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Reduced immune reaction prevents immunopathology after challenge with avian influenza virus: A transcriptomics analysis of adjuvanted vaccines

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ABSTRACT

To gain more insight in underlying mechanisms correlating to protection against avian influenza virus (AIV) infection, we investigated correlates of protection after AIV H9N2 infection and studied the contribution of different adjuvants to a protective response at host transcriptional level. One-day-old chickens were immunised with inactivated H9N2 supplemented with w/o, $Al(OH)_3$, CpG or without adjuvant. Two weeks later, birds were homologously challenged and at 1–4 days post challenge (d.p.c.) trachea and lung were collected. Birds immunised with H9N2 + w/o or H9N2 + $Al(OH)_3$ were protected against challenge infection and had lower viral RNA expression, less immune related genes induced after challenge, a lower amplitude of change of gene expression and smaller cellular influxes compared to the higher and prolonged gene expression in unprotected birds. We show that a limited number of differentially expressed genes correlates with reduced immune activation and subsequently reduced immunopathology after challenge with AIV.

1. Introduction

Avian influenza virus (AIV) infection causes problems worldwide and affects both humans and animals. Therefore, much effort is invested in development of effective vaccines. A good understanding of mechanisms that correlate to protection or AIV induced pathology may help in the development of effective vaccines and methods to diagnose the outcome of the infection at an early stage.

Much research has been performed into the interplay between a virus and the immune system to unravel mechanisms that correlate to virus induced pathology and predictors of disease progression. Persistent immune activation is a strong predictor of disease progression after HIV infection [9]. Chronic immune activation after HIV-1 infection has been described to correlate to pathogenesis of progressive HIV-1 infection [12], while in non-pathogenic SIV infection of sooty mangabeys a high immune activation was rapidly attenuated despite the presence of high viral loads [36]. Uncontrolled immune activation has also been reported for high pathogenic influenza virus. H5N1 influenza virus has been reported to induce severe lung pathology accompanied by strong and persistent induction of genes involved in innate and inflammatory immune responses in macaques [2]. A highly pathogenic infection with the 1918 H1N1 influenza virus also caused high and persistent induction of innate and inflammatory immune responses in macaques [20], mice [17] and ferrets [4]. Furthermore, highly pathogenic influenza virus infection has been described to cause early and extensive infiltration of macrophages and neutrophils in the lungs [2,31], while for low pathogenic virus infections lower numbers of macrophages and neutrophils were found in the lungs. This suggests a relationship between influenza virus virulence and host responses in which an uncontrolled host response to influenza virus infection seems to be correlated to high pathology and the severity of the infection.

Mechanisms that correlate to reduced pathology and to protection against AIV infection remain poorly defined. In mammals, innate immune cells like macrophages, dendritic cells and natural killer cells have shown to be important to control influenza virus infection at and early stage and promote adaptive response [28]. Adaptive immune cells like B- and T-cell play a crucial role in clearing the infection and neutralizing antibodies play an important protective role during infection by diminishing virus entry. In general the induction of hemagglutinin (HA) antibodies is accepted as a major correlate of protection and vaccine efficacy in chickens and human [23,34].

In this study we set out to investigate predictors of reduced immunopathology and subsequent protection after vaccination

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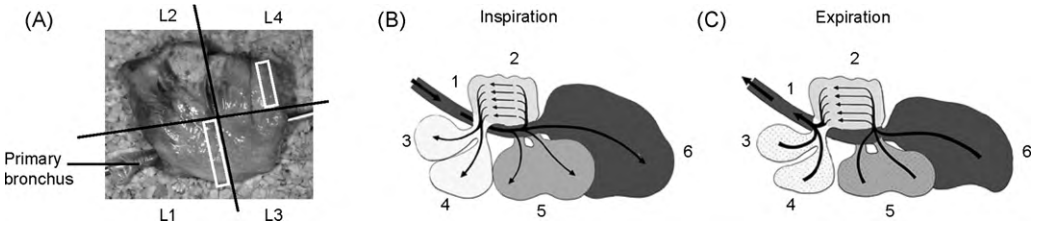

Fig. 1. (A) "Right chicken lung with a blunt probe indicating the localization of the primary bronchus." The lung was divided into four parts, L1 to L4 according to airflow and lung anatomy [32], with the primary bronchus entering the lung in L1 shown on the left side. Segment L1, containing the primary bronchus and secondary bronchi, and L4, containing the paleopulmonic parabronchi, were used for analysis. The white blocks indicate the part of the lung segments used for RNA isolation, while the adjacent part within the segment was used for immunocytochemistry. "Illustration of the unidirectional air flow pattern, which flows from caudal to cranial during (B) inspiration and (C) expiration in the avian lung. (1) Primary bronchus, (2) lung, (3) clavicular air sac, (4) cranial thoracic air sac, (5) caudal thoracic air sac, (6) abdominal air sac." Adapted and reprinted with permission [21], Copyright 2008.

and challenge with AIV H9N2. We also addressed the question whether different adjuvants contribute to a difference in immune activation. Therefore, we choose three adjuvants with a different mode of action. Water-in-oil (w/o) and hydroxide ($Al(OH)_3$) act Toll-like receptor (TLR) independent [15] and are generally associated with induction of Th2 responses [6,13,14,25]. Non-methylated CpG oligonucleotides (CpG) act TLR dependent and are associated with Th1 responses [15]. All three adjuvants have been reported to induce a protective response in birds [11,24,29,35]. In our study chickens were immunised with H9N2 vaccine in presence of w/o, $Al(OH)_3$, CpG or without adjuvant, and these birds together with non-immunised birds were challenged with live H9N2 14 days post immunisation. At 1–4 days post challenge (d.p.c.) viral RNA expression, gene expression profiles and cellular influxes were determined in trachea and lung. Together with HI and AIV nucleoprotein (NP)-specific antibody titers in serum we defined characteristics of protective responses and differences in the mode of action between the different adjuvants. Birds immunised in presence of w/o and $Al(OH)_3$ were protected and both groups had a limited number of differentially expressed genes, reduced immune activation and high HI and AIV NP-specific antibody titers. We show that a limited number of differentially expressed genes correlates with reduced immune activation and subsequently reduced immunopathology after challenge with AIV.

