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Immunomodulatory effects of progesterone on TLR3 and TLR4 mediated signalling in macrophages and dendritic cells

By

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has lead to the award of a degree.

Signed:

Date:

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Abbrevations

3β-HSD	3β-Hydroxysteroid Dehydrogenase
AAMΦ	Alternatively Activated Macrophages
AKP	Streptavidin-Alkaline Phosphatase
AMCase	Acidic Mammalian Chitinase
AP-1	Activation Protein-1
APC	Antigen Presenting Cells
BAFF	B-cell-Activating Factor belonging to the TNF family
BMDCs	Bone Marrow Derived Dendritic Cells
BMD-Macrophages	Bone Marrow Derived-Macrophages
BSA	Bovine Serum Albumin
CAMΦ	Classically Activated Macrophage
CCR2^{+}	CC-Chemokine Receptor 2
CD14	Cluster of Differentiation-14
cDCs	conventional DCs
CLP	Common lymphoid Progenitor
CMP	Common Myeloid Progenitor
CPG	Cytidine-Phosphate-Guanosine
CREB	Cyclic –AMP-Responsive-Element-Binding protein
CX3CR1	CX3C-Chemokine Receptor1
CXCL10	CXC-Chemokine Ligand 10
CYPB	Cyclophilin B
DBD	DNA-Binding Domains
DCs	Dendritic Cells
DD	Death Domain
Dexa	Dexamethasone
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone Sulphate
DHT	Dihydrotestosterone
DMEM	Dulbecco’s Modified Eagle’s Medium

DMPA	Depot Medroxyprogesterone Acetate
DTT	Dithiothreitol
E_1	Estrone
E_2	Estradiol-17β
E_3	Estriol
EBI3	Epstein-Barr Virus (EBV) -Induced gene 3
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
ELAM-1	Endothelial Leukocyte Adhesion Molecule-1
ELISA	Enzyme-Linked Immunosorbent Assay
EMCV	Encephalomyocarditis Virus
EMSA	Electrophoretic Mobility Shift Assay
ER	Estrogen Receptor
EREs	Estrogen Response Elements
ERK	Extracellular signal- Regulated protein Kinase
Flt3	Fms-like Tyrosine kinase 3
FSH	Follicle Stimulating Hormone
GIPLs	Glycoinosito-Phospholipids
GM-CSF	Granulocyte Macrophage- Colony Stimulating Factor
GMP	Granulocyte / Macrophage Progenitor
GPI-anchor	Glycosylphosphatidylinositol Anchors
GR	Glucocorticoid Receptor
GRIP1	Glutamate Receptor Interacting Protein1
hCG	human Chorionic Gonadotropin
HEPES	4-(2-Hydroxyethyl) Piperazine-1-Ethanesulfonic Acid
HEVs	High Endothelial Venules
HI-FCS	Heat Inactivated- Fetal Calf Serum
HIV	Human Immunodeficiency Virus
HLH	Helix-Loop-Helix
hPL	human Placental Lactogen
HRE	Hormone-Response Element
HSC	Hematopoietic Stem Cell

HSV	Herpes Simplex Virus
ICAM-1	Intercellular Adhesion Molecule-1
IFI	Interferon Inducible gene
IFIT1	Interferon-induced Protein with Tetratricopeptide Repeats 1
IFN γ	Interferon γ
IFNAR	IFNα Receptor
IFN-β	Interferon-β
IgM	Immunoglobulin M
IKK	IκB Kinase
IKKε	Inhibitor of kappa B Kinase Epsilon
IL-	Interleukin-
IMDM	Iscoves's Modified Dulbecco's Medium
iNOS	Inducible Nitric Oxide Synthase
IP10	IFNγ Induced Protein 10
IPS-1	IFNβ Promoter Stimulator 1
IRAK	IL-1 Receptor Associated Kinase
IRF-	IFN Regulatory Factor-
ISGF3	IFN-Stimulated Gene Factor 3
ISGs	Interferon Stimulated Genes
ISRE	IFN-Stimulated Response Elements
JAK	Janus Kinase
JNK	c-Jun amino (N)-terminal Kinase
LBD	Ligand-Binding Domains
LBP	LPS Binding Protein
LCs	Langerhans Cells
LH	Luteinizing Hormone
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat
LTβ	Lymphotoxin-β Receptor
LZ	Lucine Zipper
mAb	monoclonal Antibody
MAL	MyD88 Adaptor-Like

MAPKs	Mitogen Activated Protein Kinases
MDA5	Differentiation-Associated Gene 5
MDP	Macrophage/Dendritic cell progenitor
MEP	Megakaryocyte /Erythrocyte progenitor
MHC I	Major Histocompatability Complex class I
MHC II	Major Histocombatability Complex Class II
MKK	MAP Kinase Kinase family
MKPs	MAPK Phosphatases
MMTV	Mouse Mammary Tumour Virus
MS	Multiple Sclerosis
MyD88	Myloid Deferentiation primary- response gene88
NAK	NFκB Activating Kinase
NBD	NEMO-Binding Domain
NEMO	NFκB Essential Modifier
NFκB	Nuclear Factor-κ B
NIK	NFκB Inducing Kinase
NK	Natural Killer
NK-T cell	Natural Killer- T cell
NLK	Nemo Like Kinase
NLS	Nuclear Localisation Sequence
NO	Nitric Oxide
Norg	Norgestrel
PAMPs	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pDCs	Plasmacytoid DCs
PI3K	Phosphatidylinositol 3-kinase
PIBF	Progesterone-Induced Blocking Factor
PIN1	Peptidylproplyl Isomerase,NIMA-interacting 1
PMSF	Phenylmethylsulphonylfluoride
pNPP	p-Nitrophenyl phosphate

PolyI:C	Polyinosinic:polycytidylic acid
PR	Progesterone Receptor
PRDs	Positive Regulatory Domains
Prog	Progerterone
PRR	Pathogen Recognition Receptors
RA	Rheumatoid Arthritis
RANTES	Regulated upon Activation Normal T-cell Expressed and Secreted
RE	Response Element
RHD	Rel- Homology Domain
RHIM	Rip Homotypic Interaction Motif
RIG-I	Retinoic acid- Inducible Gene I
RIP1	Receptor-Interacting Protein 1
ROI	Reactive Oxygen Intermediates
ROS	Reactive Oxgen Species
RSV	Respiratory Syncytial Virus
SAPKs	Stress-Activated Protein Kinases
SARM	Sterile α –and Armadillo-Motifs-ontaining Protein
SAv-AKP	Streptavidin-Alkaline Phosphatase
SAv-HRP	Streptavidin-Horse Radish Peroxidase
SDS- PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophores**es**
SHRs	Steroid Hormone Receptors
SIKE	Suppressor of IKKε
SLE	Systemic Lupus Erythematosis
SOCS	Suppressor Of Cytokine Signalling
STAT	Signal Transducer and Activator of Transcription
STDs	Sexually Transmitted Diseases
T2K	TRAF2-associated Kinase
TABs	TAK1- Binding Proteins
TADs	Transactivation Domains
TAK1	Transforming Growth Facter-β-Activated Kinase 1

TBK1	TANK Binding Kinase 1
TEMED	N, N, N', N'-tetramethylethylenediamine
Th1	T helper 1
Th2	T helper 2
TIR	Toll-IL-1 Receptor Domain
TIRAP	TIR-domain-containing Adaptor Protein
TLR	Toll Like Receptors
TNF	Tumour Necrosis Factor
TBP	TATA box-Binding Protein
TRAF6	Tumour Necrosis Factor Receptor (TNFR) - Associated Factor 6
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR-domain containing adaptor protein Inducing IFNβ
Tyk2	Tyrosine Kinase 2
VCAM-1	Vascular Cell Adhesion Molecule1
VREs	Virus-Responsive Elements
WNV	West Nile Virus

Abstract

Sexual dimorphism in the immune response is at least in part related to the effects of sex hormones. The current studies investigate the ability of progesterone to manipulate signalling events in macrophages and dendritic cells following TLR3 or TLR4 ligation. Progesterone can mediate its action by binding both progesterone and glucocorticoid receptors. To differentiate these effects, norgestrel, a synthetic progesterone specific receptor agonist and dexamethasone a glucocorticoid receptor agonist were utilised. Progesterone and norgestrel, but not dexmethasone increased poly-riboinosinic-ribocytidylic acid (polyI:C), a TLR3 agonist, induced interferon regulatory factor 3 (IRF3) phosphorylation in BMDCs, suggesting that it may be mediated by progesterone receptor. Subsequent experiments, showed inhibitory effects of both progesterone and norgestrel on numerous elements downstream in the TLR3 and TLR4 signalling pathways, specifically at the transcriptional level represented by inhibition of polyI:C-induced p65 and IRF3 nuclear translocation. Progesterone also inhibited LPS-induced IRF3 nuclear translocation. Progesterone and norgestrel inhibited LPS- and polyI:C-induced ISRE DNA-binding activity and may contribute to modulation of specific target gene promoters bearingISRE consensus elements including IL-12, IL-27 and IFNβ. The effects of progesterone were evident at the level of transcription. Thus, polyI:C-induced IL-12p35, EBI3 and IFNβ mRNAexpression were inhibited by progesterone and norgestrel and LPS-induced IFNβ mRNA expression was inhibited by progesterone. Progesterone inhibited LPS- and polyI:C-induced IL-6, but not norgestrel. However, IL-12p70 production was reduced by both progesterone and norgestrel following LPS and polyI:C stimulation. These results indicate that progesterone selectively functions as an immunomodulatory agent through both progesterone and glucocorticoid receptors. Progesterone potentially has a greater ability to affect TLR3 rather than TLR4 signalling.These results could have therapeutic implications for control of the innate immune response, inflammation and infectious diseases.

CHAPTER ONE

INTRODUCTION

1.1 Immune System

1.1.1 Introduction to the immune system

The immune system has evolved a diverse collection of mechanisms to protect the host against a wide range of pathogenic microorganisms as well as normalise and eliminate toxic and allergenic substances (Chaplin, 2010). The immune response is one of the most important physiological functions that protect our bodies against infectious and non-infectious substances. The immune response includes an immediate response mediated by the innate immune system, which includes mechanisms that can recognise specific broad categories of infectious agents through pathogen associated molecular patterns (PAMPs). The adaptive immune system develops a highly specific response that is capable of recognising virtually all non-self-antigens. This arm of the immune response is also responsible for all autoimmune diseases (Janeway, 2001).

1.1.2 Innate and adaptive immune systems

The innate immunity is considered as the first line of defence and dominant ancient immune system that is present in all plants and animals (multicellular organisms). It has the ability to recognise a vast number of conserved molecular structures of microorganisms by a set of germ-line encoded receptors (Medzhitov and Janeway, 1998; Janeway, 2001; Akira, 2011). The innate immune system was once believed to lack specificity and diversity for detection of microbes, but recently the discovery of an evolutionary conserved family of receptors designated Toll like receptors (TLRs) has changed this view (reviewed by Akira, 2011). Furthermore, the innate immune system has other means to detect microbes including the complement system and certain intracellular sensors that together enables it to be efficient in discrimination among non-self, missing self and induced-self (reviewed by Hoebe *et al*., 2004). The first recognition strategy involves the ability of the host to detect conserved molecular patterns that are essential to the microorganism, but are not present in the host. The second strategy, involves the recognition of normal self-markers which are essential to the host but absent from the microorganism and functions to inhibit the

immune response. The third strategy involves recognition and marking of abnormal self for elimination by the immune system, a strategy that is effective in dealing with viral infection and cellular transformation (Reviewed by Medzhitov and Janeway, 2002; Gonzalez *et al*., 2011). However, although the innate immune system provides immediate recognition and initiates a rapid response against infection, it has been characterised as a temporary system that cannot compete with the demands for complete eradication of the microbes (Reviewed by Gonzalez *et al*., 2011).

The adaptive immune system is a second line of defence which employs a diverse set of somatically encoded receptors. This arm of immune system is usually triggered by different innate immune response mediators to mount stronger and long-lasting attacks against microbes (Gonzalez *et al*., 2011). The adaptive immune response is dependent on antigen-specific receptors expressed on T and B lymphocytes. An important feature of the adaptive response is immune memory that is possible due to the long life span of B and T cells that persist in a dormant state, but re-activate when they encounter a specific antigen for a second time (Chaplin, 2010).

1.1.3 Cellular elements of the immune response

An efficient immune response requires a combination of different subsets of leukocytes. Each subset exerts a specific job to detect or clear the pathogen. Most immune cells are derived from bone marrow progenitor cells, see Figure (1.1). Immune cell formation starts by differentiation of hematopoietic stem cells (HSC) in bone marrow into common myeloid progenitor (CMP) cells or common lymphoid progenitor (CLP) cells (Kawamoto *et al*., 1997; Kondo *et al*., 1997; Akashi *et al*., 2000). Lymphoid progenitor cells undergo further differentiation giving T cells, B cells, natural killer (NK) and NK-T cells (reviewed by Chaplin, 2010). B-cells express the B-cell receptor and can be distinguished according to the antibody type that they produce and the antigen that they detect. T-cells are defined by T-cell receptor expression (Akashi *et al*., 2000). NKs are granular lymphocytes that recognise infected or tumour cells by activating or inhibitory receptors on their surfaces (Jonsson and Yokoyama, 2009). NK-T cells have NK and T-cell activity

(Balato *et al*., 2009). The myeloid progenitors are precursors of many cell types; Megakaryocyte/Erythrocyte progenitors (MEPs) or Granulocyte/Macrophage progenitors (GMPs). MEPs differentiate to platelets and erythrocytes (Akashi *et al*., 2000; Adolfsson *et al*., 2005). GMPs differentiate to monocytes which give rise to macrophages or dendritic cells and diverse types of granulocytes like neutrophils, eosinophils, basophils and mast cells (Kumar and Jack, 2006; Chaplin, 2010). Another subgroup has been identified as a new subset of cells that share many characteristic with myloid progenitors designated as macrophage/dendritic cell progenitors (MDPs) (Fogg *et al*., 2006). MDPs differentiate to monocytes and common DC precursors (CDPs) (Varol *et al*., 2007). See Figure (1.1).

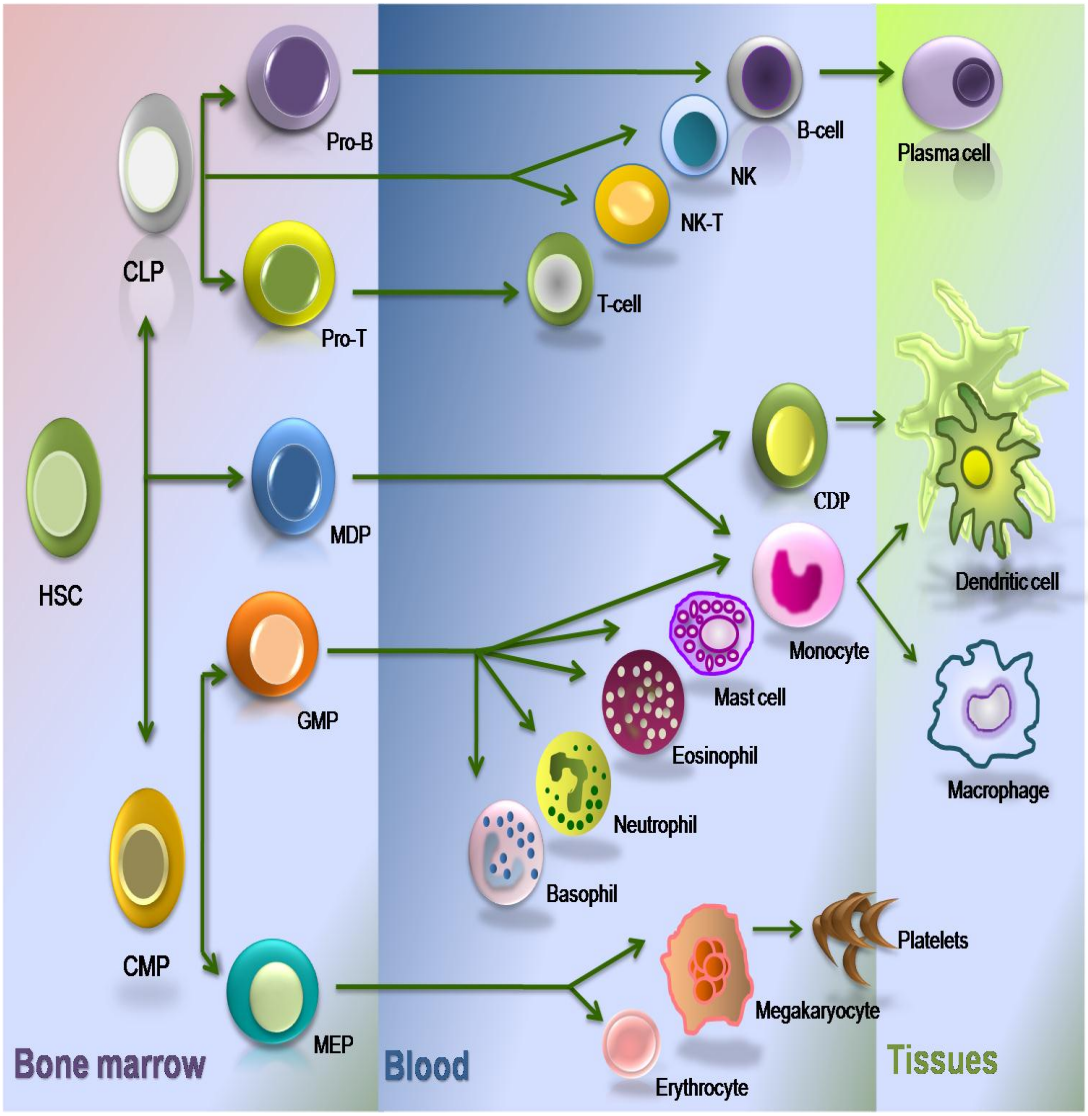


Figure 1.1. Immune cells lineage. Hematopoitic stem cells (HSCs) differentiate in the bone marrow into common lymphoid progenitors (CLPs) and common myeloid progenitor cells (CMPs). CLPs differentiate to NK, NK-T cells, Pro-B and Pro-T cells which give B-cells and T-cells respectively. CMPs differentiate to Granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs). GMPs give rise to monocytes that differentiate peripherally to different subsets of macrophages in different tissues and inflammatory DCs. GMPs also give rise to diverse types of granulocytes including mast cells, eosinophils, neutrophils and basophils. MEPs differentiate to erythrocytes and megakaryocytes which give rise to platelets. Different types of dendritic cells are derived from common DC precursors (CDPs), which are derived from HSCs in bone marrow.

1.1.4 Monocytes, macrophages and dendritic cells

Monocytes were described earliest, as the circulating division of mononuclear phagocytes that circulate with blood and settle down forming tissue macrophages in different organs such as spleen, liver, lymph nodes, lung, peritoneal cavity and the subcutaneous tissues (Van Furth and Cohn, 1968). Broadly, monocytes act as immune effector cells that are derived from bone marrow. They circulate in the blood and populate different tissues as macrophages or dendritic cells. They migrate from blood to tissues during infection following stimulation of their chemokine and adhesion receptor (reviewed by Geissmann *et al*., 2010). Monocytes are heterogenous cells that are diverse morphologically and phenotypically (Geissmann *et al*., 2003; Ziegler-Heitbrock, 2007). The myeloid progenitor cells give rise to monoblasts which are the precursor of pro-monocytes that give rise to monocytes (Gordon and Taylor, 2005). Monocytes participate in pathogen and debris clearance both by phagocytosis and by production of proinflammatory cytokines, reactive oxygen intermediates (ROI) and nitric oxide (NO) (Serbina *et al*., 2008). On the other hand, these cells contribute to chronic inflammatory diseases such as atherosclerosis and also paly role in the development of autoimmune diseases (Nahrendorf *et al*., 2007; Ingersoll *et al*., 2011). In mice, half of the blood monocytes migrate via the circulation each day in a steady state condition (Van Furth and Cohn, 1968; Van Furth *et al*., 1973). A number of effete cells end in the spleen but a considerable fraction of monocytes also enter different body tissues and differentiate either into tissue macrophages (Van Furth *et al*., 1973) or dendritic cells (Randolph *et al*., 1999). Tissue macrophages remain in the same tissue for the whole life of the individual while tissue dendritic cells transfer to draining lymph nodes via afferent lymphatic vessels, stay for a few days, mature, present antigen to T cells and die (reviewed by Muller, 2001). In mice, two monocyte populations have been identified inflammatory and resident monocytes, depending on the time they spend in blood (Geissmann *et al*., 2003). The two types of murine monocytes can be distinguished by the expression of cell-surface markers. Cells expressing CC-chemokine receptor 2 ($CCR2^{+}$), CX3C-chemokine receptor1 ($CX3CR1^{low}$) and $GR1^{+}$ are defined as inflammatory monocytes while cells expressing $CCR2^{-}$, $CX3CR1^{Hi}$ and $GR1^{-}$ are defined as resident monocytes (Geissmann *et al* ., 2003). Both types of murine

monocytes are found in equal percentage in the blood (Passlick *et al*., 1989). Human monocytes are also classified according to cell-surface markers; classical monocytes are identified as those that express $CD14^{Hi}$ and $CD16^{-}$ and represent 90-95% of the total monocytes in a healthy person (Passlick *et al*., 1989; Strauss-Ayali *et al*., 2007). Non-classical monocytes or proinflammatory monocytes express $CD14^{+}$ and $CD16^{+}$ and are associated with lower levels of the anti-inflammatory cytokine IL-10 production in response to TLR4 stimuli and higher levels of TNFα production in response to TLR2 and TLR4 agonists. This subpopulation contributes to about 5-10% of the total monocytes in a healthy person (Strauss-Ayali *et al*., 2007).

1.1.5 Macrophages

Macrophages are major phagocytic cells that differentiate from circulating monocytes. They circulate with blood to different tissues in steady state or in response to inflammation forming a wide range of tissue macrophages each with different characteristics; macrophage of the bone (osteoclasts), macrophage of the central nervous system (microglial cells), macrophage of the connective tissue (histiocytes), macrophage of the liver (kupffer cells), and also alveoli, gastrointestinal, spleen and peritoneum macrophages (Kumar and Jack, 2006). "Stimulate the phagocytes" is the concept adopted by Elie Metchnikoff a hundred years ago when he won the Nobel Prize for his description of the phagocytic process and his assumption to consider this process as a key element for the immune response (Nathan, 2008). After this, macrophages were known as immune effecter cells and the first line of the host defence. In addition, they also have important vital homeostatic roles which are often ignored by immunologists (reviewed by Mosser and Edwards, 2008). Regarding homeostatic roles of macrophages, in each day approximately 2×10^{11} erythrocytes are cleared by macrophages. Macrophages save about 3kg of iron each year for reuse by this process (Kono and Rock, 2008). Moreover, these cells contribute to the removal of dead cells and debris that are formed during tissue trauma (Kono and Rock, 2008; Kumar and Jack, 2006). The detection and removal of apoptotic cells and cellular debris is also related to macrophages homeostatic function. These vital processes are mediated by scavenger receptors or phosphatidyl serine, thrombospondin, and integrins and complement

receptors (Erwing and Henson, 2007). In conclusion, the main role of macrophages is to clear cellular debris not to serve as immune cells (reviewed by Mosser and Edwards, 2008). It has been found that the physiology of macrophages markedly changes when necrotic cellular debris is engulfed. This includes alteration of surface-protein expression and cytokine production. The detection and clearing of necrotic cellular debris and other endogenous stimuli is unlike the clearance of apoptotic cells and tissue debris. It is usually mediated via Toll-like receptors which makes the process part of the innate immune response (Kono and Rock, 2008).

1.1.6 Activated macrophage phenotypes

It is widely accepted today that macrophages can exert a remarkable plasticity that enables them to change their phenotype to respond efficiently to different environmental stimuli, see Figure (1.2). However, physiological changes of macrophages due to different environmental stimuli may increase their antimicrobial activity. They do not always enhance their immune activity. Whether these physiological changes improve the immune response or downregulate it, they must be properly regulated to avoid dangerous consequences. A number of different macrophage activation states have been recognised; innate activated macrophages, classically activated macrophages, alternatively activated macrophages and regulatory macrophages.

1.1.6.1 Innate activated macrophages

Macrophages responding to microbial stimuli alone (in the absence of IFNγ or IL-4 or IL-23) have been described as 'innate activated macrophages' (Forlenza *et al*., 2011). However, they are similar to classically activated cells in their phagocytic activity and production of inflammatory cytokines, reactive oxygen species (ROS) and nitric oxide (NO) (Gordon and Taylor, 2005; Forlenza *et al*., 2011). They differ in certain other aspects and most notably they express arginase (Menzies *et al*., 2011) and have marked but transient physiological changes got upon stimulation (Mosser and Edwards, 2008). Ligation of cell surface receptors including Toll like receptors (TLRs) by lipopolysaccharide (LPS) is implicated in producing this type of macrophages (Forlenza *et al*., 2011).

1.1.6.2 Classically activated macrophages

The classically activated macrophages (CAMΦ) or type 1 macrophages refer to macrophage populations stimulated by interferon γ (IFN γ) and tumour necrosis factor (TNF) or TLR ligand (typically LPS). This phenotype has enhanced microbicidal and tumoricidal capacity with obvious proinflammatory cytokine production (O'Shea and Murray, 2008). NK cells are the early source of IFN-γ that activates macrophages transiently. IFN-γ is produced from Th1 cells and provides persistent macrophage stimulation to produce proinflammatory cytokines which increase immune activity and give better resistance against infection (Dale *et al*., 2008). Macrophages can also be activating via MyD88- dependent TLR signalling which induces TNF release. TNF with IFNγ cooperates to autocrine stimulation of macrophages (Mosser and Zhang, 2008). Interferon-β (IFN-β) which is released by activation of the TRIF-dependent pathway through IFN regulatory factor 3 (IRF3) can replace IFN-γ as the classically activated signal (Yamamoto *et al*., 2003a). Classically activated macrophages need to be controlled. Excessive cytokines and other immune mediators production can cause severe host tissue damage and development of autoimmune diseases (Szekanecz and Koch, 2007).

1.1.6.3 Alternatively activated macrophages

Alternatively activated macrophages (AAMΦ) can also be termed as wound healing macrophages. This type of macrophage is generated in the presence of IL-4 or IL-13 and up- regulates mannose receptor expression (Stein *et al*., 1992). A major function of these cells is to convert arginine to ornithine by the arginase enzyme. Ornithine is considerd as a precursor of collagen and polyamine which are required in wound healing and tissue repair (reviewed by Varin and Gordon, 2009). Many studies have shown that AAMΦ produce chitinase and chitinase-like molecules, including (YM1) and (YM2), acidic mammalian chitinase (AMCase) and stabilin-interacting chitinase-like protein (Raes *et al*., 2002; Zhu *et al*., 2004). It was suggested initially that these molecules were associated with antifungal and antiparasitic activity of the macrophages by the direct interacting with the surface chitin of some parasites, insects and fungi. Currently, it is believed that chitin degradation activity was shown

to be lacking in YM1 and to all the family molecules (Bleau *et al*., 1999; Fusetti *et al*., 2002). Over-stimulation of the IL-4 receptor is also associated with dysregulation or overproduction of wound healing matrix such as tissue fibrosis with schistosomaisis. Macrophages that lack the IL-4 receptor or treatment with antibody that inhibits IL-4 receptor do not develop this pathogenesis (Hesse *et al*., 2001).

1.1.6.4 Regulatory macrophages

This macrophage population has features that distinguish them from CAMΦ and AAMΦ. It is believed that these cells can be generated by different stimuli and are not preferred to be grouped as one population. However, all of them share the capacity of high IL-10 production (reviewed by Mosser and Zhang, 2008). Regulatory macrophages usually require a combination of two signals. The first signal includes the immune complex (Gerber and Mosser, 2001), prostaglandins (Strassmann *et al*., 1994), glucocorticoids, apoptotic cells (Erwig and Henson, 2007), adenine nucleotide (Hasko *et al*., 2007) and IL-10 (Martinez *et al*., 2008) applied simultaneously with the second signal, TLR stimuli. These signals can produce a macrophages population with potent anti-inflammatory response (reviewed by Mosser and Edwards, 2008) depending on induction of IL-10 and down regulation of IL-12 (Gerber and Mosser, 2001). Regulatory macrophages behave as antigen presenting cells as they highly express CD80 and CD86 co-stimulatory molecules (Edwards *et al*., 2006). Some parasites, bacteria and viruses serve as the above signals to result in the development of regulatory macrophages that encourag the spread and survival of these microorganisms (Mosser and Edwards, 2008).

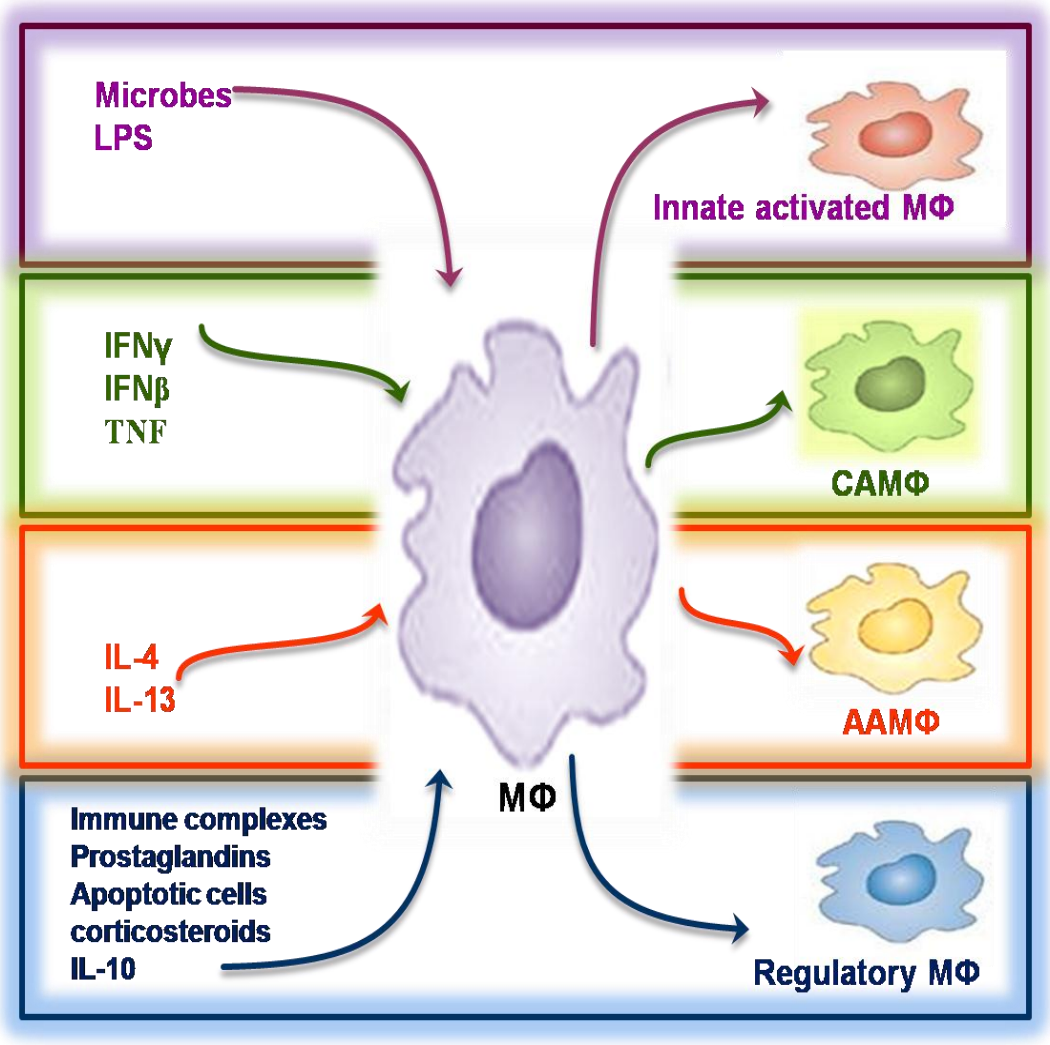


Figure 1.2. Macrophage phenotypes. Microbial stimuli including TLR agonists such as LPS produce innate activated macrophages. Interferon-β (IFN-β), interferon-γ (IFN-γ) and tumor necrosis factor (TNF) produced by innate immune or adaptive immune elements can activate macrophages classically. Interleukin-4 (IL-4) and Interleukin-13 (IL-13) produced by innate and adaptive immune cells can activate macrophages alternatively. Various types of stimuli, including immune complexes, prostaglandins, apoptotic cells, corticosteroids and interleukin-10 (IL-10) can generate regulatory macrophages.

1.1.7 Dendritic cells

Dendritic cells (DCs) are potent antigen presenting cells that represent the second major group of mononuclear phagocytes (Kumar and Jack, 2006; Geissmann *et al*., 2010). They were first recognised in 1973 and distinguished from macrophages as a novel cell population in mouse spleen (Steinman and Cohn, 1973; Steinman *et al*., 1975). Immature DCs recognised as highly active phagocytic cells respond rapidly to environmental stimuli and mature to cytokine- producing cells (Banchereau and Steinman, 1998; Mellman and Steinman, 2001). DCs play a crucial role in triggering of adaptive immunity (Steinman *et al*., 1975). This process is initiated by DCs activation via Toll Like Receptors (TLR) expressed by the cells and involve, engulfing, processing and presenting antigenic peptides to naive T cells in lymph nodes (Medzhitov and Janeway, 2002; Mellman and Steinman, 2001; Guermonprez *et al*., 2002; Fujii *et al*., 2004). Different forms of immune or tolerance responses can be achieved by the interaction of DCs and T cells rely upon DC types and their activation states to provide key signals that influence their differentiation (Shortman and Naik, 2007)

1.1.8 Dendritic cell types

It is widely documented that DCs have many distinguishable subsets, each with specialised functions and locations (Shortman and Liu, 2002; Shortman and Naik, 2007). Although, all DC subtypes possess the ability to process and present antigen to T cells, they are differ in function, migratory pattern, location, surface marker and cytokine production (Wu and Liu, 2007; Conti and Gessani, 2008). Thus different classification systems were established that gathering the cells under specific category. Most common one used the original precursor and cell surface marker as a tool to group and name them. Accordingly, most of the classification systems agree to consider macrophage and dendritic cell precursors (MDPs) as the major source of all types of DCs (Varol *et al*., 2007). MDPs differentiate to monocytes and common dendritic cell progenitors (CDPs) which give rise to plasmacytoid DCs (pDCs) and precursors of conventional DCs (cDCs) (pre-cDCs) (Naik *et al*., 2007; Onai *et al* ., 2007). Although, monocytes cannot give rise to cDCs or pDCs (Fogg *et al*., 2006;

Auffray *et al*., 2009a), they can under inflammatory conditions enter tissues and develop to cells that have the main phenotypic features and function of DCs and for this reason they are named inflammatory DCs (Geissmann *et al*., 2003; Serbina *et al*., 2008; Auffray *et al*., 2009b).

1.1.8.1 Conventional DCs (cDCs)

These cells have all the features of DCs and perform the same functions in steady state (healthy state). cDCs are derived from MDPs and pre-cDCs. Different types of DCs can be produced from different pre-DCs (Shortman and Naik, 2007). cDCs can be further divided to migratory and lymphoid DCs.

Migratory DCs

Migratory DCs leave their places in peripheral tissues and migrate to lymphoid organs via the lymphatics. These cells are also derived from MDPs and pre-DCs and are found in all peripheral tissues like skin, lung, liver, kidney and intestine (Kushwah and Hu, 2011). Most of the DCs in non-lymphatic peripheral organs are probably of the migratory cDC type (Shortman and Naik, 2007). The best example of these cells is Langerhans cells (LCs) (Schuler and Steinman, 1985). LCs are a special type of DCs found in epidermis and possess particular features that distinguish them from other DCs (Romani *et al*., 2003). These cells can also be found in the epithelial lining of reproductive organs, respiratory and digestive system (Iijima *et al*., 2007). Activation of LCs raises major histocompatibility complex class II (MHC II) and costimulatory molecule expression and enhances their migration to lymph nodes to present antigen to T cells to activate adaptive immunity (Stoitzner *et al*., 2008).

Lymphoid DCs

This subdivision of cDCs is described as resident cells that stay in lymphoid organs like lymph nodes, thymus and spleen (Kushwah and Hu, 2011). Lymphoid cDCs can be further separated into two subdivisions, $CD8^+$ that express CD8α on its surface and $CD8^-$ that not present this marker (Vremec *et al*., 1992). In mice these two subdivision show considerable differences in cytokine production (Proietto *et al*.,

2004). Upon stimulation, $CD8^+$cDCs are considered the major source of IL-12 that drives the differentiation of inflammatory Th1 cells (Hochrein *et al*., 2001). These cells are also efficient at the cross-priming process by which they can divert microbial antigen to MHC class 1 pathway (Den Haan *et al*., 2000). These features make $CD8^+$cDCs the dominant inducer of the cytotoxic T cell response against viral infections (Belz *et al*., 2004). Both upon activation or even at the steady-state $CD8^+$cDCs can be found in lymphoid tissues at T cell area (Steinman *et al*., 1997), while $CD8^-$cDCs are found in marginal areas in normal condition and only migrate to the T cell area when activated (Metlay *et al*., 1990; De Smedt *et al*., 1996).

1.1.8.2 Plasmacytoid DCs (pDCs)

Plasmacytoid DCs are also known as interferon –producing cells (IPCs) as they have the ability to produce copious amount of Type-1 interferon following the stimulation of their TLR7 and TLR9 by viruses or bacteria (Liu, 2005). Initially they were described as a small population of cells resemblings plasma cells morphologically, but they do not have B or T cell markers and home to secondary lymphoid tissues (Facchetti *et al*., 1988; Fitzgerald-Bocarsly, 1993). pDCs reach lymph nodes through high endothelial venules (HEVs) as do naive T cells. The interaction between pDCs and HEVs followed by migration of these cells from blood to peripheral lymph nodes are achieved by the expression of adhesion molecules and chemokine receptors (Yoneyama *et al*., 2004a; Diacovo *et al*., 2005). pDCs were initially regarded as precursor of DC. Currently, it is widely accepted that these professional effector cells can be further differentiated to give another professional cell type which has all the morphological and function characteristic of DCs and this accounts as a unique phenomenon in cell biology (Soumelis and Liu, 2006). Besides their production of Type 1 IFN, pDCs produce interleukin-12 (IL-12) (Dalod *et al*., 2003) and proinflammatory chemokines (Krug *et al*., 2002; Megjugorac *et al*., 2004). Moreover, pDCs express major histocompatibility complex class II (MHC II) (Villadangos and Young, 2008). Using all these mechanisms enabling pDCs to stimulate proliferation and differentiation of T cells (Colonna *et al*., 2004), promote B-cells differentiation to plasma cells (Jego *et al*., 2003; Shaw *et al*., 2010), induce NK and $CD80^+$ immune response against viral infection (Romagnani *et al*., 2005)

and induce different DC types differentiation, maturation and immune response (Yoneyama *et al*., 2005). pDCs are the only immune cells that switch from professional innate immune cells to professional antigen presenting cells linking innate and adaptive immune system (Soumelis and Liu, 2006).

1.1.8.3 Inflammatory dendritic cells (inflammatory DCs)

This group of DCs are not detectable in the steady-state and appear as a consequence of microbial infection or inflammation. Inflammatory DCs are derived from inflammatory monocytes. This type of DCs produces tumour necrosis factor (TNF) and inducible nitric- oxide synthase (iNOS) (Shortman and Naik, 2007). Generation of inflammatory DCs depends on Granulocyte Macrophage Colony Stimulating Factor (GM-CSF). Thus inflammatory monocytes of GM-CSF deficient mice fail to develop in MHC class II after they enter the spleen (Shortman and Naik, 2007). Surface markers can distinguish these cells from other types of DCs. They are recognised by the expression of a glycoprotein (MAC3) which is also found on activated macrophages, by moderate expression of CD11c and high level of CD11b and the absence of expression of CD4 and CD8 (Naik *et al*., 2006).

1.2 Toll like receptors (TLRs)

1.2.1 Introduction to TLRs

Detection of non-self (microbes) is a fundamental step to protection strategies or immunology. Immune response is usually ignited once a foreign body is recognised (Beutler, 2009), but how microbes are recognised? This question had required decades of investigation resulting in the discovery of a diverse group of germ line encoded receptors on different types of innate immune cells. These receptors were termed pattern recognition receptors (PRR) (Janeway, 2001). PRRs include several classes, each with specific functions like opsonisation, activation of complement system, or phagocytosis (Pasare and Medzhitov, 2004). Among these classes is the family of Toll-like receptors (TLRs). TLRs can be defined as conserved receptors that represent the first line of defense against wide range of invading microorganism and the key to the innate immune system (Doyle and O'Neill, 2006). These receptors

were considered initially as essential receptors of *Drosophila* defense against fungal infection and the innate immune response (Lemaitre *et al*., 1996). Their roles include the recognition of wide range of microbial structures that are conserved among microorganisms and are not found in mammals. These molecular structures are known as pathogen-associated molecular patterns (PAMPs). Detection of different PAMPs triggers a variety of signalling pathways that lead to inflammatory gene expression and the production of anti-inflammatory cytokines, chemokines and type -1 interferons α/β which help in the clearing of infectious agents (Kopp and Medzhitov, 2003; Takeda *et al.*, 2003; Kawai and Akira, 2005). Secretion of these cytokines and chemokines can contribute to activation of the adaptive immune system receptors and maturation of dendritic cells (Takeda *et al*., 2003; Kawai and Akira, 2005).

TLRs are type-1 membrane glycoproteins consisting of an extracellular domain comprised of leucine rich repeats (LRR) responsible for recognition of different types of PAMPs, a transmembrane, spacing component and a cytoplasmic portion which is similar to that of the interleukin-1 receptor (named Toll-IL-1 receptor domain abbreviated to TIR) (Takeda and Akira, 2003). Currently, 13 members of TLRs have been identified in mammals (Uematsu and Akira, 2006). TLR1-9 are present in both humans and mice, humans express TLR10 while TLR11-13 are expressed in mice only. The functions of TLR10 in humans and TLR12 and TLR13 in mice remain unclear (Kawai and Akira, 2011). TLR 1, 2, 4, 5 and 6 are expressed on the plasma membrane on cell surface. These receptors can recognise molecules derived from bacteria, fungi, and protozoa while TLR3, 7, 8 and 9 are expressed inside the cell within endocytic compartments and are responsible for the detection of nucleic acids derived from viruses or bacteria (Kumer *et al*., 2009a; Kawai and Akira, 2011).

1.2.2 TLRs and their ligands

Lipopolysaccharide LPS from gram negative bacteria, which are composed of lipid A, core oligosaccharide, and o-side chain, is recognised by TLR4 via detection of the lipid A moiety of LPS (Shimazu *et al*., 1999; Miller *et al*., 2005). Respiratory

syncytial virus (RSV) and mouse mammary tumour virus (MMTV) have been reported to be recognised by TLR4 (Kawai and Akira, 2009; Kumer *et al*., 2009a). The fungal component glucuronoxylomannans is recognised by TLR4 and the protozoa components glycoinosito-phospholipids (GIPLs) and glycosylphosphatidyl-inositol anchors (GPI-anchor) derived from *Trypanosoma (species), P.falciparium,* and *T.gondii* are detected by TLR4 and TLR2 (Kawai and Akira, 2009; Kumer *et al*., 2009a). Bacterial, fungal, protozoal and viral components are detected by TLR2 as illustrated in Table (1.1) (Akira *et al*., 2006; Kawai and Akira, 2009; Kumer *et al*., 2009a). TLR2 and TLR1 heterodimer can recognise triacyl lipopeptide while TLR2 and TLR6 dimer recognise diacyl lipopeptide structures on bacteria, mycobacteria, and mycoplasma (Takeuchi *et al*., 2001; Takeuchi *et al*., 2002, Kumer *et al*., 2009b). RSV has been reported to induce cytokines and chemokines signalling through TLR2 and TLR6 (Murawski *et al*., 2009). Double stranded RNA (dsRNA) can be detected by TLR3 (Alexopoulou *et al*., 2001), flagellin from flagelatted bacteria is detected by TLR5 (Kawai and Akira, 2011; Kumer *et al*., 2009b). TLR7 and 8 can recognise viral and CPG DNA motifs related to bacteria Genomic of DNA viruses and Genomic DNA of other microorganisms are recognised by TLR9 (Takeda *et al*., 2003; Wagner, 2009). Profilin from *T.gondii* is recognised by mouse TLR11 (Kawai and Akira, 2009; Kumer *et al*., 2009a). A summary of TLR ligands is illustrated in Table (1.1)

Table 1.1. TLRs and their Ligands (adapted from Kumar *et al*., 2009b)

Receptor	Location	PAMPs	Species	Adaptor(s)	TF(s)
TLR1 & 2	Plasma membrane	Triacyl lipopeptide	Bacteria& Mycobacteria	TIRAP, MyD88	NFκB
TLR2	Plasma membrane	Peptidoglycan, Lipoarabinomannan, Hemagglutinin, Phosphlipomannan, Glycosylphosphotidyl-Inositol mucin,	Bacteria, Mycobacteria, Virus, Fungi, Protozoa	TIRAP, MyD88	NFκB
TLR3	Endosome	ssRNA, dsRNA	Virus	TRIF	NFκB,IRF3&7
TLR4	Plasma membrane	LPS, Glycoinositolphospholipids, RSV & MMTV, Glucuronoxylomannans	Bacteria, Protozoa, Virus, Fungi	TIRAP, MyD88, TRAM & TRIF MyD88	NFκB,IRF3&7
TLR5	Plasma membrane	Flagellin	Bacteria	MyD88	NFκB
TLR6&2	Plasma membrane	Diacyl lipopeptide,	Mycoplasma	TIRAP, MyD88	NFκB
TLR7	Endosome	RSV	Virus	MyD88	NFκB, IRF7
TLR8	Endosome	ssRNA	Virus	MyD88	NFκB, IRF7
TLR9	Endosome	dsDNA (HSV), CPG motif, Genomic DNA	Virus Virus, Bacteria,	MyD88	NFκB, IRF7
TLR11*	Plasma membrane	Profillin-like molecules	Protozoa	MyD88	NFκB

TF, transcription factor; RSV, Respiratory syncytial viruse; MCMV, Murine cytomegalovirus; HSV, Herpes simplex virus; CPG, Cytidine-phosphate-guanosine; *Mice TLR11

1.2.3 TLRs signalling pathways

TLRs signalling is intiated by interaction of the cytoplasmic Toll-IL-1 receptor (TIR) domains with addition signalling proteins. TIR domains contain proline residues which are conserved in all TLR types except TLR3. Substitution of proline amino acids by histidine results in negative effects on TLRs signalling (Hoshino *et al*., 1999). Three marked conserved regions have been detected in the TIR domain through which TLRs and signal transduction adaptor proteins can assemble as protein-protein interaction (Lu *et al*., 2008). Signalling events are initially mediated through recruitment of various TIR domain containing adaptor molecules of different TLRs. These adaptor molecules include myloid deferentiation primary- response gene88 (MyD88), TIR-domain containing adaptor protein inducing IFNβ (TRIF) also known as (TICAM-1), TIR-domain-containing adaptor protein (TIRAP) also called MyD88 adaptor-like (MAL), TRIF-related adaptor molecule (TRAM) and sterile α – and armadillo-motif-containing protein (SARM) (Uematsu and Akira, 2006; O'Neill and Bowie, 2007; Kumar *et al*., 2009a; Kawai and Akira, 2011; Takeuchi and Akira, 2010). Recruitment of these adaptor molecules activate varied transcription factors and kinase proteins such as nuclear factor-κ B (NFκB), interferon regulatory factor 3 or 7(IRF3)/(IRF7), and mitogen-activated protein kinases (MAPKs) to coordinate pro-inflammatory cytokines, chemokines and Type-1 interferons production (Kumar *et al*., 2009a; Kawai and Akira, 2011; Takeuchi and Akira, 2010).

MyD88 is utilised by all TLRs except TLR3. TLR signalling involves MyD88, known as the MyD88-dependent pathway and is essential for pro-inflammatory cytokines production including expression of TNFα, IL-6, IL-12, and IL-1β and costimulatory molecules activation (Akira *et al*., 2006). TIRAP coordinates with MyD88 to stimulate the MyD88-dependent pathway in TLR1, 2, 4, and 6 signalling (Kawai and Akira, 2011; Takeuchi and Akira, 2010). MyD88 is utilised alone by TLRs 5, 7, 9, and 11.

Another intercellular signalling pathway known as the TRIF-dependent pathway has also been investigated extensively. TLR3 and TLR4 are the only TLRs that can activate this pathway through the TRIF adaptor molecule (Takeda and Akira, 2003). This pathway induces type-1 interferon production by activation of IFN

regulatory factor 3 (IRF3) and 7(IRF7) and proinflammatory cytokines (Yamamoto *et al*., 2002; Hoebe *et al*., 2003). TRAM is another adaptor utilised by TLR4 but not TLR3 to induce the TRIF-dependent pathway (Oshiumi *et al*., 2003; Yamamoto *et al*., 2003b).

1.2.3.1 The MyD88-dependent pathway

Almost all TLRs utilise this signalling pathway, in which forming a complex between the adaptor molecules and TIR-domain triggers signalling cascades downstream of these receptors and promotes transcription of a vast number of proinflammatory genes (Lu *et al*., 2008). MyD88 consists of a C-terminal region (approximately 150 aa residues) which associates with the TIR domain of TLRs and an N-terminal region (approximately 90 aa residues) which corresponds to a death domain (DD) (Bonnert *et al*., 1997). MyD88-TIR domain forms complexes involving cytosolic TLR domains.

The best example of the activation of the MyD88-dependent pathway is TLR4 signalling. LPS activates TLR4 by serial interactions of a number of proteins including; LPS binding protein (LBP), cluster of differentiation 14 (CD14) and myloid differentiation protein-2 (MD2). Firstly, LBP binds LPS and promotes interaction between LPS and CD14, CD14 in turn facilitate binding of LPS to TLR4/MD2 complex (Lu *et al*., 2008; Fitzgerald *et al*., 2004). Although, the soluble protein MD2 can associate with TLR4, it can form a complex with LPS even in the absence of TLR4 (Nagai *et al*., 2002). Pathogenic LPS activates and interacts with MyD88-TIR domain in coordination of another adaptor molecule (TIRAP/MAL) (Horng *et al*., 2001). TIRAP/MAL is utilised by TLR2 and TLR4 (Horng *et al*., 2002). These signal initiated complexes recruit IL-1 receptor associated kinase (IRAK) by the interaction of MyD88 and IRAK death domains. IRAK4 firstly activated and then IRAK1 and IRAK2 undergo phosphorylation and dissociation from receptor sites and then bind to tumour necrosis factor receptor (TNFR)-associated Factor 6 (TRAF6). TRAF6 and NFκB essential modifier (NEMO) also known as IκB kinase γ (IKKγ) are polyubiquitinated by E3 ubiquitin ligase which forms a complex with E2 ubiquitin-conjugating enzymes (Adhikari *et al*., 2007).

Ubiquitinated TRAF6 induces transforming growth facter-β-activated kinase 1 (TAK1) and TAK1-binding proteins (TABs) such as TAB1, TAB2 and TAB3 to form a complex. This complex undergoes further interactions with IKKs and complexation then ends with the phosphorylation of the IKKs (Takeda and Akira, 2004). Phosphorylated IKKs activates NF-κB through IκBα phosphorylation, ubiquitination and degradation by the cellular proteasome. Activated NF-κB translocates into the nucleus and binds a specific DNA sequences called response elements (REs). This process leads to expression of proinflammatory cytokine genes and adhesion molecules (Arancibia *et al*., 2007). The MyD88–dependent pathway is also responsible for activation of mitogen activated protein (MAP) kinases. TAK1 activates two members of the MAP kinase kinase family, (MKK3) and (MKK6), which in turn activate c-Jun- N terminal kinase (JNK) and p38 respectively that lead to ATF2-c-Jun activation and induction of activation protein-1 (AP-1) (Adhikari *et al*., 2007; Takeda and Akira, 2004). Stimulation of TLR7 and TLR9 in pDCs also recruits MyD88. MyD88 downstream of TLR7 and 9 activates IRF7 transcription factor by the recruitment of various signalling proteins like IRAK4, TRAF6, TRAF3, IRAK1 and IKKα to induce type-1 IFNs production. Other proteins such as osteopontin, phosphoinositol 3 kinase (PI3K), mTOR and p70S6K are also important for IRF7 phosphorylation (Kumar *et al*., 2009a; Kawai and Akira, 2011).

1.2.3.2 The TRIF-dependent pathway

It has been demonstrated that TLR4 has the ability to activate both NF-κB and JNK in MyD88 knockout mice but with delayed kinetics which suggested the presence and involvement of a different signalling pathway (Kawai *et al*., 1999; Takeda and Akira, 2004). This pathway was identified and is now known as TRIF-dependent pathway. TIR domain-containing adaptor inducing IFN-β (TRIF) is required by this pathway which is restricted to TLR3 and TLR4 (Akira *et al*., 2001). This signalling pathway besides its ability to activate the transcription factorsNFκB and AP-1, it activates the IRF3 pathway

Two distinct pathways have been reported to activate NFκB via TRIF. The first one is mediated through the C-terminal of TRIF which contains a Rip homotypic

interaction motif (RHIM) and interacts with receptor-interacting protein 1 (RIP1) (Meylan *et al*., 2004). A complex forms with ubiquitinated RIP1 or TRAF6 to recruit TAK1 which subsequently activates NFκB (Cusson-Hermance *et al*., 2005). The second pathway relates to the N-terminal of TRIF which interacts with TRAF6 through TRAF6-binding motifs (Sato *et al*., 2003; Meylan *et al*., 2004). TRAF6 induces TAK1 activation in a similar way to the MyD88-dependent pathway which leads to phosphorylation of IκB proteins and activation of NFκB (Sato *et al*., 2003; Meylan *et al*., 2004). One of the most important signalling pathways triggered by the TRIF-dependent pathway is the activation of the IRF3 transcription factor, see Figure (1.3). In this pathway IRF3 induces the expression of IFNβ and several IFN-inducible genes (Ogasawara *et al*., 2012). TANK binding kinase 1 (TBK1) also known as TRAF2-associated kinase (T2K) or NFκB activating kinase (NAK) and inhibitor of kappa B kinase i (IKKi), also known as IKKε, phosphorylate IRF3 by binding to TRAF3 (Oganesyan *et al*., 2006). IRF3 could also be activated via TLR3 by enhanced another tyrosine phosphorylation (not mediated by TBK1) which recruits phosphatidylinositol 3-kinase (PI3K)/Akt (Sarkar *et al*., 2004). Phosphorylated IRF3 translocates to the nucleus, binds a specific DNA sequence known as the IFN-stimulated response element (ISRE), and induces Type-1 IFNs and other IFN-inducible genes (Chau *et al*., 2008) .Figure 1.3 summarises the MyD88- and TRIF- dependent signalling pathways mobilised by TLR3 and TLR4.

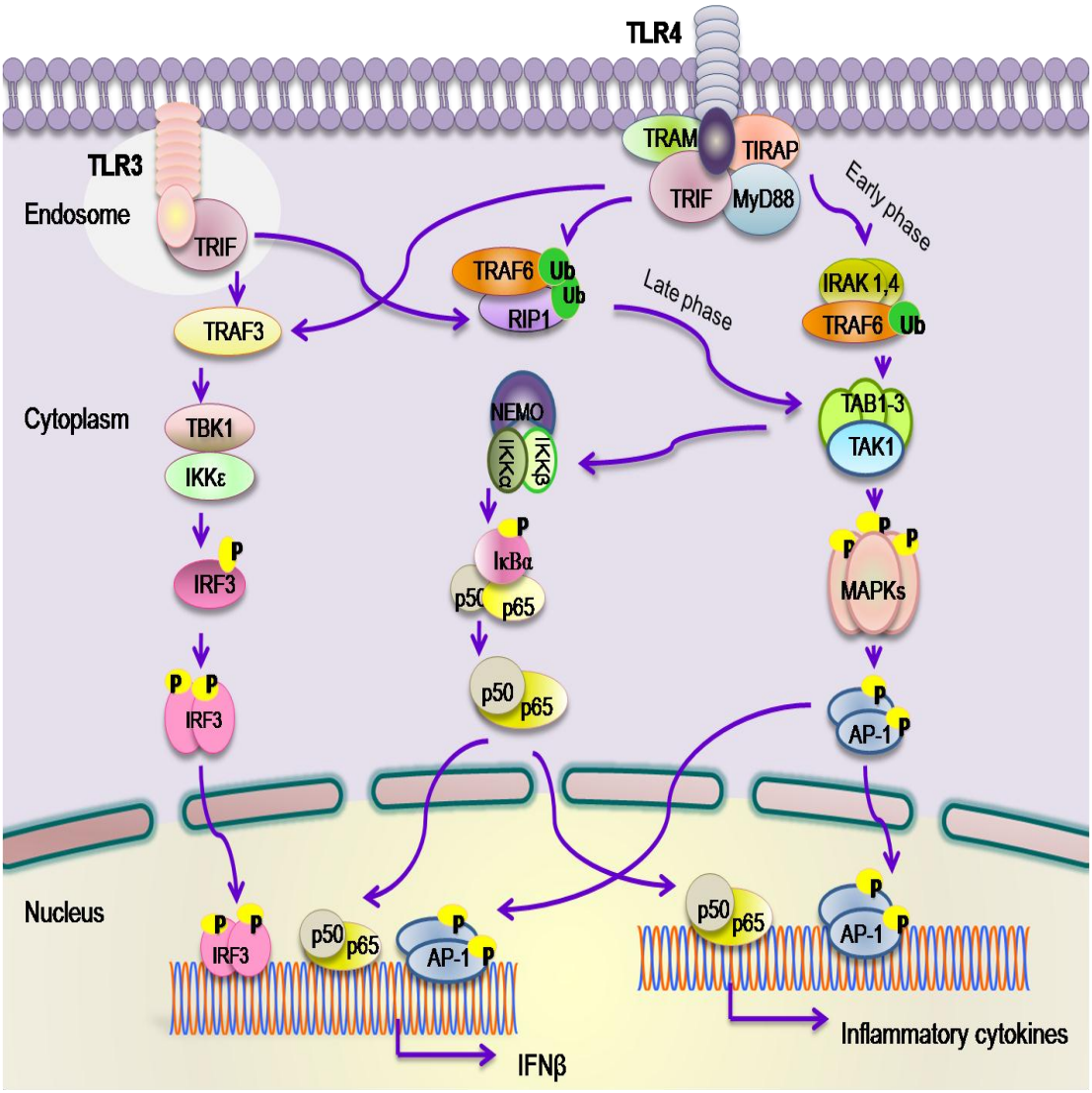


Figure 1.3. TLR3 and TLR4 signalling pathways. TLR4 activates both MyD88 and TRIF-dependent signalling. TIRAP is utilised by TLR4 togather with MyD88 to activate IRAK family members that help TRAF6 activation (early phase). TRAF6 with E3 ubiquitin ligase activates TAK1 which activates the IKK complex .IKKs (IKKα, IKKβ and NEMO) induce IκB phosphorylation (P), Ubiqutination(Ub) and then degradation. Thus, liberating p50/p65 heterodimers to translocate to the nucleus.Within this pathway TAK1 can also activate MAPKs which enhance phosphorylation of AP-1. Activated AP-1 translocates into the nucleus and together with NFκB induces the production of inflammatory cytokines. Another adaptor molecule TRAM is required by TLR4 to recruit TRIF-dependent pathway. TLR3 and TLR4 recruit TRAF3 via TRIF signalling. TRAF3 activate TBK1 and IKKε which then induce IRF3 phosphorylation. Activated IRF3 forms a homodimer, translocates into the nucleus and with NFκB and AP-1induces IFNβ production. TRIF also induces TRAF6 activation (late phase) together with RIP1 and induces TAK1 in similar manner as in MyD88- dependent signalling

1.2.4 The key elements of TLRs signalling

TLR3 and TLR4 activation trigger three important elements governing the signalling downstream of these receptors and are documented to regulate wide range of proinflammatory cytokines, chemokines and Type 1 IFNs genes. These include NFκB, IRF3 and MAPKs.

1.2.4.1 Nuclear factor kappa B (NFκB)

Sen and Baltimore in 1986 introduced NFκB as a nuclear factor that binds the k light chain enhancer in B-cells (Doyle and O'Neill, 2006). This pathway could be activated by over 200 physiological stimuli. These involve bacterial and virus products, stress genes, apoptosis regulators, enzyme, growth factors, transcriptional factors and others (Tergaonkar, 2006). As a result, this pathway can activate a large number of specific genes. The NFκB family consists of several dimeric transcription factors that form homo- or hetero-dimers, regulating the expression of many overlapped but discrete genes utilised in innate and adaptive immunity, anti-apoptosis, proliferation, stress response or cancer (Ghosh *et al*., 1998; Silverman and Maniatis, 2001; Karin and Greten, 2005; Gilmore, 2006).

In mammals, five members of NFκB have been identified: NFκB1 (p105/p50), NFκB2 (p100/p52), Rel A(p65), Rel B and c-Rel. NFκB1 and NFκB2 are considered as the large members that give rise to the smaller DNA-binding members p50 and p52 respectively. All NFκB members contain Rel- homology domain (RHD), present in the N-termini. The RHD contains a nuclear localisation sequence (NLS) which interacts with IκB proteins and with specific DNA sequence known as κB sites (Ghosh *et al*., 1998). Rel A, Rel B and c-Rel have transcription activation domains (TADs) which are present in the C-termini (Hayden *et al*., 2006). p50 and p52 lack TADs (Tong *et al*., 2004). Homodimers of p50 or p52 repress transcription because they do not contain C-terminal TADs and can only be active as transcriptional dimers when bound to Rel A, Rel B and c-Rel (Gilmore, 2006). The inhibitory IκB proteins tightly interact with NFκB members and regulate their activity. The members of the inhibitory proteins are comprised of six or more ankyrin repeats, a regulatory domain in the N-terminus and a peptide sequence which

is rich in proline (P), glutamic acid (E), serine (S), and threonine (T) known as PEST motif-containing domain in C-terminal (Jacobs and Harrison, 1998). Seven IκB proteins have been identified: IκBα, IκBβ, IκBγ, IκBε, Bcl-3, IκBζ and IκBNS. Cytoplasm to nuclear translocation of NFκB members is inhibited due to interaction with IκBα, IκBβ, IκBγ, or IκBε while Bcl-3, IκBζ and IκBNS regulate transcription by interaction with NFκB inside the nucleus (Kawai and Akira, 2007). Thus, NFκBs are present as inactive complexes in the cytoplasm bound to IκB proteins. Two signalling pathways (see Figure 1.4) have been described to activate NFκB and subsequently lead to translocation from the cytoplasm to the nucleus. These include the classical, also known as canonical pathway and an alternative pathway, also known as non-canonical pathway.

The NFκB classical (canonical) pathway

The IKKβ-dependent IκB degradation pathway is referred to as classical NFκB activation or the canonical pathway. In unstimulated cells the RelA/p50 heterodimer is inactive in the cytoplasm through interaction with IκB proteins. Upon stimulation by different inflammatory signals e.g. TLR ligands, phosphorylation of the IκB proteins occurs at specific serine residues is catalysed by the IKK complex (IKKα, IKKβ, and NEMO). IKKα and IKKβ have very similar structures, both have a kinase domain in their N-terminal regines, a lucine zipper (LZ) domain, helix-loop-helix (HLH) motif in their C-termini and a NEMO-binding domain (NBD) (Gilmore, 2006; Hayden *et al*., 2006). It has been reported that IKKβ but not IKKα is responsible of NFκB activation in TLR signalling (Karin and Greten, 2005). In contrast, termination of NFκB activation can be mediated through IKKα activity (Li *et al*., 2005). Phosphorylated IκB proteins undergo polyubiquitination and degradation by the 26S proteosome, liberating linked NFκB. As a result of NFκB activation, p50 and p65 dimers translocate to the neucleus enhancing transcription of chemokines, cytokines, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule1 (VCAM-1), endothelial leukocyte adhesion molecule 1 (ELAM-1), enzymes responsible for the production of inflammatory mediators and inhibitors of apoptosis (reviewed by Bonizzi and Karin, 2004). These molecules aid in migration of inflammatory cells where NFκB is activated in response to infection. It

has been widely accepted that engagement of all TLRs results in NFκB activation and expression of similar target genes (Medzhitov, 2001). See Figure (1.4)

The NFκB alternative (non-canonical) pathway

NFκB1/p105 and NFκB2/p100 can prevent their own nuclear translocation by interaction of their C-termini, which act as IκB proteins, with their own RHD. Processing of p100 to p52 is an important step in the alternative NFκB activation pathway which depends completely on IKKα but not IKKβ and IKKγ (Dejardin *et al*., 2002). NFκB inducing kinase (NIK) activates IKKα. IKKα-IKKα homodimer phosphorylates NFκB2/p100 at two C-terminal sites followed by ubiquitination and then degradation (Senftleben *et al*., 2001). Phosphorylation of these two sites of p100 is crucial to process it to p52. This pathway involves partial degradation only of the inhibitory C-terminal side of p100 (Xiao *et al*., 2001). Subsequently to the degradation of C-terminal, the N-terminal aspect, NFκB-p52 that contains the RHD is librated. p52 then in the form of a heterodimer with Rel B, translocates to the nucleus and to regulate target genes expression (Xiao *et al.*, 2001). The alternative pathway plays an important role in the development and maintenance of secondary lymphoid organs (Bonizzi and Karin, 2004). It is uncertain if this pathway can be activated by TLR ligands in parallel to the classical pathway but it is mobilised by activation of the lymphotoxin-β receptor (LTβ), B-cell-activating factor belonging to the TNF family (BAFF) and CD40 (hayden *et al*., 2006). See Figure (1.4).

p50-p50 homodimer mediates a third NFκB activation pathway. This dimer can only be activated through interation with the nuclear IκB molecules such as Bcl3 (Kawai and Akria, 2007).

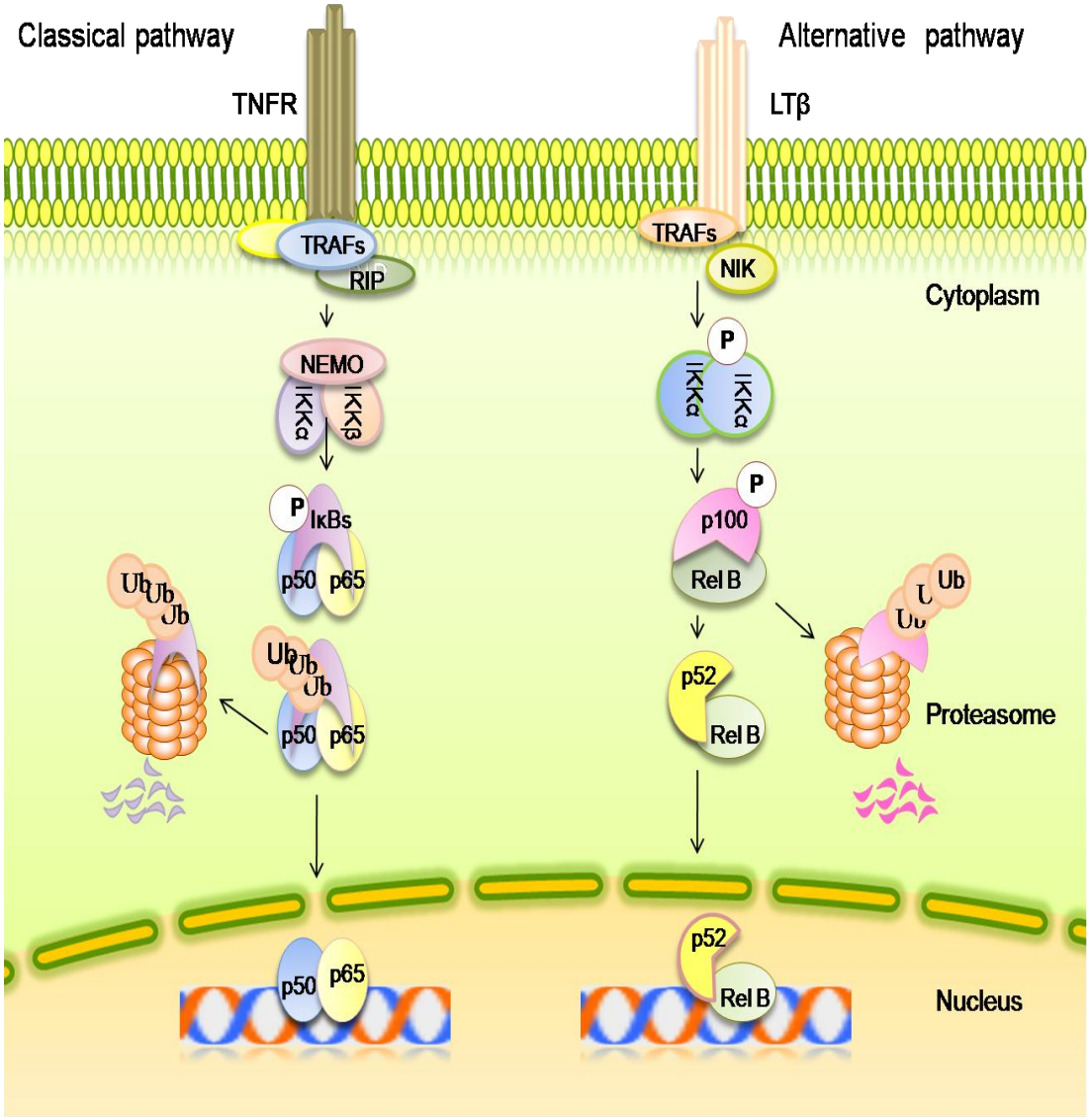


Figure 1.4. The NFκB activation pathways (Classical and Alternative). Activation of classical pathway through TLRs or the TNFR recruits adaptor molecules e.g. TRAFs and RIP. This will activate the IKK complex (IKKα, IKKβ, and NEMO). IKKβ undergoes phosphorylation (P). Phosphorylated IKKβ activates IκBα. Phosporylated IκBα undergoes polyubiquitination (Ub) and subsequent degradation liberating p65/p50 heterodimers. p65/p50 dimer translocate into the nucleus initiating transcription of target genes. The alternative pathway is activated by lymphotoxinβ or CD40 ligands which recruit TRAFs adaptor molecules that activate IKKα-IKKα homodimer. Phosphorylated IKKα dimer activates p100. Phosphorylation of the two sites of p100 is crucial to process it to p52. The forming heterodimer of p52 and RelB then translocates to the nucleus and activates transcription of target genes.

1.2.4.2 Interferon regulatory factors (IRFs)

The IRFs are a family of transcription factors involved in the expression of type 1 interferon genes. First recognition of these factors was in the 1950s from independent investigations by Isaacs and Nagano, in which they demonstrated the release of anti-viral factors by virally infected cells (Nagano and Kojima, 1954; Isaacs and Lindenmann, 1957). The IRF family is comprised of nine members (Taniguchi *et al.*, 2001). All family members play important roles in immunoregulation stimulated by TLR and other pattern recognition receptors. The induction of type 1 interferon in general is initiated by virus infection via activation of virus-responsive elements (VREs) that are located in the upstream region of type1 IFN-genes. The N-terminal of each IRF members contains a DNA-binding domain that tends to bind a distinct DNA sequence known as IFN-stimulated response element (ISRE). VREs possess two binding sites, one is for ISRE and the other is the binding site for, NFκB and activator protein 1 (AP-1) (Wathelet *et al*., 1998; Mamane *et al*., 1999). Four members of the IRF family, IRF1, 3, 5 and 7 have been implicated as positive regulators of the transcription of Type 1 IFN genes (Mamane *et al*., 1999). IRF1 was the first family member discovered and reported as inducer of Type 1 IFN gene promoters (Miyamoto *et al*., 1988). However, recently it has been reported that neither IRF1 nor IRF5 is required for virus infection mediated Type 1 IFNs production (Honda and Taniguchi, 2006).

Interferon regulatory factor 3/interferon regulatory factor 7 (IRF3/IRF7)

Both IRF3 and IRF7 are considered as the key factors of Type 1 IFN production elicited by viral infection. TLR3, whether it is in the cell surface membrane as observed in fibroblasts or in endosomes, in other cell types such as epithelial or macrophage cells, the cytoplasmic retinoic acid- inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are the receptors that induce IRF3 and IRF7 activity and therefore trigger antiviral responses upon dsRNA activation (Yoneyama *et al*., 2004b; Sasai *et al*., 2006). IRF3 is expressed constitutively in many tissues and induced exclusively by viral infection (Au *et al*., 1995). It forms either homodimer or heterodimer with IRF7, each dimer induces

different genes of the family of Type 1 IFNs. IRF3 homodimers can induce IFNβ production but not IFNα except IFNα 4 (Sato *et al*., 1998), whilst IRF7 homodimers induce both IFNα and IFNβ production (Sato *et al*., 1998; Sato *et al*., 2000). IRF3 is presents in an inactive form in the cytoplasm. It undergoes phosphorylation in a signal-dependent manner and forms a dimer that translocates to the nucleus following viral infection. This dimer interacts with co-activators, the cyclic–AMP-responsive-element-binding protein (CREB-binding protein) or p300 (Weaver *et al*, 1998; Wathelet *et al*., 1998; Suhara *et al*., 2000). The formed complex translocates into the nucleus and binds target DNA sequences. The protein kinases that are activated by virus and activate IRF3 are identified as two serine/threonine kinases; TBK1 (Bonnard *et al*., 2000) and IKKε (Shimada *et al*., 1999), both kinases phosphorylate serine residues in the C-terminus of IRF3. Cyclophilin B (CYPB) an immunophilin and a family member of the cis-trans peptidylprolyl isomerases, has also dissociated to be involved in IRF3 phosphorylation by helping TBK1-mediated phosphorylation and dimerisation of IRF3 following viral infection (Obata *et al*., 2005; Fitzgerald *et al*., 2003a). Another family member, NIMA-interacting 1(PIN1) peptidylprolyl isomerase cis-trans peptidylproplyl isomerase, enhances binding of polyubiquitin to serine- phosphorylated IRF3 and hence proteasome-dependent degradation of IRF3 to terminate IRF3 action (Saitoh *et al*., 2006). Moreover, there is another IRF3 inhibitor, suppressor of IKKε (SIKE), that inhibits IRF3 phosphorylation after viral infection by interact with IKKε and TBK1 (Huang *et al*., 2005).

Type 1 IFNs production mediated by IRF3 and IRF7 displays biphasic kinetics and involves two-step induction (Figure 1.5). The first step or the early phase of type1 IFN gene induction is mediated through IRF3 activation and the second step (late phase) represents a positive-feedback loop mediated by IRF7 activity, each phase responsible for activation of a distinct set of genes. Examples of IRF3 mediated early Type 1 IFNs induction was demonstrated in murine fibroblasts by the immediate release of IFNβ and IFN4α upon viral infection (Marie *et al*., 1998). As a result of the 2 phases, abundant production of Type 1 IFNs is apparent during viral infection. By this mechanism, IRF7 plays an essential role in Type 1 IFNs gene induction whilst IRF3 contribution is of less importance (Honda *et al*,

2005), supported by the observed severe impairment of Type 1 IFNs production in *Irf7* $^{-/-}$ mice infected by ssRNA viruses. As mentioned previously, viral infection triggers TLR signalling pathways that activates non-canonical IKK kinases, IKKε and TBK1 which induce phosphorylation of IRF3 and IRF7 (Sharma *et al*., 2003). Activated IRF3 translocates into the nucleus and induces IFNβ and IFNα4 production as the initial phase of Type 1 IFNs production which is characterised by small production of the IFNs. This initial IFN production induces conformational changes in IFNα receptor 1 and 2 (IFNAR1 and 2) which are the receptors of Type 1 IFNs. These activated receptors recruit and activate what is called Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling pathway. Jak1 and tyrosine kinase 2 (Tyr2), two janus kinases (JAK) family members mediate this pathway (Schindler *et al*., 2007). By this way signal transducer and activator of transcription1/2 (STAT1/STAT2) and IRF9 are activated and together form the IFN-stimulated gene factor 3 (ISGF3) complex (Schindler *et al*., 1992). Activated ISGF3 translocates to the nucleus and regulates transcription of specific genes among them, IRF7 (Takaoka and Taniguchi, 2003). IRF7 launches a positive feedback regulation initiating the second phase of IFNs production. IRF7 homodimers or the heterodimers of IRF7/IRF3 are responsible for the cytosolic pathway of Type 1 IFNs production which is distinguishable by the copious production of mediators (Erlandsson *et al*., 1998; Marie *et al*., 1998; Honda *et al*, 2005). The IRF3 homodimer is responsible for the activation of other genes rather than IFNs, like chemokine CXC-chemokine ligand 10 (CXCL10) (Nakaya *et al*., 2001).

