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THE AVIAN THROMBOCYTE IS A SPECIALIZED IMMUNE CELL

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THE AVIAN THROMBOCYTE IS A SPECIALIZED IMMUNE CELL

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biological Sciences

by
Farzana Ferdous
August 2014

Accepted by:
Dr. Tom Scott, Committee Chair
Dr. Charles Rice
Dr. Charlie Wei
Dr. Jeremy Tzeng

ABSTRACT

Thrombocytes are the most abundant circulating cells next to red blood cells in avian blood. Avian thrombocytes are homologous in function to mammalian platelets. Avian thrombocytes and the mammalian platelet are widely recognized contributors to inflammatory responses upon stimulation with various microbial stimulants. However, some observed responses of the avian thrombocyte depart from a standardized model ascribed to innate cells. To help unravel the role of thrombocytes in innate immunity, and possibly adaptive immunity, first we examined the surface features of chicken thrombocytes. Chicken thrombocytes constitutively express transcripts for different Toll-like receptors (TLRs) that are crucial for the innate immune response. We observed surface expression of major histocompatibility complex II, which is exclusively limited to true antigen presenting cells (APC). Furthermore, we have also detected surface expression of co-stimulatory molecules CD40, 80 and 86 on chicken thrombocytes. Expression of MHC II and co-stimulatory molecules indicates that thrombocytes are unconventional/unique immune cells that not only resemble innate effector cells in function but may have a role in affecting adaptive immunity through cellular contact and interaction with APC and lymphocytes. Next, we examined inflammatory responses when thrombocytes were stimulated *in vitro* and *in vivo* with several TLR ligands and vaccines, respectively. *In vitro* treatment of thrombocytes with various TLR ligands demonstrates a definite bacterial effect while no viral effect on gene expression of IL-6. Bacterial ligand, LPS stimulation led to release of significant amounts of active IL-6 in the thrombocyte culture supernatants. The *in vitro* treatment of thrombocytes also

indicated constitutive expression of iNOS gene expression. Although *in vitro* treatment of thrombocytes with viral TLR ligands induced significant release of nitric oxide (NO) into culture supernatant, bacterial TLR ligands did not lead to release of NO. *In vivo* treatment by vaccination with the recommended doses was not sufficient to stimulate chicken thrombocytes to induce expression of pro-inflammatory mediators. Understanding how TLRs initiate particular thrombocytic responses and the involvement of thrombocytes in antigen presentation may shed new light on adjuvant and vaccine research for vaccine development for different poultry diseases.

DEDICATION

To my loving parents Dr. Md. Ferdous Alam and Sayeeda Banu. Without your love and continuous encouragement, the completion of this degree would not have been possible. Thank you for your support and your belief in pursuing academic excellence. I dedicate this dissertation to you.

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TABLE OF CONTENTS

	Page
TITLE PAGE	i
ABSTRACT	ii
DEDICATION	iv
ACKNOWLEDGMENTS	v
LIST OF TABLES	viii
LIST OF FIGURES	ix
CHAPTER	
I. LITERATURE REVIEW	1
1.1 Overview	1
1.2 Avian Immune System	1
1.3 Thrombocytes/Platelets	10
1.4 Physiological Role of Thrombocytes/Platelets	20
1.5 Impact/Relevance	28
1.6 References	30
II. DECIPHERING THE ROLE OF CHICKEN THROMBOCYTES IN IMMUNITY	38
2.1 Introduction	38
2.2 Materials and Methods	39
2.3 Results	46
2.4 Discussion	60
2.5 Acknowledgements	66
2.6 References	67
III. BACTERIAL AND VIRAL INDUCTION OF CHICKEN THROMBOCYTE IMMUNE RESPONSES	69
3.1 Introduction	69
3.2 Materials and Methods	71
3.3 Results	78

Table of Contents (Continued)

	Page
3.4 Discussion.....	96
3.5 Acknowledgements.....	100
3.6 References.....	100
IV. CONCLUSIONS AND FUTURE STUDIES	104
4.1 Conclusions.....	104
4.2 Future Studies	105
4.3 References.....	107

LIST OF TABLES

Table	Page
1.1 Avian Toll-like receptors (TLRs) and their ligands.....	5
1.2 Different pathogen recognition receptors present in human and chicken	6
1.3 Different inflammatory, antimicrobial, and immune modulating molecules released by activated human platelets	22
2.1 Quantitative real-time polymerase chain reaction primer sets for messenger RNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH , housekeeping gene) and Toll-like receptors (TLRs) 2, 3, 4, and 7 of chickens.....	45
2.2 Percentages of chicken thrombocytes and MQ.NCSU cells positive for MHC class II, CD40, 80 and 86 identified by specific antibody labeling detected through flow cytometric analysis.....	54
2.3 RNA microarray analysis of Toll-like receptor (TLR) pathway specific molecules by using control and 1hr lipopolysaccharide (1 µg/mL) stimulation of chicken thrombocytes.....	61
3.1 Quantitative real-time polymerase chain reaction primer sets for messenger RNA of glyceraldehyde 3 phosphate dehydrogenase (GAPDH, housekeeping gene), interleukin-6 inducible nitric oxide synthase.....	75

LIST OF FIGURES

Figure	Page
1.1 Regulation of T-helper cell development by Toll-like Receptors (TLRs) on antigen presenting cells (APCs).....	9
1.2 Electron micrographs of zebrafish thrombocyte.....	12
1.3 Electron micrographs of avian thrombocyte.....	14
1.4 Appearance of a typical blood smear of Single Comb White Leghorn chicken.....	15
1.5 Electron micrographs of mammalian platelet.....	19
1.6 Activated platelets can mediate cell-cell interactions and affect immune responses.....	25
2.1 Green fluorescent image of viable chicken thrombocytes.....	47
2.2 Microscopy images of CD41/61 and 4',6-diamidino-2-phenylindole (DAPI) stained chicken thrombocytes.....	48
2.3 Tali™, image-based cytometer analysis of isolated chicken thrombocyte suspension.....	50
2.4 Flow cytometric analysis of CD41/61 labeled cells.....	51
2.5 Flow cytometric analysis of thrombocytes and MQ.NCSU macrophage cell line with mouse anti-chicken MHC II.....	53
2.6 Flow cytometric analysis of thrombocytes and MQ.NCSU macrophage cell line with mouse anti-chicken CD40.....	55
2.7 Flow cytometric analysis of thrombocytes and MQ.NCSU macrophage cell line with mouse anti-chicken CD80.....	57
2.8 Flow cytometric analysis of thrombocytes and MQ.NCSU macrophage cell line with mouse anti-chicken CD86.....	59
2.9 Relative quantification in terms of fold expression of Toll-like receptors (TLRs) 2, 3, 4, and 7.....	62

List of Figures (Continued)

Figure	Page
3.1 Relative fold expression of interleukin-6 (IL-6) in control and stimulated chicken thrombocytes.....	79
3.2 Relative fold expression of interleukin-6 (IL-6) in control and Layermune SE vaccinated chicken thrombocytes.....	80
3.3 Relative fold expression of interleukin-6 (IL-6) in control and Poluvac Maternavac vaccinated chicken thrombocytes.....	82
3.4 Stimulation index of B9 cell bioassay with supernatants from control and stimulated chicken thrombocytes.....	84
3.5 Stimulation index of B9 cell bioassay with supernatants from control and stimulated MQ.NCSU macrophages.....	85
3.6 Relative fold expression of inducible nitric oxide synthase (iNOS) in control and stimulated chicken thrombocytes.....	87
3.7 Average cycle number (C_t values) of inducible nitric oxide synthase (iNOS) gene expression in control, and stimulated chicken thrombocytes.....	88
3.8 Difference in C_t values of control, and stimulated chicken thrombocytes for inducible nitric oxide synthase (iNOS) gene.....	89
3.9 Relative fold expression of inducible nitric oxide synthase (iNOS) in control and Layermune SE vaccinated chicken thrombocytes.	90
3.10 Relative fold expression of inducible nitric oxide synthase (iNOS) in control and Poulvac Maternavac IBD-REO vaccinated chicken thrombocytes.	92
3.11 Release of nitrite from control and stimulated chicken thrombocytes	94
3.12 Release of nitrite from control and stimulated chicken macrophage cell line.....	95

CHAPTER I

LITERATURE REVIEW

1.1 Overview

Among all the cells in the blood vascular system, platelets in mammals and thrombocytes in lower vertebrates such as birds, reptiles, amphibians and fish are the source of crucial mediators in hemostatic functions. Although, these cells have been known to be primarily involved in thrombosis and hemostasis; platelets and thrombocytes have been shown recently to have a role in inflammatory functions and the immune response in general. Here, we discuss the thrombocyte of the avian immune system and its role in the immune response. We start by discussing the unique features of the avian immune system and the cells involved in innate and adaptive immunity, which will lead to a description of the origin, structure and function of thrombocytes/platelets.

1.2 Avian Immune System

The avian immune system functions in general as the mammalian immune system. The immune responses can be divided into two types: innate and adaptive. Innate immunity is less specific and it provides the first line of defense against infection while adaptive immunity is more specific and long term against an invading pathogen. There are some differences in the avian and the mammalian immune systems and in the ways each develops and becomes diverse enough to meet lifelong challenges. Below we

present some of these major differences and later discuss the avian innate and adaptive responses.

The most significant structural difference is in the lymphoid organs of the avian immune system. A primary lymphoid organ is where lymphocytes first express antigen receptors, gain phenotypic and functional maturity. The bursa of Fabricius is a specialized primary lymphoid organ involved in B-cell development of birds (Glick *et al.*, 1956; Sharma, 1991 and 1997; Ola'h *et al.*, 2014). The thymus is another primary lymphoid organ that is present in both birds and mammals. However, the mammalian thymus has only two lobes (Kindt *et al.*, 2007) while the avian thymus consists of 7-8 lobes along each side of the neck (Ola'h *et al.*, 2014) indicative of distinctly different embryological origins. T-cell differentiation takes place in the thymus (both species) while B-cell differentiation occurs in bursa of Fabricius (aves) or bone marrow (mammals). A secondary lymphoid organ is usually where lymphocytes encounter antigen, become activated, and undergo clonal expansion and differentiation into effector cells. In mammals, highly organized secondary lymphoid organs include lymph nodes, and spleen, while less organized lymphoid tissue known as mucosa-associated lymphoid tissue (MALT) includes Peyer's patches, tonsils, and the appendix (Kindt *et al.*, 2007). Although chickens lack defined lymph nodes, these birds have spleen and all mucosa-associated tissues, such as the ocular associated lymphoid tissue (the Harderian gland and the conjunctiva of the lower eyelid), the nasal-, bronchus-, genital- and gut-associated lymphoid tissues (esophageal and pyloric tonsils, Peyer's patches, cecal tonsils

and Meckel's diverticulum), and the skin and pineal-associated lymphoid tissues (Olañ *et al.*, 2014).

Another unique aspect of the avian immune system is that the immunoglobulin (Ig) diversity in birds is generated by somatic gene conversion events in which sequences derived from upstream families of pseudogenes replace homologous sequences in unique and functionally rearranged immunoglobulin heavy and light chain variable region genes (Reynaud *et al.*, 1985; McCormack *et al.*, 1991; Ratcliffe, 2006; Fella *et al.*, 2014). In humans and mice, the primary repertoire of Ig genes is created by combinatorial and junctional variations that occur during the gene rearrangement events to produce complete heavy and light chain Ig genes (Tonegawa, 1981; Kindt *et al.*, 2007; Fella *et al.*, 2014). This process mostly occurs in the bone marrow in humans and mice. Chicks are born with an incomplete immune system and maternal immunity is passed on to chicks when the embryo swallows amniotic fluid during hatch and in the absorption of the egg yolk after hatch. Chicken Ig gene rearrangement occurs only during a brief period of embryonic development and gene conversion takes place in the bursal microenvironment (Ratcliffe, 2006; Fella *et al.*, 2014) from 15-17 days of incubation until bursa of Fabricius involution at sexual maturity.

1.2.1 Innate Immune Response

In the mammalian and avian systems, the non-specific immune response includes the innate or inherent ways each resists disease. Although there are differences in some of

the effector cells or molecules that are produced, the general mechanisms involved in the innate immune response in both birds and mammals are very similar. The cells of the innate immune system have or produce molecules such as defensins, cytokines, and chemokines for effector function upon pathogen recognition. Defensins are small, cationic, natural anti-microbial peptides. These generally bind to microbial cell membranes and form pores which directly lead to microbial cell death and chemoattraction of innate effector cells to help kill the microbe (Soruri *et al.*, 2007). Cytokines are essentially molecular messengers that control and coordinate immune cells both during innate and adaptive immune responses, as well as during development and homeostasis (Kaiser, 2010). In the innate response, these molecules drive inflammatory responses and the acute phase response.

Cells associated with the non-specific, innate immune response include phagocytic cells, natural killer (NK) cells, basophils, and mast cells. The primary function of a phagocytic cell is to engulf and destroy pathogens upon identification. Common phagocytes are monocyte/macrophages and neutrophils in mammals or heterophils in birds (chicken). In recent years, researchers have shown that platelets (Clawson and White, 1971; Klinger, 1997; Klinger and Jelkmann, 2002; Elzey *et al.*, 2003) or thrombocytes (Glick *et al.*, 1964; Carlson *et al.*, 1968; Ferdous *et al.*, 2008; Hitchcock, 2009; Scott and Owens, 2008; Paul *et al.*, 2012) are also involved in innate immunity and inflammation.

Table 1.1 Avian Toll-like receptors (TLRs) and their ligands.

TLR	Other names	Agonist	Pathogen
TLR1La	TLR1.1, TLR1/6/10, TLR16		
TLR1Lb	TLR1.2	Lipoprotein,	Mycoplasma,
TLR2a	TLR2.1	Peptidoglycan	G + Bacteria
TLR2b	TLR2.2		
TLR3		dsRNA	Viruses
TLR4		LPS	G-Bacteria
TLR5		Flagellin	G-Bacteria
TLR7		Imiquimod, ssRNA	Viruses
TLR21		CpG motifs, chromosomal DNA	Bacteria and viruses
TLR15		Unknown	

(Adapted from Brownlee and Allan, 2011)

Table 1.2. Different pathogen recognition receptors (PRRs) present in human and chicken.

Pattern recognition receptor	Human	Chicken
Membrane-bound		
TLRs (signalling) recognizing cell surface PAMPs	TLR1/6/10 TLR2 TLR4 TLR5 TLR11 ^a	TLR1LA and TLR1LB TLR2A and TLR2B Present Present Absent TLR15 (predicted ^b)
TLRs (signalling) recognizing pathogen nucleic acid	TLR3 TLR7 TLR8 TLR9	Present Present Pseudogene TLR9 absent; CpG recognized by TLR21
Endocytic	Mannose receptor Glucan receptors Scavenger receptors	Present Present Present
Cytoplasmic		
NLRs		
NODs	NOD1 NOD2	Present Absent
NALPs	14 genes	All absent
RNA helicases	RIG-I MDA5 LPG2	Absent, but present in duck Present Absent

^aPresent in mice but a pseudogene in human. ^bPredicted to recognize a pathogen surface PAMP due to its pattern of LRRs.

(Adapted from Kaiser, 2010)

The innate immune system provides an important initial response against pathogens to limit or prevent infection. Most components of innate immunity are present before the onset of infection and constitute a set of disease-resistance mechanisms that include cellular and molecular components to recognize classes of molecules on a particular pathogen and some endogenous proteins. The innate effector cells are activated through pathogen recognition receptors (PRRs), which play a major role in initiating the innate immune response by recognizing exogenous and endogenous antigens (Akira *et al.*, 2001; Akira and Takeda, 2004). These cells identify pathogen-associated molecular patterns (PAMPs), which include combinations of sugars, certain proteins, particular lipid-bearing molecules, and some nucleic acid motifs expressed on the surfaces of pathogens. Toll-like receptors (TLRs) are some of the best characterized membrane-bound PRRs. Table 1.1 presents a list of avian TLRs and the ligands that activate through these PRRs (Brownlie and Allan, 2011). There are also specific cytoplasmic PRRs in both avian and mammalian systems such as nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) and RNA helicases (Table 1.2 adapted from Kaiser, 2010). Innate effector cells perform phagocytosis and killing of microbes by producing antimicrobial peptides, and other effector molecules such as reactive oxygen and nitrogen species. These cells are also involved in inflammatory responses, which include localized and systemic effects in response to tissue injury and other trauma. Tissue injury causes release of vasoactive and chemotactic factors that initiate a local increase in blood flow and capillary permeability. Increased capillary permeability allows an influx of

antimicrobial exudate and phagocytes to the sites of infection in order to destroy and remove foreign pathogens and heal damaged tissue.

In addition, the activation of the innate immune response can be a prerequisite for the triggering of acquired immunity and in some ways determines the course of an adaptive immune response. Through the recognition of pathogens or their products, the PRRs like TLRs can induce the production of cytokines such as IL-12 and IL-18 in antigen presenting cells (APCs), which function as "instructive" cytokines and drive naïve T- cells to differentiate into T-helper cell type 1 (T_H1) cells (Fig. 1.1).

1.2.2 Adaptive Immune Response

The specific immune responses include adaptive or acquired immunity characterized by specificity, heterogeneity, and memory. T and B cells are major effector cells in both avian and mammalian adaptive immune responses. Intracellular pathogens are generally cleared by cell-mediated adaptive immune responses initiated by recognition of antigens by T cells. Antigen presenting cells (APCs) play an important role in the innate immune response and linking the innate and adaptive immune response by activating naïve T-cells leading to proliferation and differentiation of effector and memory T-cells (Fig. 1.1). Professional APCs include dendritic cells (DCs), macrophages and B cells. These cells acquire microbial components during the innate response by phagocytosis, endocytosis or other similar processes. In humans, DCs bring microbial components from the site of infection to lymph nodes. In the lymph nodes, microbial antigens that are displayed on the major histocompatibility complex (MHC) of DCs are presented to T cells resulting in

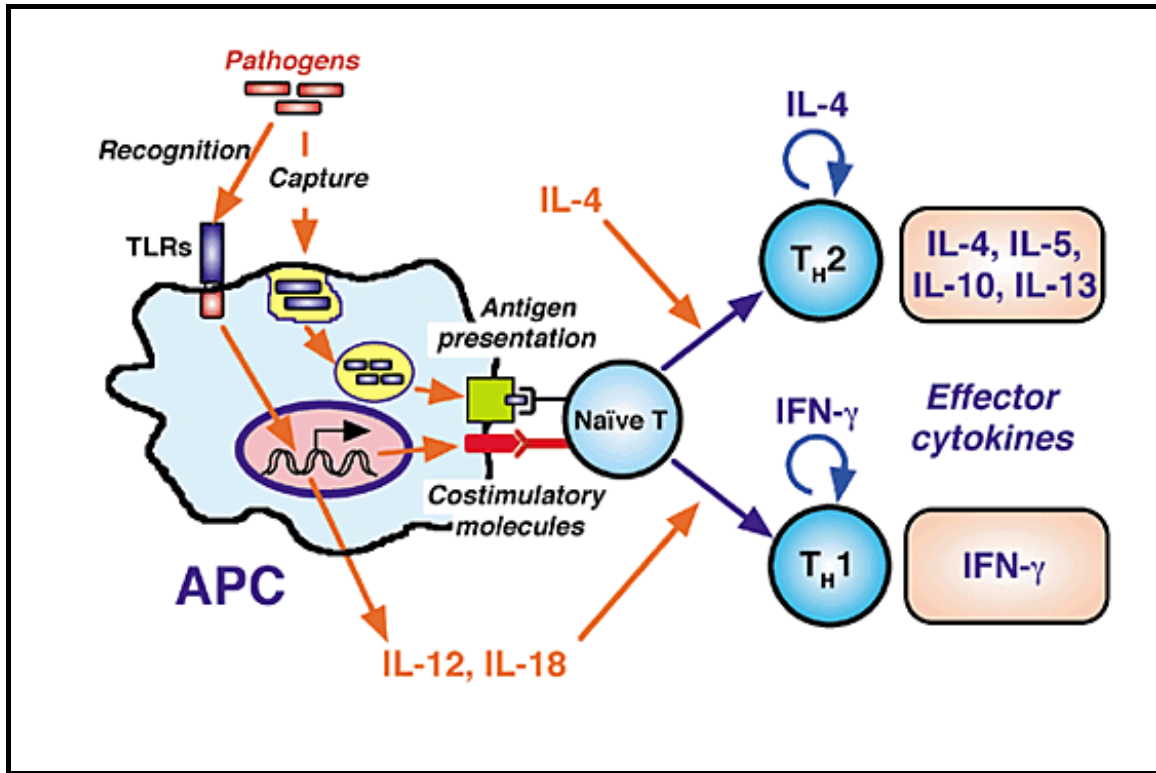


Figure 1.1: Regulation of T-helper cell development by Toll-like receptors (TLRs) on antigen presenting cells (APCs). Through recognition of pathogens or their products, TLRs can induce the production of cytokines in APCs. Pathogens are captured in multiple ways, including phagocytosis, endocytosis or *via* TLRs themselves. Captured pathogens are processed and presented to T-cells as major histocompatibility complex-antigen. For expansion of antigen-specific T cell clones, antigen presentation requires up-regulated expression of costimulatory molecules on the cell surface of APCs which can be triggered by TLR signaling (Adpated from Akira *et al.*, 2001).

T-cell activation. Extracellular pathogens are usually cleared by humoral immune responses mediated by antibodies produced by B cells and plasma cells. Antigen specific protection against various pathogens can also be achieved by passive immunity, which consists of maternal antibodies that are present at hatch in chicks. Recently, researchers have recognized the role of mammalian platelets in the adaptive immune response (Henn *et al.*, 1998; Elzey *et al.*, 2003; Elzey *et al.*, 2005). Platelets have been shown to influence DCs and thereby modulate T-cell responses. The role of avian thrombocytes in the adaptive immune system is a relatively new area of research (Tregaskes *et al.*, 2005; Paul *et al.*, 2012). Before we discuss the physiological role of thrombocytes or platelets, including the role in innate and adaptive immune responses, we will discuss thrombocytes/platelets in more details.

1.3 Thrombocytes/ Platelets

Thrombocytes/platelets are small, circulating, and the most abundant cells next to erythrocytes in blood. Although thrombocytes and platelets are homologous in function, there are some differences in their origin and morphology. Enucleated platelets are only found in mammals while nucleated thrombocytes are found in lower vertebrates such as reptiles, amphibians, fish and birds (Levin 2007). Invertebrate species have circulating nucleated cells in their hemolymph termed hemocytes that are similar in function to thrombocytes/platelets (Levin, 2007). Below we discuss thrombocytes in lower vertebrates, birds and mammals in more detail.

1.3.1 Thrombocytes in Lower Vertebrates

Historical studies of thrombocytes in lower vertebrates are focused on characterization of morphological features and the role of thrombocytes in hemostasis (Desser and Weller, 1979; Daimon *et al.*, 1987; Daimon *et al.*, 1979a; Daimon *et al.*, 1979b; Daimon and Uchida, 1985; Jagadeeswaran, *et al.*, 1999; Rey Vázquez and Guerrero, 2007). Lower vertebrate thrombocytes are, like those of higher vertebrates and platelets, the most abundant blood cells after erythrocytes (Meseguer *et al.*, 2002). The process of thrombopoiesis takes place in different tissues in adult animals, depending on the vertebrate group and species. Thrombopoiesis consists of immature and mature prothrombocytes and thrombocyte cell stages prior to the formation of the circulating thrombocyte (Esteban *et al.*, 1989). According to a review by Meseguer *et al.* (2002), thrombopoiesis occurs in the lymphomyeloid and lymphoid tissues in fish (Esteban *et al.*, 1989; Zapata *et al.*, 1996), the spleen, kidney, liver, and more rarely bone marrow in amphibians (Welsch and Storch, 1982; Zapata *et al.*, 1982), and the bone marrow or spleen in reptiles (Rowley *et al.*, 1988). Below we discuss the structure of fish thrombocytes.

