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Research paper

Cellular host transcriptional responses to influenza A virus in chicken tracheal organ cultures differ from responses in *in vivo* infected trachea

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ABSTRACT

In this study a viral infection of a tissue culture model system was compared to an *in vivo* infection, which is of importance to gauge the utility of the model system. The aim was to characterize early immune responses induced by avian influenza virus using tracheal organ cultures (TOC) as a model system. First, the *in vitro* system was optimized to ensure that the host transcription responses were only influenced by virus infection and not by differences in viral load. Upper and lower trachea both could be used in the cultures because the virus load was the same. Cilia motility was not affected in non-infected TOC and only slightly in infected TOC at 24 h post-inoculation. Gene expression profiles of early immune responses were analyzed in *in vitro* infected TOC, and were compared to the responses found in *in vivo* infected trachea. The gene expression profile in infected TOC suggested the up regulation of innate anti-viral responses that were triggered by attachment, entry and uptake of virus leading to several signalling cascades including NF-κB regulation. Genes associated with IFN mediated responses were mainly type I IFN related. Overlapping gene expression profiles between non-infected and infected TOC suggested that tissue damage during excision induced wound healing responses that masked early host responses to the virus. These responses were confirmed by real-time quantitative RT-PCR showing up regulation of IL-1β and IL-6. Microarray analysis showed that gene expression profiles of infected and non-infected TOC had a large overlap. This overlap contained many immune-related genes associated with inflammatory responses, apoptosis and immune system process and development. Infected TOC and *in vivo* infected trachea shared few significantly differentially expressed genes. The gene expression profile of infected TOC contained fewer genes which were expressed at reduced amplitude of change. Genes that were common between TOC and trachea were associated with early immune responses likely triggered by virus attachment and entry. Most of the genes were associated with IFN-mediated responses, mainly type I IFN related. Our study implicates that although the TOC model is suitable for culturing of virus and lectin or virus binding studies, it is not suitable for measuring early immune responses upon viral infection at host transcriptional level.

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1. Introduction

Avian influenza virus (AIV) causes infection of the respiratory tract, triggering a cascade of both innate and specific immune responses. In the last decade our knowledge on these host defenses against and pathogenic mechanisms of influenza virus infections has tremendously increased. It has been demonstrated that early immune responses are critical in defining the severity and pathological outcome of AIV infection in mice and macaques (Kash et al., 2006; Kobasa et al., 2007). Yet little is known about the early immune response and host factors that play a role in the induction phase of the responses to AIV in the chicken. By using *in vitro* models, experimental conditions can be highly controlled and both mechanisms of viral infection and host defenses can be studied.

Numerous respiratory epithelial cell lines have been frequently used to study influenza virus infection (Chan et al., 2005; Veckman et al., 2006). An alternative for cell lines is the use of primary cell cultures. Primary respiratory epithelial cell cultures have been developed for (Matrosovich et al., 2004), hamster (Newby et al., 2006), mouse (You et al., 2002), pig (Steimer et al., 2006) and since recently chicken (Zaffuto et al., 2008). Another alternative has been the use of organ cultures as infection model, such as lung slice cultures (Booth et al., 2004) and tracheal organ cultures (TOC), which mimic natural infection events more closely and can also be used under controlled conditions. To date early immune responses to AIV in the chicken have not been systematically characterized at global gene transcriptional levels. This report is the first one exploring the use of organ cultures for illustrating these responses at host transcriptional level.

TOC are susceptible to respiratory virus infections *in vitro* and are commonly used to study host–pathogen interaction (Schmidt et al., 1974), to grow virus, and as diagnostic tool (Cook et al., 1976). In this study an organ culture model system was compared to an *in vivo* infection which is of importance to gauge the utility of the model system. We investigated early immune responses to AIV infection and explored the value of using the *in vitro* TOC model for influenza studies by comparing responses in infected TOC with *in vivo* AIV infected trachea using a high-throughput genomics approach.

2. Materials and methods

2.1. Virus

Avian influenza A virus, subtype H9N2, isolate A/Chicken/United Arab Emirates/99 was produced in eggs according to routine procedures. Virus was kindly provided by Intervet Schering-Plough Animal Health, Boxmeer, The Netherlands.

2.2. In vitro infection model

Tracheas of 18-day-old Cobb broiler embryos (Lagerwey, Lunteren, The Netherlands) were aseptically removed and transferred to warm culture medium containing DMEM, 20 mM HEPES and penicillin/streptomycin. Tracheas were cut into 3 mm rings resulting in 6 rings per trachea. For optimization of the *in vitro* infection model, rings from 1 trachea were divided into two groups. Each group consisted of 3 rings derived from either the lower or the upper part of the trachea. These were transferred to a 12-well plate (3 rings per well; TOC) containing culture medium. TOC were inoculated with increasing concentrations of virus; 10^1–10^5 EID_{50} H9N2/ml in a total volume of 0.5 ml culture medium per well and incubated at 37 °C, 5% CO_2. After 1 h 1.5 ml culture medium was added and incubation continued. TOC were harvested at 24 h post-inoculation (p.i.) and stored in RNAlater (Ambion Europe Ltd., Huntingdon, UK) at −80 °C.

For the experimental set up, infected and non-infected TOC were incubated in either 0.5 ml culture medium or 0.5 ml culture medium containing 10^5 EID_{50} H9N2/ml. After 1 h 1.5 ml culture medium was added and incubation continued. TOC were harvested at 0, 3, 6, 15 and 24 h p.i. and stored in RNAlater at −80 °C.

