ABSTRACT

DIL, NYLA. Molecular regulation of inducible nitric oxide synthase gene in chicken macrophages. (Under the direction of Muquarrab Ahmed Qureshi).

Inducible nitric oxide synthase serves as an important mediator of macrophage immunobiology by regulating the biosynthesis of nitric oxide that in turn mediates several immunological and physiological functions. Previous studies have demonstrated that macrophages from Cornell K-strain chickens ($B^{15}B^{15}$) were hyper-responsive to *Escherichia coli* LPS whereas macrophages from GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) chickens were hypo-responsive for inducible nitric oxide synthase (iNOS) expression and activity. Furthermore, this differential iNOS induction in response to LPS was transcriptionally regulated. The current study was conducted to explore the molecular basis of strain-based differential iNOS induction in chicken macrophages. In the first study, macrophages from K-strain, GB1, GB2 chickens and MQ-NCSU macrophage cell line were exposed to LPS from three sources other than *E.coli* and both iNOS activity and expression were studied. iNOS mRNA and nitrite data showed that these strain-based differences are intrinsic in nature and not limited to the bacterial source of LPS. In addition, when surface expression of LPS-related receptors was studied via flow cytometry, macrophages from the K-strain exhibited higher numbers of LPS signal transducing molecule TLR4 constitutively in comparison to GB2 macrophages. In the second study, inducible expression of LPS related receptors as well as NFκB activation and the involvement of these proteins in iNOS induction was studied. LPS exposure induced significantly higher

numbers of inducible TLR4 and CD14 molecules on K-strain macrophages and a significantly higher corresponding increase in NFκB activation as compared with GB2 macrophages. Furthermore, blocking studies revealed that CD14, TLR4 and NFκB activation were all involved in LPS mediated iNOS induction in chicken macrophages. The objective of the third study was to examine the expression of IL-1β and to explore if LPS-mediated induction of IL-1β modulates iNOS induction in these genetic lines of chickens. Initially GB2 and K strain macrophages produced comparable IL-1β mRNA soon after LPS stimulation. However, IL-1β mRNA persisted up to 9 h only on GB2 macrophages and LPS-inducible IL-1 surface receptor expression was also found to be higher in GB2 than on K-strain macrophages. In addition, blocking of IL-1 receptor by the anti-IL-1 receptor antibody inhibited the LPS mediated iNOS induction by 50% both in K and GB2 chickens indicating that IL-1β does not contribute to differential iNOS induction in these chicken lines. Overall these studies demonstrate that strain based differences in iNOS expression in chickens are mediated via enhanced expression of LPS related receptors.

MOLECULAR REGULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE GENE IN CHICKEN MACROPHAGES

by

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DEDICATION

I dedicate this dissertation to my husband Mamoon Rashid, to my son Haroon Rashid, and to my parents Zarina and Ch. Dil Muhammad Tarar.

With faith in Allah, dedication and hard work, you can accomplish all things**.**

BIOGRAPHY

Nyla Dil was born in Lahore Pakistan, the daughter of Zarina and Ch. Dil Muhammad Tarar. Nyla obtained her high school diploma from Queen Mary College Lahore in 1988. She obtained a D.V.M. with highest rank from College of Veterinary Sciences, Lahore Pakistan in 1993. She continued in the same school and received a M.Sc. in veterinary pathology with honors and distinction in 1995. She was awarded a merit scholarship for higher studies abroad. Nyla joined the Livestock and Dairy Development Department, Pakistan as a veterinary officer and was promoted to assistant director.

In August of 1997, Nyla entered North Carolina State University as a Ph.D. candidate in the interdisciplinary graduate program of Immunology under the direction of M. A. Qureshi. She received the graduate student research paper certificate of excellence from the Poultry Science Association in 2001 for her work examining the macrophage inflammatory gene and LPS receptor expression.

On July 9, 1997, Nyla was married to Mamoon Rashid in Islamabad, Pakistan. Mamoon, who received a D.V.M. and a M.Sc. Hons from College of Veterinary Sciences Lahore Pakistan, began graduate studies in the Animal Science program at North Carolina State University in 1999, and received a MS in Animal Science in August 2001. On July 3, 1999, Nyla and Mamoon were blessed with a beautiful baby, Haroon Rashid.

After completion of her Ph.D. in immunology with a minor in biotechnology, Nyla will pursue post-doctoral research at North Carolina State University under the supervision of Dr. Janice Allen.

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Finally, to my parents, I cannot express the depth of my love and gratitude for their nurturing and belief in me and for teaching me the importance of learning and for making me who I am. To my sisters, Saima Dil and Humera Dil, for their unconditional love and support. None of this would have been possible had it not been for the guidance and inspiration of my sister Saima Dil during my undergraduate studies. To my special angel my son, Haroon Rashid for his innocent understanding and love. I owe you big time Haroon! To my husband, for his love, patience, encouragement, and help every step of the way. His understanding and love means the world to me. I could not have done it without you Mamoon! To my whole family again, thank you for being such magnificent people. I love you all.

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LIST OF ABBREVIATIONS

AEC	abdominal exudates cells
AFB1	Aflatoxin B1
AP-1	activator protein-1
Arg	arginine
BAL	bronchoalveolar lavage cells
BH_4	tetrahydrobiopterin
cDNA	complementary deoxyribonucleic acid
E. coli	*Escherichia coli*
ecNOS	endothelial constitutive nitric oxide synthase
eNOS	endothelial nitric oxide synthase
FITC	fluoroscein isothiocyanate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glycosyl phoshatidylinositol
IFN	interferon
IFN-γ	interferon-gamma
IκB	inhibitor of NFκB
IKK	IκB kinase
IL-1	interleukin-1
IL-1α	interleukin-1 alpha
IL-1 β	interleukin-1 beta

IL-1R	interleukin-1 receptor
$IL\text{-}1R_I$	interleukin-1 receptor type I
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
IRAK	IL-1 receptor associated kinase
IRF	interferon regulatory factor
Jak	Janus kinase
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
mAbs	monoclonal antibodies
macNOS	macrophage nitric oxide synthase
MAPK	mitogen activated protein kinase
MCP-1	macrophage chemotactic protein-1
mNOS	macrophage nitric oxide synthase
MQ	macrophage
mRNA	messenger ribonucleic acid
MTT	3-(5,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ncNOS	neuronal constitutive nitric oxide synthase
NCSU	North Carolina State University
NFκB	nuclear factor kappa B
NIK	NFκB inducing kinase

NLS	nuclear localization signal
NO	nitric oxide
NO_2	nitrite
NO_3	nitrate
NOS	nitric oxide synthase
NOS1	neuronal nitric oxide synthase
NOS2	macrophage nitric oxide synthase
NOS3	endothelial nitric oxide synthase
nNOS	neuronal nitric oxide synthase
OD	optical density
PAMPs	pathogen associated molecular patterns
PBS	phosphate buffer saline
PE	phycoerythrin
PMBCs	peripheral mononuclear blood cells
RHD	Rel-homology domain
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SF	*Shigella flexneri*
SIIK	Serine-threonine innate immunity kinase
SM	*Serratia marcescens*
ST	*Salmonella typhimurium*
STAT	signal transducers and activators of transcription
TIR domain	toll / interleukin-1 receptor homology domain

TLR	toll like receptor
TLR2	toll like receptor-2
TLR4	toll like receptor-4
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor- alpha
TRAF6	TNF-receptor associated kinase

INTRODUCTION

Macrophages respond to external stimuli by inducing the expression of various cytokines, adhesion molecules, and enzymes that modulate an array of immune functions. Inducible nitric oxide synthase (iNOS) is one such enzyme that catalyses the biosynthesis of nitric oxide (NO) which in turn mediates several immunological and physiological processes. The enzyme nitric-oxide synthase (NOS) catalyzes the biosynthesis of free radical nitric oxide as a by-product of an oxidative reaction using L-arginine as a substrate to make L-citruline. NO is a highly reactive, low molecular weight, short lived cytotoxic entity and is converted to nitrite and nitrate that are more stable products. There are three known isoforms of NOS: two constitutive forms, neuronal NOS and endothelial NOS, and an inducible form (iNOS) that is typically not present in the cells until the cell is exposed to immune or inflammatory stimulants. iNOS was first cloned from macrophages. Nitric oxide expression by iNOS is an important host-defense mechanism against microbial pathogens in mononuclear phagocytes.

Previous studies have demonstrated a genetic influence on the expression and activity of iNOS in chickens when macrophages from different genetic sources were stimulated with LPS from *E. coli*. These genetic differences characterized macrophages from Cornell K-strain ($B^{15}B^{15}$) and MQ-NCSU, a broiler macrophage cell line, as hyper-responders and macrophages from GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) chicken strains as hypo-responders in terms of iNOS expression and activity. While iNOS mRNA stability was found to be comparable between the hyper and hypo responder macrophages, the

differences were found in the level of transcriptional activity in macrophages from different genetic sources.

The current study was conducted to enhance our understanding of the molecular mechanisms governing the LPS-mediated differential iNOS induction in these genetic lines of chickens. Since the initial categorization of iNOS hypo or hyper responsiveness in macrophages was established by using LPS from *E. coli***,** the first objective of the current study was to determine whether these genetic differences were due to the host-based intrinsic mechanisms or are dependent upon a specific bacterial source (i.e., *E. coli* versus *Shigella flexneri, Serratia marcescens, and Salmonella typhimurium*). If these differences were found to be host-based regardless of the source of LPS, then the next objective would be to examine the role of LPS binding (i.e., CD14) and signaling molecules such as Toll like receptor (TLR) 2 and 4 in mediating iNOS expression and activity differences in macrophages from various genetic sources. Since the common pathway leading to the signal transduction for gene transcription works via NFκB activation and binding to the promoter region of the gene of interest, it was considered important to examine the NFκB binding potential after LPS stimulation for the purpose of iNOS expression and activity. Since IL-1β is another macrophage mediated, LPS inducible, potent inducer of iNOS, its expression and role in LPS-based iNOS expression and activity was examined in hypo and hyper-responder macrophages.

Therefore, the overall goal of this thesis was to examine the bacterial and host-based molecular mechanism(s), which may be responsible for imparting hypo or hyper responsiveness in macrophages from different genetic strains of chickens.

LITERATURE REVIEW

MACROPHAGES

Macrophages are extraordinarily versatile cells. They are found in some form in practically every tissue in the body, where they participate in an array of biological processes, ranging from development, to bone remodeling and wound healing. However it is as guardians of the immune system that macrophages express their full repertoire of functions; they detect, ingest and destroy infectious agents; they initiate T-cell responses by antigen presentation, and they act as effectors cells for both humoral and cell mediated responses. The immune response leads to the establishment of protective immunity (Aderem and Underhill, 1999). It is, therefore, important for the macrophage to discriminate the large numbers of potential pathogens using a restricted number of germ-line encoded receptors. This problem is compounded by the ability of microbes to mutate. This task has been accomplished by the evolution of a variety of receptors that recognize conserved motifs on pathogens that are not found in higher eukaryotes. These motifs, called pathogen-associated molecular patterns (PAMPs), have essential roles in the biology of the pathogen and are therefore not subject to high mutation rates (Janeway and Medzhitov, 1998). PAMPs are recognized and bound by pattern-recognition receptors (Janeway and Medzhitov, 1998). PAMPs include formylated peptides, and diverse bacterial cell wall components, such as lipopolysaccharide (LPS), lipopeptides, peptidoglycans, and teichoic acids (Janeway and Medzhitov, 1998). Two major classes of pattern recognition receptors exist in macrophages; those that mediate phagocytosis and

those that lead to the activation of pro inflammatoy pathways (Aderem and Underhill, 1999; Janeway and Medzhitov, 1998).

LIPOPOLYSACCHARIDES

The bacterial endotoxin lipopolysaccharide (LPS) is the major constituent of Gram-negative bacteria that activates macrophages (Ulevitch and Tobias, 1999). LPS consists of three covalently linked domains (Fig. 1). Lipid A (endotoxin), which functions as the hydrophobic anchor for LPS in the outer membrane and is the bioactive component responsible for some of the pathophysiology, associated with severe Gram-negative infections. The core region, a phosphorylated non-repeating oligosaccharide required for the function of the outer membrane as a barrier to the antibiotics. And the O-antigen polymer, an immunogenic repeating oligosaccharide of 1 to 40 units that varies greatly from strain to strain (Raetz, 1990).

LPS induction of gene expression. LPS stimulation of monocytes and macrophages induces many genes that express proinflammtory mediators, such as cytokines (for example TNF-α, IL-1, IL-6, GM-CSF), chemokines (IL-8, MCP-1), and enzymes such as iNOS. LPS responsive *cis*-acting DNA promoter elements have been characterized in the 5′ flanking region of many of these genes (Sweet and Hume, 1996). The transcription factors that bind to these response elements include NFκB protein, AP-1 proteins, and NF-IL6. These transcription factors are activated by phosphorylation, which permits the rapid induction of gene expression in response to LPS. NFκB appears

to be required for the induction of all LPS-inducible genes in monocytes (Muller et al., 1993

LPS RECEPTOR COMPLEX

CD14. CD14 is a myeloid marker antigen (Goyert et. al., 1986). The role of CD14 in LPS activation of monocytes and macrophages has been demonstrated both biochemically and genetically. For example, over expression of human CD14 in transgenic mice renders these mice hypersensitive to LPS, as evidenced by their increased susceptibility to endotoxin shock (Ferrero et al., 1993). In contrast, CD14 deficient mice are hypo-responsive to LPS and are at least 10 times less sensitive to LPS than normal mice (Haziot et al., 1995). Although CD14 is known to bind LPS, this pattern recognition receptor is anchored into the cell membrane by a glycosyl phosphatidylinositol (GPI) linkage, which suggests that it would have little signaling capacity. There is now clear evidence that Toll like receptors (TLRs) mediate the response to LPS.

Chicken CD14. Previous studies have described the expression of CD14 on the surface of chicken monocytes, macrophages and osteoclasts using monoclonal antibodies (mAbs) against human CD14 (Athanasou et al., 1992). Recently, we quantitated the expression of CD14 on the surface of chicken macrophges using anti-human CD14 mAbs via flow cytometry (Dil and Qureshi, 2002).

Toll like receptor. In Drosophila, a family of proteins known as the Toll family is central to innate defense, which involves the recognition of bacteria and fungi, and the

induction of antimicrobial peptides that kill invading organisms. Recently mammalian homologs of Toll, designated as Toll like receptors (TLRs) have been reported. Conserved throughout evolution mammalian TLRs are thought to be part of an innate response against microbial pathogens (Aderem and Ulevitch, 2000).

Toll-like receptor-2. Yang et al. (1998), suggested that TLR2 was the LPS signaling receptors in mammals. TLR2 signaling depended on lipopolysaccharide binding protein (LBP) and was enhanced by CD14. However, a criticism of both studies is that they relied on results from LPS stimulation of TLR2 over expressed in 293 human embryonic kidney cells.

Chicken TLR2. Boyd et al. (2001) have reported the identification and mapping of Chicken *TLR2*. *TLR2* lies on chicken chromosome number 4. The mapping data position *TLR2* in a conserved chromosomal segment of at least four loci which is conserved in chicken and mouse and shares homology with the q26-q35 region of the human chromosome 4. Furthermore, another research group (Fukui et al., 2001) has recently reported cloning of TLR from a chicken bursa cDNA library based on the consensus sequences of mouse and Drosophila Toll. This chicken TLR showed greatest homology to human TLR2 and like human TLR2 serves as signaling apparatus for mycoplasma.

TLR4. Other studies used a genetic approach to study the LPS signaling receptor. These studies made use of two strains of mice, C3H/HeJ and C57BL/10ScCr, which contained a mutation in the *Lps* gene that made them hypo-responsive to LPS. It was determined that defective LPS signaling in these mouse strains was due to mutation

in TLR4, indicating that the TLR4 was the LPS signaling receptor (Poltorak et al., 1998; Qureshi et al., 1999). Also, macrophages from mice with a targeted inactivation of the TLR4 gene did not respond to LPS (Hoshino et al., 1999), which confirmed the identity of TLR4 as the LPS signaling receptor. Moreover, comparison of the responses in TLR2 and TLR4-deficient mice demonstrated that TLR2 and TLR4 recognize cell wall components from Gram-positive and Gram-negative bacteria, respectively (Takeuchi, et al., 1999).

Chicken TLR4. We have recently reported the expression of TLR4 on the surface of chicken macrophages (Dil and Qureshi, 2002) and also that TLR4 signaling is involved in LPS mediated iNOS induction. At present there are no other reports of TLR4 expression in chicken.

TLR4 and iNOS induction. Recent studies have described a relationship between TLR4 and iNOS induction. Ohashi et al., (2000) have shown in bone marrow-derived macrophages from endotoxin resistant HeJ and endotoxin-responsive C3H/HeN mice that nitric oxide formation is dependent on a functional Tlr4 (gene encoding TLR4 protein). Also, Tlr4 has been shown to be an important determinant of iNOS expression in mice bronchoalveolar lavage (BAL) cells (Kleeberger et al., 2001).

NFκB AND IκB PROTEINS

The transcription factor NFκB, originally identified as a required factor for B-cell specific gene expression (Sen, 1986), is a pleiotropic transcription factor that serves as a critical regulator of inducible expression of many genes (for example iNOS, IL-1, IL-6,

IL-8). NFκB exists in the cytoplasm of the majority of cell types as homodimers or heterodimers of a family of structurally related proteins (Baldwin, 1996; Kopp and Ghosh, 1995). Each member of this family contains a conserved amino terminal region called the Rel-homology domain (RHD) within which lie the DNA binding and dimerization domains and the nuclear localization signal (NLS). To date, five proteins belonging to the NFκB family have been identified in mammalian cells: RelA (also known as p65), c-Rel, RelB, NFκB1 (p50/p105), and NFκB2 (p52/p100). Rel-A, c-Rel, and RelB are produced as transcriptionally active proteins, whereas NFκB1 and NFκB2 are synthesized as longer precursor molecules of 105 kDa and 100 kDa respectively, that are further processed to smaller, transcriptionally active forms. The classical NFκB dimer contains RelA and NFκB 1 (Baeuerle and Baltimore, 1996; May and Ghosh, 1998).

NFκB exists in the cytoplasm in an inactive form associated with inhibitory proteins termed IκBs (May and Ghosh, 1998). IκB regulates the DNA binding and sub cellular localization of Rel-NFκB proteins by masking a nuclear localization signal (NLS) near the carboxyl terminal of RHD (Henkel et al., 1992).

NFκB and iNOS. NFκB has been shown to be a required transcription factor for iNOS induction in both rodent (Xie et al., 1994) and chicken macrophages (Lin et al., 1996). Using a murine macrophage model, Goldring et al. (1998) have shown that LPS hypo-responsiveness in terms of iNOS expression and activity is mediated via the altered composition of NFκB binding to the κB sites in the promoter region of iNOS gene.

TLR4 mediated NFκB activation pathway. Signaling downstream of TLR4 is mediated by several protein complexes (Fig. 2). The first complex includes the activated TLR, an adapter molecule such as MyD88, and a SIIK (serine-threonine innate immunity kinase) such as IRAK (IL-1 receptor associated kinase). The activated TLR4 complex, in turn, activates the IKK (IκB kinase) in a step mediated by TRAF6 (TNF-receptor associated factor 6). The IKK complex includes NIK (NFκB inducing kinase), which phosphorylates and activates IKKα/β. Subsequently, activated IKKα/β phosphorylates target serines in the amino-terminal domain of IκB. Once phosphorylated, the IκB-NFκB complex become associated with an ubiquitin ligase. In the last step of this signaling cascade, phosphorylated and ubiquitinated IκB, which is still associated with NFκB in the cytoplasm, is selectively degraded by the 26S proteosome. Once IκB is degraded, the NLS of NFκB is exposed and NFκB moves to the nucleus, where it can activate target genes (Anderson, 2000).

INTERLEUKIN-1

Interleukin-1 (IL-1) is a cytokine, synthesized by activated mononuclear phagocytes that have been stimulated by LPS or by interaction with $CD4^+$ T cells, and mediates inflammation sharing many properties in common with tumor necrosis factors (TNF). IL-1 is comprised of two principal polypeptides of 17kD each with isoelectric points of 5.0 and 7.0. They are designated IL-1α and IL-1β respectively. They have the same biological activities and bind to the same receptor on cell surfaces. They are derived

by proteolytic cleavage of 33kD precursor molecules. IL-1α acts as a membrane associated substance, whereas IL-1β is found free in circulation.

IL-1 Receptor. Mammalian IL-1 binds to two receptors called type-I and type-II. These receptors have little affinity for other proinflammatory cytokines. The type-I IL receptor (IL-$1R_I$) has a cytoplasmic tail responsible for signal transduction. The type-II receptor does not transmit a signal to the cytoplasm, and serves as a decoy receptor that down regulates the inflammatory responses (Auron, 1998).

Chicken IL-1 and IL-1 Receptor. Chicken IL-1, like mammalian IL-1, has been described to induce the proliferation of thymic cells exposed to low levels of mitogen (Klasing and Peng, 1990; Bombara and Taylor, 1991). Chicken IL-1β (Weining et al., 1998) and IL-1 receptor have been molecularly characterized (Guida et al., 1992). Unlike mammalian IL-1, in chicken only IL-1β and the type-I IL-1 receptor (IL-$1R_I$) have been described. The predicted chicken IL-1 R_I protein sequence shares 61 and 64% homology with murine and human IL-1 R_I, respectively.

IL-1 and iNOS. Interleukine-1β is the critical cytokine inducing iNOS gene expression in several cell types (Chao et al., 1997; Jaimes et al., 1997; Wong et al., 1996). Wong et al., (1996) showed that IL-1β induces the transcriptional activation of iNOS expression in cultured rat pulmonary artery smooth muscle cells. Furthermore, previous studies have shown that during systemic infection, IL-1β binds to IL-$1R_I$ and induces the expression of iNOS in rat brain vasculature and perivascular areas (Wong et al., 1996).

NITRIC OXIDE SYNTHASE

Nitric oxide synthases (NOS) is a group of evolutionarily conserved cytosolic or membrane bound isoenzymes that convert the amino acid L-arginine to citrulline and nitric oxide (NO) in mammalian and nonmammalian animals as well as in plants (Stuehr, 1999; Bogdan, 2000). Currently, there are three major NOS isoforms known, that are named after the prototypic cell type from which the respective isoform was first isolated as well as after the main characteristic of their regulation.

Neuronal NOS and Endothelial NOS. The neuronal NOS (ncNOS or NOS1) and the endothelial NOS (ecNOS or NOS3) are constitutively expressed. They exist in the cell as preformed monomers, which dimerize and gain activity upon Ca^{2+} influx and binding of calmodulin (MacMicking et al., 1997; Bogdan, 2000).

Inducible NOS. The macrophage NOS (macNOS, iNOS or NOS2), in contrast, is an inducible isoform that is absent in strictly resting cells, is strongly induced by cytokines and other immunological stimuli, and is regulated on transcriptional and post-transcriptional levels involving a number of signal transduction pathways and molecules: Jak1/Stat1α/IRF-1; IκB/NF-κB; mitogen-activated protein kinases (MAPK); protein kinase C; phosphotidylinositol-3 kinase; protein tyrosine phosphatases; and protein phosphatases 1 and 2A (MacMicking et al.,1997; Bogdan, 2000).

The various NOS isoforms are also subject to regulation by the availability of L-arginine and BH_4, which are required for the formation of active NOS dimers. Virtually every nucleated cell of the immune system has been described to express one or several isoforms of NOS and most probably every type of mammalian cell is capable of

generating NO. NOS activity (NOS1, NOS2) is found in the cytosol (attached to cytoskeletal proteins) but can also be targeted to plasmalemmal caveolae (NOS3), or localized in vesicles (NOS2), or in mitochondria (Tatoyan and Giulivi, 1998).

Nitric Oxide. NO is a highly reactive, low molecular weight, short-lived free radical and is converted to nitrite and nitrate that are more stable products (Stuehr and Marletta, 1987; Nathan, 1992). NO performs many diverse and significant biological functions. In the nervous system, it acts as a novel neurotransmitter that is synthesized as needed and is not stored in synaptic vesicles. Nitric oxide does not interact with receptors on the surface of neurons but targets redox centers within neighboring neurons (Bredt and Snyder, 1992). In the vascular system, NO acts as the endothelium-derived vasodilator and thus mediates blood vessel relaxation and blood pressure (Ignarro, 1990). It can also inhibit platelet aggregation and adhesion in mammalian systems (Radomski and Moncada, 1993). In the immune system, NO is synthesized by activated macrophages and acts as a cytotoxic and tumoricidal agent (Hibbs, 1991; Marletta 1993). NO also mediates important functions in other tissues and organs such as the gastrointestinal tract (Stark and Szurszewski, 1992), liver (Muriel, 2000), pancreas (Corbett et al., 1994), kidney (Cattell and Cook, 1993), and the reproductive system.

Chicken iNOS. Unlike mammals, chickens do not use the urea cycle, and thus are dependent on exogenous sources for the NOS substrate arginine (Sung et al., 1991). Chicken iNOS have been molecularly characterized (Lin et al., 1996). The predicted iNOS protein sequence showed 66.6%, 70.4%, 54.2% and 48.7% sequence homology to mouse and human inducible NOS and to rat brain cNOS and bovine endothelium cNOS,

respectively. Also, chicken iNOS mRNA size (4.5kb) is similar to mammalian iNOS. An endotoxin regulatory region and NFκB binding site was located within 300bp of transcription start site. Chicken iNOS gene has been shown to be differentially regulated at the transcription level in macrophages from different genetic sources (Hussain and Qureshi, 1997, 1998). Macrophages from the MQ-NCSU macrophage cell line (established from a meat-type chicken) as well as from Cornell K-strain white Leghorn ($B^{15}B^{15}$) chickens expressed higher levels of iNOS mRNA as well as higher corresponding nitrite levels in the culture supernatants as compared with the macrophages from GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) chickens. Recently, Dil and Qureshi (2002) have reported that these strain-based differences in iNOS induction are intrinsic and these genetic lines of chicken also differ in their expression of LPS signal transducing molecule TLR4 and implied that differential iNOS induction was due to a differential TLR4 mediated LPS signal.

