IMPACT OF SPECIES-SPECIFIC LACTOFERRICIN PEPTIDES ON MACROPHAGE-ASSOCIATED INFLAMMATORY RESPONSES

by

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ABSTRACT

Inflammation is the body's response to injury or infection and is important for elimination of the infecting agent and healing. However, prolonged inflammation can be damaging and may lead to the development of chronic inflammatory disorders. Recently, there has been growing interest in exploiting antimicrobial peptides (AMPs) that exhibit immunoregulatory activities for treatment of inflammatory diseases. In this study, we have investigated the immunomodulatory effects of the AMP, lactoferricin, from three different species, bovine, murine, and human, with subtle differences in their amino acid sequences. Macrophages, which are a key player in the induction and propagation of inflammation, were used as a cellular model to investigate the effects of species-specific lactoferricin peptides on inflammatory processes. Bovine lactoferricin was the only one of the three peptides studied that downregulated LPS-induced pro-inflammatory cytokines, TNF-α and IL-6 in both human and murine macrophages. Lactoferricin peptides regulated inflammation through targeting of LPS-activated NF-κB and MAPK signaling pathways. The ability of lactoferricin to downregulate a macrophage-mediated inflammatory response suggests potential for the development of this peptide as a novel immunotherapeutic agent.

LIST OF ABBREVIATIONS AND SYMBOLS USED

α	Alpha
Ab	Antibody
Ala	Alanine
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
APS	Ammonium Persulfate
ATCC	American Tissue Culture Collection
ATF	Activating transcription factor
AMP	Antimicrobial Peptide
Arg	Arginine
β	Beta
β-ME	Beta-Mercaptoethanol
Bcl	B-Cell Lymphoma
Blfcn	Bovine lactoferricin
BL-Blfcn	Biotin-Labelled Bovine Lactoferricin
BMDM	Bone Marrow-Derived Macrophages
BSA	Bovine Serum Albumin
°C	Celsius
CD	Cluster of Differentiation
cDNA	Complementary DNA
CLR	C-type lectiin receptor
COX	Cyclooxygenase
CREB	cAMP response binding element
cRPMI	Complete Roswell Park Memorial Institute Medium
δ	Delta

DAMPs	Danger Associated Molecular Patterns
d	Day
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E. Coli	*Escherichia Coli*
EDTA	Ethylene Diamine Tetraacetic Acid
EEA1	Early Endosome Antigen 1
ELISA	Enzyme-Linked Immunosorbant Assay
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular-Signal-Regulated Kinase
EtOH	Ethanol
F	Forward
FBS	Fetal Bovine Serum
γ	Gamma
GAPDH	Glyceraldyhyde-3-Phosphate Dehydrogenase
GSK3	Glycogen Synthase Kinase
h	Hour
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
Hlfcn	Human lactoferricin
HRP	Horseradish Peroxidase
HUVECs	Human Umbilical Vein Endothelial Cells
IDR	Innate Defense Regulator
IFN	Interferon
IκB	Inhibitor of κB
IKK	IκB Kinase
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase

JNK	c-Jun N-Terminal Kinase
κ	Kappa
kDa	Kilodalton
LL-37	Human Cathelicidin
LPS	Lipopolysaccharide
M1	Classically-activated macrophage
M2	Alternatively-activated macrophage
MAPK	Mitogen-Activated Protein Kinase
MCP	Moncyte chemotactic protein
M-CSF	Macrophage Colony-Stimulating Factor
MEK	MAPK/ERK Kinase
MHC	Major Histocompatibility Complex
min	Minute
MIP	Macrophage Inflammatory Protein
MK2	MAPK-Activated Protein Kinase 2
MKK	MAPK Kinase Kinase
Mlfcn	Mouse lactoferricin
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myleoid Differentiation Primary Response Gene 88
NaCl	Sodium Chloride
NF-κB	Nuclear Factor κ-Light-Chain-Enhancer of Activated B Cells
NLR	NOD-like receptor
NO	Nitric Oxide
nNOS	Neuronal nitric oxide synthase
N-Terminus	Amino-Terminus
PAMPs	Pattern-associated Molecular Patterns

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
Pg/ml	Picogram/milliliter
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PMSF	Phenylmethylsulfonyl Fluoride
PRR	Pattern Recognition Receptors
q-PCR	Quantitative-Polymerase Chain Reaction
R	Reverse
Rb	Rabbit
RNA	Ribonucleic Acid
RLR	RIG-like receptor
ROS	Reactive Oxygen Species
RPMI-1640	Roswell Park Memorial Institute Medium
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
Ser	Ser
SOCS	Suppressors of Cytokine Signaling
STAT	Signal Transducer and Activator of Transcription
TAE	Tris-Acetate-EDTA
TAK1	Transforming Growth Factor Beta-Activated Kinase 1
TRAF-6	TNF Receptor-Associated Factor 6
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR-Domain-Containing Adaptor-Inducing Interferin β
TTBS	Tris-Buffered Saline and Tween-20
TEMED	*N,N,N′,N′*-Tetramethylethylenediamine
TGF	Transforming Growth Factor
Th	T Helper Cell

TLR	Toll-Like Receptor
TMB	Tetromethylbenzidine
TNF	Tumor Necrosis Factor
Trp	Tryptophan
TTP	Tristetraprolin
UV	Ultraviolet
μM	Micromolar
VIP	Vasoactive Intestinal Peptide

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CHAPTER 1

INTRODUCTION

1.1 Acute Inflammation

Inflammation is the body's innate response to invading pathogens or tissue trauma and is characterized by 5 features: redness, swelling, pain, heat, and loss of function (1, 2). Despite its well-defined physiological manifestations, inflammation on a cellular and molecular level encompasses a wide array of responses that vary from one causative agent to the next. Inflammation on a basic level involves the directed and well-regulated influx of blood plasma, proteins, and leukocytes to the site of infection or injury (1). Although both microbial infection and injury trigger an inflammatory response, the mechanism by which the former induces a reaction is far better characterized.

Exogenous stimulators of inflammation typically include pathogen-associated molecular patterns (PAMPs), which are highly conserved molecular patterns or motifs on different classes of pathogens that are recognized by pattern-recognition receptors (PRRs) on host cells (3, 4). Binding of PAMPs with a specific PRR triggers the production and release of diverse pro-inflammatory mediators, which function to combat the microbial infection.

Often times, microbial infection can be coupled with an initiating injury that disrupts tissue homeostasis, leading to the release of danger signals from cells undergoing stress or irregular cell death (5). Much like the ability of our cells to recognize PAMPs, "danger signals" released from stressed or damaged host cells can also trigger an inflammatory cascade through binding various PRRs. Non-microbial or sterile initiators of the inflammatory cascade are endogenous components that are often referred to as alarmins or danger-associated molecular patterns (DAMPs) (6). Although chemically distinct, several characteristics define endogenous stress signals of the immune system, including their release from cells undergoing non-programmed cell death, secretion from immune cells at the site of injury, recruitment and activation of immune effector cells, and finally the promotion of tissue repair and reconstruction (7).

There are several different classes of PRRs that induce a pro-inflammatory response when recognizing a PAMP or DAMP signal; these include C-type lectin receptors (CLRs), NOD-like receptors (NLRs), (RIG)-I-like receptors (RLRs), and the toll-like receptors (TLRs) (4). TLRs are the best characterized of the receptor families, responsible for the extra- and intracellular detection of inflammatory stimuli that trigger an innate immune response.

1.2 Inflammatory First Responders

The innate immune system is an important first line of defense during the onset of infection for its role in the recognition of pathogens and as a source of pro-inflammatory mediators following PRR stimulation (4). During the onset of an inflammatory assault, an assortment of stress or danger signals cues both immune and non-immune cells to mount a response that works to eradicate the source of inflammation. The first responders of inflammation are resident immune cells that include neutrophils, macrophages, mast cells, and dendritic cells. These sentinel cells recognize and respond to compromised tissue homeostasis and, through a complex system of interactions, are responsible for eradication of the underlying source of inflammation, removal of damaged tissue and microbial remnants, and finally restoration of cellular homeostatic functions (1, 8).

Mast cells, although traditionally associated with allergic reactions, are also important players in inflammatory responses (9). Mast cells are among the types of cells that can be activated through PRRs. Once activated, mast cells release granular components that include histamine and other vasoactive proteins that act on the endothelial layer to induce vascular permeability and dilation (10). In concert with vaso-targeted mediators, mast cells also release chemotactic molecules such as interleukin (IL)-8 that allow for the extravasation and infiltration of leukocytes from the blood to the site of inflammation (11, 12). Once activated, the endothelial layer of blood vessels allows for selective extravasation through the upregulation of selectins on the cell surface, thereby excluding red blood cells and allowing for leukocytes to move out of the blood stream (13).

Neutrophils that are primarily confined to the blood stream are now able to migrate to the site of inflammation along with protein rich plasma exudate (14). Neutrophils are a

subset of phagocytic granulocytes that release nonspecific toxic substances that are microbicidal. As these granular molecules are nonspecific, they can also cause significant damage to the host. These cells not only aid in the dispersal of cytotoxic, pro-inflammatory molecules they also contribute the elimination of invading pathogens through phagocytosis (15, 16). To limit the amount of cellular damage to the host due to sustained exposure to toxic granular content, neutrophils undergo apoptosis, which is a type of controlled cell death. The infiltration of additional phagocytic cells, such as macrophages, aids in the removal of cellular debris and invading pathogens. The infiltration of macrophages to aid in the removal of pathogens and apoptotic neutrophils is important for the effective progression and eventual resolution of inflammation (17, 18).

Tissue macrophages detect stress signals or PAMPs during the early onset of infection and release a variety of pro-inflammatory mediators that include tumor-necrosis factor-alpha (TNF-α), IL-1β, and IL-6, and nitric oxide, further promoting the recruitment and activation of leukocytes, but also combating the invading pathogen (19). Macrophages are not only essential for their early pro-inflammatory and leukocyte recruitment functions, but also for their key role in resolution of inflammation, wound healing, and restoration of tissue homeostasis (20–22). Tissue macrophages are important for the clearance of pathogens and the production and secretion of proteases that aid in the tissue-remodeling processes that come before fibroblast proliferation and angiogenesis (23).

1.3 Macrophage Polarization: Classical vs. Alternative

Tissue resident macrophages are derived from monocytic progenitor cells that originate in the bone marrow and circulate in the blood stream. These monocytic cells move into peripheral tissues where they differentiate into macrophages. Signals within the tissue local environment, such as growth factors (i.e. M-CSF) and cytokines, are responsible for the differentiation process from monocyte to macrophage (24, 25). Macrophages are complex cells of the innate immune system that are often organized into two subsets: classically activated M1 macrophages and alternatively activated M2

macrophages (26, 27). The differentiation of monocytes to M1 and M2 macrophages can happen concurrently (27). Although M1/M2 polarization is often thought to result in distinct cellular subsets of macrophages, these cells experience phenotypic plasticity and their polarization state may, at times, be ambiguous (27). M2 macrophages are typically thought of as anti-inflammatory and are often associated with tissue repair and growth while M1 macrophages are pro-inflammatory and cause damage to the host and invading microbes (26).

These macrophage subsets mirror the Th_1/Th_2 state of T cells in adaptive immunity, as one subset of cells is tailored toward a pro-inflammatory response and the other associated with an anti-inflammatory effect. Th_1 responses, as the drivers of cellular immunity, are often associated with the production of IFN-γ and an external viral or bacterial stimulus, while Th_2 responses which drive humoral immunity involve the presence of IL-4, IL-5, and IL-10 (28). Much like their lymphocyte counterparts, typical M1 stimuli include lipopolysaccharide (LPS) and interferon (IFN)-γ, while polarization of macrophages towards an M2 phenotype requires the presence of IL-4 and IL-13 (26, 28). This paradigm between Th_1/M1 and Th_2/M2 differentiation is dependent on the stimulus and results in two very different outcomes in the context of inflammation, its regulation, and wound healing.

TLRs are a major contributor to M1 polarization and detection of PAMPs. Bacterial lipopolysaccharide detection by TLR4 induces a pro-inflammatory response in macrophages through a myeloid differentiation primary response gene 88 (MyD88)-dependent signaling pathway (29). Stimulation by LPS induces various signaling pathways that result in a pro-inflammatory cytokine profile in these macrophages that is characterized by the production of TNF-α, IL-6, IL-1β, and IL-12 (24). Along with increased production and secretion of cytokines, M1 macrophages also have raised expression of other pro-inflammatory markers which include inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), major histocompatibility complex (MHC) molecules and costimulatory molecules (30–32).

1.4 Cytokines and Other Pro-inflammatory Mediators

Inflammation is tightly regulated by various soluble inflammatory mediators, the most important of which include cytokines. Cytokines are a diverse group of potent cell-to-cell signaling agents, which direct the course of inflammation. Cytokines present in the early inflammatory response include IL-1β, TNF-α, IL-6, IL-11, and chemokines including IL-8. Long term inflammation may still have these factors present, but also sees the production of several other cytokines including IL-4, IL-13, IL-5 and IL-10 (33). These cytokines signal in both an autocrine and paracrine fashion, initiating specific cellular functions and regulating the function and expression of cytokines as well as other inflammatory mediators. Inflammatory cytokines are produced by a wide variety of cells, but the most prevalent cytokine producers are activated macrophages (34).

TNF-α was originally identified as a circulating soluble mediator that initiates the necrosis of tumors, but perhaps even more notably is known as a key element of the acute inflammatory response (35, 36). As essential players in inflammation, activated macrophages are the primary producers of TNF-α (37). One particular target of this cytokine is the endothelium and endothelium-leukocyte interactions. TNF-α induces physiological changes in the endothelial cell that include the upregulation of specific adhesion molecules that allow for the attachment and eventual extravasation of leukocytes (38, 39). TNF-α also operates in an autocrine manner to prolong the survival of macrophages, thus sustaining further production of TNF-α and associated pro-inflammatory mediators in a TLR-induced response (40). TNF-α propagates the inflammatory response through the induction of other pro-inflammatory cytokines such as IL-6 and IL-8 (41, 42).

IL-6 is another prototypical cytokine produced during inflammation by an assortment of cells, including macrophages, T cells, endothelial cells, and dendritic cells. IL-6 plays an important role in the progression of inflammation which involves a shift from a neutrophil-dominated response to one that is dominated by monocytes and macrophages (43, 44). This is accomplished through downregulation of neutrophil-associated chemokines such as IL-8 and an increase in macrophage-associated chemokines, monocyte chemoattractant protein (MCP)-1 and MCP-2, and the increased

expression of E-selectin (a macrophage specific adhesion molecule) on endothelial cells (45).

Nitric oxide (NO) is a key regulatory mediator in inflammation. NO is produced from nitric oxide synthases, a family of enzymes that catalyze the conversion of L-arginine to L-citrulline (46). There are three isoforms of nitric oxide synthases: iNOS, endothelial nitric oxide synthase (eNOS), and neuronal nitric oxide synthase (nNOS). Of the three NOS isoforms, iNOS is produced in a variety of cells including endothelial cells, monocytes, macrophages, and mast cells, while the other NOS isoforms are localized to the endothelium and brain. The expression of iNOS is increased in response to TLR and pro-inflammatory cytokines (47). NO is an important antimicrobial mediator as this molecule is able to freely diffuse from the cytoplasm where it is produced to target microbial cells to inhibit cellular process such as DNA synthesis (48). In addition to being a potent antimicrobial agent, NO is also a key player in inflammation, acting to increase vasodilation, regulate infiltration of neutrophils, and can inhibit T-cell proliferation by causing decreased production of T-cell activating cytokines (47, 49, 50).

Although each pro-inflammatory mediator can act independently on specific cells to elicit a particular effect, they often act in concert along similar signaling pathways. Secreted cytokines act as a communication tool between cells, allowing for a coordinated inflammatory response. These protein messengers bind to particular cell surface receptors, initiating a signal cascade that confers further transcriptional control of other pro-inflammatory cytokines and soluble mediators.

1.5 Inflammatory Signaling

Inflammatory stimuli can operate through several major pathways leading to the production of pro-inflammatory mediators. One of the quintessential signaling pathways involved in an immune response is the nuclear factor-kappa B (NF-κB) pathway. NF-κB is a transcription factor composed of two subunits p65 and p50. Prior to stimulation by inflammatory triggers, NF-κB is sequestered in the cytoplasm by a member of the inhibitor of kappa-B (IκB) family (51, 52). The NF-κB pathway is activated by a variety of stimuli, including host mediators such as TNF-α and IL-1, pathogenic signatures such

as LPS and double stranded RNA, as well as heavy metals and oxidative stress. In the canonical pathway, inflammatory stimuli activate downstream IκB kinase (IKK) complexes, consisting of IKKα and IKKβ. The importance of the NF-κB pathway is highlighted by the number of stimuli from different sources that use different pathways to converge on the IKK complex and activate NF-κB. Activation of this complex leads to the phosphorylation of IκBα on two serine (Ser) residues, Ser32 and Ser36 (53). Once phosphorylated, IκBα is targeted for polyubiquitination by a member of the E3 protein ligase family and subsequent proteosomal degradation. Once degraded, IκBα no longer masks the nuclear localization signal of NF-κB so this transcription factor is free to enter the nucleus and bind to the promoter region of various genes to initiate transcription (Figure 1.1) (54–56). NF-κB regulates several genes, including those that code for TNF-α, IL-6, iNOS, chemokines, adhesion molecules, growth factors, and cyclins, thereby playing a role in immunological responses as well as acting as a regulator for cell proliferation, apoptosis, and survival (57).

Although NF-κB is a main regulator of the inflammatory response, there are several other pathways such as the mitogen-activated protein kinase (MAPK) pathway that play a role in producing pro- and anti-inflammatory mediators (Figure 1.1). The MAPK pathway is a complex and intricate signaling pathway that includes a series of mediators that act to regulate various immunological responses, including those responsible for inflammation. Much like NF-κB, a multitude of stimuli can activate the MAPKs. There are three primary groups of MAPK that contribute to the regulation of gene expression in humans; extracellular regulated kinases 1 and 2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinases (JNK). Although all belong to the larger family of kinases, these three major groups of MAPKs are distinctly regulated (58–60)

The main activators of the ERK 1/2 pathway are mitogens and growth factors, but TLR ligands and cytokines including TNF-α, are also responsible for activation of ERK 1/2 in macrophages (61, 62). Once ERK 1/2 is phosphorylated through a series of the signal transduction events, it can then translocate to the nucleus where it further propagates the signaling cascade by phosphorylating various transcription factors (63). The traditional role of ERK 1/2 is associated with cell proliferation and growth; however, this mediator is also involved in inflammatory regulation. Once activated, ERK 1/2

phosphorylates members of the activator protein-1 (AP-1) transcription factor family, which includes c-Jun, c-Fos, and ATF2 (64–66). Activation of ERK 1/2 during an inflammatory response leads to the phosphorylation of downstream elements that allow for TNF-α production and iNOS expression in macrophages (61, 67, 68).

Another MAPK that is important for in the regulation of macrophage function is p38 MAPK. This MAPK, like ERK 1/2, is activated by pro-inflammatory cytokines (TNF- α and IL-1 β), growth factors such as colony-stimulating factor-1 (CSF-1), LPS, and oxidative stress. The p38 MAPK has been implicated as a key regulatory molecule in the expression of inflammatory cytokines and mediators. Four distinct isoforms of p38 MAPK have been identified: α, β, γ, and δ (58, 69). The α and β isoforms of p38 MAPK are the most widely studied variants as they are ubiquitously expressed, with α being the predominant form associated with an inflammatory response in macrophages (70, 71). Upstream regulation of p38 MAPKs is generally dependent on the MAPK kinase kinases (MKK); a diverse group of MAPK regulators triggered by the variety of stimuli mentioned above (58,73). Once phosphorylated, p38 MAPK can elicit downstream effects on transcription factors such as cAMP response element-binding protein (CREB), activating transcription factor-1 (ATF1), and signal transducers and activators of transcription-1 (STAT-1). Operating via this pathway leads to the production of inflammatory cytokines and mediators, including TNF-α, IL-6, IL-1β, and cyclooxygenase-1 (COX-1), and the regulation of cell cycle and proliferation (72, 73) Another important substrate that p38 MAPK interacts with is MAPK-activated protein kinase-1 (MK2), a downstream mediator that facilitates the phosphorylation of tristetraprolin (TTP). TTP is an mRNA destabilizing protein that, when phosphorylated by M2K, functions to bind TNF-α mRNA, thereby inhibiting TNF-α production. The p38 MAPK pathway has also been strongly associated with the production of IL-10 in human primary monocytes (74, 75), thus demonstrating a role for p38 MAPK in the negative regulation of the inflammatory response.

JNK is the last of the three major MAPK groups. Although still involved in the inflammatory response, some studies suggest JNK operates to a much lesser extent than ERK1/2 and p38 in macrophage responses to inflammation-promoting stimuli (59, 76). JNK is activated in response to many of the same stimuli that activate ERK 1/2 and p38

MAPK, including pro-inflammatory cytokines, various endotoxins, and oxidative stress (77–79). Like p38 MAPK, JNK exists in more than one isoform. JNK1 and 2 are expressed ubiquitously in humans, while JNK3 is found exclusively in brain and heart tissue (80). JNK 1 and 2 regulate the phosphorylation and subsequent activation of various transcription factors, including members of the AP-1 transcription factor family, leading to the production of pro-inflammatory mediators (81).

Another major pro-inflammatory pathway that is commonly activated during an inflammatory response is the β-catenin signaling cascade. The β-catenin pathway has been implicated in regulating the inflammatory response induced by pathogens. Upon cellular activation with LPS, β-catenin accumulates in the cytoplasm and migrates to the nucleus following the inactivation of inhibitory glycogen synthase kinase 3 beta (GSK3β). Although β-catenin is heavily involved in the control of cell proliferation and survival, this transcription factor has also been implicated in the induction of cell migration and inflammatory mediators, IL-8 and NO (82–85). One study also demonstrates the involvement of β-catenin pathway in the production of inflammatory ROS in macrophages (82).

CREB and specificity protein-1 (SP-1) are other major transcription factors involved in the regulation of inflammatory mediators. Numerous signaling mediators including the MAPKs and protein kinase B (AKT) regulate both of these transcription factors. The transcriptional regulation these factors exert is dependent on the stimulus, which can include growth factors, cytokines, or microbial stimuli. Once activated CREB is responsible for the regulation of pro- and anti-inflammatory cytokines, and has shown an inhibitory effect on NF-κB signaling (86). SP-1-associated signaling demonstrates a role in the production of anti-inflammatory IL-10 in stimulated macrophages (87).

1.6 Immune Dampening

Inflammatory signaling is a complex and tightly regulated process that relies on forward and negative feedback loops to propagate the response, but also dampening inflammation when needed. In order to function properly the inflammatory response must be robust, swift and self-limiting. Through the release of anti-inflammatory mediators,

such as IL-10, pro-inflammatory mediators are downregulated (20, 88). Cellular debris is cleared as the stimulating pathogen is eliminated. Removal of the inflammatory stimuli is the first in the number of immunosuppressive actions of inflammatory regulation. Once the particular PAMP or DAMP is removed or cleared by invading immune cells such as neutrophils and macrophages, the inflammatory response can no longer be potentiated and production of inflammatory cytokines and mediators will halt (89).

In order for tissue homeostasis to resume, both cellular debris and pathogens must be removed by phagocytosis (8, 90). In an acute response, neutrophils rapidly undergo programmed cell death or apoptosis, and the cellular debris is cleared by macrophages. The eradication of cellular fragments clears the way for tissue repair and also contributes to the production of several anti-inflammatory mediators that further downregulate the inflammatory response (91, 92). Macrophages that have phagocytosed apoptotic neutrophils contribute to downregulation of inflammation through the release of anti-inflammatory mediators that include transforming growth factor-β (TGF-β) and IL-10 (93–95). Studies demonstrate the immunosuppressive effects of TGF-β through its ability to downregulate expression of adhesion molecules on endothelial cells and production of pro-inflammatory cytokines TNF-α and IL-8 from macrophages (96–98). TGF-β also has a significant inhibitory effect on the expression of iNOS and subsequent NO production (99). A study involving TGF-β knockout mice shows considerable inflammatory pathology in comparison to the wild type mice, indicating that TGF-β is important for dampening the immune response (100).

Upon phagocytosis of neutrophils, macrophages also increase synthesis of anti-inflammatory IL-10. The regulation of IL-10 during an inflammatory response is interesting as it is also controlled by the same regulatory elements that are responsible for the induction of pro-inflammatory mediators. IL-10 is produced following macrophage stimulation by TLR ligands such as LPS, but production is delayed in comparison to the upregulation of pro-inflammatory genes such as TNF-α that are also induced by LPS (101). Transcription factors AP-1 and NF-κB are involved in the induction of IL-10, as well as the induction of other previously mentioned pro-inflammatory signaling mediators. Previous investigations have highlighted the dual roles of NF-κB and AP-1 in

both pro-inflammatory cytokine production and IL-10 production by macrophages (102–104)

The importance of IL-10 in dampening an inflammatory response is also highlighted by numerous studies showing that IL-10 reduces the severity of sepsis and plays an essential role in the inhibition of TNF-α overexpression. IL-10 operates through the transcription factor STAT3 to reduce pro-inflammatory gene transcription and the expression of costimulatory molecules on the surface of macrophages. The exact mechanism by which IL-10 exerts its anti-inflammatory properties is yet to elucidated, but there is evidence that IL-10 upregulates suppressor of cytokine signaling-3 (SOCS-3), which inhibits the phosphorylation of IκBα and subsequent nuclear translocation of NF-κB(88, 105). Mice with SOCS-3 deficiency showed a higher incidence of M1 macrophages markers, including IL-6 and iNOS (88, 106). Since IL-10 is the main anti-inflammatory agent that is produced by macrophages, it will be the main anti-inflammatory mediator discussed in this thesis.

1.7 Dysregulation of Inflammation and Disease

When one or more of the immunosuppressive mechanisms fails, unregulated expression of pro-inflammatory mediators occurs and the inflammatory condition persists. Although evolved as an immediate immune response to pathogens, inflammation can be considered the double-edged sword of immunity. Inflammation, when regulated properly, is crucial to the removal of pathogens and cellular debris; however, when negative feedback and immunosuppressive mechanisms fail, unresolved inflammation becomes harmful to the host. The induction of inflammation due to exogenous pathogen-derived molecules or self-originating danger signals can lead to the upregulation of various pro-inflammatory mediators, removal of necrotic cells, remodeling of the damaged tissues, and eventual wound healing and regeneration at the site of trauma (107). When stimuli are not effectively cleared by the immune system, effector cells and their pro-inflammatory mediators can persist and exacerbate the immune response. Diseases and conditions that are initiated by the improper resolution of inflammation include sepsis, colitis, and cancer (108–112).

Sepsis is an exacerbated TLR-mediated response to invasion of pathogens, which can be triggered by microbial components, but can also be propagated by endogenous stimulants such as those released by necrotic or damaged cells. Sepsis is considered a hyperinflammatory response in which there is overexpression of pro-inflammatory cytokines, chemokines, and adhesion molecules on leukocytes. On a cellular level, sepsis is characterized by delayed neutrophil apoptosis, sustained leukocyte activation, and increased damage to surrounding tissue, leading to the recruitment leukocytes and increased permeabilization of the endothelium of blood vessels (113, 114). The exact underlying mechanisms of septic shock are yet to be elucidated; however, NF-κB activation is known to play an important role in the overexpression of the pro-inflammatory mediators that lead to septic shock since this transcription factor is a main regulator of cytokine and chemokines in response to TLR stimulation (115, 116). The overproduction of NO has important implications in the pathogenesis of sepsis due to NO-induced vascular permeability in endothelial cells leading to systemic hypotension. This systemic pro-inflammatory response and vasodilation can lead to harmful ischemia or inadequate supply of blood to different tissues and organs, resulting in acute organ dysfunction and eventually death (117, 118).

