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Innate antiviral responses are induced by TLR3 and TLR4 ligands in chicken tracheal epithelial cells: Communication between epithelial cells and macrophages

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ABSTRACT

The chicken upper respiratory tract is the portal of entry for respiratory pathogens including avian influenza virus (AIV). There is a paucity of information about the role of airway epithelial cells in the induction of antiviral responses in the chicken trachea. A better understanding of the role of these cells in the initiation of innate responses may improve prophylactic or therapeutic strategies for control of viral infections. The present study aimed to characterize antiviral innate responses in chicken tracheal epithelial cells (cTECs) induced by TLR ligands. The results demonstrated that stimulation of cTECs with TLR ligands induced antiviral responses, and subsequently reduced the replication of AIV in cTECs. Additionally, stimulated cTECs were able to influence the function of other cells such as macrophages. Overall, these results provided evidence that cTECs mount antiviral responses after stimulation with TLR ligands through IRF7 and NF-κB signaling pathways, leading to activation of other cells, such as macrophages.

1. Introduction

Airway epithelial cells are the main barrier to the entry of respiratory pathogens and are the primary target of respiratory viral infections, including influenza viruses, to support virus replication (Wu et al., 2016). Airway epithelial cells form a barrier as the initial line of defence by isolating the lumen and luminal surfaces from basolateral surfaces. These cells are capable of recognizing the presence of pathogens and initiating pro-inflammatory responses and cytokine production in the respiratory system (Diamond et al., 2000; Kato and Schleimer, 2007). Previous studies in mice and humans have highlighted the role of epithelial cells in detecting various pathogens and in shaping immune responses to pathogens (Weitnauer et al., 2016; Schleimer et al., 2007). The induction of innate responses at the epithelial barrier depends on identification of the presence of pathogens and their pathogen-associated molecular patterns (PAMPs) through germline-encoded pattern recognition receptors (PRRs), including Toll-Like Receptors (TLRs) (Akira and Takeda, 2004). Following activation of PRRs, the induction of type I interferons (IFNs) and interferon-stimulated genes (ISGs) is essential for protection against viral infections. The IFN system and the expression of ISGs are responsible for restraining early replication and spread of viruses (Le Bon and Tough, 2002). Contrary to mammalian species in which several members of type I IFNs have been identified, including IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, and IFN-τ, only two type I IFNs (IFN-α and –β) have been identified in avian species (Santhakumar et al., 2017). Chicken IFN-α and IFN-β can bind to the IFN receptor and induce ISGs in chickens (Qu et al., 2013). Chicken type I IFNs bind to the same receptors (IFNAR1 and IFNAR2), but they distinctively regulate the transcription of ISGs. A previous study has demonstrated that chicken IFN-α induces significantly higher expression of some ISGs, including protein kinase R (PKR) and 2′-5′-oligoadenylate synthetase (OAS) compared to IFN-β (Qu et al., 2013). Subsequently, the ISGs that are induced and the resultant antiviral responses vary in different tissues and cells following the activation of TLRs (Santhakumar et al., 2017; Karpala et al., 2008). For example, a previous study in chickens has determined the significant expression of OAS in cecal tonsils following treatment with lipopolysaccharides (LPS) from *Escherichia coli* 026: B6, however, LPS

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did not induce the expression of OAS in the trachea (Barjesteh et al., 2015). Therefore, ISGs that are induced in different tissues determines the output of host antiviral responses.

Previous studies in chickens confirmed the induction of antiviral responses in the respiratory system following TLR ligand treatment (Barjesteh et al., 2015; St Paul et al., 2012a). These studies showed that intranasal or intramuscular administration of TLR ligands including LPS from *E. coli* 026: B6, synthetic class B CpG ODNs, and polyI:C significantly reduce oral and cloacal shedding of avian influenza virus (AIV). These studies confirmed the significant expression of ISGs in the trachea and lungs following treatment with TLR ligands (Barjesteh et al., 2015; St Paul et al., 2012a). Also, a previous study using tracheal organ culture showed that TLR ligands are able to induce functional antiviral responses in the chicken trachea which may inhibit viral replication in host cells and activate macrophages (Barjesteh et al., 2016). However, activation of intracellular signaling cascades downstream of TLR activation, transcriptional activation of type I IFNs and functional antiviral responses in chicken airway epithelial cells have not been described very well. In mammalian species, sensing of PAMPs by PRRs causes the activation of transcription factors including interferon regulatory factors (IRF), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). In mammalian species, IRF7 controls the transcription of type I and III IFNs, and ISGs in epithelial cells. Therefore, it has an essential role in the induction of innate antiviral responses in the host (Bosco et al., 2016). Also, a previous study in mice demonstrated the central role of IRF7 in the activation of antiviral responses. A functional mutation in IRF7 leads to severe susceptibility to influenza virus (Honda et al., 2005; Ciancanelli et al., 2016). In addition, previous studies in chickens demonstrated the critical role of IRF7 in the regulation of antiviral responses and type I IFNs production (Wang et al., 2012; Kim and Zhou, 2015).

Further studies are required to fill the gap in our current knowledge of the underlying mechanisms for the activation of antiviral responses in chicken airway epithelial cells. In the present study, we aimed to gain a better understanding of induced antiviral responses in tracheal epithelial cells following TLR ligand treatments. We employed chicken primary tracheal epithelial cell culture to determine whether TLR3 and 4 ligand treatments can inhibit the replication of avian influenza virus in these cells. Also, we explored the possible role of IRF7 and NF-κB pathways in the induction of these responses in chickens. In addition, to confirm the effects of treated cTECs on macrophages, we explored the chemotactic effects of cTECs on macrophages, the expression of MHCII, CD86 and CD80 on macrophages and the replication of AIV in macrophages treated with cTEC supernatants.

2. Results

2.1. Avian influenza virus replicates in chicken tracheal epithelial cells

Viral replication in cTECs was assessed by examining infectious virus in cell culture supernatants using the $TCID_{50}$ assay. The results showed that the virus titer in supernatants was significantly increased by 274, 4880, 13334 and 2740-fold at 8, 18, 24 and 48 h post-infection, respectively, compared to the time of infection (time 0). Peak virus titer occurred at 24 h, and it was significantly decreased by 4.9-fold at 48 h post-infection relative to 24 h post-infection (Fig. 1).

2.2. Chicken tracheal epithelial cells express TLRs

We examined the constitutive expression TLR2, 3, 4, 5,7 and 21 in cTECs. The results confirmed that cTECs expressed TLR2, 3, 4,5, 7 and 21 at the transcript level. However, varying transcriptional levels of TLR genes were observed for different TLRs (Fig. 2).

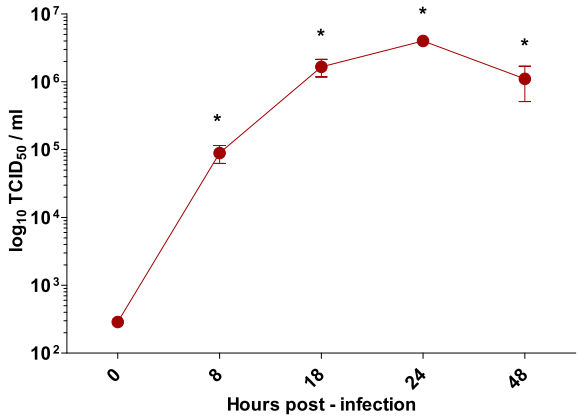

Fig. 1. AIV replication in chicken tracheal epithelial cells. Chicken tracheal epithelial cells were infected with low pathogenic H4N6 AIV at a MOI of 0.1. Cell supernatants were collected at 0, 8, 18, 24 and 48 h post-infection. This figure is representative of two separate experiments with four biological replicates per time point. Significant differences ($P \leq 0.05$) between the viral titer at a specific time point and the time of infection (time 0) are indicated by *.

