PHOSPHOINOSITIDE-BINDING MOLECULES AS POTENTIAL MULTIFUNCTIONAL ANTICANCER, ANTI-INFECTIVE AND IMMUNOMODULATORY THERAPEUTICS

Submitted by

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ABSTRACT

Multidrug resistance and adverse off-target toxicity of current therapeutics have urgently called for less toxic, more effective and multifaceted therapeutics with new modes of action. Furthermore, owing to the emerging pathological association between cancer and microbial infection, there is also expectation for combinatorial anticancer and anti-infective agents, especially for patients suffering infective agent-induced malignancies or immunocompromised anticancer treatments.

As phosphoinositides (PIPs), which are minor, yet functionally important, cellular lipids, and their metabolising enzymes crucially control multiple stages of malignant transformation and microbial infection, they offer invaluable opportunities for therapeutic targeting. Based on previous findings on a novel PIP-binding motif present in antifungal solanaceous plant defensins and on PIP-binding aminoglycosidic antibiotics, this thesis aimed to: (i) identify candidates that bind PIPs, particularly phosphatidylinositol 4,5-bisphopsphate [$PI(4,5)P_2$], and display antimicrobial as well as anticancer activity through aminoglycoside testing, computational search for plant defensin-like or HBD-2-like cationic patterns, and $PI(4,5)P_2$ bead pull-down using human hematological samples; (ii) investigate the role of PIPs and elucidate molecular mechanisms underlying the anticancer properties of a potential candidate, and (iii) determine the immunological effects, particularly ones governed by $PI(4,5)P_2$ signalling, of the selected candidate.

The study revealed an arsenal of potential PI(4,5)P2-binding proteins with speculative anticancer an antimicrobial activities. It also provides mechanistic and structural insights to lipid/$PI(4,5)P_2$ targeting on mammalian plasma membranes, particularly in PI(4,5)P2-mediated cancer cell permeabilisation. Furthermore, it enriches the knowledge of $PI(4,5)P_2$ and its signalling in immune response. Together, the knowledge acquired from this study would ultimately contribute to the exploration of PIP-binding molecules with anticancer, antimicrobial and immunomodulatory effects as a new generation of multifaceted therapeutics.

STATEMENT OF AUTHORSHIP

This thesis includes work by the author that has been published or accepted for publication as described in the text.

Except where reference is made in the text of the thesis, this thesis contains no other material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma.

No other person's work has been used without due acknowledgment in the main text of the thesis.

This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

All research procedures reported in the thesis were approved by the La Trobe University Human Research Ethics Committee (FHEC09/16) and the Australian Red Cross Blood Service Material Supply Agreement (14-11VIC-03).

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PUBLICATIONS

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Other publications

Atkin-Smith GK, Paone S, Zanker DJ, Duan M, **Phan TK**, Chen W, Hulett MD, Poon IK (2017) Isolation of cell type-specific apoptotic bodies by fluorescence-activated cell sorting. *Sci Rep* **7:** 39846.

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Oral presentations

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Poster presentations

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AWARDS

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Chapter 1

Combating pathogens and cancer by targeting phosphoinositides and their metabolism

1.1 Introduction

Cancer and infectious diseases are major causes of health and economic burdens in Australia (Australian Bureau of Statistics, 2012) and worldwide (World Health Organisation, 2012; http://www.who.int/mediacentre/factsheets/fs310/en/). They are commonly seen as distinct diseases that require different therapeutic approaches or treatment modalities. However, multiple pathological associations and similarities at the molecular, immunological and clinical levels of cancer and infectious diseases have recently been hypothesised or reported (Benharroch & Osyntsov, 2012; Glickman & Sawyers, 2012; Goldszmid *et al*, 2014). Pathogen infections have been identified as strong risk factors for specific cancers. Approximately 20% of new cancer cases in 2008 worldwide were attributable to infectious agents, predominantly viruses (**Table 1**; de Martel *et al*, 2012). Upon infection, viral oncogenicity can occur either directly via the insertion of viral oncogenes or by activating pre-existing proto-oncogenes in the host genome (Huebner & Todaro, 1969; Kim *et al*, 1991; Storey *et al*, 1998; Hu *et al*, 2004; Bouvard *et al*, 2009). Alternatively, this can occur indirectly via chronic inflammation, leading to the accumulation of mutations and hyperplasia (Levrero, 2006; Negash *et al*, 2013).

A number of bacterial and parasitic infections have been demonstrably linked to cancer. *Helicobacter pylori* potentially induces non-cardia gastric cancer and B-cell mucosa-associated lymphoid tissue (MALT) gastric lymphoma, probably via a mechanism involving chronic inflammation or direct action through bacterial virulence factors, such as cytotoxin-associated gene A (Hatakeyama & Higashi, 2005; Wundisch *et al*, 2005; Peter & Beglinger, 2007). A tentative association of *Chlamydophila psittaci* infection with MALT lymphoma of ocular adnexae has been proposed recently (Ferreri *et al*, 2009). Several case-control studies showed a high risk of cholangiocarcinoma linked to parasitic liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis* (Honjo *et al*, 2005; Choi *et al*, 2006). Schistosome-induced chronic inflammation upon infection with *Schistosoma haematobium* and *Schistosoma japonicum* may also initiate bladder and colorectal cancers, respectively (Ishii *et al*, 1994; Rosin *et al*, 1994).

Table 1.1 Global infection-related cancer incidences in 2008

Data represent number of new infection-related cancer cases (proportion of the total number of new cases attributed with a particular infectious agent. Numbers are rounded to two significant digits (adapted from de Martel *et al*, 2012).

Infectious agents	Associated cancer types	Cancer incidences
Hepatitis B and C viruses	Liver cancer, non-Hodgkin lymphomas	600,000 (5.3%)
Human papillomavirus	Cervix, penile, anal, vulvar, vaginal carcinomas	610,000 (5.4%)
Helicobacter pylori	Non-cardia gastric cancer, non-Hodgkin lymphoma of gastric region	660,000 (5.8%)
Epstein-Barr virus	Hodgkin's lymphoma, Burkitt's lymphoma, Nasopharyngeal carcinoma	110,000 (1.0%)
Human herpes virus type 8	Kapsosi's sarcoma, primary effusion lymphomas	43,000 (0.4%)
Human T-cell lymphotropic virus type 1	Adult T-cell leukaemia, lymphoma	2,100 (0.02%)
Opisthorchis viverrini Clonorchis sinensis	Cholangiocarcinoma	2,000 (0.02%)
Schistosoma haematobium	Bladder carcinoma	6,000 (0.05%)
Total		**2,000,000 (18.0%)**

Furthermore, infectious complications persistently remain a serious cause of morbidity and mortality in cancer patients, particularly those with blood malignancies, metastatic cancers and those who receive prolonged courses of chemotherapy, radiotherapy and immunotherapy or undergo stem cell transplants. Predisposing factors can be cancer-associated immunosuppression or treatment-induced adverse effects, to some extent, are likely to be accompanied with specific infectious agents (**Table 1.2**; Elting *et al*, 1997; Barnes & Stallard, 2001; Safdar & Armstrong, 2001; Uzun *et al*, 2001; Safdar *et al*, 2011; Baden *et al*, 2012; Zembower, 2014). Certain cancers cause specific immunity defects, thus increasing infection risk. Cellular immunodeficiency due to decreased leukocyte count (leukocytopenia), which includes neutropenia commonly observed in acute leukemia and non-Hodgkin's lymphoma as well as monocytopenia and defective T cell function in hairy cell leukemia, are frequently associated with bacterial (*Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Haemophilus influenzae, Mycobacterium* spp.), fungal (*Candida* spp., *Aspergillus* spp., *Cryptococcus neoformans*), protozoan (*Taxoplamsa gondii*, *Pneumocystis jirovecii*) and viral (herpes simplex virus (HSV), cytomegalovirus (CMV)) infections (Gerson *et al*, 1984; Tsiodras *et al*, 2000; Fassas *et al*, 2001; Audeh *et al*, 2003; Shaukat *et al*, 2005; Menichetti, 2010; Zembower, 2014). Patients with chronic lymphocytic leukemia or multiple lymphoma may suffer humoral immunity defects owing to non-functional γ-globulin (also known as hypogammaglobulinemia), predisposing them to infections with encapsulated bacteria

including *Streptococcus pneumoniae, H. influenzae* and *Neisseria meningitidis* (Tsiodras *et al*, 2000; Audeh *et al*, 2003; Wadhwa & Morrison, 2006; Damaj *et al*, 2009; Zembower, 2014). The immunosuppression caused by solid organ tumours may instead involve disruption of the mucosal barrier due to malignant invasion and mechanical obstruction, resulting in organ-specific infections such as staphylococci and steptrococci (skin), anaerobic bacteria and *H. influenzae* (oral cavity and nasopharynx), entrobacteriaceae (gastrointestinal tract), *Clostridium* spp. (female genital tract) and anatomical tract infections (Zembower, 2014 and references therein). Furthermore, cancer treatments have also been reported to increase the risk of infection such as meningitis and/or sepsis (craniotomy), encapsulated bacterial infections (splenectomy), CMV, *P. jirovecii* and *Aspergillus terreus* (radiotherapy for glioblastoma) and phlebitis, humoral immunity defect or zygomycosis (chemotherapeutic agents) (**Table 1.2**; Zembower, 2014 and references therein).

Current anticancer and anti-infective therapies are facing several key issues, not least the development of acquired multidrug resistance and adverse side effects (Cunha, 2001; Mader & Hoskin, 2006; Al-Benna *et al*, 2011; Bush *et al*, 2011). Of major concern is that antimicrobial resistance frequently poses a serious threat to cancer patients. A number of clinical reports have warned of increased rates of penicillin-resistant streptococci, vancomycin and daptomycin-resistant enterococci, methicillin-resistant *S. aureus*, fluoroquinolone-resistant *E. coli*, azole-resistant *Candida* spp. and, less commonly, prophylactic acyclovir and ganciclovir-resistant CMV and HPV, in patients suffering different malignancies and/or undergoing different treatments (Fan-Havard *et al*, 1991; Wingard *et al*, 1991; Tang & Shepp, 1992; Boken *et al*, 1993; Reusser, 2002; Bacon *et al*, 2003; Ghanem *et al*, 2007; Huang *et al*, 2007b; Shiomori *et al*, 2007; Cattaneo *et al*, 2008; Morris *et al*, 2008; Chemaly *et al*, 2010; Kamboj *et al*, 2010; Reish *et al*, 2013; Wudhikarn *et al*, 2013). It is therefore vital to simultaneously conduct cancer treatment and control microbial infections, achievable by combining anticancer and anti-infective therapies.

Table 1.2 Cancer and cancer treatment-associated opportunistic infections

Common associated infections are exemplarily showed for different cancer types and different treatment (adapted from Zembower, 2014).

Cancer-associated immunosuppression		
Cancer type	**Immunodeficiency**	**Opportunistic infection**
Acute leukemia Non-Hodgkin's lymphoma	Neutropenia Non-neutropenic cellular immunity	Bacteria (*S. aureus*, *Salmonella* spp., *E. coli*, *L. monocytogenes, H. influenza*) Fungi (*Aspergillus* spp., *Candida* spp.) Protozoa (*T. gondii*, *P. jirovecii*) Virus (HSV, CMV)
Hairy cell leukemia	Monocytopenia Defective T cell function	Bacteria (*Mycobacterium* spp., *Salmonella* spp., *L. monocytogenes*) Fungi (*Aspergillus* spp., *Candida* spp., *C. neoformans*) Virus (HSV, CMV)
Multiple lymphoma Lymphocytic leukaemia	Humoral immunity defect Hypogammaglobinemia	Bacteria (*S. pneumoniae, H. influenzae, N. meningitides*)
Solid tumours	Disruption of the mucosal barrier Mechanical obstruction	Skin (staphylococci, streptococci) Oral cavity and nasopharynx (anaerobic bacteria and *H. influenza*) Gastrointestinal tract (Entrobacteriaceae) Female genital tract (*Clostridium* spp.) Anatomical tract infection
Treatment-induced adverse effect		
Cancer type	**Cancer treatment**	**Opportunistic infection**
Solid tumours	Craniotomy	Meningitis and/or sepsis
	Paranasal sinuses	*Pseudomonas* meningitis
Lymphoma Leukaemia	Splenectomy	*S. pneumoniae, N. meningitides, H. influenzae*
Gynaecologic cancer	Pelvic irradiation	Genital condyloma
Glioblastoma	Chemo/radiotherapy	*P. jirovecii*, *Aspergillus terreus*, CMV
Various cancer types	Chemotherapy	Immunosuppression-associated infections (see above)

Pathogens and cancer cells are believed to share a number of molecular analogies, including, but are not limited to, mode of spread and dissemination as well as the association with immune system (Benharroch & Osyntsov, 2012). Although this hypothesis requires further investigations, together with the aforementioned pathological associations, it might imply common therapeutic and preventative approaches. Strikingly, some anticancer drugs, such as daunorubicin, doxorubicin, gallium and 5-fluorouracil, display antimicrobial activities and vice versa, possibly underlied by the similarities between cancer cells and pathogens (Rangel-Vega *et al*, 2015). In fact, a few therapeutics have been used in both anticancer and anti-infection treatment. Notable examples include adjuvant interferon therapy for hepatitis B virus (HBV) infection and HBV-associated hepatocellular carcinoma (Yang *et al*, 2016), and endostastin (an anti-angiogenic factor) for schistosomiasis and cancer neoangiogenesis/metastasis inhibition (Shariati *et al*, 2011).

This review focuses on phosphoinositides (PIPs), a family of minor, yet functionally essential, membrane phospholipids, which are exploited by both cancer cells and pathogens. It is tempting to propose another evidence of the molecular analogy between these two diseases, thereby suggesting PIPs as appropriate targets for combination therapy. The development of phosphoinositide-targeting molecules that exhibit dual antimicrobial and anticancer activities may represent a novel generation of combined anticancer and anti-infective treatments.

1.2 Distribution and metabolism of phosphoinositides

PIP is a phosphorylated phosphatidylinositol (PI), comprised of a diacylglycerol moiety linked to a D-myo-inositol ring via a phosphodiester linkage (**Figure 1.1A**). The inositol hydroxyls at positions 3, 4 or 5 are reversibly conjugated with phosphate groups, resulting in mono-phosphorylated (phosphatidylinositol 3-phosphate (PI(3)P), PI4P and PI5P), di-phosphorylated (phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$), $PI(3,5)P_2$ and $PI(4,5)P_2$) and tri-phosphorylated (phosphatidylinositol 3,4,5-trisphosphate, $PI(3,4,5)P_3$) derivatives. These seven interconvertible PIP species are distinctly distributed at the cytoplasmic side of the plasma and subcellular organelle membranes where they dynamically participate in various distinct cellular processes (**Figure 1.1B**). $PI(4,5)P_2$ and $PI(3,4,5)P_3$ are predominantly enriched at the inner membrane leaflet, particularly accumulated at sites of endocytosis (Di Paolo & De Camilli, 2006). PI(4)P is located along Golgi-endosomal trafficking axis, on exocytic vesicles and also at the plasma membrane (Dickson *et al*, 2014). On the other hand, 3-phosphate-containing PIPs (3-PIPs) are primarily associated with endosomal pathways: $PI(3)P_2$ on the limiting membrane of early endosomes and intraluminal vesicles contained within multivescular bodies (MVB), $PI(3,4)P_2$ on non-clathrin endocytic vesicles, and $PI(3,5)P_2$ at late endolysosomal membranes (Sbrissa *et al*, 2007; Cullen & Carlton, 2012). The precise location of PI(5)P pool(s) has not been comprehensively defined, although low levels of PI(5)P have been found in the nucleus, the plasma membrane, Golgi complex and sarco/endoplasmic reticulum (S/ER) (Sarkes & Rameh, 2010; Grainger *et al*, 2012).

Under certain physiological circumstances, PIPs can be synthesised at locations other than those aforementioned. For example, PI(3)P is detected in the ER during autophagosome formation (Axe *et al*, 2008). Outer plasma membrane PI(3)P is associated with oomycete and fungal internalisation through their effector proteins with the PI(3)P-binding RXLR motif (Kale *et al*, 2010). $PI(4,5)P_2$ has also been observed at sites of

membrane remodelling and ruffling, at nuclear mRNA splicing factor compartment (nuclear speckles), perinuclear vesicles, focal adhesion and epithelial cell-cell junctions (Doughman *et al*, 2003; Giudici *et al*, 2006; Ling *et al*, 2007; Mellman *et al*, 2008; van den Bout & Divecha, 2009; Gericke *et al*, 2013).

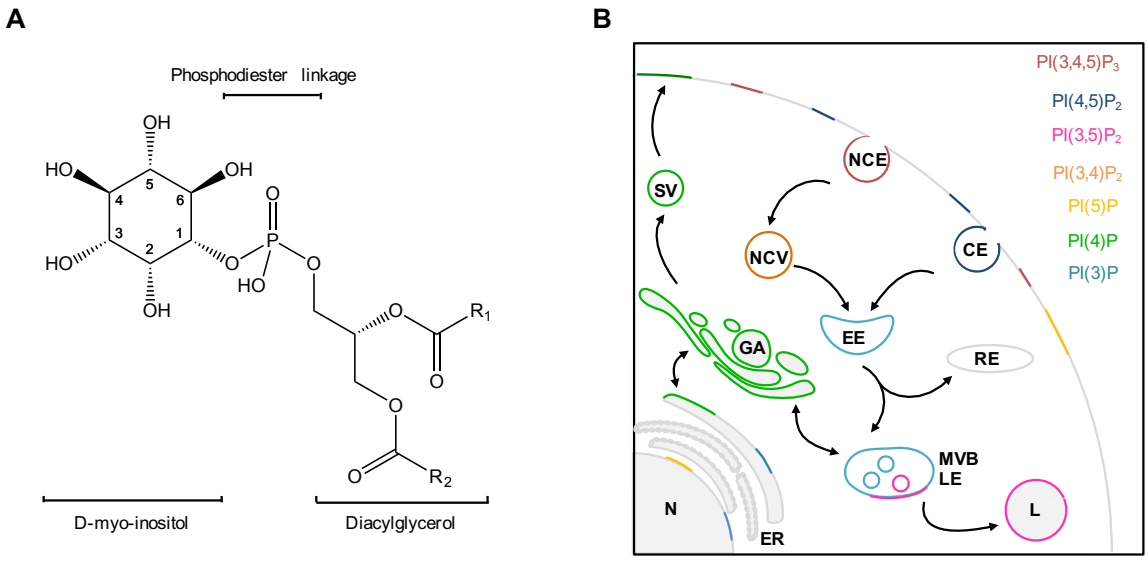

Figure 1.1 Cellular distribution of phosphoinositides

(A) Chemical structural of phosphatidylinositol, comprised of phosphodiester-linked diacylglycerol and D-myo-inositol moieties. R_1 and R_2 represent any acyl (fatty acid) chain. Hydroxyl at positions 3, 4 and 5 of D-myo-inositol ring are readily phosphorylated, giving rise to seven phosphoinositides. **(B)** Localisation of phosphoinositides on plasma and organelle membrane. This distribution map is exemplary, only showing the cellular location where a particular phosphoinositide species are prominently found. CE: clathrin-dependent endocytosis, NCE: clathrin-independent endocytosis, NEV: non-clathrin endocytic vesicles, EE: early endosomes, RE: recycling endosomes, MVB: multivesicular bodies, LE: late endosomes, L: lysosome, ER: endoplasmic reticulum, N: nucleus, GA: Golgi apparatus, SV: secretory vesicles. Black arrow (→) indicates the progression of membrane trafficking pathway.

PIP turnover at specific compartmentalised distributions is dynamically, but tightly, regulated by specific kinases and antagonistic phosphatases which add or remove phosphates from the 3, 4 or 5 positions of the *myo*-inositol ring (**Figure 1.2**). This critically allows for the rapid generation of product PIPs or removal of the precursor PIPs (Toker, 1998; Cremona *et al*, 1999; Rameh & Cantley, 1999; Di Paolo & De Camilli, 2006; Robinson & Dixon, 2006; Sbrissa *et al*, 2007; Cao *et al*, 2008; Cullen & Carlton, 2012; Grainger *et al*, 2012; Balla, 2013). Typically, these enzymes are catalytically selective for specific PIP species, and generally exist as different isoforms, despite similar catalytic activity, with isoform-specific functions and non-overlapping localisation. For

instance, $PI(4,5)P_2$ is synthesised chiefly by phosphorylation of PI(4)P by either of three PI(4)P 5-kinases (PIP5K) isoforms (α, β and γ). These PIP5Ks are mainly localised on the plasma membrane, although distinct additional subcellular distributions have also been observed. PIP5Kα localises at sites of membrane remodelling such as lamellipodia and nascent phagosomes, at sites of membrane ruffling and at nuclear speckles (Doughman *et al*, 2003; Mellman *et al*, 2008; van den Bout & Divecha, 2009). PIP5Kβ is enriched at perinuclear vesicles, and at least one spliced form of PIP5Kγ can be found at focal adhesion and epithelial cell-cell junctions (Giudici *et al*, 2006; Ling *et al*, 2007; van den Bout & Divecha, 2009; Gericke *et al*, 2013). $PI(4,5)P_2$ is alternatively produced by phosphatidylinositol 5-phosphate 4-kinases (PIP4K)-catalysed phosphorylation of PI(5)P or phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-mediated dephosphorylation of $PI(3,4,5)P_3$. However, due to the remarkably low PI(5)P level and rapid $PI(3,4,5)P_3$ turnover upon synthesis, these metabolising pathways are unlikely to functionally contribute to generation of the $PI(4,5)P_2$ pool, but respectively act instead in PI(5)P and $PI(3,4,5)P_3$ regulation or modulation (Vazquez & Devreotes, 2006; Ramel *et al*, 2011; Clarke & Irvine, 2012; Gericke *et al*, 2013).

Similarly, four mammalian PI4K isozymes, the type II (PI4KIIα and β) and the type III (PI4KIIIα and β) have distinct distribution, functions and regulations (Boura & Nencka, 2015; Dornan *et al*, 2016). The PI4KIIα, which is responsible for approximately 50% of PI(4)P production, mainly localises on the Golgi and endosomes and regulates Golgi-endosomal trafficking, Wnt3a signalling and proangiogenic vascular endothelial growth factor signalling (Wang *et al*, 2007a; Craige *et al*, 2008; Pan *et al*, 2008; Qin *et al*, 2009; Li *et al*, 2010a; Jovic *et al*, 2014). The plasma membrane-bound PI4KIIβ, although limitedly-characterised, is known to be activated by platelet-derived growth factor and to play a role in early T cell activation (Wei *et al*, 2002; Sinha *et al*, 2013). The PI4KIIIα is critical in governing ER export and production of the PI(4)P pool at the plasma membrane for $PI(4,5)P_2$ and $PI(3,4,5)P_3$ synthesis (Blumental-Perry *et al*, 2006; Balla *et al*, 2008). The PI4IIIKβ is also a Golgi network enzyme, essential for Golgi formation and function, and also for cytokinesis, lysosomal identity and ceramide synthesis (Brill *et al*, 2000; Toth *et al*, 2006; Sridhar *et al*, 2013; Dornan *et al*, 2016).

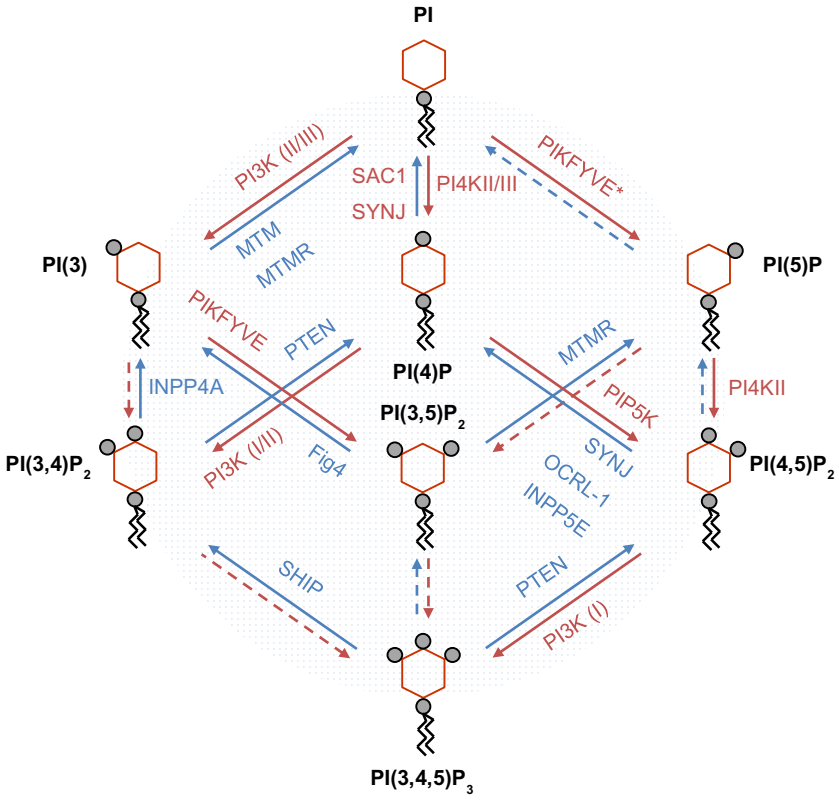

Figure 1.2 Metabolism of mammalian phosphoinositides

Generation (→) and turnover (→) of PIPs are achieved by the action of kinases and phosphatases. Multiple metabolism pathways can exist for particular PIP synthesis, though not necessarily contribute toward its cellular pool(s) but rather act as substrate PIP removal. In some instances, enzymatic conversion was only confirmed *in vitro*, as indicated by an asterisk (*). Metabolising enzymes for certain conversions, remarked by dashed arrows, have not been identified. PI3K: PIP 3-kinases, MTM: myotubularin family, MTMR: myotubularin-related protein family, PI4K: PIP 4-kinases, SAC1: suppressor of actin 1, SYNJ: Synaptojanins, PIKFYVE: FYVE-finger containing PIP kinase, INPP4A: inositol polyphosphate 4-phosphatase, Fig4: factor-induced gene 4, PTEN: phosphatase and tensin homologue deleted on chromosome 10, PIP5K: PIP 5-kinases, OCRL-1: oculocerebrorenal syndrome protein, INPP5E: inositol polyphosphate 5-phosphatase, SHIP: Src homology 2 domain-containing inositol phosphatase. Roman numerals (I, II and III) represent different classes/types of PI3Ks or PI4Ks.

These PIP metabolising kinases and phosphatases are also responsive to stimuli or changes in the cellular states such as cell cycle. For example, the majority PI(3)P is constitutively produced by the class III phosphoinositide 3-kinase (Vps34) from PI at the endocytic membranes, but is also inducibly synthesised at inner plasma membrane upon insulin stimulation (Gillooly *et al*, 2000; Maffucci *et al*, 2003). Together, spatially restricting and temporally governing the PIP metabolising enzymes are critical for PIP metabolic turnover at dedicated membrane compartments and to a particular stimulation.

1.3 Physiological functions of phosphoinositides

Despite their low abundance, PIPs and PIP metabolism by a number of PIP kinases, phosphatases and lipases are crucial to the precise spatiotemporal regulations of key cellular processes, such as signalling transduction, cytoskeleton reorganisation, membrane dynamics, cell migration, membrane trafficking, programmed cell death, ion channel activities, in which PIPs can serve as a source of second messengers or regulate membrane proteins via membrane recruitment or activity modulation (Di Paolo & De Camilli, 2006; Falkenburger *et al*, 2010). Typically, PIPs interacts with a number of PIP-binding domains (**Table 1.3**), with a cluster of basic amino acids (arginine and lysine) that provides direct contacts to the lipid phosphate groups.

1.3.1 Second messenger

Initial research into PIPs were mainly focused on their role as second messengers of transmembrane signalling. $PI(4,5)P_2$, in particular, is an essential intermediate for two important pathways: PIP-specific phospholipase C (PLC)/diacylglycerol (DAG)/inositol-1,4,5-trisphosphate (IP_3) pathway and class I PI3Ks/protein kinase B (Akt) pathway (**Figure 1.3**). Typically, stimulation of G protein-coupled receptors and receptor tyrosine kinases leads to activation of PLC, that via its $PI(4,5)P_2$-binding Pleckstrin homology (PH) domain, hydrolyses $PI(4,5)P_2$ to generate DAG and IP_3 and thus initiating two-armed intracellular signalling cascade (Essen *et al*, 1997). DAG remains at the plasma membrane to facilitate activation and membrane localisation of protein kinase C (PKC) family members, whereas cytosolic IP_3 binds the IP_3 receptor and activates a ligand-gated Ca^{2+} channel on smooth ER surface, triggering Ca^{2+} release from intracellular storage (Berridge & Irvine, 1984; Meldrum *et al*, 1991; Katan, 1996). On the other hand, phosphorylation of $PI(4,5)P_2$ by PI3K yields $PI(3,4,5)P_3$, an essential effector of Akt signalling (Rameh & Cantley, 1999). The PI3K/Akt signalling cascade is activated by B and T cell receptors, epidermal growth factor receptor (EGFR), cytokine receptors, receptor tyrosine kinases, small Ras GTPase and other PI3K-inducing stimuli.

Table 1.3 PIP-binding domains

Exemplary PIP-binding domains that are mentioned in this review are listed and described. PH (Pleckstrin homology), FERM (band 4.1-ezrin-radixin-moesin), ENTH (epsin N-terminal homology), ANTH (AP180 N-terminal homology), BAR (Bin-amphiphysin-Rvs), FYVE (Fab1-YOTB-Vac1-EEA1), PX (phox homology).

Domain	Specificity	Description	Example	Reference
PH	PI(4)P	Comprised of a seven-stranded β-barrel (by two perpendicular antiparallel β-sheets), with an PIP binding site formed by three variable loops and a C-terminal α-helix at either open ends.	FAPP1/2	Godi *et al* (2004)
	$PI(4,5)P_2$		PLCδ	Di Paolo *et al* (2006)
			Dynamin	Bethoney *et al* (2009)
	$PI(3,4,5)P_2$		Akt	Hemmings & Restuccia (2012)
FERM	$PI(4,5)P_2$	Consists of three globular lobes (F1, F2 and F3) that resemble a ubiquitin fold, an acyl-coA binding domain and a PH-phosphotyrosine (PH-PTB) fold respectively. $PI(4,5)P_2$ binding does not occur at the PH-fold lobe, but rather at F1 and F2 interface.	ERM	Hamada *et al* (2000)
			PTPL1	Abaan *et al* (2008)
ENTH	$PI(4,5)P_2$	Composed of nine α-helices (α0-8), six of which forms three consecutively-stacked helical hairpins (α1-2, α3-4 and α6-7). Upon $PI(4,5)P_2$ contact the distorted amphipathic extension at N-terminal structures into an α-helix (α0), forming the lipid binding pocket. Basic residues from surrounding helices also contribute to coordinate $PI(4,5)P_2$ head groups. The structured α0 also enable its membrane insertion, hence membrane curvature formation.	Epsins	De Camilli *et al* (2002) Ford *et al* (2002)
ANTH	$PI(4,5)P_2$	Similar to ENTH, but binds to $PI(4,5)P_2$ weakly through completely different residues, the conserved K-X(9)-K-X-(KR)(HY)) sequence, without membrane penetration.	AP180	Stahelin *et al* (2003)
BAR	$PI(4,5)P_2$	Features a 'banana-shaped' coiled coil formed by three extended α-helices, with a cluster of basic residues at the concave face for lipid binding.	Amphiphysin 2 (BIN1 variant)	Picas *et al* (2014) Gallop & McMahon (2005)
FYVE	PI(3)P	Consists of two double-stranded antiparallel β-sheets and a C-terminal α-helix, containing three defined motifs W-X-X-D, R-(RK)-H-H-R and RVC that form a PI(3)P binding site.	Vps34	Backer (2008)
			MTMRs	Cao *et al* (2007)
			PIKFYVE	Rutherford (2006)
			EEA1	Simonsen *et al* (1998)
			Hrs	Raiborg *et al* (2001)
			Syntaxins	McBride *et al* (1999)
PX	PI(3)P	Comprised of a triple-stranded β-sheet and a triple/quadruple helical subdomain, with three motifs essential for PI(3)P binding R-R-Y-X(2)-F-X(2)-L-X(3)-L, P-X(2)-P-X-K and R-(RK)-X(2)-L.	$p40^{phox}$	Ellson *et al* (2001)
			$p47^{phox}$	Zhan *et al* (2002)

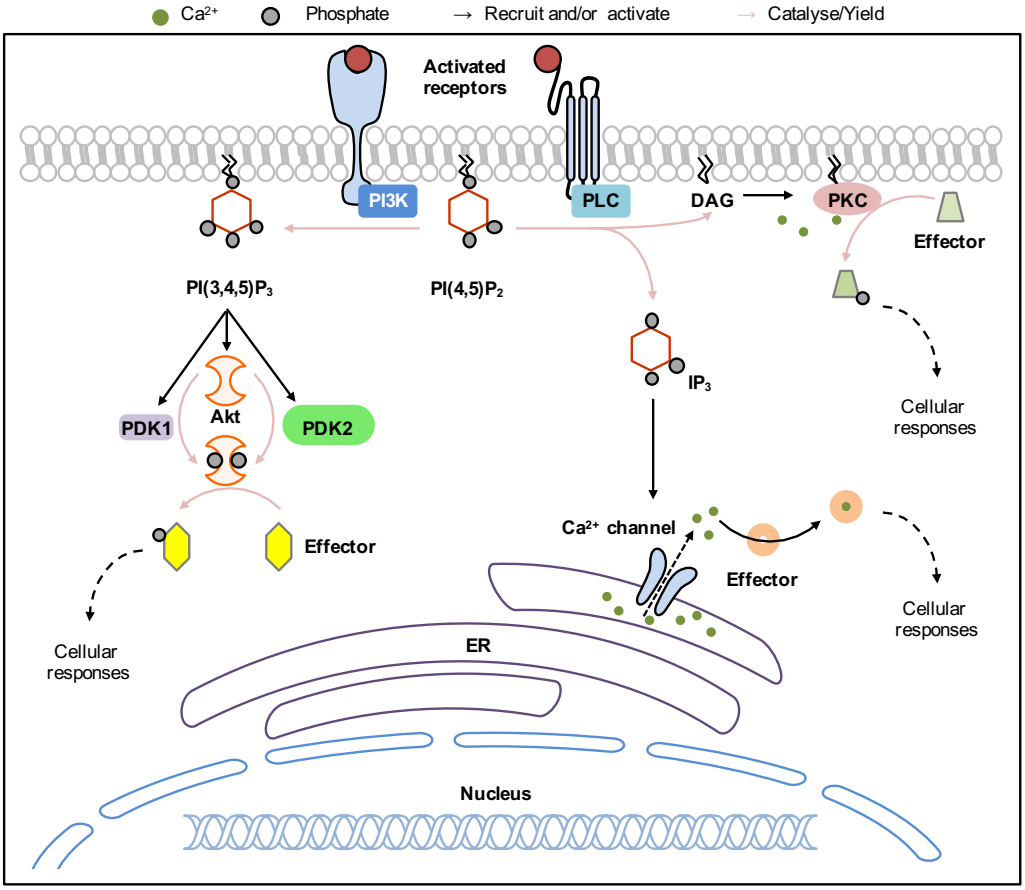

Figure 1.3 PI(4,5)P_2 as a second messenger

PI(4,5)P_2 mediates two major transmembrane signalling pathways: PLC-DAG-IP_3 and PI3K-Akt. Activated receptor recruits and induce class I PI3K to phosphorylate PI(4,5)P_2. The resultant PI(3,4,5)P_3 then recruits Akt and its activator kinases PDK1 and PDK2 (or mTORC2, ILK) can also to the plasma membrane, leading to complete phosphorylation of Akt, Subsequently, Akt regulates downstream effectors through its activated kinase activity. PLC, on the other hand, cleaves PI(4,5)P_2 into DAG and IP_3 upon stimulation. DAG remains at the plasma membrane to recruit and activate PKC to modulate its downstream effectors via its regulatory phosphorylation. IP_3 translocates to ER to excite Ca^{2+} channels, causing Ca^{2+} flux into cytoplasm, initiating Ca^{2+} signalling via Ca^{2+} binding effectors. PI3K: PIP 3-kinases, PLC: PIP-specific phospholipase C, DAG: diacylglycerol, IP_3: inositol 1,4,5-trisphosphate, PKC: protein kinase C, Akt: protein kinase B, PDK1: PIP-dependent kinase 1, PDK2: PIP-independent kinase 2, ER: endoplasmic reticulum, mTORC2: mechanistic target of rapamycin complex 2, ILK: integrin-linked kinase.

The resultant PI(3,4,5)P_3 promotes translocation of phosphoinositide-dependent kinase 1 (PDK1) and Akt to the plasma membrane, leading to Akt phosphorylation of threonine-308 and its partial activation (Rameh & Cantley, 1999). Complete Akt activation requires additional phosphorylation of serine-473, achievable by multiple proteins, including phosphoinositide-dependent kinase 2 (PDK2), mechanistic target of rapamycin complex (mTORC), integrin-linked kinase (ILK) or DNA-dependent protein kinase (DNA-PK) (Osaki *et al*, 2004; Hemmings & Restuccia, 2012). Subsequently, downstream

transduction of DAG/PKC, IP_3/Ca^{2+} and Akt signalling indispensably orchestrate many cellular and physiological processes including, but are not limited to, cell proliferation, cell growth and survival, cell division, cell migration, autophagy, immune response, muscle contraction, memory and learning (Katan, 1998; Kane *et al*, 1999; Chen & Lin, 2001; Dai *et al*, 2006; Rajaram *et al*, 2006; Cain & Ridley, 2009; Heras-Sandoval *et al*, 2014; Yu & Cui, 2016).

1.3.2 Regulators of cellular membrane-associated processes

Since the initial interest in PIP transmembrane signal transduction, PIP research has been intensively developed. PIPs have been demonstrated to regulate membrane localisation and activities of various key proteins of interplaying membrane-associated cellular functions. Notably, these include actin cytoskeleton remodelling, membrane remodelling, cell polarity and motility, membrane and vesicular trafficking processes (*e.g.* endocytosis, phagocytosis, macropinocytosis and exocytosis), and programmed cell death (apoptosis, autophagy, pyroptosis and necroptosis).

1.3.2.1 *Actin cytoskeleton remodelling*

Membrane-associated and actin-dependent motile, trafficking and morphogenetic processes are indispensably driven by actin cytoskeleton remodelling, characterised by precisely coordinated membrane detachment/attachment and actin polymerisation/ depolymerisation of actin filaments. The structure and dynamics of the cellular actin cytoskeleton are controlled by a number of actin-binding proteins (**Figure 1.4**) including: (i) regulators of filamentous polymeric (F) actin assembly and disassembly such as nucleators (*e.g.* actin-related proteins Arp2/3 complex and Wiskott-Aldrich syndrome protein WASP, formins), rulers and stabilisers (*e.g.* tropomyosin, nebulin, calponin), capping and severing proteins (*e.g.* capZ, scinderin, villin, gelsolin, fragmin, actin depolymersing factor ADF/cofilin), nucleotide exchangers (*e.g.* profilin) and actin monomer delivery (*e.g.* twinfilin, verprolin); (ii) regulators of higher-order F-actin structures including actin-bundling and crosslinking proteins (*e.g.* α-actinin, espin, filamin, transgelin); (iii) mechanical framework proteins such as myosin, actin-microtubules/actin-intermediate filament linkers (*e.g.* spectrin, plectin, tau) and actin-membrane anchors (*e.g.* dystrophin, utrophin, talin, viculin, annexins, ezrin-radixin-moesin ERM family) (Winder & Ayscough, 2005).

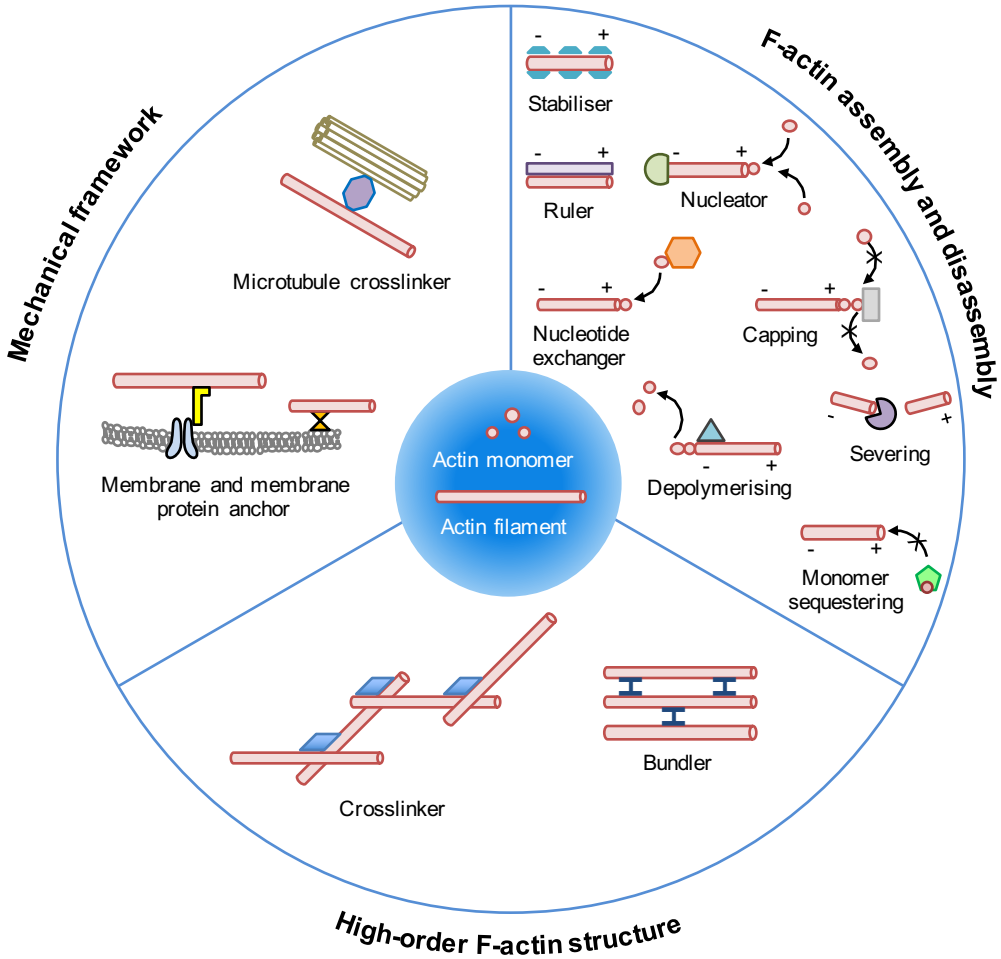

Figure 1.4 Three classes of actin binding proteins involved in regulation of structure and dynamics of actin cytoskeleton

F-actin assembly is typically initiated by nucleators that bind either to slow-growing (minus) end (*e.g.* WASP, Arp2/3) or fast-growing (plus) end (*e.g.* formin). Nuclear exchanger (*e.g.* profilin) binds actin monomer and accelerates filament elongation. Filament stabilisers and rulers (*e.g.* tropomyosin, calponin) promote stabilise and determine the filament length. By contrast, depolymerising proteins, such as actin depolymerising factor ADF/cofilin, induces actin assembly at minus end, monomer sequestering proteins (*e.g.* thymosin) bind actin monomer subunit to prevents polymerisation at plus end. Capping proteins (*e.g.* capZ) prevents both assembly and disassembly at plus end. Severing proteins (*e.g.* gelsolun) sever filaments and remains associated with newly-formed plus end. Regulators of higher-order F-actin structures, including crosslinkers (*e.g.* filamin) and bundlers (*e.g.* α-actinin, fimbrin), faciliate actin filamin crosslinking and bundling. Mechanical framework proteins (*e.g.* spectrin and ERM) provide membrane and microtubule anchoring.

