IMMUNOMODULATORY EFFECTS OF SYNTHETIC PEPTIDE IDR-1018 IN HUMAN KERATINOCYTES

by

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Abstract

The diverse immunomodulatory properties of naturally occurring host defence peptides have gained prominence over the past decade. There is large interest in creating small synthetic peptides with similar or enhanced immunomodulatory activities. The epithelia are vital components of the human innate immune system, offering protection against potential pathogens by acting as a physical barrier and actively participating in the immune response. The aim of this study was to examine the direct effects of synthetic host defence peptides on the immune response in keratinocytes, the primary cell type in skin, in the context of wound healing, and to evaluate their ability to modulate the keratinocyte immune response in the presence of other immune mediators.

The synthetic peptides HHC-36 and IDR-1018 were shown to have a positive effect on keratinocyte proliferation and caused a dose-dependent induction of IL-6 and IL-8. Neither peptide was able to influence keratinocyte migration on its own. The immunomodulatory effects of IDR-1018 in human keratinocytes were further investigated by co-stimulating cells with IDR-1018 in the presence of immune mediators or TLR agonists. Co-treatment of keratinocytes with IDR-1018 and either the TLR3 agonist poly(I:C) or IL-1β resulted in a synergistic induction of IL-8. This synergy could be seen transcriptionally 8 hours post-stimulation and was associated with increased levels of phosphorylated CREB. Synergistic IL-8 induction was not observed when IDR-1018 was given with Pam3CSK4, flagellin or GM-CSF. Pre-treatment of keratinocytes with inhibitors of p38 MAPK, NF-κB or Src-family kinases suppressed the IDR-1018-induced synergistic IL-8 production in the presence of poly(I:C) or IL-1β. PKC was shown to play a role in the synergy induced by IDR-1018 and IL-1β, but not poly(I:C). The results of this study offer insights into the immunomodulatory properties needed to effectively enhance the protective abilities of the skin, and highlight the complexity of the mechanism of action of IDR-1018.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

B2M	beta 2-microglobulin
Bis	bisindolylmaleimide I
CREB	cAMP-responsive element binding protein
DMEM	Dulbecco's modified Eagle medium
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signaling-regulated kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
GRO-α	growth regulated oncogene alpha
HaCaT	human adult low calcium temperature keratinocytes
hBD	human beta-defensin
HDP	host defence peptide
HEKa	human epidermal keratinocytes, adult
HKGS	human keratinocyte growth supplement
IFN	interferon
IL-	interleukin
IKK	IκB kinase
IRF	interferon regulatory factor
JNK	Jun N-terminal kinase
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MCP-1	macrophage chemoattractant protein-1
MEK	mitogen activated protein kinase/extracellular signaling-regulated kinase kinase

mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation protein-88
NF-κB	nuclear factor κB
PAMP	pattern associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PI3K	phosphoinositide-3 kinase
PKC	protein kinase C
Poly(I:C)	polyriboinosinic polyribocytidylic acid
PRR	pattern recognition receptor
RANTES	regulated and normal T cell expressed and secreted
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAK-1	transforming growth factor-beta-activated kinase-1
TBS	tris buffered saline
TBST	tris buffered saline + 0.1% Tween-20
TGF	transforming growth factor
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF-α	tumour necrosis factor alpha
TRAF	tumour-necrosis factor receptor-associated factor
TRIF	Toll/interleukin-1 receptor-domain-containing adaptor inducing interferon-beta

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CHAPTER 1: Introduction

1.1 Innate Immunity

All complex living organisms have a defence system to combat invading pathogens. The human defence system consists of two branches: the evolutionarily ancient, relatively non-specific innate immune system, and the highly specific adaptive immune system. The innate immune system is immediately available to respond to threats, unlike adaptive immunity which takes at least three days to become fully activated and functional (1). Moreover, an innate immune response is required to initiate the appropriate adaptive immune response (2).

The adaptive and innate immune systems employ different strategies to recognize pathogens. Adaptive immunity uses a seemingly limitless repertoire of clonally expressed, randomly generated receptors that are highly specific. Innate immunity uses germ-line encoded pattern recognition receptors (PRRs) to recognize so-called pathogen associated molecular patterns (PAMPs), a group of molecular signature molecules unique to microbes (3, 4). These microbial signatures include lipids, nucleic acids, proteins and lipoproteins that are loosely conserved in broad classes of microorganisms. They include functionally indispensable molecules, such as flagellin, lipopolysaccharide (LPS) and double-stranded RNA. As such, they have relatively conserved structures, which render them excellent triggers for the innate immune response (4, 5).

PRRs are expressed on various immune cells, such as macrophages, neutrophils and dendritic cells, but also on non-immune cells like fibroblasts and epithelial cells. PRRs are constitutively expressed, but their expression is quickly modulated in response to pathogens, cytokines and environmental stressors (6). The first PRRs to be recognized were the Toll-like receptors (TLRs). TLRs are type I membrane glycoproteins containing extracellular domains with varying numbers of leucine-rich repeats (LRRs) responsible for PAMP recognition, and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain that is homologous to the IL-1 receptor and required for downstream signaling (5). Ten TLRs have been identified in humans and are localized on the cell surface (TLRs 1, 2, 4-6, 10) or within intracellular compartments (TLRs 3, 7-9) (7). Since the discovery of TLRs, several additional classes of PRRs have been identified. These include the cytosolic Retinoic acid-inducible gene (RIG)-I-

like receptors (RLRs), the Nod-like receptors (NLRs) and the transmembrane C-type lectin receptors (CLRs).

1.1.1 Major components of innate immunity

Engagement of PRRs with their respective ligands triggers the activation of signaling pathways that generally converge at key transcription factors, like nuclear factor-κB (NF-κB), interferon regulatory factors (IRFs), activator protein 1 (AP1) and nuclear factor of activated T cells (NFAT). These transcription factors can combine with one another to induce genes crucial to mounting an immune response. The specific genes that are induced depends on the cell type involved, the type of PRR engaged and the specific adaptor molecules recruited to that PRR (8). The immune response includes the secretion of inflammatory cytokines and chemokines, type I interferons (IFNs) and host defence peptides (HDPs) which results in the recruitment of monocytes, neutrophils and other leukocytes, activation of macrophages and the induction of IFN-responsive genes, all of which contribute to the direct killing of the invading pathogens. In addition, PRR signaling causes the maturation and activation of dendritic cells, thus contributing to the activation of the adaptive immune response (7).

Because TLRs are the most frequently studied PRRs, the downstream signaling pathways for those receptors are better defined compared to other PRRs to date. In general, TLRs dimerize after ligand binding and undergo conformational changes necessary for the recruitment of TIR domain-containing adaptor molecules to the TIR domain of TLRs. These adaptor molecules propagate downstream TLR signaling. Five TIR domain-containing adaptor molecules have been identified to date: myeloid differentiation protein-88 (MyD88), TIR-domain-containing adaptor inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM), TIR-associated protein/MyD88 adaptor-like (TIRAP/Mal) and Sterile-alpha and Armadillo motif-containing protein (SARM) (9, 10). Alternate or combinatorial use of these adaptor molecules contributes to the specificity of TLR signaling and subsequent responses.

TLR signaling can be broadly separated into two predominant signaling pathways: the MyD88-dependent pathway and the TRIF-dependent (or MyD88-independent) pathway. The two pathways have different kinetics, but both result in the induction of pro-

inflammatory cytokines. The TRIF-dependent pathway induces the type I IFN response in addition to the pro-inflammatory response (11, 12). Briefly, the MyD88-dependent pathway results in early activation of the transcription factor nuclear factor-κB and the mitogen activated protein (MAP) kinases (MAPKs), culminating in the induction of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α. In this pathway, the MyD88 adaptor molecule and IL-1R-associated kinase (IRAK) family of protein kinases are recruited and cause the activation of tumour necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 catalyzes the formation of a polyubiquitin chain on IκB kinase- γ (IKK-γ)/ NF-κB essential modulator (NEMO) and causes the activation of the TGF-β-activated kinase 1 (TAK1). TAK1 phosphorylates IKK-β and mitogen activated protein kinase kinase 6 (MKK6). These modulate activation of NF-κB and the MAP kinases, resulting in the induction of pro-inflammatory cytokines (7, 11). MyD88 is used by all TLRs, except for TLR3.

TLR3 (exclusively) and TLR4 (alternatively) use the adaptor molecule TRIF to activate the alternative TRIF-dependent pathway that results in the production of IFNs and pro-inflammatory cytokines through activation of IFN regulatory factor 3 (IRF-3) and NF-κB, respectively (6, 7, 13). In this pathway, TRIF works with TRAF3 to activate TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) and inducible IκB kinase (IKK*i*/IKKε) which catalyze the phosphorylation and subsequent nuclear translocation of IRF-3 and IRF-7. This results in the production of IFNγ and IFN-inducible genes. In addition, TRIF recruits and forms a multi-protein signaling complex with TRAF6, TRAF3, Pellino-1, TRADD and receptor-interacting protein 1 (RIP1) to activate the NF-κB and MAPK pathways (5, 6).

1.1.2 Role of the epithelium in innate immunity

The epithelium is an avascular tissue that covers the exterior body surface, lines internal body cavities and forms the secretory portions of glands and their ducts. The skin which covers our exterior surfaces is the body's largest organ. It is made up of a stratified, cellular epidermis supported underneath by a dermal layer of connective tissue (14).

There are four different cell types in the epidermis: keratinocytes, melanocytes, Langerhans' cells and Merkel's cells. Of the four cell types, the keratinocyte is the predominant cell type and represents 95% of the total cells in the epidermis. Keratinocytes

produce keratin, the major structural protein of the epidermis that constitutes roughly 85% of fully differentiated keratinocytes. Keratinocytes originate from the epidermal basement membrane and move progressively towards the skin surface. As they migrate, they undergo a specialized type of apoptosis called terminal differentiation or cornification, which results in the formation of four well-defined layers in the epidermis (15).

The deepest layer in the epidermis is the stratum basale or stratum germinativum. This single-cell layer contains the stem cells from which new keratinocytes arise. The stratum spinosum is immediately above the basal cell layer and is several cells thick. Under a light microscope, the cells of this layer show numerous cytoplasmic processes or spines extending from cell to cell. The stratum spinosum is succeeded by the stratum granulosum. This layer is one to three cells thick and is named for the intracellular keratohyalin granules found within the cells of this layer (14). These granules contain the cysteine- or histidine-rich protein precursors of the protein filaggrin, which aggregates the keratin filaments into tonofibrils in a process called keratinization. Keratinization takes between two to six hours, during which the cells enter the next and final epidermal layer, the stratum corneum (16). An abrupt transition occurs between the stratum granulosum and the stratum corneum. The cornified cells in this outermost layer are the most differentiated cells in the skin. They become almost entirely filled with keratin filaments, losing their nucleus and organelles in the process (17). These cornified cells are also coated with an extracellular layer of lipids that forms the main constituent of the epidermal water barrier (18). The thickness of the stratum corneum varies throughout the body. It is thickest at sites where high amounts of friction may occur, such as the palms of the hands and the soles of the feet.

The skin has many important roles in the human body, including the regulation of body temperature, as well as sensory and autonomic functions. The structure of skin provides insight into the skin's primary function, which is to protect the body from the external environment. This includes preventing the free movement of water and electrolytes, absorbing radiation from the sun, reducing penetration by harmful chemicals and preventing the entry of microorganisms. Maintaining the integrity of the skin is critical to this barrier function, and the skin (and all epithelia) has the ability to repair any breaches through the process of wound healing. Wound healing is a complex, yet sophisticated physiological process that involves multiple cells and signaling pathways. The process can be divided into

four overlapping stages: inflammation, tissue granulation, re-epithelialization and remodelling (19).

Immediately after injury, platelets aggregate in the wound, leading to the formation of blood clots and the re-establishment of hemostasis. The inflammatory stage is initiated soon after when degranulating platelets, damaged cells and resident macrophages and mast cells release inflammatory mediators into the wound site, such as IL-1, IL-6 and IL-8, and growth factors like transforming growth factor-beta (TGF-β) and platelet-derived growth factor (PDGF) (20-22). These recruit leukocytes to the wound site, particularly neutrophils, which cleanse the wound of debris and invading bacteria, and monocytes, which become activated macrophages.

Monocytes and macrophages secrete a host of cytokines and growth factors crucial to the transition from the inflammatory stage to the formation of granulation tissue and re-epithelialization. These include IL-1, IL-6, fibroblast growth factor (FGF), epidermal growth factor (EGF), PDGF and TGF-β (21). Fibroblasts infiltrate the wound and begin to secrete various components of the extracellular matrix (ECM). These components, together with fibrinogen and fibrin from the blood clot, matrix molecules from macrophages and platelets, fibroblasts and endothelial cells, form the granulation tissue. Granulation tissue is a provisional matrix that offers a scaffold for the migration of epithelial cells and the re-establishment of the dermis. Within hours of injury, epithelial cells at the free edge of the wound proliferate and begin migrating over the provisional matrix. EGF, TGF-α and numerous members of the FGF family released by fibroblasts, macrophages and platelets have all been shown to stimulate epithelial cell proliferation and migration (23). In the final stage of wound repair, the granulation tissue is removed by migrating keratinocytes and replaced by a network of collagen and elastin fibres. The end result is not skin, but rather, scar tissue.

1.1.3 Active contribution of keratinocytes in the epithelium to immunity

As keratinocytes represent 95% of the cells in the epidermis, their primary function is often considered to be that of a physical permeability barrier. However, these cells also play an active role in the immune responses of the skin through the production and secretion of cytokines, chemokines and host defence peptides (HDPs), often referred to as antimicrobial

peptides in the literature (24-26). Studies show this chemical barrier function is co-dependent with the physical permeability barrier function, which highlights the equal importance of both roles (27, 28).

Immune mediators are barely detectable in unstimulated keratinocytes. However, upon stimulation by microbes, allergens, chemical reagents, UV light or physical trauma, keratinocytes express and secrete a wide variety of cytokines, chemokines, growth factors and host defence peptides (29). These include pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-18), chemokines (IL-8, MIP-3α, CCL2, CCL5, CXCL9-11), the anti-inflammatory cytokine IL-10, granulocyte colony-stimulating factor (G-CSF), transforming growth factor (TGF)-β, FGF-2, PDGF, the endogenous human cathelicidin LL-37 and human beta-defensins (hBDs)-1 through 4 (21, 30-32). Low expression of LL-37 and hBD-2 in the skin lesions of patients with atopic dermatitis has been linked to a higher susceptibility to skin infections by *Staphylococcus aureus* (33). On the other hand, increased levels of LL-37 can be found in the lesions of patients suffering from the non-infectious inflammatory skin diseases rosacea and psoriasis (34). LL-37 has been shown to be critical for activating the auto-inflammatory cascade in psoriasis (35).

