Differential gene-expression and host-response profiles against avian influenza virus within the chicken lung due to anatomy and airflow

Sylvia S. Reemers,1 Daphne A. van Haarlem,1 Marian J. Groot Koerkamp2 and Lonneke Vervelde1

Correspondence
Lonneke Vervelde
L.Vervelde@uu.nl

1Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands
2Genomics Laboratory, Department of Physiological Chemistry, Utrecht Medical Centre, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands

Sampling the complete organ instead of defined parts might affect analysis at both the cellular and transcriptional levels. We defined host responses to H9N2 avian influenza virus (AIV) in trachea and different parts of the lung. Chickens were spray-inoculated with either saline or H9N2 AIV. Trachea and lung were sampled at 1 and 3 days post-inoculation (p.i.) for immunocytochemistry, real-time quantitative RT-PCR and gene-expression profiling. The trachea was divided into upper and lower parts and the lung into four segments, according to anatomy and airflow. Two segments contained the primary and secondary bronchi, cranial versus caudal (parts L1 and L3), and two segments contained the tertiary bronchi, cranial versus caudal (parts L2 and L4). Between the upper and lower trachea in both control and infected birds, minor differences in gene expression and host responses were found. In the lung of control birds, differences in anatomy were reflected in gene expression, and in the lung of infected birds, virus deposition enhanced the differences in gene expression. Differential gene expression in trachea and lung suggested common responses to a wide range of agents and site-specific responses. In trachea, site-specific responses were related to heat shock and lysozyme activity. In lung L1, which contained most virus, site-specific responses were related to genes involved in innate responses, interleukin activity and endocytosis. Our study indicates that the anatomy of the chicken lung must be taken into account when investigating in vivo responses to respiratory virus infections.

INTRODUCTION

Avian influenza virus (AIV) infection is a continuing threat to both humans and birds worldwide. In order to control outbreaks, research into new intervention strategies and vaccines is ongoing. Therefore, studies into pathogenesis and host–virus interactions are being performed (Baskin et al., 2004; Kash et al., 2004; Degen et al., 2006). Host–virus interactions depend on the ability of the virus to enter host cells. The molecules used by avian and human influenza viruses to enter the respiratory tract are sialic acid linked to galactose by respectively an α-2,3 or an α-2,6 linkage, which are not distributed equally. Differences in virus entry generally correspond to variation in the type of sialic acid expressed in the respective host species (Shinya et al., 2006; Van Riel et al., 2007). The pattern of receptor distribution in chickens was shown to be less defined than in mammals (Wan & Perez, 2005). In chickens, uneven spread of low-pathogenic AIV (LPAI) has been shown for H9N2, with a preference for infecting the upper part of the respiratory tract (Nili & Asasi, 2002). Difference in distribution of virus within the respiratory tract has an impact on the host responses, as shown for infected macaques (Baas et al., 2006). Host responses to respiratory virus infections have only been investigated in the whole lung, one part of the lung or a pool of samples, without taking the anatomy and airflow, bidirectional in mammals and unidirectional in birds, through the respiratory tract into account.

Herein, we describe that gene expression within the lung of control chickens was affected by anatomy, and that airflow affected virus deposition and thereby the localized host responses. The trachea and lung of saline- and H9N2 AIV-inoculated birds were sampled 1 and 3 days post-inoculation (p.i.). The trachea was divided into upper and lower parts and the lung into four parts, based on airflow (Fig. 1). The airflow is unidirectional and ventilation is performed by the air sacs (Fedde, 1998).

Three supplementary tables showing expression of immune-related genes induced by H9N2 infection in upper trachea and lung L1 and L4 at 3 days p.i. are available with the online version of this paper.
were killed (the isolator was ventilated as before. At 1 and 3 days p.i., chickens remained in the closed isolator for 10 min, after which birds in each isolator, eight were used for the experiment described in infections.

METHODS

Infection model. Avian influenza A virus, subtype H9N2, isolate A/Chicken/United Arab Emirates/99 was provided by Intervet Schering-Plough Animal Health.

One-day-old White Leghorn chickens were housed under specific-pathogen-free (SPF) conditions and all experiments were carried out according to protocols approved by the Intervet Animal Welfare Committee.

Chickens were divided into two groups over two isolators, infected and non-infected, containing 20 birds per group. Fourteen-day-old chickens were inoculated via aerosol spray either with 20 ml 10^7 EID50 H9N2 AIV or with 20 ml saline per isolator. Of the 20 birds in each isolator, eight were used for the experiment described here. Chickens remained in the closed isolator for 10 min, after which the isolator was ventilated as before. At 1 and 3 days p.i., chickens were killed (n=4 per time point per group) and trachea and left lung were isolated and stored in RNAlater (Ambion) at −80 °C for RNA isolation and in liquid nitrogen for immunocytochemistry. The trachea was divided into upper and lower parts and the lung was divided into four pieces (Fig. 1), L1–L4.