The plasma membrane PIPs, especially PI(4,5)P_2 and PI(3,4,5)P_3, spatiotemporal control the organisation and dynamics of the cortical actin cytoskeleton by mediating signal transduction events. Apart from the aforementioned PIP signalling, the actin cytoskeleton remodelling and actin-binding proteins are also controlled by various master regulators, among which are small GTPases (**Figure 1.5**). The Rho-family small GTPases (*e.g.* Rac1,

Cdc42), are in turn regulated by $PI(3,4,5)P_3$-recruited guanine nucleotide exchange factors (GEFs) in formation of focal adhesion complexes, extension of migratory pseudopods, or specialised actin filament assembly such as stress fibres (Benard *et al*, 1999; Egami *et al*, 2014). The GTP-binding protein ADP ribosylation factor (Arf) family GTPases, such as Arf1, whose activities are often indispensably linked to the actin cytoskeleton, requires direct $PI(4,5)P_2$ binding to mediate its interaction with GTPase activating proteins (GAPs) and membrane-associated phospholipases D (PLD), leading to PLD activation (Brown *et al*, 1993; Liscovitch *et al*, 1994; Randazzo & Kahn, 1994). Activated PLD cleaves phosphatidylcholine to produce phosphatidic acid (PA), another vital membrane lipid. Vice versa, PA directly and potently activates $PI(4,5)P_2$-generating enzymes PIP5Ks. The reciprocal stimulation of PLD and PIP5Ks is believed to form a rapid positive feed-forward loop (**Figure 1.5**) for a localised and acute generation of functionally important PA and $PI(4,5)P_2$ to further influence actin remodelling and many other cellular functions (Jenkins *et al*, 1994; Divecha *et al*, 2000; Jones *et al*, 2000; Skippen *et al*, 2002). In addition, activated PLD and PLD-derived PA can facilitate GTP loading of Rac, since PLD atypically displays GEF function whilst PA can also promote membrane recruitment of unconventional GEFs (Oude Weernink *et al*, 2007; Mahankali *et al*, 2011).

Furthermore, $PI(3,4,5)P_3$, and the more rigorously characterised $PI(4,5)P_2$, are also considered as direct positive regulators of actin filament formation, by directly interacting with actin-binding proteins at cellular and subcellular organelle membranes in a local concentration-dependent manner (Saarikangas *et al*, 2010; Egami *et al*, 2014). Typically, $PI(4,5)P_2$ binding leads to conformational change-associated activation of proteins that are involved in promoting actin filament assembly (nucleators, rulers and stabilisers) and membrane anchoring while inhibits bundlers, crosslinkers, capping and severing proteins as well as other actin disassembly inducers such as profilin (Figure 1.5; Hilpela *et al*, 2004; Saarikangas *et al*, 2010). The $PI(4,5)P_2$:actin-binding protein interactions are generally mediated through canonical $PI(4,5)P_2$ binding domains, such as Pleckstrin homology (PH) and band 4.1-ezrin-radixin-moesin (FERM), or defined basic amino acid-rich clusters/motifs on their sequences (Toker, 1998; Kabachinski *et al*, 2014).

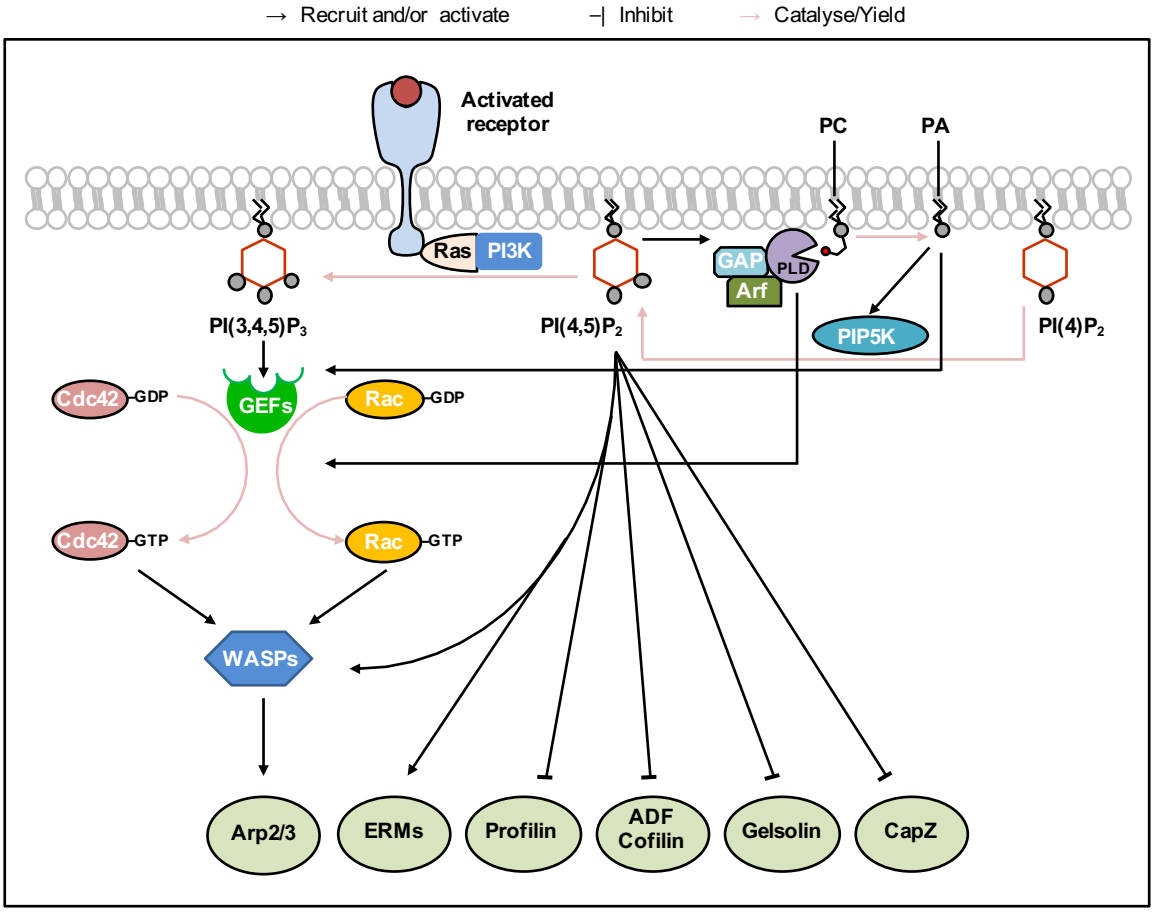

Figure 1.5 Regulation of actin remodelling by PIPs

$PI(3,4,5)P_2$, synthesised from $PI(4,5)P_2$ upon receptor-mediated stimulation of PI3K via Ras GTPase, recruits GEFs to plasma membrane. GEFs then facilitate GTP loading on Rho family GTPases Cdc42 and Rac, leading to activation of actin nucleators WASPs and subsequently, Arp2/3 complex. Furthermore, $PI(4,5)P_2$ promotes interaction of Arf GTPase, GAP and PLD, inducing PLD to cleave PC to PA which in turn activates $PI(4,5)P_2$-producing PI5Ks. As a result, a rapid positive feed-forward loop is formed for acute generation of PA and $PI(4,5)P_2$. Activated PLD and PLD-derived PA can facilitate GTP loading of Rac, since PLD atypically displays GEF function whilst PA can also promote membrane recruitment of unconventional GEFs. PI3K: PIP 3-kinases, PIP5Ks: PIP 5-kinases, Ras: Rat sarcoma GTPase, Cdc42: cell division control protein 42 homologue, Rac: Ras-related C3 botulinum toxin substrate , GEF: guanine nucleotide exchange factor, WASP: Wiskott-Aldrich syndrome protein, Arp2/3: actin-related proteins 2/3 complex, ERM: erzin, radixin and moesin, ADF: actin depolymerising factor, GAP: GTPase activating proteins, Arf: ADP ribosylation factor, PLD: phospholipase D, PC: phosphatidylcholine, PA: phosphatidic acid.

For instance, ERM family proteins, which directly connect the actin cytoskeleton to the plasma membrane, characteristically contain an N-terminal FERM domain and C-terminal actin-binding site. An interaction between these two domains effectively establishes autoinhibition, rendering these proteins inactive. The subsequent binding of $PI(4,5)P_2$ to the FERM domain leads to their activation by exposing the actin-binding site

(Matsui *et al*, 1999; Barret *et al*, 2000). On the other hand, gelsolin binds $PI(4,5)P_2$ through two basic amino-acid rich regions which also overlap with actin-binding sites, resulting in the blocking of its actin binding, and thus actin-severing and filament end-capping activity (Janmey & Stossel, 1987; Yu *et al*, 1992).

1.3.2.2 *Membrane dynamics*

Dynamic membrane remodelling and generation of membrane curvatures are critically essential for a number of cellular processes such as cell division and vesicular biogenesis for membrane trafficking. PIP (mainly $PI(4,5)P_2$ and PI(4)P) binding activity of certain number of actin-associated proteins, such as Bin-Amphiphysin-Rvs (BAR) domain superfamily, epsin/ATP180 NH_2-terminal homology (ENTH/ANTH) domain family and dynamin, are able to directly influence membrane remodelling including membrane protrusions or invaginations by manipulating PIP-rich membranes (Gallop & McMahon, 2005; McMahon & Gallop, 2005; Lemmon, 2008). The binding of BAR domain-containing proteins, such as amphiphysin 2, to PIPs promotes their membrane binding and bending to create rigid scaffolds, and affects the degree of intrinsic curvature and mechanistic variability (Gallop & McMahon, 2005; Frost *et al*, 2008; Lemmon, 2008; Wang *et al*, 2008). Similarly, PIP binding via ENTH domains also enables membrane interactions of epsins as well as induce folding of distorted N-terminal extension into an amphipathic α-helix, an important structure for ENTH-mediated membrane bending/deformation (Itoh *et al*, 2001; Ford *et al*, 2002). The interaction between dynamin and $PI(4,5)P_2$ through its PH domain not only allow its membrane translocation, but also to mediate membrane scission via $PI(4,5)P_2$ clustering (Bethoney *et al*, 2009).

1.3.2.3 *Cell polarity and cell migration*

Cell polarity, referring to the asymmetry of cellular morphology, structures and functions, is a prerequisite establishment for many specialised functions, including neuronal synaptic activity, cell differentiation, proliferation, cytokinesis and migration. PIPs, especially $PI(4,5)P_2$ and $PI(3,4,5)P_3$, and their spatial distribution are particularly crucial for initiation and maintenance of cell polarity via recruitment and regulation of downstream proteins with PIP-binding domains. In particular, not only do $PI(4,5)P_2$ and $PI(3,4,5)P_3$ regulate the actin cytoskeleton remodelling in directional cell migration (chemotaxis), but their gradient also directs cell movement via polarised formation of leading and trailing edges (Saarikangas *et al*, 2010. Stimulation of chemotactic receptors induces acute PI3K-generated $PI(3,4,5)P_3$ synthesis at the leading edge, and $PI(3,4,5)P_3$

degradation at the trailing edge by local PTEN activity (Saarikangas, 2010). However, Src homology 2 (SH2) domain-containing inositol phosphatase (SHIP) has recently showed to outperform PTEN in generating PI(3,4,5)P_3 gradient across cytoplasm to trailing edge (Nishio *et al*, 2007; Harris *et al*, 2011). Indeed, inhibition of SHIP activity severely disrupt cell polarity and asymmetric actin filament assembly, hence impairing chemotaxis (Nishio *et al*, 2007), whereas PTEN disruption only affects the speed, but not directionality, of cell movement (Lacalle *et al*, 2004; Heit *et al*, 2008). At the leading edge, PI(3,4,5)P_3 activates Rho-family GTPases (Rac1 and Cdc42), which in turn induces Arp2/3-mediated F-actin polymerisation and resultant pseudopod extension (filopodia or lamellipodia) (Benard *et al*, 1999; Le Clainche & Carlier, 2008; Suraneni *et al*, 2012). Furthermore, local PI(4,5)P_2 enrichment at the trailing end by PIP5Kβ and γ potentially stimulate actin contraction and rear retraction via activation of ERM proteins and Rho-family GTPase RhoA (Lacalle *et al*, 2004; Lokuta *et al*, 2007). Intriguingly, though preliminary, multiple lines of evidence has suggested PI(5)P, generated from PI by sequential actions of class III PI3K, PIKFYVE and MTMR3, as a direct regulator of fibroblast migration upon growth factor stimulation (Haugsten *et al*, 2013; Oppelt *et al*, 2013). Further studies are required to identify PI(5)P-binding effector proteins and to determine the distribution of PI(5)P during cell migration.

1.3.2.4 *Membrane trafficking*

Membrane trafficking is critically responsible for the uptake (endocytosis), release (exocytosis) and distribution (biosynthetic trafficking) of cargo biomolecules to various intracellular or extracellular destinations in membrane-bound compartments or vesicles. It is a fundamental event that maintains homeostasis and facilitates signalling perception, transduction and execution. PIPs, at their exclusive subcellular locations, serve as intrinsic and compartmentalised membrane signals that govern different aspects of membrane trafficking. In general, PIPs are distributed along two spatiotemporal regulated transport axes: PI(4,5)P_2 and 3-phosphate-containing-PIPs (3'-PIPs) along endocytic pathway and 4-phosphate-containing-PIPs (4'-PIPs) along Golgi complex-associated pathway.

Though membrane trafficking is an actin-dependent process, the role of PI(3,4,5)P_3 and PI(4,5)P_2, is far beyond that of classical second messengers and regulators of actin cytoskeleton remodelling. In fact, both phospholipids are directly involved in regulating the formation of endocytic vesicles, including clathrin-coated vesicles (**Figure 1.6**),

phagosomes and macropinosomes. During the early steps of clathrin-mediated endocytosis, many adaptor proteins, including AP2 and AP180 (Gaidarov & Keen, 1999; Ford *et al*, 2001; Sun *et al*, 2005b), induce the recruitment and assembly of clathrin lattice on the plasma membrane upon $PI(4,5)P_2$ binding. Membrane-deforming proteins, such as epsin, BAR-domain containing proteins, including amphiphysin, and dynamin, also through indispensable interactions with $PI(4,5)P_2$, are subsequently recruited to aid the membrane invagination, vesicle scission and Arp2/3-mediated actin polymerisation machinery, ultimately driving the internalisation of clathrin-coated vesicles (Wigge *et al*, 1997a; Wigge *et al*, 1997b; Itoh *et al*, 2001; Ford *et al*, 2002; Gallop & McMahon, 2005; Frost *et al*, 2008; Lemmon, 2008; Wang *et al*, 2008; Bethoney *et al*, 2009). Once internalised, the rapid switch of $PI(4,5)P_2$ to PI(4)P by inositol 5'-phosphatases (*e.g.* SYNJs, OCRL1) triggers the dissociation of endocytic adaptors and clathrin uncoating (Cremona *et al*, 1999; Haffner *et al*, 2000; Choudhury *et al*, 2005).

Furthermore, endocytic events of large-sized cargoes, or cellular engulfment, including phagocytosis (ingestion of large particles, microbes or apoptotic cells) and macropinocytosis (uptake of fluid-phase materials), also requires $PI(4,5)P_2$ and $PI(3,4,5)P_3$. Commonly, three major spatiotemporally PIP-regulated events must occur to proceed cargo endocytosis: (i) actin-driven formation of membrane protrusions (membrane ruffles), (ii) membrane contraction into cup-shaped domain, and (iii) closure of engulfment cup to form intracellular cargo vesicles (also referred to as phagosomes or macropinosomes; **Figure 1.7**). Initiation of phagocytosis and macropinocytosis analogously leads to transient increased $PI(4,5)P_2$ concentration, through activation of $PI(4,5)P_2$-generating PIP5Kα and γ, which stimulates localised actin polymerisation-dependent membrane ruffling and contraction to capture the target cargo (Botelho *et al*, 2000; Coppolino, 2002; Araki *et al*, 2007). Small GTPases, such as Arf1 and Rho-family GTPase, are also activated to accelerate acute $PI(4,5)P_2$ synthesis and F-actin assembly (Hoppe & Swanson, 2004; Beemiller *et al*, 2006; Egami *et al*, 2014).

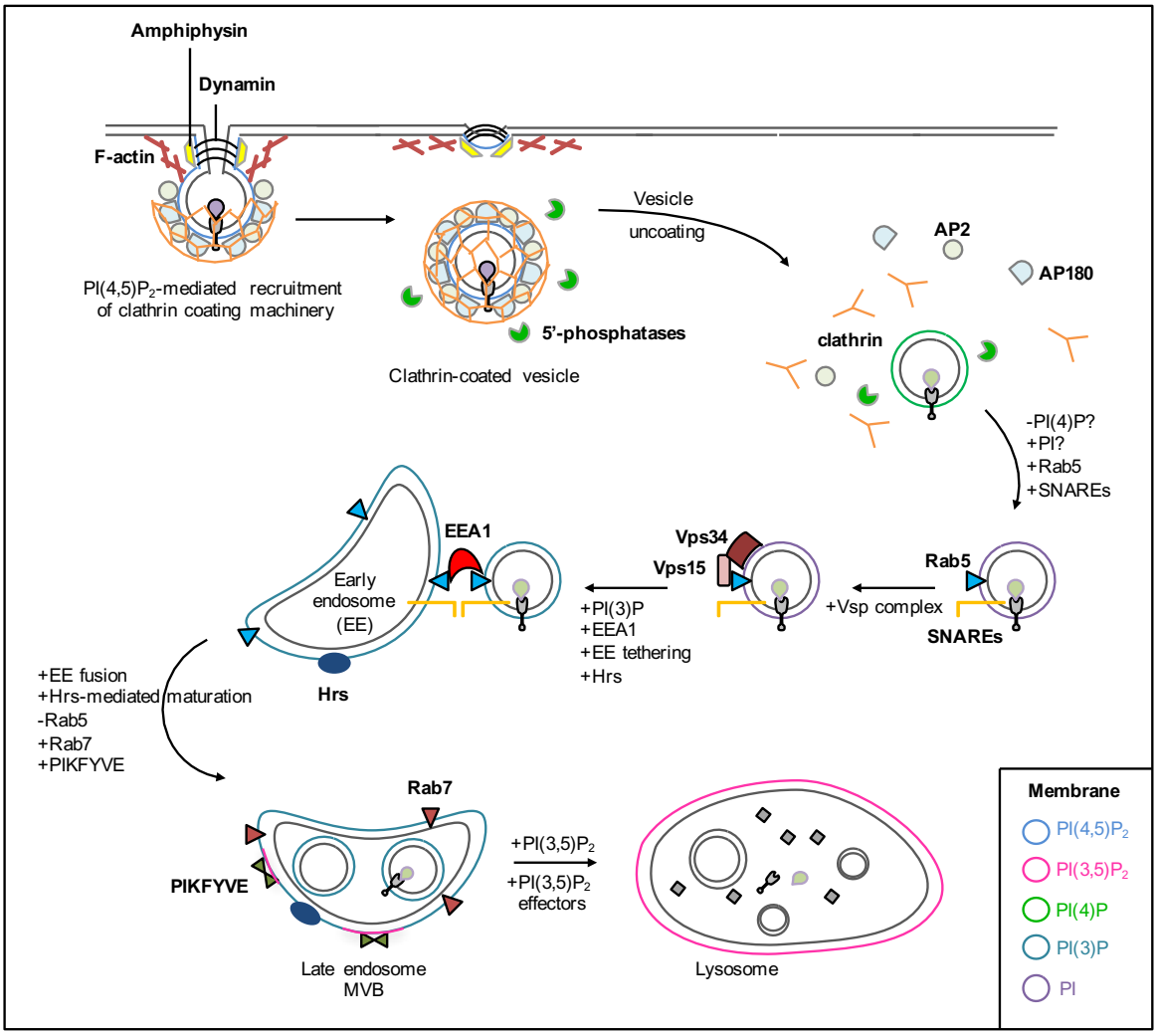

Figure 1.6 Regulation of clatharin-mediated endocytosis and endolysosomal trafficking by PIPs

Upon ligand-dependent receptor activation, $PI(4,5)P_2$ promotes membrane recruitment of clathrin adaptor proteins (AP2, AP180) for clathrin coat module assembly. $PI(4,5)P_2$ also recruits membrane-deforming amphyphysin and dynamin to the neck of clathrin pit as well as actin nucleators (WASP, Arp2/3), to induce membrane invagination, vesicle scission, ultimately driving the internalisation of the clathrin-coated vesicle. The 5'-phosphatases then dephosphorylate $PI(4,5)P_2$, causing the disassembly of clathrin machinery. Though unclear, freed uncoated vesicle possibly undergoes simultaneous PI(4)P removal and PI enrichment. Rab5 GTPase and fusion proteins SNAREs are also acquired upon entry. Rab5, through interaction with the regulatory subunit Vsp15, recruit Vps34 complex which convert PI to PI(3)P. In turn, PI(3)P recruits many endosome-specific effectors, including EEA1 and Hrs. EEA1 allows membrane tethering, and thus SNARE-mediated early endosomal fusion. Hrs promotes endosomal maturation and fusion with late endosome, accompanied by change of Rab5 to late endosome-specific Rab7. PIKFYVE is also induced by PI(3)P to generate $PI(3,5)P_2$ for recruitment of lysosome-specific $PI(3,5)P_2$ effectors.

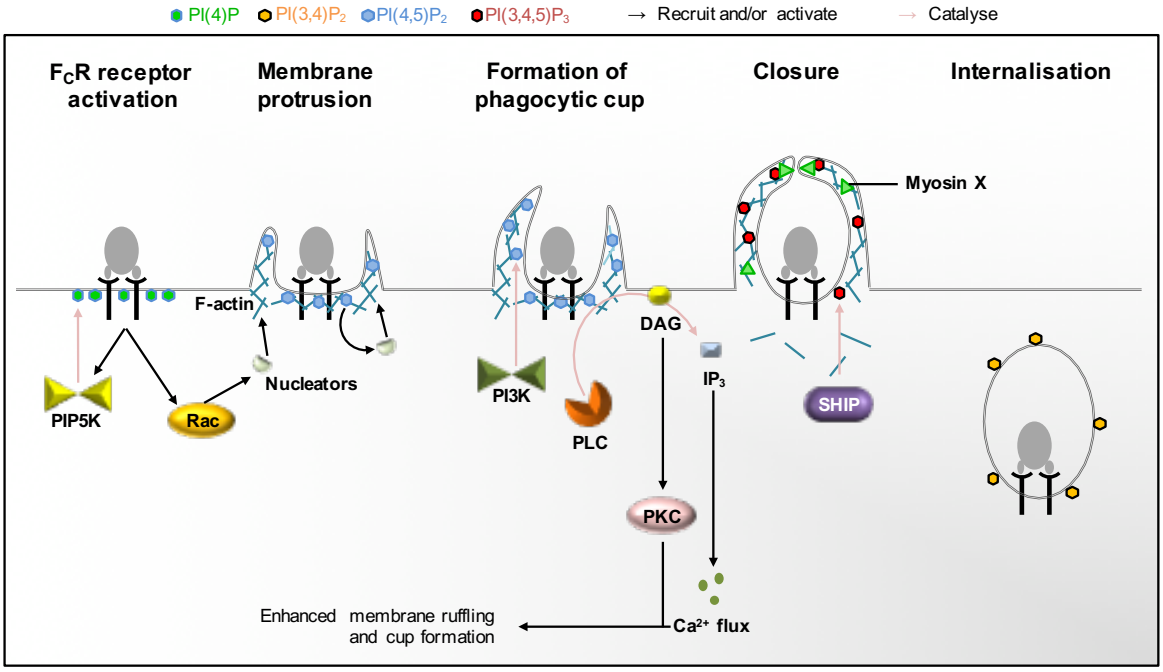

Figure 1.7 Regulation of phagocytosis by phosphoinositides

Upon stimulation of F_CR receptor by cargo, PIP5Ks are activated, converting PI(4)P to $PI(4,5)P_2$. The resultant $PI(4,5)P_2$ and activated small GTPases (*e.g.* Rac) promote actin polymerisation, leading to membrane protrusion and formation of phagocytic cup to capture target cargo. Subsequent recruitment of PI3K and PLC results in removal of $PI(4,5)P_2$, thus actin depolymerisation at the base of phagocytic cup. In addition, PLC-derived $PI(4,5)P_2$ products, DAG and IP_3, further accelerate membrane ruffling and cup formation processes via PKC activation and Ca^{2+} flux respectively. $PI(3,4,5)_3$, synthesised by PI3K, recruits myosin X for contractile force to seal the phagocytic cup. SHIP then generates $PI(3,4)P_2$ from $PI(3,4,5)_3$, completing internalisation of cargo as phagosomes. Macropinocytosis also undergoes similar PIP-regulated events, enabling the engulfment of solutes. PIP5K: PIP 5-kinase, Rac: Ras-related C3 botulinum toxin substrate, PI3K: class I PIP 3-kinase, PLC: phospholipase C, DAG: diacylglycerol, IP_3: inositol 1,4,5-trisphosphate, PKC: protein kinase C, SHIP: inositol 5-phosphatase

Sequential activities of PI3K and PLC are required to remove $PI(4,5)P_2$ respectively by phosphorylation and hydrolysis, resulting in actin disassembly from the base of phagosomes or macropinosomes (Amyere *et al*, 2000; Scott *et al*, 2005; Cheeseman *et al*, 2006). Furthermore, DAG, generated by PLC-mediated $PI(4,5)P_2$ hydrolysis, recruits and activates PKC, which is known to enhance membrane ruffling and engulfment cup formation (Larsen *et al*, 2002; Tisdale *et al*, 2014). Accumulation of $PI(3,4,5)P_3$ also promotes PH domain-containing myosins that are required for membrane extension to seal up the phagocytic/macropinocytic cup (Cox *et al*, 2002; Jiang *et al*, 2010). Finally, the conversion of $PI(3,4,5)P_3$ to $PI(3,4)P_2$ by inositol 5-phosphatase SHIP completes the cargo internalisation (Kamen *et al*, 2008; Saarikangas *et al*, 2010; Welliver & Swanson, 2012). In addition, emerging evidence has suggested the involvement of plasma

membrane PI(3)P, PI(5)P and PI(3,4)P_2 in the initiation, maturation and regulation of phagocytosis and macropinocytosis (Dewitt *et al*, 2006; Swanson, 2008; Chua & Wong, 2013; Viaud *et al*, 2014). Remarkably, during phagocytosis, PI(3)P also induces membrane localisation, assembly and activation of NADPH oxidase complex, via their PI(3)P-binding PX domain to generate reactive oxygen species-associated defense mechanism (Ellson *et al*, 2001; Bagaitkar *et al*, 2016).

Endo-lysosomal sorting and maturation of the endocytic vesicles are coordinated by 3'-PIPs in two key switches, the PI-to-PI(3)P switch and PI(3)P-to-PI(3,5)P_2 switch, in various endosomal compartments (**Figure 1.6**; Cullen & Carlton, 2012). Early endosomes are distinguished by the presence of the small GTPase Ras-related protein 5 (Rab5), Rab5 effectors (*e.g.* EEA1) and PI(3)P (Chavrier *et al*, 1990; Simonsen *et al*, 1998; Christoforidis *et al*, 1999; Zoncu *et al*, 2009; Pfeffer, 2013). Conversion of endocytic vesicles, presumably containing PI, to the PI(3)P-enriched early endosomes is achieved by the class III phosphoinositide 3-kinase (Vps34) complex, which is recruited by Rab5 through its Vps15 regulatory subunit (Christoforidis *et al*, 1999; Backer, 2008). PI(3)P subsequently recruits membrane trafficking effectors, mainly through their Fab1-YOTB-Vac1-EEA1 (FYVE) zinc-finger domain (*e.g.* early endosome antigen EEA1, hepatocyte growth factor-regulated tyrosine kinase substrate Hrs) (Simonsen *et al*, 1998; Gaullier *et al*, 1999; Rubino *et al*, 2000; Raiborg *et al*, 2001; Vieira *et al*, 2004). The FYVE domain of EEA1, required for early endosomal fusion, endosomal tethering (via interaction with *trans*-Golgi network molecules SNAREs syntaxin-6 and syntaxin-13) and directionality of endocytic transport, exhibits high affinity for PI(3)P and moderate affinity for Rab5 which allow selective recruitment of EEA1 to Rab5 and PI(3)P-positive endosome (Simonsen *et al*, 1998; McBride *et al*, 1999; Rubino *et al*, 2000). In contrast, Hrs, which is involved in endosomal sorting of ubiquitinated membrane proteins, binds to the early endosome solely dependently of PI(3)P, not Rab5 (Raiborg *et al*, 2001). The Hrs-PI(3)P interaction is also required for endosomal maturation and their fusion with late endosomes (Vieira *et al*, 2004). The succeeding compartment maturation leading to formation of late endosome/MVB involves the PI(3)P-to-PI(3,5)P_2 switch controlled by the PI(3)P 5-kinase, PIKfyve, through its PI(3)P-binding FYVE domain (Sbrissa *et al*, 2007). PI(3,5)P_2 regulates endosomal fission/fusion to maintain endomembrane homeostasis and proper endolysosomal trafficking. Impaired PI(3,5)P_2 synthesis upon genetic disruptions of PIKfyve and pharmacological inhibition, molecularly results in multiple expanded cytosolic vacuoles and leads to severe neurodegeneration and early

lethality (Ikonomov *et al*, 2001; Rutherford *et al*, 2006; Chow *et al*, 2007; Jefferies *et al*, 2008; Ikonomov *et al*, 2011; Katona *et al*, 2011; Nicholson *et al*, 2011). A number of endolysosome-localised proteins have been proposed to be recruited by PI(3,5)P_2. However, their validity as well as the identification of additional unknown PI(3,5)P_2 effectors require further studies. It is also worth noting that the turnovers of PI(3)P and PI(3,4)P_2 in these two endosomal trafficking switches by the actions of myotubularin family phosphatases are also physiologically important (Robinson & Dixon, 2006; Cao *et al*, 2007).

Recent evidence has suggested that PI(4)P, apart from being a primary source of PI(4,5)P_2 biosynthesis, directly serves as an essential regulator of secretory trafficking/exocytic pathway and Golgi morphology. The Golgi pool of PI(4)P is jointly generated by PI4KIIα and PI4KIIIβ, disruption of which interferes with proper Golgi architecture and function, as well as inhibits cargo secretion (Godi *et al*, 1999; Mills *et al*, 2003; Wang *et al*, 2003). Recruitment of PI(4)P-binding Golgi phosphoprotein 3 (GOLPH3), and subsequently, GOLPH3-binding myosin MYO18a potentially forms a structural linkage to maintain the shape of the *trans*-Golgi network and mediates efficient vesicle budding (Dippold *et al*, 2009). Often, like PI(3)P and Rab5 in early endosomes, Golgi-localised PI(4)P acts in concert with small GTPases (*e.g.* Arfs and Rabs) to specifically recruit effector proteins that facilitate cargo segregation and transport to specific locations. Both PI(4)P and Arf1 cooperatively recruit different clathrin-adaptor proteins (ADP-ribosylation factor binding proteins (GGAs), AP1 complex or epsinR), and thus enabling specified cargo selection and vesicle assembly events for particular endomembrane destinations (Mills *et al*, 2003; Wang *et al*, 2003; Wang *et al*, 2007a). On the other hand, PH domain-containing four-phosphate-adaptor proteins (FAPP1 and FAPP2) bind PI(4)P and Arf1-GFP simultaneously to facilitate export of cargo from the *trans*-Golgi to the plasma membrane (Godi *et al*, 2004). Once at the plasma membrane, PI(4,5)P_2 mediates the priming, docking and Ca^{2+}-triggered fusion of secretory vesicles via recruitment and activation of PI(4,5)P_2-binding proteins Ca^{2+}-dependent activator protein for secretion (CAPS), vesicle fusion protein Munc13 and SNAREs (Martin, 2012). Recently, it has also been demonstrated that synaptic PI(3,4,5)P_3, but not PI(4,5)P_2, is required for clustering of SNARE family member syntaxin 1A, an essential event leading to exocytosis-mediated neurotransmitter release (Khuong *et al*, 2013).

Taken together, PIPs and their spatiotemporal regulations by PIP kinases, phosphatases and lipases critically orchestrate major aspects of membrane trafficking through recruitment and activation of trafficking effectors to specified compartments.

1.3.2.5 *Programmed cell death and cell survival*

Phosphoinositides have been demonstrated to differentially affect various types of programmed cell death, including apoptosis, autophagy-dependent cell death, necroptosis and pyroptosis (**Figure 1.8**). The abovementioned PI3K-Akt signalling pathway critically confers protection against apoptosis, hence essential for cell survival. Activated Akt, through its kinase activity, induces either inhibitory or stimulatory phosphorylation of components of the apoptotic machinery. For example, the phosphorylated nuclear Forkhead transcription factors (FoxO/FH) are exported to cytosol and subsequently degraded via ubiquitin-proteasome-dependent pathway, thus resulting in the repression of FOX-activated pro-apoptotic gene expression (Tzivion *et al*, 2011; Zhang *et al*, 2011). Akt also inhibits pro-apoptotic proteins, including p73-mediated apoptotic Yes-associated protein (YAP) (Basu *et al*, 2003) and caspase cascade-stimulating Bcl-2-associated death promoter (BAD) and caspase 9 (Datta *et al*, 1997; Cardone *et al*, 1998). On the other hand, Akt-mediated phosphorylation positively regulates pro-survival/anti-apoptotic gene expression, via NF-κB induction (for c-Myb and Bcl-xL expression) (Kane *et al*, 1999) or cAMP response element binding protein (CREB) and CREB-binding protein (for Bcl-2 expression) (Du & Montminy, 1998; Pugazhenthi *et al*, 2000). In addition, Akt indirectly inhibits p53-depedent apoptosis via apoptotic murine double minute 2 (Mdm2)-mediated degradation of p53 (Ogawara *et al*, 2002). Furthermore, $PI(4,5)P_2$ is also a direct inhibitor of apoptosis initiator caspases 8 and 9 and their common effector caspase 3, independently of PI3K and Akt (Mejillano *et al*, 2001). Increased $PI(4,5)P_2$ levels by PIP5Kα overexpression exacerbates apoptotic suppression; and PIP5Kα is actually cleaved by caspase 3 *in vitro* at a consensus cleavage site, mutation of which prevents PIP5Kα inactivation and enhances apoptosis *in vivo* (Mejillano *et al*, 2001).

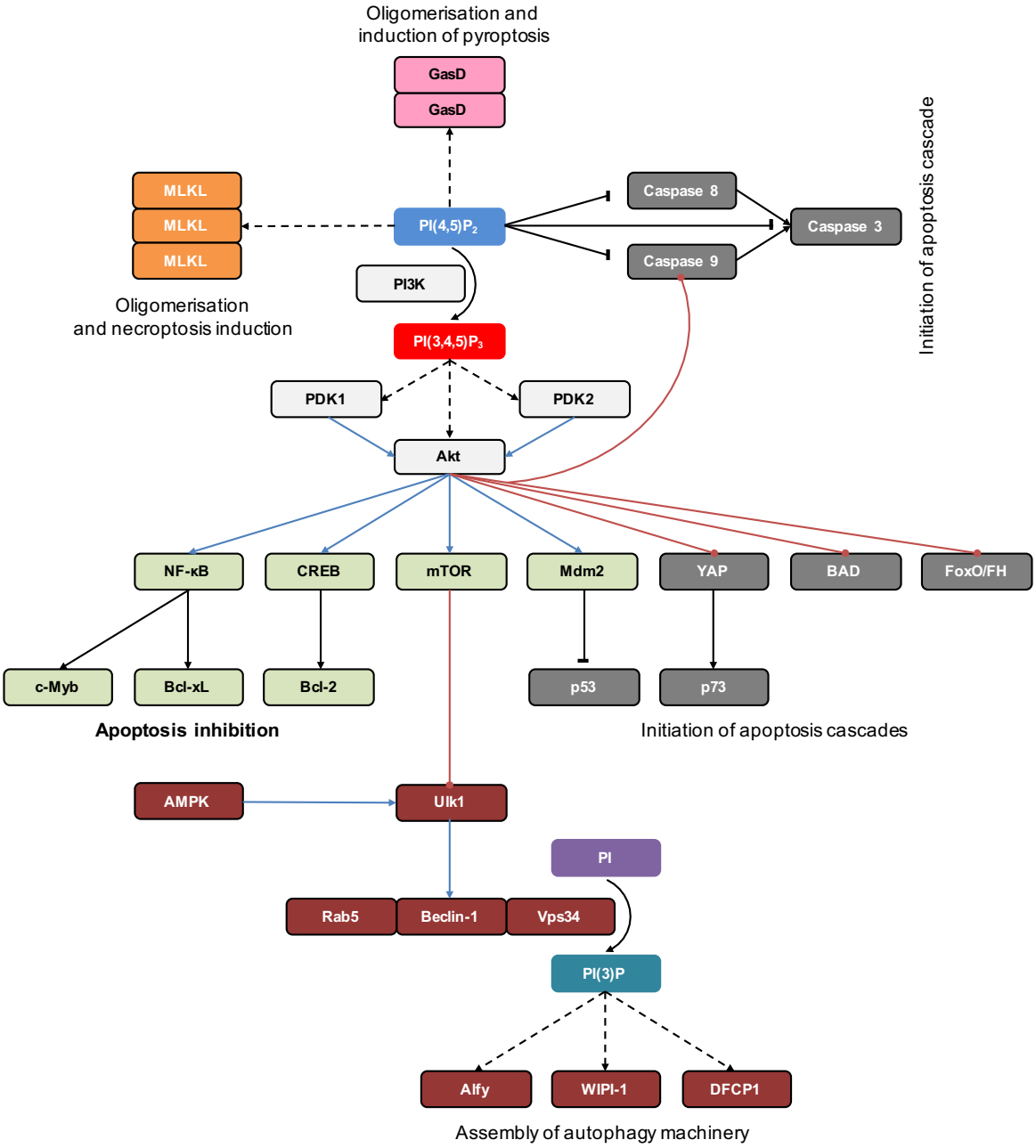

Figure 1.8 Regulation of programmed cell death and survival by phosphoinositides

Proteins involved in (**pro-)apoptosis** are in dark grey box, **anti-apoptosis and survival** in green box, **autophagy-dependent cell death** in maroon box, **necroptosis** in orange box and **pyroptosis** in magenta box. Legends for arrows/lines: kinase-catalysed PIP synthesis (curl arrow), PIP-mediated membrane recruitment (dashed arrow), direct inhibition (blunt-ended solid line), direct inhibition (solid arrow), Akt-mediated stimulatory phosphorylation (blue arrow), Akt-mediated inhibitory phosphorylation (red arrow).

Autophagy, also known as autophagocytosis, is a destructive mechanism to degrade and recycle unnecessary or dysfunctional cellular components (Takeshige *et al*, 1992; Ichimura *et al*, 2000). Autophagy is often characterised by the formation of cytoplasmic autophagic vesicles (autophagosomes) that sequester disposed constituents targeted for digestion in autolysosomes (Mizushima *et al*, 2002; Nara *et al*, 2002). Physiologically, the tight regulation of autophagy is required for proper cellular homeostasis. Autophagy also provides a defense mechanism by killing ingested bacteria (Gutierrez *et al*, 2004; Ogawa *et al*, 2005). However, under extreme pathophysiological stresses (such as amino acid starvation, growth factor withdrawal and low cellular energy level), constitutive autophagy can promote autophagic cell death (Tsujimoto & Shimizu, 2005; Green & Levine, 2014). Despite formerly being a morphologic definition, it has been recently argued as a genuine form of programmed cell death, characterised by membrane rupture, numerous autophagosomes and autolysosomes, and enlargement of major cellular organelles (Tsujimoto & Shimizu, 2005; Kroemer & Levine, 2008; Liu & Levine, 2015). Evolutionarily conserved, autophagosome biogenesis critically requires the production of PI(3)P by Vps34 kinase complexes, disruptions of which, demonstrated by genetic interference and pharmacological inhibitions, inhibit the autophagic pathway (Blommaart *et al*, 1997; Petiot *et al*, 2000; Eskelinen *et al*, 2002; Takatsuka *et al*, 2004; Axe *et al*, 2008; Juhasz *et al*, 2008). PI(3)P fundamentally provides docking platforms on the ER cytoplasmic membrane for autophagy effectors, and possibly mediates the fusion of autophagosomes and lysosomes to form autolysosomes (Axe *et al*, 2008). Particularly in human, PI(3)P is reported to specifically recruit Alfy, WD-repeat domain phosphoinositide-interacting protein 1 (WIPI-1) and FYVE domain-containing protein 1 (DFCP1) that sequesters target cargo to the autophagic machinery and forms an isolation compartment, eventually maturing into autophagosomes (Jeffries *et al*, 2004; Proikas-Cezanne *et al*, 2004; Simonsen *et al*, 2004; Walker *et al*, 2008; Filimonenko *et al*, 2010). Similar to most PIP-regulating processes, PI(3)P turnover in autophagy by Vps34 kinase and MTMR14 phosphatase (Jumpy) is critical for the initiation and termination of autophagy (Juhasz *et al*, 2008; Vergne *et al*, 2009). In contrast, the PI3K-PI(3,4,5)P_3-Akt signalling downregulates autophagy via activation of downstream Akt effector mTOR kinase which is a negative regulator of autophagy proteins (Jung *et al*, 2010; Kim *et al*, 2011). Direct phosphorylation of Unc-51-like kinase 1 (Ulk1) by mTOR at Ser757 disrupts interaction between Ulk1 and its activator AMP-activated protein kinase (AMPK) (Kim *et al*, 2011). Also of note is that AMPK-activated Ulk1 is essentially required for autophagy-specific stimulation of Vps34 complex through Ulk1-mediated

phosphorylation of Beclin-1, an enhancer component of the Vps34 complex (Russell *et al*, 2013). The Vps34-regulatory small GTPase, Rab5, has also been demonstrated to critically participate in autophagy by inhibiting mTOR (Li *et al*, 2010b) or forming autophagy-initiating complex with Beclin 1 and Vsp34 (Ao *et al*, 2014).