Human keratinocytes express a variety of TLRs, although they do not express all TLRs. Primary human keratinocytes and the spontaneously immortalized human keratinocyte cell line HaCaT have been shown to constitutively express functional TLR2, TLR3, TLR5 and TLR10. TLR6 has only been found in primary keratinocytes, which also express TLR1 to a higher degree than the HaCaT cell line (36). TLR4 expression by keratinocytes remains unclear as some studies have shown TLR4 expression by keratinocytes while others have not (36, 37).

Studies show that activation of the different TLRs in keratinocytes can have varying downstream effects. Stimulation of keratinocytes with *Staphylococcus aureus* was demonstrated to cause a TLR2-dependent translocation of NF-κB and subsequent increase in inducible nitric oxide synthase (iNOS) and IL-8 (38). The use of polyriboinosinic polyribocytidylic acid (poly(I:C)), a TLR3 agonist, to stimulate keratinocytes resulted supernatants rich in TNF-α, IL-8, IL-18 and type I IFNs. These supernatants induced maturation of human monocyte-derived immature dendritic cells. The resulting dendritic cells strongly biased naive Th cells towards a Th1 phenotype (39). Interestingly,

keratinocytes stimulated with TNF-α and IL-1β resulted in IL-8 production, but did not lead to significant amounts of IL-18, TNF-α or type I IFNs. While the supernatants from these cells were likewise able to induce maturation of human monocyte-derived immature dendritic cells, the resulting dendritic cells promoted the development of a mixed population of Th1 and Th2 cells (39). These studies, among others, prove that keratinocytes do not merely express TLRs, but that activation of TLRs on keratinocytes can initiate a variety of effective innate and adaptive immune responses (40).

1.2 Host Defence Peptides

Short amphiphilic cationic peptides can be found in virtually every life form as part of their ancient defence system (41). Despite being classically recognized as broad-spectrum antibiotics, the direct antimicrobial activity of several natural cationic peptides is rather weak under physiologically relevant conditions. Instead, it is the ability of these peptides to contribute to host defence through modulation of the innate immune response that has gained greater prominence over recent years and vastly increased their potential as effective therapeutic treatments. Thus, host defence peptide (HDP) is a more appropriate term to capture the broader contribution that this group of molecules makes to immunity.

Host defence peptides are an extremely diverse group of molecules, with large sequence variation between peptides from related species. They are generally defined as short (10-55 amino acids) peptides with an overall positive charge (+2 to +9) and ≥ 30% hydrophobic residues (42, 43). These attributes allow HDPs, under appropriate circumstances (e.g. interaction with cell membranes), to adopt a three dimensional amphipathic shape in which cationic and hydrophobic amino acids are spatially arranged into separate sections of the molecule (44). Because the diversity of HDPs is so great, they are broadly separated into four categories on the basis of their secondary structure: α-helical peptides (such as human LL-37 and silk moth cecropin); β-sheet peptides containing two to four disulfide bridges (for example, human α- and β-defensins); loop peptides with one disulfide bridge (such as bovine bactenecin); and extended peptides with unconventional structures (for example, bovine indolicin).

All biologically active HDPs are derivatives of larger precursor proteins which have undergone proteolytic cleavage. Their expression can be either constitutive or inducible,

depending on the particular species, tissue type, cell type and cell differentiation state. PAMPs, microbe-produced products, injury or inflammatory stimuli can all affect HDP expression.

The physical attributes of host defence peptides are strongly related to their activity. The cationic nature of HDPs allows them to bind to polyanionic structures at the cell surfaces surrounding microbes, such as LPS in Gram-negative bacteria and teichoic acid in the cell wall of Gram-positive bacteria. They cross the outer membrane of Gram-negative bacteria via self-promoted uptake to reach the anionic surface of the cytoplasmic membrane, where they insert into the membrane by straddling the interface between the hydrophilic head and hydrophobic fatty acyl chain portions. Once there, peptides may permeabilize the membrane and there are numerous models to explain this process (45). Although all peptides must interact with the membrane in order to reach their target, not all peptides cause membrane permeabilization. HDPs can translocate across the membrane and accumulate intracellularly, where they can attack multiple internal targets with varying potencies. Because of this, peptides are often referred to as 'dirty' drugs when compared to conventional therapies with a single target and mode of action (43).

1.2.1 Immune modulation by host defence peptides in the skin

The physical properties of host defence peptides means almost all will have antimicrobial activity when assayed *in vitro* using dilute media or buffer. However, a significant proportion of these peptides lose their antimicrobial activity when examined under physiological conditions since they are antagonized by monovalent and divalent cations, serum, and polyanions, such as heparins and glycosaminoglycans (46). Additionally, the low physiological concentrations of several HDPs indicate that direct antimicrobial activity is likely not their primary purpose (47).

More recent findings have shown that at physiological concentrations and conditions, host defence peptides contribute to defence though modulation of immune responses. The human cathelicidin LL-37, which can be found in the epithelia lining the large intestine, lung and urinary tract, in addition to being produced by keratinocytes in the epidermis, is chemotactic for monocytes, neutrophils and T cells (48, 49). Human β-defensins in the skin, large intestine and respiratory tract are chemotactic for mast cells, immature dendritic cells

and memory T cells, suggesting these molecules encourage initiation of an adaptive immune response (50, 51). Recent research shows that certain host defence peptides interact with TLR ligands directly, but most alter intracellular signal transduction pathways, thus modifying cellular responses to the ligand and potentially modulating inflammatory responses. For example, LL-37 is well known to suppress inflammatory responses induced by several bacterial ligands (e.g. LPS). Conversely LL-37 is overexpressed in psoriasis, a chronic inflammatory disease of the skin. Under these circumstances it has been proposed to bind to self-DNA, creating an aggregate structure that is detected by TLR9 on plasmacytoid dendritic cells (pDCs) leading to the production of type I IFNs. Self-DNA is not normally sensed by these cells, thus this suggests LL-37 breaks innate tolerance to self-DNA and contributes to the dysregulation of inflammation (35, 52).

Host defence peptides can also stimulate the release of cytokines, chemokines, host defence peptides or other immune mediators from cells. Human β-defensins and LL-37 stimulate epidermal keratinocytes to secrete several different molecules, including MCP-1, MIP-3α, RANTES, IP-10, IL-6, IL-8, IL-10, IL-18 and IL-20 (53). The specific profile of cytokines and chemokines that are released depends on the particular cell type. For example, LL-37 stimulates airway bronchial epithelial cells to secrete Gro-α, RANTES, IL-6 and IL-8, without inducing detectable levels of MIP-1α, CCL22 or IP-10 (54). Host defence peptides can also work with other molecules to augment or dampen immune responses in different cells. In human peripheral blood mononuclear cells (PBMCs), LL-37 synergistically enhances IL-1β- or GM-CSF-induced cytokines and chemokines, including IL-6, IL-10, MCP-1 and MCP-3. However, in the presence of IL-4, IL-12 or IFN-γ, production of these cytokines and chemokines is antagonized (55). When human monocytic cells are stimulated with LPS, low concentrations of LL-37 inhibit the expression of specific pro-inflammatory genes, such as *NFκB1*, but not anti-inflammatory genes, like the NF-κB inhibitor gene *NFκBIA*, demonstrating a potential role for LL-37 against endotoxemia (56). Thus, the ability of host defence peptides to modulate immunity can occur through multiple means.

1.2.2 The potential and development of synthetic host defence peptides as immune modulators

As our knowledge of the innate immune response continues to expand, the development of agents that modulate immunity for therapeutic purposes becomes increasingly attractive. Several therapies that target the immune system have entered clinical use, with many more entering clinical trials each year. These include TLR ligands for their immunostimulatory properties; monoclonal antibodies that can stimulate or block specific cell-surface receptors; small molecules to target signal transduction pathways in inflammation; and cytokines, chemokine or hormones (57-60).

The use of immunotherapies is not without risk. Immunosuppressive therapies, such as those used in tissue transplantation surgeries or chronic inflammatory disorders, come with an increased risk of potentially fatal infections. Similarly, immunostimulatory therapies can result in inflammation-related tissue damage or potentially fatal cytokine storms (61). Clinical experience to date with immunomodulatory therapies has taught us that effective treatment cannot be achieved through straightforward stimulation or suppression of one part of the immune system. Rather, it has become recognized that what is needed are agents that can subtly, yet precisely, modulate or polarize the immune response. In this respect, host defence peptides have gained greater prominence as potential immunomodulatory therapies due to their unique ability to act in both an immunostimulatory and anti-inflammatory manner (62).

There are several barriers to overcome before the use host defence peptides as therapeutic drugs becomes more common. They are susceptible to cleavage by proteases, which could create adverse pharmacokinetics. The cost to manufacture peptides is high, a fact that limits both the basic testing and development of therapeutic peptides. Because the mode of action of host defence peptides is complex, they have a high potential for toxicity (43). Regardless of these obstacles, an intense effort is underway to design short, synthetic peptides termed innate defence regulators (IDRs) with structures based on naturally occurring host defence peptides, immunomodulatory activity and, ideally, little cytotoxicity.

The Hancock lab recently created two large random 9-amino-acid peptide libraries through an iterative process using the amino acid composition of the most active peptides. The resulting data was then inputted into an artificial neural network model to predict the

antimicrobial activity of all 100 000 peptides in the random 9-mer peptide library. A handful of the peptides expected to exhibit high activity were subsequently synthesized and tested *in vitro* on *Pseudomonas aeruginosa* PAO1 (63). One peptide from that study, HHC-36, showed particular promise, exhibiting broad spectrum antimicrobial activity with minimal hemolytic activity. In a subsequent study examining the ability of peptide-coated surgical implants to kill bacteria and resist infection, HHC-36 was tethered to titanium implants which were inserted subcutaneously into rats, then challenged with *Staphylococcus aureus*. The presence of HHC-36 on the implants significantly decreased the amount of bacterial adherence to the implants (64). Moreover, the researchers observed that HHC-36-coated implants became encapsulated with new fibrotic tissue, suggesting the antimicrobial peptide might also engender wound healing or other immunomodulatory properties.

In a separate study, the Hancock lab performed a series of scrambling steps and amino acid substitutions to create a series of peptides based on Bac2A. Bac2A is a linear variant of the bovine peptide bactenecin, one of the smallest naturally occurring host defence peptides with moderate activity against Gram-negative bacteria and select Gram-positive bacteria. Bac2A has similar activity against Gram-negative bacteria compared to bactenecin, but has improved activity against Gram-positive bacteria (65, 66). From this study, a peptide named HH2 showed promise and was chosen as the base sequence for another series of IDRs, iteratively created through point substitutions, scrambling and deletions. From this new series, IDR-1018 displayed immense immunomodulatory promise with minimal hemolytic activity, moderate minimal inhibitory concentrations and a high reduction in LPS-induced TNF-α induction (67).

1.2 Hypothesis and experimental goals.

The aims of this study were to examine the immune modulating properties of two synthetic host defence peptides in the context of wound healing. More specifically, the goals were to determine the effects of synthetic cationic peptides in human epidermal keratinocytes that had been stimulated with immune mediators, and to examine the mechanisms underlying any immune modulating activity. By studying the effects of host defence peptides on the ability of keratinocytes to mount and maintain an immune response, valuable insight into how synthetic peptides may be used to enhance the skin's ability to protect us can be gained.

My **hypothesis** was that our synthetic host defence peptides have the ability to enhance the immune response of human keratinocytes, in the presence of specific immune mediators, through multiple pathways.

CHAPTER 2: Materials and Methods

2.1 Cell Culture

The spontaneously transformed human epithelial cell line HaCaT (human adult low calcium temperature keratinocytes) was a gift from Dr. Edward E. Putnin (University of British Columbia, School of Dentristry) (68). HaCaT cells were cultured in low glucose Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Invitrogen) and 1% L-glutamine (Life Technologies, Invitrogen). The medium was changed every two days. HaCaT cells were routinely cultivated to between 80-90% confluence in 100% humidity and 5% CO_2 at 37°C and were used between passages ten to twenty-five.

Normal primary adult human epidermal keratinocytes (HEKa) were purchased from Cascade Biologics (Portland, OR). HEKa cells were maintained in the proprietary EpiLife medium (Cascade Biologics, Invitrogen) supplemented with Human Keratinocyte Growth Supplement (HKGS) (Cascade Biologics, Invitrogen), which contains bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin and human epidermal growth factor. HEKa cells were cultured to between 80-90% confluence in 100% humidity and 5% CO_2 at 37°C before passaging, and the medium was changed every two days. HEKa cells were used between passages two to six.

2.2 Peptides, TLR agonists and inhibitors

LL-37, IDR-1018 and HHC-36 (see Table 2.1 for sequences) were synthesized using *N*-(9-fluorenyl)methoxy carbonyl chemistry (F-moc) at the Nucleaic Acid/Protein Synthesis Unit (University of British Columbia, Canada) or Genscript (Piscataway, NJ). All peptides were dissolved in endotoxin-free water (Sigma-Aldrich).

Ultrapure *Salmonella typhimurium* flagellin, poly(I:C) and Pam3CSK4 were purchased from InvivoGen (San Diego, CA). Recombinant IL-1β was obtained from BioSource (Invitrogen) while GM-CSF was obtained from R&D Systems (Minneapolis, MN).

The p38/MAPK inhibitor SB203580, JNK inhibitor SP600125, PI3K inhibitors LY294002 and Wortmannin, MAPK/ERK (MEK) kinase inhibitor PD98059, PKC inhibitor

bisindolylmaleimide, and mTOR inhibitor rapamycin were all obtained from Calbiochem (San Diego, CA). The EGFR inhibitor AG1478 was obtained from Sigma (St. Louis, MO). The NF-κB inhibitor Bay 11-7085 was purchased from Biomol (Farmingdale, NY) while Bay 11-7082, another inhibitor of NF-κB, was purchased from Calbiochem (San Diego, CA). The Src family kinase inhibitor PP2 was purchased from Invitrogen (Grand Island, NY), whereas the alternative Src family kinase inhibitor SU6656 as well as PP3, a negative control for PP2, were obtained from Calbiochem (San Diego, CA). Inhibitors were resuspended in DMSO (Sigma-Aldrich) and final concentrations of DMSO in cell culture never exceeded 0.02% (v/v). All experiments using inhibitors included DMSO vehicle controls.

Table 1. Sequences of peptides used in this study. All peptides are carboxy terminally amidated.