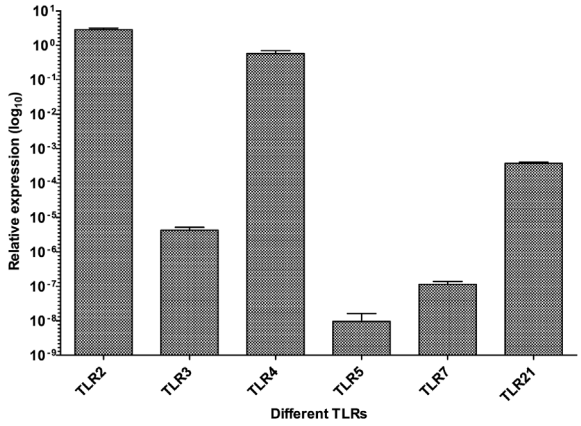

Fig. 2. Chicken tracheal epithelial cells express TLRs. Gene expression of TLR2, 3, 4,5, 7 and 21 was evaluated relative to the house-keeping gene β-actin.

2.3. TLR ligands induce antiviral responses in chicken tracheal epithelial cells

LPS and polyI:C were able to induce the expression of pro-inflammatory cytokines, IFN-β and ISGs in cTEC cells. The expression of IL-6 was significantly increased in cTECs treated with LPS at 3 h post-treatment by 63-fold. However, its expression was significantly down-regulated by 4-fold at 18 h post-treatment following LPS treatment. The expression of IL-6 in cTECs treated with polyI:C was significantly increased at 3 and 18 h of incubation by 45- and 7-fold, respectively (Fig. 3a). The expression of IL-1β in cTECs treated with LPS was significantly increased at 3 and 18 h post-treatment by 539- and 21-fold, respectively. Treatment of cTECs with polyI:C significantly induced the expression of IL-1β at 3 and 18 h post-treatment (Fig. 3b). The expression of IL-8 in cells incubated with LPS was significantly increased at 3 and 18 h post-treatment by 159- and 12-fold, respectively. In addition, the expression of IL-8 in cells incubated with polyI:C was significantly up-regulated at 3 and 18 h of incubation by 17- and 5-fold, respectively (Fig. 3c).

The expression of IRF7 in cTECs treated with either LPS or polyI:C was significantly up-regulated at 3 h post-treatment by 14- and 3-fold, respectively (Fig. 3d). The expression of IFN-α did not change in cTECs treated with either LPS or polyI:C (Fig. 3e). However, the expression of IFN-β in cTECs treated with polyI:C was significantly up-regulated at 3

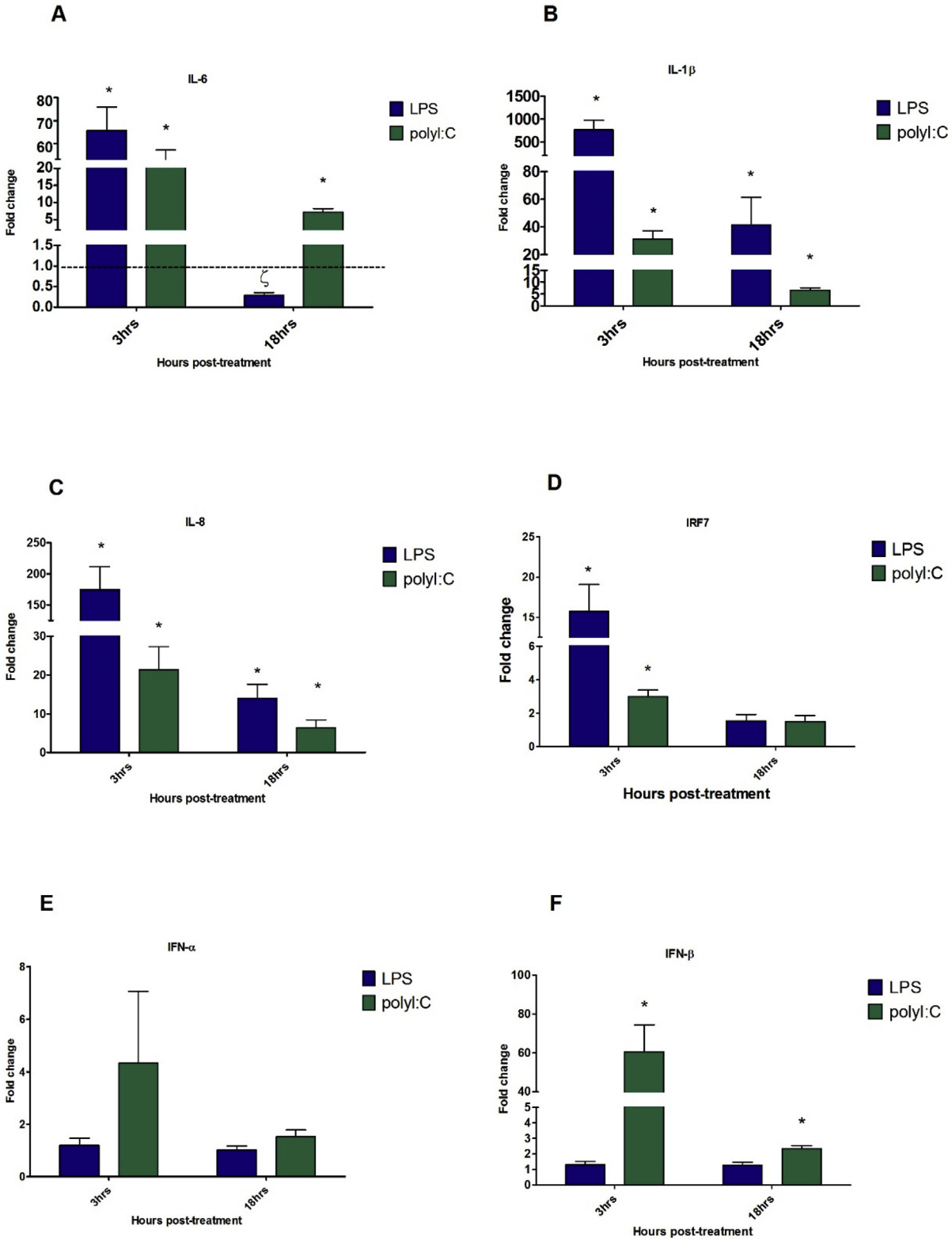

Fig. 3. mRNA expression of candidate genes in the chicken tracheal epithelial cells. a -l) represent the expression of different candidate genes induced by TLR ligands. cTECs were treated with either LPS from *E. coli* O26:B6 (1 μg/ml) and polyI:C (25 μg/ml). Control group received medium. Gene expression was determined at 3 and 18 h post-treatment using quantitative RT-PCR, relative to the housekeeping gene β-actin. Gene expression is presented as fold change relative to the medium group. Error bars represent standard errors of the means. Significant up-regulation ($P \leq 0.05$) is indicated by *. Significant down-regulation is indicated by §. There were five biological replicates in each group.