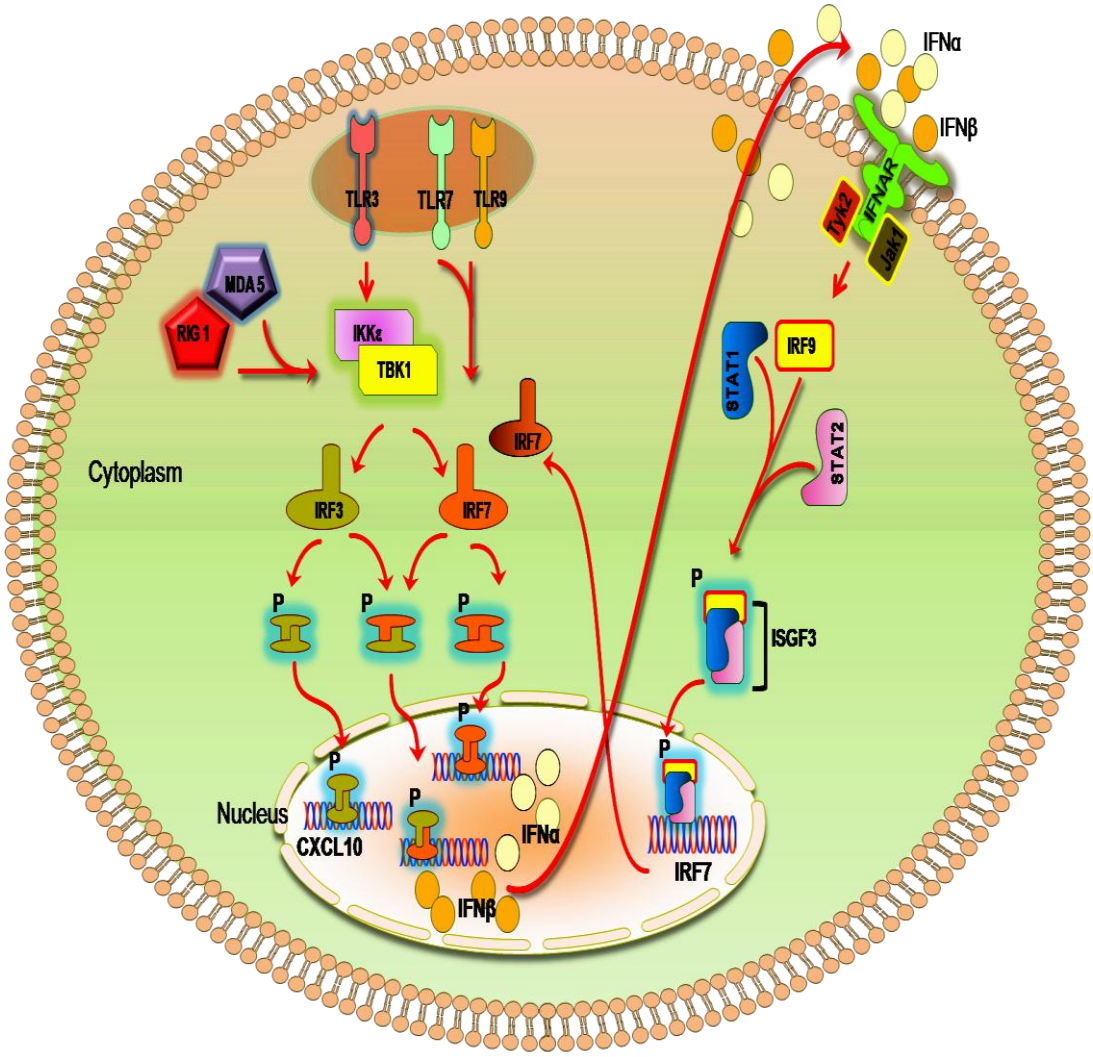


Figure 1.5. Positive-feedback regulation of Type 1 IFN production. Viral infection induces Type 1 IFN production in 2 phases mediated by TLR3/4, RIGI and MDA5 receptors by recruitment of IRF3 and IRF7. The early phase initiated by phosphorylation (P) of homo or hetero dimers of IRF3 and IRF7 which translocate into the nucleus, IRF7 may be activated via TLR7 and TLR9 as well. Different dimers are responsible for induction of different genes among them CXCL-10, IFNα and IFNβ .Although trace amount of IFNα and IFNβ are produced during this phase, it can bind and activate type 1 IFN receptors (IFNAR1 and IFNAR2 heterodimer). Acivation of Type 1 IFN receptors recruits Jak-STAT signalling pathway, by which STAT1, STAT2 and IFN9 gathering to form ISGF3. Phosphorylated ISGF3 translocates to the nucleus and regulates the transcription of IRF7 gene. Newly synthesised IRF7 can be activated by the viral infection to induce Type 1 IFNs production in copious amounst through delay phase of Type 1 IFNs production.

1.2.4.3 Mitogen activated protein kinases. (MAPKs)

The MAPKs represent a family of highly conserved serine/threonine protein kinases. They are involved in many cell processes like cell survival and death, proliferation, mitosis, metabolism, differentiation, gene expression, cellular stress and inflammatory responses (Thalhamer *et al*., 2008; Cargnello and Roux, 2011). The signal transduction of MAPKs is among the most ancient pathways that are used to regulate a wide range of different physiological processes (Widmann *et al*., 1999). These signalling pathways are activated by a vast range of stimuli such as cytokines, hormones, neurotransmitters, growth factors and cell adherence (Widmann *et al*., 1999; Kyriakis and Avruch, 2001). The MAPK protein family comprises 7 groups; conventional MAPKs which includes the extracellular signal- regulated protein kinases 1/2 (ERK 1/2), c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), p38 isoforms (α, β, γ, and δ) and ERK5 (Pearson *et al*., 2001), Whilst atypical MAPKs include ERK3/4, ERK7 and the nemo-like kinase (NLK) (Coulombe and Meloche, 2007). Each member of the family has its own activators, inhibitors and substrates (Tanoue and Nishida, 2003). MAPKs regulate gene expression or transcription by different ways; they enhance gene transcription by modulating chromatin structure, or by enhancing the activity of transcription factors such as AP-1, others by other regulatory mechanisms that include the change in stability, transcription and translation of mRNA (Wancket *et al*., 2011) Currently 14 MAPK members have been characterised but only 3 groups are studied extensively which include: ERK1/2, JNKs and p38 isoforms. Each group among conventional MAPKs is activated by sequential phosphorylation pathways (MKKK-MKK-MAPK). The MAPK kinase kinase (MAPKKK) is a Ser/Thr kinase, upon activation it activates the next kinase in the series MAPK kinase (MAPKK) by phosphorylation on two serine residues (Chang *et al*., 2003). MAPKK in turn phosphorylates MAPK on threonine and tyrosine residues in the Thr-Xaa-Tyr motif of activation loop between kinase subdomains VII and VIII of MAPK pathways which is essential for enzymatic activity and activation of ERK2 (Robbins *et al*., 1993). Last by a MAPK in such an activated series can regulate a wide range of substrates including transcription factors, other protein kinases, phospholipases and cytoskeleton-associated proteins (Whitmarsh and Davis, 1996; Widmann *et al*., 1999). See Figure (1.6).

The first member of MAPKs to be characterised was ERK1/2. The kinase activation cascade of ERK includes MAPKKKs (A-Raf, B-Raf, and Raf-1), the MAPKKs (MEK1and MEK2), and the MAPKs (ERK1 and ERK2) (Roux and Blein, 2004). ERK is recognised as comprises 2 isoforms. The two isoforms, ERK1 and ERK2, are 44 and 42 kDa proteins respectively that share 83% amino acids identity. ERK1/2 is expressed ubiquitously but to different extents (Boulton *et al*., 1991). It is activated by dual phosphorylation on threonine and tyrosine residues in activation loop motif Thr-Glu-Tyr (Wancket *et al*., 2011). Growth factors, cytokines, osmotic stress, serum and microtubule disorganisation are the main activators of this pathway (Lewis *et al*., 1998). ERK1/2 is localised in the cytoplasm and accumulates in the nucleus upon activation (Chen *et al*., 1992). ERK can phosphorylate large number of substrates at serine-threonine residues and plays an important role in cell proliferation (Yoon and Seger, 2006).

p38 is the second member of the MAPKs family. The signalling cascade for p38 activation involves MAPKKKs (MEKKs 1 to 4, (MEKK1-4), MLK2 and -3, DLK, ASK1, Tpl2 and Tak1), the MAPKKs (MEK3 and MEK6 also known as MKK3 and MKK6, respectively), and the four known p38 isoforms (α, β, γ, and δ) (Kyriakis and Avruch, 2001). It could be activated by environmental stress such as oxidative stress, UV irradiation and hypoxia and by cytokines such as TNFα (Kyriakis and Avruch, 2001). Upon activation, p38 play vital role in immune response due to its presence in many immune cellular components such as macrophages, neutrophils and T cells (Ono and Han, 2000). It participates in many activities like exocytosis, adherence and apoptosis in macrophages and neutrophiles (Ono and Han, 2000). Moreover, many studies showed that p38 is essential for T cell activation and Th1 differentiation. Sustained p38 MAPK activation leads to increase IFN-γ production in T cells stimulated by IL-12/ IL-18 (Zhang and Kaplan, 2000; Yang *et al*., 2001) and p38 kinase inhibitors block IFN-γ production by Th1 but have no effects on IL-4 production by Th2 (Rincon *et al*., 1998). It has been demonstrated that p38 MAPK regulates IFN-γ production by $CD8^+$ cells in the same way as in $CD4^+$ cells, i.e. sustained p38 activation promote IFNγ production and the opposite is also true (Merritt *et al*., 2000). See Figure (1.6)

JNK, the third member of the family is also termed a stress-activated protein kinases (SAPKs), see Figure (1.6). It is ubiquitously expressed and activated by phosphorylation of tyrosine and threonine residues in its kinase domain by dual specificity kinases MKK4 and MKK7 (Sanchez *et al*., 1994; Holland *et al*., 1997). JNK is activated by various cellular stresses, phorbol esters, heterotrimeric G-proteins, serum and growth factors (Seger and Krebs, 1995; Chang and Karin, 2001). JNK signalling controls different cellular processes such as cell differentiation, growth, transformation and apoptosis (Seger and Krebs, 1995; Chang and Karin, 2001). JNK activates transcription factors like c-jun, ATF2, p53 and c-Myc (Karin, 1995; Davis, 2000).

MAPKs activity is terminated to contribute to the coordinated gene transcription events in the cell by phosphatase-mediated dephosphorylation as a mode of deactivation. Phosphatase proteins include tyrosine, serine/threonine and dual specificity protein phosphatases. These phosphatase enzymes are known as MAPK phosphatases (MKPs) (Keyse, 1999; Keyse, 2000). 10 members of dual specificity phosphatases (MKPs) are recognised and each targets a specific MAPK substrate (Owens and Keyse, 2007).

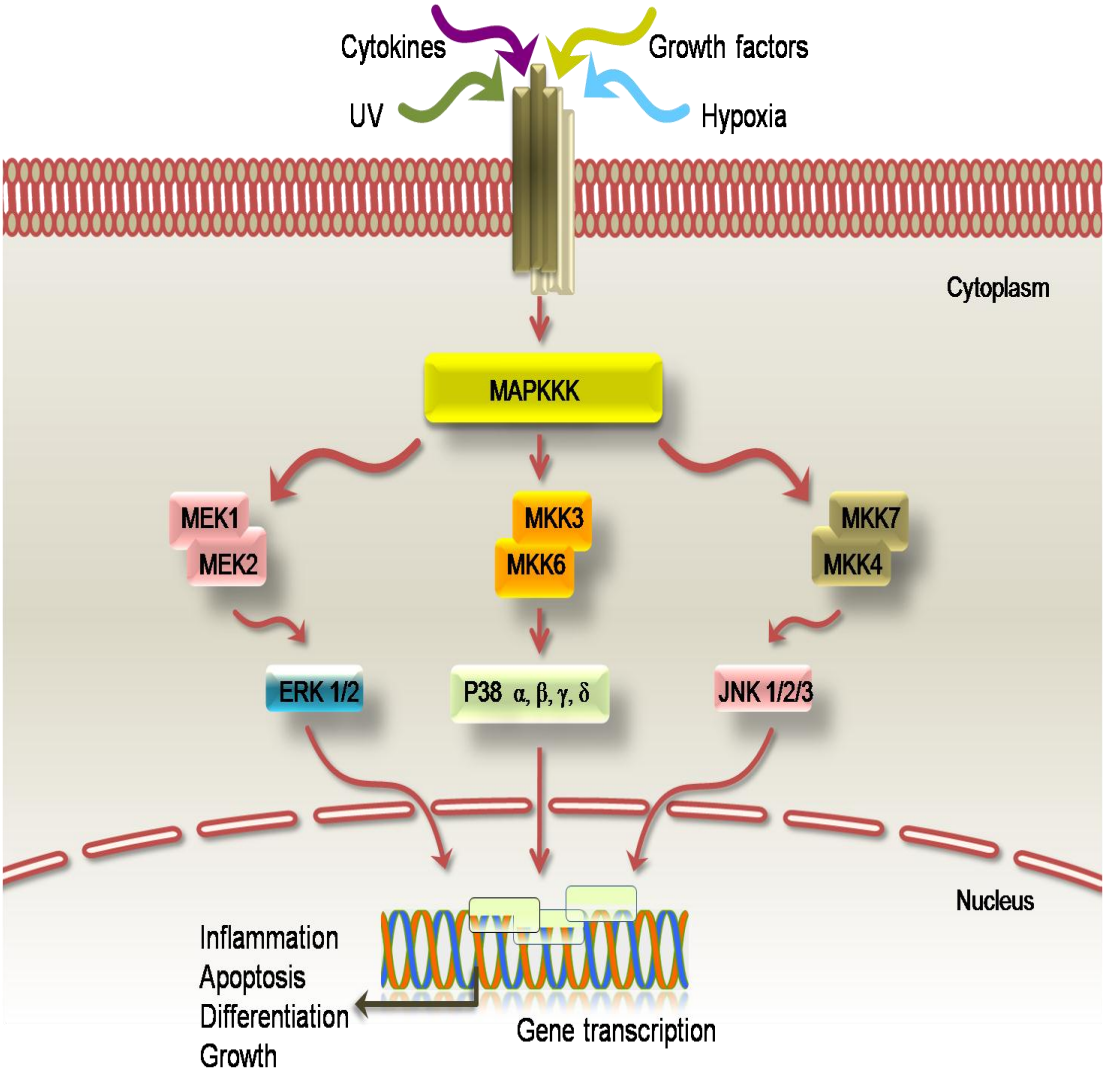


Figure 1.6. MAPKs signalling pathways. The mitogen-activated protein kinase MAPK pathways involve three tiers of protein kinases (MAPKKK, MAPKK and MAPK) and are sequtionally phosphorylated following receptors activation.Three subfamilies of MAPKs are presented to illustrate MAPKs signalling cascade, these include ERK1/2, JNK1/2/3 and p38 isoforms. ERK1/2 are activated by mitogenic stimuli such as growth factors and associated with cell proliferation, whilst, JNK and p38 are activated by stress stimuli such as UV irradiation or hypoxia and by cytokines. For this they are called stress-activated MAPKs and are associated with inflammation and apoptosis**.**

1.3 Steroid hormones

1.3.1 Introduction to steroid hormones

Steroids are a group of hormones that contain a cyclopentanophenanthrene moiety designated as steroid nucleus. The steroid nucleus is built from the fusion of 3 cyclohexane rings and one cyclopentane ring. Different steroid hormones or steroidal compounds result from variable functional group substitutions on the four ring-nucleus. They divide to 5 main groups; glucocorticoids and mineralocorticoids are synthesised in the adrenal cortex, androgens are synthesised in adrenal cortex and testes, and estrogens and progestins which are synthesised in the ovary and placenta (Beato and Klug, 2000). These hormones penetrate cell-membranes readily to enter the cytoplasm due to their non-polar structure. In the cytoplasm, steroid hormones bind their homologous receptors which act as transcription factors. Steroid hormone receptors (SHRs) are modular proteins belonging to the superfamily of nuclear receptors (Green and Chambon, 1986). These receptors remain usually in an inactive state bound to heat shock proteins but are readily activated upon hormones binding (Pratt and Toft, 1997). The hormone-receptor complex then translocates into the nucleus either as a homodimer or a heterodimer. These hormone-receptor dimers bind the hormone-response element (HRE) in DNA and induce expression and transcription of different genes. Optimal gene transcription requires the contribution of coactivators, repressor and transcription regulators (Beato *et al*., 1996; Beato and Klug, 2000). Thereby, the regulation of a wide range of genes contributes to the regulation of vital physiological processes like immune responses, metabolism, electrolytes homeostasis, pregnancy and secondary-sex characteristics. In addition, there is accumulating evidence that steroid hormones have some rapid effects (within seconds) like vasodilatation and neuronal survival. These effects described as too rapid to be involved through RNA transcription and protein synthesis and as such are classified as non-genomic, non-nuclear or non-transcriptional effects (Falkenstein *et al*., 2000; Simoncini and Genazzani, 2003). Non-genomic effects are unlikely to be mediated through the intracellular steroid receptors and could be obtained even in the absence of steroid ligands (Falkenstein *et al*., 2000; Simoncini and Genazzani, 2003).

For example, the estrogen receptor α (ERα) can be activated by epidermal growth factor through triggering MAPKs signalling (Bunone *et al*., 1996).

1.3.2 Biosynthesis of steroid hormones

Steroid hormones are synthesised under the activity of cytochrome P450 enzymes, steroid dehydrogenase, reductase and aromatase. The first step in all body steroid synthesis is the conversion of cholesterol to pregnenolone by the enzyme "Cholesterol side-chain cleavage" CYP11A. Progesterone, which is a precursor of other steroids, forms from pregnenolone through 3β-hydroxysteroid dehydrogenase enzyme 3β-HSD activity (Sanderson, 2006). This enzyme is found in all steroidogenic and nonsteriodogenic tissues unlike CYP11A enzyme which is distributed in steroidogenic tissues only. 3β-HSD enzyme is present in two isoenzymes; type one found in steroidogenic tissues while type two is found in nonsteriodogenic tissues like liver (Simard *et al*., 2005).

Androgens are produced in steroidogenic tissues only. Cholesterol gives rise to androgens by the action of $P450_c17$ enzyme through 17-hydroxylase and 17, 20-lyase enzymes activaties. Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEAS) are weak androgens. They are considered as a precursor of estrogens produced during pregnancy (Dharia *et al*., 2004). DHEA/DHEAS are converted to testosterone by the action of both 17β-HSD isoenzymes (Baker, 2002). Testosterone is converted to the potent androgen called dihydrotestosterone DHT by the action of 5α-reductase enzyme (Sanderson, 2006). Androstenedione and testosterone produce estradiol by the action of aromatase and the 17β-HSD enzyme (Labrie, 1997). It has been found that luteinizing hormone (LH) and follicle stimulating hormone (FSH) upregulate aromatase during the critical phase of the menstrual cycle. Increased estrogen production promots ovulation. On the other hand, progesterone synthesis increases during luteal phase due to rise of CYP11A and 3β-HSD activity and abolishes aromatase activity as result of LH decline activity (Sanderson, 2006). The adrenal gland produces other steroids besides sex hormones. These steroids include mineralocorticoids and glucocorticoids (Simard *et al*., 2005; Sanderson, 2006). Progesteron and 17α-hydroxyprogesterone

are converted by steroid 21 hydroxylase CYP211 to 11-deoxycorticosterone and 11-deoxycortisol. The resulting precursors are then converted to aldosterone by the action of aldosterone synthatase CYP11B2 and to cortisone by 11-β hydroxylase CYP11B1 respectively (Reincke *et al*., 1998).

1.3.3 Sex hormones

Sex-hormones are the steroid hormones that are secreted mainly from the gonads and include Estrogens, Progestins, and Androgens. All of them have important roles in the male and female reproductive systems. Besides, they exert many other functions on the nervous, cardiovascular, and immune systems. They have also an important role in the development and structural integrity of the bones (Lorenzo, 2003; Razandi *et al*., 2004). Sex steroids are synthesised in ovaries, testes, and adrenal gland or by metabolism of the original hormones in other tissues like liver, kidney and peripheral tissues (Saloniemi *et al*., 2012).

It is widely accepted that sex steroids have modulatory effects upon the immune response due to the abundant ubiquity of steroid receptors throughout innate and adaptive immune cells such as T cell, macrophages, dendritic cells, granulocytes and mast cells (Klein, 2004; Ackerman, 2006). Moreover, there are sex-based differences to immune responses (Schuurs and Verheul, 1990; Roberts *et al*., 2001; Ray *et al*., 2008). Accordingly, the incidence and severity of infectious diseases is wildly varied between males and females (Klein, 2000; Klein, 2004). Females show lower rate of bacterial, viral and parasitic infections than males (Bouman *et al*., 2005; Lee *et al*., 2012). On the other hand, elevated immune response gives rise to higher rate of autoimmune diseases among females (Ackerman, 2006; Schmidt *et al*., 2006; Gleicher and Barad, 2007).

Sex susceptibility differences give extensive evidence that sex-associated hormones have direct effects on immune system (Zuk and McKean, 1996; Roberts *et al*, 2001). And this may be more attributable specifically to the female sex-hormone estrogen and progesterone. Despite hormone type, the levels of these hormones have also a major influence on the incidence or remission of the infection. Although, sex hormone levels differ between the both sexes, females are subjected to hormonal

level fluctuations during menstruation and pregnancy. Thus, females susceptibility to diseases, especially immune-based diseases like asthma, rheumatoid arthritis and systemic lupus erythmatosis, can vary considerably according to the stage of menstrual cycle or pregnancy (Skobeloff *et al*., 1996; Whitacre, 2001). Human progesterone level is 1-2nM during follicular phase of the menstrual cycle and rise to 15-20, 35-50 and 20-40 nM in early, mid, and late luteal phase respectively. During pregnancy the concentration of hormone is (100-500nM) in peripheral circulation and up to (1-10µM) in placenta (Arck *et al*., 2007). While human serum levels of 17β-estradiol, during early follicular phase of menstruation is 0.2nM and 1-2nM in follicular phase. During pregnancy the level rises up to 130nM (Schuurs & Verheul, 1990).

1.3.3.1 The estrogens

There are two types of estrogens. Natural estrogens usually have a steroidal nucleus, and are present in three forms estradiol-17β (E_2), estrone (E_1) and estriol (E_3). Estrone and estriol form from estradiol metabolism by the liver or from androgen in peripheral tissues (Masood *et al*., 2010). Synthetic estrogens are either steroidal or non-steroidal compounds.

1.3.3.2 Estrogen receptors signalling

Estrogen receptor signailling can be considered as a representative of all nuclear receptor superfamily signalling. Estrogens usually circulate in binding form with globuline. They dissociate from binding sites, enter the cell, and exert their actions. Estrogens activities start the moment they link to their receptors in different cells. Besides, the distribution of the receptors in reproductive organs, as mentioned above, many other tissues including different immune cells widely express estrogen receptors (Couse *et al*., 1997). In humans, two types of estrogen receptors have been recognised ERα and ERβ. Although, the two isoforms have the same structure, their functions are differ (Rider and Abdu, 2001). These receptors are observed predominantly in their stabilised form with heat shock proteins. Upon stimulation, they separate from heat shock proteins, undergo dimerisation and phosphorylation (Normanno *et al*., 2005). Activated receptors, forming either homo or hetero dimers,

bind to estrogen response elements (EREs) of various gene promoters and regulate their transcription (Cunningham and Gilkeson, 2011). The estrogen response elements (EREs) consist of two half-sites separated by three nucleotides, named the spacer. Recently, numerous non-classical signalling pathways via ERα and ERβ have been suggested. These pathways are EREs- independent i.e. DNA-binding has no effects on these transcription activities. By this mechanism, ERα regulates some transcription activity through protein-protein interaction with other transcription factors, coactivators or corepressor such as AP-1, p300 and NFκB (Jakacka *et al*., 2002; Biswas *et al*., 2005). Non-genomic estrogenic effects are usually mediated by extracellular receptors (plasma membrane receptor) (Razandi *et al*., 2004; Zhao *et al*., 2008). An example of non-genomic effects is the increase of uterine blood flow by elevated uterine cAMP levels (Pietras and Szego, 1977).

1.3.3.3 Estrogens and the immune response

The estrogens have the ability to modulate both innate and acquired immune system due to the wide spread of estrogen receptors throughout the whole immune system: lymphocytes, macrophages, dendritic cells, NK, granulocytes and mast cells (Cutolo and Castagnetta, 1996; Klein, 2000; Verthelyi, 2001). Estadiol has biphasic behavior towards immune response. At lower levels, it promotes the immune response while higher levels of the hormone suppress immunity (Forsberg, 1984) e.g. peak IFNγ production occurs at lower level of estradiol (Siracusa *et al*., 2008). TNFα/β secretion is also enhanced with lower levels and is inhibitd at higher levels of the hormone (Gilmore *et al*., 1997).

The role of estrogen in innate immunity

Regulation of neutrophil function

The neutrophils are important immune cells that represent about 90-95% of total granulocytes which constitute around 65% of the total white blood cells. In addition, they are considered as the cells to leave the bloodstream earliest towards an infection site (Bouman *et al*., 2005). Estrogen decreases chemotactic activity of neutrophils

(Miyagi *et al*., 1992) and exerts anti-inflammatory response on these cells (Bouman *et al*., 2005).

Regulation of macrophage function

Estrogen receptors inhibit different members of NFκB transcription factors family by several mechanisms depending on the cell type and the stimulus (Kalaitzidis and Gilmore, 2005). The inhibitory mechanisms involve either direct interaction of ER with NFκB that prevents NFκB-DNA binding and subsequently inhibits target genes transcription or working upstream within cytoplasm to interfere with kinase activities and degradation of IκB proteins (Kalaitzidis and Gilmore, 2005). 17β-Estradiol (E_2) inhibits the inflammatory response in macrophages by inhibiting nuclear translocation and DNA-binding of NFκB. This effect is mediated through ERα and involves activation of PI3K in non-genomic signalling (Ghisletti *et al*., 2005). It has also been documented that estradiol lessens TNFα production in LPS-stimulated human macrophages by inhibiting phosphorylation and degradation of IκBα and preventing translocation of p65 (Murphy *et al*., 2010).

Regulation of dendritic cell function

Kovats and her colleagues published a body of research studying the effects of estrogen hormone on dendritic cells. They found that estrogen regulates DCs maturation, differentiation and the quality of adaptive immunity response in homeostasis and disease states. It is well known that during inflammation, elevated levels of granulocyte macrophage colony-stimulating factor (GM-CSF) induce DCs differentiation from bone marrow progenitors. They reported that physiological levels of estradiol positively regulated GM-CSF mediated differentiation of myeloid progenitor cells to $CD11b^+$ DCs. This effect was mediated through ERα but not ERβ and involves production of the transcription factor interferon- regulating factor 4 (IRF4) which in turn plays an important role in DCs maturation and differentiation. On the other hand and during homeostatic they reported that in culture supplemented with Flt3 (Fms-like tyrosine kinase 3) ligand estradiol reduced the yielded numbers of plasmacytoid and conventional DCs in a dose-dependent manner (Kovats and Carreras, 2008; Carreras *et al*., 2008; Carreras *et al*., 2010; Kovats, 2012).

Regulation of natural killer cell function

Chronic estrogen administration or elevated level of the hormone has been reported to reduce the cytotoxic activity of natural killer (NK) cells (Seaman *et al*., 1978; Ferguson and MacDonald, 1985). 17β estradiol inhibits cytotoxic activity and proliferative capacity of NK cells by modulating the phenotypes and cytokines production (Hao *et al*., 2008). It has also been reported that during pregnancy the total number of peripheral NK cells are reduced (Kuhnert *et al*., 1998; Veenstra van Nieuwenhoven *et al*., 2002). Uterine NK cell functions are mainly controlled by estrogen as they express ERβ and GR but not express PR and ERα (Henderson *et al*., 2003).

The role of estrogens in adaptive immunity

Estrogens mediate their immunomodulatery effects by binding either ERα or ERβ. Estrogen receptor α (ERα) are expressed at higher levels in $CD4^+$ T cells than B cells, whilst B cells express estrogen receptor β (ERβ) more highly. Both ERα and ERβ are expressed equivalently and at low levels in $CD8^+$ T cells (Phiel *et al*., 2005).

Although there is no difference in total lymphocyte number between males and females, the percentage of T lymphocytes in total lymphocyte count is less in males which may account to the elevated levels of testosterone in males as testosterone might enhance T cells apoptosis (McMurray *et al*., 2001). The total circulating lymphocytes count and the percent of each subtype shows no changes during menstrual phases, suggesting, niether estrogen nor progesterone has any effect on lymphocytes number during reproductive age (Bouman *et al*., 2001). Nevertheless, post-menopause women show a reduction in total lymphocyte number (Bouman *et al*., 2005). Estrogens promote $CD4^+$ Th2 and B cell immunity (Beagley and Gockel, 2003; Cutolo *et al*., 2004). It reduces thymic stromal tissue and the total number of immature lymphocytes ($CD4^+$ and $CD8^+$) during reproductive age and pregnancy of healthy females in a process called thymic involution. On the other hand, estrogen induces lymphopoesis in hepatic T cells (Tanriverdi *et al*., 2003). It has been found that low levels of estrogen enhance immunological effects of Th1 whilst higher levels mediate Th2 effects (Maret *et al*., 2003). At higher levels of the

hormone such as during the follicular menstrual phase in women and proestrus and estrus phases in mice, estrogen enhances regulatory T cells (Treg) production (Arruvito *et al*., 2007). It promotes $CD4^+$ $CD25^-$ conversion into $CD4^+$ $CD25^+$ and raises Foxp3 and IL-10 genes expression *in vitro* due to ERα expression in these cells (Tai *et al*., 2008). IL-17 production by Th17 cells has also been found to decrease at higher levels of estrogen (Wang *et al*., 2009).

Estrogen boosts B lymphocyte survival by inhibiting autoreactive B cells apoptosis (Medina *et al*., 2000). In addition, it increases antibody production and the activity of B cells (Nilsson and Carlstein, 1994). *In vitro* study showed that estrogen increased immunoglobulin M (IgM) and immunoglobulin G (IgG) production in human peripheral blood mononuclear cells (PBMCs) of both males and females (Kanda and Tamaki, 1999)

The influence of estrogen on cytokine production

Estradiol and progesterone are as potent as hydrocortisone at inhibitory cytokines at their relevant physiological concentrations (Giannoni *et al*., 2011). As estrogen mediated Th2 dominance, it stimulates IL-4, IL-5, and IL-10 secretion (Bouman *et al*., 2005; Ackerman, 2006). *In vitro*, estrogen enhances IFNγ, IL-1 and IL-10 production (Gilmore *et al*., 1997). Estradiol decreases TNFα production as one of estradiol's anti-inflammatory effects (Beagley and Gockel, 2003).

1.3.3.4 Progesterone

Progesterone is the main hormone in pregnancy. Its name is derived from the Latin word gesture which means to carry. Progesterone is required during variable reproductive stages such as ovulation, differentiation of the endometrium, implantation of fertilised ovum and growth of the placenta (Hughes, 2011). It is secreted mainly from granulosa and the corpus luteum in the ovary whilst during pregnancy it is secreted from the placenta (Tait *et al*., 2008). Progesterone classically binds cytosolic progesterone receptors (PRs) to manipulate different biological responses (Sherman *et al*., 1970). Two isoforms have been identified PR-A and PR-B (Kastner *et al*., 1990). Progesteron receptors, like other SHRs are comprised of

multiple functional domains enabling them to bind to the hormone. Hormone–receptor complex translocates to the nucleus and interacts with DNA-responsive element and other co-regulator proteins to mediate the transcription of different genes (McKenna *et al*., 1999). Progesterone also displays some rapid non-genomic effects mediated through membrane receptors (Falkenstein *et al*., 2000). Membrane progesterone receptors include mPRα, mPRβ and mPRγ are linked to GTP binding proteins (Thomas, 2008). The progestins are a group of both natural and synthetic derivative of progesterone that mimic the major biological effects of progesterone but differ pharmacokinetically and pharmacodynamically (Schindler *et al*., 2003).

1.3.3.5 Classification of progestins

Progestins are classified as; natural progestin (e.g. progesterone), retroprogesterone (e.g. dydrogesterone), progesterone derivative (e.g. medrogestone), 17-hydroxyprogesterone derivatives (pregnanes) (e.g. medroxyprogesterone acetate, megestrol acetate, chlormadinone acetate, cyproterone acetate), 17-hydroxynorprogesterone derivatives (norpregnanes) (e.g. gestonorone caproate, nomegestrol acetate), 19-norprogesterone derivatives (norpregnanes) (e.g.demegestone, promegestone, nesterone, trimegestone), 19-nortestosterone derivatives (estranes) (e.g.norethisterone, norethisterone acetate, lynestrenol, ethinodiol acetate, norethinodrel), 19-nortestosterone derivatives (gonanes) (e.g.norgestrel, levonorgestrel, desogestrel, etenogestrel, gestodene, norgestimate, dienogest). And spirolactone derivatives (e.g. drospirenone) (Pasqualini *et al*., 1998; Schindler *et al*., 2003).

1.3.3.6 Progesterone and immunity

Many studies have reported the immunomodulatory effects of progesterone, considering the hormone as a powerful modulator of numerous immune activities. Among these, a number have documented that progesterone can suppress the immune response (Miller *et al*., 1996; Miller and Hunts, 1998; Miyaura and Iwata, 2002; Butts *et al*., 2007; Jones *et al*., 2008), whereas others have linked progesterone and pregnancy to many immune-based pathological conditions (Garay *et al*., 2009; Hughes, 2011; Robinson and Klein, 2012) and some other reports have studied the

immunological roles exerted by the hormone in developing or remission of some malignant tumours (Lewis *et al*., 2004; Jin *et al*., 2012; Sivik and Jansson, 2012). Progesterone mediates its immunomodulatory effects by binding both progesterone and glucocorticoid receptors to act either agonistically or antagonistically (Cuchacovich *et al*., 1991). It has been found that the affinity to the progesterone receptor increases by introduction of a 19-nor group. Hence, norgestrel a 19-nortestosterone derivative is more active as a PR agonist (Bacelot and Kutten, 2006), see Figure (1.7).

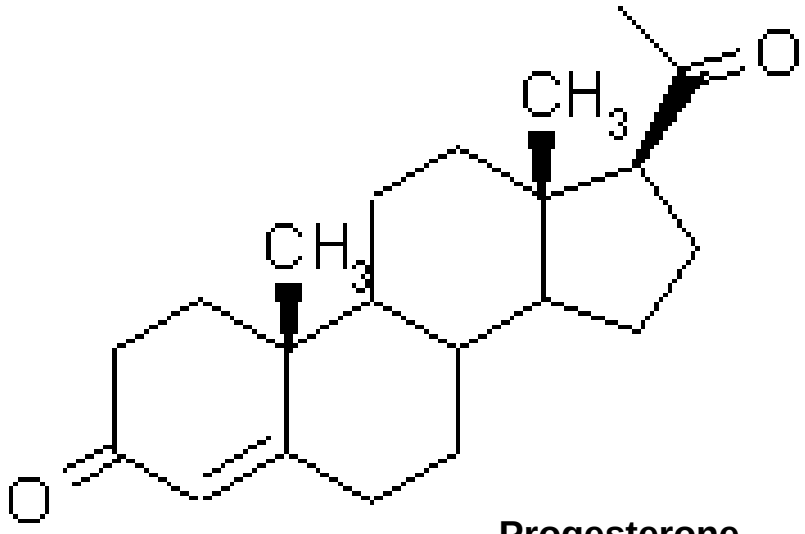
The role of progesterone in innate immunity

Regulation of macrophage function

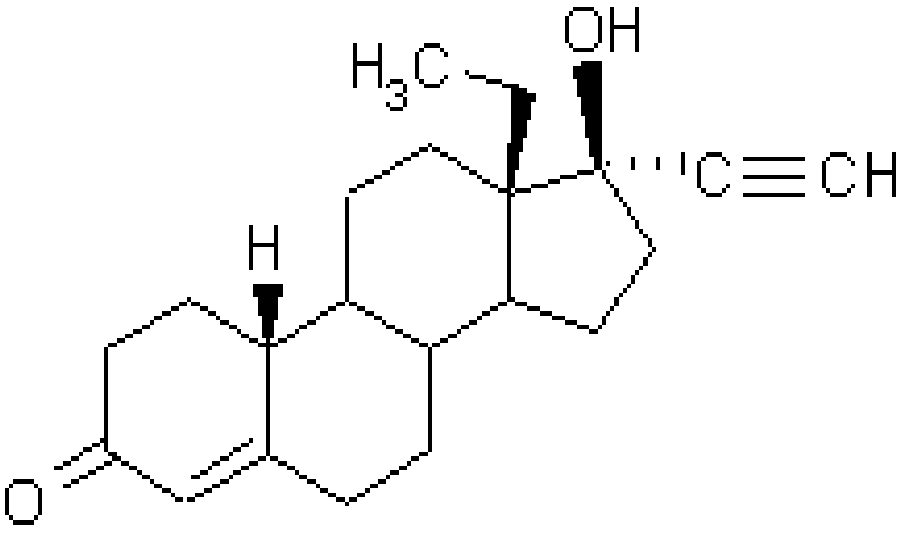
Progesterone influences macrophage function by effects on genes expression. It inhibits agonist stimulated inducible nitric oxide synthase (iNOS) gene expression and nitric oxide production by macrophages (Miller *et al*., 1998). Progesterone inhibits LPS-induced NO and IL-6 production by macrophages by inhibition of NFκB and enhance suppressor of cytokine signalling 1 (SOCS1) (Su *et al*., 2009). The same group also published that the inhibition of the NFκB pathway by progesterone plays a role in regulation of LPS- and polyI:C- induced miR-155 expression. miRNAs molecules are defined as small noncoding nucleotide that inhibit expression of genes at the post-transcriptional level. Progesterone-regulated miR-155 decreases IL-6 and IFNβ production in macrophages also by promotion of SOCS1 expression (Sun *et al*., 2012). Alternative activated macrophages, or what are known as M2 macrophages, are also subjected to immune modulation by progesterone hormone. It has been reported that progesterone down-regulates iNOS2 and arginase enzyme activity in LPS- or IL-4-stimulated BMD-macrophags and regulates selectively the expression of genes associated with alternatively activated macrophages (Menzies *et al*., 2011).

Regulation of dendritic cell function

Progesterone reduces MHC class II, CD 80 and CD 86 expression (Xu *et al*., 2011). Whilst (Yang *et al*., 2006) reported that progesterone increases MHC class II



Progesterone



Norgestrel

Figure 1.7. Progesterone and norgestrel chemical structures.

expression and CD40. Progesterone inhibits proinflammatory cytokines production by BMDCs in a dose-dependent manner. It inhibits TNFα, IL-1β and IL-12 production from splenic DCs via the progesterone receptor (Butts *et al*., 2007), But does not change IL-10 production (Butts *et al*., 2007). The immunosuppressive effects of progesterone are more obvious in BMDCs of female rats than those of males this may be as a result of higher expression of PR number per BMDC from females (Butts *et al*., 2008). Progesterone also impairs innate antiviral response in human and mouse pDCs. It inhibits IFNα production from pDCs by inhibiting IRF-7 (Hughes *et al*., 2008). Progesterone increase stimulatory capacity and IL-6 and IL-10 levels in splenic DCs (Yang *et al*., 2006)

Regulation of NK cell function

Elevated levels of progesterone during pregnancy are accompanied by a reduction in the cytotoxic activity of NK cells. On the other hand increased NK cells activity is associated with spontaneous termination of pregnancy (Roberts *et al*., 2001). Progesterone inhibits CD69 and IFNγ production in human uterine NK cells. This effect is mediated through GR as NK cells do not express PRs. Spleen NKs from mice also showed the same effects. STAT4 and IκB protein phosphorylation have also been diminished by progesterone in these cells (Guo *et al*., 2012a), indicating a suppressive role for progesterone on these cells

The role of progesterone in adaptive immunity

Progesterone promotes Th2 dominance and inhibits Th1 responses during pregnancy, as the elevated levels of the hormone are associated with induction of IL-4 and IL-5 production (Piccinni *et al*., 1995). It has been reported that the elevated levels of progesterone during midterm pregnancy can convert $CD4^+$ $CD25^-$ to $CD4^+$ $CD25^+$ T reg cells and increase their number and function (Mao *et al*., 2010). Treg cells are implicated in maintaining pregnancy tolerance by inducing anti-inflammatory response. Progesterone also inhibits antibody production from B-cell by inhibit $CD8^+$ T cell function (Lu *et al*., 2002).

1.3.3.7 Pregnancy and progesterone

Pregnancy is a state of immunological and hormonal changes that shifts the response from pro-inflammatory towards anti-inflammatory to enable the pregnancy to proceed to full term and reduce rejection of the newly created fetus (Robinson and Klein, 2012). It has been found that estrogens, progesterone, and glucocorticoids levels increased during the pregnancy course to reach the highest levels in the third trimester (Robinson and Klein, 2012). There is a growing body of evidence linking immunological changes during pregnancy to the elevated levels of these hormones. Considering that progesterone is among the most important factors in maintaining and controlling pregnancy, hence, immunological effects of progesterone during pregnancy have been summarised below.

High levels of progesterone during pregnancy up-regulate progesterone receptors (PRs) on lymphocytes (Druckmann and Druckmann, 2005). Absence or huge reduction of PR expression is usually associates with preterm-labour or miscarriages (Szekeres-Bartho *et al*., 1989). Progesterone-activated cells produce a 34 kDa immunomodulating protein, progesterone-induced blocking factor (PIBF) (Szekeres-Bartho *et al*., 1985). During the pregnancy PIBF alters the Th1/Th2 balance and promote Th2 cytokine production, like IL-4 and IL-10 (Szekeres-Bartho and Wegmann, 1996). Th2 bias mediated by PIBF production and Th2 cytokines production can also reduce Th1 cytokine production such as IL-12 (Miyaura and Iwata, 2002; Par *et al*., 2003). Any situation associated with Th1 dominance during pregnancy may cause abortion or preterm labour (Raghupathy *et al*., 2000). Accordingly, Th2 cytokines reduce NK cells activity which contributes as anti-aborative effect of the hormone (Szekeres-Bartho *et al*., 1997). In addition, decidual macrophages that are activated alternatively by Th2 cytokines (IL-4, Il-10 and IL-13), that predominant during pregnancy, showed anti-inflammatory characteristic mediated through arginase activity (Nagamatsu and Schust, 2010). It has been reported that the humoral immunity is enhanced during pregnancy with increases in total antibody production (Szekeres-Bartho, 2002). PIBF directly, or by inducing cytokines, affects B-cells and increases antibody production (Kelemen *et al*., 1996). A shift from cellular to humoral immunity during pregnancy is accompanied with

decreasing in NK cells number and activity (Veenstra van Nieuwenhoven *et al*., 2003). Another job of Th2 bias in favour of pregnancy is mediated by Th2 cytokines that stimulate the trophoblast to produce human placental lactogen (hPL) and human chorionic gonadotropin (hCG) (Dealtry *et al*., 1998). In human, hCG hormone secreted early after fertilisation, induces the migration of regulatory T cells to the fetal-maternal interface (Schumacher *et al*., 2009) and in mice the same effect appears by potentiation of regulatory T cells activity at the fetal-maternal interface (Kallikourdis and Betz, 2007).

As mentioned previously that progesterone during pregnancy increases number and immunosuppressive activity of Treg cells. Induced T regulatory cells (iTreg cells) differentiate from naïve T cells peripherally and upon activation produce TGF-β1, IL-10 and IL-35. These cells suppress the immune response and mediate the anti-inflammatory responses against inflammatory and autoimmune diseases (Andersson *et al*., 2008). Lee *et al*., (2012) utilised this to improve stability and suppressive characteristic of Treg cells by progesterone (P4) as a promising cellular therapy to treat inflammatory disorders. P4 induced iTreg cells showed more stability in inflammation and were more efficient in releasing TGF-β1 from different tissues.

1.3.3.8 Pregnancy, sex-hormones and diseases

It is widely documented that elevated levels of pregnancy-associated hormones and the subsequent immunological changes throughout the whole course of pregnancy can participate in development of many diseases. Immune-based disease such as asthma, multiple sclerosis (MS) and systemic lupus erythmatosis (SLE) are greatly influenced by reproductive state (Whitacre, 2001). Thus, this may account for the change in Th1/Th2 balance which rises due to the influence of a higher concentration of the sex hormone in decreasing this ratio during pregnancy and other menstrual phases (Ostensen, 1999). Autoimmune disorders are among these diseases. These disorders vary considerably in severity, organ or tissues targeted and the age but they share the response to immunosuppressive treatment and their dominance in females (Gleicher and Barad, 2007). For example in systemic lupus erythematosus (SLE), the

disease incidence ratio of females: males is 9:1 in adults. The ratio drops to 3-4:1 among pre-pubertal group and reaches to 1:1 in children under 5 years which may illustrate the effective role of sex hormones in governing autoimmune diseases (Hughes, 2011). The same pattern has been seen with other autoimmune diseases such as Hashimoto's thyroiditis and Grave's disease (Hughes, 2011). There is a consensus that adaptive immunity is responsible for the development of autoimmunity. Females show higher $CD4^+$ T cells with a tendency of Th2 predominance while males develop Th1 $CD4^+$ and $CD8^+$ predominance (Pennell *et al*., 2012). Though, another hypothesis links autoimmunity with X chromosome inactivation or abnormalities (Lieo *et al*., 2008). SLE, scleroderma, Sjogren's syndrome and Hashimoto's thyroiditis are Th2 dominant diseases that show more prevalence during pregnancy (Ackerman, 2006). In contrast, Th1-mediated autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS) usually alleviate during pregnancy and exacerbate in postpartum period (Buyon, 1998). It has been reported that the rate of multiple sclerosis relapse decreases during pregnancy especially in the third trimester and increases in the first 3 months after delivery to return to its pre-pregnancy rate (Confavreux *et al*., 1998)

Among other diseases affected by sex hormones is endometriosis. Endometriosis is a gynecological disease of unknown etiology. The disease is described as ectopic abnormal growth of the uterus lining associated with severe pelvic pain and infertility (Bulun, 2009). Firstly, the disease is classified as autoimmune disease. Recently, researchers suggested that the disease is a cell-mediated disorder relying upon the alteration of certain immune cells function within the peritoneum cavity of the affected woman. Women who have endometriosis are subjected to inflammation of multiple organs. Macrophages, mast cells, NK cells and DCs are all resident at the inflammed endometrial tissue. Endometrial tissues unresponsive to progesterone show predominance of estrogen role as a proinflammatory inducer for the affected immune cells. This was suggested as the disease mechanism (Herington *et al*., 2011).

High concentrations of glucocorticoids, progesterone and estrogen during pregnancy suppress the immune response systemically and locally and contribute to

the exacerbation of certain viral, bacterial, fungal and parasitic infections. It has been reported that the risk of human immunodeficiency virus (HIV) infection increases during pregnancy (Gray *et al*., 2005) and also in women who use depot medroxyprogesterone acetate (DMPA) injection for birth control. Progesterone suppresses antiviral responses through the inhibition of IFNα production and $CD8^+$ T cells function (Hel *et al*., 2010). It has been also reported that pregnant women showed more severe cases of H1N1 influenza with increase morbidity and mortality rates when compared with the general population (Klein *et al*., 2011). The high incidence rate of non-HIV sexually transmitted diseases (STDs) such as syphilis, gonorrhea and papillomavirus among females are linked to change of local immune cells function that controlled by high levels of sex hormones (Beagley and Gockel, 2003). It has been reported that for the immune cells present ubiquitously in the female reproductive tract (cervix, vagina and uterus) (Lahey *et al*., 2012). Their function and number vary considerably with the stage of the reproductive cycle. This is illustrated by the impact of the changes in sex hormones levels on their function roles (Beagley and Gockel, 2003).

With respect to parasitic infection, the susceptability of women to *T.gondii* and *P.falciprim* protozoan has been found to increase during pregnancy. In addition reactivation of the disease toxoplasmosis during pregnancy has been documented (Sensini, 2006). Severe brain inflammation and even death of female mice after *T.gondii* infection have been linked to elevated levels of the sex hormones, progesterone and estrogen (Kittas and Henry, 1980). Exaggeration of these diseases is also attributed to the anti-inflammatory environment mediated by sex hormones and their immune-suppression effects (Roberts *et al*., 2001; Robinson and Klein, 2012).

1.4 The aims of the project

Steroid hormones in general and female sex hormones, such as progesterone in particular, are capable of modulating immune responses and influencing diseases outcome especially during pregnancy when these hormones are at elevated levels. Infection and inflammation trigger a vast number of immune response elements including inflammatory cascades. TLRs are one of the first means to detect infectious agents. Previous studies have reported immune suppressive effects of progesterone including the inhibition of proinflammatory cytokine production in innate immune cells (macrophages and dendritic cells). The mechanism of action of hormones on these cells has to date only been poorly studied. The current studies aim to give a more complete picture for the molecular signalling events that account for hormonal manipulation of macrophages and dendritic cells. Specifically:

1) To determine the effect of progesterone on TLR3 and TLR4 mediated key inflammatory signalling molecules (NFκB, IRF3 and MAPKs proteins) .TLR4 is located on the surface of the cell and utilises two different signalling pathways; MyD88-dependent and TRIF-dependent pathways. It responds to a number of PAMPs present on diverse pathogens including LPS on bacteria used in these studies. In contrast, TLR3 is expressed internally and can only utilise TRIF-dependent signalling. It responds to double stranded RNA or the synthetic surrogate PAMP, poly I:C used in these studies. The use of these 2 PAMPs, LPS and poly I:C thus allows the dissection of the effects of progesterone on TLR3 and 4 as well as Myd88-dependent and independent signalling events.
2) Determine the effects of progesterone on downstream nuclear events including nuclear translocation of the proteins of interest and protein-DNA binding activity following LPS-TLR4 and poly I:C-TLR3 induced signalling events. These studies will provide mechanistic insight into previous observations where progesterone has been determined to affect macrophage and dendritic cell function.
3) Determine the effects of progesterone on further cytokine transcript and protein levels following LPS-TLR4 and polyI:C-TLR3 stimulation.

Specifically determine how progesterone affects IL-12p35, IL-23p19, IL-27p28 and EBI3 and IFNβ mRNA expression and also the levels of IL-6 and IL-12p70 production.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1. General reagents

The following suppliers were engaged for the purchase of experimental reagents as follows.

Sigma Aldrich Chemical Company Ltd. (Poole, Dorset, UK)

LPS from *Escherichia coli 055:B5*, poly I:C, progesterone, norgestrel, dexamethasone and chloroform, 2- mer**c**aptoethanol, geneticin (G418), molecular grade water, X-gal, IPTG, streptavidin-alkaline phosphate (SAv-AKP), p-Nitophenyl phosphate (pNPP), streptavidin-horse radish peroxidase (SAv-HRP), tetramethyl-benzidine (TMB). All other materials utilised during gel electrophoresis, nuclear extraction and Western blotting were also purchased from Sigma Aldrich UK.

Boehringer Mannheim (East Sussex, UK)

Bovine Serum Albumin (BSA, Fraction V).

Gibco Life Technologies Ltd (Renfrewshire, UK)

Modified Eagle's Medium (DMEM), RPMI 1640, Iscoves's Modified Dulbecco's Medium (IMDM), Fetal Calf Serum (FCS), Antibiotics; penicillin, streptomycin, L – Glutamine.

Roche diagnostics

Dithiothreitol (DTT). Quick Spin Columns for radiolabeled DNA purification

Bio-Rad Laboratories, Hertfordshire, UK.

Bio-Rad DC^{TM}protein Assay Kit, SDS-PAGE molecular weight markers.

Perkin Elmer

[γ-32P]ATP [3000 Ci/mmol].

Bender MedSystem (Vienna, Austria)

Recombinant murine IL-6.
Recombinant murine IL-12p70.

Bioline, London, UK.

Agarose powder.

Promega,UK.

Random primers, NF-κB Consensus oligonucleotide, T4 Polynucleotide, 10X T4 Polynucleotide kinase buffer, 5X Gel-shift binding buffer.

Cell Signalling technology, Inc (USA): Antibodies

Phospho-SAPK/JNK (Thr183/Tyr185; Cat. #9251S).
Phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204; Cat.#4377S).
P44/42 MAPK (Erk1/2; Cat #4695).
Phospho-IRF-3 (Ser396; Cat. #4947S).
IRF-3 (Cat. #4962).
Phospho-NFκB (p65; Ser536; Cat. #3031L)
Rabbit polyclonal Histone (H3).

Santa Cruz Biotechnology Inc. (CA, USA)

Anti-JNK-1(FL), Anti-p38 (N-20), Anti IκBα (Cat. #2607), p65 (Cat. #L0607), GAS/ISRE oligoneucliotide

Qiagen, West Sussex, UK.

QIAquick Gel Extraction kit, QIAprep spin miniprep kit, pDrive Cloning Vector

Invitrogen, UK.

Anti-phosphor-p38 (Cat. #44-684G), DNase I, primers

Fisher Scientific UK Ltd, Leicestershire, UK.

Isopropanol.

ABGene, Epsom, Surrey, UK.

Reddymix.

Stratagene, UK

AffinityScript^{TM}Multiple Temperature Reverse Transcriptase kit. Brilliant III Ultra-fast SYBR® Green

Whatman (Kent, UK)

Nitrocellulose membrane.

Corning B.V. (Netherlands)

All tissue culture plastic ware.

Nuns, Roskilde, Denmark.

Triple layer flask.

BD Biosciences, Oxford, UK.

Mose anti-IL-6 mAb (IgG clone MP5-20F3)
Anti- IL-6, Biotin,(IgG2a clone MP5-32C11).
Anti-IL12p70 (IgG2b clone 9AG)
Anti-IL12p70, Biotin, (IgG clone C15.6).
Syringes and needles.

TPP, Trasadingen, Switzerland.

Cell culture plates (6, 12, 24 and 48 well), cell scrapers
RNAse/DNAse free microcentrifuge tubes.1

2.2 Methods

2.2.1 General macrophage cell culture

L-cell conditional medium helps in differentiation of bone marrow stem cell to bone marrow derived-macrophages (BMD-macrophages). A frozen aliquot of L929 cells (ECACC, #85103115) was thawed at 37ºC and centrifuged at 200g for 5 minutes. The formed pellet was resuspended in 13 ml of D10 medium and cultured in $75cm^3$ cell culture flasks. D10 medium was composed of Dulbecco's modified eagle's medium (DMEM) with sodium pyruvate, high glucose and pyridoxine hydrochloride, 10% heat inactivated fetal calf serum (HI-FCS), 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. The cells were cultured for 4-5 days till confluent. Then the media was discarded. A 5 ml volume of cold sterile phosphate buffer saline (PBS) was added and maintained at 4ºC for 10 minutes, following this cells were gently scraped with 30 cm scraper. The cell suspension was centrifuged at

200g for 5 minutes. The pellet was resuspended in 10 ml D10 medium and used to seed ten 160 cm^3 cell culture flasks (one ml for each flask). An extra 30 ml D10 medium was added. The flasks were incubated at 37 Cº and 5% CO2 until 80-90% confluent. Nine of ten flasks were scraped as previously and resuspended in 36 ml D10 medium (4ml for each flask). 180 ml of D10 and 1ml of the cell suspension was added to each triple layer flask. The remaining flasks was used to seed a further ten 160 cm3 cell culture flasks. After 7 days the supernatant was harvested and aliquoted into 50ml tubes. These tubes were used later to differentiate of bone marrow stem cells into bone marrow derived macrophages.

2.2.1.1 The derivation of macrophages from bone marrow

Male BALB/c mice of 8-10 week old were used which were maintained and euthanised at the University of Strathclyde under Home Office Guidelines. Macrophage medium consisted of Dulbecco's modified Eagle's medium (DMEM) containing sodium pyruvate, pyridoxine hydrochloride and high glucose, with 20% (v/v) HI-FCS, 30% (v/v) L-cell conditioned medium, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. 5ml of this medium with 25-gauge needle was used to flush out bone marrow stem cells from one mouse bone. Stem cells from 2 femur and 2 tibia bones (one mouse) were collected into 50ml tubes. Another 10ml macrophage medium was added and with a 21-gauge needle the cells were aspirated 3-4 times through the needle to produce a single cell suspension. This suspension was aliquoted into petri dishes. The cells were incubated at 37º C and 5% (v/v) CO_2 for 10 days with adding extra warm macrophages medium on day 3 and 6. On day 10 the medium was discarded, 5ml of cold RPMI 1640 added and the cells scraped from the bottom and walls of the petri dishes. The suspension of cells was collected in a 50ml tube and centrifuged at 1000 rpm (350g) for 5 minutes. The pellet was resuspended and washed with RPMI 1640 for 2 times to remove any trace amount of DMEM medium. Cells were resuspended to a desired final concentration with a RPMI 1640 supplemented with 10% (v/v) HI-FCS, 2mM L-Glutamine, 100U/ml penicillin, 100µg/ml streptomycin. Macrophages were then plated as desired for experiments.

2.2.2 Dendritic cells culture

2.2.2.1 GM-CSF Conditional medium

Bone marrow derived dendritic cells (BMDCs) were differentiated in the laboratory from stem cells that were isolated from the bone marrow of BALB/c mice. Granulocyte/monocyte-colony stimulating factor (GM-CSF) enriched media obtained from X63-GM-CSF myloma cells were used to generate immature DCs from the stem cells (Lutz *et al*., 1999). X63-GM-CSF myloma cells were removed from cryo-store and thawed rapidly at 37°C until only a small pellet of ice remained and washed in 5ml of complete Iscoves's modified Dulbecco's medium (IMDM), 10% HI-FCS, 2mM L-glutamine, penicillin (100U/ml), streptomycin (100 μg/ml) and 50μM 2-mercaptoethanol. Cells were then resuspended in 15 ml complete IMDM containing 1mg/ml antibiotic G418 disulphate and cultured in 75cm^3 flask. After expansion to approximately 60 million cells, cells were washed and cultured in 200ml complete IMDM without G418 for 2-3 days. The culture supernatant was then harvested by centrifuging at 450g for 5 minutes. Supernatant was not filtered in order to avoid loss of GM-CSF activity.

2.2.2.2 Derivation of DCs from bone marrow

Bone marrow cells were flushed from the femurs and tibias of 8-10 week old, male BALB/c mice into a petri dish by using a syringe and 25-gauge needle containing 10-ml of BMDC medium RPMI1640, 10% (v/v) HI-FCS, 10% (v/v) GM-CSF enriched medium, 2mM L-glutamine. Bone marrow cells were crushed gently to make single cells suspension. The cells suspension was then collected in a flask and the volume made up to 90 ml with BMDC medium. This volume was then divided into 6 cell culture dishes, incubated at 37°C in 5% (v/v) CO_2 for 8 days. On day 4 of incubation, the cells were fed with BMDC medium containing 5% (v/v) GM-CSF enrich medium and on day 7 with medium containing 2.5% (v/v) GM-CSF. This method produced a large number of DCs largely free from contamination with other cell types. The BMDCs were plated as desired for experiments.

2.2.3 Cell treatments

Progesterone and norgestrel were initially dissolved in chloroform at a concentration of 50mg/ml. This stock solution was further diluted with complete medium (RPMI 1640 medium, 10% (v/v) HI-FCS, 2mM L-glutamine, penicillin (100U/ml), streptomycin (100 µg/ml) to give a 250µM solution for experimental use. Dexamethasone was initially dissolved in ethanol to give a 50mM solution. This stock solution was further diluted with complete medium to give a 250nM solution for experimental use. This could then be diluted as required in experiments. Solvent vehicle controls, chloroform and ethanol, were prepared by the same method. TLR ligands; LPS (1µg/ml) and polyI:C (25µg/ml) were added an hour after hormone treatments.

2.2.4 Western-Blotting

2.2.4.1 Preparation of whole cell extracts

BMD-macrophages were plated out in 12-well plate and allowed to adhere overnight. Cells were pretreated with vehicles (chloroform or ethanol) or progesterone, norgestrel or dexamethasone one hour prior to expose to LPS or polyI:C for relevant periods of time. To terminate reactions plates were placed on ice and the media was removed. Adherent cells were washed twice with ice cold phosphate buffer saline (PBS). 200µl pre-heated Laemmli's sample buffer [63mM Tris-HCL, (pH 6.8), 2mM Na4P2O7, 5mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50mM DTT, 0.007% (w/v) bromophenol blue] added. The cells were then scraped, chromosomal DNA sheared by repeatedly passage through a syringe with a 21 gauge needle and transferred into sterile Eppendorf tubes. The tubes were boiled for 5 minutes to denature proteins and samples were stored at –20 °C until further use. For BMDC, as predominantly non-adherent cells, wells were scraped into their bathing media and collected in Eppendorf tubes. Samples were then centrifuged for 1 minute at 13000g. Supernatants were discarded and pellets were resuspended in ice cold PBS. The tubes were centrifuged again for 1 minute and the supernatant aspirated. The pellets were resuspended in 200µl pre-heated Laemmli's sample buffer. Chromosomal DNA was

sheared by repeatedly passage through a syringe with a 21 gauge needle, as with macrophages. The tubes were boiled for 5 minutes to denature proteins and were stored at –20 °C until further use.

2.2.4.2 Preparation of nuclear extracts from BMDCs

Treated BMD-dendritic cells were scraped into their media, centrifuged (13000g for 1 minute) and resuspended in 1ml ice-cold PBS. After the aspiration of supernatants, cell pellets were resuspended in 400µl buffer 1 (10mM HEPES pH 7.9, 10mM KCL, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT),0.5mM phenylmethylsulfonyl fluoride (PMSF), 0.02 mg/ml aprotinin, 0.02 mg/ml leupeptin and 0.02 mg/ml pepstatin A. Resuspended cells were incubated on ice for 15 minutes. 25µl of 10 % (w/v) NP-40 was added to each tube and vortexed at full speed for 10 seconds. The tubes were centrifuged for 1 minute at 13000g. Supernatants, representing the crude cytoplasmic fractions, were removed and pellets were resuspended in 50µl of buffer 2 (20mM HPES pH 7.9, 25% (v/v) Glycerol, 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT) containing 0.5mM PMSF, 0.02 mg/ml aprotinin, 0.02 mg/ml leupeptin and 0.02 mg/ml pepstatin A. Pellets were loosened by gentle vortexing and shaken at 4ºC for 15 minutes. The tubes were sonicated on ice in bath sonicator for 2 x 30 seconds and centrifuged for 15 minutes at 13000g. Supernatants, representing crude nuclear extracts were transferred to sterile tubes and stored at – 80°C for future use.

2.2.4.3 Quantification of protein concentrations in samples

Protein concentrations were determind by Bradford assay (Bio-Rad). This relied on acidic dye binding to proteins to give different coloured solutions according to various protein concentrations. Bovine serum albumin (BSA) was used to prepare a standard curve for each assay in the concentration range of 5-20µg/ml using an appropriate buffer for dilution. All the standards and samples to be assessed were diluted with water up to 800µl, to which 200µl of the concentrate dye reagent was added in 4:1 ratio according to the manufacturer's instructions. Standards and samples were vortexed at full speed, incubated at room temperature for 15 minute at to allow colour development and then read at 595 nm using a spectrophotometer.

2.2.4.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): analysis of protein samples

Different concentrations of acrylamide gels were prepared as required. 7.5, 9, 10 and 11% (w/v) gels were prepared as resolving gels: assembled from solutions of [acrylamide:N, N'-methylenebis-acrylamide (30:0.8), 0.375M Tris (pH 8.8), 0.1% (w/v) SDS and 10% (w/v) ammonium persulfate (APS)]. Polymerisation was initiated by the addition of 0.05% (v/v) N, N, N', N'-tetramethylethylenediamine (TEMED). Prepared gel solutions were poured between 2 glass plates of a Bio-Rad protean II kit. The gel solution was then overlayed with 200 µl 0.1% (w/v) SDS. Following gel polymerisation the layer of 0.1% SDS (w/v) was removed and a stacking gel prepared by mixing component solutions of 10% (v/v) acrylamide: N,-methylenebis-acrylamide (30:0.8) in 125mM Tris (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate (APS) and 0.05% (v/v) TEMED and poured directly on the top of the resolving gel. Into the stacking gel solution and between the two glass plates a teflon comb was inserted and removed after complete polymerisation of the gel. The polyacrylamide gels were then assembled in a Bio-Rad Mini-PROTEAN II^{TM} electrophoresis tank, with reservoirs filled with electrophoresis buffer [25mM Tris, 129mM glycine, 0.1% (w/v) SDS]. Aliquots of samples (20-30µg/ml) were loaded into the gel wells using a microsyringe. Concurrently, SDS-PAGE molecular weight marker standards containing a mixture of various dye-labelled proteins already prepared in sample buffer were run to identify the protein of interest. Samples were electrophoresed at a constant voltage of 120 V, until the bromophenol dye had reached the bottom of the gel.

Transblotting

To detect the proteins using antibodies the proteins were required to be transferred onto nitrocellulose membrane by electrophoretic blotting. The gel was pressed firmly against a nitrocellulose sheet and assembled in transfer cassette sandwiched between two Whatman 3mm paper and two sponge pads. The cassette was immersed in blotting buffer [25M Tris, 19mM glycine, 20% (v/v) methanol] in a Bio-Rad Mini

Trans-Blot™ tank and a constant current of 300 mA was applied for 2 hours, whilst the tank was cooled by inclusion of an ice reservoir.

Blocking of non-specific binding

Following transfer of the proteins to the nitrocellulose membrane, the membrane was removed in a solution of 2% (w/v) bovine serum albumin (BSA) in NaTT [150mM NaCl, 20mM Tris (pH 7.4), 0.2% (v/v) Tween-20] for 2h with gentle agitation on a platform shaker to block potential non-specific antibody bindings.

Immunological detection of proteins

Nitrocellulose membranes were incubated overnight with primary antibody concentrates diluted in 0.2% BSA in NaTT. Blots were then washed for 2 hours with NaTT, changed each 15 minutes. The membranes were then incubated for a further 2 hours at room temperature with secondary horseradish peroxidase-conjugated IgG directed against the first immunoglobulin diluted to approximately 1:10000 in NaTT buffer containing 0.2% (w/v) BSA. Further washes with NaTT over 2 hours were then pursued as described previously. Protein bands were detected by incubation of blots in enhanced chemiluminescence (ECL) for 2 minutes. The membranes were then placed on an exposure cassette and covered with cling film, exposed to Kodak X-OMAT LS film for the appropriate time under darkroom conditions and developed using a KODAK M35-M X-OMAT processor.

2.2.4.5 Stripping and reprobing nitrocellulose membrane

Previously analysed nitrocellulose membranes were stored in a sealed container containing NaTT Buffer at 4°C, until reprobing was desired. Antibodies were then stripped from the nitrocellulose membrane by incubating in stripping buffer [62.5 mM Tris-HCl (pH 6.7), 100 mM beta-mercaptoethanol, 2% SDS] at 50-60°C for 45 minutes with shaking. Blots were washed with NaTT buffer three times each time for 15 minutes. At this stage blots were then re-probed with another antibody as appropriate using immunological detection protocol detailed previously.

2.2.5 Electrophoretic mobility shift assay (EMSA)

Aliquots (5-7µl) containing 5µg of nuclear proteins from nuclear extracts were incubated with 2 µl of 5x binding buffer [50mM Tris-HCL (pH 7.5), 20% (v/v) glycerol, 5mM $MgCl_2$, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, 0.25mg/ml poly (dI-dC)•poly (dI-dC)] for 20 minutes at room temperature. After that 1µl (approx. 50,000 cpm) of [γ-^{32}P]-labelled NFκB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) or ISRE consensus oligonucleotide (5′-AAGTACTTTCAGTTTCATATTACTCTA-3′) was added. The samples were incubated for another 20 minutes at room temperature, then 1µl of 10x gel loading buffer [250mM Tris-HCl (pH 7.5), 0.2% bromophenol blue, 40% (v/v) glycerol] was added. Before loading the treated samples, 4% (w/v) non-denaturating gels were electrophoresised for 30 minutes and then samples were loaded for electrophoresed for a further 45-60minutes.

2.2.5.1 Drying the gels

The gels were placed between two porous cellophane sheets in a drying frame and placed in a Hoeffer Eazy-Breeze™ gel dryer at 80ºC for 60-90 minutes. The dried gels were then exposed to Kodak X-OMAT LS film for the appropriate time at -80ºC in a metal cassette and developed by a KODAK M35-M X-OMAT processor.

2.2.5.2 Scanning and data analysis

After making scanner images (Western blotting and EMSA), the captured images were then normalised to a control and quantified using Scion Image (Scion Corp., Maryl and, USA). Densitometry data generated from immunoblots was expressed as mean ± SEM. for three separate observations. The statistical analysis as described later in data analysis section.

2.2.6 Quantitative reverse transcription real time–polymerase chain reaction (qRT–PCR)

2.2.6.1 Extraction of total RNA

BMDCs were plated in 24-well plates at $1x10^6$cells /500µl of medium. The plates were incubated at 37ºC in presence of 5% (v/v) CO_2 overnight to allow cells to adhere. Cells were then treated by vehicle or hormones (progesterone or norgestrel) one hour prior to exposed to LPS or polyI:C for relevant periods of time. Total RNA was extracted using Methodology described by Chomczynski & Sacchi (1987). Plates were centrifuged at 1000g for 5 minutes. Medium was removed and Trizol® reagent was added. Cells were scraped in Trizol and collected in an RNAse/DNAse free microcentrifuge tubes. Traizol/cells suspensions were passed through a 25 gauge needle 10 times to shear genomic DNA (gDNA). 0.5 ml of chloroform was added to each tube. The tubes were shaken vigorously and left for 2 minutes at room temperature followed by centrifugation at 10,000g for 15 minutes at 4°C. The aqueous layer was retained in new tubes and 0.5ml of ice-cold isopropanol was added. The tubes were mixed vigorously manually and incubated for 10minutes at room temperature then centrifuged for another 10 minutes at 10,000g 4°C. With a 1ml syringe and 25gague needle all isopropanol was removed carefully and the pellets washed by 1ml 70% (v/v) ethanol with flicking the tubes to dislodge the pellet from walls. The tubes were centrifuged for 15 minutes at7000g and 4°C. Ethanol was removed and the tubes were allowed to dry under a laminar flow hood for 10-15 minutes. 50µl of molecular grade water (DNase and RNase free water) was added and the tubes were incubated at 55-65 °C for 5 minutes to solubilise RNA. RNA was stored at -80 °C for future use.

2.2.6.2 Screening of RNA samples for gDNA contamination

The polymerase chain reaction (PCR) was used to ensure that extracted RNA samples were free from any gDNA contamination. Specifically, all samples were shown to be incapable of yielding a product using primers designed to amplify a 192bp region of the murine housekeeping gene TATA box-binding protein (*Tbp*).

The final volume for each reaction was 25µl, which included 12.5µl of (2x concentration) Reddymix, 25pmol of each forward and reverse primers, 10.5µl of molecular grade water and 1µl of RNA used as a template. The reactions were heated to 95ºC (1 cycle) for 3 minutes, followed by 35 cycles of 30 seconds at 95°C (denaturation), 45 seconds at 64°C (annealing), and 1 minute at 72°C (extension) before a final extension step of 72°C for 10 minutes. PCR products were separated by electrophoresis on a 1.8% (w/v) agarose gel. Agarose powder was dissolve in TBE buffer (90mM Tris, 90mM boric acid, 2mM EDTA) by using microwave. The solution was allowed to cool and 0.00003% of the fluorescent dye ethidium bromide was added. Electrophoretic separation was performed at 120V for about 45 minutes and visualised on a UV transilluminator (VWR®GenoSmart). The presence of any band equivalent to 192 bp of *Tbp* contamination with gDNA. In order to destroy any contaminating gDNA, samples were incubated with 1µl DNase I at 37ºC for 2 hours.

2.2.6.3 cDNA synthesis (reverse transcription of mRNA)

From total RNA, 2µg was added in 0.2ml RNase-free microcentrifuge tube. Molecular grade water was added to make the volume to 13.2 µl, the tubes were then incubated at 70ºC for 2 minutes to inactivate remaining DNase I. To this mixture 1µl of random primers was added. The samples were incubated at 65ºC for 5 minutes and then slowly allowed to cool to room temperature for 10 minutes allowing primers to anneal. For each samples the following were added to make the final volume of reaction 20µl, 2 µl 10X Affinity Script™ RT Buffer, 2 µl 100mM DTT, 0.8µl 100mM dNTP mix and 1µl of Affinity Script™ Multiple Temperature Reverse Transcriptase. The reaction was gently mixed and incubated at 25ºC for 10 minutes, then for 1hour at 55ºC, and finally for 15 minutes at 70ºC. Resultant cDNA samples were checked by PCR and used as template to amplify *Tbp* transcripts and the other transcripts of interest. The PCR products were visualised on agarose gel as previously described.

2.2.6.4 Real time-PCR standards preparation

Gel extraction and ligation

Oligonucleotide primers for different genes (listed in appendix A) were used to perform PCR as described previously. PCR products were separated by 1.8% agrose gel supplemented with ethidium bromide. The bands of the correct size were cut from the gel under UV visualisation. Bands of the genes of interest were purified using the QIAquick Gel Extraction kit according to the manufacturer's protocol. Purified PCR product was ligated into the cloning vector pDrive (see appendix B). 2µl of PCR product was incubated with 0.5 of pDrive and 2.5µl of ligation buffer for at least 4 hours at 4ºC or overnight.

Transformation of competent cells

Ligation product was transformed into competent DH5α *E. coli* cells. 5µl of ligation product was mixed with 50µl of the competent cells. The mixture was incubated on, ice for 20 minutes, water bath at 42ºC for 2 minutes and ice for 2 minutes. 200 µl of LB broth was added and the reaction was incubated at 37ºC for 1-1.5 hours. The transformed cells were then spread on agar plates pre-treated with 100µg/ml ampicillin, 50µg/ml X-gal and 500µM IPTG. The plates were incubated overnight at 37ºC. A single white colony was picked using a pipette tip and immersed in a 10ml LB broth supplemented with ampicillin. The broths were incubated overnight at 37ºC on an orbital shaker (225RPM). Plasmids were then purified from the culture using a QIAprep spin miniprep kit. Miniprep products were quantified by a nanodrop. Different standard concentrations for each gene were prepared by serial dilution ranging from $3x10^6$ to $3x10^1$ copies. Representative standard curves for the genes studied are shown in appendix C.

Restriction enzyme digest

Before preparing the standards and for confirmation that cDNA inserts were successfully cloned in the plasmid, 2µl of miniprep product was digested with 1µl of restriction enzyme *Escherichia coli* Restriction enzyme type 1 (EcoR1). The enzyme cut the DNA of interest to fragment of known size in a 10x buffer with a suitable salt

concentration for the enzyme. The volume of the reaction was made up to 10µl by molecular grade water and incubated for 2h at 37ºC. Products were then separated by electrophoresis on a 1.8% (w/v) agarose gel.

2.2.6.5 Quantitative RT-PCR

Each reaction included 10µl of Brilliant III ultra-Fast SYBR®Green QPCR master mix, 8µl molecular grade water, 1µl from each forward and reverse primers, and 1µl of DNA template (cDNA sample, plasmid standard or molecular water for negative control). The Stratagene Mx3000p system was used to perform all reactions. The reactions were subjected to the following programme, 1 cycle at 95ºC for 3 minutes, followed by 40 cycle at 95ºC for 0.05 minute and at (gene-specific annealing temperature), as detailed in appendix A, for 0.20 minute. The samples or standards were then subjected to 1 cycle of 1minute at 95ºC, followed by 0.30 minute at 55ºC and finally for 0.30 minute at 95ºC. From the standard curve for each gene, the number of copies was determined. Expression levels of the genes of interest were normalised to the chosen housekeeping gene *Tbp*.

2.2.7 Enzyme-linked immunosorbent assay (ELISA)

2.2.7.1 Cells treatments

BMDCs plated out in 24-well plates at $5x10^5$/500ml of medium. Plates were incubated overnight at 37ºC/5% CO_2. The cells were treated with different concentration of progesterone, norgestrel or the vehicle (chloroform)1 hour prior to expose for a relevant periods of time to LPS 1µg/ml or polyI:C 25µg/ml. Supernatants were collected after centrifugation of plates for 5 minutes at 1000g and stored at -20ºC for future use.

2.2.7.2 IL-6 assay

The concentration of IL-6 cytokine was measured in BMDCs supernatant as follows. 96-well platets were coated with capture anti IL-6 monoclonal antibody (mAb). The capture was anti-mouse (IgG clone MP5-20F3 expressed by COS-7 cells), used at a concentration of 2µg/ml prepared in PBS (pH 9.0). The plates were coated with 50µl/

well overnight at 4ºC. The plates were washed next day to remove excess capture antibody by washing buffer (0.05% Tween-20 in PBS pH 7.4) for three times and were dried well by hitting on tissues. 200µl of blocking buffer (10% FCS in PBS pH7.4) was added to each well to cover all un-bound sites on the wells and incubated for one hour at 37ºC. The plates were then re-washed for 3 times with a good drying. Samples were added at 30µl volume for each well. At the same time, and for standard curve preparation, recombinant murine IL-6 (produced by *E.coli*) was added at a concentration of 20ng/ml diluted in blocking buffer at a volume of 30µl for each well titrated in doubling dilutions from 20ng/ml- 387.5pg/ml. Samples and standards were incubated for 2 hours at 37ºC. A representative standard curve is shown in appendex D. After that the plates were washed 4 times and dried as before. Biotin labelled rat anti-mouse monoclonal Ab (IgG2a clone MP-5-32C11 expressed in COS-7 cells) was used as secondary antibody at 1µg/ml concentration diluted in blocking buffer and added as 50µl/well. The plates were incubated for one hour at 37ºC and then washed for 5 times and dried. Streptavidin-alkaline phosphatase (SAv-KP) was used as conjugate diluted as 1 in 2000 in blocking buffer and added at a volume of 50µl. Plates were later incubated for 45minutes at 37ºC before a final wash for six times. After drying, 1mg/ml of p-nitrophenyl phosphate (pNPP) diluted in glycine buffer was added at 50µl / well. The plates were then wrapped with tin foil and incubated for a while to allow color developing. Absorbance was measured at 405nm using SPECTRmax 190 microtiter plate spectrophotometer and Softmax PRO 3.0 software.

