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Molecular immunophenotyping of lungs and spleens in naive and vaccinated chickens early after pulmonary avian influenza A (H9N2) virus infection

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Abstract

In a respiratory-infection-model with the avian influenza A H9N2 virus we studied lung and splenic immune reactions in chickens using a recently developed 5K chicken immuno-microarray. Groups of chickens were either mock-immunized (referred to as non-immune), vaccinated with inactivated viral antigen only (immune) or with viral antigen in a water-in-oil (W/O) immunopotentiator (immune potentiated). Three weeks after vaccination all animals were given a respiratory infection. Immune potentiated birds developed inhibitory antiviral antibodies, showed minimal lung histopathology and no detectable viral sequences, while non-immune animals showed microscopic immunopathology and detectable virus. Immune birds, receiving antigen in saline only, showed minimal microscopic histopathology, and intermediate levels of virus detection. These classical features in the different groups were mirrored by overlapping or specific mRNA gene expression profiles in lungs and spleen using microarray analysis. To our knowledge this is the first study demonstrating pneumonia-associated lung pathology of the low pathogenic avian influenza H9N2 virus. Our data provide insights into the molecular interaction of this virus with its natural host when naive or primed by vaccination.

Keywords: Microarray; Chicken; Avian influenza H9N2

1. Introduction

Avian influenza (AI) infections are carefully monitored because of their negative effects on intensive poultry rearing systems, but also in view of occasional diseases in humans and fear for pandemic spreading. Our understanding of *in vivo* influenza pathogenesis and host immunity is limited, despite considerable experimental knowledge generated mainly in mouse and ferret models. As chickens are natural hosts for respiratory influenza infections, understanding influenza immunity in its host is not only an important issue in the poultry industry, but may also provide key insights into immune pathways required for prophylactic or therapeutic protection of other influenza hosts, and possibly even against other respiratory viruses.

Antiviral protection generally requires an orchestration of innate, and if necessary, adaptive immune cells. The tools to study immunological responses in chicken are limited when compared to those available in the mouse and human system. Recently, we and others have identified and cloned the genes that encode novel immunoregulatory response modifiers of the chicken [1–4] which, in general, show limited sequence homology to known mammalian genes**.** In addition, numerous chicken gene sequences are now available through the recently assembled chicken genome [5]. These

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sequence data allow analysis at the transcriptional level of potential immunorelevant molecules, although the effect of posttranslational gene expression cannot be measured and secure functional annotation requires confirmation in future investigations.

For the chicken study described in this paper we used the avian influenza A subtype H9N2 (isolate A/Chicken/United Arab Emirates/99). This serotype is pathotyped as low pathogenic, according to the OIE classification and certified by CVL Weybridge, but is able to produce a severe infection in the context of opportunistic secondary pathogens, such as infectious bronchitis virus (IBV) or *Mycoplasma gallisepticum* [6]. On the other hand, it was shown that H9N2 viruses evoked cross-protective CTL-based cellular immunity in chickens against a highly pathogenic H5N1 infection [7,8]. Influenza A virus is a member of the *Orthomyxoviridae* family of segmented, negative-stranded RNA viruses. Avian influenza A subtype H9N2 viruses can transmit to mammalian species, including humans [9,10]. Its transmission to humans, combined with genetic reassortment or mutation, raised anxiety for a potential pandemic spread in the human population [11,12].

Apart from classical assays, such as histopathology and antiviral antibody titers, we analyzed gene expression profiles, using a newly designed chicken immune-microarray [13]. With this cDNA microarray we studied host gene expression in the lungs and spleen of non-immune (mock-immunized), immune (immunized with inactivated H9N2 antigen in saline), and immune potentiated (immunized with inactivated H9N2 in a water-in-oil emulsion) chickens undergoing a primary respiratory infection with live H9N2. We questioned whether lung pathology and immunity-associated gene pathways could be identified in non-immune birds and whether they (partially) overlap with those in primed birds. We also aimed to identify primed memory immune pathways in immune and immune potentiated animals and questioned whether they differ from natural immune pathways in infected non-immune birds. In addition we tested whether viral replication was affected by immunization.

From our study we conclude that immune potentiated birds developed inhibitory antiviral antibodies, showed minimal lung histopathology and no detectable viral sequences, while non-immune animals showed microscopic immunopathology and detectable virus. Immune birds, receiving antigen in saline only, showed minimal overall microscopic histopathology, and intermediate levels of virus detection. These classical features were compared with host mRNA gene expression profiles in lungs and spleen using microarray analysis.

2. Materials and methods

2.1. Animals

Three-week-old chickens (S5 inbred broiler type) were derived from Hybro/Euribrid (Boxmeer, the Netherlands) and housed under SPF conditions. The animals received food and water ad libitum. In compliance with the Dutch law, all experiments were carried out according to protocols approved by the Intervet Animal Welfare Committee.

2.2. H9N2 virus and antigen preparation

Avian influenza A subtype H9N2 virus (isolate A/Chicken/United Arab Emirates/99) was produced in eggs using routine procedures. For the experimental vaccine formulation the aqueous phase containing the virus suspension, diluted in 0.01 M phosphate buffer (pH 7.2), was formalin-inactivated. This formalin-inactivated antigen preparation was formulated into saline or into an immunopotentiating mineral oil-based W/O emulsion according to standard procedures using a high speed mixing device (ultra-thurrax) [14].

2.3. Experimental immunization protocol

Distinct groups ($n = 3$) of 3-week-old chickens were i.m. vaccinated in the leg muscle (see also Table 1) with either 0.25 ml saline (naive and non-immune control groups), with 0.25 ml formalin inactivated avian influenza H9N2 antigen in saline (immune group), or with inactivated avian influenza H9N2 antigen formulated in an immunopotentiating mineral oil-based W/O emulsion (45/55, v/v) (immune potentiated group). Blood samples were taken at 0 and 3 weeks post-vaccination. At 3 weeks post-vaccination all groups, except the naive control group, were infected (challenged) via the oculo-nasal plus intratracheal route with a total dose of 10e8.8 ED50 H9N2 virus (i.e. 0.1 ml/eye, 0.1 ml/nostril, 0.2 ml into the trachea). At 1 and 5 days post-infection chickens were sacrificed and the lungs and spleens were isolated for histopathology and RNA isolation (see also Fig. 1 for a time scheme).

2.4. HI-assay for antiviral H9-antibodies

Serum levels of H9-specific antibodies were determined by a haemagglutination inhibition (HI) assay. Serial two-fold serum dilutions were prepared in microtiter plates and mixed with an equal volume containing eight haemagglutinating units/50 μl H9N2 antigen. Titers are expressed as the reciprocal of the highest dilution that gives complete inhibition of haemagglutination of chicken red blood cells (1%, v/v in buffered saline). Samples were regarded positive for inhibition of haemagglutination at a dilution ≥1:2.

Table 1
Set up of the experimental vaccination and challenge

Group	Antigen	Adjuvant	Challenge	Sacrifice date
Naive control	–	Saline	No	1 d
Non-immune control	–	Saline	Yes	1 d and 5 d p.i.
Immune	H9N2	Saline	Yes	1 d and 5 d p.i.
Immune potentiated	H9N2	W/O	Yes	1 d and 5 d p.i.

d = day(s); p.i. = post-infection (challenge).

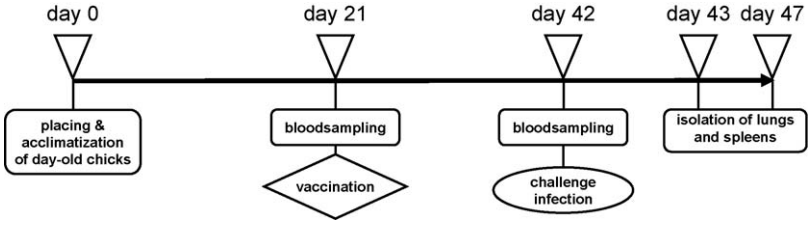

Fig. 1. Time line of the chicken study. After challenge infection, one half of the birds had a 1-day endpoint (day 43), the other half a 5-day endpoint (day 47).

2.5. Histopathology

Histopathological evaluation was performed by a certified and well-trained pathologist on haematoxylin–eosin (HE)-stained 5 μm paraffin sections of formalin-fixed lung tissue fragments dissected at 1 and 5 days post-infection from naive, non-immune, immune and immune potentiated chickens.