Circulating thrombocytes in fish are similar in size to lymphocytes and appear as round, oval, spindle, or spiked cells with long cell processes (Daimon *et al.*, 1979b; Daimon and Uchida, 1985; Jagadeeswaran, *et al.*, 1999; Rey Vázquez and Guerrero, 2007; Esteban *et al.*, 2000). The cytoplasm of fish thrombocytes contains numerous vesicles that occasionally, open to the cell surface indicating the presence of a surface

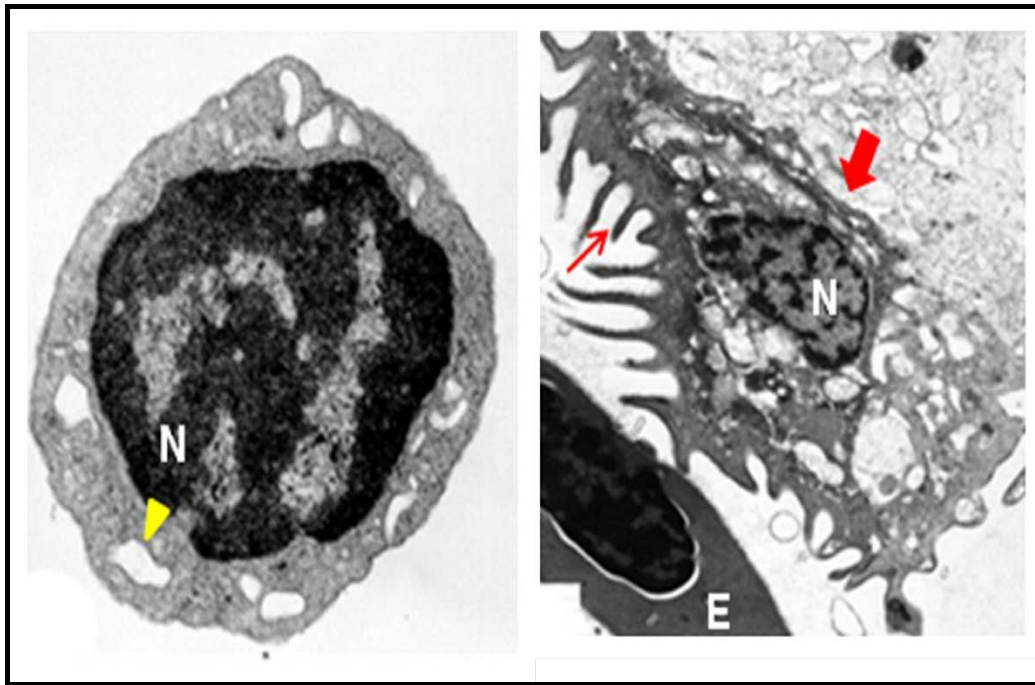


Figure 1.2: Electron micrographs of zebrafish thrombocyte. Zebrafish thrombocyte with open canalicular-like system is shown by yellow arrowhead; N: nucleus (a). An activated thrombocyte in an aggregation reaction; activated thrombocyte is shown by a thick red arrow, thrombocyte in the aggregate shows filopodia shown by a thin red arrow; E: erythrocyte (b) (Adpated from Jagadeeswaran *et al.*, 1999).

connected canalicular system (Fig. 1.2). Aggregates of thrombocytes with pseudopodia-like projections are also observed in Figure 1.2. A marginal band of microtubules maintains the shape of fish thrombocytes (Meseguer, 2002). Some mitochondria, glycogen deposits, and a Golgi apparatus are also found in these cells.

In recent years, zebrafish is one of the species where thrombocytes have been studied more extensively. Zebrafish thrombocytes are well characterized and several functional assays have been established to study the hemostatic function of these cells (Khandekar *et al.*, 2012). The zebrafish thrombocyte has been suggested to be the hemostatic homologue of the mammalian platelet due to combined morphologic, immunologic and functional evidence and conservation of major hemostatic pathways involved in platelet function and coagulation (Jagadeeswaran, *et al.*, 1999). Several researchers have demonstrated usefulness of zebrafish as a relatively more practical vertebrate genetic model to study mammalian platelet function (Jagadeeswaran, *et al.*, 1997 and 1999; Khandekar *et al.*, 2012; Thijs, *et al.*, 2012).

1.3.2 Thrombocytes in Birds

Avian thrombocytes are the most abundant white blood cell type in chicken blood (Change and Hamilton, 1979). In circulating chicken blood; the concentration of thrombocytes is 3×10^4 cells/mm³ compared to only 5×10^3 cells/mm³ for heterophils (Glick, 1958). These cells are round, oval or somewhat elongated with an irregular outline (Lucaz and Jamroz, 1961; Hodges, 1979) and occasionally, there is presence of fine pseudopodial processes (Fig. 1.3). The cytoplasm of the thrombocyte contains a few

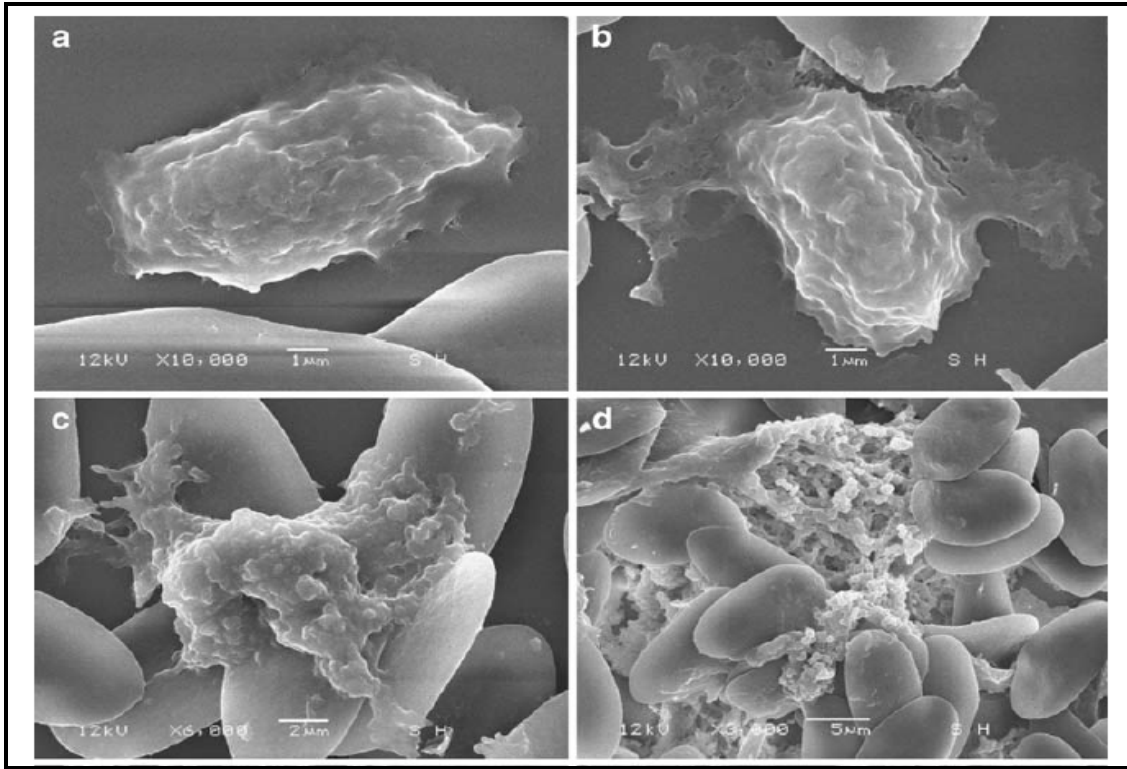


Figure 1.3: Electron micrographs of avian thrombocyte. The micrograph shows normal thrombocyte (a), spread thrombocyte (b), thrombocyte decomposed and attached to red blood cell resulting in red blood cell clumping is shown (c and d) (Adapted from Narkkong *et al.*, 2010).

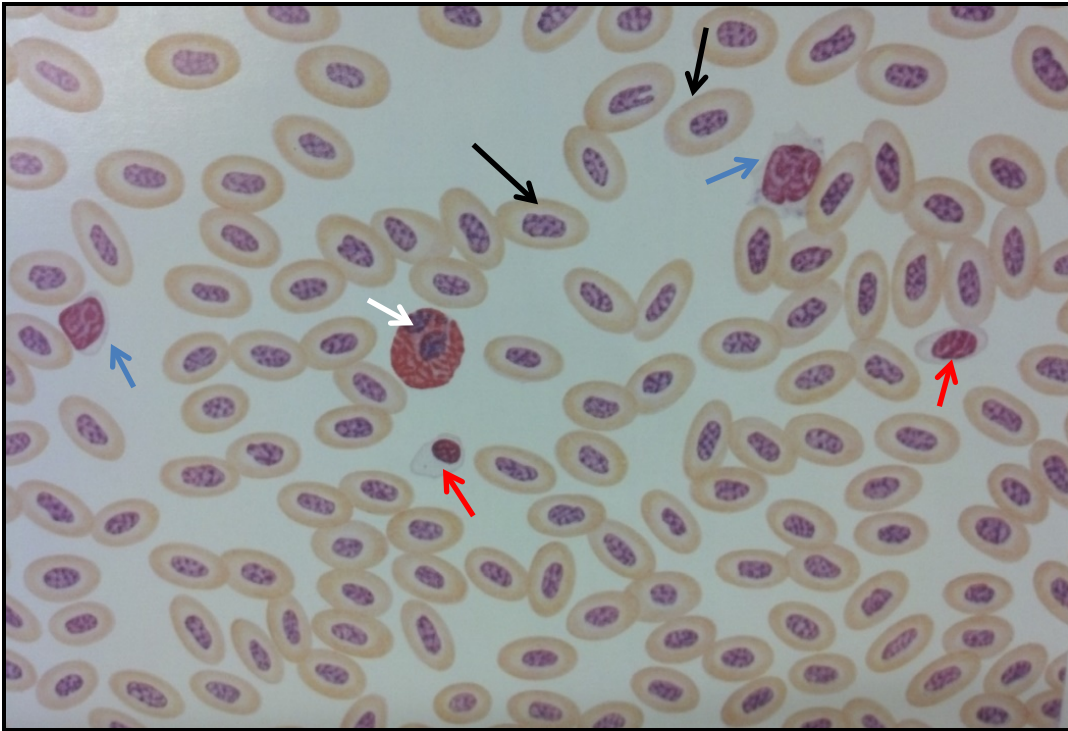


Figure 1.4: Appearance of a typical blood smear of Single Comb White Leghorn chicken It is showing red blood cells (black arrows), thrombocytes (red arrows), heterophil (white arrow), and lymphocytes (blue arrows) (adapted from Lucas and Jamroz, 1961).

small mitochondria, smooth and rough endoplasmic reticulum, and often a well-developed Golgi apparatus. The average width and length of a mature avian (chicken) thrombocyte is approximately 5 and 10 μm , respectively. Thrombocytes bear a superficial resemblance in both shape and general appearance to red blood cells (RBCs) in avian blood (Fig. 1.4). Thrombocyte cytoplasm also has microtubules at the outermost zone of the cell and large vacuoles connected to the canalicular system or opened to the surface. Morphologically these cells can be distinguished from monocytes and lymphocytes by electron microscopy and in fresh preparations by phase microscopy. Thrombocytes in peripheral blood of different avian species such as chicken, turkey and Japanese quail are very similar in terms of the ultrastructure (Sweeny and Carlson, 1968; Simpson, 1968; Nirmalan *et al.*, 1972). Maxwell (1973) also compared thrombocytes in six species of domestic bird (duck, geese, turkey, pigeon, quail and guinea-fowl) and found little difference in terms of the ultrastructure, confirming that thrombocytes are very similar in different avian species. Thrombocytes have the capacity to store a number of intra-cellular secretory granules that can be released into the circulation or translocated to the surface when activated. Avian thrombocytes are also capable of producing and releasing a vast array of bioactive proteins. Presence of large, acid phosphatase-positive granules has been observed histochemically in the cytoplasm of avian thrombocytes, which may be similar to the lysosomal granules in mammalian platelets (Carlson *et al.*, 1968).

Historically, there have been some conflicting views on the origin of avian thrombocytes (Sugiyama, 1926). One group believes that thrombocytes are descendants of lymphocytes or primitive lymphoid cells, while another group believes that these cells are of endothelial origin. Some consider thrombocytes as damaged or degenerated red blood cells (RBCs). Thrombocytes are often regarded as the antecedent of mononucleated cells that have a blast stage like that of other blood cells (Lucaz and Hamroz, 1961). According to Archer (1971), large cells in chicken bone marrow appear to be multinucleated and could be the precursors of thrombocytes. More recent research shows that thrombocytes originate from cells that resemble multipotent hematopoietic progenitors and are produced in the region where earliest intraembryonic hematopoietic cells develop (McNagny *et al.*, 1997). Chicken thrombocytes usually become morphologically distinguishable from erythrocytes on Day 3 of incubation (after 65 hr incubation). However, presence of glycogen granules can be used as a thrombocyte specific marker to detect immature thromboblats as they first emerge after 35 hr of incubation (Tahara *et al.*, 1983). Thrombocytes express surface marker thrombomucin, which is distantly related to CD34, a well-known human hematopoietic stem cell marker. In addition to thrombomucin, receptor c-myeloproliferative leukemia virus oncogene (Mpl) is also expressed on the surface of cells of the thrombocytic lineage (Bartunek *et al.*, 2008). Chicken thrombocytes can be identified with monoclonal antibodies such as 23C6 (Viertlboeck and Göbel, 2007) and K1 (Kaspers *et al.*, 1993) that recognize the intact complex formed between CD41 and CD61 (α V and β 3 integrin) and an unidentified epitope, respectively, on the surface of chicken thrombocytes.

1.3.3 Mammalian Platelets

Mammalian platelets are enucleated, small oval disc shaped cells. Although platelets lack a nucleus, these cells contain functional spliceosomes which are a multi-megadalton ribonucleoprotein complex. Spliceosomes process pre-mRNAs in the absence of nuclei during platelet formation from megakaryocytes. In addition, human platelets also contain essential spliceosome factors including small nuclear RNAs, splicing proteins, and endogenous pre-mRNAs; all the translational machinery necessary to generate their own proteins during stimulatory events (Denis *et al.*, 2005). A human platelet is usually 2 - 4 μm in diameter and reported platelet counts range from 150 - 400 $\times 10^9$ platelets per liter of blood (Yeaman, 1997; Klinger and Jelkmann, 2002; Semple *et al.*, 2011).

The platelet plasma membrane surface is generally smooth except for some periodic invaginations that delineate the entrances to the open canalicular system (Fig. 1.5). Similar to avian thrombocytes, this complex network of intertwining membrane tubes permeate the cytoplasm of platelets. Due to this system of folded membranes, platelets have an enormous surface area. When platelets are activated, an influx of calcium initiates the process of rapid change of shape from smooth discs to spiny spheres with development of finger-like filopodia and pseudopods (Semple *et al.*, 2011). The cytoplasm of a platelet is rich in actin and myosin which provides the force for this change in shape.

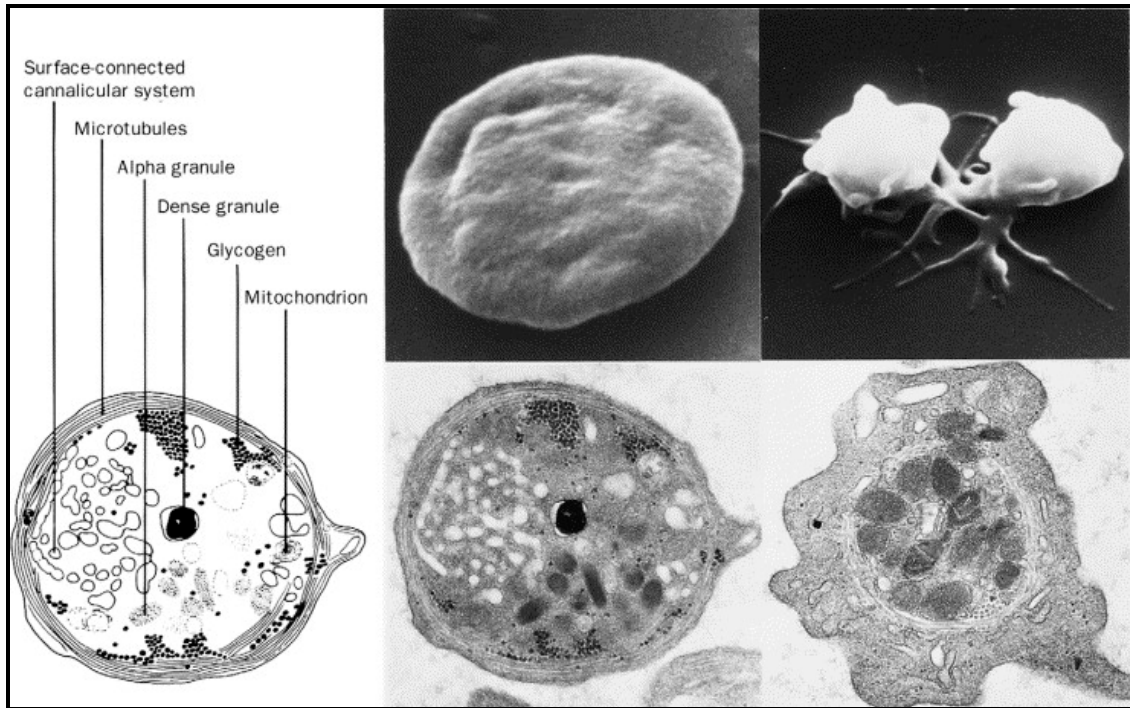


Figure 1.5: Electron micrographs of mammalian platelet. Here, top right part shows a more spherical form of resting platelet compared to an activated one with long pseudopodia (Adapted from George, 2000).

Mammalian platelets have three major types of storage granules: (i) α -granules, (ii) dense granules, and (iii) lysosomes (Yeaman, 1997; Semple *et al.*, 2011). The secretory granules found in avian thrombocytes are not as well characterized as mammalian platelets. The α -granules are the most abundant granules in mammalian platelets; and the granule protein content is derived by a combination of endocytosis and biosynthesis. These granules are involved in secreting several different bioactive proteins including coagulation factors, chemokines, adhesive proteins, mitogenic factors and regulators of angiogenesis.

The production of platelets is a complex progression of events that results with a single megakaryocyte releasing thousands of platelets into circulation. Platelets originate from the megakaryocytes of the bone marrow. Megakaryocytes produce platelets by rearranging the cytoplasm into long extensions called proplatelets that resemble strings of beads. The process of platelet morphogenesis takes place in platelet-rich plasma where megakaryocyte processes first elongate, then bead and fragment, and then curve and fuse to form disk-shaped platelets (Behnke and Forer, 1998; George, 2000). Thrombopoietin is the major hormone controlling megakaryocyte development and thus is the regulator of platelet production (George, 2000).

1.4 Physiological Role of Thrombocytes/Platelets

Although there are some differences in the origin and structure of thrombocytes/platelets in different species, these cells are very similar in their function. There has been a lot more research done with mammalian platelets, so the role of these

cells in physiological function, and capability to be involved in the immune response, is better understood compared to thrombocytes. Below we discuss the major physiological functions of platelets and thrombocytes.

1.4.1 Hemostasis

The primary physiological role of the mammalian platelet as well as the avian thrombocyte is to sense damaged vessel endothelium and accumulate at the site of the vessel injury, where these cells initiate blood clotting to block the circulatory leak. However, the rate at which clumping takes place in the avian thrombocyte is much slower compared to that of mammalian platelets (Hodges, 1979). The dense granules in platelets secrete adenosine diphosphate (ADP) and calcium, which are associated with platelet aggregation and platelet surface coagulation reactions. Avian thrombocytes do not aggregate by ADP or adenosine triphosphate (ATP) release; however thrombocytes can undergo aggregation when a micromolar concentration of serotonin is secreted from intra-cellular granules (Belamarich and Simoneit, 1973). In addition to forming an aggregate, activated platelets favor thrombin and fibrin formation.

1.4.2 Inflammation

After tissue injury, platelets take part in the process of inflammation following the hemostatic process and thrombus formation. Proteomic analysis has demonstrated that thrombin activated platelets can secrete more than 300 different proteins including pro-inflammatory cytokines, chemokines, and cell adhesive proteins (Weyrich and

Table 1.3: List of inflammatory, antimicrobial and immune modulating molecules released by activated human platelets.

Molecule	Location within platelet and mechanism of release	Activation state	Functions	Cellular targets
Histamine	Synthesized, location unknown; released during platelet activation	Preformed	Promotes allergic-type hypersensitivities	Endothelial cells, monocytes, PMNs, NK cells, T cells, B cells, eosinophils
Serotonin (also known as 5-HT)	Dense granules; released during platelet activation	Preformed	Actions on CNS; promotes coagulation and T cell activation	Monocytes, macrophages, platelets
Thromboxane A2	Plasma membrane	Synthesized	Promotes inflammation and coagulation	Platelets, macrophages, T cells
PAF	Plasma membrane	Synthesized	Promotes inflammation	Platelets, PMNs, monocytes, macrophages
PDGF	α -granules; released during platelet activation	Preformed	Promotes wound healing	Monocytes, macrophages, T cells
TGF β	α -granules; released during platelet activation	Preformed	Growth inhibition; immunosuppression	Monocytes, macrophages, T cells, B cells
CXCL7 (also known as NAP2)	Not known	Cleavage product of preformed precursor	Chemokine	PMNs
CXCL4 (also known as PF4)	α -granules; released during platelet activation	Preformed	Chemokine	PMNs, platelets
CXCL1 (also known as GRO α)	α -granules; released during platelet activation	Preformed	Chemokine	PMNs
CXCL5 (also known as ENA78)	α -granules; released during platelet activation	Preformed	Chemokine	PMNs
CCL5 (also known as RANTES)	α -granules; released during platelet activation	Preformed	Chemokine	Monocytes, eosinophils, basophils, NK cells, T cells, DCs, platelets
CCL3 (also known as MIP1 α)	α -granules; released during platelet activation	Preformed	Chemokine	Monocytes, eosinophils, basophils, NK cells, DCs
CCL7 (also known as MCP3)	Not known	Preformed	Chemokine	Monocytes, basophils, NK cells, DCs
IL-1 β (and IL-1 precursor protein)	Not known	Synthesized	T cell activation; multiple effects	Monocytes, macrophages, DCs, T cells
HMGB1	Not known	Preformed	Inflammatory gene regulation	Macrophages, PMNs, endothelial cells
Thrombocidins 1 and 2	α -granules; released during platelet activation	Preformed	Antibacterial peptides	Bacterial cells
CD40	Plasma membrane	Preformed	Co-stimulation; endothelial interactions	T cells
CD154	α -granules and plasma membrane; released and cleaved	Preformed	Co-stimulation; endothelial interactions	B cells, DCs, macrophages, monocytes, endothelial cells
TLR1, TLR2, TLR3, TLR5, TLR6, TLR7	Plasma membrane	Preformed	Pathogen detection	PMNs, DCs, macrophages, monocytes, platelets
TLR4	Plasma membrane	Preformed	Pathogen detection	PMNs, DCs, macrophages, monocytes
TLR9	Unknown internal site; upregulated on plasma membrane during platelet activation	Preformed	Pathogen detection	PMNs, DCs, macrophages, monocytes
TREM1 ligand	Plasma membrane; expression increased during platelet activation	Preformed	Pathogen detection	PMNs, DCs, macrophages, monocytes

CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; DC, dendritic cell; HMGB1, high mobility group protein B1; IL-1 β , interleukin-1 β ; NK, natural killer; PAF, platelet-activating factor; PDGF, platelet-derived growth factor; PMN, polymorphonuclear leukocyte; TGF β , transforming growth factor- β ; TLR, Toll-like receptor; TREM1, triggering receptor expressed on myeloid cells 1. The table is adapted and expanded from REF. 105.

