THE CONTRIBUTION OF CATIONIC ANTIMICROBIAL PEPTIDES TO THE HOST IMMUNE RESPONSE TO BACTERIAL INFECTION

by

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ABSTRACT

Many species produce cationic antimicrobial peptides as components of their immune systems. To combat bacterial infections, the host immune response, or treatment regime, must aim to kill the invading pathogen and manage the inflammatory response to the bacteria and its released components. Certain cationic peptides, such as the human peptide, LL-37, can be produced in large quantities at sites of infection/inflammation and their expression can be further induced by bacterial products such as lipopolysaccharide (LPS) and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α). The primary focus of this research was to further define the contribution of cationic peptides to the immune response to bacterial infection. A greater understanding of the biological characteristics of these molecules will help in evaluating their therapeutic potential. Both synthetic and natural peptides were studied here, most notably a hybrid peptide of cecropin and melittin (CEMA) and the human peptide, LL-37. I found that the cationic peptides had multifaceted activities including the ability to bind to lipoteichoic acid (LTA) and LPS and in the case of LPS, to interfere with its ability to bind LPS binding protein (LBP). The peptides were also found to be effective in neutralizing the host response to bacterial components from both Gram-negative and Gram-positive bacteria. I found that CEMA selectively inhibited macrophage gene expression in response to LPS stimulation. CEMA and LL-37 treatment of murine macrophages resulted in the up-regulation of at least 35 genes and down-regulation of at least 21 genes. Macrophage genes affected by the peptides included those involved in chemotaxis. These changes were then confirmed at the protein level in both *in vitro* and *in vivo* situations. For example, CEMA and LL-37 induced the production of chemokines and up-regulated the surface expression of chemokine receptors. When given intratracheally to mice, LL-37 increased levels of the chemokine, monocyte chemoattractant protein-1 (MCP-1) but not the pro-inflammatory cytokine, TNF-α. Thus, this study indicated that synthetic as well as natural cationic peptides produced in response to bacterial infections could contribute to the immune response by limiting the damage caused by bacterial products and by recruiting immune cells to tissue sites harboring infectious agents.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BAL	bronchoalveolar lavage
BMP	bone morphogenetic protein
BSA	bovine serum albumin
DLK	diffuse lameller keratitis
DMEM	dulbeccos modified eagle medium
ECM	extracellular matrix
ELISA	enzyme-linked immunoabsorbent assay
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FPRL1	formyl peptide receptor-like 1
GPI	glycophosphatidylinositol
HBD	human beta defensin
HNP	human neutrophil peptide
ICAM-1	intercellular adhesion molecule-1
IκB	inhibitor of NF-κB
IKK	IκBα kinase
IL-	interleukin-
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
LBP	lipopolysaccharide binding protein
M-CSF	macrophage colony stimulating factor

MAP kinase	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MIP	macrophage inflammatory protein
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium blue
MRSA	methicillin resistant *S. aureus*
NAPS	nucleic acid and protein services
NMR	nuclear magnetic resonance
NO	nitric oxide
NPN	1-*N*-phenylnaphthylamine
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PB	polymyxin B
PMN	polymorphonuclear granulocyte
RT-PCR	reverse transcriptase polymerase chain reaction
SDF-1	stromal cell-derived factor-1
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBST	TBS with 0.05% Tween 20
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumor necrosis factor
TNFR	tumor necrosis factor receptor
VCAM-1	vascular cell adhesion molecule-1

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CHAPTER 1: INTRODUCTION

1.1 Overview of cationic peptides and their functions

The role of cationic antimicrobial peptides in antimicrobial defenses has become increasingly apparent since Zeya and Spitznagel (1966) isolated the first peptide antibiotics from mammalian polymorphonuclear leukocytes (PMN) and noted their cationic nature (Zeya and Spitznagel 1966). Since then, many different peptides from a wide range of animal, plant, and bacterial species have been identified. These cationic peptides are found as components of non-specific defenses against microorganisms. In many species including amphibians, crustaceans, fish, birds, and mammals including humans, it is becoming increasingly clear that antimicrobial peptides are a major player in local innate immunity, especially at mucosal and epithelial surfaces (Andreu and Rivas 1998; Hancock and Lehrer 1998). For example, in the fruit fly *Drosophila*, cationic antimicrobial peptides are the major form of defense against infection and are induced in response to challenge by microbes or microbial components such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA). There is a growing body of evidence that their role in defense against microbes may be as important to the host as antibodies, immune cells and phagocytes (Scott and Hancock 2000).

Cationic antimicrobial peptides are well known for their ability to kill many infectious agents, including bacteria, fungi, viruses and parasites. However, the action of cationic antimicrobial peptides appears not to be limited to the direct killing of microbes. Rather, there is accumulating evidence that they have an impressive variety of additional activities that would be expected to impact on the quality and effectiveness of innate immune responses and inflammation (Gudmundsson and Agerberth 1999). These include many of the elements of inflammatory responses that are frequently ascribed to other agents. It remains to be determined whether these

peptides are central players, supportive or even bystanders in the inflammatory response. One might suspect a key role, however, based on the inducibility by infectious agents of many (but not all) peptides and the very high concentrations at sites of inflammation. For example, in the sputum of cystic fibrosis patients, defensin levels of 300 μg/ml (Soong et al. 1997) or more have been recorded and up to 170 μg/ml was observed in the plasma of septic individuals (Panyutich et al. 1993; Shiomi et al. 1993). In patients with emphysema, human neutrophil peptide (HNP) 1-3 levels are highly elevated in pleural effusions (Ashitani et al. 1998). In patients with oral inflammation and / or oral diseases, the levels of HNP-1 are significantly increased over the levels of healthy individuals (Mizukawa et al. 1999; Mizukawa et al. 1999).

Cationic antimicrobial peptides have been reported to be involved in many aspects of innate host defenses (see Figure 1 for overview) associated with acute inflammation. These include initial killing of bacterial cells to release inflammatory stimuli, mast cell degranulation leading to histamine release and consequent increase in blood vessel permeability, chemotaxis of neutrophils and T helper cells resulting in leukocyte recruitment to infection site, promotion of non-opsonic phagocytosis, inhibition of fibrinolysis by tissue plasminogen activator, thus reducing the spreading of bacteria, tissue/wound repair through proteoglycan induction (syndecan-1 and -4), promotion of fibroblast chemotaxis, and inhibition of tissue injury by inhibiting certain proteases such as furin, elastase and cathepsin (Sawyer et al. 1988; Territo et al. 1989; Hook et al. 1990; Murphy et al. 1993; Verbanac et al. 1993; Gallo et al. 1994; Panyutich et al. 1995; Chertov et al. 1997; Huang et al. 1997; Befus et al. 1999; Yang et al. 1999). Other putative roles that cationic antimicrobial peptides play in the inflammatory process, based on their *in vitro* properties, include acting as chemokines for the recruitment of monocytes and T cells, enhancement of chemokine production and the proliferative response of T helper cells leading to increased IgG but not IgA

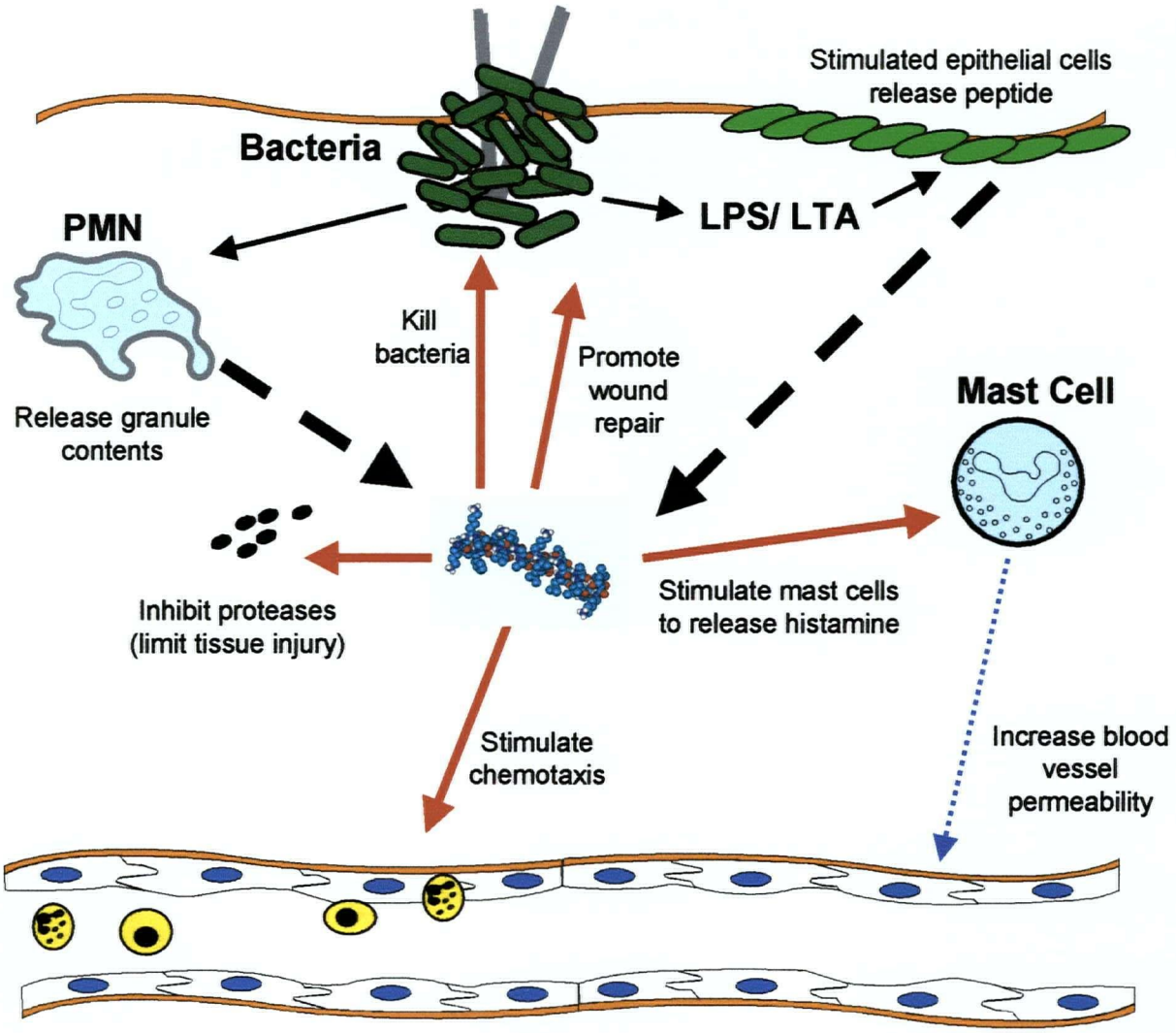


Figure 1: Overview of putative roles of cationic antimicrobial peptides in host defenses

The bold dotted lines represent release of cationic peptides, the thin black lines represent release of bacterial products and the medium lines represent activities of the peptides. The thin dotted line represents release of histamine from mast cells. This figure is adapted from Hancock and Diamond, 2000.

production, suppression of LPS-, and LTA- induced cytokine production and other responses of macrophages, and stimulation of apoptosis in macrophages and activated lymphocytes [reviewed in (Bals 2000), (Risso 2000), (Hancock and Scott 2001)].

1.1.1 Properties of cationic antimicrobial peptides

Cationic antimicrobial peptides are generally defined as being 12 to 50 amino acids in length with a net positive charge of +2 to +7 due to an excess of basic amino acids, arginine, lysine or histidine, over acidic amino acids (Hancock and Chapple 1999). Generally, 50% or more of the amino acids are hydrophobic, facilitating the interaction of such peptides with microbial membranes. Despite their small size and common physical-chemical features, cationic antimicrobial peptides appear to have arisen from multiple sources, possibly through convergent evolution, in that they have a range of folding patterns that fit into 4 broad secondary structure classes (Figure 2) (Hancock and Lehrer 1998; Hancock 2001). The most prominent classes are β-sheet peptides stabilized by 2 to 4 disulphide bridges (and occasionally containing a short α-helical stretch), and unstructured peptides that fold into amphipathic α-helices upon contact with membranes. Less common are loop peptides formed by a single disulphide bond and extended peptides with a predominance of one or two amino acids, (e.g. P, W or H). Other cationic antimicrobial peptides are apparently formed in the host by proteolytic digestion of larger cationic proteins such as lactoferrin and CAP18 (cationic antimicrobial protein). Interestingly, several cationic proteins implicated in innate immunity, such as lactoferrin, bactericidal/ permeability increasing protein, and cathepsin, have characteristics and activities analogous to cationic peptides. Generally, the best cationic peptides fold into molecules that have the charged and hydrophilic portions segregated from the hydrophobic portions resulting in either amphipathic structures or cationic double wing structures with a hydrophobic core separating two

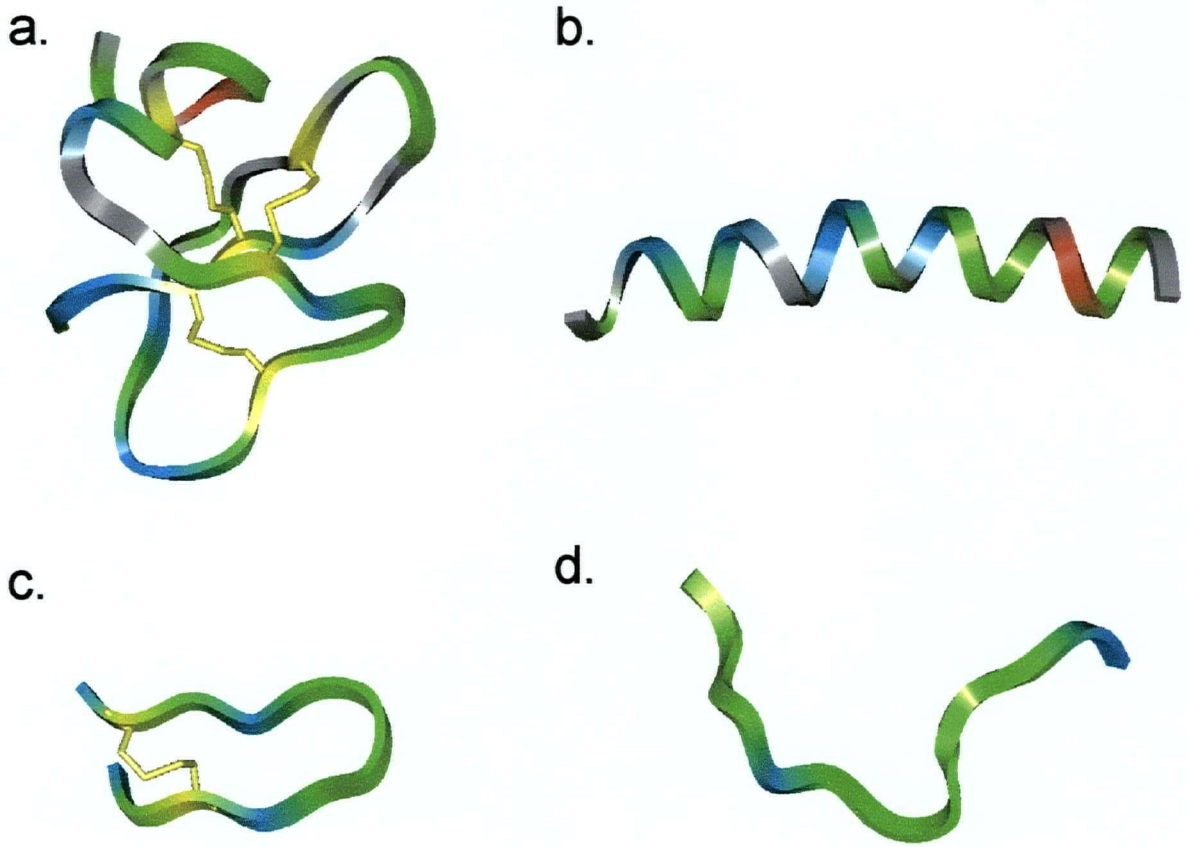


Figure 2: Different structural classes of cationic peptides

Molecular model structures of example cationic peptides from different structural classes. The charged regions of the cationic peptides are shown in black and the hydrophobic in grey. (a) HBD-2, β-sheet structure (b) magainin 2, amphipathic α-helical structure (c) bactenecin, β-turn loop structure (d) indolicidin, extended structure. This figure was taken from Hancock 2001.

charged segments.

Cationic peptides are found in most, if not all, species of life, including bacteria, fungi, plants, insects, birds, fish, crustaceans, amphibians and mammals. A single host species may contain peptides belonging to 2 to 4 different classes. For example, cattle contain sequences for 38 antimicrobial peptides (Hancock and Diamond 2000), including β-stranded α- and β-defensins, α-helical BMAP (cathelicidins) peptides, the loop peptide bactenecin, the extended peptide indolicidin, and a variety of fragments of larger proteins. There are at least three possible reasons for this variety. First, the antimicrobial spectrum of any given peptide is broad but does not extend to all microbes. Thus, a variety of peptides with overlapping ranges of targets could provide more complete immunity. Second, different peptides synergize with other peptides as well as with other effectors of innate immunity to kill microbes (Yan and Hancock 2001). Finally, peptides tend to be produced by different cell types such that a given tissue might express only a subset of these peptides.

1.1.2 Expression of defensins

Mammalian defensins are cationic peptides that contain six cysteine residues that form three characteristic disulphide bridges and can be divided into three classes (α-, β- and θ-defensins) based on the spacing of the cysteine residues and overall structure (reviewed in Bals 2000). In humans, the defensin locus is found on chromosome 8p21-23, where the genes for both α- and β-defensins are found (Liu et al. 1997). The genes for the α-defensins exhibit substantial sequence identity, as do the genes for the β-defensins even though these groups of genes are quite distinct from each other. Examination of defensin mRNA has revealed that defensins are synthesized as precursor molecules, comprising a signal sequence, a pro-region and the mature peptide (reviewed in Lehrer and Ganz 2002). After removal of the signal sequence, the pro-region

is removed to yield the mature peptide. Matrilysin (MMP7), a tissue metalloprotease, was shown to cleave the propeptide of murine cryptidins, α-defensins found in the Paneth cells of the small intestine (Wilson et al. 1999).

The Paneth cells of the small intestine express human enteric defensins (HD-5 and -6), (reviewed in Ouellette and Bevins 2001). Enteric α-defensin genes are expressed at high levels in the normal intestine of mice, humans and rats (Bevins 1999). Despite this, the expression of at least one of these genes is induced in the rat intestine after hemorrhagic shock (Condon et al. 1999). One other enteric α-defensin gene, HD-5, was found to be variably expressed in the female reproductive tract (Quayle et al. 1998), where expression in the endometrium correlated to the phase of the menstrual cycle. More recently, murine Paneth cells were found to secrete α-defensins in response to Gram-negative bacteria, Gram-positive bacteria, LPS, LTA, lipid A and muramyl dipeptide (Ayabe et al. 2000).

There are two groups of β-defensins as defined by expression patterns. Constitutively expressed β-defensins, such as human β-defensin-1 (HBD-1) in humans and bovine neutrophil β-defensins (BNBD-1 to -13), have two exons surrounding a large intron (Liu et al. 1997). HBD-1 is expressed at high levels in the genitourinary tract, and at lower levels in other tissues, but its mRNA levels do not apparently change upon microbial challenge or when host tissues are inflammed (Ganz and Lehrer 1998). In contrast, the expression of tracheal antimicrobial peptide (TAP), a β-defensin in the bovine airway, is up-regulated in primary culture systems by phorbol 12-myristate 13-actetate (Diamond et al. 1996), TNF-α (Russell et al. 1996), IL-1β, muramyl dipeptide, and lipoteichoic acid (Diamond et al. 2000) as well as numerous infectious agents and inflammatory mediators. For example, a 15-fold increase in the steady-state levels of the mRNA encoding TAP was observed upon incubation with 100 ng/ml *E. coli* LPS (Diamond et al. 1996),

indicating that airway epithelial cells respond to pathogens by the production of antimicrobial agents. A homologous β-defensin expressed in the tongue epithelium, lingual antimicrobial peptide (LAP), is also up-regulated at sites of inflammation and undergoes increased expression in airway epithelia in a coordinated fashion with TAP (Russell et al. 1996). The genes encoding both TAP and LAP are much smaller than that encoding HBD-1, mostly due to a relatively small intron in the latter.

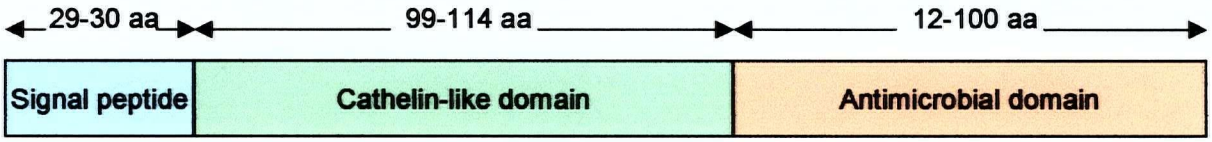
In humans, a homologue of TAP, human β-defensin-2 (HBD-2) is expressed in inflamed tissues. For example, it has been observed that primary airway epithelial cells up-regulate HBD-2 mRNA in response to IL-1β (Singh et al. 1998). A similar induction in response to either *Pseudomonas aeruginosa* LPS or TNF-α can be observed at the air-liquid interface culture of human tracheal epithelial cells. The homologous mouse β-defensin 2 (mBD2) is also up-regulated in response to LPS. The expression of defensins in the oral cavity has also been studied. Constitutive expression of HBD-1 mRNA was seen in the tongue, gingiva, parotid gland, and buccal mucosa while HBD-2 mRNA was only detected in the gingival mucosa (Mathews et al. 1999). In gingival keratinocyte cell cultures, HBD-2 expression increased 16-fold upon stimulation with IL-1β and 5-fold with LPS (Mathews et al. 1999). These patterns of defensin expression are similar to those seen at other mucosal surfaces. These data suggest that inflammation and infection mediate a peptide-based host response in mucosal tissues through transcriptional regulation.

Expression of the genes for TAP and HBD-2 is induced by bacterial LPS through an epithelial cell-expressed CD14-mediated signal transduction pathway (Diamond et al. 1996). The induction occurs via activation of the p65/p50 heterodimer of NF-κB, which migrates to the nucleus and binds to an NF-κB consensus sequence upstream from the TAP gene (Diamond et al.

2000). This pathway is known to regulate the expression of other events involved in inflammation, such as the production of pro-inflammatory cytokines. The NF-κB pathway is involved in the LPS-mediated up-regulation of the HBD-2 gene in human airway cells. Moreover, peptide induction by microbial components follows signaling pathways similar to those involved in inflammatory responses by the mammalian immune system which involves Toll-like receptor activation of NF-κB (Hetru et al. 1998).

1.1.3 Cathelicidins

The cathelicidins are a family of at least 30 structurally divergent antimicrobials from various mammalian species. The cathelicidins contain a N-terminal signal peptide, a cathelin domain, which is highly conserved and a less conserved C-terminal antimicrobial domain (see Figure 3). The cathelin domain was named for cathelin, a cysteine protease inhibitor of porcine origin (Zanetti et al. 1995)). Cathelicidins are encoded by genes of four exons, with the active mature peptide, which tends to be highly divergent in sequence and ranges in size from 12-79 amino acids, found in the fourth exon (Zhao et al. 1995a; Zhao et al. 1995b). Utilizing the high homology of the cathelin domain has enabled researchers to identify new cathelicidin sequences in numerous species, including cows, pigs, sheep, horses, mice, guinea pigs and rabbits (Figure 3). Cathelicidin peptides include protegrins from pigs, CAP18 from rabbits, bactenecin and indolicidin from cows and more recently RL-37 from monkeys. In humans, only one cathelicidin antimicrobial has been found to date, the α-helical peptide LL-37 (also known as hCAP18, human cationic antimicrobial protein) (Gudmundsson and Agerberth 1999). Cathelicidins are primarily stored in neutrophil granules and are released upon activation (Cowland et al. 1995; Zanetti et al. 1995). In contrast to defensins, cathelicidin peptides are stored as inactive propeptide precursors that, upon stimulation, are processed. The C-terminal



Species	Cathelicidin Peptide
Human	LL-37
Mouse	mCRAMP
Bovine	BMAP27,28,34, Bac5, Bac7, Indolicidin
Ovine	SMAP28,29,34
Horse	eCath1,2,&3
Guinea pig	CAP11
Rat	CRAMP
Porcine	PMAP37,36,&23, PR39, PG1,2,3,&4
Rabbit	CAP18, p15
Monkey	RL-37

Figure 3: Structure and species distribution of cathelicidins

The common structure of cathelicidins includes an N-terminal signal peptide, a cathelin domain (highly conserved), and a C-terminal antimicrobial domain (less conserved). The cathelicidin family exist in a number of mammalian species.

antimicrobial domain is cleaved off by the appropriate proteases. In porcine and bovine neutrophils, neutrophil elastase is thought to be the enzyme responsible for processing, resulting in the release of active peptides into the extracellular fluid (Panyutich et al. 1997). The human cathelicidin, hCAP18, is processed by extracellular cleavage with proteinase 3 to yield LL-37 (Sorensen et al. 2001). The antimicrobial domains of the cathelicidins have a variety of structures including β-sheet protegrins from pigs, α-helical CAP18 from rabbits and LL-37 from humans, loop-structured bactenecin and extended-structured indolicidin from cows.

In some species, cathelicidins are expressed in myeloid precursor cells, although expression of cathelicidins in mature peripheral porcine neutrophils has also been reported (Wu et al. 1999). Surprisingly, porcine cathelicidins are also expressed in a number of lymphoid tissues in young pigs (less than 4 weeks old), but such expression disappears in adults (Wu et al. 1999). The single cathelicidin from humans, LL-37, is widely expressed, and is produced by myeloid precursors (Sorensen et al. 1997), testis (Agerberth et al. 1995), by keratinocytes during inflammatory disorders (Frohm et al. 1997) and by airway epithelium (Bals et al. 1998). The promoter region of the gene encoding the arginine-rich porcine peptide PR-39 has several potential transcription factor-binding sites, including an NF-IL6-binding site, indicating that this peptide may undergo regulatory controls analogous to those of the defensins. Indeed, cathelicidins and defensins can exhibit synergistic antimicrobial activities (Nagaoka et al. 2000), indicating that they may participate in a combined host defense response.

1.1.4 Cecropin-melittin hybrid peptides

Boman et al. created the first hybrid peptides that contained sequences from both cecropin A and melittin (Boman et al. 1989). The peptides were the same size as melittin, and contained different combinations of the hydrophobic and hydrophilic regions of cecropin A and melittin.

One of these peptides contained the first 13 amino acids of cecropin A followed by the first 13 amino acids of melittin. This peptide was 100-fold more active against *S. aureus* than cecropin A (Boman et al. 1989). Unlike melittin, however, this cecropin-like peptide possessed no hemolytic activity. Piers et al produced CEME (also known as MBI-27), which contains the first eight amino acids of cecropin and the first eighteen amino acids of melittin using a bacterial expression system (Piers et al. 1993). CEME and its derivative CEMA (containing two additional charges on the C terminus) have antimicrobial activity against Gram-negative bacteria as well as a high affinity for bacterial endotoxin (LPS) (Piers et al. 1994). These hybrid peptides also have endotoxin neutralizing activity in murine macrophages and in mice (Gough et al. 1996). Table 1 on page 35 contains the amino acid sequences of cecropin-melittin hybrid peptides used in this study.

1.2 Bacterial sepsis

Systemic disease associated with the presence of pathogenic microorganisms or their toxins in the blood (i.e. septicaemia) affects hundreds of thousands of North Americans annually. The release of bacterial components during infection causes an inflammatory response, which when unchecked can lead to sepsis. Both Gram-negative bacteria, Gram-positive bacteria and their associated components can cause sepsis. Characteristic clinical manifestations associated with the presence of circulating bacteria or bacterial products include fever, and/or hypothermia, tachypnea (abnormally rapid, usually shallow, respiratory rate), tachycardia (rapid heart rate), reduced or elevated circulating PMNs, hypotension, and, multi-organ hypoperfusion leading to shock.

1.2.1 Gram-negative sepsis

Gram-negative sepsis is usually caused by the release of the bacterial outer membrane component, endotoxin (LPS). LPS is a major component of the outer leaflet of the asymmetrical

outer membrane that is characteristic of Gram-negative bacteria. It plays an important protective and structural role for these microorganisms and thus, is essential for viability. LPS is an amphipathic molecule consisting of three domains: a) the hydrophilic O-antigen side chain that extends outward from the cell, b) a core polysaccharide region consisting of various conserved components, and c) the hydrophobic lipid A tail which anchors the molecule into the membrane. Gram-negative bacteria have been grouped into S-(smooth) and R-(rough) forms. The R-form has a defect in LPS biosynthesis resulting in a lack of O-specific polysaccharide. Lipid A generally consists of a 4-phosphoglucosaminyl-(1-6)-glucosamine-1-phosphate backbone (Wilkinson 1983) to which four to six saturated fatty acids are either ester or amide linked (Karunaratne et al. 1992). These linkages can either be directly on the sugar backbone, or through the hydroxyl groups of other fatty acid chains (Kropinski et al. 1985).

Many antibiotics stimulate the release of endotoxin and thus contribute to sepsis (Cohen and McConnell 1985; Shenep et al. 1985; Prins et al. 1995a; Prins et al. 1995b). Indeed, even patients cured of bacterial infections are at immediate risk from Gram-negative sepsis. Therefore, there is substantial interest in identifying novel strategies to overcome Gram-negative sepsis, especially given the disappointing results obtained with certain other therapies such as TNF-α and LPS antibodies (reviewed in Glauser et al 1994).

1.2.2 Gram-positive sepsis

Gram-positive sepsis can be caused by the release of bacterial cell wall components such as lipoteichoic acid (LTA), peptidoglycan (PG), rhamnose-glucose polymers made by *Streptococci*, or capsular polysaccharides made by *Staphylococci* (Heumann et al. 1994; Mattsson et al. 1994; De Kimpe et al. 1995; Soell et al. 1995; Soell et al. 1995; English et al. 1996; Middelveld and Alving 2000). Two of the major Gram-positive cell wall components that are known to stimulate

the production of inflammatory mediators are PG and LTA. PG is an essential constituent of the Gram-positive cell wall whereas LTAs are associated with the cell walls of most, but not all, Gram-positive bacteria (Fischer 1988; Fischer et al. 1990). PG is usually a polymer of alternating GlcNAc and MurNAc residues with tetrapeptide side chains, cross-linked in Gram-positive bacteria by short peptides. LTAs are amphipathic compounds which typically consist of a repeating glycerol phosphate backbone that is substituted with D-alanine, sugars such as glucose, and a single lipid side chain that intercalates into the cytoplasmic membrane (Fischer et al. 1990). Both LTA and PG are released spontaneously into the culture medium during growth of Gram-positive bacteria (Soto et al. 1996). Moreover, β-lactam antibiotics such as penicillin enhance the release of LTA and PG (Horne and Tomasz 1979; van Langevelde et al. 1998). Thus, the release of LTA and PG from Gram-positive bacteria may promote septic shock during bacterial infections and during subsequent antibiotic treatment. When injected into animals, these Gram-positive cell wall components can elicit many of the characteristic features of septic shock including cytokine production, leukocytopenia, circulatory failure, multiple organ dysfunction syndrome (MODS) and mortality (Natanson et al. 1989; Wakabayashi et al. 1991; De Kimpe et al. 1995; Le Roy et al. 1996; Takada et al. 1996; Kengatharan et al. 1998). PG has also been shown to enhance the toxicity of endotoxin in animals (Takada et al. 1996). The increasing incidence of Gram-positive-induced septic shock (Bone 1994) indicates that there is a need to develop therapeutic strategies to prevent the activation of inflammatory cells by components of Gram-positive cell walls as well as Gram-negative cell walls.

Bacterial DNA has also been found to induce septic conditions including the production of TNF-α both *in vitro* and *in vivo* (Sparwasser et al. 1997). Bacterial DNA differs from mammalian DNA by having a much higher content of unmethylated cytosine-guanosine dimer sequences

(CpG DNA). In mammalian DNA there are less CpG dinucleotides in the sequence and they are methylated. The vertebrate immune system has evolved pattern recognition molecules that recognize such "CpG motifs" as foreign, and trigger protective immune responses (Krieg 2000).

1.2.3 Mechanism of sepsis

Both LPS and LTA, components of Gram-negative and Gram-positive bacteria interact with host cells via CD14, a 55 kDa protein that exists as a soluble form in blood and as a GPI-linked molecule on the surface of monocytes and macrophages (Wright et al. 1990; Weingarten et al. 1993; Arakaki et al. 1998). The interaction of LPS but not LTA with immune cells usually involves an acute-phase reactant protein, LPS-binding protein (LBP). LBP is a 60 kDa glycoprotein that forms high-affinity complexes with LPS (Tobias et al. 1986). LBP has been found to enhance LPS-macrophage interaction but it is not absolutely necessary. LBP binds LPS, and transfers it to CD14 (Tobias et al. 1994; Tobias and Ulevitch 1994; Wright 1995). LPS•CD14 complexes are thought to initiate intracellular signaling reactions by binding to Toll-like receptors (TLRs) on macrophages and other cells (Poltorak et al. 1998; Poltorak et al. 1998; Chow et al. 1999; Hoshino et al. 1999; Qureshi et al. 1999; Ulevitch 1999). LPS•CD14 complexes activate the NF-κB transcription factor as well as the ERK, JNK and p38 mitogen-activated protein kinases, all of which mediate the production of inflammatory cytokines (Lee et al. 1994; Ulevitch and Tobias 1994; DeFranco et al. 1998; Ulevitch and Tobias 1999). LTA has also been shown to bind CD14 (Cleveland et al. 1996; Arakaki et al. 1998; Sugawara et al. 1999) and signal through TLR4 (Takeuchi et al. 1999). Recent work has shown that bacterial products may signal through more than one TLR. For example, TLR2 and TLR4 both recognize bacterial peptidoglycan and the physical association between these two receptors leads to cytokine production (Ozinsky et al. 2000). While LPS appears to signal via TLR4 homodimers (Ozinsky et al. 2000), it is not known

whether LTA signals via TLR4 homodimers or via heterodimers consisting of TLR4 and another TLR. In contrast to LPS and LTA, recent work has shown that CpG DNA activates macrophages via TLR9 (Hemmi et al. 2000). TLRs signal via activation of NF-κB and mitogen-activated protein kinases, which in turn regulate the production of inflammatory cytokines. However, it appears likely that different TLR complexes activate distinct, but overlapping sets of signaling pathways which allows the cells of the innate immune system to tailor the response to the type of pathogen present (Ozinsky et al. 2000).

1.2.4 Mediators of sepsis

The physiological mechanism whereby bacteria and their products exert their effect on humans involves the release of multiple cytokines, including TNF-α, IL-1, IL-6, IL-8, IL-10, IL-15, IFN-α/β, and TGF-β (Koch 1998, van der Poll 2001). Bacterial infections, as well as antibiotic treatment, cause the release of bacterial cell wall components such as LPS, LTA and PG. These cell wall components stimulate the production of pro-inflammatory cytokines by macrophages. Local production of these inflammatory mediators recruits leukocytes to sites of bacterial infection and this is essential for combating these infections. This series of host inflammatory responses is designed to protect the host from infection. In sepsis, the generation of cytokines can themselves act as stimuli for production of additional inflammatory mediators, triggering an amplification cascade response, and resulting in systemic inflammation and tissue destruction. Systemic production of these cytokines, in particular TNF-α, during sepsis can cause systemic responses that lead to septic shock and death. TNF-α plays a significant role in sepsis and exhibits immense diversity of cellular responses mediated through membrane receptors present on nearly all cell types. Thus, both antibacterial agents, as well as agents that can limit the inflammatory response initiated by bacterial infections, are important classes of therapeutic

agents. Cationic antimicrobial peptides appear to have these properties, which indicates their potential therapeutic value in infection and sepsis.

1.3 Role of cationic peptides in the host response

There is emerging evidence for the role of cationic peptides in host defenses (Gudmundsson and Agerberth 1999; Hancock and Diamond 2000). In mammals, neutrophils are the "kamakaze" fighters of the body in that they are recruited to sites of infection, have a short half life (aproximately 14 hours) in the body and contain very potent antimicrobial activities that include both oxidative (i.e. production of toxic oxygen species, including free radicals, peroxide and hypochlorite) and non-oxidative killing mechanisms. In the latter category, defensins are the most predominant protein species in mammalian neutrophils, representing nearly 15% of total protein in these dedicated anti-infective cells. Other antimicrobial peptides are produced and secreted by other cells including epithelial cells, macrophages, paneth cells, keratinocytes etc., and are found at mucosal and epithelial surfaces and in the gut, lungs, kidneys and skin. Their induction during inflammation is consistent with a primary role in assisting and/or directing inflammatory responses (Bals et al 1999; Diamond et al 1996; Frohm et al 1997).

1.3.1 Ability of cationic peptides to kill pathogens

Cationic peptides have an unusually broad spectrum of antimicrobial activity. This includes an ability to kill or neutralize Gram-negative and Gram-positive bacteria, fungi, helminths (planaria and nematodes), protozoa (trypanosomes and plasmodia), and even enveloped viruses like human immunodeficiency virus (HIV) and herpes simplex virus (HSV). For example, HNP-1 was found to directly inactivate HSV types 1 and 2, cytomegalovirus, vesicular stomatitis virus, and influenza virus A/WSN, but not nonenveloped viruses such as echovirus type 11 and reovirus type 3 (Daher et al. 1986). Cationic peptides appear to be selective in that normal host cells are

relatively resistant to their action although certain peptides, e.g. melittin from bees, mastoparan from wasps and charybdotoxin from scorpions, are potent toxins. The basis of discrimination for the relatively non-toxic peptides appears to be the lipid composition of the target membrane. Selective peptides tend to prefer bacterial membranes that are negatively charged and free of cholesterol and which possess a large transmembrane electrical potential gradient (oriented internal negative) (Hancock and Chapple 1999). The activities of antimicrobial peptides can be reduced by a variety of relevant factors *in vivo*, including high mono- and di- valent cation concentrations, polyanions, serum, apolipoprotein A-1, serpins and proteases (Andreu and Rivas 1998; Hancock and Lehrer 1998). However, some peptides appear quite resistant to several of these agents.

The importance of cationic peptides in the host immune response to infection can be demonstrated. For example, in *Drosophilia* and other insects, peptides are produced in the fat body in response to infection or to microbial products. In *Drosophilia* it is possible to create knockouts in specific genes that control the production of several cationic peptides, in order to demonstrate the importance of these peptides in combating pathogens (Schneemann and Schaffner 1999). A knockout of the Toll receptor gene (part of the pathway of activation of the NF-κB-like transcriptional activator Dorsal, and a homologue of the TLR-receptor family in mammals), decreases the production of anti-fungal peptides like drosomycin, and makes *Drosophilia* more susceptible to the filamentous fungus *Aspergillus fumigatus*, but not to the bacterium *E. coli* (Schneemann and Schaffner 1999). Conversely, disruption of the Imd (for immune deficiency) gene affects the expression of multiple antibacterial peptides and permits three orders of magnitude more *E. coli* to grow in *Drosophilia* within the first 24 hr, without affecting susceptibility to *A. fumigatus*.

Other examples of the antimicrobial activities of cationic peptides include a wide variety of animal model studies and early clinical trials, which have demonstrated that exogenously administered natural or synthetic cationic antimicrobial peptides protect the host against local or systemic infection by bacteria and fungi (Ahmad et al. 1995; Gough et al. 1996; Steinberg et al. 1997; Gamelli et al. 1998; Kirikae et al. 1998; Loury et al. 1999; Ruissen et al. 1999). Such studies have confirmed the antibacterial (vs. both Gram-negative and Gram-positive bacteria), anti-fungal, synergistic and anti-endotoxic nature of antimicrobial peptides. Several of the synthetic cationic peptides exhibited significant protection in animals against an LD_{90} dose of the mouse pathogen, *P. aeruginosa* strain M2 (Scott et al. 1999). Recent animal model data showed systemic protection by the bacterial lantibiotic (peptide antibiotic containing the unusual amino acids lanthionine and β-methyl-lanthionine), nisin, against *Streptococcus pneumoniae* infections of mice, protection by the pig protegrin 1 against systemic *P. aeruginosa* and *S. aureus* infections and by protegrin-related peptide IB-367 against polymicrobic oral mucositis in hamsters (Loury et al. 1999). Other studies have demonstrated protection against lethal *P. aeruginosa* infections of burn wound sites in mice by the synthetic peptide D4B (Gamelli et al. 1998), and protection by the human peptide LL-37 against lethal endotoxaemia and *P. aeruginosa* infection in mice (Bals et al. 1999). Studies in coho salmon demonstrated that peptides, including the flounder peptide pleurocidin and an insect-derived cecropin-melittin hybrid peptide, were protective when given sustained parenteral administration using an osmotic pump, but were not protective when given as a single bolus injection (Jia et al. 2000). Thus the antimicrobial activity of cationic peptides has been demonstrated in a number of animal models.

Another method for demonstrating the *in vivo* activity of antimicrobial peptides is to utilize transgenic models that overexpress these peptides, thus providing increased resistance to

infections. The most detailed studies on such transgenic species have been done in plants where a rich body of data attests to the ability of hyper-expressed natural plant peptides, derived from both plant and animal species, to protect against fungal or bacterial infections (Garcia-Olmedo et al. 1998). Success in such studies appears to be a function of promoter strength (since excessively high expression can be toxic) and the lability of the peptides to plant proteases. In mammals, overexpression by adenovirus-mediated gene transfer of the human cathelicidin gene for LL-37 in the mouse airway results in the increased ability to reduce bacterial load from *P. aeruginosa* challenge and improved survival after administration of lethal doses of endotoxin (LPS) or *E. coli* (Bals et al. 1999). These studies are consistent with those demonstrating that mice made transgenic for a synthetic α-helical peptide related to the moth cecropin family Shiva 1a (Reed et al. 1997) are resistant to infection by *Brucella abortus*. Gropp et al expressed the human defensins, HBD-1 and HD-5 in eukaryotic cell lines and found the defensins localized to the cytoplasm and in the cell culture medium (Gropp et al. 1999). Overexpression of these defensins resulted in increased resistance to viral infections and infections by Gram-positive or Gram-negative bacteria. In plants, up-regulation of antimicrobial peptide gene expression also increases their resistance to pathogens (Broekaert et al. 1995). Although equivalent studies cannot be done in humans, it has been observed that patients with specific granule deficiency syndrome, completely lacking in α-defensins, suffer from frequent and severe bacterial infections (Ganz and Lehrer 1995; Andreu and Rivas 1998). Similarly a group of HIV patients with lower salivary levels of histatin peptides show a higher incidence of oral candidiasis and fungal infection (Andreu and Rivas 1998).

In vitro expression studies predict that animals with infectious and/or inflammatory conditions would exhibit an up-regulation of antimicrobial peptides. Indeed, increased levels of

antimicrobial peptides are observed in a number of clinical and laboratory-induced infectious and inflammatory states. Pigs infected by *Salmonella choleraesuis* exhibit a 3-fold increase in circulating PR-39 levels 10 to 14 days post-infection (Zhang et al. 1997). Similarly, patients with bacterial pneumonia had plasma levels of HBD-2 that were elevated four-fold (Hiratsuka et al. 1998). Experimentally induced infection of calves with *Cryptosporidium parvum* increased enteric β-defensin levels by 5-10 fold, as well as increasing the levels of other defensins (Tarver et al. 1998). Cows testing positive for infection with *Mycobacterium paratuberculosis* exhibited increased expression of β-defensins (Stolzenberg et al. 1997). Similar up-regulation of defensins in the bronchial epithelium was experimentally induced by intratracheal instillation of *Pasteurella haemolytica* into a single lobe of a cow lung, where an increase in β-defensin expression in the airway epithelium correlated with the infection (Stolzenberg et al. 1997). Similarly, intratracheal instillation of *P. aeruginosa* was sufficient to increase expression of mouse β-defensin 3 (mBD3) in the tracheal epithelium as well as in the small bowel and liver (Bals et al. 1999). In humans, inflamed intestinal epithelium exhibits very high (up to hundreds of μg/ml) levels of HBD-2 expression [relative to normal colon (O'Neil et al. 1999)], as does inflamed gingival epithelium (Mathews et al. 1999).

Genetic manipulation experiments have supported the above evidence of a role for antimicrobial peptides in host defense, though these experiments are complicated by the numerous defensin genes often expressed in the same tissues, as well as redundant defense mechanisms within the innate immune system. Most recently, an innovative technique was devised to address whether multiple defensin genes are redundant. Rather than knock-out all 20 defensins expressed in the mouse small intestine, Wilson et al. identified the single enzyme necessary for processing the preprodefensins to the active form. Genetic inactivation of this single

gene, matrilysin, prevented production of active defensin in the small intestine, and consequently led to a ten-fold increase in susceptibility to infection by virulent bacteria introduced by the oral route (Wilson et al. 1999). A recent study generated mice that were null for *Cnlp*, the gene encoding the murine cathelicidin, CRAMP. The CRAMP-deficient mice had much larger and more persistent lesions from Group A *Streptococcus* than their normal littermates and these effects appeared to be due to the lack of peptide as they had a normal recruitment of an acute neutrophil inflammatory response (Nizet et al. 2001).

1.3.2 Synergy of cationic peptides with host molecules

The innate immune response involves several different elements, including phagocytic cells such as neutrophils, macrophages and monocytes, and proteins such as complement, lysozyme, and cationic peptides. These elements have been designed to work in synergy. For example, complement activation can lead to bacteriolysis, but also serves to generate opsonins on the surface of bacteria to enhance phagocytosis. Similarly, although cationic antimicrobial peptides kill microbes independently, their killing ability can be improved in synergy with other factors. The ability of individual peptides to work synergistically with other peptides is well documented. This was first demonstrated with the frog peptides magainin and PGLa (Matsuzaki et al. 1998), and has recently been confirmed with a variety of peptides, including the mammalian peptides protegrin 1 and indolicidin (Scott and Hancock 2000). Thus, study of the kinetics of peptide interaction shows that they act cooperatively.

In addition, cationic peptides can work synergistically with lactoferrin, lysozyme and other proteins that are present in body fluids or tissues. For example, LL-37, which is co-localized in the secretory granules of neutrophils (Sorensen et al. 1997) and in the serous cells of submucosal glands of the lung (Bals et al. 1998) also demonstrates synergy with lactoferrin *in vitro* (Bals et al.

1998). It was also demonstrated that peptides promote the ability of lysozyme to lyse Gram-negative bacteria, and that there is excellent synergy between lysozyme and a range of peptides against several bacteria (Scott and Hancock 2000). Lysozyme is a slightly basic enzyme that is excluded from its target, peptidoglycan, by the outer membrane. Since lysozyme is present in most parts of the body, it is possible that such synergy is important to the action of cationic antimicrobial peptides in their natural hosts. For example, in fish challenged with bacteria, both lysozyme and cationic peptides appear to be rapidly induced (Patrzykat et al. 2001). The basis for synergy appears to be related to the mechanism by which cationic antimicrobial peptides interact with bacteria. In the case of Gram-negative bacteria, the outer membrane protects the peptidoglycan from degradation, and consequent cell lysis, by lysozyme. Cationic peptides interact with the polyanionic LPS, at sites on the LPS where divalent cations usually bind to cross bridge adjacent LPS molecules and stabilize the outer membrane (Piers et al 1994; Piers and Hancock 1994; Hancock 2001). The competitive displacement of these divalent cations (Mg^{++} and Ca^{++}) by the bulkier peptides leads to lesions in the outer membrane, permitting uptake of the permeabilizing polycationic peptide as well as other molecules, such as lysozyme.

1.3.3 Cationic peptides can modulate the host inflammatory response to sepsis

The pathogenesis of sepsis is associated with the systemic release of pro-inflammatory cytokines. Cationic peptides act on Gram-negative bacteria by initially binding to their surface polyanionic LPS. A variety of cationic antimicrobial peptides have the ability to bind LPS (Ooi et al. 1991; Hirata et al. 1994; Battafaraono et al. 1995; Levy et al. 1995; Gough et al. 1996; de Haas et al. 1998; Turner et al. 1998; Scott et al. 1999; Dankesreiter et al. 2000; Iwagaki et al. 2000). In some cases these peptides suppress the ability of LPS to stimulate the production of pro-inflammatory cytokines by macrophages (see Figure 4). Furthermore, studies with the cationic

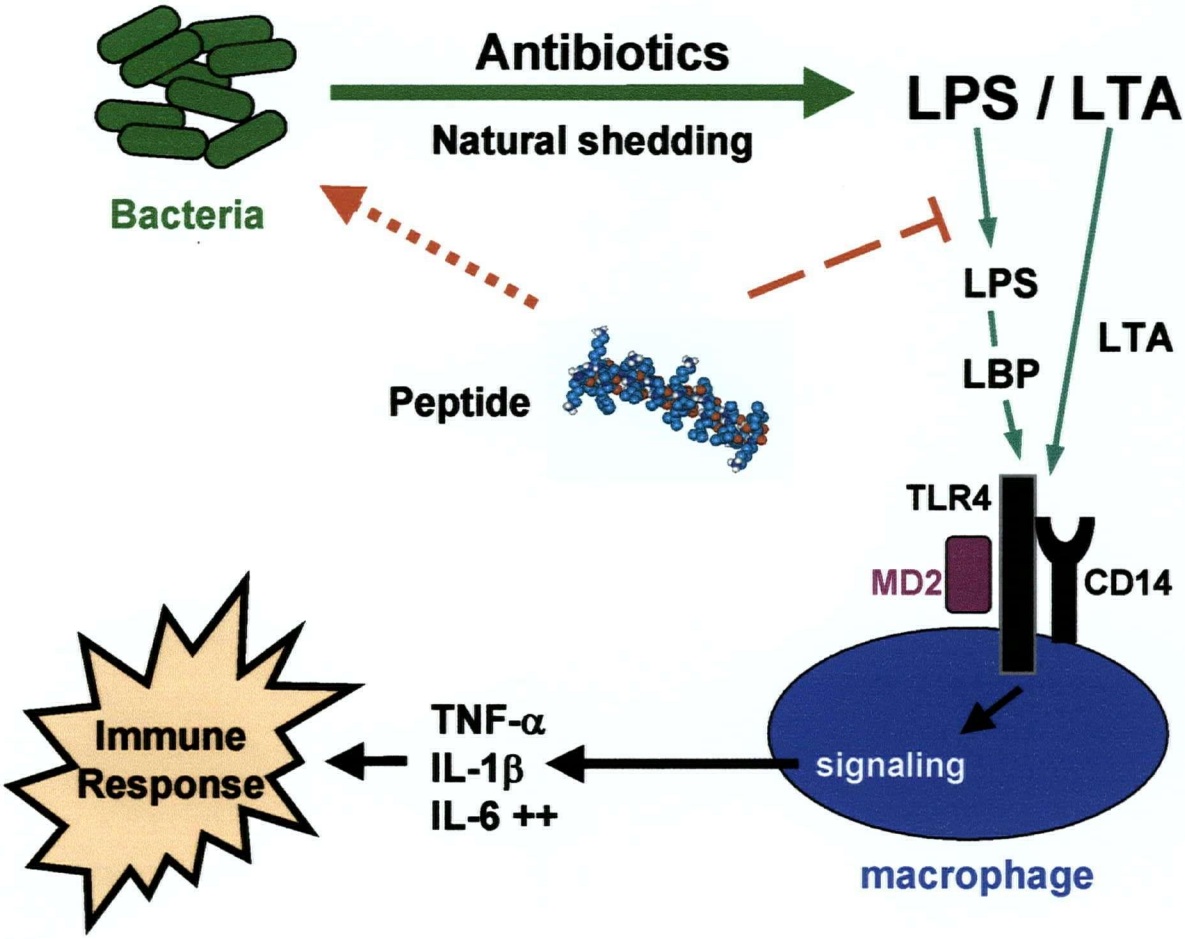


Figure 4: Hypothesized role of cationic antimicrobial peptides in neutralizing LPS- and LTA- induced production of inflammatory mediators

LPS and LTA signal an inflammatory response in part, via Toll-like receptor 4 (TLR4) macrophages. The dotted line represents the antimicrobial action of cationic peptides, and the dashed line represents the ability of cationic peptides to limit LPS- and LTA-stimulation of macrophages. The thick line represents the release of the bacterial products, LPS and LTA, from bacteria. The thin lines represent the action of LPS and LTA on macrophages. The medium black lines represent the production of cytokines by macrophages in response to LPS and LTA.

proteins bactericidal/permeability increasing protein (BPI), CAP18, CAP37 and lactoferrin showed that these molecules can antagonize LPS-induced cytokine production by macrophages (Brackett et al. 1997; Elass-Rochard et al. 1998; Hirata et al. 1994; Larrick et al. 1995; Marra et al. 1990). The cecropin-melittin hybrid peptides CEMA and CEME not only suppress cytokine production in response to LPS, but have also been shown to prevent lethal endotoxemia in the galactosamine-sensitized mouse model (Gough et al. 1996). With CEMA treatment, it was shown that the LPS-stimulated induction of the important sepsis-mediating cytokine, TNF-α, could be dramatically suppressed in the blood of galactosamine-senstized mice. Other animal model stuides have demonstrated analogous findings with diverse cationic peptides (Hirata et al. 1994; Zhang et al. 1999; Dankesreiter et al. 2000).

1.3.4 Cationic peptides have the ability to recruit inflammatory cells

Cell migration is an important host response to infection, since it is required to recruit the appropriate cells, particularly neutrophils, monocytes and lymphocytes, to the site of infection (Matsukawa et al. 2000). It is controlled by a multistep process that includes chemoattraction, cell-cell adhesion and, in some cases, transmigration through cell layers (Butcher et al. 1999). Neutrophils are important for recruitment of monocytes to sites of infection and it has been hypothesized that factors derived from neutrophils are responsible (Ward 1968; Pereira et al. 1990). CAP37, a cationic antimicrobial protein found in azurophil granules, is chemotactic for T cells, monocytes and neutrophils (Pereira et al. 1990; Chertov et al. 1996; Chertov et al. 1997). One study demonstrated that the instillation of CAP37 into rabbit airways resulted in significant monocyte infiltration in the lung (Doherty et al. 1999). CAP37 is also chemotactic for fibroblasts and can influence maturation of monocytes to macrophages (Flodgaard et al. 1991). Recently, LL-37, the human neutrophil α-helical peptide was shown to have chemotactic activity for

monocytes, T cells and neutrophils (Yang et al. 2000) as well as mast cells (Niyonsaba et al. 2002). The porcine peptide, PR-39 has Ca^{++}-dependent chemotactic activity for neutrophils that can be inhibited by pertussis toxin, which suggests that neutrophils possess a PR-39 receptor that is coupled to a G-protein pathway like other chemokine receptors (Huang et al. 1997). Similarly, guinea pig defensins are chemotactic for neutrophils but not monocytes, while the human α-defensin peptides, HNP-1 and HNP-2, stimulate chemotaxis by murine and human T cells and monocytes (Territo et al. 1989; Chertov et al. 1996). The ability of defensins to stimulate production of the chemoattractant and neutrophil activating cytokine, IL-8, in airway epithelial cells would also potentially enhance neutrophil recruitment. Conversely, IL-8 can induce the release of defensins and CAP37 when applied in conjunction with phorbol 12-myristate 13-acetate (PMA), and formylmethionyl-leucylphenylalanine (fMLP) (Chertov et al. 1996).

Human β-defensins have also been shown to be chemotactic for immature dendritic cells and memory T cells through interaction with the receptor CCR6 (Yang et al. 1999). In addition, the proform of an antimicrobial peptide, proBac7, which is released from activated neutrophils, is a monocyte-specific chemoattractant (Verbanac et al. 1993). Such chemotactic activities appear to have *in vivo* relevance since it has been demonstrated that application of the human α-defensin HNP-1 reduced *Klebsiella pneumoniae* numbers in mouse models of peritoneal infections, and this antibacterial activity was accompanied by an enhanced influx of macrophages, granulocytes and lymphocytes into the peritoneal cavity (Welling et al. 1998). The activity appeared to be mediated, in part, by leukocyte accumulation since leukocytopenic mice administrated HNP-1 did not display antibacterial activity.

Many of the cells involved in inflammatory responses come from the blood and pass through the endothelium of blood vessels. Transmigration across the blood vessel wall is

stimulated by vasodilators like histamine. Thus an important component of neutrophil recruitment is the ability of peptides such as neutrophil defensins (Befus et al. 1999), CAP-11 (Yomogida et al. 1997), LL-37 (Niyonsaba et al. 2001) and magainin-2 (Hook et al. 1990) to stimulate the activation and degranulation of tissue mast cells leading to histamine release. Magainin-2-induced release of histamine from mast cells was shown to not be a cytolytic effect of the peptide, nor was it due to the formation of anion-selective channels in the membrane of mast cells (Hook et al. 1990). Mast cells have high affinity $F_C\varepsilon$ receptors that allow them to bind IgE monomers. Thus mast cell activation and degranulation occurs through cross-linkage of surface IgE with specific antigen (Ag). Defensins were found to act in a mechanistically different fashion from Ag-IgE. They act on mast cells through a rapid G protein-dependent response that is inhibited by pertussis toxin and pre-treatment of mast cells with neuraminidase (Befus et al. 1999). This is similar to the IgE-independent activation of mast cells by venom peptides (mast cell-degranulating peptides), neuroendocrine peptides such as Substance P, vasoactive intestinal polypeptide, and somatostatin (Shanahan et al. 1985; Mousli et al. 1989; Mousli et al. 1990; Galli 1993; Befus et al. 1999). Thus these studies show that a number of cationic peptides can act as chemoattractants and in some cases through G protein-coupled receptors.

