Molecular mechanisms and effector functions of the human cathelicidin host defence peptide LL-37: Modulation of cytokine IL-32γ-induced responses and inflammatory arthritis

By

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THESIS ABSTRACT

Current therapies for chronic inflammatory diseases often abrogate the immune functions required to fight infections. Human cathelicidin host defence peptide (HDP) LL-37 selectively suppresses pathogen-induced inflammation, without compromising resistance to infections. These unique dual abilities of LL-37 make it a promising candidate as an alternative therapeutic for treating chronic inflammatory diseases. The objective of this study was to investigate the effects of LL-37 and its derivative peptide IG-19 in cytokine-mediated inflammation. I demonstrated that LL-37 and IG-19 selectively suppressed cytokine IL-32γ-induced pro-inflammatory cytokines, without compromising the production of anti-inflammatory cytokines, and chemokines in human PBMC and macrophages. However, significant quantitative differences between LL-37 and IG-19-mediated chemokine productions suggested that the mechanisms underlying the activity of these two peptides were different.

I showed that both peptides suppressed IL-32γ-mediated phosphorylation of the Src-kinase FYN(Y420), known to enhance inflammation. Contrastingly, phosphorylation of the dual phosphatase MKP-1(S359), a negative regulator of inflammation, was enhanced in response to both peptides. Similarly, both peptides increased the activity of p44/42MAPK, which phosphorylates and stabilizes MKP-1. These results suggested that MKP-1 may be a critical mediator of the immunomodulatory activity of these peptides.

Bioinformatic interrogation revealed that direct interacting protein partners of MKP-1 were overrepresented in MAPK and NF-κB signalling pathways. Both peptides enhanced the phosphorylation of p38MAPK. However, contrasting to LL-37, IG-19 did not mediate the phosphorylation of JNK MAPK and IKK-α signaling intermediates involved in inflammation.

This was consistent with observations that chemokine production was significantly lower in response to IG-19 compared to LL-37. These results suggested that IG-19 may be a better immunomodulatory therapeutic candidate compared to LL-37.

As cytokine-mediated inflammation plays critical roles in the disease pathogenesis of inflammatory arthritis, I examined the effects of exogenous administration of IG-19 in a murine model of collagen-induced arthritis. Administration of IG-19 decreased disease severity, suppressed pro-inflammatory cytokines and anti-collagen antibodies, and mitigated cartilage destruction in the CIA mice. These results provide a rationale to further develop IG-19 as a therapeutic agent for chronic inflammatory arthritis. The advantage of HDP based therapy is the potential to control inflammation without compromising the patient's ability to resolve infections.

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Thank you very much, everyone!

DEDICATION

I dedicate this work to all of my family and friends.

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LIST OF ABBREIVATIONS

$1,25(OH)_2D_3$ or Vitamin D_3	1,25-dihydroxycholeclciferol
Ab	Antibodies
ACPA	Anti-citrullinated protein antibody
AMP	Antimicrobial peptides
AP-1	Activator protein-1
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BCA	Bicinchoninic acid
Bcl-xL	B-cell lymphoma-extra large
Bid	BH3 interacting-domain death agonist
BSA	Bovine serum albumin
CAMP	Cathelicidin antimicrobial peptide
CD	Crohn's disease
CDCA	Chenodeoycholic acid
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CII	Type II Collagen
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
CRAMP	Cathelin-related antimicrobial peptide
CREB	cAMP response element-binding
DAMPs	Damage-associated molecular pattern molecules
DC	Dendritic cells
DUSP-1	Dual specificity phosphatase-1
ECL	Chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FBS	Fetal bovine serum
FPRL1/2	Formyl peptide-like receptor 1 and 2
Fyn	Proto-oncogene tyrosine-protein kinase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCRs	G-protein-coupled receptors
GRO-α	growth regulated oncogene-alpha
H & E stain	Hematoxylin and eosin stain
hBD-2	human beta-defensin-2

HBS	Hepes buffer
hCAP18	Human cationic antimicrobial peptide 18
HDP	Host defence peptide
HIV	Human immunodeficiency virus
HMGB1	High mobility group box 1
HRP	Horseradish peroxidase
i.d.	Intradermal
IBD	Inflammatory bowel disease
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IGF-1R	Insulin-like growth factor-1 receptor
IKKα/β	IκB kinase alpha/beta
IL-	Interleukin
IL-1RA	IL-1 receptor antagonist
JNK	c-Jun N-terminal kinases
LCA	Lithocholic acid
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MCSF	Macrophage colony-stimulating factor
MDM	Monocyte-derived macrophages
MDP	Muramyl dipeptide
MHC-II	Major histocompatibility complex class II
MIC	Minimum inhibitory concentration
MIP-2	Macrophage inflammatory protein-2
MKP-1	Mitogen-activated protein kinase phosphatase-1
MMPs	Matrix metalloproteinases
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaCl	Sodium chloride
NF-κB	Nuclear factor-kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
NK4	Natural killer cell transcript 4
PAMPs	Pathogen-associated molecular pattern molecules
PBMC	Peripheral blood derived mononuclear cells
PHA	Phytohaemagglutinin
PKCδ	Protein kinase Cδ
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PR3	Proteinase 3
PRRs	Pattern recognition receptors

RA	Rheumatoid arthritis
RF	Rheumatoid factor
S	serine
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
Syk	spleen tyrosine kinase
TC	Tissue culture
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor
VSV	Vesicular stomatitis virus
Y	Tyrosine

CHAPTER 1 – GENERAL INTRODUCTION

1 - 1 Innate Immune response

Innate immune responses are the first line of defense of host immune system, which recognizes and discriminates pathogens from host cells, and initiates rapid, non-specific responses against invading pathogens [1-3]. One of the key components of innate immune responses is known as inflammation, a process where the body eliminates invading pathogens, removes damaged tissues and initiates tissue repair. This is followed by anti-inflammatory responses, which acts as a regulating mechanism to resolve inflammation and return the immune system to homeostasis. Together, these processes are known as acute inflammation, which usually have a short duration, and resolve quickly once the invading pathogen or tissue damage is cleared. However, dysregulation in inflammatory responses or failure to resolve inflammation has the potential to result in chronic inflammation, a process of persistent unresolved inflammation. Chronic inflammation often leads to irreversible damages and contributes to the development of complex diseases and disorders. Hence, a tight regulation of inflammation is required for optimal functioning of the immune system.

1 - 1.1 Inflammation

Inflammation is physically characterized by the presence of pain, heat, redness and swelling, which reflects the release of chemicals to stimulate nerve endings, the increased in blood flow, activation and influx of immune cells and release of a wide variety of inflammatory effectors (such as complements, inflammatory cells, cytokines and chemokines) at the site of injuries or infection [4]. Inflammatory responses are initiated upon recognizing the presence of pathogens or damaged cells. This process is driven by innate immune cells, such as neutrophils,

eosinophils, basophils, mast cells, macrophages, fibroblasts and dendritic cells (DC), which recognize pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs) via pattern recognition receptors (PRRs) [1, 2, 5, 6]. PAMPs are usually pathogen-associated microbial components which are absent in the host, such as bacterial or viral surface proteins (eg. lipopolysaccharides [LPS]), flagellin, lipoproteins and nucleic acids [1, 2, 5]. DAMPs refer to endogenous molecules, such as adenosine triphosphate (ATP), nuclear protein high mobility group box 1 (HMGB1), self nucleic acids and uric acid, which are released by damaged or stressed cells [7-9]. PRRs recognize PAMPs and DAMPs either directly or indirectly, following which the receptors oligomerize and assemble into larger multi-subunit complexes. These complexes further activate various cascades of signaling pathways within immune cells, resulting in the release of molecules such as cytokines, chemokines and host defence peptides (HDPs), to promote recruitment of leukocytes to the site of infection and inflammation. In addition, activated immune cells also releases factors such as histamine and prostaglandins, which induces vasodilatation, increases blood flow, enhances vascular permeability, and contributes to the migration of immune cells. The secreted molecules, such as cytokines and chemokines, play essential roles in the activation and regulation of the immune system.

1 – 1.2 Cytokines and chemokines

Cytokines and chemokines are small, pleotropic proteins that play essential roles in coordinating and regulating many essential immune responses, such as mediating cell activation and differentiation, altering signaling pathways, and modulating inflammation. Every cell in the body is capable of producing and responding to cytokines and chemokines. These soluble molecules can signal through autocrine (target self), paracrine (target nearby cells) or endocrine (target cells

in a distant) mechanisms [10, 11]. Depending on the environment, more than one type of cytokines or chemokines can be released at the same time and can work synergistically to complement the functions of each other. In contrast, these molecules can also be antagonistic by inhibiting the expressions or functions of each other. These soluble proteins modulate signaling pathways that are often redundant, complex and sometimes contradictory.

Chemokines are a specific group of cytokines containing conserved cysteine patterns near the amino-terminal domain, and mainly function to influence cell trafficking and recruit immune cells, such as moonocytes, neutrophils and lymphocytes, to a specific location [12]. These molecules bind to specific G-protein-coupled chemokine receptors (GPCRs) located on the cell surface, and activate the downstream intracellular signaling cascades, which result in migration of immune cells [13]. Chemokines are divided into four major families (CC, CXC, C and CX3C) based on the location of the conserved cysteine residues near the N-terminus of the protein (where the C represents cysteine and the X represents any amino acid residues) [14]. The majority of known chemokines belong to the CC and CXC families. Chemokines from the CC family, such as the monocyte chemoattractant protein-1 (MCP-1, also known as CCL2), primarily mediate chemotactic activity towards monocytes, but can also influence T-cell chemotaxis [15]. Within the CXC chemokine family, IL-8 (also known as CXCL8), and growth regulated oncogene-alpha (GRO-α, also known as CXCL1), are known to be potent chemoatractants for granulocytes [16]. In addition, chemokines can generally be divided into two groups (inflammatory and homeostatic) based on their functions, with some chemokines having both inflammatory and homeostatic properties [17, 18]. Inflammatory chemokines are produced under inflammatory conditions, and are responsible to recruit immune cells to the sites of infections or inflammation [18]. At the target site, the recruited cells mediate various pro-

inflammatory responses to kill invading pathogens and resolve infection. Homeostatic chemokines are constitutively expressed, and guide the movement of immune cells (such as lymphocytes and dendritic cells) as part of normal tissue development and functional maintenance (such as hematopoiesis, development of lymphoid organs and immune surveillance) [17, 19].

Cytokines are generally categorized into two major types, pro-inflammatory or anti-inflammatory cytokines, based on their overall functions. Better understanding of how these soluble molecules interact to mediate different immune responses can facilitate the identification of treatment targets for immune-mediated chronic inflammatory diseases. Pro-inflammatory cytokines activate and enhance a complex network of inflammatory pathways and activate immune cells, induce the production of adhesion molecules, promote cell-mediated immune responses and induce the production of other pro-inflammatory cytokines, contributing to both acute and chronic inflammatory processes [20, 21]. Anti-inflammatory cytokines, such as IL-10, IL-1 receptor antagonist (IL-1RA) and transforming growth factor-beta (TGF-β), suppress the production of pro-inflammatory cytokines and chemokines, and thus antagonize the effects of pro-inflammatory responses, aimed to resolve inflammation and bring the immune system back to homeostasis [22].

The processes of cytokines and chemokines production are all highly regulated. The breakdown in any of the appropriate regulation processes can cause excessive or prolonged inflammatory responses, which leads to tissue damage or injury, and eventually lead to a wide variety of chronic inflammatory diseases, such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), chronic obstructive pulmonary disease (COPD), asthma and the development of cancers [4]. Hence, it is essential to appropriately modulate inflammatory responses. Over the last two

decades, many studies have shown that another family of molecules, known as host defence peptides (HDPs) is able to modulate immune responses, such as regulating the production of pro-inflammatory cytokines by influencing different signalling pathways without significantly altering the production of homeostatic chemokines, and to create an overall balanced inflammatory response that can contribute to the clearance of infection and mediate tissue repair. Modulation of pro-inflammatory cytokine-mediated cellular responses by HDPs is the general focus of my thesis.

1 – 1.3 Host Defence Peptides (HDPs)

Cationic Host defence peptides (HDPs), also known as antimicrobial peptides (AMPs), are a group of relatively small (typically less than 50 amino acids) molecules that play important roles in providing protection against invading pathogens [23]. These small molecules are amphipathic, containing both hydrophobic and hydrophilic regions, and have net positive charges of +2 to +9 at physiological pH 7.4 [24-26]. To date, there are over 2,600 known natural HDPs that have very diverse conformational structures, such as linear α-helical structure (e.g. cathelicidin), β-sheet structure with disulfide bridges (e.g. α-defensin), cyclic structures (e.g. catestatin), and extended and flexible loop structures (e.g. indolicidin) [23, 25, 27-29]. Since the discovery of the first HDP (cecropin from moths) more than 25 years ago, increasing number of HDPs has also been identified in a wide variety of organisms across different species, which include microorganisms, plants, insects, crustacean, fishes, birds, amphibians and mammals [28, 30-37]. The expression of HDPs across such diverse species suggests that these peptides may be evolutionarily conserved molecules that contribute to host defense mechanism [34, 38].

The discovery of HDPs in insects and hagfish, which lack adaptive immune systems, was the first evidence to suggest that HDPs play important roles as innate host defense mechanism

against pathogens [33, 39-41]. Further studies of HDPs showed that these peptides have broad-spectrum antimicrobial activity against bacteria, virus and parasites, and therefore these molecules are also known as antimicrobial peptides (AMP) [23-25, 28, 30, 42, 43]. Several modes of antimicrobial action involving direct interactions with microbes were proposed, such as destabilizing microbial membranes, neutralizing endotoxin, influencing microbial DNA and protein synthesis, or triggering autolysis [44, 45]. However, more recent studies have shown that the direct anti-microbial activities of certain HDPs, such as the human cathelicidin LL-37 and human β-defensin, are antagonized in the presence of physiological salt concentrations (such as 2mM Mg^{2+}, Ca^{2+}) and in the presence of host factors such as anionic polysaccharides [46, 47]. There are increasing studies demonstrating that certain HDPs contribute to the clearance of infection by influencing the host immune system, including the modulation of both the innate and adaptive immune responses to resolve infections. Previous studies have also shown that HDPs have the ability to resolve inflammation, regulate cytokine and chemokines productions, mediate cell recruitment, induce cell proliferation and activation, and influence angiogenesis [25, 27, 48-53]. Taken together, HDP-mediated immune functions contribute to an overall result of enhancing clearance of infection and balanced inflammation. The emerging theme that HDPs contribute to the clearance of infection without directly targeting microbes suggests that these peptides have the potential to overcome bacterial resistance, which has piqued interests in the use of these peptides as alternative therapeutics against antibiotic-resistant microbial pathogens.

Over the years, it has been demonstrated that human HDPs exhibit anti-infective activities against both Gram-positive and Gram-negative bacteria, virus, fungi and protozoa, as well as have pleiotropic immunomodulatory functions influencing both the innate and adaptive immunity [48, 54-58]. Human HDPs are expressed by both circulating immune cells (such as

neutrophils, lymphocytes, and monocytes) and non-circulating structural cells (such as fibrocytes and epithelial cells), and can be found in bodily fluids such as plasma, mucus, breast milk, sweat, and tears. Physiological concentration of HDPs varies greatly within the body, with higher concentrations (in the range of mg/mL) found in the granules of leukocytes and at the bottom of intestinal crypts, and lower concentration (in the range of ng/mL to μg/mL) can be found in the mucosa and in circulation [59-64]. Among the natural cationic HDPs, the two most characterized families in mammals are defensins and cathelicidins. Defensins are non-glycoslated peptides that have six cysteine residues to form intramolecular disulfide bridges and β-sheet conformation [65]. Cathelicidins are linear peptides which contain a conserved cathelin-like domain. It has a random-coil conformation in hydrophilic environment, and forms α-helical structure in hydrophobic environment [65]. My study focuses on elucidating the effects of the sole human cathelicidin peptide LL-37 within an inflammatory environment.

1 – 2 Human Cathelicidin LL-37

1 - 2.1 Cathelicidin

Cathelicidin is a family of HDPs characterized by the highly conserved cathelin-like domain located at the N-terminal, with the highly variable cathelicidin domain located at the C-terminal [49]. The term "cathelicidin" was named after the cathelin-like domain, which was first described in bone marrow myeloid cells of mammals, and was characterized as an inhibitor for the cysteine proteinase - cathepsin L [49, 66]. Due to the highly conserved cathelin-like domain among species, the genes of cathelicidin in different species were identified via sequence screening and cDNA cloning [32, 66-68]. For example, the murine cathelicidin peptide shares 67% homology with the human cathelicidin peptide [27]. Over the years, cathelicidins have been

found to be expressed in a wide variety of species, such as mammals, amphibians, avians, and fishes [69-75]. The cathelicidin peptides are processed before secretion, and the processed mature peptide among different species ranges from 12 to 71 amino acids long, and varies in structures. The number of cathelicidins expressed by each species is different [76-80]. For example, there are more than ten different cathelicidins found in bovine or porcine [80-82]. However, there is only one known cathelicidin peptide defined in human and murine, named LL-37 (leucine-leucine-37) and cathelin-related antimicrobial peptide (CRAMP) respectively [27, 71]. The human cathelicidin hCAP18-derived mature peptide LL-37 plays essential roles in providing protection against infections as well as balancing the immune system. The peptide LL-37 is the focus of my research studies in this thesis.

1 - 2.2 Transcription, secretion and processing of LL-37

The human cathelicidin is known as hCAP18, where hCAP18 refers to the pre-proprotein, human cationic antimicrobial peptide 18, which is the precursor of LL-37 [32, 71, 83]. The gene for the human cathelicidin hCAP18/LL37 is known as cathelicidin antimicrobial peptide (*camp*) [32, 83]. This gene is expressed in myeloid and lymphoid bone marrow cells and epithelial cells of skin, airways, ocular surface and intestine [32, 62, 71, 83, 84]. The *camp* gene is 1963 base pairs long, containing four exons, and is located on chromosome 3p21 [83, 85]. Exons 1 - 3 code for the signalling and the cathelin-like domain sequences, while exon 4 codes for the antimicrobial sequence. Altogether, the *camp* gene translates to an 18kDa pre-proprotein known as hCAP18, which contains an N-terminal signaling peptide (30 amino acid residue), a highly conserved cathelin-like domain (103 amino acid residue), and C-terminal peptide domain (37 amino acid residue of LL-37) as shown in Figure 1.

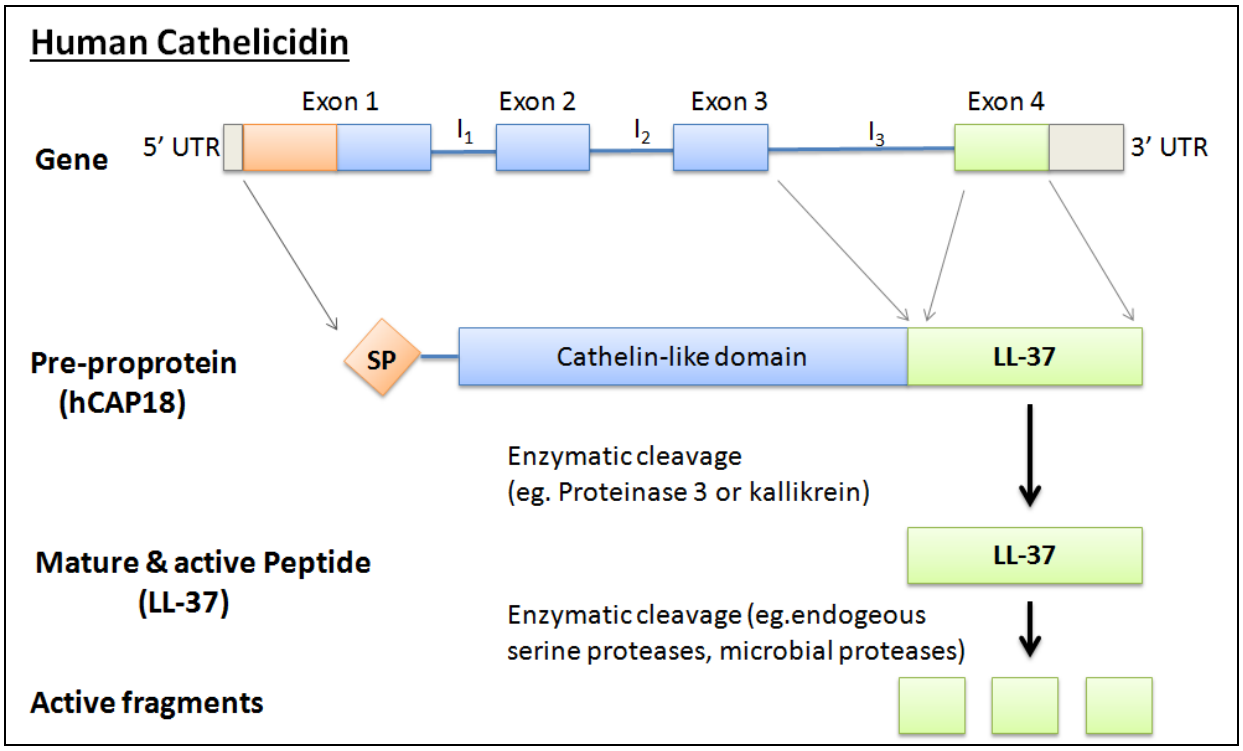


Figure 1. Gene and protein processing of human cathelicidin LL-37.
From top to bottom: structure of the human cathelicidin gene *(camp)*; structure of the pre-proprotein (hCAP18), mature peptide (LL-37) and active fragments. Figure modified from Seil et. al., Pharmaceuticals, 2010. 3(11): 3435-3460 [44].

The pre-proprotein hCAP18 is the inactive, storage form of LL-37, which can be found in granules of immune cells, as well as in the bloodstream [84, 86, 87]. The signaling peptide on the N-terminus is responsible to direct the peptide either to storage granules or to the exterior of the cells. This ability to direct hCAP18/LL37 to either storage or secretion is a key to rapid host defense. For example, human epithelial cells do not constitutively express hCAP18/LL37, and it would take several hours for epithelial cells to begin producing the peptide upon injury or infection. Therefore, storing high concentration of the precursor peptide hCAP18 in the secondary granules of granulocytes, such as neutrophils, allows for rapid response at the sites of injury or infection [84]. The cathelin-like domain is highly conserved among different species

and defines hCAP18/LL-37 as belonging to the cathelicidin family. For example, human and porcine cathelicidin share more than 60% of identical residues in the cathelin-like domain [80]. The exact function of the cathelin-like domain is still controversial, with some studies suggesting that it contributes to antimicrobial activities and inhibits cathepsin L activity, while others suggest it inhibits and regulates LL-37 activities. The mature and biologically active peptide LL-37 is produced upon enzymatic cleavage of the signaling peptide and cathelin-like domain in hCAP18 via serine proteases, such as proteinase 3 and kallikrein [88, 89]. The secreted mature peptides of cathelicidins have limited conservation in their amino acid sequences from various species.

The human peptide LL-37 is 37 amino-acid long, amphipathic, and contains both hydrophobic and hydrophilic regions [90]. The mature LL-37 peptide (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) begins with two leucines and contains no cysteine or tryptophan. At physiological pH, LL-37 has a net positive charge of +6 [83, 90]. The secondary structure of LL-37 seems to depend on the environment as well as concentration of the peptide [91, 92]. Studies have demonstrated that LL-37 has a disordered conformation in aqueous solution, and can fold into an amphipathic α-helix when in contact with lipid membranes [92, 93]. Furthermore, Johansson and colleagues have shown that LL-37 maintains a random disordered conformation at micromolar concentration in water, but will transition to α-helix conformation once its concentration increased [91]. It was reported that the α-helical structure of LL-37 is essential in its anti-endotoxin, antimicrobial and antibiofilm activities [45, 94, 95]. In addition, LL-37 was found to form aggregates in solutions and lipid bilayers, which was suggested to protect the peptide from proteolytic degradation [91, 93, 96].

However, the formation of aggregates also renders LL-37 cytotoxic at higher concentrations [91].

Enzymatic cleavages had been shown to further process LL-37 into smaller fragments (such as, RK31, KS30, LL-13 – 31 (also known as IG-19), LL29, KS22 and KR20) on the epithelial surfaces [88]. Some studies suggested that these smaller fragments retain their antimicrobial actions, while other studies have suggested the alternative cleavage of LL-37 downregulates the functions of the peptide [59, 88, 89, 97-100]. Wang et al. have established that the central helix of LL-37 is critical for antibacterial, antibiofilm and antiviral activity [45]. It was shown that IG-19, an internal region of LL-37 (also known as LL-13-31), was the shortest fragment of LL-37 that exhibited antibacterial activity against *Burkholderia thailandensis*, and was the best immunomodulatory peptide [95, 101]. Consistent with this, peptide IG-19 showed selective modulation of inflammatory responses similar to LL-37 in our screening studies using human peripheral-blood derived mononuclear cells (PBMC) [102]. Hence, I focused on examining the effects of LL-37 and its derivative peptide IG-19 on cytokine-mediated inflammatory responses in this thesis.

1 - 2.3 Expression and regulation of hCAP18/LL-37

LL-37 is expressed by a wide variety of cells and tissues, such as monocytes, lymphocytes, natural killer (NK) cells, mast cells, dendritic cells (DC), neutrophils, synovial membranes and epithelial cells of skin, gastrointestinal and respiratory tracts [69, 103-107]. It is also found in tears, wound fluid, breast milk, sweat, saliva, bronchoalveolar lavage (BAL) fluid, amniotic fluids and spermatozoa [59-64]. Extensive studies over the last decade have demonstrated that LL-37 plays multifaceted roles in immunomodulation and inflammation.

Since the development of a specific ELISA assay to quantify hCAP18/LL37 in cells, plasma and urine in 1997, a wide range of physiological concentrations of hCAP18/LL37 have been reported [108]. Sorensen et. al. have reported that in healthy donors, the amount of hCAP18 in neutrophils is 627 ng of hCAP18/ 10^6 neutrophils and the plasma level is about 1.18 μg hCAP18/mL plasma [86, 108]. It has also been reported that 2 μg/mL and 5 μg/mL of hCAP18/LL37 was detected in healthy adults and neonates airway fluids respectively. More recent studies have reported that 40 – 140 μg/mL hCAP18 was found in seminal plasma of healthy donors [109]. However, the expression of LL-37 significantly increases at the site of infection and inflammation. In the bronchoalveolar lavage fluid of infants with pulmonary infections and patients with cystic fibrosis, as much as 15 –25 μg/mL hCAP18/LL37 was detected [110, 111]. As much as 20 μg/mL of LL37 was detected in patients with lung infection [111, 112]. The expression of LL-37 can be upregulated by a wide variety of stimuli, such as cytokines, growth factors, short chain fatty acid, nutrients (e.g. vitamin D_3), bacterial and fungal products (butyrate, flagellin, DNA) and other HDPs (e.g. human beta-defensin 2 [hBD-2]) [78, 113-118]. There are also some microbes, such as *Shigella flexneri*, *Neisseria gonorrhoeae*, and *Vibrio cholera*, that downregulate the expression of LL-37 [119-121]. However, the mechanisms behind bacterial-mediated downregulation of LL-37 are not fully understood.

Recent studies have revealed that 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$ or Vitamin D_3), bile acids and butyrate can enhance the expression of the human LL-37 gene *(camp)* [113-115, 122-124]. Further studies demonstrated that sufficient amount of $1,25(OH)_2D_3$ is required to induce the production of adequate amount of LL-37 to fight infections, such as tuberculosis [104, 114, 125, 126]. In the colon, chenodeoxycholic acid (CDCA, a major primary bile acid) and its by-products lithocholic acid (LCA) and butyrate (a short-chain fatty acid) can also upregulate the

expression of LL-37 [123, 127]. It has been shown that oral administration of sodium butyrate can restore *Shigella*-mediated downregulation of LL-37 [128]. Furthermore, butyrate and $1,25(OH)_2D_3$ synergistically induce human *camp* expression in lung epithelial and myeloid cells [129].

The expression of LL-37 has been reported to be altered not only during infections, but also in autoimmune and other chronic diseases. Increased expression of LL-37 has been associated with pulmonary inflammation, rheumatoid arthritis, psoriasis, eczema, while suppression of LL-37 has been associated with skin disorders (such as atopic dermatitis), chronic ulcers, Morbus Kostmann syndrome, asthma and inflammatory bowel disease [27, 111, 130, 131]. However, the exact roles of LL-37 in chronic diseases are not well understood.

1 - 2.4 Antimicrobial functions of LL-37

Human cathelicidin LL-37 was first demonstrated to be an endogenous antimicrobial peptide which plays key roles in fighting infections. When tested *in-vitro* for antimicrobial and anti-biofilm activity, the peptide was able to kill a wide range of microorganisms, including bacteria, virus and fungi [42, 49, 51, 57, 105, 115, 132-134]. The minimum inhibitory concentration (MIC) of LL-37 ranges from 0.2–32 μM for a wide variety of Gram-positive (such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Listeria monocytogenes*) and Gram-negative bacteria (such as *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Escherichia coli*) in *in-vitro* assays [49, 132, 135-137]. Recent studies have demonstrated the ability of the peptide LL-37 and its synthetic derivatives to inhibit the formation of biofilms, using both in *in-vitro* and *in-vivo* models [56, 138-140]. The significance of the antimicrobial role of LL-37 was demonstrated in studies using mice deficient in CRAMP, a murine ortholog of LL-37 [141-143]. It was shown that mice deficient in CRAMP have increased colonization and invasion of

pathogenic bacteria on the skin, and in the urinary tract, lung and colon [141-143]. The CRAMP-deficient mice were also more susceptible to both bacterial and viral infection.

One of the first proposed modes of antimicrobial action was direct interaction of LL-37 with the pathogenic organisms due to electrostatic binding of the cationic peptide LL-37 to the negatively charged bacterial membrane [32, 43]. The amphipathic characteristic of the peptide, containing both hydrophobic and hydrophilic residues, allowed LL-37 to penetrate and disrupt the bacterial cell membrane, forming transmembrane pores and causing leakage of cell contents, which results in cell death [43]. The same study showed that *in-vitro* eukaryotic cytotoxicity was only observed at a much higher concentration (more than 25 μM) of LL-37. It was suggested that the highly asymmetric eukaryotic plasma membranes containing cholesterol, phosphatidyethanolamine, phosphatidylcholine, and sphingolipid, reduce the membrane association with LL-37 and thus protecting the host cell membranes from being disrupted. In addition, the cytotoxic effect of LL-37 is inhibited by the presence of serum, suggesting that specific components in the serum provide protection to host cells.

More recent studies have shown that the antimicrobial function of LL-37 is not merely by the direct interaction of the peptide with bacterial cell membranes. In fact, it was observed that the direct antimicrobial activities of LL-37 was reduced and antagonized in the presence of physiological concentration of salt, serum, lipoproteins and glycosaminoglycans [91, 144-146]. In addition, some microorganisms seem to have developed resistance and overcome direct interaction with LL-37 *in-vitro* [98, 99, 147]. An *in-vitro* study by Ouhara and colleagues, has shown that some pathogenic bacteria, such as methicillin-resistant *S. aureus* (MRSA), have higher resistance to LL-37 direct killing due to the higher net charge on the cell surface [148]. Other bacteria, such as *Hemophilus influenzae*, had been demonstrated to possess resistance to

LL-37 killing, by decorating its cell membrane with phosphorylcholine, a component similar to those found in host membrane [149]. A few studies have further shown that some pathogenic microbes express proteinases that can degrade and inactivate LL-37 [150-154]. However, LL-37 is essential for the protection against infections, including resistant bacteria such as MRSA-induced pneumonia and influenza A virus infection *in-vivo* [65, 100, 155, 156]. The importance of LL-37 or CRAMP in providing protection against infection has been demonstrated in many studies, showing that humans and mice deficient in their respective cathelicidin peptide are more susceptible to bacterial and viral infections [141, 156, 157]. Clinical studies have shown that patients deficient in LL-37 are more susceptible to oral infection and develop Morbus Kostmann syndrome [131]. It has also been reported that LL-37 forms part of the innate defense shield in the nasal epithelium and external auditory canal to prevent bacterial and fungal infections [137, 158]. Schaller-Bals et al. reported that LL-37 increased two to three folds in tracheal aspirates of newborn infants during pulmonary infections [111]. Furthermore, there is increasing evidence demonstrating that LL-37 mediates the clearance of infection primarily via modulating host immune functions and acts synergistically with other immune molecules, such as certain cytokines, defensins and lysozymes, thus the role of LL-37 in immunity may be more important than the direct microbicidal activity in resolving infections [23, 27, 65, 156, 159].

1 - 2.5 Immunomodulatory functions of LL-37

The cathelicidin peptide LL-37 has the ability to mediate a wide range of host immune mechanisms, including both pro-inflammatory and anti-inflammatory functions depending on the cell type, the concentration of LL-37 and the presence of other stimuli [26, 49, 50, 103, 105, 159, 160]. Numerous studies have shown that depending on the cell type and the presence of other stimuli, LL-37 is able to differentially regulate the production of cytokines and chemokines, and

regulate pathways involved in the activation and differentiation of immune cells. In addition, LL-37 also plays a role in initiating wound healing. A summary of the immunomodulatory function of LL-37 is presented in Figure 2.