A few proteins also require membrane recruitment via direct interaction with PIP, particularly $PI(4,5)P_2$, to execute programmed cell death. Mixed lineage kinase-like (MLKL) pseudokinase is an effector of tumour necrosis factor (TNF)-induced programmed necrosis, or necroptosis, characterised by calcium influx, membrane damage and release of inflammatory damage-associated molecular patterns (Sun et al, 2012; Wang et al, 2014). MLKL homotrimerises once activated, and translocates to plasma membrane by binding to $PI(4,5)P_2$ through a patch of surface basic amino acids to trigger cell death (Sun et al, 2012; Cai et al, 2014; Dondelinger et al, 2014; Wang et al, 2014). Similarly, in order to respond to bacterial infection and danger signals, caspase-cleaved gasdermin D undergoes oligomerisation, inner plasma membrane binding by preferential PIP interaction, and pore formation to trigger inflammatory cell death (pyroptosis) and inflammatory cytokine release (Liu et al, 2016).

Phosphoinositides play a mandatory role in physiological regulation of ion channels and transporter activities (Toker, 1998; Suh & Hille, 2005). Mediated mainly through electrostatic interactions, $PI(4,5)P_2$ can act directly as an agonist in a ligand-like manner, co-activate in the presence of ligands, stabilise by reducing current rundown or, albeit less common, inhibit these ion channels (Rohacs *et al*, 2005; Suh & Hille, 2005; Zhao *et al*, 2007; Hansen *et al*, 2011; Zaydman & Cui, 2014; de la Cruz *et al*, 2016; Woo *et al*, 2016). Characterised $PI(4,5)P_2$-bound ion channels include, inwardly rectifying potassium channels (K_{ir}), voltage-gated potassium channels (K_v), voltage-gated calcium channels (Ca_v), transient receptor potential (TRP) channels, and non-selective cation channel P2X receptors. Remarkably, as a stress response and pathogen defense mechanism, activation of $P2X_7$ by extracellular ATP leads to increased cell permeability, cytokine release and different forms of programmed cell death (Lammas *et al*, 1997; North, 2002; Adinolfi *et al*, 2005; Yang *et al*, 2015). $PI(4,5)P_2$, in a concentration-dependent fashion, essentially activates $P2X_7$ currents upon ATP stimulation, and indispensably induces $P2X_7$-mediated cell death, particularly apoptosis, in endothethial cells, T cells and macrophages (Zhao *et al*, 2007).

1.4 Phosphoinositides in tumourigenesis and pathogen virulence

The human body has evolved a sophisticated immune system to safeguard itself from disease-causing agents, both external (e.g. microbial pathogens) and internal (e.g. cancerous cells). However, over their long-standing history with the human host, microbial pathogens have also adopted different strategies to ensure their entry, survival and replication (Rosenberger & Finlay, 2003; Cossart & Sansonetti, 2004). Similarly, cancer cells have developed adept mechanisms to escape immunosurveillance and progress (Liu *et al*, 2009; Bunney & Katan, 2010). Being essential for almost all major cellular processes as discussed above, PIPs and their metabolizing enzymes have recently emerged as crucial target for pathogen and cancer cell virulence factors (Pizarro-Cerda & Cossart, 2004; Hilbi, 2006; Chan *et al*, 2008; Saeed *et al*, 2008; Liu *et al*, 2009; Bunney & Katan, 2010; Bishe *et al*, 2012; Pizarro-Cerda *et al*, 2015).

1.4.1 Phosphoinositides in tumorigenesis

Phosphoinositide signalling is commonly deregulated in many, if not most, cancers and promotes pathogenesis and progression. In particular, the PI(3,4,5)P_3-metabolising enzymes class IA PI3K and PTEN, respectively serving as positive and negative regulators of the PI3K-PI(3,4,5)P_3-Akt pathway, are often targeted during oncogenic activation (Liu et al, 2009; Bunney & Katan, 2010). For instance, activating mutations of p110α catalytic subunit, particularly in the helical domain (Glu542 and Glu545) and the kinase domain (His1047) of PI3K substantially enhances its activity, leading to growth factor-independent Akt activation (Huang *et al*, 2007a; Miled *et al*, 2007; Vergne *et al*, 2009). Intriguingly, the helical domain mutations were structurally illustrated to abrogate the charge-inhibitory interactions between the catalytic and regulatory subunits of PI3K, resulting in retained and prolonged membrane localisation and activity (Huang *et al*, 2007a; Miled *et al*, 2007). In addition, somatic mutations of upstream effectors of PI3K, such as receptor tyrosin kinases and Ras GTPase also lead to PI3K upregulation in various cancers (Meshinchi *et al*, 2003; Zenonos & Kyprianou, 2013). Furthermore, as an antagonist of PI3K signalling, impaired PTEN expression and function (caused by transcription repression, promoter inactivation, loss-of-function mutations in many cancers) results in abnormal PI(3,4,5)P_3 accumulation and unstrained downstream Akt signalling (Soria *et al*, 2002; Zhou *et al*, 2003; Chow & Baker, 2006). The PI3K-Akt signalling hyperactivation elevates many aforementioned PI(3,4,5)P_3-regulated cellular processes, including, but are not limited to, growth, survival, cell polarity-driven epithelial-to-mesenchymal transition (EMT) and motility, which are cancer hallmarks

(Yuan & Cantley, 2008; Chalhoub & Baker, 2009). The pathway also sustains cancer-supporting tumour microenvironment through, for example, angiogenesis (via increased vascular endothelial growth factor VGEF secretion, nitric oxide and angiopoietins) and inflammation (through inflammatory cell recruitment) (Yuan & Cantley, 2008; Karar & Maity, 2011). Increased Akt-mediated inhibitory phosphorylation, via the loss of PTEN, also causes reduced nuclear localisation and enhanced ubiquitin-dependent degradation of checkpoint kinase CHK1, resulting in cancer-favouring genomic instability with increased mutation frequencies and mutation accummulation (Puc *et al*, 2005).

Elevated activation of the PI3K-Akt pathway boosts cancer cell metabolism to fulfill their substantial nutritional demand, particularly glucose (primary energy and carbon source) and glutamine (additional energy and nitrogen source), for the maintenance of growth and survival (Mosca *et al*, 2012). Constitutively stimulated Akt phosphorylation activity influences glycolytic pathway, by inducing expression of glucose transporter 1 (GLUT-1) (Barthel *et al*, 1999; Rathmell *et al*, 2003), mitochondrial translocation of hexokinase (HK-1) (Elstrom *et al*, 2004) and allosteric activation of phosphofructokinase (PFK-1) (Deprez *et al*, 1997) that are responsible for glucose uptake and initial glycolysis. In addition, PI3K-Akt signalling also directly induces other key glycolysis activators, particularly transcriptional factors MYC (Miller *et al*, 2012) and the hypoxia inducible transcription factor 1 (HIF-1) (Harada *et al*, 2009), leading to stimulation of glycolytic gene expression whilst suppresses tumour suppressor p53-mediated inhibition of glucose uptake and glycolysis (Kim & Dang, 2006) via Mdm2 phosphorylation (Ogawara *et al*, 2002). MYC, which is encoded by a proto-oncogene c-Myc, is also a master regulator with pleiotropic effects that strongly favours cancer hallmarks, including enhanced glutamine metabolism, protein biosynthesis, metabolic transformation, cellular proliferation and metastatic capacity (Miller *et al*, 2012).

Though overshadowed as being a PI3K substrate, and despite no reported mutational activation of $PI(4,5)P_2$-generating enzymes PI5Ks (from PI(4)P) and PI4Ks (from PI(5)P) in any cancer, emerging evidence has suggested the involvement of $PI(4,5)P_2$ in tumourigenesis independently of PI3K (Thapa *et al*, 2016). Correlation of increased PI4K expression and phosphorylation by oncogenic epithelial growth factor receptor (EGFR) in triple-negative breast cancers with enhanced cancer metastasis and with decreased cancer survival, implies the pivotal role of PI5Ks in cancer progression and metatasis (Sun *et al*, 2010; Chen *et al*, 2015). PI4Ks are also overexpressed in HER2-positive breast cancer

and appear essential for tumour growth in p53-null cancers (Emerling *et al*, 2013; Sumita *et al*, 2016). In parallel, PI(4,5)P_2 is essential for cancer cell polarity, EMT, invasion and metastasis through the recruitment of focal adhesion proteins and actin polymerisation effectors (Thapa *et al*, 2016).

1.4.2 Phosphoinositides in pathogen virulence

Microbial pathogens often harness the phosphoinositide-regulated processes, by overriding host PIP-mediated processes or using their own, to promote their infection processes. From viruses to parasitic protozoans, various mechanisms have been described to (i) facilitate adhesion and entry, (ii) evade host defense response, (iii) manipulate the maturation of their intracellular replication compartment, and/or (iv) to support their assembly and spread.

1.4.2.1 *Modulation of plasma membrane phosphoinositide metabolism for invasion*

Phosphoinositides (mainly PI(4,5)P and PI(3,4,5)P_3), which regulate actin remodelling and endocytosis, as well as their metabolising enzymes, are ectopically modulated by pathogens to trigger phagocytosis or macropinocytosis. For example, internalisation of enteropathogenic *Yersinia* species into non-phagocytic cells requires the induction of Rac1-mediated PIP5Kα recruitment to the bacterial entry site through the interaction of bacterial protein invasin and host β1-integrin receptor (Isberg & Leong, 1990; Alrutz *et al*, 2001). Arf6 subsequently activates and accelerates PI(4,5)P_2 synthesis, possibly via a positive feedback loop that involves Arf6, PIP5Ks, PI(4,5)P_2 and Arf6 exchange factors (Brown *et al*, 2001; Pizarro-Cerda & Cossart, 2004). The acute increased local PI(4,5)P_2 then stimulates membrane recruitment of actin remodelling and phagocytic machineries required for bacterial uptake.

Table 1.4 Modulation of PIPs and/or PIP metabolisms by pathogens to promote their infection processes

Exemplary PIP-modulating pathogens that are mentioned in this review are listed and described. The asterisk (*) denotes pathogen effectors that require host receptors to induce their PIP modulation, the hash sign (#) denotes pathogen PIP-metabolising enzymes that act directly on host PIPs, while others modulate host PIP-metabolising enzymes. The question mark (?) denotes unidentified effector/receptor/PIP-metabolising enzyme. Refer to main text for detailed discussion and references.

Pathogen entry and invasion					
Pathogen	**Pathogen effector**	**Host receptor**	**Affected enzyme**	**PIP modulation**	**Remarks**
Yersinia spp	Invasin*	β1-integrin receptor	PI5PKα (activated)	$PI(4,5)P_2$ (synthesis induction)	$PI(4,5)P_2$-mediated actin remodelling and phagocytosis
HIV-1	Env-gp120*	CD4			Accumulated $PI(4,5)P_2$ promotes receptor binding and viral uptake
HSV-1	?	?	?		
Foot-and-mouth disease virus	?	?	?		
S. flexneri	IpgD#	-	-	$PI(4,5)P_2$ turnover	IpgD and SopB display PI 4-phosphatase activity. Breakdown of $PI(4,5)P_2$ leads to disrupted $PI(4,5)P_2$-binding actin effectors, leading to actin remodelling and membrane ruffling that favours bacterial uptake.
S. enterica	SopB (SigD)#	-	-		
L. monocytogenes	InLA* InLB*	E-cadherin Met	Class I PI3Ks (activated)	$PI(3,4,5)P_3$ (synthesis induction)	$PI(3,4,5)P_2$-dependent phagocytosis/macropinocytosis
T. cruzi	*trans*-sialidase/gp85 cruzipain	?			
S. aureus	?	?			
S. agalactiae	?	?			
P. aeruginosa	?	?			
Evasion of lysosomal degredation and/or formation of replication niche					
Pathogen	**Pathogen effector**		**Affected enzyme**	**PIP modulation**	**Remarks**
M. tuberculosis	LAM		Vps34	PI(3)P (synthesis inhibition)	LAM, a bacterial analogue of phosphoinositol, inhibits host Vps34 and, thus, PI(3)P synthesis. Consequently, endolysosomal maturation and, hence, lysosomal degradation are inhibited.
	SapM#		-	PI(3)P turnover	SapM and MptpB possess PI 3-phosphatase activity, ensuring minimal PI(3)P on *Mycoplasma*-containing vesicles.
	MptpB#				
S. enterica	SopB (SigD) #		-	PI(3)P (accumulation)	PI 3- or 4-phosphatase activities of SopB dephosphorylates $PI(3,4)P_2$ and $PI(3,5)P_2$, resulting in PI(3)P accumulation for endosomal fusion, and, hence formation of

				replication vacuoles.
L. pneumophila	SidF[#]	-	PI(4)P (accumulation)	SidF is a bacterial PIP 3-phosphatase, specifically dephosphorylating $PI(3,4)P_2$ and $PI(3,4,5)P_2$ to PI(4)P and $PI(4,5)P_2$ respectively. LpnE recruits host OCRL to dephosphorylate $PI(4,5)P_2$ to PI(4)P. Accumulated PI(4)P on *Legionella*-containing phagosomes navigates PI(4)P-binding bacterial effectors to promote formation of ER-like replicative compartment.
	LpnE	OCRL		
	SidP[#]	-	PI(3)P turnover	Bacterial SidP phosphatase converts PI(3)P to PI, hence preventing endolysosomal degradation
HCV	NS5A	PI4KIIIα	PI(4)P (synthesis induction)	Formation of ER-derived replication niche
		-	$PI(4,5)P_2$ binding	$PI(4,5)P_2$ binding stabilises viral NS5A and host Rab1 GTPase-activating protein (TBC1D20) to promote HCV replication
Genome assembly and/or egress				
Pathogen	**Pathogen effector**	**Affected enzyme**	**PIP modulation**	**Remarks**
HIV-1	Gag (MA domain)	-	$PI(4,5)P_2$ binding	Importance for plasma membrane localisation of viral components prior to assembly. For Ebola virus, $PI(4,5)P_2$ may also mediate matrix protein (VP40) oligomerisation.
Influenza A virus	Nucleoprotein			
Ebola virus	VP40			
Pathogen growth and survival				
Pathogen	**Host effector**	**Affected enzyme**	**PIP modulation**	**Remarks**
L. monocytogenes	?	Class I PI3K	$PI(3,4,5)P_3$ (synthesis induction)	Activated PI3K-Akt stimulates host survival and nutrient uptake signalling cascades.
T. cruzi	?			
HSV-1	?			
Leishmania spp	?			
P. gingivalis	?			
T. gondii	?			
G. lamblia	?			Activation of PI3K-Akt-mediated signalling via EGFR inhibits IL-12 induction
S. mansoni	?			
S. flexneri	IpgD[#]	-	$PI(4,5)P_2$ turnover, leading to PI(5)P accumulation	Accumulated PI(5)P is likely to activate EGFR at the bacterial entry sites, independently of its *bona fide* ligands, thereby activating PI3K-Akt-mTOR survival signalling. PI(5)P also recruit TOM1 to prevent EGFR recycling or degradation.
L. monocytogenes	PlcA[#]	-	PI(3)P turnover	The bacterial phosphatase PlcA depletes PI(3)P, causing blockade of PI(3)P-induced autophagy by infected host cells.
HSV-1	ICP34.5	Vps34 (via Beclin-1 binding)	PI(3)P (synthesis inhibition)	Viral effectors bind to Beclin-1, inhibiting formation of PI(3)P-synthesising Vps34 complex, hence inhibiting autophagic induction.
γ-herpesvirus	Blc-2 homologue			

HIV-1	Nef			
Influenza A	Matrix 2 protein			
A. phagocytophilum	Ats-1	Vps34 (via Beclin-1 binding)	PI(3)P (synthesis induction)	Ats-1 binds Beclin-1-Vps34 to stimulate PI(3)P synthesis, leading to induction of host mTOR-independent autophagy for nutrient acquisition.
HCV	NS4B	Vps34 (through Rab5 binding)		NS4B binds Rab5 and activates Vps34 complex to facilitate viral replication and virion production through induced formation of autophagosomes

Foodborne *Listeria monocytogenes* triggers its phagocytic uptake through PI3K-dependent manner (Braun *et al*, 1997; Bierne *et al*, 2001; Dussurget *et al*, 2004; De Santis *et al*, 2009). *L. monocytogenes* invasion-promoting protein internalins (InlA and InlB) interact with host adhesion molecule E-cadherin and hepatocyte growth factor receptor (Met; a receptor tyrosine kinase), respectively (Mengaud *et al*, 1996; Braun *et al*, 1997). InlB binding induces Met dimerisation and then autophosphorylation of its cytosolic domain that serves as a docking site for downstream signalling mediators, including class I PI3K-recruiting Gab-1, Cbl and Shc (Sun *et al*, 2005a). PI(3,4,5)P_3 synthesised by PI3K orchestrates actin polymerisation-dependent phagocytosis, as discussed above, to aid bacterial entry (Ireton *et al*, 1996). Binding of InlA to E-cadherin also stimulates PI3K recruitment (Ireton *et al*, 1996), possibly by engaging PI3K regulatory subunit (De Santis *et al*, 2009), though further investigation is required to elucidate this mechanism. In certain cell types, *L. monocytogenes* uptake is abolished upon the depletion of type II PI4K-synthesised PI(4)P, clathrin or its effectors (*e.g.* AP-1), suggesting their essential involvement (Veiga & Cossart, 2005; Pizarro-Cerda *et al*, 2007; Veiga *et al*, 2007). Similarly, the macropinocytic engulfment of protozoan parasite *Trypanosoma cruzi* is also host class I PI3K-dependent (Woolsey *et al*, 2003), although the molecular events underlying *T. cruzi*-induced PI3K activation is unresolved. Speculatively, the parasitic surface protein *trans*-sialidase/gp85 family and secreted cruzipain, which were respectively shown to stimulate the PI3K-mediated survival signalling in neuronal cells (Chuenkova *et al*, 2001) and cardiomyocyte (Aoki Mdel *et al*, 2006), may initiate the *T. cruzi* macropinocytic uptake. Activation of the PI3K-Akt signalling pathway to promote pathogen internalisation is also implicated in many other microbial pathogens, including *S. aureus* (Oviedo-Boyso *et al*, 2011), *Streptococcus agalactiae* (Burnham *et al*, 2007) and certain *P. aeruginosa* strains (Kierbel *et al*, 2005).

While the functions of PI(5)P in the physiological setting remain undercharacterised, its generation from PI(4,5)P_2 plays in important role during *Shigella flexneri* invasion (Niebuhr *et al*, 2002). The pathogen induces its uptake by non-phagocytic cells through bacterial invasion effectors IpgD (Niebuhr *et al*, 2002), which is directly injected into host cytoplasm using a type III secretion system (T3SS) (Reed *et al*, 1998). The virulence factor IpgD with its phosphatidylinositol 4-phosphatase, dephosphorylates plasma membrane PI(4,5)P_2, thus accumulating PI(5)P (Niebuhr *et al*, 2002). However, the defined role of PI(5)P in *S. flexneri*-induced cytoskeleton remodelling and endocytosis requires further study. Although IpgD-induced PI(5)P synthesis activates class I PI3K, its

inhibitor, wortmannin, does not block *S. flexneri* entry, suggesting the PI3K stimulation is merely concomitant (Niebuhr *et al*, 2002), possibly as a survival mechanism (to be discussed later). It is also possible that this $PI(4,5)P_2$ depletion disrupts $PI(4,5)P_2$-recognising actin effectors, resulting in actin detachment, membrane ruffling and cytoskeleton remodelling, which favours *S. flexneri* uptake (Pizarro-Cerda & Cossart, 2004). Likewise, $PI(4,5)P_2$ turnover at entry site, by translocated bacterial PIP phosphatase SopB (also called SigD) through T3SS, is pivotal to the invasion of gastroenteritic *Salmonella enterica* (Marcus *et al*, 2001; Terebiznik *et al*, 2002). SopB, which shares similarity with mammalian PI 4- and 5-phosphatase, is in fact a multi-specificity phosphatase. SopB also dephosphorylates soluble inositol polyphosphates in infected cells, leading to activation of Cdc42 and downstream actin cytoskeletal rearrangement (Norris *et al*, 1998; Marcus *et al*, 2001; Zhou *et al*, 2001).

Although there have been limited studies, $PI(4,5)P_2$ has also been implicated in viral adsorption and entry, sometimes independently of PI3K pathway. The earliest evidence suggesting the link between $PI(4,5)P_2$ and viral invasion was described three decades ago by Langeland and colleagues (Langeland *et al*, 1986; Langeland *et al*, 1987; Langeland *et al*, 1989). The authors demonstrated that herpes simplex virus-1 (HSV-1), but not HSV-2, induced $PI(4,5)P_2$ synthesis in host cells and sequestering of $PI(4,5)P_2$ blocked receptor binding and HSV-1 internalisation. The importance of $PI(4,5)P_2$ in HSV-1 infection may also extend to HSV-1-activated PI3K-Akt signalling to orchestrate non-professional phagocytosis in certain cell types and to maximise internalisation efficiency via increased intracellular Ca^{2+} (Tiwari & Shukla, 2012; Cheshenko *et al*, 2013). Similarly, initial binding of human immunosuppression virus 1 (HIV-1) protein Env-gp120 to T lymphocyte cluster of differentiation 4 (CD4) receptor stimulates $PI(4,5)P_2$ production through PIP5Kα, allegedly an indispensable step for HIV-1 entry (Barrero-Villar *et al*, 2008). In addition, it has been reported that $PI(4,5)P_2$ is required for β1-integrin-mediated clathrin-dependent internalisation of non-envelope foot-and-mouth disease virus (Vazquez-Calvo *et al*, 2012). Therefore, $PI(4,5)P_2$ metabolism appears to be an crucial node in viral infection, although further research is required to elucidate the viral activation of $PI(4,5)P_2$.

1.4.2.2 *Modification of endomembrane phosphoinositides for evasion of lysosomal degradation or replication*

Upon endocytic uptake, certain intracellular pathogens modify the behaviour of the endocytic vesicles to alter their maturation, hence evading lysosomal fusion and degradation and/or forming replicative niches. For example, *Mycobacterium tuberculosis* halts the maturation of carrier phagosomes by inhibiting PI(3)P-mediated machinery (Armstrong & Hart, 1971; Vergne *et al*, 2003). Mycobacterial glycosylated phosphatidylinositol analogue, lipoarabinomannan (LAM), causes the inhibition of Vps34 activating-signalling cascade, which consecutively suppresses PI(3)P synthesis, phagosomal localisation of Rab5 effector EEA1, and syntaxin 6-mediated tethering to *trans*-Golgi complex (Vergne *et al*, 2003). As a consequence, lysosomal hydrolases and vacuolar H^+-ATPase, two prominent late endolysosomal markers, cannot be recruited (Sturgill-Koszycki *et al*, 1994; Clemens & Horwitz, 1995). In addition, the PI(3)P-binding Hrs is also absent, further contributing to the maturation arrest of *M. tuberculosis* phagosomes (Vieira *et al*, 2004). Simultaneously, phosphatidylinositol mannoside (PIM), another mycobacterial phosphatidylinositol analogue, stimulates phagosomal fusion with early endosomes possibly via Rab5 modulation, thus acting in concert with LAM to abrogate phagosomal maturation (Vergne *et al*, 2004). In addition, *M. tuberculosis* secretes enzymes with phosphatidylinositol 3-phosphatase activity, SapM and MptpB, to ensure minimal presence of PI(3)P in its carrier phagosomes (Vergne *et al*, 2005; Beresford *et al*, 2007). In contrast, internalised *S. enterica* maintains high level of PI(3)P on its endocytic vesicles by direct action of the bacterial SopB, which possibly dephosphorylates $PI(3,5)P_2$ (Hernandez *et al*, 2004), or SopB-induced recruited host Vps34 (Mallo *et al*, 2008), depending on host cell types. Accumulated PI(3)P then robustly recruits its effector proteins, including EEA1, PX domain-containing retromer sortin nexin-1 (SNX-1), and SNARE vesicle-associated membrane protein 8 (VAMP8), which function in concert to continually induce homotypic fusion of nascent bacterial phagosomes and other empty PI(3)P-enriched endosomes (Scott *et al*, 2002; Dai *et al*, 2007; Bujny *et al*, 2008). This delicately programmed maturation process enables *S. enterica* to evade lysosomal fusion, to establish spacious replicative compartments as well as to, seemingly, promote efficient bacterial uptake.

Another endosomal trafficking-modifying strategy has been elegantly developed by *Legionella pneumophila* to facilitate its intracellular replication within a distinct ER-like compartment. During its initial interaction with aveolar macrophages, *L. pneumophila* translocates a vast number of effector proteins into the host cytoplasm via type IV secretion system (T4SS) (Vogel *et al*, 1998). Several of these bacterial proteins and host

effectors contribute to the maturation process, which is PI(4)P-dependent, converting the tight nascent plasma membrane-derived bacterial phagosomes to a spacious ER-like compartment for bacterial replication via fusion with ER-originated vesicles (Kagan & Roy, 2002). The PI(4)P synthesis on bacterial phagosomes is therefore critical for *L. pneumophila* infection (Weber *et al*, 2006). Bacterial PIP 3-phosphatase SidF specifically dephosphorylates PI(3,4)P_2 and PI(3,4,5)P_3 into PI(4)P and PI(4,5)P_2 (Hsu *et al*, 2012). Host 5-phosphatase OCRL is recruited, possibly by bacterial effector LpnE, to hydrolyse PI(4,5)P_2, also to PI(4)P (Weber *et al*, 2009). Host PI4KIIIβ also contributes to the generation of the PI(4)P pool on *L. pneumophila*-containing phagosome (Hubber *et al*, 2014). Meanwhile, another bacterial phosphatase, SidP, that converts PI(3)P to PI, is also required to halt the accumulation of PI(3)P, thus diverting bacterial phagosomes from canonical PI(3)P-mediated endosomal trafficking (Toulabi *et al*, 2013). PI(4)P then serves as an lipid anchor to recruit other secreted bacterial effector proteins, such as SidC, SdcA, SidM and LidA, for recruitment of ER and ER-derived vesicles (Swanson & Isberg, 1995; Kagan & Roy, 2002). SidC and its paralogue SdcA binds to PI(4)P through the PI(4)P binding of SidC (P4C), a unique 20-kDa α-helix bundled domain, to induce translocation of ER proteins to the bacterial vacuole (Weber *et al*, 2006; Luo *et al*, 2015). In addition, SidM, a GTP exchange factor, and its auxillary protein LidA recruit Rab1, another effector GTPase from Rab5-including family that is involved in vesicle trafficking between ER and Golgi (Murata *et al*, 2006; Brombacher *et al*, 2009; Stenmark, 2009). The subsequent spatiotemporal regulation of Rab1 dynamics by other *Legionella* effectors is pivotal to formation of the ER-like replicative compartments (Murata *et al*, 2006; Brombacher *et al*, 2009; Sherwood & Roy, 2013).

Likewise, hepatitis C virus (HCV) genome replication occurs within ER-derived membrane compartments formed upon membrane reorganisation via PI(4)P enrichment and PI(4)P effector recruitment (Bishe *et al*, 2012). To this end, viral non-structural effector protein NS5A directly binds and activates PI4KIIIα via its domain 1 (Lim & Hwang, 2011). It was also found that α-helix segment within the N-terminal of NS5A specifically binds to PI(4,5)P_2 via BAAPP domain (basic amino acid PI(4,5)P_2 pincer), inducing a conformational change that stabilises the interaction between NS5A and host TBC1D20 (a Rab1 GTPase-activating protein), which is required for HCV replication (Sklan *et al*, 2007). It is likely that PI(4,5)P_2 is derived via the above-mentioned PI(4)P pools. The BAAPP domain is conserved across all HCV isolates, as well as the α-helix

domain of many pathogens (Japanese encephalitis virus, Dengue virus, rhinovirus and enterovirus) and apolipoproteins (Cho *et al*, 2014).

1.4.2.3 *Exploitation of phosphoinositide-regulated secretory machineries for egress*

Certain pathogens, particularly enveloped viruses, require translocation of their propagated genomes to the inner membrane leaflet for viral packaging and budding. Generally, viruses, such as HIV-1, exploit the membrane recruitment activity of $PI(4,5)P_2$ as the determinant of virion assembly prior to their egress. $PI(4,5)P_2$-binding matrix polyprotein Gag is a major driving force of HIV-1 assembly process (Ono *et al*, 2004; Saad *et al*, 2006). Gag contains four major structural domains, each with distinct function to assist the viral packaging: membrane targeting N-terminal matrix (MA), Gag oligomer-stabilising capsid (CA), RNA-binding nucleocapsid (NC) and endosomal-sorting complex (ESCRT)-recruiting p6 domain (Chukkapalli & Ono, 2011; Bell & Lever, 2013). The MA domain features a sequestered N-terminal myristate group and a cluster of basic residues (highly basic region, HBR) ^{14}KWEKIRLRPGGKKQYKLK^{31} that forms a specific $PI(4,5)P_2$-binding pocket (Saad *et al*, 2006). It is also believed that viral RNA binds to HBR, in addition to the NC domain, to inhibit nonspecific membrane binding of MA (Yandrapalli *et al*, 2016). The bound viral RNA only release its inhibition when $PI(4,5)P_2$ contacts and competes for HBR binding (Yandrapalli *et al*, 2016). Therefore, the MA-$PI(4,5)P_2$ interaction facilitates initial membrane targeting of Gag, and concomitantly induces myristate exposure, hence strengthening its membrane anchoring (Tang *et al*, 2004; Saad *et al*, 2006). $PI(4,5)P_2$ depletion or pharmacological disruption of $PI(4,5)P_2$ binding of Gag severely decreases the efficiency of viral particle release, thereby emphasising the importance of $PI(4,5)P_2$ in viral assembly. Furthermore, $PI(4,5)P_2$, sphingolipids, and cholesterol are enriched in the HIV-1 envelope, suggesting that the viral particles egress from lipid domains (rafts) of the host plasma membrane (Chan *et al*, 2008). However, not until very recently has it been demonstrated that Gag (via its MA) is unlikely to target pre-existing rafts, but instead induces local membrane rearrangement to generate $PI(4,5)P_2$-cholesterol nanodomains upon its binding (Yandrapalli *et al*, 2016).

The mandatory role of $PI(4,5)P_2$ in viral assembly is further accentuated by recent reports on Influenza A virus and Ebola virus. Similar to HIV-1, Influenza virus A was also found to exploit $PI(4,5)P_2$ for trafficking and packaging of viral RNA-bound nucleoprotein at host plasma membrane through its second intrinsically disordered region (Kakisaka *et al*, 2016). Ebola virus, on the other hand, employs a phosphatidylserine (PS)-binding matrix

protein, called VP40, for plasma membrane binding prior to viral assembly and budding. It is commonly agreed that VP40 binds PS at inner leaflet for its membrane localisation and subsequent oligomerisation (typically, dimer to hexamer) to create building blocks of viral matrix for virion formation (Timmins *et al*, 2003; Adu-Gyamfi *et al*, 2015). However, additional inner membrane lipids may be employed by VP40, as PS later translocates to the outer membrane leaftlet of non-apoptotic host cells at viral budding sites (Adu-Gyamfi *et al*, 2015). The 'PS translocation' observation is indeed supported by the abundant PS exposure on release particles and the envelope PS-mediated viral entry through interaction with PS receptors on host phagocyte surface (Morizono & Chen, 2014; Adu-Gyamfi *et al*, 2015).

Multiple emerging evidence have recently indicated the association of VP40 with $PI(4,5)P_2$ of inner membrane leaflet and *in vitro* vesicles, essential for formation of higher order (likely, dodecamer or greater) VP40 oligomers and viral assembly (Johnson *et al*, 2016). It, however, remains unclear how PS and $PI(4,5)P_2$ structurally and mechanistically determine the VP40 oligomerisation switch, or why PS is initially required by VP40 since $PI(4,5)P_2$ can solely facilitate membrane targeting and virion formation as described for other viruses. Possibly, the need of Ebola virus for PS acquisition predisposes its priority for initial localisation to inner membrane PS via VP40, leading to membrane binding and low order oligomerisation. Potentially similar to HIV-1 Gag, membrane-bound VP40 can induce membrane reorganisation in favour of $PI(4,5)P_2$. Strikingly, computational studies showed that VP40 hexamer can enhance the clustering of $PI(4,5)P_2$ (Gc *et al*, 2016). The newly-clustered $PI(4,5)P_2$, which is more negatively-charged than PS, may then compete for VP40 binding, displacing PS and allow it freely translocate to the outer plasma membrane. As previously observed in oligomeric plant defensin NaD1–$PI(4,5)P_2$ co-crystal structure (Poon *et al*, 2014), $PI(4,5)P_2$ with its phosphate groups not only confers the electronegativity but can also serve as multi-arm proton acceptor upon hydrogen bonding, likely mediating its concentration-dependent higher-order oligomerisation of NaD1. Hence, the structural modulation of VP40 oligomerisation, potentially determined by PS-to-$PI(4,5)P_2$-binding switch, may be crucial to induce extensive VP40 oligomeric state, thus enhancing nascent viral matrix and structural integrity (Johnson *et al*, 2016).

1.4.2.4 *Manipulation of various phosphoinositide-mediated processes for pathogen growth and survival*

Microbial pathogens satisfy their high nutritional demands from the host. Intracellular residence, while rewarding invading pathogens with protective shelter from humoral and complement-mediated immune defense as well as shear stress-induced clearance, hinders their direct access to nutritional sources in host circulation, as opposed to extracellular pathogens (Eisenreich *et al*, 2013; Ribet & Cossart, 2015). Furthermore, as the proverb 'diamond cut diamond' depicts, infected host cells unceasingly defend to obliterate the unwelcomed 'guests', by lysosomal degradation, intracellular pathogen-targeting autophagy (also known as xenophagy) and programmed cell death (Tam & Jacques, 2014). Imperative to their growth and survival within host cells, intracellular pathogens must ensure sufficient nutrient replenishment and evade cellular defenses. As previously discussed, modification of PIP-regulated membrane trafficking and downstream events, such as formation of replication niche or pathogen vacuole lysis, are brilliant strategies to evade lysosomal degradation. In this section, other PIP-mediated tactics, including PI3K-Akt hyperactivation and autophagy manipulation are discussed.

One common approach to promote pathogen growth and survival is hyperactivation of the PI3K-Akt signalling pathway. For instance, some of the aforementioned pathogens (*L. monocytogenes*, *T. cruzi, S. flexneri* and HSV-1), and many others (*Leishmania* species, *Porphyromonas gingivalis*, *Taxoplasma gondii,* etc.) directly or collaterally activate PI3K, via various phosphoinositide-modulating strategies (Yilmaz *et al*, 2004; Krachler *et al*, 2011; Eisenreich *et al*, 2013). Subsequent stimulated pro-survival Akt activity potently suppresses host apoptosis and prolongs their survival, hence supporting pathogen growth and allowing the escape of apoptosis-mediated pathogen clearance (Krachler *et al*, 2011). The PI3K-Akt-dependent stimulation of glucose, glutamine and other nutrition uptake/metabolism is also exploited by pathogens, similar to cancer cells, to ensure sufficient nutritional replenishing for host cells, hence for intracellular pathogens (Eisenreich *et al*, 2013). Remarkably, certain extracellular pathogens, such as the protozoan parasite *Giardia lamblia* (Kamda & Singer, 2009) and helminth parasite *Schistosoma mansoni* (Kane *et al*, 2004), also appear to exploit the PI3K pathway to inhibit pro-inflammatory response by dendritic cells, particularly interleukin-12 induction, to evade immune defense. To perpetuate these effects, the sustenance of PI3K stimulation is thus necessary, delicately attainable by maintaining its upstream activator EGFR. As aforementioned, *S. flexneri* IpgD generates PI(5)P from plasma membrane PI(4,5)P_2 to assist its internalisation to host cells. EGFR is also recruited in an IpgD-dependent manner and, though additional investigation is required, PI(5)P production is

likely to activate EGFR at the bacterial entry sites, independently of its *bona fide* ligands, leading to PI3K and Akt stimulation (Ramel *et al*, 2011). Activated EGFR is however short-lived, as it is subjected to endocytosis, ubiquitination, and lysosomal degradation. More recently, the accumulated PI(5)P, simultaneously internalised on the cytoplasmic membrane of EGFR-containing vesicles, has been shown to recruit a novel PI5P effector, TOM1 [target of myb-1 (chicken)] through its Vps27-Hrs-STAM (VHS) domain (Boal *et al*, 2015). The PI(5)P-induced TOM1 recruitment to the signalling endosomes is crucial to prevent EGFR recycling or degradation by blocking endosomal maturation (Ramel *et al*, 2011; Boal *et al*, 2015). Active EGFR signalling therefore persists in the early endosomes, sufficiently sustaining its downstream pathways, particularly PI3K-Akt, for host cell growth and survival (Wang *et al*, 2002; Boal *et al*, 2015). Remarkably, the multidrug-resistant opportunistic bacteria *P. aeruginosa* and *Enterococcus faecalis* also appear to rely on EGFR PI3K-Akt pathway to prolong their survival by preventing host cell apoptosis in the early stage of bacterial infection (Zhang *et al*, 2004; Zou & Shankar, 2014).

As a host defense mechanism against intracellular pathogens, the infection-controlling xenophagy is preferentially inhibited by many invading microorganisms, through PIP-dependent or PIP-independent manipulations of autophagy machineries. The former approaches are often achieved via two autophagic PIP regulators, PI(3)P (also, Beclin-1-Vps34) and PI(3,4,5)P_3 (via PI3K-Akt-mTOR). For example, once internalised into host cells, *L. monocytogenes* escapes from phagosomes and enters the host cytosol. To avoid host induced xenophagy, a bacterial phosphoinositol-specific phospholipases C (PlcA), which depletes PI(3)P level *in vitro*, is required to block PI(3)P-mediated autophagic initiation (Mitchell *et al*, 2015). Multiple viral Beclin-1-binding proteins, such as HSV-1 neurovirulence factor ICP34.5 (Orvedahl *et al*, 2007), human γ-herpesvirus Blc-2 homologues (Sinha *et al*, 2008; Zhou & Spector, 2008; Kyei *et al*, 2009), HIV-1 accessory protein Nef and influenza A matrix 2 protein (Gannage *et al*, 2009) block the formation or maturation of autophagosomes. HIV-1 infection also transcriptionally suppresses Beclin-1 expression, hence inhibiting autophagy. On the other hand, the maintenance of EGFR-PI3K-Akt signalling by the protozoan *T. gondii* is essential to inhibit autophagy effectors, through the negative regulator of autophagy mTOR, hence suppressing autophagy initiation, pathogen vacuole-lysosomal fusion and lysosome-assisted microbial killing (Muniz-Feliciano *et al*, 2013).

Conversely, many pathogens favour to actively induce host autophagy for autophagosome-mediated nutrient harvest and for growth support. Necessarily, these pathogens also develop various evasive strategies to render the activated autophagy innocuous, hence sustaining the growth benefit without risking their survival. Gram-negative bacteria *Anaplasma phagocytophilum* that causes the zoonotic disease human granulocytic anaplasmosis unleashes its T4SS effector Ats-1 to initiate the ER autophagy machinery in host granulocytes through Beclin-1-Vsp34 binding (Niu *et al*, 2012). Ast-1-induced mTOR-independent autophagy is likely to promote the nutrient acquisition, evidently by elevated *Anaplasma* growth upon Ast-1 overexpression, suppressed infection by host Beclin-1 depletion, and alleviation of Vsp34 inhibitor-arrested bacterial growth by essential amino acid supplementation (Niu *et al*, 2012). HCV also induces host mTOR-independent autophagy through its non-structural effector protein NS4B, which directly interacts with host Rab5, probably to facilitate viral replication and virion production (Su *et al*, 2011). NS4B appears to form an autophagy-stimulating complex with Rab5 and Vps34, as suggested by coimmunoprecipiation. Genetic disruption of Rab5 or pharmacological inhibition of Vps34 impaired NS4B-induced autophagosome formation (Su *et al*, 2011).

1.5 Potential pharmacological targeting of phosphoinositides and phosphoinositide-metabolising enzymes

Their fundamental role in tumourigenesis and microbial infection offers promising opportunities to pharmacologically target PIP-metabolising enzymes or, potentially PIPs themselves directly.

1.5.1 Targeting phosphoinositide-metabolising enzymes

Being a pivotal node of the prevalent signalling for many cancer hallmarks, PI3K is one of the most therapeutically investigated targets. However, due to kinase homology, isoform redundancy and inter-pathway complexity, PI3K targeting has presented not only promising therapeutic opportunity but also immense challenge for cancer therapy (Marone et al, 2008; Liu et al, 2009; Bunney & Katan, 2010). First generation broad-spectrum class I PI3K inhibitors, wortmannin (covalently binds to a functionally-important lysine within PI3K catalytic site) and LY294002 (reversibly targets PI3K family members) show limited selectivity and substantial toxicity (Marone *et al*, 2008). Nonetheless, they have contributed immensely to our understanding of the biological importance of PI3K signalling and therapeutic development. As such, anticancer PI3K

signalling antagonists have progressed at an astounding pace with increased drug potency and cancer selectivity, as well as reduced toxicity (Marone *et al*, 2008; Massacesi *et al*, 2016).