(Adapted from Semple *et al.*, 2011)

Zimmerman, 2004; Semple *et al.*, 2011). Table 1.3 lists some of the platelet-associated molecules, immune functions and cellular targets of those molecules. For example, activated platelets (human) are associated with mediating the adhesion of neutrophils and dendritic cells to the site of tissue injury and also up-regulating the release of pro-inflammatory cytokines and chemokines. Avian thrombocytes are also able to release a variety of similar bioactive compounds; chemotactic factors (e.g. Macrophage Inflammatory Protein-1 β), other mediators in inflammation such as Cyclooxygenase-2 (COX-2), and prostaglandin D₂ (PGD₂), PGE₂, thromboxane A₂ synthases and leukotriene B₄ (Lam, 2002; Scott and Owens, 2008; Hitchcock, 2009; Jha *et al.*, 2005). Chicken thrombocytes express transcripts of anti-inflammatory cytokines transforming growth factor (TGF)- β and interleukin (IL)-10 (Paul *et al.*, 2012) and up-regulate expression of pro-inflammatory cytokines (IL-1 β , IL-6, IL-8 and IL-12) when stimulated with LPS (Ferdous *et al.*, 2008; Hitchcock, 2009; Scott and Owens, 2008).

1.4.3 Wound Healing

In addition to forming an aggregate and taking part in the process of inflammation, mammalian platelets express and release proteins and substances that promote tissue repair. The binding of secreted proteins within a developing fibrin mesh or to the extracellular matrix can create chemotactic gradients favoring the recruitment of stem cells, stimulating cell migration and differentiation, and promoting repair (Semple *et al.*, 2011). Chicken thrombocytes have been shown to have transcripts for platelet-derived growth factors (Horiuchi *et al.*, 2001; Horiuchi *et al.*, 2002) that may have

important roles in healing damaged tissue (Wachowicz and Krajewski, 1981; Horiuchi *et al.*, 1990).

1.4.4 Immunological Capacity

Although platelets and thrombocytes have been well recognized for their importance in homeostasis and their contribution to wound healing, their role in anti-microbial host defense and immunity have been historically underappreciated. The role of these cells in the immune response began with the evidence of their phagocytic ability, followed by their role in the inflammatory response. More recently, the presence of germ-line encoded PRRs such as TLRs have led to a new understanding of the platelet/thrombocyte role in immune responses. To date, the mammalian (human and mice) platelets have been confirmed to express TLR1-9 and the avian (chicken) thrombocytes express TLRs 2-5, 7 and 21. This characteristic strengthens the notion that platelets may have direct roles in protecting the host from infection like any other leukocytes. Figure 1.6 shows some examples of how activated platelets can affect the innate immune response. In addition to their role in the innate immune response, these cells have been shown recently to be associated with adaptive immunity.

1.4.4.1 Innate Immune Response

Although historically underappreciated for their role in anti-microbial host defense, at present platelets are regarded as an integral part of inflammation and potent effector cells of the innate immune response. Platelets are capable of binding,

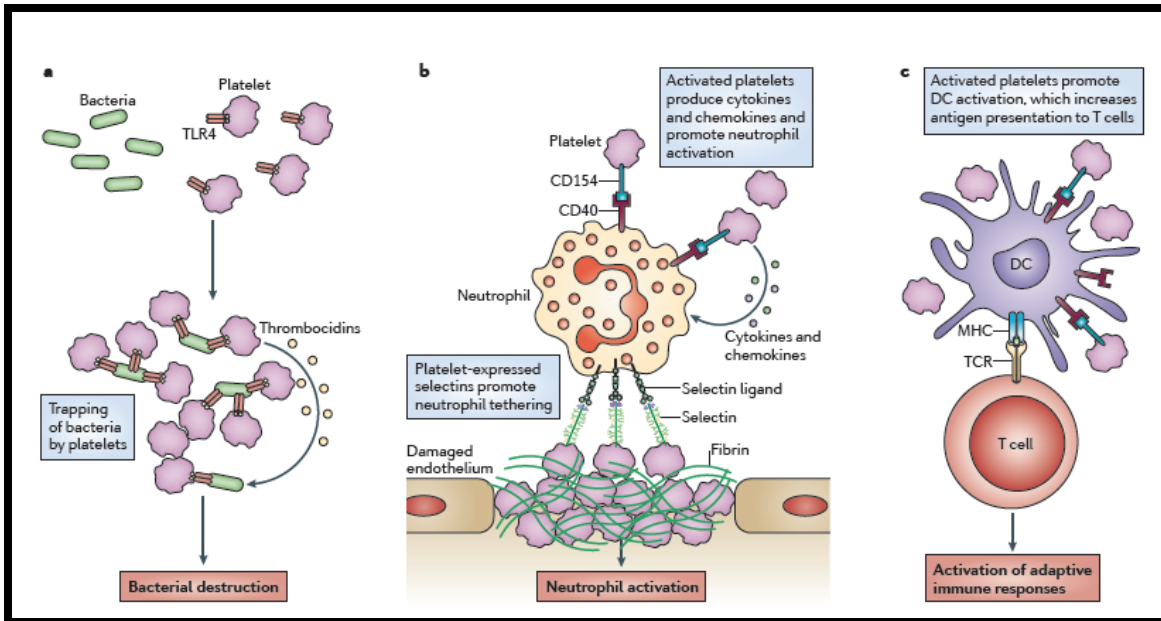


Figure 1.6: Activated platelets can mediate cell–cell interactions and affect immune responses. Platelet Toll-like receptor (TLR) expression enables activated platelets to bind and capture bacteria that may directly kill the bacteria by producing thrombocidins or by aggregating around the bacteria and ‘trapping’ them for elimination by professional phagocytes (a). Platelets can also interact with a wide variety of cells, including leukocytes (b). Activated platelets can also promote the activation of monocytes and DCs, particularly through CD40–CD154 (also known as CD40L) interactions (c) (Adapted from Semple *et al.*, 2011).

aggregating, and internalizing microorganisms which enhances clearance of pathogens from the bloodstream. For example, platelets can bind to *Plasmodium chabaudi* infected red blood cells; induce intracellular parasite killing and cell destruction (Yeaman *et al.*, 1992). These cells have been shown also to actively bind circulating bacteria and microbial products, and present those to neutrophils and cells of the reticuloendothelial system (Aslam *et al.*, 2006). Glick *et al.* (1964) first demonstrated the phagocytic ability of circulating thrombocytes. Avian thrombocytes are capable of phagocytosing bacteria (Glick *et al.*, 1964; and Carlson *et al.*, 1968), dye particles (Carlson *et al.*, 1968), and viruses (Hodges, 1979). Chang and Hamilton (1976) have suggested that thrombocytes are the primary circulating phagocytes in the chicken. Thrombocytes phagocytized about three times as rapidly as heterophils and monocytes (Chang and Hamilton, 1979a). Chang and Hamilton (1979a) have shown also that circulating thrombocytes engulfed nearly twice as many bacteria as the heterophil and monocyte together.

1.4.4.2 Adaptive Immune Response

The discovery of functional CD40 ligand (CD40L/CD154), a molecule of vital importance to the adaptive immune response on the surface of activated platelets (Henn *et al.*, 1998), has led researchers to investigate the potential modulatory capacity of platelets in bridging innate immunity to the adaptive side of immune responsiveness. CD40 and CD40L are a receptor-ligand pair with a central role in promoting interactions between lymphocytes and APCs such as DCs.

DCs are known as the most important link between innate and adaptive immunity since these cells are thought to be the only cells capable of activating naïve T-cells sufficiently for induction of a productive cellular immune response. Initially, platelets have been shown to activate DCs *in vitro* and promote T-cell responses via CD40L. Later, platelet-derived CD40L has been shown to augment CD8⁺ T-cell responses, both *in vitro* and *in vivo*, and to promote protective T-cell responses following infection with *Listeria monocytogenes* (Elzey *et al.*, 2008). Platelets can also modulate T-cell responses through non-CD40L dependent mechanisms. In a hepatitis model, platelet depletion has been shown to prevent cytotoxic T-lymphocytes entry into the liver, which protects against acute organ damage (Iannacone *et al.*, 2005). Platelet-derived CD40L has been reported to support B-cell differentiation and immunoglobulin class-switching in mice (Elzey *et al.*, 2003). During viral infections, transfer of wild-type platelets to CD40L mutant mice has been shown to transiently increase production of virus-specific IgG and protect the animals from subsequent viral infection (Elzey *et al.*, 2003). This phenomenon suggests that platelet-derived CD40L has the ability to promote protective adaptive immune responses. CD40L expressed on the surface of platelets can also interact with CD40 on endothelial cells to induce endothelial cell up-regulation of several adhesion molecules such as intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) and release of CC-chemokine ligand 2 (CCL2) (Andre *et al.*, 2002). All of which promote leukocyte recruitment to inflammatory sites. Activated platelets also release soluble CD40L. Soluble CD40L can interact with vascular endothelial cells and induce the up-regulation of E-selectin and P-selectin and the release

of pro-inflammatory cytokines, IL-6 and other tissue factors (Hammwöhner, *et al.*, 2007 and Anand *et al.*, 2003).

Avian thrombocytes also express CD40L among other biologically active surface molecules and receptors (Tregaskes *et al.*, 2005). Similar to mammalian platelets, the discovery of functional CD40L is of vital importance in the potential modulatory capacity of thrombocytes in bridging innate immunity to the adaptive side of immune responsiveness. Thrombocytes are more than innate effector cells. In addition to CD40L, a recent article indicates that chicken thrombocytes also express transcripts for CD40, CD80 and MHC II (Paul *et al.*, 2012). Therefore, chicken thrombocytes should have not only the ability to interact with APC but also have the potential to be involved in antigen presentation. Besides expressing functional CD40L, platelets and thrombocytes can also release cytokines such as IL-12 during activation by LPS (Ferdous *et al.*, 2008). Cytokines such as IL-12 are expressed by APCs that drive naïve T-cells to differentiate into T_H1 cells. Therefore, in addition to attracting APCs, platelets may be able to function like non-professional APCs.

1.5 Impact/Relevance

The complete role of thrombocytes in the physiology of the chicken immune system is still unknown. Thrombocytes are the most abundant white blood cells in avian blood and there are almost six times more thrombocytes than heterophils in avian circulation (Glick 1958; Lucas and Jamroz, 1961). It has been widely believed that other innate effector cells such as heterophils play the most dominant role in the avian innate

immune system (Kogut *et al.*, 2002; Kogut *et al.*, 2005). However due to the sheer number and the above discussion regarding the role of thrombocytes as innate effector cells, thrombocytes have the potential to play the role of a primary effector cell in the immune system. In addition these cells may have a significant role in shaping the adaptive immune response, as well.

The research reported herein enables us to better understand which pathogenic ligands are recognized by chicken thrombocyte TLRs to initiate immune responses. It may also assist in understanding involvement of chicken thrombocytes during various microbial infections. Thrombocytes have recently been shown to have the potential to be involved in antigen presentation and to interact with professional antigen presenting cells such as DCs. Understanding how TLRs initiate particular immune responses and the involvement of thrombocytes in antigen presentation may be applied to adjuvant and vaccine research for improved vaccines for different poultry diseases.

Most vaccines require a vaccine adjuvant in order to get the immune system to elicit an appropriate response. Since individual subsets of APCs often express different TLRs, it may be possible to elicit specific immune responses by selectively targeted antigen-TLR ligand fusion proteins to specific APC subsets (O'Neill *et al.*, 2009). For example, some lipid A analogues, agonists for TLR4 and CpG DNA, an agonist of TLR9 are being investigated for use as vaccine adjuvants (Romagne, 2007; Connolly and O'Neill, 2012). In mammalian models, DCs can be potent inducers of T-cell-mediated immunity and are activated through TLR ligation (O'Neill *et al.*, 2009). TLR3 agonists

such as Poly (I:C) have been demonstrated to have potential for DC-targeted vaccine efficacy (O'Neill *et al.*, 2009; Jurk and Vollmer, 2007). Therefore, there is a need for further research in this area to understand the potential interaction of thrombocytes with other immune cells and understand the role of the thrombocytes in antigen presentation for future adjuvant and thrombocyte-targeted vaccine research.

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CHAPTER II

DECIPHERING THE ROLE OF CHICKEN THROMBOCYTES IN IMMUNITY.

2.1 Introduction

Chicken thrombocytes represent the most abundant white blood cell type in chicken blood (Chang and Hamilton, 1979) and are homologous in function to enucleated mammalian platelets. Platelets and thrombocytes have been well recognized for importance in homeostasis and contribution to wound healing, however, the role of these cells in anti-microbial host defense and the immune system has been historically underappreciated.

One of the early studies by Glick *et al.* (1964) demonstrates that thrombocytes have a considerable role in antimicrobial host defense by displaying a phagocytic nature. More recently, chicken thrombocytes have been shown to release a variety of bioactive compounds such as pro- and anti-inflammatory cytokines, chemotactic factors, and other mediators in inflammation (Ferdous *et al.*, 2008; Scott and Owens, 2008; Hitchcock, 2009; Paul *et al.*, 2012; Lam, 2002; Jha *et al.*, 2005). The discovery of germ-line encoded pattern recognition receptors (PRRs) such as Toll-like Receptors (TLRs) 2-5, 7 and 21 on chicken thrombocytes adds to understanding the role of thrombocytes in the innate immune response (Scott and Owens, 2008; Paul *et al.*, 2012).

To help unravel the role of thrombocytes in innate immunity, and possibly adaptive immunity, here we examine the immune capacity surface features of chicken

thrombocytes. We focus especially on features that are shared with other immune cells; especially the MQ.NCSU chicken macrophage cell line. In doing so, the broader involvement of the thrombocyte in immunity of the chicken is explored.

2.2 Materials and Methods

2.2.1 Chickens

Female Single Comb White Leghorn (SCWL) chickens (between 16-24 weeks old) were used for this study. The chickens were housed at the Clemson University Morgan Poultry Center, Clemson, SC, which is an Institutional Animal Care and Use Committee (IACUC) approved animal facility operating under standard management practice adhering to the Association for Assessment and Accreditation of Laboratory Animal Care International criteria.

2.2.2 Thrombocyte Isolation

Syringes fitted with needles were used to collect 3 mL of whole blood from the wing vein of each chicken into 0.1 mL volume of 10% ethylene diamine tetra acetic acid (EDTA) solution. The collected blood samples were stored on ice until brought back to the laboratory. The thrombocyte isolation protocol has been modified from that used by Horiuchi *et al.* (2004). Each blood sample was diluted (1:1) with calcium and magnesium free Hanks balanced salt solution (HBSS) (Cambrex Bio Sciences Walkersville Inc., Walkersville, MD). Diluted blood samples were then layered on a lymphocyte separation

medium (Density 1.077-1.080 g/mL, Mediatech. Inc., Herdon, VA) and centrifuged at 1700 x g for 30 min at 23°C. The resultant band of cells containing the thrombocytes was collected, washed and resuspended in calcium and magnesium free HBSS. Trypan blue solution (0.4% w/v in normal saline) was used for quantification of viable cell numbers on a SPolite[®] Hemacytometer (Baxter Healthcare, McGaw Park, IL) with the aid of an upright light microscope. An additional assessment of viability of thrombocytes was determined with glass adhered cells used for microscopy studies. Isolated thrombocytes (5×10^5 cells per 500 μ L) were incubated with fluorescent vital stain, calcein AM (1 μ M, Invitrogen) at 41°C and 5% CO₂ for 30 minutes in a 4-chambered coverglass system #1.0 (Labtek). After washing, the chambers were then imaged in differential interference contrast and green fluoresce using a Nikon Ti microscope with 60x APO water emulsion objective (Clemson Light Imaging Facility, Clemson University, Clemson, SC).

2.2.3 MQ.NCSU Cell Culture

The MQ.NCSU cell line was a generous donation from Dr. Matthew Koci's laboratory (NCSU Prestage Department of Poultry Science, North Carolina State University, Raleigh, NC). These cells were cultured at 41°C and 5% CO₂ with LM Hahn medium made with a combination of Lebovitz A medium (Sigma-Aldrich, St Louis, MO) and McCoy's 5a medium (Invitrogen Life Technologies), tryptose phosphate broth (Sigma Aldrich, St Louis, MO), bovine fetal serum (HyClone[®], Logan, Utah), chicken serum (Sigma-Aldrich, St Louis, MO), glutamine, sodium pyruvate (CellGro, Mediatech Inc, Manassas, VA), 2-mercaptoethanol (Sigma-Aldrich, St Louis, MO), and a mixture of

penicillin, streptomycin, and fungizone (Lonza Walkersville Inc., Walkersville, MD) (Qureshi *et al.*, 1990).

2.2.4 *Thrombocyte and MQ.NCSU Stimulation*

The isolated thrombocytes or cultured MQ.NCSU cells (1×10^7 in 1 mL) were incubated with 1 $\mu\text{g/mL}$ of ultra-pure Lipopolysaccharide (LPS) from *Salmonella minnesota* (TLR4 ligand) or 400 $\mu\text{g/mL}$ of Polyinosinic-polycytidylic acid [Poly (I:C)], a synthetic analog of double-stranded RNA (TLR3 ligand) (InvivoGen, San Diego, CA). The cell suspension and TLR ligands were incubated in sterile 1.5 mL microcentrifuge tubes on a rocking platform (VWR, Suwanee, GA) at 41°C for 10 and 60 min.

2.2.5 *Antibodies*

The monoclonal antibodies used to label cells include mouse anti-chicken CD41/61 (MCA2240), CD40 (MCA2836), CD80 (MCA2837), CD86 (MCA2838), and a MHC class II monomorphic (MCA2171) from BioRad AbD Serotec Inc., Raleigh NC. Alexa Fluor® 488 F(ab')₂ fragment of goat anti-mouse Ig (H+L) (A11017) and Alexa Fluor® 594 of goat anti-mouse Ig (H+L) (A11005) from Molecular Probes by Life Technologies™, Grand Island, NY were used as the secondary antibodies. All primary antibodies were used at a dilution of 1:200 while the secondary antibodies were used at a dilution of 1:400.

2.2.6 *Fluorescent Microscopy*

For fluorescent microscopy, slides were prepared by thrombocyte adherence. Cell suspensions (200 μ l) containing 2×10^6 cells were allowed to adhere to glass slides for 30 min at 41°C. Adhered thrombocytes were then fixed with 4% paraformaldehyde. Monoclonal mouse anti-chicken CD41/61 at 1:200 dilution per 1×10^6 cells was used as primary antibody with Alexa® 594 goat anti-mouse Ig (H+L) at 1:400 dilution per 2×10^6 cells as secondary antibody. The thrombocytes were counterstained with 4', 6-diamidino-2-phenylindole (DAPI), at a dilution of 1:2000 per 2×10^6 cells. DAPI preferentially stains double-stranded DNA of the cellular nuclei. For all washes and dilutions of antibodies, HBSS containing 1% BSA was used unless otherwise stated.

2.2.7 *Cytometry*

For flow cytometry, 1×10^7 cells per microcentrifuge tube were used. Thrombocytes were fixed with 1% paraformaldehyde overnight, and washed before a 15 min treatment with 0.1% Triton x-100 in HBSS. The washes were completed at 16,060 $\times g$ for 3 min in a table top centrifuge. Next, a blocking step was performed by adding 10% goat serum in HBSS at room temperature for 60 min. Finally, the cells were incubated with mouse anti-chicken primary antibodies for CD41/61, CD40, CD80, CD86 or MHC II and secondary antibody, Alexa 488, respectively. Labeled samples were evaluated using a Beckman Coulter CyAn flow cytometer (Beckman Coulter, Inc., Brea, CA) located at the CTEGD Flow Cytometry Core Facility, University of Georgia (UGA),

Athens, GA. The results were analyzed with FlowJo Software at UGA and the Clemson Light Imaging Facility (CLIF, Clemson University, SC).

For image-based cytometer, each cell suspension was stained in the same way as described above for flow cytometry. The primary antibody used here was mouse anti-chicken CD41/61 and the secondary antibody was Alexa Fluor® 594 goat anti-mouse Ig (H+L). Aliquots of 25 μ L of the cell suspension (stained and unstained samples) were placed on the specific Tali™ cellular analysis slides (Life Technologies™, Grand Island, NY) for imaging. Images were analyzed using Tali™, an image based cytometer (Life Technologies™, Grand Island, NY) available at the CLIF, Clemson University, SC.

2.2.8 RNA Isolation and Quantification

For RNA isolation after thrombocyte stimulation, cells were centrifuged at 5000 x g for 2 min to pellet. The pellets were stored in 100 μ L of RNAlater (Qiagen Inc., Valencia, CA), a RNA stabilizing solution. After 24 hr at 4°C in RNAlater, the cells were centrifuged again to remove the supernatant. The cells can be stored at this point at -20°C for use later if not used immediately. The RNeasy Kit (Qiagen Inc., Valencia, CA) was used according to the manufacturer's protocol to isolate the total RNA from these samples. The RNA samples were treated with an on-column DNase, RNase-free DNase Set (Qiagen Inc., Valencia, CA) to remove any possible contamination from chicken genomic DNA. Isolated RNA samples were quantified using a Nano Drop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA).

2.2.9 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Quantitative RT-PCR was performed using a QuantiTect SYBR Green RT-PCR Kit (Qiagen Inc., Valencia, CA) and an Eppendorf Mastercycler ep *realplex* (Eppendorf North America, Westbury, NY). Primers used for GAPDH, TLR2, TLR3, TLR4 and TLR7 amplification are listed in Table 2.1. The RT-PCR mixture consisted of 0.20 μL QuantiTect RT Mix, 10 μL 2 \times QuantiTect SYBR Green RT-PCR Master Mix, 0.4 μL of 0.5 μM of each specific primer listed in Table 2.1, 8 μL of 1 ng/ μL template RNA, and 1 μL of RNase-free water to make the final reaction volume of 20 μL . The cycling profile for all the reactions was 1 cycle of 50°C for 30 min, 95°C for 15 min, and 40 cycles of 94°C for 15 s, 57°C for 20 s, and 72°C for 20 s.

2.2.10 Data Analysis

The data obtained from the qRT-PCR were analyzed using information on the cycle number (C_t values). The software determined the cycle number for each sample at which the fluorescence crosses the established threshold; this crossing point is referred as the C_t value. The sample that expresses more of a given gene product has a lower C_t value while the lower expressing sample has a higher C_t value. Relative quantification of mRNA levels has been determined through the use of the relative fold change calculation according to Pfaffl (2001). The amplification efficiencies (E) of target and housekeeping genes are required to be approximately equal to one another for this method of relative quantification (Table 2.1).