1.3.5 Other activities of cationic peptides in the immune system

There have been other strong indications that cationic peptides can interact directly with eukaryotic cells (Risso et al. 1998). Generally speaking, the interaction of most peptides with eukaryotic membranes is inhibited by the lack of negatively charged lipids on the cell surface, by the rather low membrane potential (-15mV) across the plasma membrane and by the presence of cholesterol in the plasma membranes (cf. bacteria have an abundance of anionic surface phospholipids, such as phosphatidyl glycerol and cardiolipin, and have a transmembrane potential

of –140mV and no cholesterol) (Scott and Hancock 2000). Nevertheless, there have been some indications that cationic peptides can interact with the cell surface or even enter host cells. For example, it has been shown that the peptide PR-39 rapidly enters human microvascular endothelial cells (Chan and Gallo 1998). Conversely, it was found that two bovine antimicrobial peptides of the cathelicidin family, BMAP-27 and BMAP-29, permeabilized eukaryotic cell membranes, after interacting with negatively-charged sialyl residues at the membrane surface, and caused Ca^{2+} flux into the cytosol (Risso et al. 1998). It has also been shown that defensins are potent protein kinase C inhibitors (Charp et al. 1988) suggesting an intracellular target. It is also known that some tumor cell lines can be killed by cationic peptides at relatively high concentrations. Such peptides are selectively more toxic towards tumour cells than towards non-malignant cells, although the mechanism of their activity is not fully understood (Lichtenstein et al. 1988; Lichtenstein et al. 1988; Cruciani et al. 1991; Ohsaki et al. 1992; Baker et al. 1993; Andersson et al. 1995; Risso et al. 1998). In some of these cases, the anti-tumor activity of the cationic peptides can be improved by acting in synergy with host molecules. For example, defensins, can act synergistically with hydrogen peroxide (also released from neutrophils) to lyse erythroleukemia targets (Lichtenstein et al. 1988).

Defensins interfere with the activation of neutrophil superoxide-generating NADPH oxidase (Tal and Aviram 1993; Tal et al. 1998). In some cases this property of defensins may have a negative effect. Reactive oxygen intermediates generated by the phagocyte NADPH oxidase are critically important components of host defence however; they are highly toxic and can cause significant tissue injury during inflammation. It is therefore important that their generation and inactivation are tightly regulated. For example, the implantation of foreign materials into the body results in the production of oxygen free radicals by activated neutrophils (Kaplan et al. 1992),

which protects the body from infection. Defensins appear to down-regulate the ability of the neutrophils to generate superoxide, and consequently inhibit host defenses at the site of the foreign implant (Kaplan et al. 1999). PR-39 has also been shown to inhibit NADPH oxidase activity, which it does by blocking the assembly of this enzyme, through interactions with Src homology 3 domains of the p^{47phox} cytosolic oxidase component (Shi et al. 1996). This indicates that PR-39 might also play a role in limiting excessive tissue damage during inflammation. PR-39 has also been shown to block reactive oxygen production by cultured endothelial cells and isolated, perfused rat lungs (Al-Mehdi et al. 1998). The reactive oxygen species produced by neutrophils are thought to contribute to the pathogenesis of ischemia reperfusion injury of a variety of organs. It has been demonstrated that PR-39 blocks postischemic oxidant production and venular protein leakage in rat mesenteries subjected to ischemia-reperfusion (Korthuis et al. 1999).

Another important property of cationic peptides that may allow them to modulate the immune response is the ability to inhibit certain proteases that otherwise cause injury to host cells as well as the degradation of the extracellular matrix (Mainardi et al. 1980; Mainardi et al. 1980; Mendis et al. 1990; Okrent et al. 1990; Amitani et al. 1991; Brown et al. 1992). For example histatins, histidine-rich peptides found in human saliva, inhibit the proprotein convertases (serine endoproteases belonging to the kexin/subtilisin family) furin and PC7 (Basak et al. 1997), while defensins can inactivate serpins (Panyutich et al. 1995). A cysteine proteinase thought to contribute to tissue injury in inflammation, cathepsin L, was shown to be inhibited *in vitro* by the neutrophil propeptide, proBac5 (Verbanac et al. 1993). Another study demonstrated that interactions between defensins and serine proteases released from neutrophils could regulate neutrophil-mediated injury at sites of inflammation (Van Wetering et al. 1997). For example,

defensins were found to inhibit serine protease-induced detachment of lung epithelial cells. Conversely elastase and cathepsin G both reduced IL-8 induction by defensins without affecting TNF-α-induced synthesis of IL-8 and there was no inhibitory effect of cathepsin G on the antibacterial activity of defensin (Van Wetering et al. 1997). Defensins can also be inhibited by serum proteinase inhibitors such as α_1-proteinase inhibitor (α_1-PI) (Panyutich et al. 1995) which could also serve to limit the activity of defensins. These proteinase inhibitors also serve to limit the activity of elastase and cathepsin G. Thus there appears to be complex interactions between proteases and cationic peptides at sites of infection which may serve to regulate both the proteases and cationic peptides.

Cationic peptides have also been suggested to stimulate wound healing, through promotion of re-epithelialization of damaged surfaces. This may be related in part to their mitogenic (growth promoting) activity towards epithelial cells and fibroblasts, observed with defensins at concentrations expected to be present *in vivo* during the wound healing process (Murphy et al. 1993). Defensins have also been shown to increase DNA synthesis of the leukemic cell line HL-60 (Bateman et al. 1991). Proliferative effects of α-helical peptides were seen with murine fibroblast cells (SB-37) and preimplantation embryos (Reed et al. 1992). In addition to this activity, PR-39 has been demonstrated to induce the synthesis of syndecans (major cell surface heparan and chondroitin sulfate proteoglycans), in a manner similar to that seen during wound repair (Gallo et al. 1994). Syndecans are required for cellular responses to heparin-binding growth factors and extracellular matrix components. PR-39, which was isolated from fluid accumulating in cutaneous wounds undergoing repair, was found to specifically induce the expression of syndecan-1 and syndecan-4 mRNA in cultured fibroblasts and epithelia (Gallo et al. 1994). The induction of syndecans could also contribute to the antimicrobial activity of PR-39 as bacteria

bind heparin sulfate and killing may be facilitated by immobilization of this molecule on the host cell surface. The induction of syndecan-1 by PR-39 was also found to decrease the invasiveness of human hepatocellular carcinoma cells (hepatocellular carcinomas have previously been shown to have reduced syndecan-1 expression and high metastatic potential) (Ohtake et al. 1999). Conversely the above-mentioned activities in cell recruitment, if applied to epithelial cells and fibroblasts, e.g. as seen with CAP37 chemoattraction of fibroblasts (Flodgaard et al. 1991), may be a component of the wound healing process.

1.4 Aims of this study

The importance of cationic antimicrobial peptides in the immune system is just beginning to be understood. Previous studies with the synthetic CEME-related peptides have demonstrated their potent anti-endotoxin activity. Since there is an increasing incidence of Gram-positive sepsis, the first goal of this thesis was to ascertain if the cationic peptides could reduce the production of TNF-α in response to components of Gram-positive bacteria. I hypothesized that since cationic peptides have an affinity for LPS, they could also bind LTA, a component of Gram-positive bacteria and thus reduce its ability to stimulate macrophages. Macrophage model systems commonly used to study anti-endotoxic compounds were utilized in these studies. The second goal of these studies was to ascertain the mechanism by which cationic peptides reduce stimulation of macrophages by LPS and possibly LTA. This was done in two parts. First, cationic peptides were tested for their ability to block the interaction of LBP (an important catalyst in LPS signaling) with LPS and if applicable LTA. Second, gene arrays were used to study the effect of cationic peptides on LPS-induced gene expression changes in macrophages. The third goal of this thesis was to apply knowledge gained from studying synthetic cationic peptides to further understanding the natural function of cationic peptides in the immune system. The up-regulation of certain cationic peptides in response to bacterial infection suggest that cationic peptides play an important role in the immune response to the bacteria. I hypothesized that LL-37, which is similar in structure to the CEME-related peptides, widely expressed in humans and up-regulated in response to bacteria would have the ability to modify macrophage responses to bacterial components. These studies led to a more detailed examination of the direct effect of cationic peptides on macrophages and further definition of the role of cationic peptides in the immune system. Thus, the main goal of this thesis was to understand the role of cationic peptides in the host immune response to bacterial infection and discover attributes which could be applied to the

design of new therapies to combat bacterial infection.

The results reported here have helped to further define the role of cationic antimicrobial peptides in the immune response and have resulted in a number of publications listed in Appendix C. They have also been presented orally at several international and national meetings. In addition, a number of the cationic peptides designed for this study and their potential therapeutic uses have been filed for patent protection (see Appendix C).

CHAPTER 2: MATERIALS AND METHODS

2.1 Cationic peptides

Most of the cationic peptides were synthesized at the Nucleic Acid/Protein Synthesis (NAPS) Unit at the University of British Columbia by Fmoc [(N-(9-fluorenyl) methoxycarbonyl)] chemistry using an Applied Biosystems Model 431 peptide synthesizer. The purity of the cationic peptides was found to be greater than 95% by HPLC analysis and Mass Spectrometry. Gramicidin S and human neutrophil peptide-1 were purchased from Sigma (St. Louis, MO). Human β-defensin-2 was kindly provided by Dr. Tomas Ganz (University of California, Los Angeles, CA). The amino acid sequences of the peptides appear in Table 1 using the single letter amino acid code.

2.2 Bacterial products and bacterial strains

All of the bacterial components used in this study are listed in Table 2. *E. coli* 0111:B4 (smooth) and *E. coli* J5 (rough mutant of 0111:B4) LPS were purchased from Sigma. *E. coli* O55:B5 LPS was also purchased from Sigma and biotinylated using biotin-LC-hydrazide (Pierce, Rockford, IL) according to the manufacturer's instructions. *Salmonella enterica* serovar Typhimurium LPS was purchased from Sigma. LPS was prepared (by hot phenol extraction) from *Burkholderia cepacia* isolated from the inner tubings of a STATIM^{TM} (SciCan, Mississauga, Ontario) sterilizer reservoir during an outbreak of Diffuse Lameller keratitis (DLK) (Morck et al. 2002). LTA from *S. aureus*, *S. pyogenes* and *B. subtilis*, as well as PG peptide from the cell wall of *S. aureus* (D-ala-Isoglutaminyl-L-lys-D-ala-D-ala) were purchased from Sigma. Peptidoglycan from *Micrococcus luteus* was purchased from Wako (Osaka, Japan). LTA and PG were resuspended in endotoxin free water (Sigma). The Limulus amoebocyte lysate assay (Sigma) was

Table 1: Peptide amino acid sequences

Peptide	Structure	Amino acid sequence [a]
Human β defensin-2	β-sheet	TCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP
Human neutrophil peptide-1	β-sheet	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
Gramicidin S	β-structured loop	cyclic (PFdLOVPFdLOV)
Polymyxin B	Cyclic lipopeptide	cyclized isooctanoyl BTBB(BF^{d}LBBT)^{c}
Indolicidin	Extended	ILPWKWPWWPWRR-NH_2
CP11CN[b]	Extended	ILKKWPWWPWRRK-NH_2
Bac 2A[b]	Linear	RLARIVVIRVAR-NH_2
LL-37	α-helical	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
CEME[b]	α-helical	KWKLFKKIGIGAVLKVLTTGLPALIS
CEMA[b]	α-helical	KWKLFKKIGIGAVLKVLTTGLPALKLTK
CP29[b]	α-helical	KWKSFIKKLTTAVKKVLTTGLPALIS
CP26[b]	α-helical	KWKSFIKKLTSAAKKVVTTAKPLISS
CM5[b]	α-helical	KLFKKIGIGAVLKVLTTGLPALKLTK
CM7[b]	α-helical	KLWKLFKKIGIGAVLKVLTTGLPALKLTK
CPα2[b]	α-helical	KWKKFIKKIGIGAVLKVLTTGLPALKLTKK
CP203[b]	α-helical	KWKSFIKKLTSAAKKVLTTGLPALIS
CP207[b]	α-helical	KWKSFIKKLTSVLKKVVTTAKPLISS
CP208[b]	α-helical	KKKSFIKLLTSAKVSVLTTAKPLISS

[a] Single letter amino acid code: O, ornithine; B, diaminobutyrate; F^{d}, the D enantiomer of phenylalanine.

[b] Synthetic peptides

[c] Sequences in parentheses are cyclized

Table 2: Bacterial components

Strain	Description	Reference/Source
LPS	*B. cepacia*	Dr. Simon Holland (The Eye Care Center, UBC)
	Salmonella Typhimurium	Sigma
	E. coli 0111:B4	Sigma
	E. coli J5	Sigma
	E. coli 055:B5	Sigma
	E. coli 055:B5 biotinylated	A. Vreugdenhil (Maastricht University)
LTA	*S. aureus*	Sigma
	B. subtilus	Sigma
	S. pyogenes	Sigma
PG	PG peptide from the cell wall of *S. aureus* (D-ala-Isoglutaminyl-L-lys-D-ala-D-ala) *Micrococcus luteus*	Sigma Wako
AraLAM	Non-capped lipoarabinomannan isolated from a rapid growing *Mycobacterium* species	Dr. John T. Belisle (Colorado State University)
ManLAM	Mannose-capped lipoarabinomannan isolated from *M. tuberculosis* $H_{37}R_v$	Dr. John T. Belisle (Colorado State University)

performed on the LTA and PG preparations to confirm that lots were not substantially contaminated by endotoxin. The levels of endotoxin found in the preparations were less than 1 ng/ml, and this concentration of endotoxin did not cause significant cytokine production (<0.2 ng/ml) by the RAW 264.7 cells. Heat-killed *S. aureus* was produced by boiling the cells for 10 minutes and then washing them three times with PBS. The efficacy of the heat treatment was confirmed by culturing the bacteria overnight to ensure that there was no growth. AraLAM (non-capped lipoarabinomannan) and ManLAM (mannose-capped lipoarabinomannan) from *Mycobacterium* were gifts from Dr. John T. Belisle of Colorado State University. The AraLAM was filter sterilized and the endotoxin contamination was found to be 3.8 ng per 1.0 mg of LAM as determined by Limulus amebocyte assay. ManLAM was filter sterilized and the endotoxin contamination was found to be 3.7 ng per 1.0 mg of LAM.

The bacterial strains used in this study were grown on Meuller-Hinton medium supplemented with 1.5% (w/v) agar, with the exception of *S. pyogenes*, which was grown on Todd Hewitt medium. The strains used were *Staphylococcus aureus* RN4220, ATCC 25293, and SAP0017-MRSA as well as clinical isolates received from Dr. A. Chow (Dept. Medicine, UBC), *Staphylococcus epidermidis* (a clinical isolate from Dr. A. Chow), *Streptococcus pyogenes* ATCC 19615, *Enterococcus facealis* ATCC 29212, *Bacillus subtilis* (lab strain), *Listeria monocytogenes* NCTC 7973, *Cornyebacterium xerosis* (lab strain), *Escherichia coli* UB1005 (Rocque et al. 1988) and *Burkholderia cepacia* strain ATCC25416.

2.3 Purification of recombinant LBP (rLBP)

CHO cells expressing human recombinant LBP were a gift from Dr. Peter Tobias (Scripps Research Institute, La Jolla, CA). LBP was purified by selective affinity immunosorption, from the culture medium as described previously (Vreugdenhil et al. 1999). The concentration of

purified rLBP was determined using a sandwich ELISA described by Froon et al. (Froon et al. 1995).

2.4 Determination of minimum inhibitory concentration (MIC)

The MIC of each peptide for a range of microorganisms was determined by the modified broth dilution method (Wu and Hancock 1999). Experiments were done using Mueller-Hinton medium (except *S. pyogenes* which required Todd Hewitt medium) in 96-well polypropylene microtitre plates (Costar, Cambridge, MA). Wells were inoculated with 10 µl medium containing approximately $2x10^6$-10^7 CFU/ml of the test organism. Samples of the bacterial innoculum were plated to ensure they were within this range. The MIC was determined after 18 h incubation of the plates at 37°C. The MIC was visually assessed as the lowest concentration of cationic peptide which inhibited bacterial growth.

2.5 Determination of peptide LPS and LTA binding affinity

The relative binding affinity of each peptide for LPS was determined using the dansyl polymyxin B binding assay described previously by Moore et al (Moore et al. 1986). The fluorescence was measured in a Perkin-Elmer 650-10S fluorescence spectrophotometer with an excitation wavelength of 340 nm and an emission wavelength of 485 nm using slit widths of 5 nm. A concentration of dansyl polymyxin B giving 90-100% maximum fluorescence (2.5 µM) was chosen and used in the experiments. Dansyl polymyxin B and *E. coli* O111:B4 (300 µg/mL), J5 LPS (300 µg/mL) or *S. aureus* LTA (5 µg/mL) were mixed in 1 ml of 5 mM HEPES (pH 7.2). This resulted in >90% of maximum fluorescence as measured by the fluorescence spectrophotometer. The decrease in fluorescence due to dansyl polymyxin B displacement was recorded upon the progressive addition of aliquots of 5-10 µl of each of the peptides. Addition of the peptide continued until it resulted in only a small (<5%) decrease in fluorescence. The data

were plotted as the percent displacement of the dansyl polymyxin B as a function of peptide concentration.

2.6 Measurement of LPS-LBP interactions

The anti-LBP MAb HM14 (Vreugdenhil et al. 1999), which recognizes both unbound human LBP and LBP•LPS complexes, was diluted to 10 μg/ml in PBS and adsorbed onto 96-well Nunc MaxiSorp ELISA plates (Nunc Corp., Rochester, NY) overnight at 4°C. Plates were blocked at room temperature for 1 h with 1% BSA in PBS and washed with 0.1% Tween 20 in dH_2O. rLBP (50 ng/ml) diluted in PBS / 0.1% BSA was added to the plates for 1.5 h at room temperature. After washing the plates, biotinylated LPS was added in the presence or absence of cationic peptides. Where indicated, the peptides were either preincubated with biotinylated LPS for 30 min or added to the wells at various times after the addition of biotinylated LPS. In all cases, the plates were washed 1 h after the addition of LPS. Binding of the biotinylated LPS to the immobilized LBP was detected using horseradish peroxidase-conjugated streptavidin diluted 1:2000 in PBS, 0.1% BSA. After a 1 h incubation, TMB (3,3',5'5-tetramethylbenzidine) was added as a substrate and the reactions were allowed to proceed for 15 min before being stopped by the addition of 0.18 M sulfuric acid. The absorbance at 450 nm was determined using a Molecular Devices model Vmax ELISA reader.

2.7 Cell lines and cell maintenance

The murine macrophage cell lines RAW 264.7 and J774.1 were obtained from American Type Culture Collection (Manassas, VA). The human monocytic cell line THP-1 was obtained from Dr. Rick Stokes (UBC). The human epithelial cell line A549 was obtained from Dr. David Speert (UBC). The RAW 264.7 and J774.1 macrophage cell lines and the A549 human epithelial cell line were maintained in DMEM (Dulbecco's modified Eagle medium) complete medium,

which is DMEM (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum ((FBS), 2.4 mM L-glutamine, 60 U/ml penicillin, and 60 μg/ml streptomycin (GIBCO). The THP-1 cell line was grown in suspension and maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 10% FBS and 1.0 mM sodium pyruvate. The cell lines were incubated at 37°C and 5% CO_2 in 175 cm^2 cell culture flasks (Costar, Cambridge, MA) and passaged at least twice a week. A flask with a confluent monolayer of RAW 264.7 cells was treated with 10 ml cell dissociation medium (Sigma) at 37°C for 10 min and A549 cells were treated with Trypsin-EDTA (GIBCO) at 37°C for 5 minutes to dissociate cells from the flasks. The J774.1 cells were detached using a cell scraper. The detached cells were transferred to a 50 ml centrifuge tube containing 30 ml DMEM complete medium and then centrifuged for 5 min at 1500 rpm. The supernatant was discarded and the cells were resuspended in DMEM complete medium. Viable cells were counted with trypan blue exclusion using a hemacytometer (American Scientific Products, McGraw Park, IL). A 175 cm^2 flask was seeded with 10^6 viable cells in 45 ml of DMEM complete medium and incubated at 37°C in 5% CO_2 for 7 days.

2.8 Isolation of bone marrow macrophages from mice

Bone marrow macrophages were obtained from 8- to 10-week-old BALB/c mice (Charles River Laboratories, Wilmington, MA) as previously described (Celada et al. 1984). The cells were cultured in 150-mm plates in DMEM (Sigma) supplemented with 20% FBS (Sigma) and 20% L cell-conditioned medium (supernatant from L-929 murine cells) as a source of M-CSF. Once the macrophages were 60–80% confluent, they were deprived of L cell-conditioned medium for 14–16 h to render the cells quiescent and were then stimulated with bacterial products and/or cationic peptides.

2.9 Production of inflammatory mediators by monocytes and macrophages

For some experiments, murine macrophages were seeded in 24 well plates (Costar) at a density of 10^6 cells per well in DMEM complete medium and incubated at 37°C in 5% CO_2 overnight. The medium was replaced with fresh medium, LPS was added to a final concentration of 100 ng/ml and the cells were incubated for 6-24 hr at 37°C in 5% CO_2. Cationic peptides were added at the same time as LPS. Control assays were performed to demonstrate that the highest concentrations of peptides used did not induce TNF-α production and were not cytotoxic, as judged by trypan blue exclusion and continued adherence of the cells. In other experiments, the RAW 264.7 cells were plated in 24 well dishes at 2.5×10^5 cells/well in the above medium, except that the DMEM lacked phenol red in order to prevent interference with the Griess reagent. The cells were incubated overnight and then stimulated with 100 ng/ml *Salmonella* Typhimurium LPS alone, 50 μg/ml CEMA alone, or 100 ng/ml *Salmonella* Typhimurium LPS plus 50 μg/ml CEMA. The cells were then incubated for 24 h before measuring the production of IL-1β or nitric oxide (NO) for 4 h for measuring MIP-1α production.

2.10 Production of cytokines and chemokines in epithelial cells

A549 cells were seeded in 24 well plates at a density of 10^5 cells/well in complete DMEM medium and incubated at 37°C in 5% CO_2 overnight. The medium was aspirated from the cells and replaced with fresh medium plus LPS or other bacterial products, as well as cationic peptides. After 6-24 h at 37°C in 5% $C0_2$, the supernatants were removed and the levels of cytokines or chemokines were measured in duplicate by ELISA (R&D Systems).

2.11 Chemokine production by whole blood

Blood from three donors was collected by venipuncture into tubes (Becton Dickinson, Franklin Lakes, NJ) containing 14.3 USP units heparin/ml blood. Whole blood was stimulated

with 100 ng/ml LPS and/or cationic peptides (50 μg/ml) in polypropylene tubes at 37°C for 4-6 hours. The samples were centrifuged for 10 min at 2000 x g, the plasma was collected and stored at –20°C until analyzed for levels of TNF-α, IL-1β, IL-6, IL-12, IL-8 and MCP-1 by ELISA (R&D Systems, Minneapolis, MN).

2.12 Measurement of cytokine and chemokine production

The concentrations of TNF-α in the cell supernatants were measured by ELISA (R&D Systems, USA) according to the manufacturer's directions. The samples were diluted 1:10 in calibrator diluent in order to fall into the range of the standard curve. The plates were read immediately following addition of stop solution with a Molecular Devices plate reader at an absorbance of A_{550} minus the reference A_{690}. IL-1β levels were determined using an ELISA (R&D Systems) that could detect < 10 pg/ml IL-1β. MIP-1α levels were determined using an ELISA (R&D Systems) that could detect < 31 pg/ml. These experiments were performed a minimum of three times. MIP-1α, IL-1β, and TNF-α were also measured by ELISA (R&D Systems) in the supernatants of cells used for RNA isolation.

2.13 Nitric oxide production

RAW 264.7 cells were cultured as described above and the amount of NO in the supernatant was estimated from the accumulation of the stable NO metabolite nitrite using the Griess reagent (Stuehr and Nathan 1989) (Molecular Probes, Eugene, OR). Briefly 150 μl samples or standards and 130 μl of H_2O were added to wells of a 96-well plate in duplicate. The Griess reagent (20 μl) was added to each well and the plate was incubated at room temperature for 30 min and the OD_{450} read with a spectrophotometer.

2.14 MTT assay

RAW cells (at a concentration of 5×10^4 cells/well) were plated in 96-well plates in the medium described previously (section 2.7) in the presence and absence of increasing concentrations of peptide. The plates were incubated at 37°C in 5% CO_2 for 24 or 48 h. The medium was removed and 100 µl of MTT (5 mg/ml) was added. The plates were then incubated at 37°C in 5% CO_2 for 3 h. MTT was removed and replaced with 100 µl ethanol and the plates were incubated for 15 minutes at room temperature. The plates were then read with a Molecular Devices plate reader at an absorbance of A_{570} minus the reference A_{690}.

2.15 RNA isolation

RAW 264.7 cells were plated in 150 mm tissue culture dishes at 5.6×10^6 cells/dish, incubated overnight and then incubated with microbial products and/or cationic peptides for 4 hours. After stimulation, the supernatant was removed for the measurement of cytokine production, and the cells were washed once with diethyl pyrocarbonate-treated PBS and detached from the dish using a cell scraper. Total RNA was isolated using Trizol (Gibco Life Technologies). The RNA pellet was resuspended in RNase-free water containing RNase inhibitor (Ambion, Austin, TX). To remove contaminating genomic DNA, the RNA was treated with DNase I (Clontech, Palo Alto, CA) for 1 h at 37°C. After adding termination mix (0.1 M EDTA [pH 8.0], 1 mg/ml glycogen), the samples were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), and once with chloroform. The RNA was then precipitated by adding 2.5 volumes of 100% ethanol and 1/10 volume sodium acetate, pH 5.2. The RNA was resuspended in RNase-free water with RNase inhibitor (Ambion) and stored at –70°C. The quality of the RNA was assessed by gel electrophoresis on a 1% agarose gel by looking for the presence of intact 28S and 18S RNA bands without smearing. Lack of genomic DNA contamination was assessed by

using the isolated RNA as a template for PCR amplification with β-actin-specific primers (5'-GTCCCTGTATGCCTCTGGTC-3' and 5'-GATGTCACGCACG-ATTTCC-3'). Agarose gel electrophoresis and ethidium bromide staining confirmed the absence of an amplicon after 35 cycles.

2.16 Mouse cDNA expression arrays

Atlas cDNA Expression Arrays (no. 7741-1), which consist of 588 selected mouse cDNAs spotted in duplicate on positively charged membranes, were purchased from Clontech. Details of the arrays and the methodology used can be found on the Clontech website: www.clontech.com. Briefly, ^{32}P-radiolabeled cDNA probes were prepared from 5 μg total RNA using the Moloney murine leukemia virus reverse transcriptase and pooled primers specific for the 588 genes. The ^{32}P-labeled cDNA probes were separated from unincorporated nucleotides using ChromaSpin columns and 1 x 10^6 cpm/ml of denatured probe in 5 ml of hybridization solution was used for hybridization. The gene array filters were prehybridized with ExpressHyb containing 0.5 mg/ml sheared salmon sperm DNA (Ambion) before incubating overnight at 71°C with the denatured cDNA probes in a hybridization oven at 5 rpm. The filters were washed extensively at low and high stringency conditions recommended by Clontech and then exposed to a phosphoimager screen (Molecular Dynamics, Sunnyvale, CA) for 3 days at 4°C. The image was captured using a Molecular Dynamics PSI phosphoimager. The hybridization signals were analyzed using AtlasImage 1.0 Image Analysis software (Clontech) and Excel (Microsoft, Redmond, WA). The intensities for each spot were corrected for background levels and normalized for differences in probe labeling using the average values for 5 genes observed to vary little between our stimulation conditions: β-actin, ubiquitin, ribosomal protein S29, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and Ca^{2+} binding protein. When the normalized hybridization

intensity for a given cDNA was less than 20, it was assigned a value of 20 to calculate the ratios and relative expression (Der et al. 1998).

2.17 Northern blots

RNA was isolated as described above. Northern blots were performed using the NorthernMax-Gly kit (Ambion). The RNA was separated on glyoxal/dimethylsulfoxide gels and transferred to positively-charged membranes (Ambion). The RNA was crosslinked to the filters using ultraviolet light and the filters were then baked at 80°C for 30 minutes. DNA templates from which probes were produced were generated by PCR using macrophage cDNA and the appropriate primer pairs (Table 3). Antisense cDNA probes were prepared by incubating 50 ng of the PCR product with antisense primer and modified nucleotides that facilitate repeated stripping of blots (Strip-EZ PCR, Ambion). These single-stranded PCR products were purified using Qiagen spin columns and biotinylated by incubating them with psoralen-biotin (Ambion) in the presence of 365 nm ultraviolet light. After a pre-hybridization step, the filters were incubated with biotinylated probes (3 ng in 10 ml UltraHyb or ZipHyb (Ambion)) at 45°C. Hybridization of the probes to the filter was visualized using the BrightStar nonisotopic Detection kit (Ambion) and quantitated by densitometry, with GAPDH levels used for normalization.

2.18 Immunoblot analysis

RAW 264.7 cells were plated in 6 well plates at 1.25 x 10^6 cells/5 ml and grown overnight. They were then incubated with 100 ng/ml *Salmonella* Typhimurium LPS, 1 μg/ml *S. aureus* LTA, 1 μM CpG or medium alone for 4 h. After stimulation, the supernatant was removed and the cells were washed with cold sterile PBS. After washing, the cells at a concentration of 5 x 10^7/ml were solubilized in cold Triton X-100 lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH

Table 3: Primer pairs for Northern blots

Gene	Primers
GAPDH	5′-AGAACATCATCCCTGCATCC-3′
	5′-CTGGGATGGAAATTGTGAGG-3′
IL-1β	5′-TCCAGGATGAGGACATGAGC-3′
	5′-CTTGTGCTCTGCTTGTGAGG-3′
Cyclin D1	5′-CAGCTTAATGTGCCCTCTCC-3′
	5′-GGTAATGCCATCATGGTTCC-3′
CD14	5′-CTGATCTCAGCCCTCTGTCC-3′
	5′-CAGGAGGATGCAAATGTTCC-3′

8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin). After 10 minutes on ice, detergent-insoluble material was removed by centrifugation. Protein concentrations were determined using a bicinchoninic acid assay (Pierce, Rockford, IL). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked for 1 h with 10 mM TrisHCl, pH 7.5, 150 mM NaCl (TBS)/5% skim milk powder and then incubated overnight in the cold with primary antibody in TBS/0.05% Tween 20. After washing for 30 min with TBS/0.05% Tween 20, the filters were incubated for 1 h at room temperature with 1 μg/ml secondary antibody in TBS. The filters were washed for 30 min with TBS/0.05% Tween 20 and then incubated 1 h at room temperature with horseradish peroxidase-conjugated sheep anti-mouse IgG (1:10,000 in TBS/0.05% Tween 20). After washing the filters for 30 min with TBS/0.1% Tween 20, immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection.

2.19 Migration assay

Cells were resuspended to 5 x 10^6/ml in chemotaxis medium (RPMI 1640, 10 mM Hepes pH 7.4, 0.5% BSA). Migration assays were performed in 24 well plates using 5 μm polycarbonate Transwell inserts (Costar) as described by Reif and Cyster (Reif and Cyster 2000). Briefly, peptides or controls (SDF-1, MIP-1β) were diluted in chemotaxis medium and placed in the lower chamber while 0.1 ml cells (5 x 10^6/ml) was added to the upper chamber. After 3 h at 37°C, the number of cells that had migrated into the lower chamber was determined using flow cytometry. The medium from the lower chamber was passed through a FACscan for 30 seconds, gating on forward and side scatter to exclude cell debris. The number of live cells was compared to a "100% migration control" in which 5 x 10^5 /ml cells had been pipetted directly into the lower chamber and then counted on the FACscan for 30 seconds.

2.20 Flow cytometry

To analyze cell surface expression of IL-8RB, CXCR4, CCR2, ICAM-1 and LFA-1, cells were incubated with 10-100 μg/ml of the appropriate primary antibody for 20 min and washed. IL-8RB, CXCR4, and CCR2 primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), ICAM-1 and LFA-1 antibodies were purchased from R&D Systems. The cells were then stained with the FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or FITC-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) for 20 min and washed. The cells were then resuspended in 500 μl buffer (1% FBS in PBS). The cells (10000 live events were counted) were passed through a FACscan (Becton Dickinson, Mountain View, CA) and forward and side scatter were used to gate on live cells.

2.21 Direct intratracheal instillation

Adult mice were anaesthetized with an intraperitoneal injection of Avertin (4.4 mM 2-2-2-tribromoethanol, 2.5% 2-methyl-2-butanol, in distilled water), using 200 μl per 10 g body weight. The instillation was performed using a non-surgical, intratracheal instillation method adapted from Ho and Furst 1973 (Ho and Furst 1973). Briefly, the anaesthetized mouse was placed with its upper teeth hooked over a wire at the top of a support frame with a spring pushing the thorax forward to position the pharynx, larynx and trachea in a vertical straight line. The lower jaw was held open with a loop of thread around the lower teeth and weighted. The airway was illuminated externally using a swan neck fibre optic lamp placed against the mid sternum. The tongue was moved to one side and an intubation catheter was inserted between the vocal folds at the base of the larynx and passed into the clearly illuminated tracheal lumen. With the catheter held steady, 20 μl of peptide suspension or sterile water was placed in a well at the proximal end of the catheter. A 1 ml syringe was then attached to the end of the catheter and the solution gently

instilled into the trachea with 200 µl of air. The animal was maintained in an upright position for 2 minutes after instillation to allow the fluid to drain into the respiratory tree. This procedure was performed with Dr. Donald Davidson, Department of Microbiology and Immunology, UBC.

2.22 Bronchoalveolar lavage

Mice were euthanised by intraperitoneal injection of 300 mg/kg of pentobarbital. The trachea was exposed by opening the skin, parting the submucosal glands and an intravenous catheter was passed into the proximal trachea, just below the thyroid cartilage, and tied in place with suture thread. Lavage was performed by introducing 0.75 ml sterile PBS into the lungs via the tracheal cannula and then after a few seconds, withdrawing the fluid. This was repeated 3 times with the same sample of PBS. The lavage fluid was placed in a tube on ice and the total recovery volume per mouse was approximately 0.5 ml. The bronchoalveolar lavage (BAL) fluid was centrifuged at 1200 rpm for 10 min and the clear supernatant tested for TNF-α, MCP-3 and IL-10 by ELISA (R&D Systems). This procedure was performed with Dr. Donald Davidson.

RESULTS

CHAPTER 3: Cationic peptides neutralize the biological activity of bacterial products from Gram-positive bacteria

3.1 Introduction

To combat bacterial infection, the host immune response or treatment regime must aim to kill the invading pathogen while at the same time keeping in check the inflammatory response generated by the bacteria and their released components. Components of both Gram-negative bacteria and Gram-positive bacteria stimulate the release of inflammatory mediators such as TNF-α and this response, when unchecked, can lead to sepsis. Sepsis is a potentially lethal condition associated with the activation of multiple inflammatory pathways. A number of cationic peptides not only have antimicrobial activity against a broad range of bacteria but also bind the Gram-negative bacteria component LPS, and neutralize its ability to stimulate the production of inflammatory cytokines (reviewed in Scott et al. 2000, Appendix A). Since Gram-positive bacteria also contribute to many cases of sepsis, cationic peptides were tested for their ability to modify the host response to components of Gram-positive bacteria. The release of Gram-positive bacterial cell wall components such as LTA has been shown to induce the release of inflammatory mediators (Heumann et al. 1994; Kengarthan et al. 1998). Since LTA has structural similarity (being anionic and acylated) to LPS, I hypothesized that the cationic peptides would also bind LTA and neutralize its biological activity. Therefore, I tested the affinity of the cationic peptides for LTA and tested their ability to limit LTA stimulation of TNF-α production in the murine macrophage cell line, RAW 264.7, and in whole human blood, model systems commonly used to study anti-endotoxic compounds. Thus the goal of these studies was to ascertain if the cationic peptides had a broader spectrum of anti-sepsis activity than originally thought and were able to neutralize the biological activity of LTA as well as LPS.

3.2 Cationic peptides can bind to *S. aureus* LTA

The ability of cationic peptides to bind LPS and limit its biological activity has been demonstrated in previous studies (reviewed in Scott et al. 2000). Since there has been an increase in the incidence of sepsis attributed to Gram-positive bacteria (Bone et al. 1994), it was important to study the effect of cationic peptides on components of Gram-positive bacteria as well as Gram-negative bacteria. I hypothesized that cationic peptides would bind to purified LTA *in vitro* similar to their ability to bind LPS. To test this hypothesis, the dansyl polymyxin B (DPX) fluorescence assay, that had been previously used to monitor the binding of peptides to LPS, was modified. When excited at 340 nm, DPX fluoresces at 485 nm. This fluorescence is increased when DPX binds to LPS and is reduced when cationic peptides bind to LPS and displace the DPX. A similar increase in DPX fluorescence was observed when LTA was added. Therefore, an analogous displacement assay was performed to determine whether the CEME-related peptides bound to purified LTA.

Figure 5 shows that CEME and its derivatives (for peptide sequences see Table 1 on page 35) were able to displace up to 90% of the bound DPX from purified LTA. Most of the peptides had a higher affinity for LTA than polymyxin B with CM5 and CEME being exceptions. The ability of the cationic peptides to bind LTA did not correspond to their MIC values for Gram-positive bacteria (Table 5 on page 65). For example, CEME was the most effective antimicrobial peptide of this series against Gram-positive bacteria but had a relatively low affinity for *S. aureus* LTA compared to some of the other peptides. Conversely, CP207 had the highest affinity for purified LTA but its MIC towards Gram-positive bacteria were in general 4-fold higher than CEME. Futhermore, CP208 also exhibited substantial affinity for purified LTA even though it

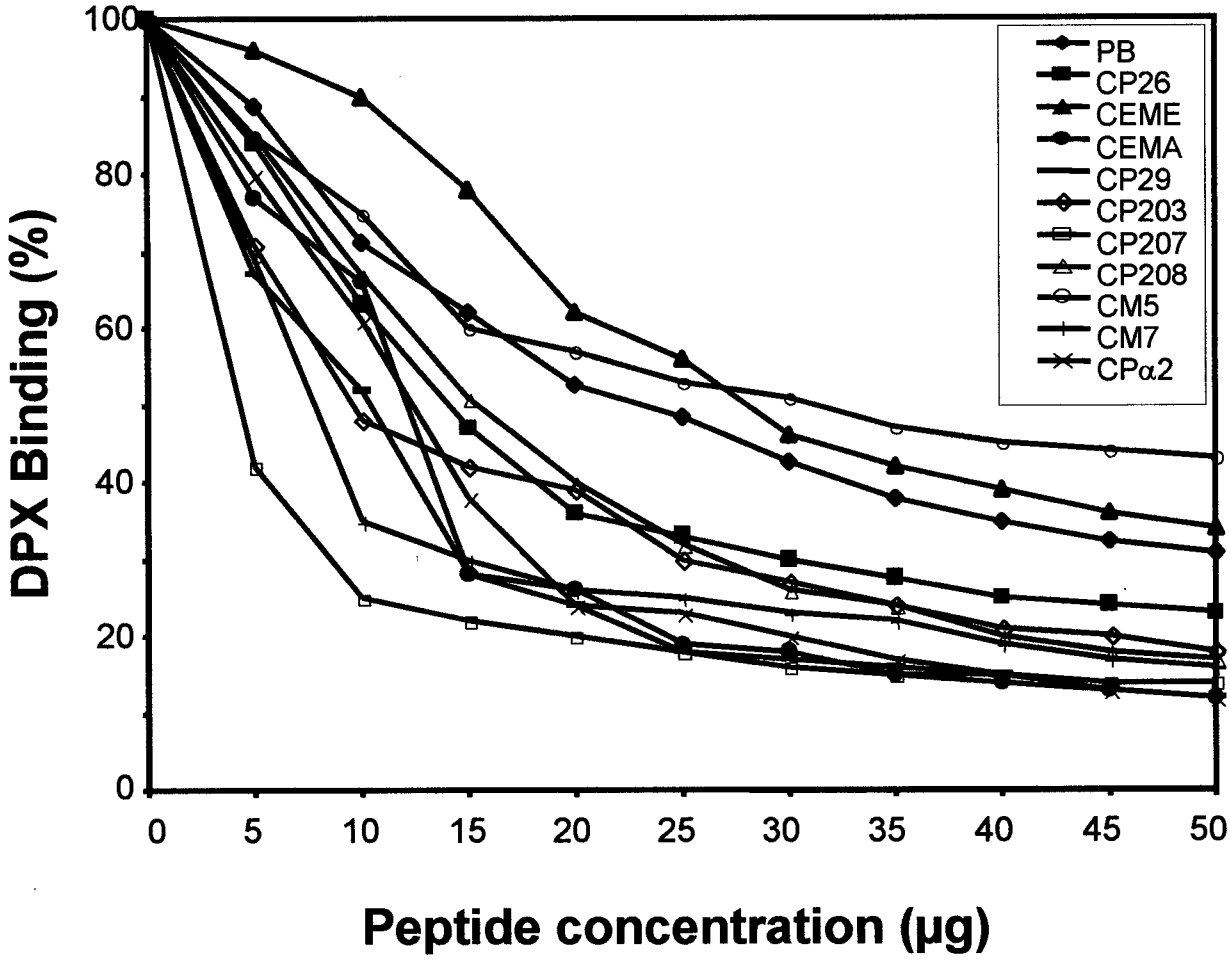


Figure 5: Cationic peptides bind *S. aureus* LTA as measured by the DPX displacement assay

Purified soluble *S. aureus* LTA (5 µg/ml) was incubated with 2.5 µM DPX and the fluorescence at 485 nm measured. Cationic peptides and polymyxin B (PB) were added in increments of 5 µg/ml and the DPX fluorescence was measured after each addition. Each data point represents the mean value for 3 independent experiments done in duplicate. The standard errors of the mean were all less than 10%.

was unable to kill Gram-positive bacteria. These results indicate that the ability to bind LTA is probably not the major mechanism by which CEME-related peptides kill Gram-positive bacteria. The exact mechanism by which cationic peptides kill bacteria is not known. Nevertheless, the binding of cationic peptides to LTA could prevent LTA that is shed from bacteria from inducing overwhelming inflammatory responses. To test this hypothesis, cationic peptides were tested for their potential to block the ability of soluble LTA to induce the production of inflammatory cytokines by macrophages.

3.3 Cationic peptides reduce LTA-induced cytokine release by macrophages

Previous studies have shown that when LTA is injected into animals it causes many of the characteristics of septic shock (Natanson et al. 1989; Wakabayashi et al. 1991; De Kimpe et al. 1995; Le Roy et al. 1996; Kengatharan et al. 1998). Consistent with this observation, LTA induces the production of inflammatory cytokines such as TNF-α by macrophages *in vitro* (Heumann et al. 1994). Therefore the cationic peptides were tested for their ability to reduce LTA-induced cytokine production by the murine macrophage cell line, RAW 264.7. As IL-6 has been associated with sepsis, the levels of this cytokine was measured in these cells along with TNF-α, which is commonly associated with sepsis. Figure 6 shows that LTA stimulated the release of TNF-α and IL-6 by RAW 264.7 cells. Maximal TNF-α and IL-6 production was observed after 6 hours, and 0.1 μg/ml *S. aureus* LTA was the minimal concentration of LTA that induced substantial cytokine production. CEMA (20 μg/ml) substantially reduced cytokine production elicited by 0.1 μg/ml or 1 μg/ml of LTA (Figure 6 and Table 4). When RAW 264.7 cells were stimulated with 10 μg/ml of LTA, CEMA was not as effective, inhibiting less than 50% of the LTA-induced TNF-α release. The production of TNF-α and IL-6 in response to 1 μg/ml of LTA (Figure 7) was completely suppressed over the entire 24-hour observation period by 20 μg/

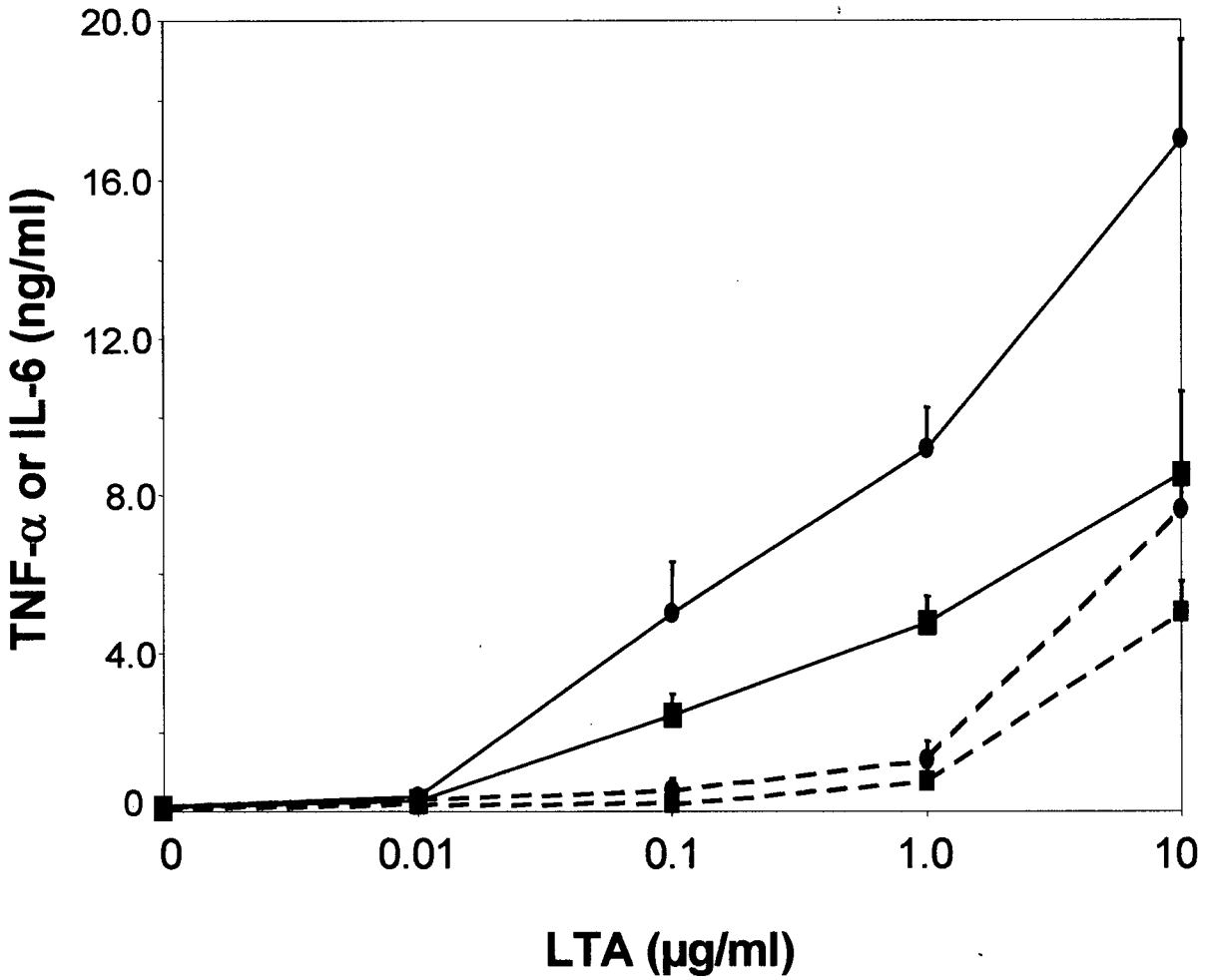


Figure 6: CEMA reduces LTA-induced TNF-α and IL-6 production by RAW 264.7 cells

RAW 264.7 cells were incubated for 6 hours with the indicated doses of *S. aureus* LTA in the absence (solid line) or presence (broken line) of 20 µg/ml of CEMA. The concentrations of TNF-α (●) and IL-6 (■) in the cell supernatant were determined by ELISA. Production of TNF-α and IL-6 by macrophages incubated in medium alone for 6 hours was less than 0.3 ng/ml. The data is presented as the mean of three experiments (done in duplicate) ± standard error.

Table 4: Cationic peptides reduce LTA-stimulated production of TNF-α and IL-6 by RAW 264.7 cells

	% Inhibition of cytokine induction ± standard error									
	100 ng/ml *E.coli* LPS[a]		100 ng/ml *S.aureus* LTA		1 µg/ml *S.aureus* LTA		1 µg/ml *B.subtilis* LTA		1 µg/ml *S.pyogenes* LTA	
Peptide	TNF-α	IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α	IL-6
PB	98	98	99 ± 1	98 ± 1	96 ± 1	93 ± 4	93 ± 2	91 ± 1	97 ± 1	99 ± 1
CP26	91	90	48 ± 19	76 ± 1	52 ± 12	50 ± 15	23 ± 17	42 ± 11	68 ± 3	78 ± 6
CEME	94	76	99 ± 1	92 ± 2	86 ± 10	92 ± 6	90 ± 2	94 ± 2	96 ± 1	98 ± 1
CEMA	90	82	94 ± 4	97 ± 2	91 ± 4	87 ± 2	86 ± 2	88 ± 1	96 ± 1	97 ± 2
CP29	98	96	99 ± 1	95 ± 3	90 ± 2	95 ± 3	76 ± 14	89 ± 4	96 ± 1	99 ± 1
CM5	42	44	65 ± 2	88 ± 1	64 ± 12	73 ± 8	77 ± 1	67 ± 3	80 ± 4	75 ± 5
CM7	99	95	99 ± 1	95 ± 1	96 ± 2	95 ± 4	83 ± 4	91 ± 6	90 ± 4	94 ± 5
CP203	98	90	98 ± 1	99 ± 1	90 ± 2	93 ± 3	70 ± 14	77 ± 12	87 ± 6	93 ± 3
CP207	97	93	92 ± 6	96 ± 1	83 ± 3	84 ± 10	72 ± 15	73 ± 12	69 ± 11	89 ± 3
CP208	0	7	46 ± 5	61 ± 1	14 ± 8	19 ± 9	26 ± 8	29 ± 5	23 ± 9	37 ± 13
CPα2	93	94	97 ± 1	89 ± 6	89 ± 5	85 ± 3	83 ± 5	88 ± 6	93 ± 1	95 ± 2

RAW 264.7 cells were cultured with the indicated concentrations of LTA from different bacteria in the presence or absence of the various CEME-related peptides (20 µg/ml) or with polymyxin B (PB; 20 µg/ml). After 6 hours, cell supernatants were collected and analyzed for TNF-α and IL-6 content by ELISA. The data are presented as percent inhibition of cytokine production ± the standard error of the mean for triplicate samples. The 100% value ranged from 16-20 ng/ml for LPS and 9-12 ng/ml for LTA. Medium only controls as well as medium plus peptide were less than 0.3 ng/ml.

[a] The values were from a separate set of experiments and the values had <10% standard deviation from the mean.

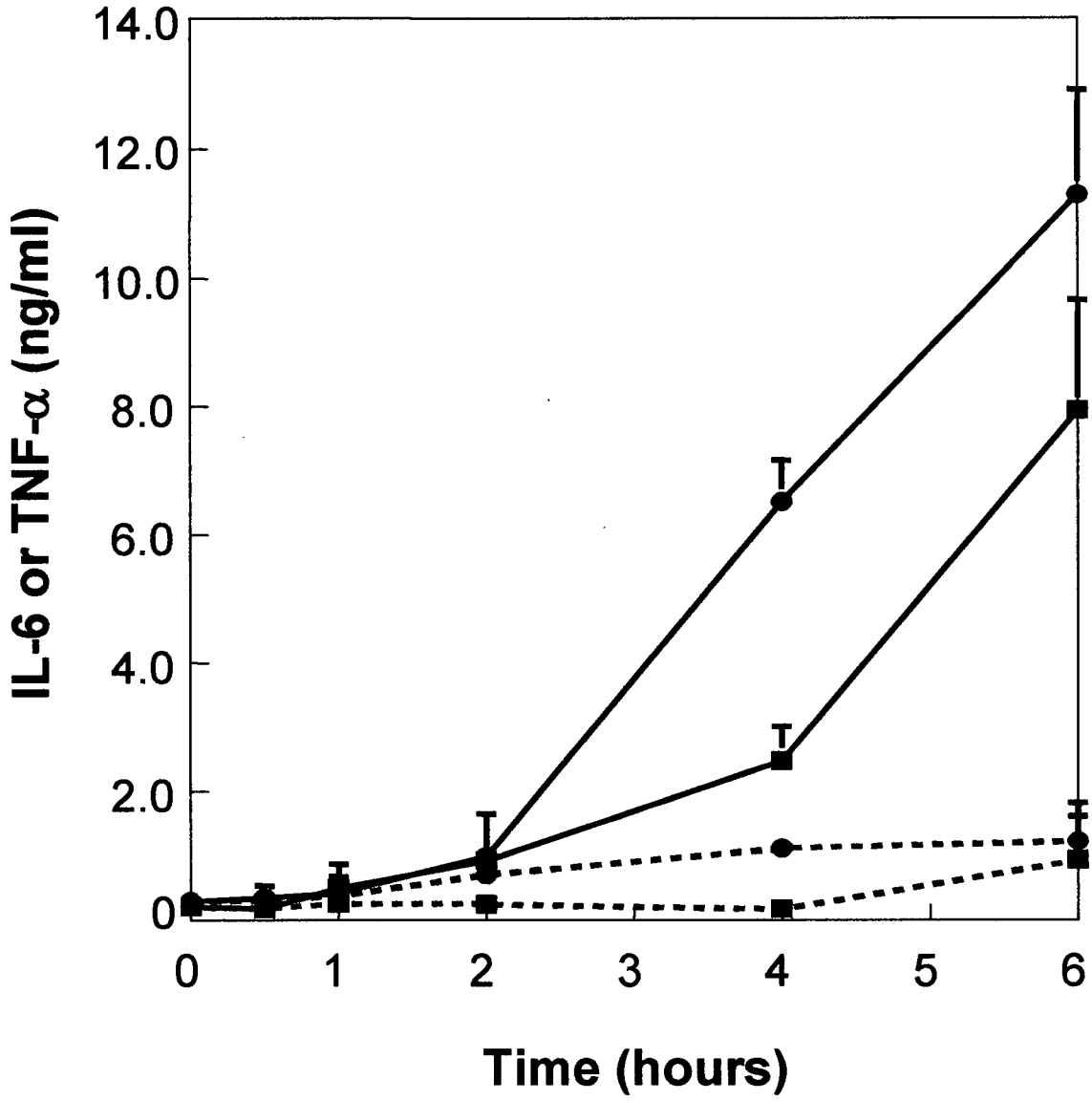


Figure 7: CEMA reduces TNF-α and IL-6 production by RAW 264.7 cells stimulated with *S. aureus* LTA over a 6 hr period

RAW 264.7 cells were stimulated with 1 μg/ml of *S. aureus* LTA for 1 to 6 hours in the absence (solid line) or presence (broken line) of 20 μg of CEMA. Concentrations of TNF-α (●) and IL-6 (■) in the cell supernatant were assayed for cytokine levels at each time point. The data is presented as the mean of three experiments (done in duplicate) ± standard error.

ml CEMA. Figure 8 shows that 10 μg/ml of CEMA was sufficient to cause nearly complete inhibition of TNF-α and IL-6 production by RAW 264.7 cells stimulated with 1 μg/ml LTA. Thus, these results show that CEMA is a potent inhibitor of LTA-induced production of inflammatory cytokines.

Having shown that CEMA can block the ability of *S. aureus* LTA to stimulate TNF-α and IL-6 production by RAW 264.7 cells, the next step was to assess whether the other cationic peptides could block LTA-stimulated cytokine production. In addition to testing their ability to block cytokine production stimulated by either 100 ng/ml or 1 μg/ml *S. aureus* LTA (Table 4), the peptides were also tested for their ability to block cytokine production induced by LTA from *B. subtilis* and *S. pyogenes* (Table 4). In this way, it could be determined whether some or all of the peptides had the ability to neutralize LTA from a variety of Gram-positive bacteria. I found that almost all of the CEME-related peptides substantially reduced LTA-stimulated TNF-α and IL-6 production (Table 4). The exceptions were CP26 and CP208, which caused only partial inhibition of cytokine production. These two peptides also have little or no antimicrobial activity towards Gram-positive bacteria. Two other structurally different peptides, indolicidin, a peptide from bovine neutrophils, and a variant (CP11CN), had only a weak ability to reduce TNF-α production by *S. aureus* LTA stimulated RAW 264.7 cells (20 μg/ml indolicidin inhibited LTA-induced TNF-α production an average of 39% and CP11CN 42%). This was analogous to their weak activity in reducing TNF-α by LPS-stimulated RAW 264.7 cells (Chapter 4, Figure 17). These peptides also have only weak to moderate antimicrobial activity against Gram-positive bacteria (Falla and Hancock 1997). Nevertheless, many of the CEME-related peptides such as CEMA and CEME were potent antagonists of LTA from a variety of Gram-positive bacteria.

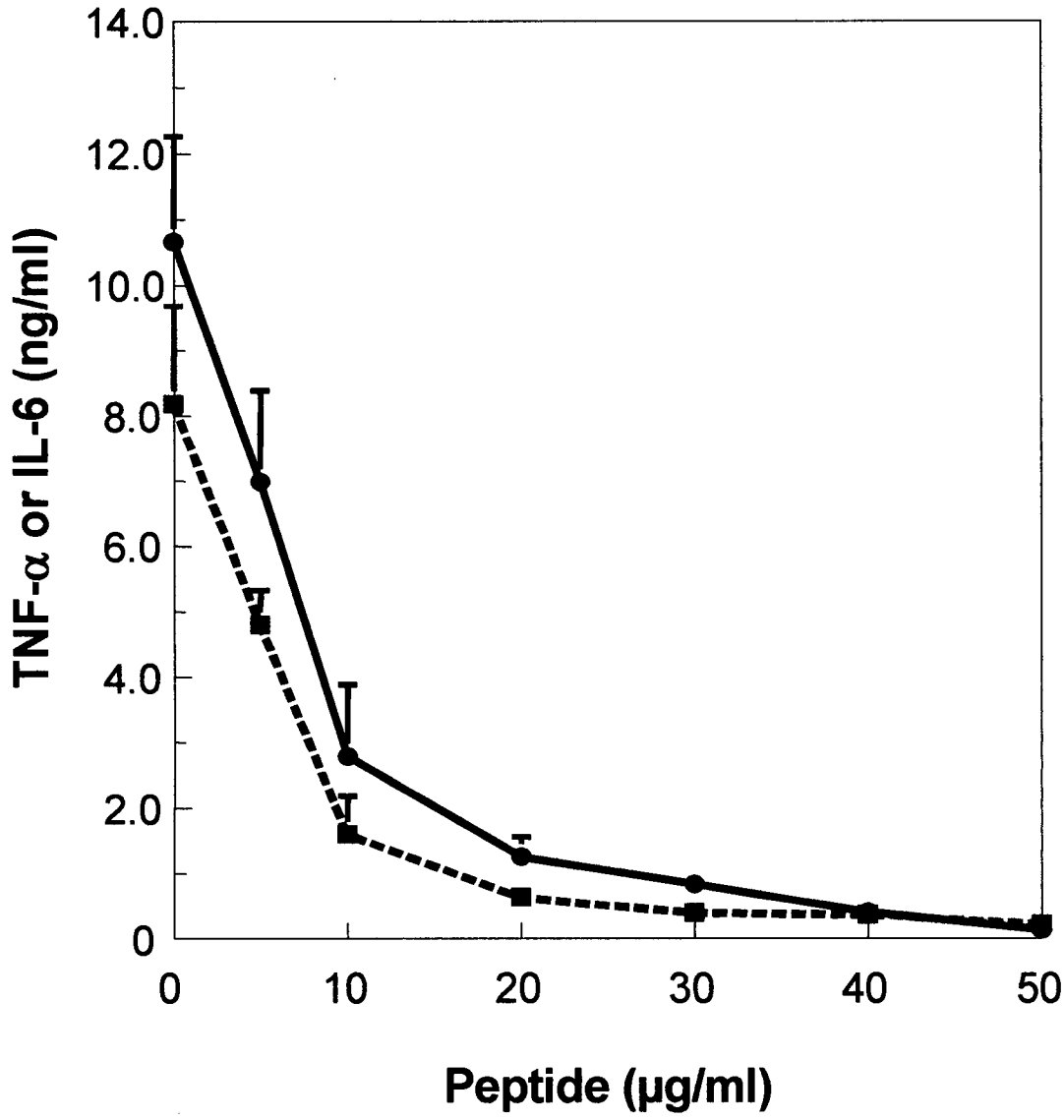


Figure 8: CEMA reduces TNF-α and IL-6 produced by RAW 264.7 cells stimulated with *S. aureus* LTA

RAW 264.7 cells were stimulated with 1 μg/ml of *S. aureus* LTA and increasing doses of CEMA for 6 hours. The concentrations of TNF-α (solid lines) and IL-6 (dotted lines) in the supernatant were measured by ELISA. The data are presented as the mean of three experiments (done in duplicate) ± standard error.

