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Crosstalk between innate and adaptive immune responses to infectious bronchitis virus after vaccination and challenge of chickens varying in serum mannose-binding lectin concentrations

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ABSTRACT

Mannose-binding lectin (MBL), a C-type collectin with structural similarities to C1q, is an innate pattern-recognition molecule that is sequestered to sites of inflammation and infections. MBL selectively binds distinct chemical patterns, including carbohydrates expressed on all kinds of pathogens. The present study shows that serum MBL levels influence the ability of chickens to clear the respiratory tract of virus genomes after an infectious bronchitis virus (IBV) infection. The primary IBV infection induced changes in circulating T-cell populations and in the specific antibody responses. Serum MBL levels also influenced IBV vaccine-induced changes in circulating T-cell populations. Moreover, addition of mannose to an IBV vaccine altered both vaccine-induced changes in circulating T-cell populations and IBV specific vaccine and infection-induced antibody responses in chickens with high serum MBL levels. These data demonstrate that MBL is involved in the regulation of the adaptive immune response to IBV.

1. Introduction

The innate immune system is considered the first line of defense against infections. The innate immune system reacts promptly to invading pathogens and instructs antigen presenting cells (APCs) to activate and secrete cytokines that, among other things, regulate T- and B cells towards an appropriate adaptive effector phenotype. Activation of APCs in response to pathogenic stimuli is mediated by unique pattern-recognition receptors (PRRs) and results in the production of pro-inflammatory cytokines and co-stimulatory molecules which participate in the activation of the adaptive immune response [1–3]. Several soluble PRRs have been characterized as LPS-binding proteins, collectins, pentraxins and alternative pathway complement components in addition to cell-associated receptors like C-type lectins, scavenger receptors, Toll-like receptors (TLRs) and various intracellular receptors. Many PRRs are highly conserved and provide the cornerstones upon which the antimicrobial immune response is built. Moreover, vaccine-induced protective immunity can be regulated by PRR stimulation by pathogen-associated molecular patterns (PAMP) in order to perform the "adjuvant effect" which is central to successful vaccinations.

The serum protein mannose-binding lectin (MBL) is an important soluble PRR that belongs to the C-type collectin family. MBL binds to mannose and other sugar residues present on the cell wall of bacteria, viruses and parasites with high affinity [4]. As these sugars do not naturally occur in higher organisms, MBL represents an important mechanism for discrimination between pathogens and host. MBL exhibits a number of innate immunological properties. By binding to, e.g. microbial cell wall antigens, MBL can act as an opsonin and mediate phagocytosis directly through MBL receptors expressed on the surface of neutrophils and monocytes [5–7]. Alternatively, MBL can activate the complement system through the 'lectin pathway' causing pathogen lysis or phagocytosis via complement receptors on phagocytic cells. In addition, MBL is important for the removal of circulating immune complexes. Knowledge of the functional role of MBL in the immunity of both humans and animals is increasing [8–12]. Individuals with MBL deficiency are reported more susceptible to viral and bacterial infections as well as

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autoimmune diseases especially during the early neonatal period or when rendered neutropenic from chemotherapy [13,14]. It has also been shown that MBL deficiency in humans has a genetic basis [15–18].

Results from our lab using chickens selected for a high or a low serum concentration of MBL have shown that a low amount of circulating MBL is associated with increased viral replication in the airway after an infectious bronchitis virus (IBV) infection [19] and reduced growth rate after an *E. coli* infection [20]. We have also shown that low MBL concentration is associated with increased numbers of *Pasteurella multocida* in the spleen in a non-selected exotic breed after an infection [21]. Finally, preliminary results with the parasite *Ascaridia galli* showed that the parasite egg burden post infection was higher in birds with low serum MBL concentration (unpublished). These results confirm that MBL, as proven in mammals, plays a major role in the outcome of various infections in chickens.

An inverse relationship between pathogen-specific antibody responses and serum MBL levels has been recorded in inbred as well as outbred chickens [19,21,22]. Recently, this was also shown in mouse models where the specific IgG response was increased in MBL-deficient mice following immunization with a Group B *streptococcus* vaccine [23] and in double MBL knockout mice injected with soluble hepatitis B surface antigen [24]. These results suggest that MBL influences the production of specific antibodies either by directing the immune response into a more cellular response (Th1 vs. Th2) by neutralizing the microorganism via the complement membrane-attack complex, and/or by inhibiting the production of pro-inflammatory cytokines via interaction with other surface receptors [13]. The latter has been shown in a human whole-blood model where a low concentration of MBL enhances the cytokine production and a high concentration suppresses the cytokine production [25].

A detailed understanding of the interaction between innate and adaptive immunity can lead to new approaches to improve vaccination efficiency, e.g. by blocking/inhibiting the MBL function. This may lead to the discovery of new adjuvants for the vaccine industry. In the future, this will be an important issue as breeding companies currently focus on selection markers that belong to the innate part of the immune system in their effort to produce more robust animals [26]. Improvement of genetically determined disease resistance is an attractive and inexpensive approach to improve the health status of our production animals. Therefore, the challenge in future vaccination strategies is to choose the optimal adjuvant design for innate robust animals. Thus, when vaccinating it may be necessary to temporarily overcome the contribution from the innate part of the immune system in order to allow the development of the adaptive immune response.

Infectious bronchitis (IB) is a highly contagious viral respiratory disease of domestic fowl occurring worldwide and causing great losses to the poultry industry due to the negative impact on the production results and the vaccination costs. The infection is extremely difficult to control because vaccination with one strain of IBV does not necessarily protect against disease caused by other strains of IBV.