Peptide	Sequence
HHC-36	KRWWKWWRR-NH_2
IDR-1018	VRLIVAVRIWRR-NH_2
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

2.3 Cell proliferation assay

HaCaT cells were seeded in 48-well plates (Costar, Corning NY) in 10% FBS, 1% L-glutamine DMEM at a concentration of $5.0x10^4$ cells/mL (0.5mL per well). After 24 hours, the indicated amounts of peptides were added to triplicate wells and incubated in 100% humidity and 5% CO_2 at 37°C. After the desired incubation period, the number of live cells in each well was counted by removing the media, rinsing the well with 1% phosphate buffered saline (PBS) (Life Technologies, Invitrogen), trypsinizing the cells with 0.25% Trypsin-EDTA (Life Technologies, Invitrogen) and staining with the vital stain Trypan blue (Life Technologies, Invitrogen). Treatments were done in triplicate and the results averaged. The experiment was repeated five times.

2.4 Cell migration assay

HaCaT cells were seeded in flat-bottom, 96-well plates (Costar, Corning NY) in 10% FBS, 1% L-glutamine DMEM at a concentration of $2.0x10^5$ cells/mL (0.1mL per well). Once a confluent monolayer had formed (48 hours, on average), an artificial wound or zone of clearing was created in each well by stamping the cell monolayer with a silicone stopper from the Oris™ Cell Migration Assay kit (Platypus Technologies, Madison WI) (see Appendix A for diagram). Each well was photographed at the time of wounding, and wound area was measured using ImageJ software. The media in each well was then replaced with 2-10% FBS, 1% L-glutamine DMEM containing the indicated concentration of peptides. Each wound was subsequently photographed and measured after 24 and 48 hours. Cell migration was assessed by calculating the percent wound closure over time, using the following equation: $((\text{Area } T_0 - \text{Area } T_x)/\text{Area } T_0) \times 100\%$.

2.5 Detection of cytokines and chemokines

HEKa cells were seeded in 48-well plates (Costar, Corning NJ) at 7000 cells/cm^2 in HKGS-containing EpiLife™ medium. Upon reaching 70-80% confluence, the cells were washed with basal EpiLife™ medium (no HKGS) and 0.5mL of basal EpiLife™ medium was added to each well. If chemical inhibitors were used, they were added to the cells after the washing step. Cells were rested for 1 hour, then treated with peptide and/or stimuli as indicated. After the desired incubation period, supernatants were collected and stored at -20°C.

The concentrations of IL-8, IL-6, MCP-1, MCP-3, TNF-α, IL-1β, RANTES and GRO-α in cell supernatants were measured using capture ELISA as per the manufacturers' suggestions. GRO-α and RANTES were purchased from R&D Systems (Minneapolis, MN). TNF-α, IL-1β and IL-6 were purchased from eBioscience (San Diego, CA). IL-8, MCP-1 and MCP-3 were obtained from BioSource International (Camarillo, CA).

2.6 Cytotoxicity assay

The cytotoxic effects of peptides, TLR agonists, immune mediators, pharmacological inhibitors or any combination thereof were monitored by measuring the level of lactate dehydrogenase (LDH) in cell supernatants with the Cytotoxicity Detection Kit (Roche,

Mississauga, ON). LDH is a stable cytoplasmic enzyme present in all cells. Its presence in cell supernatants is indicative of plasma membrane damage. 2% Triton-X-100 was used as a positive control. The percentage of LDH in cell supernatants was calculated using the following equation: ((experimental value − LDH released from untreated cells)/(maximum releasable LDH in cells by 2% Triton X-100 − LDH released from untreated cells)) × 100%.

2.7 Cell metabolism assay

Any adverse effects of peptides, TLR agonists, immune mediators, pharmacological inhibitors or any combination thereof on cells were additionally monitored using the Cell Proliferation Reagent WST-1 (Roche, Mississauga, ON). This stable tetrazolium salt is cleaved by metabolically active cells to produce a soluble formazan dye that can then be quantified using a spectrophotometer. 2% Triton-X-100 was used as a negative control and untreated cells were used as a positive control.

2.8 Western immunoblotting

HEKa cells were seeded in 24-well plates (Costar, Corning NJ) at 7000 cells/cm^2 in HKGS-containing EpiLife™ medium. Upon reaching 80-85% confluence, the cells were washed with basal EpiLife™ medium (no HKGS) and 1mL of basal EpiLife™ medium was added to each well. Cells were rested for 3 hours and subsequently treated with peptides and/or stimuli. After the desired incubation period, cells were rinsed with ice cold PBS, then solubilized on ice with 150µL NP-40 lysis buffer (1% NP-40, 20mM Tris-HCl pH=8, 137mM NaCl, 10% glycerol, 2mM EDTA) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich), scraped, and centrifuged. The total protein concentration of sample lysates was quantified using the BCA Protein Assay Kit (Pierce).

Sample lysates were denatured at 95°C for 10 minutes and resolved on a 12% SDS-PAGE. The samples were subsequently transferred onto an Immuno-blot PVDF membrane (Bio-rad) at 100V for 1 hour. Membranes were probed with specific antibodies at 1/1000 dilution in TBST (20mM Tris pH=7.4, 150mM NaCl, 0.1% Tween 20) containing 5% skim milk powder, for the length of time recommended by the manufacturer. Incubation with the appropriate second antibody (HRP-conjugated goat anti-mouse or anti-rabbit antibodies) followed. Membranes were developed with the ECL chemiluminescence peroxidise substrate

(Sigma-Aldrich) according to the manufacturer's instructions. To ensure an equal amount of protein loaded, all blots were re-probed with an anti-GAPDH or anti-total CREB antibody.

2.9 RNA isolation and real-time PCR

HEKa cells were seeded in 48-well plates (Costar, Corning NJ) at 7000 cells/cm^2 in HKGS-containing EpiLife™ medium. Upon reaching 70-80% confluence, the cells were washed with basal EpiLife™ medium (no HKGS) and 0.5mL of basal EpiLife™ medium was added to each well. Cells were rested for 2 hours, then treated with peptide and/or stimuli as indicated. After the desired incubation period, cells were rinsed with ice cold PBS, then lysed with 150µL RLT lysis buffer, containing β-mercaptoethanol, then stored at -80°C. Total RNA was isolated using the RNAeasy Mini kit (Qiagen, Maryland DC) as per the manufacturer's instructions, with the optional on-column DNase digestion step for approximately 20 minutes.

One µg of total RNA was converted to cDNA using the SuperScript III Platinum Two-Step qRT-PCR kit with SYBR Green (Invitrogen). qRT-PCR was performed on the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) using the following program, with Dissociation Curve: 50°C for 2 minutes, 95°C for 2 minutes, then 50 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

Table 2. Primer sequences used in this study.

Primer	Forward Sequence (5'-3')	Reverse Sequence (3'-5')
B2M	CTCGCGCTACTCTCTCTCTTTCT	TGCTCCACTTTTTCAATTCTCT
IL-8	CACCACACTGCGCCAACAC	CTTCTCCACAACCCTCTGCAC

Each qRT-PCR reaction mixture was a total volume of 12.5 µL. Each reaction contained 2.5 µL of 1/10 diluted cDNA template and 10 µL of a master mix consisting of 0.25µL Rox, 6.25 µL UDG, 0.5 µL of 10 µM primer mix and 3 µL nuclease-free water. Beta-2 microglobulin (B2M) was used as the housekeeping gene control. Fold changes were calculated after normalization to B2M using the comparative Ct method (69). PCR primers used in this study are listed in Table 2.

2.10 Statistical Analysis

Statistical significance was calculated using the Student's t-test. A p-value ≤ 0.050 was considered statistically significant. Values shown are expressed as a mean ± standard deviation or standard error of the mean, as indicated.

CHAPTER 3: Results

3.1 HHC-36 and IDR-1018 induced dose-dependent production of IL-6 and IL-8 by human keratinocytes

The ability of peptides HHC-36 and IDR-1018 to induce the production of chemokines and cytokines from HEKa cells was evaluated by ELISA. Treatment with HHC-36 and IDR-1018 resulted in a dose-dependent release of the pro-inflammatory cytokine IL-6 and the chemokine IL-8 from human epidermal keratinocytes (Figure 1). The production of TNF-α, IL-1β, MCP-1, MIP-3α, and RANTES was also tested, but any increases in the release of these cytokines and chemokines were small and statistically insignificant once background subtraction was performed.

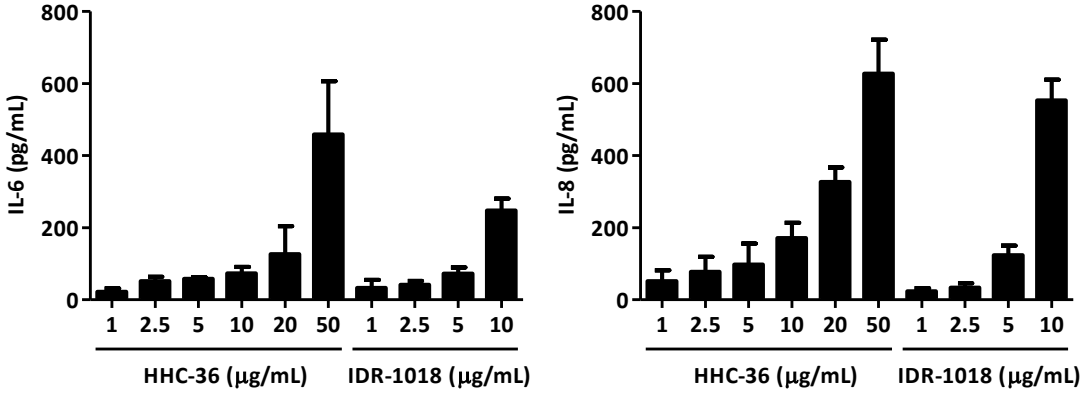

Figure 1. HHC-36 and IDR-1018 induced dose-dependent IL-6 and IL-8 production by human keratinocytes.
Sub-confluent HEKa cells were stimulated with HHC-36 (1-50 μg/mL) or IDR-1018 (1-10 μg/mL) for 24 hours. The concentrations of IL-6 and IL-8 in cell culture supernatants were measured using ELISA. The results for all samples are background subtracted. Each bar represents the mean of three independent experiments ± standard deviation.

The lactate dehydrogenase (LDH) assay was performed to measure any peptide-induced host cell toxicity. IDR-1018 was found to be cytotoxic (>10% LDH released) to HEKa cells at concentrations greater than 10 μg/mL, whereas HHC-36 was well tolerated by HEKa cells at even 50 μg/mL (Figure 2).

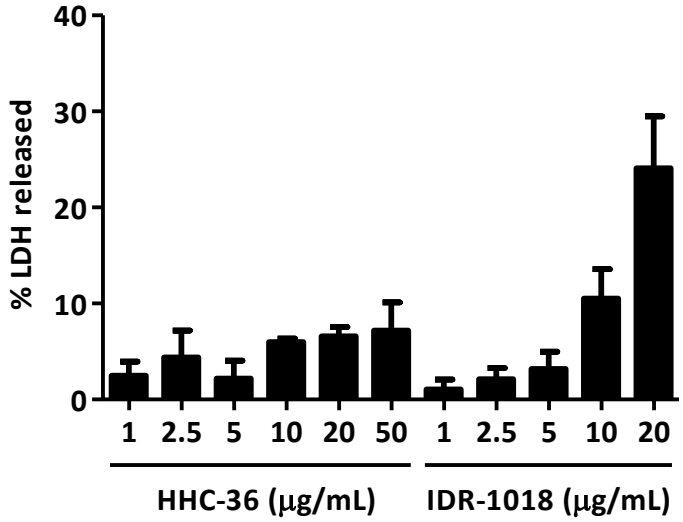

Figure 2. Cytotoxic effects of HHC-36 and IDR-1018 on human keratinocytes. Sub-confluent HEKa cells were stimulated with HHC-36 (1-50 μg/mL) or IDR-1018 (1-10 μg/mL) for 24 hours. 2% Triton-X-100 was used as a positive control. Cytotoxicity was measured by calculating the percentage of LDH released into the cell supernatants using the following equation: ((experimental value − LDH released from untreated cells)/(maximum releasable LDH in cells by 2% Triton X-100 − LDH released from untreated cells)) × 100%. Each bar represents the mean of three independent experiments ± standard deviation.

3.2 HHC-36 and IDR-1018 promote keratinocyte cell proliferation

The primary function of the skin is to act as a protective barrier. The skin's ability to rapidly and effectively close wounds is critical for it to fulfill this function. As discussed in the Introduction, one of the early stages of wound repair involves the proliferation of keratinocytes, the main cell type in the epidermis. To assess the ability of synthetic peptides HHC-36 and IDR-1018 to induce keratinocyte cell proliferation, subconfluent HaCaT cells were treated with 1 or 10 μg/mL of HHC-36, IDR-1018, or LL-37, as a positive control. The HaCaT cell line is widely used to study various aspects of keratinocyte physiology, such as growth and differentiation (70-72). When HaCaT cells are grown in organotypic cultures or transplanted into nude mice, they produce a stratified epithelium that resembles the normal epidermis to some extent (73, 74). After 24 and 48 hours of incubation with the peptides, the cells in each well were trypsinized, stained with Trypan Blue and counted under the microscope.

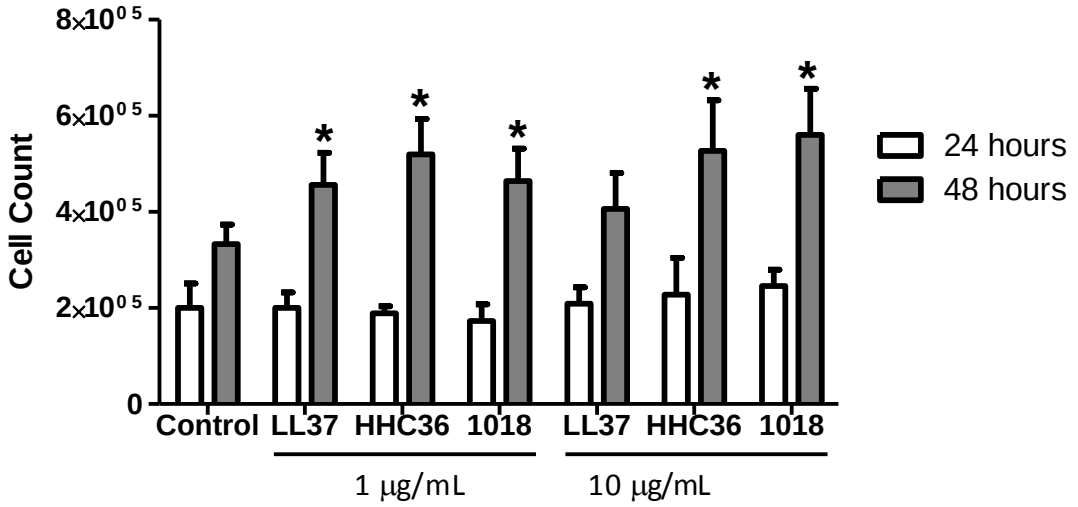

Figure 3. HHC-36 and IDR-1018 promoted HaCaT cell proliferation.
HaCaT cells were seeded into 48-well plates at a concentration of 5 x 10^4 cells/mL (0.5mL per well). After 24 hours, the cells were treated with LL-37, HHC-36 and IDR-1018 at 1 or 10 μg/mL for 24 to 48 hours, trypsinized, stained then counted under a light microscope. Results are expressed as the mean values of four independent experiments ± standard deviation. Statistical comparisons were done using a 2-tailed Student's t-test. (* indicates $p < 0.05$ relative to untreated cells at the respective time point)

After 24 hours, neither peptide had an appreciable effect on HaCaT proliferation compared to the untreated control group. After 48 hours, both HHC-36 and IDR-1018 caused significant increases in HaCaT cell numbers relative to the untreated control ($p<0.05$) (Figure 3). At low concentrations (1 μg/mL), HHC-36 and IDR-1018 increased the total number of HaCaT cells by 56% and 39%, respectively, relative to untreated cells. A higher concentration of HHC-36 (10μg/mL) did not significantly improve its effect on HaCaT proliferation, increasing the number of cells by 58% compared to untreated cells. However, the effect of IDR-1018 on HaCaT proliferation was augmented at the higher concentration and HaCaT cell numbers increased by 68% relative to untreated cells.