2.6. RNA isolation

Total RNA was isolated from lung and spleen samples using a Trizol extraction method as described by the manufacturer (Invitrogen Life Technologies, Paisley, UK) and subsequently purified using the RNeasy Midi RNA Purification kit (Qiagen Ltd., Crawley, UK). The RNA concentration was determined spectrophotometrically and its quality by using the Agilent 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). RNA was treated with DNaseI as described by the manufacturer (Ambion Ltd., Huntingdon, Cambridgeshire, United Kingdom).

2.7. Real-time quantitative RT-PCR

Presence of AI-H9 viral RNA in the lungs of naive and infected chickens was quantitated using real-time quantitative RT-PCR (Q-RT-PCR). Primer sequences for H9 and chicken GAPDH are depicted in Table 2. GAPDH is being used as the housekeeping control gene as this molecule proved to be more stable than β-actine (data not shown). Eight micrograms of total RNA was reverse-transcribed into cDNA using the Superscript II RT protocol (Invitrogen Life Technologies) in a 30-μl reaction. After dilution of the reaction mixture with 15 μl H_2O, 2 μl of template cDNA was used for each Q-PCR, which was performed in single wells of a 96-well plate (ABgene, Epsom, Surrey, United Kingdom) in a 50 μl reaction volume. The 50 μl reaction mixture contained 28.5 μl H_2O, 5.0 μl iCycler mixture (without Triton and $MgCl_2$), 5.0 μl 25 mM $MgCl_2$, 4.0 μl 2 mM dNTPs, 2.0 μl of a 10 μM forward and reverse primer mix, 2.5 μl 20× EvaGreen dye (Biotium Inc., Hayward, CA) and 1.0 μl 5 U/μl Supertaq (HT Biotechnology). Amplification and detection of specific products were performed using the iCycler (BioRad, Hercules, CA) with the following cycle profile: 1 cycle of 95 °C for 5 min, and 40 cycles of [92 °C for 10 s, 55 °C for 10 s, 72 °C for 30 s] and 1 min 72 °C for a final extension. Fluorescence was measured following each cycle and displayed graphically (iCycler iQ Multicolor Real-Time Detection System, Optical System Software, version 3.1, BioRad, Hercules, CA). The software determined a cycle threshold (C_t) value, which identified the first cycle detecting the fluorescence above the baseline for that sample or standard.

Table 2
Real time quantitative RT-PCR primers

RNA target		Primer sequence (5′–3′)	Accession No.
GAPDH	F	GTGGTGCTAAGCGTGTTATC	K01458
	R	GCATGGACAGTGGTCATAAG	
AI-H9	F	GGTCAGACATTGCGAGTAAG	AF461530
	R	CTAGCAGGCACATTCCTCAG	

The GAPDH and H9 PCR-fragments were also cloned and used in combination with the Q-RT-PCR primers to calculate the slopes from $\log_{10}$ dilution series regression lines. To correct for differences between template RNA levels between samples within an experiment, first the difference factor (DF) (normalization factor) for each sample was calculated by dividing the mean C_t value for GAPDH-specific product of a sample by the mean C_t value for GAPDH-specific product for all samples. The corrected H9 RNA per sample was calculated using the following formula [15]:

$$\frac{(40 - \text{mean H9 } C_t \text{ sample}) \times \text{H9 slope}}{\text{DF sample} \times \text{GAPDH slope}}$$

Results are expressed as standardized 40-C_t values.

2.8. cDNA microarray

Construction of the cDNA libraries, EST sequence analysis, construction of the microarray containing 5007 elements (in duplicate), RNA labeling, hybridizations and data extraction were all performed as described in the paper by Smith et al. [13]. For the hybridizations a reference design approach was used in which all samples from all treatments were pooled and compared with individual treatment samples. The reference and treatment samples were labeled using the dye flip approach, i.e. the reference and treatment samples were cross-labeled and hybridized to two arrays.

2.9. Data analysis

Data was extracted from individual slides using the Bluefuse software (BlueGnome, Cambridge, UK) after which

features with poor confidence information (confidence <0.30) were eliminated from the analysis. Background-corrected signal values were imported into the VectorNTI Xpression 3.1 software (Invitrogen Life Technologies, Paisley, UK) for comprehensive analysis. M versus A calculation, where $M = \log 2$ (sample/reference) and $A = 0.5 \log 2$ (sample × reference), Lowess normalization and finally anti-log transformation of M were applied. The [sample/reference] ratios, i.e. the anti-log $2M$ values, from slides with similar treatments were pooled and compared with other treatments using the Student's t-test. Only genes with a significant ($P < 0.05$) difference between two treatments were used for further calculations. Final gene expression fold changes were calculated by dividing the [sample/reference] ratio of a gene from a treatment by the [sample/reference] ratio of that same gene in the naive group. Only ratios with a fold change ≥1.5 are shown. The raw data files can be found at [www.ebi.ac.uk/arrayexpress].

2.10. Statistical analysis

Comparison of the levels of up- or down-regulation (microarray data) was performed with the VectorNTI Xpression 3.1 software package (see above). To analyze the Q-PCR and HI-antibody data we used analysis of variance (ANOVA) with least significant difference (L.S.D.) as multiple comparison test. Differences were considered significant at a confidence level of 95% ($P < 0.05$).

3. Results

3.1. Pathology of H9N2 infection

No evidence of gross clinical pathology was noted in infected animals, similar to earlier unpublished studies with this H9N2 virus (data not shown). The lungs of naive chickens infected with H9N2, similar to those of immune or immune potentiated birds, showed little gross pathology at macroscopic inspection during necropsy (not shown). However, upon microscopic examination by a certified and well-trained pathologist, profound differences were noted in the lungs of non-immune, immune and immune potentiated infected birds at 5 days post-infection (Fig. 2).

In non-immune infected animals the lungs showed necrosis of parabronchial epithelium, edema, and fibris exudation in airways and air capillaries indicating severe pneumonia (Fig. 2B). Next to this, diffuse infiltration of lymphoid cells

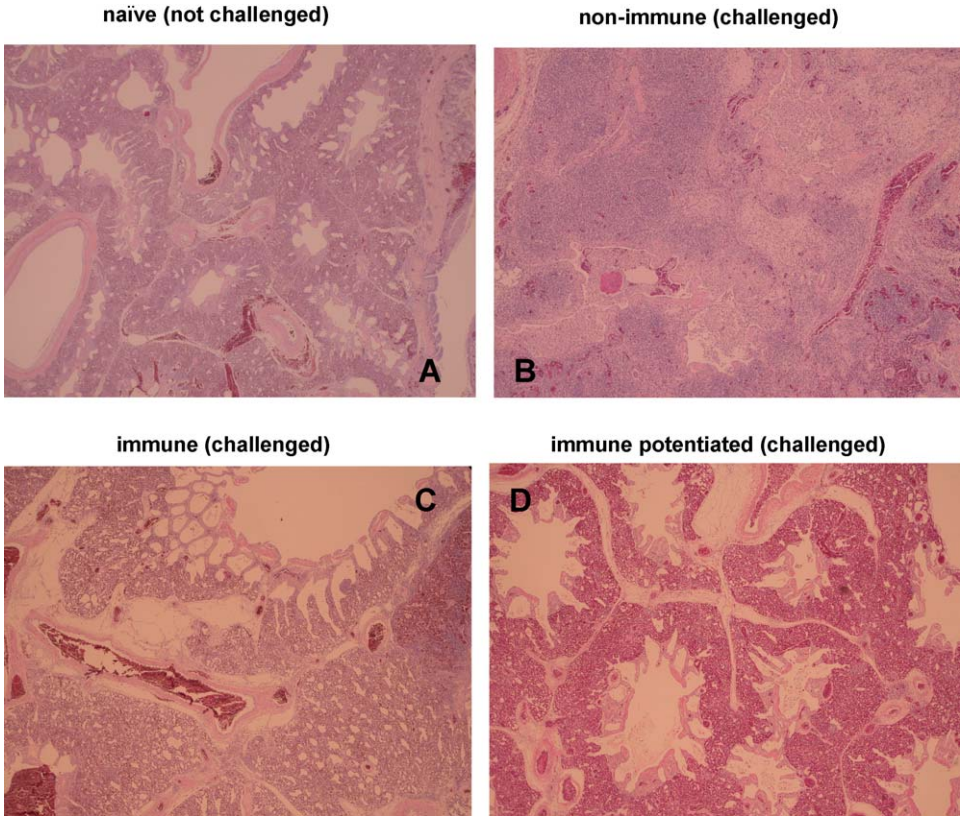

Fig. 2. Microscopic histopathology of lung tissue at day 5 after oculo-nasal-intratracheal H9N2 infection. Inoculated chickens were either naive not challenged (A), non-immune challenged (B), immunized with antigen in saline (immune) and challenged (C), or immune potentiated as a result of H9N2 antigen formulated in W/O immunopotentiator and challenged (D). Sections were stained with HE; magnification at 40×. Data representative of two experiments are shown.