The hypothesis of this study was therefore that a temporary blocking/inhibition of the MBL function by adding an MBL ligand to the vaccine would allow the adaptive immune response to produce more specific antibodies and a better development of the protective immunity. In order to address this we performed an experiment where chickens were vaccinated against IB with or without mannose added to the vaccine followed by a challenge with IBV. Immunological parameters belonging to the innate as well as the adaptive immune system were measured throughout the experimental period.

2. Materials and methods

2.1. Animals and experimental design

The experimental chickens ($n = 48$) were offspring from the AU line L10 which has been selected for low (L) vs. high (H) concentration of MBL in serum for several generations [19]. The offspring were reared together until 3 weeks of age under conventional conditions in a bio-secured IBV-free herd and then allocated to 3 different treatments groups with 8 birds from each sub-line in each group. The first group was treated with 200 μL 0.9% NaCl solution and used as a mock-vaccine control group. The second group was treated with 200 μL 0.9% NaCl solution containing one dose of live attenuated Nobilis IB Ma5 Vet (Intervet/Schering-Plough Animal Health, Ballerup, Denmark). The third group was treated with 200 μL 0.9% NaCl solution containing one dose of Nobilis IB Ma5 Vet and 100 mg/mL purified mannan from *Saccharomyces cerevisiae* (Sigma–Aldrich, Brøndby, Denmark) aiming at 100 μg mannose per gram body weight [27]. The solutions were given half nasally and half orally. At day 49 of age the birds were transferred to BSL2 facilities and randomly allocated to 6 isolators each containing 8 animals. At day 50 of age the birds were infected with 200 μL sterile allantoid solution of IBV strain Massachusetts-41 (M41; Veterinary Laboratory Agency, Weybridge, UK) containing $10^{6.5}$ ELD of IBV. The chickens were fed diets that met or exceeded NRC requirements. Feed and water were provided *ad libitum*.

2.2. Blood and swab collection

The experimental chickens were blood sampled from the jugular vein on days 0, 1, 2, 3, 4, 5, 7, 10, 14, 21 and 28 post vaccination (PV) and days 1, 2, 3, 4, 5, 7, 9, 14, 21, and 35 post infection (PI). Serum was collected from all blood sampling time points and citrate-stabilized blood for immune phenotyping was collected on days 0, 7, 14, 21, and 28 PV and days 7, 14, 21, and 35 PI. Oropharyngeal airway (OPA) swab samples were collected on days 0, 2, 3, 4, 5, and 10 PV and on days 1, 2, 3, 4, 5, 7, and 9 PI. Swab samples were kept at −20 °C until the termination of the experiment after which they were transferred to −80 °C and kept there until testing by RT-PCR.

2.3. RT-PCR of IBV

Four swab samples from each subtype, treatment and day were pooled; thus, 2 pools per group were analyzed for each time point. The tubes with the swabs were shaken vigorously for 1 h after thawing and the supernatant was collected after 10 min of centrifugation at 1000 × *g*. Purification of RNA was done according to the instructions for the RNeasy Kit from QIAGEN (Copenhagen, Denmark). The RT-PCR was carried out according to the manufacturer's instructions (TitanTM One Tube RT-PCR System, Boehringer Mannheim), which utilize avian myeloblastosis virus reverse transcriptase and a blend of *Pwo* and *Taq* DNA polymerases. Briefly, for a 50-μL reaction: 10 μL 5× reaction buffer, 2.5 μL 25 mM dithiothreitol, 0.5 μL dNTP (20 mM for each dNTP), 1 μL enzyme mixture, 100 pmol of each oligonucleotide, 5 μL of RNA and RNase-free water up to 50 μL. The stocks and mixtures were kept on ice until the transfer to the thermocycler (Abacus, Hybaid). The RT-PCR was carried out using the following program: cycle 1, 45 min at 48 °C (RT reaction); cycle 2, 95 °C for 120 s; cycle 3 to 30, 95 °C for 30 s, 60 °C for 60 s and 68 °C for 120 s; cycle 31, 68 °C for 7 min. The RT-PCR reaction was performed with the IBVN (+) and the IBVN (−) primers which identify Massachusetts/Connecticut strains:

IBVN (+): 5′-GAA-GAA-AAC-CAG-TCC-CAG-ATG-CTT-GG-3′
IBVN (−): 5′-GTT-GGA-ATA-GTG-CGC-TTG-CAA-TAC-CG-3′

The presence of the PCR products (10 μL sample and 5 μL control sample) was confirmed by agarose gel-electrophoresis, using a 1.5% SeaKem GTG (FMC) agarose and 0.1 mg/mL ethidium bromide. Each band was subjected to densiometric analyses in which raw volumes of the bands were recorded using a GeneGenius gel documentation system (SYNGENE, Cambridge, UK). The raw volume was defined as a three-dimensional size of the band where intensity was the third dimension. Data were further analyzed using the GeneTools software (SYNGENE, Cambridge, UK). Positive control PCR fragments originating from a purified IBV strain M41 were set to 100% and the experimental samples were estimated as percentages of this.