2. Materials and methods

2.1. Infection model

Avian influenza A virus, subtype H9N2, isolate A/Chicken/United Arab Emirates/99 produced in eggs using routine procedures was kindly provided by Intervet/Schering-Plough Animal Health. For the vaccine formulation used to immunise the birds the aqueous phase containing the virus suspension, diluted in 0.01 M phosphate buffer (pH 7.2), was formalin-inactivated (Intervet/Schering-Plough Animal Health).

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

Chickens were divided into six groups over six isolators, containing twenty animals per group. One-day-old chickens were immunised s.c. with 0.25 ml formalin-inactivated avian influenza H9N2 with w/o, $Al(OH)_3$, CpG or without adjuvant. The non-immunised-non-challenged (NINC) and non-immunised-challenged (NIC) groups were immunised s.c. with saline. The formalin-inactivated antigen preparation was formulated into an immunopotentiating mineral oil-based w/o emulsion according to standard procedures (HAR titer of $\geq 7.0 \log_2$; commercial H9N2 vaccin of Intervet/Schering-Plough Animal Health [8]). $Al(OH)_3$ consisted of an $Al(OH)_3$-gel (Brenntag) with a final concentration in the vaccine of 0.5%. CpG-ODN 2007 was custom made and consisted of the whole phosphorothioate backbone purified via ethanol precipitation (TibMolbiol). Per dose 40 μg CpG was used. Two weeks after the immunization blood samples were taken from each bird for measuring HI titers and AIV NP-specific antibody titers after which they were inoculated via aerosol spray with 20 ml $10^{7.7}$ EID_{50} H9N2 AIV per isolator. The NINC group was inoculated via aerosol spray with 20 ml saline. Chickens remained in the aerosol spray in a closed isolator for 10 min, after which the isolator was ventilated as before. This led to the following six experimental groups: non-immunised-non-challenged (NINC), non-immunised and H9N2 challenged (NIC), H9N2 immunized and challenged (IC), H9N2 + adjuvant w/o immunized and challenged (IC + w/o), H9N2 + adjuvant $Al(OH)_3$ immunized and challenged (IC + $Al(OH)_3$), H9N2 + adjuvant CpG immunized and challenged (IC + CpG).

At 1–4 days post challenge (d.p.c.) chickens were killed ($n = 4$ per time point per group; $n = 2$ for NIC at 4 d.p.i. due to death caused by infection of two birds) and trachea and left lung were isolated and stored in RNAlater (Ambion) at −80 °C for RNA isolation or fixed in liquid nitrogen for immunocytochemistry. The trachea was divided into upper and lower trachea of which upper trachea was used for analysis. The lung was divided into four parts L1–L4 according to anatomy and airflow (Fig. 1). Segment L1, containing the primary bronchus and secondary bronchi, and L4, containing the paleopulmonic parabronchi, were used for analysis. Selection of organ parts used for analysis was based on viral load and virus induced gene expression as previously described [32].

2.2. H9-specific HI and AIV NP-specific antibody titers

H9-specific haemagglutination inhibition (HI) titers in serum were determined by a HI assay as previously described [8].

AIV NP-specific antibody titers in serum were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, Nunc Maxisorp flatbottom plates were coated with mouse monoclonal antibody (mAb) to nucleoprotein (NP) of H9N2 at 37 °C overnight. Antibody coated plates were washed three times with PBS/Tween and blocked with 10 mM PBS + 1% BSA for 1 h at room temperature. Formalin-inactivated AIV H9N2 antigen, containing intact virions and virion subunits due to degradation of virions, was added to the plates and incubated for 1 h at 37 °C after which the plates were washed three times with PBS/Tween. Chicken sera were 2-fold serially diluted in PBS/Tween, added to the plates and incubated for 1 h at 37 °C. Plates were washed three times with PBS/Tween. HRP-conjugated rabbit anti-chicken IgG (Nordic Immunological Laboratories) was added to the plates and incubated for 30 min at 37 °C. After washing three times with PBS/Tween the plates were developed with TMB substrate for 10 min at room temperature in the dark. The reaction was stopped by adding 4N H_2SO_4 to the plates and the absorbance was measured at 450 nm.

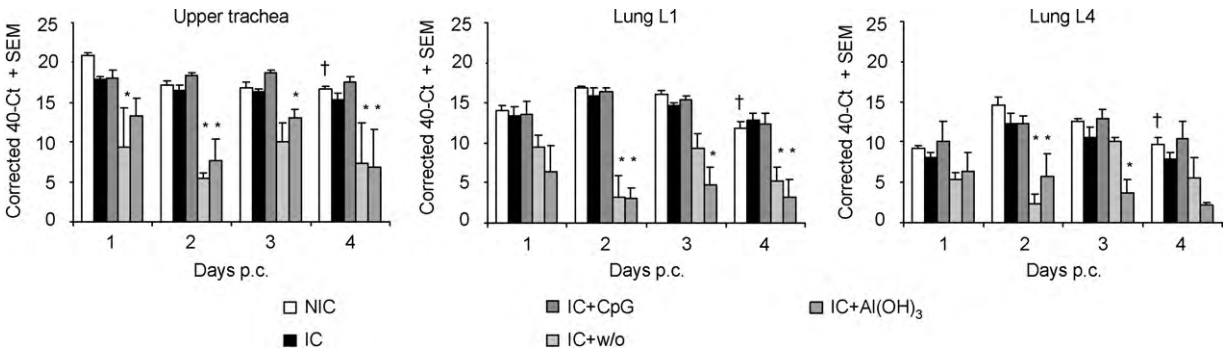

Fig. 2. Viral RNA levels in trachea and lung of birds immunised with H9N2 adjuvanted with w/o or $Al(OH)_3$ are lower compared to the other challenged groups. Highest viral RNA levels were found in the groups non-immunised-challenged (NIC), immunised-challenged (IC) and immunised with H9N2 adjuvanted with CpG. Data were expressed as means ($n = 4$, † $n = 2$) with standard error of the mean (SEM). * Indicates significant differences ($P < 0.05$) in viral RNA expression compared to NIC and IC birds.