2.2.7.3 IL-12p70 assay

ELISA for IL-12p70 had the same steps as with IL-6 with the following changes. Coating antibody was purified anti-mouse IL-12p70 Rat (IgG2b from clone 9AG expressed by CHO cells) and was prepared in carbonated buffer (1:1000 dilution) pH 9.6. For standard curve preparation, recombinant murine IL-12p70 was used at 10ng/ml concentration titrated in doubling dilutions from 10ng/ml-193.75pg/ml. A representative standard curve is shown in appendex E. Secondary antibody was biotin rat anti-mouse (IgG1, clone C15.6 expressed by CHO cells) and at 500ng/ml concentration. The conjugate used was streptavidin-horse radish peroxidase (SAv-

HRP) as 1:4000 diluted in blocking buffer. The substrate was 1% 3, 3′, 5, 5′-tetramethyl- benzidine (TMB) prepared in sodium acetate buffer pH 5.5, containing 0.0075% hydrogen peroxide. This reaction was then stopped by adding 10% sulphuric acid in a 50µl volume. The plates were read at 450nm.

2.2.8 Data analysis

For the entire experiments held in this Thesis, values were mean ± SEM for three separate observations. The statistical significance of differences between mean values from control and treated groups were determined by the one-way analysis of variance (ANOVA) followed by a Newman-Keuls using GraphPad Prism® Version 5.0 software. $P < 0.05$ was accepted as significant.

CHAPTER THREE

THE INFLUENCE OF STEROID HORMONES ON LPS AND POLYI:C STIMULATED INFLAMMATORY SIGNALLING PATHWAYS IN MACROPHAGES AND DENDRITIC CELLS

3.1 Introduction

Hundreds rather than tens of articles have reported sexual dimorphism in immune responses and generally females mount stronger responses than males. Many studies have proposed that the immunomodulatory effects of sex steroids are responsible for the elevated immune response in females. Preponderance of the female immune response over that of males is, however, a double- edged sword. Although, it may be beneficial against infective pathogens, it may result in exacerbation of some of autoimmune diseases as well as immune-mediated injuries (Bouman *et al*., 2005; Gleicher and Barad, 2007). In addition there is a growing consensus that the female sex hormones (progesterone and estrogen) are implicated in promoting and maintaining a variety of infectious, cancerous and autoimmune diseases (Fish, 2008). The levels of female sex-steroids fluctuated during pregnancy and menstruation and are accompanied usually by immunological changes (Roberts *et al*., 2001). In addition, there is accumulating evidence that many of these diseases are linked to the elevated levels of these hormones that are associated with pregnancy (Klein *et al*., 2012), and the menstrual cycle (Case and Reid, 2001; Oertelt-Prigione, 2012) as well as other endocrine disorders. However, even at their normal physiological levels, these hormones might cause immunological changes associated with some pathological conditions (Klein, 2004). The mechanisms by which sex hormones and the immune system interact are still unclear although there is a general consensus on some of the general outcomes. Higher levels of sex hormones during pregnancy shift the Th1/Th2 balance towards Th2 dominance (Szereday and Szekeres-Bartho, 1997; Ostensen, 1999). This change in immunological bias during pregnancy may be responsible for the aggravating of some autoimmune diseases such as systemic lupus erythematosis (SLE) and remission of others such as rheumatoid arthritis (Ostensen, 1999). It has been suggested that progesterone is a major mediator of Th2 predominance during pregnancy (Piccinni *et al*., 1995). This may be a result of the expression of progesterone receptors on lymphocytes during pregnancy (Szekeres-Bartho *et al*., 1990; Sader *et al*., 2005). Regarding infectious diseases, females record higher incidence levels of some infectious diseases especially with genital tract infectious pathogens such as Chlamydia, human immunodeficiency virus (HIV) and genital herpes compared with males (Butts *et al*., 2011). As mentioned previously in

the introduction, the immune-modulatory effects of steroid hormones arise from the abundant expression and availability of steroid hormone receptors throughout immune cells. In the same context reproductive activities such as ovulation, menstruation and even pregnancy are subjected to being under immunological control (Laird *et al*., 2003). Within the innate immune system, the TLRs 1-6 have been detected in cells of female reproductive tract (Pioli *et al*., 2004). Epithelial cell lines of the vagina and cervix express TLR 1, 3, 5 and 6 (Dusio *et al*., 2010). Thus, an immune-endocrine interaction may be apparent and potentially essential to the control of many normal reproductive function as well as reproductive and non-reproductive diseases.

Glucocorticoids are well known for their role as immunosuppressives and have striking effects and prompt amelioration, for example, corticosteroids in rheumatic disease (Hench *et al*., 1949). As a result of this discovery Hench with Kendall and Reichstein won the Noble prize in 1950. Since then, glucocorticoid-derivative products are commonly prescribed as potent anti-inflammatory and immunosuppressive agents for a vast array of medical problems ranging from simple allergic symptoms to complicated cases of neoplastic, autoimmune diseases and organ transplantation (Cohn, 1997). The hormone, progesterone, which has a similar structure to glucocrticoids (has a steroid nucleus), exerts its action not only by binding the PR but also the GR (Kontula, 1983; Sugino *et al*., 1997; Telleria *et al*., 1999). It has been widely documented that a number of the immunosuppressive or anti-inflammatory effects of progesterone are mediated via GRs. Steroid hormones induce signalling via steroid receptors as ligand-dependent transcription factors. Hormone-receptor complexes form dimers that translocate to the nucleus as transcription factors and bind DNA specific sequences regulating the transcription of target genes. Through protein-protein interaction these ligand–bound receptors interact with other transcription factors and change their molecular activities (Chinenov and Rogatsky, 2007; Tait *et al*., 2008). Down regulation of inflammatory responses by these hormones is believed to be mediated through transcriptional repression of different signalling pathways. In other words, they disrupt NFκB, AP-1 and IRFs pro-inflammatory transcription activities induced by different stimuli such as TLR ligands.

Innate and adaptive immune responses comprise two integrated arms of defense mechanisms working together to provide complete protection against life threatening (self and non-self) assaults. Innate immunity serves as an advanced line of defense. The majority of animal species rely on this arm during protective responses (Janeway, 2001). Although, adaptive immunity in mammalian species for example is more effective and more specific, it requires innate immune mechanisms for initiation. It is now widely accepted that TLRs are important tools of innate immune system recognition (Akira *et al*., 2006). Macrophages and dendritic cells are mononuclear phagocytes as well as antigen presenting cells of varying effectiveness. As a part of innate immunity they detect and clear potential pathogens via expression and ligation of TLRs (Kawai and Akira, 2005; Medzhitov, 2007). Stimulation of TLRs by microbe products triggers a series of intracellular signal transduction events that end in expression of different genes such as pro-inflammatory cytokines, chemokines and Type 1 interferon (α/β) production (Janeway and Medzhitov, 2002; Kawai and Akira, 2005; Medzhitov, 2007).

TLR4 signalling involves a complicated interaction, in which one or more adaptor molecules have to interact. MyD88 was the first adaptor molecule considered to mediate the full response to LPS exposure (Muzio *et al*., 1997). However, Horng *et al* (2001) reported that TLR4 can activate NFκB, MAPKs and DCs maturation in MyD88 deficient mice. Thus, it was suggested another adaptor molecule would be involved. This is now known as Mal/TIRAP and is recognised to contribute to regulating the signalling cascades downstream of TLR4 (Fitzgerald *et al*., 2001; Horng *et al*., 2001). In addition, two other adaptor molecules were shown to be involved in TLR4 signalling, namely TRIF and TRAM (Fitzgerald *et al*., 2003b; Yamamoto *et al*., 2003b). Therefore, TLR4 initiates signalling in response to a wide variety of ligands including LPS such as from gram-positive bacteria, viruses, fungi and protozoa (Kumer *et al*., 2009b; Kumer *et al*., 2011).

TLR3 was discovered more than a decade ago as a receptor mediating antiviral activity by inducing Type 1 IFNs in response to ds RNA viruses (Alexopoulou *et al*., 2001). It can also recognise ssRNA viruses during their replication and production of dsRNA such as West Nile Virus (WNV) and

encephalomyocarditis virus (EMCV) (Kumer *et al*., 2009b). Moreover, the synthetic analogue of dsRNA, polyI:C can be recognised by TLR3 and used experimentally as the receptor ligand mimetic (Matsumoto and Seya, 2008). TLR3 has been detected in myeloid DCs and intestinal epithelial cells (Muzio *et al*., 2000). In addition, many human tissues have been demonstrated to express TLR3 mRNA including placenta, brain, pancreas, heart, liver and lymph nodes (Rock *et al*., 1998; Muzio *et al*., 2000). Signalling cascade events of TLR3 transduction result in activation of IRF3, NFκB and AP-1 which in turn induce production of cytokines, chemokines and Type 1 IFNs to mediate the immune response against a wide range of self and non-self threats.

To study the effects of steroid hormones on inflammatory responses elicited by innate-cellular components, TLR3 and TLR4 have been chosen as representative of TLRs mediating signalling cascades that induce different inflammatory gene responses.

3.2 Results

The influence of steroid hormones on LPS- or polyI:C- stimulated inflammatory signalling

The inflammatory response is a major characteristic of all acute and chronic diseases. Innate immune cells, macrophages and dendritic cells are among the initial immune components that respond immediately to any external stimuli. Upon activation, diverse molecules inside these cells are recruited in a series of sequential events that results in the regulation of the expression of vast number of genes implicated in inflammation. Inflammatory signalling events within these cells have been extensively studied by means of TLR stimulation. In this study the immune-modulatory effects of progesterone hormone on inflammatory signalling has been investigated using a comparison between the classical inflammatory signalling elicited by TLR3 and TLR4 ligands to those pretreated with the hormone and stimulated by the same ligands. In order to differentiate the PR and GR effects of progesterone the influence of progesterone was compared with the synthetic

progesterone analogue norgestrel (a PR agonist) and the synthetic glucocorticoid analogue dexamethasone (a GR agonist) on the inflammatory signalling cascades induced by TLR ligands LPS and polyI:C in macrophages and dendritic cells. This study investigated the immunomodulating role of progesterone on inflammatory pathways by assessing the expression and activation of NFκB, MAP kinases and IRF3 proteins.

Macrophages

3.2.1 The NFκB signalling pathway

Classical activation of the NFκB pathway is responsible for an inflammatory response operating via a heterodimer consisting of p50 and p65. In the resting situation this dimer is held inactive by binding one member of the inhibitory protein family IκB. Stimulation of innate immune cells (macrophages) leads to phosphorylation of IκB. Phosphorylated IκB is tagged by ubiquitin proteins leading to further degradation by the proteasome pathway, and liberating NFκB from its binding site. Liberated NFκB translocates to the nucleus where it binds DNA and initiates transcription. To investigate these events, BMD- macrophage cells were exposed to LPS as a TLR4 ligand and to polyI:C as a TLR3 ligand. Different stimulation times were investigated from 5 minutes to 2 hours. Both p65 phosphorylation and IκBα degradation were assessed as indicators of NFκB activation. For comparison, cells were treated with the hormones progesterone (62.5μM), norgestrel (62.5 μM) and dexamethasone (62.5 nM) one hour prior exposure to 1μg/ml LPS or 25μg/ml polyI:C.

3.2.1.1 Agonist stimulated-p65 phosphorylation and the potential effects of hormones in macrophages

Regarding p65 phosphorylation in macrophages, both ligands LPS and polyI:C showed generally similar effects. Both initiated phosphorylation very rapidly (after 5 minutes). LPS induced peak p65 phosphorylation (Figure 3.1) at 5minutes as an approximately 6 fold (6.2206 ± 1.1438, $P < 0.001$) and this gradually declined to reach a minimum level after 2 hours. Treatment with the steroid hormones

progesterone, norgestrel and dexamethasone prior to exposure to LPS (Figure 3.2) did not influence LPS stimulated p65 phosphorylation in BMD-macrophages (5-120 min). PolyI:C exerted a maximum activation after 15 minutes (Figure 3.3) which increased approximately 5 fold (4.7804± 0.7503, $P<0.001$) and declined gradually to reach a minimum level after 2 hours of stimulation. Treatment with the hormones prior to exposureto polyI:C (Figure 3.4) did not also have any influence on p65 phosphorylation. P65 protein expression was used as a means for determining equal protein loading with both ligands.

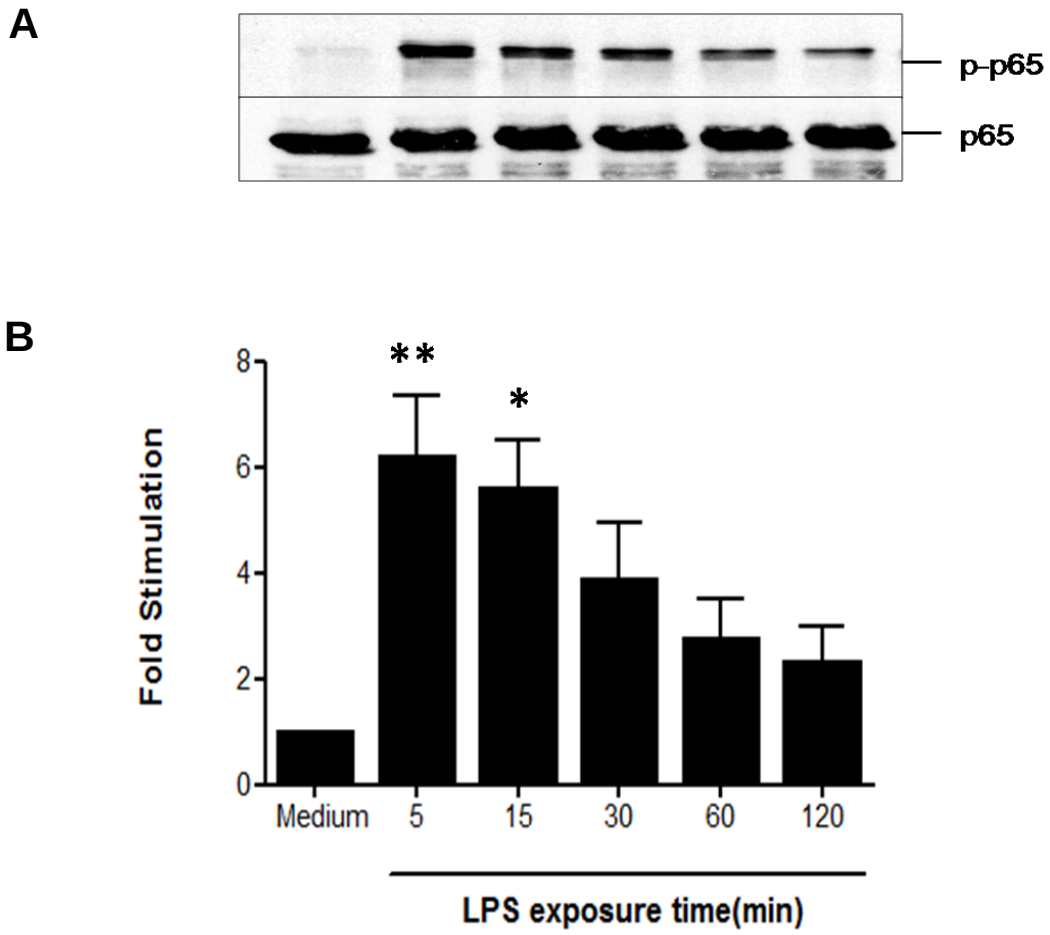


Figure 3.1. Assessment of LPS-stimulated p65 phosphorylation in BMD-macrophages. Cells were incubated in media or exposed to 1µg/ml LPS for the indicated times (min). Whole cell extracts were prepared and p65 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data were quantified by densitometry scanning and are depicted in panel B. Each value represents mean±SEM. *p<0.05 and **p<0.001 are statistically significant vs. control (Medium).

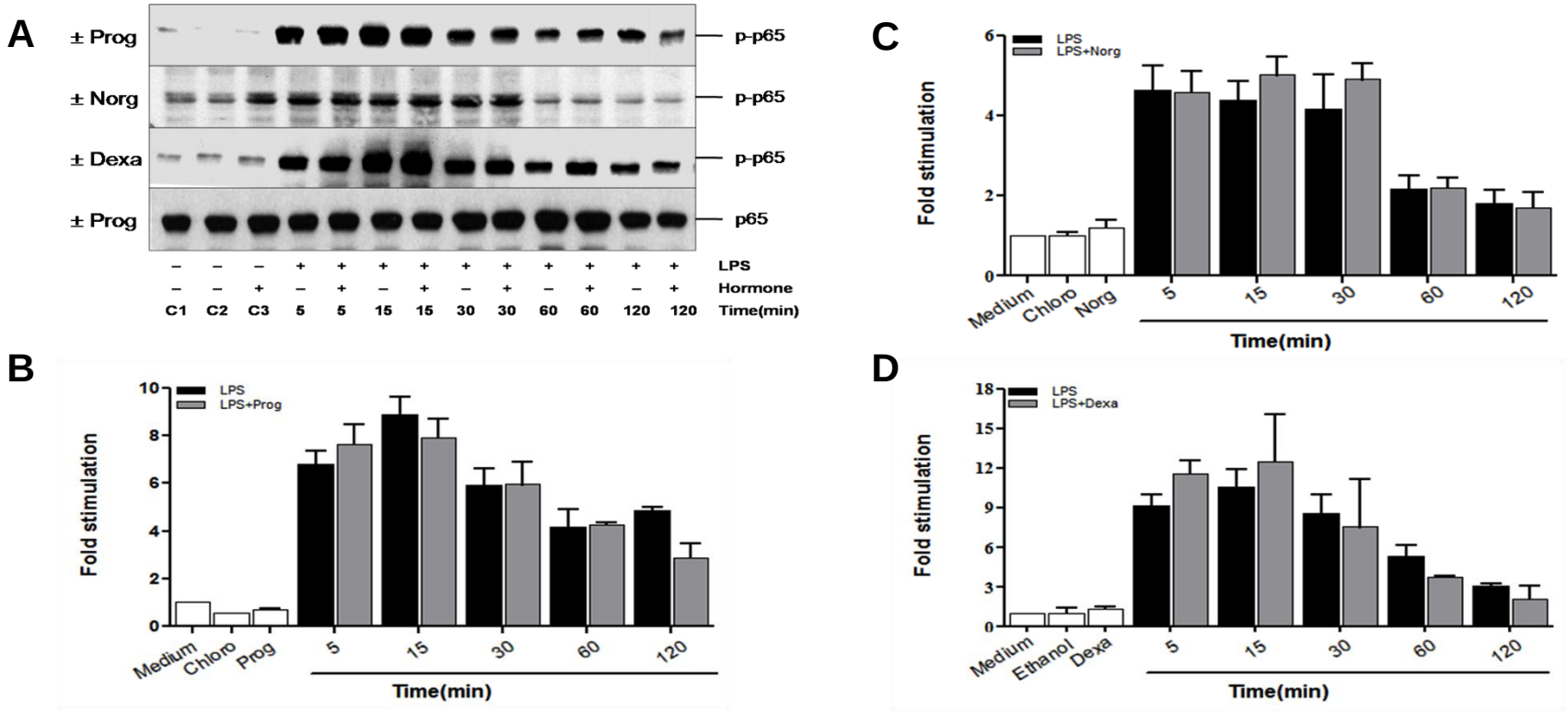


Figure 3.2. The effect of progesterone, norgestrel and dexamethasone on LPS –stimulated p65 phosphorylation in BMD-macrophages. BMD-macrophages were treated with vehicle or 62.5 μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 1μg/ml LPS for the indicated times (min). Whole cell extracts were prepared and p65 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3). C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

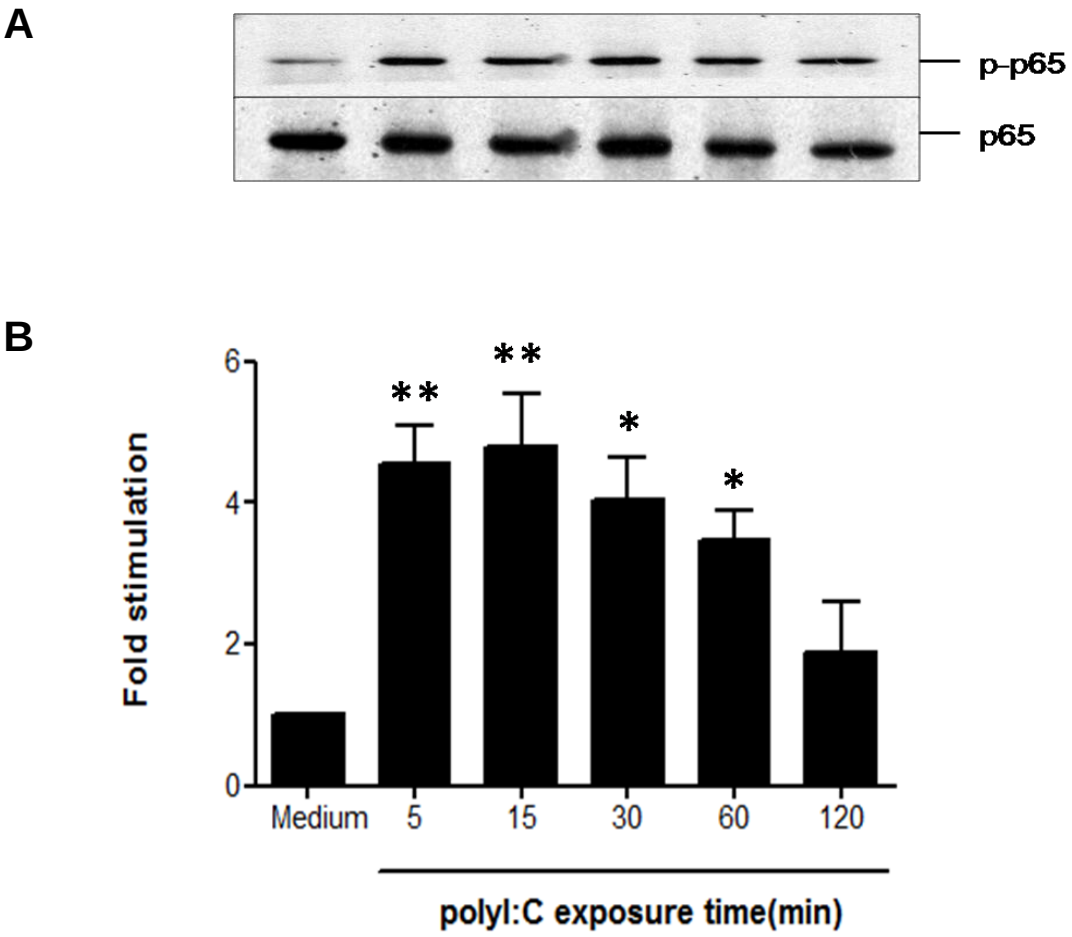


Figure 3.3. Assessment of polyI:C-stimulated p65 phosphorylation in BMD- macrophages. Cells were incubated in media or exposed to 25µg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and p65 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data were quantified by densitometry scanning and are depicted in panel B. Each value represents mean±SEM. *p<0.05 and **p<0.001 are statistically significant vs. control (Medium).

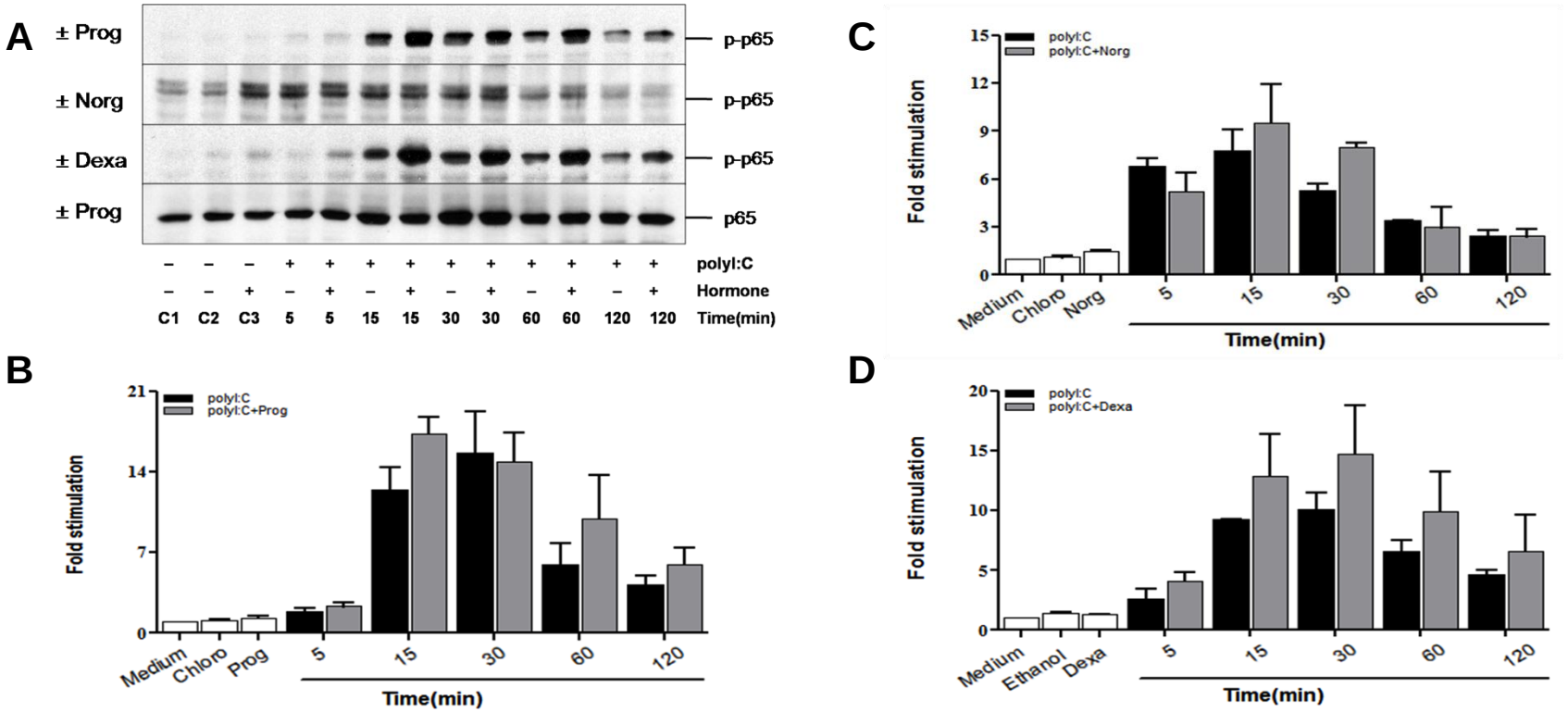


Figure 3.4. The effect of progesterone, norgestrel and dexamethasone on polyI:C–stimulated p65 phosphorylation in BMD-macrophages. BMD-macrophages were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 25μg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and p65 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section.Results in panel A are representative of three independent experiments. The associated data for experiments examining pretreatment with progesterone, norgestrel and dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3). C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

3.2.1.2 Agonist stimulated-IκBα degradation and the potential effects of hormones in macrophages

A requisite for activation of the classical NFκB cascade is IκBα degradation accompanied commonly by p65 phosphorylation. Therefore, to investigate LPS- or polyI:C- induced IκBα degradation, BMD-macrophage cells were treated with either 1μg/ml LPS or 25μg/ml polyI:C over 120 minutes. Degradation of IκBα started very rapidly within (5 minutes) after exposure of macrophages to LPS (Figure 3.5) (21.6226 fold ± 6.6686, $P < 0.0001$). IκBα protein levels then increased slightly after 30 minutes (80.8563 fold ± 5.8358, $P < 0.001$) but still there were significant inhibition for the remaining time points compared to the vehicle. Pretreatment with the steroid hormones progesterone, norgestrel and dexamethasone did not influence LPS- induced IκBα degradation (Figure 3.6). Macrophages exposed to polyI:C (Figure 3.7) showed a maximum degradatory effect after 15 minutes (35.4238 fold ± 9.43123, $P < 0.001$) and the protein expression was restored after 30 minutes to reach a peak after 60 minutes (81.03986 fold ± 8.7898). Once again pretreatment with the steroid hormones did not influence poly I:C- induced IκBα degradation (Figure 3.8), (5-120 min). p38 protein expression was used as a means for determining equal protein loading with both ligands.

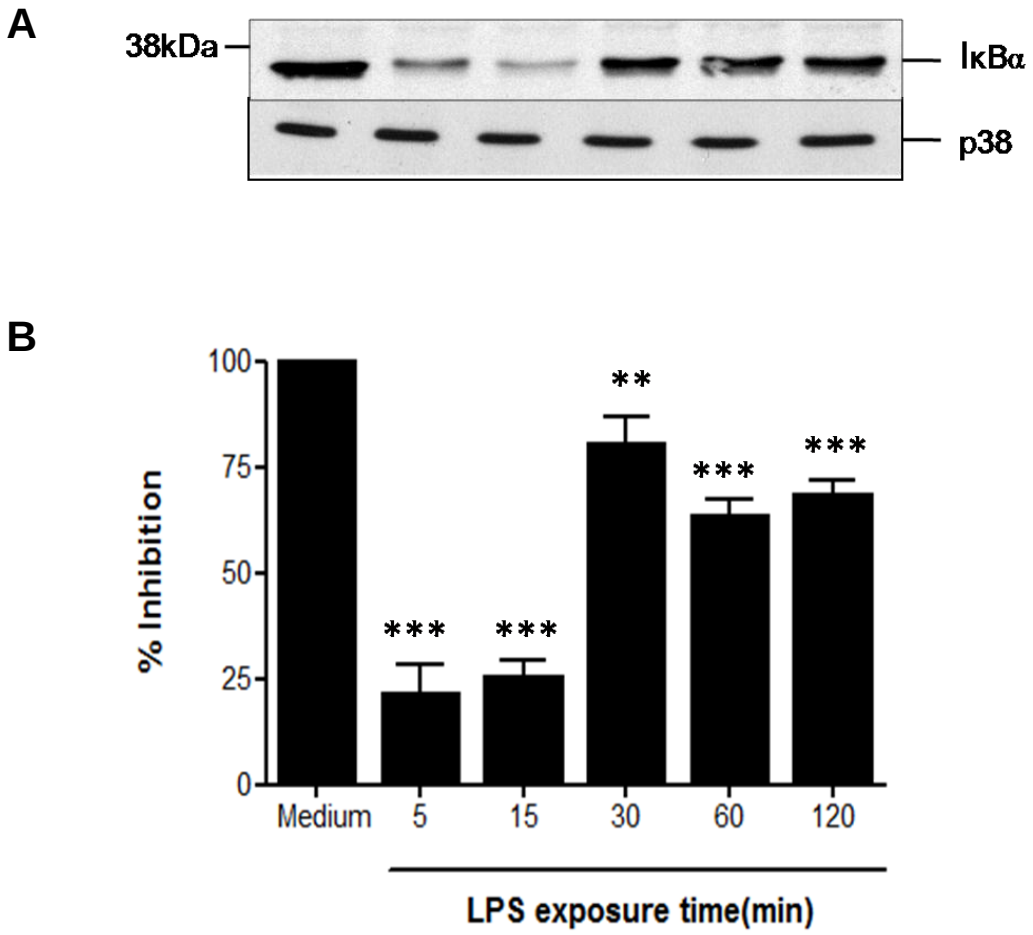


Figure 3.5. Assessment of LPS-stimulated IκBα degradation in BMD-macrophages. Cells were incubated in media or exposed to 1µg/ml LPS for the indicated times (min). Whole cell extracts were prepared and IκBα degradation was assessed by Western blotting using specific antibodies as detailed in Materials and Methods section. p38 expression was used as protein loading control. Results in panel A are representative of three independent experiments. The associated data were quantified by densitometry scanning and are depicted in panel B. Each value represents mean±SEM. **p<0.001 and ***p<0.0001 are statistically significant vs. control (Medium).

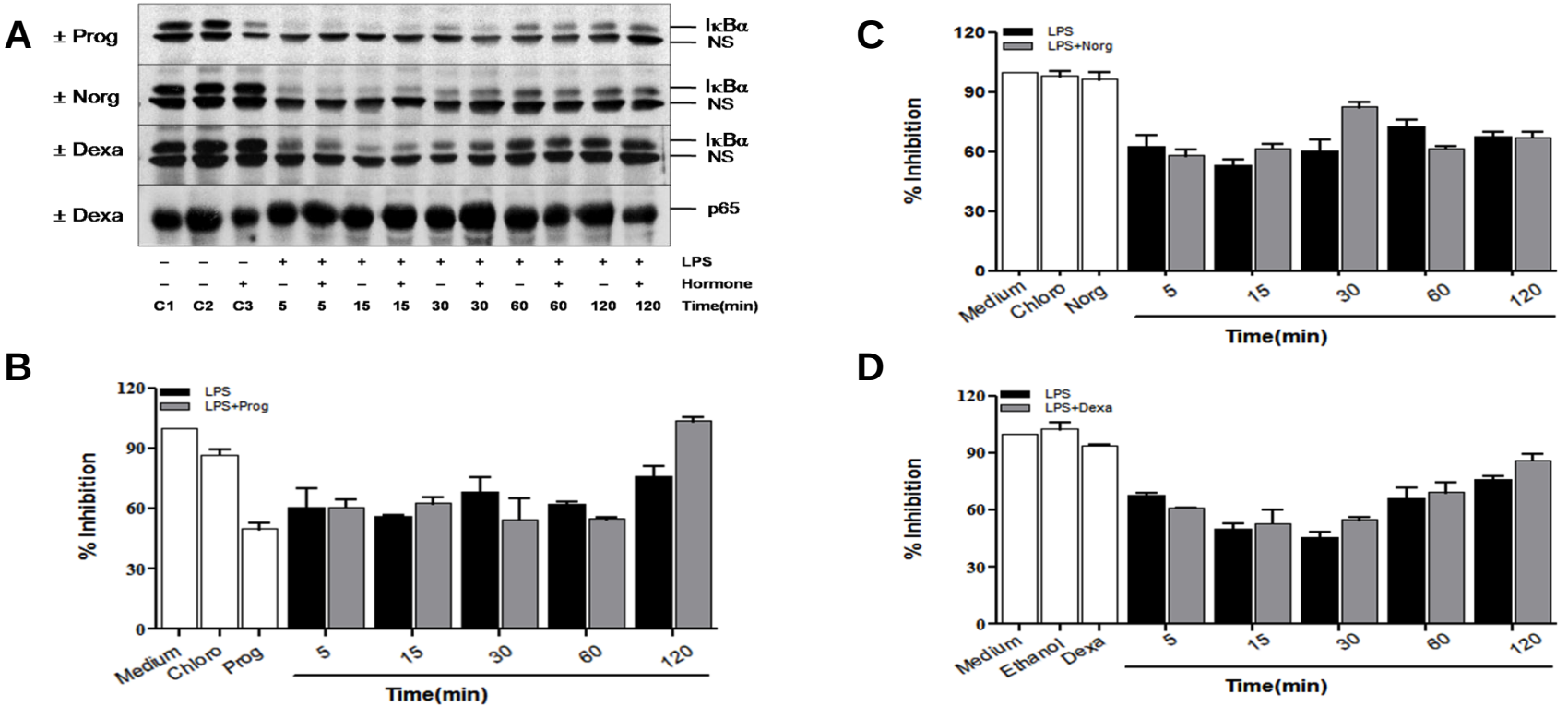


Figure 3.6. The effect of progesterone, norgestrel and dexamethasone on LPS-stimulated IκBα degradation in BMD-macrophages. BMD-macrophages were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 1μg/ml LPS for the indicated times (min). Whole cell extracts were prepared and IκBα degradation was assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. p65 expression was used as protein loading control. Results in panel A are representative of three independent experiments. The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3). C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa), NS, non specific band.

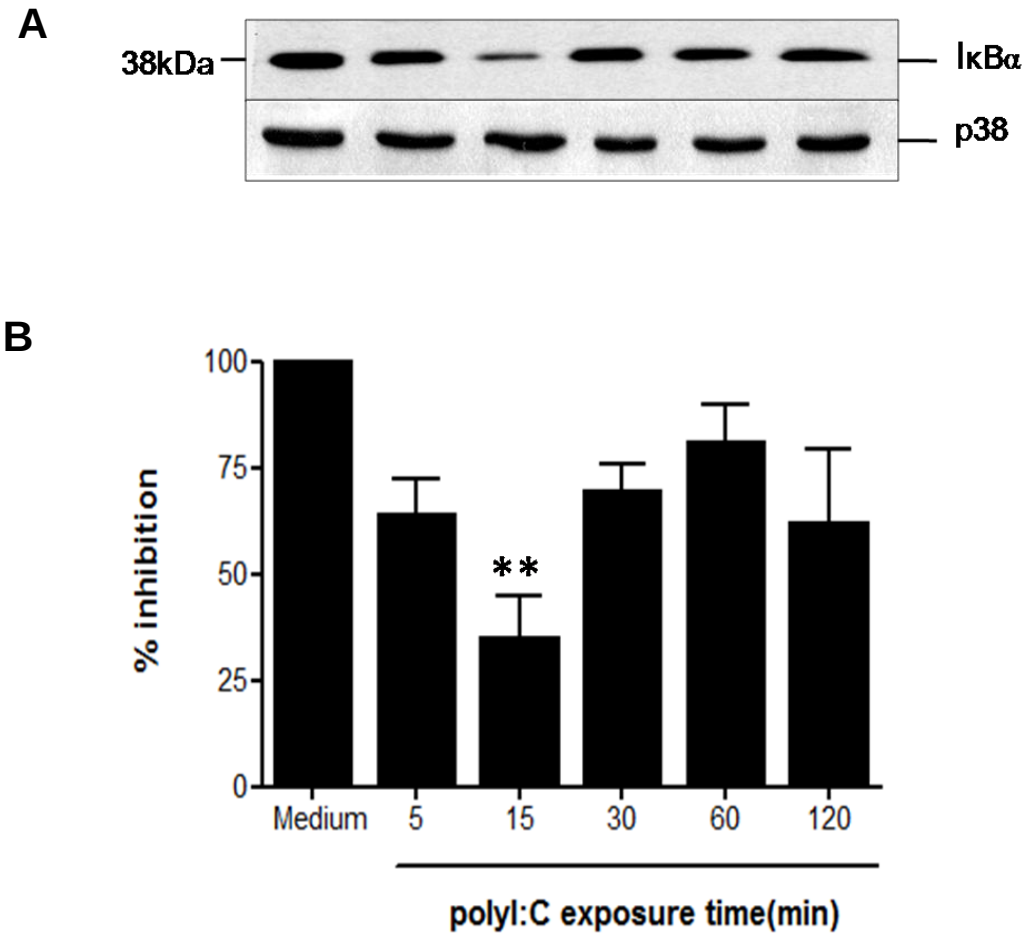


Figure 3.7. Assessment of polyI:C-stimulated IκBα degradation in BMD-macrophages. Cells were incubated in media or exposed to 25µg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and IκBα degradation was assessed by Western blotting using specific antibodies as detailed in Materials and Methods section. p38 expression was used as protein loading control. Results in panel A are representative of three independent experiments. The associated data were quantified by densitometry scanning and are depicted in panel B. Each value represents mean±SEM. **p<0.001is statistically significant vs. control (Medium).

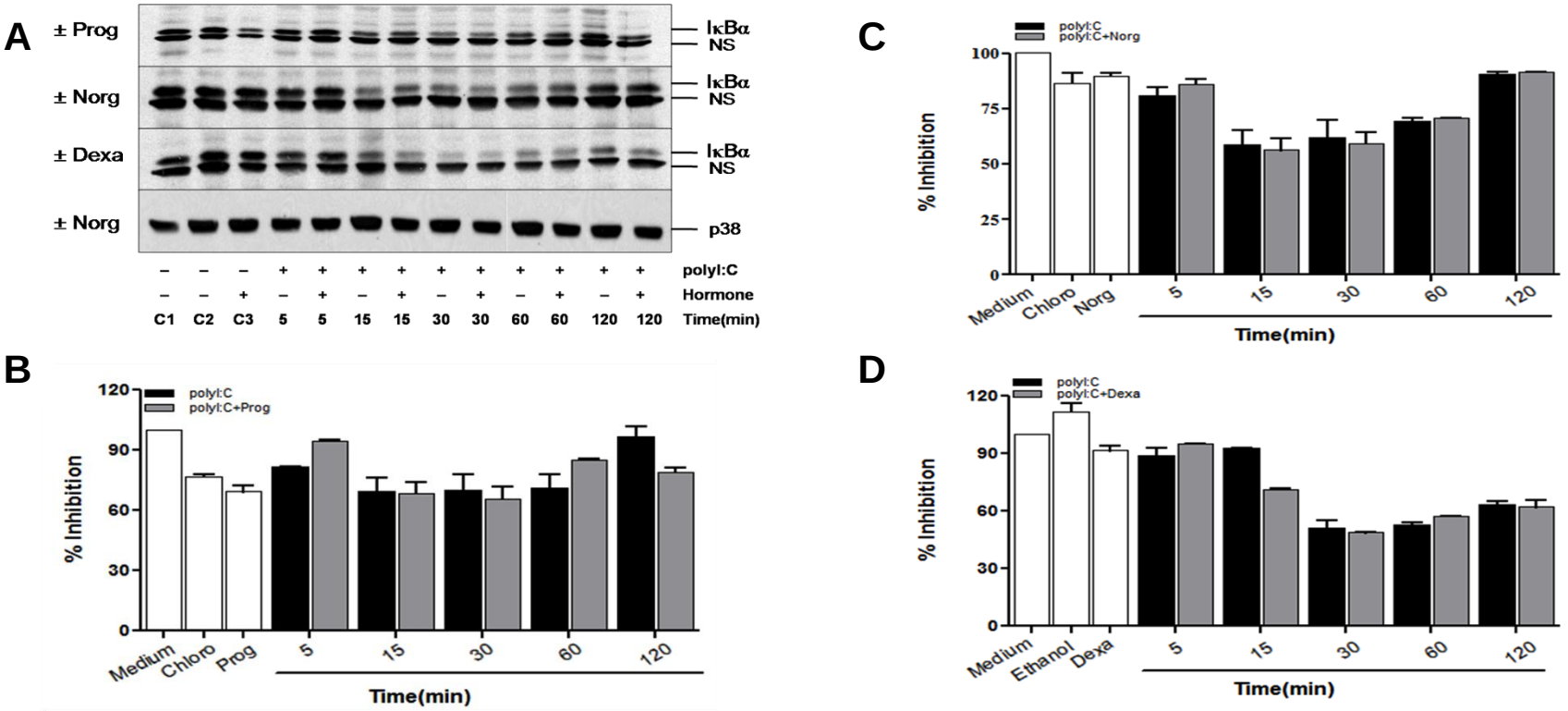


Figure 3.8. The effect of progesterone, norgestrel and dexamethasone on polyI:C-stimulated IκBα degradation in BMD-macrophages. BMD-macrophages were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 25μg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and IκBα degradation was assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. p38 expression was used as protein loading control. Results in panel A are representative of three independent experiments. The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3). C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa), NS, non specific band.

3.2.2 The MAPK signalling pathways

The MAPKs are involved in the events that regulate the expression and activation of many inflammatory genes (Thalhamer *et al*., 2008). The three subgroups of MAPK family (JNK, p38 and ERK) were assessed by means of protein phosphorylation. BMD-macrophages were exposed to 1µg/ml LPS or 25µg/ml poly I:C as TLR4 and TLR3 agonist respectively in order to follow the signalling cascades of these kinases in macrophages using different time points from 5-120 minutes. Subsequently, the potential comparative effects of progesterone, norgestrel and dexamethasone on these pathways were studied following activation with the agonists.

3.2.2.1 Agonist-stimulated JNK phosphorylation and the potential effects of hormones in macrophages

Results showed that both TLR4 and TLR3 agonists activated JNK proteins rapidly within 5 minutes. In cells exposed to LPS (Figure 3.9) JNK phosphorylation started at 5 minutes and ceased within one hour, with maximal activation at 15 minutes (16.5686 ± 3.0631 fold increase as control, $P < 0.001$). Pretreatment with progesterone, norgestrel or dexamethasone showed no effect upon JNK phosphorylation in cells exposed to LPS (Figure 3.10). Cell exposed to polyI:C (Figure 3.11) showed the same pattern of JNK phosphorylation as observed with LPS .i.e, JNK phosphorylation apparent within 5 minutes and reached the maximum level in 15 minutes post stimulation (8.5504±1.149 fold increase as control, $P < 0.0001$) returning to basal level after one hour. However, unlike LPS maximal activation was induced until 30 minutes. JNK protein expression was used as a means for determining equal loading proteins. Pretreatment with hormones also showed no influence on polyI:C- induced JNK phosphorylation (Figure 3.12).

3.2.2.2 Agonist-stimulated p38 phosphorylation and the potential effects of hormones in macrophages

Both TLR3 and TLR4 induced pathways showed similar kinetics with regard to p38 activation. Both LPS and polyI:C, which stimulate TLR4 and TLR3 respectively induced rapid and strong p38 phosphorylation within 5 minutes, reaching a maximal

effect by 15minutes, and thereafter declined by 30 minutes returning to the same levels as controls an hour post stimulation. In BMD-macrophages exposed to LPS (Figure 3.13). p38 phosphorylation was apparent at 5 minutes (4.3751±0.5537fold stimulation) and reached the maximum response at 15 minutes (7.1138±2.88466 fold, $P < 0.05$). For cells exposed to polyI:C (Figure 3.15) this phosphorylation also started at 5 minutes (8.3385 ± 0.5507 fold, $P < 0.001$) and the maximum effects was also observed by 15minutes of stimulation (10.6192±1.0784 fold stimulation, $P < 0.0001$). p38 protein expression was used as a means for determining equal protein loading with both TLR ligands. Pretreatment with progesterone, norgestrel or dexamethasone had no effect upon LPS- (Figure 3.14) nor polyI:C- (Figure 3.16) stimulated p38 phosphorylation in BMD-macrophages.

3.2.2.3 Agonist stimulated-ERK phosphorylation and the potential effects of hormones in macrophages

Macrophage ERK phosphorylation following TLR4 or TLR3 ligation followed a similar pattern to JNK and p38 over the same time frame. Phosphorylation was observed within 5 minutes and maintained for 30 minutes, thereafter declined, reaching a minimum level after 2 hours of stimulation. Macrophages treated with LPS (Figure 3.17) showed ERK phosphorylation after 5 minutes of stimulation (13.4758 ± 2.9087fold change) and reached maximal effects by 15 minutes (17.9345 ± 3.3544 fold, $P < 0.0001$). This then declined to a minimum phosphorylation levels after 2 hours post stimulation. PolyI:C effects on ERK activation (Figure 3.19). ERK phosphorylation was apparent after 5 minutes (11.5822±0.5746 fold stimulation) and reached a maximum effect by 15 minutes (14.3447 ± 2.7604 fold stimulation, $P < 0.0001$). This then sharply declined after an hour. ERK protein expression was used as a means for determining equal proteins loading with both ligands. Pretreatment with progesterone, norgestrel or dexamethasone had no effect on LPS- (Figure 3.18) nor polyI:C- (Figure 3.20) stimulated ERK phosphorylation in BMD-macrophages.

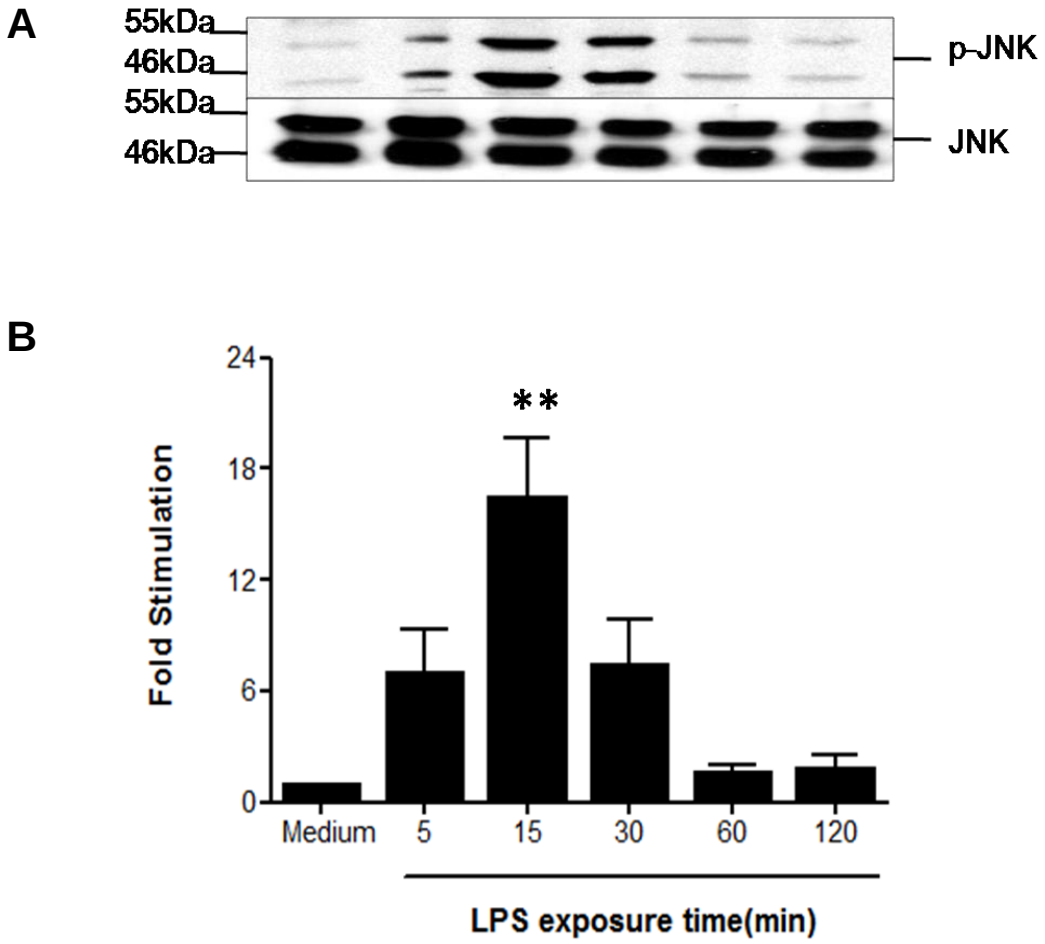


Figure 3.9. Assessment of LPS-stimulated JNK phosphorylation in BMD-macrophages. Cells were incubated in media or exposed to 1µg/ml LPS for the indicated times (min). Whole cell extracts were prepared and JNK phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data were quantified by densitometry scanning and are depicted in panel B. Each value represents mean±SEM. **p<0.001 is statistically significant vs. control (Medium).

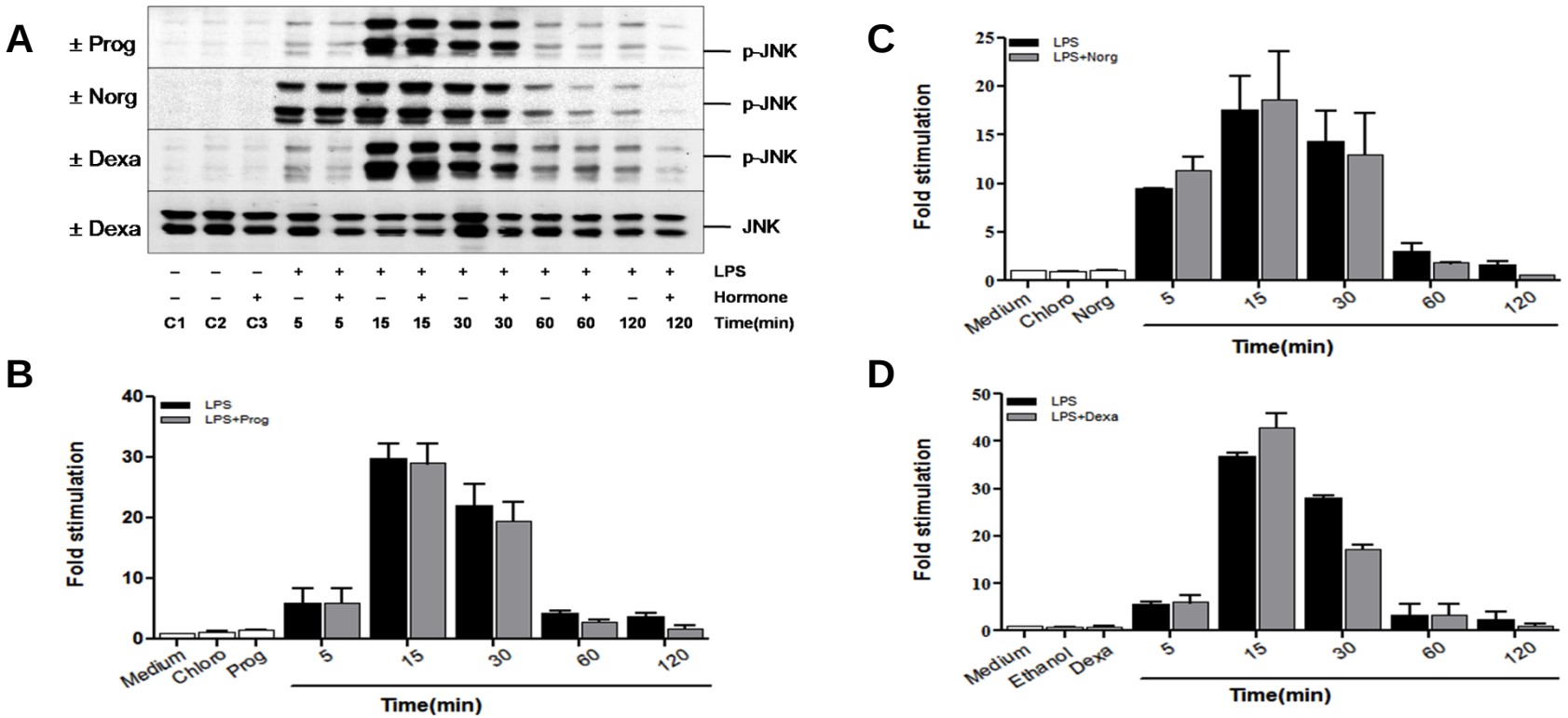


Figure 3.10. The effect of progesterone, norgestrel and dexamethasone on LPS-stimulated JNK phosporylation in BMD- macrophages. BMD-macrophages were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 1μg/ml LPS for the indicated times (min). Whole cell extracts were prepared and JNK phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data for experiments examining pretreatment with progesterone, norgestrelor dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3). C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

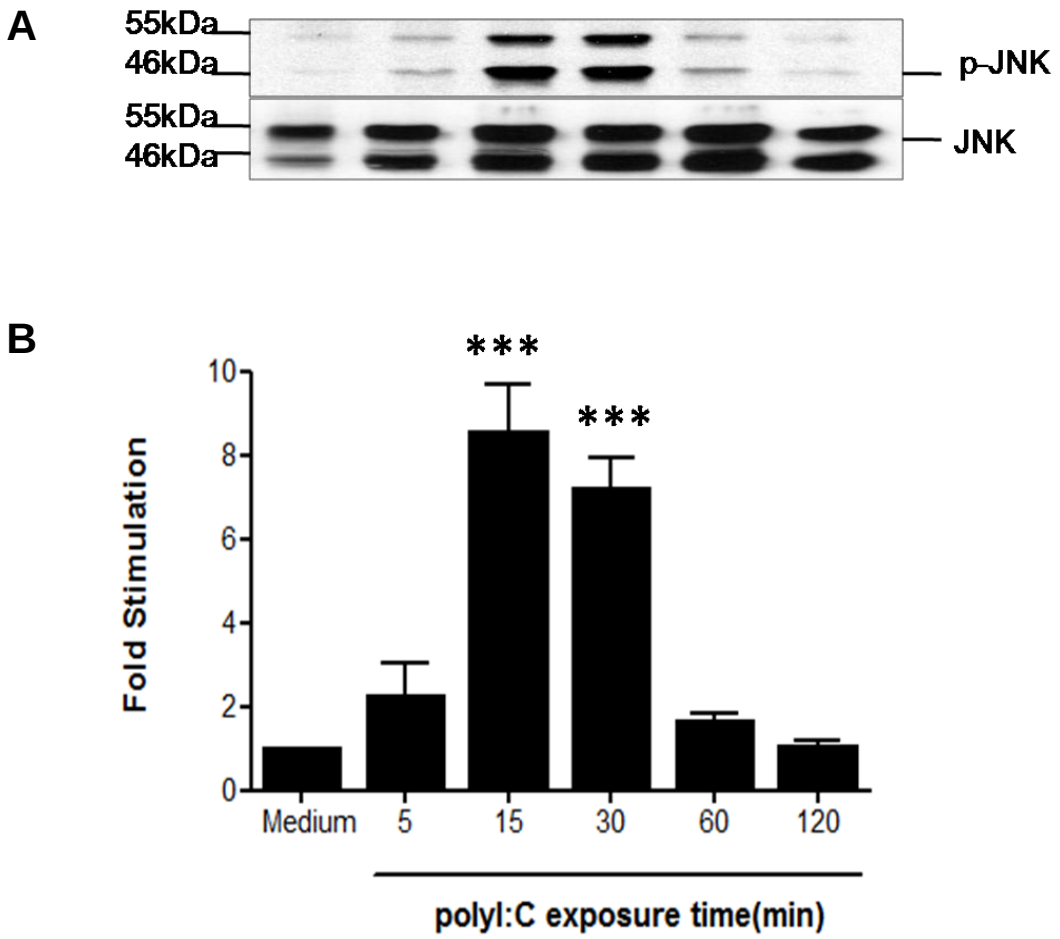


Figure 3.11. Assessment of polyI:C-stimulated JNK phosphorylation in BMD-macrophages. Cells were incubated in media or exposed to 25µg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and JNK phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data were quantified by densitometry scanning and are depicted in panel B. Each value represents mean±SEM. ***p<0.0001 is statistically significant vs. control (Medium).

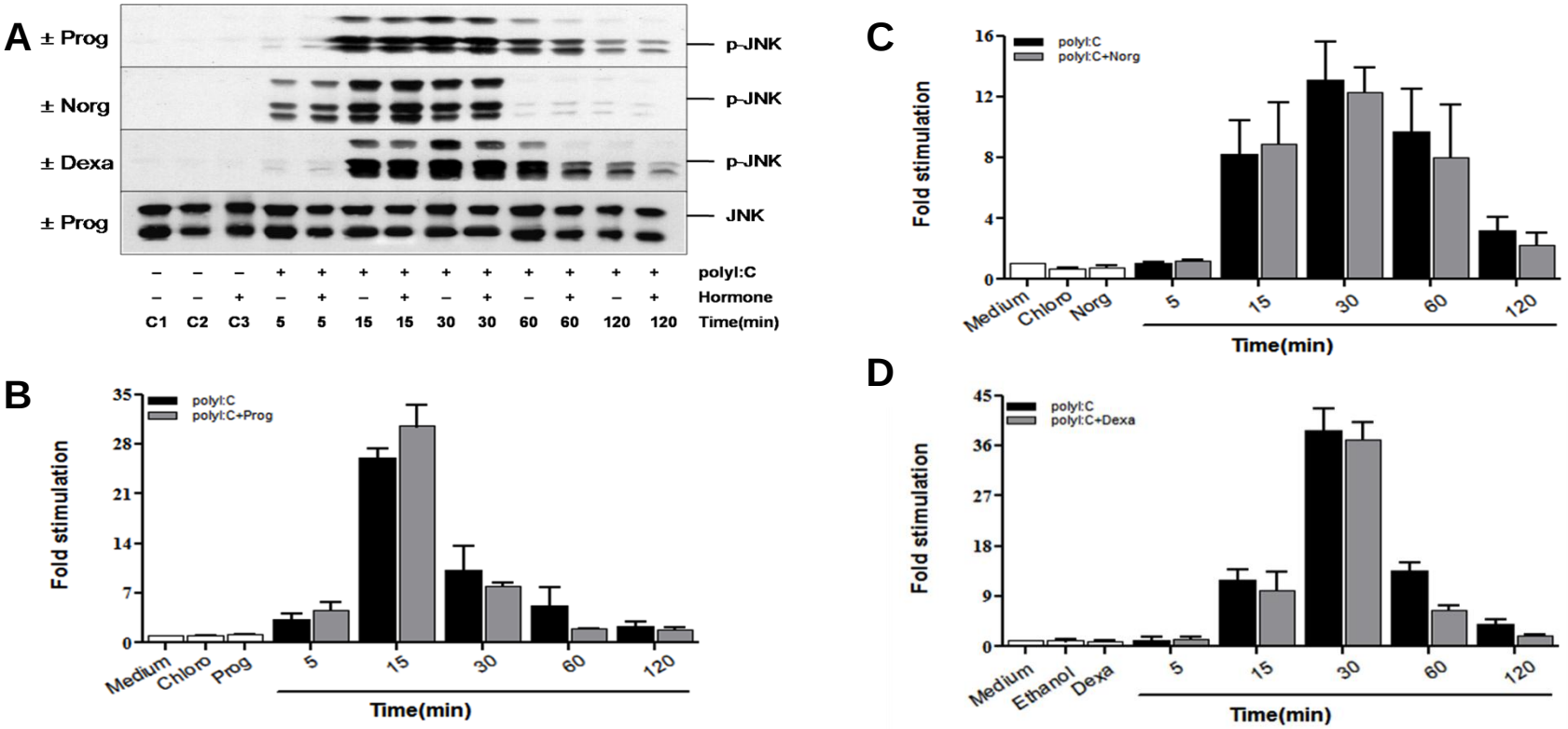


Figure 3.12. The effect of progesterone, norgestrel and dexamethasone on polyI:C-stimulated JNK phosphorylation in BMD-macrophages. BMD-macrophages were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 25μg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and JNK phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments.The associated data for experiments examining pretreatment with progesterone, norgestrel and dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3). C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

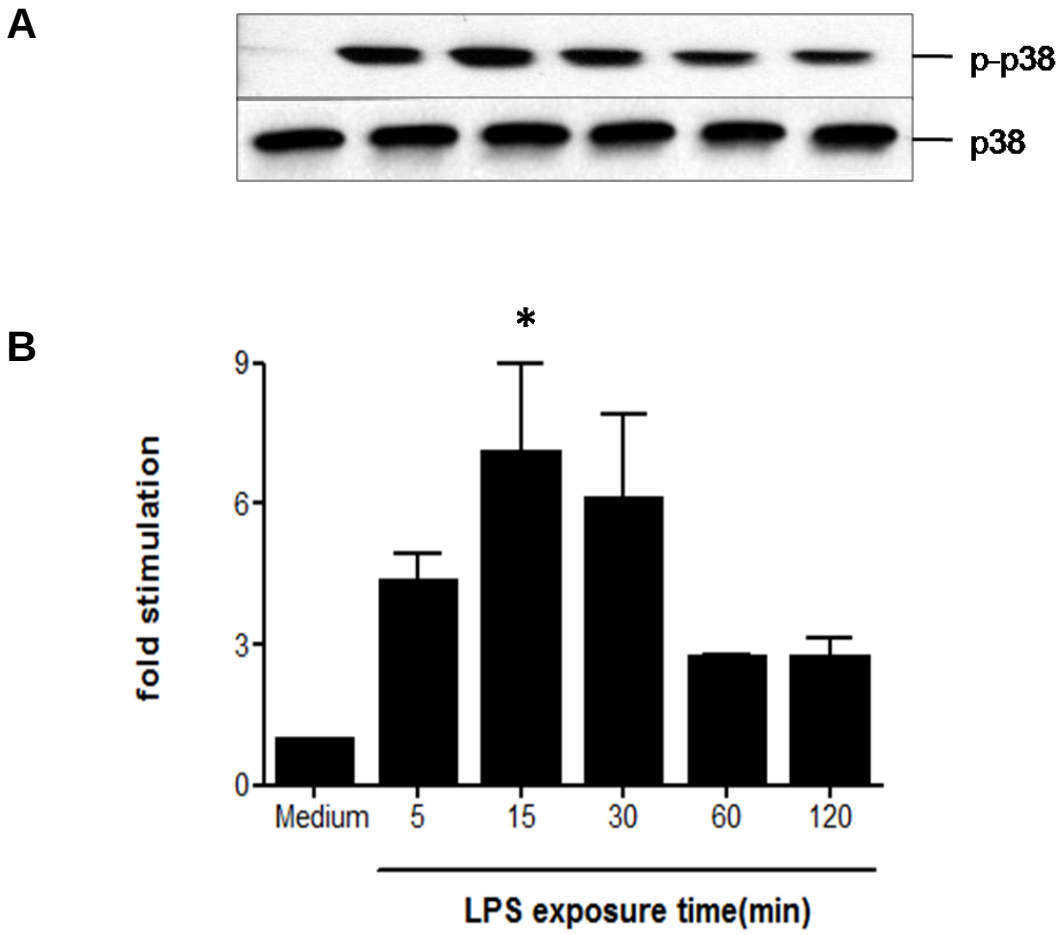


Figure 3.13. Assessment of LPS-stimulated p38 phosphorylation in BMD-macrophages. Cells were incubated in media or exposed to 1µg/ml LPS for the indicated times (min). Whole cell extracts were prepared and p38 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data were quantified by densitometry scanning and are depicted in panel B. Each value represents mean±SEM. *$p<0.05$ is statistically significant vs. control (Medium).

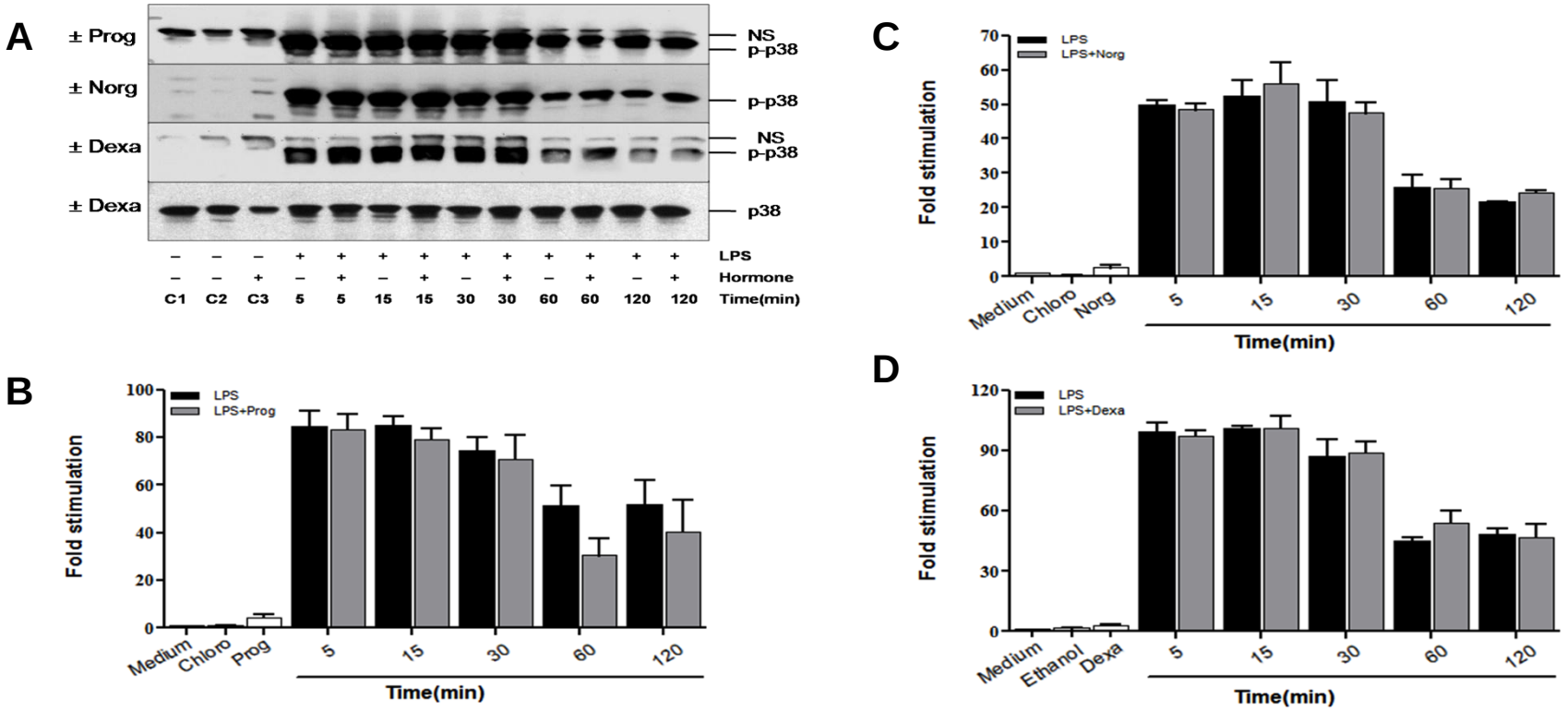


Figure 3.14. The effect of progesterone, norgestrel and dexamethasone on LPS-stimulated p38 phosporylation in BMD- macrophages. BMD-macrophages were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 1μg/ml LPS for the indicated times (min). Whole cell extracts were prepared and p38 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data for experiments examiningpretreatment with progesterone or norgestrelor dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3). C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa), NS, non specific band.

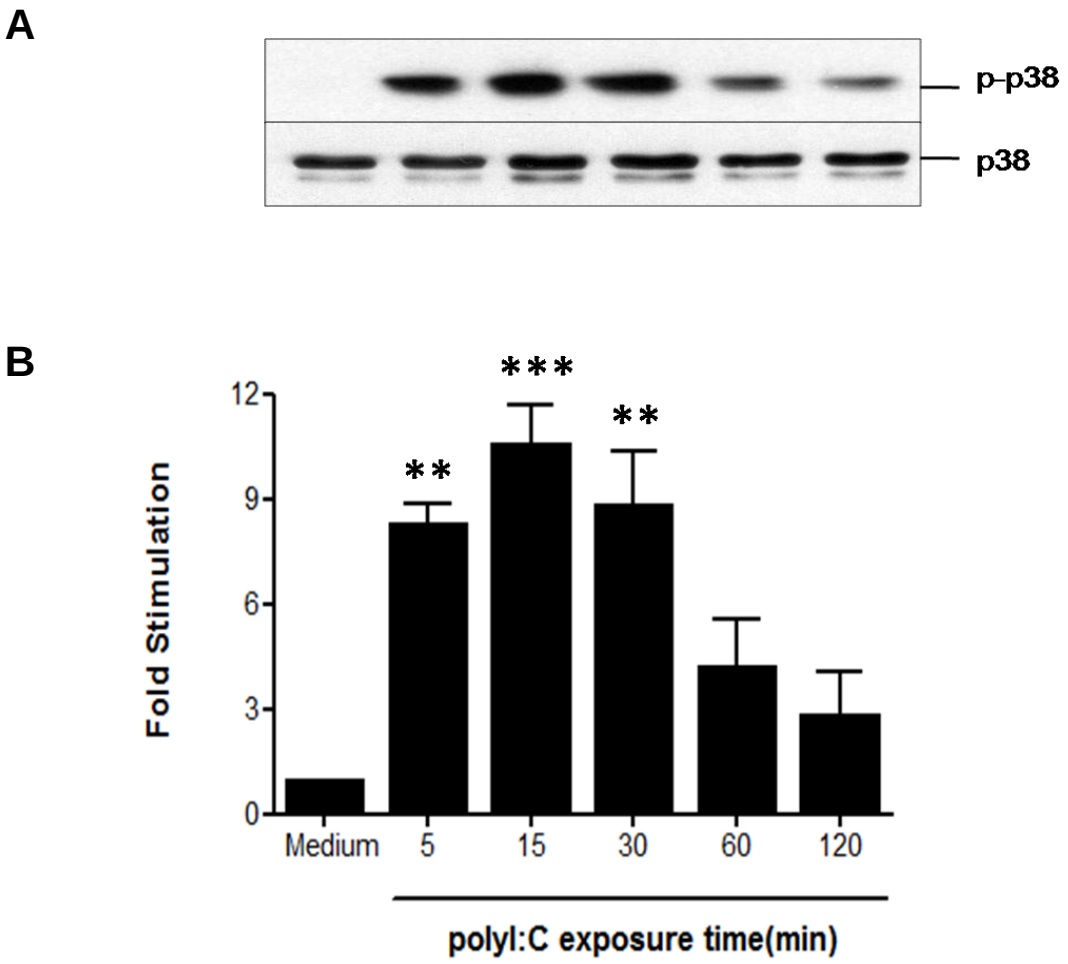


Figure 3.15. Assessment of polyI:C-stimulated p38 phosphorylation in BMD-macrophages. Cells were incubated in media or exposed to 25µg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and p38 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in Materials and Methods section. Results in panel A are representative of three independent experiments.The associated data were quantified by densitometry scanning and are depicted in panel B. Each value represents mean±SEM. **p<0.001 and ***p<0.0001 are statistically significant vs. control (Medium).

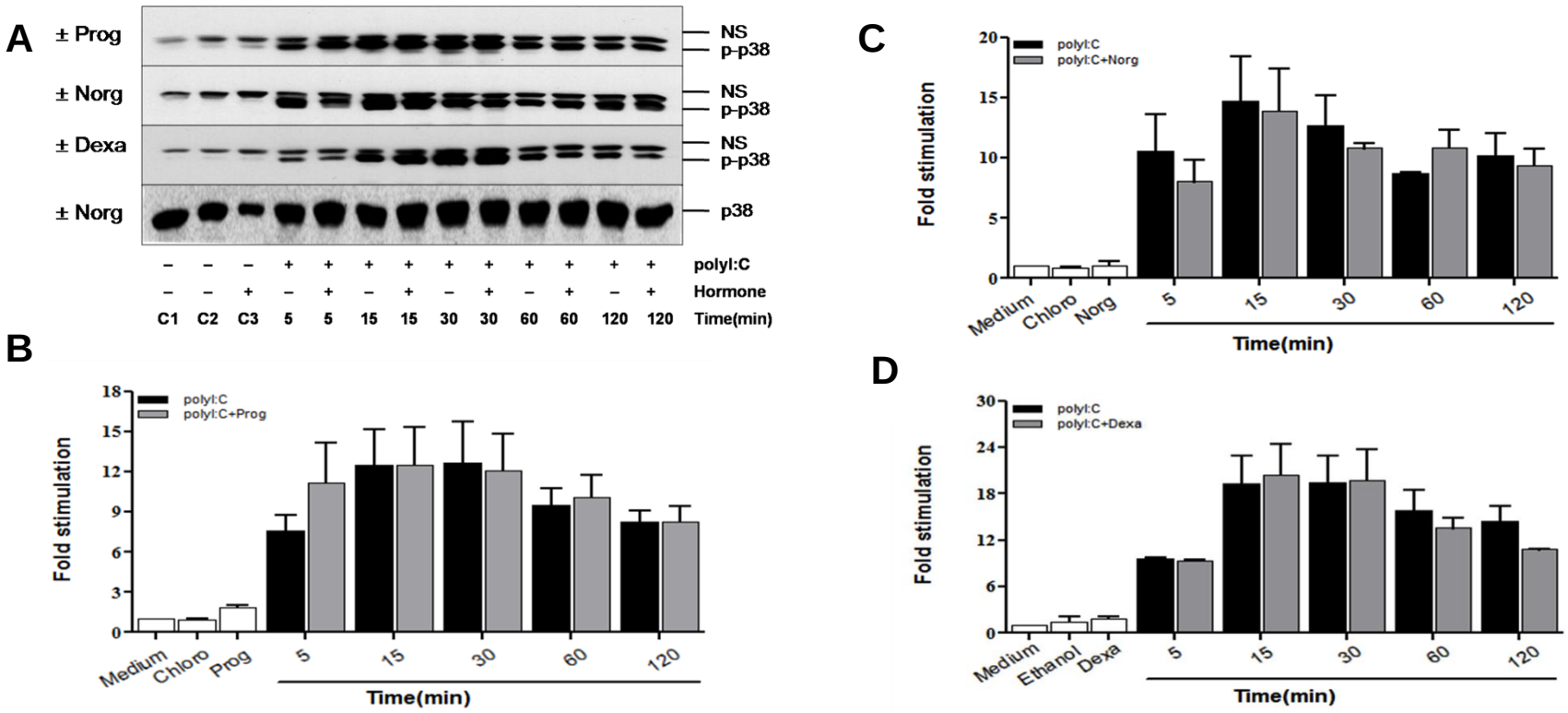


Figure 3.16. The effect of progesterone, norgestrel and dexamethasone on polyI:C-stimulated p38 phosphorylation in BMD-macrophages. BMD-macrophages were treated with vehicle or 62.5μM progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 25μg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and p38 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments.The associated data for experiments examining pretreatment with progesterone, norgestrel and dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3). C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa). NS, non specific band

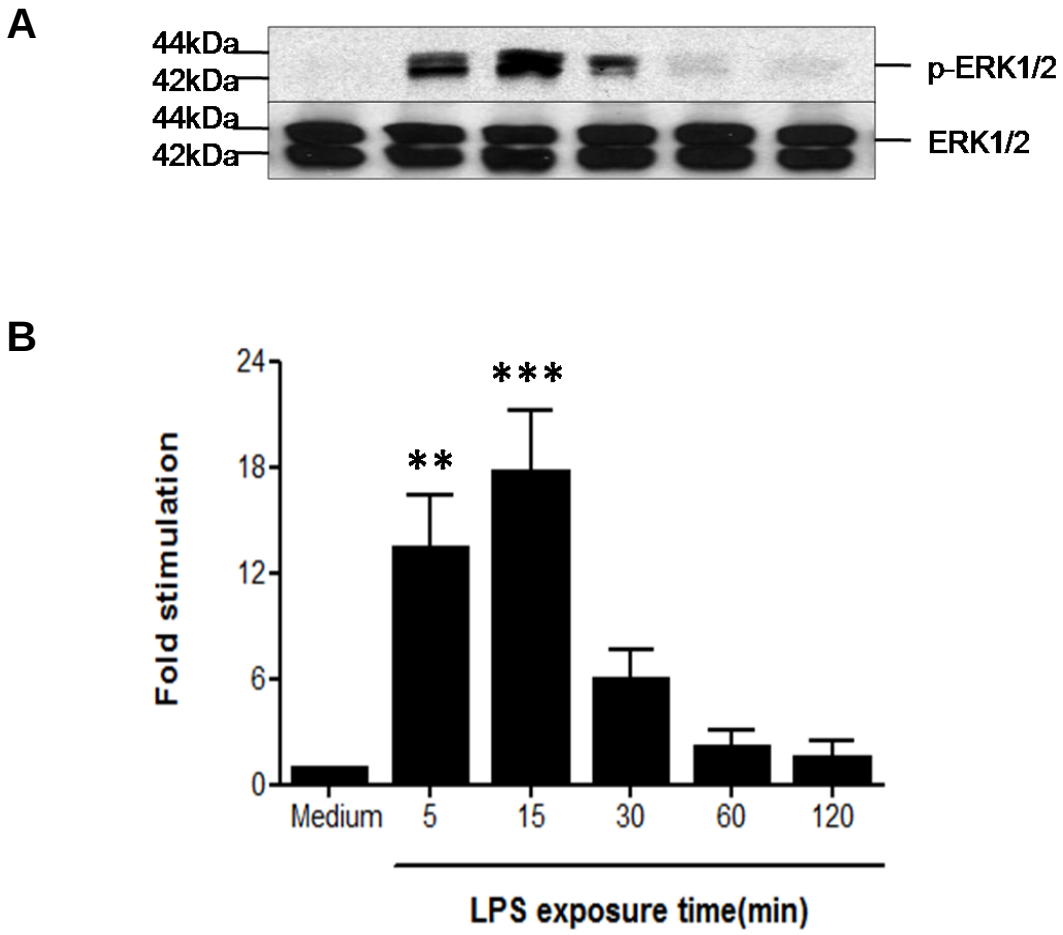


Figure 3.17. Assessment of LPS-stimulated ERK phosphorylation in BMD-macrophages. Cells were incubated in media or exposed to 1µg/ml LPS for the indicated times (min). Whole cell extracts were prepared and ERK phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data were quantified by densitometry scanning and are depicted in panel B. Each value represents mean±SEM. **p<0.001 and ***p<0.0001 are statistically significant vs. control (Medium).