Current class I PI3K inhibitors are typically ATP mimetics, that competitively and reversibly bind to the ATP-binding pocket of the p110 catalytic subunit. In general, many exhibit favourable tolerability and have passed phase I into phase II testing in multiple cancer types (Thorpe *et al*, 2015; Wang *et al*, 2015). Notably, BKM120 (Buparlisib, Norvatis Oncology) is currently in in placebo-controlled randomised phase III clinical assessment for hormone-positive HER2-negative breast cancer (United States National Institutes of Health Clinical Trials, 2016; https://clinicaltrials.gov). In addition, structural similarities between ATP-binding domains of PI3K and mTOR have warranted dual PI3K/mTOR drug development (Thorpe *et al*, 2015; Wang *et al*, 2015). The first inhibitors of mTOR were rapamycin analogues which functioned through an allosteric inhibition mechanism, making them of limited clinical use and therefore have only been approved for advanced renal cell carcinoma and soft-tissue/bone sarcomas (Liu *et al*, 2009; Wander *et al*, 2011). Their cytostatic nature, partial mTOR suppression, and negative regulatory feedback loops that activate PI3K/Akt following treatments seemingly contribute to undermine their efficacy (Sarbassov *et al*, 2005; Wan *et al*, 2007; Wander *et al*, 2011). The ATP-competitive compounds with the active site inhibition of both mTOR and class I PI3K can not only overcome the limitations posed by rapamycin-based drugs, but also show a broader activity and greater efficacy. Despite positive cancer responses, both pan-PI3K and dual PI3K/mTOR inhibitors thus far have demonstrated modest clinical activity and considerable off-target effects, possibly due to limited efficacy (Brana & Siu, 2012). For example, recent inadequate results on completed phase II trials of the combinatorial PI3K/mTOR antagonists BEZ235 (Dactosilib, Norvatis Oncology) and GDC-0980 (Apitolisib, Genentech), respectively in advanced pancreatic neuroendocrine tumour and metastatic renal cell carcinoma (United States National Institutes of Health Clinical Trials, 2016), have discouraged their further progression (Fazio *et al*, 2016; Powles *et al*, 2016). Therefore, as specific PI3K catalytic isoforms are implicated in certain malignancies, such as p110α frequently mutated in many solid tumours, p110β in PTEN-null cancers, and, potentially, p110δ in lymphomas, specific targeting PI3K isoforms have been expected to confer more precise targeting (Thorpe *et al*, 2015; Wang *et al*, 2015). Promisingly, several have entered placebo-controlled randomised phase III evaluation (United States National Institutes of Health Clinical

Trials, 2016), including PI3K-γ,δ-targeted IPI-145 (Duvelisib, Infinity) for relapsed or refractory chronic lymphocytic leukemia or small lymphocytic lymphoma (O'Brien *et al*, 2014) and PI3K-δ-specific CAL-101 (Idelalisib, Gilead Sciences) for chronic lymphocytic leukemia (Khan *et al*, 2014).

Inevitably, these PI3K therapeutic progressions are constantly challenged by mutation specificity, lack of biomarker diagnosis, complexity of PI3K signalling network, pathway cross-talks and feedback activation-mediated resistance. Furthermore, trialled single-agent and combinatorial inhibitors of PI3K pathway appear to be associated with higher risk of infection, likely attributable to functional defects of immune response rather than myelosuppression (Rafii *et al*, 2015). Optimism, however, remains high with the further advancement of PI3K inhibitors with isoform specificity and multi-pathway targeting, with the better understanding of their immunological impacts and with substantial effort in biomarker discovery.

On the other hand, targeting PI3K or other PIP-metabolising enzymes, in anti-infective therapies are disappointingly underdeveloped, despite their fundamental role in many aspects of microbial infection. Data relating to the effectiveness of PIP-metabolising enzyme inhibitors have largely been from *in vitro* studies, although suggesting therapeutic potential. Prominently, studies on blockade of PI3K signalling using PI3K-γ specific (AS-605240), dual PI3K/mTOR (BEZ235) or mTOR antagonists (*e.g.* rapamycin analogues) currently in various stages of cancer clinical evaluation have shown promising intervention of trypanosomatid infection, including *T. cruzi, T. brucei* and *Leishmania* species (Diaz-Gonzalez *et al*, 2011; Oghumu & Satoskar, 2013) and coronavirus infection (Kindrachuk *et al*, 2015). Intriguingly, BEZ235 displays sub-nanomolar potency, efficacy against cultured parasites and the ability to clear blood-present parasite content in *T. brucei*-infected animal model (Diaz-Gonzalez *et al*, 2011). These results have suggested PI3K signalling targeting as a potential starting platform for anti-infective drug discovery and new opportunities for drug repurposing.

Furthermore, in response to the emergence of resistant HCV mutants, there have been an appreciable number of studies to develop PI4KIIIα antagonists, particularly ATP mimetics, as novel anti-HCV therapeutics. As PI4IIIKα holds a pivotal host physiological role, despite potent *in vitro* inhibition of viral replication, PI4KIIIα-targeted compounds display anti-proliferative activity in lymphocytes (Bishe *et al*, 2012). Additionally,

PI4KIIIα homozygous knockout or kinase-defective knock-in, leads to a lethal phenotype with global mucosal epithelial degeneration (Vaillancourt *et al*, 2012). PI4KIIIα targeting therefore may present a toxicity-associated obstacle, probably hampering its potential therapeutic application. These findings suggest that PIP-metabolising enzymes may be therapeutically applicable when they or their specific isoforms are required by the microbial pathogens, but not by the host. Thus, therapeutic investigation of pathogen-specific PIP-metabolising enzymes, such as SapM and MptpB of *M. tuberculosis*, SopB of *S. enterica*, SidF and SidP of *L. pneumophila* requires further investigation.

1.5.2 Direct targeting of phosphoinositides

Although long-neglected, the direct targeting of PIPs with PIP-binding/sequestering molecules, may also hold promise for future therapeutic treatments. Antifungal plant defensins *Nicotiana alata* defensin 1 (NaD1) and tomato defensin TPP3, which are small host defense peptides (HDPs), display broad spectrum anticancer activity via $PI(4,5)P_2$-mediated membrane blebbing, membrane permeabilisation and eventually to necrotic lysis (Poon et al, 2014; Baxter et al, 2015). NaD1 and TPP3 have been shown to bind $PI(4,5)P_2$ via their conserved cysteine-flanked highly-positively charged β2-β3 loop (^{35}S**K**IL**RR**^{40} in NaD1 and in ^{37}S**K**LQ**RK**^{42} in TPP3) (Poon et al, 2014; Baxter et al, 2015). As a dimer, two β2-β3 loops of the plant defensin monomers form a claw-like structure to bind $PI(4,5)P_2$, which promotes defensin oligomerisation. The protein-lipid interaction involves an intensive hydrogen-bonding network provided by residues within and around the β2-β3 (Poon et al, 2014; Baxter et al, 2015). Interestingly, in a proteomic study by Lewis et al (2011), a large number of identified $PI(4,5)P_2$-binding proteins were found to possess a lysine/arginine-rich motif **K/R**-X(3,7)-**K**-X-**K/R**-**K/R**, apart from the well-characterised $PI(4,5)P_2$-binding domains, such as Pleckstrin homology (PH). The analogy between the β2-β3 loop of the plant defensins and the lysine/arginine-rich motifs of the nuclear phosphoinositide effectors is certainly conspicuous, suggesting a potentially novel $PI(4,5)P_2$-bias interacting motif. Indeed, human β-defensin 2 (HBD-2) with its cysteine-flanking P**RR**Y**K**QIGT motif also forms a claw-like shaped dimer (Hoover et al, 2000), which strikingly resemble that of the plant defensins (**Figure 1.9**). Unpublished data from Hulett laboratory (La Trobe University) demonstrated that HBD-2 is able to induce cancer membrane permeabilisation, albeit at a high concentration, and bind preferentially to PIPs, including $PI(4,5)P_2$ (Phan, 2012) . Therefore, HDPs with this motif could possibly be exploited as novel anticancer therapeutics, and even as combined anticancer/anti-infectives, considering their antimicrobial activity as well as the

indisputable importance of $PI(4,5)P_2$ in pathogen invasion. In fact, it has long been proposed that cell-permeabilising HDPs act mainly on cell membranes via receptor-independent pathway(s), potentially hindering cancer cells from acquiring drug resistance (Chen *et al*, 2014) and thus imparting HDPs with anticancer activities favourable for therapeutic development.

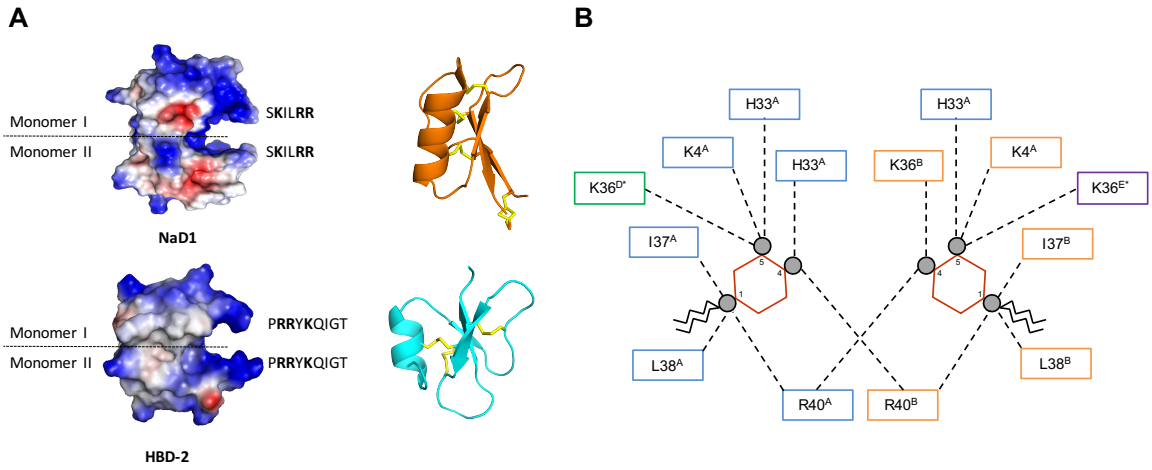

Figure 1.9 Novel phosphoinositide-targeting host defense peptides

(**A**) Dimeric (space-filled model, left) and monomeric (ribbon model, right) defensin structure. NaD1 (PDB 4CQK; Poon *et al*, 2014) and HBD-2 (PDB 1FD3; Hoover *et* al, 2000) display similar juxtaposition of α-helix and anti-parallel triple-stranded β-sheet. Both dimers show a positively-charged claw structure (grip), formed by two cationic loops of the defensin monomers. Electrostatic potential (positive (blue) and negative (red)) is indicated. The side chain of the disulfide bonds are indicated and coloured in yellow. (**B**) Schematic representation of NaD1 and $PI(4,5)P_2$ interaction within the NaD1 dimer grip, involving residues within and around the SKILRR loop from neighbouring NaD1 monomers (adapted from Poon *et al*, 2014).

The molecular basis of the specificity towards cancer cells by cationic HDPs and defensins such as NaD1 and TPP3 (Poon et al, 2014; Baxter et al, 2015) is unclear but may be associated with morphological changes of plasma membranes upon cancer transformation to influence their robust growth, motility, invasion and metastasis, as opposed to normal cells (Hoskin & Ramamoorthy, 2008). Common features of cancer cells are their increased negatively-charged phospholipid (Utsugi et al, 1991; Ran & Thorpe, 2002) and glycoprotein (Blackhall et al, 2001; Hollingsworth & Swanson, 2004) content in the membrane outer leaflet, increased membrane surface area (Yamazaki et al, 2005) and membrane fluidity (Barnett et al, 1974), all of which may contribute to enhanced affinity of membrane interaction, and cationic HDP-mediated cytotoxicity. It is also interesting to note that PIPs, particularly $PI(4,5)P_2$ and $PI(3,4,5)_3$, have key signalling

functions and crucial roles in the regulation of cell survival, growth, proliferation, invasion and metastasis, processes that are critical in tumourigenesis (Katso et al, 2001; Engelman et al, 2006; Wang et al, 2007b; Bunney & Katan, 2010; Yamaguchi et al, 2010). PIP levels could thus be expectedly upregulated in cancer cells.

The blockade of PIPs (particularly $PI(4,5)P_2$) has shown, though preliminary, some antiviral outcomes. For example, anti-$PI(4,5)P_2$ antibodies can neutralise HIV-1 by preventing virion release from host cells through disruption of Gag membrane localisation, and also triggering chemokine production, which in turn binds to chemokine receptor CCR5 to further inhibit virion binding (Matyas et al, 2010; Jobe et al, 2012). Furthermore, 30-year-old studies showed that the well-known $PI(4,5)P_2$-sequestering molecule, neomycin, can inhibit $PI(4,5)P_2$-dependent HSV entry (Langeland *et al*, 1987). The polycationic aminoglycosides constitute a large family of potent traditional protein synthesis-inhibiting antibacterial compounds, including tobramycin, kanamycin, amikacin, netilmicin, neamine and neomycin. They share a common neamine core, comprised of 1,4-O-glycosidically-linked glucosaminopyranose (ring I) and aminocyclitol (2-deoxystreptamine; ring II). The ring II is further conjugated with additional amino sugar moieties at its position 5 or 6, resulting in 4,5- and 4,6-disubstituted groups of aminoglycosidic antibiotics. The neamine core is not only responsible for bacterial 16S rRNA binding, but also readily chemically modifiable and thus favourable for drug design (Chou et al, 2004; Konno et al, 2004). Like neomycin, many other aminoglycosides appears to interact with $PI(4,5)P_2$ and neutralise its negative charge (Schacht, 1979; Marche et al, 1983; Gabev et al, 1989; Arbuzova et al, 2000; Wang et al, 2005; Jiang et al, 2006). Interesting, they also increase membrane permeability in $PI(4,5)P_2$-containing liposomes (Au et al, 1987), similar to the effect of the plant defensins. Moreover, neomycin blocks $PI(4,5)P_2$ signalling, including $PI(4,5)P_2$ hydrolysis by phospholipase C (PLC) activation (Wang et al, 2005). Kanamycin also disturbs the balance between $PI(4,5)P_2$ and $PI(3,4,5)P_3$, as well as impair PI3K/Akt signalling (Jiang et al, 2006). Despite their established ototoxicity and nephrotoxicity, aminoglycosides with their versatile neamine core, might provide a productive starting point for anticancer and anti-infective drug design via $PI(4,5)P_2$ targeting. Additional investigation is therefore required to demonstrate the importance of neamine core in $PI(4,5)P_2$ binding and anticancer/antimicrobial activities.

Targeting PIP-metabolising enzymes and PIPs represents both an opportunity and a challenge for anticancer and anti-infective therapies. However, despite promising therapeutic progression, particularly in targeting PIP-metabolising enzymes, further substantial efforts are needed. Additional understanding of isoform preference, disease-specific enzymes, immunological effects or difference in PIP profiles between normal and disease agents, is important to enable the development of PIP-targeting anticancer/anti-infective compounds with greater focused efficacy and lower toxicity.

1.6 Nature and scope of this thesis

Multidrug resistance and adverse off-target toxicity of current therapeutics have urgently called for less toxic, more effective and multifaceted therapeutics with new modes of action. Furthermore, owing to the emerging pathological association between cancer and pathogenic infection, there is also a requirement for combinatorial anticancer and anti-infective agents, especially for patients suffering infective agent-induced malignancies or undergoing immunocompromising anticancer treatments. As PIP and PIP-metabolising enzymes crucially control multiple stages of malignant transformation and microbial infection, they offer an attractive opportunity for therapeutic targeting.

Until the onset of this work, proposed or currently-developing therapeutic agents for PIP-dependent diseases such as cancer and infections mainly inhibit PIP-metabolising enzymes. This approach is, however, likely accompanied with low potency and off-target cytotoxicity owing to the existence of multiple isoforms as well as the physiological importance and complexity of pathways governed by those enzymes (Vaillancourt *et al*, 2012; Rafii *et al*, 2015; Thorpe *et al*, 2015; Wang *et al*, 2015). By contrast, directly targeting PIPs, particularly $PI(4,5)P_2$, may offer better selectivity, rendered by the potential distinction between their physiological and pathological levels. Indeed, previous findings imply a novel $PI(4,5)P_2$ binding motif present in solanaceous plant defensins with antimicrobial and anticancer activities (Poon et al, 2014; Baxter et al, 2015), and past studies on $PI(4,5)P_2$-binding aminoglycosides (Langeland *et al*, 1986; Langeland *et al*, 1987), which can possibly be exploited for therapeutic application.

The next chapter (Chapter 2) describes the effort to identify small chemical compounds and proteins (preferentially with known antimicrobial activities) that bind PIPs, particularly $PI(4,5)P_2$, and to test their anticancer effects. Aminoglycoside testing, computational search for proteins with defensin-like cationic $PI(4,5)P_2$-binding motifs,

and PI(4,5)P_2 bead pull-down using stimulated human hematological samples were conducted. An arsenal of putative PI(4,5)P_2-binding molecules was identified, potentially useful for further therapeutic development. Among potential candidates, human β-defensin 3 (HBD-3), which displays a strikingly similar cationic motif to the solanaceous plant defensins, was demonstrated to selectively kill cancer cells at low micromolar dosage.

In Chapter 3, the importance of PI(4,5)P_2 binding and the molecular mechanism was comprehensively defined for the anticancer property of HBD-3, using a series of biochemical and cell biological assays. The plant defensin-like cationic motif of HBD-3 was demonstrated to be crucial for PI(4,5)P_2 binding and cancer cytotoxicity, suggesting a structural and mechanistic conservation among defensins.

In Chapter 4, to better understand the immunological impacts by HBD-3 upon interaction with PI(4,5)P_2, chemotactic assays, cytokine release assays and signalling activation detection were conducted, with different pharmacological inhibitors. For the first time, the interaction of a HDP with PI(4,5)P_2 was found to be essential for PI3P/Akt-dependent cytokine induction, but not the chemotactic recruitment.

Finally, the Chapter 5 features concluding remarks to provide a summary of key findings and discussions of their future perspectives, particularly the potential exploration of PI(4,5)P_2-binding molecules identified in this thesis as therapeutic agents.

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Chapter 2

Identification of potential anticancer and antimicrobial phosphoinositide-binding molecules

2.1 Introduction

Despite extensive efforts in medical research and public healthcare, cancer and infectious diseases remain the leading causes of morbidity, mortality and economic loss in Australia (Australian Bureau of Statistics, 2012) and worldwide (World Health Organisation, 2012; http://www.who.int/mediacentre/factsheets/fs310/en/). These burdens are attributable to their pathological complexity and limitations of current therapeutic approaches (Al-Benna *et al*, 2011). Most conventional anticancer and antimicrobial agents show adverse side effects due to nonspecific cytotoxicity (Cunha, 2001; Mader & Hoskin, 2006). Moreover, as such drugs similarly target one or a few key biological pathways, cancer and pathologic cells can rapidly develop multidrug resistance (Al-Benna *et al*, 2011; Bush *et al*, 2011). Also of concern is that one in six new cancer cases were attributable to carcinogenic infective agents in 2012 (Plummer *et al*, 2016). In addition , cancer patients have a greater tendency to acquire severe opportunistic infections (Barnes & Stallard, 2001; Safdar & Armstrong, 2001; Uzun *et al*, 2001; Safdar *et al*, 2011; Baden *et al*, 2012; Zembower, 2014). Therefore, combined and multifaceted anticancer/anti-infective agents have been enthusiastically proposed as the new therapeutic generation (Al-Benna *et al*, 2011; Winter & Wenghoefer, 2012).

Phosphoinositides (PIPs) comprise seven natural phosphorylated derivatives of phosphatidylinositol (PI). Despite their low abundance, PIPs play important regulatory roles for diverse cellular processes, including, but is not limited to, cytoskeletal rearrangement, membrane trafficking and, cellular signalling as direct or secondary messengers (Di Paolo & De Camilli, 2006; Balla, 2013). Given their structural and functional importance, it is unsurprising that PIPs and their tightly regulated metabolism have recently emerged as exploitable targets for pathogen and cancer cell to facilitate their invasion, immunosurveillance escape, replication and proliferation (Pizarro-Cerda & Cossart, 2004; Bunney & Katan, 2010; Balla, 2013). PI(4,5)P_2 and PI(3,4,5)P_3 are localised on plasma membrane and their metabolism and/or signalling pathways are often hijacked to promote pathogen uptake by phagocytosis (*e.g. Mycobacterium tuberculosis*), macropinocytosis (*e.g. Legionella pneumophila, Trypanosoma cruzi* and *Toxoplasma gondii*), other receptor-mediated PI(4,5)P_2-associated or PI3K-Akt signalling-stimulated translocations (*e.g. Listeria monocytogenes*, *Shigella flexneri*, *Salmonella enterica*, and Ebola virus) (Pizarro-Cerda & Cossart, 2004; Saeed *et al*, 2008; de Carvalho *et al*, 2015; Pizarro-Cerda *et al*, 2015). In addition, certain pathogens manipulate endosomal PI(3)P and PI(4)P production or function to establish their replicating compartments as observed

in *S. enterica*, *L. pneumophila, S. flexneri* and hepatitis C virus (HCV) (Hilbi, 2006; Bishe *et al*, 2012; Pizarro-Cerda *et al*, 2015). $PI(4,5)P_2$ is also exploited by HIV-1 virus via its matrix protein Gag to facilitate virion release from infected host cells (Ono *et al*, 2004; Saad *et al*, 2006; Chan *et al*, 2008). Furthermore, $PI(4,5)P_2$, $PI(3,4,5)P_3$, and their metabolising kinase (PI3K) and phosphatase (PTEN), and associated signalling pathways, particularly protein kinase B (Akt), have been implicated in cancer pathogenesis and progression. PI3K signalling hyperactivation and PTEN supression promote tumour cell survival, proliferation, invasion, metastasis and angiogenesis (Liu *et al*, 2009; Bunney & Katan, 2010).

Therefore, PIPs, their metabolising enzymes and signalling pathways could be potential targets for anti-infective and anticancer therapeutics. In fact, anti-$PI(4,5)P_2$ antibody neutralises HIV-1 by preventing virion release from host cells, and also triggering chemokine production, which in turn binds to the chemokine receptor CCR5 to further inhibit virion binding (Matyas *et al*, 2010; Jobe *et al*, 2012). HCV replication is impaired upon suppression of the PI(4)P-generating enzyme PI4KIIIβ (Zhang *et al*, 2012). Furthermore, inhibition of PI3K-Akt pathway using PI3K, Akt or downstream effector mTOR inhibitors, some of which are currently advanced to clinical trials, attenuates pathogen uptake as well as arrests tumour progression (Meuillet *et al*, 2004; Hay, 2005; Faivre *et al*, 2006; Marone *et al*, 2008). However, targeting PI3K-Akt pathway presents not only promising therapeutic opportunities but also immense challenges, particularly for cancer therapy due to redundancy of PI3K isoforms and signalling pathway interconnection (Marone *et al*, 2008; Liu *et al*, 2009; Bunney & Katan, 2010).

Recently, a few PIP-binding proteins have been reported to induce cell death upon interaction with PIPs, particularly $PI(4,5)P_2$. Mixed lineage kinase-like (MLKL) pseudokinase is an effector of tumour necrosis factor (TNF)-induced programmed necrosis, or necroptosis, characterised by calcium influx, membrane damage and release of inflammatory damage-associated molecular patterns (Sun *et al*, 2012; Wang *et al*, 2014). MLKL homotrimerises once activated, and translocates to plasma membrane by binding to $PI(4,5)P_2$ through a patch of surface basic amino acids to trigger cell death (Sun *et al*, 2012; Cai *et al*, 2014; Dondelinger *et al*, 2014; Wang *et al*, 2014). Similarly, in order to respond to bacterial infection and danger signals, caspase-cleaved gasdermin D undergoes oligomerisation, inner plasma membrane binding by preferential PIP interaction, and pore formation to trigger inflammatory cell death (pyroptosis) and

inflammatory cytokine release (Liu *et al*, 2016). Furthermore, antifungal defensins *Nicotiana alata* defensin 1 (NaD1) and tomato defensin TPP3, which are host defense peptides (HDPs), from solanaceous plants, have been showed to display broad spectrum anticancer activity via PI(4,5)P_2-mediated membrane permeabilisation, membrane blebbing and eventually to necrotic lysis (Poon *et al*, 2014; Baxter *et al*, 2015). NaD1 and TPP3 have been shown to bind PI(4,5)P_2 via their conserved cysteine-flanked highly-positively charged β2-β3 loop (^{35}S**K**IL**RR**^{40} in NaD1 and in ^{37}S**K**LQ**RK**^{42} in TPP3) (Poon *et al*, 2014; Baxter *et al*, 2015). These plant defensins form dimers, of which the two β2-β3 loops form a claw-like structure to bind PI(4,5)P_2, which promote defensin oligomerisation. The protein-lipid interaction involves an intensive hydrogen bonding network provided by residues within and around the β2-β3 (Poon *et al*, 2014; Baxter *et al*, 2015). Interestingly, in a proteomic study by Lewis *et al* (2011), a large number of identified PI(4,5)P_2-binding proteins were found to possess a lysine/arginine-rich motif **K/R**-X(3,7)-**K**-X-**K/R**-**K/R**, that was distinct from the well-characterised PI(4,5)P_2-binding domains, such as Pleckstrin homology (PH). In particular, the similarity between the β2-β3 loop of the plant defensins and the lysine/arginine-rich motifs of the nuclear phosphoinositide effectors is conspicuous. Human β-defensin 2 (HBD-2) with its cysteine-flanking P**RR**Y**K**QIGT motif also forms a claw-like shaped dimer (Hoover *et al*, 2000), that shows striking resemblance to that of the plant defensins. Unpublished work from the Hulett laboratory has demonstrated that HBD-2 induces membrane permeabilisation, albeit at high μM concentrations, and binds preferentially to PIPs, including PI(4,5)P_2 (Phan, 2012). These observations suggest that PI(4,5)P_2 and other PIPs could possibly be targeted to induce tumour and pathogen cell death, but also that PIP-binding antimicrobial peptides (AMPs) may be exploited as novel multifunctional anticancer/anti-infective therapeutics.

Furthermore, polycationic aminoglycosides, which constitute a large family of potent well-characterised protein synthesis-inhibiting antibacterial compounds, also bind to phosphoinositides, neutralise their negative charge and modulate their metabolism and cellular activities (Schacht, 1979; Marche *et al*, 1983; Gabev *et al*, 1989; Arbuzova *et al*, 2000; Wang *et al*, 2005; Jiang *et al*, 2006). They share a common neamine core, comprised of 1,4-*O*-glycosidically-linked glucosaminopyranose (ring I) and aminocyclitol (2-deoxystreptamine; ring II). The ring II is further conjugated with additional amino sugar moieties at its position 5 or 6, resulting in 4,5- and 4,6-disubstituted groups of aminoglycosidic antibiotics (**Figure 2.1**). The former category includes, but is not limited

to, neomycin and paramomycin, which are structurally different only by the substitution of 6-amino ($–NH_2$) to 6-hydroxyl (–OH) group on glucosaminopyranose ring (ring I). The latter category is comprised of most clinically-used broad-spectrum antimicrobial agents, such as kanamycin, gentamicin, amikacin and tobramycin (Shalev & Baasov, 2014). With exceptions of streptomycin and hygromycin lacking the ring I, the neamine core is not only responsible for bacterial 16S rRNA binding, but also readily chemically modifiable and thus favourable for drug design (Chou *et al*, 2004; Konno *et al*, 2004). Neomycin and other aminoglycosides, including tobramycin, kanamycin, amikacin, netilmicin and neamine, were demonstrated to increase membrane permeability in $PI(4,5)P_2$-containing liposomes (Au *et al*, 1987), though only neomycin was explicitly showed to bind to $PI(4,5)P_2$ (Schacht, 1979). Moreover, neomycin blocks $PI(4,5)P_2$ signalling, including $PI(4,5)P_2$ hydrolysis by phospholipase C (PLC) activation (Wang *et al*, 2005). Kanamycin also disturbs the balance between $PI(4,5)P_2$ and $PI(3,4,5)P_3$, as well as impair PI3K/Akt signalling (Jiang *et al*, 2006). Therefore, aminoglycosides and their derivatives represent attractive candidates for drug development targeting PIP-dependent infection and cancer.

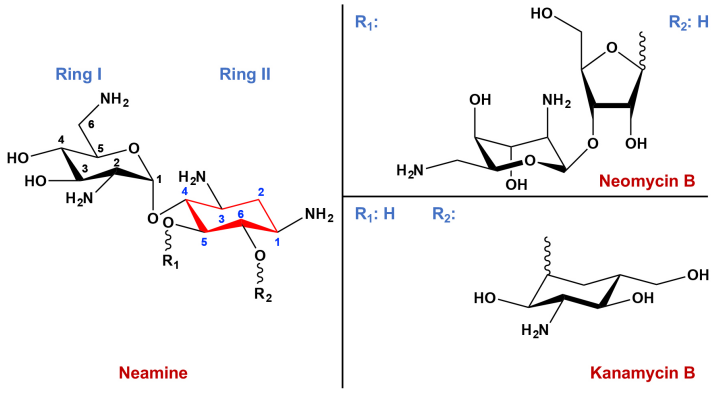

Figure 2.1 Neamine core of aminoglycosides.
Its constituent rings are explicitly showed and numbered. Neamine is flexibly conjugated with additional moieties via position 5 or 6 of rings, as exemplified by 4,5- (neomycin) and 4,6-disubstituted (kanamycin) aminoglycosides

Considering the abovementioned implications of PIPs in pathogen infection and cancer progression as well as therapeutic potential of PIP-targeting molecules, this chapter was aimed to identify candidates that bind PIP, particularly $PI(4,5)P_2$, and display antimicrobial and anticancer activity. To this end, aminoglycoside testing, computational searches for plant defensin-like or HBD-2-like cationic motifs and $PI(4,5)P_2$ bead pull-downs using human hematological samples, were employed. It was demonstrated for the first time that small $PI(4,5)P_2$-binding aminoglycoside antibiotics, particularly kanamycin and neomycin, exhibit considerable tumour cell-killing activity, hence suggesting the importance of neamine core for further anticancer drug design. Furthermore, a number of human β-defensins and arachnid toxins were found with the cationic motifs. In particular,

HBD-3 was identified as a promising candidate, due to the pattern similarity and structural analogy to plant defensins NaD1 and TPP3. Indeed, HBD-3 was shown to bind preferentially to PIPs including $PI(4,5)P_2$ *in vitro* and on the plasma membrane as well as exhibit selective anticancer activity. Additionally, a number of antimicrobial $PI(4,5)P_2$-binding candidates from $PI(4,5)P_2$ bead pull-down of human plasma and infection-mimicking stimulation were identified, implying that targeting $PI(4,5)P_2$ and other PIPs may be a natural mechanism to combat microbial infection. Taken together, this study has identified an arsenal of PIPs and $PI(4,5)P_2$-targeting molecules with both antimicrobial and anticancer activity, potentially useful for further therapeutic development.

2.2 Materials and methods

2.2.1 Cell cultures, aminoglycosides and plant defensins

Human epithelial cervical cancer (HeLa), leukemic monocyte lymphoma (U937), prostate cancer (PC3), promyelocytic leukemia (HL-60), leukemic T cell lymphoblast (Jurkat) cells were cultured in RPMI-1640 medium supplemented with 5–10% (v/v) fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, CA, USA). Human umbilical vein epithelial cell (HUVEC) cells were cultured in M199 medium (Invitrogen) supplemented with 20% (v/v) FCS, 40 μg/mL gentamicin, 4 μg/mL endothelial cell growth factor, 4 μg/mL L-glutamine and 135 μg/mL heparin, in 0.1% (w/v) gelatin-coated Corning CELLBIND flask (Corning Life Sciences, MA, USA). Adult human dermal fibroblast (AHDF) and human coronary artery smooth muscle cell (CASMC) cells were cultured using FGM™-2 BulletKit™ and SmGM™-2 BulletKit™ (Lonza, MD, USA). All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Gentamicin, kanamycin, neomycin, paromomycin and streptomycin were purchased from Sigma-Aldrich (MO, USA). All aminoglycosides were reconstituted in appropriate assay buffer or medium before use. Recombinant NaD1 was kindly provided by Dr Fung Lay (La Trobe University, Australia).

2.2.2 Preparation of human serum and peripheral blood mononuclear cells

Fresh buffy coat was obtained from the Australia Red Cross Blood Service (Melbourne, Australia) with an appropriate ethics approval (FHEC09/16) from La Trobe Human Ethics Committee. Buffy coat was diluted 1:3 in endotoxin-free phosphate buffered saline (PBS; Ca^{2+}/Mg^{2+}-free, Life Technologies, CA, USA) with endotoxin-low 2 mM

ethylenediaminetetraacetic acid (EDTA; Merck Millipore, Germany) prior to layering over Ficoll-Paque PLUS (GE Healthcare, UK) and centrifugation (400 *g*, room temperature, 30 min, no deceleration). Human serum and peripheral blood mononuclear cell (PBMC) layers were separately collected. To remove platelet contaminants, PBMCs were washed three times with PBS containing 0.5% endotoxin-free bovine serum albumin (BSA; Merck Millipore) and 2 mM EDTA at 200 *g* for 10 min.

2.2.3 Isolation of human polymorphonuclear cells

Human polymorphonuclear cells (PMNs) were isolated as described by Nauseef (2007), with few minor modifications. Briefly, fresh buffy coat, diluted 1:5 in endotoxin-free PBS supplemented with 2 mM EDTA, was mixed with 3% (w/v) aqueous *Leuconostoc mesenteroides* dextran (Sigma-Aldrich) at 1:1 volume ratio and allowed to sediment for 20 min at room temperature. The erythrocyte-depleted upper layer was collected and subjected to centrifugation at 500 *g* for 10 min at 4°C. The leukocyte pellet was subsequently resuspended in 0.9% (w/v) NaCl and overlaid on Ficoll-Paque PLUS. After centrifugation (400 *g*, room temperature, 30 min, no deceleration), the lower and denser band with enriched PMN-erythrocytes was retained and subjected to hypotonic lysis to remove erythrocytes by adding sterile miliQ water for exactly 30 s. The tonicity was promptly restored with equal volume of 1.8% NaCl. PMNs were collected by centrifuging at 500 *g* for 5 min at 4°C.

2.2.4 Lipopolysaccharide stimulation

PBMC or PMN were seeded at 1×10^6 cells/mL in 6-well plate in serum-free RPMI-1640 medium and stimulated with 50 ng/mL *Escherichia coli* lipopolysaccharides (LPS; Sigma-Aldrich) for 18 h. Culture supernatants were collected after centrifugation at 350 *g* for 10 min at room temperature.

2.2.5 Computational searches for cationic patterns

The cationic patterns, designated ‘NaD1-like’ and ‘HBD-2-like’, were designed based on cysteine-flanked β2-β3 and β1-β2 loop motifs of solanaceous plant defensins and HBD-2 respectively. Both sequence patterns were independently submitted to sequence-based ScanProsite and structure-based Protein Data Bank in Europe (PDBe) databases (**Table 2.1**). Due to the large database size, the ScanProsite search was restricted to human peptides less than 100 amino acids, which, by definition, covers the majority of HDPs.

Table 2.1 Search parameters for cationic patterns

For pattern sequences, 'C', 'R' and 'K' denote cysteine, arginine and lysine respectively. 'X' represents any amino acids. Square bracket '[]' denotes either of specified amino acids, whereas braces '{}' indicates any other amino acids, except for specified one(s). Number in bracket '()' shows the number of specified amino acids.

Parameters	ScanProsite	PDBe
Website	prosite.expasy.org/scanprosite/	www.ebi.ac.uk/pdbe/
Syntax	C-x(1,3)-[RK]-x(1,2)-[RK]-[RK]-x(0,4)-C	
	C-x(1,3)-[RK]-[RK]-x(1,2)-[RK]-x(0,4)-C	
Database	UniProt	RCSB Protein Data Bank
Organism	*Homo sapiens*	No restriction
Size	Less than 100 amino acids	No restriction
Secondary structure	Not applicable	Strand-loop-strand
Relative position	Not applicable	Pattern sequence within set structure
Other filters	Not applicable	Loop less than 9 residues

2.2.6 Sequence alignment and sequence logo

Alignment of nucleotide or protein sequences were performed using built-in ClustalW Multiple Sequence Alignment tool of BioEdit sequence alignment editor (version 5.09) software (Hall, 1999). Sequence logo of mammalian β-defensin 3 homologues were generated by submitting their sequence alignment to Weblogo tool page (http://weblogo.berkeley.eu).

2.2.7 PI(4,5)P_2 bead pull-down

PI(4,5)P_2-coated agarose beads and control beads (Echelon Biosciences, UT, USA) were prepared by calibration with ice-cold assay buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 0.25 % IGEPAL® CA-630, complete protease inhibitor cocktail (all from Sigma-Aldrich) and 150 mM NaCl) and centrifugation at 100 *g* for 2.5 min at 4°C. After passing through 0.22 μm Millex-GV syringe filter units (Merck Millipore) to remove cell and debris contaminants, 10 μg total protein from human serum or culture media of LPS-stimulated PBMCs or PMNs (estimated using BCA protein assay kit (Thermo-Fisher Scientific Pierce, IL, USA)) was added to 100 μL of PI(4,5)P_2 beads in assay buffer. At the end of 4 h incubation at 4°C, beads were washed five times with excess assay buffer prior to liquid chromatography-electrospray ionisation-tandem mass spectrometry analysis (LC-ESI-MS/MS), which was done with the assistance of Dr Pierre Farou (Comprehensive Proteomics Platform, La Trobe University). Bead samples were resuspended in digestion

buffer (8M urea, 50 mM $(NH_4)_2CO_3$ pH 8.5, 10 mM dithiothreitol (DTT)) and incubated for 5 h at room temperature. Thiol-alkylating agent iodoacetamide was then added to final concentration of 55 mM (35 min, 20°C, in the dark). The alkylated preparation was diluted to 1 M urea using 25 mM (NH4)2CO3 (pH 8.5) prior to overnight incubation with 5 μM sequencing grade trypsin (Promega) at 37°C. The digests were acidified with 1% (v/v) trifluoroacetic acid (TFA; Sigma-Aldrich), then desalted on Empore™ SDS-XC StageTips as previously described (Rappsilber *et al*, 2007). Peptides were reconstituted in 0.1% (v/v) TFA and 2% (v/v) acetonitrile (ACN) and loaded onto a C_{18} PepMap 100 μm ID × 2 cm trapping column (Thermo-Fisher Scientific) at 5 μL/min for 6 min, followed by a 6 min wash before switching in line the analytical column (Vydac MS C_{18}, 3 μm, 300 Å and 75 μm ID × 25 cm; Grace, MD, USA). Peptide separation was performed at 300 nL/min using non-linear ACN gradient of buffer A (0.1% (v/v) formic acid, 2% (v/v) ACN) and buffer B (0.1% (v/v) formic acid, 80% CAN), starting at 5% to 55% of buffer B over 120 min. Data were collected on an Orbitrap Elite (Thermo-Fisher Scientific) using MS scan range of *m/z* 300-1500, and collision-induced dissociation (CID) MS spectra were collected for 20 of the most intense ions. Returned hits were analysed by comparing $PI(4,5)P_2$ beads to respective control beads to statistically determine fold enrichments.

2.2.8 *Pichia pastoris* expression of human β-defensin 3

A DNA fragment encoding the mature HBD-3 protein was PCR amplified from human blood-derived genomic DNA using Phusion DNA polymerase (0.02 U/μL) (Finnzymes, Finland), 1× Phusion™ High-Fidelity buffer containing 1.5 mM $MgCl_2$ and dNTPs (200 μM each). PCR was performed with the following temperature profile: an initial cycle of 98°C, 30 s; 30 cycles of 98°C, 30 s, 55°C, 30 s; 72°C, 30 s; and a 10 min final extension cycle at 72°C.

The reaction product(s) were loaded directly onto a 1% (w/v) agarose gel in 1× TBE buffer (50 mM Tris base, 100 mM borate, 10 mM EDTA, pH 8.2) and subjected to gel electrophoresis. A prominent band under 200 bp was excised and purified using Wizard® SV Gel and PCR Clean-up system (Promega, WI, USA). The amplified sequence was initially cloned into the pCR2.1-TOPO vector using TOPO® TA cloning® kit (Invitrogen, CA, USA), and subsequently subcloned into the pPIC9 expression vector (Invitrogen), directly in-frame with the yeast α-mating factor secretion signal. Nucleotide sequencing was carried out to confirm identity of the recombinant sequence and

performed at the Australian Genome and Research Facility (Melbourne, Australia). Sequencing result were analysed using the BioEdit sequence alignment editor. After transformation into *Escherichia coli* TOP10 cells, the recombinant pPIC9-HBD3 plasmid was isolated using PureLink™ Quick Plasmid Miniprep kit (Invitrogen), and linearised with *Sal*I (New England Biolabs, MA, USA) to allow integration at the *his4* locus of the methylotrophic yeast *Pichia pastoris* genome. The linearised DNA was then transformed into electrocompetent yeast GS115 as described by Chang *et al* (2005), and His^+ transformants were selected by plating onto MD agar (1.34% YNB, 4×10^{-5}% biotin, 1% dextrose and 1.5% agar). Recombinant HBD-3 was expressed and purified from a single pPIC9-HBD3 transformant as previously described for a plant defensin from tomato (Lay *et al*, 2012b).

2.2.9 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining

All reagents and apparatuses used in SDS-PAGE were purchased from Thermo-Fisher Scientific, unless otherwise specified. Protein samples were heated at 70°C for 10 min, in presence of 1 × NuPAGE® LDS sample buffer and 0.1 M dithiothreitol, and directly loaded on pre-cast NuPAGE® 4-12% Bis-Tris gel and electrophoresised at 200 V for 35 min in XCell SureLock™ Electrophoresis Cell with 1 × NuPAGE® MES SDS as running buffer. The proteins were visualised by Coomassie Brilliant Blue staining (0.4% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad, CA, USA), 20% (v/v) ethanol, 7% (v/v) glacial acetic acid), and compared to molecular weight marker (SeeBlue® Plus2 pre-stained standard).