3.4 Cationic peptides reduce production of TNF-α by whole blood stimulated with LTA

The cationic peptides were also tested for their ability to inhibit LTA-induced responses in an *ex vivo* assay in which LTA and peptide were added to whole blood from human volunteers and incubated for 6 hours, at which time the plasma was separated and tested for TNF-α levels by ELISA (Figure 9). I found that the cationic peptides effectively inhibited LTA-induced TNF-α production in whole human blood although not quite as well as in the RAW 264.7 cell assay. CP29 was the most effective, inhibiting TNF-α production by 83%, whereas CP26, CP208 and CPα2 were relatively ineffective. The decreased ability of the cationic peptides to reduce LTA-induced TNF-α inhibition in whole blood could be due to the presence of heparin in the blood collecting tubes (Ogata et al. 1997). Nevertheless the cationic peptides were generally effective at reducing LTA-induced stimulation of TNF-α in human blood.

3.5 Cationic peptides limit TNF-α production by RAW 264.7 cells stimulated with other bacterial products

I next examined whether the cationic peptides could reduce the release of cytokine by macrophages in response to intact heat-killed *S. aureus,* soluble products of *S. aureus*, or *Mycobacterium* non-capped lipoarabinomannan (AraLAM). In the case of the soluble *S. aureus* products, these stimuli may more closely reflect a physiological encounter between macrophages and bacteria than the addition of purified LTA to cultures. Incubating RAW 264.7 cells with intact heat-killed *S. aureus* for 6 hr resulted in release of high levels of TNF-α, approximately 20 ng/ml TNF-α. When RAW 264.7 cells were exposed to soluble products of live *S. aureus* by culturing the macrophages and the bacteria in separate compartments of Transwell dishes, the macrophages also produced substantial amounts of TNF-α, approximately 2.2 ng/ml. At a concentration of 50 μg/ml (optimal dose, based on a does-response curve), a number of the

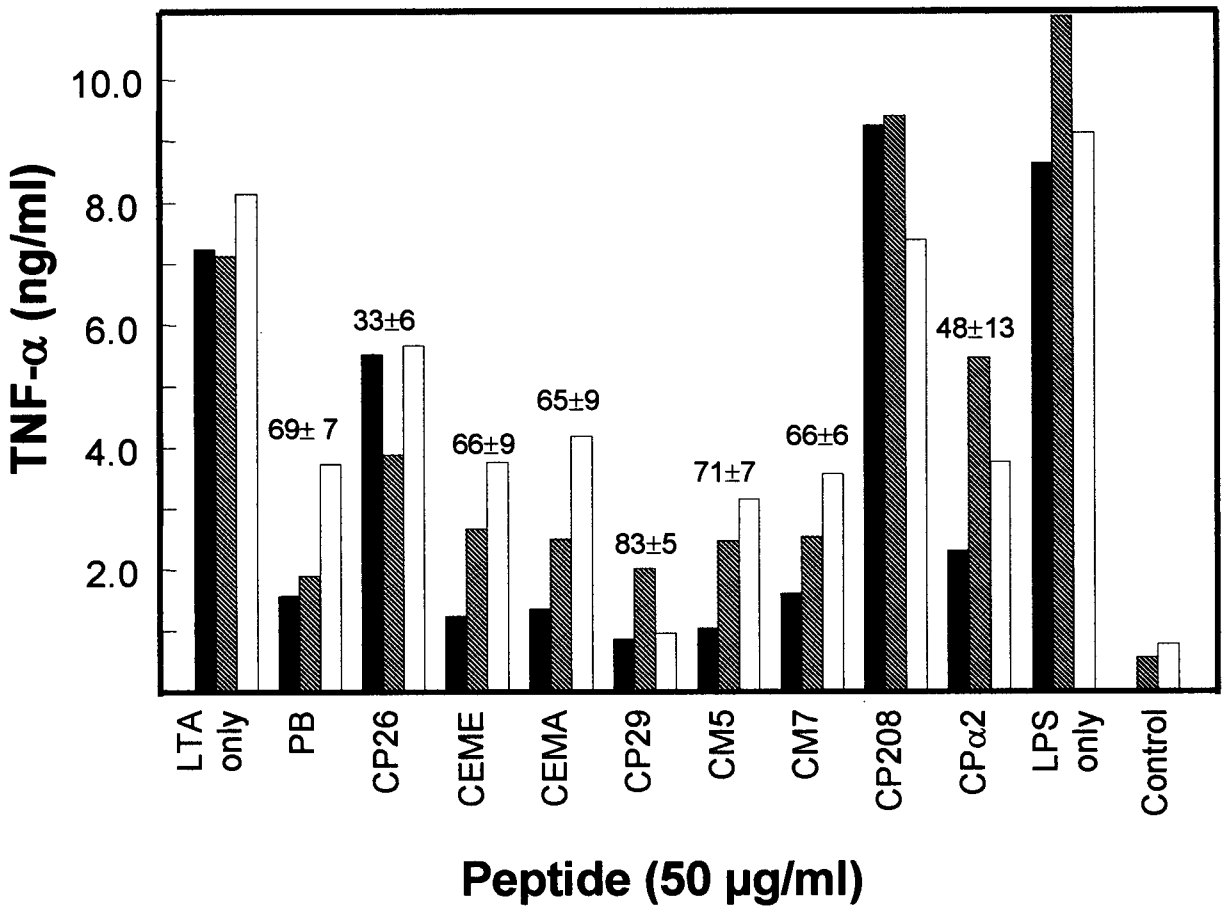


Figure 9: Cationic peptides reduce release of TNF-α in whole blood stimulated with 1 µg/ml *S. aureus* LTA

Whole human blood from three donors was stimulated with 1 µg/ml *S. aureus* LTA and 50 µg/ml peptide and the production of TNF-α by was measured by ELISA after a 6 hour incubation. The induction of TNF-α by 100 ng/ml *E. coli* 0111:B4 LPS was shown as a control. The three different bars represent the average of duplicate samples for each of the three donors. The numbers above the bars represents the percentage by which the peptides inhibited TNF-α production (mean ± standard error).

cationic peptides substantially decreased the ability of the intact heat-killed *S. aureus* and the *S. aureus* soluble products to stimulate TNF-α production (Figure 10). The CEMA, CM7, CPα2, CP29 and CP203 cationic peptides were the most effective peptides in inhibiting *S. aureus*-stimulated TNF-α production, decreasing the level of TNF-α release by more than 50%. Interestingly, the CEME peptide was somewhat less effective at blocking *S. aureus*-stimulated TNF-α production, even though it had the lowest MIC values of all the peptides for *S. aureus*. Nevertheless, many of the cationic peptides were able to reduce the ability of *S. aureus* or its products to cause TNF-α release.

Mycobacterium tuberculosis is an important human pathogen with a distinctive lipid-rich cell wall. The cell envelope of mycobacteria contains lipoarabinomannan (LAM), lipomannan and phosphatidylinositol mannosides. The ability of non-capped LAM (AraLAM) and mannose-capped LAM (ManLAM) to stimulate macrophage production of TNF-α was tested. AraLAM (isolated from a rapidly growing *Mycobacterium* species) and ManLAM (isolated from *M. tuberculosis* $H_{37}R_v$) share structural similarities with LPS. LAM and LPS are both amphipathic molecules that have a glycolipid tail. It was found in this study that AraLAM was a more potent stimulus of TNF-α production by RAW 264.7 cells than ManLAM as seen in other studies (Moreno et al. 1989; Barnes et al. 1992; Chatterjee et al. 1992). Therefore, the ability of CEMA to reduce AraLAM-induced TNF-α release by RAW 264.7 cells was examined.

I found that CEMA reduced AraLAM-induced TNF-α production by RAW 264.7 cells (Figure 11), although to a lesser extent than that seen with LTA stimulation of RAW 264.7 cells. For example, 10 μg/ml CEMA reduced the release of TNF-α production by RAW 264.7 cells in response to 1 μg/ml *S. aureus* LTA by ~75% whereas at 20 μg/ml CEMA inhibited AraLAM-

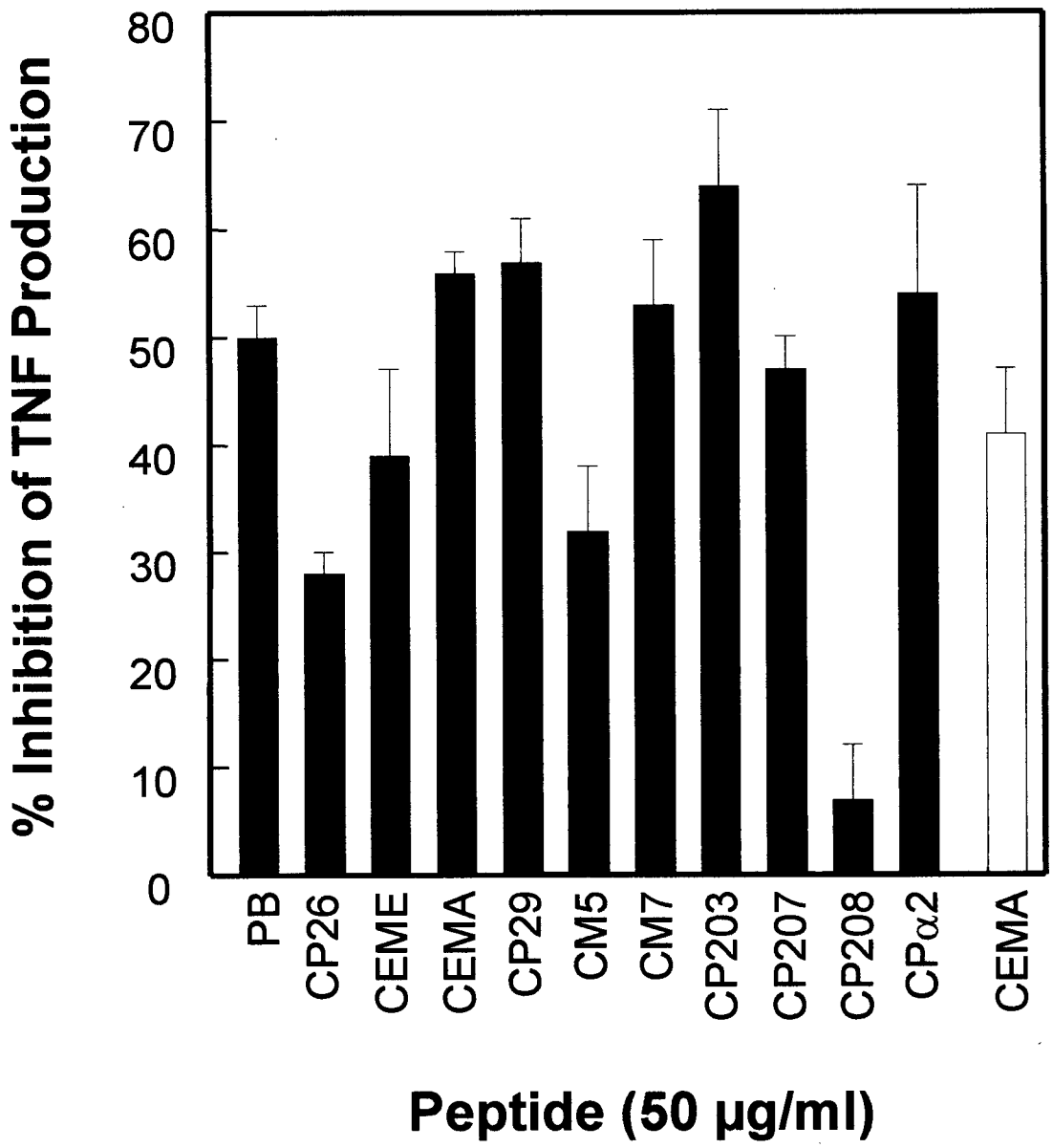


Figure 10: Cationic peptides reduce production of TNF-α by RAW 264.7 cells stimulated with heat-killed *S. aureus*

RAW 264.7 cells were incubated with boiled *S. aureus* (solid bars) or live *S. aureus* separated by a filter from the RAW 264.7 cells (open bar) for 6 hours. The supernatant was collected and measured for TNF-α by ELISA. The data are presented as percent inhibition of cytokine production ± the standard error of the mean for triplicate samples.

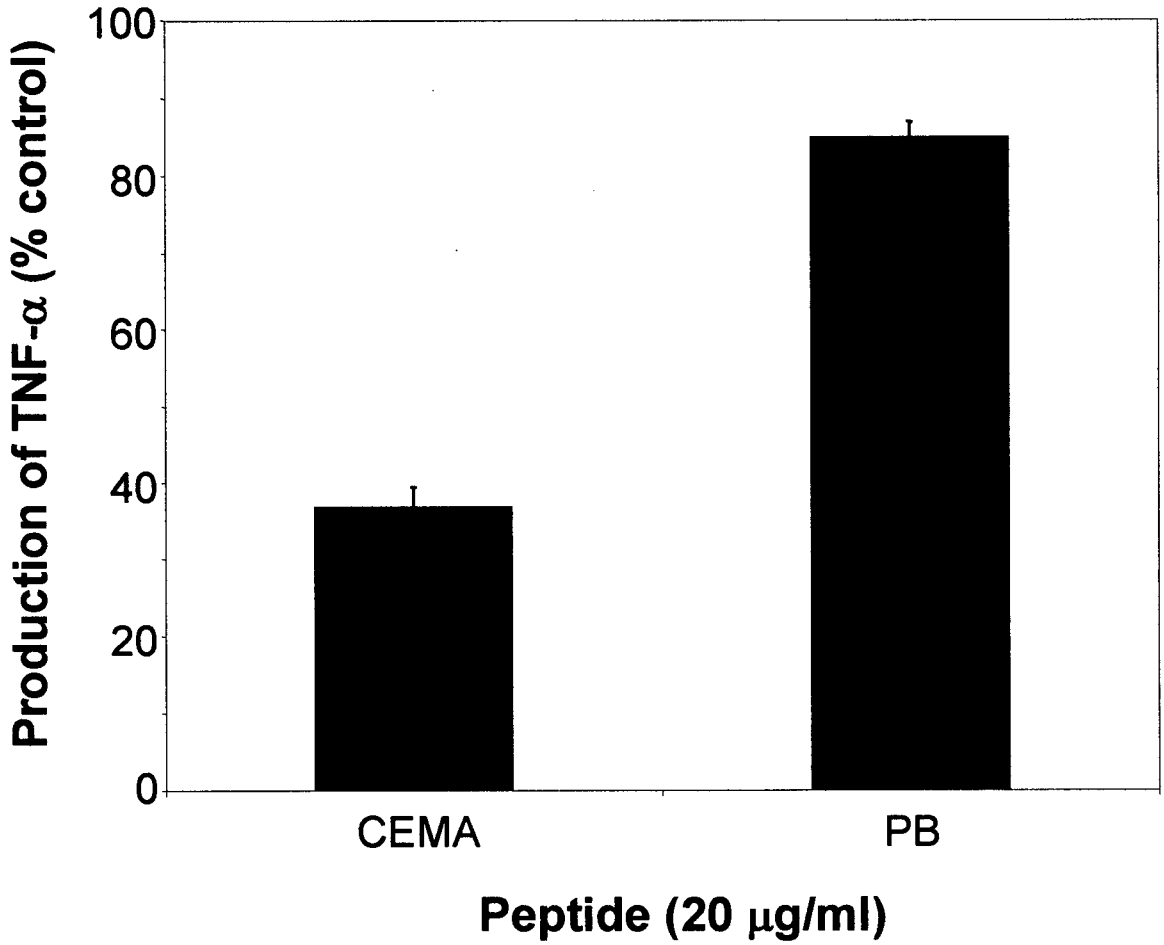


Figure 11: CEMA reduces TNF-α production by RAW 264.7 cells stimulated with *Mycobacterium* AraLAM

RAW 264.7 cells were stimulated with 1 μg/ml AraLAM in the absence or presence of 20 μg/ml CEMA or Polymyxin B (PB). The supernatant was collected and tested for levels of TNF-α by ELISA. 100% represents the amount of TNF-α resulting from RAW 264.7 cells incubated with AraLAM alone for 6 hours (2.4 ± 0.3 ng/ml). The data from duplicate samples is presented as the mean of three or more experiments ± standard error.

induced TNF-α release by only ~60%. Polymyxin B (PB) was included as a control to show that contaminating endotoxin was not a significant factor in the inhibition by peptide of AraLAM induced TNF-α. These studies demonstrated that cationic peptides can reduce TNF-α release produced by macrophages in response to a variety of bacterial products.

3.6 Cationic peptides have antimicrobial activity against Gram-positive and Gram-negative bacteria

A potential therapeutic agent for sepsis would need to not only bind the bacterial products released during infection but also kill the invading pathogen. Since the cationic peptides were effective in blocking macrophage stimulation by Gram-positive bacterial products, it was then of interest to learn if they were also effective in killing Gram-postive bacteria. CEME and some of its derivatives (for peptide sequences see Table 1 on page 35) have previously been shown to have strong antimicrobial activity against a broad range of Gram-negative bacteria (Scott et al. 1999). The aim of this study was to determine whether CEME and its derivatives were capable of killing a variety of clinically relevant Gram-positive pathogens as well as the Gram-negative bacteria, *E. coli* and *Burkholderia cepacia* (Table 5). All of the cationic peptides were inactive (MIC > 64 μg/ml) against *B. cepacia*, an opportunistic pathogen that is intrinsically resistant to many antibiotics. Otherwise, the cationic peptides tended to be effective against Gram-negative bacteria as seen in the previous study (Scott et al. 1999) and in this study with *E. coli*. The MIC of the peptides tested for *E. coli* ranged from 0.5 to 2 μg/ml, with the exception of CP208, which had an MIC of 32 μg/ml. In contrast to their strong activity against *E. coli*, we found that the antimicrobial activity of the peptides towards Gram-positive bacteria varied widely depending upon the bacterium (Table 5). In general, most or all of the CEME-related cationic peptides, with the exception of CP208, had moderate activity (4-16 μg/ml) versus *Cornyebacterium xerosis* and

Table 5: Activity of cationic antimicrobial peptides against Gram-positive bacteria, *E. coli* and *B. cepacia*

	MIC (μg/ml)[a]										
Strain	**CP26**	**CEME**	**CEMA**	**CP29**	**CP203**	**CP207**	**CP208**	**CM5**	**CM7**	**CPα2**	**PB[b]**
Staphylococcus aureus RN4220	64	4	4	8	8	16	>64	64	4	16	32
25923	>64	8	8	16	16	32	>64	>64	16	16	32
SAP0017 (MRSA[c])	64	4	4	16	8	16	>64	>64	8	16	32
Clinical isolate	>64	8	16	16	8	16	>64	>64	16	16	16
Clinical isolate	>64	4	16	16	8	16	>64	>64	16	32	16
Staphylococcus epidermidis	16	4	4	8	4	8	>64	32	8	16	16
Streptococcus pyogenes 19615	16	8	8	8	4	8	>64	16	8	16	8
Enterococcus facealis 29212	>64	32	32	64	32	64	>64	>64	16	64	>64
Bacillis subtilis	32	8	8	8	16	16	>64	64	8	16	64
Listeria monocytogenes	32	4	4	4	4	8	64	64	4	8	4
Cornyebacterium xerosis	2	4	4	4	2	2	32	4	4	4	2
Escherichia coli UB1005	1	2	2	2	2	2	32	4	2	2	0.5
Burkholderia cepacia	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64

Indicated bacterial strains were cultured with serial 2-fold dilution of the various peptides (0.5-64 μg/ml) for 18 hours as described in the Materials and Methods. The MIC represents the minimal concentration of peptide that completely inhibited growth.

[a] The values represent of the median of three experiments, the range of error was generally 2-fold from the median

[b] polymyxin B

[c] MRSA, methicillin-resistant *S. aureus*

Listeria monocytogenes while many of the peptides had moderate activity versus *S. pyogenes*, *S. epidermidis*, and a variety of *S. aureus* lab strains and clinical isolates. Even when the peptides did have antimicrobial activity, the MICs for Gram-positive bacteria were in the range of 4 to 16 μg/ml, which was considerably higher than their MICs for *E. coli*, which ranged from 0.5 to 2 μg/ml. Nevertheless, several of the CEME-related peptides, had antimicrobial activity against multiple Gram-positive bacteria.

Of the cationic peptides tested, CEME had the lowest MICs for the Gram-positive bacteria tested while CEMA and CP203 were only slightly less effective. CM7 also had reasonable antimicrobial activity against Gram-positive bacteria, although it was generally less effective than CEME, CEMA, or CP203. CM7 was also the only peptide that had activity at 16 μg/ml versus *E. faecalis*. Despite being highly effective against Gram-negative bacteria, CP29, CP207, and CPα2 had only modest antimicrobial activity against most of the Gram-positive bacteria while CP26 and CP208 had little activity.

In addition to testing CEME-like peptides, the cationic lipopeptide polymyxin B was tested for its antimicrobial activity towards Gram-positive bacteria (Table 5). Polymyxin B is considered a Gram-negative-selective drug, which has very low MICs (0.1 to 2 μg/ml) for Gram-negative bacteria such as *E. coli*. It was found that while polymyxin B had good antimicrobial activity towards *C. xerosis* and *L. monocytogenes* as well as modest activity towards *S. pyogenes*, it had minimal antimicrobial activity against other Gram-positive bacteria. Thus, while polymyxin B has antimicrobial activity towards some species of Gram-positive bacteria, it does not have as broad a range of activity as CEME and some derivatives such as CEMA and CP203.

3.7 Summary

Sepsis is a potentially lethal condition that is associated with excessive inflammation

generated in response to severe infection (van der Poll, 2001). The host immune response or treatment regime must aim to not only kill the invading pathogen but also limit the inflammatory response to the bacteria and their released components. Since previous studies have shown that synthetic α–helical cationic peptides block LPS-stimulated production of cytokines by macrophages, it was hypothesized that cationic peptides might also limit the stimulation of macrophages by components of Gram-positive bacteria. A number of cationic peptides were tested for their ability to bind LTA, to inhibit cytokine production by macrophages stimulated with components of Gram-positive bacteria and to kill Gram-positive bacteria. The cationic peptides tested were able to bind LTA and many, including CEMA were able to block production of cytokines by macrophages stimulated with LTA. The cationic peptides were also effective in blocking the stimulation of RAW 264.7 cells by heat killed *S. aureus* and *Mycobacterium* AraLAM. Many of the cationic peptides also had antimicrobial activity against Gram-positive bacteria, although a few peptides had no antimicrobial activity. Thus by killing invading pathogens, and by binding released bacterial products and limiting their ability to stimulate macrophages, cationic antimicrobial peptides could potentially inhibit the development of sepsis from bacterial infections.

CHAPTER 4: Cationic peptides inhibit the interaction of LBP and LPS

4.1 Introduction

The mechanism by which cationic antimicrobial peptides block the activation of macrophages by LPS and LTA has not been elucidated. Lipopolysaccharide binding protein (LBP), an acute-phase serum protein, is an important mediator of LPS signaling of macrophages. It functions as a lipid transfer molecule, catalyzing the transfer of LPS to the host protein CD14 phospholipids and high-density lipoprotein. A LBP-deficient mouse line ($LBP^{-/-}$) challenged with purified LPS was used to demonstrate the role of LBP as these mice were defective in their ability to mount an inflammatory response, as judged by the serum titer of TNF-α (Jack et al. 1997). It is not known what role, if any, LBP plays in LTA activation of macrophages. Because cationic peptides that block LPS-induced macrophage activation can bind to LPS, we hypothesized that these peptides could act by inhibiting the binding of LPS and possibly LTA to LBP. To test this, an ELISA-type assay that measures the ability of biotinylated LPS to bind to immobilized LBP was utilized. Using this assay, the ability of structurally diverse cationic peptides to block the binding of LPS and possibly LTA to LBP was analyzed.

4.2 LTA does not require LBP to activate macrophages

Since LTA and LPS share structural similarity and utilize overlapping signaling molecules (CD14 and TLR4), I decided to test if LBP enhances LTA signaling and whether the cationic peptides affect this process. Although the ability of LBP to enhance LPS-macrophage signaling is well documented, there is little information on its effect on LTA-macrophage signaling. One study did however show that lipoproteins actually inhibited the ability of LTA to stimulate the production of TNF-α by macrophages (Grunfeld et al. 1999).

To test whether LTA requires LBP to activate macrophages, experiments were performed in

which RAW 264.7 cells were grown in serum-free medium overnight and then incubated for 6 hours with 1 μg/ml *S. aureus* LTA in the presence or absence of 50 ng/ml human LBP in serum-free media. The addition of LBP to increasing concentrations of LTA and RAW 264.7 cells did not increase the production of TNF-α from LTA alone-stimulated cells (Figure 12). In other experiments, LTA was added to LBP and LPS in the LBP ELISA assay, an assay that measures the binding of biotinylated LPS to immobilized LBP. LTA did not appear to have any competitive effect on LPS-LBP interaction suggesting that LTA does not interact strongly with LBP. Since LBP did not enhance LTA stimulation of TNF-α production by macrophages in serum-free conditions, LBP probably does not play an essential role in LTA stimulation of macrophages and thus the effect of cationic peptides on LTA-LBP interaction was not studied.

4.3 Cationic antimicrobial peptides block the interaction of LPS with LBP

The mechanism by which cationic antimicrobial peptides block the activation of macrophages by LPS has not been elucidated. I hypothesized that the peptides may block the first step in the process, the interaction of LPS with LBP. To test this directly, an assay that measures the binding of biotinylated LPS to immobilized LBP was utilized. Figure 13 illustrates that amounts of LPS ranging from 10-500 ng/ml produced a binding curve that was approximately linear over this range. Since my previous studies had shown that the CP29 cecropin:melittin hybrid peptide (a variant of CEMA) was a potent inhibitor of LPS-induced TNF-α production by RAW 264.7 cells (Scott et al. 1999), it was used to test whether peptides could block the binding of biotinylated LPS to LBP in this assay. When 10 μg/ml CP29 was added to the assay at the same time as the LPS, it substantially reduced the ability of the LPS to bind to LBP (Figure 13). The dose-response curve for LPS binding was shifted approximately 10-fold, i.e. in the presence of peptide 10 times as much LPS was required to yield the same amount of LPS bound to LBP

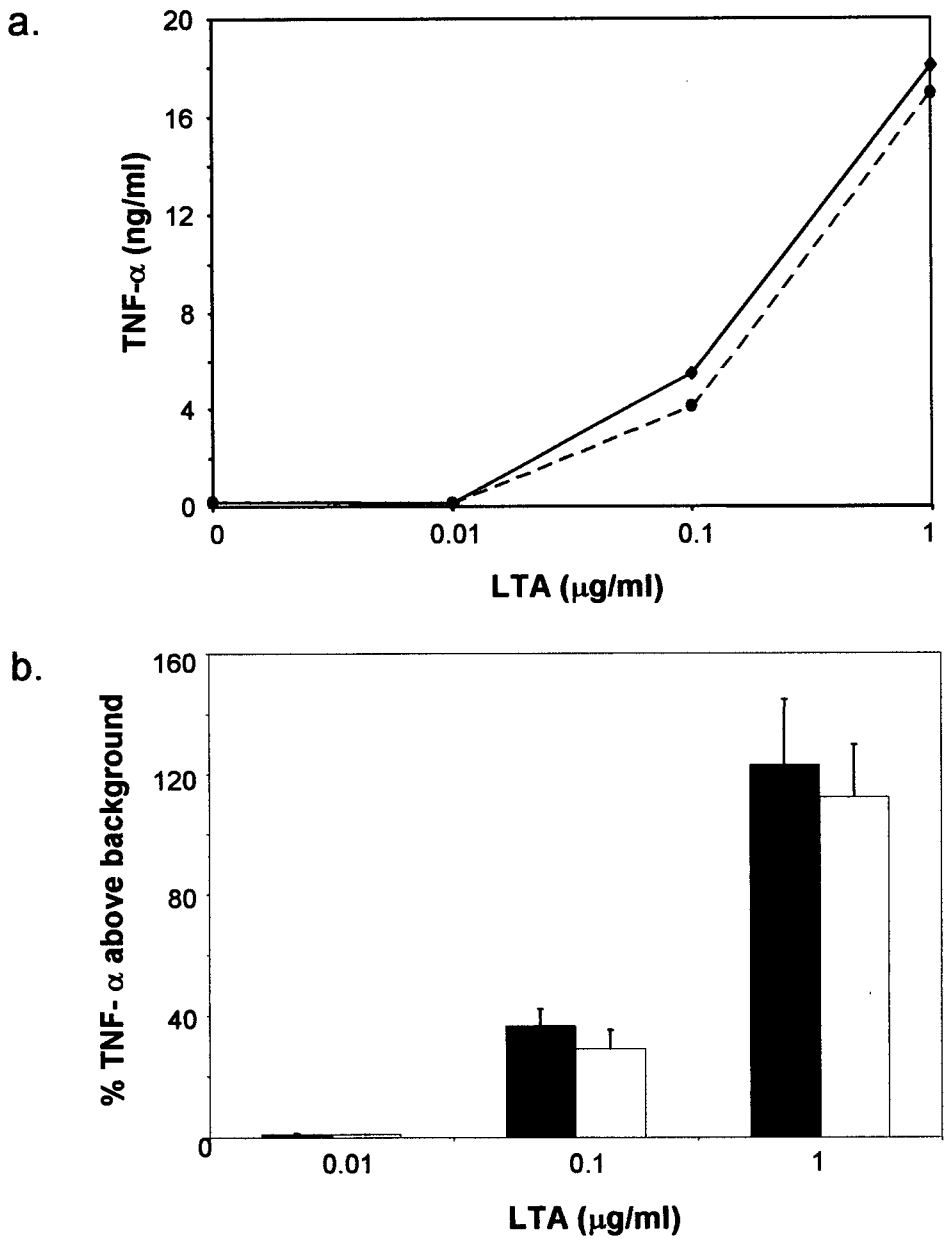


Figure 12: Effect of LBP on LTA stimulation of macrophages

RAW 264.7 cells were stimulated with *S. aureus* LTA in the absence and presence of 50 ng/ml LBP in serum-free media. The supernatant was collected and tested for levels of TNF-α by ELISA. a) The solid line represents LTA alone and the dashed line represents LTA and LBP. Representative data from one of three experiments are shown. Each point represents the average of duplicate samples. b) The solid bars represent % TNF-α above background for RAW 264.7 cells stimulated with *S. aureus* LTA alone and the open bars represent % TNF-α above background for RAW 264.7 cells stimulated with *S. aureus* LTA and 50 ng/ml LBP. The values represent the mean ± standard error for 3 independent experiments.

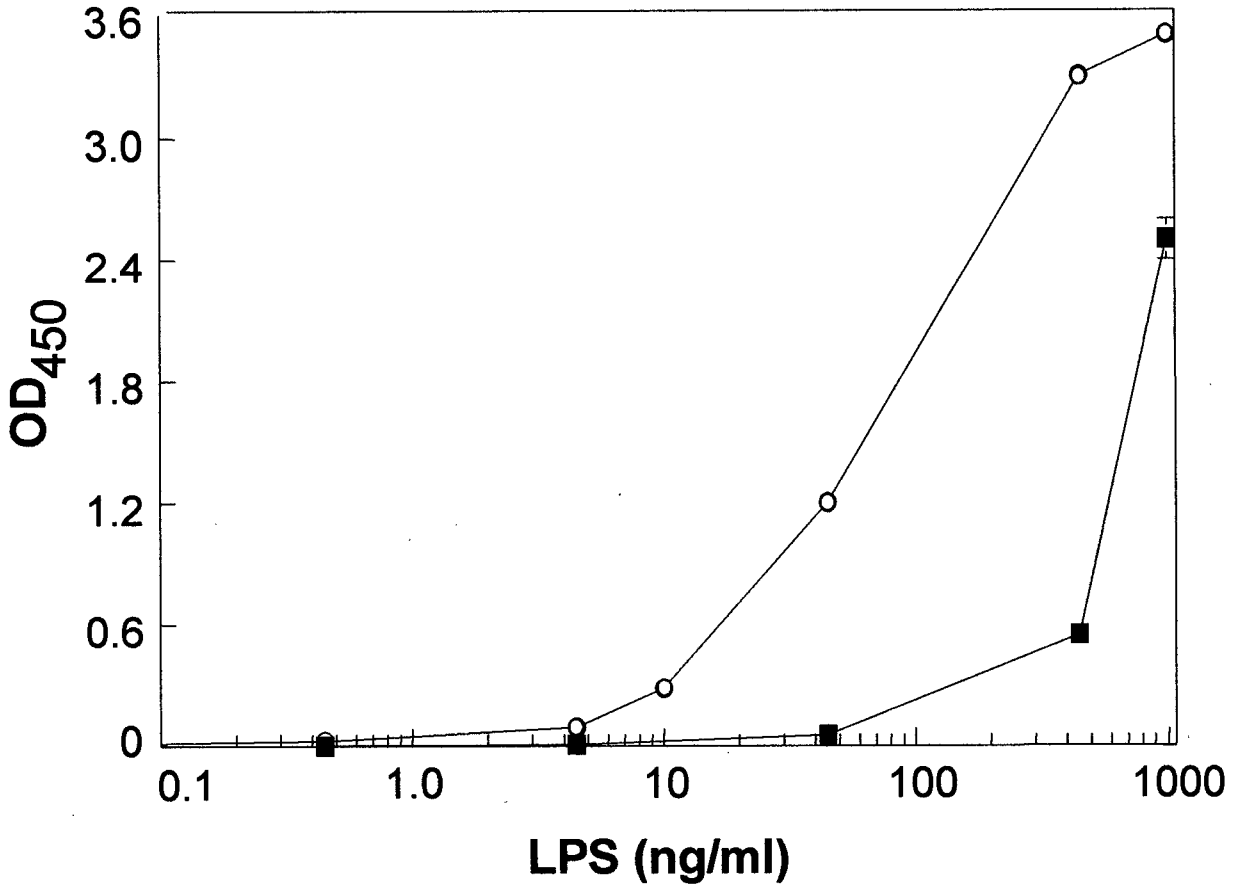


Figure 13: The binding of biotinylated LPS to immobilized LBP is inhibited by CP29

Biotinylated *E. coli* O55:B5 LPS (circles) or biotinylated LPS plus 10 μg/ml (3.4 μM) CP29 (squares) was added to wells containing recombinant LBP immobilized using anti-LBP Abs. Binding of the biotinylated LPS to the immobilized LBP was detected using horseradish peroxidase-conjugated streptavidin. The values represent the mean and standard error for the averages of duplicate samples from three independent experiments. Where no error bars are shown, they were smaller than the symbols.

compared to when the peptide was not present. CP29 reduced the binding of LPS to LBP almost completely when 45 ng/ml LPS was added to the well and by more than 80% when 450 ng/ml LPS was added to the well. Thus, the CP29 cationic peptide strongly inhibits the binding of LPS to LBP.

The dose-response and kinetic characteristics for the cationic peptide inhibition of LPS binding to LBP are shown in Figure 14. In these experiments, I analyzed the inhibition of the LPS-LBP interaction by the CP29 peptide as well as by polymyxin B, a cationic antibiotic that causes substantial inhibition of LPS-induced TNF-α production (see Figure 17). Figure 14 illustrates that as little as 10 ng/ml polymyxin B or 100 ng/ml CP29 could markedly inhibit the binding of LPS to LBP when 45 ng/ml LPS was added to the wells. The ability of polymyxin B to inhibit the LPS-LPB interaction leveled off at 60-70% inhibition when 1-10 μg/ml polymyxin B was added to the wells, whereas increasing the amount of CP29 from 1 to 10 μg/ml resulted in greater inhibition, with 10 μg/ml CP29 causing nearly complete inhibition of the LPS-LBP interaction, as seen also in Figure 13. Despite these differences, it is clear that both CP29 and polymyxin B are potent inhibitors of the LPS-LBP interaction.

It was also observed that pre-incubating the LPS with CP29 or polymyxin B before adding it to the immobilized LBP increased the ability of these peptides to block the LPS-LBP interaction at lower concentrations of CP29 or polymyxin B (Figure 14). Such that, less peptide (CP29) or polymyxin B was required to block the LPS-LBP interaction when the peptide was added to the LPS 30 min before adding the LPS to the immobilized LBP compared to when the peptide and LPS were added at the same time to the LBP-coated wells. This observation was consistent with our previous findings that CP29, polymyxin B, and other cationic peptides bind to LPS (Scott et

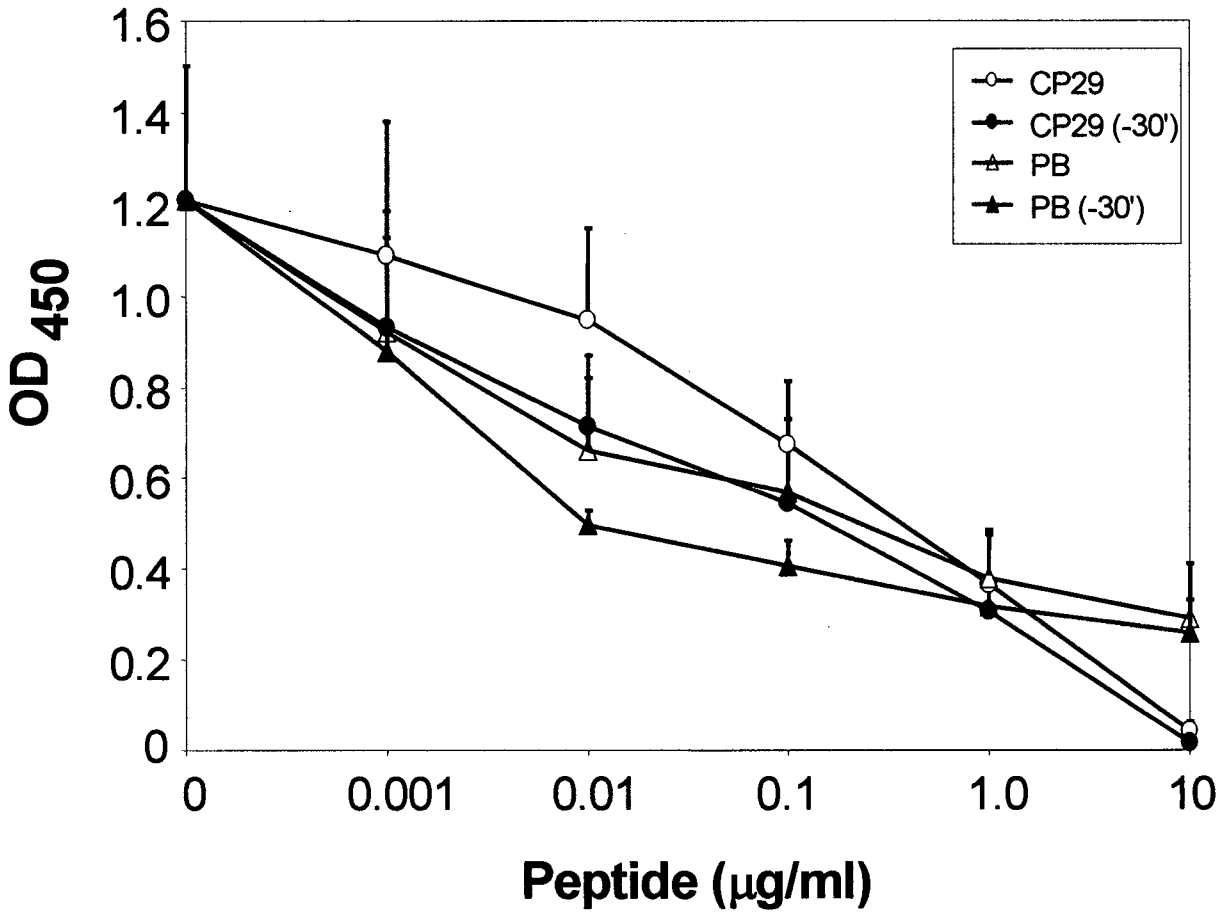


Figure 14: Effect of CP29 and polymyxin B on the binding of LPS to LBP

Biotinylated LPS (45 ng/ml) was mixed with the indicated amounts of CP29 (solid circles) or polymyxin B (PB, solid triangles) for 30 min at 37°C before being added to the immobilized LBP. Alternatively, the LPS was added to the wells containing the immobilized LBP at the same time as either CP29 (open circles) or polymyxin B (PB, open triangles), as in Figure 13. The binding of the biotinylated LPS to the LBP was measured as in Figure 13. The values represent the mean and standard error for the averages of duplicate samples from three independent experiments. Where no error bars are shown, they were smaller than the symbols.

al. 1999) and suggests that this interaction prevents LPS from binding to LBP.

While CP29 and polymyxin B could block the interaction of LPS with LBP, they did not effectively disrupt the binding of LPS to LBP once it had occurred. Figure 15 shows that when the peptides were added to the LBP-coated wells 20-30 minutes after the LPS, they were no longer able to substantially reduce the binding of LPS to LBP.

4.4 Structurally different cationic antimicrobial peptides can inhibit the LPS-LBP interaction

Cationic antimicrobial peptides have a wide variety of secondary structures (Ganz and Lehrer 1998; Hancock and Lehrer 1998). For this study, cationic peptides belonging to different structural groups (defensins, indolicidins, bactenecins, linear α-helical peptides, and cyclic peptides) were tested for their ability to inhibit LPS-LBP interaction. The human defensins HNP-1 and HBD-2, which are comprised of β-sheet structures that are stabilized by two or three disulfide bridges, were tested. HNP-1 is an α-defensin naturally produced by neutrophils, macrophages and Paneth cells while HBD-2 is an arginine-rich β-defensin that is found on mucosal surfaces. Indolicidin and an indolicidin variant, which both have an extended structure and a high proportion of two amino acids (W, P), were also tested. Of the bactenecin-like peptides, Bac 2A, a linear variant of the cow dodecapeptide bactenecin (Wu and Hancock 1999), was tested. A large structural class of cationic antimicrobial peptides comprizes amphipathic α-helical peptides, such as magainins, insect cecropins and melittin. Several synthetic melittin:cecropin hybrid peptides, which were previously shown to have significant anti-endotoxin activity (Gough et al. 1996; Scott et al. 1999) were tested. In adddition, two cyclic bacterial-derived peptide antibiotics polymyxin B and gramicidin S were tested.

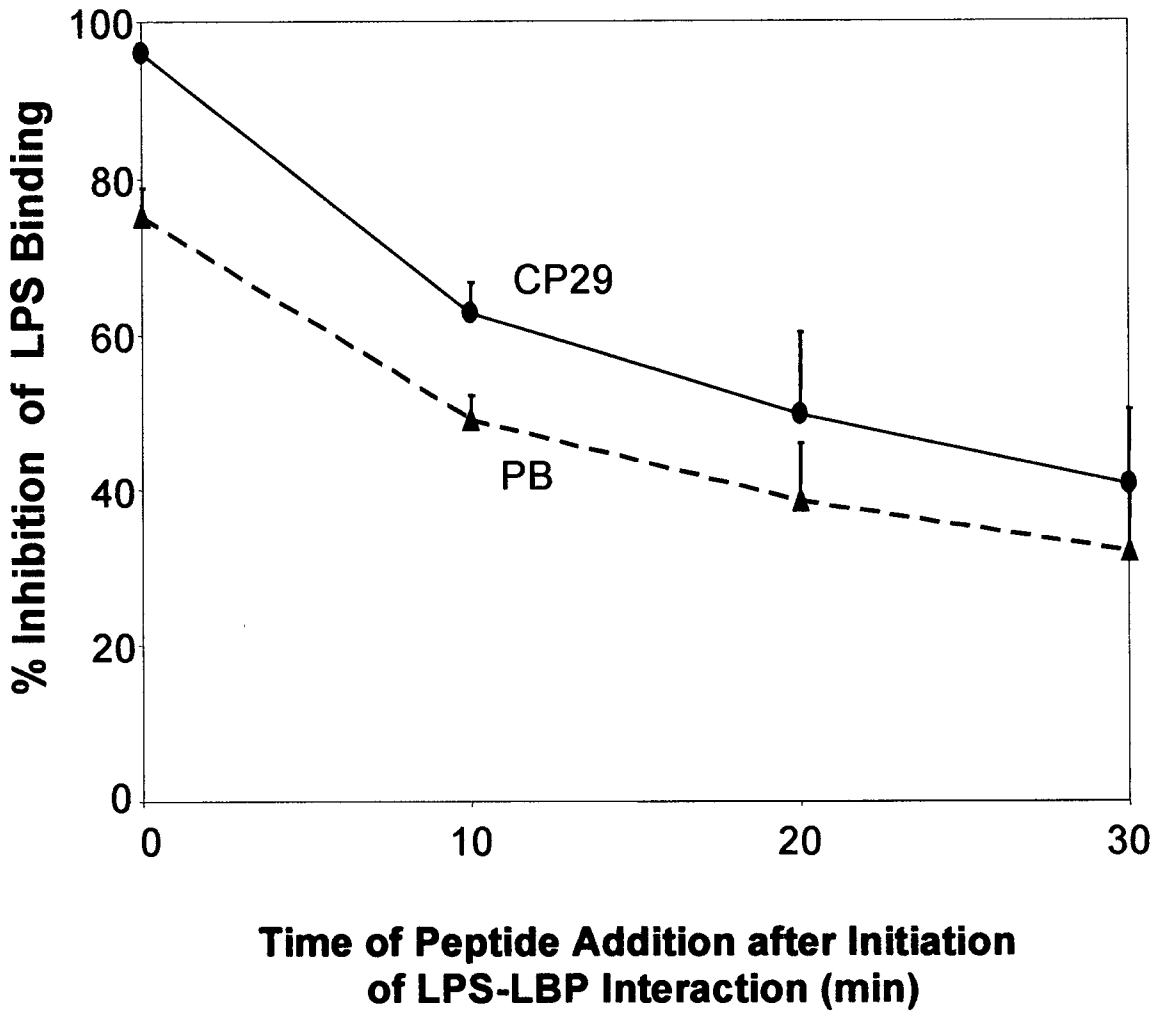


Figure 15: Effect of time of peptide addition on the binding of LPS to LBP

Biotinylated LPS (45 ng/ml) was added to wells containing the immobilized LBP. 10 μg/ml of CP29 (3.4 μM) or polymyxin B (7 μM) were added to the wells either at the same time (time 0) as the biotinylated LPS or at various times after the addition of the biotinylated LPS to the immobilized LBP. The data are presented as percent inhibition of LPS binding. The OD_{450} for the binding of biotinylated LPS (100% value) in the absence of peptides ranged from 1.4 to 1.7. The data is from duplicate samples and presented as the mean of three experiments ± standard error.

The abilities of cationic peptides belonging to these different structural groups to inhibit the LPS-LBP interaction were compared (Figure 16). At the same time, their abilities to block LPS-induced TNF-α production by the RAW 264.7 macrophage cell line were assessed (Figure 17). CP29 and CEMA, synthetic α-helical peptides, were the most effective peptides at blocking both the LPS-LBP interaction and the production of TNF-α by RAW 264.7 cells. They consistently caused a 90-95% inhibition of the LPS-LBP interaction while reducing LPS-induced TNF-α production by more than 80%. In contrast to CP29, CP208, an α-helical peptide that is related to CP29 but which is missing the tryptophan found at residue 2 of CP29, had little effect on the LPS-LBP interaction. Fully in line with this observation, this peptide did not significantly reduce LPS-induced TNF-α production. It has been previously shown that CP208 binds LPS poorly compared to CP29 and CEMA and that it has little antimicrobial activity (Scott et al. 1999). Thus, the ability of the α-helical cationic peptides to inhibit LPS-LBP interactions depends on structural features in addition to their positive charges.

Of the other structural groups of cationic peptides, Bac 2A, gramicidin S, and polymyxin B all caused substantial (55-80%) inhibition of the LPS-LBP interaction (Figure 16). For gramicidin S and polymyxin B, this correlated with their ability to block LPS-induced TNF-α production by 60-80% (Figure 17). In contrast, Bac 2A repeatedly caused only a modest (approximately 30%) inhibition of TNF-α production even though it inhibited the LPS-LBP interaction by 75% in this assay. The human neutrophil peptide α-defensin HNP-1 and the human β-defensin HBD-2, which belong to the β-sheet class of cationic antimicrobial peptides, both exhibited only a modest ability (approximately 40% inhibition) to block the LPS-LBP interaction and this correlated with their modest ability (25-40% inhibition) to block LPS-induced TNF-α production. Two other peptides,

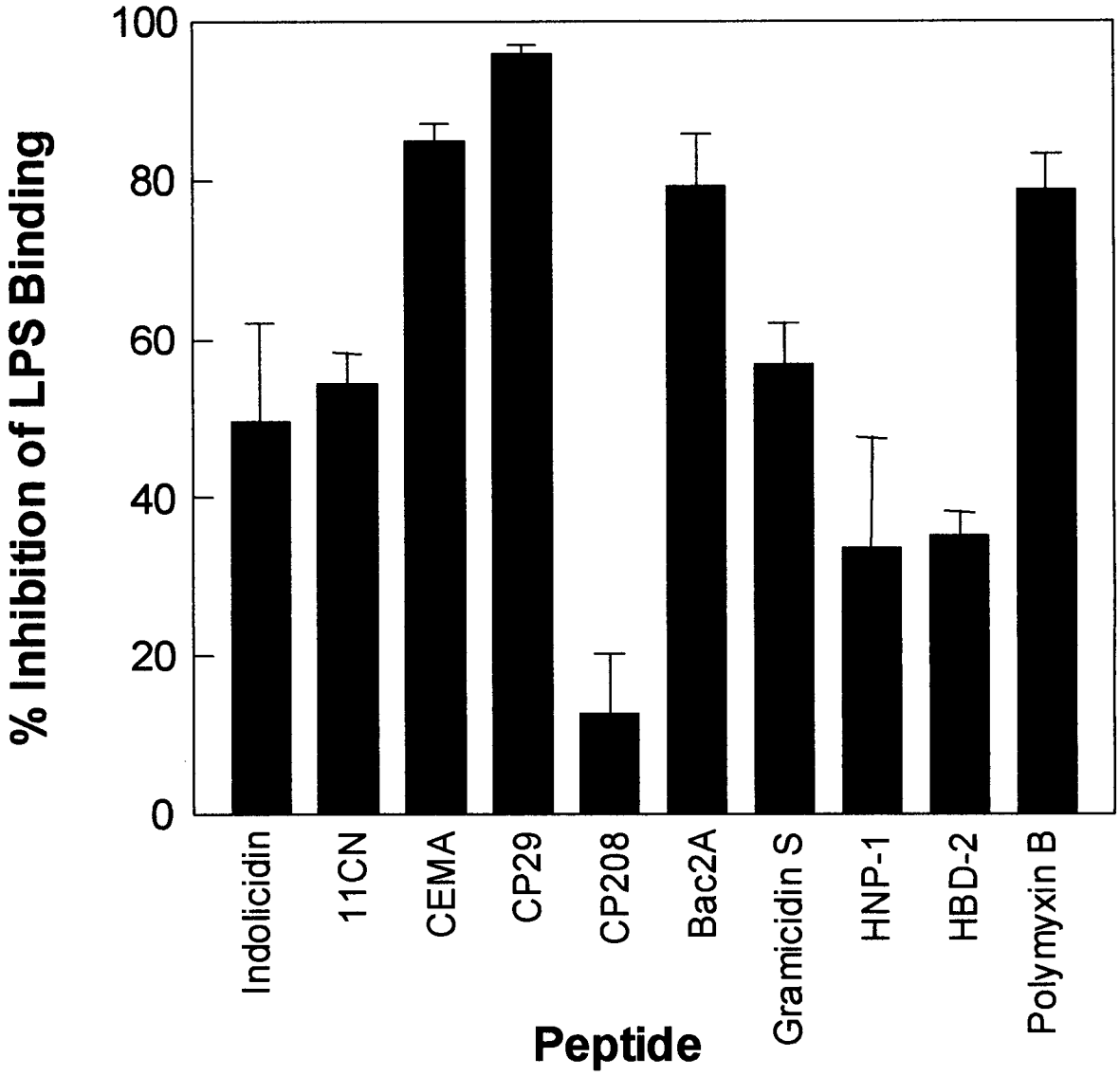


Figure 16: Inhibition of the LPS-LBP interaction by structurally different cationic peptides

Biotinylated LPS (45 ng/ml) was added to wells with LBP in the presence or absence of 10 μg/ml of the indicated cationic peptides. The peptides were added to the wells at the same time as the LPS. In the absence of peptides, the binding of the biotinylated LPS to the immobilized LBP yielded an OD_{450} of 1.1-1.7. The data are expressed as percent inhibition of LPS binding by the peptides. The values represent the mean ± standard error for three independent experiments.

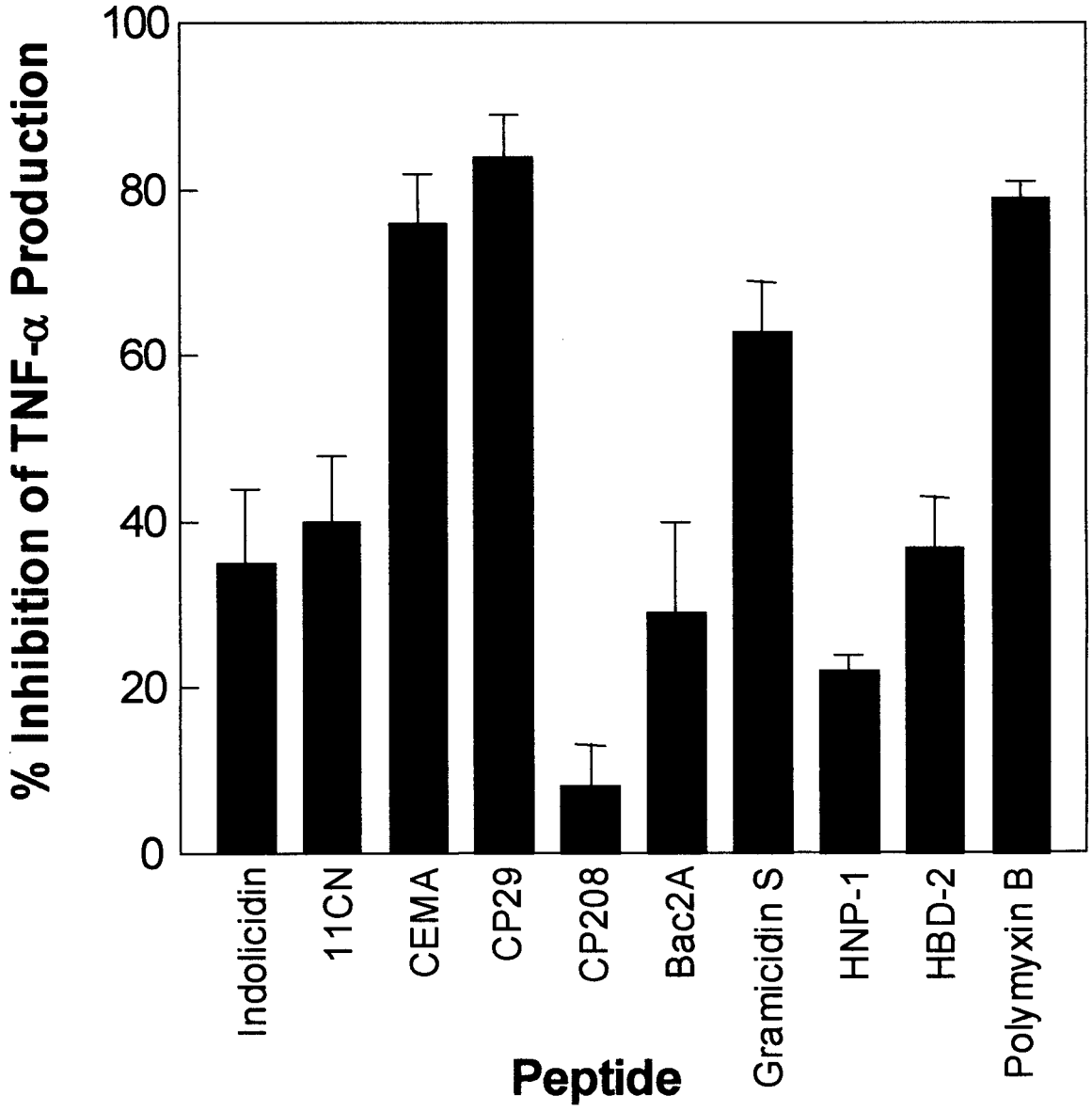


Figure 17: Inhibition of LPS-induced TNF-α production by structurally different cationic peptides

RAW 264.7 cells were incubated with 100 ng/ml *E. coli* O55:B5 LPS in the presence or absence of 20 μg/ml of the indicated peptides for 6 hours. TNF-α released into the culture supernatant was measured by ELISA. TNF-α production by unstimulated RAW 264.7 cells was always less than 0.3 ng/ml while the LPS stimulation routinely resulted in 2.4-3.2 ng/ml TNF-α in the culture supernatant. The LPS-stimulated value was used as 100% and the data are represented as percent inhibition of LPS-stimulated TNF-α production by the peptides. The values represent the mean ± standard error for 3-4 independent experiments.

indolicidin and CP11CN (a variant of indolicidin), significantly inhibited the LPS-LBP interaction (approximately 50% inhibition), but to a lesser extent than CP29. This correlated with their lower ability to inhibit TNF-α production (approximately 40% inhibition) as compared to CP29. Thus, with the exception of Bac 2A, the ability of different cationic peptides to inhibit LPS-induced TNF-α production correlated well with their ability to block the binding of LPS to LBP. The strong correlation ($r^2 = 0.921$: by linear regression analysis when the Bac 2A result was omitted) between these two properties is illustrated in Figure 18. Moreover, the nearly 1:1 correlation between the inhibition of LPS-LBP interaction and the inhibition of TNF-α release was consistent with the suggestion that the ability of the cationic peptides to block LPS-LBP interaction may be the major mechanism by which they block the ability of LPS to activate macrophages.

