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Early host responses to avian influenza A virus are prolonged and enhanced at transcriptional level depending on maturation of the immune system

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ABSTRACT

Newly hatched chickens are more susceptible to infectious diseases than older birds because of an immature immune system. The aim of this study was to determine to what extent host responses to avian influenza virus (AIV) inoculation are affected by age. Therefore, 1- and 4-week (wk) old birds were inoculated with H9N2 AIV or saline. The trachea and lung were sampled at 0, 8, 16 and 24 h post-inoculation (h.p.i.) and gene expression profiles determined using microarray analysis. Firstly, saline controls of both groups were compared to analyse the changes in gene profiles related to development. In 1-wk-old birds, higher expression of genes related to development of the respiratory immune system and innate responses were found, whereas in 4-wk-old birds genes were up regulated that relate to the presence of higher numbers of leukocytes in the respiratory tract. After inoculation with H9N2, gene expression was most affected at 16 h.p.i. in 1-wk-old birds and at 16 and 24 h.p.i. in 4-wk-old birds in the trachea and especially in the lung. In 1-wk-old birds less immune related genes including innate related genes were induced which might be due to age-dependent reduced functionality of antigen presenting cells (APC), T cells and NK cells. In contrast cytokine and chemokines gene expression was related to viral load in 1-wk-old birds and less in 4-wk-old birds. Expression of cellular host factors that block virus replication by interacting with viral factors was independent of age or tissue for most host factors. These data show that differences in development are reflected in gene expression and suggest that the strength of host responses at transcriptional level may be a key factor in age-dependent susceptibility to infection, and the cellular host factors involved in virus replication are not.

1. Introduction

Young animal are highly susceptible to opportunistic pathogens that are common in their environment. Susceptibility to disease decreases as the bird matures, suggesting that this phenomenon is due to immaturity of the immune system (Beal et al., 2005; Hume et al., 1998; Raj and Jones, 1997). Neonatal immune dysfunction has also been reported for mammals (Chelvarajan et al., 2004; Gasparoni et al., 2003; Velilla et al., 2006). At present, vaccination of young chicks is commonly practised to establish immunity in a flock. In newly hatched birds, the activation, phagocytosis and bactericidal activities of heterophils and macrophages were shown to be age-dependent in that they increase with age (Kodama et al., 1976; Kogut et al., 2002; Wells et al., 1998). T cells from 1-day-old chicks hardly proliferate in response to mitogens and produce less IFN and IL-2 (Lowenthal et al., 1994). Immunization of 1-day-old broilers with BSA resulted in a much lower and slower antibody production compared to immunization at 1 or 2 wks of age (Mast and Goddeeris, 1999).

The respiratory tract is constantly exposed to pathogens like influenza virus and to provide adequate protection, inhaled pathogens are removed by mucus, neutralizing molecules like IgA, complement and antimicrobial peptides. When virus entry is not successfully blocked, influenza virus will infect the epithelial cells resulting in the production of pro-inflammatory cytokines, chemokines and interferons (Julkunen et al., 2001). This attracts macrophages and DC, which upon activation or influenza virus infection also start producing cytokines and chemokines attracting more antigen presenting cells (APC) and lymphocytes to the place of infection. The trachea, lung and air sacs contribute to the respiratory immune system, but the lung plays a special role because it contains secondary lymphoid structures (Kothlow and Kaspers, 2008). Newly hatched birds have dispersed T cells, B cells, leukocytes and monocytes present throughout the lung. At 2 wks of age areas with lymphocyte infiltrates are found around the bifurcations of the caudal secondary bronchi. These organized structures show

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similarity to Peyer's patches and are called bronchus associated lymphoid tissue (BALT; Fagerland and Arp, 1993; Jeurissen et al., 1989). Avian BALT is not comparable to inducible BALT in mice and human (Moyron-Quiroz et al., 2004; Rangel-Moreno et al., 2006), because its presence is independent of antigenic stimulation (Reese et al., 2006). In the trachea no organized lymphoid structures have been reported, but infection with various pathogens results in lymphoid infiltration and in formation of lymphoid follicles (Gaunson et al., 2006; Matthijs et al., 2009; Reemers et al., 2009b).

For replication and transcription of the influenza virus genome, the virus uses both viral and cellular host factors. Viral factors like the polymerase complex or nucleoprotein have been known to interact with mammalian host factors like importins or histones during influenza virus infection (Engelhardt and Fodor, 2006; Naffakh et al., 2008; Nagata et al., 2008). The virus must stimulate expression of host factors that are needed for replication ongoing during infection to ensure virus multiplication. However, some host factors that interact with viral factors are used in host defense responses and can limit viral replication.

To investigate the effect of age on the early host response and on host factors affecting viral replication in the respiratory tract, we inoculated 1- and 4-week (wk) old birds with H9N2 avian influenza virus (AIV) or saline. The trachea and lung were sampled at 0, 8, 16 and 24 h post-inoculation (h.p.i.) and gene expression was studied using microarray analysis. Differences between 1- and 4-wk-old saline inoculated control birds were mainly related to tissue development and immunological maturation. Differences between 1- and 4-wk-old H9N2 inoculated birds were related to strength and the timing of host responses at transcriptional level. Expression of cellular host factors that block viral replication by interacting with viral factors was independent of age and tissue.

2. Materials and methods

2.1. Experimental design

Lohmann Brown chickens of 1 and 4 wks of age were divided into 2 groups per age, a saline and AIV inoculated group. The birds were inoculated intratracheally (i.t.) with either 0.1 ml PBS or with 0.1 ml $10^{7.7}$ EID_{50} H9N2 AIV, isolate A/Chicken/United Arab Emirates/99 (kindly provided by Intervet Schering-Plough Animal Health). At 0, 8, 16 and 24 h post-inoculation (h.p.i.) 5 birds per time point per group and per age were killed. The upper trachea and the left lung were isolated and stored in RNAlater (Ambion) at −80 °C for RNA isolation. The segment of the lung containing the primary and secondary bronchi was used for analysis. Selection of the upper trachea and the lung segment used for analysis was based on high viral load and high virus-induced gene expression as described previously (Reemers et al., 2009b). All experiments were carried out according to protocols approved by the Animal Experiment Committee of Utrecht University (Utrecht, The Netherlands).