Table 2.1: Quantitative real-time polymerase chain reaction primer sets for messenger RNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene) and Toll-like receptors (TLRs) 2, 3, 4, and 7 of chickens.

Target RNA	Accession No.	Primer Sequence (5'-3')	Efficiency (E)
GAPDH	NM_204305	F: ATGCCATCACAGCCACACAGAAGA	0.98
		R: ATGCCATCACAGCCACACAGAAGA	
TLR2	NM_001161650	F: AGAACGACTCCAACCTGGGTGGAAA	1.03
		R: AGAGCGTCTTGTGGCTCTTCTCAA	
TLR3	NM_001011691	F: GCATTGCTTGGTTTGCTAGTTGGC	0.95
		R: TGCCTTGCAGGCTGAGGTATCAA	
TLR4	NM_001030693	F: AGGGTTCCACCAGCTCTTTCATCA	1.02
		R: AAGATGGGTGGTGTGTAGAAGCCT	
TLR7	NM_001011688	F: ATCTGACGGTGTGGATCTTGGGA	1.00
		R: ACGTGCCTGCTGTATTGCTCTACT	

2.3 Results

2.3.1 *Thrombocyte Validation*

First we performed a viability assay with calcein AM in order to verify that thrombocytes adhered to glass slides were still viable. The staining method verifies green viable thrombocytes following glass adherence (Fig. 2.1). To determine if the isolated cells are an enriched population of thrombocytes, cells were stained with an antibody for a thrombocyte specific marker, CD41/61 (Fig. 2.2) and were visible through red-fluorescent Alexa® 594 (Fig. 2.2A). Chicken thrombocytes that are adhered to glass slides are shown as a monochromatic image in differential interference contrast, DIC (Fig. 2.2C) and these cells appear to be small, circular in shape with rough edges. Upon visual examination of a combined image, which is an overlay of blue (DAPI) and red labeled cells on the DIC image, most of the cells appear to be thrombocytes (Fig. 2.2D).

Results from the image based cytometer, Tali™ show that the enriched cells are on average 5 µm in diameter and 96% of those cells are positive for thrombocyte specific marker CD41/61 (Fig. 2.3). Figure 2.3 also shows the red and green fluorescence and other threshold values set for the cell analysis. Further analysis by flow cytometry shows, expression of CD41/61 on chicken thrombocytes is not affected by stimulation with LPS nor Poly (I:C). CD41/61 was positively expressed on more than 99% of control, LPS and Poly (I:C) treated chicken thrombocytes (Fig. 2.4).

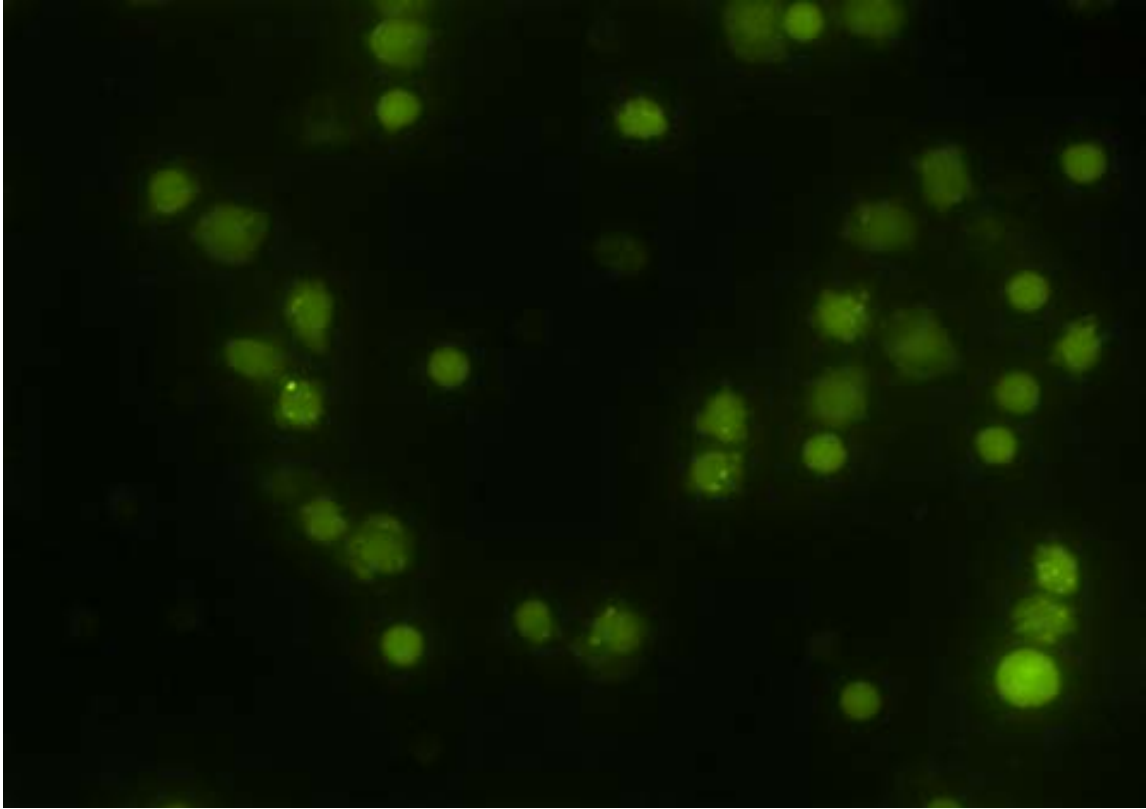


Figure 2.1: Green fluorescent image of viable chicken thrombocytes. In order to verify if thrombocytes adhered to glass slides were still viable, isolated thrombocytes (5×10^5 cells per $500\mu\text{L}$) were incubated with fluorescent vital stain, calcein AM ($1 \mu\text{M}$, Invitrogen). The cells visualized using the Nikon Ti microscope with 60x APO water emulsion objective (Clemson Light Imaging Facility, Clemson University, Clemson, SC).

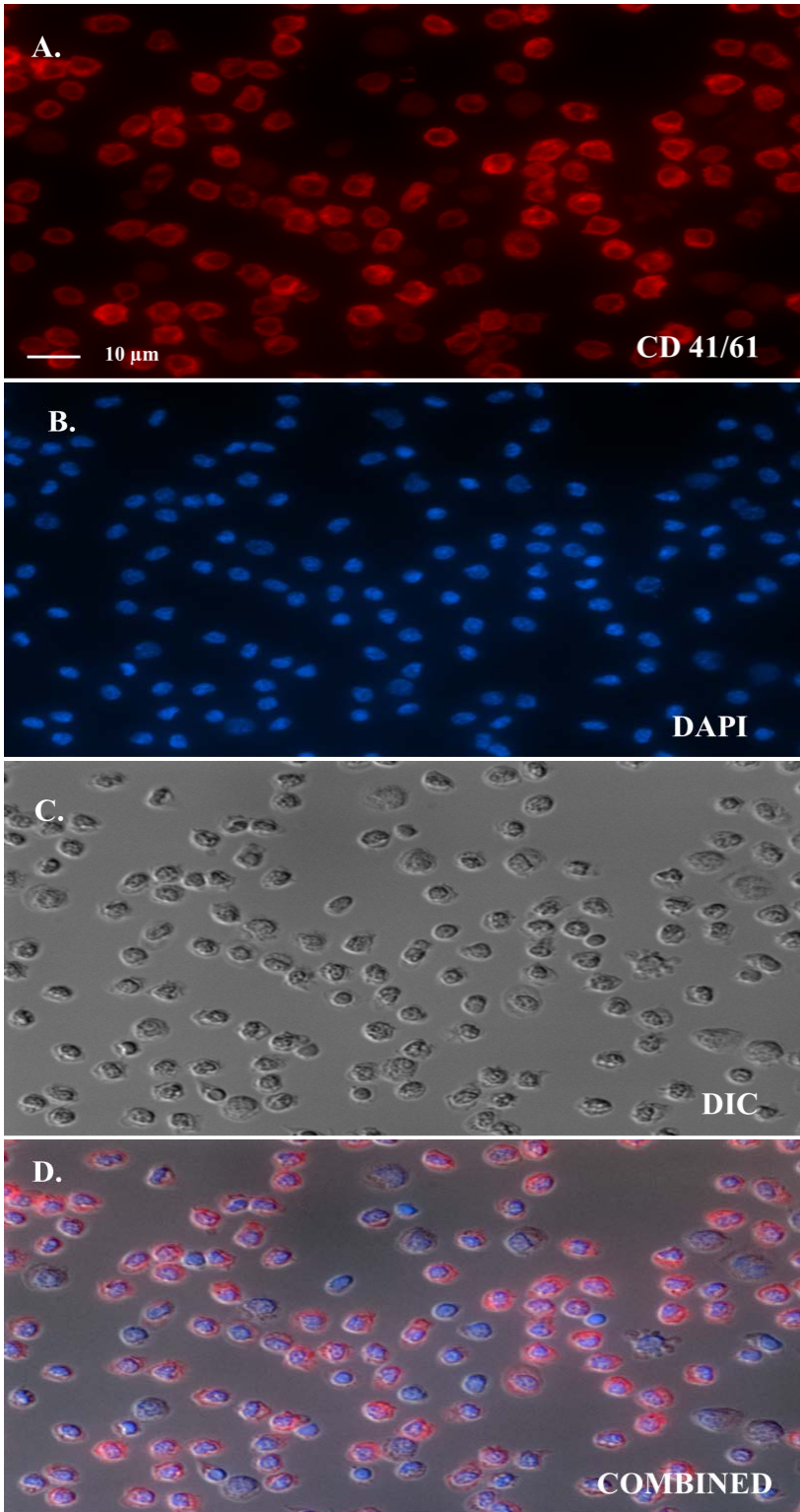


Figure 2.2: Microscopy images of CD41/61 and 4',6-diamidino-2-phenylindole (DAPI) stained chicken thrombocytes. Chicken thrombocytes visualized with (A.) Alexa Fluor® 594 labeled monoclonal mouse anti-chicken CD41/61, a thrombocyte specific marker, (B.) DAPI, a nuclear stain, and (C.) monochromatic differential interference contrast (DIC). The last image is a combined image showing all of the above (D.). The cells were visualized using the Nikon Ti microscope with 60x APO water emulsion objective (Clemson Light Imaging Facility, Clemson University, Clemson, SC).

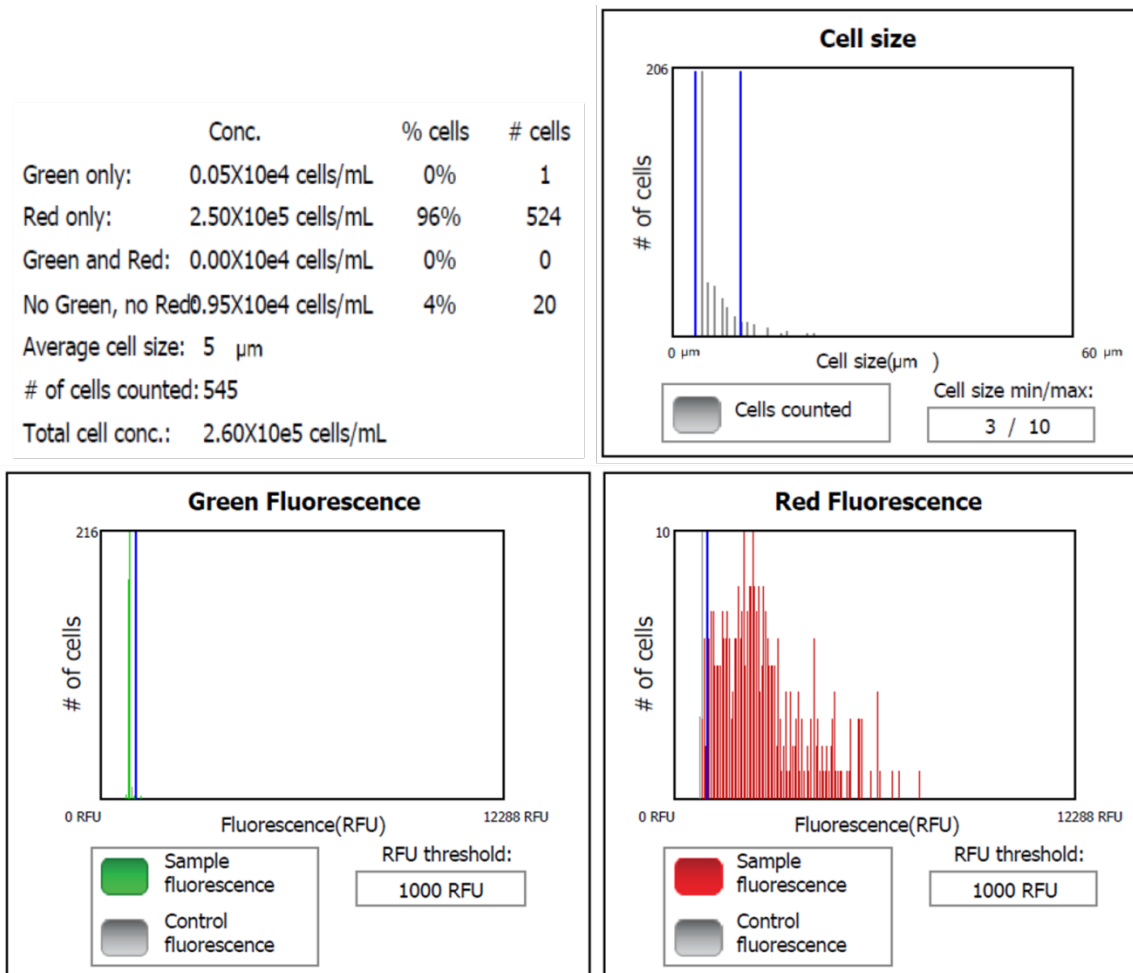


Figure 2.3: Tali™, image-based cytometer analysis of isolated chicken thrombocyte suspension. Isolated thrombocyte suspension was analyzed using, Tali™ at CLIF, Clemson University, Clemson, SC. The cell suspension was incubated with Alexa Fluor® 594 labeled monoclonal mouse anti-chicken CD41/61. The analysis shows that 96% of the cells are positive for thrombocyte specific CD41/61. There were no green fluorescent labeled cells in this experiment.

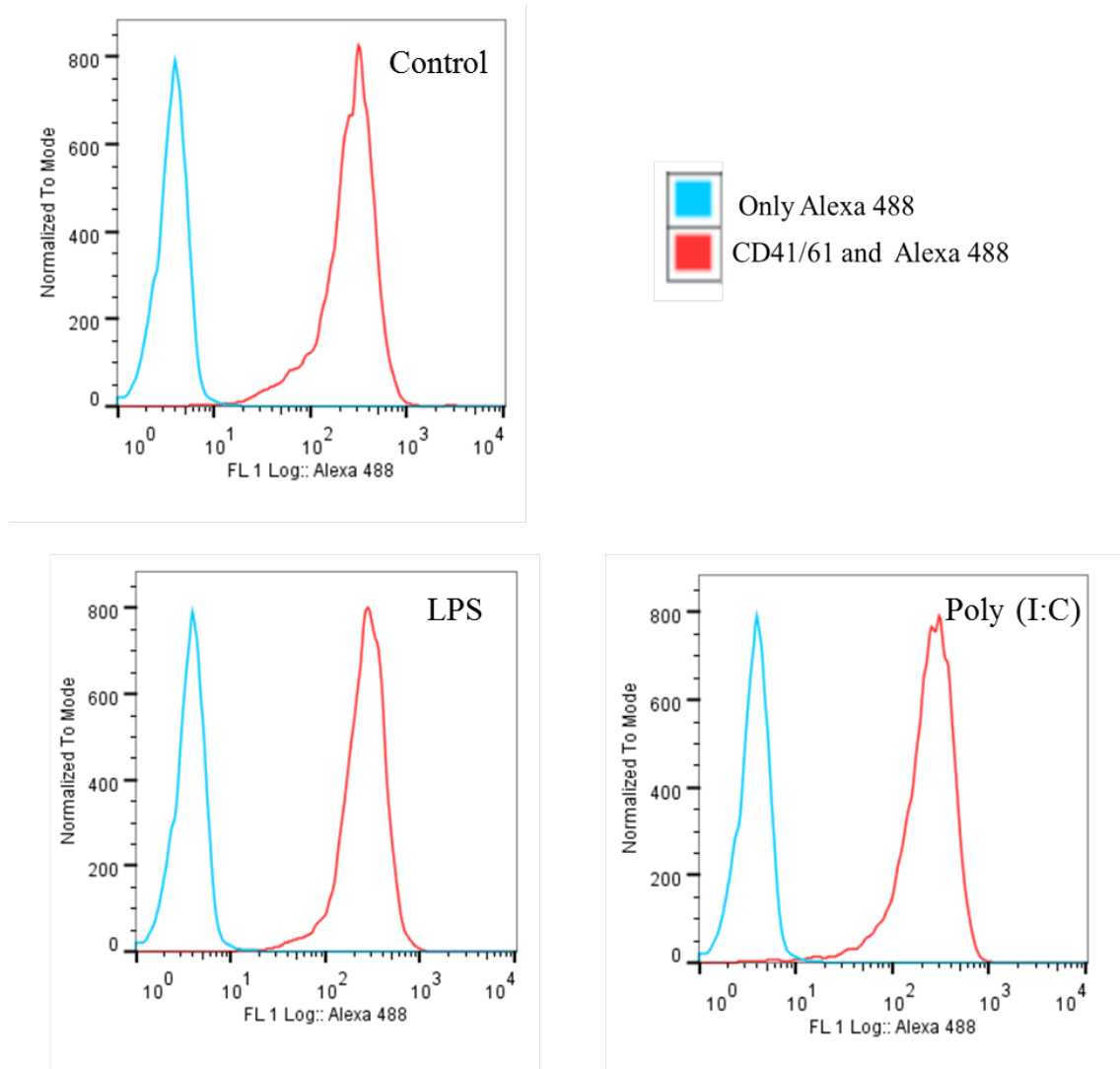


Figure 2.4: Flow cytometric analysis of CD41/61 labeled cells. The histograms shows surface expression of thrombocyte specific CD41/61 labeled with Alexa Fluor® 488 (red) compared to cells labeled with only Alexa Fluor® 488 (negative control, blue). The top panel shows histogram of control cells that were not stimulated and the bottom panel shows histograms of cells stimulated for 1 hr with 1 µg/mL of lipopolysaccharide (LPS) and 400 µg/mL of polyinosinic-polycytidylic acid [Poly (I:C)].

2.3.2 *Expression of MHC Class II*

To detect presence of the MHC II on the surface of chicken thrombocytes, we quantified the percentage of the chicken thrombocyte population expressing MHC II through cell cytometry analysis (Fig. 2.5A). We also examined percentage of the MQ.NCSU macrophage cells expressing MHC II (Fig 2.5B). Upon analysis, percent of thrombocytes and MQ.NCSU macrophages expressing MHC II appears to be very similar (Table 2.2). It is extremely interesting to find thrombocytes expressing MHC II which is exclusively limited to true antigen presenting cells (APCs). As an additional observation, treatment of both thrombocytes and macrophages with LPS and Poly (I:C) does not change dramatically the percentages of cell expressing MHC II (Table 2.2).

2.3.3 *Expression of Co-Stimulatory Molecules*

Chicken thrombocytes express co-stimulatory molecules CD40, CD80 and CD86 (Fig. 2.6-2.8). We reconfirm the reported presence of these co-stimulatory molecules on MQ.NCSU cells (Fig. 2.6-2.8). Nearly all thrombocytes express CD40 while roughly half of the macrophages were found to be positively identified with this important receptor for T-cell bearing CD40 ligand (CD40L, Table 2.2). Neither LPS nor Poly (I:C) significantly affect the expression of any of the co-stimulatory molecules on either cell type (Table 2.2).

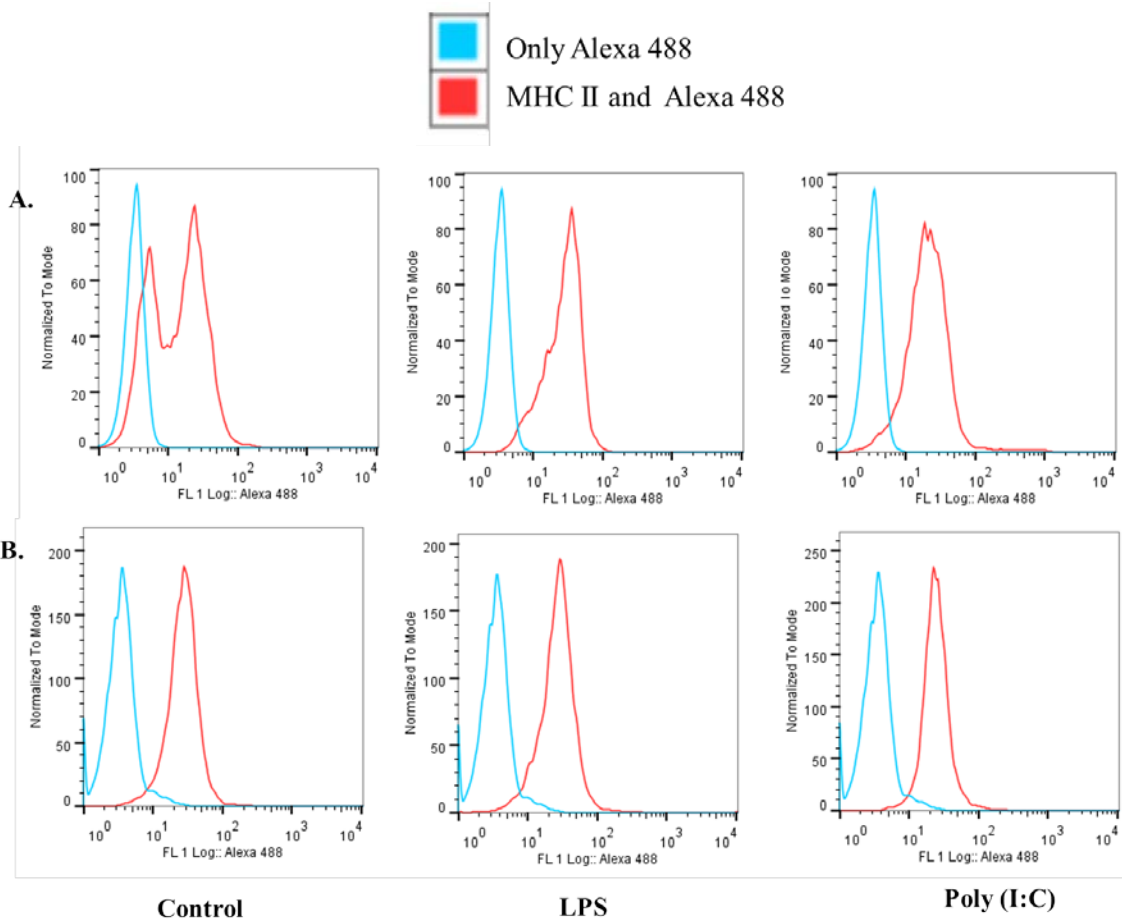


Figure 2.5: Flow cytometric analysis of thrombocytes and MQ.NCSU macrophage cell line with mouse anti-chicken MHC II. Surface expression pattern of MHC II on chicken thrombocyte (A) and MQ.NCSU cells (B) on 1 hr control (not stimulated), 1 $\mu\text{g}/\text{mL}$ of lipopolysaccharide (LPS) and 400 $\mu\text{g}/\text{mL}$ of polyinosinic-polycytidylic acid [Poly (I:C)] stimulated cells (blue= negative control and red=Alexa 488 labeled MHC II). On average 93% of the thrombocyte and 98% of the MQ.NCSU macrophage population express MHC II.