4.5 Summary

The mechanism by which cationic antimicrobial peptides blocked the activation of macrophages by LPS and LTA was investigated. Since these peptides have been shown to bind to both LTA and LPS, it was hypothesized that they inhibited the first step of LPS and possibly LTA binding to LBP. It was found that LBP did not have a substantial impact on LTA stimulation of macrophages and therefore the effect of cationic peptides on LTA-LBP interaction was not examined. Using an assay that measures the binding of biotinylated LPS to immobilized LBP, it was shown for the first time that a variety of structurally diverse cationic antimicrobial peptides block the interaction of LPS with LBP. Of the peptides tested, the α-helical peptides CP29 and CEMA had the best ability to inhibit both the LPS-LBP interaction and LPS-induced TNF-α secretion by macrophages. CP29 significantly inhibited LPS-LBP interaction at concentrations as low as 10 ng/ml whereas 10 μg/ml CP29 caused nearly complete inhibition of the LPS-LBP

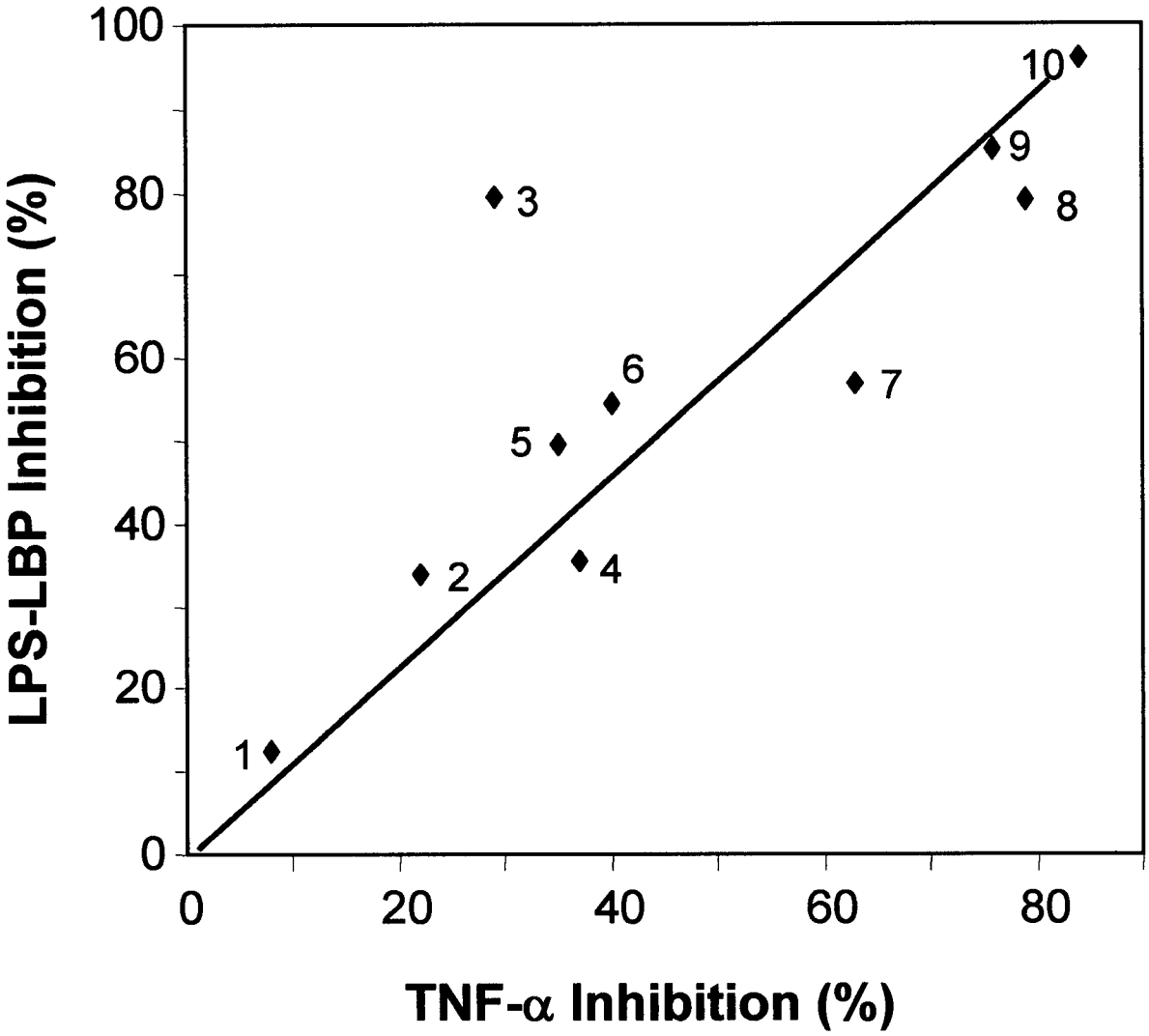


Figure 18: The ability of cationic peptides to inhibit LPS-LBP interaction correlates with their ability to inhibit TNF-α production

The data from A and B were graphed together as an XY scatter plot. The numbers represent the peptides as follows: 1=CP208; 2=HNP-1; 3=Bac 2A-NH_2; 4=HBD-2; 5=Indolicidin; 6=CP11CN; 7=Gramicidin S; 8=Polymyxin B; 9=CEMA; 10=CP29. With the values for Bac 2A-NH_2 omitted, the coefficient of correlation (r^2) between the peptide-induced inhibition of the LPS-LBP interaction and the inhibition of TNF-α release was 0.921 by linear regression analysis (with Bac 2A, 0.6793). The slope of the best-fit line was 0.965 indicating that there was nearly a 1:1 correlation between the inhibition of the LPS-LBP interaction and the inhibition of TNF-α release.

interaction. Preincubation of LPS with CP29 markedly improved its ability to inhibit LPS- LBP interaction. At a concentration of 10 μg/ml, the other cationic peptides tested including Bac 2A-NH_2, the indolicidins and gramicidin S, also inhibited both the LPS-LBP interaction and LPS-induced TNF-α production. Bac 2A-NH_2 was different from other peptides in that it caused strong inhibition of LPS-LBP interaction but had only a moderate effect on LPS-induced TNF-α production. However, in general, the relative ability of different cationic peptides to block the binding of LPS to LBP correlated strongly with their ability to block LPS-induced TNF-α production by RAW 264.7 cells. Thus, the ability of cationic peptides to block macrophage activation by LPS may be due in large part to their ability to block the binding of LPS to LBP which would presumably block the transfer of LPS to CD14 by LBP (Figure 4 on page 24), greatly decreasing the ability of LPS to activate macrophages.

CHAPTER 5: Cationic peptides selectively reduce LPS-induced gene transcription in macrophages

5.1 Introduction

The mechanism by which bacterial products such as LPS stimulate macrophages and how cationic antimicrobial peptides modify this stimulation is only partially understood. Although cationic peptides have an affinity for LPS and can inhibit LPS-LBP interaction, it is likely that there is more to their mechanism of action since they can reduce the production of TNF-α by LPS-stimulated macrophages even when added up to an hour after LPS (Gough et al. 1996; Figure 27 on page 107). The availability of gene arrays allows for a global snapshot of gene expression changes caused by the activation of macrophages by bacterial products. To gain a more complete understanding of how cationic peptides affect LPS-induced gene expression changes in macrophages, gene array technology was used to profile gene expression patterns in RAW 264.7 macrophages treated for four hours with LPS in the presence or absence of the potent anti-endotoxic, cationic peptide CEMA. The effects of LPS on macrophage gene transcription in the presence or absence of CEMA were then confirmed by several methods including Northern blots.

5.2 Bacterial products stimulate overlapping and distinct patterns of gene expression in RAW 264.7 cells

Total RNA was isolated from RAW 264.7 cells that had been incubated for 4 h with medium alone, 100 ng/ml *Salmonella* Typhimurium LPS, 1 μg/ml *S. aureus* LTA, or 1 μM CpG DNA. Bacterial DNA has a much higher content of unmethylated cytosine-guanosine dimer sequences (CpG DNA) which can induce septic conditions including the production of TNF-α both *in vitro* and *in vivo* (Sparwasser et al. 1997). The RAW 264.7 cell RNA was used to generate cDNA probes that were hybridized to Clontech Atlas gene array filters. These nylon membrane

broad-coverage arrays contain cDNAs to 588 mouse genes (catalog # 7741-1). The hybridization of the cDNA probes to each immobilized DNA was visualized by autoradiography and quantified using a phosphoimager. The changes in gene expression induced by LPS, LTA, and CpG DNA were compared, and it was found that all three of these bacterial products increased the expression of pro-inflammatory genes such as iNOS, MIP-1α, MIP-2α, IL-1β, IL-15, TNFR1 and NF-κB to a similar extent (Appendix B). It was discovered that there were also a number of genes whose expression was altered to different degrees by the three bacterial products (Appendix B). The functional significance of the transcriptional changes in RAW 264.7 cells that were stimulated with LPS, LTA and CpG DNA, were confirmed and the levels of selected mRNAs and proteins were assessed and quantified by densitometry (Appendix B). The effect of the bacterial products, LPS, LTA and CpG DNA on macrophage stimulation was also compared. ELISAs were performed on culture supernatants from RAW 264.7 cells stimulated with bacterial products to determine whether LPS, LTA, and CpG DNA increased the release of various cytokines. Consistent with the array findings, it was demonstrated that levels of NO and IL-1β secreted into the medium were significantly increased by LPS, LTA, and CpG DNA It was also found that *E. coli* LPS (eLPS), *Salmonella* Typhimurium LPS (sLPS), and *S. aureus* LTA all stimulated similar amounts of plasma TNF-α, IL-8, and IL-6. CpG also stimulated production of these cytokines, albeit to much lower levels.

5.3 CEMA inhibits LPS stimulated macrophage gene expression

Since the expression patterns of pro-inflammatory genes in the RAW 264.7 cells were similar in response to LPS, LTA and CpG DNA, only LPS was used to examine the effect of cationic peptides on macrophage gene expression changes induced by bacterial components. I previously demonstrated that many cationic antimicrobial peptides reduce the ability of LPS to

interact with LBP and stimulate the production of inflammatory cytokines by macrophages, however, the effects of these peptides on other macrophage functions have not been evaluated in detail. To discover more about how cationic peptides inhibit LPS activation of macrophages, gene expression changes in the RAW 264.7 murine macrophage cell line were studied.

RNA was extracted from RAW 264.7 cells that were cultured for 4 h with medium alone, 100 ng/ml *Salmonella* Typhimurium LPS, 100 ng/ml LPS plus 50 μg/ml CEMA, or 50 μg/ml CEMA alone. CEMA, an α-helical synthetic peptide, was chosen since it has been shown to potently inhibit cytokine production by LPS-stimulated macrophages, and protect mice from lethal endotoxemia (Gough et al. 1996). After reverse transcription, cDNA probes were hybridized to Clontech Atlas gene array filters. The hybridization of the cDNA probes to each immobilized DNA was visualized by autoradiography and quantified using a phosphorimager. Representative autoradiographic images of the gene arrays are shown in Figure 19 and the complete data sets representing the expression levels of all 588 genes in the four different cell populations can be found on the web site: http://www.cmdr.ubc.ca/arraydata1.

It was found that LPS treatment of RAW 264.7 cells resulted in increased expression of at least 57 genes (Table 6 and Table 7, columns labeled "Ratio LPS:unstimulated"). These included the genes described above encoding inflammatory cytokines such as IL-1β and IL-15, inducible nitric oxide synthase (iNOS), chemokines such as MIP-1α, MIP-1β and MIP-2α, and cell surface proteins such as Fas and CD40, and a variety of transcription factors including members of the pRb (retinoblastoma) family. Since many of these genes had been previously reported to be LPS-regulated genes (reviewed in (Ulevitch and Tobias 1995; Sweet and Hume 1996; Ulevitch and Tobias 1999)), it confirmed the validity of the array results. Several novel LPS-regulated genes

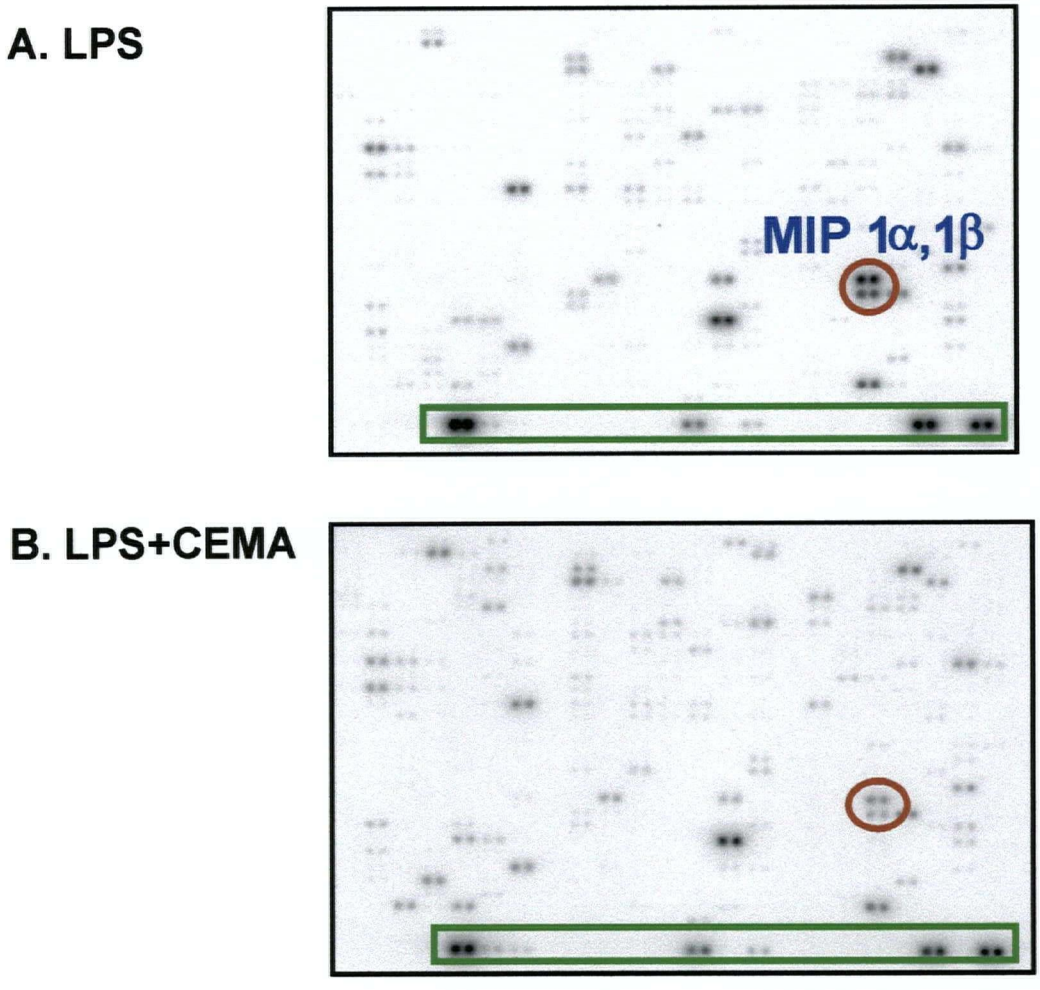


Figure 19: Effect of CEMA on LPS-induced gene expression in RAW 264.7 cells

RAW 264.7 cells were stimulated with (A) 100 ng/ml *Salmonella* LPS or (B) 100 ng/ml *Salmonella* LPS and 50 μg/ml CEMA. The RNA was isolated from the cells and used to make ^{32}P-labeled cDNA probes, which were hybridized to the Clontech Atlas arrays. After a 3-day exposure, they were analyzed using a phosphoimager and Clontech Atlas software. The cDNA spots in the green box identify the genes used for data normalization. The genes in the red circles are examples of macrophage genes whose expression was up-regulated by LPS and decreased by CEMA. These data are representative of 2 to 3 experiments. These experiments were performed in collaboration with C.M. Rosenberger.

were identified including a winged helix transcription factor called brain factor 1, Brn-3.2 POU transcription factor, PD-1 (possible cell death inducer), and HMG-14 chromosomal protein.

It was of interest to determine whether the binding of CEMA to LPS inhibited all LPS-induced changes in gene expression, or whether it selectively modulated LPS responses. Table 6 shows that when RAW 264.7 cells were cultured with LPS in the presence or absence of CEMA, CEMA reduced (30-86%) the ability of LPS to up-regulate the expression of 41 different genes. Interestingly, there was a large variation in inhibition of LPS-induced gene expression. CEMA reduced the LPS-induced up-regulation of many of the inflammation-related genes on the arrays including IL-1β, IL-15, MIP-1α and iNOS (Table 6). In addition to reducing the ability of LPS to increase the levels of cytokine mRNA, it was found that CEMA also substantially reduced the ability of LPS to induce the expression of a number of genes with other functions. In particular, LPS increased the levels of mRNA for the pRb-family retinoblastoma proteins p107 and p130 by more than 30-fold and CEMA reduced these responses by 59% (p107) and 77% (p130). CEMA also decreased the LPS-induced expression of several transcription factors including basic leucine zipper transcription factor and Brn-3.2 POU transcription factor by 80 and 70% respectively. Previous cationic peptide studies have focused on peptide-mediated inhibition of selected pro-inflammatory proteins induced by LPS. This was the first report of an antimicrobial peptide decreasing LPS-stimulated induction of genes other than pro-inflammatory genes, including genes involved in cell proliferation and apoptosis.

CEMA varied widely in its ability to reduce LPS-induced gene expression; the transcription of some genes was reduced by as much as 85% (IL-15) and that of other genes such as Stat1 and NF-E2 transcription factor was only partially inhibited (30-40%). Furthermore, CEMA did not

Table 6: Genes up-regulated by LPS in RAW 264.7 cells and reduced by CEMA

Gene/protein	Unstimulated intensity	Ratio of stimulated : unstimulated		Reduction due to CEMA (%)[3]	Accession number	Gene
		LPS[1]	LPS + CEMA[2]			
IL-1β	20	105.8	72.2	32	M15131	F4k
Fas	20	84.8	59.2	30	M83649	C3f
Egr-1	20	83.7	47.1	44	M20157	D2l
MIP-2α	20	72.7	19.3	73	X53798	F3g
CD 40	20	64.5	25.3	61	M83312	E1f
Tristetraprolin	20	62.1	13.9	78	M57422	B4k
ICE	20	59.4	22.1	63	L28095	F7a
brain factor 1	20	51.4	7.5	85	U36760	D1f
CACCC Box- binding protein BKLF	20	47.8	7.8	84	U36340	D1j
Butyrate response factor 1	20	46.7	9.0	81	M58566	D1l
MIP-1β	188	36.8	15.4	58	M35590	F3f
Basic leucine zipper transcription factor	20	32.9	6.5	80	L36435	D1e
p107	20	31.6	12.9	59	U27177	A1j
p130	20	31.4	7.2	77	U36799	A1k
iNOS	20	31.0	4.3	86	M87039	C3m
Brn-3.2 POU transcription factor	20	31.0	9.4	70	S68377	D1h
Activator –1 140-kDa subunit	20	24.7	14.1	43	X72711	C5e
IL-15	20	22.2	3.4	85	U14332	F5a
AT motif-binding factor	20	21.9	4.8	78	D26046	D1d
Thrombomodulin	20	20.5	11.5	44	X14432	F4d
Stat5a	20	19.8	8.4	58	Z48538	B4f
Adenosine A3 receptor	20	18.4	3.3	82	L20331	C2h
Glucocorticoid receptor form A	20	18.3	11.4	38	X13358	E3m
NF-E2 transcription factor	20	16.5	11.0	34	U01036	D2d

Table 6, continued

Gene/protein	Unstimulated intensity	Ratio of stimulated : unstimulated		Reduction due to CEMA (%)[3]	Accession number	Gene[4]
		LPS[1]	LPS + CEMA[2]			
MIP-1α	489	15.1	8.3	45	X12531	F3e
Syk tyrosine-protein kinase	20	12.0	7.0	42	U25685	B5d
CD40 ligand	20	11.3	3.0	74	X65453	C2n
IRF1	120	8.7	3.6	59	M21065	B7k
TNFR-1	580	7.1	4.5	37	M59378	C5d
uPAR1	121	6.7	3.8	44	X62700	B3l
I-κB β	143	6.3	2.6	59	U19799	B3n
TRAIL	151	6.2	2.9	52	U37522	C5c
Transferrin receptor	234	4.5	3.0	33	X57349	B3h
FLIP-L	188	4.0	1.7	57	U97076	C3h
TNF1 (55 kDa)	121	3.6	1.5	59	X57796	C5b
I-κB α	402	3.3	2.0	38	U36277	B3m
Stat1	858	3.2	2.2	32	U06924	B4d
TFIID Transcription factor	124	3.0	1.7	43	D01034	B4j
Stat3	188	2.7	1.1	57	U06922	B4e
PD-1	582	2.6	1.7	36	X67914	C4f
Interferon regulatory factor 2	**442**	**2.2**	**1.3**	**43**	**J03168**	**D4l**

Total RNA was isolated from unstimulated RAW 264.7 cells and cells treated for 4 hr with 100 ng/ml LPS in the presence or absence of 50 μg/ml CEMA. After reverse transcription, ^{32}P-labelled cDNA was used to probe Clontech Atlas gene array filters. Hybridization was analyzed using Atlas Image (Clontech) software. The array experiments were repeated 3 times with different RNA preparations and yielded very similar results; the average fold changes are shown above. The actual changes in the normalized hybridization intensities of the housekeeping genes ranged from 0.8-1.2 fold, validating the use of these genes for normalization. When the normalized hybridization intensity for a given cDNA was less than 20, it was assigned a value of 20 (Der et al. 1998) to calculate the ratios and relative expression levels.

[1] The ratio was calculated by dividing the intensities for cells treated with 100 ng/ml LPS by the intensities for unstimulated cells.

[2] The ratio was calculated by dividing the intensities for cells treated with 100 ng/ml LPS and 50 μg/ml CEMA by the intensities for unstimulated cells.

Table 6, continued

[3] The percent reduction by CEMA of LPS-induced gene expression intensities is represented as the ratio of LPS:unstimulated - LPS+CEMA:unstimulated divided by the LPS:unstimulated ratio.

[4] The gene classes (given by the first letter of the gene name) include Class A: oncogenes, tumour suppressors and cell cycle regulators; Class B: stress response, ion channels, transport, modulators, effectors and intracellular transducers; Class C: apoptosis, DNA synthesis and repair; Class D: transcription factors and DNA-binding proteins; Class E: receptors (growth, chemokine, interleukin, interferon, hormone, neurotransmitter), cell surface antigens and cell adhesion; Class F: cell-cell communication (growth factors, cytokines, chemokines, interleukins, interferons, hormones), cytoskeleton, motility, and protein turnover.

block the ability of LPS to increase the expression of 16 other genes (Table 7). These genes included several that are strongly up-regulated by LPS such as c-rel, mdm-2, and ICAM-1. This indicates that the peptide had a selective effect on gene induction by LPS. This was interesting since CEMA, like other cationic antimicrobial peptides, binds to LPS and inhibited its binding to LBP (Chapter 4). LBP catalyzes the transfer of LPS to CD14 and the binding of LPS to CD14 is thought to be important for most responses to LPS. Based on this model, one could predict that CEMA would globally suppress responses to LPS. Several explanations are possible as to why some LPS responses were not blocked by CEMA. One possibility was that those responses that were not blocked by CEMA did not involve the transfer of LPS to CD14 by LBP. A second explanation was that different responses had different thresholds for induction. Some genes might require a stronger LPS signal to be induced more than others. Inhibition of LPS binding to CD14 by CEMA would reduce the ability of LPS to stimulate intracellular signaling reactions. Therefore, genes that required very strong LPS signals to be induced would be inhibited by CEMA, whereas genes that require only small amounts of LPS for signaling might still be induced. A third possibility was that cationic peptides such as CEMA also act directly on macrophages to regulate signaling pathways and that this differentially affected the ability of LPS to up-regulate the expression of different genes.

5.4 Confirmation of inhibition of IL-1β and NO production by LPS-stimulated macrophages

To assess the functional significance of the gene array results, ELISAs were performed on culture supernatants from the RAW 264.7 cells. Consistent with the array findings, it was found that the levels of the chemokine MIP-1α secreted into the medium were greatly increased by LPS stimulation (cytokine concentrations of 6.3-8 ng/ml compared to <0.2 ng/ml for unstimulated

Table 7: Genes up-regulated by LPS in RAW 264.7 cells and not inhibited by CEMA

Gene/protein	Unstimulated intensity	Ratio of stimulated : unstimulated		Accession number	Gene
		LPS	LPS + CEMA		
c-rel proto-oncogene	20	24.4	30.1	X15842	A2m
Mdm2	20	22.3	22.5	X58876	A1h
HMG-box transcription factor	20	17.6	17.5	D49474	D3l
ICAM-1	20	15.9	17.0	X52264	E7l
DNA topoisomerase I	20	14.8	15.6	D10061	C5m
GA binding protein beta-2 chain	20	14.2	18.0	M74517	D3d
NF-κB binding subunit	172	4.7	5.0	M57999	D5g
Golgi 4 transporter	193	3.6	4.3	U34259	B2d
N-ras proto-oncogene	283	2.4	2.9	X13664	A5e
MAPKK1	722	2.0	2.4	L02526	B6a
CD18 β subunit	592	2.0	2.2	X14951	E5n
Mad related protein 2	193	1.9	2.1	U60530	F3h
Cyclin B1	704	1.8	2.3	X64713	A6c
DNA topoisomerase II	219	1.7	2.9	D12513	C5n
HMG-14 chromosomal protein	994	1.6	2.7	X53476	D3m
CD14	**5970**	**1.6**	**1.7**	**M34510**	**E6h**

RNA was isolated from unstimulated RAW 264.7 cells and RAW 264.7 cells treated for 4 hr with 100 ng/ml LPS in the presence or absence of 50 μg/ml CEMA (refer to Table 6 legend for details).

cells) and that CEMA at 50 μg/ml reduced this response by 46%. Levels of IL-1β (Figure 20) in the supernatant of RAW 264.7 cells incubated with LPS (100-130 pg/ml) were found to be decreased by 53 ± 5% (inhibition ± standard error) in the presence of 50 μg/ml CEMA. In whole human blood incubated with LPS and LPS plus 50 μg/ml CEMA for 4-6 hours, there was similar inhibition of LPS-induced IL-1β production by CEMA. LPS alone resulted in serum levels of IL-1β ranging from 0.56-0.94 ng/ml and CEMA inhibited this by 40 ± 3% (mean inhibition ± standard error). This again is similar to the results with the gene arrays. When the supernatants of the cells used for RNA isolation were tested for the cytokine levels of TNF-α and IL-6, CEMA inhibited the LPS-induction of these cytokines by 78% and 86% respectively, consistent with previous studies and those with other cell lines and primary macrophages (Gough et al. 1996; Scott et al. 1999).

The gene iNOS (inducible NO synthase) encodes the enzyme responsible for inducing the inflammatory mediator, NO. Since the peptide was found to also inhibit LPS-induced iNOS expression, NO levels were examined in the supernatant of the macrophage cells stimulated with LPS and LPS+CEMA by measuring the accumulation of the stable NO metabolite nitrite with the Griess reagent (Figure 20). The levels of NO were found to be increased in the presence of LPS (0-8 to 47-71 μM) and inhibited by an average of 76 ± 2% by the addition of CEMA (Figure 20). Similarly, the levels of iNOS on the gene arrays were up-regulated by LPS (31-fold) and inhibited 86% by CEMA. It should be noted that although these results demonstrated the same trends for the transcriptional array and product assays, the measurement of iNOS and IL-1β was performed at 24 h to permit the development of measurable amounts of product whereas the gene arrays examined transcriptional changes at 4 h time point.

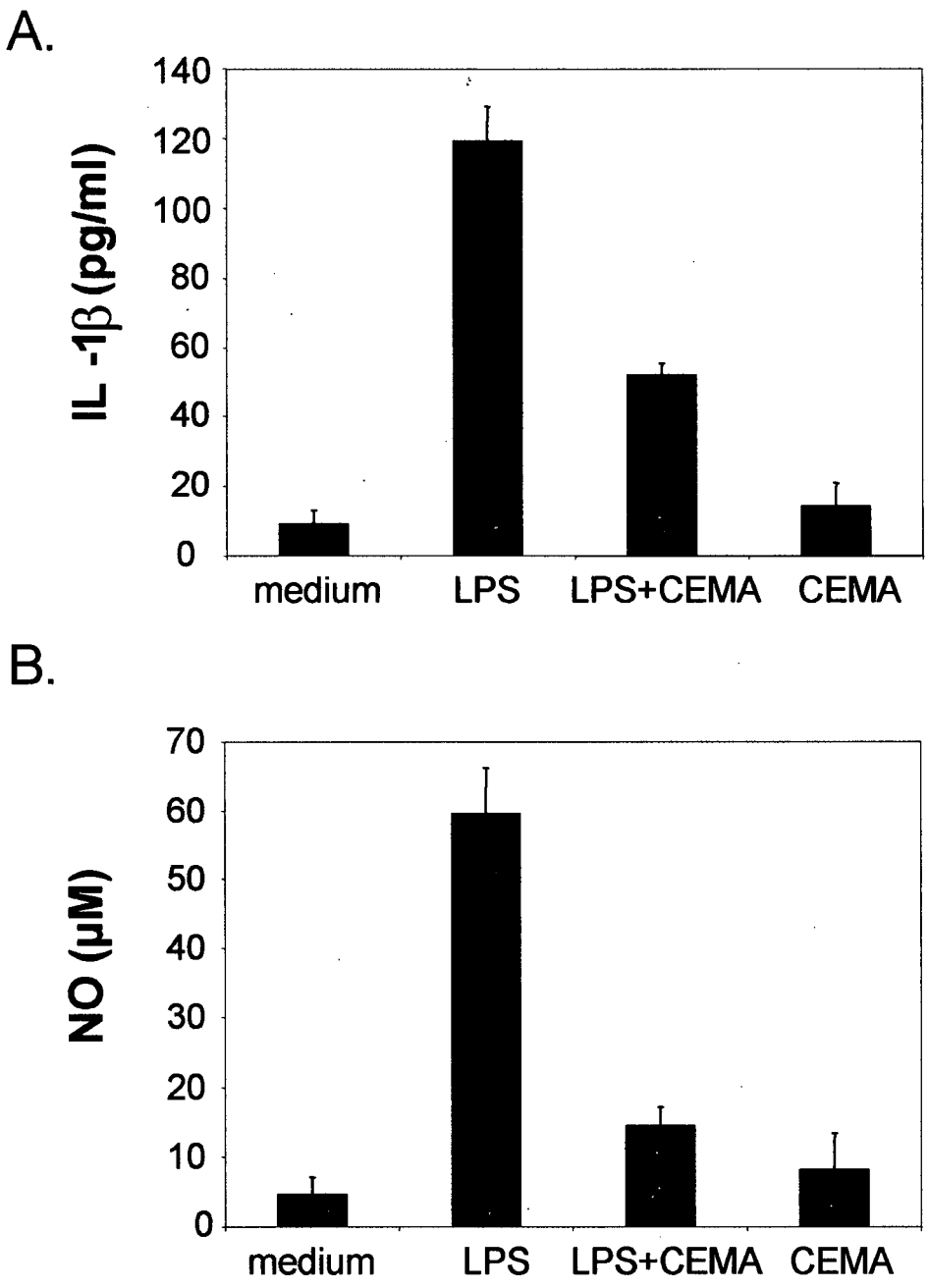


Figure 20: Effect of CEMA on LPS-induced production of IL-1β and nitric oxide (NO) by RAW 264.7 cells

RAW 264.7 cells were stimulated with 100 ng/ml *Salmonella* LPS, 100 ng/ml *Salmonella* LPS plus 50 μg/ml CEMA, 50 μg/ml CEMA or media alone in phenol-red free DMEM +10% fetal bovine serum for 24 hours. The supernatant was removed and tested for (A) IL-1β by ELISA, and (B) the amount of NO was estimated with the Griess reagent. The data presented is the average of three experiments ± standard error.

5.5 Confirmation of selected array data by Northern analysis

Although the array data was very reproducible, and some of the findings were confirmed with ELISAs, it was important to directly confirm that LPS and CEMA affected mRNA levels similarly to the levels indicated by the gene arrays. Northern blots were performed to analyze the expression of IL-1β, CD14, and cyclin D1 since these genes represent the three different scenarios observed. According to the gene array results, IL-1β mRNA levels were strongly up regulated by LPS and this response was reduced by CEMA (Table 6). Conversely CD14 mRNA levels were modestly up-regulated by LPS and this response was not blocked by CEMA (Table 7) while cyclin D1 mRNA levels were not induced by LPS but modestly up-regulated by CEMA. All of these results were confirmed by the Northern blots and the quantification of these results is shown in Figures 21 and 22. The gene arrays successfully identified multiple patterns of gene expression and demonstrated trends similar to those observed by Northern blot analysis. To demonstrate that these results were not confined to the synthetic antimicrobial peptide CEMA, LL-37 a natural human peptide was also found to inhibit LPS-induced gene expression of IL-1β (Figure 22) and MIP-2α (data not shown) in the RAW 264.7 macrophage cells to an extent similar to that observed with CEMA. Thus cationic peptides can selectively inhibit macrophage gene expression induced by LPS.

5.6 Summary

Gene arrays were used to profile global changes in gene expression in macrophages treated with bacterial products (LPS, LTA and CpG DNA) as well as macrophages treated with LPS and CEMA. The objective of this study was to better understand the mechanism by which bacterial products stimulated macrophages and to determine how cationic peptides affected this

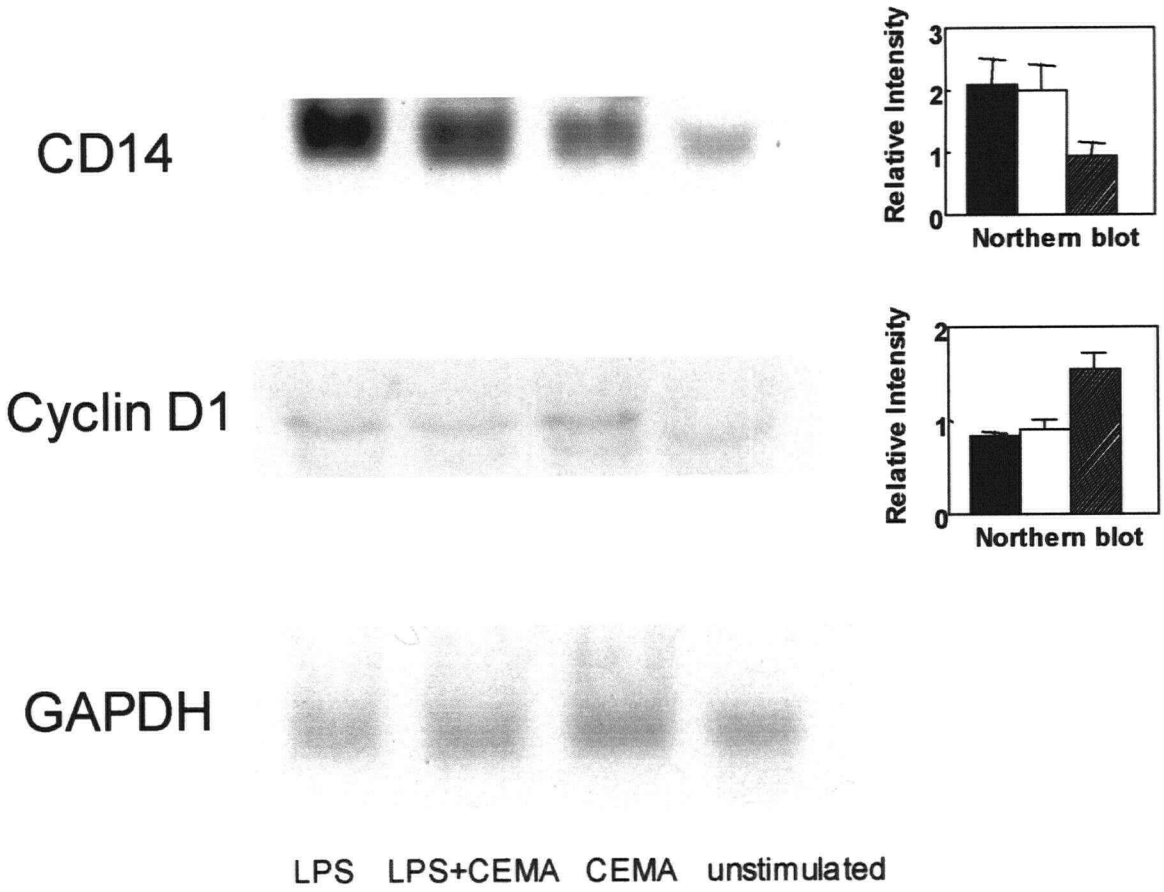


Figure 21: Effect of LPS and CEMA on RAW 264.7 cell mRNA levels as measured by Northern blot analysis

RAW 264.7 cells were stimulated with 100 ng/ml *Salmonella* LPS, 100 ng/ml *Salmonella* LPS plus 50 μg/ml CEMA, 50 μg/ml CEMA or media alone for 4 hours. Total RNA was prepared for Northern blotting and the membrane was probed progressively for CD14, cyclin D1, and GAPDH. The hybridization intensities of the Northern blots were measured with a densitometer and normalized to GAPDH to correct for inconsistencies in loading. The stimulated mRNA levels relative to unstimulated cells was calculated as the mean ± standard error from 3-4 experiments. The bar graphs show the ratios of gene expression of LPS-stimulated / unstimulated cells (solid bars), LPS-stimulated, CEMA-treated to unstimulated cells (open bars), and CEMA-treated to unstimulated cells (hatched bars). A ratio of one therefore indicates no stimulation.

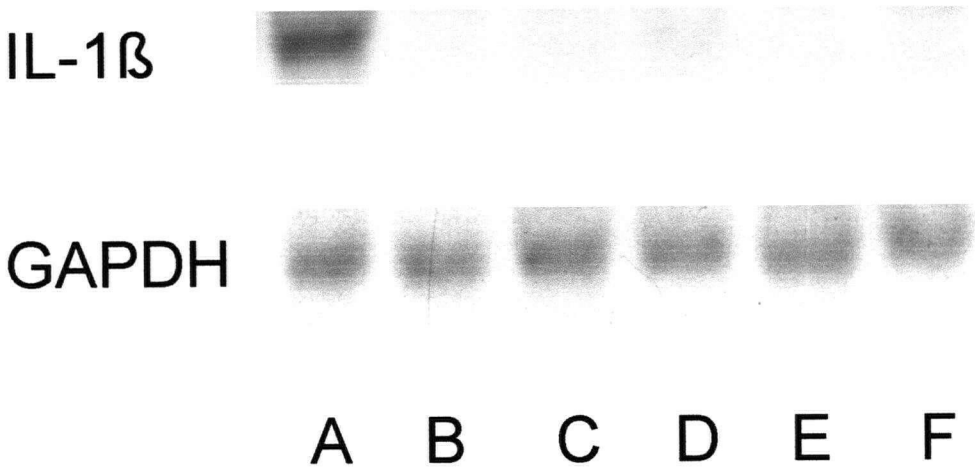


Figure 22: Effect of LPS, CEMA, and LL-37 on IL-1β mRNA levels as measured by Northern blot analysis

RAW 264.7 cells were stimulated with (A) 100 ng/ml *Salmonella* LPS, (B) 100 ng/ml *Salmonella* LPS plus 50 μg/ml CEMA, (C) 100 ng/ml *Salmonella* LPS plus 50 μg/ml LL-37, (D) 50 μg/ml LL37, (E) 50 μg/ml CEMA or (F) media alone for 4 hours. Total RNA was prepared for Northern blotting and the membrane was probed for GAPDH and IL-1β.

stimulation. It was found that a large panel of genes, largely encoding pro-inflammatory products, was similarly affected by all three of the bacterial products tested. It was also found that the cationic peptide, CEMA selectively inhibited LPS-induced changes in gene expression. While the ability of LPS to induce the expression of 41 genes was significantly inhibited by CEMA, the induction of an additional 16 genes was unaffected by CEMA even though it is known that CEMA can interfere with the first step in LPS signalling, the binding of LPS to LBP. It is clear that CEMA has effects other than interference with LPS:LBP binding since CEMA can also suppress endotoxin stimulated induction of cytokines even when added to RAW 264.7 cells up to an hour after LPS. Interestingly, the induction by LPS of inflammatory mediators was substantially inhibited by CEMA, indicating that cationic peptides may selectively down-regulate some macrophage inflammatory functions as opposed to other cellular processes.

CHAPTER 6: The human peptide, LL-37, neutralizes the biological activity of bacterial products from Gram-negative and Gram-positive bacteria

6.1 Introduction

To combat bacterial infection, the host immune response must not only eliminate the bacteria but also limit the inflammatory response to the bacteria and their released components in order to reduce the risk of sepsis. Many of the synthetic cationic antimicrobial peptides studied in this thesis have the ability to neutralize the biological activity of bacterial components from both Gram-positive and Gram-negative bacteria. It was of interest to determine if these activities could be related to the natural function of cationic peptides in the immune system. I hypothesized that LL-37, which is similar in structure to CEMA, widely expressed in humans, and up-regulated in response to bacteria (reviewed in Bals 2000) would also reduce TNF-α release by macrophages stimulated with bacterial components. Therefore, the human α-helical peptide, LL-37, was tested for its ability to reduce the host response to bacterial components in order to learn more about its role in innate immunity. Specifically, LL-37 was tested for its ability to reduce TNF-α production induced by the presence of LPS in a number of model systems. Since Gram-positive bacteria also contributes to many cases of sepsis, LL-37 was also tested for its ability to reduce TNF-α release by RAW 264.7 cells stimulated with *S. aureus* LTA. Thus the goal of these studies was to gain insight into the natural function of LL-37 and determine if this includes the ability to reduce production of TNF-α in response to bacterial components.

6.2 LL-37 reduces LPS-induced production of TNF-α by macrophages

A number of synthetic cationic antimicrobial peptides have been shown to block many LPS-induced responses and are being considered as candidate drugs for the treatment of sepsis. In a previous studies, I demonstrated that synthetic α-helical peptides, including CEME and CEMA, were able to block LPS-induced production of inflammatory cytokines by macrophages (Gough

et al. 1996; Scott et al. 1999). I hypothesized that the ability to limit the biological activity of LPS was a natural function of cationic peptides in the host immune response. I hypothesized that the naturally occurring α-helical cationic peptide LL-37, which is up-regulated in response to bacteria and inflammation would be able to neutralize the biological activity of endotoxin. Therefore, I analyzed the effects of LL-37 on macrophage responses to LPS.

LPS from *E. coli* and other Gram-negative bacteria induce high levels of TNF-α release when incubated with macrophages (Scott et al. 1999; reviewed in van der Poll 2001; Figure 23). The RAW 264.7 murine macrophage cell line was used to study the effect of LL-37 on LPS-induced production of TNF-α release. This cell line has been widely used to study the anti-endotoxin activity of various compounds. The RAW 264.7 cells were incubated with the human peptide, LL-37, along with one of three types of LPS (*E. coli, Salmonella* Typhimurium, or *B. cepacia*). Figure 23 shows that the human peptide, LL-37, substantially reduced TNF-α production by RAW 264.7 cells stimulated with *E. coli* O111:B4, *Salmonella* Typhimurium, or *B. cepacia* LPS, with the *Salmonella* Typhimurium LPS-induced TNF-α production being affected to a somewhat lesser extent. At concentrations as low as 1 μg/ml LL-37 (0.25 nM), substantial reduction of TNF-α production by *E. coli* O111:B4 LPS-stimulated macrophages was observed. LL-37 inhibition of TNF-α produced by LPS-stimulated RAW 264.7 cells was comparable to that seen with the synthetic cationic peptide, CEMA. At 20 μg/ml LL-37 and CEMA reduced TNF-α by 81% and 90% respectively (Figure 23 and Scott et al.1999). The cationic peptides, LL-37 and CEMA (at concentrations up to 100 μg/ml) when incubated with the RAW 264.7 cells and medium containing 10% bovine serum but without LPS, did not significantly increase TNF-α production above background levels. To determine if cationic peptide inhibition of TNF-α production by macrophages was a general effect, 10-100 nM phorbol

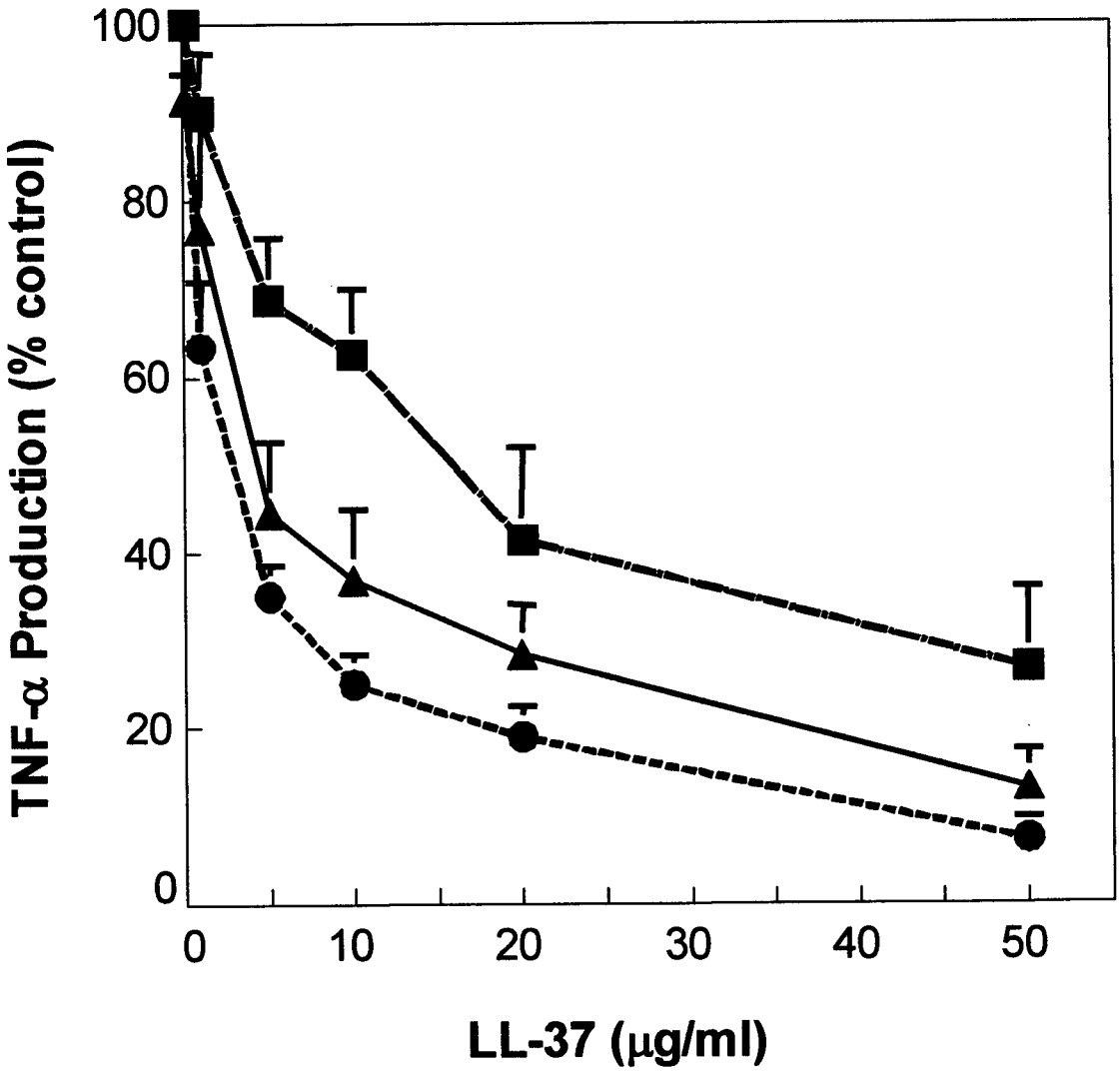


Figure 23: LL-37 reduces LPS-induced production of TNF-α by RAW 264.7 cells

RAW 264.7 cells were stimulated with (■) 100 ng/ml *Salmonella* LPS, (▲) 100 ng/ml *B. cepacia* LPS or (●) 100 ng/ml *E. coli* 0111:B4 LPS in the presence of the indicated concentrations of LL-37 for 6 hr. The concentrations of TNF-α released into the culture supernatants were determined by ELISA. 100% represents the amount of TNF-α resulting from RAW 264.7 cells incubated with LPS alone for 6 hours (*Salmonella* LPS = 34.5 ± 3.2 ng/ml, *B. cepacia* LPS = 11.6 ± 2.9 ng/ml, and *E. coli* 0111:B4 LPS = 30.8 ± 2.4 ng/ml). Background levels of TNF-α production by the RAW 264.7 cells cultured with no stimuli for 6 hours resulted in TNF-α levels ranging from 0.037 – 0.192 ng/ml. The data is from duplicate samples and presented as the mean of three experiments ± standard error.

12-myristate 13-acetate (PMA) was used to stimulate RAW 264.7 cells and it was found that the cationic peptides did not block the TNF-α production (<10% inhibition of TNF-α) caused by PMA. Similar results of cationic peptide inhibition of LPS-stimulated TNF-α production were also obtained using another macrophage cell line, J774.1 (ie. the production of TNF–α by J774.1 cells stimulated with LPS was inhibited 92% by 20 μg/ml CEMA). Thus the human peptide, LL-37 was able to reduce the release of TNF-α by a macrophage cell line stimulated with a variety of LPS types.

B. cepacia LPS is not well known for its endotoxin activity and yet it has been shown to cause DLK (Diffuse Lameller Keratitis), a rare complication of LASIK surgery, in a rabbit model (Morck et al. 2002) and clinical epidemiological and microbiological data suggest that LPS induced DLK may be an important cause of cluster or outbreak DLK (Holland et al. 2000). LASIK stands for Laser-Assisted *In Situ* Keratomileusis and is a procedure that permanently changes the shape of the cornea in order to reduce a person's dependency on glasses. It was also interesting to study the LPS from *B. cepacia* as these bacteria are resistant to killing by cationic peptides (Table 5 and Saiman et al. 2001). For these reasons Figure 24 has been included which demonstrates the ability of *B. cepacia* LPS to induce TNF-α release and that this can be inhibited by the cationic antibiotic, polymyxin B. The RAW 264.7 cell line was stimulated with a range of concentrations of *B. cepacia* LPS for 6 hours and the release of TNF-α into the culture supernatants was quantitated by ELISA (Figure 24). *B. cepacia* LPS was found to induce significant levels of TNF-α at 0.1 and 1 μg/ml. Although it induced high levels of TNF-α, it was not as potent an inducer of TNF-α as *E. coli* LPS (Figure 23, 100 ng/ml, *E. coli* LPS induced 30.8 ± 2.4 ng/ml TNF-α in RAW 264.7 cells). However, *B. cepacia* LPS has been shown to induce a stronger TNF-α response from human monocytes than *P. aeruginosa* LPS (Zughaier et al. 1999).

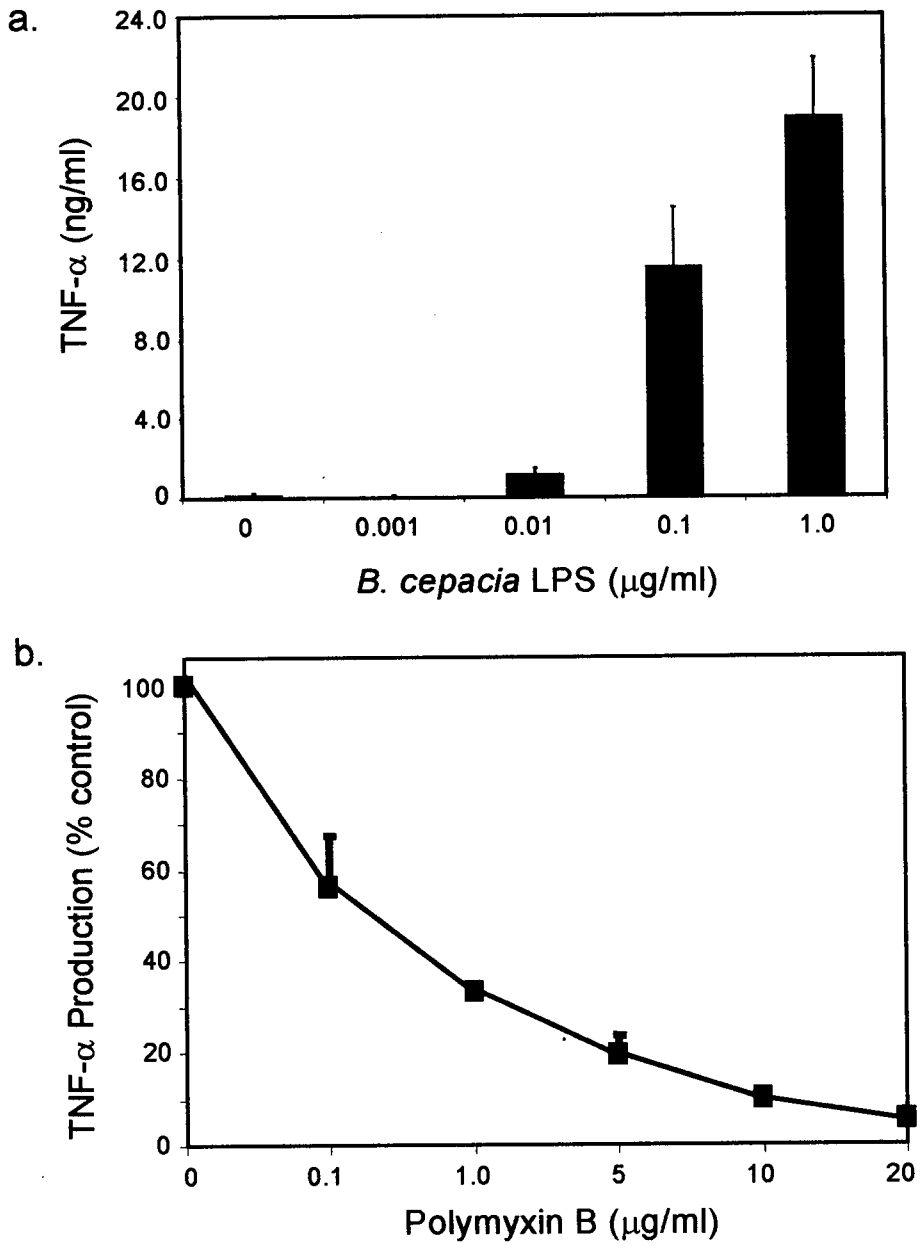


Figure 24: ***B. cepacia*** **LPS induces production of TNF-α by RAW 264.7 cells which can be reduced by polymyxin B**

RAW 264.7 cells were stimulated with a) a range of concentrations of *B. cepacia* LPS for 6 hr. and b) 100 ng/ml *B. cepacia* LPS in the presence of the indicated concentrations of polymyxin B for 6 hr. The concentrations of TNF-α released into the culture supernatants were determined by ELISA. The data from duplicate samples is presented as the mean of three experiments ± standard error. 100% represents the amount of TNF-α resulting from RAW 264.7 cells incubated with *B. cepacia* LPS alone for 6 hours (11.6 ± 2.9 ng/ml). Background levels of TNF-α production by RAW 264.7 cells cultured with no stimuli for 6 hours resulted in TNF-α levels ranging from 0.037 – 0.192 ng/ml.

Also in another study, the biological activity of highly purified *B. cepacia* LPS has been shown to be comparable to that of the LPS of *Salmonella enterica* serovar Abortus-equi (Shimomura et al. 2001). Since the cationic antibiotic, polymyxin B has a high affinity for LPS (i.e. Scott et al. 1999), it was tested to determine if it could reduce TNF-α production by RAW 264.7 cells stimulated with *B. cepacia* LPS. Polymyxin B (at a concentration as low as 1 μg/ml) was found to substantially reduce TNF-α production by RAW 264.7 cells incubated with *B. cepacia* LPS (Figure 24). This is a significant finding as it demonstrates the potential for polymyxin B to be used as a topical treatment for DLK. Subsequent to these studies, when polymyxin B (in the form of the commercially available Polytrim^{TM} (Allergan, Irvine CA)) was given to rabbits that underwent LASIK surgery, it was found to significantly limit the development of DLK in a rabbit model (Morck et al. 2002).