On each plate positive and negative control sera were included with known H9N2-NP titer. Results were expressed as mean 2log titers.

2.3. Immunocytochemistry

Virus and cellular influxes were detected in lung by immunocytochemistry as previously described [38]. Viral NP was detected with mAb to NP of H9N2 (provided by Intervet/Schering-Plough Animal Health). Macrophages and $CD4^+$ cells were detected with mAb KUL-01 [26] and CT-4 (Southern Biotech). For detection of $CD8\alpha^+$ cells a mix of mAb EP72 (Southern Biotech) and AV14 (kind gift of Dr T.F. Davison, Institute for Animal Health, Compton UK [39]) was used to avoid differences in staining due to polymorphism in the chicken CD8α molecule [3,22].

2.4. RNA isolation

Total RNA was isolated from trachea (5 mm part) and lung L1 and L4 (1 mm × 5 mm part) using the RNeasy Mini Kit (Qiagen) as previously described [33]. All RNA samples were checked for quantity using a spectrophotometer (Shimadzu) and quality using a 2100 Bioanalyzer (Agilent).

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

cDNA was generated from 500 ng RNA with reverse transcription using iScript cDNA Synthesis Kit (Biorad Laboratories B.V.).

Real-time qRT-PCR was used for detection of GAPDH and H9 hemagglutinin (HA) products using the primers and program previously described [8,33]. Expression of GAPDH mRNA, which was used as a reference gene for correction of viral RNA expression, was not affected by H9N2 AIV (data not shown). Corrections for variation in RNA preparation and sampling were performed as previously described [10]. Results are expressed in terms of the threshold cycle value (Ct) and given as corrected 40-Ct values.

To determine the statistical significance between groups and time points of trachea and lung an ANOVA with a Tukey post-hoc test was used. A P-value < 0.05 was considered significant.

2.6. Oligonucleotide microarray analysis

Microarray analysis was performed as previously described [32] using the Gallus gallus Roslin/ARK CoRe Array Ready Oligo Set V1.0 (Operon Biotechnologies). All 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 four chickens that were not included in the inoculation experiment.

Microarray arrays were analysed as previously described [32]. Briefly, slide normalisation was performed with Printtip Loess on mean data without background subtraction. Groups of replicates were analysed using ANOVA. In a fixed effect analysis, sample, array and dye effects were modelled. P-values were determined by a permutation F^2-test, in which residuals were shuffled 5000 times globally. Genes with $P < 0.05$ after family wise error correction were considered significantly differentially expressed and were selected to be included for further analysis. Visualisation and cluster-analysis were performed using GeneSpring 7.2 (Agilent Technologies). Ensembl *Gallus gallus* (assembly: WASHUC2, May 2006, genebuild: Ensembl, August 2006, database version: 47.2e) was used for gene names, description and gene ontology (GO) annotations. For pathway analysis database for annotation, visualization and integrated discovery (DAVID) 2008 was used.

2.7. Microarray data accession numbers

Primary data are available in the public domain through Arrayexpress at http://www.ebi.ac.uk/microarray-as/ae/under accession numbers E-MTAB-136 for L1, E-MTAB-137 for L4 and E-MTAB-138 for upper trachea.

3. Results

3.1. Effect of adjuvants on protection against challenge with avian influenza virus

To determine the effect of the adjuvants on viral RNA level after challenge, viral RNA expression was measured using real-time qRT-PCR (Fig. 2). IC + w/o and IC + $Al(OH)_3$ birds had significantly lower ($P < 0.05$) viral RNA expression in upper trachea, L1 and L4 compared to NIC and IC birds. There were no significant differences in the viral RNA levels in IC + CpG compared to NIC and IC birds at any time point. The viral RNA levels were significantly higher ($P < 0.05$) in all unprotected groups in lung L1, which contained the larger airways and the bifurcations to the secondary bronchi, compared to lung L4, which contained the paleopulmonic parabronchi. Based on the reduced levels of viral RNA, we denominated the groups immunized in the presence of w/o and $Al(OH)_3$ "protected" against challenge with AIV and the CpG birds "unprotected".

3.2. Global gene expression profiles

In order to determine whether the differences in viral RNA level after challenge were also reflected in gene expression profiles, microarray analysis was performed on upper trachea and lung L1 and L4 at 1–4 d.p.c. By connecting the gene expression level per gene over time, a global gene expression pattern was