Immunocytochemistry. Detection of virus nucleoprotein (NP) and cellular influxes in cryosections was performed as described previously (Vervelde et al., 1996). NP was detected with a mouse mAb to NP of H9N2 (provided by Intervet Schering-Plough). KUL-01+ cells (macrophages; Mast et al., 1998) and CD4+ cells were detected with monoclonal antibodies KUL-01 and CT-4 (Southern Biotech). For the detection of CD8α+ cells, a mixture of mAbs EP72 (Southern Biotech) and AV14 (a kind gift of Dr T. F. Davison, Institute for Animal Health, Compton, UK; Withers et al., 2005) was used to avoid differences in staining due to polymorphism in the chicken CD8α molecule (Breed et al., 1996; Luhtala et al., 1997).

RNA isolation. Total RNA was isolated from upper and lower trachea (5 mm per part of the trachea) and all four segments of the lung (a 1 × 5 mm part per segment of the lung; Fig. 1a) by using an RNeasy Mini kit, and DNase-treated by using an RNase-free DNase set (both from Qiagen) according to the manufacturer’s instructions. All RNA samples were checked for quantity by using a spectrophotometer (Shimadzu) and for quality by using a 2100 Bioanalyzer (Agilent Technologies).

Real-time quantitative (q) RT-PCR. cDNA was generated from 500 ng RNA with reverse transcription using an iScript cDNA Synthesis kit (Bio-Rad). Real-time qRT-PCR was performed by using iQ SYBR Green Supermix (Bio-Rad) and TaqMan Universal PCR Master Mix (Applied Biosystems). Primers (Invitrogen Life Technologies) and probes (Applied Biosystems) were described by Degen et al. (2006) and Eldaghyes et al. (2006). Detection of glycolaldehyde-3-phosphate dehydrogenase and H9 haemagglutinin was described by Degen et al. (2006). Primers were used at 400 nM concentration. Detection of interleukins (IL), beta interferon (IFN-β) and 28S rRNA was as described by Ariaina et al. (2008). Primers were used at 600 nM and probes at 100 nM concentration. Corrections for variation in RNA preparation and sampling were performed according to Eldaghyes et al. (2006).

A paired t-test was used to determine the statistical significance between upper and lower tracheal samples from the same bird. To determine the statistical significance between parts of the lung, an
ANOVA with a Tukey post-hoc test was used. A P-value <0.05 was considered statistically significant.

Oligonucleotide microarray analysis. For microarray analysis, the *Gallus gallus* Roslin/ARK CoRe Array Ready Oligo Set v1.0 (Operon Biotechnologies) 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 quality control and normalization purposes (Van de Peppel et al., 2003). RNA amplification and labelling were performed according to Roepman et al. (2005). All hybridizations contained 2.5 μg cRNA per channel on a HS4800Pro hybridstation (Tecan Benelux BVBA). All trachea and lung samples were co-hybridized with a trachea or lung reference sample, respectively. These reference samples consisted of RNA extracted from tracheas or lungs of four chickens that were not included in the infection experiment. Slides were scanned with a G2565AA scanner (Agilent Technologies) at 100% laser power, 30% photomultiplier tube sensitivity. Resulting image files were analysed by using Imagene 8.0 (BioDiscovery, Inc.). Within-slide normalization was performed with Printtip Loess (Yang et al., 2002) on mean data without background subtraction. Groups of replicates were analysed by using ANOVA (Wu et al., 2003). In a fixed-effect analysis, sample, array and dye effects were modelled. P-values were determined by a permutation P2 test, in which residuals were shuffled 5000 times globally. Genes with P<0.05 after family-wise error correction were considered to be statistically significantly differentially expressed and were selected to be included for further analysis. Visualization and cluster analysis were performed by using GeneSpring 7.2 (Agilent Technologies). Ensembl *G. gallus* (assembly: WASHUC2, May 2006; genebuild: Ensembl, Aug 2006; database version: 47.2e) was used for gene names, description and Gene Ontology (GO) annotations. Primary data are available in the public domain through Expression Array Manager (http://www.ebi.ac.uk/arrayexpress/?#ae=main[0]) under accession numbers E-TABM-637 for trachea and E-TABM-636 for lung.

