# *IN VIVO* EFFICACY OF NOVEL ANTIBACTERIAL AND IMMUNOMODULATORY SYNTHETIC PEPTIDES

by

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## ABSTRACT

Despite the success of modern medicine in treating infections, infectious diseases remain a major source of morbidity and mortality worldwide. The evolution of antibiotic resistant strains of bacteria means that new innovations in therapeutics must be pursued to combat this emerging threat. A novel approach is to utilize the anti-infective properties of endogenous host defence peptides by creating smaller synthetic peptides with enhanced protective activities. Some of these peptides directly kill bacteria and many display varied immunomodulatory activities, enhancing the host innate immune response to more effectively clear an infection. Here I examined the efficacy of several synthetic peptides in a murine model of invasive bacterial infection, induced by the Gram positive bacterium *Staphylococcus aureus*. Several peptides were able to significantly reduce peritoneal bacterial load *in vivo* by up to 4-logs relative to the controls, either through direct antibacterial killing or immunomodulatory activity. The latter class was studied in more detail; in particular, the peptides IDR-1 and 1002 displayed significant immunomodulatory effects *in vivo*. Both peptides were able to significantly induce the proinflammatory chemokines MCP-1, RANTES and KC, as well as increased recruitment of neutrophils and monocytes to the site of infection. These effects were not dependent on live bacteria, as heat inactivated *S. aureus* was also able to induce chemokines and cell migration. Mice that had been depleted of macrophages did not respond to peptide treatment, indicating that macrophages are an important effector cells through which immunomodulatory peptides counter infections. These results suggest that synthetic peptides have the potential to become a viable treatment option for bacterial infections.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AF647 | Alexa Fluor 647 |
| CBA | Cytometric Bead Array |
| CFU | Colony Forming Units |
| CRAMP | Cathelin-Related Antimicrobial Peptide |
| DC | Dendritic Cell |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ERK | Extracellular signal-regulated kinase |
| FACS | Fluorescence Activated Cell Scanner |
| FCS | Fetal Calf Serum |
| FITC | Fluoroscein Isothiocyanate |
| fPRL-1 | Formyl Peptide Receptor-Like 1 |
| GRO-α | Growth Regulated Oncogene |
| HIV | Human Immunodeficiency Virus |
| IDR-1 | Innate Defence Regulator |
| IFN | Interferon |
| IL- | Interleukin |
| IP | Intraperitoneal |
| IV | Intravenous |
| JNK | Jun N-terminal Kinase |
| KC | CXCL-1 |
| LPS | Lipopolysaccharide |
| LRR | Leucine Rich Repeat |
| MAPK | Mitogen Activated Protein Kinase |
| MCP-1 | Monocyte Chemoattractant Protein |
| MH | Mueller-Hinton |
| MIC | Minimum Inhibitory Concentration |
| MX-226 | Omiganan |
| MyD88 | Myeloid Differentiation Primary Response Gene 88 |
| NF-κB | Nuclear Factor-κB |

| | |
|---|---|
| NOD | Nucleotide Binding-Oligomerization Domain |
| PBMC | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate Buffered Saline |
| PBS | Phosphate Buffered Saline |
| PE | Phycoerythrin |
| QSAR | Quantitative structure-activity relationship |
| RANTES | Regulated upon Activation, Normal T-cell Expressed, and Secreted |
| SARM | Sterile Alpha and TIR Motif Containing |
| TAK | TGF-β Activated Kinase |
| TIR | Toll/IL-1 Receptor |
| TIRAP | Toll-Interleukin 1 Receptor Domain-Containing Adaptor Protein |
| TIRP | TIR containing protein |
| TLR | Toll-Like Receptor |
| TMB | 3, 3', 5, 5'-tetramethylbenzidine |
| TNF-α | Tumor Necrosis Factor |
| TRIF | TIR-domain-containing adapter-inducing interferon-β |
| VRE | Vancomycin Resistant Enterococci |

# CHAPTER 1: GENERAL INTRODUCTION

## 1.1. Antibiotic Resistant Bacteria and the Need for New Therapeutics

Infectious diseases remain a major source of morbidity and mortality in the developing world, causing a third of deaths worldwide. Diseases such as tuberculosis, cholera, HIV, influenza and others account for millions of deaths annually in developing regions of the world, such as Africa, the Middle East, India and Southeast Asia (World Health Organization). While the prevalence of fatal infectious agents is less in developed countries, infectious diseases remain a significant financial burden due to their healthcare costs (Boutayeb, 2006). Clearly, infectious diseases are a major global health concern that requires a concerted effort by the research community to develop new ways to treat emergent pathogens.

One of modern medicine's most important weapons against infectious diseases has been the use of antibiotics. One of the earliest and most successful antibiotics was Penicillin, a β-lactam isolated from the yeast *Penicillium*. Discovered in 1928 by Alexander Fleming, penicillin was originally claimed to be a miracle drug that could cure a wide range of bacterial diseases. Its manufacture and distribution helped change the course of history, as the use of penicillin in World War II helped soldiers recover from normally deadly infections caused by injuries on the battlefield (DuBose, Inaba et al. 2008).

Over the decades, several new structural families of antibiotics have been discovered and marketed. Sulfa drugs, imidazoles, tetracyclines, and fluoroquinolones were created or isolated from various organisms. Chemists were also able to alter the molecular structure of the antibiotics, creating new compounds with increased activity

and decreased toxicity. Most antibiotics work by inhibiting key enzymes that are required for bacterial homeostasis, DNA replication, or protein translation. Penicillin, for example, primarily inhibits enzymes involved in cell wall synthesis (Tipper 1979); however several other targets have also been identified (Zapun, Contreras-Martel et al. 2008).

Early researchers and clinicians observed that not all bacteria could be treated with antibiotics. Fleming himself, in his Nobel Prize acceptance speech, commented on the ease of producing penicillin resistant strains of bacteria in the laboratory (Fleming 1945). In 1947, doctors observed the first clinical infection caused by a penicillin-resistant *Staphylococcus aureus* (Barber and Rozwadowska-Dowzenko 1948). Resistance to antibiotics has increased over time with the excessive and/or improper use due to poor prescribing practices and failure of patients to complete treatment courses. In recent years, some bacterial strains have developed resistance to multiple types of antibiotics, making it extremely difficult to treat these infections.

Resistance mechanisms differ depending on both the antibiotic and strain of bacteria in question. One common mechanism is simply the alteration of the drug target. For example, *Mycobacterium tuberculosis* became resistant to the drug Rifampin through alteration of its RNA polymerase, preventing the binding of the drug to this enzyme (Morris, Bai et al. 1995). Conversely, penicillin-resistant *Staphylococcus aureus* can possess β-lactam binding proteins which competitively bind β-lactam antibiotics, preventing them from interacting with their target (Davies 1994). Some bacteria also possess efflux pumps that can non-specifically remove several types of antibiotics from the cell with high efficiency (Levy 2002)

Presently, there are several multiple antibiotic resistant strains of bacteria that are commonplace in the developed world. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a common cause of nosocomial infection, and can be responsible for severe blood infections and toxic shock. Bacteria resistant to the "last resort" antibiotic, the Vancomycin-resistant Enterococci (VREs) are also a common cause of hospital acquired infections. Multiresistant *Streptococcus pneumoniae* is also becoming more prevalent and causes many serious diseases, including pneumonia, meningitis and bacteremia (Tomasz 1994).

In the third world, the situation is even more dire, as infectious diseases thought to be declining are re-emerging. Though a large proportion of the world is infected with *Mycobacterium tuberculosis* (as much as a third by some estimates,(Tiruviluamala and Reichman 2002), infections were treatable with a long term drug cocktail. Now, multidrug resistant tuberculosis is on the rise in the developing world, rendering the already slow drug treatments ineffective.

### 1.2. Immunomodulation as a Novel Anti-Infective Treatment

The rise of antibiotic resistant bacteria means that new drugs are required to combat infections; however, research and product development in these areas has all but stopped. Many pharmaceutical companies do not consider the research and development of new antibiotics to be cost effective. Most estimates state that the time to develop a drug from discovery to marketing takes 15 years and 800 million dollars (DiMasi, Hansen et al. 2003). Indeed, the last novel class of antibiotic to be developed was the lipopeptide daptomycin in 2003; however, resistance was being observed as little as 2

years after it was made available (Hayden, Rezai et al. 2004). Thus, there is an enormous demand for new anti-infective therapies, but little research is being devoted to this area.

Researchers around the globe are investigating new mechanisms needed to combat antibiotic resistant bacteria while reducing or eliminating the chances that resistance can develop. One approach in development is the use of genetically modified bacteriophages to infect and kill bacteria while leaving host cells unharmed. This approach is beneficial as phages have evolved very potent and ancient mechanisms to infect and kill bacteria, meaning that the development of resistance is reduced, especially when cocktails of phages are employed. Unfortunately, phages usually only have a narrow spectrum of activity, and thus the infectious agent would need to be identified before phage therapy can be administered. Also, phages can be recognized by the host immune system and an antibody response can be mounted against them, potentially neutralizing their ability to bind and infect bacteria (Hermoso, García et al. 2007).

Another approach is the augmentation of the hosts' own immune system. Mammalian organisms have evolved numerous mechanisms to combat infectious pathogens, so it may be possible to develop compounds that augment immunity. There are a few immunostimulators available, but these tend to only stimulate a certain aspect of immunity. For example, the product Levamisole strongly induces type I interferon production, giving it activity against viral infections (Johnkoski JA 1996). Other naturally occurring compounds (such as Echinacea) may function as immunomodulators but there is limited data regarding their effects on the immune system (Woelkart, Marth et al. 2006). Echinacea has also been observed to induce strong allergic reactions in rare cases, which makes it a poor therapeutic choice (Huntley, Thompson Coon et al. 2005). Our lab

has taken the approach of using novel peptides, based on endogenous Host Defence Peptides, as a means to stimulate the immune system to defend against a bacterial infection.

Using peptides to stimulate immunity to combat infections is advantageous over traditional antibiotics for several reasons: A) by stimulating the multitude of weapons that have been utilized by multicellular organisms for millions of years, it is extremely unlikely that resistance can be developed, although there is some concern that this approach could select for bacteria with an intrinsic ability to evade immunity (Hornef, Wick et al. 2002); B) peptides would constitute a non-specific drug, and can theoretically be used to treat fungal, parasitic and perhaps even viral infections; C) immunomodulatory peptides can be combined with traditional antibiotic therapy to further enhance bacterial killing (Arora, Nadkarni et al. 2006); and D) some peptides display anti-inflammatory activity while upregulating other beneficial aspects of immunity, limiting the chance of developing the negative aspects of inflammation (endotoxic shock, for example) (Scott, Davidson et al. 2002); (Finlay and Hancock 2004).

### 1.3. Innate Immunity and Host Defence Peptides

The innate immune system is an ancient, evolutionarilzy conserved mechanism of host defence against microbial invaders. All multicellular organisms possess host defence mechanics, although there is significant divergence in the proteins involved in innate immunity, indicating that genes involved in host defence are important for survival and under constant evolutionary pressure (Mushegian and Medzhitov 2001). By contrast,

adaptive immunity is a relatively new mechanism, and has only been observed in vertebrate organisms.

Innate immunity relies on a variety of receptors that recognize highly conserved microbial structures (Apostolopoulos and McKenzie 2001) to identify invaders. The most heavily researched innate receptors in recent years have been the Toll-Like Receptors (TLRs) (Akira, Uematsu et al. 2006), although other families of receptors exist, such as the cytosolic nucleotide binding-oligomerization domain (NOD) receptors (Rietdijk, Burwell et al. 2008). There are 10 TLRs currently found in humans with a wide variety of expression profiles, which depends on the cell type in question. Six TLRs are extracellular, while four are found intracellularly in vesicular membranes (TLR-3, -7, -8 and -9). TLRs share similar structural homology, consisting of: an extracellular domain which contains the Leucine Rich Repeat (LRR) subdomain, which interacts with the ligand (Bell, Botos et al. 2005); a transmembrane domain that embeds into the plasma membrane; and the intracellular Toll/IL-1 Receptor (TIR) domain that recruits adaptor molecules that also have a TIR domain (Watters, Kenny et al. 2007). Most TLRs require a homodimer structure in order to function, however TLR-2 forms heterodimers with TLR-1 or TLR-6. The composition of the heterodimer changes the ligand specificity (Triantafilou, Gamper et al. 2006).

Once a receptor binds its ligand, a conformational change takes place that activates the intracellular TIR domain (Gay and Gangloff 2008), which then recruits TIR-adaptor molecules, of which 5 are currently known: myeloid differentiation primary response gene 88 (MyD88); toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP); TIR-domain-containing adapter-inducing interferon-β (TRIF); TIR containing

protein (TIRP); and sterile alpha and TIR motif containing (SARM). The TIR-adaptor molecule then recruits and phosphorylates IL-1 receptor associated kinase-4 (IRAK-4) through interaction of death domains found on both molecules. IRAK-1 is then phosphorylated, which in turn recruits tumor necrosis factor receptor (TNFR) associated factor (TRAF-6), as well as several other proteins, including tumor growth factor (TGF-β)-activated kinase (TAK-1). This complex then interacts with and phosphorylates the IκB-kinase (IKK) complex, which phosphorylates IκB (and is also ubiquitinated and subsequently degraded), an inhibitor of NF-κB. NF-κB then enters the nucleus, where it transcribes several genes involved in the inflammatory response, including cytokines and enzymes that synthesize bactericidal molecules (Kawai and Akira 2007). Refer to Figure 1 for a diagram of TLR signaling.

The above paragraph presents a simplified version of the TLR-4→ NF-κB pathway, however there is a significant amount of redundancy in the pathway. For example, a second TLR-4 pathway exists which does not require MyD88 but instead uses TRIF as an adaptor (Yamamoto, Sato et al. 2003). The MyD88-independent pathway culminates in the activation of interferon regulatory factor-3 (IRF-3), and the production of type I interferons, as well as a delayed NF-κB response (Fitzgerald, Rowe et al. 2003). In addition, the NF-κB pathway branches out to other signaling pathways, such as TAK-1 that interacts with the jun N-terminal kinase (JNK) mitogen activated protein kinase (MAPK) pathway, which is involved in a variety of maintenance and homeostatic functions (Salojin K 2007).

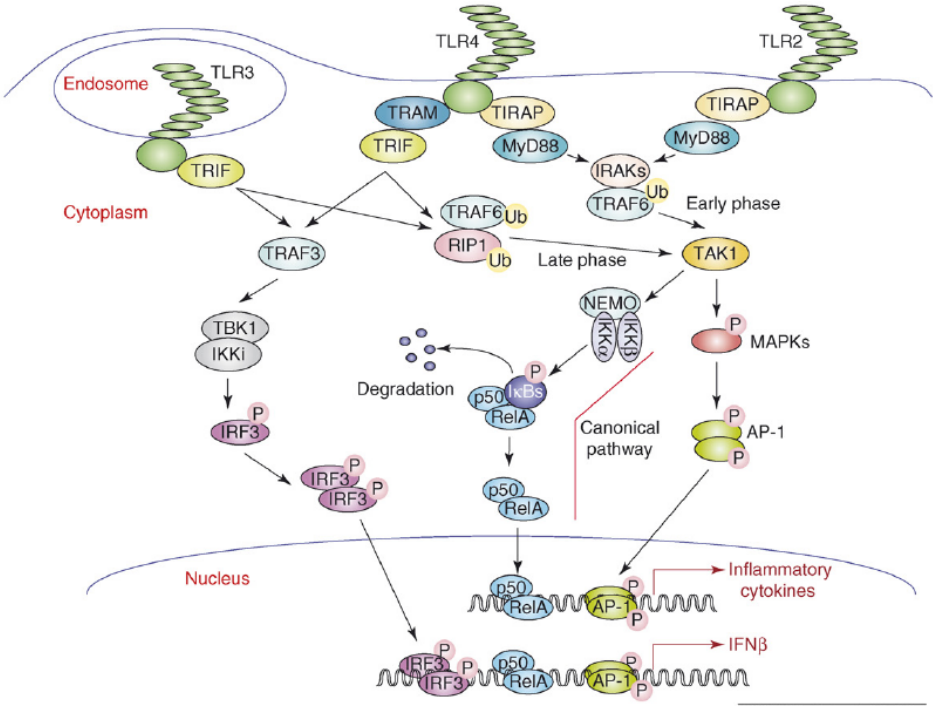


Figure 1**:** Diagram of TLR→NF-κB signaling. (Kawai and Akira 2007)

Following recognition of a pathogen, cells initiate the inflammatory response, which aids the host in combating the pathogen. When a pathogen is detected, cells release chemokines, which attract leukocytes to the site of infection (Alon and Feigelson 2002), where they phagocytose invading bacteria, and release protective molecules such as reactive oxygen/nitrogen radicals, lysozyme, and host defence peptides (Bogdan, Röllinghoff et al. 2000).

Cationic host defence peptides are an evolutionary ancient mechanism of bacterial defence. These peptides are found in virtually all multicellular organisms, and while peptides possess a significant degree of sequence and structural diversity between species, most peptides have a net positive charge and a large number of hydrophobic residues (Powers and Hancock 2003). Stored in granules in epithelial cells, neutrophils

and mast cells, these peptides were initially characterized by their ability to kill invading bacteria (Gudmundsson and Agerberth 1999). In mammals, there are two classes of peptides: defensins, of which there are numerous variants present in the human genome (Ganz 2003), and cathelicidins, of which there is only one known human member, hCAP18, also known as LL-37 (Dürr, Sudheendra et al. 2006).