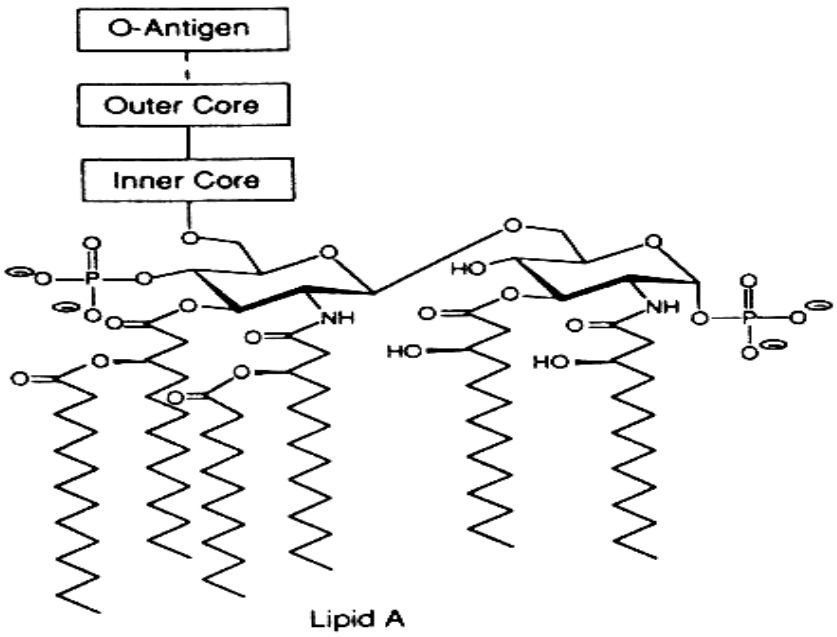
iNOS and Host Resistance. iNOS and NO have been described in a number of avian and mammalian diseases. For example, NO has been shown to be involved in inhibiting *in vitro* and *in vivo* replication of chicken tumorigenic virus, such as Marek's disease virus (Schat et al., 2001). Marek's disease is an important lypmho-proliferative disease of chicken. The Cornell K-strain genotype, which is a hyper-responder to iNOS expression and activity, is also naturally resistant to Marek's disease (Hutt and Cole, 1957; Gavora et al., 1977). Coccidiosis is yet another chicken disease producing serious economic losses due to high morbidity and mortality rate. Recent studies have reported up regulation of iNOS during infection with *Eimeria* (Laurent et al., 2001; Qureshi et al.,

2000). There was a 200 fold up-regulation of iNOS during *E. tenella* infection. iNOS gene was also up regulated during *E. maxima* infection but at a lower magnitude. Aflatoxin B1 (AFB1) is known to impair specific and non-specific immune responses. Moon et al. (1998) have shown that AFB1 reduces the LPS mediated induction of iNOS expression and activity in murine peritoneal macrophages. Furthermore, vaccinia virus morphogenesis was inhibited by interferon (IFN) induced iNOS (Esteban and Patino, 2000). Also, iNOS have been implicated in the intestinal pathology during intestinal nematodal infections (Garside et al., 2000).

In cell lines and in inbred mouse strains, resistance to microbial growth is often associated with expression of iNOS. For example, replication of ectromelia virus was restricted in IFNγ treated nitric oxide producing RAW264.7 mouse macrophage-like cells or primary peritoneal macrophages from an innately resistant strain (C57BL/6), yet it was unhindered in fibroblasts or epithelial cell in which IFNγ failed to induce iNOS (Karupiah et al., 1993). Similarly, the fungus *H. capsulatum* grew better in iNOS deficient P388D1 macrophage like cells than in RAW 264.7 (Lane et al., 1994). The protozoan pathogen *Leishmania majora* was more effectively killed by cytokine stimulated macrophages from mouse strains resistant to rather than susceptible to cutaneous leishmaniasis, a distinction reflected in their respective expression of iNOS (Liew, et al., 1991).

CURRENT STUDY

Nitric oxide has been shown to affect severity of disease in numerous animal models. NO is one of the smallest biological mediators and it can influence pathophysiology either as a cytotoxic component of nonspecific immunity or as a mediator that stimulates the signal transduction pathways. Macrophage type of inducible nitric oxide synthase mediates most of the pathophysiological functions of nitric oxide. In the past few years research groups working with mammalian systems have paid considerable attention to this small molecule. On the contrary, there are only a few scientific reports available on iNOS gene research in chickens. Previous studies have shown that macrophages from. Cornell K-strain chickens ($B^{15}B^{15}$) were hyper-responsive to *Escherichia coli* (*E. coli*) lipopolysaccharide (LPS) whereas macrophages from GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) chickens were hypo-responsive for inducible nitric oxide synthase (iNOS) expression and activity. The current study was designed to uncover the molecular basis of such genetic differences in chickens. These findings will provide new insights on the regulation of host defense mechanisms in chickens. Better understanding of the chicken immune system can be utilized to improve overall health status of poultry.



(From: Raetz, C. R. H., Ann. Rev. Biochem., 59, 1990)

Fig. 1. General structure of LPS. LPS of Enterobacteriaceae consists of three covalently linked domains: the lipid A moiety serves as the hydrophobic anchor for LPS in the outer most bacterial membranes and confers endotoxic properties to the LPS, the core region is the phosphorylated non-repeating oligosaccharide that links lipid A to the hypervariable O-antigen polymer.

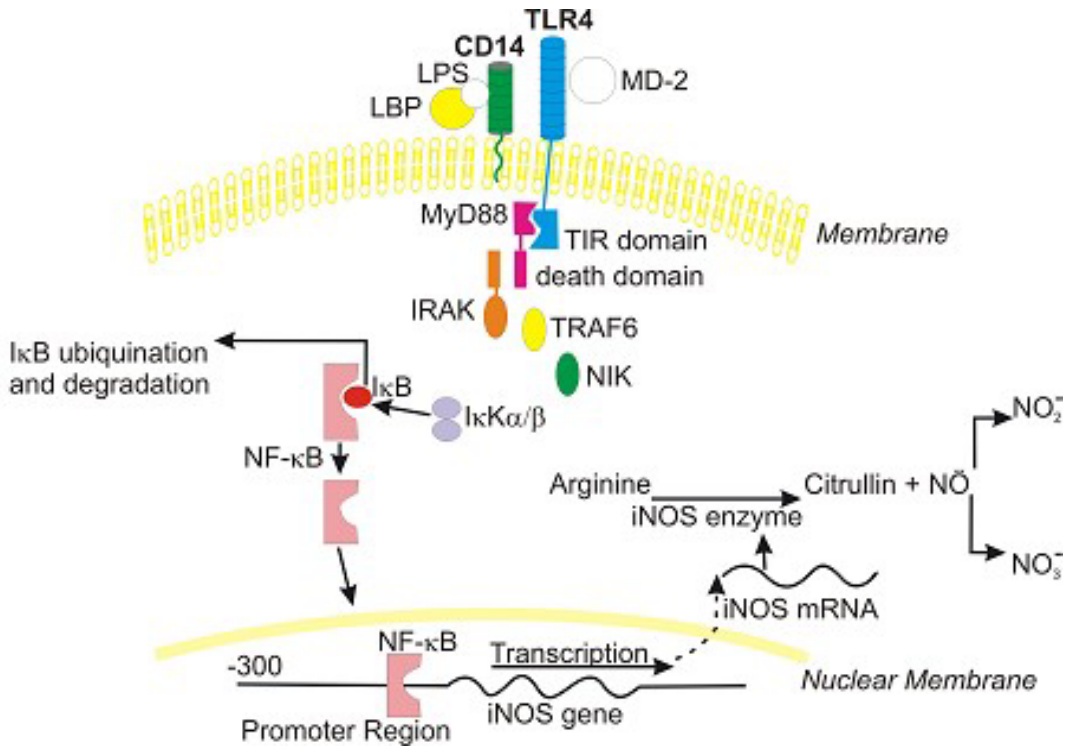


Fig. 2. Schematic representation of the signaling cascade initiated by LPS leading from the TLR4 receptors. LPS is opsonized by LBP, the lipopolysaccharide binding protein, and the opsonized LPS binds with the LPS binding receptor, the CD14 molecule. Since, CD14 lacks a cytoplasmic signaling apparatus; the signal is somehow transferred to TLR4 that triggers the activation of a cascade of adapter proteins leading to the activation and nuclear translocation of NFκB. The transcription factor NFκB binds to the κB sites in the iNOS promoter region and transcription is initiated ultimately leading to the production of iNOS enzyme which is then available to metabolize arginine in to effector molecules such as nitric oxide (NO) and citrulline.

CHAPTER 1

Differential expression of inducible nitric oxide synthase is associated with differential Toll-like receptor-4 expression in chicken macrophages from different genetic backgrounds[1]

ABSTRACT The purpose of this study was to examine iNOS gene expression and activity in macrophages from different chicken genetic lines against various bacterial LPS. Furthermore, the possible involvement of surface LPS receptors as candidates for differential iNOS gene induction in these genetic lines of chicken was also examined. Sephadex-elicited abdominal macrophages (1 x 10^6) as well as iNOS hyper-responder macrophages from a transformed chicken macrophage cell line, MQ-NCSU, were exposed to 5 μg/mL LPS from *E. coli, Shigella flexneri, Serratia marcescens, and Salmonella typhimurium.* Nitrite levels were quantitated in the culture supernatant fractions of macrophages after 24 h by the Griess method. The results showed that macrophages from K-strain ($B^{15}B^{15}$) (range from two separate trials: 31-89 μM) and MQ-NCSU (22-81 μM) were high responders whereas macrophages from both GB1 ($B^{13}B^{13}$) (15-38 μM) and GB2 (B^6B^6) (7-15 μM) chickens were low responders against all LPSs used. Northern-blot analysis revealed that K-strain macrophages expressed higher intensity of 4.5 Kb iNOS mRNA (iNOS/β-actin ratio) than macrophages from GB2 regardless of the LPS source. To elucidate possible molecular mechanism(s) involved in iNOS gene expression in these two strains of chickens, the constitutive[1]

[1] The contents of this chapter were published in Vet. Immunol. Immunopathol. 84, 191-207. 2002.

expression of LPS-related macrophage cell surface receptors, CD14, Toll-like receptor-2 (TLR2), and Toll-like receptor-4 (TLR4), was examined via flow cytometry using anti-human CD14, TLR2, and TLR4 antibodies. CD14 surface expression and intensity was not different between macrophages from K-strain or GB2 chickens. In contrast, while the overall percentage of TLR4-positive macrophages was the same (K-strain, trial 1=92%, trial 2=62%; GB2, trial 1=91%, trial 2=64%), the mean fluorescence intensity (MFI), an indicator of receptor number, was significantly higher (P=0.05) in K-strain macrophages (MFI: trial 1 = 145; trial 2 = 131) than GB2 macrophages (MFI: trial 1 = 101; trial 2 = 98). Furthermore, TLR2 (a previously thought candidate as LPS signaling molecule)-positive cell numbers were higher in K-strain than the GB2 macrophages in one of the two trials with no difference in the intensity of TLR2 expression in either trial. These findings suggest that the observed differences in iNOS expression and activity among the K-strain (hyper- responder) and GB2 (hypo-responder) chickens are, at least in part, due to differential expression of Toll-like receptor-4 (a LPS signaling molecule), leading to more intense LPS-mediated activation of K-macrophages.

Key words: Macrophage; iNOS; Chicken; Genetic lines; CD14; TLR4

INTRODUCTION

Macrophages are specialized form of bone marrow-derived mononuclear phagocytic cells and play a central role in both innate and acquired immune responses. These cells respond to external stimuli by inducing the expression of various cytokines,

adhesion molecules, and enzymes that mediate or modulate various immune responses (Klasing, 1998; Qureshi, 1998). Inducible nitric oxide synthase (iNOS) is one of these enzymes. The enzyme nitric-oxide synthase (NOS) catalyzes the biosynthesis of free radical nitric oxide (NO) as a by-product of an oxidative reaction using guanidino nitrogen group of L-arginine to make L-citruline (MacMicking et al., 1997). NO is a highly reactive, low molecular weight, short lived cytotoxic entity and is converted to nitrite and nitrate that are more stable products (Stuehr and Marletta*.,* 1987; Nathan, 1992). There are three known isoforms of NOS: two constitutive forms, neuronal NOS and endothelial NOS, and an inducible form (iNOS) which was first cloned from macrophages (MacMicking et al., 1997). The two constitutive forms are dependent on intracellular calcium, whereas the inducible form is calcium independent (Gross et al., 1995). Nitric oxide expression by iNOS is an important host-defense mechanism against microbial pathogens in mononuclear phagocytes (Hibbs et al., 1988).

Bacterial lipopolysaccharides (LPS) are immunogenic glycolipids and are a major component of the outer surface of the outer membrane of Gram negative bacteria (Raetz , 1990). LPS is a potent activator of the immune and inflammatory cells including macrophages, monocytes, and endothelial cells (Morrison and Ryan, 1979). LPS of Enterobacteriaceae consists of three covalently linked domains (David et al.,1998); the highly conserved lipid A moiety serves as the hydrophobic anchor for LPS in the outer most bacterial membranes and confers endotoxic properties to the LPS, the core region is the phosphorylated non-repeating oligosaccharide that links lipid A to the hypervariable O-antigen polymer (Wyckoff et al., 1998).

Lipopolysaccharide-dependent macrophage activation is predominantly mediated by CD14, a pattern recognition receptor, implicated in inflammatory responses to microbial components such as lipopolysaccharides, peptidoglycan and lipoarabinomannan (Wright et al., 1990; Pugin et al., 1994). There are two forms of CD14 molecule; the membrane bound, glycosylphosphatidyl inositol linked CD14 expressed on myeloid cells, and the soluble CD14 which is present in plasma. Both CD14 forms are essential in enhancing the LPS (endotoxin)-dependent cellular activation (Pugin et al., 1994). Binding of LPS to the membrane bound form of CD14 is further enhanced by another protein called LPS binding protein (Ulevitch and Tobias, 1995). Interestingly, CD14 does not serve as a transmembrane signaling molecule after LPS binding (Haziot et al., 1988). Instead, recent studies have implicated Toll like receptors (TLR) in the activation of mononuclear phagocytes by bacteria and bacterial products (Yang et al., 1998; Matsuguchi et al., 2000; Means et al., 1999; Ulevitch and Tobias, 1999). In mice, Gram negative bacterial cell wall components have been shown to activate macrophages via TLR4 whereas the mycobacterial and Gram-positive bacterial cell wall constituents activate cells via TLR2 (Takeuchi et al., 1999). The recognition of LPS results in TLR4 mediated signaling of the host cells that culminates in the induction of various pro inflammatory cytokines and cytotoxic molecules.

In chickens, the iNOS gene is known to be regulated at the transcriptional level (Hussain and Qureshi, 1998). Genetic influence on the expression and activity of iNOS in chickens has been shown when macrophages from different genetic sources were stimulated with LPS from *E. coli* (Hussain and Qureshi, 1997, 1998). These genetic

differences characterized macrophages from Cornell K-strain ($B^{15}B^{15}$) and MQ-NCSU, a broiler macrophage cell line) as hyper-responders and macrophages from GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) chicken strains as low-responders in terms of iNOS expression and activity. The objective of the current study was to determine whether these genetic differences are due to the host-based intrinsic genetic mechanism(s) or are dependent upon a specific bacterial source of LPS. Furthermore, if the true basis of these genetic differences is intrinsic then what are the molecular mechanisms that are regulating iNOS gene differentially in these genetic lines of chicken?

MATERIALS AND METHODS

Experimental Animals and Cell Line. Cornell K-strain ($B^{15}B^{15}$), GB1 ($B^{13}B^{13}$), and GB2 ($B^{6}B^{6}$) chickens were maintained at the Department of Poultry Science, North Carolina State University under the Institutional Animal Care and Use committee's approval. These chickens were fed a corn- soybean based chick starter diet with 21% crude protein. The feed and water were available for *ad libitum* consumption. The MQ-NCSU, a transformed chicken macrophage cell line was maintained in LM-Hahn's growth medium at 41C in a 5% CO_2 humidified incubator (Qureshi et al., 1990). For nitrite assay cells were collected in the log phase of growth. The final concentration was adjusted to 1 x 10^6/mL of RPMI 1640 medium supplemented with Penicillin (100 U/mL), Streptomycin (100 μg/mL), Amphotericin B (0.25μg/mL) and 5% heat-inactivated fetal bovine serum (complete medium, CM).

Reagents. Lipopolysaccharides from four different Enterobacteriaceae bacterial species were used. One mg each of *Escherichia coli* (strain O55.B5) (EC), *Shigella flexneri* (SF), *Serratia marcescens* (SM), and *Salmonella typhimurium* (ST) (Sigma Chemical Co., St. Louis, MO) was dissolved in 1 mL of sterile distilled deionized water (Mediatech, VA; less than 0.002 ng endotoxin units/mL). These LPS solutions were then aliquoted at a concentration of 1μg / μL and stored frozen at –20 until used.

Fluorescein isothiocynate (FITC) conjugated mouse anti-human CD14 monoclonal antibody was purchased from Southern Biotechnology Associates, Inc. (Birmingham, Al). Rabbit anti-TLR2 and TLR4 antisera (raised against synthetic peptides based on human TLR2 and TLR4 extra cellular domain sequence) were generously provided by Tularik Inc. (South San Francisco, CA). Fluorescein tagged anti-rabbit IgG (secondary antibody) was purchased from Vector Laboratories (Burlingame, CA).

Macrophage Isolation. At five weeks of age, 10 chicks in each group were weighed and injected intra abdominally with a 3% (wt. /vol.) Sephadex® G50 (Sigma) suspension at a dose of one mL/100 g body weight as described previously (Qureshi et al., 1986). Approximately 42 h post Sephadex injection, chicks were euthanized using CO_2 and the abdominal exudate was collected in siliconized glass tubes from each chick by abdominal lavage with cold 0.85% saline containing 0.5U/mL heparin. The tubes containing abdominal exudate cells (AEC) were allowed to sit on ice for 15 minutes to allow any unabsorbed Sephadex to settle. Twelve mL of supernatants containing cells

were then transferred to clean siliconized tubes and cells were pelleted by centrifugation at 430 g for 20 minutes. The AEC pellets were then resuspended in RPMI 1640 CM.

Macrophage culture. One mL of pooled AEC (final concentration 1 x 10^6/mL) from 10 chicks per strain and MQ-NCSU cells were added to each of the 15 wells (3 wells/LPS for each of the 4 LPS, and 3 wells for no LPS control) of a 24-well culture plate. After 2 h incubation at 41C in 5% CO_2 to allow macrophage adherence, non-adherent cells or any contaminating erythrocytes were removed by twice washing the wells with complete medium. The culture wells were then replenished with one mL of fresh complete medium. Three macrophage culture wells for each strain were then exposed to *Escherichia coli*, *Shigella flexneri*, *Serratia marcescens*, and *Salmonella typhimurium* LPS at a final concentration of 5μg/mL. Three macrophage culture wells from each strain were used as sham controls with no LPS exposure. Triplicate culture wells of complete medium alone were also included as negative controls. The culture plates were incubated for 24 h at which point supernatants were collected from each well and stored at –20 C until analyzed for nitrite levels.

Determination of Nitric Oxide production. Macrophage NO production was determined by measuring the concentration of the stable NO degradation product nitrite (NO_2^-) in the supernatant using a colorimetric assay, Griess method, as previously described (Green et al., 1982). One hundred μL of macrophage supernatant was added into each well of a flat-bottomed 96-well microtiter plate. Then an equal volume of Griess reagent (one part of 1% sulphanilamide and one part of 0.1% of nephthylethylenediaminne dihydrochloride) was added to the same wells. After 10

minutes incubation at room temperature, the plates were read at 540 nm on an ELISA plate reader (Bio-Rad, Richmond, CA) to quantify change in the color that was indicative of nitrite presence. For each sample an average of three readings was used in the final analysis. The standard curve for the nitrite assay was generated using various dilutions of 10mM stock solution of sodium nitrite in RPMI 1640 CM. The nitrite levels in culture supernatants were calculated by comparing the optical density (OD) readings against the nitrite standard curve.

Preparation of cDNA probes. The probe for iNOS was a 4.5Kb chicken iNOS cDNA (Genbank accession number U46504 provided by C.C. McCormick, Cornell University). DH5α strains of *E. coli* competent cells (provided by Dominique Robertson, North Carolina State University) were transformed with plasmid containing iNOS cDNA. Plasmid DNA was extracted from a 12 h culture of transformed cells using GenElute™ plasmid miniprep kit (Sigma). After digestion with EcoRI (Promega, Madison, WI), DNA was separated on 1% agarose gel, and the insert was purified using QIAquick gel extraction kit (Qiagen, Valencia, CA) following the manufacturer's protocols. Chicken β-actin cDNA (Oncor, Gaithersburg, MD) was also purified as described for iNOS cDNA. The probes were labeled by random prime labeling (Boehringer Mannheim, Indianapolis, IN).

Total RNA isolation and Northern blot Analysis. Total RNA was isolated from LPS stimulated or unstimulated macrophages from both K-strain and GB2 strain of chickens, using RNeasy kit (Qiagen), according to protocols supplied by the manufacturer. For Northern blot analysis, total RNA (6-20μg per lane) was resolved by

electrophoresis in a 1.2% agarose gel containing 0.66 M formaldehyde prior to being transferred to the Nytran® Plus membrane (Schleicher & Schuell, Keene, NH). The mRNA was crosslinked to the membrane with UV crosslinker (Spectrolinker; Spectronics Corporation, Westbury, NY) and then hybridized with the digoxigenin-labeled cDNA probes. Hybridization was carried out for 16 h in Dig Easy Hyb (Roche Diagnostic Corporation, Indianapolis, IN) at 45^{o} C. After hybridization the blots were washed twice (15 min each) with 2X SSC (1X SSC is 0.015 M NaCl plus 0.015 M Sodium Citrate) + 0.1 % SDS at room temperature and then twice with 0.5X SSC + 0.1 % SDS at 68^{o} C for 15 min each. The hybridized probes were then immunodetected with anti-digoxigenein-AP Fab fragments (Boehringer Mannheim) using CDP-Star ready-to-use (Roche) as a chemiluminescent substrate following the manufacturer instructions. The blots were then exposed to X-ray film for autoradiography. Digoxigenin-labeled chicken β-actin (Oncor) was used as an internal control.

Quantification of mRNA levels. The mRNA levels on blots were quantitated by densitometric analysis using the ChemiImager™ (Alpha Innotech Corporation, San Leandro, CA) scanning. The β-actin signal was used to normalize samples for variations in the amount of loaded RNA to validate comparisons of the iNOS mRNA across samples.

Flow Cytometry Analysis. Sephadex-elicited AEC were harvested from K and GB2 strains of chickens. After washing, cells were resuspended in RPMI 1640 CM and allowed to adhere to the Petri dish surface for 1 h. The cells were then recovered with a sterile rubber policeman into PBS yielding 99.5 to 100% macrophages based on

morphological criteria. After adjusting the cell concentration to 7 X 10^5/mL, 100 µl of each sample was added to a polystyrene tube (Becton-Dickinson Labware, Lincoln Park, NJ), and incubated briefly with heat inactivated normal mouse, rabbit, or goat serum followed by the addition of appropriate antibody, and incubated for 30 min on ice in the dark. Antibodies against macrophage cell surface markers were used in the following concentrations: 1:4 mouse anti-human CD14 (fluorescein isothiocyanate conjugate), or 1:100 rabbit anti-TLR2 and TLR4 antibodies (unlabeled), and 20µg/mL fluorescein goat anti-rabbit IgG (H+L). After three washes with PBS, cells were resuspended in 200µl of PBS and analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson Immunocytometry, San Jose, CA). For each sample, 15,000 events were obtained.

Statistical analysis. Data were analyzed by analysis of variance using the general linear model procedure of SAS (SAS Institute, 1995). For nitrite data, individual effects of LPS or cell source were compared using the least square means option in SAS. For each experiment at least two trials were conducted on chicks obtained from two separate hatches. Data from each trial were analyzed separately. Significance was established at $P \leq 0.05$.

RESULTS

Nitrite production by activated macrophages. Results for nitrite production by macrophages from four different genetic sources after *in vitro* stimulation with LPS from *Escherichia coli* (EC), *Shigella flexneri* (SF), *Serratia marcescens* (SM), and *Salmonella*

typhimurium (ST) from two separate trials are presented in Tables 1.1 and 1.2. Non-LPS stimulated macrophages from all four sources produced less than 3 μM nitrite/ 10^6 macrophages after 24 h of culture (data not shown). However, after stimulation with LPS there was a significant increase in nitrite production by macrophages from every genetic source. In Trial 1 (Table 1.1), MQ-NCSU macrophages had a nitrite range of 21 to 26 μM against all LPS used. Similarly, macrophages from $B^{15}B^{15}$ (Cornell K-strain) had a nitrite range of 31 to 48 μM. In contrast, nitrite levels in macrophage culture supernatants from GB1 ($B^{13}B^{13}$) and GB2 (B^6B^6) ranged from 15 to 19 and 7.4 to 7.6 μM, respectively over all LPS used. In Trial 2 (Table 1.1), MQ-NCSU macrophages had nitrite range of 67 to 81 μM against all LPS used. Similarly, macrophages from $B^{15}B^{15}$ (Cornell K-strain) had a nitrite range of 59 to 89 μM. In contrast, nitrite levels in macrophage culture supernatants from GB1 ($B^{13}B^{13}$) and GB2 (B^6B^6) ranged from 29 to 39 and 12 to 16 μM, respectively over all LPS used. Taken together, data from both trials indicate that MQ-NCSU and Cornell K-strain macrophages were high-responders whereas GB1 and GB2 macrophages were low- responders in terms of nitrite production, regardless of the source of LPS used.