3.3 HHC-36 and IDR-1018 did not directly promote keratinocyte cell migration

Cell migration is a rate limiting step in wound healing (75, 76). Keratinocytes at the wound margins actively migrate over the wound opening. Several growth factors are thought to stimulate keratinocyte migration, including epidermal growth factor, transforming growth factor α and keratinocyte growth factor (77-79), as well as other molecules like human beta-defensins and human lactoferrin (32, 80). The absence of neighbour cells at the wound edge,

otherwise known as the “free edge” effect, is also thought to promote the migration of keratinocytes.

Studies to date have used a scratch assay to demonstrate the effects of various compounds on keratinocyte migration. In this method, a confluent cell layer is scratched by some mechanical means, such as a pipette tip, and cell migration is observed through a microscope. However, this method does not allow for quantification of cell migration. In this experiment, I sought to determine and quantify whether HHC-36 or IDR-1018 could directly impact keratinocyte migration.

To do so, HaCaT cells were grown to confluence on 96-well plates and an artificial circular wound/zone of clearing was created in each well by stamping the cell layer with a silicone stamp (see Appendix A for diagram). The media in each well (DMEM containing 10% FBS) was then replaced with low (2% FBS) serum DMEM containing either LL-37, HHC-36, or IDR-1018 (1, 10 or 20 μg/mL) and incubated for 24 to 48 hours. Cell migration was assessed by photographing the entire well and digitally measuring the zone of clearing at each time point, then calculating the percent wound closure using the following equation: $((\text{Area } T_0 - \text{Area } T_x)/\text{Area } T_0) \times 100\%$

Neither peptide appeared to have a significant effect on cell migration relative to untreated cells after 24 or 48 hours (Figure 4A). Percent wound closure hovered between 15% - 30% for all treatments, including LL-37. Intriguingly, the effect of IDR-1018 on cell migration appeared to diminish as the peptide concentration went up. This is in contrast to the effect IDR-1018 had on keratinocyte proliferation.

Because previous studies have shown the promotional effects of LL-37 on keratinocyte proliferation and migration to be dependent on the presence of serum (81), this experiment was repeated using DMEM containing the normal (cell cultivation) level of serum (10% FBS). This change resulted in an increase in cell migration for all treatments, after 24 and 48 hours (Figure 4B). The presence of more serum increased percent wound closure two-fold or greater, to between 40% - 80%. However, the trend relative to untreated cells remained unchanged and neither HHC-36 nor IDR-1018 alone had an appreciable effect on keratinocyte cell migration. The diminishing effect of IDR-1018 at higher peptide concentrations remained consistent at the higher serum concentration.

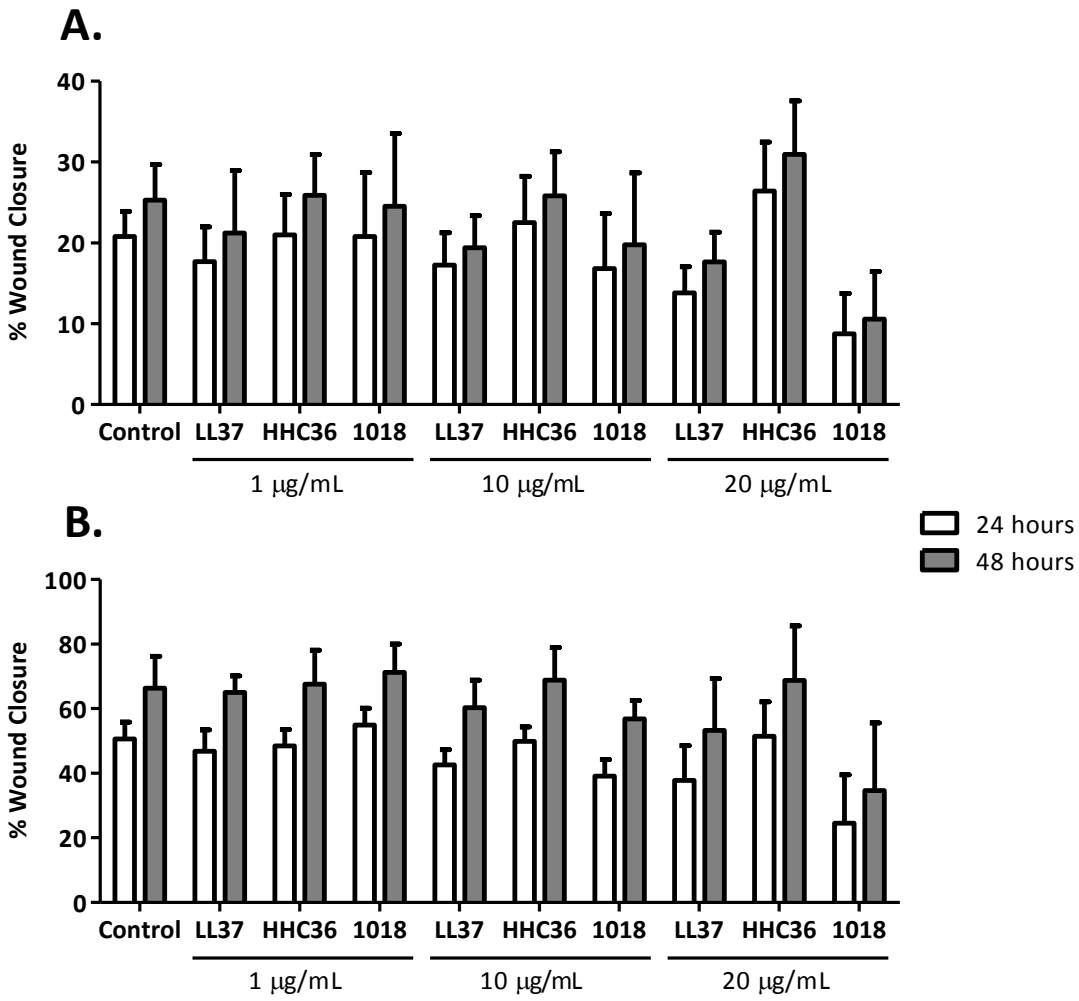

Figure 4. Direct peptide effects on keratinocyte cell migration.
A zone of clearing was created in a confluent HaCaT cell monolayer. HaCaT cells were treated with 1, 10 or 20 μg/mL of LL-37, HHC-36, or IDR-1018 in the presence of DMEM containing **A.** low serum (2% FBS) or **B.** high serum (10% FBS). Cell migration was assessed after 24 and 48 hours by digitally measuring and calculating percent wound closure using the equation ((Area T_0 – Area T_x)/Area T_0) x 100%. Results show the mean of at least three independent experiments ± standard deviation.

3.4 IDR-1018 causes synergistic production of IL-8 by human keratinocytes in presence of poly(I:C) or IL-1β, but not Pam3CSK4, flagellin or GM-CSF.

Because IDR-1018 was designed to be an immune modulator, it was hypothesized that the immune response in keratinocytes needed to first be activated in order to see the immunomodulatory effects of this peptide. As discussed in the Introduction, human keratinocytes express a number of TLRs which initiate the innate immune response upon recognition of their cognate ligands (36). Accordingly, primary HEKa cells were grown to sub-confluence, then treated with HHC-36 or IDR-1018 in combination with TLR agonists Pam3CSK4 (TLR1/2), poly(I:C) (TLR3) and flagellin (TLR5), or immune mediators GM-CSF and IL-1β. Cell-free supernatants were collected after 24 hours incubation and IL-8 levels were assayed using ELISA.

Pam3CSK4, GM-CSF, and flagellin in combination with HHC-36 or IDR-1018 did not result in synergistic IL-8 production from HEKa cell (Figure 5A-C). In contrast, treatment of HEKa cells with either poly(I:C) (Figure 6) or IL-1β (Figure 7) in combination with IDR-1018 significantly increased IL-8 release from HEKa cells compared to the sum of separate treatments. IL-6 release was also measured, however, no synergistic effects were seen (data not shown).

HHC-36, better characterized for its antimicrobial properties than any immuno-modulatory activities, displayed weaker and/or insignificant synergy with respect to IL-8 production in response to the same TLR agonists and immune mediators (data not shown). This peptide was thus not studied further in this project.

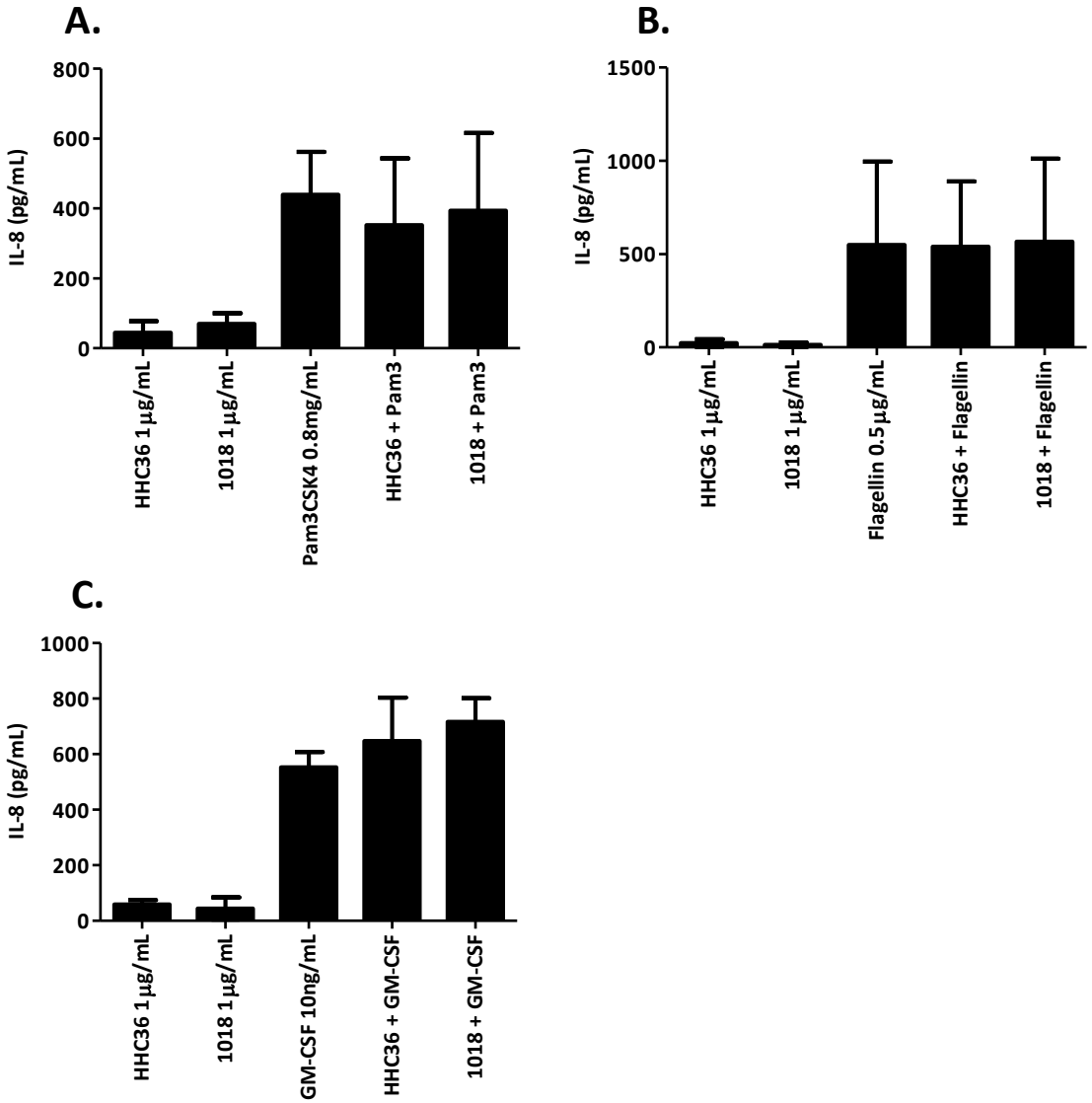

Figure 5. IDR-1018 with Pam3CSK4, flagellin or GM-CSF did not cause synergistic release of IL-8 from human keratinocytes.
Subconfluent HEKa cells were incubated with HHC-36 or IDR-1018 in combination with TLR agonists **A.** Pam3CSK4 (TLR1/2), **B.** flagellin (TLR5) or **C.** immune mediator GM-CSF. The concentration of IL-8 in cell culture supernatants were measured after 24 hours using ELISA. Background subtraction of untreated cells was performed on all data. Results show the mean of at least three independent experiments ± standard deviation.

