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Innate immune response to double-stranded RNA in American heritage chicken breeds

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ABSTRACT Backyard poultry flocks that employ heritage breeds of chicken play a crucial role in the maintenance of poultry pathogens of economic and zoonotic importance. This study examined innate immunity to viral pathogens in heritage chicken breeds using a model of viral double-stranded RNA (**dsRNA**). Following intraperitoneal injection of high molecular weight (**HMW**) -poly(I:C)/Lyovec into 4-wk-old chicks, we evaluated gene expression in peripheral blood mononuclear cells (**PBMCs**) and splenocytes. There was a significant difference across breeds in the expression of IL-4, IL-12p40, IFNγ, and B-cell activating factor (**BAFF**) in the spleen. In PBMCs, a significant difference in IFN-α expression was seen across breeds. Approximately 57% of IFN-α transcripts in PBMCs was explained by levels of expression of MDA5 transcripts. Using flow cytometry, we showed that only monocytes/macrophages (KUL01+ cells) expressed the scavenger receptor CD163. Regression analysis showed that 42% of fold change in CD163 expression on PBMCs was explained by breed ($P < 0.0004$). In general, breeds that responded to HMW-poly (I:C) by showing higher upregulation of IFNγ, IL-1β, and IL-12p40 transcripts in the spleen, and higher IFNα transcripts in peripheral blood, expressed less CD163 on blood monocytes. These findings suggest a genetic basis for the response of chickens to double-stranded RNA. Surface expression of the scavenger receptor CD163 in PBMCs following injection of high molecular weight poly(I:C) may be a rapid method to select chickens for breeding based on innate immune response to viral dsRNA.

KEY WORDS: dsRNA, cytokine transcript, CD163, American heritage chicken

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INTRODUCTION

Backyard flocks are growing in popularity in both rural and urban United States (Cadmus et al., 2019; Larsen et al., 2022). However, backyard chickens could play an important role in the emergence, spread, and maintenance of several infectious diseases by serving as reservoirs and amplifiers of pathogens (Spackman et al., 2018; Ayala et al., 2020). Most backyard poultry operations employ heritage chicken breeds derived from parent and grandparent stock of breeds recognized by the American Poultry Association prior to the mid-20th century. Unlike commercially relevant poultry lines, the immunological basis of disease resistance of backyard and fancy breed poultry has not been extensively investigated (Fulton et al., 2016).

The innate immune system is a danger sensor that maintains homeostasis by responding to cellular injury, infection, and dysregulated cellular processes. It is the first line of defense against viral infections. The innate immune response is triggered when pattern recognition receptors (**PRR**) expressed by immune cells recognize danger-associated molecular patterns found on damaged cells or unique pathogen-associated molecular patterns found on pathogens (Luecke and Paludan, 2017). A major pathogen-associated molecular pattern in RNA viruses is double-stranded RNA (**dsRNA**), an intermediate species that accumulates in the cytoplasm of infected cells during replication (Zhou and Rana, 2013; Kell and Gale, 2015). Toll-like receptor 3 (**TLR3**) and protein products of retinoic acid-inducible gene 1 (**RIG-1**) and melanoma differentiation-associated (**MDA**) gene 5 (**MDA5**) are crucial PRRs for viral dsRNA. TLR3 is a single-pass transmembrane protein localized

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to the endosomal membrane. The ligand-binding ectodomain faces the endosomal lumen. Following endocytosis, TLR3 on the endosomal membrane binds viral dsRNA to induce type I interferons (**IFN**), pro-inflammatory cytokines, and products of IFN-stimulated genes by specifically recruiting the intracytoplasmic adaptor molecule TRIF (O'Neill et al., 2013).

Retinoblastoma Inducible Gene-1 (**RIG-1**) and MDA5, otherwise called interferon-inducible helicase 1 (**IFIH1**), belong to a group of PRRs called RIG-like receptors (**RLRs**). Unlike TLR3, MDA5 and RIG-1 are found in the cytoplasm where they play a central role in the recognition of intracytoplasmic dsRNA following virus infection (Goubau et al., 2013). Following binding with dsRNA, the caspase activation and recruitment domain (**CARD**) of RIG-1 and MDA5 specifically interacts with a mitochondria-associated protein CARDIF (also known as MAVS) to induce type IFN and other proinflammatory cytokines (Yoneyama et al., 2015).