These data were also analyzed to determine the sensitivity of macrophages from individual genotypes to LPS from various sources. As shown in Table 1.2 (Trial 1), LPS from EC (47.8 μM) and ST (41.5 μM) were more stimulatory for K-strain whereas LPS from EC (26.5 μM) and SF (25.46 μM) were more stimulatory for MQ-NCSU cells. In contrast, differential LPS source sensitivity of GB1 (EC = 19.28 μM; SF = 15.26 μM;

SM = 15.35; ST = 17.21 μM) and GB2 (EC = 7.60; SF = 7.36; SM 7.60; ST = 7.57 μM) chicken macrophages was minimal. In Trial 2 (Table 1.2), K-strain was still higher for LPS from EC and ST as compared with the LPS from SF and SM whereas MQ-NCSU was highest against LPS from SF. Similar to Trial 1, differential LPS source sensitivity for GB1 and GB2 macrophages was minimal in comparison with MQ-NCSU and K-strain.

Macrophage iNOS mRNA expression. In an attempt to correlate differential nitrite levels with iNOS mRNA expression, Northern blots were performed on K-strain (high-responder) and GB2 (selected as an example of low nitrite producer) macrophage RNA after stimulation with LPS from various bacterial sources. As seen in figure 1.1, 4.5-Kb iNOS band and a 2.0-Kb β-actin band was detectable in all samples. Macrophages from K-strain chickens exhibited high intensity iNOS mRNA bands when treated with *Escherichia coli*, *Shigella flexneri*, *Serratia marcescens*, or *Salmonella typhimurium* LPS. In contrast, LPS treated GB2 strain chicken macrophages expressed barely discernable iNOS mRNA bands. The expression level of iNOS in LPS treated GB2 macrophages was very similar to the expression level of iNOS in non-LPS treated chicken macrophages. Furthermore, the level of β-actin was relatively comparable among samples. iNOS mRNA to corresponding β-actin (housekeeping gene) band density ratio (Fig. 1.1) further validates that K-strain and GB2 macrophages are hyper- and hypo-responders, respectively, for iNOS production regardless of the LPS source.

Macrophage cell surface receptors. The expression of three macrophage cell surface molecules, CD14, TLR2 and TLR4 was quantitated via flow cytometry. Since all three antibodies used in this study were against mammalian molecules, the reactivity of these antibodies against chicken macrophages was established in preliminary titration experiments using only K-strain macrophages. All three antibodies positively labeled chicken macrophages as follows: 87.44% positive with 1:4 dilution of anti-CD14; 87.02% positive with 1:100 dilution of anti-TLR2 and 93.78% positive with 1:100 dilution of anti-TLR4 antibodies. All secondary antibody and sham-treated macrophage controls had minimal background reactivity ranging from 0.52% to 1.65% (flow cytometry figures not shown). The comparison of CD14, TLR2 and TLR4 expression on GB2 and K-strain macrophages in one representative experiment is given in figure 1.2. No significant differences were observed in the overall percentage of macrophages positive for CD14, TLR2 or TLR4 between the two chicken strains. The percent positive values ranged from 44 to 46% for CD14, 94 to 95% for TLR2, and 86 to 92% for TLR4 between GB2 and K-strain macrophages. In a repeated experiment (data not shown), no difference was observed between these strains for CD14 (range: 72 to 78%) or TLR4 (range: 62 to 64%) expression. However, K-strain had relatively higher TLR2 percentage positive macrophages (79%) as compared with the GB2 macrophages (64%). In addition to percent positive macrophage quantification, the mean fluorescence intensity (MFI) (as a measure of relative numbers of receptors) of expression of these molecules on macrophages from both strains was also examined. As shown in Figure 1.3 (representative experiment), macrophages from both strains exhibited similar TLR2 (K =

128.5, GB2 = 127.1 MFI), and CD14 (K = 49.0, GB2 = 49.1 MFI) surface expression intensities. In contrast, TLR4 expression intensity was significantly higher (P = 0.05) on K-strain macrophages (145.5 MFI) as compared with the GB2 macrophages (101.4 MFI). In a repeated experiment (data not shown), the MFI values for CD14 were 55.1 and 57.0, for TLR2 were 152.6 and 150.6, and for TLR4 were 98.2 and 131.72 (P = 0.05) for GB2 and K-strain macrophages, respectively

DISCUSSION

It was reported earlier (Hussain and Qureshi, 1997, 1998) that macrophages from chickens with different genetic backgrounds exhibit differential iNOS expression and activity when stimulated with *Escherichia coli* LPS. For example, macrophages from the MQ-NCSU macrophage cell line (established from a meat-type chicken) as well as from Cornell K-strain white Leghorn ($B^{15}B^{15}$) chickens expressed higher levels of iNOS mRNA as well as higher corresponding nitrite levels in the culture supernatants as compared with the macrophages from GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) chickens. The question addressed in the current study was to investigate if these genetic differences in iNOS activity are indeed due to some host-based intrinsic genetic mechanism(s) or are dependent upon the bacterial source of LPS. Furthermore, if the true basis of these differences are intrinsic then what are the molecular mechanisms regulating theses differences. As presented in Table 1.1, both Cornell K-strain and MQ-NCSU macrophages turned out to be high-responders for nitrite levels (an indication of higher iNOS activity) not only against *E. coli* LPS but also against LPS from three additional

bacterial sources, i.e. *Shigella flexneri, Serratia marcescens,* and *Salmonella typhimurium*. Similarly, the low-responder iNOS activity genotypes GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) for *E. coli* LPS were also low responders for LPS from *Shigella flexneri*, *Serratia marcescens*, and *Salmonella typhimurium*. These data, therefore, provide strong evidence that MQ-NCSU and Cornell K-strain ($B^{15}B^{15}$) are clearly hyper-responders and GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) are hypo-responders for iNOS activity regardless of the source of LPS.

Among the iNOS hyper-responder chicken genotypes, however, the sensitivity to various LPS was not identical. For example, LPS from EC, ST and SF were more stimulatory to macrophages as compared with the LPS from *Serratia marcescens* (SM). In contrast, among the hypo-responsive genotypes (e.g., GB1 and GB2), the least amount of variation to various LPS sources was observed. These findings clearly show a differential susceptibility of macrophages to various bacterial LPS in terms of iNOS activity.

To further confirm the genetic differences in the iNOS activity, expression of iNOS mRNA after induction with different LPS sources was quantified using Northern blot analysis technique. The low nitrite producing GB2 macrophage had lower iNOS mRNA expression relative to the iNOS produced by macrophages from the high responder K-strain after 10 h stimulation with any of the four LPS used. Furthermore, level of iNOS mRNA in LPS stimulated GB2 chicken macrophages was very similar to iNOS mRNA in unstimulated chicken macrophages. Expression of the inducible form of nitric oxide synthase in non-LPS treated chicken macrophages is perhaps due to *in vivo*

inflammatory activation to which macrophages respond during Sephadex-elicitation. These experiments further confirmed that the differences in the macrophage-mediated nitrite levels in supernatant fractions of macrophage cultures are due to differential expression of iNOS mRNA regardless of the source of its induction. The true basis of such differences in iNOS enzyme activity among macrophages from various sources is not known. However, it has been reported that the increase in the iNOS mRNA in murine (Xie et al., 1992) and chicken (Hussain and Qureshi, 1997) macrophages is transcriptional in nature. Furthermore, Hussain and Qureshi (1998) showed that posttranscriptional mechanisms are not involved as a basis for the differential expression of iNOS in these chicken lines. These observations suggest that proximal events in LPS induction of iNOS should be explored as a possible cause of differential iNOS expression.

The second hypothesis tested was the possibility that LPS binding and signaling receptors may be differentially expressed on macrophages from different sources resulting in differential stimulation of macrophages leading to differential induction of the iNOS gene. This hypothesis was tested as an initial step towards trying to explore the molecular mechanisms involved in the differential susceptibility of these genetic sources of macrophages to lipopolysaccharides. CD14 is the most important LPS receptor on the surface of macrophages and is involved in the activation of these cells by LPS (Wright et al., 1990; Ziegler-Heitbrock and Ulevitch 1993; Otterlei et al., 1995). In addition, different cytokines (Dimri et al., 1994), or slight shifts in temperature (Antal-Szalmas et al., 1997) can alter the number of surface expressed CD14 on monocytes. Although

CD14 has been shown to mediate LPS induced activation of various cell types, this receptor is glycosylphosphatidyl inositol anchored and incapable of directly transducing the signals across the cell membranes (Haziot et al., 1988). It has been shown that CD14 mediates LPS activation by interaction with other signal transducing molecules, most important of which are Toll-like receptors (Yang et al., 1998; Chow et al., 1999; Hoshino et al., 1999). The Toll proteins were first described as the mediators of dorsoventral pattern formation during the early embryonic development of *Drosophila melanogaster,* and later a role for Toll proteins and Toll-like proteins in anti-microbial immune responses was found both in *Drosophila* (Medzhitov et al., 1997) and in mammals (Rock et al., 1998). Recent studies in mice have shown that TLR2 and TLR4 recognize different bacterial cell wall components. TLR2 plays a major role in Gram positive bacterial recognition where as TLR4 is the signaling receptor for Gram-negative bacterial constituents namely LPS (Takeuchi et al., 1999; Chow et al., 1999; Akashi et al., 2000). In this current study no statistical differences were observed in the constitutive expression of CD14 on chicken macrophages from different genetic sources. However, the overall number of CD14 expressed on the surface of these chicken macrophages varied between experiments perhaps due to slight temperature variation (as shown by Antal-Szalmas et al., 1997) which may have modulated CD14 expression as well as nitrite levels. Furthermore, Sephadex-elicited inflammatory response recruits a rather heterogeneous macrophage population in the abdominal cavity (Qureshi et al., 2000) that would also vary from experiment-to-experiment resulting in the observed variation in CD14 expression. Also, there was no difference in the expression of TLR2 molecule on the cell

surface. TLR4 intensity, on the other hand, was significantly (P = 0.05) higher in macrophages from K-strain than from GB2 strain of chicken. A parallel example of LPS hypo responsiveness due to defect in LPS signaling receptor exists in mammals. Poltorak et al. (1998) have reported that a missense mutation in the Tlr4 gene results in defective LPS signal transduction in C3H/HeJ and C57BL/10ScCr mice rendering them resistant to endotoxin. Qureshi et al. (1999) have observed that LPS-hypo responsive C57BL/10ScNCr strain of mice bear a homozygous deletion of Tlr4 gene and therefore, they exhibit impaired ability to respond to LPS. Also studies performed by Hu et al. (1997) suggest that distinct allelic forms of the Lps gene influence survival in birds during Gram-negative bacterial infections. Further more, Nomura et al. (2000) have recently reported that endotoxin tolerance in mouse correlates with down regulation of TLR4 on the surface of macrophages. It seems likely that differences observed in TLR4 intensity in the current study might be due to some mutations in Tlr4 gene rendering one strain of chicken highly susceptible (K) and the other one highly resistant (GB2) to LPS stimulation.

Based on the data presented in this study we conclude that LPS binds similarly to the macrophage cell surface LPS binding receptors in both K and GB2 strains of chickens. Yet, the LPS signaling receptor (TLR4) is expressed in greater numbers on K-strain chicken macrophages than on macrophages from GB2 strain of chicken. When macrophages are stimulated with LPS, the greater number of TLR4 receptors on K-strain macrophages perhaps lead to stronger signal transduction and ultimately higher iNOS

expression and activity than GB2 macrophages which express comparatively fewer TLR4 receptors (Fig. 1.4).

Whether or not the differential iNOS response between K-strain and GB1 or GB2 chickens impart differential resistance and/or susceptibility to bacterial or viral mediated infections is not known. Nitric oxide has been shown to be involved in inhibiting replication of chicken tumorigenic virus, such as Marek's disease virus (Schat et al., 2001). Interestingly, the Cornell K-strain genotype, which is a hyper-responder to iNOS expression and activity, is also naturally resistant to Marek's disease (Hutt and Cole, 1957; Gavora et al., 1977). It would be interesting to examine various immunological endpoints (such as cytokine profiles) of iNOS hypo- and hyper-responder genotypes perhaps during a disease challenge to establish a positive or negative correlation of disease susceptibility or resistance to iNOS expression and activity in chicken.

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TABLE 1.1. Nitrite production by macrophages from different genetic backgrounds after stimulation with various bacterial lipopolysaccharides (LPS): Comparison of Genotypes with respect to individual LPS source (TRIAL 1 and 2) [1].

Stimulus	**Macrophage Source[2]**									
LPS	**MQ-NCSU**		**Cornell K-strain**		**GB1**		**GB2**		**Pooled SEM[3]**	
	Trial		Trial		Trial		Trial		Trial	
	1	2	1	2	1	2	1	2	1	2
	----------(μM)[4]----------									
Escherichia coli	26.55[b]	69.02[b]	47.80[a]	88.96[a]	19.28[c]	37.71[c]	7.60[d]	15.55[d]	0.48	0.67
Shigella flexneri	25.46[b]	80.61[a]	31.05[a]	59.38[b]	15.26[c]	38.57[c]	7.36[d]	11.76[d]	1.09	7.56
Serratia marcescens	21.59[b]	67.99[a]	31.17[a]	60.95[b]	15.35[c]	29.15[c]	7.60[b]	14.52[d]	0.39	1.03
Salmonella typhimurium	22.01[b]	66.96[a]	41.56[a]	66.80[a]	17.21[b]	33.92[b]	7.57[c]	15.28[c]	2.92	2.08

1. Sepahdex- elicited abdominal exudate macrophages and cells of a chicken macrophage cell line were cultured for 24 hr (1×10^6) in the presence of 5μg/ml of individual lipopolysaccharide. Nitrite levels were measured in the culture supernatant fraction by Greiss method.

2. MQ-NCSU = a broiler macrophage cell line; Cornell K-strain = $B^{15}B^{15}$; GB1 = $B^{13}B^{13}$; GB2 = $B^{6}B^{6}$.

3. Pooled standard error of means.

4. Values represent means of nitrite levels in the culture supernatants from 3 replicate samples established from a pool of 10 chicks per macrophage source. The values within rows with different superscript letters (within each trial) are different at $P < 0.05$.

TABLE 1.2. Nitrite production by macrophages from different genetic backgrounds after stimulation with various bacterial lipopolysaccharides (LPS): Comparison of LPS source within a Genotype (TRIAL 1and 2) [1].

Stimulus	**Macrophage Source[2]**							
LPS	**MQ-NCSU**		**Cornell K-strain**		**GB1**		**GB2**	
	Trial		Trial		Trial		Trial	
	1	2	1	2	1	2	1	2
	----------(μM)[4]----------							
Escherichia coli	26.55[a]	69.02[ab]	47.80[a]	88.96[a]	19.28[a]	37.71[a]	7.60[a]	15.55[a]
Shigella flexneri	25.46[a]	80.61[a]	31.05[b]	59.38[c]	15.26[c]	38.57[a]	7.36[a]	11.76[b]
Serratia marcescens	21.59[b]	67.99[ab]	31.17[b]	60.95[c]	15.35[c]	29.15[ab]	7.60[a]	14.52[a]
Salmonella typhimurium	22.01[b]	66.96[b]	41.56[a]	66.80[b]	17.21[b]	33.92[ab]	7.57[a]	15.28[a]
Pooled SEM[3]=	1.00	4.89	2.95	1.54	0.44	6.04	0.46	0.43

1. Sephadex- elicited abdominal exudate macrophages and cells of a chicken macrophage cell line were cultured for 24 hr (1 x 10^6) in the presence of 5μg/ml of individual lipopolysaccharide. Nitrite levels were measured in the culture supernatant fraction by Greiss method.

2. MQ-NCSU = a broiler macrophage cell line; Cornell K-strain = $B^{15}B^{15}$; GB1 = $B^{13}B^{13}$; GB2 = B^6B^6.

3. Pooled standard error of means.

4. Values represent means of nitrite levels in the culture supernatants from 3 replicate samples established from a pool of 10 chicks per macrophage source. The values within columns with different superscript letters are different at $P < 0.05$.

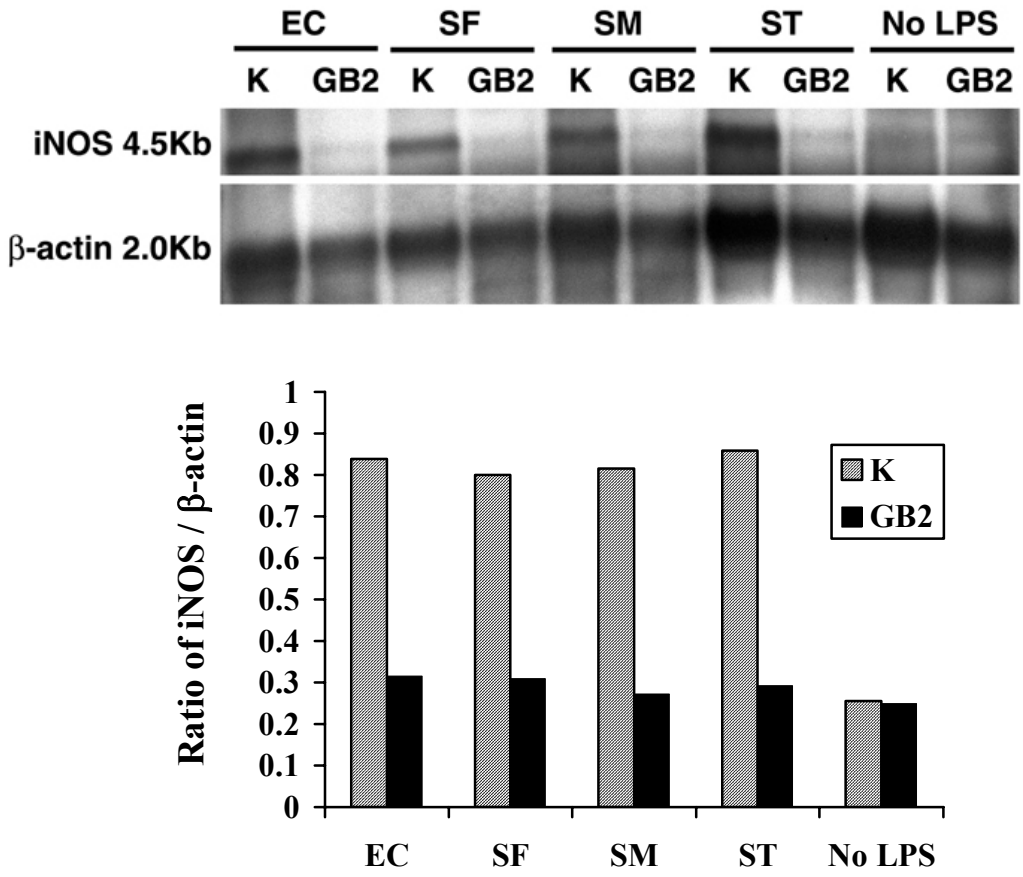

Fig. 1.1. Northern blot analysis of total RNA from LPS-treated chicken macrophages. Total RNA was obtained from K-strain and GB2-strain chicken macrophages stimulated with 5μg/mL LPS for 10 h, fractioned by denaturing agarose gel electrophoresis (15μg/lane), and transferred on to nylon membrane. Blots were hybridized with chicken iNOS and β-actin specific digoxigenin labeled probes. EC, SF, SM, and ST represent *Escherichia coli*, *Shigella flexneri*, *Serratia marcescens*, and *Salmonella typhimurium* lipopolysaccharide, respectively. No LPS denotes unstimulated negative controls. Hybridization signal at 4.5Kb represents the iNOS mRNA and hybridization signal at 2.0Kb represents β-actin (internal control) mRNA. iNOS and β-actin band intensities ratios for each lane are provided. Both iNOS and β-actin band intensities were measured in each sample by densitometry, and the iNOS / β-actin ratio was calculated (bar graph). The experiment was repeated three times and similar results were obtained.

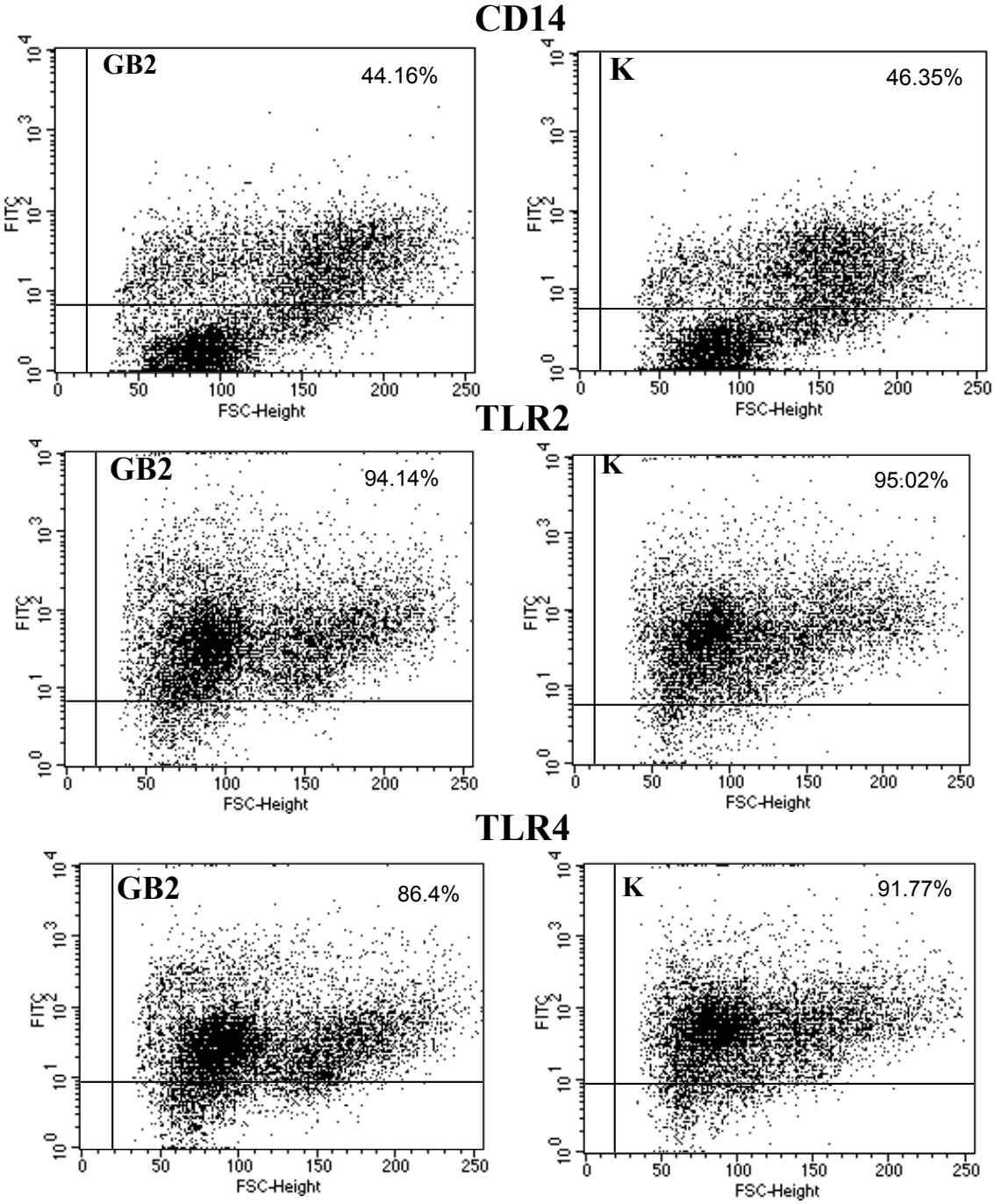

Fig. 1.2. Flow cytometric analysis of chicken macrophages. 7 x 10^5 macrophages from K- or GB2-strain of chickens were stained with 1:4 dilution of either FITC mouse anti-human CD14, or 1:100 dilution of unlabeled rabbit anti-TLR2 or anti-TLR4 antibodies followed by incubation with 20μg/mL FITC goat anti-rabbit IgG and analyzed by FACScan. Analysis gate was set on macrophages using forward and side scatter gating. The number in each panel indicates the percentage of positive cells in gated macrophage population. These trends were consistent upon repeating the experiments two times.

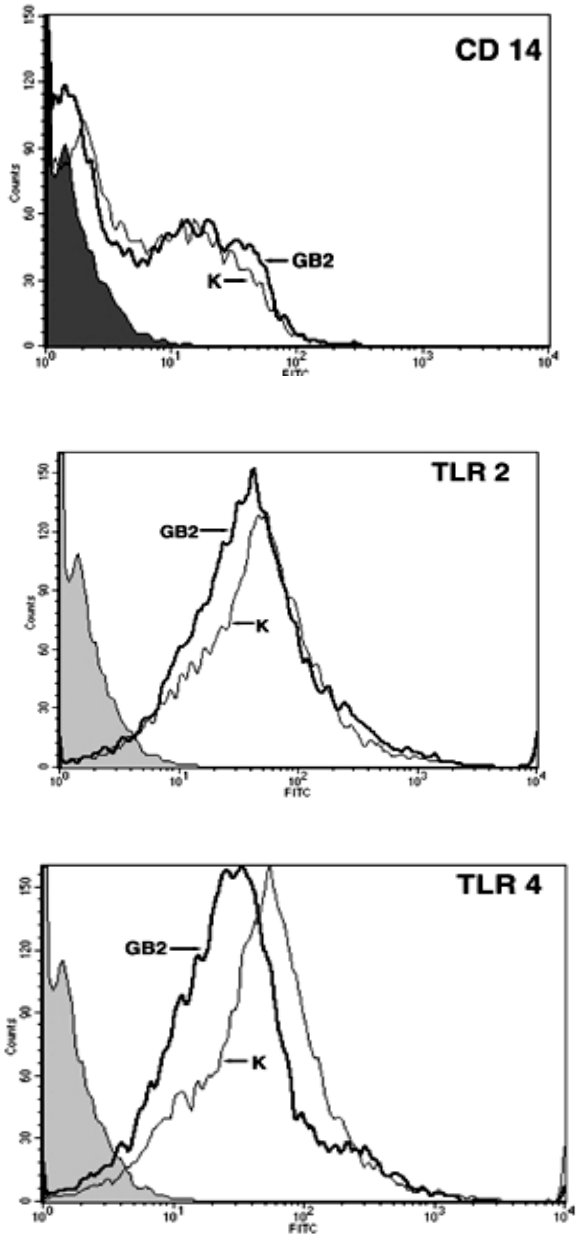

Fig. 1.3. Representative flow cytometry histograms for fluorescence intensity of CD14, TLR2, and TLR4 on the surface of chicken macrophages. 7 x 10^5 macrophages from K- or GB2-strain of chicken were stained with 1:4 dilution of either FITC mouse anti-human CD14, or 1:100 dilution of unlabeled rabbit anti-TLR2 or anti-TLR4 antibodies followed by incubation with 20μg/mL FITC goat anti-rabbit IgG and analyzed by FACScan. Shaded area represents the cells only. Thick line represents GB2-strain macrophage cell surface intensity and thin line indicates K-strain intensity. These trends were consistent upon repeating the experiments two times.

