MECHANISMS OF IMMUNE RESPONSE REGULATION BY INNATE DEFENSE REGULATOR PEPTIDES

by

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ABSTRACT

The growing threat of antibiotic-resistant bacteria necessitates the development of new anti-infective therapeutics. Innate defense regulator (IDR) peptides are a novel class of immunomodulatory agents shown to combat bacterial pathogens in murine models of infection via the augmentation of host immune functions, including the stimulation of chemokine production and enhancement of leukocyte recruitment, while suppressing bacterial-induced inflammation. Although IDR-peptides present the potential for future broad-range anti-infective agents, our limited understanding of how they modulate host immunity remains an obstacle in their development as clinical therapeutics. I hypothesized that IDR-peptides impact host immunity by modulating the immune responses of monocytes, a cell population necessary for IDR-mediated protection against infection. In this study, IDR-1002 was found to be a multi-faceted regulator of monocyte migration. IDR-1002 induced the production of monocyte-specific chemokines MCP-1 and MCP-3, as well as neutrophil-specific chemokines, IL-8 and GRO-α in human peripheral blood mononuclear cells (PBMCs), correlating with the activation of the mitogen-activated protein kinases (MAPK), p38 and extracellular-regulated kinase (ERK)-1/2, in monocytes. IDR-1002 was also found to enhance human monocyte migration towards chemokines through the enhancement of β1-integrin-mediated adhesion to fibronectin via regulation of the phosphatidylinositol-3-kinase (PI3K)-Akt signalling pathway. In addition, IDR-1002 increased monocyte responsiveness to the chemokines MIP-1α and RANTES via modulation of CCR5 expression. These results demonstrate an overall promotion of monocyte motility by IDR-1002. In contrast to the immune-strengthening effects of IDR-1002, the production of pro-inflammatory cytokines in human PBMCs stimulated with bacterial lipopolysaccharide (LPS) was suppressed by the peptide, and correlated with a suppression of LPS-induced NFκB and p38 MAPK signalling and activation of PI3K-Akt signalling in monocytes. These results demonstrate that IDR-peptides are potent modulators of human monocyte function via their extensive regulation of monocyte signalling networks, potentially accounting for their multifunctional effects on host immunity in murine models of bacterial infection.

PREFACE

Chapter 1

Sections reviewing human host defense peptides and their functions in the immune response were published as the following book chapter: Madera, L., S. Ma, and R. E. W. Hancock. 2011. Host Defense (Antimicrobial) Peptides and Proteins. In The Immune Response to Infection. S. H. E. Kauffmann, B. T. Rouse, and D. L. Sacks, eds. ASM Press, Washington, DC. p. 57-67.

Sections discussing the use of immunomodulatory agents as anti-infective therapeutic agents were included in the following publication: Nicholls, E. F., L. Madera, and R. E. W. Hancock. 2010. Immunomodulators as adjuvants for vaccines and antimicrobial therapy. *Ann N Y Acad Sci*. 1213: 46-61.

Chapter 2

Experiments demonstrating the necessity of immune signal transduction pathways (Figure 2.3) and actin/microtubule polymerization (Figure 2.4) in IDR-1002-mediated chemokine induction, as well as experiments demonstrating MAPK activation in CD14 monocytes by IDR-1002 (Figure 2.5), were included in the following publication: Nijnik, A., L. Madera, S. Ma, M. Waldbrook, M. R. Elliott, D. M. Easton, M. L. Mayer, S. C. Mullaly, J. Kindrachuk, H. Jenssen, and R. E. W. Hancock. 2010. Synthetic cationic peptide IDR-1002 provides protection against bacterial infections through chemokine induction and enhanced leukocyte recruitment. *J. Immunol*. 184: 2539-2550.

Chapter 3

Data delineating the ability of IDR-1002 to enhance monocyte migration and adhesion on fibronectin, through the PI3K-Akt-mediated activation of β1-integrins, (Figures 3.2-3.12, 3.15) are included in the following manuscript: Madera, L. and R. E. W. Hancock. 2012. Synthetic immunomodulatory peptide IDR-1002 enhances monocyte migration and adhesion on fibronectin. *J. Innate Immun.* Accepted Apr. 2012.

Chapter 4

Experiments describing the ability of IDR-1002 to selectively augment monocyte chemotaxis towards specific chemokines (Figures 4.1-4.4) are being included in the following manuscript in preparation: Madera, L. and R. E. W. Hancock. Immunomodulatory peptide IDR-1002 enhances monocyte migration towards MIP-1α and RANTES via regulation of CCR5 expression.

Chapter 5

Data describing the suppression of LPS-induced cytokine production and cellular signalling (Figures 5.1-5.3, 5.5-5.7) are being included in the following manuscript in preparation:

Madera, L. and R. E. W. Hancock. Regulation of the LPS-induced inflammatory response by the synthetic immunomodulatory peptide IDR-1002.

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ATF-2 – Activating transcription factor 2
BSA – Bovine serum albumin
CaMKII – Ca^{2+}/calmodulin-dependent protein kinase II
CBP – CREB-binding protein
CGD – Chronic granulomatous disease
CREB – cAMP response element-binding
CSF – Colony-stimulating factor
DMSO – Dimethyl sulfoxide
EGFR – Epidermal growth factor receptor
ERK-1/2 – Extracellular regulated protein kinase-1/2
ECM – Extracellular matrix
ELISA – Enzyme-linked immunosorbent assay
FBS – Fetal bovine serum
FPRL-1 – Formyl peptide receptor-like 1
GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
GPCR – G protein-coupled receptor
GSK3β – Glycogen synthase kinase 3 beta
HD – Human defensin
HDP – Host defense peptide
HNP – Human neutrophil peptide
HPF – High powered field
ICAM-1 – Intracellular adhesion molecule 1
IDR – Innate defense regulator
IL -- Interleukin
IFN – Interferon
JAK-STAT -- Janus-activated kinase-signal transducers and activators of transcription
LFA1 – Lymphocyte function –associated antigen 1
LPS – Lipopolysaccharide
mAb – Monoclonal antibody
MAPK – Mitogen-activated protein kinase
MCP-1 – Monocyte chemotactic protein-1
MCP-3 – Monocyte chemotactic protein-3
MFI – Geometric mean fluorescence intensity
MIP-1α – Macrophage inflammatory protein-1 alpha
MRSA – Methicillin-resistant *Staphylococcus aureus*
NFκB – Nuclear factor kappa B
NLR – Nod-like receptors
PBMC – Peripheral blood mononuclear cell
PBS – Phosphate-buffered saline
PECAM1 – Platelet endothelial cell adhesion molecule 1
PI3K – Phosphoinositide 3-kinase
PLC – Phospholipase C
PMA -- Phorbol myristate acetate
PTX – Pertussis toxin

RANTES – Regulated upon activation, normal T-cell expressed, and secreted
SDF-1 – Stromal cell-drived factor 1
TGFβ – Transforming growth factor beta
TLR – Toll-like receptor
TNFα – Tumor necrosis factor alpha
TNFR2 – TNFα receptor 2
VCAM-1 – Vascular cell adhesion molecule 1
VLA4 – Very late antigen 4
VRE – Vancomycin-resistant enterococci

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1 INTRODUCTION

1.1. ANTIBIOTIC RESISTANCE AND THE EMERGING BACTERIAL THREAT

The discovery and commercial development of antibacterial antibiotics in the early-to-mid-20th century heralded a new era of medicine. Over the past seven decades, the use of these therapeutic agents allowed for the effective management of infectious diseases previously seen as untreatable (1). The application of antibiotics, in synergy with improvements in hygiene and sanitation, drastically reduced the morbidity and mortality from common bacterial pathogens to the point where it was believed that infectious diseases would be fully under control. Reports of microbes resistant to specific classes of antibiotics, observed as early as the 1950s, remained largely unnoticed, obscured by the rapid discovery of novel antibiotic classes and their increasing availability. However, the extensive use of antibiotics, in both medicinal and non-medicinal settings (particularly in agriculture), has greatly accelerated the development of antibiotic-resistant pathogens via natural selection. In parallel, the discovery of new antibiotic classes has considerably slowed, the majority of antibiotics in use today being discovered prior to the 1970s. This situation has culminated in the rise of drug-resistant "superbugs," including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE), which are now significant medical menaces. Our options for treating these pathogens only continues to shrink as the use of last-resort antibiotics is met with the rapid development of resistance, foreshadowing a dire return to the pre-antibiotic era unless novel anti-infective strategies can be developed.

1.2. IMMUNE MODULATION AS AN ANTI-INFECTIVE STRATEGY

As the development of antibiotic agents fails to keep pace with the expansion of drug resistant bacteria, new strategies to combat bacterial infections are required. One such strategy under consideration is the manipulation and enhancement of natural host immune responses, or immunomodulation, to promote pathogen clearance. Dissimilar to traditional antibiotic agents that act on specific bacterial targets, immunomodulatory anti-infective agents enhance a pre-existing system that has evolved to exhibit broad spectrum antimicrobial activity (2). Utilization of the innate immune system, with its diverse

mechanisms of action, in principle will minimize the development of antimicrobial resistance since pathogens would have to counter multiple modes of attack from processes that have been effective in keeping them at bay for millions of years (2). Thus the concept of regulating innate immune responses for the purpose of fighting infection provides vast potential for the development of broadly effective anti-infective therapeutics.

The innate immune response is a highly conserved system which serves to rapidly counter microbial invasion. Through the utilization of germ-line encoded pathogen-recognition receptors such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs), the host is able recognize pathogen-associated molecular patterns (PAMPs), common motifs conserved among numerous pathogenic species (3-5). The recognition of these PAMPs, including bacterial and viral nucleic acids as well as bacterial lipid and protein structures, results in the initiation of host defense. This includes the robust recruitment of immune cells, such as neutrophils and monocytes, from the blood to the site of infection. In addition, the subsequent activation of immune cells in the infectious locale results in the generation of numerous antimicrobial mechanisms, including microbial phagocytosis, generation of reactive chemical species, and production of antimicrobial peptides (5). These multiple cascades collaborate to promote microbial clearance and resolve infection. To manage and coordinate these complex processes, the host relies on the production of cell-to-cell signalling agents, or cytokines (5). Pathogenic recognition results in the rapid production of pro-inflammatory cytokines, which serve to activate proximal immune cells. The production of chemoattractive cytokines, or chemokines, is essential for the recruitment of circulating cells to the site of infection (6). Regulatory cytokines are also essential for the termination of innate immune responses after pathogen clearance, minimizing inflammation-induced host damage (7). In summary, cytokines play a paramount role in the management of innate immune responses and, thus, are tantalizing candidates as immune modulating therapeutic agents.

Numerous immunomodulatory agents based on cytokine mediators are being developed, or have been developed, into clinical anti-infective agents. The enhancement of beneficial immune responses by immunomodulators, such as the promotion of cell recruitment and activation and/or the restoration of cellular antimicrobial functions, has been shown to promote effective pathogenic clearance. One of the best examples of this is the

development of interferon (IFN) therapy. IFNs encompass a family of endogenous proteins expressed by the host immune system primarily in response to viral infections (8). Through interactions with their cognate receptors, and downstream induction of IFN-stimulated genes, Type I IFNs inhibit protein synthesis, promote cellular apoptosis, and increase the production of RNA-digesting enzymes, all of which serve to inhibit viral replication (8). The synthesis of Type I IFN derivatives with improved activity and stability, such as PEGylated IFN-α, has led to the development of antiviral therapeutics (9). Administration of Peg-IFN-α, in combination with other antiviral drugs, is commonly done in the treatment of chronic hepatitis B virus and hepatitis C virus infections to control disease (10-13). Type II IFN, namely IFN-γ, promotes the antimicrobial functions of immune cells, enhancing the phagocytic and oxidative burst activity of macrophages and NK cells (14). Harnessing this effect, IFN-γ is currently used as an anti-infective agent in patients with chronic granulomatous disease (CGD) (15, 16). Individuals with CGD are unable to mount an effective oxidative burst response in their immune cells and consequently suffer from increased occurrence of microbial infection (17). Administration of IFN-γ significantly reduces the frequency of bacterial and fungal infections in CGD patients, a striking example of how strengthening the immune response can counter microbial infections (18). Colony stimulating factors (CSFs) represent another class of endogenous host immune mediators that have been developed into successful anti-infectives. Derivatives of granulocyte-CSF, a promoter of neutrophil proliferation and activator of antimicrobial activity, can restore neutrophil function and reduce the frequency of infection in neutropenic patients (19). Granulocyte-macrophage-CSF is also effective in restoring immune function and reducing infection in leukopenic patients, and in addition has met with some success as an anti-fungal agent in *in vitro* settings (19-21). Exogenous activators of immunity, including TLR-agonists, similarly have either been developed into antimicrobial agents, or have shown potential as anti-infective agents in animal studies (22-27). These advances have shown that modulation of the host immunity is an effective strategy to counter microbial infections in a wide variety of settings.

Although immunomodulators have seen successes as antimicrobial agents, many obstacles remain. Artificial regulation of the immensely complex system that is host immunity requires a delicate balancing act, and the modulation of immunity is a double-

edged sword. The associated inflammatory response which is necessary for effective antimicrobial immune functions must be kept in control to prevent the onset of adverse effects on the host. In certain cases, the systemic administration of immune stimulatory agents has been shown to elicit symptoms of a potentially harmful inflammatory response, sometimes termed a "cytokine storm". Administration of IFNs, as well as exogenous TLR-agonists, strengthen host anti-viral responses yet induce flu-like symptoms, including fever, hypotension, and fatigue (28-30). So in summary, the application of immunomodulatory agents must always be balanced against the risks of pushing the immune response to harmful extremes. Thus, a major goal in anti-infective immunomodulatory therapy is the development of multi-functional poly-directional immune regulators that can enhance the features of immunity responsible for microbial clearance while limiting adverse inflammatory reactions.

1.3. HOST DEFENSE PEPTIDES

Cationic host defense peptides (HDPs), also termed antimicrobial peptides, encompass a large family of peptides and represent one among many defense mechanisms utilized by our innate immune system. HDPs are widely distributed throughout many species, from plants to insects to mammals, a breadth matched by the diverse nature of these peptides in terms of size, sequence, and structure (31-33). Despite these differences, HDPs exhibit certain similarities. They are approximately 12 to 50 amino acids in length and amphipathic in nature due to the inclusion of hydrophobic, hydrophilic, and charged residues. HDPs are positively charged, hence their cationic designation, displaying a charge of +2 to +9, stemming from the presence of arginine and lysine residues. These peptides also adopt a broad range of structures, including α-helical conformations, β-sheets, extended configurations, and loop structures, and are often classified based on these characteristics.

In mammals, two major classes of HDPs can be found, the cathelicidins and defensins. Members of these peptide families can be found, constitutively expressed or induced, in the granules of many leukocytes and epithelial cells (Table 1.1) (34-36). Initial investigations revealed that these peptides exhibited *in vitro* antimicrobial activity against a broad range of organisms, including bacteria, fungi, and enveloped viruses, due to either their preferential disruptive effects on microbial membranes or on microbial intracellular targets (37, 38). The observation of antimicrobial activity, coupled with their prominent presence in

immune cells, led to the hypothesis that HDPs participated in the immune response through direct antimicrobial killing. However, research in the last decade showing the sensitivity of HDP-mediated direct killing to the presence of physiological levels of monovalent and divalent cations, indicates that this role is restricted to locales where HDPs are present in high concentrations, such as in the phagocytic granules of neutrophils (37). Further studies revealing novel HDP-mediated functions suggested instead that HDPs are comprehensive regulators of the overall immune response.

Table 1.1 Localization of human host defense peptides

Peptide Class	Members	Localization
α-defensins	HNP1-4	• Neutrophils, monocytes, lymphocytes • Paneth cells
	HD5-6	• Paneth cells
β-defensins	hBD1-4	• Epithelial cells • Monocytes
	hBD5-6	• Epididymis and airway epithelial cells • Paneth cells
Cathelicidins	hCAP18/LL-37	• Monocytes, Neutrophils, Mast cells • B- and γσ-T-lymphocytes • Epithelial cells

The ability of HDPs to regulate the immune response is extensive, impacting on numerous functional aspects of immunity (Figure 1.1). HDPs exhibit profound regulatory effects on the innate immune response. Initial invasion by pathogens results in the increased production and release of HDPs by immune cells at the site of infection. In addition to direct HDP-mediated killing in phagocytic granules, peptides have been shown to act as chemotactic factors for a number of immune cells, resulting in the further infiltration of effector cells to combat invading pathogens. Human defensins and human cathelicidin LL-37 have been shown to directly attract monocytes and neutrophils (39-41). An effective immune response relies on the rapid infiltration of these cell populations due to their potent antimicrobial functions, such as the phagocytosis of microbes, the release of damaging

reactive chemical species, and the secretion of antimicrobial enzymes. These peptides also demonstrate the ability to chemoattract T-lymphocytes and dendritic cells (41, 42), essential mediators of later-stage adaptive immune responses. Activation of immune cells by HDPs results in the production and secretion of certain cytokines, particularly chemokines, leading to secondary regulatory effects on immunity. LL-37 alone can induce the production of multiple chemokines including monocyte chemoattractant MCP-1, neutrophil chemoattractant IL-8 in human peripheral blood mononuclear cells (PBMCs) (43, 44). Likewise, human defensins are able to stimulate the production of chemokines in a variety of cell types (45, 46), further demonstrating that HDPs are direct and indirect promoters of immune cell recruitment.

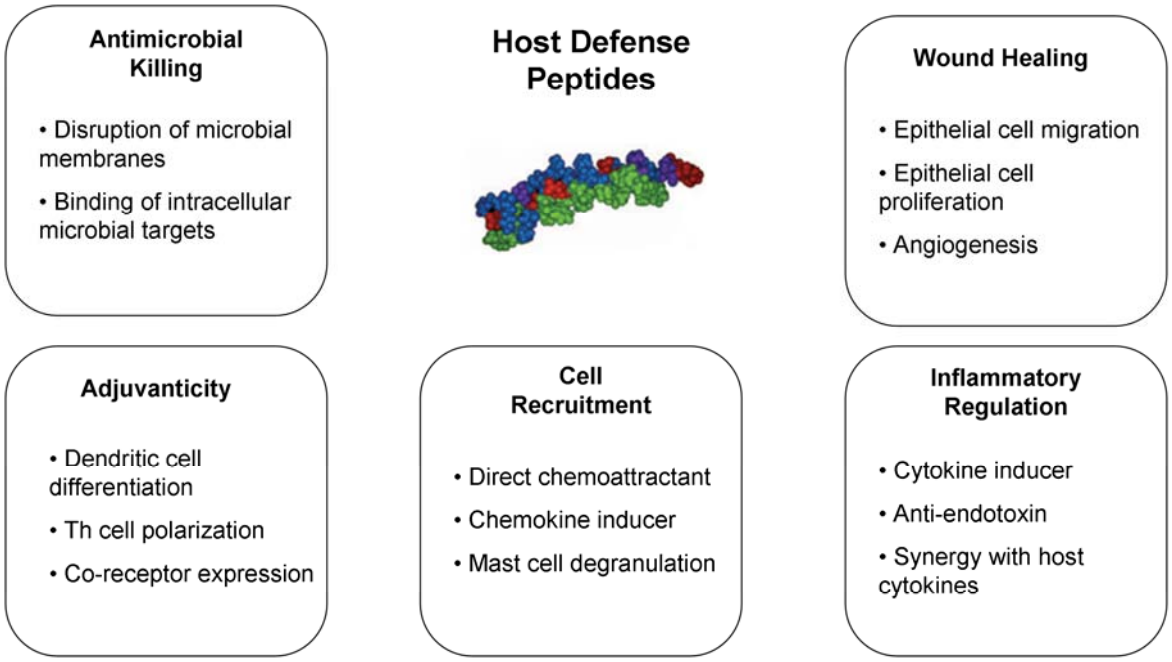

Figure 1.1 The roles of host defense peptides in immunity.

Aside from their effects on cellular recruitment, HDPs also have the capability to regulate other axes of the host inflammatory response. Certain HDPs have the capacity to propagate the inflammatory response, potentially strengthening the immune reaction against

infection. For instance, the induction of pro-inflammatory cytokines, including TNFα and IL-1β, is enhanced by human α-defensins in activated monocytes (47). Defensins are also able to elicit pro-inflammatory cytokine production in epithelial cells, keratinocytes and lymphocytes (46, 48, 49). On the other hand LL-37 does not stimulate pro-inflammatory cytokine induction but can act in tandem with intrinsic immune mediators to potentiate the host response. Thus human cathelicidin synergizes with IL-1β to promote cytokine production in PBMCs (50). In contrast, HDPs can also rein in the overstimulation of the host inflammatory response. LL-37 is well-documented as a potent suppressor of bacterial lipopolysaccharide (LPS)-induced responses and can also suppress cytokine production induced by lipoteichoic acid (44, 51-53). Consequently LL-37 is able to confer protection in rats against LPS-induced sepsis (54). Similarly, LL-37 and defensins can induce, or synergistically enhance, in a number of cell populations, the production of IL-10 (45, 49, 50), an immunosuppressive cytokine essential in the regulation of the host inflammatory response. Taken together, HDPs are complex inflammatory mediators, promoting immune activation while limiting the onset of an excessive, harmful, inflammatory response.

The later stages of the immune response, including the potential engagement of adaptive immune responses, are modulated by HDPs as well. Failing the rapid clearance of pathogens by innate immunity, the onset of slower, antigen-specific adaptive response then plays a major role (4). Adaptive responses are activated by specific pathogenic patterns in conjunction with signals generated from the innate immune response. Specifically, antigen presentation by cells such as macrophages and dendritic cells, in addition to host cytokines generated by innate immunity, stimulates the selective proliferation and activation of lymphocytes. The clonal expansion of these lymphocytes results in the generation of B-lymphocyte-mediated antibody responses and the promotion of cell-mediated immunity, such as the killing of infected cells by cytotoxic T-lymphocytes and the promotion of helper T-lymphocyte activity. HDPs have been shown to influence the growth and differentiation behaviour of a number of immune cells essential in adaptive immunity. Defensins in mouse models enhanced the CD3-mediated proliferation of CD4 T-lymphocytes, correlating with other studies showing an enhancement of antigen-specific humoral and cellular responses by defensins (55, 56). LL-37 demonstrates the capacity to upregulate co-receptor CD86 expression on dendritic cells and can promote the polarization of monocyte-derived dendritic

cell to Th_1 type responses by enhancing the production of Th_1 cytokines IL-12 and IL-6 while decreasing IL-4 secretion (57). In addition, recent studies have shown that LL-37 skews M-CSF-driven macrophage differentiation to a more reactive, pro-inflammatory state (58). Correspondingly, investigations have shown that LL-37 exhibits adjuvant properties, enhancing adaptive responses (59).

The ability of HDPs to regulate cellular growth, coupled with their ability to chemoattract cells, allows them to play a role in the resolution of infection. Defensins demonstrate the ability to promote keratinocyte migration, as well as keratinocyte proliferation, suggesting a role in the re-epithelialization of a wound area (46). Defensins can also promote extracellular matrix (ECM) formation, essential for wound restoration, by increasing the levels of collagen precursors while decreasing levels of matrix metalloproteinase-1, an enzyme involved in the breakdown and remodeling of the ECM (60). Likewise LL-37, which can be induced by wounding, is able to promote the chemoattraction of keratinocytes, suppress keratinocyte apoptosis and stimulate the proliferation of epithelial cells (61-65). LL-37 also promotes angiogenesis by endothelial cells (66). Certainly, the minimal protein expression of LL-37 in chronic epithelial ulcers and the inhibition of epithelial healing in a human skin wound model by LL-37-specific neutralizing antibodies underscores the importance of this human cathelicidin in wound healing (67).

Cationic HDPs, previously regarded as one of several families of direct antimicrobial agents in our immune arsenal, have been revealed as elaborate coordinators of host immunity. HDPs, produced or found at sites of pathogenic invasion, can promote the recruitment and activation of immune effector cells, enhancing the initial antimicrobial barrage the host utilizes to rapidly clear infection. HDPs, by virtue of their effects on the host cytokine environment, are also modulators of inflammation, the effector arm of innate immunity essential for further recruitment of immune cells and antimicrobial agents to the site of infection. Moreover, HDPs are agents of inflammatory balance, promoting necessary elements of inflammation, while suppressing microbial-mediated overstimulation, which can often result in severe host damage, and even death. The regulation of the host cytokine milieu by HDPs also results in a cascade of HDP-mediated effects, including the regulation of cellular growth, differentiation, and behaviour. This diverse, yet concerted, regulation leads to the further activation of innate anti-infective responses, enhancement of adaptive

immunity, and the promotion of wound healing and resolution. Overall cationic HDPs exhibit the characteristics of an ideal anti-infective immunomodulator, potentiating host anti-microbial responses via the multi-pronged enhancement of host immunity, yet displaying inherent regulatory properties which limit the harmful drawbacks of traditional immunomodulatory therapeutics.

The major issue is whether the complex immunomodulatory behaviour of HDPs translates into improved host morbidity and mortality during infection and whether they can be harnessed and developed into effective anti-infective therapeutics. To be sure, numerous studies have shown the importance of HDPs in infection. Dysregulation of HDP levels correlate with increased susceptibility to infectious diseases. In inflammatory skin diseases, psoriatic patients, who exhibit elevated levels of defensins and LL-37, show a decreased susceptibility to infections relative to atopic dermatitis patients who exhibit decreased HDP levels (68). Similarly, defensin and cathelicidin knock-out mice showed delayed clearance and increased levels of bacterial pathogens (69-72). Neutropenic individuals, such as those suffering from morbus Kostmann disease, are treated with cytokine G-CSF to restore circulating neutrophils with normal oxidative responses (73, 74). Yet these restored neutrophils are deficient in LL-37, an observation that correlates with chronic periodontal diseases and excessive oral inflammation in these patients (73). Numerous studies also show that the exogenous addition or overexpression of HDPs lead to improved disease outcomes. Administration of defensins in murine and porcine models leads to improved clearance of bacterial pathogens (75, 76). This defensin-mediated protection is suggested to be due to an enhancement of the immune response rather than any direct antimicrobial effect by the peptide (75, 76). These studies demonstrate that HDPs should be considered as a potential repository of immunomodulatory anti-infectives, and although some HDPs are undergoing clinical trials for such purposes, the use of HDP-based immunotherapy is still in its infancy (77, 78).

1.4. INNATE DEFENSE REGULATORS

The innate defense regulator (IDR)-peptide initiative represents an effort to utilize natural HDP as templates to develop agents with improved immunomodulatory properties,

and thus, in theory, improved anti-infective properties. Using the amino acid sequences of natural HDPs as design templates, diverse peptide libraries are generated via sequence scrambling and alteration (Figure 1.2). The generated peptide libraries were then screened *in vitro* for immunomodulatory properties associated with *in vivo* protection against infection, such as the ability to enhance chemokine production or suppress harmful endotoxin-induced pro-inflammatory responses. This process resulted in the generation of candidate IDR-peptides and furthered our understanding of structure-function relationships of peptide-mediated immunomodulation, allowing for the iterative optimization and synthesis of novel candidates.

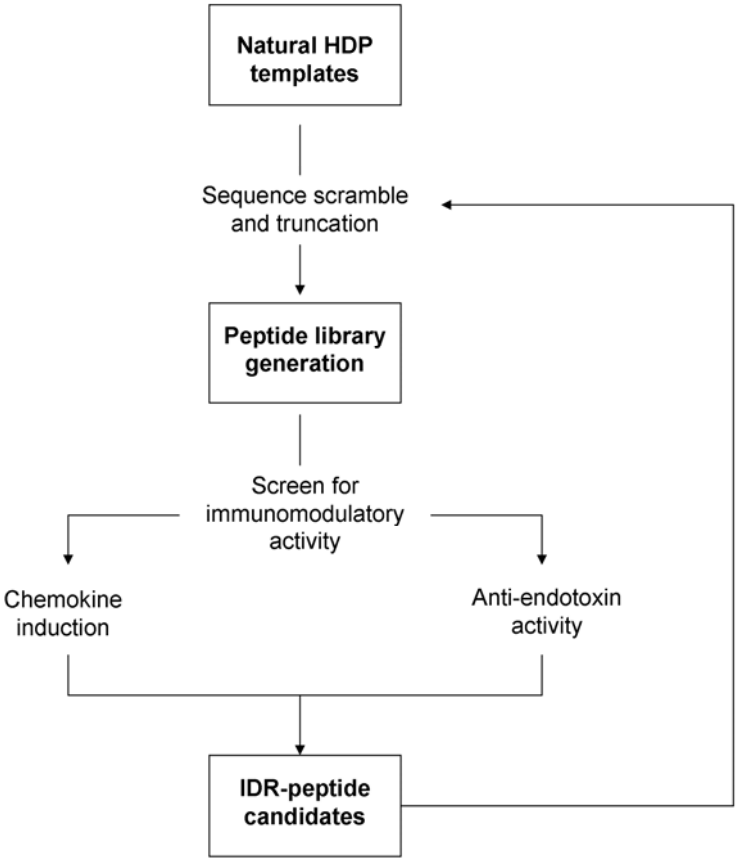

Figure 1.2 The generation of IDR-peptides from natural HDP templates.

The creation of immunomodulatory IDR-peptides has resulted in anti-infective successes and a tacit demonstration that selective modulation of the immune response is a

viable anti-infective strategy. IDR-1, a 13-amino acid peptide which exhibits the ability to enhance chemokine gene expression and protein production *ex vivo* in human PBMC models, grants protection against a range of bacterial pathogens in murine infection models(79). Both pre-treatment and post-treatment of mice with IDR-1 relative to intraperitoneal infection with methicillin-susceptible and methicillin-resistant *S. aureus*, VRE, or *Salmonella enterica* serovar Typhimurium, results in significantly enhanced bacterial clearance and reduced mortality. This protection is also seen regardless of the route of IDR-1 administration, as protection was observed via intraperitoneal, intramuscular, intravenous, or subcutaneous injection of peptide. Coupled with the observations that IDR-1 demonstrated no direct antimicrobial activity *in vitro* and has a half-life of less than 30 minutes in the blood, these results strongly suggest that this peptide primes the immune response to elicit or strengthen an immunoprotective response rather than acting directly upon bacteria. Indeed administration of IDR-1 during an infection leads to a significant change in the murine immune environment; namely an elevation of monocyte chemokine MCP-1, which correlates with an increased macrophage presence at the site of infection, and a suppression of bacterial-induced pro-inflammatory cytokines TNF-α and IL-6. Similarly in studies reported in part here, IDR-1002, a 12-amino acid derivative of bovine bactenecin which demonstrated potent MCP-1 inducing activity in preliminary human *ex vivo* PBMC studies, conferred prophylactic and therapeutic murine protection against bacterial pathogens; this effect correlating with an elevation of murine chemokines and cellular recruitment (80). Importantly, IDR-peptides conferred anti-infective protection without the certain toxic effects associated with some HDPs, such as mast cell degranulation and cellular apoptosis (79, 81-83).

These studies have demonstrated that the generation and investigation of IDR-peptides is a strategy worth pursuing in the quest to develop immunomodulatory therapeutic agents with broad-range antimicrobial activity. The ability of IDR-peptides to enhance certain aspects of the immune response has also led to other anti-infective applications beyond direct treatment of infections. IDR-peptides, as adjuvant components, result in the generation of a balanced T-helper cell type 1 and type 2 responses and elevated antibody titers in vaccine formulations against pertussis toxoid and Pseudorabies virus in murine and porcine models, respectively (84). The potential capability of IDR-peptides to regulate

inflammatory responses also makes them prospective candidates for suppression of inflammatory diseases without compromising host anti-infective responses. While the immunomodulatory range of IDR-peptides make them a gold-mine of prospective immunotherapeutics, many obstacles must be overcome prior to their use in the clinic. While the aforementioned studies demonstrate the functional efficacy of IDR-peptides as anti-infectives and adjuvants, the mechanisms by which these peptides modulate the complex process that is immunity remains inadequately understood. Apart from our rudimentary understanding that IDR-peptides can modulate levels of certain cytokines, many questions remained unanswered at the start of this thesis research, including the range of cytokines modulated by IDR-peptides and the methods by which IDR-peptides can selectively alter the cytokine environment, leading to an enhancement of chemokine production and cell recruitment while suppressing levels of specific inflammatory cytokines. Furthermore, it is unknown whether IDR-mediated immunomodulation goes beyond cytokine regulation and impacts on the wide array of cell-mediated anti-infective processes in other ways; and ultimately, whether IDR-mediated effects in animal models reflect their effects in a human system. The further characterization of peptide-mediated effects on the human immune response, as well as the elucidation of the mechanisms by which IDR-peptides modulate immunity, is essential for the further optimization and development of IDR-peptides into potent anti-infective immunotherapeutic agents, and thus is the basis of this thesis project

1.5. HYPOTHESIS AND PROJECT GOALS

Initial investigations into the immunoregulatory activities of IDR-peptides have yielded tantalizing clues as to their mechanism(s) of action. The anti-infective activity of IDR-peptides is mediated through the monocyte/macrophage population, with *in vivo* depletion of the monocyte population abolishing any protective effects of peptides in reducing bacterial load (80). In contrast, IDR-peptides conferred antimicrobial protection even after the *in vivo* depletion of neutrophils or the T- and B-lymphocyte population (79). These observations hint at the central role of the monocyte population in IDR-peptide-mediated immunomodulation. In addition, recent investigations are revealing that endogenous HDPs themselves are regulators of monocyte signal transduction networks, particularly those heavily involved in immunity. For example, LL-37 has been demonstrated

to activate the MAPK pathways, essential for cellular growth processes as well as the production of immune cytokines (43). In parallel, LL-37 is able to suppress endotoxin-induced NFκB signalling, a major pro-inflammatory signalling network. Initial studies have also suggested that IDR-peptides regulate certain aspects of immunity, e.g. chemokine induction, through these same pathways (79). As signalling pathway cascades regulate virtually all cellular functions, it is feasible that the complex immunoregulatory effects of IDR-peptides stem from their potential ability to regulate cellular signalling. Therefore, I **hypothesize** that IDR-peptides are extensive modulators of cellular immune function via their ability to regulate monocyte signalling networks. As such, this project aimed to utilize the prototypical IDR-peptide IDR-1002 to: 1. further characterize the effects of IDR-peptides on human cellular immune function, with monocytes as a focus, 2. determine the ability of IDR-peptides to modulate monocyte signalling cascades of immune-related pathways, and 3. investigate whether the functional regulatory effects of IDR-peptides arise from their regulation of monocyte signalling.

2 IDR-1002 REGULATES CYTOKINE PRODUCTION AND CELLULAR SIGNALLING IN HUMAN PBMCs

2.1. INTRODUCTION

As demonstrated by researchers in our laboratory, anti-infective peptide IDR-1002 conferred *in vivo* protection against infection by Gram-positive and –negative bacterial pathogens in murine models (80). This effect correlated with a regulation of the murine chemokine/cytokine environment by IDR-1002 using *in vivo* and *ex vivo* models, suggesting a regulation, and perhaps strengthening, of innate immune responses. Immunity is a highly complex, tightly regulated, cascade of processes which act in concert to recognize microbial invasion, recruit effector molecules to contain and combat pathogens, and repair host damage and restore the normal state at the resolution of infection. Such an orchestrated process requires regulatory elements to coordinate the variety of immune processes in order to mediate a coherent immune response. The wide array of host cytokines, many of which are rapidly produced at the onset of infection, plays an essential role this capacity as cell-to-cell signalling mediators. Cytokines regulate virtually all aspects of immune functions. Within innate immunity itself, a large repertoire of cytokines exists to initiate and mediate a rapid and effective antimicrobial response. Inflammatory cytokines, such as TNFα, IL-6, and IL-1 are required to initiate inflammation, the effector arm of innate immunity, leading to the enhanced recruitment of immune cells and antimicrobial compounds (85-87). A subset of chemoattractive cytokines, or chemokines, are responsible for directly recruiting immune effector cells, targeting cell traffic to specific sites via their generation of chemokine concentration gradients. A diverse range of chemokines attracts specific immune effector cells based on cellular receptor expression and specificity (88). As such this chemokine milieu is immensely important in the initiation of innate immune responses. In addition, cytokines such as IFNs or CSFs act as activators of effector cell functions, turning on or strengthening the antimicrobial functions of immune cells, including neutrophils, monocytes, and lymphocytes (8, 19). Due to the critical effects of cytokines on immunity, initial observations that IDR-1002 and other IDR-peptides regulate the levels of specific murine cytokines suggest that IDR-peptides are complex regulators of immunity. Therefore, an

increased understanding of the breadth of cytokines regulated by IDR-1002, as well as the mechanism by which it does so, would be invaluable in determining how IDR-regulation of immunity can lead to enhanced immune protection.

The ability of IDR-1002 to increase the levels of monocyte chemoattractant MCP-1 during an infection, and the correlative increase in monocyte recruitment to the site of infection, almost certainly play a major role in IDR-protection against infection. *In vitro* murine studies showing an induction of neutrophil chemoattractant GRO-α by IDR-1002 may also explain the enhancement of neutrophil recruitment seen during infection. However, both the extent and the mechanisms by which IDR-1002 and other IDR-peptides elicit their cytokine-modulating effects have not been elucidated. In this study, the ability of IDR-1002 itself to induce the production of an array of cytokines by human PBMCs was further investigated. Investigations into this heterogenous cell population were chosen for its relevance to the systemic administration of IDR-peptides. The cytokines investigated focused on those with profound effects on innate immunity, including effector cell chemoattractants and general inflammatory cytokines. Here, IDR-1002 was observed to stimulate the production of neutrophil chemoattractants GRO-α and IL-8 and monocyte chemoattractants MCP-1 and MCP-3 in human PBMCs. In contrast, treatment of human PBMCs with IDR-1002 elicited minimal production of pro-inflammatory cytokines IL-6 and TNF-α, demonstrating selective cytokine induction. IDR-1002 stimulation of MCP-1 induction was dependent on a wide range of immune signalling networks, including G-protein signalling pathways, PI3K, p38 and MEK-1 MAPK, and NFκB pathways, networks that are heavily involved in the regulation of cytokine production. Chemokine induction was also dependent on cellular endocytic processes, including actin and microtubule polymerization, suggesting peptide uptake was required for its cytokine regulation potentially through its effects on intracellular targets. Furthermore, this pathway dependency correlated with an ability of IDR-1002 to regulate and activate p38 and ERK-1/2 MAPK signalling in human monocytes. Overall, these results reveal that IDR-1002 is a selective regulator of chemokine production in human PBMCs, potentially reflecting an extensive regulation of monocyte signalling pathways involved in immunity.

2.2. METHODS AND MATERIALS

2.2.1. Reagents

Peptide IDR-1002 (VQRWLIVWRIRK-NH_2) was synthesized by solid phase F-moc chemistry by CPC Scientific (Sunnyvale, CA). LPS from *Pseudomonas aeruginosa* strain H103 was purified using the Darveau-Hancock method as previously described (89) and stored at 4˚C. Inhibitors used included: G_i-protein inhibitor pertussis toxin, formyl peptide receptor-like 1 antagonist WRW4, NFκB inhibitor BAY11-7082, PI3K inhibitor LY294002, MEK-1 inhibitor PD98059, p38 MAPK inhibitor SB203580 (all from Calbiochem, San Diego, CA), microtubule assembly inhibitor nocodazole, and actin polymerization inhibitor cytochalasin D (both from Sigma-Aldrich, St. Louis, MO). Inhibitors were resuspended in dimethylsulfoxide (DMSO) (Sigma-Aldrich) and stored at -20˚C. Concentrations of DMSO used never exceeded 0.02% (v/v) in cell cultures.

2.2.2. PBMC isolation

Human PBMCs were isolated as previously reported (44). Briefly, venous blood was collected from healthy volunteers using heparin-containing Vacutainer tubes (BD Bioscience, San Jose, CA) in accordance with UBC ethical approval and guidelines. Human blood was diluted in an equal amount of phosphate buffered saline (PBS) (Invitrogen, Carlsbad, CA) then layered over Ficoll-Paque Plus (Amersham, Piscataway, NJ) prior to separation by density-gradient centrifugation. The mononuclear cell layer was extracted and washed twice with PBS. Cells were then resuspended in RPMI 1640 with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Invitrogen), placed in a polypropylene tube (BD Falcon, San Jose, CA), and cultured in a humidified incubator at 37°C with 5% CO_2.

2.2.3. Measurement of cytokine production by ELISA

PBMCs at a concentration of $1x10^6$ cells/ml were seeded on tissue culture plates and placed in a humidified incubator at 37 ˚C and 5% CO_2 for 1 hour. Cells were then stimulated with the indicated concentrations of IDR-1002 and incubated for 24 hours. Treatment with 10

ng/ml of LPS was used as a positive control for stimulation of cytokine production in some experiments. In inhibitor treatments, PBMCs were pre-treated with the indicated dose of chemical inhibitors for 1 hour, prior to stimulation with 100 μg/ml of IDR-1002 for 24 hours. Samples were then centrifuged at 1000 x *g* for 10 minutes to obtain cell-free supernatants, which were then stored at -20 ˚C. Cytokine levels were measured by ELISA using anti-human MCP-1 Ab clones 5D3-F7 and 2H5, anti-human IL-6 Ab clones MQ2-13A5 and MQ2-39C3, anti-human TNFα Ab clones MAb1 and MAb11 (all from eBioscience, San Diego, CA), anti-human GRO-α Ab clones 20326 and BAF275, anti-human MCP-3 Ab clones 36320 and BAF282 (both from R&D Systems, Minneapolis, MN), and anti-human IL-8 Ab clones 893A6G8 and 790A-2862 (Invitrogen), followed by avidin HRP (eBioscience) as per manufacturer's protocols. The ELISAs were then developed using the TMB Liquid Substrate System (Sigma-Aldrich) and measured with a Power Wave X340 plate-reader (Bio-Tek Instruments, Winooski, VT). Quantification of cytokines was done by comparisons against serial dilutions of recombinant cytokine standards (R&D Systems, and eBioscience).

2.2.4. Measurement of protein phosphorylation by flow cytometry

All flow cytometry results were collected using the FACSCalibur flow cytometer in conjunction with CellQuest Pro software (BD Biosciences). $2x10^6$ PBMCs were seeded per well on tissue culture plates and placed in a humidified incubator at 37 °C and 5% CO_2 for 1 h. Cells were then stimulated with 100 μg/ml of IDR-1002 and incubated at 37 °C for 30 minutes. Following treatment, PBMCs were fixed with PBS and 4% (w/v) formaldehyde (Fisher Scientific, Pittsburgh, PA) at room temperature for 20 min in preparation for staining.

