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Kinetics of the avian influenza-specific humoral responses in lung are indicative of local antibody production

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ABSTRACT

The role and kinetics of respiratory immunoglobulins in AIV infection has not been investigated. In this study we determined the numbers of both total antibody secreting cells (ASC) and virus-specific ASC in lung, spleen, blood and bone marrow (BM) following low-pathogenic AIV infection. Antiviral humoral immune responses were induced both locally in the lung and systemically in the spleen. Responses in the lung and BM preceded responses in the spleen and in blood, with virus-specific IgY ASC already detected in lung and BM from 1 week post-primary inoculation, indicating that respiratory immune responses are not induced in the spleen, but locally in the lung. ASC present in the blood of the lungs and co-isolated during lymphocyte isolation from the lungs have no major impact on the ASC detected in the lungs based on statistical correlation.

1. Introduction

In influenza virus infections, humoral immune responses contribute to virus neutralization through binding to viral antigens. In chicken, the role of respiratory immunoglobulins (Ig) in avian influenza virus (AIV) infection has been investigated after vaccination at the level of secreted Ig in tears and serum and antibody-secreting cells (ASC) in Harderian glands (HG), but not locally at the level of ASC in the lung itself. It is known that antibodies are involved in protection against AIV as birds that are protected by vaccination have high serum titers of influenza-specific antibodies (Kumar et al., 2007; Maas et al., 2009; Nayak et al., 2009). In this study we determined numbers of both total ASC and virus-specific ASC following low pathogenic (LP) influenza infection.

The avian lung exhibits both highly organized lymphoid structures and diffusely-distributed lymphoid and myeloid cells. Organized bronchus-associated lymphoid tissue (BALT) structures develop at the junctions of the primary bronchus and the caudal secondary bronchi (Bienenstock et al., 1973; Van Alstine and Arp, 1988). In contrast to the human and mouse lung in which BALT is inducible (Kocks et al., 2007; Moyron-Quiroz et al., 2004; Tschernig and Pabst, 2000), BALT is constitutively present in the chicken lung (Bienenstock et al., 1973). It is suggested that BALT is a normal lymphoid structure in poultry, compensating for the lack of draining lymph nodes (Fagerland and Arp, 1993).

Van Ginkel et al. (2008) studied mucosal and systemic humoral immune responses following ocular influenza vaccination with a haemagglutinin (H)5-expressing replication competent adenovirus (RCA)-free human E1/E3-defective Ad5 vector. High frequencies of H5-specific IgA ASC were observed in HG, and H5-specific antibodies were found in serum and tears. Studies on avian respiratory ASC have been performed using other disease models. In Newcastle Disease Virus (NDV) infection the respiratory antiviral immunity is thought to depend on locally produced antibodies (Al-Garib et al., 2003). Following NDV infection, NDV-specific antibodies can be detected in tracheal washes (Al-Garib et al., 2003) saliva (Ewert et al., 1979) and tears. It is thought that this NDV-specific IgY is transudated from blood to tears (Toro et al., 1993), while the NDV-specific IgM and IgA are mainly produced locally in HG (Russell and Koch, 1993). Polymeric IgA associates with the polymeric Ig receptor (pIgR) (Wieland et al., 2004) and it is therefore expected to be able to transfer across epithelial cells into mucosal secretions, whereas IgY cannot be secreted in this manner.

HA-specific IgA in nasal washes from human volunteers is considered to be an important mediator in protection from influenza infection (Clements et al., 1986). In children IgG found in nasal secretions was locally produced in some cases (Johnson et al.,

Abbreviations: AIV, avian influenza virus; ASC, antibody-secreting cell; BALT, bronchus-associated lymphoid tissue; BM, bone marrow; HG, Harderian gland; IBV, infectious bronchitis virus; Ig, immunoglobulins; LP, low-pathogenic; NDV, Newcastle Disease Virus; p.p.i., post-primary inoculation; p.s.i., post-secondary inoculation; S/N, sample/negative ratio.

