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Research paper

# Rapid whole blood assay using flow cytometry for measuring phagocytic activity of chicken leukocytes

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ABSTRACT

Phagocytic activity of leukocytes in whole blood was assessed as a potential immune competence trait in chickens. A flow cytometry based whole blood phagocytosis (WBP) assay was set up and evaluated using blood from chickens homozygous for four different MHC haplotypes, B12, B15, B19 and B21. Fluorescent latex beads and two serotypes of fluorescently labelled heat-killed bacteria (*Salmonella* Infantis and *Salmonella.* Typhimurium) were evaluated as phagocytic targets. In addition, the opsonophagocytic potential (OPp) of individual sera from the birds was included in a phagocytosis assay using the HD11 chicken macrophage cell line. Results showed that both serotypes of bacteria but not the latex beads were effectively phagocytosed by leukocytes in the whole blood cultures. Differences were observed in the phagocytic capacity of monocytes and thrombocyte/lymphocytes, respectively between the different MHC lines. No significant differences on the OPp of serum was identified between MHC lines. In addition, for both phagocytic activity of leukocytes and OPp of serum large variations between individuals were observed within MHC haplotypes. No significant relationships were observed between the phagocytic activity of leukocytes and serum OPp or *Salmonella*-specific IgY levels.

In conclusion, our results suggest that the WBP assay, using a no-lyse no-wash single staining method, is a rapid and convenient method to assess phagocytic functions of different leukocyte populations.

## 1. Introduction

Phagocytic cells perform important functions in both the innate and the acquired immune responses against primarily extracellular infectious agents. Measures of functional capacity of the different phagocytic cell populations may thus prove useful as immune competence traits. In poultry rearing such traits could potentially be included for selection in breeding programmes aiming to produce robust production animals (Muir et al., 2014). There are several different methodologies available to assess phagocytic function. Methods based on flow cytometry, performed with fluorescent particles, bacteria, or yeast to assess and quantify phagocytosis, are common for *in-vitro* phagocytosis assays (Nuutila and Lilius, 2005). In the chicken three major phagocytic cell subsets in the blood have been identified: monocytes/macrophages, heterophils, and thrombocytes (Genovese et al., 2013; Wigley, 2013; Ferdous et al., 2016) and so far, functional capacity of phagocytic cells has mostly been studied in purified heterophils and monocytes (Ma et al., 2010; Chou et al., 2009; Sun et al., 2008; Andreasen et al., 1991) or in the chicken macrophage-like cell line HD11 (Ulrich-Lynge et al., 2015; de Geus et al., 2012; Naghizadeh et al., 2018). However, purifying leukocytes using media supplemented with foreign serum may eliminate many important regulatory factors with an impact on the traits studied (Millet et al., 2007). In addition, isolation of cells can alter receptor expression, activity, and viability of cells (Papp and Smits, 2007). Hence, using whole blood rather than purified cell preparations to study functional activity of phagocytic cells, may give a more reliable reflection of the actual state of the host immune system. Moreover, by combining tests to measure phagocytic capacity with phenotypic identification of cells in whole blood it would be possible to simultaneously assess the phagocytic activity of several different cell types.

Flow cytometry based whole blood phagocytosis (WBP) assays have been set up for several mammalian species, including humans (Jakovac-Strajn et al., 2011; Prosser et al., 2013; Schreiner et al., 2011; White-Owen et al., 1992). However, chicken blood has some biological

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particularities that cause additional technical difficulties. Primarily, the presence of abundant nucleated erythrocytes of ellipsoid shape makes discrimination of various cells by simple parameters for size or granulation difficult (Désert et al., 2016). Furthermore, the morphological similarity between chicken thrombocytes and lymphocytes causes further problems for basic cell identification in whole blood by flow cytometry (Beaufrère et al., 2013; Bohls et al., 2006). In addition, standard erythrocyte lysis techniques for mammalian blood are unsuitable for chicken erythrocytes mainly due to liberation of a large amount of DNA which leads to the enhanced viscosity of the sample, thus hampering further analytic procedures (Bohls et al., 2006; De Boever et al., 2010). However, Seliger et al. (2012) set up a technique allowing flow cytometric cell quantification in chicken whole blood without the necessity of prior erythrocyte lysis or separation. Hence, based on this method we aimed to set up a simple and rapid no-lyse no-wash flow cytometric WBP assay for chicken samples. To evaluate the method, we used blood samples from four different inbred chicken lines homozygous for the MHC haplotypes B12, B15, B19, and B21, respectively. The use of these lines was chosen to test if this method may detect genetic differences in phagocytic function which may enable future use of the assay as a selection trait in breeding programmes. Opsonophagocytic potential (OPp) is a measure of the general ability of serum proteins (opsonins) to opsonize a phagocytic target and may be used as a trait to assess innate immune responses to extracellular pathogens. It has previously been shown that OPp is genetically influenced (Sun et al., 2008) and reflects general robustness of the host innate immune system (Hu et al., 2005). Therefore, to identify potential correlations between phagocytic capacity and serum OPp, we concurrently tested blood samples for phagocytosis and serum OPp, the latter with an assay using the HD11 chicken macrophage cell line (Naghizadeh et al., 2018).

## 2. Material and methods

### 2.1. Animals and experimental design

Blood samples were collected from chickens of the AU/DIAS lines (Miller et al., 2004; Delaney and O'Hare, 2014). Female offspring from inbred white leghorn lines homozygous for the MHC haplotypes B12, B15, B19, and B21 were used for this experiment. The inbred chicken lines were kept and bred at our institute and MHC haplotypes were for each new generation verified using the LEI0258 microsatellite locus (McConnell et al., 1999) by a PCR-based fragment analysis (Fulton et al., 2006) as previously described (Dalgaard et al., 2005; Miller et al., 2004). In some generations, additional MHC serotyping was performed using a traditional haemagglutination technique as earlier described (Nielsen et al., 1999). Chickens in the current study were kept in a bio contained facility without outdoor access during the whole experimental period. The chickens were fed diets that met or exceeded National Research Council requirements (1994). Feed and water were provided *ad libitum*. The experiment was conducted under the protocols approved by the Danish Animal Experiments Inspectorate and complied with the Danish Ministry of Justice Law no. 382 (10$^{th}$ June 1987) and Acts 739 (6$^{th}$ December 1988) and 333 (19$^{th}$ May 1990) concerning animal experimentation and care of experimental animals following the described ethical guidelines. The license to conduct the animal experiment was obtained by Ricarda Engberg, Aarhus University (license no. 2017-15-0201-01211).

### 2.2. Sample collection

Un-stabilised blood for serum (3 ml/chicken) and stabilised blood samples (3 ml/chicken) for WBP assays were collected from the jugular vein of 6 chickens from each line (24 in total) at 16 weeks of age. Stabilised blood was collected in BD Vacutainer® Blood Collection Plasma Tube coated with 60 USP Units of Sodium Heparin (BD, Franklin Lakes, NJ, USA). Un-stabilised blood and stabilised blood samples were taken from each chicken at the same sampling occasion.

### 2.3. Whole blood phagocytosis assay

Assessment of phagocytic activity in whole blood was performed using a no-lyse, no-wash flow cytometry-based method. Briefly, 100 µl of heparinized blood diluted 10-fold in RPMI-1640 (containing 25 mM HEPES and 300 mg/l L-Glutamine; Lonza, cat. no. BE12-702 F/U1) were added per well in round bottom 96-well cell culture plates (Thermo Fisher Scientific, Slangerup, Denmark, cat. no. 163320) and mixed with anti CD45-PerCP-Cy5.5 antibody (clone UM16-6). The monoclonal CD45 antibody was obtained from AbD Serotec® Bio-Rad Laboratories, Inc (Kidlington, UK). Conjugation of anti-CD45 with PerCPCy5.5 was performed using Lightning-Link® from Innova Bioscience (Cambridge, United Kingdom, cat. no. 763-0030) according to the manufacturer's instructions. Subsequently, samples were incubated for 60 min at 41 °C with 25 µl of 200 times diluted carboxylate-modified polystyrene, fluorescent yellow-green (YG) beads (Sigma-Aldrich, St. Louis, cat. no. MKBJ8932, excitation, 470 nm; emission, 505 nm), 10 µl of FITC-*Salmonella* serotype Infantis (*S.* 123445) or 10 µl FITC- *Salmonella.* serotype Typhimurium (NCTC 06571), respectively. The bacteria were kindly donated by John Elmerdahl Olsen, Copenhagen University, Denmark. A single batch of each FITC-labelled bacteria was used in all experiments. Identical whole-blood control samples were prepared in parallel and incubated on ice for 60 min to determine background adherence of beads or bacteria to the cell surface. After incubation, the samples were placed on ice and fixed with paraformaldehyde (Thermo Fisher Scientific, United Kingdom, cat. no. 10131580) at a final concentration of 1%. Next, cultures were transferred from the 96 well plates to test tubes including 400-µl FACS buffer (i.e. 0.2% BSA, 0.2% sodium azide, 0.05% normal horse serum in PBS). The samples were analysed by flow cytometry within 1 h of transfer.