PBMCs were then washed with 0.5% (w/v) BSA in PBS and permeabilized in 90% (v/v) methanol at 4 °C for 30 min. The cells were then washed and stained at room temperature for 1 hour with anti-phospho-p38 MAPK (Thr180/Tyr182) 3D7 rabbit mAb or anti-phospho-p44/p42 MAPK (ERK-1/2, Thr202/Tyr204) E10 rabbit mAb (both from Cell Signalling Technology, Danvers, MA). Following washing, the cells were stained with a goat anti-rabbit IgG-Alexa Fluor 647 (H+L, Invitrogen) and with an anti-human CD14-Alexa Fluor 488 M5E2 mouse mAb for 30 min at room temperature. The cells were then washed and resuspended in PBS with 0.5% BSA and 0.5% formaldehyde for analysis. Monocytes

were assayed by gating on CD14 expressing cells within the PBMC population. Levels of phosphorylated p38 and p44/p42 MAPK in IDR-1002-stimulated monocytes were determined by measuring the geometric MFI levels of 1000 cells, divided by the geometric MFI levels of untreated monocytes, resulting in a fold-change-over-baseline measurement.

2.3. RESULTS

2.3.1. IDR-1002 regulation of chemokine production

Previous studies indicated that cationic peptides modulate the production of chemokines. To determine whether this effect was seen in a human system with IDR-1002, an *ex vivo* model using PBMCs isolated from human blood was established. Human PBMCs were stimulated with IDR-1002, and the levels of various chemokines in culture supernatants were measured by ELISA 24 hours after stimulation. IDR-1002 elicited an increase in the production of chemokines GRO-α and MCP-1 by human PBMCs (Figure 2.1), consistent with our results from murine *in vitro* models (80). IDR-1002 also demonstrated an ability to induce the production of chemokines IL-8 and MCP-3, a neutrophil and monocyte chemoattractant, respectively (90, 91). Significant induction of MCP-1, MCP-3, and GRO-α was seen at peptide concentrations as low as 20 μg/ml whereas significant IL-8 induction was seen as at peptide concentrations as low as 10 μg/ml. Maximal chemokine induction was seen at the highest concentration of peptide used at 100 μg/ml in all cases. These results demonstrate that IDR-1002 was capable of stimulating protein production of a wide range of chemokines in a human system. It is valuable to note that IDR-peptides at these concentrations μg/ml displayed no cytotoxic effects on human PBMCs as measured by an LDH cytotoxicity assay (data not shown). This is in contrast to natural HDPs, such as LL-37 which exhibits cytotoxicity in PBMCs (unpublished data) and in airway epithelial cells (61) at concentrations higher than 20 μg/ml.

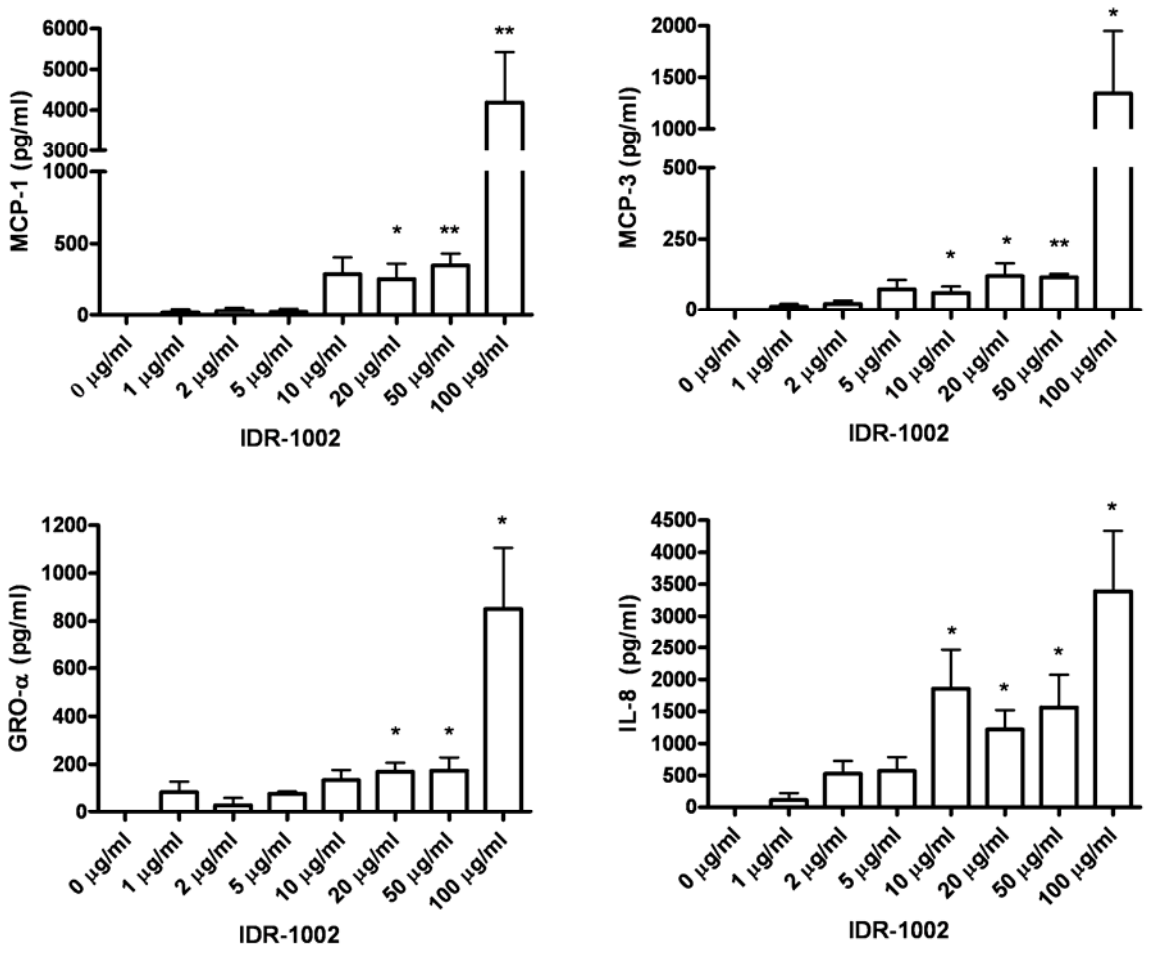

Figure 2.1 IDR-1002 stimulation of chemokine production by human PBMCs. PBMCs were stimulated with different concentrations of IDR-1002. 24 hours after stimulation, culture supernatants were collected and chemokine levels were measured by ELISA. Data are presented as the mean chemokine levels (± SE) of at least 4 independent experiments, each from independent donors. Statistical comparisons to untreated controls were done by Student's two-tailed t test. *$p < 0.05$; **$p < 0.01$.

2.3.2. IDR-1002 regulation of inflammatory cytokine production

As previously mentioned, the onset of a bacterial infection is rapidly met by the production of a wide range of cytokines, a major component of the initiation of innate immune responses. The elevation of inducible chemokines in an infection is often associated with, and caused by, an elevation of inflammatory cytokines, such as IL-6 and TNFα which act to initiate numerous inflammatory cell processes, such as the activation of immune cells and secondary production of inflammatory mediators. The ability of IDR-1002 to modulate

the production of these cytokines was investigated. Human PBMCs were stimulated with varying doses of IDR-1002 and after 24 hours cytokine levels in the culture supernatant were measured by ELISA. Interestingly, cultures of human PBMCs stimulated with IDR-1002 showed minimal production of IL-6 and TNFα even at a peptide concentration of 100 μg/ml (Figure 2.2). In contrast, stimulation of PBMCs with bacterial LPS resulted in a robust elevation of pro-inflammatory cytokine levels after 24 hours as expected. The minimal effect of IDR-1002 on IL-6 and TNFα production, despite its induction of chemokine levels, revealed the ability of IDR-1002 to selectively regulate chemokine production in human PBMCs.

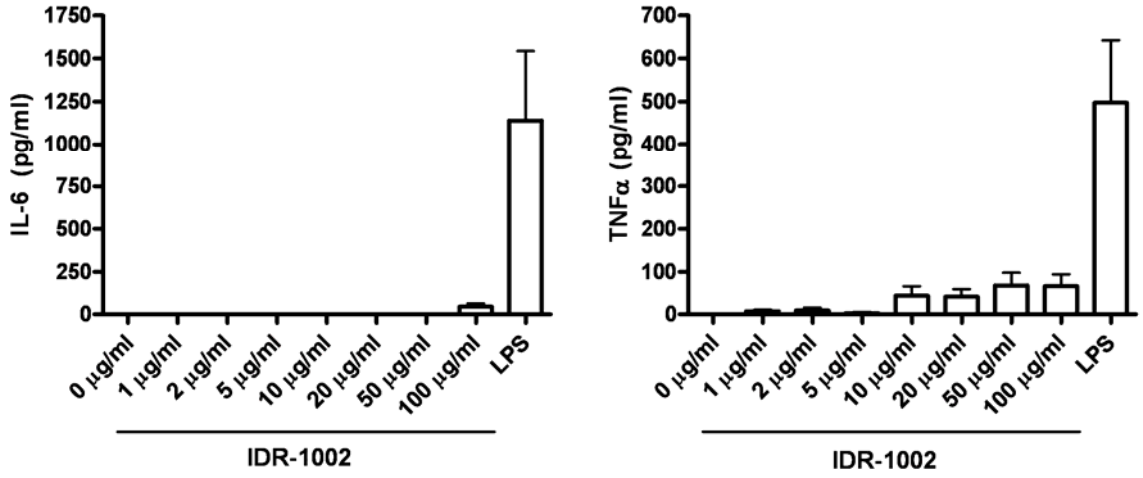

Figure 2.2 IDR-1002 stimulation of pro-inflammatory cytokine production by human PBMCs. PBMCs were stimulated with different concentrations of IDR-1002 or 10 ng/ml of LPS. Twenty four hours after stimulation, culture supernatants were collected and levels of inflammatory cytokines TNFα and IL-6 were measured by ELISA. Data are presented as the mean cytokine levels (± SE) of at least 4 independent experiments, each from independent donors.

2.3.3. Signalling pathway dependency of IDR-1002-mediated cytokine regulation

IDR-1002 induced the cellular production of numerous chemoattractants in both human and murine models. Therefore it was of interest to determine the mechanism by which it did this. The production of cytokines in response to an infection is mediated by a wide array of cellular signalling networks. In the context of immunity, these networks are

triggered upon the detection of pathogenic signatures or endogenous host signals; the resultant signalling cascades lead to the production of cytokines among other effects. To investigate whether IDR-1002 utilized any of these networks to elicit its cytokine-inducing activity, inhibitors specific for pathways essential in the immune response were utilized. Human PBMCs were pre-treated with chemical inhibitors or a DMSO vehicle control for 1 hour, prior to stimulation with IDR-1002. As before, the ability of IDR-1002 to induce PBMC production of MCP-1 was assayed by ELISA 24 hours post stimulation. Pre-treatment with a DMSO control had no significant effects on the ability of IDR-1002 to induce MCP-1 production (Figure 2.3). However, inhibition of pertussis toxin-sensitive G-protein signalling and G-protein-coupled receptor (GPCR) FPRL-1 knocked down peptide-induced MCP-1 production completely and by more than 60% respectively, an effect nearing significance in the latter case. FPRL-1 function, as well as pertussis toxin-sensitive G-protein signalling play an important role in LL-37-mediated chemotaxis and angiogenesis (40, 66). Inhibition of numerous essential immune mediators gave similar results. Inhibition of the NFκB pathway resulted in a knockdown of MCP-1 production of greater than 60% and inhibition of the MAPK family members, p38 and MEK-1, showed a near-complete knockdown of peptide-mediated MCP-1 production. A similar effect was seen in PBMCs pre-treated with a PI3K inhibitor. Parallel studies showed that, in contrast, inhibition of JAK-STAT, JNK, PKA, and CaMKII signalling had no effects on IDR-1002-induced MCP-1 production (80). The extensive inhibition of chemokine induction suggested that IDR-1002 was impacting on a diverse subset of immune signalling pathways to elicit its cytokine-regulating properties.

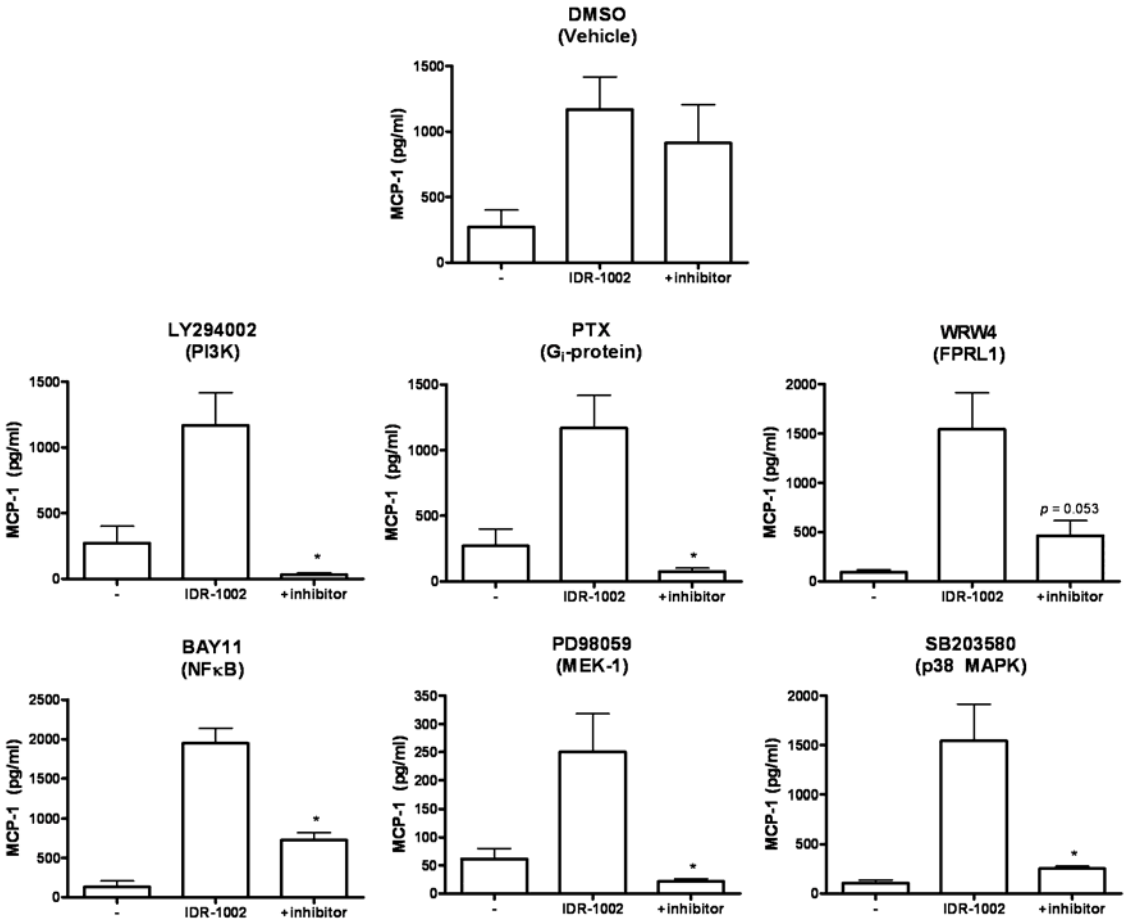

Figure 2.3 The role of immune signalling pathways in IDR-1002 induction of MCP-1 production. PBMCs were pre-treated for 1 hour with a G_i-protein inhibitor (pertussis toxin, 100ng/ml), FPRL-1 antagonist (WRW4, 10μM), PI3K inhibitor (LY294002, 10μM), NFκB inhibitor (BAY11, 1μM), MEK-1 inhibitor (PD98059, 10μM), p38 MAPK inhibitor (SB203580, 10μM), or 0.02% DMSO. PBMCs were then stimulated with 100 μg/ml of IDR-1002 for 24 hours. Levels of MCP-1 in culture supernatants were measured by ELISA. Data are presented as the mean MCP-1 levels (± SE) of at least 3 independent experiments, each from independent donors. Statistical comparisons between peptide treatments and peptide with inhibitor treatments were done by Student's two-tailed *t* test. *$p < 0.05$.

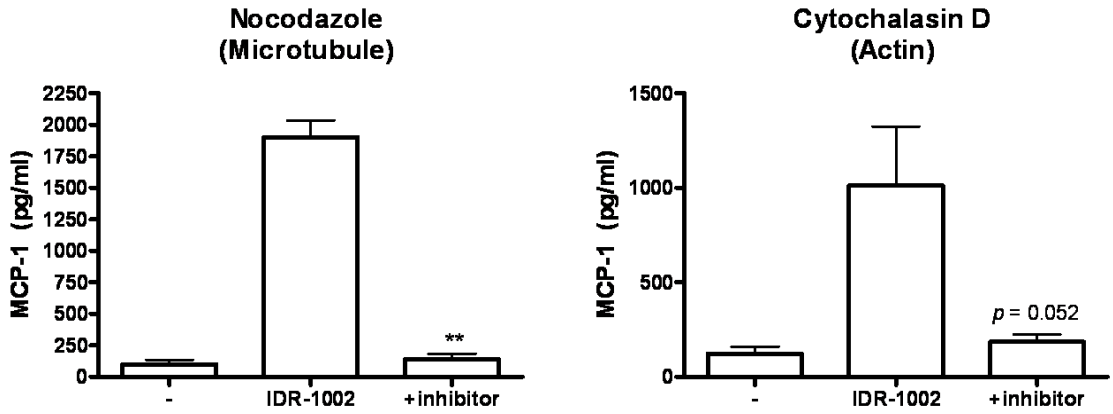

Figure 2.4 The role of actin and microtubule assembly in IDR-1002 induction of MCP-1 production. PBMCs were pre-treated for 1 hour with a microtubule assembly inhibitor (nocodazole, 500 ng/ml) or an actin polymerization inhibitor (cytochalasin D, 500 ng/ml). PBMCs were then stimulated with 100 μg/ml of IDR-1002 for 24 hours. Levels of MCP-1 in culture supernatants were measured by ELISA. Data are presented as the mean MCP-1 levels (± SE) of at least 3 independent experiments, each from independent donors. Statistical comparisons between peptide treatments and peptide with inhibitor treatments were done by Student's two-tailed *t* test. **$p < 0.01$.

Recent studies have demonstrated that LL-37 also utilizes a comparable range of signalling networks to mediate its chemokine-inducing properties (92). LL-37 and IDR-1 have also been shown to act on intracellular targets after internalization (93). It seemed likely that IDR-1002 was also internalized, allowing it to act on intracellular targets. The ability to interact with intracellular mediators as well as cell-surface components would provide a plausible explanation for the seeming promiscuity of IDR-1002 in utilizing multiple pathways. To investigate this possibility, human PBMCs were pre-treated for 1 hour with inhibitors of microtubule and actin polymerization, both important in cellular uptake processes and endocytosis, prior to stimulation with IDR-1002. As previously described, levels of MCP-1 production induced by peptide were measured by ELISA 24 hours post stimulation. The inhibition of microtubule assembly eliminated the ability of IDR-1002 to induce MCP-1 production (Figure 2.4). Similarly, inhibition of actin polymerization resulted in a strong knockdown of peptide-induced MCP-1 production, although this effect was not statistically significant. These results suggest that cytoskeletal rearrangement is necessary for IDR-1002-mediated cytokine regulation, potentially through its active uptake into the cell and downstream effects on intracellular interacting partners.

2.3.4. IDR-1002 activation of MAP Kinase signalling in human monocytes

As cellular signalling pathways played an important role in IDR-mediated chemokine induction, the ability of IDR-1002 to modulate monocyte signalling activity was examined. This specific cell population within human PBMCs was a natural target to examine for a number of reasons. In addition to the specific dependency of IDR-mediated protection on the monocyte population, the observation of increased chemokine gene expression by murine macrophages stimulated with IDR-1002 *in vitro* further suggests that IDR-1002 may modulate the host cytokine environment via regulation of monocyte/macrophage responses (80). Thus, it was hypothesized that IDR-1002 can modulate monocyte/macrophage signalling behaviour, therefore leading to these effects.

Human PBMCs were stimulated with 100 μg/ml of IDR-1002 and assessed for the levels of intracellular phosphorylated p38 (T180/Y182) and ERK-1/2 (T202/T204). Antibodies against CD14 and specific gating allowed for the measurement of intracellular phosphorylated MAPK mediators specifically in the monocyte population. After 30 minutes, monocytes stimulated with IDR-1002 exhibited a significant, approximately 2-fold increase in phospho-p38 and phospho-ERK-1/2 levels relative to unstimulated monocytes, indicative of the activation of the p38 and ERK-1/2 MAPK pathways (94, 95) (Figure 2.5). This observation supported the pathway inhibitor experiments in showing a dependency of peptide-mediated chemokine induction on the MAPK pathways, and suggested that MAPK mediators are functionally activated by IDR-1002. No significant changes in MAPK phosphorylation between peptide treatments and control treatments were seen in the CD14-negative population (data not shown). Importantly, these results demonstrated that, in principle, IDR-1002 was a regulator of monocyte signalling behaviour.

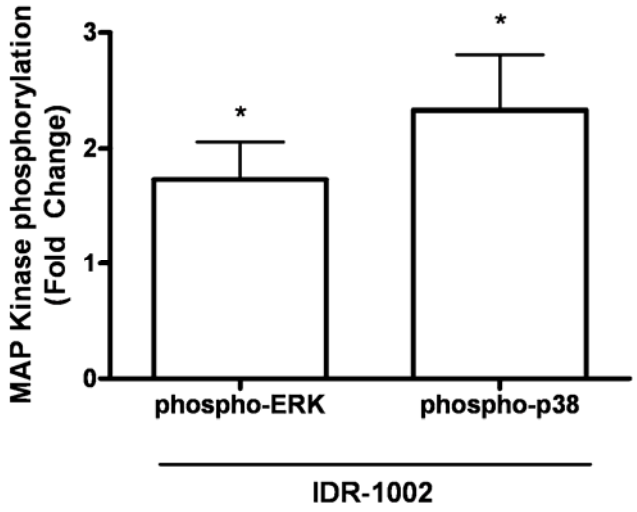

Figure 2.5 IDR-1002 activation of MAP Kinase pathways in human monocytes. PBMCs were stimulated with 100 μg/ml of IDR-1002 for 30 minutes. PBMCs were stained with phospho-specific antibodies against p38 MAPK and ERK-1/2. Monocyte levels of phosphorylated MAPK mediators were analyzed by flow cytometry, gating on the $CD14^+$ monocyte population. Data are presented as mean fold-increases in MAPK phosphorylation over untreated cells (± SE) of 4 independent experiments, each from independent donors. Statistical comparisons between IDR-1002 stimulated cells and untreated controls were done by Student's two-tailed *t* test. *$p < 0.05$.

2.4. DISCUSSION

The intent of this study was to characterize how IDR-1002 regulates the cytokine environment, furthering our understanding of how IDR-peptides can modulate host immune responses. In this investigation, IDR-1002 demonstrated an ability to selectively induce the production of cytokines by human PBMCs; specifically, IDR-1002 stimulated the production of chemokines IL-8, GRO-α, MCP-1, and MCP-3 while the production of inflammatory cytokines TNF-α and IL-6 was unaltered. These observations correlated favourably with the effects of IDR-1002 seen in murine models in studies done in collaboration with Dr Anastasia Nijnik and others (80). IDR-1002 stimulated the production of chemokines by peritoneal lavage cells in *ex vivo* models, and upregulated chemokine gene expression in *in vitro* models using bone marrow-derived macrophages. *In vivo* infection studies showed similar results. During an *S. aureus* infection, pre-treatment with IDR-1002 resulted in an

enhanced production of murine chemokines at the site of infection, while levels of inflammatory regulators TNFα and IL-6 remained unchanged, as I have shown in the human PBMC system. The very strong correspondence of effects in murine and human models further reinforced the likelihood that these observations are functionally important since the innate immune systems of these two species is reasonably divergent, especially in the chemokine arm of innate immunity (96, 97)

My investigation, in addition to supporting the observations seen *in vivo*, further expands our understanding of how IDR-1002 enhances innate immune responses. While our collaborative infection studies linked the enhancement of monocyte and neutrophil recruitment to the augmentation of MCP-1 and GRO-α production, the studies presented in this thesis suggest a multi-pronged mobilization of leukocytes to the site of infection via the production of an array of chemokines. The relationship between multiple chemokines in the context of antimicrobial innate immunity is complex. Despite the similarity in function of cell-specific chemoattractants, multiple seemingly redundant chemokines are often required for a proper antimicrobial response. For instance, in murine models of *Listeria monocytogenes* infection knockout mice for either MCP-1 or MCP-3 show impaired monocyte recruitment as well as increased susceptibility to infection, demonstrating the need for multiple chemokines for an optimal antimicrobial response (98). My study has revealed that IDR-1002 stimulates the production of a range of chemokines, each one potentially playing major roles in IDR-mediated protection against infection.

Considering the ability of IDR-1002 to induce the production of numerous chemokines without the stimulation of general inflammatory mediators, it is tempting to state that the primary immunomodulatory function of IDR-1002 is an enhancement of cell migration. This hypothesis is supported by a number of *ex vivo* and *in vitro* studies regarding IDR-1002. Investigations into leukocyte microbicidal indicators, including activation of neutrophils and macrophages, phagocytosis, and production of nitric oxide and reactive oxygen species, showed minimal regulation by IDR-1002. However indirect potentiation of antimicrobial activities by IDR-1002 cannot be discounted, for instance IDR-1002 enhanced the production of the cell-activating cytokine IFNγ during *S. aureus* infections but not in *ex vivo* or *in vitro* models (80). The implication is that IDR-1002 likely reacts differently in an infection system, potentially acting in synergy with endogenous host mediators or even

microbial components to modulate immune responses. This phenomenon is seen with both endogenous host defense peptides and IDR-peptides. LL-37, a moderate inducer of chemokine production alone, drastically enhances chemokine production and immune signalling in human PBMCs in the presence of the endogenous inflammatory mediator IL-1β or cytokine GM-CSF, correlating with an enhancement of IL-1β-induced IκBα phosphorylation and NFκB nuclear translocation (43, 50). In addition, LL-37, in combination with self-DNA, acts as a stimulator of interferon production in plasmacytoid dendritic cells (99). Similarly, synergy between IDR-HH2, a structurally-related IDR with similar chemokine inducing properties as IDR-1002, and CpG oligonucleotides results in a robust enhancement of chemokine production in human PBMCs (84, 100). However, although there may be other unreported immunomodulatory effects of IDR-1002, the results in this section advocate the modulation of chemokine production and enhancement of cell migration as a major axis in IDR-mediated immunomodulation.

Following the characterization of IDR-1002 cytokine regulation, the mechanism through which this occurs was investigated. Cellular reactions to regulatory agents, whether from endogenous agents such as cytokines or HDPs, or exogenous agents such as microbial components, are governed by complex signal transduction pathways. Regulation of cytokine gene expression and protein production are no exceptions. It was hypothesized that IDR-1002 exhibited an effect on cytokine levels in similar ways. I investigated the role of pathways known to be used by endogenous HDPs, such as G-protein-related cascades (40, 42, 46, 82, 101), and showed they were implicated in IDR-1002-mediated MCP-1 induction. Studies into downstream pathways determined that the central inflammatory signalling pathways NFκB, PI3K, p38 and ERK-1/2 MAPK were all involved in IDR-induced MCP-1 production in human PBMCs, implicating these pathways as targets for IDR-1002 regulation. This finding correlates with the observation that IDR-1 induction of chemokine production is dependent on the NFκB, PI3K, and MAPK pathways (79). The involvement of multiple pathways is not altogether unexpected as cytokine expression is dependent on the coordinated actions of numerous pathways, such as, for example, the activation of transcription factors required for optimal gene expression. This may account for the interesting observation that IDR-1002, while inducing and utilizing pro-inflammatory pathways, did not induce the production of the pro-inflammatory cytokines IL-6 and TNFα,

which are regulated by similar pathways. The signal transduction profile induced by IDR-1002 may be sufficient to promote the production of chemokines, yet insufficient to significantly induce the expression and activation of certain pro-inflammatory cytokines. As mentioned, this fine control of cytokine production likely originates from the integration of regulation by multiple signal transduction pathways on numerous host processes, such as transcription factor activity and cytokine message stability to name a couple. Our understanding of the methods by which IDR-peptides and HDPs affect immune signalling activity remains in its infancy. The upstream receptors of IDR-peptides which coordinate this IDR-induced signal regulation have not yet been fully elucidated. However, intracellular targets for IDR-peptides have been suggested, due to the necessity of actin polymerization and microtubule formation, both required for cellular uptake and endocytic processes, in IDR-1002-mediated chemokine production. It is possible that endosome formation and intracellular trafficking, mediated by actin and microtubules respectively, facilitate cellular uptake and localization of IDR-peptides whereby they act on host targets. Indeed certain intracellular IDR-peptide receptors have been identified, including the cellular scaffold protein sequestosome-1/p62 and GAPDH, both important for IDR-1-mediated activation of p38 MAP Kinase (93, 102). These observations demonstrate that the interaction of IDR-peptides with endogenous molecules can result in the downstream regulation of signalling. Further studies concerning the intracellular trafficking of IDR-peptides, and how this is affected by actin and microtubule activity, are required; these findings would be of interest in identifying downstream peptide-targets. Certain structural characteristics shared both by HDPs and synthetic IDR-peptides, including their small size, net positive charge, and amphiphilic properties, suggest the potential for numerous overlapping interacting partners. Indeed, HDPs have been shown to utilize different receptors, including chemokine receptors, to regulate different aspects of immunity (66, 103-105). Therefore, it is speculated that IDR-peptides, akin to endogenous HDPs, likely utilize a diverse range of endogenous receptors to regulate distinct aspects of the immune response, including the regulation of monocyte integrin function and adhesion.

The major aim of this chapter was to elucidate the ability of IDR-1002 to regulate cytokine production, in hopes it would increase our understanding of how IDR-peptides modulate immunity and, as a result, antimicrobial protection. This investigation has shown

that IDR-1002 is an inducer of numerous chemokines, and thus cell migration, through regulation of cellular signalling pathways. Yet, the ability of IDR-1002 to regulate chemokine production, cell migration, and cellular signalling suggests a deeper, more comprehensive, regulation of immunity by IDR-peptides. In the grand network of immunity, chemokine-mediated cellular recruitment forms the basis for a host of essential immune functions. Although the recruitment of effector cells, such as monocytes and neutrophils, is indeed required for innate immune microbial clearance, trafficking of these cells in addition to primed lymphocytes to the site of infection is required for the assertion of adaptive immune functions (106). The augmented production of RANTES, a chemoattractant and activator of T-lymphocytes (107), by IDR-1002 during *S. aureus* infection could facilitate adaptive immune responses. Similar induction by IDR-peptides of other lymphocyte chemoattractants is certainly feasible and would be of investigative interest. Proof-of-principle studies have shown that IDR-peptides, especially IDR-1002, can serve as adjuvant components in vaccine formulations, augment antigen-specific immune responses, as well as modulate T-helper-cell balance (84, 108, 109). Cell recruitment is also an essential process for wound healing and resolution. Recruitment of effector cells responsible for clearance of cellular debris and tissue remodeling is essential for wound repair (110). Mobilization of epithelial cells to wounded sites, prior to their proliferation, is necessary for wound closure (111, 112). As previously mentioned, human defensins and LL-37 are promoters of wound healing, in part due to their ability to stimulate epithelial cell migration (61, 113). Chemokines themselves process secondary functions, involved in cellular activation and growth (114, 115). Thus, through these consequential processes stemming from chemokine regulation and cell mobility, the potential for immunomodulation by IDR-1002 is vast and must be further investigated.

In summary, this study has demonstrated that IDR-1002 regulates cellular cytokine production through its modulation of cellular signalling pathways; this expands upon a proposed mechanism of IDR protection, whereby bacterial clearance is promoted due to an enhancement of leukocyte recruitment. It must be emphasized that this thesis demonstrates that the immunoregulatory effects of IDR-1002 also occur in a human system, a requisite observation in the context of clinical drug development of anti-infective IDR-peptides. In addition, the similarities in IDR-mediated cytokine regulation between murine and human

systems provides support that investigations in a murine system can yield viable insights regarding IDR-mediated immunomodulation in a human system, and vice versa. These results also expose a widespread utilization of immune signalling pathways and a modulation of monocyte signalling behaviour, a cell population necessary for IDR-mediated protection, revealing potential avenues by which IDR-peptides may modulate host immunity. Overall, this study hints at a complex regulatory role of IDR-1002 in immunity and, most importantly, provides a foundation from which further mechanistic investigations can be undertaken.

3 IDR-1002 PROMOTES HUMAN MONOCYTE MIGRATION ON FIBRONECTIN VIA INDUCTION OF INTEGRIN-MEDIATED ADHESION

3.1. INTRODUCTION

The enhanced clearance of pathogenic bacteria by IDR-1002 in murine infection models demonstrates the effectiveness in modulating host immunity to augment natural antimicrobial responses. Antimicrobial protection mediated by IDR-peptides is accompanied by the *in vivo* regulation of host cytokine production, as well as the enhancement of leukocyte recruitment to the site of infection (79, 80). In the previous chapter IDR-1002 was revealed to have extensive effects on cellular chemokine production through its modulation of numerous signal transduction pathways involved in the innate immune response. The ability of IDR-peptides to regulate cytokine production is currently under intense investigation in the hopes of further understanding their complex regulatory effects on host immunity. Indeed this aspect of IDR-regulation has been harnessed and developed for other anti-infective purposes, for instance the use of IDR-peptides as adjuvants in vaccine formulation due to their ability to boost vaccine cytokine production (84, 100, 108). It was hypothesized here that the second hallmark effect of IDR-peptides, the capability to enhance leukocyte recruitment, also stems from its ability to induce host chemokine production at the site of infection. However, while the induction of chemokine production by IDR-1002 is often observed hours after administration in *in vivo* murine models and *in vitro* human PBMC models, the promotion of leukocyte recruitment is a very rapid process, observable as soon as 1 hour after administration (80). These observations are consistent with the ability of IDR-1002 to directly regulate cellular motility, perhaps independent of its influence on host chemokine production. The study reported in this chapter thus aimed to uncover undiscovered new mechanisms whereby IDR-1002 could directly modulate monocyte migratory behaviour. The elucidation of such mechanisms not only furthers our understanding of how IDR-peptides mediate their antimicrobial protective effects, but is critical to the development of improved anti-infective immunomodulatory agents.

Trafficking of monocytes and other immune cells in response to microbial invasion is an immensely complex process. Cellular recruitment in the innate immune response is mediated by a multi-stage process that serves to draw cells from the circulatory system and rapidly direct them to specific areas where their antimicrobial immune functions are required. In the initial stages of infection the detection of microbial patterns by sentry cells, such as macrophages and dendritic cells, at the invasion site results in the production of a variety of inflammatory mediators and chemoattractive compounds (3, 5, 116). Activation of proximal endothelial cells by these inflammatory mediators leads to the increased expression of selectins and integrins, which serve to capture circulating leukocytes (117-119). Immune cells initially interact weakly with endothelial cells lining the vasculature, evidenced by their rolling along the endothelia, and are further activated by interaction with chemokines presented at the endothelial surface. This activation results in the firm adhesion and arrest of cells, preliminary to cellular transmigration or extravasation across the endothelial layer. Once past the endothelial barrier, cells then respond to a chemokine gradient and migrate towards the site of infection, culminating in a rapid influx of effector cells to the site of infection or injury. Regulation of any of these processes can have significant effects on cellular recruitment. However, the current investigation focused on monocyte adhesive behaviour and aimed to elucidate IDR-mediated effects on monocyte migration under this context.

Adhesion is a major theme in the process of cellular trafficking and, as such, mediators of cellular adhesion play a crucial role in leukocyte recruitment (Figure 3.1). Low-affinity adhesion, mediated between selectins and their receptors, is necessary for the initial capture of circulating leukocytes (118). Firm integrin-mediated adhesion is then necessary for the arrest of rolling leukocytes and allows for their extravasation into tissues. Within this environment, adhesion also plays a central role in cellular motility. The extracellular matrix (ECM) provides a tissue structural support network comprising of fibrous meshes of proteins and polysaccharides, including, but not limited to, collagen, fibronectin and glycosaminoglycans. Integrin-mediated interactions with the ECM provide a foothold from which cells can apply directional force that is essential for oriented movement (118, 120). Furthermore, cell-to-ECM interactions are essential for secondary signalling from integrin-mediated signal transduction cascades or from chemokines and growth factors bound and

presented on the matrix itself (121, 122). These signalling cascades not only serve to modulate and direct cellular movement, but also play important roles in adhesion-mediated tissue remodeling, cell growth, and wound healing. As such, the interaction between monocytes and their environment, by virtue of adhesion, plays a fundamental role in monocyte recruitment to the site of infection.

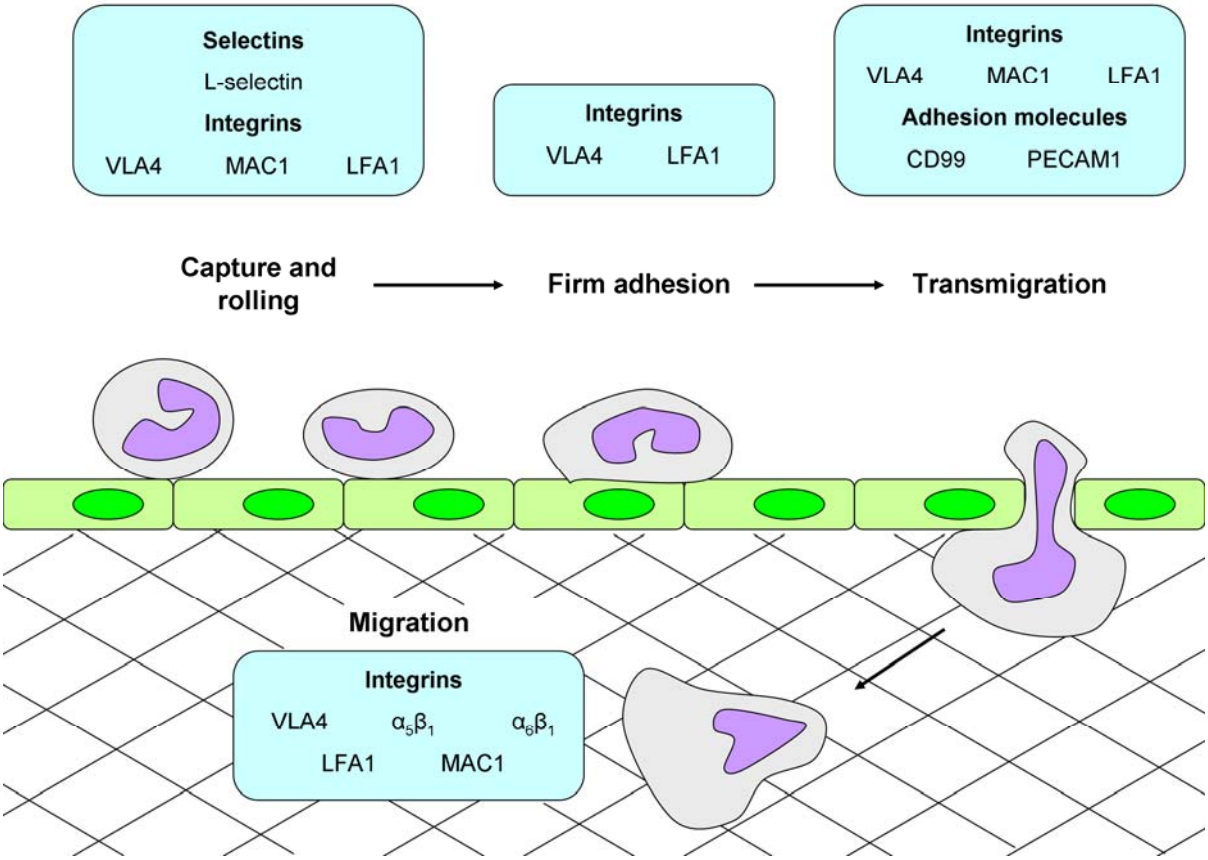

Figure 3.1 Leukocyte adhesion molecules regulating the multiple stages of cellular trafficking. This figure was partially adapted from Ley *et al.* (2007) with permission (118).

As a key process in leukocyte recruitment, the regulation of cellular adhesion by IDR-1002 was investigated as a potential mechanism for promoting monocyte migration in *in vivo* models. In this chapter, it was demonstrated that, unlike some host defence peptides, IDR-1002 possessed no direct chemokinetic or chemoattractive ability for human monocytes. However, IDR-1002 could augment monocyte migration through a membrane coated with fibronectin, an ECM glycoprotein which plays an important role in monocyte locomotion

(120, 123). Specifically, it was shown that IDR-1002 enhanced monocyte migration towards endogenous chemokines MCP-1, MCP-3, MIP-1α, and RANTES by promoting monocyte adhesion to fibronectin. The pro-adhesive effect elicited by IDR-1002 was dependent on the function of the β1-integrin family of receptors, one of the major monocyte adhesive receptors for fibronectin. These phenomena also correlated with the peptide-induced activation of β1-integrins, an effect mediated by the activation of the PI3K-Akt pathway. IDR-1002 also demonstrated the ability to promote monocyte adhesion to ICAM-1, an adhesion molecule essential in the firm adhesion between leukocytes and the endothelial surface (118). Correspondingly, IDR-1002 was shown to increase the surface expression of β2-integrin subunit CD11c, a monocyte receptor for ICAM-1 (124, 125). In addition, it was also found that promotion of monocyte adhesion to fibronectin was likely a function shared among IDR-peptides and endogenous immunomodulatory peptide LL-37. Overall, this study reveals a novel mechanism whereby IDR-1002, through its regulation of signal transduction pathways, directly enhances monocyte recruitment; namely the promotion of integrin-mediated monocyte adhesion. Moreover, the knowledge that IDR-peptides regulate monocyte adhesion provides insights into the mechanisms through which IDR-peptides potentially modulate adhesion-dependent immune processes, such as cellular growth and wound healing.

3.2. METHODS AND MATERIALS

3.2.1. Reagents

Peptides LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-NH_2), IDR-1002 (VQRWLIVWRIRK-NH_2), IDR-HH2 (VQLRIRVAVIRA-NH_2), and IDR-1018 (VRLIVAVRIWRR-NH_2), and were synthesized by solid phase F-moc chemistry by CPC Scientific (Sunnyvale, CA). IDR-1 (KSRIVPAIPVSLL-NH_2) and negative control peptide 1035 (KRWRWIVRNIRR-NH_2) were similarly synthesized by the Biomedical Research Center (University of British Columbia, Vancouver, BC, Canada). PMA was resuspended in DMSO (both from Sigma-Aldrich, St. Louis, MO) and stored at -20 ˚C. PI3K inhibitor LY294002, Akt inhibitor SH-5, p38 MAPK inhibitor SB203580, and MEK1 MAPK inhibitor PD98059 (all from Calbiochem, San Diego, CA) were resuspended in DMSO and stored at -20 ˚C. In inhibitor experiments, the concentrations of DMSO used never exceeded 0.1% (v/v)

in cell cultures. G_i-protein inhibitor, pertussis toxin, was also obtained from Calbiochem. Chemokines MCP-1, MCP-3, MIP-1α, and RANTES were obtained from R&D Systems (Minneapolis, MN).