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1986), while IgG found in nasal washes from adults was shown to be a serum transudate, with significant correlations between titers in serum and nasal wash (Wagner et al., 1987). In this study, we determined isotype-specific ASC frequencies in chicken after infection with LP H7N1 AIV using ELISPOT assay. We hypothesize that after infection with influenza virus, humoral responses will be induced in the respiratory tract and systemically in the spleen and these responses will differ in isotype that is induced, with local IgA production involved in protection against infection of the respiratory tract.

Antiviral humoral immune responses are induced both locally in the lung and systemically in the spleen. Responses in the lung precede responses in the spleen, with virus-specific IgY ASC already detected in lung from 1 week post-primary inoculation (p.p.i.). ASC present in the blood of the lungs and co-isolated during lymphocyte isolation from the lungs have no major impact on the ASC detected in the lungs based on statistical correlation.

2. Materials and methods

2.1. Chickens

Lohman Brown ED18 eggs were purchased from Verbeeks Broederij BV (Renswoude; the Netherlands) and hatched at the Utrecht University animal facilities. Animals were housed in groups and received food and water at libitum. In compliance with Dutch law, all experiments were approved by the Animal Experimental Committee of the Faculty of Veterinary Medicine of Utrecht University, the Netherlands, in accordance with the Dutch regulation on experimental animals.

2.2. Inoculum

Low pathogenic avian influenza strain H7N1 A/chicken/Italy/1067/99 (kindly provided by Drs. I Capua and W. Dundon) was propagated in SPF embryonated eggs. Allantois fluid was harvested and gross cellular debris was removed by centrifugation at 300*g* for 5 min. To determine viral titer, the virus was inoculated in SPF embryonated eggs. The titer of the virus was 1.6×10^9 EID50/ml. Virus was stored at −80 °C until further use. Chickens were inoculated with 0.1 ml intranasally and 0.1 ml intratracheally, containing 1×10^6 EID50/ml in sterile PBS. For use in ELISPOT virus was first centrifuged for 30 min at 3000*g*, then supernatant was concentrated by ultracentrifugation for 1 h at 100,000*g*. Supernatant was discarded and the pellet resuspended in PBS. Protein content was determined using a BCA protein assay kit (Pierce). The concentrated virus was aliquotted and stored at −80 °C until further use.

2.3. Experimental design

Three-week-old chickens were given a primary inoculation by the intratracheal and intranasal route. One group was sacrificed 1 week p.p.i. Three weeks p.p.i., a second group of birds was sacrificed and two groups of birds were given a secondary infection. One group was killed one week after secondary inoculation (p.s.i.) and the last group was killed 6 weeks p.s.i. At all time points a group of uninfected control chickens was included. Each group consisted of 4 birds. Whole lung including BALT tissue, spleen, bone marrow (BM), whole blood and serum were collected. Part of the lung was used to isolate RNA from; remaining part was used for ELISPOT.

2.4. Preparation of cells

Heparinised blood, lung, spleen, femur and tibia were collected. Lungs were cut into small pieces and digested with 2.4 mg/ml collagenase A (Roche) and 1 mg/ml DNAse I (Roche) solution for 30 min. at 37 °C. BM was flushed out using PBS. Single cell suspensions of spleen, lung and BM were prepared by gently squeezing through a 70 μm cell strainer. Leukocytes were isolated by density gradient centrifugation for 20 min at 1000*g* using Ficoll-Paque (GE Healthcare) and then washed two times with PBS. Cells were then resuspended in RPMI supplemented with 5% FCS, 2 mM glutamax-I, 100 U/ml penicillin and 100 μg/ml streptomycin at 2×10^6 cells/ml. After Ficoll density gradient separation, plasma phase of blood samples was stored at −20 °C and used for ELISA.