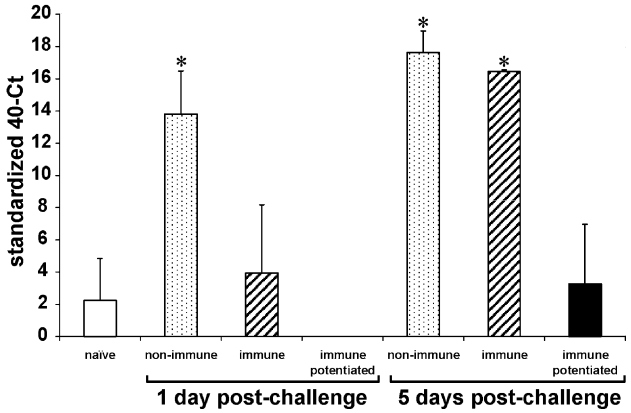

Fig. 3. Virus-specific avian influenza H9 expression levels in the lungs of chickens as measured by real time quantitative RT-PCR analysis. Results are expressed as standardized 40-C_t values corrected for variation in input RNA measured by GAPDH levels. As values are subtracted from 40, higher values represent higher levels of expression. Inoculated chickens were either naive not challenged, non-immune challenged, immunized with antigen in saline (immune) and challenged, or immune potentiated as a result of H9N2 antigen formulated in W/O immunopotentiator and challenged. Error bars are S.E.M. for three samples from each treatment group analyzed in duplo. *Significantly different ($P < 0.05$) from the naive group.

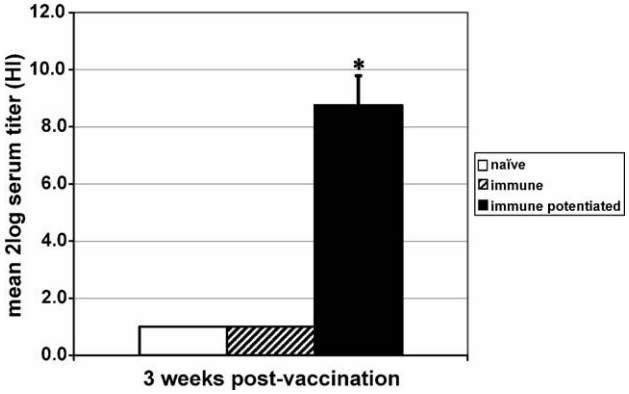

Fig. 4. H9N2 specific HI-antibody responses at 3 weeks after primary immunization. Immunized chickens were either naive (saline control), immunized with H9N2 antigen in saline only (immune), or immune potentiated as a result of H9N2 antigen formulated in W/O immunopotentiator (W/O). *, Significantly different ($P < 0.05$) from the naive and immune group.

was noticed at higher magnification (not shown). In comparison, the lungs of animals immunized before with H9N2 in saline only (immune infected birds), showed less dramatic fibrinous exudate in the airways (Fig. 2C) indicating moderate/mild pneumonia, with isolated foci of clustered immune cells (not shown). The airways of chickens immunized with H9N2 antigen formulated in an immunopotentiating W/O delivery system proved minimally affected without obstruction by fibrinous exudate (Fig. 2D). The lungs showed little recruitment of immune cells and minimal areas of lymphoid cell infiltration at higher magnification (not shown).

3.2. Detection of H9N2 virus in naive and immunized hosts

Using quantitative RT-PCR (Q-RT-PCR) employing primers specific for the H9 sequence of avian influenza we noted clear evidence of viral replication at days 1 and 5 in the lungs of non-immune infected birds (Fig. 3). The lungs from immune animals still showed H9 gene detection especially on day 5. By contrast, immune potentiated animals showed strongly reduced viral replication.

3.3. Specific antibody formation in immunized chickens

In blood samples taken 3 weeks post-vaccination, i.e. 1 day before challenge infection, H9N2-specific HI serum antibodies were measured in individual birds of all groups. Immune birds, vaccinated with H9N2 in saline, showed no detectable antibody titers, while immune potentiated animals, vaccinated with H9N2 in a W/O emulsion, showed a readily detectable serum HI titer (Fig. 4).

3.4. Immunophenotyping of host responses in lung tissue and spleen

In order to obtain more detailed information about immunity-related responses in the lungs and spleen of chickens we used microarray-assisted *ex vivo* gene expression profiling on lung and spleen tissue isolated at 1 and 5 days after oculo-nasal-intratracheal infection. For this, a reference design combined with a dye flip approach was used (see Section 2.8).

Fig. 5 gives an overview of the total amount of significantly ($P < 0.05$) changed (fold change ≥ 1.5) genes in the lungs (A–D) and spleens (E–H) between the indicated treatments relative to organs of naive uninfected birds on day 1 and day 5. In the lungs of non-immune infected birds 10.3% ($[(202 + 387) - 74]/5007 \times 100\%$) of all genes on the array show a greater than 1.5-fold regulation. This percentage drops to 9.1% and 8.3% in the immune and immune potentiated birds, respectively, suggesting that immune potentiated birds, who received antigen formulated in immunopotentiator, exhibited less immune activation to control the virus, when compared to immune and non-immune birds. For spleen we noted the same phenomena with percentages of 4.0%, 3.1% and 3.1% for non-immune, immune and immune potentiated birds, respectively. The number of genes, common in the lungs on day 1 and day 5 post-infection, ranges between 74, for the non-immune birds, and 51, for the immune potentiated birds. Remarkably, only 3 genes are common on day 1 and day 5 in the spleens of immune potentiated birds (13 for the non-immune and 17 for the immune animals). In general, when comparing the gene expression regulation in lung and spleen it is obvious that the spleen is relatively quiet. Subsequently, detailed analysis focussed on genes with an available well-known (putative) immunological annotation, which showed significant ($P < 0.05$) regulation (fold change ≥ 1.5).