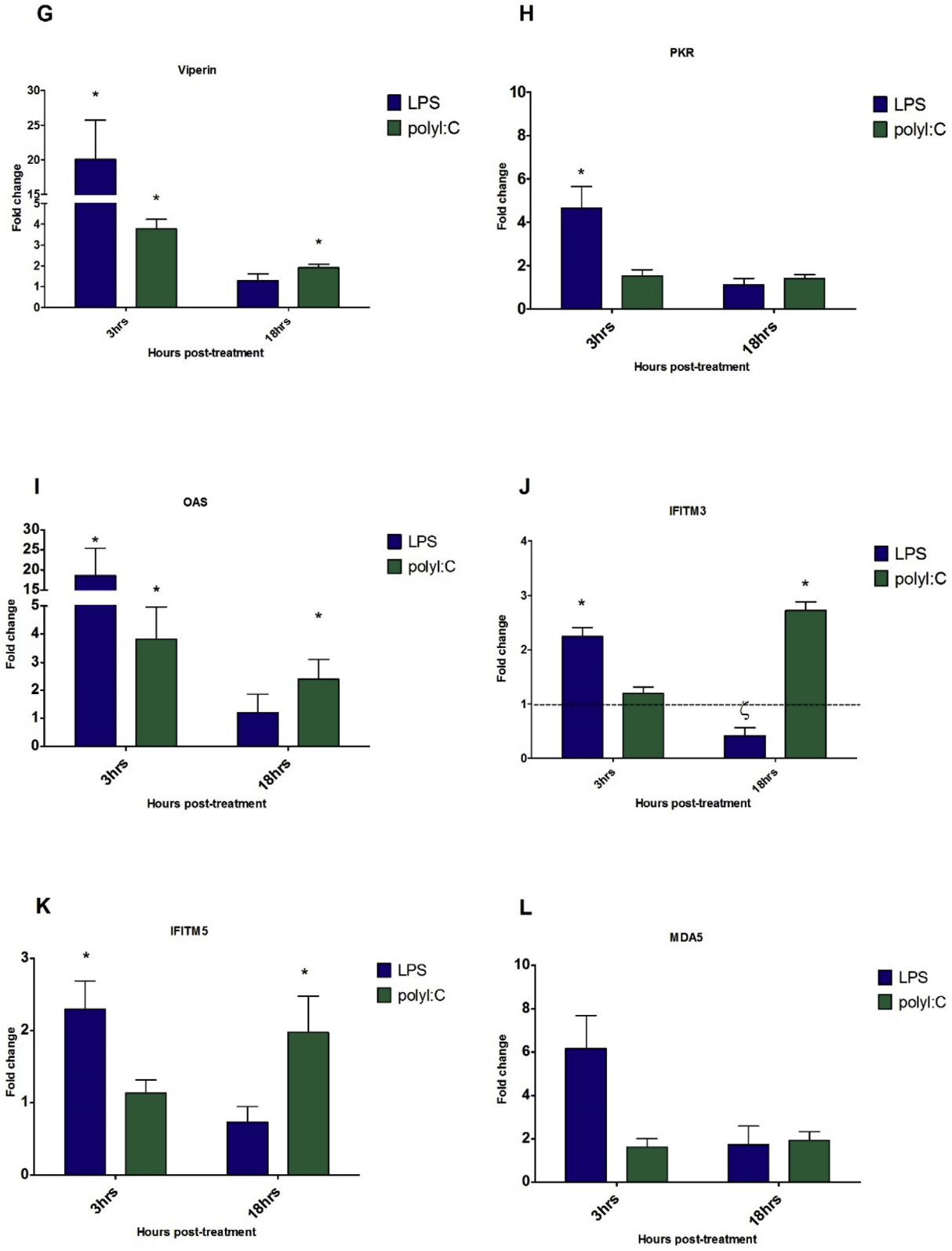

Fig. 3. (*continued*)

and 18 h post-treatment by 55- and 2.5-fold, respectively. The expression of IFN-β did not change following treatment with LPS (Fig. 3f).

LPS caused a 17-fold up-regulation of viperin expression in cTECs at 3 h post-treatment ($P \leq 0.05$). Moreover, polyI:C induced a 3.7 and 1.9-fold up-regulation of viperin at 3 and 18 h post-treatment, respectively ($P \leq 0.05$) (Fig. 3g). The expression of PKR by cTECs treated with LPS was significantly up-regulated by 4-fold at 3 h post-treatment (Fig. 3h). The expression of 2′-5′ OAS by cTECs incubated with LPS was significantly up-regulated by 15-fold at 3 h post-treatment. The expression of 2′-5′ OAS by cTECs treated with polyI:C was significantly up-regulated by 3.3- and 2.7-fold at 3 and 18 h of incubation, respectively (Fig. 3i). The expression of IFITM3 was significantly increased in cTECs

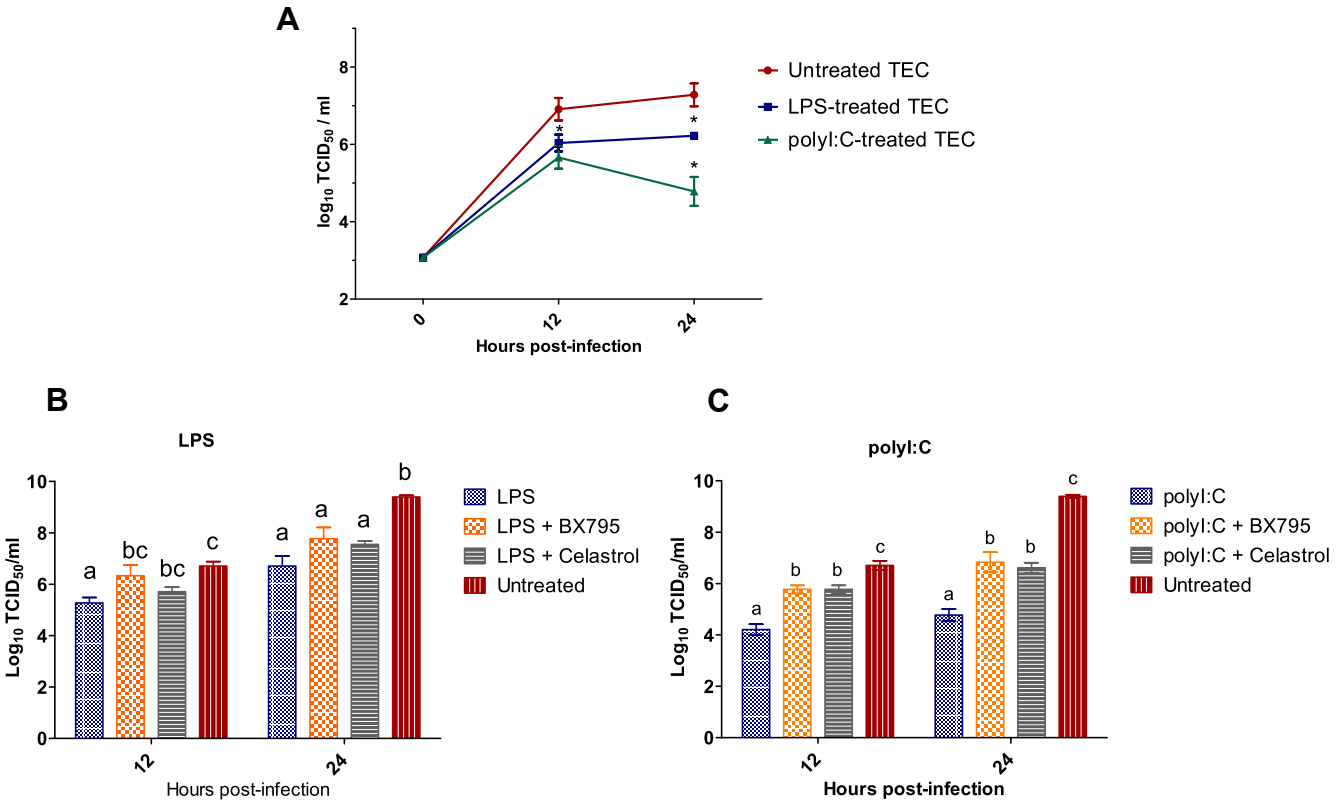

Fig. 4. Induction of antiviral responses in chicken tracheal epithelial cells through NF-ƙB and IRF3 pathways. a) TLR ligands reduced influenza virus replication in cTECs. Cells were untreated (control group) or treated with polyI:C (25 μg/ml) and LPS from *E. coli* 026: B6 (1 μg/ml) for 12 h. Cells (either treated or untreated) then were infected with a MOI of 0.1 of H4N6 avian influenza virus for 24 h. Virus titer was quantified via $TCID_{50}$ assay. b & c) IRF7 and NF-ƙB pathways play a role in the induction of antiviral responses in cTECs. Cells were untreated (control group) or treated with BX795 and celastrol as inhibitors of IRF7 and NF- κB pathways for one hour. Then, cells were treated with LPS from *E. coli* 026: B6 (1 μg/μl) (b) or polyI:C (25 μg/ml) (c) and for 12 h. Cells then were infected with a MOI of 0.1 of H4N6 avian influenza virus. Virus titer was quantified via $TCID_{50}$ assay. Different letters (a–c) above each column indicate a significant difference between groups ($P < 0.05$). When different groups have a common letter, it is indicated that there is no significant difference.

treated with LPS at 3 h post-treatment by 2.2-fold. However, LPS caused significant down-regulation of IFITM3 at 18 h post-treatment. In addition, the expression of IFITM3 by cTECs treated with polyI:C was significantly increased at 18 h of incubation by 2.7-fold (Fig. 3j). Moreover, LPS and polyI:C caused a significant up-regulation of IFITM5 in cTECs (Fig. 3k). LPS significantly up-regulated the expression of MDA5 in cTECs at 3 h of incubation by 5.28-fold (Fig.3l).