2.5. ELISPOT for determining total and virus-specific ASC

Multiscreen 96-wells filtration plates (Millipore) were coated with goat anti-chicken-IgL (Bethyl Laboratories) 10 μg/ml in 50 mM sodium bicarbonate buffer (pH = 9.6) or with 1 μg/well H7N1 virus in sodium bicarbonate buffer. Plates were kept at 4 °C for 1–3 days and blocked with PBS and 5% FCS for 2 h at 37 °C. Blocking buffer was discarded and 50 μl of cells was added, in three different concentrations, starting at 2×10^6/ml and 1:5 serially diluted. Plates were incubated overnight at 37 °C and 5% CO_2. After incubation, plates were washed three times with PBS and 0.05% Tween-20. Mouse anti-chicken-IgM (HIS-C12; Cedi Diagnostics Lelystad), alkaline phosphatase-labeled rabbit anti-chicken-IgY (-AP; ProSci Incorporated) or mouse anti-chicken-IgA (Southern Biotech) was added and incubated for 1 h at room temperature. Plates were washed 4 times. Plates incubated with anti-IgY-AP were developed with NBT/BCIP solution (Roche) and the reaction was stopped with tap water. Remaining plates were incubated with goat anti-mouse-AP (Southern Biotech) for 1 h at RT, washed and subsequently developed. ASC, visible as purple spots, were analyzed using the A.ELVIS machine and the Eli.Analyse software (v5.0) that allows for automated counting of the number of spots based on a minimum spot of 6 and a minimum spot intensity of 5 and the spot slope was set at low.

2.6. ELISA

The competitive blocking ELISA IDEXX FlockChek Avian Influenza MultiS-Screen Ab Test Kit (IDEXX) was used according to manufacturer's instructions. In short, 100 μl per well diluted sample or 100 μl per well positive or negative control serum was added to AIV-coated plates. Plates were incubated for 1 h at RT and washed. Horseradish peroxidase-labeled anti-AIV conjugate was added too detect free coating and plates were incubated for 30 min at RT. Plates were washed and color development was done using TMB substrate solution. Color development was stopped using stop solution and absorbance was read at 650 nm. Sample/Negative (S/N) ratio was calculated as follows: S/N ratio = sample absorbance/negative control absorbance.

2.7. Data analysis

All data were analyzed using Kruskal-Wallis or Mann-Whitney U test with SPSS 16.0 software. Pearson correlations were determined using SPSS 16.0 software. Graphs were prepared using Prism Graphpad 4.

3. Results

3.1. Serum antibodies are detected at 1 week post-secondary infection

The presence of anti-AIV antibodies in serum at different time points after H7N1 infection was determined using ELISA (Fig. 1). In this commercial competitive blocking ELISA, the samples

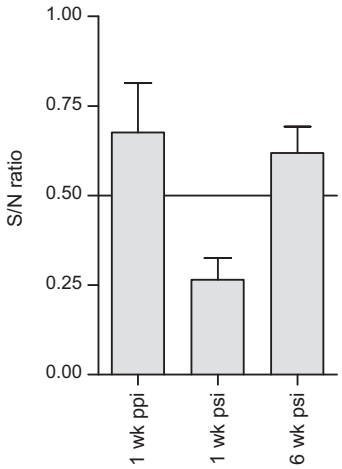

Fig. 1. Influenza-specific antibodies in serum of H7N1-infected chickens. Presence of influenza-specific antibodies in serum was determined 1 week p.p.i., 3 weeks p.p.i. (not shown), 1 week p.s.i. and also 6 weeks p.s.i. using the competitive blocking ELISA IDEXX FlockChek Avian Influenza MultiS-Screen Ab Test Kit (IDEXX). Influenza-specific serum antibodies are detected in samples with S/N ratios <0.5, samples with S/N ratios >0.5 are considered negative. Data are depicted as mean S/N ratio + SEM.

containing AIV-specific antibodies have a sample/negative (S/N) ratio <0.5. Therefore, samples with low S/N ratios contain more AIV-specific antibodies than samples with higher S/N ratios. At 1 week p.p.i. AIV-specific antibodies were only detected in 1 out of 4 chickens. Three weeks p.p.i., all chickens were negative for AIV-specific antibodies (not shown). After secondary infection with the same H7N1 strain, AIV-specific antibodies were detected in all chickens at 1 week p.s.i. Six weeks p.s.i. antibody levels were already decreased below the detection limit in 3 out of 4 chickens.

