fifty µL 1% sulfanilamide (Merck, Darmstadt, Germany) was added in each well mixed with 50 µL 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (VWR) at room temperature for 5 min. The nitrite concentration was determined by measuring optical density at 550 nm. Sodium nitrite (Sigma) was used as a standard to accurately determine the nitrite concentration in the cell supernatant.

2.7. Gene expression

HD11 cells were incubated with APEC at 41 °C for 3 h and subsequently treated with 500 µg/mL gentamicin for 1 h as described above. After 4 h incubation, total RNA was extracted by Trizol (Ambion, Carlsbad, CA) reagent according to manufacturer's instructions. RNA (500 ng) was reverse transcribed by the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer's instructions. Primers and probes were designed and produced by Eurogentec (Seraing, Belgium) (Table 1). Quantitative real time PCR

Table 1
Primer and probe sequences for qPCR.

Gene		5'→3'sequence
GAPDH	Forward	GTCAACCATGTAGTTCAGATCGATGA
	Reverse	GCCGTCCTCTCTGGCAAAG
	Probe	AGTGGTGGCCATCAATGATCCC
28S	Forward	GACGACCGATTTGCACGTC
	Reverse	GGCGAAGCCAGAGGAAA
	Probe	AGGACCGCTACGGACCTCCACCA
IFN-β	Forward	CCTCCAACACCTCTTCAACACG
	Reverse	TGGCGTGTGCGGTCAAT
	Probe	AGCAGCCCACACACTCCAAAACACT
IL-1β	Forward	GCTCTACTAGTCGTGTGTGATGAG
	Reverse	TGTCGATGTCCCGCATGA
	Probe	CCACACTGCAGCTGGAGGAAGCC
IL-6	Forward	GTCGAGTCTCTGTGCTAC
	Reverse	GTCTGGGATGACCACTTC
	Probe	ACGATCCGGCAGATGGTGA
IL-8	Forward	GCCCTCCTCCTGGTTTCA
	Reverse	CGCAGCTCATTCCCCATCT
	Probe	TGCTCTGTCGCAAGGTAGGACGCTG
IL-10	Forward	CATGCTGCTGGGCCTGAA
	Reverse	CGTCTCCTTGATCTGCTTGATG
	Probe	CGACGATGCGGCGCTGTCA

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.

2.8. Confocal microscopy

HD11 cells were seeded on a 12 mm coverslip in a 24-well plate and incubated overnight. Cells were incubated with GFP-APEC for 3 h at 41 °C as described previously. After three wash steps with RPMI 1640-glutaMAX, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature (RT). Subsequently, cells were incubated with 50 mM NH_4Cl in PBS for 10 min at RT and blocked with 5% normal goat serum in PBS for 1 h. Then, cells were stained with *E. coli* antiserum (Cuperus et al., 2016) (1:500) for 1 h. After the wash steps, cells were incubated with Donkey anti-Rabbit Alexa 647 (Jackson ImmunoReasearch, West Grove, PA, USA) (1:100) 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.

2.9. Statistical analysis

Data are represented as mean ± SEM of three independent experiments for each group (n = 3) and were analyzed by a T-test for two groups or by one-way ANOVA with post-hoc *t*-test for more than two groups. $p \leq 0.05$ were considered significant. Bio-Rad CFX Manager 3.0 software was used for qPCR data analysis. All the graphs were made using GraphPad Prism® 5.0.

3. Results

3.1. Effect of temperature on the growth of E. coli

It is well known that the optimal culture temperature is 37 °C for some bacterial species. However, enteric bacteria are specifically adapted to their hosts' body temperature. Since a chicken's body temperature is close to 41 °C, we determined whether the temperature can affect the growth of APEC. For this, two *E. coli* strains (APEC and K88) were simultaneously cultured at 37 °C and 41 °C. As shown in Fig. 1, both *E. coli* strains reached the logarithmic phase faster at 41 °C than 37 °C, resulting in higher OD values at 2 h and 3 h. Interestingly, at 5 h and 6 h, the final OD of K88 was significantly lower at 41 °C compared to 37 °C.

3.2. Effect of temperature on phagocytic capacity and viability of HD11 cells

The effect of temperature and incubation time on the phagocytic capacity of HD11 cells was tested. HD11 cells were incubated with APEC at 37 °C and 41 °C and the number of intracellular bacteria was determined after 1–5 h. As shown in Fig. 2A, the number of bacteria increased over time, and at all time points more bacteria were phagocytosed at 41 °C. In the same experiment, viability of HD11 cells after the infection was tested with trypan blue. No significant reduction in viability of HD11 was observed (Fig. 2B) at any time point. Based on these results, an incubation time of 4 h at 41 °C was chosen as the optimal assay condition for subsequent experiments.