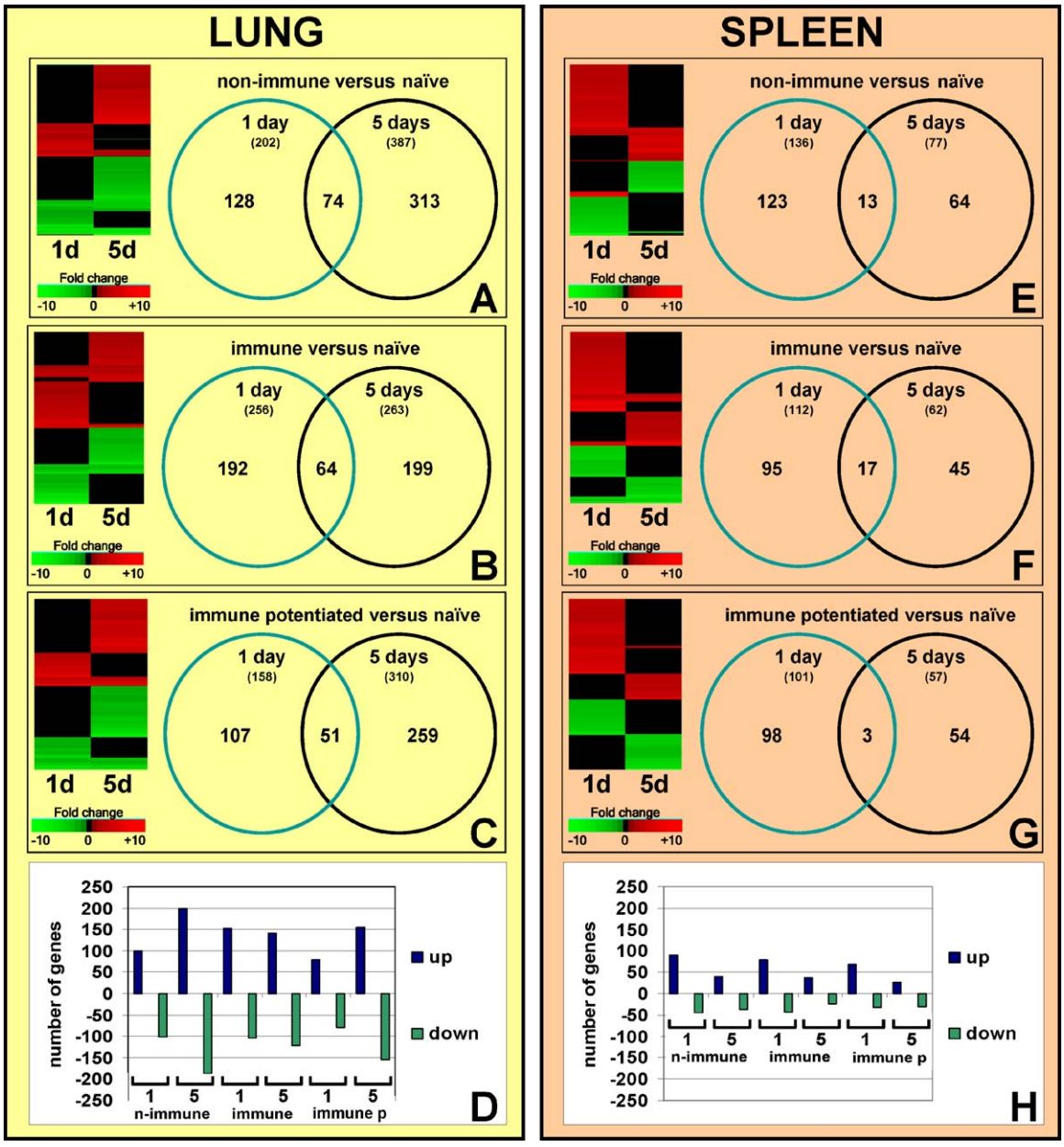

Fig. 5. Analysis of host responses to avian influenza H9N2. Patterns of gene expression in whole lungs and spleens of non-immune (n-immune), immune and immune potentiated (immune p) chickens at 1 or 5 days post-infection with H9N2 influenza virus, via the oculo-nasal and intratracheal route, are shown. (A–C, E–G) Venn diagrams show overlap of genes with $\geq$1.5-fold ($P < 0.05$) regulation. The heat maps were made using an unweighted average hierarchical clustering algorithm and the Euclidean distance matrix. Genes shown in red were up-regulated, while genes shown in green were down-regulated. (D and H) Total numbers of up- or down-regulated genes are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.5. Microarray-assisted immunogenomic analysis

Using the reference design approach, we examined significant gene expression differences of non-immune infected, immune infected and immune potentiated infected animals when compared to naive uninfected (saline) hosts. Conspicuous genes in the lungs and spleen, with a well-known immunological annotation, are depicted in Tables 3 and 4, respectively. Measured gene responses were divided into those associated with known innate immune pathways and genes involved in adaptive immune pathways. As the functional annotation of chicken genes lags dramatically behind similar work achieved in mammals (i.e. mainly mice), we can only assume that most of the chicken genes, which are printed on the micro-array, function in a similar manner as the mammalian molecules. As the chicken genome was released just recently [5], it may take years before all putative orthologues have been characterized and functionalized. The critical reader should therefore be aware of the fact that if an annotation suggests that a chicken gene is an

Table 3
Regulation of well-known immunologically relevant genes in lungs of AI-H9N2 infected chickens

Gene	Accession number	Non-immune vs. naive		Immune vs. naive		Immune potentiated vs. naive	
		1 d	5 d	1 d	5 d	1 d	5 d
Innate immune pathway							
Chemokines and receptors							
Chemokine receptor-like 2 protein	BU444213	1.7 (0.016)	1.8 (0.047)	1.7 (0.036)			2.4 (0.014)
Chemokine AH221	AM064266		2.6 (0.0001)		1.9 (0.002)		
Chemokine AH294 (RANTES)	BU397782		2.6 (0.049)				
Chemokine-like factor	AM066499			−1.6 (0.036)			
CXCL12 (chemokine)	AM064247					2.3 (0.017)	
SCYa4 (chemokine)	BU299262	−2.2 (0.048)					
SCYa13 (TNF inducible gene)/MCP-4 chemokine	BU397023	3.0 (0.019)		3.5 (0.025)			
Macrophage/myeloid cells							
MARCO (scavenger receptor)	AM064405	2.6 (0.048)		1.7 (0.043)		3.2 (0.026)	2.1 (0.038)
MIF (macrophage migration inhibitory factor)	BU438017	−1.5 (0.048)			−1.9 (0.047)		
MCSF-R (macrophage colony stimulating factor) receptor	BU432910				1.9 (0.016)		
NRAMP2 (natural resistance-assoc. macrophage protein)	BU203948				−1.8 (0.003)		
Toll-pathway							
TLR2 (Tol-like receptor 2) type 1	BU374739	2.9 (0.042)	10.3 (0.050)	4.6 (0.002)	5.5 (0.002)	3.9 (0.024)	
TLR4 (Tol-like receptor 4)	BU475859	−1.8 (0.048)					
NF-κB1 nuclear factor	AM063779	−1.6 (0.016)	−1.8 (0.001)	−1.6 (0.031)		−1.8 (0.011)	
IFN-pathway							
IFP35 (IFN-induced 35 kDa protein)	BU135331	2.3 (0.005)		2.5 (0.009)			
IRF3 (IFN-regulatory factor 3)	AM070266		−1.7 (0.029)				
IRF4 (IFN-regulatory factor 4)	AM072251	2.5 (0.048)	2.6 (0.026)	2.7 (0.036)	3.5 (0.008)		2.7 (0.016)
IRF5 (IFN-regulatory factor 5)	AI981854						−2.9 (0.048)
IRF8 (IFN-regulatory factor 8)	AM065010			2.4 (0.036)	1.9 (0.012)	1.7 (0.018)	
Heat shock proteins (HSPs)							
Hsp70	AM070074		6.1 (0.008)	4.5 (0.008)		5.5 (0.042)	3.3 (0.027)
Hsp70 protein 12B	AM065354			−1.6 (0.009)			
Hsc70.II (heat shock cognate 70.II protein)	AM069279	1.8 (0.024)					1.8 (0.024)
Hsp90α	AM068283					1.7 (0.044)	1.6 (0.034)
Complement factors							
Complement C3	AM067441		1.8 (0.027)	1.9 (0.026)		1.6 (0.037)	
Complement inhibitory factor H	AM063703	−1.6 (0.048)					
Pro-inflammatory cytokines							
IL-6	AI982185	2.2 (0.009)		2.1 (0.002)			
Proteases							
Granzyme M (NK cell granular protease)	AM071672	2.0 (0.023)		2.0 (0.027)	1.8 (0.041)	2.7 (0.005)	
Adaptive immune pathway							
Antigen presentation							
Cathepsin S	AM067017	2.7 (0.037)		2.1 (0.042)			
Class I alpha	AM068770		3.4 (0.003)		2.3 (0.038)		2.4 (0.002)
Class I B–F heavy	AM067119		2.6 (0.026)				1.7 (0.008)
Class I minor	AM068728		2.0 (0.019)				
Class II B–LBII-beta	AM068054					−1.6 (0.029)	
Cytokines and receptors							
Cytokine receptor common beta chain precursor	AM066518	−1.5 (0.048)			−1.8 (0.035)		−1.6 (0.043)
IL-2Rb	AM064890				1.6 (0.002)		
IL-4R	BU324362			−1.5 (0.021)			
IL-13R2	BU341330			2.9 (0.009)	3.7 (0.013)		

Table 3 (*Continued*)