2.2. RNA isolation

The trachea (5 mm part) and lung (1 × 5 mm part) were homogenized (Mixer Mill 301, Retsch) and total RNA was isolated using the RNeasy Mini Kit and DNase treated using the RNase-free DNase Set following manufacturer's instructions (Qiagen). All RNA samples were checked for quantity using a spectrophotometer (Shimadzu) and quality using a 2100 Bioanalyzer (Agilent).

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

cDNA was generated from 500 ng RNA using reverse transcription using iScript cDNA Synthesis Kit (Biorad Laboratories B.V.). Real-time qRT-PCR was used for detection of GAPDH, viral H9 hemagglutinin (HA), interleukins (IL-1β, IL-8, IL-18), interferon alpha (IFN-α) and 28S as previously described (Reemers et al., 2009a). Amplification and detection of specific GAPDH and viral H9 HA products was achieved using iQ SYBR green supermix (Biorad). For amplification and detection of IL-1β, IL-8, IL-18, IFN-α and 28S TaqMan Universal PCR Master Mix (Applied Biosystems) was used.

2.4. Statistical analysis qRT-PCR data

To determine the statistical significance in viral RNA expression between time points within an age group and between age groups within a time point in the trachea and lung an ANOVA with a Tukey post hoc test was used. To determine the statistical significance in cytokine mRNA expression between control birds and H9N2 inoculated birds within a time point and age group in the trachea and lung an ANOVA was used. Correlations between viral RNA and cytokine mRNA expression were based on the Pearson correlation coefficient (r) and determined using SPSS 15.0 software. A p-value < 0.05 was considered significant.

2.5. Oligonucleotide microarray analysis

Microarray analysis was performed as described previously (Reemers et al., 2009b) using the *Gallus gallus* Roslin/ARK CoRe Array Ready Oligo Set V1.0 (Operon Biotechnologies). All the trachea and lung samples were co-hybridised with, respectively a trachea or lung reference sample. These reference samples consisted of pooled RNA extracted from tracheas or lungs of 4 chickens that were not included in this experiment.

Ensembl *Gallus gallus* (assembly: WASHUC2, May 2006, genebuild: Ensembl, August 2006, database version: 47.2e) was used for gene names and description. For analysis of gene lists and Gene Ontology (GO) analysis Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 was used. Primary data are available in the public domain through Expression Array Manager at http://www.ebi.ac.uk/microarray-as/aer/?#aemain[0] under accession number E-TABM-771 for the lung and E-TABM-772 for the trachea.

3. Results

3.1. Age-dependent gene expression in control birds

Before we compared virus-induced host responses between 1- and 4-wk-old birds we determined the effect of age on gene expression in control birds. Differences would affect virus-induced gene profiles and a direct comparative study between the age groups would not be possible. The number of genes in the lung that differed significantly between 1- and 4-wk-old control birds at 0, 8, 16 and 24 h.p.i. were, respectively 230, 167, 74 and 227 genes, and in the trachea 58, 104, 47 and 19 genes. On each set of genes Gene Ontology (GO) analysis was performed using DAVID and the resulting top three functional groups of every gene set were depicted in Table S1 in the supplementary data. These functional groups were mainly related to tissue development, but most functional groups contained genes that also play a role in immune responses. Furthermore, several functional groups relating to immune related processes like immune system response, lymphocyte activation and chemotaxis were also significantly differentially expressed between 1- and 4-wk-old control birds, but did not belong to the top 5 of functional groups found. The number of immune related genes that were significantly differentially expressed between 1- and 4-wk-old control birds was determined. The immune related category was based on the GO annotations host–pathogen interaction, external stimulus and immune response. The number of

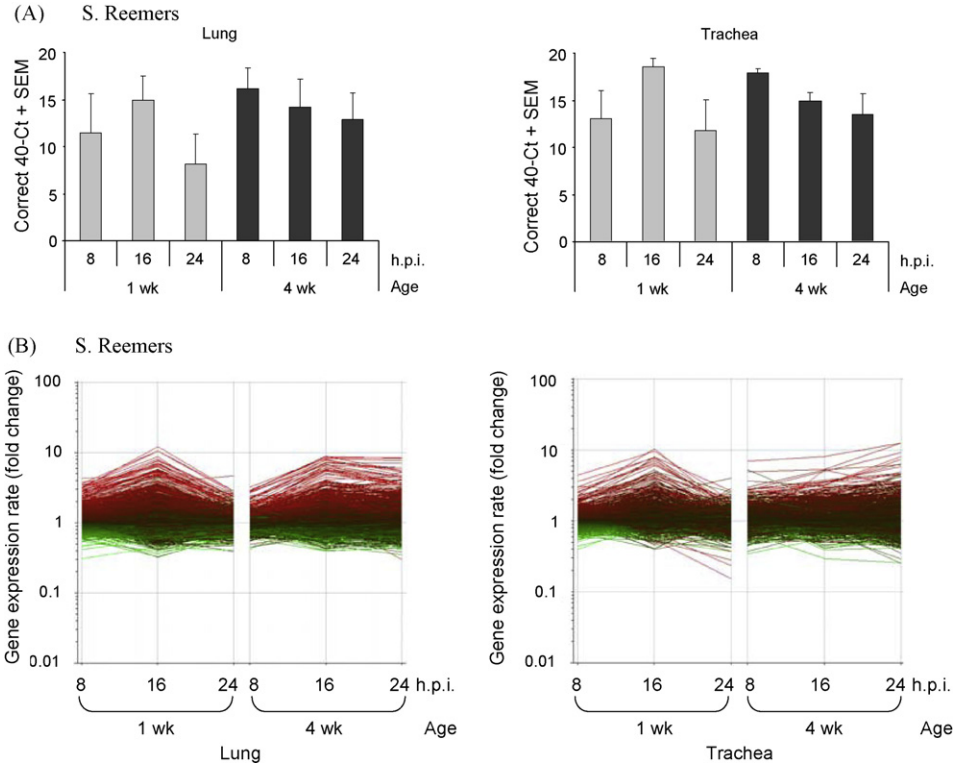


Fig. 1. (A) Viral RNA expression in the lung and trachea of 1- and 4-wk-old H9N2 inoculated birds. Viral RNA expression was determined using qRT-PCR ($n = 4$) and data were expressed as means with standard error of the mean (SEM). (B) Gene expression patterns of global genes induced after H9N2 inoculation in the lung and trachea. Gene expression of global genes was determined using microarray analysis. Gene expression rates in 1- and 4-wk-old H9N2 inoculated birds were compared to gene expression rates in age and time matched control birds ($n = 4$). Red indicated up regulated genes and green down regulated genes. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

immune related genes at 0, 8, 16, and 24 h.p.i. were in the lung, respectively 34, 33, 13 and 38 genes and in the trachea 7, 17, 4 and 4 genes (Supplementary data Tables S2 and S3). In the lung these genes were involved in antigen presentation/binding, apoptosis, cell differentiation and proliferation, chemotaxis, innate immune response, protein folding and binding and signal transduction. In the trachea less genes were differentially expressed than in the lung, but the ones expressed were involved in similar biological processes. Most genes were expressed at a higher rate in 4-wk-old birds in both the lung and trachea. Genes higher expressed in the lung and trachea of 1-wk-old birds were mostly involved in apoptosis, cell adhesion and proliferation, innate immune responses, protein binding and folding, and signal transduction.