TOC harvested at 0 h were non-infected controls harvested directly after preparation and were not cultured or inoculated. Infected and non-infected TOC originated from the same trachea and were time matched during harvesting, which enabled us to control for variations in physiology and bird to bird variation thus only to detect changes due to virus infection.

2.3. In vivo infection model

One-day-old White Leghorns were housed under SPF conditions and all experiments were carried out according to protocols approved by the Intervet Animal Welfare Committee.

Chickens were divided into 2 groups over 2 isolators, infected and non-infected, containing 20 animals per group. Fourteen-day-old chickens were inoculated via aerosol spray with 20 ml $10^{7.7}$ EID_{50} H9N2 AIV. The control group was inoculated via aerosol spray with 20 ml saline. Aerosol spray was made with an aerographer and compressor at a pressure of 1.5 atm, resulting in droplets with an average diameter of 50 μm. Chickens remained in the aerosol spray in a closed isolator (0.79 m^3) for 10 min, after which the isolator was ventilated as before. At day 1 p.i. chickens were killed ($n = 5$ per time point per group) and the upper part of the tracheas were isolated and stored in RNAlater at −80 °C.

2.4. RNA isolation

TOC or trachea, stored in RNAlater, were homogenized (Retsch Mixer Mill 301, Fisher Scientific, Ochten, The Netherlands) and total RNA was isolated using the RNeasy Mini Kit and DNase treated using the RNase-free DNase set following manufacturer's instructions (Qiagen Benelux B.V., Venlo, The Netherlands). Purified RNA was eluted in 30 μl RNase-free water and stored at −80 °C. All RNA samples were checked for quantity using a spectrophotometer (Shimadzu, 's Hertogenbosch, The Netherlands) and quality using a 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). Isolated RNA

was used for both real-time quantitative RT-PCR and microarray analysis.

2.5. Real-time quantitative reverse transcription-PCR (qRT-PCR)

cDNA was generated with reverse transcription using iScript cDNA Synthesis Kit (Biorad Laboratories B.V., Veenendaal, The Netherlands).

Real-time qRT-PCR was performed with a MyiQ Single-Color real-time PCR Detection System (Biorad) using iQ SYBR green supermix (Biorad) and the TaqMan Universal PCR Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Primers (Invitrogen-Life Technologies, Merelbeke, Belgium) and probes (Applied Biosystems) were designed according to previously published sequences (Sijben et al., 2003; Rothwell et al., 2004; Degen et al., 2006; Eldaghayes et al., 2006).

Amplification and detection of specific GAPDH and AI-H9 hemagglutinin (HA) products were achieved with SYBR green, 400 nM primers and using the following cycle profile: one cycle of 95 °C for 5 min, 40 cycles of 92 °C for 10 s, 55 °C for 10 s and 72 °C 30 s. For detection of the cytokines and 28S, product specific probes were used according to the cycle profile described in Ariaans et al. (2008). Primers were used at 600 nM and probes at 100 nM concentration.

To generate standard curves GAPDH, AI-H9 HA and IL-4 PCR-fragments were cloned and used to generate $\log_{10}$ dilution series regression lines. RNA from LPS stimulated HD11 cells was used to generate standard curves for IL-10 and RNA from ConA stimulated splenocytes was used for standard curves of the remaining cytokines and 28S. GAPDH was used as a reference gene for correction of AI-H9 HA mRNA expression and 28S as reference gene for the cytokines. Corrections for variation in RNA preparation and sampling were performed according to Eldaghayes et al. (2006). Results are expressed in terms of the threshold cycle value (Ct) and given as corrected 40-Ct values.

A paired *t*-test was used to determine the statistical significance between infected and non-infected TOC samples from the same bird. To determine the statistical significance between infected and non-infected trachea and between TOC and trachea at 24 h an ANOVA with a Tukey post hoc test was used. For both tests a *p*-value <0.05 was considered significant.

2.6. Microarray analysis

For microarray analysis the *Gallus gallus* Roslin/ARK CoRe Array Ready Oligo Set V1.0 (Operon Biotechnologies, Cologne, Germany) was used. The array was spotted onto Codelink activated slides (GE Healthcare) and contains 20,460 oligo probes representing chicken genes, and 3828 control spots, used for QC and normalisation purposes (Van de Peppel et al., 2003). RNA amplification and labelling was performed according to Roepman et al. (2005). All hybridizations contained 2.5 μg cRNA per channel on a HS4800 Pro hybstation (Tecan Benelux BVBA, Giessen, The Netherlands).

For determining early responses in TOC to H9N2 infection, infected samples were co-hybridized with non-infected samples of the same trachea. In order to investigate the gene expression profile over time in infected TOC, a 0 h non-infected control reference sample was co-hybridized with infected and non-infected TOC samples at 24 h p.i. The 0 h non-infected control reference sample was created by pooling equal amounts of RNA extracted from 0 h non-infected control TOC samples.

For the *in vivo* experiment, infected and non-infected trachea samples were co-hybridized with a trachea reference sample. This reference sample consisted of pooled RNA extracted from tracheas of 4 chickens that were not included in the infection experiment.