Colitis is an autoimmune disorder that is characterized by severe inflammation of the colon. Colitis is initiated by both environmental and genetic factors, including intolerance to the natural microbiota of the gut (119). Much like inflammation leading to septic shock and many other inflammatory conditions, colitis is marked by the prolonged activation of NF-κB and subsequent increased synthesis of pro-inflammatory cytokines: TNF-α, IL-1β and IL-6. NO and ROS also contribute to the pathogenesis of colitis through intestinal cellular damage and induction of apoptosis (111, 120, 121). Chronic unregulated inflammation of the gut has been linked to the development of colon cancer, which identifies carcinogenesis as another consequence of long-term dysregulated inflammation (122, 123).

Inflammatory disorders that have been linked to carcinogenesis include colitis, smoking-induced lung inflammation, and UV associated skin inflammation. Inflammation is considered to be a hallmark of cancer development, with pro-inflammatory mediators such as TNF-α, IL-6, and ROS having a major influence on the

induction and advancement of cancer (124–127). The dysregulated functioning or overactivaton of NF-κB has been associated with the progression of several types of cancer. One study of a hepatocellular carcinoma demonstrates that inhibition of the IκBα and NF-κB signaling pathway impeded the progression of cholestatic hepatitis to tumor development (128). Activation of NF-κB also results in the upregulation of anti-apoptotic genes such as B-cell lymphoma 2 (BCL2) (129, 130).

Initiation of tumor growth occurs when there is clonal expansion of somatic cells with genomic changes resulting from various DNA mutations (131). Chronic inflammation, through continuous activation of immune cells at the site of trauma, contributes to the generation and proliferation of tumor cells (132). The release of cytokines, chemokines, and growth factors from immune effector cells, such as infiltrating macrophages, influences the progression of inflammation and also promotes angiogenesis and cancer development (133). The inability of the immune system to gain control of an inflammatory response and allow for wound healing can increase the risk for the development of cancer. The flurry of pro-inflammatory mediators released from effector cells promotes further inflammation, further exacerbating an already existing condition. Inflammation and angiogenesis are connected in several ways, including the interaction of immune cells with endothelial cells and the common stimuli that trigger both of these processes. A study by Kimura *et al*., demonstrates that inflammatory stimuli released from tumor-associated macrophages propagate the production of pro-angiogenic cytokines along with the growth factors needed for tumor cell and endothelial cell proliferation (134). DNA damage generated by the prolonged presence of ROS is one main contributing factor of inflammation-based tumorigenesis (135, 136). Reactive oxygen or nitrogen intermediates are capable of oxidizing DNA or interfering with DNA repair mechanisms, both of which can lead to inactivating mutations in tumor suppressor genes (137).

The balance between appropriate pro-inflammatory and efficient anti-inflammatory responses is critical to maintain homeostasis and prevent the development of destructive and potential harmful chronic conditions. The immune system is a complex and strictly regulated system that therefore requires regulatory agents to maintain this delicate balance.

1.8 Antimicrobial Peptides

A complex system such as inflammation also requires complex regulatory molecules with diverse functions. Such regulators include antimicrobial peptides (AMPs), which are a group of small (~30-100 amino acids) cationic molecules associated with the innate immune system (138). AMPs have amphipathic properties, containing both hydrophilic and hydrophobic amino acid residues allowing for their solubility in aqueous environments as well as their insertion into the lipid-rich cellular membranes (139). More than a thousand AMPs have been isolated from different species, from insects to mammals. These evolutionary adapted and diverse molecules are categorized based on their amino acid composition and secondary structure. AMPs are classified into 4 major groups: α-helices, β-sheets, extended structures, and a mixed structure consisting of two or more of these configurations. The α-helical and β-sheet structures are the best characterized and studied groups of the AMPs (140). Their mechanism of action is not through surface receptor interactions on microbes, but rather by insertion into the microbial membrane, leading to pore formation, disruption of ion gradients, disruption of the cellular membrane and, ultimately, cell death (138, 140). As these peptides are cationic, they are able to electrostatically interact with negatively charged components on a microbial cell membrane such as LPS or lipoteichoic acid on Gram-negative and Gram-positive bacteria, respectively. Although a definite model of membrane disruption is yet to be determined, leading theories of action include the carpet model, toroidal pore model, the barrel-stave model, and a detergent-like effect (141–145). There is evidence suggesting that AMPs are able to interact with the hydrophobic tails of the lipid membrane due to their amphipathic nature. In some cases these AMPs are able to transverse the cell membrane and disrupt cellular machinery through DNA and RNA binding, which also leads to cell death (146). In addition, AMPs are able to neutralize viral pathogens through either disruption of the viral envelope or binding to structures such as glycoproteins, rendering the virus incapable of attaching to and entering host cells (147, 148). Thus, AMPs demonstrate broad antimicrobial activities, as they are able to protect the host from a wide variety of pathogens using diverse mechanisms of actions, and thus are an integral aspect for the innate immune system.

Since the innate immune system is the first line of defense for protecting the host against invading pathogens, it is not surprising that these molecules are most highly concentrated in cells and tissues of the body that are most often exposed to pathogens. AMPs can be produced constitutively or induced upon TLR stimulation by invading pathogens. Within humans, the cells that are the most common source of AMPs include epithelial cells, phagocytes, and lymphocytes (149). Isolation of AMPs from sources other than humans, or even mammals, has been gaining considerable attention because of their antimicrobial effects and ability to shape immune responses.

1.9 AMPs and Immunomodulation

AMPs were first studied for their direct antimicrobial properties; however, more recently these peptides have been documented to have an immunomodulatory role in both innate and adaptive immunity that includes regulation of pro- and anti-inflammatory cytokines and other inflammatory mediators, activation of immune cells, and enhancement of several other immunological processes (138, 149–153).

AMPs can act as direct chemotactic agents or indirectly influence chemotaxis through the upregulation of chemokines. In increased concentrations, some AMPs such as the human cathelicidin, LL-37, can act as a chemoattractant, facilitating the infiltration of monocytes and neutrophils to the site of infection or inflammation (154). LL-37 also acts synergistically with the TLR-5 ligand, flagellin, to increase the production of the neutrophil chemoattractant, IL-8, from human keratinocytes (155). Defensins also show influence on migration of immune cells (156). One study, in particular, demonstrates the role of α-defensins in the induction of the CC chemokines macrophage inflammatory protein (MIP)-1α and MIP-1β production by human macrophages (157).

The ability of AMPs to influence myeloid cell differentiation establishes the ability of these peptides to bridge innate-adaptive immunity (158). Both cathelicidins and defensins influence monocyte-derived dendritic cell differentiation, which indirectly affects T-cell polarization (159). The increased expression of Fc receptors, CD32 and CD64, on macrophages is seen following treatment with human neutrophil peptides

(HNP 1-3); this peptide also enhances phagocytic properties of these antigen presenting cells (160).

Possibly the most widely studied and well-defined immunomodulatory characteristic of AMPs is suppression of pro-inflammatory responses. A number of studies highlight the ability of AMPs to downregulate pro-inflammatory cytokine production in both PAMP and DAMP-induced inflammation. Studies focusing on LL-37 show that this peptide decreases microbial-induced pro-inflammatory TNF-α and IL-6 in myeloid-derived cells (151, 161, 162). Melanocortins, a family of neuropeptides, demonstrate considerable anti-inflammatory effects in bacteria-induced inflammation, but also control inflammation triggered by endogenous monosodium urate crystals (163–165). Anti-inflammatory and immunosuppressive properties of AMPs have been described both *in vitro* and *in vivo*. A study by Brown *et al.* (161) shows the anti-inflammatory effects of intratracheal instilled cathelicidin in a mouse lung infection model. *In vivo* studies of β-defensins also show similar effects, downregulating TNF-α synthesis in a mouse endotoxin model (166).

One of the most interesting aspects of immunosuppression by AMPs is the selective manner in which these peptides can influence an inflammatory response. Various investigations demonstrate the selective pattern of peptide-targeted signaling (167). For example, cathelicidins modulate TLR-mediated inflammatory signaling through inhibition of various mediators along inflammatory pathways (168). One study by Mookherjee *et al*. highlights the inhibition of nuclear translocation of NF-κB, thereby blocking the upregulation of pro-inflammatory cytokines (169). Both human cathelicidin and β-defensins target p38 and ERK MAPK signaling in keratinocytes, mast cells, and macrophages (170–172).

The development of AMPs as novel therapeutic agents is gaining wide attention because of their role as selective immunoregulators. The ability to activate certain inflammatory pathways while inhibiting others suggests a role in immune balance and regulation while simultaneously maintaining their function as potent antimicrobials. Several AMPs and their synthetic derivatives have already entered clinical trials, including vasoactive intestinal peptide (VIP) for sepsis treatment and ghrelin, a peptide

produced in the human GI tract, for treatment of chronic respiratory inflammation (173–175).

1.10 Lactoferrin and Lactoferrin-Derived Peptides

Of particular interest in the development of immunotherapeutic agents is the diverse lactoferrin-derived, small cationic peptide, lactoferricin. Lactoferricin, which is the pepsin-cleaved product of lactoferrin, an 80 kDa, iron-binding polypeptide belonging to the larger transferrin protein family (176–178). Transferrins are a large family of iron-binding glycoproteins that contribute to iron homeostasis (179). Lactoferrin is found in exocrine secretions including bile, saliva, and tears, as well as in the secondary granules of neutrophils, which can be released during an inflammatory response (180, 181). Lactoferrin was named based on the source from which it was first isolated, milk. Lactoferrin is found in milk from many species, but is most highly concentrated in human milk (1 g/L), while bovine milk is a lesser, but still significant source of lactoferrin (0.1 g/L) (181, 182). Lactoferrin deficiency, also know as neutrophil-specific granule defiency, is associated with recurrent infections and immunodeficiency (183). Clinical studies of the effects of oral administration of lactoferrin on patients with colorectal cancer show a inhibition of polyp growth and better prognosis than those patients who did not receive lactoferrin (184). It is suspected that the enzymes that cleave lactoferrin into smaller peptides, including lactoferricin, function within the human gastrointestinal tract and site of infection or inflammation (185).

Lactoferricin is the 25 amino acid, N-terminus portion of lactoferrin, produced in an acid-pepsin hydrolysis reaction. Lactoferricin is rich in basic amino acids and also contains both hydrophobic and hydrophilic residues. Once cleaved from lactoferrin, lactoferricin takes on an anti-parallel beta-sheet structure (186). The amphipathic and cationic properties of lactoferricin contribute to its broad-spectrum antimicrobial effects. Lactoferricin is a more potent antimicrobial agent than its parent peptide (187–189). Lactoferrin mediates its antimicrobial effects through sequestration of iron, while lactoferricin does not retain its larger polypeptide's iron binding capacity, but rather, depends on its cationic and amphipathic properties to elicit an antimicrobial effect.

Lactoferricin binds negatively charged components on bacterial membranes, such as LPS, and causes disruption in membrane integrity in susceptible bacteria such as *Escherichia coli* (*E. coli*) (190). Lactoferricin also has strong antiviral properties. Several studies have highlighted the competitive nature of lactoferricin for heparin sulfate binding sites on host cells, thereby inhibiting viral entry and subsequent infection of host cells (191, 192).

Differing amino acid sequences between lactoferricin peptides derived from different species (Table 1.1), has been the driving force behind several studies comparing their antimicrobial efficacies. One study in particular from Vorland *et al*. (193) demonstrates that bovine lactoferricin has more potent antimicrobial activities in comparison to lactoferricin from human, caprine, and mouse sources. Bovine lactoferricin has the lowest minimal inhibitory concentration (MIC), and the only lactoferricin that is active against *E. coli* and *Staphylococcus aureus*. The authors attributed the antimicrobial potency of bovine lactoferricin to its greater hydrophobicity and overall higher positive charge in comparison to its counterparts from other species (193, 194).

1.11 Lactoferricin as an Immunomodulatory Agent

Lactoferrin and lactoferricin peptides also have as the ability to act as immmunomodulatory agents. Most studies of lactoferrin and lactoferricin have focused on the bovine and human homologs as they are the most biologically relevant and demonstrate potent anti-tumor, anti-metastatic, and immunomodulatory properties in a variety of cell types (Table 1.2). Lactoferrin and lactoferricin display highly diverse roles as immunomodulatory agents. Studies implicate lactoferrin in the enhancement of certain immunological responses, including phagocytosis by antigen-presenting cells, increased expression of costimulatory molecules by macrophages, and boosting T-cell responses through upregulation of T-cell activating cytokines (177, 195, 196). A study by Hayworth also shows the attenuating effect of lactoferrin on Staphylococcal entertoxin B-induced T cell proliferation and cytokine production (197).

The most widely studied immunomodulatory effect of these peptides is their ability to suppress an *in vitro* and *in vivo* pro-inflammatory response. A number of studies show that bovine lactoferrin attenuates a pro-inflamamtory response induced by microorganisms (198–200). One study that examined an inflammatory response mediated by THP-1 monocytes shows that bovine lactoferrin downregulates LPS-stimulated TNF-α cytokine production in a NF-κB-dependent manner (201). Studies of human lactoferrin show the significance of this peptide as an anti-inflammatory agent. A study by Håversen *et al*. (202) highlights the use of human lactoferrin in attenuating IL-1β-driven inflammatory responses in a mouse model of colitis.

Although less studied, a few investigations have emphasized the use of lactoferrin-derived-lactoferricin as a potent immunomodulatory agent. Bovine lactoferricin is an effective anti-inflammatory and anti-catabolic agent, mitigating the production of pro-inflammatory mediators, IL-6 and iNOS, in human chondrocytes (203). *In vitro* and *ex vivo* evidence shows that lactoferricin is an important downregulator of LPS-induced inflammation in nucleus pulposus cells derived from the intervertebral disc (204). A study with THP-1 monocytes demonstrate that bovine lactoferricin inhibits the production of IL-6 more strongly than its parent peptide (205) In addition to the ability of bovine lactoferricin to decrease LPS-induced cytokine production, this peptide can also upregulate anti-inflammatory cytokines IL-10, IL-11, and IL-4 (203). Evidence suggests lactoferricin exerts its anti-inflammatory effects in an ERK and p38 MAPK-dependent manner (203, 206). Studies of human lactoferricin also show capacity for influencing anti-inflammatory responses through upregulation of IL-10 and the downregulation of bacterial-induced TNF-α production in macrophages (207–209).

Lactoferrin and its derived peptides undoubtedly play a role in immunity beyond their role as broad-spectrum antimicrobials. Bovine lactoferricin displays greater antimicrobial properties in comparison to its peptide equivalent in other species, but whether this greater potency also translates to superior immunomodulatory properties remains unknown.

1.12 Rationale and Objectives

The development of lactoferricin peptides as immunotherapeutic agents in inflammatory conditions is an intriguing idea considering their diverse role in immunological processes. Lactoferricin peptides demonstrate a wide variety of immunomodulatory and anti-cancer properties, but few studies have addressed their influence on a macrophage-associated inflammatory response, and no investigations have addressed the possible differential efficacies of lactoferricin derived from different species. Macrophages are a primary source of cytokines at the site of inflammation, thus making them an ideal cell model for studying inflammation and possible therapeutic interventions. Given the accumulating evidence of bovine lactoferricin as an immunomodulatory agent and the most potent antimicrobial agent of the species-specific lactoferricin, it was hypothesized this peptide would have the greatest effect on macrophage-associated inflammation. This investigation focused on the impact of lactoferricin peptides derived from bovine, murine, and human sources on macrophage inflammatory processes.

Figure 1.1 The major inflammatory signaling cascades triggered during an LPS-induced inflammatory response via TLR-4. *Nuclear Factor-κB* (NF-κB) *pathway*: binding of lipopolysaccharide (LPS) to Toll-like receptor-4 (TLR-) induces the recruitment of cytosolic adaptor proteins TIR-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM), and myeloid differentiation primary response gene 88 (MYD88) to the cytoplasmic tail of the TLR-4. This leads to the activation of the IκB kinase (IKK) complex composed of IKKα, IKKβ, and IKKγ. The IKK complex then phosphorylates inhibitor κBα (IκBα), which sequesters NF-κB in the cytoplasm. Once phosphorylated, IκBα is promptly ubiquinated and directed to proteosomal degradation. NF-κB is then liberated and can freely translocate in the nucleus where it can bind to the promoter region of various genes coding for proinflammatory cytokines and mediators (TNF-α, IL-1β, IL-6, iNOS). *Mitogen-activated protein kinase (MAPK) pathway*: Activation of TLR-4 by LPS induces the recruitment of several adaptor proteins (TRIF, TRAM, MyD88), which results in TNF receptor-associated factor 6 (TRAF6) activation. TRAF6 then activates transforming growth factor β-activated kinase 1 (TAK1), a mediator upstream of the MAPKs, which in turn activates several MAPKs, including mitogen-activated protein kinase kinase 1/2 (MEK1/2), MAPK kinase kinase 3/6 (MKK 3/6), and MAPK kinase kinase (MKK 4/7), which go on to phosphorylate and activate extracellular signal-regulated kinases 1/2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinases (JNK) respectively. Activation of ERK 1/2, p38 MAPK, and JNK regulates the activation of several subunits (c-Fos, c-Jun, ATF2) that comprise the transcription factor, activator protein-1 (AP-1), which like NF-κB, regulates the expression of several proinflammatory genes (51, 210–212).

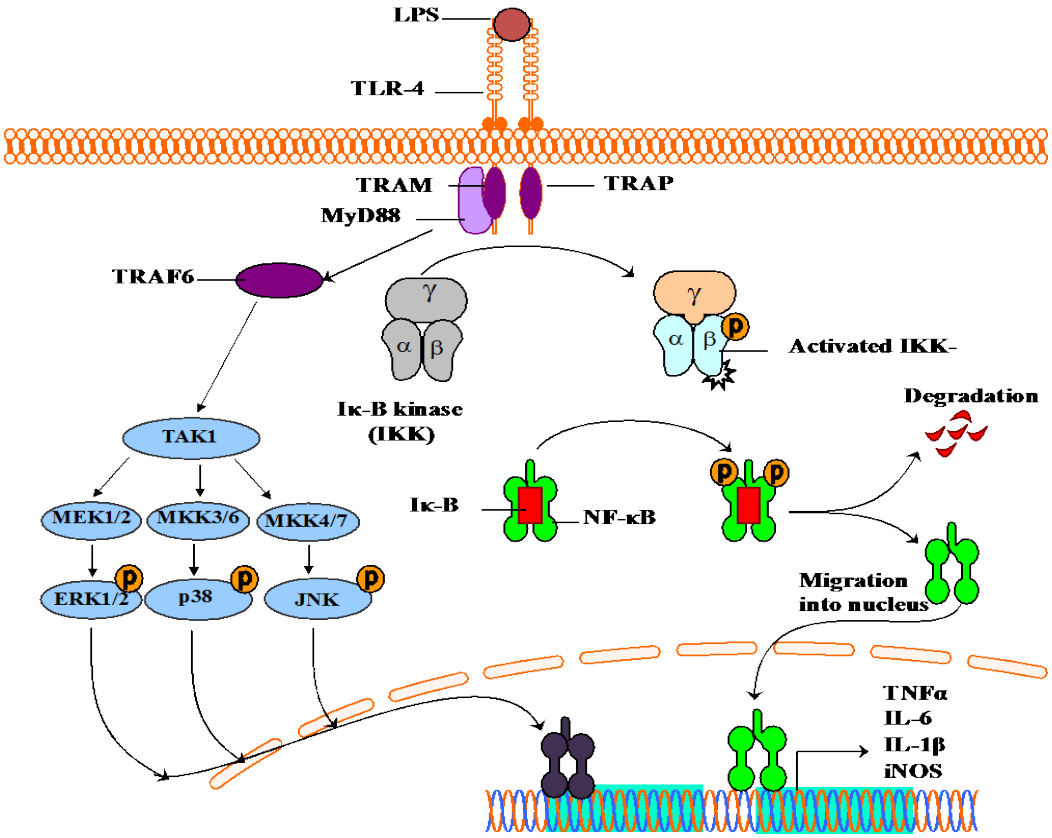

LPS
TLR-4
TRAM
TRAP
MyD88
TRAF6
γ
α
β
Iκ-B kinase
(IKK)
γ
α
β
p
Activated IKK-
Degradation
TAK1
MEK1/2
MKK3/6
MKK4/7
ERK1/2
p38
JNK
p
Iκ-B
NF-κB
p
p
Migration
into nucleus
TNFα
IL-6
IL-1β
iNOS

Figure 1.1

Table 1.1 Amino acid sequences of bovine, murine, and human lactoferricin. The 25-amino acid residue sequence of lactoferrcin from bovine, murine, or human origin. Highlighted residues (in green) represent the presence of a disulfide bond that exists between two cysteine residues in each peptide. Adapted from Vorland *et al*. (193).

Lactoferricin	**Amino acid sequence**
Bovine	NH_2-PHE-LYS-CYS-ARG-ARG-TRP-GLN-TRP-ARG-MET-LYS-LYS-LEU-GLY-ALA-PRO-SER-ILE-THR-CYS-VAL-ARG-ARG-ALA-PHE-COOH
Murine	NH_2-GLU-LYS-CYS-LEU-ARG-TRP-GLN-ASN-GLU-MET-ARG-LYS-VAL-GLY-GLY-PRO-PRO-LEU-SER-CYS-VAL-LYS-LYS-SER-SER-COOH
Human	NH_2-THR-LYS-CYS-PHE-GLN-TRP-GLN-ARG-GLN-MET-ARG-LYS-VAL-ARG-GLY-PRO-PRO-VAL-SER-CYS-ILE-LYS-ARG-ASP-SER-COOH

Table 1.2 The immunomodulatory effects of bovine and human lactoferrin and lactoferricin peptides on different cell types.

Peptide and Origin	Immunomodulatory Effects	Reference
Bovine lactoferrin	↓ LPS-induced TNF-α in osteoblasts ↓ flagellin-induced IL-1β in osteoclasts ↓ TNF-α, IL-6, and GM-CSF in squamous cell carcinoma cell line ↓IL-6 in LPS-treated THP-1 cells ↑ phagocytosis of BMDM, THP-1 and U937 cells, and neutrophils ↓oxidative stress/apoptosis in U937 cells ↑CD40 in RAW 264.7 cells ↑IL-12 in peritoneal macrophages ↓T-cell proliferation and secretion of IL-5 ↓TNF- α and IL-6 in blood of *E.coli* infected mice ↓ TNF- α and IL-6 in carrageenan-induced inflammation in rat feet	(199–201, 213–219)
Bovine lactoferricin	↓iNOS and IL-6 in nucleus pulposus cells of human intervertebral disc ↓IL-1β, IL-6, iNOS, TLR-2 in chondrocytes ↑IL-4 and IL-10 in chondrocytes ↑IL-10 in articular cartilage ↓IL-6 in LPS-stimulated THP-1 cells ↑thymidine uptake by mitogen-activated splenocytes	(201, 203–206, 220)
Human lactoferrin	↓ICAM, E-selectin and TNF-α in HUVECs ↑IL-8, CXCL10, IL-10 in human DCs ↓PGE_2 secretion by breast milk macrophages ↓IFNγ in human DCs ↓IL6 in LPS-stimulated THP-1 cells ↓inflammation in dextran sulfate model of colitis in mice ↓enteritis in *Shigella flexneri* treated rabbits ↓epithelial cell proliferation	(201, 202, 221–228)
Human lactoferricin	↓myeloperoxidase activity in primary human macrophages ↑IL-10 in primary human macrophages ↓ TNF-α in LPS-stimulated human mononuclear cells ↓TNF-α in macrophages stimulated with lipid A	(207–209, 229)

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents

Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), β-mercaptoethanol (β-ME), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffer saline (PBS), phenylmethylsulfonyl fluoride (PMSF), Roswell Park Memorial Institute 1640 medium (RPMI), Dulbecco's Modified Eagle's Medium (DMEM), phorbol 12-myristate 13-acetate (PMA), and Triton-X-100 were purchased from Sigma-Aldrich Canada (Oakville, ON). Fetal bovine serum (FBS), 10,000 U/ml penicillin/10,000 μg/ml streptomycin solution, 200 mM L-glutamine, 1M 4-(2-hydroyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution, and 0.4% trypan blue dye solution were obtained from Invitrogen Canada (Oakville, ON). Acrylamide/bis-acrylamide (29:1, 30% solution, ammonium persulfate (APS), paraformaldehyde (PFA), sodium dodecyl sulfate (SDS), tetramethylethylenediamine (TEMED), Tris base, and Tween-20 were acquired from Bio-Shop Canada Inc. (Burlington, ON). Ethylene diamine tetraacetic acid (EDTA) was purchased from EM 46 Industries Inc. (Hawthorne, NY). Luminata^{TM} Forte Western HRP substrate and p38 MAPK inhibitor III were purchased from EMD Millipore (Etobicoke, ON). Anhydrous ethyl alcohol (EtOH) was obtained from Commercial Alcohols (Brampton, ON). Bio-Rad Protein Assay Dye Reagent was obtained from Bio-Rad Laboratories Inc. (Mississauga, ON). Lactoferricin peptides (>95% purity) were synthesized and purchased from American Peptide and dissolved in serum-free RPMI and kept at -80 º C until further use (Sunnyvale, CA).

2.2 Antibodies

Rabbit (Rb) anti-phospho p38 MAPK (pTpY180/182), Rb anti-p38 MAPK, Rb anti-p65, Rb anti-phospho p44/42 MAPK (ERK1/2) (pTpY202/204), Rb anti- p44/42 MAPK (ERK1/2), Rb anti-phospho-IκBα (Ser32), Rb anti- IκBα, Rb anti-phospho-c-Jun (Ser63), Rb anti-c-Jun and Rb anti-β-actin (HRP conjugate) were all purchased from cell signaling technology (Beverly, MA). Secondary antibody, HRP conjugated donkey anti-

rabbit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ELISA TNF-alpha, IL-6, and IL-10 capture and biotin-conjugated detection antibodies were purchased from eBioscience (San Diego, CA). Anti- early endosomal antigen 1 (EEA1) antibody was purchased from Abcam (Toronto, ON).

2.3 Cell Lines

RAW 264.7 mouse macrophage-like cells and L929 mouse fibrosarcoma cells were purchased from ATTC ® (Manassas, VA). THP-1 human monocytic-like cells were kindly provided by Dr. Brent Johnston (Dalhousie University, Halifax, NS).

2.4 Culture Medium and Conditions

THP-1 and RAW 264.7 cell lines were maintained at 37°C in a humidified 5% CO_2 incubator cultured in RPMI 1640 medium supplemented with 10% heat inactivated (56°C for 30 min) FBS, 2 mM glutamine, 100 µg/ ml streptomycin, 100 U/ml penicillin, and 5 mM HEPES buffer (7.4 pH), referred to as complete RPMI (cRPMI). L929 cells were maintained at 37°C in a humidified 10% CO_2 incubator cultured in DMEM supplemented with 10% heat inactivated (56°C for 30 min) FBS, 2 mM glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, and 5 mM HEPES buffer (7.4 pH). Cells were cultured in 75 mm^2 tissue culture flasks (Fisher Scientific, Waltham, MA) and passaged at 80-90% confluency. RAW 264.7 cells were passaged using a 25 cm cell scraper (VWR, Mississauga, ON).