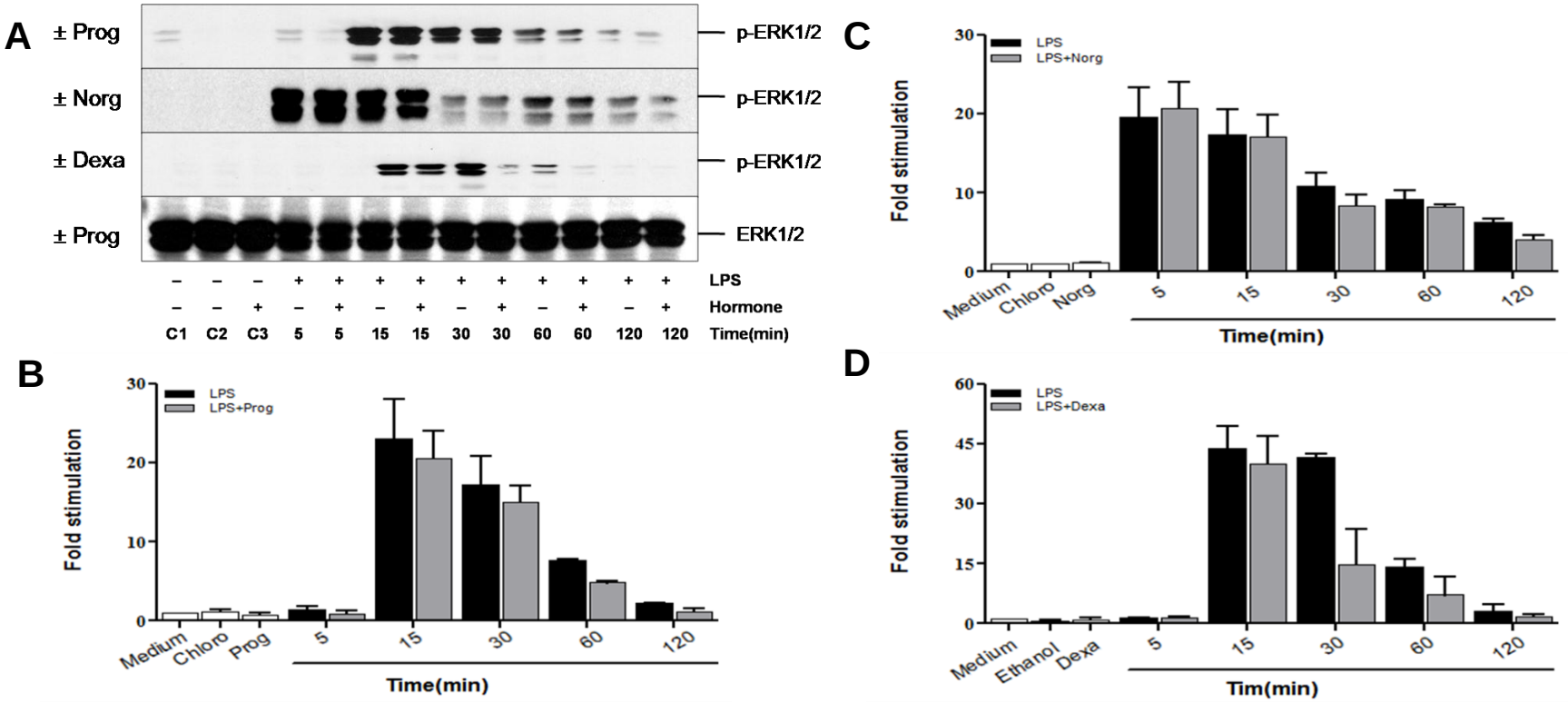


Figure 3.18. The effect of progesterone, norgestrel and dexamethasone on LPS-stimulated ERK phosporylation in BMD- macrophages. BMD-macrophages were treated with vehicle or 62.5μM progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 1μg/ml LPS for the indicated times (min). Whole cell extracts were prepared and ERK phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments.The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3).C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg or Dexa)

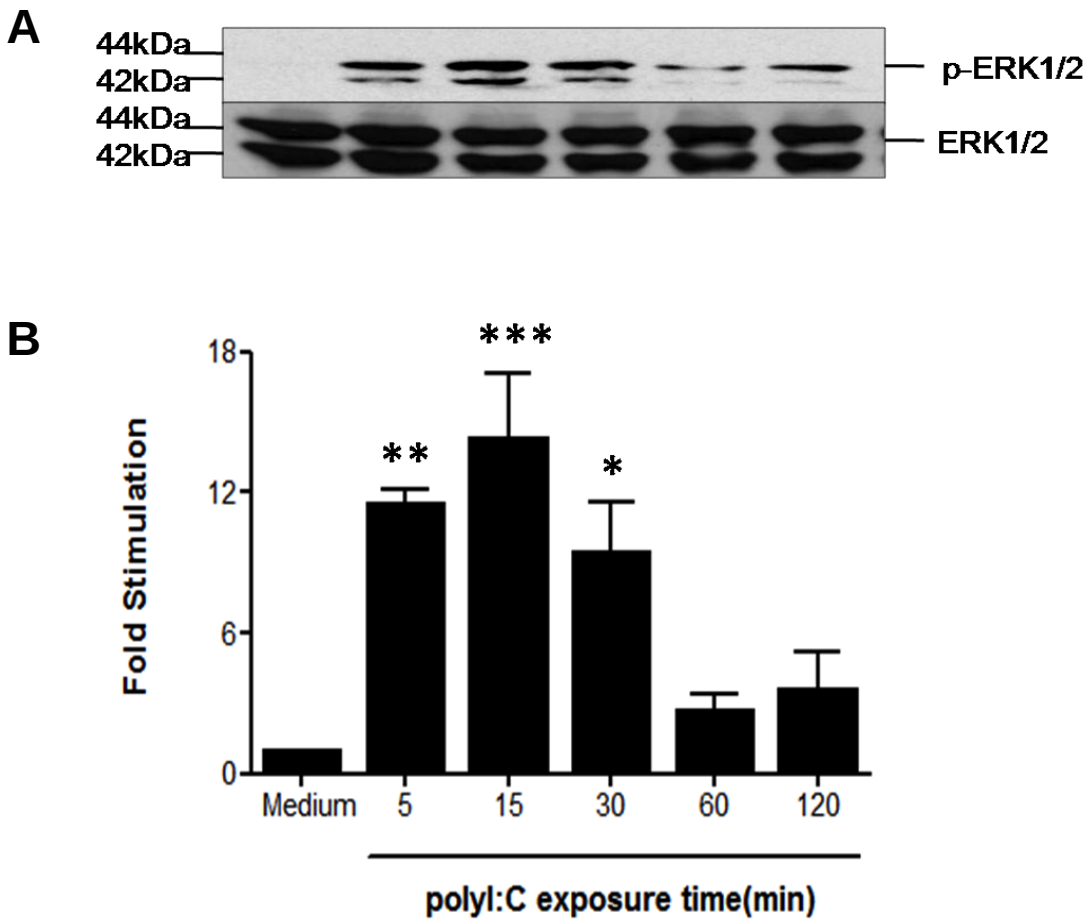


Figure 3.19. Assessment of polyI:C-stimulated ERK phosphorylation in BMD-macrophages. Cells were incubated in media or exposed to 25µg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and ERK phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in Materials and Methods section. Results in panel A are representative of three independent experiments.The associated data were quantified by densitometry scanning and are depicted in panel B. Each value represents mean±SEM.*p<0.05, **p<0.001 and ***p<0.0001 are statistically significant vs. control (Medium).

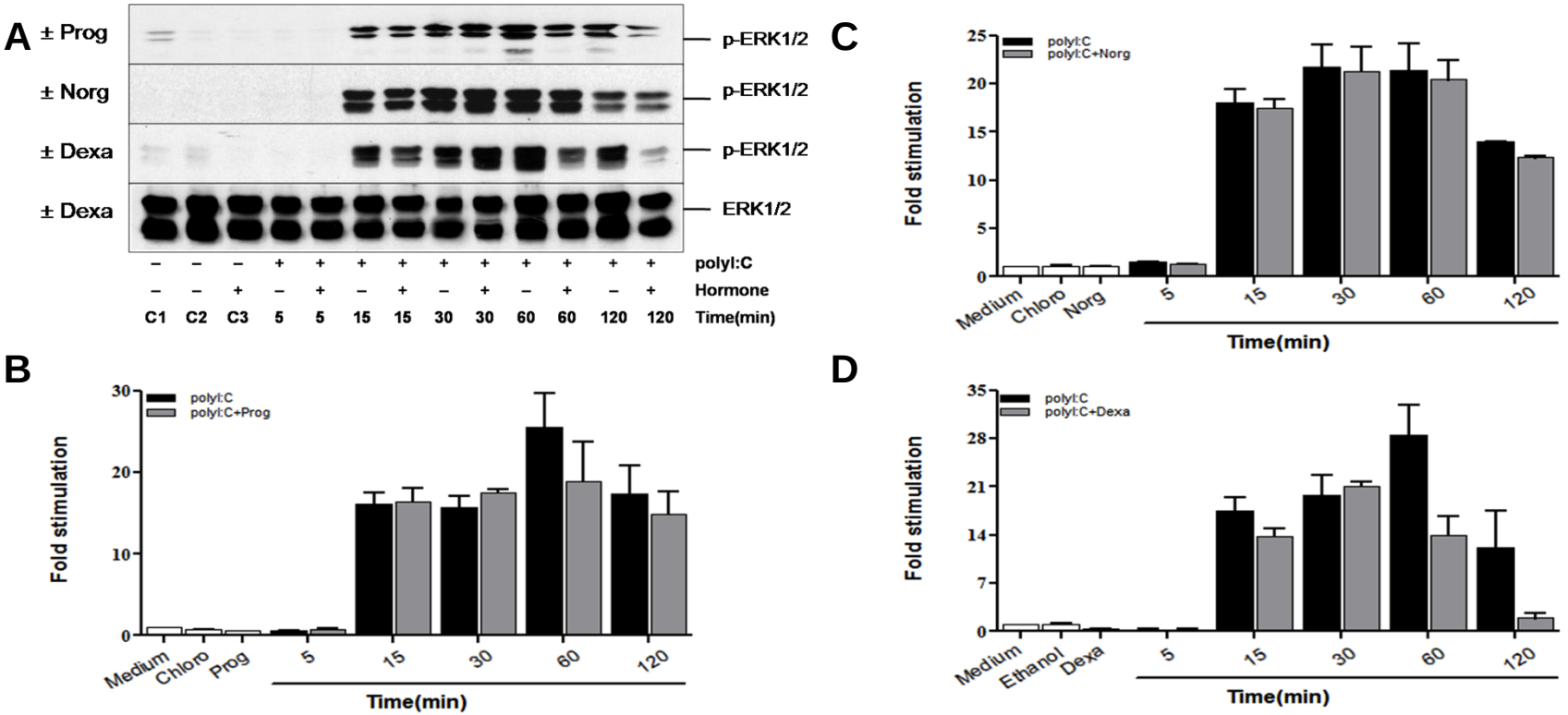


Figure 3.20. The effect of progesterone, norgestrel and dexamethasone on polyI:C-stimulated ERK phosphorylation in BMD-macrophages. BMD-macrophages were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 25μg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and ERK phosphorylation was assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3). C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg or Dexa).

3.2.3 Agonist stimulated-IRF3 phosphorylation and the potential effects of hormones in macrophages

In the TLR MyD88 independent pathway, which is restricted to TLR3 and TLR4, TRIF adaptor molecule activation leads to the phosphorylation of the transcription factor IRF3 which plays an essential role in IFN-β production (Kawai *et al*., 1999; Akira *et al*., 2001). To investigate this pathway in innate immune cells BMD-Macrophages were stimulated with 1µg/ml LPS as a TLR4 ligand or with 25µg/ml polyI:C as a TLR3 ligand. Different stimulation times were set starting from 30 minutes and ending after 8 hours. IRF3 phosphorylation was induced in macrophages approximately an hour post stimulation with LPS (Figure 3.21), and by 2 hours there was a significant difference compared with control cells (14.1725 ± 3.2230 fold stimulation, $P < 0.05$) which was maintained through 4 to 8 hours. For cells treated with poly I:C (Figure 3.23), IRF3 phosphorylation was intiated earlier than observed with LPS stimulation, was significant by 30 minutes and highly elevated levels were sustained from 2-8 hours post stimulation. IRF3 protein expression was used as a means for determining equal protein loading with both ligands. Pretreatment with progesterone, norgestrel or dexamethasone had no effect for the cells stimulated with LPS (Figure 3.22) nor with polyI:C (Figure 3.24).

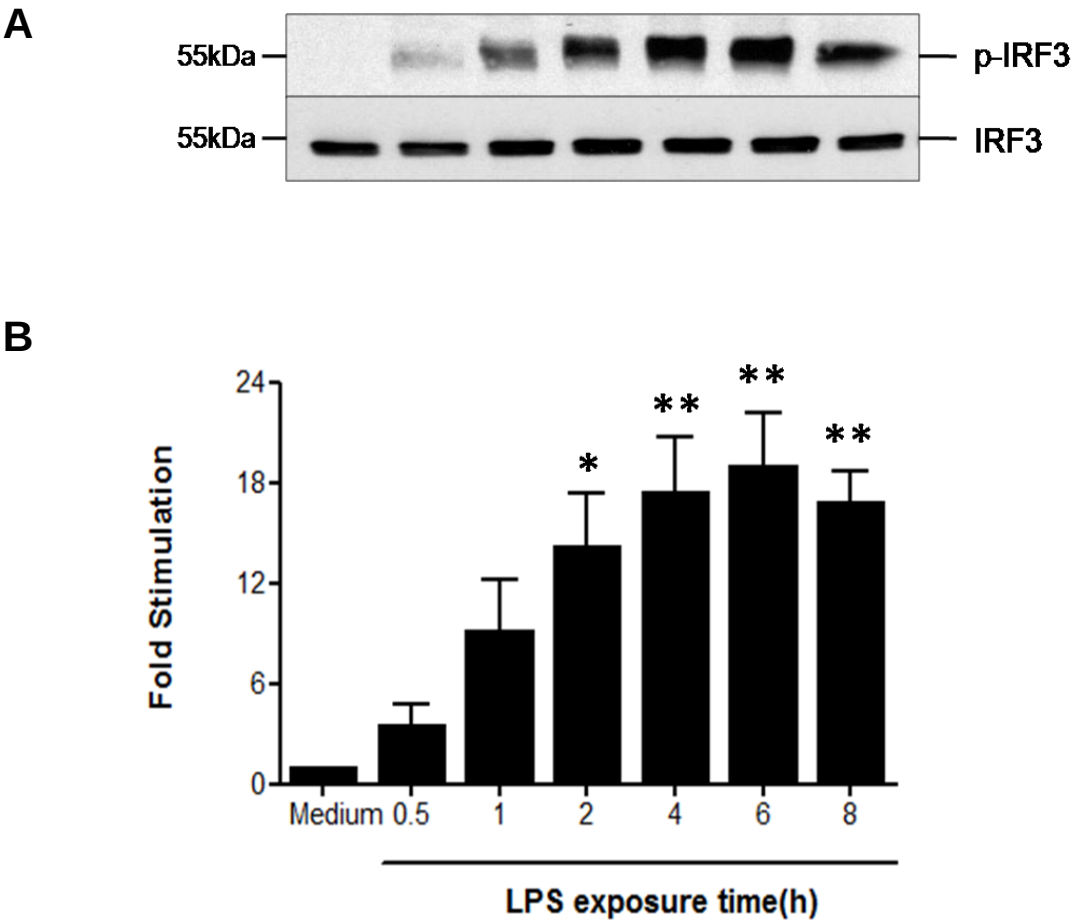


Figure 3.21. Assessment of LPS-stimulated IRF3 phosphorylation in BMD-macrophages. Cells were incubated in media or exposed to 1µg/ml LPS for the indicated times (h). Whole cell extracts were prepared and IRF3 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data were quantified by densitometry scanning and are depicted in panel B.Each value represents mean±SEM. *p<0.05 and **p<0.001 are statistically significant vs. control (Medium).

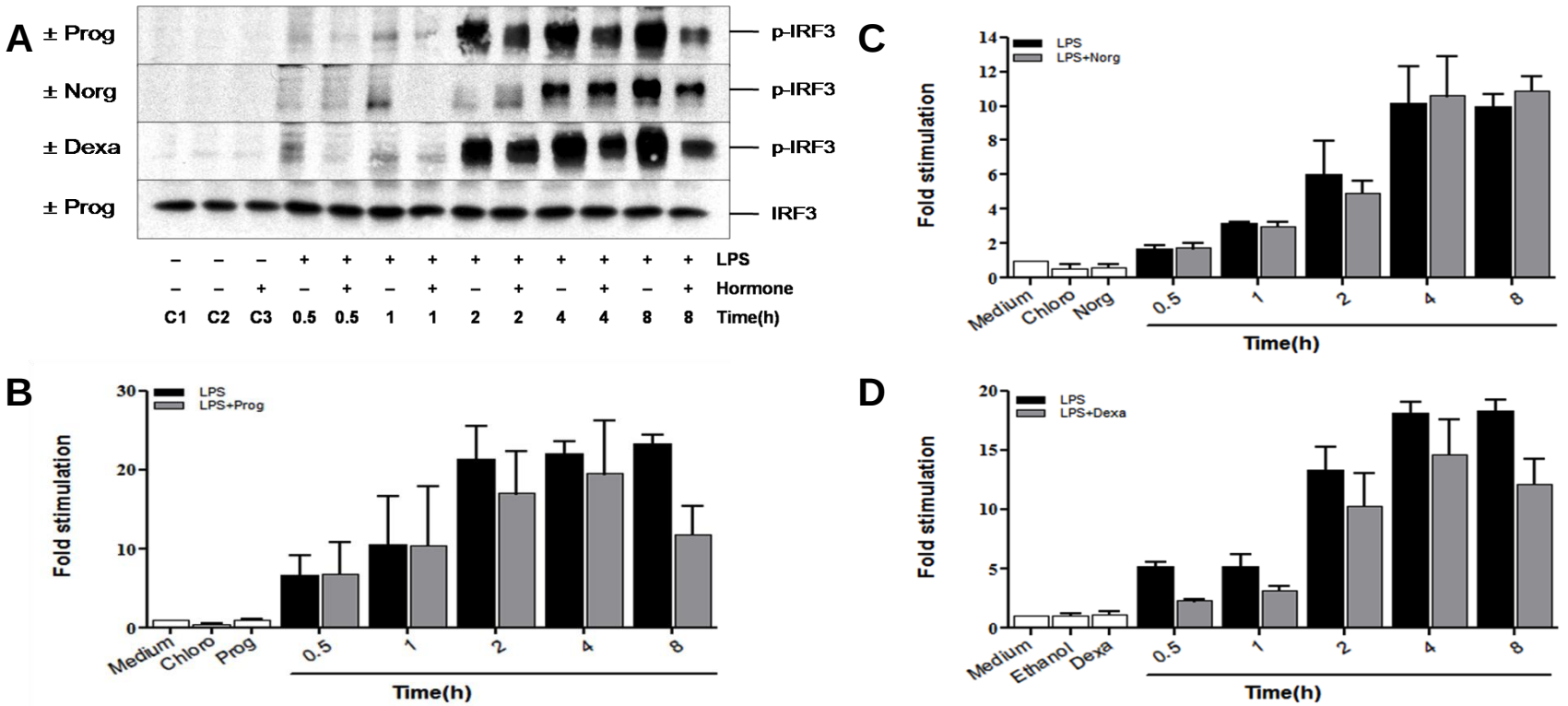


Figure 3.22. The effect of progesterone, norgestrel and dexamethasone on LPS-stimulated IRF3 phosporylation in BMD- macrophages. BMD-macrophages were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 1μg/ml LPS for the indicated times (h). Whole cell extracts were prepared and IRF3 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments and associated data for experiments examining pretreatment with progesterone, norgestrel ordexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3). C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

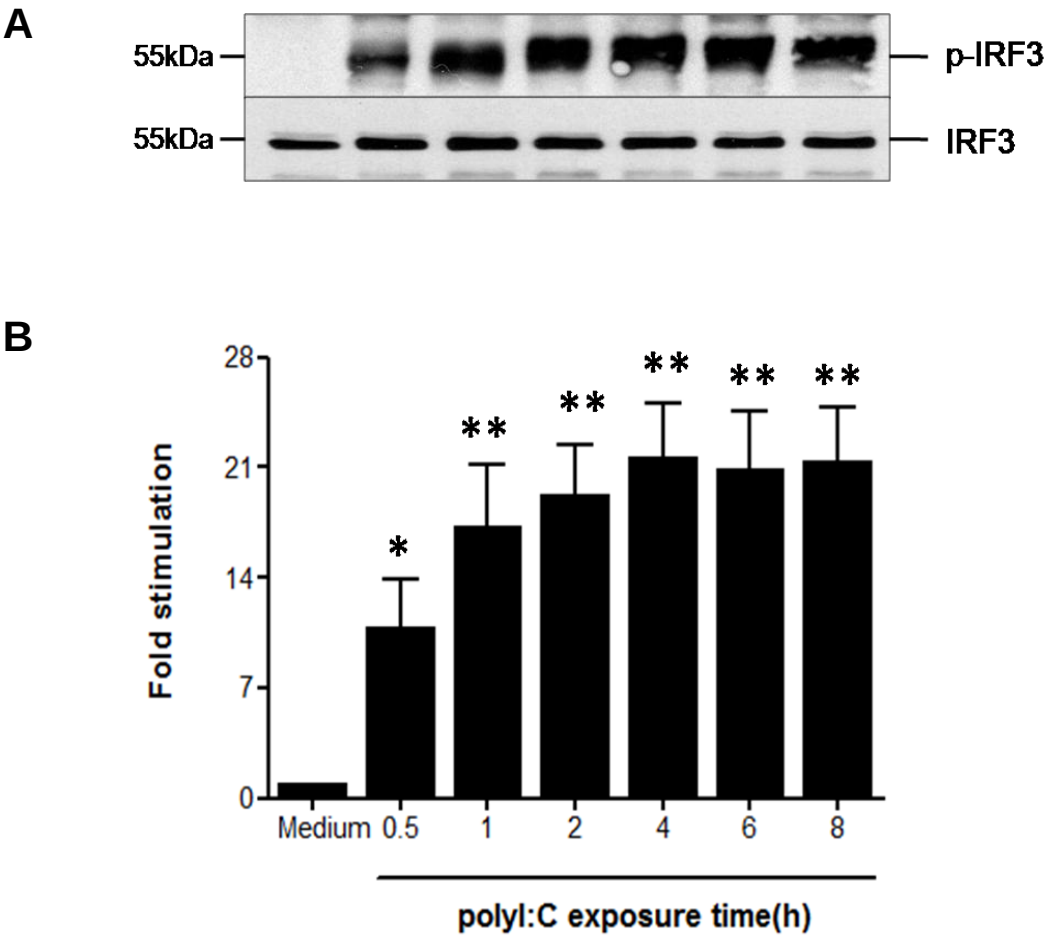


Figure 3.23. Assessment of polyI:C-stimulated IRF3 phosphorylation in BMD-macrophages. Cells were incubated in media or exposed to 25µg/ml polyI:C for the indicated times (h). Whole cell extracts were prepared and IRF3 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data were quantified by densitometry scanning and are depicted in panel B. Each value represents mean±SEM. *p<0.05 and **p<0.001 are statistically significant vs. control (Medium).

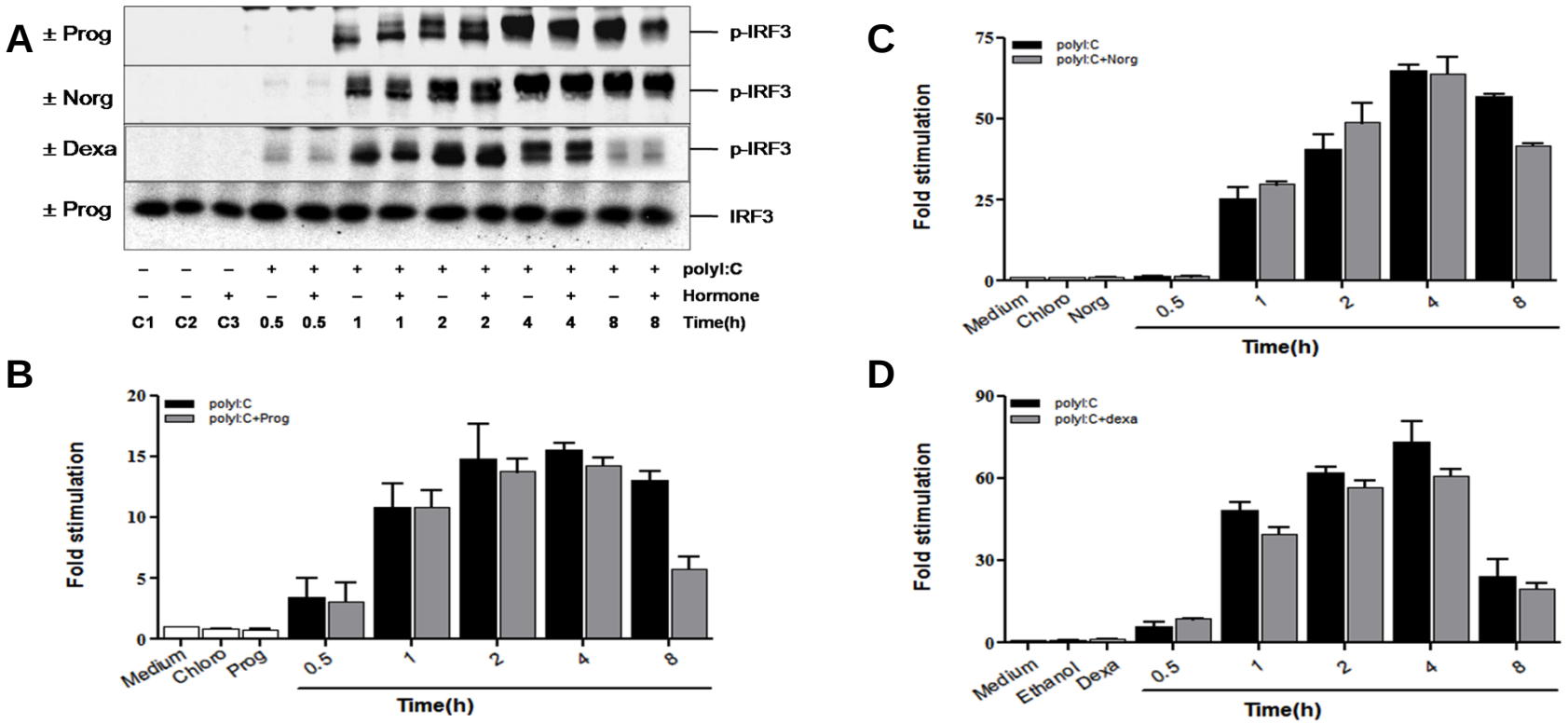


Figure 3.24. The effect of progesterone, norgestrel and dexamethasone on polyI:C-stimulated IRF3 phosphorylation in BMD-macrophages. BMD-macrophages were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 25μg/ml polyI:C for the indicated times (h). Whole cell extracts were prepared and IRF3 phosphorylation was assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments.The associated data for experiments examining pretreatment with progesterone, norgestrel and dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3). C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

Dendritic cells

Given that DCs are major APCs linking the innate and adaptive immune systems and also share a role with macrophages as being part of the first line of defense against pathogens, the current study has been extended to investigate the potential immune-modulatory roles of progesterone on inflammatory signalling in these cells. Consequently, BMDCs were treated either with 1µg/ml LPS or 25µg/ml polyI:C, as TLR4 and TLR3 ligands respectively and were studied to establish appropriate experimental conditions to then allow studies with hormones. Cells were then pretreated with 62.5µM of progesterone, 62.5µM of norgestrel or 62.5nM of dexamethasone, then exposed either to LPS or polyI:C for the selected time points identified to study the potential effects of the hormones and whether these effects would be consistent with observation made in the macrophage cell setting.

3.2.4 The NFκB signalling pathway

3.2.4.1 Agonist stimulated p65 phosphorylation and the potential effects of hormones in BMDCs

BMDCs exposed to LPS alone displayed rapid p65 phosphorylation (within 5 minutes) that was sustained up to 2 hours (data not shown). Pretreatment of LPS stimulated BMDCs with either progesterone, norgestrel or dexamethasone did not influence LPS-induced p65 phosphorylation in these cells (Figure 3.25). For cells exposed to polyI:C alone, the p65 phosphorylation pattern was slightly different from that in cells exposed to LPS, however, it also started rapidly (within 5 minutes), it continued to increase up to 2 hours post- stimulation (data not shown) Pretreatment with the hormones also had no influence on polyI:C induced p65 phosphorylation (Figure 3.26). p65 protein expression was used for determining equal protein loading.

3.2.4.2 Agonist stimulated-IκBα degradation and the potential effects of hormones in BMDCs

In order to expand the investigation into NFκB signalling, assessment of agoinst-stimulated IκBα degradation was performed. BMDCs treated with LPS alone showed rapid IκBα degradation which was identified within 5 minutes post-stimulation. IκBα expression was restored gradually after 30 minutes to reach normal levels after 120 minutes (data not shown). Pretreatment with the steroid hormones progesterone, norgestrel or dexamethasone had no influence on LPS induced IκBα degradation (Figure 3.27) except a slight inhibition exerted by the three hormones after 120 minutes which was not significant. Cells treated with polyI:C alone showed IκBα degradation with a kinetic profile similar to that observed in cells exposed to LPS . Results showed a rapid IκBα degradation, within 5 minutes which was restored gradually after 30 minutes to reach normal control levels after 2 hours (data not shown). Pre-treatment with hormones had no influence on polyI:C induced IκBα degradation (figure 3.28). p65 protein expression was used for determining equal protein loading with both ligands.

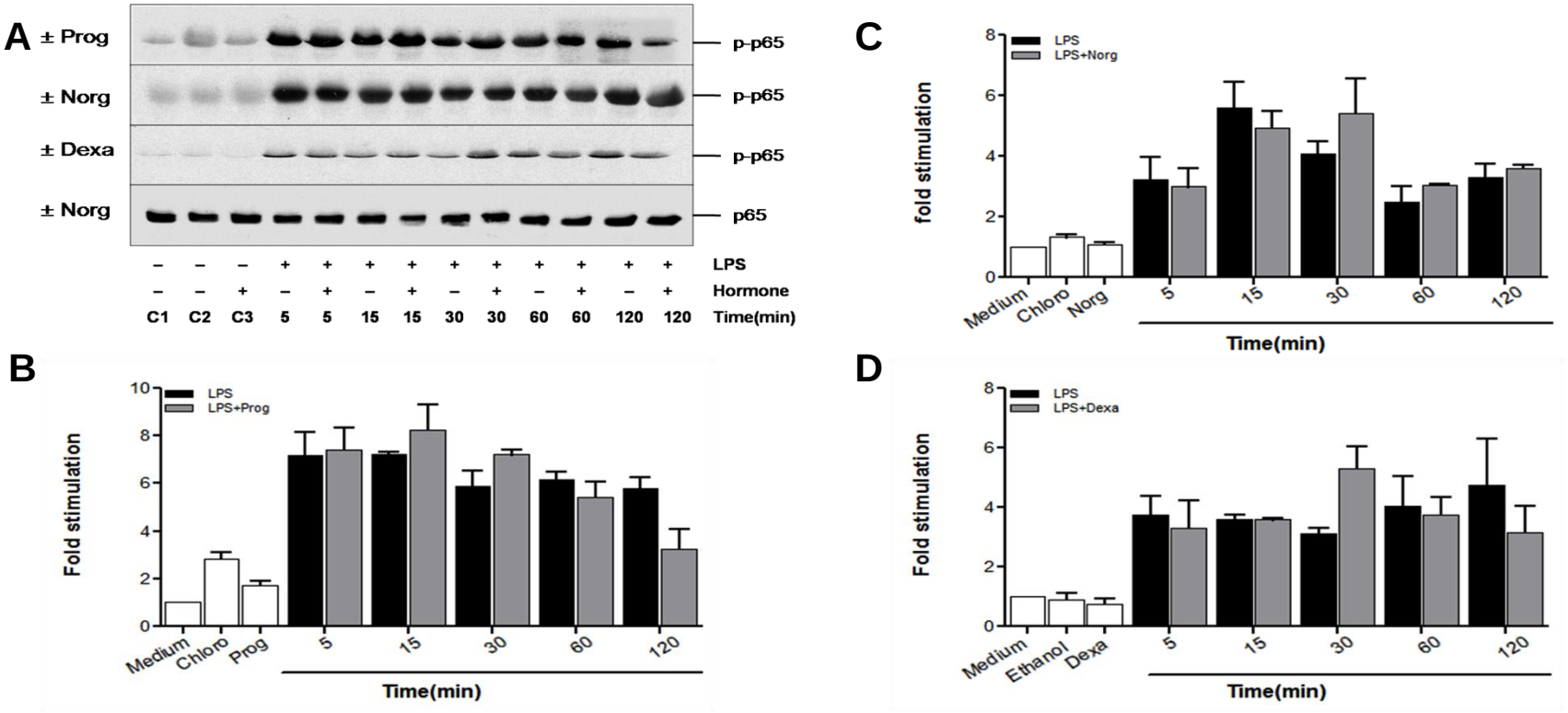


Figure 3.25. The effect of progesterone, norgestrel and dexamethasone on LPS–stimulated p65 phosphorylation in BMDCs. BMDCs were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 1μg/ml LPS for the indicated times (min). Whole cell extracts were prepared and p65 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments.The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3).C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

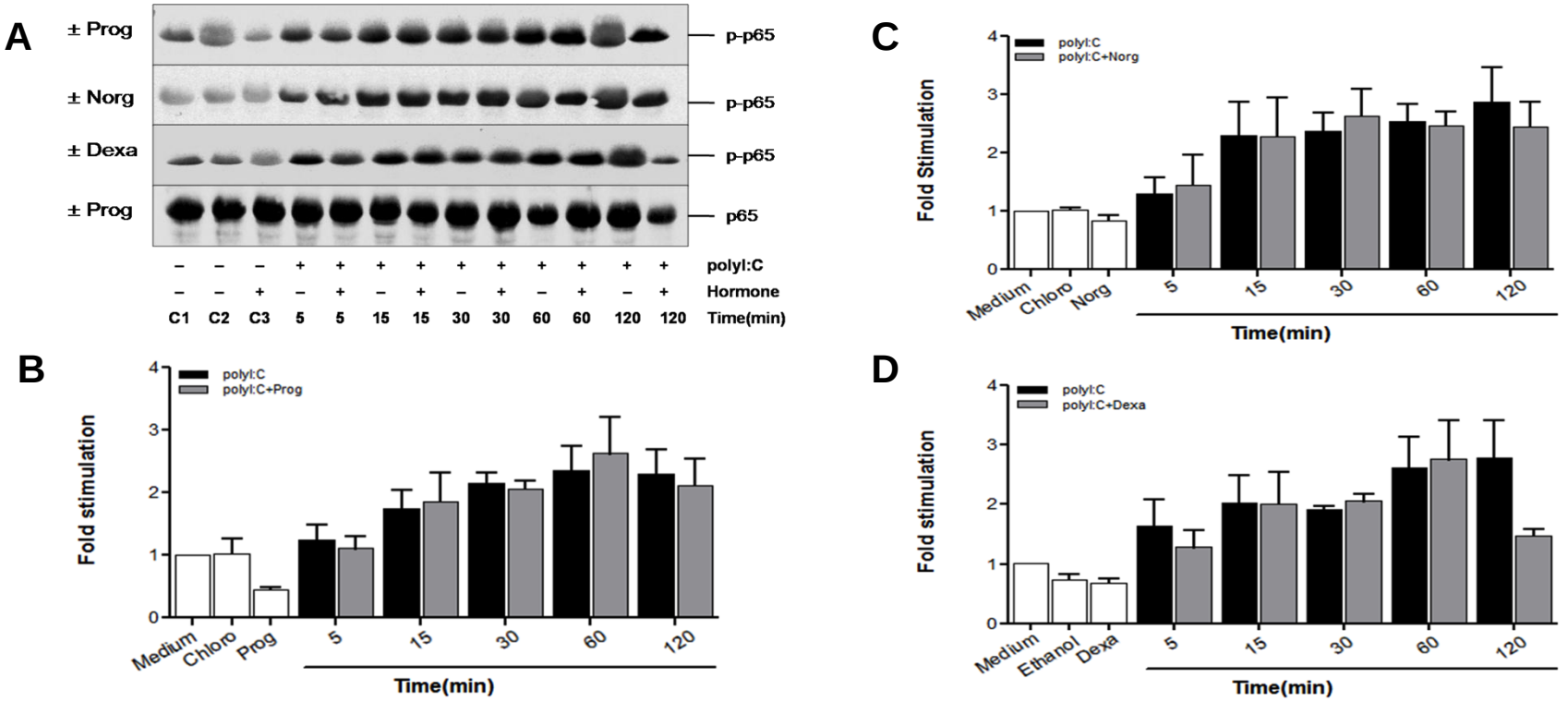


Figure 3.26. The effect of progesterone, norgestrel and dexamethasone on polyI:C-stimulated p65 phosphorylation in BMDCs. BMDCs were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 25μg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and p65 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3).C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

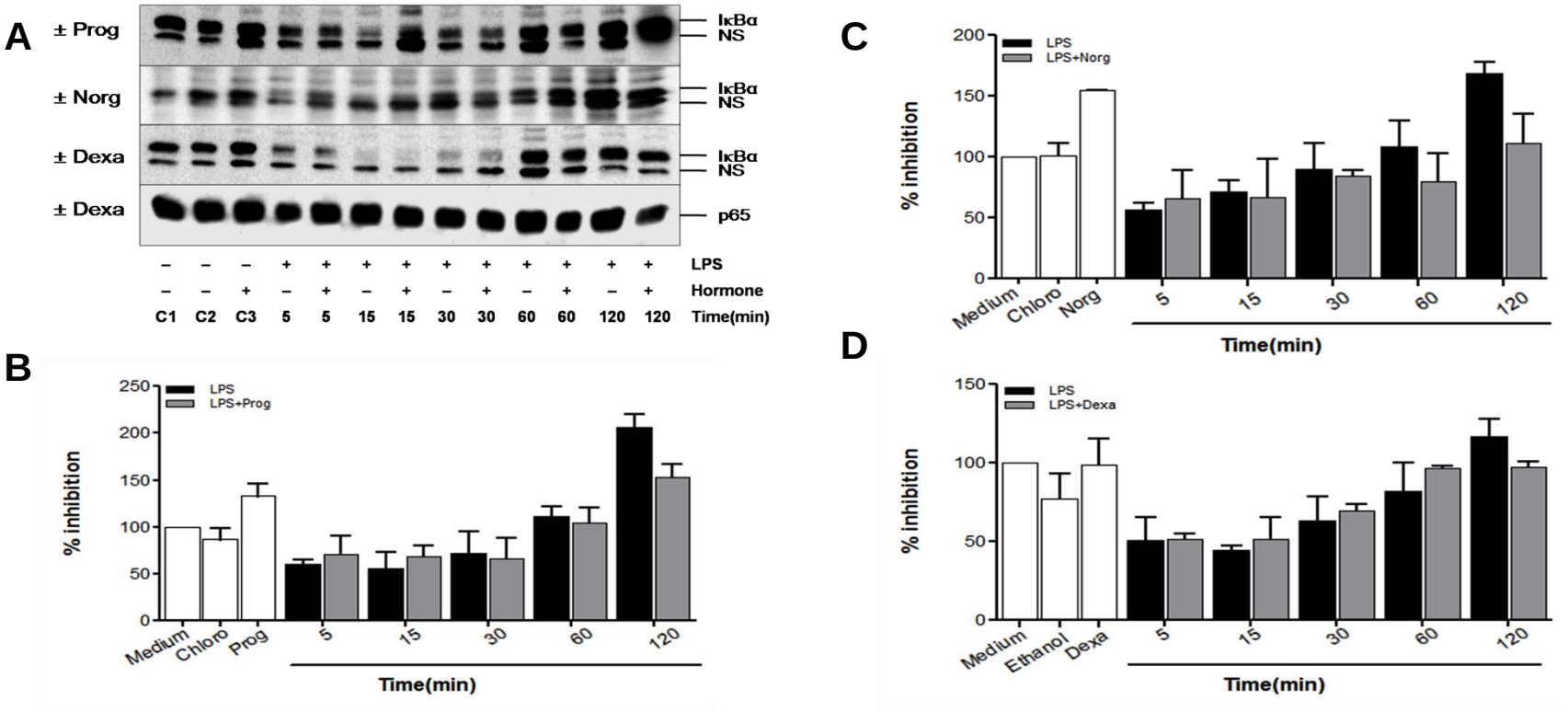


Figure 3.27. The effect of progesterone, norgestrel and dexamethasone on LPS-stimulated IκBα degradation in BMDCs. BMDCs were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 1μg/ml LPS for the indicated times (min). Whole cell extracts were prepared and IκBα degradation was assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. p65 expression was used as protein loading control.Results in panel A are representative of three independent experiments.The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and Drespectively (n=3).C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa), NS,non specific band.

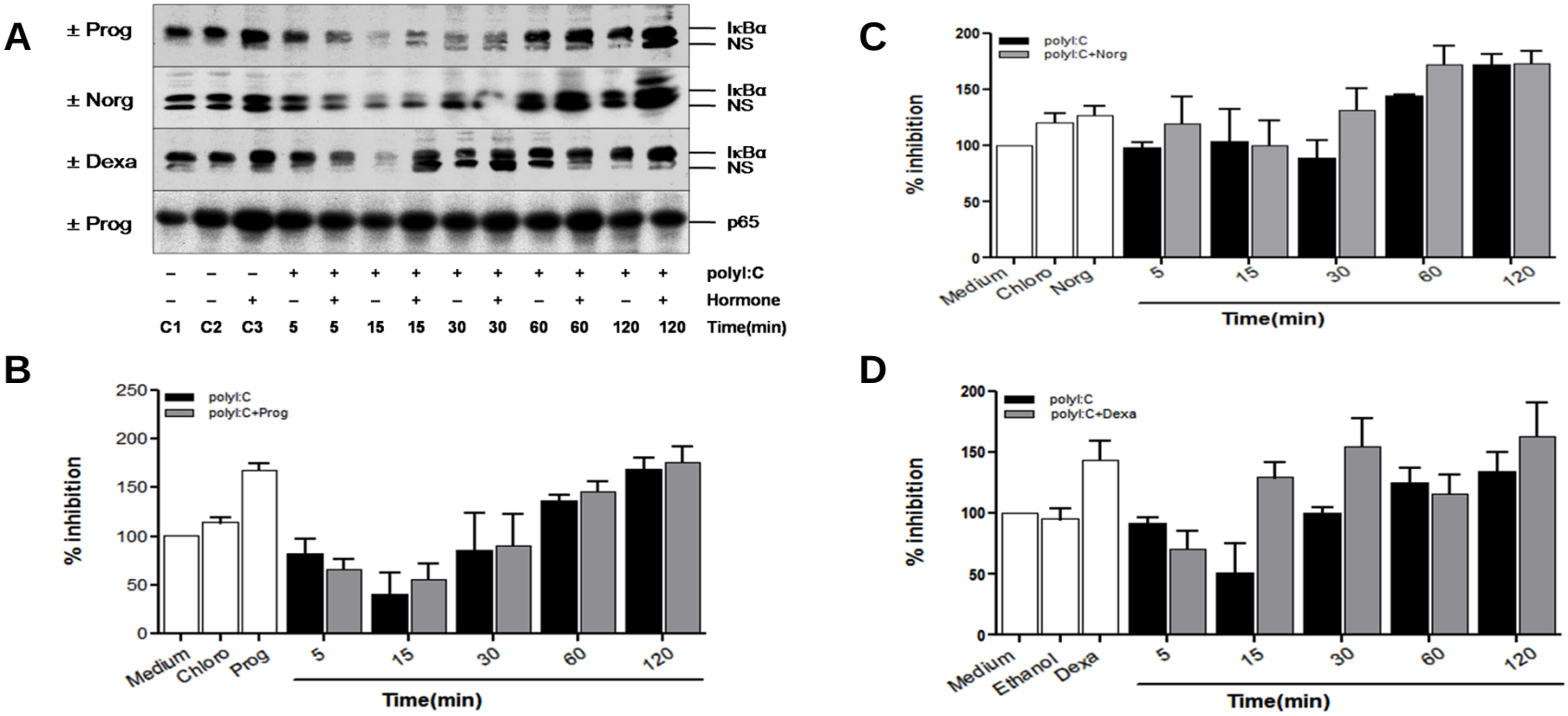


Figure 3.28. The effect of progesterone, norgestrel and dexamethasone on polyI:C-stimulated IκBα degradation in BMDCs. BMDCs were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 25μg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and IκBα degradation was assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section, p65 expression was used as protein loading control. Results in panel A representative of three independent experiments and associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3).C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa), NS,non specific band.

3.2.5 The MAPKs signalling.pathways

BMDCs were treated in a manner similar to previous experiments to assess JNK, p38 and ERK protein expression and phosphorylation induced by LPS or polyI:C and to study also the potential effects of proesterone, norgesterone or dexamethasone on these proteins following TLR -ligation.

3.2.5.1 Agonist-stimulated JNK phosphorylation and the potential effects of hormones in BMDCs

BMDCs exposed to LPS alone induced rapid JNK phosphorylation, this was observed within 5 minutes, which was maximal by 15-30 minutes and declined sharply after 60 minutes (data not shown). Pretreatment with hormones had no influence on LPS- induced JNK phosphorylation (Figure 3. 29). PolyI:C treated cells showed a slight delay in JNK phosphorylaion in compared with LPS treated cells. PolyI:C induced JNK phosphorylation within 15 minutes post stimulation, which was maximal by 30 minutes and declined gradually after 60 minutes (data not shown). Pretreatment of cells with hormones had no effect on polyI:C induced JNK phosphorylation (Figure 3.30). JNK protein expression was used for determining equal protein loading with both ligands.

3.2.5.2 Agonist-stimulated p38 phosphorylation and the potential effects of hormones in BMDCs

BMDCs exposed to LPS alone induced rapid BMDC p38 phosphorylation; this was observed after 5 minutes stimulation and sustained up to 120 minutes (data not shown). Pretreating the BMDC with the hormones did not influence LPS induced p38 phosphorylation (Figure 3.31). PolyI:C treatment alone induced p38 phosphorylation in BMDC 15 minute post stimulation and this was sustained up to 120 minutes (data not shown). Pretreatment with hormones had no influence on polyI:C induced p38 phosphorylation (Figure 3.32). p38 protein expression was used for determining equal protein loading.

3.2.5.3 Agonist-stimulated ERK phosphorylation and the potential effects of hormones in BMDCs

BMDCs exposed to LPS alone induced rapid ERK phosphorylation, this was observed within 5 minutes, was maximal between 5-15 minutes and declined gradually until 120 minutes post-stimulation (data not shown). Pretreatment with hormones had no influence on LPS induced ERK phosphorylation (Figure 3. 33). Cells treated with polyI:C showed a slight delay ERK phosphorylation compared with LPS treated cells. polyI:C induced BMDC ERK phosphorylation by 15 minutes post stimulation and this declined gradually after 30 minutes (data not shown). In cells pretreating with hormones there was no influence of the hormones on polyI:C induced ERK phosphorylation (Figure 3.34). ERK protein expression was used for determining of equal protein loading with both ligands.

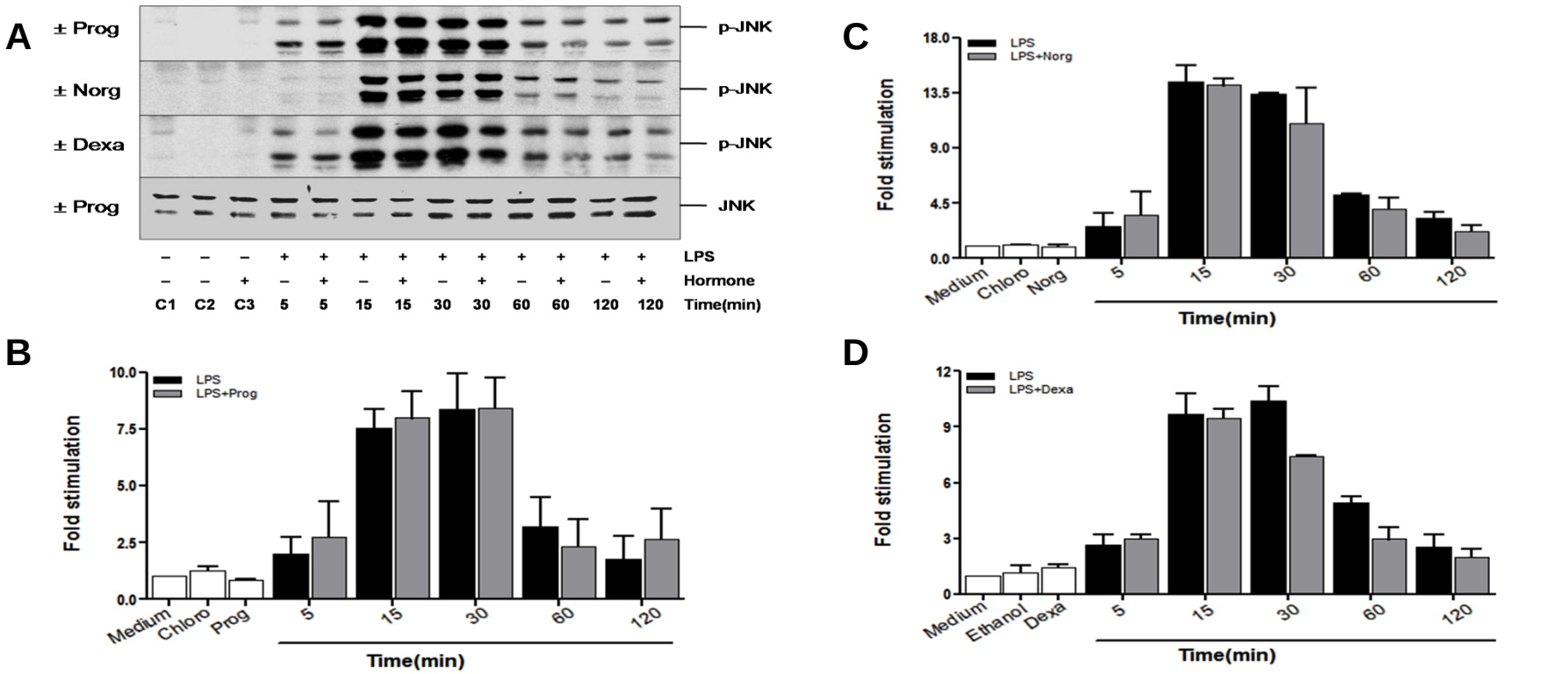


Figure 3.29. The effect of progesterone, norgestrel and dexamethasone on LPS –stimulated JNK phosphorylation in BMDCs. BMDCs were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 1μg/ml LPS for the indicated times (min). Whole cell extracts were prepared and JNK phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments.The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3).C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

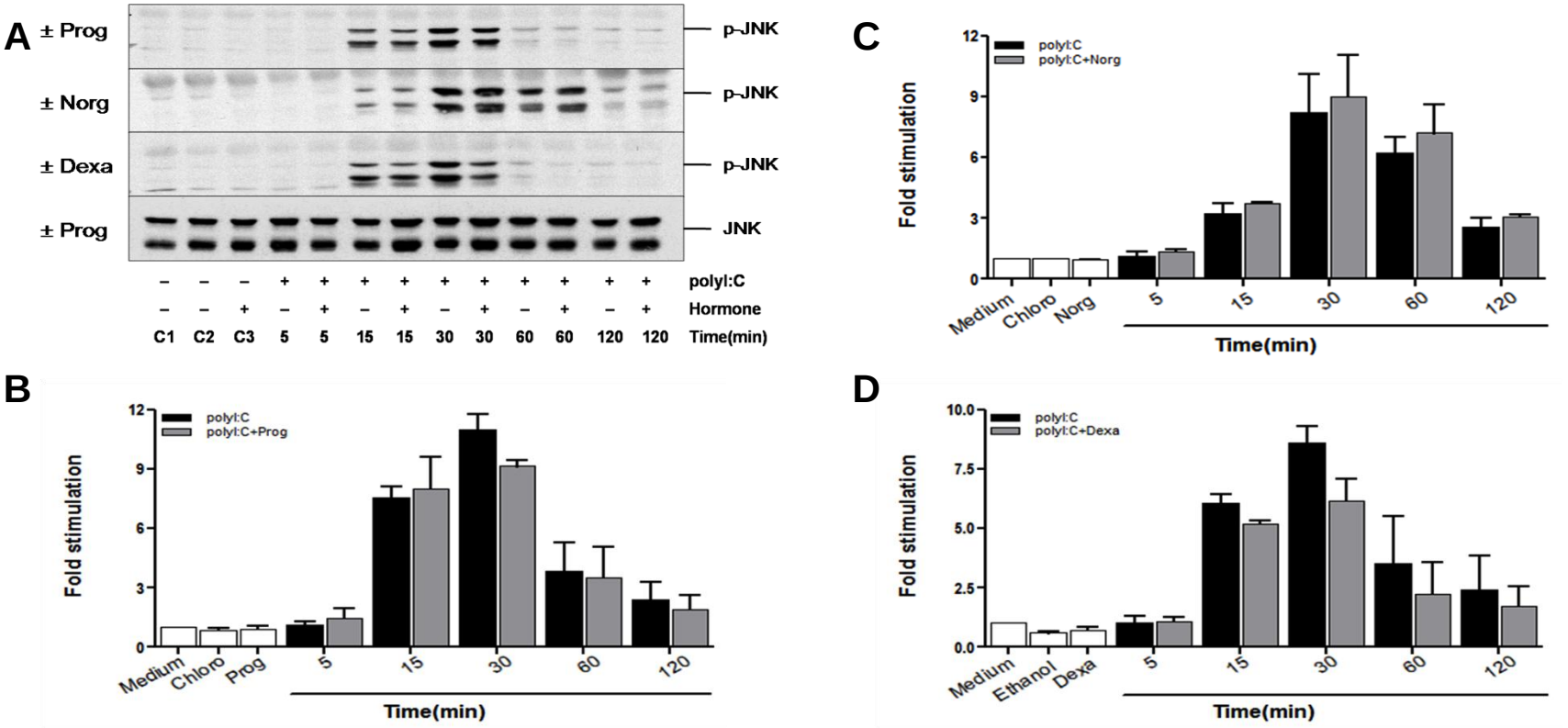


Figure 3.30. The effect of progesterone, norgestrel and dexamethasone on polyI:C-stimulated JNK phosphorylation in BMDCs. BMDCs were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to25μg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and JNK phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments.The associated data for experiments examining pretreatment with progesterone, norgestrel ordexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3).C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

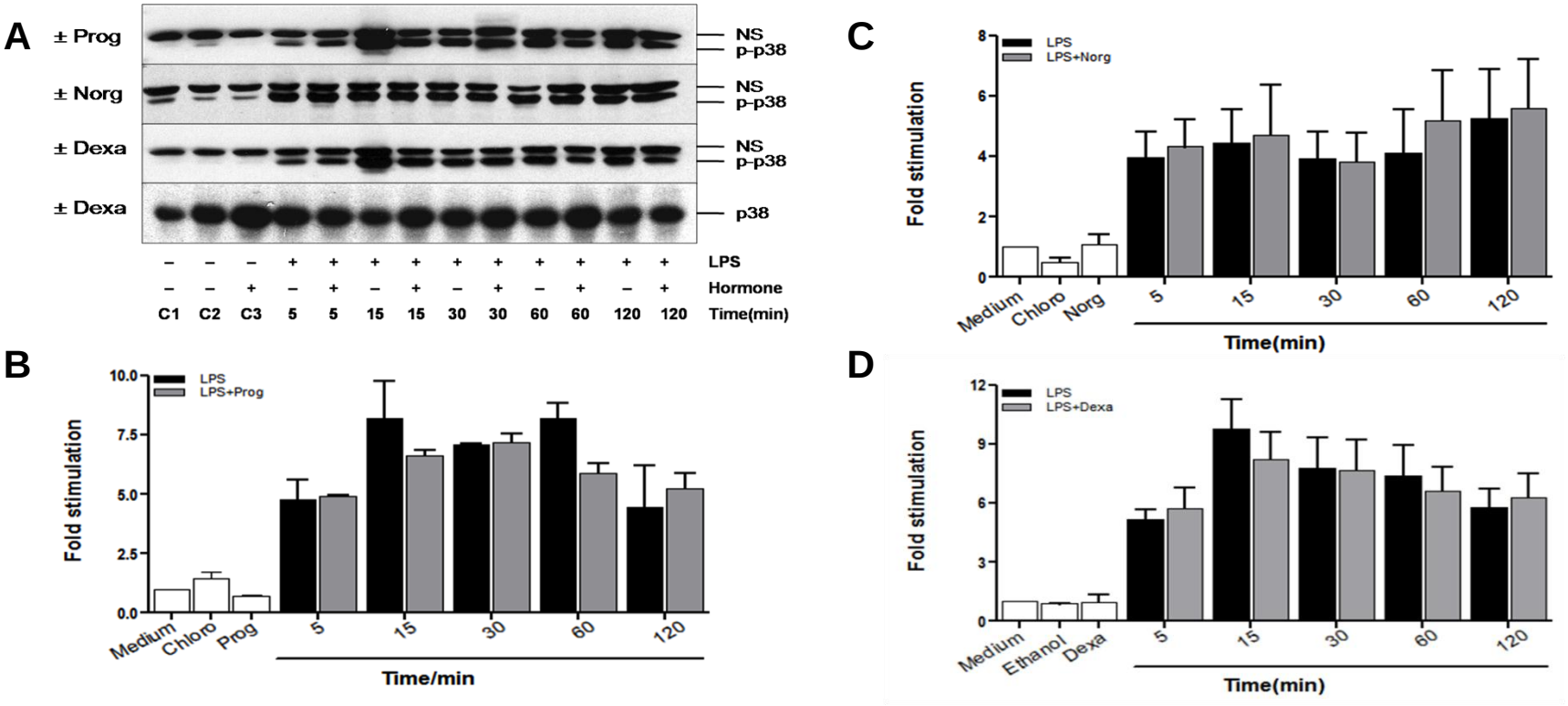


Figure 3.31. The effect of progesterone, norgestrel and dexamethasone on LPS –stimulated p38 phosphorylation in BMDCs. BMDCs were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 1μg/ml LPS for the indicated times (min). Whole cell extracts were prepared and p38 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments.The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3).C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa). NS, non specific band.

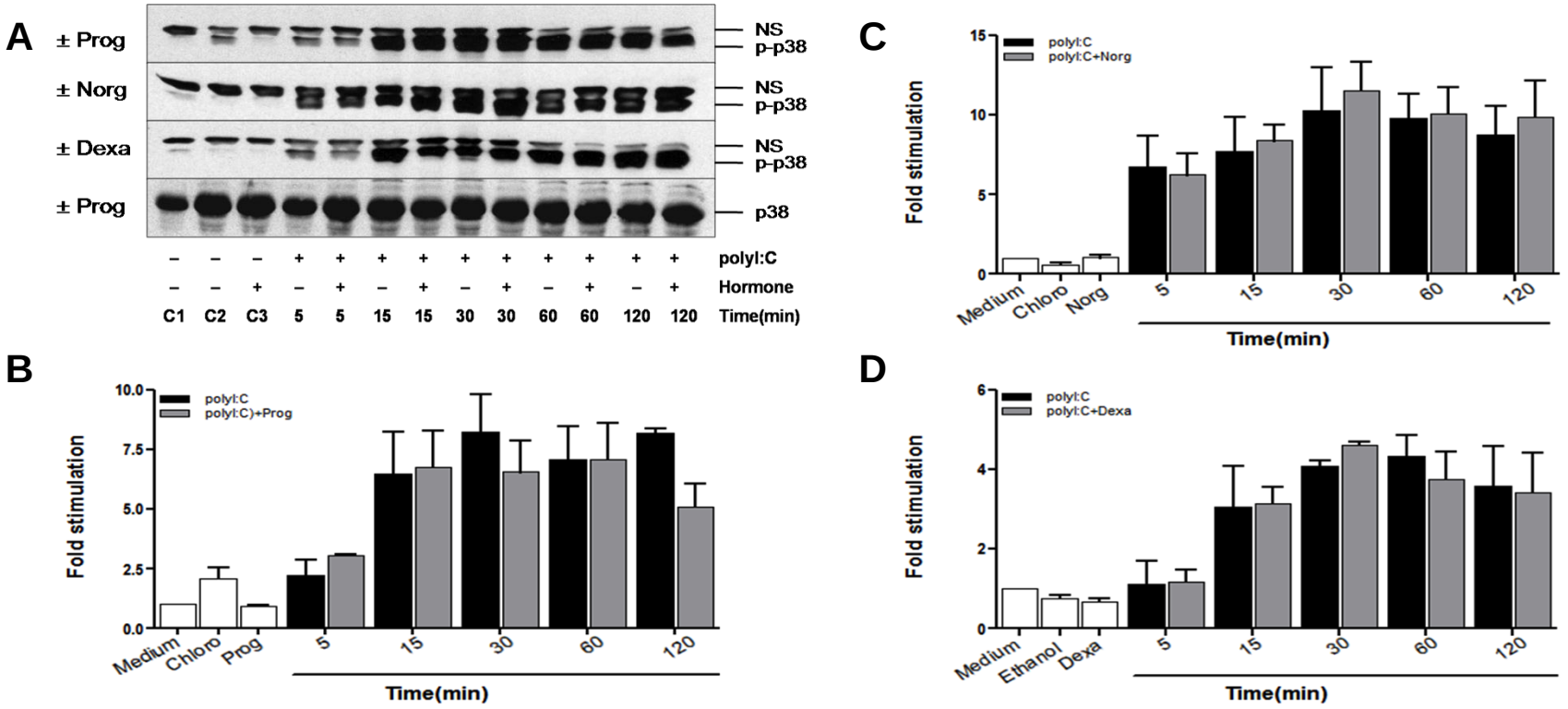


Figure 3.32. The effect of progesterone, norgestrel and dexamethasone on polyI:C-stimulated p38 phosphorylation in BMDCs. BMDCs were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 25μg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and p38 phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. . Results in panel A are representative of three independent experiments. The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3).C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa). NS, non specific band.

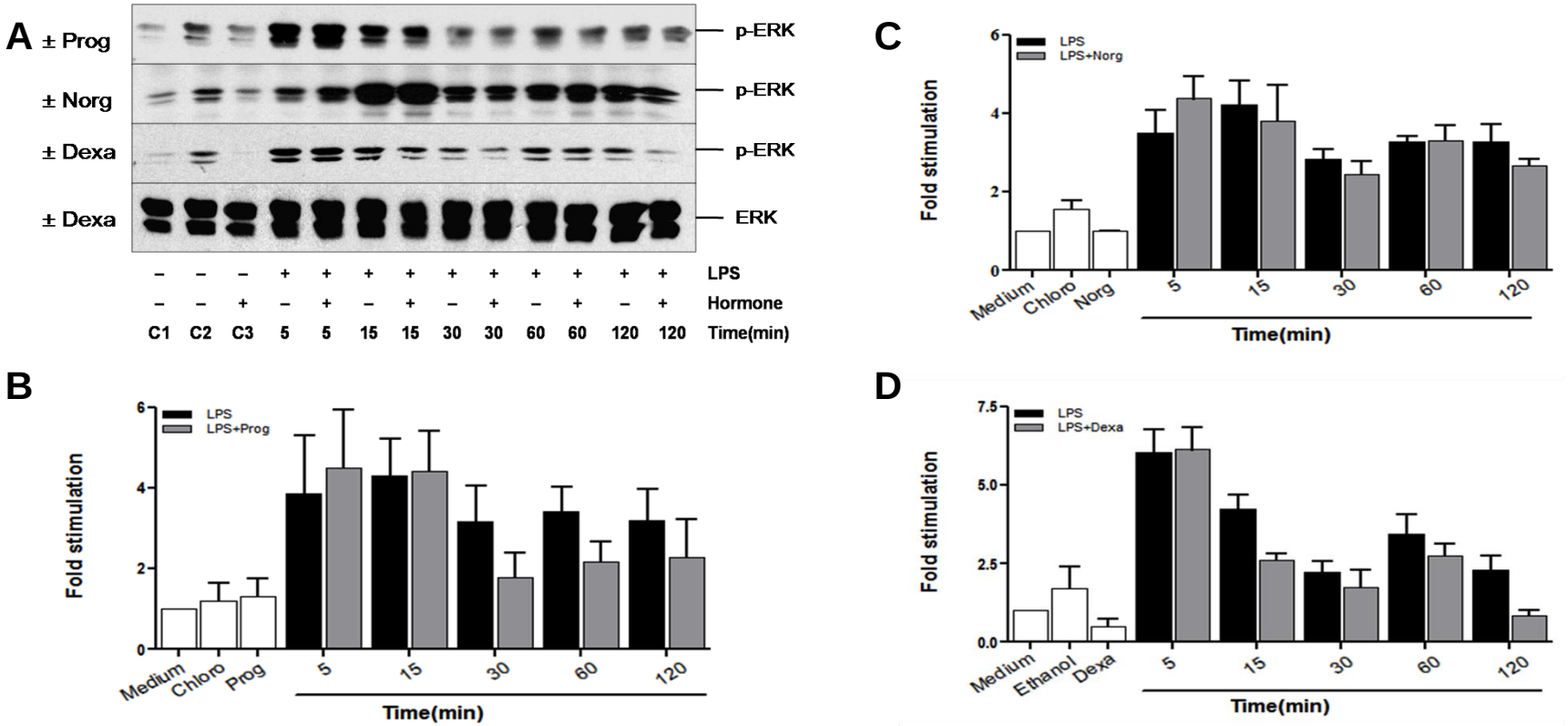


Figure 3.33. The effect of progesterone, norgestrel and dexamethasone on LPS –stimulated ERK phosphorylation in BMDCs. BMDCs were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 1μg/ml LPS for the indicated times (min). Whole cell extracts were prepared and ERK phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments.The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3).C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

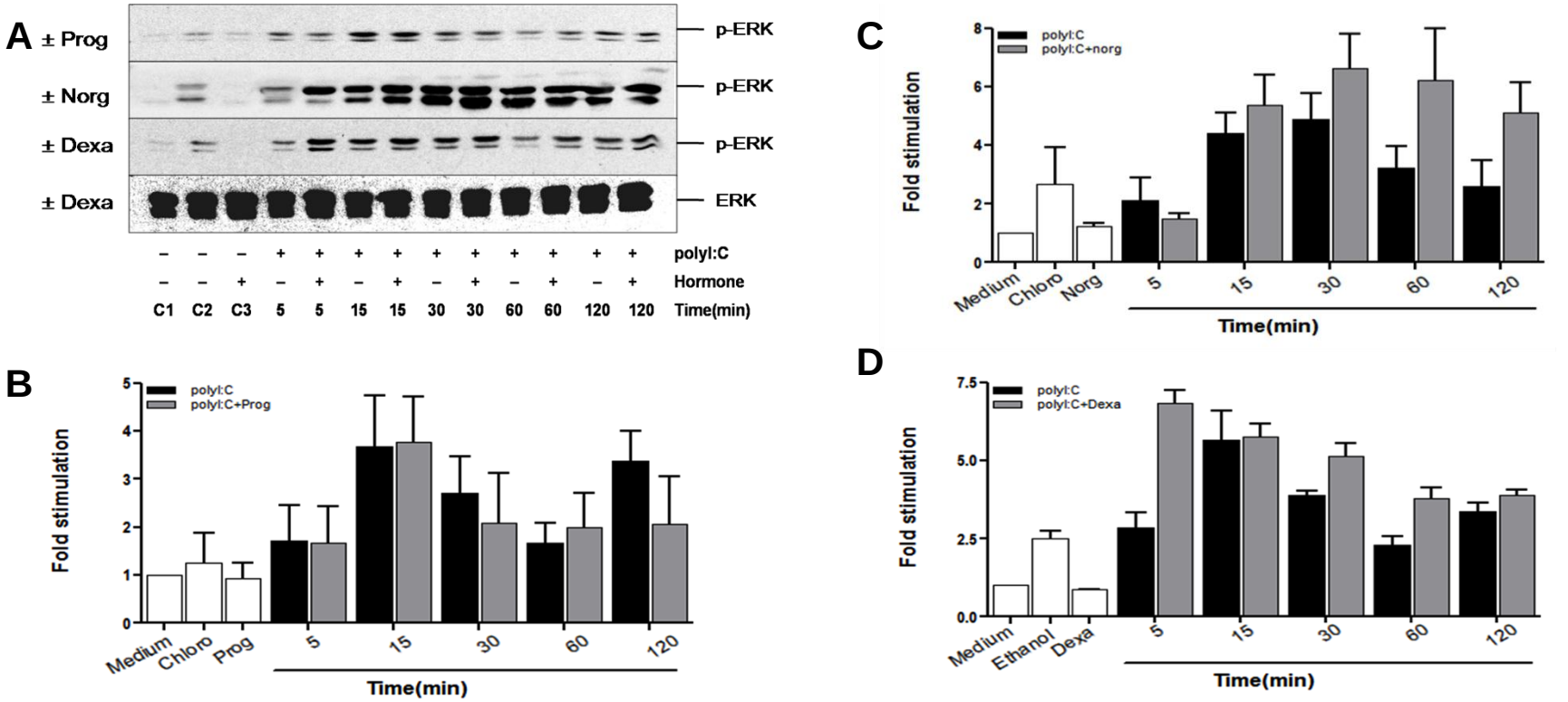


Figure 3.34. The effect of progesterone, norgestrel and dexamethasone on polyI:C-stimulated ERK phosphorylation in BMDCs. BMDCs were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 25μg/ml polyI:C for the indicated times (min). Whole cell extracts were prepared and ERK phosphorylation and expression were assessed by Western blotting using specific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments. The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3).C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

3.2.6 IRF3 signalling pathway

3.2.6.1 Agonist stimulated IRF3 phosphorylation and the potential effects of hormones in BMDCs

As in previous experiments, signalling events of the protein of interest were studied by means of TLR3 and TLR4 ligation to trigger signalling cascades. The effects of progesterone, norgestrel and dexamethasone hormones on IRF3 phosphorylation were studied by comparing with agonist treated cells alone. In experiments assessing IRF3 status, the selected time points were quite different from previous experiments. It was observed that the kinetics of IRF3 phosphorylation were much slower in comparison to NFκB and MAPK pathways. And this was therefore monitored from 0.5-8 hours. For cells treated with LPS alone, LPS induced IRF3 phosphorylation was observed after a half an hour, this increased gradually for 1-2 hours then declined gradually between 6-8 hours (data not shown). Pretreatment with progesterone, norgestrel or dexamethasone had no effect on LPS induced IRF3 activation (Figure 3.35). BMDCs treated with polyI:C alone induced approximately the same activation pattern as LPS over the time points examined. In contrast, however, pretreatment with progestrone and norgestrel but not dexamethasone induced prolongation of the IRF3 phosphorylation induced by polyI:C (Figure 3.36). A significant increase in polyI:C induced IRF3 phosphorylation was observed in cells pretreated with progesterone at 2hours post stimulation (22.6389 ± 1.1589 fold stimulation, $P < 0.05$) compared to cells treated with agonist alone (12.098 ± 1.97 fold stimulation). For cells pretreated with norgestrel and exposed to polyI:C IRF3 phosphorylation was also maximal and significantly greater than poly I:C alone stimulated cultures at 2hours (14.4139 ± 1.6497, $P < 0.05$) compared to cells treated with agonist alone (7.314 ± 1.30 fold stimulaton). IRF3 protein expression was used for determining equal protein loading with the both ligands.

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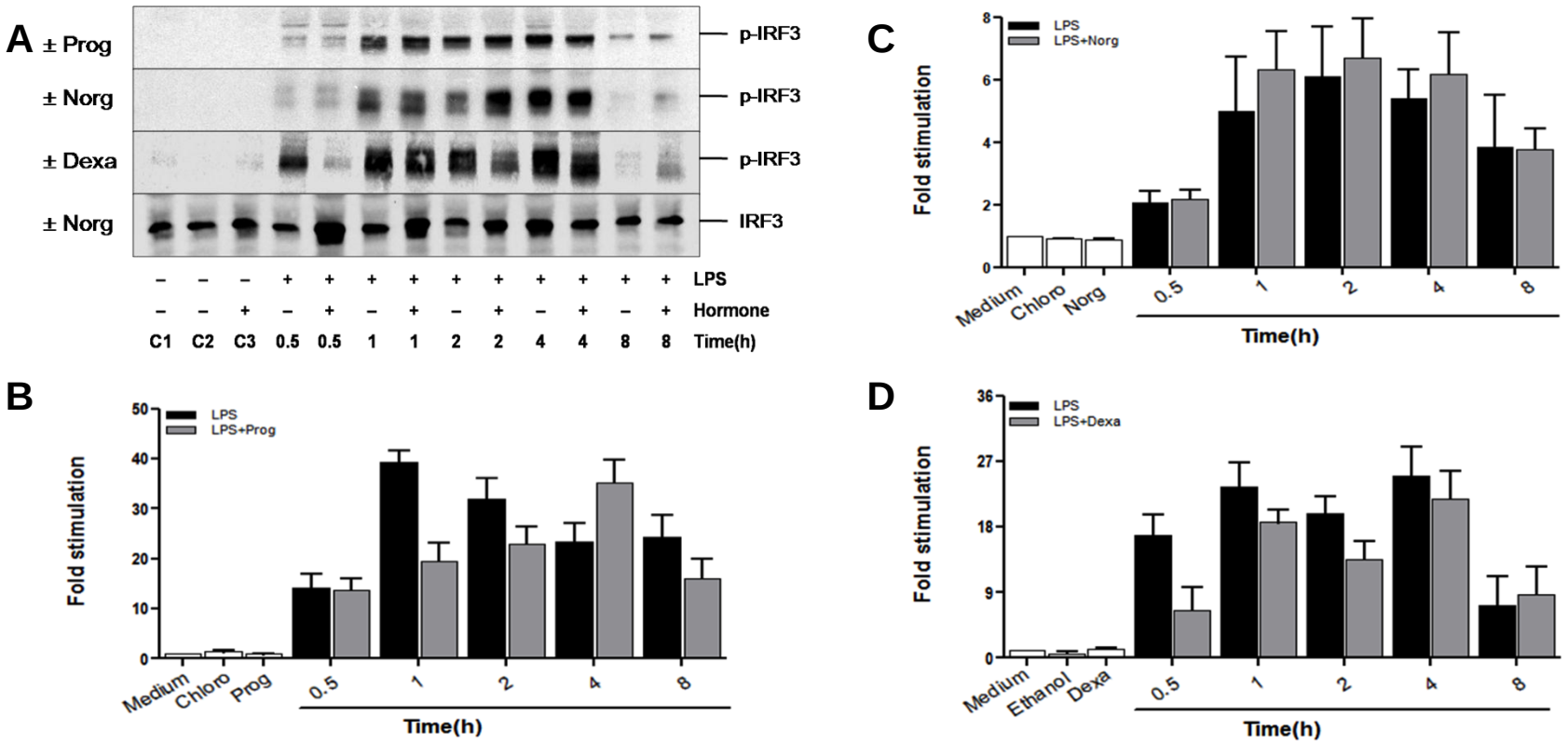


Figure 3.35. The effect of progesterone, norgestrel and dexamethasone on LPSstimulated IRF3 phosphorylation in BMDCs. BMDC were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 1μg/ml LPS for the indicated times (h). Whole cell extracts were prepared and IRF3 phosphorylation and expression assessed by Western blotting usingspecific antibodies as detailed in the Materials and Methods section. Results in panel A are representative of three independent experiments.The associated data for experiments examining pretreatment with progesterone, norgestrel or dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively (n=3).C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).