2.4. Induction of antiviral responses in chicken tracheal epithelial cells through NF-ƙB and IRF7 pathways

Treatment of cTECs with either LPS or polyI:C significantly reduced viral replication in these cells. Treatment of cTECs with LPS significantly reduced virus titer in cTEC supernatants by 28 and 528-fold at 12 and 24 h post-infection, respectively, compared to untreated, infected cTECs. Also, virus titer in cTECs treated with polyI:C was significantly lower at 12 and 24 h post-infection by 323 and 43791-fold, respectively, compared to untreated, infected cTECs (Fig. 4A).

The results demonstrated that treatment of cTECs with signaling pathway inhibitors including BX795 (IRF7 inhibitor) and celastrol (NF-κB inhibitor) restrained the antiviral activity of LPS and polyI:C. Virus titer was increased by 10.8-fold in cTECs received treated with BX795 and LPS at 12 h post-infection compared to cTECs treated with LPS only. Also, virus titer was increased by 2.8-fold in cTECs treated with celastrol and LPS at 12 h post-infection compared to cTECs treated with LPS only (Fig. 4b). Virus titer was higher in the supernatants of cTECs treated with BX795 and polyI:C by 37 and 111.8-fold at 12 and 24 h post-infection, respectively, compared to cTECs treated with polyI:C alone. Virus titer was higher in the supernatants of cTECs treated with celastrol and polyI:C by 37 and 71-fold at 12 and 24 h post-infection, respectively, compared to cTECs treated with polyI:C only. Moreover, virus titer in cTECs treated with BX795 and polyI:C was significantly lower than untreated, infected cTECs by 8.6-and 392-fold at 12 and 24 h post-infection (Fig. 4c).

2.5. Products of TLR ligand stimulated chicken tracheal epithelial cells limit AIV replication in chicken macrophages

Treatment of chicken macrophages with culture supernatants from cTECs stimulated with TLR ligands significantly limited AIV replication in these cells, demonstrating antiviral activity of cTECs against AIV. Culture supernatants of cTECs treated with LPS (SupTEC-LPS) significantly reduced virus titer in macrophage supernatants by 16.5-fold compared to the infected, untreated macrophages. Treatment of chicken macrophages with SupTEC-polyI:C significantly reduce virus titer in macrophage supernatants by 14.5-fold compared to the infected, untreated macrophages. However, culture supernatants of cTECs treated with TLR ligands and inhibitors including BX795 and celastrol did not reduce virus titer in macrophage supernatants (Fig. 5).

2.6. TLR ligand stimulated chicken tracheal epithelial cells activate chicken macrophages

Culture supernatants of cTECs treated with either LPS or polyI:C significantly increased macrophage migration by 1.43 and 1.37-fold, respectively. BX795 did not interfere with chemotactic effects of culture supernatants of cTECs treated with either LPS or polyI:C on macrophages, but celastrol interfered with their chemotactic effects as there

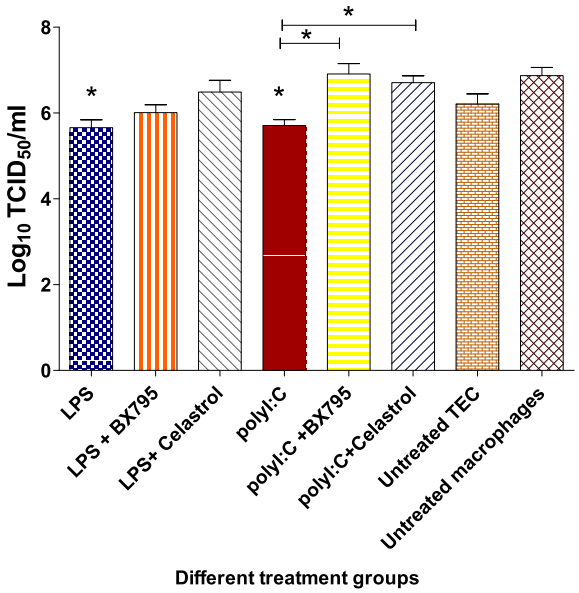

Fig. 5. Treated cTECs reduced influenza virus replication in a chicken macrophage cell line (MQ-NCSU). MQ-NCSU cells were untreated or treated with supernatants from cTECs treated with LPS from *E. coli* O26:B6 or polyI:C. Cells then were infected with a MOI of 1 of H4N6 avian influenza virus. Virus titer was quantified via $TCID_{50}$ assay. cTEC supernatants were collected 48 h post-treatment with TLR ligands.

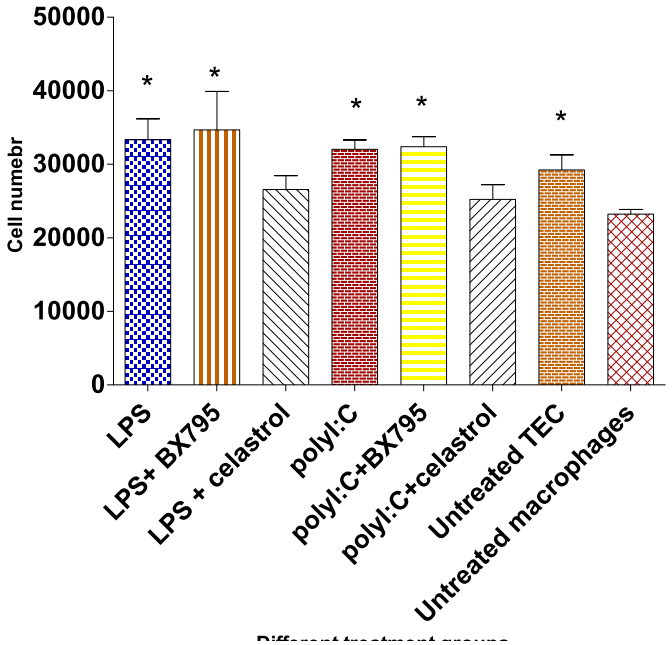

Fig. 6. Migration of chicken macrophages after culturing with supernatants from cTECs treated with TLR ligands. Macrophages were cultured with supernatant from cTECs treated with LPS from *E. coli* O26:B6, polyI:C or treated with supernatant from cTECs that received only medium (untreated TEC). cTECs were treated with inhibitors one hour before treatment with TLR ligands.

was a significant difference in cell numbers between untreated macrophages and macrophages treated with celastrol and TLR ligands (Fig. 6).