2.2.10 Western transfer and immunoblotting

Following SDS-PAGE, protein gels were transferred onto nitrocellulose membrane (Bio-Rad, Germany), using XCell II™ Blot Module with NuPAGE® transfer buffer. The membrane was blocked with 5% (w/v) skim milk in PBS containing 0.1% (v/v) Tween®-20, before incubating with polyclonal rabbit anti-HBD-3 antibody (0.25 µg/mL; Abcam, MA, USA). Bound antibody was detected using donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase (0.25 µg/mL; Abcam) and enhanced chemiluminescence detection reagents (GE Healthcare Biosciences).

2.2.11 Circular dichroism (CD) spectroscopy

Aqueous solution (300 µL) of HBD-3 at 150 µg/mL was prepared in 1 mm quartz cuvettes. Far-UV CD spectra were recorded in triplicate at 1 nm increments with 2 s

averaging time between 190 and 250 nm, using an AVIV 420 CD spectrometer. After normalising against blank water scan, data were analysed using the CONTILL algorithm on the SP29 database from the CDPro software (Sreerama & Woody, 2000).

2.2.12 Proton nuclear magnetic resonance spectroscopy (NMR) and two-dimensional nuclear Overhauser effect spectroscopy (2D NOESY)

The proton NMR and 2D NOESY experiments were performed by Dr Mark Hinds (La Trobe University) as described elsewhere (Kumar *et al*, 1980; Gong *et al*, 2007; Tawani & Kumar, 2015). Briefly, HBD-3 was dissolved in 20 mM sodium acetate (pH 5.5) and 5% heavy water D_2O. Proton NMR and 2D NOESY spectra were acquired in a Bruker Avance IIID spectrometer (Bruker Corporation, MA) equipped with cryoprobes, operating at 700 MHz, 298 K and mixing time of 150 ms (for 2D NOESY).

2.2.13 Anti-PI(4,5)P_2 antibody blocking assay

U937 cells at 1×10^6 cells/mL were treated with 10 mM aminoglycosides for 3 h, or 15 μM defensins for 30 min in serum-free RPMI 1640 medium containing 0.1% BSA (Sigma-Aldrich), followed by 20 min fixation with 2% (v/w) paraformaldehyde (Sigma-Aldrich), 10 min lysis with 0.5% (w/v) saponin (Sigma-Aldrich), and then 30 min blocking with 3% (w/v) BSA. Three 5-min centrifugal washes with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at 500 *g* were also included between steps. Fluorescein isothiocyanate (FITC)-conjugated mouse anti-PI(4,5)P_2 IgM (Echelon Biosciences) or IgM control (GeneTex, Irvine, CA) was subsequently used at 10 μg/mL for 30 min prior to flow cytometry analysis, using BD FACSCanto II Flow Cytometer and BD FACSDiva Software v6.1.1 (BD Biosciences, San Jose, CA). For each flow cytometry reading, 10,000 cells, gated appropriately based on forward scattering and side scattering, were recorded. The resultant data were processed using FlowJo software (Tree Star, San Carlos, CA) to determine mean FITC fluorescence intensity.

2.2.14 Annexin V blocking assay

Blocking of annexin V binding assay was conducted similarly to anti-PI(4,5)P_2 antibody blocking assay. However, fixed and lysed cells were instead stained with annexin V-FITC (BD Biosciences, CA, USA) diluted 1:200 in 1× annexin V binding buffer for 10 min prior to flow cytometry analysis.

2.2.15 Cell viability assay

Different concentrations of aminoglycosides or HBD-3 were added to cells seeded in 96-well plates (Greiner Bio-One, Austria) in appropriate complete medium. Initial plating cell density was pre-optimised to avoid confluence at endpoint. After 48 h, cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, MO) for adherent cells (HeLa, PC3, HUVEC, AHDF, CASMC) or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, Madison, WI) coupled with phenzine methosulfate (PMS) (Promega) for suspension cells (U937, HL-60, Jurkat, PBMC) prior to absorbance measurement at 570 nm and 490 mm, respectively. Absorbance readings of untreated control wells was designated as 100% cell viability. IC_{50} values were determined using GraphPad Prism 5 (GraphPad Software, CA, USA).

2.2.16 Inhibition of NaD1-induced propidium iodide uptake assay

U937 cells suspended at 1×10^6 cells/mL in serum-free medium containing 0.1% (w/v) BSA were pre-treated with 10 mM aminoglycosides followed by three centrifugal washes with PBS at 500 *g* for 5 min prior to incubation with 10 μM NaD1 at 37°C for 30 min. Nucleic acid stain propidium iodide (PI) was then added to a final concentration of 1 μg/mL and cells subjected to flow cytometry analysis. Data were processed to determine PI-positivity, which reflects the level of membrane permeabilisation.

2.2.17 Protein-lipid overlay assay

Protein-lipid overlay assays using Membrane strip^{TM} or PIP strip^{TM} (Echelon Biosciences, Salt Lake City, UT) were performed using 1 μg/mL proteins as described previously (Poon *et al*, 2014) . Lipid binding was immunodetected using a combination of rabbit anti-HBD-3 (50 μg/mL) and horseradish peroxidase-conjugated donkey-anti-rabbit Ig (10 μg/mL) antibodies. Chemiluminescence signal intensity was quantitated by densitometry analysis using ImageJ (National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/).

2.3 Results

2.3.1 $PI(4,5)P_2$ binding and inhibition of NaD1-induced membrane permeabilisation by aminoglycosides

In addition to its essential regulatory roles for a great number of diverse cellular processes, $PI(4,5)P_2$ is a key membrane target in cell death mediated by necrosis,

necroptosis or pyroptosis (Dondelinger *et al*, 2014; Poon *et al*, 2014; Baxter *et al*, 2015; Liu *et al*, 2016). In particular, the solanaceous plant defensins NaD1 and TPP3 selectively induce membrane permeabilisation and cytolysis in tumour cells (Poon *et al*, 2014; Baxter *et al*, 2015). Neomycin, a highly positively-charged aminoglycoside antibiotic, also binds $PI(4,5)P_2$ with high affinity and neutralises its negativity (Schacht, 1976; Schacht, 1978; Gabev *et al*, 1989; Arbuzova *et al*, 2000). Therefore, it was of interest to investigate its ability to bind $PI(4,5)P_2$ and to induce membrane permeabilisation of neomycin and other aminoglycosides (gentamicin, kanamycin, paromomycin and streptomycin).

A flow cytometry-coupled immunodetection assay using FITC-conjugated anti-$PI(4,5)P_2$ IgM was first used to determine if aminoglycosides interacted with $PI(4,5)P_2$ on tumour cell membrane. This assay was based on the premise that the interaction of aminoglycosides with $PI(4,5)P_2$ would block antibody binding to the lipid at inner plasma membrane leaflet. Indeed, NaD1, neomycin (as positive controls), and kanamycin blocked anti-$PI(4,5)P_2$ antibody binding to U937 cells, as indicated by the significant decrease of overall fluorescence intensity, compared to the untreated control (**Figure 2.2A**). Gentamincin, streptomycin, and, surprisingly, paramomycin whose structure closely resembles neomycin, only differing by the substitution of an aminomethyl ($–CH_2NH_2$) to a hydroxymethyl ($–CH_2OH$) group on glucosaminopyranose ring, did not show any change in FITC signal, suggesting minimal $PI(4,5)P_2$ binding. An isotype control was also included to indicate background binding.

None of aminoglycosides used displayed any membrane permeabilisation effect on U937 cells as suggested by minimal PI uptake after 30 min treatment (data not shown). However, kanamycin, neomycin and streptomycin were able to delay and/or diminish U937 cell membrane disruption by NaD1 (**Figure 2.2B**), with PI positivity reduced by approximately 25%, 30% and 15% respectively. Neither gentamicin nor paramomycin showed any apparent change. These data might support the correlation between $PI(4,5)P_2$ binding and blocking of NaD1-induced permeabilisation, particularly in kanamycin and neomycin.

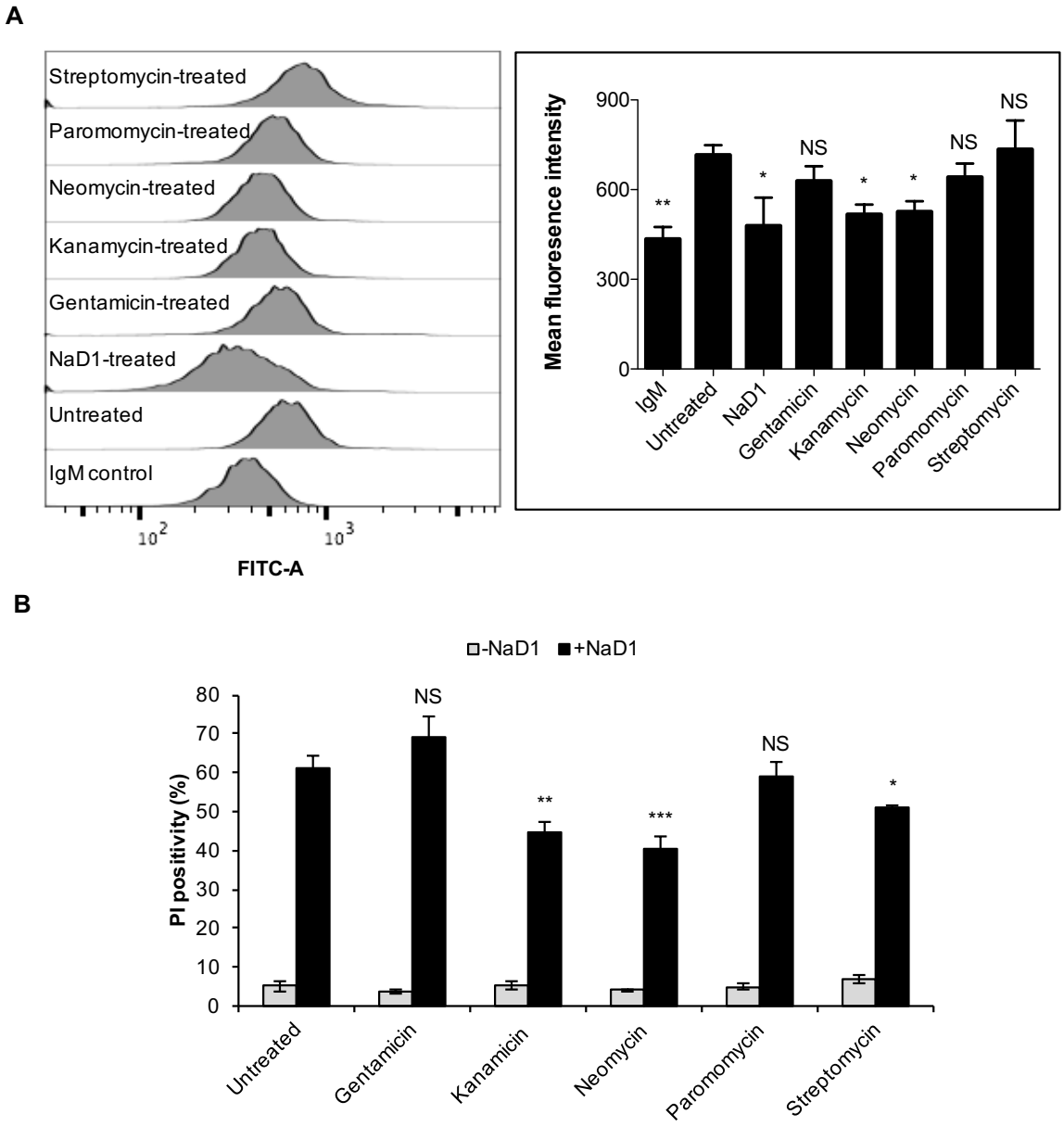

Figure 2.2 Binding of aminoglycosides to PI(4,5)P_2

(A) Blocking of anti-PI(4,5)P_2 antibody binding to plasma membrane PI(4,5)P_2 by aminoglycosides. Untreated or aminoglycoside-treated U937 cells were fixed, lysed and stained with FITC-conjugated anti-PI(4,5)P_2 IgM prior to flow cytometry analysis. (B) Inhibitory effect of aminoglycosides on membrane permeabilisation by NaD1. U937 cells were treated with aminoglycosides prior to flow cytometry-based PI uptake assay. Data in **A** and **B** represent mean ± SEM of three independent experiments, each with triplicates. P-values in **A** were calculated against the untreated control whist those in **B** were compared against NaD1 only-treated sample. NS, not significant; *, $p<0.05$; **, $p<0.01$, unpaired t-test.

2.3.2 Aminoglycoside cytotoxicity on primary and tumour cells

$PI(4,5)P_2$-binding plant defensins NaD1 and TPP3 possess specific tumour cell cytotoxicity (Poon *et al*, 2014; Baxter *et al*, 2015). Therefore, tetrazolium-based cell viability assays were performed on a number of different human tumour and primary cell lines, to investigate the effect of aminoglycosides on cell viability. All five aminoglycosides displayed cytotoxicity in the low millimolar range against all tested cell lines (**Figure 2.3**). Generally, kanamycin and neomycin were more potent on tumour cells than primary cells. In fact, IC_{50} values of kanamycin on the tumour cell lines HeLa, PC3, and U937 cells are 10-20 times lower than those on primary AHDF cells and PBMCs. Neomycin also exhibited a degree of tumour cell selectivity, albeit slightly less obvious, with IC_{50} values of 1–2 mM for tumour cells as opposed to 4-10 mM on primary cells. Paramomycin, streptomycin, and, particularly, gentamicin did not appear to display any pronounced specificity.

2.3.3 Database search for cationic putative 'lipid binding' patterns

$PI(4,5)P_2$ binding, membrane disruption and cell lytic effects of the plant defensins NaD1 and TPP3 are mediated through the highly-conserved cysteine-flanked cationic loops (S**K**IL**RR** and S**K**LQ**RK**, respectively) between β2 and β3 strands ((Poon *et al*, 2014; Baxter *et al*, 2015). Two cationic loops in a plant defensin homodimer form a claw-like structure with a $PI(4,5)P_2$-binding grip. Strikingly, HBD-2 also displays a similar dimeric cationic grip with its cysteine-flanked P**RR**Y**K**QIG motifs (Hoover *et al*, 2000). Coincidentally, it was demonstrated by unpublished work from the Hulett laboratory that HBD-2 binds preferentially to PIPs, including $PI(4,5)P_2$, and also induces bleb-associated membrane permeabilisation (Phan, 2012). Therefore, computational searches for the cationic patterns were undertaken to identify potential $PI(4,5)P_2$-binding candidates, using ScanProsite and PDBe search tools.

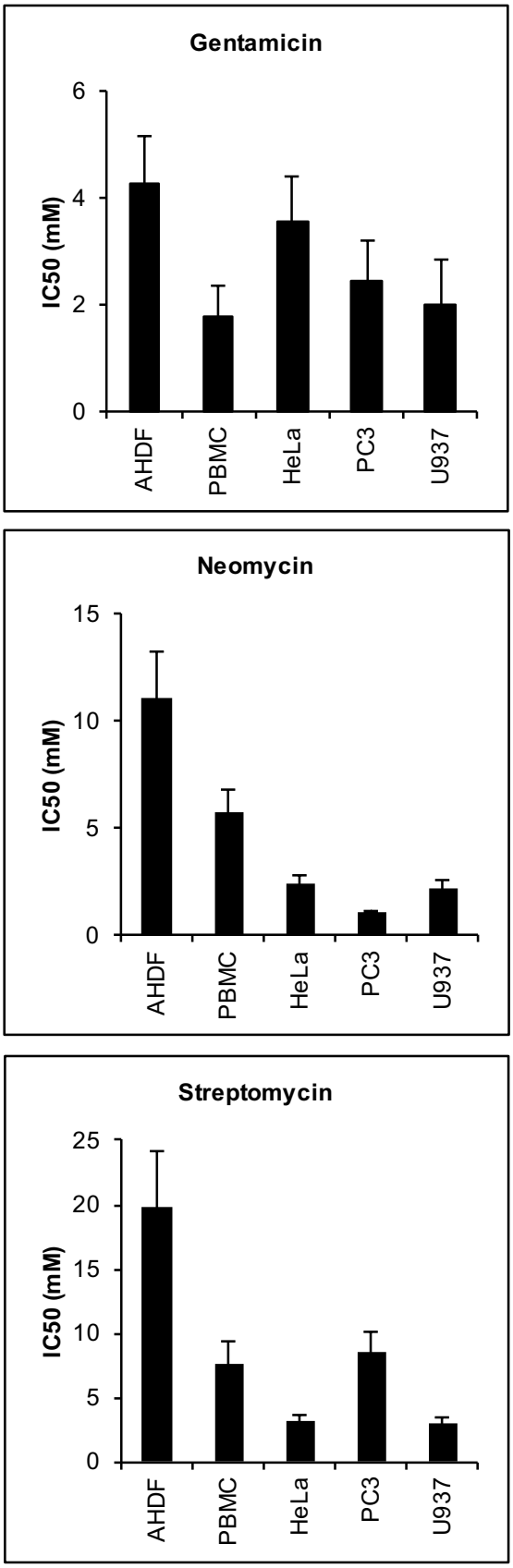

Figure 2.3 Cancer and primary cell cytotoxicity of aminoglycosides

Estimated IC_{50} of HBD-3 on different cancer (HeLa, PC3 and U937) and normal (HUVEC, ADHF and PBMC) cells, determined by tetrazolium-coupled cell viability assay. Data were normalised against untreated control, which was arbitrarily assigned as 100% cell viability. Data represent mean ± SEM of three independent experiments.

Remarkably, the majority of the search results, for both plant defensin-, or NaD1-like, and HBD-2-like patterns, are human β-defensins and scorpion toxins (**Table 2.2** and **2.3**). ScanProsite reveals HBD-3 for the NaD1-like pattern and HBD-9, HBD-14 and HBD-23 for the HBD-2-like pattern. Also, LEAP2 is another antimicrobial peptide identified possessing the cationic pattern, although with increased positivity in the motif due to two additional basic amino acids. The remaining hits, including ESRα, CKMT1 and protamine P1, albeit propitious, have not been reported with antimicrobial or anticancer activity. In agreeance, the PDBe search also identified HBD-3, suggesting that its cationic pattern (^{34}STRGRK^{39}), as defined by search parameters, localises within its strand β2-loop-strand β3 secondary structure. Similarly, nematode antibacterial factor ASABF and antiviral human θ-defensin 2 appear to have HBD-2-like patterns within their specified structures. Additionally, cardiolipin-binding β-2-glycoprotein 1 (Borchman *et al*, 1995), or apolipoprotein H, and a number of arachnid toxins (psalmotoxin-1, huwentoxin-4 and charybdotoxin) are also identified. It should be noted that, the HBD-2-like cationic pattern (S**RK**T**R**W) of huwentoxin-4 partially resembles the S**K**IL**RR** loop of NaD1, particularly the serine residue which is highly conserved among plant defensins.

HBD-3 is a promising candidate, due to the pattern similarity (as showed by ScanProsite search) and structural homology (as showed by PDBe search). Indeed, as suggested by sequence alignment, although HBD-3, NaD1 and TPP3 share relatively low sequence identity and differ in disulfide connectivity patterns and secondary structure organisation, the conservation of their cysteine-flanked cationic β2–β3 loops (ST**R**G**RK**, S**K**IL**RR** and S**K**LQ**RK** respectively), including overall loop charge (+3) and basic residue arrangement, are apparent (**Figure 2.4A**). Also, K32 that precedes the loop in HBD-3 is the equivalent to key PI(4,5)P_2-interacting residues H33 in NaD1 and H34 in TPP3, with potential H-bonding residues (R, K, S or T) at position 32 also observed amongst other mammalian homologues (**Figure 2.4B**), implying a functional importance. The HBD-2-like motifs of other β-defensin (HBD-9, HBD-14 and HBD-23), are not aligned as well with that of HBD-2 (**Figure 2.4B**). Nevertheless, it is worth noting that, based on sequence alignment, they are located within predicted loop regions, particularly the cationic motif of HBD-9 (**RRR**M**K**) which might also span between β2 and β3 strands.

Table 2.2 Consolidated search results for cationic patterns using ScanProsite database

ScanProsite search, against Uniprot database, was performed using the cationic motif patterns C-x(1,3)-[RK]-x(1,2)-[RK]-[RK]-x(0,4)-C and C-x(1,3)-[RK]-[RK]-x(1,2)-[RK]-x(0,4)-C, which were designed based on the plant defensins, including NaD1, and HBD-2 respectively. Search results were restricted to human peptides less than 100 amino acids in length. Refer to main text for further comments and references.

	Full name	Short name	UniProt entry	Motif sequence	MW (kDa)	Functions
'NaD1-like' pattern	Human β-defensin 3	HBD-3	P81534	CSTRGRKC	5.1	Antimicrobial, immune modulation
	Liver-expressed antimicrobial peptide 2	LEAP-2	Q969E1	CITRLCRKRRC	4.6	Antimicrobial
	Estrogen receptor alpha isoforms	ESRα	P03372	CTIDKNRRKSC	9.0	Regulation of gene expression
	Mitochondiral creatine kinase U-type	CKMT1	P12532	CTRVFRKGTC	10.4	Cellular energy transduction
	Testis-specific prion protein	-	Q86SH4	CNRKSKKIYC	8.6	Unknown
'HBD-2-like' pattern	Human β-defensin 2	HDB-2	O15263	CPRRYKQIGTC	4.3	Antimicrobial, immune modulation
	Human β-defensin 9	HBD-9	Q30KR1	CRRRMKC	7.4	Antimicrobial
	Human β-defensin 14	HBD-14	Q30KQ6	CTKRRYGRC	5.2	Antimicrobial, LPS neutralisation
	Human β-defensin 23	HBD-23	Q8N688	CSKKERVYVYC	5.9	Antimicrobial
	Sperm protamine P1	-	P04553	CQTRRRAMRC	6.8	DNA packaging
	Metallothionein-1H	MT-1H	P08294	CKCKKCKCTSC	6.0	Metal binding
	Scrapie-responsive protein 1	ScRG-1	O75711	CYRKILKDHNC	11.1	Nervous system development

Table 2.3 Consolidated search results for cationic pattenrs using PDBe databases

PDBe search, against Protein Database Bank, was performed using the cationic motif patterns C-x(1,3)-[RK]-x(1,2)-[RK]-[RK]-x(0,4)-C and C-x(1,3)-[RK]-[RK]-x(1,2)-[RK]-x(0,4)-C, designed based on the plant defensins, including NaD1, and HBD-2 respectively. Search results were restricted to loop-strand-loop secondary structure, which contains the query motifs. Refer to main text for further comments and references.

	Full name	Short name	PBD	UniProt entry	Motif sequence	MW (kDa)	Functions
'NaD1-like' pattern	*Nicotiana alata* defensin 1	NaD1	4AAZ	Q8GTM0	CSKILRRC	5.3	Antifungal, anticancer
	Petunia hybirda defensin 1	PhD1	1N4N	Q8H6Q1	CSKILRRC	5.2	Antifungal
	Tomato pistil predominant 3 defensin	TPP3	4UJ0	Q40128	CSKQLRKC	5.4	Antifungal, anticancer
	Human β-defensin 3	HBD-3	1KJ6	P81534	CSTRGRKC	5.1	Antimicrobial, immune modulation
	Human β-2-glycoprotein 1	Apo-H	1QUB	p02749	CKNKEKKC	36.2	Cardiolipin binding
	Tarantula psalmotoxin-1	PcTx1	4FZ0	P60514	CWKRRRSFEVC	4.7	Ion channel blocking
'HBD-2-like' pattern	Tarantula huwentoxin-4	HWTX-4	1MB6	P83303	CSRKTRWC	4.1	Ion channel blocking
	Scorpion charybdotoxin	CTX	2CRD	P13487	CMNKKCRC	4.3	Ion channel blocking
	Nematode antibacterial factor	ASABF	2D56	P90683	CEKRGGRTC	7.9	Antibacterial
	Human β-defensin 2	HBD-2	1FD3	O15263	CGRRICRC	4.3	Antimicrobial, immune modulation
	Human θ-defensin 2	HTD-2	2LZI	-	CGRRICRC	2.0	Antiviral

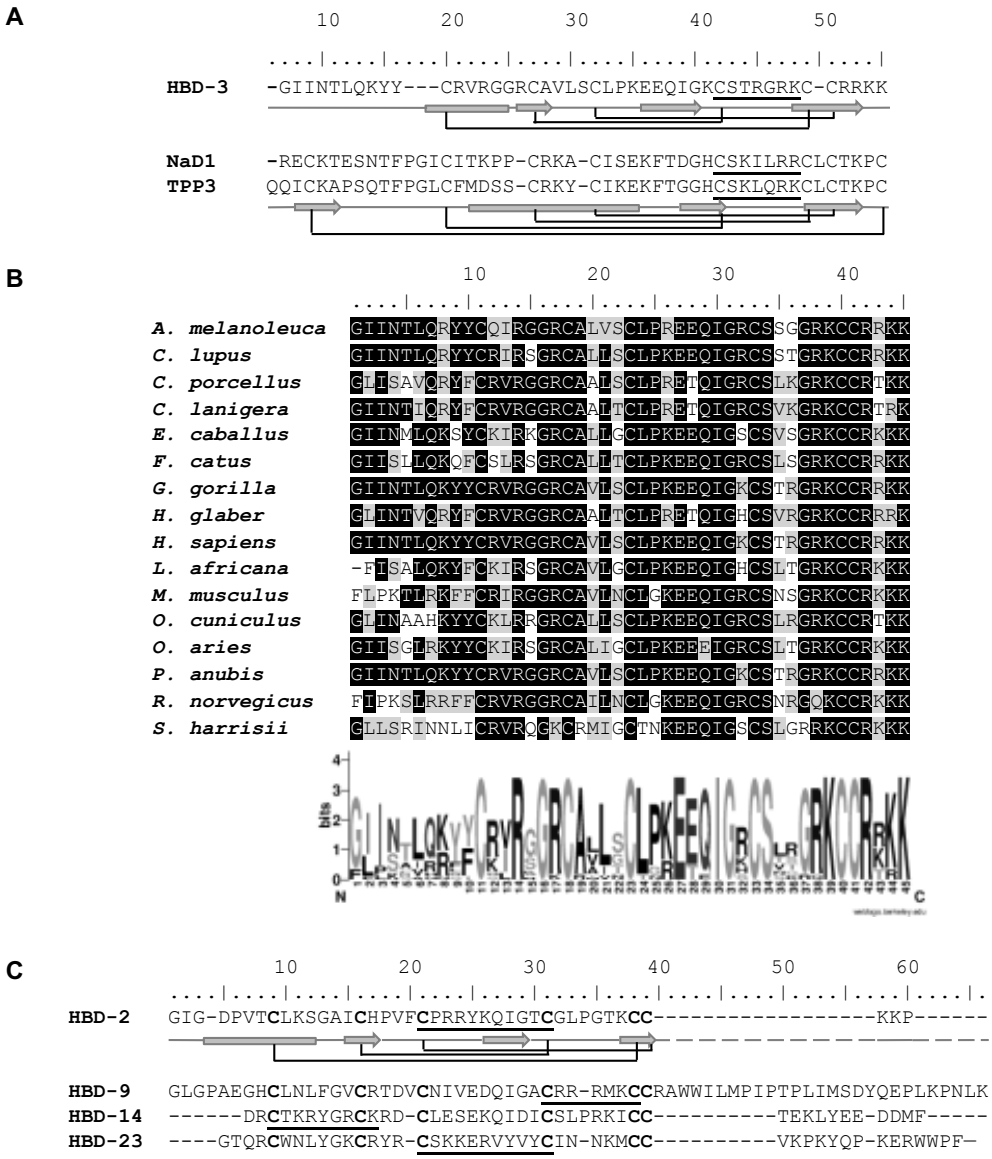

Figure 2.4 Potential sequence and structural similarity of defensins from database searches

(A) Schematic illustration of mature structures of defensins with 'KILRR' pattern . **(B)** Sequence alignment and sequence logo of mammalian β-defensin 3 homologues, generated using WebLogo (http://weblogo.berkeley.edu). **(C)** Sequence comparison of defensins with 'RRYK' pattern. Amino acid sequences were aligned according to conserved cysteine residues, which form intramolecular disulfide bonds (connecting lines). Secondary structures (α-helix, block; β-strand, arrow; loop, thin line), cationic patterns (underlined) and conserved residues of interest (bolded) are shown explicitly.

2.3.4 PI(4,5)P_2 pull-down assay

A number of different domains and motifs have been reported to bind to PI(4,5)P_2. Indeed, PI(4,5)P_2-mediated cell death, as induced by gasdermin D (Liu *et al*, 2016) or MLKL (Dondelinger *et al*, 2014) do not appear to be dependent on the aforementioned cationic motifs. Thus, to identify novel PI(4,5)P_2-binding antimicrobial molecules, direct PI(4,5)P_2 pull-down assays were performed using human serum as well as LPS-stimulated monocyte and neutrophil culture supernatants, simulating bacterial infection, which are well-documented to induce production and secretion of HDPs (Ayabe *et al*, 2000; Becker *et al*, 2000). Statistically-significant hits, determined by LC-ESI-MS/MS, could be grouped into different categories based on their reported functions (Tables **2.4**, **2.5** and **2.6)**. Among the identified proteins, the pull-down of apolipoprotein A-1 (apoA-1) with PI(4,5)P_2 has been experimentally confirmed recently, showing that PI(4,5)P_2, effluxed via a floppase-mediated mechanism, directly binds to apoA-1 and mediate high-density lipoprotein assembly (Gulshan *et al*, 2016). The remainder are predominated by antimicrobial, immunomodulatory and membrane-permeabilising proteins, including dermicidin, platelete basic protein (CXC chemokine 7), proteins S100-A7/A9, azurocidin, bactericidal/permeability increasing protein, human neutrophil defensin 1 (HNP-1) and, possibly, gasdermin A (Elsbach, 1998; Gudmundsson & Agerberth, 1999; Schittek *et al*, 2001; Donato *et al*, 2013; Chen *et al*, 2014; Wang *et al*, 2016). Cysteine and serine inhibitors, such as serpins and cystatins (Law *et al*, 2006; Ochieng & Chaudhuri, 2010), were also commonly obtained across all three samples. Fibrinogen, fibronectin, thrombospondin-1 are important clot coagulation-promoting proteins (Vickers *et al*, 1987; Singh *et al*, 2010). Other identified binding proteins, bleomycin hydrolase, catalase and suprabasin, all found in human serum, are not well-characterised.

2.3.5 Cloning and expression of mature HBD-3

As mentioned earlier (section 1.3.1), HBD-3 possesses a strikingly similar β2-β3 cationic motif to the solanaceous plant defensins. It is therefore of interest to investigate its role in anticancer and PI(4,5)P_2 binding. HBD-3 is a highly positively-charged (+11) HDP, inducibly expressed and secreted by epithelial cells, non-epithelial tissues, monocytes and neutrophils (Garcia *et al*, 2001; Harder *et al*, 2001; Harder *et al*, 2004; Sorensen *et al*, 2006) and is arguably the most potent antimicrobial of the β-defensins (Schibli *et al*, 2002; Chen *et al*, 2007; Schroeder *et al*, 2011). HBD-3 exhibits broad-spectrum antibacterial, antifungal and antiviral activities (Garcia *et al*, 2001; Harder *et al*, 2001;

Quinones-Mateu *et al*, 2003b; Feng *et al*, 2005; Leikina *et al*, 2005; Sun *et al*, 2005; Pazgier *et al*, 2006). HBD-3 is also chemoattractive and activates antigen presenting cells as well as induces chemokine expression, crucially contributing to the integration of innate and adaptive immune responses (Funderburg *et al*, 2007; Nagaoka *et al*, 2008; Ferris *et al*, 2013; Petrov *et al*, 2013). Proposed cellular targets of HBD-3, including bacterial lipid II (Sass *et al*, 2010), monocytic phosphatidylserine (Lioi *et al*, 2012), and Toll-like, CC and CXC chemokine receptors (Funderburg *et al*, 2007; Nagaoka *et al*, 2008; Rohrl *et al*, 2010; Feng *et al*, 2013) has been challenged recently (Lee *et al*, 2015).

Table 2.4 Significantly-enriched hits for PI(4,5)P_2 pull-down using human serum

PI(4,5)P_2-conjugated agarose beads or control beads were incubated with human serum, prior to urea digestion and subsequent LC-ESI-MS/MS. Only statistically-significant hits from the pull-down are presented below, with fold enrichment determined by comparing the PI(4,5)P_2 beads to the control beads of three independent experiments. Refer to main text for further comments and references.

Protein names	UniProt entry	Fold difference	P-value	MW (kDa)	Functions
α-1-microglobulin	P02760	PI(4,5)P_2 bead only	-	39.0	Trypsin inhibitor
α-2 globin chain	D1MGQ2	2.2	0.042	15.2	Oxygentransport
Apolipoprotein A-1	P02647	2.4	0.022	28.5	Cholesterol transport
Bleomycin hydrolase	Q13867	PI(4,5)P_2 bead only	-	52.6	Unknown
Calmodulin-like protein 5	Q9NZT1	2.14	0.028	15.9	Calcium binding
Catalase	P04040	PI(4,5)P_2 bead only	-	59.8	Hydrogen peroxide scarvenging
Cystatin-A	P01040	PI(4,5)P_2 bead only	-	14.5	Thiol protease inhibitor
Cystatin-S	P01036	PI(4,5)P_2 bead only	-	11.0	Thiol protease inhibitor
Dermcidin	P81605	1.76	0.027	11.3	Antimicrobial, proteolysis
Platelet basic protein	P02775	3.02	0.035	10.3	Immune modulation, antimicrobial
Protein S100-A7	P31151	1.79	0.027	11.5	Calcium binding, antimicrobial
Protein S100-A9	P06702	4.23	0.005	39.0	Calcium binding, immune modulation, antimicrobial
Serpin A12	Q8IW75	PI(4,5)P_2 bead only	-	47.2	Serine protease inhibitor
Serpin B3	P29508	PI(4,5)P_2 bead only	-	44.6	Cysteine protease inhibitor
Suprabasin	Q6UWP8	2.88	0.035	60.5	Epidermal differentation
Zinc-α-2-glycoprotein	P25311	PI(4,5)P_2 bead only	-	34.3	Lipid degradation

Table 2.5 Significant hits for PI(4,5)P_2 pull-down using culture media of LPS-stimulated PBMCs

PI(4,5)P_2-conjugated agarose beads or control beads were incubated with culture media of LPS-stimulated PBMCs, prior to urea digestion and subsequent LC-ESI-MS/MS. Only statistically-significant hits from the pull-down are presented below, with fold enrichment determined by comparing the PI(4,5)P_2 beads to the control beads of three independent experiments. Refer to main text for further comments and references.

Protein names	UniProt entry	Fold difference	P-value	MW (kDa)	Functions
14-3-3 protein ζ/δ	P63104	PI(4,5)P_2 bead only	-	27.7	Protein kinase C inhibitor
14-3-3 protein σ	P31947	2.06	0.044	27.7	Protein kinase C inhibitor
Cystatin-S	P01036	PI(4,5)P_2 bead only	-	11.0	Thiol protease inhibitor
Fibrinogen	P02671	PI(4,5)P_2 bead only	-	95.0	Blood coagulation, immune modulation
Fibronectin	P02751	PI(4,5)P_2 bead only	-	262.6	Cell adhesion, cell motility
Serpin B3	P29508	4.09	0.001	44.6	Cysteine protease inhibitor
Thrombospondin-1	P07996	PI(4,5)P_2 bead only	-	129.4	Cell interaction
Zinc-α-2-glycoprotein	P25311	PI(4,5)P_2 bead only	-	34.3	Lipid degradation

Table 2.6 Significant hits for PI(4,5)P_2 pull-down using culture media of LPS-stimulated neutrophils

PI(4,5)P_2-conjugated agarose beads or control beads were incubated with culture media of LPS-stimulated PBMCs, prior to urea digestion and subsequent LC-ESI-MS/MS. Only statistically-significant hits from the pull-down are presented below, with fold enrichment determined by comparing the PI(4,5)P_2 beads to the control beads of three independent experiments. Refer to main text for further comments and references.

Protein names	UniProt entry	Fold difference	P-value	MW (kDa)	Functions
Azurocidin	P20160	PI(4,5)P_2 bead only	-	26.9	Antimicrobial, chemoattraction
Bactericidal permeability-increasing protein	P17213	3.48	0.002	53.9	Antimicrobial
Cathepsin D	P07339	1.59	0.048	44.6	Aspartate protease
Cystatin-A	P01040	5.47	0.013	14.5	Thiol protease inhibitor
Cystatin-S	P01036	4.57	0.002	11.0	Thiol protease inhibitor
Gasdermin A	Q96QA5	PI(4,5)P_2 bead only	-	49.4	Pyroptosis regulator
Neutrophil defensin 1	P59665	2.56	0.017	3.4	Antimicrobial, immune modulation
Serpin B3	P29508	4.42	0.001	44.6	Cysteine protease inhibitor

Although HBD-3 was reported to have an anti-metastatic effect on head, neck and colon cancer cells (Wang *et al*, 2012; Uraki *et al*, 2014), its direct anticancer mechanism generally remains poorly defined. In order to examine anticancer activity and the mechanistic conservation between HBD-3 and the plant defensins, molecular cloning and *P. pastoris* expression of HBD-3 were performed. Initially, the exonic mature HBD-3-encoding sequence was amplified from human blood-derived genomic DNA by PCR using forward and reverse primers that incorporated *Xho*I and *Not*I restriction sites for subsequent subcloning (**Figure 2.5A, 2.5B**). The most abundant amplified DNA fragment under 200 bp was separated by agarose gel electrophoresis (**Figure 2.5C**) and ligated into the pCR2.1-TOPO vector before subcloning into pPIC9 vector for expression in *P. pastoris* strain GS115 cells. The sequence identity and integrity of the cloned HBD-3 gene in pPIC9 was confirmed by DNA sequencing (**Figure 2.6A**). Protein expression from transformed yeast was performed in BMG/BMM media for 4 days with daily methanol induction and cleavage of the yeast α-mating factor secretion signal upon synthesis was carried out to promote secretion of mature HBD-3 into culture medium (**Figure 2.6B**). The highly positively-charged nature (+11) of HBD-3 was exploited for purification using cation-exchange chromatography.

2.3.6 Biochemical and structural characterisation of HBD-3

Purified HBD-3 was subjected to a number of quality control tests to assure its purity, integrity and topology. SDS-PAGE analysis with Coommassie Brilliant Blue staining (**Figure 2.7A**) and Western blotting (**Figure 2.7B**) using anti-HBD-3 antibody revealed a distinct single band at under 6 kDa. CD spectra (**Figure 2.7C**) showed the dip at 195–200 nm, concernedly suggesting a random coil conformation. However, CD analysis suggested a structural composition of 4% α-helix, 42% β-strand, 20% turn and 34% unordered structure, which is accordant with the predetermined NMR structural values (PDB: 1KJ6) of 11%, 47%, 11% and 31% respectively. To resolve this issue, proton NMR (**Figure 2.7D**) and 2D NOESY (**Figure 2.7E**) were performed. Both proton NMR and 2D NOESY spectra show pronounced alpha proton (H^{α}-H^{α}) resonances at 5.0-5.6 ppm (downfield of water in 2D NOESY), reconfirming the β-sheet rich structure, while weak amide proton (H^{N}-H^{N}) NOEs indicates little α-helix. The broad, yet poorly-dispersed, inter-residue amide-alpha (H^{N}-H^{α}) proton region at 7–9 ppm is indicative of internal dynamics. Calculated diffusion constant of 1.56×10^{-10} m^2/s is consistent with expected monomeric molecular weight of HBD-3 at experimented conditions.

A

*Xho*I

Forward primer 5′-CTCGAG**AAAAGA**GGAATCATAAACACATTACAGAA-3′

*Not*I

Reverse primer 5′-GCGGCCGCTTATTTCTTTCTTCGGCAGCATTTT-3′

B

caaatccatagggagctctgccttaccattgggttcctaattaactgagtgagtgggtgtgttctgcatg
gtgagaggcattggaatgatgcatcagaaaacatgtcataatgtcatcactgtaatatgacaagaattgc
agctgtggctggaacctttataaagtgaccaagcacacctttttcatccagtctcagcgtggggtgaagcc
tagcagctATGAGGATCCATTATCTTCTGTTTGCTTTGCTCTTCCTGTTTTTGGTGCCTGTTCCAggtaa
M R I H Y L L F A L L F L F L V P V P

gatgggctgggaaatctaaggattgatctaattgagaatatataattcagagtcagtatttctccatcct
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aaaagcaagggatgagttatttgaggaattccacaagccttgtacgtgtaccaaaagccttcctaaaacc
tttccgtgtgtgctgttttgtcattgca*GGTCATGGA*GGAATCATAAACACATTACAGAAATATTATTGC
G H G **G I I N T L Q K Y Y C**

AGAGTCAGAGGCGGCCGGTGTGCTGTGCTCAGCTGCCTTCCAAAGGAGGAACAGATCGGCAAGTGCTCGA
R V R G G R C A V L S C L P K E E Q I G K C S T

CGCGTGGCCGAAAATGCTGCCGAAGAAAGAAATAAaaaccctgaaacatgacgagagtgttgtaaagtgt
R G R K C C R R K K *
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C

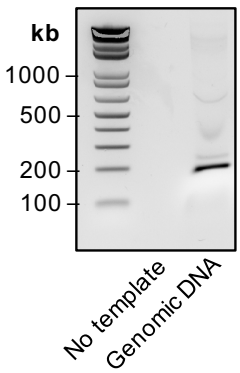

Figure 2.5 PCR amplification of genomic DNA encoding mature HBD-3

(A) HBD-3 oligonucleotide primers with incorporated restriction sites (underlined) and proteolytic cleavage site (bolded). (B) DNA sequence encoding HBD-3 within a part of chromosome 8p23.1 with untranslated regions and intron (grayed) and propeptide-encoding sequence, on which primer binding sites are underlined. Only one strand with the polarity of mRNA is presented. Translated HBD-3 propeptide, shown in single letter code, is given below the nucleotide sequence, and mature sequence is bolded. The stop codon is indicated with an asterisk (*) (C) Electrophoresis of PCR product using 1% (w/v) agarose gel in 0.5× TBE buffer.