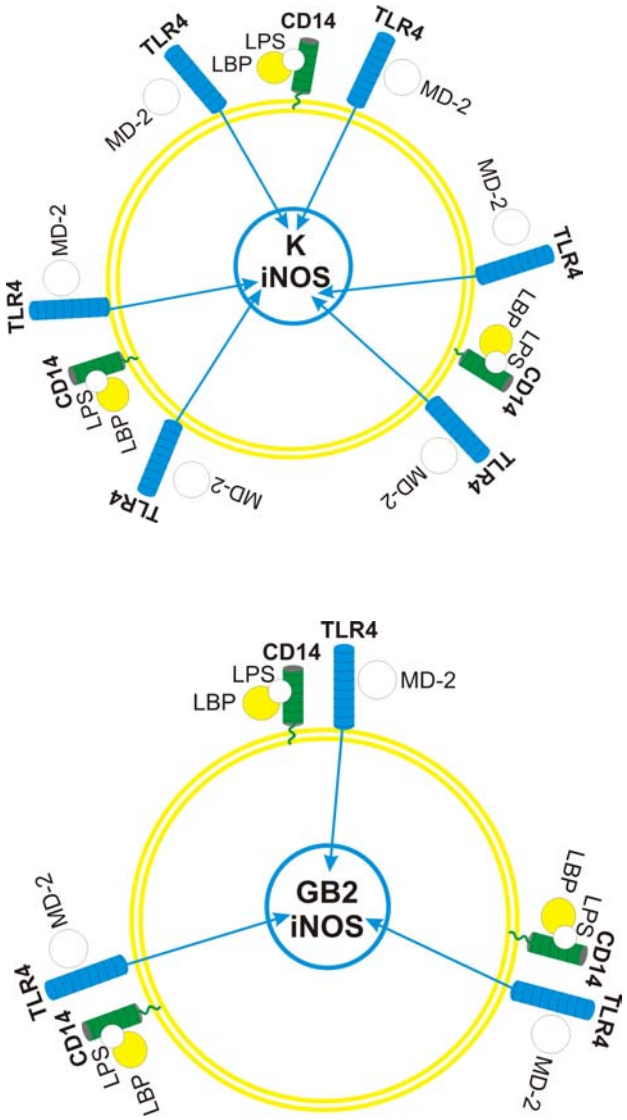

Fig. 1.4. Schematic representation of the differential TLR4 mediated LPS signaling in K and GB2 macrophages. The iNOS low responder GB2 chicken (lower panel) express fewer TLR4 LPS signaling molecules on the surface of macrophages as compared with the iNOS hyper responder K strain chicken that express significantly higher number of TLR4(upper panel). This means that more intense LPS mediated signal is induced in K macrophages leading to higher iNOS expression and activity as compared with GB2.

CHAPTER 2

Involvement of LPS related receptors and nuclear factor Kappa-B in differential expression of inducible nitric oxide synthase in chicken macrophages from different genetic backgrounds

ABSTRACT Macrophages from Cornell K-strain chickens ($B^{15}B^{15}$) are hyper- and from GB2 chickens ($B^{6}B^{6}$) are hypo-responders to LPS-mediated iNOS expression and activity. The molecular mechanism(s) responsible for this differential expression is not yet fully understood. We have previously reported that macrophages from K (iNOS hyper responder) and GB2 (iNOS hypo responder) chickens differ in constitutive expression of TLR4 but not in CD14 molecules. The objectives of the current study was to determine if the iNOS differences between K and GB2 macrophages are possibly due to differential expression of LPS-induced TLR4, CD14 and/or NFκB. The results showed that Sephadex-elicited, adherence purified K macrophages expressed more TLR4 and CD14 receptors ($P \leq 0.05$) at 6 and 12 h post LPS stimulation than GB2 macrophages as measured by flow cytometry. In addition, pre-incubation of macrophages from a transformed chicken macrophage cell line, MQ-NCSU, with 50 μg / mL anti CD14 and anti TLR4 antibodies significantly reduced whereas pre incubation with 100 μg / mL completely blocked LPS-mediated iNOS activity as measured by nitrite levels. Furthermore, the amount of nuclear bound NFκB was found to be significantly greater in K than in GB2 macrophages at 30 minute post LPS stimulation. This nuclear localization of NFκB as well as iNOS activity was completely inhibited by pretreatment of

macrophages with 50μM MG132, a proteosome inhibitor, both in K and GB2 macrophages. Taken together, these findings suggest that a differential and perhaps stronger LPS mediated signaling via CD14, TLR4 and NFκB is responsible for the heightened iNOS gene induction in K-strain (hyper-responder) macrophages than in GB2 (hypo-responder) chickens.

Key words: Macrophage; iNOS; Chicken; LPS; Genetic lines; CD14; TLR4; NFκB

INTRODUCTION

Macrophages are versatile cells found in every tissue in the body. They must perform a number of cellular functions that allow them to kill invading microorganisms and neoplastic cells. To this effect, macrophages use a variety of cytotoxic effectors. One such effector is nitric oxide (NO) (Nathan, 1992). Nitric oxide is generated by nitric oxide synthases (NOS), a group of evolutionarily conserved isoenzymes that convert the amino acid L-arginine to citrulline and NO (Bogdan, 2000). There are three major genetically distinct types of NOS that are named after the cell types from which they were first isolated and cloned: neuronal NOS or NOS 1; mNOS or inducible NOS or NOS 2; and endothelial NOS or NOS 3 (Moncanda et al., 1997). These isoforms differ with respect to the main mode of regulation, their key function, the average amount of NO produced, and the tissue expression pattern *in vivo* (Stuehr, 1999). Although neuronal and endothelial NOS do exhibit a modest degree of regulation at the expression level,

inducible NOS (iNOS) is the major isoform and is expressed in macrophages and other cell types only after induction by bacterial lipopolysaccharides (LPS) and other immunologic or inflammatory stimuli (Stuehr and Marletta, 1985; Rao, 2000).

LPS induced activation of monocytes and macrophages is mainly mediated through CD14, a glycosylphoshatidyl-inositol (GPI)-anchored membrane glycoprotein (Ulevitch and Tobias 1999; Wright et al., 1990; Wright, 1995). However, CD14 lacks a transmembrane and cytoplasmic domain and is not believed to have intrinsic signaling capabilities. Until recently a receptor that directly transduces an activation signal in response to LPS has remained elusive. However, recent studies in mammals have indicated that Toll-like receptor-4 (TLR4) mediates response to LPS by functioning as the transmembrane component of the LPS receptor complex (Poltorak et al., 1998; Hoshino et al., 1999; Qureshi et al., 1999).

LPS induced signal transduction is thought to entail binding to specific cellular receptors, which trigger intracellular signaling cascades leading to the activation of the transcription factor nuclear factor κB (NFκB) (May and Ghosh, 1998). NFκB is composed of two subunits, p50 and p65, and is normally sequestered in cytoplasm via association with IκB (inhibitor of κB) protein (May and Ghosh, 1998; Sha 1998). NFκB can be activated by exposure of cells to bacterial endotoxins, cytokines, shear forces, oxidative stress, UV irradiation, and other physiological and non-physiological stimuli (Baeuerle and Henkel, 1994). The translocation of NFκB into the nucleus is controlled by the targeted phoshorylation and subsequent degradation of IκB (Chen et al., 1995). In the nucleus, free active NFκB dimer (p50/p65) binds to the κB sites in the promoter region of

its target genes and typically up regulates the expression of gene products. NFκB has been shown to be involved in induction of iNOS gene expression in both mammals (Xie et al., 1994) and chicken (Lin et al., 1996).

Studies have shown that iNOS induction in response to LPS is transcriptionally regulated (Hussain and Qureshi, 1998: Xie et al 1993: Lowenstein et al., 1993). Furthermore, in chicken, iNOS expression and activity has been shown to be influenced by genetic background (Hussain and Qureshi, 1997, 1998) that characterize macrophages as hyper- (Cornell K-strain; $B^{15}B^{15}$) and hypo- (GB1; $B^{13}B^{13}$ and GB2; $B^{6}B^{6}$) iNOS responders. Our previous studies have shown that these observed differences in iNOS gene expression and activity are intrinsic regardless of the bacterial source of LPS. In addition, the iNOS hyper-responder K-strain macrophages expressed significantly higher numbers of TLR4, the LPS signaling receptor, constitutively as compared with macrophages from GB2 chickens (Dil and Qureshi, 2002). The objective of the current study was to further investigate the role of LPS binding (CD14) and signal transducing (TLR4) proteins in the differential iNOS induction in these genetic lines. To this end, inducible expression of CD14 and TLR4 and LPS induced activation of NFκB as well as involvement of these proteins in the LPS stimulated iNOS induction in chicken macrophages was studied.

MATERIALS AND METHODS

Experimental Animals. Cornell K-strain ($B^{15}B^{15}$) and GB2 ($B^{6}B^{6}$) chickens were maintained at the Department of Poultry Science, North Carolina State University

under the Institutional Animal Care and Use committee's approval. These chickens were fed a corn-soybean based chick starter diet with 21% crude protein. The feed and water were available for *ad libitum* consumption.

Reagents. Lipopolysaccharide (LPS) from *Escherichia coli* (*E. coli,* strain O55.B5, Sigma Chemical Co., St. Louis, MO) was dissolved in sterile distilled deionized water (Mediatech, VA; less than 0.002 ng endotoxin units/mL). The LPS stock solution was stored frozen at –20 until used.

Purified unlabelled and fluorescein isothiocynate (FITC) conjugated mouse anti-human CD14 monoclonal antibody was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Functional grade purified and phycoerythrin (PE), anti-human Toll-like receptor 4 (TLR4) monoclonal antibody, and mouse IgG2a isotype control antibody were purchased from eBioscience (San Diego, CA). Rabbit polyclonal NFκB p65 antibody and rabbit IgG isotype control antibody were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). FITC conjugated anti-rabbit monoclonal antibody was purchased from Sigma Chemical Co. (Saint Louis, MO).

The proteosome inhibitor MG132 (Z-Leu-Leu-Leu-H) (Peptide institute, Osaka, Japan) was generously provided by Janice Allen (North Carolina State University).

Macrophages. At five weeks of age, 5-7 chicks in each group were weighed and injected intra abdominally with a 3% (wt. /vol.) Sephadex® G50 (Sigma) suspension at a dose of one mL/100 g body weight as described previously (Qureshi et al., 1986). Approximately 42 h post Sephadex injection, chicks were euthanized using CO_2 and the abdominal exudate was collected in siliconized glass tubes from each chick by abdominal

lavage with cold 0.85% saline containing 0.5U/mL heparin. The tubes containing abdominal exudate cells (AEC) were allowed to sit on ice for 15 minutes to allow any unabsorbed Sephadex to settle. Twelve mL of supernatants containing cells were then transferred to clean siliconized tubes and cells were pelleted by centrifugation at 430 g for 20 minutes. The AEC pellets were then resuspended in RPMI 1640 complete medium (CM: RPMI 1640 medium supplemented with 100 U/mL Penicillin, 100 μg/mL Streptomycin, 0.25μg/mL Amphotericin B and 5% heat-inactivated fetal bovine serum).

The MQ-NCSU, a transformed chicken macrophage cell line was maintained in LM-Hahn's growth medium at 41C in a 5% CO_2 humidified incubator (Qureshi et al., 1990). For nitrite assay and receptor studies cells were collected in the log phase of growth and maintained in CM.

Macrophage Culture Set Up for CD14 and TLR4 Blocking Study. In order to ensure the specificity of anti-human CD14 and TLR4 antibodies as well as to determine the involvement of these LPS receptors in LPS-mediated iNOS induction, MQ-NCSU macrophages were seeded in a 24-well culture plate (Corning Inc., Corning, NY) at a viable cell concentration (determined by trypan blue exclusion) of 1 X 10^6/mL of CM per well. 50 μg / mL or 100 μg / mL of either of purified anti-CD14 or anti-TLR4 or isotype-matched control antibody was added to appropriate culture wells 1 h prior to the LPS stimulation. LPS was added to these wells at a concentration of 1μg / mL. No LPS exposed controls were included. After 24 h incubation at 41 C, 5% CO_2, the culture supernatants were removed, aliquoted, and stored at –20 C until analyzed for nitrite analysis.

Macrophage Culture Set Up for NFκB Activation Blocking Study. To assess the involvement of NFκB activation in iNOS gene induction, pooled AEC from 5 chickens per strain were added in a 24 well culture plate at a viable cell concentration of 1 x 10^6/mL of CM per well. Culture plates containing AEC were incubated for 2 h at 41C in 5% CO_2 to allow macrophage adherence. After incubation, non-adherent cells or any contaminating erythrocytes were removed by twice washing the wells with CM. The culture wells were then replenished with one mL of fresh CM. Next, 50μM of MG132, a proteosome inhibitor, was added to the appropriate wells 1 h prior to LPS stimulation. Macrophage viability against 25 and 50 μM of MG132 was determined by an MTT assay (Mosmann, 1983) to assess any MG132 mediated toxicity. LPS was added to these wells at a concentration of 1μg / mL. No LPS exposed controls were also included. After 24 h incubation at 41 C, 5% CO_2, the culture supernatants were removed, aliquoted, and stored at –20 C until analyzed for nitrite analysis.

Determination of Nitric Oxide production. Nitric Oxide (NO) production was measured by determination of nitrite levels (as a stable end product of NO) in the culture supernatants using a colorimetric assay, Griess method, as previously described (Green et al., 1982). One hundred μL of macrophage supernatant was added into each well of a flat-bottomed 96-well microtiter plate. Then an equal volume of Griess reagent (one part of 1% sulphanilamide and one part of 0.1% of nephthylethylenediaminne dihydrochloride) was added to the same wells. After 10 minutes incubation at room temperature, the plates were read at 540 nm on an ELISA plate reader (Bio-Rad, Richmond, CA) to quantify change in the color that was indicative of nitrite presence. For

each sample an average of three readings was used in the final analysis. The standard curve for the nitrite assay was generated using various dilutions of 10mM stock solution of sodium nitrite in RPMI 1640 CM. The nitrite levels in culture supernatants were calculated by comparing the optical density (OD) readings against the nitrite standard curve.

Quantification of CD14 and TLR4 Inducible Expression. Sephadex-elicited AEC from K and GB2 strains were allowed to adhere to the Petri dish surface for 1 h. The cells were then recovered with a sterile rubber policeman into CM yielding 99.5 to 100% macrophages based on morphological criteria. After adjusting the cell concentration to 5 X 10^6/mL in siliconized glass tubes, 1μg/mL of LPS was added and 100 μl of each sample was transferred to polystyrene tubes (Becton-Dickinson Labware, Lincoln Park, NJ) and incubated for indicated periods of time at 41 C, 5% CO_2. At the end of each incubation period, samples were incubated with heat inactivated normal mouse serum for 2-3 minutes followed by the addition of appropriate antibody, and incubated for 30 min on ice in the dark. Antibodies against macrophage cell surface markers were used in the following concentrations: 1:4 mouse anti-human CD14 (fluorescein isothiocyanate conjugate), or 1:4 mouse anti-human TLR4 (Phycoerytherin) conjugate antibodies. After three washes with PBS, cells were resuspended in 200μl of PBS and analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson Immunocytometry, San Jose, CA). For each sample, 15,000 events were obtained.

Quantification of DNA bound NFκB. Adherence purified macrophages from GB2 and K stain were analyzed through flow cytometry to quantify nuclear binding of NFκB (an estimate of NFκB activation) as described previously (Foulds, 1997). This technique exploits the ability of bacterial endotoxins to stimulate NFκB DNA binding (Muller et al., 1993) and the ability of NFκB inhibitors to neutralize this activation (Wang et al., 1999). Briefly, adherence purified macrophages from each strain were stimulated by adding 1μg/mL *E.coli* LPS to 100 μl ($5 \text{ X } 10^5$) of cells in polystyrene tubes and incubating the tubes at 41 C for 30 min. In three tubes from each strain, cells were pretreated with MG132 (50 μM) for 1 h prior to LPS stimulation and three tubes from each strain were used as sham controls with no LPS exposure. After incubation the cells were washed twice with PBS at 200 g for 5 min each and then prepared for staining using reagents contained in the Cycle test PLUS DNA reagent kit (Becton Dickinson) following the manufacturer's protocols. Next, 50 μl of rabbit polyclonal NFκB p65 antibody or control antibody was added, the tubes incubated at room temperature for 10 min, followed by a further 10 min incubation with 2.5 μl FITC conjugated anti-rabbit monoclonal antibody . 200 μl of cold propidium iodide (PI) was then added to the nuclei and the preparation was incubated for another 10 min and kept on ice until analyzed by flow cytometry.

Statistical analysis. Data from experiments involving nitrite analysis and flow cytomtery were analyzed by analysis of variance using the general linear model procedure of SAS (SAS Institute, 1995). $P \leq 0.05$ was accepted as the level of

significance. All experiments were repeated at least two times on chickens obtained from separate hatches. Data from each trial were analyzed separately.

RESULTS

Role of CD14 and TLR4 in LPS induction of iNOS. Figure 2.1 illustrates nitrite production by LPS-stimulated macrophages with or without blocking with 50μg/mL of anti-CD14 or TLR4 antibodies. Macrophages stimulated with 1μg/ml LPS produced 97.14 μM of nitrite as compared with 1.76 μM produced by sham-treated macrophages ($P \leq 0.05$). However, this increase in nitrite production was inhibited by almost 60% on pre treating the cells with 50μg/ml of either purified mouse anti-human CD14 (32.4 μM) or TLR4 (39.7 μM) for 1h before the addition of LPS. Pre treating the cells with mouse isotype matched control antibody did not affect the nitrite level (97.1 μM) relative to LPS-stimulated cells. In a separate experiment, the pretreatment of the macrophages with 100μg/ml of either CD14 or TLR4 antibody completely inhibited nitrite production (Fig 2.1) whereas all sham, LPS or isotype control macrophage cultures had expected levels of nitrite in their supernatants.

Effect of LPS treatment on CD14 and TLR4 expression. A preliminary time course study was conducted to see if CD14 and TLR4 proteins were inducible by LPS in chicken macrophages. The incidence of CD14 (Table 2.1) or TLR4 (Table 2.2) positive MQ-NCSU macrophages after exposure to 1μg/ml LPS did not differ from unstimulated controls at any time point post LPS stimulation in either of the two experiments. On the contrary, the mean fluorescence intensity (MFI) (as a measure of relative numbers of

receptors) of expression of both CD14 and TLR4 was significantly higher ($P < 0.05$) in LPS-stimulated macrophages at 6 and 12 h post stimulation as compared with the sham-treated control macrophages (Table 2.3 and 2.4). Based on these results the 6 and 12 h of LPS exposures were chosen to compare the expression of these molecules on macrophages from K and GB2 strain.

The comparison of CD14 and TLR4 expression on K and GB-strain macrophages in two separate experiments is given in Figure 2.2. No significant differences were observed in the overall percentage of macrophages positive for CD14 (Fig. 2.2 A) or TLR4 (Fig. 2.2 B) between the two chicken strains without or with 6 and 12 h post LPS stimulation in two separate experiments. On the contrary, relative number of these molecules on the surface of macrophages as measured by the mean fluorescence intensity (MFI) showed differences between K and GB2 macrophages. As shown in Figure 2.3 A, macrophages from both strains exhibited comparable CD14 surface expression intensities without LPS stimulation (K=161.05, GB2=167.34). However at 6 and 12 h post LPS stimulation CD14 expression intensity was significantly higher ($P < 0.05$) on K-strain macrophages (6h = 421.01, 12h = 403.50) as compared with GB2 macrophages (6h = 302.75, 12h = 245.68). On the other hand, TLR4 expression intensity was significantly higher ($P < 0.05$) on K-strain macrophages without LPS stimulation (58.47) or at 6h (77.08) and 12h (66.33) post LPS stimulation as compared with the GB2 (no LPS=30.23, 6 h LPS = 43.76, 12 h LPS = 53.86) macrophages. In a repeated experiment (Fig 2.3 B) the MFI values for CD14 were: no LPS = 63.82 and 67.92, 6 h LPS = 208.93 and 169.50, 12 h = 70.95 and 49.98 for K and GB2 macrophages, respectively. The MFI values for

TLR4 were: no LPS = 64.63 and 40.17, 6 h LPS = 116.62 and 49.03, 12 h LPS = 73.62 and 43.68 for K and GB2 macrophages, respectively.

Flow cytometric detection of NFκB. The results from two separate experiments conducted on chickens obtained from separate hatches are given in Figure 2.4. There was no difference in the Mean Channel Fluorescence (MCF) value of nuclear bound NFκB in K (MCF = 372.24) and GB2 macrophages (MCF = 373.66) without LPS stimulation. However, after treatment with LPS for 30 minutes (Experiment 1), DNA bound NFκB intensity was significantly (P = 0.05) higher in K-strain macrophages (MCF = 1148.53) as compared with GB2 macrophages MCF = 725.90). This nuclear expression of NFκB in chicken macrophages was inhibited by pre-treating cells with MG132 (a proteosome inhibitor that blocks the nuclear translocation of NFκB) (K = 499.70 and GB2 = 488.98). In a repeated experiment (Experiment 2), the MCF values for DNA bound NFκB on 30 minute LPS stimulation were 1241.88 and 676.23 (P = 0.05) for K and GB2 macrophages, respectively. Pretreating the cells with MG132 resulted in inhibition of nuclear expression of NFκB (K = 373.92 and GB2 = 370.10).

Effect of NFκB Activation Inhibition on LPS induced nitrite formation. MG132, a proteosome inhibitor, at concentrations of 25 and 50μM was not cytotoxic to macrophage viability as measured by the MTT assay (data not shown). The results of two separate experiments in which macrophages from two separate chicken hatches were pretreated with 50μM MG132 are given in Figure 2.5. As expected, LPS- stimulated K-strain macrophages produced significantly higher nitrite levels (Exp. 1 = 88.63 μM, Exp.

2 = 65.94 μM) than GB2 macrophages (Exp. 1 = 15.77 μM, Exp. 2 = 11.76 μM). However, pretreatment of macrophages with 50 μM MG132 completely suppressed the LPS-induced increase in nitrite production in culture supernatants of both K (Exp. 1 = 7.10 μM, Exp. 2 = 6.34 μM) and GB2 (Exp. 1 = 6.94 μM, Exp. 2 = 6.13 μM) macrophages.

DISCUSSION

Previous studies have categorized chicken macrophages from different genetic backgrounds into hyper- and hypo-responders based on differential LPS-induced iNOS expression and activity (Hussain and Qureshi, 1997, 1998). The iNOS hyper-responder group includes an egg type chicken strain, the Cornell K-strain ($B^{15}B^{15}$), as well as MQ-NCSU, a macrophage cell line derived from a meat-type chicken of unknown haplotype (Qureshi et al., 1990). The hypo responder group includes GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) egg type chickens. Hussain and Qureshi (1998) also reported that differential iNOS expression and activity occurs at the transcriptional level and that the post-transcriptional changes, such as iNOS mRNA degradation, do not account for these genotype differences. Recently it was reported that these observed genetic differences are intrinsic in nature such that they are not limited to the bacterial source of LPS (Dil and Qureshi, 2002). Dil and Qureshi (2002) also reported that iNOS hyper responder K strain macrophages also exhibited significantly higher number of TLR4, the LPS signaling receptor, than GB2 macrophages implying that a differential stimulation effect might be responsible for these differences.

The objective of the current study was to further characterize the role of LPS receptors and NFκB in the differential iNOS induction in chicken macrophages. In this regard, the involvement of LPS receptors in the LPS induction of iNOS in chicken macrophages was investigated initially. As presented in Figure 2.1, monoclonal antibodies (mAb) directed against human CD14 and TLR4 proteins, inhibited the LPS stimulated iNOS induction (measured as nitrite levels) by chicken macrophages, in an antibody concentration dependent manner. Pretreatment of MQ-NCSU cells with 50 μg of either of anti-CD14 or anti-TLR4 antibody produced partial blocking where as 100 μg pretreatment completely blocked the LPS induced chicken iNOS activity. Pretreatment with isotype matched control antibody did not affect the nitrite levels at all. These findings are consistent with mammalian studies that demonstrate the involvement of these cell surface proteins in LPS induction of inflammatory genes. Akashi et al. (2000) showed that mAbs against TLR4 blocked the LPS-induced TNF production by PBMCs. IL-6 production by human gingival fibroblasts was strongly blocked on pretreating the cells with HTA125 (mAb against TLR4) (Tabeta et al., 2000). Similarly, antibodies to CD14 block LPS stimulated activation of macrophages (Wright et al., 1990). Furthermore, Ohashi et al., (2000) have shown in bone marrow-derived macrophages, from endotoxin resistant HeJ and endotoxin-responsive C3H/HeN mice, that nitric oxide formation is dependent on a functional Tlr4 (gene encoding TLR4 protein). Tlr4 has been shown to be an important determinant of iNOS expression in mice bronchoalveolar lavage (BAL) cells (Kleeberger et al., 2000). In addition, Akashi et al (2000) demonstrated that signaling via TLR4 for LPS mediated NFkB activation is dependent on

CD14. Using resonance energy transfer (RET) microscopy, Jiang et al., (2000) demonstrated that LPS promoted physical proximity between CD14 and TLR4 resulting in LPS signaling and ultimately leading to nuclear translocation of NFkB. Our findings from this study clearly demonstrate that both CD14 and TLR4 are required for LPS mediated iNOS induction in chicken macrophages at 1μg/ ml concentration of the LPS used. This experiment also showed that mammalian mAbs were capable of recognizing LPS-related molecules on the surface of chicken macrophages. This was not surprising, as both CD14 and TLR4 are parts of the primitive more conserved innate immune system. Chicken and mouse Tlr2 have been mapped to an evolutionarily conserved chromosomal segment that shares homology with the human chromosomal segment (Boyd et al., 2000).