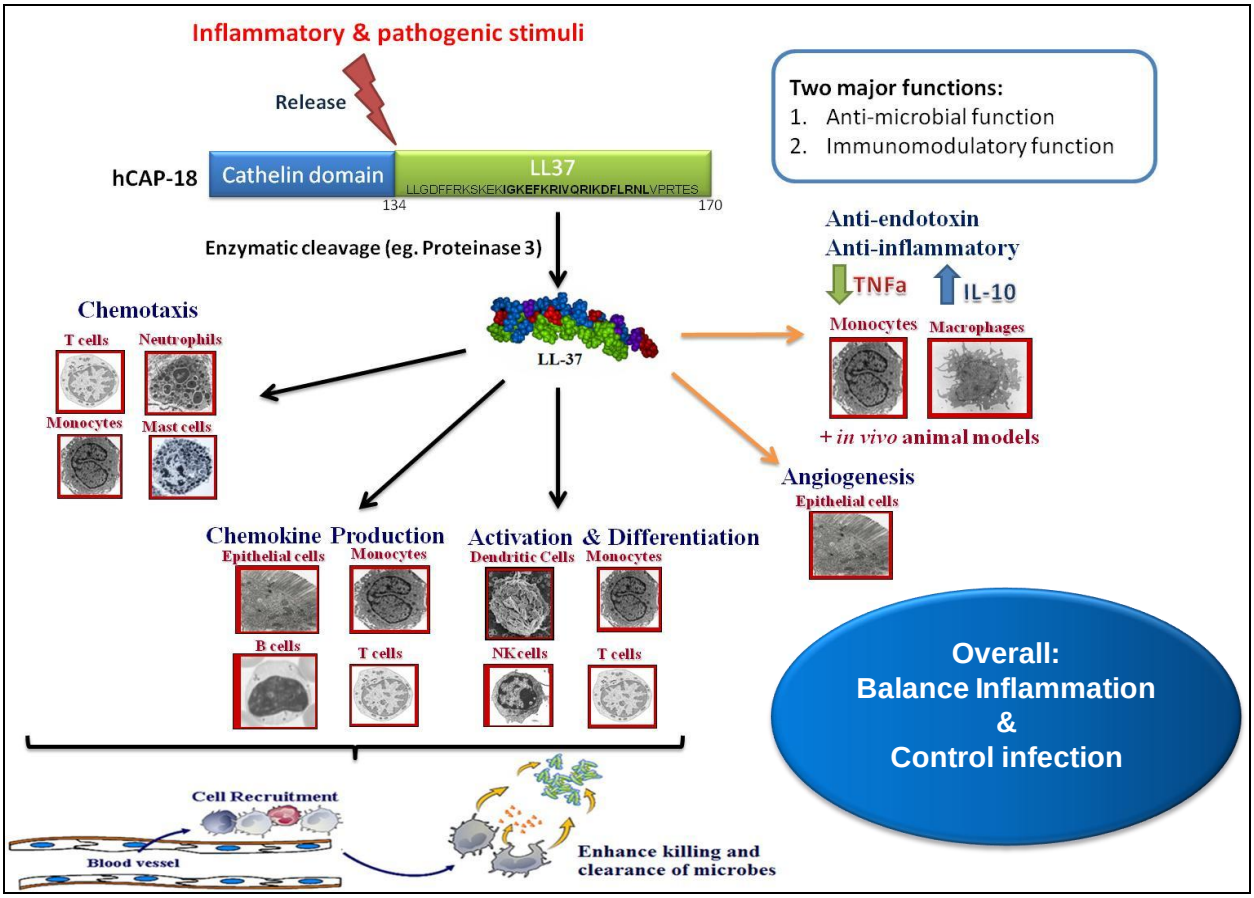


Figure 2. Immunomodulatory functions of LL-37.
Human cathelicidin peptide is expressed and stored as the preproprotein hCAP18. In the presence of inflammatory or pathogenic stimuli, hCAP-18 is secreted and processed to the active form LL-37 by serine proteases. The peptide LL-37 modulates a variety of immune functions to fight infections and contribute to an overall balanced inflammation. Figure is modified from Hancock et. al., Nature Biotechnology, 2006. 24(12): 151 – 7 [161].

Peptide LL-37 is known to act as a direct chemoattractant for neutrophils, monocytes, mast cells and T-cells by interacting with formyl peptide-like receptor 1 and 2 (FPRL1/2), a G protein-coupled receptor, to induce mobilization of neutrophils, eosinophils and monocytes [51, 105,

162]. Zhang et al. showed that LL-37 may act as a direct ligand for CXCR2 to facilitate neutrophil migration [163]. LL-37 is also known to induce the production of chemokines (such as GRO-α, IL-8, MCP-1 and MIP-1) and enhance the expression of chemokine receptors (such as CCR2 and CXCR4), which indirectly facilitate chemotaxis of immune cells to sites of infection or inflammation [46, 49-51, 69, 103, 164]. It had been suggested that 10 μM (46 μg/mL) of LL-37, which is a higher concentration usually only found at sites of infection and inflammation, induces chemotaxis in monocytes [105]. Unlike the antimicrobial activity, the chemotactic activity of LL-37 was not inhibited by physiological concentrations of serum [145, 162].

Another function of LL-37 is to mediate immune cell activation and differentiation, which contributes to pro-inflammatory responses and enhances the clearance of pathogens. For example, LL-37 was shown to downregulate the production of anti-inflammatory cytokine IL-10, upregulate the production of pro-inflammatory cytokine IL-12p40, and direct toward an M1 phenotype during macrophage differentiation [160, 165]. Contrary to that, other studies have shown that LL-37 upregulates the gene expression and protein production of IL-10 in the presence of pathogenic stimuli, discussed below [166]. LL-37 induces mast cell degranulation, leading to the release of inflammatory mediators such as histamine and prostaglandins, which increase vascular permeabilization and allow cell infiltration [167, 168]. The peptide significantly induced the expression of phagocytic receptors and pro-inflammatory cytokines in DC [169]. During viral infection, LL-37 stabilizes and enhances activation of Toll-like receptor 3 (TLR3) and TLR5, resulting in enhanced production of pro-inflammatory cytokines against viral infection [170]. Consistent with this, LL-37 mediates the release of tumor necrosis factor-alpha (TNF-α), IL-1β and IL-18 when exposed to LPS-primed monocytes and macrophages [46, 171,

172]. Kahlenberg et al. have demonstrated that LL-37 activates caspase-1, which plays an important role in the release of IL-1β and IL-18 [172]. Furthermore, LL-37 can suppress neutrophil apoptosis by down-regulating Bid (BH3 interacting-domain death agonist, a proapoptotic protein) and upregulate Bcl-xL (B-cell lymphoma-extra large, an antiapoptoic protein) [173]. Prolonging the life-span of neutrophils contributes to enhance inflammation and elimination of pathogens. Overall, these studies demonstrated that LL-37 possesses pro-inflammatory properties in the presence of pathogenic stimuli, which contribute to the antimicrobial functions of LL-37, required to clear infections. Consistent with this, deficiency of LL-37 has been associated with increased susceptibility to infection [27, 42, 49, 141-143] .

In addition, LL-37 is able to control inflammation by suppressing excessive pro-inflammatory cytokine productions (such as TNF-α, IL-1β) and increasing anti-inflammatory cytokine (such as IL-10 and IL-19) production [49-51, 67, 103, 165, 174]. At very low physiological concentrations (≤ 1 μg/mL) which are typically found in mucosal surfaces, LL-37 can inhibit LPS-induced TNF-α in monocytes [166]. This inhibitory effect increases to more than 95% with and increased concentration of LL-37 (20 μg/mL) which is similar to that found at sites of infections and inflammation. The peptide LL-37 can suppress toxin A-mediated inflammatory cytokine production and provide protection against endotoxin-induced sepsis in murine models [175, 176]. LL-37 can also significantly inhibit cell activation, proliferation and production of pro-inflammatory cytokines (such as TNF-α and IL-12) on interferon (IFN)-γ-induced monocytes, macrophages, and DC [177]. Further, LL-37 was able to downregulate signaling through the TLR4 receptor complex in both DC and macrophages, leading to lower levels of pro-inflammatory cytokine production in the presence of LPS [166, 178]. Anti-inflammatory properties of LL-37 have been shown by its antagonistic effect on the production of IFN-γ, TNF-

α, IL-4 and IL-12 in various cell types [167]. Overall, these studies showed that LL-37 enhances anti-inflammatory responses in an environment containing pro-inflammatory stimuli, such as infections and/ or increased production of specific pro-inflammatory immune mediators.

A study by Elssener et. al. have demonstrated the ability of LL-37 to induce either pro-inflammatory or anti-inflammatory responses based on the environment [171]. The experiment utilized freshly isolated monocytes or monocytes primed by 1 ng/mL LPS for 3h. LL-37 treatment of freshly isolated monocytes did not induce the release of IL-1β, but blocked LPS-induced IL-1β. On the other hand, LL-37 treatment of LPS-primed monocytes induced the processing and release of IL-1β by interacting with the purinergic receptor $P2X_7$ [171]. These results are consistent with other studies which demonstrated that LL-37 can neutralize endotoxin (such as LPS and lipoteichoic acid) - induced pro-inflammatory responses, providing protection against septic shock both *in-vitro* and *in-vivo* [67, 101, 166, 174, 175, 179-181]. Overall, LL-37 is able to modulate immune responses to enhance clearance of pathogens, selectively suppress pro-inflammatory responses, maintain certain chemokine responses and enhance anti-inflammatory responses to control inflammation [165, 166, 182].

LL-37 is also involved in wound healing, by inducing release of other host defence peptides, enhancing proliferation of epithelial cells and migration of keratinocytes, promoting angiogenesis, and mediating re-epithelialization [158, 183-185]. The importance of LL-37 in re-epithelialization was shown by Heilborn and colleagues, where they demonstrated that re-epithelialization was inhibited in a dose-dependent manner when specific antibodies against LL-37 were used [186]. The same study has also observed that chronic ulcers patients have lower levels of LL-37, suggesting that LL-37 might be required for wound healing. In addition, a

clinical trial has shown that synthetic LL-37 (0.5 and 1.6 mg/mL) can be safely used as a topical treatment to enhance wound healing in patients suffering from chronic leg ulcers [187].

Recent studies have suggested that LL-37 may play a role during reproduction [188-190]. High concentrations of hCAP-18 have been reported to be associated with spermatozoa, and high expression of the *camp* gene was found in epididymis and spermatids [190]. It was hypothesized that hCAP18 may contribute as antimicrobial peptide or modulate the mobility of the sperm during fertilization. A recent study found that LL-37 at 3.6 μM concentration can inhibit mouse sperm from fertilizing the egg, without damaging the female reproductive tract [190].

The ever-expanding list of immunomodulatory functions of LL-37 suggests that the peptide probably acts through more than one receptor or pathway to modulate the immune system and enhance clearance of infection. A recent review by Dr. Robert E. W. Hancock has shown a protein-protein interaction network of the human cathelicidin LL-37 and its 16 interacting protein and receptors, which further interact with more than 1,000 secondary effector proteins (Figure 3) [27]. This interacting network embodies the complexity of how one peptide can modulate such a diverse range of functions and mediate pleiotropic effects on different cell types depending on the environment, the peptide concentration and the presence of other stimuli [27].

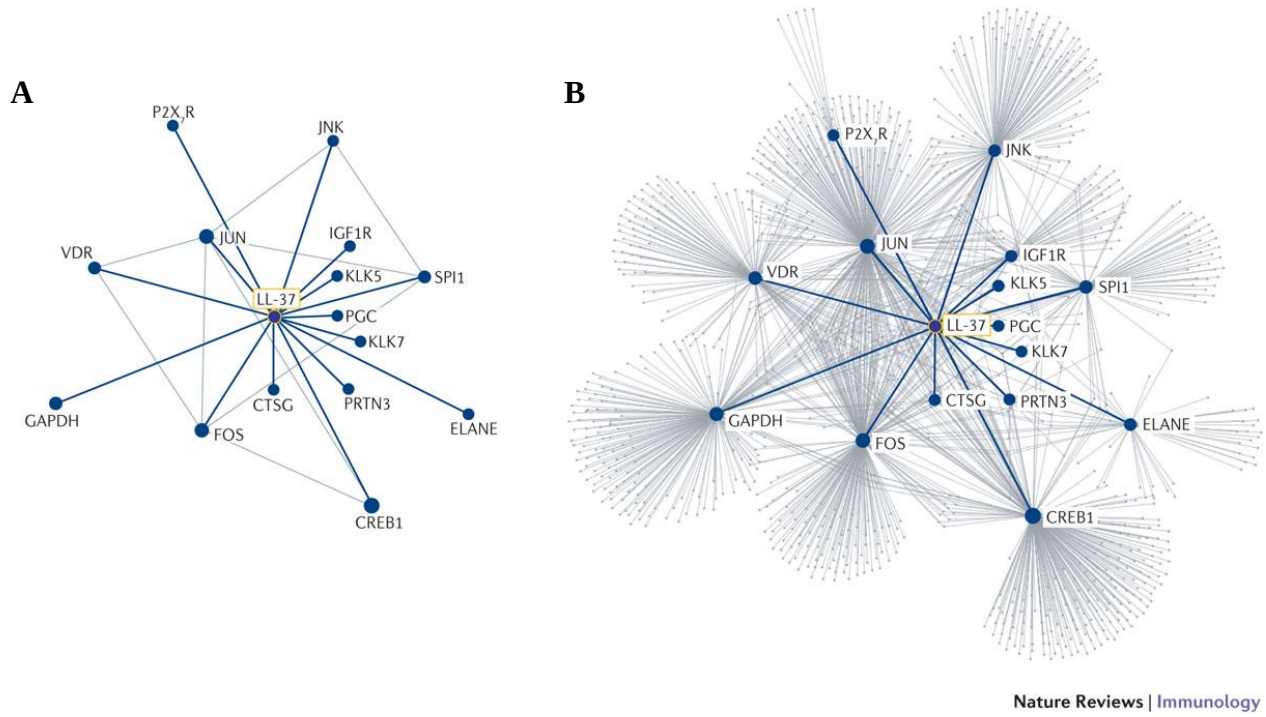


Figure 3. LL-37 interacts directly and indirectly with a broad range of genes and proteins. **(A)** A network interaction diagram showing zero-order interactions of the cathelicidin LL-37. These interacting proteins have diverse functions within the cell, including glucose metabolism and cytoskeletal dynamics (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)), transcription (JUN, FOS, cAMP-responsive element-binding protein 1 (CREB1), vitamin D3 receptor (VDR) and SPI1)) and inflammation (cathepsin G (CTSG)). (**B**) LL-37 has more than 1,000 first-order interaction partners (that is, direct interactors and proteins known to interact with the direct interactors (indicated by grey dots)), highlighting the complexity of the actions of the host defence peptide LL-37 within the cell. Network diagrams were created using NetworkAnalyst[191]. ELANE, neutrophil elastase; IGF1R, insulin-like growth factor 1 receptor; KLK, kallikrein; MAPK, mitogen-activated protein kinase; $P2X_7R$, $P2X_7$ purinergic receptor; PGC, PPAR-γ co-activator; PRTN3, proteinase 3. Figure is from Hancock et. al., Nature Reviews Immunology, 2016. 16(5): 321-34 [27].

1- 2.6 Role of LL-37 in human diseases

Chronic inflammatory Skin diseases

Epithelia are consistently exposed to the surrounding environment, and play an essential role in the innate immune system as part of the first line of defense against invading pathogens. It contains three distinctive barriers: (i) a chemical barrier which contains low pH and various cationic host defence peptides (such as LL-37), (ii) a physical barrier which involves the stratum corneum and (iii) a cellular barrier which involves resided cells (such as epithelial keratinocytes) and other infiltrating immune cells (such as neutrophils, and natural killer cells) [192]. Human cathelicidin LL-37 is produced by both epithelial cells as well as infiltrating immune cells during infection or inflammation, and is only active on the epithelia upon proteolytic cleavage by serine proteases, such as kallikrein [88]. Under normal and healthy conditions, the expression of LL-37 is barely detectable in keratinocytes [61]. However, the expression of LL-37 is significantly increased during infection or injury. This significant increase in LL-37 concentration enhances innate immune responses to fight infection and contributes to wound healing via different mechanisms, such as mediating the production of cytokines and chemokines, enhancing proliferation of endothelial cells and inducing angiogenesis. However, studies have shown that dysregulated expression, function or processing of LL-37 could contribute to the pathogenesis of chronic inflammatory skin diseases, such as atopic dermatitis, rosacea and psoriasis [61].

It has been demonstrated that the expression of LL-37 by keratinocytes and in lesional skin was significantly suppressed in atopic dermatitis patients, which correlates with increased susceptibility to infections [61, 193]. It was suggested that the use of certain pharmaceuticals may contribute to the inhibition of LL-37 production, resulting in a dampened immune response against pathogens. Studies have shown that increased production and activation of serine

proteases, such as kallikrein, would increase the proteolytic rate of converting hCAP18 into LL-37 in rosacea patients [88, 194]. It was suggested that the increased level of LL-37 would enhance immune responses to demodex mites found on the skin of rosaea patients and contribute to the pathogenesis of the disease. However, the mechanisms of LL-37 in the pathogenesis of these chronic inflammatory skin diseases are still unclear.

Psoriasis is a common chronic inflammatory disease characterized by skin inflammation, keratinocyte proliferation, enhanced type I interferon (IFN) production and over-expression of LL-37 [192, 195, 196]. As much as 1.5 mg/mL LL-37 was detected in skin lesion of psoriasis patients [193]. Gilliet and Lande suggested that LL-37 plays a pathogenic role in psoriasis by contributing to the inappropriate recognition of self-nucleic acid [197]. However, the mechanisms in which LL-37 contributes to psoriasis is controversial. On one hand, the LL-37/self-DNA complex triggers excessive pro-inflammatory responses, such as enhanced production of IFN, activation of myeloid DC and auto reactive T-cells and enhanced proliferation of keratinocytes [122, 195, 198, 199]; on the other hand, studies have shown that LL-37/ self-DNA complex blocks the activation of inflammasome, which results in suppression of IL-1β production [122, 198]. It was suggested that LL-37 shifts the balance of psoriatic skin pathology from inflammatory mediators (IL-1β) to type I IFN-driven, and breaking self-tolerance might also be involved [195, 196]. To date, the exact mechanisms of LL-37 contributing to the pathogensis of psoriasis is still under study.

Rheumatoid Arthritis

It had been found that the expression of LL-37 and rCRAMP, an ortholog of LL-37 in rats, is elevated in the synovial membranes of rheumatoid arthritis (RA) patients, and in the blood and

synovial membranes of rats with pristine-induced arthritis [200, 201]. Some studies suggested that increased level of LL-37 is associated with the pathogenesis of arthritis, while others suggested the peptide is upregulated to control the disease by suppressing inflammation, and some other studies have suggested that the peptide does not correlate with the disease progression at all [130, 201, 202]. Immunohistochemistry was used to demonstrate that local expression of LL-37 was strongly enhanced in the synovial membrane of RA patients [201]. It was found that LL-37 was mainly expressed by granulocytes and macrophages. Consistent with this, the same study has also shown that rCRAMP was mainly expressed in inflammatory cells, such as macrophages, fibroblasts and granulocytes, of pristine-induced arthritis rats. In addition, the level of rCRAMP and IFN-α also increased as arthritis progressed in peripheral blood [201]. Other studies suggested that LL-37 contributes to the pathogenesis of RA via enhanced recruitment of immune cells, which may contribute to the enhanced joint swelling and elevated levels of inflammatory cytokines [201]. In contrast, it was also shown that LL-37 inhibits osteoclastogenesis, a process which involves the breakdown of bone tissue, suggesting that LL-37 may play a protective role in RA [203]. To further complicate the matter, a recent study, comparing 92 RA patients with 67 sex-matched healthy controls, showed that there was no correlation between LL-37 and the pathogenesis of RA [202]. It should be noted that this particular study measured serum level of LL-37, while the previous study mentioned above measured the local concentration of the peptide in the synovial membrane of RA patients. The contrasting conclusions raised the question of whether LL-37 mediated its effects in RA locally. Overall, the role of LL-37 in RA remains unclear. Hence, there is a need for more in-depth studies to elucidate the role and molecular mechanisms of LL-37 in RA.

Respiratory diseases

Local level of the human cathelicidin peptide LL-37 was found to be elevated in patients with chronic obstructive pulmonary diseases (COPD) [110, 204-206]. There is evidence which showed correlation between the increasing levels of LL-37 to the progression of COPD. Studies have shown that LL-37 induced small airway remodeling and enhanced expression of mucus [207]. It was found that LL-37-induced IL-8 production contributes to airway epithelial cell apoptosis [204]. It was suggested that LL-37 mediates chemotactic activity and induces pro-inflammatory responses in the lung, which may contribute to the pathogenesis of COPD [208]. In addition, it was shown that elevated levels of peptidylarginine deiminases (PADIs) correlated with increased levels of citrullination of LL-37 in COPD patients [209]. This process suppressed the antimicrobial activities of LL-37 against bacteria, such as *S. aureus* [209]. Interestingly, the amount of LL-37 in the BAL fluid seems to change over the course of the disease. During the earlier-stages of COPD (GOLD stage I – II), the level of LL-37 in the BAL fluid was increased when compared to healthy controls [210]. In contrast, during the late-stages of COPD (GOLD stage III –IV), the level of LL-37 in the BAL fluid decreased when compared to healthy controls [210]. However, the exact mechanism of this peptide in COPD and how that relates to the progression of the disease at various stages is not well understood.

There is limited knowledge on the role of LL-37 in asthma, and studies showed conflicting expression patterns of LL-37 in asthmatic patients. Studies have shown that the expression of LL-37 was suppressed in asthmatic patients, and suggested a negative correlation between LL-37 and the development of asthma [205]. However, more recent studies showed that protein level of LL-37 was increased in eosinophils of asthma patients, and suggested that the peptide may indirectly contribute to the development of asthma by recruiting and activating immune cells

(such as eosinophils, neutrophils and mast cells) and thus induce the release of effector molecules (such as IL-8, histamine and neutrophils extracellular traps) [162, 211-214]. Further studies are required to elucidate the role of LL-37 in asthma at various stages and for the different forms of the disease.

Cancer

Peptide LL-37 plays contradictory roles in various cancers. It was shown that LL-37 can promote or inhibit tumor growth depending on the type of cancer, specific tissues or cells involved, and the presence of receptors and other stimuli in the environment. For example, LL-37 is over-expressed in breast, ovarian and lung cancers. However, the protein level of LL-37 was found to be suppressed in colon cancer tissues and the mRNA level of LL-37 was downregulated in gastric cancer tissues [215-217]. Hence, some studies have suggested that LL-37 can be used as a biomarker to diagnose certain types of cancers, such as ovarian and colon cancer [93, 216, 218]. In contrast, other studies have shown that direct interaction between LL-37 and cancer cells might play a role in anti-tumor effect. For instance, Utsugi et al. has shown that cancer cell membranes have elevated phosphatidylserine content [219]. The author suggested that the overall cell membrane net charge is more negative than healthy cells, which makes cancer cells more susceptible to cytotoxic activities of cationic peptides such as LL-37 [219]. Other mechanisms proposed include interaction of the peptide with different receptors. For example, it was shown that the interaction between LL-37 and FPRL-1 mediate contrasting activities on different cell types. On one hand, it was found that LL-37 interacts with immune cells, such as NK cells and T-lymphocytes, enhances immune surveillance, recognition and elimination of cancer cells, which inhibits the progression of gastric cancer [220]. The peptide LL-37 was also found to interact with cancer cells, and mediate apoptotic or other pathways which results in cell

death [221]. On the other hand, LL-37 mediates recruitment of mesenchymal stromal cells, which contributes to the progression of ovarian cancer [222]. It is known that LL-37 interacts and activates the purinergic recepor P2X7, which increases the secretion of pro-inflammatory cytokines IL-1β and IL-8, which subsequently contributed to the suppression of certain cancer cells [223, 224]. Furthermore, LL-37 can also bind and activate insulin-like growth factor-1 receptor (IGF-1R), which induces cell proliferation and contributes to metastatic cancer [225]. These contrasting roles of LL-37 in different cancers suggested that additional studies to elucidate molecular mechanism of the peptide in specific cancers are required. The peptide may play different role in different cancers based on the tumor type and the stage of the disease.

CHAPTER 2 – RATIONALE AND HYPOTHESIS

2 – 1 Study Rationale

Inflammatory responses are essential for the body to fight pathogens and clear damaged tissues. Various pro-inflammatory cytokines (such as TNF-α and IL-1β) contribute to the activation and enhancement of inflammatory responses, while anti-inflammatory cytokines regulate and resolve inflammation. Hence, these processes are required to be tightly regulated to maintain the balances between pro-inflammatory and anti-inflammatory responses, essential for effective clearance of pathogen and bring the immune system back to homeostasis. A breakdown in the regulation of the inflammatory process leads to the loss of balance between pro-inflammatory and anti-inflammatory cytokines. This results in excessive or uncontrolled inflammatory responses, which results in chronic inflammation.

The pro-inflammatory cytokine IL-32 was found to contribute in the amplification and continuation of cytokine-mediated chronic inflammation by inducing the feed-forward loop of the production of other pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1β and macrophage inflammatory protein-2 (MIP-2) [226-228]. The level of IL-32 is directly associated with severity and persistence of chronic inflammatory diseases, such as RA, asthma and IBD [229-231]. As IL-32 is critical in mediating chronic inflammation, this study employed IL-32 as the stimulus for the study of cytokine-mediated inflammatory responses.

Current biologic therapies for chronic inflammatory diseases often aim to directly target and neutralize pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 [230-234] such as Infliximab (neutralizing anti-TNF monoclonal antibody), Anakinra (IL-1 receptor antagonist), and Tocilizumab (IL-6 receptor blocking monoclonal antibody)[235]. However, these therapies

are effective in only a proportion of patients and often contain side effects, such as compromised immune functions, which results in increased susceptibility to infections in the host [235-238]. Similarly, long term standard treatment with steroids also increases the risk of infection in chronic inflammatory diseases such as arthritis and asthma [239, 240]. Hence, there is a need to develop alternate therapeutic strategies which can control chronic inflammatory diseases, without comprising effective host immune functions required to resolve infections.

Previous studies have shown that the human cathelicidin LL-37 has the ability to selectively suppress endotoxin-induced inflammatory responses, and enhance anti-inflammatory responses, without compromising the necessary innate immune responses to eliminate pathogens [46, 47]. The levels of LL-37 are differentially altered during chronic inflammatory diseases. For example, LL-37 was found to be suppressed in Crohn's disease, but was elevated in psoriasis [122, 195, 241, 242]. However, the role of the peptide LL-37 in chronic inflammatory diseases is not well understood. Smaller fragments of LL-37 processed via enzymatic cleavage, such as IG-19, are less cytotoxic, retain antimicrobial actions and exhibit similar immunomodulatory functions as LL-37 in the presence of LPS [59, 89, 97-99, 101, 243, 244] . Therefore, in this study I have screened LL-37-derived peptides and have identified some lead peptide candidates that show promises as selective anti-inflammatory agents, in particular in modulating the cytokine IL-32γ-induced pro-inflammatory responses [245]. My overall research focus was to elucidate the immunomodulatory roles of the human host defence peptide LL-37 and its synthetic derivative peptides on pro-inflammatory cytokine IL-32γ-induced immune responses.

2 – 2 Global Hypothesis

I hypothesized that the human host defence peptide LL-37 and its derivative peptide IG-19 will suppress cytokine IL-32γ-induced inflammatory responses. The selected synthetic derivative peptide IG-19 will control the disease process in chronic inflammatory diseases, such as rheumatoid arthritis (RA).

2 – 3 Specific Aims

Specifically, this study aimed to (1) define the effects of the peptide LL-37 and its synthetic derivative peptide IG-19 on chronic inflammatory cytokine IL-32γ-induced cellular responses, (2) to delineate the mechanisms underlying the immunomodulatory functions of peptides LL-37 and IG-19, and (3) to investigate the effects of exogenous administration of the peptide IG-19 in a murine model of inflammatory arthritis.

CHAPTER 3 – GENERAL MATERIALS AND METHODS

3 – 1 Ethics Statement

All the experimental procedures were approved by the Human Research Ethics Board of the University of Manitoba, Winnipeg, Manitoba, Canada. A written informed consent for collecting blood to isolate human peripheral blood mononuclear cells (PBMC) was obtained from all healthy donors. The animal experimental procedures were approved by the University of Manitoba Animal Care Ethics Board.

3 – 2 Reagents

3 - 2.1 Chemicals and tissue culture reagents

Human PBMC and macrophage-like THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1 mM sodium pyruvate (all purchased from Gibco®, Life Technologies, Burlington, ON, Canada), which will be referred as complete RPMI-1640 medium (methods discussed below). Phorbol-12-myristate-13-acetate (PMA) obtained from Sigma-Aldrich, Canada (Oakville, Ontario) was used to differentiate monocytic THP-1 cell into plastic-adherent macrophage-like cells. Versene solution (Gibco®, Life technologies, Burlington, ON, Canada), formulated as 0.2g EDTA(Na_4) per liter of phosphate buffered saline, was used to detach adherent cells from tissue culture plates. Phosphate buffered saline (PBS) was also purchased from Gibco®, Life technologies (Burlington, ON, Canada). Lipopolysaccharide (LPS) from *Escherichia coli 0111:B4*, protease inhibitor mixture,endotoxin-free E-Toxate^{TM} water, bovine serum albumin (BSA), Tween®20 and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Sigma-Aldrich (Oakville,

Ontario). Triton X-100, Tris, sodium chloride (NaCl), Ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), sodium fluoride, sodium orthovanadate, sodium pyrophosphate, and phenylmethylsulfonyl fluoride (PMSF) were also purchased from Sigma-Aldrich (Oakville, Ontario).

3 - 2.2 Peptides and cytokines

Sequences of the human cathelicidin HDP LL-37, LL-37-derived smaller peptides, and the scrambled LL-37 (sLL-37) control peptide used in this study are shown in **Table I.** These peptides were synthesized by CPC Scientific (Sunnyvale, CA). The peptides obtained were high performance liquid chromatography-purified and quality control (mass spectrometry) analysis provided by the company indicated >95% peptide purity. The peptides obtained were amidated ($-NH_2$) at the C-terminal to increase stability. The synthetic peptides were reconstituted in endotoxin-free E-Toxate^{TM} water to obtain desired concentrations, aliquoted and stored at -20°C until needed. The peptides were used within three months after reconstitution. Recombinant human IL-32γ, IL-8, TNF-α, IL-1β, and macrophage colony-stimulating factor (MCSF) were all obtained from R&D Systems (Minneapolis, MN, USA).

Table I. Peptide Sequences

Peptide	Sequence
LL-37	**LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES**
IG-19	**IGKEFKRIVQRIKDFLRNL**
VP-6	VPRTES
LL-12	LLGDFFRKSKEK
RK-25	RKSKEKIGKEFKRIVQRIKDFLRNL
sLL-37	**RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL**

3 - 2.3 Antibodies

Antibodies (Ab) against human actin was purchased from Millipore (Darmstadt, Germany).Primary antibodies against phospho-IKKα/β (Ser176/180), total-IKK, phospho-p38 MAPK (Thr180/Tyr182), phospho-DUSP-1/MKP-1 (Ser 359), total MKP-1, phospho-SAPK/JNK (Thr183/ Tyr185), total-JNK, phospho-AKT-1 (T308), total-AKT, phospho-p44/42 MAPK, total p44/42 MAPK were all purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase (HRP)-linked anti-rabbit IgG and anti-mouse IgG secondary Abs were obtained from Cell Signaling Technology (Danvers, MA).

3 – 3 METHODS

3 - 3.1 Isolation and culture of human peripheral blood mononuclear cells (PBMC)

Venous blood was collected from healthy volunteers using BD Vacutainer® (367874) containing sodium heparin as an anticoagulant, in accordance with a protocol approved by the University of Manitoba Ethics Review Board. Fresh human blood was diluted 1:1 with complete RPMI1640 medium, containing 1mM sodium pyruvate and 10% (v/v) FBS. Human PBMC was separated over Ficoll-Paque^{TM} Premium (GE Healthcare, Buckinghamshire, UK) using SepMate^{TM} tubes (Stemcell Technologies, Vancouver, BC) according to the manufacturer's instructions. Briefly, Ficoll-Paque^{TM} Premium was pre-layered into the bottom of the SepMate^{TM} tubes through the insert. Diluted blood was carefully pipetted into the SepMate^{TM} tube and centrifuge for 1000x g for 10min at room temperature (RT). The top layer containing the enriched PBMC was collected into a new centrifuge tube and washed twice in complete RPMI 1640 medium (300 x g for 10min). The isolated PBMC were seeded (1 x 10^6 cells/ mL/ well) into 24 well tissue culture

(TC) plates and rested for one hour at 37°C in a humidified 5% CO_2 incubator before stimulations.

3 - 3.2 Culture of human monocyte-derived macrophages (MDM)

Human monocyte-derived macrophages (MDM) were generated by seeding 3 x 10^6 human PBMC/ well containing 1 mL complete RPMI-1640 medium, into 24-well TC plates and incubated for 16h at 37°C in a humidified 5% CO_2 incubator. Non-adherent cells were removed, and the adherent cells were differentiated into MDM in complete RPMI-1640 medium supplemented with 50 ng/mL of recombinant human MCSF for additional six days. Half of the media (500 μL) was replaced every 48h with fresh RPMI-1640 medium containing 50 ng/mL of recombinant human MCSF. On the day of stimulation, the culture medium was replaced with fresh complete RPMI-1640 medium (1 mL per well) and the MDM were rested for 1h at 37°C in a humidified 5% CO_2 incubator before stimulations.

3 - 3.3 Culture of human macrophage-like THP-1 cells

Human monocytic THP-1 cells obtained from American Type Culture Collection (ATCC TIB-202) were cultured in complete RPMI 1640 medium and maintained at 37°C in a 5% CO_2 humidified incubator for a maximum of six passages, as previously described by us [166]. Briefly, THP-1 cells at a density of 1.1 x 10^5 cells/cm^2 in 24-well TC plates, 1.4 x 10^5 cells/cm^2 in 60mm TC Petri plates or 1.6 x 10^5 cells/cm^2 in 6-well TC plates were treated with 52.5 – 270 ng/mL PMA respectively, for 24h, which resulted in plastic-adherent macrophage-like cells. Growth area per well within a 24-well TC plate is 1.9cm^2, 60mm TC Petri plate is 21cm^2 and 6-well TC plate is 9.5 cm^2. Volume of complete RPMI 1640 medium used was 1 mL per well in 24-well TC plates, 4 mL per well in 60mm Petri plate, and 3 mL per well in a 6-well TC plate. The plastic-adherent THP-1 cells were rested in fresh complete RPMI 1640 medium for an

additional 24 h, before stimulation. On the day of stimulation, macrophage-like THP-1 cells were rested in fresh complete RPMI 1640 medium for 1 h at 37°C in a humidified 5% CO_2 incubator prior to stimulations.