2.4. Measurement of serum MBL

The ELISA measuring the concentration of total MBL in serum was performed as previously described [22,28]. Briefly, microtiter plates (Nunc A/S, Roskilde, Denmark) were coated with 5 μg/mL of the monoclonal anti-chicken MBL antibody HYB182-1 (State Serum Institute, Copenhagen, Denmark) and non-specific binding was blocked before 100 μL of serum dilutions was added to the wells and incubated. Bound MBL was detected with 1 μg/mL of biotinylated HYB182-1. The horseradish peroxidase (HRP) conjugated streptavidin (DAKO, Glostrup, Denmark) was used for visualization. MBL concentrations in serum samples were calculated by linear regression to a standard curve with known concentrations of MBL.

2.5. Serum antibody titers against IBV

The ProFLOK® IBV ELISA Test Kit from Synbiotics (San Diego, CA) was used to measure IgG specific antibody titers against IBV in serum. The ELISA was performed according to the kit manual. Briefly, 96-well microtiter plates coated with IBV antigen were incubated with 50 μL diluted serum samples (1:5) or with the positive and negative controls included in the kit, followed by incubation with a horseradish peroxidase conjugated affinity purified antibody from a pool of serum from goats immunized with chicken IgG (H+L). 2.2′-Azinodi (3-ethyl benzthiazoline sulfonic acid; ABTS) was used as chromogen and 5% sodium dodecyl sulfate (SDS) as stop solution. The result was monitored as optical density (OD) at 405 nm and the antibody titer was calculated from the following equation format: SP = (sample absorbance) − (average normal control absorbance)/corrected positive control absorbance according to the kit manual.

2.6. Flow cytometry

The distribution of different T-cell subset was measured in a whole blood protocol for immunophenotype staining. Fifty microliters of citrate-stabilized blood was mixed with 50 μL antibody solution containing 2 μL FITC-CD8α (3-298), RPE-CD4 (CT4), SPRD-CD3 (CT3), and APC-CD45 (LT40) in FACS-buffer (0.2% BSA, 0.2% sodium azide, 0.05% normal horse serum in PBS). The cell suspension was incubated at 4 °C for 15 min in a 4-mL tube in darkness, washed once in 2 mL FACS-buffer and centrifuged at 295 × *g* for 5 min. The supernatant was discarded and the cells were resuspended in 100 μL FACS-buffer. All flow cytometric analyses were performed on a BD FACSCanto (Beckton Dickinson) equipped with a 488 nm blue laser and a 633 nm red laser. A minimum of 80,000 cells in the storage gate (FSC/SSC-defined lymphocyte gate) was collected from each sample using the FACSDiva software. Single-stained compensation controls as well as negative fluorescence minus one (FMO) controls were included. All monoclonal antibodies were obtained from Southern Biotech (Birmingham, AL).

2.7. Ethical aspects

License to conduct the animal experiment was obtained from the Danish Ministry of Justice, Animal Experimentation Inspectorate by Helle R. Juul-Madsen. The experiment was conducted according to the ethical guidelines.

2.8. Statistics

The model used was as follows: $y = \mu + G_i + S_j + G \times S_{ij} + e$, where μ = overall mean, G = fixed effect of the ith genetic group, S = fixed effect of the jth sampling day, and e = residual—not explained. The analysis of variance was performed by the GLM procedure of the SAS software (1994).

3. Results

3.1. Experimental outline

Chickens from L10, selected for high (H) or low (L) MBL in serum, were vaccinated at 21 days of age with a commercial attenuated live IBV vaccine followed by a challenge infection with the Massachusetts strain of IBV (M41). The chickens were divided into three groups: mock-vaccinated (group 1), vaccinated with IBV vaccine without mannose added (group 2), vaccinated with IBV vaccine containing mannose (group 3). All chickens were challenged with IBV 28 days PV.

3.2. Replication of vaccine and virus

The replication of the vaccine virus PV and the M41 virus PI in the OPA was monitored by a Massachusetts/Connecticut strain specific RT-PCR on swabs sampled from days 0 to 10 PV and days 1 to 9 PI. Group 1 was tested only after infection (Fig. 1a). The PCR-amplified products were subjected to densitometry analysis and adjusted to a positive control containing purified M41 which was set to 100%. The adjusted percentages of virus genomes are shown in Fig. 1b. The vaccine virus was detected in the OPA from days 2 to 10 peaking at days 4 to 5 PV, but no difference was observed between the two groups (groups 2 and 3) PV. After infection with M41 no viral genomes were detected in the vaccinated chickens (groups 2 and 3). Unvaccinated chickens (group 1), on the other hand, had a very high replication of viral genomes from days 1 to 9 PI. At day 7 PI it appeared that the number of viral genomes had decreased in H-chickens, but still not in L-chickens. At day 9 PI all unvaccinated chickens had cleared the virus from the OPA.

3.3. The MBL acute phase response

The MBL acute phase response in serum samples was measured during the whole experimental period (Fig. 2). No response was seen PV after mock vaccination in group 1, neither in H-chickens nor in L-chickens. However, in groups 2 and 3 a weak acute phase response was observed after vaccination—most pronounced in L-chickens. The response peaked at days 2–3 PV followed by a decrease in MBL serum level from days 3 to 10 PV. At day 10 the MBL level was below the baseline seen at day 0 PV (approx. 40–65% decrease). The MBL levels subsequently increased to a higher level than the baseline just before infection (approx. 26–37% increase)—most pronounced in H-chickens. This increase was not age-dependent since the mock-vaccinated group (both H and L) had identical MBL levels at day 0 PV and day 0 PI. As expected, the mock-vaccinated group (group 1) had a higher MBL acute phase response after infection than the two vaccinated groups (groups 2 and 3) upon challenge infection. L-chickens peaked at day 2 PI, whereas H-chickens peaked twice at days 2 and 5, respectively. Subsequently