Gene	Accession number	Non-immune vs. naive		Immune vs. naive		Immune potentiated vs. naive	
		1 d	5 d	1 d	5 d	1 d	5 d
IL-20Ralpha	BU241765			−2.2 (0.036)			−2.0 (0.048)
IFN-γR2	BU294744			1.9 (0.015)			
IL-15	BU202444				−2.0 (0.019)		
IK cytokine	AM070735				−1.9 (0.017)		
CD and T cell markers							
CD2 (expression: T cells, thymocytes, NK cells)	AM068706		−1.7 (0.029)				
CD200 (expression: DCs, activated T cells)	AI981679					1.7 (0.006)	
CD3 (expression: thymocytes, T cells)	AM070515	1.5 (0.038)	2.3 (0.24)	1.7 (0.013)	2.7 (0.00006)		1.8 (0.004)
CD4 (expression: Th cells, monocytes, macrophages)	AM075583			2.3 (0.012)			
CD5 (expression: thymocytes, T cells, subset of B cells)	AM071524		2.4 (0.013)		2.4 (0.001)		2.4 (0.012)
CD8 (expression: subset thymocytes, cytotoxic T cells)	AM069615			2.2 (0.032)			
TCRzeta (T cell receptor zeta)	AM069278				2.0 (0.029)		
Transcription factors							
GATA-3 (Th2 transcription factor)	AM064179	2.0 (0.036)		2.1 (0.003)			
Cystatin F (cytokine-like nuclear factor)	AM070770					2.0 (0.034)	
Cytokine signaling suppressors							
SOCS1(suppressor of cytokine signaling 1)	BU218362	−1.6 (0.048)					
SOCS5 (suppressor of cytokine signaling 5)	BU326390	1.7 (0.038)					
B cell							
Ig J chain	AM064723		3.3 (0.048)	3.7 (0.036)	12.2 (0.011)	3.4 (0.036)	5.2 (0.009)
Ig mu chain C region	AM067797		2.2 (0.050)		2.2 (0.024)		
Ig light chain VJC region	AM064528		2.5 (0.003)		2.8 (0.005)		2.5 (0.001)
Ig lambda chain C region	AM072032		2.5 (0.002)		2.2 (0.01)		2.3 (0.024)
Miscelaneous							
Apoptosis							
anti-apoptotic NR13	AM071765				1.5 (0.037)		
Death receptor 6 (mediates apoptosis; TNF family member)	AM066244		2.0 (0.028)	2.1 (0.039)			
FasL decoy receptor 3 (inhibits FasL/TNF apoptosis)	AM064070			1.5 (0.028)			
TNF-related							
TRAF1 (TNF receptor-associated factor 1)	AI982046		1.5 (0.021)				
TRAF2 (TNF receptor-associated factor 2)	BU455745			1.8 (0.009)			
TRAF5 (TNF receptor-associated factor 5)	BU455745			2.1 (0.029)	2.1 (0.031)		

Regulation (≥1.5-fold; $P < 0.05$) of well-known immunologically relevant genes in the lungs of non-immune, immune or immune potentiated chickens at 1 or 5 days post-infection with avian influenza H9N2 when compared to naive control birds. *P* values are between parentheses.

orthologue it might not function in a similar manner as in mammals.

3.5.1. Innate immune responses

3.5.1.1. Chemokines and receptors. Chemotactic migration of leukocytes largely depends on adhesive interactions with the substratum and recognition of a chemoattractant gradient provoked by chemokines. Increased expression of the TNF inducible gene SCYa13 (mcp-4 chemokine or small inducible A13 cytokine precursor), was noted on day 1 only in both the lungs and spleens of non-immune and immune infected birds (Tables 3 and 4). In the lungs of non-immune and immune infected birds chemokine receptor like protein 2 was also significantly up-regulated at day 1 in both groups, and at day 5 in the non-immune group, while the chemokines AH221 and AH294 (RANTES), but only in the non-immune group, were

Table 4
Regulation of well-known immunologically relevant genes in spleens of AI-H9N2 infected chickens

Gene	Accession no.	Non-immune vs. naive		Immune vs. naive		Immune potentiated vs. naive	
		1 d	5 d	1 d	5 d	1 d	5 d
Innate immune pathway							
Chemokines and receptors							
Chemokine AH221	AM064266	2.8 (0.026)	−1.7 (0.020)	2.8 (0.0020)	2.0 (0.036)		
CXC-R4 (chemokine receptor)	AM069849					−1.6 (0.018)	
SCYa13 (TNF inducible gene)/MCP-4 chemokine	BU397023	3.5 (0.008)		3.7 (0.0003)			
Macrophage/myeloid cells							
MARCO (scavenger receptor)	AM064405		−1.6 (0.026)				
Toll-pathway							
TLR2 (Tol-like receptor 2) type 1	BU374739				3.0 (0.014)		
IFN-pathway							
IFP35 (IFN-induced 35 kDa protein)	BU135331	2.1 (0.0004)		1.8 (0.001)			
Heat shock proteins (HSPs)							
Hsp70	AM070074	1.8 (0.020)					
Complement factors							
Complement C1r-like proteinase	AM065161	1.5 (0.019)	1.7 (0.050)	1.9 (0.006)			
Complement C1qB	AM065730	2.0 (0.048)		2.0 (0.024)			
Complement C1qC	AM067767		−1.6 (0.017)				
Adaptive immune pathway							
Antigen presentation							
Cathepsin S	AM067017	1.9 (0.014)		1.8 (0.001)	2.5 (0.020)		1.6 (0.036)
Class I B-F heavy	AM067119				1.7 (0.037)		
Cytokines and receptors							
Cytokine receptor common beta chain precursor	AM066518		1.6 (0.035)				
IL-13R2	BU341330			3.1 (0.020)			
IFN-γR2	BU294744	1.6 (0.020)					
CD and T cell markers							
CD200 (expression: DCs, activated T cells)	AI981679			1.6 (0.042)			
CD226 (expression: Th1 cells)	AI980296		−1.8 (0.028)				
CD5 (expression: thymocytes, T cells, subset of B cells)	AM071524	−2.1 (0.022)					
CD84 (expression: monocytes, platelets, circ. B cells)	AM070961					−1.5 (0.048)	
Transcription factors							
GATA-3 (Th2 transcription factor)	AM064179			2.1 (0.006)			
B cell							
Ig J chain	AM064723	−1.5 (0.048)					
Ig rearranged heavy chain VDJ region	BU398082	−4.8 (0.048)					
Ig lambda chain C region	AM072032	−1.5 (0.031)					
T cell activation and differentiation							
Ly6e (SCA-2/TSA1)	AM071100	3.6 (0.004)					
Miscelaneous							
Apoptosis							
Bcl-2-like	AM071676			1.6 (0.048)			

Regulation (≥1.5-fold; $P < 0.05$) of well-known immunologically relevant genes in the spleens of non-immune, immune or immune potentiated chickens at 1 or 5 days post-infection with avian influenza H9N2 when compared to naive control birds. *P* values are between parentheses.

up-regulated at day 5 only (Table 3). In the spleen of immune birds AH221 is up-regulated on day 1 and 5, and only on day 1 in non-immune birds (Table 4). Remarkably, limited up-regulation of chemokines and chemokine receptors was noted in the lungs of immune potentiated infected birds except for the CXCL12 chemokine, which is up-regulated at day 1, and chemokine receptor like protein 2 at day 5 (Table 3).

3.5.1.2. Macrophage/myeloid cells. The scavenger receptor MARCO (macrophage receptor with collagenous structure), a molecule involved in specific recognition and defense against pathogens as well as differentiation of monocytes into dendritic cells, proved selectively expressed at day 1 in non-immune, immune and immune potentiated infected birds, and also at day 5 post-infection in immune potentiated birds (Table 3). In MARCO-deficient mice it was shown that this molecule plays an important role in mounting an efficient and appropriately regulated innate immune response against inhaled particles and airborne pathogens [16].

3.5.1.3. Toll-pathway. The recognition of microbes by innate immune cells is crucial for initiation of antigen presentation and down-stream activation of adaptive immune reactions. Toll-like receptors (TLRs) are known to recognize various components of invading pathogens. TLR4, the receptor for bacterial lipopolysaccharide (LPS), is down-regulated in non-immune infected birds at day 1 (Table 3). Interestingly, TLR2 type 1 [17], the receptor for bacterial cell wall components, was up-regulated in non-immune and immune infected animals at days 1 and 5, and at day 1 in immune potentiated birds only. The selective up-regulation of TLR2 type 1 expression with concomitant decrease of TLR4 expression at day 1 in non-immune birds maybe due to either influx of distinct innate immune cells, such as antigen presenting cells, or selective regulation of receptor expression on one cell population. TLR2 type 1 is also up-regulated in the spleens of immune birds on day 5 (Table 4). In parallel with the up-regulated MARCO receptor and TLR2 type 1 mRNA molecules, all groups showed increased expression of granzyme M, a granular protease associated with NK cells at day 1.