Table 2.2: Percentages of chicken thrombocytes and MQ.NCSU cells positive for MHC class II, CD40, 80 and 86 identified by specific antibody labeling detected through flow cytometric analysis. Surface expression of these molecules were examined on cells that were stimulated with 1 µg/mL of lipopolysaccharide (LPS), 400 µg/mL of polyinosinic-polycytidylic acid [Poly (I:C)] for an hour and control (no stimulation) cells.

Antibody	% Positive Thrombocytes			% Positive MQ.NCSU cells		
	Control	LPS	Poly (I:C)	Control	LPS	Poly (I:C)
MHC II	84.5	99.0	96.1	98.6	97.9	99.2
CD40	91.9	92.1	90.1	49.0	45.6	56.6
CD80	99.7	99.7	99.8	99.3	99.3	99.2
CD86	99.2	94.3	98.8	91.9	90.6	89.0

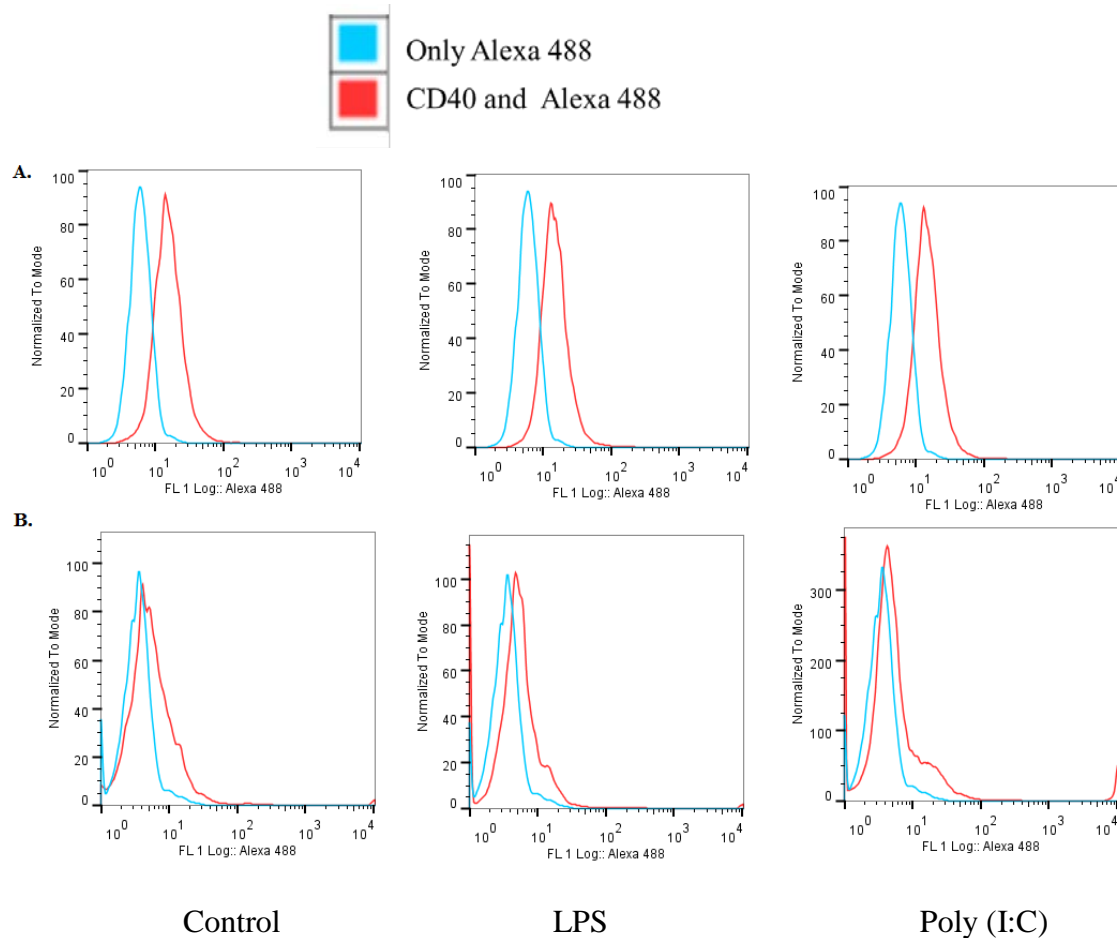


Figure 2.6: Flow cytometric analysis of thrombocytes and MQ.NCSU macrophage cell line with mouse anti-chicken CD40. Surface expression of CD40 on chicken thrombocyte (A) and MQ.NCSU cells (B) on 1 hr control (no stimulation), 1 $\mu\text{g}/\text{mL}$ of lipopolysaccharide (LPS) and 400 $\mu\text{g}/\text{mL}$ of polyinosinic-polycytidylic acid [Poly (I:C)] stimulated cells (blue= negative control and red=Alexa 488 labeled CD40). Although the curves appear to be similar, on average 91% of the thrombocyte and 50% of the MQ.NCSU macrophage population express CD40. The shift of the CD40 positive population compared to the negative population is much lower than that observed for

MHC II and other co-stimulatory molecules. Mean intensity unit comparison reveals the reason for the smaller shift to the right. It indicates that the number of CD40 receptors present per cell is lower on both thrombocytes and MQ.NCSU macrophages compared to MHC II, CD80 and CD86.

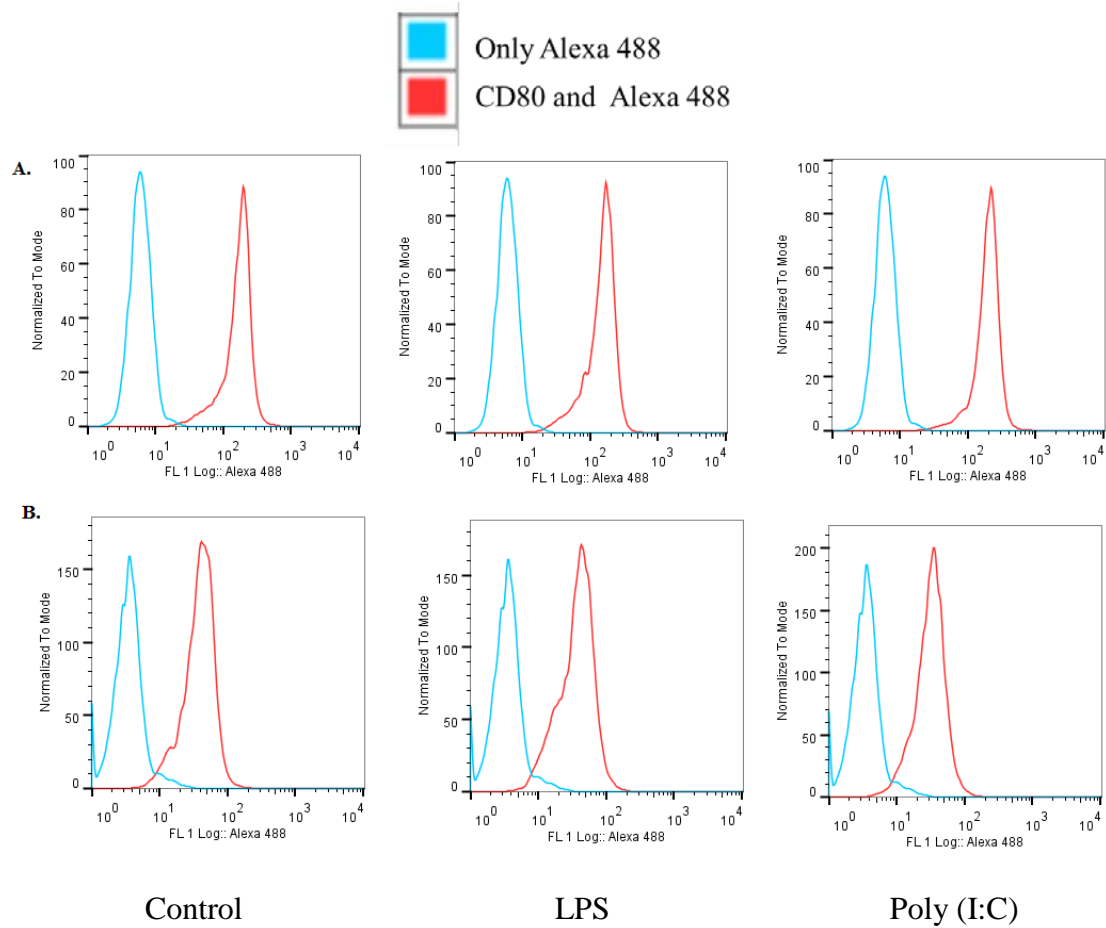


Figure 2.7: Flow cytometric analysis of thrombocytes and MQ.NCSU macrophage cell line with mouse anti-chicken CD80. Surface expression of CD80 on chicken thrombocyte (A) and MQ.NCSU cells (B) on 1 hr control, 1 $\mu\text{g}/\text{mL}$ of lipopolysaccharide (LPS) and 400 $\mu\text{g}/\text{mL}$ of polyinosinic-polycytidylic acid [Poly (I:C)] stimulated cells (blue= negative control and red=Alexa 488 labeled CD80). Thrombocytes and MQ.NCSU macrophages both were over 99% positive for CD80, but the average mean intensity value for thrombocyte was 175 while MQ.NCSU macrophages were 35. This

indicates that there are less CD80 receptors per MQ.NCSU macrophage than thrombocytes.

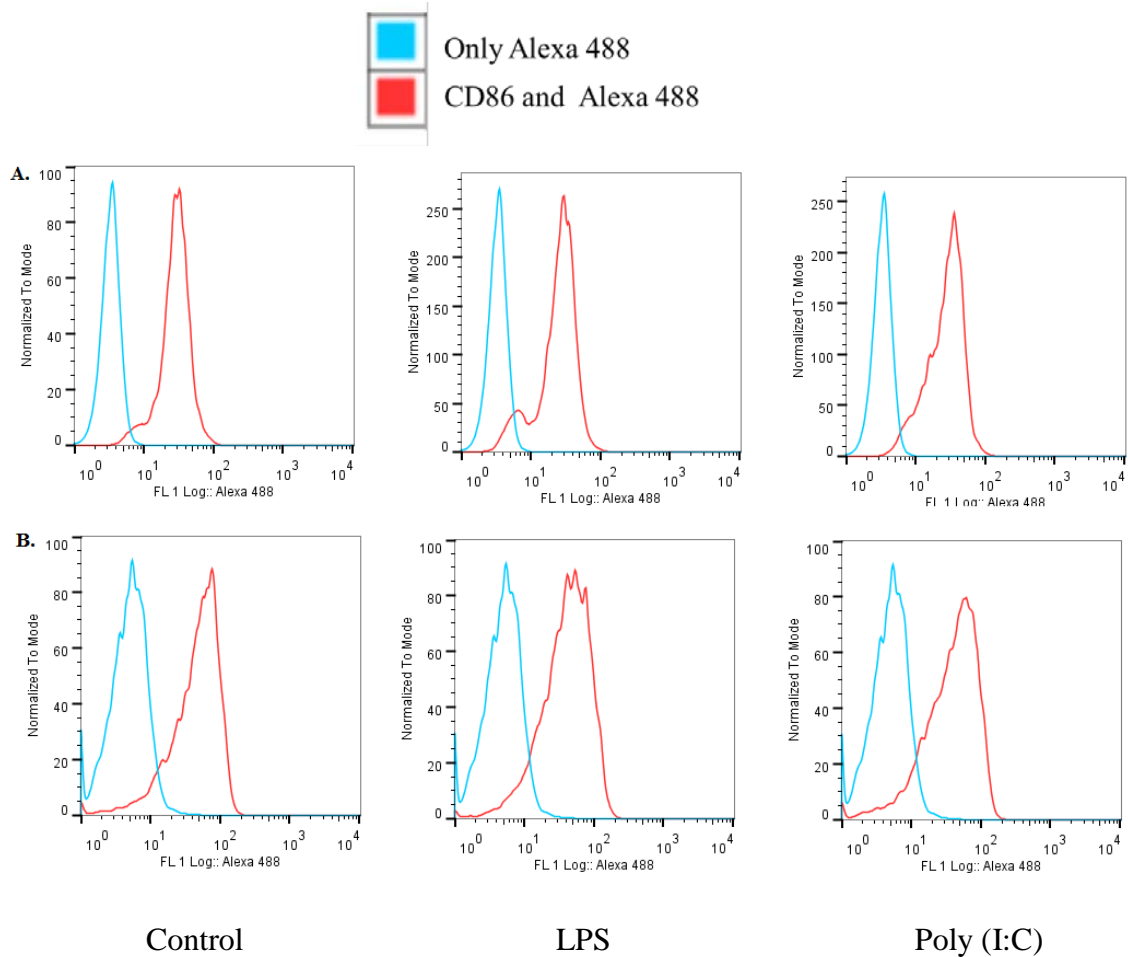


Figure 2.8: Flow cytometric analysis of thrombocytes and MQ.NCSU macrophage cell line with mouse anti-chicken CD86. Surface expression of CD86 on chicken thrombocyte (A) and MQ.NCSU cells (B) on 1hr control (no stimulation), 1 μ g/mL of lipopolysaccharide (LPS) and 400 μ g/mL of polyinosinic-polycytidylic acid [Poly (I:C)] stimulated cells (blue= negative control and red=Alexa 488 labeled CD86). Thrombocytes were 97% and MQ.NCSU macrophages 91% positive for CD80, but the average mean intensity value indicate that there was twice as much CD86 receptors on MQ.NCSU than thrombocytes.

2.3.4 Expression of TLRs

Previous work from our laboratory has shown that chicken thrombocytes respond to LPS stimulation which takes place through TLR4 to activate MAP kinase and NF- κ B pathways (Scott and Owens, 2008). We wanted to further verify if thrombocytes express other TLRs and if those TLRs can be stimulated with other TLR ligands such as Poly (I:C) (TLR3 ligand). A preliminary assessment of the gene expression in thrombocytes was done through a RNA microarray at the University of Delaware (C. Keeler) for Toll-like receptor pathways genes. Examples of some of the TLR specific up-regulated genes are shown in Table 2.3. These results were used to guide our decisions on “likely-suspect” TLRs to examine. We show that chicken thrombocytes express transcripts of TLR2, TLR3, TLR4, and TLR7 (Fig. 2.9). One hour LPS and Poly (I:C) stimulation appears to increase expression of these TLRs indicating a generalize, non-specific expression in thrombocytes. Antibodies for chicken TLRs are not available so we have not been able to report a specific identification of the receptor via cell surface expression, although thrombocytes do respond to TLR ligands and gene expression is detectable.

2.4 Discussion

In order to perform experiments and study the characteristics of chicken thrombocytes, it was essential for us to have an enriched population of thrombocytes. Chicken thrombocytes can be identified with monoclonal antibodies such as 23C6 (Viertlboeck and Göbel, 2007) and K1 (Kaspers *et al.*, 1993), that recognize the intact

Table 2.3: RNA microarray analysis of Toll-like receptor (TLR) pathway specific molecules by using control and 1hr lipopolysaccharide (1 µg/mL) stimulation of chicken thrombocytes.

Gene	Fold Change from Control
Interleukin 1β (IL1-β, IL1B)	5.756
Inducible nitric oxide synthase	5.744
TLR5	4.266
Major histocompatibility complex class I glycoprotein	2.868
Nuclear factor kappa-light-chain-enhancer of activated B cells p50 subunit	2.683
C-fos proto-oncogene	2.669
Interleukin 6	2.385
V-jun	1.947
TLR3	1.737

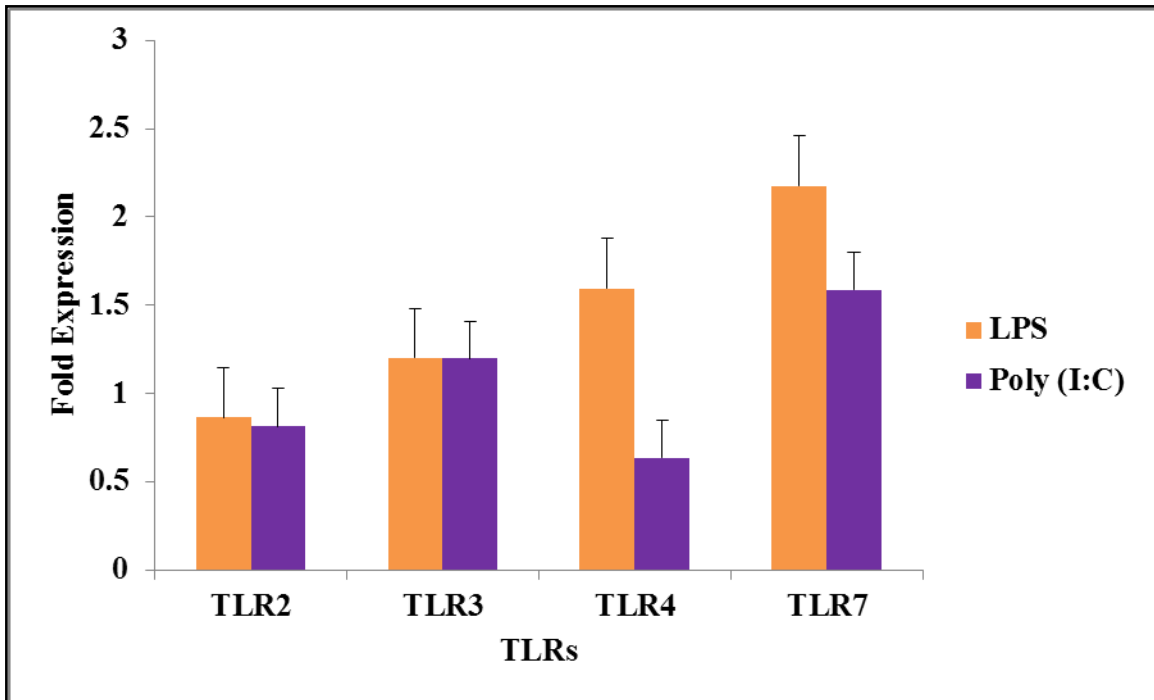


Figure 2.9: Relative quantification in terms of fold expression of Toll-like receptors (TLRs) 2, 3, 4, and 7. Fold expression values are calculated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene) according to Pfaffl (2001) using the cycle number obtained from the quantitative real-time polymerase chain reaction and amplification efficiencies of target and housekeeping gene. One hour stimulation with lipopolysaccharide (LPS, 1 $\mu\text{g}/\text{mL}$) and polyinosinic-polycytidylic acid [Poly (I:C), 400 $\mu\text{g}/\text{mL}$) appears to increase expression of these TLRs compared GAPDH.

complex formed between CD41 and CD61 (α V and β 3 integrin) and an unidentified epitope, respectively, on the surface of chicken thrombocytes. Here we used mouse anti-chicken CD41/61, clone 11C3 that recognizes the chicken integrin complex which is a heterodimeric cell surface protein. We have consistently observed enriched populations of thrombocytes using our isolation procedure (Fig. 2.2-2.4). It was reassuring to see that stimulation with LPS or Poly (I:C) did not change the surface expression of this integrin complex (Fig. 2.4) that could have potentially changed through TLR activation and led to an altered ability for thrombocytes to bind to specific integrin ligands.

In our search to find relevant immune molecules expressed on the surface of chicken thrombocytes, we found expression of MHC II and co-stimulatory molecules CD40, CD80 and CD86 on the majority of thrombocytes. Expression of MHC II is a unique finding for chicken thrombocytes since mammalian platelets are devoid of MHC class II molecules (Semple, 2011). Generally, dendritic cells (DCs, professional APCs) express both class I and class II MHC molecules while macrophages and B cells must be activated to express class II. In our studies, we found chicken thrombocytes to express class II MHC even in the absence of cellular stimulation. Interestingly, treatment of thrombocytes with TLR ligands did not change the expression profile for neither thrombocytes nor MQ.NCSU macrophage cells. Although it has been previously reported that thrombocytes express transcripts for MHC II and the transcripts for MHC II were significantly up-regulated in response to LPS and CpG ODN (Paul *et al.*, 2012), we did not observe a change in expression of MHC II molecules on chicken thrombocytes when stimulated with LPS or Poly (I:C) (Table 2.2).

We have also observed expression of co-stimulatory molecules CD40, CD80 and CD86 on chicken thrombocytes and MQ.NCSU macrophages. Although the percent positive cells for these receptors were similar (Table 2.2), the actual number of receptors per cell indicated by mean intensity values were often very different. For example, thrombocytes and MQ.NCSU macrophages both were over 99% positive for CD80, but the average mean intensity value for thrombocyte was 175 while MQ.NCSU macrophages were 35. Based on comparison of mean fluorescence intensity, expression of CD40 per cell appeared to be lower on thrombocytes and MQ.NCSU macrophages when compared to CD80 and CD86. Presence of CD40 on primary chicken macrophage and the chicken HD11 macrophage cell line has been previously shown by Chen *et al.* (2010). Interestingly, the distribution of fluorescence intensity is tighter in the macrophage cell line compared to primary macrophages (Chen *et al.*, 2010), and similar to what we observe for the MQ.NCSU macrophage cell line.

DCs and most B-cell lineage also express CD40 that binds to complementary ligand, CD154 (CD40L) on T cells (Kindt *et al.*, 2007). The interaction between CD40/CD154 can influence cytokine production (such as IL-12). The cytokine IL-12 expressed by APCs can influence T-cell behavior and drive naïve T-cells to differentiate into T-helper type 1 (T_H1) cells. The CD40/CD40 ligand (CD154) interaction also influences B-cell growth, differentiation and isotype switching. Thus, CD40 plays a critical role in regulation of cell-mediated immunity as well as antibody-mediated immunity.

Our observation shows constitutive expression of CD80 and CD86 on chicken thrombocytes. The number of receptors for CD80 appears to be higher than that of CD86 per thrombocyte, while number of receptors for CD86 appears to be higher than that of CD80 per MQ.NCSU macrophage. DCs known as major APCs constitutively express CD80 and CD86.. DCs are the most important link between innate and adaptive immunity since they are thought to be the only cell capable of activating naïve T-cells sufficiently for induction of a productive cellular immune response. Following antigenic stimulation, DCs mature by increasing their surface expression of MHC class II and T-cell co-stimulatory molecules.

We examined gene expression of several TLRs on chicken thrombocytes. Expression of different chicken TLRs have been studied by Iqbal *et al.* (2005) in a wide range of isolated chicken immune cell types (such as blood monocyte derived macrophages, heterophils, B cells, and different populations of T-cell subsets) and cultured cells (such as the DT40 cell line, which is derived from an avian leukosis virus-transformed pre-B-cell, and the HD11 cell line that is a retrovirally transformed macrophage-like cell line). According to Iqbal *et al.* (2005), TLR1/6/10, TLR3, TLR4 and TLR5 specific RT-PCR products were detected in all immune cell populations although the level of expression was different for the different cell subset. Chicken TLR2 type 2 mRNA was detected with most cell subsets and chTLR2 type 1 was expressed on a more restricted subset of cells (heterophils and specific T-cell subsets). HD11 chicken macrophage cells also express mRNA for chicken TLR15 and TLR21 (Ciraci and Lamont, 2011).