3.3. Intracellular and extracellular APEC

In order to confirm the intracellular localization of APEC in our experimental set-up, confocal microscopy was performed. After HD11 incubation with GFP-APEC, the bacteria were stained with an anti-*E. coli* antibody. Because cells were not permeabilized, only extracellular APEC was labeled with this antibody enabling a distinction between phagocytosed intracellular and adhered extracellular APEC. As shown in Fig. 3, intracellular bacteria, only GFP stained bacteria (green) are indeed present in HD11 cells, as well as extracellular bacteria (yellow/red), qualitatively confirming the results shown in Fig. 2.

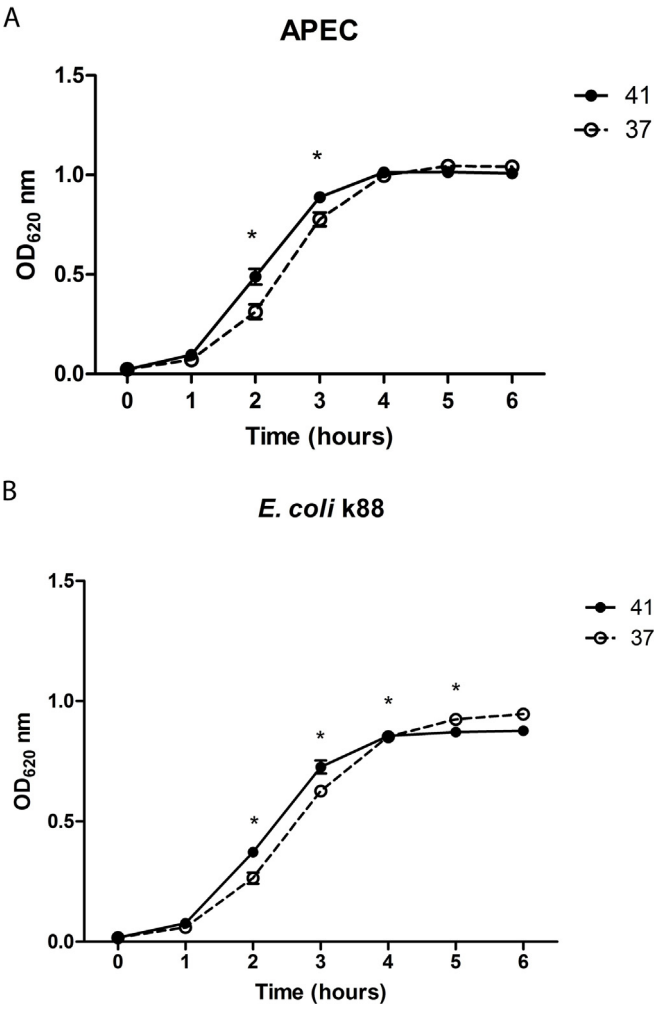

Fig. 1. Temperature dependency of growth of *E. coli* strains. (A) APEC. (B) *E. coli* k88. Data are shown as mean ± SEM of three independent experiments (in triplicate per experiment) for each group (n = 3). * indicates significant difference ($P \leq 0.05$) between 41 °C and 37 °C for a single time point using a student's T-test.

3.4. Killing of APEC by HD11 macrophages

HD11 cells are capable of phagocytizing*S. typhimurium*, *S. enteritidis* and *Listeria monocytogenes* (Jarvis et al., 2017; Wisner et al., 2011). Once phagocytized, the number of viable intracellular bacteria decreased over the next 24 h (He et al., 2012b). To determine whether macrophages are also capable of killing intracellular APEC, viability of APEC after phagocytosis was checked at several time points (Fig. 4). At 4 h, a lower number of APEC was present in the macrophage compared to *S. enteritidis* (Fig. 4A), demonstrating a difference of HD11 uptake capacity towards different strains. At 6, 7 and 8 h, a significantly decreased number of APEC was observed (Fig. 4A), indicating that HD11 are able to kill APEC, contrary to *S. enteritidis*. This observation indicated that *S. enteritidis* is more resistant to HD11 killing.