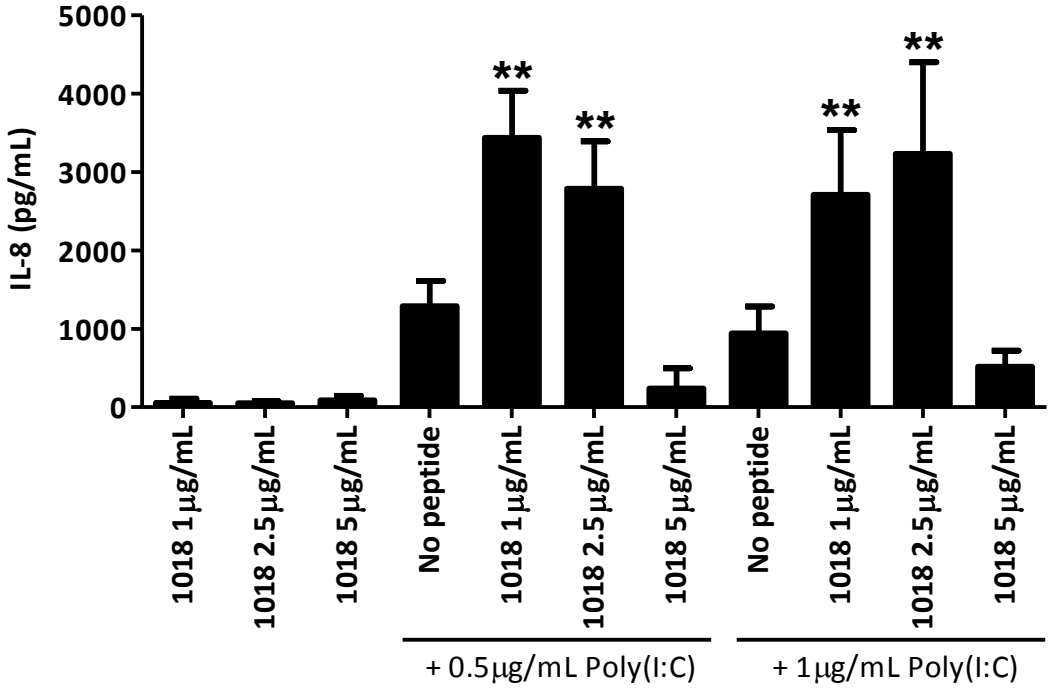

Figure 6. IDR-1018 and poly(I:C) synergistically induced IL-8 from human keratinocytes.

Sub-confluent HEKa cells were treated with IDR-1018 and/or poly(I:C) for 24 hours. IL-8 levels in cell culture supernatants were assayed using ELISA. Background subtraction was performed on all data. The simultaneous treatment of IDR-1018 with poly(I:C) had a synergistic effect on IL-8 release compared to the sum of the IL-8 released when cells were treated with each compound separately. Results are expressed as the mean values of at least three independent experiments ± standard deviation. Statistical comparisons were done using a 2-tailed Student's t-test. (** indicates $p < 0.01$ relative to the sum of separate treatments)

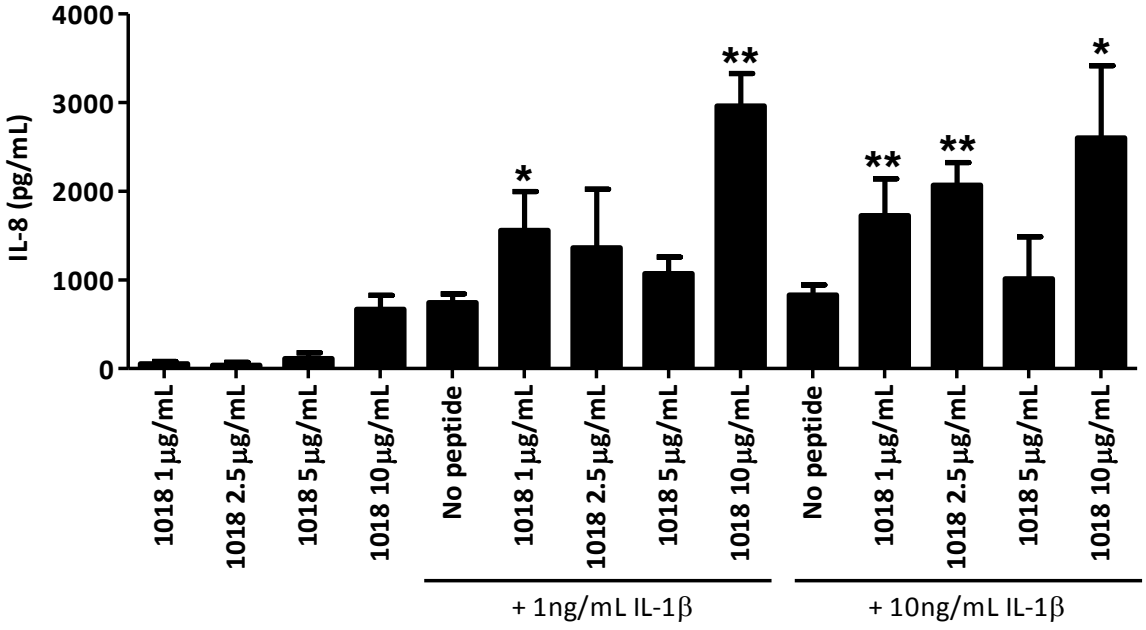

Figure 7. IDR-1018 and IL-1β synergistically induced IL-8 from human keratinocytes. HEKa cells were grown to sub-confluence and incubated with IDR-1018 and/or IL-1β for 24 hours. IL-8 levels in cell culture supernatants were assayed using ELISA. Background subtraction was performed on all data. The simultaneous treatment of the two compounds had a synergistic effect on IL-8 release when compared to the sum of the IL-8 released when IDR-1018 and IL-1β were treated separately. Results are expressed as the mean values of at least three independent experiments ± standard deviation. Statistical comparisons were done using a 2-tailed Student's t-test. (* indicates $p < 0.05$ and ** indicates $p < 0.01$ relative to the sum of separate treatments)

The LDH assay was performed to determine whether the observed synergy was accompanied by any cytotoxic effects on the cells. The addition of poly(I:C) substantially increased LDH release from keratinocytes in the presence of IDR-1018, thus lower concentrations of IDR-1018 were used for subsequent experiments involving poly(I:C) (Appendix B). In contrast, the addition of IL-1β did not substantially increase LDH release from cells co-treated with IDR-1018 (Appendix C). Thus, a higher concentration of IDR-1018 (10 µg/mL) and IL-1β (10 ng/mL) were used for subsequent experiments, as the synergistic effects of these two molecules was consistently strong at these concentrations.

3.5 IDR-1018 increases IL-8 expression by human keratinocytes when co-stimulated with poly(I:C) or IL-1β.

The effects of IDR-1018 on the transcriptional regulation of IL-8 was measured over a period of 8 hours using qRT-PCR. Sub-confluent HEKa cells were rinsed with serum free medium and rested for 2 hours prior to the addition of IDR-1018 and poly(I:C) or IL-1β for 4 to 8 hours.

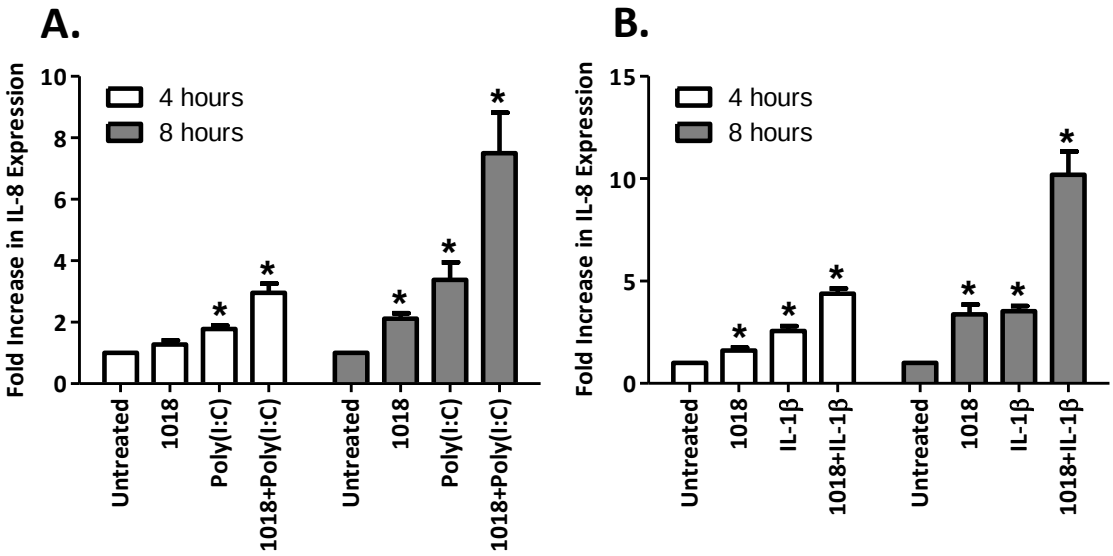

Figure 8. IDR-1018 up-regulated IL-8 gene expression in human keratinocytes in the presence of poly(I:C) and IL-1β.
Sub-confluent HEKa cells were treated with **A.** IDR-1018 (1 μg/mL) and poly(I:C) (1 μg/mL) or **B.** IDR-1018 (10 μg/mL) and IL-1β (1 ng/mL) for 4 to 8 hours. IL-8 gene expression was measured using qRT-PCR. Fold changes in expression were normalized to beta-2 microglobulin (housekeeping gene) and are compared to the gene expression in untreated cells using the comparative Ct method. Results represent the mean of at least four independent experiments ± SEM. (* indicates $p < 0.05$ relative to the corresponding untreated control at a given time point)

As shown in Figure 8A, a low (1 μg/mL) concentration of IDR-1018 alone increased IL-8 expression 4 hours post-stimulation by a detectable, but statistically insignificant amount. By 8 hours, the effect of IDR-1018 alone reached statistical significance, resulting in a 2.1-fold increase in IL-8 expression. A higher (10 μg/ml) concentration of IDR-1018 alone increased IL-8 expression by 1.6- to 3.4-fold after 4 to 8 hours stimulation (Figure 8B). Both poly(I:C) and IL-1β induced statistically significant fold increases in IL-8 expression after 4

to 8 hours incubation. When keratinocytes were co-treated with IDR-1018 and either poly(I:C) or IL-1β, there was a significant increase in IL-8 expression after 4 hours, although the effects of co-treatment were additive rather than synergistic. Synergistic IL-8 induction could be seen more clearly 8 hours post-stimulation, at which time IDR-1018 resulted in a 7.5-fold increase in IL-8 expression in the presence of poly(I:C) and a 10.2-fold increase in IL-8 expression when co-stimulated with IL-1β.

3.6 IDR-1018-induced synergistic IL-8 release is regulated by p38 MAP kinase, but not ERK1/2 or JNK signaling.

Human keratinocytes activate multiple signal transduction pathways in response to various stimuli. The MAPKs (p38, extracellular signaling-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK)) have been shown to play a major role in the regulation of the keratinocyte inflammatory response (53, 82). To examine whether these pathways play a role in the observed synergy between IDR-1018 and poly(I:C) or IL-1β, sub-confluent HEKa cells were pre-treated with the pharmacological inhibitors SB203580, PD98059 or SP600125 (inhibitors of p38 MAPK, MAPK/ERK kinase (MEK) and JNK, respectively) for one hour before treatment with IDR-1018 and poly(I:C) or IL-1β. Supernatants were collected after 24 hours incubation and IL-8 levels were measured using ELISA.

The p38 pathway was shown to play a major role in the regulation of synergistic IL-8 release with both poly(I:C) and IL-1β. The p38 inhibitor SB203580 resulted in an 82% decrease in IL-8 release with the poly(I:C) agonist and an 85% decrease in IL-8 production with IL-1β (Figure 9). Additionally, SB203580 caused 73%, 86% and 59% reductions in the amounts of IL-8 induced by either IDR-1018, poly(I:C) and IL-1β alone, respectively. Pre-treatment with inhibitors of MEK (which phosphorylates ERK1/2) or JNK had no effect on IL-8 release with either poly(I:C) or IL-1β, suggesting that these pathways do not contribute to the observed synergy.

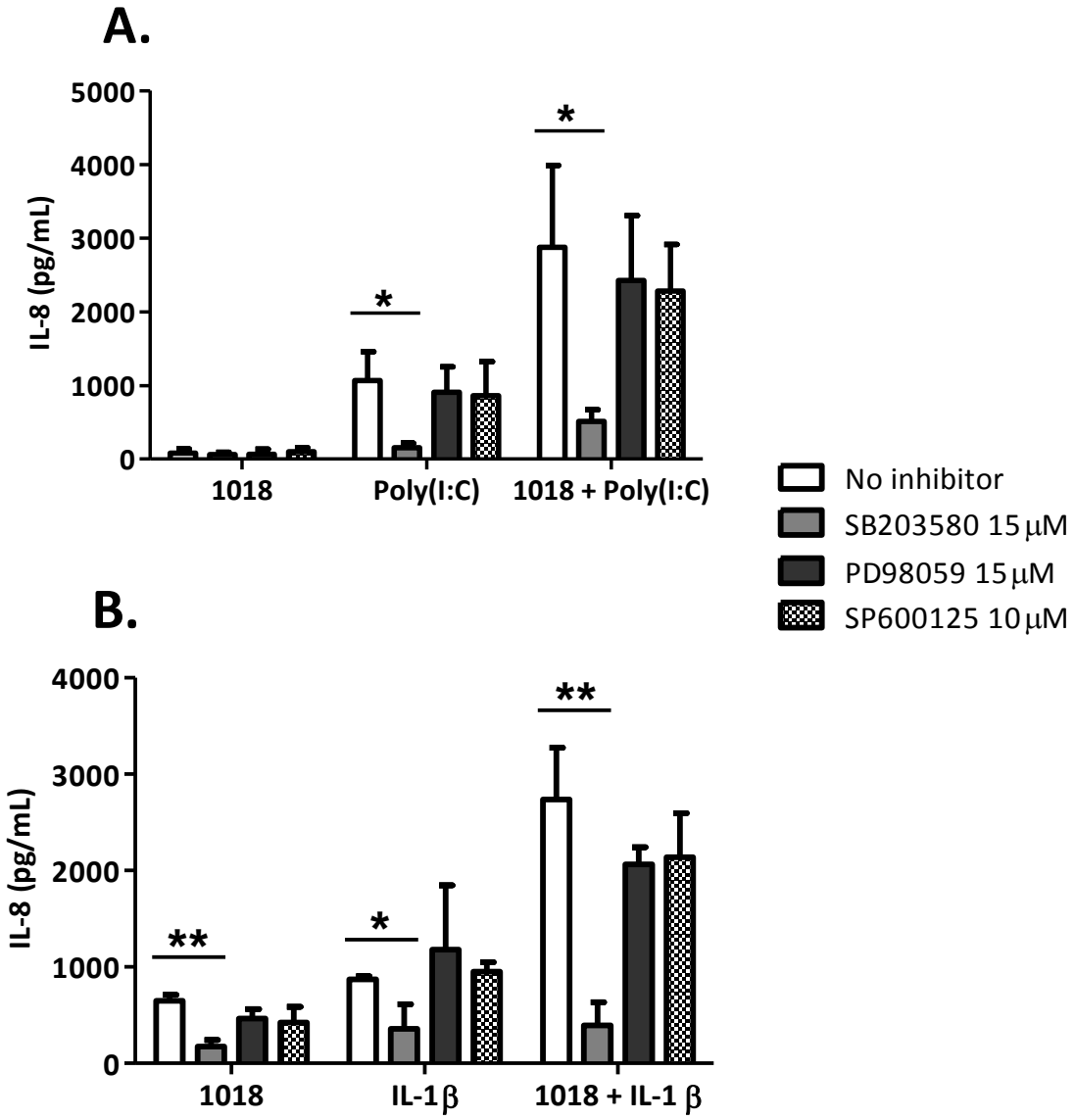

Figure 9. Involvement of MAPK pathways in poly(I:C)- and IL-1β-induced synergy with IDR-1018.