Next, we studied whether these receptor proteins are inducible in response to LPS in the chicken system and, if so, do iNOS hyper- and hypo-responder chicken macrophages differ in inducible expression of these proteins. A time course study was conducted using MQ-NCSU cells. The results showed that although the overall number of percent positive macrophages remains the same, the surface expression intensity of both of TLR4 and CD14 is increased on stimulation with LPS. The maximum induction of these proteins was achieved at 6 or 12 h post LPS stimulation. These observations are in accordance with previous studies that have described the LPS induced up-regulation of CD14 and TLR4 in various cell types (Landmann et al., 1996; Merchant et al., 1992; Tabeta et al., 2000). Based on the results obtained from this preliminary study 6 and 12 h LPS stimulation time was selected to compare the up-regulated expression of CD14 and

TLR4 on the surface of macrophages obtained from iNOS hyper responder K-strain and hypo-responder GB2 chickens. The overall percentage of macrophages positive for either CD14 or TLR4 obtained from either source did not differ, and no statistical difference were observed in the constitutive expression of CD14 between the iNOS hyper- and hypo- responder macrophages, which is consistent with our previous study (Dil and Qureshi, 2002). However, constitutive expression intensity of TLR4 as well as inducible expression intensity of both CD14 and TLR4 was significantly ($P < 0.05$) higher in K strain macrophages in comparison with GB2 macrophages at either of the two time points post LPS stimulation. CD14 expression intensity varied between experiments perhaps due to slight temperature variations (as shown by Antal-Szalmas et al., 1997). These findings clearly demonstrated that these LPS related receptors are expressed differentially both constitutively (TLR4) and inducibly (TLR4 and CD14) on the surface of chicken macrophages. It seems likely that a greater number of LPS binding and signaling receptors on the surface of K strain macrophages, perhaps, could lead to stronger signal transduction and ultimately higher iNOS expression and activity than in the GB2 macrophages that express fewer LPS related receptors.

Since there was an effort to relate differential expression of LPS related receptors to differential LPS-induced iNOS response, it was very important to assess LPS induced activation of signaling molecules in these chicken macrophages. NFκB served as the best candidate in this regard, as it has been shown to be a required nuclear factor to activate the chicken iNOS gene expression by LPS (Lin et al., 1996), and LPS signaling via TLR4 is known to lead to NFκB activation (Akashi et al., 2000). NFκB activation is achieved

through the signal-induced proteolytic degradation of IκB in the cytoplasm. LPS induced signaling via TLR4 initiate a signaling cascade leading to activation of IκB kinases (Guha and Mackman, 2001) which phosphorylate IκB at specific amino terminal serine residues (Regnier et al., 1997). Phosphorylated IκB is then selectively ubiquitinated by an E3 ubiquitin ligase (Alkalay et al., 1995). In the last step of this signaling cascade, phosphorylated and ubiquitinated IκB (Fig. 2.6) (which is still associated with NFκB in the cytoplasm) is selectively degraded by 26S proteosome thereby freeing NFκB to translocate to the nucleus (Chen et al., 1995). The LPS-induced NFκB activation was analyzed by quantifying the DNA-bound NFκB via flow cytometry as described by Foulds (1997). In non-LPS treated chicken macrophages from either K or GB2 chickens, minimal DNA binding activity of NFκB transcription factor was observed. LPS stimulation induced a significant increase in the NFκB DNA-binding activity that was significantly ($P \leq 0.05$) higher in K strain macrophages as compared with the GB2 macrophages in either of the two experiments (Figure 2.4). However, pretreatment of the macrophages with the proteosome inhibitor, MG132 completely abrogated the LPS induced NFκB activation as well as iNOS induction in macrophages from either of the two sources (Figure 2.5). Previous studies have demonstrated that the proteosome inhibitor MG132, blocks cytokine induced degradation of IκB (NFκB inhibitor) thereby preventing activation and nuclear translocation of NFκB (Palombella et al., 1994; Wang et al., 1999), and it also inhibited the expression of multiple NFκB dependent proinflammatory genes (Wang et al., 1999). Therefore, it is possible that increased

nuclear binding of the transcription factor NFκB in macrophages from iNOS hyper responder genotype (i.e., K-strain) chickens may be an important reason for hyper responsiveness to LPS-induced signaling.

The data presented in this study demonstrated that the genetic differences observed in the iNOS expression and activity are due to a differential stimulation effect making one strain hyper-responsive and other hypo-responsive to LPS induction of iNOS. C3/HeJ strain of mouse is an example of bacterial endotoxin hypo responsiveness in mammalian system. In C3H/HeJ mice a single point mutation in LPS locus (that encodes Tlr4) modified the cytoplasmic domain of Tlr4 at an evolutionarily conserved residue that results in defective endotoxin response (Poltorak et al., 1998). Whereas in C57BL/10ScCr mice, no Tlr4 mRNA is made owing to deletion of the locus due to a null mutation (Poltorak et al., 1998). Furthermore LPS tolerance in mice has been correlated with down-regulation of surface TLR4 expression on macrophages leading to a significant reduction in NFκB DNA-binding activity and ultimately resulting in reduced IL-6 production (Nomura et al., 2000). In addition the work of Hu et al (1997) have provided some evidence suggesting that in chickens, distinct allelic forms of the *Lps* gene influence survival during endotoxin mediated infections. An attractive hypothesis to explain these findings could be that mutations in the Tlr4 gene result in varying expression of TLR4 on the surface of chicken macrophages from different genetic sources and differential LPS signaling via these differentially expressed receptors leads to hyper and hypo responsiveness in iNOS expression and activity.

In spite of the considerable advances in TLR4 work in mammalian systems, chicken TLR4 has not been cloned yet. Our previous study (Dil and Qureshi, 2002) was the first report describing the expression of TLR4 on the surface of chicken macrophages. Our current study is the first report describing a relationship between TLR4 mediated LPS signaling and iNOS induction in chicken macrophages. It remains to be seen whether strain-dependent variation in LPS responses in terms of iNOS induction in the chicken may be traced to the Tlr4 gene.

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Table 2.1. Incidence of LPS-inducible CD14 positive chicken macrophages.

Treatment	Percent Positive Macrophages[1]	
	Experiment 1	**Experiment 2**
No LPS	86.86 ± 4.84	93.95 ± 1.09
30 Min in LPS	88.92 ± 4.84	--
1 h LPS	82.92 ± 4.84	90.37 ± 1.09
2 h LPS	87.84 ± 4.84	---
3 h LPS	89.33 ± 4.84	94.78 ± 1.09
6 h LPS	91.49 ± 4.84	98.88 ± 1.09
12 h LPS	93.48 ± 4.84	99.54 ± 1.09
24 h LPS	----	93.34 ± 1.09

[1]MQ-NCSU (5×10^5) macrophages were treated with 1μg / ml *E. coli* LPS. The cells were then stained with 1: 4 dilution of FITC mouse anti-human CD14 and analyzed by FACScan. Analysis gate was set on macrophages using forward and side scatter gating. Values represent mean ± SE of percentage of positive cells in gated macrophage population. Data were obtained from 3 replicate samples at each time point.

Table 2.2. Incidence of LPS-inducible TLR4- positive chicken macrophages.

Treatment	Percent Positive Macrophages[1]	
	Experiment 1	**Experiment 2**
No LPS	88.37 ± 4.54	90.16 ± 2.07
30 Min LPS	84.66 ± 4.54	---
1 h LPS	86.73 ± 4.54	88.33 ± 2.07
2 h LPS	88.28 ± 4.54	---
3 h LPS	88.04 ± 4.54	90.72 ± 2.07
6 h LPS	88.76 ± 4.54	90.79 ± 2.07
12 h LPS	86.92 ± 4.54	90.78 ± 2.07
24 h LPS	----	89.45 ± 2.07

[1]MQ-NCSU (5 x $10^{5)}$ macrophages were treated with 1μg/ml *E. coli* LPS. The cells were then stained with 1: 4 dilution of PE mouse anti-human TLR4 and analyzed by FACScan. Analysis gate was set on macrophages using forward and side scatter gating. Values represent mean ± SE of percentage of positive cells in gated macrophage population. Data were obtained from 3 replicate samples at each time point.

Table 2.3. Intensity of staining of MQ-NCSU macrophages for CD14 expression.

Treatment	Mean Fluorescent Intensity[1]	
	Experiment 1	**Experiment 2**
No LPS	23.64 ± 0.81	62.21 ± 3.55
30 Min in LPS	24.92 ± 0.81	---
1 h LPS	19.88 ± 0.81	61.39 ± 3.55
2 h LPS	23.57 ± 0.81	---
3 h LPS	21.50 ± 0.81	96.11 ± 3.55*
6 h LPS	49.03 ± 0.81*	169.95 ± 3.55*
12 h LPS	79.75 ± 0.81*	194.77 ± 3.55*
24 h LPS	----	57.78 ± 3.55

[1]MQ-NCSU (^{5} x 10^5) macrophages were treated with 1μg/ml *E. coli* LPS. The cells were then stained with 1: 4 dilution of FITC mouse anti-human CD14 and analyzed by FACScan. Analysis gate was set on macrophages using forward and side scatter gating. Values represent mean ± SE of mean fluorescence intensity (MFI) (indicative of receptor number) in gated macrophage population. Data was obtained from 3 replicate samples at each time point. * $P < 0.05$ compared with No LPS.

Table 2.4. Intensity of staining of MQ-NCSU macrophages for TLR4 expression.

Treatment	**Mean Fluorescent Intensity**[1]	
	Experiment 1	**Experiment 2**
No LPS	30.49 ± 1.14	27.16 ± 0.77
30 min LPS	31.84 ± 1.14	---
1 h LPS	32.16 ± 1.14	29.02 ± 0.77
2 h LPS	31.30 ± 1.14	---
3 h LPS	36.00 ± 1.14	27.18 ± 0.77
6 h LPS	46.36 ± 1.14 *	36.54 ± 0.77*
12 h LPS	32.18 ± 1.14	43.12 ± 0.77*
24 h LPS	----	25.00 ± 0.77

[1]MQ-NCSU (^{5} x 10^5) macrophages were treated with 1μg/ml *E. coli* LPS. The cells were then stained with 1: 4 dilution of PE mouse anti-human TLR4 and analyzed by FACScan. Analysis gate was set on macrophages using forward and side scatter gating. Values represent mean ± SE of mean fluorescence intensity (MFI) (indicative of receptor number) in gated macrophage population. Data was obtained from 3 replicate samples at each time point. * P=0.05 compared with No LPS.

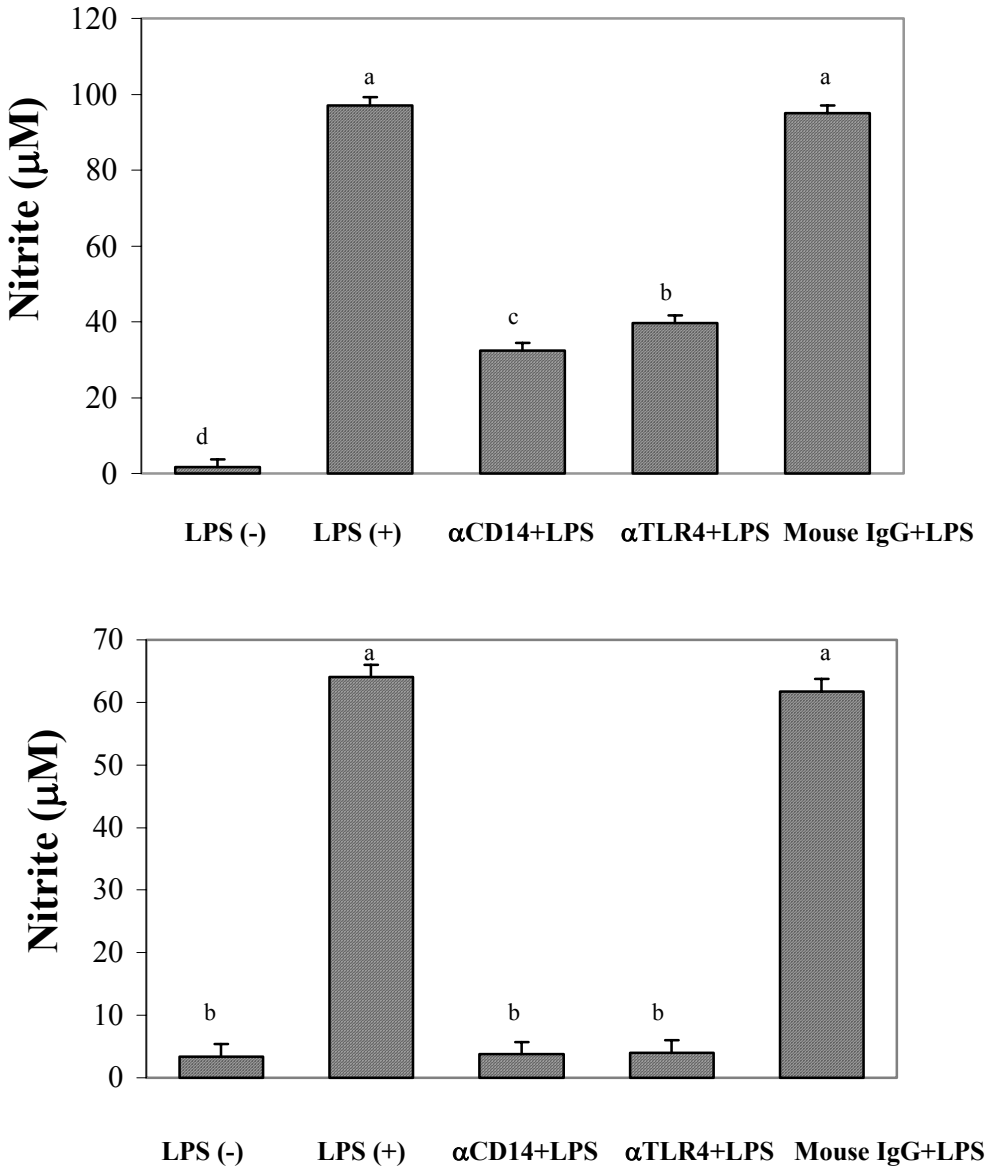

Fig. 2.1. Blocking of CD14 and TLR4 receptors on the surface of chicken macrophages by anti human CD14 and TLR4 antibodies: **1** x 10^6 chicken macrophages (MQ-NCSU) were treated with 1μg / mL *E. coli* LPS for 24 h. Cells in the anti-CD14, anti-TLR4 treated samples and control sample were pre incubated for 1 h with 50μg / mL (upper panel) or 2 h with 100 μg / mL (lower panel) of purified mouse anti human TLR4, CD14 or the mouse isotype matched control antibody prior to the addition of LPS. Nitrite production was measured in the culture supernatants using Griess method. Each bar represents the mean nitrite levels (μM) $\pm$ the SE in the culture supernatant from three replicate wells. Non-overlapping superscript letters on bars indicate significant difference at $P < 0.05$

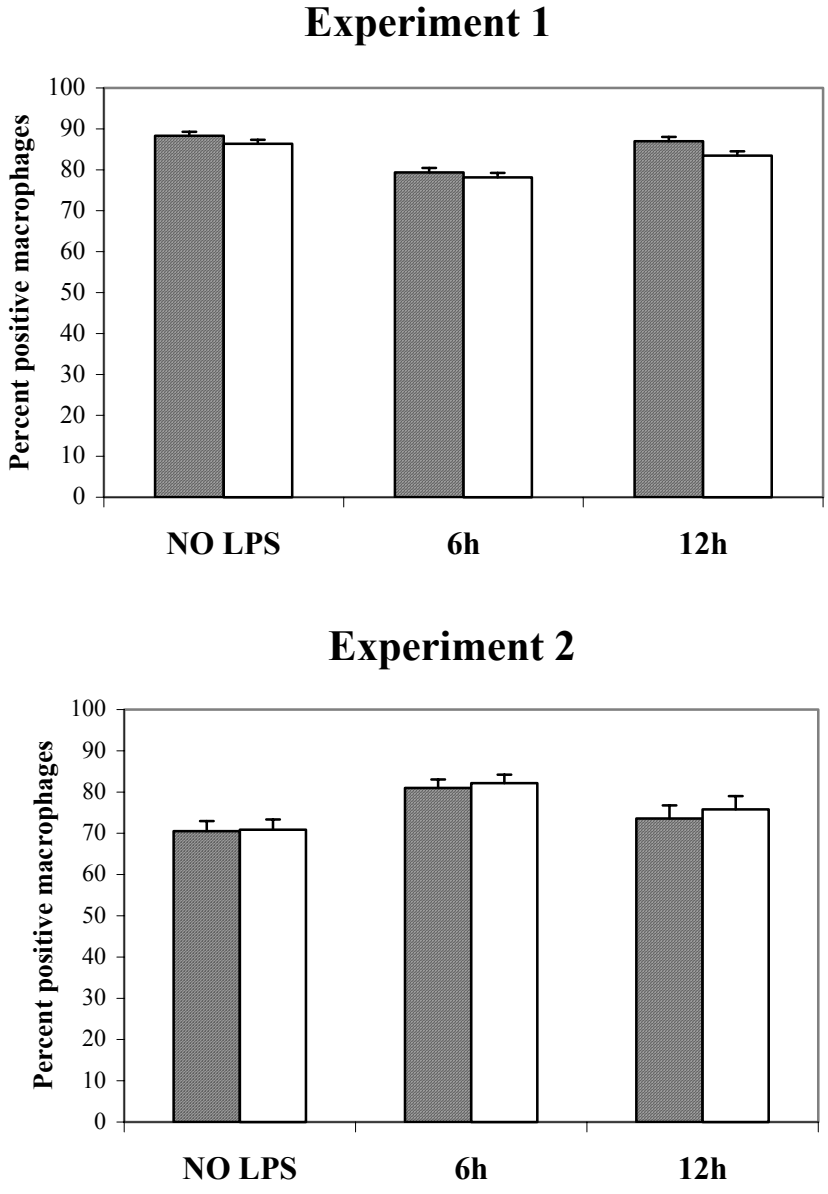

Fig. 2.2A. Quantification of LPS-inducible CD14: 5 x 10^5 macrophages from K- or GB2 strain of chickens were treated with 1μg / ml *E. coli* LPS for 6 and 12 h. The cells were then stained with 1: 4 dilution of FITC mouse anti-human CD14 and analyzed by FACScan. Analysis gate was set on macrophages using forward and side scatter gating. Stripped bars correspond to K strain and open bars correspond to GB2 chicken. Each bar represents mean $\pm$ SE of percentage of positive cells in gated macrophage population. Data were obtained from 3-4 individual chickens per strain.

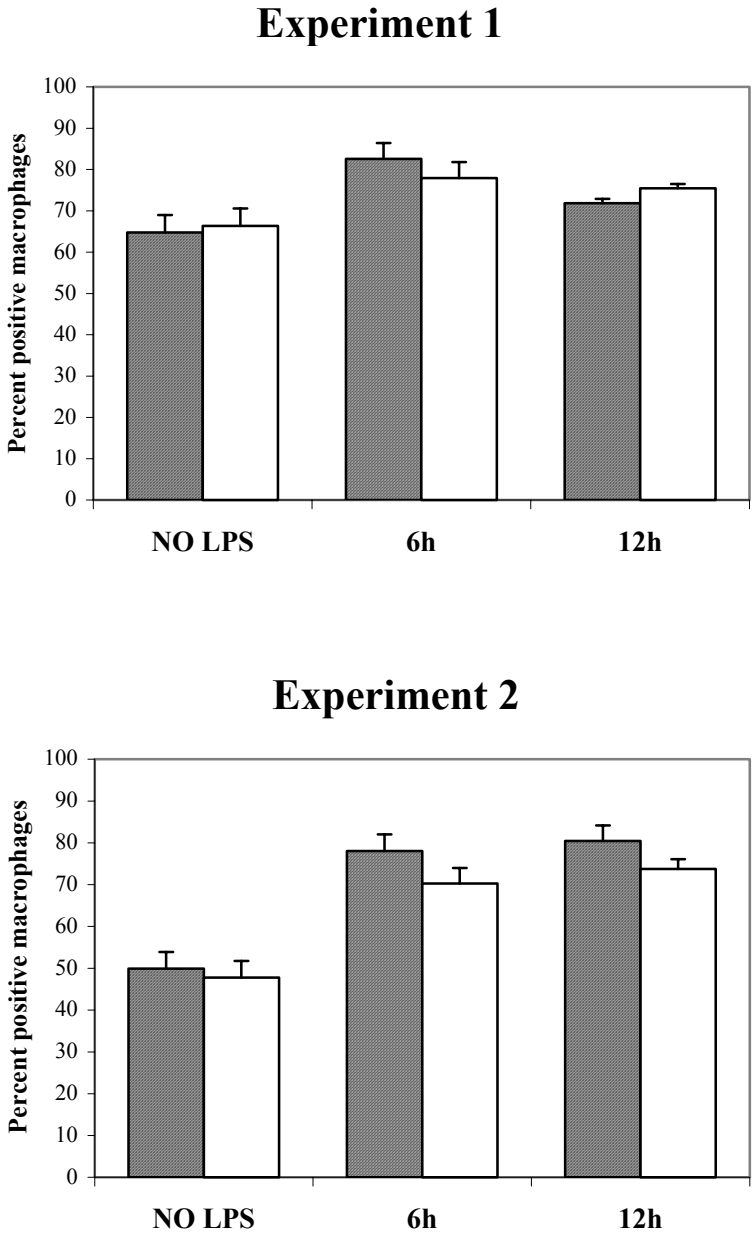

Fig. 2.2 B. Quantification of LPS-inducible TLR4: 5 x 10^5 macrophages from K- or GB2 strain of chickens were treated with 1μg / ml *E. coli* LPS for 6 and 12 h. The cells were then stained with 1: 4 dilution of PE mouse anti-human TLR4 and analyzed by FACScan. Analysis gate was set on macrophages using forward and side scatter gating. Stripped bars correspond to K strain and open bars correspond to GB2 chicken. Each bar represents mean $\pm$ SE of percentage of positive cells in gated macrophage population. Data were obtained from 3-4 individual chickens per strain.

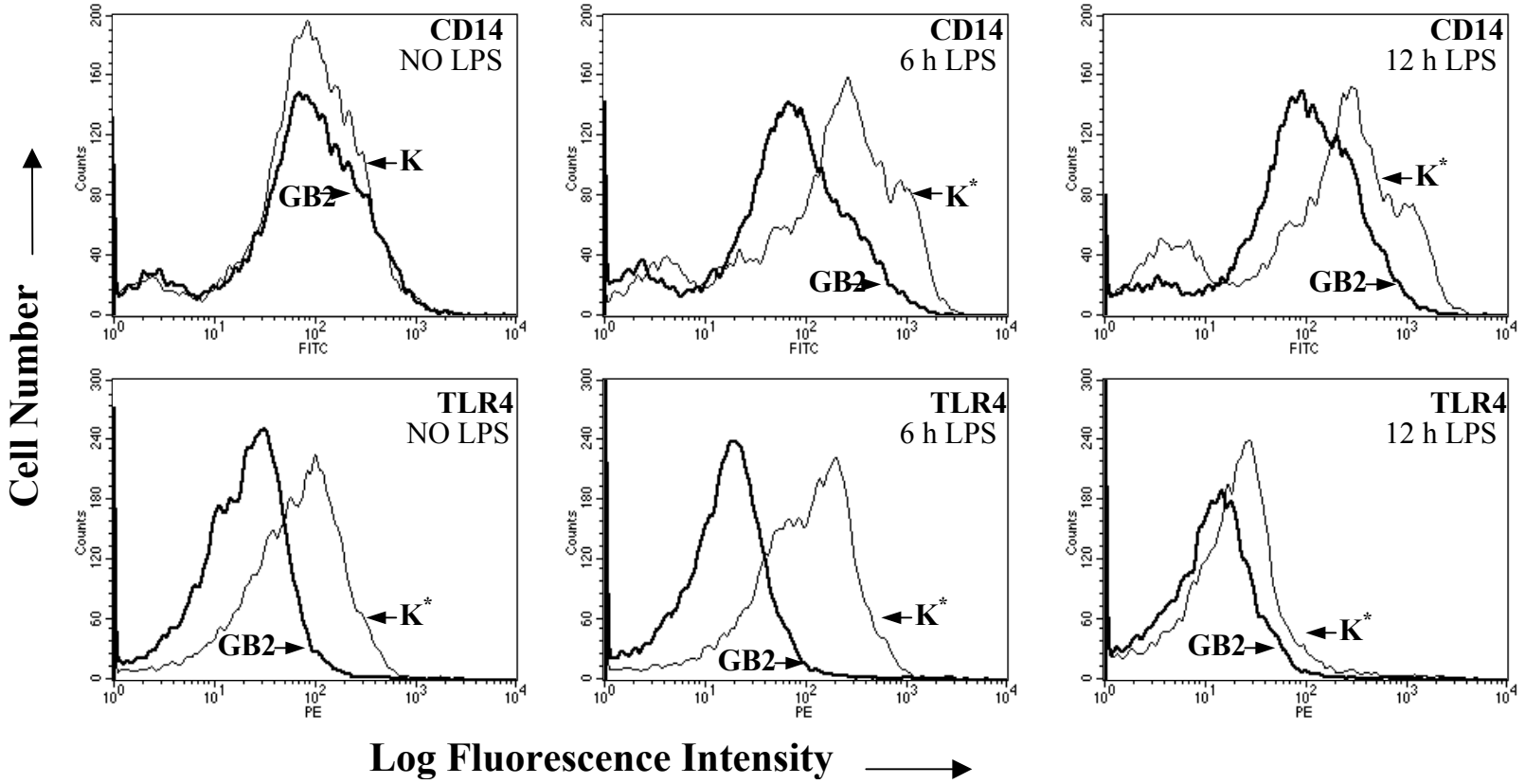

Fig. 2.3A. Representative flow cytometry histograms for fluorescence intensity of CD14 (upper panel) and TLR4 (lower panel) on the surface of chicken macrophages: 5 x 10^5 macrophages from K- or GB2 strain of chickens were treated with 1μg / ml *E. coli* LPS for 6 and 12 h. The cells were then stained with 1:4 dilution of either FITC mouse anti-human CD14 or PE mouse anti-human TLR4 and analyzed by FACScan. Thick line represents GB2 strain macrophage cell surface intensity and thin line indicates K-strain intensity. * $P < 0.05$ compared with GB2.