2.5 Isolation and Differentiation of Murine BMDMs

C57BL/6 female mice purchased from Charles River Laboratories (Wilmington, MA), at 8-10 weeks of age were sacrificed by cervical dislocation and bone marrow was collected from the tibias via aseptic flushing with PBS (pH 7.2) and a 26_G3/8 PrecisionGlide ® Needle (Becton Dickinson & Co, Mississauga, ON). Cells were forced into a single cell suspension using an 18_G1 PrecisionGlide ® needle pressing the needle against the inside of the 15 ml tube. Cells were then centrifuged at 500 x g for 5 min and

resuspended in 0.2% NaCl to lyse red blood cells, followed by the addition of equal parts 1.6% NaCl, and finally equal parts PBS to return the solution to isotonic conditions. Cells were centrifuged again at 500 x *g* for 5 min.

BMDMs were differentiated over 7 d by culturing in cRPMI containing 15% (v/v) L929-conditioned DMEM as a source of M-CSF. After 3 d of culture, the cells were fed with fresh BMDM medium. At 6 d, culture medium and non-adherent cells were removed and the remaining cells fed with fresh BMDM medium. At 7 d macrophages were removed from cell culture plates, counted, and seeded accordingly in cRPMI.

2.6 Cell Seeding

Both cell lines and primary cells were seeded 1 d prior to treatment to allow for adherence to plastic. For all cell types, cells were treated in cRPMI. THP-1 monocytic-like cells were treated with 200 ng/ml PMA in order to cause cells to differentiate into macrophages and adhere to the plate overnight prior to any additional treatment. For experiments involving p38 MAPK inhibitor (30 μM), cells were treated with the compound for 1 h prior to peptide treatments. The inhibitor concentration was maintained during peptide treatment.

2.6.1 THP-1 Human Monocytic-like Cell Line

For ELISA experiments cells were seeded in 24-well plates at 2.5×10^5 cells/well in 1 ml of medium. Cells were plated at 6×10^5 cells/well in a 6-well plate for quantitative real-time polymerase chain reaction (q-PCR) experiments. For MTT assays, cells were plated in 96-well plates at 1.5×10^4 cells/well in 100 μl of medium. For immunofluorescent assays, cells were seeded on 10 mm glass coverslips placed in a 12-well plate at 1.5×10^5 cells/well in 1 ml medium. Once adhered, cells were cultured overnight in serum-free medium to allow for cell growth arrest. For western blot experiments cells were seeded at 1×10^6 cells in 75 mm^2 tissue culture flasks and left for 36-48 h until 90% confluency was reached, then cells were treated with 200 ng/ml PMA overnight and serum-starved an additional night prior to treatment.

2.6.2 RAW 264.7 Murine Macrophage-like Cell Line

For ELISA experiments, RAW 264.7 cells were seeded in 24-well plates at $1.5x10^5$ cells/well in 1 ml of medium. Cells were plated at $2.5x10^5$ cells/well in a 6-well plate for q-PCR. For MTT assays cells were seeded at $1x10^4$ cells/well in a 96-well plate in 100 µl of medium. For western blot experiments cells were seeded at $1x10^6$ cells/well in 10 cm^2 cell culture plates (Thermo Fisher) and left for 36-48 h until 90% confluency was reached, then they were serum-starved overnight prior to treatment. For immunofluorescent assays, cells were seeded on 10 mm glass coverslips placed in a 12-well at $1x10^5$ cells/well in 1 ml medium.

2.6.3 Bone Marrow-Derived Macrophages

BMDM cells were seeded in 24-well plates at $2.5x10^5$ cells/well in 1 ml of medium. Cells were plated at $6x10^5$ cells/well in a 6-well plate for quantitative real-time polymerase chain reaction (q-PCR) experiments For MTT assays, cells were plated in 96-well plates at $1.5x10^4$ cells/well in 100 µl medium.

2.7 Trypan Blue Viability Assay

Cell viability was determined via trypan blue assay, as the blue dye does not penetrate the membrane of intact or undamaged cells (230). The ratio of dead cells, stained in blue, to viable cells can be determined using this assay. Cells were seeded in a 24-well plate, left overnight, and then treated. After 24 h of treatment with increasing concentrations (0-10 µM) of bovine, mouse or human lactoferricin peptides, cells were detached using 1 ml of 10 mM EDTA and 50 µl of the cell suspensions were then diluted 1:2 in trypan blue dye and 10 µl of that dilution was added to a hemocytometer (Hausser Scientific, Horsham, PA). Once counted and recorded, the percent cell viability was determined through normalization of treatments to the untreated control.

2.8 MTT Cell Metabolism Assay

The metabolic activity of cells treated with increasing concentrations of each lactoferricin peptide was determined using an MTT assay, in which MTT is reduced from its yellow tetrazole form to purple formazan via dehydrogenases in metabolically active cells (231, 232). After 24 h of treatment with lactoferricin peptide, 10 µl of MTT solution (5 mg/ml in PBS) was added to each well and the cells were again incubated for 2 h at 37°C in a humidified 5% CO_2 incubator. After the 2 h incubation period with MTT, the 96-well plates were centrifuged at 500 x *g* for 5 min. The supernatants were then discarded and 100 µl of DMSO was added to each well to dissolve the formazan crystal, producing a purple solution. Plates were then shaken for 5 min at 550 rpm on a Microplate Genie (Montreal Biotech Inc., Montreal, QC). The absorbance at 570 nm was read on an Expert 96 microplate reader (Biochrom ASYS, Cambridge, UK). The absorbance reading of each well is proportional to dehydrogenase activity, and thus is indicative of the number of metabolically active cells in each well. Percent viability was determined by normalization to the untreated control (100% viable cells), and calculated using the formula ([T/C] x 100), where T is the absorbance value of the treatment well, and C is absorbance value of the untreated control.

2.9 Enzyme-Linked Immunosorbent Assay

Supernatants were collected from THP-1, RAW 264.7, and BMDM cell cultures for detection of TNF-α, IL-6, and IL-10 using a sandwich enzyme-linked immunosorbent assay (ELISA) Read-SET-Go!® ELISA kit from eBioscience (San Diego, CA) according to the manufacturer's instructions. Cells were treated with medium alone, 100 ng/ml LPS alone or in combination with 5 µM lactoferricin peptides (bovine, murine, human). A subset of experiments was also conducted using cadmium chloride ($CdCl_2$), purchased from Sigma Aldrich. For these experiments cells were treated with medium alone, 10 µM $CdCl_2$ alone, or in combination with 5 µM lactoferricin peptides. Flat-bottom plates (96-well) were coated with capture antibody, diluted in 1:250 in 1x PBS, and left at 4°C overnight. Plates were then washed 3 times in wash buffer (1x PBS and 0.05% Tween-20) and coated with 200 µl of assay diluent (1x PBS and 2% BSA [w/v]) and left at room

temperature for 1 h. Supernatants being analyzed for TNF-α were collected after 6 h, all other supernatants were collected after 24 h of treatment. Samples were diluted 1:4, loaded into the plate and incubated for 2 h at room temperature. The plate was washed 3 times in wash buffer and tapped dry. The plate was loaded with 100 µl biotin-labeled secondary antibody diluted 1:250 in assay diluent and incubated for 1 h at room temperature. The plate was washed again, loaded with an avidin-HRP diluted 1:250 in assay diluent, and incubated in the dark for 30 min. The plate was washed once more, tapped dry, and 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added. After 1 min, 50 µl of 0.3 M H_2SO_4 was then added to the plate to stop the reaction and the absorbance values at 450 nm were determined using a ELx800 UV universal microplate reader (BioTek Instruments, Winooski, VT), Digiread software, and SOFTmax® PRO software (version 4.3; Molecular Devices Corp., Sunnyvale, CA).

2.10 Griess Assay

Supernatants were collected from RAW 264.7 cells and BMDMs for detection of stable nitrite ion in solution as an indicator of nitric oxide production using Griess reagent purchased from Sigma Aldrich as per the manufacturers instructions. Cells were seeded in a 24-well plate and left overnight. Cells were then treated with 500 ng/ml of LPS alone or in combination with 5 µM lactoferricin (bovine, murine, or human) for 24 h. Supernatants were then collected and added to an equal volume of Griess reagent in a 96-well plate. Sodium nitrite purchased from Sigma Aldrich was used to make a standard curve. Plates were then incubated at room temperature in the absence of light for 5 min. Absorbance values at 570 nm were determined using a ELx800 UV universal microplate reader (BioTek Instruments, Winooski, VT), Digiread software, and SOFTmax® PRO software (version 4.3; Molecular Devices Corp., Sunnyvale, CA).

2.11 RNA Isolation

RNA was harvested using an RNeasy Mini Kit purchased from Qiagen (Valencia, CA). Cells were lysed in the 6-well plate in which they were seeded and treated, using

350 µl Buffer RLT. The solution was then transferred into a 1.5 ml eppendorf tube and mixed with equal volume of 70% ethanol and transferred to an RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8000 x g for 15 s. The flow-through was then discarded and 350µl of Buffer RW1 was added to the column and centrifuged at 8000 x g for 15 s. The flow-through was discarded and 80 µl of DNase diluted in Buffer RDD was added to each column and left at room temperature for 15 min, to eliminate any contaminating DNA. The column were again washed with 350 µl of Buffer RW1 and centrifuged at 8000 x g for 15 s. The flow-through was discarded and 500 µl Buffer RPE was added to the spin column and centrifuged at 8000 x *g* for 15 s. The flow-through was discarded and 500 µl Buffer RPE was added to the column and centrifuged at 8000 x g for 2 min. The flow-through was discarded one final time and 35 µl of RNase-free water was used to elute the RNA. The purity and concentration of each RNA sample was determined using a NanoVue Plus Spectrophotometer (GE Healthcare Life Sciences; Piscataway Township, NJ). The purity of the sample was based on the A280/A260 ratio with the value between 1.7-2.0 being acceptable. Samples were then stored at -80°C for future use.

2.12 cDNA Synthesis

Approximately 500 ng RNA, isolated as described in section 2.10, was then reverse transcribed using an iScript™ cDNA synthesis kit (Bio-Rad Laboratories; Hercules, CA) according to the manufacturers instructions. The iScript reaction mix (2 µl) and iScript reverse transcriptase (0.5 µl) was added to RNA template and nuclease-free water to a final volume of 10 µl and final concentration of RNA template 50 ng/µl. The reaction was incubated in a Bio-Rad T100™ Thermocycler using the following reaction protocol: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. Once synthesized, the cDNA was stored at -20°C for future use.

2.13 Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reactions (q-PCR) were conducted using the SsoFast EvaGreen™ Supermix® (Bio-Rad Laboratories). The cDNA samples were diluted 1:4 in pyrogen-free water. Primer mixes of 100 nM for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TNF-α, IL-6, iNOS, and IL-10 (Table 2.1) (Intergrated DNA Technologies; Skokie, IL) were made from 10 µl of both the forward and reverse primers added to 80 µl of water. A 1 µl sample of diluted cDNA was then added to a master mix solution containing 5 µl EvaGreen Supermix, 3 µl pyrogen-free water, and 1 µl primer mix in a final volume of 10 µl. Negative controls did not contain any cDNA. Reactions were conducted in triplicate using a Stratagene Mx3005p q-PCR system (Agilent Technologies, Santa Clara, CA) and a Rotor-Gene 6000 q-PCR machine (Qiagen, Valencia, CA). Cycling conditions consisted of a 30 s activation step at 95°C, followed by 40 amplification cycles for 5 s at 95°C and 30 s at an annealing temperature specific to each primer set used (refer to Table 1). To confirm that the q-PCR reaction had produced the specific and intended products, a melt curve analysis was conducted using MxPro q-PCR Software (Agilent Technologies, Santa Clara, CA) cycle threshold (CT) values, indicating the number of cycles it takes for the fluorescent signal to surpass the background fluorescence (233). The relative amounts of amplicons were determined by normalizing the CT values of the target gene to the endogenous control, GAPDH. These values were then normalized to the untreated control values, giving the expression fold values.

2.14 Primer Optimization/Efficiency

For each primer set that was purchased, primer optimization was done to determine the optimal annealing temperature and concentration of cDNA template to be used during the q-PCR reaction. For temperature optimization, samples are set up in a similar manner to q-PCR with a ratio of 5:3:1:1 for EvaGreen, H_2O, primer mix, and cDNA, respectively. The cDNA used for primer optimization is pooled from several samples to prevent r due to inadequate cDNA template. Once mixed thoroughly, 10 µl of the solution with components listed above were distributed into 250 µl tubes. Using a

Bio-Rad T100™ Thermo Cycler, a temperature gradient ranging from 50-60°C was established with each individual tube at a different temperature during the annealing step. Once the incubation cycles were complete, 10% BlueJuice ™ Gel Loading Buffer [w/v] (Fisher Scientific, Waltham, MA) was added to each sample. To prepare the 1.5% agarose gel, agar was added to 100 ml 1x TAE, swirled, and microwaved for 90 s. Once the agar had dissolved, the flask was removed from the microwave and left on the bench top until cool, then the gel was poured into a mold with a 10-well comb and left to set for 10 min. Once polymerized, 10 µl of the sample was mixed with loading buffer and loaded into a well, with each well containing a sample that was incubating at a different annealing temperature during the q-PCR cycling. The cast with the gel was mounted in the gel apparatus, 500 ml of 1x TAE was added, and the system was set at 100 V for 45 min. Once the samples migrated 75 percent of the way down the gel, the cast and gel was removed from the apparatus, imaged using a Kodak Image Station 4000 mm Pro, and analyzed using Carestream Software (Rochester, NY). Primer efficiency was conducted in a similar manner to the instructions outlined in section 2.12, except increasing dilutions of cDNA were used (1:1, 1:4, 1:8, and 1:16 and dissociation curves were analyzed to determine the dilution at which the primers work optimally.

2.15 Protein Isolation

Treated THP-1 cells, RAW 264.7 cells, and BMDMs treated with medium only, bovine, murine, or human lactoferricin alone [5 µM], LPS [100 ng/ml], or a combination of lactoferricin peptides and LPS were lifted from tissue culture flasks using 5 ml of 10 mM EDTA and a cell scraper and collected in tubes that were centrifuged at 500 x g for 5 min. The supernatant was then discarded and cell pellets were resuspended in 1 ml of cold PBS, transferred to 1ml eppendorf tubes, and centrifuged at 1000 x *g* for 5 min. The supernatant was discarded and the pellet resuspended in 40 µl of cold lysis buffer composed of 50 mM Tris-HCL, 0.25% sodium deoxycholate (w/v), 150 nM NaCl, 50 mM Na_2HPO_4, 0.1% NP-40 (v/v), 5 mM EGTA, and 5 mM EDTA, mixed with protease and phosphatase inhibitors (5 µg/ml pepstatin A, 5 µg/ml leupeptin, 1 mM PMSF, 1 mM DTT, 10 mM NaF, 10 µg/ml aprotinin, 10 µM PAO, and 100 µM Na_3VO_4). Samples

were then incubated on ice on for 15 min and centrifuged at 14,000 x *g* for 10 min. Supernatants containing protein were then collected and stored at -80°C for future use.

2.16 Protein Quantification

A Bradford assay was used to quantify total protein concentrations from each sample by adding 2.5 µl of each sample to 500 µl of a 1 in 4 dilution of BioRad Protein Assay Dye Reagent in ddH_2O. BSA was used to generate a protein standard curve that ranged from 2.5-40 µg/ml. Each of the diluted standards and samples were then plated in triplicate in a 96-well flat-bottom plate and absorbance was measured at 570 nm with an Expert 96 Microplate Reader. Using the protein standard curve, the concentration of protein in each sample was calculated and diluted in lysis buffer to equalize protein amounts amongst each sample. The proteins in each sample were then denatured by the addition of SDS-PAGE sample loading buffer (200 mM Tris HCl [pH 6.8], 15% v/v β-mercaptoethanol, 30% v/v glycerol, 6% w/v SDS, and 0.01% w/v bromophenol blue) and placed in a heating block at 95°C for 5 min. If not used immediately, samples were stored at -80°C until future use.

2.17 Western Blotting

Equal amounts of protein sample (10 µg) and 5 µl of pre-stained BluEye protein ladder (Froggabio; North York, ON) were combined and loaded onto a 12% polyacrylamide gel (12% acrylamide, 0.1% [w/v] sodium dodecyl sulfate [SDS], 375 mM Tris-HCl [pH 8.8], 0.15% tetramethylethylenediamine (TEMED) [v/v], and 0.1% ammonium persulfate (APS)[w/v])) and a 4% polyacrylamide stacking gel (4% acrylamide, 0.1% SDS [w/v], 125 mM Tris-HCl [pH 6.8], 0.15% TEMED [v/v], and 0.1% APS [w/v]). Proteins were separated at 200 V for 1 h in SDS-PAGE running buffer (20 mM Tris-HCl [pH 8.3], 200 mM glycine, and 0.1% SDS [v/v]). Proteins from the acrylamide gel were then transferred to a nitrocellulose membrane using the iBlot® dry blotting system (Life Technologies, Burlington, ON) according to the manufacturers instructions. Membranes were blocked for 1 h at room temperature with 5% BSA [w/v]

in Tris-buffered saline (20 mM Tris-HCl [pH 7.6], 200 mM NaCl) containing 0.05% Tween-20 [v/v] (TTBS). Once blocked, membranes were incubated overnight with the specified primary antibody at 4°C. Membranes were washed with TTBS every 5 min for 45 min, followed by incubation with the specified HRP-conjugated secondary antibody at room temperature for 1 h. Membranes were then washed again in TTBS for 45 min, with changes in the wash every 5 min. After the final wash membranes were incubated with Luminata™ Forte Western HRP Substrate (Millipore; Taunton, MA) for 1 min, exposed to X-ray film (Sci-Med Inc.; Truro, NS), and then developed using a Kodak X-OMAT 1000A automated X-ray developer. In order to account for any variation of loading between protein samples, membranes were also reprobed for β-actin. Image Studio™ Software (LI-COR®; Guelph, ON) was using to determine the relative intensity of each band through densitometry. The ratio of actin normalized to phospho-protein was compared to that of total protein normalized to phospho-protein and subsequently normalized to the medium control.

2.18 Immunofluorescence

Imaging of cells stained with fluorescent antibodies was used to determine nuclear localization of inflammatory transcription factor NF-κB. Cells were grown on coverslips that were placed in a 12-well plate, left for 24 h, serum-starved overnight, and treated with peptide and LPS for 4 h. Medium was then removed from the cells, which were then incubated with 4% paraformaldehyde for 15 min at room temperature. Coverslips were washed 3x 5 min in PBS and allowed to try overnight. Slides were then incubated for 1 h in blocking buffer (1x PBS, 5% goat serum [w/v], and 0.3% Triton X-100 [v/v]). Following blocking, cells were incubated with anti-p65 antibody diluted 1:100 in antibody dilution buffer at 4°C overnight. From this point on all incubations were done in the absence of light. Cells were then washed 3x 5 min in PBS and incubated for 1 h at room temperature in goat-anti-rabbit secondary antibody conjugated to Alexa Fluor ® 488 diluted 1:500 in antibody dilution buffer. Cells were again washed 3x 5 min in PBS and incubated at room temperature for 10 min in 30 μM 4',6-diamidino-2-phenylindole (DAPI) diluted 1:100 in PBS. The cells were washed a final 3x for 5 min in PBS and

coverslips were lifted from the plate. Dako Fluorescent Mounting Medium was then applied, and each coverslip was mounted to individual Fisherbrand Superfrost® Plus slides. Visualization of slides was done using a Zeiss Axioplan II Motorized Microscope (Zeiss Canada, North York, ON) and AxioVision 4.8 Microscopy Software.

2.19 Confocal Immunofluorescence

Confocal imaging of cells was performed to determine colocalization between endosomal marker EEA1 and biotinylated bovine lactoferricin peptide. RAW 264.7 cells were grown on coverslips that were placed in a 12-well plate, left for 24 h, serum-starved overnight, and treated with biotinylated bovine lactoferricin peptide for 30 min. Medium was then removed from the cells, which were incubated with 4% paraformaldehyde for 15 min at room temperature. Coverslips were then washed 3x 5 min in PBS and left to dry overnight at room temperature. Slides were then incubated for 1 h in normal blocking buffer or in blocking buffer without Triton X, to avoid permeabilization when testing for peptide accumulation on the cell surface. Following blocking, cells were incubated with mouse anti-EEA1-antibody diluted 1:500 overnight in antibody dilution buffer at 4°C. Cells were then washed 3x 5 min in PBS and incubated for 1 h at room temperature in goat-anti-rabbit secondary antibody conjugated to Alexa Fluor ® 555 and streptavidin-conjugated to Alexa Fluor ® 488 (Thermo Fisher Scientific), both diluted 1:1000 in antibody dilution buffer. Cells were washed 3x 5 min in PBS and incubated at room temperature for 10 min in 30 μM DAPI diluted 1:500 in PBS. The cells were washed a final 3x for 5 min in PBS and coverslips were lifted from the plate Dako medium was applied and each coverslip was mounted to a Fisherbrand Superfrost® Plus slides. Slides were visualized using an LSM710 Zeiss Confocal Microscope and AxioVision 4.8 Microscopy Software.

2.20 Statistical Analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA) with a Dunnett's post-test, using GraphPad Prism Software (GraphPad Software Inc.; La

Jolla, CA). Data was considered significantly different when the *p* value was less than 0.05 (indicated by *); when the *p* value was greater than 0.05, data were considered non-significant.

Table 2.1 List of human and mouse primer sequences used for q-PCR experiments.

Human Primers	**Primer Sequence**
GAPDH	F-5′CAACGGATTTGGTCGTATTGG-3’ R-5’GGCAACAATATCCACTTTACCAGAGT-3’
TNF-α	F-5’CCAGGCAGTCAGATCATCTTCTC-3’ R-5’AGCTGGTTATCTCTCAGCTCCAC-3’
IL-6	F-5’ GTGCCTCTTT GCTGCTTTCAC-3’ R- 5’ GGTACATCCTCGACGGCATCT-3’
Mouse Primers	
GAPDH	F-5’CCACTTCAACAGCAACTCCCACTCTTC’3 R-5’TGGGTGGTCCAGGGTTTCTTACTCCTT’3
TNF-α	F-5’CATCTTCTCAAAATTCGAGTGACAA’3 R-5’GCACCTCAGGGAAGAGTCTG’3
IL-6	F- 5'-GCCAGAGTCCTTCAGAGAGATACAG-3' R- 5’ GAATTGGATGGTCTTGGTC-CTTAGC-3'
iNOS	F-5’CAGCTGGGCTGTACAAACCTT’3 R-5’TGAATGTGATGTTTGCTTCGG’3

CHAPTER 3

RESULTS

3.1 Low-dose lactoferricin peptide treatment is non-toxic for macrophages

Previous studies involving the non-antimicrobial properties of lactoferricin have mainly focused on the treatment of various cancer cells as this peptide has potent anti-proliferative, anti-angiogenic, and anti-metastatic properties for cancer cells (234–237). Few studies, however, have addressed different species-specific lactoferricin immunomodulatory properties in innate immune cells such as macrophages. In order for these peptides to have functional immunomodulatory influences, they must be administered in sub-lethal doses to ensure any regulation of inflammatory cytokines is not due to cell stress or death. A non-cytotoxic dose of each bovine (Blfcn), murine (Mlfcn), and human (Hlfcn) lactoferricin peptide was determined using MTT assays, in which MTT is reduced to purple formazan by mitochondrial reductases as an indication of the number of viable cells (231, 238). Low concentrations (2.5-10 μM) of lactoferricin peptides were not cytotoxic to RAW 264.7, THP-1, or BMDM cells (Figure 3.1 A-C). Loss of cell viability in RAW 264.7 and BMDMs was observed with treatment of concentrations of bovine lactoferricin at 50 μM and above. Concentrations of bovine lactoferricin at and above 120 μM were toxic to THP-1 cells (Figure 3.1D).

Trypan blue assays were also conducted in parallel with MTT experiments because MTT assays do not discriminate between cytostatic and cytotoxic events. The trypan blue exclusion assay is based on the principle that intact cell membranes exclude trypan blue dye. Dead cells will take up of the dye and therefore will appear blue in comparison to viable cells (230). Trypan blue assays were conducted using RAW 264.7, THP-1, and BMDM cells to determine working concentrations of lactoferricin peptides that are not cytotoxic. Not surprisingly, results mirrored that of MTT assays (Figure 3.1 A-C), in which concentrations of peptide used did not cause any significant cell death compared to the untreated control macrophages (Figure 3.1 E). Given the results of the cell viability assays, previous studies on lactoferricin treated cells (201, 204, 206), and data from this current study (Table 3.1), it was determined that 5 μM of either Blfcn, Mlfcn, and Hlfcn would be suitable for studying the immunomodulatory effects of these

peptides. This coincides with other studies of AMPs and their immunomodulatory properties on a variety of different cells, as typical treatment concentrations range from 1 μM-30 μM (150, 151, 209, 239–241).

3.2 Lactoferricin peptides decrease pro-inflammatory cytokines in LPS-stimulated macrophages

The focus of this investigation was to determine and compare the effect of each species-derived lactoferricin peptide on an inflammatory response in macrophages. TNF-α and IL-6 are prominent cytokines that are present at the site of infection or injury and contribute to the pathogenesis of inflammation (242–244). These cytokines also contribute to the development and maintenance of several chronic inflammatory conditions such as arthritis, atherosclerosis, and psoriasis (33, 245–249), thus making them ideal candidates for this investigation. LPS is an effective inducer of inflammatory cytokine production in monocytic cells (250–252) and was chosen as a stimulatory agent of inflammation for all experiments, unless otherwise indicated.

To determine the effect of three different species-specific lactoferricin peptides on TNF-α production in macrophages, RAW 264.7 cells, THP-1 cells, and BMDMs were treated with 100 ng/ml (LPS) alone, or in combination with 5 μM Blfcn, Mlfcn, or Hlfcn peptides. Once treated, supernatants were collected at 6 h, as several studies show peak production of TNF-α in LPS-stimulated macrophages occurs at this time (253–255). Supernatants were then used in ELISA assays to determine the relative levels of TNF-α in comparison to the untreated controls. Results demonstrate that Blfcn significantly reduced the production of TNF-α in RAW 264.7 cells, THP-1 cells, and BMDMs co-treated with LPS in comparison to LPS treatment alone (Figure 3.2 A-C). Mlfcn and Hlfcn caused a decrease in LPS-induced TNF-α production by LPS-stimulated THP-1 cells, but not in mouse macrophages (Figure 3.2 B).

To determine if lactoferricin peptides also had an influence on IL-6 production, another pro-inflammatory cytokine produced by macrophages, RAW 264.7 cells, THP-1 cells, and BMDMs were incubated with LPS alone or in combination with Blfcn, Mlfcn, or Hlfcn for 24 h and supernatants collected for ELISA analysis. LPS-stimulated RAW

264.7 and BMDM cells treated with Blfcn showed a significant decrease in IL-6 production in comparison to the LPS control (Figure 3.3A, C, $p < 0.05$). All three lactoferricin peptides were able to decrease LPS-induced IL-6 production in THP-1 cells (Figure 3.3 B, $p < 0.05$).

It should be noted that treatment with lactoferricin without LPS stimulation did not influence the production of TNF-α or IL-6 in RAW 264.7 cells, THP-1 cells, or BMDMs (Table 3.2 and Table 3.3).

3.3 Lactoferricin peptides reduce pro-inflammatory cytokine mRNA expression in macrophages

Since lactoferricin peptides decreased both TNF-α and IL-6 protein production in macrophages (Figure 3.2 and Figure 3.3), the ability of lactoferricin peptides to downregulate pro-inflammatory cytokine mRNA expression was also investigated. A series of q-PCR experiments were conducted to determine the effects of lactoferricin on pro-inflammatory cytokine mRNA expression in LPS-stimulated macrophages. RAW 264.7, THP-1, and BMDM cells were treated with 100 ng/ml LPS, or the combination of LPS and either 5 μM Blfcn, Mlfcn, or Hlfcn for 4 h prior to RNA isolation, as peak mRNA production is at 4 h post-LPS treatment (256, 257).