Sub-confluent HEKa cells were incubated with inhibitors of p38 (SB203580 10 μM), MAPK/ERK (PD98059 15 μM) or JNK (SP600125 10 μM) for one hour prior to the addition of **A.** IDR-1018 (1 μg/mL) and poly(I:C) (1 μg/mL) or **B.** IDR-1018 (10 μg/mL) and IL-1β (1 ng/mL). Supernatants were collected after 24 hours and ELISA was performed to measure the concentrations of IL-8. Results show the mean of at least three independent experiments ± standard deviation. Background subtraction of untreated cells was performed on all data. (* indicates $p < 0.05$ and ** indicates $p < 0.01$ relative to the corresponding No Inhibitor control)

3.7 IDR-1018 synergy is regulated by Src-family kinase and NF-κB signaling.

The Src-family tyrosine kinases have been previously shown to play a role in the synergistic production of IL-8 by human keratinocytes co-stimulated with the endogenous human cathelicidin LL-37 and the TLR5 agonist flagellin (83). To determine whether the Src-family kinases played a role in the observed synergy with IDR-1018, sub-confluent HEKa cells were pre-treated with PP2, a relatively selective inhibitor of the Src family of protein tyrosine kinases (84). After one hour pre-treatment, the cells were stimulated with the addition of IDR-1018 and poly(I:C) or IL-1β for 24 hours, and IL-8 levels were measured *via* ELISA.

Pre-treatment with PP2 caused a 63% decrease in synergistic IL-8 production when HEKa cells were co-stimulated with IDR-1018 and poly(I:C) (Figure 10A). PP2 also caused a slight, but statistically insignificant decrease in the amount of IL-8 induced by poly(I:C) alone. When cells were co-treated with IDR-1018 and IL-1β, the presence of PP2 resulted in a 76% decrease in IL-8 production compared to the No Inhibitor control, in addition to statistically significant reductions in the amount of IL-8 induced by IDR-1018(70%) and IL-1β (41%) alone (Figure 10B). Interestingly, this experiment was performed using an alternative Src-family kinase inhibitor, SU6656 (85). SU6656 has been described as a potent and selective inhibitor of Src family kinases, however it did not cause detectable inhibition of IL-8 secretion in this experiment.

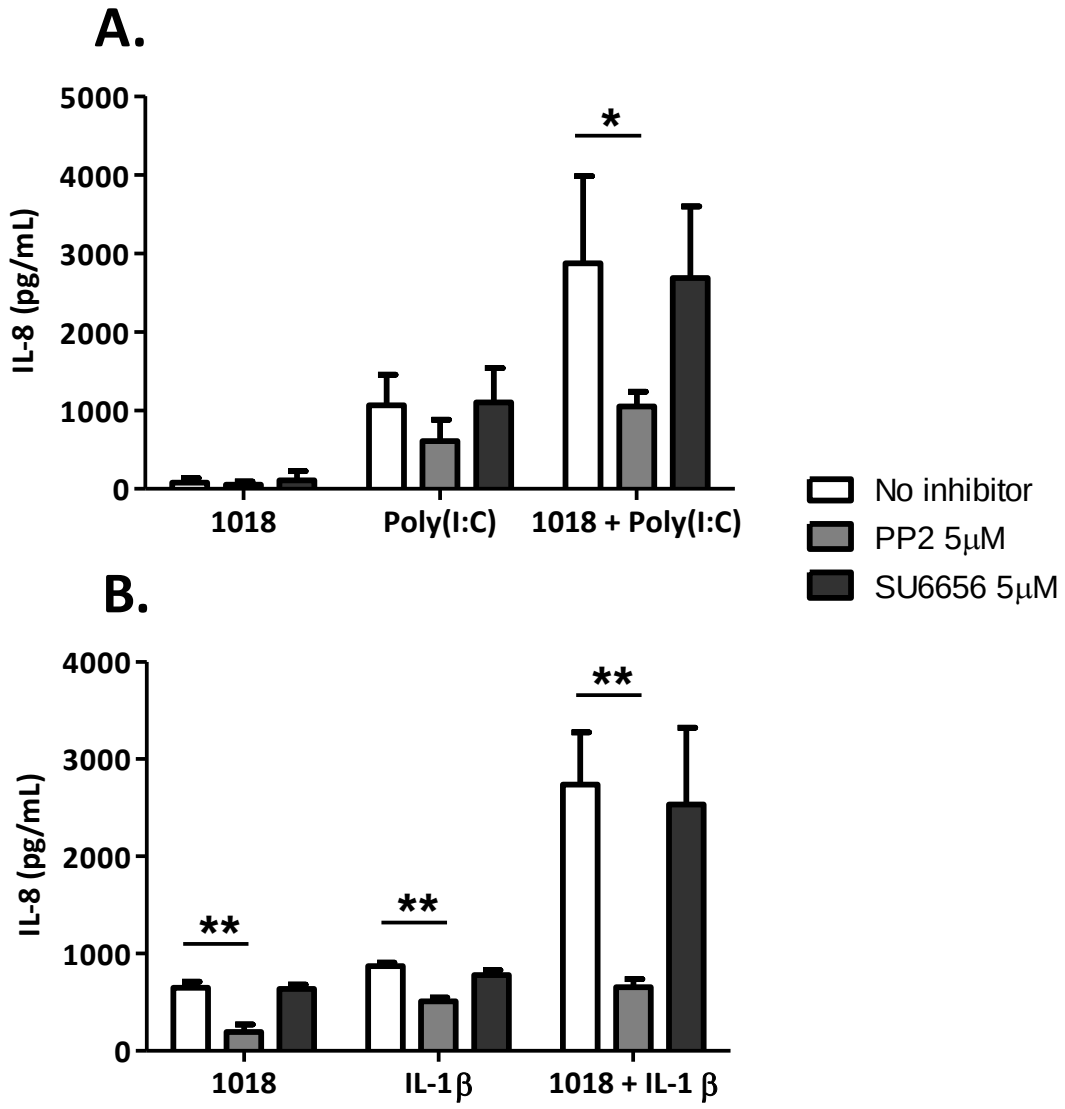

Figure 10. The Src-family kinase inhibitor PP2 suppressed IL-8 release in keratinocytes co-stimulated with IDR-1018 and poly(I:C) or IL-1β.
Sub-confluent HEKa cells were pre-treated with the Src-family kinase inhibitors PP2 (5 μM) and SU6656 (5 μM) for one hour prior to co-stimulation with **A.** IDR-1018 (1 μg/mL) and poly(I:C) (1 μg/mL) or **B.** IDR-1018 (10 μg/mL) and IL-1β (1 ng/mL). Supernatants were collected after 24 hours and IL-8 levels were measured using ELISA. Results are the mean of at least three independent experiments ± standard deviation. Background subtraction of untreated cells was performed on all data. (* indicates $p < 0.05$ and ** indicates $p < 0.01$ relative to the corresponding No Inhibitor control)

TLR signaling results in the phosphorylation and translocation of NF- κB, which causes pro-inflammatory genes to be expressed. To determine whether this signaling

pathway was important to achieve synergistic production of IL-8 with IDR-1018 and poly(I:C) or IL-1b, sub-confluent HEKa cells were pre-treated with Bay 11-7085, an inhibitor of NF- κB, for one hour, then stimulated with IDR-1018 in the presence or absence of poly(I:C) or IL-1β.

Keratinocytes pre-treated with Bay 11-7085 displayed an 89% decrease in the synergistic IL-8 production following IDR-1018 and poly(I:C) co-treatment, and a dramatic 95% decrease after IDR-1018 and IL-1β co-stimulation (Figure 11). Additionally, this inhibitor significantly decreased the amount of IL-8 released when cells were stimulated with either IDR-1018 (92%), poly(I:C) (80%) or IL-1β (93%) alone.

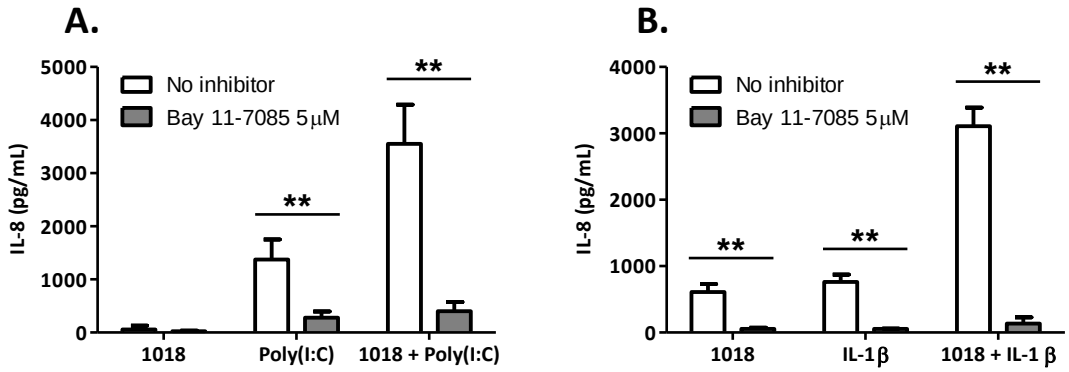

Figure 11. NF-κB signaling played a major role in synergistic IL-8 production. Sub-confluent HEKa cells were pre-treated with Bay 11-7085 for one hour before the addition of **A.** IDR-1018 (1 μg/mL) and poly(I:C) (1 μg/mL) or **B.** IDR-1018 (10 μg/mL) and IL-1β (1 ng/mL). Supernatants were collected after 24 hours and IL-8 levels were measured using ELISA. Results are the mean of at least three independent experiments ± standard deviation. Background subtraction of untreated cells was performed on all data. (** indicates $p < 0.01$ relative to the corresponding No Inhibitor control)

3.8 PKC regulates IL-1β-induced synergistic IL-8 release, but not poly(I:C) induced synergy.

The protein kinase C (PKC) family of serine/threonine kinases comprises a major signaling pathway that has been implicated in several processes in human keratinocytes, such as keratinocyte differentiation and intercellular communication during wound repair (86, 87). To see if this pathway is implicated in the activity of IDR-1018, HEKa cells were incubated with bisindolylmaleimide I (Bis), a highly selective inhibitor of PKC, for one hour.

Subsequently, the cells were co-stimulated with IDR-1018 and poly(I:C) or IL-1β for 24 hours and IL-8 levels were measured.

Interestingly, the presence of Bis resulted in a strong (92%) inhibition of IL-8 production when HEKa cells were stimulated with IDR-1018 and IL-1β, suggesting this pathway plays a role in the IL-1β-induced effects (Figure 12B). The effects of IDR-1018 and IL-1β alone on IL-8 levels were likewise reduced by 68% and 69%, respectively. However, the same inhibitor had no effect on the synergy induced with the poly(I:C) agonist, suggesting a difference in the combination of pathways IDR-1018 activates in the presence of poly(I:C) versus IL-1β.

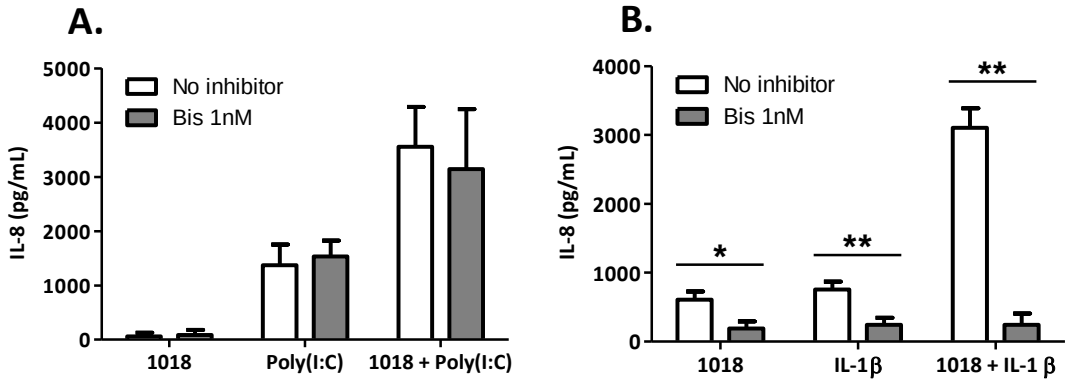

Figure 12. Protein kinase C mediated synergistic production of IL-8 by keratinocytes co-treated with IDR-1018 and IL-1β, but not poly(I:C).
Sub-confluent HEKa cells were pre-treated with the PKC inhibitor bisindolylmaleimide I (1 nM) for 1 hour before co-stimulation with **A.** IDR-1018 (1 μg/mL) and poly(I:C) (1 μg/mL) or **B.** IDR-1018 (10 μg/mL) and IL-1β (1 ng/mL). IL-8 concentrations in supernatants were measured by ELISA after 24 hours. Results show the mean of at least three independent experiments ± standard deviation. Background subtraction of untreated cells was performed on all data. (* indicates $p < 0.05$ and ** indicates $p < 0.01$ relative to the corresponding No Inhibitor control)

The phosphoinositide-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is a key signaling cascade during normal wound healing (88). In keratinocytes, it plays a regulatory role in IL-22-induced proliferation, is activated in response to various cytokines, growth factors and hormones (89). Additionally, PI3K is a potential early intermediary of MAP kinase activation in response to inflammatory stimuli (90). The significance of the PI3K/Akt/mTOR pathway in the immune modulating activities of IDR-

1018 was investigated using LY294002, a specific inhibitor of PI3K, Wortmannin, an alternative PI3K inhibitor, and rapamycin, a potent inhibitor of mTOR. None of these inhibitors had an effect on the synergistic IL-8 production produced with IDR-1018 and either poly(I:C) or IL-1β (Appendices D and E) thus, this pathway is unlikely to play a key role in the synergistic release of IL-8 by IDR-1018 and these agents.

The stimulation of human keratinocytes by LL-37 and numerous hBDs induces the phosphorylation of epidermal growth factor receptor (EGFR). Activation of EGFR is necessary for hBD-mediated keratinocyte proliferation and migration. To see if this pathway was important for IDR-1018-induced synergistic induction of IL-8, the EGFR inhibitor AG 1478 was employed. Treatment with AG 1478 did not have an effect on synergistic IL-8 production when keratinocytes were co-stimulated with IDR-1018 and poly(I:C) or IL-1β (Appendix F), suggesting this pathway does not play a key role in the observed effects.