3.2. Highest numbers of total ASC at one week post-secondary infection

We examined the effects of AIV infection on antibodies produced in lung and systemically in spleen. Therefore, both total and virus-specific ASC ELISPOT were performed. To enumerate possible contamination of blood co-isolated in lung, we also performed ELISPOT using lymphocytes isolated from blood. In human and mouse, BM is a niche for long-living plasma cells (Radbruch et al., 2006; Tokoyoda et al., 2010) and therefore we also performed ELISPOT on lymphocytes isolated from BM.

Following primary infection both at 1 and 3 week p.p.i. (not shown), no differences were found in total non-specific ASC frequencies either in the lung or in the spleen, blood and BM (Fig. 2A–C).

In lung, the secondary infection resulted in increased numbers of IgM ($p = 0.01$) ASC at 1 week p.s.i., which decreased to control levels at 6 weeks p.s.i. (Fig. 2A). In contrast to IgM, no differences were observed in IgY ASC numbers (Fig. 2B) and IgA was increased at 6 weeks p.s.i. ($p = 0.008$) (Fig. 2C).

In spleen, at 1 week p.s.i. increased numbers of ASC of all isotypes ($p < 0.05$ for all isotypes) were detected (Fig. 2A–C). Although the numbers of IgM and IgY ASC were decreased at 6 weeks after secondary infection, IgY ASC numbers were still significantly higher ($p = 0.03$) compared to control spleen (Fig. 2B). Splenic IgA ASC numbers remained high after secondary infection (Fig. 2C).

In blood no significant differences in ASC numbers in response to secondary AIV infection were found due to variation within the group, though increased numbers of IgM and IgY ASC were found at 1 week p.s.i. (Fig. 2A–C).

In BM, secondary infection resulted in significantly increased ASC numbers of all isotypes and numbers remained elevated 6 weeks p.s.i. (Fig. 2A–C).

3.3. Antiviral ASC responses in lung and BM precede responses in spleen and blood

We determined frequencies of H7N1-specific ASC in the different organs using an H7N1-specific ELISPOT. After primary infection, virus-specific ASC are located both in lung and in spleen (Fig. 3A–C). In the lung H7N1-specific IgM and IgY ASC were significantly increased at 1 week p.p.i. (Fig. 3A and B). IgM ASC were elevated at 1 week p.p.i. in spleen and lung but not significantly different from control birds. In spleen, H7N1-specific IgM ASC were also significantly increased in 3 out of 4 chickens at 1 week p.p.i. In contrast to the lung, IgY ASC were not detected at that time point in spleen and blood (Fig. 3B). In BM both IgM and IgY ASC were significantly increased at 1 week p.p.i. (Fig. 3A and B).

Three weeks p.p.i., no H7N1-specific ASC were detected in any of the organs sampled (data not shown).

In the lung after secondary AIV infection numbers of IgM ASC were not affected, while H7N1-specific IgY ASC numbers were increased at 1 week p.s.i. In lung, ASC numbers decreased to control levels at 6 weeks p.s.i. (Fig. 3B). There was no prevalence of numbers of lung IgA ASC compared to lung IgY ASC and within a bird there was no correlation between numbers of H7N1-specific IgY and numbers of H7N1-specific IgA ASC in lung.