Slides were scanned with a G2565AA scanner (Agilent Technologies) at 100% laser power, 30% PMT. Resulting image files were analyzed using Imagene 8.0 (BioDiscovery, Inc., El Segundo, USA). Within slide normalisation was performed with Printtip Loess on mean data without background subtraction. Groups of replicates were analyzed using ANOVA (R version 2.2.1/MAANOVA version 0.98-7, http://www.r-project.org/). In a fixed effect analysis, sample, array and dye effects were modelled. *p*-values were determined by a permutation F2-test, in which residuals were shuffled 5000 times globally. Genes with $p < 0.05$ after family wise error correction were considered significantly differentially expressed and were selected to be included for further analysis. Visualisation and cluster-analysis was performed using GeneSpring 7.2 (Agilent Technologies). Ensembl *Gallus gallus* (assembly: WASHUC2, May 2006, genebuild: Ensembl, August 2006, database version: 47.2e) was used for gene names, description and GO-annotations.

In accordance with proposed MIAME standards primary data are available in the public domain through Expression Array Manager at http://www.ebi.ac.uk/arrayexpress/?#ae=main[0] under accession number E-MTAB-12.

3. Results

3.1. In vitro TOC infection model

The TOC infection model had to be optimized to ensure that host transcription responses were only influenced by virus infection and not by differences in viral load.

To ensure that the inoculation dose would result in consistent viral load of the TOC, AI-H9 HA mRNA levels were measured with real-time qRT-PCR. TOC infected with 10^3 EID_{50}/ml had significant differences in H9 mRNA levels between the replicates and between TOC of the upper and lower part of trachea (data not shown). There was no significant difference in AI-H9 HA mRNA levels between TOC of the upper and lower part of trachea for TOC infected with a dose of 10^4 and 10^5 EID_{50}/ml. However, based on standard deviations TOC infected with 10^5 EID_{50}/ml had a 0.4 Ct smaller difference between the replicates than TOC infected with 10^4 EID_{50}/ml (data not shown). Based on these results an infection dose of 10^5 EID_{50}/ml was used for further experiments and TOC of upper and lower part of trachea were divided randomly.

In order to determine the kinetics of AI-H9 HA mRNA expression in TOC, infected and non-infected TOC were harvested at 0, 3, 6, 15 and 24 h p.i. AI-H9 HA mRNA was

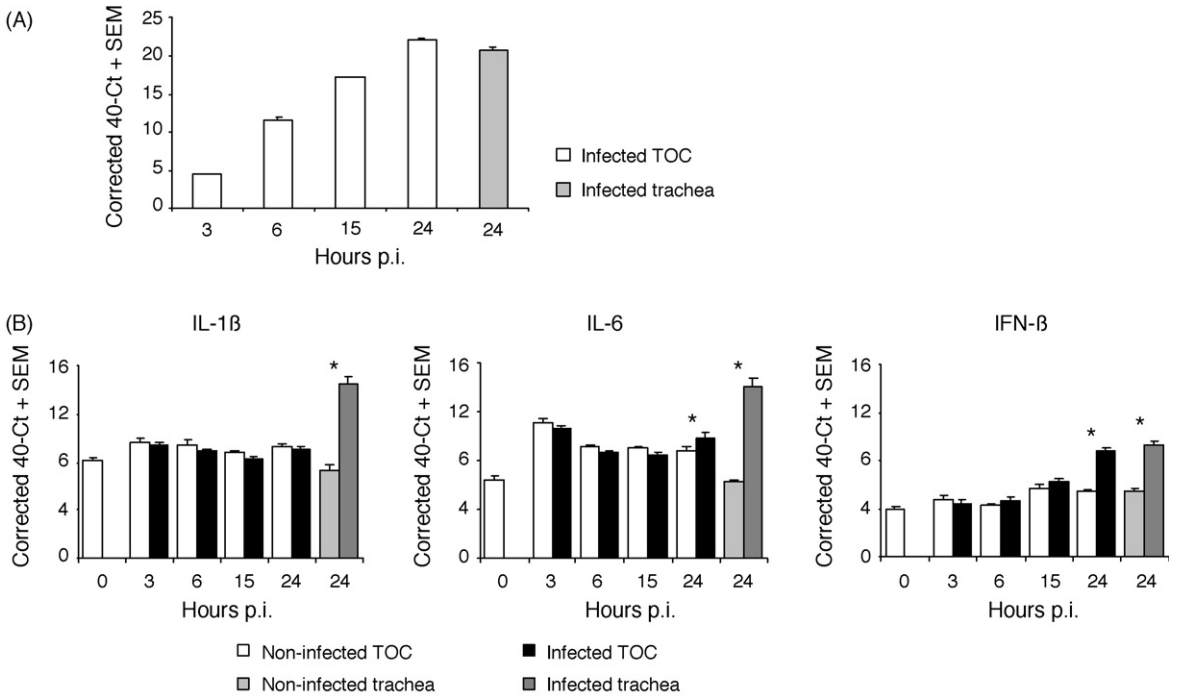

Fig. 1. (A) Real-time qRT-PCR of AI-H9 HA mRNA levels normalised to GAPDH over time in TOC infected *in vitro* with 10^5 EID_{50}/ml H9N2 and at 24 h p.i. in trachea of birds spray inoculated with $10^{7.7}$ EID_{50}/ml H9N2. (B) Real-time qRT-PCR of mRNA levels normalised to 28S of IL-1β, IL-6 and IFN-β over time in infected and non-infected TOC and at 24 h p.i in trachea of infected and non-infected birds (*$p < 0.05$). Results are presented as 40-Ct and errors bars show SEM for pooled expression level in four TOC (from four birds) and four tracheas per treatment.

detected from 3 h. p.i. and levels increased over time (Fig. 1A). The differences between replicates were very small at every time point. Therefore, differences in responses caused by a difference in viral load will not affect the gene expression within a treatment group.