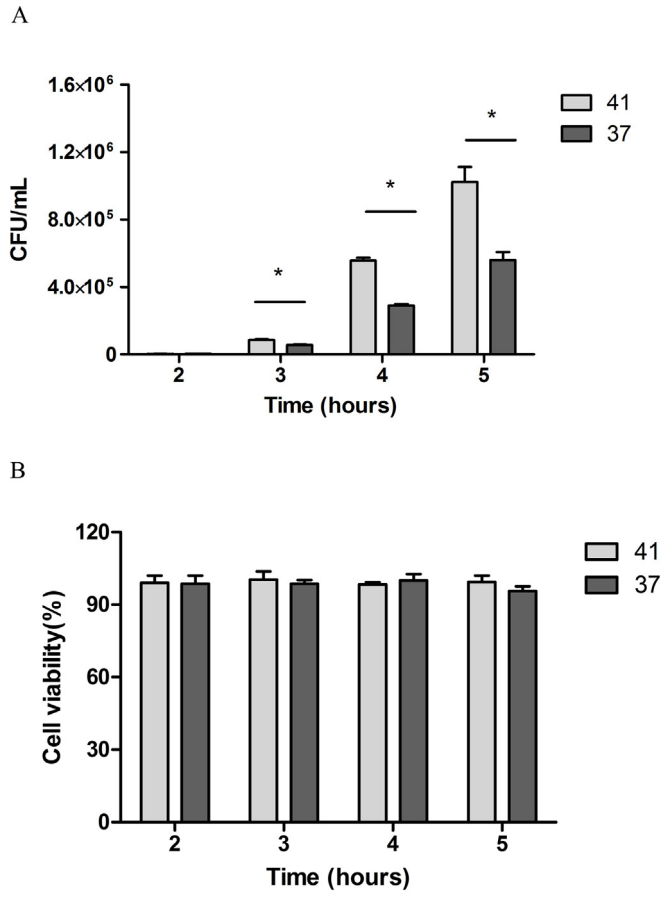

Fig. 2. Temperature dependency of phagocytosis of APEC by HD11 cells. (A) number of phagocytosed APEC in HD11 cells at 2–5 h post-incubation at 37 °C and 41 °C. (B) Viability of HD11 cells at 2–5 h post incubation. Data are shown as mean ± SEM of three independent experiments for each group (in triplicate per experiment). * indicates significant difference ($P \leq 0.05$) in bacterial number between 37 °C and 41 °C using a student's T-test.

3.5. NO production

Phagocytosis of APEC and *S. enteritidis* induced a significant NO production in HD11 cells, with APEC giving higher levels of NO compared to *S. enteritidis* (Fig. 5). In order to get a first indication whether bacteria had to be viable to activate cells, heat-killed APEC and *S. enteritidis* was also used in these experiments. Although the absolute amount of NO was lower for heat-killed bacteria, they were still able to induce a significant amount of NO. These results indicate that activation of HD11 cells is partially depending on bacterial strain and viability but that bacterial products are responsible for most activation.

3.6. Cytokine expression

Besides NO production, activation of macrophages can lead to an increased expression of cytokines that further modulate the immune response in response to a bacterial infection. Quantitative real-time PCR (qRT-PCR) was used on selected genes (Table 1) to evaluate the effect of APEC on the immune response in HD11 cells. In addition, to evaluate the effect of viability of APEC on cytokines expression in HD11 cells, heated-killed APEC was also used in these experiments. At 4 h post-infection, APEC strongly up-regulated the expression of pro-inflammatory cytokines IL-1β and IL-6, inflammatory cytokine IL-8 and anti-inflammatory cytokine IL-10 in HD11 cells (Fig. 5), whereas IFN-β was not affected. Similar results were also found for *S. enteritidis* stimulation in HD11, indicating that HD11 cells respond similarly towards both bacteria. Interestingly, addition of heat killed APEC (or *S. enteritidis*) to HD11 cells resulted in a lower expression of IL-1β, IL-6 and IL-8, compared to viable APEC, but expression of IL-10 was not affected. Overall these results show that HD11 cells are capable of a strong cytokine production after phagocytosis of APEC, comparable to *S. enteritidis*, and that viability of APEC affects most but not all cytokines indicating different signaling pathways could be involved for the production of these mRNAs.

4. Discussion

Avian pathogenic *Escherichia coli* (APEC) can infect different kinds of birds including chickens, turkeys, and ducks, and causes systemic infections called avian colibacillosis due to immunosuppression and damage of the immune system (Antao et al., 2008). Despite of the identification of some virulent genes involved in bacterial adhesion and invasion that contribute to APEC pathogenesis (Kemmett et al., 2013), the pathogenic mechanism of APEC is still unknown as no specific virulence gene has been identified for the entire APEC pathogenesis in avian host. Most studies have focused on the role of virulence in APEC pathogenesis, but little research is performed on the interaction of APEC with host innate immune cells. Macrophages are quickly increased upon APEC infection response to APEC infection (Ariaans et al., 2008). Therefore, we studied the interaction between APEC and macrophages as an important step to determine the initial host response to APEC infection.