3.9 IDR-1018 induced CREB phosphorylation when co-stimulated with poly(I:C) or IL-1β.

The synergistic production of IL-8 by IDR-1018 in combination with poly(I:C) or IL-1β was determined to be dependent on the activation of numerous pathways, including p38 MAPK, NF- κB, Src-family kinase and PKC. To attempt to provide a more detailed understanding of the mechanisms underlying the observed synergy, the activation of a downstream protein in these pathways was examined.

cAMP-responsive element binding protein (CREB) is a transcription factor that regulates several biological processes, including cell proliferation, cell differentiation and has more recently been shown to induce transcription of immune-related genes (91). Short wavelength radiation activates CREB in human keratinocytes, and several different kinases promote CREB phosphorylation, including protein kinase A and protein kinase C (92). LL-37 has been shown to cause an increase in CREB phosphorylation in the presence of IL-1β in PBMCs (55). Phosphorylated CREB may inhibit NF-κB signaling, possibly through competition with the NF-κB subunit RelA for the CREB co-activators CREB-binding protein (CBP) and p300, which help mediate NF-κB activity (93). To determine whether IDR-1018-induced synergy involves CREB activation, subconfluent HEKa cells were serum starved for three hours, then co-treated with IDR-1018 and either poly(I:C) or IL-1β. After 15 to 30

minutes incubation, nuclear extracts were obtained and activation of CREB was assessed by immunoblotting against phosphorylated CREB.

Co-stimulation of keratinocytes with IDR-1018 and poly(I:C) resulted in the phosphorylation of CREB after 15 minutes (Figure 13A). IDR-1018 was also able to induce CREB activation in the presence of IL-1β after 30 minutes incubation (Figure 13B).

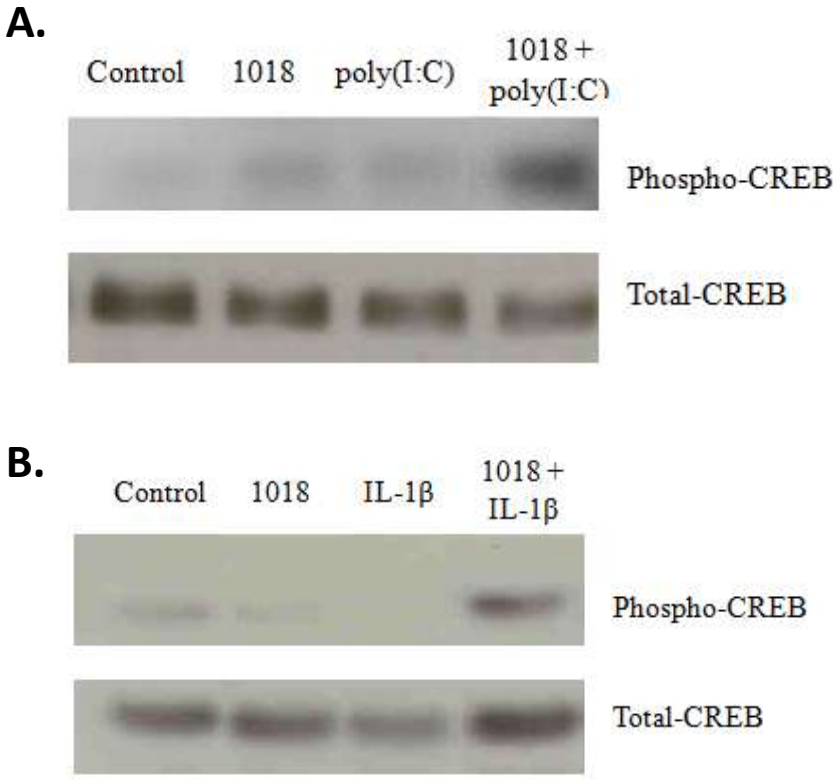

Figure 13. IDR-1018 induced CREB phosphorylation in presence of poly(I:C) or IL-1β in human keratinocytes.
Sub-confluent HEKa cells were treated with **A.** IDR-1018 (1 μg/mL) and/or poly(I:C) (1 μg/mL) or **B.** IDR-1018 (2.5 μg/mL) and/or IL-1β (1 ng/mL) for 15 to 30 minutes post-stimulation. Nuclear extracts were analyzed for activation of CREB by SDS-PAGE and Western immunoblotting, using antibodies against phosphorylated CREB (upper panel) or total CREB (lower panel, housekeeping gene). One gel, representative of at least three independent experiments, is shown for each panel.

CHAPTER 4: Discussion

In the current study, the immunomodulatory abilities of the synthetic peptide IDR-1018 were assessed in human keratinocytes. IDR-1018 has been recently shown to assist in wound healing (91). As keratinocytes are the predominant cell type in the epidermis of the skin, the results of this study have important implications for the biological process of wound healing as well as the treatment of skin-related inflammatory disorders.

In this study, IDR-1018 and another synthetic peptide, HHC-36, were found to induce IL-6 and IL-8 from human keratinocytes in a dose-dependent manner. Host defence peptides vary in their ability to induce immune responses in cells; the specific cytokine and chemokine profile is dependent on both the cell type and the particular host defence peptide. For example, the endogenous human cathelicidin LL-37 induces Gro-α, RANTES, IL-6 and IL-8 from human bronchial epithelial cells, but stimulates human keratinocytes to release a wide variety of pro- and anti-inflammatory cytokines and chemokines, including MCP-1, MIP-3α, RANTES, IP-10, IL-6, IL-8, IL-10, IL-18 and IL-20 (32, 53, 54). The abilities of IDR-1018 and HHC-36 to stimulate production of both a pro-inflammatory cytokine (IL-6) and a chemoattractant for neutrophils and T cells (IL-8) from human keratinocytes speaks to the potential of these peptides as important modulators of the innate immune response of the skin (94). Despite the array of cytokine and chemokines measured in this study, there are many more that could be looked at. A high throughput transcriptional (e.g. RNA-Seq) and/or proteomic analysis of host defence peptide effects on normal or wounded human keratinocytes could provide a more comprehensive picture of the pathways and processes potentially affected by these peptides.

Both peptides were also observed to have a positive effect on the proliferation of the keratinocyte cell line, HaCaT. HaCaT is a spontaneously transformed human epithelial cell line derived from normal human adult skin (68). They can differentiate into a reasonably structured epithelium in organotypic culture conditions and form a differentiated epithelium with the essential characteristics of a regular epidermis when transplanted into nude mice (73, 74). Because of this, HaCaT cells are widely used as substitutes for normal human keratinocytes. Other peptides, such as LL-37 and hBD-2, -3 and -4 have previously been shown to increase keratinocyte proliferation (32). Several growth factors have also been

proven to be strong inducers of keratinocyte proliferation, several of which are released from fibroblasts present in the underlying dermis (95, 96). Thus, it would be interesting to further examine the effects of IDR-1018 and HHC-36 on keratinocyte proliferation indirectly by observing peptide-mediated effects on the release of growth factors from fibroblasts.

On its own, neither IDR-1018 nor HHC-36 was shown to significantly enhance keratinocyte migration over an artificially created wound *in vitro*. However, other investigators have previously shown that LL-37 increases keratinocyte migration (97, 98), a finding that was not reflected by the peptides used in the current study. This suggests there may be an issue with the experimental design. A new cell migration assay (Oris™ Cell Migration Assay) was used with slight modifications in this study. Instead of seeding cells with the stopper already in place, the stopper was used as a stamp to create an artificial wound after a confluent monolayer had been formed. Additionally, the assay calls for cells to be fluorescently stained to facilitate tracking and measuring whereas in this study, the cells were simply photographed at various time points and digitally measured using free computer software. Fluorescent staining would likely increase the sensitivity of this assay and perhaps allow for differences at earlier time points to be seen, which was not the case in the present study.

IDR-1018 has been shown by our collaborators to induce a higher rate of re-epithelialization in non-diabetic mice compared to LL-37 and HB-107, another synthetic peptide with previously characterized wound healing abilities (99). HHC-36-coated implants were observed to cause notable increases in new tissue growth in *Staphylococcus aureus* infected wounds in a study done in rats (64). Because these were *in vivo* studies, it is possible that the peptides do not directly stimulate keratinocyte migration and instead, exert their effect on other cells which may then release factors that promote keratinocyte migration. To study any peptide-mediated effects on keratinocyte migration, it would be interesting to treat PBMCs or fibroblasts with host defence peptides and use the resulting supernatants in a keratinocyte migration assay. Alternatively, the direct chemotactic abilities of IDR-1018 and HHC-36 could be examined using the more standard Boyden chamber assay (100).

IDR-1018, designed to be an immune modulator, was able to synergistically induce IL-8 production from human keratinocytes in the presence of the TLR3 agonist poly(I:C) or immune mediator IL-1β. This finding was not observed in the presence of Pam3CSK4,

flagellin or GM-CSF. qRT-PCR analysis showed that this synergistic IL-8 production could be seen transcriptionally 8 hours post-stimulation. HHC-36, originally selected for further development due to its direct antimicrobial activities, was unable to induce any synergy from human keratinocytes in the presence of other agents. The observed synergy suggests IDR-1018 can work cooperatively with naturally occurring agents during the innate immune response to enhance the activation of human keratinocytes. As incidents of resistance to antibiotics or other therapies continue to rise, this finding may play an important role in the search for effective treatment combinations.

Several investigators have reported that human host defence peptides can act in synergy with other antibacterial agents or peptides to enhance their antimicrobial or immunomodulatory effects. The antimicrobial activity of human β-defensin 3 against a variety of oral bacteria has been shown to be enhanced when given in combination with metronidazole, amoxicillin, chlorhexidine or lysozyme; these antibacterial agents all exert different modes of action (101). hBDs-1, -2, -3 and -4 and LL-37 can synergistically induce IL-18 secretion from human keratinocytes when treated in groups of two to five peptides (53). In PBMCs, LL-37 synergistically and selectively enhances the production of cytokines and chemokines in the presence of IL-1β and GM-CSF (55). To further examine the potential of IDR-1018 as a therapeutic agent, it would be of interest to examine the peptide's ability to enhance the effects of other host defence peptides, such as LL-37 and the human β-defensins, and relevant antibiotics.

A panel of pharmacological inhibitors was screened to elucidate which biological pathways contributed to the ability of IDR-1018 to synergistically induce IL-8 from keratinocytes. p38 MAPK, NF-κB and Src-family tyrosine kinases were found to play a role in the synergy seen between IDR-1018 and both poly(I:C) and IL-1β. PKC was additionally shown to play a role in the IL-1β, but not poly(I:C), synergy with IDR-1018. The activity of CREB, a downstream transcription factor in several of these pathways, was also examined and found to have increased phosphorylation when keratinocytes were co-stimulated with IDR-1018 and either poly(I:C) or IL-1β.

The number of commercially available protein kinase inhibitors has exploded over the past decade. A large majority of these inhibitors are described as being highly specific for at least one protein kinase. The specificity of some inhibitors, such as SB 203580, has been

validated *in vivo* by showing that the effects of the inhibitor disappear in cells that express drug-resistant mutants of the targeted protein kinase (102). Protein kinase inhibitor profiling studies have suggested the specificities of several commonly used inhibitors to be much less selective than previously thought (103, 104). In the present study, the protein kinase inhibitor PP2 caused a significant decrease in IDR-1018-induced IL-8 synergy in the presence of both IL-1β and poly(I:C). PP2 has been shown to be a potent inhibitor of the Src family of protein kinases and does not discriminate between the different members of the family (84). However, this inhibitor has been shown to inhibit other protein kinases, including p38α MAPK, although this inhibition occurred with 3- to 10-fold lower potency (104). SU6656, another potent inhibitor of the Src family of enzymes, did not have an effect on the synergistic IL-8 production observed in the present study. Like PP2, SU665 has been shown to have significant non-specific effects, although its specific targets differed from those of PP2. The non-specific effects of SU6656 have been shown to be of almost equal potency as its inhibition of the Src family kinases (103, 104). Thus, it is possible that the inhibitory effects of PP2 on the IDR-1018-induced synergy is caused by the non-specific effects of PP2 rather than its inhibition of the Src family kinases.

That multiple pathways appear to play a role in IDR-1018-induced synergy in the presence of a TLR agonist or immune mediator is not wholly surprising. LL-37 is likewise known to use multiple immune pathways and transcription factors, and host defence peptides in general are considered to be 'dirty' drugs that affect multiple internal cell targets through various mechanisms (43, 55). Future experiments could examine the effects on IL-8 production when inhibitors are used in combination. Additionally, the activity of other relevant transcription factors, such as IRFs and AP-1, could be examined to gain a better idea of the downstream effects of IDR-1018.

In summary, the findings in this study have shown the ability of two synthetic peptides to have a positive effect on the production of cytokines and chemokines from and proliferation of human keratinocytes. The ability of one of these peptides, IDR-1018, to synergistically induce the chemokine IL-8 from keratinocytes in the presence of the TLR3 agonist poly(I:C) or immune mediator IL-1β highlights the potential of this peptide to bolster host immunity through subtle modulation of the existing immune response. Multiple immune pathways were shown to be involved in the observed synergistic production of IL-8, which

suggests not only that the mechanism of action of IDR-1018 is complex, but that there are multiple ways that this peptide might exert its immunomodulatory effects that will no doubt be the subject of future studies. The results of this study have implications towards our understanding of how synthetic peptides may be used in the future to enhance the vitally important protective function of keratinocytes in the skin.