3.2.2. Cell isolation and culture

Human PBMCs were isolated as previously reported (44). Briefly, venous blood was collected from healthy volunteers using heparin-containing Vacutainer tubes (BD Bioscience, San Jose, CA) in accordance with UBC ethical approval and guidelines. Human blood was diluted in an equal amount of PBS (Invitrogen, Carlsbad, CA) then layered over Ficoll-Paque Plus (Amersham, Piscataway, NJ) prior to separation by density-gradient centrifugation. The mononuclear cell layer was extracted and washed twice with PBS. The monocyte population was enriched by negative selection magnetic bead purification from the PBMCs using the Easy-Sep Human Monocyte Enrichment Kit (Stemcell Technologies, Vancouver, BC, Canada) as per the manufacturer's instructions. Monocytes were then resuspended in RPMI 1640 with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Invitrogen), placed in a polypropylene tube (BD Falcon, San Jose, CA), and cultured in a humidified incubator at 37 °C with 5% CO_2. The human monocytic cell line THP-1(126), from the American Type Culture Collection (ATCC TIB-202, Manassas, VA), was cultured in the same media and conditions, as stated above, for a maximum of five passages.

3.2.3. Monocyte adhesion assay

Each well of polystyrene tissue-culture plates (BD Falcon) were coated overnight at 4 °C with 50 μg/ml of human plasma fibronectin (Calbiochem) or 2 μg/ml of recombinant human ICAM-1 (R&D Systems) per well. The wells were then washed with PBS prior to blocking with PBS containing 1% (w/v) BSA (Roche, Basel, Switzerland) for 1 hour at 37 °C. Wells were washed again with PBS prior to use. Wells without fibronectin or ICAM-1 were also blocked with 1% BSA as controls for non-specific adhesion.

Human blood monocytes and THP-1 cells were resuspended in RPMI 1640 with 1% FBS at a density of $5x10^5$ cells/ml for 1 hour. For PI3K-Akt inhibition studies, cells were then pre-treated with chemical inhibitors, or a DMSO vehicle control, at the indicated concentrations for 1 h. Similarly for β1-integrin blocking studies, cells were pre-treated with an anti-human β1-integrin functional blocking mAb (P4C10, Millipore, Billerica, MA) at the indicated concentrations or 20 μg/ml of a mouse IgG1 isotype Ab (MG1-45, Biolegend, San Diego, CA) for 1 h. $5x10^4$ cells were added to each well and stimulated with the indicated treatments. phorbol myristate acetate (PMA) at 50 ng/ml was used as a positive control for monocyte-fibronectin binding in some experiments. The cells were incubated at 37 °C for 3 h, unless otherwise stated. The cells were then removed and the wells washed twice with PBS prior to upside-down centrifugation at 50x g for 5 min to remove non-adherent cells. Wells were washed twice with PBS and adherent cells were stained with the Diff-Quik Staining Kit (VWR Scientific Products, Radnor, PA) as per the manufacturer's instructions.

Measurement of adhesion levels were done by averaging the counts of cells over six 200x magnification high power fields (HPF) under phase-contrast microscopy with a light microscope. Counts per HPF of adhered cells on non-protein-coated, BSA-blocked wells were subtracted from counts of protein-coated wells for each treatment to assess fibronectin-specific or ICAM-1-specific monocyte adhesion.

3.2.4. Flow cytometry to determine integrin levels and Akt phosphorylation

All flow cytometry results were collected on a FACSCalibur flow cytometer in conjunction with CellQuest Pro software (BD Biosciences). THP-1 cells were resuspended in RPMI 1640 with 1% FBS at a density of $1x10^6$ cells/ml. $2x10^5$ cells per well were then seeded on tissue culture plates and placed in a humidified incubator at 37 °C and 5% CO_2 for 1 h. For inhibitor studies, THP-1 cells were then pre-treated with chemical inhibitors, or a DMSO vehicle control, at the indicated concentrations for 1 hour. Cells were then stimulated with the indicated treatments for the indicated time-points and incubated at 37 °C for 3 h. Following treatment, THP-1 cells were then fixed with PBS and 4% (w/v) formaldehyde (Fisher Scientific, Pittsburgh, PA) at room temperature for 20 min in preparation for staining.

To investigate integrin surface expression, THP-1 cells were washed using PBS with 0.5% BSA and stained at room temperature for 1 hour with an anti-human CD29 mouse IgG1-Alexa Fluor 647 (TS2/16, Biolegend) Ab, an anti-human CD11c mouse IgG1 Alexa Fluor 647 (3.9, Biolegend) Ab, or a monoclonal Ab (mAb) specific for the active conformation of human β1-integrins (HUTS-4, Millipore). THP-1 cells were also stained, in parallel, with a mouse IgG1 Ab conjugated to Alexa Fluor 647 (MOPC-21, Biolegend) or a mouse IgG2b isotype Ab (MPC-11, Biolegend) as respective isotype controls. For the determination of activated β1-integrin levels, THP-1 cells were then washed and further stained with a goat anti-mouse IgG-DyLight 649 (Poly4053, Biolegend) Ab for 30 min at room temperature. Cells were then washed and resuspended in PBS with 0.5% BSA and 0.5% formaldehyde for analysis. Protein expression levels for each sample were determined by measuring the geometric mean fluorescence intensity (MFI) levels of 1000 cells, subtracted by the geometric MFI levels of their isotype-stained counterparts. The displayed fold-change-over-basal values were calculated by dividing the corrected MFI values of each treatment by the MFI values of the untreated sample of their respective time-point and inhibitor conditions.

To investigate the intracellular levels of phosphorylated Akt, THP-1 cells were washed with 0.5% BSA and permeabilized in 90% (v/v) methanol at 4 °C for 30 min. The cells were then washed and stained at room temperature for 1 hour with phospho-Akt (Ser-473) 193H12 rabbit mAb or a DA1E rabbit IgG isotype mAb (both from Cell Signalling Technology, Danvers, MA). Following washing, the cells were stained with a goat anti-rabbit IgG-Alexa Fluor 647 (H+L, Invitrogen) for 30 min at room temperature. The cells were then washed and resuspended in PBS with 0.5% BSA and 0.5% formaldehyde for analysis. Levels of phosphorylated Akt for each sample were determined by measuring the geometric MFI levels of 1000 cells, subtracted by the geometric MFI levels of their corresponding isotype-stained samples. The displayed fold-change-over-basal values were calculated by dividing the corrected MFI values of each treatment by the MFI values of the untreated sample of their respective time-point and inhibitor conditions.

3.2.5. Fibronectin migration assay

All migration assays were performed using a 48-well microchemotaxis chamber (Neuro Probe). Freshly enriched human peripheral blood monocytes were adjusted to RPMI 1640 with 1% FBS and incubated at 37 °C for 1 h. For inhibitor studies, monocytes were pre-treated with chemical inhibitors, or a DMSO vehicle control, at the indicated concentrations for 1 h. Similarly in β1-integrin blocking experiments, monocytes were pre-treated with 20 μg/ml of a HUTS-4 blocking Ab or an isotype-matched mouse IgG1 control Ab for 1 h. $5x10^4$ monocytes were added to the upper wells of the chamber and indicated concentrations of chemokines in RPMI 1640 with 1% FBS were added to the lower wells. Lower wells containing RPMI 1640 and 1% FBS were used as negative controls for migration. Treatment with IDR-1002 was done by adding peptide to both upper and lower wells to avoid a peptide concentration gradient. The upper and lower wells were separated by a polycarbonate membrane with 5-μM-diameter pores. Membranes were pre-coated with 50 μg/ml of fibronectin at 4°C overnight, washed with PBS, and air-dried prior to use. After 1 hour of incubation non-migrated monocytes were removed by PBS washing and scraping with a rubber blade. Adhered cells on the underside of the membrane were stained with the Diff-Quik Staining Kit

Control experiments assessing the effects of IDR-1002 on monocyte migration independent of chemokines were similarly done. To investigate any direct chemotactic properties of IDR-1002, different concentrations of IDR-1002 were added to the lower wells of the chemotaxis chamber and separated from the monocyte-containing chamber with a non-coated polycarbonate membrane. Likewise, migration experiments investigating the chemotactic or chemokinetic properties of IDR-1002 in a fibronectin system were done by the addition of the indicated amount of peptide to both the upper and lower wells in chemokinesis experiments, and lower wells alone in chemotaxis experiments. The wells were then separated with a fibronectin-coated polycarbonate membrane and processed in the method stated above.

Migration for each treatment was measured by averaging the number of migrated cells per HPF over five fields with each treatment condition done in duplicate. Fold-change over control values were calculated by dividing the average cell count per HPF of each treatment by the average cell count per HPF of basal migration, in media alone.

3.3. RESULTS

3.3.1. Monocyte chemotactic and chemokinetic properties of IDR-1002

To investigate the mechanisms underlying the enhancement of monocyte migration by IDR-1002, an *in vitro* model using human monocytes was established. Initially, the ability of IDR-1002 to directly chemoattract monocytes across an uncoated membrane filter was investigated. This was done by the addition of IDR-1002 to the lower wells of a chemotaxis chambers with the placement of monocytes in the upper wells, separated by a porous polycarbonate membrane. As shown in Figure 3.2, varying concentrations of IDR-1002 elicited no significant change in migration after 1 hour compared to baseline migration towards media-only controls, demonstrating a lack of monocyte chemotactic activity by IDR-1002.

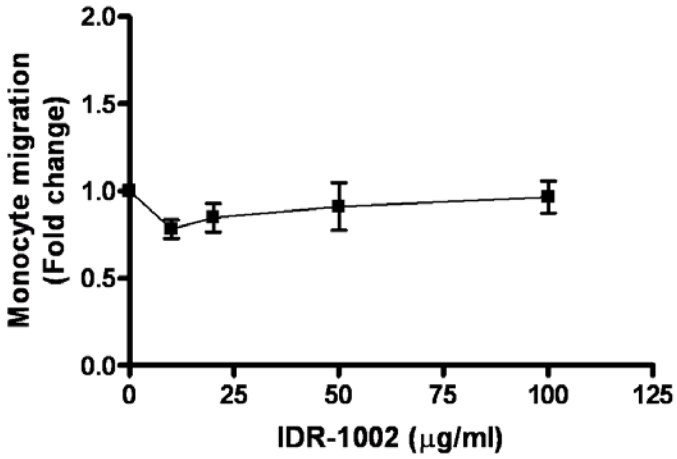

Figure 3.2 Monocyte chemotactic properties of IDR-1002. Human monocytes were assessed for the ability to migrate towards various concentrations of IDR-1002 through a non-coated polycarbonate membrane after 1 hour. Data are presented as mean fold-increases migration over baseline migration towards media alone (± SE) of at least 3 independent experiments, each from independent donors.

The effects of IDR-1002 on monocyte migration through a fibronectin-coated membrane were then assessed. Similarly, after 1 hour, a range of IDR-1002 concentrations in the lower wells of a chemotaxis chamber did not alter monocyte migration across a fibronectin-coated membrane compared to baseline migration towards media-only controls

(Figure 3.3A). Baseline migration of monocytes was estimated to be nearly 2% of the starting monocyte population. This result further demonstrates the minimal monocyte chemotactic properties of IDR-1002. The addition of IDR-1002 to both upper and lower wells also failed to elicit any changes in monocyte migration compared to baseline migration, indicating minimal chemokinetic activity by IDR-1002 as well (Figure 3.3B). Overall, these observations demonstrate that IDR-1002 does not directly modulate monocyte migratory activity.

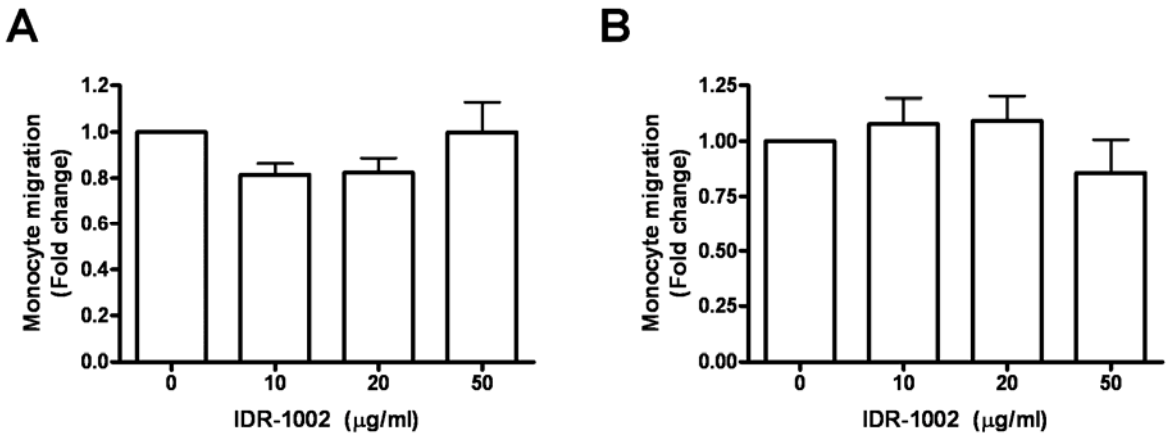

Figure 3.3 IDR-1002 regulation of monocyte migration on fibronectin. (A) The chemotactic properties of IDR-1002 were assessed by the addition of the indicated concentrations of peptide to the lower wells of the chemotaxis chamber, whereas (B) the chemokinetic properties of IDR-1002 were assessed by the addition of peptide to both upper and lower wells. Monocytes were then measured for their ability to migrate through a fibronectin-coated membrane after 1 hour. Data are presented as mean fold-increases in migration over monocyte migration towards media alone (± SE) of 3 independent experiments, each from independent donors.

3.3.2. IDR-1002 enhancement of human monocyte migration on fibronectin

It was then hypothesized that IDR-1002 would regulate the migratory activity of monocytes towards natural mediators of chemotaxis expressed during an infection, such as host chemokines. Therefore the effects of IDR-1002 in cooperation with molecules that are known to promote cell migration were then examined. It was observed that in the presence of fibronectin, IDR-1002 substantially enhanced the ability of human blood monocytes, to migrate towards a range of chemokines. The degree of monocyte migration across a

fibronectin-coated membrane was determined by microscopy after 1 h. As shown in Figure 3.4, varying concentrations of monocyte chemokines MCP-1, MCP-3, MIP-1α, and RANTES increased the migration of monocytes across a fibronectin-coated membrane compared to baseline migration of monocytes in media alone, as expected. In the presence of IDR-1002 the numbers of migrating monocytes towards the chemokines were significantly increased by up to five fold of baseline migration levels, demonstrating an enhancement of monocyte chemotactic activity. In conjunction with the lack of chemotactic or chemokinetic activity by IDR-1002, the observed promotion of migration by IDR-1002 was indicative of the synergistic enhancement of monocyte migratory behaviour on fibronectin towards chemokines.

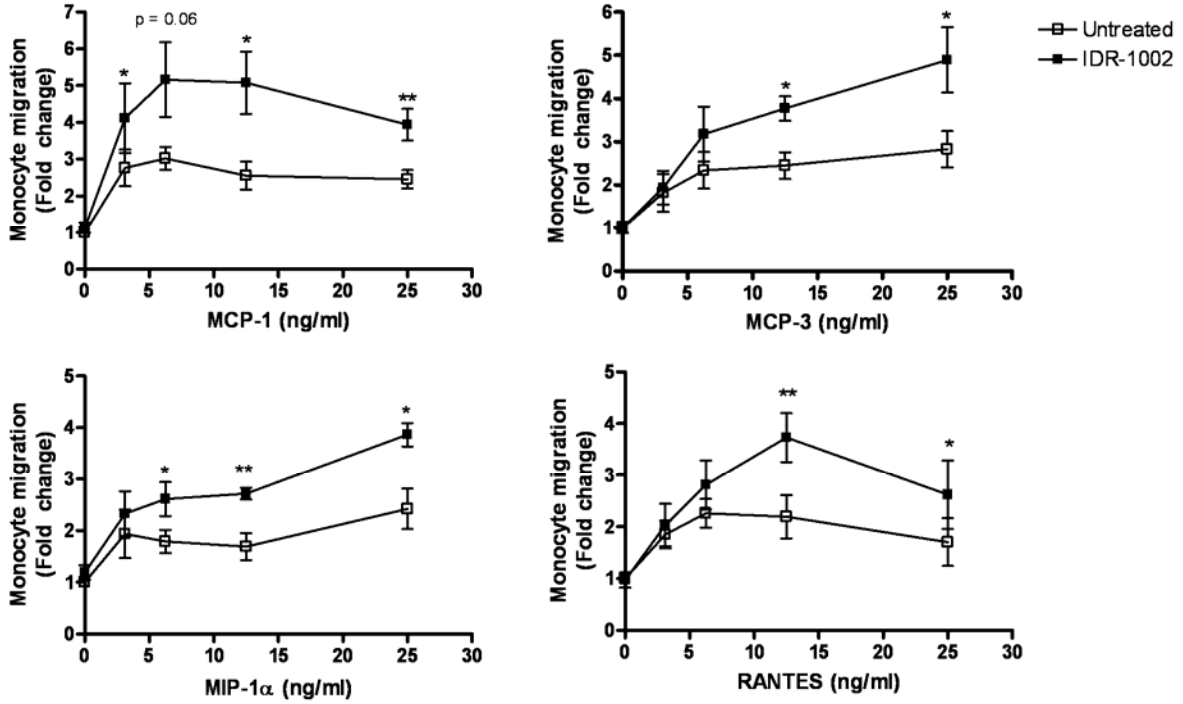

Figure 3.4 IDR-1002 enhancement of monocyte migration towards chemokines on fibronectin. IDR-1002 (20 μg/ml) demonstrated an enhancement of monocyte chemotaxis towards varying levels and types of monocyte chemokines (3.125, 6.25, 12.5, 25 ng/ml) through a fibronectin-coated membrane after 1 hour. Data are presented as mean fold-increases in monocyte migration over baseline migration towards media alone (± SE) of at least 4 independent experiments, each from independent donors. Statistical comparisons between migration levels of IDR-1002 stimulated cells and non-peptide-stimulated cells were done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$.

As IDR-1002 displayed an enhancement of chemotaxis towards all tested chemokines, the ability of IDR-1002 to promote general monocyte-fibronectin interactions was then investigated. Fibronectin is a glycoprotein component of many tissue networks, such as the extracellular matrix. Cellular adhesion to fibronectin is known to facilitate monocyte migration through a number of environments including the basement membrane of the endothelia and the ECM (123). The interactions between monocytes and surrounding substrates, such as endothelial surfaces and ECM components, are highly dependent on the actions of the integrin family of heterodimeric receptors. Monocyte adhesion to fibronectin is primarily mediated by the β1 family of integrins, which include the monocyte adhesion receptors VLA4 and α5β1 (127, 128). Pre-treatment of monocytes with 20 μg/ml of an inhibiting antibody of β1-integrins abolished the enhancing effect of IDR-1002 on monocyte chemotaxis towards MCP-1 and MCP-3, compared to monocytes pre-treated with an isotype-matched antibody control (Figure 3.5). Inhibition of β1-integrin function greatly reduced, but did not eliminate, the augmentation of monocyte migration towards MIP-1α and RANTES by IDR-1002, suggesting alternative mechanisms through which IDR-1002 promotes monocyte migration. Overall, these results show that the β1-integrins play a large role in the promotion of monocyte chemotaxis by IDR-1002, likely through the enhanced interaction between monocytes and the fibronectin-coated membrane. IDR-1002 did not significantly alter migration of isotype-antibody-treated or β1-integrin-treated monocytes in the absence of chemokines (data not shown).

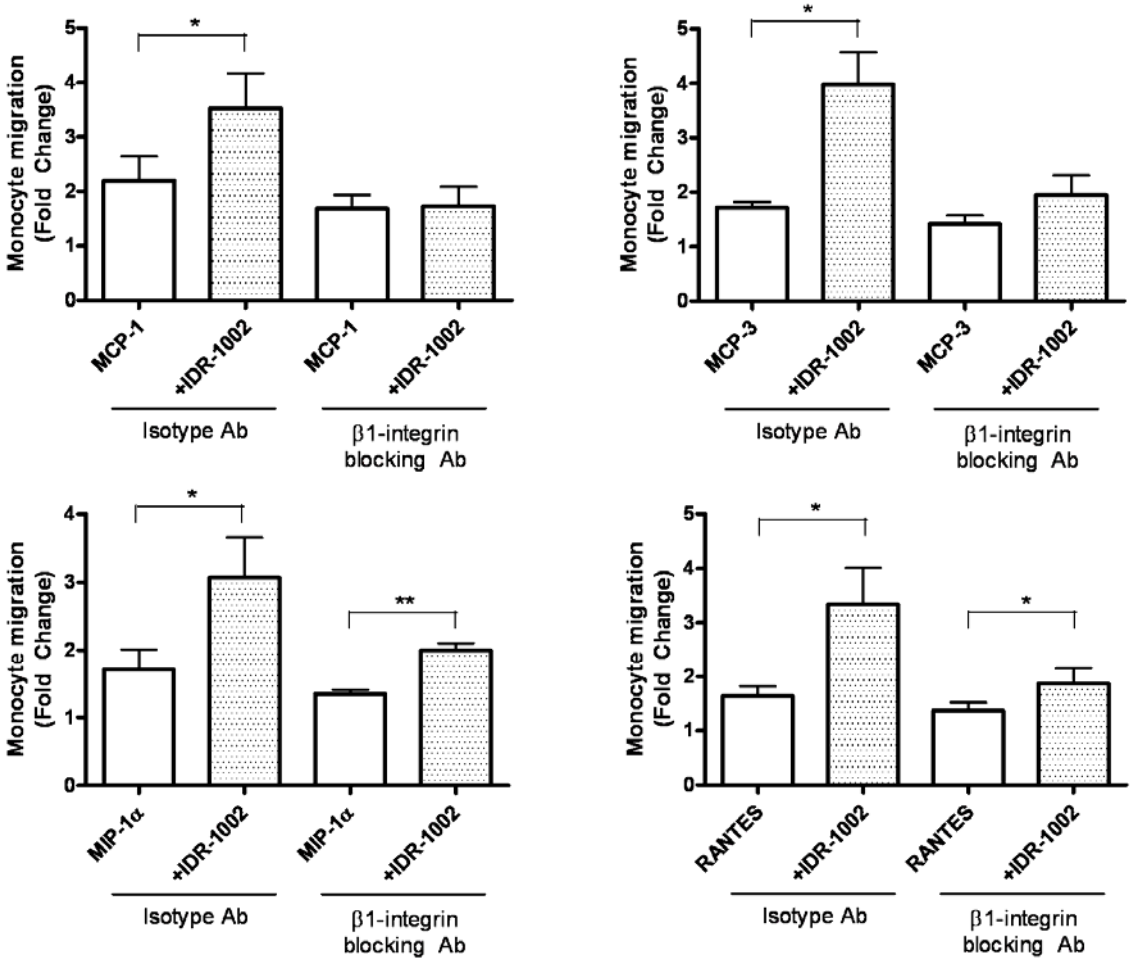

Figure 3.5 The role of β1-integrins in IDR-1002-mediated enhancement of monocyte migration. Monocytes were pre-treated with a β1-integrin inhibiting antibody or an isotype-matched control antibody (20 μg/ml) for 1 hour. Monocytes were then assessed for their ability to migrate towards chemokines (12.5 ng/ml) through a fibronectin-coated membrane for 1 hour with or without IDR-1002 (20 μg/ml) treatment. Data are presented as mean fold-increases in migration over isotype-treated monocyte migration towards media alone (± SE) of at least 4 independent experiments, each from independent donors. Statistical comparisons were done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$.

3.3.3. IDR-1002 promotion of β1-integrin-mediated monocyte adhesion to fibronectin

To elucidate the potential regulation of β1-integrin-mediated binding by IDR-1002, it was investigated whether IDR-1002 could promote monocyte adhesion to fibronectin. Human blood monocytes or THP-1 cells were treated with varying doses of IDR-1002 and assessed for their ability to adhere to fibronectin-coated plates. The degree of adhesion at 3

hours was determined by microscopy after the removal of unattached cells. IDR-1002 treatment induced a dose-dependent and very substantial increase in adhesion to fibronectin of either blood monocytes or THP-1 cells, indicating a promotion of monocyte-fibronectin interactions (Figure 3.6A). Although blood monocytes demonstrated increased sensitivity to lower IDR-1002 concentrations compared to a THP-1 model, the adhesion patterns of IDR-1002-stimulated monocytes and THP-1 cells were similar. Thus, THP-1 cells were selected as an alternative cell model to further investigate monocyte adhesion behaviour.

Monocyte adhesion and migration is a rapid event. Monocyte adhesive interactions with various substrates can be differentially regulated in as little as a few minutes (129, 130). The rapid regulation of adhesion in response to extracellular stimuli, such as chemokines, is a necessity for substrate-mediated monocyte motility, which requires the coordinated engagement and disengagement of adhesion receptors to their environmental ligands. Thus the temporal profile of IDR-1002-mediated adhesion to fibronectin was determined. THP-1 cells on fibronectin-coated plates were stimulated with IDR-1002 for varying amounts of time prior to removal of non-adherent cells. Time-course adhesion experiments showed that IDR-1002-induced THP-1 adhesion to fibronectin was rapid, with significant adhesion observed as early as 15 minutes. A continued increase in THP-1 adhesion was evident up to 3 hours after IDR stimulation (Figure 3.6B).

Next it was determined whether the adhesive activity of IDR-1002 was mediated specifically through the binding activity of the β1-integrins. THP-1 cells were pre-treated with varying doses of a β1-integrin inhibiting antibody 1 hour prior to stimulation with IDR-1002. The addition of a β1-integrin inhibiting antibody resulted in a dose-dependent inhibition of THP-1 adhesion to fibronectin-coated plates, fully abrogating IDR-1002-mediated adhesion at an antibody concentration of 20 μg/ml (Figure 3.7). In contrast, the pre-treatment of THP-1 cells with 20 μg/ml of an isotype-matched antibody had no effect on peptide-mediated adhesion. These results suggest that the IDR-1002-induced adhesion of THP-1 cells to fibronectin is dependent on β1-integrin activity, supporting the previous observation that β1-integrins play a large role in peptide-mediated enhancement of monocyte chemotaxis on fibronectin.

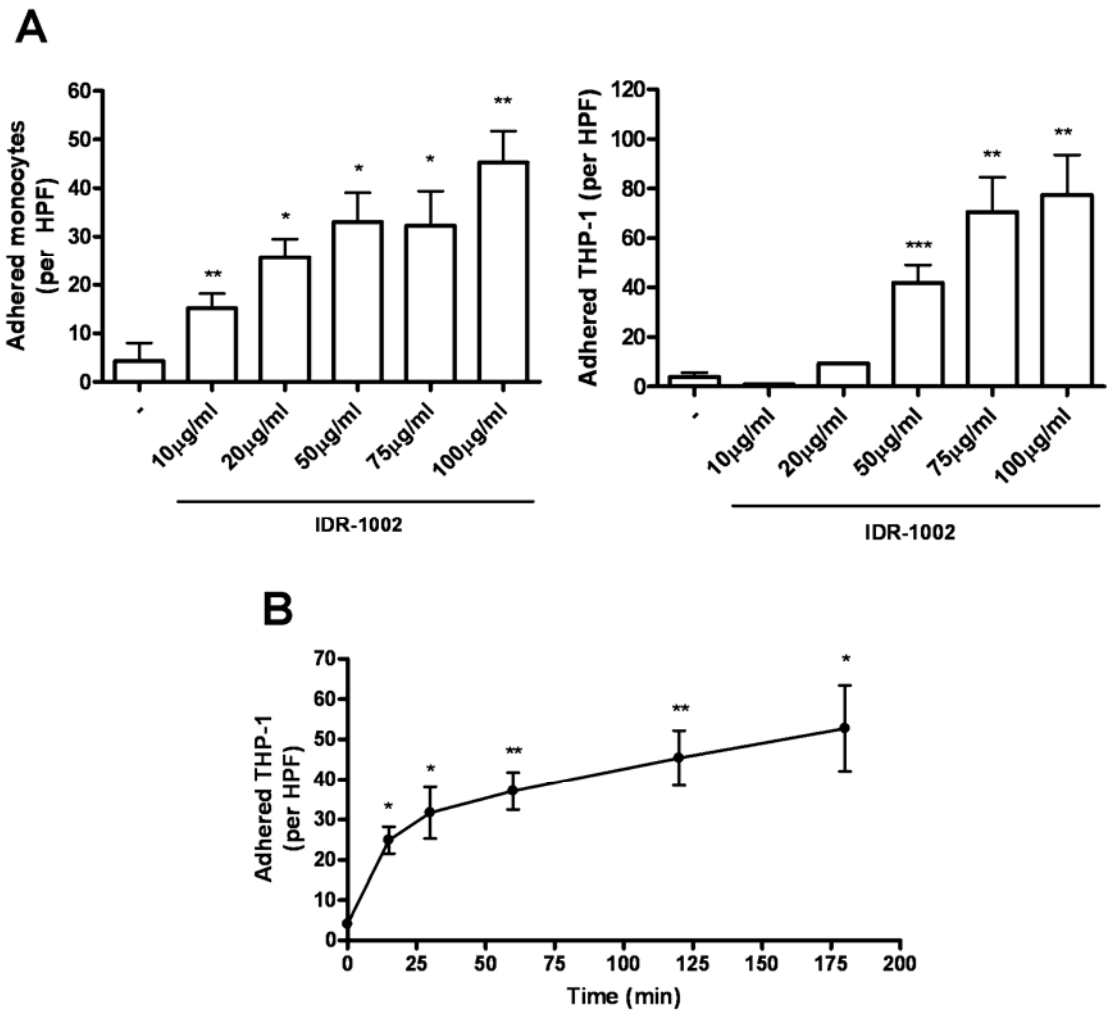

Figure 3.6 IDR-1002 regulation of monocyte adhesion on fibronectin. (A) Monocytes or THP-1 cells were stimulated with various concentrations of IDR-1002 for 3 hours and assessed for their ability to adhere to fibronectin-coated plates. (B) Time course effects of IDR-1002 (50 µg/ml) on THP-1 adhesion to fibronectin. Data are presented as the mean of adhered cells per HPF (± SE) of at least 3 independent experiments. In experiments using primary monocytes, each experiment utilized cells from an independent donor. Statistical comparisons to untreated cells were done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

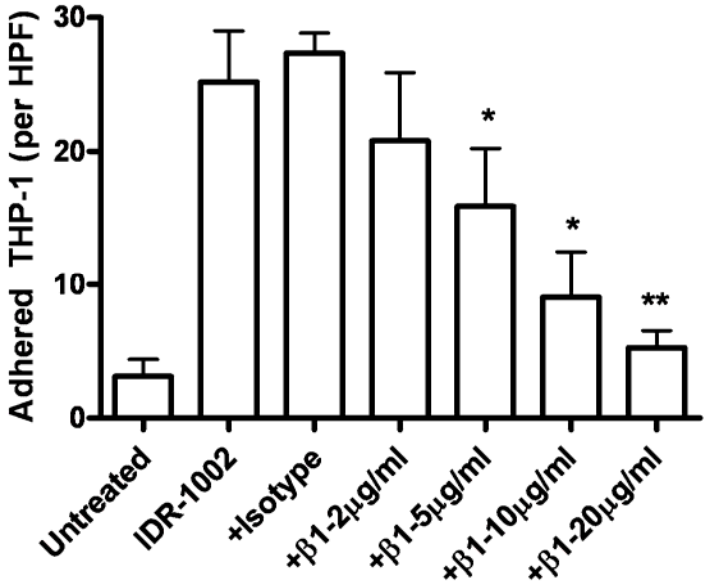

Figure 3.7 The role of β1-integrins in IDR-1002 promotion of THP-1-fibronectin adhesion. THP-1 cells were pre-treated with a β1-integrin inhibiting antibody or an isotype-matched control antibody (20 μg/ml) for 1 hour. Cells were then stimulated with IDR-1002 (50 μg/ml) for 3 hours and assessed for their ability to adhere to fibronectin-coated plates. Data are presented as the mean of adhered cells per HPF (± SE) of 3 independent experiments. Statistical comparisons to IDR-1002 stimulations of isotype-treated THP-1 cells were done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$.

3.3.4. IDR-1002 regulation of β1-integrin expression and activation in THP-1 cells

Due to the β1-integrin-dependency of peptide-mediated effects on monocyte-fibronectin adhesion and migration, the regulation of β1 integrins by IDR-1002 was assessed. The effects of IDR-1002 on the overall expression levels of β1-integrins on monocyte surfaces were investigated by measuring, in THP-1 cells, levels of CD29, the β-subunit of β1-integrin heterodimers. THP-1 cells were stimulated with IDR-1002 for various time intervals prior to assessment of β1-integrin levels. Over the course of 4 hours, there was no substantial change in overall β1-integrin levels (Figure 3.8A). However, integrin function can be differentially regulated via multiple processes, including the conformational change of resting integrins to a higher-affinity state, termed integrin activation (118). THP-1 cells stimulated with IDR-1002 displayed a transient increase in levels of activated β1-integrins, as

determined by staining with the HUTS-4 antibody, which specifically targets β1-integrin in its activated conformation (131). Integrin activation was detectable as early as 15 minutes and peaked at 30 minutes, showing a 50% increase over unstimulated controls (Figure 3.8B). Levels of β1-integrin activation decreased after 60 minutes before returning to unstimulated levels after 4 hours. Thus while IDR-1002 had no major effect on the expression of total β1-integrin surface levels, it induced the transient activation of β1-integrins from a low-affinity state to a high-affinity one.

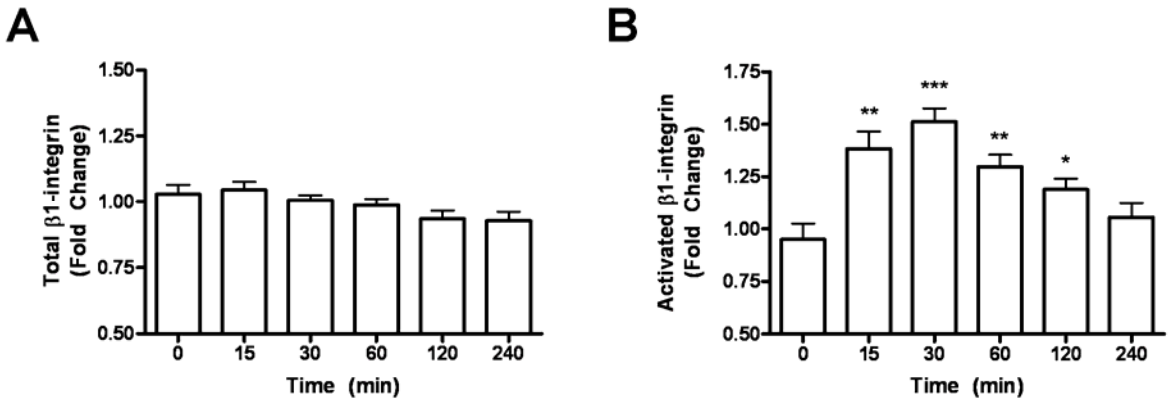

Figure 3.8 IDR-1002 regulation of β1-integrins on THP-1 surfaces. Time course effects of the levels of (A) total β1-integrin or (B) the activated isoform of β1-integrin on THP-1 cells stimulated with IDR-1002 (50μg/ml). Detection of integrin levels was done by staining with an anti-CD29 antibody or a HUTS-4 antibody, which detects the activated conformation of β1-integrins, and measured by flow cytometry. Data are presented as mean fold increases of β1-integrin expression over unstimulated controls (± SE) of at least 5 independent experiments. Statistical comparisons were done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.3.5. The role of the PI3K-Akt pathway in IDR-1002 enhancement of adhesion

The PI3K-Akt pathway has been shown to play a major role in regulating cellular adhesion (128, 132-137). As IDR-1002-mediated chemokine regulation in human PBMCs is dependent on the PI3K pathway (80), the ability of IDR-1002 to activate the PI3K-Akt pathway in human monocytes, and whether this resulted in the regulation of monocyte adhesion, was then investigated. PI3K activation was determined by measuring the levels of Akt Ser473 phosphorylation in THP-1 cells. THP-1 cells were stimulated with IDR-1002 for

varying time intervals prior to assessment of intracellular phospho-Akt levels. IDR-1002-stimulated THP-1 cells exhibited a rapid increase in Akt phosphorylation, peaking at 15 minutes with a 2-fold increase in phosphorylation over unstimulated controls (Figure 3.9A). Elevated phospho-Akt levels persisted until 4 hours post-treatment exhibiting a similar kinetics profile to that of IDR-1002-induced β1-integrin activation. This effect was abrogated in THP-1 cells pre-treated for 1 hour with LY294002, a PI3K-specific inhibitor, demonstrating a PI3K-dependent activation of Akt by IDR-1002 (Figure 3.9B).

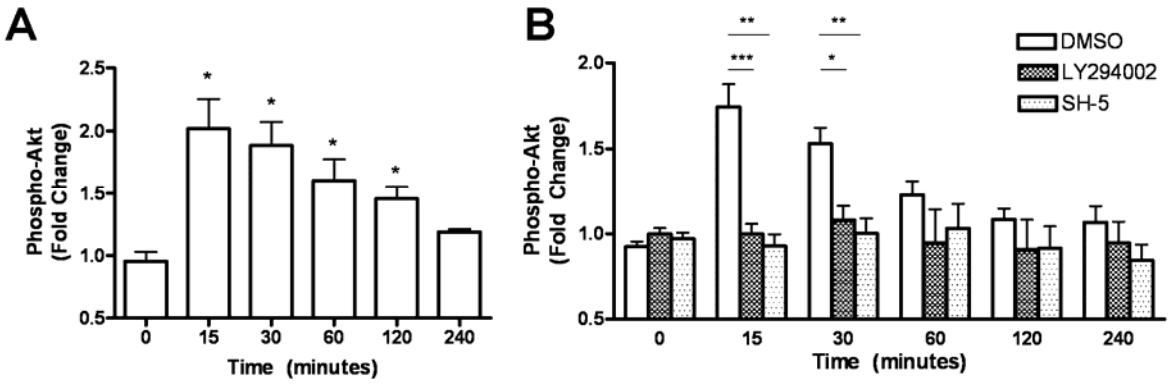

Figure 3.9 IDR-1002 activation of the PI3K-Akt pathway in THP-1 cells. (A) Time course effects on Akt Ser-473 phosphorylation in THP-1 cells stimulated with IDR-1002 (50 μg/ml). (B) The effects of PI3K-Akt inhibitors on IDR-1002-mediated Akt phosphorylation in THP-1 cells. THP-1 cells were pre-treated for 1 hour with PI3K-inhibitor LY294002 (10 μM), Akt-inhibitor SH-5 (10 μM), or 0.1% DMSO prior to IDR-1002 stimulation (50 μg/ml). Phospho-Akt levels were assessed by intracellular staining with an anti-phospho-Akt antibody and detected by flow cytometry. Data are presented as the mean fold increase of Akt phosphorylation over untreated controls (± SE) of at least 5 independent experiments. Statistical comparisons were done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

To determine the involvement of the PI3K-Akt pathway in IDR-1002-mediated monocyte adhesion to fibronectin, adhesion experiments were repeated using THP-1 cells pre-treated with PI3K inhibitor LY294002 and Akt-specific inhibitor SH-5 for 1 hour prior to stimulation with IDR-1002. The addition of a PI3K or an Akt inhibitor reduced peptide-induced monocyte adhesion to fibronectin in a dose-dependent manner compared to a DMSO pre-treatment control (Figure 3.10A, B). Complete elimination of the adhesive response by either inhibitor occurred at concentrations of 10 μm, a non-toxic concentration capable of fully inhibiting Akt activation induced by IDR-1002. To assess whether other pathways

required for IDR-mediated immunomodulation are involved in IDR-1002-induced adhesion, namely the MAPK pathways (79, 80), THP-1 cells were pre-treated with a p38 MAPK inhibitor SB203580 or an MEK-1 inhibitor PD98059 prior to stimulation IDR-1002. Inhibition of p38 had no effect on peptide-induced THP-1 fibronectin adhesion (Figure 3.10C), while MEK-1 inhibition, at high inhibitor concentrations, led to a 40% reduction in adhesion (Figure 3.10D). In the absence of peptide stimulation, none of the inhibitors alone affected THP-1 adhesion to fibronectin at any concentration used (data not shown). Overall it could be concluded that activation of the PI3K-Akt pathway by IDR-1002 is necessary for peptide-mediated monocyte adhesion to fibronectin.