All three lactoferricin peptides dampened LPS-induced TNF-α mRNA expression in RAW 264.7 cells (Figure 3.4A; $p < 0.01$). Similar to cytokine data shown in Figure 3.2 B, all three species-specific lactoferricin peptides downregulated TNF-α mRNA expression in LPS-stimulated THP-1 macrophages (Figure 3.4B; $p < 0.05$). However, in BMDM cells, Blfcn was the only lactoferricin peptide that was able to downregulate LPS-induced TNF-α mRNA expression (Figure 3.4C; $p < 0.05$).

The transcriptional control of IL-6 was also investigated in RAW 264.7 and THP-1 cells. RAW 264.7 cells treated with combination Blfcn and LPS had decreased IL-6 mRNA expression in comparison to cells treated with LPS alone (Figure 3.5 A; $p < 0.05$). THP-1 cells cotreated with LPS and lactoferricin peptides had a similar pattern of

reduced IL-6 mRNA expression that correlated with IL-6 protein profiles; however, the reduced expression was not statistically significant (Figure 3.5 B, NS).

3.4 Lactoferricin peptides increase anti-inflammatory IL-10 cytokine production in RAW 264.7 cells

LPS also promotes the synthesis and release of anti-inflammatory IL-10 (258). Other studies involving the parent peptide of lactoferricin, lactoferrin, have demonstrated the capacity for this peptide to induce anti-inflammatory IL-10 in a variety of cells types, including intraepithelial lymphocytes, mesenteric lymph node cells, and in mouse models of rheumatoid arthritis (228, 259). RAW 264.7 cells were treated with 100 ng/ml LPS alone or in combination with either 5 μM Blfcn, Mlfcn, Hlfcn peptide for 24 h. Supernatants were collected and an ELISA was conducted to determine the IL-10 production among treatments. Results demonstrated that Blfcn, and Mlfcn in combination with LPS, has an increased production of IL-10 by RAW 264.7 cells in comparison to LPS treatment alone (Figure 3.6A, $p < 0.05$).

The increase in IL-10 production in peptide-treated RAW 264.7 cells stimulated with LPS prompted the investigation of IL-10 production RAW 264.7 cells treated with peptide alone. Interestingly, there was an increase in IL-10 production in comparison to untreated controls following treatment of the three species-specific lactoferricin peptides (Figure 3.6 B, $p < 0.05$).

A similar experiment was then conducted using THP-1 cells treated with either Blfcn, Mlfcn, or Hlfcn for 24 h. ELISA analysis on collected supernatants showed no significant difference in IL-10 production in THP-1 cells treated with lactoferricin peptides in comparison to the untreated control (Figure 3.6 C, NS)

3.5 Lactoferricin peptides decrease nitric oxide production in LPS-stimulated macrophages

The increased production of nitric oxide by macrophages is a typical response to a pro-inflammatory stimulant such as LPS (Figure 3.7 A). To determine if lactoferricin

peptides inhibited the LPS-stimulated production of nitric oxide, RAW 264.7 cells and BMDMs were incubated with 500 ng/ml LPS or the combination of LPS and different lactoferricin peptides for 24 h. Supernatants were tested for the presence of nitric oxide using Griess reagent. A significant decrease in LPS-induced nitric oxide production was observed in RAW 264.7 cells with the addition of either Blfcn or Hlfcn (Fig 3.7B, $p < 0.01$). A decrease in nitric oxide production upon LPS-stimulation was observed with the addition of Blfcn, Mlfcn, or Hlfcn in BMDMs (Figure 3.7 C, $p < 0.05$).

3.6 Lactoferricin peptides decrease inducible nitric oxide synthase expression in LPS-stimulated macrophages

NO is a byproduct of the conversion of L-arginine to L-citrulline by iNOS by a variety of cell types (46). The two other isoforms of nitric oxide synthases, eNOS and nNOS are primarily located in endothelial and neuronal cells, as the names indicate (260). iNOS is the primary producer of NO in macrophages (261), and given lactoferricin peptides are able to decrease LPS-stimulated NO production in RAW 264.7 and BMDM cells, it was important to determine whether these peptides could also downregulate iNOS expression.

RAW 264.7 and BMDM cells were treated with 500 ng/ml LPS alone or in combination with 5 μM Blfcn, Mlfcn, or Hlfcn for 4 h prior to RNA isolation. Levels of iNOS expression were normalized to the LPS control as iNOS mRNA was below the level of detection in untreated cells. A significant decrease in LPS-induced iNOS mRNA expression was seen in RAW 264.7 cells treated with either Blfcn or Hlfcn (Figure 3.8 A, $p < 0.05$). When LPS-stimulated BMDMs were treated with either Mlfcn or Hlfcn there was a significant reduction in iNOS expressing in comparison to LPS treatment alone (Figure 3.8 B, $p < 0.05$). iNOS expression experiments demonstrated the capacity for lactoferricin peptides to downregulate another prominent pro-inflammatory marker in macrophages.

3.7 Biotin-labeled bovine lactoferricin enters the cell through an endosomal-independent mechanism

The mechanism by which lactoferricin peptides are able to downregulate a strong pro-inflammatory response induced by LPS in macrophages is still unclear. Previous studies suggest that lactoferrin and its derived peptides may elicit their effects by an intracellular action in a wide variety of cell types (262–264). One study in particular, by Jiang *et al*. (265) demonstrates the uptake of lactoferrin in Caco-2 cells is through a receptor-associated clathrin-mediated endocytic-dependent mechanism.

To determine if the smaller lactoferricin peptide enters cells in a similar fashion to its larger parent peptide, cells were treated with biotin-labeled Blfcn (BL-Blfcn) and strepavidin-conjugated Alexa Fluor 488 for visualization using confocal microscopy. To determine if lactoferricin entered the cell and whether this occurred in an endosomal-mediated process, the cells were incubated with nuclear stain DAPI (Figure 3.9 A) and observed for colocalization between EEA-1, an endosomal marker (Figure 3.9 B), and BL-Blfcn (Figure 3.9 C). Results demonstrated there is no colocalization, as there was little to no overlap between the fluorescent markers (Figure 3.9 D and E). However, distinct puncta of BL-Blfcn was observed in cells, indicating that lactoferricin may be entering the cells in an endosomal-independent process (Figure 3.9 D and E). As a control, cells were treated in the same manner as in Figure 3.9 A-E, but no Triton-X was added to blocking or antibody dilution buffers (Figure 3.9 F). The absence of Triton-X, a membrane-permeabilizing agent, was used as a control to demonstrate that biotin-conjugated bovine lactoferricin was internalized into the cell and not bound to the cell surface.

RAW 264.7 cells stimulated with 500 ng/ml LPS with the addition of BL-Blfcn had no significant decrease in NO production after 24 h in comparison to cells treated with non-biotinylated Blfcn (Figure 3.9 G).

3.8 Lactoferricin peptides reduce expression of phosphorylated IκBα in LPS-stimulated macrophages

The binding of LPS to TLR-4 induces rapid and specific pro-inflammatory mediators via NF-κB and associated signaling pathways outlined in Chapter 1.5. To determine whether lactoferricin peptides influenced LPS-induced pro-inflammatory signaling cascades, RAW 264.7 and THP-1 cells were treated with LPS alone or in combination with Blfcn, Mlfcn, or Hlfcn and cell lysates were collected for western blot analysis. Cells were treated for 4 h, 1 h, or 30 min, cell lysates were collected, and western blot analysis of phospho-IκBα levels was conducted.

Since the time course experiment showed that Blfcn reduced LPS-induced phospho-IκBα levels in RAW 264.7 cells at 1 h but not at 30 min or 4h (Figure 3.10 A), subsequent western blot analysis was conducted after 1 h incubation with peptide, LPS, or combination treatment. Treatment of RAW 264.7 cells with the combination of LPS and lactoferricin peptides led to a decrease in phospho-IκBα levels in comparison to LPS treatment alone, whereas treatment with peptide alone had no significant effect on phospho-IκBα levels (Figure 3.10 B and C $p < 0.01$). In a similar manner to RAW 264.7 cell treatments, there was a significant decrease in LPS-induced phospho-IκBα expression in comparison to LPS alone treatment in THP-1 cells following treatment with Blfcn, Mlfcn, or Hlfcn (Figure 3.10 D and E, $p < 0.01$).

3.9 Lactoferricin peptides inhibit the nuclear translocation of NF-κB in LPS-stimulated macrophages

Phosphorylation of IκBα leads to the proteosomal degradation of IκBα and release of transcription factor, NF-κB, into the nucleus, allowing for the expression of a number of pro-inflammatory genes, including those that code for TNF-α, IL-6, and iNOS (57, 266). To determine whether lactoferricin peptides inhibit the nuclear translocation of NF-κB, RAW 264.7 and THP-1 cells were treated with LPS alone, or in combination with Blfcn, Mlfcn or Hlfcn for 1 h and stained with anti-p65 (a subunit of NF-κB) antibody

and the nuclear stain DAPI. Cells were visualized using fluorescent microscopy. The results showed that a strong pro-inflammatory response corresponded to prominent nuclear translocation of p65, as seen in LPS–treated macrophages (Figure 3.11 A and B). RAW 264.7 cells treated with Blfcn, Mlfcn, or Hlfcn had varying degrees of reduced nuclear localized p65 in comparison to the LPS control. Blfcn in particular had the strongest inhibitory effect of p65 translocation in comparison to human and mouse lactoferricin peptides (Figure 3.11 A). Nuclear translocation of p65 in THP-1 cells was also inhibited with lactoferricin treatment (Figure 3.11 B). These results demonstrated that species-specific lactoferricin treatments inhibited the LPS-induced translocation of NF-κB into the nucleus of murine and human macrophages. To exclude the possibility that there is increased cytosolic p65 of NF-κB in lactoferricin-treated cells in comparison to cells treated with LPS alone, western blot analysis of total p65 expression was conducted. There was no significant change in total p65 expression levels among treatments; indicating peptide treatment did not affect total NF-κB expression (Figure 3.11 C).

Although NF-κB is a prominent transcription factor induced by LPS, it is not the only inflammatory transcription factor induced by this TLR-4 agonist. β-catenin is another transcription factor that is activated upon LPS-stimulation, leading to the expression a number of pro-inflammatory mediators such as IL-6 and IL-8, as well as having a possible regulatory role in NF-κB-dependent gene transcription (267). Production of IL-6, as mentioned previously is an important mediator that propagates the inflammatory response and IL-8 is a potent neutrophil chemoattractant (248, 257). Immunofluorescent microscopy experiments were therefore conducted to determine if LPS-induced nuclear translocation of β-catenin was inhibited by lactoferricin treatment. In contrast to the inhibition of p65 nuclear translocation, there was no effect on LPS-induced β-catenin nuclear translocation in lactoferricin-treated RAW 264.7 cells (Figure 3.12).

3.10 Lactoferricin peptides reduce ERK phosphorylation in LPS-stimulated macrophages

ERK 1/2 MAPK is phosphorylated upon TLR-4 stimulation with LPS leading to the induction of inflammatory mediators by macrophages (61, 62, 268, 269). To determine if lactoferricin peptides also target MAPK signaling pathways, western blot analysis was conducted using lysates from RAW 264.7 and THP-1 cells treated with lactoferricin alone, LPS alone, or the combination of LPS and lactoferricin. RAW 264.7 cells treated with either Blfcn or Mlfcn showed a significant reduction in LPS-induced phospho-ERK 1/2 levels in comparison to LPS treatment alone (Figure 3.13 A and B, $p < 0.05$). THP-1 cells showed a similar trend of reduced phospho-ERK 1/2, demonstrating a decrease in LPS-induced ERK 1/2 levels with peptide treatment (Fig 3.13 C and D, $p < 0.05$).

3.11 Lactoferricin peptides decrease c-Jun phosphorylation in LPS-stimulated macrophages

As a result of LPS-induced MAPK signal transduction, AP-1 transcription factor is induced in macrophages (59, 76). AP-1 is a heterodimeric transcription factor composed of ATF, Fos and c-Jun subunits (270). Upon AP-1 activation by external stimuli, c-Jun, a principle subunit of AP-1, is phosphorylated (271). Western blot analysis of phospho-c-Jun expression in lactoferricin-treated, LPS-stimulated RAW 264.7 cells demonstrated a significant reduction in phospho-c-Jun levels in comparison to cells treated with LPS alone (Fig 3.14 A, $p < 0.05$). However, LPS-stimulated THP-1 macrophages treated in a similar manner did not show a significant decrease in phospho-c-Jun expression following lactoferricin treatment (Figure 3.14 B).

3.12 Lactoferricin peptides promote p38 MAPK phosphorylation in macrophages

The p38 MAPK signaling pathway is a complex pathway that has been associated with the induction of both pro- and anti-inflammatory molecules (75, 272, 273). Given that there was an increase in IL-10 production in lactoferricin-treated macrophages and

production of IL-10 is associated phospho-p38 MAPK levels, western blot analysis was done to determine if lactoferricin affects p38 MAPK activation. Western blot analysis showed a significant increase in phospho-p38 MAPK levels in lactoferricin treated RAW 264.7 and THP-1 cells in comparison to untreated controls (Figure 3.15 A-D, $p < 0.05$).

3.13 Lactoferricin peptides show no significant decrease in IL-10 when treated with p38 MAPK inhibitor SB203528

p38 MAPK is responsible for both pro-inflammatory mRNA degradation and IL-10 production in macrophages (75, 273, 274). Given that there was a significant increase in p38 MAPK phosphorylation (Figure 3.15 A-D) and IL-10 production (Figure 3.6 B) in RAW 264.7 cells treated with lactoferricin peptides, ELISA experiments of IL-10 production were conducted using supernatants collected from RAW 264.7 cells treated with lactoferricin peptides and p38 MAPK specific inhibitor, SB203528. However, no significant decrease in lactoferricin-stimulated IL-10 production was observed when LPS alone and peptide-treated cells were incubated with p38 MAPK-specific inhibitor (Figure 3.16, NS).

3.14 Lactoferricin peptides decrease cadmium-induced TNF-α production in macrophages

Macrophages are known to produce an inflammatory response in the presence of heavy metals through the release of various mediators and increased oxidative stress. For example, $CdCl_2$ is a potent carcinogenic that also induces a potent pro-inflammatory response in macrophages exposed to subcytotoxic concentrations of the metal (275–277). As expected, an increase in pro-inflammatory cytokine TNF-α was observed when THP-1 cells were exposed to $CdCl_2$ (Figure 3.17 A). The next experiment determined whether species-specific lactoferricin peptides were able to downregulate a pro-inflammatory response induced by $CdCl_2$. RAW 264.7 and THP-1 cells were treated with $CdCl_2$ alone or in the presence of Blfcn, Mlfcn, or Hlfcn, for 24 h, supernatants were collected and used for ELISA analysis to test for the presence of TNF-α. Results showed a significant

decrease in $CdCl_2$-induced TNF-α production by RAW 264.7 and THP-1 cells treated with Blfcn, Mlfcn, or Hlfcn (Figure 3.17 B and C, $p < 0.05$). There was also a significant decrease in TNF- α mRNA expression in $CdCl_2$-treated RAW 264.7 cells (Figure 3.17 D, $p < 0.05$). Lactoferricin peptides therefore downregulated pro-inflammatory TNF-α induced by nonmicrobial $CdCl_2$.

3.1 Low concentrations of lactoferricin peptides are not cytotoxic to mouse and human macrophages (A) RAW 264.7 cells **(B)** THP-1 cells and **(C)** BMDMs were incubated for 24 h with the indicated concentrations of either bovine lactoferricin (Blfcn), murine lactoferricin (Mlfcn), or human lactoferricin (Hlfcn). The MTT colorimetric assay was used to determine cell viability. At 20 h post treatment MTT solution was added and cells were incubated for an additional 4 h. The percent viability is relative to the untreated control. The data shown are the mean of 3 independent experiments ± SEM; none of the treatments were significantly different from the untreated controls, as determined by ANOVA with a Dunnett's multiple comparisons post-test. **(D)** RAW 264.7, THP-1, and BMDM cells were incubated with the indicated concentrations of Blfcn for 24 h and a MTT assay was conducted as in **A-C**. The percent viability is relative to the untreated control. The data shown are the mean of 2 independent experiments. **(E)** Cells were treated as in **A-C** for 24 h prior to the addition of trypan blue dye and counted. The percent viability is relative to the untreated control for one experiment.

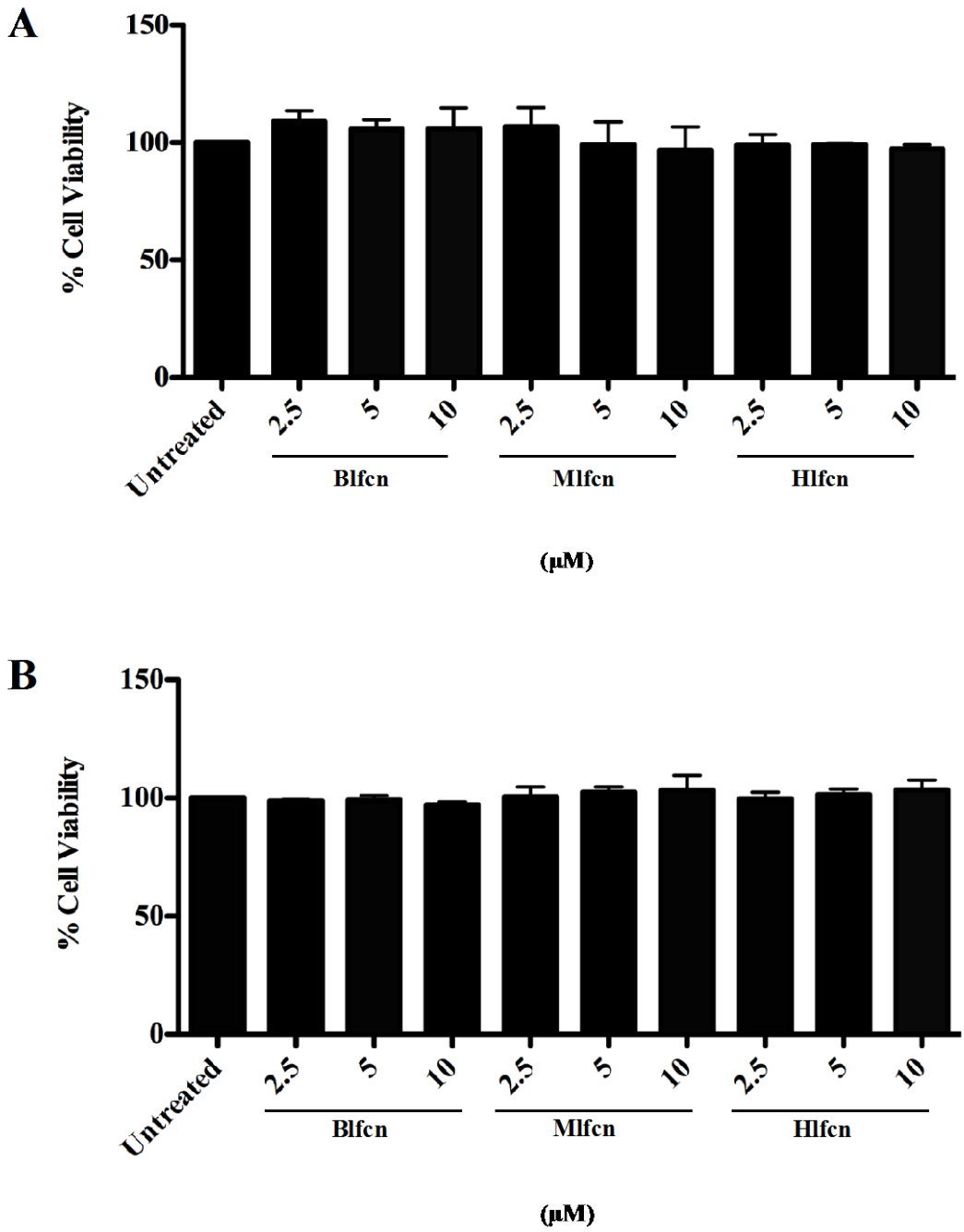

A
150
100
50
0
% Cell Viability
Untreated
2.5
5
10
2.5
5
10
2.5
5
10
Blfcn
Mlfcn
Hlfcn
(µM)
B
150
100
50
0
% Cell Viability
Untreated
2.5
5
10
2.5
5
10
2.5
5
10
Blfcn
Mlfcn
Hlfcn
(µM)

Figure 3.1

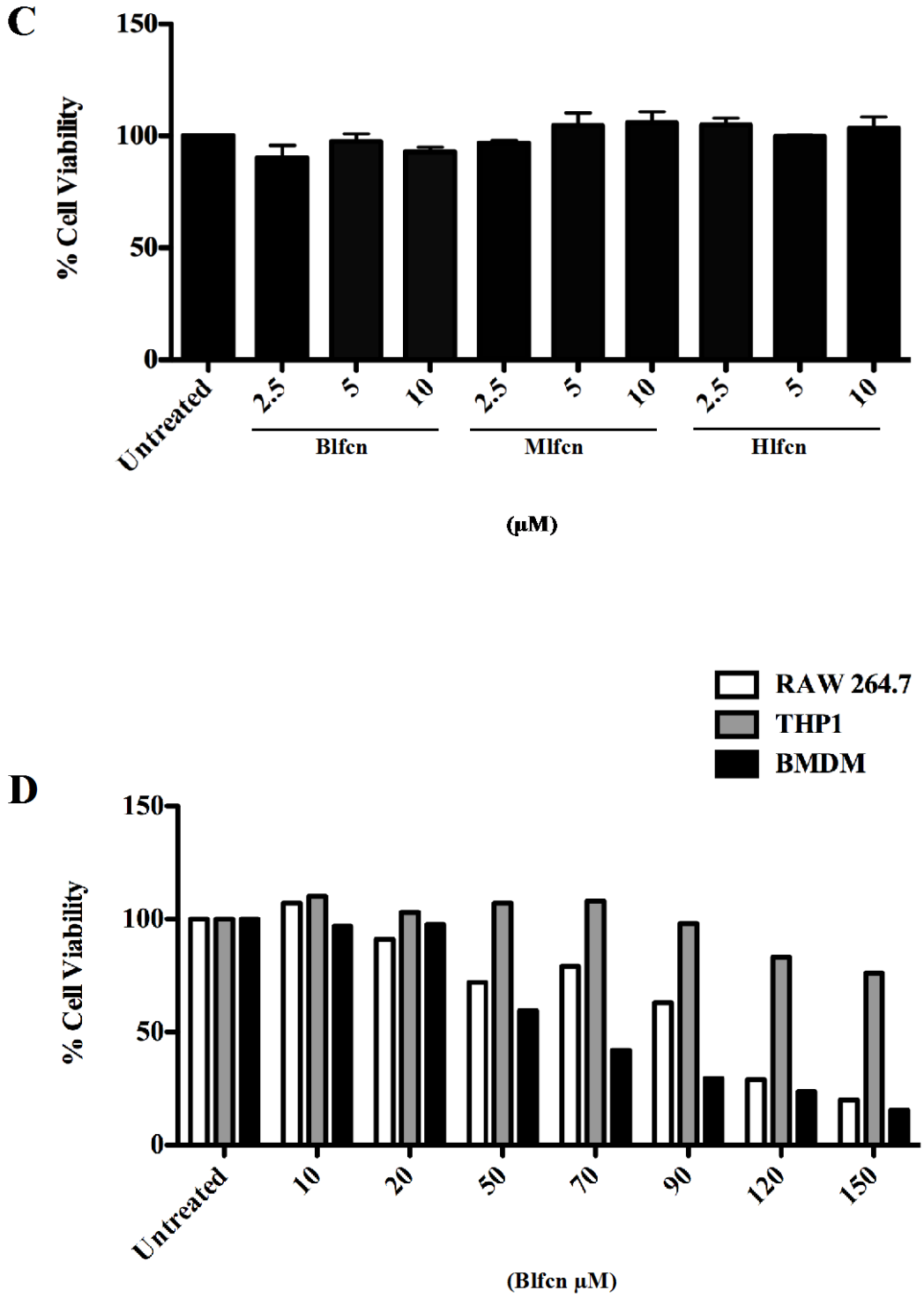


Figure 3.1 (continued)

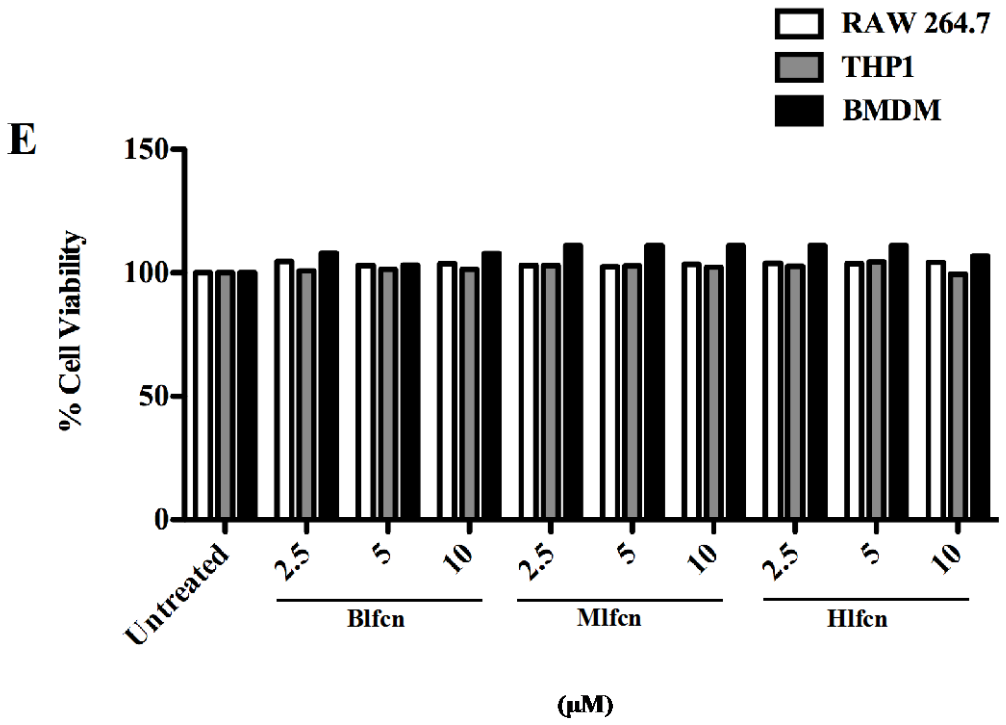


Figure 3.1 (continued)

3.2 Species-specific lactoferricin peptides decrease LPS-induced TNF-α cytokine production in macrophages. (A) RAW 264.7 cells **(B)** THP-1 cells and **(C)** BMDMs were treated with 100 ng/ml LPS alone or in combination with 5 μM Blfcn, Mlfcn, or Hlfcn for 6 h. Supernatants were collected and used in ELISA assays to detect TNF-α production. Levels of TNF-α production are relative to untreated control treatments. Data are the mean of 4 **(A)** or 3 **(B and C)** independent experiments ± SEM. Data are normalized to untreated controls: 47 pg/ml, 90 pg/ml, and 35 pg/ml, respectively; * denotes $p < 0.05$ compared to LPS positive control as determined by ANOVA with Dunnett's multiple comparisons post-test. a.u. denotes arbitrary units

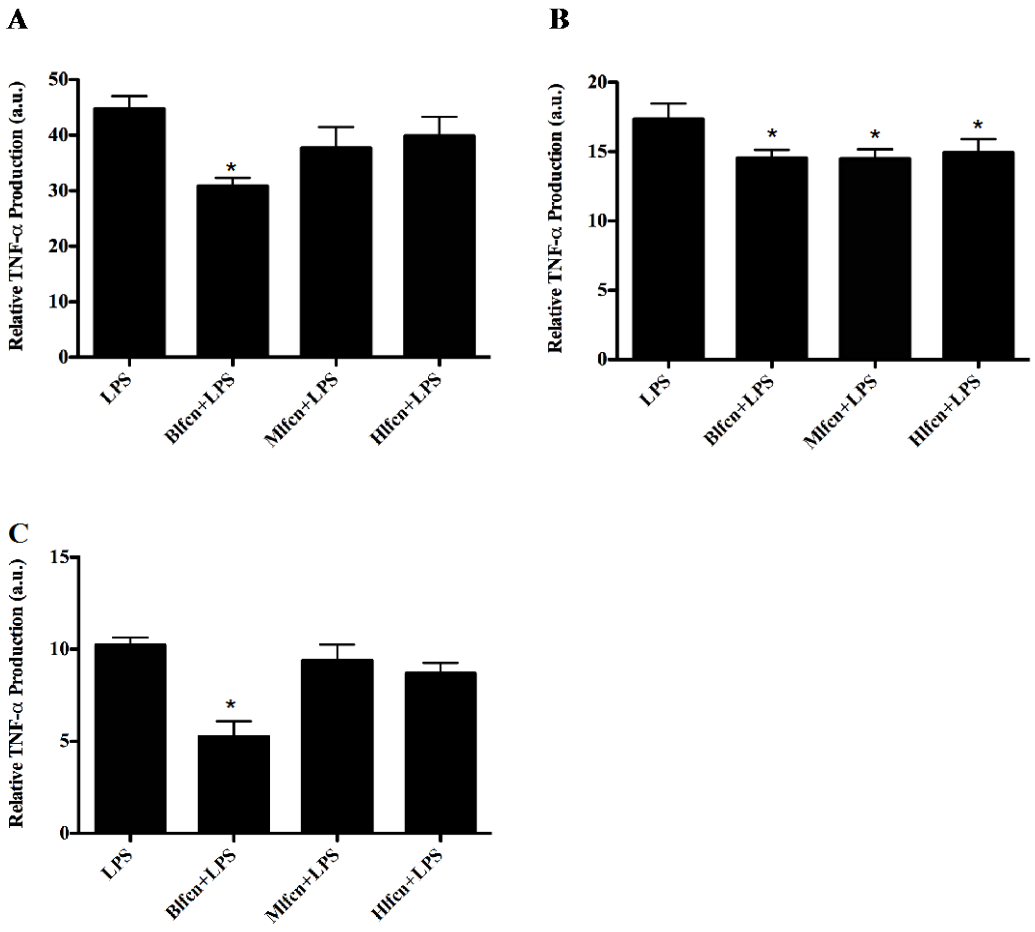

A
Relative TNF-α Production (a.u.)
50
40
30
20
10
0
*
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS
B
Relative TNF-α Production (a.u.)
20
15
10
5
0
*
*
*
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS
C
Relative TNF-α Production (a.u.)
15
10
5
0
*
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS

Figure 3.2

3.3 Species-specific lactoferricin peptides decrease LPS-induced IL-6 cytokine production in macrophages (**A**) RAW 264.7 cells (**B**) THP-1 cells and (**C**) BMDMs were treated with 100 ng/ml LPS alone or in combination with 5 μM Blfcn, Mlfcn, or Hlfcn for 24 h. Supernatants were collected and used in ELISA assays to detect levels of IL-6 production. Levels of IL-6 production are relative to untreated control treatments. Data are the mean of 3 (**A**) or 4 (**B** and **C**) independent experiments ± SEM. Data **(B)** and **(C)** are normalized to untreated controls: 85 pg/ml and 153 pg/ml, respectively; * denotes $p < 0.05$ and ** $p < 0.01$ compared to LPS positive control as determined by ANOVA with Dunnett's multiple comparisons post-test. a.u. denotes arbitrary units.