Initially it was thought that LL-37 functions by direct microbial killing as it is possesses antimicrobial properties *in vitro*. However, further research into LL-37 has revealed that it has only a modest level of antimicrobial activity in bacterial killing assays in physiological salt conditions (Bowdish, Davidson et al. 2005). Yet, patients with genetic disorders in which the peptide is not properly activated, suffer higher chances of skin infections compared to people who have a functional LL-37 (Pütsep, Carlsson et al. 2002). Also decreased levels of LL-37 in the skin of patients with atopic dermatitis are associated with increased incidence of skin infections (Ong, Ohtake et al. 2002). Additionally, mice lacking the LL-37 homolog cathelin related antimicrobial peptide (CRAMP) are more susceptible to skin infections than those with CRAMP (Nizet, Ohtake et al. 2001). Given that direct microbial killing is diminished in physiological salt concentrations, this raises the possibility that the peptides may have an indirect antimicrobial function in host defence.

Recent research has concluded that LL-37 is a multifaceted modulator of innate immunity (Bowdish, Davidson et al. 2006). LL-37 has been shown to upregulate several host defence mechanisms (summarized in Figure 2), including: induction of chemokines to attract leukocytes to the site of infection (Yu, Mookherjee et al. 2007); action as a monocyte chemoattractant itself (Tjabringa, Ninaber et al. 2006); facilitation of dendritic

cells maturation (Davidson, Currie et al. 2004); and enhancement of wound healing (Shaykhiev, Beisswenger et al. 2005) and angiogenesis (Koczulla, von Degenfeld et al. 2003). LL-37 has been shown to possess a number of anti-inflammatory activities, most notably its ability to reduce tumor necrosis factor-α (TNF-α) secretion from macrophages in response to TLR stimulation by conserved bacterial signature molecules such as lipopolysaccharide (LPS) (Scott, Davidson et al. 2002). In systemic infections, high levels of TNF-α secretion causes cellular necrosis, organ damage and wide-spread permeabilization of blood vessels, causing fluid influx into the tissues and rapid loss of blood pressure (Galanos and Freudenberg 1993). LL-37, it seems, reduces the potentially harmful effects of inflammation while increasing the responsiveness of host cells involved in the resolution of infection.

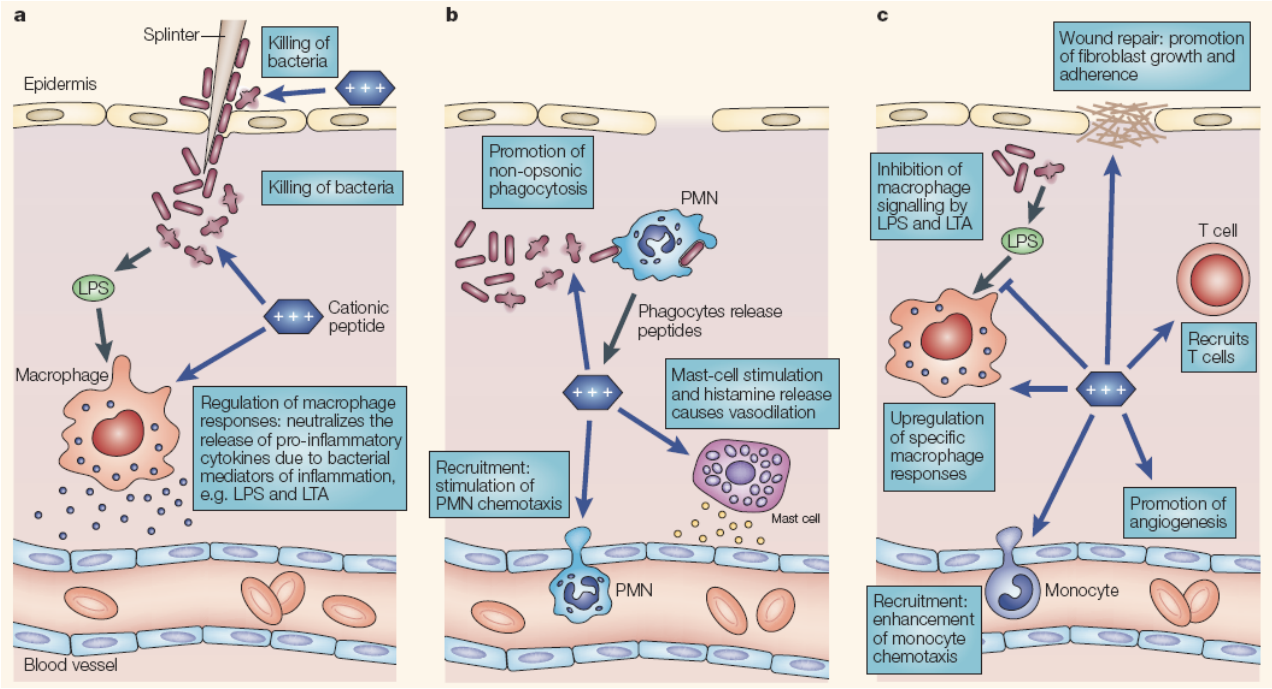


Figure 2: Summary of observed physiological roles of LL-37. (Finlay and Hancock 2004)

Research into the molecular mechanisms of LL-37 activity has shown that the peptide works through a variety of mechanisms. Initially, it was shown that LL-37 binds to the fPRL-1 receptor with high affinity (Yang, Chen et al. 2000; Tjabringa, Ninaber et al. 2006). This receptor, found on neutrophils, monocytes and T cells, is also the receptor for bacterial peptides bearing the formyl-methionine residue, however it is not required for LL-37's chemotactic activity (Niyonsaba, Iwabuchi et al. 2002; Bowdish, Davidson et al. 2004). It is also known that LL-37 modulates TLR4→NF-κB pathway signaling at some point, and this is responsible in part for LL-37's anti-endotoxin activity (Mookherjee, Brown et al. 2006). Lastly, it has been shown that LL-37 was capable of activating the extracellular signal-regulated kinase (ERK) and p38 MAPK pathways in monocytes (Bowdish, Davidson et al. 2004).

### 1.4. Hypothesis and Experimental Goals

With such varied host defence activities observed for LL-37 and other peptides, our lab has hypothesized that it is possible to use peptides as novel therapeutics to treat a wide range of infections. Previous experiments have used LL-37 itself in animal models of sepsis (Torossian, Gurschi et al. 2007), but it is not cost-efficient to manufacture large quantities of this peptide for mass-distribution. Being 37 residues long, chemically synthesizing large amounts LL-37 would be expensive and inefficient. While recombinant production of LL-37 in bacteria is possible, it is a difficult and time consuming process (Moon, Henzler-Wildman et al. 2006). LL-37 also shows some toxicity, in the form of induction of apoptosis (Barlow, Li et al. 2006) and histamine release (Niyonsaba, Someya et al. 2001). Also, administering LL-37 in the blood of mice

can lead to precipitation of the peptide and clogging of the capillaries, leading to rapid death (unpublished observations). Therefore, investigators have taken the approach to create new, smaller peptides that retain the activities of the natural peptides.

The aim of these studies was to determine whether novel synthetic antimicrobial and immunomodulatory peptides are capable of reducing the bacterial load in a mouse model of invasive *Staphylococcus aureus* infection. Novel peptides with enhanced activities as determined by *in vitro* assays were generated using a variety of high throughput methods. However it was unknown if these novel peptides would display protective abilities *in vivo.* Peptides are created with natural amino acids, and are thus susceptible to proteases found in the tissues and bloodstream, digesting and rendering them inactive shortly after injection (unpublished observations, Inimex Pharmaceuticals). I also investigated if immunomodulatory peptides can influence innate immunity *in vivo* by examining changes in cytokine and chemokine secretion, and cell recruitment changes to the site of infection. Lastly, I investigated the mechanism of peptide mediated immunomodulation by examining how monocyte-depleted animals respond to peptide treatment and subsequent infection.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1. Peptides

Peptides HH-2, HH-17, HH-18 and CRAMP were synthesized at the Biomedical Research Centre (UBC, Vancouver, British Columbia). Peptides 1012 and 1002 were synthesized at GenScript. All peptides were synthesized using Fmoc chemistry to a purity greater than 95% as assayed by HPLC. Peptide IDR-1 is licensed by Inimex Pharmaceuticals, Inc. Compound 5 and 5NHis$_{6,12}$ were generously provided by Dr. Annelise Barron and Ann Czyeweski of Stanford University (refer to Figure 3B for peptoid structure). All other peptides were developed in house by members of the Hancock Laboratory. Refer to Table 1 for peptide sequences.

Table 1: List of peptides and sequences studied

| **Peptide** | **Sequence** |
|---|---|
| **IDR-1** | KSRIVPAIPVSLL |
| **HHC-10** | KRWWKWIRW |
| **HHC-36** | KRWWKWWRR |
| **Compound 5** | N/A |
| **5NHis$_{6,12}$** | N/A |
| **HH-2** | VQLRIRVAVIRA |
| **HH-17** | KIWVRWK |
| **HH-18** | IWVIWRR |
| **1002** | VQRWLIVWRIRK |
| **1012** | IFWRRIVIVKKF |
| | |

A) Peptide B) Peptoid

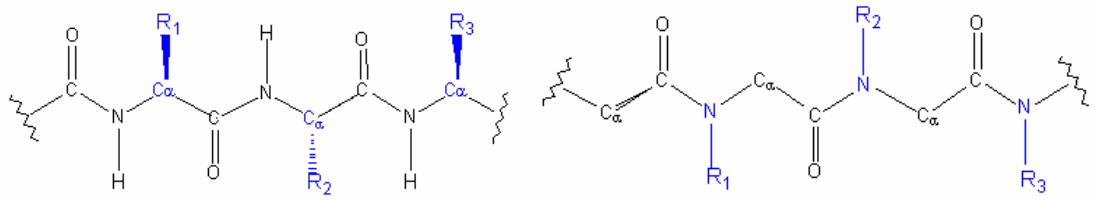

Figure 3: Chemical structure of poly-N-substituted glycine peptoids A) Normal peptide B) Peptoid.

## 2.2. Animal Infection Model

Female CD-1 mice were obtained from Harlan Laboratories. Female C57Bl/6J mice were obtained from Jackson Laboratories. All mouse experiments were performed in accordance with the University of British Columbia's ethical approval and guidelines under animal care certificate #A04-0020.

Bacteria were prepared by inoculating 5 ml of sterile Mueller-Hinton (MH) broth (Difco# 275730) with a single colony of *Staphylococcus aureus* (ATCC #25923). The tube was placed in a rotary shaker overnight at 37 $^{o}$C. The following morning, bacteria were diluted 1 in 5 in MH broth and allowed to grow for 1.5 hours, or until the bacteria reached an optical density of approximately 0.250 (following a 1/10 dilution) at 600 nm and 1 cm light path. The subculture was then diluted 1/10 in Porcine Mucin (Sigma # M2378, 5% w/v in Saline [0.9% NaCl in water]) and mixed thoroughly. A sample was withdrawn, serially diluted and plated on MH agar to determine the amount of bacteria administered. This procedure normally resulted in values of approximately 5.0 x $10^{8}$ colony forming units (CFU) per mouse.

Mice were infected intraperitoneally (IP) with 200 µl of bacteria/mucin mixture. Depending on the experiment, peptide was administered by the listed route either 4 hours before or 4 hours after infection. Peptides were dissolved in sterile saline to the

concentrations listed in each experiment. The infection was allowed to proceed for 4 to 24 hours before euthanasia via $CO_2$ asphyxiation and cervical dislocation. Peritoneal lavage was obtained by washing the peritoneal cavity with 5 ml of PBS. Blood was collected via cardiac puncture and deposited into tubes with 1.3 mg Ethylenediaminetetraacetic acid (EDTA). The peritoneal lavage and blood were then serially diluted and plated onto MH agar plates for bacterial enumeration.

### 2.3. Heat-Killed *Staphylococcus aureus*

A single colony of *Staphylococcus aureus* was inoculated into 5 ml of MH broth. The culture was incubated at 37 $^{o}$C in a rotary shaker overnight. The following day, the overnight culture was centrifuged twice (2000 RPM for 10 minutes), washed in PBS, and resuspended in sterile saline. The bacterial suspension was then boiled for 30 minutes to kill the bacteria and a sample was withdrawn and plated onto MH Agar to confirm that there were no viable bacteria in the solution. Mice were injected IP with 200 µl of this suspension.

### 2.4. Liposomal Clodronate Macrophage Depletion Model

This method was adapted from Biewenga *et al*, Cell & Tissue Research (1995), 280:189-196. Clodronate was purchased from Sigma-Aldrich (Cat# D4434). A solution of 10 mg/ml clodronate in Phosphate Buffered Saline (PBS) was mixed with a phosphatidylcholine/cholesterol (3:1 molar ratio) lipid solution dissolved in Chloroform, and left to incubate for 2 hours at 37 $^{o}$C. Vesicles were formed by drying this mixture by vacuum desiccation overnight, and subsequently resuspending the lipid film in 5 ml

saline. The vesicles were then additionally purified by size-exclusion chromatography using Sephadex G-50 beads (Sigma# G50150). Before each experiment, the vesicles were sonicated for approximately 30 minutes to reduce the size of any large vesicles that may have formed by vesicle fusion.

Four days before the mice were to be infected 200 μl of the purified liposome solution was injected IP into mice. Elimination of macrophages and recovery of the neutrophil population was confirmed by fluorescence-assisted cell sorting (FACS) analysis of whole blood.

**2.3. Chemokine and Cytokine Assays**

The concentration of MCP-1, RANTES, and KC in the blood and lavage collected from treated mice was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems) were performed according to manufacturer's instructions as described. Briefly, a flat bottomed 96 well plate (Costar #2592) was coated overnight at room temperature with 100 μl of the appropriate dilution of primary antibody in assay diluent (10% Fetal Calf Serum in PBS, v/v). The following morning, the plate was washed 3 times with wash buffer, and incubated in 300 μl of assay diluent for 2 hours at room temperature. Then the plate was washed 3 times in wash buffer (0.05% Tween-20, v/v) and 100 μl of diluted samples and standard curve were added to the plate and negative control wells that only contained assay diluent were also included. The dilution used depended on the sample and ELISA performed, but in most cases it was found that a 1/5 to 1/10 dilution was appropriate. The exception was MCP-1, which required a much higher dilution (1/50) to obtain appropriate results. The samples, standards and negative controls

(blanks) were incubated for 2 hours on a rotary shaker at room temperature. The plate was washed 3 times in wash buffer and 100 μl of the appropriate biotinylated secondary antibody in assay diluent added, and the mixture left to incubate for 2 hours at room temperature, with shaking and protection from light. The plate was then washed 3 times in wash buffer to remove the secondary antibody, then 100 μl of the horseradish peroxidase in assay diluent solution added to each well. Following the 20 minute incubation (shaking and protected from light), the plate was washed and 100 μl of substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB, Sigma # T8665) added to each well. The plate was incubated in the dark for approximately 20 minutes, or until a strong blue colour was visible at the higher concentrations of the standard curve. The reaction was terminated by adding 50 μl of 2N $H_2SO_4$. Finally, the plate was read on a spectrophotometer at 450 nm to determine colour development. The colour development in sample wells was compared to that of the standards to calculate the concentration of analyte.

The mouse inflammation Cytometric Bead Array (CBA, Beckton-Dickinson # 552364) kit was used as an alternative to ELISA for determining multiple analytes. This kit contained reagents to analyze the mouse cytokines TNF-α, interleukin-6 (IL-6) interleukin 10 (IL-10), interleukin-12 p70 subunit (IL-12p70), interferon gamma (IFN-γ), and monocyte chemoattractant protein (MCP-1), and used beads of varying sizes that bound to its specific substrates which were then quantified using fluorescent activated cell scanning (FACScan). Briefly, beads and samples (including standards) were mixed in a tube and allowed to incubate for 2 hours at room temperature in the dark, during which time the FACScan machine is calibrated and the detection reagent made.

Following the incubation, the phycoerythrin (PE) detection reagent in assay diluent was added to each sample tube and again allowed to incubate for 2 hours at room temperature in the dark. Then, the tubes were centrifuged at 1000 RPM for 10 minutes and the supernatant carefully decanted. Samples were then washed in wash buffer, centrifuged again, and resuspended in 300 μl of wash buffer. Samples were analyzed on the FACScan machine using BD CellQuest software, and the concentrations of analytes was determined using FCAP Array software (developed by Soft Flow Inc.)

**2.4. Flow Cytometry**

Peritoneal lavage fluid was centrifuged and washed in cold PBS, and cell numbers were determined by staining with crystal violet and counting under light microscope with a hemocytometer. $5.0 \times 10^5$ cells were aliquotted into a 96-well round bottom plate and the well was filled with (Hanks Balanced Salt Solution, supplemented with 2% FCS, 0.2% Sodium Azide and 20 mM HEPES), approximately 300 μl total. The plate was centrifuged at 1000 RPM for 10 minutes and this wash was repeated twice before the cells were resuspended in the primary antibody, pre-diluted in staining buffer (refer to Table 2 for the list of antibodies used). The cells were then incubated with antibody for 20 minutes at room temperature in the dark on ice. If more antibodies were needed, the centrifugation was repeated and cells resuspended the secondary and subsequent antibody solutions. Once the cells had been stained with all antibodies, the plate was centrifuged again and the cells fixed for 20 minutes with 0.5% formaldehyde in PBS. Cells were then left overnight at 4 $^{o}$C protected from light. The following day, cells were analyzed via FACS using BD Cell Quest software.