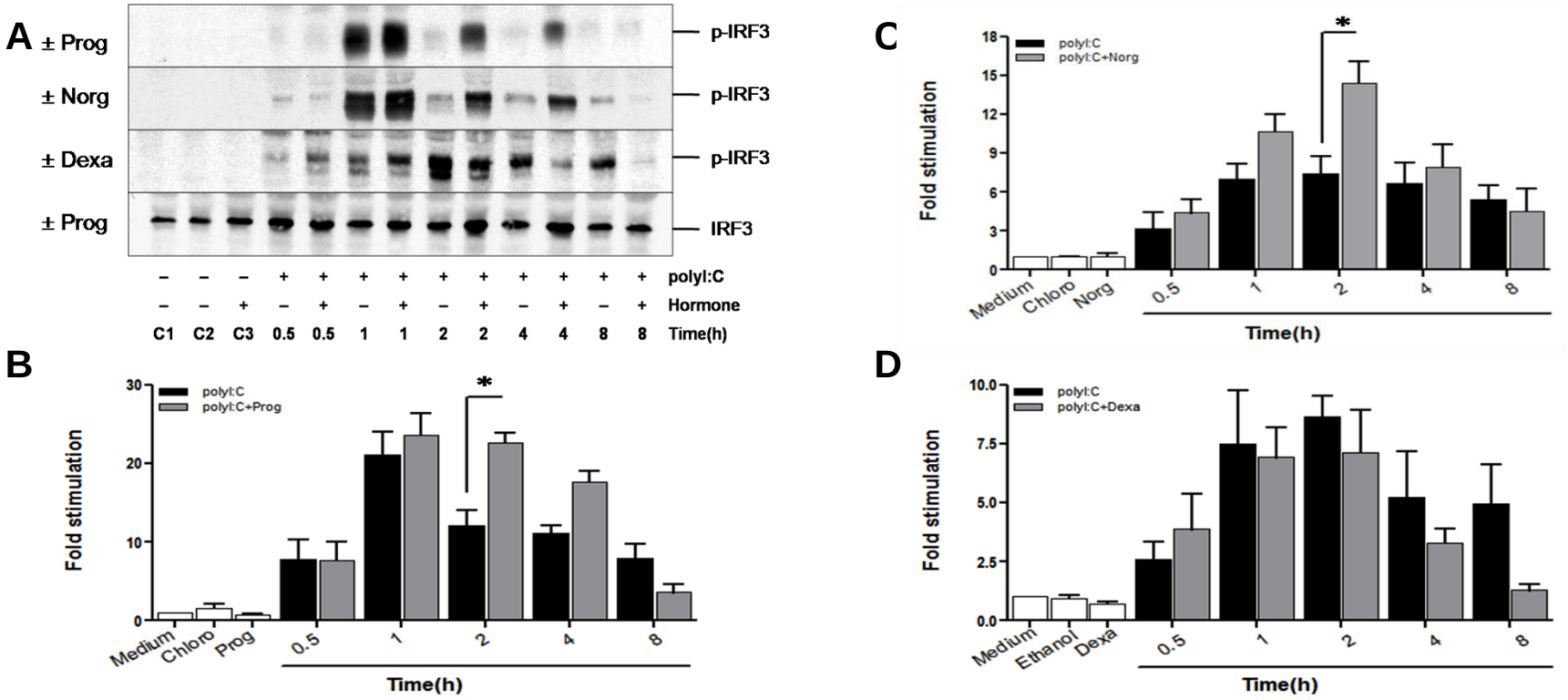


Figure 3.36. The effect of progesterone, norgestrel and dexamethasone on polyI:C stimulated IRF3 phosphorylation in BMDCs.BMDC were treated with vehicle or 62.5μM of progesterone (Prog) or norgestrel (Norg) or 62.5nM dexamethasone (Dexa) for one hour prior to exposure to 25μg/ml polyI:C for the indicated times (h). Whole cell extracts were prepared and IRF3 phosphorylation and expression assessed by Western blotting usingspecific antibodies as detailed in the Materials and Methods section. Results in panel Aare representative of three independent experiments.The associated data for experiments examining pretreatment with progesterone, norgestrel and dexamethasone were quantified by scanning densitometry and are depicted in panels B, C and D respectively(n=3). C1, media, C2, vehicle (ethanol or chloroform), C3, hormone (Prog, Norg, or Dexa).* $p<0.05$; statistically significant vs. agonist alone.

3.3 Discussion

In intial experiemts polyI:C and LPS, as TLR3 and TLR4 ligands respectively, induced rapid NFκB activation with a slight delay following poly I:C compared with LPS treatment. It has been found that deficiencies of RelA and IKKβ result in an increase in the chance of infectious diseases (Alcamo *et al*., 2001). This is due to the essential role of IKKβ in IκB degradation. This involves the classical activation pathway of NFκB which causes the liberation of p65 which then translocates to the nucleus initiating an immune response (Alcamo *et al*., 2001). Stimulation of different TLRs by different pathogens can trigger classical NFκB signaling (Medzhitov, 2001). Phosphorylated p65 could be found in unstimulated cells and further increased upon stimulation by a broad range of stimuli (Naumann *et al*., 1994). It has been well established that there are five serine residues within the p65 NFκB subunit which are phosphorylated in response to different stimuli (Zhong *et al.*, 1998). One of these is serine 536 which was investigated by Sakurai *et al.*, (1999) and Doyle and O Neill, (2006). They found that phosphorylation of serine 536 *in vivo* and *in vitro* related to IKKα or IKKβ over expression. Also, LPS is known as a potent IKK inducer. That explains the rapid and high phosphorylation rate induced by LPS. Yang *et al*. (2003) found that IKKβ is essential for serine 536 phosphorylation of p65 in mouse embryonic fibroblasts (MEFs) under LPS but not TNF stimulation, while IKKα plays only a partial role in serine 536 phosphorylation. They also found that p65 transcriptional activity increased as a result of phosphorylation on serine 536. There is a large body of evidence for the essential role of TRIF in MyD88-independent signalling. Major impairment of TLR3 mediated NFκB activation occurs in TRIF $^{-/-}$ cells. In contrast, normal NFκB and MAP kinase activation occurs in TRIF $^{-/-}$ embryonic fibroblasts stimulated via TLR4 (Yamamoto *et al*., 2003b). For TLR4, the MyD88-dependent pathway leads to an early phase of NFκB and MAP kinase activation. However, MyD88-independent signalling induces the late phase of NFκB and MAP kinase activation which is demonstrated in MyD88$^{-/-}$ cells (Kawai *et al*., 2001).The role of LPS in the activation of NFκB and MAPKs via TLR4 is well understood. In addition, many studies explain the essential role of MAPKs in the innate immune response and in inflammatory cytokine production (Zhang and Dong,

2005). It has also been found that dsRNA has the ability to activate MAPKs signalling via TLR3. Activated MAPKs take part in regulation of inflammatory genes (Andrade *et al*., 2004). IL-1 expression is regulated by ERK (Maggi *et al*., 2003). Moreover, activated MAPKs induce COX-2 expression (Faour *et al*., 2003). This study showed that the three MAPK groups investigated were activated rapidly via both TLR4 and TLR3 within 5-15 minutes. The same finding was reported by Steer *et al*., (2006). They found that polyI:C caused rapid and high levels of phosphorylation for the 3MAPKs, this activation is transient for p38 and prolonged for both ERK and JNK.

The latter results of this section showed no influence of the three hormones upon the TLR3– and TLR4-stimulated NFκB and MAPKs pathways in both macrophages and dendritic cells, assessed at the level of IκB degradation, p65 (Ser-536) phosphorylation and MAP kinase phosphorylation respectively. Previous studies from our lab have demonstrated that progesterone can modulate macrophage and dendritic cell cytokine production and therefore it was anticipated that these pathways would be modulated by the steroid hormone pretreatments. For example, in macrophages Jones *et al*, (2008) reported a significant reduction in LPS-induced NO production, mediated through glucocorticoid receptors GRs, and down-regulation of LPS-induced IL-12 production, mediated through both progesterone receptor PR and GR. In dendritic cells Jones *et al*., (2010) reported a concentration-dependent reduction of LPS- and polyI:C- induced IL-6 production mediated through GRs and down-regulation of total IL-12 and IL-12p70 production, mediating through GR and PR. As mentioned previously in the introduction many other studies have reported similar results; Miller *et al*. (1996) reported that progesterone can cause time and dose–dependent inhibition of nitric oxide production by BMDM and macrophage cell lines (RAW 264.7, J774). Su *et al*. (2009) reported that progesterone can inhibit TLR4- and TLR9- mediated IL-6 and NO production by macrophages whilst Butts *et al* (2007) reported that progesterone inhibited proinflammatory cytokine production by BMDCs in a concentration-dependent manner including expression of TNFα, IL-1β and IL-12. In human and mouse pDCs. Progesterone also inhibits IFNα production by inhibiting IRF-7 (Hughes *et al*., 2008).

Potentially linked to cytokine production in these settings, there is a growing body of evidence for the crucial roles of NFκB, MAPKs and IRF3, and molecules associated with their activation in the induction of genes that express pro-inflammatory cytokines, chemokines, Type-1 IFNs and adhesion molecules. Consistent with coordinated regulation of gene expression by these signalling mechanisms TNFα, IL-1β, IL-2, IL-6, IL-8, RANTES, IFNβ, GM-CSF, ICAM-1 and VCAM-1, all contain sites for NFκB and /or AP-1 in the promoter or regulatory region of their genes (De Bosscher *et al*., 2003). It has also been reported that the promoter region of p40 subunit gene of IL-12 in human and mouse contain sites for NFκB, IRF-1, c-Rel and interferon consensus binding protein (ICBP) (Murphy *et al*., 1995; Ma *et al*., 1996). Additionally, the IFNβ gene also contains consensus binding sites for NFκB, IRF3 and ATF-c-Jun transcription factors in its promoter (Yoneyama *et al*., 1998; Grandvaux *et al*., 2002). Therefore, despite the reported immune-modulatory effects of progesterone in the above publications and also the understanding of the expected regulatory elements that govern coordinated pro-inflammatory gene expression, the hormone pretreatment again did not display modulatory effects against any of the chosen markers assayed for each of the signalling pathways. Failing to demonstrate any influence of the hormones on these molecules may be due to the the timing of hormones treatments and perhaps the level of receptor expression and/or that of other required molecules in these cells. However, it must also be noted that each of the pathways were examined by one or two markers only. It was not inconceivable that the hormones may have effects at sites further downstream in these pathways, Given that the molecular mechanism by which steroid hormones mediated their effects, in both the cytoplasmic and nuclear compartments, and involves different types of co-activators and /or co-repressor molecules in parallel to various protein-protein interactions, particularly in relation to nuclear events and the coordinated regulation of gene transcription; this required further investigation.

Progesterone mediates its action either by binding GR or PR. Both receptors have close similarity in their structure. Their DNA-binding domains (DBD) that recognise a specific DNA sequences are identical by 90% and their ligand-binding domains (LBD) that bind different steroids are identical by 55% (Evans, 1988). In

the nucleus they bind to the same hormone response elements (HRE). Despite all that each hormone has different biological roles and regulates different genes (Szapary *et al*., 2008). Glucocorticoid and progesterone as lipophilic molecules can cross cell membrane easily and bind their cytoplasmic receptors which reside in cytoplasm linking to other proteins. Ligand-bound receptor regulates gene expression either by protein-DNA binding or by interacting with other transcription factors like NFκB, AP-1 or IRF3 and through protein-protein interaction change transcription activity of these factors (Ogawa *et al*., 2005). Again, these molecular mechanisms require further investigation as potential means of regulating nuclear gene transcription, in concert with NFκB, MAP kinase and IRF3 pathways.

In contrast to the NFκB and MAP kinase cascade it was not apparent in the macrophage setting but clear in DCs, both progesterone and norgestrel, showed prolongation of polyI:C-induced IRF3 (396) phosphorylation in dendritic cells. Dexamethasone in DCs did not demonstrate this effect. Thus, PRs were considered to mediate this effect. DCs respond to viral infection or to the synthetic dsRNA via TLR3 and MDA5 receptors. Antiviral immune response involves activation of IRF3 and production of Type 1 IFNs (Sasai *et al*., 2006; Perrot *et al*., 2010). Many viruses cause morbidity by interfering or down-regulation of host antiviral responses. West Nile Virus (WNV) disrupted host antiviral response by inhibiting polyI:C-induced IRF3 and NFκB nuclear translocation and inhibited TLR3-dependent IL-6 production (Wilson *et al*., 2008). Therefore, previous results related to the ability of progesterone to down-regulation of proinflammatory cytokines or Type 1 IFNs production may be exerted downstream preventing related transcription factors from nuclear translocation or DNA binding. Although, progesterone and norgestrel increased polyI:C-induced IRF3 phosphorylation, possible interpreted to represent increased activity with a positive modulatory outcome, elevated IRF3 level may act as a repressor of consequent transcription events. Strikingly, Dahlberg *et al*., (2006) reported overexpression of IRF3 inhibited IL-12p35 production by repressing promoter activity in macrophages in response to Theirler's virus. The role of hyper-phosphorylation of IRF3, and importantly whether this relates to increased IRF3 activity, as either an activator or repressor of transcription, remains to be defined and understood more clearly. The potential sustained phosphorylation and/or activation

of IRF3 and potential function as an immunomodulator will remain obscure unless further study is pursued to examine both downstream gene regulation and also the upstream kinases/phosphatases that regulate IRF3 phosphorylation status. Furthermore, the hormone immunomodulatory effect, if mediated through PRs, suggests that a more developed study of any protein-protein interactions with IRF3 and/or components of the IRF3 signalling pathway and protein-DNA interactions that may activate or repress the transcription activity at the promoter regions of individual target genes is required.

CHAPTER FOUR

THE INFLUENCE OF PROGESTERONE AND NORGESTREL HORMONES ON LPS- AND POLYI:C- INDUCED P65/ RELA AND IRF3 NUCLEAR TRANSLOCATION AND DNA-BINDING IN BMDCs

4.1 Introduction

Dendritic cells are potent innate immune cells, equipped with different types of pathogen recognition receptors (PRR) (Ilchmann *et al*., 2012). Upon activation by varied numbers of PAMP, DCs convert to potent antigen presenting cells (APC) (Mellman and Stienman, 2001). DCs as APCs induce adaptive immunity by various mechanisms. All DCs subtypes share the ability to process and present the engulfed antigen (Juretic *et al*., 2004). Besides, they release chemokines, cytokines and Type 1 IFNs. These characteristics enable DCs to promote inflammatory responses and overcome invading pathogens. Plasmacytoid DCs (pDCs) are the major source of Type 1 IFNs following viral infection (Soumelis and Liu, 2006; Dalod *et al*., 2003; Irla *et al*., 2010), whilst, splenic $CD8^+$ DCs are major producer of IL-12 (Tailor *et al*., 2006). Other DC subtypes contribute to immune responses as inducers of either anti-inflammatory or pro-inflammatory cytokines (Tailor *et al*., 2006).

TLRs, an important group of PRRs, are expressed on many cell types including myeloid cells such as BMDCs (Dearman *et al*., 2009). TLRs elicit innate immune responses through diverse signalling pathways targeting a vast number of inflammatory genes. TLR3 utilises TRIF-dependent signalling whilst TLR4 utilises MyD88- and TRIF-dependent signalling pathways, to induce complicated downstream signalling arising from their responding to a wide range of stimulating ligands. As a result, these pathways initiate the transcription of a considerable number of genes. Both receptors induce NFκB and MAPKs pathways that regulate proinflammatory gene expression that is common to all TLRs. In addition, they induce exclusively IRF3 activation which contributes to the regulation of genes that mediate the antiviral response. Type 1 IFNs or specifically IFNβ mediates this response. IRF3, the key player in IFNβ production, can be induced not only by TLRs but also by the cytoplasmic helicases retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) receptors. Post dsRNA viral infection, signalling downstream from these receptors recruits the IFNβ promoter stimulator-1 (IPS-1) adaptor protein which is also called mitochondrial antiviral signalling (MAVS) or CARD adaptor inducing IFNβ (Cardif), (Yoneyama *et al*., 2004b; Loo *et al*., 2008; Perrot *et al*., 2010). Although, IPS-1 is an adaptor molecule

utilised by RIG-I and MDA5 receptors, it may be involved in TLR signalling by linking to TRAF3 inducing IRF3 and IRF7 activation (Seth *et al*., 2005; Saha *et al*., 2006).

It is widely accepted that TANK binding kinase 1 (TBK1) and IKKε are kinases essential for IRF3 and IRF7 phosphorylation aside of their earlier discovered role in NFκB activation downstream of TLR3- and TLR4-TRIF signalling (Barton and Medzhitov, 2003; Hemmi *et al*., 2004). In order to act efficiently, TBK1 and IKKε desire to assemble with scaffold proteins. Three scaffold proteins have been proposed TANK, NAP1 and SINTBAD (Chau *et al*., 2008). IRF3 phosphorylation and Type 1 IFNs induction require all of these scaffold proteins to be assembled (Ryzhakov and Randow, 2007).

IRF3 is a 55kDa, 427 amino acid phosphoprotein that under resting condition presents in an inactive form in the cytoplasm (Servant *et al*., 2001). IRF3 has multiple phosphorylation sites within its C-terminus which are targeted by IKKε and TBK1; Ser-396, Ser-398, Ser-402, Ser-405 and Thr-404 (Lin *et al*., 1998) and also on Ser-385 and Ser-386 (Servant *et al*., 2002). N-terminal phosphorylation of IRF3 has also been reported to be induced by several agents including LPS (McCoy *et al*., 2008). Although, each site is crucial for distinct roles, all of them are important in IRF3 activation and subsequent regulation of gene transcription. It has been proposed (Clement *et al*., 2008), that IRF3 undergoes serial phosphorylation events starting with Ser-396 and Ser-402 sites, followed by Ser-404 and Ser-405. IRF3 becomes hyperactivated due to the phosphorylation of Ser-396 and Ser-339, which then initiates homodimerisation and translocation into the nucleus. This results in binding to CBP and p300, and then binds positive regulatory domain I and III in the promoter region of Type 1 IFNs (IFNβ). IRF3 action is terminated by an ubiquitin-proteasome mediated mechanism resulting in degradation of IRF3 (Ronco *et al*., 1998). Phosphorylated IRF3 downstream of TLR3/4 mediates production of Type 1 IFNs in 2 phases (see Chapter 1, Figure 1.5). Each phase involves regulation of distinct genes. Primary response genes, which are induced in early phase include; regulated upon activation normal T cell expressed and secreted (RANTES), IFNγ induced protein 10 (IP10), interferon stimulated genes (ISGs) (Servant *et al*., 2002; Doyle *et*

al., 2002; Bonjardim, 2005), Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) (Smith and Herschman, 1996; Doyle *et al*., 2002) and IFNβ (Taniguchi and Takaoka, 2002; Sasai *et al*., 2006). The expression of secondary response genes are induced by early Type 1 IFNs production that binds to Type 1 IFN receptor (IFNAR). Downstream signalling of IFNAR involves induction of Janus kinase (JAK), tyrosine kinase 2 (Tyk2) and Janus kinase 1 (Jak1) which promote signal transducer and activator of transcription1 and 2 (STAT1) and (STAT2) phosphorylation and with IRF9 form the IFN stimulatory gene factor 3 complexes (ISGF3). This phase which is known as the late phase is responsible for induction of additional genes including interferon- induced GTP binding protein (Mx1), interferon inducible gene 1 (IFI1), interferon inducible gene 204 (IFI204) and IRF7 (Doyle *et al*., 2002).

Four regulatory cis elements (transcriptional enhancers) have been distinguished in the promoter region of the human IFNβ, these are the positive regulatory domains (PRDs) I, II, III, and IV (Leblance *et al*., 1990; Kim and Maniatis, 1997). IRF family members bind to PRD I and III, while NFκB binds PRD II and ATF-2/ c-Jun to PRD IV. Upon viral infection the three transcription factors (NFκB, IRF3 and AP-1) with the CBP and p300 coactivator and the high-mobility group protein HMG-1(Y) assemble as a specific enhanceosome to initiate transcription (Maniatis *et al*., 1998).

It has long been recognised that steroid hormones such as progesterone may generate immune modulatory responses and this may be achieved by impacting on key cell signalling events. TLR signalling has also been utilised extensively as a means to probe by which mechanism progesterone hormone may modulate inflammatory responses as a component of innate immunity. The synthetic progestin, norgestrel is a gonane progestin. Chemically, it is 19-nortestosterone derivative used clinically in contraceptive preparations mediates its action through the progesterone receptor (Kontula *et al*., 1983; Petit-Topin *et al*., 2009). Relative binding affinity of norgestrel to human PR is 323% that of progesterone itself and 7.5% that of cortisol to human glucocorticoid receptor (Sitruk-Ware, 2006).

The influence of progesterone hormone on TLR3/4 inflammatory signalling has been studied in both BMD-macrophages and BMDCs. Results showed no influence of progesterone hormone on these pathways, as assessed at the level of MAPKs (p38, JNK and ERK) and IκBα/p65 RelA phosphorylation. However, this study identified prolonged polyI:C- stimulated IRF3 phosphorylation in DCs pretreated with progesterone or norgestrel hormone. This effect was observed for progesterone and norgestrel but not dexamethasone, suggesting that progesterone mediates prolongation of IRF3 phosphorylation through progesterone receptor (PR). Many of the immune modulatory effects of progesterone may be mediated through GRs. However, GR expression is more abundant in lymphoid and non-lymphoid tissues (Miller and Hunt, 1998; Butts *et al*., 2011, Robinson and Klein, 2012).

Within the TLRs family, IRF3 activation is restricted to TLR3 and TLR4 activation. Both receptors utilise the TRIF adaptor molecule to recruit similar but not identical pathways. TRAM is another adaptor molecule required by TLR4 for IRF3 activation (Fitzgerald *et al*., 2003b; Yamamoto *et al*., 2003b). Dimers of phosphorylated IRF3 translocates to the nucleus, binds ISRE and initiating the transcription of target genes such as interferon –inducible protein-10 (IP-10), and IFNβ. IFNβ production requires binding of transcription factors to the enhancer of the gene promoter. These include NFκB, IRF3 and ATF-c-Jun (Servant *et al*., 2002). IFNβ production relies intially on IRF3 which plays a critical role (Tsuchida *et al*., 2009). This selectivity defines TLR3 and TLR4 as the only TLRs able to induce IFNβ production in mediating an antiviral response (Fitzgerald *et al*., 2003b).

In previous results, norgestrel showed some immunomodulatory effects like progesterone, and these effects may have been mediated through progesterone receptors. In the current section these effects were examined further in an attempt to better understand the molecular mechanisms that regulate translocation and DNA binding and to link to future experiments studying these effects at the transcription level (gene expression). In this component of the experimental work, potential nuclear translocation of IRF3 and associated DNA binding activity were examined to investigate the potential implication of prolonged IRF3 phosphorylation as observed in Chapter 3 and to develop further understanding of the potential mechanism of

regulation by progesterone/ norgestrel hormones. This was pursued in parallel with NFκB status as a comparative approach.

4.2 Results

4.2.1 LPS and polyI:C induced p65/RelA nuclear translocation in BMDCs

Nuclear extracts from BMDCs exposed either to 1µg/ml LPS or 25µg/ml polyI:C for varying times (0.5-6h) were examined for nuclear translocation of p65/RelA. The nuclear expression of Histone (H3) protein was also examined as a sample loading control.

LPS and polyI:C induced p65/RelA nuclear translocation (Figure 4.1). Following exposure to 1µg/ml LPS (panel A), p65/RelA translocation was increased after half an hour and sustained up to 2 hours with a maximally increase of approximately 3-fold (2.869 fold ± 0.38, $P < 0.001$). In nuclear extracts from DCs treated with 25µg/ml polyI:C (panel B) a significant increase in p65/ RelA nuclear translocation was also observed after half an hour, maximally by 2 h (approximately 8-fold) and sustained until 4 h (7.988 fold ± 1.03, $P < 0.001$). For subsequent experiments with hormones, a 2-hour period of stimulation with each agonist; LPS or polyI:C was selected.

4.2.2 The influence of progesterone and norgestrel hormones on LPS or polyI:C induced p65/RelA nuclear translocation in BMDCs

High concentrations, 62.5µM of both hormones to reflect the local concentration evident in placenta during pregnancy were utilised to study the effects of progesterone on inflammatory signalling. As an alternative, a concentration of 0.1µM, which is commensurate with peripheral level of the hormone in mice during pregnancy (McCormack and Greenwald, 1974; Schuurs and Verheul, 1990; Butts *et al*., 2007), was also used. Therefore, in this aspect of the study, an attempt was made to investigate the effects of progesterone pretreatment on LPS or polyI:C induced

p65/RelA translocation into the nucleus under conditions putatively reflecting hormone concentration locally and peripherally. BMDCs were pretreated with hormones (progesterone or norgestrel, 0.1 and 62.5µM) for 1 hour, and then exposed to 1µg/ml LPS or 25µg/ml polyI:C for 2 hours. Samples were collected, nuclear extracts were prepared and assessed by Western blotting for p65/ RelA nuclear translocation.

Significant reduction of LPS induced p65 translocation in nuclear extracts from BMDCs pretreated with the higher concentration (62.5µM) of both hormones was observed (Figure 4.2). Pretreatment with progesterone (panel B) and norgestrel (panel C) reduced LPS induced translocation when compared with the agonist alone (LPS) ($P < 0.05$). This significant reduction could not be taken in consideration as the vehicle control showed a slight elevation in comparing with medium controls (panels B and C). However, the difference between the vehicle and medium control is insignificant (panel B). The agonist (LPS + vehicle) showed a significant elevation in p65/RelA nuclear translocation in comparing with the vehicle control (2.629 ± 0.494, $P < 0.05$). The higher concentration (62.5µM) of the both hormones did not show significant reduction when compared with LPS+ vehicle.

Both progesterone and norgestrel hormones reduced significantly polyI:C-induced p65/RelA nuclear translocation (Figure 4.3). Pretreatment with progesterone (panel B) at higher concentration (62.5µM) reduced significantly polyI:C- induced p65/RelA translocation into the nucleus, approximately 3- fold (3.058 fold ± 0.12, $P < 0.001$) compared with polyI:C + vehicle. Also polyI:C+vehicle showed a significant (4.451 fold ± 0.51, $P < 0.0001$) increase in the induction of p65/RelA translocation into the nucleus when compared with the vehicle control. Norgestrel (panel C) showed the same influence as progesterone for the higher hormone concentration (62.5 µM). It reduced significantly polyI:C- inducted p65/RelA translocation into the nucleus relative to cells treated with polyI:C + vehicle (1.275 fold ± 0.146, $P < 0.001$). PolyI:C + vehicle, itself induced significantly p65 /RelA nuclear translocation (2.18 fold ± 0.266, $P < 0.05$).

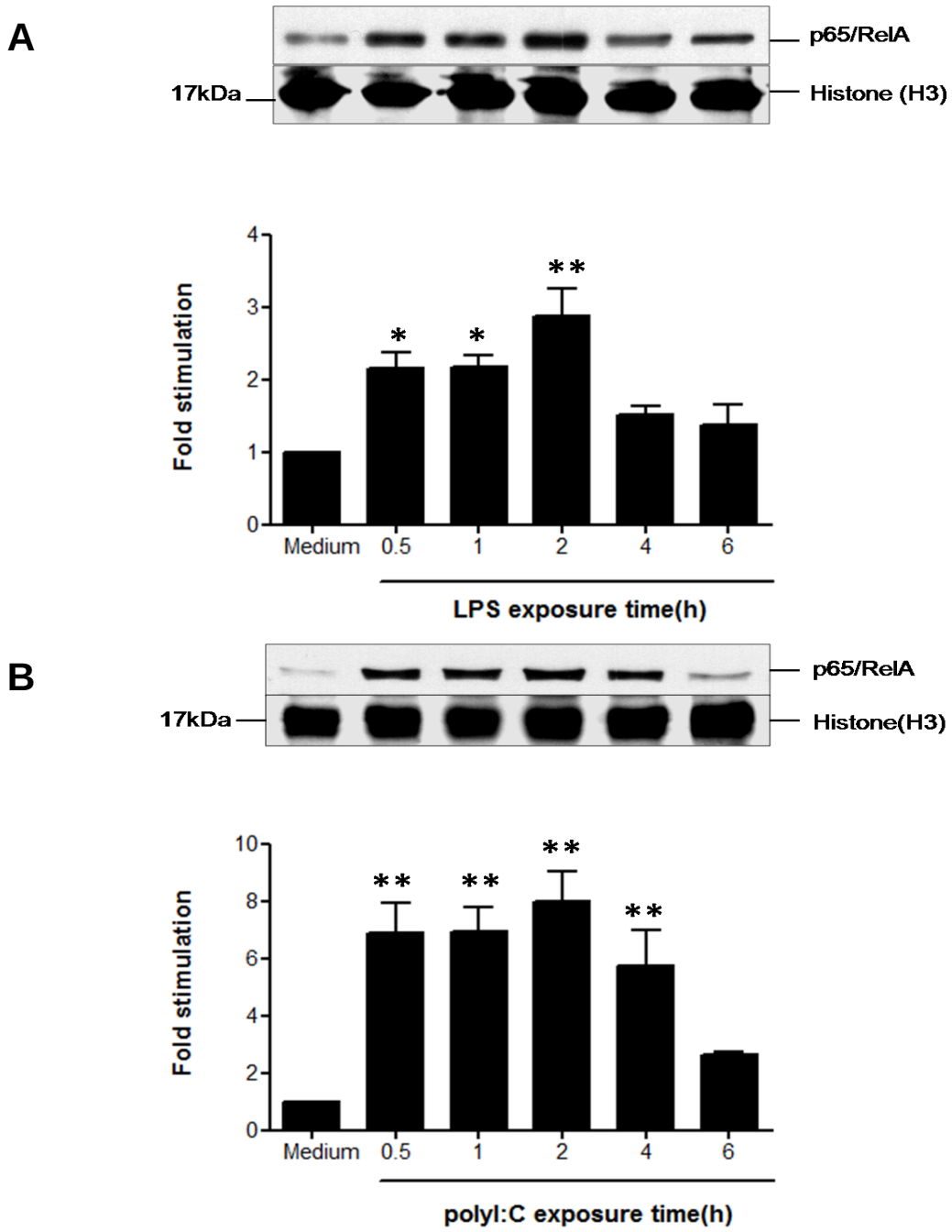


Figure 4.1. LPS or polyI:C induced p65/RelA nuclear translocation in BMDCs. BMDCs were exposed to 1µg/ml LPS (A) or 25µg/ml polyI:C (B) for indicated times (h). Nuclear extracts were prepared as outlined in Methods section and then assessed for LPS or polyI:C induced p65/RelA translocation by Western blotting. Upper panels of A and B are representative of three independent experiments. Nuclear Histone (H3) expression was used as loading control. The associated data were quantified by scanning densitometry and are depicted in lower panels of A and B. Each value represents the mean ± SEM from 3 independent experiments. *p<0.05 and **p<0.001 are statistically significant vs. control (Medium).

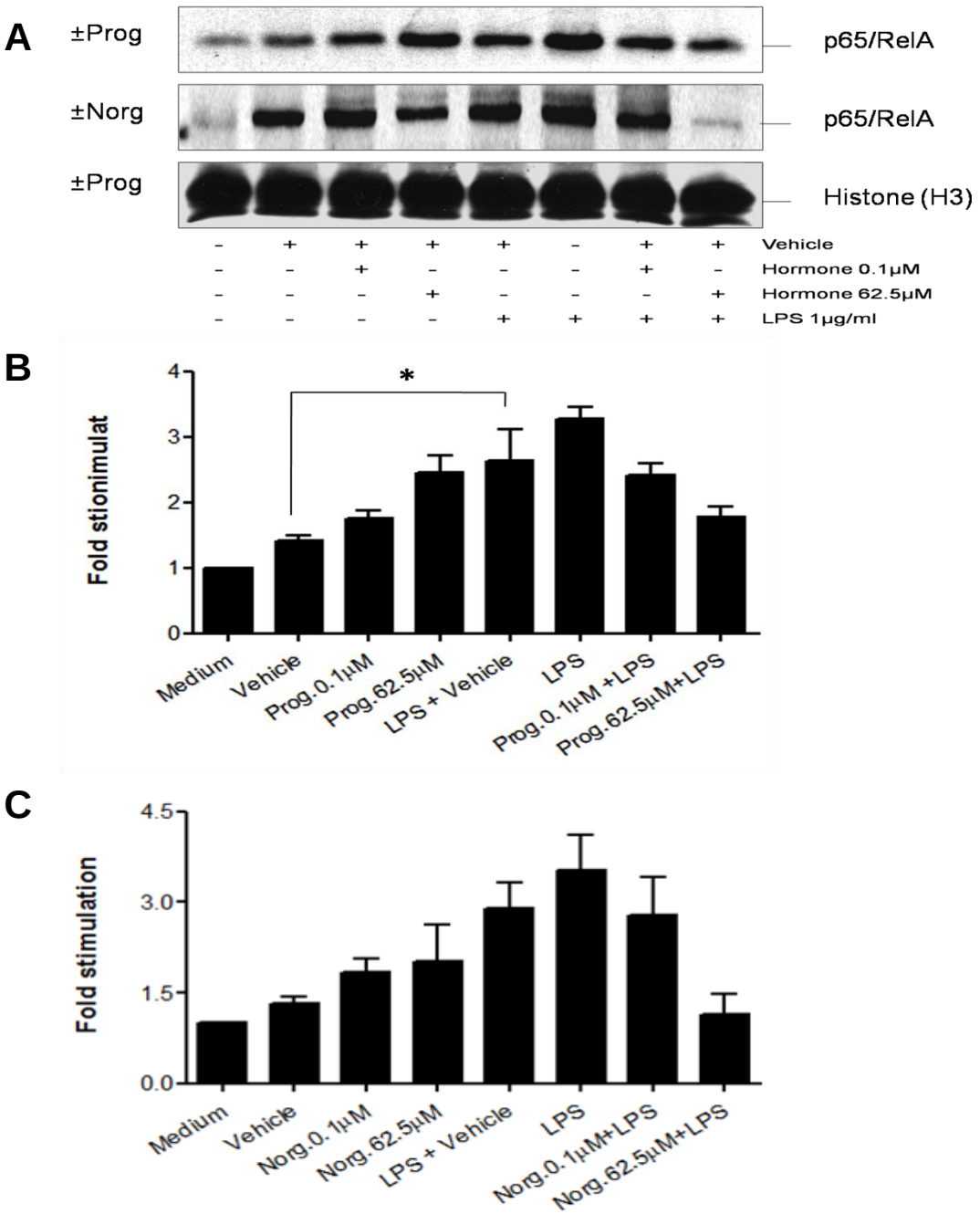


Figure 4.2. The influence of progesterone and norgestrel hormones pretreatment on LPS induced p65/RelA nuclear translocation in BMDCs. BMDCs were treated with vehicle, 0.1µM and 62.5µM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 1µg/ml LPS for 2h. Nuclear extracts were prepared as outlined in Methods section and then assessed for p65/RelA nuclear translocation by Western blotting. Results in panel A are representative of three independent experiments, nuclear Histone (H3) expression was used as loading control. The associated data for experiments examining pretreatment with progesterone and norgestrel were quantified by scanning densitometry and are depicted in panels B and C (n=3). *p <0.05 is statistically significant vs. vehicle control.

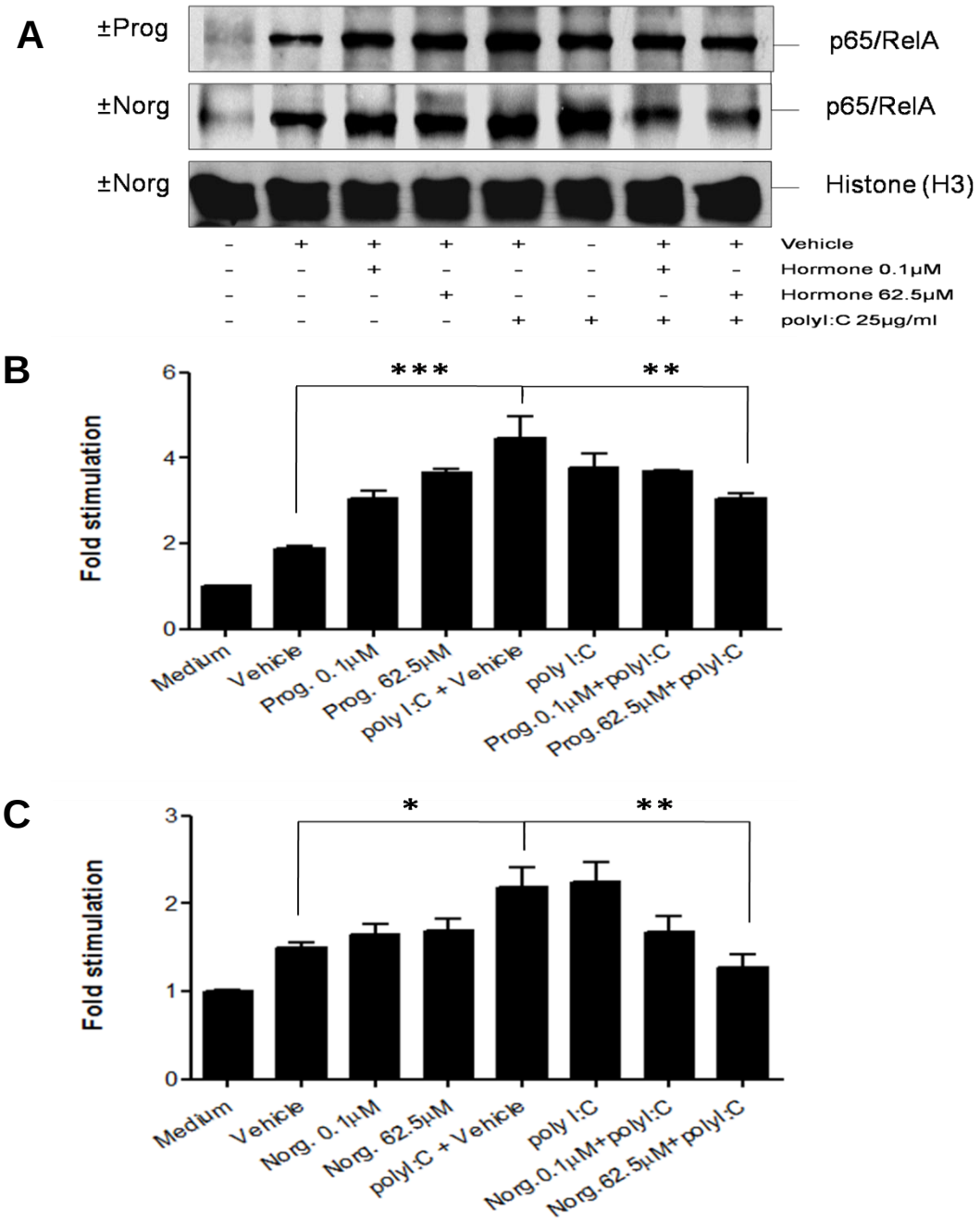


Figure 4.3. The influence of progesterone and norgestrel hormones pretreatment on polyI:C induced p65/RelA nuclear translocation in BMDCs. BMDCs were treated with vehicle, 0.1µM and 62.5µM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 25µg/ml poly I:C 2 h. Nuclear extracts were prepared as outlined in Methods section and then assessed for polyI:C induced p65/RelA nuclear translocation by Western blotting. Results in panel A are representative of three independent experiments, nuclear Histone (H3) expression was used as loading control. The associated data for experiments examining pretreatment with progesterone and norgestrel were quantified by scanning densitometry and are depicted in panels B and C (n=3). *p<0.05, **p<0.001 and***p<0.0001 are statistically significant vs. polyI:C+vehicle agonist.

4.2.3 LPS and polyI:C induced IRF3 nuclear translocation in BMDCs

Nuclear extracts from BMDCs exposed either to 1µg/ml LPS or 25µg/ml poly I:C for varying times (0.5-6h) were examined for nuclear translocation of IRF3. The nuclear expression of Histone (H3) protein was also examined as sample loading control. LPS induced IRF3 nuclear translocation (Figure 4.4, panel A), a significant increase in nuclear translocation of IRF3 was observed after 2 hour of stimulation with 1µg/ml LPS (1.612175 ± 0.10 fold, $P < 0.05$). In nuclear extracts from DCs exposed to 25µg/ml polyI:C (Figure 4.4, panel B) , significant elevation of IRF3 nuclear translocation was also observed after a half an hour, maximally by 2 hour and sustained until 4 hours, approximatly 3 fold (2.9091± 0.3833 fold, $P < 0.0001$).

4.2.4 The influence of progesterone and norgestrel hormones on LPS or polyI:C induced IRF3 nuclear translocation in BMDCs

The agonist (LPS+vehicle) showed significant increase of induced IRF3 translocation in comparsion to the vehicle control (3.7094 ± 0.46 fold, $P < 0.001$) (Figure 4.5, panel B). A significant reduction of LPS- induced IRF3 translocation in nuclear extracts from BMDCs pretreated with the higher progesterone concentration (62.5µM) was also observed when compared to LPS + vehicle (1.733 ± 0.19 fold, $P < 0.001$), however, the difference between the vehicle (chloroform) and medium control was insignificant. A slight reduction in LPS- induced IRF3 nuclear translocation for the both norgestrel concentration (0.1 and 62.5µM) were also observed but were insignificant (Figure 4.5, panel C).

A significant increase in polyI:C induced IRF3 nuclear translocation was observed for BMDCs exposed to poly I:C+vehicle in comparsion to the vehicle control (3.1158 ± 0.1 fold, $P < 0.001$) (Figure 4.6, panel B) . A significant reduction of polyI:C- induced IRF3 nuclear translocation for BMDCs pretreated with the higher concentration of progesterone (62.5µM) was also observed in comparison to cells exposed to poly I:C + vehicle (2.0373 ± 025 fold, $P < 0.05$).

A significant increase in IRF3 translocation in nuclear extracts from BMDCs exposed to polyI:C+vehicle in comparison to the vehicle control (5.1755 ± 0.56 fold, $P < 0.05$) (Figure 4.6, panel C) was also observed. Furthermore, a significant reduction of polyI:C- induced IRF3 transloction in nuclear extracts for BMDCs pretreated with the higher concentration of norgestrel (62.5µM) was observed when compared to cells exposed to poly I:C + vehicle (2.0454 ± 071 fold, $P < 0.05$).

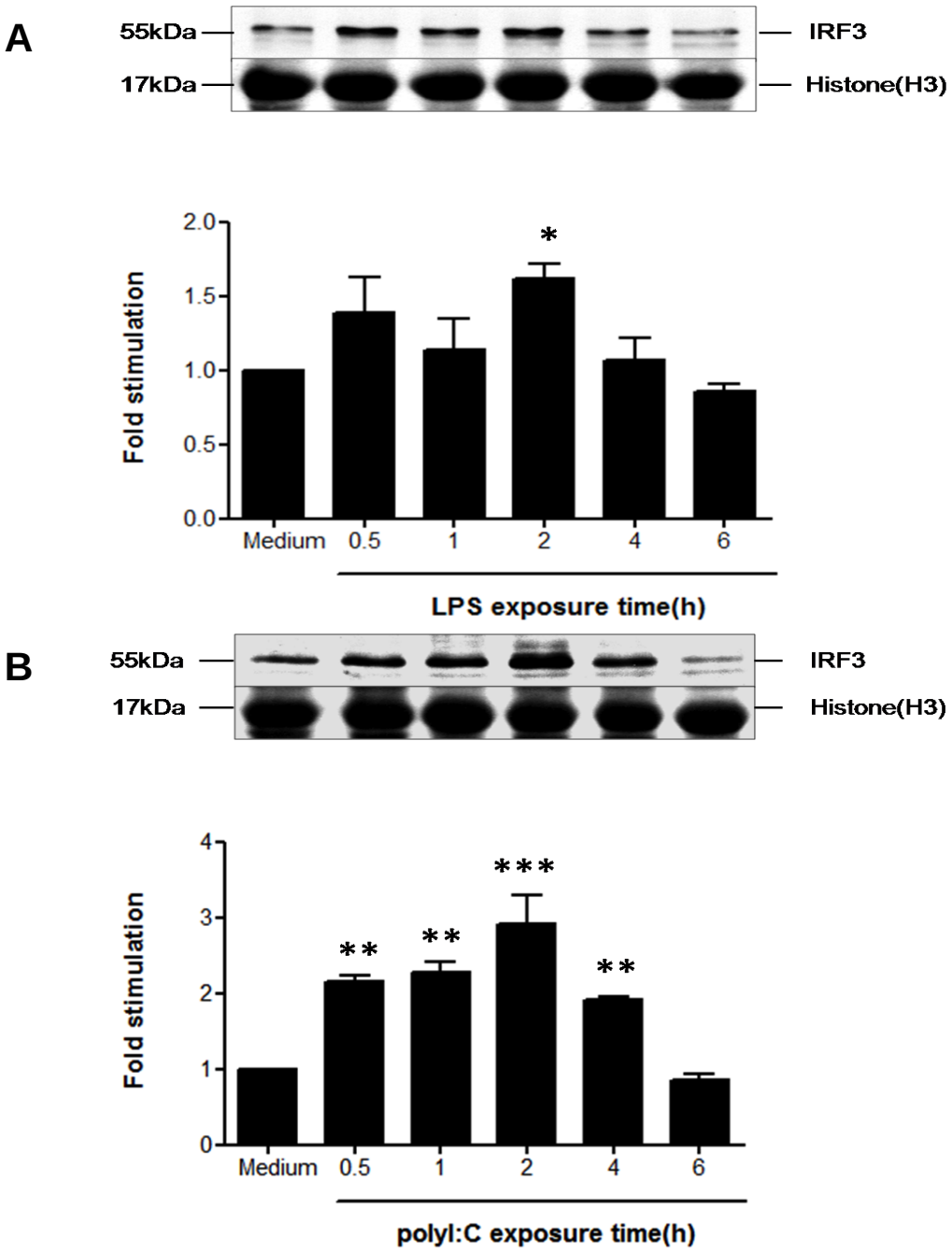


Figure 4.4. LPS or polyI:C induced IRF3 nuclear translocation in BMDCs. BMDCs were exposed to 1µg/ml LPS (A) or 25µg/ml poly I:C (B) for indicated times (h). Nuclear extracts were prepared as outlined in Methods section and then assessed for LPS or poly I:C induced IRF3 nuclear translocation by Western blotting. Upper panels of A and B are representative of three independent experiments. Nuclear Histone (H3) expression was used as loading control. The associated data were quantified by scanning densitometry and are depicted in lower panels of A and B. each value represents the mean ± SEM from 3 independent experiments. *p<0.05; **p<0.001 and ***p<0.0001 are statistically significant vs. control (Medium).

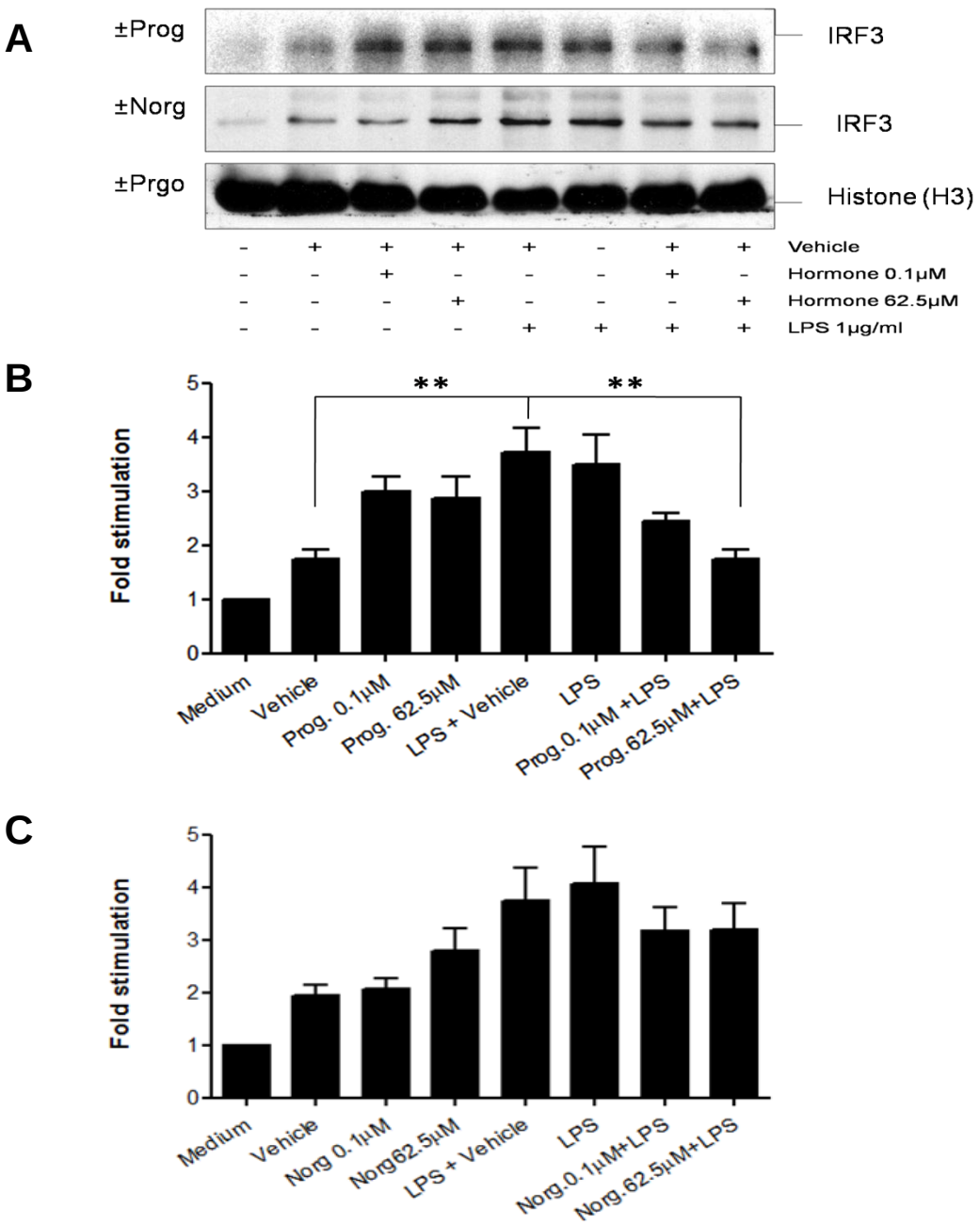


Figure 4.5. The influence of progesterone and norgestrel hormones pretreatments on LPS induced IRF3 nuclear translocation in BMDCs. BMDCs were treated with vehicle, 0.1µM and 62.5µM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 1µg/ml LPS for 2h. Nuclear extracts were prepared as outlined in Methods section and then assessed for LPS induced IRF3 nuclear translocation by Western blotting. Results in panel A are representative of three independent experiments, Nuclear Histone (H3) expression was used as loading control. The associated data for experiments examining pretreatment with progesterone and norgestrel were quantified by scanning densitometry and are depicted in panels B and C (n=3). **p<0.001 is statistically significant vs. LPS+vehicle agonist.

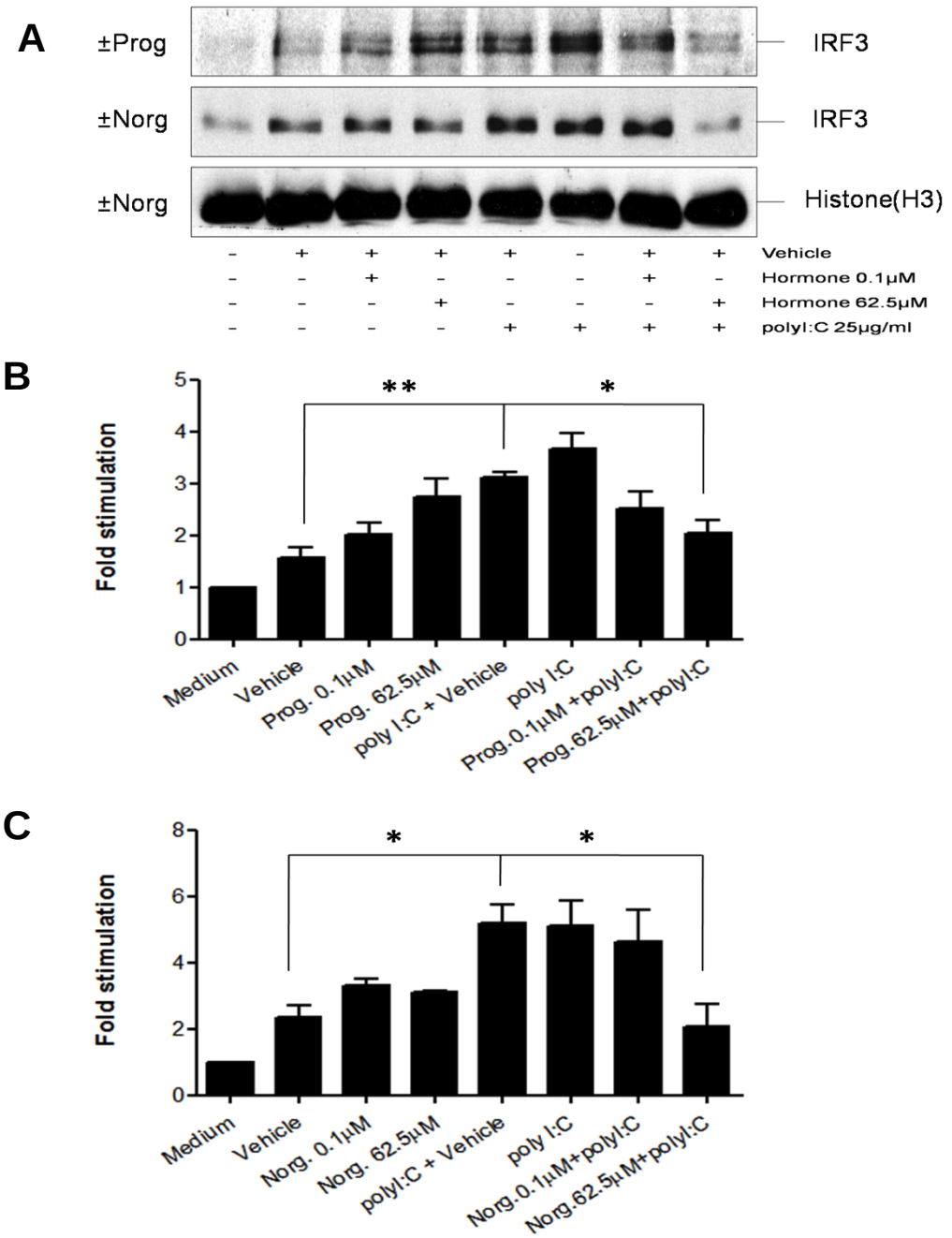


Figure 4.6. The influence of progesterone and norgestrel hormones pretreatment on polyI:C induced IRF3 nuclear translocation in BMDCs. BMDCs were treated with vehicle, 0.1µM and 62.5µM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 25µg/ml poly I:C for 2h. Nuclear extracts were prepared as outlined in Methods section and then assessed for poly I:C induced IRF3 nuclear translocation by Western blotting. Results in panel A are representative of three independent experiments, Nuclear Histone (H3) expression was used as loading control. The associated data for experiments examining pretreatment with progesterone and norgestrel were quantified by scanning densitometry and are depicted in panels B and C (n=3). *p<0.05 and **p<0.001 are statistically significant vs. polyI:C+vehicle agonist.

Protein DNA- binding activity.

Gel shift assay or electrophoretic mobility shift assay (EMSA) is the technique used to assess the ability of the transcription factors (NFκB) and (IRF3) to bind DNA sequence representative of consensus binding sites from some inflammatory genes. Subsequently, the influence of the sex hormones progesterone and norgestrel on these sequence-specific DNA binding factors could be assessed. The first part of this experimental work (Figures 4.1- 4.6) investigated the influence of the sex hormones progesterone and the synthetic progestin norgestrel on LPS- or polyI:C- induced p65/RelA and IRF3 nuclear translocation. For further analysis, protein DNA-binding activity was also studied. Translocation into the nucleus does not give accurate estimation of the effects on gene transcription, whilst protein-DNA binding activity provides more details. Usually increased binding activity suggests increased transcriptional activity.

Nuclear extracts of BMDCs were pretreated with progesterone or norgestrel hormones for one hour prior to exposure to either LPS or polyI:C. Nuclear extracts subjected to assessment were incubated either with radiolabeled NFκB oligoneocleotide or radiolabeled ISRE olignocleotide probes to examine the associated NFκB- or IRF3- DNA binding activities respectively. The main principle of the EMSA technique relies upon the higher molecular weight protein-DNA complexes electrophoresing move slowly through non-denatured polyacrylamide gel relative to free DNA.

4.2.5 LPS or polyI:C stimulated NFκB DNA- binding activity in BMDCs

Nuclear extracts from BMDCs exposed either to 1μg/ml LPS or 25μg/ml poly I:C for varying times (0.5-6h) were examined for NFκB DNA- binding activity. LPS induced NFκB DNA- binding activity with the time, within 30 minutes up to 2 hours and then declined (Figure 4.7, panel A). Maximal binding was observed as a 1.7-fold increase at 2h (1.731 ± 0.14 fold, $P < 0.001$). For BMDCs exposed to polyI:C (Figure 4.7, panel B), a significant increase in polyI:C induced NFκB DNA- binding activity from a half an hour, maximally at 4 hour, approximately a 1.8 fold increase

compared to control (medium) and sustained up to 6 hour (1.7806 ± 0.084 fold, $P < 0.001$). For subsequent experiments with hormones, a 2 hour period of stimulation with each agonist; LPS or polyI:C was selected.

4.2.6 The influences of progesterone and norgestrel hormone on LPS or polyI:C stimulated NFκB DNA- binding activity in BMDCs

BMDCs were pretreated with hormones progesterone or norgestrel, using 2 different concentrations (0.1 and 62.5µg/ml) one hour prior to exposure to either 1µg/ml LPS or 25µg/ml polyI:C for 2 hours. Samples were collected, nuclear extracts were prepared and assessed by EMSA for NFκB DNA- binding activity.

Effects of the hormones (progesterone and norgestrel) on BMDCs exposed to LPS examined NFκB DNA-binding activity (Figure 4.8) were unlike preceding experiments which investigated the effects of hormones on LPS induced p65 translocation into the nucleus (figure 4.2). Nuclear translocation experiments showed slight inhibition (although it was insignificant) of LPS induced p65 translocation with both hormones, whilst DNA-binding activity induced by LPS, experiments showed slight elevation with both hormones. BMDCs treated with progesterone and exposed to LPS (Figure 4.8, panel A) showed slight elevation in NFκB DNA-binding activity while with norgestrel hormone (Figure 4.8, panel B) showed significant elevation of NFκB DNA-binding activity for cells treated with 0.1µM concentration of the hormone in comparsion to cells treated with LPS+vehicle (4.945 ± 0.62 fold, $P < 0.05$). LPS+vehicle agonist showed significant elevation in LPS induced NFκB DNA-binding activity in comparison to vehicle (2.6607 ± 0.363 fold, $P < 0.001$ panel A), (3.1117 ± 0.37, $P < 0.001$ panel B).

In BMDCs pretreated with hormones and exposed to polyI:C (Figure 4.9), both hormones (progesterone panel A and norgestrel panel B) showed insignifigant effects on polyI:C induced NFκB DNA-binding activity in comparison to the agonist polyI:C+vehicle. PolyI:C + vehicle showed significant elevation in NFκB DNA-

binding activity in comparing with vehicle (1.950 ± 0.034 fold, $P < 0.001$ panel A) and (3.481 ± 0.393 fold, $P < 0.001$ panel B)

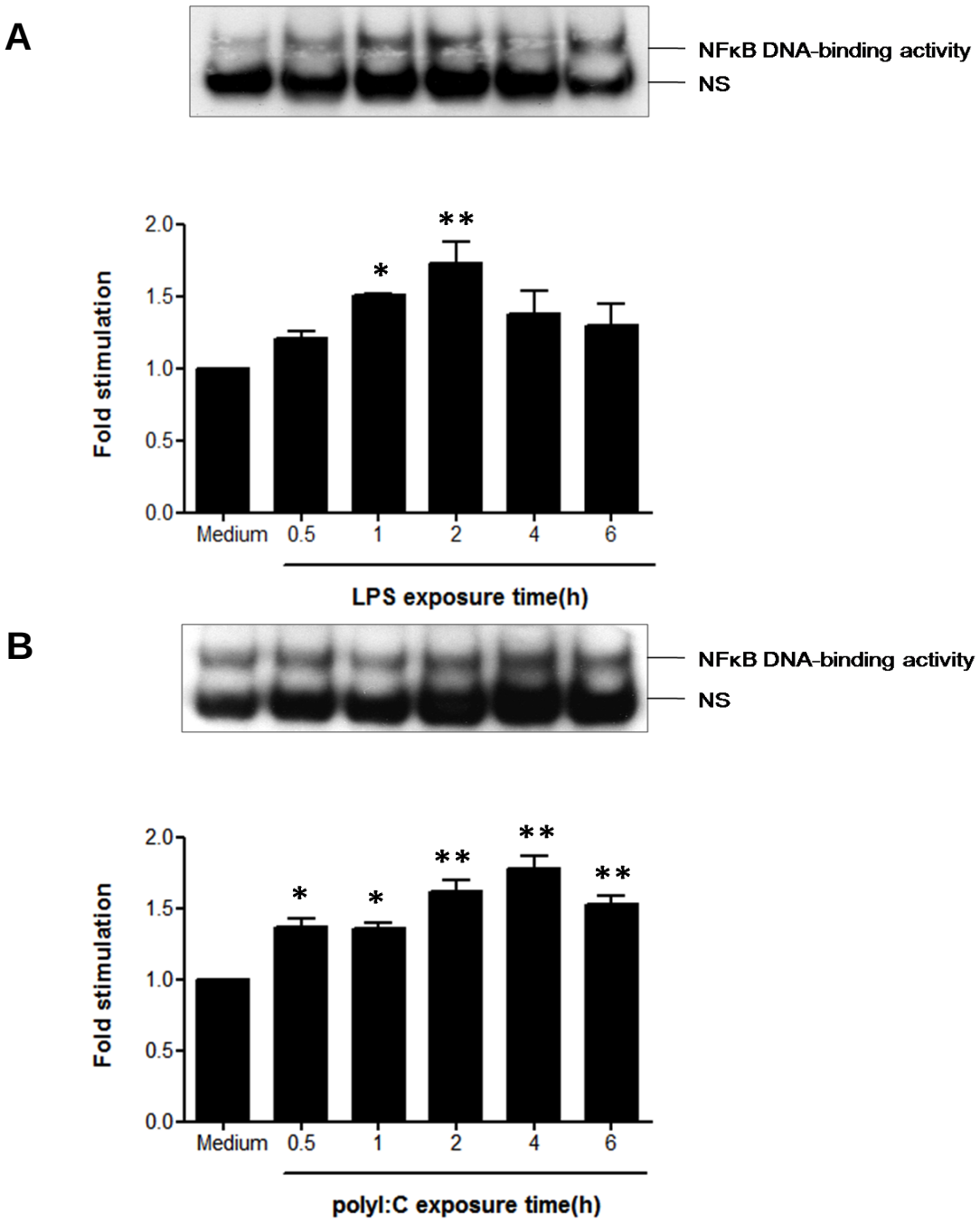


Figure 4.7. LPS or polyI:C stimulated NFκB DNA-binding activity in BMDCs. BMDCs were exposed to 1µg/ml LPS (A) or 25µg/ml polyI:C (B) for indicated times (h). Nuclear extracts were prepared as outlined in Method section and then LPS or polyI:C induced NFκB DNA-binding activity were determined by EMSA. Upper panels of A and B are representative of three independent experiments. The associated data were quantified by scanning densitometry and are depicted in lower panels of A and B. Each value represents the mean ± SEM from 3 independent experiments. *p<0.05 and **p<0.001 are statistically significant vs. control (Medium). NS, non specific band.

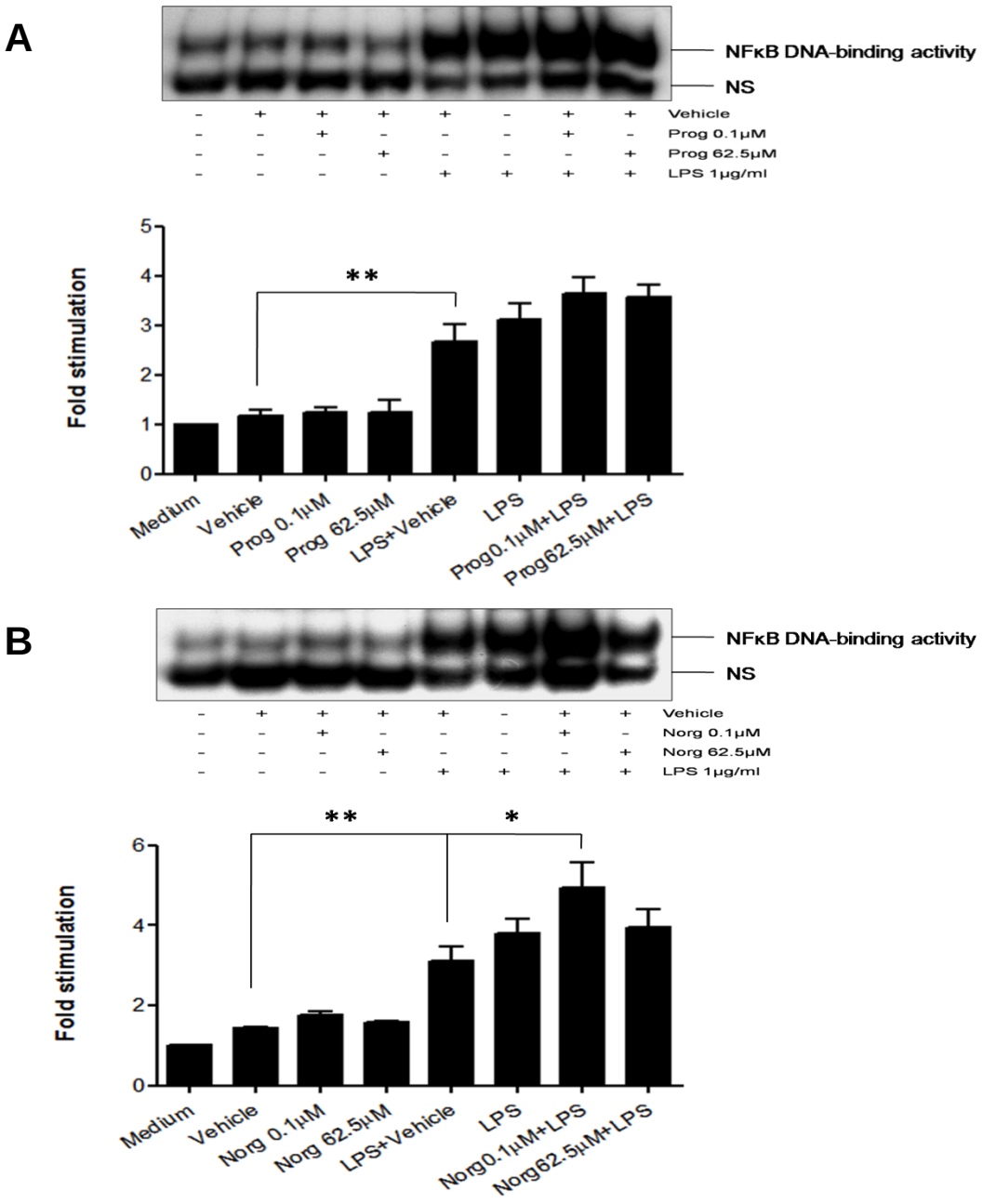


Figure 4.8. The influence of progesterone and norgestrel hormones pretreatment on LPS stimulated NFκB DNA-binding in BMDCs.BMDCs were treated with vehicle, 0.1μM and 62.5μM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 1μg/ml LPS for 2h. Nuclear extracts were prepared as outlined in Methods section. LPS induced NFκB DNA-binding activity was determined by EMSA. Upper panels of A and B are representative of three independent experiments**.** The associated data for experiments examining pretreatment with progesterone or norgestrel were quantified by scanning densitometry and are depicted in lower panels of A and B. Each value represents the mean±SEM from 3 independent experiments. *p<0.05 and **p<0.001 are statistically significant vs. LPS+vehicle. NS, non specific band.

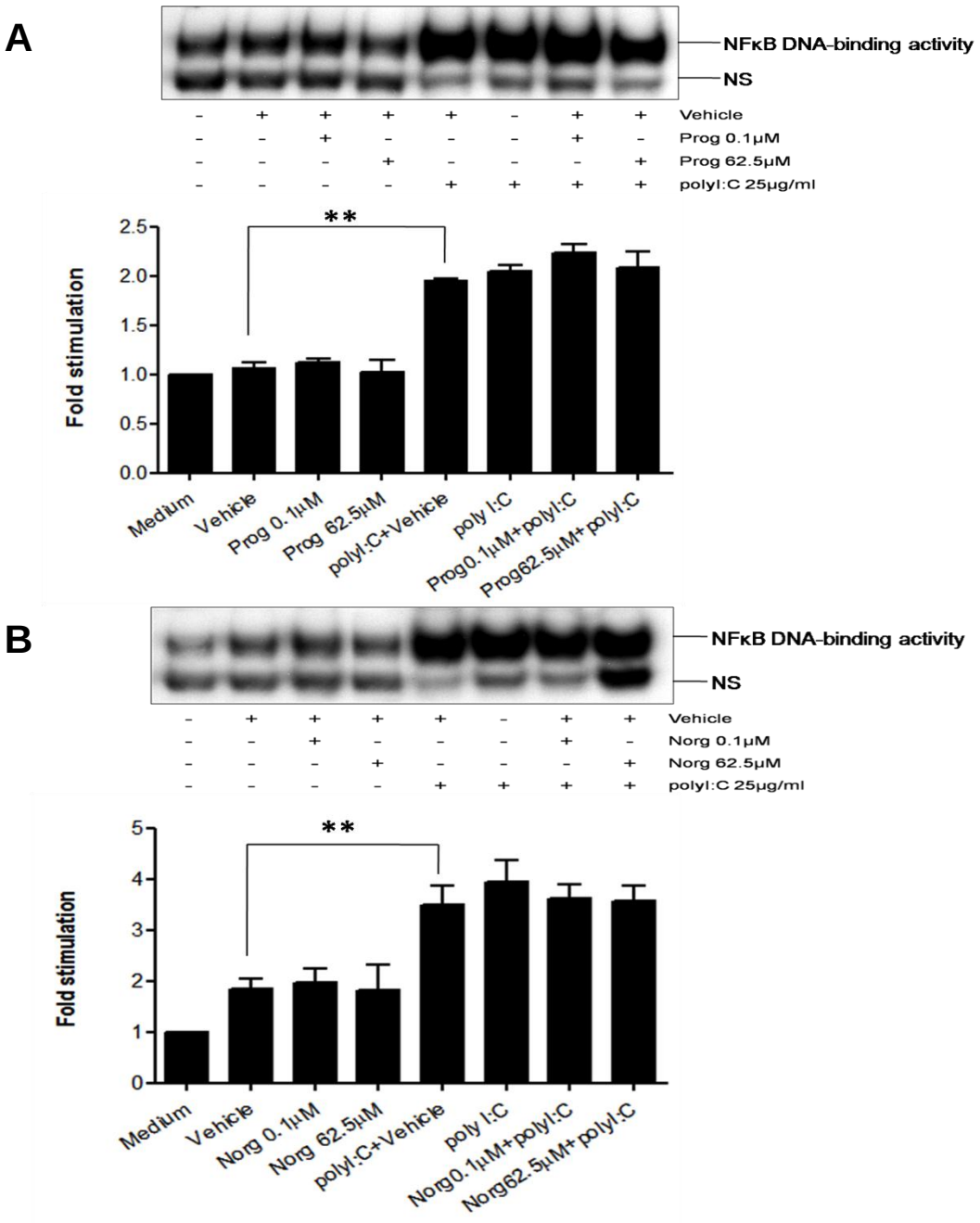


Figure 4.9. The influence of progesterone and norgestrel hormones pretreatment on polyI:C stimulated NFκB DNA-binding in BMDCs. BMDCs were treated with vehicle, 0.1μM and 62.5μM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 25μg/ml polyI:C for 2h. Nuclear extracts were prepared as outlined in Methods section. PolyI:C induced NFκB DNA-binding activity was determined by EMSA. Upper panels of A and B are representative of three independent experiments. The associated data for experiments examining pretreatment with progesterone or norgestrel were quantified by scanning densitometry and are depicted in lower panels of A and B. Each value represents the mean ± SEM from 3 independent experiments. **p<0.001 is statistically significant vs. polyI:C+vehicle. NS, non specific band.

4.2.7 LPS or polyI:C stimulated ISRE DNA- binding activity in BMDCs

Following the investigation of IRF3 nuclear translocation, protein –DNA binding activity was assessed with respect to the ISRE consensus sequence which is the specific DNA sequence targeted by IRF3 and exists in the promoter of many genes regulated by IRF3. Nuclear extracts from BMDCs exposed either to 1µg/ml LPS or 25µg/ml poly I:C for varying times (0.5-6h), were examined for ISRE DNA-binding activity.

LPS induced ISRE DNA- binding activity (Figure 4.10, panel A) increased significantly after 2 hour and sustained up to 4 hour and then declined. Cells exposed to LPS for 2h displayed a 1.4 fold increase vs. control (medium) (1.433 ± 0.057 fold, $P < 0.05$). poly I:C induced ISRE DNA-binding activity increased significantly after an hour and was sustained up to 6 hours (Figure 4.10, panel B). Cells exposed to polyI:C for 2h showed a 2-fold increase vs. control (medium) (2.152 ± 0.281 fold, $P < 0.05$). For subsequent experiments with hormones, a 2 h period of stimulation with each agonist; LPS or polyI:C was selected.

4.2.8 The influences of progesterone and norgestrel hormone on LPS or polyI:C stimulated ISRE DNA- binding activity in BMDCs

BMDCs were pretreated with hormones progesterone or norgestrel, using 2 different concentrations (0.1 and 62.5µgM) one hour prior to exposure to either 1µg/ml LPS or 25µg/ml polyI:C for 2 hours. Samples were collected, nuclear extracts were prepared and assessed by EMSA for ISRE DNA- binding activity.

Effects of the hormones (progesterone and norgestrel) on BMDCs exposed to LPS exerted reduction of ISRE DNA-binding activity (Figure 4.11). The higher concentration of progesterone (Figure 4.11, panel A) inhibited significantly LPS-induced ISRE DNA-binding activity in comparison with LPS + vehicle (1.281± 0.095, $P < 0.001$). The higher norgestrel concentration (Figure 4.11, panel B) also inhibited LPS induced ISRE DNA-binding activity in comparison to LPS + vehicle

(1.594 ± 0.090, $P < 0.05$). Both hormones at higher concentration (62.5µM) reduced polyI:C- induced ISRE DNA-binding activity (Figure 4.12) relative to polyI:C+ vehicle. This represented a reduction from 2.13 to 1.29 for progesterone, panel A (1.292 ± 0.173, $P < 0.05$) and from 2.43 to 1.66 for norgestrel, panel B (1.662 ± 0.127, $P < 0.05$) which was appreciated as 39% and 32% inhibition respectively.

