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## Adjuvant effects of mannose-binding lectin ligands on the immune response to infectious bronchitis vaccine in chickens with high or low serum mannose-binding lectin concentrations



Rikke M. Kjærup<sup>a</sup>, Tina S. Dalgaard<sup>a</sup>, Liselotte R. Norup<sup>a</sup>, Ingrid-Maria Bergman<sup>a</sup>, Poul Sørensen<sup>b</sup>, Helle R. Juul-Madsen<sup>a,\*</sup>

<sup>a</sup> Department of Animal Science, Aarhus University, Blichers Allé 20, P.O. Box 50, DK-8830 Tjele, Denmark

<sup>b</sup> Department of Molecular Biology and Genetics, Aarhus University, Blichers Allé 20, P.O. Box 50, DK-8830 Tjele, Denmark

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### ABSTRACT

Mannose-binding lectin (MBL) plays a major role in the immune response as a soluble pattern-recognition receptor. MBL deficiency and susceptibility to different types of infections have been subject to extensive studies over the last decades. In humans and chickens, several studies have shown that MBL participates in the protection of hosts against virus infections. Infectious bronchitis (IB) is a highly contagious disease of economic importance in the poultry industry caused by the coronavirus infectious bronchitis virus (IBV). MBL has earlier been described to play a potential role in the pathogenesis of IBV infection and the production of IBV-specific antibodies, which may be exploited in optimising IBV vaccine strategies. The present study shows that MBL has the capability to bind to IBV *in vitro*. Chickens from two inbred lines (L10H and L10L) selected for high or low MBL serum concentrations, respectively, were vaccinated against IBV with or without the addition of the MBL ligands mannan, chitosan and fructooligosaccharide (FOS). The addition of MBL ligands to the IBV vaccine, especially FOS, enhanced the production of IBV-specific IgG antibody production in L10H chickens, but not L10L chickens after the second vaccination. The addition of FOS to the vaccine also increased the number of circulating CD4+ cells in L10H chickens compared to L10L chickens. The L10H chickens as well as the L10L chickens also showed an increased number of CD4-CD8 $\alpha$ - $\gamma\delta$  T-cells when an MBL ligand was added to the vaccine, most pronouncedly after the first vaccination. As MBL ligands co-administered with IBV vaccine induced differences between the two chicken lines, these results indirectly suggest that MBL is involved in the immune response to IBV vaccination. Furthermore, the higher antibody response in L10H chickens receiving vaccine and FOS makes FOS a potential adjuvant candidate in an IBV vaccine.

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### Introduction

The first line of host defence against pathogens involves the innate immune system. Pathogens have specific microorganism-associated molecular patterns (MAMPs) that are recognised by pattern-recognition receptors (PRRs). Two kinds of PRRs exist: surface PRRs and soluble PRRs. PRRs trigger intracellular signalling

casades upon MAMP recognition culminating in activation of anti-presenting cells and production of co-stimulatory molecules as well as pro-inflammatory cytokines. The production of co-stimulatory molecules and pro-inflammatory cytokines initiates the early host response to infection and also partakes in the activation and shaping of the adaptive immune response (Crozet et al. 2009; de Visser and Coussens 2005; Hoffmann et al. 1999). Several soluble PRRs have been described, and an example of such is mannose-binding lectin (MBL). MBL has various functions in, for example, complement activation, promotion of complement-independent opsonophagocytosis, modulation of inflammation, recognition of altered self-structures and apoptotic cell clearance (Dommett et al. 2006). MBL is a collectin consisting of multiple identical polypeptide chains oligomerised into different sizes. The chains are made up of four distinct domains. These are a cysteine-rich N-terminal domain, a collagenous domain, a neck domain, and a calcium-dependent carbohydrate-recognition domain (CRD) at the C-terminal (Takahashi 2011). It is the CRD that permits MBL

**Abbreviations:** CRD, carbohydrate-recognition domain; CTL, cytotoxic T-lymphocyte; FOS, fructooligosaccharide; IB, infectious bronchitis; IBV, infectious bronchitis virus; MBL, mannose-binding lectin; OPA, oropharyngeal airway; MAMP, microorganism-associated molecular patterns; PBS+, Gibco® DPBS with 0.1% BSA; PRR, pattern-recognition receptors; PV, post vaccination; qRT-PCR, real-time quantitative reverse transcription PCR; SARS, severe acute respiratory syndrome; SAV-HRP, Streptavidin Horseradish Peroxidase; TMC, N,N,N-trimethylated chitosan.

\* Corresponding author. Tel.: +45 8715 7837.

E-mail addresses: [RikkeM.Kjaerup@agrsci.dk](mailto:RikkeM.Kjaerup@agrsci.dk) (R.M. Kjærup), [Helle.JuulMadsen@agrsci.dk](mailto:Helle.JuulMadsen@agrsci.dk) (H.R. Juul-Madsen).

to bind in a  $\text{Ca}^{2+}$ -dependent manner to MAMPs such as polysaccharides. In fact, terminal sugars, such as D-mannose, L-fucose and N-acetyl-D-glucosamine, found on the surface of many microorganisms, contain equatorial 3- and 4-hydroxyl groups to which MBL binds. MBL does not bind to D-galactose and sialic acid found on the surface of many animal cells. Beside sugars, MBL has also been found to bind to phospholipids, nucleic acids, and non-glycosylated proteins (Ip et al. 2009). The MBL genes in human (Madsen et al. 1994, 1995; Steffensen et al. 2000; Sumiya et al. 1991), porcine (Juul-Madsen et al. 2011a; Lillie et al. 2007), bovine (Liu et al. 2011) and chickens (Kjærup et al. 2013) have been found to have polymorphisms resulting in wide variations in MBL serum levels in the organisms.

Over the last decades MBL deficiency and the influence on the susceptibility to different types of infections have been subject to extensive studies as reviewed (Heitzeneder et al. 2012; Mayilyan 2012; Takahashi 2011). Some results indicate that MBL deficiency may actually be beneficial with regard to disease, for example visceral leishmaniasis (Santos et al. 2001). However, most results suggest that MBL deficiency leads to a weaker immune response. In humans, several studies have shown that MBL participates in the protection of hosts against virus infections, such as infections with influenza A virus (Chang et al. 2010), Hepatitis C virus (Brown et al. 2010), Ebola virus (Michelow et al. 2011), and severe acute respiratory syndrome (SARS) coronavirus (Ip et al. 2005; Zhou et al. 2010). Thus, MBL in chickens may also play a role in the pathogenesis of chicken virus infections and the production of antibodies as suggested by Juul-Madsen et al. (2007).

Selective breeding of chickens for low or high serum MBL concentrations has been performed for several generations at our department as published by Juul-Madsen et al. (2007). This has resulted in two distinct chicken lines designated high (L10H) or low (L10L) with mean serum MBL concentrations of 33.4  $\mu\text{g}/\text{mL}$  serum (L10H) and 7.6  $\mu\text{g}/\text{mL}$  serum (L10L) (F14 generation, unpublished). Studies using these chicken sublines as well as outbred chickens have shown an inverse relationship between the MBL concentrations and the pathogen-specific antibody response (Juul-Madsen et al. 2007; Schou et al. 2008). Studies in mice have shown that MBL deficiency may result in a higher IgG antibody response after infections (Carter et al. 2007) and vaccinations (Guttormsen et al. 2009). From these results it can be hypothesised that basal MBL plasma levels may influence specific humoral immune responses. This explanation for this may be that either: (1) MBL pushes the immune response into a more cellular response (Th1 vs. Th2); (2) MBL efficiently neutralises the pathogen via the complement membrane-attack complex and no adaptive immune response is needed; or (3) MBL influences the pro-inflammatory cytokine production via interaction with surface receptors, such as toll-like receptors (Ip et al. 2008).

Infectious bronchitis (IB) is a highly contagious disease of economic importance in the poultry industry with symptoms such as sneezing, tracheal rales, and coughing. Furthermore, IB may cause a decline in egg quality and production in layers (Raj and Jones 1997). IB is caused by the coronavirus infectious bronchitis virus (IBV) which is highly able to genetically mutate and recombine. As a result, there is a continuous development of new strains throughout the world. Different strains can co-circulate within a region, and the severity of the disease varies from strain to strain and from flock to flock (Capua et al. 1999; Cavanagh 2007; Cook et al. 2012). Consequently, applied vaccines sometimes provide insufficient protection, as vaccination with one strain of IBV may not be protective against other strains.