Since our group had already demonstrated that expression of the TLR4 transcript is up-regulated when chicken thrombocytes are stimulated with a bacterial TLR ligand (LPS) (Scott and Owens, 2008), we next verified expression of TLR2, TLR3 and TLR7. We also examined if 1 hr LPS or Poly (I:C) stimulation can change the expression pattern of these TLRs. Poly (I:C) is a synthetic analog of double-stranded RNA (dsRNA), a molecular pattern associated with viral infection. Poly (I:C) is recognized by TLR3 inducing the activation of NF- κ B and the production of cytokines. Although we have detected transcripts for TLR3 in chicken thrombocytes, there appears to be no significant change in the expression of TLR3 due to stimulation with Poly (I:C) (Fig. 2.4).

Chicken thrombocytes constitutively express transcripts for different TLRs which is crucial for the innate immune response. This chapter also provides evidence that thrombocytes could be non-professional APCs and be involved in adaptive immunity.

2.5 Acknowledgements

We want to sincerely thank Dr. Matthew Koci for approving to donate the MQ.NCSU cell line and Dr. Rizwana Ali for all her assistance regarding the transfer process, her suggestions and assistance with the troubleshooting and establishment of this cell line for this research (NCSU Prestage Department of Poultry Science, North Carolina State University, Raleigh, NC). We would like to thank Dr. Terri Bruce and MS. Rhonda Powell (CLIF, Clemson University, SC) for assistance with fluorescent microscopy and Dr. Julie Nelson (CTEGD Flow Cytometry Core Facility, University of Georgia, Athens,

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CHAPTER III

BACTERIAL AND VIRAL INDUCTION OF CHICKEN THROMBOCYTE IMMUNE RESPONSES.

3.1 Introduction

The role of platelets as mediators and effector inflammatory cells has been a focus of research in the past few decades (Capron *et al.*, 1987; Page, 1989; Herd and Page, 1994; Klinger, 1997). As inflammatory cells, activated platelets express receptors for adhesive proteins, different cytokines and other mediators that make direct contact with endothelial cells, granulocytes, monocytes and lymphocytes to either stimulate or inhibit functions of other cellular partners in inflammation (Bazzoni *et al.*, 1991; Klinger and Jelkmann, 2002). Platelets also contain cytoplasmic granules that store a vast array of bioreactive molecules, which play an integral part in inflammation (George, 2000; Elzey *et al.*, 2005; Semple *et al.*, 2011). With the discovery of functional CD40 ligand (CD154) expression (Hen *et al.*, 1998; Martinson *et al.*, 2004; Chakrabarti *et al.*, 2005), platelets extend potential modulatory capacity from innate immunity to adaptive immunity.

Since enucleated platelets have such a crucial role in different aspects of the immune response, nucleated thrombocytes might also have a significant role in avian immune responses. Chicken thrombocytes have been shown to release β -thromboglobulin (Wachowicz and Krajewski, 1981), thrombocyte-derived growth factors (Horiuchi *et al.*, 1990), lysosomal compounds (Carlson *et al.*, 1968; Daimon and Uchida, 1982), chemotactic factors (Lam, 2002), platelet-derived growth factor-A and -B chains

(Horiuchi *et al.*, 2001; Horiuchi *et al.*, 2002) and some of the enzymes from the arachidonic acid cascade [cyclooxygenase-2 (COX-2), and prostaglandin D₂ (PGD₂), PGE₂, thromboxane A₂ synthases and leukotriene B₄] (Scott and Owens, 2008; Hitchcock, 2009; Jha *et al.*, 2005). Our lab has demonstrated that chicken thrombocytes respond to lipopolysaccharide (LPS) (Ferdous *et al.*, 2008; Scott and Owens, 2008). This LPS stimulation takes place through Toll-like receptor (TLR) 4 and mitogen-activated protein (MAP) kinase (ERK, MEK1 and p38 MAPK) and nuclear factor- κ light-chain-enhancer of activated B cells (NF- κ B) pathways (Scott and Owens, 2008). Thrombocytes also express transcripts for TLRs 2, 3, 4, 7 (previous chapter) and 21 (Paul *et al.*, 2012). Activation of the TLR4 pathway results in gene expression of pro-inflammatory cytokines interleukin (IL)-6, IL-1 β , IL-8, and IL-12 (Scott and Owens, 2008; Ferdous *et al.*, 2008; Hitchcock, 2009). According to another group of researchers, thrombocytes constitutively express transcripts for both pro- and anti-inflammatory cytokines for anti-viral responses and antigen presentation (Paul *et al.*, 2012). Other recent studies, in the case of H5N1 Avian Influenza, virus replicated to a high titer in chicken thrombocytes and caused up-regulation of TLR3 and several cell adhesion molecules (Schat *et al.*, 2012).

Here, we have examined inflammatory responses when thrombocytes are stimulated *in vitro* and *in vivo*. For the *in vitro* study, chicken thrombocytes were stimulated with bacterial ligands; LPS from Gram-negative bacteria (TLR4 ligand), and lipoteichoic acid (LTA) from Gram-positive bacteria (TLR-2 ligand), and viral ligands; polyinosinic-polycytidylic acid [Poly (I:C)], a synthetic analog of double-stranded RNA (ds RNA)

(TLR3 ligand), and thymidine homopolymer phosphorothioate ODN [Poly(dT)] a single-stranded RNA product (TLR 7/8 ligand). Chicken thrombocytes express TLRs and therefore, should participate in immune responses to bacterial and viral TLR ligands. For the *in vivo* stimulation, we examined thrombocytes from chickens vaccinated with a bacterial or viral vaccine. For this experiment, we have used a Gram negative bacterial vaccine for *Salmonella enteritidis* and a combination of dsDNA viral vaccine for infectious bursal disease (IBD) and reovirus (REO). It is probable that thrombocytes of bacterial or viral vaccine immunized chickens are activated and express pro-inflammatory genes compared to unvaccinated chickens. The rationale behind this hypothesis is that the vaccines will activate thrombocytes through TLRs and induce gene expression and production of pro-inflammatory cytokines and mediators.

3.2 Materials and Methods

3.2.1 Chickens and Vaccines

Female Single Comb White Leghorn (SCWL) chickens (between 16-26 weeks old) were used for this study. The chickens were housed at the Clemson University Morgan Poultry Center, Clemson, SC, which is an Institutional Animal Care and Use Committee (IACUC) approved animal facility operating under standard management practice adhering to the Association for Assessment and Accreditation of Laboratory Animal Care International criteria. The chickens were vaccinated according to manufacturer's protocol with a killed bacterial vaccine for *Salmonella enteritidis* (LAYERMUNE® SE, Ceva Biomune, Lenexa, KS) and a killed viral vaccine for IBD and

reovirus (Poulvac Maternavac IBD-REO, Zoetis Marietta, Marietta, GA). As a control, chickens were injected with sterile phosphate buffer saline (PBS). Blood samples were collected from vaccinated and unvaccinated chickens on Day 0 before vaccination and on Day 3, 5 and 7 post-vaccination for blood collection and thrombocyte isolation.

3.2.2 Thrombocyte Isolation and In Vitro Stimulation

Syringes fitted with needles were used to collect 3 mL of whole blood from the wing vein of each chicken into 0.1 mL volume of 10% ethylene diamine tetra acetic acid (EDTA) solution. The collected blood samples were stored on ice until brought back to the laboratory. The thrombocyte isolation protocol has been modified from that used by Horiuchi *et al.* (2004). Each blood sample was diluted (1:1) with calcium and magnesium free Hanks balanced salt solution (HBSS) (Cambrex Bio Sciences Walkersville Inc., Walkersville, MD). Diluted blood samples were then layered on a lymphocyte separation medium (Density 1.077-1.080 g/mL, Mediatech. Inc., Herdon, VA) and centrifuged at 1700 x g for 30 min at 23°C. The resultant band of cells containing the thrombocytes was collected, washed and resuspended in calcium and magnesium free HBSS. Trypan blue solution (0.4% w/v in normal saline) was used for quantification of viable cell numbers on a SPolite[®] Hemacytometer (Baxter Healthcare, McGaw Park, IL) with the aid of an upright light microscope.

The isolated thrombocytes (1×10^7 in 1 mL) were incubated with 1 µg/mL of ultra pure LPS from *Salmonella minnesota*, 400 µg/mL of purified LTA from *Staphylococcus aureus*, 400 µg/mL of Poly (I:C) and 50 µM of Poly (dT) (InvivoGen, San Diego, CA).

The cell suspension and TLR ligands were incubated in sterile 1.5 mL microcentrifuge tubes on a rocking platform (VWR, Suwanee, GA) at 41°C for 10 and 60 min.

3.2.3 MQ.NCSU Culture and Stimulation

The MQ.NCSU cell line was a generous donation from Dr. Matthew Koci's laboratory (NCSU Prestage Department of Poultry Science, North Carolina State University, Raleigh, NC). These cells were cultured at 41°C and 5% CO₂ with LM Hahn medium made with a combination of Lebovitz A medium (Sigma-Aldrich, St Louis, MO) and McCoy's 5a medium (Invitrogen Life Technologies), tryptose phosphate broth (Sigma Aldrich, St Louis, MO), bovine fetal serum (HyClone[®], Logan, Utah), chicken serum (Sigma-Aldrich, St Louis, MO), glutamine, sodium pyruvate (CellGro, Mediatech Inc, Manassas, VA), 2-mercaptoethanol (Sigma-Aldrich, St Louis, MO), and a mixture of penicillin, streptomycin, and fungizone (Lonza Walkersville Inc., Walkersville, MD)(Qureshi *et al.*, 1990). The MQ.NCSU macrophages (1×10^7 in 1 mL) were incubated with 1 µg/mL of LPS, 400 µg/mL of LTA, 400 µg/mL of Poly (I:C) and 50 µM of Poly (dT) (InvivoGen, San Diego, CA) as described above for *in vitro* thrombocyte stimulation.

3.2.4 RNA Isolation and Quantification

For RNA isolation after thrombocyte stimulation, cells were centrifuged at 5000 x g for 2 min to pellet. The pellets were stored in 100 µL of RNAlater (Qiagen Inc.,

Valencia, CA), a RNA stabilizing solution. After 24 hr at 4°C in RNAlater, the cells were centrifuged again to remove the supernatant. The cells can be stored at this point at -20°C for use later if not used immediately. The RNeasy Kit (Qiagen Inc., Valencia, CA) was used according to the manufacturer's protocol to isolate the total RNA from these samples. The RNA samples were treated with an on-column DNase, RNase-free DNase Set (Qiagen Inc., Valencia, CA) to remove any possible contamination from chicken genomic DNA. Isolated RNA samples were quantified using a Nano Drop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA).

3.2.5 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Quantitative RT-PCR was performed using a QuantiTect SYBR Green RT-PCR Kit (Qiagen Inc., Valencia, CA) and an Eppendorf Mastercycler ep *realplex* (Eppendorf North America, Westbury, NY). Primers used for GAPDH, IL-6 and iNOS amplification are listed in Table 3.1. The RT-PCR mixture consisted of 0.20 µL QuantitTect RT Mix, 10 µL 2× QuantitTect SYBR Green RT-PCR Master Mix, 0.4µL of 0.5 µM of each specific primer listed in Table 3.1, 8 µL of 1 ng/µL template RNA, and 1 µL of RNase-free water to make the final reaction volume of 20 µL. The cycling profile for all the reactions was 1 cycle of 50°C for 30 min 95°C for 15 min, and 40 cycles of 94°C for 15 s, 57°C for 20 s, and 72°C for 20 s.

Table 3.1: Quantitative real-time polymerase chain reaction primer sets for messenger RNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene), interleukin-6 inducible nitric oxide synthase.

Target RNA	Accession No.	Primer Sequence (5'-3')	Efficiency (E)
GAPDH	NM_204305	F: ATGCCATCACAGCCACACAGAAGA	0.98
		R: ATGCCATCACAGCCACACAGAAGA	
IL-6	AJ30954	F: ATGTGCAAGAAGTTCACCGTGTGC	0.91
		R: TTCCAGGTAGGTCTGAAAGGCGAA	
iNOS	NM_204961.1	F: ATGGGAACAGAGATTGGAGTGCGA	1.01
		R: TACAACAGCTCGGTCCTTCCACAA	

3.2.6 NO Assay

The Griess Reagent System (Promega Corporation, Madison, WI) was used to investigate nitric oxide (NO) formation by measuring nitrite, which is one of two primary, stable and nonvolatile breakdown products of NO. A Nitrite Standard reference curve was prepared for each assay for accurate quantitation of experimental samples according to the manufacture's protocol. The nitrite concentration of the reference curve ranged from 0-100 μM . The assay was performed as suggested in the the manufacture's protocol. Briefly, 50 μL of each experimental sample (supernatant from control and stimulated chicken thrombocytes and MQ.NCSU cells) were added to wells of a 96-well plate in triplicate. Using a multichannel pipettor, 50 μL of the Sulfanilamide Solution was then added to all experimental samples and wells containing the dilution series for the Nitrite Standard reference curve. Following 10 min incubation at room temperature in dark, 50 μL of the *N*-1-naphthylethylenediamine dihydrochloride (NED) solution was added to all wells. A purple/magenta color forms immediately after addition of the NED solution. After another 10 min of incubation at room in dark, plates were read on a Power Wave X Microplate Scanning Spectrophotometer (Bio-Tek[®] Instruments, Inc., Winooski, VT) at 520 nm.

3.2.7 B9 Cell Bioassay

The bioactivity of IL-6 in supernatants of control and stimulated chicken thrombocytes and MQ.NCSU cells was assessed according to their ability to promote growth of the IL-6-dependent murine hybridoma B9 cells (Helle *et al.*, 1991; Rath *et al.*,

1995). Controls included recombinant chicken IL-6 (AbD Serotec, Raleigh, NC), ligand spiked samples and B9 cells only with RPMI-1640 medium. Recovered cells were adjusted to 1×10^5 cells/mL with IL-6-free cell culture medium following a viability determination by trypan blue exclusion. Aliquots of 100 μ l of different IL-6 sources and 100 μ L of B9 cell suspension (1×10^4 cells) were added to appropriate wells. The plate containing the cells were then placed in a humidified 5% CO₂ incubator at 37°C for 92 hr. Then, 50 μ L of MTT (5 mg/mL, tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide, Sigma Chemical Co., St. Louis) were added to each well and incubated again for 4 hr in the incubator. At conclusion of incubation, the supernatant from each well was removed and 150 μ L of DMSO (Dimethylsulfoxide, EMD Chemical Inc., Darmstadt, Germany) was added to dissolve dye crystals. Plates were read on a Power Wave X Microplate Scanning Spectrophotometer (Bio-Tek[®] Instruments, Inc., Winooski, VT) at 570 and 650 nm.

3.2.8 Statistical Analysis

A series of complete random designed experiments with a 2×5 factorial arrangement of treatments was used where treatments were 2 response times (10 and 60 min) and 5 TLR ligands [control (or no ligand), LPS, LTA, Poly (I:C) and Poly (dT)] for the *in vitro* study. For the *in vivo* vaccine study, a complete random design was used where the treatments were vaccine (Layermune SE or Poulvac Maternavac IBD-REO) and no vaccine (PBS). Analysis of variance was performed with the General Linear Model

procedure of SAS 9.1 (SAS Manual 2000), and hypothesis testing is conducted using $\alpha = 0.05$.

3.3 Results

3.3.1. Interleukin-6 (IL-6) Gene Expression and Release

In vitro treatment of thrombocytes with various TLR ligands demonstrates a definite bacterial effect while no viral effect on gene expression of IL-6. Among the two bacterial TLR ligands used, a dramatic LPS effect was observed at 60 min while a LTA effect is less dramatic but apparent at both 10 and 60 min (Fig. 3.1). However, both viral TLR ligands Poly (I:C) and Poly (dT) did not induce detectable IL-6 gene expression by 60 min (Fig. 3.1). Immunizations with Layermune SE and Poulvac Maternavac IBD-REO do not induce an effect on IL-6 gene expression (Fig. 3.2 and 3.3) in the time frame post immunizations.

In order to examine if there was any difference in release of IL-6 at the protein level, the B9 cell bioassay was performed with culture supernatants. IL-6 activity in supernatants was observed for 60 min LPS treated thrombocytes (Fig. 3.4). Other ligands did not affect thrombocyte release of IL-6 (Fig. 3.4) compared to control. A comparison study with the MQ.NCSU macrophage cell line did not reveal induced release of IL-6 even at 6 and 24 hours of treatment (Fig. 3.5). Thrombocytes are extremely reactive to LPS, but not other ligands with regard to IL-6, and thus, provide a ready source for IL-6

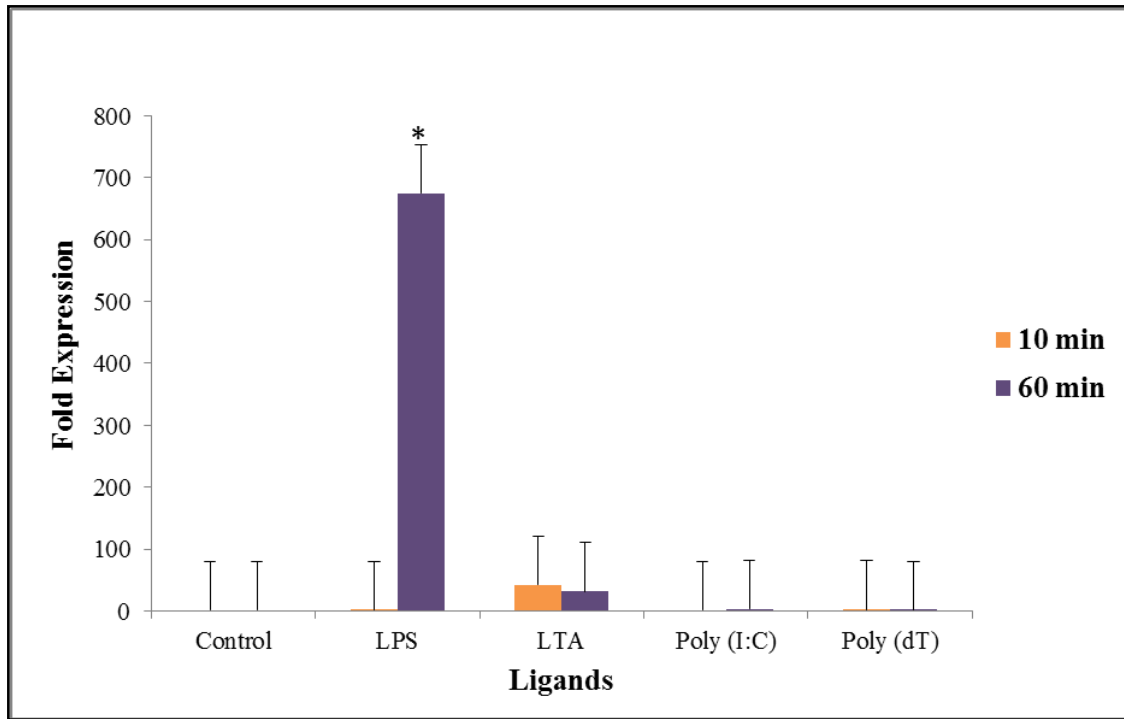


Figure 3.1: Relative fold expression of interleukin-6 (IL-6) in control and stimulated chicken thrombocytes. Thrombocytes were stimulated for 10 min and 60 min with lipopolysaccharide (LPS, 1 $\mu\text{g}/\text{mL}$), lipoteichoic acid (LTA, 400 $\mu\text{g}/\text{mL}$), polyinosinic-polycytidylic acid [Poly (I:C), 400 $\mu\text{g}/\text{mL}$] and homopolymer phosphorothioate ODN [Poly(dT), 50 μM]. Fold expression values are calculated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene) according to Pfaffl (2001) using the cycle number obtained from the quantitative real-time polymerase chain reaction and amplification efficiencies of target (IL-6) and housekeeping gene (GAPDH). Only the LPS stimulation at 60 min showed significant increase of IL-6. Error bars show \pm SEM with 6 samples. * Statistically significant difference when compared against respective control ($p \leq 0.05$).

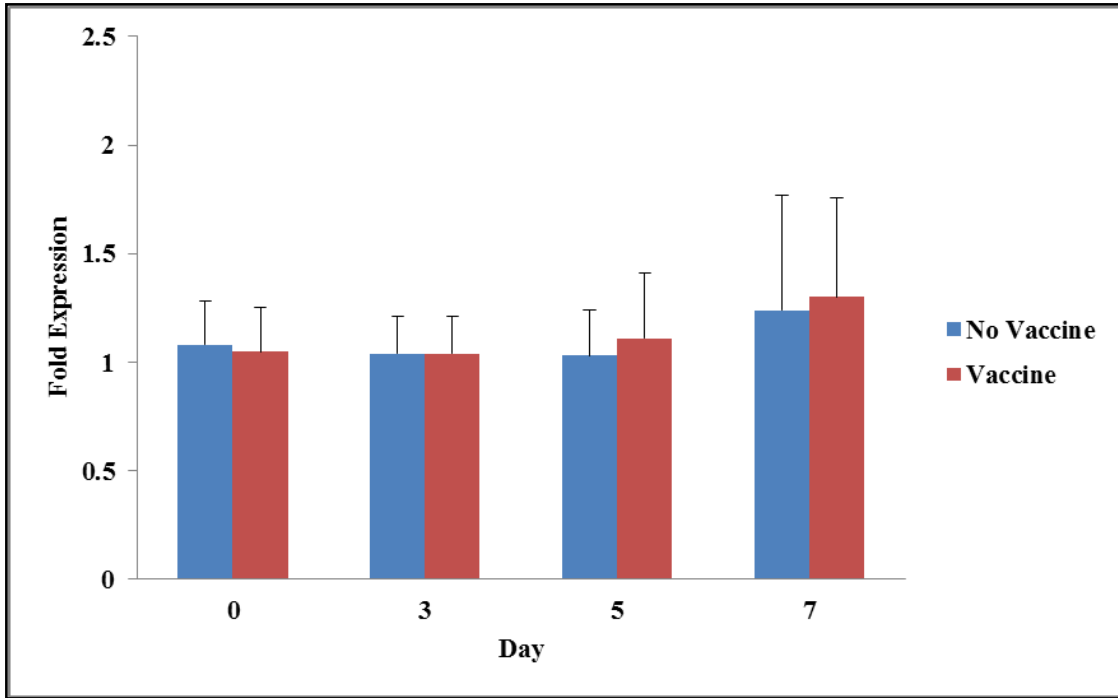


Figure 3.2: Relative fold expression of interleukin-6 (IL-6) in control and Layermune SE vaccinated chicken thrombocytes. The chickens were vaccinated according to manufacturer's protocol with a killed bacterial vaccine for *Salmonella enteritidis* (LAYERMUNE[®] SE, Ceva Biomune, Lenexa, KS) and as control, chickens were injected with sterile phosphate buffer saline (PBS). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using thrombocytes isolated from blood samples collected from vaccinated and unvaccinated chickens on Day 0 before vaccination and on Day 3, 5 and 7 post-vaccination. Fold expression values are calculated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene) according to Pfaffl (2001) using the cycle number obtained from the qRT-PCR and amplification efficiencies of target (IL-6) and housekeeping gene (GAPDH). No

statistical difference was observed in IL-6 expression in thrombocytes collected from vaccinated and control chickens. Error bars show \pm SEM with 4 samples.