As a first step, the effect of temperature on the phagocytic activity of macrophages was determined. HD11 cells are often used at 37 °C (Xie et al., 2003; Lavric et al., 2008; Jarvis et al., 2017) but the chicken body temperature is closer to 41 °C, which implies that the latter temperature would better resemble the *in vivo* situation. Indeed, some clear differences were observed at the higher temperature *E. coli* (APEC and non-APEC) grew faster at 41 °C (Fig. 1) indicating that the temperature was non-specific for the growth of APEC. On the other hand, HD11 cells phagocytized more *E. coli* at 41 °C (Fig. 2), demonstrating that HD11 cells have higher phagocytic activity at 41 °C. This is in line with available literature on the effect of temperature on phagocytic activity of immune cells in cold-blooded species. Phagocytic activity has been reported to decline in fish at low (< 15 °C) or high (37 °C) temperature (Mondal and Rai, 2001). Activity of other immune cells and antibody binding activity also reduced at decreased body temperature (Collazos et al. 1995, 1996; Maniero and Carey, 1997). In a mice model experiment, macrophages exposed to an acute cold environment (4 °C for 24 h) were suppressed in their activity (Kizaki et al., 1996). These studies imply that temperature plays an important role in remaining immune response and function of immune cells, which suggests that chicken macrophages should be used at 41 °C in order to resemble *in vivo* activity as much as possible.

The mononuclear phagocytic cells are part of the first line defense against invading pathogens. Activated macrophages can secrete a series of cytokines and chemokines and kill microorganisms by phagocytosis (Smith et al., 2005; Flannagan et al., 2009; Aderem and M. Underhill, 1990; Sansonetti, 2001). For mammalian macrophages, many studies on phagocytic activity have been described, but the available data for chicken macrophages is limited. For chicken macrophage studies, phagocytosis of different bacteria has been investigated, such as *Salmonella typhimurium* (Wisner et al., 2011), *Listeria monocytogenes* (Jarvis et al., 2017) and *Mycoplasma synoviae* (Lavric et al., 2008), but the research about APEC is lacking. Therefore, we studied phagocytosis of APEC in chicken macrophages (HD11). Our data showed that HD11 cells are relatively slow at uptake of APEC with relatively low numbers of APEC at 2 h post-infection after addition of the bacteria. However, longer incubation times significantly increased the number of phagocytosed APEC, although still much lower than *Salmonella* (Fig. 2). In initial studies to optimize *E. coli* uptake by HD11 cells, the effect of addition of chicken serum on phagocytosis of HD11 cells was tested but