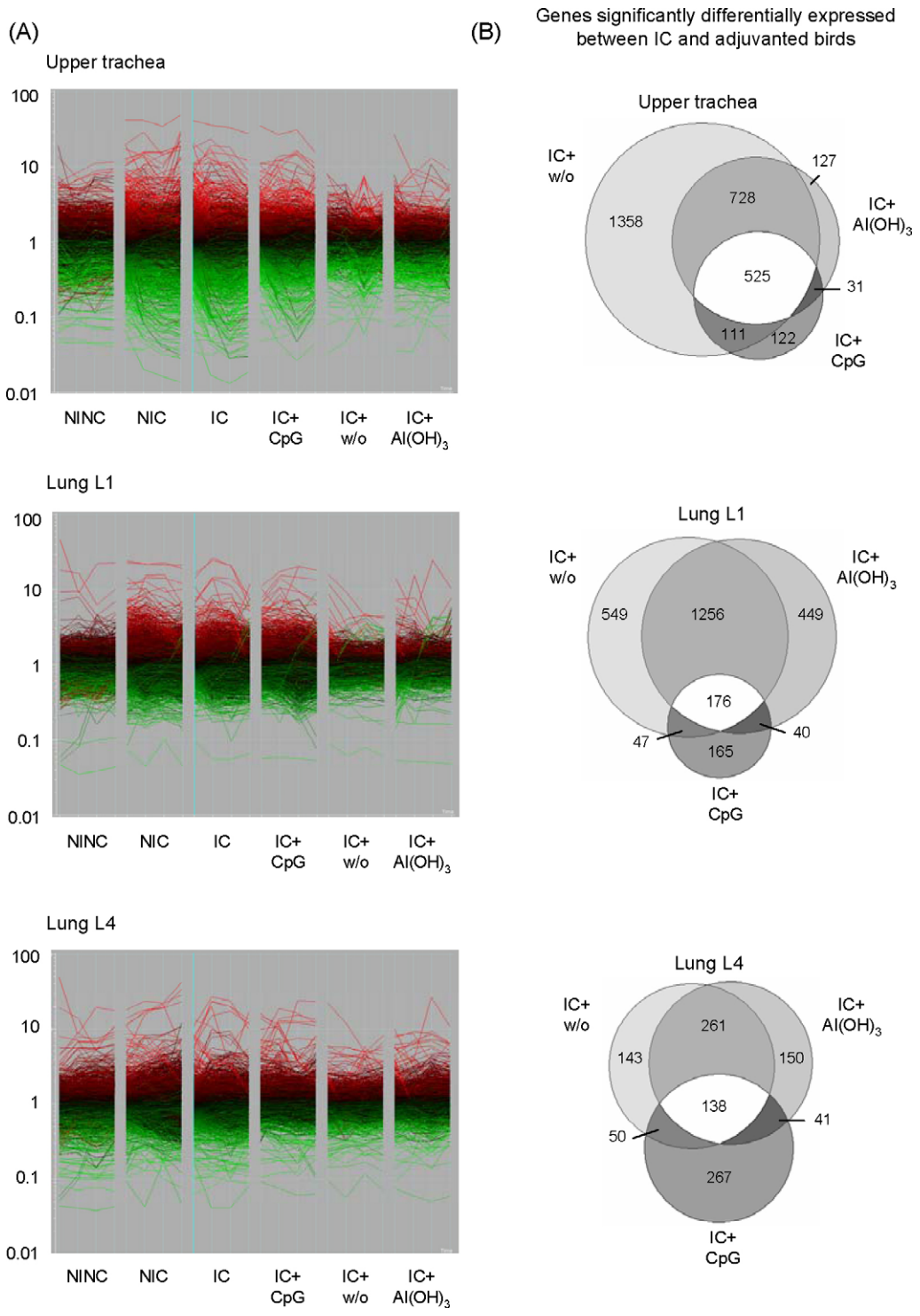

Fig. 3. (A) A lower amplitude of change in global gene expression patterns over time was found in upper trachea, lung L1 and L4 of birds with low viral RNA levels (IC + w/o and IC + $Al(OH)_3$) versus birds with high viral RNA levels (NIC, IC and IC + CpG) birds ($n = 4$, except for NIC 4 d.p.c. $n = 2$). Red indicates up regulation and green down regulation. Gene expression rates of individual birds are compared to expression rates in a reference sample consisting of pooled lungs or pooled trachea of uninfected birds. (B) Venn diagrams showing genes significantly differentially expressed at any day post challenge between IC birds and IC + CpG, IC + w/o and IC + $Al(OH)_3$ birds in upper trachea, lung L1 and L4 ($n = 4$). The numbers within the segments indicate the number of genes that were significantly differentially expressed between the IC group and an adjuvanted group. Most overlap in gene expression was found between the groups IC + w/o and IC + $Al(OH)_3$ with low viral RNA levels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

created reflecting the global host response. Based on the expression patterns, the different treatment groups fell into two groups: one group with low amplitude of change and one group with high amplitude of change (Fig. 3A). The gene expression patterns of the NIC, IC and IC + CpG group showed that many genes were significantly differentially expressed with high amplitude of change. The IC + w/o and IC + $Al(OH)_3$ group, and the non-challenged NINC group had a gene expression pattern with low amplitude of change. In the trachea more genes were expressed at a higher amplitude of change compared to lung L1 and L4, but the global gene expression