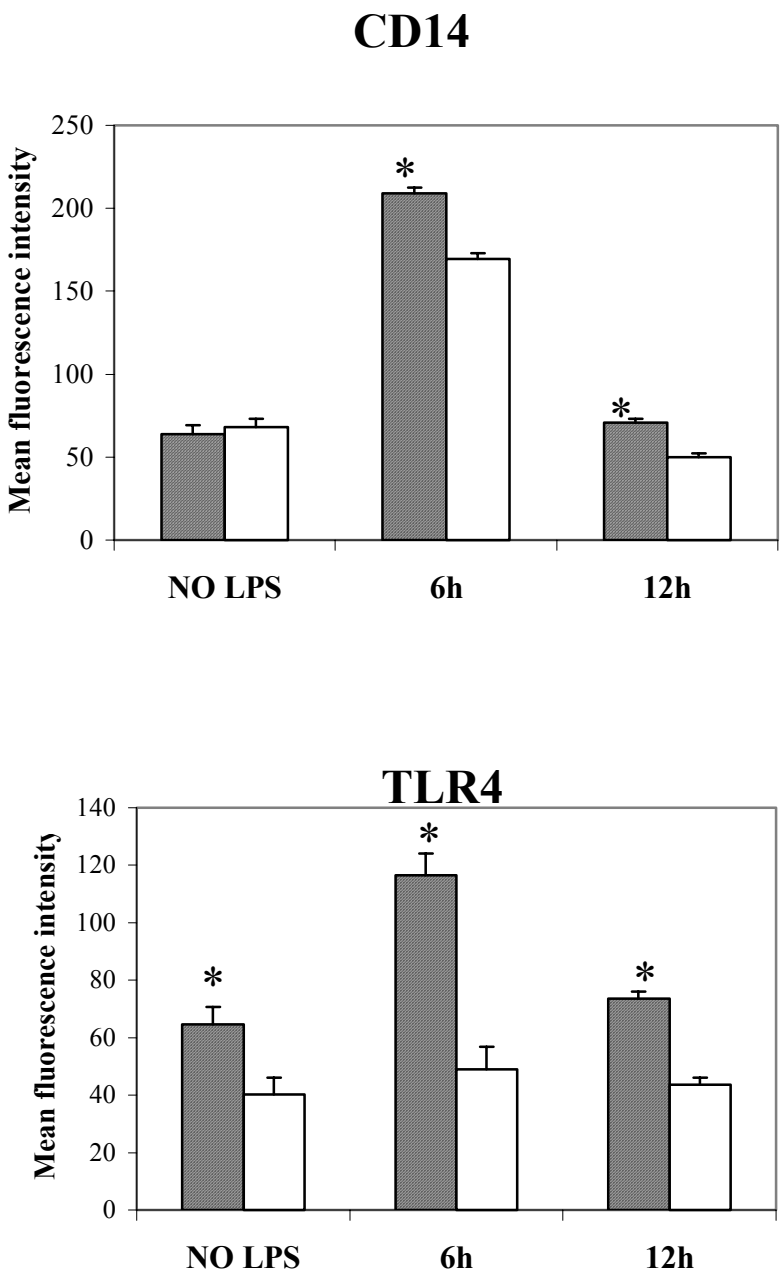

Fig 2.3B. Fluorescence intensity of CD14 and TLR4 on the surface of chicken macrophages: 5 x 10^5 macrophages from K- or GB2 strain of chicken were treated with 1μg / ml *E. coli* LPS for 6 and 12 h. The cells were then stained with 1:4 dilution of either FITC mouse anti-human CD14 or PE mouse anti-human TLR4 and analyzed by FACScan. Stripped bars correspond to K strain and open bars correspond to GB2 chickens. Each bar represents the mean fluorescence intensity (MFI) $\pm$ the SE from 3-4 individual chickens per strain. * $P < 0.05$ compared with GB2.

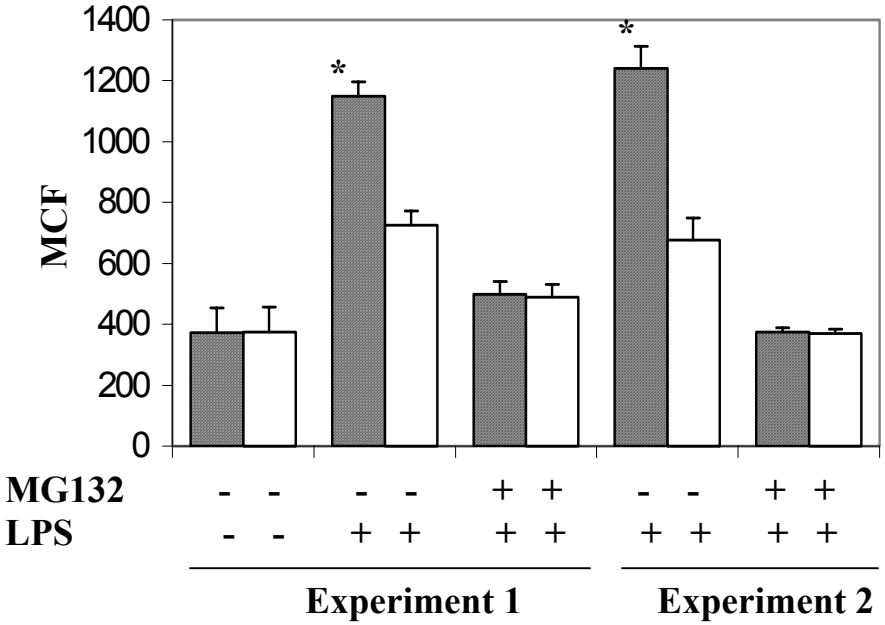

Fig. 2.4. Effect of LPS stimulation on NFκB activation in GB2 and K strain macrophages: 5 x 10^5 macrophages from either source were incubated at 41 C, 5% CO_2 for 30 min in the presence of 1μg / ml *E.coli* LPS with or without 50μM MG132 pretreatment (1h). Cells were prepared for staining and then stained with 50 μl of polyclonal NFκB p65 antibody followed by incubation with 2.5 μl FITC anti-rabbit monoclonal antibody and finally 10 min incubation with 200 μl of cold propidium iodide. The cells were then analyzed by flow cytometry. Stripped bars correspond to K strain and open bars correspond to GB2 chickens. Each bar represents the mean channel fluorescence (MCF) $\pm$ the SE from 3-4 individual chickens per strain. *P < 0.05 compared with GB2.

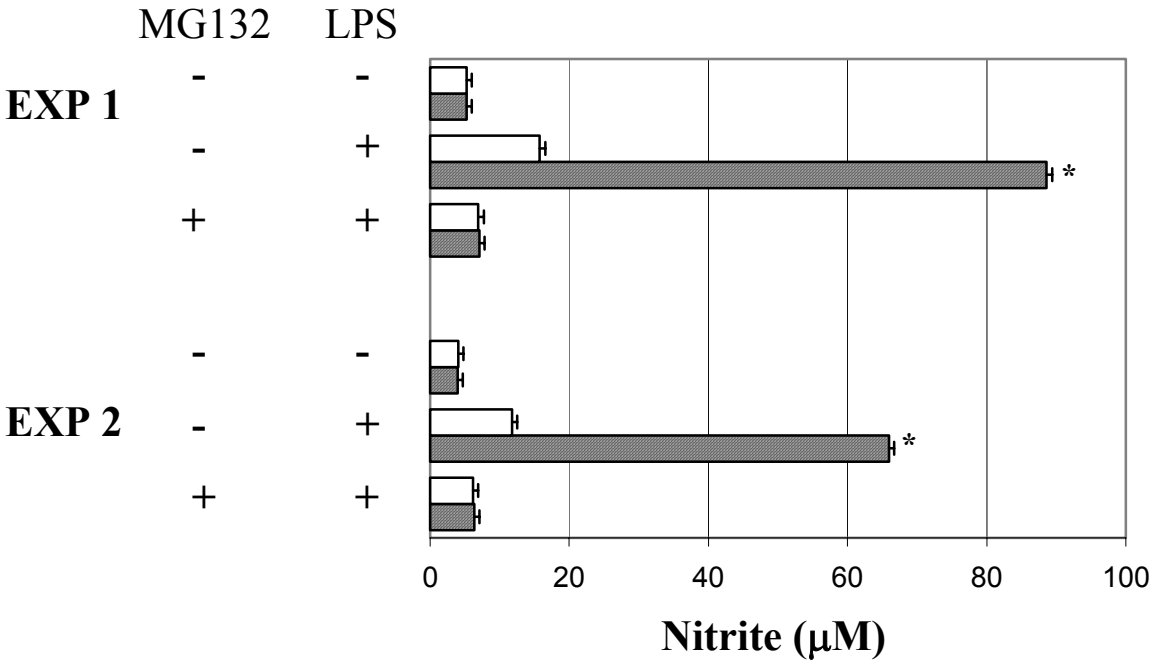

Fig. 2.5. Effect of inhibition of NFκB activation on nitrite production from GB2 and K-strain macrophages: Sephadex-elicited abdominal exudate macrophages (1 X 10^6) from either source were cultured for 24 h in the presence of 1 μg / ml of *E. coli* LPS with or without 50 μm MG132 pretreatment (1 h). Nitrite levels were measured in the culture supernatant fraction by Griess method in two independent experiments. Stripped bars correspond to K strain and open bars correspond to GB2 chickens. Each bar represents means of nitrite level $\pm$ SE in the culture supernatant from 3 replicate samples from a pool of 5-7chicks per group. *$P < 0.05$ compared with GB2.

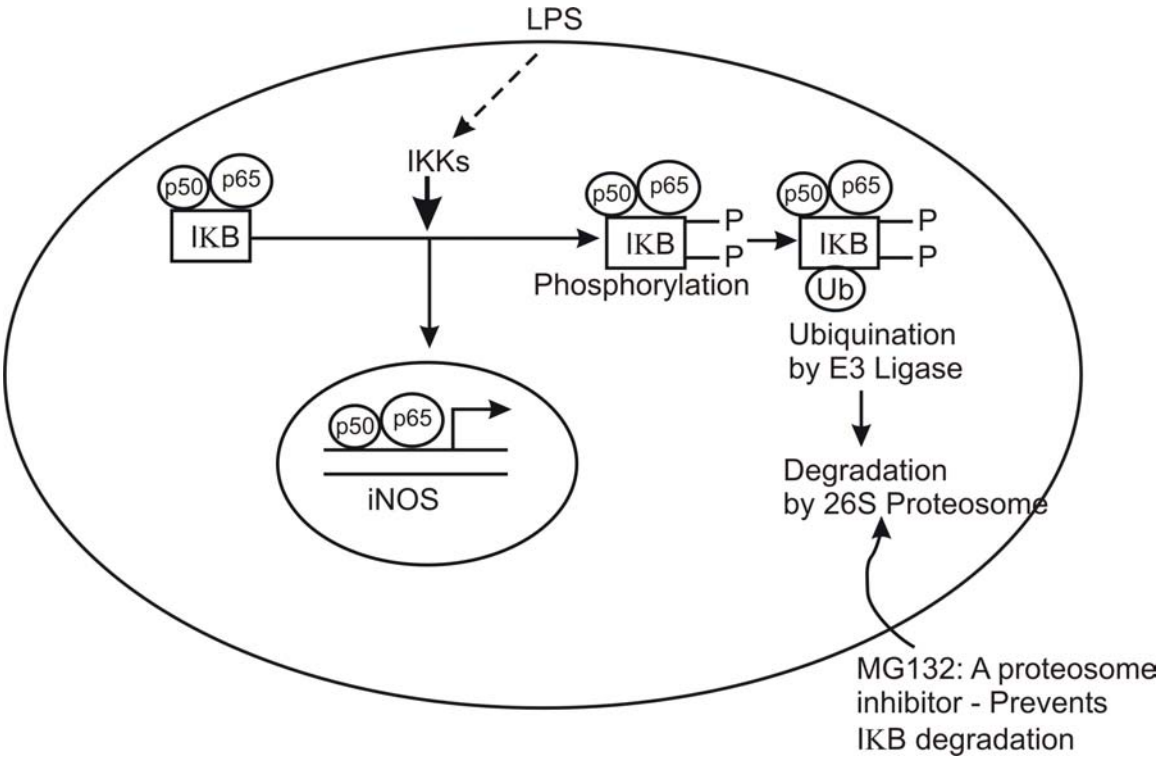

Fig. 2.6. Schematic representation of LPS mediated NFκB activation. NFκB is sequestered in the cytoplasm bound with its inhibitor IκB. Signal induced activation of I kappa kinases (IKKs) results in the phosphorylation of IκB. Once phosphorylated by the IKKs, IκB is ubiquitinated by E3 ligase, facilitating its degradation by proteosome. Released NFκB dimer p50/p65 can then translocate to the nucleus and affect the target genes. The proteosome inhibitor MG132, blocks signal induced degradation of IκB thereby preventing activation and nuclear translocation of NFκB.

CHAPTER 3

Interleukin-1β does not contribute to genetic strain-based differences in iNOS expression and activity in chicken macrophages.

ABSTRACT The objective of the current study was to examine the expression of IL-1β in macrophages from iNOS hypo (GB2, B^6B^6) and hyper (K-strain, $B^{15}B^{15}$) responsive chicken lines and to examine if LPS-mediated induction of IL-1 modulates iNOS induction in these genetic lines of chicken. Sephadex-elicited macrophages were stimulated with 1 μg / ml of *E. coli* LPS. The iNOS mRNA, determined by RT-PCR, followed the expected expression profile, i.e; K-strain macrophages had higher iNOS mRNA as compared with the macrophages from GB2 genotype chickens. On the contrary, IL-1 mRNA was initially comparable between K and GB2 chickens at 3 h LPS stimulation but persisted up to 9 h only in GB2 macrophages. The LPS-inducible IL-1 surface receptor expression, measured by flow cytometry, was found to be higher in GB2 than on K-strain macrophages. Blocking of IL-1 receptor by the anti-IL-1 receptor antibody significantly reduced the LPS-mediated iNOS expression by 50% over the LPS-stimulated controls as quantified by competitive RT-PCR. Furthermore, iNOS activity, determined by the nitrite quantification in macrophage culture supernatants, was also reduced to 50%. However, this magnitude of inhibition was similar in both K and GB2 macrophages. While these observations suggest that IL-1 is involved in mediating LPS-induced iNOS expression and activity, the differential response of GB1 and K-strain macrophages in terms of LPS-induced iNOS expression and activity is certainly not due to a defect in IL-1 receptor function. Instead, this study supports the previous observation

that differential (enhanced) expression of CD14 and TLR4 as well as preferential binding of NFkB may be responsible for hyper iNOS responsiveness in K-strain macrophages versus the low responder GB2 chicken macrophages.

Key words: Macrophage; iNOS; Chicken; Genetic lines; IL-1β; IL-1R_I

INTRODUCTION

Macrophages represent a system of widely dispersed cells that are able to recognize and destroy invading microorganisms and altered host components such as apoptotic cells (Gordon, 1998). They are also the key regulatory cells involved in initiating and directing the immune and inflammatory responses. Stimulation of macrophages with LPS leads to a rapid and transient expression of genes encoding proteins with immunomodulatory activities (Ziegler-Heitbrock, 1989). Inducible nitric oxide synthase (iNOS) and Interleukin 1 (IL-1) are two examples of such gene-products. Nitric Oxide synthase (NOS) is the enzyme responsible for the synthesis of nitric oxide (NO) from L-arginine (MacMiking et al., 1997). NO is a highly reactive, low molecular weight, cytotoxic effector molecule (Stuehr and Marletta et al., 1987). There are three known isoforms of NOS: two constructive forms, neuronal NOS and endothelial NOS, and an inducible form (iNOS), which was first identified in macrophages after stimulation with pro-inflammatory cytokines (MacMiking et al., 1997). Nitric oxide

production by iNOS is an important-host defense mechanism against microbial pathogens in macrophages (Hibbs et al., 1988).

Interleukine-1β is the critical cytokine which induces iNOS gene expression in several cell types (Chao et al., 1997; Jaimes et al., 1997; Wong et al., 1996). IL-1 binds to two receptors, type-I IL-1 receptor (IL-$1R_I$) and type-II receptor. IL-$1R_I$ has a cytoplasmic tail responsible for signal transduction. The type-II receptor does not transmit a signal to the cytoplasm, and serves as a decoy receptor that down-regulates the inflammatory response (Pruitt et al, 1996; Auron, 1998).

Previous studies have shown that chicken macrophages obtained from different genetic sources differ in their iNOS gene expression and activity at the transcription level (Hussain and Qureshi, 1997, 1998). This difference in iNOS induction has categorized chickens into hyper, such as K strain ($B^{15}B^{15}$), and hypo, such as GB2 ($B^{6}B^{6}$) strain, iNOS responders based on macrophage sensitivity to LPS. We have reported previously that iNOS hyper responder chicken macrophages also constitutively express a higher number of Toll like receptor 4 (TLR4), the LPS signaling molecule, which might be responsible for differential LPS mediated induction of iNOS (Dil and Qureshi, 2002). Later studies provided additional evidence in support of this concept of differential stimulation effect by showing that inducible TLR4 as well as CD14 differs significantly in these chicken macrophages. We also showed that NFκB activation in iNOS hyper responder macrophages is significantly higher than hypo responder macrophages (unpublished data). Since IL-1 is a potent inducer of iNOS gene expression, the objective of the current study was to study the expression of IL-1β in macrophages from iNOS

hypo and hyper responsive chicken lines and to examine whether LPS-mediated induction of IL-1 modulates iNOS induction in these genetic lines of chicken.

MATERIAL AND METHODS

Experimental Animals. Cornell K-strain ($B^{15}B^{15}$) and GB2 ($B^{6}B^{6}$) chickens were maintained at the Department of Poultry Science, North Carolina State University under the Institutional Animal Care and Use Committee's approval. These chickens were fed a corn- soybean based chick starter diet with 21% crude protein. The feed and water were available for *ad libitum* consumption.

Reagents. Lipopolysaccharide (LPS) (*Escherichia coli* (Serotype O55.B5) obtained from Sigma Chemical Co. (St. Louis, MO) was dissolved in sterile distilled deionized water (Mediatech, VA; less than 0.002 ng endotoxin units/mL) and stored frozen at –20 C until used. Rabbit anti-chicken IL-1 receptor ($IL\text{-}1R_I$) antibody was generously provided by Kirk Klasing (University of California, Davis, CA). FITC conjugated anti-rabbit IgG monoclonal antibody was purchased from Sigma Chemical Co. (Saint Louis, MO). The competitor cDNA fragment was kindly provided by Karel A. Schat (Cornell University Ithaca, NY).

Macrophage Source. Sephadex-elicited macrophages: At five weeks of age, 5-7 chicks in each group were weighed and injected intra-abdominally with a 3% (wt. /vol.) Sephadex® G50 (Sigma) suspension at a dose of one mL/100 g body weight as described previously (Qureshi et al., 1986). Approximately 42 h post Sephadex injection, chicks were euthanized using CO_2 and the abdominal exudate was collected in siliconized glass

tubes from each chick by abdominal lavage with cold 0.85% saline containing 0.5U/mL heparin. The tubes containing abdominal exudate cells (AEC) were kept on ice for 15 minutes to allow any unabsorbed Sephadex to settle. Twelve mL of supernatants containing cells were then transferred to clean siliconized tubes and cells were pelleted by centrifugation at 430 g for 20 minutes. The AEC pellets were then resuspended in RPMI 1640 complete medium (CM: RPMI 1640 medium supplemented with 100 U/mL Penicillin, 100 μg/mL Streptomycin, 0.25μg/mL Amphotericin B and 5% heat-inactivated fetal bovine serum).

Transformed Macrophage Cell line: The MQ-NCSU, a transformed chicken macrophage cell line (Qureshi et al., 1990) was maintained in LM-Hahn's growth medium at 41C in a 5% CO_2 humidified incubator. For receptor studies, cells were collected in the log phase of growth and maintained in CM.

Detection of iNOS and IL-1β mRNA expression by RT-PCR. Total RNA was isolated from LPS stimulated or unstimulated Sephadex elicited macrophages from both K- and GB2 strains of chickens, using RNeasy kit (Qiagen, Inc., Valencia, CA) and the RNase-free DNase kit (Qiagen) following manufacturer's instructions. RNA was eluted in 100 μL of nuclease-free water and quantified by spectrophotometry. Subsequently, samples were reverse transcribed and amplified by the Titan^{TM} One Tube RT-PCR kit (Roche Molecular Biochemicals, Mannheim, Germany) in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf Scientific Inc., Westbury, NY). Each 50 μL RT-PCR reaction included: 0.2mM dNTP mix, 5 mM DTT, 5 U RNase inhibitor, 1 μL Titan^{TM} enzyme mix, 1 X RT-PCR buffer, 2 mM $MgCl_2$, (Master mix) 0.4 μM each upstream and

downstream primer, and 1.5 μg of the appropriate RNA sample. Primers for chicken iNOS (5'-AATGCTGTGCCCATGGCAGTTTGCA-3'; 5'-CACCTCAAGGAGCATGTTGGCAACA-3'), chicken IL-1β (5'-ACAGAGATGGCGTTCGTTCCCGA-3'; 5'-TCAGCTCGACGCTGTCGATGT-3') and chicken β-actin (5'-CCCCCGTGCTGTGTTCCCATCTATCG-3'; 5'-GGGTGCTCCTCAGGGGCTACTCTCAG-3') were synthesized by Sigma-Genosys (Cambridge, UK), using sequences published previously (Xing and Schat, 2000). Prior to the addition of the master mix, primers and RNA were incubated at 68^{o} C for 2.5 min and placed immediately on ice. Next, the master mix, containing the enzymes and buffer, was added, and the complete reaction was incubated at 50^{o} C for 30 minutes for reverse transcription. The following procedure was utilized for amplification: 1 cycle of 94^{o} C for 2 min; 35 cycles of 94^{o} C for 30 sec, 65^{o} C for 30 seconds, and 68^{o} C for 1 min (with the addition of 5 sec / cycle for cycles11 through 35); and a final elongation step of 68^{o} C for 5 min. RT-PCR reactions with iNOS, IL-1β and β-actin primers were conducted in separate tubes using the same RNA sample simultaneously in the thermocycler. Samples were stored at –20^{o} C until analysis.

PCR products were thawed and 5 μL of loading dye (50% bromophenol blue, 50% glycerol) was added to each. Subsequently, PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide (0.5 μg/mL) for 1 h at 150 volts. Low DNA mass ladder (Roche) was utilized as a molecular weight marker. DNA bands were visualized and densitometric analysis was done on an UV transilluminator (Chemilmager 4400, Alpha Innotech, San Leandro, CA).

Quantification of IL-1R$_I$ expression by Flow cytometry analysis. Sephadex-elicited AEC from K and GB2 strains were allowed to adhere to the Petri dish surface for 1 h. The cells were then recovered with a sterile rubber policeman into CM yielding 99.5 to 100% macrophages based on morphological criteria. After adjusting the cell concentration to 5 X 10^6/mL in siliconized glass tubes, 1μg / mL of LPS was added and 100 μl of each sample was transferred to polystyrene tubes (Becton-Dickinson Labware, Lincoln Park, NJ) and incubated for 6 and 12 h at 41 C, 5% CO_2. At the end of each incubation period, samples were incubated with heat inactivated normal rabbit serum for 2-3 minutes followed by the addition of 1:4 dilution of rabbit anti-chicken IL-1R$_I$ antibody for 30 min on ice. This was followed by the addition of 1:16 dilution of FITC-labeled anti-rabbit IgG monoclonal antibody for 30 min incubation on ice and in the dark. After three washes with PBS, cells were resuspended in 200μl of PBS and analyzed by flow cytometry using a FACScan flow cytometer (Becton-Dickinson Immunocytometry, San Jose, CA). For each sample, 15,000 events were obtained.

IL-1R$_I$ blockage and iNOS mRNA expression by Competitive RT-PCR. Semi quantitative analysis of iNOS mRNA was performed by a competitive RT-PCR as described before (Sun et al., 1996, Xing and Shat, 2000). In brief, an equivalent amount of RNA samples were co amplified with an added constant concentration of the Multi Sequence Segment (MSS, competitor cDNA) with equal efficiencies in the same PCR tubes. The competitor and target shared the same iNOS sense and anti sense primers used for target amplification. Reverse transcription and amplification, gel electrophoresis of

PCR products and visualization were performed as described above. After PCR products were visualized the target / competitor ratio was calculated for semi quantification.

Macrophage Culture Set Up for IL-1R$_I$ blocking Study. In order to determine the involvement of IL-1 stimulation in iNOS gene induction, pooled AEC from 5 chickens per strain were added in a 24 well culture plate at a viable cell concentration of 1 x 10^6/mL of CM per well. Culture plates containing AEC were incubated for 2 h at 41C in 5% CO_2 to allow macrophage adherence. After incubation, non-adherent cells or any contaminating erythrocytes were removed by twice washing the wells with CM. The culture wells were then replenished with one mL of fresh CM. Next, a 1: 20 dilution of chicken anti- IL-1R$_I$ was added to the appropriate wells for 30 min at 41 C, 5% CO_2. Afterwards, cells were washed twice with CM and one ml of fresh media was added to the wells. LPS was added to these wells at a concentration of 1μg / mL. Sham controls (no LPS exposure) were also included. After 24 h incubation at 41 C, 5% CO_2, the culture supernatants were removed, aliquoted, and stored at –20 C until analyzed for nitrite analysis.

Determination of Nitric Oxide production. Macrophage NO production was determined by measuring the concentration of the stable NO degradation product nitrite (NO_2^-) in the supernatant using a colorimetric assay, Griess method, as previously described (Green et al., 1982). One hundred μL of macrophage supernatant was added into each well of a flat-bottomed 96-well microtiter plate. Then an equal volume of Griess reagent (one part of 1% sulphanilamide and one part of 0.1% of nephthylethylenediaminne dihydrochloride) was added to the same wells. After 10

minutes incubation at room temperature, the plates were read at 540 nm on an ELISA plate reader (Bio-Rad, Richmond, CA) to quantify change in the color that was indicative of nitrite presence. For each sample an average of three readings was used in the final analysis. The standard curve for the nitrite assay was generated using various dilutions of 10mM stock solution of sodium nitrite in RPMI 1640 CM. The nitrite levels in culture supernatants were calculated by comparing the optical density (OD) readings against the nitrite standard curve.