Table 2**:** List of Antibodies used in FACS experiments

| **Antibody** | **Clone** | **Fluorophore** | **Supplier** |
|---|---|---|---|
| Ly6C | HK1.4 | FITC | Abcam |
| F4/80 | C1:A3-1 | PE | Cederlane |
| Ly6G | 1A8 | PE | BD Biosciences |
| GR-1 | RB6-8CD | AF647 | Biolegend |
| CD40 | HM40-3 | AF647 | Biolegend |
| CD80 | 16-10A1 | AF647 | Biolegend |
| CD86 | GL1 | AF647 | Biolegend |
| I-A/I-E | M5/114.12.2 | AF488 | Biolegend |

### 2.5. Statistical Analysis

All statistics were performed by using a non-parametric t-test (Mann-Whitney test) and significant results were those calculated as having a p value of less than 0.05. Statistics were analyzed using Graphpad Prism 4.0 software (GraphPad Software Inc).

# CHAPTER 3: DIRECTLY ANTIMICROBICIDAL PEPTIDES REDUCE PERITONEAL BACTERIAL LEVELS IN A *STAPHYLOCOCCUS AUREUS* INFECTION MODEL

## 3.1. Introduction

Certain endogenous host defence peptides, such as pig protegrin and crab polyphemusin are able to potently and directly kill bacteria (Miyata, Tokunaga et al. 1989). Investigation into their mechanism of action suggests that peptides interact with the bacterial plasma membrane lipid bilayer, integrating into the membrane and either disrupting membrane integrity by forming channels or translocating across the membrane to attack internal targets (Powers and Hancock 2003). These peptides may be active against a variety of bacterial species and after optimization, have the potential to be used as a broad spectrum antibiotic. Indeed, currently there are certain directly antimicrobial peptides in clinical trials. Several (Pexiganan, Omiganan and Iseganan) have been developed as topical treatments or catheter coating antibiotics, although a few are being developed for systemic administration (Neuprex) (Gordon, Romanowski et al. 2005).

However, there are several challenges in developing peptide therapeutics that must be addressed. One is stability, both in storage and when the peptide is administered into the body. The peptide bond is susceptible to hydrolysis and thus long-term storage may steadily degrade the peptide into inactive byproducts. Furthermore, proteases found in the tissues and bloodstream would quickly digest peptides. Studies examining peptide lifetime *in vivo* found that peptides are undetectable approximately 15 minutes following intravenous injection (unpublished observations, Inimex Pharmaceuticals).

Another issue is cost of goods and manufacturing. Endogenous peptides vary in length, with a usual range of 25-100 amino acids. Chemically synthesizing large amounts of peptide is costly, and in synthesizing longer peptides, errors in synthesis are more likely to occur. While techniques exist for recombinant synthesis of longer peptides such as LL-37 in *E. coli* (Moon, Henzler-Wildman et al. 2006) or yeast (Hong, Lee et al. 2007), this peptide has relatively poor antimicrobial activity, and thus it is possible that by recombinantly synthesizing more active peptides there may be toxicity issues to the producing strain and difficulty in purification to homogeneity.

To address these issues, researchers around the globe are using a variety of methods to produce smaller, more stable peptides with increased activity for therapeutic use. This section deals with two types of antimicrobial peptides: the HHC peptides, which were designed using an artificial neural network computer program which trains itself to recognize important patterns amongst residues and sequences for determining antibacterial activity and creates new peptide sequences using the information learned; and the peptidomimetics, that are created with an altered backbone structure that renders the peptide-like molecule resistant to protease degradation.

## 3.2. Results

### 3.2.1: Quantitative Structure-Activity Relationship (QSAR) Peptides

In an attempt to design more active antimicrobial peptides our laboratory, in collaboration with Dr. Artem Cherkasov, utilized an artificial neural network computer program, and a variety of "training sets" of data on peptides to predict peptide sequences that would confer strong antimicrobial activity. Two hundred 9-mers were synthesized

and assayed for antimicrobial activity against *Pseudomonas* and 20 of these made in larger amounts and tested against, using *in vitro* Minimum Inhibitory Concentration (MIC) assays, including known antibiotic resistant strains. Being only nine residues, the costs of large scale synthesis of these peptides would be much less than larger natural peptides. The most active peptides, HHC-10 and HHC-36, were chosen for use in the mouse infection model. HHC-10, in particular, had potent antimicrobial activity against a wide range of bacteria strains equivalent to that of certain broad spectrum commercial antibiotics and the clinically developed peptide MX-226 (Table 3).

Table 3**:** Excerpt of results from MIC screening to determine peptide antimicrobial activity. Not all HHC peptides shown. Data courtesy of Dr. Havard Jensen.

| | | **Physical Properties** | | | **MIC (μM)** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Peptide** | **Sequence** | **NC** | **HF** | **HM** | **PA** | **A** | **D** | **E** | **H** | **I** | **J** | **K** | **L** | **M** | **N** | **O** |
| Bac2A | RLARIVVIRVAR | 4 | 0.67 | 4.9 | 35 | 48 | 192 | 95 | 3.0 | 24 | 24 | 24 | 192 | 192 | 24 | 24 |
| HHC-8 | KIWWWWRKR | 4 | 0.56 | 2.1 | 4.5 | 5.9 | 47 | 47 | 3.0 | 94 | 12 | 5.9 | 189 | 47 | 5.9 | 5.9 |
| HHC-9 | RWRRWKWWL | 4 | 0.56 | 2.1 | 2.7 | 2.9 | 23 | 5.8 | 0.7 | 5.8 | 5.7 | 2.9 | 46 | 11 | 2.9 | 2.9 |
| **HHC-10** | **KRWWKWIRW** | **4** | **0.56** | **4.7** | **1.4** | **0.8** | **3.0** | **5.9** | **0.4** | **3.0** | **1.5** | **1.5** | **25** | **6.2** | **3.0** | **1.5** |
| HHC-20 | WRWWKIWKR | 4 | 0.56 | 4.8 | 4.8 | 5.9 | 47 | 12 | 0.8 | 12 | 5.9 | 5.9 | 94 | 24 | 3.0 | 3.0 |
| **HHC-36** | **KRWWKWWRR** | **5** | **0.44** | **5.9** | **4.4** | **0.7** | **5.7** | **11** | **1.4** | **11** | **5.4** | **2.7** | **174** | **22** | **2.9** | **1.4** |
| MX-226 | ILRWPWWPWRRK | 4 | 0.67 | 3.4 | ND | 19 | 38 | 76 | 19 | 9.6 | 38 | 38 | 153 | 76 | 9.6 | 19 |
| Tobramycin | | | | | | 1.1 | >274 | 8.6 | 8.6 | 1.1 | 137 | 274 | 69 | 34 | 0.5 | >274 |
| Imipenem | | | | | | 6.68 | 428 | 3.34 | 428 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 |
| Ceftazidime | | | | | | 3.61 | >231 | 57.7 | 0.45 | 57.7 | 231 | >231 | >231 | >231 | 28.9 | 115 |
| Ciprofloxacin | | | | | | 0.38 | 193 | 1.51 | 0.75 | 0.38 | >386 | >386 | 0.4 | 193 | 0.38 | 6.04 |

**Column legends**: **NC** represents net charge at pH 7.0, hydrophobicity expressed as hydrophobic fraction **HF** and hydrophobic moment **HM**. **PA** represents an estimated MIC (μM) derived from luminescence readings (Rel.$IC_{50}$) for *P. aeruginosa* strain H1001 *fliC::luxCDABE*; Remaining columns give MIC values (μM) measured in 3-5 replicates for **A**, *P. aeruginosa* wild type strain H103; **B**. *P. aeruginosa* multidrug resistant strain from Brazil #9,; **E,** *P. aeruginosa* Liverpool epidemic strains LES400; **H**, *P. maltophilia* ATCC13637; **I**, Constitutive Class C chromosomal β-lactamase expressing *Enterobacter cloacae* 218R. **J,K,** Extended-spectrum β-lactamase-producing (ESBL) *E. coli* (clinical strains 63103 and 64771); **L,M**, ESBL resistant *Klebsiella pneumonia* (clinical strain 61962 and 63575); **N**, *S. aureus* ATCC25923; **O**, Methicillin resistant *S. aureus* strain C623.

To observe if HHC-10 and HHC-36 were capable of reducing bacterial load *in vivo*, two groups of five female CD-1 mice were infected intraperitoneally (IP) with *S. aureus*. Four hours following infection, mice were treated IP with 4 mg/kg of peptide dissolved in saline. The infection was allowed to proceed for a total of 24 hours, after which mice were euthanized and samples (peritoneal lavage and blood) collected. The samples were serially diluted and plated on agar plates for bacterial enumeration. Under the animal facility protocol, death could not be used as an endpoint; however occasionally animals would succumb to the infection before the time of euthanasia. These animals were assigned the highest observed bacterial counts in the experiment.

To determine whether our novel peptides were equal to or exceeded current antimicrobial peptides in their efficacy, peptide HHC-10 was compared to the commercial peptide Omiganan (MX-226). This peptide is in phase 3 clinical trials for use for prevention of catheter associated infections, having shown statistically significant efficacy in Phase IIIa clinical trials, and is considered the most clinically advanced antimicrobial peptide available. Mice were infected IP with *S. aureus* in mucin, and 4 hours later MX-226 was injected in both IP and IV routes of administration. Twenty-four hours following infection, mice were euthanized, peritoneal lavage and blood (via cardiac puncture) samples obtained and plated for bacterial enumeration. Figure 4 demonstrates that Omiganan did not significantly reduce either peritoneal or blood bacterial levels in either the IV or IP route, signifying that the QSAR method can improve antibacterial activity over peptides currently in clinical trials.

Next, I examined the *in vivo* efficacy of the novel synthetic peptides HHC-10 and HHC-36. Mice were infected IP with *S. aureus* in mucin, and 4 hours later peptides

HHC-10 and -36 was injected in the IP route of administration. Twenty-four hours following infection, mice were euthanized, peritoneal lavage collected and plated for bacterial enumeration. Figure 5 depicts that mice treated with the peptides HHC-10 and -36 demonstrated an approximately 2 to 2.5-log, statistically-significant reduction in bacterial numbers in the peritoneal cavity. Of particular note was the correlation of *in vitro* and *in vivo* efficacy. HHC-10 was the more effective peptide against the majority of bacteria tested *in vitro* (Table 3), and *in vivo* HHC-10 was slightly more effective than HHC-36. This experiment was repeated several times and potent antibacterial activity was observed each time.

However, these results only demonstrated local protection, and since *S. aureus* is an invasive infection, an antibiotic needs to be able to be administered and be efficacious systemically. To assess if peptide HHC-10 could be used via a route of administration different from the site of infection, mice were infected intraperitoneally but treated intravenously (IV, via lateral tail vein) with peptide. Twenty-four hours following infection, mice were euthanized, peritoneal lavage and blood collected, and plated for bacterial enumeration. Figure 6 demonstrates that peptide HHC-10, when administered via the IV route, was capable of significantly reducing bacterial load in both the peritoneum and blood, indicating that the peptide was capable of crossing from the blood into the peritoneal cavity. Given that peptides are extremely labile *in vivo,* it is remarkable that HHC-10 had the potency to kill a high number of bacteria in the short time that it was present. This experiment was repeated twice and intravenous activity observed both times.

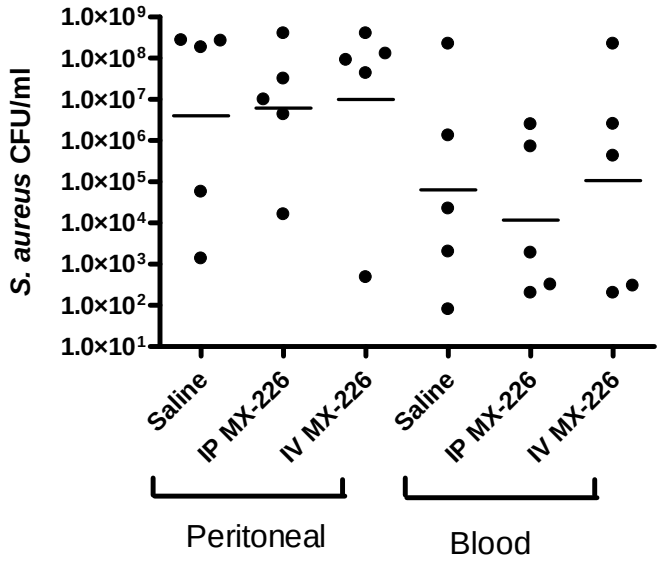


Figure 4**: Synthetic peptide MX-226 does not display significant antibacterial effect in vivo.** Female CD-1 mice were infected intraperitoneally with approximately 1.0 x $10^9$ CFU of *S. aureus.* Four hours later, mice were treated with 4 mg/kg (~100 μg/mouse) of peptide MX-226, via IV or IP routes. Twenty-four hours post-infection, mice were euthanized and samples collected. The bars represent the Geometric Mean, as calculated by Graphpad Prism 4.0.

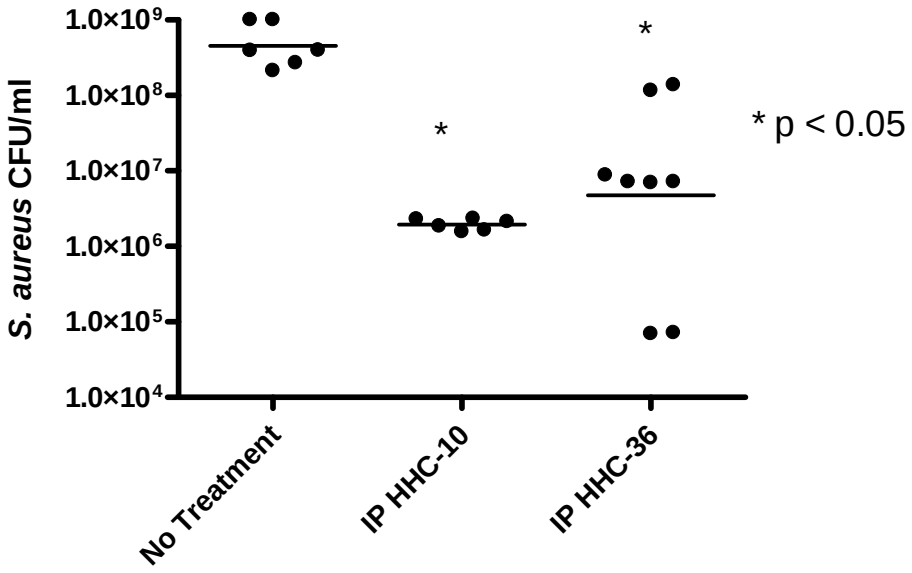


Figure 5**: Synthetic peptides HHC-10 and HHC-36 significantly reduced peritoneal bacteria levels.** Female CD-1 mice were infected with approximately 1.0 x $10^9$ CFU of *S. aureus* intraperitoneally. Four hours later, mice were treated with an IP dose of 4 mg/kg peptide or saline. Mice were euthanized after 24 hours of infection and peritoneal contents diluted and plated on MH agar. Mice that died from the infection were assigned the highest colony counts observed in the experiment. The bars represent the Geometric Mean, as calculated by Graphpad Prism 4.0. This graph is representative data from a series of 3 independent experiments.

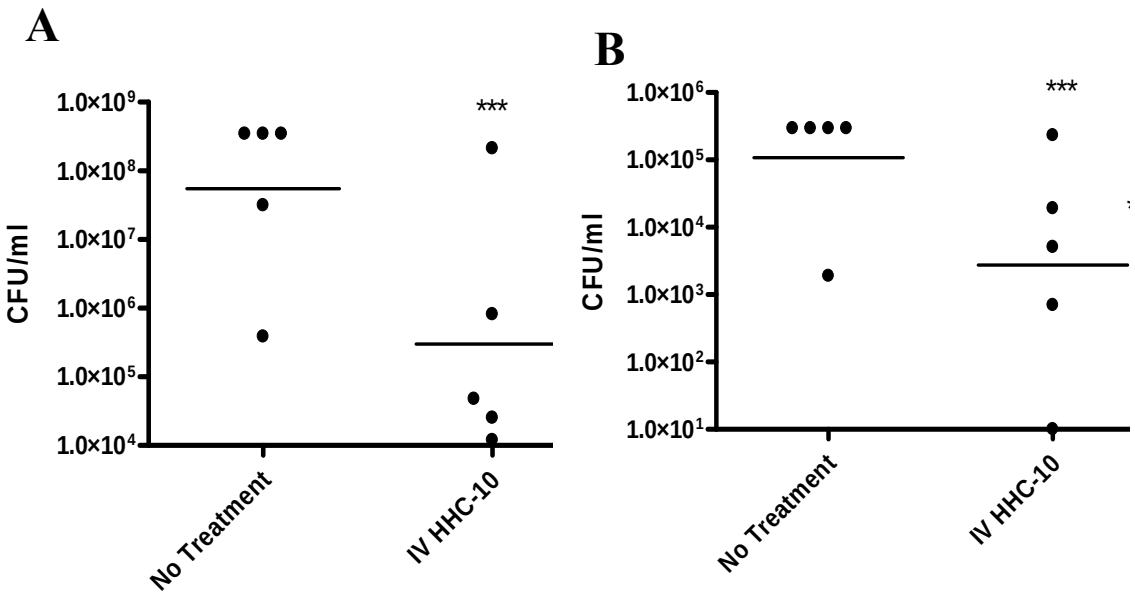


Figure 6**: Synthetic peptide HHC-10 reduced peritoneal and blood S. aureus when administered intravenously.** Female CD-1 mice were infected with approximately 5.0 x $10^8$ CFU IP. Four hours later, mice received a single dose of 200 μg of peptide HHC-10 IV (tail vein). Mice were euthanized 24 hours post-infection, and peritoneal lavage and blood collected. Panel A depicts peritoneal bacterial counts, Panel B depicts blood bacterial levels. Mice that died from the infection were assigned the highest colony counts observed in the experiment. The graph is representative data from 2 independent experiments. The bar represents geometric mean, as calculated by Graphpad Prism 4.0. *** represents a P value of $< 0.05$.