RESULTS

Responses in upper and lower trachea are similar

Trachea of control birds at 3 days p.i. was used to analyse global and immune-related gene expression. The immune-related category was based on the Gene Ontology (GO) terms host–pathogen interaction, external stimulus and immune response. Gene expressions were depicted in a scatter plot in which upper and lower trachea were plotted against each other (Fig. 2a). Only a few genes were statistically significantly expressed (P<0.05) with ≥2-fold difference (Table 1). These genes are involved in clathrin-independent vesicular transport, nuclear organization, energy metabolism and calcium binding.

IL-1β, IL-6 and IFN-β mRNA expression was measured in the trachea of control birds at 3 days p.i. (Fig. 2b) and no significant differences in expression were found between upper and lower trachea. In the trachea of control birds, the lamina propria consisted of one to three cell layers with no lymphoid infiltrates. Few CD4⁺, CD8⁺ and mainly KUL-01⁺ cells were found in the lamina propria of both upper and lower trachea (Fig. 2e).

Upon infection, we found that the virus RNA level in upper trachea was significantly higher than in lower trachea at both 1 and 3 days p.i. (Fig. 2c). Samples from 3 days p.i. were used to analyse cytokine expression and global gene expression. The difference in virus RNA level did not result in significant differences in IL-1β, IL-6 and IFN-β mRNA expression between upper and lower trachea (Fig. 2b), but in infected birds, the mRNA levels of IL-1β and IL-6 mRNA increased by approximately 6 Cₐ values compared with control birds. IFN-β mRNA expression also increased, but to a lesser extent (approx. 2 Cₐ values). Gene expressions in upper and lower trachea of infected birds were plotted against each other in a scatter plot (Fig. 2d). Inoculation with H9N2 resulted in differential gene expression of approximately 1700 genes compared with control birds, but only a few genes were expressed with a statistically significant ≥2-fold difference (Table 1). Three genes were downregulated: cysteine- and glycine-rich protein 3, cardiac phospholamban and a chemokine gene. The upregulated genes were involved in metabolic processes, metalloproteinase activity, regulation of cytokinesis and endothelial-cell activation.

At 1 and 3 days p.i., virus was located in the epithelial cells and the mucoid glands. The lamina propria was slightly swollen at 1 day p.i., with influxes of CD4⁺ and KUL-01⁺ cells, whereas at 3 days p.i., the lamina propria consisted of multiple cell layers due to cellular influxes. KUL-01⁺ cells were located in the epithelial cell layer and in the lamina propria; these cells had a round appearance, indicative of activation, whereas in the submucosa, these cells had a dendritic appearance with branched projections. Influxes of CD8⁺ cells were found in the lamina propria from 3 days p.i. and were smaller than the CD4⁺ cell influxes. All cellular influxes were localized and not found...
AIV distribution and host response differ within lung
throughout the whole trachea, with no differences between upper and lower trachea (Fig. 2e).

**Differential responses in lung segments L1, L2, L3 and L4**

Lung was divided into four pieces according to unidirectional airflow and lung anatomy. In this study, the airflow through the air sacs is ignored for simplicity, because it does not affect the airflow through the lung. Global and immune-related gene expression in lung L1–L4 of control birds at 3 days p.i. was compared by hierarchical clustering. L1 clustered with L3 and L2 clustered with L4 for both global and immune-related genes (Fig. 3a), coinciding with airflow and lung anatomy.

No significant difference in IL-1β and IFN-β mRNA expression between L1, L2, L3 and L4 was found (Fig. 3b). However, IL-6 mRNA expression in L1 and L3 was significantly higher than in L2 and L4.

In a non-infected lung, the KUL-01+ and CD8α+ cells were distributed throughout the lung in the parabronchi, interparabronchial septa and lamina propria of larger airways. KUL-01+ and CD8α+ cells were found

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**Table 1. Genes expressed significantly at ≥2-fold difference in upper versus lower trachea at 3 days p.i. in control and infected birds**

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*Expression ratio in lower trachea dived by expression ratio in upper trachea.
†Immune-related gene.