To determine if the cationic peptides affected the viability of the RAW 264.7 cells, several experiments were performed. To assess possible toxicity of the cationic peptides on the RAW cells, trypan blue staining was performed. At concentrations up to 100 μg/ml of LL-37 or CEMA, there was no apparent cytotoxicity as visualized by low levels of trypan blue stained cells (comparable to cells with medium alone) and the continued adherence to tissue culture plates of cells incubated with peptide for 24 hours. The MTT (3- 4 5-dimethylthiazol-2-yl 2 5-diphenyltetrazolium bromide) assay (first described (Mosmann 1983)) was performed to determine if the cationic peptides affected cell viability. The RAW 264.7 cells were incubated with increasing concentrations of peptide for 24, 48, and 72 hours and stained with MTT. The principle of the MTT assay is metabolically active cells will cleave the yellow tetrazolium salt, MTT to purple formazan crystals which can be solubilized in ethanol and read with an ELISA plate reader. Figure 25 demonstrates that at 24 hours, 125 μg/ml of LL-37, CEMA and CP208 did

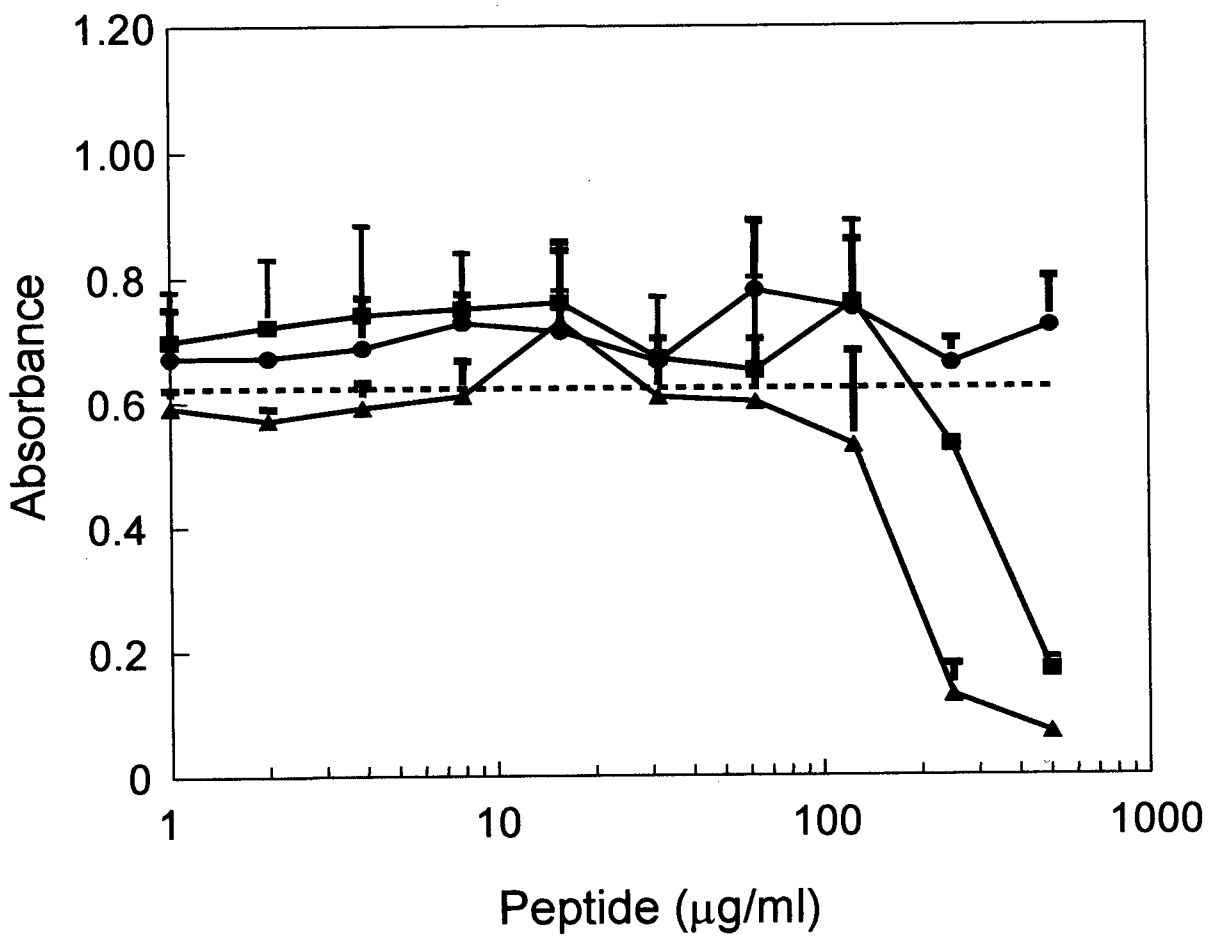


Figure 25: Effect of cationic peptides on RAW 264.7 cell viability as measured by the MTT assay

RAW 264.7 cells were incubated with increasing concentrations of LL-37 (■), CEMA (▲), CP208 (●) and media alone (--) for 24 hours. The supernatant was removed and the cells were stained with MTT. After a 3 hour incubation, the dye was removed and replaced with ethanol to solubilize the formazan crystals. The absorbance was then read with a plate reader. The dotted line represents the average absorbance for 12 wells of RAW 264.7 cells incubated with medium alone. The reduced absorbance levels below the level of medium alone indicates decreased cell viability. The data is presented as the mean of two experiments ± standard error.

not substantially affect the metabolic activity of the RAW 264.7 cells. These results were similar at 48 hours.

It was important to see if the inhibition of LPS-induced TNF-α production by LL-37 could be reproduced in other macrophage cells. The anti-endotoxin activity of several cationic peptides was confirmed in murine bone marrow-derived macrophages cells, in that LL-37 and CEMA significantly reduced TNF-α production (>90%) by bone marrow-derived macrophages from BALB/c mice that had been stimulated with 100 ng/ml *E. coli* 0111:B4 LPS (Figure 26a). These experiments also demonstrated that LL-37 alone at concentrations up to 100 μg/ml did not induce TNF-α production by the bone marrow-derived macrophages (Figure 26b). As IL-6 is also a known inflammatory mediator of sepsis, the levels of this cytokine were also measured in these cells and very similar results to those seen with TNF-α were found (10 μg/ml LL-37 or CEMA resulted in 98 ± 1.5% and 99 ±1% reduction of LPS-induced TNF-α release respectively). These experiments were performed in the presence of serum, which contains LBP. Therefore we examined the kinetics of antagonism of LPS-induction of TNF-α production (Figure 27). Addition of LL-37 to macrophage cultures one hour after addition of 100 ng/ml *E. coli* LPS still resulted in substantial (70%) reduction of TNF-α production. Similar results had been previously obtained with CEMA (Gough et al. 1996). Thus both synthetic and natural cationic peptides can inhibit LPS-induced TNF-α production even when added an hour after LPS, suggesting that the peptides may be doing more than simply binding to LPS.

Consistent with the ability of LL-37 to prevent LPS-induced production of TNF-α *in vitro*, LL-37 partially protected mice against lethal endotoxic shock induced by a high concentration of LPS (Table 8). CD-1 mice were sensitized to LPS with an injection of galactosamine. Mice that

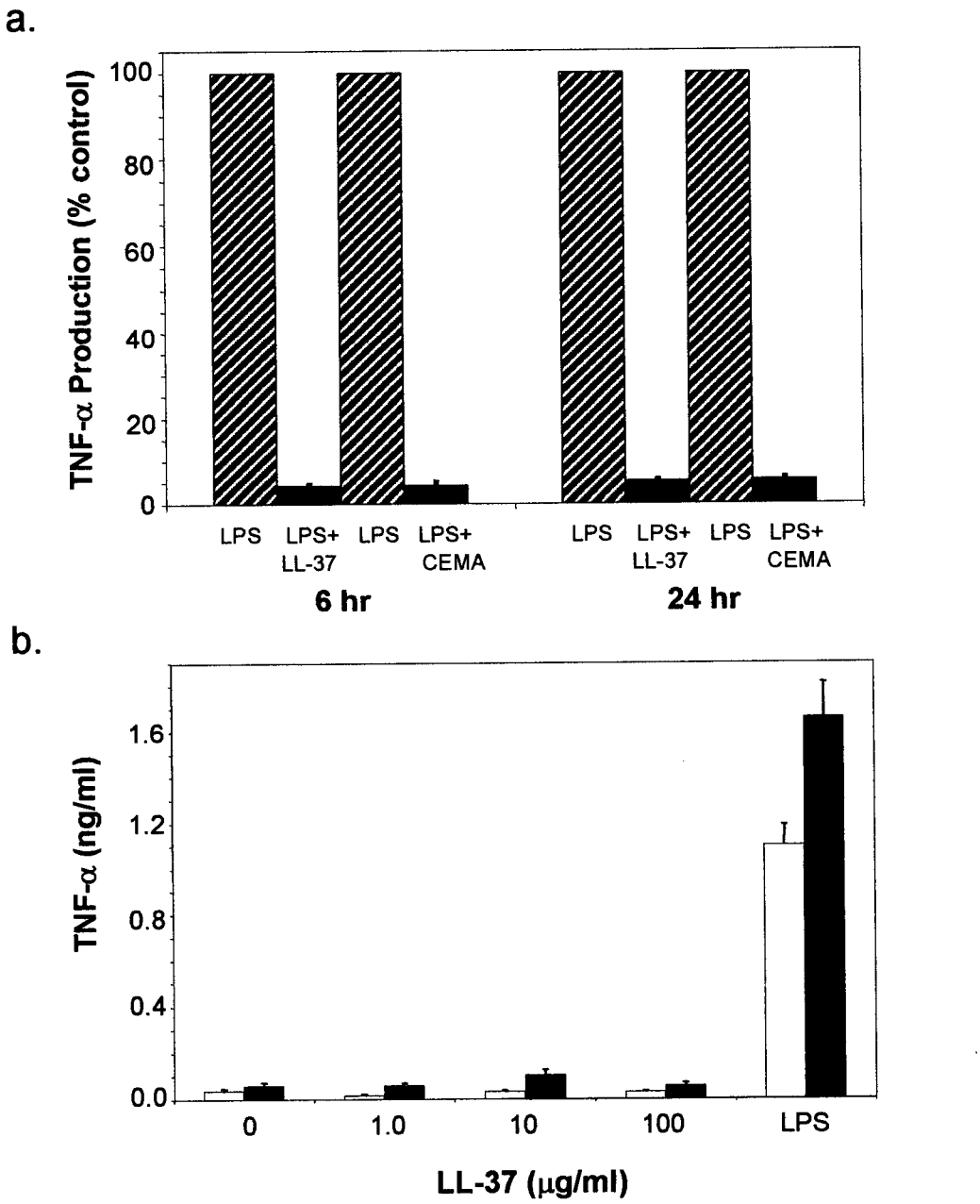


Figure 26: LL-37 reduces LPS-induced production of TNF-α and does not itself induce TNF-α by murine bone marrow-derived macrophages

Bone marrow-derived macrophages were cultured for either 6 h or 24 h with a) 100 ng/ml *E. coli* 0111:B4 LPS in the presence or absence of 20 μg/ml of peptide or b) increasing amounts of LL-37 alone (6h-open bars, 24h-solid bars). The supernatant was collected and tested for levels of TNF-α by ELISA. 100% represents the amount of TNF-α resulting from duplicate wells of bone marrow-derived macrophages incubated with LPS alone for 6 h (1.1 ± 0.09 ng/ml) or 24 h (1.7 ± 0.2 ng/ml). The data from duplicate samples is presented as the mean of three experiments ± standard error for a) and the mean of two experiments ± standard error for b).

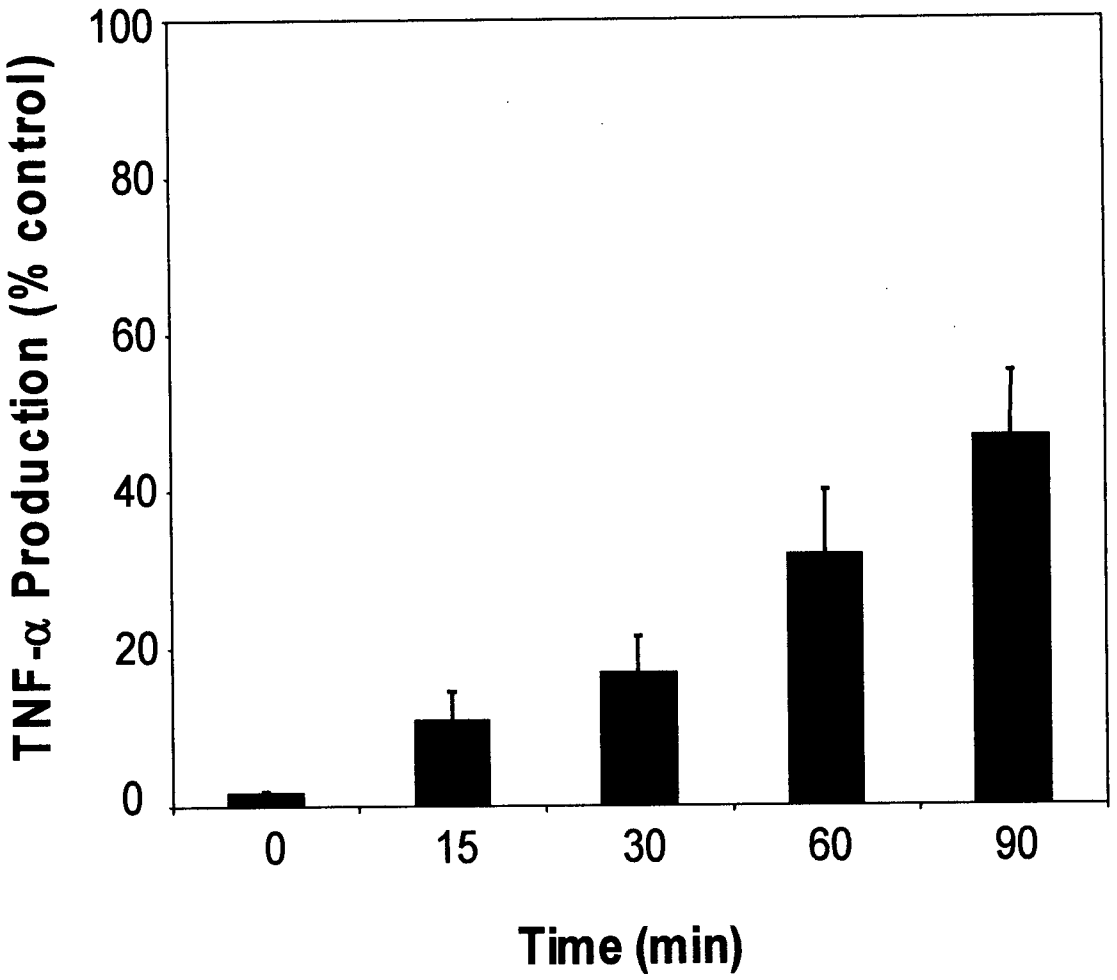


Figure 27: LL-37 reduces LPS-induced production of TNF-α by RAW 264.7 cells even when added after the addition of LPS

LL-37 (20 μg/ml) was added at various times after the addition of 100 ng/ml *E. coli* 0111:B4 LPS to RAW 264.7 cells. The supernatant was collected after 6 hours and tested for levels of TNF-α by ELISA. 100% represents the amount of TNF-α resulting from RAW 264.7 cells incubated with LPS alone for 6 hours (30.8 ± 2.4 ng/ml). The average of duplicate samples is presented as the mean of three experiments ± standard error.

Table 8: Protection against lethal endotoxemia in galactosamine-sensitized CD-1 mice by LL-37

D-Galactosamine treatment	*E. coli* 0111:B4 LPS	LL-37 or buffer	Total mice	Survival post endotoxin shock
0 mg	3 µg	PBS	5	5 (100%)
20 mg	3 µg	PBS	12	0 (0%)
20 mg	3 µg	LL-37	12	6 (50%)

CD-1 mice (9 weeks-old) were sensitized to endotoxin by intraperitoneal injection of galactosamine (20 mg in 0.1 ml sterile PBS). Endotoxic shock was then induced by intraperitoneal injection of *E. coli* 0111:B4 LPS (3 µg in 0.1 ml PBS). LL-37 (200 µg/mouse = 8mg/kg) was injected at a separate intraperitoneal site 15 min after injection of LPS. The survival of the mice after 48 hr was determined.

were then injected with 3 μg of *E. coli* 0111:B4 LPS died within 4-6 hours. When 200 μg of LL-37 was injected 15 min after the LPS, 50% of the mice survived (Table 8). CEMA has also been found to increase survival of mice injected with LPS and neutropenic mice challenged with *P. aeruginosa* (Gough et al. 1996). Potentially then, both natural and synthetic cationic peptides can protect against the toxic effects of LPS *in vivo*.

It has been shown that cationic peptides are active inhibitors of LPS stimulation in macrophage cell lines, in mouse primary cells and in mice. Since LL-37 is a widely expressed (ie. neutrophils, epithelium) human cationic peptide, it was of interest to study its activity in a human experimental system. LL-37 and CEMA along with other cationic peptides were then examined for their ability to inhibit LPS-induced production of cytokines in whole human blood (Figure 28). The cationic peptides tested, with the exception of CM3, substantially inhibited the production of TNF-α and IL-1β in whole blood stimulated with *E. coli* LPS for 6 hours. In contrast, at these concentrations, the cationic peptides had little to no effect on LPS-induced IL-8 production (ie. CP29, CM7, and CEMA inhibited TNF-α production in whole blood stimulated with LPS by an average of only 18%, 13% and 2% respectively). Although the cationic peptides were not as active in whole blood, they could still reduce the LPS-mediated induction of select pro-inflammatory mediators. The decreased ability of the peptides to reduce LPS-induced TNF-α inhibition in whole blood could be due to the presence of heparin in the blood collecting tubes (Ogata et al. 1997). Nevertheless, the human peptide, LL-37, as well as, synthetic peptides were able to blunt the biological activity of LPS in a wide range of systems including cell lines, primary cells, whole blood and mice.

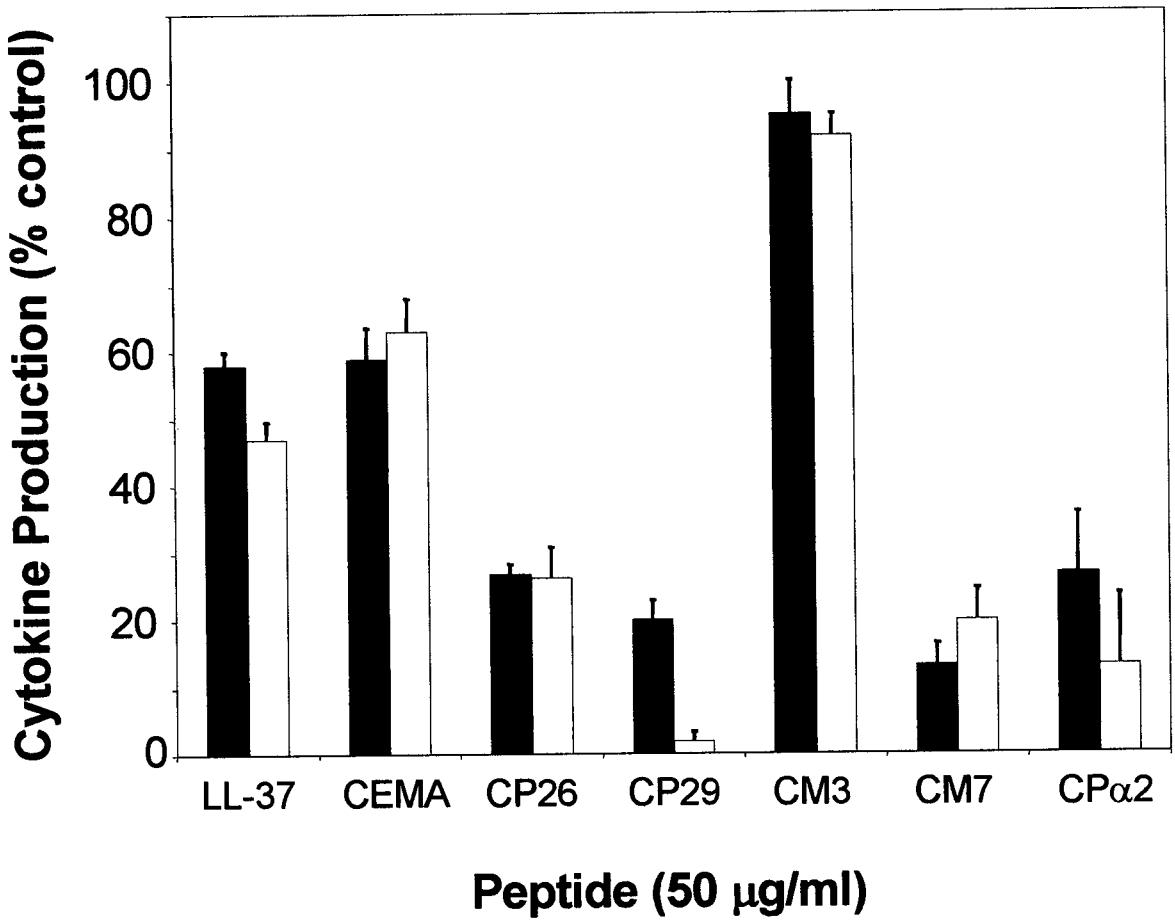


Figure 28: Cationic peptides reduce levels of TNF-α and IL-1β in whole blood stimulated with 100 ng/ml *E. coli* 0111:B4

Whole human blood was incubated with *E. coli* 0111:B4 LPS in the presence or absence of 50 μg/ml peptide for 6 hr. The concentrations of TNF-α (solid bars) and IL-1β (open bars) were measured by ELISA. The bars represent the amount of TNF-α or IL-1β production (mean ± standard error) from duplicate samples of blood from two separate donors incubated with LPS and peptide. 100% cytokine production represents the amount of TNF-α or IL-1β resulting from whole blood incubated with LPS alone for 6 hours (TNF-α = 7.3 ± 1.5 ng/ml, IL-1β = 0.8 ± 0.1 ng/ml). The amount of TNF-α in unstimulated whole blood was 0.298 ± 0.09 ng/ml and there was no detectable IL-1β in unstimulated whole blood.

6.3 LL-37 reduces TNF-α release by macrophages stimulated with *S. aureus* LTA and *Mycobacterium* AraLAM

Previous studies have shown that when LTA is injected into animals it causes many of the characteristics of septic shock (Natanson et al. 1989; Wakabayashi et al. 1991; De Kimpe et al. 1995; Le Roy et al. 1996; Kengatharan et al. 1998). Consistent with this observation, LTA also induces the production of inflammatory cytokines by macrophages *in vitro* (Heumann et al. 1994). Since many of the synthetic CEME-related peptides tested were potent inhibitors of LTA-induced cytokine production, the natural human peptide, LL-37, was also tested for its ability to inhibit *S. aureus* LTA induced TNF-α production by RAW 264.7 cells. LL-37 did indeed reduce induction of TNF-α in RAW 264.7 cells by the Gram-positive bacterial product, LTA (Figure 29). At a concentration of 1 μg/ml, LL-37 was able to substantially reduce (>75%) the induction of TNF-α production by 1 μg/ml *S. aureus* LTA. Thus, both natural and synthetic cationic peptides can reduce the ability of the major cell wall components released from both Gram-negative (LPS) and Gram-positive (LTA) bacteria to stimulate inflammatory responses.

I next examined whether LL-37 could block the induction of cytokine production by *Mycobacterium* non-capped lipoarabinomannan (AraLAM). The cell envelope of mycobacteria contains lipoarabinomannan (LAM), lipomannan and phosphatidylinositol mannosides. Both non-capped LAM (AraLAM) and mannose-capped LAM (ManLAM) stimulate macrophage production of TNF-α although AraLAM is a more potent stimulus of TNF-α production (Chapter 3; Moreno et al. 1989; Barnes et al. 1992; Chatterjee et al. 1992). Therefore, the ability of LL-37 to block AraLAM-induced cytokine production in RAW 264.7 cells was examined. I found that LL-37 reduced AraLAM-induced TNF-α production by RAW 264.7 cells (Figure 30), although to a lesser extent than with LTA stimulation. For example, 1 μg/ml LL-37 was able to reduce the

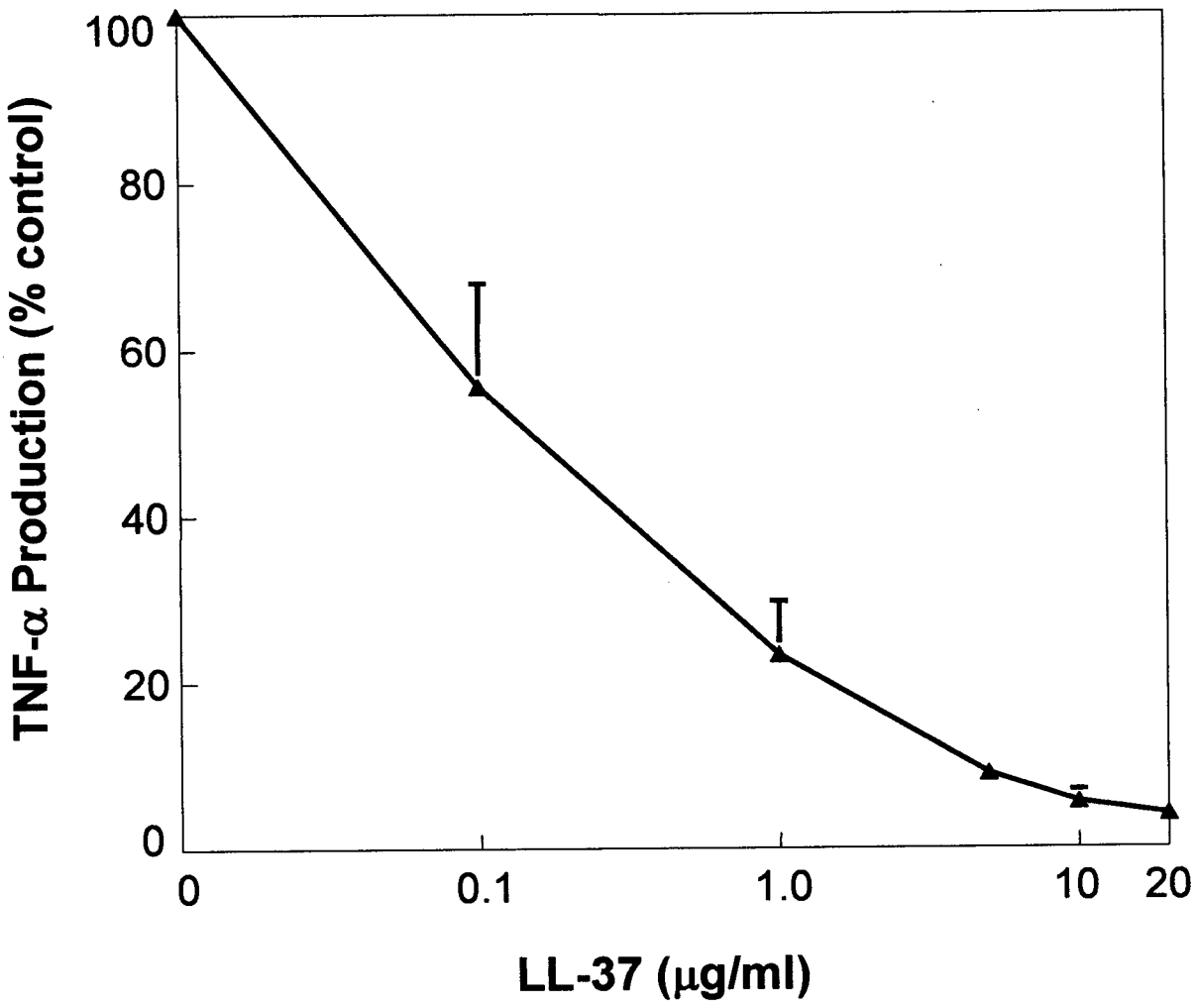


Figure 29: LL-37 reduces TNF-α production by RAW 264.7 cells stimulated with *S. aureus* LTA

RAW 264.7 cells were stimulated with 1 μg/ml *S. aureus* LTA and increasing concentrations of LL-37. The supernatant was collected and tested for levels of TNF-α by ELISA. 100% represents the amount of TNF-α resulting from RAW cells incubated with *S. aureus* LTA alone for 6 hours (8.7 ± 3.2 ng/ml). The data is presented as the mean of three experiments (done in duplicate) ± standard error.

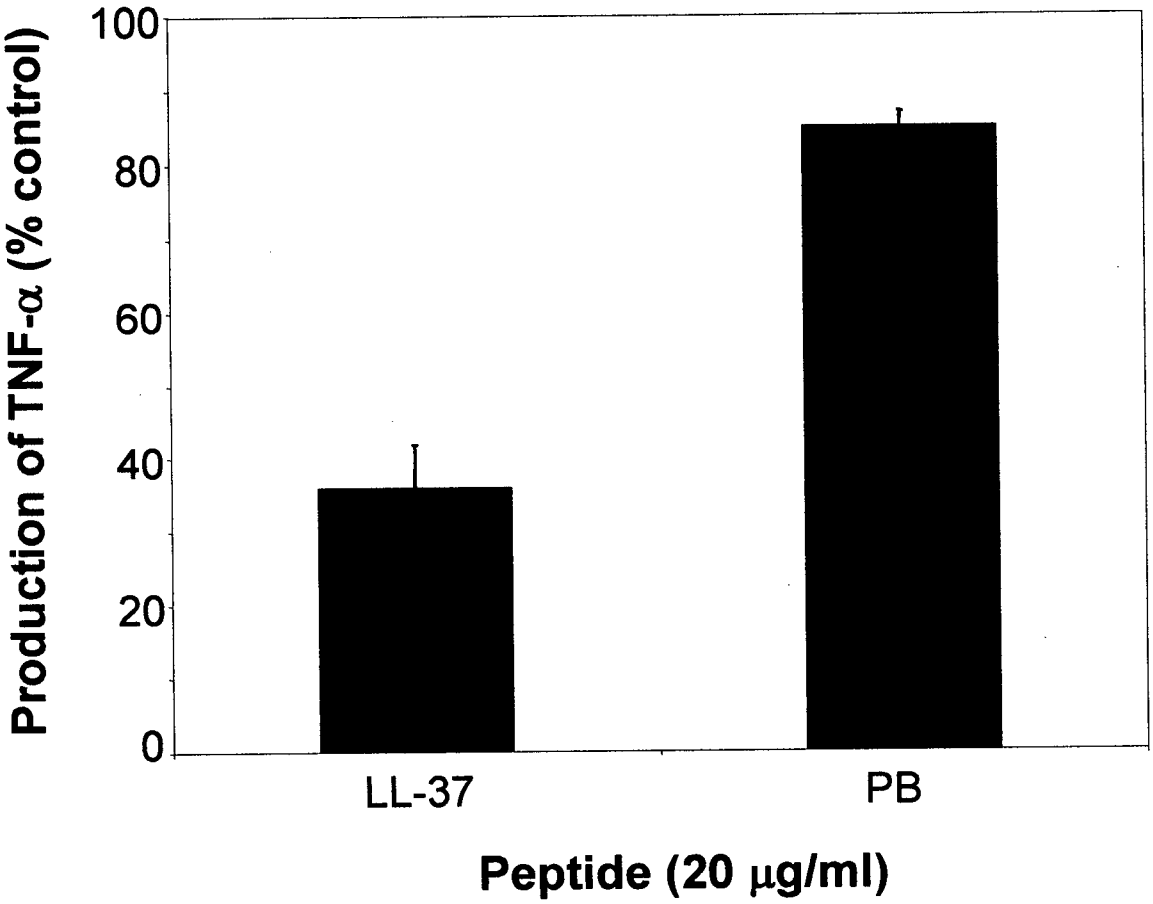


Figure 30: LL-37 reduces TNF-α production by RAW 264.7 cells stimulated with *Mycobacterium* AraLAM

RAW 264.7 cells were stimulated with 1 μg/ml AraLAM in the absence or presence of 20 μg/ml LL-37, or Polymyxin B (PB). The supernatant was collected and tested for levels of TNF-α by ELISA. 100% represents the amount of TNF-α resulting from RAW 264.7 cells incubated with AraLAM alone for 6 hours (2.4 ± 0.3 ng/ml). The data from duplicate samples is presented as the mean of three or more experiments ± standard error.

induction of TNF-α production by 1 μg/ml *S. aureus* LTA by ~75% whereas at 20 μg/ml LL-37 reduced AraLAM-induced TNF-α production by only ~60%. Polymyxin B (PB) was included as a control to show that contaminating endotoxin was not a significant factor in the inhibition by peptide of AraLAM induced TNF-α. These studies have demonstrated that both natural and synthetic cationic peptides could potentially modulate the host immune response to a variety of bacterial products released upon infection.

6.4 Summary

The role of cationic peptides in the immune system is just beginning to be understood. Since previous studies have shown that synthetic α–helical cationic peptides reduce LPS-stimulated production of cytokines by macrophages, it was hypothesized that this might be a natural function of cationic peptides in the immune system. The widely expressed human α–helical peptide, LL-37, was tested for its ability to modify macrophage responses to LPS. LL-37 reduced LPS-induced macrophage stimulation of pro-inflammatory cytokines. LL-37, when added to macrophage cell lines, primary macrophages, and whole human blood stimulated with LPS, was able to reduce the production of TNF-α. This was seen even when the peptide was added up to an hour after LPS. LL-37 was also able to reduce mortality associated with high doses of LPS in a mouse model of sepsis. LL-37 was also effective in reducing the release of TNF-α by RAW 264.7 cells stimulated with *S. aureus* LTA and *Mycobacterium* AraLAM. Thus by binding released bacterial components and limiting their ability to stimulate macrophages, both natural and synthetic peptides could potentially inhibit the development of sepsis from bacterial infections. These results thus far provide insight into the natural functions of cationic antimicrobial peptides as well as their therapeutic potential for the treatment of sepsis caused by both Gram-negative and Gram-positive bacteria.

CHAPTER 7: Both synthetic and human cationic peptides directly effect host cells

7.1 Introduction

Both synthetic and natural cationic peptides have potent anti-endotoxin activities. It was shown thus far that cationic peptides can not only bind to bacterial products and reduce their ability to induce TNF-α, but can also selectively modify LPS-induced macrophage gene transcription. The possibility that peptides act directly on macrophages, as opposed to merely neutralizing LPS, prompted the study of whether treating RAW 264.7 cells with cationic peptides alone caused any changes in gene expression. The ability of CEMA and LL-37 to directly alter gene transcription in RAW 264.7 cells was studied. It was found that CEMA and LL-37 induced the expression of overlapping and distinct sets of genes. Several genes up-regulated by the peptides that are involved in chemotaxis were further studied. It was discovered that cationic peptides can up-regulate chemokine and chemokine receptor gene expression, increase secretion of chemokines and the surface expression of chemokine receptors, and increase chemokine release *in vivo* in the mouse lung. This suggests that synthetic as well as natural cationic peptides produced in response to bacterial infections may directly regulate macrophage function and promote the recruitment of other immune cells to combat bacterial infection.

7.2 CEMA directly modifies macrophage transcriptional responses

To gain a more complete understanding of cationic peptides activities, gene array technology was used to profile gene expression patterns in RAW 264.7 macrophages treated with CEMA or medium alone. RAW 264.7 cells were treated with 50 μg/ml of CEMA for 4 h, the RNA isolated, and ^{32}P-radiolabeled cDNA probes were prepared and hybridized to mouse gene arrays (Figure 31). Table 9 shows that CEMA treatment of RAW 264.7 cells up-regulated the

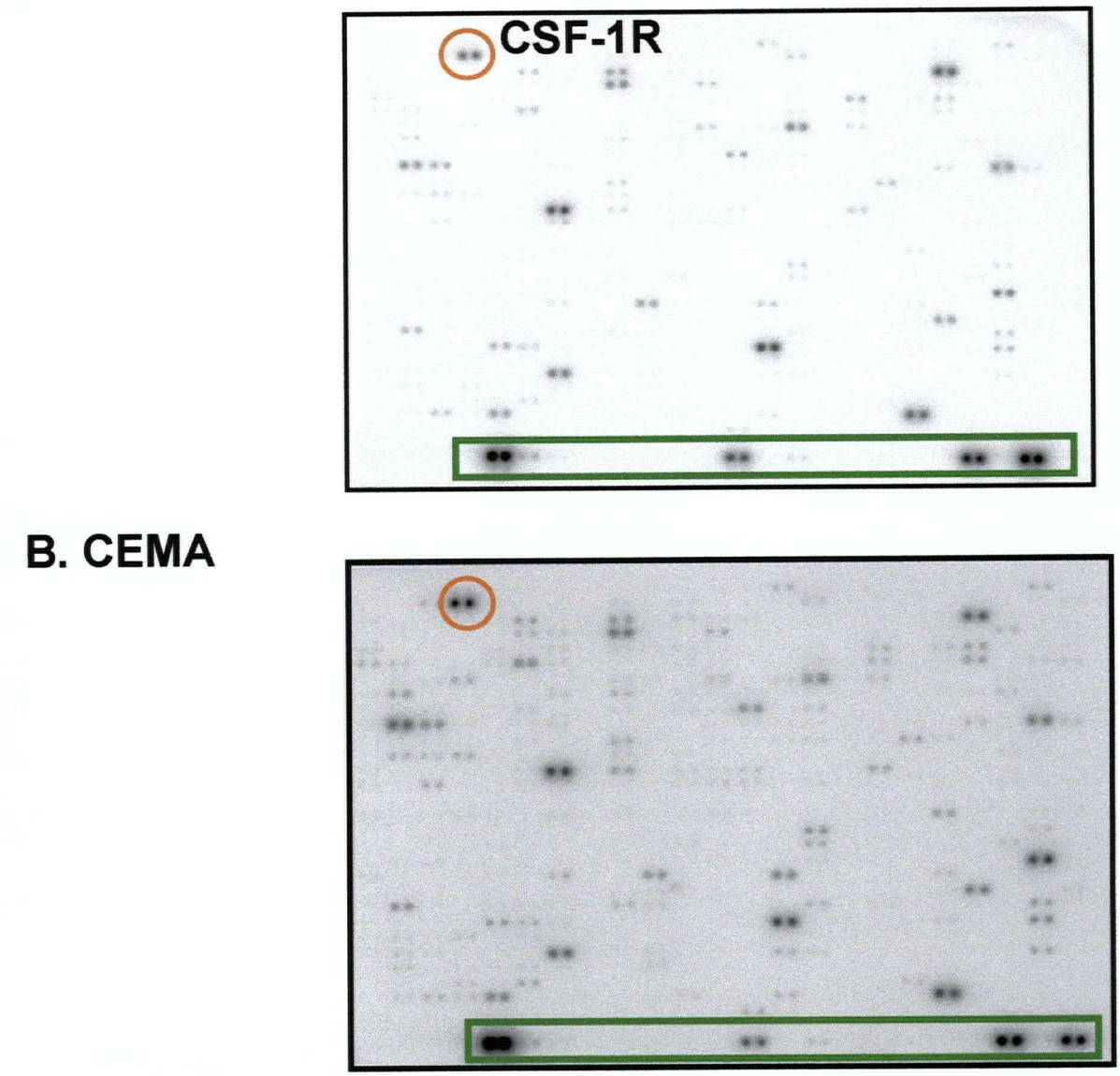


Figure 31: CEMA alters gene expression in RAW 264.7 cells.

RAW 264.7 cells were stimulated with (A) media alone for 4 hours, or (B) 50 µg/ml CEMA. The RNA was isolated from the cells and used to make ^{32}P-labeled cDNA probes, which were hybridized to the Clontech Atlas arrays. After a 3-day exposure, they were analyzed using a phosphoimager and Clontech Atlas software. These data are representative of 2 to 3 experiments. The cDNA spots in the green box identify the genes used for data normalization. An example of a gene up-regulated by CEMA is shown in the orange circle.

Table 9: Genes up-regulated by CEMA treatment of RAW 264.7 cells

Gene/protein	Unstimulated intensity	Ratio CEMA: unstimulated	Accession number	Gene
unconventional myosin VI	**20**	**35.4**	**U49739**	**F6e**
p21/Cip1/Waf1; cdk-inhibitor protein 1	20	27.9	U09507	A7e
Rab-3b ras-related protein	20	24.7	Y14019	F6c
p27kip1; G1 cyclin-Cdk inhibitor	20	23.9	U10440	A7f
Jun-D transcription factor	20	22.7	J05205	A3g
Egr-1 transcription factor	20	18.3	M20157	D2l
glucocorticoid receptor form A	20	18.3	X13358	E3m
NFAT 1 transcription factor	20	16.7	U02079	D7a
H-ras proto-oncogene	20	16.5	Z50013	A5c
TGF-β receptor type 1	20	16.2	D25540	E2k
α_6 integrin	20	15.8	X69902	E7d
p19ink4; cdk4 and cdk6 inhibitor	161	4.0	U19597	A7d
Shc transforming adaptor protein	176	3.2	U15784	A5f
MDR1; multidrug resistance protein	138	3.0	M14757	B1g
Ikaros transcription factor	156	2.9	L03547	D4l
Growth factor receptor	157	2.8	M98547	E2f
Elk-1 ets-related proto-oncogene	123	2.8	X87257	A3a
PD-1 possible cell death inducer	582	2.7	X67914	C4f
SnoN; ski-related oncogene	148	2.6	U36203	E2j
Insulin-like growth factor-IA	572	2.5	X04480	F3a
c-ErbA oncogene	147	2.5	X51983	A2g
Kruppel-like factor LKLF	207	2.4	U25096	D4m
Lymphotoxin receptor	194	2.4	U29173	E2g
c-myb proto-oncogene protein	181	2.4	M16449	A2k
ERCC5 excision repair protein	148	2.4	D16306	C6f
Mad related protein 2	193	2.3	U60530	F3h
Vimentin	2702	2.2	X51438	F6d
Csk; c-Src-kinase	199	2.2	U05247	B4n
UPAR	194	2.1	X02389	F7f
Cyclin B2	511	2.0	X66032	A6d
MAC-1 alpha subunit	494	2.0	X07640	E6j
c-Jun proto-oncogene	426	2.0	J04115	A2l
Cyclin B1	704	1.9	X64713	A6c

Table 9, continued

Gene/protein	Unstimulated intensity	Ratio CEMA: unstimulated	Accession number	Gene
CD44	1345	1.8	M27129	E6e
CSF-1 receptor	4455	1.7	X68932	A4b
Cyclin D1	**1489**	**1.7**	**S78355**	**A6f**

Total RNA was isolated from unstimulated RAW 264.7 cells and RAW 264.7 cells treated for 4 hr with 50 μg/ml CEMA. After reverse transcription, ^{32}P-labelled cDNA was used to probe Clontech Atlas gene array filters. Hybridization was analyzed using Atlas Image (Clontech) software. The array experiments were repeated 2-3 times with different RNA preparations and yielded very similar results; the average fold changes are shown above.

expression of 35 different genes. The genes most strongly affected by CEMA (by 2 to 35-fold) included cyclin-dependent kinase (cdk) inhibitors, the anti-inflammatory cytokine transforming growth factor-β type I subunit (TGF-β1) receptor, NF-AT1 transcription factor, Jun-D and Egr-1, which controls monocyte development and also appears necessary for maintenance of macrophage differentiation (Table 9). CEMA also up-regulated the expression of the urokinase plasminogen activator receptor (uPAR) which is widely expressed on different cell types including hematopoietic cells and has been shown to be involved in cell adhesion, chemotaxis, receptor clustering, and changes in cell shape (Blasi 1997). CEMA also up-regulated a number of genes involved in cell adhesion including α_6 integrin and MAC-1 and to a lesser extent α_4 integrin, α_5 integrin, and CD44 (Table 9). There were a number of genes in the RAW 264.7 cells involved in apoptosis that were affected by treatment with CEMA. For example, CEMA up-regulated PD-1 (possible cell death inducer) (Table 9) and CEMA down-regulated the expression of a number of other apoptosis-related genes (Table 10) including those encoding the apoptosis inhibitors BAG-1, Bcl-2 (both suppressed 2.5 fold), A20 zinc finger protein (suppressed 10 fold). This data might help explain the results of a previous study, which found a cecropin-melittin hybrid peptide to have an apoptotic effect on a murine macrophage cell line (Velasco et al. 1997). From the genes affected by treatment with CEMA, it appears that the peptide most notably affected the expression of genes from three families with functions in cell proliferation, apoptosis and cell adhesion.

7.3 LL-37 directly modifies macrophage transcriptional responses

I hypothesized that the natural human peptide, LL-37, could also directly affect macrophage gene expression as was observed with CEMA. Therefore gene array studies were performed to determine the transcriptional responses of macrophages to LL-37. RNA was extracted from RAW

Table 10: Genes down-regulated by CEMA treatment of RAW 264.7 cells

Gene/Protein	Unstimulated intensity	Ratio CEMA: unstimulated[1]	Accession number	Gene
A20 zinc finger protein	**242**	**0.08**	**U19463**	**C2e**
clusterin	133	0.15	L08235	C3b
MSH2 DNA repair protein	128	0.16	U21011	C7a
NADPH-cytochrome P450 reductase	183	0.27	D17571	C4a
inhibin beta A subunit	240	0.32	X69619	F2h
Bcl-2	174	0.38	M16506	C1h
thrombopoietin	393	0.43	L34169	F4e
BAG-1	1339	0.44	U17162	C1e
neuronal death protein	651	0.46	D83698	C4b
MmLim15	263	0.49	D64107	C6l
PA6 stromal protein	885	0.51	X96618	C6a
TNF 55	121	0.51	X57796	C5b
PCNA	343	0.52	X53068	C7b
IL-2 receptor gamma chain	499	0.55	L20048	E3c
DNA-polymerase delta subunit	352	0.56	Z21848	C6b
YY1 TF	509	0.57	L13968	D7k
T-lymphocyte activated protein	1413	0.60	M31042	D6h
c-Akt	1486	0.65	M94335	C2k
I-κB alpha chain	402	0.65	U36277	B3m
Bax	814	0.67	L22472	C1g
MHR23A	574	0.69	X92410	C6i

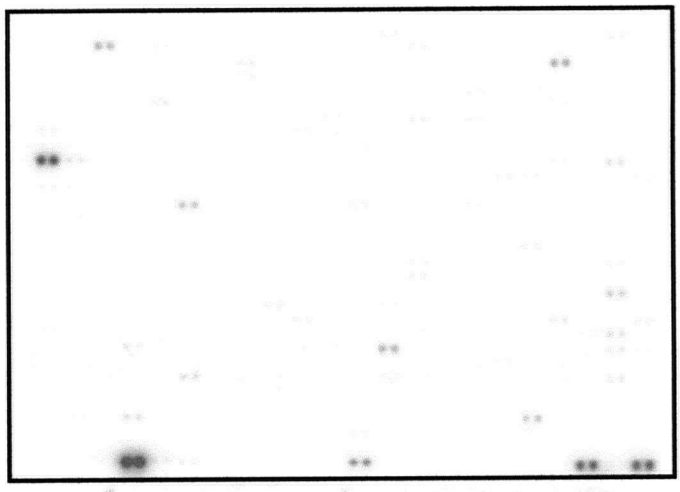
Total RNA was isolated from unstimulated RAW 264.7 cells and cells treated for 4 h with medium alone or 50 μg/ml CEMA. After reverse transcription, ^{32}P-labelled cDNA was used to probe Clontech Atlas gene array filters.

[1] The ratio was calculated by dividing the intensities of hybridization spots for cells treated with 50 μg/ml CEMA by the intensities for unstimulated cells.

264.7 cells that were cultured for 4 h with medium alone, or 50 μg/ml LL-37 alone and converted into P^{32}-labelled cDNA. The hybridization of the cDNA probes to each immobilized DNA was visualized by autoradiography and quantified using a phosphoimager. Representative autoradiographic images of the gene arrays are shown in Figure 32. Table 11 shows that LL-37 treatment of RAW 264.7 cells up-regulated the expression of at least 30 different genes. The genes up-regulated by LL-37 were mainly from two categories: one that encodes receptors (growth, chemokine, interleukin, interferon, hormone, neurotransmitter), cell surface antigens and cell adhesion and another one that includes genes for cell-cell communication (growth factors, cytokines, chemokines, interleukin, interferons, hormones), cytoskeleton, motility, and protein turnover factors. The specific genes up-regulated included those encoding chemokine MCP-3, the anti-inflammatory cytokine IL-10, macrophage colony stimulating factor, and receptors such as IL-1R-2 (a putative antagonist of productive IL1 binding to ILR1), PDGF receptor B, NOTCH4, LIF receptor, LFA-1, TGFβ receptor 1, G-CSF receptor, and IFNγ receptor. The gene array data suggested that LL-37 up-regulates the expression of the chemokine receptors IL-8RB, CXCR-4 and CCR2 by 10, 4 and 1.4 fold above unstimulated cells respectively.

LL-37 also up-regulated the expression of genes encoding several metalloproteinases, and inhibitors thereof, including the bone morphogenetic proteins (BMPs) BMP-1, BMP-2 and BMP-8a, tissue inhibitor of metalloproteinases (TIMPs) TIMP2 and TIMP3. As well, LL-37 up-regulated specific transcription factors, including JunD, and the YY and LIM-1 transcription factors, and kinases such as Etk1 and Csk demonstrating its widespread effects. It was also discovered from these gene array studies that LL-37 down-regulated at least 20 genes in RAW 264.7 cells (Table 12). The genes down-regulated by LL-37 included DNA repair proteins and several inflammatory mediators such as MIP-1β, oncostatin M and IL-12. There were only a few

A. Unstimulated



B. LL-37

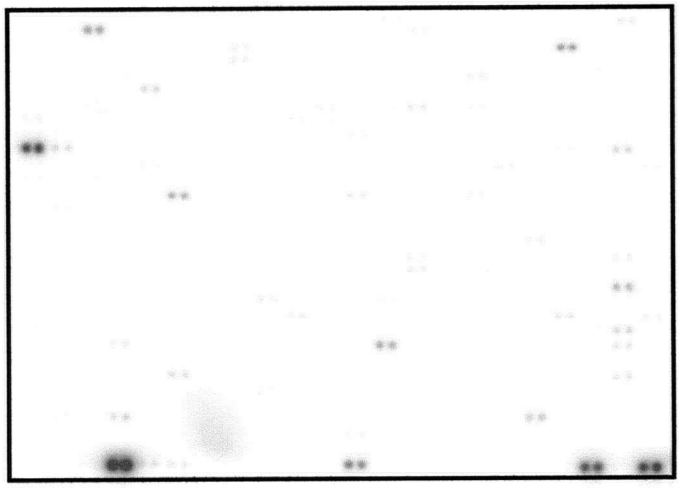


Figure 32: LL-37 modifies gene expression in RAW 264.7 macrophage cells.

RAW 264.7 cells were stimulated with (A) media alone for 4 hours, or (B) 50 μg/ml LL-37. The RNA was isolated from the cells with Trizol, DNase treated and used to make ^{32}P-labeled cDNA probes, which were hybridized to the Clontech Atlas arrays. After a 3-day exposure, they were analyzed using a phosphoimager and Clontech Atlas software. These data are representative of 3 experiments.

Table 11: Genes up-regulated by LL-37 treatment of RAW 264.7 cells

Gene/protein	Gene function	Unstimulated intensity	Ratio LL-37: unstimulated[1]	Accession number	Gene
Etk1	Tyrosine-protein kinase receptor	20	43.3	M68513	B2l
PDGFRB	Growth factor receptor	24	25.4	X04367	E2i
NOTCH4	proto-oncogene	48	17.8	M80456	A5h
IL-1R2	Interleukin receptor	20	15.8	X59769	E2n
MCSF	Macrophage colony stimulating factor 1	85	13.7	X05010	A5g
BMP-1	Bone morphogenetic protein (growth factor)	20	13.6	L24755	F1b
MCP-3	Chemokine	56	13.5	S71251	E1k
LIFR	Leukemia inhibitory factor (cytokine) receptor	20	12.2	D26177	E1l
BMP-8a	Bone morphogenetic protein (growth factor)	20	11.8	M97017	F1f
GCSFR	Granulocyte colony-stimulating factor receptor	20	11.0	M58288	E1j
IL-8RB	Chemokine receptor	112	9.5	D17630	E3h
IL-10	Interleukin	168	4.2	M37897	F4l
C5aR	Chemokine receptor	300	4.0	S46665	E1g
CXCR-4	Chemokine receptor	36	4.0	D87747	B3d
L-myc	Oncogene	208	3.5	X13945	A3h
Cyclin D3	Cyclin	327	3.0	U43844	A6h
YY (NF-E1)	Transcription factor	593	2.4	L13968	D7k
TIMP3	Protease inhibitor	259	2.3	L19622	F7n
PAI2	Protease inhibitor	154	2.2	X19622	F7i
JunD	Transcription factor	534	2.1	J050205	A3g
LIM-1	Transcription factor	295	2.1	Z27410	D6m
SLAP	Src-like adaptor protein	315	2.0	U29056	B5c
IFNGR	Interferon gamma receptor	308	2.0	M28233	E2m
BMP-2	Bone morphogenetic protein (growth factor)	186	1.9	L25602	F1c
LFA-1	Cell adhesion receptor	340	1.7	X14951	E5n
TIMP2	Protease inhibitor	136	1.7	X62622	F7m

Table 11, continued

Gene/protein	Gene function	Unstimulated intensity	Ratio LL-37: unstimulated[1]	Accession number	Gene
Mas	Proto-oncogene	131	1.7	X67735	A5l
TGFβR1	Growth factor receptor	1038	1.6	D25540	E2k
Csk	c-src kinase	489	1.6	U05247	B4n

Total RNA was isolated from unstimulated RAW 264.7 cells and cells treated for 4 hr with 50 μg/ml LL-37. After reverse transcription, ^{32}P-labelled cDNA was used to probe Clontech Atlas gene array filters. The array experiments were repeated 3 times with different RNA preparations and the average fold change is shown above.

[1] The ratio was calculated by dividing the intensities for cells treated with 50 μg/ml LL-37 by the intensities for unstimulated cells.

Table 12: Genes down-regulated by LL-37 treatment of RAW 264.7 cells

Gene/protein	Gene function	Unstimulated intensity	Ratio LL-37: unstimulated	Accession number	Gene
XRCC1	DNA repair protein	227	0.12	U02887	C7n
XPAC	DNA repair protein	485	0.17	X74351	C7m
voltage-gated sodium channel	Voltage-gated ion channel	257	0.24	L36179	B2f
PMS2 DNA	DNA repair protein	200	0.30	U28724	C7d
IGFBP6	Growth factor	1291	0.39	X81584	F2i
THAM	Enzyme involved in protein turnover	342	0.39	X58384	E7f
Ung1	DNA repair protein	535	0.40	X99018	C7l
MIP-1β	Cytokine	327	0.42	M23503	F3f
oncostatin M	Cytokine	1127	0.44	D31942	F3n
bcl-x	Apoptosis protein	142	0.44	L35049	C1j
MmRad52	DNA repair protein	371	0.44	Z32767	C6n
orphan receptor	Nuclear receptor	224	0.47	U11688	E1b
uromodulin	glycoprotein	363	0.47	L33406	F4i
IL-12 p40	Interleukin	601	0.48	M86671	F4n
nucleobindin	Golgi resident protein	367	0.48	M96823	D5j
Tob1	Antiproliferative factor	956	0.50	D78382	A7n
NF-A3	Transcription factor	583	0.50	M34381	D5k
N-cadherin	Cell adhesion receptor	238	0.50	M31131	E7k
KRT18	Intermediate filament proteins	318	0.52	M11686	F5i
Integrin α_6	Cell adhesion receptor	287	0.52	X69902	E7d
GLUT1	Glucose transporter 1 (facilitated diffusion proteins)	524	0.53	M23384	B2e

RNA was isolated from unstimulated RAW 264.7 cells and RAW 264.7 cells treated for 4 hr with 50 μg/ml LL-37. After reverse transcription, ^{32}P-labelled cDNA was used to probe Clontech Atlas gene array filters. The array experiments were repeated 3 times with different RNA preparations and the average fold change is shown above.

genes commonly up- or down-regulated by both CEMA and LL-37 (see Section 8.1.4).

7.4 LL-37 induces chemokine expression

The gene array studies indicated that LL-37 increased the expression of chemokine genes in RAW 264.7 cells. This suggests that LL-37 may induce macrophages to produce chemokines, which could in turn recruit additional immune cells to the sites of infection. Thus, I attempted to confirm the up-regulation of chemokines in several different systems. First the effect of LL-37 on RAW 264.7 cell production of MCP-1 was examined (MCP-1 was not represented on our arrays but has similar activity to MCP-3 for which no ELISA is available). Both MCP-1 and MCP-3 belong to the β(C-C) chemokine family and have chemotactic activity for monocytes, eosinophils, NK cells, basophils, T lymphocytes and even neutrophils and both bind CCR2 (reviewed in (Van Coillie et al. 1999)). The small differences between the 2 chemokines tend to lie in the minimal effective concentrations for chemotaxis and the lesser-studied non-chemotactic functions (Van Coillie et al. 1999). For example, MCP-1 has a protective effect in a murine model of peritoneal sepsis. (Matsukawa et al. 1999).

When RAW 264.7 cells were stimulated with increasing concentrations of LL-37, they produced significant levels of MCP-1 in their supernatants, as determined by ELISA (Figure 33). RAW 264.7 cells stimulated with peptide concentrations ranging from 20-50 μg/ml for 24 hr produced substantial levels of MCP-1 (200-400 pg/ml above background). When the cells were stimulated with 100 μg/ml of LL-37, high levels of MCP-1 (>1000 pg/ml above background) were produced. The ability of LL-37 to induce human MCP-1 in whole blood was also tested. Whole human blood from three separate donors was incubated with LL-37 for 4 hours, the samples were centrifuged, and the serum was removed and tested for hMCP-1 by ELISA. Although there was substantial production of human MCP-1 in response to LL-37 by all three

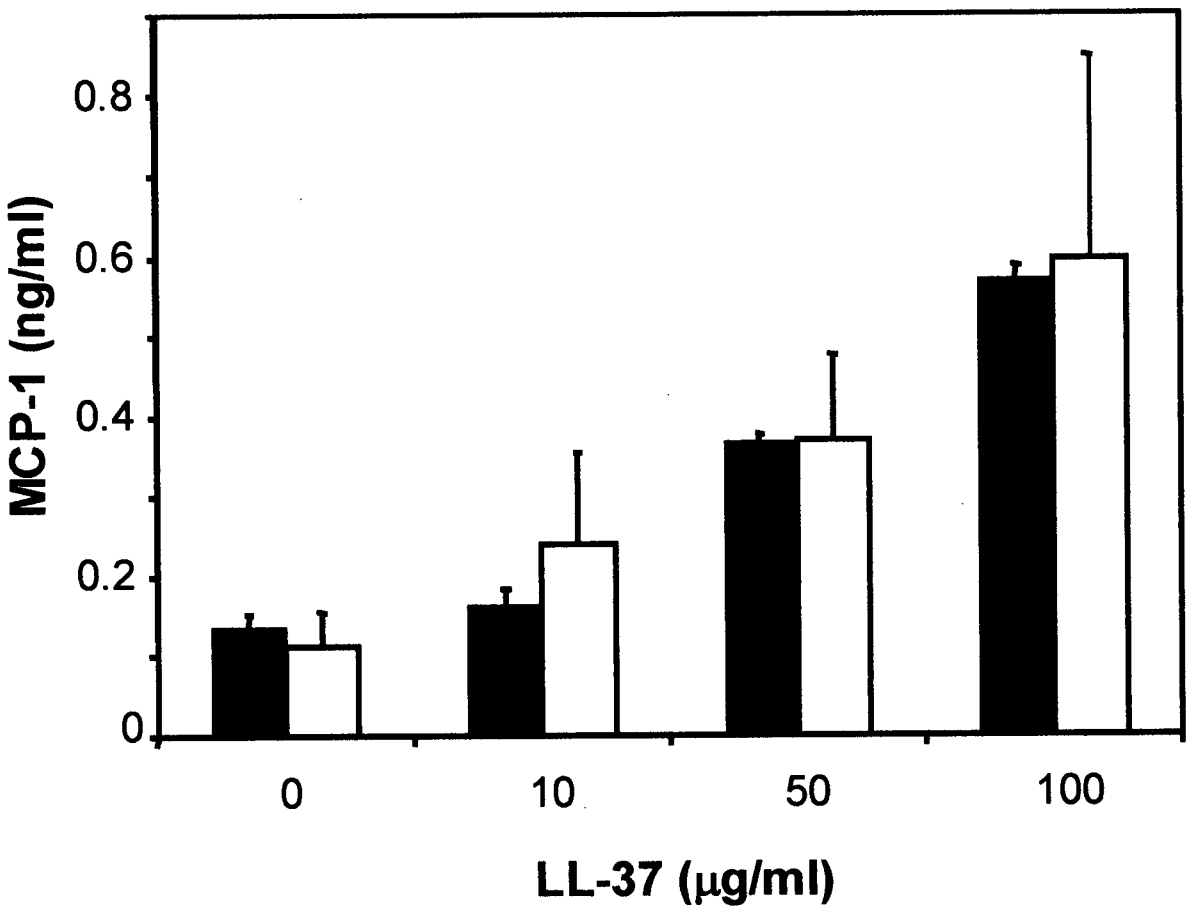


Figure 33: LL-37 induces MCP-1 production in RAW 264.7 cells and whole human blood.