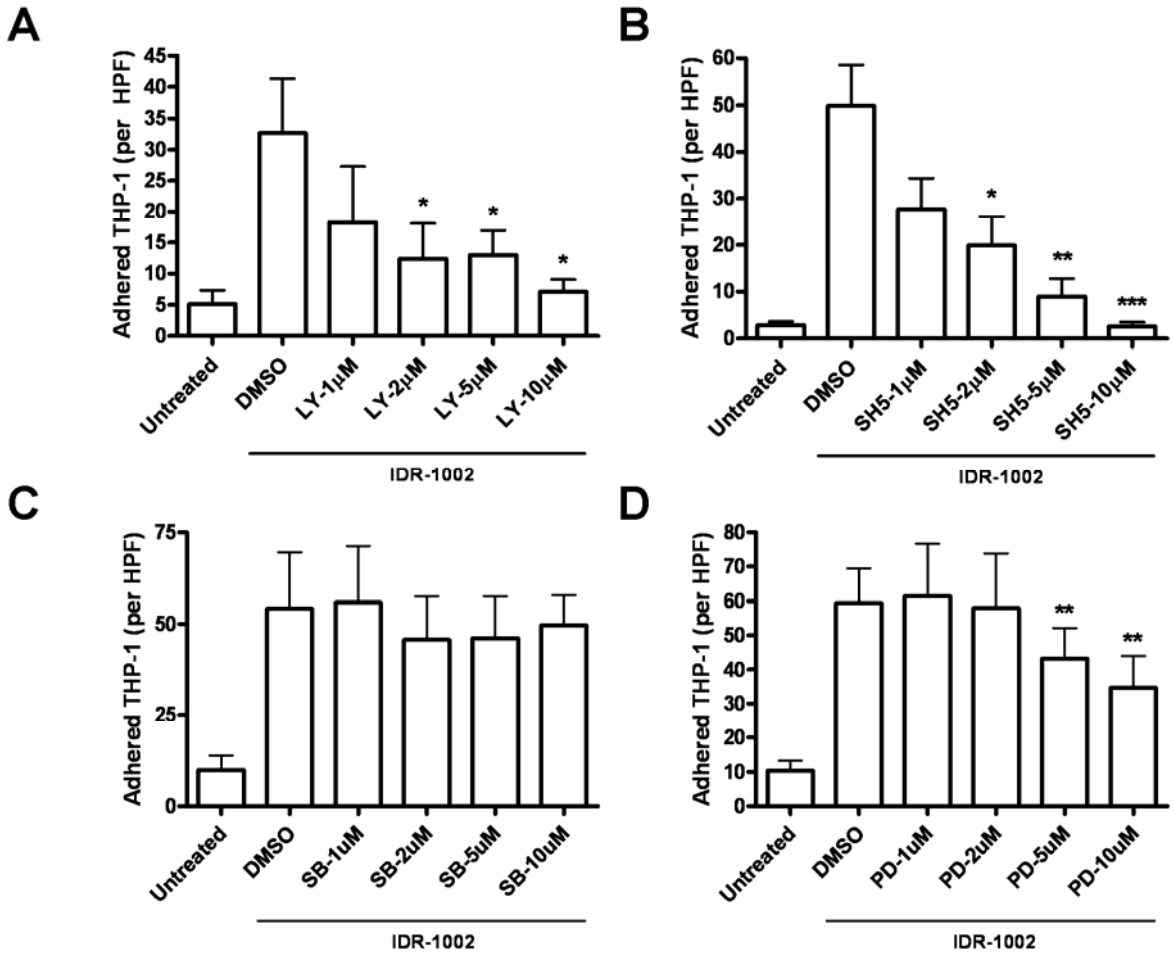

Figure 3.10 The role of the PI3K-Akt pathway in IDR-1002 promotion of THP-1 adhesion. Dose-dependent effects of a (A) PI3K-inhibitor LY294002, (B) Akt-inhibitor SH-5, (C) p38 MAPK-inhibitor SB203580, or (D) MEK-1 inhibitor PD98059 on IDR-1002-induced THP-1 adhesion. THP-1 cells were pre-treated for 1 hour with 10 μM of inhibitors or 0.1% DMSO prior to a 3 hour stimulation with IDR-1002 (50 μg/ml). THP-1 cells were then assessed for their adherence to fibronectin-coated plates. Data are presented as the mean of adhered THP-1 cells per HPF (± SE). Statistical comparisons against IDR-1002 stimulations of DMSO pre-treated cells were done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.3.6. The role of the PI3K-Akt pathway dependency in IDR-1002 activation of β1-integrins and monocyte migration on fibronectin

Both PI3K and Akt have been demonstrated to be inside-out signalling activators of various integrin classes, including β1-integrins (134, 138-140). As IDR-1002 promotion of THP-1 adhesion to fibronectin was shown to be PI3K-Akt-dependent, the role of the PI3K-Akt pathway in IDR-1002-induced β1-integrin activation was then investigated. THP-1 cells pre-treated with 10μm of LY294002 or SH-5, prior to stimulation with IDR-1002, exhibited no increase in β1-integrin activation compared to non-peptide-stimulated controls, as measured by HUTS-4 antibody binding (Figure 3.11A). DMSO pre-treated THP-1 cells, stimulated with IDR-1002, presented transient activation of β1-integrins over unstimulated controls. As pertussis toxin-sensitive G_i-protein-mediated signal transduction was necessary for IDR-1002 induction of chemokines (80), and also necessary for many chemokine-mediated functions (141), the role of this signalling axis in IDR-mediated β1-integrin activation was also investigated. THP-1 cells pre-treated with 100 ng/ml of pertussis toxin, prior to IDR-1002 stimulation for 30 minutes, exhibited an increase in β1-integrin activation similar to THP-1 cells without pertussis-toxin treatment (Figure 3.11B). Taken together these results indicate that IDR-1002 promotes THP-1 binding to fibronectin via the PI3K-Akt pathway, likely through the inside-out activation of β1-integrins.

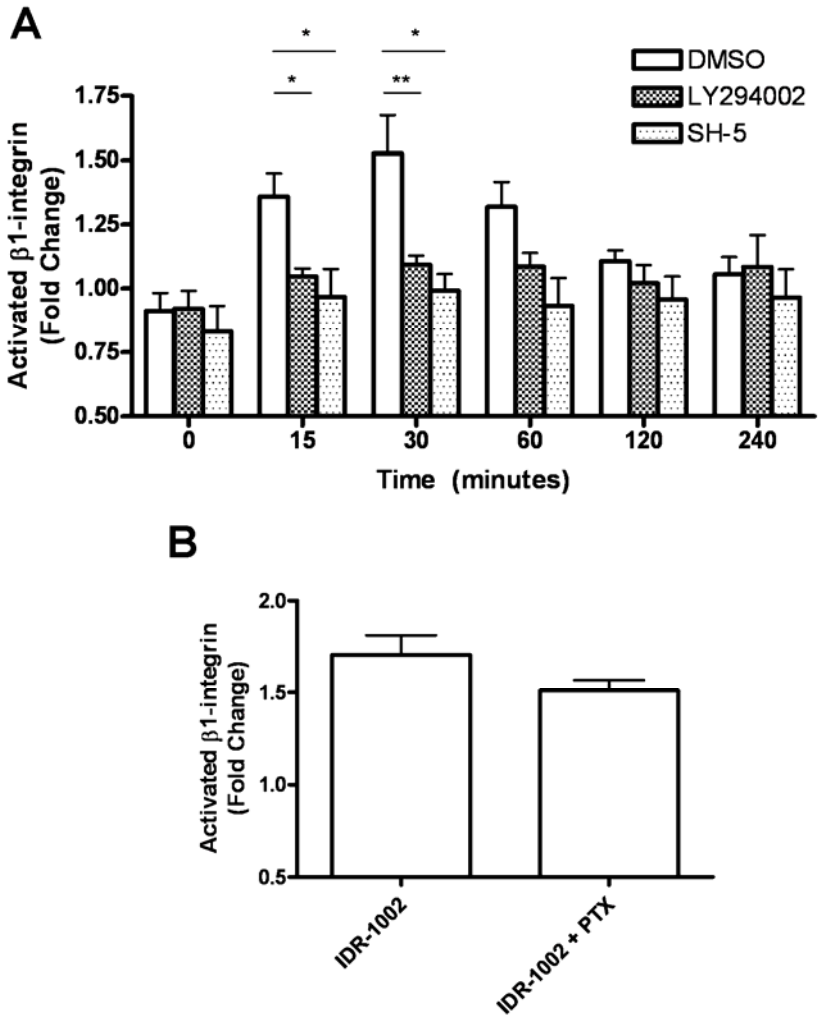

Figure 3.11 The role of the PI3K-Akt pathway in IDR-1002 activation of β1-integrins. (A) The effect of PI3K-Akt inhibitors on IDR-1002 induction of THP-1 β1-integrin activation. THP-1 cells were pre-treated for 1 hour with PI3K-inhibitor LY294002 (10 μM), Akt-inhibitor SH-5 (10 μM), or 0.1% DMSO prior to stimulation with IDR-1002 (50 μg/ml). (B) The effect of G_i-protein inhibition on IDR-1002 induction of β1-integrin activation. THP-1 cells were pre-treated for 1 hour with pertussis toxin (500 ng/ml) prior to a 30 minute stimulation with IDR-1002 (50 μg/ml). Activated β1-integrins were measured by flow cytometry detection of the HUTS-4 antibody. Data are presented as the mean fold-increase of β1-integrin activation over untreated controls (± SE) of at least 4 independent experiments. Statistical comparisons were done by Student's two-tailed *t*-test. *$p < 0.05$, **$p < 0.01$.

Having demonstrated that IDR-1002 utilized the PI3K-Akt pathway to promote monocyte interaction with fibronectin via β1-integrin activation, it was then investigated whether this pathway played a role in the peptide-mediated enhancement of monocyte chemotaxis. Monocytes were pre-treated with 10 μm of LY294002 or SH-5 for 1 hour prior

to chemotaxis towards monocyte chemokines. The presence of LY294002 or SH-5 reduced baseline spontaneous monocyte migration by 53% and 70%, respectively, compared to DMSO pre-treatment controls ($p < 0.05$, $n = 3$). In the absence of chemokines, IDR-1002 did not significantly alter monocyte migration in each respective inhibitor treatment (data not shown). Similar to previous experiments, monocytes pre-treated with DMSO migrated towards chemokines through a fibronectin-coated membrane, an effect significantly enhanced in the presence of IDR-1002 (Figure 3.12). However after LY294002 and SH-5 pre-treatment, IDR-1002 had a minimal or no effect on monocyte chemotaxis towards chemokines, indicating that the PI3-Akt pathway was essential for the enhancement of monocyte chemotaxis by IDR-1002.

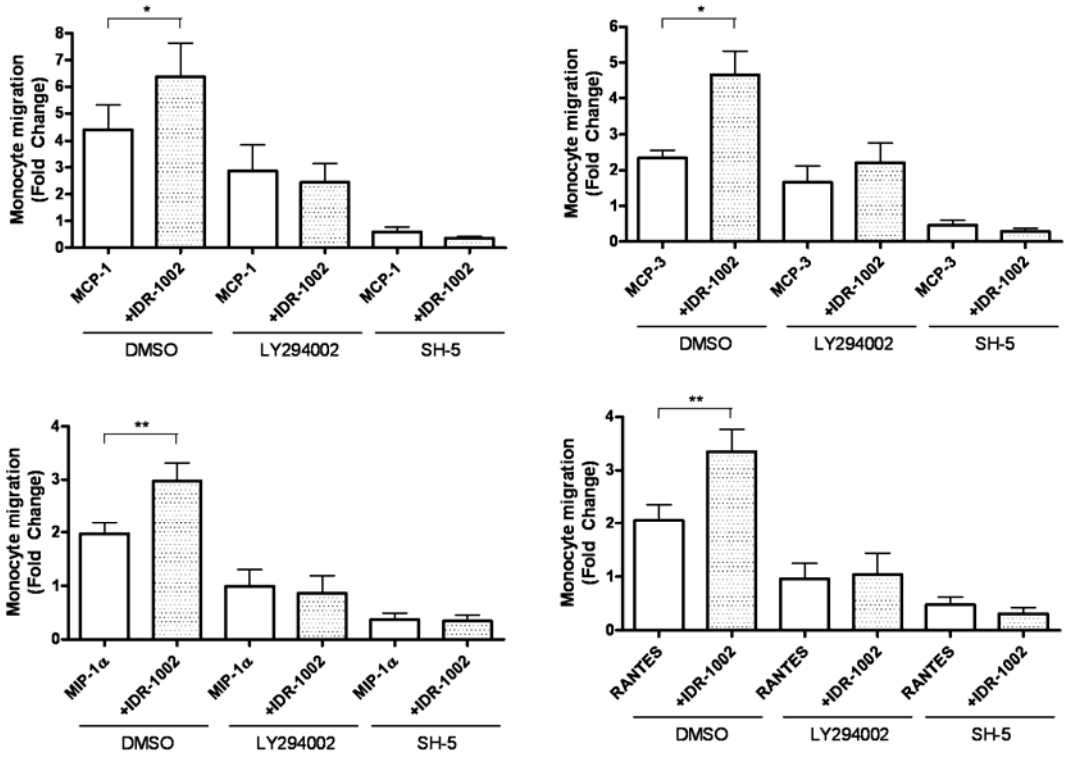

Figure 3.12 The role of the PI3K-Akt pathway in IDR-1002 enhancement of monocyte migration. Monocytes were pre-treated for 1 hour with PI3K-inhibitor LY294002, Akt-inhibitor SH-5, or 0.1% DMSO. Monocytes were then assessed for their ability to migrate towards chemokines (12.5 ng/ml) for 1 hour through a fibronectin membrane with or without IDR-1002 (20 μg/ml) treatment. Data are presented as the mean fold-increases in migration over the migration of DMSO-pre-treated monocytes towards media alone (± SE) of at least 3 independent experiments, each from independent donors. Statistical comparisons were done by Student's two-tailed *t* test. *p < 0.05; **p < 0.01.

3.3.7. IDR-1002 promotion of monocyte adhesion to ICAM-1

As it was determined that IDR-1002 enhanced monocyte migration on fibronectin through an enhancement of integrin-mediated adhesion, the ability of IDR-1002 to influence monocyte adhesion to another integrin ligand, ICAM-1, was investigated. ICAM-1, an endothelial surface glycoprotein, is an adhesion molecule which plays an important role in cellular recruitment during an inflammatory response (118). Through its interaction with integrins of cells in the bloodstream, ICAM-1 facilitates the firm adhesion of rolling cells to the endothelial layer**,** a prerequisite for cellular transmigration across the endothelia and further localization to the site of infection. To ascertain the effects of IDR-1002 on ICAM-1 adhesion, THP-1 cells were stimulated with varying concentrations of peptide and measured for their ability to adhere to ICAM-1-coated plates. After 3 h, IDR-1002 demonstrated the ability to induce THP-1 adhesion to ICAM-1 in a dose-dependent manner (Figure 3.13). Significant adhesion was observed at peptide concentrations as low as 20 μg/ml. This demonstrates that the promotion of monocyte adhesion by IDR-1002 is not limited to fibronectin, but occurs with other adhesive ligands that play an essential role in cellular recruitment.

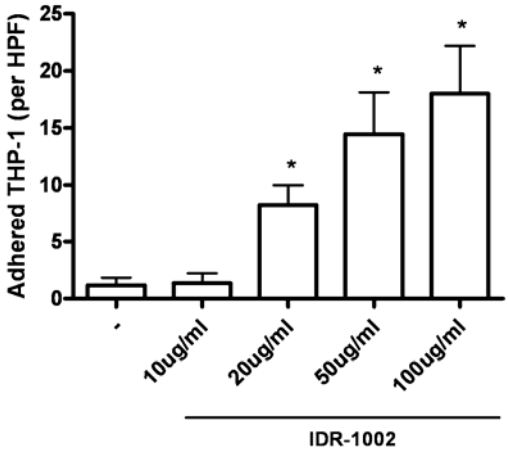

Figure 3.13 IDR-1002 promotion of THP-1 adhesion to ICAM-1. THP-1 cells were stimulated with varying concentrations of IDR-1002 for 3 hours and assessed for their adherence to ICAM-1-coated plates. Data are presented as the mean THP-1 adherence (± SE) of 3 independent experiments. Statistical comparisons to untreated THP-1 cells were done by Student's two-tailed *t* test. *$p < 0.05$.

3.3.8. IDR-1002 regulation of integrin CD11c expression on THP-1 cells

Whereas cellular adhesion to fibronectin is mediated by the β1-integrin family of receptors, adhesion to ICAM-1 is generally regulated by the β2-integrin family (124). Similar to the β1-integrins, the β2-integrin receptor complex consists of a heterodimer of an α- and β-receptor subunits. As was done for the β1-integrins, the ability of IDR-1002 to regulate the monocyte surface expression of β2-integrins was investigated. THP-1 cells, stimulated with IDR-1002 over a course of time, were assayed for the surface expression of CD11c, one of the β2-integrin α-subunits expressed by monocytes. IDR-1002 upregulated the surface expression of CD11c as early as 15 minutes (Figure 3.14). This increase peaked at 30 minutes, where an approximate 50% increase over baseline levels of CD11c was observed, and elevated levels of CD11c were sustained up to 2 hours post stimulation.

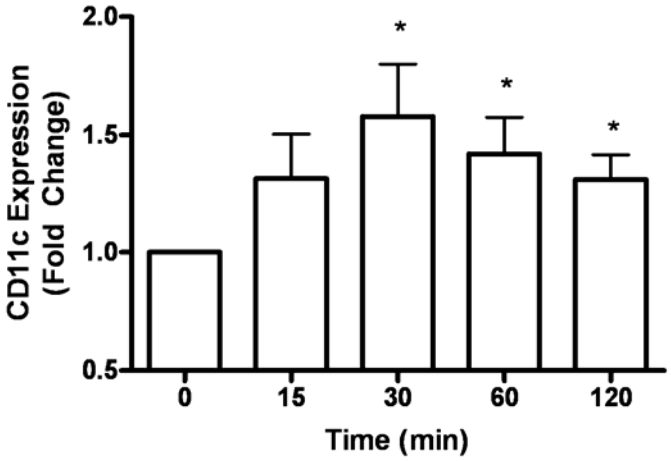

Figure 3.14 IDR-1002 regulation of CD11c expression on THP-1 cells. Time course effects of IDR-1002 stimulation (50 μg/ml) on CD11c surface expression by THP-1 cells. CD11c levels were measured by the detection of anti-CD11c antibodies using flow cytometry. Data are presented as the mean fold-increases in CD11c expression over untreated cells (± SE) of 5 independent experiments. Statistical comparisons to untreated cells were done by Student's two-tailed *t* test. *$p < 0.05$.

3.3.9. Regulation of monocyte adhesion by IDR-peptides

Immunomodulatory IDR-peptides exhibit many functional similarities. Anti-infective peptides IDR-1 and IDR-1002 confer *in vivo* protection against bacterial infections through modulation of innate immune responses, including the regulation of chemokine/cytokine responses and recruitment of monocytes to the site of infection (79, 80). Likewise, IDR-1018 and IDR-HH2 also demonstrate the ability to modulate the host cytokine/chemokine environment (84, 142), while both IDR-1002 and IDR-HH2 promote adjuvant activity in formulation (100, 108). It was of interest to determine whether the ability to promote monocyte adhesion demonstrated by IDR-1002 was shared with other IDR-peptides as well. Human monocytes or THP-1 cells were stimulated with IDR-peptides or human cathelicidin LL-37, a host defense peptide with similar immune modulatory functions, and investigated for their ability to adhere to fibronectin-coated plates after 3 h. LL-37 was utilized at a concentration of 20 μg/ml as higher concentrations have been associated with observable cytotoxicity in human PBMCs as measured by a lactate dehydrogenase release assay; in contrast IDR-peptides elicited minimal cytotoxicity up to concentrations of 100 μg/ml (unpublished data). Treatment with PMA was utilized as a positive control for monocyte-fibronectin adhesion (143). As shown in Figure 3.15, immunomodulatory peptides LL-37, IDR-1, IDR-1002, IDR-1018, and IDR-HH2 promoted monocyte and THP-1 adhesion to fibronectin to varying degrees. IDR-1002 was the most potent adhesion-inducing peptide, promoting monocyte adhesion exceeding that of PMA-treatment. 1035, a negative control peptide with no immunomodulatory properties as measured by its lack of enhancement of chemokine production in human PBMCs (unpublished), did not elicit monocyte or THP-1 adhesion above baseline levels. These results indicate that promotion of monocyte adhesion to fibronectin is a shared property between synthetic immunomodulatory IDR peptides, with IDR-peptides stimulating monocyte adhesion at levels comparable to, or exceeding, natural inducers of adhesion.

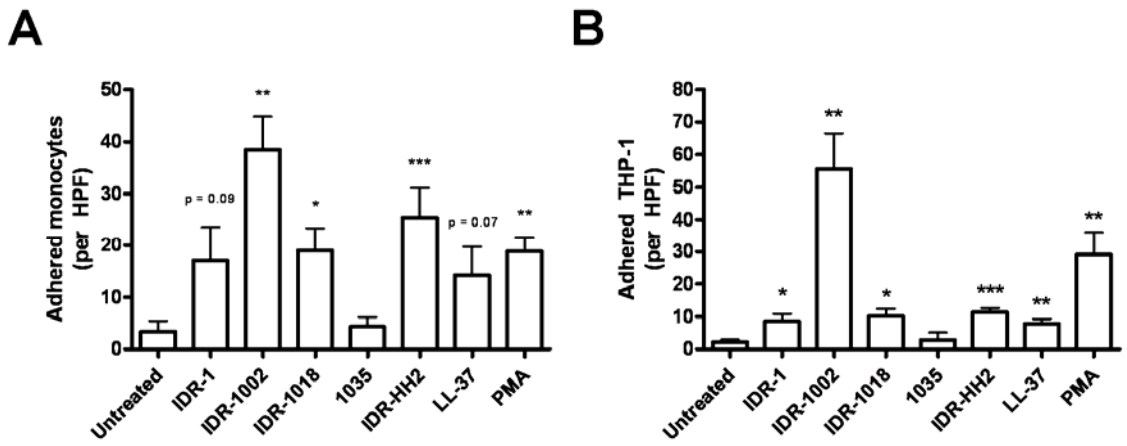

Figure 3.15 IDR-peptide induction of monocyte adhesion to fibronectin. (A) Monocytes or (B) THP-1 cells were stimulated with IDR-peptides, negative control peptide 1035 (100 μg/ml), or LL-37 (20 μg/ml) for 3 hours and assessed for their adherence to fibronectin-coated plates. Stimulation with 50 ng/ml of PMA was used as a positive control for monocyte fibronectin adhesion. Data are presented as the mean of adhered cells (± SE) of at least 4 independent experiments. In experiments using monocytes, each independent experiment utilized cells from an independent donor. Statistical comparisons to untreated cells were done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.4. DISCUSSION

The aim of this study was to identify novel mechanisms by which IDR-1002 enhances monocyte recruitment, a hallmark effect in IDR-mediated protection against bacterial infection (79, 80) and its deployment in adjuvant formulations (100, 108). In this chapter, it was demonstrated that IDR-1002, a peptide which displayed neither direct chemokinetic nor chemoattractive activity, enhanced human monocyte migration towards a wide range of chemokines through a fibronectin matrix. This augmentation by IDR-1002 on monocyte recruitment correlated with a promotion of monocyte and THP-1 cell adhesion to fibronectin, a major ECM component. Recruitment of leukocytes to the site of infection is a highly orchestrated and complex event, of which cellular adhesion plays a major role. Mediators of cell recruitment produced during an infection, must not only stimulate cellular movement, but also promote cellular attachment to the surrounding environment (118). Cellular extensions of chemokine-stimulated leukocytes would generate little net movement without an adhesive foothold on which to apply directional force (120). Many studies have

shown that leukocyte adhesion to the endothelial surface is a fundamental step in subsequent cellular extravasation and transmigration into tissues (118). Further localization of leukocytes within tissues also depends on cellular adhesion to the ECM to facilitate movement. Here, it was shown that inhibition of β1-integrin, the major monocyte adhesion receptor for fibronectin, not only eliminated IDR-1002 promotion of monocyte fibronectin adhesion, but also greatly reduced the ability of the peptide to enhance monocyte recruitment on a fibronectin membrane. These results also correlated with a rapid activation of β1-integrins on THP-1 surfaces by IDR-1002. Taken together, these results demonstrate that IDR-1002, through its promotion of β1-integrin-mediated adhesion, augments monocyte movement towards chemotactic signals through fibronectin. Thus it is feasible that *in vivo* administration of IDR-1002 during a bacterial infection would prime monocytes for enhanced adhesion to fibronectin, and thus, tissue migration towards chemotactic mediators produced in abundance at the site of infection. Furthermore, the ability of other immunomodulatory peptides to promote monocyte adhesion to fibronectin suggests similar mechanisms of enhancement may be shared among IDR-peptides.

The regulation of integrin-mediated cellular adhesion is an enormously complex process that involves the actions and interactions of numerous intracellular signalling networks. A diverse host of signalling mediators have been shown to modulate integrin function, including those of the MAP Kinase and PI3K-Akt pathways, GPCR and phospholipase C (PLC) networks, and the small GTPases Rho and Rap (118, 132-136). Many of these pathways and networks have been implicated as necessary for certain immunomodulatory effects of natural HDPs and IDR-peptides (50, 79, 80). Thus the role of signal transduction pathways in IDR-1002 promotion of fibronectin adhesion and β1-integrin activation was investigated. In this study, it was observed that the ability of IDR-1002 to induce β1-integrin activation and promote monocyte adhesion and migration on fibronectin was highly dependent on its activation of the PI3K-Akt pathway, demonstrating a link between the modulation of signalling by IDR-peptides and the regulation of integrin function. Interestingly, the inhibition of the p38 and MEK1 MAP Kinase pathways, both involved in monocyte integrin regulation and IDR-1002-mediated chemokine regulation in PBMCs, showed no or comparatively minor effects on monocyte adhesion. Similarly, inhibition of G_i-protein function by pertussis toxin, involved in both IDR-1002-mediated

cytokine induction or chemokine-mediated responses, had no effect on peptide-mediated activation of β1-integrins. This underscores the complexity by which IDR-peptides might modulate specific signalling networks to regulate distinct aspects of immunity.

Although the current study focused on the ability of IDR-1002 to utilize the PI3K-Akt pathway in modulating β1-integrin affinity via 'inside-out' activation, there are other mechanisms through which this pathway can regulate integrin function. The PI3K-Akt pathway is known to promote the recycling of internalized integrins to the cell surface (144, 145), although the lack of effect by IDR-1002 on total surface β1-integrins levels makes this an unlikely explanation for IDR-1002-enhanced adhesion. However, we cannot exclude the role of PI3K-Akt-mediated increases in integrin avidity via its promotion of integrin mobility and clustering (146, 147). Taking into consideration the multiple effects of IDR-peptides and natural peptides on cellular signalling, as well as the complexity of interactions within signalling networks, IDR-1002 may affect other signalling networks through cross-talk. For example, PI3K in various cell models has been shown to activate various small GTPases and PLC, which are among the best characterized mediators of integrin activation (148-152). Similarly, other signalling pathways used by IDR-1002 are known to impact the PI3K-Akt pathway, including the GPCR-induced networks. Despite these complexities, it is clear that the PI3K-Akt pathway is central to IDR-1002 modulation of β1-integrin function and subsequent enhancement of monocyte migration and adhesion to fibronectin.

Monocyte adhesion and migration on fibronectin within tissues is only one process coordinating overall leukocyte infiltration. This study provides insights into other mechanisms by which IDR-1002 potentially promotes monocyte mobilization to infectious sites. The β1-integrin activating properties of IDR-1002 may have implications at other stages of monocyte recruitment. VLA4, a β1-integrin member, is a well known receptor for VCAM-1 on endothelial cells and plays a major role in cellular rolling, firm adhesion, and transmigration across the endothelia (153, 154). In addition, with many similarities between the regulation of β1-integrins and other integrin family members, it is conceivable that IDR-peptides may affect other stages of integrin-mediated adhesion and migration. Indeed it was observed that IDR-1002 promotes THP-1 adhesion to ICAM-1, an interaction essential for monocyte arrest and adhesion on the endothelial layer (118). This effect correlated with an increase in the total surface expression of CD11c, one of numerous β2-integrin α-chain

subunits responsible for monocyte adhesion to ICAM-1. It remains to be seen whether the promotion of ICAM-1 adhesion by IDR-1002 is β2-integrin-dependent and whether IDR-1002 regulates the expression and activation of other β2-integrin family members. However, these results imply that modulation of integrin-mediated adhesion by IDR-1002 extends beyond enhancing monocyte adhesion and migration on fibronectin. More so, these results suggest a possible promotion of monocyte endothelial arrest and transmigration by IDR-1002, potentially contributing to the enhancement of monocyte recruitment seen in *in vivo* models.

In summary, it was established that IDR-1002 can promote the adhesion and migration of monocytes to fibronectin through enhanced β1-integrin function via activation of the PI3K-Akt pathway. It was also established that regulation of monocyte adhesion is a common function among IDR-peptides. This study thus identified novel functions of IDR-peptides in promoting integrin-mediated monocyte adhesion to fibronectin and synergistically enhancing monocyte migration towards host chemokines. Furthermore, it was demonstrated that IDR-1002 modulation of monocyte adhesion may extend to other substrates, possibly through regulation of other integrin families, further expanding the repertoire of immune regulating effects of IDR-peptides. Monocyte integrin-mediated adhesion not only has implications in monocyte recruitment, but far-reaching regulatory functions in many aspects of the host immune response. Dynamic signal transduction activity induced by integrin-mediated adhesion can play a role in monocyte phagocytosis, growth, cytokine production, and macrophage-differentiation (155-157). Understanding how IDR-peptides regulate monocyte migration, adhesion, and integrin function through its effect on cellular signalling networks will aid in the development and optimization of novel agents with improved anti-infective and immunomodulatory functions.

4 IDR-1002 ENHANCES HUMAN MONOCYTE CHEMOTAXIS TOWARDS RANTES AND MIP-1α VIA REGULATION OF CCR5 SURFACE EXPRESSION

4.1. INTRODUCTION

The synthesis of anti-infective IDR-peptides embodies one of many efforts in the development of anti-infective immunomodulatory agents. As the effectiveness of IDR-peptides in conferring anti-bacterial protection has been demonstrated, focus has shifted to understanding the mechanisms through which they regulate immune responses, allowing for the development of improved immunotherapeutics. While not entirely unexpected, investigations into their mechanisms of action reveal levels of complexity comparable to those exhibited by endogenous regulators of innate immunity, such as cytokines and HDPs. As shown in Chapter 2, IDR-1002 acted as a selective regulator of the host cytokine environment through modulation of host signal transduction pathways. In the previous chapter, IDR-1002 was demonstrated to have the ability to directly regulate monocyte integrin function and monocyte adhesion to multiple substrates, through the activation of monocyte immune signalling networks. To add to this complexity, certain observations in Chapter 3 suggest that IDR-1002 may play a distinct role in enhancing monocyte behaviour towards certain chemokines.

Chemokines, as previously discussed, are one of the primary regulators of cellular trafficking in many host processes, from development and homeostasis to the inflammatory antimicrobial immune response. The intricate ability to direct the movement of distinct cell populations to specific locales arises from the legion of chemokines utilized by the host system. In the human system alone, more than 40 chemokines have been identified (88). These distinct, yet often functionally-overlapping, molecules can be sorted into numerous subsets which possess distinct chemoattractive profiles for specific leukocyte populations (88). During antimicrobial invasion, the localized production of many inducible chemokines is essential for the rapid infiltration of monocytes, neutrophils, and lymphocytes to the infectious site. The presence of a range of chemokines not only provides a functional redundancy necessary for a robust immune response, but also is necessary for the optimal

migration of many specific cell populations (158, 159). Studies have also reported the cross-regulation and synergy between different chemokines in cellular immune function (159-161). Furthermore, delicate fine tuning of leukocyte traffic can occur based on changes in sensitivity to certain chemokines. As an example, altered chemokine sensitivity of dendritic cells is necessary for their outflow from inflamed tissues into lymph nodes where they can prime T-lymphocytes (162). Primed and differentiated T-lymphocytes exit the lymph nodes through similar mechanisms (163, 164). Altered chemokine sensitivity is also observed during the monocyte-to-macrophage differentiation process (165). In addition to their ability to attract cells, chemokines possess many secondary regulatory effects. These include the ability of chemokines to modulate cellular attachment to substrates and regulate cellular proliferation; through regulation of these events, chemokines play important roles in homeostatic and regenerative processes, such as tissue remodeling, angiogenesis, and wound healing (114, 115, 166, 167). Thus it is clear that changes in cellular response to chemokines can have profound consequent effects on host antimicrobial immunity.

In this chapter, the modulation by IDR-1002 of chemokine-mediated monocyte responses was investigated. An examination of how IDR-1002 influences chemokine responses might yield a novel mechanism through which IDR-peptides enhance monocyte recruitment. Furthermore, due to the pleiotropic effects of chemokines on immunity, this investigation will further our understanding of how IDR-1002 regulates different aspects of immunity. In this study, it was demonstrated that stimulation with IDR-1002 enhanced human monocyte chemotaxis towards MIP-1α and RANTES but not MCP-1 and MCP-3. It must be noted that, in contrast to observations Chapter 3, this result was observed in a fibronectin-free model, thus displaying an adhesion-independent enhancement of monocyte chemotaxis by IDR-1002. This selective enhancement of chemotaxis by IDR-1002 correlated with the increased surface expression of CCR5, but not CCR2, on monocytes. Furthermore, the enhancing properties of IDR-1002 on chemotaxis towards MIP-1α and RANTES were fully dependent on CCR5 function. This investigation also demonstrated the potential of IDR-1002 to reinforce downstream activation of p38 MAPK by MIP-1α and RANTES. In summary, this chapter revealed a distinct mechanism through which IDR-1002 promoted monocyte recruitment by selectively augmenting monocyte responsiveness to chemokines.

Moreover, the ability of IDR-1002 to enhance chemokine functions suggested a novel axis of IDR-peptide regulation of the antimicrobial immune response.

4.2. METHODS AND MATERIALS

4.2.1. Reagents

Peptide IDR-1002 (VQRWLIVWRIRK-NH_2) was synthesized by solid phase F-moc chemistry by CPC Scientific (Sunnyvale, CA). Pertussis toxin was obtained from Calbiochem (San Diego, CA) and stored at -20 ˚C. Chemokines MCP-1, MCP-3, MIP-1α, and RANTES were obtained from R&D Systems (Minneapolis, MN).

4.2.2. Cell isolation and culture

Human PBMCs were isolated as previously reported (44). Briefly, venous blood was collected from healthy volunteers using heparin-containing Vacutainer tubes (BD Bioscience, San Jose, CA) in accordance with UBC ethical approval and guidelines. Human blood was diluted in an equal amount of PBS (Invitrogen, Carlsbad, CA) then layered over Ficoll-Paque Plus (Amersham, Piscataway, NJ) prior to separation by density-gradient centrifugation. The mononuclear cell layer was extracted and washed twice with PBS. The monocyte population was enriched by negative selection magnetic bead purification from the PBMCs using the Easy-Sep Human Monocyte Enrichment Kit (Stemcell Technologies, Vancouver, BC, Canada) as per the manufacturer's instructions. Monocytes were then resuspended in RPMI 1640 with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Invitrogen), placed in a polypropylene tube (BD Falcon, San Jose, CA), and cultured in a humidified incubator at 37 °C with 5% CO_2.

4.2.3. Monocyte chemotaxis assay

All migration assays were performed using a 48-well microchemotaxis chamber (Neuro Probe). Freshly enriched human peripheral blood monocytes were adjusted to RPMI 1640 with 1% FBS and incubated at 37 °C for 1 h. For inhibitor studies, monocytes were pre-treated for 1h with 20 μg/ml of an anti-human CCR5-blocking mouse IgG2b Ab (45531,

R&D Systems), an isotype-matched Ab control (MG2b-57, Biolegend, San Diego, CA), or 100 ng/ml of pertussis toxin. Monocytes ($5x10^4$ per well) were stimulated with 20 μg/ml of IDR-1002 and added to the upper wells of the chamber. Indicated concentrations of chemokines in RPMI 1640 with 1% FBS were added to the lower wells. Lower wells containing RPMI 1640 and 1% FBS were used as negative controls. The upper and lower wells were separated by a polycarbonate membrane with 5-μm-diameter pores. After 1 hour of incubation non-migrated monocytes were removed by PBS washing and scraping with a rubber blade, and adhered cells on the underside of the membrane were stained with the Diff-Quik Staining Kit.

Migration for each treatment was measured by averaging the number of migrated cells per 400x magnification HPF over five fields, with each treatment condition done in duplicate. Fold-change-over-control values were calculated by dividing the average cell count per HPF of each treatment by the average cell count per HPF of the untreated control treatments.

4.2.4. Flow cytometry to determine CCR surface expression levels

All flow cytometry results were collected using a FACSCalibur cytometer in conjunction with CellQuest Pro software (BD Biosciences). Freshly enriched human PBMCs were adjusted to RPMI 1640 with 1% FBS. Then $2x10^6$ PBMCs per well were seeded on tissue culture plates and placed in a humidified incubator at 37 °C and 5% CO_2 for 1 h. Cells were then stimulated with 20 μg/ml of IDR-1002 and incubated at 37 °C for the indicated time-points. Following treatment, monocytes were then fixed with PBS and 4% (w/v) formaldehyde (Fisher Scientific, Pittsburgh, PA) at room temperature for 20 min in preparation for staining.

To investigate CCR surface expression, PBMCs were washed using PBS with 0.5% (w/v) BSA and stained at room temperature for 1 hour with an anti-human-CCR5 rat IgG2A-AlexaFluor®647 (HEK/1/85a) Ab, an anti-human-CCR2 mouse IgG2b-AlexaFluor®647 Ab (TG5/CCR2), or an isotype mouse IgG1-AlexaFluor®647 Ab (MOPC-21) (all from Biolegend). In conjunction, PBMCs were also stained with an anti-human-CD14-Alexa Fluor 488 M5E2 mouse mAb. PBMCs were then resuspended in PBS with 0.5% BSA and 0.5%

formaldehyde for analysis. The monocyte population was investigated by gating on the CD14 expressing cell population and CCR expression levels for each sample were determined by measuring the geometric mean fluorescence intensity (MFI) levels of 1,000 monocytes, with the geometric MFI levels of isotype-stained samples subtracted at each respective time point. The displayed fold-change-over-basal values were calculated by dividing the corrected MFI values of each treatment by the MFI values of the untreated sample of their respective time-point conditions.

4.2.5. Measurement of protein phosphorylation by flow cytometry

PBMCs ($2x10^6$ per well) were seeded on tissue culture plates and placed in a humidified incubator at 37 °C and 5% CO_2 for 1 h. PBMCs, with or without a 15 minute pre-treatment with 20 μg/ml of IDR-1002, were then stimulated with 12.5 ng/ml of MIP-1α or RANTES, and incubated at 37 °C for 5 minutes. Following treatment, PBMCs were fixed with PBS and 4% (w/v) formaldehyde (Fisher Scientific, Pittsburgh, PA) at room temperature for 20 min in preparation for staining. PBMCs were then washed with 0.5% BSA in PBS and permeabilized in 90% (v/v) methanol at 4 °C for 30 min. The cells were then washed and stained at room temperature for 1 hour with anti-phospho-p38 MAPK (Thr180/Tyr182) 3D7 rabbit mAb (Cell Signalling Technology, Danvers, MA). Following washing, the cells were stained with a goat anti-rabbit IgG-Alexa Fluor 647 (H+L, Invitrogen) and with an anti-human-CD14-Alexa Fluor 488 M5E2 mouse mAb for 30 min at room temperature. The cells were then washed and resuspended in PBS with 0.5% BSA and 0.5% formaldehyde for analysis. Monocytes were assayed by gating on CD14 expressing cells within the PBMC population. Levels of phospho-p38 MAPK stimulated monocytes were determined by measuring the geometric MFI levels of 1000 cells, subtracted by the geometric MFI levels of untreated monocytes.

4.3. RESULTS

4.3.1. Selective enhancement of human monocyte migration towards chemokines by IDR-1002

Indications of β-integrin-independent enhancement of monocyte chemotaxis towards certain chemokines by IDR-1002 in Chapter 3 led to the hypothesis that IDR-1002 can regulate the monocyte response to specific chemokines. The ability of IDR-1002 to promote monocyte responses to endogenous chemokines may present an alternative mechanism by which peptide enhances monocyte recruitment *in vivo*. To investigate the effects of IDR-1002 on monocyte responses to chemokines, peptide-stimulated human monocytes were assessed for their ability to migrate towards various monocyte-specific chemokines after 1 hour. As expected, chemokines MCP-1, MCP-3, MIP-1α, and RANTES induced up to a 3-fold increase in monocyte chemotaxis across a porous membrane relative to spontaneous monocyte chemotaxis towards media-containing wells (Figure 4.1). Stimulation of monocytes with IDR-1002 did not significantly alter monocyte migratory behaviour towards chemokines MCP-1 and MCP-3. In contrast IDR-1002 stimulation of monocytes resulted in a significant enhancement of migration towards MIP-1α and RANTES, exhibiting a 5-fold increase in monocyte migration over media-only controls, up to a 2-fold increase over chemokine-induced monocyte migration. The results show that IDR-1002 is able to enhance monocyte chemotaxis to certain endogenous chemokines. Furthermore, these observations suggest that IDR-1002 can selectively alter monocyte sensitivity to endogenous mediators produced by the host during infection.

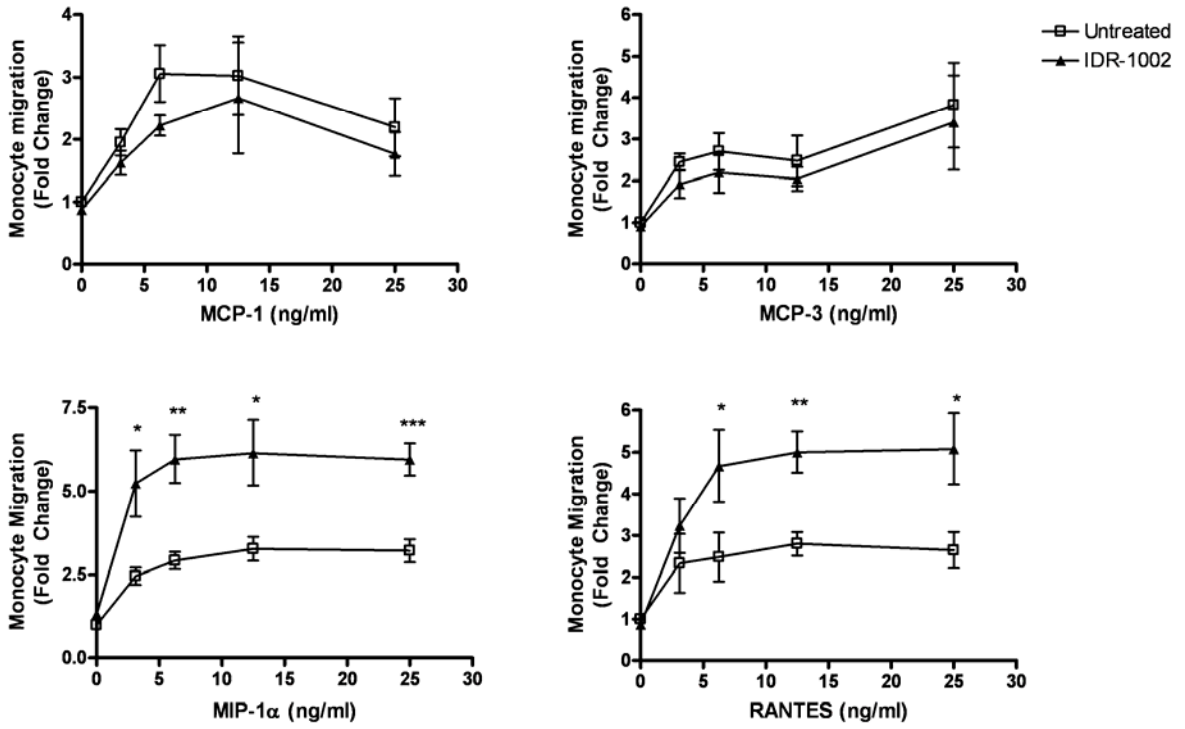

Figure 4.1 IDR-1002 enhancement of monocyte chemotaxis towards chemokines. Effects of IDR-1002 stimulation (20 μg/ml) on monocyte chemotaxis towards chemokines (3.125, 6.25, 12.5, 25 ng/ml) through a non-coated membrane were observed after 1 hour. Data are presented as the mean fold-increases in monocyte migration over baseline migration towards media alone (± SE) of at least 4 independent experiments, each from independent donors. Statistical comparisons between IDR-1002-stimulated monocytes and untreated monocytes were done by Student's two-tailed *t* test. *p < 0.05; **p < 0.01; ***p < 0.001.