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**Fig. 3.** (a) Hierarchical clustering of expression of global and immune-related genes in lung L1–L4 in control birds at 3 days p.i. Genes in red were upregulated and those in green were downregulated. (b) IL-1β, IL-6 and IFN-β mRNA expression in lung L1–L4 at 3 days p.i. Bars with different letters were significantly different (P<0.05). (c) Virus RNA levels in infected birds at 1 and 3 days p.i. Bars within a time point with different letters were significantly different (P<0.05). (d) Hierarchical clustering of expression of global and immune-related genes in lung L1–L4 in infected birds at 3 days p.i. Genes in red were upregulated and those in green were downregulated. (e) Cryosections of lung L1–L4 stained for virus NP at 3 days p.i. The parabronchi of the different parts of the lung were affected to a different extent. L indicates the luminal side of a parabronchus. Bar, 75 μm. (f) Cryosections of lung L1 stained for virus NP and for KUL-01+, CD4+ and CD8α+ cells at 3 days p.i. Upon infection, KUL-01+ cells (macrophages) change from dendritic (arrows) to round (arrowheads) morphology. Bar, 75 μm.
AIV distribution and host response differ within lung

(a) Global genes
(b) Immune-related genes

(c) Virus RNA expression
(d) Cytokine mRNA expression

(e) Control
(f) Infected

http://vir.sgmjournals.org
closer to the lumen than CD4+ cells, and the number of KUL-01+ cells exceeded those of CD4+ and CD8α+ cells (Fig. 3f).

After inoculation (Fig. 3c) at both 1 and 3 days p.i., no significant differences in virus RNA levels were found between L1 and L3 or between L2 and L4. However, at both time points, virus RNA level was higher in L1 and L3 than in L2 and L4, being significantly different at 1 day p.i. for L2 and at both time points for L4. The effect of uneven virus distribution on IL-1β, IL-6 and IFN-β mRNA expression is shown at 3 days p.i. (Fig. 3b). IL-1β and IL-6 mRNA levels were similar in L1 and L3, but significantly higher than in L2 and L4. In all four parts of the lung, IL-1β and IL-6 mRNA expression was significantly higher in infected birds than in control birds. The IFN-β mRNA levels in infected birds were low and not significantly different between L1, L2, L3 and L4, nor were they different in control birds.

A hierarchical clustering of both global and immune-related gene expression was performed on infected birds at 3 days p.i. (Fig. 3d). In infected birds, the same clustering was observed as in control birds, but the clustering of L1/ L3 and of L2/L4 in infected birds was even stronger for both global and immune-related genes, as the branches leading to the L1/L3 and L2/L4 clusters were longer and the branches leading to the individual lung parts were shorter compared with the cluster tree in control birds.

By using immunocytochemistry, virus deposition and cellular influxes in the lung parts were studied (Fig. 3e). In both L1 and L3, virus-infected areas were found in the primary and secondary bronchi and adjacent parabronchi. At 3 days p.i., more virus was located in the parabronchi than at 1 day p.i. In L2 and L4, infected areas were located in the parabronchi and an increase over time was seen. The largest infected areas were found in L1, followed by L3; L4 and L2 were similar and contained fewer and smaller infected areas (Fig. 3e). At 1 day p.i., influxes of KUL-01+, CD4+ and CD8α+ cells were seen in both L1 and L4 in the bronchi and parabronchi (Fig. 3f). Cellular influxes colocalized with virus-infected areas, and the number of cells correlated positively with the size of the virus-infected areas. At 1 day p.i., KUL-01+ cells in infected areas were mainly round, whereas in non-infected areas they were dendritic in morphology. The number of KUL-01+, CD4+ and CD8α+ cell influxes increased over time.

**Differences between lung L1 and L4**

In both control and infected birds, gene expression differed most between L1 and L4. These significantly differentially expressed genes are depicted in a graph showing three major GO categories and an immune-related category (Fig. 4a). In control birds, more genes were expressed at a higher rate in L1 than in L4 for all four categories. Immune-related genes that differed significantly between L1 and L4 are depicted in a heat map (Fig. 4b). Genes expressed at a higher rate in L1 were mainly involved in cell adhesion and motility, e.g. VTN and TSPAN1, T cell- and B cell-related, e.g. ITFG1 and PIGR, or involved in chemotactic activities, e.g. CCL20, CX3CR1 and CHIA. In infected birds, more genes were expressed at a higher rate in L4 than in L1 for the molecular function, cellular component and biological processes categories, but not for immune-related genes. Immune-related genes that differed significantly between L1 and L4 are depicted in a heat map (Fig. 4c). These genes were mainly involved in lymphocyte activation, apoptosis, chemokine and IL activity. Genes expressed at a higher rate in L1 were mainly involved in lymphocyte activation, mainly T cell-related, such as CD3E, CD28 and ICOS, and chemokine and IL activity, e.g. CCL19, SOCS1, CXCL1, IL-1β, IL-7Rα and IL-16. In L4, mainly genes involved in apoptosis, stress responses and major histocompatibility complex (MHC) pathways were expressed at a higher level than in L1, e.g. BCLX, BNIP3L, HSP70, HSPB2 and MHC class II.