3 - 3.4 Cell stimulation and cytotoxicity assays

For all experiments, human PBMC, human MDM, and plastic-adherent macrophage-like THP-1 cells were rested in fresh complete RPMI 1640 for at least 1h at 37°C in a 5% CO_2 humidified incubator before stimulation. Cells were stimulated with IL-32γ (20 ng/mL) in the presence and absence of 5 μM peptides (LL-37, IG-19, or sLL-37) at 37°C, 5% CO_2 for the various time points as indicated. TC supernatants were centrifuged at 250 x g for 5 min,to obtain cell-free TC supernatants, which were subsequently aliquoted and stored at -20°C until required.

Cellular cytotoxicity was determined by monitoring the release of the enzyme lactate dehydrogenase (LDH) in fresh TC supernatants after each stimulation, using a colorimetric LDH detection assay kit from Roche Diagnostic (Laval, QC, Canada), according to the manufacturer's instructions. Briefly, equal volume of cell-free TC supernatant was incubated with LDH substrate mix, containing catalyst diaphorase/NAD^+ and tetrazolium salt INT, for at least 30min at room temperature, in the dark. The release of lactate dehydrogenase, a cytoplasmic enzyme released due to damage to the plasma membrane, was measured at 490nm. The percentage of cytotoxicity was determined as $(OD_{sample} - OD_{blank}) / (OD_{total} - OD_{blank}) \times 100\%$, where OD_{blank} correspond to optical density (OD) of LDH released in the absence of cells and stimuli, and OD_{total} refer to optical density of 100% LDH released (i.e. 100% cell death) in the presence of 2% (v/v) Triton X-100.

Haemolytic activity of the peptides was also determined in human erythrocytes [246]. Briefly, human erythrocytes were isolated from freshly collected blood, centrifuged at 800 x g for 10min, and washed with 20 mM HEPES buffer (Hepes buffered saline [HBS], pH-7.4, containing 150 mM NaCl). Erythrocytes were diluted with HBS at 2.5% (vol/vol), followed by incubation with various peptides (LL-37, IG-19 and sLL-37) for 30 min at 37°C. After centrifugation at 800 x g for 10 min, the supernatant was carefully transferred to a new plate, and the release of hemoglobin was measured at 577nm. The percentage of hemolysis was determined as (OD_{sample} - OD_{blank})/(OD_{total}-OD_{blank}) x 100%, where OD_{blank} correspond to optical density (OD) of hemolysis in the absence of peptides, and OD_{total} refer to optical density of 100% hemolysis in the presence of 2% (v/v) Triton X-100.

3 - 3.5 RNA isolation and quantitative real time reverse-transcription polymerase chain reaction (qRT-PCR)

THP-1 cells were differentiated into plastic-adherent macrophage-like cells with PMA as previously described and rested for 24h. Macrophage-like THP-1 cells were stimulated for 1, 2, 4 and 6h with peptides (5 μM) in the presence or absence of IL-32γ (20 ng/mL). Cells were lysed using Buffer RLT Plus lysis buffer from the Qiagen RNeasy Plus kit (California, USA), containing β-mercaptoethanol. Total RNA was isolated using the Qiagen RNeasy Plus kit and eluted in RNase-free water (Ambion, Thermo Fisher Scientific, Wilmington, DE, USA) as per the manufacturers' instructions. Total RNA concentration and purity were assessed by NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA). Differential mRNA expression was analyzed using SuperScript III Platinum Two-Step qRT-PCR kit with SYBR Green (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. The ABI PRISM 7300 Real-Time PCR System was used for qRT-PCR and the data was analyzed

using the 7500 System SDS software (Applied Biosystems, Foster City, CA, USA). Briefly, up to 1 µg of total RNA was reverse transcribed into a 20 µL reaction volume for 10 min at 25°C, followed by 50 min at 42°C, and the reaction was terminated by incubating the reaction mixture at 85°C for 5 min. The cDNA samples were incubated with 1 µl per sample of 2U RNase H (Ambion, Thermo Fisher Scientific, Wilmington, DE, USA) at 37°C for 20 min. cDNA was aliquoted and stored at -20°C until needed. For qRT-PCR amplification, a reaction mix (total volume of 12.5 µL) containing 2.5 µL of 1/5 - 1/10 diluted cDNA template, 6.25 µL of Platinum SyBr Green qPCR-Super-Mix UDG with Rox reference, 0.5 µL of 10 µM primer mix, and 3.25 µL of RNase-free water were used. Product specificity was determined by melting curve analysis. Fold changes of mRNA expression were calculated after normalization to 18sRNA, and using the comparative Ct method [247]. The primers used for qRT-PCR are listed in Table II.

Table II. Sequence of human primers used for qRT-PCR

Gene	**Sequence (5' - 3')**
18sRNA -L	GTAACCCGTTGAACCCCATT
18sRNA -R	CCATCCAATCGGTAGTAGCG
DUSP-1-L	TTCAACGAGGCCATTGACTT
DUSP-1-R	CCTGGCAGTGGACAAACAC

3 - 3.6 Enzyme-linked immunosorbent assay (ELISA)

Human PBMC, human monocyte-derived macrophages (MDM), and plastic-adherent macrophage-like THP-1 cells were stimulated with IL-32γ (20 ng/mL) in the presence and absence of 5 µM peptides (LL-37, IG-19, or sLL-37) and incubated at 37°C, 5% CO_2 for 24 h, 48 h, or 72 h. Production of cytokines and chemokines were monitored in cell-free TC supernatant using specific ELISAs. The production of TNF-α, IL-1β, IL-6 and MCP-1 were monitored using specific antibody pairs from eBioscience (San Diego, CA), and interleukin-1 receptor antagonist (IL-1RA), IL-8, GRO-α and IL-10 were monitored using antibody pairs from R&D Systems, as

per the manufacturers' instructions. Briefly, capture Ab against cytokines or chemokines was coated onto Corning™ Costar™ 96-well EIA/RIA plates (Corning, Thermo Fisher Scientific, Wilmington, DE, USA) for 24 h. Washing buffer (PBS containing 0.05% Tween20) was used to washed off excessive Ab or samples in between each steps. This is followed by blocking with 3% BSA (bovine serum albumin) to reduce background for 1 h at room temperature or 24 h at 4°C. Serial dilution of respective recombinant human cytokines and chemokines were used to establish standard curves for the determination of cytokine and chemokine productions.

3 - 3.7 Kinome analysis

The kinome studies were performed in collaboration with Dr. Scott Napper at the Vaccine and Infectious Disease Organization – International Vaccine Centre (VIDO-InterVac, University of Saskatchewan).

Human monocytic THP-1 cells were differentiated to plastic adherent macrophage-like THP-1 cells as described above. Cells were rested for 24 h, followed by media changed to RPMI containing 10% (v/v) FBS. THP-1 cells were stimulated with IL-32γ (20 ng/mL) for 15 min. Cell lysates were obtained by incubating with lysis buffer (containing 20 mM Tris-HCl with pH-7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, protease inhibitor mixture (Sigma-Aldrich), and 1% (v/v) Triton X-100) on ice for 10 min [248]. Cell free lysate was obtained by micro-centrifugation for 10 min at 4°C, follow by determining the protein concentration using micro-Bradford assay. A mixture of cell lysate and activation mix (50 μM ATP, 0.05% v/v Brij-35, 0.25 mg/mL BSA, 2 mCi/mL [γ-^{32}P]ATP, 50% glycerol and 60 mM $MgCl_2$) was incubated with an array of peptides containing 300 selected phosphorylation events for quantifying global kinase activity for 2 h at 37°C. The peptides of interested were synthesized and printed in a grid pattern

on a block in triplicate, by JPT Peptide Technologies [248]. The full list of the 300 selected peptides can be found in the National Center for Biotechnology Information's Gene Expression Omnibus database (http://www-ncbi-nlm-nih-gov.uml.idm.oclc.org/geo/query/acc.cgi) under series accession number GSE28649. Phosphorylation events were detected by exposing the peptide array to a phosphoimager screen, using ImageQuant TL v2005 software, for 1 week. Background intensity for each spot was calculated from the average pixels of four regions in the immediate vicinity of each spot [248]. A global normalization was done using the GeneSpring software.

3 - 3.8 Phospho-p44/42 mitogen activated protein kinase (MAPK) activity assay

Total cell lysates were obtained by sonicating cells on ice in the cell lysis buffer from the p44/42 MAPK activity assay kit (Cell Signaling Technology), containing triton X-100 and glycol octylphenol ether plus 1 mM PMSF (a serine protease inhibitor). Total protein concentration was determined using a micro-bicinchoninic acid (microBCA) assay from Thermo Scientific, and a serial dilution of bovine serum albumin (BSA, Sigma-Aldrich) was used to form the standard curve. Kinase activity specific to p44/42 MAPK was monitored by determining the phosphorylation of p44/42 MAPK substrate Elk-1, using the p44/42 MAPK activity assay kit as per the manufacturer's instructions. Briefly, 150 µg of total protein was used for immunoprecipitation with an immobilized phospho-p44/42 MAPK antibody at 4°C overnight with gentle rocking. The eluent was incubated with p44/42 MAPK substrate Elk-1 in the presence of ATP at 30°C for 30min. Subsequent phosphorylation of Elk-1 substrate was determine by probing immunoblots with anti-phospho Elk-1 (S383) antibody. Horseradish peroxidase-linked secondary antibodies were used for detection, and Amersham ECL detection system (GE Healthcare, Baied'Urfe, QC, Canada) were used to develop membranes.

3 - 3.9 Western blotting to assess MKP-1, FYN, p38 AKT and IKK protein expression and phosphorylation

Total cell lysates were prepared in lysis buffer containing 20 mM Tris-HCl pH-7.5, 150 mM NaCl, 1mM EDTA, 1mM EGTA, 1mM sodium fluoride, 1mM sodium orthovanadate, 2.5mM sodium pyrophosphate, protease inhibitor cocktail (Sigma) and 1% (v/v) Triton X-100.Total protein concentration was determined using micro-BCA protein assay (Thermo Scientific) with BSA (Sigma-Aldrich) standard curve. Equal amounts of protein were resolved on 4 - 12% NuPAGEBis-Tris gels (Invitrogen, Burlington, ON, Canada), and transferred to nitrocellulose membrane (Millipore, Billerica, MA). Membranes were blocked with TBST (20 mM Tris-HCl pH-7.5, 150 mM NaCl, 0.1% Tween-20) containing 3% BSA for 1 h, at room temperature. Phosphorylation of MKP-1 (SerS359), FYN (Tyr420), AKT (Thr308), p38 MAPK (Thr180/ Tyr182) and IKKα/β were determined using anti-human phospho-site-specific antibodies (Cell Signaling Technology, Danvers, MA) in TBST with 1% BSA. Antibodies against total- MKP-1, FYN, AKT, IKK and β-actin (Cell Signaling Technology) in TBST with 1% BSA were used to detect protein loading amounts. Horseradish peroxidase-linked secondary antibodies were used for detection, and Amersham chemiluminescent (ECL) detection system (GE Healthcare, Baied'Urfe, QC, Canada) was used to develop membranes. Using AlphaEaseFC (FluorChem 8800, Genetic Technologies Inc., Miami, FL, USA), densitometry was measured for each band to quantify the amount of phosphorylated proteins, followed by normalizing to the band density of the non-phosphorylated forms of the respective proteins for each sample.

3 - 3.10 PathScan® Sandwich ELISA to quantitatively assess phosphorylation of JNK (Thr183/Tyr185)

Plastic adherent macrophage-like THP-1 cells grown on 60 mm culture dishes were stimulated for the appropriate time points. Cell lysis buffer (containing 20 mM Tris-HCl pH-7.5, 150 mM

NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin) from the PathScan® phospho-SAPK/JNK (Thr183/Tyr185) ELISA kit (Cell Signaling Technology, Danvers, MA) containing 1mM PMSF was used to lyse the cells. Total protein concentration was quantified using micro-BCA assay. Equal amounts of total protein for each cell lysate samples was incubated in microwell strips that are pre-coated with antibody against total SAPK/JNK protein. Following extensive washes, phospho-SAPK/JNK (Thr183/Tyr185) antibody was added to detect the captured phospho-protein. Anti-rabbit IgG, HRP-linked antibody was added to recognize the detection antibody. The quantity of phospho-SAP/JNK (Thr183/Tyr185) protein is proportional to the magnitude of light emission upon addition of chemiluminescent reagent.

3 - 3.11 Flow cytometry – determination of major cell types within the PBMC population which produce TNF-α in response to IL-32γ

Freshly isolated human PBMC (1 x 10^6 cell/ mL) were stimulated with IL-32γ (20 ng/mL) in the presence and absence of 5 μM peptides (LL-37, IG-19, or sLL-37) for 18 h at 37°C, 5% CO_2. BD GolgiPlug^{TM}, a protein transport inhibitor containing brefeldin A (BD Bioscience, Mississauga, ON, Canada) was added to the PBMC during the last 12 h of stimulation, to inhibit the release of cytokines. Unstimulated cells were used as a negative control and cells stimulated with lipopolysaccharide (LPS, 10 ng/mL) were used as a positive control. Adherent cells were detached using Versene (Gibco®, Life technologies, Burlington, ON, Canada). Adherent and non-adherent cells were washed with cold PBS, followed by two washes with staining buffer (PBS + 3% FBS). Cells were incubated in human FcR-binding inhibitor (eBioscience) on ice for 20 min, followed by staining with antibodies against specific extracellular cell markers from eBioscience (anti-human CD3-phycoerythrin/Cy7, anti-human CD14-eFluor450 and anti-human CD19-allophycocyanin) on ice for 30 min in the dark. BD Cytofix/Cytoperm Solution (BD

Bioscience) was used to fix and permeabilize cells according to the manufacturer's protocol, followed by incubation with anti-human TNF-α-FITC antibody (eBioscience) on ice for 30min, in the dark. The stained cells were washed with BD Perm/Wash Buffer (BD Bioscience) twice, resuspended in staining buffer and analysed by flow cytometer. UltraCompeBeads from eBioscience were used for compensation. FACSCanto II cytometer and FACSDiva software (Becton Dickinson, Mountain View, CA) were used to acquire cell samples. Data were analyzed using the FlowJo software (Treestar, CostaMesa, CA).

3 - 3.12 Murine model of collagen-induced arthritis (CIA)

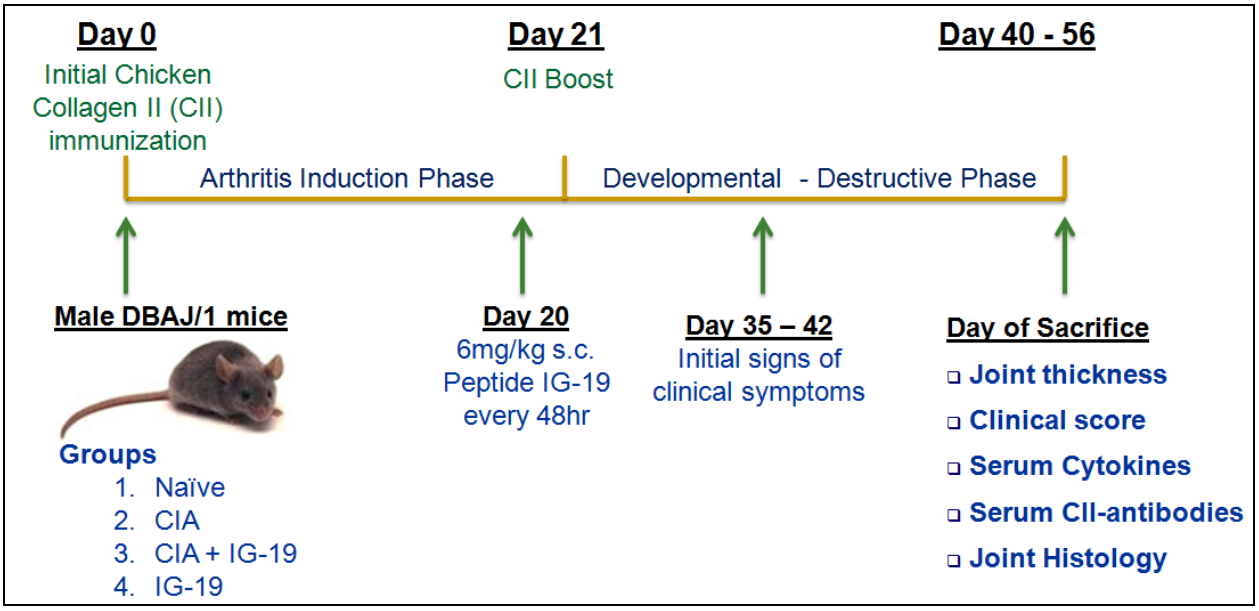


Figure 4. Murine model of collagen-induced arthritis (CIA)

3 - 3.12.1 Murine model of collagen-induced arthritis (CIA)

All murine experimental procedures were approved by the University of Manitoba Animal Care Ethics Board. Highly susceptible male DBA/1J mice (6 - 7 weeks old, obtained from Jackson

laboratories, were injected with 100 μg of chicken collagen II (CII, Chondrex Inc.) emulsified in complete Freund's adjuvant (Figure 4). CII injections were administered intradermal (i.d.) at the base of the tail (1 - 2 cm below the base of the tail). On day 21, a boost of chicken CII emulsified in incomplete adjuvant was administrated as described above [249, 250]. The peptide IG-19 (CPC Scientific, CA, USA), resuspended in sterile saline, was administrated at 6 mg/kg per mouse by s.c. injections on the hind limbs, from day 20 onwards and subsequently every 48 h. Disease progression was monitored by measuring joint thickness using digital calipers (Tresna Corporation) every 48 h from day 22 onwards [251, 252]. The mice were sacrificed 8 weeks after the initial CII challenge. Disease severity was monitored using a clinical score (shown in

Table III) as described by Galligan et al [252]. A clinical score, range 0 – 16 (shown in

Table III), was assigned to each mouse by summing the scores of each paw in a blinded manner. Mice were sedated with isofluorane, and blood was collected via cardiac puncture. Joints were collected and were fixed in 10% buffered formalin for histology.

Table III. Clinical scores

Clinical score (per paw)	Signs/ Symptoms
0	Normal, no signs of swelling
1	Mild swelling confined to the joint
2	Mild swelling extending from ankle to the tarsals
3	Moderate swelling extending from ankle to metatarsal joints
4	Severe swelling encompass the ankle, foot and digits, or ankylosis of the limb

3 - 3.12.2 ELISA and flow cytometry

Blood was collected by cardiac puncture on the day of sacrifice and allowed to coagulate at room temperature for 2 h. Serum was obtained after centrifugation at 10,000rpm for 10min, aliquoted

and stored at -20°C. Serum levels of anti-chicken CII antibody and anti-mouse CII antibody were monitored by ELISA (Chondrex Inc., Redmond, WA, USA) as per the manufacturer's recommendation. The production of murine cytokines (IL-2, IL-4, IL-6, IFN-γ, TNF-α, IL-17A, and IL-10) were analyzed in serum using the multiplex BD^{TM} CBA Th1/Th2/Th17 cytokines kit as per manufacturer's instructions [245].

3 - 3.12.3 Histology

On the day of sacrifice, the ankle joints were collected and fixed in 10% buffered formalin for 24 h at room temperature. The bones were decalcified in 10% EDTA (pH-7.4) for 14 days at 4°C, followed by dehydration in increasing ethanol concentrations. The tissues were embedded in paraffin and 5 μM sagittal serial sections were obtained. Hematoxylin and eosin (H &E) staining was used to evaluate cellular infiltration. Safranin-O staining was used to determine the loss of proteoglycans, an indication of cartilage degradation. Images of the sections were obtained by the Zeiss imager M2, and processed using the Zen 2011 software. Histology scoring was assigned to the stained sections, in a blinded manner, as described by Van Holten et al. [253]. The extent of infiltration and arthritis was evaluated using a scoring system (Table VII) previously published by Sun et al. and Nishikawa et al. [254, 255].

Table IV. Histology scores

Histology Score	Signs/ Symptoms
0	Normal synovium
1	Synovial membrane hypertrophy and cell infiltration
2	Pannus formation and cartilage erosion
3	Major erosion of the cartilage
4	Loss of joint integrity

3 - 3.13 Statistical analysis

3 - 3.13.1 Statistical analysis for macrophage-like THP-1 cell line *in-vitro* studies

Statistical significance was determined by using GraphPad Prism software (GraphPad software, CA, USA), with Student's paired t-test. A p-value of less than 0.05 was considered to be statistically significant.

3 - 3.13.2 Statistical analysis for *ex-vivo* human PBMC and MDM studies

Statistical analysis was performed by using GraphPad Prism software (GraphPad software, CA, USA), using independent t-test for unequal variance. A p-value of less than 0.05 was considered to be statistically significant.

3 - 3.13.3 Statistical analysis for murine model of collagen-induced arthritis

Statistical significance was determined using independent t-test for unequal variance. The trend for number of affected limbs over time was analyzed using the Turkey honest significant difference (HSD) test. A p-value of less than 0.05 was considered to be statistically significant.

CHAPTER 4 – HUMAN CATHELICIDIN LL-37 AND ITS SYNTHETIC DERIVATIVE PEPTIDE IG-19 MODULATE INTERLEUKIN-32γ-INDUCED INFLAMMATORY RESPONSES.

4 – 1 Introduction

Inflammation is an important innate immune response that is responsible for the resolution of infection and injuries, the process of which is regulated for the restoration and maintenance of the body's homeostasis. Amplified, prolonged and dysregulated inflammatory responses is a key factor in the development of chronic inflammation and contributes to the pathogenesis and progression of chronic inflammatory diseases, such as rheumatoid arthritis (RA) and inflammatory bowel diseases (IBD). Chronic inflammatory diseases, such as arthritis, pose major economic and health burden of approximately $6.4 billion (http://www.cihr-irsc.gc.ca/e/27028.html). Current available therapies for chronic inflammatory diseases suppress important pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α) and interleukin (IL)-1β, which are required to induce immune responses to resolve tissue injury and to clear infections. Therefore, patients on these therapies develop increased susceptibility to opportunistic infections and development of neoplasms. This highlights the significance of finding alternative therapeutic strategies that can selectively control chronic inflammation without compromising normal immune functions required to resolve infection and tissue damages.

A recently described inflammatory cytokine, known as IL-32, plays key roles in the amplification and persistence of chronic inflammation [256-258]. It has been demonstrated that the level of IL-32 is directly associated with the severity and persistence of chronic inflammatory diseases [228-231]. Inhibition of IL-32 using small interfering RNA (siRNA) suppressed downstream pro-inflammatory cytokine production and reduced inflammatory responses [259].

Therefore, the cytokine IL-32 is being explored as a therapeutic target for chronic inflammatory diseases. The first objective of my project was to elucidate the effects of the human HDP LL-37 and its synthetic derivative peptides on IL-32-induced responses in immune cells.

4 – 1.1 Pro-inflammatory cytokine IL-32

The cytokine interleukin (IL) - 32, also known as natural killer cell transcript 4 (NK4), was first described in 1992 by Dahl and colleagues as a transcript that was highly expressed in mitogen-activated T-cells, and IL-2-activated natural killer (NK) cells [260]. Production of the NK4 protein was also upregulated in activated T-cells, monocytes and epithelial cells [21, 260, 261]. Cycloheximide inhibited the expression of phytohaemagglutinin (PHA)-induced NK4 expression in human PBMC, which suggested that NK4 may be an early activated gene [260]. It was reported that NK4 does not share homology of coding sequence with any known cytokine family and its biological function was not reported until 2005. Kim and colleagues were the first to describe the gene structure, regulation and function of NK4. Using microarray studies, they first identified that NK4 was one of the most upregulated genes in an IL-18 responsive cell line [21]. Further, Kim et al. showed that the NK4 protein had typical pro-inflammatory cytokine properties, such as inducing the production of other pro-inflammatory cytokines (e.g. TNF-α, IL-6, IL-12 and IL-8), and they subsequently renamed the NK4 molecule as IL-32 [21]. However, the structure of IL-32 has not been defined to date, and the gene encoding IL-32 does not have significant sequence homology of any known cytokine encoding genes. Therefore, IL-32 does not belong to any of the known cytokine family. Furthermore, there are no receptors identified to date for IL-32.

4 – 1.2 Gene expression and isoforms of IL-32

Human IL-32 mRNA is expressed in both immune cells and tissues, such as monocytes, macrophage, lymphocytes, NK cells, fibroblasts, keratinocytes, epithelial cells and endothelial cells [257, 260, 262, 263]. The expression of IL-32 can be induced by a wide variety of pathogen-related stimuli, such as LPS, muramyl dipeptide (MDP) and double-stranded RNA, as well as pro-inflammatory cytokines, such as TNF-α, IL-1β, IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) [21, 264]. Moon et al. have shown that spleen tyrosine kinase (Syk), protein kinase Cδ (PKCδ) and c-Jun N-terminal kinase (JNK) pathways may play a role in the regulation of TNF-α-induced IL-32 expression in synovial fibroblasts [226]. Silencing any one of these pathways either by siRNA or inhibitors of Syk, PKCδ and JNK suppressed the expression of IL-32. Several studies involving viral infections showed that cyclooxygenase-2 (COX-2), an enzyme which catalyzes the formation of prostaglandins, is an upstream regulatory factor for the production of IL-32 [265-267]. It was shown that suppression of COX-2 using a selective COX-2 inhibitor (NS398) decreased the expression of IL-32 [267]. Li et al. have also demonstrated that over-expression of IL-32 suppressed the activity of COX-2, which suggested that there is a feedback regulatory mechanism between IL-32 and COX-2 [267]. Jeong et al. have demonstrated that GM-CSF-induced the production of IL-32 through the activation of caspase-1 in eosinophils [268, 269]. In addition, IL-32 is essential for the production of GM-CSF-induced inflammatory cytokines IL-6, IL-8 and TNF-α, suggesting a feed-forward mechanism between GM-CSF and IL-32 in the amplification of inflammatory mediators [269].

The gene encoding human IL-32 contains eight exons and is located on chromosome 16 p13.3 [21]. There are six known splice variants of the gene (IL-32α, IL-32β, IL32γ, IL32δ, IL-32ε and IL-32ζ) [270, 271]. Based on the sequence, it was reported that the isoform IL-32γ is the original

variant identified by Dahl as NK4 in 1992 [260]. The different isoforms of IL-32 are derived from splicing IL-32γ at the mRNA level [270, 271]. Proteinase 3 has been described as one of the proteinases that can bind and cleave IL-32, specifically binding with IL-32-α and IL-32-γ [272, 273]. The homologs of IL-32 have been identified in most mammals except rodents [21, 274-276]. Among different species, human and equine IL-32 has the highest homology (31.8% identity), followed by bovine, ovine and swine. The exact functions of each isoform are not yet well defined, with IL-32α and IL-32γ being the most well characterized isoforms of IL-32.

Among all the isoforms, IL-32α is the most abundant transcript. It is also the shortest isoform (14.9kDa), which lacks exon 3 and 7. Studies have found that IL-32α is highly expressed by epithelial cells in inflammatory bowel disease (IBD) and Crohn's disease (CD) patients [277]. It was shown that the expression of IL-32α is significantly increased in the presence of IL-1β, IFN-γ, and TNF-α. Furthermore, TNF-α and IFN-γ synergistically induce the expression of IL-32α in epithelial cells. Interestingly, IL-32α has no effect on TNF-α- mediated IL-8 transcript in HT-29 cells [263].

Although IL-32α is the most abundant transcript, IL-32γ is the longest (26.7kDa) and the most biologically active isoform, with respect to induction of cytokine production, cell activation, and cell death [21, 267, 270, 271, 278]. IL-32γ is also the only variant that contains a signal sequence and lacks a transmembrane region [260, 279]. This suggests that IL-32γ can be secreted via the classical endoplasmic reticulum/ Golgi-dependent pathway. In order to identify the most biologically active isoform, single-nucleotide mutation was used to create splice variations (splice-resistant and spliceable) of IL-32γ, followed by over-expressing the IL-32γ variants in macrophage-like THP-1 cell line and in RA synovial fibroblasts [270]. Heinhuis et al. demonstrated the effects of the splice variants by measuring the production of downstream

cytokines [270]. They showed that over-expression of the splice-resistant IL-32γ induced the maximum amount of pro-inflammatory cytokines (such as IL-1β and IL-6) when compared to control and the other spliceable variants [270]. Consistent with this, the authors also demonstrated that over-expression of the splice-resistant IL-32γ enhanced greater joint swelling and more cell infiltration to the synovium when compared to the spliceable IL-32γ, in an animal model of RA [270]. In addition, Li and colleagues have demonstrated that out of all the IL-32 isoforms, IL-32γ exhibit the strongest antiviral activity against H5N1 influenza virus infection in Madin-Darby canine kidney cells [278]. Previous studies have suggested that splicing IL-32γ into alternative isoforms, such as IL-32β or IL-32α, may contribute to the regulation of IL-32 activities and thus suppress production of pro-inflammatory cytokines (IL-1β and IL-6), resulting in reduced inflammatory responses [270, 271]. Several studies have shown that the production of pro-inflammatory cytokines directly correlate with the increase in IL-32γ expression [257, 270, 271, 280, 281]. Taken together, these studies demonstrate that IL-32γ is the most biologically active isoform of IL-32. Therefore, in this study I have used recombinant IL-32γ to assess the effects of cationic peptides LL-37 and its derivatives on IL-32γ-induced responses in immune cells.

4 – 1.3 Expression and regulation of IL-32

IL-32 is produced by a wide variety of immune cells (such as lymphocytes, NK cells, epithelial cells, endothelial cells, monocytes, macrophages and DC), as well as synovial fibroblasts, stromal cells, pancreatic cells and myofibroblasts [257, 260, 262, 263, 276], and found in body fluids such as bronchoalveolar lavage (BAL) fluid, synovial fluid and serum [257, 270, 282]. Recent studies have shown that IL-32 can be secreted by both primary cells and cell lines [264, 271, 279, 283]. It had been demonstrated that in healthy volunteers, the concentration of IL-32

detected within the BAL fluid (4.21 ± 1.13 pg/mL) , sputum supernatant (3.59 ± 0.66 pg/mL) and serum (4.63 ± 1.03 pg/mL) were relatively low [282]. However, IL-32 transcripts and protein can be significantly upregulated in the presence of pathogenic stimuli (such as LPS, muramyl dipeptide (MDP), and double-stranded RNA) and pro-inflammatory cytokines (such as IL-1β, IL-2, IL-18, IFN-γ or TNF-α) [21, 257, 276, 284, 285]. Consistent with this, levels of IL-32 increased significantly in patients infected with pathogens (such as *Mycobacterium tuberculosis*, influenza A virus, and HIV-1 virus) and in patients with chronic inflammatory diseases (such as RA, psoriasis, COPD, and IBD) [229, 259, 267, 274, 285-292]. It was shown that the average serum level of IL-32 in HIV-seropositive patients was 178.58 ± 36.81 pg/mL [286]. The concentration of IL-32 detected within the BAL fluid (22.46 ± 2.48 pg/mL) , sputum supernatant (19.66 ± 1.69 pg/mL) and serum (26.77 ± 2.56 pg/mL) were significantly higher in COPD patients when compared to healthy volunteers [282]. Gui et al. has shown that increased level of IL-32 correlated with increased disease severity by measuring serum levels of IL-32 from highly active RA patients (107 ± 42 pg/mL), moderately active RA patients (66 ± 27 pg/mL), and stable RA patients (21 ± 9 pg/mL) [228].

The presence and correlation of increasing IL-32 expression with the severity of numerous chronic inflammatory diseases makes it appealing to understand the role of this pro-inflammatory cytokine using various *in-vivo* models. Unfortunately, as mentioned earlier, due to the lack of a defined homolog of IL-32 found in rodents, it has been difficult to study the role of IL-32 in various chronic inflammatory diseases using the well established animal models of chronic inflammatory diseases that utilize rodents. However, recent studies have developed transgenic models that allow rodents to expresses human IL-32 [293, 294]. For example, there are IL-32γ transgenic (IL-32γ-TG) mice which express the human IL-32γ. In addition, many

studies have also used transfection models of IL-32 in different cell types to elucidate the biological function of the cytokine. The following section will discuss some of the known functions of IL-32 in immunity and disease defined to date.

4 - 1.4 Immune functions of IL-32

IL-32 is a multifunctional cytokine which is known to enhance the production of pro-inflammatory and anti-inflammatory cytokines and chemokines, mediate differentiations of immune and structural cells, and induce apoptosis and cell death (summarized in Figure 5) [21, 287, 295-298].

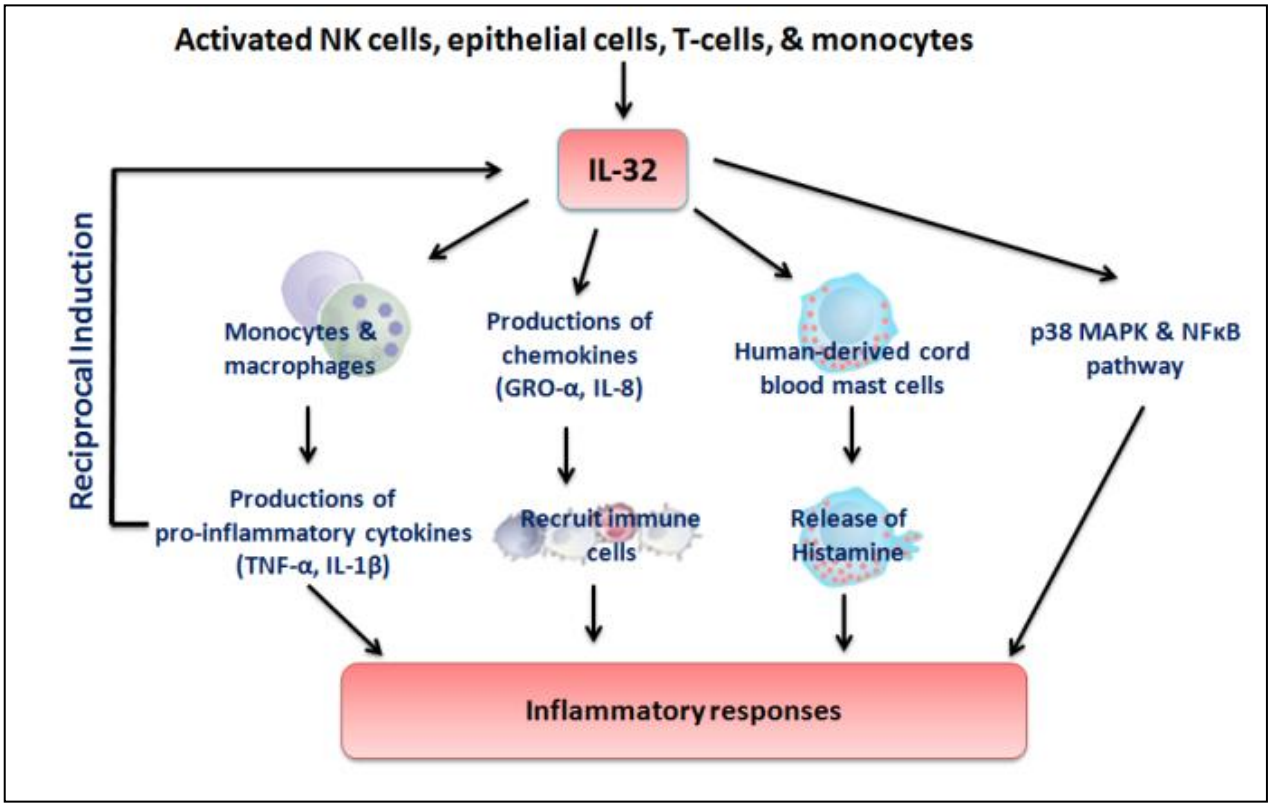


Figure 5. Immune functions of IL-32. Pro-inflammatory cytokine IL-32 is secreted by activated immune cells. It contributes to inflammatory responses by enhancing a variety of downstream immune functions, such as inducing the production of other pro-inflammatory cytokines, enhancing the recruitment of immune cells, induce the release of histamine and activating signalling pathways (e.g. p38 MAPK).