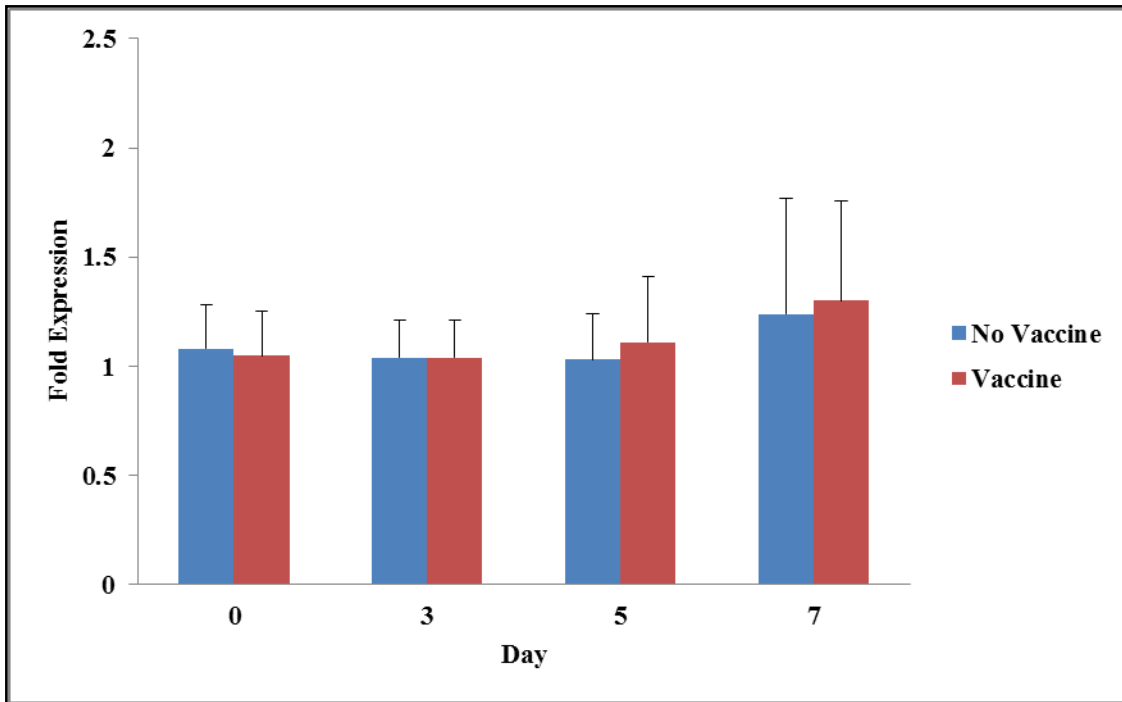


Figure 3.3: Relative fold expression of interleukin-6 (IL-6) in control and Poulvac Maternavac vaccinated chicken thrombocytes. The chickens were vaccinated according to manufacturer's protocol with a killed viral vaccine for IBD and reovirus (Poulvac Maternavac IBD-REO, Zoetis Marietta, Marietta, GA) and as control, chickens were injected with sterile phosphate buffer saline (PBS). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using thrombocytes isolated from blood samples collected from vaccinated and unvaccinated chickens on Day 0 before vaccination and on Day 3, 5 and 7 post-vaccination. Fold expression values are calculated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene) according to Pfaffl (2001) using the cycle number obtained from the qRT-PCR and amplification efficiencies of target (IL-6) and housekeeping gene (GAPDH). No

statistical difference was observed in IL-6 expression in thrombocytes collected from vaccinated and control chickens. Error bars show \pm SEM with 5 samples.

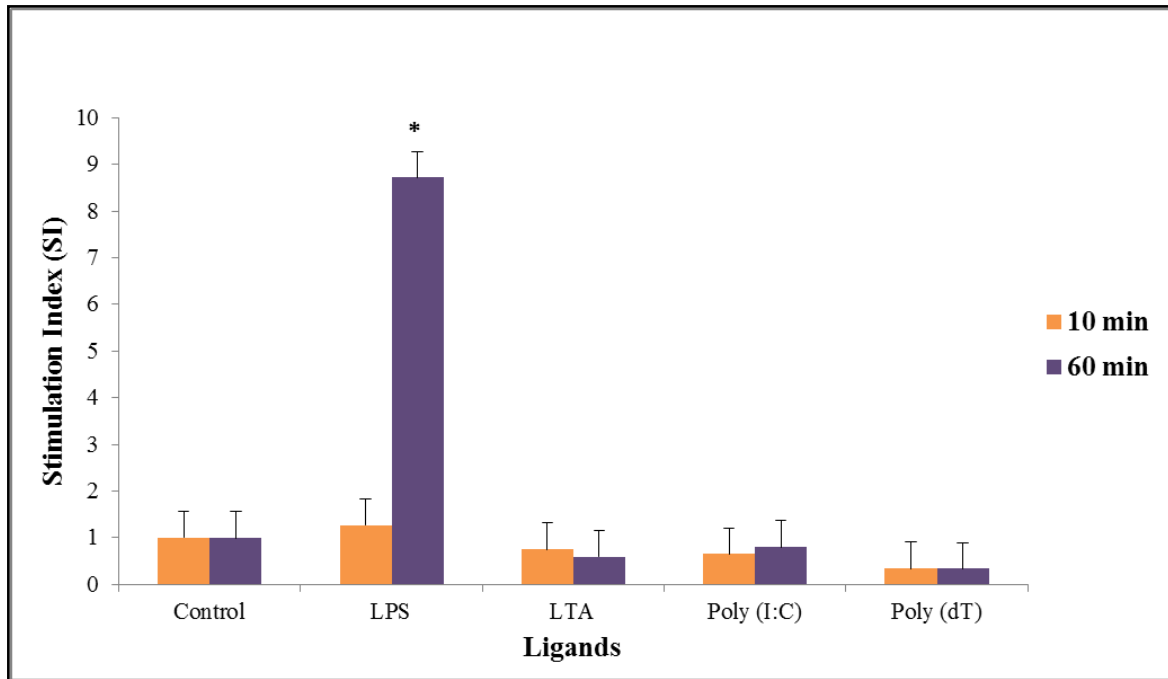


Figure 3.4: Stimulation index of B9 cell bioassay with supernatants from control and stimulated chicken thrombocytes. The bioactivity of IL-6 in supernatants of control and stimulated chicken thrombocytes was assessed according to their ability to promote growth of the IL-6-dependent murine hybridoma B9 cells. Thrombocytes were cultured with lipopolysaccharide (LPS, 1 $\mu\text{g}/\text{mL}$), lipoteichoic acid (LTA, 400 $\mu\text{g}/\text{mL}$), polyinosinic-polycytidylic acid [Poly (I:C), 400 $\mu\text{g}/\text{mL}$] and homopolymer phosphorothioate ODN [Poly(dT), 50 μM] for 10 min and 60 min. LPS stimulation for 60 min released enough IL-6 to show a significant increase in stimulation index compared to any other ligands at 10 min or 60 min. Error bars show $\pm\text{SEM}$ with 5 samples. * Statistically significant difference when compared against respective control ($p \leq 0.05$).

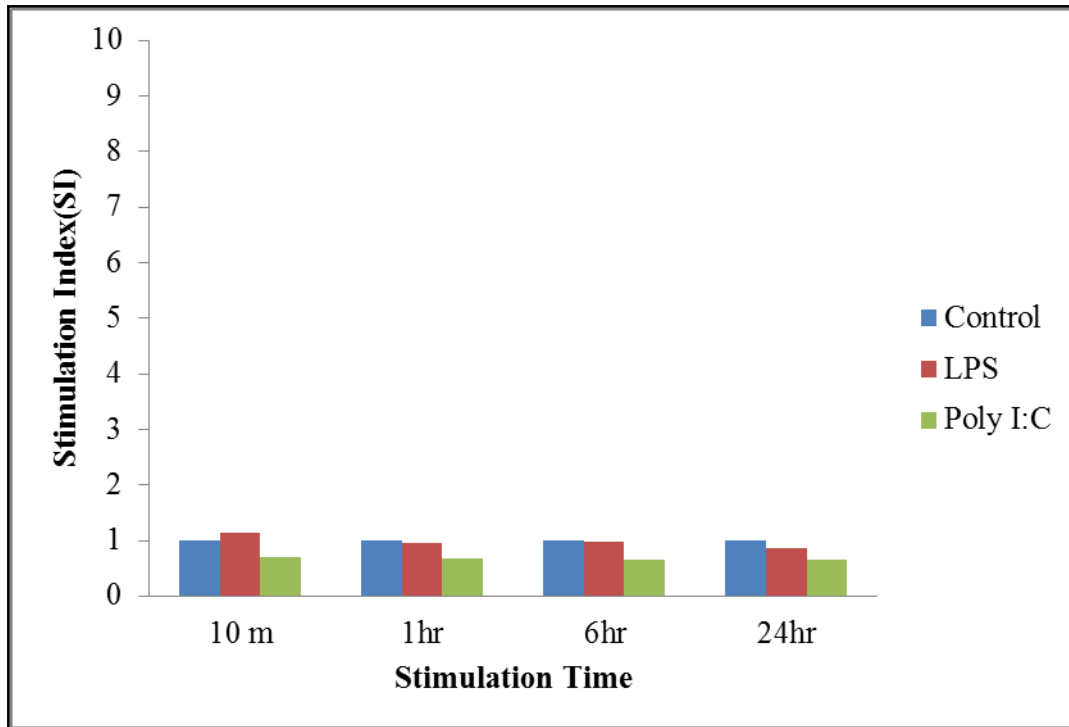


Figure 3.5: Stimulation index of B9 cell bioassay with supernatants from control and stimulated MQ.NCSU macrophages. The bioactivity of IL-6 in supernatants of control and stimulated MQ.NCSU chicken macrophage cells was assessed according to their ability to promote growth of the IL-6-dependent murine hybridoma B9 cells. The MQ.NCSU chicken macrophage cells were cultured with lipopolysaccharide (LPS, 1 $\mu\text{g}/\text{mL}$), and polyinosinic-polycytidylic acid [Poly (I:C), 400 $\mu\text{g}/\text{mL}$] 10 min, 1 hr, 6hr and 24hr. The MQ-NCSU macrophage cell line does not show significant increase or decrease of IL-6 release at 10 min, 1hr, 6hr or 24 hr when stimulated with LPS or Poly (I:C).

that can be systemically released and lead to drastic consequences under circumstances of gram-negative bacterial exposure. This exposure would apparently have to be blood-borne and in excess of the exposure provided through subcutaneous immunization with a bacterial vaccine that may not have sufficient TLR ligand (i.e. LPS) concentration to induce *in vivo* induction of thrombocytes.

3.3.7 Inducible Nitric Oxide Synthases (iNOS) Gene Expression and Nitric Oxide (NO) Release

In vitro treatment of thrombocytes with TLR ligands did not induce a dramatic rise in gene expression of iNOS (Fig. 3.6). C_t values for iNOS gene are around mid-twenties (Fig. 3.7) whereas the C_t values for the housekeeping gene are around low-twenties (not shown). Also, there is not much difference in the delta C_t values (Fig. 3.8), which are the difference in C_t values from the housekeeping gene. Expression of iNOS appears to be constitutive in thrombocytes. Immunization with Layermune SE and Poulvac Maternavac IBD-REO do not induce an effect on gene expression of iNOS post-vaccination on Day 0, 3, 5 and 7 (Fig. 3.9 and 3.10).

We have also examined nitrite (NO_2^-) formation by performing the Griess Reagent Assay that allows spectrophotometric detection of NO_2^- formed by spontaneous oxidation of nitric oxide (NO). Interestingly, *in vitro* treatment of thrombocytes with both viral TLR ligands Poly (I:C) and Poly (dT) induce significant release of NO into culture supernatant at both 10 and 60 min (Fig. 3.11). This is an almost instantaneous event as observed in a preliminary study where NO release was detected in supernatants of

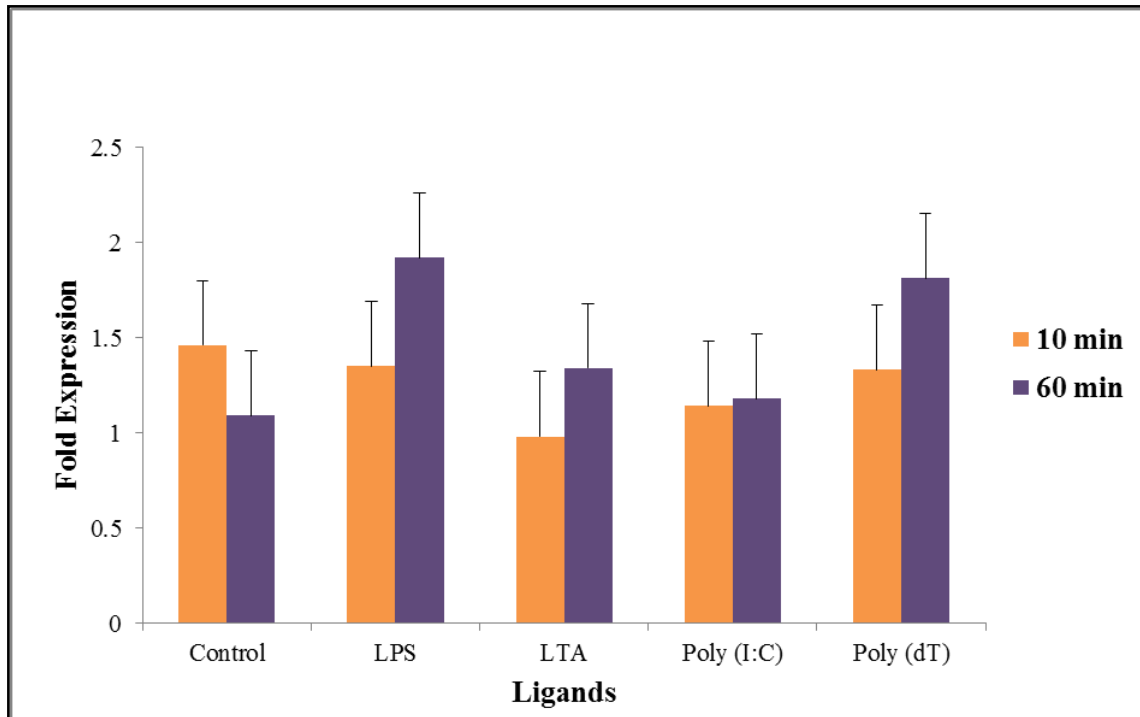


Figure 3.6: Relative fold expression of inducible nitric oxide synthase (iNOS) in control and stimulated chicken thrombocytes. Thrombocytes were stimulated for 10 min and 60 min with lipopolysaccharide (LPS, 1 $\mu\text{g}/\text{mL}$), lipoteichoic acid (LTA, 400 $\mu\text{g}/\text{mL}$), polyinosinic-polycytidylic acid [Poly (I:C), 400 $\mu\text{g}/\text{mL}$] and homopolymer phosphorothioate ODN [Poly(dT), 50 μM]. Fold expression values are calculated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene) according to Pfaffl (2001) using the cycle number obtained from the quantitative real-time polymerase chain reaction and amplification efficiencies of target (iNOS) and housekeeping gene (GAPDH). No significant changes were observed in gene expression of iNOS at any time point due to stimulation with any bacterial or viral ligands. Error bars show \pm SEM with 6 samples.

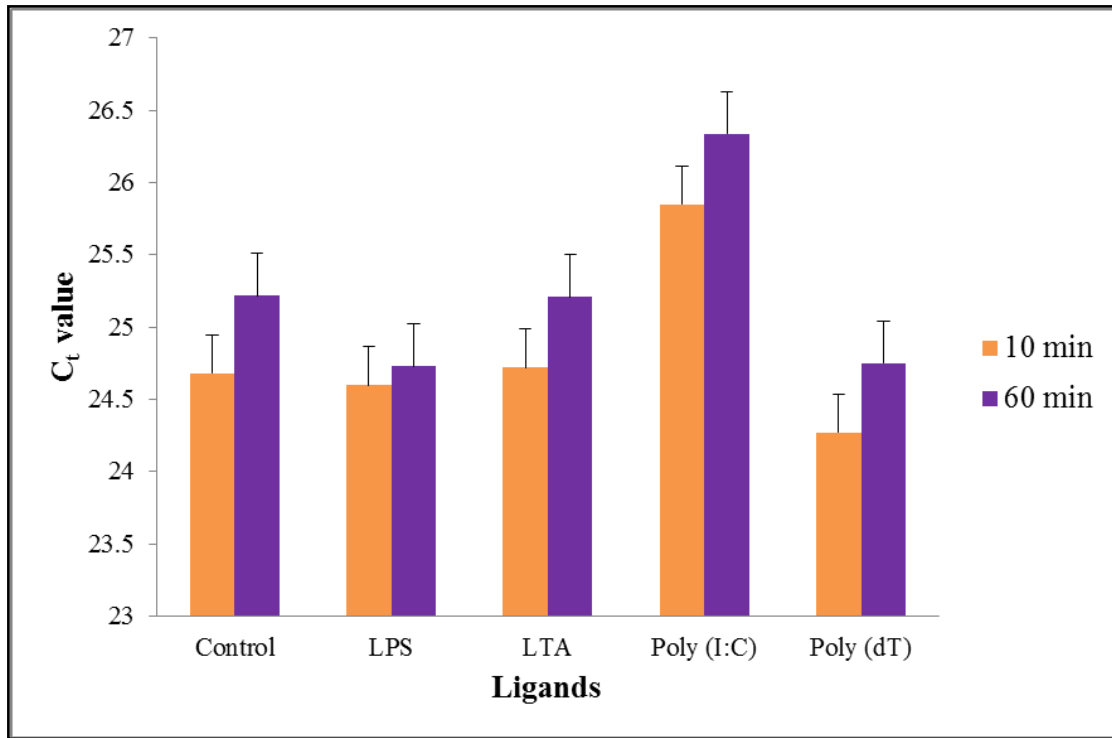


Figure 3.7: Average cycle number (C_t values) of inducible nitric oxide synthase (iNOS) gene expression in control, and stimulated chicken thrombocytes. For quantitative real-time polymerase chain reaction, the cycle number for each sample at which the fluorescence crosses the established threshold is referred as the C_t value. Thrombocytes were stimulated for 10 min and 60 min with lipopolysaccharide (LPS, 1 $\mu\text{g}/\text{mL}$), lipoteichoic acid (LTA, 400 $\mu\text{g}/\text{mL}$), polyinosinic-polycytidylic acid [Poly (I:C), 400 $\mu\text{g}/\text{mL}$] and homopolymer phosphorothioate ODN [Poly(dT), 50 μM]. C_t values for iNOS gene are around mid-twenties whereas the C_t values for the housekeeping gene is around low-twenties (not shown here). Error bars show $\pm\text{SEM}$ with 6 samples.

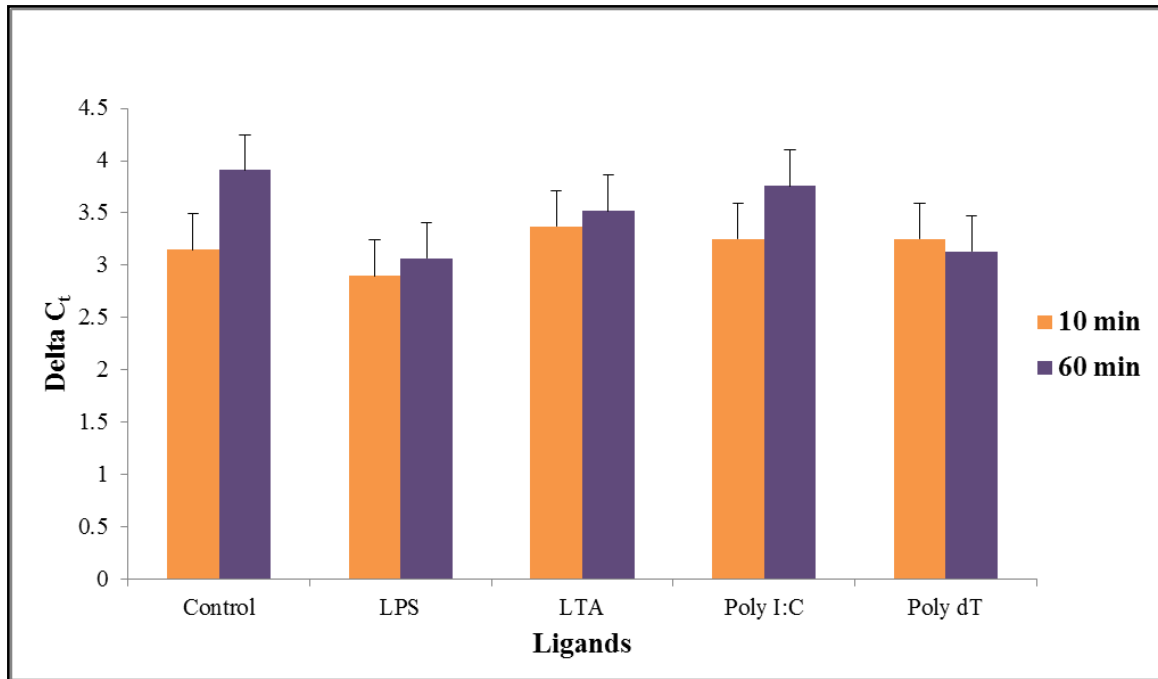


Figure 3.8: Difference in C_t values of control, and stimulated chicken thrombocytes for inducible nitri oxide synthase (iNOS) gene. For quantitative real-time olymerase chain reaction, the cycle number for each sample at which the fluorescence crosses the established threshold is referred as the C_t value. The delta C_t values are difference in C_t between the housekeeping gene, GAPDH and iNOS. The delta C_t values within the treatment did not change significantly among the different treatments compare to housekeeping gene. This indicates that iNOS appears to be constitutively expressed in thrombocytes. Error bars show \pm SEM with 6 samples.

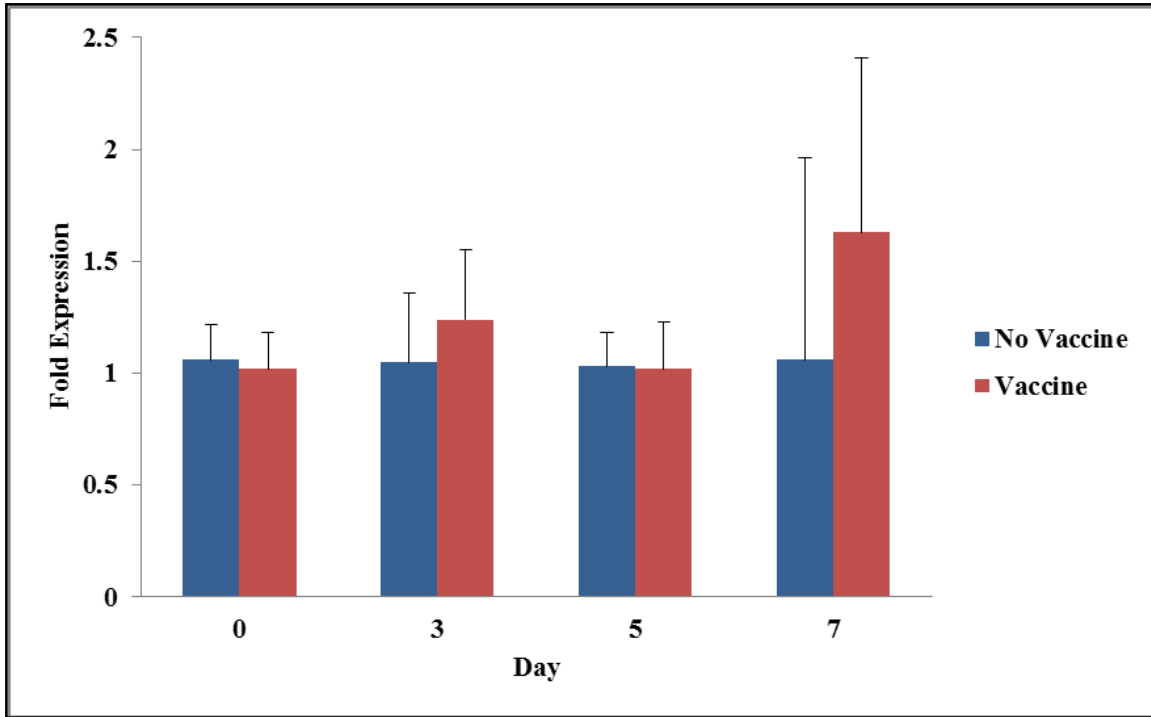


Figure 3.9: Relative fold expression of inducible nitric oxide synthase (iNOS) in control and Layermune SE vaccinated chicken thrombocytes. The chickens were vaccinated according to manufacturer’s protocol with a killed bacterial vaccine for *Salmonella enteritidis* (LAYERMUNE[®] SE, Ceva Biomune, Lenexa, KS) and as control, chickens were injected with sterile phosphate buffer saline (PBS). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using thrombocytes isolated from blood samples collected from vaccinated and unvaccinated chickens on Day 0 before vaccination and on Day 3, 5 and 7 post-vaccination. Fold expression values are calculated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene) according to Pfaffl (2001) using the cycle number obtained from the qRT-PCR and amplification efficiencies of target (iNOS) and housekeeping gene (GAPDH). Relative

fold expression of iNOS in control (no vaccine, PBS injected) and Layermune SE vaccinated chicken thrombocytes show no significant differences. Error bars show \pm SEM with 4 samples.