**Induction of host responses against H9N2 in upper trachea and lung L1 and L4**

To quantify chicken responses against H9N2 in the respiratory tract at the transcriptional level, immune-related genes that were significantly differentially expressed between control and infected birds were analysed. Most genes were significantly differentially expressed in trachea and lung L1 (respectively 244 and 272 immune-related genes), whereas in lung L4, there were 86 significantly differentially expressed immune-related genes. Genes were divided into functional groups based on GO interpretations, of which the groups containing most genes are depicted in Fig. 5. The complete gene sets are listed in Supplementary Tables S1–S3, available in JGV Online.

A shortened gene list, indicating the location in the respiratory tract at which genes were significantly induced due to H9N2 infection, is depicted in Table 2. This list is based on the functional groups shown in Fig. 5 and depicts a global overview of the location of expression and expression rate of genes (up or down) within a functional group. The most striking facts are that many fewer genes were expressed differentially in lung L4, and most genes were upregulated independently of the location and functional group.

In trachea and both lung L1 and L4, genes involved in chemokine and cytokine signalling were most commonly expressed, with the different regions sampled sharing many similar genes. In contrast to the upregulation of chemokine-related genes, several genes related to growth factors were downregulated.

Of the genes involved in innate responses and IFN signalling, only the genes involved negatively in IFN signalling (FLN29, USP18) and related to IFN-γ signalling (IRF10) were expressed in trachea, lung L1 and L4. Other
genes involved in IFN-γ signalling (IFI35, MIME, IFI30) and type I IFN signalling (IRFs) were expressed at certain locations, rather than in trachea and both lung segments. Genes involved in NF-κB signalling were expressed in trachea and L1, but in L4, no genes related to NF-κB signalling were found.

Genes involved in antigen presentation were all upregulated. MHC class IV or B-G, which is only expressed in lung L1, and ASB2, which was only expressed in trachea, were downregulated.

Upregulation of genes that supported an increased T-cell response was mostly observed in lung L1 and trachea. Several genes were expressed exclusively in L1 and were involved in co-stimulation (CD86, CTLA4, PDL2), T-cell activation (ZAP70, TXK), T-cell proliferation (IGSF2, TNFRSF4) and CD3 signal transduction (TRAT1). Many genes involved in B-cell activity were expressed especially in lung L1 and trachea. All B cell-related genes were upregulated except for BRAG, which was expressed only in trachea, and PIGR, which were both downregulated. In lung L4, genes related to T- and B-cell responses were rarely expressed.

Several genes involved in cell migration and adhesion were expressed exclusively in trachea (CD9, THBS4) or in lung
infection with infectious bronchitis virus or Newcastle to pathogens have been described for the chicken. Upon Kothlow & Kaspers, 2008), only the humoral responses pathogens are present in these tissues (reviewed by 2006). Although cells capable of rapid responses against 2142 Journal of General Virology contact with aerosolized virus or particles (Corbanie et al., 2001; Etchart et al., 2006), showing that, also within the nasal compartment of mice, sampling of specific parts of the tissue will result in a different outcome, as shown for antibody production, but which will probably also be found at the host transcriptional level.

In our study, the trachea was divided into upper and lower parts, as H9N2 LPAI has a preference for infecting the upper part of the respiratory tract (Nili & Asasi, 2002), which coincided with our data at both 1 and 3 days p.i. However, only minor differences in gene expression were seen between upper and lower trachea in both control and infected birds, suggesting that gene expression within the trachea was not affected by a difference in virus deposition. Lung was divided into four pieces according to unidirectional airflow and lung anatomy. Lung L1 was most similar to L3 and L2 resembled L4. In this paper, we report that the differences in anatomy and airflow were reflected upon gene expression in control birds and, upon infection with H9N2, host responses in the different parts of the lung were even more diverse. Furthermore, airflow and anatomy affected virus deposition within the lung. During inhalation, air flows through the primary bronchus, bypassing the cranially located openings of the medioventral secondary bronchi, and flows into the caudal air sacs or through the mediodorsal and lateroventral secondary bronchi (Fig. 1). This caudal-to-cranial flow pattern is also evident during expiration. In the caudal part of L1 and L3, which contains the bifurcations to the mediodorsal and lateroventral secondary bronchi, virus RNA levels were significantly higher and virus-infected areas were larger than in L2 and L4. This correlates with results of studies on deposition of airborne microspheres in the avian respiratory tract, which indicated that airborne particles are not distributed uniformly in the lung and deposition is influenced by particle size and size of the airway, and that particles are intercepted at bifurcations within the respiratory tract (Hayter & Besch, 1974; Mensah & Brain, 1982; Corbanie et al., 2006). Differences in virus deposition in infected birds enhanced the differences in gene expression already seen in control birds. This coincided with the finding in the lung of macaques that differences in H1N1 influenza virus mRNA result in differences in gene expression (Baas et al., 2006). A functional consequence of the flow pattern in the avian lung, resulting in an uneven spread of virus, may explain the absence of bronchus-associated lymphoid tissue (BALT) in the cranial part of the lung. Although organized BALT nodules are not observed before the third week after hatching, a similar time-course of BALT development in SPF and conven-

L1 (MAEA, VCAM1). Genes involved in monocyte activity were equally expressed in all three parts of the respiratory tract, sharing similar genes.