### 2.4. Assay for opsonophagocytic potential of serum

The chicken macrophage-like cell line HD11 was maintained, and opsonisation assay plates were set up, according to Naghizadeh et al., (2018). The serum OPp assay was performed by measuring HD11 cell phagocytosis activity of opsonised beads and bacteria as described previously (Naghizadeh et al., 2018) with a few modifications. As the first step, 25 µl of 200 times diluted YG beads or 10 µl of FITC-labelled heat-killed bacteria (*S.* Iinfantis, or *S.* Typhimurium), respectively, were added to RPMI-1640 supplemented with 25% of serum samples and incubated at room temperature (RT) for 30 min in the dark. Subsequently, 25 µl of the opsonised YG beads or 10 µl of the opsonised FITC-labelled heat-killed bacteria (*S.* Iinfantis and *S.* Typhimurium, $OD600_{nm} = 1$) was added to a final volume of 100 µl and incubated at 41°C for one hour in a 5% $CO_2$ humidified incubator. In parallel, a control plate was incubated at 4°C. After washing 3x with PBS, the cells were harvested by adding 22 µl pre-warmed 2 mM EDTA to each well containing 200 µl PBS and violently pipetting after 10 min incubation at 41°C. The cells were fixed immediately by adding paraformaldehyde to a final concentration of 1%, where after the cells were transferred to test tubes containing 300 µl FACS buffer.

### 2.5. Flow cytometry

The distribution of different leukocyte subsets was analysed in peripheral blood using a single staining (CD45), no-lyse no-wash method, as described by Seliger et al. (2012). The cell populations were distinguished based on CD45 positive cells and leukocyte subset were gated only on forward scatter (FSC), and side scatter characteristics (SSC) (Fig. 1a, b).

The HD11 cells were gated only according to their FSC and SSC characteristics (suppl. Fig. 1). The gated subsets were subsequently analysed in a dot plot (Fig. 1c) where gates were set according to FITC

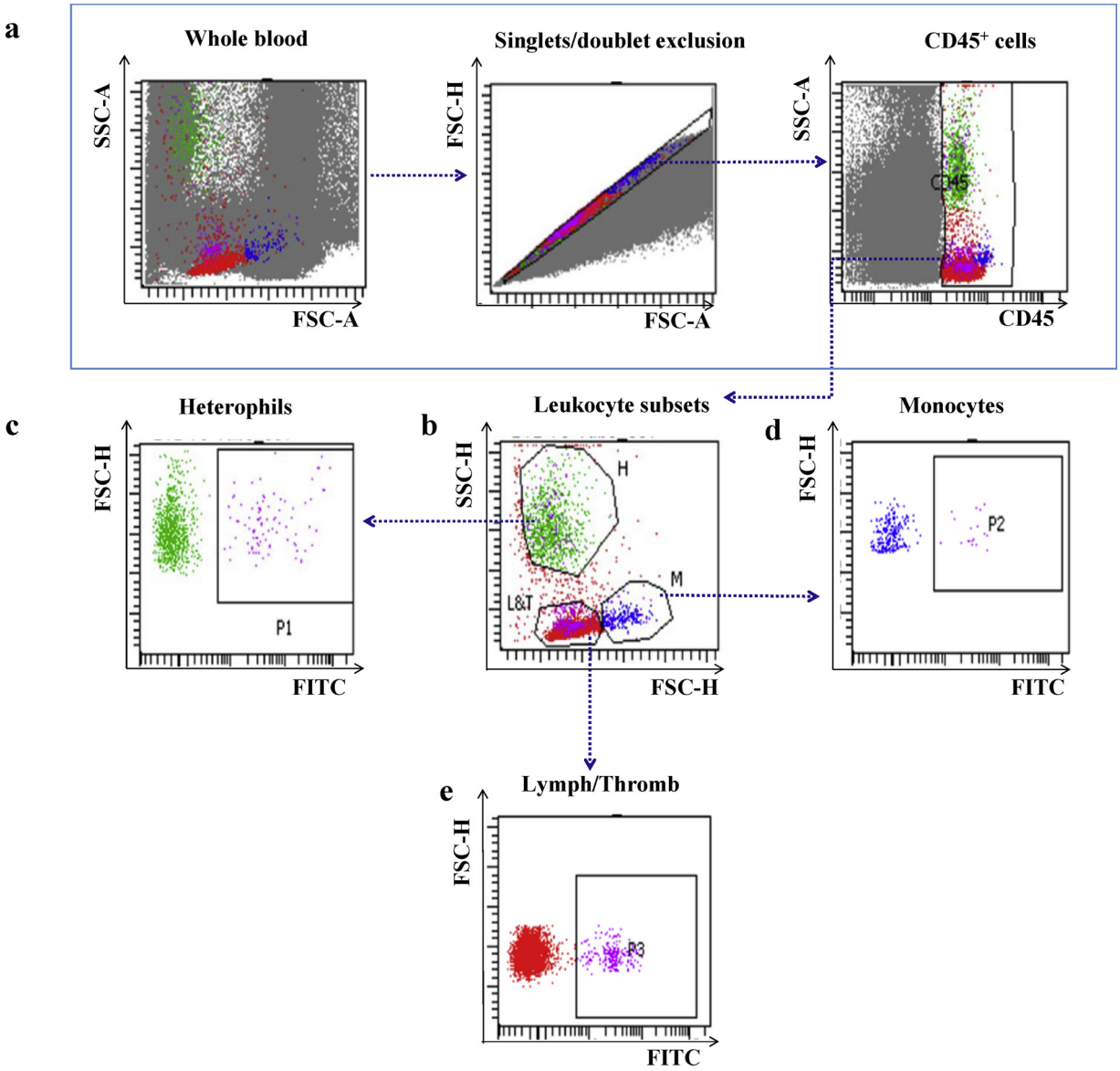


**Fig. 1.** Gating strategies for whole blood flow cytometry analysis of a representative chicken whole blood sample. (a) No-lyse no-wash whole blood staining of leukocytes with anti-chicken-CD45-PerCP. (b) FSC/SSC dot plot showing CD45$^+$ cells and subset gating (H: Heterophils, M: Monocytes and L&T: Lymphocytes/Thrombocytes). (c) Representative dot-plots of the FITC positive population of heterophils, (d) monocytes, and (e) lymphocytes/thrombocytes.

positive/or negative cells, i.e. phagocytosis positive/or negative cells. 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. Each sample was acquired and recorded for 1 min at a medium flow rate corresponding to in average $6 \times 10^3$ CD45+ or HD11 cells. All data were analysed using the FACSDiva software. Phagocytic activity was expressed as percentage cells being active in phagocytosis combined with the amount of internal fluorescence. In this study in the negative 4°C control we observed a few events with high background fluorescence therefore the integrated mean fluorescence intensity (iMFI) (Darrah et al., 2007) was used as a phagocytic score and the iMFI was determined based on MFI of FITC positive cells and calculated as follows: (percentage of FITC positive cells × MFI). MFI and percentage of FITC positive HD11 cells and leukocyte subset were also shown in suppl. Figs.2 and 3, respectively

### 2.6. Detection of antibodies to S. Infantis and S. Typhimurium

The levels of *S.* Infantis and *S.* Typhimurium specific IgY in serum were measured by ELISA. In brief, diluted heat-killed bacteria (1:25 in Na-carbonate-bicarbonate buffer pH 9.6) were added to flat-bottomed 96-well ELISA plates (Thermo Fisher Scientific, Slangerup, Denmark, cat. no. 163320) and after an overnight incubation step at 4°C, the plate supernatant was discarded and blocking buffer (PBS with 1% BSA, 10 mg/ml PBS) was added to each well. Plates were washed three times with 250 µl washing buffer (PBS with 0.1% BSA, pH 7.4) after 1 h incubation at RT on a shaking table (400 rpm), and subsequently 100 µl of serum samples (1:200 diluted in PBS with 0.1% BSA, pH 7.4) was added to each well and incubated at RT on a shaker at 400 rpm for 90 min. Plates were washed three times with washing buffer, and 100 µl/well of horseradish peroxidase conjugated goat anti-chicken IgG (FC; AA129 P, Serotec, Oxford, UK; 1:30000 dilution in PBS with 0.1% BSA) was added. After 1 h plates were washed three times and 100 µl of Tetramethylbenzidine substrate (Clinical Science Product Inc, USA, cat. no. 01016-1-1000) was added. Finally, the reaction was stopped by adding 100 µl of 1 N $H_2SO_4$. Absorbance was measured at 450 nm–650 nm and result were presented as optical density (OD).