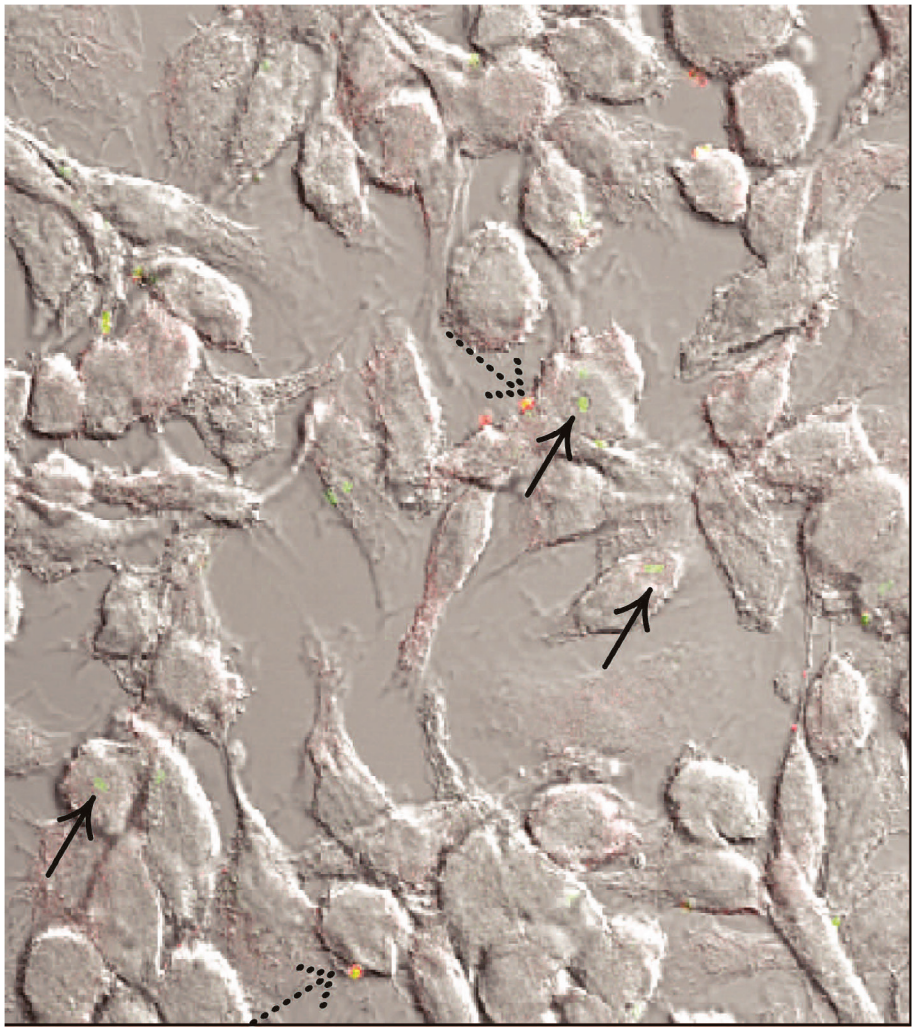

Fig. 3. Localization of APEC in HD11 cells. Cells were incubated with GFP-APEC for 3 h. Subsequently, cells were washed and fixed, but not permeabilized. Extracellular APEC were labeled with rabbit anti E. coli antibody and Donkey anti-Rabbit Alexa 647 (red). HD11 cells appeared grey under the DIC channel. Intracellular bacteria appear green (black arrows), while extracellular bacteria appear as red/yellow (dashed arrows).

this had no effect (data not shown). Similarly, low numbers of phagocytosed (non-avian pathogenic) *E. coli* by HD11 macrophages were also observed by Wisner et al. where *E. coli* DH5α was used as a control group for their *Salmonella* studies, although these authors suggested that this was due to fast killing of *E. coli* (Wisner et al., 2011). These differences in phagocytosis indicate that macrophages have different phagocytic capacity to different bacterial strains. After all, phagocytosis is a complex process involving a diverse set of receptors that can stimulate phagocytosis. Different phagocytosed microbes have a different capacity to affect cells. In our study, it is visually shown for the first time that HD11 can indeed phagocytize APEC (Fig. 3) as intracellular APEC was clearly observed under the fluorescent microscopy (Fig. 3), indicating that a lack of uptake is not likely the cause of APEC virulence.

It is well known that macrophages can ingest and kill bacteria. Our results clearly indicated that APEC was killed by HD11 at 6 h post-infection while *Salmonella enteritidis* survived at all the time points. It has been reported that *Salmonella* can secrete different virulence factors in host cells to invade, survive and replicate within these host cells (Malik-Kale et al., 2011; Ibarra and Steele-Mortimer, 2009), explaining the observed survival of *Salmonella*. In addition, *Salmonella* has been shown to delay the phagolysosomal maturation and neutralize radical oxygen and nitrogen species (ROS and NOS) as defense mechanisms against host cells (Haraga et al., 2008; Aussel et al., 2011; Henard and Vazquez-Torres, 2011). APEC apparently does not have such an evasion strategy although it must be said that after 6 h, the number of APEC did not further change.

NO plays an important role in the host defense against microbial infection (Alam et al., 2008). It can be produced by activated monocytes or macrophages, and act as effector molecules to kill invading pathogens. Previously, *S. enteritidis*-induced NO production was described in chicken macrophages (Okamura et al., 2005; Babu et al., 2006). Similarly, our results showed that live and, to lesser extent, heat-killed bacteria induced NO production (Fig. 5), demonstrating that NO production might partially depend on bacterial viability. Interestingly, even though HD11 cells took up lower numbers of APEC, compared to *S. enteritidis*, NO production was higher, indicating that APEC is a relatively strong inducer of NO. One study actually showed that (viable) *S. enteritidis* was able to inhibit production of NO in HD11 cells (He et al., 2012b) probably as an evasion mechanism, but the exact mechanism was unknown. The discrepancy with our results could be related to strain dependency since the same study showed that other *Salmonella* serovars induced a strong NO response in HD11 cells. Overall, chicken macrophages seem to be able to produce a strong response towards APEC, comparable to, or even higher than *Salmonella*.