Vaccine efficacy may be improved by the use of adjuvants. Good candidates for vaccine adjuvants are carbohydrates since they are mostly of low toxicity and high biocompatibility and furthermore play major roles within the immune system (Petrovsky and Cooper

2011). Carbohydrates such as mannan (Liu et al. 2012), chitosan (Rauw et al. 2010), and fructooligosaccharide (FOS) (Benyacoub et al. 2008) have previously been used in vaccines or diets as modulators of the immune response. These three carbohydrates are potential MBL ligands owing to their content of sugar units. The hypothesis of this study was that immunity after IBV vaccination may be improved after temporarily inhibition of the MBL function. This was achieved by adding an MBL ligand (mannan, chitosan, or FOS) to the vaccine given to chickens and thereby creating an artificial MBL deficiency during vaccination. Innate as well as adaptive immunological parameters were measured throughout the experimental period.

## Materials and methods

### Chemicals and reagents

All chemicals were obtained from Sigma–Aldrich, Ballerup, Denmark, except when noted. DreamTaq™ Master Mix was obtained from Qiagen, and oligonucleotide primers and probes were obtained from Eurofins MWG Operon, Ebersberg, Germany.

### Purified MBL

Purified chicken MBL was bought from the Department of Cancer and Inflammation Research, University of Southern Denmark. It was purified from chicken serum as previously described (Laursen et al. 1998a).

### MBL-IBV binding assay

The binding capacity of MBL to IBV was measured using ELISA. Dilutions of purified MBL and serum samples from L10L or L10H chickens were made with or without addition of saccharides. BioWhittaker® Veronal buffer (Lonza, Walkersville, MD, USA; cat. no. 12-624E) adjusted to a final concentration of 5 mM  $\text{MgCl}_2$  and 10 mM  $\text{CaCl}_2$  was used for diluting the samples. For the titration of MBL binding, concentrations of 0, 1, 2, 4, 8, 16, 24 and 32  $\mu\text{g}/\text{mL}$  purified MBL were used, and 5  $\mu\text{L}$  serum samples were diluted 1:50. The concentrations of the saccharides added were as follows: mannan 100 mg/mL; FOS 100 mg/mL; chitosan 10 mg/mL; galactose 9 mg/mL; and EDTA 20 mM. One-hundred microlitres of the dilutions and saccharides were mixed in a Nunc 96-well polypropylen MicroWell plate (Thermo Fisher Scientific, Slangerup, Denmark; cat. no. 442587) and incubated for 5 min before the samples were transferred to a 96-well microtitre plate coated with IBV antigen (from The ProFLOK® IBV Antibody Test Kit from Synbiotics Corporation, San Diego, CA, USA; cat. no. 96-6506). Wells receiving only buffer were used as negative controls. All dilutions were added in triplicates. The plate was then incubated at room temperature for 1 h. After a washing step with Gibco® DPBS supplemented with calcium and magnesium (Life Technologies Europe BV, Nærum, Denmark; cat. no. 14080-048) and pH adjusted to 7.4 followed by supplementation with 0.1% BSA (hereafter called PBS+), the wells were incubated for 45 min at room temperature with 1  $\mu\text{g}/\text{mL}$  of biotinylated monoclonal mouse anti-cMBL (BioPorto Diagnostics A/S, Gentofte, Denmark; cat. no. HYB 182-01) in PBS+. After another washing step Streptavidin Horseradish Peroxidase (SAV-HRP) (BD Biosciences, Albertslund, Denmark; cat. no. 554066) diluted 20,000-fold in PBS+ was added. After 30 min of incubation and washing with PBS+, the presence of SAV-HRP was detected by adding 100  $\mu\text{L}$  of substrate solution (<0.05%, wt/wt; 3,3',5,5'' tetramethylbenzidine). Colour development was stopped with a 1 M  $\text{H}_2\text{SO}_4$  solution. The colour development was determined by

reading the absorbance at 405 nm with absorbance at 650 nm as reference.

#### *Animal material and in vivo experimental design*

Generation 13 from the two AU inbred sub-lines L10L and L10H (Juul-Madsen et al. 2007) was used in this study ( $n = 72$ ). Chickens from L10 consist of 67.5% UM-B19 and 33.5% White Cornish (Laursen et al. 1998b). The offspring were reared together in a bio-secured IBV-free environment until they were 3 weeks of age and then allocated into 4 different treatment groups with 9 birds from each subline in each group. The first treatment group was treated with 100  $\mu$ L deionized water containing one dose of live attenuated Nobilis<sup>®</sup> IB Ma5 (MSD Animal Health, Ballerup, Denmark; Danish product license no. 8674) per animal. The second treatment group was treated with 100  $\mu$ L deionized water containing one dose of live attenuated Nobilis IB Ma5 Vet and 200 mg/mL purified mannan from *Saccharomyces cerevisiae* per animal aiming at 100  $\mu$ g mannan per gram body weight (Juul-Madsen et al. 2011b). The third treatment group was treated with 100  $\mu$ L deionized water containing one dose of live attenuated Nobilis IB Ma5 Vet and 50 mg/mL deacylated chitosan from shrimp shells per animal. The fourth treatment group was treated with 100  $\mu$ L deionized water containing one dose of live attenuated Nobilis IB Ma5 Vet and 200 mg/mL FOS (Orafti<sup>®</sup>P95 from Alsiano A/S, Birkerød, Denmark) per animal aiming at 100  $\mu$ g FOS per gram body weight. All solutions were shaken before nasally applied to the chickens. After three weeks, the four treatment groups were vaccinated again with the same amount of vaccine and saccharides as for the first vaccination. The chickens were fed diets that met or exceeded NRC requirements. Food and water were provided ad libitum.

The experimental procedures were conducted under the protocols approved by the Danish Animal Experiments Inspectorate and complied with the Danish Ministry of Justice Law no. 382 (10 June 1987) and Acts 739 (6 December 1988) and 333 (19 May 1990) concerning animal experimentation and care of experimental animals.

#### *Blood and swab collection*

Serum was collected from blood samples (0.5–0.7 mL) taken from the jugular vein or the wing vein from the experimental chickens on days 0, 1, 2, 3, 4, 5, 7, 14, 21, 22, 23, 24, 25, 26, 28, 35, 42, 49 and 56 post vaccination 1 (PV1). Heparin-stabilised blood for immunophenotyping was collected once a week on days 0, 7, 14, 21, 28, 35, 42, 49 and 56 PV1 and the collected blood (0.5–0.7 mL) was divided into one serum tube and one heparin tube. Oropharyngeal airway (OPA) swab samples were collected on days 0, 2, 3, 4, 5, 7, and 23 PV1. Swab samples were kept in 0.5 mL virus media (20.4% Gibco<sup>®</sup> Penicillin-Streptomycin (Life Technologies Europe BV, Nærum, Denmark; cat. no. 15140-122); 74% BioWhittaker<sup>®</sup> PBS (Lonza, Verviers, Belgium; cat. no. BE17-517Q); 5% BioWittaker<sup>®</sup> foetal bovine serum (Lonza, Verviers, Belgium; cat. no. DE14-801F); and 0.01% phenol red (Merck, Darmstadt, Germany, cat. no. 1072410005) at  $-20^{\circ}\text{C}$  until testing by real-time quantitative reverse transcription PCR (qRT-PCR) after the termination of the experiment.

#### *MBL haplotype determination*

MBL haplotypes were determined by means of the TaqMan<sup>®</sup> SNP genotyping technique (Applied Biosystems, Foster City, CA, USA). Two assays were designed, using the Custom TaqMan<sup>®</sup> Assay Design Tool according to the instructions on the website (<https://www5.invitrogen.com/custom-genomic-products/tools/genotyping/>). These assays distinguish between the CG and TA alleles of SNP1 and the GGGG and AGGA alleles of SNP2 (Kjærup

et al. 2013). The assays were validated and run on 384-well microtitre plates in an ABI Prism 7900HT Sequence Detection System instrument, using version 2.2 of the SDS software. A total reaction volume of 10  $\mu$ L was applied, each sample containing 5  $\mu$ L TaqMan<sup>®</sup> Universal PCR MasterMix (Applied Biosystems, Life Technologies Europe BV, Nærum, Denmark; cat. no.4364338), 0.25  $\mu$ L assay mix, 3.75  $\mu$ L H<sub>2</sub>O, and 1  $\mu$ L DNA at a concentration of 5 ng/ $\mu$ L. To ensure optimal clustering, 24 positive controls, representing animals of known genotypes, were included in each run, together with four non-template (negative) controls. Amplification was obtained through an initial incubation period of 10 min at  $95^{\circ}\text{C}$  followed by 40 cycles of denaturation for 15 s at  $92^{\circ}\text{C}$  and annealing/extension for 1 min at  $60^{\circ}\text{C}$ . After amplification, end-point reads were carried out, and analysis was performed with 2-cluster calling enabled in the SDS software version 2.2.