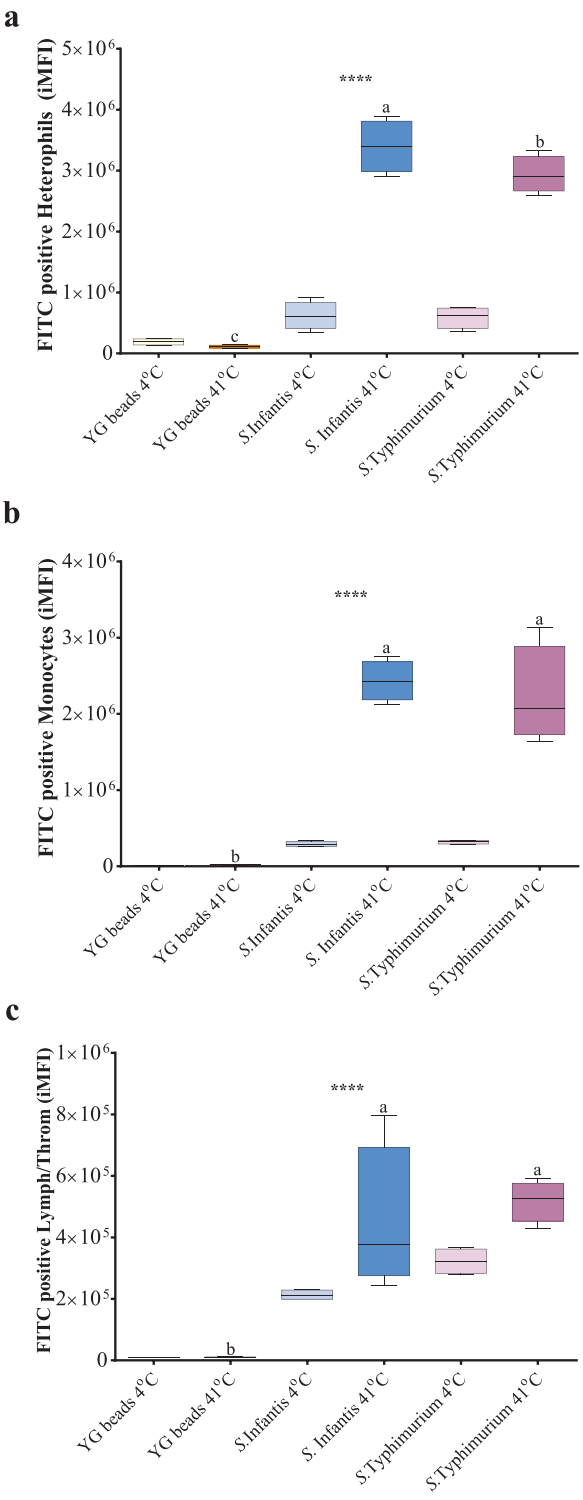


**Fig. 2.** WBP activity shown as average of all the examined MHC haplotypes determined by flow cytometry. (a) FITC integrated mean fluorescence intensity (iMFI) of heterophils at 4°C and 41°C. (b) iMFI of monocytes 4°C and 41°C. (c) iMFI of lymphocytes/thrombocytes 4°C and 41°C. The phagocytic score or integrated iMFI was calculated as follows: (percentage of FITC positive cells × MFI). Results are presented as box and whisker plots showing the median, with 25–75 percentile range as the box and 5–95 percentiles as the whiskers. Boxes with different superscript letter were statistically significantly different. Statistics: Two − way ANOVA ****p < 0.00001.

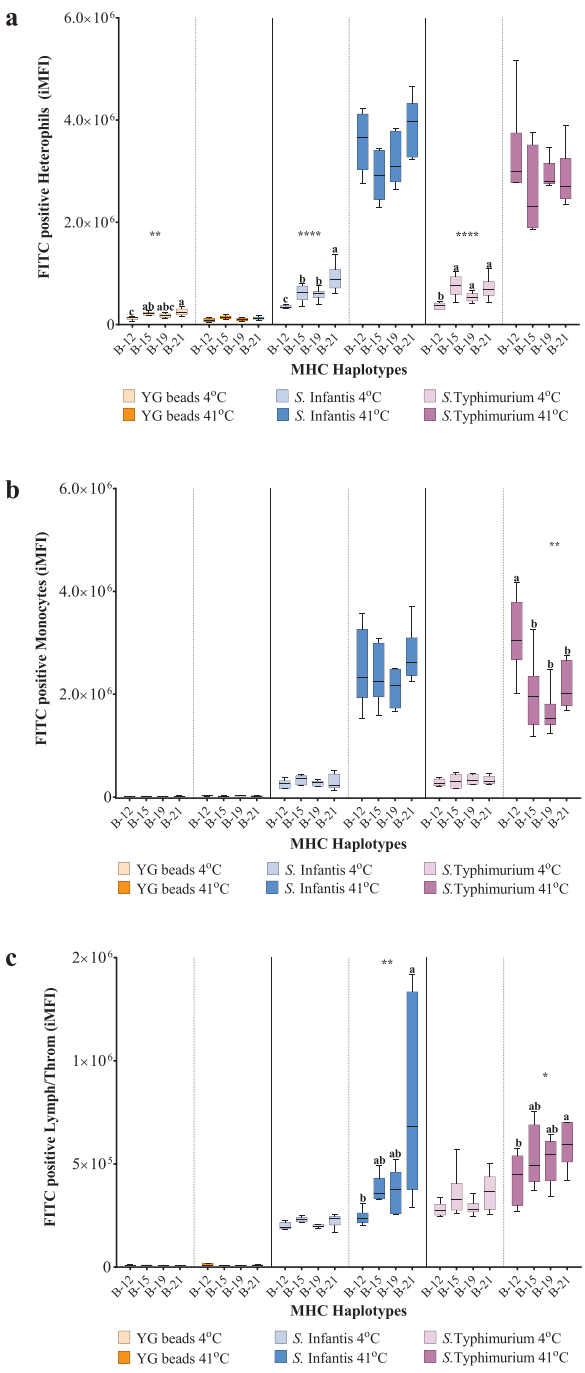


**Fig. 3.** WBP activity of four different MHC haplotypes determined by flow cytometry. (a) FITC integrated mean fluorescence intensity (iMFI) of heterophils at 4°C and 41°C. (b) iMFI of monocytes at 4°C and 41°C. (c) iMFI of lymphocytes/thrombocytes at 4°C and 41°C. The phagocytic score or integrated iMFI was calculated as follows: (percentage of FITC positive cells × MFI). Results are presented as box and whisker plots showing the median, with 25–75 percentile range as the box and 5–95 percentiles as the whiskers. Statistics: Boxes with different superscript letter were statistically significantly different. Statistics: Two − way ANOVA *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00001.

### 2.7. Statistics

Unpaired two-tailed Student's t-tests and two-way ANOVA were used to assess statistically significant differences between groups. D'Agostino-Pearson omnibus test were used to determine normal distribution of data sets. Data with non-normal distribution were log10

transformed prior to statistical analysis. All statistical analyses were performed with Graph Pad Prism version 7.0C (Graph Pad Software, San Diego, CA). Correlations between traits were assessed by Spearman correlation coefficient (r), and P values and sample sizes (n) are reported. Correlations greater than |0.6| were considered as strong correlations, whereas correlations between |0.3| and |0.6| were considered as weak correlations. P value of ≤ 0.05 was considered statistically significant in all cases.