To ensure that the tracheal rings were viable in non-infected TOC and to investigate if influenza virus infection affected cilia motility like avian pneumoviruses and influenza viruses in respiratory epithelium do (Diaz-Rodriguez and Boudreault, 1982; Naylor and Jones, 1994) we microscopically examined the motility of cilia in both infected and non-infected TOC at 0, 3, 6, 15 and 24 h p.i. No difference in cilia motility was detected in non-infected TOC. In infected TOC at 24 h p.i., 10–20% of the epithelial cell layer showed no cilia motility, whereas in non-infected TOC at 24 h of culturing the whole epithelial cell layer showed cilia activity (data not shown).

3.2. Expression of genes relevant for immune responses to H9N2 infection in TOC

In order to elucidate the expression of genes involved in host defenses in H9N2 infected TOC, infected and non-infected TOC within the same time point were compared using microarray analysis. The data were consistent and even small differences were measured as significantly differentially expressed. At 3, 6 and 15 h p.i. no significantly differentially expressed genes were found, but at 24 h p.i. 108 genes were significantly differentially expressed. Of these genes, 106 genes were up regulated and 2 genes were down regulated (supplemental Table S1). Based on the GO terms host–pathogen interaction, external stimulus and immune response, we created an immune-related term that was used for selection of immune-related genes. Of the 108 genes, 22 were immune-related genes that were divided into GO terms using GSEA to obtain more details on the biological processes they were involved in (Table 1). All 22 immune-related genes were up regulated and 8 of these genes are associated with IFN mediated responses like ISG12-2. STAT4, OASL, MDA5, LY6E are induced by type I IFNs, while CYSLTR2 is induced by IFN-γ. On the other hand SOCS1 is able to inhibit IFN-γ expression and USP18 can inhibit signal transduction pathways triggered by type I IFNs. When looking at the cellular position based on GO terms of the immune-related genes LY6E, ISG12-2, CYSLTR2, DGKE, CD8A, STAB1, TIMD4 and TLR5 reside in a membrane, PTK2B, OASL, MDA5 and SOCS1 reside in the cytoplasm and STAT4, USP18 and ZEB1 reside in the nucleus. IL19 and MASP2 are secreted molecules. There was no significant difference in gene expression between infected TOC and non-infected TOC at 3, 6 and 15 h p.i. This suggested that tissue excision and culturing caused host responses in non-infected TOC similar to host responses caused by H9N2 infection in infected TOC. Therefore the gene expression profiles of infected and non-infected TOC within a 24 h period were determined by comparing infected or non-infected TOC at 24 h to non-infected control TOC at 0 h. The comparison of non-infected TOC at 24 h of culturing with non-infected control TOC at 0 h represented would healing responses. Responses in infected TOC at 24 h p.i. compared to non-infected control TOC at 0 h represented host

Table 1
Immune-related genes significantly differentially expressed ($p < 0.05$) in H9N2 infected compared to non-infected TOC at 24 h p.i. with associated GO terms. All genes were up regulated.

GO annotation	Gene name																					
	STAT4[a]	PTK2B	OASL[a]	MDA5[a]	LY6E[a]	ISG12-2[a]	CYSLTR2[a]	CCDC18	DGKE	GENE A[a,b]	GENE B[c]	CD8A	IL19	MASP2	SOCS1[a]	STAB1	TIAL1	TIMD4	TLR5	TRAF3IP2	USP18[a]	ZEB1
Membrane					x	x	x		x			x				x		x	x			
Cytoplasm		x	x	x											x							
Nucleus	x																				x	x
Extracellular region													x	x								
Signal transduction	x	x			x		x						x		x				x	x		
Protein binding	x			x					x			x			x				x			
Immune response			x	x								x	x	x								
Apoptosis		x		x									x				x					
Response to virus			x	x																		
Defense response																x	x					
T cell activation												x						x				
Inflammatory response																x			x			
JAK-STAT cascade	x														x							
Regulation of NF-κB cascade																			x	x		
Cell adhesion		x																				
Cell proliferation		x																				
Lysosome																	x					
Response to stress		x																				
Antigen presentation/ processing												x										
Complement activation														x								
Defense response to bacterium																x						
Cell adhesion																x						
Cell–cell signalling																x						
Ubiquitin-dep. protein catabolic process																					x	
Neg. reg. epithelial cell differentiation																						x
Transport								x														

[a] Genes involved in interferon mediated responses.
[b] GENE A: ENSGALG00000006384.
[c] GENE B: ENSGALG00000007208.