4.3.2. Regulation of CCR5 surface expression on human monocytes

Cellular responses to chemokines are mediated by a diverse range of chemokine receptors expressed on cell surfaces. The variegated expression of chemokine receptors between different cell populations dictates their ability to respond to specific chemokines within the host chemokine milieu. Within the same cell population, the regulation of receptor expression, coupled with the promiscuous binding of chemokine receptors to an assortment of chemokines (Table 4.1) can result in the desensitization or enhancement of cellular responses to specific sets of chemokines. It is through these mechanisms that the immune response can direct cellular trafficking to different locales at different stages of the immune

response. It seemed feasible that IDR-1002 might selectively enhance monocyte chemotaxis towards MIP-1α and RANTES by modulating chemokine receptor expression. To investigate this, PBMCs were stimulated with IDR-1002 at various time intervals and the $CD14^+$ monocyte population was assessed for the surface expression of CCR2, an MCP-1 and MCP-3 receptor, and CCR5, a MIP-1α and RANTES receptor. IDR-1002 induced a rapid upregulation of CCR5 surface expression by human monocytes (Figure 4.2). Significant CCR5 upregulation by IDR-1002 was observed as early as 15 minutes and remained elevated at 60 minutes post stimulation. In contrast, monocyte surface expression of CCR2 was slightly decreased by IDR-1002 stimulation, an effect that was not statistically significant. This observation demonstrates that IDR-1002 promoted CCR5 expression on monocyte surfaces and this potentially explained the selective enhancement of monocyte chemotaxis towards MIP-1α and RANTES.

Table 4.1 Monocyte chemokine receptors and their ligands.

Receptor	**Chemokine Ligands** (88, 168)
CCR1	MCP-3 (CCL7), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5)
CCR2	MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13)
CCR5	MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5)
CCR8	CCL1
CXCR1	IL-8 (CXCL8)
CXCR2	IL-8 (CXCL8), GRO-α (CXCL1), GRO-β (CXCL2), GRO-γ (CXCL3)
CXCR4	SDF-1 (CXCL12)
CX_3CR1	Fractalkine (CX_3CL1)

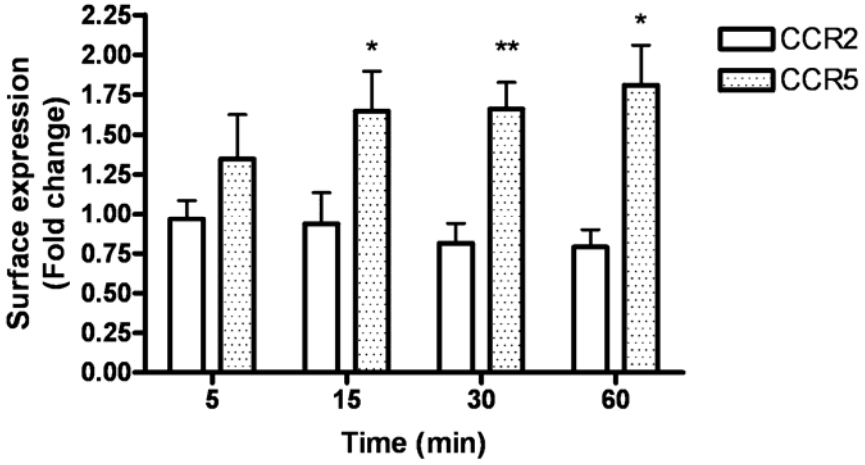

Figure 4.2 IDR-1002 regulation of monocyte CCR surface expression. PBMCs were stimulated with IDR-1002 (20 μg/ml). Time course determination of monocyte CCR levels was done by flow cytometric detection of anti-CCR antibodies, gating on the $CD14^+$ monocyte population. Data are presented as the mean fold-increases of CCR expression over unstimulated cells (± SE) of 3 independent experiments, each from an independent donor. Statistical comparisons to unstimulated cells were done by Student's two-tailed *t* test. *$p < 0.05$, **$p < 0.01$.

4.3.3. The role of CCR5 in IDR-1002 enhancement of monocyte migration

To investigate the link between IDR-1002 enhancement of monocyte chemotaxis towards MIP-1α and RANTES and the promotion of monocyte CCR5 surface expression, the chemotaxis studies were repeated in a CCR5-inhibited system. Monocytes were pre-treated with CCR5-inhibiting antibody or an isotype-matched control antibody for 1 hour, prior to stimulation with IDR-1002. Levels of spontaneous monocyte migration towards media-only wells were not altered between the two antibody treatments, nor did IDR-1002 influence spontaneous monocyte migration in either system, correlating with the lack of chemokinetic activity seen in Chapter 3 (Figure 4.3). Chemotaxis towards MIP-1α and RANTES by isotype-Ab-treated monocytes was observed, as expected. Similar to the effects seen in the initial chemotaxis studies, migration towards MIP-1α was significantly enhanced with IDR-1002 stimulation, as was migration towards RANTES, an effect nearing significance. However, inhibition of CCR5 eliminated any promotion of chemotaxis mediated by IDR-

1002. Interestingly, CCR5-inhibition resulted in only a slight, non-significant, decrease in monocyte chemotaxis towards both MIP-1α and RANTES in the absence of peptide. It is possible that, in this system, monocyte chemotaxis towards these chemokines is primarily mediated, or compensated for, by other chemokine receptors and, as such, is less sensitive to CCR5 inhibition. Compensation of chemotaxis is possible due to the functional redundancy of the chemokine-chemokine receptor system. This redundancy stems from the ability of chemokines to engage multiple chemokine receptors, and vice versa. Thus during CCR5-inhibition, monocyte chemotaxis towards MIP-1α and RANTES is likely compensated by the engagement of chemokines to alternate receptors, such as CCR1 (165, 169, 170).

To determine whether IDR-1002 was promoting monocyte chemotaxis through modulating chemokine receptor function, inhibition experiments were repeated after monocyte pre-treatment for 1 hour with pertussis toxin. G-protein coupled receptor-mediated signalling is a major signal transduction axis triggered by the vast majority of chemokines (141). Briefly, chemokine binding to their specific receptor results in the activation of receptor-bound heterotrimeric G-protein. This bound G-protein is often the pertussis toxin-sensitive $G\alpha_i$-protein. Upon activation, the dissociation of Gα from the Gβγ subunit results in the activation of PLC, the generation of inositol triphosphate and diacylglycerol, calcium flux, and the subsequent activation of protein kinase C. The generation and activation of these secondary signalling mediators leads to subsequent activation of numerous signalling pathways, including the PI3K and MAPK pathways. These signalling events serve to coordinate actin re-organization, integrin-mediated adhesion, and overall cell motility. Baseline migration of monocytes was inhibited by more than 50% with pertussis toxin treatment (Figure 4.3). As expected, migration of pertussis toxin-treated monocytes towards chemokines did not differ from migration towards media-only wells, signifying an elimination of chemotaxis. IDR-1002 treatment also had no effect on monocytes pre-treated with pertussis toxin with respect to chemotactic ability and migration. These results indicate that IDR-1002 enhancement of migration potentially stems from a strengthening of chemokine receptor-mediated signalling rather than through another means.

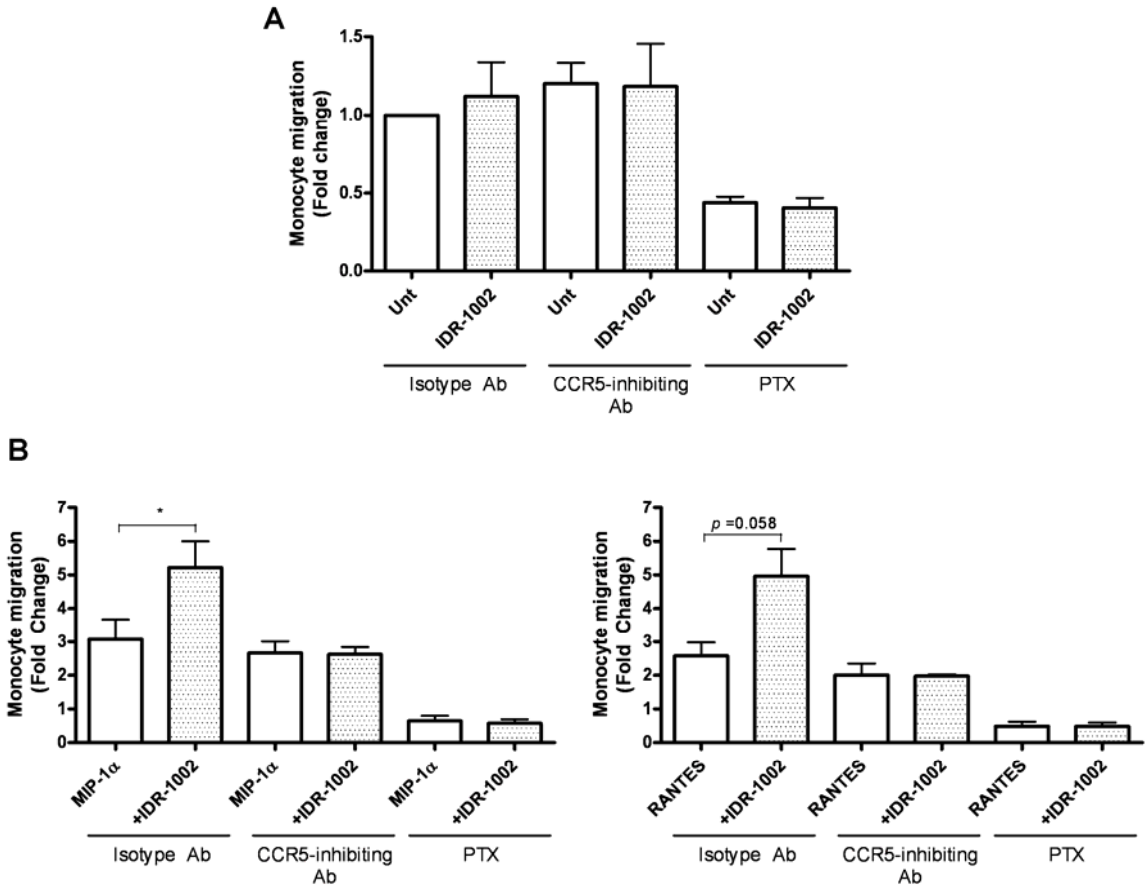

Figure 4.3 The role of CCR5 and Gi-proteins in IDR-1002 enhancement of monocyte chemotaxis. Monocytes were pre-treated for 1 hour with a CCR5-inhibiting antibody (20 μg/ml), an isotype-matched control antibody (20 μg/ml), or pertussis toxin (100 ng/ml) prior to stimulation with IDR-1002 (20 μg/ml). The ability of monocytes to migrate (A) spontaneously or (B) migrate towards host chemokines (12.5 ng/ml) was assessed after 1 hour. Data are presented as the mean fold-increases of migration over baseline migration of isotype-treated monocytes (± SE) of at least 3 independent experiments, each from independent donors. Statistical comparisons were done by Student's two-tailed *t* test. *p < 0.05.

4.3.4. IDR-1002 reinforcement of chemokine-induced p38 MAPK activation

As IDR-1002 was found to augment monocyte chemotaxis towards MIP-1α and RANTES in a CCR5-dependent manner, the effects of IDR-1002 on CCR5 chemokine-mediated signalling was then investigated. Chemokines, through their interaction with chemokine receptors, are potent activators of multiple signal transduction pathways, including the G_i-protein signalling axis as well as the PI3K and MAPK pathways (141, 171). It is through these receptor-mediated signalling pathways that chemokines exert their effects

on motility and adhesion. The ability of IDR-1002 to influence monocyte expression of CCR5 may result in an enhancement of chemokine-mediated signalling and downstream function. To investigate this, PBMCs were pre-treated with IDR-1002 for 15 minutes prior to 5 minutes of stimulation with MIP-1α and RANTES. The levels of intracellular phosphorylated p38 MAPK (T180/Y182) in the $CD14^+$ monocyte population were assessed. MIP-1α and RANTES induced a rapid phosphorylation of p38 MAPK (Figure 4.4). IDR-1002 pre-treated monocytes also exhibited modestly elevated phospho-p38 MAPK levels, consistent with observations that IDR-1002 is an activator of monocyte MAPK pathways. Chemokine stimulation of IDR-1002 pre-treated monocytes resulted in a greater than 2-fold enhancement of p38 MAPK phosphorylation compared to peptide or chemokines alone. These results demonstrate the enhancement of MIP-1α- and RANTES-induced signal transduction activity in human monocytes.

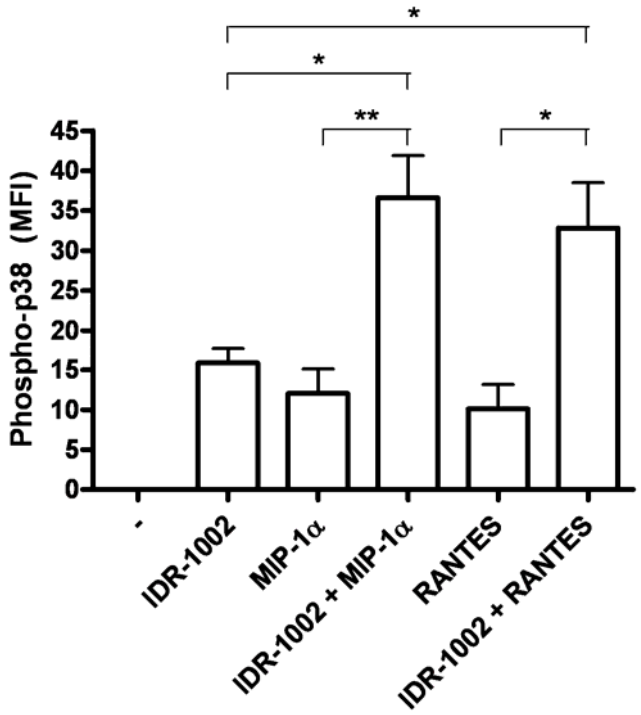

Figure 4.4 IDR-1002 regulation of chemokine-induced p38 MAPK activation. PBMCs were pre-treated with IDR-1002 (20 μg/ml) prior to stimulation with MIP-1α and RANTES (both 12.5 ng/ml). After 5 minutes, monocyte p38 MAPK activation was assessed by flow cytometric detection of intracellular phosphorylated p38 MAPK, gating on the $CD14^+$ monocyte population. Data are presented as the mean MFI values over unstimulated monocytes of 4 independent experiments, each from independent donors. Statistical comparisons were done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$.

4.4. DISCUSSION

It was demonstrated in this study that IDR-1002 enhanced human monocyte chemotaxis towards chemokines MIP-1α and RANTES. The synergistic effect on monocyte migration between IDR-1002 and certain endogenous chemokines has significant implications in the role of IDR-peptides during an anti-infective immune response. As mentioned in previous chapters, the onset of microbial infection results in the production of endogenous immune modulating agents, including a diverse range of inflammatory cytokines. MIP-1α and RANTES, and others belonging to the subset of inducible chemokines, are rapidly produced by immune cells in response to endogenous inflammatory

mediators (158, 172, 173). Similarly, TLR-targetted microbial signatures can also directly stimulate cellular production and secretion of a range of chemokines (158, 174). These effects culminate in the generation of multiple chemokine gradients that are collectively responsible for the targeting and trafficking of monocytes and other immune cells to the local site of infection. Ultimately, cellular sensitivity to these assorted gradients dictates the degree to which immune cells are recruited. It is feasible that the priming of monocytes by IDR-1002, for enhanced migration within a MIP-1α and RANTES gradient, contributes to the promotion of monocyte recruitment seen *in vivo*. Thus, this investigation revealed a novel mode of action through which IDR-1002 modulated monocyte behaviour and potentially enhanced host anti-infective responses.

The selective enhancement of monocyte migration to MIP-1α and RANTES, but not MCP-1 or MCP-3, was initially puzzling and demanded further investigation. In terms of function, members of the sub-family of chemoattractive cytokines are highly similar. Chemokines, through engagement with their G-protein-coupled receptors, cause the activation of pertussis-toxin-sensitive G-protein coupled receptor signalling and the generation of secondary signalling molecules, such as inositol triphosphate and diacylglycerol (141). These effects further lead to the activation of downstream signalling pathways, such as the MAPK and PI3K pathways. This results in the regulation of cellular shape through cytoskeletal arrangements and coordination of movement. Despite the high similarity between the general functions of chemokines and consequences of their action, different chemokines play distinct roles in immunity, directing specific cell populations to various locales at different temporal stages of an anti-infective response (166, 175). The method by which the immune system orchestrates these highly similar cytokines into complex multi-stage directors of cellular trafficking relies, in large part, on differential expression of chemokine receptors on cell surfaces. Increased surface expression of specific receptors can enhance cellular responses to their specific chemokine ligands, whereas internalization or downregulation of chemokine receptors is a common method of desensitizing cells to specific chemokines (162, 165). Thus I proposed that the selective enhancement of migration towards specific chemokines by IDR-1002 resulted from a modulation of chemokine receptor expression. In this study, IDR-1002-stimulated monocytes were shown to demonstrate upregulated surface expression of CCR5, while CCR2 expression

remained unchanged. An upregulation of CCR5 expression, and subsequent enhancement of downstream receptor-induced signal transduction, would account for the selective enhancement by IDR-1002 of monocyte migration towards CCR5-chemokines. Consistent with this interpretation, IDR-1002 augmentation of chemotaxis towards MIP-1α and RANTES was eliminated in the presence of a CCR5-inhibiting antibody, further supporting the hypothesis that IDR regulation of chemokine receptors leads to this observed enhancement. This study could not exclude the potential regulation of other MIP-1α and RANTES receptors expressed by monocytes, such as CCR1. However, it is interesting to note that although monocyte chemotaxis is mediated by multiple chemokine receptors, as evidenced by the very slight reduction of chemotaxis to the chemokines themselves when CCR5 was blocked, promotion of chemotaxis towards these chemokines by IDR-1002 was fully dependent on CCR5.

Although this study demonstrates the regulation of CCR5 surface presentation by IDR-1002, potentially leading to a promotion of chemotaxis towards CCR5-chemokines, the mechanism of regulation is currently unknown. Regulation of chemokine receptor mRNA expression can lead to the alteration of the levels of receptor proteins synthesized, an effect utilized by many endogenous cytokines to coordinate cell sensitivity to chemokines. While the modulation of chemokine receptor gene transcription and protein synthesis by IDR-1002 is certainly possible, the rapid effect on receptor expression and enhancement of chemotaxis are consistent with a postulated post-translational mode of regulation. The level of chemokine receptors on cell surfaces is largely dependent on the balance between internalization and receptor recycling (176). Chemokine receptors undergo basal levels of internalization mediated by multiple endocytic pathways, an effect which is greatly increased by ligand binding and is the major mode of chemokine desensitization (176, 177). Internalized chemokine receptors are then either sent into degradative pathways or trafficked back to the plasma membrane in a re-sensitized state. It is possible that IDR-1002 promotes rapid receptor expression by acting on this process, whether by limiting chemokine receptor uptake or promoting recycling receptors to the membrane. Actin polymerization, an essential process for CCR5 movement and recycling, is also essential for IDR-mediated chemokine induction (80, 176, 178). There is a possibility that IDR-1002 modulates monocyte actin polymerization and actin-mediated cytoskeletal rearrangement, as reinforced by the fact that

the proposed IDR receptor p63/SQSTM-1 has a role in actin mobilization in cells. This would then likely result in downstream effects on chemokine receptor steady-state levels. The mode by which IDR-1002 influences CCR5 expression, without affecting CCR2 expression, also remains unknown. This selective regulation may originate from slight differences in the trafficking behaviour between the two receptors. CCR5 seems to be predominantly recycled, accumulating in early endosomes that are recycled in a perinuclear location following internalization (179). CCR5 ligand binding does not affect the basal rate of receptor degradation, and indeed agonist-bound CCR5 can be repeatedly internalized and quickly recycled to the plasma membrane instead of being directed towards degradative lysosomal compartments (180, 181). In contrast, CCR2 can be found within lysosomal compartments as early as 30 minutes following ligand binding and restoration of CCR2 surface levels occurs at a relatively slower pace (182, 183). Perhaps the slight differences in trafficking mechanisms and degradative behaviour between chemokine receptors, coupled with possible effects on receptor compartment mobilization by IDR-1002, resulted in the observed upregulation of CCR5 but not CCR2.

This study focused on the ability of IDR-1002 to regulate chemokine receptor surface expression as a mechanism for enhancing monocyte chemotaxis. However, regulation of chemokine/chemokine receptor function can occur on many levels. Direct receptor modifications, such as through phosphorylation by G protein-coupled receptor kinases, can impact on receptor sensitivity and activity (184, 185). In addition, modulation of receptor-mediated downstream signal transduction might result in changes to chemokine-mediated behaviour, such as chemotaxis. The signal transduction cascades regulating these processes overlap with those impacted by IDR-1002, including calcium flux-mediated signal transduction and those regulated by the PI3K-Akt, MAPK, and G protein pathways (80, 141, 171). It seems highly likely that the modulation of these networks by IDR-1002, in addition to modulation of receptor expression, impacts on chemokine-mediated functions. The observation that IDR-1002 enhancement of monocyte chemotaxis is abolished in the presence of pertussis toxin supports the suggestion that IDR-1002 may be reinforcing chemokine receptor-mediated signalling or acting through other GPCR-mediated mechanisms. Similarly, the finding that IDR-1002 enhanced chemokine-induced p38 MAPK activation suggests a potential additive effect between IDR-1002- and chemokine-mediated

signal transduction events. Whether this enhancement stems from the upregulation of monocyte CCR5 expression by IDR-1002 or from the enhancement of downstream signalling events, or a combination of both, remains to be seen. Further investigation regarding the possible cross-talk between IDR-mediated signalling and chemokine-induced pathways is needed to determine the extent of regulation that IDR-1002 exerts on chemokine function. A synergistic enhancement of chemokine signal transduction might however account for the observed increase in monocyte chemotaxis.

In summary this chapter revealed a novel mechanism by which IDR-1002 enhanced monocyte recruitment; namely the enhancement of CCR5-mediated chemotaxis towards host chemokines MIP-1α and RANTES by IDR-1002. It is proposed that this peptide-mediated effect stems from the selective promotion of CCR5 expression on monocyte surfaces and a potential reinforcement of chemokine-mediated signal transduction pathways. This study not only revealed a third regulatory axis of IDR-1002 on monocyte mobilization, in addition to an enhancement of β1-integrin-mediated monocyte adhesion and induction of chemokine production, but presents novel avenues through which IDR-peptides may regulate immunity. Synergy between IDR-1002 and chemokines, both of which are complex immunomodulators, may have noteworthy ramifications on the anti-infective immune response. Chemokines, in addition to mediating recruitment, are highly involved, directly and indirectly, in many aspects of immunity, including cellular growth, wound healing, and differentiation (114, 167), processes that are also influenced by HDPs. It would be of great interest to determine the effects of IDR-peptides on cell growth and differentiation, and whether this stems from their ability to enhance chemokine-mediated responses. Overall, this study further characterizes the augmenting effects of IDR-1002 on monocyte recruitment and hints at the ability of IDR-peptides to modulate an array of immune processes through the cooperation with host chemokines.

5 IDR-1002 REGULATES THE INFLAMMATORY RESPONSE IN LPS-STIMULATED HUMAN PBMCs VIA MODULATION OF LPS-INDUCED SIGNALLING

5.1. INTRODUCTION

In the search for immunomodulatory antimicrobial therapeutics, IDR-peptides have been discovered and characterized as complex regulatory agents with diverse effects on the immune response, akin to their natural HDP counterparts. IDR-peptides have been shown as potent, selective inducers of chemokine production in murine and human experimental models. Additionally, in previous chapters IDR-1002 was demonstrated to have an ability to influence monocyte behaviour, specifically through the modulation of integrin-mediated adhesive responses as well as the selective regulation of monocyte chemokine receptors, both influencing monocyte motile behaviour. Parallel studies regarding IDR-peptide function have also suggested the ability of peptides to modulate macrophage differentiation and to promote re-epithelialization, similar to human cathelicidin LL-37 (unpublished data from Hancock lab members N. Afacan, O. Pena, J. Pistolic, and L. Wong). Thus through these investigations, it is clear that IDR-peptides, analagous to endogenous HDPs, are complex regulators of the innate immune process, including inflammation. However, the regulation of bacterial-induced inflammation by IDR-peptides has been only modestly explored.

During an infection, pro-inflammatory mediators induced by microbial signatures work hand-in-hand with other immune mediators to synergistically increase cellular recruitment, activation, differentiation, antimicrobial mechanisms, and wound healing, etc. A characteristic feature of some HDPs is their ability to enhance these functions while selectively limiting the potentially harmful aspects of the induced inflammatory response. This effect was seen with the natural HDP, LL-37, and the synthetic peptide IDR-1, which enhance chemokine production and macrophage recruitment, functions necessary to augment murine anti-infective responses, while suppressing the levels of pro-inflammatory cytokines like TNFα and IL-6 (40, 44, 50, 79). Similarly, the enhancement of the murine anti-infective response by IDR-1002 was not associated with an increase in these inflammatory mediators, a result mirrored by my studies in a human *in vitro* PBMC model (80). These observations

strongly suggest that the suppression of bacterial-induced pro-inflammatory responses is among the repertoire of IDR-peptide immunomodulatory functions and is of great interest in the development of these anti-infective immune modulators. Thus, this Chapter aimed to characterize how IDR-1002 selectively modulated the inflammatory response induced by LPS, one of the principal mediators of bacterial-induced inflammation.

LPS, a generally conserved structure found on the outer membrane of Gram-negative bacteria, acts as one of the most potent inducers of inflammation during infection. Following its recognition by TLR4, a member of the TLR-family of innate pathogen-recognition receptors, LPS triggers the activation of inflammatory signal transduction networks (186). These include the activation of central inflammatory cascades, such as the NFκB and MAPK pathways, resulting in the rapid generation of a wide range of pro-inflammatory mediators, including the cytokines, TNFα, IL-6 and IL-1 (5, 186). The production of these cytokines serves numerous functions. Pro-inflammatory cytokines are necessary for the activation of host cells, including the activation of endothelial cells to promote the recruitment of effector cells and the activation of the acute-phase response which results in microbial opsonization and activation of the complement system (5, 118). In addition, this inflammatory milieu generated, directly or indirectly, by LPS serves to activate antimicrobial cellular responses, consisting of an enhancement of phagocytosis and the generation of damaging reactive oxygen and nitrogen species (187, 188). Overall, these essential processes, triggered in part by bacterial LPS, cooperate to form a rapid and effective antibacterial response. However, in some situations the immune response cannot effectively clear bacterial pathogens. A major risk of using immunomodulatory agents to augment an insufficient anti-infective response lies in the potential disruption of inflammatory balance. Specifically, the exogenous strengthening of innate anti-infective responses, and an associated intensification of the pre-existing inflammatory response, may lead to damaging side effects. Indeed, the development of systemic immune boosting agents, such as natural and synthetic TLR agonists, have been limited by the threat of these risk factors, and even current immune therapies, such as IFN therapy, have elicited symptoms of harmful inflammatory responses and must be carefully controlled (189-192). In this context, the HDP-like ability of IDR-peptides to enhance innate immune functions while potentially modulating bacterial-induced inflammation is of significant interest. Thus an investigation of how IDR-1002 modulates LPS-induced

inflammation is an essential stepping stone in the therapeutic development of safe and effective anti-infective immune mediators.

In this Chapter, IDR-1002 was shown to be a potent suppressor of LPS-induced inflammatory responses in human PBMCs. The PBMC population was chosen for its relevance in bacterial-induced sepsis and its role in mediating IDR-1002-induced responses. IDR-1002 at low concentrations was able to knock down the production of a range of cytokines by LPS-stimulated PBMCs. This suppression of responses correlated with the IDR-1002 regulation of LPS-induced signal transduction activity in human monocytes, a cell population significant in the coordination of the inflammatory response; Specifically, IDR-1002 caused the suppression of LPS-induced p65 NFκB and p38 MAPK activation, pathways essential for the production of inflammatory cytokines. Furthermore, it was found that the regulation of LPS-induced inflammatory responses in PBMCs by IDR-1002 involved the PI3K-Akt network, a pathway known to play a role in the suppression of LPS-induced TLR signalling. Specifically, the ability of IDR-1002 to suppress LPS-induced PBMC cytokine production was partially dependent on the Akt pathway. As well, these effects correlated with an additive reinforcement of LPS-induced PI3K-Akt signalling by IDR-1002. Additionally, the presence of PI3K- and Akt-specific inhibitors partially abrogated the ability of IDR-1002 to suppress LPS-induced activation of p38 MAPK and its downstream target ATF-2. Overall, this study demonstrated the ability of IDR-1002 to suppress LPS-induced responses in human PBMCs and indicated this correlated with the modulation by this peptide of LPS-induced monocyte signalling activity. The PI3K-Akt pathway, shown to be involved in other IDR-mediated functions, was also identified as one of several possible mechanisms by which IDR-1002 elicits its anti-endotoxic effects, potentially through its regulatory cross talk with other TLR4-activated pathways. An understanding that IDR-1002 could regulate endotoxin-induced inflammatory responses, in conjunction with previous findings demonstrating peptide-mediated enhancement of other innate immune processes, is valuable in the development of anti-infective agents which act by selectively enhancing the beneficial aspects of the immune response.

5.2. METHODS AND MATERIALS

5.2.1. Reagents

Peptide IDR-1002 (VQRWLIVWRIRK-NH_2) was synthesized by solid phase F-moc chemistry by CPC Scientific (Sunnyvale, CA). LPS from *Pseudomonas aeruginosa* strain H103 was purified using the Darveau-Hancock method as previously described (89) and stored at 4˚C. PI3K inhibitor LY294002 and Akt inhibitor SH-5 (both from Calbiochem, San Diego, CA) were resuspended in DMSO (Sigma-Aldrich) and stored at -20˚C. Concentrations of DMSO used never exceeded 0.1% (v/v) in cell cultures.

5.2.2. PBMC isolation

Human PBMCs were isolated as previously reported (44). Briefly, venous blood was collected from healthy volunteers using heparin-containing Vacutainer tubes (BD Bioscience, San Jose, CA) in accordance with UBC ethical approval and guidelines. Human blood was diluted in an equal amount of PBS (Invitrogen, Carlsbad, CA) then layered over Ficoll-Paque Plus (Amersham, Piscataway, NJ) prior to separation by density-gradient centrifugation. The mononuclear cell layer was extracted and washed twice with PBS and then resuspended in RPMI 1640 with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Invitrogen), placed in a polypropylene tube (BD Falcon, San Jose, CA), and cultured in a humidified incubator at 37°C with 5% CO_2.

5.2.3. Measurement of pro-inflammatory cytokine production by ELISA

Anti-endotoxic effects of IDR-1002 were investigated by seeding $1x10^6$ PBMCs on tissue culture plates and placing them in a humidified incubator at 37 ˚C and 5% CO_2 for 1 h. Cells were then stimulated with 10 ng/ml of LPS, IDR-1002 at 10 μg/ml, or a combination of both, and incubated for 24 hours. Samples were then centrifuged at 1000 x *g* for 10 minutes to obtain cell-free supernatants, which were then stored at -20 ˚C. Cytokine levels were measured by ELISA using anti-human-MCP-1 Ab clones 5D3-F7 and 2H5, anti-human-IL-6 Ab clones MQ2-13A5 and MQ2-39C3, anti-human-TNFα Ab clones MAb1 and MAb11 (all from eBioscience, San Diego, CA), anti-human-GRO-α Ab clones 20326 and BAF275 (R&D

Systems, Minneapolis, MN), and anti-human-MIP-1α Ab clones A122B14D7 and A122B10E11 (Invitrogen), followed by avidin HRP (eBioscience) as per manufacturer's protocols. The ELISAs were then developed using the TMB Liquid Substrate System (Sigma-Aldrich) and colour development measured using a Power Wave X340 plate-reader (Bio-Tek Instruments, Winooski, VT). Quantification of cytokines was done by comparisons with results for serial dilutions of recombinant cytokine standards (R&D Systems, and eBioscience).

In Akt inhibitor studies, $1x10^6$ PBMCs were seeded on tissue culture plates and placed in a humidified incubator at 37 ˚C and 5% CO_2 for 1 h. PBMCs were then treated with the indicated concentrations of Akt inhibitor SH-5, or a 0.1% (v/v) DMSO vehicle control treatment, for 1 hour prior to a 24 hours stimulation with 10 ng/ml LPS and 10 μg/ml IDR-1002. Levels of pro-inflammatory cytokines in the culture supernatants were then assayed by ELISA as described above. Cytokine levels are presented as the % of LPS-induced cytokine production, calculated by the following equation, $[(Cytokine_{LPS+IDR-1002})/(Cytokine_{LPS}) * 100]$, for each inhibitor condition.

5.2.4. Flow cytometry to measure intracellular protein phosphorylation

All flow cytometry results were collected using a FACSCalibur cytometer in conjunction with CellQuest Pro software (BD Biosciences). PBMCs ($2x10^6$ per well) were seeded into tissue culture plates and placed in a humidified incubator at 37 °C and 5% CO_2 for 1 h. Cells were then stimulated with 10 ng/ml of LPS, 10 μg/ml of IDR-1002, or a combination of both, and incubated at 37 °C for the indicated time points. Following treatment, PBMCs were fixed with PBS and 4% (w/v) formaldehyde (Fisher Scientific, Pittsburgh, PA) at room temperature for 20 min in preparation for staining. PBMCs were then washed with 0.5% (w/v) BSA in PBS and permeabilized in 90% (v/v) methanol at 4 °C for 30 min. The cells were then washed and stained at room temperature for 1 hour with anti-phospho-NFκB p65 (Ser536) 93H1 rabbit mAb, anti-phospho-p38 (Thr180/Tyr182) 3D7 rabbit mAb, anti-phospho-Akt (Ser473) 193H12 rabbit mAb, or an anti-phospho-GSK3β 5B3 rabbit mAb (all from Cell Signalling Technology, Danvers, MA). Following washing, the cells were stained with a goat anti-rabbit IgG-Alexa Fluor 647 Ab (H+L, Invitrogen) for 30

min at room temperature. Cells were then washed again and stained with an anti-human CD14-Alexa Fluor 488 M5E2 mouse mAb (Biolegend) for 30 min at room temperature. The cells were then washed and resuspended in PBS with 0.5% BSA and 0.5% formaldehyde for analysis. Monocytes were assayed by gating on CD14 expressing cells within the PBMC population. Levels of phosphorylated p38 MAPK, phospho-p65 NFκB, phospho-Akt, and phospho-GSK3β in monocytes were determined by measuring the geometric MFI levels of 1000 cells, subtracted by the geometric MFI levels of untreated monocytes.

In PI3K-Akt inhibitor studies, $2x10^6$ PBMCs were seeded per well on tissue culture plates and placed in a humidified incubator at 37 °C and 5% CO_2 for 1 h. Cells were then pre-treated with 10 μM of PI3K inhibitor LY294002 or 5 μM Akt inhibitor SH-5 for 1 hour prior to stimulation with 10 ng/ml of LPS, 10 μg/ml of LPS, or a combination of both, for the indicated time points. Following treatment, PBMCs were fixed with PBS and 4% (w/v) formaldehyde (Fisher Scientific) at room temperature for 20 min in preparation for staining. PBMCs were then washed with 0.5% BSA in PBS and permeabilized in 90% (v/v) methanol at 4 °C for 30 min. The cells were then washed and stained at room temperature for 1 hour with anti-phospho-ATF-2 (Thr71) 11G2 rabbit mAb or anti-phospho-p38 (Thr180/Tyr182) 3D7 rabbit mAb (both from Cell Signalling Technology). Following washing, the cells were stained with a goat anti-rabbit IgG-Alexa Fluor 647 Ab (H+L, Invitrogen) and an anti-human CD14-Alexa Fluor 488 M5E2 mouse mAb (Biolegend) for 30 min at room temperature. The cells were then washed and resuspended in PBS with 0.5% BSA and 0.5% formaldehyde for analysis. Monocytes were assayed by gating on CD14 expressing cells within the PBMC population. Levels of phosphorylated phospho-p38 and phospho-ATF-2 in monocytes were determined by measuring the geometric MFI levels of 500 cells, subtracted by the geometric MFI levels of untreated monocytes. The % change of MFI between LPS-1002-induced phosphorylation and LPS-induced phosphorylation (Δ%) was calculated by the following equation, $[(1\text{-}MFI_{LPS+IDR\text{-}1002})/(MFI_{LPS}) * 100]$, for each time-point and inhibitor condition.

5.3. RESULTS

5.3.1. IDR-1002 regulation of LPS-induced cytokine production in human PBMCs

Cytokine mediators, produced in response to an infectious insult, play an essential role in the propagation and coordination of the inflammatory response. As such, to elucidate

peptide regulation of the LPS-mediated inflammatory response, this study focused on the effects of IDR-1002 on LPS-induced cytokine production. PBMCs, freshly isolated from human blood, were stimulated with 10 ng/ml of *P. aeruginosa* LPS, 10 μg/ml IDR-1002, or a combination of the two. After a 24 hour stimulation period, culture supernatants were harvested and measured for the levels of inflammatory cytokines by ELISA. As expected, endotoxin-stimulated PBMCs exhibited a potent production of pro-inflammatory mediators IL-6 and TNFα whereas IDR-1002 stimulation alone had no effect on inflammatory cytokine production (Figure 5.1). However, the co-stimulation of PBMCs with LPS and IDR-1002 resulted in a significant suppression of IL-6 and TNFα of 60% and 95% respectively suggesting a peptide-mediated suppression of LPS-induced responses.

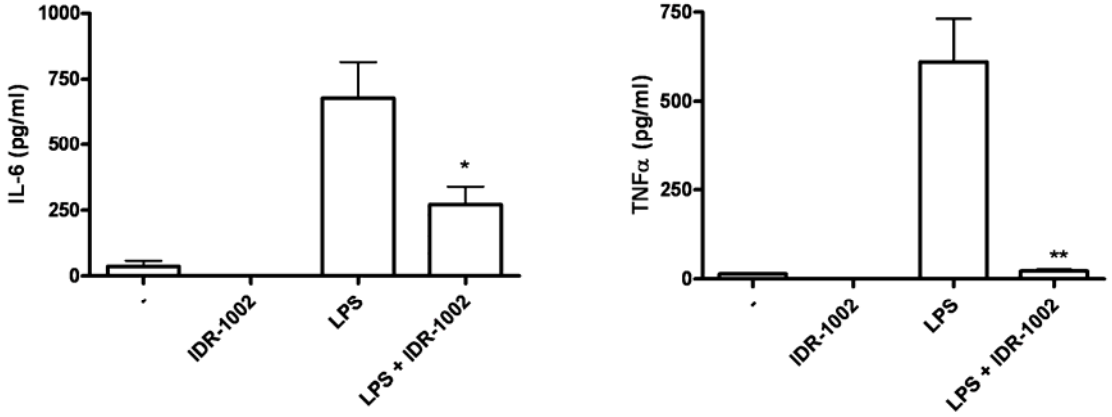

Figure 5.1 IDR-1002 regulation of LPS-induced cytokine production by human PBMCs. PBMCs were stimulated with IDR-1002 (10 μg/ml), LPS (10 ng/ml) or a combination of both. After 24 hours, levels of cytokines IL-6 and TNFα in culture supernatants were measured by ELISA. Data are presented as the mean cytokine production (± SE) of 5 independent experiments, each from individual donors. Statistical comparison against LPS-stimulated PBMCs were done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$.

It was then investigated whether this suppressive effect was seen in the production of other LPS-induced cytokines. Thus the experiment was repeated with the assessment of LPS-induced chemokine production. Stimulation with a low dose of IDR-1002 alone did not result in any change in PBMC chemokine production (Figure 5.2). As expected, LPS-stimulation

resulted in the robust production of chemokines. The co-stimulation of PBMCs with LPS and IDR-1002, resulted in a significant suppression of MCP-1, and GRO-α, while suppression of MIP-1α was near significant. Interestingly, the degree of suppression by IDR-1002 varied between cytokines, with a near-complete suppression seen with TNFα whereas suppression of MCP-1 production was only approximately 50%. It must be noted that chemokine production by LPS and peptide-stimulated PBMCs was at a higher level than that of PBMCs stimulated with peptide alone (Figure 5.2**,** Figure 2.1), suggesting a balancing of LPS-induced chemokine responses by IDR-1002. Overall, these results demonstrate a widespread modulation of LPS-induced cytokine production by IDR-1002 in human PBMCs. These results, in turn, indicate a function of IDR-1002 in moderating LPS-mediated inflammation.

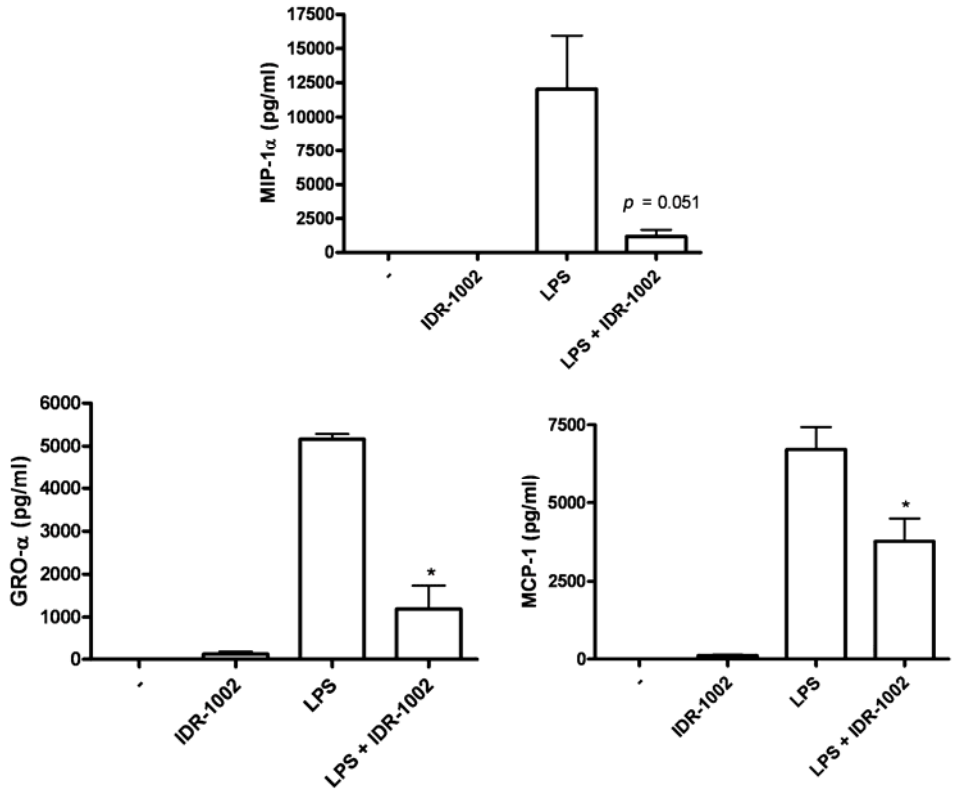

Figure 5.2 IDR-1002 regulation of LPS-induced chemokine production by human PBMCs. PBMCs were stimulated with IDR-1002 (10 μg/ml), LPS (10 ng/ml) or a combination of both. After 24 hours, levels of chemokines MIP-1α, GRO-α, and MCP-1 in culture supernatants were measured by ELISA. Data are presented as the mean cytokine production (± SE) of 3 independent experiments, each from individual donors. Statistical comparison against LPS-stimulated PBMCs were done by Student's two-tailed *t* test. *p < 0.05.