## 3. Results

### 3.1. Flow cytometric assessment of phagocytic activity in whole blood

A single staining (CD45) flow cytometric protocol using a no-lyse, no-wash method, was used to quantify the phagocytic activity of leukocyte subpopulations in chicken peripheral blood. The leukocyte subsets were identified as shown in Fig. 1. The phagocytic activity of leukocyte subsets was compared between treatment groups (the three different phagocytosis targets; Fig. 2) and MHC lines (B12, B15, B19 and B21; Fig. 3). Among the three different targets tested, phagocytosis of *S.* Infantis and *S.* Typhimurium bacteria was shown for heterophils, monocytes and thrombocytes/lymphocytes in whole blood while the YG beads were not phagocytosed by any of the leukocyte populations (Fig. 2). Comparison of phagocytosis in whole blood cultures from chickens of different MHC haplotypes showed that monocyte phagocytosis of *S.* Typhimurium was significantly higher for chickens of the B12 haplotype than for chickens of the other haplotypes ($P < 0.005$) (Fig. 3b). Monocyte phagocytosis of *S.* Infantis did not differ between the different MHC haplotypes ($P > 0.05$) (Fig. 3b). For lymphocytes/thrombocytes, chickens of the B21 haplotype showed the highest phagocytosis of both *S.* Infantis ($P < 0.005$) and *S.* Typhimurium bacteria ($P < 0.05$), respectively, compared to chickens of the other MHC haplotypes (Fig. 3c). For heterophils there were no significant differences between the MHC haplotypes in phagocytosis of any of the bacteria (Fig. 3a). By and large the haplotype differences shown in iMFI are mirrored in the separate measurements of percentages and MFI as shown in supplementary Fig. 4. However, the statistical significance of the haplotype differences were slightly different when looking at differences in the numbers of phagocytic cells or in the amount of phagocytosis per cell. Interestingly, for heterophils, no MHC line differences where observed in iMFI or frequencies of phagocytosing cells but the number of bacteria phagocytosed per heterophil differed significantly between MHC lines as shown in supplementary Fig. 4.

More importantly, these results displayed a wide variation in the phagocytosis potential of the leukocyte subsets between individual chickens within the same line. Moreover, *S.* Typhymurium and *S.* Infantis showed higher adhesion (4°C control) to the different leukocyte subsets than to the YG beads.

### 3.2. Leukocyte subset proportions

The proportions of heterophils, monocytes and lymphocytes/thrombocytes out of $CD45^+$ leukocytes were compared between the three different targets and the different MHC haplotypes (Figs. 4 and 5). Regardless of MHC haplotypes, the proportions of heterophils and monocytes decreased after incubation at 41 °C with both types of bacteria compared to YG bead as well as compared to parallel cultures at 4°C (Fig. 4a, b; $P < 0.00001$) and the proportions of lymphocytes/thrombocytes were hence significantly increased in these cultures (Fig. 4c; $P < 0.00001$). Thus, it seems that phagocytosis by heterophils and monocytes were associated with cell depletion. In addition, results showed that the highest and the lowest proportions of heterophils were observed in blood from the B12 and B21 haplotypes, respectively (Fig. 5a; $P < 0.00001$). The proportions of heterophils in blood from chickens of B15 and B19 haplotypes were similar to each other and lower than those of B12 chickens and higher that those of B21 chickens.

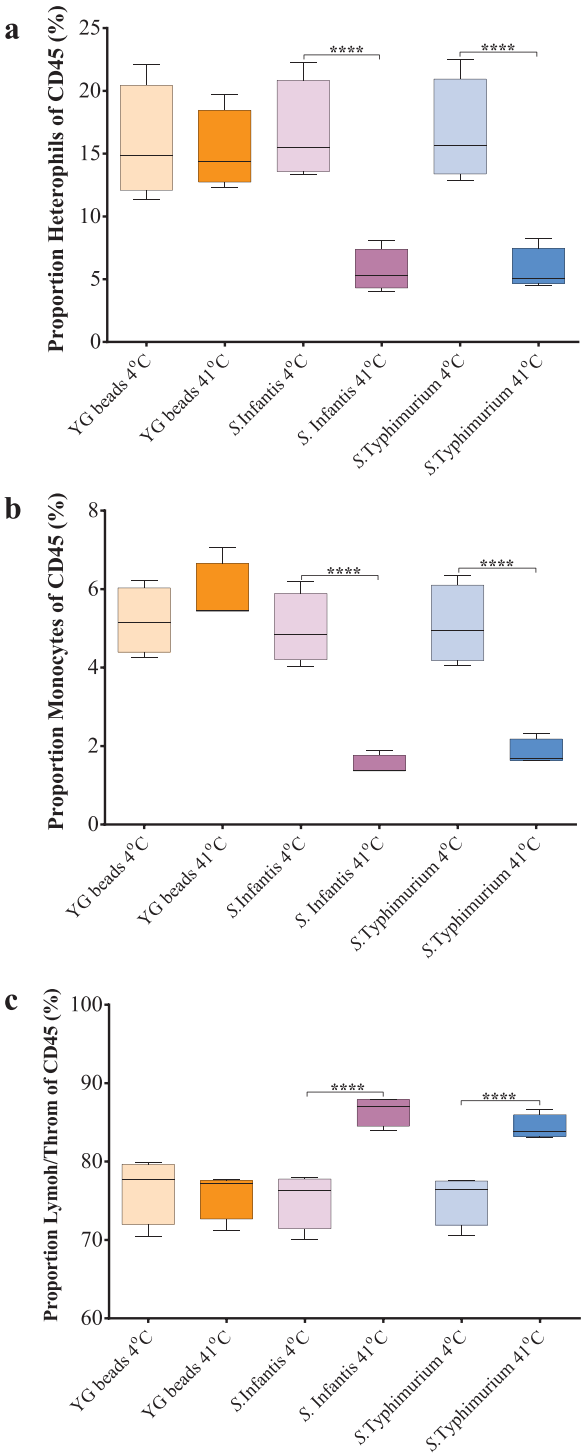


**Fig. 4.** Proportions of leukocyte subsets of all the examined MHC haplotypes after one hour of incubation with YG beads or *Salmonella* bacteria 4°C and 41°C. The relative leukocyte subsets percentages were calculated as proportions of all CD45-positive blood cells. (a) Proportion of heterophils. (b) Proportion of monocytes. (c) Proportion of lymphocytes/thrombocytes. Results are presented as box and whisker plots showing the median, with 25–75 percentile range as the box and 5–95 percentiles as the whiskers. Boxes with different superscript letter were statistically significantly different.Statistics: Two − way ANOVA **** $P < 0.00001$.

Generally, chickens of the B21 haplotype had the highest proportion of monocytes compared to the other MHC haplotypes (Fig. 5b; $P < 0.05$), while the proportions of lymphocytes/thrombocytes were significantly lower for the B12 line compared to the other MHC haplotypes