### 3.2.2. Peptidomimetics (Peptoids)

One method researchers are using to improve the longevity of peptides in mammalian systems *in vivo* is constructing peptides using chemically altered amino acids or backbone structures. This approach creates peptides with approximately the same 3-dimensional structure and configuration as a natural peptide, but with different biochemistry, making them much more resistant to endogenous proteases. With peptides lasting longer *in vivo,* they would have more time to exert their effects on bacteria, and thus offer better activity against infections.

One approach researchers are using is to design peptides with D-amino acids. Most proteases are unable to cleave D-amino acid proteins, so a D-amino acid-containing host defence peptide should be resistant to proteases and persist in the active form longer when administered *in vivo*. However, altering the stereochemistry of amino acids changes the 3-D structure of the peptide, potentially affecting its interactions with receptor proteins and biological activity. In order to create peptides that are resistant to proteases but closely resemble natural peptides in their 3-D structure, with the same position of amino acid side chains relative to the peptide backbone, a "retro-inverso" approach can be used. This involves making a peptide with D-amino acids, positioned in reverse order compared to the natural peptide. Using an established antimicrobial peptide sequence, and the "retro-inverso" approach, the novel peptide will have approximately the same 3-D structure and activity as the template but much longer half *in vivo* half-life can be produced (Taylor, Otero et al. 2000).

Another approach utilized by researchers at Stanford University has been using peptoids comprised of poly-N-substituted glycines with amino acid-like constituents in

which the side group is moved from the chiral carbon to the adjacent nitrogen of the peptide bond (refer to Figure 3 for a structural comparison to normal amino acids). This alters the physical space surrounding the peptide bond while still retaining an overall 3-dimensional peptide-like structure needed for antimicrobial activity.

Stanford university scientist Dr. Annelise Barron has designed numerous peptidomimetics (or peptoids) using these altered amino acids. Based on the sequence of the naturally occurring peptide magainin, these peptoids display similar antimicrobial properties as the parent peptide but are highly protease resistant (Chongsiriwatana, Patch et al. 2008). They have observed in *in vitro* assays a high level of antimicrobial activity against a broad range of bacteria.

In collaboration with Dr. Barron and graduate student Ann Czeweski, I attempted to determine if these compounds were effective *in vivo*. Two compounds were tested, Compound 5, and a chemical derivative, $5NHis_{6,12}$. Both were effective in *in vitro* killing assays, with the latter possessing greater activity. $5NHis_{6,12}$ was also less toxic in an *in vitro* hemolysis assay than Compound 5.

These peptoids have never been used *in* vivo, and without a preexisting knowledge of their efficacy, I initially used a relatively low dose of 4 mg/kg, or approximately 100 μg per mouse. Surprisingly, this concentration proved to be lethal for the $5NHis_{6,12}$ compound, which was purported to be the less hemotoxic compound. Even in the absence of an infection, the compound was lethal approximately 2 hours following IP injection. Compound 5, while active and capable of reducing bacterial load *in vivo*, produced a milder adverse reaction in the treated animals after the injection. Mice became visibly ill following injection with Compound 5, showing signs such as

decreased movement, hunched abdomen, closed eyes and mild piloerection. However, these symptoms were resolved 2 hours following injection, and the mice remained free of symptoms for the duration of the experiment. Mice were infected IP with *S. aureus* in mucin, and 4 hours later peptoid was injected by both the IP and IV routes of administration. Twenty-four hours following infection, mice were euthanized, peritoneal lavage and blood samples obtained and plated for bacterial enumeration. The data presented in Figure 7 demonstrates that the peptoid Compound 5 was able to significantly reduce peritoneal bacterial load by 2 to 2.5-log, despite or perhaps in association with the toxic properties observed.

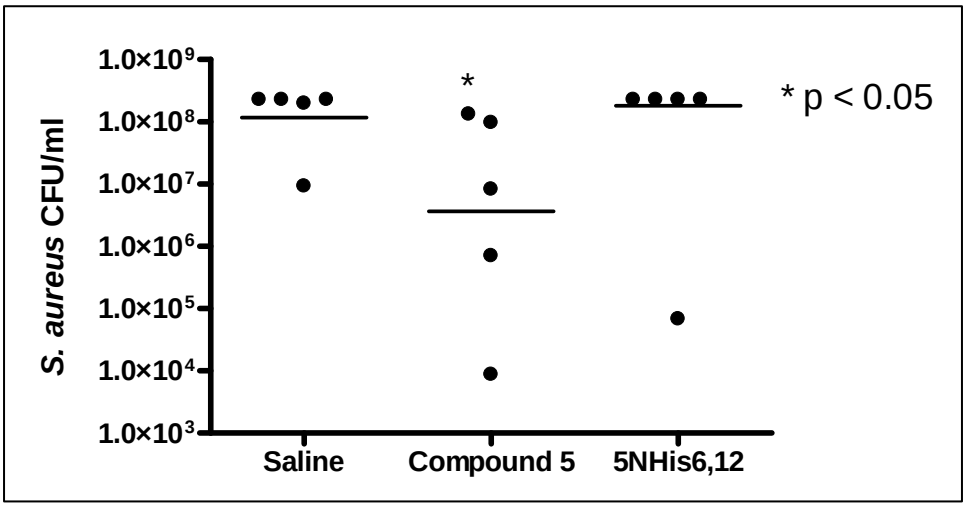


Figure 7**: Peptidomimetic (peptoid) Compound 5 significantly reduces peritoneal bacteria count.** Female CD-1 mice were infected intraperitoneally with approximately 1.0 x $10^9$ CFU of *S. aureus.* Four hours later, mice were IP treated with 4 mg/kg (~100 μg/mouse) of peptoid. Mice were euthanized 24 hours post-infection and sample collected. Compound 5 results significant to a P value of 0.05. Compound $5NHis_{6,12}$ showed significant toxicity. Animals that died during the course of the experiment were assigned the highest colony counts observed in the experiment. The bars represent the Geometric Mean, as calculated by Graphpad Prism 4.0. This graph is representative data from a series of 3 independent experiments (only Compound 5 was used in repeat experiments) and protective activity observed each time.

### 3.3. Discussion

The data observed from these experiments shows that synthetic antimicrobial peptides can significantly reduce bacterial loads *in vivo* in a *S. aureus* model of invasive infection. HHC-10 was capable of reducing bacterial loads by nearly 3-logs fold, and was superior to the commercially developed peptide Omiganan, which did not cause any significant reduction of bacterial numbers. HHC-10 was also capable of reducing peritoneal bacterial load when administered intravenously, indicating that the peptide was capable of crossing from the blood into the peripheral tissues and retaining enough activity to kill a significant number of bacteria. It should be mentioned that Omiganan is being developed as a topical antibiotic, and is not intended for systemic use. Therefore, it is perhaps not surprising that the peptide is not able to reduce bacterial load in this infection model. Future experiments should compare the activity of HHC-10 and HHC-36 to a conventional antibiotic *in vivo.*

These results highlight a proof-in-principle experiment that shows that by analyzing structure-function relationships of peptide antimicrobial activity, new peptide sequences with heightened antimicrobial activity can be designed. Theoretically, the neural network procedure can be used to optimize any peptide activity, including immunomodulation. As the peptide sequences in Table 3 depict, the novel peptides possess a high number of positively charged (Arginine and Lysine) and hydrophobic aromatic residues (Tryptophan), supporting the hypothesis that antimicrobial peptides interact with negatively charged components on the bacterial membrane surface while the hydrophobic residues interact with the hydrophobic interior of the bacterial membrane.

This multifaceted interaction is believed to lead to disruption of membrane integrity, facilitating the death of the bacterium (Powers and Hancock 2003).

The experiments performed with Compound 5 and $5NHis_{6,12}$ demonstrated that protease resistant antimicrobial peptoids can reduce bacterial load *in vivo.* These peptoids would not be as readily degraded as natural peptides *in vivo,* resulting in a longer half-life and thus a longer exposure time to bacteria, theoretically increasing bacterial clearance. Compound 5 was capable of reducing peritoneal *S. aureus* by approximately 2.5 logs, relative to the control, similar values to those observed with HHC-10. However, issues arose as these compounds appeared to have significant toxicity *in vivo,* despite *in vitro* toxicity experiments showing no significant toxicity. Compound $5NHis_{6,12}$ showed lethal toxicity 2 hours following injection in all animals, indicating that a hemolysis assay is not sufficient for estimating *in vivo* toxicity. Mice injected with Compound 5 showed initial signs of distress but recovered shortly afterward. The mechanism of toxicity was not investigated. Given that $5NHis_{6,12}$ induced death within 2 hours following an IP injection, I suspect that the peptoid might have significant nephro or hepatotoxicity. This could be tested by injecting animals, sacrificing them and examining the kidneys and liver by tissue sectioning and histochemistry for evidence of tissue damage.

In conclusion, the novel synthetic peptides HHC-10 and HHC-36 were all capable of significantly reducing bacterial load *in vivo*. HHC-10 also demonstrated efficacy in an IV route of injection. These results signify that by analyzing structure-function relationships of antimicrobial peptides, new peptides with optimized activity can be designed. In addition, the peptidomimetic Compound 5 was also capable of significantly reducing bacterial load, however further work is needed to reduce *in vivo* toxicity.

# CHAPTER 4: IMMUNOMODULATORY PEPTIDES ENHANCE BACTERIAL CLEARANCE IN A MURINE MODEL OF INVASIVE *S. AUREUS* INFECTION BY SELECTIVELY MODULATING THE INNATE IMMUNE RESPONSE

## 4.1. Introduction

Direct antimicrobial killing is not the only mechanism by which synthetic host defence peptides can protect against bacterial infections. Considerable research over the years has shown that endogenous host defence peptides also act as immunomodulators, enhancing certain aspects of the immune response, while limiting the potentially harmful side effects of inflammation (Mookherjee, Rehaume et al. 2007).

As mentioned previously, these immunomodulatory properties were first observed with the human cathelicidin LL-37 (Scott, Davidson et al. 2002), and its mouse homologue CRAMP (e.g. refer to Figure 8 for my data suggesting that CRAMP reduces TNF-α secretion). Indeed, it appears that immunomodulation is LL-37's primary role in host defence. Experiments examining the antimicrobial properties of LL-37 found that the peptide does not demonstrate any significant killing activity in physiological salt solution (Bowdish, Davidson et al. 2005). It seems therefore that immunomodulation might be the main mode by which LL-37 acts against infections *in vivo.*

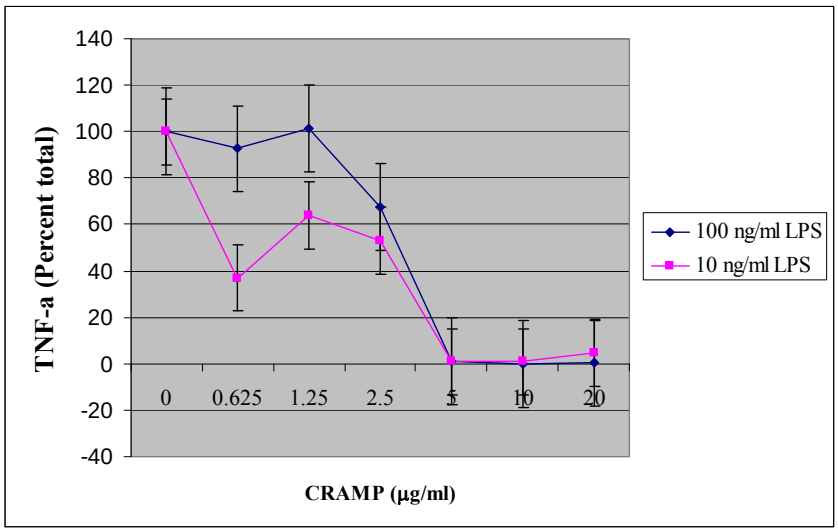


Figure 8**: The Murine cathelicidin CRAMP reduces TNF-α secretion from LPS stimulated RAW cells.** 5.0 x $10^5$ cells were simultaneously stimulated with the listed concentrations of CRAMP and LPS for 24 hours. Supernatant concentration of TNF-α was determined by ELISA. The data was normalized, with 100% being the amount of TNF-α released in response to LPS without any CRAMP added (normal values are approximately 800 pg/ml). The graph is the combination of 3 independent experiments.

Similar to the antimicrobial peptides described in the previous chapter, our lab has endeavored to create smaller, more effective immunomodulatory peptides by utilizing high-throughput synthesis and screening of peptide variants. A known peptide sequence was used as a template molecule, in this case a linear derivative of the bovine cathelicidin Bactenecin, Bac2A. This peptide is one of the smallest peptides in which immunomodulatory functions have been observed (Bowdish, Davidson et al. 2005). Using robotic synthesis on cellulose sheets (peptide arrays), each position of the template peptide was replaced with every other amino acid, and the novel peptides were synthesized on a cellulose membrane (Hilpert, Winkler et al. 2007).

To assay for activity, each peptide spot was initially used in a high-throughput screening assay using *Pseudomonas aeruginosa* containing a gene cassette incorporating the *lux* luminescent reporter (Hilpert and Hancock 2007). Because *lux* requires ATP from

living cells in order to luminesce, peptide-mediated killing of bacterial cells can be followed by measuring luminescence over time. This technique allows for rapid screening of peptide activity, rather than lengthy and sometimes inaccurate MIC assays (Figure 9).

| Original amino acid | | Substituted amino acid | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| | | A | C | D | E | F | G | H | I | K | L | M | N | P | Q | R | S | T | V | W | Y |
| 1 | R | 0.15 | 0.27 | 0.34 | 0.41 | 0.18 | 0.15 | 0.24 | 0.17 | 0.14 | 0.16 | 0.35 | 0.34 | 0.17 | 0.33 | 0.13 | 0.29 | 0.25 | 0.21 | 0.06 | 0.20 |
| 2 | L | 0.17 | 0.08 | 0.33 | 0.21 | 0.13 | 0.06 | 0.10 | 0.12 | 0.06 | 0.13 | 0.18 | 0.18 | 0.15 | 0.16 | 0.05 | 0.10 | 0.17 | 0.12 | 0.06 | 0.09 |
| 3 | A | 0.13 | 0.09 | 0.18 | 0.16 | 0.04 | 0.12 | 0.09 | 0.07 | 0.05 | 0.09 | 0.14 | 0.12 | 0.14 | 0.09 | 0.03 | 0.17 | 0.11 | 0.15 | 0.04 | 0.11 |
| 4 | R | 0.31 | 0.35 | 0.49 | 0.41 | 0.45 | 0.46 | 0.35 | 0.59 | 0.11 | 0.75 | 0.39 | 0.28 | 0.25 | 0.26 | 0.13 | 0.30 | 0.31 | 0.27 | 0.25 | 0.29 |
| 5 | I | 0.31 | 0.05 | 0.42 | 0.30 | 0.29 | 0.26 | 0.23 | 0.13 | 0.10 | 0.21 | 0.23 | 0.26 | 0.33 | 0.26 | 0.08 | 0.22 | 0.20 | 0.17 | 0.06 | 0.20 |
| 6 | V | 0.25 | 0.06 | 0.44 | 0.47 | 0.09 | 0.43 | 0.20 | 0.20 | 0.12 | 0.20 | 0.26 | 0.30 | 0.75 | 0.25 | 0.15 | 0.29 | 0.23 | 0.13 | 0.07 | 0.13 |
| 7 | V | 0.37 | 0.06 | 0.25 | 0.20 | 0.17 | 0.17 | 0.09 | 0.05 | 0.03 | 0.11 | 0.20 | 0.10 | 0.60 | 0.05 | 0.05 | 0.26 | 0.09 | 0.13 | 0.19 | 0.11 |
| 8 | I | 0.48 | 0.06 | 0.75 | 0.75 | 0.14 | 0.50 | 0.23 | 0.13 | 0.15 | 0.18 | 0.38 | 0.40 | 0.31 | 0.31 | 0.16 | 0.42 | 0.52 | 0.13 | 0.16 | 0.16 |
| 9 | R | 0.39 | 0.09 | 0.75 | 0.75 | 0.38 | 0.23 | 0.41 | 0.48 | 0.18 | 0.41 | 0.27 | NF | 0.41 | 0.40 | 0.13 | 0.41 | 0.31 | 0.49 | 0.22 | 0.19 |
| 10 | V | 0.61 | 0.06 | 0.75 | 0.75 | 0.21 | 0.39 | 0.21 | 0.11 | 0.14 | 0.16 | 0.22 | 0.29 | 0.22 | 0.41 | 0.18 | 0.41 | 0.33 | 0.13 | 0.08 | 0.13 |
| 11 | A | 0.13 | 0.04 | 0.21 | 0.23 | 0.12 | 0.08 | 0.08 | 0.06 | 0.06 | 0.08 | 0.09 | 0.12 | 0.18 | 0.13 | 0.05 | 0.10 | 0.10 | 0.10 | 0.13 | 0.07 |
| 12 | R | 0.38 | 0.75 | 0.75 | 0.75 | 0.75 | 0.33 | 0.20 | 0.27 | 0.19 | 0.47 | 0.47 | 0.25 | 0.42 | 0.32 | 0.13 | 0.40 | 0.29 | 0.37 | 0.75 | 0.54 |

Figure 9**: Results of luminescence killing assay with newly derived synthetic peptides.** The X-axis represents the original parent peptide, Bac2a. The Y-axis shows the amino acid substitution at each position of the parent peptide. The corresponding values indicate the relative killing activity. Values in white indicate no change in activity from the parent. Values in grey signify a modest increase in activity, while black values represent a large increase in activity. These data were obtained by Dr Kai Hilpert in our laboratory and are published in Nature Protocol. (Hilpert and Hancock 2007)

For the studies described here, I was interested in immunomodulatory activity, rather than direct antimicrobial killing. Therefore, numerous peptides that displayed only moderate antimicrobial activity were chosen for screening in chemokine secretion assays. Freshly isolated human peripheral blood mononuclear cells (PBMCs) were incubated

with 20 or 100 μg/ml of novel peptide for 24 hours, after which the culture supernatant was analyzed by ELISA for monocyte chemokines MCP-1 and MCP-3, and neutrophil chemokines GRO-α, and IL-8.