In most in vivo and in vitro studies that investigated the signaling pathways that resulted in IFN production following the binding of dsRNA to TLR3 or RIG-1/MDA5, polyinosinic-polycytidylic acid (poly I:C) was used as a synthetic analog of viral dsRNA (Zevini et al., 2017). While endosomal TLR3 signaling is induced by naked poly(I:C) (De Miranda et al., 2009), cytosolic receptors RIG-1 and MDA5, are specifically activated using poly(I:C) combined with LyoVec, a transfection reagent (De Miranda et al., 2009; Farias-Jofre et al., 2023). Transfection of poly(I:C) complexed to LyoVec (Invivogen, CA), a proprietary cationic phosphonolipid mimics the specific induction of IFN through binding to intracytoplasmic dsRNA sensors RIG-1 and MDA5 (Kato et al., 2008). This action of LyoVec is most likely related to the ability of phosphonolipids to enhance endosomal escape for intracytoplasmic delivery of poly I:C (Floch et al., 1997; Guillaume-Gable et al., 1998; Alvarez-Benedicto et al., 2022).

More recent studies used an avian-specific CRISPR/Cas9 system to generate single and double TLR3 and MDA5 knockouts (**KO**) of the chicken embryo fibroblast cell line DF1 (Lee et al., 2020). In this model, poly (I:C) treatment did not induce IFNs in TLR3-MDA5 double KO cells, supporting the importance of TLR3 and MDA5 in the innate response of chicken cells to dsRNA. In another study using the same approach, IFN transcripts were not upregulated following treatment of TLR3-MAVS KO DF1 cells with poly I:C, whereas they were upregulated in wild-type and TLR3-STING KO DF1 cells (Shin et al., 2023). Put together, these studies highlighted the central role played by MDA5-MAVS signaling in poly(I:C)-induced innate response in chicken cells and further supported the use of LyoVec/poly(I:C) as a specific activator of MDA5-MAVS signaling.

Previous reports in human subjects showed strong correlations between mutations in the MDA5 (*IFIH1*) gene and susceptibility or resistance to type 1 diabetes, an autoimmune disease related to inappropriate activation of the Th1 immune response (Looney et al., 2015). Other studies have associated mutations in this gene with a wide spectrum of conditions (Miner and Diamond, 2014; Rice et al., 2014; Todd, 2014; Bursztejn et al., 2015). These findings suggest that the IFIH1/MDA5 gene, is subject to selection pressure in human populations. Interestingly, chickens have only MDA5 but not RIG-1 (Cornelissen et al., 2012; Cornelissen et al., 2013). It is conceivable that, compared to humans with both MDA5 and RIG-1, chicken MDA5 will be subject to a stronger selection pressure. Consequently, we hypothesized that specific activation of the MDA5 pathway in different breeds of chickens may result in significant differences in their response as evidenced by differences in the upregulation of cytokine/chemokine RNA transcripts. The data reported herein supported our hypothesis and suggested that CD163-positive mononuclear phagocytic cells may play a crucial role in the differences observed.

MATERIALS AND METHODS

Experimental Chickens

Newly hatched unvaccinated male chickens (breeds: White Leghorn; Rhode Island Red; Red Ranger Broiler; Barred Plymouth Rock; Ameraucana; Black Australorp; New Hampshire; Blue Andalusian; Black Star; Delaware; Red Star; and Brown Leghorn) were purchased from Murray McMurray Hatchery Inc. (Webster City, IA) and housed in battery cages (Labex, Worcester, MA) in a controlled-temperature room at the Poultry Research Laboratory, Tuskegee University. Chicks were fed a commercial starter diet (CP 25%, ME 3200 kcal/kg) and autoclaved water *ad libitum*. Experiments were performed after the birds were 4-wk old. All procedures were approved by the Tuskegee University Animal Care and Use Committee (**TUACUC**). The Guidelines for Care and Use of Laboratory Animals published by the Institute for Laboratory Animal Research (**ILAR**, 1996 edition) were followed in keeping the experimental chickens for the duration of this study (http://www.nap.edu/openbook.php?recod_id=5140).