5.3.2. IDR-1002 modulation of LPS-induced NFκB and p38 MAPK signalling in human monocytes

The ability of LPS to rapidly induce a wide range of inflammatory mediators relies on its potent activation of TLR4-mediated signalling pathways. Engagement of LPS, and its associated binding complex, with TLR4 results in the activation of several discrete immune pathways. The NFκB and p38 MAPK pathways play major roles in this signalling cascade. Included in their diverse roles in regulating cellular behaviour and immune functions, these two pathways trigger the activation of a variety of different transcription factors that are collectively necessary for the robust transcription of many cytokines (193-195). In addition, the p38 MAPK pathway regulates mRNA stability for a variety of cytokines (196, 197). These processes serve an essential role in regulating the production of cytokines during an inflammatory response. Accordingly, the ability of IDR-1002 to modulate LPS-induced activation of these signalling pathways was investigated. PBMCs were stimulated with LPS, IDR-1002, or a combination of both, over a time course and monocyte signal transduction activity assessed in the population by gating of responses with anti-CD14 antibodies. Intracellular phosphorylated p38 MAPK (T180/Y182) and phosphorylated p65 NFκB (Ser536) were monitored as indicators of p38 MAPK and p65 NFκB activation respectively. As expected, LPS induced the rapid phosphorylation of p65 NFκB and p38 MAPK, peaking at 15 and 30 minutes respectively (Figure 5.3). Phosphorylation of these two signalling mediators was sustained for up to 60 minutes post-stimulation. IDR-1002, which alone had no significant effects on p38 MAPK or p65 NFκB phosphorylation, significantly suppressed LPS-induced phosphorylation of these two mediators. While studies in Chapter 2 showed an activation of p38 MAPK (Figure 2.5) that effect was seen with a much higher IDR-1002 concentration (100 μg/ml), demonstrating both the chemokine-inducing and pathway-activating functions of IDR-peptides are dose-dependent. These results demonstrate that, in addition to suppressing LPS-induced cytokine production in human PBMCs, a low level of IDR-1002 is able to limit LPS-induced signal transduction in human monocytes.

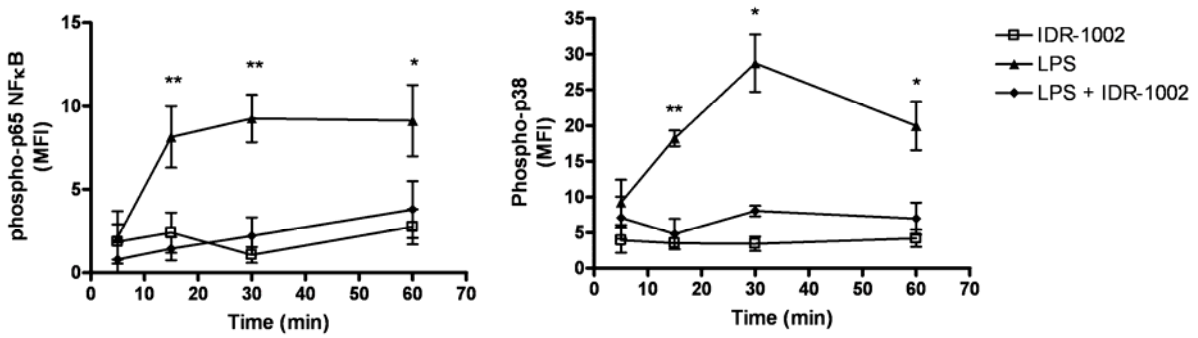

Figure 5.3 IDR-1002 regulation of LPS-induced NFκB and p38 MAPK activation. Time course effects on p65 NFκB (Ser 536) and p38 MAPK (T180/Y182) phosphorylation. PBMCs were stimulated with IDR-1002 (10 μg/ml), LPS (10 ng/ml), or a combination of both. Levels of phosphorylated proteins in monocytes were measured by the flow cytometric detection of anti-phospho-p38 and –p65 antibodies, gating on the $CD14^+$ monocyte population. Data are presented as the mean MFI over unstimulated controls (± SE) of at least 4 independent experiments, each from independent donors. Statistical comparisons between LPS-stimulations and LPS-IDR-1002-stimulations were done by Student's two-tailed *t* test. *p < 0.05; **p < 0.01.

5.3.3. The role of Akt in IDR-1002-mediated cytokine suppression in human PBMCs

The above experiments showed that IDR-1002 suppressed both LPS-induced cytokine production and LPS-induced monocyte signal transduction activity, suggesting a regulation of LPS-induced inflammatory responses. However, the mechanism through which IDR-1002 regulateds LPS-mediated activity needed to be elucidated. Here I focused on the role of the PI3K-Akt pathway in the anti-endotoxic activity of IDR-1002. While the role of the PI3K-Akt pathway activation in TLR-mediated inflammation remains a controversial topic, numerous studies have described an important negative regulatory role of this pathway in LPS-induced inflammatory responses (198-200). Akt regulation of TLR signalling acts through multiple modes, including the suppression of LPS-induced MAPK and NFκB signalling, as well the limitation of transcription factor activity necessary for inflammatory cytokine production (Figure 5.4) (198-201). As a mediator of several IDR immunomodulatory functions, the PI3K-Akt pathway was a natural target of investigation into the anti-inflammatory properties of IDR-1002. To test the involvement of Akt in this context, human PBMCs were pre-treated with varying doses of the Akt-specific inhibitor SH-

5, or a 0.1% DMSO-vehicle control, for 1 hour. PBMCs were then stimulated for 24 hours with LPS with or without the addition of IDR-1002. Culture supernatants were then harvested and assessed for levels of inflammatory cytokines by ELISA. Similar to previous experiments, in DMSO control conditions IDR-1002 suppressed LPS-induced PBMC production of IL-6, TNFα, MIP-1α, MCP-1, and GRO-α (Figure 5.5**A**). However, in the presence of increasing concentrations of an Akt-inhibitor, the ability of IDR-1002 to suppress LPS-induced cytokine production was increasingly diminished. This effect varied between cytokines tested. In the presence of 5 μM of SH-5, a significant abrogation of IDR-1002 suppression was seen for the production of IL-6, MIP-1α, and MCP-1. Although a compromising of IDR-mediated suppression was also seen with TNFα and GRO-α, this effect was not statistically significant. Furthermore, with the exception of MCP-1, inhibition of Akt did not completely eliminate the ability of IDR-1002 to suppress LPS-induced cytokine production. LPS-stimulated PBMCs in the presence of the Akt inhibitor showed only a slightly higher, but not statistically significant, production of the cytokines MIP-1α, IL-6, and TNFα compared to those pre-treated with DMSO, whereas no significant changes were observed with MCP-1 and GROα (Figure 5.5B). Overall these results demonstrated that IDR-1002 suppression of LPS-induced cytokine production was only partially dependent on the PI3K-Akt pathway.

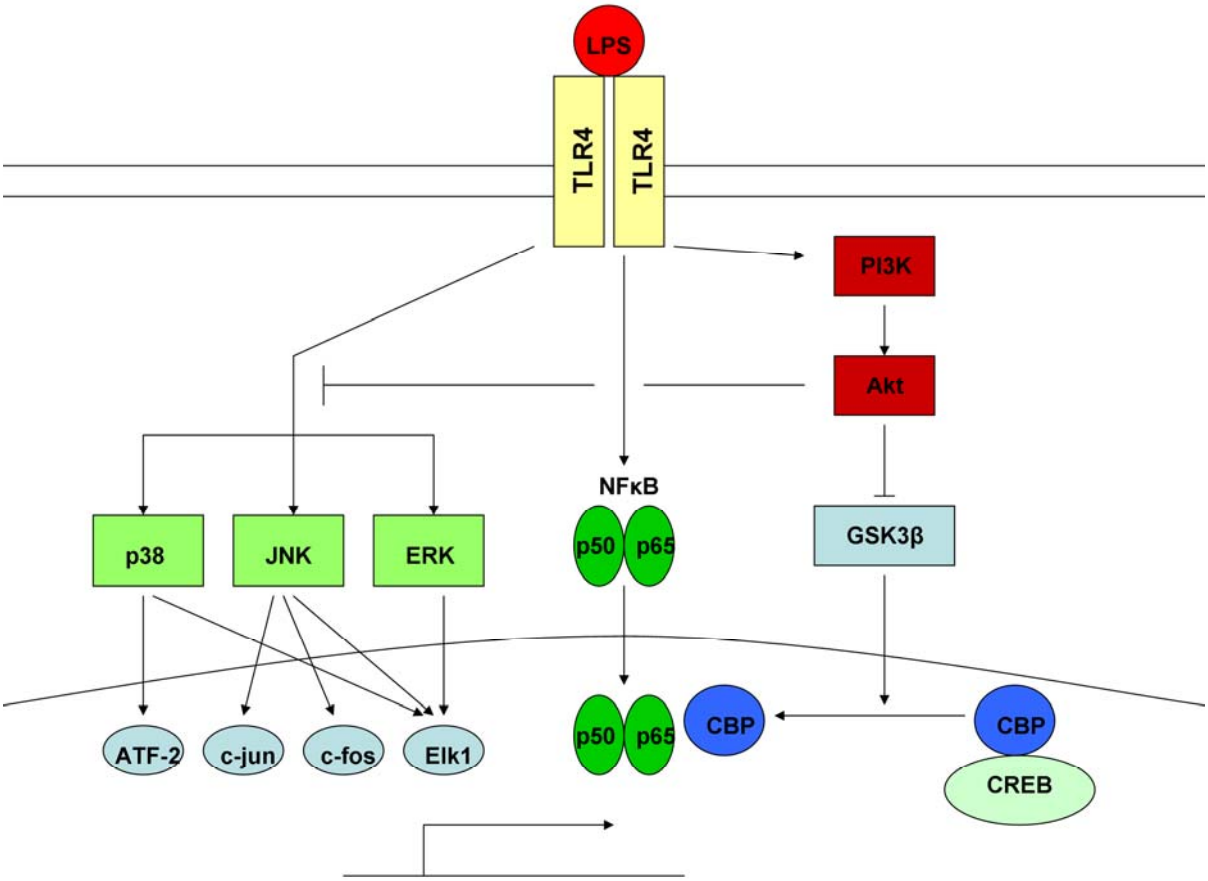

Figure 5.4 Regulation of LPS-TLR4 signalling and transcription by the PI3K-Akt pathway. PI3K-mediated activation of Akt results in the suppression of LPS-induced MAPK signalling activity. Akt, through inactivation of GSK3β, leads to the limitation of CBP association with p65 NFκB, resulting in impaired p65 NFκB transcriptional activity. Through these activities, the PI3K-Akt pathway limits LPS-induced production of pro-inflammatory mediators. This figure was partially adapted from Guha and Mackman (2002) with permission (199).

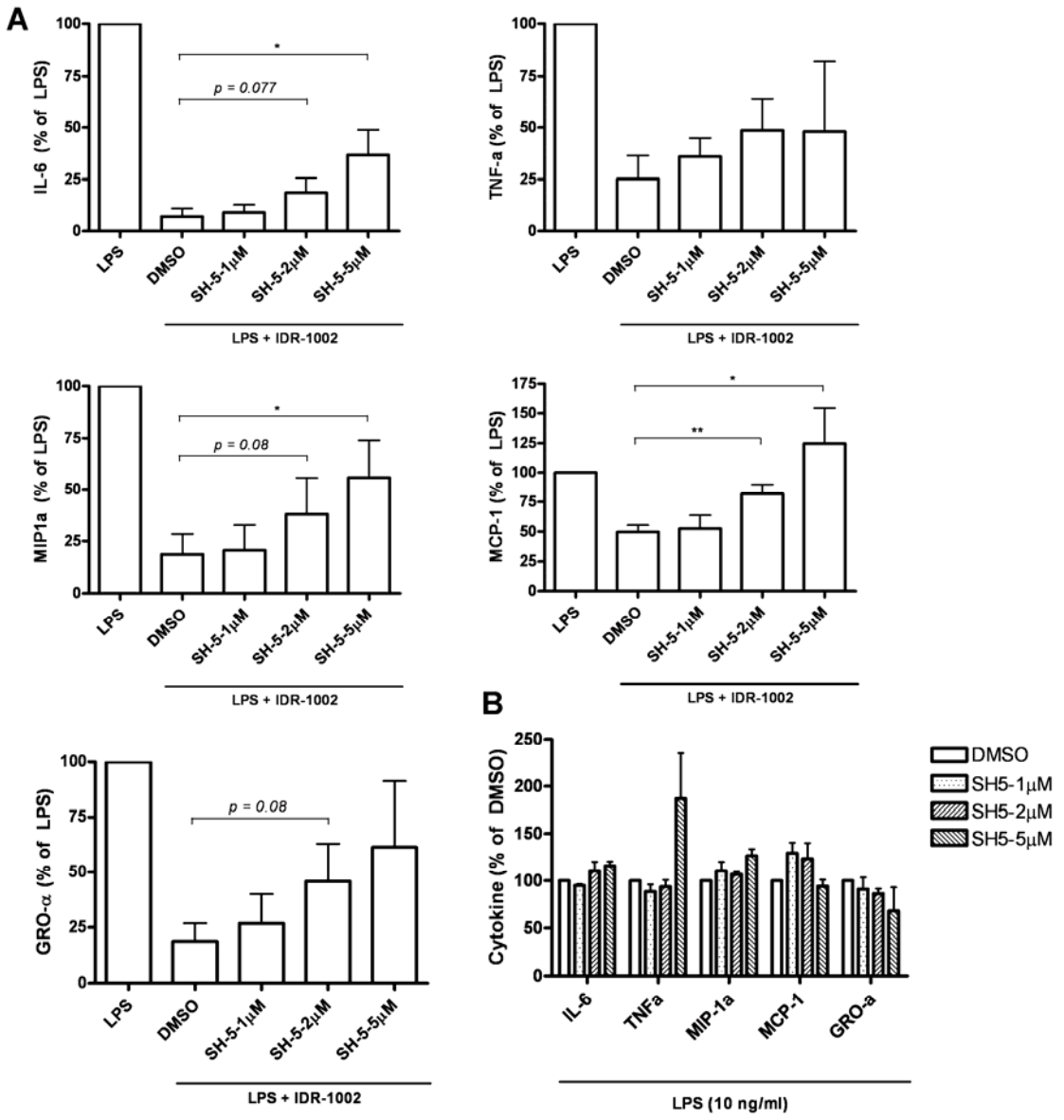

Figure 5.5 The role of Akt in IDR-1002-mediated anti-endotoxicity. PBMCs were pre-treated with the indicated concentrations of Akt-inhibitor SH-5 or 0.1% DMSO. (A) PBMCs were then stimulated with LPS (10 ng/ml) with or without IDR-1002 (10 μg/ml) for 24 hours prior to measurement of cytokine levels by ELISA. Data are presented as the mean % of cytokines relative to LPS-stimulation in each inhibitor condition (± SE) of at least 3 independent experiments, each from independent donors. (B) To assess the effects of Akt-inhibitor on LPS-mediated effects alone, PBMCs were stimulated with LPS (10 ng/ml) for 24 hours prior to measurement of cytokine levels by ELISA. Data are presented as the mean % of cytokines relative to LPS-stimulation of DMSO controls (± SE) of at least 3 independent experiments, each from independent donors. Statistical comparisons were done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$.

5.3.4. Regulation of LPS-induced PI3K-Akt signalling activity by IDR-1002 in human monocytes

Due to the role of the Akt pathway in IDR-1002-mediated anti-endotoxicity, focus was turned to the effects of this peptide on LPS-induced PI3K signalling. IDR-1002 was shown above to activate and utilize the PI3K-Akt pathway to exert certain of its immunomodulatory functions. Thus the interplay of regulation by IDR-1002 and LPS on the PI3K-Akt pathway likely plays a role in peptide modulation of LPS-induced inflammation. PBMCs were stimulated with LPS, IDR-1002, or the combination, over a varying time periods. Activation of the PI3K-Akt pathway in human monocytes was measured by assessing the levels of intracellular phosphorylated Akt (Ser473) in the $CD14^+$ monocyte population. LPS-treatment alone resulted in the increasing activation of Akt over the course of an hour compared to unstimulated controls (Figure 5.6A). IDR-1002 stimulation, correlating with findings in Chapter 3, significantly induced the transient phosphorylation of Akt in human monocytes, peaking at 15 and 30 minutes before diminishing by 60 minutes. The combination treatment of both IDR-1002 and LPS showed an additive effect on Akt phosphorylation in human monocytes. To further investigate the effects of IDR-1002 on LPS-mediated PI3K-Akt signalling, phosphorylation of GSK3β, a downstream target of Akt, was then evaluated. Both LPS and IDR-1002 stimulation separately resulted in the increased Ser9 phosphorylation of GSK3β compared to unstimulated controls (Figure 5.6B). As with Akt phosphorylation, the combination of LPS and IDR-1002 led to an additive phosphorylation of GSK3β. Overall, these results stand in strong contrast to the suppression of LPS-induced p38 MAPK and p65 NFκB signalling by IDR-1002 and demonstrate that LPS-induced PI3K-Akt pathway activation is elevated in the presence of IDR-1002. Interestingly, these results indicate a selective regulation of LPS-induced signal transduction by IDR-1002; this is exhibited by a suppression of LPS-activated inflammatory pathways by IDR-1002, whereas the regulatory PI3K-Akt pathway is reinforced.

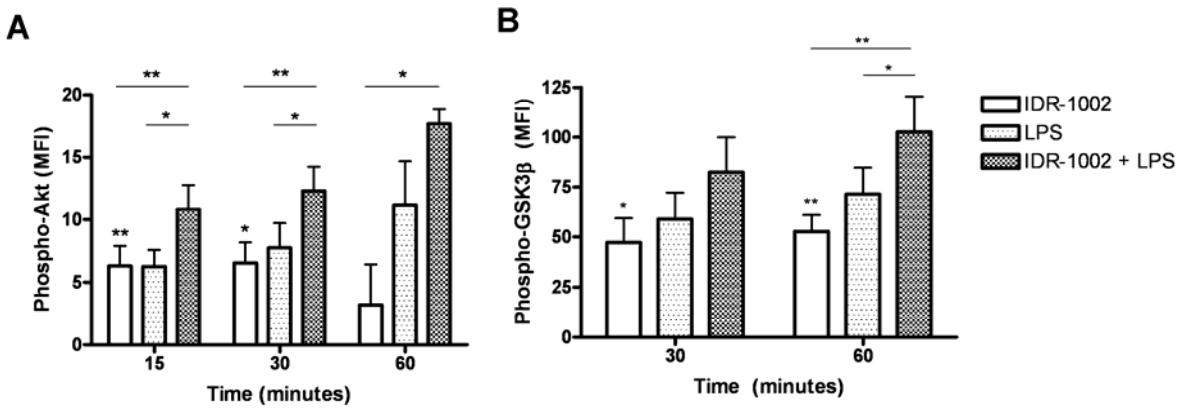

Figure 5.6 IDR-1002 regulation of LPS-induced PI3K-Akt signalling in monocytes. Time course effects on Akt (Ser 473) and GSK3β (Ser 9) phosphorylation. PBMCs were stimulated with IDR-1002 (10 μg/ml), LPS (10 ng/ml), or the combination of both. Measurement of monocyte protein phosphorylation was done by flow cytometric detection of anti-phospho-Akt and –GSK3β antibodies, gating on the $CD14^+$ monocyte population. Data are presented as the mean MFI over unstimulated controls (± SE) of at least 5 independent experiments, each from independent donors. Statistical comparisons were done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$.

5.3.5. The role of the PI3K-Akt pathway in IDR-1002 modulation of p38 MAPK signalling in human monocytes

As mentioned previously, the PI3K-Akt pathway can negatively regulate LPS-TLR4-mediated inflammation in part through its suppression of LPS-induced MAPK signalling. As the PI3K-Akt pathway was shown to be partially involved in IDR-mediated suppression of LPS-induced pro-inflammatory cytokine production, as well as being activated by both LPS and IDR-1002, the involvement of the PI3K-Akt pathway in IDR-1002 suppression of LPS-induced p38 MAPK signalling was then investigated. PBMCs were pre-treated for 1 hour with 10 μM of the PI3K-inhibitor LY294002 or 5 μM of the Akt-inhibitor SH-5 prior to stimulation with LPS, IDR-1002, or the combination of both. At the indicated time points, the intracellular levels of phosphorylated p38 MAPK of the monocyte population was assessed. Similar to initial signalling experiments, at 30 and 60 minutes, LPS alone strongly induced p38 MAPK phosphorylation in monocytes (Figure 5.7A). p38 MAPK phosphorylation in monocytes pre-treated with LY294002 or SH-5 did not significantly differ from that in DMSO vehicle controls. In both DMSO and inhibitor conditions, stimulation with a low concentration of IDR-1002 failed to elicit noticeable monocyte p38

MAPK. In DMSO control conditions, however, the addition of IDR-1002 to LPS-stimulated cells suppressed LPS-induced p38 MAPK phosphorylation at both tested time points. In the presence of a PI3K-inhibitor the suppressive effect of IDR-1002 was partially, but significantly, abrogated. Similarly, in the presence of an Akt-inhibitor, the ability of IDR-1002 to suppress LPS-induced p38 MAPK phosphorylation in monocytes was eliminated. These results demonstrate that the ability of IDR-1002 to regulate LPS-induced MAPK signalling is partially dependent on IDR-1002-activation of the PI3K-Akt pathway.

The consequent effects of IDR-1002 on LPS-induced p38 MAPK signalling were then investigated. Activating transcription factor (ATF)-2 is one of the downstream targets of p38 MAPK member of the cAMP response element binding (CREB)/ATF family of transcription factors (202, 203). ATF-2 binding sites are found in the promoter regions of numerous cytokines (202, 204, 205). Indeed studies have shown that ATF-2 plays a role in LPS-induced gene transcription and is involved in the production of a wide range of inflammatory cytokines, including TNFα, IL-6, and IL-1 (202, 203, 206). Thus it is feasible that the IDR-1002 activation of the PI3K-Akt pathway, and subsequent regulation of LPS-induced p38 MAPK activation, leads to the suppression of downstream ATF-2 activity, potentially contributing to the suppression of LPS-induced cytokine production. To investigate this, ATF-2 activation in monocytes, treated similarly as above, were determined through the measurement of phosphorylated ATF-2 (Thr71). LPS-stimulated monocytes exhibited an increase of ATF-2 phosphorylation at 30 and 60 minutes (Figure 5.7B). Although LPS-induced ATF-2 phosphorylation was slightly decreased with the addition of the PI3K-specific inhibitor, and slightly increased with an Akt-specific inhibitor, these effects were not significant. IDR-1002, which alone had no significant effects on ATF-2 phosphorylation in any of the conditions, was able to moderately suppress LPS-induced ATF-2 phosphorylation in monocytes. This effect was eliminated in the presence of a PI3K-specific inhibitor. In the presence of an Akt-specific inhibitor, the anti-endotoxic activity of IDR-1002 was significantly abrogated only at 30 minutes but was unchanged at 60 minutes. Overall, these results demonstrate that the ability of IDR-1002 to suppress the LPS-induced p38 MAPK signal cascade partially stems from its activation of the PI3K-Akt pathway.

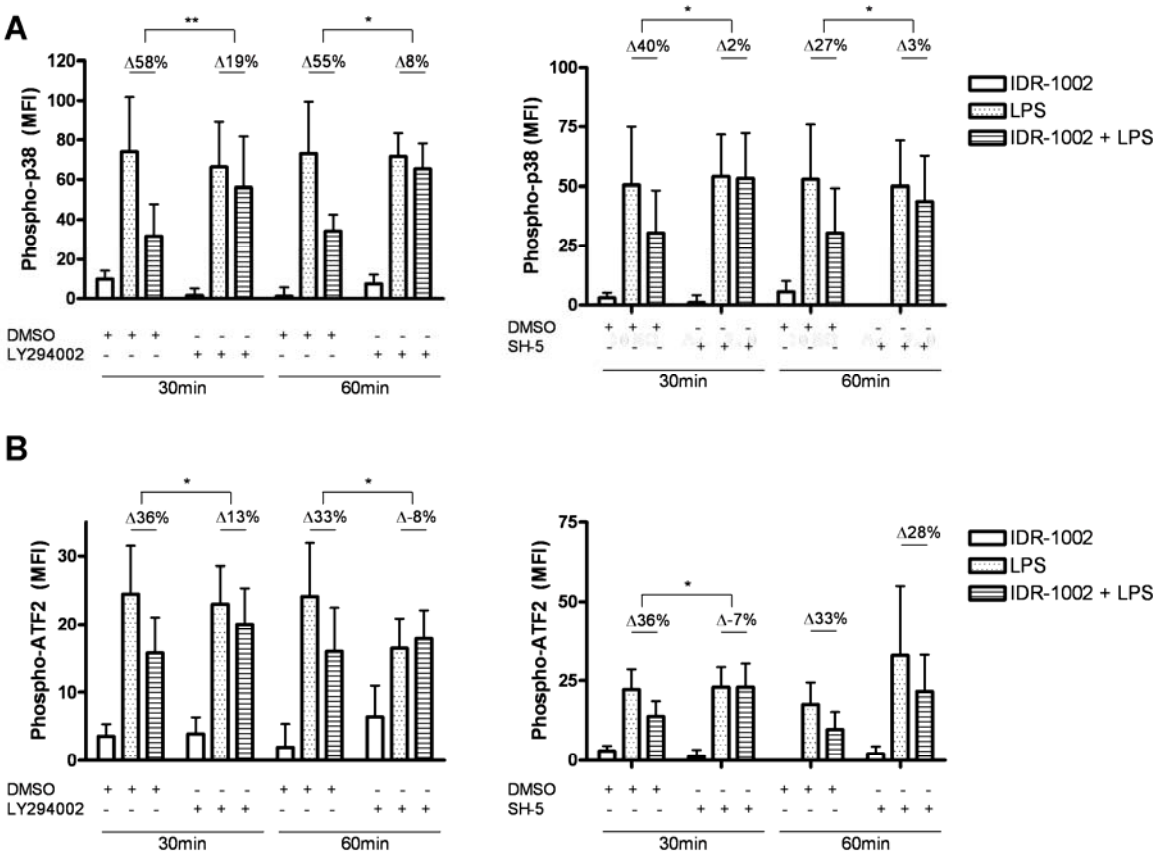

Figure 5.7 The role of the PI3K-Akt pathway in the suppression of LPS-induced p38 MAPK signalling by IDR-1002. PBMCs were pre-treated for 1 hour with PI3K-inhibitor LY294002 (10 μM) or Akt-inhibitor SH-5 (5 μM) prior to stimulation with IDR-1002 (10 μg/ml), LPS (10 ng/ml), or both. Measurement of (A) phosphorylated p38 MAPK (T180/Y182) or (B) phosphorylated ATF-2 (Thr 71) was done by flow cytometric detection of anti-phospho-antibodies, gating on the $CD14^+$ monocyte population. Data are presented as the mean MFI over untreated samples (± SE) of at least 3 independent experiments, each from an independent donor. Statistical comparisons between % reduction of LPS-induced phosphorylation was done by Student's two-tailed *t* test. *$p < 0.05$; **$p < 0.01$.

5.4. DISCUSSION

In this chapter, it was demonstrated that IDR-1002 could suppress LPS-induced cytokine and chemokine production by human PBMCs. The cytokine cascade induced by host exposure to LPS and other TLR agonists plays a major role in the initiation of the inflammatory response. However, the dysregulation of inflammatory cytokine levels can result in diseases of uncontrolled inflammation. TNFα has been implicated in a wide range of inflammatory disorders, including psoriasis, rheumatoid arthritis, inflammatory bowel

disease, and atherosclerosis (207-211). Similarly, dysregulation of IL-6 has been shown to play a role in arthritis and atherosclerosis, as well as chronic inflammatory and autoimmune diseases (210, 212). Excessive levels of chemokines, resulting in exaggerated leukocyte infiltration and activation, also play a role in arthritis and cardiovascular diseases with underlying inflammatory causes (213-215). Indeed in cases of bacteremia and sepsis, it is the overwhelming cytokine storm induced by LPS and other bacterial components that result in shock, leading to physiological damage and possibly death (216). It is this risk of cytokine dysregulation which poses a major obstacle in the development and administration of systemic immune enhancing mediators. In this context, the ability of IDR-1002 to suppress LPS-induced cytokine production, while maintaining moderate levels of chemokines, is an important finding in the development of therapeutic immunomodulatory agents which can boost immunity in a safe manner. It is also interesting to note that suppression of LPS-induced cytokine production is varied, as evinced by the range of suppression between cytokines observed in peptide-LPS co-stimulations. These results suggest a selective balancing of the LPS-induced inflammatory response by IDR-1002, allowing moderate production of specific mediators while limiting the excessive production of potentially harmful cytokines. It must be noted that the suppression of pro-inflammatory cytokines is seen with low levels of IDR-1002 (10 μg/ml), whereas chemokine-induction and *in vivo* protection are seen at higher concentrations (100 μg/ml in *in vitro* models). It would be of interest to investigate the effects of higher IDR-1002 concentrations on LPS-induced responses. It is feasible that in this scenario, chemokine-induction would be maintained, or perhaps enhanced, whereas pro-inflammatory cytokine production would be regulated. This moderating effect is observed in *in vivo* infection models in which IDR-peptides promote chemokine production and leukocyte recruitment while maintaining or suppressing inflammatory cytokines induced by bacteria (79, 80). A precedent for this selective immune modulation is found with endogenous HDPs. Human LL-37 exhibits the ability to protect rats against Gram-negative bacteria-induced sepsis by limiting cytokine-mediated inflammation, yet promoting bacterial clearance (54, 217). Cathelicidins of various species, which possess potent anti-inflammatory properties, also demonstrate the promotion of antimicrobial clearance in various infection models (218, 219). Thus this study expands the immunoregulatory repertoire of IDR-peptides in demonstrating a role in moderating LPS-

induced inflammatory responses, similar to HDPs. It is noteworthy however that the administration of cathelicidins in these animal models is limited by peptide toxicity (217, 218), whereas administration of IDR-peptides at much higher concentrations is well tolerated (79, 80).

In searching for the anti-inflammatory mechanisms of IDR-peptides, this study further demonstrated that IDR-1002 is a suppressor of LPS-induced signal transduction in human monocytes. The potency of LPS in inducing strong inflammatory responses depends on its ability to activate a wide range of signal transduction cascades. Activation of the MAPK family of signalling mediators and the NFκB network plays a central role in mediating LPS-induced responses. The resulting activation of multiple downstream transcription factors, including NFκB and AP-1 transcription subunits, leads to the widespread gene expression of inflammatory and immune mediators (220-222). In this study, I showed that IDR-1002 impacts on this process by limiting the ability of LPS to induce NFκB p65 phosphorylation in monocytes, thus limiting the full transcriptional activity of p65-containing heterodimer complexes (223, 224). Additionally, LPS-induced phosphorylation of p38 MAPK in monocytes, necessary for the activation of this enzyme, is also suppressed by IDR-1002. These observations suggest that the suppression of LPS-induced signalling is an anti-endotoxic mechanism of IDR-1002, and potentially other IDR-peptides. It must be noted that IDR-1002 at higher doses has been shown to activate the MAPK pathways in human monocytes, leading to the induction of chemokine production without the consequent production of pro-inflammatory mediators (80). LPS-mediated inflammatory responses depend on the cooperation between multiple signal transduction cascades; this synergistic cooperation is required for the full expression and production of inflammatory mediators (225). It seems likely that the modulation of certain LPS-induced signalling pathways, paired with the activation of others, by IDR-peptides, is what leads to the suppression of inflammatory responses while maintaining or promoting specific aspects of immunity. Again, this effect can be generally observed with endogenous LL-37, a known activator of the MAPK pathways yet a potent suppressor of LPS-induced signalling (43, 44). It must also be noted that LL-37 exhibits the ability to selectively modulate LPS-induced gene transcription, rather than simply suppressing LPS-mediated responses (44). It is likely therefore that the complex modulation of LPS-induced gene transcription by LL-37, and

perhaps IDR-peptides, may originate from the fine tuning of cellular signal transduction pathways. Taken together, these observations demonstrate that IDR-1002 is not simply a suppressor of LPS function, but a complex regulator of the LPS-induced inflammatory programme.

Consistent with previous findings that IDR-1002 is a regulator of monocyte signalling activity, peptide modulation of LPS responses was found to be partially dependent on signalling pathway cross-talk. Specifically, this study has demonstrated that the elevation of the PI3K-Akt pathway by IDR-1002 during LPS-induced inflammation contributes to the anti-endotoxic effects of the peptide. The PI3K-Akt signalling axis is known to regulate TLR-mediated inflammatory responses in several ways. Studies have demonstrated a negative regulatory role of PI3K and Akt on p38-mediated signalling, and subsequently on p38-mediated responses induced by LPS (199, 226, 227). It is interesting that in this study, inhibition of Akt exhibited only a mild enhancement of LPS-induced cytokine production and had almost no effect on LPS-induced p38 MAPK signalling. These observations are in contrast to the reported findings of Guha and Mackman (199) which demonstrated a significant promotion of LPS-induced MAPK activation and TNFα production by PI3K-inhibited PBMCs. This discrepancy may have arisen from the differences in LPS-stimulations between studies; specifically the use of 10 μg/ml LPS in the reported study compared to the 10 ng/ml used here, a 1000-fold difference. Perhaps the regulatory effects of the PI3K-Akt pathway on LPS-induced signal transduction is dose-dependent, engaging only after a signal threshold is reached with higher doses. However, here it was found, through the use of PI3K- and Akt-specific inhibitors, that IDR-1002 suppression of LPS-induced monocyte p38 MAPK activation indeed stemmed partly from this peptide's activation of the PI3K-Akt pathway. This correlated with observations that IDR-1002 suppression of ATF-2 activation, a downstream target of p38 MAPK, was also partially dependent on the PI3K-Akt pathway. Taken together, these results indicate that IDR-1002 activation, and its reinforcement of PI3K-Akt signalling, negatively regulates LPS-induced activation of the p38 MAPK pathway. Akt can also limit LPS-mediated responses through phosphorylation and inactivation of its target GSK3β (Figure 5.4). Optimal NFκB-induced gene transcription depends on the association of the p65 complex with nuclear co-activator CREB-binding protein (CBP) (228). Due to the limiting amounts of nuclear CBP, GSK3β can enhance p65

NFκB-mediated gene transcription by inhibiting CREB association with CBP (229, 230). Inactivation of GSK3β, through serine phosphorylation by Akt or chemical inhibitors, promotes CREB-CBP binding and CREB transcriptional activity, while reducing p65-CBP interactions and NFκB-mediated transcription (229). This is manifested by the decreased production of LPS-induced, NFκB-controlled cytokines and an increase in CREB-controlled cytokines, such as anti-inflammatory cytokine IL-10 (229). It seems likely that IDR-1002 reinforcement of LPS-induced GSK3β phosphorylation shifts CBP-binding activity, resulting in the observed reduction of pro-inflammatory cytokines by this peptide. Moreover, inhibition of GSK3β has been shown to increase nuclear translocation of the transcription factors C/EBPβ and CREB, which are important in IL-10 production (231). Overall, these results show that IDR-1002 modulation of the PI3K-Akt pathway plays a role in its regulatory effects on LPS-induced inflammation.

In summary, this chapter demonstrates the ability of IDR-1002 to suppress LPS-induced pro-inflammatory cytokine production and signal transduction activity, revealing a novel role for IDR-peptides in modulating immune responses. Moreover, IDR-1002 activation of the PI3K-Akt pathway during an LPS-induced inflammatory response correlated with an Akt-dependent suppression of inflammatory cytokine production and p38 MAPK signalling. Research into the regulatory roles of IDR-peptides and HDPs in LPS-induced inflammation is still in its infancy. Recently the perception of anti-endotoxic HDPs, previously considered by some authors to be simple endotoxin-binding agents, has evolved into an understanding of HDPs as intricate regulators of TLR-mediated inflammation (44, 50, 232). The partial role of the PI3K-Akt pathway in IDR-mediated anti-endotoxic activity demonstrated in this study expands on the potential mechanisms for this regulation and suggests a similar complexity whereby HDPs and IDR peptides influence inflammation through multiple mechanisms some of which are undoubtedly yet to be identified. This complexity is further compounded by observations that HDPs and IDR-peptides differentially regulate inflammatory responses induced by different TLR agonists. For example, while inflammatory responses induced by LPS and lipoteichoic acid are suppressed by LL-37, the production of certain pro-inflammatory cytokines induced by CpG DNA is enhanced by this peptide (44, 53). Indeed a similar synergy between IDR-peptides and CpG DNA is being exploited for vaccination strategies, using IDR-peptides and CpG

oligodeoxynucleotides as immune-boosting adjuvants in single dose vaccine formulations (84, 100, 108, 109). This study, by identifying potential mechanisms through which IDR-1002 moderate LPS-induced responses, is a step forward in revealing the extent of regulation that IDR-peptides exert on inflammation. The understanding of how IDR-peptides alter inflammation induced by bacteria and other pathogens (e.g. Achtman, A.H., S. Pilat, C.W. Law, D.J. Lynn, L. Janot, M. Mayer, S. Ma, J. Kindrachuk, B.B. Finlay, F.S.L. Brinkman, G.K. Smyth, R.E.W. Hancock and L. Schofield. 2012. Effective adjunctive therapy by an innate defense regulatory peptide in a pre-clinical model of severe malaria. Science Transl. Med. in press.), is essential for the development of anti-infective and adjuvant agents that selectively augment innate immune responses yet limit harmful inflammatory responses.

6 DISCUSSION – MODULATION OF HOST IMMUNITY BY IDR-1002

The threat of bacterial pathogens that are becoming increasingly resistant to our arsenal of conventional antimicrobial weapons, has spurred demands for the advancement of novel treatment options. In recent years the use of immunomodulators to enhance host immune responses has been proposed as a strategy to ward off bacterial infection (2). The development of IDR-peptides (4), mimetics of endogenous HDPs, represents a major beachhead in the artificial improvement of immune system regulators to combat infectious diseases. IDR-peptides have demonstrated the ability to enhance antimicrobial responses in a variety of applications, ranging from the prophylactic and therapeutic protection of the murine host against bacterial pathogens to the enhancement of vaccine-mediated responses in numerous animal models (79, 80, 84, 100, 108). Unlike classical immunomodulatory agents, IDR-peptides augment relevant antimicrobial aspects of host immunity while limiting harmful inflammatory responses. These initial successes have yielded the promise of potent, immunomodulatory agents that can counter infection by antibiotic-resistant pathogens. However, a lack of understanding of how IDR-peptides exert their seemingly complex regulatory effects, especially how they translate their regulatory effects to a human system, would represent a major obstacle in their advancement as clinical therapeutic agents. Thus the aim of this thesis was to elucidate the extent of regulation by IDR-peptides in a human *in vitro* cellular system and determine the mechanisms by which they would influence human immunity. The ultimate goal of this project was to provide an increased understanding of how these peptides influence the human immune response to enable the design and development of improved anti-infective immunotherapeutics.

6.1. IDR-1002 REGULATION OF MONOCYTE MIGRATION

In this thesis, significant progress was made in elucidating the mechanisms through which IDR-peptides mediate their observed functions. During bacterial invasion IDR-peptides promote cellular recruitment to the site of infection (79, 80). The available data, published in part by me and my colleagues (80), indicate that the ability to enhance leukocyte

recruitment, particularly of monocytes, is a primary function of IDR-mediated antimicrobial activity. The ability of IDR-1002 to protect against *S. aureus* infection correlated with a peptide stimulation of chemokine production and enhancement of monocyte and neutrophil recruitment to the site of infection. In direct comparison with IDR-1, IDR-1002 exhibited a stronger chemokine inducing effect correlating with a greater than five-fold reduction in the protective dose of IDR-1002, suggesting a principal role of chemokines, and thus cell recruitment, in peptide-mediated protection.

Given this proposed cardinal effector function in IDR-mediated protection, I aimed here to reveal the mechanisms by which IDR-peptides regulate human monocyte mobility. It was discovered that IDR-1002 likely utilizes a multi-pronged approach in regulating monocyte motile behaviour as outlined in Figure 6.1. In Chapter 2, IDR-1002 was characterized as promoting the production of chemoattractive molecules through the modulation of immune signalling pathways. The capacity of IDR-1002 to stimulate human cellular production of monocyte and neutrophil chemokines likely plays a major role in the recruitment of effector cells. These effects correlated with murine *in vitro* and *in vivo* models, performed in collaboration, which showed that IDR-peptides, including IDR-1002, stimulated chemokine production by murine cells and during protection against bacterial infections (79, 80). Thus IDR-peptides can indirectly promote leukocyte recruitment to infection locales by enhancing the generation of chemokine-mediated gradients. It was also found that IDR-1002 could regulate monocyte mobility by more direct methods. Cellular adhesion events play a central role in directing cellular traffic from the bloodstream into peripheral areas (118). In Chapter 3, IDR-1002 was shown to enhance monocyte chemotaxis towards host chemokines through an adhesive substrate, as would occur *in vivo* in tissue environments, by directly promoting monocyte integrin-mediated adhesion. It was shown that IDR-1002 promotion of β1-integrin function resulted in monocyte adhesion to fibronectin, a major component of the ECM, and this subsequently promoted monocyte migration through this substrate. This study also demonstrated a potential regulation of monocyte β2-integrin function and monocyte adhesion to ICAM-1, an important endothelial adhesion molecule for cellular transmigration (233-235). While this unique finding had not been shown for other IDR peptides and HDPs previously, data was presented to indicate that although IDR-1002 demonstrates better activity than other peptides, this seems to be a

general mechanism shared amongst these anti-infective molecules. Furthermore, in Chapter 4, it was revealed that IDR-1002 could potentiate monocyte responses to endogenous chemokines. Specifically, it was shown that IDR-1002 stimulation of monocytes resulted in enhanced migration of monocytes towards a subset of chemokines and potentially enhanced chemokine-induced signal transduction activity, acting through the chemokine receptor CCR5. These phenomena implicated a priming of monocyte motility and sensitivity towards inducible chemokines produced at the site of infection, further enhancing monocyte recruitment, and potentially activation.