Statistical analysis. Data were analyzed by analysis of variance using the general linear model procedure of SAS (SAS Institute, 1995). Each experiment was repeated using chicks obtained from two separate hatches. Data from each experiment were analyzed separately. Significance was established at $P \leq 0.05$.

RESULTS

iNOS and IL-1β mRNA expression. Macrophages from GB2 and K-strain were stimulated with *E. coli* LPS for different times and iNOS as well as IL-1β mRNA was examined using RT-PCR. iNOS mRNA was abundant in RNA from K-strain macrophages, whereas it was quite low in RNA from GB2 macrophages at 2, 3, 6, 9, 10, and 12 h post LPS stimulation in two separate experiments (Fig 1 A and B). The expression of iNOS within K- or GB2 strain macrophages was rather stable up to 9 h and then declined around 12 h post-LPS stimulation (Fig 1B). Interestingly, comparable expression of IL-1β mRNA was detected in K and GB2 macrophages at 2 and 3 h post

LPS stimulation in two separate experiments (Fig 2A and B). However, IL-1β mRNA expression was more persistent in GB2 macrophages and remained detectable at 6 and 9 h post LPS stimulation whereas IL-1β mRNA was present only in negligible amounts after 3 h LPS stimulation in K strain macrophages. Furthermore, IL-1β expression in LPS stimulated K or GB2 macrophages decreased overtime.

IL-1R_I expression on chicken macrophages. In order to determine if the persistence in IL-1β mRNA in GB2 versus K-strain macrophages is perhaps due to a differential autocrine induction through IL-1 receptors, the constitutive and inducible expression of IL-1R_I on GB2 and K macrophages was quantitated via flow cytometry. A preliminary time course study was conducted to determine the inducibility of the surface IL-1R_I using MQ-NCSU macrophage cell line. While the incidence of IL-1R_1-positive macrophages after exposure to 1μg / ml LPS did not differ from unstimulated controls at any time point post LPS stimulation in either of the two experiments (Table 1), the Mean Fluorescence Intensity (MFI), an indicator of IL-1R_1 receptor numbers, increased significantly ($P < 0.05$) over time suggesting the inducibility of IL-1R_1 receptor (Table 2). Based on this study 6 and 12 h LPS exposure as well as no LPS treatments were chosen to compare the expression of IL-1R_I on K and GB2 macrophages. No significant differences were observed between K and GB2 macrophages in the percentage of macrophages positive for IL-1R_I without or with 6 and 12 h post LPS stimulation (Fig 3). IL-1R_I percent positive values ranged from 85 - 87% without LPS stimulation, 92 - 96% at 6 h, and 90 - 94% at 12 h post LPS stimulation between GB2 and K stain macrophages. The experiment was repeated on macrophages from a different hatch of chickens with

similar results (Fig 3). The MFI data from these experiments is shown in figure 4. Macrophages from both strains exhibited similar IL-$1R_I$ intensities without LPS stimulation (K = 794.71, GB2 = 889.82) or at 12 h post LPS stimulation (K = 853.9, GB2 = 942.7). However, at 6 h post LPS stimulation, IL-$1R_I$ expression intensity was significantly ($P < 0.05$) higher in GB2 macrophages (1243.55) as compared with K macrophages (923.84). The experiment was repeated on macrophage from a different hatch of chickens with similar results (Fig 4).

IL-$1R_I$ blockage and iNOS mRNA expression. iNOS mRNA levels were measured semi quantitatively via competitive RT-PCR in K and GB2 macrophages after blocking IL-$1R_I$ with chicken anti- IL-$1R_I$ antibody (Fig 5). A 30 min pre incubation of the chicken macrophages with 1:20 dilution of anti- IL-$1R_I$ antibody resulted in a 50% decrease in iNOS mRNA in both GB2 and K-strain macrophages. Furthermore, macrophages from K-strain exhibited higher iNOS mRNA levels with or without pre incubation with anti- IL-$1R_I$ as compared to GB2 macrophages.

IL-$1R_I$ blockage and nitrite formation. Incubation of macrophages with rabbit anti- chicken IL-$1R_I$ antibody, at a 1:20 dilution significantly decreased LPS induced iNOS activity (measured as nitrite levels) by 50% relative to the LPS control (Fig. 6). Unstimulated macrophages from K and GB2 chicken produced comparable nitrite levels (K= 4.12 μM trial 1, 4.77 μM trial 2; GB2 = 3.96 μM trial 1, 5.53 μM trial 2) whereas significantly higher ($P < 0.05$) levels of nitrite were elicited from the LPS- stimulated K-strain macrophages (67.99 μM trial 1, 77.48 μM trial 2) than GB2 macrophages (15.55 μM trial 1, 21.22μM trial 2). However, pretreatment of macrophages with 1:20 dilution

of anti- IL-1R_I antibody significantly reduced the LPS-induced nitrite production in similar fashion in K and GB2 macrophages (K= 37.65 μM trial 1, 41.07 μM trial 2; GB2 = 9.00 μM trial 1, 11.98 μM trial 2). Pre incubating the cells with normal rabbit serum did not significantly affect the iNOS activity (data not shown).

DISCUSSION

Strain based differences in LPS induced iNOS expression and activity have been described in chicken macrophages (Hussain and Qureshi 1997, 1998). For example, macrophages from Cornell K strain white leghorn ($B^{15}B^{15}$) chicken expressed higher levels of iNOS mRNA as well as higher corresponding nitrite levels in the culture supernatants as compared with macrophages from GB2 ($B^{6}B^{6}$) chicken. Recently, we reported that these observed genetic differences are not limited to the bacterial source of LPS and but a differential Toll-like receptor 4 was associated with differential iNOS expression (Dil and Qureshi, 2002). While further exploring the molecular mechanism involved in differential iNOS induction, it was observed that K strain macrophages not only express higher number of TLR4 constitutively, inducible expression of TLR4 as well as of CD14 was also significantly higher in K strain macrophages than GB2 macrophages. Furthermore, this differential expression of LPS receptors on chicken macrophages resulted in increased NF κB activation in K strain macrophages in comparison to GB2 macrophages (Dil and Qureshi, 2002).

The current study attempted to examine an additional pathway of iNOS induction, i.e., via IL-1β, which is a potent inflammatory cytokine. The assumption was that IL-1

involvement might further explain the observed differences between the iNOS hypo and hyper responder chicken strains. The data clearly showed that iNOS mRNA expression, as measured by RT-PCR, was variable between K (hyper) and GB2 (hypo responder) chickens. These results are in accordance with differential iNOS mRNA expression in these chicken lines observed via northern blot analysis (Dil and Qureshi, 2001). When total RNA obtained from same macrophages samples was analyzed via RT-PCR using IL-1β primers, no difference was observed in IL-1β mRNA expression between K and GB2 at either 2 or 3 h post LPS stimulation. Interestingly, IL-1β mRNA was found to be persistent up to 9 h post LPS stimulation in GB2 macrophages whereas no IL-1β mRNA could be detected beyond 3 h of LPS stimulation in K-strain macrophages (Fig. 2). In an attempt to understand the molecular mechanisms behind this differential IL-1β induction we examined the surface expression of IL-1 receptor. As presented in Figure 4, GB2 macrophages seemed to have relatively higher numbers of IL-$1R_I$ (measured as MFI) as compared with K strain macrophages both statistically (6 h post-LPS stimulation) or numerically both with and without LPS stimulation. Since IL-1 has been shown to induce its own synthesis in mononuclear phagocytes (Fig. 7) and other cell types in an autocrine manner (Cruse and Lewis, 1999; Stanimicrovic et. al., 2001), the higher incidence of IL-1 receptors on GB2 macrophages implies that a stronger IL-1β mediated induction of IL-1β occurs in GB2 macrophages in autocrine, juxtacrine or paracrine fashion. It is possible that upon initial stimulation with LPS, both iNOS and IL-1β genes are expressed at higher levels in K macrophages as compared with GB2 macrophages. However, since the second stimulus of IL-1β expression (IL-1β itself) is transducing stronger signal through

higher number of receptors in GB2 macrophages (shown also by the higher induction of IL-1β mRNA in GB2 macrophages, (Fig 2), it compensates for the lesser first stimulation (LPS) effect. This is clearly evident in IL-1β mRNA levels which are very comparable between K and GB2 macrophages at 3 h post LPS stimulation (Fig 2). Moreover, tumor necrosis factor (TNF) might also have a role in this differential IL-1β induction as both IL-1 and TNF have been shown to be mutually stimulatory (Dinarello et al., 1986; Muegge and Durum, 1989). Therefore, iNOS hypo responsiveness in GB2 macrophages could not be explained by the enhanced or persistent expression of IL-1β message in GB2 versus K-strain macrophages.

IL-1 has been described as a potent inducer of iNOS in several cell types (Corbett et al., 1994). Also, Wong et al., (1996) showed that IL-1β induces the transcriptional activation of iNOS expression in cultured rat pulmonary artery smooth muscle cells. There is evidence in the literature that IL-1β binds to IL-$1R_I$ during systemic infection and induces the expression of iNOS in rat brain vasculature and perivascular areas (Wong et al., 1996). Therefore, the last series of experiments involved IL-1 receptor blocking studies. Again, the hypothesis was that if IL-1 was involved in differential iNOS induction between K and GB2 macrophages, the blocking of IL-1 receptor would abrogate those differences. This assumption was based on murine studies in which blockade of IL-1 receptor with anti IL-1 receptor monoclonal antibodies attenuated the host inflammatory responses (Gershenwald et al., 1990). The current study showed that IL-1 receptor blockade did reduce LPS-induced iNOS expression and activity in both GB2 and K-strain macrophages (Figures 5 and 6). Interestingly, this 50% reduction in

expression and activity was comparable in macrophages from both genotypes. Therefore, given the fact that GB2 macrophages are low responders as compared with the macrophages from K-strain chickens, this low responsiveness was certainly not due to a defect in IL-1 mRNA expression or receptor function. This study supports previous findings (Dil and Qureshi, 2002) that LPS-induced differences in iNOS expression and activity in K and GB2 strain chickens are more likely due to the differences in expression of LPS binding and signaling molecules, such as CD14 and TLR4.

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Table 3.1. Incidence of IL-1 R_I on chicken macrophages.

Treatment	Percent Positive Macrophages[1]	
	Experiment 1	**Experiment 2**
No LPS	92.08 ± 1.38	96.68 ± 0.64
1 h LPS	93.46 ± 1.38	97.76 ± 0.64
3 h LPS	96.41 ± 1.38	96.18 ± 0.64
6 h LPS	95.94 ± 1.38	97.91 ± 0.64
12 h LPS	94.94 ± 1.38	96.92 ± 0.64
24 h LPS	96.79± 1.38	95.65 ± 0.64

[1]MQ-NCSU (5×10^5) macrophages were treated with 1μg / ml *E.coli* LPS. The cells were then stained with 1: 4 dilution of rabbit anti-chicken IL-1R followed by 1:16 dilution of FITC mouse anti-rabbit IgG and analyzed by FACScan. Analysis gate was set on macrophages using forward and side scatter gating. Values represent mean ± SE of percentage of positive cells in gated macrophage population. Data were obtained from 3 replicate samples at each time point.

Table 3.2. Intensity of staining of MQ-NCSU macrophages for IL-1R_I expression.

Treatment	Mean Fluorescent Intensity	
	Experiment 1	**Experiment 2**
No LPS	44.09 $\pm$ 7.12 [d]	54.79 $\pm$ 8.93 [d]
1 h LPS	56.18 $\pm$ 7.12 [d]	65.00 $\pm$ 8.93 [d]
3 h LPS	72.04 $\pm$ 7.12 [d]	79.66 $\pm$ 8.93 [d]
6 h LPS	124.85 $\pm$ 7.12 [b] *	137.76 $\pm$ 8.93 [b] *
12 h LPS	179.23 $\pm$ 7.12 [a] *	167.42 $\pm$ 8.93 [a] *
24 h LPS	104.12 $\pm$ 7.12 [c] *	110.34 $\pm$ 8.93 [c] *

5 x 10^5 MQ-NCSU macrophages were treated with 1µg / ml *E.coli* LPS. The cells were then stained with 1: 4 dilution of rabbit anti-chicken IL-1R followed by 1:16 dilution of FITC mouse anti-rabbit IgG and analyzed by FACScan. Analysis gate was set on macrophages using forward and side scatter gating. Values represent mean $\pm$ SE of mean fluorescence intensity (MFI) (indicative of receptor number) in gated macrophage population. Data was obtained from 3 replicate samples at each time point. * p=0.05 compared with No LPS. Values within columns with different superscript letters (within each experiment) are different at $p < 0.05$.

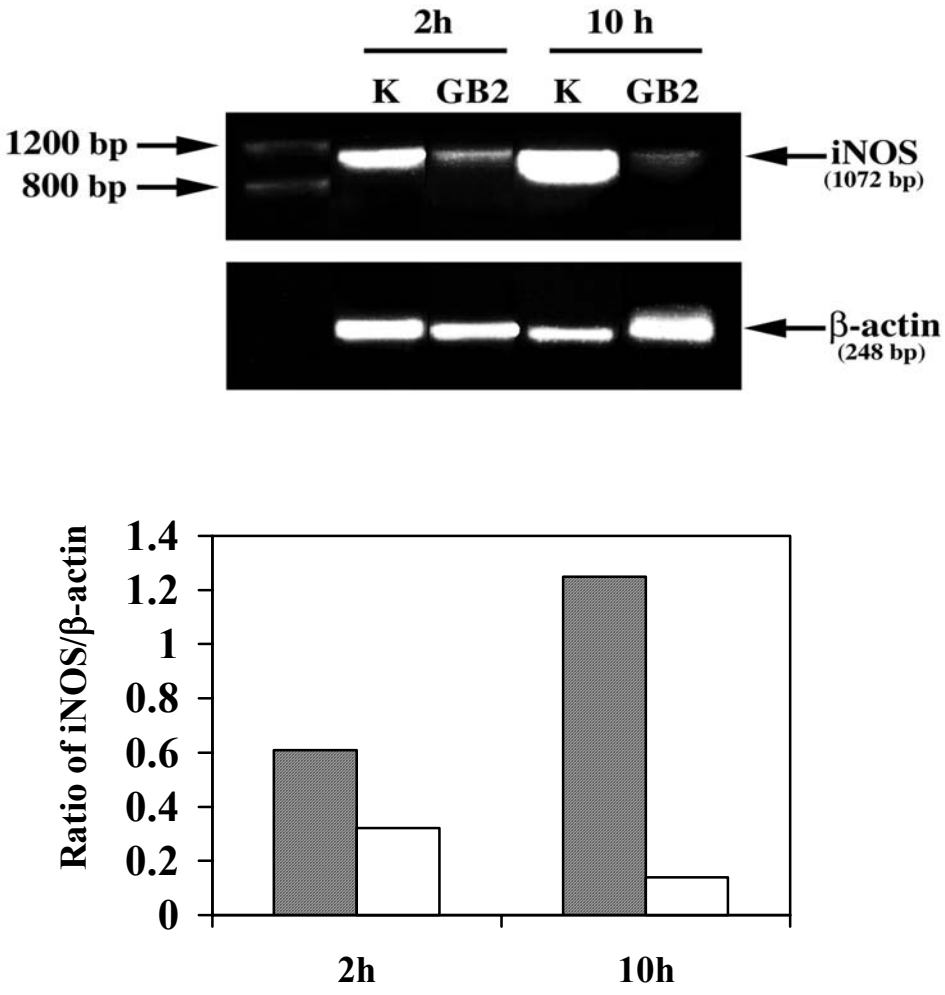

Fig 3.1A. Detection of iNOS mRNA in Sephadex-elicited K and GB2 strain macrophages exposed to 1μg / ml *E. coli* LPS for 2 and 10 h by RT-PCR. Low DNA Mass Ladder is shown in the far left lane. Approximate molecular weight of the iNOS or β-actin band is shown (upper panel). Bar graph of the ratio of densitometric readings of the iNOS mRNA divided by the density of the house keeping gene β-actin. Stripped bars correspond to K strain and open bars correspond to GB2 chickens (lower panel).

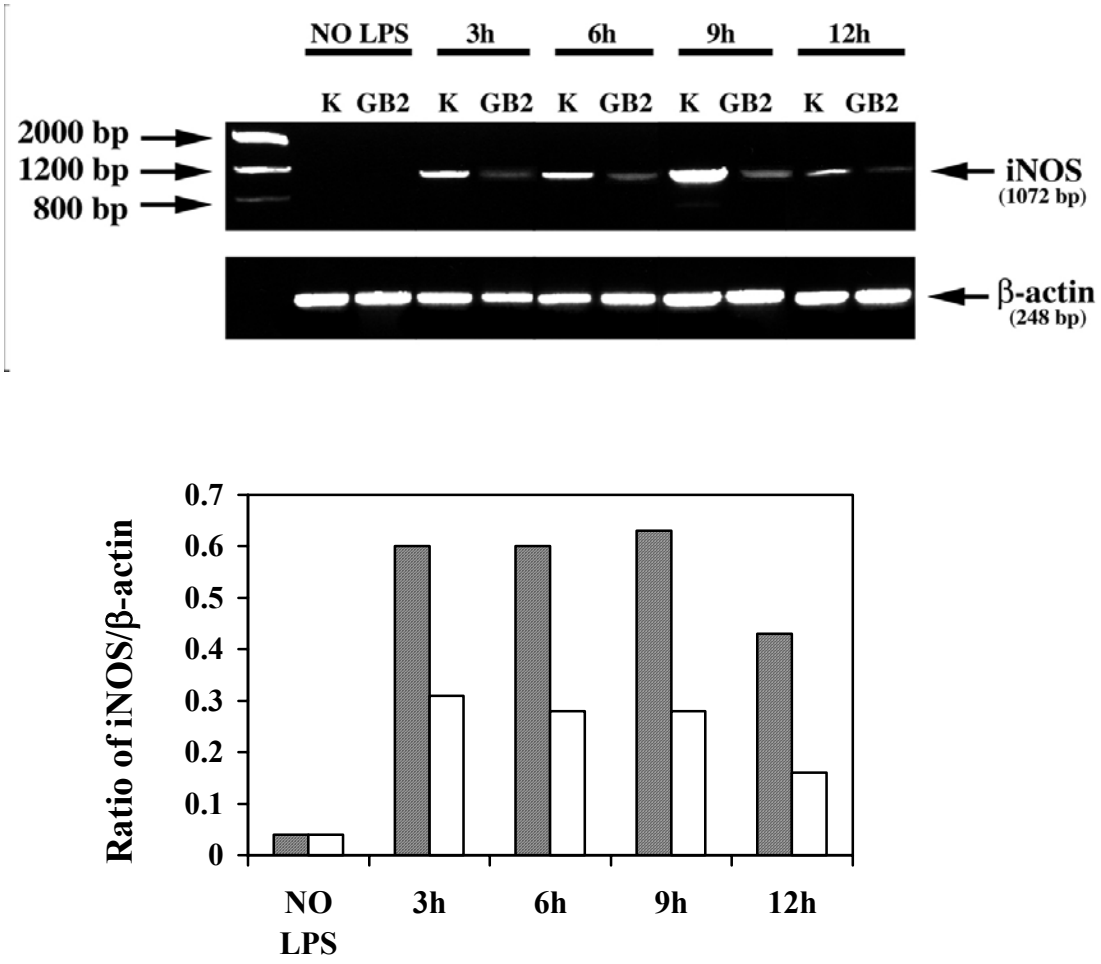

Fig 3.1B. Detection of iNOS mRNA in Sephadex-elicited K and GB2 strain macrophages exposed to 1μg / ml *E. coli* LPS for 3, 6, 9, and 12 h or unexposed macrophages by RT-PCR. Low DNA Mass Ladder is shown in the far left lane. Approximate molecular weight of the iNOS or β-actin band is shown (upper panel). Bar graph of the ratio of densitometric readings of the iNOS mRNA divided by the density of the house keeping gene β-actin. Stripped bars correspond to K strain and open bars correspond to GB2 chicken (lower panel).

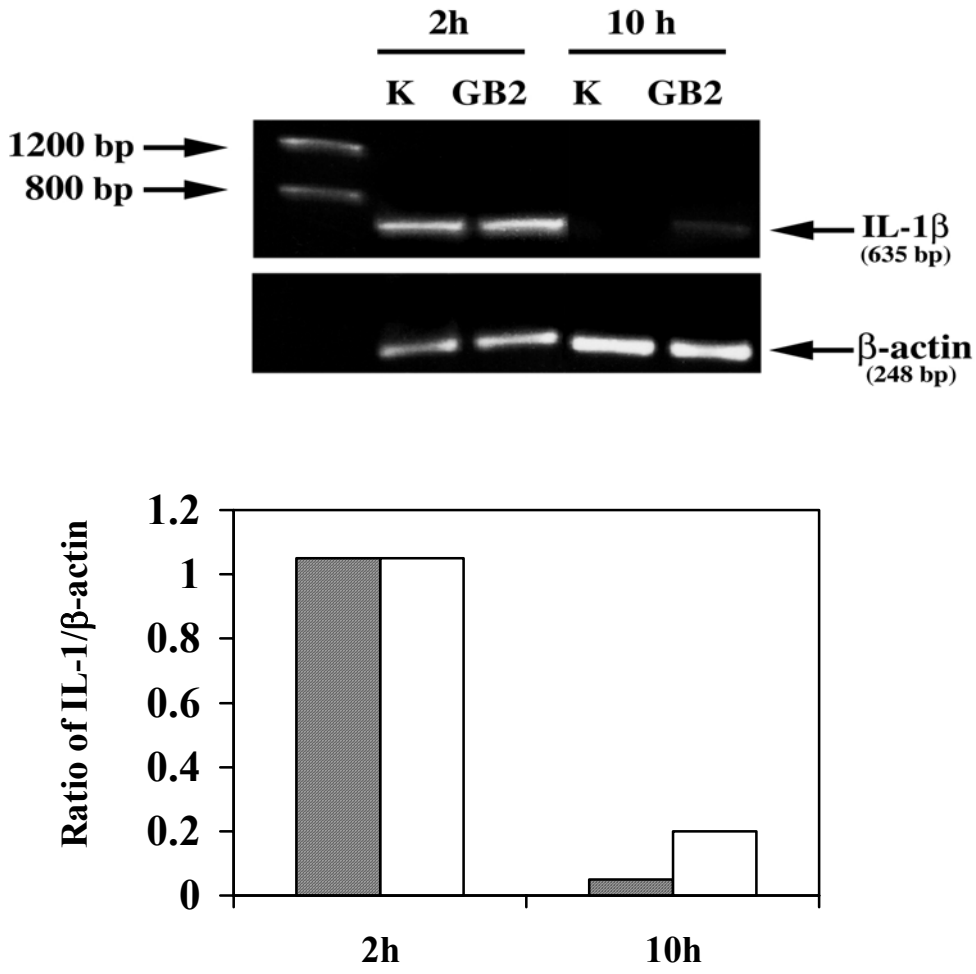

Fig 3.2A. Detection of IL-1β mRNA in Sephadex-elicited K and GB2 strain macrophages exposed to 1μg / ml *E. coli* LPS for 2 and 10 h by RT-PCR. Low DNA Mass Ladder is shown in the far left lane. Approximate molecular weight of the IL-1β or β-actin band is shown (upper panel). Bar graph of the ratio of densitometric readings of the IL-1β mRNA divided by the density of the house keeping gene β-actin. Stripped bars correspond to K strain and open bars correspond to GB2 chicken (lower panel).

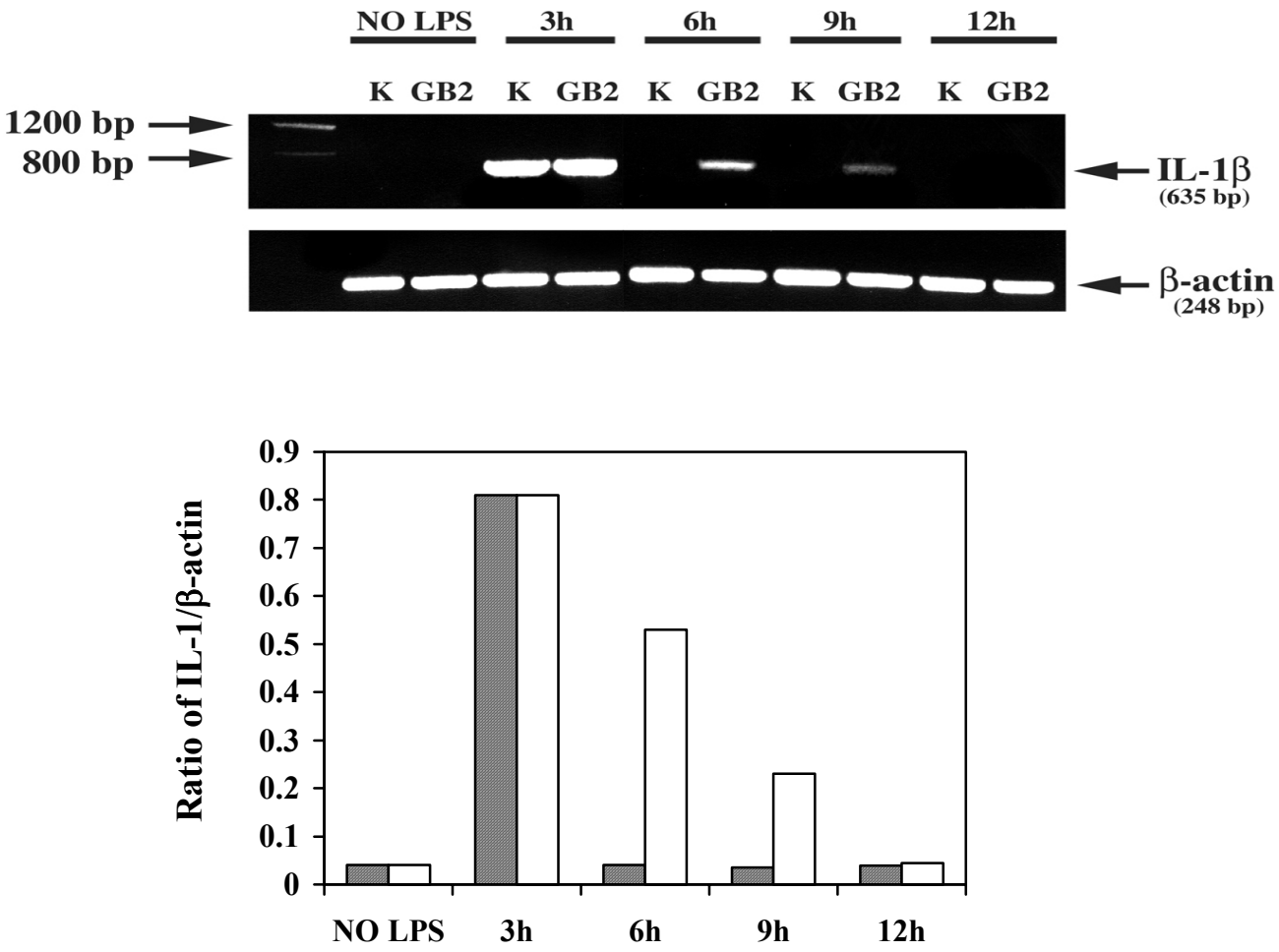

Fig 3.2B. Detection of IL-1β mRNA in Sephadex-elicited K and GB2 strain macrophages exposed to 1μg / ml *E. coli* LPS for 3, 6, 9, and 12 h or unexposed macrophages by RT-PCR. Low DNA Mass Ladder is shown in the far left lane. Approximate molecular weight of the IL-1β or β-actin band is shown (upper panel). Bar graph of the ratio of densitometric readings of the IL-1β mRNA divided by the density of the house keeping gene β-actin. Stripped bars correspond to K strain and open bars correspond to GB2 chickens (lower panel).