A

α-factor

Sequenced CGACAACTTGAGAAGATCAAAAAACAACTAATTATTCGAAGGATCCAAACGATGAGATTTCCTTCAATTTTTACTGCAGTTTTATTCGCA
M R F P S I F T A V L F A
Reference ----------ATGAGATTTCCTTCAATTTTTACTGCAGTTTTATTCGCA

Sequenced GCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGAT
A S S A L A A P V N T T T E D E T A Q I P A E A V I G Y S D
Reference GCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGAT

Sequenced TTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATT
L E G D F D V A V L P F S N S T N N G L L F I N T T I A S I
Reference TTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATT

*Xho*I Signal cleavage

Sequenced GCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGGAATCATAAACACATTACAGAAATATTATTGCAGAGTCAGAGGCGGCCGGTGT
A A K E E G V S L E K R **G I I N T L Q K Y Y C R V R G G R C**
Reference GCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGGAATCATAAACACATTACAGAAATATTATTGCAGAGTCAGAGGCGGCCGGTGT

Sequenced GCTGTGCTCAGCTGCCTTCCAAAGGAGGAACAGATCGGCAAGTGCTCGACGCGTGGCCGAAAATGCTGCCGAAGAAAGAAATAAGCGGCC
A V L S C L P K E E Q I G K C S T R G R K C C R R K K *
Reference GCTGTGCTCAGCTGCCTTCCAAAGGAGGAACAGATCGGCAAGTGCTCGACGCGTGGCCGAAAATGCTGCCGAAGAAAGAAATAAGCGGCC

Sequenced CAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCGAAT

Reference ----------

B

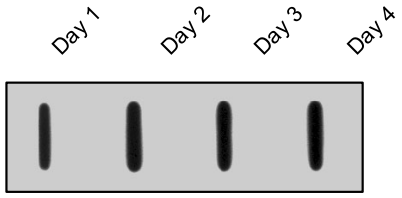

Figure 2.6 Expression of mature HBD-3 in *P. pastoris*

(A) Confirmation of recombinant pPIC9-HBD3 sequence, showing sequence comparison of experimental and reference pPIC9-HBD3 DNA. The amino acid sequence is presented between two nucleotide sequences with mature HBD-3 bolded. The stop codon is indicated with an asterisk (*). The beginning of α-factor, *XhoI* restriction site and signal cleavage are also explicitly highlighted. (B) Slot blot, showing HBD-3 expression in *P. pastoris*. Supernatants from expression culture at different time points were applied on nitrocellulose membrane followed by immunoblotting using rabbit-anti-HBD-3 and HRP-conjugated donkey-anti-rabit antibodies (0.25 μg/mL).

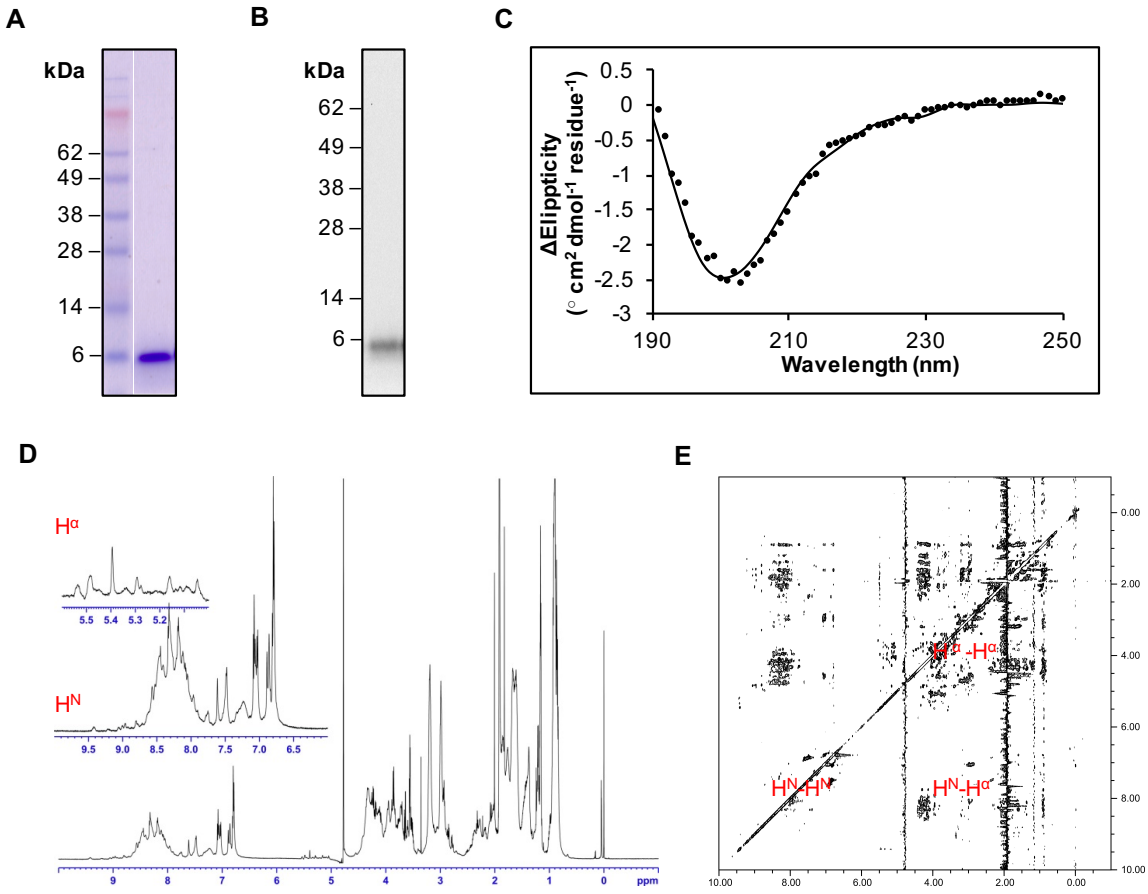

Figure 2.7 Quality control of recombinantly-expressed HBD-3

(A) Reducing and denaturing SDS-PAGE analysis (2 µg protein/lane) followed by Coomassie Brilliant Blue staining, **(B)** Immunoblotting (250 ng protein/lane) using rabbit-anti-HBD-3 and HRP-conjugated donkey-anti-rabbit antibodies (0.25 µg/mL), **(C)** CD spectrum of 150 µg/mL aqueous solution of HBD-3 was recorded in 1 nm increments with a 2 s averaging time from 190 to 250 nm using a 1 mm quartz cuvette. Raw data (solid black circle) were fitted using the CONTILL algorithm on the SP29 database from the CDPro software package. The nonlinear best fit resulted in a RMSD of 0.069 with structural composition of 4% α-helix, 42% β-strand, 20% turn and 33.4% unordered structure**. (D)** Proton NMR and **(E)** 2D NOESY spectra of HBD-3 were obtained in a Bruker Avance IIID spectrometer (Bruker Corporation, MA) equipped with cryoprobes, operating at 700 MHz, 298 K and mixing time of 150 ms (for 2D NOESY), in 20 mM sodium acetate (pH 5.5) and 5% heavy water D_2O.

2.3.7 Binding of HBD-3 to PI(4.5)P_2 *in vitro* and on cellular membranes

The anticancer activity of NaD1 was shown to be mediated by its interaction with PIPs, particularly PI(4,5)P_2 (Poon *et al*, 2014). Therefore, protein-lipid overlay assays using commercially available Membrane™ and PIP™ strips (**Figure 2.8A)** were performed to study the binding of HBD-3 to different functionally important lipids. Among membrane lipids, HBD-3 showed greater relative binding intensity toward phospholipids, especially PA, PIPs including (PI(4)P, PI(4,5)P_2 and PI(3.4,5)P_3), PS, cardiolipin, and, to a lesser extent, PG. Weaker interactions with sphingolipids (sphingomyelins and sulfatide) were also detected. The PIP™ strip further indicated the binding preference of HBD-3 towards, most strongly, PA and all phosphoinositides, but not other lipids, including phosphatidylinositol.

Anti-PI(4,5)P_2 antibody blocking assay showed a significant reduction of FITC signal of U937 cells treated with HBD-3, similar to the well-known PI(4,5)P_2 sequeestering neomycin control, further supporting the interaction of HBD-3 and cellular PI(4,5)P_2 (**Figure 2.8B**). In contrast, no difference in annexin-V binding was observed in untreated, HBD-3-treated and neomycin-treated U937 cells, suggesting the availability of PS after these treatments (**Figure 2.8C**). In other words, it is unlikely that HBD-3 or neomycin binds to cellular PS.

2.3.8 Tumour-cell selective cytotoxicity of HBD-3

To investigate the cytotoxic effects of HBD-3 on cell viability, tetrazolium-based assays were performed on a number of different human tumour and primary cell lines. HBD-3 showed dose-dependent cytotoxicity on tumour lines HeLa, HL-60, Jukat, U937 and PC3, at low micromolar concentrations with IC_{50} values from 8–20 μM (**Figure 2.9**). Primary cells, HUVEC and particularly AHDF and CASMC, were less susceptible to HBD-3 treatment (IC_{50} values from 30–65 μM). No discrimination between adherent and suspension cells was observed.

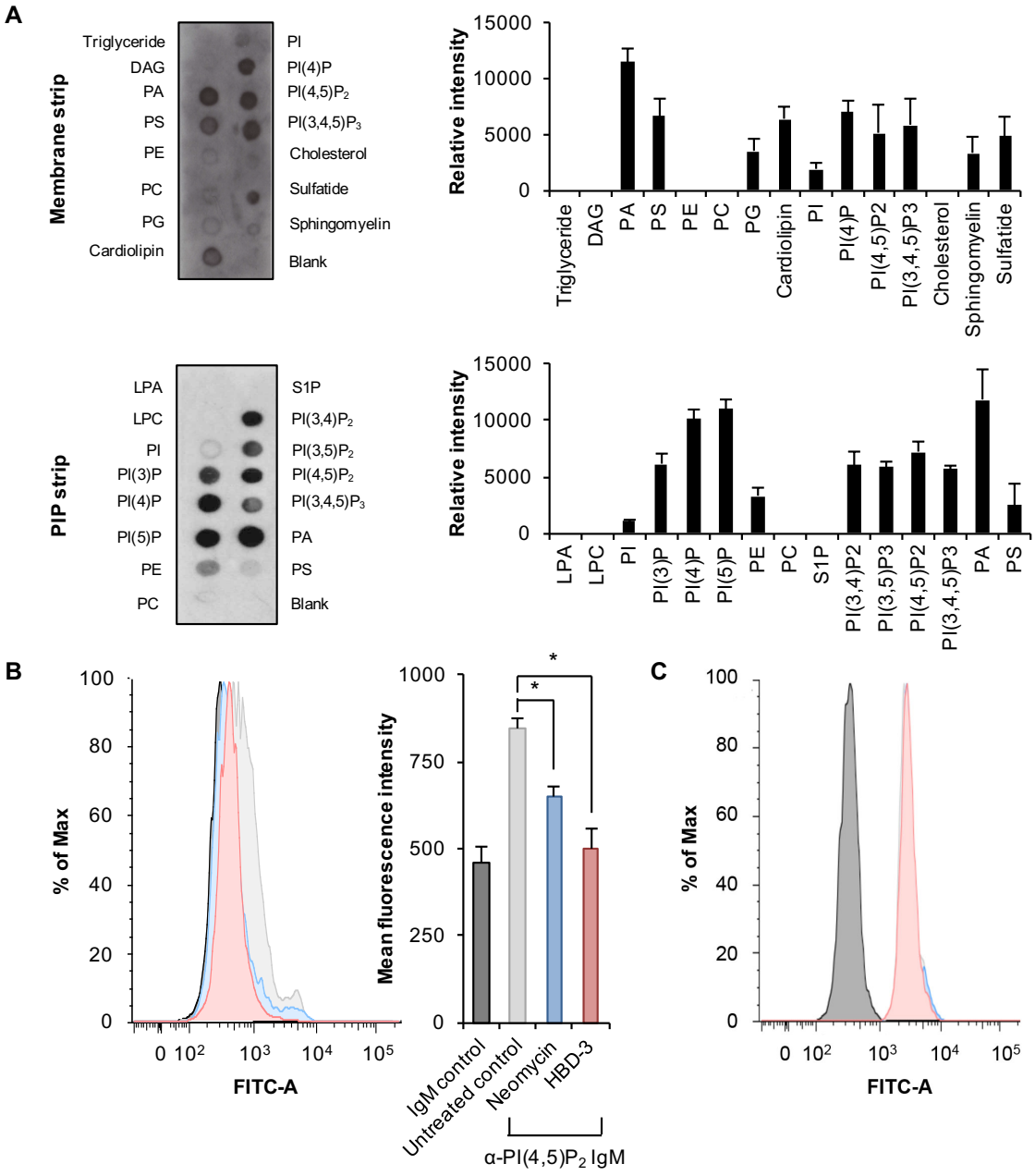

Figure 2.8 Preferential binding to phosphoinositides, particularly PI(4,5)P_2, by HBD-3

(A) Immunodetection of lipid binding by HBD-3 on membrane strip™ and PIP strip™. Relative binding intensity was determined by densitometry analysis of chemiluminescence signals. **(B)** Blocking of anti-PI(4,5)P_2 antibody binding to plasma membrane PI(4,5)P_2 by HBD-3. Untreated and treated U937 cells were fixed, lysed and stained with FITC-conjugated anti-PI(4,5)P_2 IgM prior to flow cytometry analysis. Data in **A** and **B** represent mean ± SEM of three independent experiments. NS, not significant; *, $p<0.05$; **, $p<0.01$, unpaired t-test. **(C)** Unsuccesful blocking of Annexin V binding to plasma membrane PS by HBD-3. Data are representative of three independent experiments.

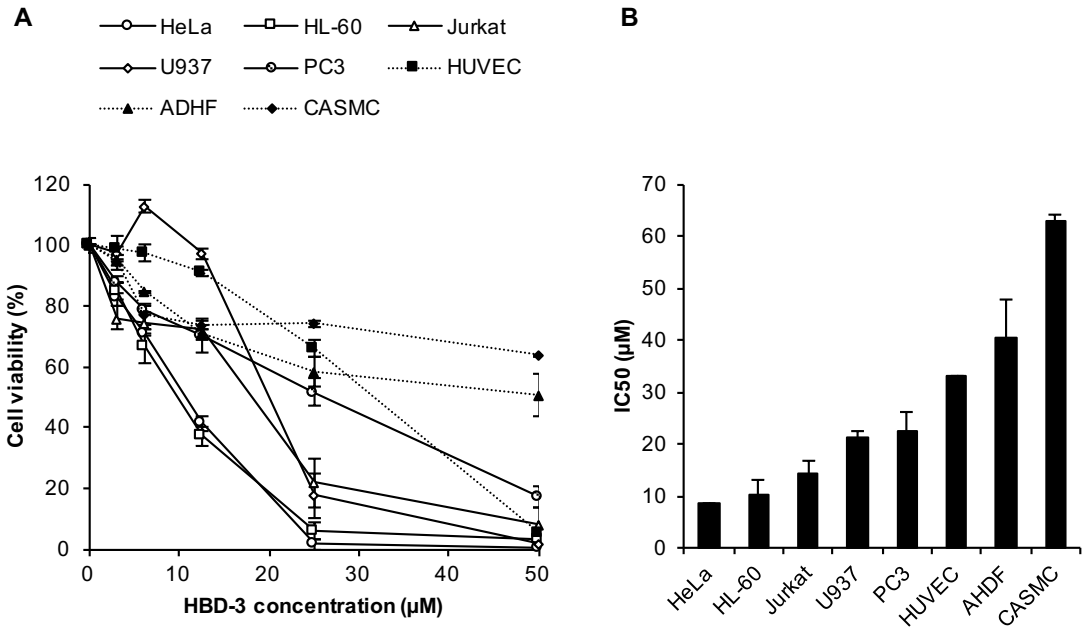

Figure 2.9 Cancer-specific cytotoxicity of HBD-3.

(A) Dose-independent cytotoxic effect and **(B)** estimated IC_{50} of HBD-3 on different cancer (HeLa, HL-60, Jurkat, U937 and PC3) and normal (HUVEC, ADHF and CASMC) cell lines, determined by tetrazolium-coupled cell viability assay. Data were normalised against untreated control, which was arbitrarily assigned as 100% cell viability. Data represent mean ± SEM of three independent experiments.

2.4 Discussion

As tumour cells and a number of microbial pathogens manipulate PIP metabolism and signalling to promote their invasion, survival and progression, PIPs, particularly $PI(4,5)P_2$, represent promising therapeutically exploitable targets (Pizarro-Cerda & Cossart, 2004; Bunney & Katan, 2010; Balla, 2013). In fact, a number of studies have demonstrated the ability to neutralise pathogens, to lyse (tumour) cells and/or to trigger inflammatory responses of $PI(4,5)P_2$-targeting molecules, including anti-$PI(4,5)P_2$ antibodies, solanaceous plant defensins and MLKL (Matyas *et al*, 2010; Dondelinger *et al*, 2014; Poon *et al*, 2014; Baxter *et al*, 2015). Therefore, $PI(4,5)P_2$-binding antimicrobial and anticancer agents were proposed as novel combined and multifaceted therapeutics. As a proof of concept, it is essential to identify new candidates that preferentially bind $PI(4,5)P_2$ and simultaneously display antimicrobial and anticancer activity, prior to elucidating the role of $PI(4,5)P_2$ underlying these functions.

2.4.1 Neamine core and 6-amino group on ring I as structural prerequisites for $PI(4,5)P_2$ binding and undeniably selective anticancer activity of aminoglycosides

In this chapter, aminoglycoside antibiotics, particularly kanamycin and neomycin, were demonstrated to bind $PI(4,5)P_2$ on cellular membranes by competing with the plant defensin NaD1 and leading to a reduction of its membrane permeabilisation, as well as displaying somewhat selective tumour cell-killing activity, albeit only observed at millimolar dose. Kanamycin and neomycin shares a strikingly similar neamine core, compared to gentamicin whose ring I lacks 3,4-hydroxyl groups and carries an additional 6-methyl group adjacent to the 6-amino, or streptomycin that does not have ring I. In particular, the 6-amino group in ring I appears to be indispensable for the interaction with $PI(4,5)P_2$, which is noticeably impaired upon substitution by an hydroxyl as observed for paramomycin or due to possible steric hindrance by additional residues (*e.g.* 6-methyl group) as in gentamicin. Replacing the 6-amino with the hydroxyl substituent does in fact profoundly affect the inhibitory effect of kanamycin on bacterial ribosomes due to disrupted hydrogen bonding with an adenine at ribosomal decoding A site (Salian *et al*, 2012). It is also possible that, in addition to ionic interaction (Gabev *et al*, 1989), the 6-amino group provides the hydrogen bonding between neomycin and $PI(4,5)P_2$ that was demonstrated by Wu *et al* (2011). These data also show direct $PI(4,5)P_2$ binding and hence further support the finding that neamine, neomycin, kanamycin and other 4,6-disubstituted aminoglycosides tobramycin and amikacin disrupt membrane permeability in $PI(4,5)P_2$-containing liposome (Au *et al*, 1987). However, as indicated by the PI uptake assay results, neither kanamycin nor neomycin induces membrane permeabilisation in U937 cells. This discrepancy highly likely results from the shorter incubation time (30 min) in PI uptake assay as compared to 10 hour period by Au *et al*. Unsurprisingly, the liposome permeabilisation effect by the aminoglycosides were recorded to be below 5% per hour (Au *et al*, 1987), which might explain the modest cytotoxicity against tumour cells of these aminoglycosides.

The correlation between $PI(4,5)P_2$ binding and anticancer activity, although requires further investigation, is undeniable, as none of gentamicin, paramomycin and streptomycin, show significant $PI(4,5)P_2$ binding nor apparent selective cytotoxicity. Therefore, the neamine core with the 6-amino group on its ring I are structurally crucial for the interaction with $PI(4,5)P_2$, and, probably the selective cytotoxicity against tumour cells. It is unclear why kanamycin, compared to neomycin, shows dramatically lower cytotoxicity against primary cell ADHF and PBMC, and thus displays more pronounced selectivity (10-fold difference; **Figure 2.3**). Molecular interactions by additional glycosyl substituent(s) outside the neamine core, which remains undermined, may have

contributed to such discrimination. Despite their disappointingly low potency, aminoglycosides reveal a structural basis for future therapeutic development. Future drug design should retain the neamine core and its 6-amino group in ring I while manipulating 5- or 6-substituted residues in ring II to generate higher $PI(4,5)P_2$ binding affinity and substantially greater potency and tumour cell specificity.

2.4.2 Human β-defensins with cationic motifs

Human β-defensins, which belong to a broader defensin superfamily also including α- and θ-defensins, are prominent cysteine-rich HDPs constitutively and inducibly expressed in keratinocytes and epithelial cells of various organs (reviewed by Pazgier *et al*, 2006). Majority of the database search results are β-defensins, which are likely to contain cationic motifs that may direct phospholipid binding. Apart from the under-characterised HBD-9, like many other HBDs, HBD-2, -3, -14 and -23 display broad spectrum antimicrobial and immunomodulatory effects, thus not only contributing to the first line of pathogen defense but also providing a link between innate and adaptive immunities (Harder *et al*, 2001; Motzkus *et al*, 2006; Pazgier *et al*, 2006; Rohrl *et al*, 2010; Yu *et al*, 2013). HDB-2 and -3, the most intensively studied and arguably the most potent β-defensins, electrostatically bind and disrupt plasma membrane integrity, leading to a leakage of intracellular content (Lichtenstein *et al*, 1988; Feng *et al*, 2005; Ouhara *et al*, 2005; Krishnakumari *et al*, 2009). HBD-3 can also bind to the bacterial cell wall lipid II building block and inhibit cell wall synthesis (Sass *et al*, 2010). Despite ongoing research efforts, the molecular detail for interaction between defensins and cellular membrane is still unresolved. In an attempt to computationally identify prospective candidates for further investigation of their membrane permeabilisation and lipid binding, this thesis chapter reveals that a number of human β-defensins exhibit either NaD1-like (HBD-3) or HBD-2-like cysteine-flanked cationic motifs (HBD-9, HBD-14, HBD-23). To date, the role(s) of cationic motifs in the β-defensins remain undetermined for any reported functions.

Upon HBD sequence alignment, their cationic motifs are located in different loop regions and seem poorly aligned against one another (**Figure 2.4 A** and **C**). However, it is worth noting that HBD-3 possesses a strikingly analogous cationic loop (ST**R**G**RK**) to the solanaceous plant defensins (S**K**IL**RR** in NaD1 and S**K**LQ**RK** in TPP3), all spanning between the β2 and β3 strands. Despite being classified into distinct superfamilies including *cis*-defensins (plant, fungi, mollusc, and arthropod defensins as well as

similarly-folded neurotoxins) and *trans*-defensins (vertebrate defensins, mollusc big defensins and anemone defensin-like proteins), defensins typically undergo extensive convergent evolution (Shafee *et al*, 2016a; Shafee *et al*, 2016b). Both defensins superfamilies convergently adopt compact cysteine disulfide-stabilised cores featuring an antiparallel double- or triple-stranded β-sheet, and typically, with an α-helix. Functionally important residues may be located in the core or in the displayed loops spanning between two defined secondary structures; however, they are typically highly solvent-exposed basic amino acids to facilitate anionic ligand binding (Ganz, 2003; Poon *et al*, 2014; Shafee *et al*, 2016b). Owing to the remarkably similar structural and functional features, vertebrate β-defensins (including human) were initially believed to be more closely related to plant defensins than to vertebrate α-defensins (Hughes, 1999; Thomma *et al*, 2002). However, as described above, this has recently been suggested as incorrect, with human and plant defensins being assigned to distinct superfamilies having likely arisen by convergent evolution (Shafee *et al*, 2016a). Probably, the analogous cationic loops of solanaceous plant defensins and human β-defensins displayed on the compact disulfide-stabilised core may have independently evolved to adopt a number of converged functions. The positively-charged loops can interact with negatively-charged lipids such as PIPs on plasma membrane, essentially leading to membrane disruption by NaD1, TPP3 and, speculatively, HBD-3 (Shafee *et al*, 2016b).

2.4.3 PI(4,5)P_2 binding and selective anticancer activity of HBD-3

For all functional studies described in this chapter, HBD-3 was recombinantly produced using a pPIC9 vector and methanol-inducible *P. pastoris* expression system. Compared to a bacterial expression system, use of this system was in part favoured by the fact that yeast is a eukaryote, thus would promote correct protein folding (Cregg *et al*, 1985; Buckholz & Gleeson, 1991). Moreover, cloning downstream of the alcohol oxidase (5'-AOX) promoter, α-mating factor secretion signal and *Kex*2 cleavage site of the pPIC9 vector (Cregg *et al*, 1985; Buckholz & Gleeson, 1991) enables production of high levels of secreted soluble protein. By exploiting its highly basic nature (+11), the secreted HBD-3 protein was successfully purified from the culture supernatant using cation-exchange chromatography. SDS-PAGE followed by Coomassie staining and immunoblotting analysis with anti-HBD-3 antibodies, which reveal a single solid band slightly under 6 kDa, assure the monomeric product with correct size (expected at 5.155 kDa) and minimal degradation and contamination. Although CD spectroscopy suggests a random coil structure as showed by the typical dip at 195–200 nm, the structural

composition of the recombinantly expressed HBD-3 is accordant with the available NMR structural data showing a β-sheet core and an unstructured N-terminus with little α-helix (PDB: 1KJ6; Schibli *et al*, 2002). Moreover, this CD spectrum is also consistent with the previously reported oxidised synthetic and native HBD-3, whose structure becomes more ordered upon an organic modifier (trifluoroacetic acid) or membrane mimetic environment (Boniotto *et al*, 2003; Kluver *et al*, 2005; Liu *et al*, 2008; Scudiero *et al*, 2010). Proton NMR and 2D NOESY spectra as well as calculated diffusion constant reaffirm the β-sheet-rich and α-helix-poor monomeric structure as indicated by the diagnostic H^{α}- H^{α} resonance at 5.0-5.6 ppm and the lack of H^{N}-H^{N} NOEs. There might also be some internal dynamic process due to broad and poorly dispersed H^{N}-H^{α} resonances at 7-9 ppm, possibly contributing to the random coil effect observed by CD spectroscopy. It is interesting to note that HBD-3 may display a pH-dependent dimerisation, as the NMR and SDS-PAGE analyses in this study (performed at pH 5.5-7.2) consistently suggested a monomeric state for HBD-3, whereas previous studies conducted at lower pH (4-4.19) showed the presence of HBD-3 dimers (Schibli *et al*, 2002). Two glutamate residues (E28 and E29) with pKa of 4.15 may have crucially contributed to the formation of HBD-3 at lower pH. Taken together, the recombinantly expressed HBD-3 is of high purity, of expected size, identity and of appropriate fold, and thus suitable for further experiments.

The high similarity of the cationic β2-β3 loops in HBD-3 and solanaceous plant defensins encouraged the investigation of the lipid binding activity by HBD-3. The protein-lipid overlay assays were performed, consistently revealing a strong preference toward phospholipids, particularly PA, PIPs, cardiolipin, PS and, modestly, PG; however, the magnitude of relative binding intensity is not proportionally related to the negativity of the lipids. The PIP strip emphasises interactions of HBD-3 with PA and PIPs, but not their non-phosphorylated form, PI. Moreover, as suggested by the blocking of anti-$PI(4,5)P_2$ antibody and annexin V binding assays, HBD-3 binds to $PI(4,5)P_2$, but not PS, on cellular membranes despite having comparable binding intensity on the membrane strip. Therefore, lipid binding activity of HBD-3 might not solely dependent on ionic interaction. The HBD-3:lipid interaction may therefore be initiated by electrostatic forces, but maintained by structural binding as previously suggested (Hoover *et al*, 2000a; Hoover *et al*, 2000b; Pazgier *et al*, 2006; Pazgier *et al*, 2006). At this stage, it is unclear why HBD-3, compared to the solanaceous plant defensins, appears to bind most strongly to PA, although its interactions with PIPs are also relatively strong. The available solution

structure and predicted dimeric form of HBD-3 (Schibli *et al*, 2002) are of limited use to in explaining the molecular basis of these lipid preferences as they do not account for the potential extensive conformational change upon lipid interaction of HBD-3 as suggested by a number of former studies (Boniotto *et al*, 2003; Liu *et al*, 2008; Scudiero *et al*, 2010). However, it is tempting to speculate that other factors, such as overall structures, the dimension of lipid binding 'pocket' (as observed in NaD1; Poon *et al*, 2014) or steric hindrance caused by non-basic residues within the β2-β3 loop may contribute to the difference in lipid binding preference. Ultimately, crystallographic data of HBD-3, preferentially in presence of lipids, is needed to define the structural relationship between cationic motifs and lipid specificity. The functional importance of lipids, particularly $PI(4,5)P_2$, binding and the relevance of the β2-β3 cationic motif will be discussed in the next chapter.

This study has also reported the selective tumour cell-killing activity of HBD-3 against a broad spectrum of epithelial and haemotological tumour cell lines at low micromolar concentrations. Many cationic host defense peptides (CHDPs), to which HBD-3 and other defensins belong, have previously been showed to selectively kill tumour cells by various mechanisms. For example, melittin, an α-helical CAP from European honeybee (*Apis mellifera*), is lytic to human leukaemic tumour cells, primarily by inserting into the membrane bilayer and forming barrel-stave pores (Gauldie *et al*, 1978; Killion & Dunn, 1986; Sui *et al*, 1994). Bovine lactoferricin triggers either necrotic membrane disruption or apoptosis depending on the target cell type, and also inhibits xenografted tumour growth and metatasis (Yoo *et al*, 1997a; Yoo *et al*, 1997b; Eliassen *et al*, 2002; Mader *et al*, 2005; Eliassen *et al*, 2006). Human α-defensins HNP-1, -2 and -3, the best characterised members of human α-defensins, cause membrane permeabilisation via ion-permeable channel formation, induce DNA damage and suppress DNA synthesis in tumour cells (Lichtenstein *et al*, 1986; Lichtenstein *et al*, 1988; Kagan *et al*, 1990; Lichtenstein, 1991; Muller *et al*, 2002; Nishimura *et al*, 2004). HNPs are also cytolytic to normal human epithelial cells, fibroblasts and leukocytes (Lichtenstein *et al*, 1986; Nishimura *et al*, 2004). Interestingly, HBD-3 is also cytolytic to normal cells but at higher concentrations than to tumour cells. These data identify HBD-3 as the first human defensin demonstrated to display relatively selective, broad-ranged and direct anticancer activity.

The molecular basis of the specificity towards tumour cells by CHDPs and defensins such as HBD-3, NaD1 and TPP3 (Poon *et al*, 2014; Baxter *et al*, 2015) is unclear but may be associated with morphological changes of plasma membranes upon tumour transformation to influence their robust growth, motility, invasion and metastasis, as opposed to normal cells (Hoskin & Ramamoorthy, 2008). Common features of tumour cells are their increased negatively-charged phospholipid (Utsugi *et al*, 1991; Ran & Thorpe, 2002) and glycoprotein (Blackhall *et al*, 2001; Hollingsworth & Swanson, 2004) content in the membrane outer leaflet, disrupted membrane asymmetry (Fadeel & Xue, 2009), increased membrane surface area (Yamazaki *et al*, 2005) and membrane fluidity (Barnett *et al*, 1974), all of which may contribute to enhanced affinity of membrane interaction, and therefore CAP-mediated cytotoxicity. It is also interesting to note that PIPs, particularly $PI(4,5)P_2$ and $PI(3,4,5)_3$, have key signalling functions and crucial roles in the regulation of cell survival, growth, proliferation, invasion and metastasis, processes that are critical in tumourigenesis (Katso *et al*, 2001; Engelman *et al*, 2006; Wang *et al*, 2007; Bunney & Katan, 2010; Yamaguchi *et al*, 2010). PIP levels could thus be expectedly upregulated in tumour cells. The importance of PIPs in HBD-3 cytotoxicity against tumour cells remains to be elucidated in the next chapter.

2.4.4 Other host defense peptides with PIP binding potential

Apart from HBDs, a number of different HDPs, the majority being cationic, were also identified from all search approaches, including liver-expressed antimicrobial peptide 2 (LEAP2 with CIT**R**LC**RKRR**C motif; ScanProsite search), human θ-defensin 2 (retrocycin-2 or HTD-2 with CG**RR**IC**R**C loop; PDBe search), dermcidin, platelet basic protein (PPBP), azurocidin, bactericidal/permeability increasing protein (BPI), human neutrophil defensin 1 (HNP-1), gasdermin A, S100-A7 ($PI(4,5)P_2$ bead pull-down). Like many other HDPs, the majority of these proteins exhibit broad spectrum antibacterial, antifungal and antiviral activities, and modulate immune response against pathogenic invasion (Campanelli *et al*, 1990; Wasiluk *et al*, 1991; Krause *et al*, 2003; Ericksen *et al*, 2005; Glaser *et al*, 2005; Zou *et al*, 2007; Csosz *et al*, 2015). A few of these proteins such as BPI, dermcidin, gasdermin A, S100-A7 and HNP-1 were reported to bind to bacterial/fungal membrane and/or to induce membrane perturbation, though through different mechanisms (Levy, 2000; Varkey & Nagaraj, 2005; Paulmann *et al*, 2012; Ding *et al*, 2016). The neutrophil BPI, as the name suggests, with its highly-positively charged N-terminal moiety causes sequential damage to bacterial outer and inner membranes respectively via LPS binding-induced diavent cation displacement and time-dependent

progression of uncharacterised inner membrane rupture leading to bacteria killing (Gazzano-Santoro *et al*, 1992; Wiese *et al*, 1997; Levy, 2000). On the other hand, dermcidin, a constitutively-expressed HDP of sweat glands, and its post-secretory proteolytically-processed derivatives, (*e.g.* anionic DCD-1L) preferentially interact with negatively charged bacterial phospholipids and form Zn^{2-}stabilised oligomers leading to ion channel formation and disruption of membrane potential (Paulmann *et al*, 2012; Becucci *et al*, 2014). Similarly, binding of gasdermin A, which is a member of gasdermin family, to bacterial membrane lipids cardiolipin and PE, is thought to be mediate its antibacterial activity (Ding *et al*, 2016). The α-defensin HNP-1 also causes membrane disruption and depolarisation by concentration-dependent mechanisms, ranging from membrane thinning and destabilisation, oligomeric pore formation as well as membrane partitioning as HNP-1 level increases (Lehrer *et al*, 1988; Hill *et al*, 1991; Wimley *et al*, 1994; Bonucci *et al*, 2013). As suggested by a former study on its orthologues rabbit neutrophil defensins, HNP-1 may also require cardiolipin to induce bacterial membrane permeabilisation (Hristova *et al*, 1997; Bonucci *et al*, 2013). S100A7 (psoriasin), a member of S100 family containing calcium-binding EF hand motifs, acts prominently as an antimicrobial peptide by forming transmembrane pores to permeabilise bacterial cells (Michalek *et al*, 2009). It is possible that S100A7 can bind to phospholipids since several S100 proteins require specific binding with phospholipids, *e.g.* S100-A13:PS interaction, and the cell membrane to achieve their cellular functions (Malmendal *et al*, 2005; Kathir *et al*, 2007).

Surprisingly, the HDPs identified by $PI(4,5)P_2$ pull-down may not only be restricted to membrane-permeabilising activity as suggested by the detection of serine/cysteine protease inhibitors (serpins and cystatins), especially in PBMCs and neutrophils stimulated with *E. coli* LPS. Serine and cysteine proteases secreted by microbial pathogens, including *E. coli* pathotypes, are known to be virulence factors during pathogenicity to remove host proteins, particularly those involved in cytoskeleton stability, autophagy or immune response (Yongqing *et al*, 2011; Ruiz-Perez & Nataro, 2014). Therefore, host anti-protease serpins and cystatins can act as innate defense molecules against microorganisms (Irving *et al*, 2002; Kopitar-Jerala, 2006; Aboud *et al*, 2014; Wahlmuller *et al*, 2014). Several cell-penetrating serpins (A4, A5 and B12) were showed to bind to PA, cardiolipin and PIPs, leading to membrane remodelling (Wahlmuller *et al*, 2014). The direct interaction of PIPs, in particular, by serpins activates Akt signalling pathway via stimulation of the PIP-dependent-5-phosphotase SHIP2

(Wahlmuller *et al*, 2014). On the other hand, cystatins and membrane-pore forming HDP cathelicidins are structurally similar, arising from a common ancestral cystatin scaffold fold (Zhu, 2008), implying potential membrane permeabilisation by cystatins.

However, the anticancer properties or the relevance of $PI(4,5)P_2$ or other PIPs in abovementioned HDP activities generally remains largely undetermined. It is also worth noting that none of the proteins revealed by bead pull-down experiments appear to possess the cationic motifs of interest. In fact, $PI(4,5)P_2$ or PIP-interacting determinants are not necessarily restricted to said cationic motifs. The diversity of candidates and, provisionally, $PI(4,5)P_2$ binding show promise for broadening therapeutic development, although further experimental investigations are clearly needed to verify the authenticity and the importance of these protein:PIP/$PI(4,5)P_2$ interactions. Nevertheless, the involvement of PIPs, including $PI(4,5)P_2$, shows strong possibility for some candidates, especially for gasdermin A and, particularly, HNP-1 whose tumour cell killing effect was recently reported. The N-terminal domain of gasdermin A, similar to the closely-related gasdermin D, may execute infection-responding pyroptosis through binding to intracellular PIPs, mainly $PI(4,5)P_2$, homooligomerisation and formation of membrane-disrupting pores to trigger immune cell death (Ding *et al*, 2016). This phenomenon only occurs once the gasdermin A fragment is delivered cytosolically, but not administered extracellularly, thus further emphasising the importance of $PI(4,5)P_2$ (Ding *et al*, 2016). It is, however, not unexpected to detect gasdermin A in LPS-stimulated neutrophil culture media, possibly due to the above-mentioned antimicrobial activity. Nevertheless, the physiological relevance of these observations remains unanswered because, unlike gasdermin D which is activated by caspase-mediated cleavage (Liu *et al*, 2016), gasdermin A is unknown to be preoteolytically processed *in vivo*. On the other hand, $PI(4,5)P_2$ binding of HNP-1 may contribute to its antiviral properties. HNP-1 and, intriguingly, HBD-2 as well as HBD-3, but not HBD-1, significantly inactivate both CCR5-tropic and CXCR4-tropic HIV replication at physiological concentration (Shiomi *et al*, 1993; Jia *et al*, 2001). These defensins are generally believed to exercise their inhibitory effect at both entry point (via, *e.g.* virion binding and down-modulation of HIV coreceptors), and post-entry events by an undefined mechanism (Quinones-Mateu *et al*, 2003a; Sun *et al*, 2005; Seidel *et al*, 2010; Demirkhanyan *et al*, 2012). The post-entry inhibition, which could be achieved even after 12 h post-infection, occur after reverse transcription and integration (Sun *et al*, 2005; Seidel *et al*, 2010). Hypothetically, their interaction with $PI(4,5)P_2$ may interfere with HIV matrix protein Gag to inhibit viral

assembly and budding as demonstrated with an anti-PI(4,5)P_2 monoclonal antibody (Matyas *et al*, 2010; Jobe *et al*, 2012). Furthermore, HNP-1 can suppress protein kinase C (PKC) activation during HIV and influenza A virus infection (Chang *et al*, 2005; Salvatore *et al*, 2007). Meanwhile, PI(4,5)P_2 antagonists, such as neomycin, can mask PI(4,5)P_2 from hydrolysis by phospholipase C, which is vital for PKC activation (Wang *et al*, 2005).

To date, among the candidate proteins, only HNP-1 has been showed to exhibit low micromolar tumour cell killing activity via a two-phase mechanism (Lichtenstein *et al*, 1988; Lichtenstein, 1991). The initial phase is associated with outer membrane binding, possibly initiated by electrostatic interaction, followed by oligomerisation and pore formation leading to rapid loss of membrane potential and membrane integrity (Lichtenstein *et al*, 1988; Hill *et al*, 1991; Lichtenstein, 1991; Wimley *et al*, 1994). However, these initial effects appear insufficient for subsequent cell lysis, which may require HNP-1 internalisation and an intracellularly-acting second phase (Lichtenstein *et al*, 1988; Lichtenstein, 1991). A recent study on cellular morphology and membrane ultrastructure using atomic force microscopy (Gaspar *et al*, 2015) suggested that HNP-1 might induce apoptosis upon translocation to cytoplasm, although further apoptotic characterisations are required to support this claim. Intriguingly, although no membrane blebbing was recorded possibly due to cell fixation prior to imaging, HNP-1-treated tumour cells showed reduced cell membrane roughness, indicating the detachment of cytoskeletal network from the plasma membrane (Gaspar *et al*, 2015). This observation, albeit dependent on many factors, is similar to enzymatic modification or sequestration of the inner membrane PI(4,5)P_2 (Sheetz *et al*, 2006; Keller *et al*, 2009) and also to the proposed molecular mechanism underlying the PI(4,5)P_2-dependent tumour cell lysis by the plant defensin NaD1 (Poon *et al*, 2014).

Characterising the provisional participations of PI(4,5)P_2 discussed here, particularly for gasdermin A and HNP-1 (as well as HBD-2 and HBD-3) functions, will not only expand the understanding of PI(4,5)P_2 importance in physiological and disease settings but also potentially open innovative strategies for drug development.

2.4.5 Arachnid toxins with the cationic loop

In addition to human β-defensins, the cationic search using PDBe database also unveiled a few arachnid neurotoxins with the cysteine-flanked cationic loops spanning between

two β-strands. Tarantula psalmototoxin-1 (PcTx1), huwentoxin-4 (HWTX-4) and scorpion charybdotoxin (CTX) are potent and selective inhibitors of acid-sensing ion channels (ASICs, also known as proton-gated sodium channels), voltage-gated sodium (Na_V) channels and calcium-activated potassium (maxi-K) channels, respectively (Garcia *et al*, 1994; Escoubas *et al*, 2000; Peng *et al*, 2002). Majority of cysteine-rich arachnid toxins are generally structured around two main scaffolds, the cysteine-stabilised α/β (CSαβ) motif and the inhibitor cysteine knot (ICK) motif (Pallaghy *et al*, 1994; Norton & Pallaghy, 1998; Mouhat *et al*, 2004). The CSαβ motif, as observed in CTX (Cohen *et al*, 2008), many scorpion toxins as well as plant and insect defensins (Lay & Anderson, 2005), comprises an α-helix and a double- or triple-stranded antiparallel β-sheet (in a βαββ configuration) that are stabilised by three or four disulfide bonds. On the other hand, the ICK motif, as displayed by PcTx1 (Escoubas *et al*, 2003), HWTX-4 (Sermadiras *et al*, 2013), other arachnid and mollusc toxins and knottin-type HDPs (Craik *et al*, 2001; Barbault *et al*, 2003; Tam *et al*, 2015), features an embedded ring formed by a double- or triple-stranded β-sheet and two disulfide bridge, through which the third one penetrates.