Activation of Innate Immune Response in Different Breeds of Chicken by Poly(I:C) in Vivo

The experiment was conducted in 3 trials. Each trial consisted of 3 experimental birds and 3 control birds. We evaluated twelve different breeds of chickens; for each breed, 3 experimental birds were treated with high molecular weight polyinosinic-polyctidylic acid-poly(I:C) (HMW)/Lyovec—(Invivogen, San Diego, CA) while 3 control birds were treated with Lyovec control reagent without dsRNA obtained from the same suppliers. For this purpose, poly(I:C) (HMW)/Lyovec was reconstituted with 500 μL endotoxin-free water to contain 200 μg/mL of HMW poly(I:C). Using an insulin syringe, 0.1 mL of this solution was injected intraperitoneally under the sternum so that each bird received 20 μg of HMW poly(I:C) delivered in LyoVec. After 18 h, birds

were euthanized using carbon dioxide. Blood was collected into anticoagulated tubes and the spleen was harvested into a tube containing 2 mL of RPMI 1640 with 1% FBS and antibiotics. Splenocytes were prepared using sterile cell strainers (MidSci, St Louis, MO) as previously described (Brisbin et al., 2011).

Real-Time PCR to Quantify mRNA Transcript Expression

Total RNA was extracted from 5 million splenocytes using the Qiagen RNeasy Mini Kit (Qiagen, Germantown, MD) with on-column DNAse treatment to remove genomic DNA (Qiagen). The concentration and quality of the RNA extracted was evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Frederick, MD). The QuantiTect Reverse Transcription Kit (Qiagen) was used to synthesize cDNA from the RNA sample with random primers. Based on our initial results of RNA transcript profiling in HD11 chicken macrophage cell line treated with poly(I:C) (HMW)/Lyovec, we used the RT^2 Profiler PCR Array System (Qiagen) to evaluate the expression of CCL4, IL-12p40, IFNγ, B-cell activating factor (BAFF), IL-1β, and IL-4 in spleen samples from birds treated with HMW poly(I:C) and birds treated with Lyovec control reagent as previously described (Abo-Samaha et al., 2023). In addition to splenocytes, we extracted mRNA from 200 μL of whole blood, synthesized complementary DNA (**cDNA**), and used the QuantiTect SYBR Green PCR Kit (Qiagen) to quantify IFNα, 28s rRNA, and MDA5 transcripts using a Stratagene Mx3000P real-time cycler (Agilent Technologies). Primers and real-time PCR conditions for quantifying IFNα and 28s rRNA transcripts were previously described (Gimeno and Cortes, 2011). The following primers were used to amplify MDA-5 transcripts: MDA-F (5'-AAT TGC TAT GGT GCA GGC CC—3) and MDA-R (5'-TGG ACA CGT CGA ATG GCC TTA—3). Each sample was run in triplicates. The Ct of 28s rRNA was used as the housekeeping gene. Fold change in relative mRNA transcript level was determined using the $2^{-\Delta\Delta Ct}$ method (Agilent Technologies, Santa Clara, CA).

Flow Cytometry

We asked what cell types might be associated with the expression of IFNα and MDA5 expression in whole blood samples of chickens following HMW/poly(I:C) injection. Having previously demonstrated the effects of HMW/poly(I:C) on the expression of cytokine transcripts in the chicken macrophage cell line HD11, we focused on macrophages. After 18 h postinjection, birds were euthanized and blood was collected by cardiac venipuncture into an EDTA tube, mixed well, and kept on ice before processing. To stain for specific markers, 100 μL of blood was diluted with 100 μL of Phosphate buffered saline containing 0.1% albumin (PBS+) in a 5-mL flow cytometry tube. Following the addition of antibodies, this mixture was incubated in the dark on ice (4°C) for 30 min. Erythrocytes were lysed by adding 1.5 mL of sterile double-distilled water and pipetting until RBC lysis was evident (color change to wine red). The tube was filled up to 4 mL with PBS+ and centrifuged for 5 min at 300 g. This wash step was repeated twice. Stained cells were resuspended in 500 μL of PBS+. Antibody reactivity was analyzed using a FACS Calibur flow cytometer (BD Biosciences/Cytotek). Forward and side scatter thresholds on linear scales were first adjusted using unstained cells, without gating out populations with very low side and forward scatter properties (these populations often contain membranes of lysed erythrocytes, cell-free nuclear materials, and thrombocytes). Fluorescence from the green channel (**FL1**), yellow channel (**FL2**), and red channel (**FL3**) was set using cells stained with conjugated antibodies alone (antimouse-IgG-FITC, antimouse IgG-PE, and antimouse IgG-PE-Cy5). The photomultiplier tube (**PMT**) and fluorescence gain on each channel was adjusted such that these negatively-stained cells were within 10^0 to 10^1 fluorescent units. For multiparameter acquisition where fluorescence values from the 3 channels were combined, the spectral overlap was minimized by adopting the fluorescence-minus-one strategy (Leggat et al., 2013). Antibodies used include mouse antichicken MHC Class-II-FITC (Clone 2G11, IgG1k), mouse antichicken monocyte/macrophage-PE (clone KUL01) (Southern Biotech, Birmingham, AL), and mouse antihuman CD163-FITC (clone Ber-Mac3; MBL, Japan) following validation in chicken macrophages. Analysis of flow cytometry data was carried out using the Flow Jo software version 10.90 (Flowjo Inc., Ashland, Oregon).