RAW 264.7 cells (solid bars) or whole human blood (open bars) were stimulated with increasing concentrations of LL-37 for 4 hr. The human blood samples were centrifuged and the plasma was removed and tested for MCP-1 by ELISA along with the supernatants from the RAW cells. The RAW cell data presented is the mean of three or more experiments ± standard error and the human blood data represents the mean ± standard error from three separate donors.

donors, there was substantial variation in donor response to the peptide, as indicated by the large standard errors in Figure 33.

Since LL-37 is expressed in the human airway and the release of LL-37 is up-regulated in epithelial cells in response to bacterial products (Bals et al. 1996), the effect of LL-37 on chemokine induction was also examined in A549 human epithelial cells. This pulmonary type II epithelial cell line was derived from an individual with alveolar cell carcinoma (Leiber et al. 1976). The A549 cells were stimulated with various concentrations of cationic peptide and the levels of IL-8 and MCP-1 were measured by ELISA. IL-8, an important chemokine secreted by epithelial cells (and others), is a member of the α (CXC) chemokine family and is a potent activator and chemoattractant of neutrophils, basophils and T cells (Leonard and Yoshimura 1990). Substantial but low levels of IL-8 were secreted by A549 cells in response to 20 μg/ml LL-37 (Figure 34). In response to 100 μg/ml LL-37, the A549 cells secreted a high level of IL-8 (>1 ng/ml). LL-37 also induced substantial levels of IL-8 in whole human blood (Figure 34) although the background levels observed were quite high. CEMA also induced high levels of IL-8 in A549 cells (100 μg/ml resulted in 0.97 ± 0.05 ng/ml IL-8) but not a variant of CEMA, CPα2. CPα2 did not to cause induction of IL-8 (average levels of IL-8 [0.08 ± 0.004 ng/ml)] and in fact A549 cells incubated with CPα2 had lower than background levels [0.172 ± 0.03 ng/ml]) of IL-8. This suggests that the induction of chemokines is specific to select cationic peptides.

Since LPS is a known stimulus of IL-8 production and LL-37 neutralized the responses of LPS, I studied the effect of LL-37 on LPS-induced IL-8 production in A549 cells (Figure 35). This cell line does not produce TNF-α so the effects of cationic peptides on LPS-induced TNF-α production in epithelial cells was not examined. It was found, that at low concentrations of LL-37 (1-20 μg/ml), that the peptide reduced LPS-induced IL-8 production, but at high concentrations of

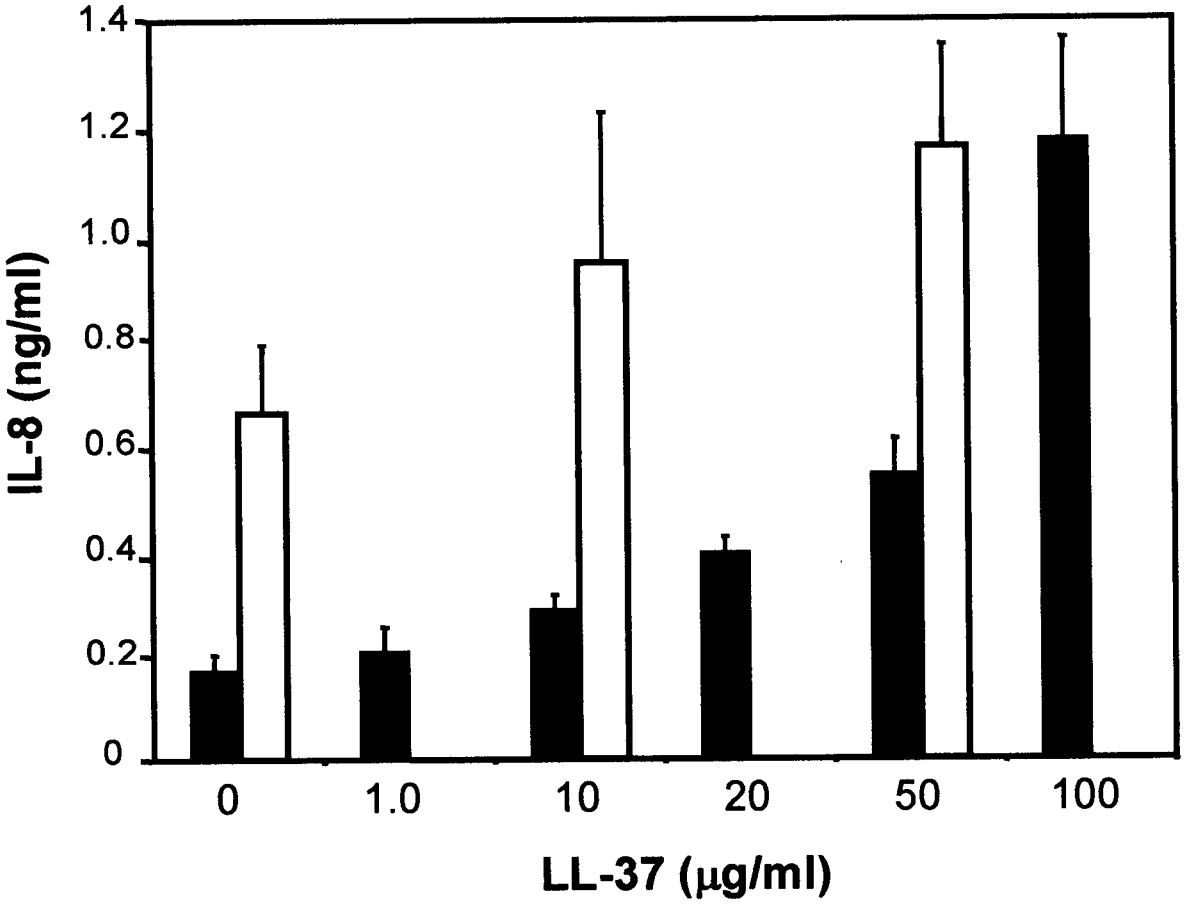


Figure 34: LL-37 induces IL-8 production by A549 epithelial cells and whole human blood.

A549 cells (solid bars) or whole human blood (open bars) was stimulated with increasing concentrations of LL-37 for 24 and 4 hr respectively. The human blood samples were centrifuged and the serum was removed and tested for IL-8 by ELISA along with the supernatants from the A549 cells. The A549 cell data presented is the mean of three or more experiments ± standard error and the human blood data represents the mean ± standard error from three separate donors.

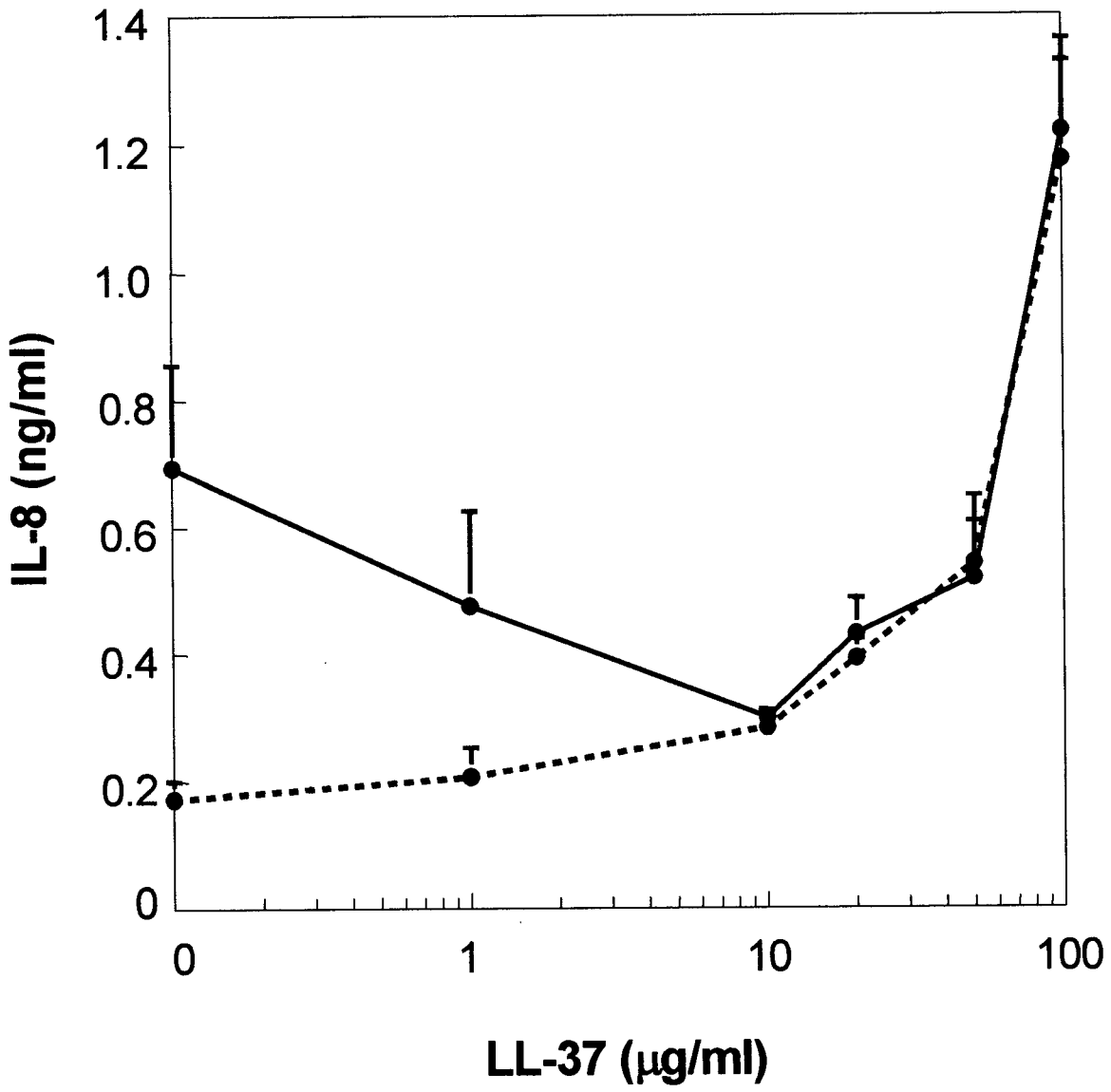


Figure 35: Low concentrations of LL-37 inhibit LPS-induced production of IL-8 by A549 cells and induce IL-8 at high concentrations.

A549 cells were stimulated with 100 ng/ml *E. coli* O111:B4 LPS and LL-37 (solid line) or LL-37 alone (broken line) for 24 hours. The supernatant was removed and tested for IL-8 by ELISA. The data is from duplicate samples and presented as the mean of three experiments ± standard error.

LL-37 (50-100 μg/ml), there was an increase in IL-8 production independent of LPS. These results indicate that LL-37 may have differential roles in the immune system depending on the concentration found at the site of infection. The A549 cell line also produces human MCP-1, however, the basal levels are very high (1.08 ± 0.12 ng/ml). The effect of peptide alone and LPS + peptide on A549 cell production of MCP-1 was tested. LL-37 inhibition of LPS-induced MCP-1 in the A549 cells was highest at 10 μg/ml LL-37, which resulted in an average inhibition of 52 ± 6%. There were only minor increases in MCP-1 production (from the high background levels), by LL-37 or CEMA (Figure 36 and data not shown). Interestingly, CPα2 did not induce MCP-1, but it did substantially reduce the levels of MCP-1 from background levels (from 1.08 ± 0.12 to 0.032 ± 0.02 ng/ml), analogous to the results seen with IL-8.

In other experiments LL-37 and CEMA were tested for their ability to directly cause the migration of monocytes, and T cells using a transwell migration assay. At concentrations of 100 ng/ml – 300 μg/ml, neither of these peptides caused significant migration of the cell lines tested. For example, migration experiments with the human T lymphocyte cell line, Jurkat, the positive control [100 ng/ml of SDF-1 (stromal cell-derived factor)] resulted in 36 ± 4% migration while up to 300 μg/ml of LL-37 or CEMA did not cause migration of the Jurkat cells. Similar migration experiments were performed with THP-1 monocytes and again no migration (<1%) was observed in response to 300 μg/ml LL-37, CEMA or indolicidin (except Bac 2A which resulted in 100% migration). However, by using human peripheral neutrophils, monocytes, and T cells, LL-37 was chemotactic (Yang et al. 2000). Recently LL-37 was also found to induce mast cell chemotaxis (Niyonsaba et al. 2002). Thus LL-37 not only induces the release of chemokines but also is chemotactic for mast cells and peripheral neutrophils, monocytes, and T cells.

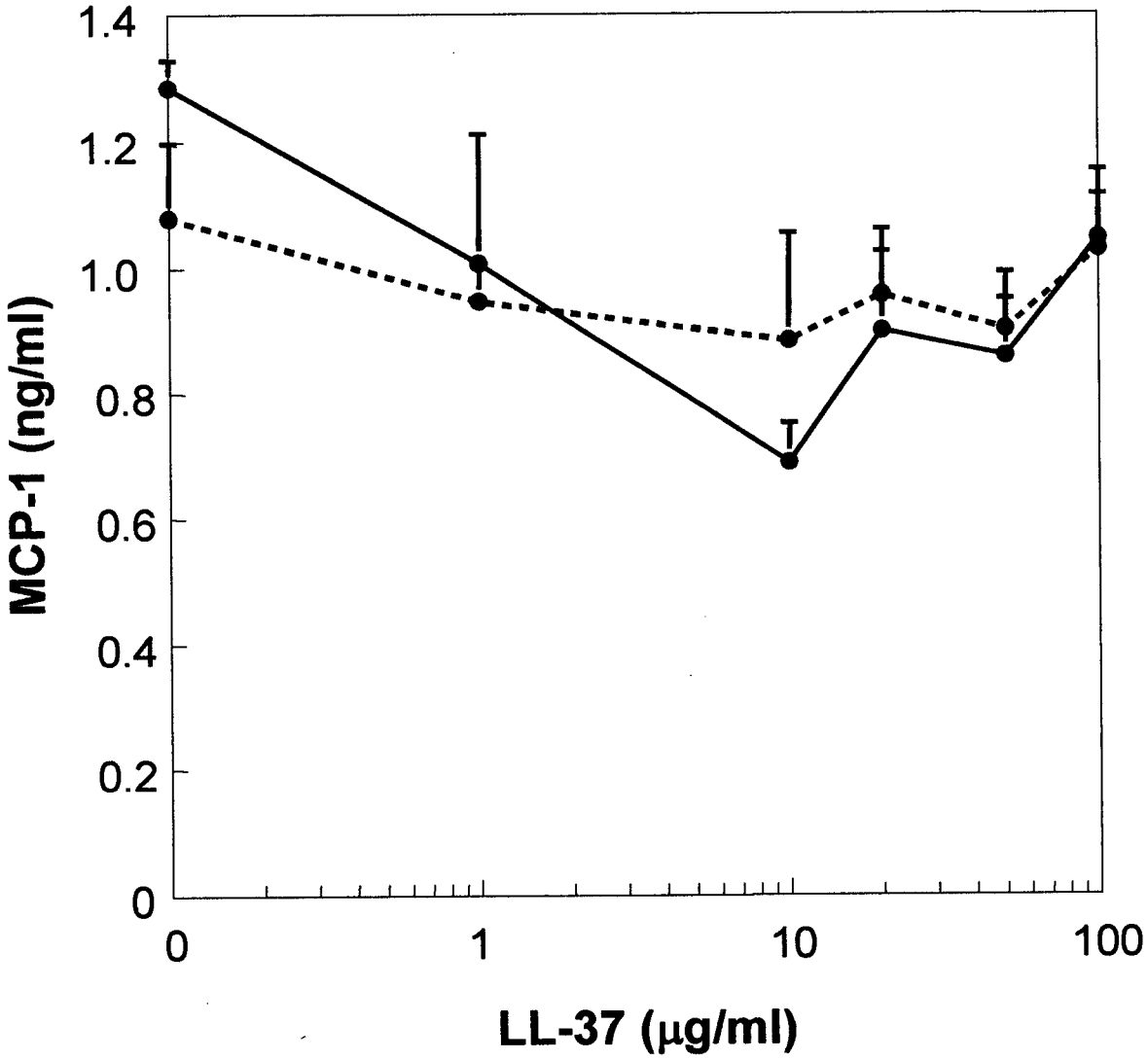


Figure 36: LL-37 does not substantially impact on MCP-1 production by A549 cells.

A549 cells were stimulated with 100 ng/ml *E. coli* O111:B4 LPS and LL-37 (solid line) or LL-37 alone (broken line) for 24 hours. The supernatant was removed and tested for MCP-1 by ELISA. The data is from duplicate samples and presented as the mean of three experiments ± standard error.

7.5 Cationic peptides increase surface expression of chemokine receptors

The gene array data suggested that LL-37 up-regulates the expression of the chemokine receptors IL-8RB, CXCR-4 and CCR2 by 10, 4 and 1.4 fold above unstimulated cells respectively. To confirm the gene array data, the surface expression of these receptors on RAW 264.7 cells cultured with or without peptide for 4 hr was examined by flow cytometry. To analyze cell surface expression of IL-8RB, CXCR-4, and CCR2 RAW 264.7 cells were stained with the appropriate primary antibody followed by FITC-conjugated secondary antibody. The cells were analyzed using a FACscan, counting 10,000 events and gating on forward and side scatter to exclude cell debris. A representative FACS plot is shown in Figure 37. When 100 μg/ml of LL-37 was incubated with RAW 264.7 cells for 4 hr, IL-8RB was up-regulated an average of 2.4-fold above unstimulated cells, CCR2 was up-regulated 1.8-fold above unstimulated cells and CXCR-4 was slightly up-regulated (1.6-fold above unstimulated cells) (Table 13). Thus these results showed a similar trend but different absolute values of the array data. It was also found that the synthetic peptides, CEMA and Bac 2A increased the surface expression of IL-8RB, CXCR-4, and CCR2.

Since LL-37 was also found to increase expression of LFA-1 on the gene arrays, the effect of cationic peptides (LL-37, CEMA, and Bac 2A) on surface expression of this molecule was also tested by FACs analysis. Of the peptides tested, only Bac 2A significantly increased the surface expression of LFA-1.

7.6 LL-37 up-regulates MCP-1 but not TNF-α levels in BAL fluid of peptide treated mice

BALB/c mice were given LL-37 or endotoxin-free water by intratracheal instillation and the levels of MCP-1 and TNF-α were examined in the bronchoalveolar lavage (BAL) fluid after

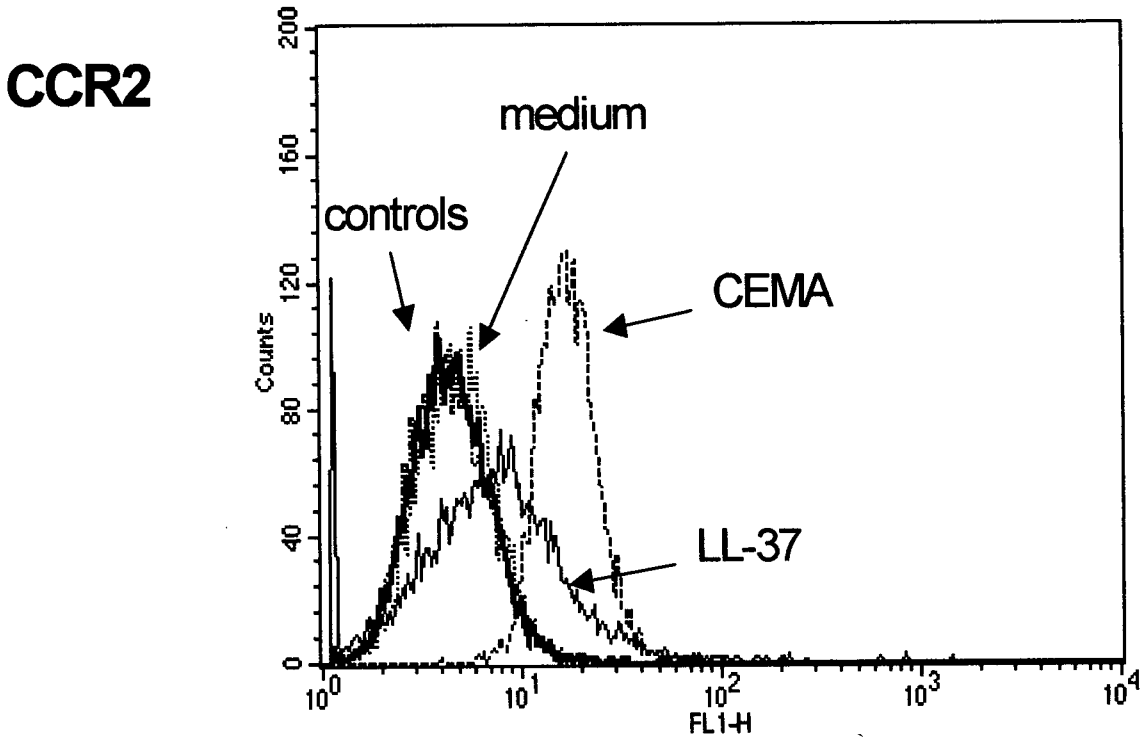


Figure 37: Representative FACs plot of RAW 264.7 cells treated with LL-37 and CEMA.

RAW 264.7 cells were incubated with or without 100 μg/ml LL-37 or CEMA for 4 hr. The cells were washed and stained with CCR2 primary antibody and then FITC-labeled secondary antibody. The cells were analyzed using a FACscan and forward and side scatter were used to gate on live cells. The figure above is a representative FACS plot. The controls represent cells that were not stained and cells stained with secondary antibody only.

Table 13: Increased surface expression of CXCR-4, IL-8RB and CCR2 in response to cationic peptides

		Relative Change in Chemokine Receptor Expression		
Peptide	**Concentration (μg/ml)**	**IL-8RB**	**CXCR-4**	**CCR2**
LL-37	10	1.0	1.0	1.0
	50	1.3 ± 0.05	1.3 ± 0.03	1.3 ± 0.03
	100	2.4 ± 0.6	1.6 ± 0.23	1.8 ± 0.15
CEMA	50	1.6 ± 0.1	1.5 ± 0.2	1.5 ± 0.15
	100	3.6 ± 0.8		4.7 ± 1.1

RAW 264.7 cells were stimulated with LL-37 or CEMA for 4 hr. The cells were washed and stained with the appropriate primary and FITC-labeled secondary antibodies. The data shown represents the average (fold change of RAW 264.7 cells stimulated with peptide from media) ± standard error.

3-4 hr. It was found that the mice treated with 50 μg/ml LL-37 secreted increased levels of MCP-1 over mice given water or anesthetic alone (Figure 38). This was not a general pro-inflammatory response to LL-37 since peptide did not significantly induce more TNF-α than mice given water or anesthetic alone. This is analogous to the findings in other model systems such that LL-37 did not induce TNF-α production by RAW 264.7 cells and bone marrow-derived macrophages treated with LL-37 (up to 100 μg/ml). Differential cell counts of the BAL fluid were performed by Dr. Donald Davidson. There was not a significant difference in inflammatory cell (PMN) infiltration in the LL-37 treated mice from the untreated mice. This may be due to the time period tested and a longer time interval would possibly yield more significant results (Doherty et al. 1999). Nevertheless, the up-regulation of chemokine by LL-37 was confirmed *in vivo* in a mouse model. LL-37 selectively induced the production of the chemokine, MCP-1, without inducing the production of the inflammatory mediator TNF-α. Since LL-37 can also protect against lethal endotoxaemia in a mouse model, there is an emerging picture of LL-37 as a factor that can selectively limit bacterial-induced damage and also help mount an immune reponse by recruiting leukocytes to fight bacterial infections.

7.7 Summary

The results presented here along with a separate published study (Yang et al. 2000) indicate that LL-37 may have a role in the host immune response by enhancing the recruitment of leukocytes to help clear bacterial infections and prevent the onset of sepsis. This could be done in part by up-regulating the chemokines, IL-8 and MCP-1 and also by up-regulating the surface expression of chemokine receptors such as IL-8RB, CXCR-4 and CCR2. The function of LL-37 could depend on the concentration of the peptide. For instance, at low peptide concentrations [ie. 1.2 μg/ml found in plasma, (Sorenson et al. 1997)], LL-37 could synergize with other

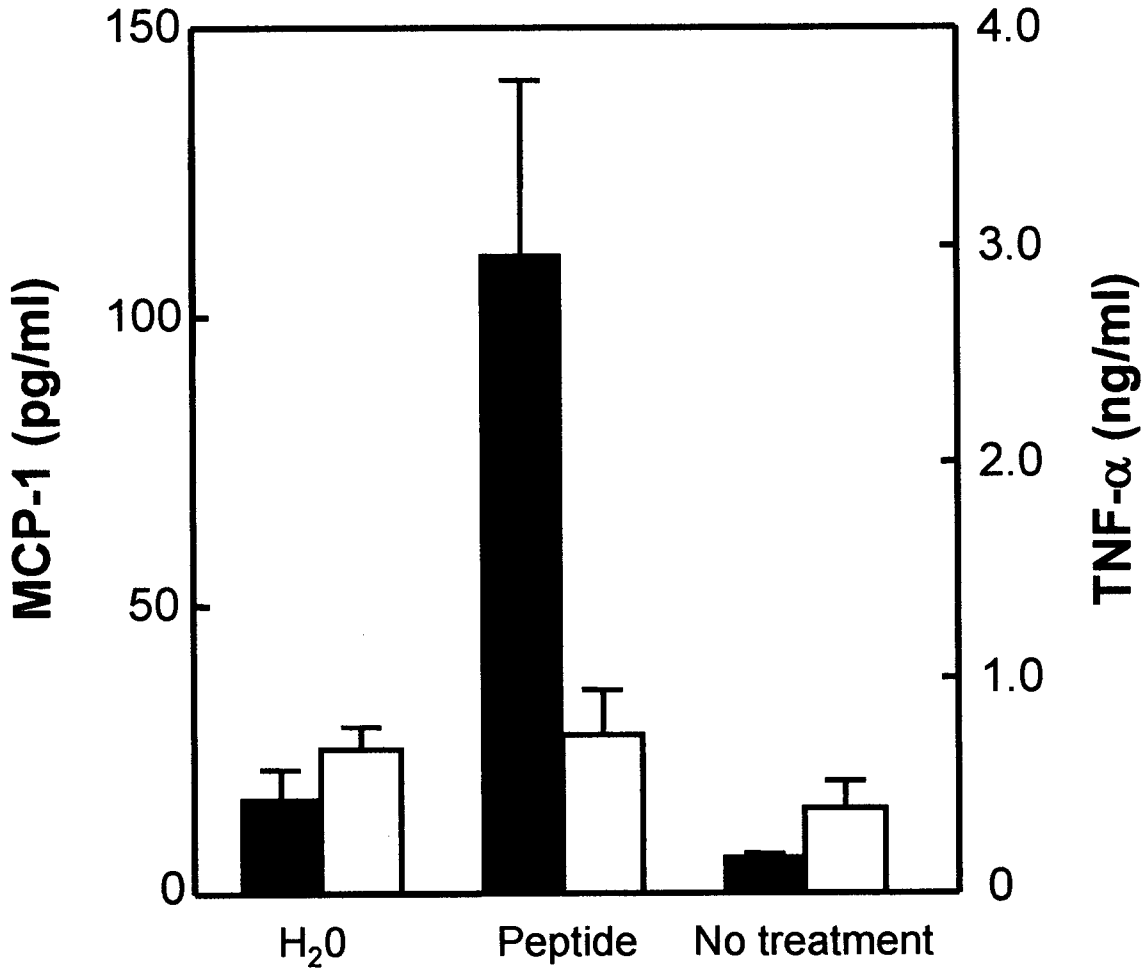


Figure 38: LL-37 increases production of MCP-1 but not TNF-α in murine bronchial alveolar lavage fluid.

BALB/c mice were anaesthetised with Avertin and given intratracheal instillation of 50 μg/ml LL-37 or water or no instillation (no treatment). The mice were monitored for 4 hours, anaesthetised and the BAL fluid was isolated and analyzed for MCP-1 (solid bars) and TNF-α (open bars) concentrations by ELISA. The data shown is the mean for 4 or 5 mice for each condition ± standard error. This experiment was performed with Dr. Donald Davidson.

antimicrobial factors to kill bacteria (Yan and Hancock 2001; Nagaoka et al. 2000) and limit the TNF-α release induced by released bacterial products (Chapter 6). If this does not contain the invading pathogen, the bacteria or resulting inflammatory stimuli would induce higher concentrations of LL-37 (Frohm Nilsson et al. 1999) which could then enhance chemotaxis of immune cells to help control the bacterial infection. The activities of LL-37 appears to involve the regulation of a large number of genes, some of which are known to have anti-inflammatory and some pro-inflammatory roles. Importantly these studies resulted in the novel finding that LL-37 induces chemokine production and surface expression of chemokine receptors, and can to do this when instilled into the lungs of mice. Thus the activities of LL-37 in the host immune response may include it acting directly as a chemoattractant and / or indirectly by increasing expression of chemokines and chemokine receptors.

CHAPTER 8: DISCUSSION

8.1 Cationic peptides may have multiple roles in the immune system

We have numerous defenses against the invasion of microorganisms. Amongst this arsenal are cationic peptides. Over 400 antimicrobial peptides have been identified in plants, insects and animals (Yang et al. 2001). Many of our microbial barriers such as skin keratinocytes, epithelial cells, and neutrophils contain cationic peptides such as LL-37. Cationic peptides comprise a major component of the neutrophil arsenal. Three of the α-defensins (HNP-1, -2, -3), which are stored as processed mature peptides in the azurophil granules account for 5% of the total cellular protein and 30-50% of the azurophil granule protein (Lehrer et al. 1993). In many species including humans, cationic antimicrobial peptides are up-regulated and found in significant concentrations after microbial challenge and other inflammatory stimuli (reviewed in (Gennaro and Zanetti 2000; Hancock and Scott 2000; Scott and Hancock 2000; Yang et al. 2001)). Their broad-spectrum antimicrobial activity has been well documented. More recently, there has been an interest in other peptide properties which would suggest they have a much more encompassing role in the host immune response than previously thought. Some of these peptide properties include mast cell degranulation leading to histamine release, stimulating the chemotaxis of neutrophils, monocytes, immature dendritic cells and T helper cells, tissue/wound repair, and inhibition of tissue injury by inhibiting certain proteases such as furin, elastase and cathepsin (Befus et al. 1999; Hook et al. 1990; Huang et al. 1997; Territo et al. 1989; Chertov et al. 1997; Yang et al. 1999; Gallo et al. 1994; Verbanac et al. 1993). This study examined the antimicrobial, anti-sepsis and chemotactic activity of α-helical cationic peptides, mainly the synthetic peptide, CEMA and the natural human peptide, LL-37.

8.1.1 Cationic peptides can kill a range of bacteria

In this study, a number of α-helical peptides were tested for their ability to kill *E. coli*, *B. cepacia* and Gram-positive bacteria. In other studies, their activity against other Gram-negative bacteria has been well documented (Andreu et al. 1992; Gough et al. 1996; Bals et al. 1998; Johansson et al. 1998; Turner et al. 1998; Bals et al. 1999; Friedrich et al. 1999; Scott et al. 1999; Brogden et al. 2001; Zhao et al. 2001). Many of the peptides tested in this study had antimicrobial activity against Gram-positive bacteria, although a few peptides had no antimicrobial activity. The cationic peptides tested had better antimicrobial activity against Gram-negative bacteria although some of the peptides were still active against Gram-positive bacteria. It is likely that structural differences among these peptides determine whether or not they can interact with the surface molecules of various Gram-positive and Gram-negative bacteria. The different abilities of the various cationic peptides to kill Gram-positive bacteria may point to structural features that are important for this process. CP26 and CP29 were designed to have increased amphipathicity relative to CEME. Although CP29 still had reasonable antimicrobial activity towards Gram-positive bacteria, it was less effective than CEME. CP26, while being very effective against *E. coli*, had no antimicrobial activity towards Gram-positive bacteria. Thus an amphipathic nature (refer to Figure 2 on page 5 for an example of an amphipathic, α–helical structure), by itself, is not sufficient for these cationic peptides to kill Gram-positive bacteria. Except for three conservative amino acid changes, the major difference between CP26 and CP29 is that the C-terminus of CP29 is identical to that of CEME (PALIS; single letter code) while the C-terminus of CP26 is PLISS. Consistent with the idea that the C-terminal PALIS sequence confers greater activity towards Gram-positive bacteria, CP203, which also has a C-terminal PALIS sequence, was somewhat more potent than CP207, which has a C-terminal PLISS sequence (see Table 1 on page 35 for peptide sequences). Despite having the same C-terminal PLISS sequence as CP26,

CP207 had modest activity against Gram-positive bacteria while CP26 did not. These two peptides differ only at two amino acids at positions 12 and 13 where CP207 has a valine and a leucine while CP26 has two alanine residues. The presence of the larger hydrophobic side chains at residues 12 and 13 in CP207 may therefore be important for activity against Gram-positive bacteria. Both CEME and CEMA have an alanine and a valine at positions 12 and 13 suggesting that one larger side chain may be sufficient. CP203, while having a PALIS sequence at the C-terminus, has two alanine residues at positions 12 and 13. Thus, the CEME-like peptides that have the best antimicrobial activity against Gram-positive bacteria have a C-terminal PALIS sequence and valines or leucines instead of alanines at positions 12 and 13.

The antibacterial activity of the natural human peptide, LL-37 was not included in this study, as it has been found to have weak antimicrobial activity (Yan and Hancock 2001). The CEME-related peptides have much better antimicrobial activity than LL-37. Although LL-37 has some antimicrobial activity against *E. coli* (4 μg/ml), it is ineffective against *P. aeruginosa*, MRSA, and *E. faecalis* (MIC > 64 μg/ml) (Yan and Hancock 2001). Since LL-37 can be found in nonkeratinized squamous epithelia of the mouth and tongue of humans (Frohm Nilsson et al. 1999), its antibacterial activity of LL-37 against a number of oral bacteria was tested. Guthmiller et al found that LL-37 was inactive against a range of oral bacteria tested including *P. gingivalis*, *S. sanguis*, *C. tropicalis* and *A. actinomycetemcomitans* (Guthmiller et al. 2001). In some instances however, LL-37 has been shown to synergize with other natural substances and peptides to kill bacteria (Nagaoka et al. 2000; Yan and Hancock 2001). In another study, the overexpression of LL-37 augmented the murine host response to *P. aeruginosa* infection (Bals et al. 1999). This suggests that the primary role of LL-37 may not be antimicrobial although it may have some activity in conjunction with other peptides or when expressed at high levels.

Although the mechanism of action of the CEME-related peptides against Gram-positive bacteria is not clearly understood, it does not appear to be related to their ability to bind LTA as the relative ability of these peptides to bind LTA did not correspond to their MIC values. Even though many of the peptides had a higher affinity for *S. aureus* LTA than did CEME, it was the most effective peptide at killing *S. aureus* and other Gram-positive bacteria. While the ability of the peptides to bind LTA may not be important for their antimicrobial activity it is likely to be important for reducing the ability of shed LTA to stimulate inflammatory reactions.

8.1.2 Cationic peptides can neutralize the biological activity of LTA and other Gram-positive bacterial products

Sepsis is associated with the presence of pathogenic microorganisms or cell wall components in the blood. The release of bacterial components from Gram-negative or Gram-positive bacteria during infection causes an inflammatory response, which when unchecked can lead to sepsis. The release of LTA from Gram-positive bacteria, which can be enhanced by antibiotic treatment, has been shown to stimulate the production of inflammatory mediators such as TNF-α. LTA, at high doses, has been shown to stimulate cytokine production by the murine macrophage cell line RAW 264.7 (Grunfeld et al. 1999). In this study, it was shown that the CEME-related peptides were able to substantially reduce production of TNF-α and IL-6 by RAW 264.7 cells. LL-37, a natural human peptide was also found to substantially reduce LTA-induced TNF-α production, suggesting this may be a natural activity of this peptide. The cationic antimicrobial peptides were able to block cytokine production by macrophages stimulated with LTA from different species of Gram-positive bacteria with two exceptions. CP26 was relatively ineffective in reducing the production of TNF-α and IL-6 by LTA-stimulated RAW 264.7 cells and it also had virtually no antimicrobial activity against Gram-positive bacteria. This same peptide has a much lower MIC against Gram-negative bacteria and is also much more effective at

inhibiting cytokine production in response to LPS. CP208 also did not substantially reduce LTA-induced cytokine production and had no antimicrobial activity against Gram-positive bacteria. In order to examine the activity of the peptides in a model system closer to humans, a whole blood assay was used. The cationic peptides were effective in whole human blood at reducing release of TNF-α in response to LTA. Although the levels of inhibition were lower than in the RAW 264.7 cell assay, this was not completely surprising as blood has many factors, including proteins and proteases, which could bind and/or inhibit cationic peptide activity. Another factor is heparin, which was used as an anticoagulant in the blood collecting tubes and since it has a net negative charge it could bind the cationic peptides and lower their activity (Ogata et al. 1997). One other issue in these experiments is the levels of cytokines in the whole blood assay would be different than cells in tissue culture since in whole blood the cytokines could be bound to receptors on other cells in the blood.

Polymyxin B was also found to inhibit LTA-induced cytokine production, partly in contrast to the findings of other researchers (Wakabayashi et al. 1991; English et al. 1996). This could be due to differences in the concentrations of polymyxin B, used in this study, which were much higher (20-50 μg/ml) than those used previously (5-10 μg/ml). It is reasonable to assume that LTA and polymyxin B could interact as suggested by the DPX (dansyl polymyxin displacement) assay based on the negatively charged lipid structure of LTA and the cationic structure of polymyxin B. Since LTA, like LPS, has both an anionic and lipidic nature, it seems reasonable that it should be able to interact with polymyxin B and dansyl polymyxin, although the kinetics of binding of DPX to LTA suggested a lower affinity than observed for DPX:LPS binding. It has previously been demonstrated that antibiotics and other peptides with no charge do not displace DPX (Moore et al. 1986). Thus, these peptides not only have antimicrobial activity against both

Gram-negative and Gram-positive bacteria but also reduce the ability of the major cell wall components released from these bacteria (LPS, LTA) to stimulate inflammatory responses.

8.1.3 Cationic peptides neutralize the biological activity of LPS in part by inhibiting LBP-LPS interaction and in part by selectively inhibiting LPS-induced transcriptional responses

Many cationic peptides including bactenecin, indolicidin, and cecropin:melittin hybrids have been shown to bind to LPS (Ooi et al. 1991; Piers et al. 1994; Hirata et al. 1995; Gough et al. 1996; Brackett et al. 1997; Falla and Hancock 1997; Turner et al. 1998; de Haas et al. 1999; Scott et al. 1999; Zhang et al. 1999). Cationic peptides have also been shown to block the ability of LPS to stimulate macrophage inflammatory functions (VanderMeer et al. 1995; Gough et al. 1996; Brackett et al. 1997; Kirikae et al. 1998; Scott et al. 1999; Zhang et al. 1999). In this thesis, I investigated the mechanism by which cationic antimicrobial peptides block the activation of macrophages by LPS. Using an assay that measures the binding of biotinylated LPS to immobilized LBP, it was shown for the first time that structurally diverse cationic antimicrobial peptides can block the interaction of LPS with LBP. Of the peptides tested, the α-helical peptide CP29 had the best ability to reduce both the LPS-LBP interaction and LPS-induced TNF-α release by macrophages. CEMA also had excellent ability to reduce both the LPS-LBP interaction and LPS-induced TNF-α release by macrophages. CP29 significantly inhibited LPS-LBP interaction at concentrations as low as 10 ng/ml (3.4 nM), whereas 10 μg/ml (3.4 μM) CP29 caused nearly complete inhibition of the LPS-LBP interaction. It is interesting to note the activity of CP29 at 10 ng/ml, as this is a five fold lower concentration than the LBP used. Preincubation of LPS with CP29 markedly improved its ability to inhibit LPS-LBP interaction at lower concentrations of peptide. At a concentration of 10 μg/ml, many of the other cationic peptides tested including Bac 2A, the indolicidins and gramicidin S, also inhibited both the LPS-LBP

interaction and LPS-induced TNF-α production. Bac 2A was different from the other peptides in that it caused moderate inhibition of LPS-LBP interaction but had only a minor effect on LPS-induced TNF-α production. One possibility is that Bac 2A, compared to the other peptides, is less stable in tissue culture medium over the 6 hour time course of the TNF-α stimulation (and therefore less able to block the LPS-LBP interaction) than it is in the one hour incubation in the ELISA measuring the binding of LPS to LBP. Otherwise, the relative ability of different cationic peptides to block the binding of LPS to LBP correlated strongly with their ability to block LPS-induced TNF-α production by RAW 264.7 cells. Thus, the ability of cationic peptides to block macrophage activation by LPS may be due in part to their ability to block the binding of LPS to LBP. This would presumably block the transfer of LPS to CD14 by LBP, greatly decreasing the ability of LPS to activate macrophages. Since LBP did not enhance LTA stimulation of TNF-α production by macrophages in serum-free conditions, LBP probably does not play an essential role in LTA stimulation of macrophages and thus the effect of cationic peptides on LTA-LBP interaction was not studied.

Le Roy et al. demonstrated that mAbs that block either the binding of LPS to LBP or the binding of LBP to CD14 are potent inhibitors of LPS toxicity *in vivo* and also block LPS-induced TNF-α production by RAW 264.7 cells (Le Roy et al. 1999). Together with my data, these results argue that inhibiting the LPS-LBP interaction is likely to be a very specific and efficient way to reduce or prevent LPS-induced inflammatory responses. The use of cationic antimicrobial peptides, in combination with anti-LBP mAbs may be a very potent anti-endotoxin treatment. In addition to providing potential anti-endotoxin therapies, cationic peptides may naturally limit inflammatory responses to bacterial components as it has been shown that bacteria and their components can induce production of cationic peptides (Russell et al. 1996).

However, this is not the complete story. Cationic peptide inhibition of LPS-LBP interaction did not fully explain the anti-endotoxin effects of peptides, especially since peptide could be added up to an hour after LPS to macrophages in the presence of serum containing LBP and still significantly reduce TNF-α production. I was not able to show that cationic peptides could reverse interaction of LPS-LBP once bound and so that would not explain these results. To further explore the mechanism by which cationic peptides neutralize LPS-inducted macrophage stimulation, gene arrays were used to profile changes in gene expression in macrophages treated with LPS in the absence or presence of the cationic peptide, CEMA. It was demonstrated that CEMA selectively inhibited LPS-induced changes in gene expression in RAW 264.7 macrophage cells. While the ability of LPS to induce 41 genes was substantially inhibited by CEMA, the increase in gene expression of an additional 16 genes was unaffected by CEMA. The indication that cationic peptides might do more than interfere in LPS transfer to macrophages is consistent with the results demonstrating that delayed addition of CEMA and LL-37 to RAW 264.7 cells still permitted reduction of endotoxin-stimulated induction of cytokines. Interestingly, the induction by LPS of inflammatory mediators was inhibited by CEMA, indicating that cationic peptides may selectively down-regulate macrophage inflammatory functions as opposed to other cellular processes.

Although these results have contributed to the current knowledge of the anti-endotoxin activity of cationic antimicrobial peptides, I wished to relate these results to the natural function of cationic peptides in the human immune response. Since HNP-1 and HBD-2 did not have very good anti-endotoxin activity (Figure 17 on page 78), I chose to study the widely expressed human peptide, LL-37. It is similar in structure to the CEME-related peptides in that they are amphipathic α-helices. LL-37 is derived from the cationic protein, CAP18 which itself and

fragments of have been shown to have anti-endotoxin activity (Hirata et al. 1995; Larrick et al. 1994; Bals et al. 1999). LL-37 did indeed have potent anti-endotoxin activity against several different types of LPS and at concentrations similar to that found in the humans (Sorenson et al. 1997; Lysenko et al 2000). LL-37 was active in the mouse model system commonly used to study potential anti-endotoxin therapies (and used to study the CEME-related peptides) and also in whole human blood. Thus part of the natural function of LL-37 may in fact be to bind released bacterial components and prevent an overwhelming inflammatory response which can lead to sepsis.

8.1.4 Cationic peptides directly modify transcriptional responses of macrophages

The possibility that CEMA acts directly on macrophages, as opposed to merely neutralizing LPS, prompted us to determine whether treating RAW 264.7 cells with CEMA or the human peptide, LL-37 alone caused any changes in gene expression. This lead to the very exciting finding that both the natural peptide, LL-37 as well as the synthetic peptide, CEMA, can directly influence macrophage gene expression by up- or down-regulating the expression of a wide variety of genes, including receptors, cell surface antigens, transcription factors, growth factors, cytokines, and chemokines. At least 35 genes were up-regulated and at least 21 genes were down-regulated in the RAW 264.7 cells after treatment with either LL-37 or CEMA. There were several genes that were up-regulated by both CEMA and LL-37 including the transcription factor, Jun-D, the c-src tyrosine kinase, Csk and TGFβR1, a receptor for TGFβ1 which is a growth inhibitor and a chemoattractant for neutrophils. Both peptides up-regulated genes involved in cell cycle, although not necessarily the same genes. For example LL-37 up-regulated cyclin D3 and CEMA up-regulated the expression of three cell cycle inhibitors, $p21^{Cip1}$, $p27^{kip1}$, and $p19^{ink4}$. These results are analogous to those observed with lactoferrin, an iron-binding glycoprotein synthesized

by epithelial cells and polymorphonuclear cell precursors, that contains an antimicrobial cationic peptide domain called lactoferricin. It was found that lactoferrin treatment of human breast carcinoma cells caused an increase in expression of the cdk inhibitor $p21^{Cip1}$ (Damiens et al. 1999). The effect of CEMA on cell proliferation is consistent with the anti-cancer properties observed for some cationic peptides, including CEMA (Cruciani et al. 1991; Baker et al. 1993). Such peptides are selectively more toxic towards tumor cells than towards non-malignant cells although the mechanism of their differential activity is not fully understood (Cruciani et al. 1991; Baker et al. 1993; Risso et al. 1998). These intriguing results involving the effects of cationic peptides on cell proliferation are being followed up by Dr. Donald Davidson.

CEMA and LL-37 both down-regulated a number of DNA repair protein and apoptosis genes although not necessarily the same genes. CEMA down-regulated the expression of the apoptosis inhibitors BAG-1 and Bcl-2. This data might help explain the results of a previous study, which found a cecropin-melittin hybrid peptide to have an apoptotic effect on a murine macrophage cell line (Velasco et al. 1997). The possiblity that cationic peptides effect apoptosis is being followed by a graduate student.

There were a number of genes, which were only induced by one or the other peptide. For example, LL-37 but not CEMA up-regulated several bone morphogenetic protein (BMP) genes including BMP-2, which induces bone formation. Recently BMP-2 produced by macrophages was implicated in bone healing (Champagne et al. 2002). In 1997, rhBMP-2 was used for the first time in clinical trials in patients undergoing spinal fusion. All eleven patients who had been implanted with rhBMP-2 achieved successful fusion within 6 months from the time of surgery (10 of these 11 patients had achieved their fusions within 3 months of surgery) (Boden et al. 2000).

There have been some reports that demonstrate that cationic peptides permeabilize

eukaryotic cells (Risso et al. 1998). Risso et al found that two antimicrobial peptides, BMAP-27 and BMAP-29 permeabilize eukaryote cell membranes and possibly interact with negatively charged sialyl residues on the membrane, causing Ca^{2+} flux into the cytosol (Risso et al. 1998). This could provide a potential mechanism for how cationic peptides might alter macrophage signalling or gene expression. Human cationic peptides have also been shown to bind to receptors on a number of different cell types (Niyonsaba et al. 2002; Yang et al. 1999; Yang et al. 2000). While the mechanism of cationic peptide acting on macrophage gene expression warrants further investigation, the current study clearly showed for the first time that cationic antimicrobial peptides directly influence expression of a large number of diverse genes in macrophages. The mechanism by which such peptides regulate gene expression was not studied here, but it is possible that cationic antimicrobial peptides interact with cell surface receptors and/or can permeabilize cell membranes and enter cells to directly influence signalling pathways, as previously suggested (Shi et al. 1996). Given the potential use of cationic antimicrobial peptides as antibacterial agents and anti-inflammatory agents, the effects of these peptides on macrophages and other host cells warrant further investigation.

8.1.5 Cationic peptides promote migration of immune cells

Cell migration is an important component of the host response to infection. It is required to recruit the appropriate cells, particularly neutrophils and monocytes, to the site of infection (Matsukawa et al. 2000). It is a process that involves chemoattraction, cell-cell adhesion and, in some cases, transmigration through cell layers. Neutrophils are important for the recruitment of monocytes to sites of infection and it was been hypothesized that factors derived from neutrophils were responsible (Ward 1968; Pereira et al. 1990). Indeed, the human neutrophil peptides HNP-1 and HNP-2, have chemotactic activity towards murine and human T cells and monocytes (Territo

et al. 1989; Chertov et al. 1996). A number of other cationic peptides have also been found to be chemotactic. HBD-2 selectively attracts a subset of T cells as well as immature dendritic cells (Yang et al. 1999). PR-39, a porcine cathelicidin is capable of inducing chemotaxis of porcine neutrophils and mobilizing Ca^{2+} (Huang et al. 1997). In other studies, LL-37, was chemotactic for monocytes, neutrophils, T cells and mast cells (Yang et al. 2000; Niyonsaba et al. 2002).

In this study LL-37 and CEMA were found to stimulate the expression and release of the chemokines, IL-8 and MCP-1, representing the CXC and C-C chemokine families. The concentration of peptide needed to induce significant amounts of chemokines (50 μg/ml or 1×10^{-5} M) is in the range estimated to be produced in response to inflammatory stimuli (Frohm et al. 1997; Frohm Nilsson et al. 1999). The ability of LL-37 to induce chemokine release was observed in a number of different models, including epithelial and macrophage cell lines representing cells that produce LL-37 (epithelial) and those that would be at the site of infection (macrophage). LL-37 also increased release of chemokines *in vivo*, in the mouse lung and *ex vivo*, in whole human blood. This suggests that LL-37 can contribute to the host immune response indirectly by promoting the recruitment of leukocytes to help clear bacerial infections. An increase in MCP-1 levels has a protective effect in a murine model of sepsis (Matsukawa et al. 1999). LL-37 was also found to be directly chemotactic for human peripheral neutrophils, monocytes, and T cells by utilizing formyl peptide receptor-like 1 (FPRL1) (Yang et al. 2000). Synthetic cationic peptides, such as CEMA can also cause increased release of chemokines. However, it is a specific property of select peptides, as the bovine cathelicidin peptide, indolicidin did not cause significant production of IL-8 or MCP-1.

The levels of chemokine release induced by the cationic peptides were in the range of biologically significant concentrations. For example, the minimal effective concentrations for

chemotaxis by MCP-1 are in the nM range (0.1-3 nM) for most cell types (monocytes, basophils, NK cells, and T lymphocytes) (Van Coillie et al. 1999). The range of concentrations of IL-8 leading to chemotactic activity towards PMNs is from 0.1-5 nM (Ludwig et al. 1997). The higher concentrations of peptide (50 μg/ml) induced about 1 ng/ml of IL-8, which is about 0.1 nM and in the range for chemotactic activity. We also found that synthetic and natural cationic peptides, LL-37, CEMA and Bac 2A all up-regulated the surface expression of chemokine receptors CXCR-4, IL-8RB and CCR2. Interestingly, two of these are receptors for IL-8 and MCP-1 which were also up-regulated by cationic peptides. When these effects are combined, the result could be a more powerful chemotactic stimulus (see Figure 39).

8.2 Different peptides have different activities

Many studies have demonstrated specific immune activities for individual peptides, but rarely look at a wide range of peptide structures and activities. From this study and certain others it is becoming clear that cationic peptides have a broad spectrum of activities and that the peptides can vary greatly in their activities. In this study it was demonstrated that although many peptides are antimicrobial, they also have other specific activities (see Figure 40 for overview). Some peptides, such as CEMA have a wide range of activities including neutralizing macrophage responses to bacterial products, directly modifying macrophage transcriptional responses, and inducing chemokines. Other peptides such as the bovine cathelicidin, indolicidin, have specific activities such as moderately reducing macrophage production of TNF-α and IL-6 in response to *E. coli* LPS. A linear variant of another bovine peptide, Bac 2A has very weak anti-endotoxin activities but has potent activities relating to chemotaxis such as inducing chemokine release, increasing surface expression of chemokine receptors, and direct chemotactic activity towards T cells. The human cathelicidin, LL-37, is a weak antimicrobial but has potent activity in reducing

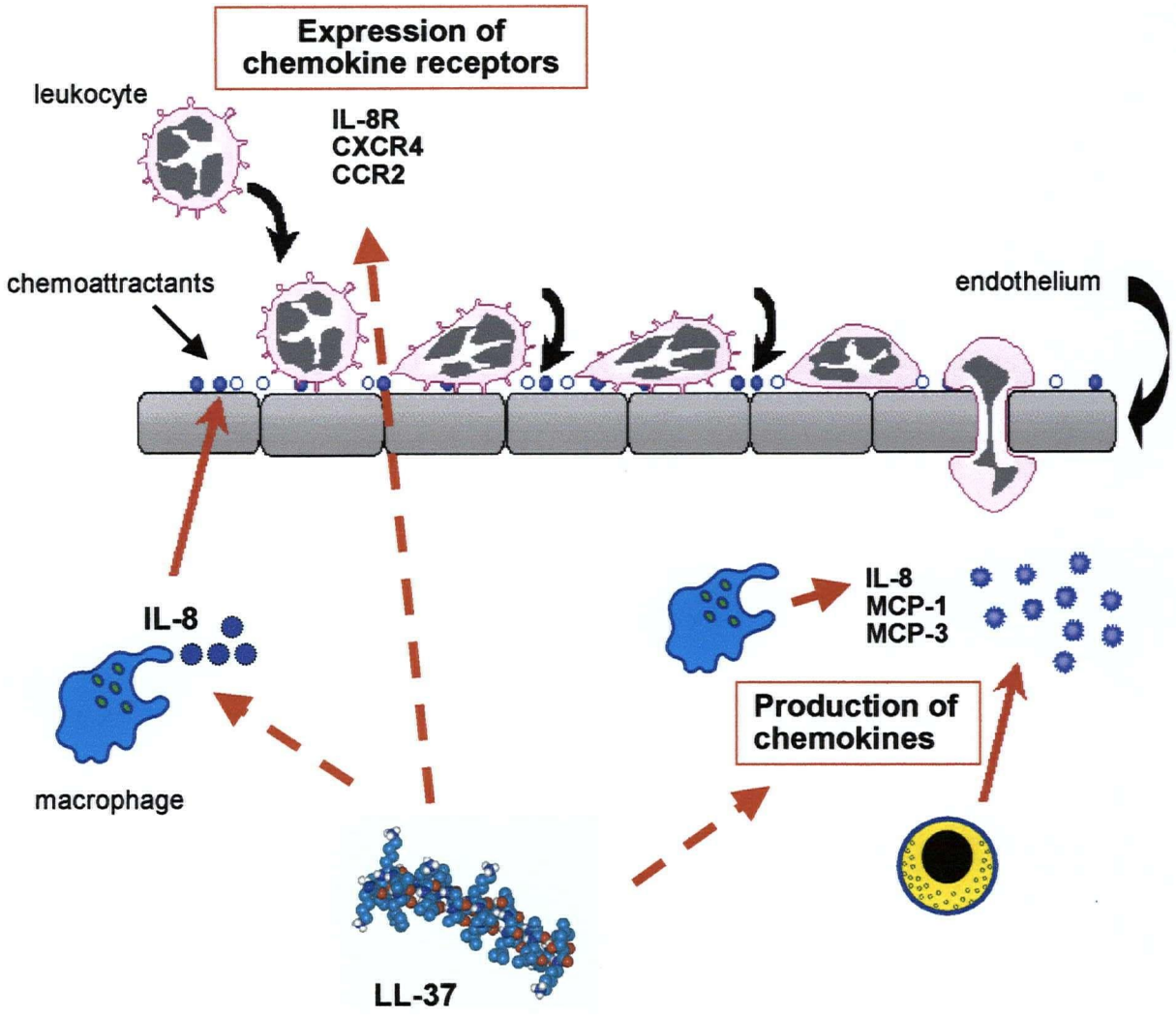


Figure 39: Promotion of leukocyte migration by cationic peptides

A schematic diagram of chemotaxis of leukocytes across an endothelial layer and the influence of cationic peptides. The red dotted line represents the effects of cationic peptides. This figure is adapted from http://www.med.virginia.edu/medicine/basic-sci/biomed/ley/index.html.