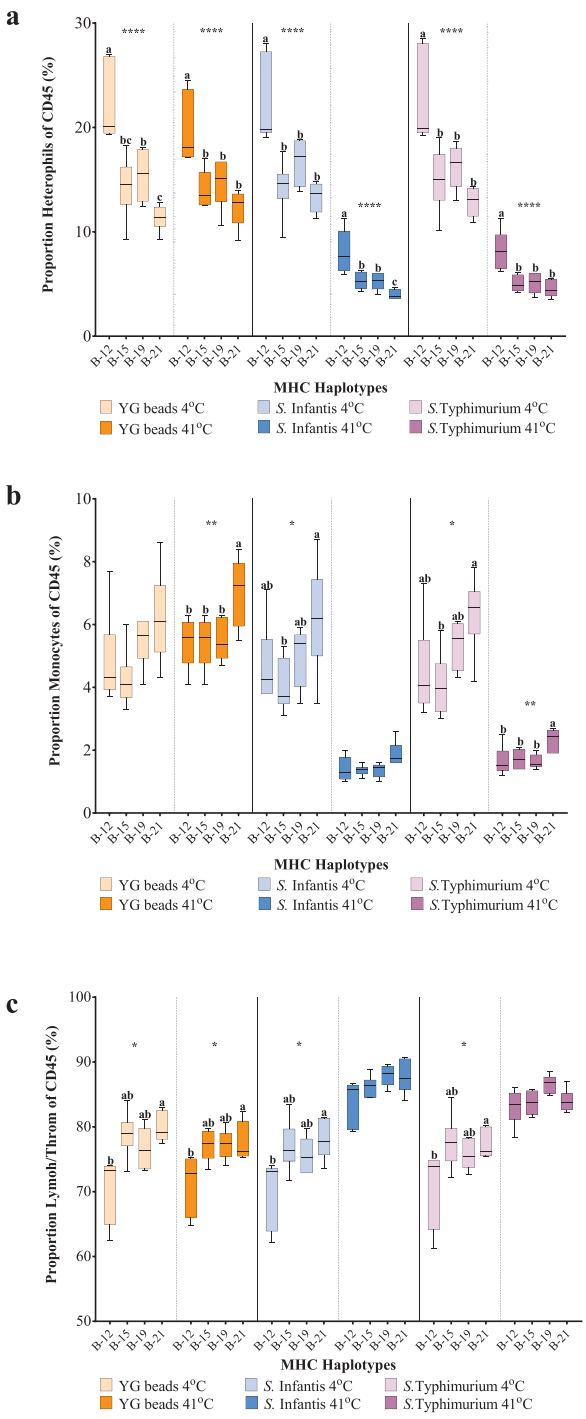


**Fig. 5.** Proportions of leukocyte subsets of four different MHC haplotypes after 1 h of incubation with YG beads and bacteria at 4°C and 41°C. The relative leukocyte subsets percentages were calculated as proportions of all CD45-positive blood cells. (a) Proportion of heterophils. (b) Proportion of monocytes. (c) Proportion of lymphocytes/thrombocytes. Results are presented as box and whisker plots showing the median, with 25–75 percentile range as the box and 5–95 percentiles as the whiskers. Boxes with different superscript letter were statistically significantly different. Statistics: Two − way ANOVA *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00001.

(P < 0.05).

### 3.3. Opsonophagocytic potential of serum

The OPp of serum from the four chicken lines was determined in an

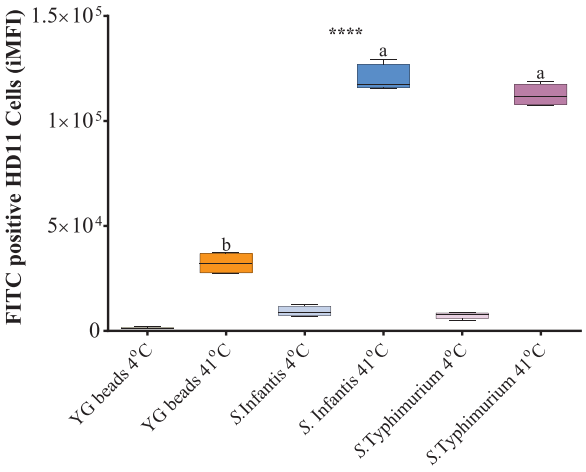


**Fig. 6.** Serum OPp of all the examined MHC haplotypes determined by flow cytometry. FITC integrated mean fluorescence intensity (iMFI) of HD11 cells at 4°C and 41°C. The phagocytic score or integrated iMFI was calculated as follows: (percentage of FITC positive cells × MFI). Results are presented as box and whisker plots showing the median, with 25–75 percentile range as the box and 5–95 percentiles as the whiskers. Boxes with different superscript letter were statistically significantly different. Statistics: Two − way ANOVA *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001.

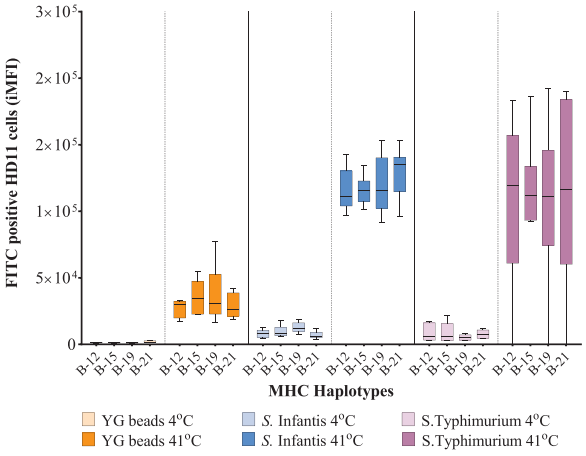


**Fig. 7.** Serum OPp of four different MHC haplotypes determined by flow cytometry. FITC integrated mean fluorescence intensity (iMFI) of HD11 cells at 4°C and 41°C. The phagocytic score or integrated iMFI was calculated as follows: (percentage of FITC positive cells × MFI). Results are presented as box and whisker plots showing the median, with 25–75 percentile range as the box and 5–95 percentiles as the whiskers. Statistics: Two-way ANOVA.

opsonophagocytosis assay using HD11 cells incubated with opsonised bacteria or opsonised YG beads (Figs. 6 and 7). Similar to the results of phagocytic activity of leukocyte subpopulations in chicken peripheral blood, the total number of ingested *S*. Infantis and *S*. Typhimurium bacteria by HD11 cells were significantly greater than YG beads (Fig. 6; P < 0.00001). The YG beads, however, were to some extent phagocytosed by the HD11 cells which was not the case for any of the leukocyte subsets in whole blood. Moreover, a large variation in the amount phagocytosis positive HD11 cells was found between sera from individuals within each MHC line but no significant differences were found between sera from the different MHC haplotypes (Fig. 7). Moreover, in the separate measurements of percentages and MFI as shown in supplementary Fig. 5 no differences between MHC lines were found. No correlation was observed between the phagocytic capacity of the leukocyte subsets and serum OPp (Fig. 8).

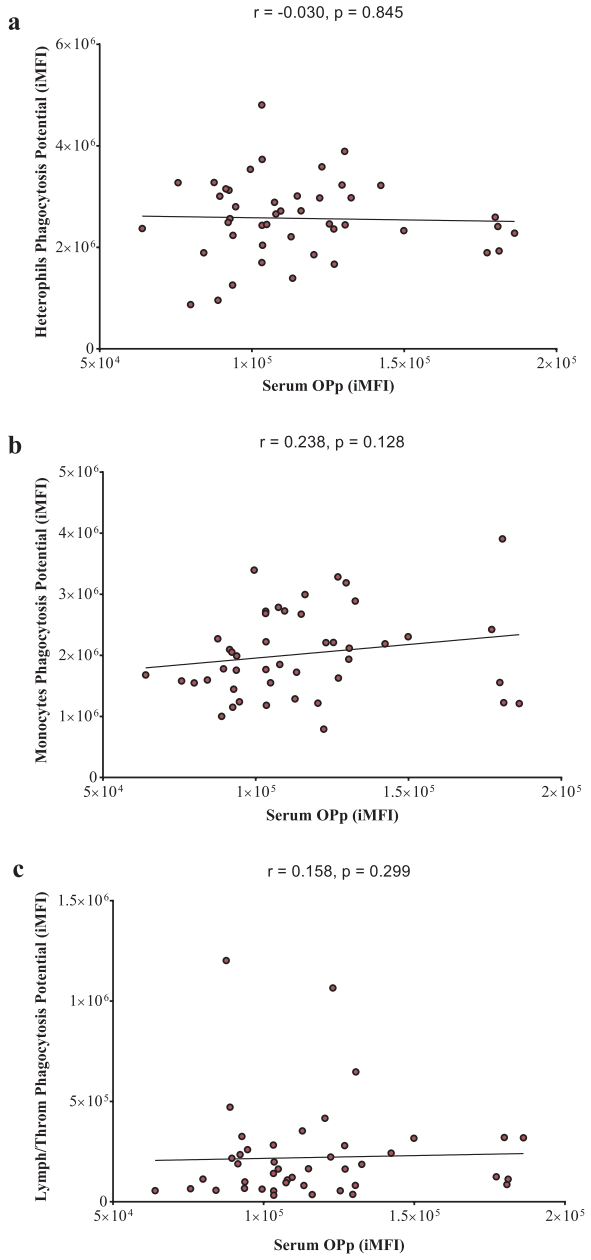


**Fig. 8.** Correlations between WBP activity (iMFI; y-axis) and serum OPp (iMFI; x-axis) of all examined chickens. (a) Correlation between heterophil phagocytosis potential (y-axis) and serum OPp (x-axis). (b) Correlation between monocyte phagocytosis potential (y-axis) and serum OPp (x-axis). (c) Correlation between lymphocyte/thrombocyte phagocytosis potential (y-axis) and serum OPp (x-axis). The integrated mean fluorescence intensity (iMFI) was calculated as follows: (percentage of FITC positive cells × MFI). Pearson correlation coefficients (r) and P-values are shown in each panel.