Data Analysis

Data analysis was carried out using IBM SPSS Statistics (version 26) Predictive Analytics software. The normality of numerical data was evaluated using Shapiro−Wilk, Kolmogorov−Smirnov, Cramer−von Mises, and Anderson−Darling methods. A P-value > 0.05 was interpreted as nondeparture from normality. A one-way analysis of variance (ANOVA) was conducted to determine if differences exist in gene expression across the different breeds of chickens examined. This was then followed by t test and chi-square tests to determine differences between groups when ANOVA identified significant differences across groups. In addition, after criterion coding of the categorical variable "breed," a multiple regression analysis was carried out to determine the predictors of IFN-α expression following the injection of poly(I:C). In every case, the null hypothesis of no difference was rejected if $P < 0.05$.

RESULTS AND DISCUSSION

Chicken Breeds Differentially Respond to dsRNA Stimulation in Vivo

Naked poly(I:C) was previously shown to increase the systemic expression of interferon-inducible genes

Table 1. Expression of proinflammatory cytokine transcripts among 12 chicken breeds. Birds were injected with 20 μg/bird Lyovec/HMW Poly(I:C) via the intraperitoneal route. Spleen samples were harvested after 18 h. RNA was prepared from splenocytes (5×10^6 cells) and probed with cytokine-specific primers using the RT2 Array System. Experiments were conducted in 3 trials containing 3 experimental and 3 control birds for each breed. Nucleic acid for 3 treated birds or 3 control birds were pooled per trial so that each data point represents 9 samples per treatment/control.

Breed	IL-4	IL-12p40	IFNγ	CCL4	IL-1β	BAFF
White Leghorn	$0.83 \pm 0.91^{a,b}$	$0.94 \pm 0.95^{a,b,c}$	0.68 ± 0.93^{b}	1.56 ± 1.01	1.07 ± 0.90	$0.67 \pm 1.37^{a,b,c}$
Rhode Island Red	$1.22 \pm 1.24^{a,b}$	$1.25 \pm 1.12^{a,b,c}$	1.07 ± 0.91^{b}	0.52 ± 1.46	0.78 ± 1.26	$0.60 \pm 2.05^{a,b,c}$
Red Ranger Broiler	$1.33 \pm 1.17^{a,b}$	$1.10 \pm 1.22^{a,b,c}$	1.21 ± 1.46^{b}	1.81 ± 1.64	1.40 ± 1.00	$1.39 \pm 1.64^{a,b}$
Barred Plymouth Rock	1.89 ± 0.34^{a}	$1.84 \pm 0.43^{a,b}$	0.95 ± 0.21^{b}	1.35 ± 1.42	1.59 ± 0.37	$1.45 \pm 0.64^{a,b}$
Ameraucana	$0.48 \pm 0.72^{a,b,c}$	$0.66 \pm 0.59^{a,b,c}$	0.38 ± 0.68^{b}	-0.08 ± 0.25	0.30 ± 0.97	$0.36 \pm 0.86^{b,c}$
Black Australorp	$-0.86 \pm 0.35^{b,c,d}$	$-0.88 \pm 0.35^{b,c,d}$	-1.34 ± 0.33^{c}	0.11 ± 0.80	-1.32 ± 0.28	$-2.97 \pm 0.03^{d,e}$
New Hampshire	-2.17 ± 0.79^{d}	$-0.55 \pm 1.34^{b,c,d}$	2.90 ± 0.35^{a}	0.95 ± 0.74	0.76 ± 2.56	$1.58 \pm 1.27^{a,b}$
Blue Andalusian	$-1.14 \pm 2.11^{b,c,d}$	3.05 ± 2.57^{a}	0.99 ± 0.14^{b}	0.59 ± 2.40	2.01 ± 1.92	3.11 ± 0.41^{a}
Black Star	-2.28 ± 1.20^{d}	-2.66 ± 0.34^{d}	-3.01 ± 0.28^{d}	-3.19 ± 3.56	-2.10 ± 1.19	-3.68 ± 0.42^{e}
Delaware	$-0.41 \pm 0.83^{a,b,c,d}$	$0.28 \pm 0.83^{a,b,c}$	$-0.27 \pm 0.90^{b,c}$	0.52 ± 0.86	-0.45 ± 0.85	$-0.93 \pm 1.18^{b,c,d}$
Red Star	$-1.79 \pm 0.37^{c,d}$	$-1.18 \pm 0.57^{c,d}$	$-1.65 \pm 0.30^{c,d}$	-0.55 ± 0.54	-1.38 ± 0.28	$-1.27 \pm 0.18^{c,d,e}$
Brown Leghorn	$0.00 \pm 1.22^{a,b,c,d}$	$-0.16 \pm 1.62^{b,c,d}$	$-0.23 \pm 0.86^{b,c}$	-0.80 ± 0.65	-0.17 ± 1.40	$-0.52 \pm 0.69^{b,c,d}$
P value	**0.0177**	**0.0244**	**0.0003**	0.2531	0.1024	**0.0014**