#### *qRT-PCR of IBV*

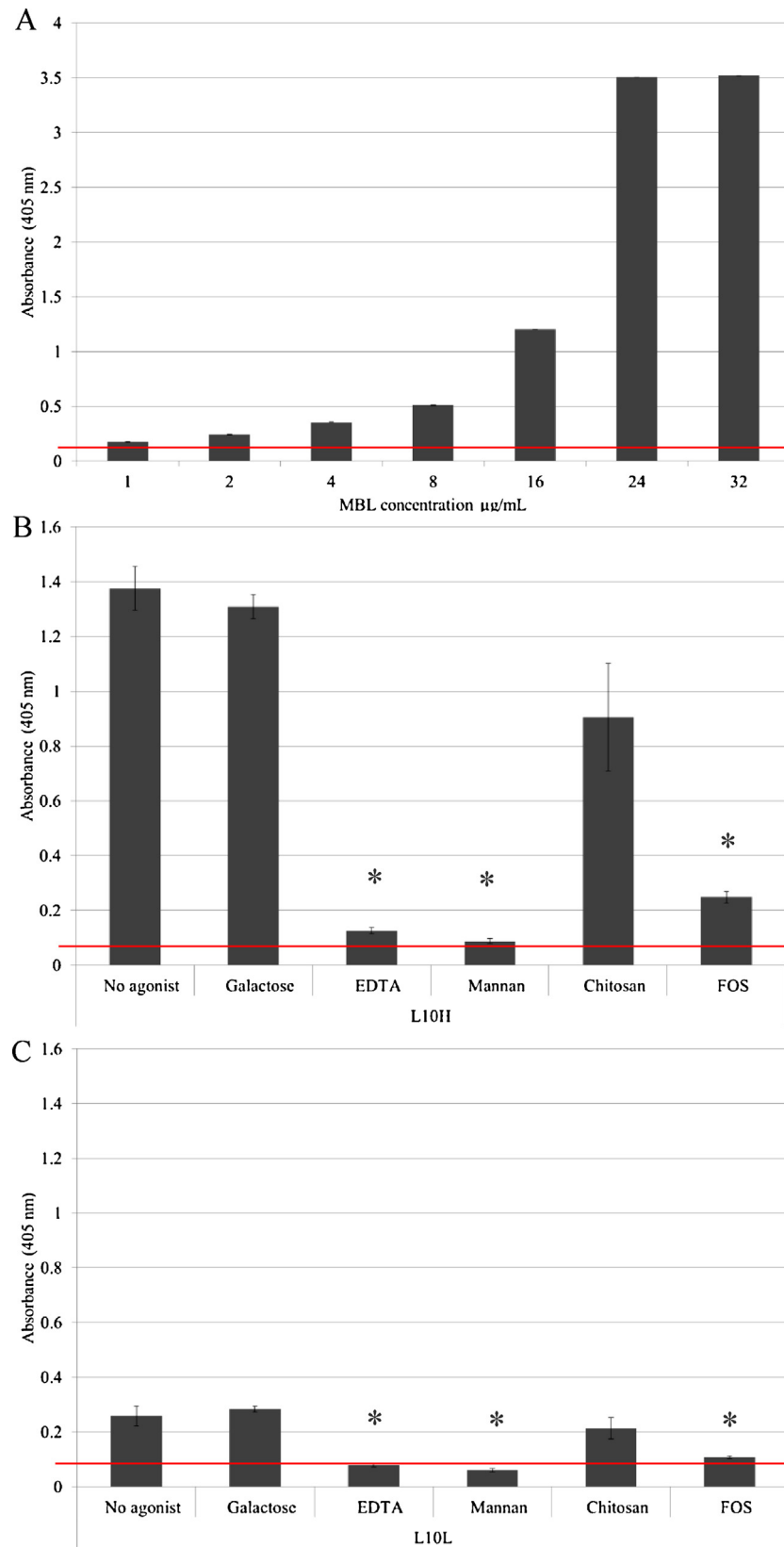
Forty-seven microlitres of three swab samples from each subline, treatment group and day were pooled; thus, 3 pools per subline were analysed for each time point. RNA purification was done using the QIAamp<sup>®</sup> Viral RNA Mini kit (Qiagen, Copenhagen, Denmark; cat. no. 52904) according to the manufacturer's instructions. The reverse transcription PCR was carried out using the High-Capacity cDNA Archive kit (Applied Biosystems, Life Technologies Europe BV, Nærum, Denmark; cat. no. 436881) according to the manufacturer's instructions with 25  $\mu$ L of RNA. The real time quantitative reverse transcription PCR (qRT-PCR) reaction was performed with the forward primer IBV5'GU391 (5'-GCTTTTGAGCCTAGCGTT-3'), the reverse primer IBV5'GL533 (5'-GCCATGTTGTCCTGTCTATTG-3') and the dual-labelled probe IBV5'G probe (5'-FAM-CACCACCAGAACCTGTACCTC-BHQ1-3') previously described to amplify at least 15 different strains of IBV (Callison et al. 2006). The total reaction volume was 20  $\mu$ L containing 11  $\mu$ L TaqMan<sup>®</sup> Universal PCR Master Mix (Applied Biosystems, Life Technologies Europe BV, Nærum, Denmark; cat. no. 4304437), 0.9  $\mu$ M primers, 0.125  $\mu$ M probe and 0.9  $\mu$ L cDNA as template. The reaction was performed in an ABI Prism 7900HT Sequence Detection System at  $50^{\circ}\text{C}$  for 2 min,  $95^{\circ}\text{C}$  for 10 min with optic off; 40 cycles of  $95^{\circ}\text{C}$  for 15 s followed by  $60^{\circ}\text{C}$  for 1 min with optics on. Standard curves were included in each qRT-PCR run and were generated from dilutions of cDNA originating from purified RNA from the live attenuated Nobilis IB Ma5 Vet. The standard curves were made as 5-fold dilutions starting at one dose, corresponding to minimum  $10^3$  EID<sub>50</sub> according to the manufacturer's instructions. Results are expressed as viral load according to the standard curve. However, the viral load is only a measure of viral RNA, not true viral replicates. Each qRT-PCR experiment contained triplicate no-template controls, test samples and dilution series of Nobilis IB MA5 Vet cDNA.

#### *ELISA measurements*

The IgG-specific antibody titres against IBV in serum were measured as previously described (Juul-Madsen et al. 2011b) using the ProFLOK<sup>®</sup> IBV Antibody Test Kit from Synbiotics Corporation (San Diego, CA, USA; cat. no. 96-6506) according to the manufacturer's instructions.

#### *Flow cytometry*

The absolute numbers of different T-cell subsets in peripheral blood were measured once a week using a no-lyse no-wash flow cytometric method. Fifty microlitres of 25 times diluted heparin-stabilised blood was mixed with 50  $\mu$ L antibody solution containing 1  $\mu$ L anti-CD3-FITC (clone CT3), 0.25  $\mu$ L anti-CD4-RPE (clone CT4),



**Fig. 1.** MBL-IBV binding as measured by ELISA. MBL-IBV binding is expressed as absorbance at 450 nm. (A) Dose-dependent binding of purified MBL to IBV. The results for MBL concentrations 1–8  $\mu\text{g/mL}$  are shown as means  $\pm$  SEM. The results for MBL concentrations 16–32  $\mu\text{g/mL}$  are shown as singlets. The vertical line indicates the value for no MBL-IBV binding. (B) Inhibition of L10H serum MBL-IBV binding. Results are shown as mean values  $\pm$  SEM. The vertical line indicates the value for no MBL-IBV binding. The asterisks indicate a statistically significant difference from MBL-IBV binding without addition of ligand ( $P < 0.01$ ). (C) Inhibition of L10L serum MBL-IBV binding. Results are shown as mean values  $\pm$  SEM. The vertical line indicates the value for no MBL-IBV binding. The asterisks indicate statistically significant difference from MBL-IBV without addition of ligand ( $P < 0.01$ ).



and 0.25  $\mu\text{L}$  anti-CD8 $\alpha$ -Cy5 (clone 3-298) in FACS buffer (0.2% BSA, 0.2% sodium azide and 0.05% normal horse serum in PBS). All monoclonal antibodies were obtained from Southern Biotech (Birmingham, AL, USA). The samples were incubated at room temperature for 20 min in a 4 mL tube in darkness, and immediately before acquisition 400  $\mu\text{L}$  FACS-buffer and 25  $\mu\text{L}$  Flow-Count™ Fluorospheres (Beckman Coulter Ireland, Mervue, Galway, Ireland; cat. no. 7547053) were added.