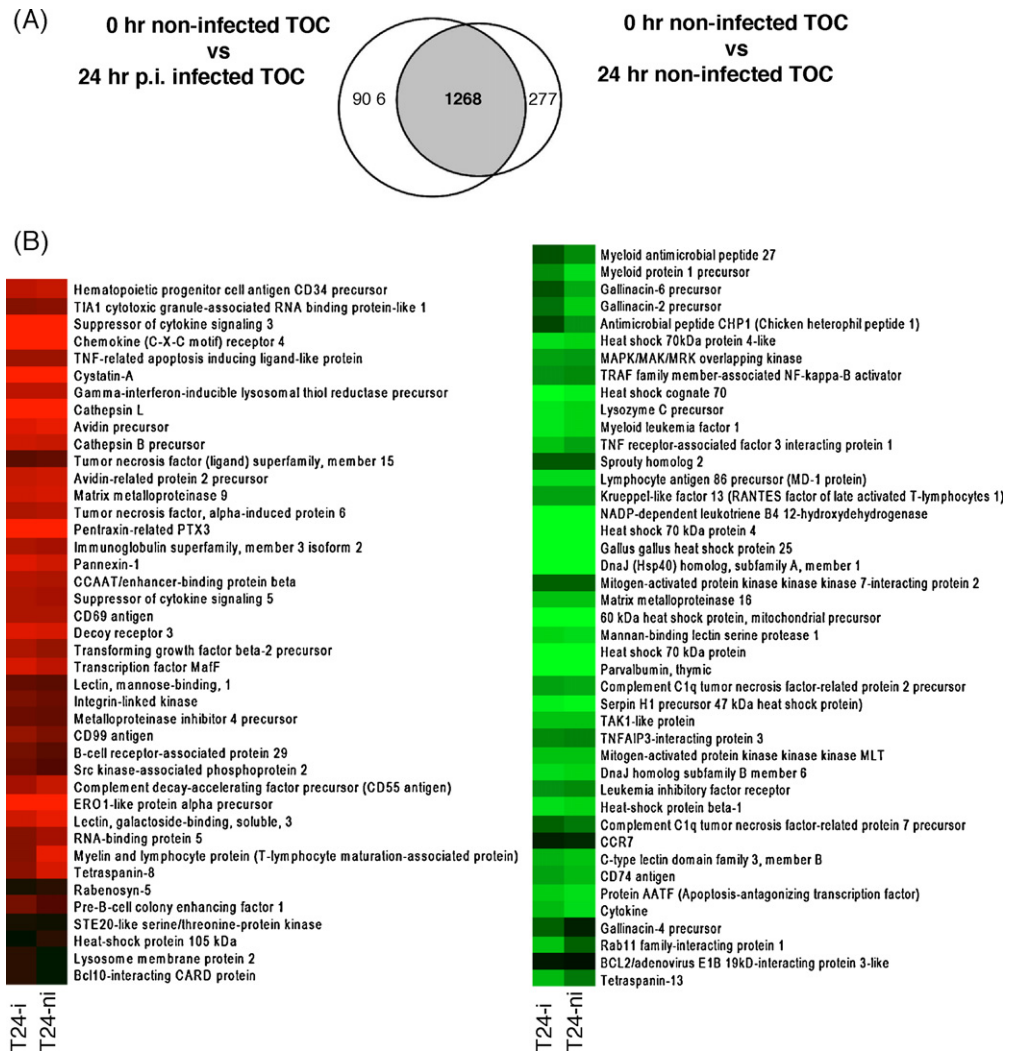

Fig. 2. (A) Venn diagram of significantly differentially expressed genes ($p < 0.05$) involved in host responses to H9N2 infection and in wound healing in TOC on the left and host responses involved in wound healing on the right ($n = 4$). The grey area represents the overlapping genes between the expression profiles and indicates the overlap between responses to H9N2 infection and wound healing responses. The numbers in the circles indicate the altered genes for each condition. (B) Overlap of 93 immune-related genes between responses to H9N2 infection in infected TOC and wound healing responses in non-infected TOC. Red represents up regulation and green down regulation in infected (T24-i) and non-infected (T24-ni) TOC at 24 h p.i. compared to non-infected control TOC at 0 h.

responses to H9N2 infection and wound healing responses. The overlap between significantly differentially expressed genes in the gene expression profile of infected TOC in one set and in the gene expression profile of wound healing in non-infected TOC in the other set represents the overlap in responses caused by wound healing and virus infection and was depicted in a Venn diagram (Fig. 2A). An overlap of 1268 genes between the profiles was found, containing 84 genes involved in immune responses, of which 41 genes were up regulated and 43 genes were down regulated (Fig. 2B). Immune-related genes were divided into GO terms to obtain more details on the biological processes they were involved in. GO terms that mostly contained down regulated genes could be divided into three categories containing relating GO terms. Most genes are associated with response to stress, chemical and biotic stimuli, defense to pathogens and regulation of NF-κB. The other two categories consisted of GO terms negative regulation of apoptosis and negative regulation of programmed cell death, and protein folding and protein binding. Together these genes suggest that cells are not responding to stimuli, protein production has come to a hold and apoptosis and programmed cell death are no longer inhibited. Death of the cells can either be induced via virus infection or tissue damage due to preparation and culturing of TOC. GO terms that contained more up regulated than down regulated genes could also be divided into categories containing relating GO terms. Response to wounding and to external stimuli indicated wound healing responses of the TOC due to tissue damage caused by excision and culturing of TOC and to virus infection. This resulted in cell death as suggested by up regulation of

genes involved in regulation of apoptosis and programmed cell death, anti-apoptosis and negative regulation of cellular process. Genes associated with immune system processes and development were also up regulated suggesting the initiation of an immune response. This response involved genes that are related to inflammatory responses, leukocyte migration and differentiation, defense responses, hemopoiesis, hemopoietic or lymphoid organ development and carbohydrate binding. These suggested cell attraction, migration and adhesion and phagocytosis of virus or cell debris by MΦs inducing an inflammatory response. Virus and cell debris were likely degraded in lysosomes or lytic vacuoles suggested by the up regulation of genes that were part of the GO terms lysosome, lytic vacuole and vacuole. Besides cell death, the TOC structure was possibly also renewed as suggested by up regulation of genes associated with cell development, positive regulation of cell differentiation, organelle part and regulation of developmental processes. Up regulation of genes involved in protein metabolic process, cellular protein process and lipid raft was in accordance with this and suggested an active communication in and between cells. These data showed a large overlap of host responses, particularly early immune responses, between H9N2 infection and wound healing.