Each value represents mean ± SD for 3 trials consisting of 3 pooled bird samples/treatment/breed. Means within the same column bearing different superscripts are statistically different ($P < 0.05$).

Significant differences are highlighted as *P*-values in bold.

BAFF, B cell activating factor; CCL4, Chemokine (C-C motif) ligand 4; IFN-γ, Interferon Gamma; IL-4, Interleukin 4, IL-12p40; IL-1β, Interleukin-1β.

through the TLR-3 pathway when injected into day-old chicks via the intramuscular route (Parvizi et al., 2012b). We asked whether the Lyovec-protected high molecular weight (**HMW**) Poly(I:C), which signals through the MDA 5 pathway would upregulate in vivo the expression of some of the genes activated by this synthetic dsRNA species in vitro. We evaluated the effects of HMW poly(I:C) on the expression of transcripts in splenocytes using real-time PCR. We focused on the expression of the following chemokines, cytokines, and growth factors: IL-4, IL-12p40, IFNγ, IL1-β, CCL4, and BAFF. These transcripts were chosen because they were the most significantly upregulated in a preliminary in vitro experiment using HD11 macrophage cells (Abo-Samaha et al., 2023). Although IL-12p40 is a constituent of the bioactive cytokines IL-12 and IL-23, it was investigated in this study as a surrogate for IL-12 without considering the expression of the heterodimeric partner IL-12p35. Expression of IL-4, IL-12p40, IFNγ, and BAFF did not deviate from normal distribution. A significant difference was found across breeds in the expression of the following transcripts: IL-4 ($P = 0.018$), IL-12p40 ($P = 0.024$), IFNγ ($P = 0.0003$), and BAFF ($P = 0.0014$) (Table 1). However, no statistically significant difference was found in the expression of IL-1β ($P = 0.102$) and CCL4 ($P = 0.253$) across the groups. Of the 12 breeds examined, the following breeds of chickens consistently showed higher fold increases compared to untreated birds in IL-4, IL-12p40, IFN-γ, and BAFF transcripts than other breeds following activation with HMW poly(I:C): White Leghorn, Rhode Island Red, Red Ranger Broiler, and Barred Plymouth Rock. The strongest upregulation of IL-4 was observed in Barred Plymouth Rock. Conversely, the New Hampshire breed showed the highest level of upregulation of IFNγ among the breeds examined. In general, no significant increase in IL-4, IL-12p40, and IFNγ was found in the Black Star breed following treatment with HMW poly(I:C). As an adjuvant known to promote an environment that leads to the generation of a Th1 type response, it was not surprising that HMW poly(I:C) led to the upregulation of IFNγ and IL-12p40 transcripts in this study. However, it was surprising that this expected Th1-promoting effect of poly(I:C) was only observed in 4 breeds of chickens, suggesting that response to immunopotentiation may be genetically determined. Multiple MHC haplotypes and novel MHC recombinants have been found in a study that employed better-characterized heritage breeds of chickens than those used in our study, supporting a genetic basis for immunity in this group of chickens (Fulton et al., 2016). Indeed, a study that examined the innate immune response to the Newcastle Disease Virus by measuring mRNA transcripts assigned a role to genetic differences (Schilling et al., 2018). Although treatment of hens with poly(I:C) was shown to have a distinct transgenerational imprint on gene expression patterns up to the F2 generation, the effect of breed on the immunopotentiating activity of this synthetic analog of viral dsRNA has not been extensively explored (Liu et al., 2022). Our study suggests that the role of poly(I:C) as an adjuvant may depend on the breed of chickens. Previous studies examining the use of poly(I:C) as an adjuvant for vaccination against Marek's disease with HVT-RD vaccination produced disparate results when splenic transcripts were compared in meat versus egg-type birds (Parvizi et al., 2012a; Bavananthasivam et al., 2018; Ding et al., 2022; Boone et al., 2023). Nonetheless, the same 4 breeds of chickens that responded to poly(I:C) with significant increases in IL-12p40B and IFNγ transcripts showed upregulation of transcripts for the canonical Th2 cytokine, IL-4, and BAFF, a cytokine that is crucial to the development, maturation, and survival of B cells (Kothlow et al., 2010; Yang et al., 2015). Apart from genetics, it has been suggested that the pattern of transcript upregulation following poly(I:C) injection or vaccination with HVT-RD may be dependent on the organ or cells examined and the type of cytokine transcripts under investigation (Boone et al., 2023).