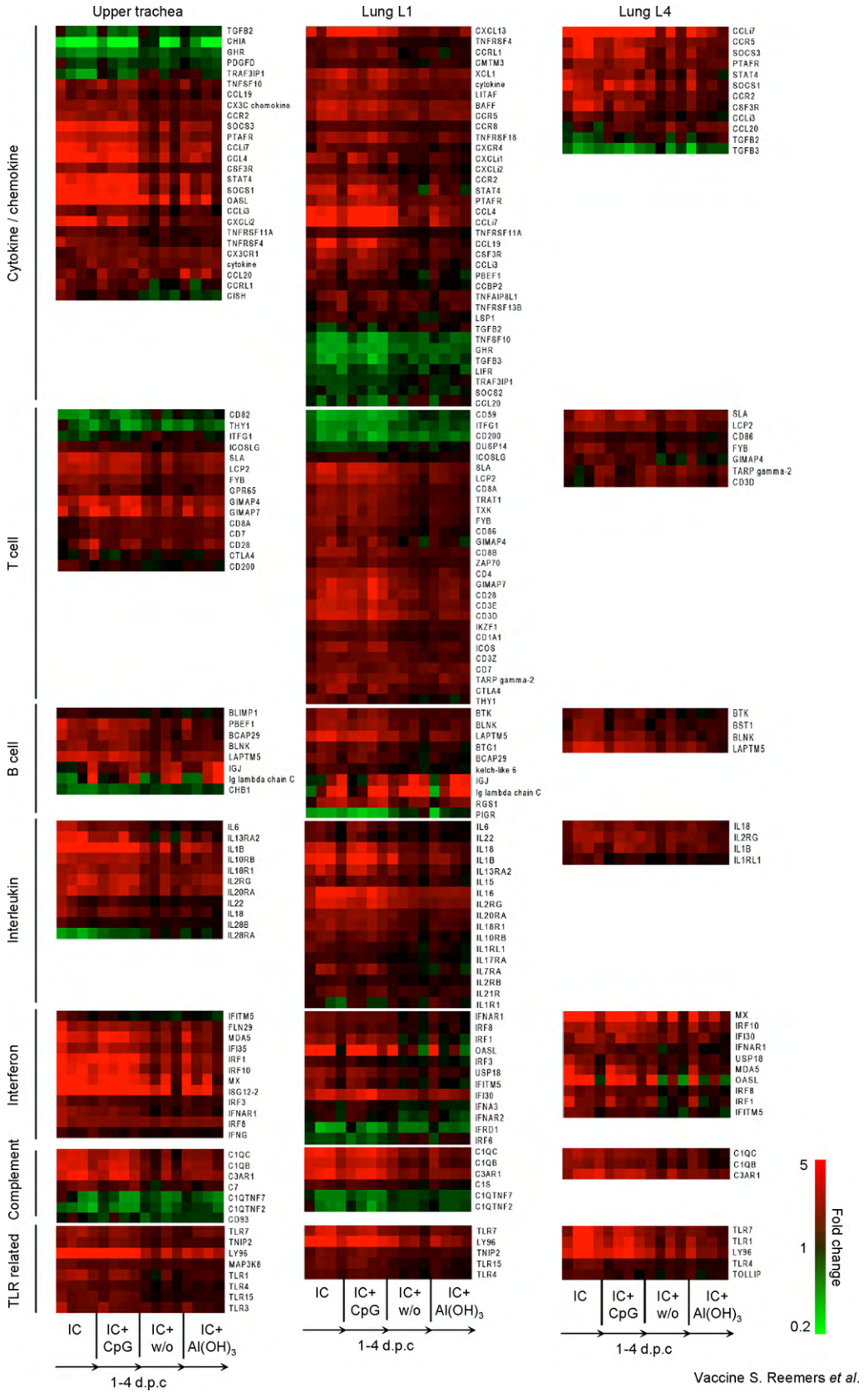

Fig. 4. Lower expression rates of immune related genes were associated with low viral RNA levels in IC + w/o and IC + Al(OH)$_3$ groups. Heatmaps over time showing genes significantly differentially expressed at any day post challenge between IC birds and IC + w/o and/or IC + Al(OH)$_3$ birds in upper trachea, lung L1 and L4 ($n = 4$). Gene expression rates in the heatmaps are the result of comparing expression in challenged birds to expression in non-challenged birds (NINC). Red indicates up regulation and green down regulation. Genes were divided into functional groups based on GO annotations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

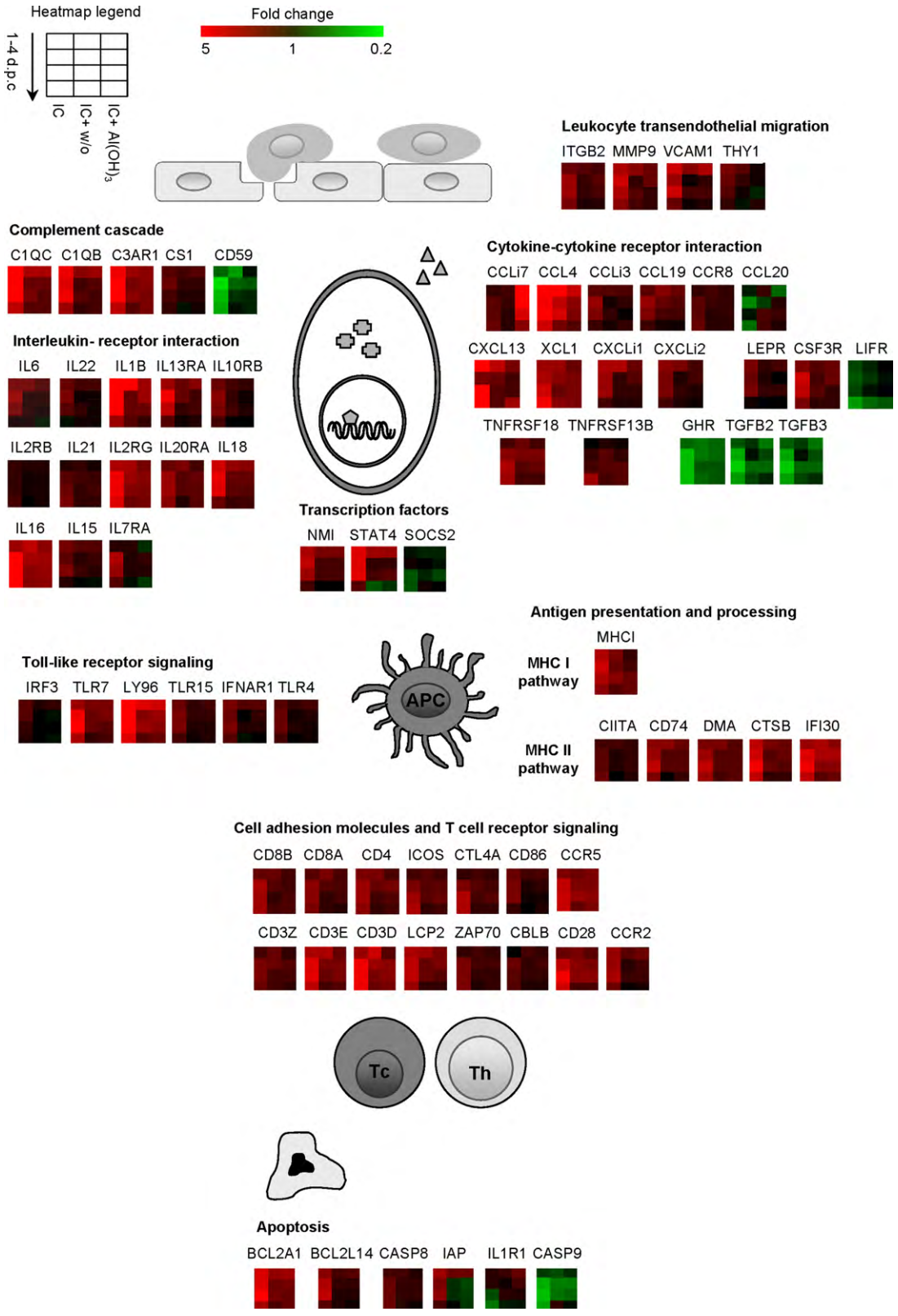

Fig. 5. Brief and low expression of significantly differentially expressed pathways is associated with reduced immune activation and subsequent immunopathology. Pathways showing genes significantly differentially expressed at any day post challenge between IC birds and both IC + w/o and IC + Al(OH)$_3$ birds in lung L1 ($n = 4$). Gene expression rates in the gene individual heatmaps are the result of comparing expression in IC + w/o, IC + Al(OH)$_3$ and IC birds to expression in NINC birds. Red indicates up regulation and green down regulation. Pathways analysis was performed using DAVID and based on KEGG. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

patterns of trachea, lung L1 and to a lesser extent in L4 showed a similar trend. Thus, gene expression patterns can act as predictor of viral RNA levels after challenge, which often correlate to protection.