3.3. Cytokine mRNA expression in infected and non-infected TOC

In order to confirm the microarray data on the early responses induced by viral entry and replication, we measured mRNA expression of different cytokines using real-time qRT-PCR. mRNA levels of infected TOC were compared to non-infected TOC within the same time point. In infected TOC IL-6, IL-8, IFN-α and IFN-β were significantly up regulated at 24 h p.i., but no significant changes were detected in IL-1β, IL-4, IL-10, IL-18 and IFN-γ levels compared to non-infected TOC (Fig. 1B and data not shown). IL-6, IL-8 and IL-10 mRNA levels were also up regulated from 3 h of culturing in non-infected TOC compared to non-infected control TOC at 0 h and remained elevated up to 24 h of culturing (Fig. 1B, and data not shown). With microarray analysis IL-6, IL-8, IFN-α and IFN-β were not significantly differentially expressed in infected TOC compared to non-infected TOC. Real-time qRT-PCR is more sensitive than microarray analysis and differences in IL-6, IL-8, IFN-α and IFN-β levels between infected and non-infected TOC measured with real-time qRT-PCR were small, which explain why these cytokines were not found with microarray analysis. Moreover, for real-time qRT-PCR a paired *t*-test was used to determine significant differences, which rules out biological differences, because infected and non-infected TOC from the same animal were compared. For microarray analysis, stringent statistics with multiple testing was used, which does not take into account that infected and non-infected TOC hybridized together on a slide were from the same animal. Resulting from this the small differences were significant for real-time qRT-PCR, but not for microarray analysis, and are not shown in the gene lists.

3.4. Comparison of in vitro and in vivo gene expression after H9N2 infection

To compare responses in *in vitro* infected TOC with *in vivo* infected trachea, results should not be affected by differences in viral load between TOC and trachea. AI-H9 HA mRNA levels of infected trachea at 24 h p.i. were compared with TOC at 24 h p.i. (Fig. 1A). There was no significant difference in AI-H9 HA mRNA level between infected TOC and trachea at 24 h p.i.

Scatterplots were made of the expression ratio of infected samples compared to a reference sample on one axis and of non-infected samples to a reference sample on the other axis for both TOC and trachea (Fig. 3A–C). For trachea a broad scatterplot was seen (Fig. 3A), suggesting a high amplitude of change in infected trachea compared to non-infected trachea. Whereas for TOC the response is more moderate with a scatterplot close to the diagonal 1-fold indication line (Fig. 3B and C), suggesting a low amplitude of change between infected and non-infected TOC. Moreover, the expression profile of infected trachea involved more significantly differentially expressed genes than that of infected TOC (Fig. 3A–C white dots), and few genes were shared between both expression profiles. Responses in infected samples were compared to non-infected samples at 24 h for both TOC and trachea representing responses to H9N2 infection. A Venn diagram was generated of significantly differentially expressed genes to elucidate the overlap between the expression profile of H9N2 infection in TOC in one set and in trachea in the other set (Fig. 3D). A small overlap in response was seen between TOC and trachea consisting of 34 genes that were all up regulated in both TOC and trachea. Of these 34 genes, 10 were immune-related which were divided into GO terms to obtain more details of the biological processes they were involved in (Table 2). Of the 10 immune-related genes, which were all up regulated in both TOC and trachea, 8 are associated with IFN mediated responses. All genes are related to type I IFN mediated responses, except for SOCS1 which is able to inhibit IFN-γ. Genes involved in IFN mediated responses were expressed at a >6-fold change in the trachea whereas responses of these genes in TOC were 3–6-fold lower compared to trachea. These 10 immune-related genes were related to small parts of several core immune responses induced by virus infection. Together they suggested that several signal transduction cascades were triggered by attachment and entry of virus in the epithelial cells which likely lead to defense responses via activation of the NF-κB cascade. Possibly, viral Ags were processed in APC or epithelial cells for Ag presentation on the cell surface. In TOC, fewer genes were differentially expressed compared with trachea and at lower amplitude of change.