In spleen, H7N1-specific IgY and IgA ASC were significantly increased at 1 week p.s.i. and similar to the lung, numbers decreased again at 6 weeks p.s.i. (Fig. 3B and C). In blood, H7N1-specific IgY ASC numbers increased significantly at 1 week p.s.i. (Fig. 3B). Furthermore, low, but significantly higher than the control, numbers of H7N1-specifc IgY ASC are present in BM at 1 week p.s.i. (Fig. 3B), and at 6 weeks after secondary infection H7N1-specific ASC numbers are back to control levels in all organs, except for BM and spleen IgA ASC, which remained elevated (Fig. 3A–C).

3.4. Lung ASC numbers correlate to BM ASC, but not to spleen and blood ASC and serum antibody

In chicken it is not known where an antiviral humoral immune response is induced following AIV infection. We determined correlation coefficients of virus-specific serum antibody and virus-specific ASC (total of all virus-specific ASC per organ) in the different organs to get more information on where responses are induced (Fig. 4). Spleen ASC, blood ASC and serum antibody showed a significant positive correlation with each other (all p values <0.05), but not with lung and BM ASC (all p values >0.5). Furthermore, lung ASC and BM ASC showed a significant positive correlation ($p = 0.01$). Blood ASC and lung ASC showed no significant correlation ($p = 0.9$), suggesting that blood contamination in the lung does not have a major impact on ASC numbers found in the lung.

4. Discussion

In this study we determined the kinetics of the humoral immune responses after both primary and secondary LP H7N1 infection and we determined whether these responses are induced in the respiratory tract or systemically in spleen. To do so, we determined serum antibodies using ELISA and enumerated ASC numbers using a total and an AIV-specific ELISPOT in lung, blood, spleen, and BM at 1 and 3 weeks p.p.i. and at 1 and 6 weeks p.s.i. The choice of time points was based on previous studies. After inoculation with the model antigen Human Serum Albumin, Parmentier et al. (2008) show that humoral immune responses peak at around day 7 post-primary and secondary inoculation and responses are decreased, but still elevated 3 weeks post-inoculation. Furthermore, sampling to detect IBV-specific IgM is optimal between 4 and 8 days post-infection (De Wit et al., 1998). Pei and Collisson (2005) observed IgY ASC