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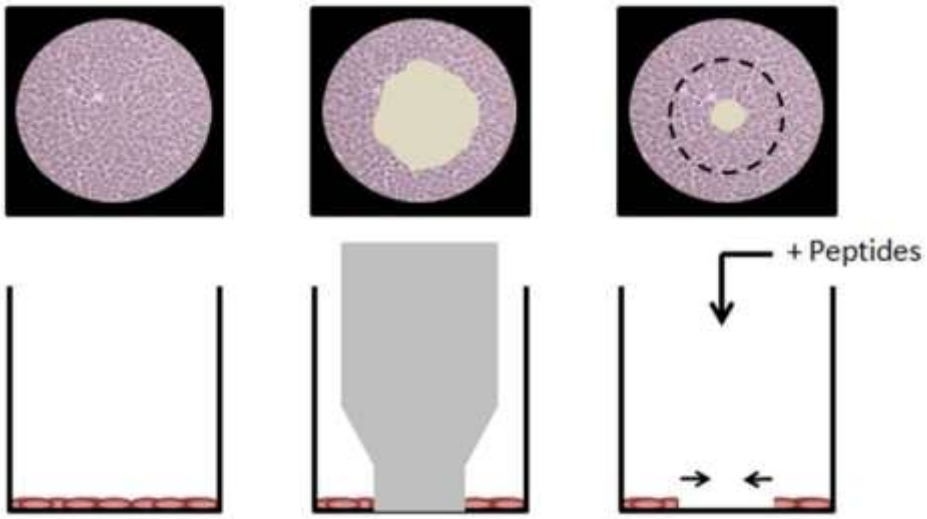
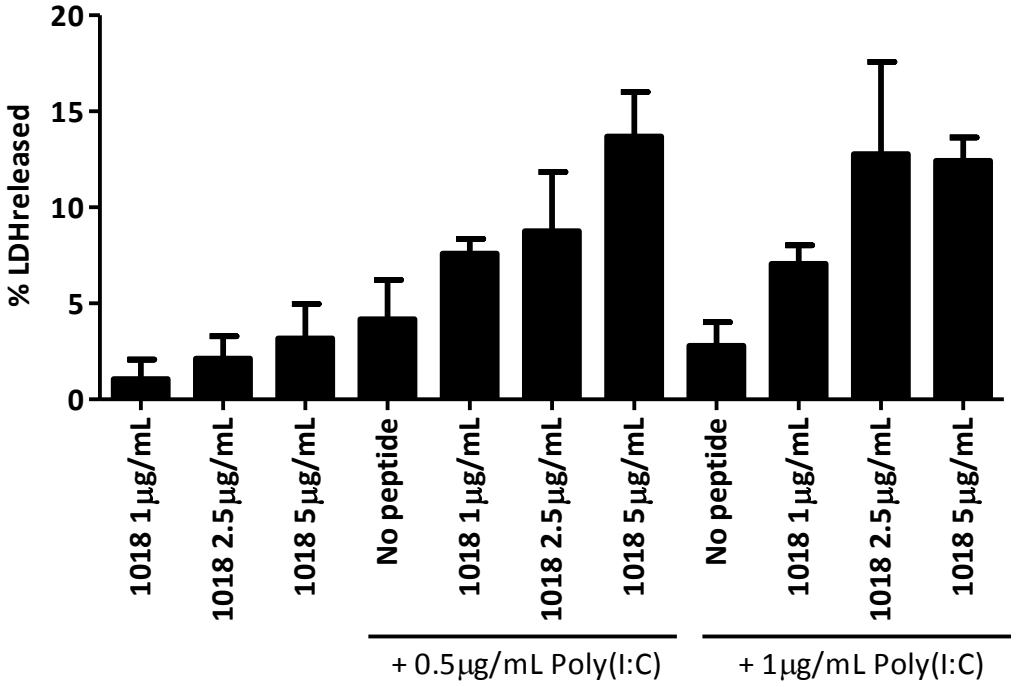
Appendices

Appendix A: Modified cell migration assay.



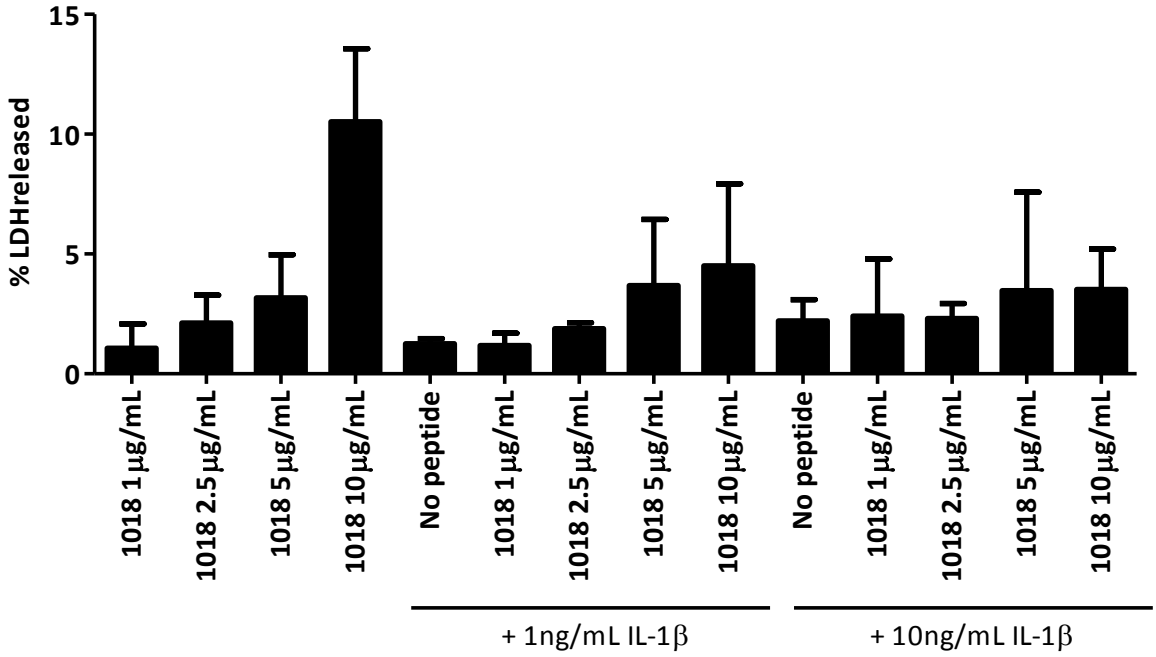
The Oris™ Cell Migration Assay was slightly modified to measure migration of keratinocytes. An artificial wound was created by stamping a confluent monolayer of HaCaT cells with a silicone stopper. The media in each well was then replaced with media containing the indicated concentration of peptide. Keratinocyte migration was measured over 48 hours by photographing the entire well and measuring the wound area using digital software. Percent wound closure was calculated using the following equation: ((Area T_0 – Area T_x)/Area T_0) x 100%.

Appendix B: Cytotoxic effects of IDR-1018 and poly(I:C) on human keratinocytes.



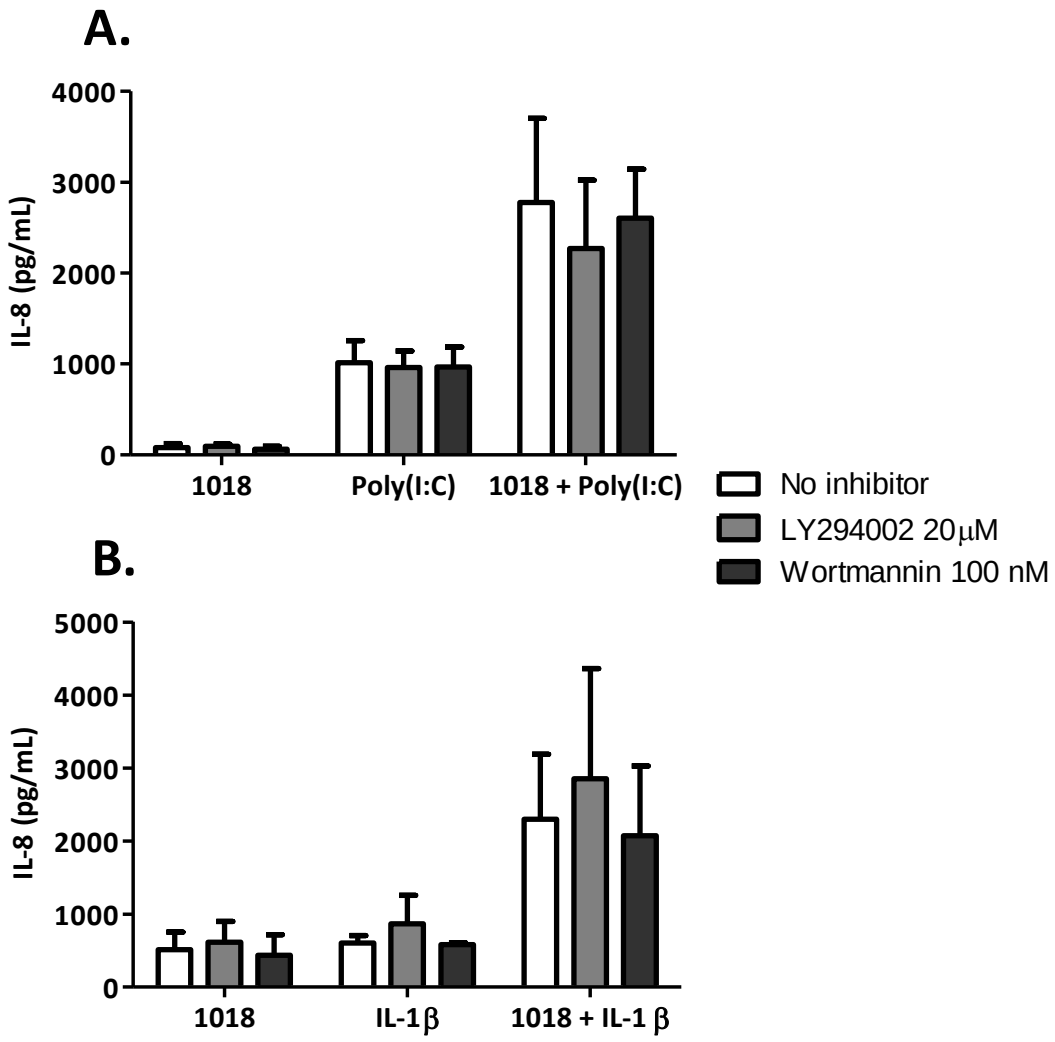
Sub-confluent HEKa cells were stimulated with IDR-1018 (1 µg/mL) with or without poly(I:C) (0.5 or 1 µg/mL) for 24 hours. 2% Triton-X-100 was used as a positive control. Cytotoxicity was assessed by calculating the percentage of LDH released into the supernatant using the following equation: ((experimental value − LDH released from untreated cells)/(maximum releasable LDH in cells by 2% Triton X-100 − LDH released from untreated cells)) × 100%. Each bar represents the mean of three independent experiments ± standard deviation.

Appendix C: Cytotoxic effects of IDR-1018 and IL-1β on human keratinocytes.



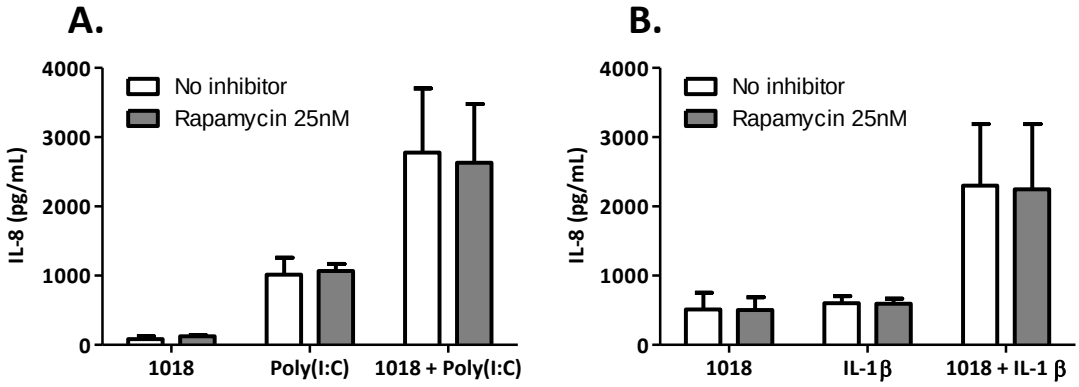
Sub-confluent HEKa cells were stimulated with IDR-1018 (10 µg/mL) with or without IL-1β (1 or 10 ng/mL) for 24 hours. 2% Triton-X-100 was used as a positive control. Cytotoxicity was assessed by calculating the percentage of LDH released into the supernatant using the following equation: ((experimental value − LDH released from untreated cells)/(maximum releasable LDH in cells by 2% Triton X-100 − LDH released from untreated cells)) × 100%. Each bar represents the mean of three independent experiments ± standard deviation.

Appendix D: Involvement of PI3K pathways in poly(I:C)- and IL-1β-induced synergy with IDR-1018



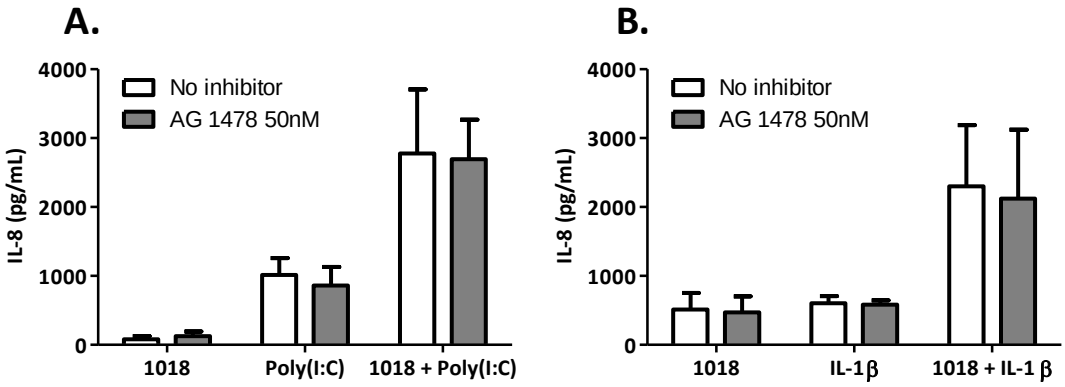
Sub-confluent HEKa cells were incubated with PI3K inhibitors LY294002 (20 μM) or Wortmannin (100 nM) for one hour prior to the addition of **A.** IDR-1018 (1 μg/mL) and poly(I:C) (1 μg/mL) or **B.** IDR-1018 (10 μg/mL) and IL-1β (1 ng/mL). Supernatants were collected after 24 hours and ELISA was performed to measure the concentrations of IL-8. Results show the mean of at least three independent experiments ± standard deviation. Background subtraction of untreated cells was performed on all data.

Appendix E: mTOR does not play a role in poly(I:C)- and IL-1β-induced synergy with IDR-1018



Sub-confluent HEKa cells were pre-treated with Rapamycin for one hour before the addition of **A.** IDR-1018 (1 μg/mL) and poly(I:C) (1 μg/mL) or **B.** IDR-1018 (10 μg/mL) and IL-1β (1 ng/mL). Supernatants were collected after 24 hours and IL-8 levels were measured using ELISA. Results are the mean of at least three independent experiments ± standard deviation. Background subtraction of untreated cells was performed on all data.

Appendix F: Inhibition of EGFR does not affect poly(I:C)- and IL-1β-induced synergy with IDR-1018



Sub-confluent HEKa cells were incubated with the EGFR inhibitor AG 1478 (50 nM) for one hour prior to the addition of **A.** IDR-1018 (1 μg/mL) and poly(I:C) (1 μg/mL) or **B.** IDR-1018 (10 μg/mL) and IL-1β (1 ng/mL). Supernatants were collected after 24 hours and ELISA was performed to measure the concentrations of IL-8. Results show the mean of at least three independent experiments ± standard deviation. Background subtraction of untreated cells was performed on all data.