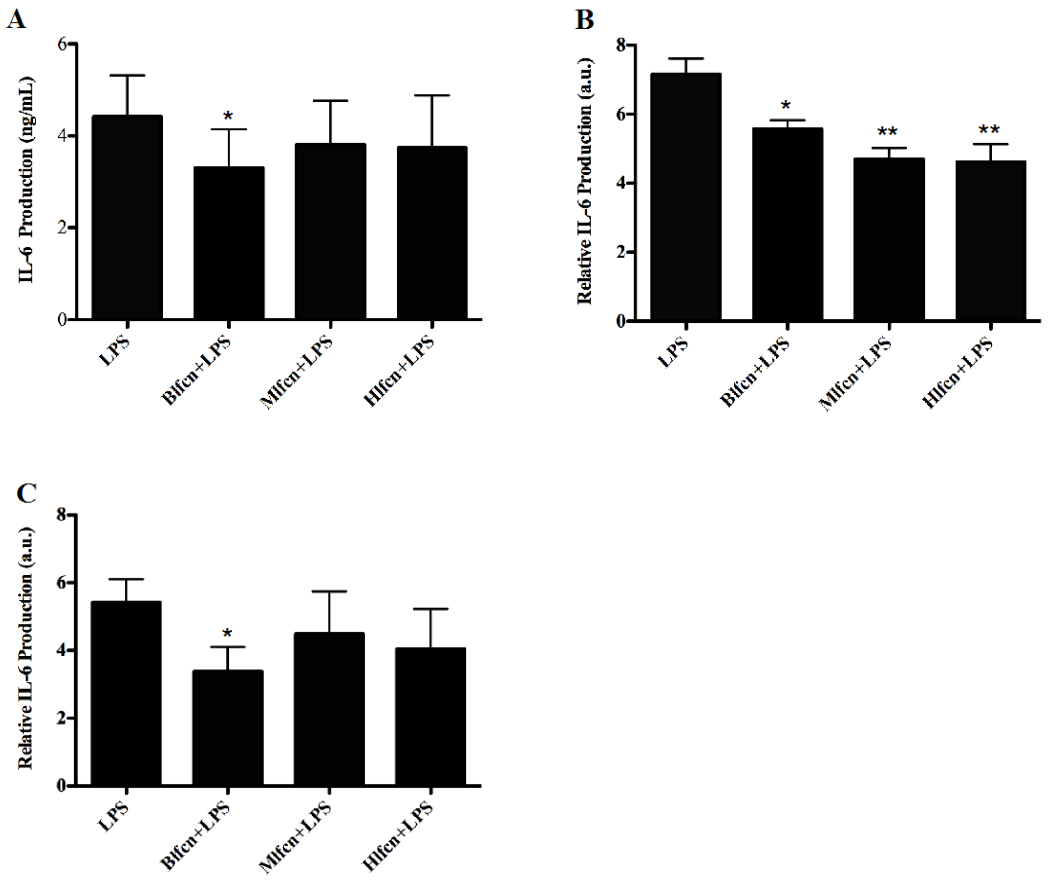

A
IL-6 Production (ng/mL)
0
2
4
6
*
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS
B
Relative IL-6 Production (a.u.)
0
2
4
6
8
*
**
**
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS
C
Relative IL-6 Production (a.u.)
0
2
4
6
8
*
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS

Figure 3.3

3.4 Species-specific lactoferricin peptides downregulate LPS-induced TNF-α mRNA expression in macrophages. (A) RAW 264.7 cells **(B)** THP-1 cells and **(C)** BMDMs were treated with 100 ng/ml LPS alone or in combination with 5 μM Blfcn, Mlfcn, or Hlfcn for 4 h. RNA was isolated for cDNA synthesis and then q-PCR was performed to determine expression levels of TNF-α relative to untreated control treatments. Data are the mean of 4 **(A)** or 3 **(B and C)** independent experiments ± SEM; * denotes $p < 0.05$ and ** $p<0.01$ compared to LPS positive control as determined by ANOVA with Dunnett's multiple comparisons post-test. a.u. denotes arbitrary units.

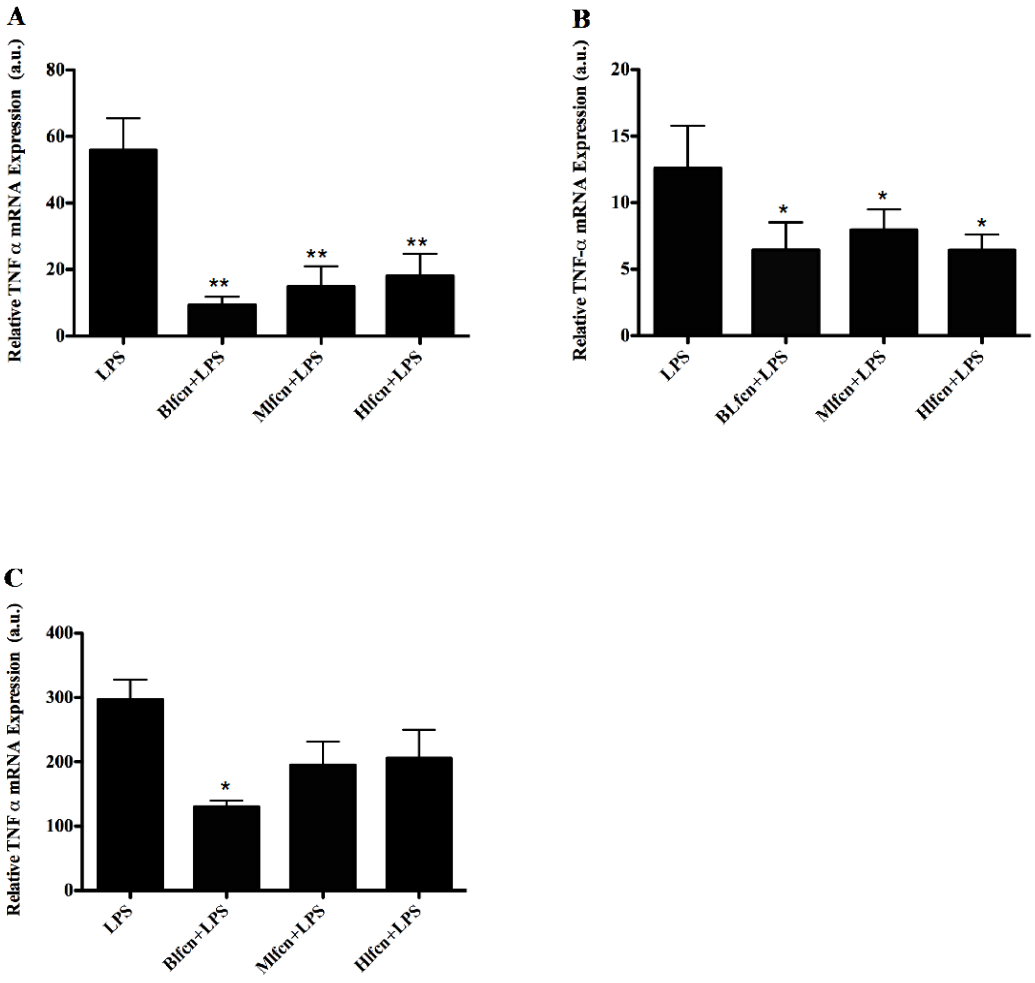

A
Relative TNF α mRNA Expression (a.u.)
80
60
40
20
0
**
**
**
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS
B
Relative TNF-α mRNA Expression (a.u.)
20
15
10
5
0
*
*
*
LPS
BLfcn+LPS
Mlfcn+LPS
Hlfcn+LPS
C
Relative TNF α mRNA Expression (a.u.)
400
300
200
100
0
*
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS

Figure 3.4

3.5 Species-specific lactoferricin peptides downregulate LPS-induced IL-6 mRNA expression in macrophages. (A) RAW 264.7 cells and **(B)** THP-1 cells were treated with 100 ng/ml LPS alone or in combination with 5 μM Blfcn, Mlfcn, or Hlfcn for 4 h. RNA was isolated for cDNA synthesis and then q-PCR was performed to determine expression levels of IL-6 relative to untreated control treatments. Data are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ compared to LPS positive control as determined by ANOVA with Dunnett's multiple comparisons post-test. a.u. denotes arbitrary units.

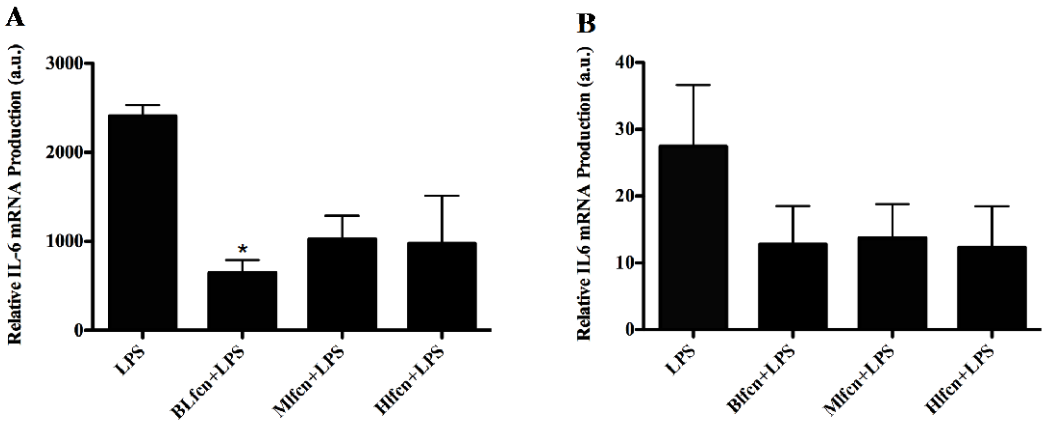

A
Relative IL-6 mRNA Production (a.u.)
3000
2000
1000
0
*
LPS
BLfcn+LPS
MIfcn+LPS
HIfcn+LPS
B
Relative IL6 mRNA Production (a.u.)
40
30
20
10
0
LPS
BIfcn+LPS
MIfcn+LPS
HIfcn+LPS

Figure 3.5

3.6 Species-specific lactoferricin peptides increase IL-10 cytokine production in non-stimulated RAW 264.7 cells. (A) RAW 264.7 cells were treated with 100 ng/ml LPS alone or in combination with 5 μM Blfcn, Mlfcn, or Hlfcn for 24 h. **(B)** RAW 264.7 cells and **(C)** THP-1 cells with 5 μM Blfcn, Mlfcn, or Hlfcn for 24 h. Supernatants were collected and used in ELISA assays to detect IL-10 production. Levels of IL-10 production are relative to untreated control treatments. Data are the mean of 4 **(A** and **B)** or 3 **(C)** independent experiments ± SEM. Data are normalized to untreated controls: 147 pg/ml, 157 pg/ml, and 142 pg/ml respectively; * denotes $p < 0.05$ compared to the untreated control as determined by ANOVA with Dunnett's multiple comparisons post-test. a.u. denotes arbitrary units.

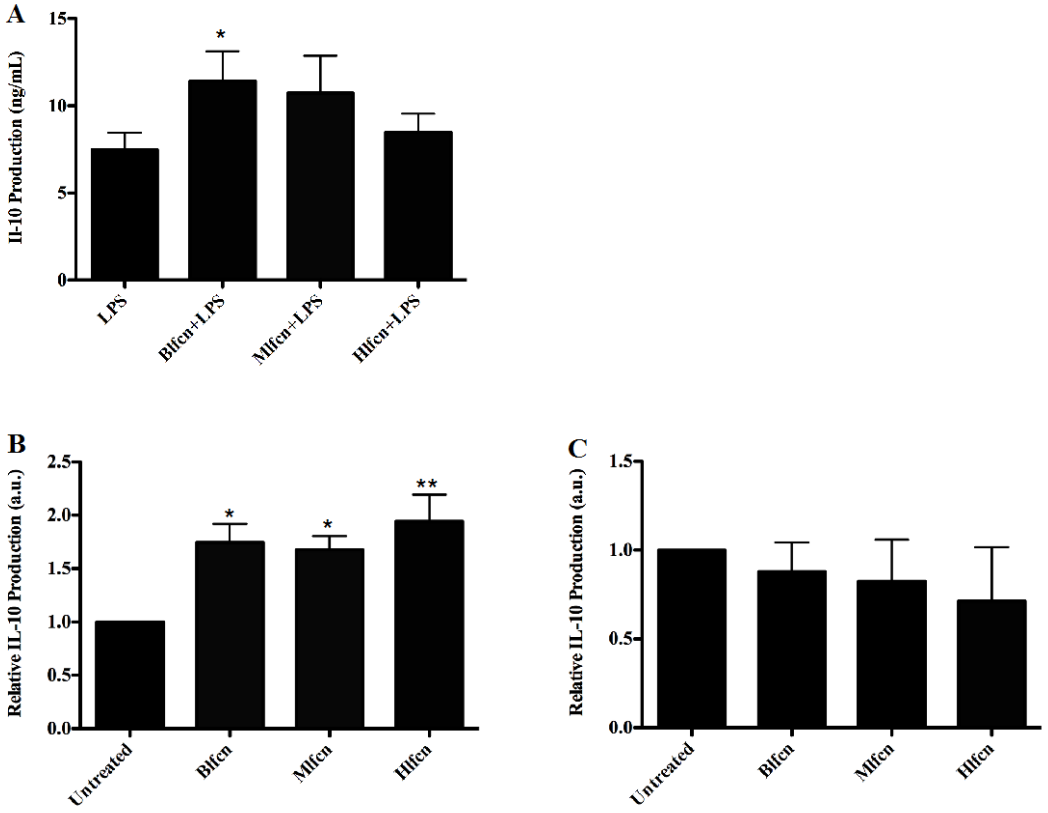

A
Il-10 Production (ng/mL)
15
10
5
0
*
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS
B
Relative IL-10 Production (a.u.)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
**
Untreated
Blfcn
Mlfcn
Hlfcn
C
Relative IL-10 Production (a.u.)
1.5
1.0
0.5
0.0
Untreated
Blfcn
Mlfcn
Hlfcn

Figure 3.6

3.7 Lactoferricin peptides decrease nitric oxide production in LPS-stimulated macrophages (A) RAW 264.7 cells were treated with increasing concentrations of LPS as indicated for 24 h. Supernatants were collected and used in a Griess assay to measure nitric oxide production. **(B)** RAW 264.7 cells and **(C)** BMDMs were treated with 500 ng/ml of LPS alone or in combination with 5 μM Blfcn, Mlfcn, or Hlfcn for 24 h. Supernatants were collected and used in a Griess assay to measure nitric oxide production. Data are the mean of 3 **(A)** or 4 **(B** and **C)** independent experiments ± SEM; * denotes $p < 0.05$ and ** $p < 0.01$ compared to LPS treatment as determined by ANOVA with Dunnett's multiple comparisons post-test. BDL= below detectable levels.

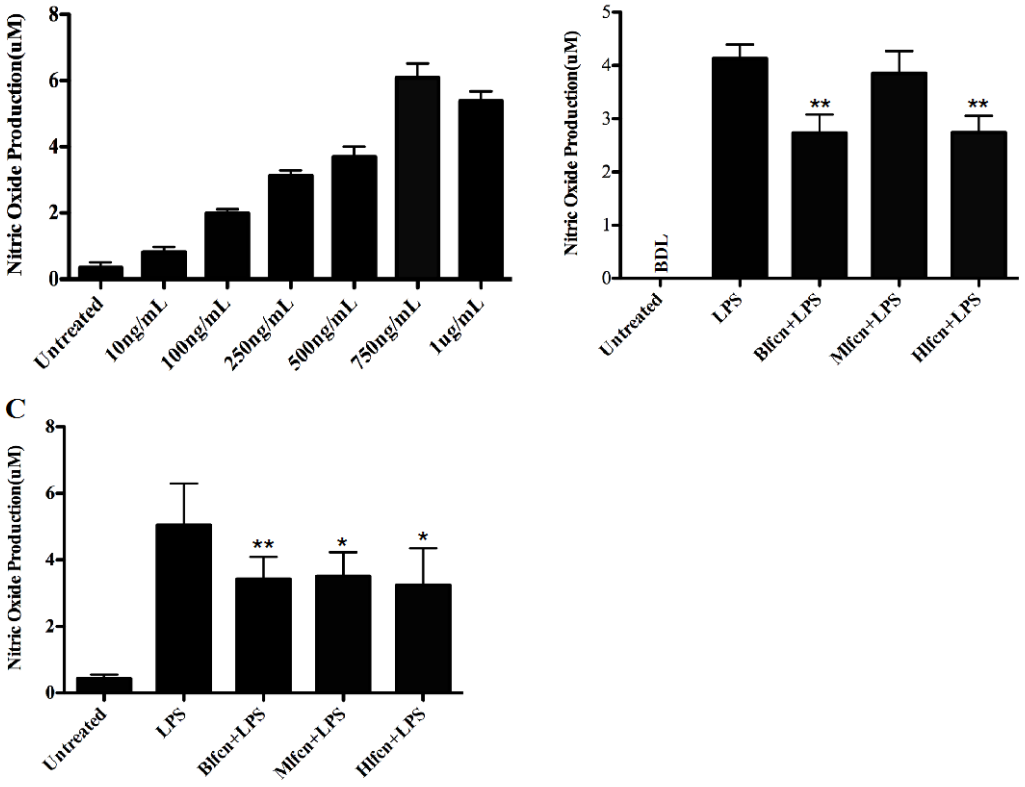

Nitric Oxide Production(uM)
8
6
4
2
0
Untreated
10ng/mL
100ng/mL
250ng/mL
500ng/mL
750ng/mL
1ug/mL
Nitric Oxide Production(uM)
5
4
3
2
1
0
BDL
**
**
Untreated
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS
C
Nitric Oxide Production(uM)
8
6
4
2
0
**
*
*
Untreated
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS

Figure 3.7

3.8 Lactoferricin peptides decrease inducible nitric oxide synthase expression in LPS-stimulated macrophages (A) RAW 264.7 cells and **(B)** BMDMs were treated with 500 ng/ml of LPS alone or in combination with 5 μM Blfcn, Mlfcn, or Hlfcn for 4 h. RNA was isolated for cDNA synthesis, and q-PCR was performed to determine levels of iNOS expression. Data are expressed as a relative value of LPS induced iNOS expression. Data are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ and ** $p < 0.01$ compared to LPS positive control as determined by ANOVA with Dunnett's multiple comparisons post-test. a.u. denotes arbitrary units.

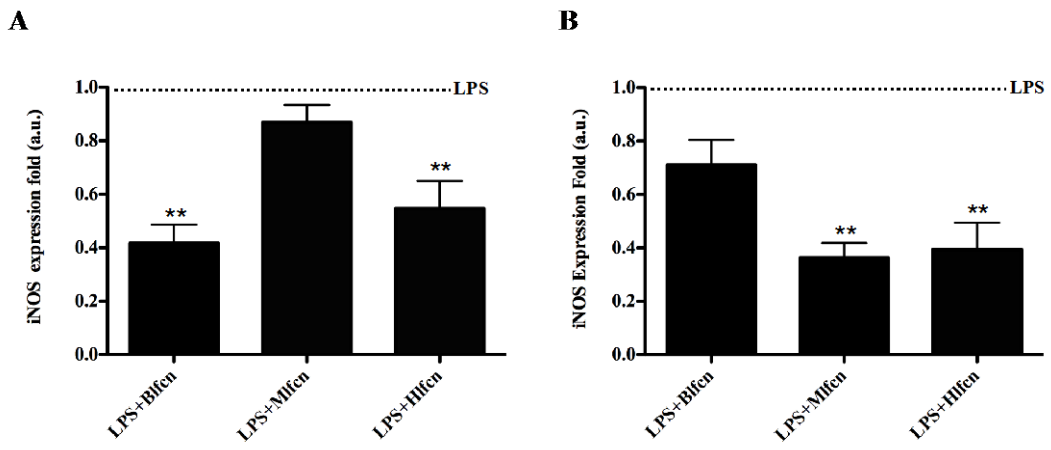
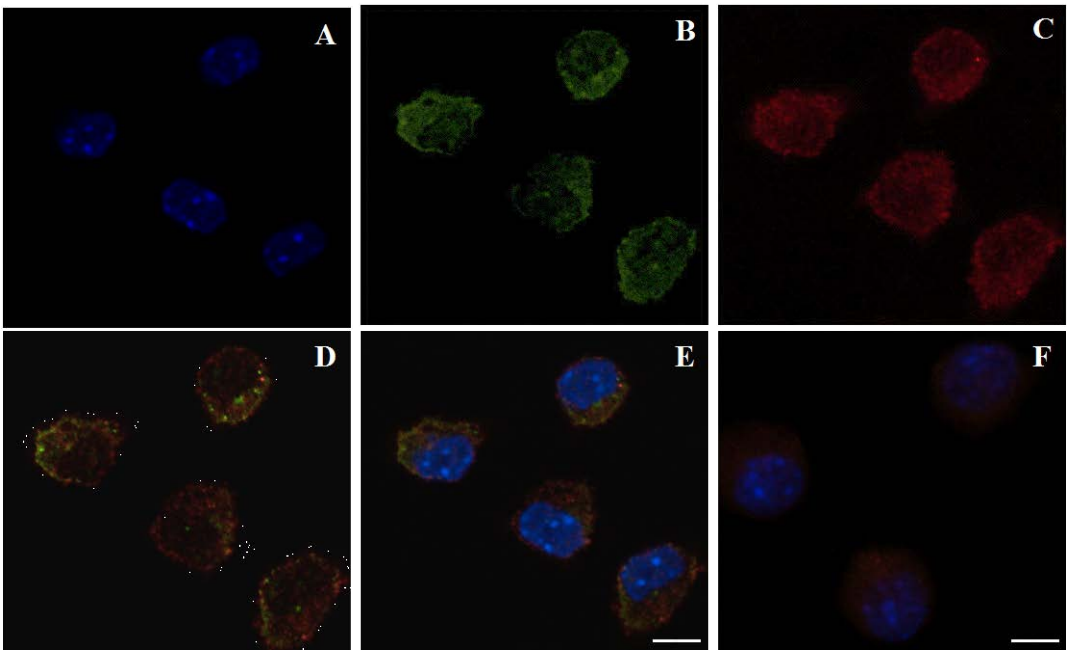

A
B
iNOS expression fold (a.u.)
iNOS Expression Fold (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
LPS
**
LPS+Blfcn
LPS+Mlfcn
LPS+Hlfcn

Figure 3.8

3.9 Biotinylated bovine lactoferricin enters the cell through an endosomal-independent pathway (A) RAW 264.7 cells were stained with 30 µM DAPI **(B)** treated with 5 µM BL-Blfcn for 30 min and incubated with strepavidin-conjugated Alexa Fluor 488 and **(C)** with an anti-EEA-1 antibody and anti-mouse antibody conjugated to Alexa Fluor 555. **(D)** Overlay of **B** and **C. (E)** Overlay of **A, B,** and **C. (F)** RAW 264.7 cells were treated and incubated as in **A-D,** but incubated with blocking and antibody dilution buffer contained no Triton-X. Scale bar represents 10 µm. **(G)** RAW 264.7 cells incubated with 500 ng/ml LPS alone, LPS and 5 µM Blfcn, or LPS and 5µM BL-Blfcn for 24 h. Supernatants were collected and used in a Griess assay to detect nitric oxide production. * denotes $p < 0.05$ compared with LPS treatment as determined by ANOVA and Dunnett's multiple comparisons post-test.