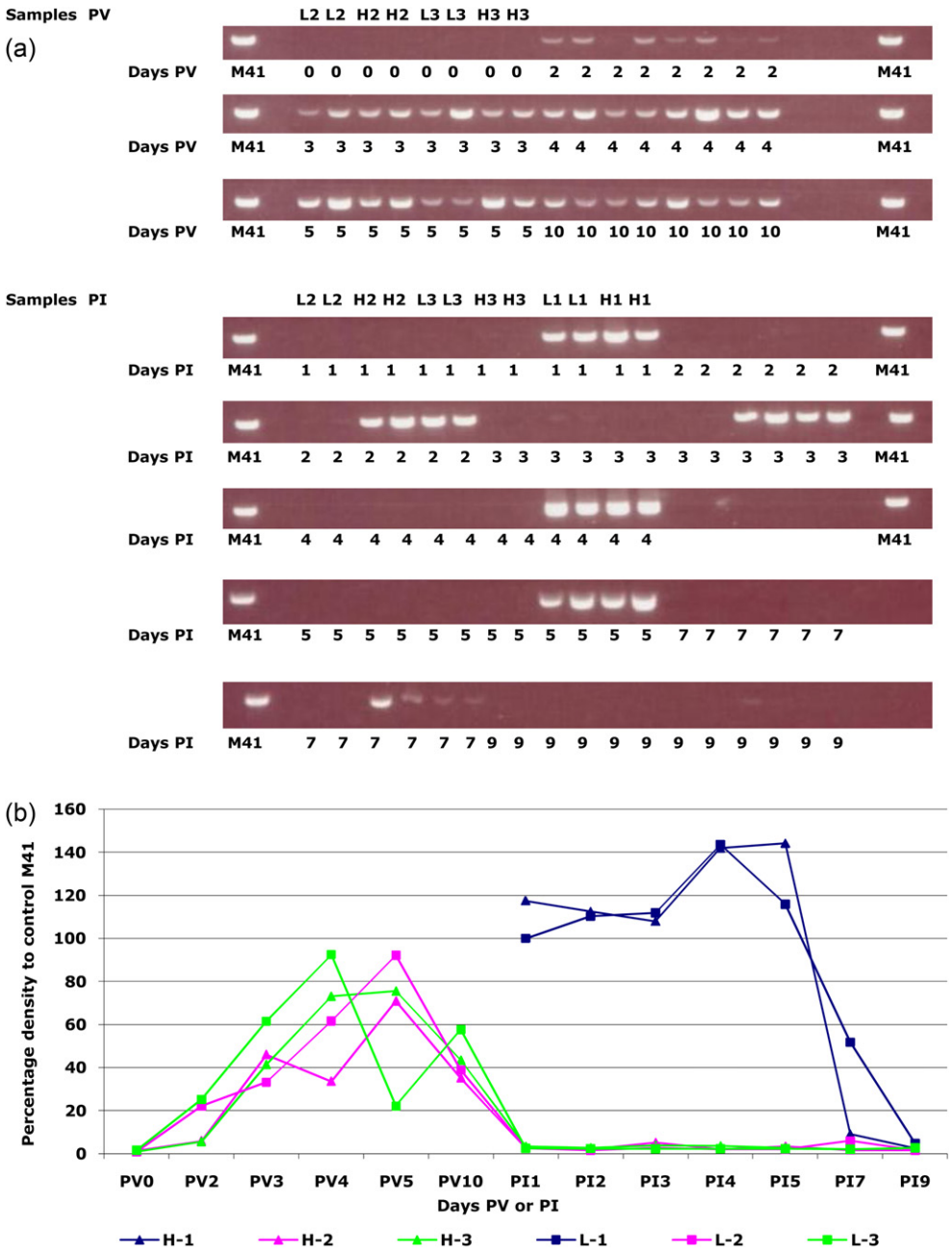

Fig. 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) profiles of IBV genomes detected in oropharyngeal airway swab samples. The IBV genomes were visualized by ethidium-bromide-stained 1.5% agarose gels. (A) The first and last samples in each row are the positive M41 control except for the last two rows where the M41 control is missing at the right side. The first three rows show samples post vaccination (PV) with a series of 8 samples from the days indicated. The last five rows show samples post infection (PI) with a series of 12 samples from the day indicated. (B) The relative percentage density of virus load in oropharyngeal airway swab samples collected PV and PI. H or L indicates the sub-lines with high or low serum concentration of MBL, respectively; 1, 2 or 3 indicates the treatment groups '−vaccination − mannose' (1), '+vaccination − mannose' (2), and '+vaccination + mannose' (3).

both sub-lines in group 1 showed decreasing amounts of MBL until day 9 PI (approx. 29–94%) after which an increase was observed. Both sub-lines in group 1 ended the experiment with higher levels of serum MBL after the infection than before the infection (approx. 41–58%), which is comparable to what was observed for groups 2 and 3 PV.