These results indicated that gene expression levels differed significantly between 1- and 4-wk-old control birds due to development of the tissue itself and maturation of the immune system. Therefore, host responses induced by H9N2 inoculation in 1- and 4-wk-old birds could only be compared indirectly by comparing expression patterns between control and H9N2 inoculated birds within an age group.

3.2. Early gene expression patterns after H9N2 inoculation

In the lung and trachea of all H9N2 inoculated birds viral RNA was detected using qRT-PCR (Fig. 1A). There was no significant difference in viral RNA levels between 1-wk and 4-wk-old birds in both the lung and trachea at any time point.

To determine the effect of H9N2 inoculation at transcriptional level over time, we compared gene expression rates in H9N2 inoculated birds to age matched control birds within a time point. Differences in gene expression rates were given as fold change and depicted over time generating gene expression patterns. For the lung and trachea gene expression patterns were generated for global genes (Fig. 1B) and immune related genes (data not shown, but patterns similar to those of global genes). After inoculation, genes were mostly up regulated and not down regulated in both the lung and trachea independent of age. In the lung more genes were up regulated than in the trachea, but gene expression patterns in the lung and trachea were similar. The biggest difference was found between the age groups. In 1-wk-old birds gene expression was most affected by H9N2 inoculation at 16 h.p.i. and the amplitude of change declined at 24 h.p.i. In 4-wk-old birds the effect of H9N2 inoculation on gene expression increased over time and gene expression was affected most at 16 and 24 h.p.i. Therefore the overall gene expression pattern in response to H9N2 inoculation differed between 1- and 4-wk-old birds. Genes involved in development (based on GO annotation terms developmental process, developmental maturation, multicellular organismal development, anatomical structure development) did not follow this expression pattern and did not differ in expression between H9N2 inoculated birds and age matched control birds in both age groups (data not shown).

3.3. Early gene expression after H9N2 inoculation

In order to determine early responses to H9N2 inoculation in the respiratory tract we compared gene expression in H9N2 inoculated birds to age matched control birds within a time point. The number of global and immune related genes significantly differentially induced after H9N2 inoculation at 8, 16 and 24 h.p.i. in the lung and trachea of 1- and 4-wk-old birds were depicted in Fig. 2A. In the lung and trachea more genes were differentially expressed after H9N2 inoculation in 4- compared to 1-wk-old birds, except for global and immune related genes in the trachea at 16 h.p.i.

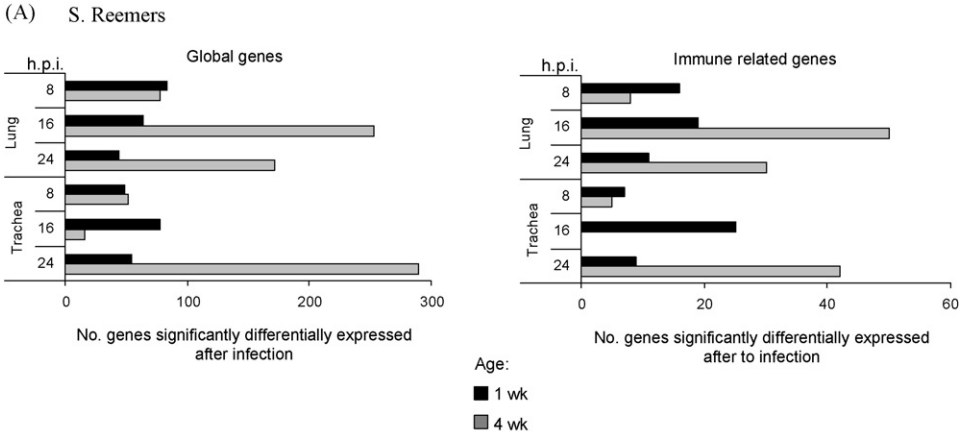
Table 1
Immune related genes induced after H9N2 inoculation in the lung of 1- and 4-wk-old birds at 8, 16 and 24 h.p.i.