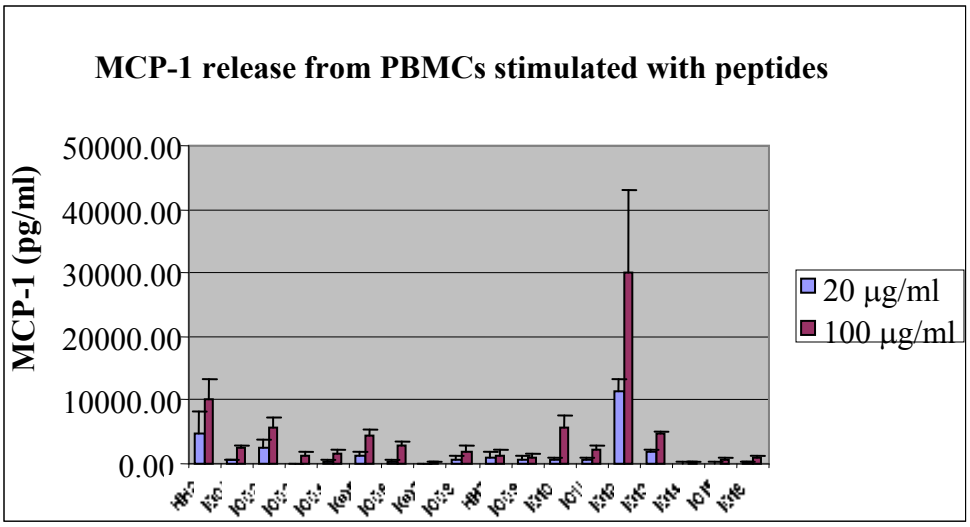


Figure 10**: MCP-1 release from human PBMCs stimulated with synthetic peptide for 24 hours.** PBMCs were isolated and seeded at 5.0 x $10^5$ into a tissue culture plate. Cells were stimulated with 20 or 100 μg/ml of peptide for 24 hours. Culture supernatant were centrifuged to remove any floating cells. Supernatant was assayed for chemokine levels by ELISA. Only MCP-1 ELISA is shown as representative data. Data courtesy of Melissa Elliot.

Peptides that stimulated a large release of chemokines were then chosen for use in the mouse model of invasive *Staphylococcus aureus*. To eliminate any possibility of direct antimicrobial killing, the protocol was changed slightly, and peptide was administered 4 hours prior to infection. This ensures that, due to the action of host proteases, no freely circulating peptide would be present in the animal at the time of infection, and any reduction of bacterial load could be attributed to an enhancement of the animal's immune system.

## 4.2. Results

### 4.2.1. Peptide Innate Defence Regulator (IDR-1)

The following results were previously published in Scott *et al*, Nature Biotechnology (2007) Apr 25 (4):465-72, and performed by me or in collaboration with others as named.

The first synthetic peptide that was used as a novel immunomodulator in animal models was Innate Defence Regulator-1 (IDR-1). Developed by the Hancock Lab and licensed to Inimex Pharmaceuticals (Vancouver, BC), IDR-1 was the focus of a large, cross-institutional study examining its immunomodulatory activity.

First, the ability of IDR-1 to protect animals from an infection was determined. Mice were pretreated with 24 mg/kg of IDR-1 IP for 4 hours, following a 24-hour infection with *S. aureus.* Mice were euthanized, peritoneal lavage collected and plated for bacterial enumeration. Figure 11 demonstrates that IDR-1 significantly reduced peritoneal bacterial load *in vivo*, by approximately 3-fold. This effect was observed when IDR-1 was administered both 24 hours pre-infection and 4 hours post-infection. In addition, mice that were treated with peptide appeared healthier, with less signs of illness or distress, a phenomenon observed with the other immunomodulatory peptides studied (data not shown).

To investigate the mechanisms mediating protective activity or IDR-1, I also analyzed the presence of chemokines and cytokines in mouse peritoneal lavage. Mice were pretreated with 24 mg/kg of IDR-1 for 4 hours and infected with *S. aureus* for 24 hours. Mice were euthanized and peritoneal lavage collected, centrifuged and analyzed for the presence of cytokines and chemokines by ELISA. While the data in Figure 12 was very variable, there was a statistically significant induction of the anti-inflammatory

cytokine IL-10 and a non-significant trend towards increased induction of the monocyte chemokine MCP-1.

To address whether the cytokine upregulation observed was due to an increase in gene expression, human PBMCs were isolated and stimulated with 200 μg/ml of IDR-1 for 4 hours. RNA was isolated and analyzed for gene expression by qPCR. Figure 13 demonstrates that IDR-1 was capable of significantly increasing gene expression of a number of inflammatory chemokines and cytokines. Chemokines MCP-1, MCP-3, and GROα were all significantly upregulated, while the proinflammatory cytokines IL-6 and IL-19 were also upregulated. The anti-inflammatory cytokine IL-10 was also significantly upregulated by a factor of 5, supporting the data observed in Figure 12.

Lastly, the cellular contents of the peritoneal lavage in the presence and absence of IDR-1 was analyzed. Mice were administered 24 mg/kg of peptide IP at the listed times, then infected with *S. aureus* IP at time = 0. Mice were euthanized 24 hours post-infection and peritoneal lavage obtained and analyzed for cellular numbers by histochemistry and counting under a light microscope. Figure 14 demonstrates that IDR-1 caused a non-significant trend towards an increase in the numbers of macrophages found in the peritoneal cavity. These results possibly suggest that IDR-1 was capable of increasing cellular numbers at the site of injection. It is unknown at this point if this influx of cells was due to the observed increase in chemokine stimulation, or whether IDR-1 itself acted as a chemoattractant.

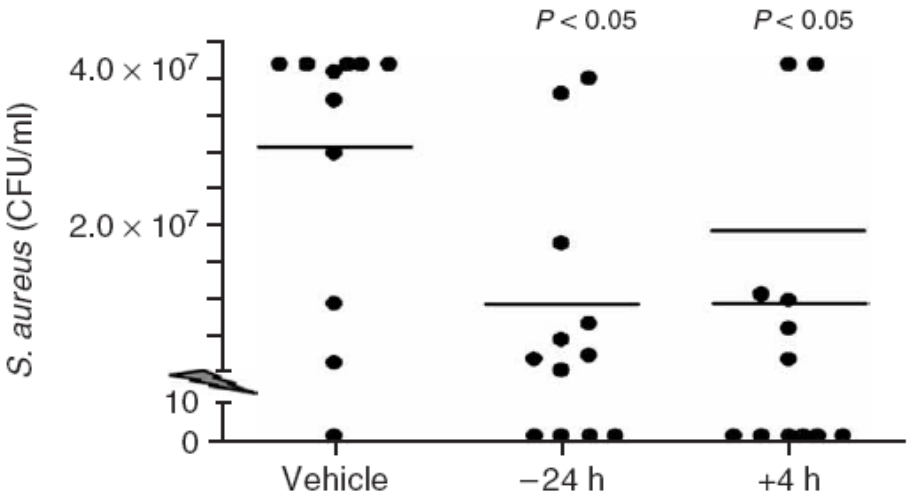


Figure 11**: Peptide IDR-1 significantly reduced bacteria levels in vivo.** Female CD-1 mice were IP pretreated with 24 mg/kg of IDR-1 at the listed times, and infected 4 hours later with ~1.0 x $10^9$ CFU of *S. aureus*. The infection proceeded for 24 hours before euthanasia and sample collection. Peritoneal contents were diluted and plated on MH agar. Animals that died from the infection are assigned the highest colony counts observed in the experiment. Results are the combination of 2 independent experiments. The bar represents the geometric mean, as calculated by GraphPad Prism 4.0.

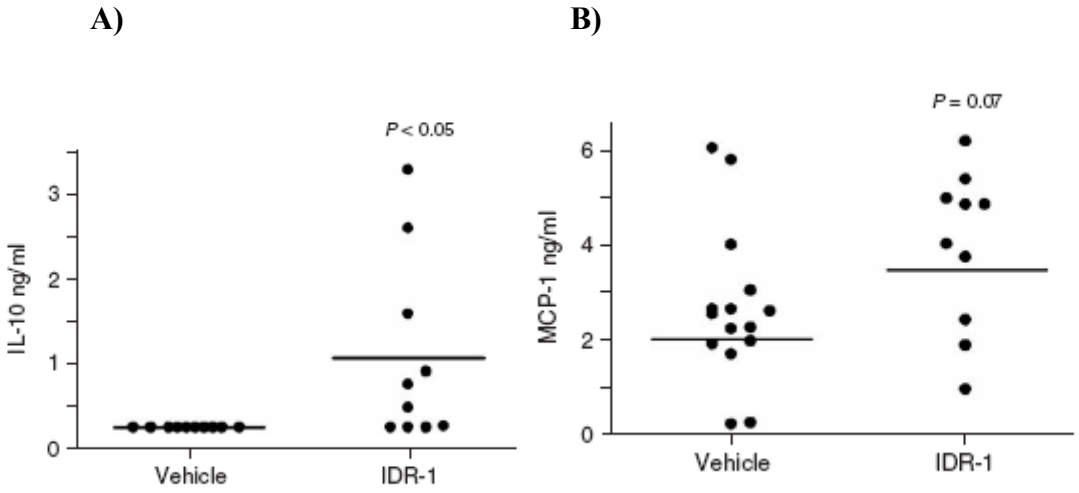


Figure 12**: IDR-1 increased MCP-1 and IL-10 release in vivo.** CD-1 mice were pretreated with IDR-1 for 4 hours, followed by a 24 hour infection with *S. aureus*. Mice were then euthanized and peritoneal lavage and blood collected. Cytokines and Chemokines were analyzed by ELISA. Panel *A* shows IL-10 concentration. Panel *B* shows MCP-1 concentration. The bar represents arithmetic mean, as calculated by GraphPad Prism 4.0. Data was the combination of 2 independent experiments.

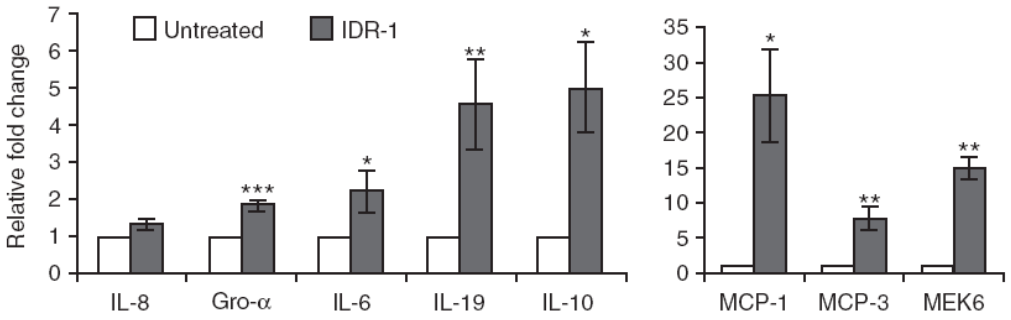


Figure 13**: Peptide IDR-1 selectively altered expression of pro-inflammatory genes in human PBMCs.** Freshly isolated human PBMCs were stimulated with 200 μg/ml of IDR-1 for 4 hours. RNA was isolated and analyzed by qPCR. Data is normalized to GAPDH and relative to unstimulated controls. Results show are mean +/- SEM, and was consolidated from 4 independent donors (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Experiments performed by Neeloffer Mookherjee.

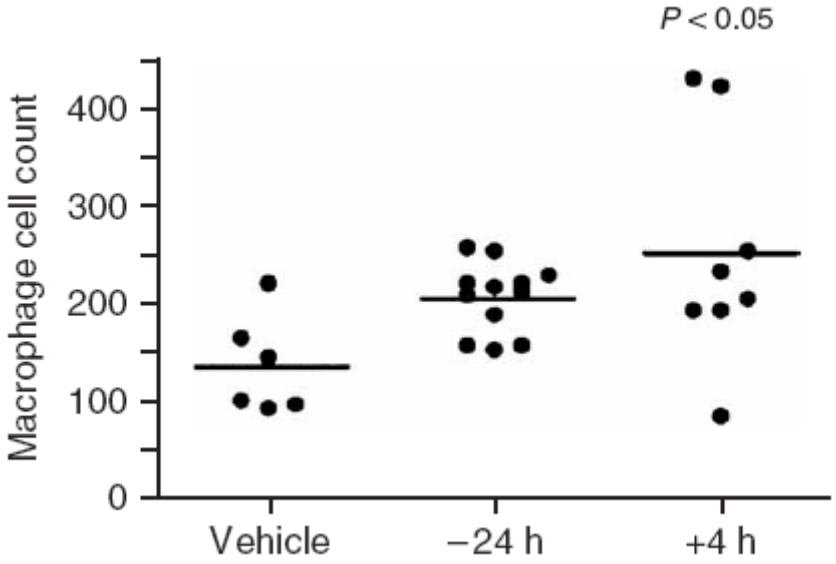


Figure 14**: IDR-1 caused an increase in peritoneal monocytes.** Mice were injected with 24 mg/kg IDR-1 IP at the listed times. At time = 0, mice were infected with approximately 5.0 x $10^8$ CFU *S. aureus* for a total of 24 hours. Mice were then euthanized and peritoneal lavage obtained. Monocyte numbers were determined by cytospin fractionation, Wright-Giemsa staining and counting under a light microscope. Measurements were performed by Edie Dullaghan in conjunction with the studies in Figure 12.

### 4.2.2. HH-peptides

Despite the proven activity of IDR-1 *in vivo*, it required a very high dose of peptide for protective immunomodulatory activity to occur. *In vitro* studies with IDR-1 showed that a concentration of 200 μg/ml was required for any activity (as illustrated in Figure 13). In mice, the optimal dosing for effective protection was 24 mg/kg. Compared to traditional antibiotics, which have an effective dose of as low as 1 mg/kg, IDR-1 is perhaps not optimal in its potency. In addition, the legal rights to IDR-1 belonged to Inimex Pharmaceuticals, and there were limits to the experiments that we could do and publish with IDR-1.

To address these issues, we endeavored to create new immunomodulatory peptides with increased activity. New peptides would help in understanding the structure-function relationships that govern immunomodulatory activity, as well as to produce smaller and more effective peptides to lower the cost of manufacturing large quantities. The first series of new peptides were designed by Dr. Kai Hilpert, using amino acid substitution and high-throughput screening (as detailed above). This first batch of novel peptides were called the HH-peptides, and are based on a variety of template peptides, including the bactenicin derivative Bac2A.

After *in vitro* screening for chemokine release as outlined above, peptides HH-2 and HH-18 displayed the highest activity, and were thus tested for their ability to protect against infection in an *S. aureus* peritonitis model. HH-17 is a sequence scramble of HH-18, and was used as a negative control, as it did not release a significant level of chemokines in the *in vitro* screen. Mice were pretreated IP with 8 mg/kg HH-peptides, and infected IP 4 hours later with *S. aureus.* Twenty-four hours after infection, mice were

euthanized, peritoneal lavage collected, and plated for bacterial enumeration. Figure 15 demonstrates that HH-2 demonstrated greatly improved activity over IDR-1. At 8 mg/kg, a third of the dose of IDR-1, HH-2 was capable of reducing bacteria load by up to 4-log fold. Indeed, some mice had completely cleared the infection and no bacteria were recovered. HH-18 showed some activity but there was significant variability in the data. Curiously, the negative control peptide HH-17 demonstrated a non-significant trend towards minor protection, although this might reflect the inherent variability of this animal model.