3.4. The IBV specific IgG antibody titer

The IBV specific IgG antibody titers were measured by ELISA once a week during the experiment (Fig. 3). As expected, chickens from group 1 were negative for IBV antibodies until after the challenge infection where seroconversion was detected 14 days PI. At the termination day of the experiment a difference between the two sub-lines was observed in IBV titers where L-chickens appeared to have the highest titers although not significantly. In group 2, L-chickens tended to have higher antibody titers 14 days PV ($p = 0.07$). After the challenge infection a high production of antibodies was observed already 7 days PI in both sub-lines after which the titer decreased. However, no differences in antibody titers between the 2 sub-lines were observed PI due to the large variation between birds. In group 3, on the other hand, L-chickens showed a clear antibody response PV with a higher mean antibody titers at 21 days PV ($p = 0.06$) and at 28 days PV ($p = 0.03$) compared

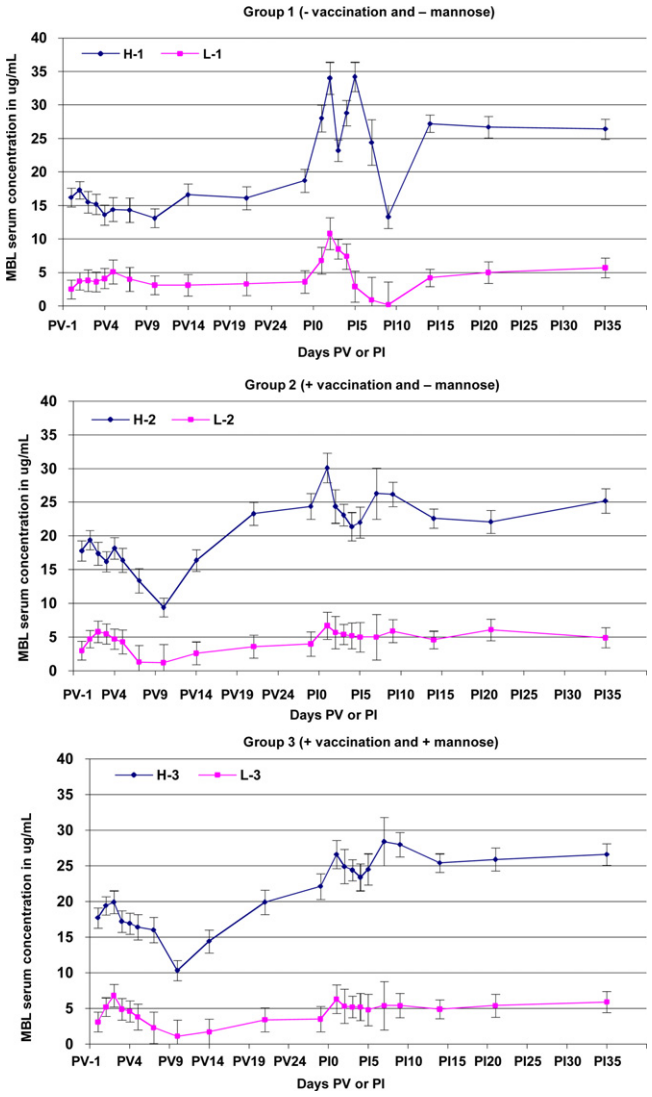

Fig. 2. The MBL serum concentration in L and H-chickens from day 0 to day 28 post vaccination (PV) with live attenuated IBV strain Ma5 vaccine and from day 1 post infection (PI) to day 35 PI with live IBV strain M41. The upper panel shows group 1, the middle panel group 2, and the lower panel group 3. Results are shown as mean values ($n = 8$) ± SE.

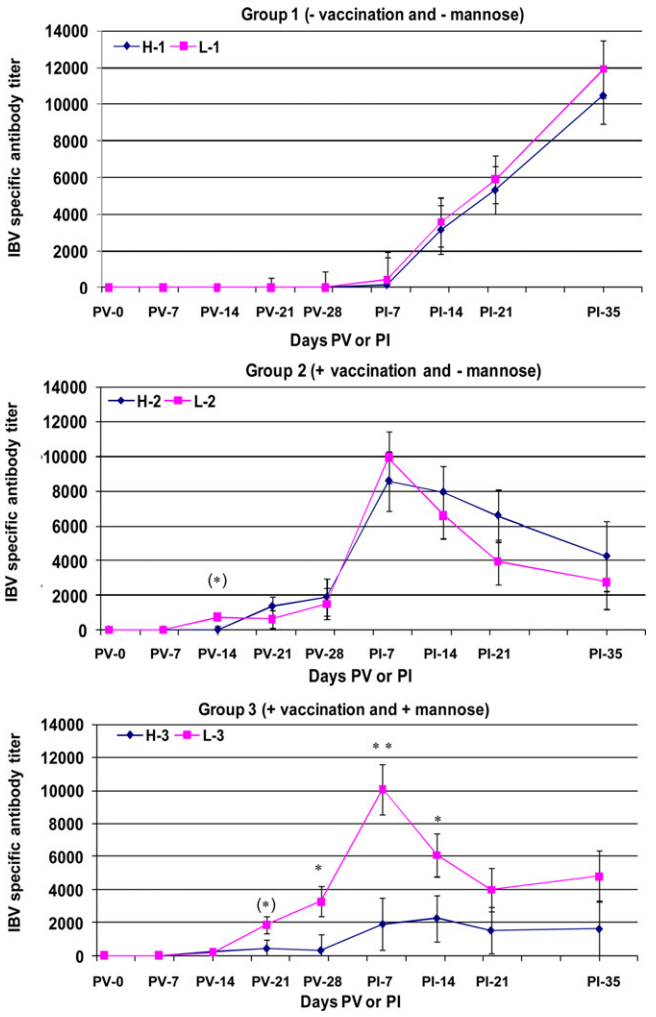

Fig. 3. The specific IBV antibody titer in L and H-chickens measured from days 0 to 28 post vaccination (PV) with live attenuated IBV strain Ma5 vaccine and from days 7 to 35 post infection (PI) with live IBV strain M41. The upper panel shows group 1, the middle panel group 2, and the lower panel group 3. Results are shown as mean values ($n = 8$) ± SE. Statistically significant differences between H and L-chickens are shown by stars. *Indicates $p < 0.05$ and **indicates $p < 0.01$.

to H-chickens. After the challenge infection, the difference between L- and H-chickens was even more pronounced with L-chickens displaying significantly higher antibody titers than H-chickens 7–14 days PI ($p = 0.0006$ and $p = 0.05$).