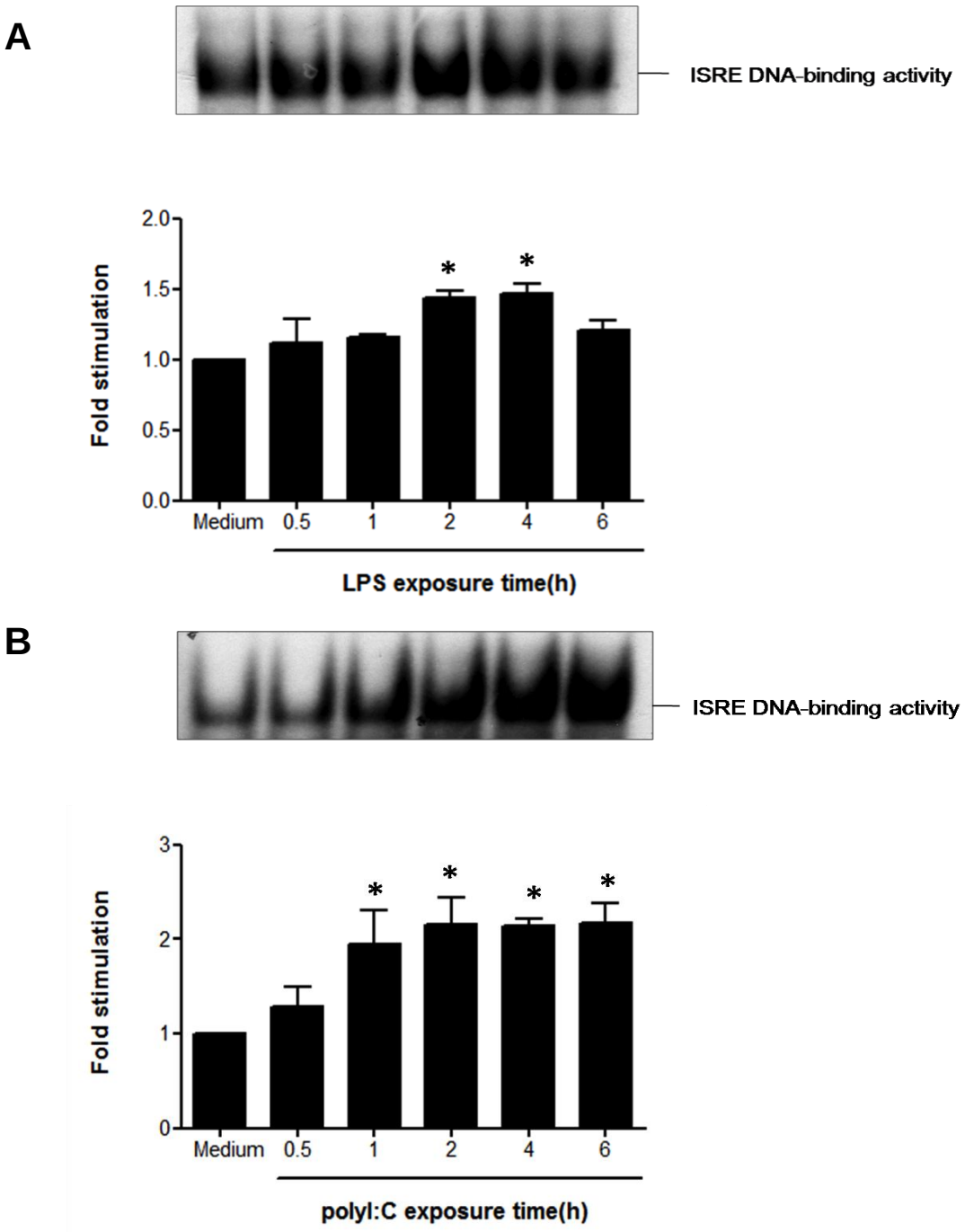


Figure 4.10. LPS or polyI:C stimulated ISRE DNA-binding activity in BMDCs. BMDCS were exposed to 1µg/ml LPS (A) or 25µg/ml polyI:C (B) for indicated times (h). Nuclear extracts were prepared as outlined in Methods section and then LPS or polyI:C induced ISRE DNA-binding activity were determined by EMSA. Upper panels of A and B are representative of three independent experiments. The associated data were quantified by scanning densitometry and are depicted in lower panels of A and B. Each value represents the mean ± SEM from 3 independent experiments. *$p<0.05$ is statistically significant vs. control (Medium).

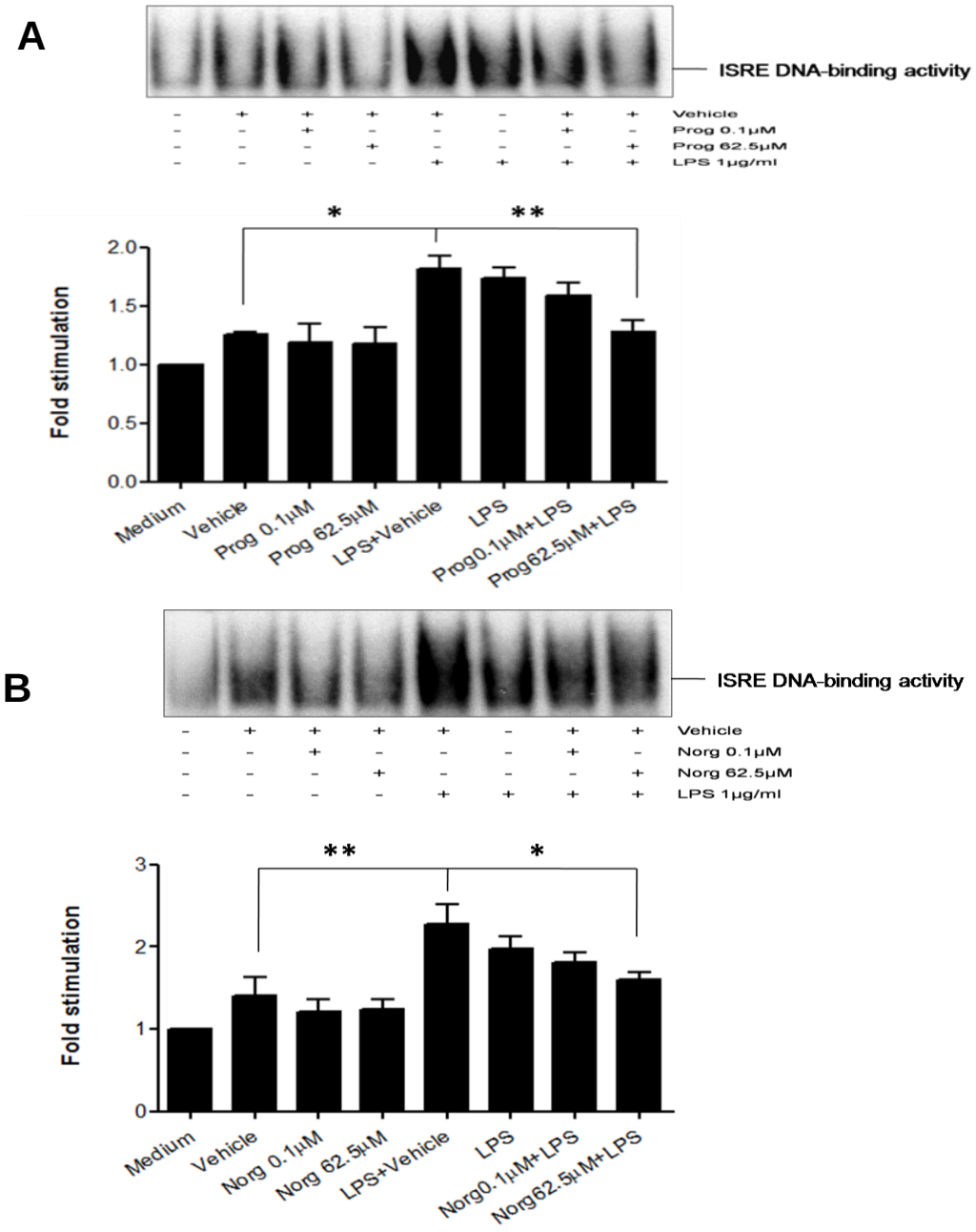


Figure 4.11. The influence of progesterone and norgestrel hormones pretreatment on LPS stimulated ISRE DNA-binding in BMDCs. BMDCs were treated with vehicle, 0.1μM and 62.5μM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 1μg/ml LPS for 2h. Nuclear extracts were prepared as outlined in Methods section. LPS induced ISRE DNA-binding activity was determined by EMSA. Upper panels of A and B are representative of three independent experiments. The associated data for experiments examining pretreatment with progesterone or norgestrel were quantified by scanning densitometry and are depicted in lower panels of A **a**nd B. Each value represents the mean ± SEM from 3 independent experiments. *p<0.05 and **p<0.001 are statistically significant vs. LPS+vehicle**.**

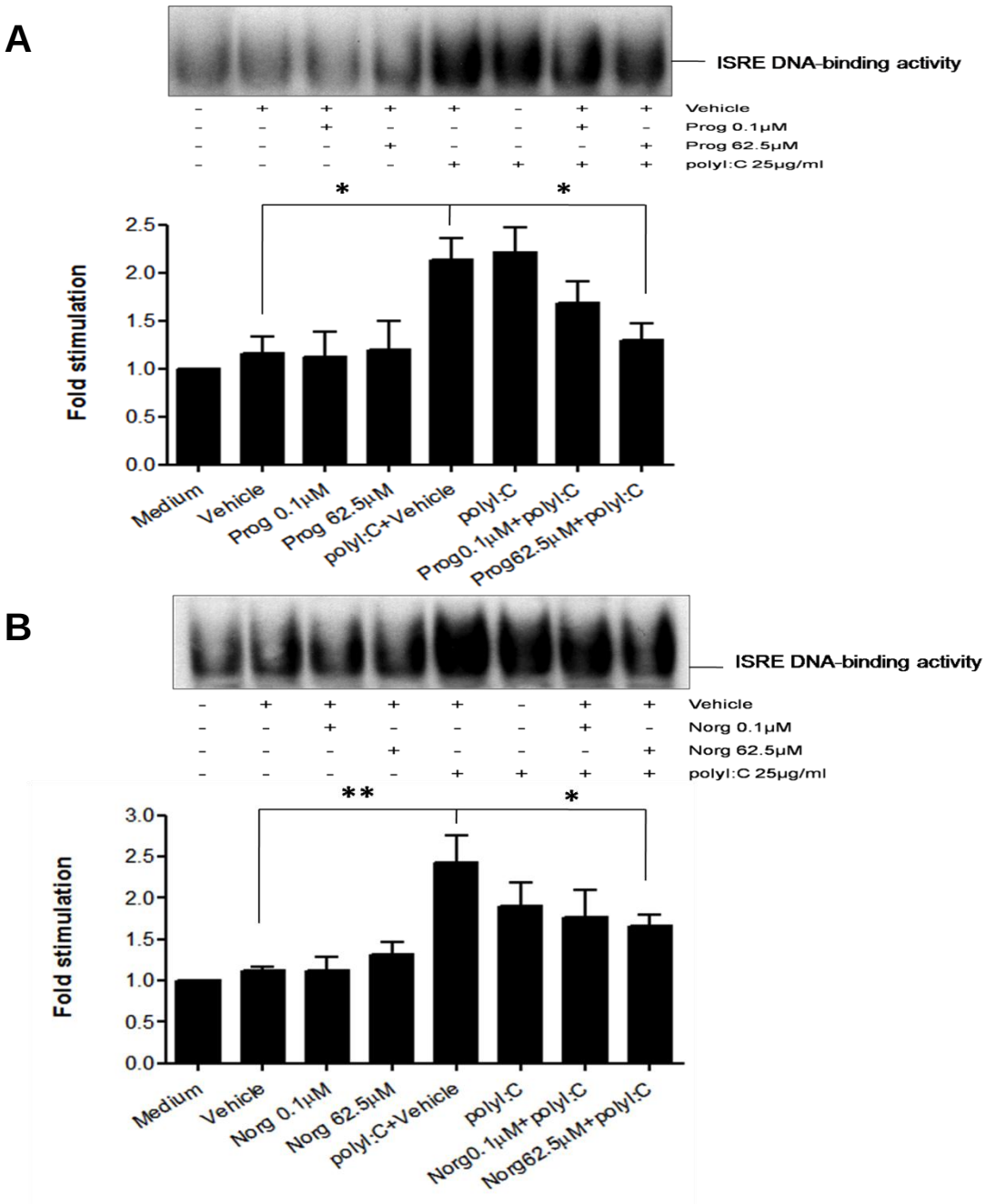


Figure 4.12. The influence of progesterone and norgestrel hormones pretreatment on polyI:C stimulated ISRE DNA-binding in BMDCs. BMDCs were treated with vehicle, 0.1μM and 62.5μM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 25μg/ml polyI:C for 2h. Nuclear extracts were prepared as outlined in Methods section. PolyI:C induced ISRE DNA-binding activity was determined by EMSA. Upper panels of A and B are representative of three independent experiments**.** The associated data for experiments examining pretreatment with progesterone or norgestrel were quantified by scanning densitometry and are depicted in lower panels of A and B. Each value represents the mean ± SEM from 3 independent experiments. *p<0.05 and **p<0.001 are statistically significant vs. polyI:C+vehicle.

4.3 Discussion

Dendritic cells are among the cellular immune components of female reproductive system. In particular they reside at the maternal- fetal interface as a result of their tendency to populate mucosal sites such as skin, gut and deciduas (Tagliani and Erlebacher, 2011). DCs as efficient innate immune cells serve dual roles during pregnancy. These roles involve protecting the mother against infection by activation of the immune response and maintaining pregnancy by induction of tolerance at the maternal-fetal interface (Blois *et al*., 2007; Collins *et al*., 2009). Progesterone hormone mediated its classic action by binding cytoplasmic progesterone receptor, inducing dimerisation and translocation into the nucleus to regulate target genes expression (Ismail *et al*., 2003). It has been reported that PRs expression is higher in female BMDCs than in males (Butts *et al*., 2008). In addition, elevated progesterone levels during metaestrus and diestrus phases of murine menstrual cycle are also associated with upregulation of PRs expression (Butts *et al*., 2011). The immune modulating effects of progesterone have been widely documented. Among these effects, the number of langerhans DCs has been reported to increase in vaginal epithelium in response to progesterone hormone (Wieser *et al*., 2001). Thus, DCs become a target of progesterone hormone especially when progesterone levels exceed physiological levels, such as during pregnancy.

As mentioned previously, IRF3 plays an essential role in Type 1 IFNs induction which in turn mediates innate and adaptive antiviral response. Activation of IRF3 is not the end of the story. Nuclear translocation and then DNA-binding both have the same importance as IRF3 phosphorylation in mediating immune response. During this course of study, results showed that polyI:C induced stronger and longer extended IRF3 nuclear translocation than LPS and this effect was maximally after 2h of stimulation by both agonists. The same findings were reported by Navarro and David, (1999) and Kawai *et al* (2001), as they found that LPS induces IRF3 translocation and ISRE binding at 2h after stimulation. polyI:C also induced stronger and long lasting p65 translocation than LPS, however, both agonists induced p65 translocation maximally after 2h of stimulation. These differences may attribute to the signalling pathways of the two receptors. TLR4 utilises two different adaptor

molecules to activate NFκB by two different mechanisms; the early phase relies upon MyD88 and the delayed phase of activation relies upon the TRIF adaptor molecule and associated signalling (Fitzgerald *et al*., 2004), whilst TLR3 activates NFκB by TRIF-dependent pathway only (Jiang *et al*., 2004). Each pathway links to different related proteins and activating kinases (Kumar *et al*., 2011). In addition, even with TRIF-dependent pathway, TLR4 specifically utilises the adaptor–related protein TRAM. TRAM is essential to TLR4 mediated cytokine and IFNβ production and up-regulation of surface markers (Yamamoto *et al*., 2003b). TLR3 had been reported to induce a greater intensity and a longer duration of antiviral gene expression than TLR4 (Doyle *et al*., 2003). NFκB and IRF3 are the key factors in regulating these gene expression events (Doyle *et al*., 2002). Moreover, TLR3 is well known to activate by dsRNA and its synthetic analogue (poly I:C), mediating a potent antiviral response by inducing proinflammatory cytokines, Type 1 IFNs and DCs maturation (Matsumoto and Seya, 2008; Marshall-Clarke *et al*., 2007).

During current section of the study, the effects of progesterone hormone were compared with norgestrel as an attempt to investigate any potential effects mediated through the progesterone receptor only. The experiments pursued examined the influence of progesterone on LPS- or polyI:C- induced p65/RelA and IRF3 translocation , results showed, both hormones inhibited LPS- and polyI:C- induced p65/RelA and IRF3 nuclear translocation. However, the inhibition of p65/RelA and IRF3 translocation appeared more intense via TLR3 which may be mediated through progesterone receptor as both progesterone and norgestrel inhibit poly I:C-induced p65/RelA and IRF3 translocation. Failure of IRF3 and NFκB to translocate may mean retarded transcription of target genes such as cytokines production, chemokines and DCs maturation. Hormones mediated inhibition of NFκB and IRF3 translocation were clearer when cells were stimulated by polyI:C as a TLR3 related response. As TLR3 expression and activation in DC plays vital role in induction of antiviral responses, activation of DCs and induction of adaptive immunity (Gauzzi *et al*., 2010; Jelinek *et al*., 2011), therefore, it could be suggested that the inhibitory mechanisms of progesterone may be mediated by inhibition of IRF3 and NFκB translocation which have a key role in mounting antiviral response. (Butts *et al*., 2011) reported that elevated levels of progesterone hormone inhibit TNFα, IL-1β and

IL-12 cytokines production that are mediated through PRs in DCs. Besides, it interferes with STAT1 activation as a non-genomic effect of the hormone. Similarly Jones *et al* (2010) reported that progesterone downeregulates IL-6 and IL-12 in BMDCs. STAT1 is involved in late phase of Type 1 IFNs production. It incorporates in autocrine/ paracrine loop of Type 1 IFNs production by assembling with STAT2 and IRF9 to form ISGF3 heterodimer (Doyle *et al*., 2002; Honda *et al*., 2006). So further investigations may be required such as measure STAT1, STAT2 and IRF9 expression or phosphorylation as suggestive proteins involved in a pathway may be targeted by progesterone as one of its immune- modulatory mechanisms. It has been found that women on hormonal contraceptive containing medroxyprogesterone acetate (MPA) may reactivate latent herpes simplex virus (HSV) by inhibiting $C8^+$ T cell (Cherpes *et al*., 2008). Another study (Morrison *et al*., 2010) reported that risk of HIV acquisition increases in women use depot-medroxyprogesterone contraceptive. Thus, progesterone may contribute in disturbing antiviral response.

The experiments studying the NFκB and ISRE DNA-binding activity showed that both LPS and poly I:C induced binding activity and poly I:C treated cells showed long lasting effect up to 6 hours. The following experiments examined the effects of the hormones on NFκB DNA-binding activity, results showed quite different pattern with both LPS and poly I:C from previous experiments examined the influence of hormones on p65/RelA translocation. Results showed no effects of the hormones on LPS- or poly I:C- induced NFκB DNA-binding activity with exception of norgestrel which evoked increase of LPS-induced NFκB DNA-binding activity. On the other hand, the influence of both hormones on ISRE DNA-binding activity induced by LPS or poly I:C were consistent with previous results examined the effects of hormones on IRF3 tranlocation induced by the two stimulants, as the outcome was inhibition. Nuclear translocation and protein DNA-binding activity are usually expected to be consistent with each other, but this is not always true. It is difficult to expect the mechanism behind the differences between hormones influences on translocation of p65 and NFκB DNA-binding activity. This may also extend to the previous results that showed progesterone and norgestrel hormones increase IRF3 phosphorylation induced by polyI:C. Increase activity usually account as induction but it may also be repression. On the other hand, later result showed that

both hormones inhibit IRF3 and p65 nuclear translocation induced by polyI:C and also they inhibited ISRE DNA-binding activity induced by polyI:C. Other investigations such as Chromatin immunoprecipitation (ChIP) or by measuring the transcriptional activity of these transcription factors by transfection of BMDC with NFκB- or ISRE- linked luciferase plasmid and perform reporter assays may be more precise in assessement the influences of the hormone on these transcription factors DNA- binding activity.

It is now widely accepted that IRF3 has a specific autoinhibitory features as displayed in the crystal structure that keeps it inactive in the cytoplasm and masks its transcriptional activity (Lin *et al*., 1998). The C-terminal aspect of IRF3 contains the IRF association domain (IAD) which is responsible for IRF3 transcriptional activity. The crystal structure of IRF3, which is described as a network of N-terminal/ C-terminal intramolecular interactions, covers the active site of IAD domain and areas responsible for binding with CBP/p300 (Qin *et al*., 2005). Though, CBP and p300 are potent coactivators for many transcription factors and cooperate in transcription of various genes (Goodman and Smolik, 2000; Blobel, 2002), activation of IRF3 results in unfolds of the autoinhibitory structure enabling it to form homo- or hetero-dimers, translocation to the nucleus and interaction with CBP/p300 before binding specific DNA sequences. There are two sites for IRF phosphorylation; site (1) includes Ser 385 / Ser 386 while site (2) includes Ser/ Thr 396-405 (Panne *et al*., 2007). Dual phophorylation on both sites is required for optimal IRF3 activity, site 2 for alleviation of the autoinhibitory and site 1 for IRF3 dimerisation (Panne *et al*., 2007). Another study by Mori *et al*., (2004) reported that site (1) is essential for IRF3 activity. While, (Lin *et al*., 1998) considered site (2) is critical for full IRF3 activation. Hence, the site(s) of IRF3 phosphorylation affect its nuclear transloction, interaction with other proteins and DNA-binding activity. So, extend the experiments to examine IRF3 phosphorylation in nuclear extract (post translocation) in response to progesterone treatment may provide more details about individual amino acids phosphorylation that related to activity which may include repression instade of activation effect. Besides, in the current study the mechanism of progesterone receptor –transcription factor interaction have to be taken in consideration in order to explain the inhibitory role of the hormones on IRF3 translocation and DNA-binding

activity. However, these remain to be examined in an experimental setting. All steroid hormone receptors (SHRs) have similar structures and mostly possess similar binding and interaction mechanism (Griekspoor *et al*., 2007). Several molecular mechanisms have been identified for SHRs and their regulation transcription. Most SHRs have the potential to interact with other proteins and transcription factors using multiple mechanisms at the same time (Beato and Klug, 2000). It has been reported that IRF3-dependent gene transcriptions could be regulated by IRF3- Glutamate receptor interacting protein (GRIP1) interaction, an IRF3 and glucocorticoid receptor compete to bind GRIP1 that functions as corepressor protein member of p160 family. GRs are ligand-dependent transcription factors bind GRIP1 and repress transcription activity of AP1 and NFκB. Upon activation GRs displace IRF3 from GRIP1 binding site and repress AP1 and NFκB at the same promoter of target gene (Reily *et al*., 2006). According to the present results, the exact mechanism by which progesterone receptor and IRF3 interacts to regulate genes could not be predicted and requires more investigations at the level of gene transcription, genes outcome and molecular mechanism of protein-protein interactions.

CHAPTER FIVE

THE INFLUENCE OF PROGESTERONE AND NORGESTREL ON TLR3 AND TLR4 SIGNALLING OUTCOMES IN BONE MARROW DERIVED DENDRITIC CELLS (GENE EXPRESSION AND PROTEIN LEVELS)

5.1 Introduction

TLR signalling is of significance as a major participator in developing inflammatory responses and its implication in prognosis of many diseases, in particular autoimmune diseases (Wang *et al*., 2012) and cardiovascular diseases (Mullick *et al*., 2006). However, even with appropriate inflammatory responses, excessive activation of TLRs can lead to serious conditions such as septic shock (Verstak *et al*., 2007). With the availability of comprehensive data regarding the agonist action of the TLR signalling network, much interest has been given towards understanding what may represent drug targets in disease (Li *et al*., 2009). Hence, during the course of the present study, many components of TLR signalling pathways have been investigated to determine whether sex hormones act through them to mediate their immunomodulatory effects. Thus, during previous chapters of the thesis, the effects of sex hormones on the initial TLR signalling components such as kinases and transcription factors in the cytosol have been studied. This was followed by a study of the effects of the hormones on these components in the nucleus. In the current chapter the effects of hormones on the outcomes of TLR signalling on downstream products such as cytokines production have been studied. Activation of various TLRs can trigger a total of ten signalling pathways which control five output objectives, these outputs include, NFκB, cAMP response element (CRE) /AP-1, IRF3, IRF7 and reactive oxygen species (ROS) (Li *et al*., 2009). These five components regulate a large number of genes implicated in infection and inflammatory responses, cell survival and proliferation, apoptosis and wound healing processes (Richard *et al*., 2012). As mentioned previously in the introduction, many studies have reported the modulatory effects of the hormone, progesterone on NFκB and ROS. Furthermore, the current study has demonstrated modulatory effects of progesterone and its synthetic analogue norgestrel on IRF3 and NFκB. IRF3 and/or NFκB have been reported to regulate many inflammatory genes. Among these are the IL-12 family of cytokines, IL-6, and IFNβ that have been chosen for investigation in this Chapter because of their importance as inflammatory mediators.

Cytokines are critical mediators of immune responses and coordinate with other immune components to combat microbial infections. IL-12 family members are important cytokines that mediate both innate and adaptive immune responses. Family members include IL-12, IL-23, IL-27 and IL-35 (Gee *et al*., 2009; Gabay and McInnes, 2009; Van Wanrooij *et al*., 2012; Vignali and Kuchroo, 2012). There is a growing consensus that the MyD88-dependent NFκB pathway activates genes encoding IL-12p35, IL-23p19, IL-27p28 and EBI3 subunits of this family of cytokines (Carmody *et al*., 2007; Wirtz *et al*., 2005), see Figure 5.1. Furthermore, the TRIF-dependent-IRF3 pathway was found to be involved in p35 and p28 gene transcription (Molle *et al*., 2007; Molle *et al*., 2010). All family members show similarity in their subunits, receptors and signalling (Gee *et al*., 2009; Gabay and McInnes, 2009; Van Wanrooij *et al*., 2012; Vignali and Kuchroo, 2012). IL-12, IL-23 and IL-27 are secreted mainly by APCs such as macrophages and dendritic cells (Gee *et al*., 2009; Van Wanrooij *et al*., 2012; Vignali and Kuchroo, 2012) while IL-35 is secreted by naturally occurring regulatory T cells (nTreg) (Collison *et al*., 2007; Vignali *et al*., 2008).

The first member of the family to be discovered was IL-12 which is a heterodimer comprising IL-12p35 and IL-12p40 which are held together covalently by a disulphide bound. Gene expression of each subunit is regulated independently but biologically active cytokine requires the presence of both components (Langrish *et al*., 2004). The IL-12p40 gene is highly inducible and a large quantity of p40 mRNA is produced relative to p35 mRNA (Vaidyanathan *et al*., 2003) i.e. much more than required to bind p35. Excess p40 forms homodimers or monomers that act as natural inhibitors to IL-12 (Gillessen *et al*., 1995). In response to infection, IL-12 could be secreted by phagocytic and B-cells but to a lesser extent than APCs (Gee *et al*., 2009; Van Wanrooij *et al*., 2012). IL-12 promotes differentiation of naïve T cells to Th1 cells and induces optimal proinflammatory responses (Hölscher, 2004; Gee *et al*., 2009; Van Wanrooij *et al*., 2012). In addition, IL-12 induces the generation and promotes the activity of cytotoxic T and NK cells (Trinchieri, 2003). IL-12 promotes its biological effects by binding to its heterodimer receptor that is composed of the IL-12Rβ1 and IL-12Rβ2 subunits (Gee *et al*., 2009; Van Wanrooij *et al*., 2012).

IL-23 is a proinflammatory heterodimeric cytokine like IL-12. It consists of p40 and a p19 subunit, which is structurally related to the p35 subunit. The two subunits are linked covalently by a disulphide bound (Oppmann *et al*., 2000). IL-23 promotes $CD4^+$ Th17 cells and enhances IL-17 production (Aggarwal *et al*., 2003). In addition, it induces the proliferation of memory T cells (Aggarwal *et al*., 2003). IL-23 binds IL-12Rβ1 and the IL-23R but not the IL-12Rβ2 subunit (Parham *et al*., 2002).

IL-27 is also a heterodimer cytokine composed of Epstein-Barr virus (EBV) - induced gene 3 (EBI3) which is related to the IL-12p40 subunit and p28 which is structurally related to IL-12p35 (Pflanz *et al*., 2002; Crabe *et al*., 2009). Unlike IL-12 and IL-23, the two subunits of IL-27 are not linked by disulphide bounds (Pflanz *et al*., 2002). Thus each subunit can be produced separately from different cells to be bound extracellularly. However, for efficient biological activity both subunits have to be produced from the same cell (Pflanz *et al*., 2002). IL-27 interacts with the heterodimer receptor composed of WSX1 and gp130 to exert its effects (Villarino *et al*., 2004). Initially, IL-27 was considered a proinflammatory cytokine that mediates Th1 responses (Mayer *et al*., 2008). In later studies, immunosuppressive properties were reported for IL-27 (Stumhofer and Hunter, 2008).

EBI3 and IL-12p35 are linked together to form the anti-inflammatory cytokine IL-35 (Devergne *et al*., 1997). It inhibits Th17 cell development and promotes regulatory T cell proliferation. IL-35 suppresses immune responses that aggravate inflammatory diseases such as arthritis and inflammatory bowel disease (Niedbala *et al*., 2007).

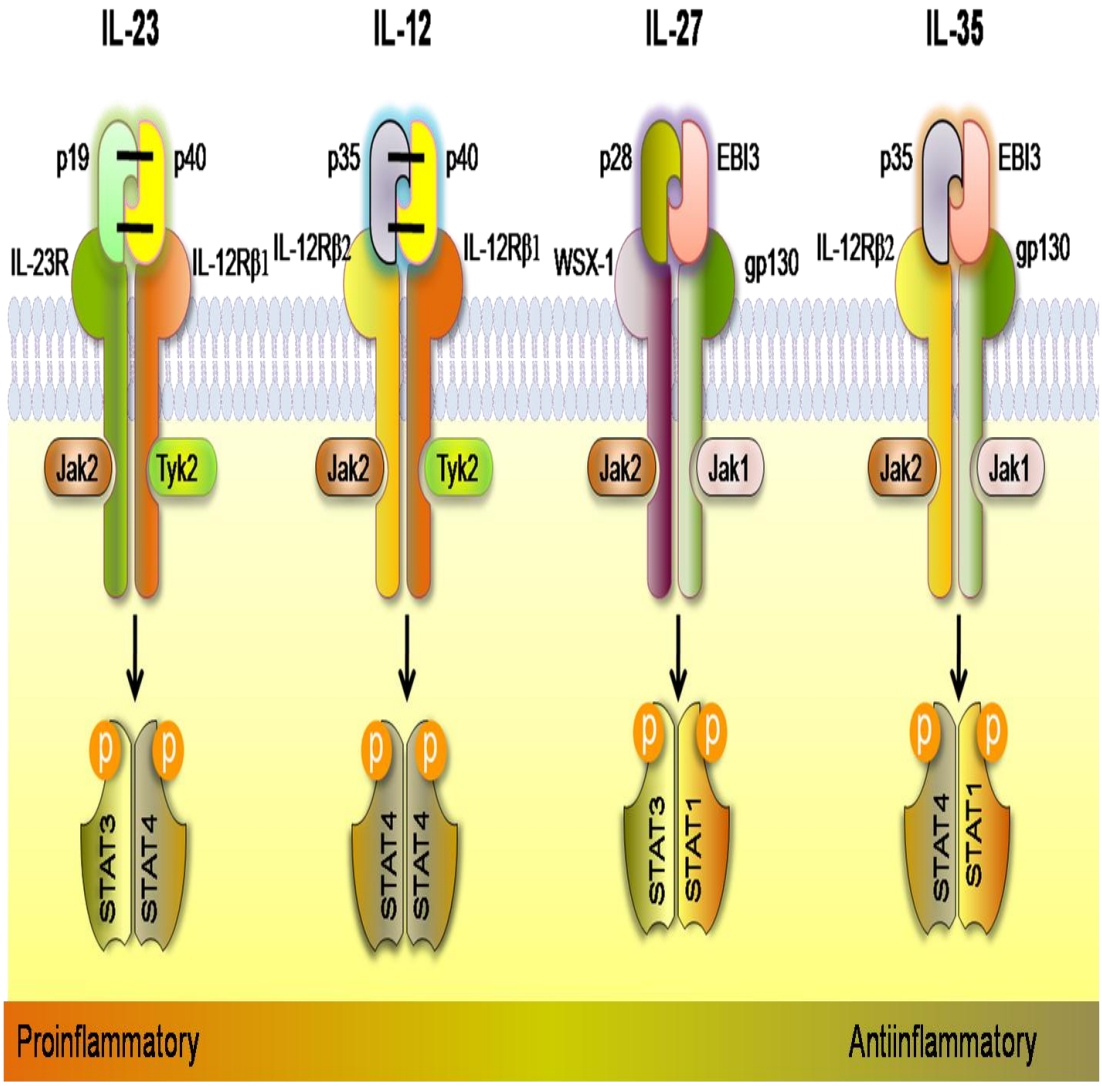


Figure 5.1. IL-12 family cytokines (adapted from Vignali and Kuchroo, 2012). All are present as heterodimers. IL-12 and IL-23 share the p40 β-chain subunit while IL-27 and IL-35 share the EBI3 β-chain subunit. The p35 α-chain subunit is shared between IL-12 and IL-35. Disulphide bonds link the two subunits of IL-12 and IL-23 which are recognised as proinflammatory cytokines. IL-27 and IL-35 lack this bond between their subunits and both cytokines display anti-inflammatory characteristics. IL-12 binds to its heterodimer receptor that consists of 2 linked sub-units IL-12Rβ1 and IL-12Rβ2. IL-12Rβ1 also binds another subunit IL-23R to form the IL-23 receptor while IL-12Rβ2 binds to the gp130 receptor subunit to form the IL-35 receptor. IL-27 receptor comprises the binding of WSX-1 and gp130. Signalling downstream from these receptors involves activation of the Jak/STAT pathway. Jak2 kinase together with Tyk2 phosphorylates the STAT3/STAT4 heterodimer and STAT4 homodimer following ligation of the IL-12 and IL-23 receptors respectively. Jak2 together with Jak1 phosphorylates STAT3/STAT1and STAT4/STAT1 heterodimers following ligation of the IL-27 and IL-35 receptors respectively.

Signalling events following ligation of the IL-12 family cytokine receptors are similar and mediated by the Jak/STAT family (Ihle, 1995) (see Figure 5.1). Jak2 and either Jak1 or Tyk2 are recruited to activate different STAT homo or heterodimers downstream from the receptors. The signalling cascade downstream of the IL-12 receptor is mediated by the homodimers of STAT4, IL-23 signalling is mediated via the heterodimers of STAT3/STAT4, IL-27 signalling is mediated via the heterodimers of STAT1/STAT3, and IL-35 mediated signalling events by phosphorylation of the heterodimers of STAT1/STAT4 (Gee *et al*., 2009; Gabay and McInnes, 2009; Van Wanrooij *et al*., 2012; Vignali and Kuchroo, 2012). See Figure 5.1.

Other inflammatory mediators studied in the current chapter are IL-6 and IFNβ. IL-6 is a member of the type 1 cytokine family that includes IL-12, IL-23 and IL-27. It shares specific structural motifs (Four-helix bundles) and uses gp130 receptor subunit alongwith other family members (Stumhofer *et al*., 2010). Although generally known as a proinflammatory cytokine secreted from both immune and non-immune cells it exerts many non-immune effects (Rohleder *et al*., 2012). The IL-6 receptor comprises IL-6Rα and gp130. Signalling downstream from the receptor is mediated through the Jak/STAT pathway by activation of the STAT3 homodimer (Guo *et al*., 2012b).

It is widely documented that IFNβ production in response to viral infection requires the interaction of IRF3, NFκB and AP-1 with different positive regulatory domains in the promoter region of the IFNβ gene (Maniatis *et al*., 1998; Yoneyama *et al*., 1998; Levy *et al*., 2002). Signalling of IFNβ, as with other Type1 IFNs is achieved in 2 steps. Initial trace secreted amounts of Type 1 IFNs produced upon viral infection, bind to Type 1 IFN receptors by either autocrine or paracrine mechanisms (IFNAR1 and IFNAR2). Downstream receptor signalling includes activation of IFN-stimulated gene 3 (ISG3) which translocates to the nucleus and induces a large number of molecules, designated as IFN inducible genes, that control many cell processes such as growth, survival, and protein synthesis as well as the release of Type 1 IFNs and induction of DC maturation (Uematsu and Akria, 2007).

In this Chapter the effects of progesterone and its synthetic analogue norgestrel were examined on the expression of proinflammatory cytokines associated with the NFκB and IRF3 signalling pathways in BMDCs following TLR4 and TLR3 ligation. Comparative expression of inflammatory cytokine genes and their products gave details as to the immune-modulatory effects of progesterone

5.2 Results

The influence of progesterone on the mRNA expression of the IL-12 family of cytokines in BMDCs.

As mentioned previously, each member of the IL-12 family of cytokines consists of two subunits regulated separately by two different genes. Progesterone mediated effects on TLR-stimulated IL-12, IL-23, and IL-27 gene expressions were examined by analysis of component subunits, IL-12p35, IL-23p19, IL-27p28 and EBI3 respectively. IL-35 is secreted by regulatory T cells and, therefore, was excluded from the study.

5.2.1 LPS- and polyI:C- induced IL-12p35 mRNA expression in BMDCs

IL-12p70 is a proinflammatory cytokine produced from APC in response to different stimuli. It consists of the p35 and p40 subunits (Grumont *et al*., 2001). It has been found that p35 mRNA expression in monocytes and DCs increases in response to the same stimuli that induces p40 expression and IL-12 production (Schulz *et al*., 2000). As mentioned above any excess of p40 needs an equivalent amount of p35 to produce IL-12. Otherwise, the excess will be utilised to inhibit IL-12 production by forming p40 monomer or homodimers (Gillessen *et al*., 1995). During this study p35 mRNA expression has been examined instead of p40 mRNA expression because it comprises a subunit of both IL-12 and IL-23.

BMDCs were exposed to 1μg/ml LPS for 2, 4 and 6h (Figure 5.2, panel A). Results showed a significant elevation of IL-12p35 mRNA compared to untreated

cells at 2h (22.73 ± 6.82, $P < 0.05$) control (4.33 ± 3.01), 4h (25.45 ± 2.02, $P < 0.05$) control (6.33 ± 2.50), and most significantly at 6h (60.86 ± 4.57, $P < 0.0001$) control (7.33 ± 2.00). Stimulation with 25µg/ml polyI:C over the same period (Figure 5.2, panel B), resulted in a similar profile of p35 mRNA expression in comparison with LPS stimulated cultures although expression was significantly greater at 4 and 6 h. There was a significant increase in polyI:C induced IL-12p35 mRNA compared to untreated cells after 4h of stimulation (59.58 ± 6.77, $P < 0.0001$) control (6.33 ± 2.50) and after 6h (85.21 ± 9.35, $P < 0.0001$) control (7.33 ± 2.00).

5.2.2 The influence of progesterone and norgestrel on LPS and polyI:C induced IL-12p35 mRNA expression in BMDCs

Two concentrations (0.1 and 62.5 µM) of progesterone and norgestrel were used to be consistent with previous experiments. Cells were pretreated with either progesterone or norgestrel one hour prior to expose to 1µg/ml LPS or 25µg/ml polyI:C for 2, 4 and 6 hours. Expression levels of p35 mRNA were quantified to examine the effects of the hormones by comparison with the agonist (LPS or polyI:C). Although there was no significant difference between IL-12p35mRNA expression in cells incubated in media alone or exposed to the vehicle (Chloroform), comparisons were carried out with agonist plus vehicle to exclude any influences of the vehicle on the results as with previous experiments. During the current set of experiments hormone controls did not reveal any significant differences to the medium or vehicle controls and consequently they were excluded from the final figures to aid clarity of presentation. Progesterone and norgestrel treatment had no influence on LPS induced p35 mRNA expression after 2 and 4h of stimulation (Figure 5.3, panel A and B). However, pretreatment of cells with high concentration of norgestrel (62.5µM) showed a significant reduction of LPS- induced p35 mRNA expression, after 6 hours (41.87 ± 5.73, $P < 0.05$) compared to agonist (LPS+vehicle) (66.99 ± 5.35) (Figure 5.3, panel C). For cells treated with polyI:C, both concentrations of norgestrel and the high concentration of progesterone, all inhibited IL-12p35 mRNA expression significantly 6h post stimulation with polyI:C (Figure 5.4, panel C). The results were, norgestrel (0.1µM) (89.43 ± 5.29, $P < 0.05$), norgestrel (62.5µM) (69.02 ± 9.02, $P < 0.05$) and progesterone (62.5µM) (75.45 ±

10.65, $P < 0.05$) compared with agonist (polyI:C + vehicle) (94.74 ± 4.63). On the other hand, neither hormone had any influence on polyI:C induced p35mRNA expression at 2 and 4h after stimulation (Figure 5.4, panel A and B)

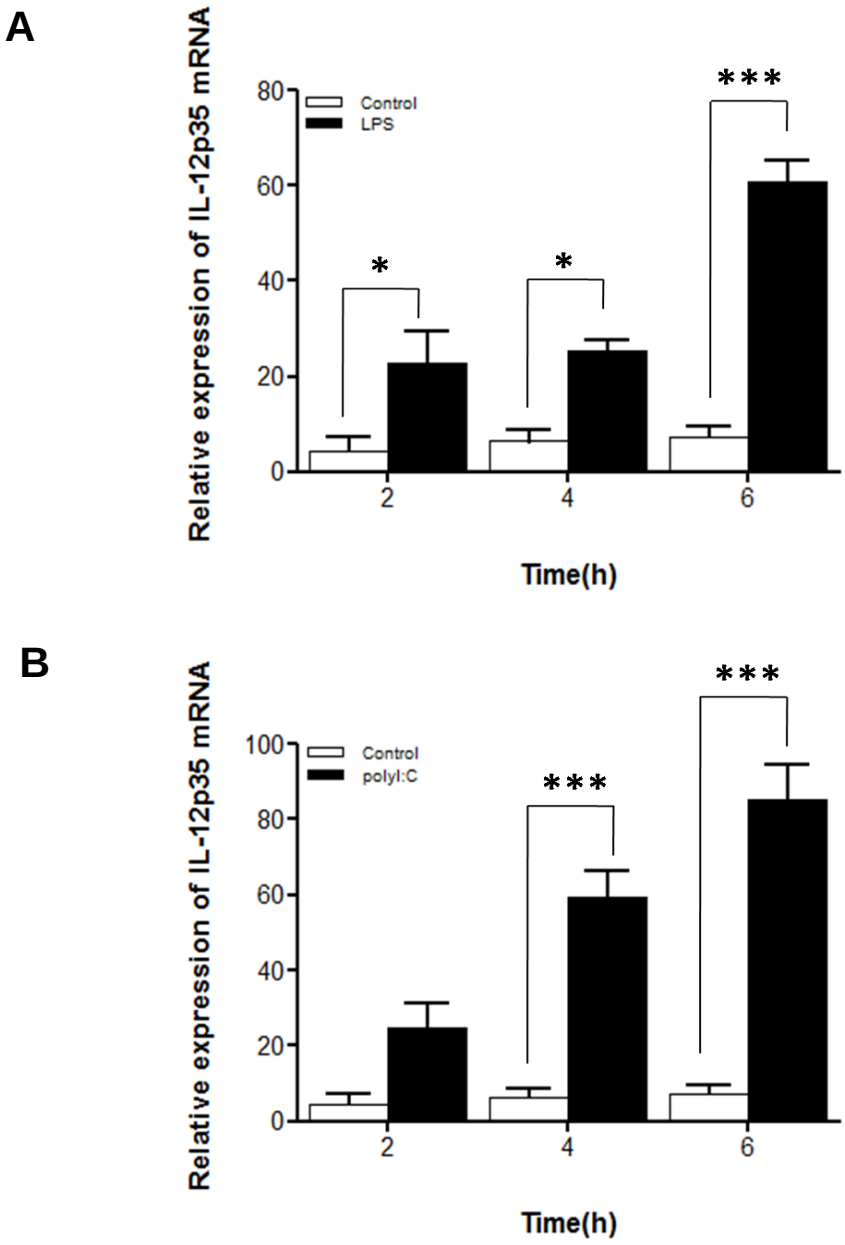


Figure 5.2. LPS- and polyI:C- induced IL-12p35 mRNA expression in BMDCs. Cells were left untreated (control) or exposed either to 1µg/ml LPS (A) or 25µg/ml polyI:C (B) for the indicated times (h). IL-12p35 mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for IL-12p35 mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean± SEM from 3 independent experiments. *P< 0.05 and ***P< 0.0001 are statistically significant vs. control.

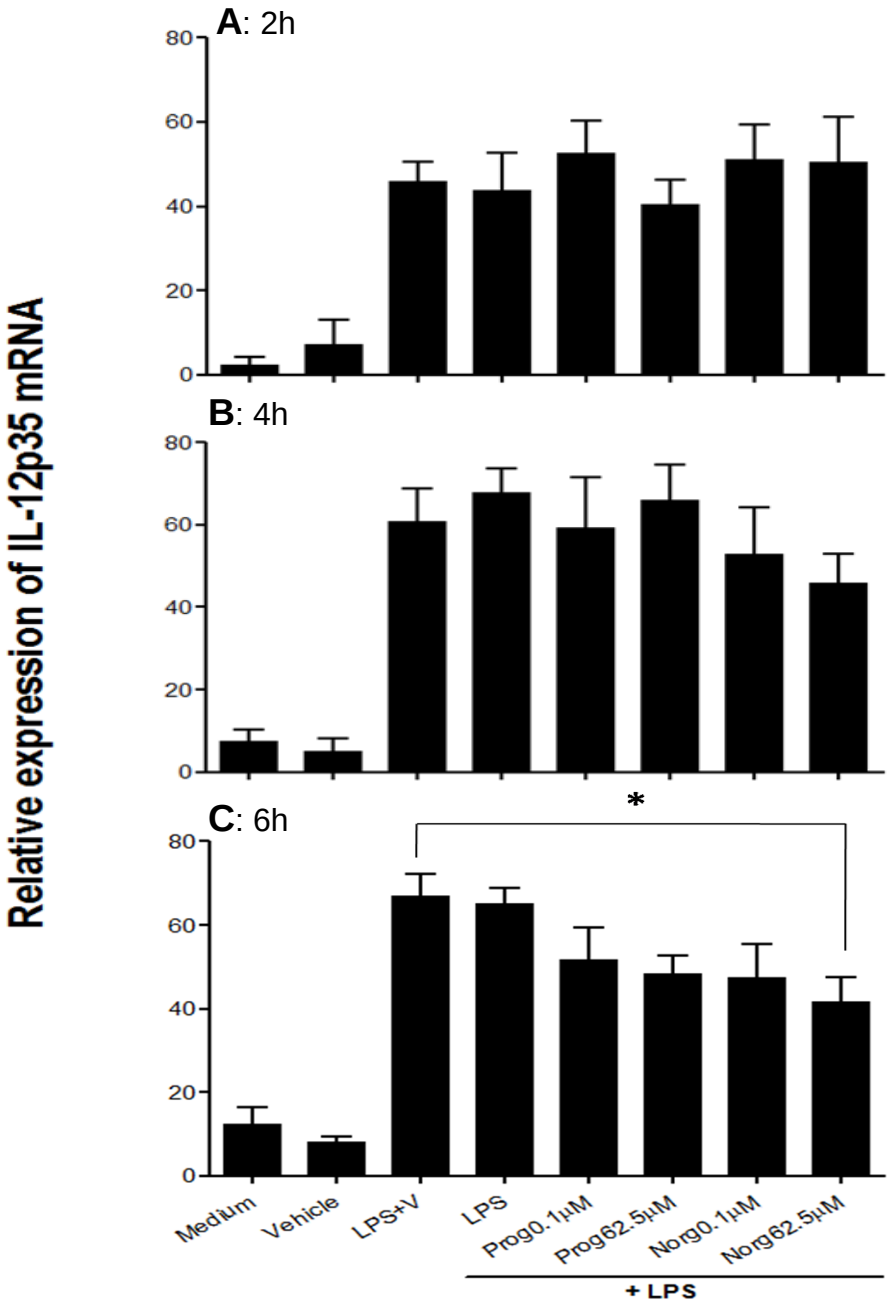


Figure 5.3. The influence of progesterone and norgestrel on LPS-induced IL-12p35 mRNA expression in BMDCs. Cells were treated with vehicle (V), 0.1µM and 62.5µM of either progesterone (Prog) or norgestrel (Norg) one hour prior to exposure to 1µg/ml LPS for 2h (A), 4h (B) or 6h (C). IL-12p35 mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for IL-12p35 mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean±SEM from 3 independent experiments. *P< 0.05 is statistically significant vs. agonist plus vehicle (LPS+V).

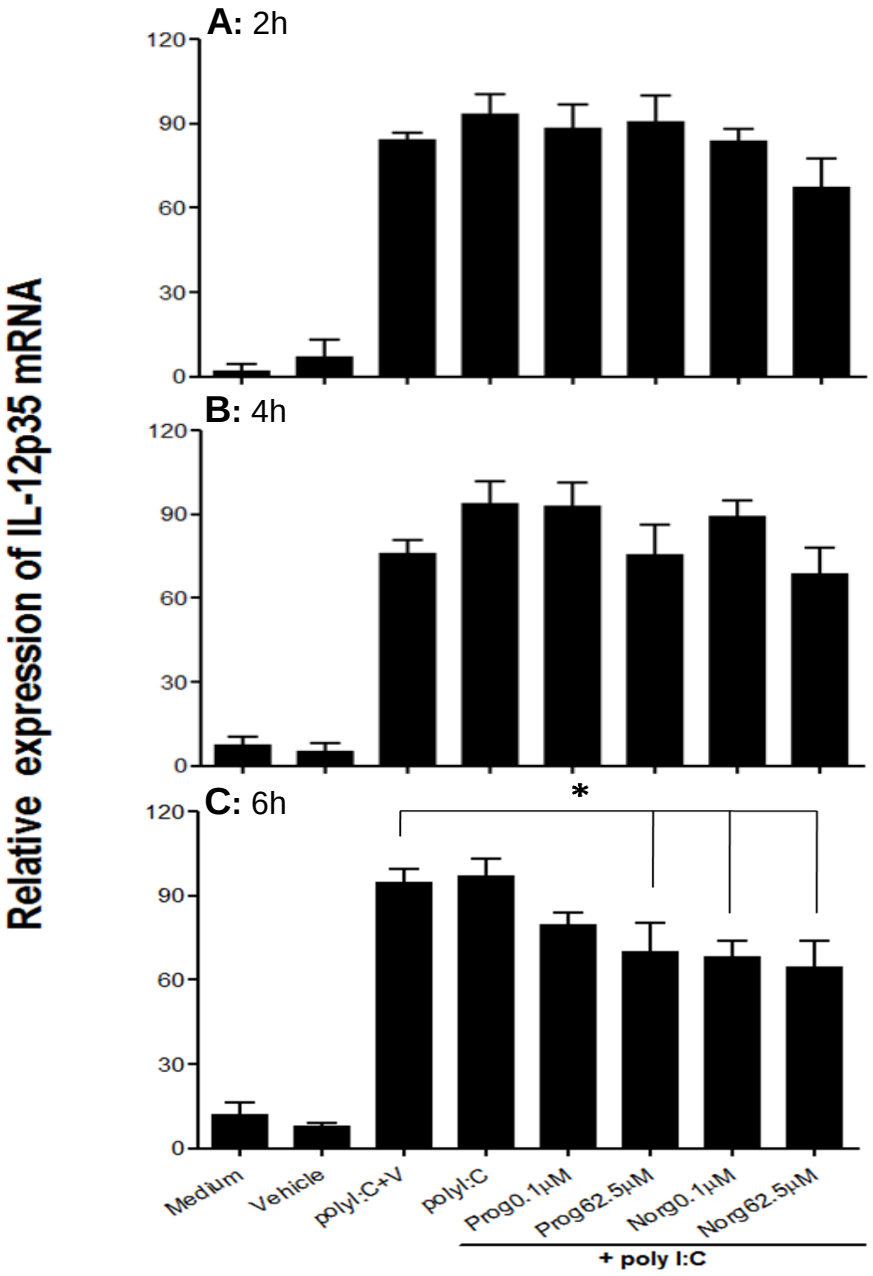


Figure 5. 4. The influence of progesterone and norgestrel on polyI:C-induced IL-12p35 mRNA expression in BMDCs. Cells were treated with vehicle (V), 0.1µM and 62.5µM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 25µg/ml polyI:C for 2h (A), 4h (B) or 6h (C).IL-12p35 mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for IL-12p35 mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean±SEM from 3 independent experiments. *P< 0.05 is statistically significant vs. agonist plus vehicle (polyI:C+V).

5.2.3 LPS- and polyI:C- induced IL-23p19 mRNA expression in BMDCs

Bioactive IL-23 cytokine production requires the expression of both the p19 and p40 subunits within the same cells (Carmody *et al*., 2006). In the present study p19 mRNA has been examined as a unique subunit involved with IL-23 production.

BMDCs were exposed to 1µg/ml LPS for 2, 4 and 6h (Figure 5.5, panel A). Results showed significant elevation of IL-23p19 mRNA copies compared with that of untreated cells after 4h of stimulation (45.18 ± 4.59, $P < 0.001$), control (10.67 ± 4.21) and after 6h (80.25 ± 9.87, $P < 0.0001$), control (8.87 ± 2.23). For cells exposed to 25µg/ml polyI:C for the same time intervals (Figure 5.5, panel B), result also showed a significant increase in IL-23p19 mRNA levels in comparison with untreated cells after 2h of stimulation (43.49 ± 10.86, $P < 0.001$) control (6.48 ± 2.11), after 4h (64.71 ± 4.58, $P < 0.0001$) control (10.67 ± 4.21) and after 6h (77.95 ± 12.05, $P < 0.0001$) control (8.87 ± 2.23).

5.2.4 The influence of progesterone and norgestrel on LPS-and polyI:C-induced IL-23p19 mRNA expression in BMDCs

BMDCs were treated with two concentrations of progesterone and norgestrel as in previous experiments prior to expose to LPS for 2, 4 and 6h (Figure 5.6, panel A, B and C) or polyI:C for the same times (Figure 5.7, panel A, B and C). Results showed that neither of the hormones had any influence on LPS- or polyI:C- induced IL-23p19 expression over the relevant time points.

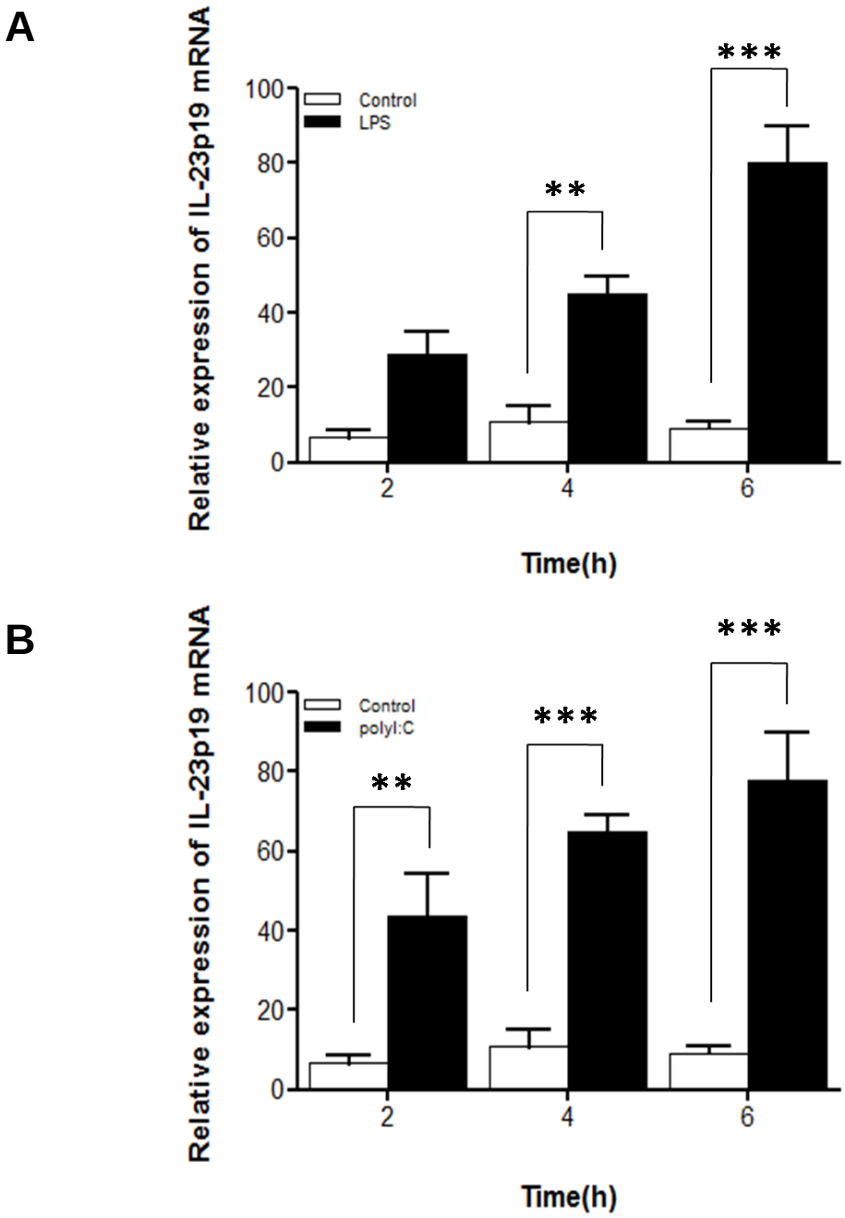


Figure 5.5. LPS- and polyI:C- induced IL-23p19 mRNA expression in BMDCs. Cells were left untreated (control) or exposed either to 1µg/ml LPS (A) or 25µg/ml polyI:C (B) for the indicated times (h). IL-23p19 mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for IL-23p19 mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean±SEM from 3 independent experiments. **P< 0.001 and ***P< 0.0001 are statistically significant vs. control.

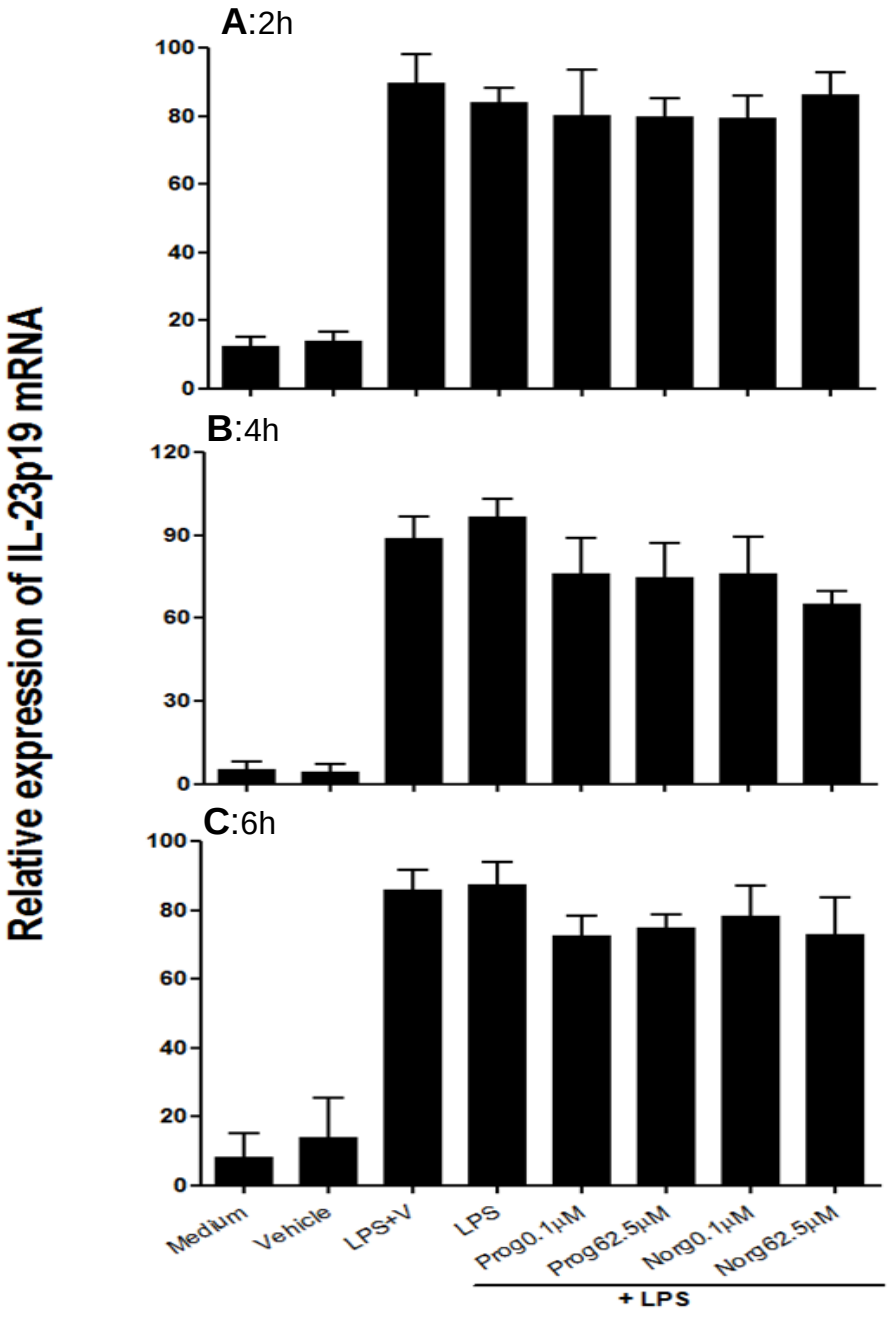


Figure 5.6. The influence of progesterone and norgestrel on LPS-induced IL-23p19 mRNA expression in BMDCs. Cells were treated with vehicle (V), 0.1µM and 62.5µM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 1µg/ml LPS for 2h (A), 4h (B) or 6h (C).IL-23p19 mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for IL-23p19 mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean ±SEM from 3 independent experiments.

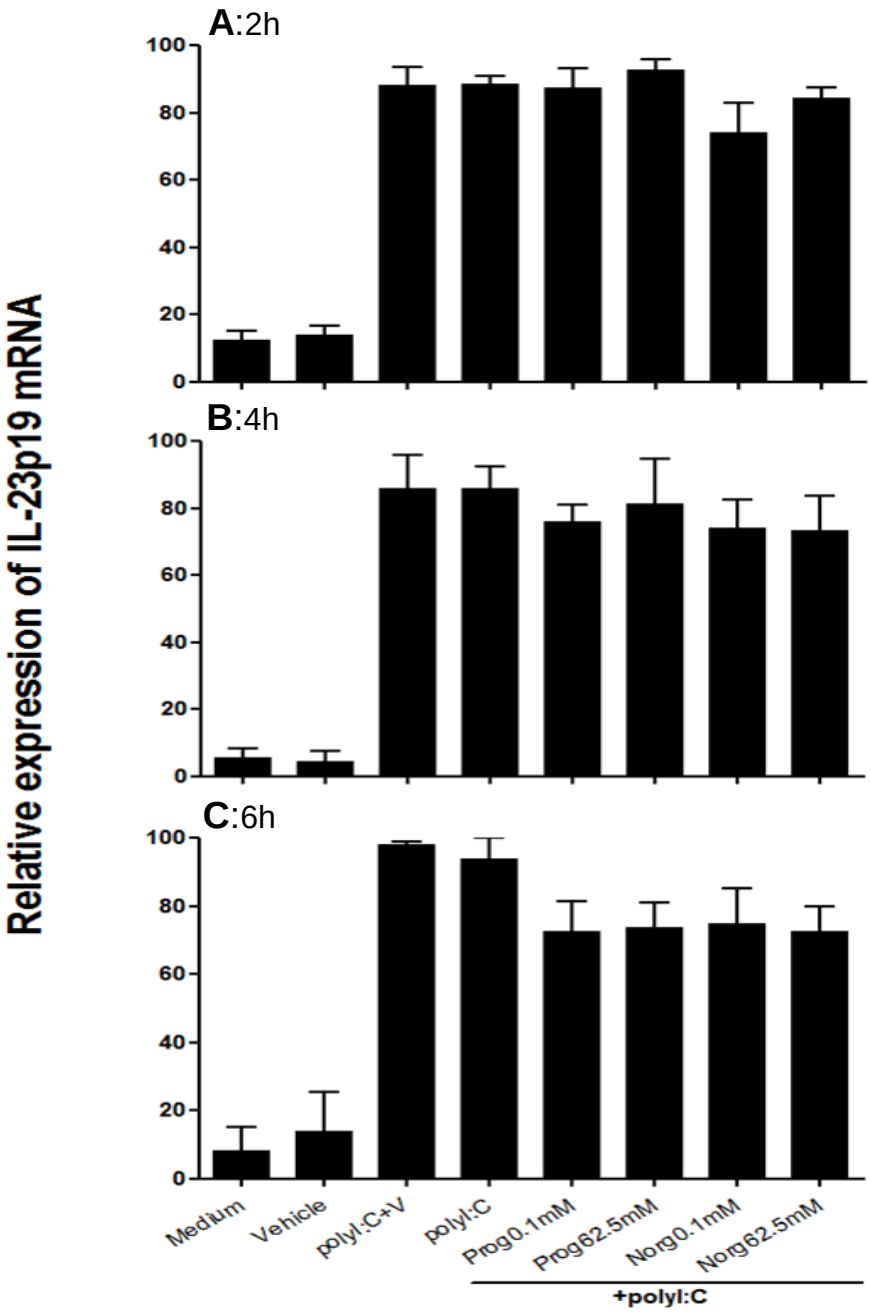


Figure 5.7. The influence of progesterone and norgestrel on polyI:C-induced IL-23p19 mRNA expression in BMDCs. Cells were treated with vehicle (V), 0.1µM and 62.5µM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 25µg/ml polyI:C for 2h (A), 4h (B) or 6h (C).IL-23p19 mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for IL-23p19 mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean ±SEM from 3 independent experiments.

5.2.5 LPS- and polyI:C- induced IL-27p28 mRNA expression in BMDCs

IL-27 comprises the p28 and EBI3 cytokine subunits. It is produced mainly from activated DCs (Siegemund *et al*., 2007). TLR3 and TLR4 activation selectively induces IL-27p28 gene expression through the IRF3 signalling pathway. Autocrine stimulation of type 1 IFNR by initial priming with Type 1 IFNs (α and β) secreted in response to TLR ligandsis required for optimal IL-27p28 expression (Molle *et al*., 2010).

BMDCs were exposed to 1µg/ml LPS for 2, 4 or 6h (Figure 5.8, panel A) to examine the ability of LPS to induce IL-27p28 gene expression. Results showed significant elevation in IL-27p28 mRNA expression by cells stimulated with LPS for 2h (41.80 ± 4.91, $P < 0.0001$), 4h (94.10 ± 7.95, $P < 0.0001$) or 6h (90.46 ± 5.35, $P < 0.0001$) compared to cells incubated in media, for 2h (1.48 ± 2.08), 4h (1.94 ± 4.24) and 6h (1.81 ± 2.64). For cells stimulated with 25µg/ml polyI:C for 2, 4 or 6h (Figure 5.8, panel B), results showed a significant elevation in polyI:C-induced IL-27p28 mRNA expression after 4 hours of stimulation (42.04 ± 3.41, $P < 0.0001$) and 6 hours (66.85 ± 10.25, $P < 0.0001$) compared with medium control, for 4h (1.94 ± 4.24) and 6h (1.81 ± 2.64). Overall LPS was able to induce greater IL-27p28 mRNA expression compared with polyI:C.

5.2.6 The influence of progesterone and norgestrel on LPS- and polyI:C- induced IL-27p28 mRNA expression in BMDCs

BMDCs were treated with progesterone or norgestrel one hour prior to exposure to LPS for 2, 4 or 6h (Figure 5.9, panels A, B and C) to assess the influence of both hormones on LPS- induced IL-27p28 mRNA expression. The results showed that neither of the hormones had an effect on LPS-or polyI:C- induced IL-27p28 mRNA expression (Figure 5.10, panels A, B and C).

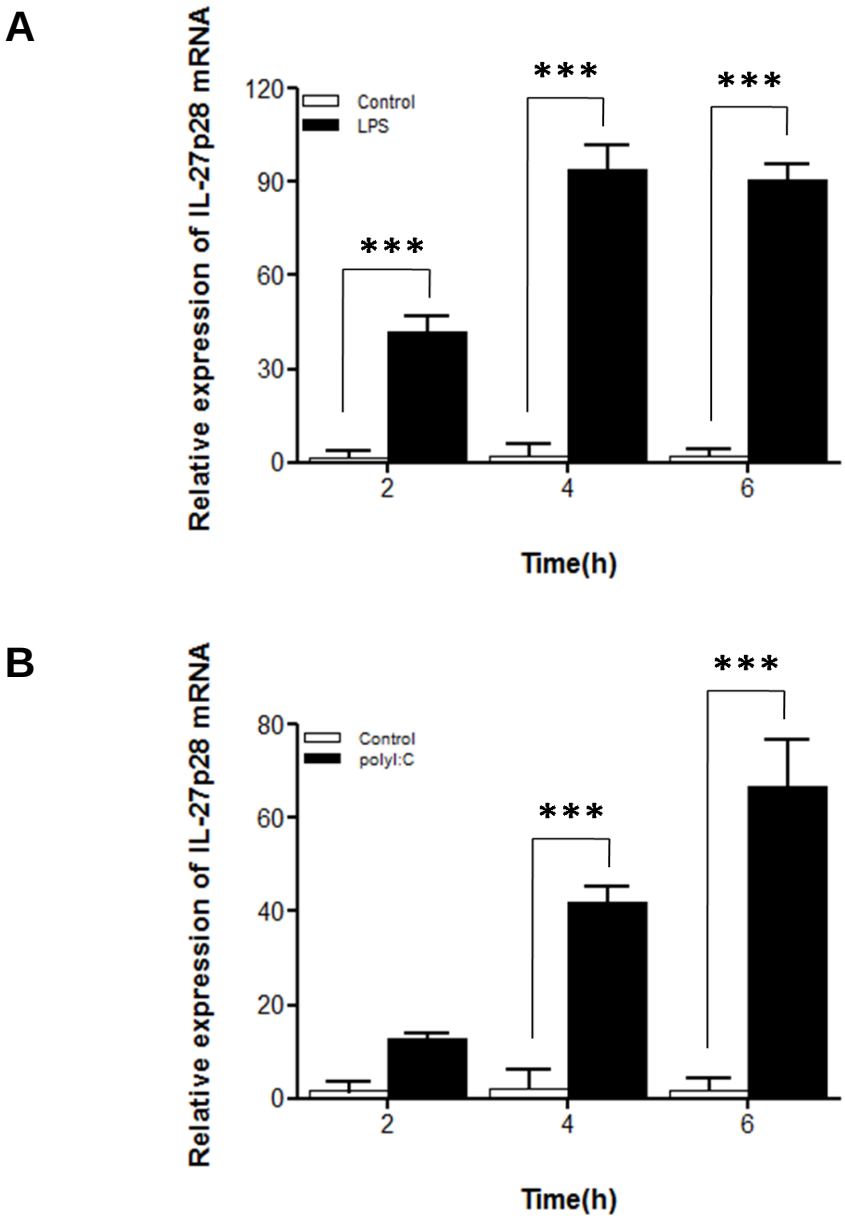


Figure 5.8. LPS- and polyI:C- induced IL-27p28 mRNA expression in BMDCs. Cells were left untreated (control) or exposed either to 1µg/ml LPS (A) or 25µg/ml polyI:C (B) for the indicated times (h). IL-27p28 mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for IL-27p28 mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean ±SEM from 3 independent experiments. ***P< 0.0001 is statistically significant vs. control.

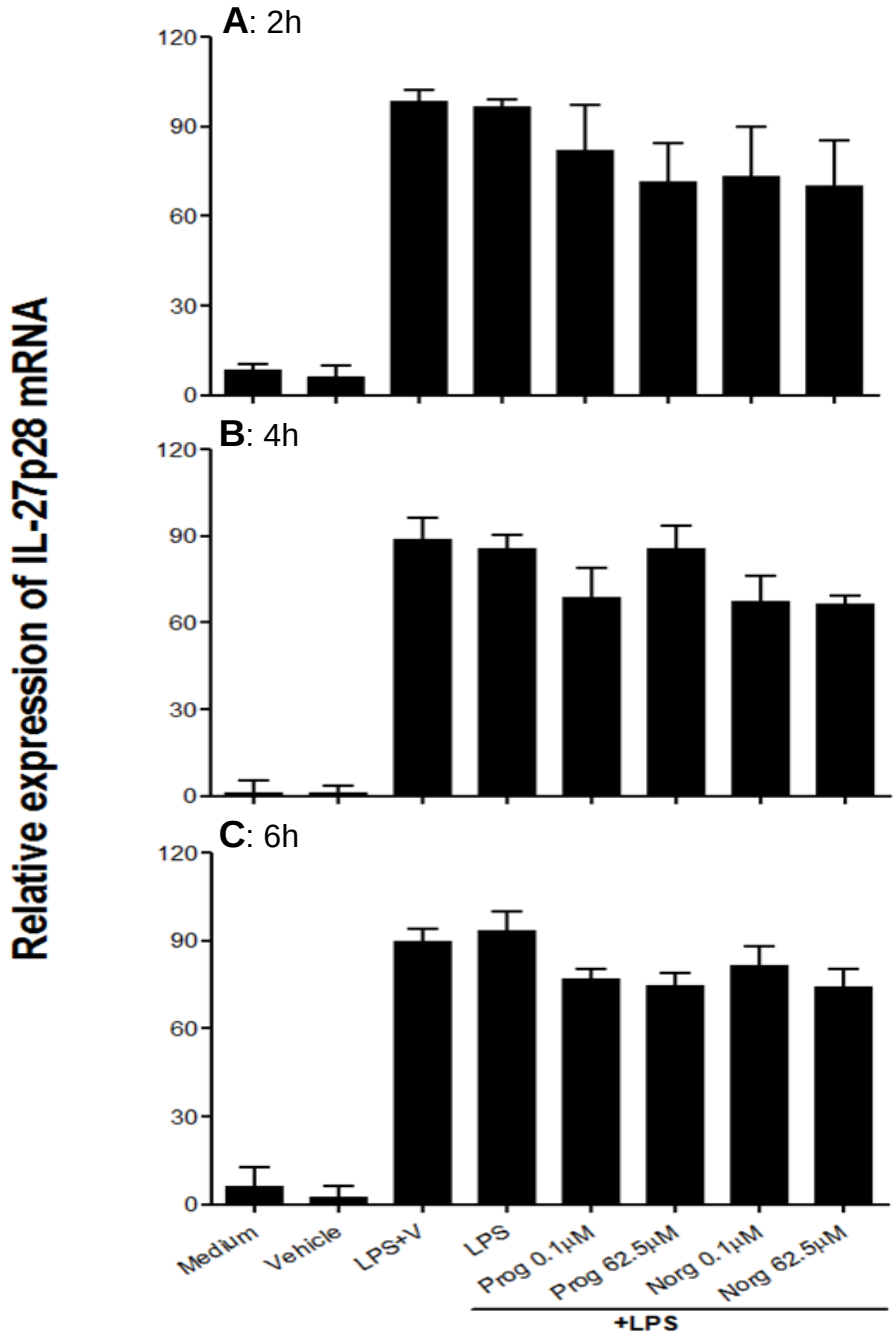


Figure 5.9. The influence of progesterone and norgestrel on LPS-induced IL-27p28 mRNA expression in BMDCs. Cells were treated with vehicle (V), 0.1µM and 62.5µM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 1µg/ml LPS for 2h (A), 4h (B) or 6h (C).IL-27p28 mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for IL-27p28 mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean ±SEM from 3 independent experiments.