To determine if HH-2 and HH-18 could change the expression of cytokines and chemokines in animals, a study was conducted to look at the effect of these peptides on several chemokines and cytokines involved in the inflammatory response. The mouse inflammation Cytometric Bead Array (CBA), a bead based kit that uses FACS to measure protein levels, was utilized. The analytes in this kit were TNF-α, MCP-1, IL-12, IFN-γ, IL-10 and IL-6. Figure 16 demonstrates that while there was a non-significant trend in reduction of TNF-α, HH-2 or -17 did not significantly alter other cytokine or chemokine levels *in vivo*, in contrast to earlier observations from the *in vitro* experiments and results from peptide IDR-1. This may be due to several reasons. First, in the *in vitro* experiments there was no stimulation from an inflammatory agent such as LPS, whereas the bacteria in the animal model would act as a potent inflammatory stimulus. Another reason might be that *in vivo* there is a diverse range of cell types, including resident macrophages, dendritic cells (DCs), lymphocytes, γδ T cells and other populations, that would be exposed to peptides, whereas the *in vitro* experiment only blood PBMCs were treated with peptide. There could also be cell to cell communication that changes or dampening

of the peptide response *in vivo.* Lastly, the proteolytic byproducts of peptides could have activity of their own, raising the possibility that a certain peptide fragment is the active component. However, despite the lack of apparent chemokine induction by HH-2, we have succeeded in improving peptide immunomodulatory and protective activity *in vivo*, demonstrating that the peptide array method could be used to optimize peptide activity.

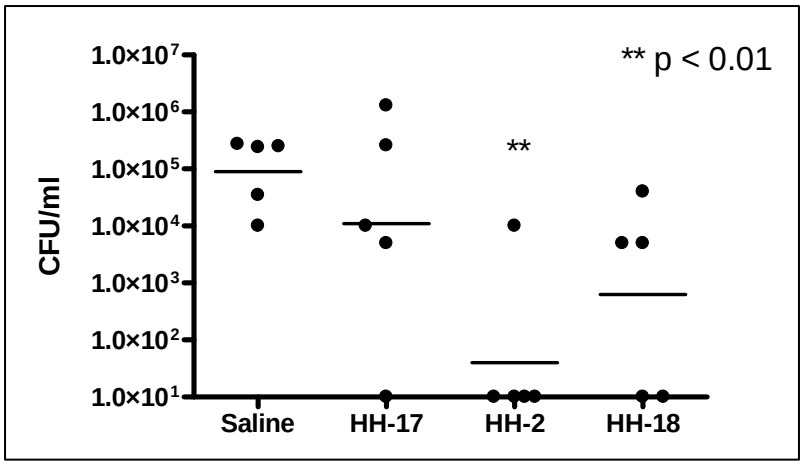


Figure 15**: Synthetic immunomodulatory peptide HH-2 reduced bacterial load in vivo.** Mice were pretreated with 4 mg/kg peptide (IP), and infected 4 hours later with approximately 1.0 x $10^9$ CFU *S. aureus*. Twenty-four hours post infection, mice were euthanized and peritoneal lavage taken, and plated on MH agar. Animals that died from the infection are assigned the highest colony counts observed in the experiment. The graph is representative data from a series of 2 independent experiments.

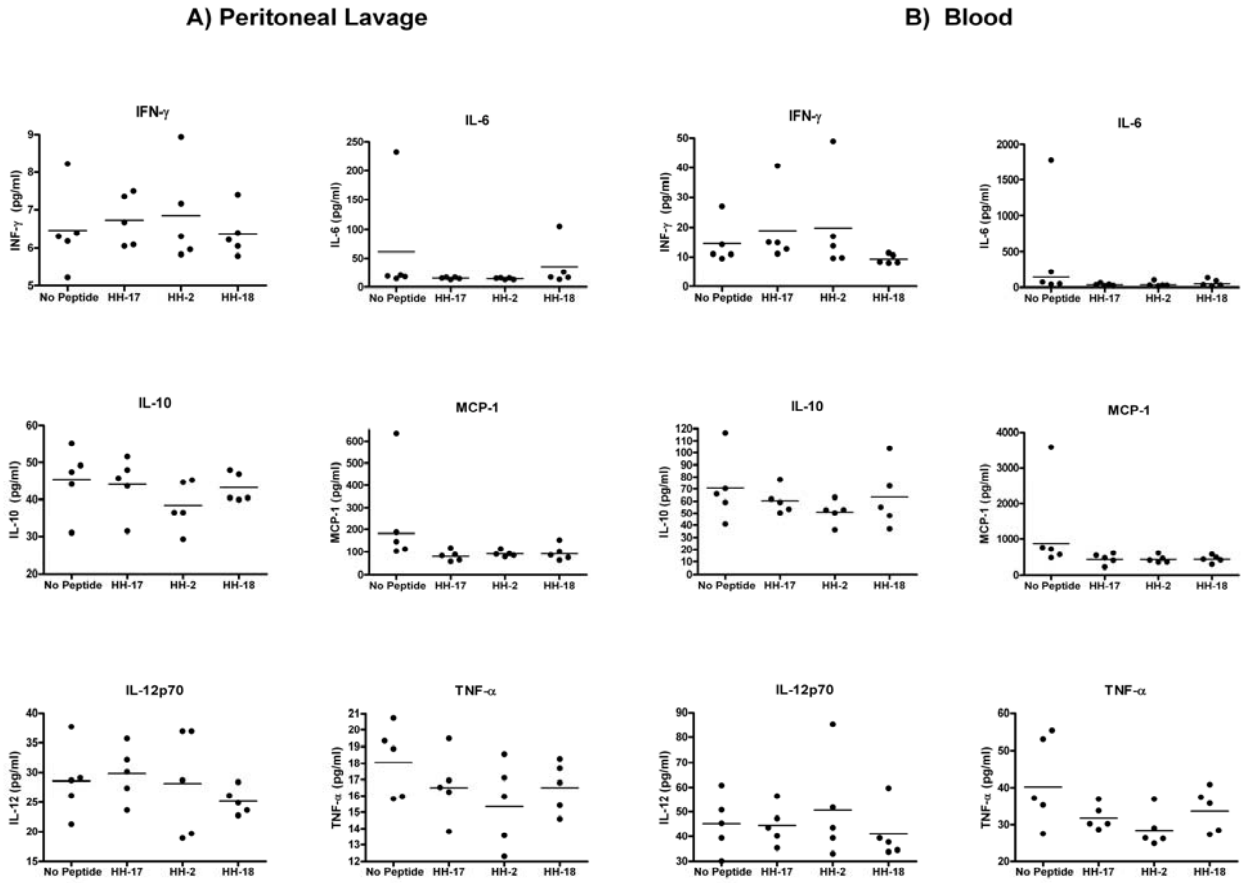


Figure 16**: HH peptides did not alter the regulation and release of proinflammatory cytokines and chemokines in vivo.** Female CD-1 mice were pretreated IP with 8 mg/kg of peptide for 4 hours, then infected for 24 hours with ~1.0 x $10^9$ CFU *S. aureus*. Animals were euthanized and peritoneal lavage and blood taken. *A* depicts results from peritoneal lavage, *B* depicts results from serum. Samples were analyzed by Cytometric Bead Array.

### 4.2.3. 1000-Series Peptides

To further optimize peptide activity, the peptide array procedure (Hilpert, Winkler et al. 2007) was repeated again, this time using HH-2 as a template. The resulting batch of peptides was called the 1000-series, consisting of dozens of 12-mers. These were again screened for antimicrobial activity, followed by PBMC chemokine secretion. The chemokine screen (Figure 10) showed that peptide 1012 stimulated the release of 3-4 times more MCP-1 than the parent peptide, HH-2. However, most of the other novel peptides induced equivalent or less chemokine secretion compared to HH-2.

For the first animal experiment utilizing these new peptides, peptides 1012 and 1002 were utilized. Figure 17 depicts that peptide 1002 demonstrated a non-significant trend towards reducing peritoneal *S. aureus* levels. Two animals treated with peptide 1002 completely cleared the infection. The standard of health of all animals treated with 1002 was also dramatically increased over the other peptides. In contrast, peptide 1012, despite having stimulated a huge amount of MCP-1 secretion from PBMCs in initial experiments, did not significantly reduce bacterial counts *in vivo*. While this might indicate that *in vitro* chemokine release and *in vivo* protection do not necessarily correlate and many other parameters need to be assessed to fully correlate *in vitro* immunomodulatory activity of peptides in our assay with *in vivo* protective activity, this result was clouded by batch to batch inconsistencies in 1012's ability to induce chemokines in human PBMC. Interestingly, the parent peptide HH-2 did not show similar levels of protection as shown in Figure 15, possibly due to the use of an outbred mouse strain (CD-1) as the host. The different genetic backgrounds between animals may change peptide activity.

I next investigated the activity of peptide 1002 in the C57BL/6J inbred mouse strain, to observe if animal genetics plays a role in peptide mediated immunomodulation. These mice are reportedly more susceptible to infections, so the protocol was altered slightly. Less bacteria were used (approximately $1.0 \times 10^8$ CFU/mouse), and no mucin was used with the bacterial solution. Figure 18 demonstrates that peptide 1002 showed a trend in reduction of bacterial load in the C57BL/6J strain, however the data was not statistically significant. With 4 animals that had completely cleared the infection, the data possibly indicates that 1002 is more effective in C57BL/6J mice, although this may be due to the lack of mucin used in this experiment. *S. aureus* binds to mucin and temporarily protects the bacteria from host defences, giving the bacteria time to establish and invade other tissues (Shuter, Hatcher et al. 1996). A lack of mucin would allow host neutrophils, resident macrophages, and non-cellular antimicrobial agents earlier access to the bacteria and thus might lead to more effective clearance, creating a less harmful infection. Indeed, the control mice showed only mild symptoms of infection at the time of euthanasia, indicating that the infection was much less severe compared to earlier experiments (data not shown).

I then determined if peptide 1002 was capable of modulating inflammatory cytokine and chemokine secretion *in vivo*. In addition to the previous analytes in Figure 16, I also examined the chemokine RANTES via ELISA. Figure 19 demonstrates that there was a small but significant change in some inflammatory mediators *in vivo* in the presence of peptide 1002. Interestingly, the type II interferon IFN-γ was significantly upregulated by a factor of 2-fold. This cytokine is involved in macrophage activation and promotion of the $T_{H1}$ response, and is secreted by NK and T cells, indicating a possible

target cell population for 1002. In addition, the monocyte chemokine RANTES was also significantly upregulated. MCP-1 was slightly but not significantly upregulated with 1002. Lastly, similar to HH-2, TNF-α was slightly but not significantly downregulated by 1002, implying that this peptide might have some anti-inflammatory activity *in vivo.*

RANTES is produced by CD8+ T-cells and is chemotactic towards T-cells as well as leukocytes; thus peptide 1002 might have a possible role in stimulating adaptive immunity. To examine whether leukocyte cell populations in the peritoneum were altered in response to peptide, the experiment was repeated with the aim of observing cell populations recruited into the peritoneum. Unlike the infection model, mice were euthanized and samples collected after 4 hours of infection, rather than the full 24 hour infection, since it was found that after 24 hours there were no detectable differences in peritoneal cell populations. Figure 20 demonstrates that peptide 1002 changed cell recruitment in the presence of infection. Figure 20A depicts total cell numbers isolated from the peritoneum (as determined by light microscopy), and it was determined that peptide 1002 caused a significant increase in cells, relative to the bacteria alone control. Figure 20B depicts the peritoneal neutrophil population, as determined with FACScan by staining for GR-1. Peptide 1002 caused a trend towards an increase in neutrophil numbers, however the data was not statistically significant. Figure 20C depicts monocyte populations by staining for F4/80, and peptide 1002 caused a significant increase in monocyte numbers. Lastly, Figure 20D depicts resident macrophage numbers as determined by $F4/80^{high}$ and $GR\text{-}1^{negative}$. While the data was not significant, there was a slight trend towards less resident macrophages in the presence of 1002 relative to the bacteria alone control. The decreased recovery of resident macrophages in the peptide-

treated mice may be the result of macrophage adherence to the walls of the peritoneal cavity. Alternatively, this might indicate that peptide-stimulated cells were leaving the site of injection, most likely migrating to the draining lymph nodes. There, they could release factors such as cytokines and chemokines to affect cell recruitment. It would be of interest to isolate the parathymic and the mesenteric lymph nodes draining the peritoneum, to examine if any increased cell migration and differentiation occurs there following peptide treatment.

It was previously reported that LL-37 was capable of increasing surface expression of dendritic cell maturation markers CD11c, and changing cell morphology (Davidson, Currie et al. 2004). Macrophage maturation markers CD80, CD86 and MHC-II were examined to compare macrophage maturation status between the control and peptide-treated animals; however no significant effects were observed. One explanation could be that cells require a longer exposure to peptide in order for any such changes to take place. Davidson *et al.* used a 7-day stimulation with LL-37, so perhaps 1002 might affect DC morphology at a later time point. Further research on this matter is needed.

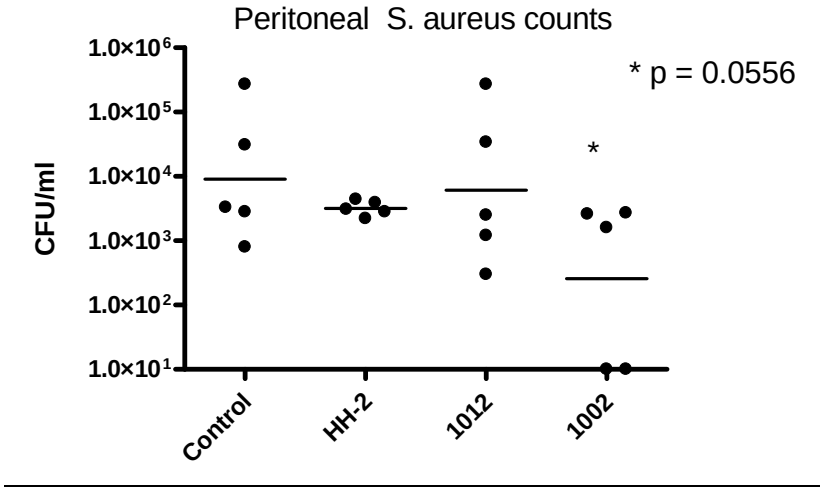


Figure 17**: Testing HH-2 derivative peptides 1012 and 1002 in an in vivo infection model.** Female CD-1 mice were IP treated with 4 mg/kg peptides. Four hours later, mice were infected IP with approximately 1.0 x $10^9$ CFU of *S. aureus.* Twenty-four hours post-infection, mice were euthanized and samples collected. Lavage was diluted in sterile PBS and plated on MH agar plates. 1002 pre-treated animals showed a strong trend but not significant trend towards reduced bacterial levels in the lavage. The graph is representative data from a series of 3 independent experiments.

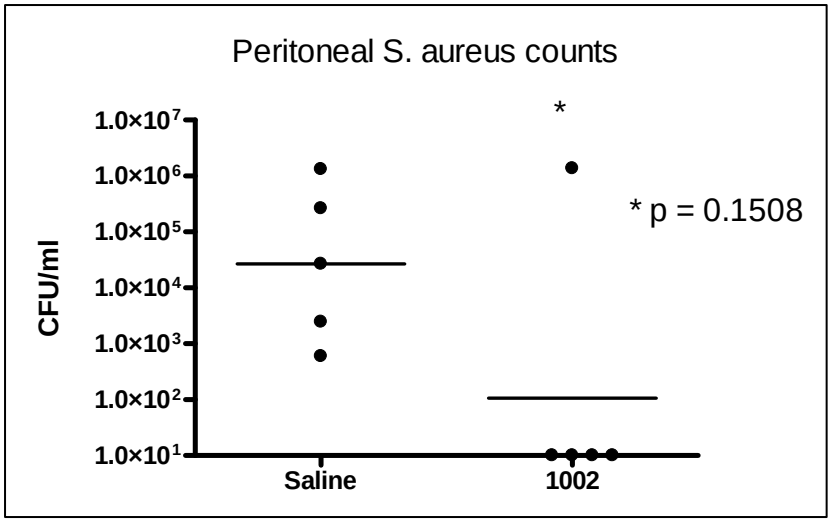


Figure 18: **Peptide 1002 reduced bacteria counts in inbred mouse strain C57Bl/6J**. Ten female C57Bl/6J mice were pretreated with 200 μg of peptide 1002 IP. Four hours later, mice were infected with ~1.0 x $10^8$ CFU of *S. aureus,* also IP. Twenty-four hours post-infection, mice were euthanized and samples collected. Lavage was diluted in sterile PBS and plated on MH agar plates. The bar represents geometric mean, as determined by GraphPad Prism 4.0. Data not significant to a confidence level of 95%. The graph is representative data from a series of two independent experiments