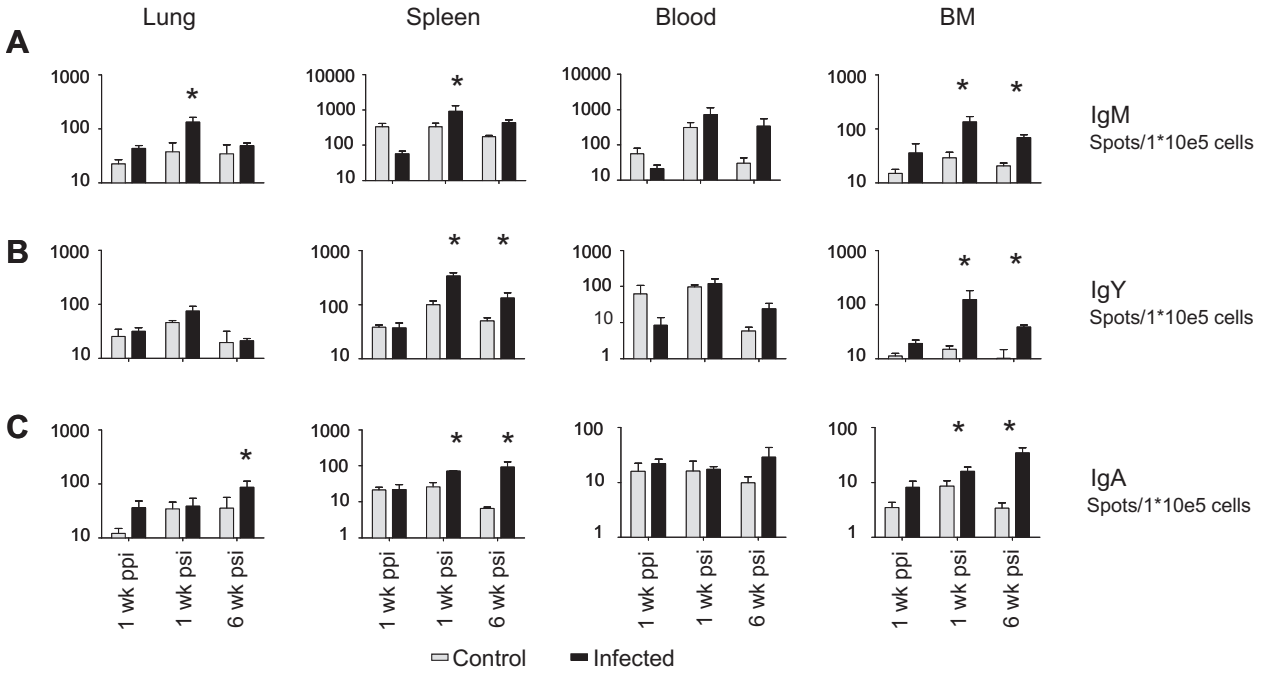

Fig. 2. Total ASC numbers after primary and secondary infection. ASC numbers were determined 1 and 3 weeks p.p.i., and 1 and 6 weeks p.s.i. using ELISPOT. Data of 3 weeks p.p.i. are not shown because there were no differences when compared to control animals. (A) IgM ASC (B) IgY ASC. (C) IgA ASC. Data are depicted as mean + SEM. $^{*}P < 0.05$ for ASC numbers in infected chickens compared to control chickens.