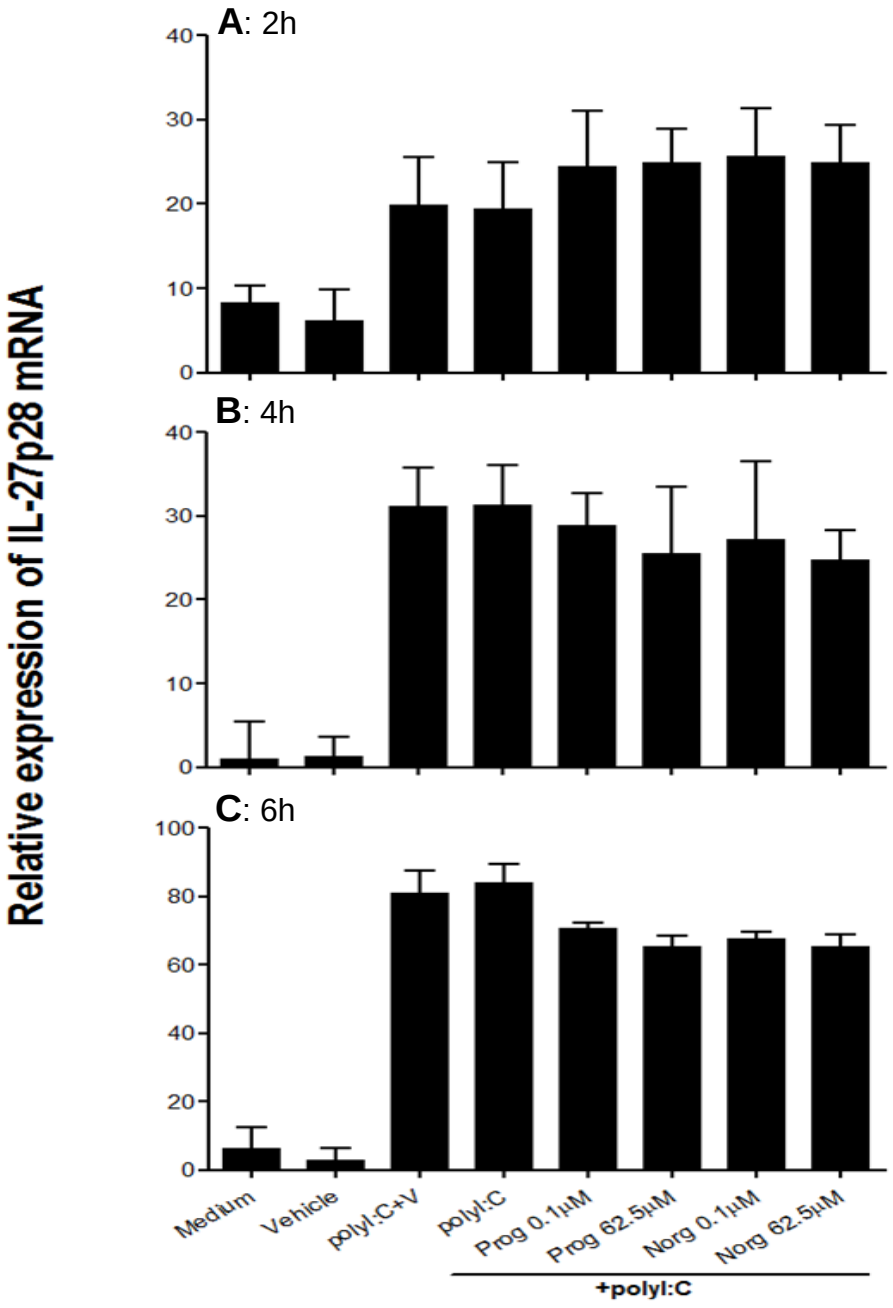


Figure 5.10. The influence of progesterone and norgestrel on polyI:C-induced IL-27p28 mRNA expression in BMDCs. Cells were treated with vehicle (V), 0.1µM and 62.5µM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 25µg/ml polyI:C for 2h (A), 4h (B) or 6h (C).IL-27p28 mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for IL-27p28 mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean ±SEM from 3 independent experiments.

5.2.7 LPS- and polyI:C- induced EBI3 mRNA expression in BMDCs

EBI3 binds p28 to produce IL-27. In addition, it binds IL-12p35 to form IL-35 (Yang *et al*., 2008). It is expressed in dendritic cells and induced by TLR activation (Wirtz *et al*., 2005).

BMDCs were exposed to 1µg/ml LPS for 2, 4 or 6h (Figure 5.11, panel A) to assess the ability of LPS to induce IL-27 EBI3 mRNA expression. Result showed a significant elevation in LPS-induced EBI3 mRNA expression at 2h (23.36 ± 3.52, $P < 0.05$) 4h (67.84 ± 5.74, $P < 0.0001$) and 6h (97.04 ± 6.96, $P < 0.0001$) compared with controls, at 2h (4.39 ± 3.01), at 4h (3.46 ± 1.42) and at 6h (7.40 ± 2.58). For cells exposed to 25µg/ml polyI:C for the same time intervals (Figure 5.11, panel B) EBI3 mRNA expression was increased at 2h (26.21 ± 6.72, $P < 0.05$), 4h (40.86 ± 2.74, $P < 0.001$) and 6 h (75.54 ± 10.23, $P < 0.0001$) compared with controls, at 2 hours (4.39 ± 3.01), at 4h (3.46 ± 1.42) and at 6h (7.40 ± 2.58).

5.2.8 The influence of progesterone and norgestrel on LPS- and polyI:C- induced EBI3 mRNA expression in BMDCs

BMDCs were pretreated with hormones and exposed to LPS (Figure 5.12, panels A, B and C). Both hormones (progesterone and norgestrel) inhibited LPS-induced EBI3 mRNA expression after 4 and 6 hours of stimulation compared with agaonist plus vehicle. High concentrations of both hormones exerted this effect at 4 hours,{progesterone (60.0 ± 6.56, $P < 0.05$) and norgestrel (54.09 ± 6.25, $P < 0.05$)} compared to LPS+vehicle (90.77 ± 9.91) and after 6 hours of stimulation, {progesterone(52.47 ± 5.25, $P < 0.05$) and norgestrel (53.77 ± 5.72, $P < 0.05$)} compared to LPS+vehicle (80.73 ± 2.78). Both hormones had similar effects for cells exposed to polyI:C (Figure 5.13, panels A, B and C) compared with stimulant. High concentrations of progesterone and norgestrel significantly reduced polyI:C-induced EBI3 mRNA expression at 4 hours of stimulation, progesterone (54.07 ± 4.52, $P < 0.05$) and norgestrel (48.44 ± 6.14, $P < 0.05$) compared to polyI:C+vehicle (77.75 ± 2.38). The hormones exerted similar influences on polyI:C stimulated cultures after 6

hours of stimulation{progesterone (54.52 ± 6.09, $P < 0.05$) and norgestrel (53.60 ± 7.96, $P < 0.05$)}compared to agonist (polyI:C+vehicle) (83.75 ± 3.30).

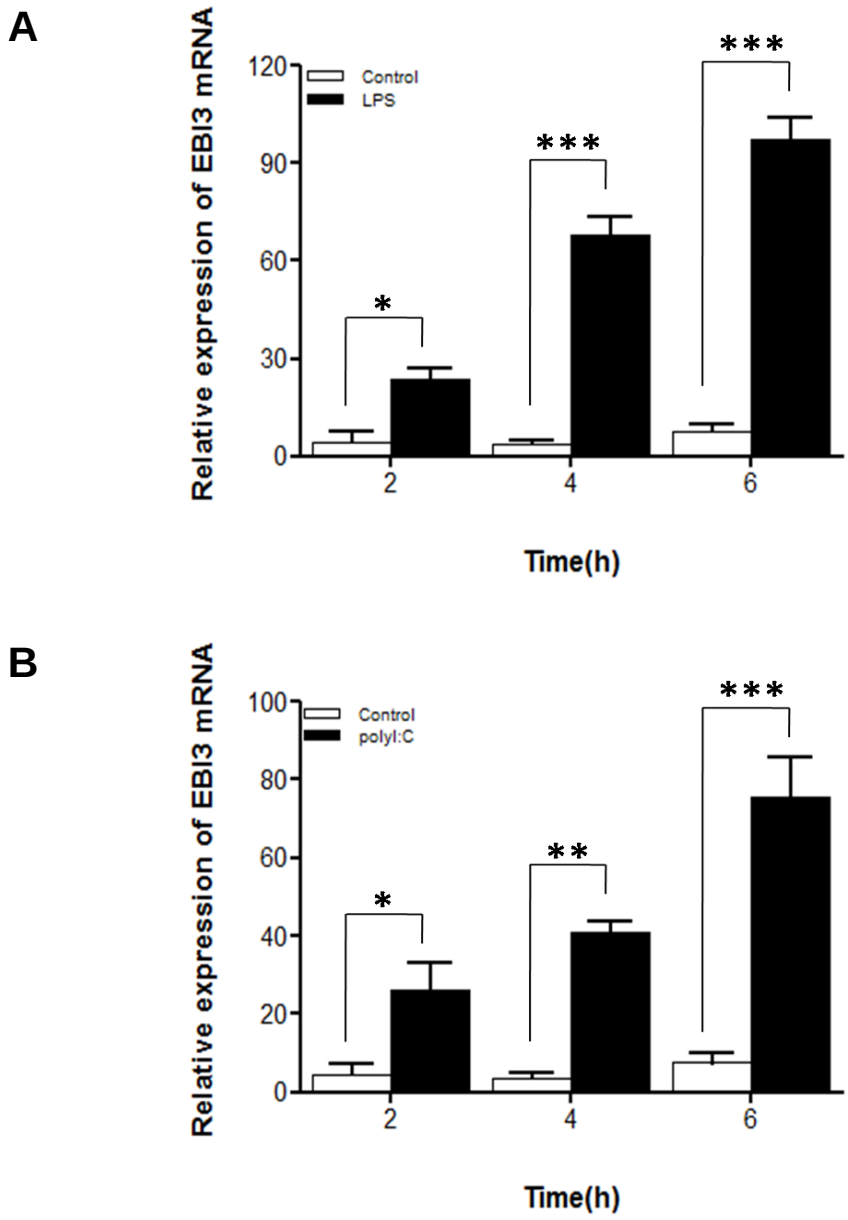


Figure 5.11. LPS- and polyI:C- induced EBI3 mRNA expression in BMDCs. Cells were left untreated (control) or exposed either to 1µg/ml LPS (A) or 25µg/ml polyI:C (B) for the indicated times (h). EBI3 mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for EBI3 mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean ±SEM from 3 independent experiments. *P< 0.05, **P< 0.001 and ***P< 0.0001 are statistically significant vs. control.

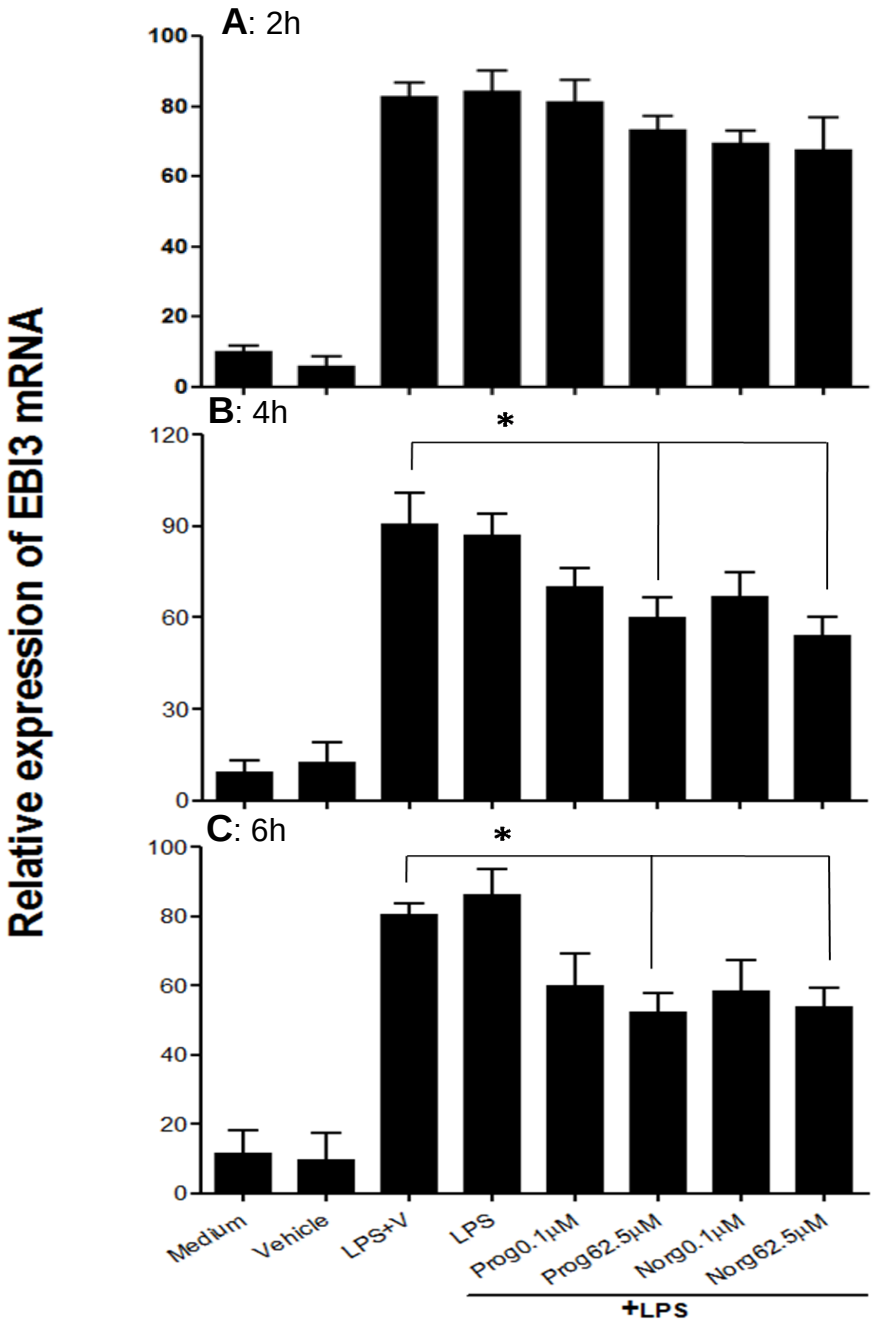


Figure 5.12. The influence of progesterone and norgestrel on LPS-induced EBI3 mRNA expression in BMDCs. Cells were treated with vehicle (V), 0.1µM and 62.5µM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 1µg/ml LPS for 2h (A), 4h (B) or 6h (C). EBI3 mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for EBI3 mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean ±SEM from 3 independent experiments. *P< 0.05 is statistically significant vs. agonist plus vehicle (LPS+V).

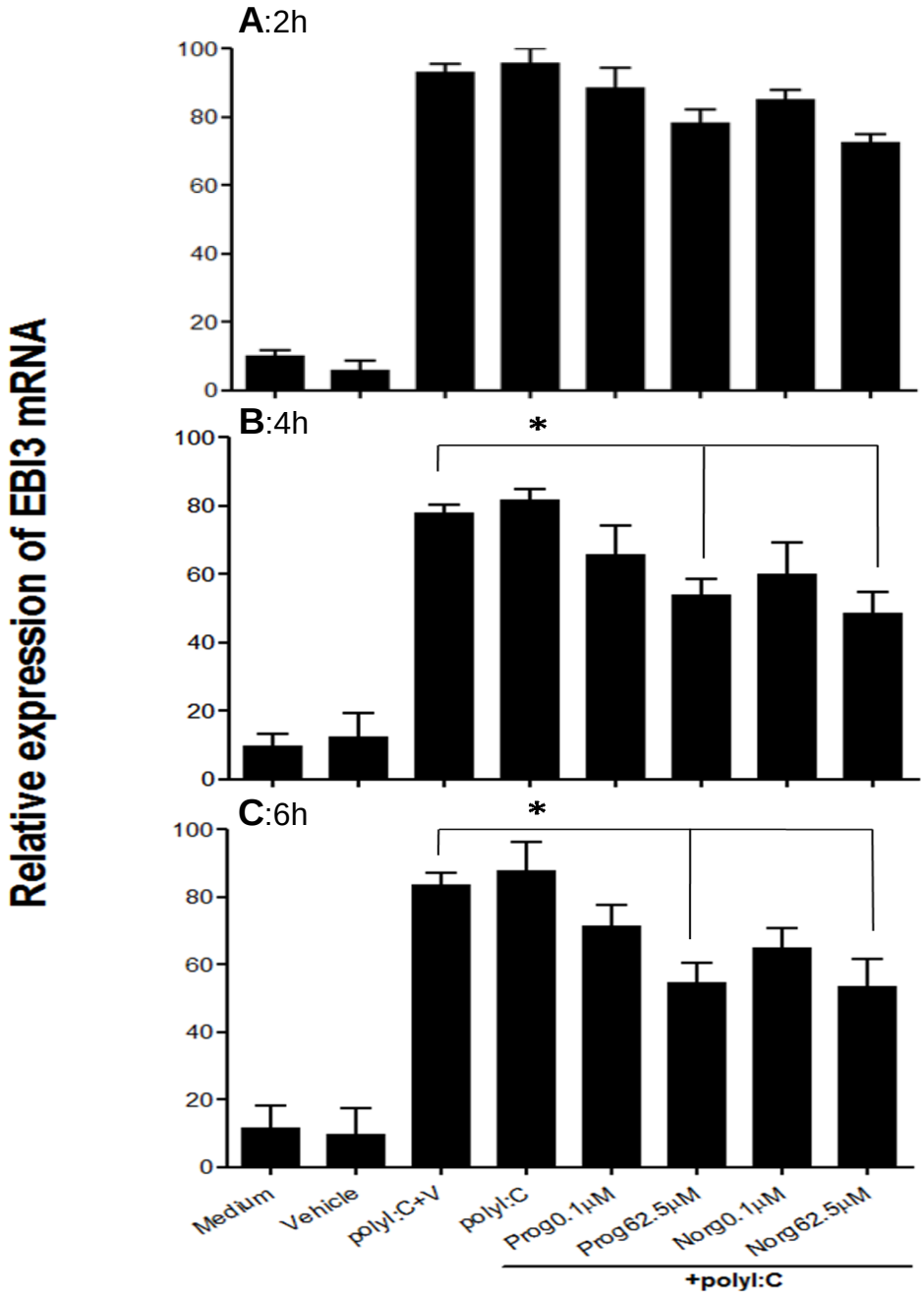


Figure 5.13. The influence of progesterone and norgestrel on polyI:C-induced EBI3 mRNA expression in BMDCs. Cells were treated with vehicle (V), 0.1µM and 62.5µM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 25µg/ml polyI:C for 2 hours (A), 4h (B) or 6h (C).IL-23p19 mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for EBI3 mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean±SEM from 3 independent experiments. *P< 0.05 is statistically significant vs. agonist plus vehicle (polyI:C+V).

The influence of progesterone on IFNβ mRNA expression

IFNβ production is one of the important outcomes of TLR3 and TLR4 in mediating anti-viral inflammatory responses. As well as the involvement of both IRF3 and NFκB transcription factors in the promoter of IFNβ gene. The study attempted to evaluate any potential immunomodulatory effect of progesterone on the gene expression of this mediator.

5.2.9 LPS- and polyI:C- induced IFNβ mRNA expression in BMDCs

The cells were exposed to 1μg/ml LPS to assess LPS-induced IFNβ mRNA expression in BMDCs after 2, 4 and 6 hours (Figure 5.14, panel A). Maximum expression compared with control cultures was demonstrated after 2 hours (89.10 ± 10.88, $P < 0.0001$), control (2.51 ± 1.24), that declined after 4 hours. For cells exposed to 25μg/ml polyI:C, maximum expression of IFNβ mRNA was also observed after 2 hours (94.64 ± 3.68, $P < 0.0001$) compared to control .

5.2.10 The influence of progesterone and norgestrel hormones on LPS- and polyI:C-induced IFNβ mRNA expression in BMDCs

The potential influence of progesterone and norgestrel on LPS- or polyI:C- induced IFNβ mRNA expression was also assessed, again at the same time points of 2, 4 and 6 hours the cells were treated with the hormones one hour prior to exposure to the agonists LPS or polyI:C. BMDCs treated with hormones and exposed to LPS (Figure 5.15, Panel A, B and C). Significant reduction of LPS-induced IFNβ mRNA expression was achieved for the cells treated with the higher concentration of progesterone for 6 hours (55.97 ± 4.50, $P < 0.05$) compared with agonist (LPS+vehicle) (80.48 ± 5.82). Significant reductions in polyI:C-induced IFNβ mRNA expression were mediated by both concentrations of each hormone 6hours post stimulation (Figure 5. 16, panels A, B and C). The results were as follows, cells treated with 0.1μM progesterone (69.81 ± 7.52, $P < 0.05$), 62.5μM progesterone (55.12 ± 3.51, $P < 0.05$), norgestrel 0.1μM (59.79 ± 5.78, $P < 0.05$) and 62.5μM

norgestrel (52.54 ± 8.12, $P < 0.05$) compared with agonist (polyI:C+vehicle) (87.79 ± 3.65).

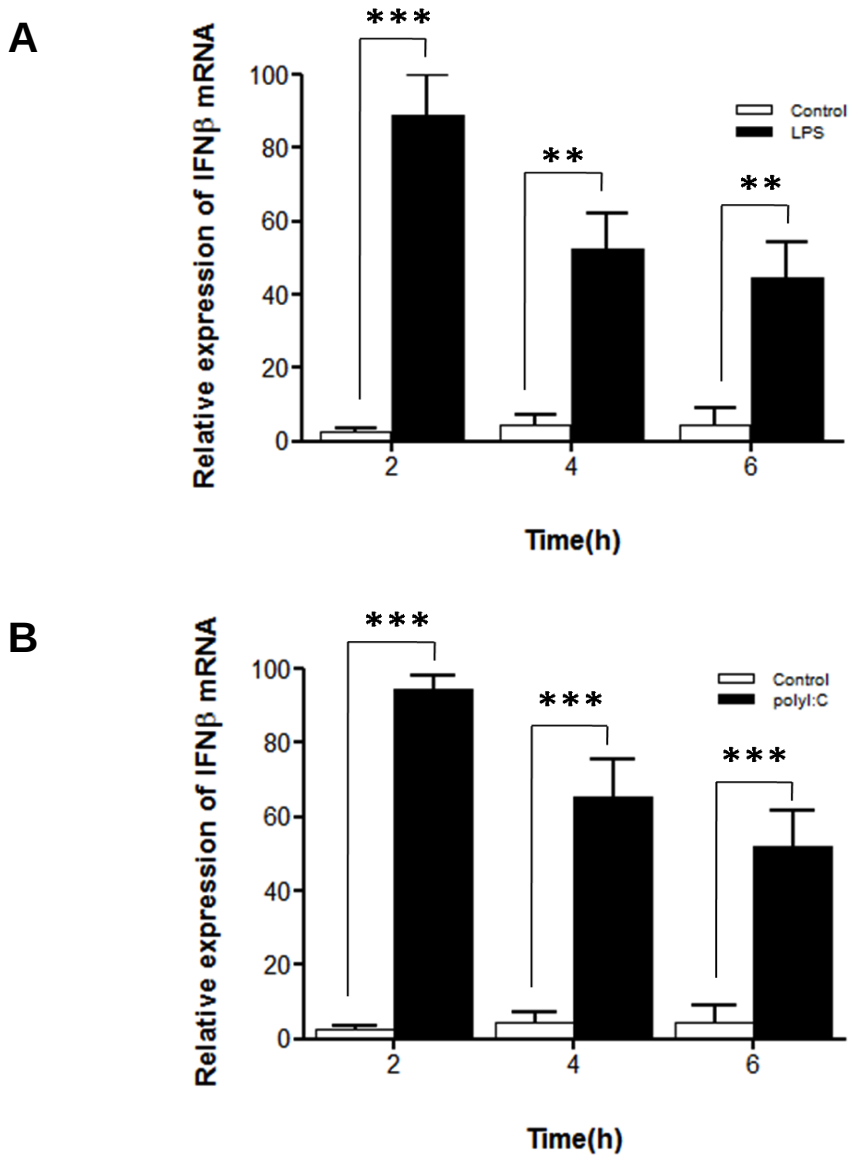


Figure 5.14. LPS- and polyI:C- induced IFNβ mRNA expression in BMDCs. Cells were left untreated (control) or exposed either to 1µg/ml LPS(A) or 25µg/ml polyI:C (B) for the indicated times (h). IFNβ mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for IFNβ mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean±SEM from 3 independent experiments. **p<0.001 and ***P<0.0001 are statistically significant vs. control.

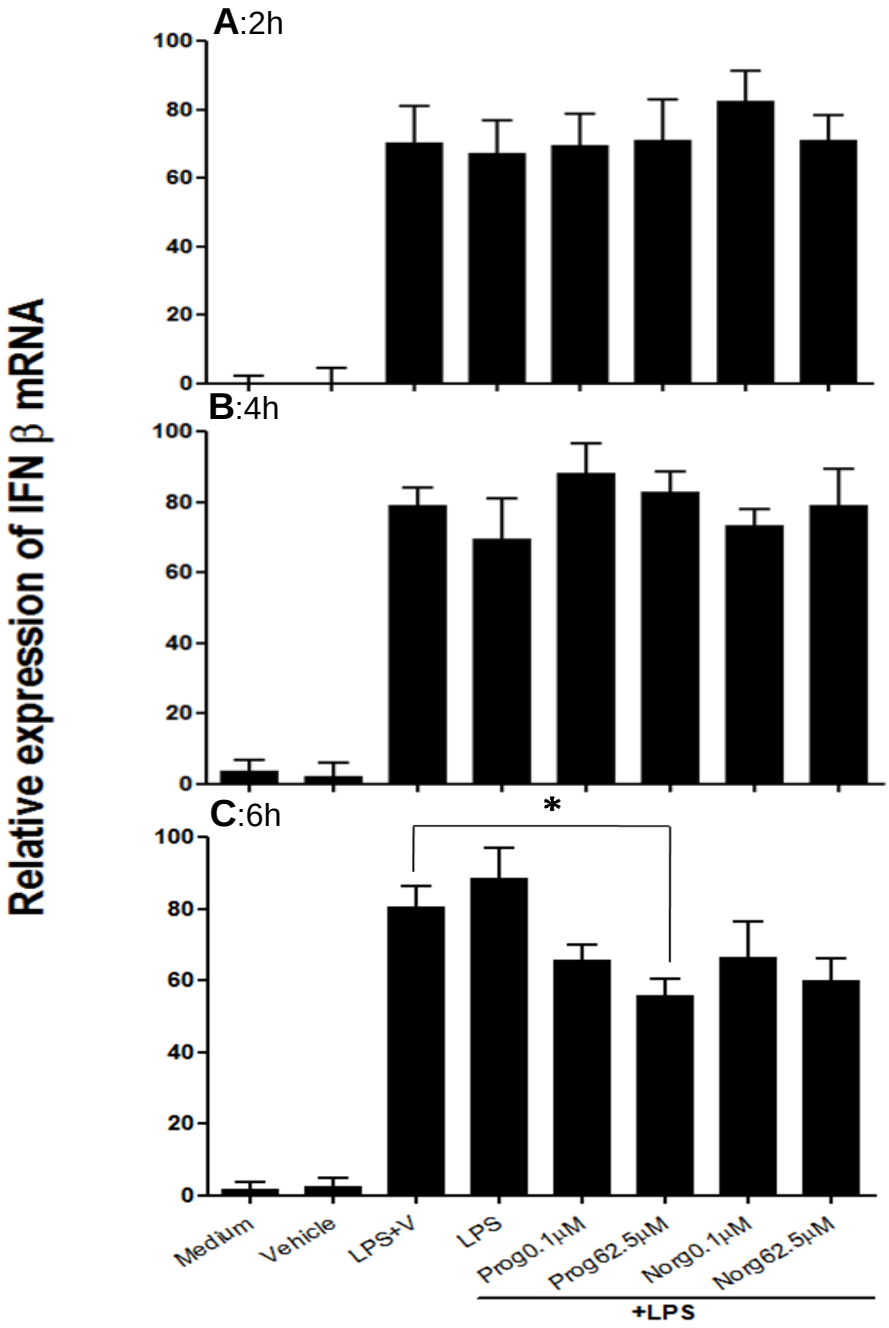


Figure 5.15. The influence of progesterone and norgestrel on LPS-induced IFNβ mRNA expression in BMDCs. Cells were treated with vehicle (V), 0.1μM and 62.5μM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to exposure to 1μg/ml LPS for 2h (A), 4h (B) or 6h (C). IFNβ mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for IFNβ mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean ±SEM from 3 independent experiments. *P<0.05 is statistically significant vs. agonist plus vehicle (LPS+V).

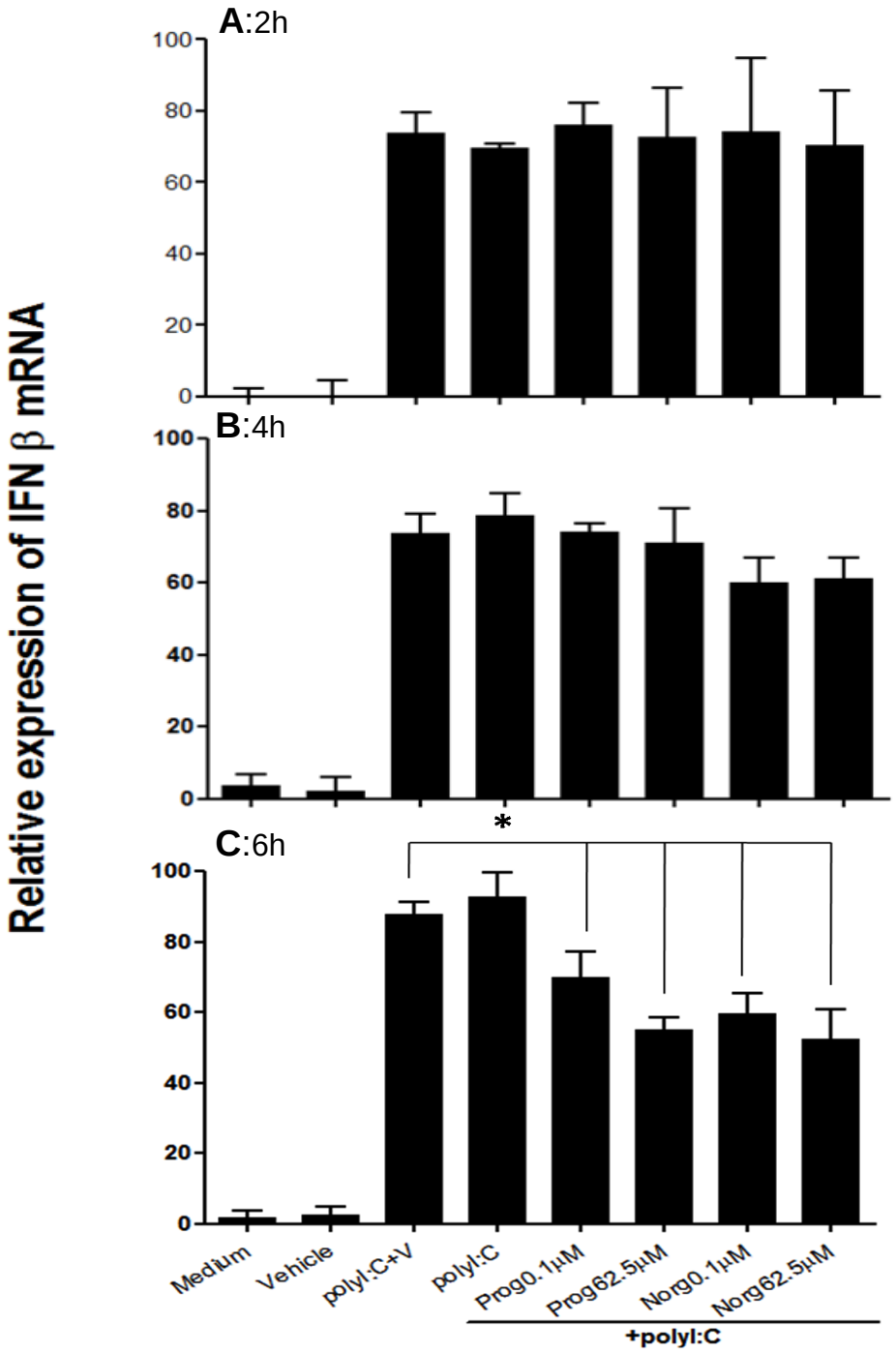


Figure 5.16. The influence of progesterone and norgestrel on polyI:C-induced IFNβ mRNA expression in BMDCs. Cells were treated with vehicle (V), 0.1μM and 62.5μM of either progesterone (Prog) or norgestrel (Norg) hormones one hour prior to expose to 25μg/ml polyI:C for 2h (A), 4h (B) or 6h (C). IFNβ mRNA expression was assessed by RT-PCR as detailed in the Methods section. Expression levels for IFNβ mRNA were normalised to the housekeeping gene *Tbp.* The maximum expression for each experiment was considered as a 100% and all other values were calculated in comparison to it. Each value represents the mean± SEM from 3 independent experiments. *P<0.05 is statistically significant vs. agonist plus vehicle (LPS+V).

The influence of progesterone and norgestrel on IL-6 and IL-12 cytokine production

In order to further assess the ability of progesterone and its analogue norgestrel to modulate inflammatory responses, IL-6 and IL-12p70 cytokines production was investigated. These cytokines, again are important mediators of inflammatory responses mounted by DCs and induced by LPS and polyI:C (Akira *et al*., 2006; Kim *et al*, 2012).

5.2.11 The influence of progesterone and norgestrel on LPS-induced IL-6 production in BMDCs

BMDCs were treated with varying concentrations (0.001µM - 100µM) of progesterone or norgestrel one hour prior to exposure to 1µg/ml LPS. Supernatants were collected after 8h, 24h and 48h of incubation to assess IL-6 levels. Higher concentrations (1, 10 and 100µM) of progesterone after 48 hours from exposure to LPS (Figure 5. 17, panel C) showed significant reduction in LPS-induced IL-6 production in comparison to LPS+vehicle (14.46 ± 1.55), the results were (6.70 ± 1.86, $P < 0.05$) for 1µM of progesterone that represents approximately a 54% inhibition, (7.05 ± 1.45, $P < 0.05$) for 10µM of progesterone approximately 51% inhibition and (5.35 ± 1.57, $P < 0.05$) for 100µM of progesterone approximately 63% inhibition.The cells treated with norgestrel and subjected to the same conditions did not show a reductionon LPS-induced IL-6 production (Figure 5.18, panel A, B and C).

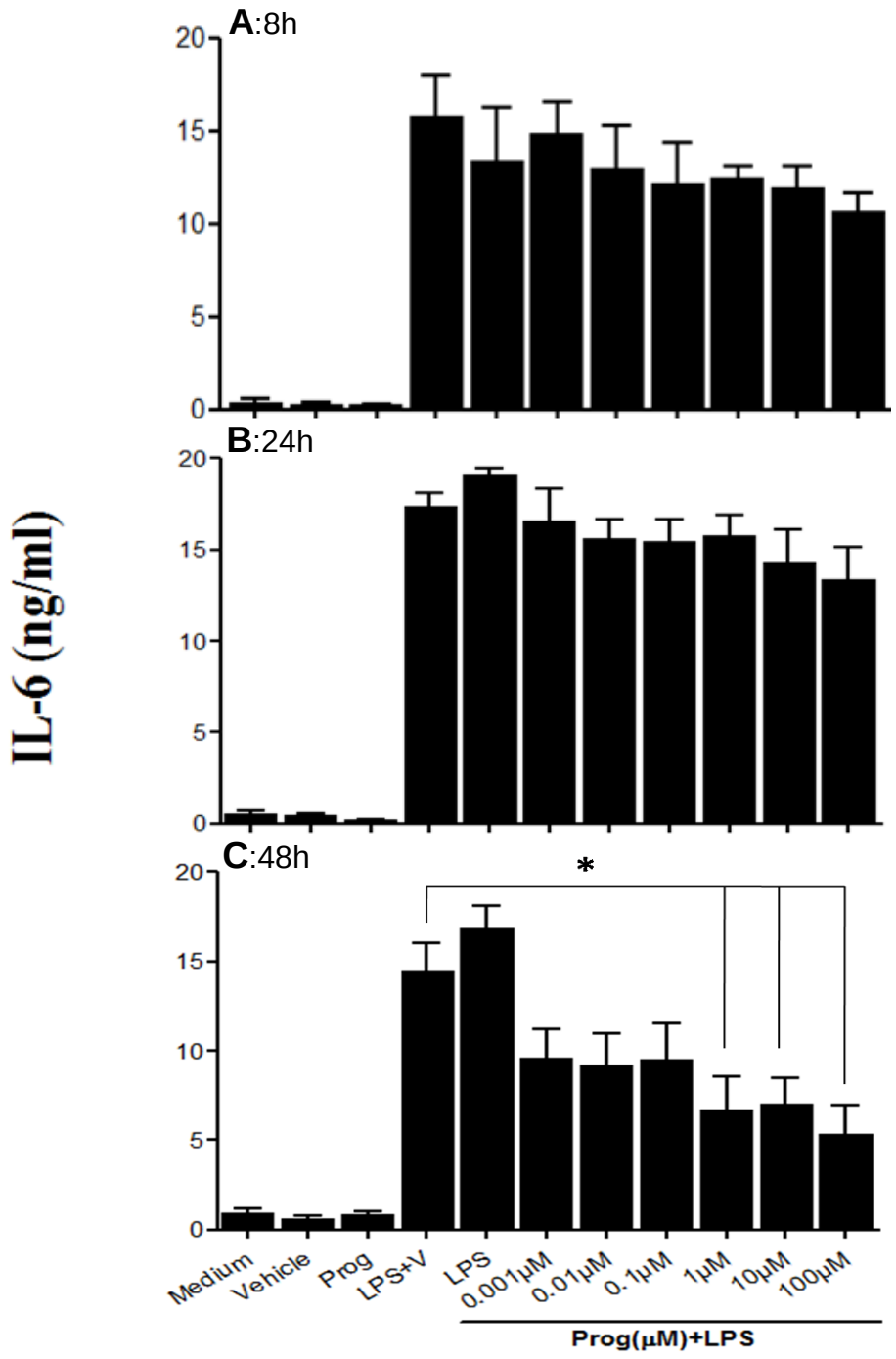


Figure 5.17. The influence of progesterone on LPS-induced IL-6 production in BMDCs. Cells were treated with vehicle (V), 10-fold increasing concentrations (0.001µM-100µM) of progesterone (Prog) hormone one hour prior to exposure to 1µg/ml LPS for 8h (A), 24h (B) or 48h (C). Supernatants were collected and IL-6 levels were assessed by ELISA as detailed in the Methods section. Each value represents the mean±SEM from 3 independent experiments. *P<0.05 is statistically significant vs. agonist plus vehicle (LPS+V).

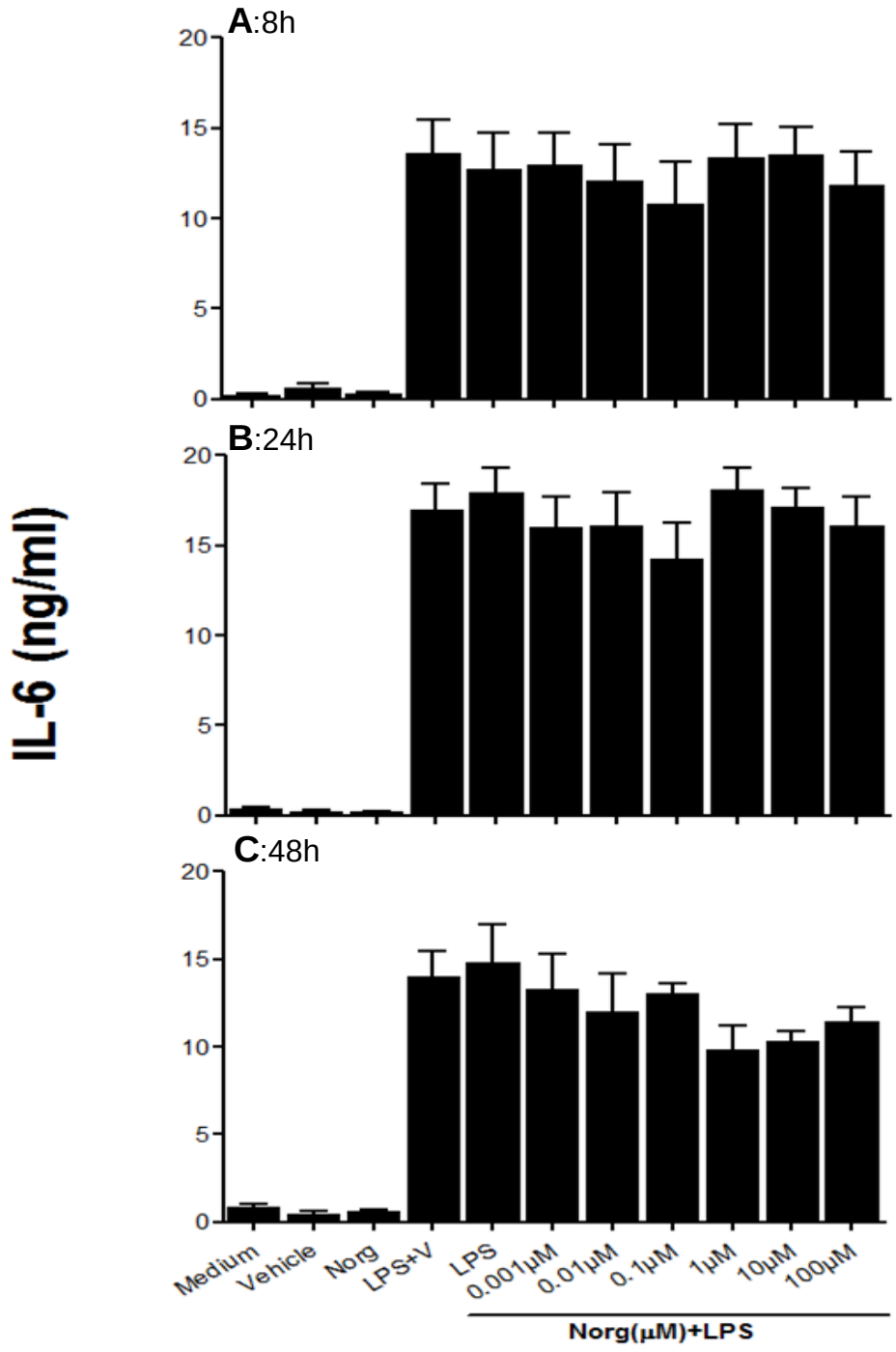


Figure 5.18. The influence of norgestrel on LPS-induced IL-6 production in BMDCs. Cells were treated with vehicle (V), 10-fold increasing concentrations (0.001µM-100µM) of norgestrel (Nrog) hormone one hour prior to exposure to 1µg/ml LPS for 8h (A), 24h (B) or 48h (C). Supernatants were collected and IL-6 levels were assessed by ELISA as detailed in the Methods section. Each value represents the mean±SEM from 3 independent experiments.

5.2.12 The influence of progesterone or norgestrel on polyI:C-induced IL-6 production in BMDCs

As with previous experiments, BMDCs were treated with varying concentrations (0.001µM - 100µM) of progesterone or norgestrel one hour prior to stimulation with 25µg/ml polyI:C. Supernatents were collected after 8, 24 and 48hours of incubation to assess IL-6 levels. 24 hour post stimulation with polyI:C (Figure 5.19, panel B) the two higher concentrations of progesterone (10 and 100µM) exerted an inhibition effect on polyI:C-induced IL-6 production compared to polyI:C+vehicle (11.39 ± 0.17), 10µM of progesterone reduced polyI:C induced IL-6 production by approximately 30% (8 ± 0.81, $P < 0.05$) and 100µM of progesterone reduced polyI:C- induced IL-6 production by approximately 39% (6.96 ± 0.29, $P < 0.05$). The same effect was observed after 48 hours of stimulation with polyI:C (Figure 5.19, panel C). 10µM of progesterone reduced polyI:C induced IL-6 production by approximately 37% (6.83 ± 0.50, $P < 0.05$) and 100µM of progesterone reduced polyI:C- induced IL-6 production by approximately 45% (6.96 ± 0.29, $P < 0.05$) compared to polyI:C+vehicle (10.89 ± 0.58). BMDCs treated with norgestrel prior to polyI:C for 8, 24 and 48 hours (Figure 5.20, panel A, B and C) showed no influence of the hormone on polyI:C-induced IL-6 production.

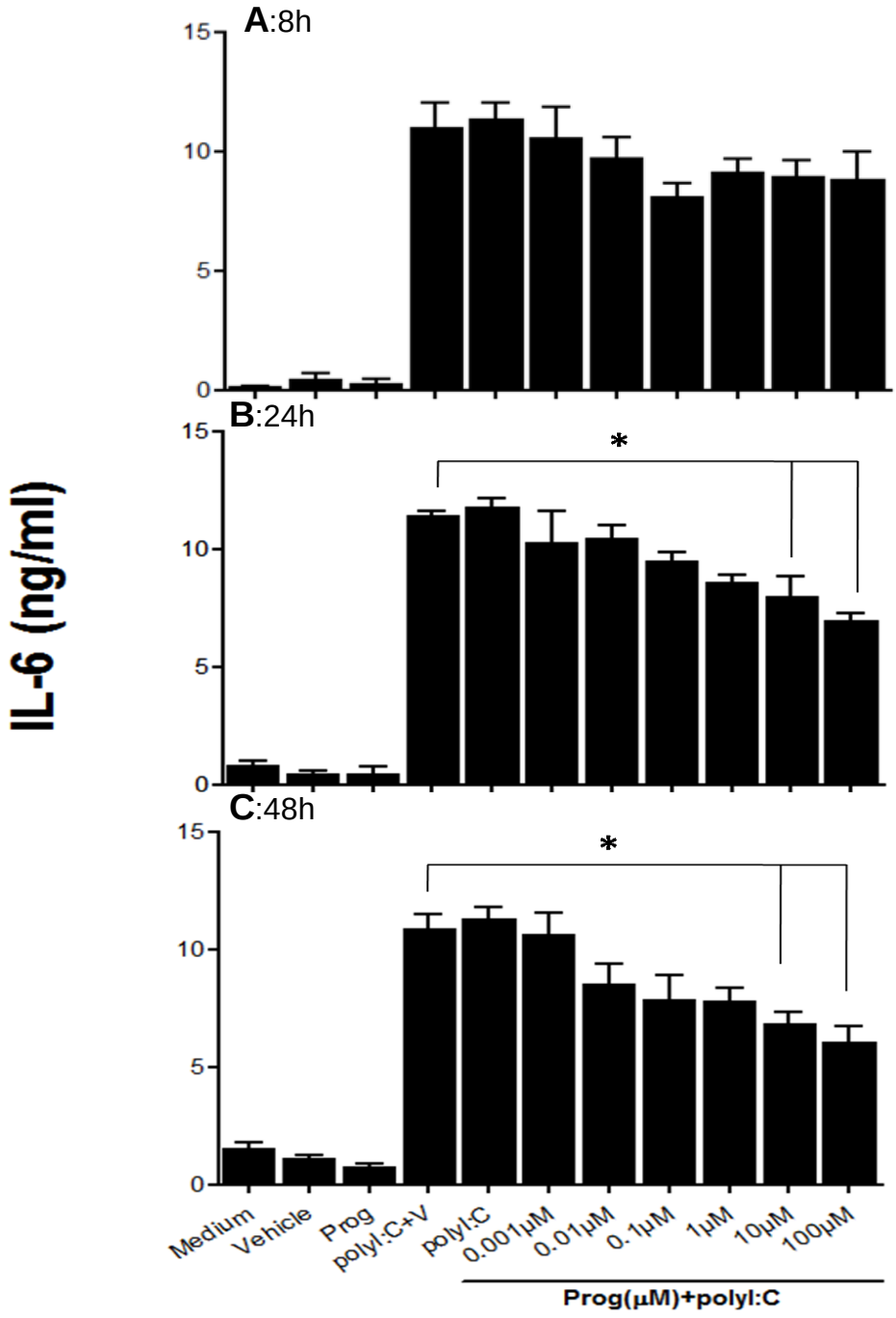


Figure 5.19. The influence of progesterone on polyI:C-induced IL-6 production in BMDCs. Cells were treated with vehicle (V), 10-fold increasing concentrations (0.001µM-100µM) of progesterone (Prog) hormone one hour prior to exposure to 25µg/ml polyI:C for 8h (A), 24h (B) or 48h (C). Supernatants were collected and IL-6 levels were assessed by ELISA as detailed in the Methods section. Each value represents the mean±SEM from 3 independent experiments. *P< 0.05 is statistically significant vs. agonist plus vehicle (polyI:C+V).

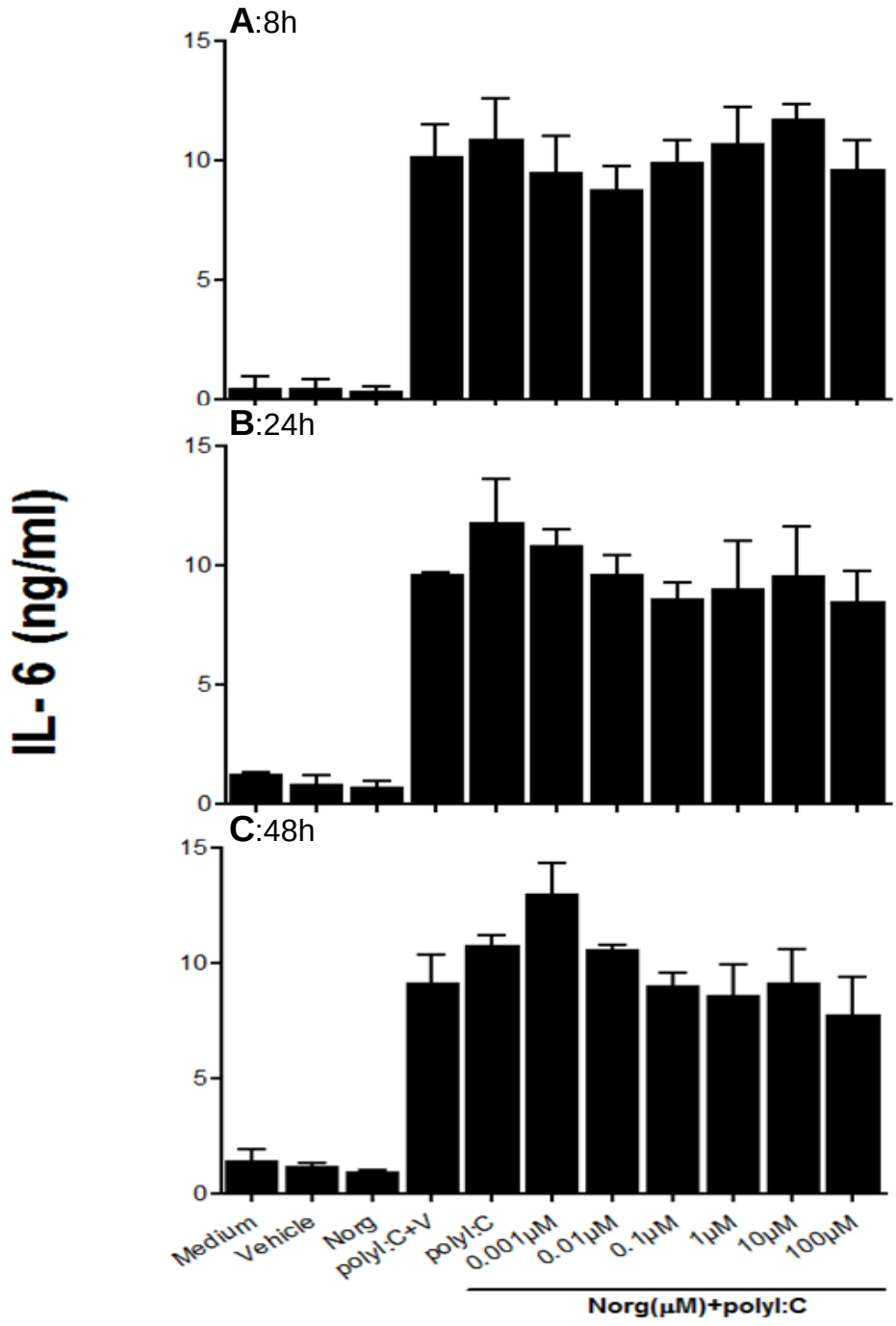


Figure 5.20. The influence of norgestrel on polyI:C-induced IL-6 production in BMDCs. Cells were treated with vehicle (V), 10-fold increasing concentrations (0.001µM-100µM) of norgestrel (Nrog) hormone one hour prior to exposure to 25µg/ml polyI:C for 8h (A), 24h (B) or 48h (C). Supernatants were collected and IL-6 levels were assessed by ELISA as detailed in the Methods section. Each value represents the mean±SEM from 3 independent experiments.

5.2.13 The influence of progesterone and norgestrel on LPS-induced IL-12p70 production in BMDCs

BMDCs were treated with varying concentrations range from 0.001-100µM of progesterone or norgestrel one hour prior to stimulation with 1µg/ml LPS for 8, 24 or 48 hours to assess IL-12p70 production. Higher concentration of progesterone (100µM) showed significant reduction in LPS-induced IL-12p70 production for 24hours (Figure 5.21, panel B) post stimulation in comparison to LPS+ vehicle (5.89 ± 0.17). It was about 47% inhibition (3.10 ± 0.381, $P < 0.05$). At 48 hours (Figure 5.21, panel C), progesterone reduced significantly LPS- induced IL-12p70 production for the concentrations 1-100µM. The effect of progesterone became more potent with the higher concentrations that approximated to 55% inhibition (3.23 ± 0.433, $P < 0.0001$) compared to LPS+vehicle (7.21 ± 0.38) .

Norgestrel also inhibited LPS-induced IL-12p70 production (Figure 5.22, panel B) 24 hours post stimulation, approximately 53% inhibition (2.90 ± 0.346, $P < 0.05$) compared with LPS+vehicle (6.19 ± 0.69). For cells stimulated with LPS for 48 hours (Figure 5.22, panel C), only the highest concentration (100µM) of norgestrel exerted an inhibition effect on LPS-induced IL-12p70 production in comparison with LPS+ vehicle (7.18 ± 0.54) approximately 37% inhibition (4.52 ± 0.935, $P < 0.05$) was observed. Norgestrel was less potent as an inhibitor for LPS-induced IL-12p70 production after 48 hours of stimulation in comparison with progesterone.

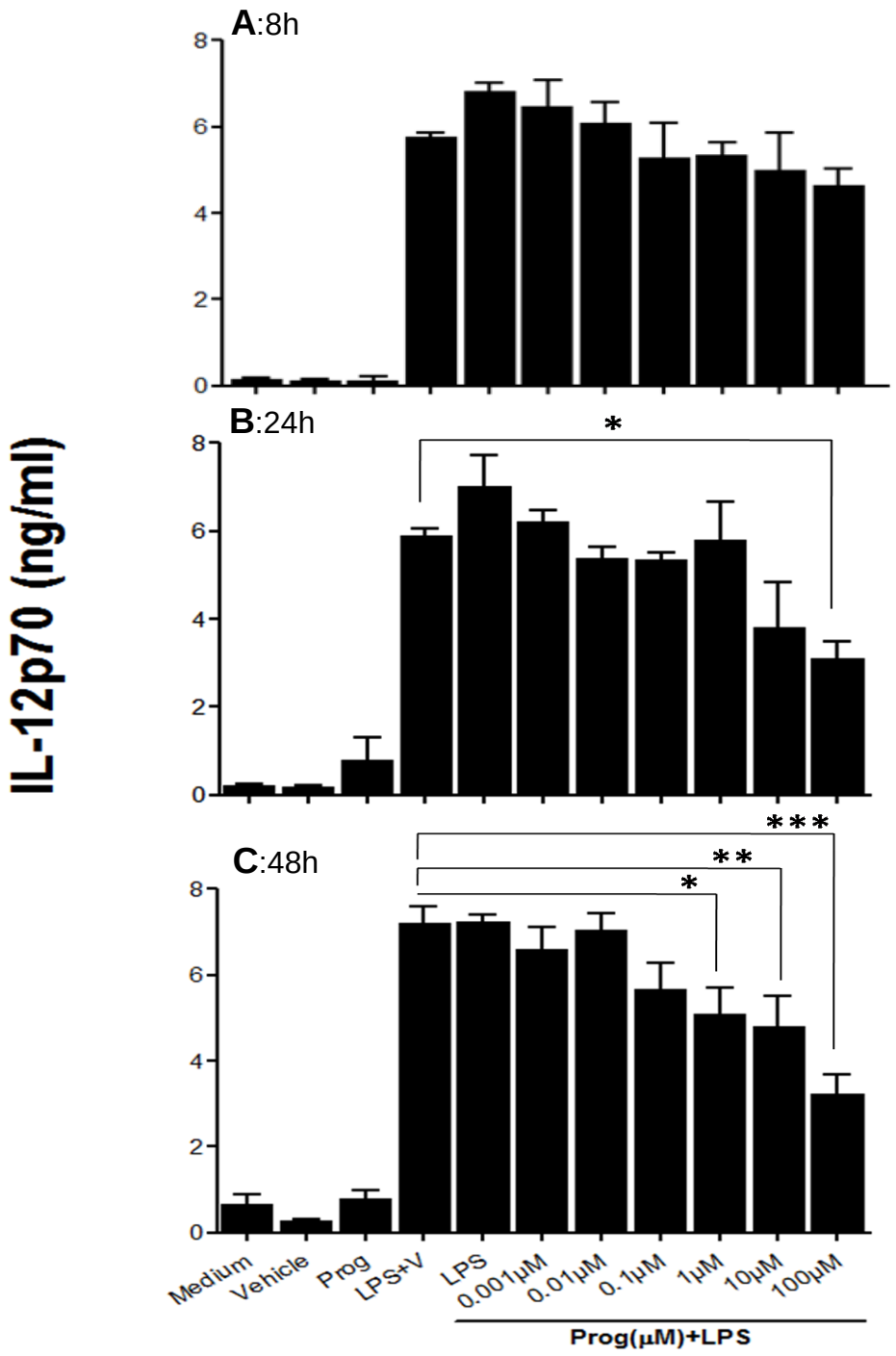


Figure 5.21. The influence of progesterone on LPS-induced IL-12p70 production in BMDCs. Cells were treated with vehicle (V), 10-fold increasing concentrations (0.001μM-100μM) of progesterone (Prog) hormone one hour prior to exposure to 1μg/ml LPS for 8h (A), 24h (B) or 48h (C). Supernatants were collected and IL-12p70 levels were assessed by ELISA as detailed in the Methods section. Each value represents the mean±SEM from 3 independent experiments. *P<0.05, **P<0.001 and ***P<0.0001 are statistically significant vs. agonist plus vehicle (LPS+V).

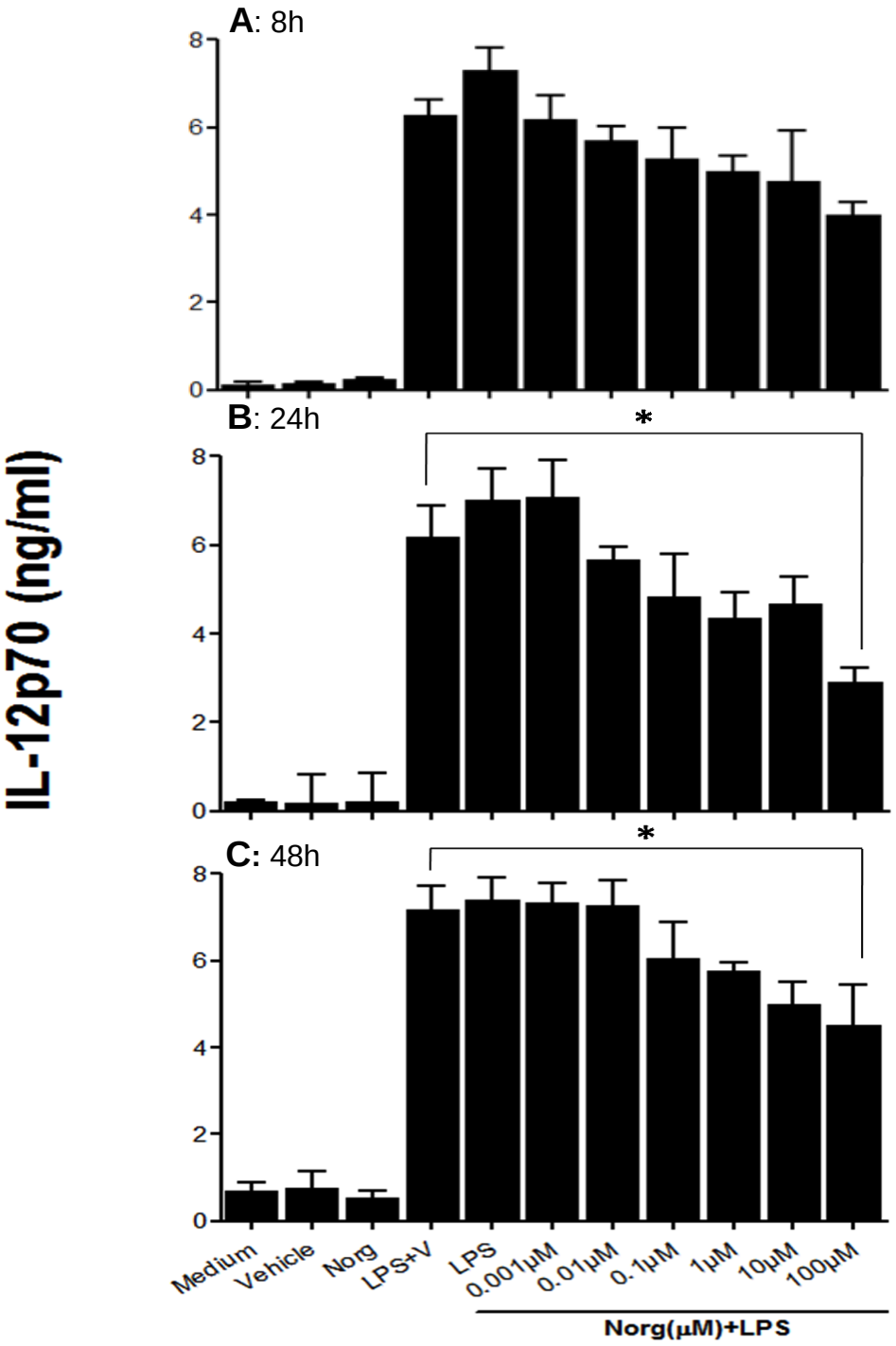


Figure 5.22. The influence of norgestrel on LPS-induced IL-12p70 production in BMDCs. Cells were treated with vehicle (V), 10-fold increasing concentrations (0.001μM-100μM) of norgestrel (Norg) hormone one hour prior to exposure to 1μg/ml LPS for 8h (A), 24h (B) or 48h (C). Supernatants were collected and IL-12p70 levels were assessed by ELISA as detailed in the Methods section. Each value represents the mean±SEM from 3 independent experiments. *P<0.05 is statistically significant vs. agonist plus vehicle (LPS+V).

5.2.14 The influence of progesterone and norgestrel on polyI:C-induced IL-12p70 production in BMDCs

BMDCs were treated with varying concentrations (0.001µM-100µM) of progesterone or norgestrel one hour prior to stimulation with 25µg/ml polyI:C. Supernatants were collected after 8h, 24h and 48h of incubation to assess IL-12p70 production. The higher concentration (100µM) of progesterone after 24 and 48 hours from exposure to polyI:C (Figure 5.23, panels B and C) showed significant reduction of polyI:C-induced IL-12p70 production compared to the agonist (polyI:C+vehicle). For 24 h exposure the result was approximately 35% reduction (0.56 ± 0.10, $P < 0.05$) compared to polyI:C+vehicle (1.18 ± 0.06) and for cells exposed to 48h the result was approximately 51% reduction (0.44 ± 0.073, $P < 0.05$) compared to polyI:C+vehicle (0.91 ± 0.12). This effect was also observed with norgestrel and involved two concentrations for the cells treated with norgestrel prior to polyI:C for 24h (Figure 5.24, panels B). 10µM norgestrel reduced polyI:C-induced IL-12p70 production by approximately 38% (0.75 ± 0.08, $P < 0.05$) and 100µM of norgestrel reduced polyI:C-induced Il-12p70 by approximately 53% (0.57 ± 0.1, $P < 0.001$) compared with polyI:C+vehicle (1.21 ± 0.1). The higher concentration of norgestrel produced a significant reduction in polyI:C-induced IL-12p70 production for cells incubated for 48h (Figure 5.24, panel C), approximately 44% reduction (0.496 ± 0.054, $P < 0.05$) compared with polyI:C +vehicle (0.88 ± 0.11).

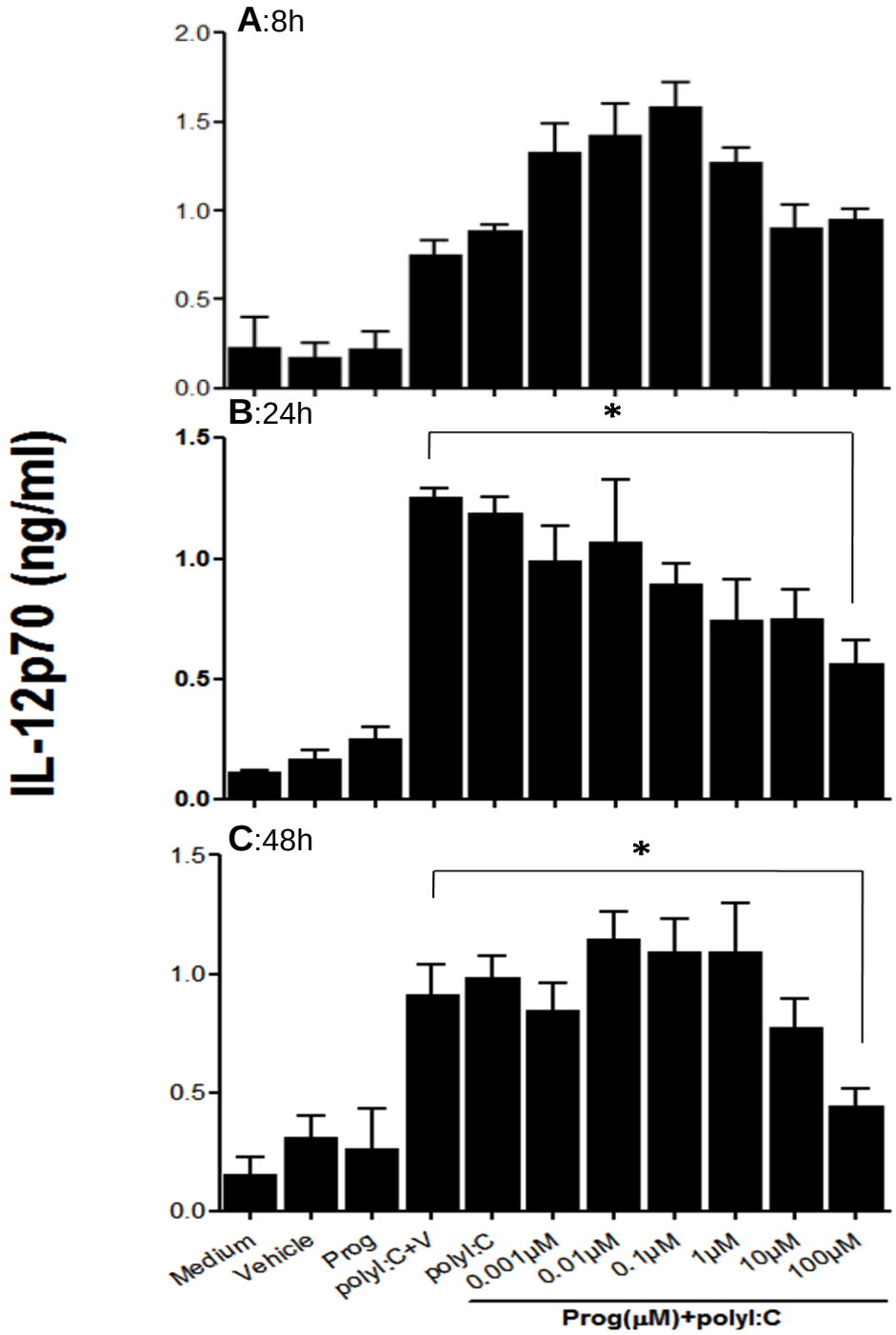


Figure 5.23. The influence of progesterone on polyI:C-induced IL-12p70 production in BMDCs. Cells were treated with vehicle (V), 10-fold increasing concentrations (0.001μM-100μM) of progesterone (Prog) hormone one hour prior to exposure to 25μg/ml polyI:C for 8h (A), 24h (B) or 48h (C). Supernatants were collected and IL-12p70 levels were assessed by ELISA as detailed in the Methods section. Each value represents the mean±SEM from 3 independent experiments. *P<0.05 is statistically significant vs. agonist plus vehicle (polyI:C+V).

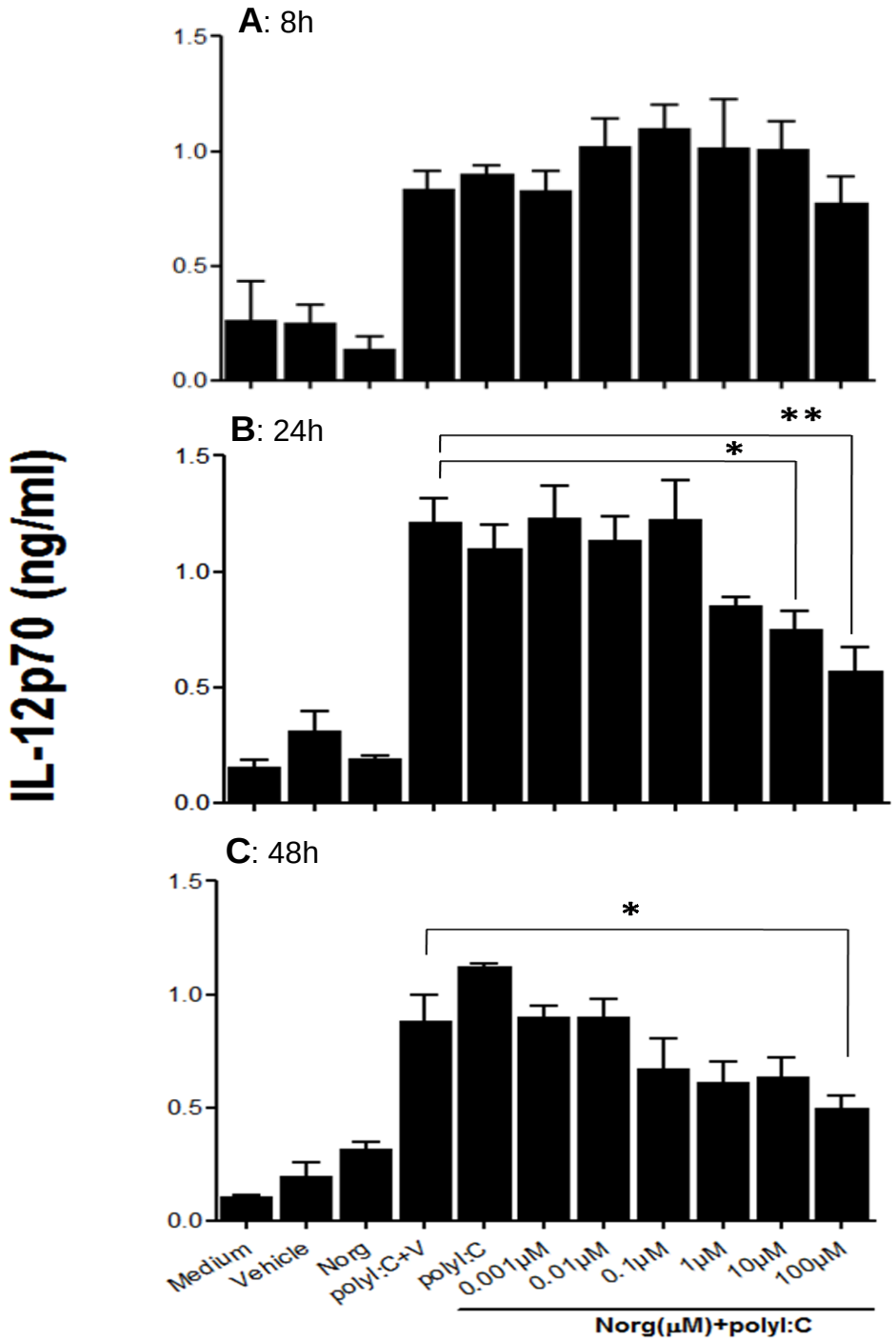


Figure 5.24. The influence of norgestrel on polyI:C-induced IL-12p70 production in BMDCs. Cells were treated with vehicle (V), 10-fold increasing concentrations (0.001µM-100µM) of norgestrel (Norg) hormone one hour prior to expose to 25µg/ml polyI:C for 8h (A), 24h (B) or 48h (C). Supernatants were collected and IL-12p70 levels were assessed by ELISA as detailed in the Methods section. Each value represents the mean±SEM from 3 independent experiments. *P<0.05 and **P<0.001 are statistically significant vs. agonist plus vehicle (polyI:C+V).

5.3 Discussion

Studying specific signalling pathway outcomes that include mediator production or regulation of transcriptional processes may provide clear and comprehensive mechanisms towards helping in the evaluation of the effects of drugs or chemicals. The present study has monitored known inflammatory signalling pathways, and examined the potential influences of progesterone on various aspects of these pathways. In the current chapter and as an attempt to link with all aspects of the experiments in chapters 3 and 4 and their impact on down stream events, the effects of progesterone on the expression of targeted gene and production of key cytokines were investigated.

In regard to progesterone influence on IL-12 family cytokine gene expression, results showed some inhibitory effects of the hormone on IL-12p35 and on EBI3 mRNA expression but no influence on IL-23 p19 and IL-27p28 mRNA expression. Both progesterone and norgestrel hormones reduced polyI:C-induced IL-12p35mRNA expression whilst only norgestrel at higher concentration reduced LPS-induced IL-12p35mRNA expression. It is unclear how norgestrel (PR- agonist) mediated an effect not mediated by progesterone that activates both PR and GR. So a simple explanation may relate to technical factors during experiments or to the variation in results over 3 experiments which impacted on the net statistical analysis that showed no influence of progesterone hormone on LPS-induced IL-12p35mRNA expression. Results from previous Chapters showed sustained polyI:C-induced IRF3 phosphorylation by progesterone and norgestrel; progesterone inhibited LPS-induced IRF3 nuclear translocation and progesterone/norgestrel inhibited polyI:C-induced IRF3 nuclear translocation, and both hormones reduced LPS- and polyI:C- induced ISRE DNA-binding activity. A study by Goriely *et al* (2006) reported a putative IRF3 binding site named as ISRE-1 in the promoter region of IL-12p35 gene suggesting a potential role for IRF3 in IL-12p35 gene expression. As mentioned previously the sustained IRF3 phosphorylation by progesterone and norgestrel hormone may be considered as a repressive effect. Subsequent results reinforced these observations and the hormones also inhibited LPS- and polyI:C-induced IRF3 nuclear translocation and ISRE DNA-binding activity. So all these events may have

contributed to the inhibitory effect of the hormones on the induction of IL-12p35 expression.

The cytokine IL-27 like other IL-12 family members, IL-12 and IL-23 are rapidly secreted by APC upon TLR activation (Villarino *et al*., 2004; Crabe *et al*., 2009). IL-27 has a complex role as either a pro-inflammatory (Yoshida *et al*., 2001; Villarino *et al*., 2008) or an anti-inflammatory mediator (Stumhofer and Hunter, 2008). Furthermore, each subunit of this cytokine (p28 and EBI3) has an independent function and can bind to other proteins forming additional bioactive mediators (Crabe *et al*., 2009). Results presented here demonstrated the inhibitory effects of both progesterone and norgestrel on LPS or polyI:C-induced EBI3 mRNA. The hormones, however, had no influence on the induction of p28mRNA. Consequently, it is difficult to determine whether there is a reduction in total IL-27 production. Nevertheless, what is of major significance in the context of progesterone playing an anti-inflammatory role is that many immune modulatory effects linked to IL-27, such as increased organ injury or even mortality in WSX-1 deficient mice infected with *Trypanosoma cruzi*or or *Toxoplasma gondii* as a result of excessive proinflammatory cytokines production (Hamano *et al*., 2003; Villarino *et al.*, 2004), can be mediated by the EBI3 homodimer or the EBI3 heterodimer complexed with other proteins such as IL-12p35 in IL- 35. In addition there is attenuation of Th2 responses associated with a reduction in NK T cells number in EBI3 deficient mice (Nieuwenhuis *et al*., 2002). P28 and EBI3 subunits exhibit different patterns of transcription (Stumhofer *et al*., 2010). Whilst p28 utilises c-Rel and IRF3 for optimum LPS-induced IL-27p28 mRNA expression and protein production (Liu *et al*., 2007; Molle *et al*., 2007), NFκB p50 and p65 are required for EBI3 mRNA expression. Therefore, the inhibition effect mediated by both hormones would be achieved by targeting EBI3 instead of IL-27p28 and may involve different protein-protein or protein-transcription factor interaction mechanisms depending on associated protein involved with each subunit gene and with steroid hormone receptors.

Both LPS and polyI:C induced IFNβ mRNA expression after 2 hours of stimulation that seemed to decline with time in both cases. The higher concentration of progesterone (62.5 μM) reduced LPS-induced IFNβ mRNA expression at 6 hours

post stimulation, at the same time, both concentrations of progesterone and norgestrel reduced polyI:C-induced IFNβ mRNA expression. Hence, this progesterone inhibitory effect was more efficient against TLR3 stimulated induction and would appear to be mediated via the PR as norgestrel also exerted this effect. These findings would support the previous results that showed both hormones targeted some of the signalling events activated by TLR3 more clearly and more pronounced extent than activated by TLR4 and it was notable that both hormones reduced polyI:C-induced p65 and IRF3 nuclear translocation. However, progesterone treatment but not norgesteral reduced LPS-induced IRF3 translocation and IFNβ mRNA expression, which may indicate that this effect is mediated through GRs. In addition both hormones also reduced LPS- and polyI:C- induced ISRE DNA-binding activity. Overall these events serve to induce a reduction in IFNβ mRNA expression as the promoter region of this gene requires the assembly of IRF3/IRF7, ATF-2/c-Jun and NFκB for optimal activity (Servant *et al*., 2002; Sasai *et al*., 2005; Panne *et al*., 2007). Although IRF3 and IRF3-ISRE DNA-binding activity may be forms for hormones effects there remains the requirement to investigate the role of the upstream kinases, TBK1 and IKKε that are responsible for phosphorylation of IRF3. Furthermore, it remains to be determined whether the inhibitory effects of progesterone and norgestrel are in fact due to modulation of kinase expression and/or activity. This would be pursued further with the recent availability of kinase inhibitory molecules e.g. MRT67307.