Therefore, we examined changes in type I interferon and MDA5 transcripts in blood samples.

Expression of IFN-α in Blood and Spleen Correlates With MDA-5 Expression

We examined the expression of IFN-α and MDA5 transcripts in the spleen and PBMCs of different chicken breeds in response to HMW poly(I:C). Fold increases in IFN-α expression in the spleen of treated versus untreated birds were not statistically different among chicken breeds (Table 2). However, there was a statistically significant difference in the fold increase in the expression of IFN-α in PBMCs across breeds ($P < 0.0001$) (Table 2). The highest increase of IFN-α transcript in PMBCs was observed in Rhode Island Red ($\log_{10}$3.36 x) while Black Australorp showed the lowest expression of IFN-α. Similarly, there was a statistically significant difference in the expression of MDA5 in spleen samples ($P = 0.002$) and PBMCs ($P = 0.004$) from different chicken breeds (Table 2). White Leghorn and Brown Leghorn showed the highest expression of MDA5 from the spleen while New Hampshire showed the lowest expression. Also, higher expression of MDA5 was observed in PBMCs of the Rhode Island Red breed ($\log_{10}$4.91 x), while the lowest expression was recorded for Black Australorp.

A similar study quantified MDA5 and IFN-α transcripts in the blood of chickens infected with avian reovirus and showed that these transcripts increased as viremia increased up to 72 h, confirming that they can indeed be used as markers of activation of innate immunity by dsRNA in chickens (Xie et al., 2019). The significantly higher amount of MDA5 and IFN-α in the PBMCs of White Leghorn and Rhode Island Red breeds, compared to other breeds in our study, is consistent with breeds that are more readily activated by viral dsRNA than other breeds. Thus, the breed-dependent pattern of Th1 and Th2 transcripts initially identified in splenocytes is not restricted to tissues but is also present in the blood.

In addition, we observed a direct correlation between the levels of MDA5 and IFN-α transcripts in the spleen ($P < 0.0001$; adjusted R-Square = 0.57). Thus, 57% of IFN-α mRNA level in the spleen can be explained by upregulation of MDA5 mRNA. Similarly, IFN-α level in PBMCs is significantly associated with the expression of MDA5 in PBMCs ($P < 0.0001$; adjusted R-square = 0.50). Although the data suggested the presence of other mechanisms that might lead to an increase in type I interferon transcripts following exposure to dsRNA, this finding confirmed that injection of poly(I:C) is a reliable in vivo model to investigate dsRNA-induced MDA5-dependent interferon pathway in chickens. Previous in vitro studies showed that silencing the chicken MDA5 resulted in a reduction of IFN-β mRNA expression in DF-1 and HD-11 cells primed with poly I:C or AIV (Kang et al., 2022; Liniger et al., 2012; Lee et al., 2020). Our data supported these in vitro results using an in vivo model of early immune response to dsRNA in chickens.