IL-32 has no homology with other known cytokines, but exhibits the typical properties of pro-inflammatory cytokines. Studies have shown that IL-32 induces the production of pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1β, IL-6 and IL-8, via activated nuclear factor-kappa B (NF-κB), p38 mitogen-activated protein kinase (MAPK) and activator protein-1 (AP-1) signaling pathways [261, 294, 298, 299]. In turn, these pro-inflammmatory cytokines are known to reciprocally induce the production of IL-32 via activating NF-κB MAPK, cAMP response element-binding (CREB), capase-1, and caspase-3 [278, 285, 300]. This feed-forward relationship between IL-32 and other pro-inflammatory cytokines, especially TNF-α and IL-1β, prolongs and amplifies inflammatory responses thus contributing to the pathogenesis of chronic inflammation diseases such as RA, IBD, COPD and atherosclerosis [229, 230, 257, 264, 277, 282, 283, 291, 301]. The importance of this feed-forward loop was demonstrated by several studies involving the use of inhibitors and siRNA. Using RA synovial fibroblasts and human macrophage-like THP-1 cells, Heinhuis et al. showed that stimulation with TNF-α significantly upregulated the expression of IL-32γ [257]. In turn, the over-expression of IL-32γ stabilized the mRNA transcripts of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-8, resulting in the increased production of these cytokines [271, 302]. IL-32γ-TG mice express higher amounts of pro-inflammatory cytokines and chemokines [303]. In various diseases, increased levels of IL-32 have been shown to correlate with increased production of pro-inflammatory cytokines and chemokines [228, 257, 268, 274, 277]. We have previously demonstrated that inhibiting the TNF-receptor (TNF-R1) suppressed IL-32γ-induced GRO-α and IL-8 production [304]. In addition, we have also shown that IL-32-mediated the phosphorylation of p300 at Ser^{1834}, which is a transcriptional coactivator involved in the translocation of AKT and TNF-α-mediated activation of the PI3K/AKT pathway [305].Collectively, these studies suggest that IL-32-

mediated responses are at least in part dependent on the TNF pathway. In contrast, silencing of IL-32γ using siRNA diminished DAMPs or PAMPs -induced TNF-α, IL-6 IL-1β, and IL-8 [256, 257]. Similarly, LPS or PMA- induced pro-inflammatory cytokines and chemokines production were diminished in monocytic cells upon silencing IL-32 using short hairpin RNA (shRNA) [258]. Importantly, the direct correlation between IL-32 and TNF has been shown in RA patients, where patients on anti-TNF-α treatments have significantly lower amounts of IL-32 proteins in their synovial tissue [257].

IL-32 is also known to induce cellular differentiation in a wide variety of cells. For examples, IL-32γ had been shown to induce the differentiation of monocytes into macrophages or DC [298]. It was further shown that IL-32γ-differentiated DC have enhanced production of pro-inflammatory cytokines IL-12 and IL-6, and skewed the proliferation of T-cells toward Th1 and Th17 phenotype [298]. Both Th1 and Th17 cells induce inflammatory responses and contribute to chronic inflammatory diseases such as IBD and collagen-induced arthritis [306, 307]. Moreover, Kim et. al. have recently demonstrated that IL-32γ can lead to the differentiation of the CD14+ monocytes to osteoclasts, which are giant multinucleated cells that degrade bone tissue and contributes to the pathogenesis of RA [296].Together, these studies have clearly demonstrated that the cytokine IL-32 plays a critical role in the pathogenesis of various chronic inflammatory diseases, as follows.

4 – 1.5 Role of IL-32 in chronic inflammatory diseases

Respiratory diseases

IL-32 was found to be elevated in the serum of COPD, asthmatic and allergic rhinitis patients [268, 282, 283, 291, 308]. These studies have demonstrated that IL-32 can act as an intracellular mediator, and can induced the production of IFNγ and TNF-α in human bronchial epithelial cells

[308]. Meyer et al. demonstrated that silencing of IL-32 enhanced the expression of endothelial growth factor and platelet-derived growth factors [308]. In addition, the supernatant from these experiments enhanced angiogenesis. The authors suggested that IL-32 may play a role in the inhibition of angiogensis [308]. Jeong et al. have shown that IL-32 concentrations increased significantly in the nasal mucosa of allergic rhinitis patients [268]. In addition, the level of IL-32 was found to correlate with the production of IL-1β, IL-18 and GM-CSF in these patients.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic disease that has been characterized with chronic inflammation and increase in oxidative stress, which result in cartilage and bone degradation. It has reported that the concentration of IL-32 is elevated in RA patients when compared to osteoarthritis (OA) patients [228, 229, 302]. Elevated levels of IL-32 positively correlated with the increased expression of pro-inflammatory cytokines production, increased disease severity and cartilage destruction in RA models [228, 302]. Multiple studies have demonstrated that IL-32-induced pro-inflammatory cytokines, specifically TNF-α, plays a major roles in the pathogenesis of RA. It is also known that TNF-α is a potent inducer of endogenous IL-32γ [229, 257, 261, 274, 296, 309]. As discussed above (Figure 5) the feed-forward mechanisms of IL-32γ with other pro-inflammatory cytokines results in the amplification and persistence of inflammatory responses in chronic disease such as RA. This auto-inflammatory loop enhances the recruitment and activation of immune cells, induces the differentiation of osteoclasts, and mediates the destruction of cartilage and bone in RA [261]. *In-vivo* studies have shown that injection of IL-32 into the joints of naïve mice leads to recruitment of immune cells, increased production of pro-inflammatory cytokines, increased joint swelling and cartilage destruction and exacerbates collagen-induced arthritis [226, 229, 296, 310]. However, when IL-32 was injected

into TNF-α deficient mice, the degree of joint swelling and immune cell recruitment was significantly decreased [229] this clearly demonstrating that IL-32 likely mediates pro-inflammatory responses through TNF-α pathway in RA. Consistent with this, anti-TNF-α treatment in RA patients have shown to decrease the expression of IL-32 in the synovial knee biopsies [230, 257].

4 – 2 Hypothesis and Rationale

As discussed above previous studies have demonstrated that IL-32 is a key player in the induction of pro-inflammatory responses and contributes to the progression and pathogenesis of various chronic inflammatory diseases. Therefore, molecules that can regulate IL-32 or its downstream targets might serve as potential therapeutic agents for chronic inflammatory diseases. As HDP LL-37 and its synthetic derivatives have the ability to selectively suppress LPS-induced pro-inflammatory responses in blood-derived cells, I hypothesized that LL-37 and its synthetic derivative peptides will regulate IL-32γ-induced inflammatory responses in peripheral blood-derived mononuclear cells (PBMC) [166].

4 – 3 Results

4 – 3.1 Peptides LL-37 and IG-19 significantly suppressed IL-32γ-induced pro-inflammatory cytokine production in human PBMC.

Previous studies have shown that cytokine IL-32 induces the production of pro-inflammatory cytokines, such as TNF-α and IL-1β, in PBMC [167, 245, 261, 304]. Out of the six isoforms, IL-32γ had been shown to be the most biologically active [271]. Kim et al. had shown that as much as 20 ng/mL of IL-32γ was found in the synovial fluid of active rheumatoid arthritis patients

[296]. Therefore, I have used 20 ng/ml IL-32γ for stimulating PBMCs in this study. I monitored the production of IL-32γ-induced pro-inflammatory cytokines in the presence or absence of 5 μM of cationic peptides (LL-37 or its synthetic derivative peptides, Table I). The concentration of 5 μM peptides was chosen based on previous studies, which showed that 5 μM of LL-37 and it synthetic derivative peptides exhibit immunomodulatory functions without inducing excessive cellular cytotoxicity [101, 166, 304, 311, 312].

From my initial screening studies in the THP-1 macrophage-like cell line, I showed that the peptides IG-19 and RK-25, internal regions of LL-37 (amino acid 13 – 31 and amino acid 7 – 31, respectively), significantly suppressed IL-32γ-induced pro-inflammatory cytokine TNF-α (appended Supplementary Figure 1). Although both these peptides derived from internal regions of LL-37 did not exhibit significant amount of cellular cytotoxicity in human macrophage-like THP-1 cells, the peptide IG-19 exhibited significantly less cellular cytotoxicity when compared to RK-25 (appended Supplementary Figure 2). In addition, peptide IG-19 is a shorter peptide (19 amino acids) compared to RK25. Therefore, based on the results of my initial screening of various LL-37-derived peptides in human macrophage-like THP-1 cells, I focused on the peptides LL-37 and its derivative IG-19 to further investigate the effects of these peptides on cytokine IL-32γ-induced pro-inflammatory cytokines. I also used a scrambled version of LL-37 (sLL-37) as a negative control, since it does not exhibit immunomodulatory functions similar to LL-37. Next, I evaluated the effects of these peptides in primary human PBMC. I showed that both peptides LL-37 and IG-19 did not mediate hemolysis in human erythrocytes (appended Supplementary Figure 3). Moreover, when compared to the parental peptide LL-37, the peptide IG-19 was relatively less cytotoxic to human erythrocytes at both concentrations of 5 and 10 μM (appended Supplementary Figure 3).

I further demonstrated that that LL-37 and its derivative peptide IG-19 abrogated IL-32γ-induced TNF-α, IL-1β and IL-6 pro-inflammatory cytokine production in human PBMC (Figure 6). In contrast, the scrambled peptide sLL-37 did not suppresse IL-32γ-induced either TNF-α, IL-1β or IL-6 production in PBMC. Data from this study was partially published in the Journal of Immunology [102]. Increased samples ($n \geq 9$) were obtained since last publication. Therefore, I have re-analyzed and provided the entire dataset in this thesis (Figure 6).

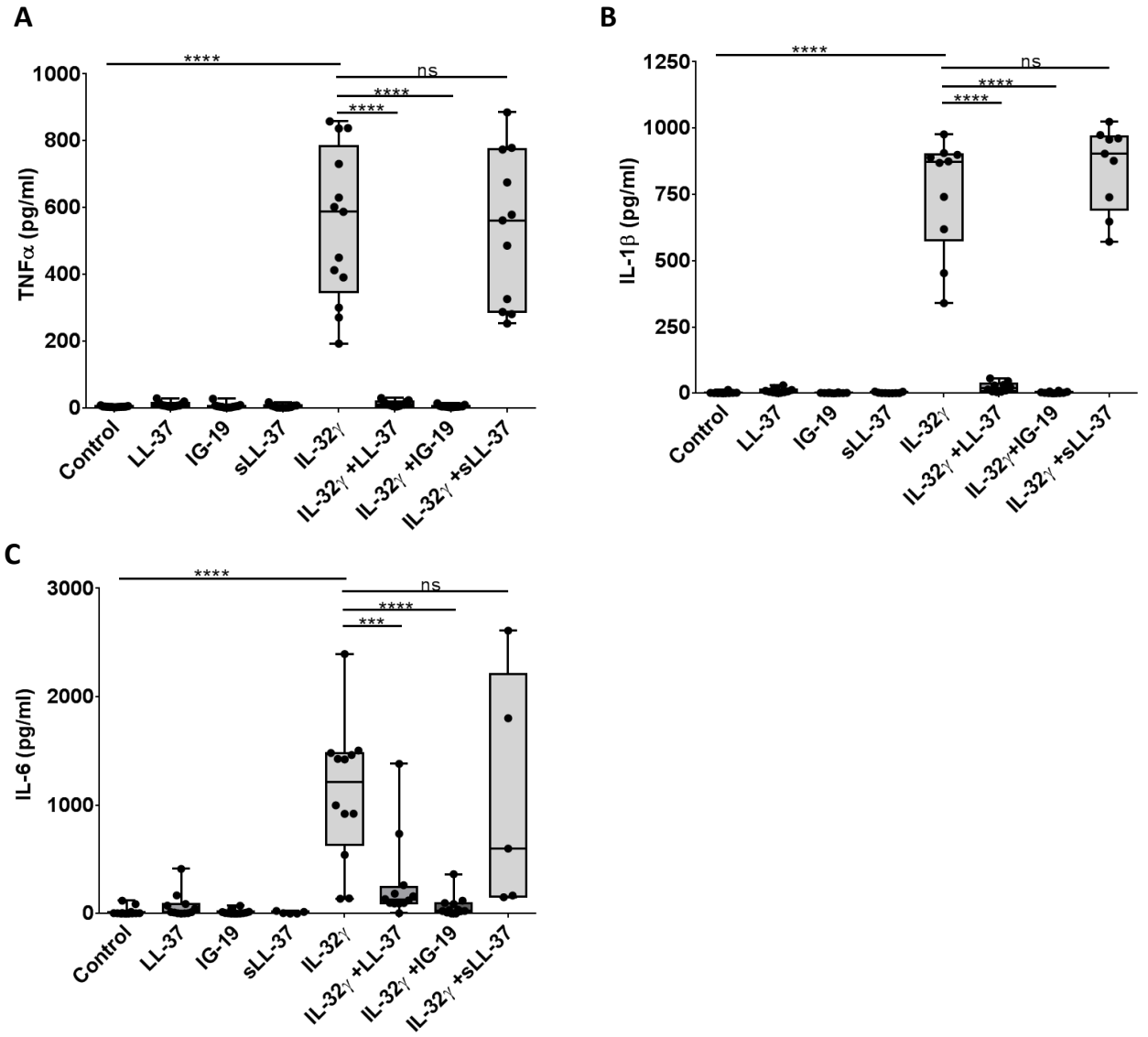


Figure 6. LL-37 and IG-19 suppressed IL-32γ-induced pro-inflammatory cytokines in human PBMC.

Human PBMC were stimulated with 20 ng/mLIL-32γ ± 5 μM peptides (LL-37, IG-19 or sLL-37) for 24 h. Tissue culture supernatants were monitored for the production of pro-inflammatory cytokines (**A**) TNF-α, (**B**) IL-1β and (**C**) IL-6 by ELISA. Results are shown in box and whisker plot, with each point representing an independent donor ($n \geq 9$). Statistical analyses were performed using the Wilcoxon-Mann-Whitney test. Significance represented by p-values (** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$, ns = not significant).

4 – 3.2 Target cells that respond to IL-32γ within the PBMC population.

To identify the target cells within the PBMC population which respond to IL-32γ, I stimulated human PBMC with IL-32γ (20 ng/mL), in the presence and absence of 5μM peptides (LL-37, IG-19 or sLL-37) for 18 h, in the presence of BD GolgiPlug. Unstimulated PBMC was used as negative control, while lipopolysaccharide (LPS) stimulated PBMC were used as positive control. The production of TNF-α was detected using anti-human TNF-α-FITC antibody. Major contributors of IL-32γ-induced TNF-α were identified via the presence of cell surface markers, using antibodies against CD3 (for T-cells), CD14 (for monocytes) and CD19 (for B cells). I showed that following stimulation of PBMC with IL-32*γ*, $CD14^{+}$ monocytic cells were the primary cell type that produced TNF-*α* (Figure 7). IL-32γ-induced TNF-α was not detected in either CD3+ T cells or CD19+ B cells (7). Further studies using a cell marker antibody against CD4 (for T cells) also showed that CD4+ T cells were not the major contributor of IL-32γ-induced TNF-α within the PBMC population (data not shown).

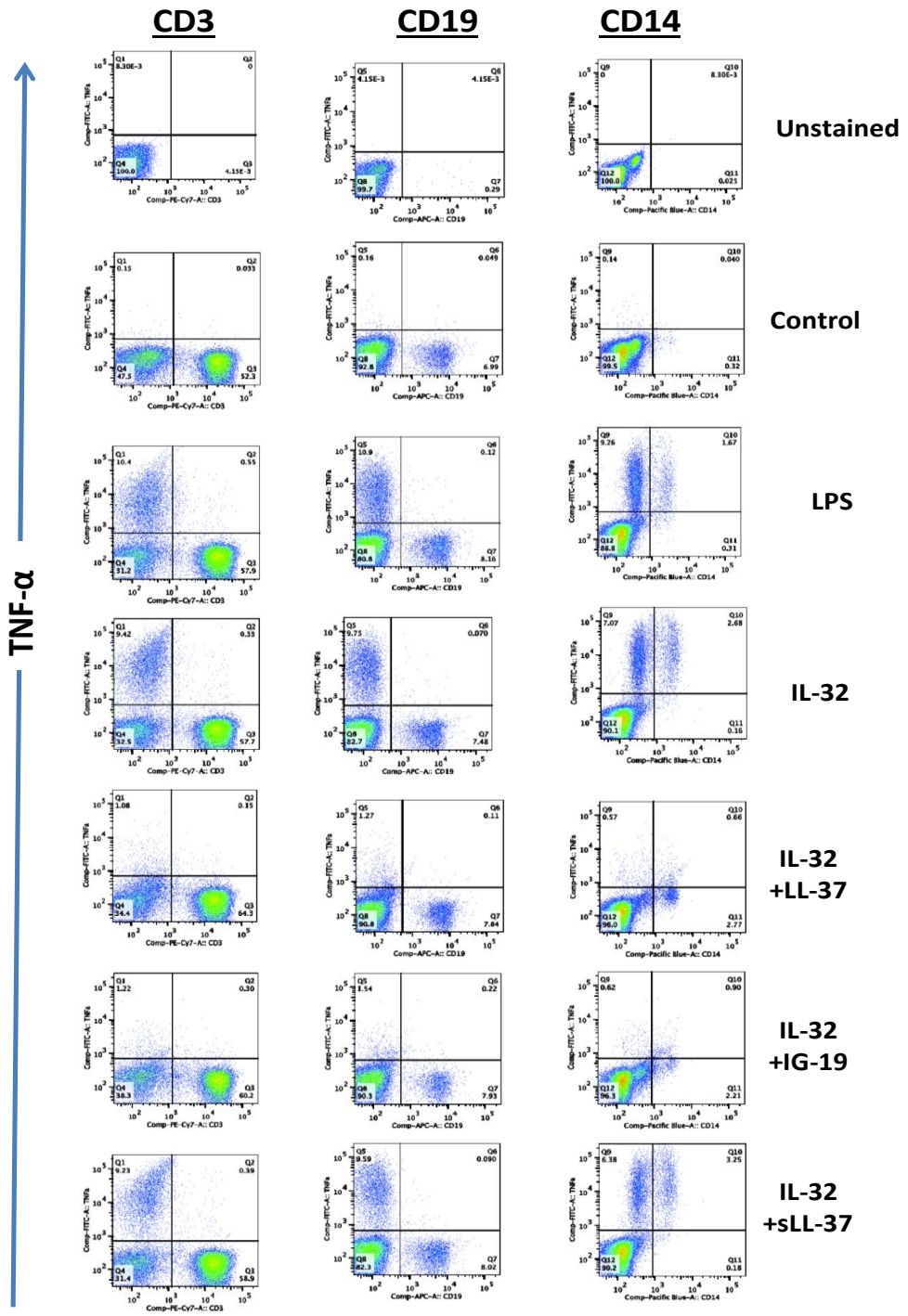
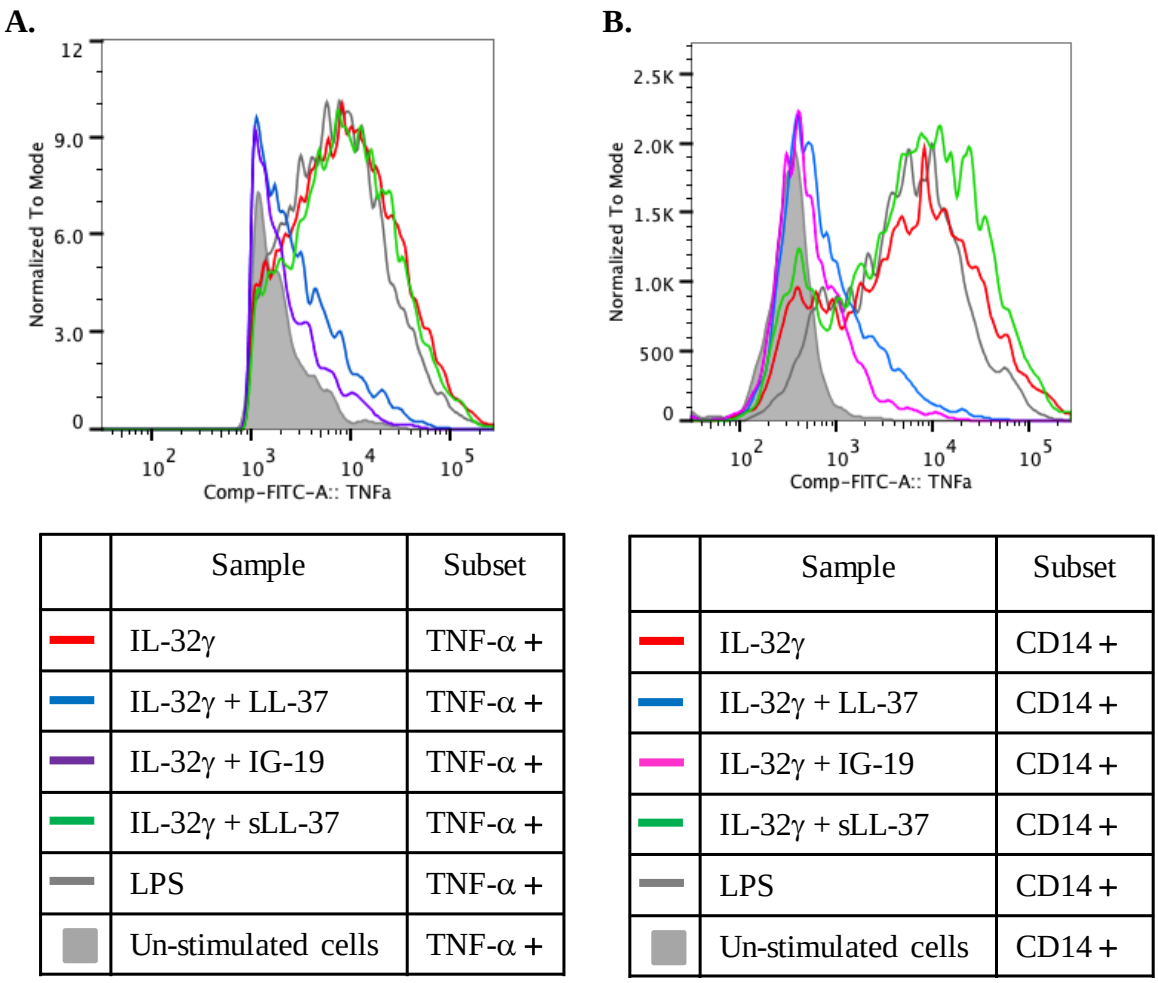


Figure 7. CD14+ monocytic cells are major contributors to IL-32γ-induced TNF-α production.

Human PBMC were stimulated with IL-32*γ* (20 ng/mL) in the presence and absence of 5 μM LL-37, IG-19 or sLL-37. The cells were stimulated with bacterial LPS as a positive control. After 18 hr of incubation at 37°C, cells were stained with antibodies for specific surface markers, fixed and permeabilized, followed by staining with anti-TNF-*α* antibody. Figure is modified from Choi et. al., Immunology, 2014. 143(1):68-80 [102].

I further analyzed the data by forming gates on subpopulations, TNF-α+ and CD14+ cells, to investigate the effects of the peptides on IL-32γ-induced TNF-α production. Peptides LL-37 and IG-19, but not sLL-37, suppressed IL-32γ-induced TNF-α in both TNF-α+ and CD14+ cells (Figure 8). This is consistent with the ELISA results shown in Figure 6. This is also consistent with previous studies demonstrating that monocytes and macrophages, which are both CD14+cells, are key players of cathelicidin-derived peptide mediated responses [243, 244]. Based on these results, I focused on further examining the effects of the peptides LL-37 and IG-19 on downstream cytokines and chemokines production in human macrophages.



	Sample	Subset
	IL-32γ	TNF-α +
	IL-32γ + LL-37	TNF-α +
	IL-32γ + IG-19	TNF-α +
	IL-32γ + sLL-37	TNF-α +
	LPS	TNF-α +
	Un-stimulated cells	TNF-α +

	Sample	Subset
	IL-32γ	CD14 +
	IL-32γ + LL-37	CD14 +
	IL-32γ + IG-19	CD14 +
	IL-32γ + sLL-37	CD14 +
	LPS	CD14 +
	Un-stimulated cells	CD14 +

Figure 8. Peptides LL-37 and IG-19 suppressed IL-32γ-induced TNF-α production in human PBMC.

Human PBMC were stimulated with IL-32γ (20 ng/mL) in the presence and absence of LL-37, IG-19 or sLL-37 (5 μM each). The cells were stimulated with bacterial LPS as a positive control. After 18 hr of incubation at 37°C, cells were stained with antibodies for specific surface markers, fixed and permeabilized, followed by staining with anti-TNF-α antibody. (**A**) TNF-α^+ cells were gated, and (**B**) CD14$^+$ cells were gated. Figure is from Choi et. al., Immunology, 2014. 143(1):68-80 [102].

4 – 3.3 LL-37 and IG-19 significantly suppressed the production of pro-inflammatory cytokines in macrophages.

We further evaluated the effects of the peptides LL-37 and IG-19 on IL-32γ-induced pro-inflammatory cytokines in PMA-differentiated, plastic-adherent THP-1 cells and human MDM.

These cells were stimulated with 20 ng/mL of IL-32γ, in the presence or absence of 5μM peptides (LL-37, IG-19 or sLL-37). Tissue culture (TC) supernatants were collected after 24hr and used to monitor the production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6. Peptides LL-37 and IG-19, but not sLL-37, significantly suppressed IL-32γ-induced TNF-α, IL-1β and IL-6 production in macrophage-like THP-1 cells (Figure 9A). IL-32γ-induced TNF-α and IL-1β production was also suppressed in human MDM in the presence of both peptides LL-37 and IG-19, but not sLL-37, (Figure 9B). However, in human MDMs, IL-32γ-induced IL-6 was significantly suppressed by LL-37, but not the peptides either IG-19 or sLL-37. These results showed that the peptides mediated similar responses in both human PBMC and macrophages, specifically the suppressive effects of the peptides LL-37 and IG-19 on IL-32γ-induced pro-inflammatory cytokines between human PBMC and macrophage-like THP-1 cells. Based on these results, I further investigated the effects of the peptides on anti-inflammatory cytokines and chemokines production in human PBMC and macrophage-like THP-1 cells.

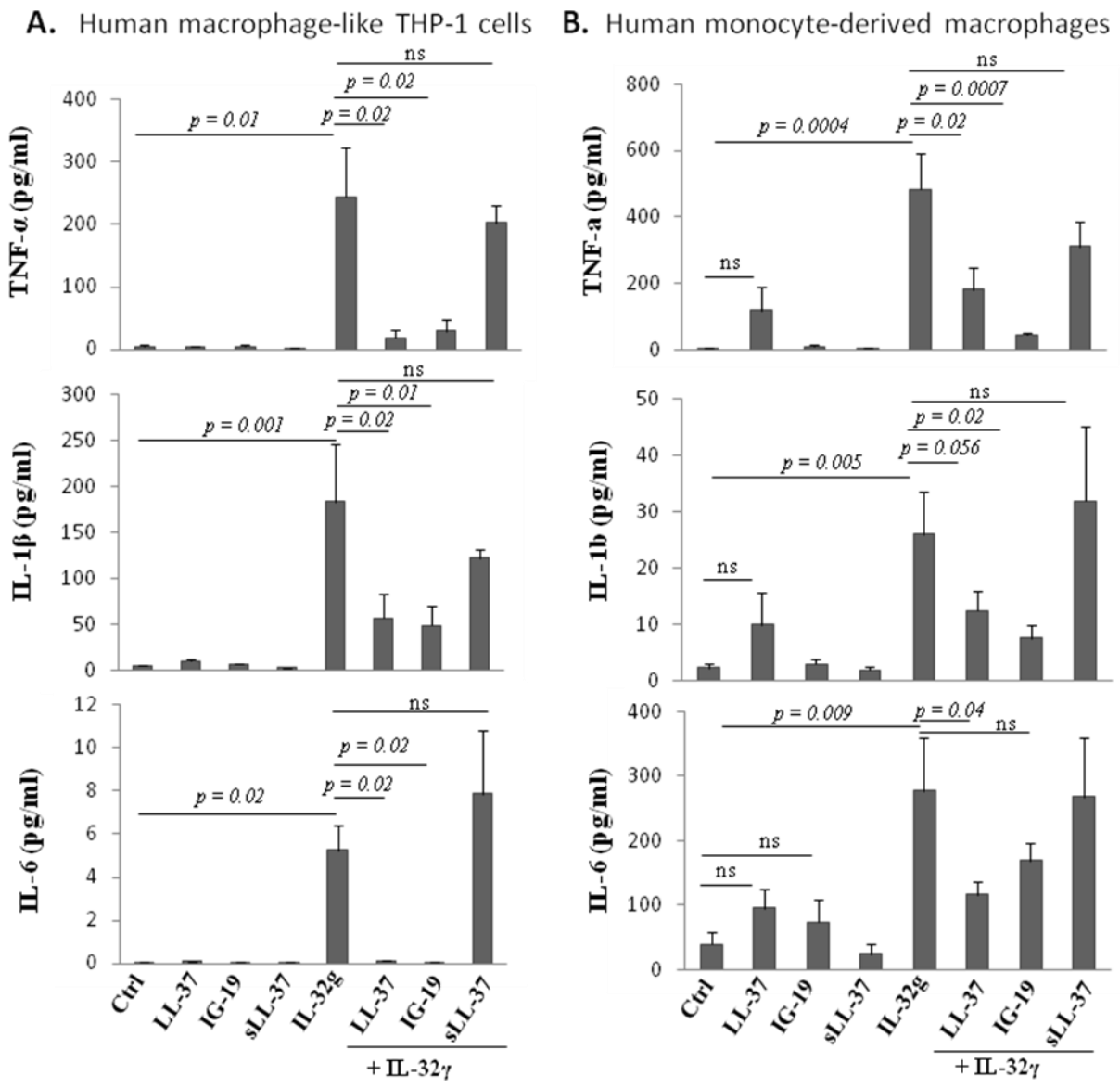


Figure 9. Peptides LL-37 and IG-19 suppressed IL-32γ-induced pro-inflammatory cytokines in macrophages.

(**A**) Differentiated THP-1 macrophages and (**B**) human monocyte-derived macrophages (MDM) were stimulated with IL-32*γ* (20 ng/mL) in the presence and absence of 5 μM of LL-37, IG-19, or sLL-37, for 24 hr. Tissue culture supernatants were monitored for production of tumour necrosis factor-*α* (TNF-*α*), IL-1*β* and IL-6 by ELISA. Results shown are an average of eight independent experiments with THP-1 cells, and at least six independent biological experiments performed with MDM isolated from different donors, ± standard error. The *P*-values are calculated using Student's paired *t*-test (NS = non-significant). Figure is modified from Choi et. al., Immunology, 2014. 143(1):68-80 [102].

4 – 3.4 LL-37 and IG-19 significantly induced the production of anti-inflammatory cytokines in human PBMC and macrophage-like THP-1 cells.

We examined the effects of the peptides LL-37 and IG-19 on the production of anti-inflammatory cytokines IL-1 receptor antagonist (IL-1RA) and transforming growth factor-beta 1 (TGF-β1) in macrophage-like THP-1 cells and human PBMC. The cells were stimulated with 5μM peptides (LL-37, IG-19 or sLL-37), in the presence or absence of 20 ng/mL of IL-32γ. Tissue culture (TC) supernatants were collected after 48 h to monitor the production of anti-inflammatory cytokines IL-1RA and TGF-β1 via ELISA. Peptides LL-37 and IG-19 significantly induced the production of IL-1RA in both human PBMC and macrophage-like THP-1 cells (Figure 10). In contrast, sLL-37 did not significantly induce the production of IL-1RA. IL-32γ-induced IL-1RA was not suppressed by the peptides in both human PBMC and macrophage-like THP-1 cells.