We determined which genes were significantly differentially expressed in IC + w/o, IC + $Al(OH)_3$ and IC + CpG compared to IC birds to determine adjuvant unique genes. Venn diagrams were made to show the number of genes that were overlapping or unique to the adjuvant (Fig. 3B). Most overlap in genes was seen between the protected IC + w/o and IC + $Al(OH)_3$ birds. Innate defense and interferon related genes were differentially expressed in all adjuvanted groups (in white), but are not correlated to viral RNA levels. The number and the genes expressed differed between locations within the respiratory tract, with lung L1 and L4 being most similar.

3.3. Immune related gene expression profiles

To determine immune related genes which correlate to the difference in viral RNA levels in unprotected versus protected birds, gene expression profiles of IC and IC + CpG birds with high viral load and of IC + w/o and IC + $Al(OH)_3$ birds with low viral load were compared to the non-immunised-non-challenged NINC birds. An immune related gene category was created based on the GO terms host-pathogen interaction, external stimulus and immune response. Immune related genes that were significantly differentially expressed in the groups with low viral RNA levels compared to the IC group with high viral RNA level were selected, divided into functional groups according to the GO annotations, and depicted in heatmaps (Fig. 4). In general these immune related genes expressed in birds with low viral RNA levels were also significantly differentially expressed in birds with high viral RNA levels, but at a much lower expression level in birds with low viral RNA levels. Of these genes, 44 genes were significantly differentially expressed throughout the respiratory tract, trachea, lung L1 and L4, in birds with low compared to birds with high viral RNA levels (IC group; Supplemented data Fig. S1) and related mainly to defense and inflammatory responses and cell differentiation. Again these genes were induced at a lower expression level in IC + w/o and IC + $Al(OH)_3$ birds with a low viral RNA level in all three parts of the respiratory tract, with two exceptions; CCL20 and TGFB2. In lung L4 differences in gene expression level between birds with high or low viral RNA levels were less profound than in L1 and trachea, which correlated to the lower viral RNA expression in L4.

3.4. Pathway analysis of immune related genes

Next, we studied expression of genes within immune related pathways that were significantly differentially expressed in birds with low viral RNA levels compared to birds with high viral RNA levels to obtain a general signature that relates to protection induced after challenge.

Genes involved in leukocyte transendothelial migration were only expressed on 1 d.p.c in birds with low viral RNA levels, but continually in birds with high viral RNA levels, suggesting that in birds only a short period of cellular recruitment occurred. This expression pattern was also seen in the other pathways that were part of the general signature of protection (Fig. 5). Based on differential gene expression the signature of protection after challenge mainly consisted of increased gene expression at 1 d.p.c in birds with low viral RNA levels after which gene expression declined. In contrast, in birds with high viral RNA levels stronger and prolonged gene expression over time was found.

Genes differentially expressed in the complement and coagulation cascade were mainly involved in the classical pathway and followed the expression pattern of the general signature of protection, except for CD59.

In the cytokine-cytokine receptor interaction pathway several genes of many subfamilies were induced. In contrast to the other cytokine and chemokine gene subsets, growth factors were increasingly down regulated in birds with high viral RNA levels, whereas in birds with low viral RNA levels expression was less down regulated.

Subsequently we looked for pathways that were significantly differentially expressed in either IC + w/o or IC + $Al(OH)_3$ birds compared to IC birds. Not one immune related pathway was uniquely significantly induced within an adjuvant group in either w/o or $Al(OH)_3$ immunised birds. However, within the pathways described above, unique genes were found within an adjuvant group (Supplemented data Fig. S2). For w/o 41 unique genes were found and for $Al(OH)_3$ 35 genes. Again expression rates of the these genes were lower in IC + w/o or IC + $Al(OH)_3$ birds compared to IC birds. In conclusion, although IC + w/o or IC + $Al(OH)_3$ birds expressed some unique genes, the expression pattern of these genes remained similar to the general gene expression signature related to low viral RNA levels.

3.5. Effect of adjuvants on recruitment of leukocytes in the lung

Gene profiling indicated that after AIV challenge a low viral RNA expression associated with a low amplitude of change in gene expression. Next, we analysed cell influxes in the respiratory tract using immunocytochemical staining for viral NP, KUL-01$^+$ (macrophages), CD4$^+$ and CD8α^+ cells.

In general, independent of the treatment, virus infected cells were detected at 1 d.p.c. in the larger airways and in adjacent parabronchi in L1. Thereafter more virus infected cells were found in the adjacent parabronchi. In L4 less and smaller virus infected areas were found than in L1, corresponding to the viral RNA levels. In birds with low viral RNA levels (IC + w/o and IC + $Al(OH)_3$), less and smaller virus infected areas in L1 were found compared to the birds with high viral RNA levels (NIC, IC and IC + CpG), but the location of infected areas was similar. No differences were found between these groups, the number and size of virus infected areas were similar in IC and IC + CpG birds to those seen in NIC birds for both L1 and L4. The staining of NP is a measure for virus replication, in contrast to the H9 viral RNA levels which represent the presence of virus replicating and non-replicating. Based on the NP-stained lung sections, the differences in the amount of replicating virus correlated to the differences found in viral RNA expression between the challenged groups and lung L1 and L4.

Influxes of KUL-01$^+$, CD4$^+$ and CD8α^+ cells were seen in lung L1 and L4 in all birds from 1 d.p.c. Independent of the adjuvant, larger influxes of KUL-01$^+$ macrophages and CD4$^+$ cells were seen in L1 than in L4 correlating to the number of virus infected cells. The cell influxes co-localised with virus-infected areas. The size of the CD4$^+$ and KUL-01$^+$ cell influxes correlated to the number of virus infected cells, resulting in fewer influxes in birds with low viral RNA levels and least NP positive cells. Influxes of CD8α^+ cells did not differ between protected and unprotected birds. Influxes of CD8α^+ cells were less dense than KUL-01$^+$ and CD4$^+$ cell influxes (Fig. 6), but also co-localised with virus infected areas.