Functional group	1-wk-old						4-wk-old					
	8 h.p.i.		16 h.p.i.		24 h.p.i.		8 h.p.i.		16 h.p.i.		24 h.p.i.	
	Gene	Ratio	Gene	Ratio	Gene	Ratio	Gene	Ratio	Gene	Ratio	Gene	Ratio
Anti-apoptosis					HSP70	2.06						
					HSP90B1	2.67	HSP90B1	0.63				
					HSPA5	2.86						
Antigen presentation/binding	CTSB	1.48										
	MHC II	0.62										
Apoptosis									BCAP29	1.82		
							DAP	0.75				
	FASL	1.68										
			IAP	3.58					IAP	2.33	IAP	2.43
	PAK2	1.60										
					PDCD1	0.64						
											TGFB2	0.66
											TGFB3	0.57
Calcium ion binding									FAM20C	0.67		
Cell adhesion							CTNNA1	0.76				
									MSLN	0.56		
	SDC4	0.65										
									SDC4	2.13		
Cell division									SGOL1	0.67		
Cell proliferation									PBEF1	2.70	PBEF1	2.25
							PCNA	0.71	PCNA	0.65		
									PDL1	3.42	PDL1	2.66
					PDPN	0.54	PDPN	0.64			PDPN	0.47
									TPX2	0.61	TPX2	0.56
Chemotaxis and cytokine activity									CCLi7	2.81		
									CCRL1	2.05		
									IL18	3.06		
Complement									C1QB	2.52		
									C1S	1.52		
	C3AR1	1.87										
Defense response	BPI	1.44										
Development									IFITM5	1.66		
Inflammatory response									MYD88	1.93		
									TLR1	1.88		
									TLR3	2.11		
Innate immune response											CMAP27	1.52
Intracellular signalling									DGKE	2.99		
									SOCS1	6.68		
Mannose binding	BSG	1.53										
Protein aa dephosphorylation											DUSP1	0.62
Protein binding			FLN29	2.46								
									FLN29	2.54	FLN29	1.82
			PHF11	3.43					PHF11	2.82	PHF11	2.60
											SH3YL1	0.64
Protein folding									DNAJA1	2.06		
					HSC70	2.38					HSC70	1.70
					HSP60	1.85					HSP60	1.59
					HSPA4L	1.75					HSPA4L	1.44
Protein modification			OASL	7.58					OASL	7.89	OASL	5.17
Proteolysis	CTSL	1.56										
			MMP2	0.54							MMP2	0.49
Respiratory burst									NCF1	2.23		
Response to DNA damage					FANCL	0.70	FANCL	0.73	FANCL	0.65		
Response to stress					HSP105	1.78					HSP105	1.63
			HSP25	3.61	HSP25	4.65						
Response to virus			IFI35	2.51					IFI35	2.81	IFI35	1.86
			ISG12-2	5.52					ISG12-2	6.64		
			MDA5	3.93					MDA5	2.78	MDA5	3.20
			MX	3.86					MX	5.79		
RNA binding											RALY	0.69
Signal transduction			LEPR	2.54					LEPR	2.84	LEPR	2.28
									LY6E	1.91		
									LY96	3.30		
											RGS18	1.88
	SIRP-B1	1.39										
	SPRY3	0.71										
	TNFRSF11B	0.66							TNFRSF11B	2.08		
Sugar binding									Galectin CG-16	0.67		
Transcription			IRF1	2.85					IRF1	2.39	IRF1	2.23
									IRF10	3.29	IRF10	2.59
									IRF2	1.61		
									IRF3	1.49		
			IRF8	1.75					IRF8	1.83		

Table 1 (*Continued*).

Functional group	1-wk-old						4-wk-old					
	8 h.p.i.		16 h.p.i.		24 h.p.i.		8 h.p.i.		16 h.p.i.		24 h.p.i.	
	Gene	Ratio	Gene	Ratio	Gene	Ratio	Gene	Ratio	Gene	Ratio	Gene	Ratio
			NMI	2.59					NMI	2.50	NMI	1.86
									STAT4	4.88	STAT4	4.25
Transport	LAPTM5	1.58										
Ubiquitin-dep catabolic process			USP18	3.20					USP18	3.07	USP18	2.14
Miscellaneous							BRI3BP	0.74				
	MHC B-G	0.31										
			TNIP3	1.85								
	TRAF3IP3	3.04										

Immune related genes significantly differentially induced after H9N2 inoculation in the lung of 1- and 4-wk-old birds at 8, 16 and 24 h.p.i. were determined using MAANOVA. A p-value < 0.05 was considered significant. Ratio represents the fold change expression rate in H9N2 inoculated birds compared to age and time matched control birds. Genes are divided into functional groups based on GO analysis using DAVID.



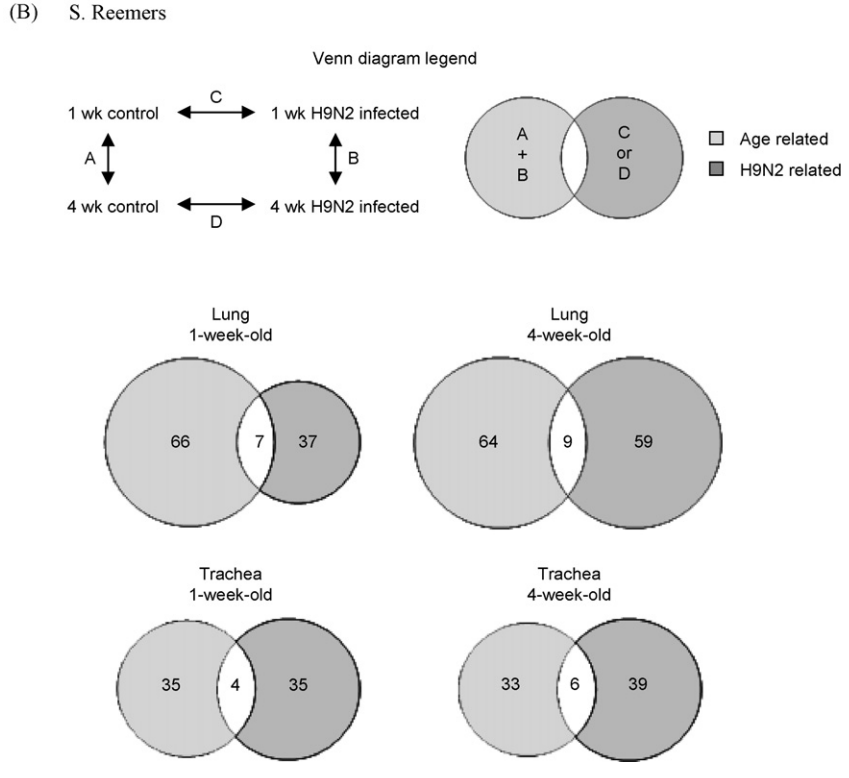


Fig. 2. (A) The number of global and immune related genes significantly differentially expressed after H9N2 inoculation in the lung and trachea. Gene expression of H9N2 inoculated birds were compared to age and time matched control birds ($n = 4$) using microarray analysis and significance was determined with MAANOVA ($p < 0.05$). (B) Overlap between age related and H9N2 related gene expression in the trachea and lung of both age groups depicted in venn diagrams. The age related gene set consists of genes significantly differentially expressed between 1- and 4-wk-old birds within a time point and treatment group in the lung and trachea (comparison A + B; $n = 4$). The H9N2 related gene set consists of genes significantly differentially expressed between control and H9N2 inoculated birds within a time point and age group in the lung and trachea (comparison C or D; $n = 4$). Gene sets were obtained with microarray analysis and significance was determined using MAANOVA ($p < 0.05$).

Table 2
Immune related genes induced after H9N2 inoculation in the trachea of 1- and 4-wk-old birds at 8, 16 and 24 h.p.i.