Infection with H9N2 mostly caused upregulation of genes involved in the regulation of apoptosis, mainly via caspase activity. Most genes involved in apoptosis were expressed in trachea and lung L1, whereas in lung L4, few apoptosis-related genes were expressed.

Several genes involving oxidative stress were upregulated, but mainly in trachea and lung L1. Lung L4 only expressed p40-phox. Gene expression related to complement activity mainly seemed to occur via the classical route. In L4, only CD93 and ITGB2 were expressed.

**DISCUSSION**

To be able to study host responses to respiratory viruses, we first wanted to know whether sampling certain locations would affect the outcome of the study. The aim of this study was firstly to determine whether gene expression differed between upper and lower trachea and between different parts of the lung in control birds and whether this was affected by H9N2 AIV infection, and secondly to study the early host responses against LPAI H9N2.

Besides the trachea and lung, other structures, such as the paranasal organs, paraocular Harderian gland (HG) and the conjunctiva-associated lymphoid tissue, come into contact with aerosolized virus or particles (Corbanie et al., 2006). Although cells capable of rapid responses against pathogens are present in these tissues (reviewed by Kothlow & Kaspers, 2008), only the humoral responses to pathogens have been described for the chicken. Upon infection with infectious bronchitis virus or Newcastle disease virus, an increase in virus-specific IgA antibody-forming cells (AFCs) in the HG is found (Russell & Koch, 1993; Van Ginkel et al., 2008), whereas most IgG-AFCs reside in the spleen (Russell & Koch, 1993). In mice, intranasal immunization with influenza virus results in local, long-term specific antibody production. These AFCs reside in larger numbers in the diffuse region, rather than in the organized region, of the nasal-associated lymphoid tissue (Liang et al., 2001; Etchart et al., 2006), showing that, also within the nasal compartment of mice, sampling of specific parts of the tissue will result in a different outcome, as shown for antibody production, but which will probably also be found at the host transcriptional level.
### Table 2. Immune-related genes induced by H9N2 infection in upper trachea (UT) and lung L1 and L4 at 3 days p.i.

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| Cytokines and chemokines
| BAFF | E16852 | + | + | PTX3 | E09694 | + | + | + |
| CCL4 | E00951 | + | + | USP18 | E13057 | + | + | + |
| CCLI3 | E00956 | + | + | CAD11 | E05278 | - | - | - |
| CCLI7 | E14585 | + | + | CD34 | E01177 | - | - | - |
| CCR5 | E11732 | + | + | CD9 | E17274 | - | - | - |
| CXCL2 | E11668 | + | + | CFS3R | E02112 | + | + | + |
| GHR | E14855 | - | - | THBS4 | E14804 | - | - | - |
| NNI | E12480 | + | + | + | MAEA | E13310 | - | - | - |
| SOCS3 | E07189 | + | + | + | RGS18 | E21143 | + | + | + |
| STAT4 | E07651 | + | + | + | IL1B | E00534 | + | + | + |
| TGFβ3 | E10346 | + | + | + | MIF | E12480 | + | + | + |
| Innate immune and IFN response
| AVID | E23622 | + | + | + | CCL1 | E16113 | - | - | - |
| AVR2 | E02441 | + | + | + | FLN29 | E04802 | + | + | + |
| CCLI2 | E16166 | + | + | + | GAL4 | E19843 | + | + | + |
| IFI30 | E03389 | + | + | + | IFI35 | E02832 | + | + | + |
| IRF1 | E06785 | + | + | + | IRF10 | E06448 | + | + | + |
| IRF3 | E14297 | + | + | + | IRF8 | E05757 | + | + | + |
| MARCO | E12119 | + | + | + | MDA5 | E10899 | + | + | + |
| MIME | E04732 | - | - | - | MHC B-G | E24357 | - | - | - |
| MMP7 | E17184 | + | + | + | MHC I | E00178 | + | + | + |
| MX | E16142 | + | + | + | MHC II β | E00141 | + | + | + |
| OASL | E13723 | + | + | + | TSPAN8 | E10152 | + | + | + |