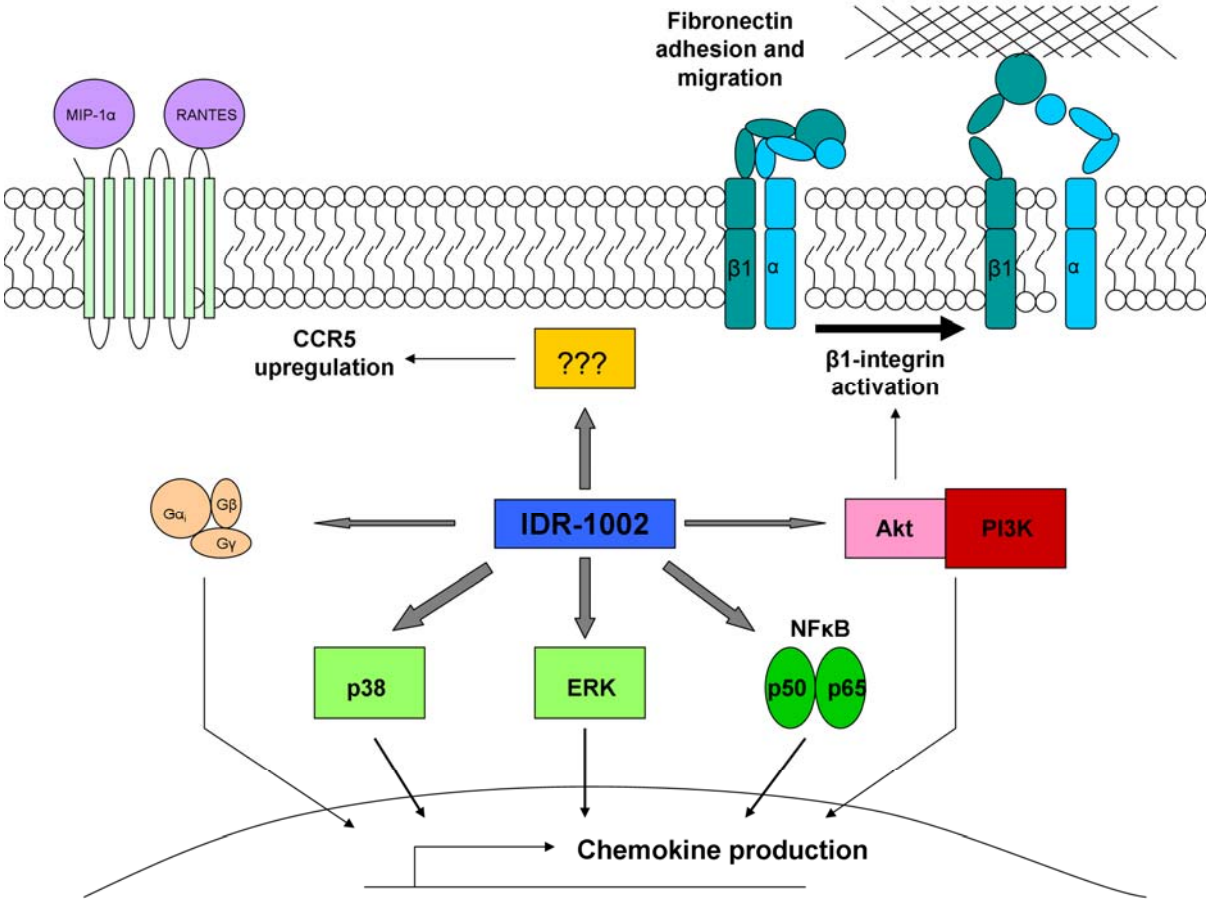

Figure 6.1 Distinct mechanisms by which IDR-1002 promoted monocyte migration. IDR-1002 is proposed to utilize a multi-pronged approach in enhancing monocyte migration. IDR-1002 was able to induce monocyte fibronectin migration via PI3K-Akt-mediated β1-integrin activation. IDR-1002 was also able to induce chemokine production via activation of multiple signalling pathways. Furthermore, IDR-1002 was able to enhance monocyte responses to CCR5 chemokines via upregulation of CCR5 expression through a yet-undetermined mechanism.

Thus it was shown that IDR-1002 was able to exploit and augment natural host mechanisms in multiple ways to regulate human monocyte motility. It is highly likely, that the varied effects of IDR-1002 act in synergy to promote recruitment. As an example, the priming of monocytes by IDR-1002 for enhanced sensitivity to MIP-1α and RANTES, coupled with the increased levels of inducible chemokines at the site of infection, would likely lead to a significant promotion of monocyte recruitment. In addition, IDR-mediated reinforcement in monocyte-substrate interactions may serve to increase recruitment even further, culminating in the strong enhancement of monocyte recruitment seen in *in vivo* infection models. It would be of interest to see how these regulatory effects change in an inflammatory setting similar to that seen in bacterial invasion. Whereas low doses of IDR-1002 have been shown to moderate cytokine production induced by LPS in *in vitro* human systems, the effects of high doses of IDR-1002 in this circumstance on chemokine levels is currently unknown. Murine *in vivo* models suggest that peptide-induced chemokine production is retained, if not enhanced, during an inflammatory response (79, 80). The moderation of bacterial-induced inflammation by IDR-1002, in parallel with increased monocyte responses to chemokines, would allow for an enhancement of monocyte migration without an associated increase in inflammatory mediators. Characterizing these peptide-mediated effects in various pathogen-induced inflammatory settings may be a useful next step in verifying the mechanism of monocyte regulation by IDR-peptides.

In regulating monocyte motility through multiple mechanisms, IDR-1002 strongly resembles endogenous host chemokines. Chemokines are potent inducers of cell recruitment not only because of their direct chemoattracting capabilities, but due to their ability to influence each stage of cellular recruitment. Through their activation of target cells, chemokines are responsible for the enhanced interaction between cells and their environment, thus playing important roles in firm adhesion of cells to the endothelial layer, extravasation into the tissues, and cellular localization within the tissue environment (6, 118). These molecules are also enhancers and inducers of secondary cytokine production, leading to the potential strengthening of chemokine-mediated responses (236, 237). Additionally, chemokines can regulate cellular sensitivity to other chemokines through cross regulation of chemokine receptors or downstream signal transduction pathways (159, 161, 238). Other endogenous cytokines act in similar fashion. IL-6, itself a potent inducer of cytokine and

chemokine production, has recently been shown to promote monocyte chemotaxis and transmigration through its modulation of monocyte integrin-mediated adhesion (239). Therefore IDR-1002 is a complex modulator of the immune response. Indeed it is possible that IDR-1002 utilizes additional mechanisms to influence monocyte recruitment. Potential avenues of investigation would include IDR-peptide effects on the endothelial transmigration process, the expression of leukocyte adhesion molecules, and selectin-mediated cell capture/rolling, all processes extensively regulated by endogenous cytokines. Studies into these activities may reveal novel axes of regulation of monocyte migration by IDR-peptides.

6.2. IDR-1002 REGULATION OF INFLAMMATORY RESPONSES

One of the noteworthy effects of IDR-peptides is their ability to enhance innate immune responses, namely the augmentation leukocyte recruitment, without promoting potentially undesirable aspects of the inflammatory response *in vivo*. This thesis project has made progress in characterizing this phenomenon from multiple angles. Firstly, it was revealed that IDR-1002, intrinisically, enhanced only selective aspects of the immune response. In Chapter 2 at concentrations sufficient to strongly induce chemokine production, IDR-1002 was shown to have minimal effects on the production of inflammatory cytokines TNFα and IL-6, consistent with murine *ex vivo* and *in vivo* observations. Furthermore, at similar concentrations, IDR-1002 also demonstrated the ability to regulate monocyte function, including the modulation of adhesive behaviour, sensitivity to endogenous mediators, and chemotaxis. Overall, these observations reinforced findings that IDR-peptides are selective complex regulators of immunity. Indeed it is due to these properties that IDR-peptides are considered as promising candidates for anti-infective, immunomodulatory therapeutics. However, since the ultimate aim is to utilize IDR-peptides in response to, or prophylactically prior to, pathogen invasion, it was important to characterize the effects of peptides on pathogen-mediated inflammatory responses. In this regard, this thesis research has made advances in characterizing IDR-regulation of inflammatory responses induced by bacterial LPS, one of the prime inducers of inflammation during a bacterial infection. In Chapter 5, the presence of a low dose of IDR-1002 was sufficient to suppress LPS-induced cytokine production by human PBMCs as well as LPS-induced signal transduction activity

by human monocytes. Thus it was revealed that, in addition to its enhancement of monocyte functions, IDR-1002 possessed the ability to moderate LPS-induced pro-inflammatory responses. This ability to antagonize bacterial inducers of inflammation was proposed to be responsible for the moderation of inflammation seen in *in vivo* infection models. Interestingly, the production chemokines, believed to play an essential role in leukocyte recruitment and IDR-mediated protection, by LPS-stimulated PBMCs was moderately suppressed by low levels of IDR-1002. This observation differs from the enhancement of chemokine levels and leukocyte recruitment by IDR-1002 seen in murine models of acute, Gram-negative bacterial infection. It is feasible that the interplay between IDR-1002 and the wide variety of other numerous bacterial components, results in the augmentation of chemokine production and the moderation of pro-inflammatory cytokine levels.

Indeed, while LPS-induced pro-inflammatory responses play a major role in inducing inflammation in infections by Gram-negative bacteria, LPS constitutes only one of many bacterial components with a substantial ability to induce host inflammatory responses. The initiation of innate immunity depends on the host repertoire of germline-encoded receptors, including the TLRs and NLRs, which recognize a wide array of pathogen-associated signatures. In bacteria alone, these signatures can include bacteria-specific DNA, cell wall and envelope components, and lipidic structures (195). The engagement of these bacterial motifs with host receptors results in the activation of signal transduction activity and the consequent robust activation of the inflammatory response. Thus, the inflammatory response in response to bacterial infection can be imagined as multiple parallel reactions to numerous pathogenic signature molecules. Inflammation collectively represents the remarkable ability of the host to coordinate and orchestrate these processes to generate a coherent response. This is well demonstrated by observations of synergy between multiple TLR ligands. TLR8 synergizes with TLR3 or TLR4 to enhance cytokine production by dendritic cells and enhanced transcription factor activity in dendritic cells and macrophages (240). Stimulation of TLR2 and TLR4 also results in the synergistic production of TNFα by murine macrophages. In this complex scheme, the characterization of the endotoxin-regulating effects of IDR-1002 addresses only one aspect of peptide-regulation of inflammation (241). A next step in understanding IDR-mediated inflammatory regulation would be the elucidation of how IDR-peptides interact with other bacterial components, as well as the

variety of endogenous mediators produced during inflammation. Progress has been made in this regard, revealing a complex effect of IDR-peptides on bacterial-induced inflammation. Similar to the regulation of LPS-induced responses, parallel studies into IDR-peptide function have shown a suppression of inflammatory cytokine production induced by flagellin and peptidoglycan (unpublished data). In contrast, IDR-peptides have been well reported to synergistically enhance the production of inflammatory cytokines by CpG DNA motifs (84). As mentioned previously, it is the exploitation of this effect that enables IDR-peptides to be utilized to enhance adaptive immune responses in vaccine formulations (84, 100, 108, 109). Further studies may reveal additional layers of complexity. It may be that this duality of IDR-peptide regulation of inflammation also exists with endogenous molecules. IDR-peptides may suppress the functions of inflammatory mediators, such as IL-6 or TNFα, or perhaps synergize with others, much like the effects of LL-37 on IL-1-mediated responses (50). Additional studies need to be done regarding the regulatory effects of IDR-peptides on other inflammatory factors and, more importantly, how these multiple axes of regulation converge to modulate inflammation as a whole. Thus, although *in vivo* models of bacterial infection demonstrate a modulation of inflammation by IDR-peptides, much more needs to be done to fully understand the complex underlying effects of IDR-peptides on inflammatory processes. This understanding is critical to enable the optimal use of IDR-peptides in inflammatory settings, either to prevent harmful undesired effects or to develop novel anti-infective applications of IDR-mediated immunotherapy.

6.3. SIGNALLING PATHWAY REGULATION AND IDR-1002 MECHANISM OF ACTION

A major objective of this thesis project was to chart the extent of IDR-peptide regulation of the immune response. Over the course of this thesis, much has been done to characterize the functions of IDR-peptides in modulating host anti-infective immunity. These studies have revealed the numerous roles of IDR-peptides in the regulation of host cytokine production and the promotion of a host of monocyte functions. They also revealed the ability of IDR-peptides to suppress endotoxin-mediated inflammation. Identification of these processes significantly furthers our understanding of how IDR-peptides selectively modulate

host immune responses to enhance anti-infective immunity. Of equal importance, however, is the determination of the mechanisms through which they do carry out these functions. In this study, IDR-peptides were identified as complex regulators of monocyte signal transduction pathways.

Substantial networks of signal transduction pathways are responsible for the regulation of virtually all cellular functions. These intricate webs allow for the incorporation of environmental signals and the subsequent development of appropriate cellular responses, such as growth, maturation, activation, movement, death, and a host of other specialized cellular functions. As mentioned above, subsets of this overall network are essential for the initiation and coordination of the immune response. It was found in this thesis that multiple regulatory functions of IDR-peptides originate from their manipulation of these immune signalling networks. In Chapter 2, it was demonstrated that IDR-1002 is a regulator of monocyte signal behaviour; specifically, it was shown that IDR-1002 triggered the activation of MAP Kinase pathways. Even more striking was the development that IDR-1002 utilized a diverse range of immune networks to induce chemokine production by PBMCs, including the NFκB, PI3K, MAP Kinases, and G-protein signalling networks. The ability of IDR-1002 to modulate signal transduction, and its effects on immune functions, were further characterized in following chapters. In Chapter 3, a central role of the PI3K-Akt signalling axis, rather than the MAP Kinase pathways, was identified in IDR-1002 regulation of monocyte β1-integrin activation. Furthermore, in Chapter 5, it was shown that IDR-1002 not only suppressed the inflammatory activity of bacterial endotoxin, but partially utilized signalling pathway cross-talk to do so; namely through IDR-mediated activation of the PI3K-Akt pathway-mediated to partially suppress LPS-induced p38 MAPK signalling in monocytes. Overall, these findings conclusively demonstrate an activation and utilization of diverse immune signalling pathways by IDR-1002 to mediate a wide range of immune functions. In a broader sense, these developments strongly suggest that the selective immunoregulation exhibited by IDR-peptides is due to their complex regulation of signal transduction networks.

While these discoveries certainly advance our understanding regarding IDR-peptide mechanisms of action, they only scratch the surface of IDR-mediated regulation. With IDR-peptides revealed as extensive regulators of immune signalling cascades, a next step is the discovery of their upstream receptor(s). Preliminary efforts in this regard have focused on

receptors known to be utilized by endogenous HDPs due to certain structural similarities shared by HDPs and IDR-peptides, such as their overall cationic charge and significant presence of hydrophobic residues. In Chapter 2 an antagonist of FPRL-1, a G protein-coupled receptor which mediates LL-37 chemotactic activity (242), was found to inhibit IDR-1002 induction of chemokines. This effect correlated with the observation that elimination of G_i-protein signalling abrogated IDR-1002 chemokine induction and enhancement of monocyte chemotaxis. While suggestive of FPRL-1 usage by IDR-peptides, it is plausible that other G protein-coupled receptors may contribute to peptide signalling. Human beta defensins, for example, have been reported to bind CCR6 to mediate chemotaxis (242). However, just as IDR-peptides utilize multiple signalling pathways to regulate distinct immune functions, IDR-peptides may trigger the activation of multiple receptors to mediate their complex signalling cascades. In recent years, immunomodulatory HDPs have shown to act in this manner. LL-37, mediates cellular chemotaxis and angiogenesis via FPRL-1, induces cytokine release via interaction with purinergic receptor $P2X_7$ and GAPDH (66, 93, 103, 242, 243) and utilizes $P2X_7$ to promote fibroblast proliferation (244). Defensins, in addition to triggering CCR6, have been reported to interact with TLRs resulting in activation of antigen presenting cells (105, 245).

A further compounding factor is the reported ability of HDPs to indirectly activate other receptors. LL-37, through its matrix metalloprotease-dependent release of ligands, can transactivate EGFR resulting in the promotion of keratinocyte migration, activation of epithelial cells, and corneal wound healing (63, 104, 246). Defensins can also promote TNFα-mediated signalling by upregulation of TNFR2, conceptually similar to the enhancement of CCR5 signalling by IDR-1002 through receptor upregulation (247).

It is highly likely IDR-peptides exhibit the same degree of complexity concerning receptor interactions. Findings in Chapter 2 demonstrating the necessity of cellular uptake processes for peptide function suggests intracellular targets for IDR-peptides. Indeed, multiple intracellular interacting partners have been identified for IDR-1. IDR-1 is able interact with the cytoplasmic scaffold protein sequestosome-1 to mediate downstream p38 MAPK activation (102). Similarly IDR-1 induction of cytokine gene expression is dependent on its interaction with intracellular GAPDH (93). Studies using labeled IDR-1002 have shown rapid cellular uptake of peptide, suggesting intracellular targets as a definite

possibility for IDR-1002 (248). Further studies are required to elucidate the role of cellular uptake in IDR-peptide-mediated immunomodulation. Investigating the involvement of distinct endocytic pathways in peptide uptake, including clathrin-mediated endocytosis or caveolin-mediated endocytosis, may grant insight into IDR-peptide subcellular localization and, thus, potential interacting targets. Although these findings provide important insight into peptide-induced signal transduction, our understanding of IDR-peptide and HDP receptors is still immature. Much more remains to be elucidated regarding peptide-receptor interactions. Furthermore, additional efforts are required to discover how these interactions regulate the numerous immune functions affected by IDR-peptides and endogenous HDPs alike.

A major aim of this project was to characterize the extent of regulation exerted by IDR-peptides on host immunity. However, given the evidence that IDR-peptides are regulators of major immune signalling hubs, the implications regarding immune regulation are staggering. Each signalling pathway axis modulates numerous aspects of the immune response. In this study alone IDR-1002 was shown to utilize the PI3K pathway to regulate cytokine production, integrin activation, and TLR pathway suppression. This pathway also plays a major role in cytoskeletal and membrane rearrangement, phagosome formation, and immune cell development and function (249-251). The p38 and ERK-1/2 MAPK, both activated by IDR-peptides, also regulate cellular growth, motility, and differentiation (141, 252, 253), all of which are essential processes in wound healing, and cellular survival. It is a near certainty that modulation of these crucial immune pathways by IDR-peptides has extensive downstream regulatory effects on the innate and adaptive immune response. Efforts are underway to characterize these as yet unreported functions of IDR-peptides, and include investigations into peptide-mediated regulation of host antiviral responses, macrophage differentiation and maturation, and wound healing processes. In summary, this project has revealed the utilization of immune signal transduction pathways by IDR-peptides to elicit numerous regulatory effects. This knowledge not only furthers our comprehension of peptide mechanism of action, but highlights and expands the immunomodulatory potential of IDR-peptides.

6.4. IDR-1002 REGULATION AND CELLULAR SPECIFICITY

This thesis has focused on the human monocyte population as a key regulatory target of IDR-1002. The attention to monocytes was based on the observation that this cell population is essential for IDR-mediated antimicrobial protection (79, 80). In addition, monocytes play a critical role not only in generating directly antimicrobial functions, but in coordinating the overall inflammatory response. In this thesis project, IDR-1002 was shown to affect the immune functions of monocytes and monocyte-containing populations. In Chapter 2 the ability of IDR-1002 to induce chemokine production by PBMCs, correlating with an activation of monocyte MAPK signalling, was observed. In Chapters 3 and 4, IDR-1002 was shown to impact monocyte chemotaxis via the regulation of monocyte integrin-mediated adhesion and monocyte sensitivity to endogenous cytokines. IDR-1002 also suppressed LPS-mediated inflammatory responses in PBMCs, correlating with a suppression of LPS-induced signal transduction in monocytes. Characterization of this multifunctional regulation of monocyte responses by IDR-1002 in this thesis elucidates the key role of this population in IDR-mediated antimicrobial protection. However monocytes are but one player, albeit a highly important one, in the overall immune response.

Endogenous HDPs are able to mediate their diverse immunomodulatory effects due to their ability to regulate the responses of numerous immune cells. LL-37, a regulator of cytokine production by PBMCs, can promote angiogenesis through its effects on endothelial cells (50, 66). The cathelicidin can also modulate the responses of keratinocytes and epithelial cells to inflammatory stimuli and regulate dendritic cell differentiation (57, 254). Similarly, human defensins are chemoattractive for T-lymphocytes and dendritic cells, can induce cytokine production by numerous immune cells, and also trigger the degranulation of mast cells (45, 46, 255, 256). This multifaceted regulation by HDPs stems, in part, from the highly similar mechanisms of regulation between different cell populations, namely the immune signal transduction pathways. As an example, LL-37 utilizes the PI3K and MAPK pathways to regulate cytokine release in both mast cells and human PBMCs (43, 50, 257). Through similar pathways, LL-37 promotes endothelial cell growth and wound healing of corneal epithelial cells (66, 246). Utilization of similar signal transduction pathways by IDR-peptides strongly suggests a regulatory role in other cell populations. Evidence of this is found in emerging studies. Recently, IDR-1002 was shown to selectively regulate

inflammatory behaviour and modulate IL-1-mediated responses in synovial fibroblasts (248). Preliminary investigations have shown that IDR-peptides can modulate neutrophil adhesive, chemotactic, and antimicrobial functions (unpublished data). Additionally, there is evidence IDR-peptides can modulate epithelial cell cytokine production in synergy with microbial stimulants (unpublished data). Thus, while the monocyte population may be a keystone of IDR-mediated bacterial clearance, IDR-peptide regulation of other cell populations are certain to mediate a wide array of immune functions

6.5. CONCLUDING REMARKS

The end goal of understanding IDR-peptide function is the development of novel therapeutic agents that can fine tune the immune response to enhance host defenses against pathogens. The development of immunomodulatory HDP-derivatives offers an improvement over classical immunomodulators which, due to their relatively narrow regulatory range, can only be used in limited scenarios. While numerous *in vivo* murine models have shown the efficacy of IDR-peptides in enhancing bacterial clearance with reduced toxicities compared to endogenous HDPs, the development of IDR-peptides is a recent initiative and thus clinical development of IDR-peptides remains in its infancy. However, IMX942, an IDR-1 derivative which exhibits broad range antimicrobial protection in animal models, has demonstrated a strong safety profile in Phase I clinical trials and is set for Phase II trials (http://www.inimexpharma.com). A potential obstacle in the clinical development of IDR-peptides is the relative lack of understanding of how IDR-peptides mediate their antimicrobial functions. This thesis project addresses many aspects of this obstacle. This investigation not only expanded the known array of immunomodulatory functions exhibited by IDR-peptides, but also elucidated the mechanisms by which peptides manipulate immune responses. Furthermore, the studies in this project demonstrated a similarity of IDR-mediated responses between a human cell system and *in vivo* animal models. Overall, the results reported here provide essential insights for the development of novel IDR-peptides with improved immunomodulatory capabilities and the application of these peptides in numerous clinical settings. Thus the findings presented in this thesis are an invaluable contribution to

the development of IDR-peptides as promising, novel, broad-range anti-infective therapeutic agents.

REFERENCES

1. Davies, J. and D. Davies. 2010. Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* 74: 417-433.

2. Nicholls, E. F., L. Madera, and R. E. Hancock. 2010. Immunomodulators as adjuvants for vaccines and antimicrobial therapy. *Ann. N. Y. Acad. Sci.* 1213: 46-61.

3. Medzhitov, R. and C. Janeway Jr. 2000. Innate immunity *N. Engl. J. Med.* 343: 338-344.

4. Hancock, R. E., A. Nijnik, and D. J. Philpott. 2012. Modulating immunity as a therapy for bacterial infections. *Nat. Rev. Microbiol.* 10: 243-254.

5. Medzhitov, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature* 449: 819-826.

6. Allen, S. J., S. E. Crown, and T. M. Handel. 2007. Chemokine: receptor structure, interactions, and antagonism *Annu. Rev. Immunol.* 25: 787-820.

7. Mege, J. L., S. Meghari, A. Honstettre, C. Capo, and D. Raoult. 2006. The two faces of interleukin 10 in human infectious diseases. *Lancet Infect. Dis.* 6: 557-569.

8. Sadler, A. J. and B. R. Williams. 2008. Interferon-inducible antiviral effectors. *Nat. Rev. Immunol.* 8: 559-568.

9. Jo, Y. W., Y. S. Youn, S. H. Lee, B. M. Kim, S. H. Kang, M. Yoo, E. C. Choi, and K. C. Lee. 2006. Long-acting interferon-alpha 2a modified with a trimer-structured polyethylene glycol: preparation, in vitro bioactivity, in vivo stability and pharmacokinetics. *Int. J. Pharm.* 309: 87-93.

10. Sarrazin, C., C. Hezode, S. Zeuzem, and J. M. Pawlotsky. 2012. Antiviral strategies in hepatitis C virus infection. *J. Hepatol.* 56 Suppl 1: S88-100.

11. Esforzado, N. and J. M. Campistol. 2012. Treatment of hepatitis C in dialysis patients. *Contrib. Nephrol.* 176: 54-65.

12. Ratnam, D., A. Dev, T. Nguyen, V. Sundararajan, H. Harley, W. Cheng, A. Lee, F. Rusli, R. Chen, S. Bell, S. Pianko, and W. Sievert. 2011. The Efficacy and Tolerability of Pegylated Interferon-alpha-2a in Chronic Hepatitis B: A Multicenter Clinical Experience. *J. Gastroenterol. Hepatol.*

13. Sonneveld, M. J., V. Rijckborst, Y. Cakaloglu, K. Simon, E. J. Heathcote, F. Tabak, T. Mach, C. A. Boucher, B. E. Hansen, S. Zeuzem, and H. L. Janssen. 2012. Durable hepatitis B surface antigen decline in hepatitis B e antigen-positive chronic hepatitis B patients treated with pegylated interferon-alpha2b: relation to response and HBV genotype. *Antivir Ther.* 17: 9-17.

14. Billiau, A. and P. Matthys. 2009. Interferon-gamma: a historical perspective. *Cytokine Growth Factor Rev.* 20: 97-113.

15. Naderi Beni, F., F. Fattahi, A. Mirshafiey, M. Ansari, M. Mohsenzadegan, M. Movahedi, Z. Pourpak, and M. Moin. 2012. Increased production of nitric oxide by neutrophils from patients with chronic granulomatous disease on interferon-gamma treatment. *Int. Immunopharmacol.*

16. Errante, P. R., J. B. Frazao, and A. Condino-Neto. 2008. The use of interferon-gamma therapy in chronic granulomatous disease. *Recent. Pat. Antiinfect Drug Discov.* 3: 225-230.

17. Holland, S. M. 2010. Chronic granulomatous disease. *Clin. Rev. Allergy Immunol.* 38: 3-10.

18. Segal, B. H. and L. R. Romani. 2009. Invasive aspergillosis in chronic granulomatous disease. *Med. Mycol.* 47 Suppl 1: S282-90.

19. Page, A. V. and W. C. Liles. 2011. Colony-stimulating factors in the prevention and management of infectious diseases. *Infect. Dis. Clin. North Am.* 25: 803-817.

20. Biesma, B., E. G. de Vries, P. H. Willemse, W. J. Sluiter, P. E. Postmus, P. C. Limburg, A. C. Stern, and E. Vellenga. 1990. Efficacy and tolerability of recombinant human granulocyte-macrophage colony-stimulating factor in patients with chemotherapy-related leukopenia and fever. *Eur. J. Cancer* 26: 932-936.

21. Baltch, A. L., L. H. Bopp, R. P. Smith, W. J. Ritz, and P. B. Michelsen. 2008. Anticandidal effects of voriconazole and caspofungin, singly and in combination, against Candida glabrata, extracellularly and intracellularly in granulocyte-macrophage colony stimulating factor (GM-CSF)-activated human monocytes. *J. Antimicrob. Chemother.* 62: 1285-1290.

22. Cluff, C. W., J. R. Baldridge, A. G. Stover, J. T. Evans, D. A. Johnson, M. J. Lacy, V. G. Clawson, V. M. Yorgensen, C. L. Johnson, M. T. Livesay, R. M. Hershberg, and D. H. Persing. 2005. Synthetic toll-like receptor 4 agonists stimulate innate resistance to infectious challenge. *Infect. Immun.* 73: 3044-3052.

23. Airhart, C. L., H. N. Rohde, G. A. Bohach, C. J. Hovde, C. F. Deobald, S. S. Lee, and S. A. Minnich. 2008. Induction of innate immunity by lipid A mimetics increases survival from pneumonic plague. *Microbiology* 154: 2131-2138.

24. Mark, K. E., L. Corey, T. C. Meng, A. S. Magaret, M. L. Huang, S. Selke, H. B. Slade, S. K. Tyring, T. Warren, S. L. Sacks, P. Leone, V. A. Bergland, and A. Wald. 2007. Topical resiquimod 0.01% gel decreases herpes simplex virus type 2 genital shedding: a randomized, controlled trial. *J. Infect. Dis.* 195: 1324-1331.

25. Horsmans, Y., T. Berg, J. P. Desager, T. Mueller, E. Schott, S. P. Fletcher, K. R. Steffy, L. A. Bauman, B. M. Kerr, and D. R. Averett. 2005. Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic hepatitis C infection. *Hepatology* 42: 724-731.

26. Flynn, B., V. Wang, D. L. Sacks, R. A. Seder, and D. Verthelyi. 2005. Prevention and treatment of cutaneous leishmaniasis in primates by using synthetic type D/A oligodeoxynucleotides expressing CpG motifs. *Infect. Immun.* 73: 4948-4954.

27. Garland, S. M. 2003. Imiquimod. *Curr. Opin. Infect. Dis.* 16: 85-89.

28. Goldstein, D., P. Hertzog, E. Tomkinson, D. Couldwell, S. McCarville, S. Parrish, P. Cunningham, M. Newell, M. Owens, and D. A. Cooper. 1998. Administration of imiquimod, an interferon inducer, in asymptomatic human immunodeficiency virus-infected persons to determine safety and biologic response modification *J. Infect. Dis.* 178: 858-861.

29. Festi, D., L. Sandri, G. Mazzella, E. Roda, T. Sacco, T. Staniscia, S. Capodicasa, A. Vestito, and A. Colecchia. 2004. Safety of interferon beta treatment for chronic HCV hepatitis *World J. Gastroenterol.* 10: 12-16.

30. Sokal, E. M., A. Bourgois, X. Stephenne, T. Silveira, G. Porta, D. Gardovska, B. Fischler, and D. Kelly. 2010. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection in children and adolescents *J. Hepatol.* 52: 827-831.

31. Yeung, A. T., S. L. Gellatly, and R. E. Hancock. 2011. Multifunctional cationic host defence peptides and their clinical applications *Cell Mol. Life Sci.* 68: 2161-2176.

32. Hancock, R. E., K. L. Brown, and N. Mookherjee. 2006. Host defence peptides from invertebrates--emerging antimicrobial strategies *Immunobiology* 211: 315-322.

33. Brown, K. L. and R. E. Hancock. 2006. Cationic host defense (antimicrobial) peptides *Curr. Opin. Immunol.* 18: 24-30.

34. Menendez, A. and B. Brett Finlay. 2007. Defensins in the immunology of bacterial infections *Curr. Opin. Immunol.* 19: 385-391.

35. Durr, U. H., U. S. Sudheendra, and A. Ramamoorthy. 2006. LL-37, the only human member of the cathelicidin family of antimicrobial peptides *Biochim. Biophys. Acta* 1758: 1408-1425.

36. Zanetti, M. 2004. Cathelicidins, multifunctional peptides of the innate immunity *J. Leukoc. Biol.* 75: 39-48.

37. Bowdish, D. M., D. J. Davidson, and R. E. Hancock. 2005. A re-evaluation of the role of host defence peptides in mammalian immunity *Curr. Protein Pept. Sci.* 6: 35-51.

38. Jenssen, H., P. Hamill, and R. E. Hancock. 2006. Peptide antimicrobial agents *Clin. Microbiol. Rev.* 19: 491-511.

39. Territo, M. C., T. Ganz, M. E. Selsted, and R. Lehrer. 1989. Monocyte-chemotactic activity of defensins from human neutrophils *J. Clin. Invest.* 84: 2017-2020.

40. De, Y., Q. Chen, A. P. Schmidt, G. M. Anderson, J. M. Wang, J. Wooters, J. J. Oppenheim, and O. Chertov. 2000. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells *J. Exp. Med.* 192: 1069-1074.

41. Agerberth, B., J. Charo, J. Werr, B. Olsson, F. Idali, L. Lindbom, R. Kiessling, H. Jornvall, H. Wigzell, and G. H. Gudmundsson. 2000. The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations *Blood* 96: 3086-3093.

42. Yang, D., O. Chertov, S. N. Bykovskaia, Q. Chen, M. J. Buffo, J. Shogan, M. Anderson, J. M. Schroder, J. M. Wang, O. M. Howard, and J. J. Oppenheim. 1999. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6 *Science* 286: 525-528.

43. Bowdish, D. M., D. J. Davidson, D. P. Speert, and R. E. Hancock. 2004. The human cationic peptide LL-37 induces activation of the extracellular signal-regulated kinase and p38 kinase pathways in primary human monocytes. *J. Immunol.* 172: 3758-3765.

44. Mookherjee, N., K. L. Brown, D. M. Bowdish, S. Doria, R. Falsafi, K. Hokamp, F. M. Roche, R. Mu, G. H. Doho, J. Pistolic, J. P. Powers, J. Bryan, F. S. Brinkman, and R. E. Hancock. 2006. Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. *J. Immunol.* 176: 2455-2464.

45. Boniotto, M., W. J. Jordan, J. Eskdale, A. Tossi, N. Antcheva, S. Crovella, N. D. Connell, and G. Gallagher. 2006. Human beta-defensin 2 induces a vigorous cytokine response in peripheral blood mononuclear cells. *Antimicrob. Agents Chemother.* 50: 1433-1441.

46. Niyonsaba, F., H. Ushio, N. Nakano, W. Ng, K. Sayama, K. Hashimoto, I. Nagaoka, K. Okumura, and H. Ogawa. 2007. Antimicrobial peptides human beta-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. *J. Invest. Dermatol.* 127: 594-604.

47. Chaly, Y. V., E. M. Paleolog, T. S. Kolesnikova, I. I. Tikhonov, E. V. Petratchenko, and N. N. Voitenok. 2000. Neutrophil alpha-defensin human neutrophil peptide modulates cytokine production in human monocytes and adhesion molecule expression in endothelial cells *Eur. Cytokine Netw.* 11: 257-266.

48. Sakamoto, N., H. Mukae, T. Fujii, H. Ishii, S. Yoshioka, T. Kakugawa, K. Sugiyama, Y. Mizuta, J. Kadota, M. Nakazato, and S. Kohno. 2005. Differential effects of alpha- and beta-

defensin on cytokine production by cultured human bronchial epithelial cells *Am. J. Physiol. Lung Cell. Mol. Physiol.* 288: L508-13.

49. Kanda, N., M. Kamata, Y. Tada, T. Ishikawa, S. Sato, and S. Watanabe. 2011. Human beta-defensin-2 enhances IFN-gamma and IL-10 production and suppresses IL-17 production in T cells *J. Leukoc. Biol.* 89: 935-944.

50. Yu, J., N. Mookherjee, K. Wee, D. M. Bowdish, J. Pistolic, Y. Li, L. Rehaume, and R. E. Hancock. 2007. Host defense peptide LL-37, in synergy with inflammatory mediator IL-1beta, augments immune responses by multiple pathways. *J. Immunol.* 179: 7684-7691.

51. Scott, A., S. Weldon, P. J. Buchanan, B. Schock, R. K. Ernst, D. F. McAuley, M. M. Tunney, C. R. Irwin, J. S. Elborn, and C. C. Taggart. 2011. Evaluation of the ability of LL-37 to neutralise LPS in vitro and ex vivo *PLoS One* 6: e26525.

52. Suzuki, K., T. Murakami, K. Kuwahara-Arai, H. Tamura, K. Hiramatsu, and I. Nagaoka. 2011. Human anti-microbial cathelicidin peptide LL-37 suppresses the LPS-induced apoptosis of endothelial cells *Int. Immunol.* 23: 185-193.

53. Scott, M. G., D. J. Davidson, M. R. Gold, D. Bowdish, and R. E. Hancock. 2002. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J. Immunol.* 169: 3883-3891.

54. Cirioni, O., A. Giacometti, R. Ghiselli, C. Bergnach, F. Orlando, C. Silvestri, F. Mocchegiani, A. Licci, B. Skerlavaj, M. Rocchi, V. Saba, M. Zanetti, and G. Scalise. 2006. LL-37 protects rats against lethal sepsis caused by gram-negative bacteria. *Antimicrob. Agents Chemother.* 50: 1672-1679.

55. Lillard, J. W.,Jr, P. N. Boyaka, O. Chertov, J. J. Oppenheim, and J. R. McGhee. 1999. Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. *Proc. Natl. Acad. Sci. U. S. A.* 96: 651-656.

56. Tani, K., W. J. Murphy, O. Chertov, R. Salcedo, C. Y. Koh, I. Utsunomiya, S. Funakoshi, O. Asai, S. H. Herrmann, J. M. Wang, L. W. Kwak, and J. J. Oppenheim. 2000. Defensins act as potent adjuvants that promote cellular and humoral immune responses in mice to a lymphoma idiotype and carrier antigens. *Int. Immunol.* 12: 691-700.

57. Davidson, D. J., A. J. Currie, G. S. Reid, D. M. Bowdish, K. L. MacDonald, R. C. Ma, R. E. Hancock, and D. P. Speert. 2004. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. *J. Immunol.* 172: 1146-1156.

58. van der Does, A. M., H. Beekhuizen, B. Ravensbergen, T. Vos, T. H. Ottenhoff, J. T. van Dissel, J. W. Drijfhout, P. S. Hiemstra, and P. H. Nibbering. 2010. LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature. *J. Immunol.* 185: 1442-1449.

59. An, L. L., Y. H. Yang, X. T. Ma, Y. M. Lin, G. Li, Y. H. Song, and K. F. Wu. 2005. LL-37 enhances adaptive antitumor immune response in a murine model when genetically fused with M-CSFR (J6-1) DNA vaccine. *Leuk. Res.* 29: 535-543.

60. Oono, T., Y. Shirafuji, W. K. Huh, H. Akiyama, and K. Iwatsuki. 2002. Effects of human neutrophil peptide-1 on the expression of interstitial collagenase and type I collagen in human dermal fibroblasts. *Arch. Dermatol. Res.* 294: 185-189.

61. Shaykhiev, R., C. Beisswenger, K. Kandler, J. Senske, A. Puchner, T. Damm, J. Behr, and R. Bals. 2005. Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 289: L842-8.

62. Chamorro, C. I., G. Weber, A. Gronberg, A. Pivarcsi, and M. Stahle. 2009. The human antimicrobial peptide LL-37 suppresses apoptosis in keratinocytes. *J. Invest. Dermatol.* 129: 937-944.

63. Tokumaru, S., K. Sayama, Y. Shirakata, H. Komatsuzawa, K. Ouhara, Y. Hanakawa, Y. Yahata, X. Dai, M. Tohyama, H. Nagai, L. Yang, S. Higashiyama, A. Yoshimura, M. Sugai, and K. Hashimoto. 2005. Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. *J. Immunol.* 175: 4662-4668.

64. Aberg, K. M., M. Q. Man, R. L. Gallo, T. Ganz, D. Crumrine, B. E. Brown, E. H. Choi, D. K. Kim, J. M. Schroder, K. R. Feingold, and P. M. Elias. 2008. Co-regulation and interdependence of the mammalian epidermal permeability and antimicrobial barriers. *J. Invest. Dermatol.* 128: 917-925.

65. Frohm, M., B. Agerberth, G. Ahangari, M. Stahle-Backdahl, S. Liden, H. Wigzell, and G. H. Gudmundsson. 1997. The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J. Biol. Chem.* 272: 15258-15263.

66. Koczulla, R., G. von Degenfeld, C. Kupatt, F. Krotz, S. Zahler, T. Gloe, K. Issbrucker, P. Unterberger, M. Zaiou, C. Lebherz, A. Karl, P. Raake, A. Pfosser, P. Boekstegers, U. Welsch, P. S. Hiemstra, C. Vogelmeier, R. L. Gallo, M. Clauss, and R. Bals. 2003. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J. Clin. Invest.* 111: 1665-1672.

67. Heilborn, J. D., M. F. Nilsson, G. Kratz, G. Weber, O. Sorensen, N. Borregaard, and M. Stahle-Backdahl. 2003. The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. *J. Invest. Dermatol.* 120: 379-389.

68. Ong, P. Y., T. Ohtake, C. Brandt, I. Strickland, M. Boguniewicz, T. Ganz, R. L. Gallo, and D. Y. Leung. 2002. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.* 347: 1151-1160.

69. Huang, L. C., R. Y. Reins, R. L. Gallo, and A. M. McDermott. 2007. Cathelicidin-deficient (Cnlp -/-) mice show increased susceptibility to Pseudomonas aeruginosa keratitis. *Invest. Ophthalmol. Vis. Sci.* 48: 4498-4508.

70. Nizet, V., T. Ohtake, X. Lauth, J. Trowbridge, J. Rudisill, R. A. Dorschner, V. Pestonjamasp, J. Piraino, K. Huttner, and R. L. Gallo. 2001. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 414: 454-457.

71. Morrison, G., F. Kilanowski, D. Davidson, and J. Dorin. 2002. Characterization of the mouse beta defensin 1, Defb1, mutant mouse model. *Infect. Immun.* 70: 3053-3060.

72. Wilson, C. L., A. J. Ouellette, D. P. Satchell, T. Ayabe, Y. S. Lopez-Boado, J. L. Stratman, S. J. Hultgren, L. M. Matrisian, and W. C. Parks. 1999. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* 286: 113-117.

73. Putsep, K., G. Carlsson, H. G. Boman, and M. Andersson. 2002. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *Lancet* 360: 1144-1149.

74. Carlsson, G. and A. Fasth. 2001. Infantile genetic agranulocytosis, morbus Kostmann: presentation of six cases from the original "Kostmann family" and a review. *Acta Paediatr.* 90: 757-764.

75. Welling, M. M., P. S. Hiemstra, M. T. van den Barselaar, A. Paulusma-Annema, P. H. Nibbering, E. K. Pauwels, and W. Calame. 1998. Antibacterial activity of human neutrophil defensins in experimental infections in mice is accompanied by increased leukocyte accumulation. *J. Clin. Invest.* 102: 1583-1590.

76. Elahi, S., R. M. Buchanan, S. Attah-Poku, H. G. Townsend, L. A. Babiuk, and V. Gerdts. 2006. The host defense peptide beta-defensin 1 confers protection against Bordetella pertussis in newborn piglets. *Infect. Immun.* 74: 2338-2352.

77. Easton, D. M., A. Nijnik, M. L. Mayer, and R. E. Hancock. 2009. Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends Biotechnol.* 27: 582-590.

78. Hancock, R. E. and H. G. Sahl. 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* 24: 1551-1557.

79. Scott, M. G., E. Dullaghan, N. Mookherjee, N. Glavas, M. Waldbrook, A. Thompson, A. Wang, K. Lee, S. Doria, P. Hamill, J. J. Yu, Y. Li, O. Donini, M. M. Guarna, B. B. Finlay, J. R. North, and R. E. Hancock. 2007. An anti-infective peptide that selectively modulates the innate immune response. *Nat. Biotechnol.* 25: 465-472.

80. Nijnik, A., L. Madera, S. Ma, M. Waldbrook, M. R. Elliott, D. M. Easton, M. L. Mayer, S. C. Mullaly, J. Kindrachuk, H. Jenssen, and R. E. Hancock. 2010. Synthetic cationic peptide IDR-1002 provides protection against bacterial infections through chemokine induction and enhanced leukocyte recruitment. *J. Immunol.* 184: 2539-2550.