Besides intracellular killing of pathogens, professional phagocytes play an important role in modulating the immune response through expression of cytokines and chemokines. Therefore, we explored the

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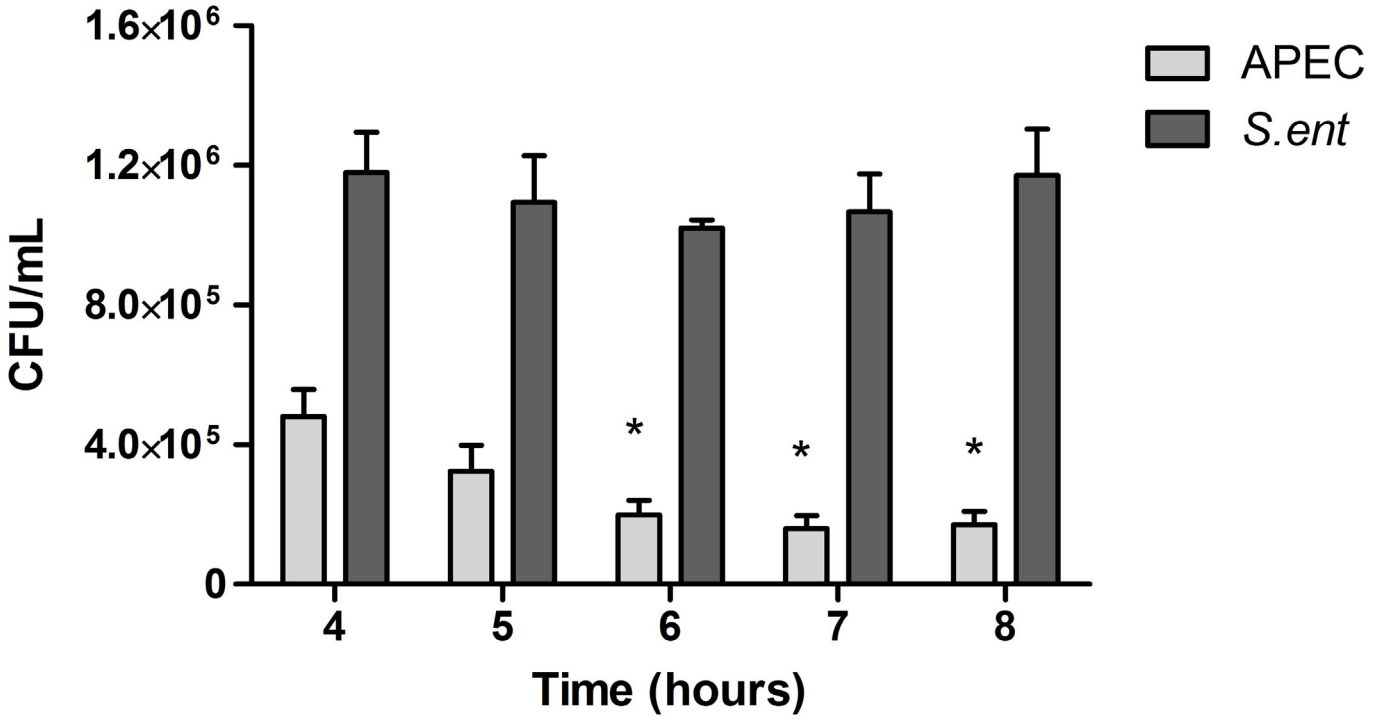