B-cell numbers were only measured at week 9 PV1. The protocol was as described above but using 1  $\mu\text{L}$  anti-Bu-1-RPE (clone AV20) in FACS buffer. Bu-1 positive macrophage and monocyte subsets were avoided by FSC/SSC gating on small lymphocytes.

All flow cytometric analyses were performed on a BD FACSCanto™ (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm blue laser and a 633 nm red laser. Using the FACSDiva software each sample was acquired and recorded for 1 min at medium flow rate. Single-stained compensation controls as well as negative fluorescence minus one (FMO) controls were included and titration of all antibodies was performed prior to the experiment in order to determine the optimal staining concentrations. The count of cells in the samples was calculated according to the manufacturer's instructions as:  $\text{cells}/\mu\text{L} = (\text{total number of cells counted} \times \text{dilution factor} \times \mu\text{L fluorospheres added} \times \text{assayed concentration of fluorospheres}) / (\text{total number of fluorospheres counted} \times \text{total volume})$ .

#### Ethics statement

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.

#### Statistics

All data, except for the viral load measured by qRT-PCR, were found to be normally distributed.

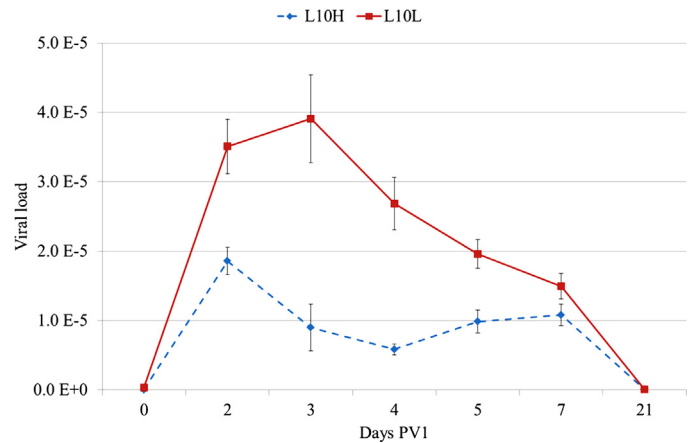
To analyse the data from the MBL-IBV binding assay the values from no addition of MBL ligand were compared with the values from each of the specific ligands added using the analysis of variance principle. In cases of significant effect ( $P < 0.05$ ) the given MBL ligand was considered to affect the MBL-IBV binding.

The viral loads were, due to pooled samples (only 3 pools per group), only tested for subline differences. The viral loads were far from being normally distributed and so an  $\chi^2$ -test was used to test if the sublines had an effect on the viral load. The test was performed on data from days 1 to 7 PV1. For test of the subline effect the observation was distributed into three viral load categories: viral load = 0,  $0 < \text{viral load} < 0.12 \times 10^{-5}$ , and viral load  $> 0.12 \times 10^{-5}$ . The  $\chi^2$ -test tested the null hypothesis: that the observations were distributed equally over the sublines for all categories.

The model used for the IBV-specific IgG antibody titre measured by ELISA was:  $y_{ijk} = \mu + T_i + W_j + L_k + TWL_{ikj} + e_{ijk}$ , where  $\mu$  = overall means,  $T_i$  = fixed effect of treatment  $i$ ,  $W_j$  = fixed effect of week  $j$  PV1,  $L_k$  = fixed effect of line  $k$ . The various interaction effects and  $y_{ijk}$  and  $e_{ijk}$  were expected to be normally distributed. To avoid weeks with little or no antibody titre, the antibody titre was only statistically analysed from week 2 to 9.

The model used for the absolute counts of T-cell subsets measured by flow cytometry was:  $y_{ijk} = \mu + T_i + W_j + L_k + LW^{jk} + TL_{ik} + TWL_{ikj} + e_{ijk}$ , where  $\mu$  = overall means,  $T_i$  = fixed effect of treatment  $i$ ,  $W_j$  = fixed effect of week  $j$  PV1, and  $L_k$  = fixed effect of line  $k$ . The various interaction effects and  $y_{ijk}$  and  $e_{ijk}$  were expected to be normally distributed.

The model used for the absolute counts of B-cells measured by flow cytometry was:  $y_{ij} = \mu + T_i + L_j + TL_{ij} + e_{ij}$ , where  $\mu$  = overall means,  $T_i$  = fixed effect of treatment  $i$ ,  $L_j$  = fixed effect of line  $j$ . The



**Fig. 2.** Viral load in OPA sampled from days 0 to 21 PV1 as measured by IBV-specific qRT-PCR. The average viral load for L10H and L10L chickens is shown as mean values for all four groups  $\pm$  SEM.

various interaction effects and  $y_{ij}$  and  $e_{ij}$  were expected to be normally distributed.

The analysis of the variance was performed by the GLM procedure of the SAS software (SAS Institute Inc. 2009).

## Results

### MBL-IBV binding assay

An ELISA protocol was used for measuring the binding of MBL to IBV. The results (Fig. 1) showed that purified MBL binds to IBV-coated plates in a dose-dependent manner (Fig. 1A). MBL in serum samples was also shown to bind to IBV (Fig. 1B and C). This binding was inhibited by adding MBL ligands. Thus, the addition of mannan and FOS reduced the MBL-IBV binding significantly ( $P < 0.01$ ) for L10H serum samples. Addition of chitosan only showed a tendency for inhibition of MBL-IBV binding ( $P = 0.0513$ ). For the MBL in L10L serum the binding was significantly inhibited by mannan and FOS ( $P < 0.01$ ), but not by chitosan. Addition of EDTA to the serum samples inhibited the MBL-IBV binding significantly ( $P < 0.01$ ) in both sublines, indicating that the binding is calcium dependent. On the other hand the negative control, galactose, had no influence on the MBL-IBV binding.

### Determination of MBL haplotypes

Chickens were tested for their MBL haplotype as described by Kjærup et al. (2013) and were determined through the TaqMan® SNP genotyping technique (data not shown). All L10L chickens were homozygous for the A1 haplotype. All L10H chickens were homozygous for the A3 haplotype, except for five chickens which were heterozygous A1/A3 and were found in groups 1 ( $n = 2$ ), 3 ( $n = 2$ ) and 4 ( $n = 1$ ).

### Viral load

IBV-specific qRT-PCR was used for monitoring the presence of IBV genomes in the OPA swabs sampled from days 0 to 21 PV1 (Fig. 2). Three swab samples from each subline were pooled based on treatment group and day. The results were statistically analysed regardless of treatments (Table 1), since three measurements per subline, treatment group and day were considered insufficient for statistics. In general, the L10L chickens had a higher viral load than the L10H chickens. The viral load in L10H peaked at day 2 PV1, whereas in L10L it peaked at days 2–3 PV1. The viral load in both

**Table 1**  
Chi-square test of viral load in L10H and L10L chickens combined for all groups ( $P < 0.001$ ).

Viral load category $\times 10^{-5}$	Line		
	H	L	Total
0	55	54	109
0 < viral load < 0.12	140	63	203
0.12 < viral load	57	135	192
Total	252	252	504

L10H and L10L reached 0 at day 21 PV1. An  $\chi^2$ -test was used for statistical analysis to test if the sublines had an effect. L10H and L10L had the same number of chickens with no IBV genomes during the experiment (Table 1). L10H had a significantly larger number of chickens with low viral load ( $< 0.12 \times 10^{-5}$ ) than L10L. Contrary to this, L10L had a significantly larger number of chickens with high viral load ( $> 0.12 \times 10^{-5}$ ) than L10H ( $P < 0.01$ ). Hence, the viral loads indicated that L10H chickens were less severely affected by the infection than L10L chickens.