Functional group	1-wk-old						4-wk-old					
	8 h.p.i		16 h.p.i.		24 h.p.i.		8 h.p.i		16 h.p.i.		24 h.p.i.	
	Gene	Ratio	Gene	Ratio	Gene	Ratio	Gene	Ratio	Gene	Ratio	Gene	Ratio
Activation of MAPK activity											C1QTNF2	0.58
Anti-apoptosis					HSP70	2.26					HSP70	2.05
					HSP90B1	1.70						
					HSPA5	2.34					HSPA5	2.89
Apoptosis											BCL2A1	1.81
			CIDEA	0.51								
	TRAIL	1.70										
B cell marker											Bu-1	1.50
Calcium ion binding											PCP4	0.69
											PRVT	0.25
Cell adhesion											CD34	0.60
	CD47	1.34										
			CDH28	0.57								
			LGALS3BP	1.70								
											SDC4	1.83
											TINAG	0.55
Cell proliferation	BTG1	1.34										
	PBEF1	1.56									PBEF1	2.26
											PDPN	0.61
Chemotaxis and cytokine activity			CCLi7	8.07							CCLi7	2.45
					CMTM3	0.61						
											TRAIL-like	0.67
Complement											C1S	1.58
Defense response							LYG	2.35				
Development			IFITM5	2.95								
Inflammatory response											LY86	0.59
			MYD88	1.93								
			TLR3	1.92								
Intracellular signalling							ASB9	0.67			ASB9	0.69
Lysozome organisation	PSAP	1.40										
Miscellaneous											IGSF3	0.55
Protein binding			PHF11	2.71							PHF11	2.41
Protein folding											DNAJA1	1.83
											DNAJB9	1.33
					HSC70	2.04					HSC70	2.10
											HSP60	1.76
											HSPA4L	1.63
Protein modification			OASL	7.88								
Respiratory burst			NCF1	1.82								
Response to stress					HSP105	1.61					HSP105	2.24
											HSP25	3.22
											RPS6KA	1.61
Response to virus			IFI35	2.25								
			IRF3	1.43								
			MDA5	3.55							MDA5	2.70
			MX	3.26								
Signal transduction					ASB2	0.63						
			LEPR	1.99							LEPR	1.69
			LY6E	2.36								
							MARCO	1.59				
											SPRY2	0.71
Sugar binding	CD69	1.69										
T cell proliferation					PDL2	0.64						
											TIMD4	1.37
Transcription											IRF1	2.64
											IRF10	2.29
											NARG1	1.54
			NMI	2.50								
							SOCS3	2.69			SOCS3	4.51
			STAT4	4.20							STAT4	3.35
Ubiquitin-dep catabolic process			USP18	3.32								
Vesicle trafficking											CLEC3B	0.57
Miscellaneous											IGSF3	0.55

Immune related genes significantly differentially induced after H9N2 inoculation in the trachea of 1- and 4-wk-old birds at 8, 16 and 24 h.p.i. were determined using MAANOVA. A p-value < 0.05 was considered significant. Ratio represents the fold change expression rate in H9N2 inoculated compared to age and time matched control birds. Genes are divided into functional groups based on GO analysis using DAVID.

Immune related genes significantly differentially expressed after H9N2 inoculation were depicted in Table 1 for the lung and Table 2 for the trachea. Since basal gene expression levels differed between 1- and 4-wk-old birds in both the lung and trachea, a direct comparison between host responses to H9N2 inoculation could not be performed. Instead we performed an indirect comparison by comparing both gene lists. However, the comparison of gene lists obtained from gene expression in H9N2 inoculated birds and age

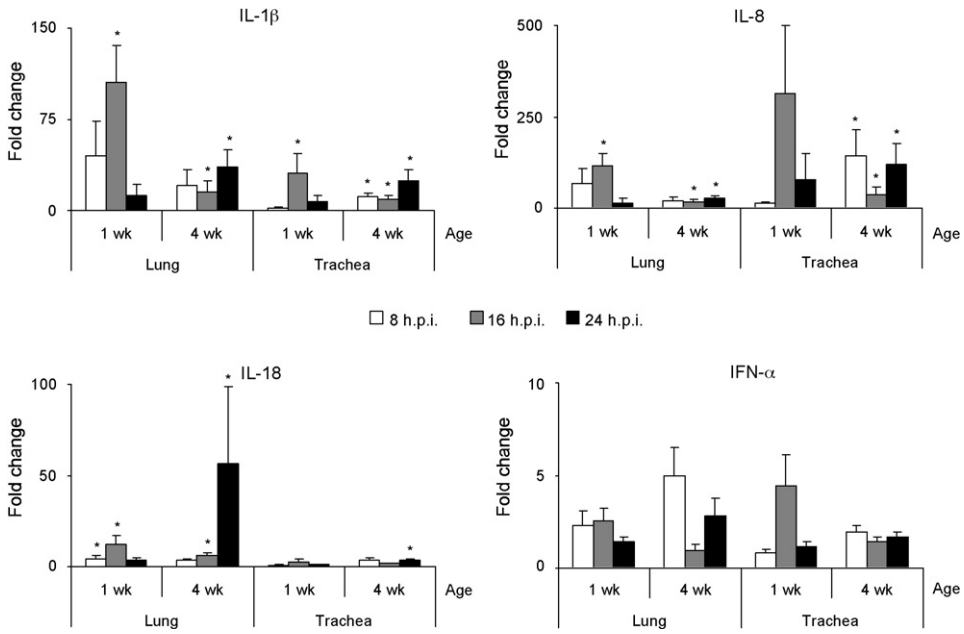


Fig. 3. Cytokine mRNA expression in the lung and trachea of 1- and 4-wk-old birds in response to H9N2 inoculation. IL-1β, IL-8, IL-18 and IFN-α mRNA expression was depicted as fold change in H9N2 inoculated compared to time and age matched control birds in the lung and trachea. Cytokine mRNA expression was determined using qRT-PCR ($n = 4$) and data were expressed as means with standard error of the mean (SEM) with asterisk (*) indicating a significant difference ($p < 0.05$) in cytokine expression between H9N2 inoculated birds and age and time matched control birds.