The recruitment of leukocytes in the lung corresponded to the gene profiles of birds with low and high viral RNA levels. Less virus entry into the lung resulted in smaller influxes of macrophages and CD4$^+$ cells, linked to absence of immunopathology after challenge therefore having fewer and lower gene expression in birds with low viral RNA levels.

3.6. Effect of adjuvants on humoral responses

Since the cause of the lower viral load in the respiratory tract might be prevention of viral entry by virus-specific antibodies, H9-specific HI titres were measured in blood samples taken two

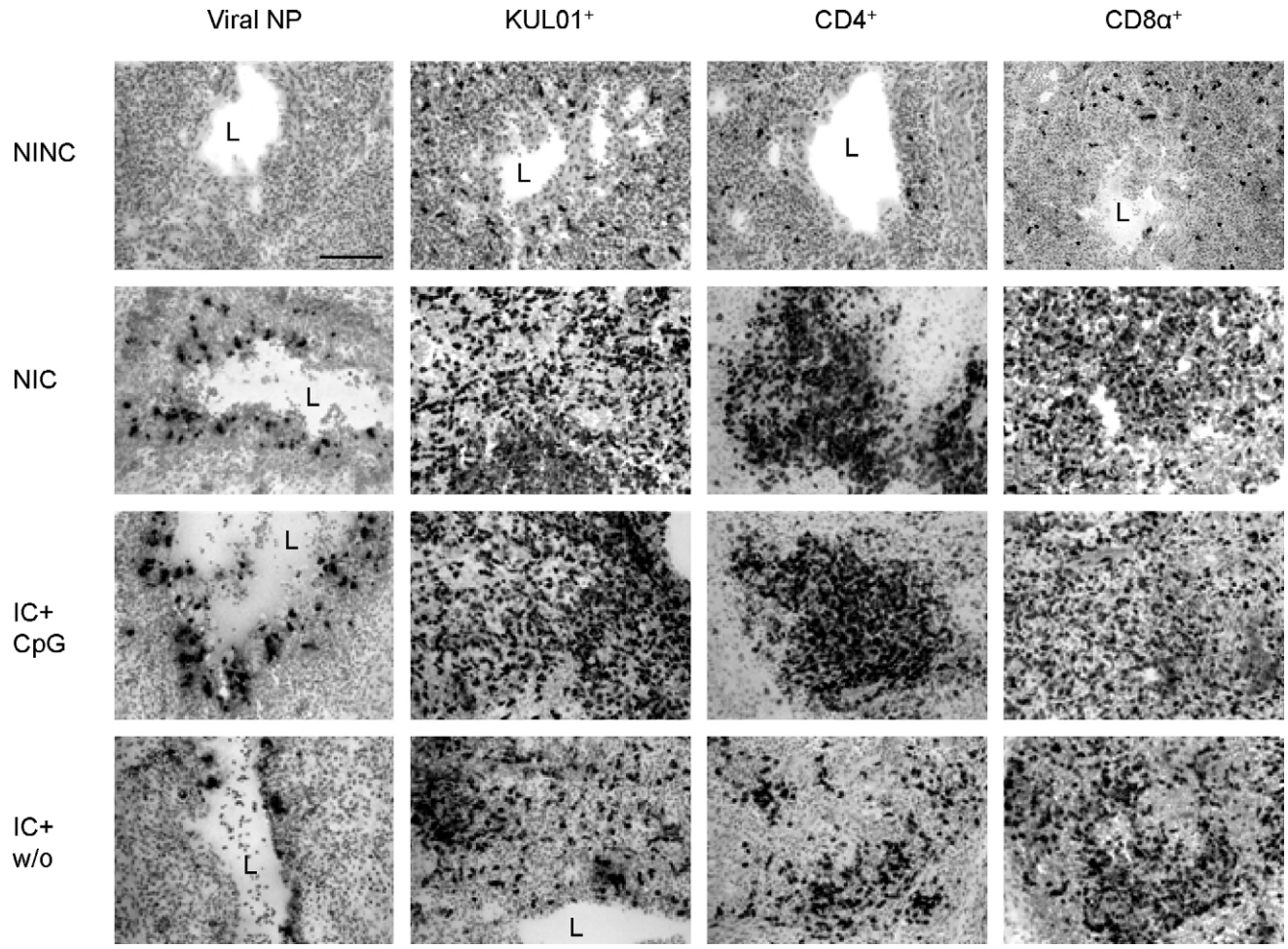

Fig. 6. Less influx of CD4$^+$ cells and KUL-01$^+$ macrophages in lung of birds with low viral RNA levels after H9N2 challenge (IC + w/o). Cryosections of lung L1 of non-immunised-non-challenged (NINC) birds, non-immunised-challenged (NIC) birds, immunised-challenged (IC + CpG) birds and immunised-challenged (IC + w/o) birds stained for viral NP and KUL-01$^+$ macrophages at 2 d.p.i. and for CD4$^+$ and CD8α$^+$ cells at 4 d.p.c. L indicates luminal side of a parabronchus. Bar is 100 μm.

weeks after immunisation prior to challenge (Fig. 7A). A significant increase in HI titer was only found in IC + w/o and IC + Al(OH)$_3$ birds. IC + Al(OH)$_3$ birds had the highest HI titer of 15, which was significantly higher compared to IC + w/o birds (titer of 5). However, this was to be expected because the maximum antibody titer post-vaccination is generally delayed in w/o vaccinated birds when compared to Al(OH)$_3$ vaccinated birds. AIV NP-specific antibody titers were also measured (Fig. 7B) and a significant increase was found in IC + w/o and IC + Al(OH)$_3$ birds which had low viral RNA levels.

4. Discussion

Avian influenza virus (AIV) infection has severe consequences, not only for the worldwide economy but also for human health. As no vaccines have been found yet that protect against a broad range of influenza virus strains much effort is invested in development of new vaccines and improvement of vaccine efficacy.