### *3.4. Correlation between Salmonella specific IgY levels and phagocytic activity*

The *Salmonella*-specific IgY levels for both of *S.* Infantis and *S.* Typhimurium in the serum samples were measured by ELISA. Chickens from all four MHC haplotypes were positive for both types of *Salmonella* antibodies (data not shown). The results were identical irrespective of antigen (S. Infantis *and* S. Typhimurium). Correlations between phagocytic activities with the average *salmonella* specific IgY levels in serum (both of *S.* Infantis and *S.* Typhimurium) were computed (Fig. 9). No statistically significant correlations were found between phagocytosis activity of either heterophils, monocytes or HD11 cells and *Salmonella* specific IgY antibody levels. However, the lymphocyte/thrombocyte phagocytosis activity was positively but weakly correlated with *Salmonella* IgY levels in serum (Fig. 9c; $P < 0006$).

## 4. Discussion

Phagocytosis as a first line of host defense is influenced by the genetic background of the animal and therefore phagocytosis assays may be reflective of the immunocompetence of the individual (Sun et al., 2008). In the present study, we established a no-lyse no-wash, whole blood assay to assess the phagocytic activity of different leukocyte populations under conditions resembling those *in vivo*. The assay was evaluated using blood from chickens of four different MHC haplotypes. We identified significant differences in phagocytic activity of some leukocyte populations between inbred MHC lines (Fig. 3). In line with our results, others have shown that MHC haplotypes may influence the susceptibility of chickens to infection with various bacteria, e.g. *Pasteurella multocida*, *Salmonella* Enteritidis and *Staphylococcus aureus* (Liu et al., 2003, 2002; Schou et al., 2010) where phagocytosis would be expected to be a major defence mechanism. However, the large variation between individuals with the same MHC haplotypes shown in the present study suggests that non-MHC genes may impact the trait as well (Behnke et al., 2003; Schou et al., 2010). Thus, the assay proved useful to detect phagocytic activity simultaneously in different leukocyte populations and we could identify MHC line associated influence on this trait.

To elucidate whether these results were linked to the phagocytic ability of leukocytes or to serum components, we evaluated the potential correlation between phagocytic activity and serum OPp in the samples. The results showed no correlation between serum OPp and phagocytosis of the bacteria by leukocytes (Fig. 8). These results may indicate that the WBP assay mainly reflects the individual's cellular activity.

In addition, contrary to expectations and despite the presence of anti-salmonella IgY in serum, levels of total *Salmonella* IgY did not correlate either with leukocyte phagocytic activity or with serum OPp (Fig. 9). This is in accordance with our previous study where no correlations were found between IgY levels and serum OPp of three different commercial layer lines used in organic production (Naghizadeh et al., 2018; Ulrich-Lynge et al., 2015). Contrasting with the present results, the binding of specific antibodies to *Salmonella* has been shown to enhance bacterial uptake by phagocytes mainly due to an increase in the number of phagocytosed bacteria per macrophage (Muniz-Junqueira et al., 1997; Eguchi and Kikuchi, 2010). Since the experiment was carried out in a bio secure facility and the chickens were not exposed to *Salmonella* bacteria, the observed IgY binding *Salmonella* in the ELISA cannot be regarded as indicating *Salmonella* infection. These IgY levels might therefore, be raised due to interfering cross-reacting antigens which are common to several gram-negative bacteria (e.g., lipopolysaccharide) and may not have a strong enough avidity to *Salmonella* to mediate phagocytosis. Furthermore, insufficient amount of pre-existing opsonins such as IgY, due to the dilution of blood (1:10), to support activation of the classical complement pathway and subsequently uptake of antigens by phagocytes in the *in vitro* assay, could be another explanation to the lack of correlation between *Salmonella* IgY levels and phagocytic activity.

The present data also showed that both the adherence (4°C control) and the total number of ingested bacteria by leukocytes (41°C) were significantly greater than that of YG beads (Fig. 3). Phagocytic activity and particle internalization has been reported to be dependent on both particle size and Fc ligand density (Pacheco et al., 2013). The action of size-dependent inhibitory factor could also explain why there is a consistently low average number of internalized latex beads per cell compared to bacteria. On the other hand, it has been reported that the Toll-Like Receptor (TLR) ligands specifically promote the phagocytosis of bacteria e.g. *S. aureus* and *E. coli,* while displaying negligible effects

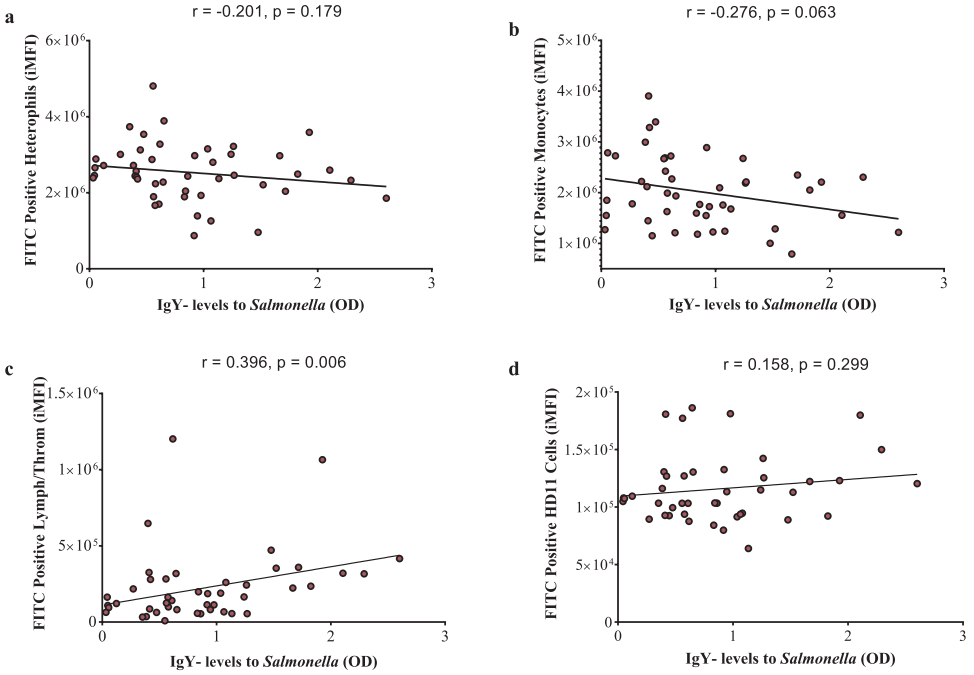


**Fig. 9.** Correlation analysis between *Salmonella* specific IgY levels in serum (x-axis, OD) and HD11 cell / WBP activity of all samples determined by flow cytometry. (a) Correlation between integrated mean fluorescence intensity (iMFI) of HD11 cells (y-axis) and *almonella*S specific IgY levels in serum. (b) Correlation between iMFI of heterophils (y-axis) and serum-specific *Salmonella* IgY levels (x-axis, OD). (c) Correlation between iMFI of monocytes (y-axis) and *Salmonella* specific IgY levels in serum (x-axis, OD). (d) Correlation between iMFI of lymphocytes/thrombocytes (y-axis), and *Salmonella* specific IgY levels in serum (x-axis, OD). The phagocytic score or iMFI was calculated as follows: (percentage of FITC positive cells × MFI). Pearson correlation coefficients (r) and P-values are shown in each panel.

on nonbacterial targets e.g. latex beads (Doyle et al., 2004). Moreover, the expression of a series of leukocyte adhesion molecules, including surface molecules and soluble adhesion molecules (Ala et al., 2002) which have been shown to be positively related to phagocytosis capacity (Sun et al., 2008) could be another explanation. Therefore, these properties of leukocytes and differences in antigens size would explain not only the blood cells higher adhesiveness but also their higher phagocytosis activity.