We also compared mRNA levels of IL-1β, IL-6 and IFN-β in TOC with trachea (Fig. 1B). These cytokines were significantly up regulated in trachea, whereas in TOC only IL-6 and IFN-β were significantly up regulated. The mRNA levels of IL-1β and IL-6 were higher in infected trachea compared to infected TOC, whereas these levels were lower in non-infected trachea compared to non-infected TOC. Thus differences between IL-1β and IL-6 mRNA levels

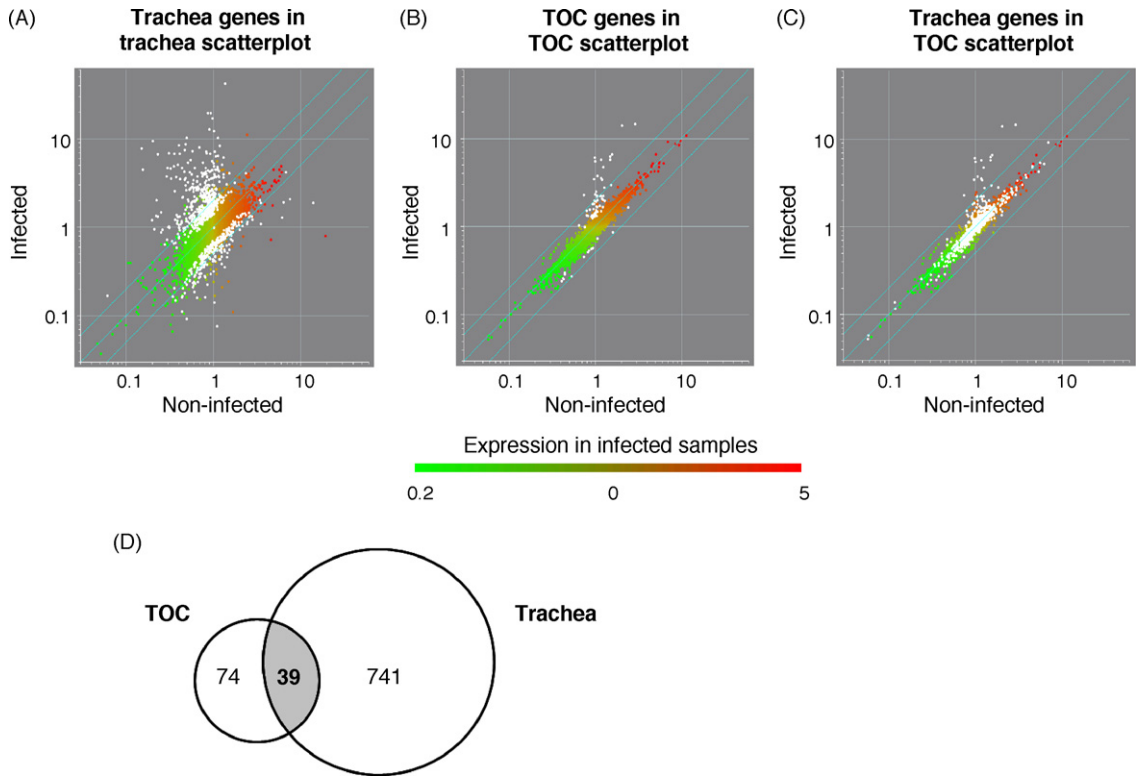

Fig. 3. (A–C) Scatterplots of host responses to H9N2 infection in TOC and trachea at 24 h p.i. ($n = 4$). The intensities for gene expression depicted in fold change in infected samples compared to a reference sample on the *y*-axis and in non-infected samples compared to a reference sample on the *x*-axis. The middle diagonal blue line represents a 1-fold change and the outer two diagonal lines a 2-fold change in infected compared to non-infected samples. (A) A scatterplot of trachea with the significantly differentially expressed genes ($p < 0.05$) in the trachea given in white. (B) A scatterplot of TOC with the significantly differentially expressed genes ($p < 0.05$) in the TOC given in white. (C) A scatterplot of TOC with the significantly differentially expressed genes ($p < 0.05$) in the trachea given in white. (D) Venn diagram of significantly differentially expressed genes ($p < 0.05$) involved in host responses to H9N2 infection in TOC and trachea at 24 h p.i. ($n = 4$). The grey area represents the overlap between the expression profile of H9N2 infection of TOC and trachea. The numbers in the circles indicate the altered genes for each condition.

Table 2
Immune-related genes significantly differentially expressed ($p < 0.05$) in H9N2 infected compared to non-infected samples at 24 h p.i. in TOC and trachea with associated GO terms. All genes were up regulated.

	Gene name									
	TRAF3IP2	TIMD4	LY6E[a]	STAT4[a]	USP18[a]	MDA5[a]	SOCS1[a]	ISG12-2[a]	OASL[a]	GENE C[a,b]
Fold change[c]										
TOC	2.2	1.3	2.0	1.7	2.0	1.8	1.7	4.4	4.6	7.6
Trachea	1.6	1.6	6.1	7.4	7.7	8.5	11.6	29.2	29.3	50.7
GO annotation										
Membrane		x	x					x		
Cytoplasm						x	x		x	
Nucleus				x	x					
Signal transduction	x		x	x			x			
Protein binding				x		x	x			
Immune response						x			x	
Response to virus						x			x	
JAK-STAT cascade				x			x			
Apoptosis						x				
Ubiquitin-dep. protein catabolic process					x					
T cell activation		x								
Regulation of NF-κB cascade	x									

[a] Genes involved in interferon mediated responses.
[b] GENE C: ENSGALG00000006384.
[c] Fold changes represent the difference in expression levels in infected TOC and trachea compared to non-infected samples.

in non-infected compared to infected samples were larger in trachea than in TOC, suggesting that wound healing responses in the TOC masked early immune responses which was not found in trachea.