Notwithstanding the different frameworks, both CSαβ and ICK-type neurotoxins, and remarkably HDPs, contain compact disulfide bond-stabilised cores that allow the exposure of flexible inter-cysteine loops spanning between two secondary structures. The loops, with different lengths and composition, are highly evolvable, leading to functional modification or neofunctionalisation for specific interactions with new targets (Bouhaouala-Zahar *et al*, 2002; Kimura *et al*, 2012; Shafee *et al*, 2016b). For example, the length of certain loops of arachnid toxins, apart from functional ion channel-binding signatures, are typically shorter than their defensin counterparts, reducing steric hindrance and thus enabling peptide-channel interaction (Zhu *et al*, 2014; Zhu *et al*, 2015; Shafee *et al*, 2016b). Indeed, a small loop deletion converts an insect defensin to a scorpion potassium channel (K_v)-binding signature, leading to K_v inhibition with same mechanism and high affinity (Zhu *et al*, 2014). Considering this evolvability, it is also possible that certain toxins, such as CTX, PcTx1 and HWTX-4, may have evolved loop compositions to the cationic motifs like solanaceous plant defensins, possibly to also bind $PI(4,5)P_2$ and/or other negatively-charged membrane lipids. In fact, it has recently been reported that lipid interaction is required for TRPV1 cation channel inhibition by the ICK-type tarantula toxin DkTx (Gao *et al*, 2016). Its finger-like inter-cysteine loops penetrate the lipid bilayer and form a phospholipid-channel-toxin tripartite complex to stabilise the

open state of the channel (Gao *et al*, 2016). The cationic loop motifs may therefore be extended beyond host defense peptides to other peptide classes, including neurotoxins.

2.4.6 Other potential roles of $PI(4,5)P_2$ binding in biological processes implicated by $PI(4,5)P_2$ pull-down

Though antimicrobial and/or anticancer activities of many $PI(4,5)P_2$ bead pulled-down proteins remains undetermined and unpredictable, $PI(4,5)P_2$ may play certain roles in their reported functions, such as blood coagulation. The blood coagulation (or blood clotting) pathway is characterised by platelet activation and the induction of coagulation cascade that involves a series of serine proteases, leading to thrombin generation and, subsequently, fibrin deposition and maturation via thrombin-mediated cleavage of fibrinogen (Delvaeye & Conway, 2009). Fibronectin and thrombospondin-1 also copolymerise with fibrin, possibly to mediate the interaction of fibrin matrix with aggregated platelets and to modulate clot network structure (Chow *et al*, 1983; Bale *et al*, 1985). Strikingly, thrombin-stimulated human platelets, only in presence of added fibrinogen, showed a large decrease of membrane $PI(4,5)P_2$, but not any other PIPs, which could not be attributed to increased $PI(4,5)P_2$ degradation due to insufficient hydrolysis products (Vickers *et al*, 1987). Therefore, it was suggested that $PI(4,5)P_2$ may specifically associate with polymerising fibrin (Vickers *et al*, 1987). Surprisingly, over the last two decades, this suggestion has been overlooked despite growing knowledge in blood coagulation. The detection of pivotal clotting matrix proteins fibrinogen, fibronectin and thrombospondin-1 by $PI(4,5)P_2$ pull-down from LPS-stimulated PBMC culture media, might re-emphasise the association of $PI(4,5)P_2$ with fibrin clot. $PI(4,5)P_2$ might directly bind and induce the co-polymerisation of clot matrix proteins, as showed for $PI(4,5)P_2$-induced oligomerisation and/or fibril formation of the fibroblast growth factor 2 (Steringer *et al*, 2012), MLKL (Dondelinger *et al*, 2014), solanaceous plant defensins (Poon *et al*, 2014; Baxter *et al*, 2015) and amyloid β (Knight & Miranker, 2004). Furthermore, it may also highlight the role of blood coagulation in host defense against invading microorganisms. Indeed, the blood coagulation and the innate immune system are remarkably integrated e.g. the fibrin matrix prevents infection by microbe trapping (Delvaeye & Conway, 2009; Esmon *et al*, 2011). Except fibrinogen, fibronectin and thrombospon-1 were consistently showed to be induced by stimulated cultured monocytes and PBMCs (DiPietro & Polverini, 1993; Kitamura *et al*, 2000). It is therefore possible that pathogens stimulate host leukocytes to express and secret fibrin, fibronectin and thrombospondin-1, in addition to the serum source. Upon thrombin cleavage and

platelet contact, binding of newly-formed fibrin with $PI(4,5)P_2$ can lead to recruitment of fibronectin, thrombospondin-1 and possibly other platelet clot matrix proteins, and co-polymersation to form fibrin network to trap the invading microorganisms. Once elucidated, this mechanism might not only resolve the role of $PI(4,5)P_2$ in blood coagulation, but also imply that $PI(4,5)P_2$-binding therapeutics could be used as anticoagulation in blood clotting-related diseases.

2.5 Conclusion

This chapter has identified an arsenal of potential PIP and $PI(4,5)P_2$-binding proteins. Many are HDPs, with or without cationic motifs, displaying broad-spectrum antimicrobial activity, positive net charge and membrane penetrating effect. These are highly favourable properties for combinatorial attack of pathogen and tumour cells, with potential therapeutic specificity by targeting of intracellular molecules, including PIPs. In particular, the potent antimicrobial HBD-3 has been demonstrated to selectively kill tumour cells at low micromolar concentrations as well as confirmed to preferentially bind $PI(4,5)P_2$ and PIPs *in vitro* and on cellular membrane. This has instigated further investigation of the provisional $PI(4,5)P_2$-dependent anticancer activity of HBD-3 in the next chapter. Moreover, the results identify putative PIP-binding cationic loops beyond HDPs to neurotoxins, suggesting the role of $PI(4,5)P_2$ and PIPs in ion channel modulation. Intriguingly, the role of $PI(4,5)P_2$ in innate defense blood clot formation is also suggested and worth investigating to define the use of $PI(4,5)P_2$-binding molecules in blood clot-related diseases. In summary, this chapter provides potential new opportunities for exploring future multifaceted drug development of $PI(4,5)P_2$ and PIP-targeting molecules.

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Chapter 3

Human β-defensin 3 contains an oncolytic motif that binds $PI(4,5)P_2$ to mediate cancer cell permeabilisation

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3.1 Introduction

Naturally occurring host defense peptides (HDPs), are an important component of host innate immunity, representing the first line of chemical defense against invading pathogens. Defensins are a prominent family of cationic HDPs (CHDPs) that are ubiquitously expressed in plant, fungi, invertebrates and vertebrates. Despite limited amino acid sequence identities and different disulfide frameworks (6–10 cysteines) amongst defensin subfamilies, their tertiary structures share remarkably similar folds that feature a triple-stranded antiparallel β-sheet (or equivalent pattern thereof) constrained by intramolecular disulfide bridges (Boman, 1995; Broekaert *et al*, 1995; Ganz, 2003). Defensins and many other CHDPs display diverse functions and mechanisms of action, including multimodal and multi-target microbicidal effects, and immunomodulatory activities (Ganz, 2003; Brogden, 2005; Jenssen *et al*, 2006; Ulm *et al*, 2012; Gwyer Findlay *et al*, 2013). Additionally, a number of CHDPs have been found to exhibit specific anticancer activity against solid and/or hematological cancers, and hence represent a potential therapeutic strategy to counter current issues of adverse side-effects and multidrug resistance (Hoskin & Ramamoorthy, 2008; Gaspar *et al*, 2013; Mulder *et al*, 2013).

Nicotiana alata defensin 1 (NaD1), a potent antifungal peptide from the flowers of the ornamental tobacco, was reported to selectively kill a broad spectrum of cancer cells *in vitro* at low micromolar concentrations (Poon *et al*, 2014). The underlying mechanism was described to involve the entry of NaD1 into the cell followed by binding to PI(4,5)P_2, leading to membrane permeabilisation, membrane blebbing and eventually to cell lysis (Poon *et al*, 2014). Similarly, Baxter *et al* (2015) recently demonstrated PI(4,5)P_2 specificity and cancer cell cytotoxicity for the related tomato defensin TPP3, suggesting a shared molecular target and mechanism of action for these defensins.

PI(4,5)P_2 is one of seven phosphorylated derivatives of phosphatidylinositol, which are collectively known as PIPs. Despite their low abundance, they play important regulatory roles for diverse cellular processes, including cellular signalling, cytoskeletal rearrangement and membrane trafficking (Di Paolo & De Camilli, 2006; Falkenburger *et al*, 2010; Balla, 2013). NaD1 and TPP3 have been showed to bind PI(4,5)P_2 via their cysteine-flanked highly-positively charged β2-β3 loop (residues 36–40 in NaD1 and residues 38–42 in TPP3) (Poon *et al*, 2014; Baxter *et al*, 2015). As a dimer, two β2-β3

loops of NaD1 monomers form a claw-like structure with PI(4,5)P_2 accommodated in the binding grip. The protein-lipid interaction involves an intensive hydrogen bonding network provided by residues within and around the β2-β3 loop. Defects in PI(4,5)P_2 binding effectively lead to severe impairment of the anticancer activity of NaD1 (Poon *et al*, 2014). Equivalently, the importance of the β2-β3 loop, in PI(4,5)P_2 binding was also recently reported for TPP3 (Baxter *et al*, 2015).

The β2-β3 loop of NaD1 and TPP3 is highly conserved among class II defensins of solanaceous plants, and interestingly, is also shared with human β-defensin 3 (HBD-3) as described in the previous chapter. HBD-3 is inducibly expressed and secreted by epithelial cells, several non-epithelial tissues, monocytes and neutrophils (Garcia *et al*, 2001; Harder *et al*, 2001; Harder *et al*, 2004; Sorensen *et al*, 2006) and is arguably the most potent antimicrobial of the β-defensins (Schibli *et al*, 2002; Chen *et al*, 2007; Schroeder *et al*, 2011). HBD-3 exhibits broad-spectrum antibacterial, antifungal and antiviral activities (Garcia *et al*, 2001; Harder *et al*, 2001; Quinones-Mateu *et al*, 2003; Feng *et al*, 2005; Leikina *et al*, 2005; Sun *et al*, 2005; Pazgier *et al*, 2006). HBD-3 is also chemoattractive and activates antigen presenting cells as well as induces chemokine expression, crucially contributing to the integration of innate and adaptive immune responses (Funderburg *et al*, 2007; Nagaoka *et al*, 2008; Ferris *et al*, 2013; Petrov *et al*, 2013). HBD-3 has been proposed to interact with bacterial lipid II (Sass *et al*, 2010), monocytic phosphatidylserine (Lioi *et al*, 2012), and different subsets of Toll-like, CC and CXC chemokine receptors (Funderburg *et al*, 2007; Nagaoka *et al*, 2008; Rohrl *et al*, 2010; Feng *et al*, 2013). However, it should be noted that the biological involvement of many of these targets in HBD-3 activities has been challenged in recent years (Lee *et al*, 2015). In addition, an anti-metastatic effect on head, neck and colon cancer cells has been reported (Wang *et al*, 2012; Uraki *et al*, 2014). However, the anticancer mechanism of HBD-3 remains poorly defined, and not until the previous thesis chapter was the direct and relatively selective cancer cytotoxicty of HBD-3 reported at low micromolecular range.

In this report, it is demonstrated for the first time that a human CHDP, HBD-3, binds phosphoinositides and that the interaction with PI(4,5)P_2 in particular, is critical for the cancer cell killing activity of this defensin. The data support the importance of a cationic β2-β3 loop for PIP, specifically PI(4,5)P_2, binding, that contributes to a conserved

mechanism of cancer cell/pathogen cytolysis among innate molecules with NaD1-like cationic motif. This study identifies PIP and PI(4,5)P_2-binding CHDPs as a potential new generation of multifaceted therapeutics, particularly as anticancer agents.

3.2 Materials and methods

3.2.1 Cell lines and cultures

Human epithelial cervical cancer (HeLa) and leukemic monocyte lymphoma (U937) were cultured as per specified in section 2.2.1.

3.2.2 Propidium iodide uptake assay

Cells suspended at 1×10^6 cells/mL in serum-free medium containing 0.1% (w/v) BSA were treated with defensins at 37°C for 30 min. PI was added to final concentratin of 1 μg/mL prior to flow cytometry analysis. For lipid inhibition assays, 15 μM HBD-3 was pre-incubated with 45 μM synthetic lipids, including L-α-phosphatidic acid (PA), L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), L-α-phosphatidylserine (PS), L-α-phosphatidylinositol-4-phosphate (PI(4)P), L-α-phosphatidylinositol-3,5-bisphosphate (PI(3,5)P_2), L-α-phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2) and L-α-phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)$P)_3$. All lipids were sourced from Avanti Polar Lipids (AL, USA). For neomycin inhibition experiments, U937 cells at 1×10^6 cells/mL were pre-treated with 10 mM neomycin, followed by three centrifugal washes with phosphate buffered saline (PBS) at 500 *g* for 5 min, prior to treatment with HBD-3 or human cathelicidin LL-37 (Abcam).

3.2.3 Cell viability assay

Cell viability assays for HBD-3 mutants were performed as per section 2.2.16.

3.2.4 ATP release assay

ATP release assay was conducted using an ATP bioluminescence assay kit (Roche Diagnostics, Germany). U937 cells were suspended at 1×10^6 cells/mL in PBS containing 0.1% (w/v) BSA, and mixed with luciferase/luciferin reagents at a ratio of 4:5 in black 96-well microtiter plates (Corning Life Sciences). HBD-3 was simultaneously added to the mixture to desired concentrations and the level of ATP release measured immediately as bioluminescence emission signal intensity for 30 min with 30 s intervals. HeLa cells (5×10^4 cells/well) were seeded overnight in RPMI-1640 supplemented with 10% FCS in clear-bottom black 96-well plates (Corning Life Sciences). Seeded HeLa cells were then

washed with PBS at 300 *g* for 5 min and added with PBS containing 0.1% (w/v) BSA, immediately before addition of luciferase/luciferin reagents and HBD-3. For neomycin inhibition experiments, overnight-seeded HeLa cells were treated with various concentration of neomycin for 3 h prior to three centrifugal PBS washes.

3.2.5 Labelling of HBD-3 with BODIPY FL EDA

Lyophilised HBD-3 was resuspended to 5 mg/mL in activation buffer (0.1 M MES, 0.5 M NaCl, pH 6). Ten-fold and 25-fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysulfosuccinimide (Thermo Scientific Pierce, Rockford, IL) was then added, respectively. After 15 min incubation, the pH was adjusted to 7.2 using 20× PBS, prior to reaction with five-fold molar excess of BODIPY FL EDA (Molecular Probes, Eugene, OR) for 3 h. Labelled HBD-3 was then eluted from free label using a PD-10 desalting column (GE Healthcare, UK). Elution fractions were subjected to semi-quantification of relative protein amount and fluorescence intensity, using NanoDrop^{TM} 2000 UV-VIS spectrophotometer (Thermo-Fisher Scientific). Concurrence of protein peak with a moderate first fluorescence peak along with appearance of strong second fluorescence peak indicated the successful labelling of HBD-3 and removal of free BODIPY FL EDA dye.

3.2.6 Confocal laser scanning microscopy (CLSM)

Live imaging was performed on a Zeiss LSM-780 confocal microscope using a 63× oil immersion objective in a 37°C incubator with 5% CO_2. Adherent HeLa cells were cultured overnight on coverslips whereas suspension U937 cells were immobilised onto 0.01% (w/v) poly-L-lysine-coated coverslips. Both cell types were prepared in serum-free RPMI 1640 medium containing 0.1% (w/v) BSA and 2 μg/mL PI. HBD-3 or BODIPY FL EDA-labelled HBD-3 was added directly to the imaging chamber to final concentration of 15 μM, via a capillary tube. In certain experiments, prior to imaging, cells were pre-stained with the membrane dye PKH67 as per manufacturer's instructions (Sigma-Aldrich) or transfected with expression vectors carrying GFP only or GFP-tagged Pleckstrin homology domain of phospholipase C-delta 1 (GFP-PH-PLC(δ)) (kindly offered by Christina Mitchell, Monash University, Australia), using Lipofectamine 3000 reagent (Invitrogen) as per manufacturer's instructions. For CLSM involving transfections, images were taken as 4×4 tiles over 30 min, and the resultant data for GFP and GFP-PH transfected cells were analysed by counting PI-positive cells. For neomycin

inhibition experiments, after immobilizing onto coverslip, U937 cells were pre-treated with 10 mM neomycin prior to PKH67 staining and HBD-3 treatment.

3.2.7 ATP-encapsulated liposome leakage assay

Liposomes were generated as described previously (Zhang *et al*, 2001) using natural PI(4,5)P_2 (porcine brain, in chloroform:methanol:water at 20:9:1 molar ratio) and PC (chicken egg, in chloroform) purchased from Avanti Polar Lipids. PC only or PC:PI(4,5)P_2 (95:5 molar ratio) solutions were dried under a stream of nitrogen gas followed by overnight vacuum-drying. The lipid films were rehydrated to a final concentration of 5 mg/mL in 20 mM HEPES (pH 7.2) containing 5 mg/mL adenosine-5-triphosphate disodium salt (ATP; Sigma-Aldrich) at 37°C for 1 h. After three subsequent cycles of freezing (liquid nitrogen) and thawing (25°C), multilamellar liposomes were extruded 15–20 times through a mini-extruder (Avanti Polar Lipids). Free ATP was removed by three centrifugal washes with 20 mM HEPES at 16,500 *g* prior to the ATP release assay. Liposomes treated with 1% Triton X-100 were included as positive control and assigned as total lysis, whereas HEPES only control served as background reading. The level of ATP release at a particular time point was determined by quotient of the corrected reading (after subtracting background) of the sample at that time point and corrected reading of total lysis, as per the following equation:

$$\%Lysis_t = \frac{Sample_t - HEPES_t}{Triton\ X - HEPES} \times 100$$

3.2.8 Cross-linking studies

HBD-3 at 1 mg/mL was incubated with 200 μM, 400 μM and 800 μM PI(4,5)P_2, approximately corresponding to 1:1, 1:2 and 1:4 molar ratio respectively, at room temperature for 30 min. Bis[sulfosuccinimidyl] suberate (BS^3) in PBS was then added to final concentration of 6.25 mM for 30 min at room temperature to cross-link protein complexes. In certain experiments, HBD-3 at various concentrations was directly incubated with BS^3 without PI(4,5)P_2. Samples were subjected to reducing and denaturing SDS-PAGE (as per section 2.2.9) prior to silver staining using SilverQuest™ silver staining kit (Thermo-Fisher Scientific).

3.2.9 Thioflavin T spectroscopic assay

HBD-3, NaD1 (as a positive control) at 1 mg/mL or buffer only (as a negative control) was incubated with 200 μM, 400 μM and 800 μM PI(4,5)P_2 for 30 min at room

temperature. Samples (10 μL) were added to black 96-well microtiter plates prior to simultaneous addition of 190 μL of 20 μM thioflavin T (Sigma-Aldrich) in PBS. Fluorescence intensity was recorded at excitation and emission wavelengths of 440 nm and 485 nm, respectively.

3.2.10 Transmission electron microscopy (TEM)

The coating of grids with prepared samples and subsequent TEM was conducted by Dr Christopher Adda (La Trobe University). HBD-3 at 0.9 mg/mL was allowed to react with 0.23 mM PtdIns(4,5)P_2 (Avanti Polar Lipids) for 30 min at room temperature, upon which samples (10 μL) were then applied to mesh copper grids coated with a thin layer of carbon for 2 min. Excess material was removed by gentle blotting and samples were negatively stained twice with 10 μl of 2% (w/v) uranyl acetate solution (Electron Microscopy Sciences, PA, USA). The grids were air-dried and viewed using a FEI Tecnai TF30 transmission electron microscope operated at 300 kV.

3.2.11 Site-directed mutagenesis

Alanine mutations of residues within and preceding the cationic motif of HBD-3 was performed using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, CA, USA). Briefly, PCR primers carrying introduced mutations were generated using PrimerX (http://www.bioinformatics.org/primerx/) with manufacturer-recommended criteria (i.e. melting temperature lower than 78°C, GC content greater than 40%, 25–45 bases in length, terminating in G or C, and GCT or GCC as alanine codons for *Pichia pastoris*). Mutagenesis PCRs were performed on the recombinant plasmid pPIC9-HBD3 (from section 2.2.8) at 0.4 ng/μL in the presence of *PfuUltra* high-fidelity DNA polymerase (0.05 U/μL), primers (2.5 ng/μL each), dNTP mix (1:50 dilution) and 1× reaction buffer with the following temperature profile: an initial cycle of 95°C, 30 s; 30 cycles of 95°C, 30 s, 55°C, 1 min; 68°C, 12.5 min; and a 5 min final extension cycle at 68 °C. *Dnp*I endonuclease was added to final concentration of 0.2 U/μL to digest the parental recombinant plasmid for 2 h at 37°C. *Dnp*I-treated PCR products were then transformed into *Escherichia coli* XL-1 Blue and sequenced to confirm mutation identity. *P. pastoris* transformation, expression and purification of HBD-3 mutants were subsequently conducted as per section 2.2.8. Successfully-purified mutants were subjected to SDS-PAGE (as in section 2.2.9) and immunoblotting analysis (section 2.2.10) for quality assurance.

3.2.12 Fungal growth inhibition assay

The ability of HBD-3, HBD-3(K32A) and HBD-3(K39A) to inhibit the growth of *Candidas albicans* (strain ATCC90028) and *Fusarium graminearum* (strain PH-1) was examined by Dr Mark Bleackley (La Trobe University) as described in Hayes *et al* (2013) and van der Weerden *et al* (2008), respectively. Briefly, *C. albicans* were grown overnight in YPD media (30°C, 250 rpm). Cell number was then determined using a hematocytometer, followed by dilution of cell culture to 5×10^3 cells/mL in half-strength potato dextrose broth (PDB; Becton Dickinson, NJ, USA). *F. graminearum* spores were isolated from sporulating cultures growing in half-strength PDB by filtration through sterile muslin. Spores were counted and adjusted to 5×10^4 spores/mL in half-strength PDB. Diluted *C. albicans* cells or *F. graminearum* spores (80 µL) were added to the wells of sterile 96-well plate along with 20 µL of sterile-filtered protein solution to final concentrations of 0-10 µM. After 24 h, cell growth was determined by measuring absorbance at 595 nm in a SpectraMAX M5e plate reader (Molecular Devices, Sunnyvale, CA).

3.2.13 Immunofluorescence microscopy

HeLa cells (5×10^5 cells/well) were seeded on sterilised-coverslips overnight in RPMI-1660 supplemented with 10% FCS. Cells were washed three times with PBS prior to addition of 15 µM HBD-3, HBD-3(K32A) or HBD-3(K39A) in serum-free RPMI-1640 containing 0.5% BSA. After 30 min incubation at 37°C, cells were stained with 1 µg/mL PI dye for 5 min prior to 20 min fixation with 2% (v/w) paraformaldehyde, 10 min lysis with 0.5% (w/v) saponin, and then 30 min blocking with 3% (w/v) BSA. Rabbit anti-HBD-3 antibody (2.5 µg/mL), and then Alexa Fluor 488-conjugated goat-anti-rabbit IgG (2.5 µg/mL; Abcam) were added, followed by fluorescence imaging using Zeiss LSM-780 confocal microscope with a 63× oil immersion objective.

3.2.14 Protein-lipid overlay assay

Protein-lipid overlay assay for HBD-3(K32A) and HBD-3(K39A) mutants were done as per described in section 2.2.18.

3.2.15 Anti-PI(4,5)P_2 antibody blocking assay

Blocking of anti-PI(4,5)P_2 antibody binding by HBD-3(K32A) and HBD-3(K39A) mutants were performed as per described in section 2.2.14. To confirm the relevance of these HBD-3:lipid interactions on the ability of HBD-3 to permeabilise cancer cell

membranes, lipid inhibition of HB3-mediated PI uptake by U937 cells was examined. Among all tested lipids, PI(4,5)P_2 and PI(3,4,5)P_3 showed the greatest inhibitory activity, with ~50% and 100% reduced PI-positivity, respectively (**Figure 5C**). A much lower inhibition was also observed for PS, with ~15% reduction. Other lipids, including PC, PE, and particularly PI(4)P and PI(3,5)P_2, did not show any significant inhibition. Interestingly, PA, the lipid suggested to bind most strongly to HBD-3 by lipid protein overlay assays, did not show any inhibitory activity but instead enhanced permeabilisation.

3.3 Results

3.3.1 Membrane blebbing and membrane permeabilisation of cancer cells induced by HBD-3

As previously reported, the plant defensins NaD1 and TPP3 bind PI(4,5)P_2 to trigger membrane blebbing and membrane permeabilisation (Poon *et al*, 2014; Baxter *et al*, 2015). In fact, PI(4,5)P_2 sequestering has long been associated with blebbing formation (Raucher *et al*, 2000; Di Paolo & De Camilli, 2006; Sato & Frank, 2014). As HBD-3, with the analogous β2-β3 cationic motif, was demonstrated to bind to PI(4,5)P_2 *in vitro* and on cell membrane (section 2.3.7). Therefore, flow cytometry-based assays using the membrane impermeable dye PI were conducted to examine the ability of HBD-3 to induce membrane permeabilisation. HBD-3 was able to permeabilise all cell types tested to varying levels in a dose-dependent manner (**Figure 3.1A**). Upon HBD-3 treatment at concentrations higher than 10 μM, the cancer cells were more readily permeabilised than the primary cells. For example, approximately 85% of U937 cells, compared to 14.5% of HUVEC cells, treated with 25 μM HBD-3 were PI-positive. Even at the highest dose tested (50 μM HBD-3), less than 20% of cells for each primary cell type compared to at least 80% of cells for the cancer lines, were permeabilised. Furthermore, cytoplasmic ATP was rapidly released from U937 and HeLa cells by HBD-3 in a similar concentration-dependent manner (**Figure 3.1B**). Together, these data suggest that HBD-3 causes cancer death via rapid, and, to certain extents, selective membrane permeabilisation.

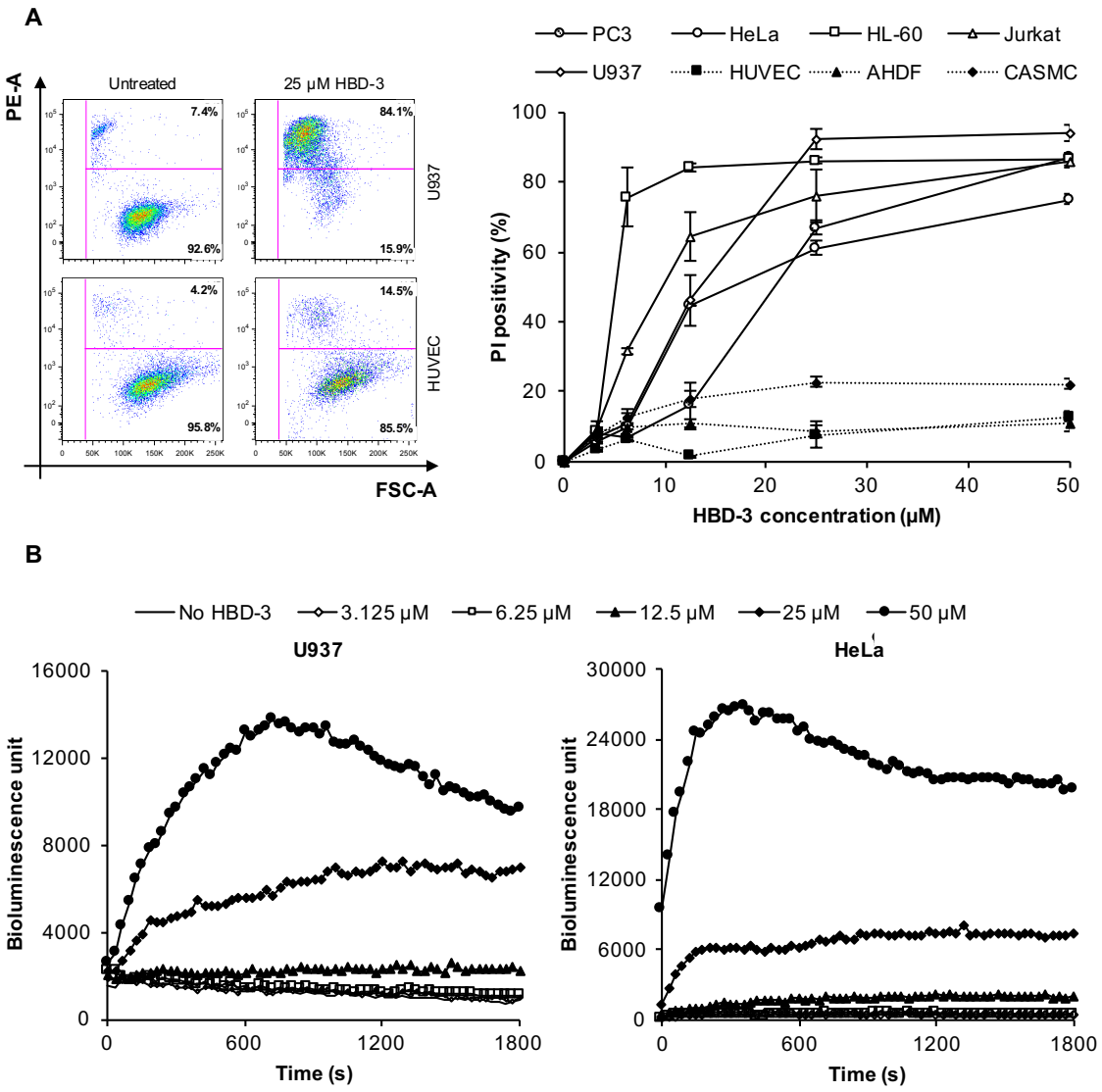

Figure 3.1 Cancer-specific induction of membrane permeabilisation by HBD-3

(A) Flow cytometry-based PI uptake assay of different cancer and primary cell lines treated with or without HBD-3. Cells were gated based on forward scatter (FSC-A) and side scatter (SSC-A). Level of permebilisation was expressed as PI positivity, detected by PE-A signal. Data represent mean ± SEM of three independent experiments. **(B)** ATP bioluminescence assay of U937 and HeLa cells treated with HBD-3 titrations. Level of cytoplasmic ATP released was detected as bioluminescence emission signal intensity. Data are representative of three independent experiments.

Using confocal laser scanning microscopy (CLSM), the morphology of HBD-3 treated U937 and HeLa cells was visualised in the presence of the membrane stain PKH67 and permeabilisation indicator PI. HBD-3 induced the formation of large membrane blebs (>10 μm) in the PI-positive cells (**Figure 3.2A**). To further monitor the kinetics and localisation of membrane permeabilisation process, a time-course CLSM study using BODIPY FL-labelled HBD-3 was performed on U937 cells (**Figure 3.2B**). Initially, at

2.5 min post-addition, HBD-3 accumulated locally on the outer plasma membrane, before gaining entry through the accumulation point. HBD-3 then appears to target and bind to the inner membrane leaflet, and potentially other cytoplasmic membranes, including the nuclear envelope at 4.5 min. PI staining, indicating membrane disruption, subsequently coincided with initiation of membrane blebbing. PI signal intensification and bleb expansion were further observed from 10 min onward. No membrane accumulation of HBD-3 and bleb formation was detected on viable (PI-negative) cells.

3.3.2 The role of PI(4,5)P_2 binding in HBD-3-induced cytolysis

To investigate the potential relationship between PI(4,5)P_2 binding and HBD-3-mediated membrane permeabilisation, liposome leakage assays were conducted by measuring the release of encapsulated ATP by bioluminescence. Whilst HBD-3 minimally induced ATP release from ATP-encapsulated PC only liposomes, a much higher level of ATP was released from PC liposomes containing 5% PI(4,5)P_2. This liposome permeabilisation effect was concentration-dependent, as increasing ATP release levels were observed with increasing HBD-3 concentration (**Figure 3.3A**).

To confirm the relevance of other HBD-3:lipid interactions on the ability of HBD-3 to permeabilise cancer cell membranes, lipid inhibition of HB3-mediated PI uptake by U937 cells was examined. Among all tested lipids, PI(4,5)P_2 and PI(3,4,5)P_3 showed the greatest inhibitory activity, with ~50% and 100% reduced PI-positivity, respectively (**Figure 3.3B**). A much lower inhibition was also observed for PS, with ~15% reduction. Other lipids, including PC, PE, and particularly PI(4)P and PI(3,5)P_2, did not show any significant inhibition. Interestingly, PA, the lipid suggested to bind most strongly to HBD-3 by lipid protein overlay assays, did not show any inhibitory activity but instead enhanced permeabilisation.

The importance of PI(4,5)P_2 binding was further demonstrated by the competitive inhibition of HBD3-mediated membrane permeabilisation with neomycin (**Figure 3.4A**). Compared to the untreated control, U937 cells pre-incubated with neomycin were significantly less susceptible to HBD-3 treatment, but not with LL-37, another CAP that exhibits non-PI(4,5)P_2-dependent detergent-like (Oren *et al*, 1999; Porcelli *et al*, 2008; Sood *et al*, 2008) and/or pore-forming (Lee *et al*, 2011) membrane disruption.

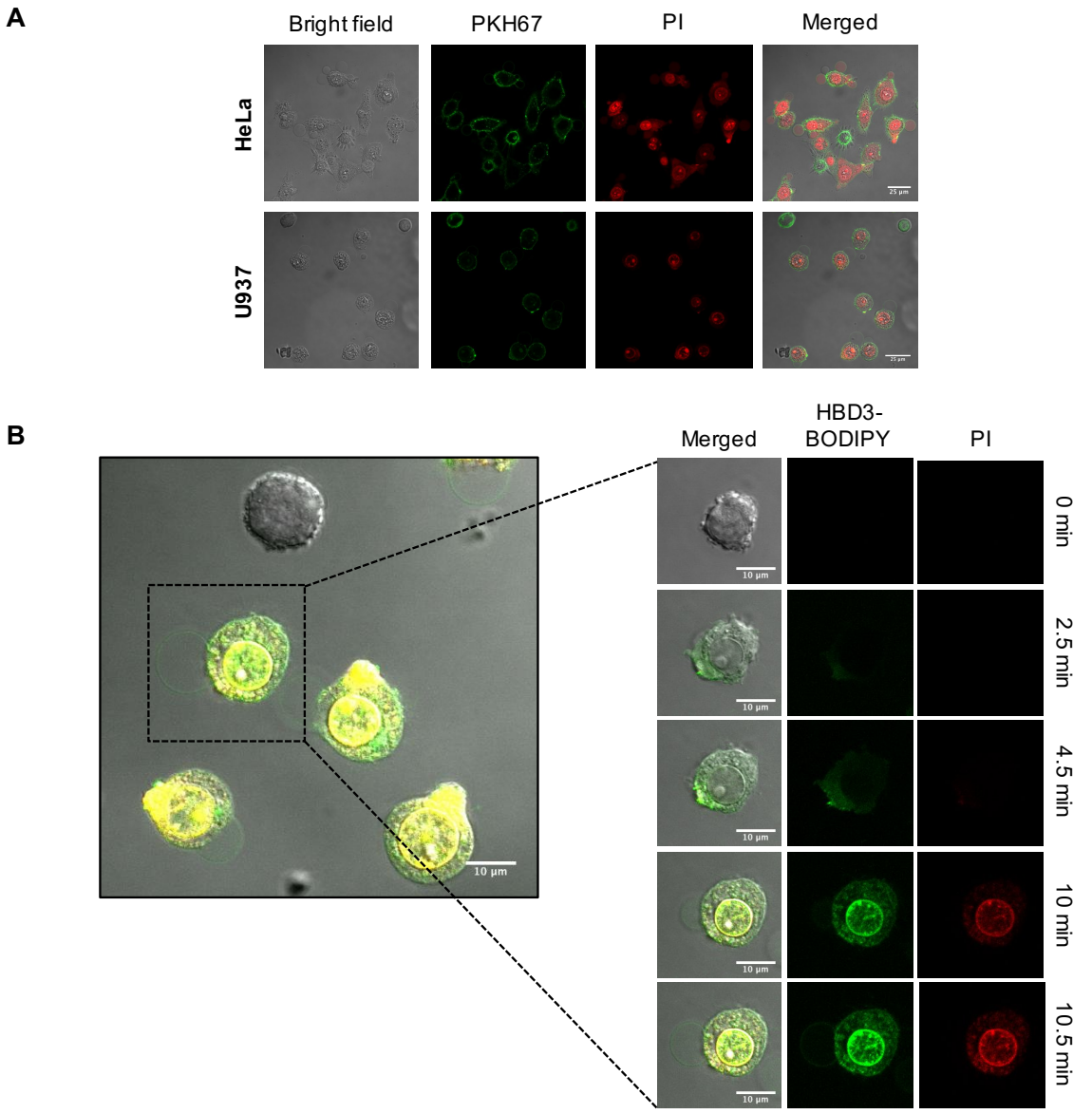

Figure 3.2 Bleb-associated membrane permeabilisation of HBD-3

(A) CLSM imaging of PKH67-prestained HeLa and U937 cells at 15 min after addition of 15 µM HBD-3. **(B)** Time-lapse CLSM imaging of U937 cells treated with 15 µM HBD3-BODIPY FL EDA. Scale bars represent 10 µm. Data in **A** and **B** are representative of three independent experiments.

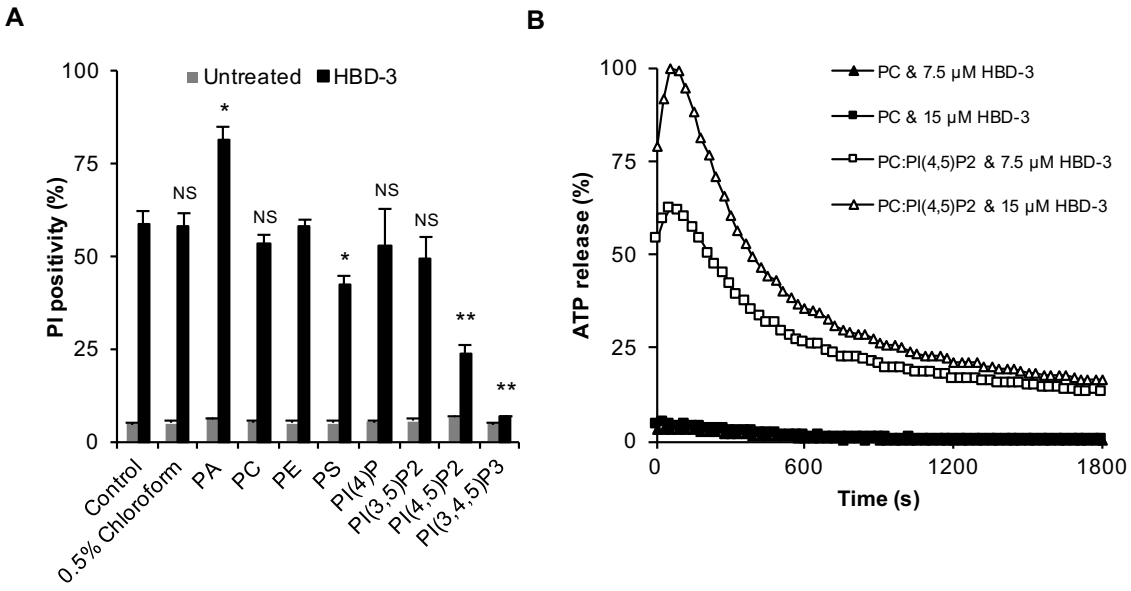

Figure 3.3 Importance of PI(4,5)P_2 binding in HBD3-mediated membrane permeabilisation

(A) Inhibitory effect of lipids on HBD3-mediated membrane permeabilisation. HBD-3 (15 µM) were pre-incubated with lipids (1:2 molar ratio) prior to flow cytometry-based PI uptake assay. Data represent mean ± SEM of three independent experiments. NS, not significant; *, $p<0.05$; **, $p<0.01$; unpaired t-test. **(B)** HBD3-induced ATP release of ATP-encapsulated PC and PC:PI(4,5)P_2 liposomes. Level of ATP released was detected as bioluminescence signals and normalised against HEPES only (background) and triton-X 100 treated (100% lysis) control. Data are representative of three independent experiments.

Nevertheless, the inhibitory effect of neomycin was overcome at higher concentrations of HBD-3, suggesting competition between neomycin and HBD-3 for PI(4,5)P_2 binding. Moreover, neomycin also inhibits ATP release from HeLa cells, in dose-dependent manner, upon HBD-3 treatment (**Figure 3.4B**). To investigate if these inhibition effects are led by blocking of HBD-3 entry, neomycin-preincubated U937 cells treated with labelled HBD-3 were monitored using CLSM (**Figure 3.4C**). HBD-3-resistant cells, as indicated by PI negativity, displayed HBD-3 signal within cytoplasm, but not at inner plasma membrane as described earlier, suggesting that neomycin indeed hinders PI(4,5)P_2 binding of HBD-3 without masking the initial membrane interaction or affecting its internalisation.

Similarly, blocking PI(4.5)P_2 by endogenously overexpressing a GFP-tagged PI(4,5)P_2-sequestering PH domain from PLC(δ) also caused reduced HBD-3 activity as demonstrated by the 2.5-fold lower number of PI-positive HeLa cells upon HBD-3 treatment compared to GFP only-transfected cells (**Figure 3.4D**).