The WBP assay was technically challenging especially when using fluorescent bacteria. The proportions of heterophils and monocytes were markedly reduced after 1 h incubation at 41°C with both types of *Salmonella* compared to the YG bead as well as compared to parallel incubation at 4°C (Figs. 4 and 5). Possibly, this observation was due to apoptosis occurring in the heterophils and monocytes. *in vivo,* a variety of innate cells are involved in the control of bacterial infection through phagocytosis and production of inflammatory mediators. Interaction of antigens with macrophages and dendritic cells via TLR or enhanced uptake of antigens via scavenger receptors lead to secretion of several cytokines e.g. IL-12 and IL-23 to induce IFN-γ production through Th1 cells and natural killer cells (Oanh and McSorley, 2015). It has been reported that IFN-γ can induce apoptosis through stimulation of the macrophages to uptake excessive number of bacteria (Oanh and McSorley, 2015). Heterophils can also synthesize and produce IL-18 and IFN-γ to orient host acquired immunity toward a Th1 response to *Salmonella* (Kogut et al., 2005). It is questionable if the current short one hour assay would be influenced by *de novo* produced cytokines. However, release of pre-synthesised cytokines present in granulae from e.g. heterophils may influence the assay conditions.

Moreover, avian thrombocytes express various cytokines (IL-1b, IL-6, and IL-12) in response to stimulation with various TLR ligands (St.Paul et al., 2012), This raises the possibility that thrombocytes can act as immunological sensors in circulating blood and may have a significant influence on the uptake of particulate antigens by phagocytes (Nagasawa et al., 2014; St.Paul et al., 2012). The observed reduction in the proportions of monocytes and heterophils after 1 h incubation with bacteria at 41 °C could also be attributed to the expression of the integrin family of adhesion molecules on these cells and therefore, adhesion of the cells onto the wall of cell culture plates.

According to the Figs. 2c and 3c, cells with a lymphocyte/thrombocyte phenotype actively phagocytosed *S.* Typhimurium and *S.* Infantis bacteria similar to those observed for heterophil and monocyte cells. Thrombocytes are CD45 positive, however they show low expression of CD45. Moreover, in whole blood staining the auto fluorescence of the CD45 negative population overlays the signal of the CD45-low thrombocytes (Seliger et al., 2012). Therefore, the majority of the cells in the lymphocyte/thrombocyte gate of the current study is supposedly of the lymphocyte phenotype. However, the assay could be extended to include antibodies directed to specific thrombocyte and lymphocyte markers in order to investigate the relative importance of the different cell subsets in detail.

Altogether, these data indicate the potential of using whole blood rather than isolated peripheral blood mononuclear cells or heterophils to address poultry immunocompetence. The WBP assay benefits from maintaining the possibility of the complex interaction between the cells and soluble components working together in the *in vivo* situation. Our results showed that latex beads were not efficiently phagocytosed by leukocytes in the whole blood assay which is contrary to the HD11 chicken macrophage cell line that readily phagocytosed the latex beads albeit less efficiently than the bacteria. Obviously, the assay also offers the possibility to study direct interactions between phagocytic cells and different species of live bacteria in specific infections models. The whole blood method is far less laborious and avoids cell loss due to separation and washing steps. Consequently, the application of this method is valuable in avian medicine and research. Furthermore, our assay meets the advantages described for other FACS assays, e.g. rapidity and the possibility of testing large numbers of samples in a short time. Altogether, although some differences were found between different MHC haplotypes, the large inter-individual variations in the phagocytosis potential of whole blood suggest that other genetic factors influence leukocyte phagocytic potential. This underlines the complexity of immunocompetence and the challenges in defining relevant traits for genomic studies in relation to poultry robustness.

## 5. Disclosure statement

No potential conflict of interest was reported by the authors.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.vetimm.2018.11.014.

## References


Ala, A., Dhillon, A.P., Hodgson, H.J., 2002. Role of cell adhesion molecules in leukocyte recruitment in the liver and gut. Int. J. Exp. Pathol. 84, 1–16. https://doi.org/10.1046/j.1365-2613.2003.00235.x.

Andreasen, C.B., Latimer, K.S., Harmon, B.G., Glisson, J.R., Golden, J.M., Brown, J., 1991. Heterophil function in healthy chickens and in chickens with experimentally induced staphylococcal tenosynovitis. Vet. Pathol. 28, 419–427.

Beaufrère, H., Ammersbach, M., Tully Jr, T.N., 2013. Complete blood cell count in Psittaciformes by using high-throughput image cytometry: a pilot study. J. Avian Med. Surg. 27, 211–217. https://doi.org/10.1647/1082-6742-27.3.211.

Behnke, J.M., Iraqi, F., Menge, D., Baker, R.L., Gibson, J., Wakelin, D., 2003. Chasing the genes that control resistance to gastrointestinal nematodes. J. Helminthol. 77, 99–110. https://doi.org/10.1079/JOH2003174.

Bohls, R.L., Smith, R., Ferro, P.J., Silvy, N.J., Li, Z., Collisson, E.W., 2006. The use of flow cytometry to discriminate avian lymphocytes from contaminating thrombocytes. Dev. Comp. Immunol. 30, 843–850. https://doi.org/10.1016/j.dci.2005.10.008.

Chou, S.H., Chung, T.K., Yu, B., 2009. Effects of supplemental 25-hydroxycholecalciferol on growth performance, small intestinal morphology, and immune response of broiler chickens. Poult. Sci. 88, 2333–2341.

Dalgaard, T.S., Vitved, L., Skjødt, K., Thomsen, B., Labouriau, R., Jensen, K.H., Juul-Madsen, H.R., 2005. Molecular characterization of major histocompatibility complex class I (B-F) mRNA variants from chickens differing in resistance to Marek's disease. Scand. J. Immunol. 62, 259–270. https://doi.org/10.1111/j.1365-3083.2005.01652.x.

Darrah, P.A., Patel, D.T., De Luca, P.M., Lindsay, R.W., Davey, D.F., Flynn, B.J., Hoff, S.T., Andersen, P., Reed, S.G., Morris, S.L., Roederer, M., Seder, R.A., 2007. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat. Med. 13, 843–850. https://doi.org/10.1038/nm1592.

De Boever, S., Croubels, S., Demeyere, K., Lambrecht, B., De Backer, P., Meyer, E., 2010. Flow cytometric differentiation of avian leukocytes and analysis of their intracellular cytokine expression. Avian Pathol. 39, 41–46. https://doi.org/10.1080/03079450903473574.

de Geus, E.D., Jansen, C.A., Vervelde, L., 2012. Uptake of particulate antigens in a nonmammalian lung: phenotypic and functional characterization of avian respiratory phagocytes using bacterial or viral antigens. J. Immunol. 188, 4516–4526. https://doi.org/10.4049/jimmunol.1200092.

Désert, C., Merlot, E., Zerjal, T., Bed'hom, B., Härtle, S., Le Cam, A., Roux, P.F., Baeza, E., Gondret, F., Duclos, M.J., Lagarrigue, S., 2016. Transcriptomes of whole blood and PBMC in chickens. Comp. Biochem. Physiol. – Part D Genomics Proteomics 20, 1–9. https://doi.org/10.1016/j.cbd.2016.06.008.

Doyle, S.E., O'Connell, R.M., Miranda, G.A., Vaidya, S.A., Chow, E.K., Liu, P.T., Suzuki, S., Suzuki, N., Modlin, R.L., Yeh, W.-C., Lane, T.F., Cheng, G., 2004. Toll-like receptors induce a phagocytic gene program through p38. J. Exp. Med. 199, 81–90. https://doi.org/10.1084/jem.20031237.

Eguchi, M., Kikuchi, Y., 2010. Binding of *Salmonella*-specific antibody facilitates specific T cell responses via augmentation of bacterial uptake and induction of apoptosis in macrophages. J. Infect. Dis. 201, 62–70. https://doi.org/10.1086/648615.

Ferdous, F., Saski, C., Bridges, W., Burns, M., Dunn, H., Elliott, K., Scott, T.R., 2016. Transcriptome Profile of the Chicken Thrombocyte: New Implications as an Advanced Immune Effector Cell. PloS One 11, e0163890.

Fulton, J.E., Juul-Madsen, H.R., Ashwell, C.M., McCarron, A.M., Arthur, J.A., O'Sullivan, N.P., Taylor, R.L., 2006. Molecular genotype identification of the Gallus gallus major histocompatibility complex. Immunogenetics 58, 407–421. https://doi.org/10.1007/s00251-006-0119-0.

Genovese, K.J., He, H., Swaggerty, C.L., Kogut, M.H., 2013. The avian heterophil. Dev. Comp. Immunol. 41, 334–340. https://doi.org/10.1016/j.dci.2013.03.021.