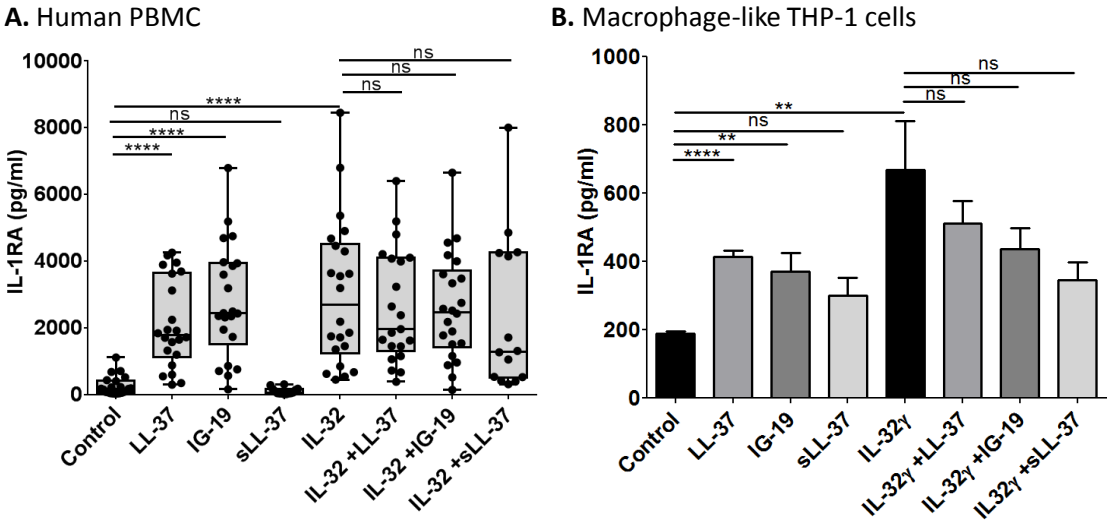


Figure 10. LL-37 and IG-19 significantly induced the production of IL-1RA.
Human PBMC and macrophage-like THP-1 cells were stimulated with 5 μM peptides (LL-37, IG-19 or sLL-37) for 48 h. Tissue culture supernatants were used to monitor for the production of anti-inflammatory cytokine IL-1RA. (**A**) PBMC results are shown in box and whisker plot, with each point representing an independent donor (n ≥ 14). (**B**) Macrophage-like THP-1 results

are shown in bar graphs, representing average of at least 6 independent experiments ($n \geq 6$) ± standard error (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$, **** $p<0.0001$).

The effects of LL-37 and its synthetic derivative peptide IG-19 on the production of anti-inflammatory cytokine TGF-β1 was also examined in human PBMC and macrophage-like THP-1 cells. As mentioned above, the cells were stimulated with 5 μM peptides LL-37, IG-19 or sLL-37, in the presence or absence of 20 ng/mL of IL-32γ for 48 h. TC supernatants were subjected to acid activation using 1N HCl, followed by neutralization with 1N NaOH to disassociate the latency associated peptide (LAP) from the mature form of TGF-β1. The presence of the matured form of TGF-β1 in TC supernatant was monitored using ELISA. Peptides LL-37 and IG-19, but not sLL-37, significantly induced the production of TGF-β1 in human PBMC (Figure 11). However, TGF-β1 production was not detected in macrophage-like THP-1 cells.

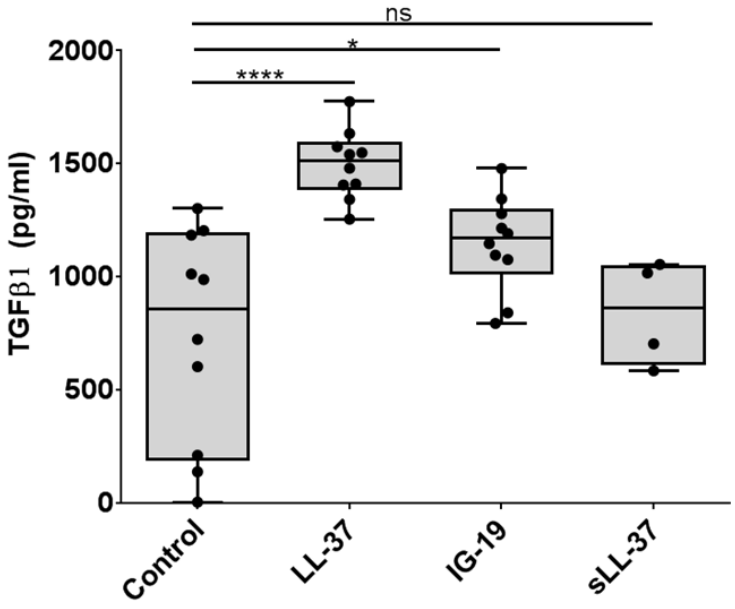


Figure 11. LL-37 and IG-19 induced the production of TGF-β1 in human PBMC.
Human PBMC were stimulated with 5 μM peptides (LL-37, IG-19 or sLL-37) for 48 h. TC supernatants were used to monitor for the production of TGF-β1 in human PBMC. Results are shown in box and whisker plot, with each point representing an independent donor ($n \geq 4$). (* $p<0.05$, **** $p<0.0001$).

4 – 3.5 LL-37 and IG-19 significantly mediated the production of chemokines in human PBMC and macrophage-like THP-1 cells.

I further examined the effect of the peptides LL-37 and IG-19 on chemokine productions in human PBMC and macrophage-like THP-1 cells. The cells were stimulated with 5μM peptides (LL-37, IG-19 or sLL-37). Tissue culture (TC) supernatants were collected after 24 h to monitor the production of chemokines GRO-α, IL-8 and MCP-1 by ELISA. Peptides LL-37 and IG-19 induced the production of chemokines GRO-α, IL-8 and MCP-1 in both human PBMC and macrophage-like THP-1 cells (Figure 12). The control peptide sLL-37 did not significantly induce the production of these chemokines. IL-32γ-induced chemokines were not significantly suppressed by the peptides (data not shown). Interestingly, I also found that there were significant quantitative differences between LL-37 and IG-19-induced chemokines in both PBMC and THP-1 cells (Figure 12). The peptide IG-19-mediated production of chemokines GROα and IL-8 were significantly lower than that produced in response to LL-37 in both PBMC and THP-1 cells (Figure 12). Peptide IG-19 mediated production of MCP-1 was significantly lower than that produced in response to LL-37 in THP-1 cells.

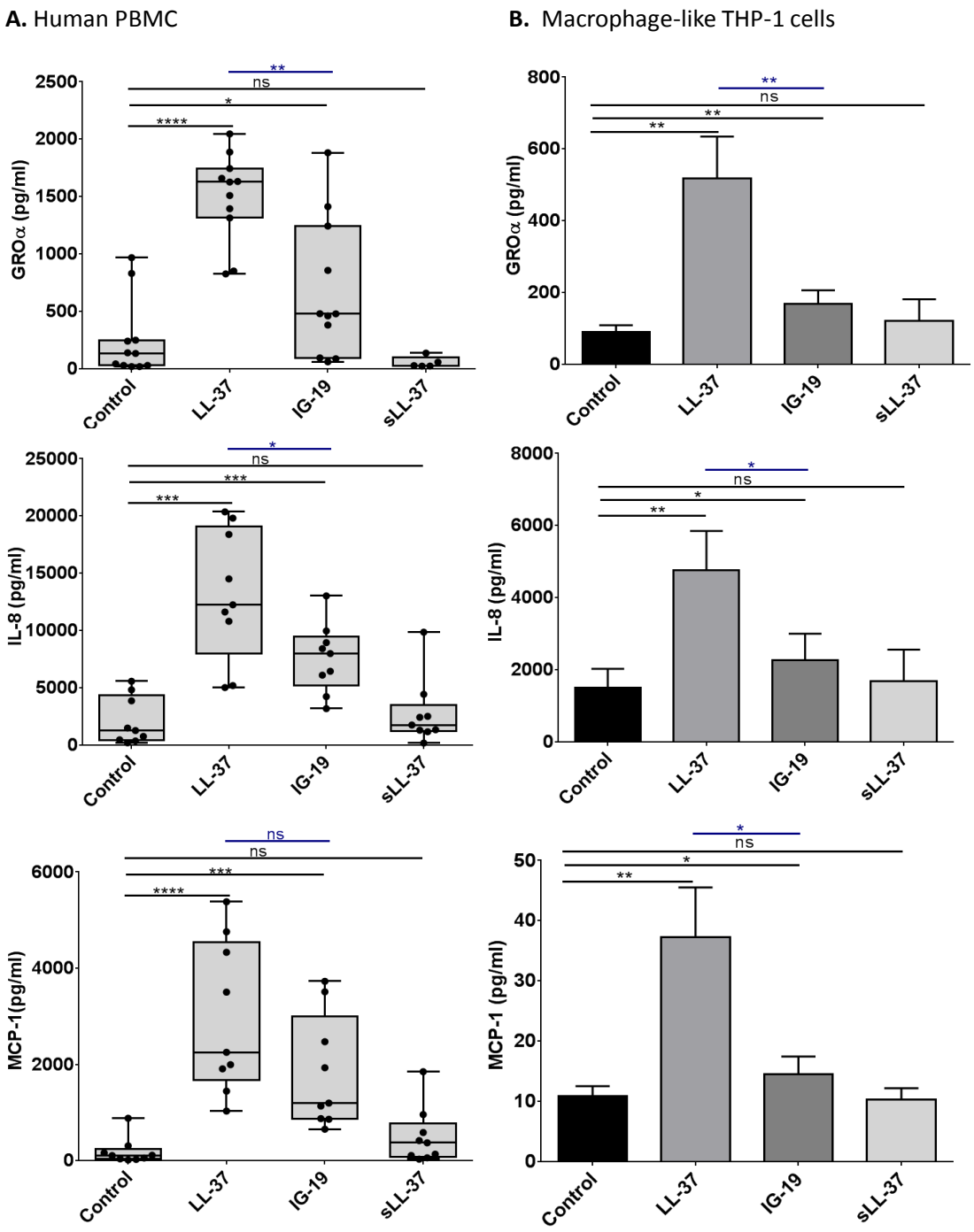


Figure 12. LL-37 and IG-19 induced the production of chemokines.

Human PBMC and macrophage-like THP-1 cells were stimulated with 5 μM peptides (LL-37, IG-19 or sLL-37) for 24 h. Tissue culture supernatants were used to monitor for the production of chemokines (GROα and IL-8) via ELISA. **(A)** PBMC results are shown in box and whisker plot, with each point representing an independent donor ($n \geq 5$). **(B)** Macrophage-like THP-1 results are shown in bar graphs, representing average of at least 3 independent experiments ($n \geq 3$) ± standard error (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$, **** $p<0.0001$).

4 – 4 Discussion

The human cathelicidin peptide LL-37 and some of its synthetic derivative peptides play important roles in host defense against pathogens [59, 88, 89, 97-100]. In the presence of pathogenic stimuli, these peptides were shown to selectively suppress pro-inflammatory responses, enhance anti-inflammatory responses and provide protection in septic shock [42, 52, 67, 147, 179, 246, 288]. More recent studies have demonstrated that the human cathelicidin LL-37 and its derivative peptides mediate immune responses, such as cell proliferation and migration, wound healing, angiogenesis and the release of cytokines and chemokines [26, 49-51, 165, 166]. However, the role of LL-37 and its synthetic derivative peptides in cytokine-mediated inflammation is not well defined. Therefore, I investigated the effects of LL-37 and its synthetic derivative peptides on IL-32γ-induced inflammatory responses in human PBMC and macrophages.

The pro-inflammatory cytokine IL-32 is elevated and directly associated with the pathogenesis and persistence of a wide variety of chronic inflammatory diseases [257, 270, 271, 280, 281]. I have shown that IL-32γ significantly induced pro-inflammatory cytokine production such as TNF-α, IL-1β and IL-6 in human PBMC, MDM and macrophage-like THP-1 cells (Figure 6). I have also demonstrated that CD14+ monocytic cells are the major cell type within the PBMC population to produce TNF-α in response to IL-32 (Figure 7). This is consistent with previous studies demonstrating that the cytokine IL-32γ induces the production of pro-inflammatory cytokines (TNF-α and IL-1β) in mononuclear cells, and these form a feed-forward loop which amplifies and prolongs inflammation to contribute to the development of chronic inflammatory diseases [21, 231, 257, 268, 274, 313, 314]. I further showed that that the peptides LL-37 and its synthetic derivative peptide IG-19 significantly suppressed IL-32γ-induced pro-inflammatory

cytokine production in PBMC and CD14+ monocytic cells (Figure 8). Silencing or inhibiting IL-32γ or downstream pro-inflammatory cytokine production induced by IL-32, have been suggested as a therapeutic strategy for chronic inflammatory diseases [229, 256, 257]. Therefore, the results in this study suggest that cathelicidin-derived peptides may be beneficial in the control of IL-32-induced pro-inflammatory responses and to treat chronic inflammatory diseases [274].

A caveat in therapeutic strategies directly targeting IL-32 is that complete suppression of IL-32-induced responses might results in serious side effects similar to current available therapies which target and completely suppresses TNF-α. Similar to other pro-inflammatory cytokines, it was demonstrated that IL-32 and IL-32γ-induced pro-inflammatory cytokines, such as TNF-α and IFNγ, play important roles in providing protection against various infections [259, 286, 315, 316]. Therefore, completely suppressing all IL-32γ-induced inflammatory responses could compromise the immune system and potentially increase susceptibility to infections. Chemokine production is considered to be a pro-inflammatory response required for infection control, as chemokines are required to attract leukocytes to the site of infections. In this study, I have demonstrated that the peptides LL-37 and IG-19 selectively suppress IL-32γ-induced pro-inflammatory cytokines such as TNF-α and IL-1β production, without neutralizing chemokine production in PBMC or macrophages. The peptides alone can mediate chemokine production in PBMC and macrophages. This selective mode of action of cathelicidin-derived peptides makes them attractive as immunomodulatory molecules that selectively suppress IL-32γ-induced inflammatory responses, without compromising host defense functions such as chemokine production that is required to control infections. These results are similar to previous studies that have shown that LL-37 can selective suppress endotoxin-induced TNF-α, without compromising chemokine responses [165, 166, 175]. However, my results also demonstrated that there were

quantitative differences in chemokine production in response to IG-19 compared to LL-37, with IG-19-mediated chemokine response being significantly lower than that with LL-37 (Figure 12). These results suggest that the peptide IG-19 exhibits anti-inflammatory properties similar to LL-37, in controlling IL-32γ-induced inflammation, but exhibits overall modest pro-inflammatory responses such as chemokine induction, compared to LL-37. In addition, my initial screening studies in THP-1 macrophage cell line showed that IG-19 had less cytotoxic effect compared to LL-37. Taken together, these results suggest that the two peptides may mediate the anti-inflammatory effects by different downstream mechanisms, and that the peptide IG-19 may be a better therapeutic agent compared to the parent peptide LL-37.

The results in this study have also shown that the peptides LL-37 and IG-19 mediated the production of the anti-inflammatory cytokine IL-1RA, without suppressing IL-32γ-induced IL-1RA in PBMC and macrophages. IL-1RA is an endogenous antagonist of IL-1β, and is known to be used as treatments for chronic inflammatory diseases [317]. Consistent with this, IL-32-induced IL-1β was suppressed by LL-37 and IG-19 in my studies (Figure 6 and Figure 9), which indicates that one of the mechanisms by which the peptides mediate their anti-inflammatory function may be blocking interaction of IL-1β with the receptor. I also demonstrated that the peptides induced the production of TGF-β1 in human PBMC. TGF-β is known to mediate immune responses differentially, depending on the stimuli in the environment. For example, in the presence of IL-6, TGF-β-mediates the differentiation of T helper 17 (Th17) cells, which enhance inflammation [318]. In contrast, TGF-β is known to promote the differentiation of regulatory T-cells (Tregs), which mediates anti-inflammatory responses [319]. As the peptides inhibit IL-32γ-induced IL-6 production (Figure 6 and Figure 9), I speculate that these peptides will not skew the response to a Th17 phenotype in an inflammatory environment with elevated

IL-32. In contrast, peptides LL-37 or IG-19-mediated production of TGF-β may promote anti-inflammatory responses by increasing the number of Tregs. In preliminary data, I have shown that indeed the peptides LL-37 and IG-19 increased the number of Tregs (data will be discussed in chapter 7). Investigating the effects and mechanisms associated with cathelicidin peptide mediated TGF-β and Tregs was beyond the scope of this thesis and will be pursued as a future direction (discussed in chapter 7).

The results from these studies overall indicate that the peptides LL-37 and IG-19 selectively regulate IL-32γ-mediated inflammation, suggesting a potential role for these peptides to control immune-mediated inflammation in chronic inflammatory diseases. Interestingly, there were significant quantitative differences of peptide-induced chemokine production, this suggested that LL-37 and IG-19 may mediate different downstream effects by different mechanisms. Hence, I further investigated the signaling mechanisms that underlie the functions of the peptides LL-37 and IG-19, using the human macrophage-like THP-1 cells.

CHAPTER 5 –PEPTIDES LL-37 AND IG-19 REGULATE IL-32γ-MEDIATED PROTEIN PHOSPHORYLATION

5 – 1 Introduction

Protein phosphorylation is one of the most common types of signal transduction, a process which transmits signals to a variety of intracellular targets and modulates downstream responses, such as cellular differentiation and proliferation, migration, and inflammation [320]. This process involves protein kinases, which transfers the phosphate from an ATP to specific phosphorylation sites on amino acids (usually serine (Ser, S), threonine (Thr, T), or tyrosine (Tyr, Y) residues) within a protein [321]. It had been demonstrated that protein kinases can be use as drug targets for a wide variety of diseases, such as chronic inflammatory diseases and cancers [322]. Since it was demonstrated that the expression of IL-32 is significantly elevated and directly associated with the pathogenesis of chronic inflammatory diseases, we investigated the effects of IL-32γ-mediated downstream protein phosphorylation.

Despite the accumulating interests in the role of pro-inflammatory cytokine IL-32γ in host immune defense and chronic inflammatory diseases, relatively little is known about the downstream signaling mechanisms of IL-32γ. As discussed in the previous chapter, IL-32γ plays important roles in inducing and prolonging inflammatory responses, and inhibiting IL-32 or its downstream effects could be beneficial for treating chronic inflammatory diseases. Therefore, elucidating the downstream signaling mechanisms of IL-32γ would allow us to identify potential protein targets, which can be used to suppress excessive inflammatory responses. In addition, my data showed that HDP LL-37 and its synthetic derivative peptide IG-19 can selectively suppress IL-32γ-induced inflammatory cytokines, without altering IL-32γ-induced anti-inflammatory cytokines or chemokines. These results suggested that the peptides LL-37 and IG-19 can impact

downstream protein targets of IL-32γ, resulting in selective control of inflammatory responses. Therefore, I further investigated the protein phosphorylation profile induced by IL-32γ, and the effects of the peptides LL-37 and IG-19 on IL-32γ-mediated protein phosphorylation.

5 – 2 Hypothesis and Rationale

Previous studies have showed that production of pro-inflammatory cytokines, chemokines and anti-inflammatory cytokines involve the modulation of signaling pathways, such as extracellular signal-regulated kinase (ERK, p44/42 MAPK), c-Jun N-terminal kinases (JNK), p38 mitogen-activated protein kinase (MAPK), and nuclear factor-κB (NF-κB) signaling pathways [280]. Since I have shown that HDP LL-37 and IG-19 selectively suppressed IL-32γ-induced protein production, I hypothesized that the peptides would also selectively alter IL-32γ-mediated protein phosphorylation events. In addition, my results have also shown that the peptides alone induced anti-inflammatory cytokines and chemokines productions, and that there are quantitative differences between LL-37 and IG-19-induced chemokine productions. Therefore, I hypothesized that the peptides alone will mediate similar downstream protein phosphorylations involved in anti-inflammatory responses, and in contrast differentially alter signaling pathways involved in chemokine production.

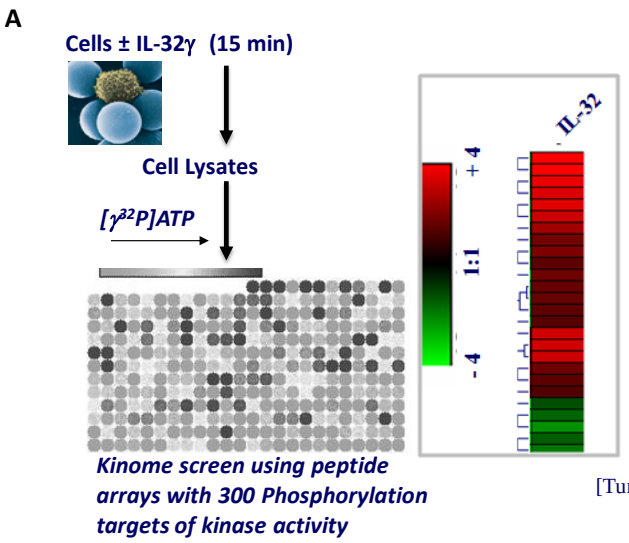
5 – 3 Results

5 – 3.1 Protein phosphorylations induced in the presence of IL-32γ

Since not much is known about the downstream signaling mechanism of IL-32γ, our lab had previously identified specific protein targets that are phosphorylated in the presence of IL-32γ, using a kinome array. This work was previously published in the Journal of Immunology in

collaboration with Dr. Scott Napper (Vaccine and Infectious Disease Org, Saskatoon, Canada), I am a co-author on this publication, the first author is Emily Turner-Brannen from Dr. Mookherjee's lab [304]. In this publication, I established that the optimum dose of cytokine IL-32γ required to elicit inflammatory responses in macrophages is 20 ng/ml. I showed that IL-32γ-mediated the nuclear translocation of NF-κB subunit p65 in macrophages. I was also involved in the functional validation of two protein phosphorylation targets, p300 and DAPK1, of IL-32γ.

Macrophage-like THP-1 cells were stimulated with 20 ng/mL of IL-32γ for 15 min. Cell lysates were collected and analysed on a peptide array containing 300 peptides (printed in triplicate) which allowed the detection of phosphorylation events at specific sites for different kinases, as previous published by Jalal et. al. (Figure 13A) [248]. Comparative kinome analysis was used to identify protein targets which are phosphorylated more than 1.5 folds, with $p \leq 0.05$, when compared to unstimulated cells [248]. Our data identified several proteins that were phosphorylated in response to IL-32γ [102, 304]. The results of the kinome analyses were deposited in NCBI's Gene Expression Omnibus database under series accession number GSE28649). From this previous study, I selected three phospho-protein targets, proto-oncogene tyrosine-protein kinase (FYN, Y420), protein kinase B (AKT-1, T308), and mitogen-activated protein kinase phosphatase-1 (MKP-1, S359), to further investigate the effects of the peptides LL-37 and IG-19 on IL-32γ-mediated downstream signaling mechanisms. These three protein targets were selected because all three proteins showed phosphorylation changes of more than 2.5-fold ($p<0.01$) in response to IL-32 compared to un-stimulated cells (Figure 13B).



B

Phosphoprotein	Kinase Target	IL-32 Relative Fold change	P <
Fyn	Y420	3.43	0.01
Akt1	T308	3.26	0.01
MKP-1	S359	2.51	0.01

[Turner-Brannen E. et al., J Immunol, 2011 Jun 15;186(12):7127-35.]

Figure 13. Kinome analysis of protein phosphorylations in the presence of IL-32γ.
(A) Schematic of kinome screen using peptide arrays representing 300 phosphorylation targets of kinase activity. (B) Protein targets that were phosphorylated in the presence of 20 ng/mL IL-32γ after 15 min of stimulation, using human macrophage-like THP-1 cells. Differentially phophorylated targets were defined as ≥ 1.5-fold increase, with $p < 0.05$, in phosphorylation compared with that in unstimulated cells. Figure is modified from Choi et. al., Immunology, 2014. 143(1):68-80 [102].

5 – 3.2 LL-37 and IG-19 alter IL-32γ-mediated specific protein phosphorylation

I used western blots to validate the kinome data, and demonstrated that FYN (Y420), AKT-1 (T308) and MKP-1 (S359) were phosphorylated after 15 min of stimulation in macrophage-like THP-1 cells with 20 ng/mL IL-32γ (Figure 14). I further showed that the peptides LL-37 and IG-19 suppressed IL-32γ-mediated phosphorylation of FYN (Y420). Phosphorylation of FYN (Y420) is known to induce downstream inflammatory responses [323]. In contrast, IL-32γ-mediated phosphorylations of MKP-1 (S359) and AKT-1 (T308) were not significantly suppressed by either peptides LL-37 or IG-19 (Figure 14). Furthermore, the peptides LL-37 and IG-19 alone enhanced the phosphorylation of MKP-1 (S359) and AKT-1 (T308) when compared to unstimulated cells. Phosphorylation of MKP-1 (S359) and AKT-1 (T308) are known to

contribute to the negative regulation of downstream inflammatory responses [324-329]. These data were published in Choi et. al., Immunology, 2014. 143(1):68-80 [102].

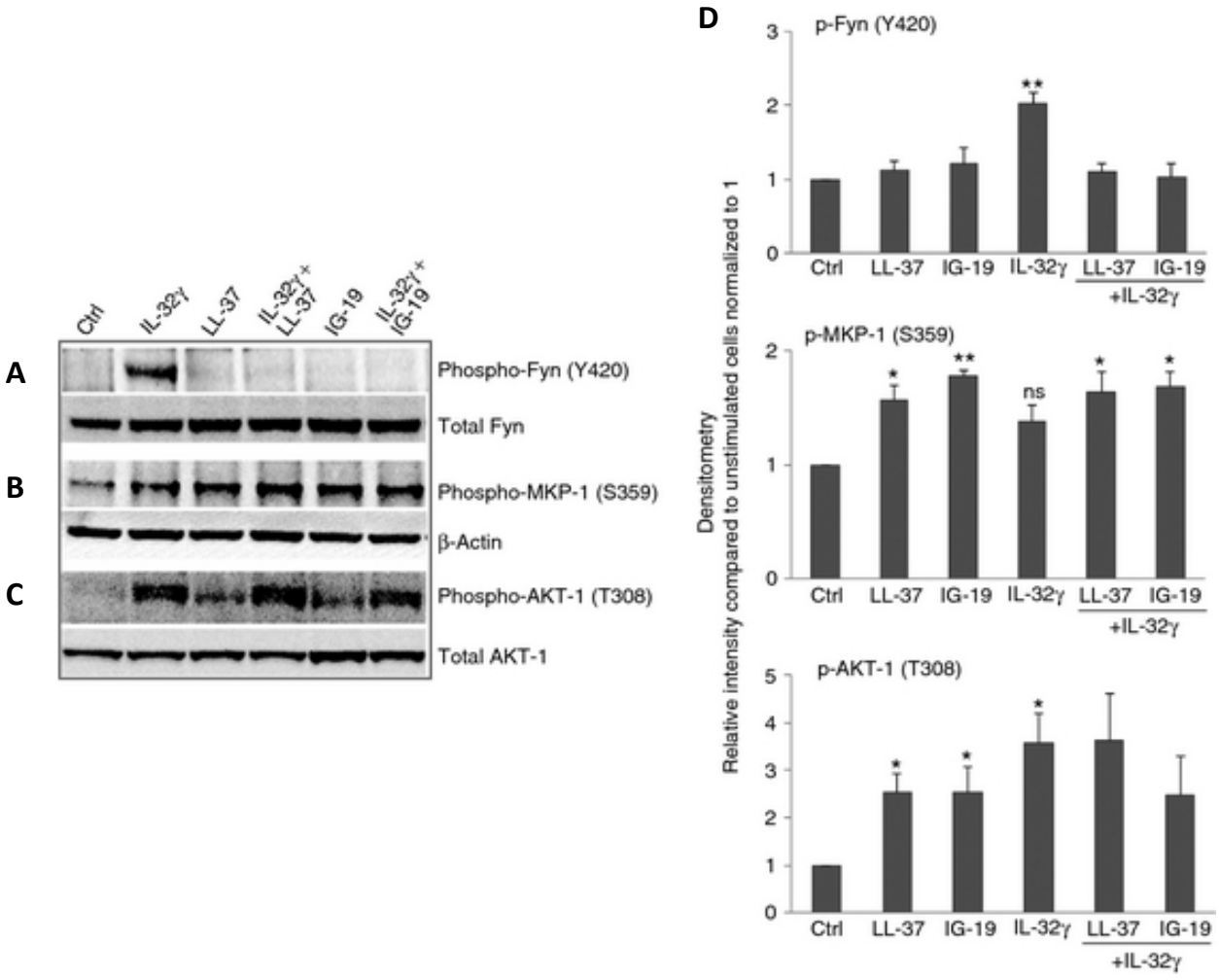


Figure 14. Protein phosphorylation in response to IL-32γ, in the presence or absence of peptides LL-37 and IG-19.

Differentiated THP-1 macrophages were stimulated with IL-32*γ* (20 ng/mL) in the presence or absence of either LL-37 or IG-19 (5 μM each) for 15 min. Cell lysates were probed in Western blots with specific antibodies to human (A) phospho-Fyn (Y420), (B) phospho-MKP-1 (S359) and (C) phospho-AKT-1 (T308). Antibodies to the non-phosphorylated forms of Fyn and AKT-1, and *β*-actin were used as paired loading control. The immunoblots shown are representative of at least three independent experiments. (D) Densitometry, ratio of band density of the phosphorylated proteins over unstimulated cells (*y*-axis), was calculated after normalization to band density of loading paired controls of the respective proteins for each sample. Results

represent an average of at least three independent experiments. The *P*-values are calculated using Student's paired *t*-test (*$P < 0.05$, **$P < 0.01$, NS = non-significant, compared with unstimulated control cells). Figure is modified from Choi et. al., Immunology, 2014. 143(1):68-80 [102].

Since both peptides LL-37 and IG-19 exhibited anti-inflammatory effects in the presence of IL-32γ, and significantly mediated the phosphorylation of MKP-1, we further interrogated the direct interacting protein partners of the dual phosphatase MKP-1 using bioinformatics approaches. Using InnateDB Biomolecular Network Analysis (http://www.innatedb.ca), we observed that the direct interacting protein partners of MKP-1 are over represented in several key signaling pathways, namely p44/42 MAPK, JNK, NFκB and p38 MAPK (Figure 15). Based on this bioinformatics analyses, I further selected to evaluate the effects of the peptides LL-37 and IG-19 focused on these four signaling pathways i.e. p44/42 MAPK, JNK, NFκB and p38 MAPK.

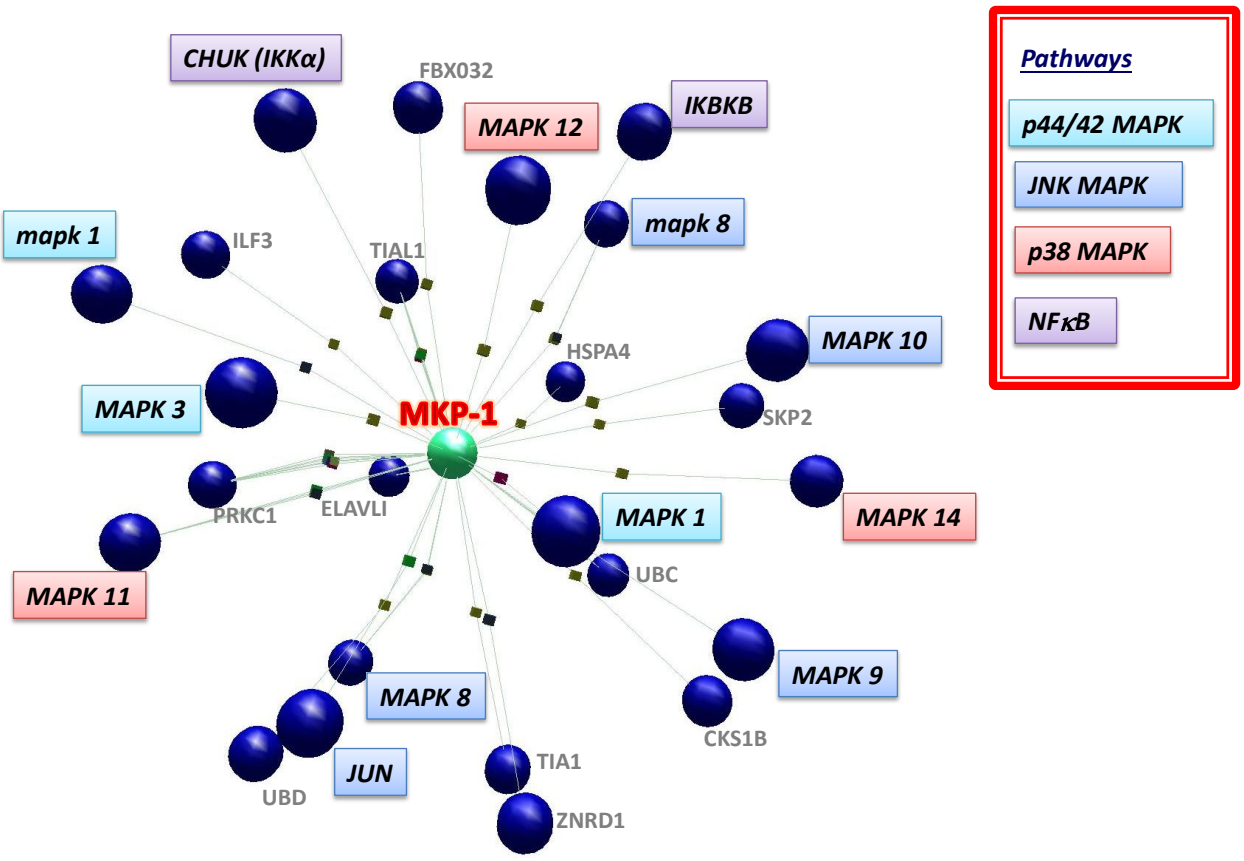


Figure 15. Interacting protein network of dual phosphatase MKP-1.
InnateDB Biomolecular Network Analysis (http://www.innatedb.ca) of direct interacting protein partners of MKP-1. Unweighted interaction between MKP-1 and proteins is visualized as a 3D figure, using BioLayout Express 3D Version 2.2. MKP-1 interacting proteins involve in key signaling pathways were represented with different colors: p44/42 MAPK (teal), JNK (blue), p38 MAPK (red) and NFκB (purple).

5 – 3.3 Peptides LL-37 and IG-19-mediated downstream MAPK and NFκB signaling responses

5 – 3.3.1 Peptide LL-37 and IG-19- mediated p44/42 MAPK activity

It is known that p44/42 MAPK, also known as ERK1/2, is an upstream kinase which mediates the phosphorylation and stabilization of MKP-1 [326]. I demonstrated that IL-32γ-mediated the phosphorylation of p44/42 MAPK was not suppressed by the peptides LL-37 and IG-19 in THP-

1 macrophages (Figure 16A). Furthermore, phosphorylation of p44/42 MAPK was also induced in response to the peptides LL-37 and IG-19 alone compared to unstimulated cells (Figure 16A).