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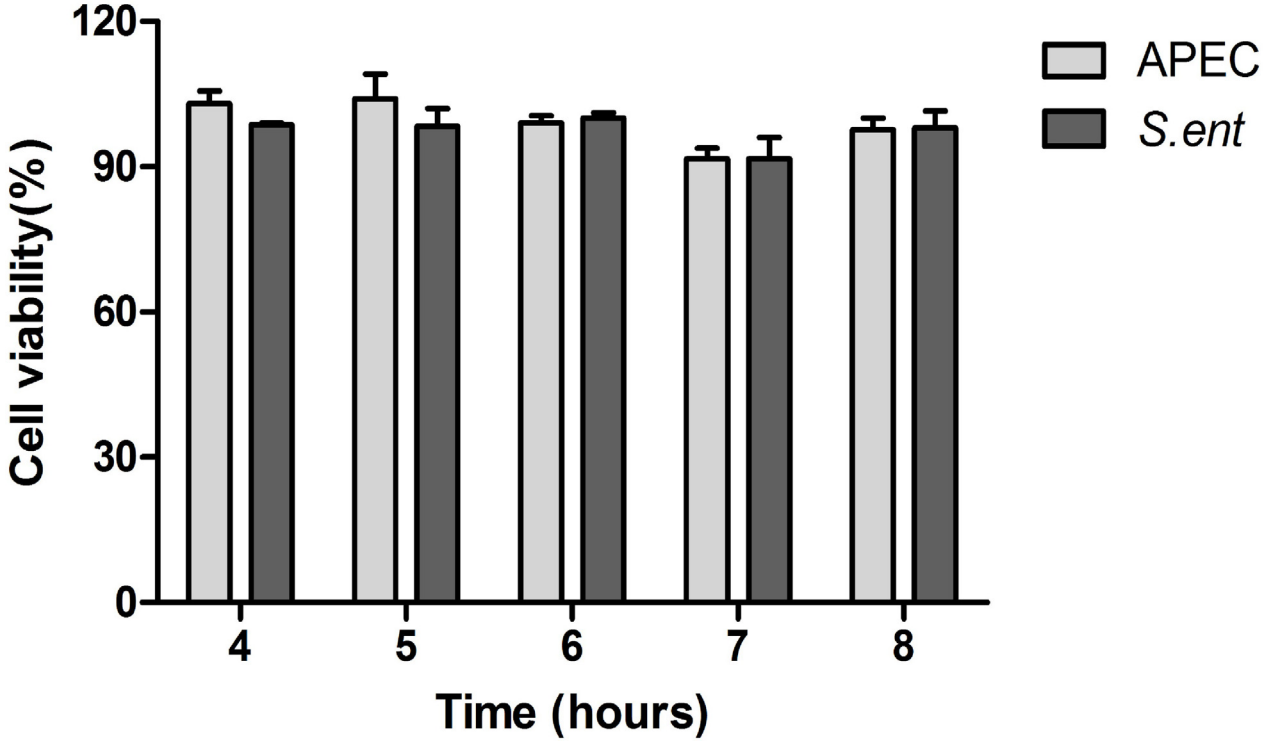

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Fig. 4. Survival of phagocytized bacteria in HD11 cells. (A) Viable APEC and *S. enteritidis* in HD11 cells. (B) Viability of HD11 cells. Data are shown as mean ± SEM of three independent experiments for each group (in triplicate). * indicates significant difference ($P \leq 0.05$) in bacteria compared with 4 h incubation using one-way ANOVA with post-hoc T-test.

initial immune response induced by APEC after 4 h incubation with chicken macrophages. Our results showed that both APEC and *S. enteritidis* significantly induced the expression of IL-1β, IL-6 and IL-8 (Fig. 5). The induction of IL-6 and IL-1β in this study is consistent with other observations that APEC induced increased expression of IL-6 and IL-1β in chicken monocyte derived macrophages (Lavric et al., 2008). In another study *S. enteritidis* infection of chickens led to increased expression of IL-8 and IL-1β even through this was measured in the cecum (Crhanova et al., 2011). The surfaces of *E. coli* and *S. enteritidis* have a variety of MAMPs (microbial associated molecular patterns), like LPS and flagellin, interacting with toll-like receptors (TLR) on the macrophage surface. The observation of induced pro-inflammatory cytokines IL-8 and IL-1β as well as inflammatory cytokine IL-6 is likely explained by activation of TLR signaling (Iqbal et al., 2005; Kogut et al., 2005). However, we observed that heat-killed APEC induced a lower amount of these three cytokines compared to live APEC, indicating that the level of cytokines expression induced by APEC is partially determined by bacteria and the presence of MAMPs. Interestingly, with respect to anti-inflammatory cytokine IL-10 expression, live APEC and heat-killed APEC induced almost the same level of cytokine expression, but *Salmonella* induced a higher expression, suggesting that this process might depend on amount and nature of MAMPs and is not necessarily bacterium-specific. Poly (I:C) and CpG-ODN have been reported to upregulate chicken IL-10 in chicken monocytes, indicating the occurrence of immune regulation to control excessive inflammation (He et al., 2012a). Finally, there was not much difference for IFN-β