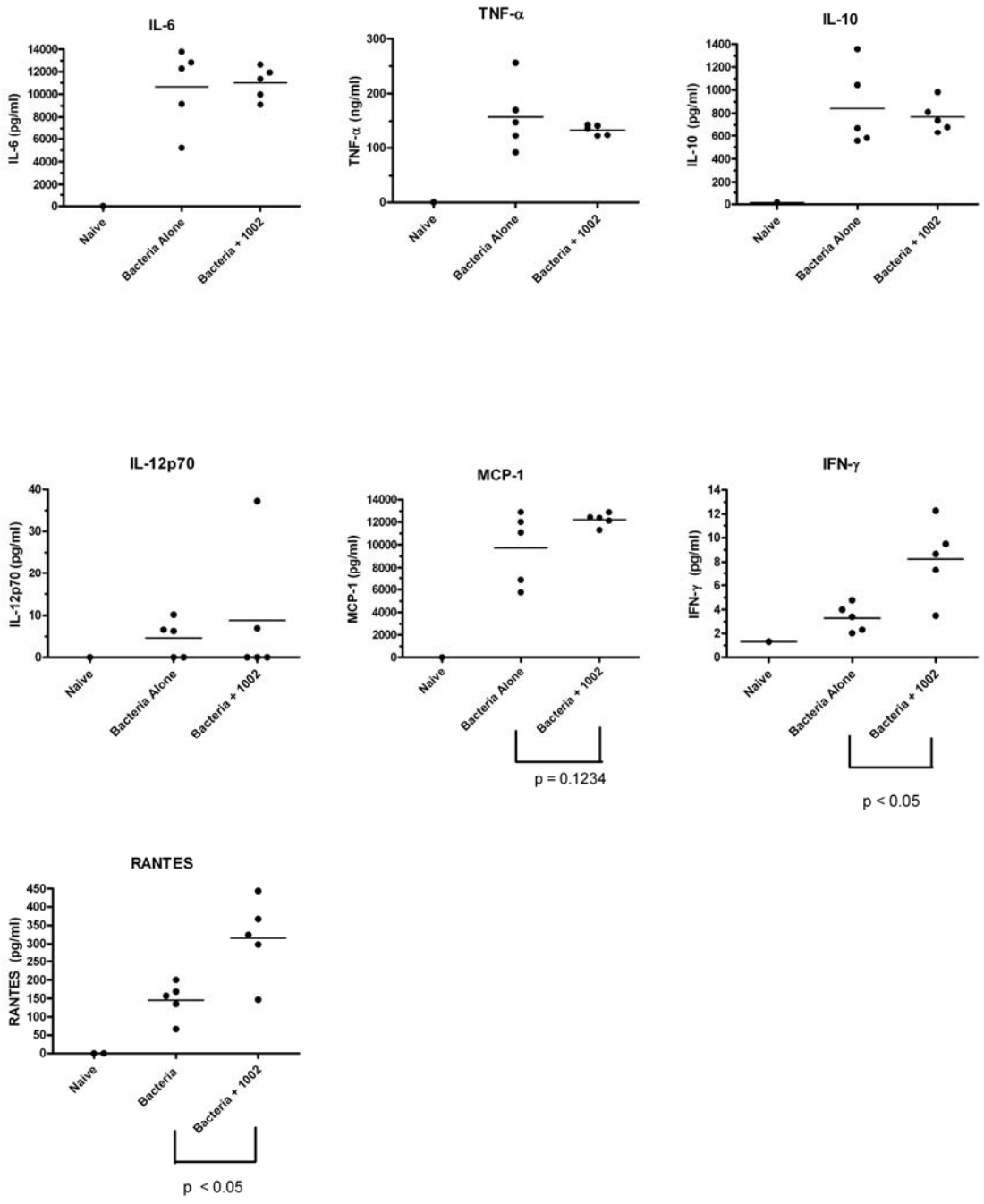


Figure 19**:** ***In vivo*** **peritoneal chemokine and cytokine production in the presence and absence of peptide 1002.** Mice were pretreated IP with 4 mg/kg of peptide 1002. Four hours later, mice were IP infected with ~1.0 x $10^9$ CFU/ml *S. aureus*. The infection proceeded for 4 hours before euthanasia and sample collection. Samples were spun and the supernatant withdrawn and analyzed by Cytometric Bead Array (Mouse Inflammation Kit, BD). RANTES analyzed via ELISA. The bar represents the arithmetic mean as calculated by GraphPad Prism 4.0.

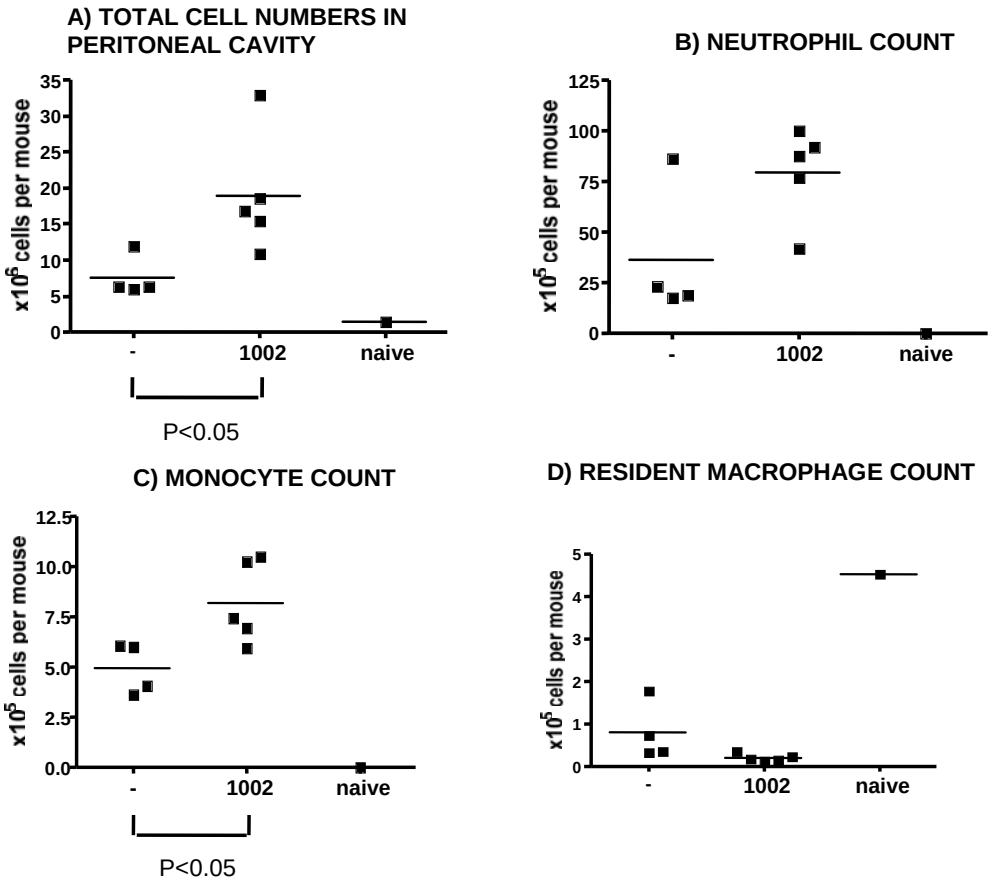


Figure 20**: Peptide 1002 increased peritoneal cell recruitment of both neutrophils and monocytes.** CD-1 mice were pretreated with 4 mg/kg 1002 for 4 hours, then infected with ~1.0 x $10^9$ CFU of *S. aureus* for 4 hours. Mice were euthanized and peritoneal contents washed with 5 ml PBS. Cells were Crystal Violet stained and counted on a light microscope. Cells were then diluted to 5.0 x $10^5$ cells and stained with labeled antibodies for FACS analysis. *A* Total cell counts from the peritoneum. Cells were stained with Crystal Violet and counted under a light microscope. *B* Neutrophil counts of peritoneal cells. Cells were identified by gating by GR-1 and forward scatter (FSC)-low. Data not significant to a confidence level of 95%. *C* Monocyte counts of peritoneal cells. Cells were gated with F4/80 and FSC-high. *D* Resident macrophages from the peritoneum. Cells identified as being F4/$80^{high}$ and GR-$1^{low}$. Data not significant to a confidence level of 95%. This experiment was repeated twice, and representative data shown.

### 4.2.4: Heat-killed *Staphylococcus aureus*

*Staphylococcus aureus* possesses numerous toxins, including hemolysins, superantigens, and porins (Gordon and Lowy 2008), which could possibly influence an animals' response to peptide 1002. To confirm the previous results and to strengthen the hypothesis that peptide 1002 possesses immunomodulatory activity *in vivo*, the above experiment was repeated using bacteria that had been heat-inactivated. This approach generated a potent inflammatory response without the negative outcomes of an infection. An overnight culture of *Staphylococcus aureus* was centrifuged, washed in saline, and boiled for 30 minutes to destroy any viable bacteria and inactivate any toxins by denaturation. This process was effective at killing viable bacteria, reducing the ~1.0 x $10^9$ CFU/ml of an overnight culture to the detection limit of less than 10 CFU/ml. The animal injections were identical to those in Figure 20, involving a 4 hour peptide pre-treatment, followed by 4 hour treatment with the inactivated bacteria. Figures 21 depicts data showing that cell recruitment to the peritoneum did not depend on the presence of live bacteria. As before, monocytes were significantly increased with the presence of the peptide (Figure 21B), while neutrophils showed an increasing but not statistically significant trend (Figure 21C).

Lastly, the chemokine changes previously observed were confirmed. Figure 22 demonstrates that the pro-inflammatory neutrophil chemokine KC (mouse GRO-α homolog, Figure 22B) was significantly upregulated. MCP-1 (Figure 22A) showed an increasing trend, however the data was not statistically significant. Taken together, these results suggest that peptide 1002 is a true modulator of the inflammatory response,

working in combination with inflammatory signals to further enhance cell recruitment and clearance of the pathogen.

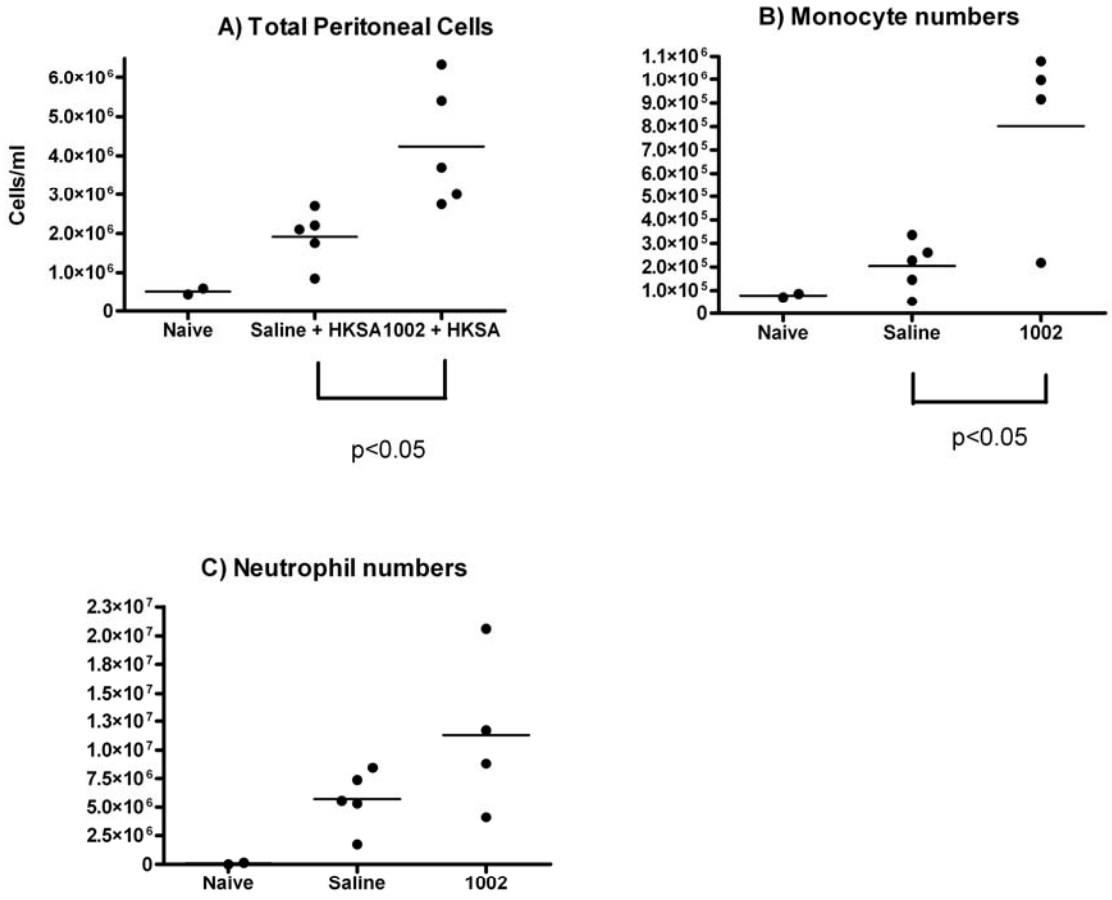


Figure 21**: Peptide 1002 increased cell recruitment to the peritoneal cavity following stimulation with heat-killed S. aureus.** C57Bl/6J mice were treated with 200 μg of peptide 1002 IP 4 hours prior to heat killed *S. aureus* injection. Four hours after killed heat-killed bacteria injection, mice were euthanized and peritoneal samples withdrawn. Total peritoneal cells were counted by Crystal Violet staining and counting under a light microscope. Monocyte and Neutrophil determination by FACS staining (F4/80 and GR-1 staining, respectively). *A* Total peritoneal cell counts *B* Peritoneal monocyte counts, *C* peritoneal neutrophil counts (not significant to a 95% degree of confidence). This experiment was repeated twice and representative data shown.

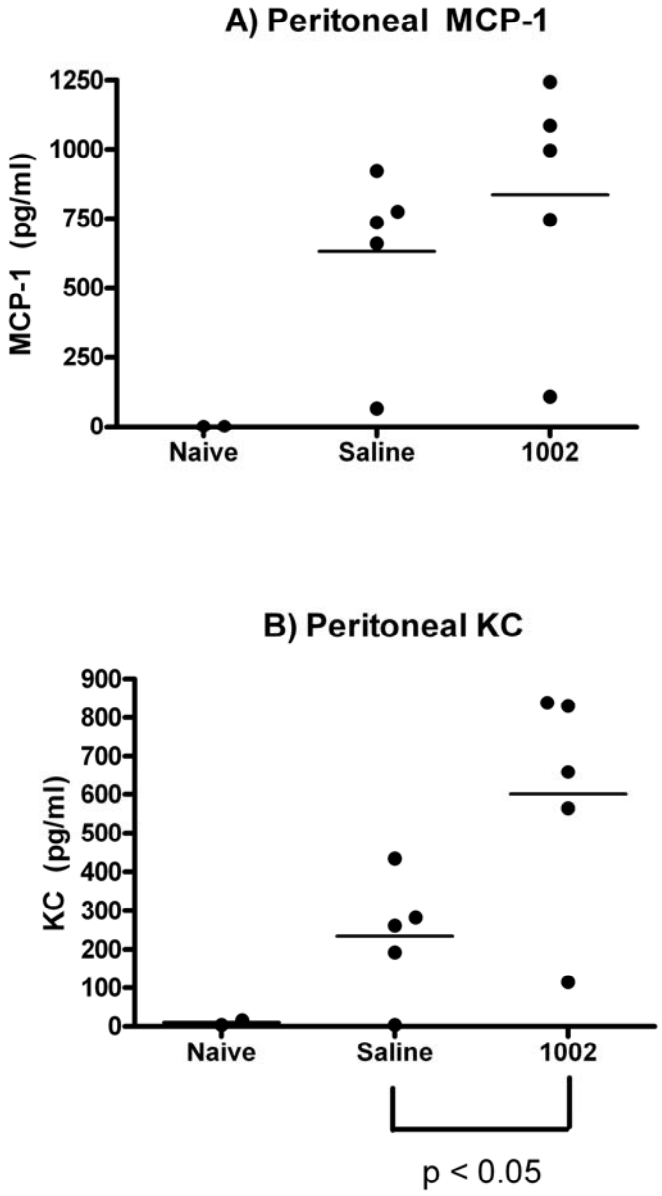


Figure 22**: Peptide 1002 increased secretion of Chemokines MCP-1 and KC from mice injected with heat-killed S. aureus.** C57Bl/6J mice were treated with 200 μg of peptide 1002 IP one hour prior to heat killed *S. aureus* injection. Four hours after killed heat-killed bacteria injection, mice were euthanized and peritoneal samples withdrawn. Chemokine levels determined via capture ELISA. This experiment was repeated twice, and representative data is shown.

**4.3.: Discussion**

These results, observed with 3 different peptides, support the concept that synthetic peptide immunomodulators have the potential to become a novel therapeutic for the treatment of bacterial diseases. All three were capable of reducing *S. aureus* loads *in vivo*; however, the next generation peptides HH-2 and 1002 were far more effective at reducing bacterial loads at lower doses.

As mentioned previously, immunomodulatory peptides were screened based on their ability to induce secretion of pro-inflammatory chemokines (MCP-1, GROα and IL-8) in human PBMCs. IDR-1 was capable of inducing MCP-1, however, peptide HH-2 was not able to induce any significant chemokine secretion *in vivo* relative to the no-peptide control. In contrast, peptide 1002 was able to significantly upregulate several proinflammatory cytokines and chemokines, including the monocyte chemokine RANTES and IFN-γ, a cytokine with potent monocyte activation activity. IFN-γ is produced by many cell types, including $Th_1$ cells, NK cells and some DC subsets. These results suggest several cell populations that may be the primary target population of peptide-mediated immunomodulation. A future experiment on this matter might be to use intracellular staining of IFN-γ in combination with traditional cell surface markers to observe which cell population is upregulating its IFN-γ production and/or secretion following peptide 1002 stimulation. Following that, the cell population can be purified by flow cytometry and *in vitro* experiments performed with the aim of better understanding peptide 1002 interaction with cells.