Early Innate Response to dsRNA Correlates with Presence of CD163+ Monocytes

Finally, we asked which cell populations were responsible for the increase in MDA5 and IFN-α transcripts in blood samples of chickens exposed to dsRNA. First, we probed 3 populations of cells in PBMCs with antibodies specific for monocyte/macrophages (KUL01) and MHC class II and identified monocytes as KUL01+/MHC II+ cells with high forward and side scatter. We then examined monocytes with an antibody that specifically detected the scavenger receptor CD163, a marker that has been used to classify monocytes/macrophages into classical (M1; CD163-) or alternatively-activated (M2; CD163+) phenotypes in other animal species. Our results showed that a population of KUL01+ chicken monocytes could be defined by the surface expression of CD163 (Figure 1). Across breeds, there was no significant difference in the upregulation of surface expression of CD163 following HMW poly(I:C) treatment (Table 3;

Table 2. Fold increase in MDA5 and IFN-α transcripts from splenocytes and PBMCs of 12 different chicken breeds, following treatment with HMW Poly I:C.

Breed	Spleen		PBMCs	
	MDA5	IFNα	MDA5	IFNα
White Leghorn	0.95 ± 0.05^{a}	1.45 ± 0.04	$-0.25 \pm 0.65^{b,c}$	$1.31 \pm 0.03^{b,c}$
Rhode Island Red	$0.17 \pm 0.44^{a,b,c}$	0.14 ± 0.11	4.91 ± 0.52^{a}	3.36 ± 0.54^{a}
Red Ranger Broiler	$-0.91 \pm 1.56^{c,d,e}$	-0.12 ± 4.69	$-1.38 \pm 0.32^{b,c,d}$	$-1.76 \pm 1.08^{e,f}$
Barred Plymouth Rock	$-0.20 \pm 0.40^{a,b,c}$	0.70 ± 0.20	$-3.34 \pm 0.37^{c,d}$	$-4.81 \pm 0.97^{h,i}$
Araucana Ameraucana	$-1.11 \pm 0.51^{c,d,e}$	-1.65 ± 2.13	$1.61 \pm 2.62^{a,b}$	$2.42 \pm 0.54^{a,b}$
Black Australorp	$0.76 \pm 0.27^{a,b}$	2.34 ± 0.69	-4.74 ± 0.51^{d}	-5.99 ± 0.45^{i}
New Hampshire	-2.47 ± 0.09^{e}	-3.34 ± 0.14	$-2.91 \pm 2.90^{c,d}$	$-4.01 \pm 0.09^{g,h}$
Blue Andalusian	$-0.003 \pm 0.71^{a,b,c}$	1.19 ± 2.26	$2.74 \pm 2.16^{a,b}$	$-0.91 \pm 0.37^{d,e}$
Black Star	$-0.75 \pm 0.43^{b,c,d}$	0.57 ± 1.36	$2.43 \pm 1.83^{a,b}$	$-2.82 \pm 0.07^{f,g}$
Delaware	$-2.07 \pm 0.86^{d,e}$	-2.43 ± 0.23	N/A	N/A
Red Star	$0.40 \pm 0.73^{a,b,c}$	1.12 ± 0.38	$2.23 \pm 3.18^{a,b}$	$-0.73 \pm 0.69^{d,e}$
Brown Leghorn	1.07 ± 0.68^{a}	0.12 ± 1.38	$0.45 \pm 0.42^{b,c}$	$-0.02 \pm 1.15^{c,d}$
P value	**0.002**	0.14	**0.004**	**<0.0001**

Each value represents mean±SD for 3 trials consisting of 3 pooled bird samples/treatment/breed. Means within the same column bearing different superscripts are statistically different ($P < 0.05$).

IFN-α, Interferon-alpha; MDA5: Melanoma Differentiation-Associated Gene 5; N/A, Not Available.