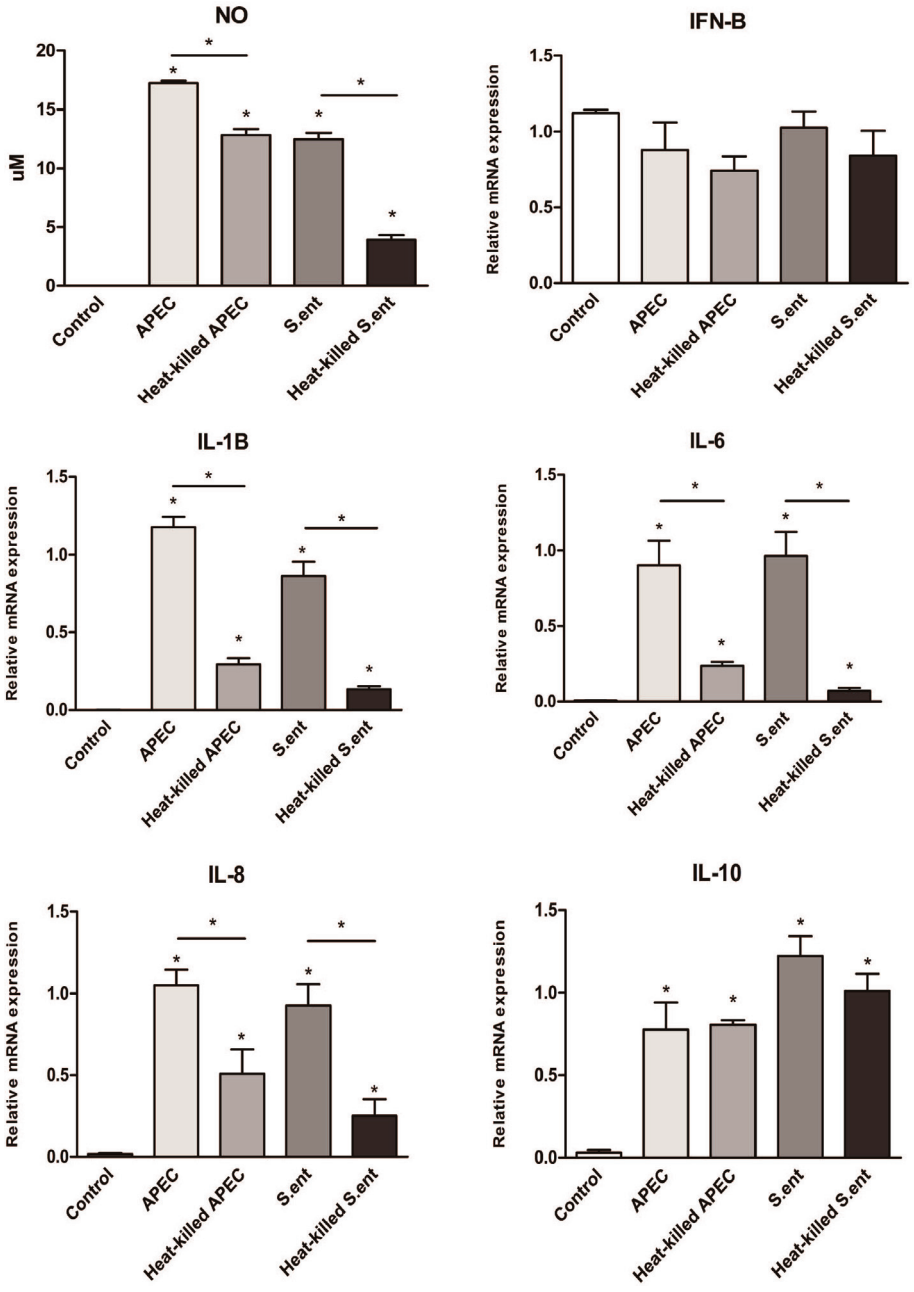

Fig. 5. NO production and cytokine expression of HD11 cells after incubation with bacteria. HD11 cells were incubated with live or heat-killed APEC and *S. enteritidis* for 18 h. Subsequently, nitrite (metabolite of produced NO) content in cell culture media was determined. For cytokines expression, HD11 cells were incubated with APEC and *Salmonella* for 4 h. Relative mRNA expression of cytokines in HD11 cells was determined using qPCR. Data are shown as mean ± SEM of three independent experiments (in triplicate) for each group. * indicates a significant difference compared to the control, or (indicated with horizontal bars) between specific treatment groups using one-way ANOVA with post-hoc T-test.

expression in this study, which is in agreement with published studies on LPS and bacterial DNA stimulation of HD11 cells, contrary to viral infection of HD11 cells, did also not IFN-β production (Gou et al., 2015; Lee et al., 2015; He et al., 2012a). These results suggest that APEC can induce a strong inflammatory response by the expression of some cytokines, but not for all the cytokines due to different signaling pathways.

In summary, we investigated the interaction of APEC with chicken macrophage HD11 cells. APEC was efficiently phagocytized by HD11 cells at 41 °C and subsequently killed. Phagocytosis resulted in a clear pro-inflammatory immune response including production of NO and pro-inflammatory cytokines, indicating that in principle chicken macrophages are capable of an appropriate immune response towards APEC infection. However, although the HD11 cell line is a well-established macrophage cell line, phagocytosis of APEC by chicken primary macrophages needs to be further explored to obtain extra confirmation of our current findings.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

All data is available within the article and its supplementary information files, and from the corresponding author on reasonable request.

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