matched control birds could still contain genes that are differentially expressed due to age and not to virus inoculation. To distinguish between gene expression induced by the virus and gene expression only resulting from aging of the birds we made venn diagrams (Fig. 2B). Within a diagram one circle contains genes from the comparison of gene expression in H9N2 inoculated birds to the age matched control birds; H9N2 related genes in 1-wk-old or in 4-wk-old birds. The other circle contains genes from the comparison of gene expression in 1–4-wk-old control birds, combined with the comparison of gene expression in 1–4-wk-old H9N2 inoculated birds; age related genes linked to development and age related genes after virus inoculation. The overlap between H9N2 related and age related gene expression in the venn diagram was analyzed, and this overlap indicated the genes that pollute the true H9N2 related genes since they were differentially expressed between the age groups independent of H9N2 inoculation. There were few genes found in the overlap between H9N2 related and age related gene expression in both age groups and tissues. No genes were shared between the overlaps in the trachea and lung. The overlaps in 1- and 4-wk-old birds shared only 2 genes (PDPN, HSPA4L) in the lung and 1 gene (STAT4) in the trachea. Genes within the overlaps were not part of similar functional groups. Thus differences found in host responses after H9N2 inoculation between 1- and 4-wk-old birds were more related to H9N2 inoculation and hardly affected by differences in age related genes expression in control birds.

Therefore an indirect comparison of host responses between the age groups by comparing the lists of genes significantly differentially expressed after H9N2 inoculation could be performed (Tables 1 and 2). In both the lung and trachea no large differences in gene expression rates between both age groups were found, only more genes were significantly differentially expressed and relatively more genes were down regulated in 4-wk-old birds after H9N2 inoculation. Furthermore, more genes involved in innate response like complement and Toll-like receptors (TLRs) were induced in the lung of 4-wk-old birds after H9N2 inoculation. In contrast, in control birds more genes involved in innate response were expressed in 1-wk-old birds. The difference in number of genes induced after H9N2 inoculation between the age groups is larger in the lung than in the trachea. In the lung, functional groups containing most genes in both infected groups were apoptosis, response to virus, transcription, signal transduction and protein folding. Functional groups containing more than 1 gene that were mainly expressed in 1-wk-old birds were antigen presentation/binding at 8 h.p.i. and anti-apoptosis at 24 h.p.i. Functional groups containing more than 1 gene that were mainly expressed in 4-wk-old birds were cell proliferation from 8 to 24 h.p.i., and chemotaxis and cytokine activity and inflammatory responses at 16 h.p.i.

In the trachea, functional groups containing most genes in both 1- and 4-wk-old birds were, anti-apoptosis, cell adhesion and signal transduction, but genes were expressed at different time points (Table 2). A functional group containing more than 1 gene that was mainly expressed in 1-wk-old birds was response to virus at 16 h.p.i. However, at 16 h.p.i. no genes were significantly differentially expressed after H9N2 inoculation in 4-wk-old birds, which seemed to be caused by large variation within this group. Up regulation of genes related to response to virus were seen at 16 h.p.i., but in individual birds and not consistent in the whole group. A functional group containing more than 1 gene that was mainly expressed in 4-wk-old birds was protein folding at 24 h.p.i. One should be aware that gene ontology analysis in general does not give any information about the direction in which respective processes/pathways are altered.

3.4. Cytokine mRNA expression levels

Microarray analysis suggested IL-1β, IL-8 and IL-18 mRNA expression was up regulated and IFN-α expression was not affected after H9N2 inoculation in 1- and 4-wk-old birds. Although up regulation of IL-1β, IL-8 and IL-18 genes was rarely significant because of the very strict microarray statistics that was applied, we did see an up regulation from 8 to 24 h.p.i. of up to 4-fold change compared to age matched control birds. The qRT-PCR data did show a significant up regulation in H9N2 inoculated compared to control birds for IL-1β, IL-8 and IL-18 in the lung and trachea of 1- and 4-wk-old birds at several time points (Fig. 3). In both the lung and trachea

Table 3
Significant correlations between viral RNA expression and cytokine mRNA expression in the lung and trachea of 1- and 4-wk-old birds at 8, 16 and 24 h.p.i.

Tissue	Age	Time (h.p.i.)	IL-1β		IL-8		IL-18		IFN-α	
			r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Lung	1 wk	8, 16, 24	0.95	1.92E−06	0.94	5.90E−06	0.87	2.40E−04	0.59	0.042
	4 wk	16	–		–		–		−0.96	0.034
Trachea	1 wk	8, 16, 24	0.58	0.047	0.65	0.023	–		0.61	0.036
	4 wk	24	0.99	0.014	0.97	0.034	–		–	

Correlations between viral RNA and cytokine mRNA expression were based on the Pearson correlation coefficient (*r*) and determined using SPSS 15.0 software. A *p*-value for 2-tailed significance (*p*) < 0.05 was considered significant.

IFN-α mRNA expression was not significantly different between H9N2 inoculated and control birds in both qRT-PCR and microarray data. The difference in significances between microarray and qRT-PCR data are caused by differences in the statistics that were used. Based on qRT-PCR and microarray analysis, IL-1β, IL-8 and IL-18 mRNA expression in the lung and trachea was up regulated in a pattern similar over time (data not shown). mRNA expression in 1-wk-old birds peaked at 16 h.p.i., whereas it peaked in 4-wk-old birds at 24 h.p.i.

To determine whether mRNA expression was related to viral RNA expression, correlation coefficients were calculated between viral RNA expression and cytokine mRNA expression in both the lung and trachea. We first determined this correlation for all cytokines per age group over time, because if a correlation between cytokine mRNA and viral RNA expression is found, this means they correlate at every time point. In 1-wk-old birds there was a significant strong positive correlation between viral RNA expression and mRNA expression of IL-1β, IL-8, IL-18 and IFN-α in the lung and mRNA expression of IL-1β, IL-8 and IFN-α in the trachea (Table 3). In 4-wk-old birds there was no correlation between viral RNA expression and expression of these cytokines mRNAs over time in both the lung and trachea. Therefore we determined correlations within a time point. In 4-wk-old birds there was a significant strong positive correlation in the trachea at 24 h.p.i. between viral RNA expression and IL-1β and IL-8 mRNA expression. In the lung at 16 h.p.i. a significant strong negative correlation was found for IFN-α mRNA expression (Table 3).