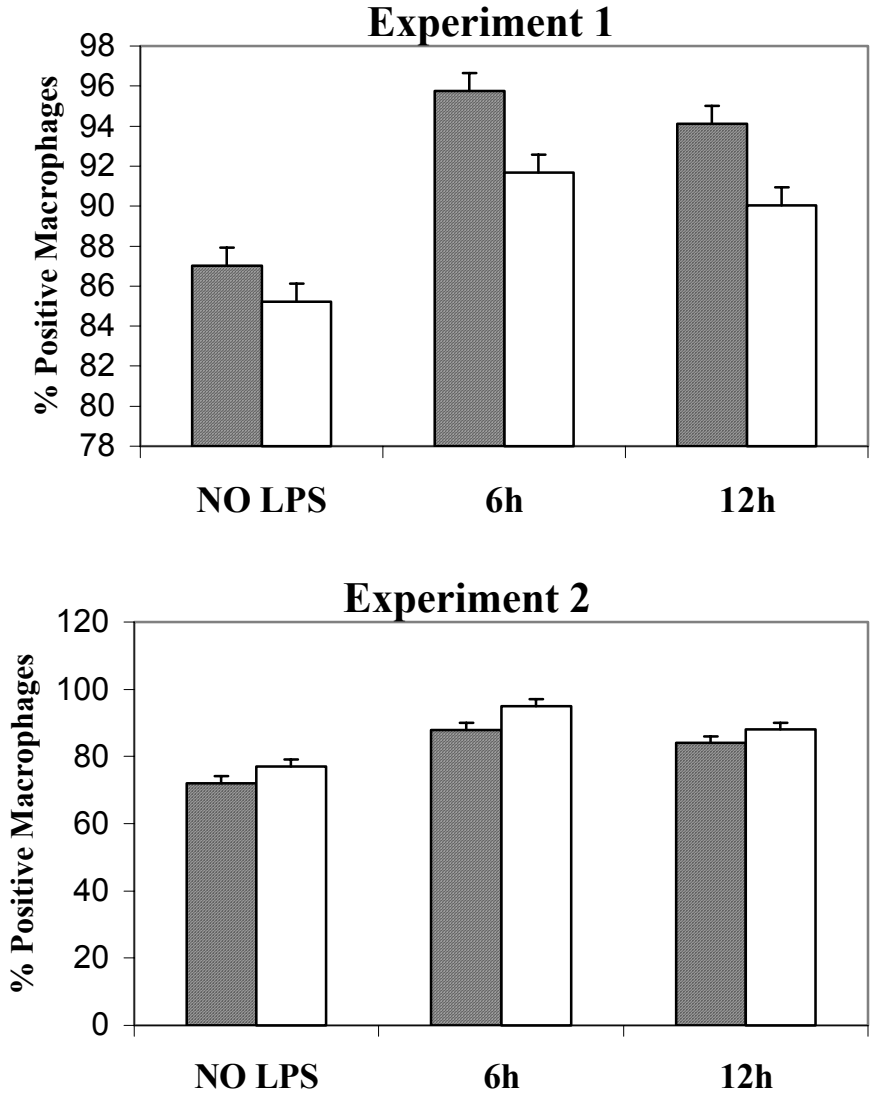

Fig. 3.3. Incidence of IL-1R_I on the surface of chicken macrophages. 5 x 10^5 macrophages from K- or GB2-strain of chicken were treated with 1μg / ml *E. coli* LPS for 6 and 12 h. The cells were then stained with 1: 4 dilution of rabbit anti chicken IL-1R_I followed by 1:16 dilution of FITC-labeled mouse anti-rabbit IgG and analyzed by FACScan. Analysis gate was set on macrophages using forward and side scatter gating. Stripped bars correspond to K strain and open bars correspond to GB2 chicken. Each bar represents mean $\pm$ SE of percentage of positive cells in gated macrophage population. Data were obtained from 3-4 individual chickens per strain. Experiments 1 and 2 were conducted on chickens obtained from two separate hatches.

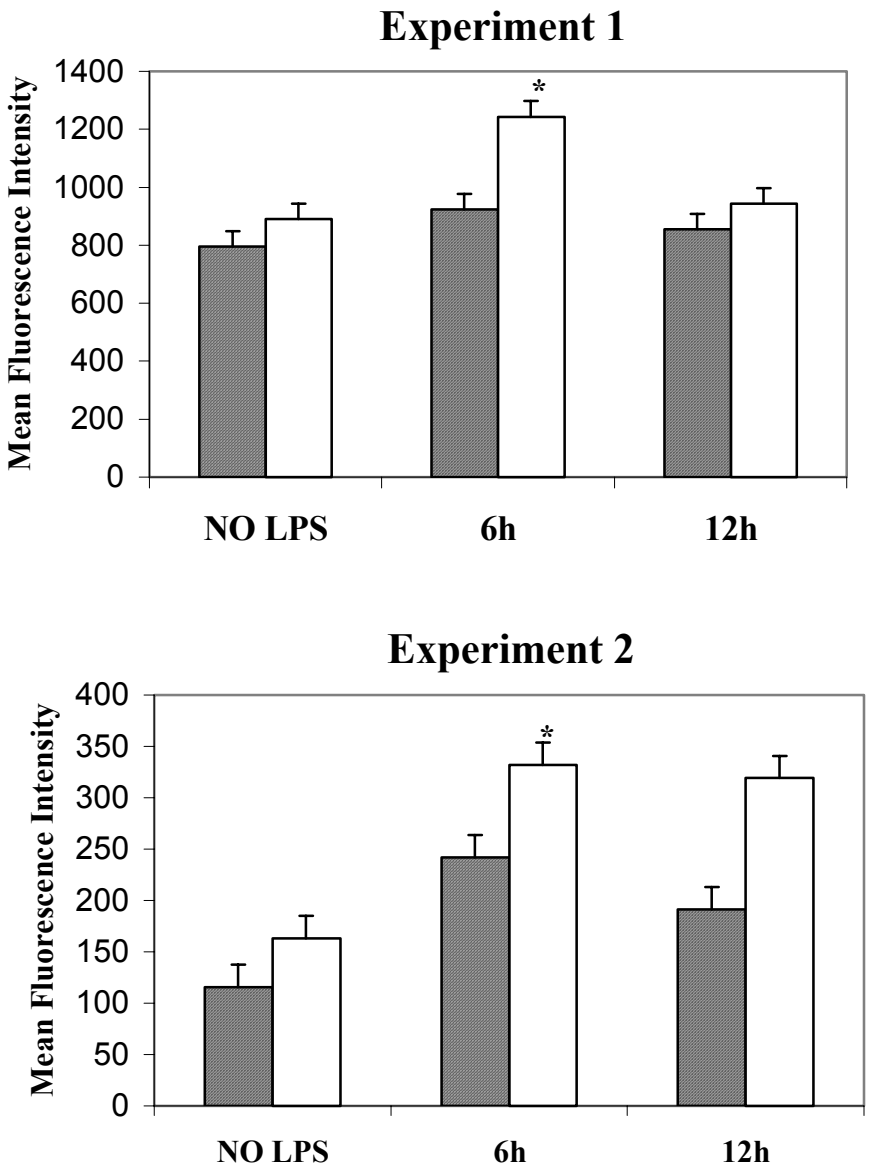

Fig 3.4. Fluorescence intensity of IL-$1R_I$ on the surface of chicken macrophages. 5 x 10^5 macrophages from K- or GB2-strain of chicken were treated with 1μg /ml *E. coli* LPS for 6 and 12 h. The cells were then stained with 1: 4 dilution of rabbit anti chicken IL-$1R_I$ followed by 1:16 dilution of FITC-labeled mouse anti-rabbit IgG and analyzed by FACScan. Stripped bars correspond to K strain and open bars correspond to GB2 chickens. Each bar represents the mean fluorescence intensity (MFI) $\pm$ the SE from 3 - 4 individual chickens per strain. Experiments 1 and 2 were conducted on chickens obtained from two separate hatches. * = $P < 0.05$ compared with GB2.

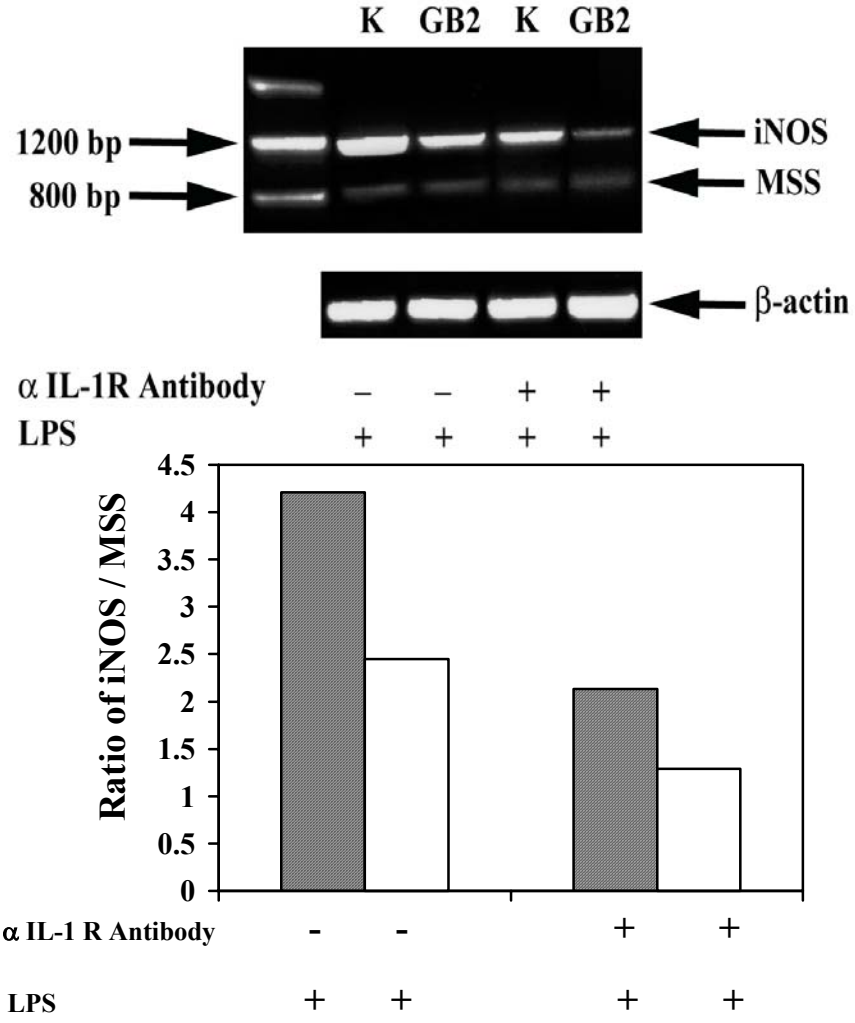

Fig 3.5. Semi quantification of iNOS mRNA after blocking the IL-1R_I in Sephadex-elicited K and GB2 macrophages by competitive RT-PCR. 1.5 μg of total RNA from each pooled sample was added to each tube containing a standardized amount (0.98 pg / μl) of competitor for the same iNOS sense and antisense primers and co amplified (35 cycles). The real iNOS bands and the competitor bands are indicated by arrows. Both real target (iNOS) and competitor band volumes were measured in each sample by densitometry and the iNOS / competitor product ratio was calculated (bar graph). Stripped bars correspond to K strain and open bars correspond to GB2 chicken.

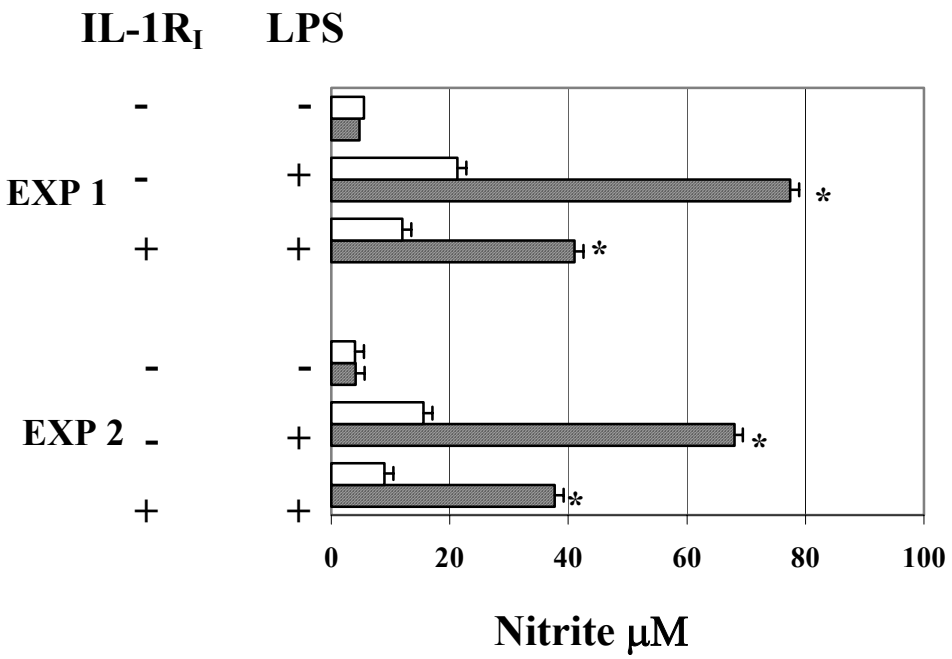

Fig. 3.6. Effect of IL-1R$_I$ blockage on LPS mediated nitrite production from GB2 and K-strain macrophages. Sephadex-elicited abdominal exudate macrophages (1 X 10^6) from either source were cultured for 24 h in the presence of 1 μg / ml of *E. coli* LPS with or without 1 : 20 dilution of IL-1R$_I$ pretreatment (30 min). Nitrite levels were measured in the culture supernatant fraction by Griess method in two independent experiments. Stripped bars correspond to K strain and open bars correspond to GB2 chicken. Each bar represents means of nitrite level $\pm$ SE in the culture supernatant from 3 replicate samples established from a pool of 5-7chicks per group. * = $P < 0.05$ compared with GB2.

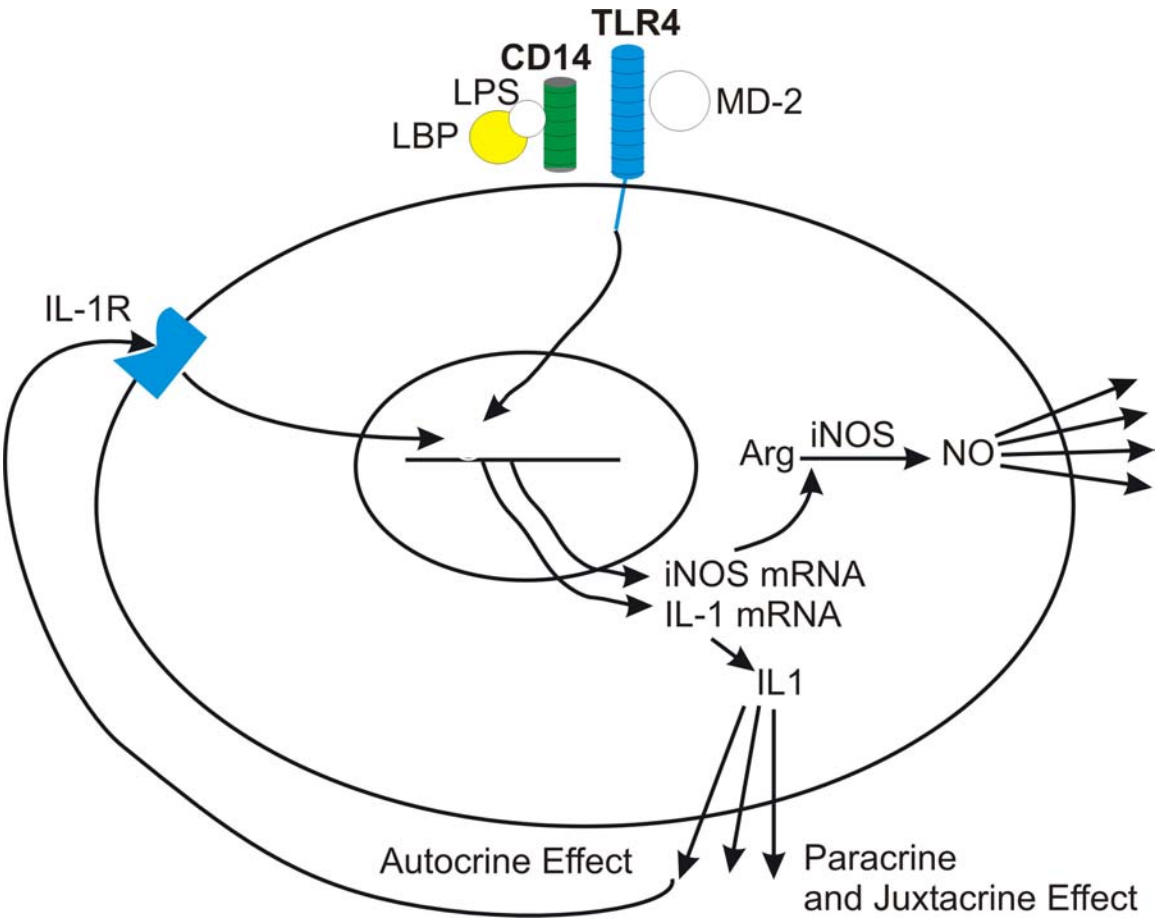

Fig. 7. Schematic representation of LPS and IL-1 mediated induction of macrophage iNOS and IL-1 genes. Receptor mediated LPS signaling in macrophages leads to the up regulation of iNOS and IL-1 genes. IL-1 acts in autocrine, paracrine, and juxtacrine fashion through type I IL-1 receptors and induces further synthesis of itself and iNOS.

SUMMARY AND CONCLUSIONS

The purpose of this research was to understand the molecular mechanisms responsible for differential iNOS gene expression and activity in different genetic lines of chickens. The bases of these studies were the findings of Hussain and Qureshi (1997, 1998) that iNOS expression and activity in chicken macrophages is influenced by genetic background. This led to the categorization of chicken lines as hyper- (Cornell K-strain; $B^{15}B^{15}$) and hypo- (GB1; $B^{13}B^{13}$ and GB2; $B^{6}B^{6}$) iNOS responders. However, these studies utilized only one source of LPS, i.e., from *E. coli* to show genetic influence on the expression and activity of iNOS. There was a possibility that these observed genetic differences were only limited to stimulation with this particular LPS. Thus, the first objective of the current study was to determine whether these genetic differences were due to the host-based intrinsic genetic mechanism(s) or were dependent upon a specific bacterial source of LPS. To accomplish this objective LPS from *E. coli* and three other bacterial sources was used to stimulate iNOS induction, activity and expression which were studied via nitrite assay and northern blot analysis for iNOS mRNA. Sephadex-elicited abdominal macrophages from K, GB1 and GB2 chicken as well as macrophages from a transformed chicken macrophage cell line, MQ-NCSU, were exposed to 5 μg / mL LPS from *E. coli, Shigella flexneri, Serratia marcescens*, and *Salmonella typhimurium* and *nitrite* levels were quantitated in the culture supernatant. Results showed that K-stain macrophages were significantly ($P < 0.05$) high responders whereas macrophages from both GB1 and GB2 chickens were low responders against all LPSs used. When iNOS gene expression was studied via northern-blot analysis, K-strain macrophages expressed higher intensity

of 4.5 Kb iNOS mRNA (iNOS/β-actin ratio) than macrophages from GB2 chickens regardless of the LPS source. These data provide strong evidence that differences in LPS stimulated iNOS induction in these genetic lines of chicken were an intrinsic and host-based phenomenon. To elucidate possible molecular mechanism(s) involved in iNOS gene expression in hypo and hyper responder strains of chickens, the constitutive expression of LPS-related macrophage cell surface receptors, CD14 and Toll-like receptor-4 (TLR4), was examined via flow cytometry using anti-human CD14, and TLR4 antibodies. The surface expression intensity of CD14, the LPS binding protein, was not found to be different between macrophages from either source. In contrast, the surface expression intensity of TLR4, the LPS signaling molecule, was significantly higher ($P < 0.05$) in K-strain macrophages than in GB2 macrophages suggesting that the observed differences in iNOS expression and activity among the K-strain (hyper- responder) and GB2 (hypo-responder) chickens were perhaps due to this differential expression of TLR4 leading to more intense LPS-mediated activation of K-macrophages. It must be pointed out that this report was the first to quantitate and describe the role of TLR4 in LPS-mediated iNOS induction in avian species.

In the second phase of this study, the role of CD14 and TLR4 proteins in the differential iNOS induction in these genetic lines was explored by examining the LPS inducible expression of these proteins as well as NFκB activation. The results from these experiments demonstrate that Sephadex-elicited, adherence purified K-strain macrophages expressed more TLR4 and CD14 receptors ($P < 0.05$) at 6 and 12 h post LPS stimulation than GB2 macrophages as measured by flow cytometry. In addition,

blockade of these receptors on transformed chicken macrophage cell line, MQ-NCSU, with anti human CD14 and anti TLR4 antibodies completely blocked LPS-mediated iNOS activity as measured by nitrite levels. Another significant finding from this study was the level of DNA bound NFκB measurement which was found to be significantly greater in K than in GB2 macrophages at 30 minutes post LPS stimulation. This nuclear localization of NFκB as well as iNOS activity was completely inhibited by pre-incubating macrophages with 50μM MG132 (a proteosome inhibitor that blocks the NFκB nuclear transfer and localization) both in K and GB2 macrophages. These studies therefore suggest that a differential and perhaps stronger LPS mediated signaling via CD14, TLR4 and NFκB was responsible for the heightened iNOS gene induction in K-strain (hyper-responder) macrophages than in GB2 (hypo-responder) chickens.

Lastly, the expression of IL-1β in macrophages from iNOS hypo-responder GB2 and hyper-responder K strain chickens was examined. The main objective of this study was to determine if LPS-mediated induction of IL-1β modulates iNOS induction in these genetic lines of chicken. Sephadex-elicited macrophages were stimulated with LPS and iNOS and IL-β mRNA was studied by RT-PCR. As expected, K-strain macrophages had higher iNOS mRNA as compared with the macrophages from GB2 genotype chickens Interestingly, IL-1 mRNA was initially comparable between K and GB2 chickens at 3 h post-LPS stimulation but persisted up to 9 h only in GB2 macrophages. Furthermore, the LPS-inducible IL-1 surface receptor expression as measured by flow cytometry was found to be higher in GB2 than on K-strain macrophages. This surface expression of receptors was numerically different constitutively and at 12 h LPS stimulation and

significantly ($P < 0.05$) different at 6 h LPS stimulation. In addition, blocking of IL-1 receptor by the anti-IL-1 receptor antibody significantly reduced the LPS-mediated iNOS expression by 50% over the LPS-stimulated controls as quantified by competitive RT-PCR. Also, iNOS activity as determined by the nitrite quantification in macrophage culture supernatants was reduced to 50%. However, this magnitude of inhibition was similar in both K and GB2 macrophages. These observations suggested that although IL-1 is involved in mediating LPS-induced iNOS expression and activity, the differential response of GB2 and K-strain macrophages in terms of LPS-induced iNOS expression and activity is certainly not due to a defect in IL-1 receptor function.

In conclusion, this study suggested that differential expression of LPS binding and signaling molecules as well as preferential binding of NFκB leads to stronger stimulus dependent expression of iNOS in K macrophages versus the low responder GB2 chicken macrophages. Whether or not, the differential iNOS response between K-strain and GB2 chickens imparts differential resistance and/or susceptibility to bacterial or viral mediated infections, is not known. However, nitric oxide has been shown to be involved in inhibiting replication of chicken tumorigenic viruses, such as Marek's disease virus (Schat et al., 2001). Interestingly, the Cornell K-strain genotype, which is a hyper-responder to iNOS expression and activity, is also naturally resistant to Marek's disease (Hutt and Cole, 1957; Gavora et al., 1977).). It would be interesting to examine various immunological endpoints (such as cytokine profiles e.g., IL-6, TNF) of iNOS hypo- and hyper-responder genotypes perhaps during a disease challenge to establish a positive or

negative correlation of disease susceptibility or resistance to iNOS expression and activity in chicken.

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