2.7. Activated chicken tracheal epithelial cells affect the expression of MHC-II, CD86 and CD80 on chicken macrophages

In order to examine the effects TLR ligand treated cTECs have on the activation of macrophages, we measured the expression of MHC-II, CD86, and CD80 on chicken macrophages co-cultured with treated cTECs. Cells were stained with anti-chicken Monocyte/Macrophage (clone KUL01), anti-chicken CD80, anti-chicken CD86, and anti-chicken major histocompatibility complex (MHC) class II antibodies for flow-cytometry analysis. Live cells were detected by the Live/Dead® Fixable Near-IR dead cell stain. In the next step, macrophages were gated based on the KUL01 marker. Supernatants from cTECs treated with LPS significantly reduced the expression of MHC-II and CD86, (30% and 37%, respectively), on chicken macrophages while supernatants from cTECs treated with polyI:C significantly increased the expression of MHC-II and CD86 on chicken macrophages by 12% and 16%, respectively. Celastrol reduced the effects of LPS on chicken macrophages. In macrophages co-cultured with cTECs treated with LPS and celastrol, the expression of MHC-II and CD86 was down-regulated by 53% and 63%, respectively. Also, cTECs treated with celastrol and polyI:C significantly down-regulated the expression of MHC-II and CD86 on chicken macrophages by 19% (Fig. 7).

3. Discussion

Accumulating evidence suggests an essential role of airway epithelial cells is to maintain a barrier function, to initiate host antiviral responses, and to defend against viral infections (Iwasaki et al., 2017; Vareille et al., 2011). Chicken airway epithelial cells, particularly in the trachea, are permissive and susceptible to AIV infection (Shen et al., 2010; Esnault et al., 2011; Samiento et al., 2008; Petersen et al., 2012). In response to viral replication, airway epithelial cells are able to actively respond to the AIV infection (Esnault et al., 2011; Samiento et al., 2008; Jang et al., 2015). A previous study demonstrated that chicken lung epithelial cells are able to induce the expression of interferons, chemokines, and cytokines very early after low pathogenic AIV infection (Esnault et al., 2011). In order to determine the induced antiviral responses in chicken tracheal epithelial cells against AIV, we first confirmed productive replication of low pathogenic AIV in tracheal epithelial cells. We showed that tracheal epithelial cells, without adding exogenous trypsin, support AIV replication. In agreement with previous studies, AIV replicated in chicken tracheal epithelial cells quickly with increasing virus titer over time, demonstrating susceptibility of tracheal epithelial cells to AIV infection (Shen et al., 2010; Samiento et al., 2008).

Previously, we highlighted the possible application of TLR ligands as antiviral agents against AIV infections (Barjesteh et al., 2015). We showed that intranasal treatment of chickens with TLR ligands significantly reduce AIV shedding from infected chickens. Subsequently, we demonstrated local innate responses in the chicken trachea stimulated with TLR ligands (Barjesteh et al., 2016). Despite this information, relatively little is known about the induction of antiviral responses by tracheal epithelial cells in chickens and their major roles in host protection against AIV infection. In this study, we selected two different ligands, LPS from *E. coli* and polyI:C, aiming to induce antiviral responses against low pathogenic AIV. Here, we have provided several lines of evidence that TLR ligands act as antiviral agents as they induce antiviral responses against AIV in chicken tracheal epithelial cells. First, treatment of tracheal epithelial cells with both TLR ligands resulted in the induction of mRNA transcripts for pro-inflammatory cytokines and antiviral genes including IFN-β and other antiviral mediators, such as viperin, IFITM3 and 5. Second, treatment of tracheal epithelial cells with both TLR ligands resulted in significant reduction of viral replication in chicken tracheal cells. Third, we confirmed functional and mechanistic aspects of antiviral activities of tracheal epithelial cells following treatment with TLR ligands.

Prior to characterizing TLR ligand induced antiviral responses, we first set out to define the transcriptional expression of TLRs in tracheal epithelial cells. The expression of TLR 2, 3, 4, 5, 7 and 21 was detected in chicken tracheal epithelial cells. Upon recognition of TLR ligands by

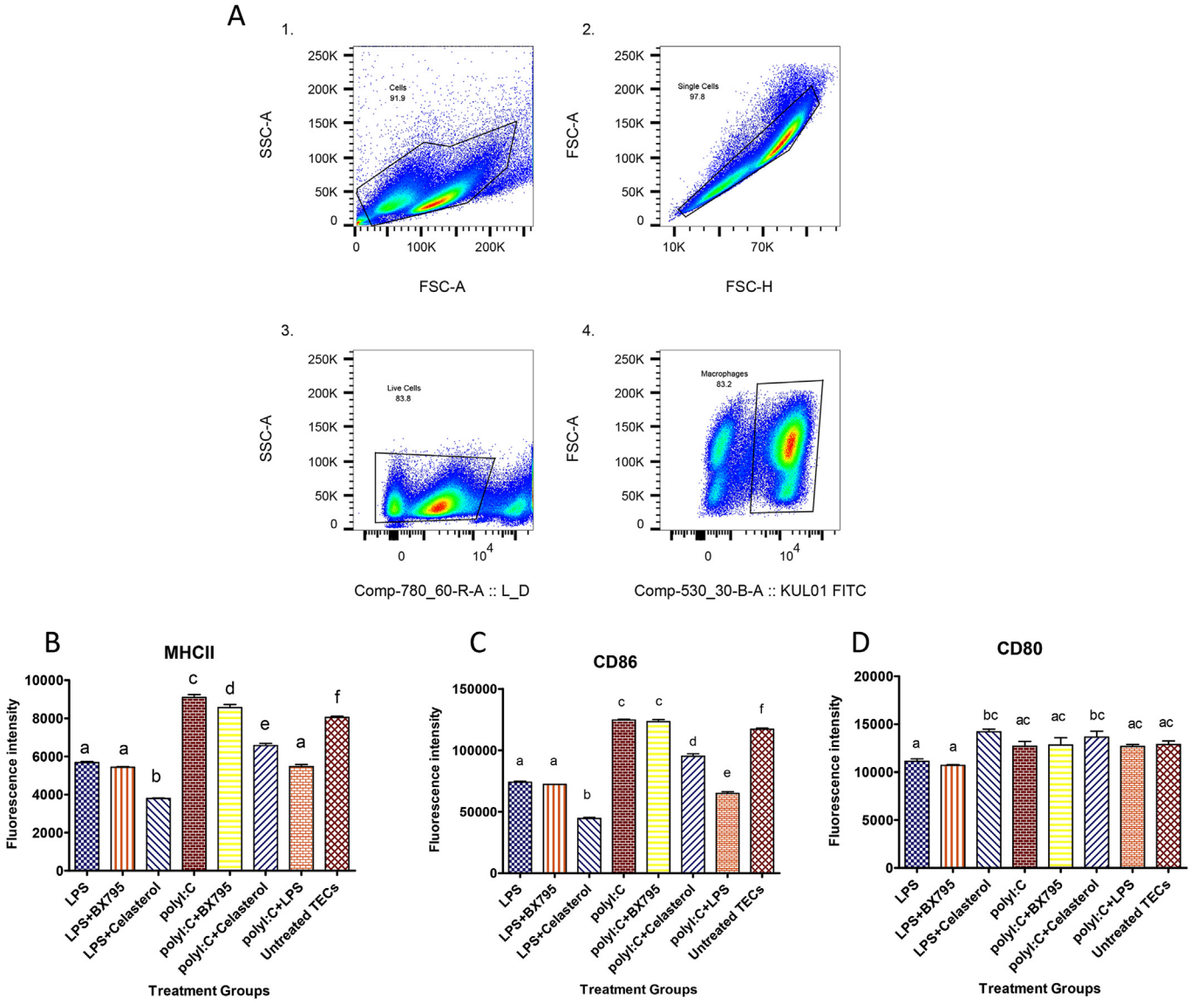

Fig. 7. Expression of surface markers on macrophages co-cultured with cTECs treated with TLR ligands. Macrophages were co-cultured with cTECs treated with LPS from *E. coli* O26:B6, polyI:C or cTECs that received only medium (untreated TEC). cTECs were treated with inhibitors one hour before treatment with TLR ligands. Isolated cells were stained with anti-CD80, -CD86 and –MHC-II antibodies, and then analyzed by flow cytometry; a) Gating on live cells and macrophages. b) MHC-II expression on chicken macrophages, c) CD86 expression on chicken macrophages and d) CD80 expression on chicken macrophages.