3.5. Host gene expression hijacking by influenza

To determine the effect of H9N2 inoculation on mRNA expression of host factors that interact with viral factors we compared their gene expression rate in H9N2 inoculated with control birds within a time point. Results were depicted in Table 4. Mini chromosome maintenance complex (MCM) related genes were down regulated after H9N2 inoculation while other genes affected by H9N2 inoculation were up regulated. MCM2 and MCM4 were down regulated after H9N2 inoculation at an early stage at 8–16 h.p.i. in the lung of both 1- and 4-wk-old birds and the trachea of 1-wk-old birds. Interferon-induced GTP-binding protein Mx (MX) was up regulated at 16 h.p.i. in the lung of 1- and 4-wk-old birds and in the trachea of 1-wk-old birds. HSP70 and HSC70 were both up regulated in a later stage after H9N2 inoculation at 24 h.p.i. in both the lung and trachea of 1- and 4-wk-old birds. The effect of H9N2 inoculation on expression of these genes was independent of age and organ. DEAD box helicase related genes DDX3, DDX18 and DDX50 were only up regulated in the trachea of 4-wk-old birds at 24 h.p.i., while in 1-wk-old birds or in the lung expression of DDX genes were not affected by H9N2 inoculation. Expression of other genes coding for host factors known to bind to viral factors and being involved in influenza virus replication like importins (or karyopherin), SFPQ/CPSF, core histones, RACK I and ERK were not significantly differentially expressed. Genes coding for other host factors that play a role in influenza virus replication like CRM1 (exportin-1), BAT1/UAP56 (DEAD box helicase), NXP-2, RanBP5, eIF-4GI, PAB II, Tat-SF1 were not annotated on this microarray.

4. Discussion

Previous studies have shown age-dependent development of resistance to infection in both mammals and birds (Hume et al., 1998; Mukiibi-Muka and Jones, 1999; Velilla et al., 2006). In mammals neonatal immune responses seem to be dominated by T helper cell type 2 (Adkins, 1999), with for example reduced numbers of dendritic cells and impaired antigen presenting cell (APC) function (Velilla et al., 2006). In birds the genetic background also plays an important role in susceptibility to pathogens like *Salmonella enterica*. However, at young age both susceptible and resistant lines were highly susceptible to infection (Beal et al., 2005). Here we describe the differences in gene expression in early host responses to AIV in the respiratory tract between 1- and 4-wk-old birds and investigated the effect of age on gene expression of cellular host factors that interact with viral factors of which some are needed for viral replication.

Before we compared virus-induced host responses between 1- and 4-wk-old birds we determined the effect of age on gene expression in control birds. Differences in gene expression between 1- and 4-wk-old control birds mainly related to tissue developmental processes and immune related functional groups. Most of these immune related genes were expressed in a higher rate in 4- compared to 1-wk-old control birds and most likely related to the higher number of leukocytes present in the respiratory tract of 4-wk-old birds. Genes expressed at higher rate in 1-wk-old birds are likely an indication for the ongoing development of the respiratory immune system, which results in differences in cellular composition of the respiratory tract. Genes involved in innate immune responses were also higher expressed in 1-wk-old control birds and may indicate that protective responses in young birds are more dependent on innate responses (Levy, 2007). In the trachea fewer genes were significantly differentially expressed between 1- and 4-wk-old control birds compared to the lung. For the trachea constitutive lymphoid tissue has not been described unlike for the lung (Kothlow and Kaspers, 2008), which would explain the lower number of differentially expressed genes between 1- and 4-wk-old control birds.

Difference in virus deposition within the respiratory tract has an influence on gene expression (Baas et al., 2006; Reemers et al., 2009b). Since no significant differences in viral RNA expression were found between 1- and 4-wk-old birds, differences in gene expression after H9N2 inoculation are caused by differences in host response between the age groups. To emphasize that these differences were not a direct effect of differences in cellular composition of the tissue alone, we showed that these differences were not affected by differences in gene expression between the age groups in control birds and therefore are directly related to H9N2 inoculation. Although genes involved in innate responses were expressed at higher rate in 1-wk-old control birds, after H9N2 inoculation genes involved in innate responses were more induced in 4-wk-old birds. In the lung, more genes related to

Table 4
Effect of H9N2 inoculation on gene expression of proteins that can interact with influenza virus in the lung and trachea of 1- and 4-wk-old birds at 8, 16 and 24 h.p.i.

Host factor	Interacting viral factor	Proposed function	Host factor chicken	Lung						Trachea						Age
				1 wk			4 wk			1 wk			4 wk			h.p.i.
				8	16	24	8	16	24	8	16	24	8	16	24	
Karyopherin-α 2-β	NP	Nuclear import RNP	KPNA2	–	–	–	–	–	–	–	–	–	–	–	–	
MxA	NP	Inhibit nuclear import RNP	MX	–	↑	–	–	↑	–	–	↑	–	–	–	–	
ERK	M1		ERK	–	–	-	–	–	–	–	–	–	–	-	–	
RACK1	M1	M1 phosphorylation	RACK1	–	–	–	–	–	–	–	–	–	–	–	–	
Hsp70	NP/RNP	Nuclear export RNP	Hsp70	–	–	↑	–	–	–	–	–	↑	––	-	↑	
Hsc70	M1	Inhibit nuclear export, RNP, M1, NP	Hsc70	––	–	↑	–	–	↑	–	–	↑	–	–	↑	
SFPQ/CPSF	NS1	Inhibits nuclear export cellular mRNAs, not viral mRNAs	CPSF4	–	–	–	–	–	–	–	–	–	–	–	–	
			CPSF5	–	–	–	–	–	–	–	–	–	–	–	–	
			CPSF6	–	–	–	–	–	–	–	–	–	–	–	–	
MCM	PA	Replication	MCM2	↓	–	–	↓	↓	–	↓	–	–	–	–	–	
			MCM3	–	–	–	–	–	–	–	–	–	–	–	–	
			MCM4	–	–	–	↓	↓	–	↓	–	–	–	–	–	
			MCM5	–	–	–	–	–	–	–	–	–	–	–	–	
			MCM6	–	––	–	–	–	–	–	–	–	–	–	–	
Histone proteins	M1, RNP	Nuclear export RNP	H2AFZ	–	–	–	–	–	–	–	–	–	–	–	–	
			H2B-VIII	–	–	–	–	–	–	–	–	–	–	–	–	
			H2B-V	–	–	–	–	–	–	–	–	–	–	–	–	
			H3-IX	–	–	–	–	–	–	–	–	–	–	–	–	
			H4-I	–	–	–	–	–	–	–	–	–	–	–	–	
DEAD box RNA helicase (DDX3, DDX5)	Polymerase complex	Nuclear export RNP	DDX3	–	–	–	–	–	–	–	–	–	–	–	↑	
			DDX18	–	–	–	–	–	–	–	–	–	–	–	↑	
			DDX50	–	–	–	–	–	–	–	–	–	–	–	↑	
			DDX[a]	–	–	–	–	–	–	–	–	–	–	–	–	