A
B
C
D
E
F

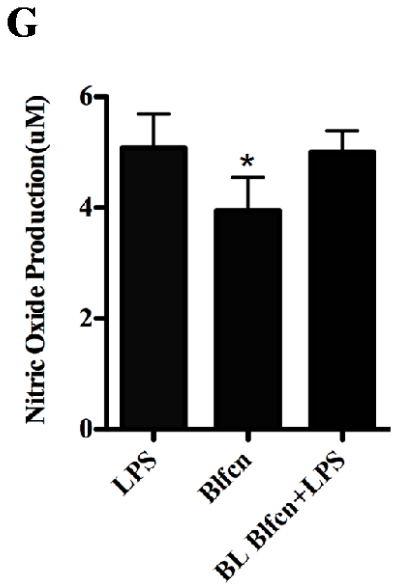

G
Nitric Oxide Production(uM)
6
4
2
0
*
LPS
Blfcn
BL Blfcn+LPS

Figure 3.9

3.10 Lactoferricin peptides reduce levels of phosphorylated IκBα in LPS-stimulated macrophages (A) RAW 264.7 cells were treated with the indicated concentrations of Blfcn and 100 ng/ml LPS for the indicated times. Cell lysates were collected and protein expression was determined by western blotting. Nitrocellulose membranes were probed with the indicated antibodies and corresponding secondary antibodies. Data shown are from one time-course experiment. **(B), (C)** RAW 264.7 and **(D), (E)** THP-1 cells were incubated with 100 ng/ml LPS, 5 μM Blfcn, Mlfcn, or Hlfcn, or a combination of LPS and one species-specific lactoferricin peptide as indicated. Cell lysates were collected after 1 h and used in western blotting as described in **(A)**. **(B)**, **(D)** One representative western blot and **(C)**, **(E)** the mean density of phosphorylated IκBα normalized to untreated controls and to total IκBα and β-actin from 4 (RAW 264.7 cells) or 3 (THP-1 cells) independent experiments ± SEM; ** denotes $p < 0.01$ and *** denotes $p < 0.001$ compared to LPS alone treatments as determined by ANOVA with Dunnett's multiple comparisons post-test. a.u. denotes arbitrary units.

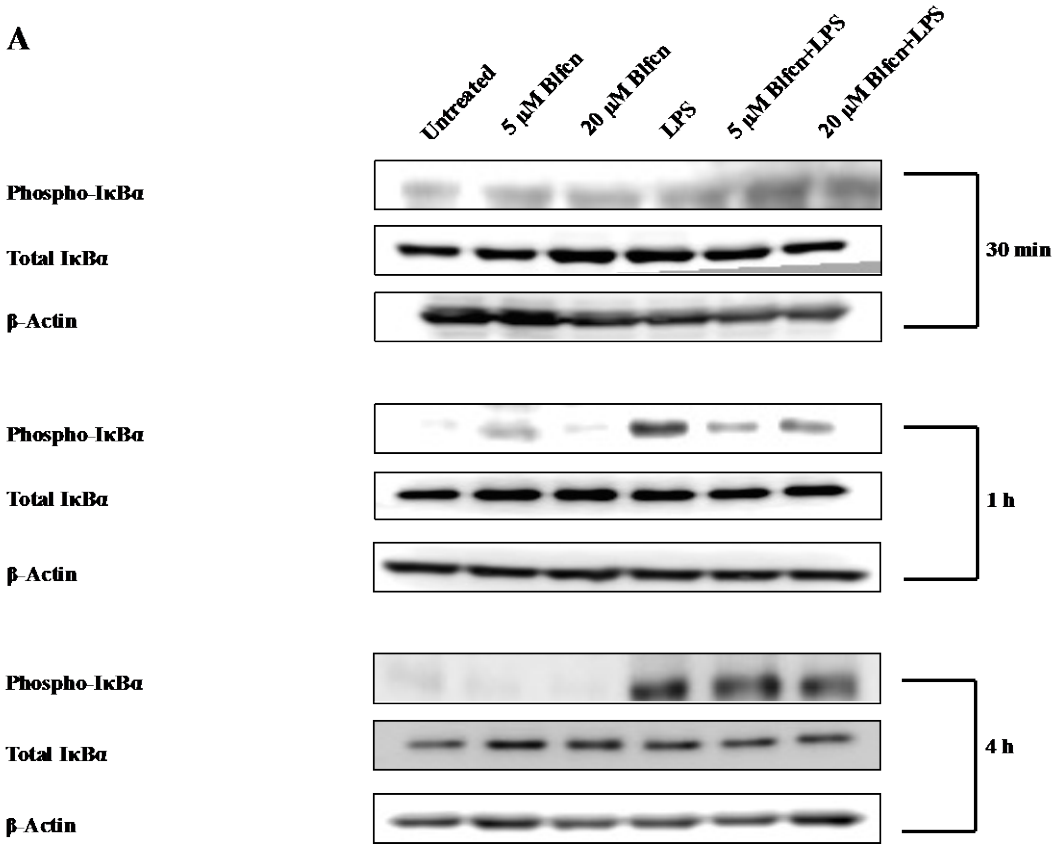

A
Untreated
5 μM Blfcn
20 μM Blfcn
LPS
5 μM Blfcn+LPS
20 μM Blfcn+LPS
Phospho-IκBα
Total IκBα
β-Actin
30 min
Phospho-IκBα
Total IκBα
β-Actin
1 h
Phospho-IκBα
Total IκBα
β-Actin
4 h

Figure 3.10

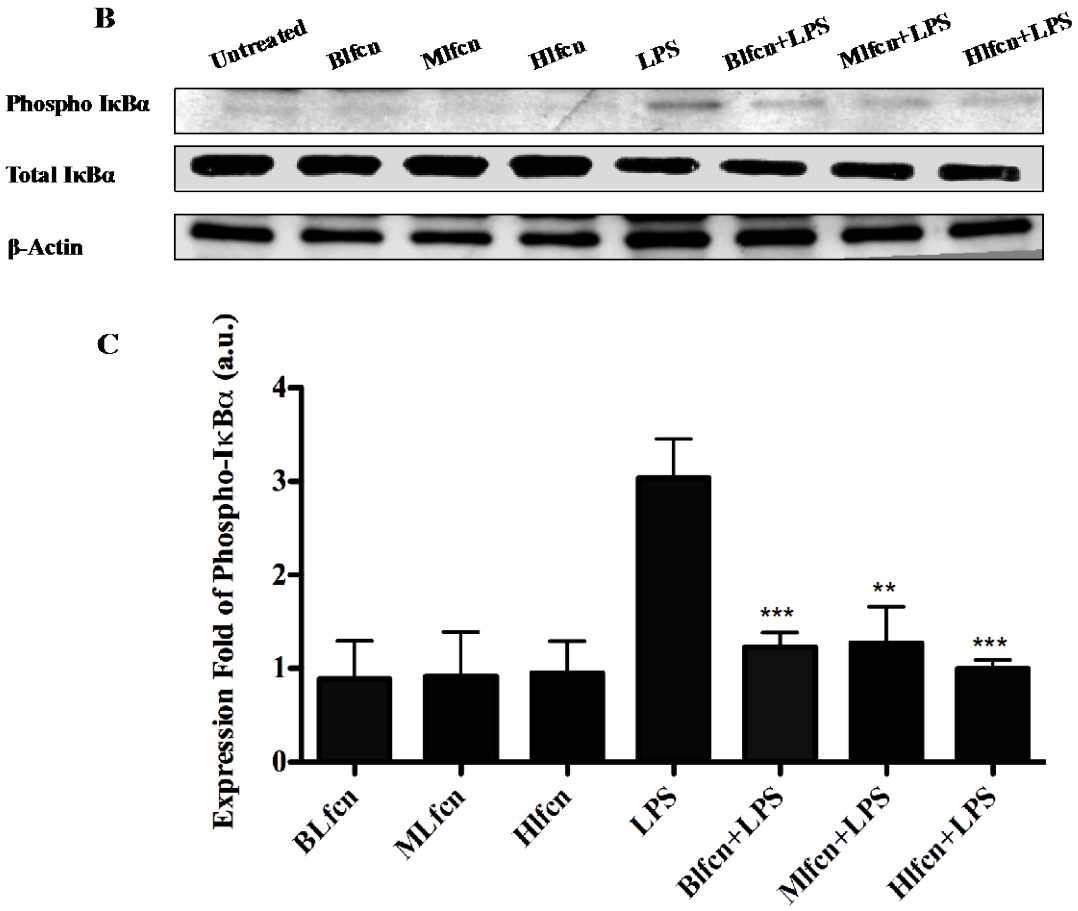


Figure 3.10 (continued)

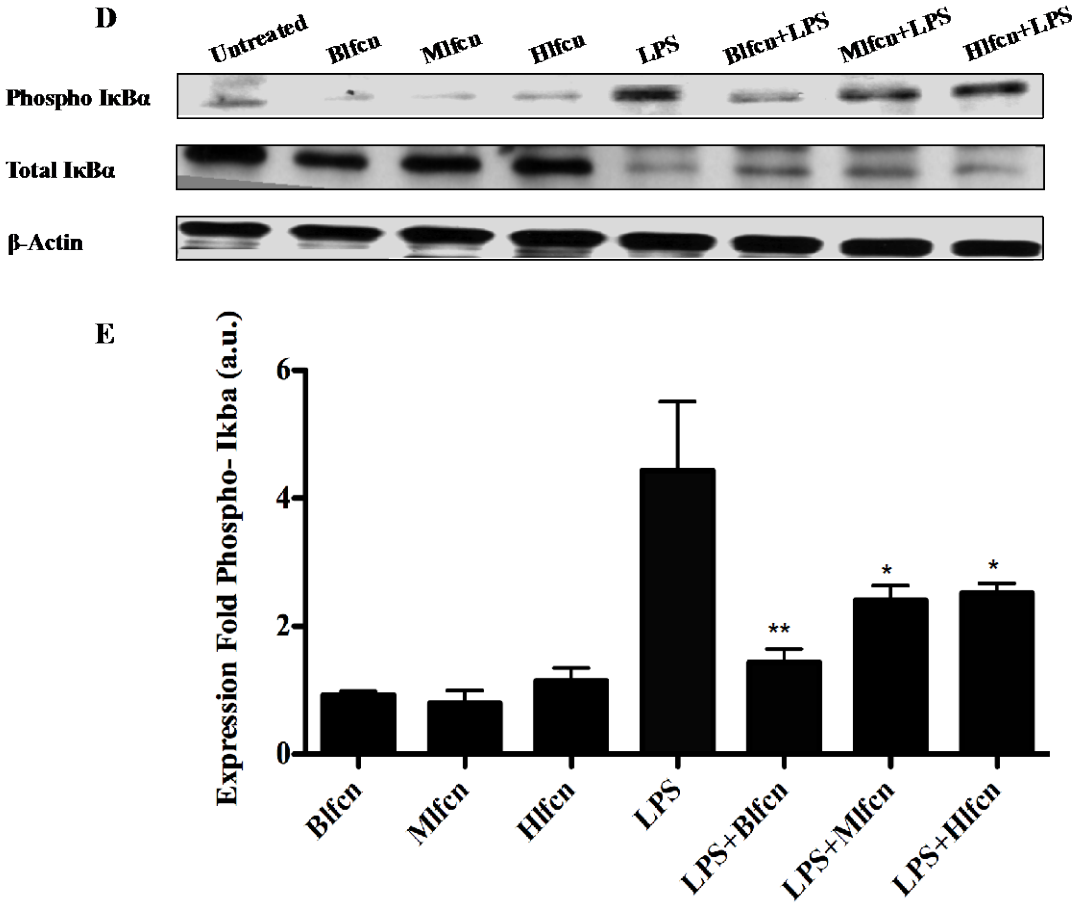


Figure 3.10 (continued)

3.11 Lactoferricin peptides inhibit the nuclear translocation of NF-κB in LPS-stimulated macrophages (A) RAW 264.7 cells and **(B)** THP-1 cells were treated with 100 ng/ml LPS alone or in combination with 5 μM Blfcn, Mlfcn, or Hlfcn for 1 h. Cells were fixed and incubated with nuclear stain, DAPI (30 μM), and rabbit anti-p65 antibody with secondary goat-anti-rabbit Alexa Fluor 488 ® conjugate, then imaged using fluorescent microscopy. Images are representative of two independent experiments. Scale bar represents 40 μm. **(C)** RAW 264.7 and **(D)** THP-1 cells were treated as in **(A)** and **(B)**, cell lysates were collected and protein expression was determined using western blots. Nitrocellulose membranes were probed with antibodies against total p65 and β-actin, and appropriate secondary antibodies.

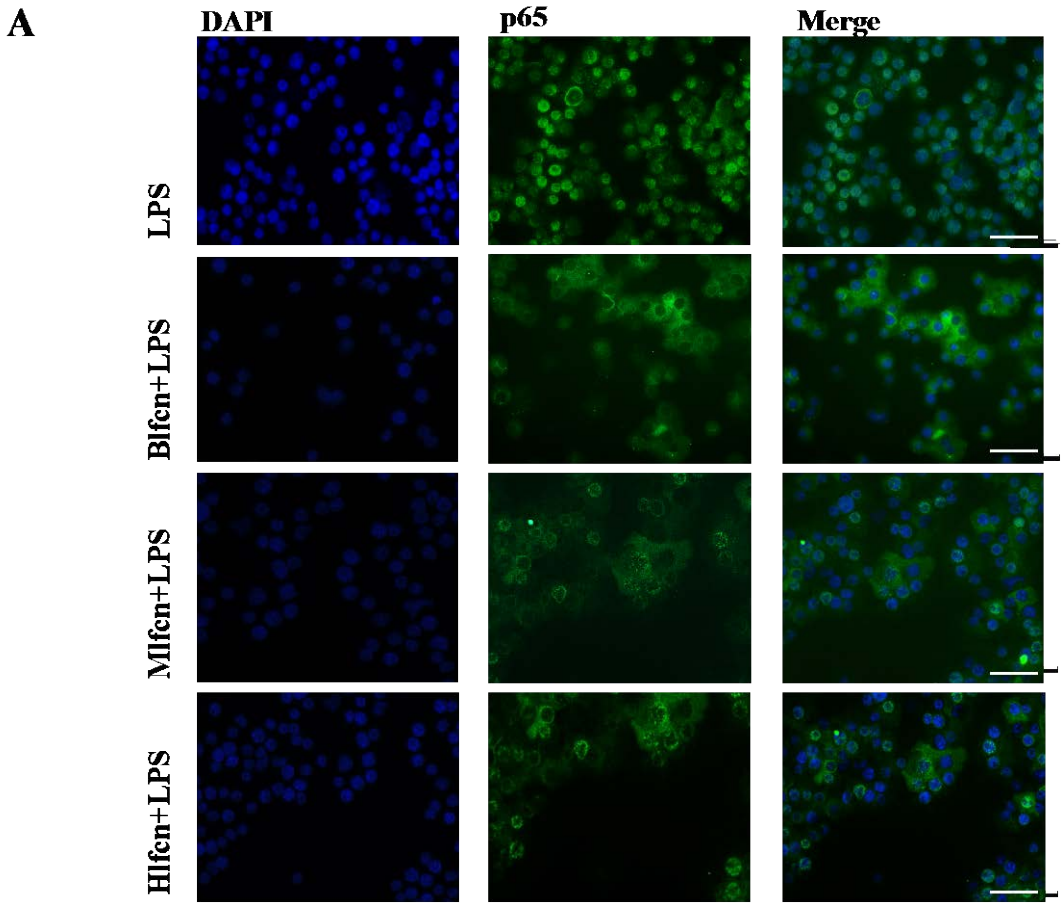


Figure 3.11

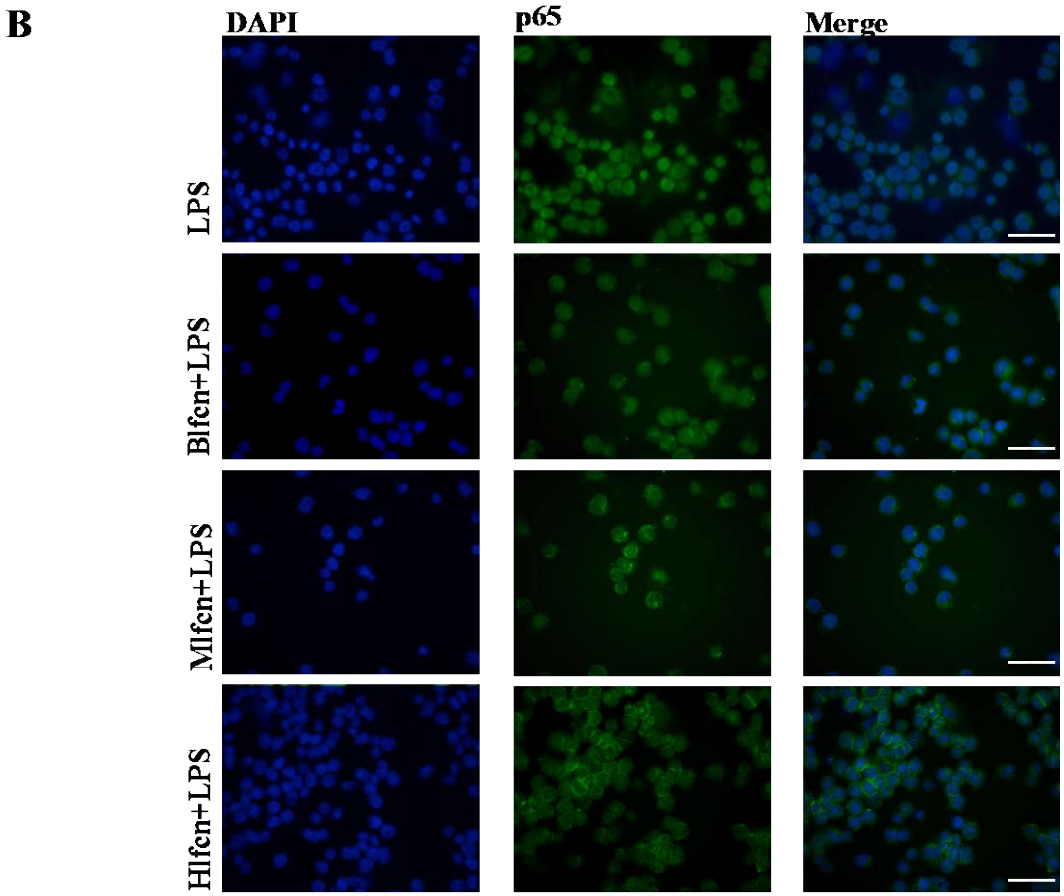


Figure 3.11 (continued)

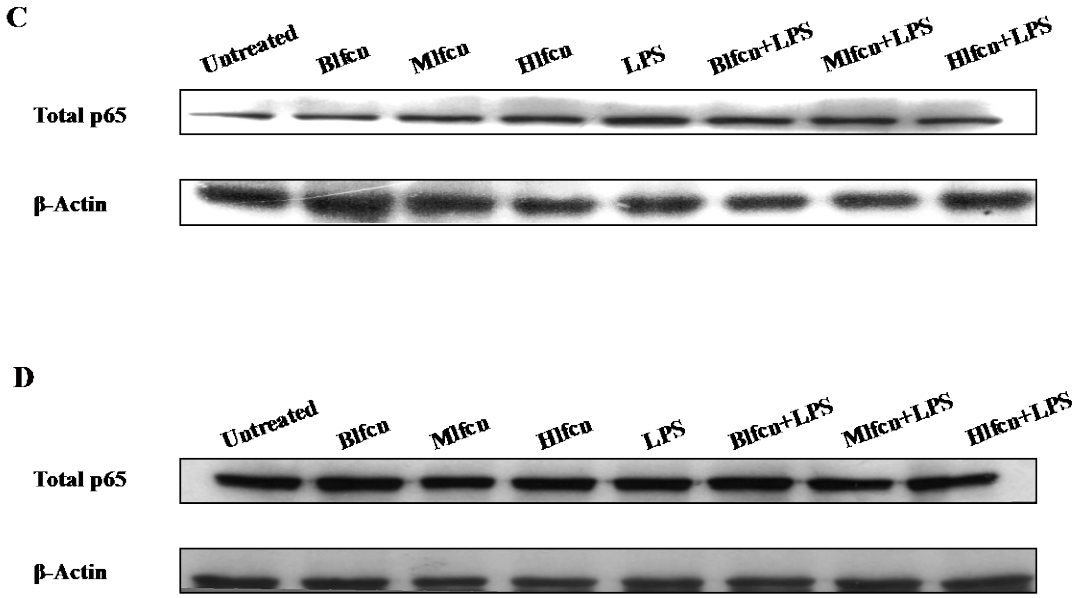


Figure 3.11 (continued)

Fig. 3.12 Bovine lactoferricin does not target the LPS-activated β-catenin signaling pathway. Nuclear localization of β-catenin was observed using fluorescent microscopy. RAW 264.7 cells were treated with 100 ng/ml LPS alone or in combination with the indicated concentrations of Blfcn for 1 h. Cells were fixed and incubated with nuclear stain, DAPI (30 μM), and rabbit anti-β-catenin antibody with secondary goat-anti-rabbit Alexa Fluor 488 ® conjugate and imaged. Images are representative of two independent experiments. Scale bar represents 30 μm.

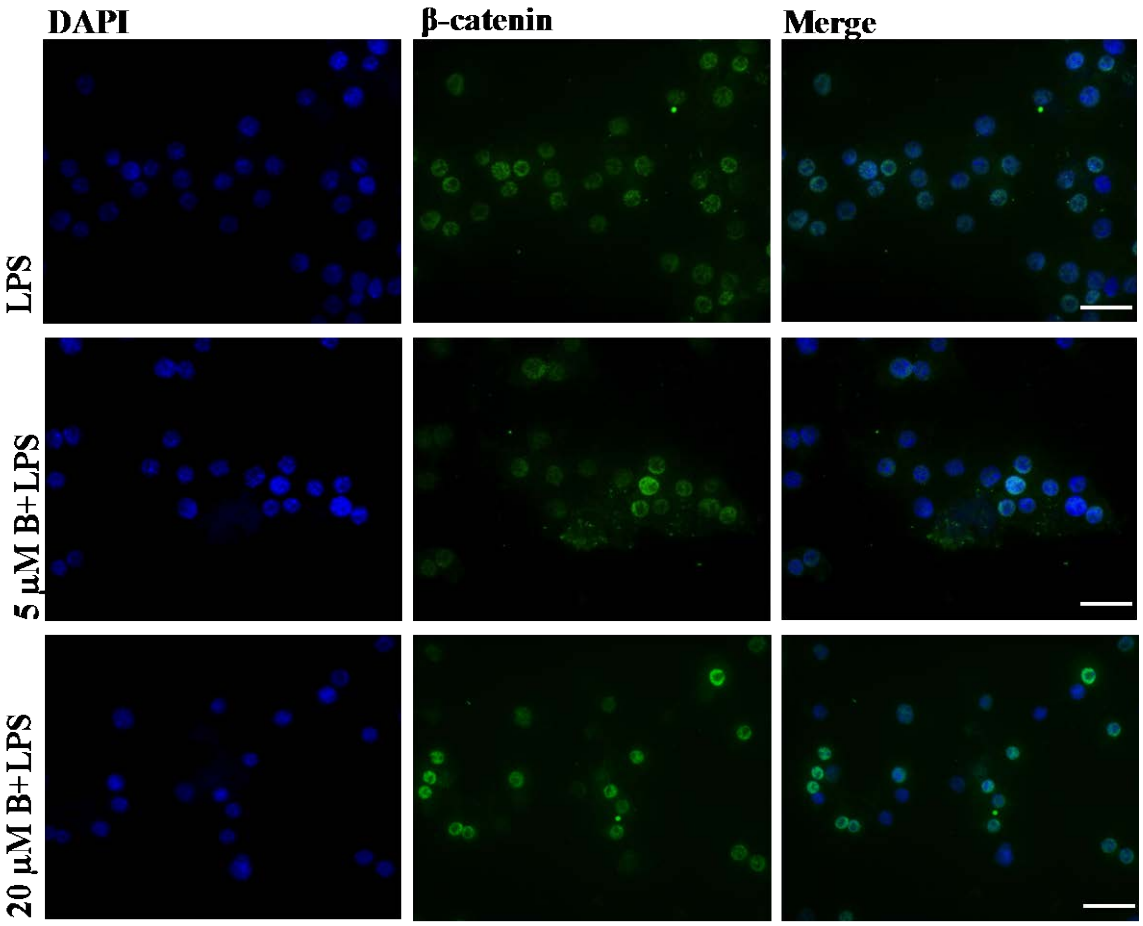


Figure 3.12

3.13 Lactoferricin peptides reduce ERK phosphorylation in LPS-stimulated macrophages. (A), (B) RAW 264.7 and **(C), (D)** THP-1 cells were incubated with 100 ng/ml LPS, 5 μM Blfcn, Mlfcn, or Hlfcn, or a combination of LPS and one peptide as indicated. Cell lysates were collected and was protein expression was determined using western blotting. Nitrocellulose membranes were probed with the indicated antibodies and the appropriate secondary antibodies. **(A), (C)** Data shown are one representative western blot. **(B)**, **(D)** The mean density of phosphorylated ERK 1/2 normalized to untreated controls and to total ERK 1/2 and β-actin from 3 independent experiments ± SEM; * denotes $p < 0.05$ compared to LPS alone treatments as determined by ANOVA with Dunnett's multiple comparisons post-test. a.u. denotes arbitrary units.