Phosphorylation is required for the activity of the p44/42 MAPK. Therefore, I determined IL-32γ-mediated activity of p44/42 MAPK, in the presence or absence of the peptides, using a p44/42 MAPK activity assay from Cell Signaling Technology. Human macrophage-like THP-1 cells were stimulated with 20 ng/mL of IL-32γ, in the presence or absence of 5 μM peptides (LL-37 or IG-19) for 15 min. Total cell lysate was subject to immunoprecipitation with antibody against phopho-p44/42 MAPK. The eluate was then incubated with p44/42 MAPK substrate Elk-1, in the presence of ATP. Activated p44/42 MAPK is known to phosphorylate the downstream substrate Elk-1 in the presence of ATP, and phosphorylated Elk-1 is representative of p44/42 MAPK activity [330]. Therefore, immunoblots were probed with antibody against phospho-Elk-1 (Ser383) to monitor the activity of p44/42 MAPK (Figure 16B). I showed that phosphorylation of Elk-1 (Ser383), indicative of p44/42 activity, was enhanced in response to the peptides LL-37 and IG-19, compared to unstimulated cells (Figure 16B). In addition, both the peptides did not suppress IL-32γ-mediated p44/42 MAPK activity (Figure 16B). These data supports that the peptides LL-37 and IG-19 enhance the phosphorylation and activation of p44/42 MAPK, which will subsequently allow the kinase to phosphorylate and stabilize the dual specificity phosphatase MKP-1 (S359) [331].

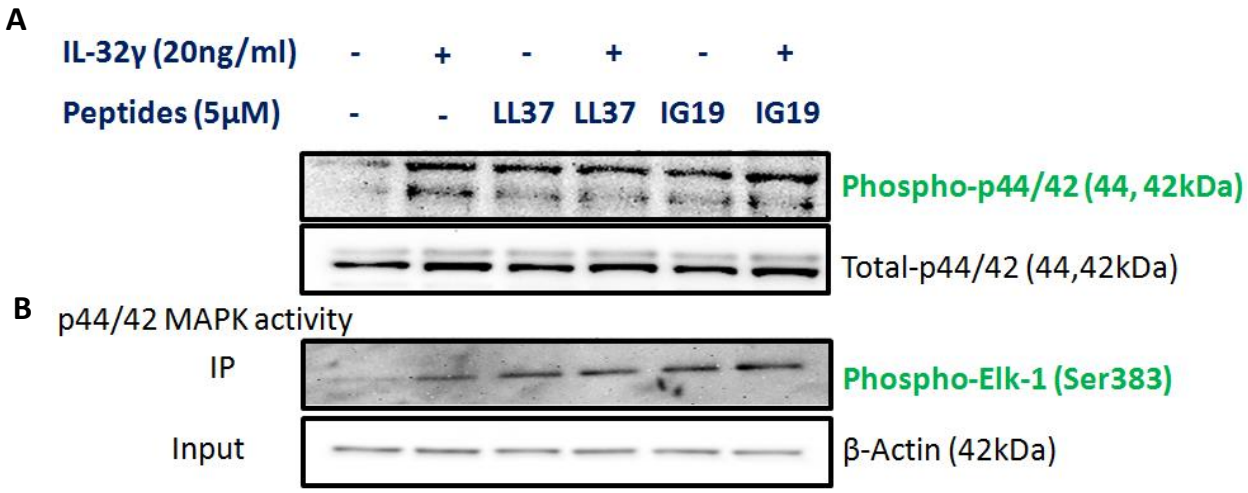


Figure 16. LL-37 and IG-19 stimulation increased phosphorylation and activity of p44/42 MAPK.

Human macrophage-like THP-1 cells were stimulated with IL-32γ (20 ng/mL) ± 5 μM peptides (LL-37 or IG-19) for 15min. (A) Using western blots, amount of protein phosphorylation were detected using phospho-p44/42 MAPK antibodies. Antibody against total – p44/42 MAPK was used as loading control. Results are representation of at least three independent experiments ($n \geq 3$). (B) Activation of p44/42 MAPK was detected using p44/42 MAPK activity assay kit (Cell Signaling Technology). Equal amounts of total protein were immunoprecipitated with a monoclonal antibody to phosphor-p44/42 MAPK (T202/ Y204). The immunoprecipiated eluates were incubated with p44/42 MAPK substrate Elk-1 in the presence of ATP, and the activity of p44/42 MAPK was evaluated by monitoring the phosphorylation of the substrate Elk-1 using an anti-phospho-Elk-1 (S383) specific antibody. Antibody against β-actin was used as loading control. Results shown are representative of at least three independent experiments (n = 3). Panel B of this figure is modified from Choi et. al., Immunology, 2014. 143(1):68-80 [102].

5 – 3.3.2 Peptides LL-37, but not IG-19, enhanced phosphorylation of JNK

To assess the effect of the peptides on JNK (c-Jun N-terminal kinases) signaling pathway, I stimulated human macrophage-like THP-1 cells with 20 ng/mL IL-32γ, in the presence and absence of 5 μM peptide (LL-37 or IG-19). Using western blots, I showed that IL-32γ enhanced the phosphorylation of JNK (T183/Y185) compared to unstimulated cells (Figure 17A). In the presence of either LL-37 or IG-19, IL-32γ-mediated phosphorylation of JNK was not

significantly suppressed. Interestingly, the peptide LL-37, but not IG-19, significantly enhanced the phosphorylation of JNK (Figure 17). Consistent with this western blot data, I also demonstrated that the peptide LL-37 but not IG-19, enhanced the phosphorylation of JNK (T183/Y185), probing with phospho-JNK (T183/Y185) in a PathScan® phospho-SAPK/JNK (T183/Y185) Sandwich ELISA (Figure 17B) as a second line of evidence. However, in the PathScan® ELISA analyses IL-32γ did not significantly enhance phospho-JNK compared to naïve, which was not consistent with the western blot data. Nevertheless, compared to all other stimuli, the peptide LL-37, but not IG-19, significantly enhanced the phosphorylation to JNK (T183/Y185), which was consistent in both data sets from western blot analyses and the PathScan® sandwich ELISA.

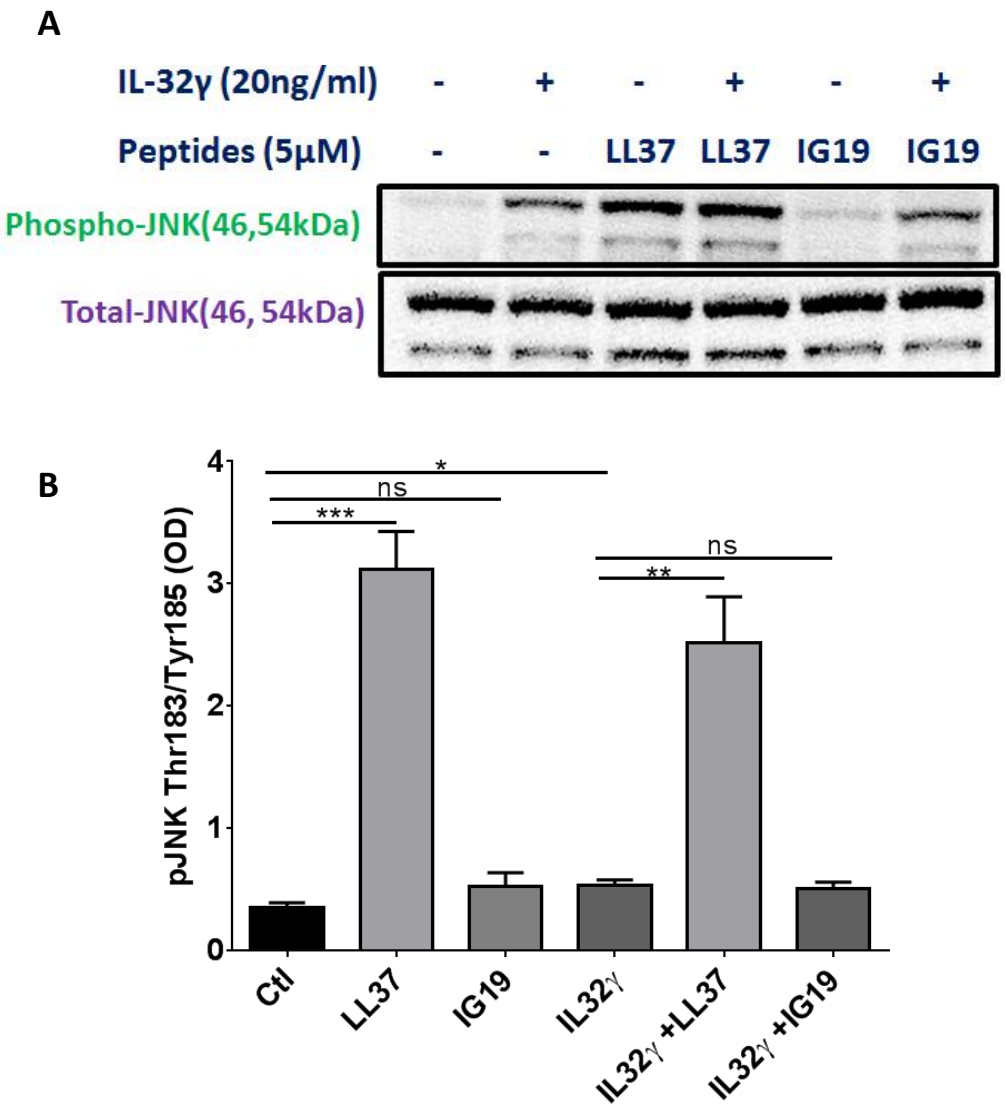


Figure 17. Peptide LL-37 mediated phosphorylation of JNK.

Human macrophage-like THP-1 cells were stimulated with IL-32γ (20 ng/mL) ± 5μM peptides (LL-37 or IG-19) for 30min. (A) Amount of protein phosphorylation were detected using phospho-JNK (T183/Y185) antibodies, and detection of total – JNK was used as loading control. Results are representations of at least three independent experiments (n = 3). (B) A PathScan® phospho-SAPK/JNK (T183/Y185) Sandwich ELISA was used to quantify the phosphorylation of JNK. Upon 30min of stimulation, cell lysate were incubated in a 96 well plate, pre-coated with phospho-SAPK/JNK rabbit Ab. Followed by incubating with total SAPK/JNK mouse (detection antibody), anti-mouse IgG, HRP-linked antibody, HRP substrate and TMB. The magnitude of optical density (OD) for the developed color is proportional to the quantity of phospho-SAPK/JNK protein. Results represent average of three independent experiments (n = 3) ± standard error (* $p<0.05$, ** $p<0.005$, *** $p<0.005$).

5 – 3.3.3 Peptides LL-37, but not IG-19, enhanced the phosphorylation of IKKα/β

To understand the effects of LL-37 and IG-19 on the NFκB pathway, I assessed the phosphorylation patterns of IKKα/β (S176/180). The IKKα/β is a complex which targets and phosphorylates IκB, allowing the release of NFκB to dimerize, enter into the nucleus and induce the expression of pro-inflammatory cytokines and chemokines [332-334]. I showed that IL-32γ-mediated the phosphorylation of IKKα/β. In the presence of the peptides either LL-37 or IG-19, IL-32γ-mediated phosphorylation of IKKα/β (S176/180) was not suppressed (Figure 18). Similar to JNK, the phosphorylation of IKKα/β (S176/180) was significantly enhanced in the presence of LL-37, but not in the presence of the peptide IG-19 (Figure 18). These data suggested that LL-37, but not IG-19, is able to mediate the phosphorylation of IKKα/β (S176/180), which allows NFκB to translocate to the nucleus and induce downstream pro-inflammatory responses.

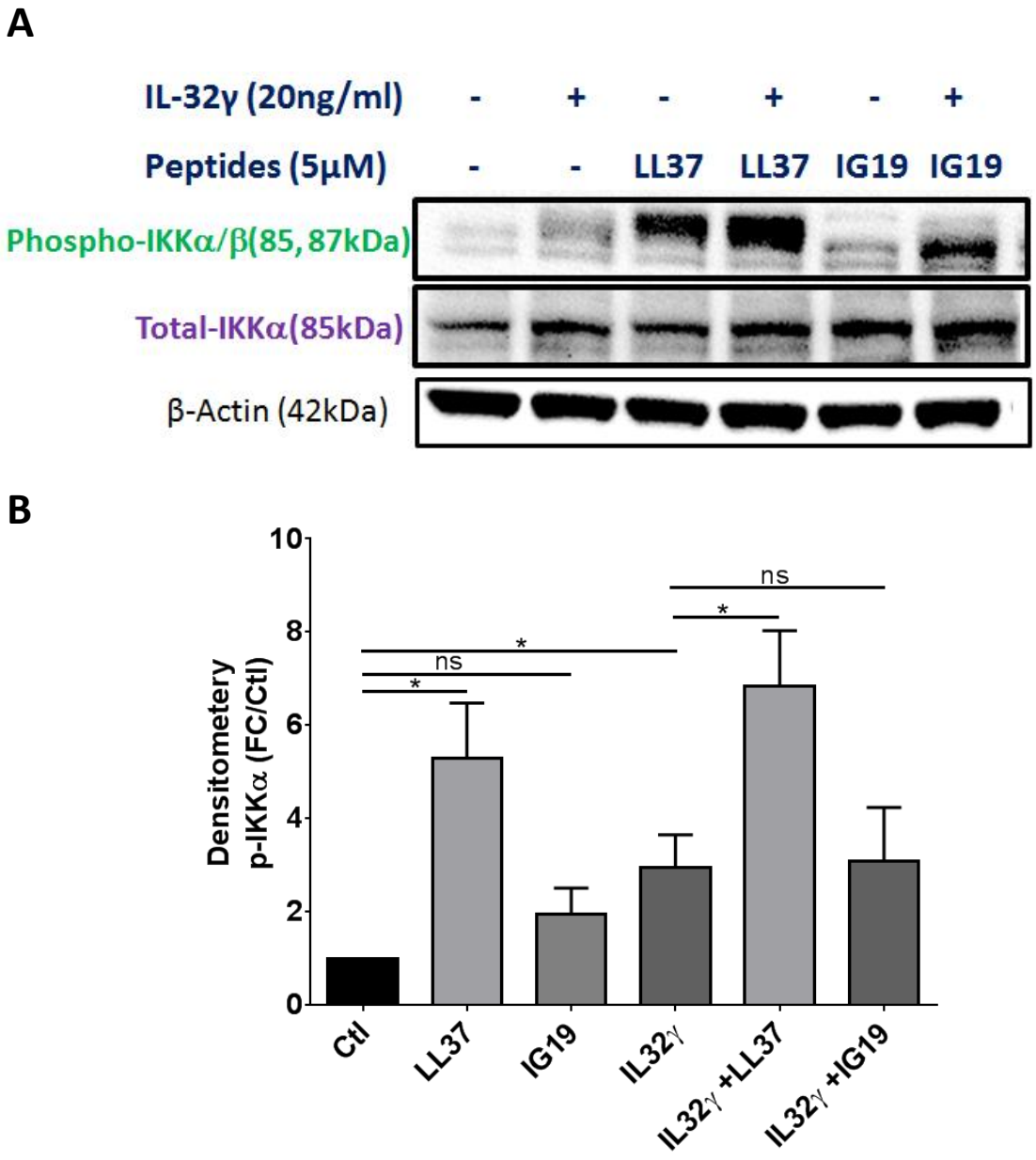


Figure 18. LL-37, but not IG-19, mediated phosphorylation of IKKα/β.
Human macrophage-like THP-1 cells were stimulated with IL-32γ (20 ng/mL) ± 5μM peptides (LL-37 or IG-19) for 30min. (A) Amount of protein phosphorylation were detected using phospho-IKKα/β (S176/180) Ab. Antibodies against total IKKα and β-actin were used as loading control. Blot shown is a representative of at least three independent experiments (n = 3). (B) Densitometry was measured with the ratio of band density of the phospho-IKKα/β over unstimulated cells, after normalization to band density of loading paired controls of the respective proteins for each sample. Results represent average of three independent experiments (n = 3) ± standard error (* $p < 0.05$).

5 – 3.3.4 Peptides LL-37 and IG-19 enhanced the phosphorylation of p38 MAPK

To evaluate the effects of LL-37 and IG-19 on the p38 MAPK pathway, human macrophage-like THP-1 cells were stimulated with 5μM of the peptides LL-37 or IG-19, in the presence or absence of 20 ng/mL IL-32γ. Cell lysate were used to monitor the phosphorylation patterns of p38 MAPK via Western Blot. I showed that IL-32γ-mediated the phosphorylation of p38 MAPK (T180/ Y182) after 15 min of stimulation (Figure 19). In the presence of the peptides either LL-37 or IG-19, IL-32γ-mediated phosphorylation of p38 MAPK (T180/ Y182) was not suppressed (Figure 19). The phosphorylation of p38 MAPK (T180/ Y182) was significantly enhanced in the presence of the peptides LL-37 and IG-19 alone (Figure 19). Studies have shown that p38 MAPK pathway plays important roles in mediating inflammation and maintaining homeostasis, such as cytokine and chemokine production, cellular activation, proliferation and differentiation. My data suggested that the peptides LL-37 and IG-19 - induced downstream responses, such as the induction of chemokine and anti-inflammatory cytokine productions, may involve the phosphorylation of p38 MAPK.

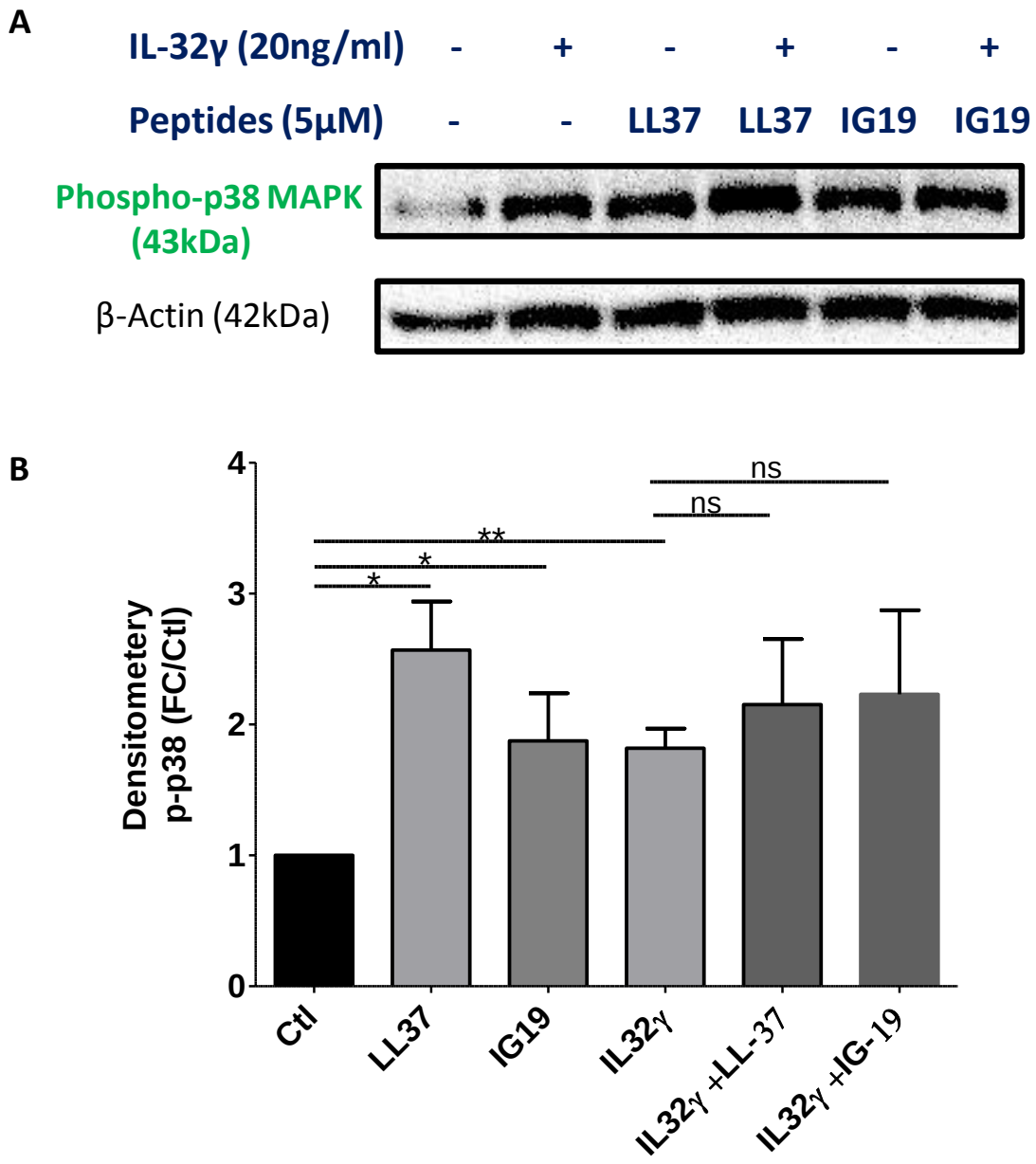


Figure 19. Peptides LL-37 and IG-19 enhanced phosphorylation of p38 MAPK.
Human macrophage-like THP-1 cells were stimulated with IL-32γ (20 ng/mL) ± 5µM peptides (LL-37 or IG-19) for 30min. (A) Amount of protein phosphorylation were detected using phospho-p38 MAPK (T180/Y182) Ab. Antibodies against β-actin were used as loading control. Blot shown is a representative of at least three independent experiments (n = 3). (B) Densitometry was measured with the ratio of band density of the phospho-p38 MAPK over unstimulated cells, after normalization to band density of loading paired controls of the respective proteins for each sample. Results represent average of three independent experiments (n = 3) ± standard error (* $p < 0.05$, ** $p<0.005$).

5 – 4 Discussion

Inflammatory response is an essential part of the immune host defense. During infection or inflammation, extracellular stimuli activates immune cells by receptors that senses PAMPs or DAMPs, mediates a complex network of highly regulated downstream signaling cascades, which results in the induction of appropriate inflammatory responses. Defective regulation of these signaling cascades can lead to chronic inflammatory diseases. It has been well documented that IL-32γ-induced pro-inflammatory responses contributes to chronic inflammatory diseases, such as RA, IBD and COPD [229, 259, 267, 274, 285-292]. This study focused on IL-32γ-mediated downstream molecular mechanisms, and the effects of the peptides (LL-37 and IG-19) on IL-32γ-mediated signaling pathways. By understanding the signaling pathways, we can identify specific protein targets of IL-32γ for diagnostic and therapeutic purposes. In addition, this knowledge is essential for elucidating the differential mechanisms underlying the activities of the cathelicidin-derived peptides LL-37 and IG-19, which had been shown to possess both pro-inflammatory and anti-inflammatory properties

My results showed that both peptides (LL-37 and IG-19) selectively suppressed IL-32γ-mediated pro-inflammatory responses in human PBMC and macrophages (chapter 4). This suggested that the peptides may regulate IL-32γ-induced responses via altering IL-32-mediated downstream signaling pathways. However, I also demonstrated that there were quantitative differences in chemokine production in response to the peptides LL-37 and IG-19, with LL-37-mediated chemokine production being significantly higher compared to that in response to IG-19, in human PBMC and macrophages (Figure 12). This suggested that there must be a differences between LL-37 and IG-19-mediated downstream signaling mechanisms. Previous studies have shown that macrophages are critical cell types required for the activity of cathelicidin-derived

peptides [243, 244]. There are several studies that have shown that LL-37 induces the production of chemokines in macrophages, including in macrophage-like THP-1 cells [46, 47, 50, 57, 102, 103, 165, 166, 335]. This is consistent with studies that have shown that LL-37 exhibits chemotactic properties for leukocytes [46, 50, 103, 162, 214, 336]. Previous studies have also shown that macrophages are one of the key cell types expressing LL-37 in both RA patients and a pristine-induced arthritis model in rats [201]. The author of this study also suggested that the chemotactic property of LL-37 may contribute to the development of arthritis. The quantitative differences of chemokine production in response to LL-37 and IG-19 demonstrated in my results (Figure 12) suggests that IG-19 is able to induce similar anti-inflammatory responses of LL-37, but may induce pro-inflammatory responses, such as production of inflammatory chemokines, relatively less when compared to LL-37. Therefore, by investigating the signaling mechanisms of the peptides LL-37 and IG-19, in the presence and absence of IL-32γ, we can further understand the differential activities of the peptides within an inflammatory environment.

One of the major challenges we faced is that not much was known about IL-32γ-induced downstream signaling mechanisms. Therefore, in collaborations with Dr. Scott, our lab identified protein targets that are phosphorylated in the presence of IL-32γ using a kinome analysis. We screened the phosphorylation of 300 peptides, in human macrophage-like THP-1 cells [248, 304]. I selected three protein targets (FYN Y420, AKT-1 T308 and MKP-1 S359) which were significantly phosphorylated in the presence of IL-32γ (more than 1.5 folds, with $p \leq 0.05$) compared to unstimulated cells, to further assess the effects of the HDP LL-37 and IG-19 on IL-32γ-mediated downstream signaling mechanisms. The protein tyrosine kinase FYN protein belongs to the Src family kinases (SFK), which plays important roles in mediating inflammatory responses [323, 337]. The protein FYN is located at the inner layer of the cell membrane, and is

activated by tyrosine phosphorylation [338, 339]. Studies have demonstrated that human monocytes and macrophages express FYN in the presence of inflammatory stimuli, such as LPS [340, 341]. In addition, Smolinska et. al. showed that over-expression of FYN in human macrophages significantly increased the production of pro-inflammatory cytokine TNF-α, and moderately increased in the production of pro-inflammatory cytokine IL-6 [342]. Fyn knockout mice were shown to have increased proportion of anti-inflammatory M2 macrophages, reduced macrophage infiltration and reduced adipose tissue inflammation [337]. These studies demonstrated that FYN plays an important role in mediating downstream inflammatory responses. Consistent with these, my study showed that the pro-inflammatory cytokine IL-32γ-mediated the phosphorylation of FYN at site Y420, which suggested that IL-32γ may induce downstream inflammatory responses via FYN phosphorylation. The results in this study also showed that both peptides, LL-37 and IG-19, suppressed IL-32γ-mediated phosphorylation of FYN (Y420). This suggested that one of the mechanisms underlying the anti-inflammatory property of these peptides in the presence of IL-32γ is mediated via suppressing FYN (Y420) phosphorylation. Furthermore, the peptides LL-37 and IG-19 alone did not enhance the phosphorylation of FYN, indicating that the peptides do not contribute to acute pro-inflammatory responses downstream of the FYN pathway such as production of TNF-α, which is consistent with my results described in chapter 4.

In contrast, I demonstrated that the peptide LL-37 and IG-19 did not suppress IL-32γ-mediated phosphorylation of the serine/threonine protein kinase AKT-1(T308). The peptides by themselves enhanced the phosphorylation of AKT-1 (T308), compared to unstimulated cells. Protein kinase B, also known as AKT, is a family of serine/ threonine kinases (AKT-1, AKT-2 and AKT-3) which are known to play important roles in the regulation of many cellular

processes, such as cell migration, survival, proliferation and differentiation, actin remodeling and cytokine productions. The importance of AKT-1 in the regulation of inflammatory responses was demonstrated in a study which showed that AKT-1 knockout macrophages have a pro-inflammatory M1 phenotype, and are hyper-sensitive to LPS stimulation [343]. Recent studies showed that phosphorylation of AKT-1(T308) plays a role in suppressing LPS-induced downstream NFκB signaling cascades, resulting in suppressing inflammatory cytokine productions and enhancing anti-inflammatory cytokine production [344-348]. Consistent with these studies, Mookherjee et. al. showed that the peptide LL-37 suppressed the nuclear translocation of NFκB subunits p50 and p65 by >50%, and consequently suppressed downstream TNF-α production, in the presence of TLR4 agonists [166]. Taken together, the results from my studies suggest that the regulatory mechanisms of the peptide LL-37 and IG-19 involves the signaling pathway mediated by the phosphorylation of AKT (T308), and this may in part intervene in the nuclear translocation of NFκB subunits (discussed further below).

In this study, I have also assessed the effects of the peptides on IL-32γ-mediated phosphorylation of mitogen kinase phosphatase-1 (MKP-1). I have shown that the peptides LL-37 and IG-19 did not suppress IL-32γ-mediated phopshorylation of MKP-1 (S359). Moreover, phospho-MKP-1 (S359) is significantly enhanced in response to the peptides alone (Figure 14). MKP-1 is also known as dual specificity phosphatase 1, it is a negative regulatory kinase for downstream inflammatory responses. MKP-1 is encoded by *dusp1*, an immediate early gene, which was reported to be induced by p44/42 MAPK (also known as ERK1/2) and JNK-1 MAPK [349, 350]. In addition, p44/42MAPK has been shown to induce the phosphorylation at S359 site on MKP-1, which stabilizes the MKP-1 protein and prevents proteosomal degradation [326, 351, 352]. In turn, the phosphorylated MKP-1 (S359) acts as a critical negative feed-back mechanism, which

dephosphorylates and inactivates p44/42 MAPK, JNK, p38 MAPK and NFκB, resulting in the suppression of downstream pro-inflammatory responses [327-329, 353-355]. *In-vivo* knockout MKP-1 mice model showed enhanced inflammatory responses to pathogen stimulations, and accelerated disease progression in a rheumatoid arthritis mouse model [327, 356, 357]. These studies clearly define the regulatory or anti-inflammatory role of the protein MKP-1, which is mediated by the phosphorylation at S359. As my results demonstrate that the peptides LL-37 and IG-19 significantly enhance the phosphorylation of MKP-1 (S359), it suggests that MKP-1 may play a critical role in the anti-inflammatory mechanisms of LL-37 and IG-19. To elucidate this further, we used a bioinformatics approach and identified several proteins in critical pathways (p44/42 MAPK, JNK, p38 MAPK and NFκB) that are known to interact with MKP-1, therefore I assessed the effects of the peptides on these signaling pathways.

My results showed that both LL-37 and IG-19-mediated the phosphorylation and activation of p44/42 MAPK, and did not suppress IL-32γ-mediated phosphorylation and activation of p44/42 MAPK. Therefore, I have clearly demonstrated that the peptides LL-37 and IG-19 can mediate the activity of the upstream kinase p44/42 MAPK, which in turn induces the phosphorylation of MKP-1 (S359), a dual phosphatase known to mediate anti-inflammatory mechanisms. A previous study by Mookherjee et al had demonstrated using microarray analyses that LL-37 can induce the expression of *dusp-1*, the gene encoding MKP-1. Therefore, I also assessed *dusp-1* mRNA expression and showed that both the peptides LL-37 and IG-19 enhance the mRNA expression of dusp-1 (appended Supplementary Figure 4). Taken together, these studies suggested that MKP-1 is a critical mediator of the anti-inflammatory functions of the HDP LL-37, and its synthetic derivative peptide IG-19.

Interestingly, when I assessed the effects of the peptides LL-37 and IG-19 on JNK and NFκB pathways, I observed differences between the effects of the two peptides on these signaling pathways. I showed that the peptide LL-37, but not IG-19, significantly enhances the phosphorylation of JNK and IKKα/β compared to unstimulated cells (Figure 17 and Figure 18). IKKα/β is a part of the IκB kinase (IKK) complex that requires phosphorylation to mediate nuclear translocation of specific NFκB subunits and downstream signaling. As JNK and NFκB signaling are known to mediate downstream pro-inflammatory responses, my results suggest that the synthetic peptide IG-19 suppresses IL-32γ-induced inflammatory responses, without exhibiting significant pro-inflammatory functions similar to the parental peptide LL-37 [314, 358]. As discussed above it has been previously shown that LL-37 can result in nuclear translocation of specific NFkB subunits, which is consistent with my data that shows that IKKα/β phosphoryaltion is enhanced in response to LL-37 [165].

In addition, I have also shown that both of the peptides LL-37 and IG-19 mediated phosphorylation of p38 MAPK. This is consistent with several studies that have also demonstrated LL-37-mediated phosphorylation of p38 MAPK in monocytes [162]. It was suggested that LL-37-mediated phosphorylation of p38 MAPK plays important roles in the secretion of chemokines IL-8, MCP-1 in human monocytes [46, 359]. This correlates with my results, showing that both peptides LL-37 and IG-19 alone induced the production of chemokines IL-8, MCP-1 and GRO-α, and that the peptides did not suppress IL-32γ-induced chemokines (Figure 12).

Taken together, my results shown that synthetic peptide IG-19 can activate the signaling pathways mediated by MKP-1 resulting in anti-inflammatory mechanisms, similar to LL-37. However, IG-19 does not significantly enhance the activity of pro-inflammatory pathways such

as JNK and NFkB, which is in contrast to LL-37. Consistent with this, the peptide IG-19-induced production of inflammatory chemokines is quantitatively less compared to that in response to LL-37. These results taken together with my observations that the peptide IG-19 is relatively less cytotoxic compared to LL-37 (supplementary data), I speculate that the peptide IG-19 may be a better anti-inflammatory peptide candidate compared to LL-37, for treating chronic inflammatory diseases, such as RA. Therefore, I further assessed the effects of IG-19 using a murine model of collagen-induced arthritis (CIA) as discussed in the next chapter.

CHAPTER 6 – HUMAN CATHELICIDIN LL-37 DERIVED PEPTIDE IG-19 CONFERS PROTECTION IN A MURINE MODEL OF COLLAGEN-INDUCED ARTHRITIS

6 – 1 Introduction

6 – 1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by persistent inflammation affecting tissues found locally at the joints (such as joint synovial tissues), as well as tissues located on other internal organs (such as the heart, blood vessels, and kidneys). Prolong and persistent inflammation leads to progressive destruction of cartilage, bones and ligaments, resulting in disability, decreased quality of life and premature mortality related to other comorbidities (such as cardiovascular diseases) [130, 201, 360-365]. In developed countries, RA affects approximately 0.5 % - 1.0 % of the population worldwide, and poses major economical and health burdens [365, 366]. RA affect individuals at any age, with females three times more susceptible to the disease than males [365]. The pathologic process in RA is still not well understood, but it is composed of both cellular and humoral immune responses, and involves both innate and adaptive immunity. McInnes and Schett have summarized several key features of the developmental stages of RA as shown in Figure 20 [367]. Although the cause of RA is still unclear, both genetic (such as polymorphism in the PTPN22 gene) and environmental factors (such as smoking or microbial infection) have been suggested to contribute to the development of RA [365, 367-370]. It was found that rheumatoid factor and anti-citrullinated peptide antibodies (ACPAs) are detectable years before the development of clinical signs of inflammatory arthritis, (such as joint swelling, pain, and stiffness), and this period is known as the pre-clinical or pre-articular stage [367, 371]. Unknown triggers further induce inflammation, which plays key roles in mediating the pathogenesis of RA and contributing to comorbidity.