3.5.1.4. IFN-pathway. Upon viral infection, production of type-I interferon (IFN) and IFN-induced genes is a typical innate defense response resulting in an antiviral state of non-infected neighboring target cells. Type I IFN is also known to act as an important immune inducer and immunoregulatory factor. A number of genes associated with an activated type I IFN-pathway were noted especially in the lungs of non-immune and immune infected chickens (Table 3). Activated genes included IFN associated IRF4/MUM1 (interferon regulatory factor 4), – also involved in controlling B-cell proliferation and differentiation, and proliferation of mitogen-activated T cells – showed similar up-regulation at days 1 and 5 after infection in non-immune and immune hosts (Table 3). In addition, type I IFN-pathway transcription factor IRF8, showed up-regulation specific for lungs of chickens immunized with antigen only at days 1 and 5 after infection and in immune potentiated birds at day 1 (Table 3). The IFN-induced protein IFP35 showed selectively increased expression at day 1 in the lungs and spleens of non-immune and immune infected birds (Tables 3 and 4). The IFN pathway genes IFP35, IRF4 and IRF8 noted in lungs of chickens receiving antigen only (immune infected), suggest that viral replication was not completely blocked as a result of immunization with antigen in saline only. This hypothesis is consistent with Q-PCR detection of the viral haemagglutinin gene (see above). However, in H9N2-infected chickens immunized with antigen in immunopotentiating adjuvant, the immune potentiated group, only IRF4 and IRF8 were up-regulated, on days 5 and 1, respectively (Table 3).

3.5.1.5. Heat shock proteins (HSPs). The main function of heat shock proteins (HSPs) is to act as chaperones of nascent or abnormally folded proteins. However, from an immunological point of view HSPs have obtained increasing interest since they may act as endogenous immune-activating "danger" signals. Hsp70 and Hsp90α expression was clearly up-regulated at days 1 and 5 in immune potentiated birds (Table 3), while Hsc70II showed up-regulation in, especially lungs of, non-immune chickens on day 1.

3.5.1.6. Complement factors. The complement system consists of several plasma proteins, i.e. complement factors, that act together to attack extracellular pathogens. Up-regulation of complement C3 mRNA was noted only in non-immune infected birds at day 5 and in immune and immune potentiated infected birds at day 1 (Table 3). Complement inhibitory factor H was down-regulated in non-immune animals on day 1 after challenge only. Remarkably, in spleens of non-immune and immune infected birds, but not in immune potentiated animals, complement C1 proteins, i.e. C1r-like and C1qB, are commonly up-regulated (Table 4).

3.5.1.7. Pro-inflammatory cytokines. Secretion of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL-1) and IL-6, is one of the early reactions of the host against infection. A combined action of these cytokines causes fever, leukocytosis and the production of acute phase proteins. At 1 day after infection, lungs of non-immune and immune infected birds, show up-regulated gene expression of interleukin-6 (IL-6) (Table 3). However, expression of this pro-inflammatory cytokine was not altered in the lungs of immune potentiated animals.

3.5.2. Adaptive immune responses

3.5.2.1. Antigen presentation. Increased antigen presentation to CD8 positive T cells was suggested by the up-regulation of class I alpha, class B–F heavy and class I minor chains at day 5 after challenge in the lungs of non-immune birds. This was, to a lower extent, also observed in lungs of immune and immune potentiated animals (Table 3).

Cathepsin S, a key protease responsible for the removal of the invariant chain from MHC class II molecules, is up-regulated at day 1 in the lungs and spleens of non-immune and immune infected birds. However, at day 5 cathepsin S is only up-regulated in the spleens of immune and immune potentiated birds (Tables 3 and 4).

3.5.2.2. CD markers and cytokine receptors. Up-regulated expression of T cell-specific CD3 genes was noted in lungs of non-immune and immune infected birds at days 1 and 5 and in immune potentiated birds at day 5 only (Table 3). Remarkably, increased expression of CD4 and CD8 was evident especially in immune birds at day 1. These animals also showed evidence of active up-regulation of cytokine receptor expression, such as IL-2Rb on day 5, IL-13R2 on days 1 and 5, and IFN-γR2 on day 1 (Table 3). Up-regulation of the cytokine receptors IL-13R2 and IFN-γR2 was also noted on day 1 in spleens of immune and non-immune birds, respectively (Table 4). Immune potentiated infected birds showed no evidence of increased cytokine receptor expression in lungs and spleen. Interestingly, no significant changes in the corresponding cytokine genes, such as IL-2, IFN-γ and IL-4, were noted in any group on any point in time in lungs and spleens when using the micro-array approach. Unfortunately, the genes encoding IL-13, an important Th-2 cytokine, and IL-20, a member of the IL-10 family of molecules associated with putative regulatory T cell function, are not included on this version of the microarray.

3.5.2.3. Transcription factors. At day 1 after infection lungs of non-immune and immune birds, and spleens of immune birds, show up-regulation of GATA-3, which is associated with Th-2 type T cell immune responses in mammals (Tables 3 and 4). Lungs and spleens of immune potentiated infected birds showed no altered GATA-3 gene expression at any time-point. However, an increased expression of cystatin F (leukocystatin) an immune cell restricted cysteine proteinase inhibitor, which may play a role in antigen presentation [18], was observed at day 1 in lungs only (Table 3).

3.5.2.4. B cells. Influx onto or (re)activation of B cells in infected lungs was suggested by the up-regulation of the genes encoding various Ig chains especially at 5 days after infection of non-immune, immune and immune potentiated animals (Table 3). In all groups Ig chain expression concurred with increased expression of CD5, a mammalian marker of many B-1a but also B-2 B cells. The profound increase of CD5 in immune birds on day 5 may be explained by a booster effect as a result of the challenge infection. For immune potentiated birds the level of responsiveness was more modest, likely in view of pre-existing virus-neutralizing antibodies in the circulation, which may immediately neutralize (part of) the challenge infection.

3.5.2.5. Apoptosis and TNF-related gene expression. Influenza virus has been shown to induce apoptosis in cells that are permissive for viral replication and cells that do not support viral replication. Influenza induced apoptosis likely represents part of an overall beneficial immune response, but, on the other hand, could also be a mechanism involved in disease pathogenesis. In our study, granzyme M, a representative of a new perforin-dependent cell death pathway playing a significant role in cell death mediated by NK cells [19], is up-regulated at day 1 after infection in the lungs of all groups (Table 3). In addition, the anti-apoptotic protein NR13, a member of the Bcl-2 family, is up-regulated at day 5 in immune infected lungs which co-incides with up-regulation of granzyme M. TRAF-1 (tumor necrosis factor-receptor associated factor-1) is an adaptor and signal transduction molecule known to interact with the cytoplasmic tail of TNF receptor 2 thereby linking it to different signal transduction pathways. TRAF1 inhibits antigen-induced apoptosis in CD8 + lymphocytes [20]. This molecule showed increased expression in lungs of non-immune chickens at 5 days after infection (Table 3). This coincided with up-regulation of MHC class I chain genes. TRAF2 and TRAF5 showed up-regulation at day 1 in lungs of immune infected birds, coinciding with CD3, CD4, CD8 and the FASL decoy receptor 3 and death receptor 6 expression. Again, lungs of immune potentiated infected birds showed no TRAF1, TRAF2 or TRAF5 expression.

4. Discussion

Influenza virus infections may cause inflammatory pneumonia in all susceptible species. Control of an acute influenza virus infection in naive hosts is thought to be based on innate and subsequent adaptive cytotoxic T lymphocyte (CTL) and B cell activity [21,22]. By contrast, readily available virus neutralizing antibodies are believed to be largely responsible for protection of immunized hosts, but not essential [22], as demonstrated in murine models. However, the precise role of host immune elements in the control of acute avian influenza is unknown. In addition, the potential contribution of host immune elements in pulmonary immunopathology in birds is largely undefined. In this study we investigated the influence of a respiratory infection of inbred broiler chickens with the low pathogenic avian influenza H9N2 virus on lung pathology and gene expression. We demonstrate for the first time lung pathology (pneumonia) of the low pathogenic H9N2 virus and insights into the molecular interaction with its naive natural host. In addition we examined molecular early host responses to respiratory influenza A H9N2 virus infection in immunized chickens infected via the respiratory route.