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| Cell migration, macrophage responses
| BAFF | E16852 | + | + | + | MAEA | E13310 | - | - | - |
| TLR3 | E13468 | + | + | + | RGS18 | E21143 | + | + | + |
| TLR4 | E07001 | + | + | + | IL1B | E00534 | + | + | + |
| TLR7 | E16590 | + | + | + | MIF | E12480 | + | + | + |
| TLR15 | E08166 | + | + | + | CCL1 | E16166 | + | + | + |
| CARD9 | E01889 | + | + | + | CCL1 | E16166 | + | + | + |
| CCL4 | E00951 | + | + | + | MAV | E13310 | - | - | - |
| CCL7 | E14585 | + | + | + | RGS18 | E21143 | + | + | + |
| CCR5 | E11732 | + | + | + | IL1B | E00534 | + | + | + |
| CXCL2 | E11668 | + | + | + | MAV | E13310 | - | - | - |
| GHR | E14855 | - | - | - | RGS18 | E21143 | + | + | + |
| NMI | E12480 | + | + | + | IL1B | E00534 | + | + | + |
| SOCS3 | E07189 | + | + | + | MAV | E13310 | - | - | - |
| STAT4 | E07651 | + | + | + | RGS18 | E21143 | + | + | + |
| TGFβ3 | E10346 | + | + | + | IL1B | E00534 | + | + | + |

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| Apoptosis, oxidative stress, complement
| USP18 | E13057 | + | + | + | MAEA | E13310 | - | - | - |
| CARD9 | E01889 | + | + | + | RGS18 | E21143 | + | + | + |
| CCL4 | E00951 | + | + | + | IL1B | E00534 | + | + | + |
| CCL7 | E14585 | + | + | + | MAV | E13310 | - | - | - |
| CCR5 | E11732 | + | + | + | RGS18 | E21143 | + | + | + |
| CXCL2 | E11668 | + | + | + | IL1B | E00534 | + | + | + |
| GHR | E14855 | - | - | - | MAV | E13310 | - | - | - |
| NMI | E12480 | + | + | + | RGS18 | E21143 | + | + | + |
| SOCS3 | E07189 | + | + | + | IL1B | E00534 | + | + | + |
| STAT4 | E07651 | + | + | + | MAV | E13310 | - | - | - |
| TGFβ3 | E10346 | + | + | + | RGS18 | E21143 | + | + | + |

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</table>
| Innate immune and IFN response
| AVID | E23622 | + | + | + | MAEA | E13310 | - | - | - |
| AVR2 | E02441 | + | + | + | RGS18 | E21143 | + | + | + |
| CCLI2 | E16166 | + | + | + | IL1B | E00534 | + | + | + |
| CCLI7 | E14585 | + | + | + | MAV | E13310 | - | - | - |
| CCR5 | E11732 | + | + | + | RGS18 | E21143 | + | + | + |
| CXCL2 | E11668 | + | + | + | IL1B | E00534 | + | + | + |
| GHR | E14855 | - | - | - | MAV | E13310 | - | - | - |
| NMI | E12480 | + | + | + | RGS18 | E21143 | + | + | + |
| SOCS3 | E07189 | + | + | + | IL1B | E00534 | + | + | + |
| STAT4 | E07651 | + | + | + | MAV | E13310 | - | - | - |
| TGFβ3 | E10346 | + | + | + | RGS18 | E21143 | + | + | + |
| Innate immune and IFN response
| AVID | E23622 | + | + | + | MAEA | E13310 | - | - | - |
| AVR2 | E02441 | + | + | + | RGS18 | E21143 | + | + | + |
| CCLI2 | E16166 | + | + | + | IL1B | E00534 | + | + | + |
| CCLI7 | E14585 | + | + | + | MAV | E13310 | - | - | - |
| CCR5 | E11732 | + | + | + | RGS18 | E21143 | + | + | + |
| CXCL2 | E11668 | + | + | + | IL1B | E00534 | + | + | + |
| GHR | E14855 | - | - | - | MAV | E13310 | - | - | - |
| NMI | E12480 | + | + | + | RGS18 | E21143 | + | + | + |
| SOCS3 | E07189 | + | + | + | IL1B | E00534 | + | + | + |
| STAT4 | E07651 | + | + | + | MAV | E13310 | - | - | - |
| TGFβ3 | E10346 | + | + | + | RGS18 | E21143 | + | + | + |
tional chickens was found (Fagerland & Arp, 1993) and infections with pathogenic micro-organisms increased the number of BALT nodules significantly (Van Alstine & Arp, 1988). This probably resulted in more differential expression with a higher fold change of genes involved in cell adhesion and motility, chemotactic and IL activities and T and B cell-related genes in L1 compared with L4, in both control and infected birds.