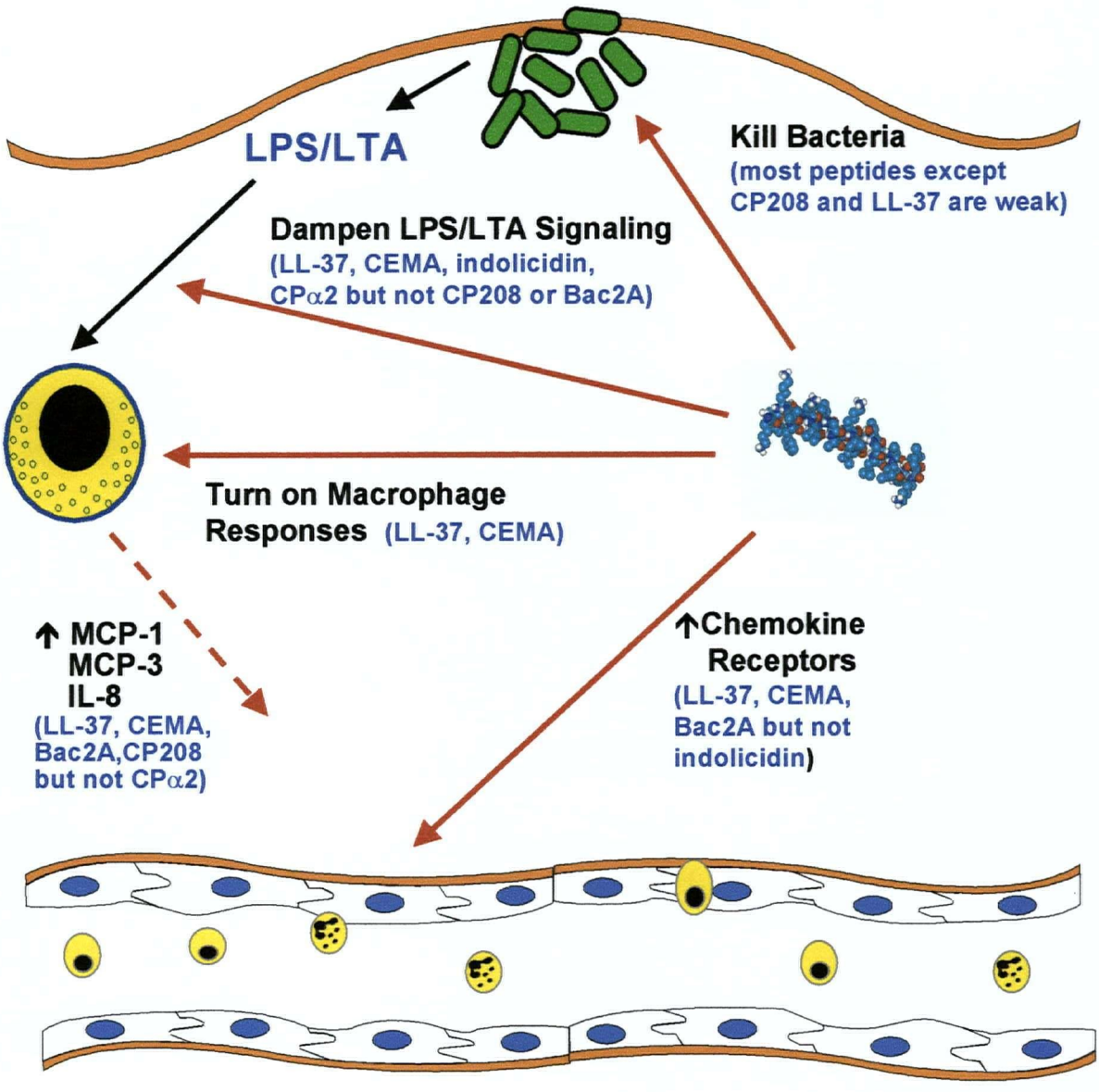


Figure 40: Different cationic peptides have different activities

Cationic peptides have a wide range of activities that can vary between peptides. LL-37 demonstrated all of the activities shown with the exception of antimicrobial activity which has been shown elsewhere to be weak unless in combination with other host factors. CEMA, however, demonstrated all of these activities. Other cationic peptides such as indolicidin and Bac 2A have more specific activities.

bacterial stimulation of macrophages at low concentrations and at higher concentrations it induces chemokine production and surface expression of chemokine receptors. Another interesting observation that suggests peptides can have different but overlapping functions is the effect of LL-37 and CEMA on gene transcription in RAW 264.7 cells that was discussed above. There were some genes that were similarly affected by both peptides but there were also a number of genes selectively affected by each peptide. A possible explanation for the different activities of LL-37 could be explained by its ability to bind different receptors. For instance, LL-37 has been shown to bind formyl peptide receptor-like 1 (FPRL1) which can be found on neutrophils and T cells (Yang et al. 2000). Another study found that LL-37 can bind both high- and low-affinity receptors on mast cells (Niyonsaba et al. 2002). HBD-2, another human cationic peptide can bind CCR6 (Yang et al. 1999). Thus, cationic peptides have a wide range of activities that can vary greatly between peptides and this could be explained in part, by their ability to bind different host cell receptors.

8.3 Role of peptide concentration

An interesting aspect of this study was the discovery that LL-37 could have different activities at different concentrations. Precedence for this was demonstrated by the studies with defensins. Defensins require high concentrations for antimicrobial activity but much lower concentrations for chemotactic activity (reviewed in Lehrer and Ganz 2002). This was also clearly demonstrated in Figure 35 on page 130 in which at low concentrations (up to 20 μg/ml), LL-37 inhibited LPS-induced IL-8 production and at higher concentrations LL-37 substantially increased production of IL-8 above background levels. There was also a general trend from the data presented in this study, in which only low concentrations were needed to neutralize bacterial product stimulation of inflammatory mediators where higher concentrations were needed for

increasing chemokine production. It should be noted that at all the concentrations used here, LL-37 and CEMA did not impair cell viability (Figure 25 on page 104). From Figure 35, it could be hypothesized that LL-37 is binding to LPS and preventing its interaction with the host cells and thus production of IL-8 by LPS. The close aposition of the LL-37 alone and LPS+LL-37 curves above 20 μg/ml suggest that the enhanced production of IL-8 in both cases is probably due to LL-37. In relation to the host immune response one could hypothesize that at low basal concentrations of LL-37 found at epithelial barriers (ie. 21 μg/ml on the mucosal surface of the nasopharynyx, Lysenko et al 2000), the peptides play a sentry role, killing microbes, and soaking up LPS. If the numbers of bacteria become too high, large increases in LL-37 production by the epithelial cells are induced by bacteria, bacterial products and inflammatory stimuli (Frohm Nilsson et al. 1999). The peptide could then play a role in recruiting more immune cells to help manage the bacterial infection.

8.4 Bacterial resistance to cationic peptides

A number of bacteria including Gram-positive and Gram-negative bacteria can modify their cell surface, resulting in increased resistance to cationic peptides. Due to their cationic nature, antimicrobial peptides interact with negatively charged components on the surface of bacteria via electrostatic interactions. There have been two mechanisms found in *S. aureus* which affect the sensitivity of the bacteria to cationic peptides. One affects the *dlt* operon that is responsible for transfer of D-alanine into teichoic acids (Peschel et al. 1999). Inactivation of this operon results in an increased negative charge of the cell due to the lack of D-alanine and a consequent increased binding of cationic proteins and an increased sensitivity to cationic peptides (Peschel et al. 1999). Second, a novel virulence factor, MprF was found to modify phosphatidylglycerol with L-lysine, resulting in a reduced negative charge of the membrane surface, which would make binding of the

cationic peptide more difficult (Peschel et al. 2001). Unlike conventional antibiotics such as penicillin, acquisition of resistance by a sensitive microbial strain against cationic peptides occurs with surprisingly low frequency. The best described resistance mechanism in Gram-negative bacteria involves the modification of LPS. The positively charged peptides interact with negative charges in the LPS. The PhoPQ regulon of many Gram-negative bacteria is a two-component regulatory system that uses a sensor (PhoQ) and a transcriptional regulator (PhoP). PhoP/PhoQ can affect LPS composition through modulation of the PmrAB two-component regulatory system. PmrAB controls at least 8 genes, whose functions include the addition of ethanolamine and 4-aminoarabinose (both positively charged). Addition of 4-aminoarabinose to LPS confers resistance to polymyxin B (Helander et al. 1994; Nummila et al. 1995; Gunn et al. 1998). Incorporation of the palmitoyl chain confers resistance to some cationic antimicrobial peptides (Guo et al. 1998). LPS modification in Gram-negative bacteria plays a significant role during infection in resistance to host antimicrobial factors, avoidance of immune system recognition, and maintenance of virulence phenotypes. In this study, the Gram-negative bacteria, *B. cepacia*, which is resistant to many antibiotics, (Goldmann and Klinger 1986; Prince 1986) was found to be resistant to killing by all the cationic peptides tested. This however, is probably not due to the inability of peptides to interact with *B. cepacia* LPS, since both CEMA and LL-37 could effectively reduce its stimulation of TNF-α production by macrophages. The structure of *B. cepacia* LPS is unusual in the KDO portion of the inner core. The KDO (3-deoxy-D-*manno*-octulosonic acid) is replaced by a KO (D-*glycero*-D-*talo*-2-octulosonic acid) to form a KO-KDO structure in the inner core region rather than the KDO-KDO structure of usual LPS (Isshiki et al. 1998). This LPS is also unique in that although it has potent TNF-α and IL-6 stimulatory activity similar to other enterobacterial LPS, it lacks the ability to induce IL-1β (Shimomura et al. 2001). The unusual structure of *B. cepacia* LPS may interfere with the interaction of cationic peptides

with the bacterial surface (as as seen with the previous study by Moore et al. 1986) while still permitting cationic peptides to bind the released LPS.

8.5 Therapeutic potential of cationic peptides

There is no question that, with the increasing antibiotic resistance problem, there is a need to develop new classes of antibiotics (Hancock and Lehrer 1998). Cationic antimicrobial peptides have many of the desirable features of a novel antibiotic class. For instance, they have a broad spectrum of activity, kill bacteria rapidly, are unaffected by classical antibiotic resistance mutations, do not easily select antibiotic resistant variants, show synergy with classical antibiotics, and are active in animal models. There is also a notable lack of effective therapies for treating sepsis. Many new strategies, including neutralizing antibodies to LPS and TNF-α as well as various endotoxin-binding factors, have been tested with limited success (Baumgartner et al. 1990; Greenman et al. 1991; Ziegler et al. 1991; Warren et al. 1993; Rogy et al. 1994; Abraham et al. 1995; Fisher et al. 1996; Opal and Cross 1999). Cationic peptides that are antibacterial, can reduce bacterial product stimulation of macrophages and recruit immune cells to the site of infection provide a potential avenue for anti-sepsis therapies either alone or in combination with other neutralizing factors. In addition to the scientific results reported here a number of these cationic peptides and peptide effects have been filed for patent protection (see Appendix 3) and may therefore impact these therapeutic areas.

The results of this study demonstrate that the synthetic CEME-related α-helical peptides tested are not only effective for Gram-negative sepsis but may also have potential therapeutic value for Gram-positive sepsis. These cecropin::melittin hybrids have demonstrated a broad range of activities including antibacterial activity, anti-endotoxin activity, the ability to synergize with other antibiotics and efficacy in animal models of infection (Gough et al. 1996; Scott et al. 1999).

Although there have been many new therapies for Gram-negative sepsis examined in the recent years, very little progress has been achieved in finding new therapies for Gram-positive sepsis. In this study, it was have shown that CEME-related peptides can kill Gram-positive and Gram-negative bacteria, can bind LTA and LPS, and can reduce cytokine release by macrophages stimulated with a variety of bacterial products. The effects of the cationic peptides on soluble LPS and LTA are significant since bacteria release LPS and LTA during normal growth and their release can be enhanced by antibiotics (Cohen and McConnell 1985; Prins et al. 1995; Soto et al. 1996; van Langevelde et al. 1998). Although the CEME-related peptides lack the potency of some antibiotics, they are able to bind bacterial products released by Gram-positive and Gram-negative bacteria and reduce their ability to activate macrophages, possibly in part by down-regulating the pro-inflammatory response which when unchecked can lead to multi-organ damage and sepsis. As well the cationic peptides can stimulate host responses to recruit immune cells to help fight bacterial infections. Thus, α-helical peptides may be an important tool for treating bacterial infections and preventing sepsis by themselves or in concert with other antibiotics.

Many issues regarding the therapeutic potential of cationic peptides remain to be solved. For example, these peptides have relatively high molecular weights compared to most antibiotics, and will have to be produced recombinantly to keep prices reasonable. While several processes for doing this have been described, to my knowledge they have not yet been successfully performed on an industrial scale. Another issue is toxicity. Some cationic antimicrobial peptides are very toxic for mammalian cells (e.g. wasp venom mastoparan), while others show little or no acute cytotoxicity. In this study, the peptides were not found to be cytotoxic at 125 μg/ml, a concentration higher than their active concentrations. However, more subtle negative consequences have not been studied, although one could assume, based on the presence of natural

peptides *in vivo* at concentrations of e.g. 100 μg/ml HNP-1 in plasma of septic patients (Panyutich et al. 1993) and 44 μg/ml in the saliva of an individual with peritonitis (Shiomi et al. 1993), that they can be tolerated at high levels. Most pharmaceutical effort has been devoted to the development of topically applied agents due to the uncertainty surrounding the long-term toxicology of the cationic peptides (Zasloff 2002). Another issue related to their use as therapeutics would be their lability to proteases in the body. In this regard, there are strategies for protecting the peptides from proteases, including liposomal incorporation or chemical modification.

With this in mind there are a number of promising clinical trials involving cationic peptides underway. The protegrin derivative IB-367 (Intrabiotics) is in Phase III clinical trials for oral mucositis, a polymicrobial ulcerative disease of cancer patients. The peptide MBI-226 (Micrologix) is in Phase III clinical trials for sterilization of catheter insertion sites, thus preventing serious infections due to colonization of such catheters by skin bacteria. Micrologix also has another peptide in Phase II clinical trials for acne. The Phase III clinical trials with Pexiganan (Magainin) for topical treatment of infected diabetic foot ulcers have been completed although it was not approved. A Histatin analogue (Demegen) is in Phase II clinical trials for oral treatment of gingivitis. The other potential therapeutic avenue for cationic peptides is the introduction of peptide genes into plants or animals (Bals et al. 1999; Osusky et al. 2000; DeGray et al. 2001). Thus cationic antimicrobial peptides are not only important components of the innate defences of all animals against infections, but synthetic variants thereof hold great potential as a weapon against antibiotic resistant bacteria. The sequence and structural diversity offered by peptides will provide many possibilities for drug design.

8.6 Final remarks

In order to combat bacterial infection, the host immune response or treatment regime must aim to kill the invading pathogen and prevent the onset of sepsis by managing the inflammatory response to bacteria and their released components. This study revealed that both synthetic cationic antimicrobial peptides and the natural cationic peptide LL-37, which is up-regulated in response to bacterial infection, could contribute to the immune response by limiting the inflammatory damage caused by bacterial products and by recruiting leukocytes to sites harboring infectious bacteria. The findings from this study can be applied in two ways. First, the results with the human peptide LL-37 can further our understanding of its biological function in the host immune response. Second, the data from the synthetic cationic peptides and LL-37 could be applied to the design of new treatment regimes to fight bacterial infections. Thus, this study has contributed to our knowledge of the role of cationic peptides in the host immune response and may aid the development of new therapeutics to treat bacterial infections.

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APPENDIX A: Cationic Antimicrobial Peptides and Their Multifunctional Role in the Immune System

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Cationic Antimicrobial Peptides and Their Multifunctional Role in the Immune System

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ABSTRACT: Many species of life contain cationic antimicrobial peptides as components of their immune systems. The antimicrobial activity of these peptides has been studied extensively and many peptides have a broad spectrum of activity against not only Gram negative and Gram positive bacteria but also against antibiotic-resistant bacteria, fungi, viruses, and parasites. Such cationic antimicrobial peptides can also act in synergy with host molecules, such as other cationic peptides and proteins, lysozyme and also conventional antibiotics, to kill microbes. It has been found that certain peptides are produced in large quantities at sites of infection/inflammation and their expression can be induced by bacterial products such as endotoxic lipopolysaccharide (LPS) and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α). These peptides often have a high affinity for bacterial products such as LPS, allowing them to modulate the host response and reduce the inflammatory response in sepsis. More recently, they have been found to interact directly with host cells to modulate the inflammatory process and innate defenses.

KEY WORDS: cationic peptides, antimicrobial, inflammation, lipopolysaccharide, innate immunity.

I. INTRODUCTION

Many potential pathogens can enter mammalian hosts daily through inhalation, ingestion or contact with other infected organisms. The adaptive humoral and cellular immune responses have minimal impact on whether these pathogens will lead to infections, but rather are important in pathogen-specific protection once a particular organism has multiplied in the body. In contrast, innate immunity plays an important role in a host's ability to avoid the progression of a potential pathogen from a threat to an infection.

In the last decade, the role of cationic peptides in antimicrobial defenses has become increasingly apparent, and there is a growing body of evidence that their role in defense against microbes may be as important to the host as antibodies, immune cells and phagocytes. In the fruit fly *Drosophila*, for example, cationic antimicrobial peptides are well recognized as the major form of defense against infection and are induced, in response to challenge by microbes or microbial signaling molecules like lipopolysaccharide (LPS), by a regulatory pathway similar to that used by the mammalian immune system (involving Toll receptors and the transcription factor; NFκB).[1] In amphibians, crustaceans, fish, birds, mammals and man, it is becoming increasingly clear that antimicrobial peptides are a major player in local innate immunity,

especially at mucosal and epithelial surfaces. [2; 3]

Cationic antimicrobial peptides are well known for their ability to kill many infectious agents, including bacteria, fungi, viruses and parasites, and they have been called "Nature's antibiotics". However, the action of cationic antimicrobial peptides appears not to be limited to the direct killing of microbes. Rather, there is accumulating evidence that they have an impressive variety of additional activities that would be expected to impact on the quality and effectiveness of innate immune responses and inflammation.[4] These include many of the elements of inflammatory responses that are frequently ascribed to other agents. In many cases it still remains to be determined whether these peptides are central players, supportive or even bystanders. One might suspect a key role, however, based on the inducibility by infectious agents of many (but not all) peptides and the very high concentrations that have been recorded at sites of inflammation. In the sputum of cystic fibrosis patients for instance, levels of 300 μg/ml [5] or more have been recorded and up to 170 μg/ml was observed in the plasma of septic individuals.[6,7] In patients with empyema, HNP1-3 levels were highly elevated in pleural effusions.[8] In patients with oral inflammation and / or oral diseases, the levels of HNP-1 are significantly increased over the levels of healthy individuals.[9, 10]

Cationic antimicrobial peptides have been reported to be involved in many aspects of innate host defenses associated with acute inflammation, including initial lysis of bacterial cells to release inflammatory stimuli, mast cell degranulation leading to histamine release and consequent increase in blood vessel permeability (vasodilation), increasing chemotaxis of neutrophils and T helper cells resulting in leukocyte recruitment to the infection site, promotion of non-opsonic phagocytosis, inhibition of fibrinolysis by tissue plasminogen activator, thus reducing the spreading of bacteria, tissue/wound repair through the induction of the proteoglycans, syndecan-1 and -4, promotion of fibroblast chemotaxis, and inhibition of tissue injury by inhibiting certain proteases such as furin, elastase and cathepsin.[11-21] If acute inflammatory responses are insufficient to result in bacterial clearance, then chronic inflammation and the adaptive immune responses are initiated. Some roles that cationic antimicrobial peptides may play in this process, based on their *in vitro* properties, include acting as chemokines for recruitment of monocytes and T cells, enhancement of chemokine production and of the proliferative response of T helper cells leading to increased IgG but not IgA production, suppression of LPS-, LTA- and bacterial-CpG-DNA-induced cytokine production and other responses of macrophages, induction of specific macrophage genes, and stimulation of apoptosis in macrophages and activated lymphocytes (see below), resulting in the potential elimination of infected cells.

Cationic peptides are clearly very ancient elements of the immune responses of numerous species of life,[3] and the induction pathways for peptides in vertebrates, insects and plants are highly conserved. Furthermore, it is becoming increasingly apparent that cationic antimicrobial peptides have many potential roles in inflammatory responses, which responses represent an orchestration of mechanisms of innate immunity. In this review we discuss selected aspects of the recent literature on cationic antimicrobial peptides that are consistent with an important role for these peptides in the early defenses against infection. The reader is also referred to a series of excellent recent reviews on the same or related topics. [4,22-34]

II. PROPERTIES OF CATIONIC ANTIMICROBIAL PEPTIDES

Although there is a continuum of cationic molecules ranging from peptides to proteins, we generally define cationic antimicrobial peptides as being 12 to 50 amino acids in length with a net positive charge of +2 to +7 due to an excess of basic amino acids, arginine, lysine or histidine, over acidic amino acids.[26] Generally speaking, 50% or more of their amino acids are hydrophobic, reflecting the interaction of such peptides with microbial membranes as part of their mechanism of action. Despite their small size and common physical-chemical features, cationic antimicrobial peptides appear to have arisen from multiple sources, possibly through convergent evolution, in that they have a range of folding patterns that fit into 4 broad secondary structure classes[3] (e.g. Table 1). The most prominent classes are β-sheet peptides stabilized by 2 to 4 disulphide bridges (and occasionally containing a short α-helical stretch), and unstructured peptides that fold into amphipathic α-helices upon contact with membranes. Less common are extended peptides with a predominance of one or two amino acids, e.g. P, W or H, and loop peptides formed by a single disulphide bond. Other cationic antimicrobial peptides are apparently formed in the host by proteolytic digestion of larger cationic proteins such as lactoferrin and CP18. Interestingly, several cationic proteins implicated in innate immunity, such as lactoferrin, bactericidal/permeability increasing protein, and cathepsin, have characteristics and activities that are reminiscent of cationic peptides. Generally speaking, the best cationic peptides fold into molecules that have the charged and hydrophilic portions segregated from the hydrophobic portions; either amphipathic structures or cationic double wing structures with a hydrophobic core separating two charged segments.

Such peptides are found in all species of life, including bacteria, fungi, plants, insects, birds, crustaceans, amphibians and mammals. A single host species may contain peptides from 2 to 4 different classes, including a number of variant peptides from any given class (www.univ.trieste.it/~tossi). For example, cattle are known to contain 38 antimicrobial peptides, including β-stranded α- and β-defensins, α-helical BMAP peptides, the loop peptide bactenecin, and the extended peptide indolicidin, as well as a variety of fragments of larger proteins. There are at least three possible reasons for this variety. Firstly, the antimicrobial spectrum of any given peptide tends to be incomplete, indicating that others may cover deficiencies inherent in any given peptide. This property may also extend to the non-antimicrobial activities of peptides as we have observed that two different α-helical peptides, CEMA and LL-37, induced the expression of distinct but overlapping subsets of genes in macrophages (Scott and Hancock, unpublished data). Secondly, different peptides show synergy with each other against microbes. Thirdly, peptides tend to be produced by different cell types such that a given tissue might express only a subset of these peptides.

III. EXPRESSION OF ANTIMICROBIAL PEPTIDES

Antimicrobial peptides are generally encoded individually by genes, which comprise highly homologous gene families, although some defensins may be alternate processing forms produced from a single gene. The antimicrobial peptide gene families are localized as clusters on the chromosome, indicating the co-evolution of different subclasses,[35] characterized by tissue of expression and inducibility. In

humans, the defensin locus is found on chromosome 8p21-23, where the genes for both α- and β-defensin are found.[36] The genes for α-defensins demonstrate substantial sequence identity, as do the genes for the β-defensins even though these groups of genes are quite diverse from each other. Examination of defensin mRNA has revealed that defensins are synthesized as precursor molecules, comprising a signal sequence, a pro-region and the mature peptide. After removal of the signal sequence, the pro-region is removed to yield the mature peptide. Each defensin family demonstrates substantial conservation of both amino acid and mRNA sequences in the region encoding the signal sequence; whilst in the mRNA this sequence identity extends into the 5'-untranslated segment which contains a region with greater than 90% identity amongst defensins of unrelated species.

The cathelicidins are a family of structurally divergent antimicrobial peptides from diverse species. Their characteristic feature is a high level of sequence identity in the 5' region,[25] termed the cathelin domain, since it is also found in cathelin, a cysteine protease inhibitors of porcine origin.[37] Cathelicidins are encoded by genes of four exons, with the active mature peptide, which tends to be highly divergent in sequence and ranges in size from 12-79 amino acids, found in the fourth exon.[38,39] Utilizing the high homology of the cathelin domain has enabled researchers to identify new cathelicidin sequences in numerous species, including cows, pigs, sheep, horses, mice, guinea pigs and rabbits. On the sheep, cow and pig chromosomes, cathelicidin genes are found as clusters. In contrast to the situation with defensins, cathelicidin peptides are stored as inactive propeptide precursors that, upon stimulation, are processed. In porcine and bovine neutrophils, neutrophil elastase is thought to be the enzyme responsible for processing, resulting in release of active peptides into the extracellular fluid.[40] Cathelicidin peptides include β-sheet protegrins from pigs, α-helical CAP18 from rabbits, loop-structured bactenecin and extended-structure indolicidin from cows. In humans only one cathelicidin has been found to date, the α-helical peptide LL-37 (also known as hCAP18).[4] Its gene is located on chromosome 3.

Both the tissue of expression and the inducibility or constitutively of cationic peptide genes reflects the gene structure. For example, the Paneth cells of the small intestine express human enteric defensins (HD-5 and -6), which are encoded by two-exon genes.[35] These appear to be the evolutionary precursor to the genes of myeloid defensins (HNP-1/3, 2 and 4) that are encoded by three exons, and they appear to be under the control of hematopoietic regulatory elements, resulting in constitutive expression in the promyelocyte.[41] In contrast, the genes for individual epithelial defensins are either constitutively expressed or inducible. For example, enteric α-defensin genes are expressed at high levels in the normal intestine of mice, humans and rats.[22] Despite this, the expression of at least one of these genes is induced in the rat intestine after hemorrhagic shock.[42] One other enteric α-defensin gene, HD-5, was found to be variably expressed in the female reproductive tract,[43] where expression in the endometrium was found to correlate to the phase of the menstrual cycle.

There are two groups of β-defensins as defined by expression patterns. Constitutively expressed β-defensins, such as human β-defensin (HBD)-1 in humans and the 13 bovine neutrophil β-defensins (BNBD-1 to -13), have two exons surrounding an intron.[36,44] HBD-1 is expressed at high levels in the genitourinary tract, and at lower levels in other tissues, but its mRNA levels do not apparently change

upon microbial challenge or when host tissues enter the inflammatory state.[25] In contrast, the expression of tracheal antimicrobial peptide (TAP), a β-defensin in the bovine airway, is up-regulated in primary culture systems including phorbol 12-myristate 13-actetate,[45] TNF-α,[46] IL-1β, muramyl dipeptide, and lipoteichoic acid[47] by numerous infectious agents and inflammatory mediators. For example, a 15-fold increase in the steady-state levels of the mRNA encoding TAP was observed upon incubation with 100 ng/ml *E. coli* LPS,[45] indicating that airway epithelial cells are able to respond to pathogens by the production of antimicrobial agents. A homologous β-defensin expressed in the tongue epithelium, lingual antimicrobial peptide (LAP), is also up-regulated at sites of inflammation and undergoes an increase in expression in the airway in a coordinated fashion with TAP.[46] The genes encoding both TAP and LAP are much smaller than that encoding HBD-1, mostly due to a relatively small intron.

In humans, a homologue to TAP, human β-defensin-2 (HBD-2) was observed to be expressed in psoriatic skin,[48] and subsequently found to be expressed in other inflamed tissues. For example, it has been observed that primary airway epithelial cells up regulate the mRNA for HBD-2 in response to IL-1β.[49] A similar induction in response to either *Pseudomonas aeruginosa* LPS or TNF-α can be observed at the air-liquid interface culture of human tracheal epithelial cells. The homologous mouse β-defensin 2 (mBD2) is also up-regulated in response to LPS. The expression of defensins in the oral cavity has also been studied. Constitutive expression of HBD-1 mRNA was seen in the tongue, gingiva, parotid gland, and buccal mucosal while HBD-2 mRNA was only detected in the gingival mucosa.[79] In gingival keratinocyte cell cultures, HBD-2 expression was found to be induced 16-fold upon stimulation with IL-1β and 5-fold with LPS.[79] These patterns of defensin expression are similar to that seen at other mucosal surfaces. These data suggest that inflammation and infection mediate a peptide-based host response in mucosal tissues through transcriptional regulation.

Expression of the genes for TAP and HBD-2 is induced by bacterial LPS through an epithelial cell-expressed CD14-mediated signal transduction pathway.[45,50] The induction occurs via activation of the p65/p50 heterodimer of NF-κB, which migrates to the nucleus and binds to an NF-κB consensus sequence upstream from the TAP gene.[47] This pathway, which presumably involves the entire LPS-signaling pathway of mammalian cells, is known to regulate the expression of several events involved in inflammation, such as the production of pro-inflammatory cytokines. A similar pathway is activated in the LPS-mediated up regulation of the HBD-2 gene in human airway cells.

In most species, cathelicidins are expressed in myeloid precursor cells, although expression of some cathelicidins in mature peripheral porcine neutrophils has been reported.[51] Surprisingly, porcine cathelicidins are also expressed in a number of lymphoid tissues in young pigs (less than 4 weeks old), but such expression disappears in adults.[51] The single cathelicidin from humans, LL-37 demonstrates widespread expression, and can be produced in be produced in myeloid precursors,[52] testis,[53] human keratinocytes during inflammatory disorders[54] and airway epithelium.[55] The promoter region of the gene encoding the arginine-rich porcine peptide PR-39 exhibits several potential transcription factor binding sites, including NF-IL6, indicating that this peptide may undergo regulatory controls analogous to those of the defensins. Indeed, cathelicidins and defensins exhibit

synergystic antimicrobial activities,[48] indicating that they participate in a combined host defense response.

IV. CATIONIC PEPTIDES DIRECTLY KILL PATHOGENS IN THE HOST

Cationic peptides have unusually broad spectra of activity. The only defining characteristic of their diverse set of targets is the possession of a membrane. This can include an ability to kill or neutralize Gram-negative and Gram-positive bacteria, fungi (including yeasts), parasites (including planaria, nematodes, trypanosomes and plasmodia), and even enveloped viruses like human immunodeficiency virus (HIV) and herpes simplex virus (HSV). For example, HNP-1 was found to directly inactivate HSV types 1 and 2, cytomegalovirus, vesicular stomatitis virus, and influenza virus A/WSN, but not nonenveloped viruses such as echovirus type 11 and reovirus type 3.[56]

The peptides are selective in that normal host cells are relatively resistant to the action of such cationic peptides, but it should be mentioned that certain cationic antimicrobial peptides, e.g. melittin from bees, mastoparan from wasps and charybdotoxin from scorpions, are potent toxins. The basis of discrimination for the relatively non-toxic peptides appears to be the lipid composition of the target membrane (selective peptides tend to prefer membranes that are negatively charged and free of cholesterol) and the possession of a large transmembrane electrical potential (oriented internal negative).[26] The activities of antimicrobial peptides can be reduced by a variety of relevant factors that exist in vivo, including high mono- and di- valent cation concentrations, polyanions, serum, apolipoprotein A-1, serpins and proteases.[2; 3] However, some peptides appear quite immune to several of these agents.

The case for a primary role for antimicrobial peptides in host defenses is becoming increasingly convincing. In mammals, neutrophils are the "kamakaze" fighters of the body in that they are recruited to sites of infection, have a short half life (aproximately 14 hours) in the body and contain very potent antimicrobial activities that include both oxidative (i.e. production of toxic oxygen species, including free radicals, peroxide and hypochlorite) and non-oxidative killing mechanisms. In the latter category, defensins are the most predominant protein species in mammalian neutrophils, representing nearly 15% of total protein in these dedicated anti-infective cells.[57] Other antimicrobial peptides are produced and secreted by other cells including epithelial cells, macrophages, paneth cells, kerationocytes etc., and are found at mucosal and epithelial surfaces and in the gut, lungs, kidneys and skin. Their induction during inflammation is consistent with a primary role in assisting and/or directing inflammatory responses. In *Drosophilia* and other insects they are produced in the fat body in response to infection or to microbial products. In such species it is possible to create knockouts in specific genes that control the production of several cationic peptides, in order to demonstrate the importance of these peptides in combating pathogens. For example a knockout in the Toll receptor gene (part of the pathway of activation of the NFκB-like transcriptional activator Dorsal, and a homolog of the TLR-receptor family in mammals), decreases the production of anti-fungal peptides like drosomycin, and makes *Drosophilia* more susceptible to the filamentous fungus *Aspergillus fumigatus*, but not to the bacterium *E. coli*.[58] Conversely, knockout of the Imd (for immune deficiency) gene, affects the expression of multiple antibacterial peptides and permits three orders of magnitude more *E. coli* to grow in *Drosophilia* within the first

24 hr, but does not affect susceptibility to *A. fumigatus*.

To demonstrate the basic ability of cationic antimicrobial peptides to protect hosts, one can add these peptides to infected animals. A wide variety of animal model studies and early clinical trials have demonstrated that exogenously added natural and synthetic cationic antimicrobial peptides will protect against local or systemic infection by bacteria and fungi.[59-65] Such studies have confirmed the antibacterial (vs. both Gram-negative and Gram-positive bacteria), anti-fungal, synergistic and anti-endotoxic nature of antimicrobial peptides. Examples of the *in vivo* antimicrobial activity of natural peptides in mouse models can be seen in Table 2. Several of these peptides demonstrated significant protection in animals against an LD_{90} dose of the mouse pathogenic *Pseudomonas aeruginosa* strain M2. Recent animal model evidence has also demonstrated systemic protection by the bacterial, lantibiotic peptide nisin against *Streptococcus pneumoniae* infections of mice, protection by the pig protegrin 1 against systemic *P. aeruginosa* and *S. aureus* infections and by protegrin-related peptide IB-367 against polymicrobic oral mucosaitis in hamsters,[63] protection against lethal *P. aeruginosa* infections of burn wound sites in mice by the synthetic peptide D4B,[60] and protection by the human peptide LL-37 against lethal endotoxaemia and *P. aeruginosa* infection in mice.[62]. Studies in coho salmon demonstrated that peptides, including the flounder peptide pleurocidin and an insect-derived cecropin-melittin hybrid peptide, were protective when administered gradually using a device called an osmotic pump, but were not protective when given as a single large-dose injection.[66]

Another method for demonstrating the *in vivo* activity of antimicrobial peptides is to utilize transgenic means to over-express these peptides, thus providing increased resistance to infections. The most detailed studies on such transgenic species have been done in plants where a rich body of data attests to the ability of hyper-expressed natural plant peptides, derived from both plant and animal species, to protect against fungal or bacterial infection.[67] Success in such studies appears to be a function of promoter strength (since to high expression can be toxic) and the lability of the peptides to plant proteases. In mammals, over expression, by adenovirus-mediated gene transfer, of the human cathelicidin gene for LL-37 in the mouse airway results in the increased ability to reduce bacterial load from *P. aeruginosa* challenge and improved survival after administration of lethal doses of endotoxin (LPS) or *E. coli*.[68] These studies are consistent with those demonstrating that mice made transgenic for a synthetic α-helical peptide related to the moth cecropin family, Shiva 1a,[69] are resistant to infection by *Brucella abortus*. Gropp et al expressed the human defensins, HBD-1 and HD-5 in eukaryotic cell lines and found the defensins localized to the cytoplasm and in the cell culture medium.[70] Over-expression of these defensins resulted in increased resistance to viral infections and infections by Gram positive or Gram negative bacteria. In plants, up regulation of antimicrobial peptide gene expression also increases their resistance to pathogens.[71] Although equivalent studies cannot be done in humans, it has been observed that patients with specific granule deficiency syndrome, completely lacking in α-defensins, suffer from frequent and severe bacterial infections.[2,57] Similarly a group of HIV patients with lower salivary levels of histatin peptides showed a higher incidence of oral candidiasis and fungal infection.[2] Other studies have connected the chronic lung infections of cystic fibrosis patients with the high salt concentrations in the airways, caused by the mutation in the cystic fibrosis

transmembrane regulator/chloride channel, since the antimicrobial substances secreted by lung epithelial cells tend to be salt sensitive.[72] However this conclusion remains controversial.

In vitro expression studies predict that animals with infectious and/or inflammatory conditions would exhibit an up-regulation of antimicrobial peptides. Indeed, increased levels of antimicrobial peptides are observed in a number of clinical and laboratory-induced infectious and inflammatory states. Pigs infected by *Salmonella choleraesuis* demonstrated a 3-fold increase in circulating PR-39 levels 10 to 14 days post-infection.[73] Similarly, patients with bacterial pneumonia had plasma levels of HBD-2 that were elevated four-fold.[74] Experimentally induced infection of calves with *Cryptosporidium parvum* increased enteric β-defensin levels by 5-10 fold, as well as increasing the levels of other defensins.[75] Cows testing positive for infection with *Mycobacterium paratuberculosis* (the causative agent of Johne's disease) exhibited increased expression of β-defensins.[76] Similar up-regulation of defensins in the bronchial epithelium was experimentally induced by intratracheal instillation of *Pasteurella haemolytica* into a single lobe of a cow lung, where an increase in β-defensin expression in the airway epithelium correlated with the infection [53]. Similarly, intratracheal instillation of *P. aeruginosa* was sufficient to increase expression of mouse β-defensin 3 (mBD3) in the tracheal epithelium, as well as in the small bowel and liver [54]. In humans, inflamed intestinal epithelium exhibits very high (up to hundreds of μg/ml) levels of HBD-2 expression relative to normal colon [55], as does inflamed gingival epithelium [28].

Genetic manipulation experiments have supported the above evidence for a role for antimicrobial peptides in host defense, though these experiments are complicated by the numerous defensin genes often expressed in the same tissues, as well as redundant defense mechanisms within the innate immune system. The first example of such a study was performed in *Drosophila* as discussed above. Most recently, an innovative technique was devised to address the issue of multiple defensin genes. Rather than attempting to knock-out all 20 defensins expressed in the mouse small intestine, Wilson et al. identified the single enzyme necessary for processing the preprodefensins to the active mature form. Genetic inactivation of this single gene (matrilysin) prevented production of active defensin in the small intestine, and consequently led to a ten-fold increase in susceptibility to infection by virulent bacteria introduced by the oral route [56].

Platelets play a significant role in innate host defenses. One of the principal reasons for this appears to be because they possess antimicrobial peptides called platelet microbicidal peptides. In this regard, *Staphylococcus aureus* and *S. epidermidis* strains that are susceptible to these peptides have a reduced propensity to cause endocarditis in humans compared to peptide-resistant isolates [57].

Taken together, these results provide a compelling rationale for the importance of cationic antimicrobial peptides in the defenses of all complex eukaryotic organisms against infectious agents.

V. CATIONIC PEPTIDES CAN ACT IN SYNERGY WITH HOST MOLECULES

The innate immune response involves several different elements, including phagocytic cells such as neutrophils, macrophages and monocytes, and proteins such as complement, lysozyme, and cationic peptides. These elements have been designed to work in synergy. For example, complement activation can lead to bacteriolysis, but also serves to generate

opsonins on the surface of bacteria to enhance phagocytosis. Similarly, although cationic antimicrobial peptides have the ability to kill microbes independently, these activities can be improved in synergy with other factors. The ability of individual peptides to work synergistically with other peptides is well documented. This was first demonstrated with the frog peptides magainin and PGLa,[82], and has recently been confirmed with a variety of peptides, including the mammalian peptides protegrin 1 and indolicidin (Table 3). Study of the kinetics of interaction of peptides shows that they act cooperatively. Thus, we assume that two peptides can act together, resulting in positive cooperativity.

In addition, cationic peptides can work synergistically with lactoferrin, lysozyme and other proteins that are present in body fluids or tissues. For example, LL-37, which is co-localized in the secretory granules of neutrophils[52] and in the serous cells of submucosal glands of the lung,[55] also demonstrates synergy with lactoferrin *in vitro.*[55] Our own early studies with rabbit defensins led us to question whether peptides always acted on their own or in synergy with other host defense components.[17] The ability of rabbit defensins to permeabilize the outer membrane of *P. aeruginosa* was observed to increase as the pH was lowered (as would occur in the phagolysosome after ingestion of *P. aeruginosa* by neutrophils), even though the antibacterial activity of defensins is antagonized at low pH. Therefore, we considered the possibility that peptides actually worked in synergy with the protein lysozyme, a slightly basic enzyme that is excluded from its target, the peptidoglycan, by the outer membrane. Indeed it was demonstrated that peptides promote the ability of lysozyme to lyse Gram-negative bacteria, and that there is excellent synergy between lysozyme and a range of peptides against several bacteria (Table 4). Since lysozyme is present is most parts of the body, it is possible that such synergy is critical to the action of cationic antimicrobial peptides in their natural hosts. Indeed, in fish challenged with bacteria, both lysozyme and cationic peptides appear to be rapidly induced (Patrykzat et al., submitted).

The basis for synergy appears to be related to the mechanism by which cationic antimicrobial peptides interact with bacteria. In the case of Gram negative bacteria, the outer membrane protects the peptidoglycan from degradation, and consequent cell lysis, by lysozyme. Cationic peptides interact with and cross the outer membrane by the mechanism known as self promoted uptake.[83] Thus the initial interaction of the polycationic peptides is with the polyanionic LPS, at sites on the LPS where divalent cations usually bind to cross bridge adjacent LPS molecules and stabilize the outer membrane. The competitive displacement of these divalent cations (Mg^{++} and Ca^{++}) by the bulkier peptides leads to lesions in the outer membrane, permitting uptake of the permeabilizing polycationic peptide as well as other molecules, such as lysozyme. Thus the uptake of molecules such as lysozyme is promoted.

Since it is known that cationic peptides also show synergy with a diverse range of conventional antimicrobials,[26] one suspects that there will be other molecules, especially at sites of inflammation, that will show synergy with cationic peptides. Interestingly, antimicrobial peptides can show synergy with conventional antibiotics against not only Gram-negative[84] bacteria, but also Gram-positive bacteria[85] that do not have an outer membrane. Therefore there must be another action of cationic peptides that promotes synergy with other molecules and that is independent of outer membrane actions. We suspect this is a permeabilizing action on the cytoplasmic membrane. All cationic antimicrobial peptides, by virtue of

their possession of a hydrophobic domain, are able to interact with bacterial cytoplasmic membranes.[26] Although some feel that this explains the mechanism of killing by cationic peptides (i.e. through disruption of the integrity of membranes), we and others have observed that there is not a strong correlation between the ability of peptides to completely depolarize membranes and their ability to kill cells.[86] Nevertheless, we have observed that many peptides cause partial disruption of the permeability barrier of the cytoplasmic membrane at low concentrations and it is possible that such permeabilization of the cytoplasmic membrane also plays a role in synergy against bacteria. Other studies have observed synergy of cationic peptides with conventional antimicrobials against fungi and *Cryptosporidium*, so it seems possible that relevant synergy between cationic antimicrobial peptides and other host molecules also occurs for these infectious agents.

VI. CATIONIC PEPTIDES CAN MODULATE THE HOST INFLAMMATORY RESPONSE TO SEPSIS

Sepsis is associated with the presence of pathogenic microorganisms or their toxins in the blood. It can result from infections with either Gram negative or Gram positive bacteria. Gram negative sepsis is usually caused by the release of a bacterial outer membrane component, endotoxin (LPS). The mechanism by which LPS activates macrophages is now fairly well understood. Lipopolysaccharide binding protein (LBP), an acute phase reactant that is present in the blood, binds LPS, and transfers it to CD14, a protein that exists as a soluble form in blood and as a GPI-linked molecule on the surface of monocytes and macrophages.[87-90] LPS•CD14 complexes are thought to initiate intracellular signaling reactions by binding to Toll-like receptors (TLRs) on macrophages and other cells.[91-96] LPS•CD14 complexes activate the NFκB transcription factor as well as the ERK, JNK and p38 mitogen-activated protein kinases, all of which mediate the production of inflammatory cytokines.[97-100] The systemic release of cytokines, in particular the pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, has been proposed to lead to septic shock and death. Cationic peptides act on Gram-negative bacteria by initially binding to their surface polyanionic LPS. A variety of cationic antimicrobial peptides have been shown to have the ability to bind LPS.[61,84,101-110] A number of these cases these peptides have also been shown to suppress the ability of LPS to stimulate the production of pro-inflammatory cytokines by macrophages. Furthermore, studies with the cationic proteins bactericidal/permeability increasing protein (BPI), CAP18, CAP37 and lactoferrin have indicated that these molecules have an analogous ability to antagonize the ability of LPS to stimulate cytokine production in macrophages.[17,111-116] The cecropin-melittin hybrid peptides CEMA and CEME not only suppress cytokine production in response to endotoxic LPS, but also been shown to prevent lethal endotoxemia in the galactosamine-sensitized mouse model.[61] With CEMA treatment, it was shown that the LPS-stimulated induction of the important sepsis-mediating cytokine, TNF-α, could be dramatically suppressed in the blood of galactosamine-sensitized mice. Other animal model studies have demonstrated analogous findings with diverse cationic peptides.[103,104,117] CEMA and other peptides are also able to antagonize the cytokine-inducing activities of LPS in whole human blood.[118,119]

Recently we have shown that cationic peptides can inhibit the interaction of LPS with LBP, and this correlates with their

ability to inhibit LPS-induced production of TNF-α by macrophages (Figure 1).[120] However, the ability of the peptides to inhibit LPS•LBP interaction does not fully explain the anti-LPS effect of the peptides, especially since the peptides proved capable of blocking LPS•LBP complex formation, but could not dissociate pre-formed complexes after 15 min of interaction. Significantly, peptides can inhibit TNF-α production by macrophages that have been pre-incubated with LPS for an hour (Figure 2),[61] at which time the LPS should have been fully bound by LBP and should have already stimulated the regulatory cascade leading to activation of the NFκB transcription factor. This was further investigated by utilizing gene array technology to profile gene expression patterns in RAW 264.7 macrophages treated with LPS and CEMA. We found that CEMA selectively inhibited LPS-induced gene expression.[121] For example, while CEMA inhibited LPS-induced expression of 36 genes including those encoding pro-inflammatory molecules such as iNOS, IL-1β, and MIP-1α, it had little or no effect on the ability of LPS to induce the expression of 18 other genes, including c-rel, mdm-2, and ICAM-1 (intracellular adhesion molecule). In addition to selectively inhibiting LPS-induced gene expression, we found that CEMA itself induced the expression of a distinct set of 35 genes, including those coding for the LIM-1 transcription factor, the type I subunit of the transforming growth factor-β (TGF-β) receptor, and cell cycle inhibitors such as $p19^{ink4}$ (cdk4 and cdk 6 inhibitor) (Figure 3). Analogous results were achieved with the human α-helical peptide LL-37 (Scott and Hancock, ms. in preparation[119]). Thus while it is not yet proven, it seems possible that cationic antimicrobial peptides are able to modulate the toxic effects of endotoxin, both by preventing the binding of LPS to serum LBP and by acting directly on the cells that normally respond to LPS.

There have been other strong indicators that cationic peptides can interact directly with eukaryotic cells. Generally speaking, the interaction of most peptides with eukaryotic membranes is inhibited by the lack of negatively charged lipids on the surface of such cells, by the rather low membrane potential (-15mV) across the plasma membrane of eukaryotic cells and by the presence of cholesterol in the plasma membranes of such cells (cf. bacteria that have an abundance of anionic surface phospholipids, such as phosphatidyl glycerol and cardiolipin, and have a transmembrane potential of −140mV and no cholesterol). Thus there have been virtually no reports that reliably demonstrate a cytolytic or direct cytotoxic effect of antimicrobial peptides on host cells. However it is possible that peptides interact with the surface of host cells or even enter these cells. For example, it has been shown that peptide PR-39 rapidly enters human microvascular endothelial cells.[122] Conversely, it was found that two bovine antimicrobial peptides of the cathelicidin family, BMAP-27 and BMAP-29, permeabilized eukaryotic cell membranes, after interacting with negatively-charged sialyl residues at the membrane surface, and caused Ca^{2+} flux into the cytosol.[123] This could provide a potential mechanism as to how cationic peptides could alter macrophage signalling or gene expression. Alternatively it has been demonstrated that defensins are among the most potent protein kinase C inhibitors identified to date,[124] and inhibition of this enzyme would be expected to dramatically influence signalling responses in all mammalian cells. It is also known that some tumor cell lines can be killed by cationic peptides at relatively high concentrations. Such peptides are selectively more toxic towards tumour cells than towards non-malignant cells, although the mechanism of

their activity is not fully understood.[123,125-130] In some of these cases, the anti-tumor activity of the cationic peptides can be improved by acting in synergy with host molecules such as defensins, which can act synergistically with hydrogen peroxide (also release from neutrophils) to lyse erythro-leukemia targets.[127]

Gram-positive sepsis is also presumed to be due to the release of bacterial cell wall components. A number of Gram-positive cell wall constituents including lipoteichoic acid (LTA),[131] peptidoglycan,[132] *Streptococcus* rhamose-glucose polymers,[133] and *Staphylococcus* capsular polysaccharide[134] have been shown to stimulate the production of inflammatory mediators *in vitro*. When injected into animals, these Gram-positive cell wall components can elicit many of the characteristic features of septic shock including cytokine production, leukocytopenia, circulatory failure, multiple organ dysfunction syndrome (MODS) and mortality.[135-140] Peptidoglycan has also been shown to enhance the toxicity of endotoxin in animals.[135] The increasing incidence of Gram-positive-induced septic shock[141] indicates that there is a need to develop therapeutic strategies to prevent the activation of inflammatory cells by components of Gram-positive cell walls.

In addition to their ability to bind to LPS, cationic peptides were also shown to bind to the Gram-positive surface molecule LTA.[142] LTA, like LPS, has both a polyanionic and lipidic nature, and thus was able to interact with the cationic reporter molecule dansyl polymyxin (DPX) as well as to cationic peptides, although the kinetics of binding indicated a lower affinity for peptide binding to LTA compared to LPS.[142] LTA from different species of Gram-positive bacteria stimulated TNF-α and IL-6 production by the murine macrophage cell line RAW 264.7, and cationic peptides from several structural classes were able to significantly inhibit this production of cytokines.[142] The peptides were also effective in human blood at reducing the production of TNF-α in response to LTA, although the levels of inhibition were lower than observed in *in vitro* studies with RAW macrophage cells.[142]

Thus, the multifunctional activities of cationic peptides include the ability to bind LPS and LTA circulating in the blood or at the site of infection and the ability to reduce the host inflammatory response to Gram negative and Gram positive bacteria, that when unchecked can lead to the lethal condition, sepsis.

VII. CATIONIC PEPTIDES HAVE THE ABILITY TO RECRUIT INFLAMMATORY CELLS

Cell migration is an important host response to infection, since it is required to recruit the appropriate cells, particularly neutrophils, and lymphocytes, to the localized site of infection. It is controlled by a multistep process that includes chemoattraction, cell-cell adhesion and, in some cases, transmigration through cell layers.[143] Neutrophils have been demonstrated to be important for recruitment of monocytes to sites of infection and it has been hypothesized that factors derived from neutrophils were responsible.[144,145] CAP37, a cationic antimicrobial protein found in azurophil granules, is chemotactic for T cells, monocytes and neutrophils.[15,145,146] One study demonstrated that the instillation of CAP37 into rabbit airways resulted in significant monocyte infiltration in the lung.[147] CAP37 is also chemotactic for fibroblasts and can influence maturation of monocytes to macrophages.[148] LL-37, a human neutrophil α-helical peptide has been found to have chemotactic activity for T cells and neutrophils.[4] The porcine peptide, PR-39 has Ca^{++}-dependent chemotactic

activity for neutrophils that could be inhibited by pertussis toxin, but this peptide does not chemoattract mononuclear cells.[13] Similarly, guinea pig defensins are also chemotactic for neutrophils but not monocytes, while the human α-defensin peptides, HNP-1 and HNP-2, have been shown to have or to stimulate chemotactic activity towards murine and human T cells and monocytes.[14,146] Defensins have been shown to stimulate production of IL-8,[149] the chemoattractant and neutrophil activating cytokine, in airway epithelial cells, which would also potentially enhance neutrophil recruitment. Conversely, IL-8 can actually induce the release of defensins and CAP37 when applied in conjuntion with phorbol 12-myristate 13-acetate (PMA), and formylmethionyl-leucylphenylalanine (fMLP).[146]

Human β-defensins has also been shown to be chemotactic for immature dendritic cells and memory T cells through interaction with the receptor CCR6.[16] In addition, the proform of an antimicrobial peptide, proBac7, which is released from activated neutrophils, has been shown to be a monocyte-specific chemoattractant.[21] Such chemotactic activities appear to have *in vivo* relevance since it has been demonstrated that application of the human α-defensin HNP-1 reduced *Klebsiella pneumoniae* numbers in mouse model peritoneal infections, and this antibacterial activity was accompanied by an enhanced influx of macrophages, granulocytes and lymphocytes into the peritoneal cavity.[150] The activity appeared to be in part mediated by leukocyte accumulation since leukocytopenic mice administrated HNP-1 did not display antibacterial activity

Many of the cells involved in inflammatory responses come from the blood and pass through the endothelium of blood vessels. Transmigration across the blood vessel wall is stimulated by vasodilators like histamine. Thus an important component of neutrophil recruitment is the ability of peptides such as neutrophil defensins,[11] CAP-11[151] and magainin-2,[12] to stimulate the activation and degranulation of tissue mast cells leading to histamine release. Magainin-2 induced release of histamine from mast cells was shown to not be a cytolytic effect of the peptide, nor was it due to the formation of anion-selective channels in the membrane of mast cells.[12] Mast cells have high affinity $F_C\varepsilon$ receptors that allow them to bind IgE monomers. Thus the best studied known method of mast cell activation and degranulation is through cross-linkage of surface IgE with specific antigen (Ag). Defensins were found to act in a mechanistically different fashion from Ag-IgE and act on mast cells through a rapid G protein-dependent response that is inhibited by pertussis toxin and pre-treatment of mast cells with neuraminidase.[11] This is similar to the IgE-independent activation of mast cells by venom peptides (mast cell-degranulating peptides), neuroendocrine peptides such as substance P, vasoactive intestinal polypeptide, and somatostatin.[11, 152-155]

Once pro-inflammatory cells have found their way to the site of infection, they usually localize themselves through adherence to the tissues. Guinea pig defensins were shown to induce the aggregation of guinea pig neutrophils and increase the expression of the integrins ICAM-1 (CD54) as well as CD11b and CD11c on human neutrophils.[156] These latter receptors, CD11b and CD11c, contribute to the adhesion reaction of leukocytes via an interaction with CD54.[157, 158] Our own studies have demonstrated that the α-helical peptide, CEMA (cecropin-melittin hybrid), is able to upregulate transcription of the gene for ICAM-1 in murine macrophages after a 4 hour incubation with the peptide.[121] Thus it appears that cationic peptides could

contribute to the inflammatory response by attracting immune cells and enhancing their adhesion and transmigration through the endothelium.

In other studies, we also observed that rabbit neutrophil defensins were able to double the effectiveness of non-opsonic phagocytosis of *P. aeruginosa* by unelicited rabbit alveolar macrophages.[17] Other studies have indicated that defensins increase the cytotoxic activity of natural killer cells.[159] Conversely others have indicated that defensins actually reduce the efficiency of complement-mediated, but not antibody-mediated phagocytosis of model particles.[160] It was demonstrated that guinea pig defensins inhibited superoxide anion generation during phagocytosis of complement-opsonized particles and that although they inhibited complement-dependent phagocytosis, they did not inhibit binding of complement-opsonized particles to neutrophils.[156]

VIII.OTHER ACTIVITIES OF CATIONIC PEPTIDES IN THE IMMUNE SYSTEM

Apoptosis is the normal process of programmed cell death, whereby the body disposes of genetically damaged or unwanted cells. The ability to carry out apoptosis appears to be inherent to all cells, although the susceptibility to apoptosis varies considerably and is influenced by external and cell-autonomous events. The major possible role for apoptosis in infection would be in the enhanced death of infected host cells, thus decreasing the replication or survival of viruses or intracellular pathogens (e.g. parasites or *Chlamydia*) contained within these cells. In this regard it has been demonstrated that BMAP-27 and -28, a cecropin melittin hybrid, and the lactoferrin hydrolysis product, lactoferricin are capable of enhancing apoptosis in macrophage cell lines, transformed cell lines, fresh hematopoietic tumor cells, and *in vitro* activated human lymphocytes.[123,161]

Reactive oxygen intermediates generated by the phagocyte NADPH oxidase are critically important components of host defence. However, they are highly toxic and can cause significant tissue injury during inflammation. It is therefore important that their generation and inactivation are tightly regulated. Defensins have been shown to interfere with the activation of neutrophil superoxide-generating NADPH oxidase.[162; 163] In some cases this property of defensins may have a negative effect. For example, the implantation of foreign material into the body results in the production of oxygen free radicals by activated neutrophils,[164] which serves to protect the body from infection. Defensins appear to down-regulate the ability of the neutrophils to generate superoxide, and consequently inhibit the host defense at the site of the foreign implant.[160]

PR-39 has also been shown to inhibit NADPH oxidase activity, which it does by blocking the assembly of this enzyme through interactions with Src homology 3 domains of the p^{47phox} cytosolic oxidase component.[165] This indicates that PR-39 might also play a role in limiting excessive tissue damage during inflammation. PR-39 has also been shown to block reactive oxygen production by cultured endothelial cells and isolated, perfused rat lungs.[166] The reactive oxygen species produced by neutrophils are thought to contribute to the pathogenesis of ischemia re-perfusion of a variety of organs. It has been demonstrated that PR-39 blocks postischemic oxidant production and venular protein leakage in rat mesenteries subjected to ischemia-reperfusion.[167]

Another important property of cationic peptides that may allow them to modulate the immune response is the inability to inhibit certain proteases that are able to otherwise cause injury to epithelial and

endothelial cells as well as the degradation of the extracellular matrix.[168-173] For example histatins, histidine-rich peptides found in human saliva, inhibit the proprotein convertases (serine endoproteases belonging to the kexin/subtilisin family) furin and PC7,[174] while defensins can inactivate serpins.[20] A cysteine proteinase thought to contribute to tissue injury in inflammation, cathepsin L, was inhibited *in vitro* by the neutrophil propeptide, proBac5.[21] One other study demonstrating that cationic peptides may serve to down-regulate the inflammatory response was that of Schluesener et al.,[175] who demonstrated that indolicidin, and to a lesser extent bactenecin, were selectively cytotoxic to T lymphocytes suggesting that they might limit clonal expansion of T lymphocytes during ongoing immune responses. They also showed that the effect was selective to these peptides as several defensins and cecropin P1 did not affect T lymphocyte viability or proliferation. One study demonstrated that interactions between defensins and serine proteases released from neutrophils could regulate neutrophil-mediated injury at sites of inflammation.[176] For example, defensins were found to inhibit serine protease-induced detachment of lung epithelial cells. Conversely elastase and cathepsin G both reduced IL-8 induction by defensins without affecting TNF-α-induced synthesis of IL-8, however, there was no inhibitory effect of cathepsin G on the antibacterial activity of defensin.[176] The observed increased expression of cell adhesion molecules that is induced by defensins on neutrophils if extrapolated to epithelial cells would provide a possible mechanism for resistance to elastase or cathepsin G-induced detachment of epithelial cells. Cell adhesion molecules play an important role in the attachment of epithelial cells to extracellular matrix components (ECM). Another possible mechanism for inhibition of cell detachment by elastase would be through inhibition of protein kinase C as demonstrated by defensins,[124] since elastase-induced synthesis of IL-8 by lung epithelial cells has been shown to be partly mediated by protein kinase C,[177] indicating that elastase may act by affecting signal transduction pathways involving protein kinase C. It should be noted that defensins can be inhibited by serum proteinase inhibitors such as α_1-proteinase inhibitor (α_1-PI)[2,0] which could also serve to limit the activity of defensins. These proteinase inhibitors also serve to limit the activity of elastase and cathepsin G.