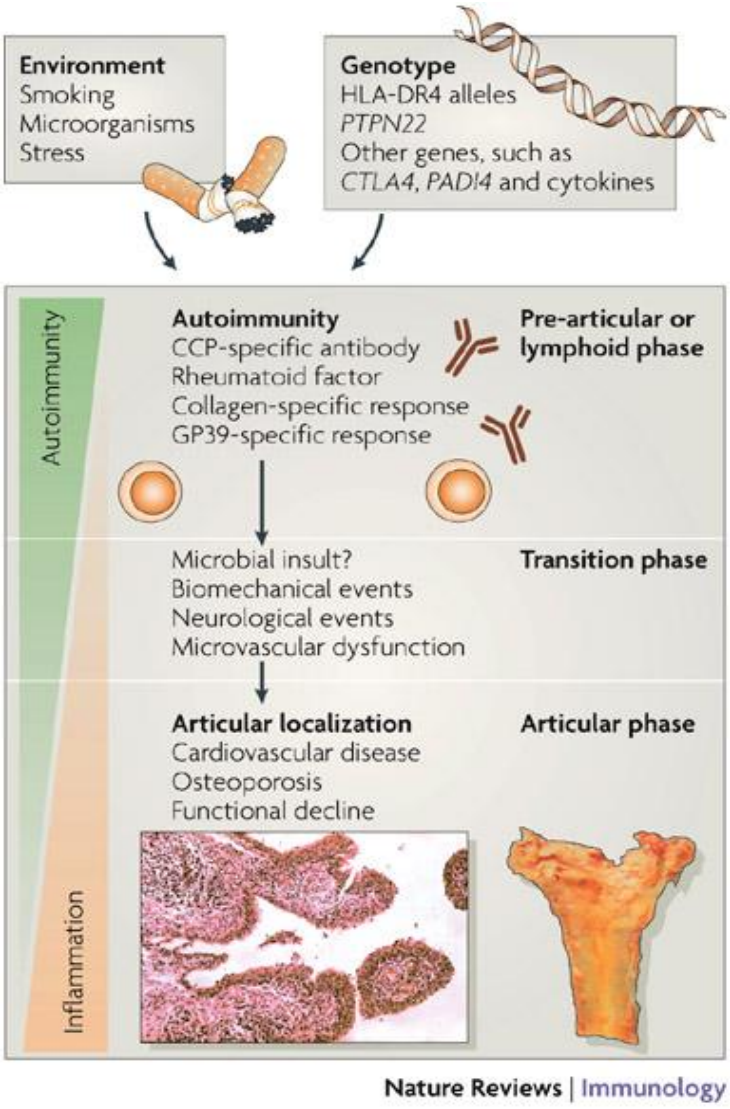


Figure 20. A contextual framework for the pathogenesis of rheumatoid arthritis.
Autoimmune processes are predicted to occur up to years prior to the clinical onset of disease and represent a pre-articular or lymphoid phase of disease. Transition to the articular phase, which corresponds to the clinical manifestation of the disease, is initiated by ill-defined processes, such as biomechanical and neurological events. Thereafter inflammation-driven pathogenesis occurs, which leads to joint destruction and increases co-morbidity, including cardiovascular disease and osteoporosis. Autoimmune processes may defer to inflammatory pathways as the disease progresses. CCP, cyclic citrullinated peptide; CTLA4, cytotoxic T-lymphocyte antigen 4; GP39, cartilage glycoprotein 39; PADI4, peptidyl arginine deiminase, type IV; PTPN22, protein tyrosine phosphatase, non-receptor type 22. Figure is from McInnes et. al., Nature Review Immunology, 2007. 7(6): 429 – 42 [367].

Persistent inflammation is a key factor which leads to joint swelling, pain, stiffness, bone loss and eventually lead to joint deformation and loss of function in RA [201, 372]. Pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-32, are some of the essential elements responsible in sustaining and prolonging inflammation and contributing to the destruction of joints [130, 201, 365, 373]. Pro-inflammatory cytokines contributes to inflammation by enhancing infiltration of immune cells to local joints (forming synovial pannus), inducing differentiation and activation of osteoclasts, and mediate the secretion of proteolytic enzymes (such as matrix metalloproteinases [MMPs]), resulting in cartilage degradation and eventually bone destruction and loss of function in arthritis (

Figure 21) [130, 201, 365, 373]. It was shown that IL-32 contributes to the persistency of inflammation by strongly inducing the production of other pro-inflammatory cytokines (such as TNF-α, IL-1β and IL-6) and chemokines via mitogen activated protein kinases [229, 261]. Pro-inflammatory cytokines (such as TNF-α, IL-1β and IL-6) also have the ability to induce the production of IL-32, leading to reciprocal induction and resulting in escalating and persisting inflammation [229, 261]. Current therapies for RA, such as disease-modifying anti-rheumatic drugs (DMARDs) and TNF inhibitors, aim to suppress inflammation by targeting the production of pro-inflammatory cytokines (such as TNF-α and IL-1β) involved in the acute phase of inflammation. However, complete suppression of pro-inflammatory cytokines leads to enhance susceptibility to infections. Therefore, there is a need to develop alternative therapies for RA which can control cytokine-mediated inflammation, without compromising immune functions required to fight infections. We proposed that cathelicidin-derived synthetic peptides have the potential to selectively suppress inflammation without compromising the ability to fight infections.

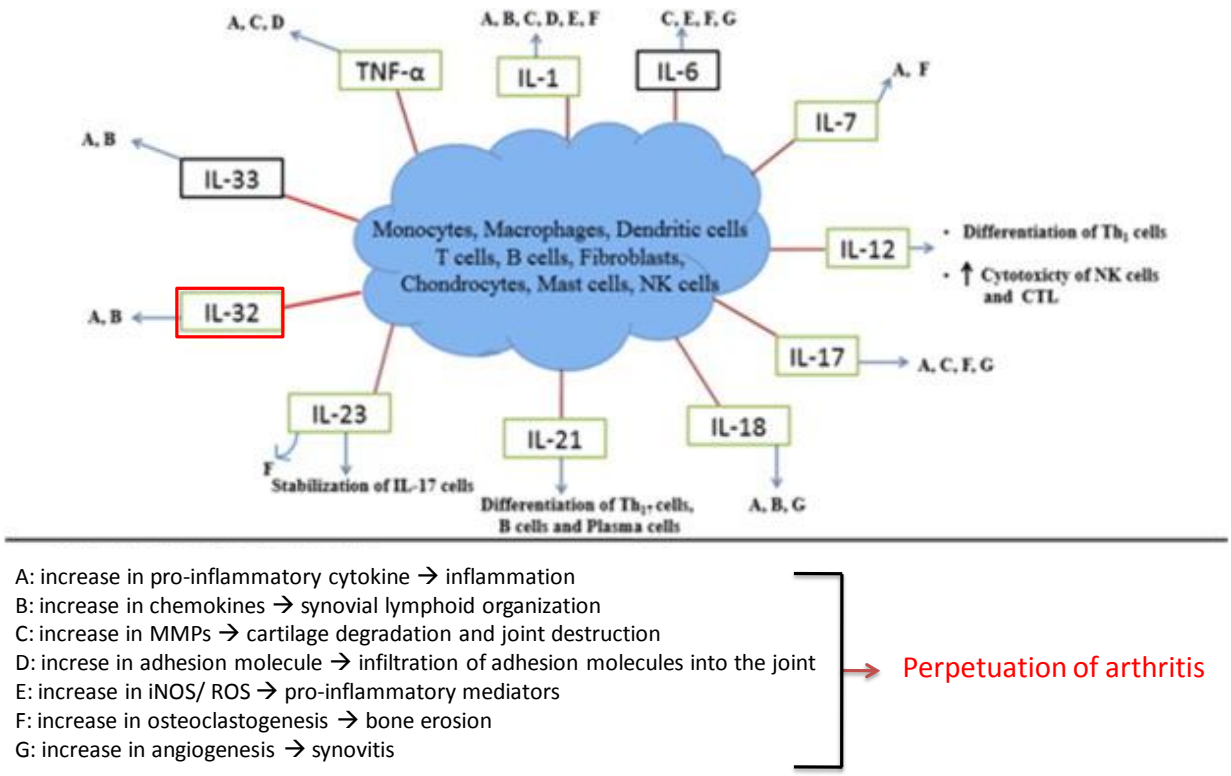


Figure 21. **Role of pro-inflammatory cytokines in the pathogenesis of rheumatoid arthritis.** Cytokines may be pleiotropic, redundant or ambivalent which operates in a co-ordinated manner leading to the inflammation and joint destruction, characteristic of RA. Green box represents pro-inflammatory cytokines while black box represents ambivalent cytokines whose pro-inflammatory functions are shown due to relative simplicity. Figure is modified from Mateen et. al., Clinica Chimica Acta, 2016. 455: 161-71 [373].

Several studies have previously reported the elevated expression of LL-37 in RA patients [200, 201, 374]. Hoffman et. al. demonstrated that the expression of LL-37 was increased locally in the synovial membranes in early RA and long-term RA patients, compared to healthy subjects [201]. However, the role of LL-37 in RA is not yet understood. Our results showed that LL-37 and its synthetic derivative peptide IG-19 have the potential to selectively suppress cytokine-mediated inflammation, without compromising essential mechanisms to fight infections (such as

productions of chemokines which recruit immune cells). Therefore, in collaboration with postdoctoral fellow Dr. Leola Chow, we have evaluated the effects of the peptide IG-19 in a murine model of collagen-induced arthritis (CIA).

6 – 1.1 Collagen-induced arthritis (CIA) mouse model

Collagen-induced arthritis (CIA) in DBA/1J mice is one of the most commonly used animal models to study RA. Type II collagen (CII), a major component of the cartilage, was identified as one of the potential candidate antigens for RA, as both anti-type II collagen antibodies and CII-specific T-cells have been detected in RA patients [375]. The development of CIA involves immunization of male DBA/1J mice with bovine or chicken CII in complete Freund's adjuvant (CFA), followed by a booster injection of CII in incomplete Freund adjuvant on 21 days later [249, 250]. The use of bovine or chicken CII and adjuvant solution results in a breach of tolerance and allows the generation of auto-antibodies toward self collagen. Signs of arthritis with clinical, histological and immunological features similar to human RA typically develop 35 to 42 days after the initial injection (Table V) in the CIA mice [250, 376-379].

Table V. Similarities and differences between human rheumatoid arthritis and collagen-induced arthritis in DBA/1 mice.

Similarities between human RA and murine CIA model

- Polyarticular
- Synovitis (inflammation of synovial membrane)
- Cartilage and bone erosion
- Inflammatory cells infiltration
- Pannus formation
- Production of pro-inflammatory cytokines, RF, anti-CII antibodies and ACPA
- Predisposition to arthritis is associated with certain MHC-II haplotypes

Differences between human RA and murine CIA model

Human RA	Murine CIA model
- Symmetric joint affected	- Asymmetric joint affected
- Exacerbation and remission stages	- Exacerbation stage only
- Greater incidence in females	- Greater incidence in males

Similar to human, susceptibility to arthritis is associated with the expression of specific class II histocompatibility complex molecule (MHC-II), which is referred as H-2 complex, in mice [380, 381]. It was reported that DBA/1J mice expressing H-2^q haplotype have the highest susceptibility for CIA [381]. In addition, the development of CIA share several pathogenic events similar to those found in RA patients in both immune-mediated inflammatory phase and destructive phase; infiltration by immune cells (e.g. macrophages, lymphocytes and neutrophils) into the joint, formation of pannus, and increased production of pro-inflammatory cytokines (such as TNF-α and IL-1β), rheumatoid factor, anti-CII antibodies, and anti-citrullinated peptide antibodies (Table V) [378, 379, 382, 383]. However, there are also some limitations and pathological differences between the murine model of CIA and human RA (Table V). For example, CIA is a relatively acute model, which lacks remission and exacerbation phases as seen in RA patients. Arthritic incidence often occurs in asymmetric joints in the CIA mice, whereas in RA patients it is often symmetrical. Furthermore in contrast to human RA, greater severity and incidence of arthritis occur in males compared to females in the CIA animal model.

6 – 2 Hypothesis and Rationale

Currently available therapies for RA include corticosteroids, disease-modifying anti-rheumatic drugs (DMARDs) and inhibitors of inflammatory cytokines (such as TNF-α), aimed to suppress inflammation and slow down the progression of the disease. However, despite the effectiveness in controlling the progression of the RA, patients on these therapies often suffer from compromised immune system and have an increased risk of infection [230, 253, 384]. Hence, there is a need to identify alternative therapies which can suppress cytokine-mediated pro-inflammatory responses, without compromising the necessary immune functions.

My results showed that the synthetic derivative peptide IG-19 selectively suppressed cytokine IL-32γ-induced pro-inflammatory responses. In addition, peptide IG-19 mediated a reduced amount of chemokine production and did not exhibit pro-inflammatory functions similar to the parental LL-37. Therefore, I hypothesized that IG-19 will be able to reduce disease severity in the mouse model of CIA by suppressing inflammatory responses, such as pro-inflammatory cytokine production and antibodies against CII.

6 – 3 Results

This work was performed in collaboration with Dr. Leola Chow, former Postdoctoral Fellow in the Mookherjee lab, and is published in Chow et. al., Molecular Immunology. 2014. 57(2): 86 – 92 [385]. I am a joint first author in this publication with Dr. Chow.

6 – 3.1 Peptide IG-19 reduced disease severity in the CIA mice

As described in "Materials and methods", disease progression was monitored in a blinded manner every 48 h by caliper measurements of joint thickness for each limb after day 20, and disease severity was monitored by assigning a clinical score to each paw (shown in table III, as previously described by Galligan et. al.) on the day of sacrifice [252]. The final clinical score reported was the sum of the scores from all four paws of each mouse, with a maximum score of 16 representing the most severe form of CIA.

Table III. Clinical scores

Clinical score (per paw)	Signs/ Symptoms
0	Normal, no signs of swelling
1	Mild swelling confined to the joint
2	Mild swelling extending from ankle to the tarsals
3	Moderate swelling extending from ankle to metatarsal joints
4	Severe swelling encompass the ankle, foot and digits, or ankylosis of the limb

As expected, administration of CII elicited a high incidence of arthritis in DBA/1J mice, with disease severity represented by a median clinical score of four (Figure 22B). In contrast, only two out of seven mice developed some arthritic symptoms in the CIA + IG-19 group, with delayed kinetics (Figure 22A). In addition, the severity of arthritic symptoms in CIA mice with peptide IG-19 was significantly lower than CIA mice, as indicated by a median clinical score of zero (Figure 22B). These results suggested that the peptide IG-19 delayed the progression of the disease and reduced the disease severity of arthritis.

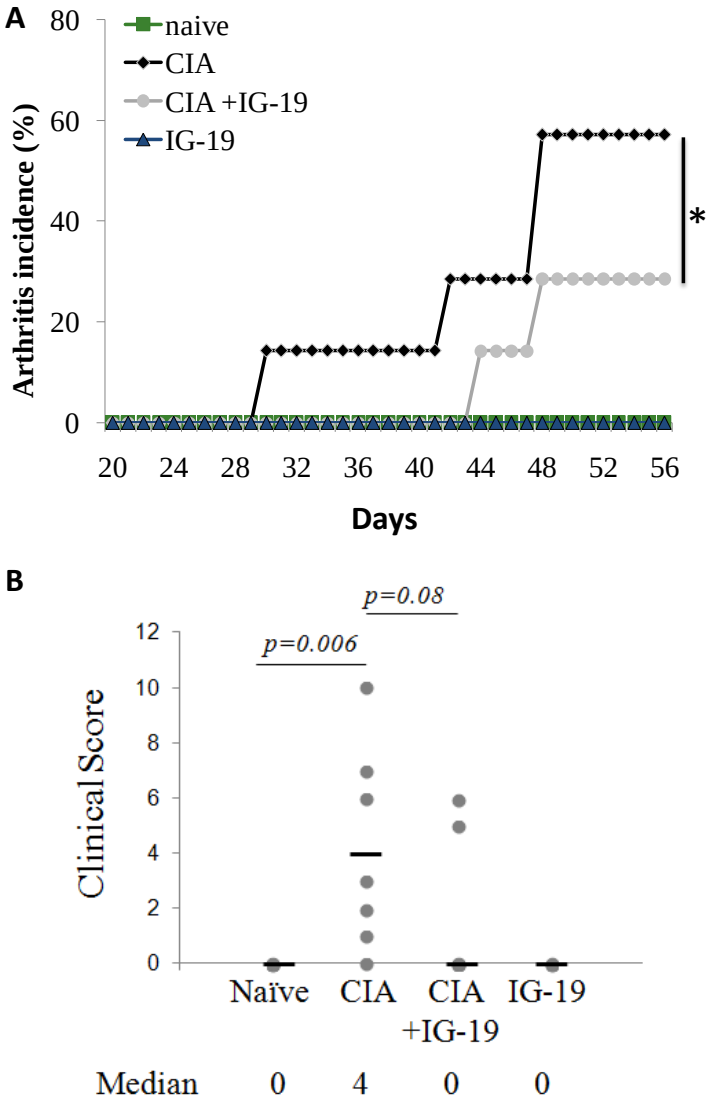


Figure 22. Peptide IG-19 delayed disease progression and reduced the disease severity in the CIA mice.

DBA/1 mice were challenged with type II chicken collagen, and the peptides IG-19 was administered by i.d. injections from day 20 onwards every 48 h. Mice were sacrificed 8 weeks after initial collagen challenge. (A) Percentage of arthritis incident was assessed based on the progression of affected limbs from day 20 onwards. (B) Disease severity was monitored using a clinical score (shown in table III) as described by Galligan et al [252]. A clinical score, range 0 – 16, is assigned to each mouse by summing the scores of each paw in a blinded manner. Statistical significance for the trend of the number of affected limbs over time was analyzed using the Tukey HSD test (*$p < 0.05$). Figure is modified from Chow et. al., Molecular Immunology. 2014. 57(2): 86 – 92 [385].

6 – 3.2 Peptide IG-19 significantly reduced the production of anti-collagen type II (CII) antibodies in CIA mice

Type II collagen is known to be expressed exclusively in the articular cartilage of the joint and is one of the major autoantigens of RA [379]. In the CIA mouse model, immunizing DBA/1J mice with CII in the presence of complete Freund's adjuvant elicit signs and symptoms similar to those found in RA patients (Table V) [249]. It was shown that CII-immunized DBA/1J mice have increased production of CII-specific antibodies when compared to naïve mice [249]. The quantity of CII-specific antibody production directly correlates with the development of RA. Therefore, we monitored the serum concentration of antibody against CII in this study to evaluate the autoimmune response to CII. We observed that DBA/1J mice challenged with type II chicken collagen have significantly higher amount of anti-mouse type II collagen antibodies when compared to naive mice (Figure 23). Administration of the peptide IG-19 to CIA mice significantly suppressed the production of anti-mouse CII antibodies by ~70% (p-value=0.006), and the peptide IG-19 alone did not elicit the production of anti-mouse CII antibodies (Figure 23). These results suggested that administration of IG-19 suppressed the production of anti-CII autoantibodies and subsequent development of arthritis in the CIA mice. We further monitored the effect of IG-19 on the production of cytokines in the CIA mice.

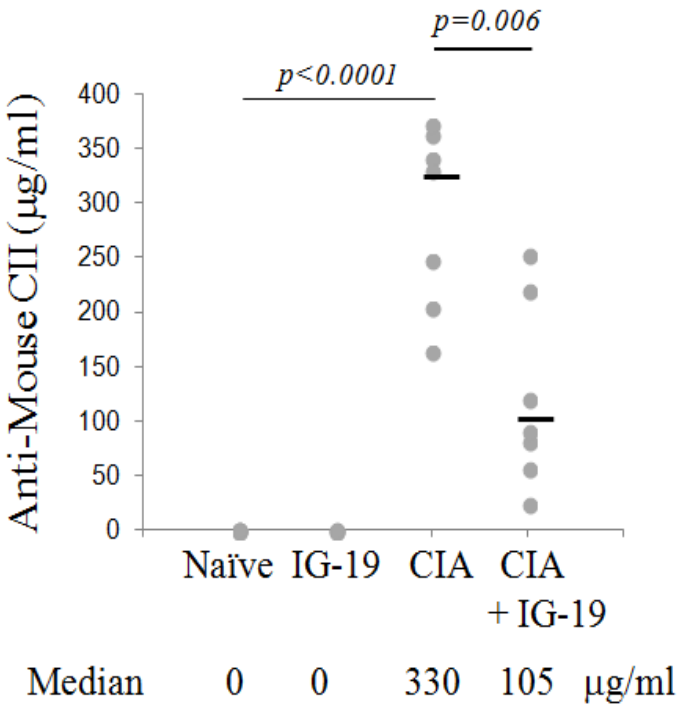


Figure 23. Peptide IG-19 significantly reduced serum concentration of antibody against type II collagen in CIA mice.
DBA/1J mice were challenged with type II chicken collagen, and the peptides IG-19 was administered by i.d. injections from day 20 onwards every 48 h. Mice were sacrificed 8 weeks after initial collagen challenge. The concentrations of anti-mouse collagen type II antibodies were monitored in the serum obtained on the day of sacrifice by ELISA. Independent *t*-tests for unequal variance were used to determine p-values. Figure is from Chow et. al., Molecular Immunology. 2014. 57(2): 86 – 92 [385].

6 – 3.3 Peptide IG-19 altered serum cytokines in CIA mice

Alteration and imbalance of cytokine production plays major roles in the development of RA, as previously described in Figure 21 [373]. Therefore, we monitored the serum concentration of cytokines on the day of sacrifice using a multiplex flow cytometry. Administration of peptide IG-19 alone did not mediate a statistically significant increase of cytokines IFN-γ, TNF-α, IL-10, IL-6, IL-17A, IL-2 or IL-4. However, we observed that peptide IG-19 mediated a ~1.7-fold increase in IL-10 production (Figure 24). There was significant increase in the serum

concentration of IFN-γ, TNF-α and IL-6, in the CIA mice compared to the naive mice on the day of sacrifice (Figure 24). Administration of the peptide IG-19 in CIA mice significantly decreased the serum concentration of TNF-α by more than 60%, and IL-10 by about 50%, when compared to the CIA mice. Although not statistically significant (p = 0.07), administration of IG-19 also reduced serum concentration of IFN-γ by 40% in CIA mice. These results showed that administration of the peptide IG-19 altered the production of important cytokines (TNF-α, IL-10 and IFN-γ) known to contribute to be involved in the disease pathogenesis and the development of RA.

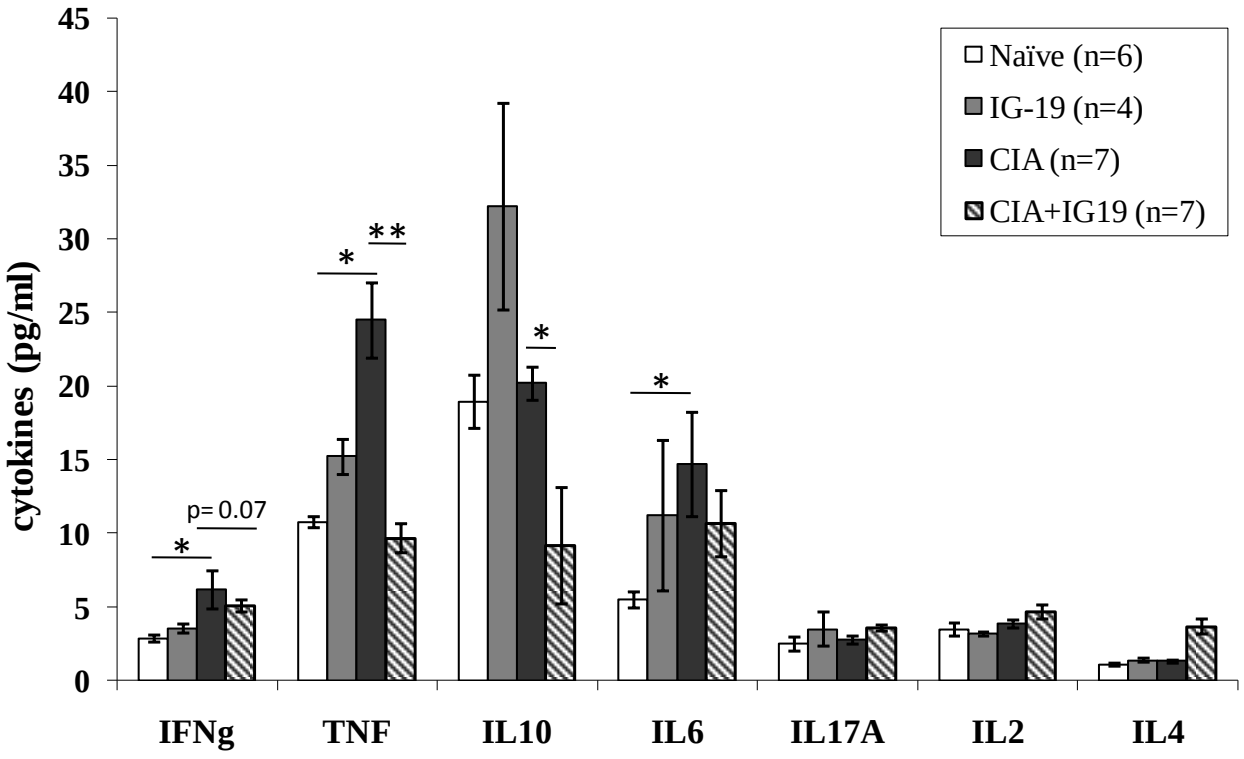


Figure 24. Peptide IG-19 alters serum concentrations of cytokines in CIA mice.
DBA/1 mice were challenged with type II chicken collagen, and the peptides IG-19 was administered by i.d. injections from day 20 onwards every 48 h. Mice were sacrificed 8 weeks after initial collagen challenge. Circulating levels of a panel of Th1/Th2/Th17 cytokines (IL-2, IL-4, IL-6, IFN-γ, TNF-α, IL-17A, IL-10) were monitored in the serum collected on the day of sacrifice using multiplex BD™ CBA preconfigured kit employing the FACS Calibur™ flow

cytometer. Results shown represent average ± standard error. Independent t-tests for unequal variance was used to determine the p-values (* $p < 0.05$, ** $p < 0.005$). Figure is from Chow et. al., Molecular Immunology. 2014. 57(2): 86 – 92 [385].

6 – 3.4 Peptide IG-19 significantly reduced cellular infiltration and cartilage degradation in the joints of CIA mice

We showed that the administration of peptide IG-19 delayed the progression of arthritis and reduced disease severity in CIA mice (Figure 22). We further monitored the histological features of the articular joints, such as cellular infiltration and cartilage degradation, on the day of sacrifices. Hematoxylin and eosin stain (H & E stain) was used to monitor cellular infiltration, where hematoxylin solution resulted in the nuclei of cells stained blue, while eosin Y (a counter-stain) colored the cytoplasm of cells, cartilage matrix, bone and extracellular proteins bright pink [386]. In our study, we observed that there was significant increase in the amount of infiltrating cells in CIA mice, when compared to naïve mice (Figure 25A). Administration of IG-19 to CIA mice reduced the amount of infiltrating cells into the joint (Figure 25A). Administration of IG-19 alone did not mediate significant amount of cellular infiltration. Cartilage degradation was monitored using Safranin O stain, which stains proteoglycan (a major component of cartilage) orange to red, nuclei of cells black, and cytoplasm or underlying bone grey or green [386]. We observed that CIA mice showed significant amount of cartilage erosion and pannus formation compared to the naïve mice (Figure 25B). CIA mice with the most severe signs of arthritis (with a clinical score of 6) showed complete loss of joint integrity. CIA mice treated with the peptide IG-19 was protected from cartilage erosion, even in the mouse with the most severe signs of arthritis (with a clinical score of 6). Exogenous administration of the peptide IG-19 prevented cartilage erosion in the CIA mice, and the peptide alone did not mediate cartilage erosion (Figure 25B). Histology sections were assigned a score, in a blinded manner, using a standardized

histology score to account for cumulative evaluate cellular infiltration and tissue damage, and biological variability (Table IV). Administration of the peptide IG-19 significantly decreased the histology score compared to the CIA mice (Figure 25C). These results suggested administration of peptide IG-19 provided protection against cartilage degradation, and reduced cellular infiltration in the CIA mice.

Table IV. Histology scores

Histology Score	Signs/ Symptoms
0	Normal synovium
1	Synovial membrane hypertrophy and cell infiltration
2	Pannus formation and cartilage erosion
3	Major erosion of the cartilage
4	Loss of joint integrity

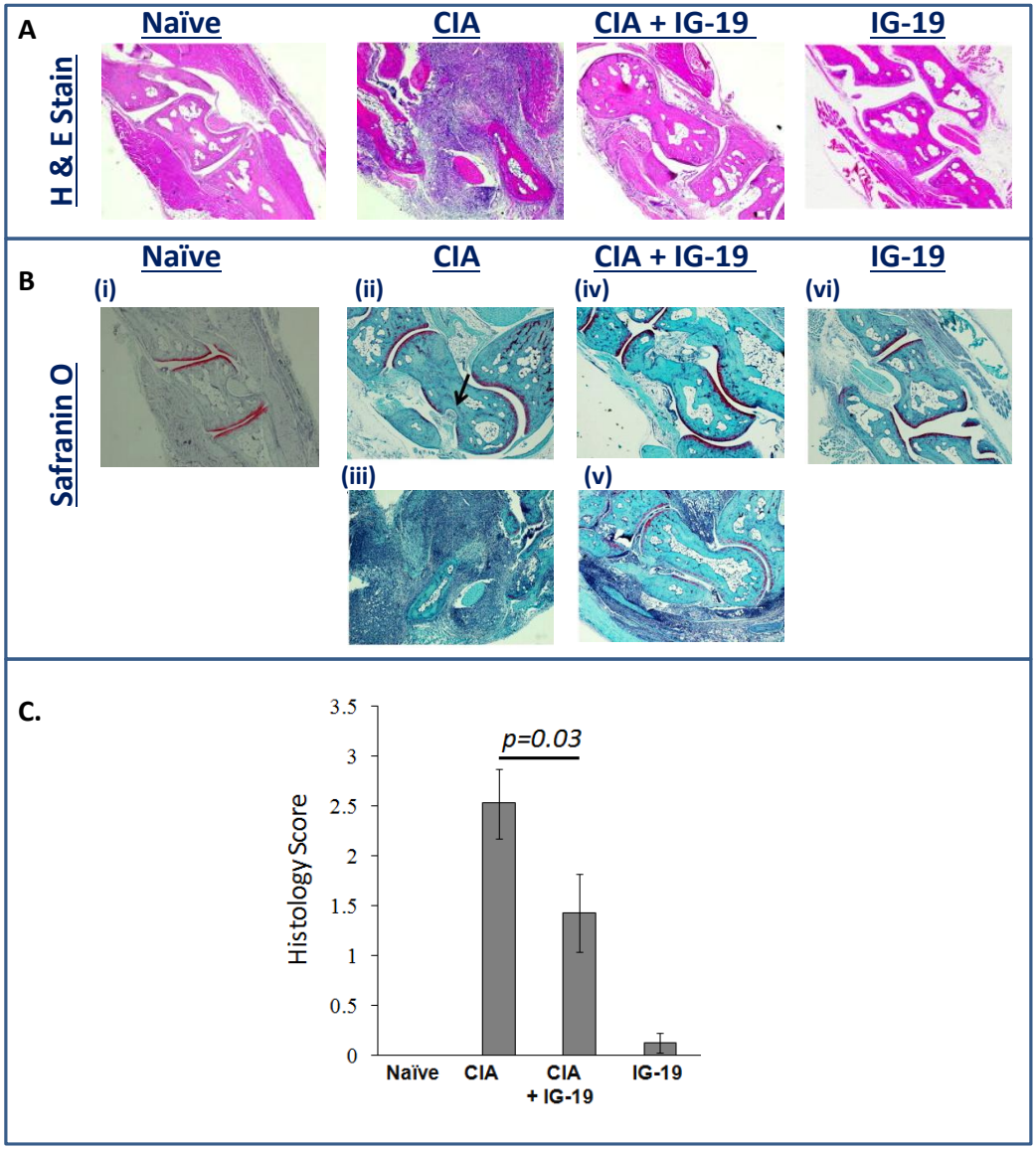


Figure 25. Peptide IG-19 significantly reduced cellular infiltration and cartilage degradation in the joints of CIA mice.

(A) Cellular infiltration of the joint was monitored by H & E stains. Paraffin embedded sagittal sections (5 µm) of hind ankle joints obtained on the day of sacrifice were stained with H & E. Images shown are representative of sections from naïve (***n*** = 7), CIA (***n*** = 7), CIA + IG-19 (***n*** = 7) and IG-19 (***n*** = 4). (B) Proteoglycan depletion, an indication for cartilage destruction, in the joints was monitored by Safranin O stain. Paraffin embedded sagittal sections (5 µm) of hind ankle joints obtained on the day of sacrifice were stained with Safranin O. Images shown are representative of sections from (i) naïve, ***n*** = 7, (ii and iii) CIA, ***n*** = 7, (iv and v) CIA + IG-19, ***n*** = 7, and (vi) IG-19, ***n*** = 4. Pannus formation is indicated by an arrow in (ii). The sections shown in (iii) and (v) are both from mouse with a clinical score of 6. The images were processed using a Zeiss imager M2 using the Zen 2011 software. (C) The stained sections from (A) and (B) were scored in a blinded manner, using a standardized histology score as indicated in Table IV (previously published by Sun et al. and Nishikawa et al. [254, 255]), to assess the extent of cell infiltrates and tissue damage. Independent ***t***-tests for unequal variance were used to determine the *p*-values. Figure is from Chow et. al., Molecular Immunology. 2014. 57(2): 86 – 92 [385].