4. Discussion

Early immune responses are important in defining the pathological outcome of AIV infection and have been demonstrated to differentiate the highly pathogenic and low pathogenic AIV H1N1 infection in mice (Perrone et al., 2008). Yet in chicken, little is known about these responses against AIV. In this study we investigated early immune responses upon viral infection at host transcriptional level in the chicken and explored the use of TOC as an *in vitro* model system. The virus strain H9N2 is important as it has regularly been detected in land-based poultry and it is able to make cross-species infection (Butt et al., 2005). TOC were susceptible to H9N2 infection *in vitro* and AI-H9 HA mRNA levels increased over time, indicating not only viral entry but also replication. Several respiratory viruses such as influenza, infectious bronchitis virus and turkey rhinotracheitis virus (or avian pneumovirus) are known to cause ciliostasis in respiratory epithelium (Diaz-Rodriguez and Boudreault, 1982; Naylor and Jones, 1994; Cavanagh et al., 2007). Macroscopical examination revealed that ciliostasis was detected in 10–20% of the epithelium of infected TOC at 24 h p.i. Pathogenesis of influenza virus infection has been investigated in equine and duck TOC (Kocan et al., 1977; O'Niell et al., 1984), where scattered loss of cilia and disruption of the epithelial cell layer was reported. Our results in chickens are consistent with these studies.

Immune responses to influenza infection in the respiratory tract have been studied extensively *in vitro* in human and mouse. Elevated expression levels of inflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8, IL-18, IFN-α and IFN-β have been reported as early as 6 h p.i. depending on the strain of influenza used (Julkunen et al., 2001; Chan et al., 2005). In our *in vitro* study also elevated expression levels of IL-6, IL-8, IFN-α and IFN-β were found in infected TOC at 24 h p.i., suggesting the initiation of an acute host response by induction of an increased MHC class I expression and anti-viral activity by IFNs and inflammatory effects of IL-6 and IL-8 mobilizing and activating lymphocytes.

Genomics tools are more and more used to study host–pathogen interactions (Kash et al., 2006). Our study was focused on gene expression profiles of early responses to low pathogenic AIV using the TOC model system. Responses in infected and non-infected TOC at 24 h showed very similar gene expression profiles. In both cultures, genes associated with responses to external stimuli and stress, regulation of NF-κB, protein folding and processing, apoptosis, and immune system process and development were significantly differentially expressed, suggesting the initiation of an immune response. Genes related to inflammatory responses found in the gene profile of non-infected TOC corresponded to those found using real-time qRT-PCR in non-infected TOC from 3 h of culturing. IL-6, IL-8 and IL-10 expression levels were up regulated in non-infected TOC. In mammals, IL-6, IL-8 and IL-10 are known to be expressed in damaged tissue (Yamamura et al., 1992). As expected, excision of the trachea into rings caused damage and resulted in up regulation of IL-6, IL-8 and IL-10. The preparation of TOC likely induced strong inflammatory responses, largely masking an early immune response triggered by AIV infection. Culturing of TOC several days before infection will most likely not improve the inflammatory responses induced by preparation of the TOC. Cilia activity slowly changed over a period of 5 days with a maximum loss of 20%, but histopathological changes in the TOC were found (data not shown). Still several genes associated with innate anti-viral responses were significantly differentially up regulated in the gene expression profile of infected compared to non-infected TOC at 24 h. Together these genes related to a virus induced immune response in which attachment and entry of virus in the epithelial cells triggered several signal transduction cascades leading to defense responses via activation of the NF-κB cascade, inflammatory responses and complement activation via the lectin pathway. Via these responses chemokines and IFNs can be secreted, likely triggering expression of IFN-induced genes and attracting APC and T cells, residing in the lamina propria of the TOC, to the place of infection. Virus might have been taken up by APC and transported to lysosomes where the virus is degraded or virus is processed in the endosomes for Ag presentation on the cell surface.

To validate whether early immune responses to AIV in *in vitro* infected TOC are similar to *in vivo* responses at host transcriptional level, a comparison was made with *in vivo* H9N2 infected trachea. Higher levels of IL-1β and IL-6 were found in trachea, which could be caused by an influx of MΦs, that cannot occur in TOC. Differences in fold change and the small overlap in genes found between TOC and trachea are most likely caused by masking of the immune responses in TOC due to wound healing responses, although some genes are involved in both wound healing and immune responses to pathogens. The number of genes that were in common in host response to H9N2 between TOC and trachea was very low. These genes up regulated and related to innate anti-viral responses, likely triggered by attachment and entry of virus leading to several signal transduction cascades. Most of the common genes are associated with IFN mediated responses. These were mainly type I IFN related corresponding with the up regulation in both TOC and trachea of IFN-α and IFN-β. These findings suggest that these common immune responses are more involved in host responses to AIV infection than in wound healing responses and likely play a dominant role.

There is a clear need for detailed knowledge of the mechanisms of early immune responses in the induction phase of AIV infection to enable modulating of the outcome. Studies conducted in *in vitro* respiratory models can play an important role in elucidating host–pathogen interactions. However, tissue culture model systems have to be compared to *in vivo* infections to gauge the utility of the model system. This study showed that host responses in TOC are masked by wound healing responses and thus

influence any study into host transcriptional responses. Although TOC and trachea shared similar immune-related genes, which may play a dominant role in immune responses to influenza virus infection, the number of shared genes significantly differentially expressed in both TOC and trachea was very low. Therefore the results obtained from organ cultures or primary cells for investigating any host responses at transcriptional level should be interpreted with caution. This makes TOC a good *in vitro* model for culturing of virus, and lectin or virus binding studies, but not for studying gene expression profiles of host responses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetimm.2009.04.021.

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