3.5. Flow cytometric assessment of T-cell subsets

A flow cytometric protocol was used for staining T-cell subsets in whole blood. The T-cell subsets were identified via FSC/SSC gating on small lymphocytes, identification of $CD45^{hi}$ positive cells (lymphocytes), followed by identification of CD3 positive cells and finally by sub-dividing the CD3 positive cells into single CD4 positive cells, single CD8α positive cells and double positive CD4CD8α cells (Fig. 4a). The results are presented in Fig. 4b. In group 1, no difference in the percentage of CD8α+ cells was found before infection, but the percentage of these cells in L-chickens was higher than in H-chickens 14 days PI ($p = 0.04$) and a similar tendency was seen 21 days PI ($p = 0.07$). In group 2, the percentage of CD8α+ cells in L-chickens was higher than in H-chickens at 21–28 days PV ($p < 0.05$) and at 7–21 days PI ($p < 0.04$). In group 3, the percentage of CD8α+ cells in L-chickens was higher than in H-chickens at 35 days PI ($p = 0.004$) only. No differences in the percentage of CD4+ cells including CD4+CD8α+ double positive cells were found between the sub-lines for any of the treatments. CD4 single positive cells and CD4CD8α double positive cells were combined due to individual differences in the percentage of double positive cells which originate from CD4 positive cells (data not shown). Finally, major differences between the sub-lines were found in cells that were CD3 positive, but CD4 and CD8α negative. In group 1, the percentage of CD4−CD8α− cells in H-chickens was higher than in L-chickens at 14–21 days PI ($p < 0.03$) and a similar tendency was seen at 35 days PI ($p = 0.08$). In group 2, the percentage of CD4−CD8α− cells in H-chickens was higher than in L-chickens at 14–28 days PV ($p < 0.04$), and finally the percentage of CD4−CD8α− cells in H-chickens was higher than in L-chickens at 21–28 days PV (0.001) and at 14–35 days PI in group 3.

4. Discussion

Following primary IBV infection (group 1) a very high level of virus replication was observed in the oropharyngeal airway of all the birds. However, 7 days PI the virus was cleared from the