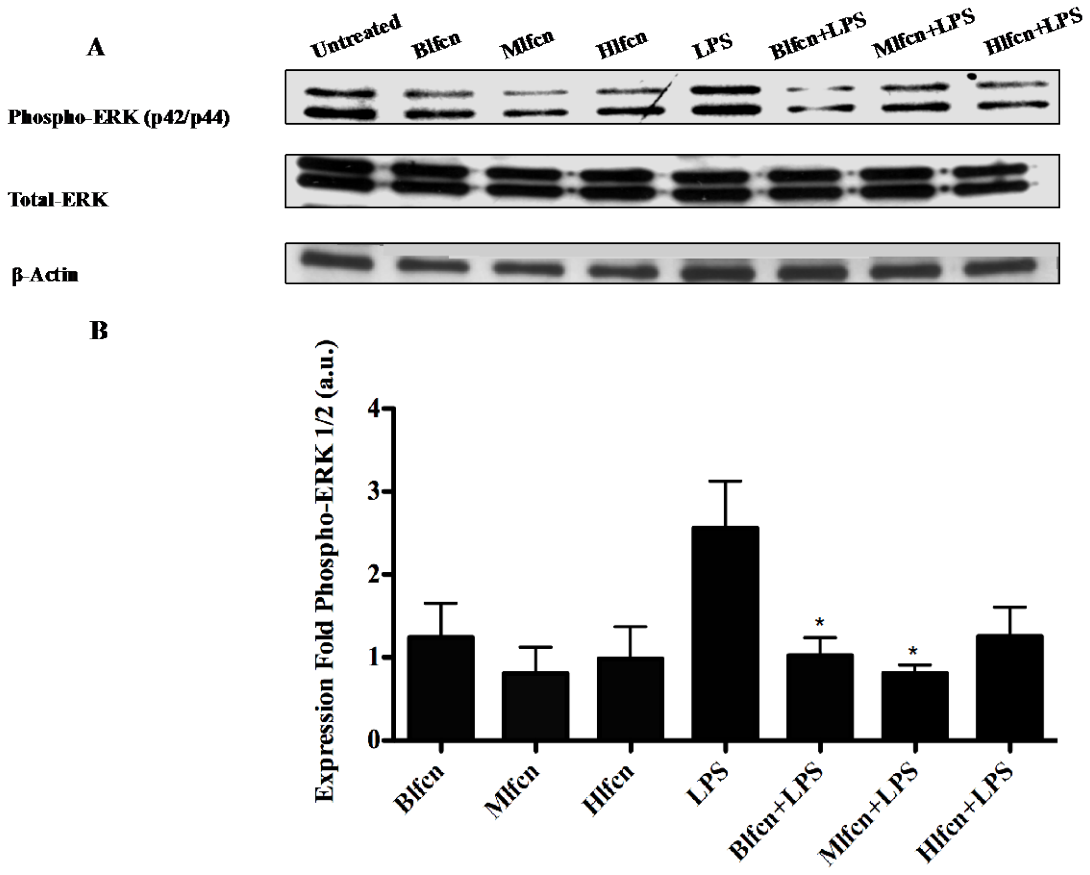

A
Untreated
Blfcn
Mlfcn
Hlfcn
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS
Phospho-ERK (p42/p44)
Total-ERK
β-Actin
B
Expression Fold Phospho-ERK 1/2 (a.u.)
0
1
2
3
4
*
*
Blfcn
Mlfcn
Hlfcn
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS

Figure 3.13

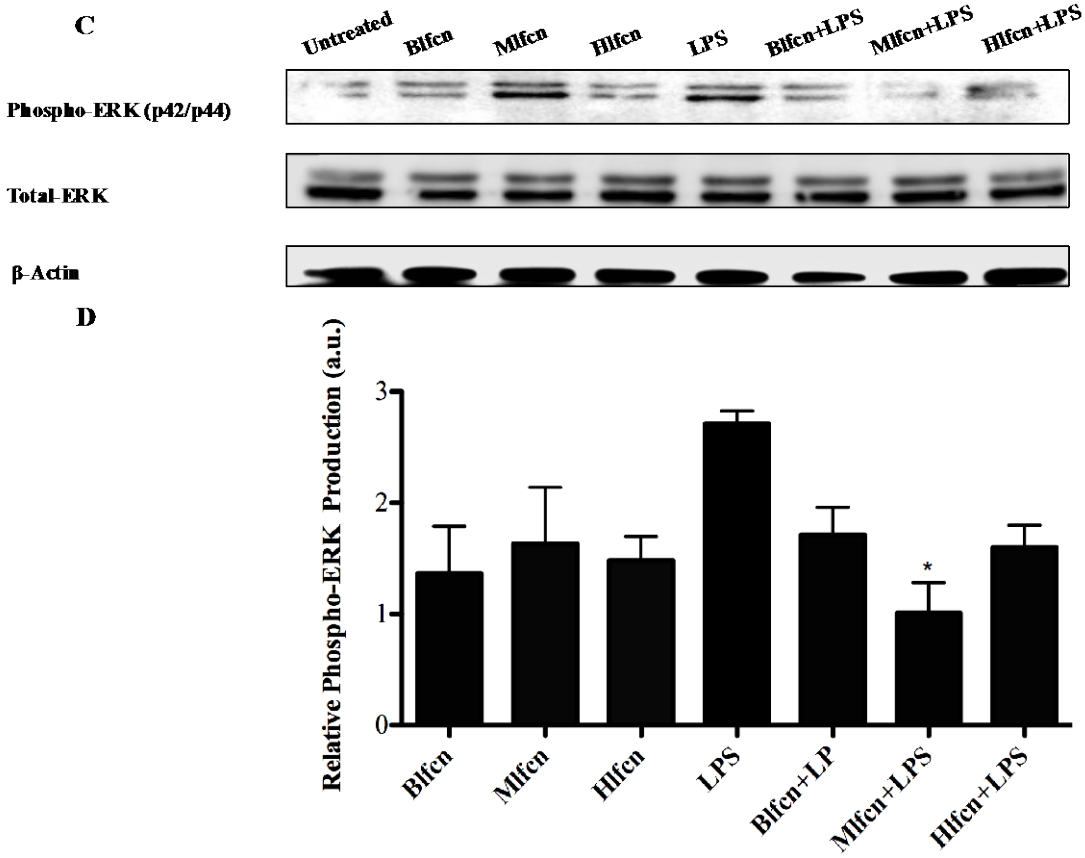


Figure 3.13 (continued)

3.14 Lactoferricin peptides inhibit c-Jun phosphorylation in LPS-stimulated macrophages (A), (B) RAW 264.7 and **(C), (D)** THP-1 cells were incubated with 100 ng/ml LPS, 5 μM Blfcn, Mlfcn, or Hlfcn, or a combination of LPS and one species-specific lactoferricin peptide as indicated. Cell lysates were collected and protein expression was determined using western blotting. Nitrocellulose membranes were probed with the indicated antibodies and the appropriate secondary antibodies. **(A), (C)** Data shown are from one representative western blot. **(B)**, **(D)** The mean density of phosphorylated c-Jun normalized to untreated controls and to total c-Jun and β-actin from 4 (RAW 264.7 cells) or 3 (THP-1 cells) independent experiments ± SEM. a.u. denotes arbitrary units.

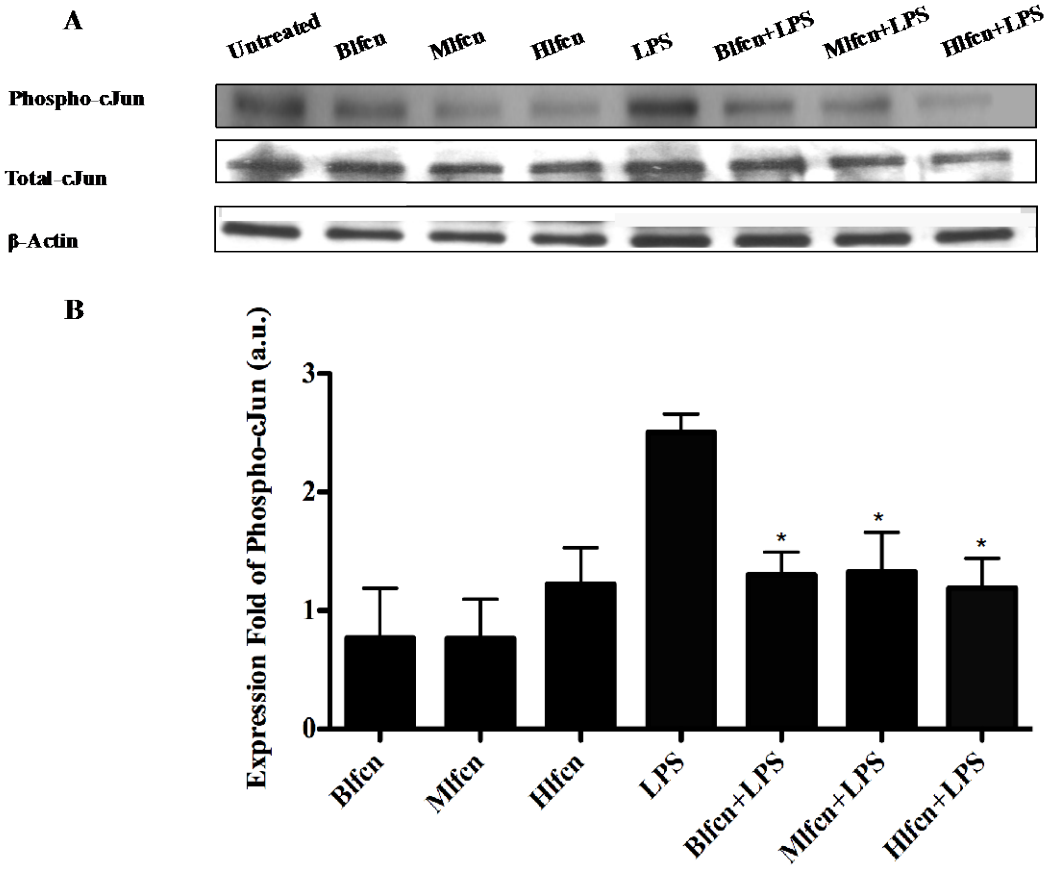

A
Untreated
Blfcn
Mlfcn
Hlfcn
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS
Phospho-cJun
Total-cJun
β-Actin
B
Expression Fold of Phospho-cJun (a.u.)
0
1
2
3
*
*
*
Blfcn
Mlfcn
Hlfcn
LPS
Blfcn+LPS
Mlfcn+LPS
Hlfcn+LPS

Figure 3.14

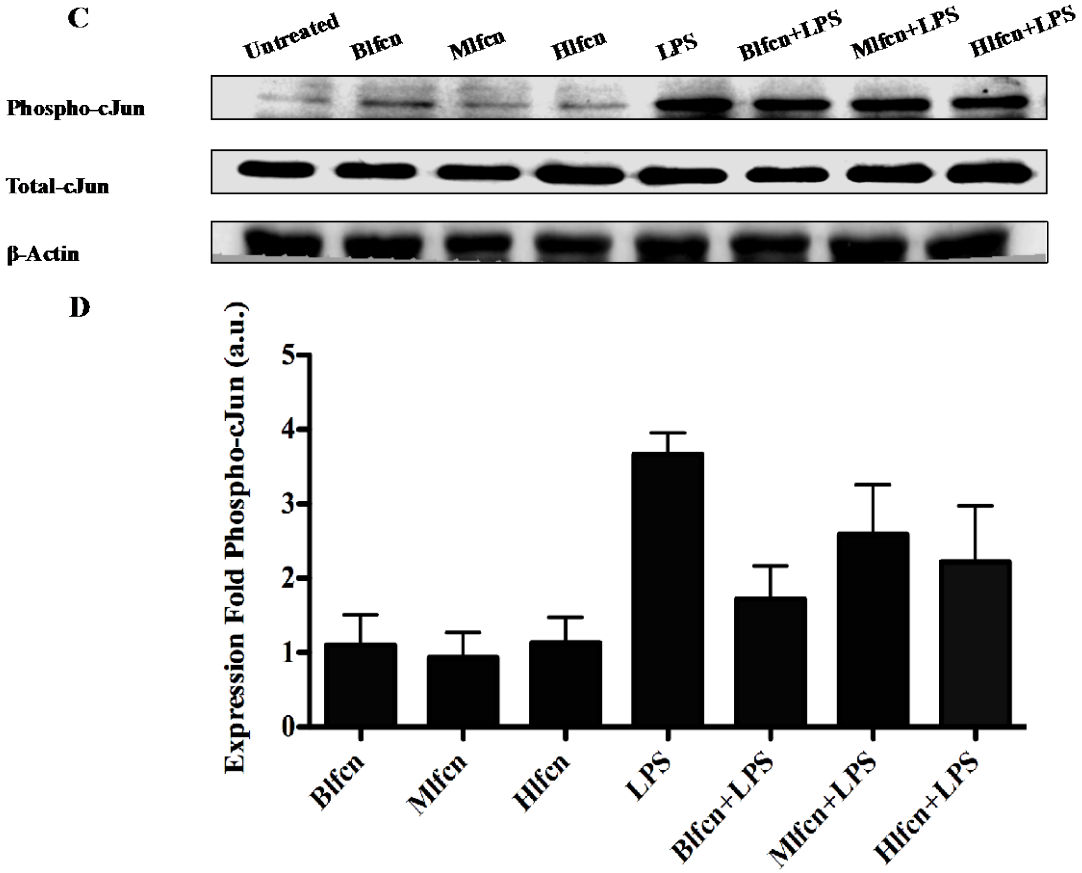


Figure 3.14 (continued)

3.15 Lactoferricin peptides promote p38 MAPK phosphorylation in macrophages. (A), (B) RAW 264.7 and **(C), (D)** THP-1 cells were incubated with 5 μM Blfcn, Mlfcn, or Hlfcn for 1 h. Cell lysates were collected and protein expression was determined using western blotting. Nitrocellulose membranes were probed with the indicated antibodies and the appropriate secondary antibodies. **(A), (C)** Data shown are from one representative blot. **(B)**, **(D)** The mean density of phosphorylated p38 MAPK normalized to untreated controls and to total p38 MAPK and β-actin from 4 independent experiments ± SEM; * denotes $p < 0.05$, and ** denotes $p < 0.01$ compared to LPS alone treatments as determined by ANOVA with Dunnett's multiple comparisons post-test. a.u. denotes arbitrary units.

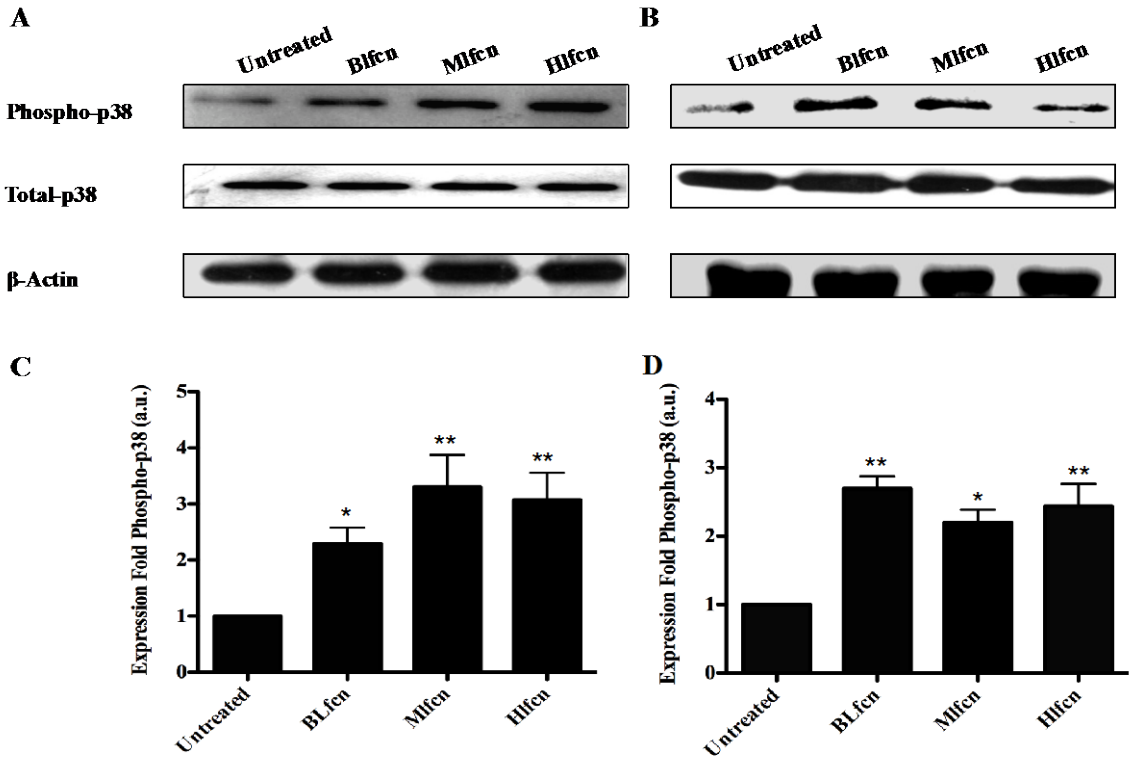

A
Untreated
Blfcn
Mlfcn
Hlfcn
Phospho-p38
Total-p38
β-Actin
B
Untreated
Blfcn
Mlfcn
Hlfcn
C
Expression Fold Phospho-p38 (a.u.)
0
1
2
3
4
5
*
**
**
Untreated
BLfcn
Mlfcn
Hlfcn
D
Expression Fold Phospho-p38 (a.u.)
0
1
2
3
4
**
*
**
Untreated
BLfcn
Mlfcn
Hlfcn

Figure 3.15

3.16 Lactoferricin peptides do not upregulate IL-10 via p38 MAPK activation. RAW 264.7 cells were incubated with 20 μM p38 MAPK inhibitor, SB203528, for 1 h and then treated with 5 μM Blfcn, Mlfcn, or Hlfcn or 100 ng/ml LPS as a positive control for 24 h. Supernatants were collected and an ELISA was used to determine IL-10 production. Data shown are the mean ± SEM from 3 independent experiments. ANOVA with a Tukey multiple comparisons post-test showed no significance.

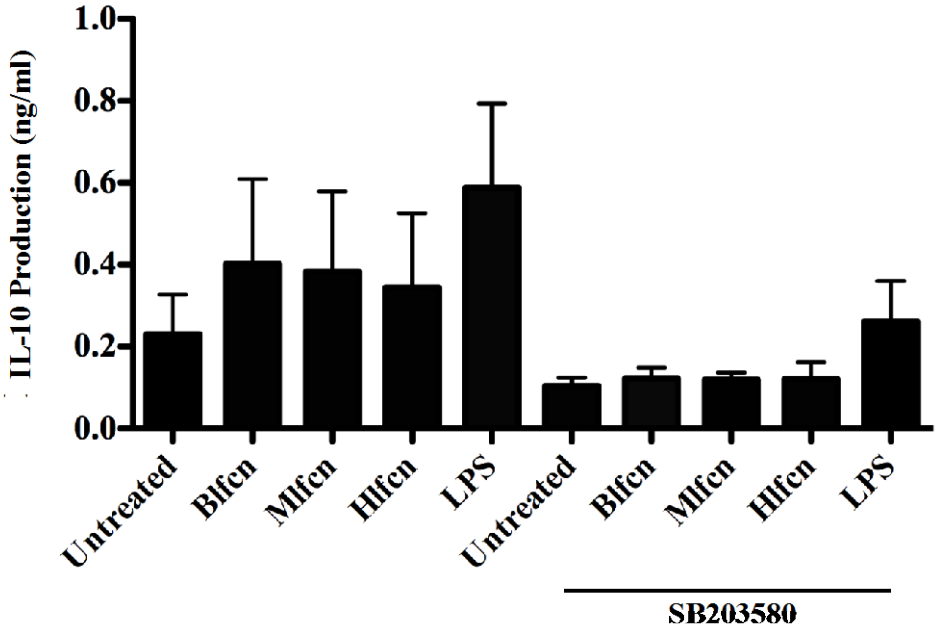

IL-10 Production (ng/ml)
1.0
0.8
0.6
0.4
0.2
0.0
Untreated
Blfcn
Mlfcn
Hlfcn
LPS
Untreated
Blfcn
Mlfcn
Hlfcn
LPS
SB203580

Figure 3.16

3.17 Lactoferricin peptides decrease cadmium-induced TNF-α production in macrophages. (A) THP-1 cells were treated with increasing concentrations of $CdCl_2$ for 24 h, supernatants were collected and ELISA was used to determine levels of TNF-α production. **(B)** THP-1 cells and **(C)** RAW 264.7 cells were treated with 10 μM $CdCl_2$ alone or in combination with 5 μM Blfcn, Mlfcn, or Hlfcn, for 24 h. Supernatants were collected and ELISA was used to determine relative levels of TNF-α production. **(D)** RAW 264.7 cells were treated as in **(C)** for 4 h. RNA was isolated, cDNA was synthesized and q-PCR was performed to determine relative levels of TNF-α expression normalized to untreated control. Data show are the mean of 2 **(A)** 3 **(B** and **D)** or 5 **(C)** independent experiments ± SEM. ELISA data was normalized to untreated controls: 108 pg/ml and 118 pg/ml, respectively; * denotes $p < 0.05$, ** denotes $p < 0.01$ compared to LPS control as determined by ANOVA with the Dunnett's multiple comparisons post-test. a.u. denotes arbitrary units.

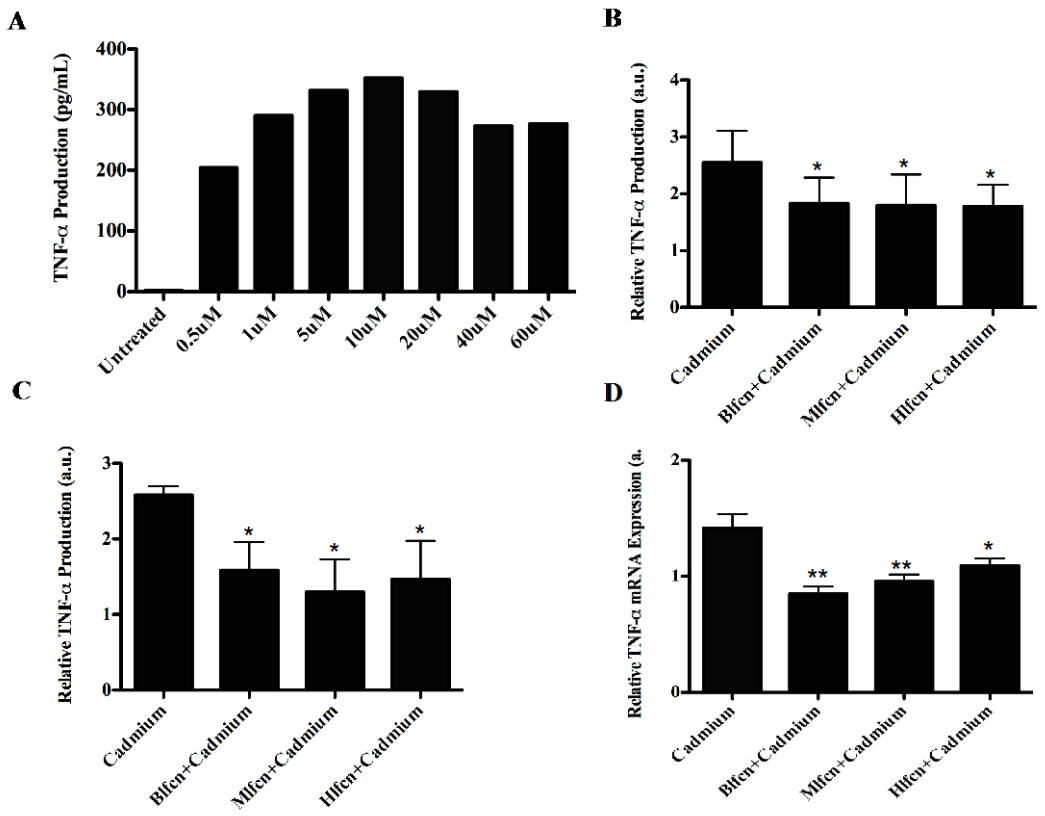

A
TNF-α Production (pg/mL)
400
300
200
100
0
Untreated
0.5uM
1uM
5uM
10uM
20uM
40uM
60uM
B
Relative TNF-α Production (a.u.)
4
3
2
1
0
Cadmium
Blfcn+Cadmium
Mlfcn+Cadmium
Hlfcn+Cadmium
C
Relative TNF-α Production (a.u.)
3
2
1
0
Cadmium
Blfcn+Cadmium
Mlfcn+Cadmium
Hlfcn+Cadmium
D
Relative TNF-α mRNA Expression (a.
2
1
0
Cadmium
Blfcn+Cadmium
Mlfcn+Cadmium
Hlfcn+Cadmium

Figure 3.17

Table 3.1 The effects of varying concentrations of bovine lactoferricin on TNF-α and IL-6 cytokine production of LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were stimulated with LPS and simultaneously treated with the indicated concentrations of Blfcn. Supernatants were collected after 6 and 24 h and used in ELISA assays to detect TNF-α and IL-6 production, respectively. Table is representative of two independent experiments.

Treatment	**TNF-α (pg/ml)**	**IL-6 (pg/ml)**
Untreated	63	44
100ng/ml LPS	3120	4006
2.5 μM Blfcn+LPS	2925	3401
5 μM Blfcn+LPS	1853	3178
10 μM Blfcn+LPS	2125	3454

Table 3.2 Lactoferricin peptides do not affect TNF-α production in non-stimulated macrophages. RAW 264.7, THP-1, and BMDM cells were treated with 5 μM Blfcn, Mlfcn, or Hlfcn for 6 h. Supernatants were collected and used in ELISA assays to determine production of TNF-α (pg/ml). Table is representative of two independent experiments.

Treatment	**RAW 264.7**	**THP-1**	**BMDM**
Untreated	84	78	18
Blfcn	75	94	12
Mlfcn	91	80	10
Hlfcn	111	96	9

Table 3.3 Lactoferricin peptides do not affect IL-6 production in non-stimulated macrophages. RAW 264.7, THP-1, and BMDM cells were treated with 5 μM Blfcn, Mlfcn, or Hlfcn for 24 h. Supernatants were collected and used in ELISA assays to determine production of IL-6 (pg/ml). Table is representative of two independent experiments.

Treatment	**RAW 264.7**	**THP-1**	**BMDM**
Untreated	25	107	91
Blfcn	29	96	111
Mlfcn	21	82	109
Hlfcn	20	95	107

CHAPTER 4

DISCUSSION

AMPs are an evolutionary conserved class of innate effector molecules. Traditionally, studies have focused on the direct antimicrobial properties of these cationic peptides, but more recently the focus has shifted towards their direct immmunomodulatory features. Studies show the capacity of AMPs to have direct interactions with the immune system, facilitating a wide variety of processes including immune regulation and dampening, as well as enhancement, and activation. Previous studies of lactoferricin have mainly focused on the direct antimicrobial action of this AMP and its parent peptide, lactoferrin. A previous investigation compared the potency of lactoferricin derived from several species in terms of eliminating a pathogen (193), but no study has done a direct comparison of how different species-specific lactoferricin peptides influence inflammatory processes in macrophages. This is the first study to demonstrate the differential immunomodulatory efficacy of species-specific peptides and uncover several potential inflammatory pathways influenced by lactoferricin in macrophages.

4.1 Lactoferricin peptides are non-toxic to macrophages

In order to appropriately observe the immunomodulatory effects of lactoferricin peptides in macrophages, Blfcn, Mlfcn, and Hlfcn had to be administered in sub-cytotoxic doses to ensure any influence on inflammatory mediator production was not due to a reduction in viable cells. MTT and trypan blue assays showed no loss in cell viability in RAW 264.7 cells, THP-1 cells, or BMDMs treated with lactoferricin peptides at low concentrations (Figure 3.1 A-D). This is consistent with other studies that have investigated the cytotoxicity of AMPs for mammalian cells and showed that relatively low concentrations of AMPs are non-cytotoxic to mammalian macrophages (153, 278–283). AMPs elicit their antimicrobial effects through interactions with negatively charged components on a prokaryote membrane, such as LPS in Gram negative bacteria. Eukaryotic cells are far more resistant to lysis by AMPs owing to a relative lack of

negatively charged components on their membrane and the neutral charge of membrane phospholipids (153, 284, 285).

Given that low dose administration of lactoferricin peptides did not affect the viability of RAW 264.7 cells, THP-1 cells, or BMDMs, and previous studies of bovine lactoferricin used concentrations < 20 μM (235, 286–289), it was determined that 5 μM lactoferricin would be sufficient to study the effects these peptides on macrophage inflammatory processes. Although serum concentrations of lactoferrin vary based on an individual basis (250-900 ng/ml) and can be increased during an inflammatory event, the concentration of lactoferricin used in this study are approximately 10 fold above that of its parent peptide found naturally in the body (290). However, rats that ingested bovine lactoferrin, in the form of milk, were found to have levels of lactoferricin in the small intestine that were as high as 20 μM, indicating that concentrations used in this study were physiologically relevant (291).

4.2 Lactoferricin peptides decrease pro-inflammatory mediator production by LPS-stimulated macrophages

A previous study demonstrates the ability of both bovine lactoferricin and lactoferrin to downregulate IL-6 production in THP-1 monocytes (201). However, the present investigation is the first study to explore the influence of lactoferricin from three different species on macrophage-associated inflammation.

Given that TNF-α is a prototypical pro-inflammatory cytokine released at the onset of inflammation, the production of this cytokine was evaluated in lactoferricin-treated macrophages. The resulting data suggested that human macrophages are more susceptible to the anti-inflammatory effects of lactoferricin from all three species (Figure 3.2 B). Blfcn decreased TNF-α production in mouse BMDMs and RAW 264.7 cells, but Mlfcn and Hlfcn had no significant effect on TNF-α production (Figure 3.2 A and C). In contrast, there was decreased LPS-induced TNF-α production by human THP-1 cells treated with all three peptides. A similar effect was seen on the production of IL-6 by LPS-stimulated macrophages (Figure 3.3 A-C). Blfcn consistently downregulated pro-inflammatory cytokine production in all cells tested.