TLRs, activation of intracellular signaling pathways including NF-kB and IRF7 pathways results in type I IFN and pro-inflammatory responses. The results presented here showed that LPS and polyI:C induced IL-1β, IL-6 and IL-8 expression in chicken tracheal epithelial cells, which is in disagreement with induction of pro-inflammatory cytokines in human airway epithelial cells induced by TLR ligands. A previous study has shown that IL-6 production is induced only by polyI:C in human airway epithelial cells and LPS is not able to induce IL-6 expression in these cells. In addition, both LPS and polyI:C induce the production of IL-8 in human tracheal epithelial cells while LPS cannot induce IL-8 in human bronchial epithelial cells (Ioannidis et al., 2013; Sha et al., 2004). Several possibilities may explain these differences between mammalian and chicken epithelial cells responding to LPS and polyI:C. TLR-mediated signaling responses may be differentially regulated in chickens and mammalian species. Additionally, differences in human studies and the present study could be related to differences in the design and time course of experiments. For example, in studies with human airway epithelial cells, the production of IL-6 and -8 was quantified at the protein level at 24 h post-treatment with TLR ligands, while in the present study, we determined the expression of transcripts for pro-inflammatory cytokines at 3 and 18 h post-treatment with TLR ligands.

Infiltration and activation of macrophages in the respiratory system may limit the spread of viral infection in the host during the early stages. Therefore, we verified the functional activity of supernatants of cTECs treated with TLR ligands. To this end, we determined the chemotactic effects of supernatants from cTECs treated with TLR ligands on chicken macrophages. We showed that supernatants from cTECs treated with TLR3 and 4 ligands increased macrophage migration toward TEC supernatants. We conclude that the induction of pro-inflammatory responses in cTECs through the NF-κB pathway following TLR ligand treatment can recruit macrophages to the site of infection.

While polyI:C significantly induced the expression of IFN-β in chicken tracheal epithelial cells, LPS did not induce the expression of type I IFNs. However, both TLR ligands induced the expression of ISGs, such as viperin, PKR, OAS, IFTIM3 and IFITM5 in chicken tracheal epithelial cells. Significant expression of these ISGs by LPS without the induction of type I IFNs underlines IFN-independent ISG activation

which has been reported previously (Grandvaux et al., 2002; Diamond and Farzan, 2013). Moreover, our results demonstrated that antiviral responses by cTECs treated with TLR ligands limited AIV in chicken macrophages. In fact, supernatants from cTECs treated with TLR ligands may contain IFNs, ISGs or other active antiviral components that interfere with AIV replication inchicken macrophages. NF-κB and IRF7 pathway inhibitors reduced antiviral activities of TLR ligands which confirms the importance of these pathways in extrinsic antiviral activities of TLR ligands.

In addition, the results presented here showed that both ligands significantly limited AIV replication in chicken tracheal epithelial cells. However, polyI:C was more effective at limiting AIV replication compared to LPS which might be associated with the profile of induced antiviral response genes by polyI:C. Our results underlined the existence of differential time-based and quantitative induction profiles of ISGs and type I IFNs between TLR ligands which may have affected the replication of AIV in treated cTECs. The expression of most of the candidate genes induced by polyI:C lasted for 18hrs, whereas the expression of most of the LPS induced candidate genes were sustained for a shorter period of time. Previous studies in chickens and other species have highlighted the essential role of the NF-κB pathway in the induction of inflammatory responses (Chiang et al., 2009; Peroval et al., 2013; Crippen, 2006). Also, a previous study has defined the role of the NF-κB pathway in antiviral responses in which NF-κB in cooperation with the IRF-3 pathway supports the expression of IFN-β in stimulated cells. In the present study, NF-κB and IRF7 inhibitors efficiently increased influenza virus titers in TLR ligand stimulated cTECs which confirmed the role of these two pathways in the induction of antiviral responses against influenza virus. However, these inhibitors did not completely block antiviral activities of TLR ligands, suggestingother pathways are stimulated by TLR ligands, such as the MAPK pathway, which may contribute to the induced antiviral responses (Peroval et al., 2013; Arthur and Ley, 2013).

Furthermore, we determined the effects of cTEC responses on chicken macrophages. We showed that cTECs treated with TLR ligands are able to affect macrophage functions which could be useful for boosting innate responses. Macrophages exposed to supernatants from TECs treated with polyI:C mostly showed a pro-inflammatory phenotype, marked by significant expression of CD86 and MHC-II. However, macrophages exposed to supernatants from TECs treated with LPS mostly showed an anti-inflammatory phenotype, marked by a significant decrease in the expression of CD86 and MHC-II. Differences in macrophage responses following treatment with TLR ligands may affect T cell responses. For example, previous studies showed an association between the polarization of macrophages and T cell responses (Blom et al., 2016). The supernatants from cTECs treated with TLR ligands may affect the polarization of macrophages in chickens. However, future studies are required to examine the polarization of these cells. Therefore, choosing a specific TLR ligand could help to shift macrophage responses as well as adaptive immune responses. Further studies are required to determine the interactions between these two cell types in chickens.

Taken together, our findings extended previous observations concerning antiviral activities of TLR ligands in the chicken respiratory system. We also demonstrated that in an *in vitro* setting, stimulated tracheal epithelial cells could interact with macrophages and influence their function. These findings provide a better understanding of innate immune responses against AIV and underlying mechanisms in chicken tracheal epithelial cells.

4. Materials and methods

4.1. Ethics statement

This study was conducted in compliance with the guidelines of the Canadian Council on Animal Care including animal care, procedures, and program management. All experiments were approved by the Animal Care Committee of the University of Guelph (Animal Utilization Protocol number 3284). Specific pathogen-free (SPF) eggs were purchased from the Animal Disease Research Institute, Canadian Food Inspection Agency (Ottawa, ON). In this study, SPF 10-day-old embryonated chicken eggs were inoculated with H4N6 AIV.

4.2. Avian influenza virus

A low pathogenic avian influenza virus (LPAIV), A/Duck/Czech/56 (H4N6), was used in the present study. We previously confirmed that it replicates in chicken macrophages and TOCs (Barjesteh et al., 2014, 2016). The virus was propagated in 11-day-old embryonated chicken eggs by inoculation through the allantoic cavity (Szretter et al., 2006). Briefly, embryonated chicken eggs were candled, and embryos were inoculated with 100 µl of allantoic fluid containing 0.2 hemagglutination units (HAU) of H4N6. The allantoic fluid was harvested after 72 h, and the virus titer was determined using end-point dilution in the Madin-Darby canine kidney (MDCK) cells and expressed as 50% tissue culture infective dose ($TCID_{50}$)/ml according to the Reed-Muench formula (WHO, 2002).

4.3. Primary tracheal epithelial cell isolation

Chicken tracheal epithelial cells were isolated from 4-6-week-old SPF chickens as previously described with some modifications (Shen et al., 2010, 2011; Samiento et al., 2008). Briefly, tracheas were aseptically collected from chickens. Tracheas were washed twice with warm Hanks' balanced salt solution (HBSS) to remove excess mucus. The connective tissues surrounding the trachea were removed by careful dissection. Tracheas were digested with protease from *Streptomyces griseus* (Sigma–Aldrich, Oakville, ON, Canada) (1.5 g/ml) in Medium 199 (Sigma–Aldrich, Oakville, ON, Canada) supplemented with 25 mM HEPES buffer, 200 U/ml penicillin, 80 µg/ml streptomycin and incubated at 37 °C for 2hrs. Detached cells from tracheal epithelium were collected by removing the digested trachea from protease solution and transferring it to a conical tube containing Medium 199. The tube was inverted several times. Then, the trachea was removed from the tube. The tube was centrifuged at 400 × *g* for 5 min at 4 °C. Cells were incubated in small flasks at 37 °C in 5% CO2 for 5 h. Then, unattached cells were collected and were seeded into 24-well cell culture plates, pre-coated with 250 µl of 5% matrigel gel solution per well, at a viable cell density (determined by Trypan blue exclusion) of 5×10^5 cells/ml in DMEM-F12 (HBSS, Gibco, Burlington, ON, Canada) containing 10% FBS, 200 U/ml penicillin, and 80 µg/ml streptomycin, 10% chicken embryo extract, 25 mM HEPES, 2 mM L-glutamine, 50 mM 2- Mercaptoethanol and MEM non-essential amino acids for 4 days. Isolated tracheal epithelial cells were stained with Vimentin and Pan-cytokeratin to ensure that they are epithelial cells.