In this study we set out to investigate predictors of reduced immunopathology and subsequent protection after vaccination and challenge with low pathogenic AIV H9N2 and studied the role of the different immune potentiators w/o, Al(OH)$_3$ and CpG. This was investigated using microarrays, and gene expression profiles were studied in more detail using qRT-PCR and immunocytochemical analysis of the lung. Immunisation of day-old birds in with H9N2 + w/o or H9N2 + Al(OH)$_3$ provided protection based on low viral RNA expression and high HI titers against homologues H9N2 challenge two weeks later, while immunisation with H9N2 + CpG did not. Although detection of antibodies against the internal viral NP protein in vaccinated chickens are not considered to correlate to reduction of the virus load after challenge [23], in our study the NP-titers were significantly increased in birds with low viral RNA levels. In contrast, the presence of antibodies against the surface proteins of AIV measured in HI tests is indicative for a reduction of virus excretion. Two weeks after vaccination of birds with inactivated H7N7 + w/o a HI titer of 16 would lead to protection after homologues challenge [23]. In our study a HI titer of 16 was found in birds with low viral RNA levels, moreover, a reduction in viral RNA level of 100–1000-fold was found in these birds, suggesting that the vaccination with w/o or Al(OH)$_3$ had been successful. When differences in gene expression patterns of birds with low and high viral RNA levels were compared, no specific sets of genes were associated with viral RNA levels and HI titer. Previous studies report subsets of genes defining disease pathology of influenza virus infection in mammals [2,5,30], but no gene subsets have been described relating to protection. Differences in the patterns of gene expression were observed. In birds with low viral RNA levels gene expression rates in lung and trachea were lower and diminished after 1 d.p.c., while gene expression patterns and pathway analysis showed stronger and prolonged gene expression in birds with high viral RNA levels. These data suggest that short-term activation of the immune system after challenge is beneficial for the host pre-

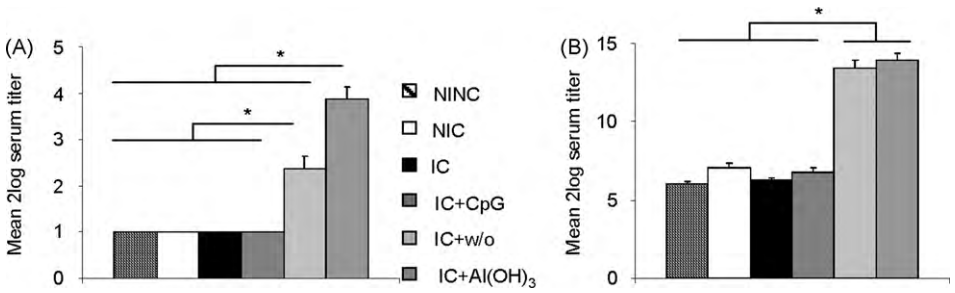

Fig. 7. IC + w/o and IC + $Al(OH)_3$ birds had a significant increase in HI titer (A) and AIV NP-specific antibody titer (B) in contrast to NIC, IC and IC + CpG birds, coinciding with low viral RNA levels. Serum was collected at 14 days after immunisation prior to challenge of all birds ($n = 16$ per group). Data are expressed as mean 2log titers with standard error of the mean (SEM). *Indicates a significant difference ($P < 0.05$).

venting immunopathology induced by overaction of the immune system. Indeed immunocytochemistry showed smaller influxes of KUL-01$^+$ macrophages and CD4$^+$ cells in the lung of protected birds compared to unprotected birds which parallels the microarray data. Thus, rather than finding certain genes or pathways that are predictors of reduced immunopathology and subsequent protection after vaccination and challenge with AIV H9N2, we conclude that the absence of immunopathology induced by overactivation of the immune system is associated with protection.

That reduced immune activation is associated with protection against progressive infection has been reported previously for other viral infections. For example, persistent immune activation after HIV-1 infection has been shown to correlate with progressive disease and immunopathology [12] and non-pathogenic SIV infection in sooty mangabeys was associated with a balanced immune activation despite the presence of high levels of viral replication [36]. Uncontrolled immune activation is also reported for high pathogenic influenza virus. The broad tissue specificity and systemic replication relates to the pathogenicity of H5N1 viruses in animals. Virus induced cytokine dysregulation contributes to disease severity, in that high viral load results in intense inflammatory responses and fatal outcome [2,7,18,31]. Thus, although activation of the immune system is necessary in order to get an anti-viral immune response, too much activation may lead to immunopathology and progressive disease.

In this study a significant difference between the effects of the three adjuvants was found based on viral RNA levels and HI titers. We showed that significant reduction in viral RNA levels after H9N2 challenge was only obtained after immunisation in the presence of immunopotentiators w/o and $Al(OH)_3$. These adjuvants are known to induce a humoral response [14,16] providing high virus-specific antibody responses. Furthermore, w/o emulsions have been demonstrated to enhance cellular immune responses [1,37]. Immunisation in the presence of CpG, which induces activation of macrophages, NK cells and antigen specific CTL did not result in protection [19]. In our study the differences between the adjuvants is most likely explained by the difference in HI titer; adjuvants that induce neutralizing antibodies prevent viral entry. In other studies addition of CpG to *E. coli* bacterin or recombinant plasmids DNA vaccine encoding the VP2 gene of IBDV did provide protection from mortality and induced increased antigen specific antibody titers [10,24]. The difference found with our study may be due to the age of the birds that were used in the different studies. We immunised day-old birds which were challenged 14 days later, while the other studies immunised birds from 7 or 10 days of age and challenged birds at 21 or 30 days of age. Previous data showed that immunization of 1-day-old broilers with BSA resulted in a much lower and slower antibody production compared to immunization at 1 or 2 weeks of age [27]. This suggests that age of the vaccinated birds may be an important factor in the choice of adjuvant, which is highly relevant for the poultry industry.

In conclusion, in this study we show that a limited number of differentially expressed genes correlates with reduced immune activation and subsequently reduced immunopathology after challenge with AIV.

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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.vaccine.2010.06.099.

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