Cationic peptides have been suggested to stimulate wound healing, through promotion of re-epithelialization of damaged surfaces. This may be related in part to their mitogenic (growth promoting) activity towards epithelial cells and fibroblasts, observed with defensins at concentrations expected to be present *in vivo* during the wound healing process.[18] Defensins have also been shown to increase DNA synthesis of the leukemic cell line HL-60.[178] Proliferative effects of α-helical peptides were seen with murine fibroblast cells (SB-37) and preimplantation embryos.[179] In addition to this activity, PR-39 has been demonstrated to induce the synthesis of syndecans (major cell surface heparan and chondroitin sulfate proteoglycans), in a manner similar to that seen during wound repair.[19] Syndecans are required for cellular responses to heparin-binding growth factors and extracellular matrix components. PR-39, which was isolated from fluid accumulating in cutaneous wounds undergoing repair, was found to specifically induce the expression of syndecan-1 and syndecan-4 mRNA in cultured fibroblasts and epithelia.[19] The induction of syndecans could also contribute to the antimicrobial activity of PR-39 as bacteria bind heparin sulfate and killing may be facilitated by immobilization of this molecule on the host cell surface. The

induction of syndecan-1 by PR-39 was also found to decrease the invasiveness of human hepatocellular carcinoma cells (hepatocellular carcinomas have previously been shown to have reduced syndecan-1 expression and high metastatic potential) [165]. Conversely the above-mentioned activities in cell recruitment, if applied to epithelial cells and fibroblasts, e.g CAP37 chemoattraction of fibroblasts,[126] would be a component of the wound healing process.

The classical pathway of complement activation involves the complement components C1, C4, and C2 in the generation of C3/C5 convertase. C1 is composed of C1q, C1r, and C1s and is able to initiate activation of the classical pathway. Defensins have also been shown to interact with activated C1, C1q, and C1-inhibitor.[181; 182] A subsequent study then found defensins (HNP-1) to actually inhibit activation of the classical complement pathway by inhibition of C1q haemolytic pathway.[183] This may be yet another facet of cationic peptides' ability to modulate the inflammatory response, and points to a potentially complex intervention in inflammation.

IX. CATIONIC PEPTIDES AS THERAPEUTIC AGENTS

There is no question that, with the increasing antibiotic resistance problem, there is a need to develop new classes of antibiotics. Cationic antimicrobial peptides have many of the desirable features of a novel antibiotic class.[184] In particular they have a broad spectrum of activity, kill bacterial rapidly, are unaffected by classical antibiotic resistance mutations, do not easily select antibiotic resistant variants, show synergy with classical antibiotics (Table 4), neutralize endotoxin and are active in animal models. Despite this, many issues remain to be solved. For example, these peptides have relatively high molecular weights compared to most antibiotics, and may have to be produced recombinantly to keep prices down. While several processes for doing this have been described, to our knowledge they have not yet been successfully performed on an industrial scale. Another issue is toxicity. Some cationic antimicrobial peptides are very toxic for mammalian cells (e.g. wasp venom mastoparan), while others show little or no acute cytotoxicity. However, more subtle toxicities have not been studied, although we assume, based on the presence of natural peptides in vivo at concentrations of e.g. 44 μg/ml in the saliva of an individual with peritonitis, that they can be tolerated at high levels. Another issue would be their lability to proteases in the body. In this regard, there are strategies for protecting the peptides from proteases, including liposomal incorporation or chemical modification.

With this in mind there are two very promising clinical trials underway. The protegrin derivative IB-367 (Intrabiotics Inc., CA) is being examined for its potential against oral mucosaitis, a polymicrobial ulcerative disease of cancer patients. The peptide MBI-226 (Micrologix Biotech. Inc, Vancouver, Canada) is being investigated for sterilizing catheter insertion sites, thus preventing serious infections due to colonization of such catheters by skin bacteria. Results from clinical trials to date have indicated efficacy and MBI-226 have been given fast track status by the FDA. Thus cationic antimicrobial peptides are not only important components of the innate defences of all animals against infections, but synthetic variants thereof hold great potential as a weapon against antibiotic resistant bacteria. The great sequence and structural diversity offered by peptides (i.e. 20 possible amino acids in each position) will provide many possibilities for drug design.

X. CONCLUSIONS

It is becoming clear that cationic antimicrobial peptides are an important and significant component of host defenses against infection. Many such peptides are encoded by mammals and are inducible under specific conditions that reflect infection, utilizing signal transduction pathways that are conserved in many eukaryotes and are also used in induction of other innate defenses. The importance of such peptides in defense against infections is attested to by experiments utilizing transgenic animals engineered to produce reduced or elevated amounts of peptides, and by the ability of exogenously-added peptides to protect against infections and sepsis. While direct antimicrobial action is an obvious effect of such peptides on infectious agents, the peptides appear to be involved in the orchestration of many aspects of innate immunity and the inflammatory response. Thus peptides are an integral and important component of early host defenses against infection.

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TABLES AND FIGURES

TABLE 1
Examples of the Primary Amino Acid Sequences of Cationic Antimicrobial Peptides

Peptide	Structural class	Sequence[a]
Rabbit α-Defensin (NP-1)	**β-sheet**	**VVC$_1$AC$_2$RRALC$_3$LPRERRAGFC$_3$RIRGRIHLC$_2$C$_1$RR**
Human β-Defensin 1	β-sheet	DHYNC$_1$VSSGQC$_2$LYSAC$_3$PIFTKIQGTC$_2$YRGKAKC$_1$C$_3$K
Human β-Defensin 2	β-sheet	MRVLYLLFSFLFIFLMPLPGVFGGIGDPVTC$_1$LKSGAIC$_2$ HPVFC$_3$PRRYKQIGTC$_2$GLPGTKC$_1$C$_3$KKP
Human NP-1 (α-defensin)	β-sheet	AC$_1$YC$_2$RIPAC$_3$IAGERRYGTC$_3$IYQGRLWAFC$_2$C$_1$
Pig Protegrin 1	β-sheet	Rggrlc$_1$yc$_2$rrrfc$_2$vc$_1$vgr*
Human LL-37	A-helical	LLGDFFrkskekigkefkrivqrikdflrnlvprtes*
Human Histatin 5	A-helical	DShakrhhgykrkfhekhhshrgy*
Pig Cecropin P1	α-helical	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR
Cattle Indolicidin	Extended	ILPWKWPWWPWRR*
Pig PR39	Extended	RRRprppylprprpppffpprlpprippgfpprfpprfp*
Cattle Bactenecin	**loop**	**RLC$_1$RIVVIRVC$_1$R**

[a] One letter amino acid code with the following additions: Positively charged residues at neutral pH are bolded; brackets represent amino acids that are cyclized; superscript d represents the D-enantiomer, all other amino acids are L-form; the subscript numbers represent amino acids that are joined by cysteine disulphides. The star at the end of a peptide implies that the peptide is known to be amidated at its carboxy terminus.

TABLE 2
Protection Against Infection in Mice by Exogenously Added or Transgenically Expressed Cationic Antimicrobial Peptides

Peptide[a] (Ref.)	Microbial infection	Animal model[b]	Improvement in survival (peptide administered)
Pig protegrin-1 [65]	Methicillin resistant *Staphylococcus aureus* (MRSA)	IP/IP	0 → 100% (0.5mg/kg)
	MRSA	IV/IV	33 → 93% (5mg/kg)
	Pseudomonas aeruginosa	IP/IP	27 → 100% (0.5mg/kg)
	Vancomycin resistant *Enterococcus faecalis*	IV/IV	33 → 87% (2.5mg/kg)
Cow indolicidin [35] in liposomal formulation	*Asperigillus fumigatus*[c]	IV/IV	0 → 30% (40mg/kg)
Human neutrophil defensin 1 [131]	*Klebsiella pneumonia*	IP/IV	2-log reduction in bacterial counts (0.02 mg/kg)
Human LL-37, expressed from transgene [44]	*Escherichia coli*	IP/TG	10 → 75%
	Endotoxic LPS	IP/TG	0 → 85%
Human LL-37[d]	Endotoxic LPS	IP/IP	0 → 66% (8mg/kg)

[a] Reference is provided as a superscript.
[b] Route of administration of the infectious agent/Route of adminsitration of the peptide; IP means intraperitoneal, IV means intravenous; TG means by delivery of an adenovirus encapsulated transgene.
[c] This organism is a fungus, all others described are bacteria.
[d] Yan Hong, M.G. Scott, and R.E.W. Hancock, unpublished data.

TABLE 3
Synergy Between the Cationic Peptides Pig Protegrin 1 and Cow Indolicidin

	Minimal inhibitory concentration (μg/ml)			
	E. coli		*P. aeruginosa*	
Peptide	**Peptide alone[a]**	**Peptides in combination[b]**	**Peptide alone[a]**	**Peptides in combination[b]**
Protegrin	4	0.5	8	2
Indolicidin	16	2	64	4

[a] Peptide alone: the MIC of the peptide against *E. coli* or *P.aeruginosa*.

[b] Performed by checkerboard titration assays in which one peptide was diluted in one dimension (i.e., the rows of a microtitre plate) and the other peptide was diluted in the other dminesion (i.e., the columns of a microtitre plate), prior to addition of bacteria and 18 hours of incubation. Thus we demonstrate the lowest concentrations (μg/ml) of the two peptides, which synergistically killed bacteria when the peptides were added together.

TABLE 4

Synergy Between the Cationic Peptides and Lysozyme[a]

	Lowest minimal inhibitory concentrations in combination (*ug*/ml)			
	E. coli		*P. aeruginosa*	
Peptide	**Peptide**	**Lysozyme**	**Peptide**	**Lysozyme**
CP-1	0.04	7.8	0.65	7.8
CP-3	0.04	15.6	1.3	15.6

[a] Lowest concentrations (μg/ml) showing synergy. Usual MICs are 1-4 μg/ml for cationic peptides and >1 mg/ml for lysozyme

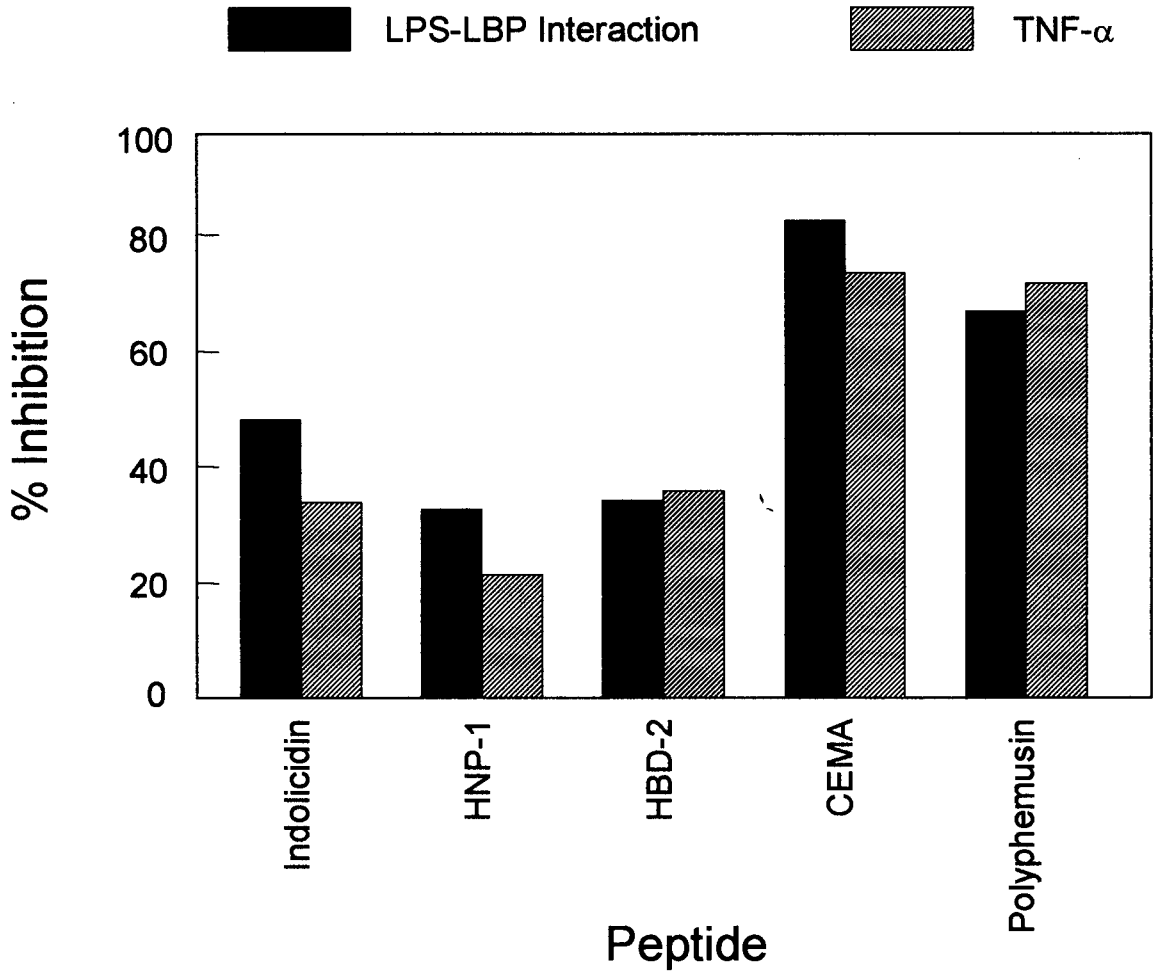

FIGURE 1. Inhibition of the LPS-LBP interaction and LPS-induced TNF-α production by structurally different cationic peptides. For measurement of LPS-LBP interaction, biotinylated LPS (45 ng/ml) was added to wells with immobilized LBP in the presence or absence of the indicated cationic peptides (10 μg/ml). The peptides were added to the wells at the same time as the LPS, and residual LPS binding was assessed by ELISA. For measurement of TNF-α inhibition, RAW 264.7 cells were incubated with *E. coli* O55:B5 LPS (100 ng/ml) in the presence or absence of the indicated peptides (20 μg/ml) for 6 hours. TNF-α released into the culture supernatant was measured by ELISA [97]. The data presented is taken from Scott et al [97] or from the authors' unpublished studies. The peptides are cow indolicidin, human neutrophil peptide 1 (HNP-1), human beta defensin 2 (HBD-2), synthetic variant cecropin-melittin hybrid CEMA and horseshoe crab polyphemusin.

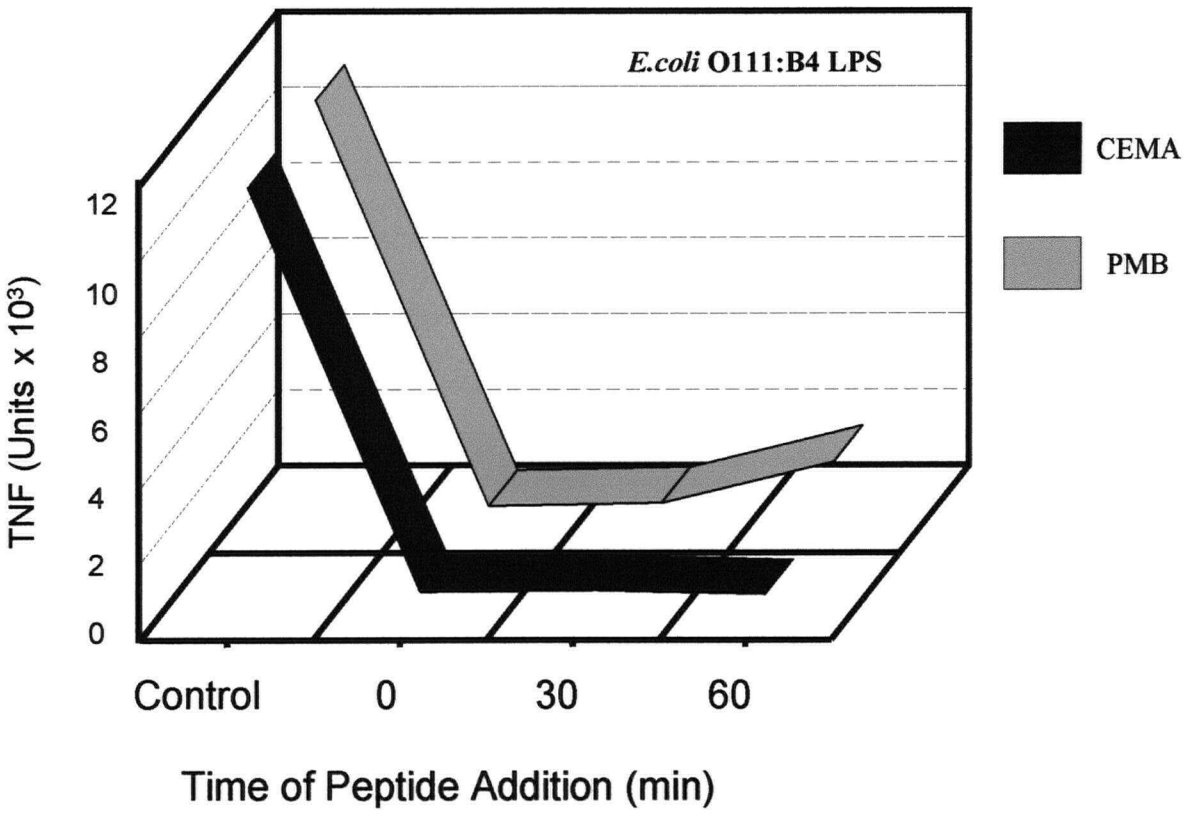

FIGURE 2. Suppression by CEMA and polymyxin B of *E.coli* 0111:B4 LPS-induced TNF-α in the RAW macrophage cell line. CEMA (5.52 μM) or 10.74 μM polymyxin B were added at the same time as LPS, 30 minutes after and 60 minutes post LPS addition to a mouse macrophage cell line in tissue culture [37]. Results are presented as mean of TNF-α levels in the macrophage supernatant.

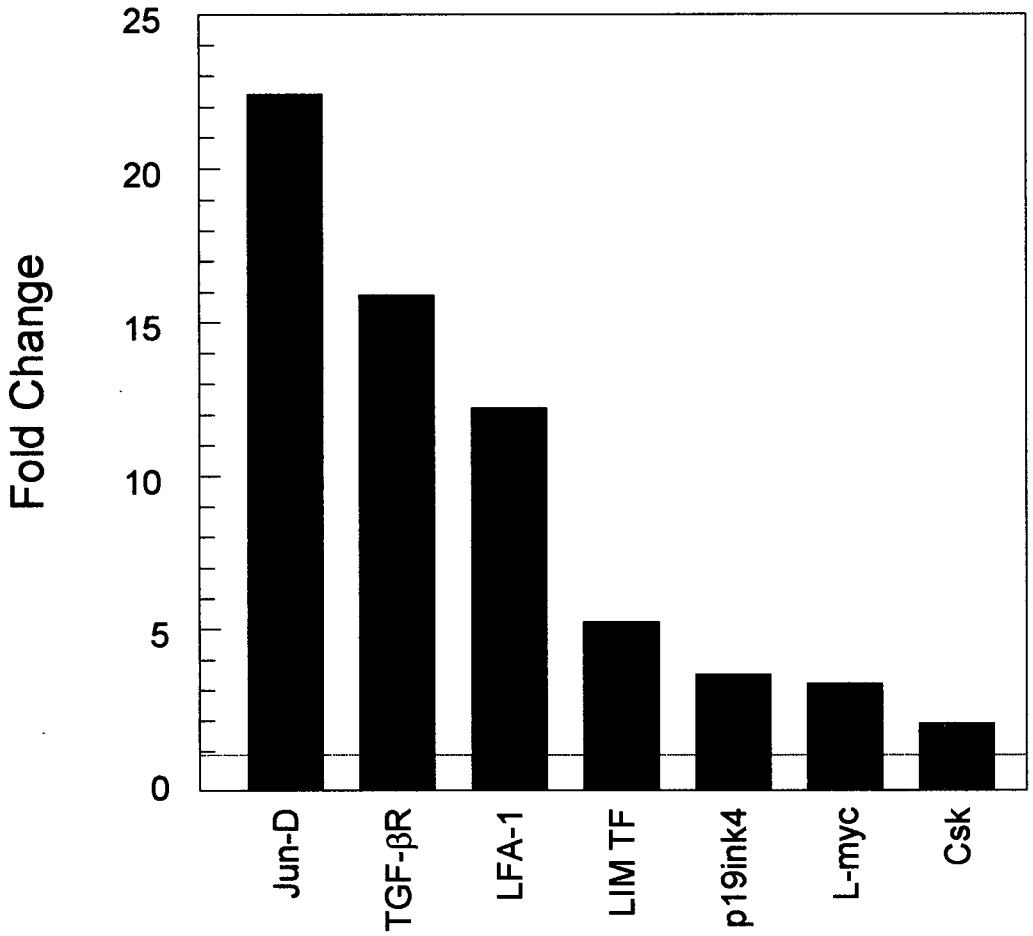

FIGURE 3: Cationic peptides upregulate the expression of RAW macrophage genes as studied by gene array ananlysis. RAW 264.7 cells were stimulated for 4 hours with media alone or 50 μg/ml CEMA. The RNA was isolated from the cells with Trizol, DNase treated and used to make ^{32}P labeled cDNA probes, which were hybridized to the Clontech Atlas arrays. After a 3-day exposure, they were analyzed using a phosphoimager and Clontech Atlas software. The results shown are a subset of the 35 induced genes and are presented as the fold change in macrophage gene transcription in cells treated with peptide compared to untreated cells. The results are taken from Scott et al, 2000 [98]. The genes include those encoding the transcription factor JunD, the type I subunit of the transforming growth factor-β receptor(TGF-βR), the cell adhesion molecule LFA-1, LIM-1 transcription factor (LIM-1TF), the cell cycle inhibitors $p19^{ink4}$ (cdk4 and cdk 6 inhibitor), the proto-oncogene L-myc, and the Src kinase Csk. The dashed line indicates no alteration in transcriptional levels as seen for the 6 control housekeeping genes.

APPENDIX B: Lipopolysaccharide, lipoteichoic acid, and CpG DNA elicit pro-inflammatory gene expression responses in macrophages

B.1 Introduction

The mechanism by which the bacterial products stimulate macrophages is only partially understood. Recently, the bacterial products LPS, LTA, and CpG DNA have been shown to stimulate macrophages by signaling through Toll-like receptors (TLR). LPS and LTA signals via TLR4 (Ozinsky et al. 2000) while CpG DNA activates macrophages via TLR9 (Hemmi et al. 2000; Bauer et al. 2001). TLRs signal via activation of NF-κB and MAP kinases, which in turn regulate the production of inflammatory cytokines. To gain a more complete understanding of how bacterial products such as LPS, LTA and CpG DNA activate macrophages, gene array analysis was performed to profile gene expression patterns in RAW 264.7 macrophages treated with the bacterial products. The availability of gene arrays allows for a global snapshot of gene expression changes caused by the activation of macrophages by bacterial products. The effect of these bacterial products on macrophage gene transcription was confirmed by Northern and Western blotting as well as by comparing their ability to stimulate the production of inflammatory mediators.

B.2 Bacterial products stimulate overlapping and distinct patterns of gene expression in RAW 264.7 cells

RNA was isolated from RAW 264.7 cells that had been incubated for 4 h with medium alone, 100 ng/ml *Salmonella* Typhimurium LPS, 1 μg/ml *S. aureus* LTA, or 1 μM CpG DNA. The RNA was used to generate labelled cDNA probes that were hybridized to Clontech Atlas gene array filters. The hybridization of the radio-labelled cDNA probes to each immobilized DNA was visualized by autoradiography and quantified using a phosphorimager. Representative

autoradiographic images of the gene arrays are shown in Figure B1.

It was found that LPS treatment of RAW 264.7 cells resulted in increased expression of more than 60 genes (eg. Table B1 and Table B2). These included genes encoding pro-inflammatory proteins such as IL-1β, inducible nitric oxide synthase (iNOS), MIP-1α, MIP-1β, MIP-2α, and a variety of transcription factors. The changes in gene expression induced by LPS, LTA, and CpG DNA were compared, and it was found that all three of these bacterial products increased the expression of pro-inflammatory genes such as those encoding iNOS, MIP-1α, MIP-2α, IL-1β, IL-15, TNFR1 and NF-κB to a similar extent (Table B1). Table B1 includes 19 genes that were up-regulated by the bacterial products to a similar extent (their ratios differed by less than 1.5 fold between the bacterial products). There were also at least 3 genes that were down-regulated by LPS, LTA and CpG to similar extents. It was discovered that there were a number of genes whose expression was altered to different degrees by the three bacterial products (Table B2). Table B2 includes many of the genes whose expression was different by more than around two fold between one or more bacterial products. The use of a 2-fold change cutoff for differentially expressed genes was somewhat arbitrary and does not necessarily indicate biological significance. However, some of the differences were quite dramatic, eg. cylin A1, cyclin G2 and thrombopoietin.

LTA appeared to have the largest subset of genes that were changed more than with LPS or CpG. Some of these genes included Jun-D, protease nexin-1, Elk-1 and cyclins G2 and A1. There were only a few genes whose expression was changed more by LPS or CpG. These results indicate that although LPS, LTA, and CpG DNA stimulate largely overlapping gene expression responses, they also exhibit differential abilities to regulate certain genes. Thus, LPS, LTA, and

Table B1: Genes expressed to similar extents after stimulation by the bacterial products LPS, LTA, and CpG DNA

Gene/protein	Unstimulated intensity	Ratio of stimulated : unstimulated[a]			Accession number	Gene[b]
		LPS	LTA	CpG		
IL-1β	20	82	80	55	M15131	F4k
tristetraprolin	20	77	64	90	M57422	B4k
MIP-2α	20	73	77	78	X53798	F3g
MIP-1β	188	50	48	58	M35590	F3f
ICE	20	49	57	50	L28095	F7a
iNOS	20	37	38	45	M87039	C3m
TGFβ	20	34	40	28	X57413	F4g
c-rel oncogene	20	20	21	15	X15842	A2m
MIP-1α	489	19	20	26	X12531	F3e
IL-15	20	14	15	12	U14332	F5a
TNFR1	580	10	13	11	M59378	C5d
TRAIL	151	6	6	6	U37522	C5c
NF-κB	172	3.8	3.5	3.4	M57999	D5g
I-κB (alpha subunit)	402	3.2	3.5	2.7	U36277	B3m
MAPKAP-2	194	3	3.8	2.5	X76850	B5n
Stat 1	858	2.4	3	3.2	U06924	B4d
CD18	592	2	2	2	X14951	E5n
NF-2	543	1.9	2.4	2.8	X60671	A1f
CD14	5970	1.6	2	1.4	M34510	E6h
c-Fms	4455	0.5	0.7	0.5	X68932	A4b
DNA polymerase	352	0.5	0.6	0.6	Z21848	C6b
B-myb	614	0.4	0.6	0.5	X70472	A2f

Total RNA was isolated from unstimulated RAW macrophage cells and cells treated for 4 h with 100 ng/ml *Salmonella* LPS, 1 μg/ml *S. aureus* LTA or 1 μM CpG DNA. After reverse transcription, ^{32}P-labelled cDNA was used to probe Clontech Atlas gene array filters. Hybridization was analyzed using Atlas Image (Clontech) software. The array experiments were repeated 2-3 times with different RNA preparations and yielded very similar results; the average fold changes are shown above. The actual changes in the normalized hybridization intensities of the housekeeping genes ranged from 0.8-1.2 fold, validating the use of these genes for normalization. When the normalized hybridization intensity for a given cDNA was less than 20, it was assigned a value of 20 (Der et al. 1998) to calculate the ratios and relative expression levels.

[a] The ratio was calculated by dividing the intensities of hybridization spots for cells treated with 100 *Salmonella* ng/ml LPS, 1μg/ml *S. aureus* LTA or 1 μM CpG by the intensities for unstimulated cells.

[b] The gene classes (given by the first letter of the gene name) include Class A: oncogenes, tumour suppressors and cell cycle regulators; Class B: stress response, ion channels, transport, modulators, effectors and intracellular transducers; Class C: apoptosis, DNA synthesis and repair; Class D: transcription factors and DNA-binding proteins; Class E: receptors (growth, chemokine, interleukin, interferon, hormone, neurotransmitter), cell surface antigens and cell adhesion; Class F: cell-cell communication (growth factors, cytokines, chemokines, interleukins, interferons, hormones), cytoskeleton, motility, and protein turnover.

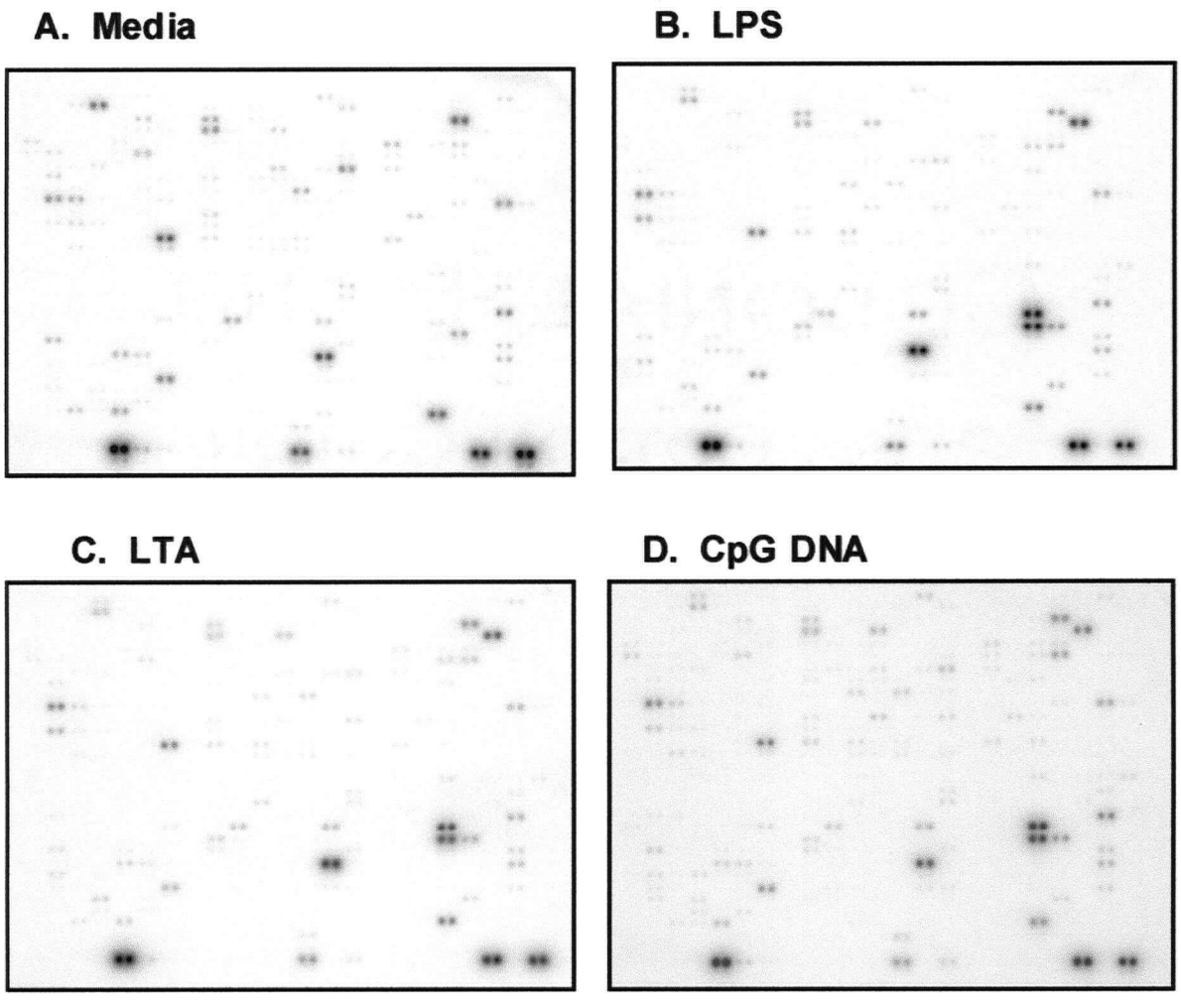

Figure B1: Effect of LPS, LTA and CpG DNA on gene expression in RAW 264.7 cells

RAW 264.7 cells were stimulated with (A) media alone for 4 hours, (B) 100 ng/ml *Salmonella* LPS (C) 1 μg/ml *S. aureus* LTA, or (D) 1 μM CpG DNA. The RNA was isolated from the cells with Trizol, DNase treated and reverse transcribed to make ^{32}P-labeled cDNA probes, which were hybridized to the Clontech Atlas arrays. After a 3-day exposure, they were analyzed using a phosphoimager and Clontech Atlas software. These data are representative of 2 to 3 experiments.

Table B2: Genes that were differentially regulated by the bacterial products LPS, LTA, and CpG DNA

Gene/protein	Unstimulated intensity	Ratio of stimulated : unstimulated			Accession number	Gene
		LPS	LTA	CpG		
hepatocyte growth factor	**20**	**1.0**	**23**	**1.0**	**X72307**	**F2e**
hepatoma transmembrane kinase ligand	20	1.0	21	1.0	L38847	F2f
thrombopoietin	393	0.3	3	0.5	L34169	F4e
Nur77	289	1	4	3	J04113	C4d
H-ras proto-oncogene	20	7	21	5	Z50013	A5c
Cyclin A1	20	4	12	2	X84311	A6b
Cyclin G2	20	5	14	2	U95826	A6l
Elk-1	123	2	4	1	X87257	A3a
Jun-D	20	18	39	20	J05205	A3g
Fas 1 receptor	20	71	80	42	M83649	C3f
ICAM-1	20	17	23	9	X52264	E7i
Leukemia inhibitory factor (LIF)	20	55	59	102	X06381	F3d
CACCC Box-binding protein BKLF	20	38	7	7	U36340	D1j
interferon inducible protein 1	**272**	**10**	**4**	**4**	**U19119**	**D4k**

See Table B1 for details of how these experiments were performed.

CpG DNA appear to induce overlapping yet distinct patterns of gene expression.

B.3 Confirmation of selected gene array results

To confirm and assess the functional significance of the transcriptional changes in RAW 264.7 cells that were stimulated with LPS, LTA and CpG DNA, the levels of selected mRNAs and proteins were assessed and quantified by densitometry (Table B3). Northern blots using CD14, vimentin, and tristetraprolin-specific probes confirmed similar expression after stimulation with all 3 bacterial products. In these experiments, RAW 264.7 cells were incubated with the bacterial products or media alone for 4 h, the RNA was collected and Northern blots were performed. Densitometry was done on the Northern blots and the data was normalized to GAPDH. The Northern blot results demonstrated that LPS, LTA and CpG DNA up-regulated CD14 mRNA levels to a similar extent (2.2, 1.8 and 1.5 fold respectively), as observed in the gene arrays (1.6, 2, and 1.4). Interestingly, although both LPS and LTA are known ligands of CD14, CpG-TLR signaling appears to occur independently of CD14 (Bauer et al. 2001). Tristetraprolin, which is involved in TNF-α mRNA stabilization (Carballo et al. 1998), was up-regulated to similar extents by the bacterial products as seen with both the gene arrays and Northern blots.

The up-regulation of leukemia inhibitory factor (LIF) by CpG DNA in the gene array analysis was also selected to confirm whether the levels of the corresponding proteins exhibited the same changes. LIF, which belongs to the IL-6 family of cytokines, can have both anti-inflammatory and pro-inflammatory properties (Gadient and Patterson 1999). It is commonly used to maintain embryonic cell lines by suppressing differentiation. The protein expression levels of LIF were examined by Western blot analysis and confirmed the preferential stimulation of LIF (a member of the IL-6 family of cytokines) by CpG. The gene array studies demonstrated

Table B3: Confirmation of array data with bacterial products

	Relative levels			
Bacterial product	**Untreated**	**LPS**	**LTA**	**CpG**
CD14[a] (mRNA)	**1.0**	**2.2 ± 0.4**	**1.8 ± 0.2**	**1.5 ± 0.3**
Vimentin[a] (mRNA)	1.0	1.2 ± 0.07	1.5 ± 0.05	1.3 ± 0.07
Tristetraprolin[a] (mRNA)	1.0	5.5 ± 0.5	5.5 ± 1.5	9.5 ± 1.5
LIF[b] (protein)	**1.0**	**2.8 ± 1.2**	**2.7 ± 0.6**	**5.1 ± 1.6**

[a] Total RNA was isolated from unstimulated RAW macrophage cells and cells treated for 4 hr with 100 ng/ml *Salmonella* LPS, 1 μg/ml *S. aureus* LTA, 1 μM CpG DNA or media alone and Northern blots were performed the membrane was probed for GAPDH, CD14, vimentin, and tristetraprolin as described previously. The hybridization intensities of the Northern blots were compared to GAPDH to look for inconsistencies in loading. These experiments were repeated at least three times and the data shown is the average relative levels of each condition compared to media (as measured by densitometry) ± standard error.

[b] RAW 264.7 cells were stimulated with 100 ng/ml *Salmonella* LPS, 1 μg/ml *S. aureus* LTA, 1 μM CpG DNA or media alone for 24 hours. Protein lysates were prepared, run on SDS PAGE gels and western blots were performed to detect LIF (R&D Systems). These experiments were repeated at least three times and the data shown is the relative levels of LIF compared to media (as measured by densitometry) ± standard error.

that CpG DNA up-regulated LIF over unstimulated cells by 102 fold, whereas LPS and LTA up-regulated it by only 59 and 55 fold. The immunoblots demonstrated that LPS and LTA resulted in a 2.8 and 2.7 fold increase over media alone levels whereas CpG DNA treatment of RAW cells resulted in a 5.1 fold increase. The relative change of CpG to LPS stimulations was thus 1.9 on the gene arrays and 1.8 on the immunoblots. The relative change of CpG to LTA was 1.7 on the gene arrays and 1.9 on the immunoblots. This demonstrates that gene expression changes on the gene array could be confirmed in part at the protein level. Other confirmatory experiments demonstrated that LPS up-regulated the expression of expression of MIP-2α (data not shown), and IL-1β (Figure B2) mRNA and down-regulation of DP-1 (data not shown) and cyclin D (Figure 28) mRNA as assessed by Northern blot analysis. Thus the gene array data was validated by confirming the expression changes of a number of macrophage genes and gene expression changes < 2-fold could be confirmed [ie. CD14 and cyclin D1 (Figure 21)].

B.4 Bacterial products stimulate the production of inflammatory mediators

The effect of the bacterial products, LPS, LTA and CpG DNA on macrophage stimulation was studied. ELISAs were performed on culture supernatants from RAW 264.7 cells stimulated with bacterial products to determine whether LPS, LTA, and CpG DNA increased the secretion of various cytokines. Consistent with the array findings, it was demonstrated that levels of the pro-inflammatory cytokine IL-1β secreted into the medium were significantly increased by LPS, LTA, and CpG DNA, with LPS being the most potent, followed by LTA and then CpG DNA (Figure B2). The levels of NO were quantified in the culture supernatant of the macrophage cells stimulated with LPS, LTA and CpG DNA for 24 hours by measuring the accumulation of the stable NO metabolite nitrite with the Griess reagent. The gene iNOS (inducible NO synthase)

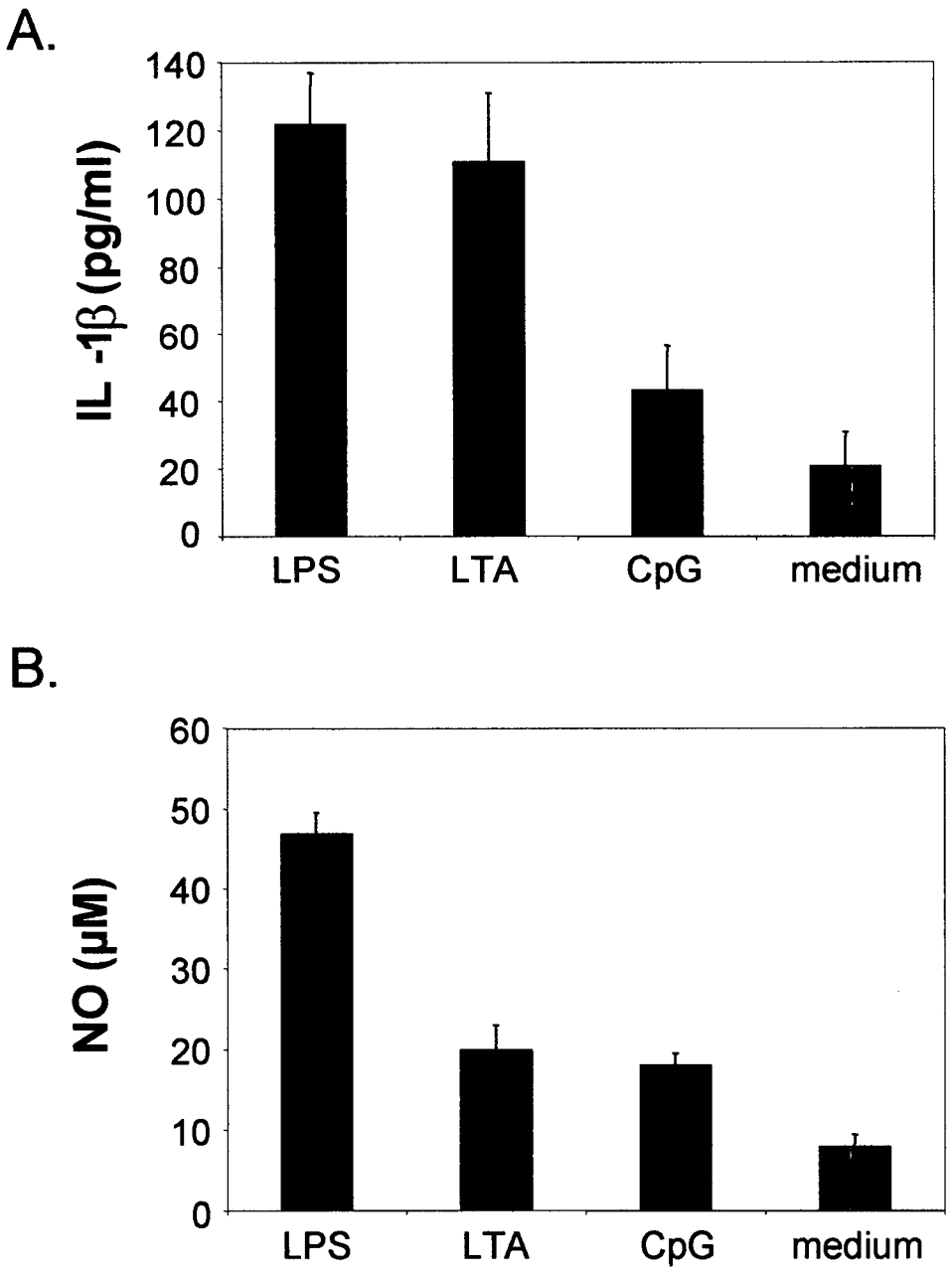

Figure B2: Induction of cytokine production in RAW 264.7 cells by LPS, LTA, and CpG DNA

RAW 264.7 cells were stimulated with 100 ng/ml *Salmonella* LPS, 1 μg/ml *S. aureus* LTA, 1 μM CpG DNA or medium alone in phenol-red free DMEM + 10% fetal bovine serum for 24 hours. The supernatant was removed and tested for (A) IL-1β by ELISA, and (B) the amount of NO formed in the supernatant as estimated from the accumulation of the stable NO metabolite nitrite with the Griess reagent. The data represent the average of three experiments ± standard error.

encodes the enzyme responsible for inducing the inflammatory mediator, NO. Again, all three bacterial products stimulated the production of NO, with LPS being the most potent, followed by LTA and CpG (Figure B2). The bacterial products also stimulated pro-inflammatory cytokine production in whole human blood (Figure B3), a clinically relevant ex vivo system. It was found that *E. coli* LPS (eLPS), *S. typhimuirum* LPS (sLPS), and *S. aureus* LTA all stimulated similar amounts of serum TNF-α, IL-8, and IL-6. CpG DNA also stimulated production of these cytokines, albeit to lower levels.

B.5 Summary

It was of interest to better understand the mechanism by which bacterial products stimulated macrophages. Gene arrays were used to profile global changes in gene expression in RAW 264.7 cells treated with bacterial products (LPS, LTA and CpG DNA). It was found that a large panel of genes, largely encoding pro-inflammatory products, was similarly affected by all three of the bacterial products tested. We observed here that the three bacterial products tested, LPS, LTA and CpG DNA, had a similar ability to stimulate the expression of a number of pro-inflammatory genes despite their acting through different TLRs. There were also a number of genes from diverse categories, whose expression was altered to varying degrees by the bacterial products. Although it was difficult to come to any conclusions, these studies show that bacterial products that signal through different TLRs induce similar gene expression changes in macrophages as well as a smaller, unique subset of gene expression changes. The gene array hybridization studies performed here have permitted a more comprehensive analysis of both conserved and distinct host gene expression responses to diverse bacterial components, a subset of which were confirmed by other methods. The ability of these bacterial products to elicit similar pro-

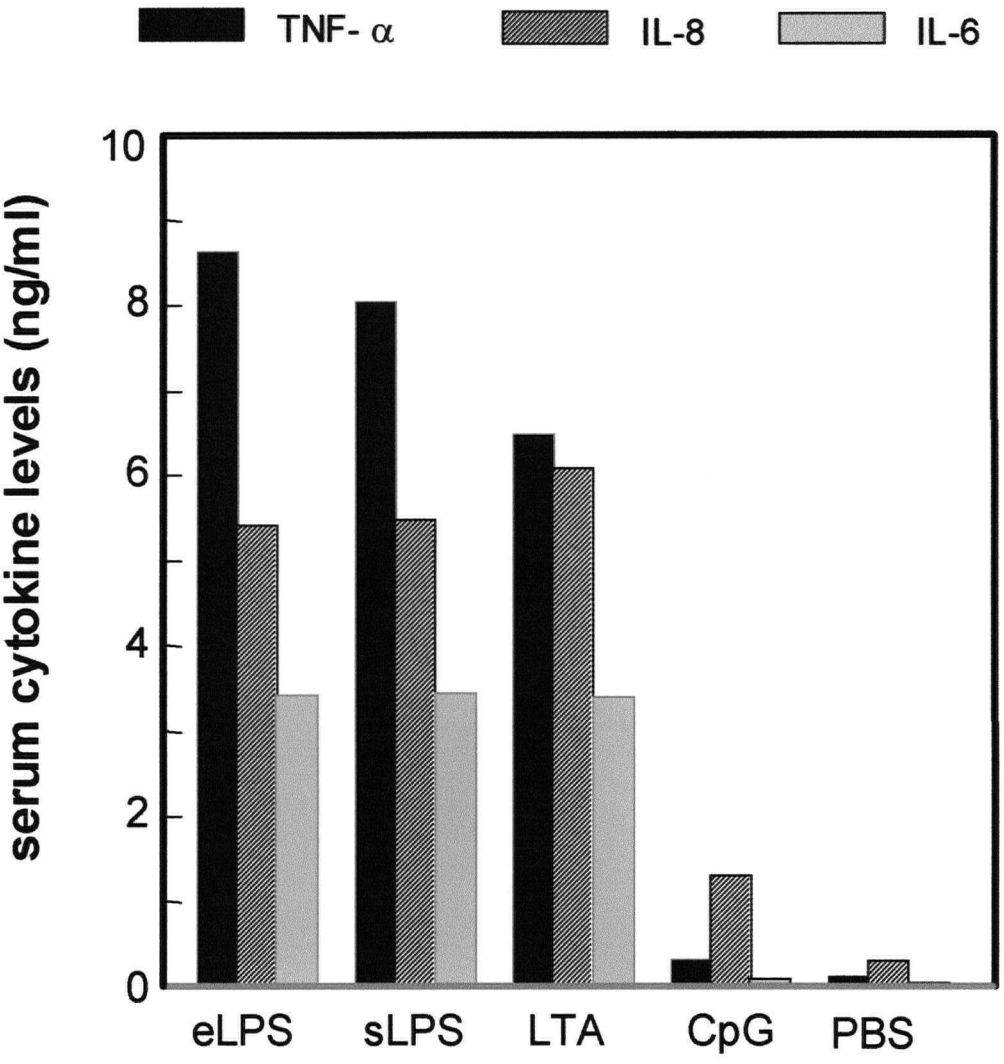

Figure B3: Induction of cytokine production in human whole blood by LPS, LTA, and CpG DNA

Human whole blood was stimulated with 100 ng/ml *Salmonella* LPS, 1 μg/ml *S. aureus* LTA, 1 μM CpG DNA or PBS for 6 hours. The levels of cytokine in the serum were measure by ELISA. The data represent the average of 2-3 donors.

inflammatory responses allows us to hypothesize that a general innate immune response is mounted against the bacterium as a whole, as well as more specific response that may be more suited to clearance of a particular bacterium involving specialized responses to specific bacterial products.

APPENDIX C: Publications arising from this graduate work

Peer-reviewed journals

2002 **Scott, M.G.**, Davidson, D.J., M.R. Gold, and R.E.W. Hancock. 2002. The human antimicrobial peptide, LL-37, is a multi-functional modulator of innate immune responses. J. Immunol. submitted

contribution: all of the experiments except the animal studies which were conducted with the expertise of Dr. Donald Davidson

2002 **Scott, M.G.**, M. Bains, C.M. Rosenberger, M.R. Gold, and R.E.W. Hancock. 2002. Lipopolysaccharide, lipoteichoic acid, and CpG DNA elicit pro-inflammatory gene expression responses in macrophages. submitted.

contribution: executed array and confirmation studies with the help of C.M. Rosenberger on the arrays and M. Bains did some of the western blots

2002 Morck, D.W., S.P. Holland, H. Ceri, R.E.W. Hancock, **M.G. Scott,** V. Nugyen, and E.J. Keith. 2002. Polymyxin and the Prevention of Diffuse Lameller Keratitis. submitted.

contribution: conducted studies with *B. cepacia* LPS and determined its ability to stimulate RAW 264.7 cells and inhibition by polymyxin

2000 **Scott, M.G.** and R.E.W. Hancock. 2000. Cationic Antimicrobial Peptides and their Multifunctional Role in the Immune System. Crit. Rev Immunol. 20(5): 407-31.

2000 Zhang, L., **M.G. Scott,** H. Yan, L.D. Mayer, and R.E.W. Hancock. 2000. Interaction of polyphemusin I and its structural analogs with bacterial membranes, lipopolysaccharide and lipid monolayers. Biochem. 39(47): 14504-14.

contribution: anti-endotoxin studies with polyphemusin

2000 **Scott, M.G.,** C.M. Rosenberger, M.R. Gold, and R.E.W. Hancock. 2000. An α-helical cationic antimicrobial peptides selectively modulates macrophage responses to LPS and directly alters macrophage gene expression. J Immunol. 165(6): 3358-65.

contribution: conducted all of the experiments with the help of C.M. Rosenberger on the arrays and northern blots

2000 Rosenberger, C.M., **M.G. Scott,** M.R. Gold, R.E.W. Hancock, and B.B. Finlay. 2000. Salmonella typhimurium Infection and Lipopolysaccharide Stimulation Induce Similar Changes in Macrophage Gene Expression. J. Immunol. 164(11): 5894-5904.

contribution: helped with the arrays, Northern blots and ELISAs

2000 Hancock, R.E.W. and **M.G. Scott**. 2000. Antimicrobial Peptides in Animal Defenses. Proc. Natl. Acad. Sci. 97(16): 8856-61.

2000 **Scott, M.G.**, A.C.E. Vreugdenhil, W.A. Buurman, R.E.W. Hancock, and M.R. Gold. 2000. Cutting edge: cationic antimicrobial peptides block the binding of LPS to LPS-Binding Protein (LBP). J Immunol. 164(2): 549-53.

contribution: performed all the experiments and the reagents and expertise were provided by A.C.E. Vreugdenhil and W.A. Buurman

1999 Hancock, R.E.W., **M.G. Scott**, C.Friedrich, L.Zhang, H.Yan, P.Nair, A.Patrzykat, and A.Rozek. 1999. Cationic peptide antibiotics for use in treatment of Pseudomonas aeruginosa infections of cystic fibrosis patients. Clin. Microbiol. Infect. 5:S52-S53.

contribution: performed antimicrobial studies

1999 **Scott, M.G.**, M.R. Gold, and R.E.W. Hancock. 1999. Interaction of Cationic Peptides with Lipoteichoic Acid and Gram-Positive Bacteria. Infect. Immun. 67: 6445-6453.

contribution: conducted all of the experiments

1999 Friedrich, C., **M.G. Scott**, D.N. Karunaratne, H. Yan, and R.E.W. Hancock. 1999. Salt-resistant alpha-helical cationic antimicrobial peptides. Antimicrob Agents Chemother. 43: 1542-8.

contribution: conducted the killing assays, some antimicrobial studies and some permeabilization experiments

Patents

1996 Novel cationic peptide antimicrobials and methods for screening. Hancock, R.E.W., T. Falla and **M. Gough**. US provisional application 60/002,687, filed August 23, 1995. CIP serial no 08/702,054, filed August 23, 1996.

1996 Novel cationic peptide antimicrobials and methods for screening. Hancock, R.E.W., T. Falla and **M. Gough**. PCT patent WO97/08199, filed August 23, 1996.

1999 Anti-endotoxic, antimicrobial peptides. R.E.W.Hancock, **M. Gough**, A. Patrykzat, X. Jia, and D. Woods, US provisional patent filed August 27, 1998; Full US patent application 09/143, 124 filed Aug 27, 1999

1999 Anti-endotoxic, antimicrobial peptides. R.E.W.Hancock, **M. Gough**, A. Patrykzat, X. Jia, and D. Woods, PCT patent PCT/US99/19646 filed Aug 27, 1999.

2001 Effectors of Innate Immunity. R.E.W. Hancock, B.B. Finlay, **M.G. Scott**, D. Bowdish, C.M. Rosenberger, J.P. Powers U.S. provisional patent No 60/336,632, filed Dec. 3, 2001.

Oral Presentations

1999 Cationic Antimicrobial Peptides Inhibit Bacterial Product Stimulation of Macrophages. Canadian Society for Immunology, Canada.

1999 Biological Properties of Structurally Related α-helical Cationic Antimicrobial Peptides. Eukaryotic Antibiotic Peptides, Spain.

1999 Comparison of Structurally Related Peptides in their Ability to Neutralize the Biological Activity of LPS. The University of Maastricht.

2000 Use of Gene Arrays to Study Host Pathogen Interactions. The Centre for Microbial Diseases and Host Defense Research, University of British Columbia.

2000 The Role of Cationic Antimicrobial Peptides in the Immune Response to Bacterial Infection. University of British Columbia

2001 Antimicrobial peptides cause transcriptional up-regulation of multiple genes in macrophages. Gordon Conference: Antimicrobial Peptides, USA.

Abstracts

2002 The human antimicrobial peptide, LL-37, is a multi-functional modulator of innate immune responses. **Scott, M.G.**, Davidson, D. J., Gold, M. R.., Speert, D. P. and Hancock, R. E. W. Keystone Symposia on Innate Immunity.

2001 Evidence for the multi functional role of the human cationic peptide, LL-37 in the immune response to bacterial infection. **Scott, M.G**. D.J. Davidson, M. Bains, K.C. Crookhall, D.P. Speert and R.E.W. Hancock. North American Cystic Fibrosis Conference.

2001 Cationic antimicrobial peptides directly activate macrophages and are effectors of innate immunity. **Scott, M.G.**, C.M. Rosenberger, M.R. Gold, B. Brett Finlay, and R.E.W. Hancock. The Centre for Microbial Diseases and Host Defense Research.

2001 Expression profiling of macrophage responses to *Salmonella typhimurium*, lipopolysaccharide, and interferon-γ using gene arrays. Rosenberger, C.M., **M.G. Scott**, M.R. Gold, R.E.W. Hancock, and B. Brett Finlay. Macrophage Activation and Deactivation: Links between innate and acquired immunity.

2000 Bacterial product stimulation of macrophages is inhibited by cationic antimicrobial peptides. **Scott, M.G.**, A.C.E. Vreugdenhil, C.M. Rosenberger, B. Brett Finlay, W. A. Buurman, M.R. Gold, and R.E.W. Hancock. International Endotoxin Society Meeting.

2000 Polymyxin B prevention of bacterial endotoxin-induced diffuse lamellar keratitis in a rabbit model. Morck, D.W., S.P. Holland, H. Ceri, R.E.W. Hancock, **M.G. Scott**, M.R. Walter, and V. Nguyen. ASM Biofilms.

1999 Cationic antimicrobial peptides inhibit bacterial product stimulation of macrophages. **Scott, M.G.**, C.M. Rosenberger, B. Brett Finlay, R.E.W. Hancock and M.R. Gold. Canadian Society for Immunology.

1999 Bacterial product stimulation of macrophages is inhibited by cationic antimicrobial peptides. **Scott, M.G.**, C.M. Rosenberger, B. Brett Finlay, R.E.W. Hancock and M.R. Gold. Cold Spring Harbor: Microbial Pathogensis and Host Response.

1999 Identification of murine macrophage genes induced by *S. typhimurium* infection using gene arrays. Rosenberger, C.M., **M.G. Scott**, M.R. Gold, R.E.W. Hancock, and B. Brett Finlay. Cold Spring Harbor: Microbial Pathogensis and Host Response.