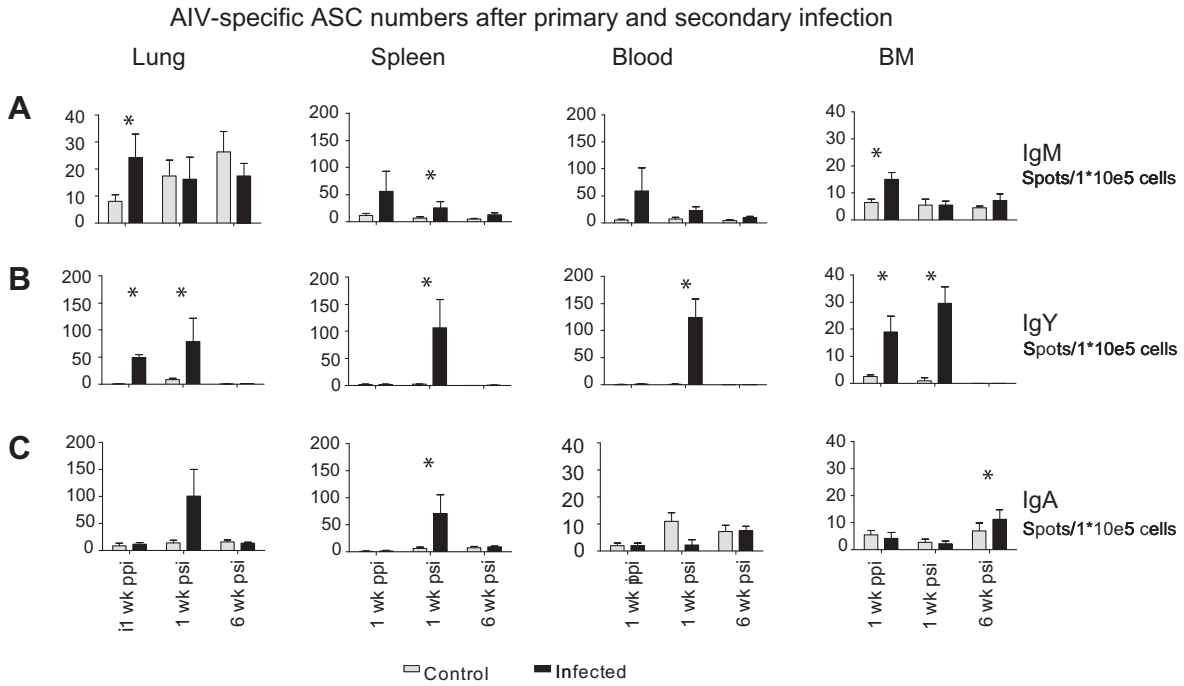

Fig. 3. H7N1-specific ASC numbers after primary and secondary infection. AIV-virus specific ASC numbers were determined 1 and 3 weeks p.p.i., and 1 and 6 weeks p.s.i. using ELISPOT. Data of 3 weeks p.p.i. are not shown because there were no differences when compared to control animals. (A) IgM ASC (B) IgY ASC. (C) IgA ASC. Data are depicted as mean + SEM. $^{*}P < 0.05$ for ASC numbers in infected chickens compared to control chickens.

from 3 days p.p.i and serum IgY from 7 days p.p.i. with IBV. In their study the antibody titers reached maximum levels at 2 weeks p.i. and remained positive until at least week 10 p.i. In vivo, most plasma cells that are generated in a given immune reaction disappear from the secondary lymphoid organs within a few days to weeks. In a secondary immune response in mice, 10–20% of the plasma cells that are generated survive in the bone marrow for more than 2 weeks. The persisting antibody-secreting cells can survive in vivo for long periods and can be detected in BM for at least the next 60 days (reviewed by Radbruch et al., 2006). To test whether

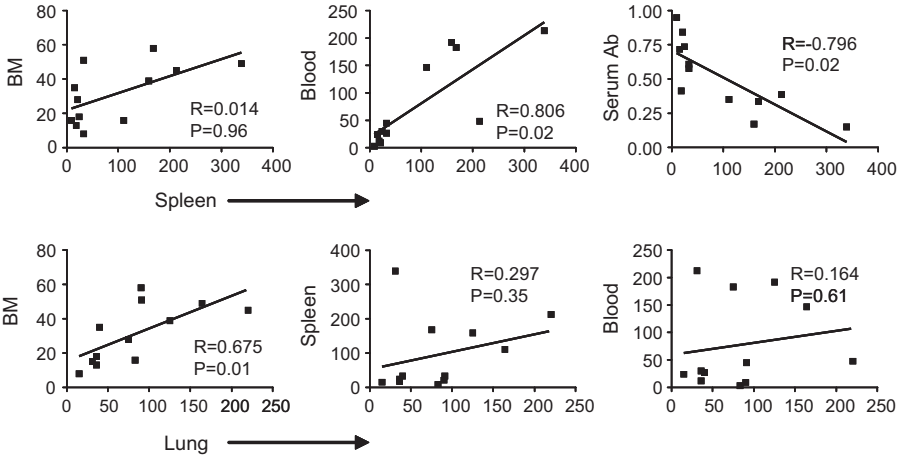

Fig. 4. Correlation of virus-specific serum antibody and virus-specific ASC. Pearson's correlations were determined using totals of AIV-specific ASC in each organ and using S/N ratios of AIV serum ELISA. Each data point depicts an individual chicken.

long-living plasma cells were present in our experiments we also included the 6 weeks p.s.i. time point.

It is already known, that the duration of antibody responses after infection with LP AIV are highly affected by virus strain (Ladman et al., 2008, 2010), dose (Lu and Castro, 2004) and chicken strain (Ladman et al., 2008). In the experiments performed here, chickens were infected with a low virus dose (10^5 EID50/chicken) which could have resulted in a relatively low serum response.