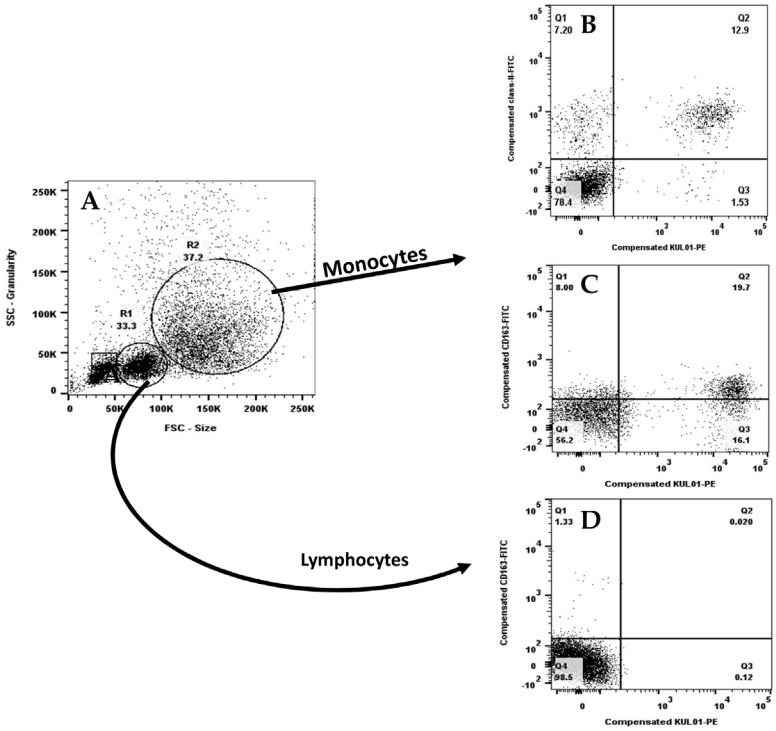

Figure 1. Expression of the scavenger receptor CD163 on chicken monocytes. A. Two populations (R1 and R2) are identifiable based on forward scatter(size) and side scatter (granularity). Cells in the R1 region are presumed to be lymphocytes while R2 region reacted with the monocyte/macrophage-specific antibody KUL01 (B and C), expressed MHC class II (B) and CD163 (C). Cells in the R1 region (mainly lymphocytes) reacted with neither KUL01 nor anti-CD163 antibody (D).

$P = 0.96$). Nonetheless, breeds such as White Leghorn and Rhode Island Red that showed significantly higher upregulation of proinflammatory cytokines appeared to show a reduction in surface CD163 expression after 18 h of activation. Conversely, breeds such as Delaware, Red Star, and Brown leghorn that did not show a significant increase in proinflammatory cytokines showed a higher level of surface CD163 expression when compared to untreated birds. These findings suggested that the expression of proinflammatory cytokines was negatively correlated with the expression of CD163 on the surfaces of macrophages. We subsequently carried out a regression analysis using data from individual birds across all breeds. Our results showed that 42% of the reduction in CD163 expression following activation could be explained by breed in our model ($P < 0.0004$). Our findings are similar to those of previous studies that associated CD163 expression with the release of anti-inflammatory and angiogenic products from macrophages (Zwadlo et al., 1987; Kodelja et al., 1997). In general, surface expression of CD163 has been shown to occur in late inflammation and was associated with downregulation of the inflammatory response and angiogenesis (Buechler et al., 2000). The importance of CD163 expression in macrophage function has not been fully explored in this study. A major limitation of the study is the sample size. However, if our findings are confirmed by future studies that employ a larger number of chickens, changes in surface expression of CD163 on blood monocytes following exposure to synthetic dsRNA could be a quick approach in live birds to identify breeds of chickens that are most likely to produce a robust innate immune response to viral dsRNA.

Table 3. Fold change in surface expression of CD163 on KUL01 +MHCII+ cells. The level of CD163 expression is expressed as the median fluorescence intensity (log MFI) of treated cells divided by log MFI of untreated cells.

Breed	ΔMFI
White Leghorn	−0.22 ± 0.23
Rhode Island Red	−0.48 ± 1.15
Red Ranger Broiler	0.29 ± 0.07
Barred Rock	0.19 ± 0.30
Araucana Ameraucana	0.16 ± 0.01
Black Australorp	−0.08 ± 0.26
New Hampshire	−0.04 ± 1.32
Blue Andalusian	0.41 ± 1.20
Black Star	−0.02 ± 0.56
Delaware	0.57 ± 0.72
Red Star	0.54 ± 0.78
Brown Leghorn	0.78 ± 1.90
P value	0.96

Each value represents mean+SD for 9 birds/breed. No statistically significant difference was observed across breeds in birds not injected with HMW poly(I:C) ($P = 0.96$). However, regression analysis of all tested birds as individuals showed that an increased in surface expression of CD163 is related to breed ($R^2 = 0.42$; $P < 0.0004$). Note that numbers are in a logarithmic scale.

ΔMFI = MFI of treated birds/MFI of untreated birds.

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DISCLOSURES

The authors declare that they have no competing interests.

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