Results in the current chapter demonstrated inhibition of LPS- or polyI:C-induced IL-6 production by progesterone but not norgestrel, suggesting that this effect was mediated via GRs similar findings have been reported by Kim *et al*, (2012). These workers reported that progesterone attenuated both IL-6 mRNA expression and protein levels induced by LPS in uterine cervical fibroblast. Once again, results showed that the hormone inhibited polyI:C-induced IL-6 production earlier and to a greater extent than LPS- IL-6 induction.This can be attributed to the specificity of the mode of interaction between the steroid hormone receptors and kinases, transcription factors and other proteins related to TLR3 signalling as discussed previously.Lesmeister *et al.*, (2005) reported that steroid hormone (estrogen and progesterone) inhibitory effects on TLR3 function were not related to a

decrease in TLR3 mRNA or protein but they were a result of hormone receptor-dependent effects.

Both hormones have been demonstrated to reduce LPS- and polyI:C-induced IL-12p70 production in BMDCs after 24 and 48 hours of incubation. It has been reported that IL-12p70 is secreted by DCs in response to TLR3 and TLR4 ligation as part of antimicrobial responses (Johanson *et al*., 2011). Bioactive IL-12p70 production is limited by p35 availability (Snijders *et al*., 1996; Grumont *et al*., 2001). Type 1 IFNs have been found to induce IL-12p70 production in immature DCs (Heystek *et al*., 2003; Gautier *et al*., 2005). The results presented here demonstrated the inhibitory effects of the hormones on both IL-12p35 and IFNβ gene expression both of which would contribute to IL-12p70 production. Butts *et al*., (2011) and Xu *et al*., (2011), have documented similar immune suppressive effects induced by progestrone on DC-IL-12 production.

In summary during the current chapter of the study progesterone was shown to induce numerous immune suppressive effects on some of the important TLR3 and 4 signalling outcomes. Some of these effects have been shown to be mediated via the PR such as inhibition of IL-12p35 mRNA expression, EBI3 mRNA expression and IL- 12p70 production. In contrast reduction of DC IL-6 production was likely to be mediated through the GR as norgestrel could not mediate this effect.

CHAPTER SIX

GENERAL DISCUSSION

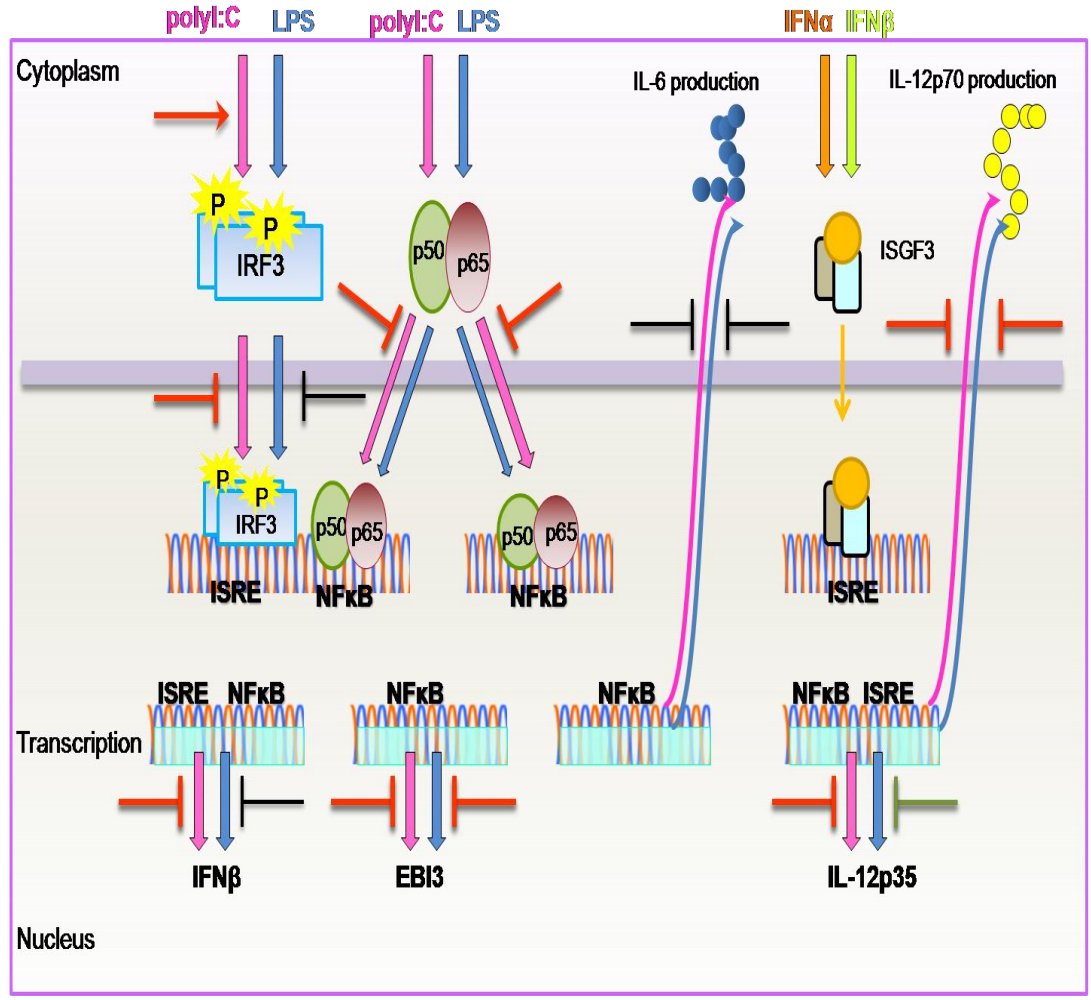
Discussion

The maintenance of optimum disease management claims to address sex-based differences both in disease pathogenesis and in the pharmacodynamic and pharmacokinetic of therapies. Sex biased differences in the outcome of diseases have been attributed to chromosomal differences between the sexes, differential expression of steroid hormones and anatomical differences (Fish, 2008; Pennell *et al*., 2011). Progesterone is one of the steroid hormones that has the ability to modify immune responses and influence disease outcomes (Butts *et al*., 2011; Xu *et al*., 2011). Its effects are most notable at higher concentrations associated particularly with specific menstruation stages and pregnancy (Su *et al*., 2009; Xu *et al*., 2011). There is a shift towards an anti-inflammatory Th2 immunological status during pregnancy that becomes dominant by the third trimester (Sykes *et al*., 2012). There is a growing consensus implicating steroid hormones such as progesterone and estrogens favouring the development expansion Th2 and Treg cells which is necessary for seccessful pregnancy (Robinson and Klein, 2012) The results described herein demonstrate that progesterone has a direct immunomodulatory effect on dendritic cells which is also likely to affect downstream T cell functions.

The majority of the results generated during the current study (see summary of results, Figure 6.1) showed that progesterone displays suppressive effects on multiple immune responses induced by TLRs. These suppressive effects were evident following both TLR3 and TLR4 ligation although they were accentuated following TLR3 ligation.

The role of TLR3-mediated signalling has been gaining increased attention. It is known to mediate anti-viral responses through the TRIF/ IRF3/ IFNβ signalling pathway (Uematsu and Akira, 2007; Gauzzi *et al*., 2010). A TLR3 ligand has been reported to have a protective role against sexually transmitted viral infections, like herpes simplex virus-2 (HSV-2) (Ashkar*et al*., 2004). It has also been reported that pretreatment with polyI:C significantly reduces herpes simplex virus-1 (HSV-1) encephalitis by inducing immune gene expression (Boivin*et al*., 2008). However, there is also a growing interest in the employment of TLRs signalling in developing therapies. TLR3 agonists have recently emerged as a promising adjuvant in vaccine-

based immunotherapy for cancer treatment. In addition, it has been found that the TLR3 agonist, polyI:C inhibits cancer growth by triggering apoptosis mechanism in these cells (Cheng and Xu, 2011).



Induction mediated by TLR3 ligand polyI:C Induction mediated by TLR4 ligand LPS

Induction mediated by prog and Norg Inhibition mediated by Norg

Inhibition mediated by Prog and Norg Inhibition mediated by Prog

Figure 6.1. Summary of the effects of hormones (progesterone and norgestrel) on the inflammatory signalling elements in BMDCs. Progesterone (Prog) and norgestrel (Norg) increased polyI:C-induced IRF3 phosphorylation. Both hormones showed reduction of polyI:C-induced IRF3 and p65 nuclear translocation whilst progesterone alone reduced LPS-induced IRF3 nuclear translocation. Both hormones reduced polyI:C- and LPS- induced ISRE DNA-binding activity (data not presented in figure). Both hormones also reduced polyI:C-induced IL-12p35mRNA, EBI3mRNA and IFNβmRNA expression and LPS-induced EBI3mRNA expression. Norgestrel alone reduced LPS-induced IL-12p35mRNA and progesterone alone reduced LPS-induced IFNβ expression. Progesterone reduced polyI:C- and LPS-induced IL-6 cytokine production and the both reduced polyI:C- and LPS-induced IL-12p70 cytokine production.

Boosting TLR3/IRF3 signalling in BMDCs has been exploited in developing vaccines to induce specific cytotoxic T cell response and IFNγ production to inhibit tumour growth in mice (Wang *et al*., 2011). In the same context, a TLR3 agonist in combination with other TLR agonists have been utilised in generation of DCs used in the treatment of acute myeloid leukaemia (Beck *et al*., 2011). It has also been reported that polyI:C can improve the inhibitory effect of an anti-cancer drug by inhibiting cell proliferation in oral cancer (Park *et al*., 2012). The results obtained in this thesis demonstrate that progesterone levels might influence the success of these therapies.

Clearly progesterone is unlikely to be administered to humans as an immune-modulator due to its other more widely known effects. However, the data obtained here demonstrate that it is possible to modulate TLR3-mediated signalling potentially through phosphorylation of IRF3 and this could potentially be exploited in the future.

The mechanism by which progesterone and norgestrel sustain TLR3-mediated IRF3 phosphorylation requires further investigation upstream in the IKK-IRF pathway to elucidate the site of potential regulation and also down-stream to support whether this effect mediates selective repression or activation of target genes. In a related manner one possible investigation could include examine the influence of hormones on expression and phosphorylation of TBK1 and IKKε kinases which are responsible of IRF3 phosphorylation. Furthermore, the recent availability of small molecule kinase inhibitors, namely MRT67307 (Clark *et al.,* 2011), targeting both TBK1 and IKKε would allow further investigation into how these kinases may potentially be involved in mediating the immunomodulatory effects of progesterone on IRF3 status. Additioanal cell staining/imaging and chromatin immunoprecipitation (ChIP) techniques may provide extra details regarding nuclear translocation and/or DNA-binding for IRF3 and/or NFκB transcription factors. Furthermore, the measurement of NFκB or ISRE transcriptional activity by transfection of DCs with a transcription factor-linked luciferase plasmid would enable the study of the influence of the hormones on the transcriptional activity of these proteins.

Another important field to study to help in the further elucidation of the mode of action of progesterone on immune responses is progesterone-progesterone receptor signalling. As mentioned previously, DCs express PRs and these receptors are subject to increased expression as hormone levels increase (Butts *et al*., 2011). Classical interaction includes binding of the hormone-receptor complex to a specific DNA sequence, named the progesterone response element (PRE), in the promoters of PR-target genes (Chen *et al*., 2011). Moreover, the progesterone-receptor complex can interact with other transcription factors as they bind their response elements in target genes and modulate transcriptional activity at these genes. Transcription factors that interact with the progesterone-PR complex include, NFκBs, specificity protein 1(Sp1), STAT5 and AP-1 (Chen *et al*., 2011). Thus, by using the available molecular techniques such as EMSA or ChIP, mode, time and type of interaction should be examined. Besides, this may allow the clear identification of the co-activator and co-repressor proteins that complexes with the PR and PR complexes at the transcription level.

One of the important outcomes of this study is the identification of the inhibitory effects of progesterone on IL-12p35 expression and IL-12p70 protein production, which have key roles in inducing both innate and adaptive immunity. Mature IL-12 induces NK cells proliferation, differentiation of Th1 cells and activation of CD8+ cells (Biron *et al*., 1999; Trinchieri, 1998; Trinchieri, 2003) and DCs have emerged as major *in-vivo* and *in-vitro* producer of IL-12 cytokine (Grumont *et al*., 2001). Regulation of IL-12 production is complex and requires regulation of IL-12p35 and IL-12p40 expression at the transcriptional, post-transcriptional and post-translational levels (Trinchieri, 1998; Trinchieri, 2003). Further analysis of the promoter region of the IL-12p35 gene is required to determine the molecular mechanism by which steroid hormones reduce its expression. This is further complicated by the fact there are four isoforms for the gene (I-IV) and it is also regulated at both transcriptional and translational levels (Babik *et al*., 1999). Kollet and Petro, (2006) documented that a region in the IL-12p35 promoter of murine macrophage is induced by c-Rel and IRF1 when cells are co-stimulated by LPS and IFNγ. Hence, expansion of investigations to consider further other

transcription factors and their locus/loci in the IL-12p35 promotor region in murine DCs and potential regulation by progesterone would be relevant.

Overall the studies described in this thesis demonstrate that progesterone influences TLR-mediated signalling events and ultimately the function of dendritic cells. The results provide potential insight into immunomodulation during pregnancy and control of TLR-mediated signalling and inflammatoryevents. They also suggest that progesterone levels should be taken into account when designing therapies that rely on TLR agonists.

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APPENDICES

APPENDIX A:

Primer sequences

Primer Name	Primer Sequence	Size of amplified portion	Annealing Temperature ˚C
Tbp Forward	AAC AGC AGC AGC AAC AAC AGC AGG	192	64
Tbp Reverse	TGA TAG GGG TCA TAG GAG TCA TTG G		
IL-12p35 Forward	CTT AGC CAG TCC CGA AAC CT	123	60
IL-12p35 Reverse	TTG GTC CCG TGT GAT GTC T		
IL-23p19 Forward	AAG TTC TCT CCT CTT CCC TGT CGC	209	60
IL-23p19 Reverse	TCT TGT GGA GCA GCA GAT GTG AG		
IL-27p28 Forward	ATC TCG ATT GCC AGG AGT GA	128	64
IL-27p28 Reverse	GTG GTA GCG AGG AAG CAG AGT		
EBI3 Forward	GCC ATG CTT CTC GGT ATC C	101	60
EBI3 Reverse	GAG CCT GTA AGT GGC AAT GA		
IFNβ Forward	GCA CTG GGT GGA ATG AGA CT	135	60
IFNβ Reverse	AGT GGA GAG CAG TTG AGG ACA		

Table A. primer sequences, size of amplified portions and annealing temperatures. Oligonucleotide primer used in RT-PCR were obtained from former publication that include the following, Tbp (manzies *et al*., 2011), IL-12p35 (Goriely *et al*., 2006), IL-23p19 (Carmody *et al*., 2007), IL-27p28 and EBI3 primers (Molle *et al*., 2007). IFNβ primers designed using MacVector™ 7.0 software (Oxford Biomolecular, UK).

APPENDIX B:

pDrive cloning vector

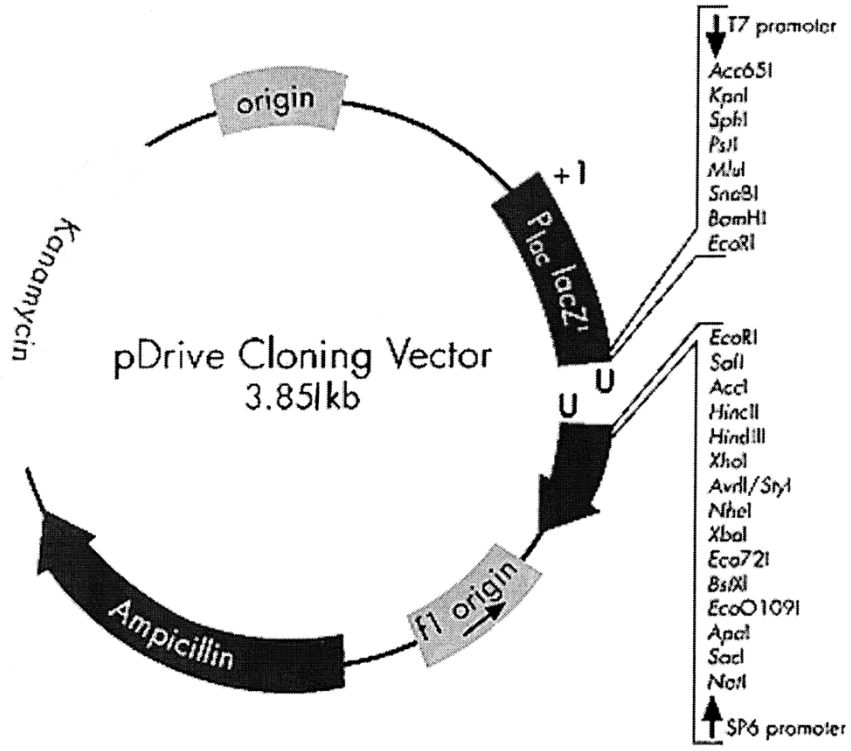
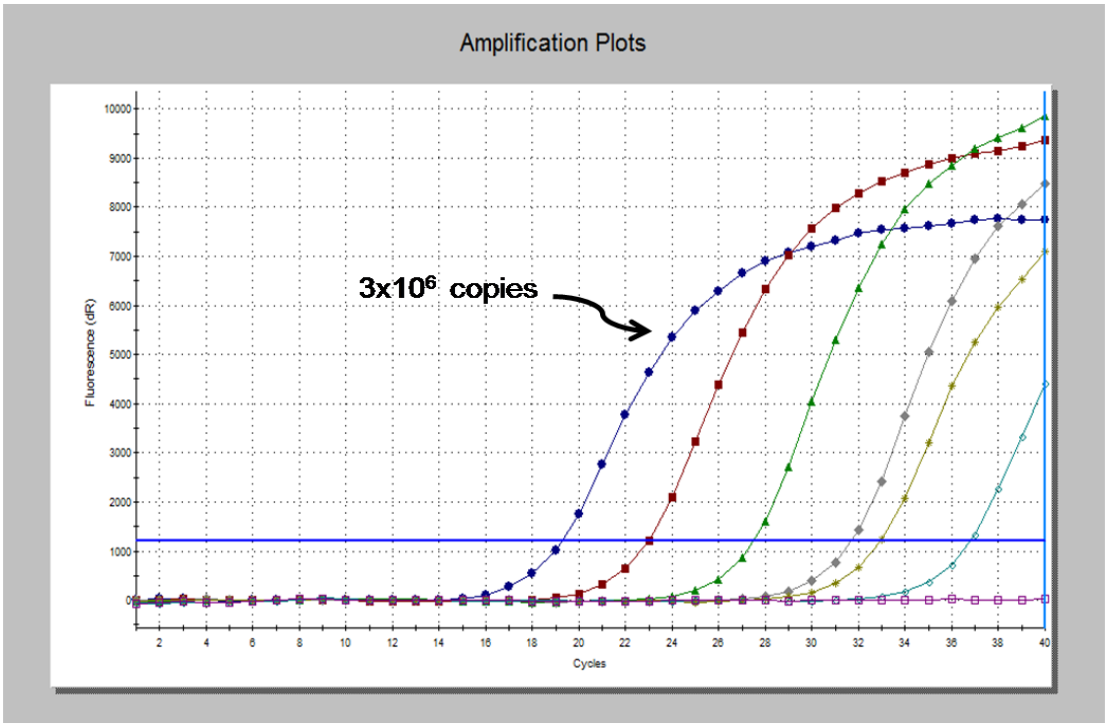


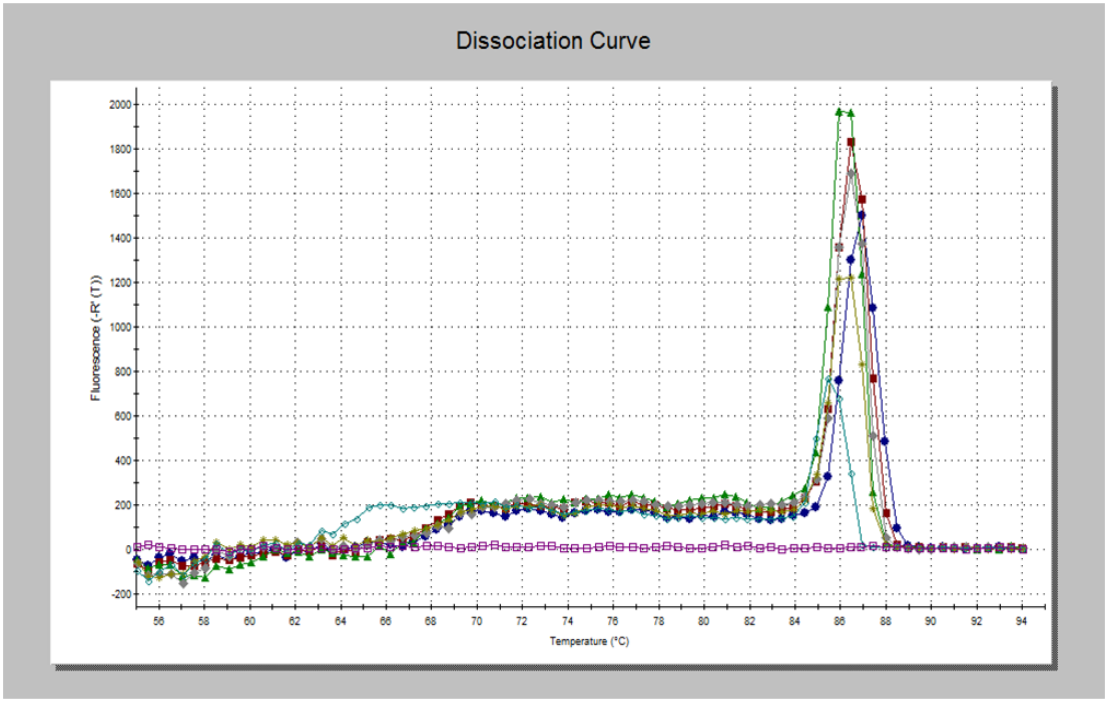
Figure B. pDrive cloning vector map and sites of restriction enzyme.

APPENDIX C:

Quantitative real time reverse transcription–polymerase chain reaction (qRT–PCR) standard curves

A





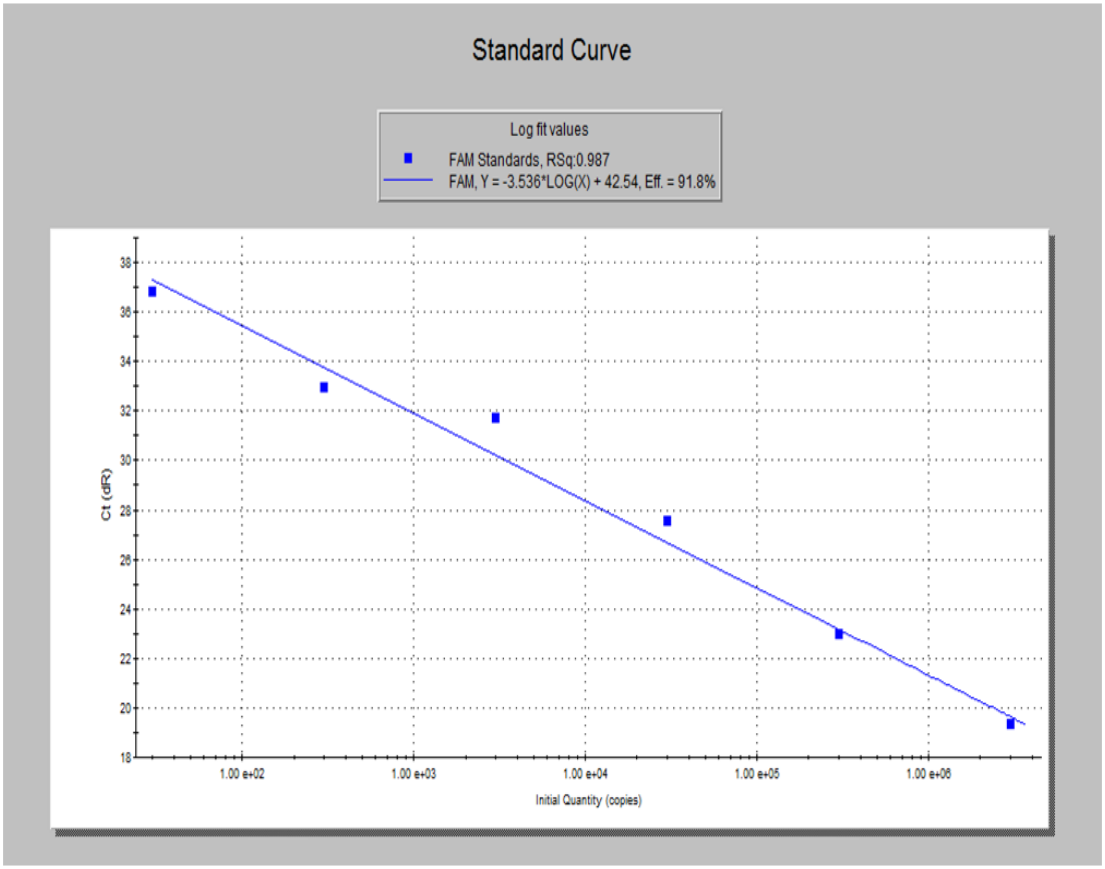
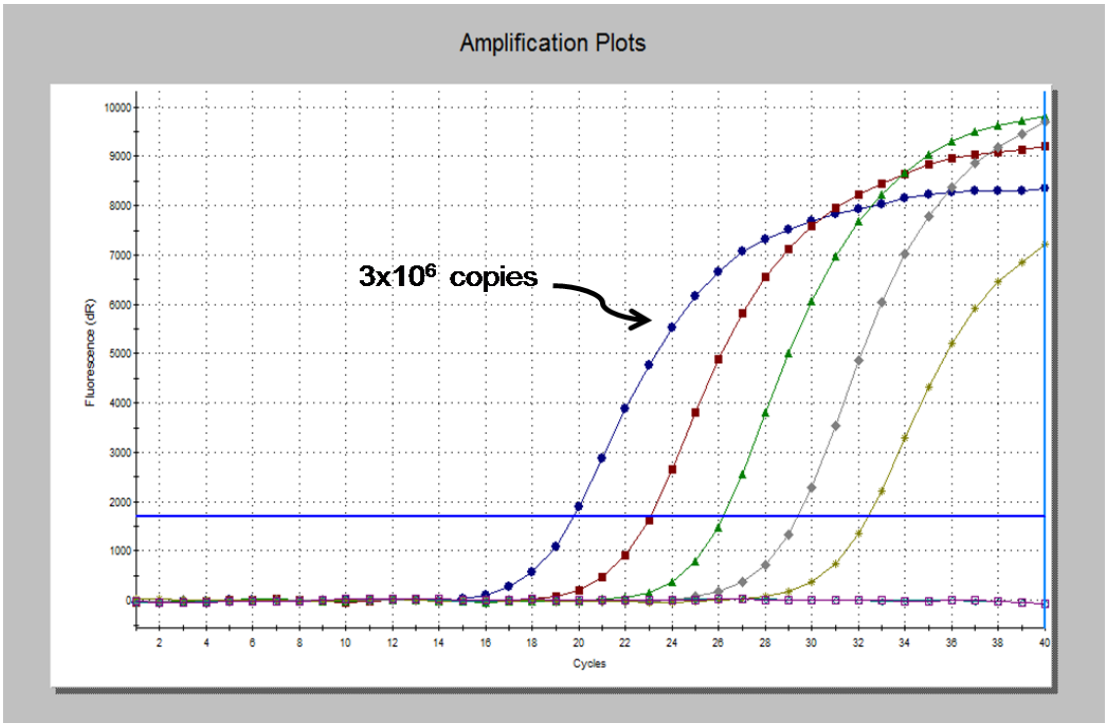
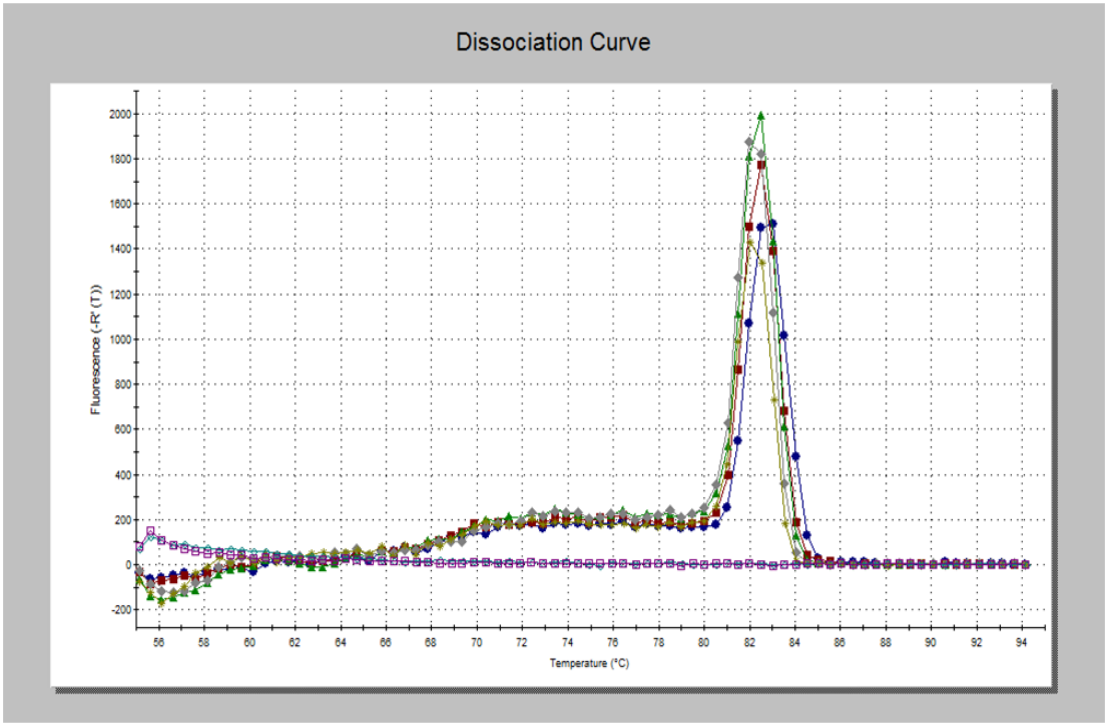


Figure C1. Standard curve of Tbp transcripts. A portion of Tbp was amplified by RT-PCR using the associated primers listed in Table (A). Standardes ranging from $3x10^1$ to $3x10^6$ copies were prepared as described in Methods section. An amplication curve as shown in panel (A) indicates the order by which DNA is amplified to a detectable level. The presence of a single peak on dissociation curve as shown in panel (B) indicates that a single specific product has been amplified and the Tbp standard curve with efficiency value 91.8% and R^2 = 0.987 shown in panel (C).


Amplification Plots
3x10⁶ copies
Fluorescence (dR)
Cycles


Dissociation Curve
Fluorescence (-R' (T))
Temperature (°C)

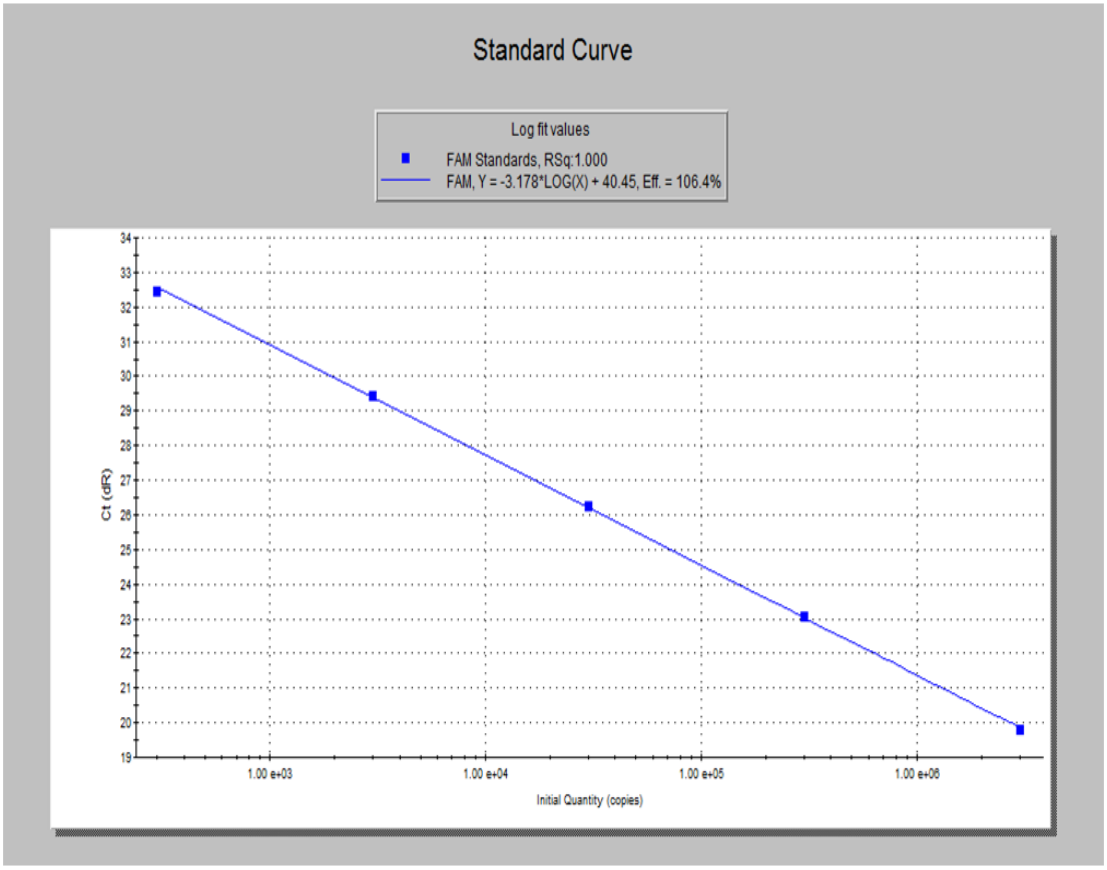
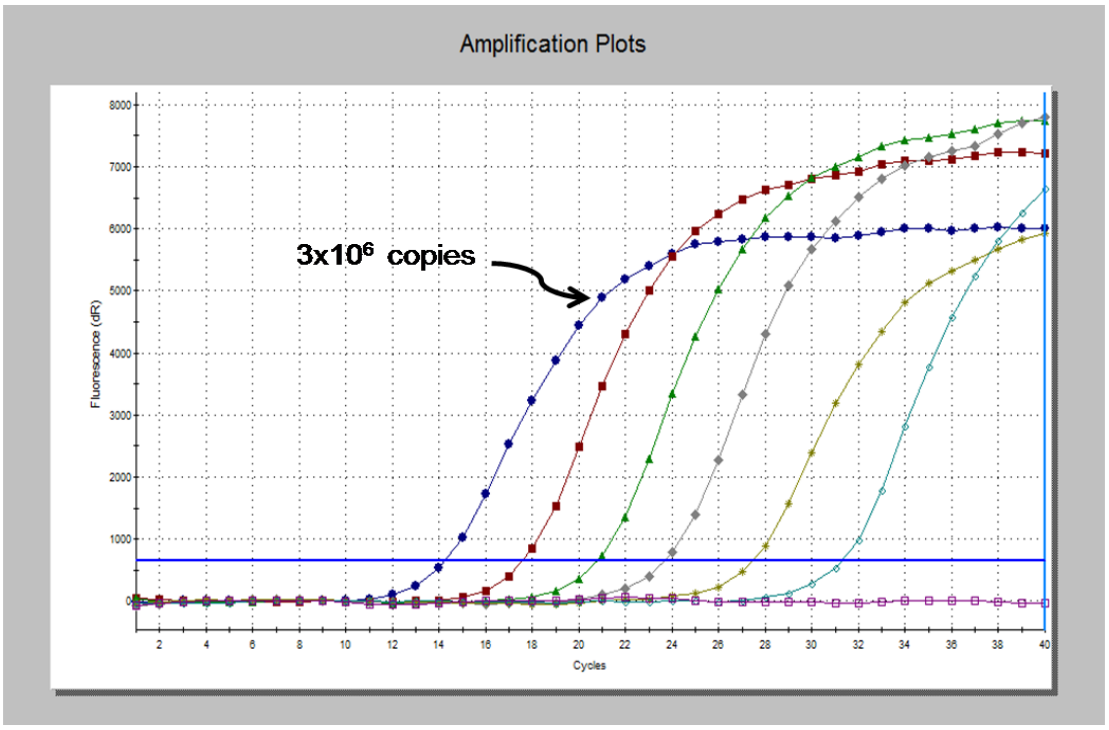
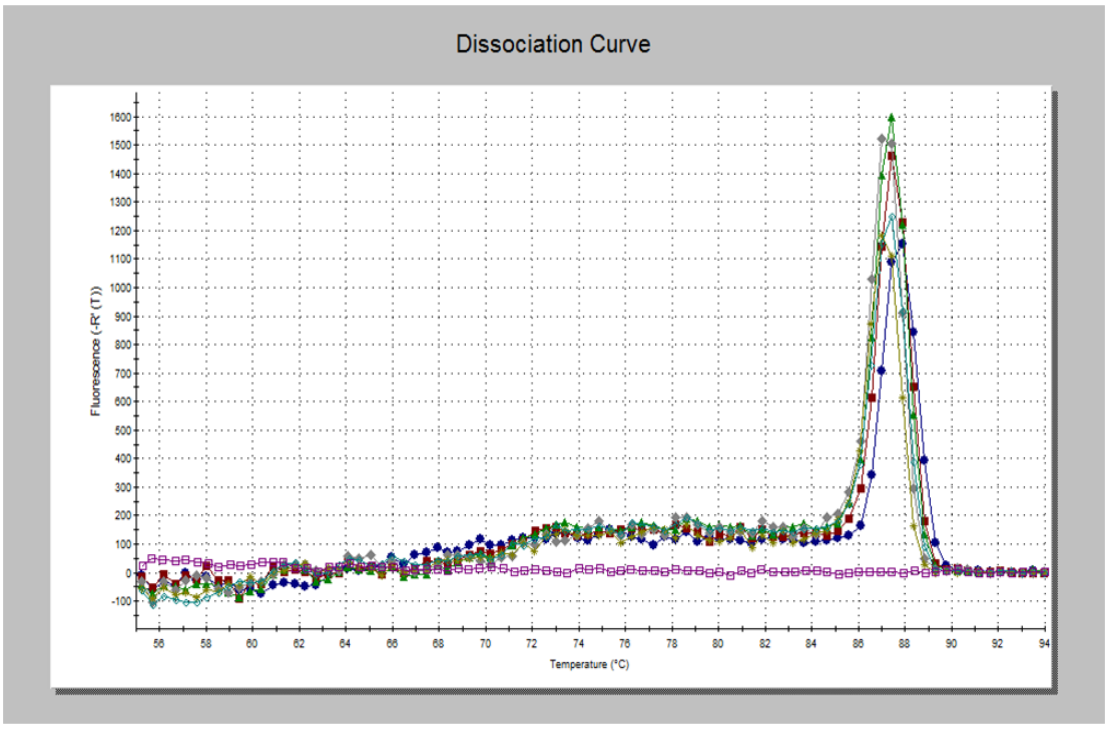


Figure C2. Standard curve of IL-12p35 transcripts. A portion of IL-12p35 was amplified by RT-PCR using the associated primers listed in Table (A). Standardes ranging from $3x10^1$ to $3x10^6$ copies were prepared as described in Methods section. An amplication curve as shown in panel (A) indicates the order by which DNA is amplified to a detectable level. The presence of a single peak on dissociation curve as shown in panel (B) indicates that a single specific product has been amplified and the IL-12p35 standard curve with efficiency value 106.4% and R^2 = 1.000 shown in panel (C).


Amplification Plots
3x10⁶ copies
Fluorescence (dR)
Cycles

B


Dissociation Curve
Fluorescence (-R' (T))
Temperature (°C)

C

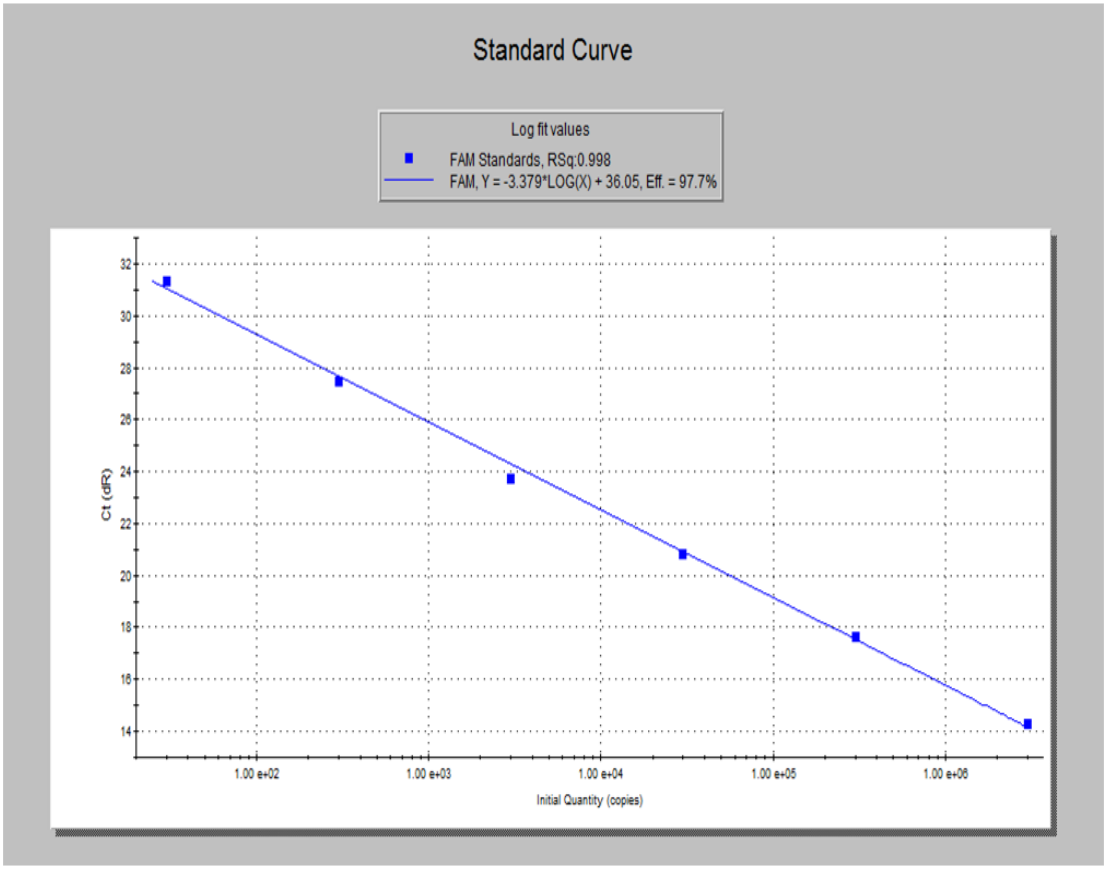
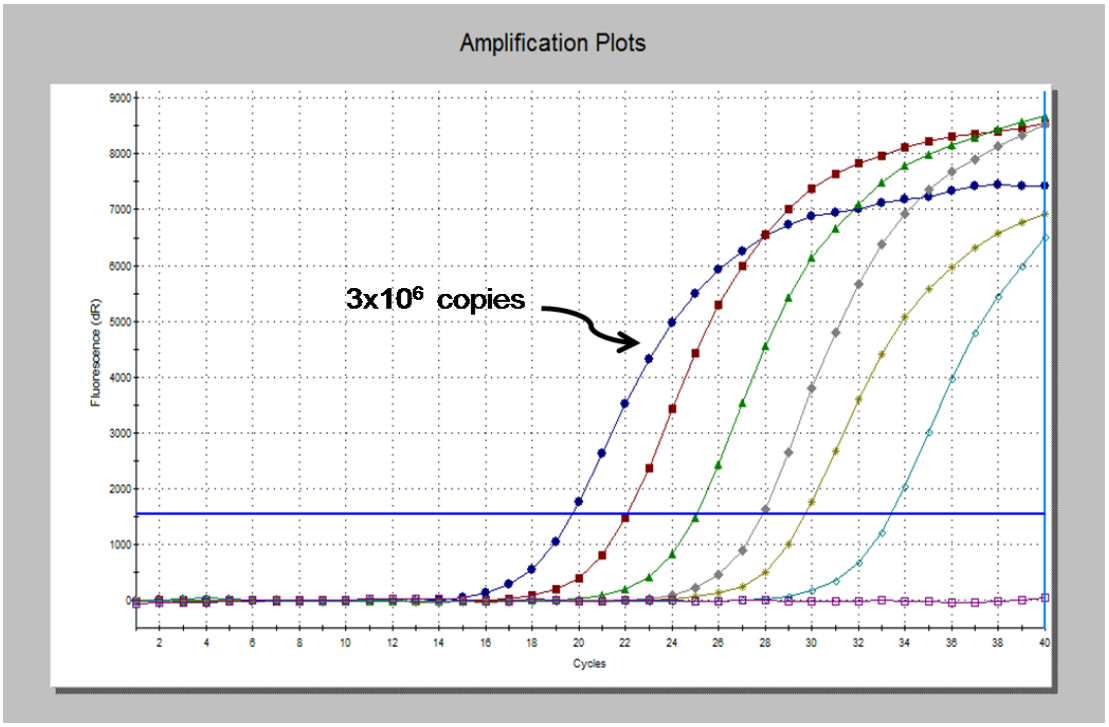
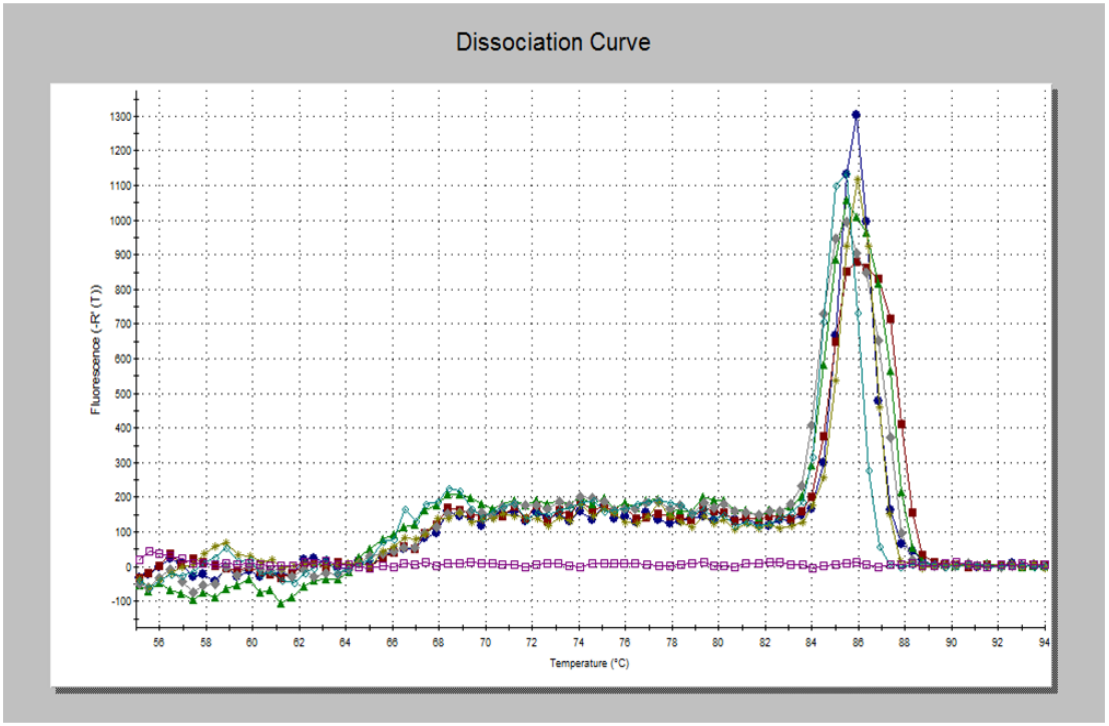


Figure C3. Standard curve of IL-23p19 transcripts. A portion of IL-23p19 was amplified by RT-PCR using the associated primers listed in Table (A). Standardes ranging from $3x10^1$ to $3x10^6$ copies were prepared as described in Methods section. An amplication curve as shown in panel (A) indicates the order by which DNA is amplified to a detectable level. The presence of a single peak on dissociation curve as shown in panel (B) indicates that a single specific product has been amplified and the IL-23p19 standard curve with efficiency value 97.7% and R^2 = 0.998 shown in panel (C).

A


Amplification Plots
3x10⁶ copies
Fluorescence (dR)
Cycles

B


Dissociation Curve
Fluorescence (-R' (T))
Temperature (°C)

C

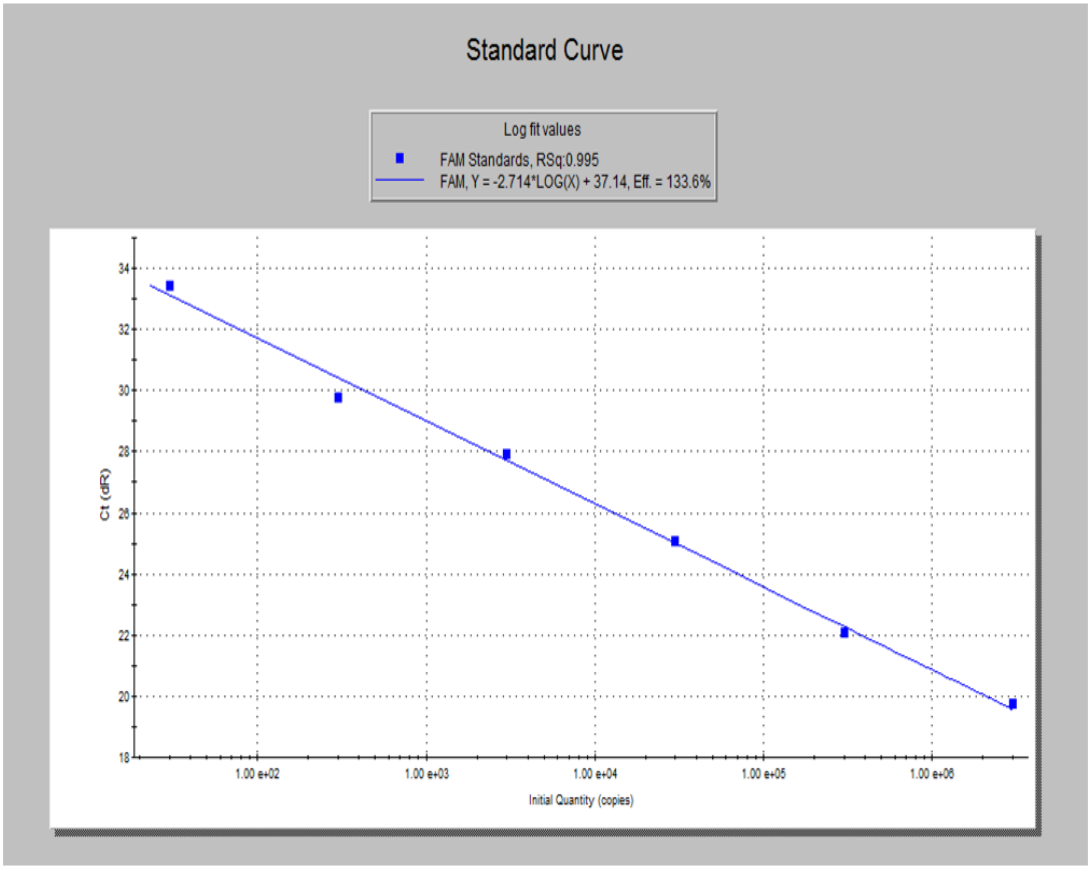
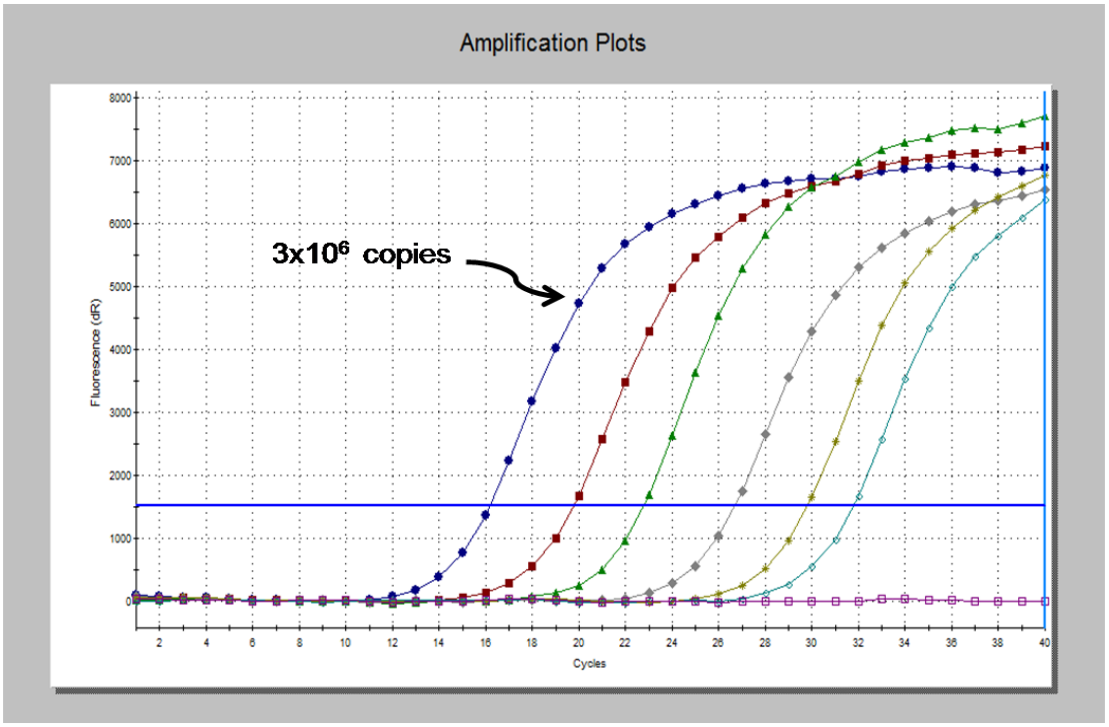
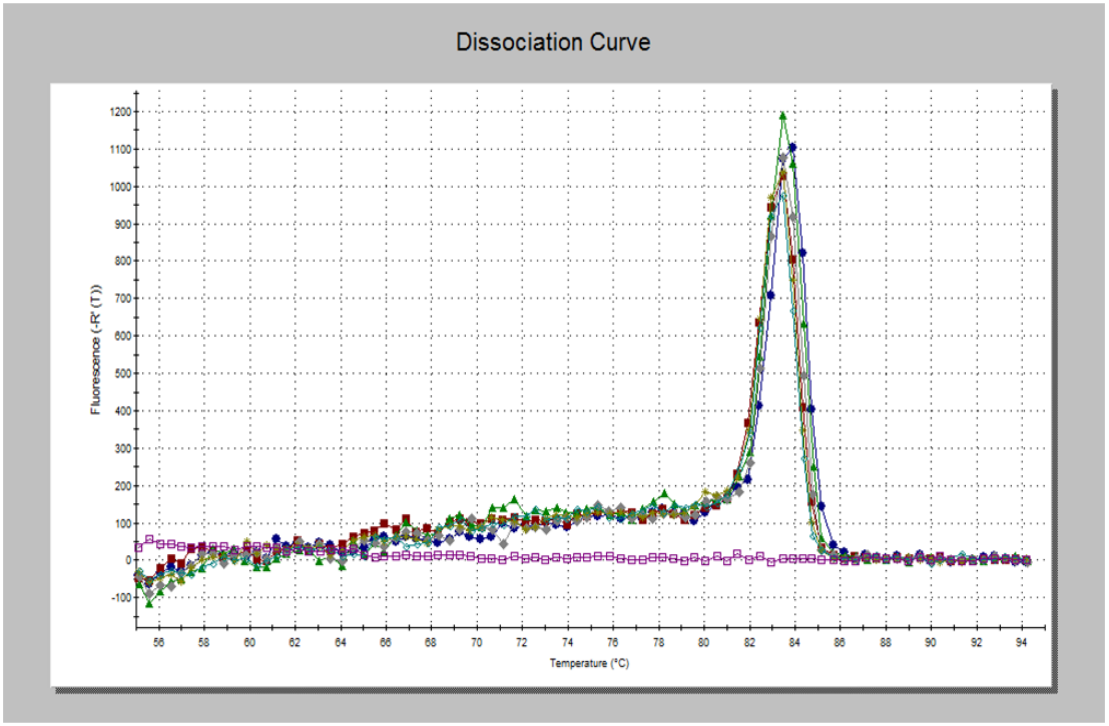


Figure C4. Standard curve of IL-27p28 transcripts. A portion of IL-27p28 was amplified by RT-PCR using the associated primers listed in Table (A). Standardes ranging from $3x10^1$ to $3x10^6$ copies were prepared as described in Methods section. An amplication curve as shown in panel (A) indicates the order by which DNA is amplified to a detectable level. The presence of a single peak on dissociation curve as shown in panel (B) indicates that a single specific product has been amplified and the IL-27p28 standard curve with efficiency value 133.6% and $R^2 = 0.995$ shown in panel (C).

A


Amplification Plots
3x10⁶ copies
Fluorescence (dR)
Cycles

B


Dissociation Curve
Fluorescence (-R' (T))
Temperature (°C)

C

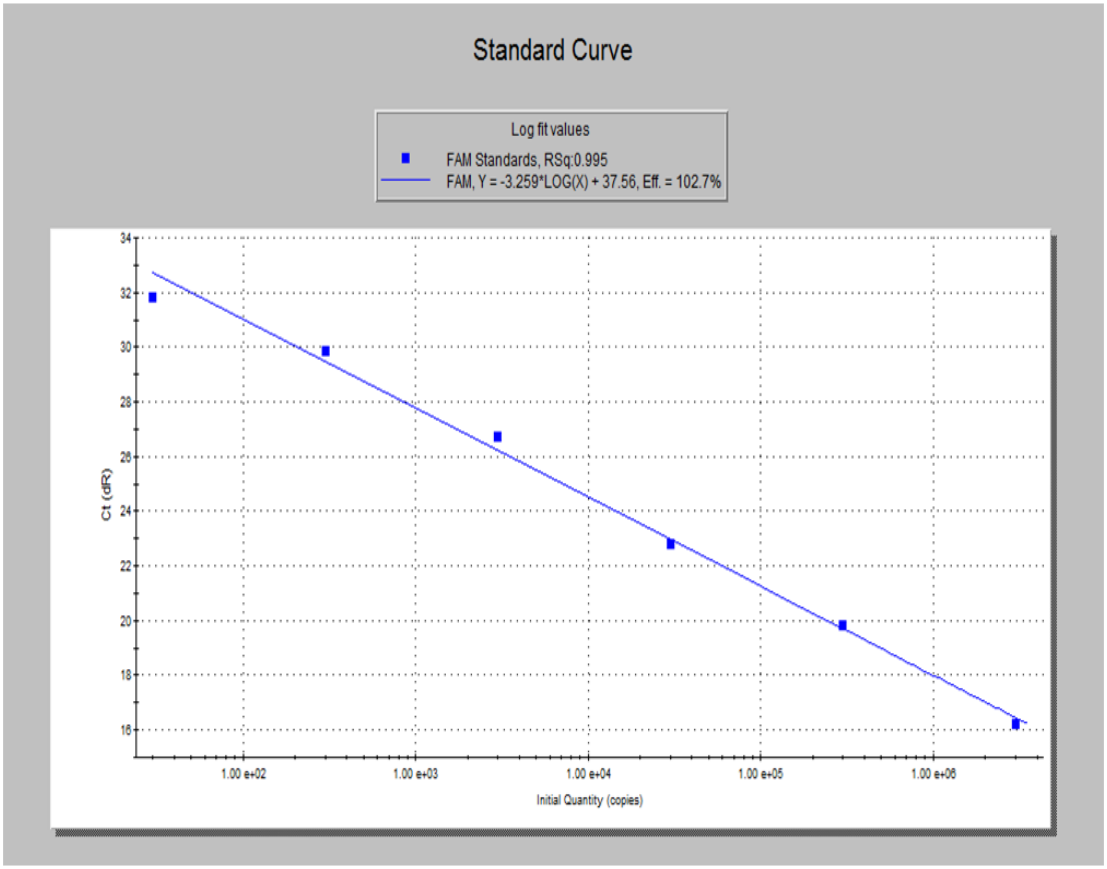
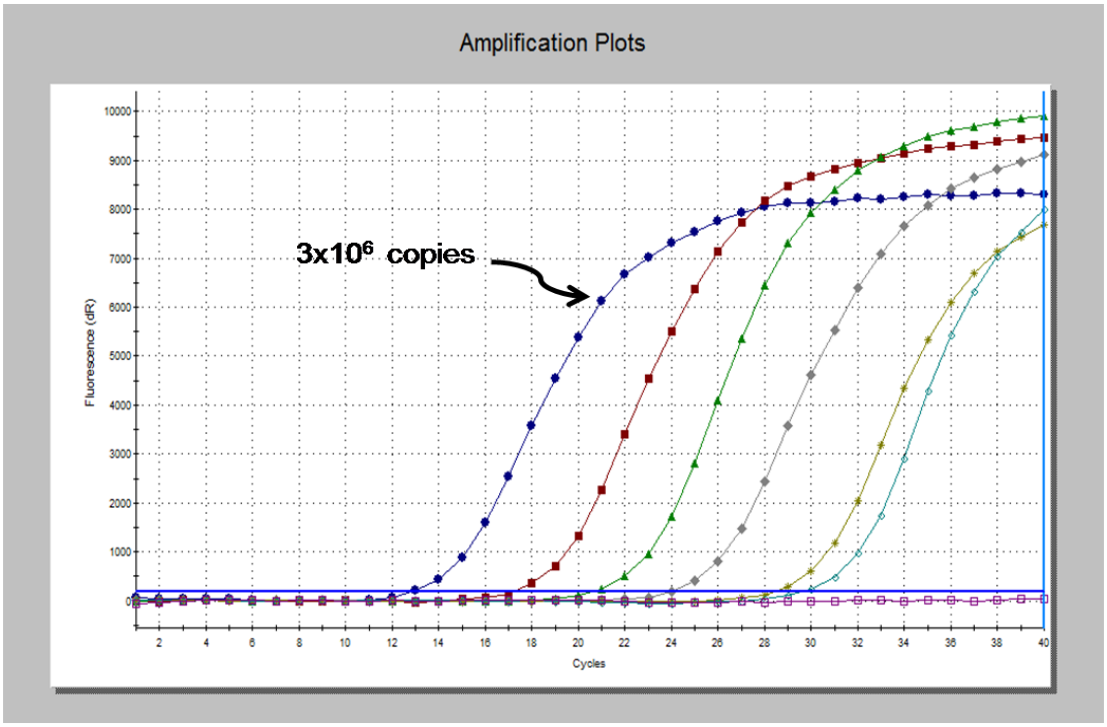
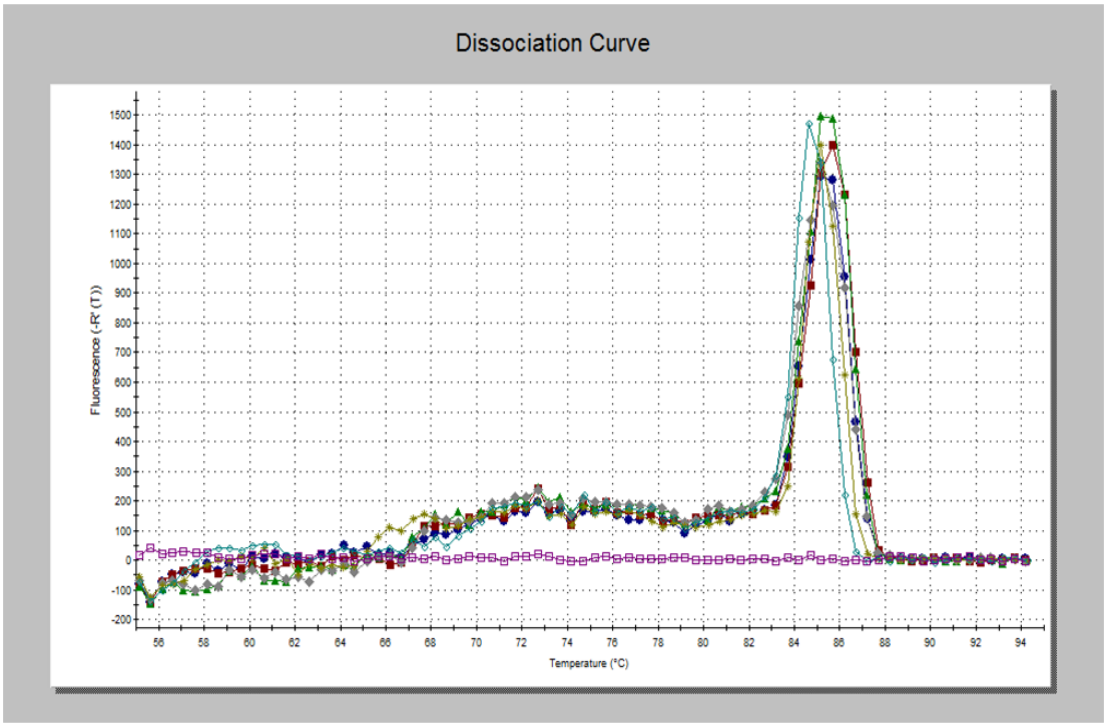


Figure C5. Standard curve of EBI3 transcripts. A portion of EBI3 was amplified by RT-PCR using the associated primers listed in Table (A). Standardes ranging from $3x10^1$ to $3x10^6$ copies were prepared as described in Methods section. An amplication curve as shown in panel (A) indicates the order by which DNA is amplified to a detectable level. The presence of a single peak on dissociation curve as shown in panel (B) indicates that a single specific product has been amplified and the EBI3 standard curve with efficiency value 102.7% and R^2 = 0.995 shown in panel (C).

A


Amplification Plots
3x10⁶ copies
Fluorescence (dR)
Cycles

B


Dissociation Curve
Fluorescence (-R' (T))
Temperature (°C)

C

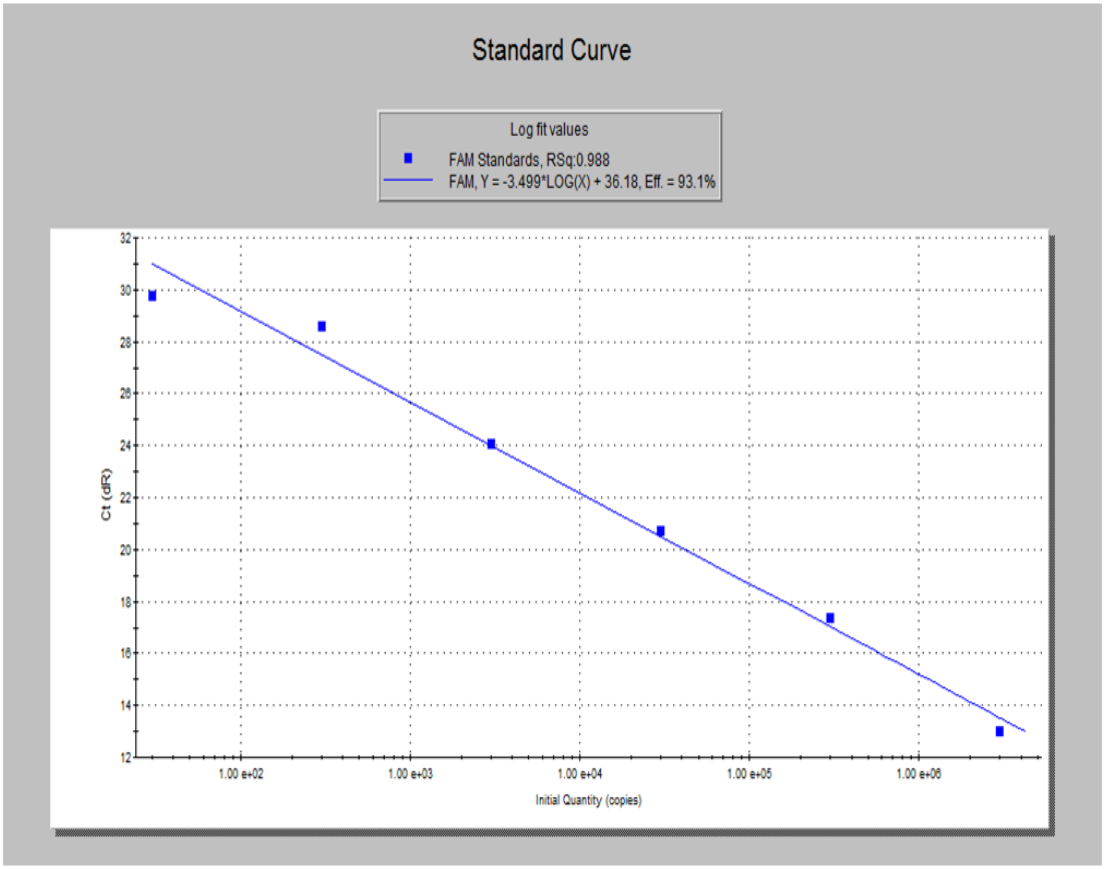


Figure C6. Standard curve of IFNβ transcripts. A portion of IFNβ was amplified by RT-PCR using the associated primers listed in Table (A). Standardes ranging from $3x10^1$ to $3x10^6$ copies were prepared as described in Methods section. An amplication curve as shown in panel (A) indicates the order by which DNA is amplified to a detectable level. The presence of a single peak on dissociation curve as shown in panel (B) indicates that a single specific product has been amplified and the IFNβ standard curve with efficiency value 93.1% and R^2 = 0.988 shown in panel (C).

APPENDIX D:

IL-6 Standard Curve

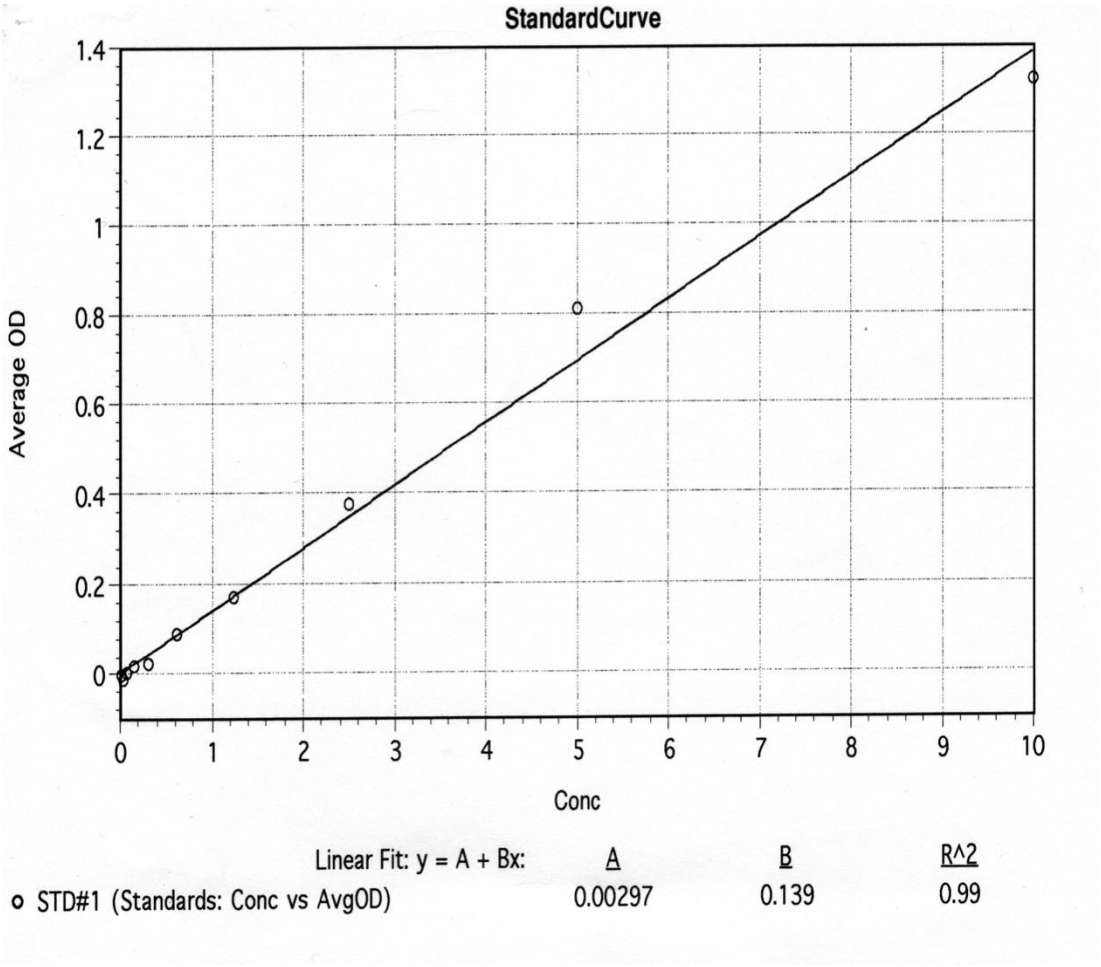


Figure D. IL-6 standard curve

APPENDIX E:

IL-12p70 Standard Curve

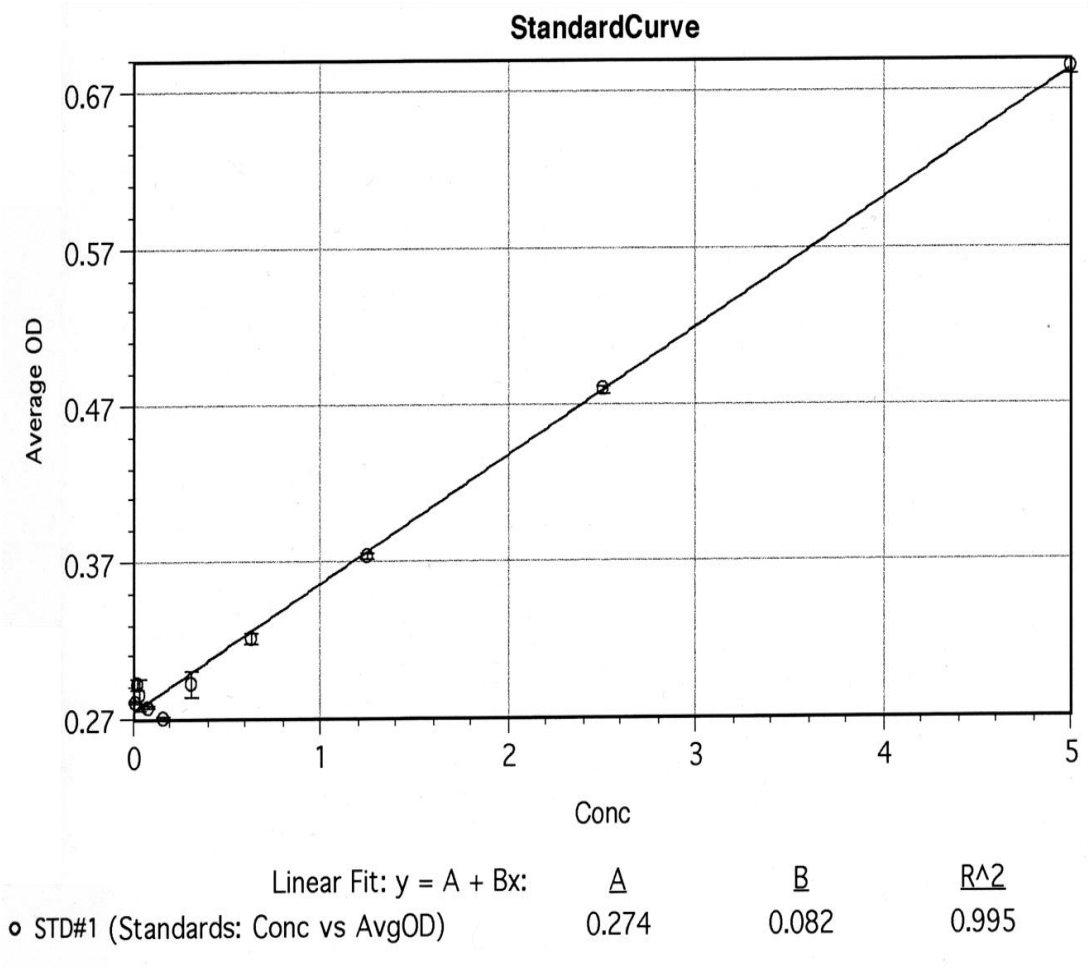


Figure E. IL-12p70 standard curve