81. Kindrachuk, J., E. Scruten, S. Attah-Poku, K. Bell, A. Potter, L. A. Babiuk, P. J. Griebel, and S. Napper. 2011. Stability, toxicity, and biological activity of host defense peptide BMAP28 and its inversed and retro-inversed isomers. *Biopolymers* 96: 14-24.

82. Niyonsaba, F., A. Someya, M. Hirata, H. Ogawa, and I. Nagaoka. 2001. Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells *Eur. J. Immunol.* 31: 1066-1075.

83. Lau, Y. E., D. M. Bowdish, C. Cosseau, R. E. Hancock, and D. J. Davidson. 2006. Apoptosis of airway epithelial cells: human serum sensitive induction by the cathelicidin LL-37. *Am. J. Respir. Cell Mol. Biol.* 34: 399-409.

84. Kindrachuk, J., H. Jenssen, M. Elliott, R. Townsend, A. Nijnik, S. F. Lee, V. Gerdts, L. A. Babiuk, S. A. Halperin, and R. E. Hancock. 2009. A novel vaccine adjuvant comprised of a synthetic innate defence regulator peptide and CpG oligonucleotide links innate and adaptive immunity. *Vaccine* 27: 4662-4671.

85. O'Neill, L. A. 2000. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense *Sci. STKE* 2000: re1.

86. Sethi, G., B. Sung, and B. B. Aggarwal. 2008. TNF: a master switch for inflammation to cancer *Front. Biosci.* 13: 5094-5107.

87. Hodge, D. R., E. M. Hurt, and W. L. Farrar. 2005. The role of IL-6 and STAT3 in inflammation and cancer *Eur. J. Cancer* 41: 2502-2512.

88. Viola, A. and A. D. Luster. 2008. Chemokines and their receptors: drug targets in immunity and inflammation *Annu. Rev. Pharmacol. Toxicol.* 48: 171-197.

89. Darveau, R. P. and R. E. Hancock. 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough Pseudomonas aeruginosa and Salmonella typhimurium strains. *J. Bacteriol.* 155: 831-838.

90. Hammond, M. E., G. R. Lapointe, P. H. Feucht, S. Hilt, C. A. Gallegos, C. A. Gordon, M. A. Giedlin, G. Mullenbach, and P. Tekamp-Olson. 1995. IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors. *J. Immunol.* 155: 1428-1433.

91. Van Damme, J., P. Proost, J. P. Lenaerts, and G. Opdenakker. 1992. Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family. *J. Exp. Med.* 176: 59-65.

92. Mookherjee, N., P. Hamill, J. Gardy, D. Blimkie, R. Falsafi, A. Chikatamarla, D. J. Arenillas, S. Doria, T. R. Kollmann, and R. E. Hancock. 2009. Systems biology evaluation of immune responses induced by human host defence peptide LL-37 in mononuclear cells. *Mol. Biosyst* 5: 483-496.

93. Mookherjee, N., D. N. Lippert, P. Hamill, R. Falsafi, A. Nijnik, J. Kindrachuk, J. Pistolic, J. Gardy, P. Miri, M. Naseer, L. J. Foster, and R. E. Hancock. 2009. Intracellular receptor for human host defense peptide LL-37 in monocytes. *J. Immunol.* 183: 2688-2696.

94. Raingeaud, J., S. Gupta, J. S. Rogers, M. Dickens, J. Han, R. J. Ulevitch, and R. J. Davis. 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.* 270: 7420-7426.

95. Rubinfeld, H. and R. Seger. 2005. The ERK cascade: a prototype of MAPK signaling. *Mol. Biotechnol.* 31: 151-174.

96. Zlotnik, A., O. Yoshie, and H. Nomiyama. 2006. The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biol.* 7: 243.

97. Mestas, J. and C. C. Hughes. 2004. Of mice and not men: differences between mouse and human immunology. *J. Immunol.* 172: 2731-2738.

98. Jia, T., N. V. Serbina, K. Brandl, M. X. Zhong, I. M. Leiner, I. F. Charo, and E. G. Pamer. 2008. Additive roles for MCP-1 and MCP-3 in CCR2-mediated recruitment of inflammatory monocytes during Listeria monocytogenes infection *J. Immunol.* 180: 6846-6853.

99. Lande, R., J. Gregorio, V. Facchinetti, B. Chatterjee, Y. H. Wang, B. Homey, W. Cao, Y. H. Wang, B. Su, F. O. Nestle, T. Zal, I. Mellman, J. M. Schroder, Y. J. Liu, and M. Gilliet. 2007. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide *Nature* 449: 564-569.

100. Gracia, A., M. Polewicz, S. A. Halperin, R. E. Hancock, A. A. Potter, L. A. Babiuk, and V. Gerdts. 2011. Antibody responses in adult and neonatal BALB/c mice to immunization with novel Bordetella pertussis vaccine formulations. *Vaccine* 29: 1595-1604.

101. Niyonsaba, F., K. Iwabuchi, A. Someya, M. Hirata, H. Matsuda, H. Ogawa, and I. Nagaoka. 2002. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis *Immunology* 106: 20-26.

102. Yu, H. B., A. Kielczewska, A. Rozek, S. Takenaka, Y. Li, L. Thorson, R. E. Hancock, M. M. Guarna, J. R. North, L. J. Foster, O. Donini, and B. B. Finlay. 2009. Sequestosome-1/p62 is the key intracellular target of innate defense regulator peptide. *J. Biol. Chem.* 284: 36007-36011.

103. Elssner, A., M. Duncan, M. Gavrilin, and M. D. Wewers. 2004. A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. *J. Immunol.* 172: 4987-4994.

104. Tjabringa, G. S., J. Aarbiou, D. K. Ninaber, J. W. Drijfhout, O. E. Sorensen, N. Borregaard, K. F. Rabe, and P. S. Hiemstra. 2003. The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. *J. Immunol.* 171: 6690-6696.

105. Biragyn, A., P. A. Ruffini, C. A. Leifer, E. Klyushnenkova, A. Shakhov, O. Chertov, A. K. Shirakawa, J. M. Farber, D. M. Segal, J. J. Oppenheim, and L. W. Kwak. 2002. Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science* 298: 1025-1029.

106. Pals, S. T., D. J. de Gorter, and M. Spaargaren. 2007. Lymphoma dissemination: the other face of lymphocyte homing *Blood* 110: 3102-3111.

107. Appay, V. and S. L. Rowland-Jones. 2001. RANTES: a versatile and controversial chemokine *Trends Immunol.* 22: 83-87.

108. Garlapati, S., N. F. Eng, T. G. Kiros, J. Kindrachuk, G. K. Mutwiri, R. E. Hancock, S. A. Halperin, A. A. Potter, L. A. Babiuk, and V. Gerdts. 2011. Immunization with PCEP microparticles containing pertussis toxoid, CpG ODN and a synthetic innate defense regulator peptide induces protective immunity against pertussis. *Vaccine* 29: 6540-6548.

109. Brown, T. H., J. David, E. Acosta-Ramirez, J. M. Moore, S. Lee, G. Zhong, R. E. Hancock, Z. Xing, S. A. Halperin, and J. Wang. 2012. Comparison of immune responses and protective efficacy of intranasal prime-boost immunization regimens using adenovirus-based and CpG/HH2 adjuvanted-subunit vaccines against genital Chlamydia muridarum infection. *Vaccine* 30: 350-360.

110. Brancato, S. K. and J. E. Albina. 2011. Wound macrophages as key regulators of repair: origin, phenotype, and function *Am. J. Pathol.* 178: 19-25.

111. Gilles, C., M. Polette, J. M. Zahm, J. M. Tournier, L. Volders, J. M. Foidart, and P. Birembaut. 1999. Vimentin contributes to human mammary epithelial cell migration *J. Cell. Sci.* 112 (Pt 24): 4615-4625.

112. Iizuka, M. and S. Konno. 2011. Wound healing of intestinal epithelial cells *World J. Gastroenterol.* 17: 2161-2171.

113. Carretero, M., M. J. Escamez, M. Garcia, B. Duarte, A. Holguin, L. Retamosa, J. L. Jorcano, M. D. Rio, and F. Larcher. 2008. In vitro and in vivo wound healing-promoting activities of human cathelicidin LL-37 *J. Invest. Dermatol.* 128: 223-236.

114. Gillitzer, R. and M. Goebeler. 2001. Chemokines in cutaneous wound healing *J. Leukoc. Biol.* 69: 513-521.

115. Strieter, R. M., M. D. Burdick, B. N. Gomperts, J. A. Belperio, and M. P. Keane. 2005. CXC chemokines in angiogenesis *Cytokine Growth Factor Rev.* 16: 593-609.

116. Medzhitov, R. 2008. Origin and physiological roles of inflammation. *Nature* 454: 428-435.

117. Adams, D. H. and S. Shaw. 1994. Leucocyte-endothelial interactions and regulation of leucocyte migration *Lancet* 343: 831-836.

118. Ley, K., C. Laudanna, M. I. Cybulsky, and S. Nourshargh. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* 7: 678-689.

119. Pober, J. S. and W. C. Sessa. 2007. Evolving functions of endothelial cells in inflammation *Nat. Rev. Immunol.* 7: 803-815.

120. Parsons, J. T., A. R. Horwitz, and M. A. Schwartz. 2010. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nat. Rev. Mol. Cell Biol.* 11: 633-643.

121. Abram, C. L. and C. A. Lowell. 2009. The ins and outs of leukocyte integrin signaling *Annu. Rev. Immunol.* 27: 339-362.

122. Schultz, G. S. and A. Wysocki. 2009. Interactions between extracellular matrix and growth factors in wound healing *Wound Repair Regen.* 17: 153-162.

123. Matias-Roman, S., B. G. Galvez, L. Genis, M. Yanez-Mo, G. de la Rosa, P. Sanchez-Mateos, F. Sanchez-Madrid, and A. G. Arroyo. 2005. Membrane type 1-matrix metalloproteinase is involved in migration of human monocytes and is regulated through their interaction with fibronectin or endothelium *Blood* 105: 3956-3964.

124. Frick, C., A. Odermatt, K. Zen, K. J. Mandell, H. Edens, R. Portmann, L. Mazzucchelli, D. L. Jaye, and C. A. Parkos. 2005. Interaction of ICAM-1 with beta 2-integrin CD11c/CD18: characterization of a peptide ligand that mimics a putative binding site on domain D4 of ICAM-1 *Eur. J. Immunol.* 35: 3610-3621.

125. Al-Numani, D., M. Segura, M. Dore, and M. Gottschalk. 2003. Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on human THP-1 monocytes stimulated by Streptococcus suis serotype 2 *Clin. Exp. Immunol.* 133: 67-77.

126. Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer* 26: 171-176.

127. Hemler, M. E. 1990. VLA proteins in the integrin family: structures, functions, and their role on leukocytes. *Annu. Rev. Immunol.* 8: 365-400.

128. Hart, R. and D. R. Greaves. 2010. Chemerin contributes to inflammation by promoting macrophage adhesion to VCAM-1 and fibronectin through clustering of VLA-4 and VLA-5. *J. Immunol.* 185: 3728-3739.

129. Chan, J. R., S. J. Hyduk, and M. I. Cybulsky. 2003. Detecting rapid and transient upregulation of leukocyte integrin affinity induced by chemokines and chemoattractants. *J. Immunol. Methods* 273: 43-52.

130. Chan, J. R., S. J. Hyduk, and M. I. Cybulsky. 2001. Chemoattractants induce a rapid and transient upregulation of monocyte alpha4 integrin affinity for vascular cell adhesion molecule 1 which mediates arrest: an early step in the process of emigration. *J. Exp. Med.* 193: 1149-1158.

131. Luque, A., M. Gomez, W. Puzon, Y. Takada, F. Sanchez-Madrid, and C. Cabanas. 1996. Activated conformations of very late activation integrins detected by a group of antibodies (HUTS) specific for a novel regulatory region (355-425) of the common beta 1 chain. *J. Biol. Chem.* 271: 11067-11075.

132. Gerszten, R. E., E. B. Friedrich, T. Matsui, R. R. Hung, L. Li, T. Force, and A. Rosenzweig. 2001. Role of phosphoinositide 3-kinase in monocyte recruitment under flow conditions. *J. Biol. Chem.* 276: 26846-26851.

133. Harokopakis, E., M. H. Albzreh, M. H. Martin, and G. Hajishengallis. 2006. TLR2 transmodulates monocyte adhesion and transmigration via Rac1- and PI3K-mediated inside-out signaling in response to Porphyromonas gingivalis fimbriae. *J. Immunol.* 176: 7645-7656.

134. Ferreira, A. M., H. Isaacs, J. S. Hayflick, K. A. Rogers, and M. Sandig. 2006. The p110delta isoform of PI3K differentially regulates beta1 and beta2 integrin-mediated monocyte adhesion and spreading and modulates diapedesis. *Microcirculation* 13: 439-456.

135. Navarro, A., B. Anand-Apte, Y. Tanabe, G. Feldman, and A. C. Larner. 2003. A PI-3 kinase-dependent, Stat1-independent signaling pathway regulates interferon-stimulated monocyte adhesion. *J. Leukoc. Biol.* 73: 540-545.

136. Yamamoto, H., Y. Naito, M. Okano, T. Kanazawa, H. Takematsu, and Y. Kozutsumi. 2011. Sphingosylphosphorylcholine and lysosulfatide have inverse regulatory functions in monocytic cell differentiation into macrophages. *Arch. Biochem. Biophys.* 506: 83-91.

137. Thamilselvan, V., D. H. Craig, and M. D. Basson. 2007. FAK association with multiple signal proteins mediates pressure-induced colon cancer cell adhesion via a Src-dependent PI3K/Akt pathway. *FASEB J.* 21: 1730-1741.

138. Katsumi, A., T. Naoe, T. Matsushita, K. Kaibuchi, and M. A. Schwartz. 2005. Integrin activation and matrix binding mediate cellular responses to mechanical stretch. *J. Biol. Chem.* 280: 16546-16549.

139. Byzova, T. V., C. K. Goldman, N. Pampori, K. A. Thomas, A. Bett, S. J. Shattil, and E. F. Plow. 2000. A mechanism for modulation of cellular responses to VEGF: activation of the integrins. *Mol. Cell* 6: 851-860.

140. Somanath, P. R., E. S. Kandel, N. Hay, and T. V. Byzova. 2007. Akt1 signaling regulates integrin activation, matrix recognition, and fibronectin assembly. *J. Biol. Chem.* 282: 22964-22976.

141. Mellado, M., J. M. Rodriguez-Frade, S. Manes, and C. Martinez-A. 2001. Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation *Annu. Rev. Immunol.* 19: 397-421.

142. Wieczorek, M., H. Jenssen, J. Kindrachuk, W. R. Scott, M. Elliott, K. Hilpert, J. T. Cheng, R. E. Hancock, and S. K. Straus. 2010. Structural studies of a peptide with immune modulating and direct antimicrobial activity. *Chem. Biol.* 17: 970-980.

143. Semel, A. C., E. C. Seales, A. Singhal, E. A. Eklund, K. J. Colley, and S. L. Bellis. 2002. Hyposialylation of integrins stimulates the activity of myeloid fibronectin receptors. *J. Biol. Chem.* 277: 32830-32836.

144. Li, J., B. A. Ballif, A. M. Powelka, J. Dai, S. P. Gygi, and V. W. Hsu. 2005. Phosphorylation of ACAP1 by Akt regulates the stimulation-dependent recycling of integrin beta1 to control cell migration. *Dev. Cell.* 9: 663-673.

145. Roberts, M. S., A. J. Woods, T. C. Dale, P. Van Der Sluijs, and J. C. Norman. 2004. Protein kinase B/Akt acts via glycogen synthase kinase 3 to regulate recycling of alpha v beta 3 and alpha 5 beta 1 integrins. *Mol. Cell. Biol.* 24: 1505-1515.

146. Krauss, K. and P. Altevogt. 1999. Integrin leukocyte function-associated antigen-1-mediated cell binding can be activated by clustering of membrane rafts. *J. Biol. Chem.* 274: 36921-36927.

147. Gilcrease, M. Z., X. Zhou, and K. Welch. 2004. Adhesion-independent alpha6beta4 integrin clustering is mediated by phosphatidylinositol 3-kinase. *Cancer Res.* 64: 7395-7398.

148. Gratacap, M. P., B. Payrastre, C. Viala, G. Mauco, M. Plantavid, and H. Chap. 1998. Phosphatidylinositol 3,4,5-trisphosphate-dependent stimulation of phospholipase C-gamma2 is an early key event in FcgammaRIIA-mediated activation of human platelets. *J. Biol. Chem.* 273: 24314-24321.

149. Cozzolino, M., V. Stagni, L. Spinardi, N. Campioni, C. Fiorentini, E. Salvati, S. Alema, and A. M. Salvatore. 2003. p120 Catenin is required for growth factor-dependent cell motility and scattering in epithelial cells. *Mol. Biol. Cell* 14: 1964-1977.

150. Huang, S., L. Y. Chen, B. L. Zuraw, R. D. Ye, and Z. K. Pan. 2001. Chemoattractant-stimulated NF-kappaB activation is dependent on the low molecular weight GTPase RhoA. *J. Biol. Chem.* 276: 40977-40981.

151. Woulfe, D., H. Jiang, R. Mortensen, J. Yang, and L. F. Brass. 2002. Activation of Rap1B by G(i) family members in platelets. *J. Biol. Chem.* 277: 23382-23390.

152. Pasquet, J. M., R. Bobe, B. Gross, M. P. Gratacap, M. G. Tomlinson, B. Payrastre, and S. P. Watson. 1999. A collagen-related peptide regulates phospholipase Cgamma2 via phosphatidylinositol 3-kinase in human platelets. *Biochem. J.* 342 (Pt 1): 171-177.

153. Meerschaert, J. and M. B. Furie. 1995. The adhesion molecules used by monocytes for migration across endothelium include CD11a/CD18, CD11b/CD18, and VLA-4 on monocytes and ICAM-1, VCAM-1, and other ligands on endothelium. *J. Immunol.* 154: 4099-4112.

154. Alon, R., P. D. Kassner, M. W. Carr, E. B. Finger, M. E. Hemler, and T. A. Springer. 1995. The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. *J. Cell Biol.* 128: 1243-1253.

155. de Fougerolles, A. R., G. Chi-Rosso, A. Bajardi, P. Gotwals, C. D. Green, and V. E. Koteliansky. 2000. Global expression analysis of extracellular matrix-integrin interactions in monocytes *Immunity* 13: 749-758.

156. Wesley, R. B.,2nd, X. Meng, D. Godin, and Z. S. Galis. 1998. Extracellular matrix modulates macrophage functions characteristic to atheroma: collagen type I enhances acquisition of resident macrophage traits by human peripheral blood monocytes in vitro *Arterioscler. Thromb. Vasc. Biol.* 18: 432-440.

157. Shi, C. and D. I. Simon. 2006. Integrin signals, transcription factors, and monocyte differentiation *Trends Cardiovasc. Med.* 16: 146-152.

158. Mantovani, A. 1999. The chemokine system: redundancy for robust outputs *Immunol. Today* 20: 254-257.

159. Gouwy, M., S. Struyf, P. Proost, and J. Van Damme. 2005. Synergy in cytokine and chemokine networks amplifies the inflammatory response *Cytokine Growth Factor Rev.* 16: 561-580.

160. Kuscher, K., G. Danelon, S. Paoletti, L. Stefano, M. Schiraldi, V. Petkovic, M. Locati, B. O. Gerber, and M. Uguccioni. 2009. Synergy-inducing chemokines enhance CCR2 ligand activities on monocytes *Eur. J. Immunol.* 39: 1118-1128.

161. Gouwy, M., S. Struyf, S. Noppen, E. Schutyser, J. Y. Springael, M. Parmentier, P. Proost, and J. Van Damme. 2008. Synergy between coproduced CC and CXC chemokines in monocyte chemotaxis through receptor-mediated events *Mol. Pharmacol.* 74: 485-495.

162. Sallusto, F., P. Schaerli, P. Loetscher, C. Schaniel, D. Lenig, C. R. Mackay, S. Qin, and A. Lanzavecchia. 1998. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation *Eur. J. Immunol.* 28: 2760-2769.

163. Sallusto, F., E. Kremmer, B. Palermo, A. Hoy, P. Ponath, S. Qin, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells *Eur. J. Immunol.* 29: 2037-2045.

164. Kim, C. H., K. Nagata, and E. C. Butcher. 2003. Dendritic cells support sequential reprogramming of chemoattractant receptor profiles during naive to effector T cell differentiation *J. Immunol.* 171: 152-158.

165. Kaufmann, A., R. Salentin, D. Gemsa, and H. Sprenger. 2001. Increase of CCR1 and CCR5 expression and enhanced functional response to MIP-1 alpha during differentiation of human monocytes to macrophages *J. Leukoc. Biol.* 69: 248-252.

166. Raman, D., T. Sobolik-Delmaire, and A. Richmond. 2011. Chemokines in health and disease *Exp. Cell Res.* 317: 575-589.

167. Yadav, A., V. Saini, and S. Arora. 2010. MCP-1: chemoattractant with a role beyond immunity: a review *Clin. Chim. Acta* 411: 1570-1579.

168. Rossi, D. and A. Zlotnik. 2000. The biology of chemokines and their receptors *Annu. Rev. Immunol.* 18: 217-242.

169. Weber, C., K. S. Weber, C. Klier, S. Gu, R. Wank, R. Horuk, and P. J. Nelson. 2001. Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and T(H)1-like/CD45RO(+) T cells *Blood* 97: 1144-1146.

170. Blanpain, C., R. Buser, C. A. Power, M. Edgerton, C. Buchanan, M. Mack, G. Simmons, P. R. Clapham, M. Parmentier, and A. E. Proudfoot. 2001. A chimeric MIP-1alpha/RANTES protein demonstrates the use of different regions of the RANTES protein to bind and activate its receptors *J. Leukoc. Biol.* 69: 977-985.

171. Curnock, A. P., M. K. Logan, and S. G. Ward. 2002. Chemokine signalling: pivoting around multiple phosphoinositide 3-kinases *Immunology* 105: 125-136.

172. Rathanaswami, P., M. Hachicha, M. Sadick, T. J. Schall, and S. R. McColl. 1993. Expression of the cytokine RANTES in human rheumatoid synovial fibroblasts. Differential regulation of RANTES and interleukin-8 genes by inflammatory cytokines *J. Biol. Chem.* 268: 5834-5839.

173. Marfaing-Koka, A., O. Devergne, G. Gorgone, A. Portier, T. J. Schall, P. Galanaud, and D. Emilie. 1995. Regulation of the production of the RANTES chemokine by endothelial cells. Synergistic induction by IFN-gamma plus TNF-alpha and inhibition by IL-4 and IL-13 *J. Immunol.* 154: 1870-1878.

174. Sallusto, F., B. Palermo, D. Lenig, M. Miettinen, S. Matikainen, I. Julkunen, R. Forster, R. Burgstahler, M. Lipp, and A. Lanzavecchia. 1999. Distinct patterns and kinetics of chemokine production regulate dendritic cell function *Eur. J. Immunol.* 29: 1617-1625.

175. Shi, C. and E. G. Pamer. 2011. Monocyte recruitment during infection and inflammation. *Nat. Rev. Immunol.* 11: 762-774.

176. Neel, N. F., E. Schutyser, J. Sai, G. H. Fan, and A. Richmond. 2005. Chemokine receptor internalization and intracellular trafficking *Cytokine Growth Factor Rev.* 16: 637-658.

177. Vroon, A., C. J. Heijnen, and A. Kavelaars. 2006. GRKs and arrestins: regulators of migration and inflammation *J. Leukoc. Biol.* 80: 1214-1221.

178. Mueller, A. and P. G. Strange. 2004. Mechanisms of internalization and recycling of the chemokine receptor, CCR5 *Eur. J. Biochem.* 271: 243-252.

179. Oppermann, M. 2004. Chemokine receptor CCR5: insights into structure, function, and regulation *Cell. Signal.* 16: 1201-1210.

180. Delhaye, M., A. Gravot, D. Ayinde, F. Niedergang, M. Alizon, and A. Brelot. 2007. Identification of a postendocytic sorting sequence in CCR5 *Mol. Pharmacol.* 72: 1497-1507.

181. Mueller, A., E. Kelly, and P. G. Strange. 2002. Pathways for internalization and recycling of the chemokine receptor CCR5 *Blood* 99: 785-791.

182. Garcia Lopez, M. A., A. Aguado Martinez, C. Lamaze, C. Martinez-A, and T. Fischer. 2009. Inhibition of dynamin prevents CCL2-mediated endocytosis of CCR2 and activation of ERK1/2 *Cell. Signal.* 21: 1748-1757.

183. Handel, T. M., Z. Johnson, D. H. Rodrigues, A. C. Dos Santos, R. Cirillo, V. Muzio, S. Riva, M. Mack, M. Deruaz, F. Borlat, P. A. Vitte, T. N. Wells, M. M. Teixeira, and A. E. Proudfoot. 2008. An engineered monomer of CCL2 has anti-inflammatory properties emphasizing the importance of oligomerization for chemokine activity in vivo. *J. Leukoc. Biol.* 84: 1101-1108.

184. Aramori, I., S. S. Ferguson, P. D. Bieniasz, J. Zhang, B. Cullen, and M. G. Cullen. 1997. Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor *EMBO J.* 16: 4606-4616.

185. Kohout, T. A., S. L. Nicholas, S. J. Perry, G. Reinhart, S. Junger, and R. S. Struthers. 2004. Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7 *J. Biol. Chem.* 279: 23214-23222.

186. Miyake, K. 2004. Innate recognition of lipopolysaccharide by Toll-like receptor 4-MD-2. *Trends Microbiol.* 12: 186-192.

187. Smith, J. A. 1994. Neutrophils, host defense, and inflammation: a double-edged sword. *J. Leukoc. Biol.* 56: 672-686.

188. Zhang, X. and D. M. Mosser. 2008. Macrophage activation by endogenous danger signals. *J. Pathol.* 214: 161-178.

189. Fletcher, S., K. Steffy, and D. Averett. 2006. Masked oral prodrugs of toll-like receptor 7 agonists: a new approach for the treatment of infectious disease. *Curr. Opin. Investig Drugs* 7: 702-708.

190. Kanzler, H., F. J. Barrat, E. M. Hessel, and R. L. Coffman. 2007. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. *Nat. Med.* 13: 552-559.

191. Iorio, R., P. Pensati, S. Botta, S. Moschella, N. Impagliazzo, P. Vajro, and A. Vegnente. 1997. Side effects of alpha-interferon therapy and impact on health-related quality of life in children with chronic viral hepatitis. *Pediatr. Infect. Dis. J.* 16: 984-990.

192. Okanoue, T., S. Sakamoto, Y. Itoh, M. Minami, K. Yasui, M. Sakamoto, K. Nishioji, T. Katagishi, Y. Nakagawa, H. Tada, Y. Sawa, M. Mizuno, K. Kagawa, and K. Kashima. 1996. Side effects of high-dose interferon therapy for chronic hepatitis C. *J. Hepatol.* 25: 283-291.

193. Zarubin, T. and J. Han. 2005. Activation and signaling of the p38 MAP kinase pathway. *Cell Res.* 15: 11-18.

194. Kumar, S., J. Boehm, and J. C. Lee. 2003. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat. Rev. Drug Discov.* 2: 717-726.

195. Akira, S. and K. Takeda. 2004. Toll-like receptor signalling. *Nat. Rev. Immunol.* 4: 499-511.

196. Patil, C., X. Zhu, C. Rossa Jr, Y. J. Kim, and K. L. Kirkwood. 2004. p38 MAPK regulates IL-1beta induced IL-6 expression through mRNA stability in osteoblasts. *Immunol. Invest.* 33: 213-233.

197. Winzen, R., M. Kracht, B. Ritter, A. Wilhelm, C. Y. Chen, A. B. Shyu, M. Muller, M. Gaestel, K. Resch, and H. Holtmann. 1999. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *EMBO J.* 18: 4969-4980.

198. Xu, C. Q., B. J. Liu, J. F. Wu, Y. C. Xu, X. H. Duan, Y. X. Cao, and J. C. Dong. 2010. Icariin attenuates LPS-induced acute inflammatory responses: involvement of PI3K/Akt and NF-kappaB signaling pathway. *Eur. J. Pharmacol.* 642: 146-153.

199. Guha, M. and N. Mackman. 2002. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J. Biol. Chem.* 277: 32124-32132.

200. Schabbauer, G., M. Tencati, B. Pedersen, R. Pawlinski, and N. Mackman. 2004. PI3K-Akt pathway suppresses coagulation and inflammation in endotoxemic mice. *Arterioscler. Thromb. Vasc. Biol.* 24: 1963-1969.

201. Fang, H., R. A. Pengal, X. Cao, L. P. Ganesan, M. D. Wewers, C. B. Marsh, and S. Tridandapani. 2004. Lipopolysaccharide-induced macrophage inflammatory response is regulated by SHIP. *J. Immunol.* 173: 360-366.

202. Reimold, A. M., J. Kim, R. Finberg, and L. H. Glimcher. 2001. Decreased immediate inflammatory gene induction in activating transcription factor-2 mutant mice. *Int. Immunol.* 13: 241-248.

203. Marin, V., C. Farnarier, S. Gres, S. Kaplanski, M. S. Su, C. A. Dinarello, and G. Kaplanski. 2001. The p38 mitogen-activated protein kinase pathway plays a critical role in thrombin-induced endothelial chemokine production and leukocyte recruitment. *Blood* 98: 667-673.

204. Tsai, E. Y., J. Jain, P. A. Pesavento, A. Rao, and A. E. Goldfeld. 1996. Tumor necrosis factor alpha gene regulation in activated T cells involves ATF-2/Jun and NFATp. *Mol. Cell. Biol.* 16: 459-467.

205. Dendorfer, U., P. Oettgen, and T. A. Libermann. 1994. Multiple regulatory elements in the interleukin-6 gene mediate induction by prostaglandins, cyclic AMP, and lipopolysaccharide. *Mol. Cell. Biol.* 14: 4443-4454.

206. Hirose, N., T. Maekawa, T. Shinagawa, and S. Ishii. 2009. ATF-2 regulates lipopolysaccharide-induced transcription in macrophage cells. *Biochem. Biophys. Res. Commun.* 385: 72-77.

207. Tobin, A. M. and B. Kirby. 2005. TNF alpha inhibitors in the treatment of psoriasis and psoriatic arthritis. *BioDrugs* 19: 47-57.

208. Feldmann, M. 2002. Development of anti-TNF therapy for rheumatoid arthritis. *Nat. Rev. Immunol.* 2: 364-371.

209. Campbell, L. A., T. Nosaka, M. E. Rosenfeld, K. Yaraei, and C. C. Kuo. 2005. Tumor necrosis factor alpha plays a role in the acceleration of atherosclerosis by Chlamydia pneumoniae in mice. *Infect. Immun.* 73: 3164-3165.

210. Tedgui, A. and Z. Mallat. 2006. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol. Rev.* 86: 515-581.

211. Waetzig, G. H., D. Seegert, P. Rosenstiel, S. Nikolaus, and S. Schreiber. 2002. p38 mitogen-activated protein kinase is activated and linked to TNF-alpha signaling in inflammatory bowel disease. *J. Immunol.* 168: 5342-5351.

212. Ishihara, K. and T. Hirano. 2002. IL-6 in autoimmune disease and chronic inflammatory proliferative disease. *Cytokine Growth Factor Rev.* 13: 357-368.

213. Sasayama, S., M. Okada, and A. Matsumori. 2000. Chemokines and cardiovascular diseases. *Cardiovasc. Res.* 45: 267-269.

214. Tarzami, S. T. 2011. Chemokines and inflammation in heart disease: adaptive or maladaptive? *Int. J. Clin. Exp. Med.* 4: 74-80.

215. Szekanecz, Z., J. Kim, and A. E. Koch. 2003. Chemokines and chemokine receptors in rheumatoid arthritis. *Semin. Immunol.* 15: 15-21.

216. Rittirsch, D., M. A. Flierl, and P. A. Ward. 2008. Harmful molecular mechanisms in sepsis. *Nat. Rev. Immunol.* 8: 776-787.

217. Fukumoto, K., I. Nagaoka, A. Yamataka, H. Kobayashi, T. Yanai, Y. Kato, and T. Miyano. 2005. Effect of antibacterial cathelicidin peptide CAP18/LL-37 on sepsis in neonatal rats. *Pediatr. Surg. Int.* 21: 20-24.

218. Giacometti, A., O. Cirioni, R. Ghiselli, F. Mocchegiani, G. D'Amato, R. Circo, F. Orlando, B. Skerlavaj, C. Silvestri, V. Saba, M. Zanetti, and G. Scalise. 2004. Cathelicidin peptide sheep myeloid antimicrobial peptide-29 prevents endotoxin-induced mortality in rat models of septic shock. *Am. J. Respir. Crit. Care Med.* 169: 187-194.

219. Giacometti, A., O. Cirioni, R. Ghiselli, C. Bergnach, F. Orlando, G. D'Amato, F. Mocchegiani, C. Silvestri, M. S. Del Prete, B. Skerlavaj, V. Saba, M. Zanetti, and G. Scalise. 2004. The antimicrobial peptide BMAP-28 reduces lethality in mouse models of staphylococcal sepsis. *Crit. Care Med.* 32: 2485-2490.

220. Jeon, Y. J., S. H. Han, Y. W. Lee, M. Lee, K. H. Yang, and H. M. Kim. 2000. Dexamethasone inhibits IL-1 beta gene expression in LPS-stimulated RAW 264.7 cells by blocking NF-kappa B/Rel and AP-1 activation. *Immunopharmacology* 48: 173-183.

221. Krappmann, D., E. Wegener, Y. Sunami, M. Esen, A. Thiel, B. Mordmuller, and C. Scheidereit. 2004. The IkappaB kinase complex and NF-kappaB act as master regulators of lipopolysaccharide-induced gene expression and control subordinate activation of AP-1. *Mol. Cell. Biol.* 24: 6488-6500.

222. Guha, M. and N. Mackman. 2001. LPS induction of gene expression in human monocytes. *Cell. Signal.* 13: 85-94.

223. Jiang, X., N. Takahashi, N. Matsui, T. Tetsuka, and T. Okamoto. 2003. The NF-kappa B activation in lymphotoxin beta receptor signaling depends on the phosphorylation of p65 at serine 536. *J. Biol. Chem.* 278: 919-926.

224. Sakurai, H., H. Chiba, H. Miyoshi, T. Sugita, and W. Toriumi. 1999. IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. *J. Biol. Chem.* 274: 30353-30356.

225. Lu, Y. C., W. C. Yeh, and P. S. Ohashi. 2008. LPS/TLR4 signal transduction pathway *Cytokine* 42: 145-151.

226. Gratton, J. P., M. Morales-Ruiz, Y. Kureishi, D. Fulton, K. Walsh, and W. C. Sessa. 2001. Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells *J. Biol. Chem.* 276: 30359-30365.

227. Kim, A. H., G. Khursigara, X. Sun, T. F. Franke, and M. V. Chao. 2001. Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1 *Mol. Cell. Biol.* 21: 893-901.

228. Sheppard, K. A., D. W. Rose, Z. K. Haque, R. Kurokawa, E. McInerney, S. Westin, D. Thanos, M. G. Rosenfeld, C. K. Glass, and T. Collins. 1999. Transcriptional activation by NF-kappaB requires multiple coactivators. *Mol. Cell. Biol.* 19: 6367-6378.

229. Martin, M., K. Rehani, R. S. Jope, and S. M. Michalek. 2005. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat. Immunol.* 6: 777-784.

230. Parry, G. C. and N. Mackman. 1997. Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-kappaB-mediated transcription. *J. Immunol.* 159: 5450-5456.

231. Huang, W. C., Y. S. Lin, C. Y. Wang, C. C. Tsai, H. C. Tseng, C. L. Chen, P. J. Lu, P. S. Chen, L. Qian, J. S. Hong, and C. F. Lin. 2009. Glycogen synthase kinase-3 negatively regulates anti-inflammatory interleukin-10 for lipopolysaccharide-induced iNOS/NO biosynthesis and RANTES production in microglial cells *Immunology* 128: e275-86.

232. Gustafsson, A., S. Sigel, and L. Ljunggren. 2010. The antimicrobial peptide LL37 and its truncated derivatives potentiates proinflammatory cytokine induction by lipoteichoic acid in whole blood. *Scand. J. Clin. Lab. Invest.* 70: 512-518.

233. Yang, L., R. M. Froio, T. E. Sciuto, A. M. Dvorak, R. Alon, and F. W. Luscinskas. 2005. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow *Blood* 106: 584-592.

234. Takahashi, M., U. Ikeda, J. Masuyama, S. Kitagawa, T. Kasahara, M. Saito, S. Kano, and K. Shimada. 1994. Involvement of adhesion molecules in human monocyte adhesion to and transmigration through endothelial cells in vitro *Atherosclerosis* 108: 73-81.

235. Issekutz, A. C., D. Rowter, and T. A. Springer. 1999. Role of ICAM-1 and ICAM-2 and alternate CD11/CD18 ligands in neutrophil transendothelial migration *J. Leukoc. Biol.* 65: 117-126.

236. Karpus, W. J., N. W. Lukacs, K. J. Kennedy, W. S. Smith, S. D. Hurst, and T. A. Barrett. 1997. Differential CC chemokine-induced enhancement of T helper cell cytokine production *J. Immunol.* 158: 4129-4136.

237. Locati, M., U. Deuschle, M. L. Massardi, F. O. Martinez, M. Sironi, S. Sozzani, T. Bartfai, and A. Mantovani. 2002. Analysis of the gene expression profile activated by the CC chemokine ligand 5/RANTES and by lipopolysaccharide in human monocytes *J. Immunol.* 168: 3557-3562.

238. Ali, H., R. M. Richardson, B. Haribabu, and R. Snyderman. 1999. Chemoattractant receptor cross-desensitization *J. Biol. Chem.* 274: 6027-6030.

239. Clahsen, T. and F. Schaper. 2008. Interleukin-6 acts in the fashion of a classical chemokine on monocytic cells by inducing integrin activation, cell adhesion, actin polymerization, chemotaxis, and transmigration. *J. Leukoc. Biol.* 84: 1521-1529.

240. Makela, S. M., M. Strengell, T. E. Pietila, P. Osterlund, and I. Julkunen. 2009. Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells *J. Leukoc. Biol.* 85: 664-672.

241. Sato, S., F. Nomura, T. Kawai, O. Takeuchi, P. F. Muhlradt, K. Takeda, and S. Akira. 2000. Synergy and cross-tolerance between toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways *J. Immunol.* 165: 7096-7101.

242. Yang, D., O. Chertov, and J. J. Oppenheim. 2001. Participation of mammalian defensins and cathelicidins in anti-microbial immunity: receptors and activities of human defensins and cathelicidin (LL-37). *J. Leukoc. Biol.* 69: 691-697.

243. Montreekachon, P., P. Chotjumlong, J. G. Bolscher, K. Nazmi, V. Reutrakul, and S. Krisanaprakornkit. 2011. Involvement of P2X(7) purinergic receptor and MEK1/2 in interleukin-8 up-regulation by LL-37 in human gingival fibroblasts. *J. Periodontal. Res.* 46: 327-337.

244. Tomasinsig, L., C. Pizzirani, B. Skerlavaj, P. Pellegatti, S. Gulinelli, A. Tossi, F. Di Virgilio, and M. Zanetti. 2008. The human cathelicidin LL-37 modulates the activities of the P2X7 receptor in a structure-dependent manner. *J. Biol. Chem.* 283: 30471-30481.

245. Funderburg, N., M. M. Lederman, Z. Feng, M. G. Drage, J. Jadlowsky, C. V. Harding, A. Weinberg, and S. F. Sieg. 2007. Human -defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. *Proc. Natl. Acad. Sci. U. S. A.* 104: 18631-18635.

246. Yin, J. and F. S. Yu. 2010. LL-37 via EGFR transactivation to promote high glucose-attenuated epithelial wound healing in organ-cultured corneas. *Invest. Ophthalmol. Vis. Sci.* 51: 1891-1897.

247. Biragyn, A., M. Coscia, K. Nagashima, M. Sanford, H. A. Young, and P. Olkhanud. 2008. Murine beta-defensin 2 promotes TLR-4/MyD88-mediated and NF-kappaB-dependent atypical death of APCs via activation of TNFR2. *J. Leukoc. Biol.* 83: 998-1008.

248. Turner-Brannen, E., K. Y. Choi, D. N. Lippert, J. P. Cortens, R. E. Hancock, H. El-Gabalawy, and N. Mookherjee. 2011. Modulation of interleukin-1beta-induced inflammatory responses by a synthetic cationic innate defence regulator peptide, IDR-1002, in synovial fibroblasts. *Arthritis Res. Ther.* 13: R129.

249. Stephens, L., C. Ellson, and P. Hawkins. 2002. Roles of PI3Ks in leukocyte chemotaxis and phagocytosis *Curr. Opin. Cell Biol.* 14: 203-213.

250. Koyasu, S. 2003. The role of PI3K in immune cells *Nat. Immunol.* 4: 313-319.

251. Okkenhaug, K. and B. Vanhaesebroeck. 2003. PI3K in lymphocyte development, differentiation and activation *Nat. Rev. Immunol.* 3: 317-330.

252. Dong, C., R. J. Davis, and R. A. Flavell. 2002. MAP kinases in the immune response *Annu. Rev. Immunol.* 20: 55-72.

253. Zhang, Y. L. and C. Dong. 2005. MAP kinases in immune responses *Cell. Mol. Immunol.* 2: 20-27.

254. Filewod, N. C., J. Pistolic, and R. E. Hancock. 2009. Low concentrations of LL-37 alter IL-8 production by keratinocytes and bronchial epithelial cells in response to proinflammatory stimuli. *FEMS Immunol. Med. Microbiol.* 56: 233-240.

255. Chen, X., F. Niyonsaba, H. Ushio, M. Hara, H. Yokoi, K. Matsumoto, H. Saito, I. Nagaoka, S. Ikeda, K. Okumura, and H. Ogawa. 2007. Antimicrobial peptides human beta-defensin (hBD)-3 and hBD-4 activate mast cells and increase skin vascular permeability. *Eur. J. Immunol.* 37: 434-444.

256. Yang, D., Q. Chen, O. Chertov, and J. J. Oppenheim. 2000. Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells. *J. Leukoc. Biol.* 68: 9-14.

257. Niyonsaba, F., H. Ushio, M. Hara, H. Yokoi, M. Tominaga, K. Takamori, N. Kajiwara, H. Saito, I. Nagaoka, H. Ogawa, and K. Okumura. 2010. Antimicrobial peptides human beta-defensins and cathelicidin LL-37 induce the secretion of a pruritogenic cytokine IL-31 by human mast cells. *J. Immunol.* 184: 3526-3534.