4.4. Chicken macrophage cell line culture

The chicken macrophage cell line (MQ-NCSU) was kindly provided by Dr. Juan Carlos Rodriguez (University of Prince Edward Island, Canada). This cell line was derived from spleen cells of a chicken infected with the JM/102 W strain of Marek's disease virus (Qureshi et al., 1990). MQ-NCSU cells were maintained in 1:1 combination of Mc Coy's 5 A modified medium and L-15 Leibovitz medium supplemented with 8% fetal bovine serum (FBS), 10% chicken serum, 1% tryptose phosphate broth, 1% sodium pyruvate, 2 mM L-glutamine, 200 U/ml penicillin, 80 µg/ml streptomycin, and 50 µg/ml gentamicin at 41 °C and 5% CO_2 in a humidified incubator.

4.5. Avian influenza virus infection of tracheal epithelial cells

Cultured tracheal epithelial cells were washed with warm DMEM-

F12 on the fourth day of isolation to remove debris and detached cells, and the medium was replaced with DMEM-F12 supplemented with 200 U/ml penicillin, 80 μg/ml streptomycin, 50 μg/ml gentamicin, 25 mM HEPES and 7.5% (Bovine serum albumin) BSA. Cells were infected at a multiplicity of infection (MOI) of 0.1 with H4N6 AIV. After infection, cells were washed twice after 1.5 h, and fresh medium was added to the culture. The virus titer in supernatants was measured using a $TCID_{50}$ assay at different time points (0, 8, 18, 24 and 48 h) post-infection.

4.6. TLR ligands

PolyI:C (TLR 3 ligand) was purchased from Invivogen (San Diego, California, USA). Lipopolysaccharide from *Escherichia coli* 026:B6 (TLR 4 ligand) was purchased from Sigma–Aldrich (Oakville, Ontario, Canada).

4.7. Tracheal epithelial cell treatment with TLR ligands and infection with avian influenza virus

cTECs were stimulated with polyI:C (25 μg/ml), LPS from *E. coli* 026:B6 (1 μg/ml) or medium for 12 h. Then, cells were infected at a multiplicity of infection (MOI) of 0.1 with H4N6 AIV. Subsequently, cells were washed twice 1.5 h post-infection, and fresh medium was added to the culture. Virus titer in supernatants was measured using a $TCID_{50}$ assay at 12 and 24 h post-infection.

4.8. Inhibition of NF-κB and IRF7 pathways in chicken tracheal epithelial cells

Cultured cTECs were treated with 1 μM celastrol (Sigma–Aldrich, Oakville, ON, Canada) and 5 μM BX795 (Sigma–Aldrich, Oakville, ON, Canada) for one hour to block NF-κB and IRF7 pathways, respectively. Then cells were treated with TLR ligands and infected with AIV. as described above. Subsequently, cells were washed twice 1.5 h post-infection, and fresh medium was added to the culture. Virus titer in supernatants was measured using a $TCID_{50}$ assay at 12 and 24 h post-infection.

4.9. Gene expression in tracheal epithelial cells stimulated with TLR ligands

Tracheal epithelial cells were seeded into 24-well cell culture plates and cultured as described above. Cells were stimulated with either polyI:C (25 μg/ml) or LPS from *E. coli* 026: B6 (1 μg/ml). The control groups were received cell culture medium only. Cells were collected for RNA extraction at 3 and 18 h post-treatment. There were five biological replicates (isolated cells from five chickens) in each group.

4.10. RNA extraction and cDNA synthesis

Total RNA was extracted with Trizol reagent (Life Technologies, Burlington, Ca), according to the manufacturer's recommendations. Total RNA was treated with the DNA Free DNAse kit (Ambion, Austin, TX), and 1 μg of RNA was used for cDNA synthesis using Superscript II First Strand Synthesis kit (Life Technologies, Burlington, Ca) and oligo-dT primers, according to the manufacturer's protocol.

4.11. Real-time PCR

Quantitative real-time PCR was performed on diluted cDNA (1:10 in DEPC treated water) using SYBR green dye in a LightCycler 480 II (Roche Diagnostics GmbH, Mannheim, DE) as previously described (Barjesteh et al., 2015, 2016). Briefly, the amplification conditions consisted of pre-incubation for 10 min at 94 °C, followed by 45 cycles of 95 °C for 10 s, 55–64 °C annealing for each of the primers for 5 s and elongation and signal acquisition (single mode) at 72 °C for 10 s. Melting curve analysis was done in three steps; 95 °C for 10 s, cooling to 65 °C for 1 min and heating to 97 °C. Specific sequences of primers was described previously (Barjesteh et al., 2015, 2016) (Table 1).

4.12. Stimulation of chicken macrophages with supernatants collected from tracheal epithelial cells

In order to determine antiviral activities of tracheal epithelial cells, these cells were stimulated with TLR ligands as described above. After 1 h of stimulation with TLR ligands, tracheal epithelial cells were washed twice with complete medium and then incubated at 37 °C in fresh medium. In the groups with NF-κB and IRF7 inhibitors, cells were received 1 μM celastrol and 5 μM BX795 one hour prior to TLR ligand treatment. Treated cells were washed twice one hour after TLR ligand treatment to remove residues of either inhibitors or TLR ligands.

TEC supernatants were collected after 48 h of stimulation with TLR ligands, and bioactive antiviral activity was assessed by treating MQ-NCSU cells with culture supernatants before infecting them with AIV. Chicken macrophages were treated with supernatants for 6 h before AIV infection. Cells were infected with H4N6 AIV as described previously (Barjesteh et al., 2014). Briefly, MQ-NCSU cells were seeded at 5×10^5 cells/ml into 24-well cell culture plates in DMEM supplemented with 10% heat-inactivated FBS, 200 U/ml penicillin, and 80 μg/ml streptomycin and 50 μg/ml gentamicin for 2 h incubation at 40 °C. Cells were then treated with supernatants for 6 h. Cells were infected with H4N6 AIV at a multiplicity of infection (MOI) of 1.0; residual virus was removed one-hour post-infection by washing twice with warm medium. Virus titer in macrophage supernatants was measured using a $TCID_{50}$ assay at 12 h post-infection.

To measure the chemotactic effect of tracheal epithelial cells on chicken macrophages, macrophages were stimulated with supernatants from tracheal epithelial cells that were previously stimulated with TLR ligands as described above. The chemotactic ability of supernatants from treated tracheal epithelial cells was measured by QCM™ Chemotaxis 5 μm 96-Well Cell Migration Assay (EMD Millipore, Mississauga, ON). Briefly, 150 μl of TEC supernatants were added to the feeder tray provided by the kit. MQ-NCSU cells were seeded at 1×10^5 cells/well in 100 μl culture medium into 96-well migration chamber supplied by the kit. The migration chamber was placed on the top of the feeder tray in which cells can migrate from the migration chamber toward the feeder tray. Cells were incubated at 41 °C and 5% CO_2 in a humidified incubator for 8 h. For the negative control, culture medium without TEC supernatants was added to the feeder tray. Migrated cells into the feeder tray were subsequently lysed and detected by the patented CyQuant GR dye provided by the kit. The green fluorescent dye binds to cellular nucleic acids and displays strong fluorescence enhancement. Lysed cells were transferred into a new 96-well plate suitable for fluorescence measurement, and the plate was read with a fluorescence plate reader using 480/520 nm filter set.