Hu, B.T., Yu, X., Jones, T.R., Kirch, C., Harris, S., Hildreth, S.W., Madore, D.V., Quataert, S.A., 2005. Approach to validating an opsonophagocytic assay for Streptococcus pneumoniae. Clin. Diagn. Lab. Immunol. 12, 287–295.

Jakovac-Strajn, B., Ihan, A., Kopitar, A.N., Malovrh, T., 2011. Phagocytic activity in blood and proliferation of peripheral blood lymphocytes during the perinatal period in primiparous sows. J. Anim. Physiol. Anim. Nutr. (Berl). 95, 328–334. https://doi.org/10.1111/j.1439-0396.2010.01058.x.

Kogut, M.H., Rothwell, L., Kaiser, P., 2005. IFN-γ priming of chicken heterophils upregulates the expression of proinflammatory and Th1 cytokine mRNA following receptor-mediated phagocytosis of *Salmonella enterica* serovar Enteritidis. J. Interferon Cytokine Res. 25, 73–81. https://doi.org/10.1089/jir.2005.25.73.

Liu, W., Miller, M.M., Lamont, S.J., 2002. Association of MHC class I and class II gene polymorphisms with vaccine or challenge response to *Salmonella* enteritidis in young chicks. Immunogenetics 54, 582–590. https://doi.org/10.1007/s00251-002-0495-z.

Liu, W., Kaiser, M.G., Lamont, S.J., 2003. Natural resistance-associated macrophage protein 1 gene polymorphisms and response to vaccine against or challenge with *Salmonella* enteritidis in young chicks. Poult. Sci. 82, 259–266.

Ma, H., Ning, Z.H., Lu, Y., Han, H.B., Wang, S.H., Mu, J.F., Li, J.Y., Lian, Z.X., Li, N., 2010. Monocytes-macrophages phagocytosis as a potential marker for disease resistance in generation 1 of dwarf chickens. Poult. Sci. 89, 2022–2029.

McConnell, S.K.J., Dawson, D.A., Wardle, A., Burke, T., 1999. The isolation and mapping of 19 tetranucleotide microsatellite markers in the chicken. Anim. Genet. 30, 183–189. https://doi.org/10.1046/j.1365-2052.1999.00454.x.

Miller, M., Bacon, L., Hala, K., Hunt, H., Ewald, S., Kaufman, J., Zoorob, R., Briles, W.E., 2004. 2004 Nomenclature for the chicken major histocompatibility (B and Y) complex. Immunogenetics 56, 261–279. https://doi.org/10.1007/s00251-004-0682-1.

Millet, S., Bennett, J., Lee, K.A., Hau, M., Klasing, K.C., 2007. Quantifying and comparing constitutive immunity across avian species. Dev. Comp. Immunol. 31, 188–201. https://doi.org/10.1016/j.dci.2006.05.013.

Muir, W.M., Cheng, H.W., Croney, C., 2014. Methods to address poultry robustness and welfare issues through breeding and associated ethical considerations. Front. Genet. 5, 1–11. https://doi.org/10.3389/fgene.2014.00407.

Muniz-Junqueira, M.I., Prata, A., Tosta, C.E., 1997. Factors influencing phagocytosis of *Salmonella* typhimurium by macrophages in murine schistosomiasis. Rev. Soc. Bras. Med. Trop. 30, 101–106. https://doi.org/10.1590/S0037-86821997000200003.

Nagasawa, T., Nakayasu, C., Rieger, A.M., Barreda, D.R., Somamoto, T., Nakao, M., 2014. Phagocytosis by thrombocytes is a conserved innate immune mechanism in lower vertebrates. Front. Immunol. 5, 36–39. https://doi.org/10.3389/fimmu.2014.00445.

Naghizadeh, M., Wattrang, E., Kjaerup, R.B., Bakke, M., Shih, S., Dalgaard, T.S., 2018. In vitro phagocytosis of opsonized latex beads by HD11 cells as a method to assess the general opsonization potential of chicken serum. Avian Pathol. 1–10.

Nielsen, O.L., Jensenius, J.C., Jørgensen, P.H., Laursen, S.B., 1999. Serum levels of chicken mannan-binding lectin (MBL) during virus infections; indication that chicken MBL is an acute phase reactant. Vet. Immunol. Immunopathol. 70, 309–316. https://doi.org/10.1016/S0165-2427(99)00090-2.

NRC, 1994. Nutrient Requirements of Poultry, 9th rev. ed. Natl. Acad. Press, Washington, DC.

Nuutila, J., Lilius, E.M., 2005. Flow cytometric quantitative determination of ingestion by phagocytes needs the distinguishing of overlapping populations of binding and ingesting cells. Cytom. Part A 65, 93–102. https://doi.org/10.1002/cyto.a.20139.

Oanh, H.P., McSorley, S.J., 2015. Protective host immune responses to *Salmonella* infection. Futur. Microbiol. 101–110. https://doi.org/10.2217/fmb.14.98.Protective.

Pacheco, P., White, D., Sulchek, T., 2013. Effects of microparticle size and Fc density on macrophage phagocytosis. PLoS One 8 e60989.

Papp, Z., Smits, J.E.G., 2007. Validation and novel applications of the whole-blood chemiluminescence assay of innate immune function in wild vertebrates and domestic chickens. J. Wildl. Dis. 43, 623–634. https://doi.org/10.7589/0090-3558-43.4.623.

St. Paul, M., Paolucci, S., Barjesteh, N., Wood, R.D., Schat, K.A., Sharif, S., 2012. Characterization of chicken thrombocyte responses to toll-like receptor ligands. PLoS One 7, 2–10. https://doi.org/10.1371/journal.pone.0043381.

Prosser, A., Hibbert, J., Strunk, T., Kok, C.H., Simmer, K., Richmond, P., Burgner, D., Currie, A., 2013. Phagocytosis of neonatal pathogens by peripheral blood neutrophils and monocytes from newborn preterm and term infants. Pediatr. Res. 74, 503–510. https://doi.org/10.1038/pr.2013.145.

Schou, T.W., Labouriau, R., Permin, A., Christensen, J.P., Sørensen, P., Cu, H.P., Nguyen, V.K., Juul-Madsen, H.R., 2010. MHC haplotype and susceptibility to experimental infections (*Salmonella* Enteritidis, *Pasteurella multocida*or *Ascaridia galli*) in a commercial and an indigenous chicken breed. Vet. Immunol. Immunopathol. 135, 52–63. https://doi.org/10.1016/j.vetimm.2009.10.030.

Schreiner, L., Huber-Lang, M., Weiss, M.E., Hohmann, H., Schmolz, M., Schneider, E.M., 2011. Phagocytosis and digestion of pH-sensitive fluorescent dye (Eos-FP) transfected *E. Coli* in whole blood assays from patients with severe sepsis and septic shock. J. Cell Commun. Signal. 5, 135–144. https://doi.org/10.1007/s12079-010-0112-0.

Seliger, C., Schaerer, B., Kohn, M., Pendl, H., Weigend, S., Kaspers, B., Härtle, S., 2012. A rapid high-precision flow cytometry based technique for total white blood cell counting in chickens. Vet. Immunol. Immunopathol. 145, 86–99. https://doi.org/10.1016/j.vetimm.2011.10.010.

Sun, S.F., Pan, Q.Z., Hui, X., Zhang, B.L., Wu, H.M., Li, H., Xu, W., Zhang, Q., Li, J.Y., Deng, X.M., Chen, J.W., Lian, Z.X., Li, N., 2008. Stronger in vitro phagocytosis by monocytes-macrophages is indicative of greater pathogen clearance and antibody levels in vivo. Poult. Sci. 87, 1725–1733. https://doi.org/10.3382/ps.2007-00202.

Ulrich-Lynge, S.L., Dalgaard, T.S., Norup, L.R., Kjærup, R.M., Olsen, J.E., Sørensen, P., Juul-Madsen, H.R., 2015. The consequence of low mannose-binding lectin plasma concentration in relation to susceptibility to *Salmonella* Infantis in chickens. Vet. Immunol. Immunopathol. 163, 23–32. https://doi.org/10.1016/j.vetimm.2014.11.003.

White-Owen, C., Alexander, J.W., Sramkoski, R.M., Babcock, G.F., 1992. Rapid whole-blood microassay using flow cytometry for measuring neutrophil phagocytosis. J. Clin. Microbiol. 30, 2071–2076.