The flow cytometry experiments shown in Figures 20 and 21 showed that peptide 1002 was able to significantly increase monocyte cell recruitment into the peritoneum.

There was a trend of neutrophil recruitment into the peritoneum but the results were not statistically significant. It is currently unknown if this was due to the increased chemokine production or whether 1002 had direct chemotactic activity. There are a number of *in vitro* chemotactic assays that could be used to test this, using an appropriate cell line or harvested primary cells. Since chemotaxis is mediated through G-protein coupled receptors, inhibitors such as pertussis toxin can be used to observe if these receptors are required for chemotaxis. Alternatively, inhibitors specific for certain chemokine receptors could be used, such as the receptor fPRL-1, which has previously been shown to bind LL-37 (Yang, Chen et al. 2000), but that interaction is not necessary for chemotaxis (Bowdish, Davidson et al. 2004).

An interesting phenomenon observed with most peptides is that mice treated with peptide appeared significantly healthier than the control mice (unpublished observations). Symptoms of infection such as piloerection, decreased movement speed, closed eyes, hunched abdomen, and diarrhea were all typically less severe in peptide-treated animals. Even more interesting is that an analogous phenomenon has been observed in tissue culture experiments with live bacteria (unpublished observations). Epithelial or monocytic cells pretreated with peptide and infected with bacteria afterwards are not destroyed as quickly as the control cells, with fewer detached cells, and better maintenance of proper cell morphology. This may indicate that peptides may function in part through ubiquitous cell homeostasis and/or stress response pathways. *Ex vivo* analysis of cells harvested from peptide treated animals with techniques such as Western Blot or qPCR could be used to determine which pathways are responding to peptide treatment.

These results further strengthen the peptide array approach to generating novel peptides with enhanced activity. By using an already proven immunomodulatory peptide such as HH-2 as a template, we were able to generate new novel peptides with even greater activity. Peptide 1002 was able to induce significant levels of RANTES and IFN-γ, and caused a non-significant increasing trend in MCP-1 and a decreasing trend in TNF-α secretion. Furthermore, 1002 caused a significant increase in monocyte numbers recruited into the peritoneum, as well as a non-significant increasing trend in neutrophil numbers, and a slight non-significant decrease in resident macrophages. Taken together, these results indicate 1002 is a potent immunomodulator and further investigation into its mechanism of action is warranted.

# CHAPTER 5: MACROPHAGES ARE A CRUCIAL COMPONENT OF PEPTIDE MEDIATED IMMUNOMODULATION

## 5.1. Introduction

One observation made in the IDR-1 paper (Scott, Dullaghan et al. 2007) was that immunomodulatory peptides are able to exert their effects over a long period of time. IDR-1 was capable of reducing bacteria loads when administered up to 48 hours prior to infection. In addition, the peptide was capable of working systemically. In a thigh-wound model, mice infected in one thigh and treated with peptide in the opposite thigh still led to a reduction in bacterial load. Given that peptides are degraded in serum within 15 minutes (Unpublished observations, Inimex Pharmaceuticals), it is currently unknown how peptides could continue to exert their effects after they are degraded.

We hypothesize that peptides are priming certain cell populations (possibly after uptake into these cells), changing their functions and activities, such that they migrate to the sites of infection and/or go on to stimulate or prepare other cells for infection, essentially enhancing certain immune functions including protective responses while preventing excessive inflammation.

There are a variety of cells that could be the cellular target of immunomodulatory peptides. Neutrophils are a key cell population in inflammation, and are involved in phagocytosing pathogens, chemotaxis to the site of infection and releasing antimicrobial compounds. However, they are a very short-lived cell population with a turnover rate of around 24 hours, so they seem to be unlikely candidates to carry the peptide signal to other parts of the body.

Another major cell population in inflammation are the monocytes, a leukocyte that can mature into a diverse range of effector cells. Monocytes exist mostly in the blood but cross into the tissues following an inflammatory signal. There, they differentiate into macrophages, phagocytes that digest pathogens, migrate to the lymph nodes, and participate in antigen presentation to $T_H$ cells. Furthermore, they are a longer lived cell population, with a turnover rate of several weeks.

Based on the physiology of monocytes, I hypothesized that immunomodulatory peptides somehow interacted with a cell of the monocytic lineage, altering its biology and activity into a "primed" state. These cells might then circulate through the body, and somehow interact with other cells, possibly through soluble factors, and reducing the amount of time it takes for cells to respond to an infection.

To test this hypothesis, we depleted animals of monocytes using the compound Clodronate. When Clodronate is encapsulated in liposomes and injected, monocytes phagocytose the particles and hydrolyse them intraceullarly. When Clodronate reaches a certain cytoplasmic concentration, apoptosis is triggered, killing the phagocyte and thus eliminating the cell population (Van Rooijen 1989). To account for any neutrophils that are also killed in the process, the liposomes are administered 5 days before the peptide is administered, to allow the neutrophil population time to replenish.

### 5.2. Results

This procedure was first attempted with peptide IDR-1. There were issues with the procedure that needed to be optimized. The dose of the liposomes and the purification protocol needed to be optimized to reduce adverse effects on the animals. Subsequent

refinement of the liposome purification process and lowering of the dose was tried and although mice became ill immediately after injection, they recovered afterwards. Elimination of monocytes, in the face of retention of a normal neutrophil population was confirmed by FACS. Figure 23 depicts that monocyte depleted animals did not respond to peptide treatment, and they did not show any significant bacterial reduction in contrast to non-clodronate treated infected mice.

To confirm these findings, the experiment was repeated in C57BL/6J mice, with peptide 1002. Figure 24 confirmed the results observed with IDR-1. Compared to figure 18, monocyte depleted animals did not respond to peptide 1002 treatment. Interestingly, depleted mice treated with 1002 appeared much worse health wise, and several died in the peptide group, compared to none in the control group, in addition to having higher bacterial counts. It was clear that peptide 1002 did not reduce bacterial levels in monocyte-depleted animals, supporting the hypothesis that a population of monocytic lineage are an effector cell in peptide-mediated immunomodulation.

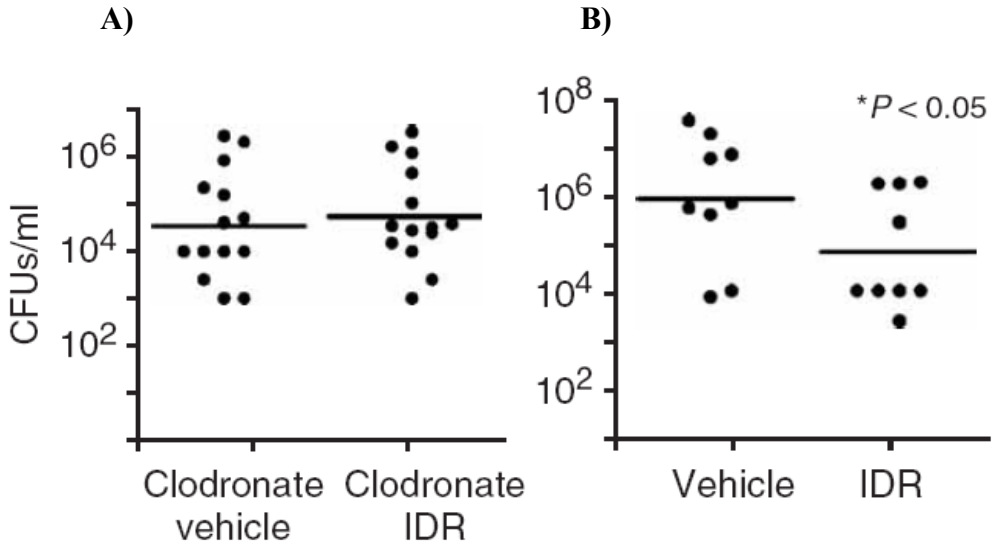


Figure 23**: Peptide IDR-1 did not protect monocyte-depleted animals from a *S. aureus* infection.** Female CD-1 mice were depleted with macrophages by an IP injection of Liposomal Clodronate. Four days following liposome injection, mice were pretreated with 24 mg/kg IDR-1 and infected with *S. aureus* 4 hours later. *A* depicts bacteria numbers from macrophage depleted animals, *B* bacteria numbers from normal animals. Mice were euthanized 24 hours post infection. Macrophage depletion in the peritoneum and blood was confirmed by FACS. Data is the combination of two independent experiments. The bar represents the geometric mean, as calculated by GraphPad Prism 4.0.

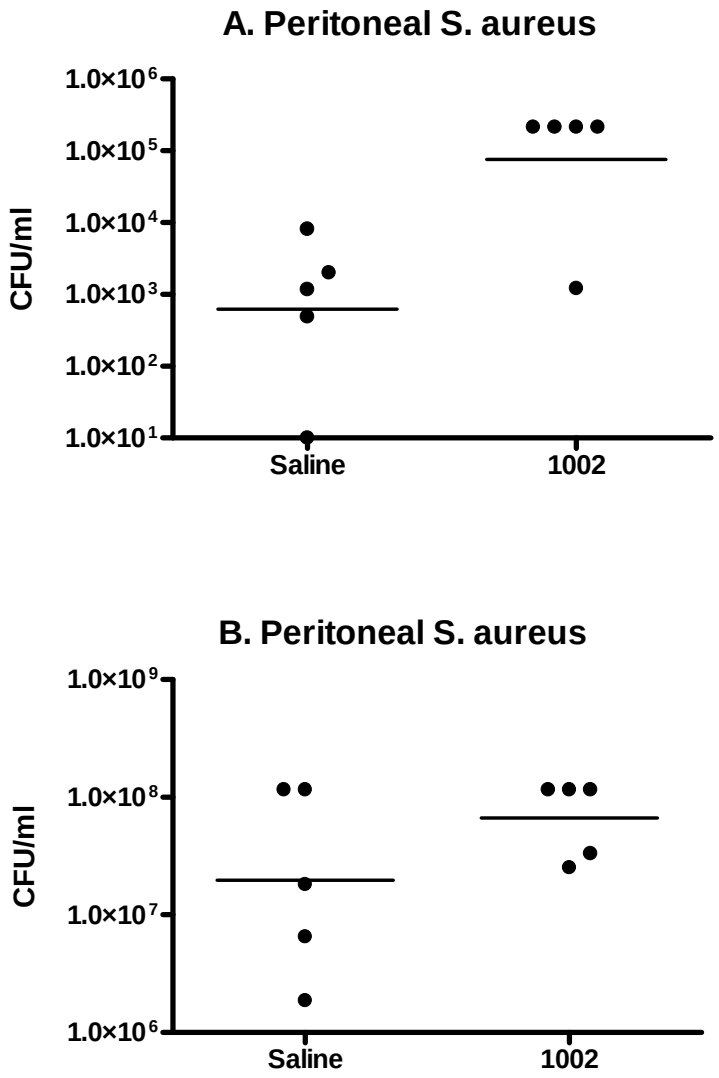


Figure 24**: Peptide 1002 did not protect macrophage depleted C57Bl/6J mice from a *S. aureus* infection.** Female C57Bl/6J mice were depleted with macrophages by an IP injection of Liposomal Clodronate. Four days following liposome injection, mice were pretreated with 200 μg of peptide 1002 and infected with ~1.0 x $10^9$ CFU *S. aureus* 4 hours later. Mice were euthanized 24 hours post infection. Animals that died from the infection are assigned the highest observed colony counts in the experiment. Figure *A* and *B* are the results of 2 identical experiments. Monocyte depletion in the peritoneum and blood was confirmed by FACS.

### 5.3. Discussion

These results support my hypothesis that monocyte-lineage cells are a key effector cell for peptide mediated immunomodulation. Mice that lack functional macrophages were not able to reduce bacterial loads when pretreated with peptide. Indeed, the results in Figure 24 suggested that depleted mice treated with peptide were in worse condition than the control mice. This is reflected both in bacterial numbers but also in the animals' health. Survivors in the peptide group were in very poor health, and many appeared to be close to death. It is unknown why this happened; however it might be that the peptide possessed toxic activity, and without monocytes to possibly uptake and sequester the peptide, the peptides' toxicity becomes apparent.

Monocytes are a diverse cell population, with a variety of activation states and surface markers. Most monocytes circulate in the blood, but some exist in the tissues, responding quickly to invading pathogens. During the inflammatory response, most monocytes enter the tissues approximately 24 hours following infection, but new evidence suggests that another monocyte population rapidly enters the tissues following infection and participates in the recruitment of neutrophils (Henderson, Hobbs et al. 2003). Research is underway to identify which specific monocyte population is the key effector population, which may further explain how immunomodulators can function over long periods of time.

# CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

My results indicate that synthetic peptides, antimicrobial or immunomodulatory, are capable of reducing bacterial load in a murine infection model. This strengthens the potential of peptides as novel therapeutic agents against bacterial infections. Table 6.1 presents a summary of the peptides studied here and the animal model data achieved.

The results with the designed short antimicrobial peptides showed that it is possible to use computer models to predict and design peptide sequences with superior activity. Novel peptide HHC-10 demonstrated superior antimicrobial activity over MX-226, a peptide in clinical trials, and was capable of reducing peritoneal bacteria load when administered intravenously. This powerful new technology gives researchers the capability to design better, more efficient compounds. Furthermore, this process can theoretically be used for any peptide compound, and efforts are underway to use the QSAR/neural network process on immunomodulatory peptides, to determine if their efficacy can be improved via this method.

In addition, the peptoid derivative Compound 5, with a chemically altered amino acid backbone, also displayed significant antimicrobial activity in animals. Given that these compounds are created with altered amino acids, they are in principle highly resistant to endogenous proteases, promoting peptide stability and thus a longer active period *in vivo.* However it is worth noting that they had issues with toxicity and where not especially better than the lab-designed antimicrobial peptides so it is as yet unclear if they will offer any major advantages.

We also investigated the activity of immunomodulatory peptides, the primary activity of which is not the direct killing of bacteria, but rather the enhancement of the

cells of the innate immune system. This activity prepares the immune system towards an infection, allowing for a heightened and more rapid response. Three immunomodulatory peptides were studied; IDR-1, HH-2 and 1002. All three were able to reduce bacterial load *in vivo*, with the later 2 being more effective. Peptide 1002 also induced significant chemokine and cytokine secretion profiles, enhancing the release of chemokines MCP-1 and RANTES, and the cytokine IFN-γ. Furthermore, peptide 1002 enhanced recruitment of both neutrophils and monocytes to the site of infection, while also possibly enhancing the migration of resident monocytes away from the peritoneum. This observation was not dependent on live bacteria, peptide 1002 was capable of inducing chemokine levels and significantly increasing monocyte numbers in the peritoneum when animals were stimulated with heat-inactivated *S. aureus*. We examined monocyte and DC maturation markers, however no cell differentiation events were observed.

Lastly, we briefly examined the mechanism of activity for two immunomodulatory peptides, IDR-1 and 1002. We hypothesized that macrophages are a key effector cell in immunomodulation; thus we used macrophage-depleted mice to observe if they were capable of responding to peptide treatment. Depleted mice did not show any reduction of peritoneal bacterial numbers in response to peptide, supporting the hypothesis that macrophages mediate the peptide signal *in vivo*.

As mentioned in the text, mice would occasionally succumb to an infection and die prematurely. Our practice was to assign these animals the highest bacteria count observed among surviving animals. Another approach in interpreting the data would be to express the data as a percentage of peptide treated animals that display a certain number of CFU/ml less than the control (for example 2 logs lower). Another potential readout

would be to use animal health, as peptide treated animals appear much healthier than controls, however care must be used to reduce any bias for this subjective readout.

These results show that synthetic peptides have the potential to become a novel therapeutic for the treatment of bacterial diseases, although significant challenges need to be addressed before clinical trials can be pursued. Issues like peptide stability, toxicity, efficacy, storage and method of delivery need to be addressed before further and more detailed *in vivo* experiments must be conducted. Research is underway at several collaborating institutions to address these issues, and have shown promising results. These results offer a potential novel source of anti-infective treatments, and with further research and development, could be included in the arsenal of weapons to combat bacterial infections.

Table 4: **Summary of peptides studied and *in vivo* activity observed.**

| Peptide | Sequence | Mode of Activity | In Vivo protection | In Vivo TNF-α reduction | Chemokine/Cytokine upregulation[1] | Activity dependant on macrophages? |
|---|---|---|---|---|---|---|
| **IDR-1** | KSRIVPAIPVSLL | Immunomodulator | Yes | Yes | IL-10, MCP-1 | Yes |
| **HHC-10** | KRWWKWIRW | Antimicrobial | Yes | ND | ND | ND |
| **HHC-36** | KRWWKWWRR | Antimicrobial | Yes | ND | ND | ND |
| **Compound 5** | N/A | Antimicrobial | Yes | ND | ND | ND |
| **5NHis$_{6,12}$** | NA | Antimicrobial | No (Lethal Toxicity) | ND | ND | ND |
| **HH-2** | VQLRIRVAVIRA | Immunomodulator | Yes | Yes | No significant regulation | ND |
| **HH-17** | KIWVRWK | Immunomodulator | No | No | No significant regulation | ND |
| **HH-18** | IWVIWRR | Immunomodulator | Yes | No | No significant regulation | ND |
| **1002** | VQRWLIVWRIRK | Immunomodulator | Yes | No | KC, MCP-1, IFN-γ, RANTES | Yes |
| **1012** | IFWRRIVIVKKF | Immunomodulator | No | No | ND | ND |

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