4.13. Tracheal epithelial cell and macrophage co-culture

Chicken tracheal epithelial cells were treated with inhibitors or TLR ligands as described above. After 4 h of stimulation with TLR ligands, tracheal epithelial cells were washed twice with complete medium and then MQ-NCSU cells were added at 5×10^5 cells/well on top of the treated cTECs. Cells were harvested at 24 h post co-culture to measure surface markers on MQ-NCSU cells.

4.14. Flow cytometry

Both TECs and MQ-NCSU cells were harvested at 24 h post co-culture using cold 4 °C PBS. Harvested cells were plated on 96 well round-bottom plates with each well containing 1×10^6 cells in 50 μl FACS buffer. The following primary antibodies were used; 0.5 μg mouse anti-chicken CD80 (IgG2a, Abd Serotec) and 0.5 μg mouse anti-chicken CD86 (IgG1, Abd Serotec) Primary antibodies were added to each well

Table 1
Primer sequences used for real-time PCR.

Target gene	Primer sequence	Annealing Temp. (°C)	Reference
β-actin	F: 5′-CAACACAGTGCTGTCTGGTGGTA-3′ R: 5′-ATCGTACTCCTGCTTGCTGATCC-3′	60	St Paul et al., (2011)
IL-6	F: 5′-CGTGTGCGAGAACAGCATGGAGA-3′ R: 5′-TCAGGCATTTCTCCTCGTCGAAGC-3′	60	St. Paul et al. (2012b)
IL-8	F: 5′-CCAAGCACACCTCTCTTCCA-3′ R: 5′-GCAAGGTAGGACGCTGGTAA-3′	64	St Paul et al. (2011)
IL-1β	F: 5′-GTGAGGCTCAACATTGCGCTGTA-3′ R: 5′-TGTCCAGGCGGTAGAAGATGAAG-3′	64	St Paul et al. (2011)
IRF7	F: 5′-CTCCCCTCCTCCAAAAGCTG-3′ R: 5′-CTGGGAGCGAAGGAGGAATG-3′	60	Barjesteh et al. (2014)
IFN-α	F: 5′-ATCCTGCTGCTCACGCTCCTTCT-3′ R: 5′-GGTGTTGCTGGTGTCCAGGATG-3′	64	St Paul et al. (2011)
IFN-β	F: 5′-GCCTCCAGCTCCTTCAGAATACG-3′ R: 5′-CTGGATCTGGTTGAGGAGGCTGT-3′	64	St Paul et al. (2011)
viperin	F: 5′-GGAGGCGGGAATGGAGAAAA-3′ R: 5′-CAGCTGGCCTACAAATTCGC-3′	60	Barjesteh et al. (2015)
PKR	F: 5′-TGGTACAGGCGTTGGTAAGAG-3′ R: 5′-GAGCACATCCGCAGGTAGAG-3′	60	Barjesteh et al. (2015)
OAS	F:5′-AGAACTGCAGAAGAACTTTGTC-3′ R:5′-GCTTCAACATCTCCTTGTACC-3′	60	St Paul et al. (2011)
IFITM3	F: 5′-CACACCAGCATCAACATGCC-3′ R: 5′-CCTACGAAGTCCTTGGCGAT-3′	60	Barjesteh et al. (2015)
IFITM5	F: 5′-CTTCGGAGTGATGGCCACTT-3′ R: 5′-AAATTACAGCCCTCGCCGAA-3′	60	Barjesteh et al. (2014)
MDA5	F: 5′-GCAAAACCAGCACTGAATGGG-3′ R: 5′-CGTAAATGCTGTTCCACTAACGG-3′	60	Barjesteh et al. (2015)
TLR2	F: 5′- ATCCTGCTGGAGCCCATTCAGAG -3′ R:5′- TTGCTCTTCATCAGGAGGCCACTC -3′	60	St. Paul et al., (2012b)
TLR3	F:5′- TCAGTACATTTGTAACACCCCGCC -3′ R: 5′- GGCGTCATAATCAAACACTCC -3′	64	St. Paul et al., (2012b)
TLR4	F: 5′- TGCCATCCCAACCCAACCACAG -3′ R: 5′- ACACCCACTGAGCAGCACCAA -3′	60	St. Paul et al., (2012b)
TLR5	F:5′- TTCTTGCAACCTCACAGGTGTTCC -3′ R: 5′- CAGGTCCAAGACACGAAGATT -3′	60	St. Paul et al., (2012b)
TLR7	F: 5′- TTCTGGCCACAGATGTGACC -3′ R: 5′- CCTTCAACTTGGCAGTGCAG -3′	64	St. Paul et al., (2012b)
TLR21	F: 5′- CCTGCGCAAGTGTCCGCTCA -3′ R: 5′- GCCCCAGGTCCAGGAAGCAG -3′	60	St. Paul et al., (2012b)

for 30 min on ice and protected from the light. Then, the cells were washed 3 times by adding 100 μl/well of cold PBS containing 10% bovine serum albumin. Cells were centrifuged at 400 × *g* for 5 min at 4 °C. Cells were incubated with fluorochrome-labeled secondary antibodies including anti-mouse IgG1-APC, anti-mouse IgG2a- PE-Cy7, 0.25 μg of FITC conjugated mouse anti-chicken Monocyte/Macrophage (clone KUL01, Southern Biotech, Birmingham, Alabama), 0.05 μg of PE-conjugated anti-chicken major histocompatibility complex (MHC) class II antibody (Southern Biotech, Birmingham, Alabama) and Live/Dead® Fixable Near-IR dead cell stain kit (Life Technologies, Eugene, Oregon, USA) for 30 min on ice. Again, the cells were washed 3 times by adding 100 μl/well of ice-cold PBS containing 10% BSA. Cells were centrifuged at 400 × *g* for 5 min 4 °C. Cells were fixed with paraformaldehyde (PFA) at a final concentration of 2% in PBS for 20 min at room temperature and then washed again with cold PBS containing 10% BSA. Cells were analyzed by BD FACSCanto™ II Flow Cytometer (Beckton Dickinson Biosciences, San Jose, CA, USA), and FlowJo v10 was used for analysis of the data. Furthermore, negative controls including unstained macrophages and single-colour controls consisting of cells stained with one of the aforementioned fluorochromes which served as compensation controls forinclusion in the analysis. (Fig. 7). There were three biological replicates for each group.

For Flow cytometry data analysis, initial gating involved a two-step process of FSC-W versus FSC-H followed by SSC-W versus SSC-H in order to eliminate doublets and to avoid false positive stains. Then, live cells were detected by the Live/Dead® Fixable Near-IR dead cell stain. In the next step, macrophages were gated based on the KUL01 marker. Subsequently, each individual marker was visualized against SSC-A.

4.15. Statistical analysis

To examine whether AIV replicates in chicken tracheal epithelial cells, statistical analysis was performed using a two-tailed Student's t-test to compare viral titers between different time points and the time of infection. Relative expression of all genes was calculated relative to the housekeeping gene β-actin using the LightCycler 480 software (Roche Diagnostics). Relative expression data represent geometric mean fold-change of 5 replicates compared to the medium control group ± standard error. For gene expression, statistical significance was calculated using a two-tailed *t*-test. For all analyses, $P \leq 0.05$ was considered statistically significant. To examine whether TLR ligand treatment of chicken tracheal epithelial cells altered viral replication, statistical analysis was performed by using a one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. To examine whether TLR ligand treatment of chicken tracheal epithelial cells altered surface markers on macrophages, statistical analysis was performed using a one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. To determine whether stimulated chicken tracheal epithelial cells by TLR ligands activated chicken macrophages, statistical analysis was performed using a two-tailed student's t-test to compare the number of migrated macrophages between different groups.

Author contributions

NB and SS conceived and designed the experiments; NB performed the experiments; KTA collaborated in primary cell isolation. RK collaborated in Flow cytometry experiment and Flow cytometric data analysis. NB analyzed the data; NB and SS wrote the paper.

Conflicts of interest

The authors declare no conflict of interest.

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