By analysing host responses in lung L1 and L4 separately, gene-expression patterns were not diluted and could be used to define overall and site-specific responses to H9N2 infection. After comparing gene expression in trachea, lung L1 and L4, we found that most changes occurred in trachea and L1. Most genes expressed in L4 were also expressed in trachea and L1, indicating these were part of a common host response, independent of the amount of virus RNA. Most of these shared responses have been described previously as being part of a common response to pathogens (Jenner & Young, 2005; Pennings et al., 2008) and involve upregulation of genes related to chemokine activity and inflammatory responses, as described in primate and rodent influenza models (Pennings et al., 2008). These correspond with increased IL-1β and IL-6 mRNA expression and the massive influx of KUL-01+ cells in trachea and lung of infected birds. Trachea and L1 had an overlap in gene expression that was not shared by L4, indicating that these responses were affected by virus load.

Several site-specific responses were measured upon infection. Toll-like receptors (TLRs) contribute to innate responses, of which TLRs 3 and 7–9 recognize nucleosides that are important in virus recognition. In chicken, TLR9 has not been identified, but it is speculated that the two TLR7 splice variants, TLR15 or TLR21, may elicit responses to mammalian TLR9 ligands (Philbin et al., 2005; Jenkins et al., 2009). After infection with H9N2, upregulation of TLR3 was found in trachea, as was also found at the mRNA level in lung and brain of chickens upon infection with high-pathogenic AIV (Karpala et al., 2008). TLR1 and TLR7 were upregulated in trachea, lung L1 and L4. Both TLR1 and TLR7 are upregulated in human primary macrophages infected with influenza or Sendai viruses (Miettinen et al., 2001), indicating that upregulation of these genes is probably part of a common host response to viruses. TLR15 expression has been reported to remain unchanged after in vitro H9N2 infection (Xing et al., 2008), in contrast to our data, which show that, upon H9N2 infection in vivo, TLR15 was upregulated in L1.

Several genes were especially expressed in L1 and were involved in innate responses, IL activity and vesicle trafficking. Molecules involved in innate immunity to pathogens, such as CCL1, PTX3 and GAL4, were only upregulated in L1. C-type lectins and PTX3 are known to have antiviral activity against influenza virus (Hogenkamp et al., 2008; Reading et al., 2008). In mice, β-defensins such as GAL4 are upregulated in the airway epithelial cells due to influenza virus infection (Chong et al., 2008).

ANXA6, DDEF2, PICALM and ZFYVE20 are involved in vesicle trafficking, such as endocytosis and phagocytosis, and were only expressed in L1; in contrast to most genes, they were downregulated. Changes in expression of these genes have not previously been described in a virus-infection model, although the endocytosis pathway is known to play an important role in the entry of influenza virus into host cells (Lakadamyali et al., 2004). Whether downregulation of these genes was induced by host cells to inhibit virus entry or by antigen-presenting cells to control dissemination of virus remains to be investigated.

Although not described in this paper, the avian respiratory tract also contains multiple air sacs that work as bellows to enable ventilation of the lungs. Upon inoculation with infectious bronchitis virus or influenza virus, reduced lucency of the air sacs and cellular influxes containing CD4+, CD8+, γδ-TCR+ and KUL-01+ cells and heterophil infiltrates are found (Perkins & Swayne, 2002; Matthijs et al., 2009). It is highly likely that the airflow also affects responses in the air sacs, because only certain air sacs will be exposed to the inhaled air containing the virus (Fig. 1).

To our knowledge, we are the first to give an overview of host responses to AIV at transcriptional level in the trachea and in the lung, taking into account the effect of anatomy and airflow through the respiratory tract. Gene expression within the lungs of control birds already differed significantly. Moreover, airflow affected virus deposition and subsequently gene expression, indicating that sampling at specific sites within the lung affects the outcome of studies of respiratory infections in chickens. Changes in gene expression and cellular influxes were significantly more pronounced in the parts of the respiratory tract where virus deposition was highest. These findings suggest not only that responses against AIV are affected locally, but also that most pathogens that are not distributed evenly throughout an organ will induce localized responses; careful sampling of the organ will be essential.

ACKNOWLEDGEMENTS

We thank Peter van de Haar, Dik van Leenen, Cheuk Ko and Linda Bakker for their technical assistance and Dr Winfried Degen for collaboration on the in vivo experiment. This work was supported by a BSIK VIRGO consortium grant (no. 03012), the Netherlands.

REFERENCES