When considering the observed levels of virus-inhibiting antibodies, as well as histopathology and viral Q-PCR data, we anticipated that immune potentiated birds, immunized with formalin-inactivated antigen formulated in W/O immunopotentiator, needed less immune activation in their lungs to control the virus, when compared to immune birds, receiving antigen only. Conversely, in view of the observed

immunopathology in the lungs and absence of antiviral antibodies at the moment of infection we foresaw more vigorous immune responsiveness following pulmonary infection of non-immune birds, which exhibited viral Q-PCR positive lungs.

Indeed, the patterns of gene expression noted in the different groups suggest most intense reactions in non-immune animals, with a partial overlap in the immune birds immunized with inactivated antigen in saline only. Similar to our naive H9N2 infected chickens, the lungs of H1N2 infected macaques, when analyzed by microarrays, showed induction of IFN and B cell activation pathways, though with more overt clinical signs [23]. By contrast, immune potentiated chickens showed insignificant responses for a number of known innate and adaptive immune elements that were noted in the less immune and naive birds, including TNF- and apoptosis-related genes as well as proinflammatory IL-6, and certain chemokines. Remarkably, in this immune potentiated group no significant changes in any known immunoregulatory cytokine or cytokine receptor gene were noted at any point in time.

Based largely on studies in mice, the first line of defense against influenza virus has been shown to involve natural antibodies produced by B-1 cells [24], which do not increase IgM production nor proceed to immunoglobulin isotype switching. Indeed, we noted up-regulated expression of CD5, a marker of many B-1a cells in mammals, in the lungs of all groups at day 5. In addition, granulocytes, such as neutrophils, are prominent in acutely infected murine lungs and believed to control initial viral replication. Indeed, granzyme M, an NK cell specific protease was significantly expressed at day 1 in the lungs of all groups. Recent studies in IL-1 receptor deficient mice, however, suggest no major contribution for neutrophils in the clearance of influenza [25]. We noted no increased expression of chicken IL-1 mRNA in any of the treated groups. However, increased expression of IL-6, another pro-inflammatory mediator, was noted in lungs of non-immune and immune (antigen-primed) birds at day 1 only.

As yet, the gene encoding for tumor necrosis factor alpha (TNF-α) has not been identified in the chicken and is therefore not available for monitoring. However, TRAF1, TRAF2, TRAF5, the TNF family member Death receptor 6 and the FasL decoy receptor 3, showed up-regulation especially in lungs of immune birds. In mice TNF-α is involved in recruitment of inflammatory cells to the lung and the severity of disease, while leaving virus clearance unaffected [26]. In the same model the initiation of infection-induced lung injury proved to depend on TNF-α expressed by Ag-specific CD8+ T cells, recognizing Ag expressed by alveolar epithelial cells [27]. TNF-α neutralization *in vivo* or TNF receptor (p55) gene deletion inhibited weight loss and lethality [27]. This was associated with minimal macrophage infiltration and reduced expression of the murine monocyte chemokine attractant gene-1 (MCP-1), at least one of the chemokines produced by target epithelial cells [27]. *In vivo* neutralization of MCP-1 significantly reduced inflammation and immunopathology. In our studies evidence for macrophage gene expression was noted for MARCO in the lungs of non-immune, immune and immune potentiated infected birds at day 1, and also at day 5 in immune potentiated birds (Table 3). The chemokine AH294 (RANTES), which is produced by influenza infected epithelial cells [28], and selectively up-regulated especially in non-immune infected lungs at day 5 (Table 3), has been reported to facilitate activation and extravasation of macrophages [29]. Genes associated with MHC class I presentation were evident especially in the non-immune infected group at day 1 and day 5, but less prominent, and possibly less needed, in the immune and immune potentiated group.

Collectively, our microarray data show that in all groups IFN pathway genes are evident, although somewhat less in immune potentiated birds, possibly explained by inhibited viral replication. The up-regulation of IFN pathway genes suggests that expression of these genes may be important in initiating the antiviral response. Remarkably, however, we could neither detect MX (a protein induced by IFN that inhibits influenza virus and which is highly polymorphic in chicken) nor IFN-γ. In addition, all groups show up-regulated genes involved in MHC-class I presentation, CD5 and B cell immunoglobulin genes. Up-regulation of MHC class I genes on day 5 are typical for lungs of non-immune infected birds. Certain innate and adaptive immune reactions are overlapping in both non-immune infected birds as well as immune infected chickens, as reflected by up-regulated chemokine receptor expression, and expression of TLR2 type 1, IFP35, IRF4, IL-6 as well as GATA-3. When compared to other groups the lungs of chickens immunized with antigen in saline only (immune), show relatively more CD marker genes, IL-13R2, IFN-γR2 and IL-2R2, hence more evidence for adaptive immunoregulation pathways, as well as TNF and apoptosis-related genes. By contrast, in the lungs of immune potentiated birds innate response genes are rather limited except for certain heat shock proteins, granzyme M and MARCO, which, together with circulating antibodies and T cells, are likely sufficient to control the challenge infection. Remarkably, in the spleens of immune potentiated birds regulation of innate and adaptive immune response genes are limited to only cathepsin S up-regulation at day 5.

Studies in mice revealed that in general Th-1 type immunity involving IFN-γ producing T helper 1 cells [30] and CD8 + CTL [31] are involved in the control of pulmonary influenza infection. However, the pulmonary environment generally promotes type-2 immune reactions [32]. Nasal-pulmonary immunization of BALB/C mice with influenza antigen alone induces increased Th-2 cytokine mRNA expression (IL-4 and IL-5) in lung lymphocytes, which are mainly CD4+ cells, but with no differences in Th-1 cytokine mRNA (IFN-γ and IL-2) expression [32]. Conflicting murine data exist on the role of IL-18. While in one study intranasal influenza virus infection of IL-18 deficient mice resulted in increased mortality as a result of reduced IFN-γ levels

and poor NK cell activation [33], Van der Sluis et al. [34] showed that IL-18 deficient mice had reduced viral loads and enhanced CD4+ T cell activation and normal IFN-γ levels monitored by intracellular staining. Interestingly, however, studies in mice with null mutations of the receptors for IFN-α/β, IFN-γ or both IFNs showed no major differences in the control of acute or secondary intranasal influenza virus infection [21], suggesting a general redundancy of the IFN system to pulmonary influenza immunity. Also, IFN-γ deficient mice show almost no impaired resistance to intranasal influenza infection [35], a skewing towards Th-2 characteristic IL-4 and IL-5 production and associated IgG1 antibody isotype. Our microarray data revealed profound up-regulation of the Th-2 response associated IL-13R2 and GATA-3 genes, but unfortunately no clear evidence for selective recruitment or activation of Th-1 or Th-2 polarized cytokines, possibly to a lack of detection sensitivity. Some key Th-2 associated chicken cytokine genes like IL-13 and IL-4 were unknown until very recently and only IL-4 is fortunately included on this version of the microarray; although not significantly regulated.

In summary, respiratory infection using H9N2 influenza A virus is useful to study respiratory immune reactions to low-pathogenic influenza virus in lungs and spleen of naive or immunized chickens. Evaluation of our preliminary microarray data suggests gene pathways unique to or common among the differently immune groups. In that respect prominent class I presentation genes are typical in lungs of non-immune infected birds. Both non-immune and immune (antigen only) infected birds show similarities in lung expression of chemokines, TLR2 type 1, and IL-6. All groups showed up-regulation of IFN pathway genes, together with genes involved in MHC class I antigen presentation, as well as genes encoding CD5 and Ig. In the lungs of immune potentiated birds we noted minimal innate responses, limited to HSPs, which, likely together with existing antibodies and effectively primed T cells, are sufficient to control the pulmonary infection. Also, in the spleens of immune potentiated birds well-known immune responses at the molecular level are not detectable. Lungs of birds that received antigen only (immune) typically show prominent expression of CD4 and CD8 genes, as well as genes encoding IL-13R, IFNγR, TRAF2 and TRAF5. Further *in vivo* studies addressing the functional contribution of selected up-regulated gene products, their association with certain cell types and transcriptional regulation will help to delineate influenza virus-induced immunopathology and (cross-protective) immunity. Such information may reveal new targets for disease intervention in the natural host or may provide a model for other species including man.