AIV-specific IgY ASC were already detected in lung and BM at 1 week p.p.i. In blood and spleen only AIV-specific IgM ASC were detected at that time point. The low numbers of AIV-specific ASC in blood and spleen are in line with the low serum responses observed in the birds. Following secondary infection AIV-specific IgM ASC were rarely detected, due to isotype switching and differentiation of B cells to IgY and IgA ASC. Indeed IgY and IgA responses were dominant in our experiments consistent with results found in influenza-infected mice (Fazekas et al., 1994; Justewicz et al., 1995), and horses (Nelson et al., 1998) and with kinetics of humoral immune responses in different primary and secondary infection models in chicken (Al-Garib et al., 2003; Beal et al., 2004; Marcos-Atxutegi et al., 2009; Pei and Collisson, 2005). Serum IgY is detected from 7 days following *Ascaridia galli* infection (Marcos-Atxutegi et al., 2009) and also *Salmonella typhimurium* infection (Beal et al., 2004). When a secondary *Salmonella typhimurium* infection is given, IgY and IgA serum responses predominate over IgM responses (Beal et al., 2004), consistent with a secondary immune response.

Serum responses and also AIV-specific ASC numbers declined very rapidly. This might suggest that H7N1 infection generates short-lived plasma cells. Generation of short-lived plasma cells has been described in a mouse vesicular stomatitis virus model, where plasma cells were transferred to naïve mice. In the presence of antigen, high numbers of ASC are maintained, while in the absence of antigen ASC numbers decline rapidly (Ochsenbein et al., 2000).

Lack of correlation between AIV-specific ASC numbers in blood and lung and also the kinetics of AIV-specific IgY responses suggested that ASC present in the blood of the lungs and co-isolated during lymphocyte isolation from the lungs have no major impact on the ASC detected in the lungs based on statistical correlation. This is indirect evidence that the majority of the ASC found in lung originates in the lung. In chicken it is not known were a respiratory immune response is induced following influenza infection, but based on our data we hypothesize that respiratory immune responses could be induced in BALT tissue and that the responses found in spleen reflect the more systemic response at later time points. AIV-specific ASC numbers in lung showed highly significant correlation to H7N1-specific ASC numbers in BM, while responses in blood correlated to responses in spleen. This suggests that the humoral immune response is induced both locally in lung and systemically in spleen and responses observed in lung do not originate in spleen. Furthermore, these results suggest that lung plasma cells could also migrate to BM or that lung APC could migrate to BM and induce immune responses in BM after which mature ASC migrate back to the lung tissue. Possibly the BM is a target site for induction of respiratory immune responses more than the spleen, as also previously described for NDV infection (Russell and Koch, 1993). Besides an increase in virus-specific ASC, also an increase in total ASC was observed. Only a fraction of total ASC is directed against H7N1 AIV, so the remainder is likely the result from bystander activation as described for human plasma cells isolated from peripheral blood. ASC numbers against the recall antigens tetanus toxoid, difteria toxoid, *Toxoplasma gondii* extract, measles, rubella, mumps and cytomegalovirus were enumerated after a boost vaccination and an increase in antigen-specific ASC was always accompanied by an increase in total IgG ASC (Benson et al., 2009; Bernasconi et al., 2002). The increase in total ASC could be a way for B memory cells to enter the pool of plasma cells in an antigen-independent manner and in this way a constant level of plasma cells and serum antibodies with a broad spectrum of specificities could be maintained throughout an animal's life-span (Bernasconi et al., 2002).

In conclusion, in this study we showed the kinetics of the humoral immune responses in chicken after LP H7N1 infection. Responses were maximal at 1 week p.s.i. and declined at 6 weeks p.s.i. As expected, IgM responses predominated during primary infection. Antiviral IgA and IgY humoral immune responses were induced both locally in the lung and systemically in the spleen. Responses in the lung preceded responses in the spleen, with virus-specific IgY ASC already detected from 1 week p.p.i., indicating that respiratory immune responses were not induced in the spleen but locally in the lung.

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