#### IBV-specific IgG antibody titre

The IBV-specific IgG antibody titres were measured using ELISA (Fig. 3). A few chickens were non-responders and remained seronegative. These chickens were two L10H from treatment groups 1 ( $n = 1$ ) and 4 ( $n = 1$ ), and four L10L from treatment groups 1 ( $n = 1$ ), 3 ( $n = 1$ ) and 4 ( $n = 2$ ). They were excluded from the statistical analysis of the IBV-specific IgG titre. These chickens responded as the other chickens in all other parameters. Therefore, the measurements for these chickens were maintained in the statistical analysis of all other parameters.

When comparing the sublines, group 4 was the only group where L10H and L10L differed significantly from each other in the IBV-specific IgG antibody titre ( $P < 0.01$ ). In this group the antibody titres in L10H chickens were higher than the antibody titres in L10L chickens after the second vaccination (week 3) and for the rest of the experimental period.

The antibody titres showed no difference between the L10L chickens in the four treatment groups. On the other hand, the antibody titre in L10H showed differences between the treatment groups at week 4 (group 1  $\neq$  groups 3 and 4; and group 4  $\neq$  groups 2 and 3), week 5 (group 4  $\neq$  groups 1, 2 and 3), week 6 (group 4  $\neq$  groups 1, 2 and 3) week 7 (group 4  $\neq$  group 1), week 8 (group 4  $\neq$  group 1) and week 9 PV1 (group 4  $\neq$  group 1), where  $P < 0.04$ . In summary, the IBV-specific IgG antibody titres were the same for L10L chickens between different treatments, whereas the addition of an MBL ligand to the vaccine, especially FOS, seemed to enhance the production of IBV-specific IgG antibody in L10H chickens, but not in L10L chickens.

#### Flow cytometric assessment of lymphocyte subsets

An absolute count flow cytometric protocol using a no-lyse no-wash method, as described by Seliger et al. (2012) was used for quantifying T-cell subsets in whole blood. The T-cell subsets were identified by FSC/SSC gating on small lymphocytes, followed by identification of CD3+ cells (T-cells) and finally by subdividing the CD3+ cells into total CD4+ cells (i.e. CD4+CD8 $\alpha$ + and CD4+CD8 $\alpha$ -), CD4-CD8 $\alpha$ + cells, and CD4-CD8 $\alpha$ - cells (Fig. 4A and B). CD4+CD8 $\alpha$ + and CD4+CD8 $\alpha$ - cells were combined as total CD4+ cells, due to individual differences in the counts of CD4+CD8 $\alpha$ + cells between animals which have an heritable origin (Hala et al. 1992). The results are presented in Figs. 5 and 6.

Comparing the counts between the two sublines (L10H and L10L) within each treatment group (Fig. 5), the numbers of total

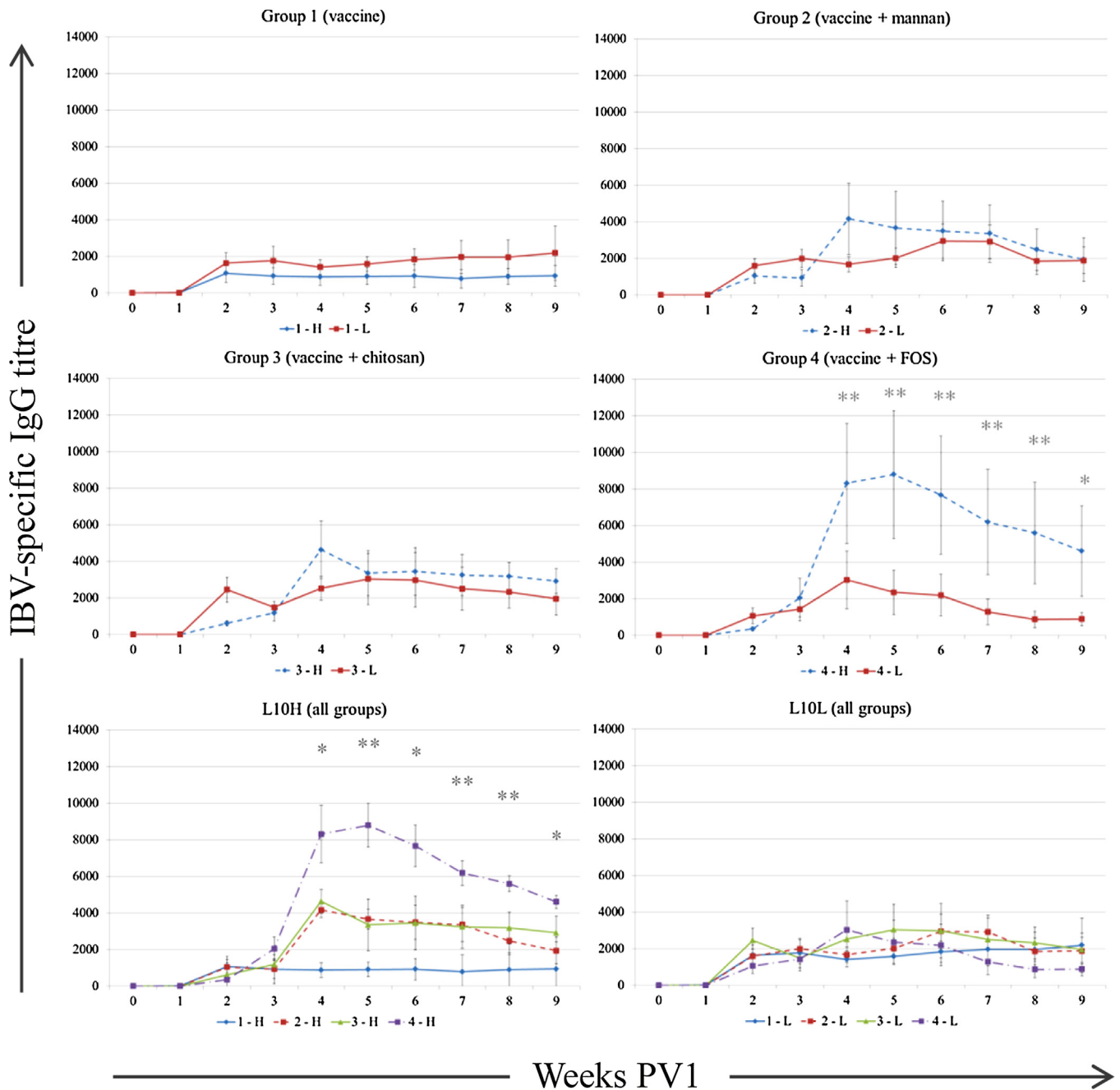
**Table 2**  
Number of B-cells per  $\mu$ L blood at week 9 PV1. Staining and gating of cell populations were performed according to Fig. 4A and C. Results are shown as mean values  $\pm$  SEM.

Treatment group	Subline		P-values between sub-lines
	L10H	L10L	
1. Vaccine	5495 $\pm$ 491	8015 $\pm$ 654	0.001
2. Vaccine + mannan	6075 $\pm$ 260	7236 $\pm$ 503	0.078
3. Vaccine + chitosan	6297 $\pm$ 519	7450 $\pm$ 465	0.151
4. Vaccine + FOS	6092 $\pm$ 685	7280 $\pm$ 584	0.078

CD4+ cells showed no difference between L10H and L10L in groups 1 and 3. In group 2 the counts of total CD4+ cells in L10H were significantly higher than in L10L at week 6 PV1 ( $P = 0.03$ ). A more pronounced difference between L10H and L10L was seen in group 4, where the counts of total CD4+ cells were significantly higher for L10H than L10L at weeks 2, 5, 6, 7, 8 and 9 PV1 ( $P \leq 0.03$ ). Counts of CD4-CD8 $\alpha$ - cells for L10H were higher than L10L in groups 3 and 4 at week 1 PV1 ( $P < 0.05$ ), whereas counts of CD4-CD8 $\alpha$ - cells were higher for L10H than L10L in group 2 at weeks 0, 1, and 2 PV1 ( $P \leq 0.02$ ). CD4-CD8 $\alpha$ + cells only differed between L10H and L10L within groups in group 1 at week 5 ( $P = 0.02$ ). Briefly, the addition of FOS to the vaccine increased the number of total CD4+ cells in L10H chickens compared to L10L chickens, and the L10H chickens showed a significant increased number of CD4-CD8 $\alpha$ - cells after the first vaccination when an MBL ligand was added to the vaccine compared to L10L chickens.