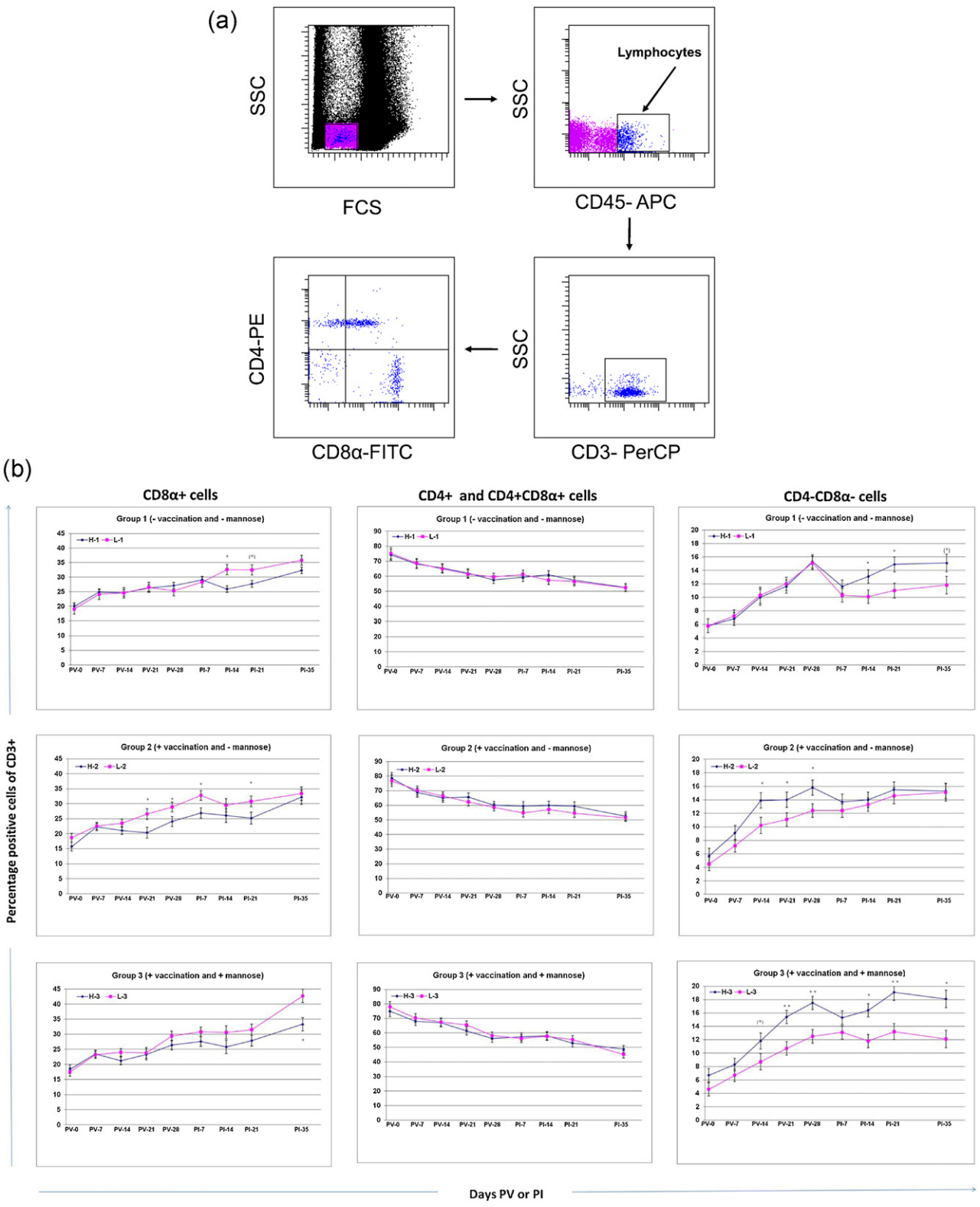

Fig. 4. Whole blood flow cytometry. (A) A flow diagram showing the gating strategy. First, small lymphocytes were gated on FCS/SSC dot plots, then $CD45^{high}$ (lymphocytes) were identified, followed by gating on CD3 positive cells. The CD3 positive cells were finally divided into CD4 or CD8 positive cells. (B) The CD8α+ percentage of CD3+ cells in the left column, the CD4+ percentage plus the CD4+CD8α+ percentage of CD3+ cells in the middle column, and the percentage of the CD4−CD8α− percentage of CD3+ cells is shown to the right. The upper panels show group 1, the middle panels group 2, and the lower panels group 3. Results are shown as mean values ($n = 8$) ± SE. Statistically significant differences between H and L-chickens are shown by stars. *Indicates $p < 0.05$ and **indicates $p < 0.01$.

respiratory tract of H-chickens whereas clearance did not occur in L-chickens until 9 days PI (Fig. 1) indicating that animals with low MBL concentration in serum harbor this virus longer than animals with high MBL concentrations as previously suggested [19].

The addition of mannose to the vaccine did not alter its capacity to induce protective immunity since no replicating virus was found in samples from the vaccinated group after infection with the IBV strain M41 even though we used a very high dose of virus

for the challenge. Recently, new field strains of IBV have been isolated in domestic poultry in China (QX) and Europe (QX-like IBV) causing severe losses to both the layer and the broiler industry [29] because vaccination is no longer fully protective against these new strains. With hindsight, one of these strains may have been a better choice as challenge virus since both groups of animals were protected against infection with the M41 strain. In order to detect any effect of MBL and mannose in relation to vaccine-induced protection against IBV, a more virulent strain should therefore be used in further experiments.

By analogy with previous results [19], the MBL acute phase response was observed after IBV vaccination and infection followed by a decrease to below the baseline level [19] (Fig. 2). In the present study, this response was most pronounced in group 1 which was only challenge-infected. The acute phase response was much weaker after the challenge infection in the two vaccinated groups (groups 2 and 3) probably due to the adaptive memory response. No influence of mannose addition to the vaccine was observed for serum MBL levels. The decrease in serum MBL after the vaccination or the infection to below the baseline level may be due to a feedback mechanism from other parts of the immune system. This feedback mechanism may have occurred at the MBL transcriptional level, or alternatively by transfer of MBL from the blood to infected tissues thereby lowering the concentration of MBL in the blood as proposed by Valdimarsson et al. [30] or simply because MBL has been used for opsonization and thus has been engulfed by phagocytic cells. The decrease was then followed by an increase to above baseline level in all three groups which we have no explanation for. Age can be left out as an explanation at least after the vaccination since there was no increase in the mock-vaccinated group.

Previously, we have shown in inbred as well as outbred animals that there was a negative correlation between the concentration of MBL in serum and the ability to produce specific antibodies after an infection [19,21,22] which may be caused by an interplay between the innate and the adaptive immune system. In this study, we found no difference between the specific antibody titers in H- and L-chickens in group 1 after infection. This difference may be attributed to the time point chosen for termination of the experiment, i.e. too early, or the titer of the virus (it was calculated to $10^{6.5}$ ELD), i.e. too high, On the other hand, we did find differences between the two sub-lines in group 3, which received mannose together with the vaccine, both PV and PI (Fig. 3). H-chickens receiving mannose in combination with the vaccine showed very low specific IBV IgG antibody titers compared to L-chickens in the same treatment group, whereas the titers of L- and H-chickens were very similar in group 2 that did not receive mannose. This indicates that high serum levels of MBL influence the production of specific antibodies and that addition of mannose to such high birds MBL with high MBL-levels alters their antibody production in a negative way. Likewise, one may likewise speculate that in birds with constant low levels of serum MBL compensatory mechanisms for induction and regulation of antibody responses are activated and therefore mannose addition does not affect antibody production in L-chicken. The direct involvement of other mannose specific receptors can be ruled out since no difference was found in the L-chickens from groups 2 and 3 presuming that these receptors are genetically identical in the two sub-lines.

As mentioned earlier, a higher specific IgG response was found in MBL-deficient mice following immunization with a Group B *streptococcus* vaccine [23] suggesting that MBL plays an inhibiting role in IgG antibody production. Further, it was shown that the increase in antibodies was restricted to T-cell-dependent antigens and that the antibodies were of the IgG1 subtype which has high affinity for Fc receptors on phagocytotic cells indicating that MBL plays a role in regulating the adaptive immune response through signaling in antigen presenting cells.