6 – 4 Discussion

In this study, we investigated the effect of the human cathelicidin LL-37-derived peptide IG-19 in the development of inflammatory arthritis using the CIA murine model. The CIA model was chosen due to several important pathological similarities shared with human RA, which include: the development of synovitis and pannus, cartilage and bone erosion, infiltration of immune cells, and increased production of pro-inflammatory cytokines, rheumatoid factors, antibodies against type II collagen and ACPA (Table V). Although there are a few caveats, the CIA model was previously optimized in our lab and was readily available to be used.

Using the CIA model, we demonstrated that the administration of the peptide IG-19 delayed disease progression and reduced disease severity in CIA mice (Figure 22). Disease progression was monitored by the percentage of affected limbs over time, while disease severity was indicated by a standardized clinical score based on the degree of joint inflammation [252]. We showed that administration of the peptide IG-19 in CIA mice significantly reduced the production of antibodies against mouse type II collagen (Figure 23) and pro-inflammatory cytokine TNF-α (Figure 24). In addition, the peptide IG-19 moderately suppressed serum IFN-γ in CIA mice, which is a pro-inflammatory cytokine known to play important role in the pathogenesis of arthritis (Figure 24) [378]. The levels of CII-specific antibodies and pro-inflammatory cytokines (TNF-α and IFN-γ) have been shown to directly correlate with the incidence and disease severity of CIA [20, 250, 387, 388]. The cytokine TNF-α is one of the main contributors to the pathogenesis of RA, by mediating the proliferation of lymphocytes and inducing the production of other pro-inflammatory cytokines and chemokines, which results in enhancing and prolonging inflammation [20, 334, 388]. In addition, TNF-α also upregulates the production of collagenase, MMPs and prostaglandins, which triggers bone reabsorption and

contributes to bone destruction [20, 334, 388]. The ability of IG-19 to significantly suppress the production of TNF-α suggested that the peptide can control the disease progression by reducing inflammatory responses. These results are consistent with previous studies that have demonstrated that the anti-inflammatory effects of the parental peptide LL-37 is also primarily by suppressing the production of TNF-α in various models of infections [47, 160, 166, 180]. Also, a previous study demonstrated that the peptide IG-19 suppresses the production of endotoxin-induced pro-inflammatory cytokines in an *in-vitro* model of challenge with TLR ligands [101]. This is the first study to demonstrate that the peptide IG-19 can suppress TNF-α and IFN-γ production in an *in-vivo* model of arthritis.

We also showed that administration of the peptide IG-19 moderately induced the production of anti-inflammatory cytokine IL-10 (Figure 24). It is known that IL-10 is an anti-inflammatory cytokine which downregulates the activation of immune cells, suppresses the production of pro-inflammation cytokines (such TNF-α, IL-6, INF-γ and IL-1β), and inhibits the production of MMPs and prostaglandins, which results in the suppressing inflammation and preventing bone destruction [389-392]. The results in this study are consistent with previous studies that have demonstrated that LL-37 can induce the production of IL-10, however these previous studies were all in *in-vitro* models [165].

The protective effects of peptide IG-19 were further demonstrated in this study by histological assessment of the joints, where administration of IG-19 significantly reduced cellular infiltration and cartilage degradation in the CIA mice (Figure 25). Overall, our result suggested that the peptide IG-19 alleviates arthritic symptoms in CIA mice by suppressing inflammatory responses, which contributes to the prevention of cartilage degradation and bone erosion. This is the first study to demonstrate the anti-inflammatory effects of the peptide IG-19 *in-vivo* in a murine

model of inflammatory disease, thus establishing the proof-of-principal that the synthetic peptide IG-19 may be beneficial to control chronic inflammatory diseases. Our results suggested that the peptide IG-19 can be used as a potential therapeutic for chronic inflammatory diseases, such as RA, due to its ability to balance inflammation without suppressing the necessary immune functions against infections. The ability of cathelidin-derived peptides to control inflammation and infections makes them better therapeutic strategy compared to the current available therapies for chronic inflammatory diseases such as RA.

CHAPTER 7 – OVERALL CONCLUSIONS, SIGNIFICANCE AND FUTURE DIRECTIONS

7 – 1 Overall conclusions and significance

Inflammation is an essential host defence process, which is responsible to fight infections and resolve injuries. Dysregulation of inflammatory responses results in chronic inflammation and the development of chronic inflammatory diseases, such as RA, COPD and IBD. Many current therapies for chronic inflammatory diseases, such as RA, neutralize critical pro-inflammatory cytokines TNF-α and IL-1β that play important roles in inducing immune responses against infections [393, 394]. The suppression of these critical pro-inflammatory cytokines is associated with increase susceptibility to infections and the potential to develop cancers. There is also the predicament of patients with poor responses to available current therapies, and those who become unresponsive to the available biological therapies over time. This highlights the significance of finding alternate therapeutic strategies that can selectively control chronic inflammation without compromising normal immune functions to fight infections.

My study showed that the human cathelicidin peptide LL-37 and its synthetic derivative peptide IG-19 can selectively control cytokine IL-32γ-mediated inflammatory responses without neutralizing specific immune functions such as chemokine induction that is required to recruit leukocytes required to fight infections. I showed that the peptides LL-37 and IG-19 suppressed IL-32γ-induced production of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in both human PBMC and macrophages, by targeting the cytokine-induced downstream signaling cascade. In addition, the peptides by themselves were able to induce the production of critical anti-inflammatory cytokine IL-1RA in human PBMC and macrophages. These results suggested that both of these peptides exhibit anti-inflammatory responses in the presence of the pro-inflammatory cytokine IL-32γ. Both LL-37 and IG-19 induced the production of chemokines (IL-8, MCP-1 and GRO-α) in human PBMC, and did not neutralize IL-32γ-induced chemokines. This demonstrated that the peptides LL-37 and IG-19 does not dampen the recruitment of immune cells, which are required to fight infections and resolve inflammation. Overall, my *in-vitro* results suggested that the peptide LL-37 and its derived peptide IG-19 have the potential to control cytokine IL-32-mediated inflammatory responses, induce anti-inflammatory cytokines, and does not neutralize chemokine responses that are required to fight infections.

I further investigated the effects of the peptides LL-37 and IG-19 on IL-32γ-mediated protein phosphorylation events involved in regulating downstream inflammatory responses. My results showed that both peptides LL-37 and IG-19 suppressed IL-32γ-mediated phosphorylation of Fyn (Y420), which is a key pro-inflammatory mediator. In contrast, both the peptides mediated the phosphorylation of the dual phosphatase MKP-1 (S359), and did not suppress IL-32γ-mediated MKP-1 (S359). My results suggest that phospho-MKP-1 (S359) may be a critical mediator of the anti-inflammatory functions of LL-37 and IG-19. The significance of these results is that

phosphorylation of MKP-1 (S359) can be used further as a readout to develop and screen other immunomodulatory peptides or small molecules for the control of chronic inflammation, specifically for diseases where IL-32 is known to contribute to disease pathogenesis and severity.

In my further studies, I showed that there were differences between LL-37 and IG-19, specifically in the signaling cascades involving MAPK (p44/42 and JNK MAPK) and NFκB pathways. These results provided insights to the molecular mechanisms of the anti-inflammatory activity of these peptides. Both LL-37 and IG-19 mediated the phosphorylation of p44/42 MAPK, which is known to phosphorylate and stabilize MKP-1 (S359). However, the peptide LL-37, but not IG-19, mediated the phosphorylation of JNK MAPK and IKKα/β, which are known to be involved in downstream pro-inflammatory responses. These results were consistent with my findings that there were quantitative differences between LL-37 and IG-19-induced chemokine responses in PBMC and macrophages. LL-37-induced chemokine production was relatively higher than that compared to IG-19. These results suggested that IG-19 exhibits a 'balanced' inflammatory response, which is different from the peptide LL-37 which has both pro- and anti-inflammatory responses. However, this raises the question if the synthetic peptide IG-19 has the potential to resolve infections similar to LL-37. Previous studies have demonstrated that the synthetic derivative peptide IG-19 is less cytotoxic than LL-37, retains the antimicrobial actions, and exhibits similar immunomodulatory functions as LL-37 in the presence of endotoxin [59, 89, 97-99, 101, 243, 244]. Furthermore, IG-19 is a relatively smaller peptide (19 amino acid with a molecular weight at 2372.84 g/mol) when compared to the peptide LL-37 (37 amino acids with a molecular weight at 4493.33 g/mol), and therefore the cost of production will be less with IG-19 compared to LL-37. Altogether, these results suggested that compared to the natural peptide LL-37, the peptide IG-19 may be a more cost effective and

better candidate for treating chronic inflammatory diseases such as RA. Based on my thesis, I propose the model that the peptide IG-19 exhibit similar anti-inflammatory functions, but does not mediate pro-inflammatory functions similar to LL-37 (Figure 26).

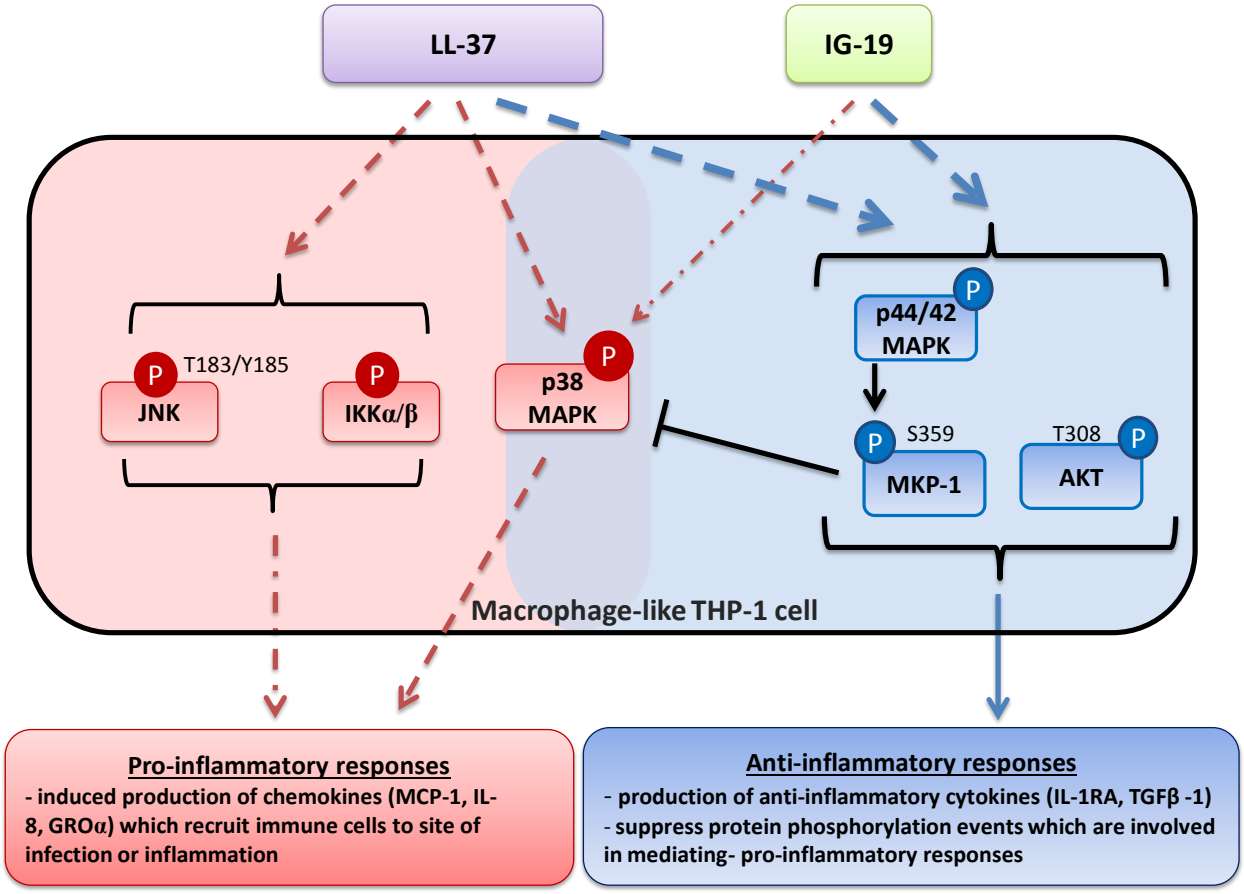


Figure 26. Differential protein phosphorylation mediated by the peptides LL-37 and IG-19. Human cathelicidin peptide LL-37 mediated phosphorylation on protein targets involved in both pro-inflammatory responses (shown in red) and anti-inflammatory responses (shown in blue) in human macrophage-like THP-1 cells. Peptide IG-19 mediated phosphorylation on protein targets involved in anti-inflammatory responses (p44/42 MAPK, MKP-1 and AKT-1) similar to LL-37. Peptide IG-19 mediated phosphorylation of p38 MAPK, but did not mediate phosphorylation of JNK and IKKα/β as peptide LL-37.

As cytokine-mediated inflammation is directly linked to the severity of chronic inflammatory diseases including rheumatoid arthritis (RA), we further investigated the effect of the peptide IG-19 *in-vivo* using a murine model of collagen-induced arthritis (CIA). We demonstrated that the

administration of the peptide IG-19 decreased disease severity, prevented infiltration of immune cells into the joints, decreased serum levels of anti-collagen antibodies and pro-inflammatory cytokines, and protected cartilage destruction in the CIA mice. These results demonstrated that the peptide IG-19 has the potential to regulate immune-mediated inflammation and control disease progression in a murine model of CIA. Overall, my studies provided a rationale to further examine the potential to use cathelicidin-derived peptides as therapeutic for chronic inflammatory diseases such as RA.

7 – 2 Future Directions

In order to further evaluate the potential of the peptide IG-19 as a therapy for cytokine-mediated chronic inflammatory diseases, the following questions remains to be addressed:

1. What are the receptors and direct interacting proteins of the peptide IG-19?

   To date, there is limited data on cellular targets of cathelicidin-derived peptide IG-19. I propose to employ metabolic labeling (SILAC) along with quantitative proteomics to identify the interacting protein targets for the peptide IG-19. The peptide will be synthesized with a carboxy-terminal biotin tag which permits the identification of interacting proteins using affinity tag pull-down followed by mass spectrometry analysis. Dr. Mookherjee has successfully used this experimental approach to identify an intracellular target GAPDH for the peptide LL-37 [335]. Once the interacting protein partners of IG-19 are identified, the direct interaction of these proteins with the peptide can be validated using biochemical techniques such as surface plasmon resonance (SPR). The results obtained from such approaches will provide molecular information of how the peptide IG-19 can selectively control inflammatory responses, without comprising the

ability to induce essential anti-infective functions, such as chemokine productions. In addition, the identified protein targets can be further used to screen libraries of small molecules for immunomodulatory properties similar to IG-19.

2. What are the effects of the peptides LL-37 and IG-19 on other cell types, such as regulatory T-cells (Tregs)?

   Based on my results, the peptides LL-37 and its synthetic derivative peptide IG-19-induced the production of anti-inflammatory cytokine TGF-β in human PBMC (Figure 11). The cytokine TGF-β is also important for the initiation and maintenance of Foxp3 expression, and suppressive functions of Tregs [395]. Regulatory T-cells plays essential roles in suppressing and regulating inflammation. My preliminary results showed that upon stimulation with peptides LL-37 or IG-19, the percentage of CD25+Foxp3+ Tregs within the CD4+ population significantly increased (Figure 27). This suggested that the peptides might induce the population of Tregs, which can contribute to suppress inflammatory responses. However, questions such as (i) whether the peptides induced the proliferation or differentiation, and (ii) whether these CD4+CD25+Foxp3+ Tregs process anti-inflammatory effects still remain to be addressed.

   (i) To establish whether the peptides mediate Tregs proliferation, I propose to isolate T-cells from the human PBMC population, and label them with carboxyfluorescein succinimidyl ester (CFSC). This is followed by stimulating the cells with 5μM peptides LL-37, IG-19 or sLL-37. Over the course of 72 h, cells are stained with antibodies against surface makers of Tregs, and cell proliferations will be monitored by flow cytometry. To determine whether the peptides mediate differentiation of

Tregs, naive T-cells will be isolated, followed by stimulation with the peptides (LL-37, IG-19 and sLL-37). Upon 72 h, cells will be collected and stained with antibodies against CD4, CD25, Foxp3, TIGIT and FcRL3 to determine whether the naive T-cells have differentiated into Tregs.

(ii) Recent studies from Dr. Ciriaco A. Piccirillo showed that some activated T-effector cells also express Foxp3 [396]. He showed that regulatory T cells can be distinguished from other T-effector cells via the expression of Helio, an lkaro family transcription factor. He showed that the Helios+ Tregs can be identified via the surface makers TIGIT and FcRL3 [396]. I propose to use the above mentioned makers (TIGIT and FcRL3) to identify whether the increased CD4+CD25+Foxp3+ Tregs in response to the peptides also exhibit the additional regulatory markers. In parallel, functional assays can be performed to determine whether the peptide-induced Tregs can suppress the proliferation of CD4+ T cells as mentioned by McMurchy et. al [397].

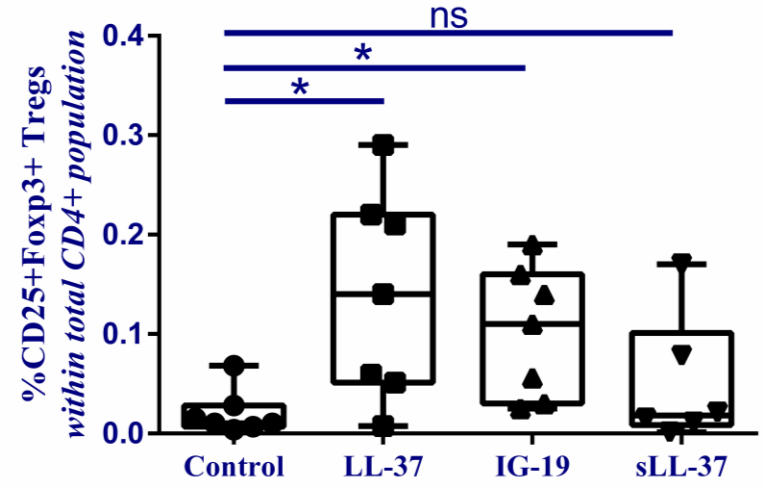


Figure 27. Peptides LL-37 and IG-19 increased the percentage of CD4+CD25+Foxp3+ regulatory T cells.
Human PBMC were stimulated with 5 μM peptides (LL-37, IG-19 or sLL-37). After 48 hr of incubation at 37°C, cells were stained with antibodies for specific surface marker CD4 (Brilliant Violet 510) and CD25 (Brilliant Violet 515), fixed and permeabilized, followed by staining with antibody against Foxp3 (APC). FACSCanto II cytometer and FACSDiva software were used to acquire cell samples. Data were analyzed using the FlowJo software. Results are shown in box and whisker plot, with each point representing an independent donor ($n \geq 6$). (* $p<0.05$).

3. What is the minimum effective dosage and the therapeutic window for using IG-19 as a therapy for chronic inflammatory diseases?

   My *in-vivo* data have established that the peptide IG-19 provided protection in a murine model of CIA when administrated one day before the boost. The murine model of CIA as described before in Figure 4 can be further used to establish the optimal dosage and therapeutic window of using IG-19 as a potential therapy for RA. A series of dose from 1.5. to 6 mg/Kg of IG-19 can be used to establish the minimum optimal concentration of the peptide that can provide protection against CIA. Further experiments can be performed by administrating the optimal concentration of the peptide IG-19 at various time points to establish optimal therapeutic window: Prophylactic (peptide IG-19 is administrated before immunization and every 48 h until the CII boost on day 21), Therapeutic (peptide IG-19 is administrated every 48 h on the onset of arthritic symptoms). These experiments are currently being performed in the Mookherjee Lab.

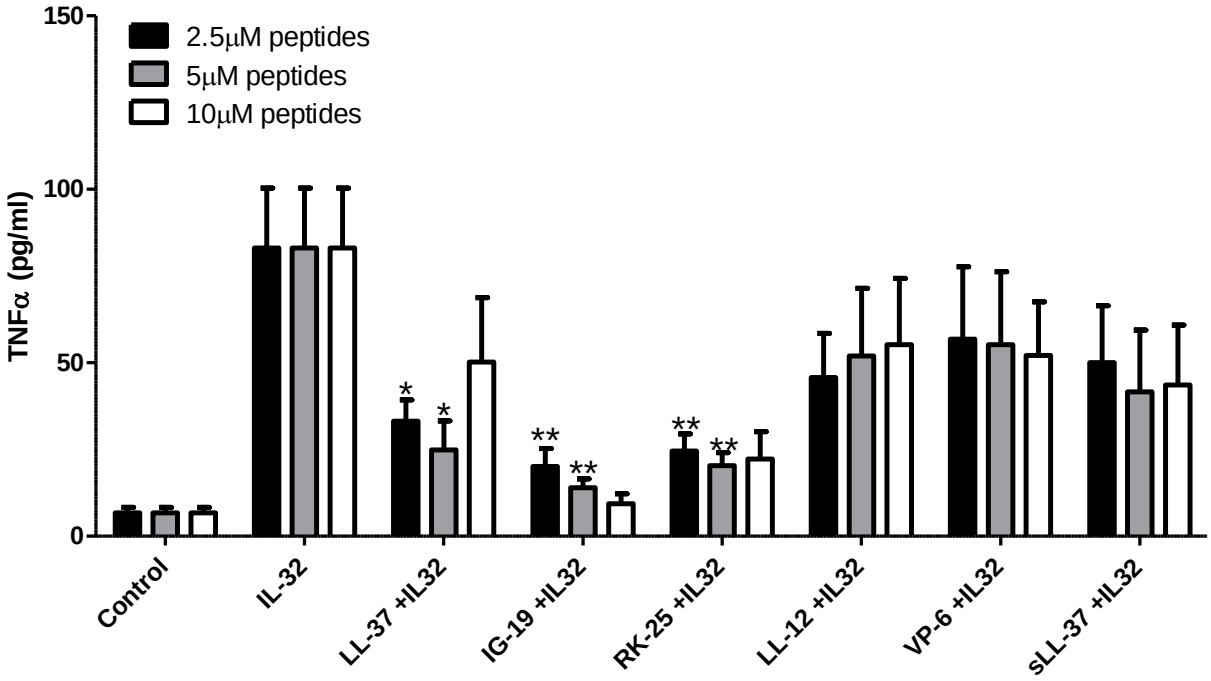
4. Does the enantiomer D-IG-19 have similar effects as IG-19?

   One of the major challenges of using small peptides as therapeutic is the stability. This challenge can be overcome by changing the conformation of the peptide. It was previously demonstrated the enantiomer of D-LL-37 was found to be more resistant to protease degradation, and have similar activity to LL-37 in inhibiting biofilm formation [224, 398]. Therefore, establishing similar functions between IG-19 and its enantiomer D-IG-19 would provide a protease-resistant therapeutic candidate to treat chronic inflammatory diseases.

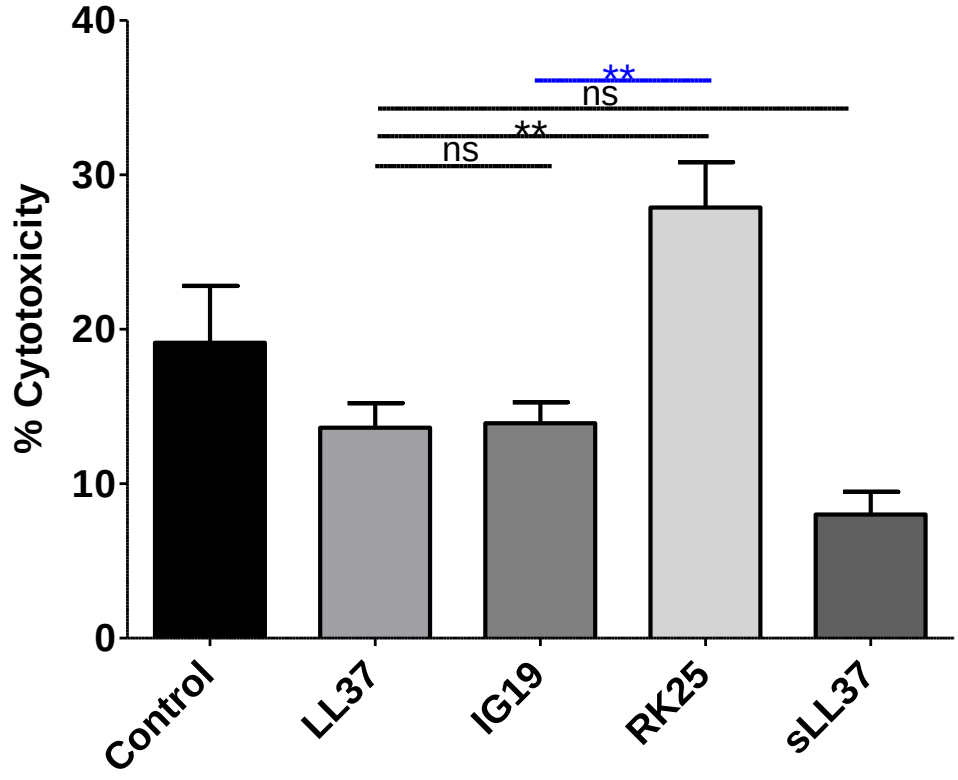
5. To investigate the role of peptide IG-19 using human IL-32γ-transgenic mice.

   One major caveat of the *in-vivo* mouse model of CIA is that wild-type mice do not express the cytokine IL-32γ. Therefore, the ability of the peptide IG-19 to control cytokine IL-32γ-mediated inflammatory responses cannot be specifically assessed in the current CIA murine model. This can be overcome with the recent development of IL-32γ-transgenic mice [293]. The IL-32γ-transgenic mice were developed by microinjecting the ORF of IL-32γ cDNA into mouse zygotes of C57BL/6 strains. It was previously showed that collagen-induced arthritis can be established in C57BL/6 mice [399]. However, it is important to note that the incidence and severity of collagen-induced arthritis in C57BL/6 strains mice are less than those in DBA1/J mice. The transgenic mice were normal in size and did not have obvious physical abnormalities [293]. It was observed that human IL-32γ was detectable in peripheral blood, liver, stomach, skeletal muscle, heart and pancreas of the transgenic mice. Compared to wild type mice, IL-32γ-trangenic mice has higher levels of TNF-α in the serum, colon and rectum [293]. Since our data showed that the peptide IG-19 has the ability to suppress IL-32γ-induced pro-inflammatory responses, I hypothesized that administration of IG-19 would control the development of CIA in IL-32γ-transgenic mice.

CHAPTER 8 – APPENDIX

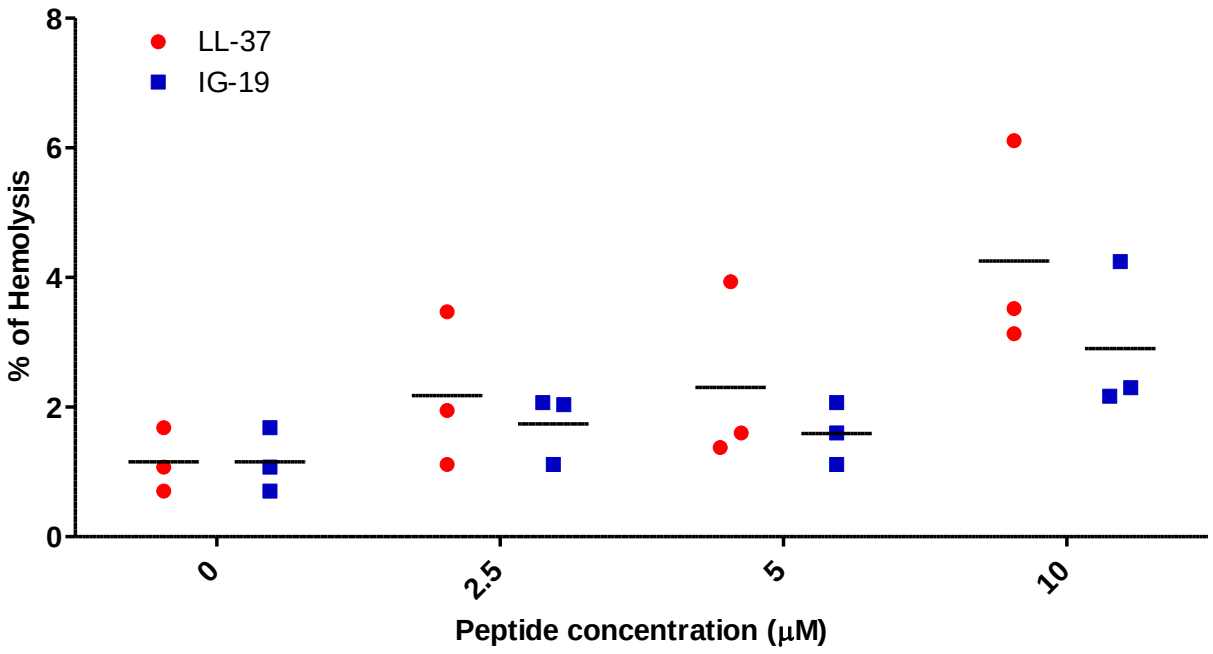


Supplementary Figure 1. LL-37-derived peptides IG-19 and RK-25 suppressed IL-32γ-mediated TNF-α similar to the peptide LL-37 in human macrophage-like THP-1 cells.
Differentiated THP-1 macrophages were stimulated with IL-32*γ* (20 ng/mL) in the presence and absence of LL-37, IG-19, RK-25, LL-12, VP-6 or sLL-37, at different concentrations (2.5, 5 and 10 μM) for 24 hr. Tissue culture supernatants were monitored for production of tumour necrosis factor-*α* (TNF-*α*), by ELISA. Results shown are an average of at least three independent experiments with THP-1 cell, ± standard error. The *P*-values are calculated using Student's paired *t*-test (*p< 0.05, **p<0.005).



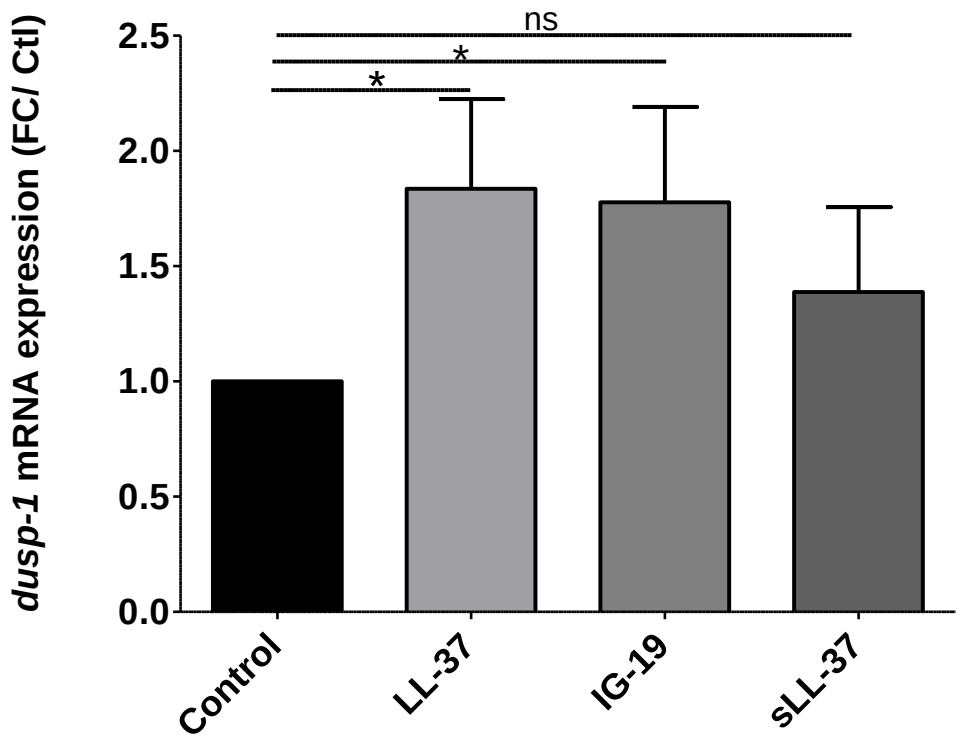
Supplementary Figure 2. Human cathelicidin peptide LL-37 and its synthetic derivative peptides did not mediate significant amount of cellular cytotoxicity in human macrophage-like THP-1 cells.

Differentiated THP-1 macrophages were stimulated with 5 μM of LL-37, IG-19, RK-25 or sLL-37, for 24 hr. Tissue culture supernatants were monitored for cellular cytotoxicity by measuring the release of lactate dehydrogenase. Results shown are an average of at least three independent experiments with THP-1 cell, ± standard error. The *P*-values are calculated using Student's paired *t*-test (*p< 0.05, **p<0.005).



Supplementary Figure 3. Human cathelicidin peptide LL-37 and its synthetic derivative peptides did not mediate significant amount of hemolysis in human erythrocytes.

Human erythrocytes, isolated from freshly collected blood, were stimulated with various concentration (0, 2.5, 5 and 10 μM) of LL-37 or IG-19, for 30 min. Haemolytic activity of the peptides was also determined by monitoring the release of hemoglobin in the supernatant, measured at 577nm. Results shown are the percentage of hemolysis, as determined by the equation: $(OD_{sample} - OD_{blank})/(OD_{total}-OD_{blank}) \times 100\%$, where OD_{blank} correspond to optical density (OD) of hemolysis in the absence of peptides, and OD_{total} refer to optical density of 100% hemolysis in the presence of 2% (v/v) Triton X-100. Each point represents an independent experiment (n = 3), with a line representing the median.



Supplementary Figure 4. Human cathelicidin peptide LL-37 and IG-19 significantly enhanced *dusp-1* mRNA expression.

Differentiated THP-1 macrophages were stimulated with 5 μM of LL-37, IG-19, or sLL-37, for 6 hr before RNA was isolated. Differential mRNA expression was monitored using qRT-PCR. Fold changes of mRNA expression over control (no stimuli) were calculated after normalization to 18sRNA. Results shown are an average of at least three independent experiments with THP-1 cell, ± standard error. The *P*-values are calculated using Student's paired *t*-test (*$p < 0.05$).

CHAPTER 9 - REFERENCES

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