The comparison of counts between treatment groups within each subline (L10H and L10L) is presented in Fig. 6. The counts of total CD4+ cells showed no difference between treatment groups neither for the L10H nor L10L sublines, except for the L10L subline at week 6 PV1 (group 3  $\neq$  group 4) where the L10L chickens in treatment group 3 showed a significantly higher count of total CD4+ cells than L10L chickens in treatment group 4 ( $P = 0.03$ ). The numbers of CD4-CD8 $\alpha$ + cells for the L10H sublines differed significantly between treatment groups at weeks 6–9 PV1 (group 1  $\neq$  group 3), where the L10H chickens in treatment group 3 had significantly lower numbers than the L10H chickens in treatment group 1 ( $P < 0.05$ ). Besides this, the numbers of CD4-CD8 $\alpha$ + cells differed significantly for L10H between treatment groups at week 6 PV1 (group 2  $\neq$  group 4), where  $P = 0.01$ . The numbers of CD4-CD8 $\alpha$ + cells for L10L only differed significantly between treatment groups at week 3 PV1 (group 1  $\neq$  groups 2 and 4), where  $P = 0.04$ . The number of CD4-CD8 $\alpha$ - cells differed significantly for the L10H sublines between the treatment groups at week 1 (group 1  $\neq$  groups 2 and 4), week 2 (groups 1  $\neq$  2 and 5), week 3 (group 1  $\neq$  2), week 5 (group 1  $\neq$  3), and week 6 PV1 (group 1  $\neq$  3), where  $P \leq 0.04$ . The number of CD4-CD8 $\alpha$ - cells for L10H in treatment group 1 was lower than the other treatment groups at weeks 1, 2, 3, 5 and 6 PV1. Besides this, the number of CD4-CD8 $\alpha$ - cells differed significantly for L10L between the treatment groups at week 1 (group 1  $\neq$  3), week 2 (group 1  $\neq$  4), and week 5 PV1 (group 1  $\neq$  3) where  $P \leq 0.05$ . The L10L chickens in treatment group 1 were lower in the number of CD4-CD8 $\alpha$ - cells than the other treatment groups at weeks 1, 2, and 5 PV1. Briefly, the addition of an MBL ligand had no influence of total CD4+ cells within a L10H or L10L subline. However, the addition of chitosan to the vaccine decreased the number of CD4-CD8 $\alpha$ - cells in the L10H chickens at week 6 to 9 PV1. Also, the L10H chickens as well as the L10L chickens also showed an increased number of CD4-CD8 $\alpha$ - $\gamma\delta$  T-cells when an MBL ligand was added to the vaccine, most pronouncedly after the first vaccination.

The B-cells were identified by FSC/SSC gating on small lymphocytes, followed by identification of Bu-1 positive cells (Fig. 4A and C). Results are shown in Table 2. A significant difference between



**Fig. 3.** IBV-specific IgG antibody titres as measured by ELISA. Results are shown as mean values  $\pm$  SEM. The asterisks indicate statistically significant differences ( $P < 0.05$ ) between groups or sublines. The double asterisks indicate statistically significant differences ( $P < 0.01$ ) between groups or sublines. H or L indicates the sublines with high or low MBL serum concentration, respectively; 1, 2, 3 or 4 indicates the treatment groups “vaccine” (1), “vaccine + mannan” (2), “vaccine + chitosan” (3) or “vaccine + FOS” (4).

L10H and L10L chickens were observed in treatment group 1 where the absolute counts of B-cells were significantly lower for L10H chickens than for L10L chickens ( $P = 0.001$ ). No significant differences were observed between L10H and L10L chickens in the other treatment groups.

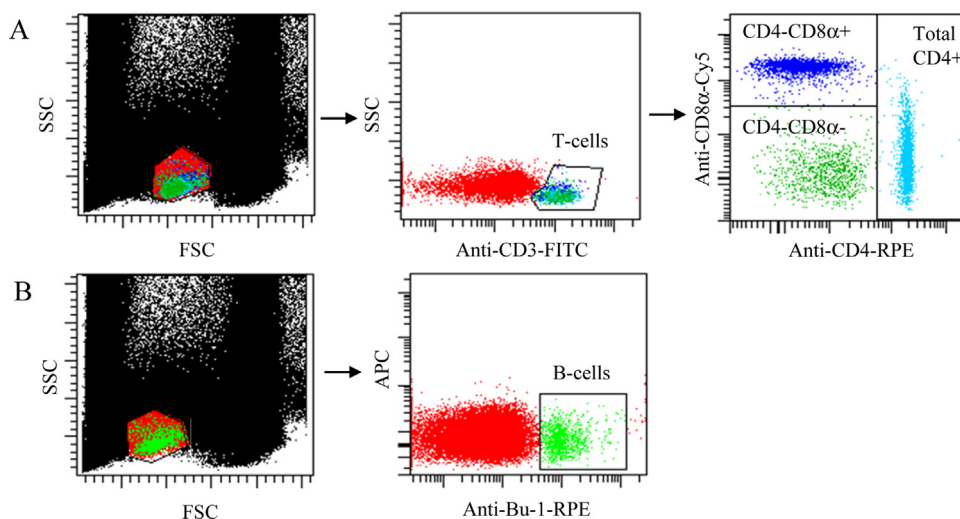
**Discussion**

The main purpose of the present study was to investigate innate and adaptive immune responses following IBV vaccination when temporarily inhibiting MBL function by co-administering MBL ligands. The binding of pathogens by PRRs, such as MBL, is an important step to initiate the early host response to infection. If MBL efficiently neutralises the pathogen via the complement

membrane-attack complex, no adaptive immune response is needed. Thus, poor adaptive memory is obtained and the purpose of the vaccine is unachieved.

To our knowledge the current study is the first to show that MBL binds to IBV. Binding was shown to occur through the CRD of MBL since binding was inhibited by EDTA as well as mannan. Previous studies have shown that human MBL binds to several viruses, such as HIV (Saifuddin et al. 2000) and SARS (Ip et al. 2005). These bindings have also been shown to occur through the CRD of MBL. Further, the current study shows that MBL binds to IBV in a dose-dependent manner. Besides this, it also provides evidence that FOS is an MBL ligand with the potential to inhibit MBL-IBV binding. FOS consists of a chain of fructose units with a terminal glucose unit (Sabater-Molina et al. 2009), and MBL is known to bind to both





**Fig. 4.** Detection of lymphocytes in whole blood using a no-lyse no-wash flow cytometry procedure. (A) 3-Colour gating strategy for T-cell subsets. The gating strategy is shown for the identification of T-cell subsets through small lymphocyte gating. Heparin-blood was stained with anti-CD3-FITC, anti-CD8 $\alpha$ -Cy5, and anti-CD4-RPE. (B) Gating strategy for B-cells. The gating strategy is shown for the identification of B-cells through small lymphocyte gating. Heparin-blood was stained with anti-BU-1-RPE. The Flow-Count™ fluorospheres were identified by FSC/Cy5-5 gating.