Supporting the observations with pro-inflammatory cytokines, lactoferricin was also able to downregulate expression of TNF-α and IL-6 mRNA in macrophages (Figure 3.4 and 3.5). As before, Blfcn exhibited the most potent anti-inflammatory effect, suppressing LPS-induced mRNA expression in murine BMDMs and RAW 264.7 macrophages as well as human THP-1 macrophages. The TNF-α mRNA expression profile mirrored that of cytokine production in THP-1 cells; however, this was not the case for RAW 264.7 cells. Possible reasons for this discrepancy include regulation of LPS-induced TNF-α on multiple levels (post-transcriptional or translational modifications), a transient effect on mRNA synthesis with lactoferricin administration, or inaccurate correlation between mRNA expression and protein production (292). A time course experiment of TNF-α cytokine and mRNA expression would help to determine the full effects of lactoferricin peptides, and to what extent the effects are transient.

NO is another important pro-inflammatory mediator produced by LPS-stimulated macrophages (46). As with pro-inflammatory cytokine production, lactoferricin peptides demonstrated a decrease in LPS-induced NO production in RAW 264.7 cells and BMDMs, with Blfcn and Hlfcn having the greatest inhibitory effect (Figure 3.7). Expression profiles of iNOS, the enzyme that synthesizes NO from the conversion of arginine to citrulline in the cytoplasm (46), mirrored that of NO production in RAW 264.7 cells and BMDMs (Figure 3.8). NO production in THP-1 cells was not investigated as previous studies have shown that these cells do not produce detectable levels of NO when stimulated with LPS or other microbial products (293, 294).

THP-1 cells require PMA stimulation to initiate monocyte to macrophage differentiation. PMA belongs to a group of phorbol ester organic compounds that activate the protein kinase C (PKC) pathway, allowing for growth and differentiation of THP-1 monocytes to a macrophage-like phenotype (295, 296). PMA treatment induces increased expression of surface markers CD11b and CD14, increased phagocytosis, and decreased proliferation (297). Studies also show that PMA causes upregulation of TLR receptors, increased sensitivity to LPS treatments, and promotion of TNF-α, IL-1β, and ROS production (297–300). A study by Carter *et al*. (301) also highlights the importance of ERK-1/2 and JNK signaling in PMA-treated THP-1 monocytes to prime these cells for LPS stimulation. It is important to note that the use of PMA in these experiments, given

its role as an inflammatory stimulant, may have interfered with some of the signaling pathways targeted by LPS and peptide treatments.

There are several possible reasons the differential effects of lactoferricin peptides from different species on pro-inflammatory mediator production by macrophages. As seen in Table 1.1, the amino acid composition between lactoferricin peptides from bovine, murine, and human sources contain subtle differences. The functional diversity that exists between each of these peptides could lie in their different amino acid compositions. Although no studies have directly looked at the structure-function between these three particular peptides in an immunomodulatory context, several investigations highlight the importance of peptide structure in host defense and antimicrobial effects of AMPs (302–304). For example, several studies demonstrate the importance of tryptophan (Trp) and arginine (Arg) residues in the antimicrobial and membrane penetrating capabilities of small AMPs (305–308). The relatively bulky side chain of Trp residues are also important for the antimicrobial properties of Blfcn, as substitution of this residue with alanine (Ala) resulted in decreased antimicrobial activity (309, 310). Arg and Trp are common among similarly structured membrane-penetrating proteins, as they allow for AMPs to intercalate into phospholipid membrane of host cells (311). A study of human cathelicidin LL-37 shows that substitution of the Trp residues of the peptide with phenylalanine (Phe) greatly inhibits the ability of this peptide to interact with zwitterionic phospholipids (306). The highly hydrophobic Trp amino acid has a high affinity for the membrane interface, suggesting the importance for this amino acid in entering into the heavily hydrophobic membrane interspace (306). Another investigation also shows that among the three lactoferricin peptides investigated in the current study, Blfcn has the greatest antimicrobial activity (193). This could be attributed to the two Trp residues within Blfcn in comparison to Mlfcn and Hlfcn, which each only contain one Trp residue.

Eukaryotic cells are composed of zwitterionic phospholipids on the outer membrane leaflet, which give the membrane a neutral charge. Studies show that Arg residues are able to interact with these zwitterionic phospholipids while other positively charged amino acid residues, such as lysine (also present in lactoferricin), interact exclusively with negatively charged molecules such as those on microbial membranes (312). Blfcn contains 5 Arg residues, Mlfcn contains 2 Arg residues, and Hlfcn contains 4

Arg residues. The variant composition of these cationic peptides may alter their ability to interact with the plasma membrane of mammalian cells. In a recent study of amphibian-derived temporin AMP, structural analogs of this peptide were synthesized with the addition of Trp and Arg residues in place of non-polar, neutral amino acids such as glycine. Analogs with increased Trp and Arg content have a greater capacity to decrease LPS-induced iNOS expression and TNF-α production in RAW 264.7 cells (307) Whether or not the presence of these amino acids or a substitution of these residues would also affect the anti-inflammatory properties of lactoferricin is yet to be determined; however, this could explain the more potent anti-inflammatory effect observed with Blfcn treatment. A simple experiment replacing neutral amino acids such as Ala or lysine with Arg or Trp in lactoferricin peptides would help to uncover whether these residues are also important for the anti-inflammatory effects lactoferricin has shown to elicit in macrophages.

4.3 Lactoferricin enters the cell to selectively target pro-inflammatory signaling

Once it was determined that lactoferricin decreased the production of pro-inflammatory mediators in macrophages, the next step was to determine whether this effect was mediated by extracellular action or through a mechanism involving cellular uptake of the peptide. Previous studies demonstrate the capacity of AMPs to translocate through the lipid-rich plasma membrane via both an endocytosis-dependent and independent process (308, 313, 314). Mechanisms of action of AMPs is dependent on cell type and the specific AMP that is being studied (152). A few studies demonstrate that macrophage and monocyte cell uptake of various AMPs is mediated by cell surface receptors or the passive translocation of peptide across the membrane, allowing AMPs to then interact with intracellular receptors (315–319). A study of the human cathelicidin, LL-37, shows that this peptide can translocate across the cellular membrane and bind with GAPDH to elicit downstream immunomodulatory effects in monocytes (318). A previous study exploring the cell penetrating mechanism of human lactoferrin reveals that this peptide enters THP-1 monocytes via clathrin-mediated endocytosis (320). Results from the current study suggest that lactoferricin is able to transverse the cell membrane in

an endosomal-independent manner as there was no co-localization between endosomal marker (EEA-1) and BL-Blfcn in RAW 264.7 cells (Figure 3.9). The parent peptide, lactoferrin, is a much larger molecule (703 amino acids) in comparison to its pepsin hydrolysate, lactoferricin (25 amino acids). A much larger peptide may not be able to passively diffuse through the plasma membrane and may require endocytosis in order to enter the cell, whereas smaller amphipathic peptides are able to passively cross cell membranes (264, 314, 321, 322).

A control experiment in which RAW 264.7 cells were incubated in buffers lacking Triton-X further demonstrated that BL-Blfcn is internalized within the cell and not bound to the cell surface. The lack of Triton-X in blocking and antibody dilution buffers leaves the cell membrane impermeable to streptavidin, thereby unable to bind internalized BL-Blfcn. It must be noted that a subsequent experiment revealed that, in contrast to native Blfcin, BL-Blfcn was unable to downregulate NO production in LPS-stimulated RAW 264.7 cells, suggesting that addition of a biotin label to a small cationic peptide may alter its immunomoculatory activity (Figure 3.9 G). It is therefore possible that biotinylation of lactoferricin inhibits the ability of this peptide to interact with LPS or signal transduction pathway mediators or decreases the affinity of peptide for its binding substrate, the identity of which is yet to be elucidated.

To determine how lactoferricin peptides might alter an inflammatory response once inside the cell, several well-known inflammatory pathways were investigated using western blot analysis. The most prominent of the inflammatory signaling pathways induced by LPS is the IκBα-NF-κB pathway (57). As mentioned previously, NF-κB is sequestered in the cytoplasm by inhibitory IκBα. Upon TLR-4 stimulation by LPS, through a series of signal transduction events, IκBα is phosphorylated and targeted for proteosomal degradation. NF-κB is liberated and able to translocate into the nucleus and bind to the promoter site of various genes that encode inflammatory mediators (52, 57). In this study, Blfcin transiently inhibited IκBα phosphorylation in LPS-stimulated macrophages, with the greatest suppressive effect seen by 1 h post-treatment (Figure 3.10 A). All subsequent experiments on cellular signaling were therefore performed after one hour of treatment with peptide. All three lactoferricin peptides significantly decreased phospho-IκBα expression in both LPS-stimulated RAW 264.7 and THP-1 cells (Figure

3.10 B-E). Peptide treatment in the absence of LPS stimulation also did not affect IκBα signaling. Previous studies of AMPs have also revealed similar downregulating effects on phospho-IκBα in macrophage cell lines (323–325).

As phospho-IκBα is indicative of a pro-inflammatory response due the release of NF-κB into the nucleus, NF-κB nuclear translocation was then monitored in LPS-stimulated macrophages. Results demonstrated the ability of all three lactoferricin peptides to attenuate LPS-driven NF-κB nuclear localization in RAW 264.7 and THP-1 cells (Figure 3.11 A and B). These observations are consistent with other studies demonstrating AMP regulation of inflammatory responses via inhibition of NF-κB nuclear translocation (169, 326, 327).

The β-catenin pathway is also involved in inflammation; signaling along this pathway results in upregulated expression of genes that control cellular growth, proliferation, and synthesis of pro-inflammatory cytokines IL-6 and IL-12 (84, 328, 329). This pathway is also induced by LPS as well as by AMPs such as α-defensins and cathelicidins (82, 330, 331). However, the present study shows that lactoferricin peptides do not alter LPS-induced activation of the β-catenin signaling pathway in macrophages (Figure 3.12).

Past studies implicate MAPK signaling in AMP-mediated immunomodulation and the selective manner in which these peptides can induce a response (171, 265, 318, 330). The present study shows that LPS-induced activation of ERK-1/2 signaling is decreased in lactoferricin-treated RAW 264.7 and THP-1 cells (Figure 3.13 A-D). Another AMP, melittin, isolated from bees, also decreases ERK-1/2 phosphorylation in LPS-stimulated RAW 264.7 cells (332). ERK-1/2 induces further downstream signaling components such as c-Jun and c-Fos subunits of the AP-1 transcription factor family in macrophages (66, 333, 334). Lactoferricin peptides were also able to decrease expression of phosphorylated c-Jun in macrophages (Figure 3.14 A-D). This is consistent with an anti-inflammatory role of lactoferricin peptides as AP-1, like NF-κB, regulates the transcription of pro-inflammatory mediators when cells are stimulated by LPS (270, 335). Although activation of JNK was not investigated in this current study, it is anticipated that

lactoferricin peptides would also decrease JNK phosphorylation as this MAPK is an important mediator upstream of c-Jun (270).

Given the ability of these peptides to have an inhibitory effect on the phosphorylation of two different inflammatory pathways, it is possible that lactoferricin has an inhibitory effect on a common mediator that may act upstream of both NF-κB and ERK 1/2. Such common mediators could include any of the adaptor proteins that propagate the LPS-induced TLR-4 response, such as TRAF 6, TRIF, or TRAM.

The ability of lactoferricin peptides to increase phosphorylation of p38 MAPK in macrophages suggests that these peptides may operate via this pathway to elicit an anti-inflammatory effect (Figure 3.15 A-D). Although LPS also operates through p38 MAPK during a pro-inflammatory response, p38 MAPK has also been linked to anti-inflammatory effects. The direct anti-inflammatory role of p38 MAPK involves the regulation of IL-10 production through signaling interactions with SP-1 (336, 337). The indirect role of p38 MAPK in downregulating inflammation is through its ability to cause destabilization of pro-inflammatory mRNA through activation of TTP, an mRNA destabilizing protein (74, 75).

The indication that AMPs can selectively modulate a host immune response is not a novel concept. Other AMPs also show selectivity towards host cell signaling pathways. For example, a study of IDR-1 peptides show that this peptide can interact with p38 MAPK, but does not affect NF-κB activation in RAW 264.7 cells (315). The human cathelicidin, LL-37, is another AMP that is able to selectively downregulate inflammation by signaling along particular inflammatory pathways to allow for decreased pro-inflammatory cytokine synthesis and increased anti-inflammatory cytokine production (169, 327, 338). The exact molecular mechanism by which different AMPs have different effects on signaling pathways has yet to be uncovered. Some studies have suggested an intracellular receptor that leads to downstream effects, while another study shows that AMP, α-defensin, was able to directly inhibit PKC phosphorylation in neutrophils (152, 318, 339).

4.4 Lactoferricin increases IL-10 production in non-stimulated RAW 264.7 cells

An important aspect of downregulating an inflammatory response is the upregulation of anti-inflammatory cytokines such as IL-10. All three lactoferricin peptides increased IL-10 production in RAW 264.7 cells, but failed to do so in THP-1 cells (Figure 3.6 B and C). This discrepancy between these two results could be attributed to the priming of THP-1 cells with PMA, which directly activates PKC (340). PMA also activates MAPK pathways both directly and indirectly (341, 342). The indirect activation of MAPKs, specifically ERK-1/2 and JNK, by PMA is associated with the induction of ROS in monocytes, which activates MAPK pathways (295, 341, 343). It is possible that exposure to PMA prior to LPS-peptide treatment may result in tolerance to other stimuli by activating the same pathways.

Western blot analysis of p38 MAPK showed an increase in phospho-p38 MAPK in lactoferricin-treated RAW 264.7 and THP-1 cells in the absence of LPS stimulation (Figure 3.15 A-D). A previous study of lactoferrin found that administration of oral human lactoferrin results in the induction of IL-10 in a rat model of arthritis (344). Another study of the parent peptide lactoferrin demonstrates the ability of this peptide to induce activation of p38 MAPK in osteoblasts (345). Since p38 is heavily involved in the inducible expression of IL-10 in macrophages (258, 273, 346, 347), an experiment was performed to determine whether IL-10 production in lactoferricin-treated RAW 264.7 cells occurred in a p38 MAPK-dependent manner. While the effect was not statistically significant, lactoferricin-mediated upregulation of IL-10 was consistently suppressed in the presence of a p38 MAPK inhibitor, suggesting a possible role of this pathway in lactoferricin-mediated inhibition of inflammation (Figure 3.16). As mentioned previously, p38 MAPK exists in 4 distinct isoforms α, β, γ, and δ. Amino acid sequence homology ranges from 60-70% between each isoform, each of which has differing levels of expression in various cell types (70, 348, 349). In macrophages, the main isoforms of p38 MAPK are α and γ (348). The p38 MAPK inhibitor used in this current study is specific for α and β isoforms as their ATP-binding pocket contains a threonine residue that interacts with SB203528, preventing ATP binding and downstream effects of p38 activation (69). The anti-p38 MAPK antibody that was used detects all four isoforms.

Therefore, it is possible that p38 MAPK γ, which is not inhibited by SB203528, could be involved in lactoferricin-induced IL-10 production in RAW 264.7 cells.

Alternatively, these peptides could be operating through another distinct signaling pathway. Other transcription factors that have been implicated in the production of IL-10 production include SP-1 and CREB (86, 336). Further exploration of mediators along these signaling pathways would help to elucidate the role of lactoferricin peptides in increased IL-10 production in macrophages.

4.5 Peptides decrease cadmium-induced TNF-α production in macrophages

$CdCl_2$ is a heavy metal carcinogen that induces inflammatory mediators in a variety of immune cell types (350). $CdCl_2$ induces mitochondrial-generated ROS in macrophages and neutrophils, which can cause the induction of pro-inflammatory cytokines such as TNF-α (344, 345). Glutathione is a major antioxidant produced in plant and animal cells that is able to reduce potentially harmful reactive oxygen species (346). Studies demonstrate the importance of glutathione in protecting the host from the damaging effects of cadmium through reduction in ROS (350, 353). AMPs such as human and murine-derived cathelicidins, peptides show anti-oxidant properties by reducing harmful oxidative burden, similar to glutathione (327, 354). Given that $CdCl_2$ leads to the production and ROS, which induces TNF- α, and all three peptides are able to downregulate $CdCl_2$-induced TNF-α production in mouse and human macrophages (Figure 3.17 B-C), it is possible that the lactoferricin peptides act as anti-oxidants. One study also shows the importance of disulfide bridges between cysteine residues (a feature of all three lactoferricin peptides) in AMPs found in frog skin in anti-oxidation. This study also demonstrates that the number of disulfide bridges and cysteines residues correlates to the overall anti-oxidant effect of these peptides (355). Given that each lactoferricin peptide decreases $CdCl_2$-induced TNF-α production in macrophages and studies show the importance of cysteine residues and disulfide bridges in anti-oxidant properties of AMPs, lactoferricin peptides could be operating in an anti-oxidative capacity in $CdCl_2$-treated macrophages.

Alternatively, L-arginine is a natural anti-oxidant, and as mentioned previously, a precursor of NO production. Lactoferricin peptides demonstrated a decrease in NO production in LPS-stimulated macrophages, indicating less arginine was being converted to citrulline and NO. The increased presence of arginine in these cells, due to lactoferricin treatment, could account for the anti-inflammatory influence of these peptides in $CdCl_2$-stimulated macrophages.

A contributing factor to the decrease in pro-inflamamtory cytokine production by macrophages in the presence of lactoferricin peptides is the ability of positively charged lactoferricin to bind negatively charged LPS, thereby inhibiting endotoxin binding to TLR-4. Other studies have highlighted the importance of endotoxin binding of AMPs to inhibit inflammation (323, 356), but given the selective manner in which lactoferricin targets cell signaling and its ability to decrease cadmium-induced TNF-α production, it is likely lactoferricin mediates its an anti-inflammatory effect by operating in a manner other than simply binding to LPS and preventing TLR-4 stimulation. However, the molar concentration of LPS used in this experiment is <0.5 μM and is possible that lactoferricin peptides are overwhelming LPS molecules to inhibit a pro-inflammatory response. In order to address this concern, an additional experiment using an alternative TLR agonist, such as endogenous high mobility protein, could be used to distinguish between the anti-inflammatory effects of lactoferricin simply binding LPS and having a direct immunomodulatory effect. Alternatively, cells could also be pretreated with LPS, washed in PBS, and treated with lactoferricin peptides to differentiate between peptide-LPS binding and independent anti-inflammatory effects.

4.6 Limitations of this study

One major limitation of this current study is the absence of data obtained using human primary cells. Both mouse and human macrophage lines (RAW 264.7 and PMA-differentiated THP-1 cells) and primary BMDMs of murine origin were used to determined the effects of lactoferricin peptides on LPS-induced inflammation, but the use of peripheral blood mononuclear cells (PMBC) would have been useful to confirm findings with THP-1 cells.

It is also unclear what effect an *in vivo* environment would have on peptide efficacy as these peptides are sensitive to pH, and are vulnerable to proteolytic cleavage by serum proteases. Another concern is the *in vivo* half-life of these peptides. Although lactoferricin peptides are somewhat less vulnerable to degradation due to their cyclic nature that results from the disulfide bond (305), it is still a concern going forward with mouse models of inflammation and eventual clinical trials.

The use of cell lines, and THP-1 cells in particular, is another limitation of this study. THP-1 monocytic cells are an appropriate model for *in vitro* studies of vascular monocytes and macrophages (357). However, their use in this study may have been hampered by the induction of various inflammatory signals by prior exposure to PMA, which could have interfered with LPS-stimulation and lactoferricin-induced anti-inflammatory effects.

4.7 Future Directions

Future directions of this current study include additional exploration of inflammatory signaling pathways and the full range of anti-inflammatory properties of these AMPs, and particularly Blfcn. It would also be interesting to explore the anti-inflammatory effects of lactoferricin peptides on additional cadmium-induced inflammatory responses and whether this involves an anti-oxidant effect of these peptides.

The inclusion of a dose response would be helpful to determine if higher concentration of Mlfcn and Hlfcn would be just as effective in downregulating a pro-inflammatory response as 5 μM of Blfcn.

The use of proteosomal inhibitor, MG-132, would be useful to determine if lactoferricin peptides operate upstream of IκBα or inhibit the actual trafficking of phospho- IκBα by the E3 ligase complex to allow for proteosomal degradation. This experiment would be useful for narrowing the exact influence these peptides have on NF-κB pro-inflammatory signaling.

There are several other transcription factor involved in the regulation of inflammatory responses, including SP-1 and CREB (86, 251, 258). Determining the effect of lactoferricin peptides on these transcription factors may help uncover the particular pathways by which these peptides impact to allow for reduction in inflammation. SP-1 would be of particular interest as this transcription factor is heavily linked to IL-10 production in macrophages (258, 336).

Lactoferricin-treated cells showed a decrease in M1-specific markers, including TNF-α and iNOS, and an increase in the M2-specific marker IL-10, indicating these peptides may alter macrophage phenotype. Examination of additional M1 and M2 markers, such as IL-1β and arginase-1, respectively, would have confirmed the influence of lactoferricin peptides on the phenotypic plasticity of macrophages and would be an interesting option to explore.

Although *in vivo* model of inflammation was beyond the scope of this project, *in vivo* experiments would be useful to determine whether these peptides can have an anti-inflammatory effect in an animal model. Given that these peptides displayed anti-inflammatory properties in an LPS-induced response, an animal inflammation model such as the carrageenan-air pouch model would offer some insight into the potential effects of lactoferricin peptides in an acute *in vivo* inflammatory response (358, 359). A chronic inflammatory setting such as a mouse model of colitis would be suitable for determining whether the immunomodulatory effects of lactoferricin on macrophage production of TNF-α and other inflammatory mediators play a key role in shaping this inflammatory response (360–362).

Finally, enhancement of the current lactoferricin peptides, specifically Blfcn, through synthetic alteration of amino acid sequence may lead to a peptide that is more potent as an immune regulator. A few studies show the benefits to using synthetic peptides to inhibit inflammation and as a more potent antimicrobial agents (363–366). Generation of synthetic peptides with increased hydrophobicity and greater positive charge that may result in better membrane insertion and enhanced effects on signaling pathways. There is already evidence to suggest that Trp and Arg amino acid residues are important for the antimicrobial and anti-inflammatory activities of AMPs; therefore, it

would be interesting to see if increasing the content of these residues in lactoferricin would alter its immunomodulatory properties.

4.8 Conclusions

Inflammation is a complicated process consisting of pro-inflammatory and anti-inflammatory components that allow for an appropriate response to microbial pathogens and other insults. Proper inflammation results in eradication of the source of stimulation, the clean up of pathogenic and cellular debris, and repair of tissue damage in order to return to homeostasis. When these tightly regulated mechanisms fail, inflammation persists and can become destructive to the host. To circumvent the development of chronic inflammation or address an already existing inflammatory condition, immune regulators are required. Regulators that target a plethora of pro-inflammatory mediators benefit the host and may offer a protective effect in a wide variety of inflammatory diseases such as sepsis and colitis. Such regulators can include certain AMPs, which offer great potential as therapeutic agent owing to their relatively non-toxic and non-mutagenic properties in eukaryotic cells.

Of the three lactoferricin peptides used, Blfcn consistently had the greatest anti-inflammatory effects. Blfcn demonstrated potent immunomodualtory effects in macrophages through downregulation of LPS-induced TNF-α, IL-6, iNOS, and NO, as well as the increase in IL-10 synthesis.

Although there is still much to be explored, this study has shown the selective influence of species-specific lactoferricin peptides on major macrophage-associated inflammatory processes. This suggests that the development of lactoferricin peptides, especially of bovine origin, may have therapeutic potential in the context of downregulating excessive or inappropriate inflammatory responses.

Figure 4.1 Schematic of outlined anti-inflammatory effects of lactoferricin in macrophages. Lactoferricin peptides inhibit LPS-induced phosphorylation of IκBα and nuclear translocation of NF-κB. This subsequently inhibits the synthesis and release of proinflammatory mediators TNF-α, IL-6, and NO. Lactoferricin peptides prevent the cadmium-induced production of TNF-α. Lactoferricin decreases LPS-induced phosphorylation of ERK and c-Jun, while increasing the phosphorylation of p38 MAPK.

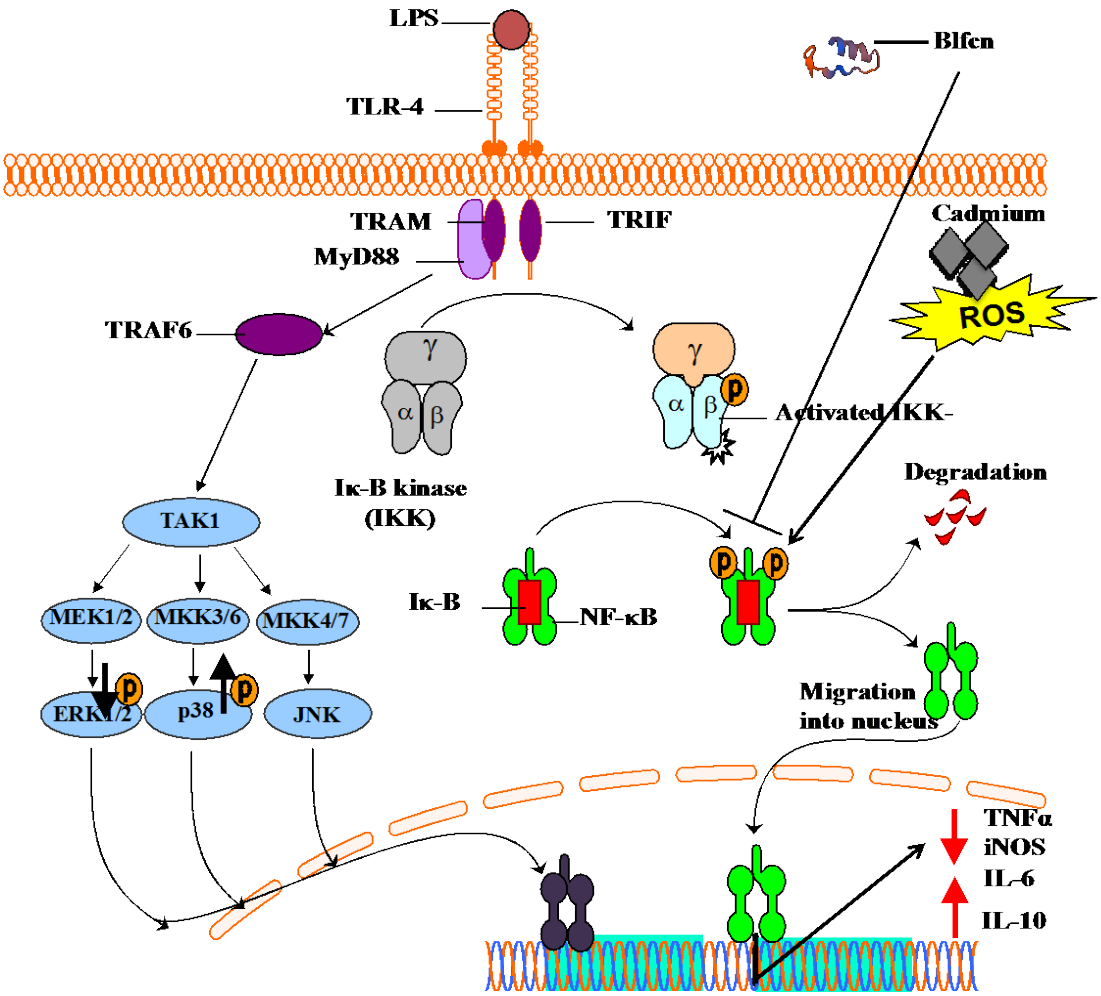

LPS
TLR-4
Blfcn
TRAM
TRIF
MyD88
Cadmium
ROS
TRAF6
γ
α
β
γ
α
β
p
Activated IKK-
Iκ-B kinase
(IKK)
Degradation
TAK1
Iκ-B
NF-κB
p
p
MEK1/2
MKK3/6
MKK4/7
ERK1/2
p38
JNK
p
p
Migration
into nucleus
TNFα
iNOS
IL-6
IL-10

Figure 4.1

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