Recently, this was supported in a paper by Zhou et al. (2010) [31] who showed a direct MBL-mediated neutralization of severe acute respiratory syndrome-coronavirus (SARS-CoV) by blocking the binding of the virus specific S glycoprotein (SARS-S) to the DC-SIGN receptor (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin-related molecule) suggesting that MBL most likely recognizes an overlapping set of high-mannose-content glycans on the S glycoprotein and thereby competes with DC-SIGN for binding. DC-SIGN is known to bind to different pathogens and triggers intracellular signaling cascades that affect TLR signaling and thereby adaptive immune responses (reviewed in [32]). The specific modulation of the immune responses is dependent on the pathogen involved and can lead to the inhibition or promotion of T helper type 1 (Th1) polarization, Th2 responses and/or induction of regulatory T cell differentiation [33]. In chickens it has been shown that the S protein of IBV alone is sufficient to induce protection [34].

For a better understanding of the humoral response, it will be necessary to measure the titer of IBV-specific IgM antibodies, because it has been shown that MBL is intimately involved in priming the humoral Ab response in mice [35]. Carter et al. (2007) [35] found a profound abrogation of antigen-specific IgM levels in *B. malayi*-infected MBL-A deficient mice in addition to higher levels of some IgG isotypes, thus suggesting that MBL-A plays a critical role in the level of IgM production and/or in the regulation of the degree of class switching to IgG isotypes. However, it should be mentioned that even though the H-chickens have a very low specific antibody titer in this study, the chickens were protected against the infection as no M41 virus was replicating in the OPA (Fig. 1) indicating that antibodies may not be decisive for protection against IBV as previously suggested by others [36].

In order to address cellular immune responses, phenotyping of three different general circulating T-cell populations: CD8α+^{high}, CD4+CD8α^{dim}+/− and CD4−CD8α− were performed during the present experiment (Fig. 4). Within these populations the majority of the CD8α+^{high} cells in the circulation are αβ T-cell receptor (TCR) T-cells expressing the CD8αβ heterodimer on their surfaces (Fig. 4a), i.e. they have the cytotoxic T-lymphocyte (CTL) phenotype. The CD4+ cells are considered "classical" T-helper cells, whereas the majority of the CD8+^{dim} positive cells also carry the CD4 molecule on the cell surface and hence are double CD4CD8 positive αβ TCR T-cells expressing the CD8αα homodimer. The function of the latter population is still unknown in the chicken but in the pig they have been identified as memory cells [37]. In the circulation, chicken CD4−CD8− is dominated by γδ TCR expressing T-cells [38,39]. Chickens belong to a group of animals that can have 20–50% of γδ T-cells in the circulation [40], which suggests that γδ T-cells play an important role in the chicken immune system. However, the function of chicken γδ T-cells is still largely unknown, but in vitro studies have shown that they can have cytotoxic and immuno-regulatory properties. Moreover, it is not known if chicken γδ T-cells recognize classical peptide MHC antigens or bind to alternative antigens. In mammals, the function of γδ T-cells is likewise not firmly identified; studies in humans [41] and cattle [42] have for instance revealed the existence of several subpopulations of γδ T-cells, that recognize both MHC and non-MHC-associated antigens. These studies have suggested a number of functions such as killer cells, classical T cells with central memory, immune-modulators through cytokine production and as antigen presenting cells.

Overall, the CD4+CD8α^{dim}+/− cells were not influenced by vaccination or infection in the present study. However, for H-chickens a decrease in CD8α+^{high} cells – which in most cases was complemented by an increase in CD4−CD8α− cells – was observed after IBV vaccination and infection (Fig. 4b). This increase in

CD4−CD8− cells was most pronounced in those H-chicken where mannose was added to the vaccine (group 3). Interestingly, it has recently been shown in humans that γδ T-cells can proliferate, express memory markers, and produce cytokines in response to infection with *Candida albicans* which contains mannose structures [41]. Our results (Figs. 3 and 4b) indicate a role of CD4−CD8− γδ T-cells in the defense against IBV especially in chickens with low levels of specific antibodies to the virus, e.g. as killer cells. An alternative explanation is that the CD8α+^{high} αβ T-cells, i.e. CTL's, in these chickens have been translocated from the circulation to the respiratory organs, because it has been shown by adoptive transfer that αβ T-cells bearing CD8 molecules are critical in protecting chickens from IBV infections [36]. This could, however, be addressed by absolute rather that relative counting the different T-cell subtypes in the blood which will be obvious to do in the next experiment.

In conclusion, MBL seems to be associated with clearance of IBV as previously indicated [19]. The acute MBL response peaked 2–3 days PV or PI followed by a down regulation which was most pronounced in H-chickens. The specific antibody titer was highest in L-chickens and mannose seems to inhibite the production of specific antibodies, but only in the H-chickens. The H-chickens, on the other hand, showed decreases in CD4−CD8α+ cells and increases in CD4−CD8α− γδ T-cells after vaccination and infection which suggested that antibodies and T-cells have complementary functions in the protection against IBV. These results indicate that MBL is involved in the regulation of the adaptive immune response to IBV. MBL should therefore be considered when designing future vaccines in relation to inhibition or promotion of T-helper type 1 (Th1) polarization, Th2 responses and/or induction of γδ T-cells. More research is needed to elucidate the importance of MBL including studies with other MBL ligands.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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