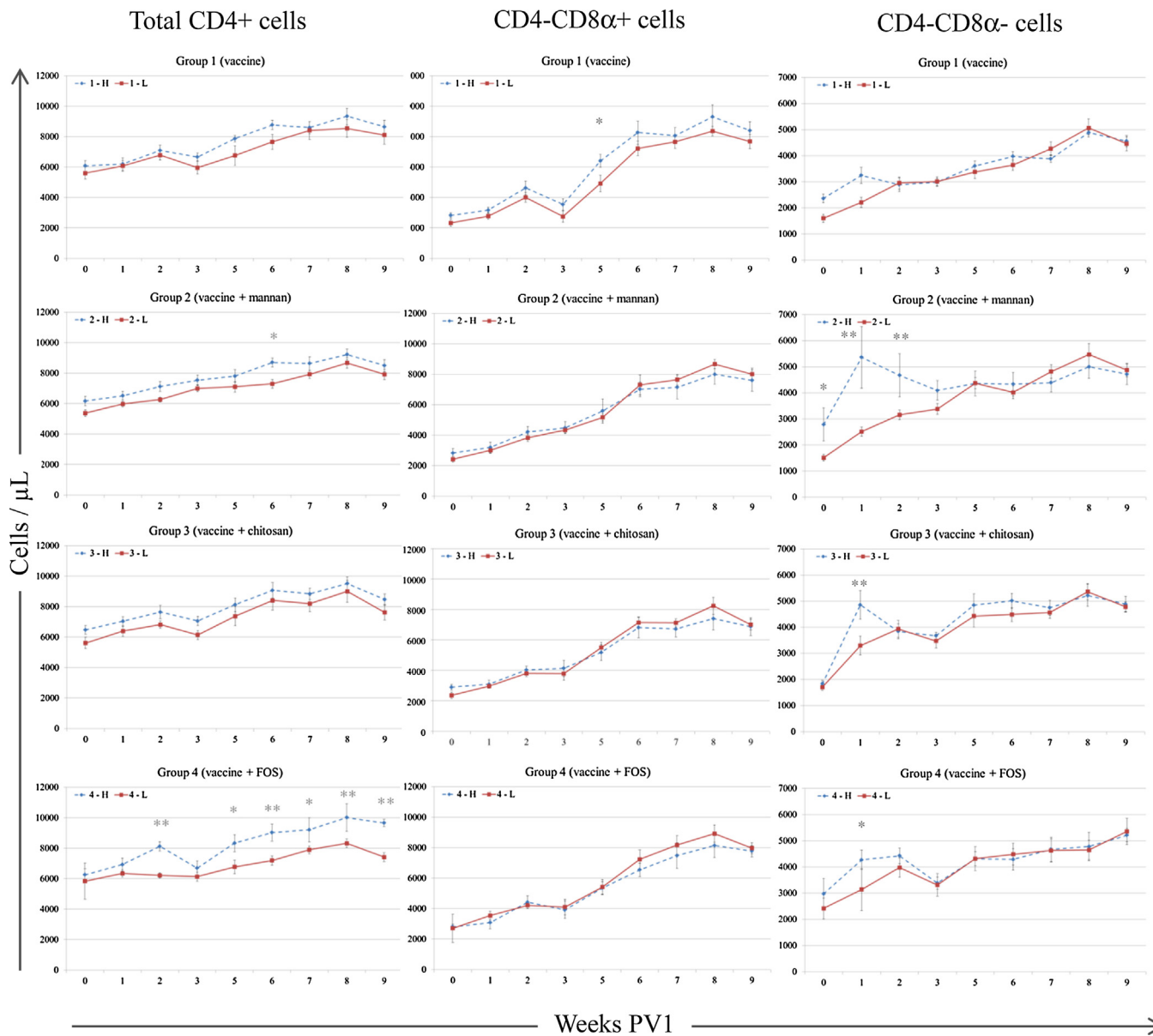
molecules (Epstein et al. 1996; Takahashi et al. 2011). In the serum samples from L10H chickens chitosan showed a tendency to inhibit the MBL-IBV binding ( $P < 0.0513$ ) indicating that chitosan may also be an MBL ligand. Chitosan is a highly basic polysaccharide obtained by deacetylation of chitin which is a linear polymer of N-acetyl-D-glucosamine (Ravi Kumar 1999), which is known to be an MBL ligand (Epstein et al. 1996). Chitosan is insoluble in water, which is why there is an uncertainty concerning the precise amount of chitosan added in the binding assay and to the vaccine given to the chickens. In a previous study both chitosan and its derivative N,N,N-trimethylated chitosan (TMC) were used as adjuvants in vaccines in raccoons (Fry et al. 2012). This study showed a higher number of responders to the vaccine when TMC was added instead of chitosan. TMC is water-soluble and have the same immunogenic and adhesive properties as chitosan (Kotze et al. 1997), which is why, with hindsight, that TMC instead of chitosan probably would have been a better choice as a possible MBL ligand added to the IBV vaccine to avoid the uncertainty about the concentrations used.

Following the first IBV vaccination the presence of viral genomes was observed in the OPA of all the birds. However, the viral loads were lower for L10H chickens than L10L chickens (Fig. 2), indicating that L10H chickens were less severely affected by the infection than L10L chickens. Previously, Juul-Madsen et al. (2011b) did not find any statistical difference in virus load between L10L and L10H chickens after vaccination. Some of the incongruence between the current study and the vaccination part of the previous study could be explained by the larger animal groups in the current study, giving a more accurate outcome. Besides, the estimation of viral load using qRT-PCR is more accurate than estimating the viral load by gel. The higher viral load in the L10L does, however, support the previous suggestions that MBL is associated with susceptibility to IBV infection (Juul-Madsen et al. 2007; Juul-Madsen et al. 2011b).

The IBV-specific IgG antibody titres after the first vaccination did not differ between sublines (L10H and L10L) or treatment. After the second vaccination (week 3) of chickens receiving vaccine alone no boosting effect of the vaccine was found in the two sublines. On the other hand, the IBV-specific IgG antibody titres after the second vaccination for L10H chickens receiving an MBL ligand together with the vaccine increased more than the antibody titre for L10H receiving vaccine alone (Fig. 3). This was most pronounced for the L10H chickens receiving vaccine and FOS. This is also in contrast to the challenge study by Juul-Madsen et al. (2011b), where the

titre was lower for L10H chickens receiving mannan together with the vaccine compared with chickens receiving vaccine alone – we have no explanation for that. The antibody titres were the same for the L10L chickens between treatment groups. This, in combination with the results of the MBL-IBV binding assay, where the MBL-IBV binding was clearly inhibited by the addition of ligands in serum from L10H but not L10L, implies that the combination of high MBL serum concentrations and the addition of an MBL ligand have an impact on the development of IBV-specific IgG antibodies. An increased antibody titre has earlier been reported in chickens vaccinated against *Eimeria* (Janardhana et al. 2009) and in mice vaccinated and infected with *Salmonella* (Benyacoub et al. 2008) when feeding a diet containing FOS. The dramatic increase in the antibody titre after vaccination with FOS may be beneficial in breeding, since the transfer of maternal antibodies to offspring is of importance in protecting the newly hatched chicks. This protection would be increased by an increase in the amount of antibodies in the dams, since there is a direct relationship between IBV-specific IgG levels in the dam and those in her offspring (Hamal et al. 2006).

Flow cytometry-based methods for counting absolute numbers of peripheral blood cells have previously been described (Burgess and Davison 1999; Seliger et al. 2012). In the current study flow cytometry was used to identify phenotypic differences between the chicken sublines vaccinated with or without the addition of MBL-ligand. No general differences were observed in the amount of total CD4+ cells for both sublines and between treatment groups. Nor was any difference between the two sublines within each treatment group observed in groups 1, 2 and 3. However, the addition of FOS to the vaccine gave a higher number of total CD4+ cells in the L10H chickens than in the L10L chickens (Fig. 5). Despite of this, no difference in the amount of B-cells at week 9 PV1 (Table 2) was observed in this group, indicating that the increase may have been caused by Th1-cells instead of Th2-cells. This is supported by a study by Guo et al. (2008) which indicates that Th1-cells are activated immediately after IBV infection. The response of CD4-CD8 $\alpha$ + cells, mostly cytotoxic T-lymphocytes (CTL), is crucial for the elimination of the virus from local infection sites (Collisson et al. 2000; Guo et al. 2008; Juul-Madsen et al. 2011b). An increased number of CD4-CD8 $\alpha$ + cells were indeed observed in the current study both PV1 and PV2 which may support the suggestions by Collisson et al. (2000) that CTLs are important in the protection against IBV. However, this increase was repressed in L10H chickens treated with