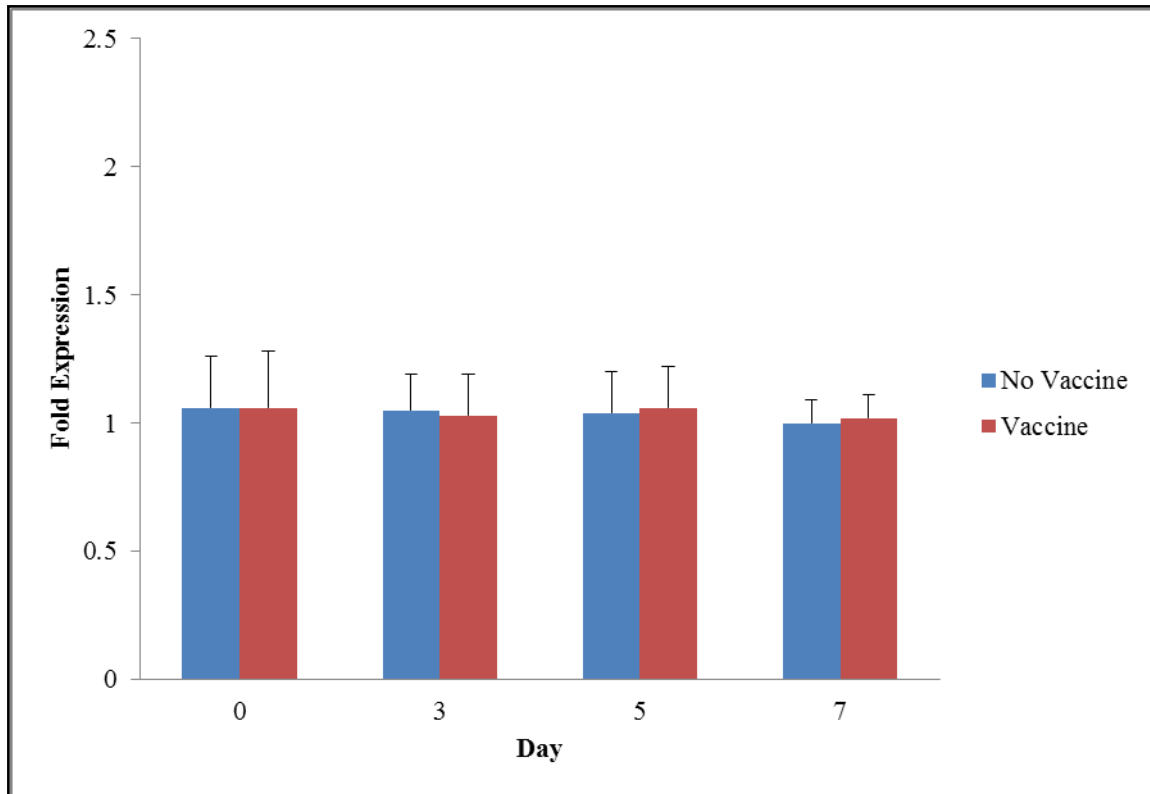


Figure 3.10: Relative fold expression of inducible nitric oxide synthase (iNOS) in control and Poulvac Maternavac IBD-REO vaccinated chicken thrombocytes. The chickens were vaccinated according to manufacturer’s protocol with a killed viral vaccine for IBD and reovirus (Poulvac Maternavac IBD-REO, Zoetis Marietta, Marietta, GA) and as control, chickens were injected with sterile phosphate buffer saline (PBS). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using thrombocytes isolated from blood samples collected from vaccinated and unvaccinated chickens on Day 0 before vaccination and on Day 3, 5 and 7 post-vaccination. Fold expression values are calculated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene) according to Pfaffl (2001) using the cycle number

obtained from the qRT-PCR and amplification efficiencies of target (iNOS) and housekeeping gene (GAPDH). Relative fold expression of iNOS in control (no vaccine, PBS injected) and Poluvac Maternavac vaccinated chicken thrombocytes show no significant differences. Error bars show \pm SEM with 5 samples.

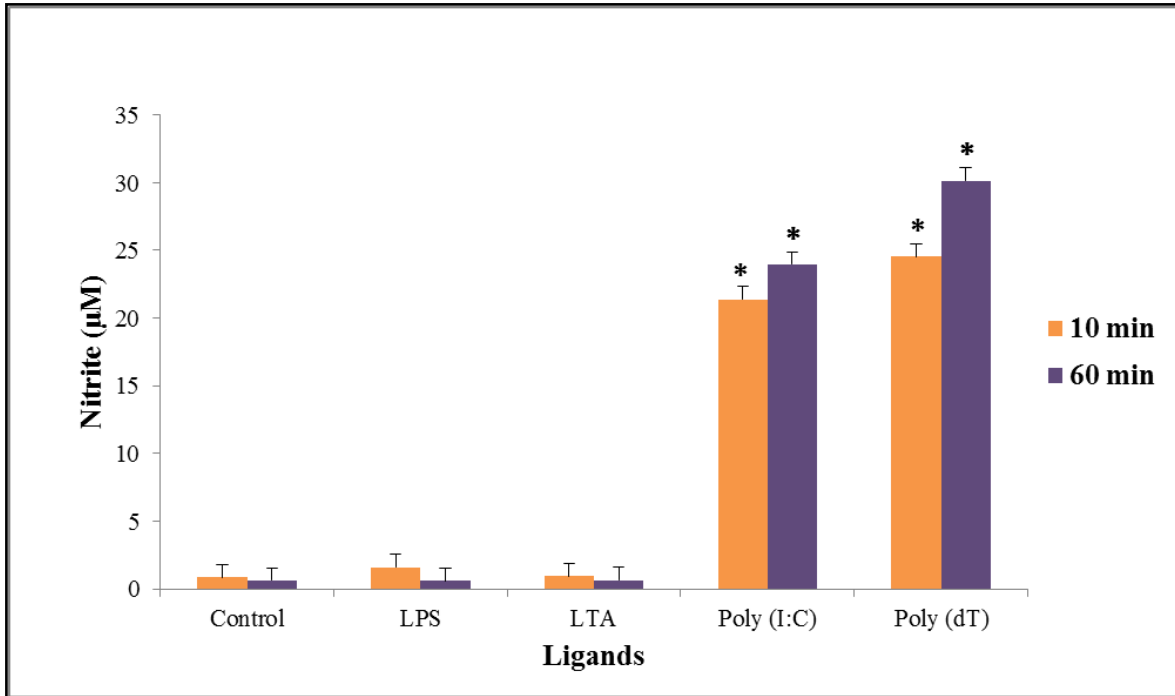


Figure 3.11: Release of nitrite from control and stimulated chicken thrombocytes.

The Griess Reagent System (Promega Corporation, Madison, WI) was used to investigate nitric oxide formation by measuring nitrite release in thrombocyte culture supernatants. Thrombocytes were stimulated for 10 min and 60 min with lipopolysaccharide (LPS, 1 µg/mL), lipoteichoic acid (LTA, 400 µg/mL), polyinosinic-polycytidylic acid [Poly (I:C), 400 µg/mL) and homopolymer phosphorothioate ODN [Poly(dT), 50 µM.. Both viral Toll-like receptor (TLR) ligands Poly (I:C) and Poly (dT)) induce significant release of NO into culture supernatant at both 10 and 60 min whereas bacterial TLR ligands LPS and LTA do not. Error bars show \pm SEM with 6 samples. * Statistically significant difference when compared against respective control ($p \leq 0.05$).

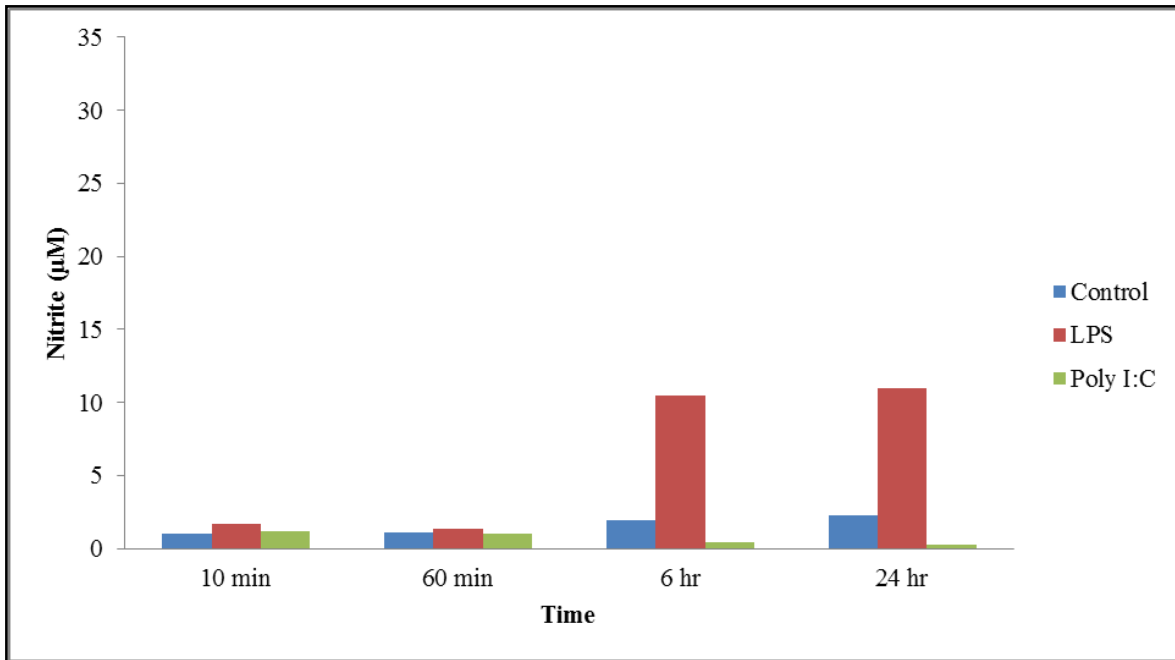


Figure 3.12: Release of nitrite from control and stimulated chicken macrophage cell line. The Griess Reagent System (Promega Corporation, Madison, WI) was used to investigate nitric oxide (NO) formation by measuring nitrite release in MQ.NCSU macrophage cell culture supernatants. The MQ.NCSU macrophages were stimulated for 10 min , 60 min, 6hr and 24 hr with lipopolysaccharide (LPS, 1 µg/mL), and polyinosinic-polycytidylic acid [Poly (I:C), 400 µg/mL). The MQ-NCSU cells when stimulated with LPS released significant NO only after 6 hr whereas Poly (I:C) did not lead to release of much NO even after 24 hr.

thrombocytes treated with Poly (I:C) and immediately sampled for NO detection. While the MQ.NCSU macrophage cells did not respond to TLR ligand treatment with detectable IL-6 release; LPS, but not Poly (I:C), caused release of NO at 6 and 24 hours of culture (Fig. 3.12). It is interesting that the macrophages did respond to LPS treatment with the resultant NO release, while thrombocytes responded to viral ligands and did so in a very short time of exposure to ligands in culture.

3.4 Discussion

We have determined that chicken thrombocytes express genes for TLRs 2, 3, 4, and 7, which is evidence that these cells are programmed for recognition of bacterial and viral ligands that induce TLR pathways to inflammatory products. Here, we have examined inflammatory responses when thrombocytes are stimulated with bacterial [LPS (TLR4 ligand), and LTA (TLR2 ligand)] and viral [Poly (I:C) (TLR3 ligand), and Poly(dT) (TLR 7/8 ligand)] ligands. Chicken thrombocytes showed significant increase in expression of the IL-6 gene by 60 min when stimulated with LPS, which agrees with previous reports from our laboratory (Scott and Owens, 2008). Conversely, Gram-positive ligand (LTA) did not induce very much IL-6 gene expression while the two viral ligands did not affect gene expression at all. The latter bacterial result was unexpected (Fig. 3.1) based on responses of chicken heterophils, which are known avian innate immune cells that have been studied extensively by Kogut's group. Chicken heterophils are functionally similar to mammalian neutrophils and these cells constitutively express several TLRs (Kogut *et al.*, 2005). Bacterial TLR agonists such as LPS, peptidoglycan,

flagellin and synthetic bacterial lipoprotein, all induce an up-regulation of expression of mRNA of the pro-inflammatory cytokines IL-1 β , IL-6, and IL-8 in heterophils. Viral ligands Poly (I:C) and the guanosine analog, loxoribine both induce down-regulation of these cytokine mRNAs in chicken heterophils (Kogut *et al.*, 2005), which makes the thrombocyte viral responses consistent with that of the heterophil.

Thrombocytes from chickens vaccinated with Layermune SE did not show any significant difference in expression of IL-6 on Day 0, 3, 5 and 7 (Fig. 3.2). There could be several reasons for this. The first reason may be that it takes longer for the immune system to respond to the vaccine as opposed to isolated thrombocytes in cell culture. We tested the serum antibody titer against Salmonella, which was negative for most of the samples tested on those days indicating the greater complexity of the entire immune system to generate a positive antibody titer in the blood. The second reason may be contact between purified LPS and thrombocytes *in vitro* is very different from any bacterial LPS of a vaccine containing killed bacteria. If thrombocytes were collected from chickens directly exposed to pathogenic bacteria, IL-6 gene expression may look similar to what we observe for thrombocytes stimulated with LPS in culture. Thrombocytes from chickens vaccinated with Poulvac Maternavac IBD-REO also did not show any significant difference in expression of IL-6 which could be also for similar reasons (Fig. 3.3) or related to the same inability of viral TLR ligands to induce changes in IL-6 gene expression of *in vitro* thrombocytes.

Since mRNA expression for a pro-inflammatory cytokine or an enzyme does not guarantee the expression of the functional protein, we examined the release of the pro-inflammatory mediators. The bioactivity of IL-6 in the supernatant of stimulated chicken thrombocytes was examined using an assay with IL-6-dependent murine hybridoma B9 cells. Results from the B9 assay indicated that by 1 hr, there was a significant amount of active IL-6 in the supernatants of stimulated chicken thrombocytes (Fig. 3.4). The time that it took to release the IL-6 from thrombocytes may be dependant on if the protein needs to be formed or is pre-formed. Based on our preliminary research, IL-6 may be synthesized upon stimulation with LPS, and, therefore, takes up to an hour to be released. In the case of the macrophage cell line (MQ-NCSU), we did not observed IL-6 production even after 24 hr (Fig. 3.5). In a reported study, plasma IL-6 has been found to be elevated at 12 and 24 hr when broiler chicks were administered LPS compared to control chickens (Xie *et al.*, 2000). After 48 hr, LPS-treated chicken plasma had very little IL-6 activity, which was comparable with that of saline-treated chickens. Xie *et al.* (2000) also found changes in plasma IL-6 activity to have a strong correlation with the blood heterophil levels at time points after LPS injection.

We have also examined gene expression of inducible nitric oxide synthase (iNOS), which is involved in the metabolism of L-arginine to L-citrulline and production of NO. Endotoxins or cytokines such as LPS and interferon (IFN)- γ are classic inducers of iNOS mRNA and lead to nitric oxide production in chicken phagocytes within hours of exposure (Hussein and Qureshi, 1997; Qureshi *et al.*, 1994). C_t values for control chicken thrombocytes suggest a constitutive level of iNOS gene expression (Fig. 3.8 and

3.9). Regardless of stimulation with bacterial endotoxins LPS and LTA or synthetic analogs of viral toxins Poly (I:C) and Poly (dT), we did not observe a ligand effect. There was also no significant difference seen in iNOS expression in thrombocytes vaccinated with Layermune SE and Poulvac Maternavac IBD-REO (Fig. 3.9 and 3.10).

However, Hussein and Qureshi (1997) reported that macrophage iNOS expression peaked between 6 and 12 hr after stimulation with LPS. Although expression of iNOS has not been directly examined in chicken heterophils, in-direct measure of iNOS activity has been examined by determination of nitrite release. We detected nitrite release by chicken thrombocytes exposed to both viral analogs soon after treating the cells in culture (Fig. 3.11), whereas bacterial ligand did not result in nitrite production. This is different from what has been reported in the literature where chicken heterophils, monocytes and macrophages (HD11 cell line) have been shown to produce NO in response to several killed bacteria and LPS from *Salmonella enteritidis* (Crippen *et al.*, 2003). Another difference observed here is that very limited stimulation with Poly (I:C) and Poly (dT) resulted in significant nitrite production. When we performed a similar study with a macrophage cell line (MQ-NCSU), we saw LPS stimulation after 6 hr resulted in significant NO, whereas Poly (I:C) did not release much NO even after 24 hr (Fig. 3.12). Crippen *et al.* (2003), have shown it takes 24 hr of LPS stimulation to observe minimal levels of nitrite production from chicken heterophils. The thrombocyte rapid release of nitrite observed could be due to pre-packaged presence of nitrite in chicken thrombocytes, if indeed gene expression of iNOS is constitutive in nature leading to a steady state of NO production in the cell.

Chicken thrombocytes demonstrated differential response when stimulated with bacterial or viral TLR ligands *in vitro*. The responses observed by thrombocytes are similar to other avian innate effector cells. Vaccination with recommended doses of Layermune SE and Poulvac Maternavac vaccine is not sufficient to stimulate chicken thrombocytes to induce expression of pro-inflammatory mediators.

3.5 Acknowledgements

We would like to thank Dr. Kristi Moore Dorsey and Dr. Jon Schaeffer for their generous gift of the Layermune SE vaccine (Ceva Biomune, Lenexa, KS) and the Poulvac Maternavac IBD-REO vaccine (Zoetis Marietta, Marietta, GA) for our experiment here at Clemson University. We also would like to thank Dr. Gwen Slacum, BioChek USA Corp., Millsboro, DE for analyzing the serum antibody titer for the *in vivo* study samples.

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CHAPTER IV

CONCLUSIONS AND FUTURE STUDIES

4.1 Conclusions

Chicken thrombocytes are immune cells. These cells are involved in the innate immune response by producing pro-inflammatory mediators such as IL-6 and NO. Thrombocytes also express genes for several TLR. However, thrombocytes respond differentially to bacterial and viral TLR ligands. *In vitro* treatment of thrombocytes with various TLR ligands demonstrates a definite bacterial effect while no viral effect on gene expression of IL-6. Bacterial ligand, LPS stimulation led to release of significant amounts of active IL-6 in the thrombocyte culture supernatants by 1 hr. *In vitro* treatment of thrombocytes with various TLR ligands also indicated constitutive expression of iNOS gene expression. Although *in vitro* treatment of thrombocytes with both viral TLR ligands Poly (I:C) and Poly (dT) induce significant release of NO into culture supernatant at both 10 and 60 min, no bacterial ligands led to release of NO. *In vivo* treatment by vaccination with the recommended doses of Layermune SE (for *Salmonella enteritidis*) and Poulvac Maternavac vaccine (for infectious bursal disease and reovirus) was not sufficient to stimulate chicken thrombocytes to induce expression of pro-inflammatory mediators.

Chicken thrombocytes are also capable of being involved in the adaptive immune response. It is extremely interesting to find thrombocytes expressing major MHC II which is exclusively limited to true APCs. Significant surface expression of MHC II was

detected in control and TLR ligand stimulated chicken thrombocytes. Moreover, chicken thrombocytes also express co-stimulatory molecules CD40, CD80 and CD86, which provides evidence that thrombocytes could be non-professional APCs.

4.2 Future Studies

We need to understand how thrombocytes are involved in immune responses when exposed to significant challenges by bacterial or viral antigens. We have tried immunizing chickens with a bacterial (Layermune SE) and viral (Poulvac Maternavac IBD-REO) vaccines to examine if that would lead to stimulation of chicken thrombocytes *in vivo*. We used the recommended dose for vaccination. However, we sampled the chickens on Day 3, 5 and 7, which may be too early to see significant antibody responses. We did not observe any difference in gene expression of pro-inflammatory mediators in the thrombocytes. Future experiments can accommodate for these issues by vaccinating chickens with a higher dose of antigens and use of alternative sampling times post-immunization.

In a review, Wu and Kaiser (2011) discussed the importance of understanding both the site and mechanism of antigen presentation in the bird, in order to rationally design and manipulate viral- or -DNA-vectored vaccines, for example by including host genes that would target the vaccines to APCs such as DCs. To understand the role of thrombocytes in antigen presentation, experiments need to be designed to examine the interaction of thrombocytes with other lymphocytes and APCs. Designing such experiments will be an elaborate task. In order to study the T-cell-polarizing abilities of

thrombocytes, naïve CD4⁺ T-cells need to be isolated or sorted, which can be difficult due to lack of chicken specific antibodies and reagents. Thrombocytes culture techniques need to be studied and modified since these cells are usually short-lived in culture. Thrombocytes will need to be stimulated with antigenic peptides such as LPS and more detailed cytokine and chemokine gene expression needs to be investigated. For example IL-12 production leads to T helper cell type 1 (T_H1) polarization, while CC-chemokine ligand 2 (CCL2) favors T_H2 polarization.

Here we have shown that chicken thrombocytes express MHC II (Chapter 2). Expression of MHC II is important as the first signal for stimulation of the T-cell. An antigen-specific signal is mediated through the T-cell receptor (TCR) triggered by MHC class-II-associated peptides processed from pathogens after internalization through specialized PRRs (Kapsenberg, 2003). We have also shown here that thrombocytes express co-stimulatory molecules CD80 and CD86 (Chapter 2), which are involved in the second signal. This signal is mainly mediated by triggering of CD28 on the T cell by CD80 and CD86 that are expressed by APCs like DCs after ligation of PRRs (Kapsenberg, 2003). We have previously observed that LPS stimulation leads to up-regulation of gene expression of IL-12 in chicken thrombocytes (Ferdous *et al.*, 2008) which can be important for the final polarizing signal for T-cell activation. Thus, chicken thrombocytes should have the ability to be involved in antigen presentation and activation of T-cell polarization.

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