Differences in gene expression between control birds and H9N2 inoculated birds of proteins that can interact with influenza virus were identified using MAANOVA within time and age in the lung and trachea. A p-value < 0.05 was considered significant. Significant differential expression in H9N2 inoculated compared to control birds is indicated with (↑) for up regulation and (↓) for down regulation. Genes expression that did not differ significantly after H9N2 inoculation was indicated with (–). DDX[a] stands for gene DDX10, DDX24, DDX25, DDX26, DDX29, DDX31, DDX32, DDX41, DDX42, DDX46, DDX55, and DDX59.

cell proliferation, chemotaxis and cytokine activity, inflammatory response and transcription were expressed in 4-wk-old birds. This suggests a different host response at transcriptional level in 1- compared to 4-wk-old birds likely relating to age related differences in immune responses. APC play an important role during innate immune responses. Neonatal APC from mice and humans have been reported to be less effective in supporting proliferation of T cells (Petty and Hunt, 1998) probably due to lower expression of MHCI, MHCII and costimulatory molecules (Hunt et al., 1994). Furthermore, they are defective in cytokine production upon LPS stimulation or influenza virus infection (Chelvarajan et al., 2004; Zhou et al., 2006) and require a higher level of activation than adult APC (Petty and Hunt, 1998), which has been proposed to be due to defects in TLR signalling (De Wit et al., 2003; Velilla et al., 2006). In our study MHCI and MHCII were less expressed in 1-wk-old birds which suggest a similar immaturity of APC as found in mice. Cytokine genes and TLR signalling related genes were more expressed in 4-wk-old birds, while MHCII was down regulated in 1-wk-old birds after H9N2 inoculation likely relating to functional impairment described for mammalian APC.

In the trachea the largest and clearest differences between 1- and 4-wk-old birds were the time points of expression and the number of genes expressed, but no large differences between functional groups were found, unlike in the lung. This possibly indicates that maturity of the respiratory immune system might have less effect on the trachea than on the lung which could correlate to the lack of constitutive lymphoid tissue in the avian trachea.

Elevated expression levels of inflammatory cytokines and chemokines due to influenza infection have been reported as early as 6 h.p.i. depending on the influenza strain (Chan et al., 2005; Julkunen et al., 2001). Also in this study up regulation of IL-1β, IL-8 and IL-18 mRNA expression was found after H9N2 inoculation in both age groups. The level of viral RNA expression correlated with the mRNA expression levels of IL-1β, IL-8, IL-18 and IFN-α over time in 1-wk-old birds, but for 4-wk-old birds a correlation was only seen at later time points after H9N2 inoculation. Although the mean IFN-α mRNA expression was not significantly up regulated, there were correlations between viral RNA en IFN-α mRNA expression for individual birds at both 1- and 4-wk of age. These data indicate that induction of gene expression of inflammatory cytokines and chemokines after H9N2 inoculation is more related to viral load in 1-wk-old birds compared to 4-wk-old birds.

Influenza viral proteins bind to several mammalian host proteins which promote viral replication or induce host responses (Engelhardt and Fodor, 2006; Naffakh et al., 2008; Nagata et al., 2008). MCM2 and MCM4 are part of the minichromosome maintenance complex which is proposed to activate virus genome replication at the early phase of infection when there is no newly synthesised viral nucleoprotein (NP) present (Nagata et al., 2008). These genes were down regulated early after H9N2 inoculation which may be an attempt of the host to block viral replication. At 16 h.p.i. blocking of viral replication is possibly enhanced by up regulation of MX, which for humans binds to viral NP and this binding likely prevents nuclear import of incoming viral ribonucleoprotein (RNP) (Naffakh et al., 2008; Turan et al., 2004). In the chicken some MX proteins are found to have a similar inhibitory function while others do not depend on breed and virus strain, for which a possible explanation is the high polymorphism in the chicken MX gene (Benfield et al., 2008; Ko et al., 2002). For chickens HSP70 and HSC70 were up regulated at 24 h.p.i. possibly preventing assembly of new virions, since HSP70 prevents binding of viral matrix protein M1 to RNP (Hirayama et al., 2004) and HSC70 binds to viral M1 (Nagata et al., 2008) resulting in inhibition of nuclear export. A common response of the host to block viral replication seemed to occur, because gene expression of MCMs, MX, HSP70 and HSC70 was regulated independent of age and tissue. Interestingly DDX3, DDX18 and DDX50 were only up regulated in the trachea of 4-wk-old birds at 24 h.p.i. when newly assembled RNPs are exported to be packaged into progeny virions. The influenza polymerase complex is known to interact with DDX3, which plays an important role in RNA nuclear export and cytoplasmic mRNA localisation (Jorba et al., 2008), promotes export of HIV-1 RNAs from the nucleus to cytoplasm (Ishaq et al., 2008) and is required for HCV RNA replication (Ariumi et al., 2007). This possibly indicate that DDX is may be needed for influenza virus replication at a later stage after virus inoculation when newly assembled RNPs are exported to be packaged into progeny virions and expression is possibly age and tissue related.

In summary, gene expression in control birds and host responses to AIV inoculation in the trachea and especially the lung indicates correlation with the development and maturation of the respiratory immune system. Differences in immune related gene expression after H9N2 inoculation in the lung likely related to the higher levels of stimulation needed to activate neonatal host responses and age-dependent functionality of leukocytes. However, expression of most cellular host factors that block viral replication by interacting with viral factors is independent of age. These findings suggest that the strength of virus-induced host responses is affected by maturation of the respiratory immune system and may be a key factor in age-dependent host responses to infection. However, the differences found at transcriptional level were not yet translated to differences in viral load between the age groups, due to the time frame in which we measured the responses. This study shows multiple factors could be involved in neonatal impaired response, such as functional impairment APC, NK cells and T cells and more research into the contribution of these factors is needed to get a better understanding of the functional capability of the neonatal immune system and the relation to susceptibility.

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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.molimm.2010.03.008.

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