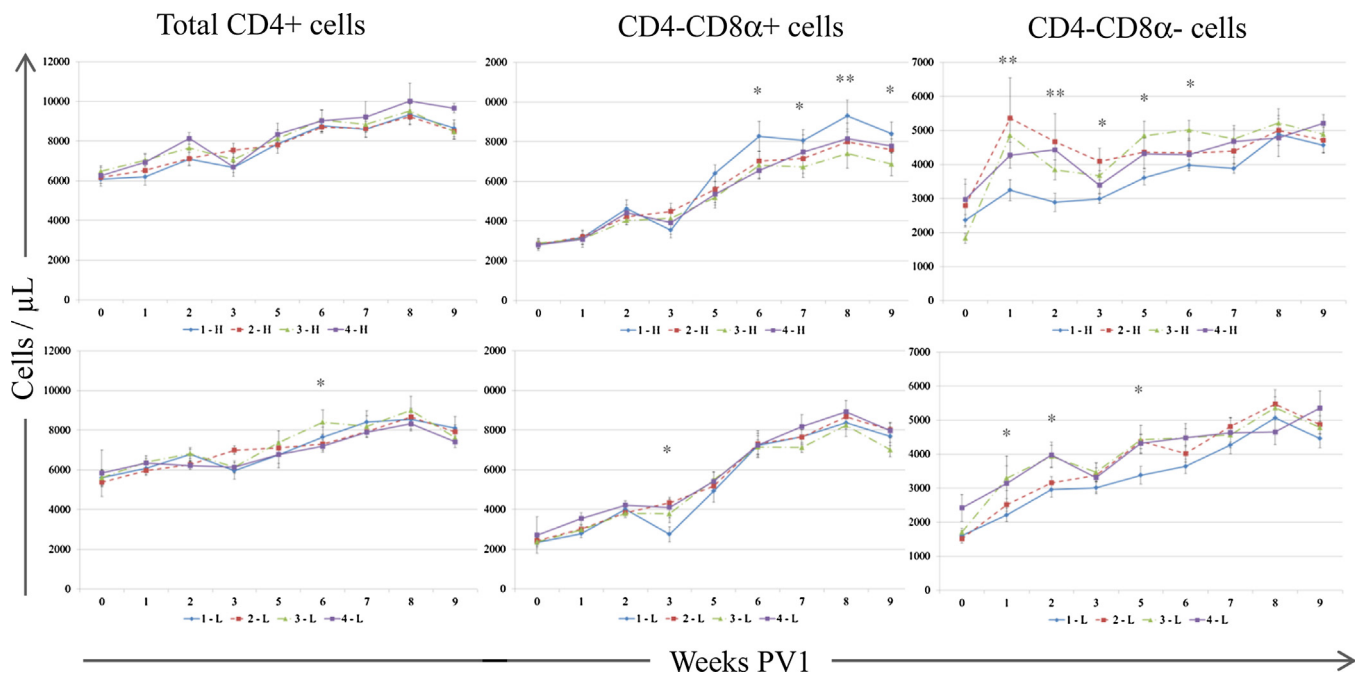


**Fig. 5.** Effect of sublines on T-cell subsets within treatment groups. Staining and gating of cell populations were performed according to Fig. 4A and B. The absolute counts of total CD4<sup>+</sup> cells are shown in the left column, the absolute counts of CD4–CD8 $\alpha$ <sup>+</sup> cells are shown in the middle column, and the absolute counts of CD4–CD8 $\alpha$ <sup>–</sup> cells are shown in the right column. The upper panels show the comparison of sublines in group 1, the second upper panels the comparison of sublines in group 2, the second lower panels the comparison of sublines in group 3, and the lower panels the comparison of sublines in group 4. Results are shown as mean values  $\pm$  SEM. The asterisks indicate statistically significant differences ( $P < 0.05$ ) between groups. The double asterisks indicate statistically significant differences ( $P < 0.01$ ) between groups. H or L indicates the sublines with high or low MBL serum concentration, respectively; 1, 2, 3 or 4 indicates the treatment groups “vaccine” (1), “vaccine + mannan” (2), “vaccine + chitosan” (3) or “vaccine + FOS” (4).

vaccine and an MBL ligand at weeks 6–9 PV1 (Fig. 6). As this study does not contain a mock vaccine group, it cannot be ruled out that the increase is age-dependent. Involvement of other receptors specific for the used MBL ligands may have influenced some of the differences observed in the current study. However, MBL has an influence as the difference was more pronounced for the L10H chickens than L10L chickens for both the IBV-specific IgG antibody titres and the numbers of CD4–CD8 $\alpha$ <sup>+</sup> and CD4–CD8 $\alpha$ <sup>–</sup> cells.

The chicken CD3+CD4–CD8 $\alpha$ <sup>–</sup> cells in circulation are mostly  $\gamma\delta$  T-cells which may contribute with up to 60% of the circulating T-cells in a healthy adult chicken (Daugaard et al. 2010) indicating that they may play an important role in the chicken immune system. For the three groups receiving vaccine and an MBL ligand, the  $\gamma\delta$  T-cell response was more pronounced in the L10H chickens than in the

L10L chickens PV1 (Fig. 5). There was also a tendency ( $P = 0.0517$ ) for this in the group receiving vaccine alone. This increase was also observed in the study by Juul-Madsen et al. (2011b). This study showed a higher increase in the percentage of  $\gamma\delta$  T-cells after IBV vaccination in the group receiving vaccine and mannan. In the current study no difference was observed in the  $\gamma\delta$  T-cell response PV2 between L10H and L10L chickens within treatment groups (Fig. 5). These inconsistencies may be explained by the use of live virus in the previous study versus the use of attenuated live virus in the current study as already suggested. However, at least a tendency for a higher number of  $\gamma\delta$  T-cells was observed in the current study until week 6 PV1 for L10H chickens receiving vaccine and MBL ligand compared to L10H chickens receiving vaccine alone (Fig. 6). The same was observed for L10L chickens receiving vaccine and chitosan or FOS until week 3 PV1 and at week 5



**Fig. 6.** Effect of treatment on T-cell subsets within each subline. Staining and gating of cell populations were performed according to Fig. 4A and B. The absolute counts of total CD4+ cells are shown in the left column, the absolute counts of CD4–CD8 $\alpha$ + cells are shown in the middle column, and the absolute counts of CD4–CD8 $\alpha$ – cells are shown in the right column. The upper panels show the comparison of the L10H in the different groups, and the lower panels the comparison of the L10L in the different groups. Results are shown as mean values  $\pm$  SEM. The asterisks indicate statistically significant differences ( $P < 0.05$ ) between groups. The double asterisks indicate statistically significant differences ( $P < 0.01$ ) between groups. H or L indicates the sublines with high or low MBL serum concentration, respectively; 1, 2, 3 or 4 indicates the treatment groups “vaccine” (1), “vaccine + mannan” (2), “vaccine + chitosan” (3) or “vaccine + FOS” (4).

PV1 for L10L treatment groups receiving vaccine and MBL ligand (Fig. 6). The function of chicken  $\gamma\delta$  T-cells is still under debate, but studies have indicated that  $\gamma\delta$  T-cells respond to pathogens and provide a protective immune response after immunisation with both live attenuated and non-attenuated *Salmonella* strains (Berndt et al. 2006). The results of the current study may support these indications since our results showed that CD4–CD8 $\alpha$ – $\gamma\delta$  T-cells are increased after IBV vaccination when MBL is inhibited.

A difference in the amount of B-cells at week 9 PV1 (Table 2) was observed between the L10H and L10L chickens receiving only vaccine, but no difference was observed for IBV-specific antibody titres in this group. In the treatment group receiving vaccine and FOS a major difference between L10H and L10L chickens was observed for the IBV-specific antibody titres, even though only a tendency ( $P = 0.078$ ) for a difference in the amount of B-cells at week 9 PV1 was observed between these chickens. These findings support the suggestions made by Guttormsen et al. (2009) that an increased IgG response in MBL-deficient mice is not only attributed to an increase in the number of B-cells. The increase in IgG may also have been caused by Ig class switching, as previously observed in mice for dietary FOS by Nakamura et al. (2004). Previous challenge studies have shown an up regulation of IgG in species with low amount of MBL in both mice (Guttormsen et al. 2009) and chickens (Juul-Madsen et al. 2007). Likewise, Ruseva et al. (2009) argue that genetic environment influences the modifying effect of MBL. We cannot conclude whether the different amounts of B-cells at week 9 PV1 observed in chickens receiving vaccine alone in the current study was caused by vaccine or age. If the difference was caused by the vaccine, the MBL ligands eliminate this difference.

In conclusion, the IBV-specific IgG antibody titres were the same for L10L chickens between different treatments, whereas the addition of an MBL ligand to the vaccine, especially FOS, seemed

to enhance the production of IBV-specific IgG antibody in L10H chickens, but not in L10L chickens. The addition of FOS to the vaccine also increased the number of total CD4+ cells in L10H chickens, in combination with an unchanged amount of B-cells at week 9 PV1 compared to L10L chickens. The L10H chickens as well as the L10L chickens also showed an increased number of CD4–CD8 $\alpha$ – $\gamma\delta$  T-cells when an MBL ligand was added to the vaccine, most pronouncedly after the first vaccination, suggesting that CD4–CD8 $\alpha$ – $\gamma\delta$  T-cells may also play a role in the immune response against IBV. These results indicate, as previously suggested by Juul-Madsen et al. (2011b) that MBL is involved in the adaptive immune response to IBV vaccination. MBL inhibition may therefore be beneficial to achieve high antibody response during vaccinations. Further studies are needed to elucidate whether the addition of MBL ligands to IBV vaccine gives a better immune protection against IBV infection.

#### Conflict of interest statement

The authors declare that they have no conflicts of interest.

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