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References

[1] Degen WG, van Daal N, van Zuilekom HI, Burnside J, Schijns VE. Identification and molecular cloning of functional chicken IL-12. J Immunol 2004;172(7):4371–80.
[2] Degen WG, Daal N, Rothwell L, Kaiser P, Schijns VE. Th1/Th2 polarization by viral and helminth infection in birds. Vet Microbiol 2005;105(3/4):163–7.
[3] Rothwell L, Young JR, Zoorob R, Whittaker CA, Hesketh P, Archer A, et al. Cloning and characterization of chicken IL-10 and its role in the immune response to Eimeria maxima. J Immunol 2004;173(4):2675–82.
[4] Avery S, Rothwell L, Degen WGJ, Schijns VEJC, Young J, Kaufman J, et al. Characterisation of the first non-mammalian T2 cytokine gene cluster, the cluster contains functional single-copy genes for IL-3, IL-4, IL-13 and GM-CSF, a gene for IL-5 which appears to be a pseudogene, and a gene encoding another cytokine-like transcript, KK34. J Interferon Cytokine Res 2004;24(10):600–10.
[5] Hillier LW, Miller W, Birney E, Warren W, Hardison RC, Ponting CP, et al. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature 2004;432(7018):695–716.
[6] Nili H, Asasi K. Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. Avian Pathol 2002;31(3):247–52.
[7] Seo SH, Webster RG. Cross-reactive, cell-mediated immunity and protection of chickens from lethal H5N1 influenza virus infection in Hong Kong poultry markets. J Virol 2001;75(6):2516–25.
[8] Seo SH, Peiris M, Webster RG. Protective cross-reactive cellular immunity to lethal A/Goose/Guangdong/1/96-like H5N1 influenza virus is correlated with the proportion of pulmonary CD8(+) T cells expressing gamma interferon. J Virol 2002;76(10):4886–90.
[9] Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, et al. Human infection with influenza H9N2. Lancet 1999;354(9182):916–7.
[10] Lin YP, Shaw M, Gregory V, Cameron K, Lim W, Klimov A, et al. Avian-to-human transmission of H9N2 subtype influenza A viruses, relationship between H9N2 and H5N1 human isolates. Proc Natl Acad Sci USA 2000;97(17):9654–8.
[11] Li KS, Xu KM, Peiris JS, Poon LL, Yu KZ, Yuen KY, et al. Characterization of H9 subtype influenza viruses from the ducks of southern China, a candidate for the next influenza pandemic in humans? J Virol 2003;77(12):6988–94.
[12] Choi YK, Ozaki H, Webby RJ, Webster RG, Peiris JS, Poon L, et al. Continuing evolution of H9N2 influenza viruses in Southeastern China. J Virol 2004;78(16):8609–14.
[13] Smith J, Speed D, Hocking PM, Talbot RT, Degen WG, Schijns VE, et al. Development of a chicken 5 K microarray targeted towards immune function. BMC Genom 2006;7:49.
[14] Jansen T, Hofmans MP, Theelen MJ, Schijns VE. Structure-activity relations of water-in-oil vaccine formulations and induced antigen-specific antibody responses. Vaccine 2005;23(8):1053–60.
[15] Hangalapura BN, Kaiser MG, Poel JJ, Parmentier HK, Lamont SJ. Cold stress equally enhances in vivo pro-inflammatory cytokine gene expression in chicken lines divergently selected for antibody responses. Dev Comp Immunol 2006;30(5):503–11.
[16] Arredouani M, Yang Z, Ning Y, Qin G, Soininen R, Tryggvason K, et al., The scavenger receptor MARCO is required for lung defense

against pneumococcal pneumonia and inhaled particles. J Exp Med 2004; %19; 200(2), 267–272.
[17] Fukui A, Inoue N, Matsumoto M, Nomura M, Yamada K, Matsuda Y, et al. Molecular cloning and functional characterization of chicken toll-like receptors. A single chicken toll covers multiple molecular patterns. J Biol Chem 2001;276(50):47143–9.
[18] Nathanson CM, Wasselius J, Wallin H, Abrahamson M. Regulated expression and intracellular localization of cystatin F in human U937 cells. Eur J Biochem 2002;269(22):5502–11.
[19] Kelly JM, Waterhouse NJ, Cretney E, Browne KA, Ellis S, Trapani JA, et al. Granzyme M mediates a novel form of perforin-dependent cell death. J Biol Chem 2004;279(21):22236–42.
[20] Zapata JM, Reed JC. TRAF1: lord without a RING. Sci STKE 2002;2002(133):PE27.
[21] Price GE, Gaszewska-Mastarlarz A, Moskophidis D. The role of alpha/beta and gamma interferons in development of immunity to influenza A virus in mice. J Virol 2000;74(9):3996–4003.
[22] Graham MB, Braciale TJ. Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. J Exp Med 1997;186(12):2063–8.
[23] Baskin CR, Garcia-Sastre A, Tumpey TM, Bielefeldt-Ohmann H, Carter VS, Nistal-Villan E, et al. Integration of clinical data, pathology, and cDNA microarrays in influenza virus-infected pigtailed macaques (*Macaca nemestrina*). J Virol 2004;78(19): 10420–32.
[24] Baumgarth N, Herman OC, Jager GC, Brown L, Herzenberg LA, Herzenberg LA. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. Proc Natl Acad Sci USA 1999;96(5):2250–5.
[25] Schmitz N, Kurrer M, Bachmann MF, Kopf M. Interleukin-1 is responsible for acute lung immunopathology but increases survival of respiratory influenza virus infection. J Virol 2005;79(10):6441–8.
[26] Hussell T, Pennycook A, Openshaw PJ. Inhibition of tumor necrosis factor reduces the severity of virus-specific lung immunopathology. Eur J Immunol 2001;31(9):2566–73.
[27] Xu L, Yoon H, Zhao MQ, Liu J, Ramana CV, Enelow RI. Cutting edge, pulmonary immunopathology mediated by antigen-specific expression of TNF-alpha by antiviral CD8+ T cells. J Immunol 2004;173(2):721–5.
[28] Matsukura S, Kokubu F, Kubo H, Tomita T, Tokunaga H, Kadokura M, et al. Expression of RANTES by normal airway epithelial cells after influenza virus A infection. Am J Respir Cell Mol Biol 1998;18(2):255–64.
[29] Julkunen I, Melen K, Nyqvist M, Pirhonen J, Sareneva T, Matikainen S. Inflammatory responses in influenza A virus infection. Vaccine 2000;19(Suppl 1):S32–7.
[30] Baumgarth N, Kelso A. In vivo blockade of gamma interferon affects the influenza virus-induced humoral and the local cellular immune response in lung tissue. J Virol 1996;70(7):4411–8.
[31] Eichelberger M, Allan W, Zijlstra M, Jaenisch R, Doherty PC. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. J Exp Med 1991;174(4):875–80.
[32] Jones HP, Hodge LM, Fujihashi K, Kiyono H, McGhee JR, Simecka JW. The pulmonary environment promotes Th2 cell responses after nasal-pulmonary immunization with antigen alone, but Th1 responses are induced during instances of intense immune stimulation. J Immunol 2001;167(8):4518–26.
[33] Liu B, Mori I, Hossain MJ, Dong L, Takeda K, Kimura Y. Interleukin-18 improves the early defence system against influenza virus infection by augmenting natural killer cell-mediated cytotoxicity. J Gen Virol 2004;85(Pt. 2):423–8.
[34] Van Der Sluijs KF, Van Elden LJ, Arens R, Nijhuis M, Schuurman R, Florquin S, et al. Enhanced viral clearance in interleukin-18 gene-deficient mice after pulmonary infection with influenza A virus. Immunology 2005;114(1):112–20.
[35] Graham MB, Dalton DK, Giltinan D, Braciale VL, Stewart TA, Braciale TJ. Response to influenza infection in mice with a targeted disruption in the interferon gamma gene. J Exp Med 1993;178(5): 1725–32.