3.3.3 PI(4,5)P_2 binding by HBD-3 does not induce oligomerisation

PI(4,5)P_2 binding promotes oligomerisation and fibril formation of the plant defensins NaD1 and TPP3, as observed in chemical cross-linking experiments, TEM and X-ray crystallography (Poon *et al*, 2014; Baxter *et al*, 2015). The oligomeric plant defensin:PI(4,5)P_2 complexes were shown to be important for subsequent membrane blebbing and membrane rupture. The ability of HBD-3 to oligomerise in the presence of PI(4,5)P_2 was therefore investigated. Unexpectedly, as revealed by BS^3 chemical cross-linking, HBD-3 appears to self-oligomerise in solution, however, remained unaltered in the presence of excess PI(4,5)P_2 (**Figure 3.5A**). The self-oligomerisation of HBD-3 appeared to be concentration-dependent (Figure **3.5B**). Furthermore, as NaD1-PIP2 fibrils have been shown to bind Thioflavin T (a benzothiazole that displays enhanced fluorescence upon interaction with amyloid) (Khurana *et al*, 2005; Biancalana & Koide, 2010) (Hulett laboratory, unpublished) the ability of HBD-3:PI(4,5)P_2 to bind this dye was assessed. No fibrils could be detected in HBD-3:PI(4,5)P_2 samples by thioflavin T (**Figure 3.5B**). In contrast, control experiments showed an increase thioflavin T fluorescence signal of NaD1 in the presence of increasing concentrations of PI(4,5)P_2, suggesting PI(4,5)P_2-induced fibril formation as consistent with the previous report (Poon *et al*, 2014). TEM analysis of NaD1:PI(4,5)P_2 samples also did not detect any defined fibrils, but did show amorphous aggregation of HBD-3 regardless of PI(4,5)P_2 presence (**Figure 3.5C**). These data suggest that, in contrast to the solanaceous plant defensins, the binding of HBD-3 to PI(4,5)P_2 does not induce formation of oligomeric defensin:lipid complexes.

3.3.4 Generation of HBD-3 mutants

The anticancer activity and PI(4,5)P_2 binding of NaD1 and TPP3 was mapped to a cationic loop region between strands β2 and β3 (Becker *et al*, 2000; Poon *et al*, 2014; Baxter *et al*, 2015). As described, HBD-3 also possesses a β2-β3 loop with a similar stretch of basic residues to NaD1 and TPP3. Most importantly, residues K32 and K39 on HBD-3 are respectively equivalent to H33 and R40 in NaD1, and H34 and K41 in TPP3. These residues act as hydrogen bond donors in NaD1 and, potentially, in TPP3 with the phosphate groups of PI(4,5)P_2. Furthermore, R40 of NaD1 and K41 of TPP3 have been proposed as critical for cooperative PI(4,5)P_2 binding (i.e. linking multiple PI(4,5)P_2 molecules) and thus establishing plant defensin-PI(4,5)P_2 oligomerisation.

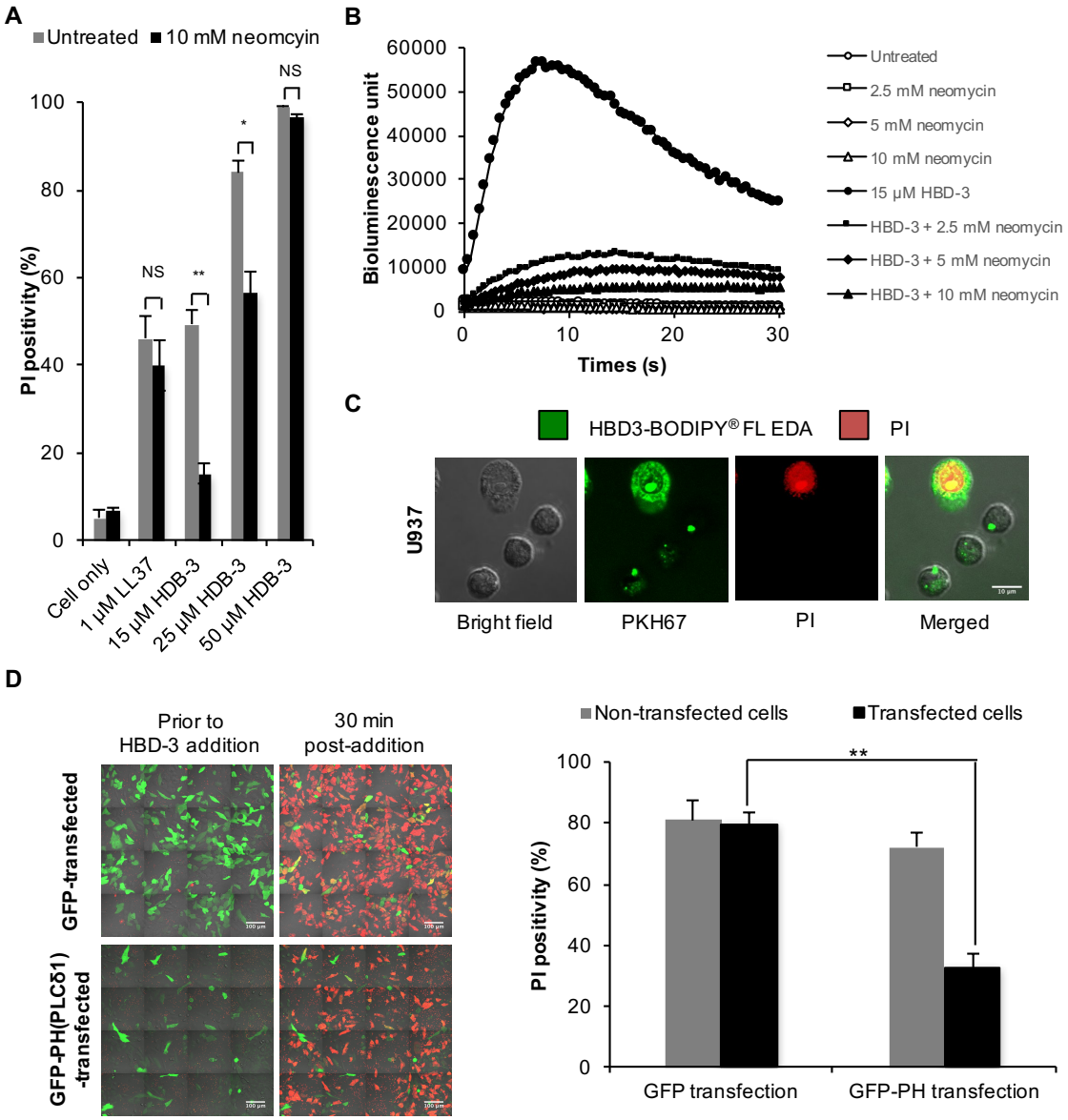

Figure 3.4 Inhibitory effect of PI(4,5)P_2-binding molecules on HBD3-mediated membrane permeabilisation

(A) Inhibitory effect of neomycin on membrane permeabilisation by HBD-3. U937 cells were treated with neomycin prior to flow cytometry-based PI uptake assay. Data represent mean ± SEM of three independent experiments. **(B)** ATP bioluminescence assay of neomycin-preincubated HeLa cells treated with 15 μM HBD-3. Level of cytoplasmic ATP released was detected as bioluminescence emission signal intensity. **(C)** CLSM imaging of U937 cells, pretreated with 10 mM neomycin for 3 h, at 15 min after addition of 15 μM HBD3-BODIPY FL EDA. Data in **B** and **C** are representative of three independent experiments. **(D)** Inhibitory effect of PH domain on on membrane permeabilisation by HBD-3. GFP and GFP-PH transfected HeLa cells were treated with HBD-3, followed by confocal imaging and PI positive counting. Data represent mean ± SEM of five independent experiments, each with three field of view containing at least 30 (GFP-PH) or 100 (GFP only) transfected cells. NS, not significant; *, $p<0.05$; **, $p<0.01$; unpaired t-test.

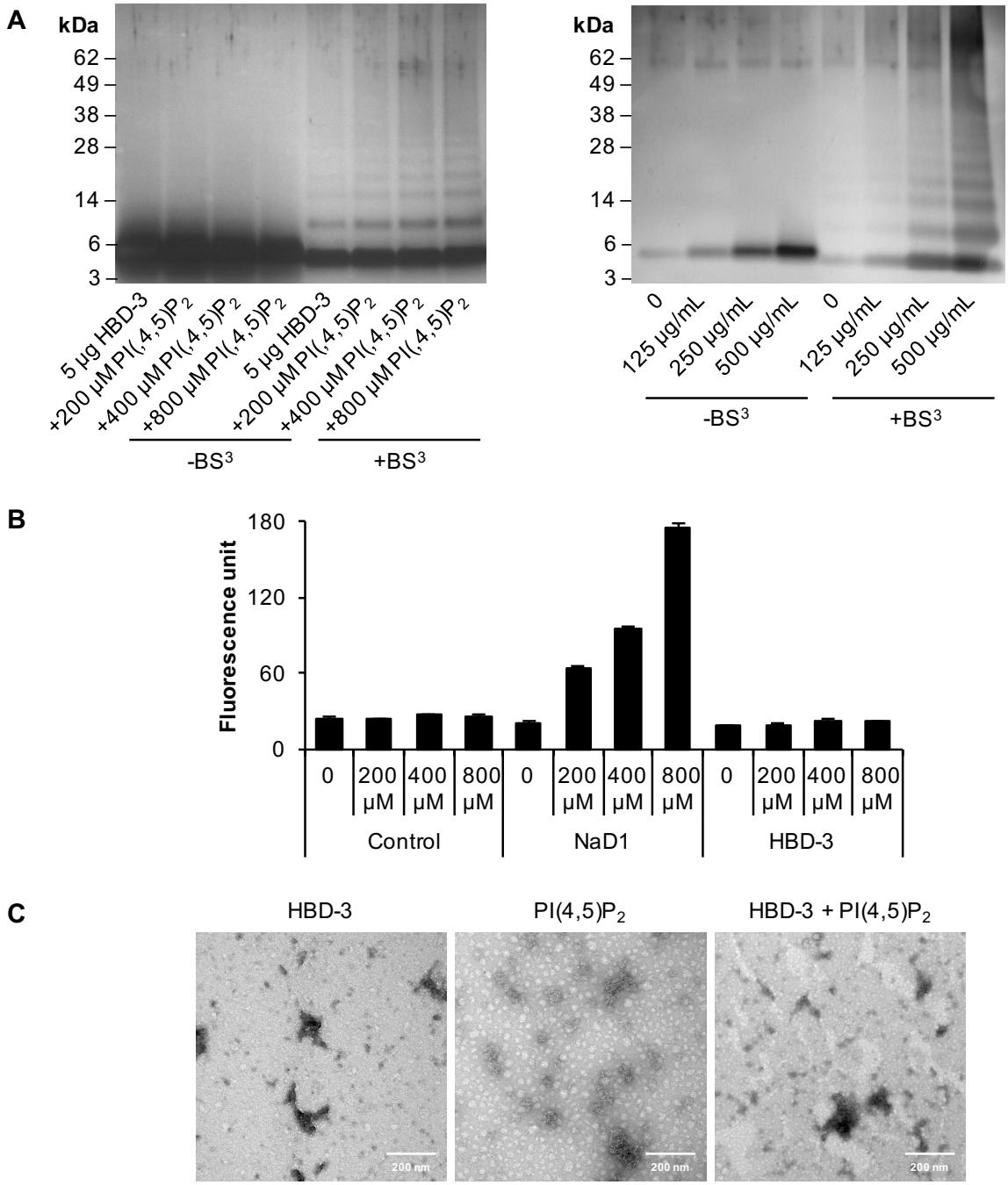

Figure 3.5 PI(4,5)P_2 binding by HBD-3 does not induce oligomerisation

(A) Protein-protein chemical cross-linking of HBD-3 in presence of PI(4,5)P_2 (left panel) and HBD-3 titration only (right panel), showing that HBD-3 self–oligomerises, independetly of PI(4,5)P_2, but rather seemingly concentration-dependent. HBD-3 incubated with PI(4,5)P_2 at 1:1, 1:2, 1:4 molar ratios or HBD-3 only at indicated concentrations were cross-linked with BS^3 (6.25 mM) prior to SDS-PAGE and silver staining. **(B)** Thioflavin T spectroscopic assay of 500 µg/mL defensins incubated with or without various concentrations of PI(4,5)P_2 and **(C)** TEM analysis of 900 µg/mL HB-3 with 230 µM PI(4,5)P_2, showing no fibril formed upon PI(4,5)P_2 binding of HBD-3. The plant defensin NaD1 was included in **B** as a positive control. Data in **A** and **C** are representative of three independent experiments. Data in **B** represent mean ± SEM of three independent experiments.

To study the importance of these residues in HBD-3, site-directed alanine mutagenesis of K32, S34, T35, R36, R38 and K39 were generated using forward and reverse primers with corresponding mutations (**Figure 3.6A**). The correct identity of each mutation was confirmed by nucleotide sequencing prior to *P. pastoris* expression and purification (**Figure 3.6B**). The expressed mutants were of high purity and at expected size (**Figure 3.7B**), and detectable by anti-HBD-3 antibody in native (**Figure 3.7A**) and reduced and denatured conformation (**Figure 3.7C**). However, it should be pointed out that the HBD-3(S34A) also showed second lighter band at around 10 kb, possibly indicating dimer formation. Nevertheless, these quality control tests indicated that HBD-3 mutants are structurally similar to native HBD-3.

3.3.5 Impaired activity on cancer cells, but neither on fungal growth inhibition nor internalisation, by HBD-3 mutations

Cell viability assays revealed that, among all tested mutants, HBD-3(K32A) and HBD-3(K39A) had consistently reduced cancer cell cytotoxicity on both U937 and HeLa cells, compared to wild-type HBD-3. HBD-3(K39A) displayed the greatest impairment, with four-fold and two-fold higher IC_{50} for U937 and HeLa cells, respectively (**Figure 3.8A**). Similarly, there was a reduction in the ability of the HBD-3(K32A) and HBD-3(K39A) mutants to permeabilise, consistently observed in both U937 and HeLa cells as measured by PI uptake (**Figure 3.8B**). HBD-3(K32A) causes approximately two-fold decrease in PI positivity, while HBD-3(K39A) abolishes the membrane permeabilisation effect, with PI positivity close to untreated controls. Other mutants do not show any impairment whatsoever in either used cancer cell lines.

HBD-3(K32A) and HBD-3(K39A) mutants, however, show no effect on HBD-3-induced inhibition of either *C. albicans* or *F. graminearum* fungal growth, with unchanged IC_{50} of around 7.5 μM and 2 μM respectively (**Figure 3.9A**). In addition, immunofluorescence microscopy imaging suggest that the two mutations only affect membrane permeabilisation without impairing internalisation as indicated by anti-HBD-3 signal, but not PI staining, in mutant-treated U3937, compared to wild-type (**Figure 3.9B**).

A

Mutation	Forward primer (5'-3')	Reverse primer (5'-3')
HBD-3(K32A)	GAGGAACAGATCGGC**GCT**TGCTCGACGCGTGGC	GCCACGCGTCGAGCA**AGC**GCCGATCTGTTCCTC
HBD-3(S34A)	GAACAGATCGGCAAGTGC**G**C**T**ACGCGTGGCCGAAAATGC	GCATTTTCGGCCACGCGT**A**G**C**GCACTTGCCGATCTGTTC
HBD-3(T35A)	GATCGGCAAGTGCTCG**GC**T**T**CGTGGCCGAAAATGC	GCATTTTCGGCCACG**A**G**C**CGAGCACTTGCCGATC
HBD-3(R36A)	GGCAAGTGCTCGACG**GC**TGGCCGAAAATGCTG	CAGCATTTTCGGCCA**GC**CGTCGAGCACTTGCC
HBD-3(R38A)	GCTCGACGCGTGGC**GC**AAAATGCTGCCGAAG	CTTCGGCAGCATTTT**GC**GCCACGCGTCGAGC
HBD-3(K39A)	CTCGACGCGTGGCCGA**GC**ATGCTGCCGAAGAAAG	CTTTCTTCGGCAGCAT**GC**TCGGCCACGCGTCGAG

B

```
                10        20        30        40        50        60        70        80
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
HBD-3          GGAATCATAAACACATTACAGAAATATTATTGCAGAGTCAGAGGCGGCCGGTGTGCTGTGCTCAGCTGCCTTCCAAAGGA
               G  I  I  N  T  L  Q  K  Y  Y  C  R  V  R  G  G  R  C  A  V  L  S  C  L  P  K  E
HBD-3(K32A)    GGAATCATAAACACATTACAGAAATATTATTGCAGAGTCAGAGGCGGCCGGTGTGCTGTGCTCAGCTGCCTTCCAAAGGA
               G  I  I  N  T  L  Q  K  Y  Y  C  R  V  R  G  G  R  C  A  V  L  S  C  L  P  K  E
HBD-3(S34A)    GGAATCATAAACACATTACAGAAATATTATTGCAGAGTCAGAGGCGGCCGGTGTGCTGTGCTCAGCTGCCTTCCAAAGGA
               G  I  I  N  T  L  Q  K  Y  Y  C  R  V  R  G  G  R  C  A  V  L  S  C  L  P  K  E
HBD-3(T35A)    GGAATCATAAACACATTACAGAAATATTATTGCAGAGTCAGAGGCGGCCGGTGTGCTGTGCTCAGCTGCCTTCCAAAGGA
               G  I  I  N  T  L  Q  K  Y  Y  C  R  V  R  G  G  R  C  A  V  L  S  C  L  P  K  E
HBD-3(R36A)    GGAATCATAAACACATTACAGAAATATTATTGCAGAGTCAGAGGCGGCCGGTGTGCTGTGCTCAGCTGCCTTCCAAAGGA
               G  I  I  N  T  L  Q  K  Y  Y  C  R  V  R  G  G  R  C  A  V  L  S  C  L  P  K  E
HBD-3(R38A)    GGAATCATAAACACATTACAGAAATATTATTGCAGAGTCAGAGGCGGCCGGTGTGCTGTGCTCAGCTGCCTTCCAAAGGA
               G  I  I  N  T  L  Q  K  Y  Y  C  R  V  R  G  G  R  C  A  V  L  S  C  L  P  K  E
HBD-3(K39A)    GGAATCATAAACACATTACAGAAATATTATTGCAGAGTCAGAGGCGGCCGGTGTGCTGTGCTCAGCTGCCTTCCAAAGGA
               G  I  I  N  T  L  Q  K  Y  Y  C  R  V  R  G  G  R  C  A  V  L  S  C  L  P  K  E

                       90        100       110       120       130       140
                      ....|....|....|....|....|....|....|....|....|....|....|....
HBD-3          GGAACAGATCGGCAAGTGCTCGACGCGTGGCCGAAAATGCTGCCGAAGAAAGAAATAAGCGGCC
                E  Q  I  G  K  C  S  T  R  G  R  K  C  C  R  R  K  K  *  A  A
HBD-3(K32A)    GGAACAGATCGGCGCTTGCTCGACGCGTGGCCGAAAATGCTGCCGAAGAAAGAAATAAGCGGCC
                E  Q  I  G  A  C  S  T  R  G  R  K  C  C  R  R  K  K  *  A  A
HBD-3(S34A)    GGAACAGATCGGCAAGTGCGCTACGCGTGGCCGAAAATGCTGCCGAAGAAAGAAATAAGCGGCC
                E  Q  I  G  K  C  A  T  R  G  R  K  C  C  R  R  K  K  *  A  A
HBD-3(T35A)    GGAACAGATCGGCAAGTGCTCGGCTCGTGGCCGAAAATGCTGCCGAAGAAAGAAATAAGCGGCC
                E  Q  I  G  K  C  S  A  R  G  R  K  C  C  R  R  K  K  *  A  A
HBD-3(R36A)    GGAACAGATCGGCAAGTGCTCGACGGCTGGCCGAAAATGCTGCCGAAGAAAGAAATAAGCGGCC
                E  Q  I  G  K  C  S  T  A  G  R  K  C  C  R  R  K  K  *  A  A
HBD-3(R38A)    GGAACAGATCGGCAAGTGCTCGACGCGTGGCGCAAAATGCTGCCGAAGAAAGAAATAAGCGGCC
                E  Q  I  G  K  C  S  T  R  G  A  K  C  C  R  R  K  K  *  A  A
HBD-3(K39A)    GGAACAGATCGGCAAGTGCTCGACGCGTGGCCGAGCATGCTGCCGAAGAAAGAAATAAGCGGCC
                E  Q  I  G  K  C  S  T  R  G  R  A  C  C  R  R  K  K  *  A  A
```

Figure 3.6 Site-directed mutagenesis of HBD-3

(A) Oligonucleotide primers used to generate HBD-3 mutations, with mutated nucleotides in bold. **(B)** Sequencing confirmation of HBD-3 mutants. The amino acid sequence is presented below nucleotide sequences with mutated nucleotides (underlined) and corresponding alanine (bolded). The stop codon is indicated with an asterisk (*).

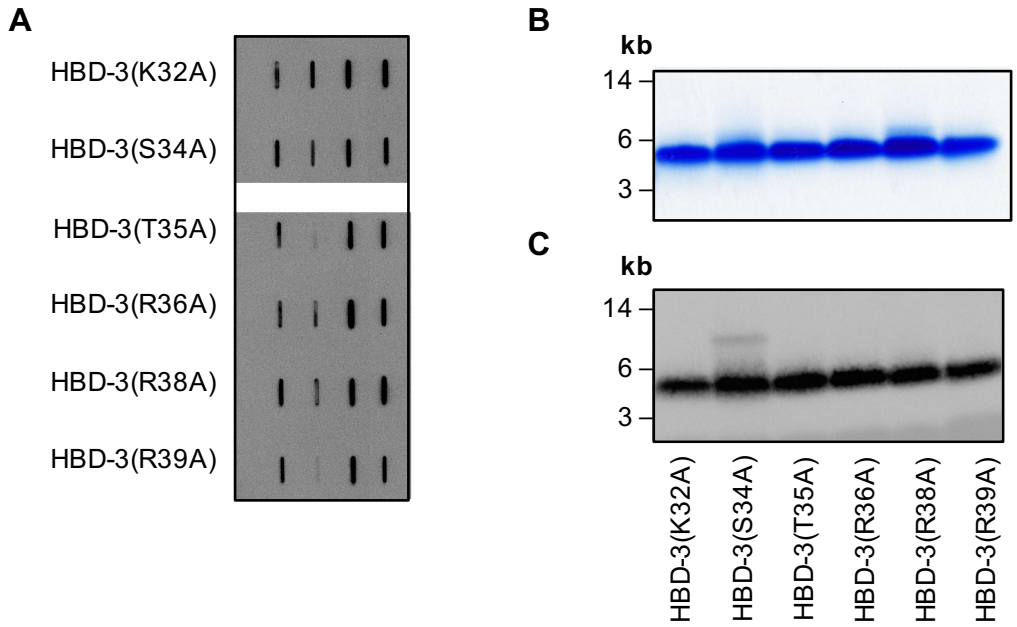

Figure 3.7 Expression and quality control of HBD-3 mutants

(A) Slot blot, showing expression of HBD-3 mutants in *P. pastoris*. Supernatants from expression culture at different time points were applied on nitrocellulose membrane followed by immunoblotting using rabbit-anti-HBD-3 and HRP-conjugated donkey-anti-rabit antibodies (0.25 µg/mL). **(B)** Reducing and denaturing SDS-PAGE analysis (3 µg protein/lane) followed by Coomassie Brilliant Blue staining. **(C)** Immunoblotting (250 ng protein/lane) using rabbit-anti-HBD-3 and HRP-conjugated donkey-anti-rabit antibodies (0.25 µg/mL).

3.3.6 Impaired PI(4,5)P_2 binding by HBD-3 mutations

Using PIP^{TM} strips (**Figure 3.10A**), HBD-3(K32A) was showed to maintain the lipid binding specificity of wild-type HBD-3 but at much lower levels; the exception being its interaction with PA that was substantially retained. In contrast, HBD-3(K39A) showed a pronounced loss of PIP binding while maintaining binding to PA. The importance of K32 and K39 to the binding of PI(4,5)P_2 was also specifically demonstrated by the reduced ability of alanine mutants to block anti-PI(4,5)P_2 antibody binding to U937 cells when compared to wild-type HBD3-treated cells. This was more evident for HBD-3(K39A) as anti-PI(4,5)P_2 antibody binding to treated cells was essentially the same as that of the untreated control (**Figure 3.10B**). Furthermore, the ability HBD-3(K32A) or HBD-3(K39A) to induce ATP release from ATP-encapsulated PC:PI(4,5)P_2 liposomes was also impaired in similar manner (**Figure 3.10C**).

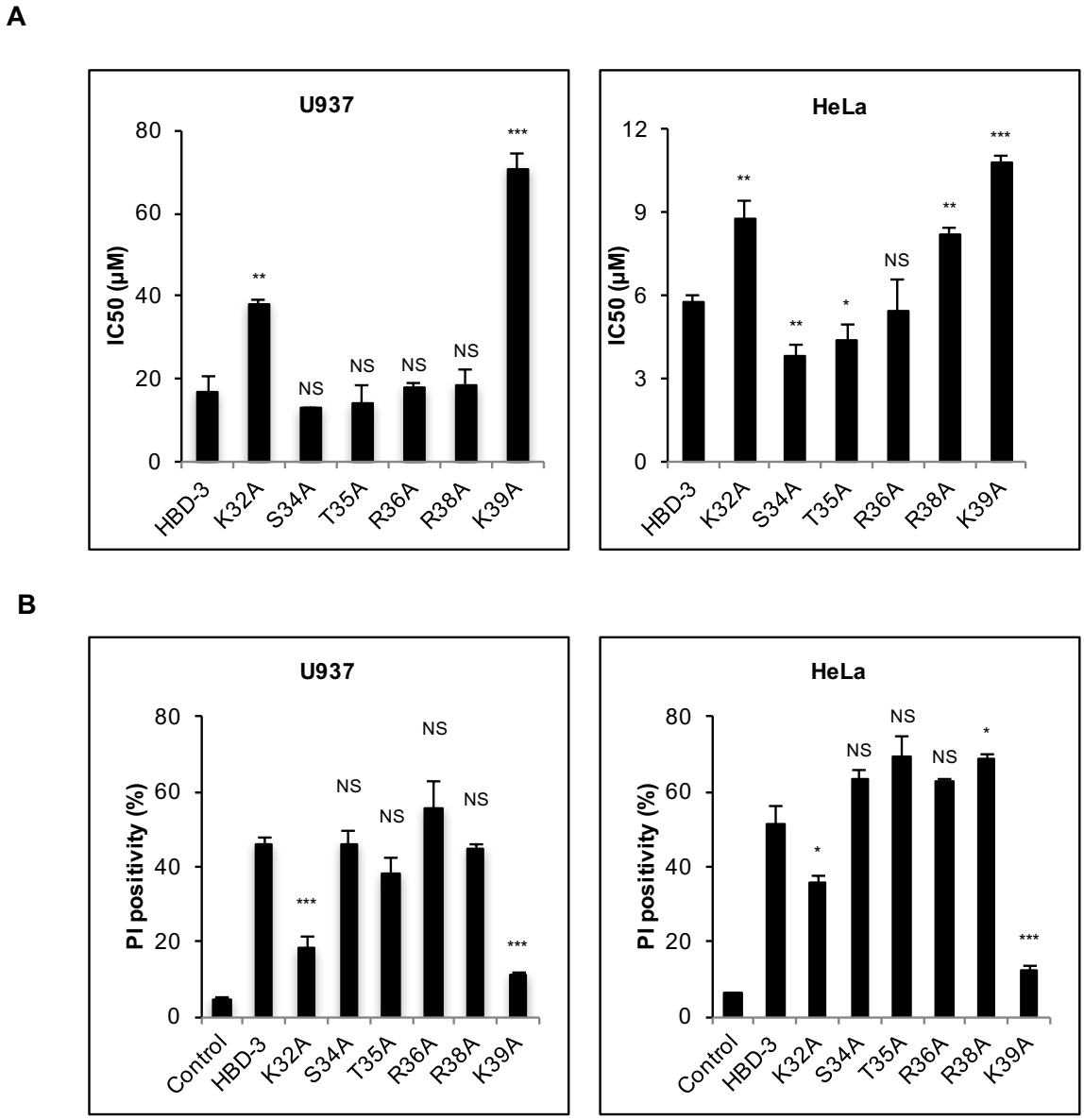

Figure 3.8 Effect of mutations on HBD-3 activity on cancer cells

(A) IC_{50} of HBD-3 and mutants on U937 and HeLa cells, determined by tetrazolium-coupled cell viability assay. Data were normalised against untreated control, which was arbitrarily assigned as 100% cell viability. **(B)** Membrane permeabilisation effect (by flow cytometry-based PI uptake assay) of HBD-3 and its mutants at 15 μM on U937 and HeLa cells. Data represent mean ± SEM of three independent experiments. NS, not significant; *, $p<0.05$; **, $p<0.01$; unpaired t-test.

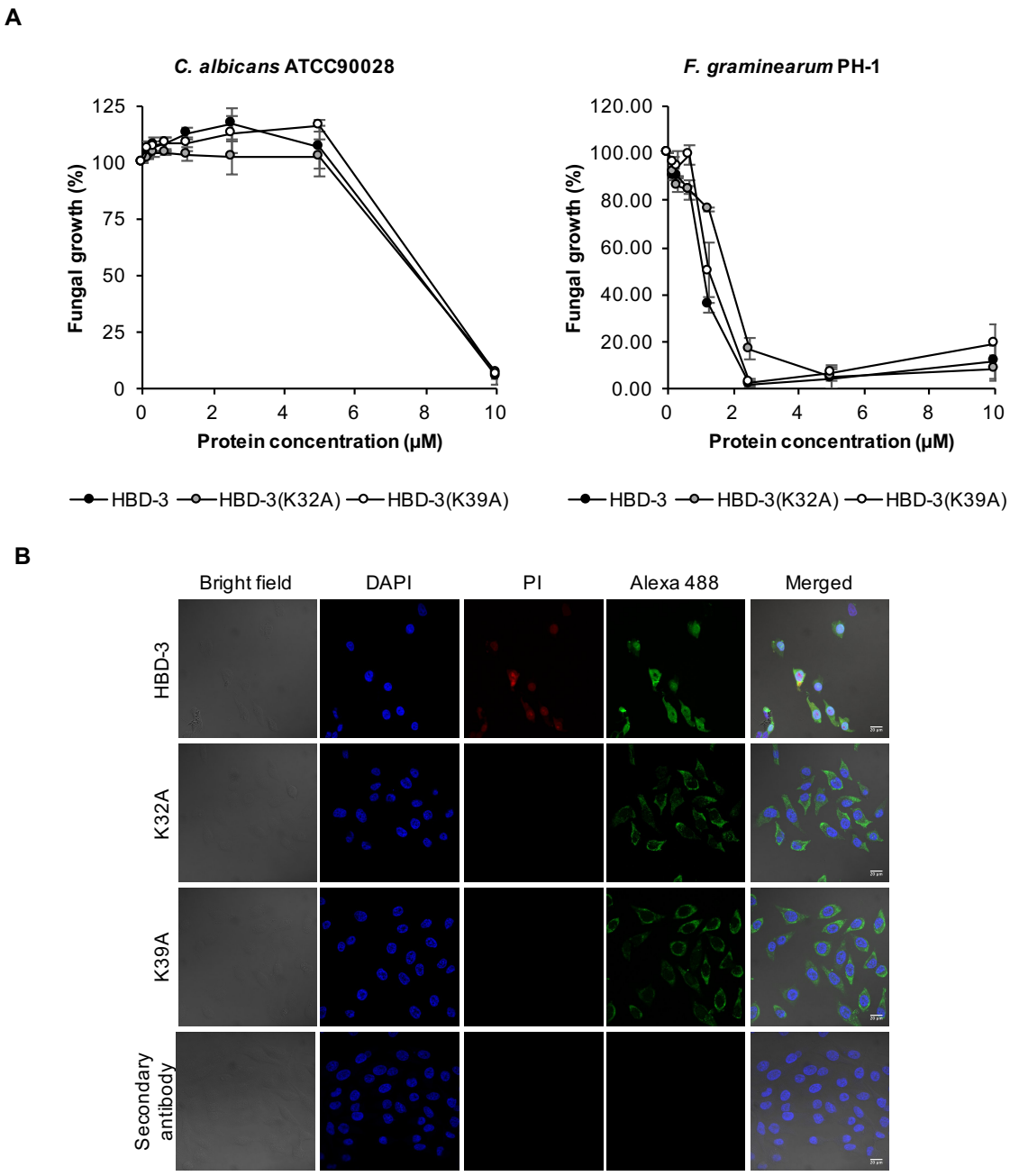

Figure 3.9 Antifungal activity and mammalian cell entry of HBD-3 by K32A and K39A mutagenesis

(A) Similar inhibitory effects of HBD-3 and mutants on fungal growth. *C. albicans* (5×10^3 cells/mL) or *F. gramunearum* (5×10^4 spores/mL) in half-strength PDB medium (80 µL) were incubated with 20 µL of protein solution at final concentrations of 0-10 µM for 24 h. Fungal growth was determined by measuring absorbance at 595 nm. **(B)** Immunofluorescence microscopy on U937 cells, seeded on poly-L-lysine-coated coverslips, were treated with HBD-3 or mutants for 30 min prior to PI staining, fixation, lysis and staining with rabbit-anti-HBD-3 and Alexa 488-conjugated goat-anti-rabbit antibodies (2.5 µg/mL).

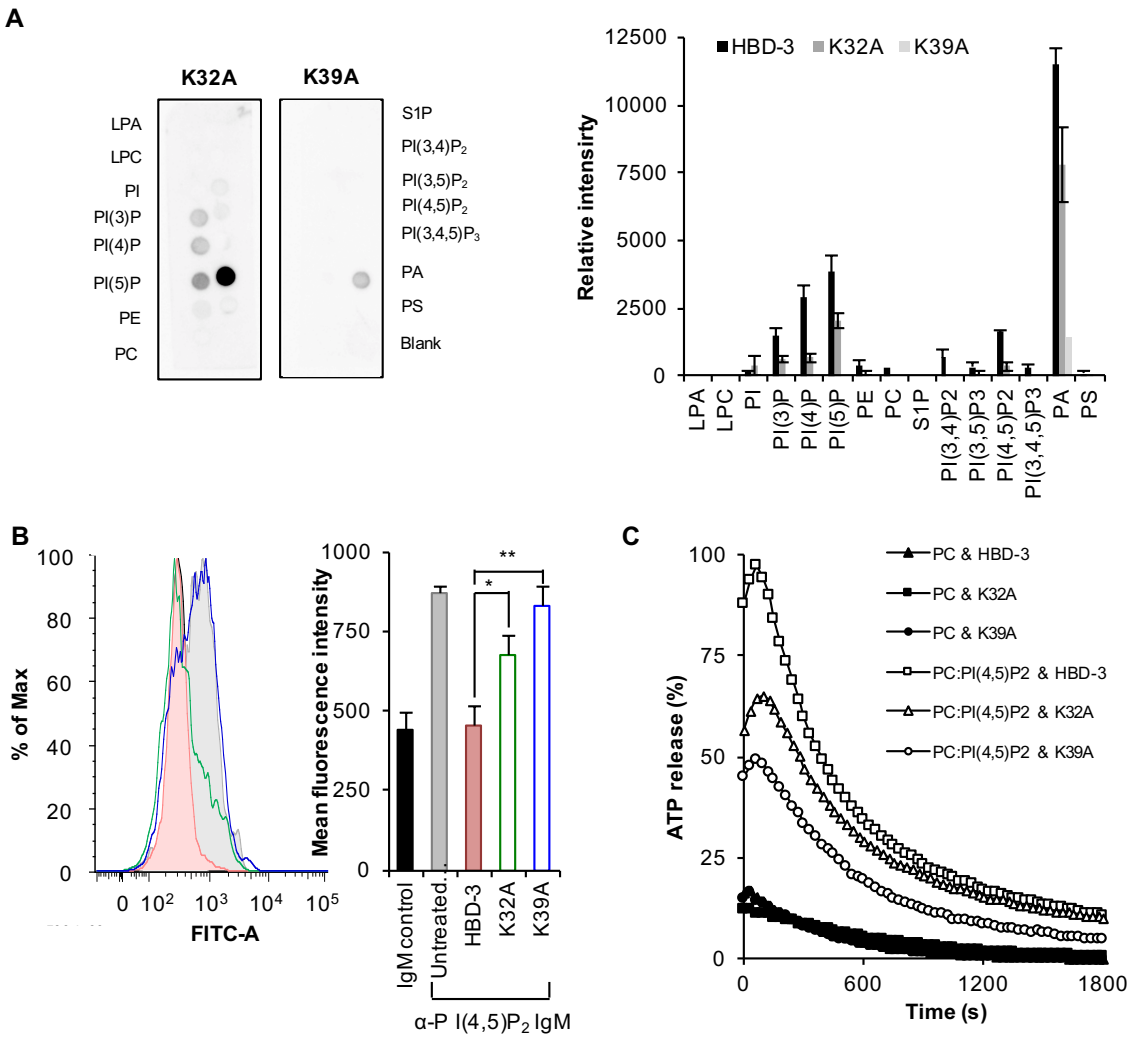

Figure 3.10 Mutation-induced reduced lipid binding.

(A) Immunodetection of lipid binding by HBD-3 mutants on and PIP strip^{TM}. Relative binding intensity was determined by densitometry analysis of chemiluminescence signals, in comparison of HBD-3. All assays were simultaneously processed, under same experimental conditions. **(B)** Blocking of anti-PI(4,5)P_2 antibody binding to plasma membrane PI(4,5)P_2 by HBD-3 and mutants. Data in **A** and **B** represent mean ± SEM of three independent experiments. *, $p<0.05$; **, $p<0.01$; unpaired t-test. **(C)** ATP release of ATP-encapsulated PC and PC:PI(4,5)P_2 liposomes by 15 μM HBD-3 mutants. Data are representative of three independent experiments.

3.4 Discussion

CHDPs, of which defensins are a major family, are important innate defense and immunomodulatory molecules, employed by most living organisms to combat pathogenic invasions and orchestrate host immune responses. In addition, a number of defensins and other CHDPs have also recently been suggested to have anticancer activity. HBD-3 of the human β-defensin subfamily displays potent activity against fungi, bacteria and viruses, as well as acts as an indispensable effector of immunity (Garcia *et al*, 2001; Harder *et al*, 2001; Quinones-Mateu *et al*, 2003; Feng *et al*, 2005; Leikina *et al*, 2005; Sun *et al*, 2005; Pazgier *et al*, 2006; Funderburg *et al*, 2007; Nagaoka *et al*, 2008; Ferris *et al*, 2013; Petrov *et al*, 2013). However, the precise molecular basis underlying these functions of HBD-3, particularly the anticancer activity, remains unclear. Indeed, to date, HBD-3 has only been showed to inhibit cancer cell migration by suppressing vascular endothelial growth factor in head and neck cancer cells (Wang *et al*, 2012) or down-regulating MTA2 (metastasis-associated 1 family, member 2) expression of colon cancer cells (Uraki *et al*, 2014).

In this study, the cytotoxicity of HBD-3 on cancer cell membrane has been demonstrated to involve bleb-associated membrane permeabilisation and cell lysis. PIPs, particularly PI(4,5)P_2 on the plasma membrane, have been identified as lipid targets, to which HBD-3 binds and exerts its membrane disrupting effect. It is likely that HBD-3 cancer cell cytotoxicity is initiated by local concentration of HBD-3 at the cell surface, possibly by electrostatic attraction, leading to plasma membrane weakening at accumulation point(s), which allows HBD-3 internalisation. Once it enters the cytoplasm, HBD-3 binds to PI(4,5)P_2 on the inner leaflet of the plasma membrane, and possibly to other PIPs of subcellular organelle membranes, causing membrane disruption, bleb formation and ultimately cell lysis. Indeed, these effects could be delayed and/or inhibited by synthetic PI(4,5)P_2 or PI(4,5)P_2-sequestering molecules such as PH-PLC(δ) and neomycin. Furthermore, neomycin, which inhibits HBD-3-induced PI uptake and ATP release, but not HBD-3 internalisation, while has no inhibitory effect on detergent-like and/or pore forming LL-37, implies the particular importance of PI(4,5)P_2 binding in HBD-3 activity on cancer cell.

In addition to PIPs, HBD-3 was also suggested to bind to other phospholipids such as PA and PS (section 2.3.7). It was interesting to note that added synthetic PA actually

enhanced the cancer cell permeability activity of HBD-3. It is possible that the exogenously added PA might have induced conformational change, promoted local accumulation or internalisation of HBD-3, thus enhancing its cytotoxicity. Previous studies have suggested that HBD-3 induces membrane damage in PS-enriched monocytes but not PS-deficient lymphocytes (Loi et al, 2012), implying that HBD-3 may interact with PS to mediate such effects. Indeed, in our study, HBD-3 was suggested to bind PS in protein-lipid overlay assays and exogenously added PS was showed to have a minor but significant inhibitory activity on the cell permeability function of HBD-3. However, because blocking of annexin V binding assay (section 2.3.7) show no difference in cellular PS signal in controls and HBD-3-treated U937 cells, it is likely that PS is not directly involved in mediating membrane permeabilisation, as PI(4,5)P_2 does, but would rather contribute to membrane negativity and hence initial electrostatic interaction. Nevertheless, in the context of antimicrobial function, it is tempting to speculate the ability of HBD-3 to interact with a diverse array of lipids, including PIPs, PA and PS, may impart the versatility to combat a wide range of different microbial pathogens.

It is also possible that HBD-3 dimerises and adopts a similar cationic grip to the plant defensins to accommodate PI(4,5)P_2. In fact, Schibli *et al* (2002) proposed a dimer model of HBD-3 based on NMR data, although this was not consistent with the NaD1 or TPP3 'cationic grip' PIP-binding dimeric conformation. Nevertheless, HBD-3 might undergo a conformational change upon lipid binding because free HBD-3 is highly dynamic, particularly around the β2-β3 loop region (section 2.3.6). It remains puzzling that, although NaD1 and TPP3 bind PI(4,5)P_2 and oligomerise to form fibrils *in vitro*, a similar effect was not observed for HBD-3 binding of PI(4,5)P_2 by cross-linking experiment, thioflavin T spectroscopic assay or transmission electron microscopy. Interestingly, it was recently suggested using a molecular dynamics simulation that HBD-3 may be able to self-oligomerise, as consistent with the chemical-crosslinking experiment, but was unable to lyse, in the context of a bacterial membrane surface (Zhao *et al*, 2015). Further study is therefore needed to determine whether oligomerisation is important for permeabilisation function of HBD-3. It would also be interesting to examine the biological relevance of HBD-3 self-oligomerisation, as reported for human α-defensin HD-6 which self-assembles and forms bacteria-entrapping nanonets (Chu *et al*, 2012).

In addition, it is unclear how HBD-3 gains entry into cancer cells. Possibly, HBD-3 is taken up via receptor-dependent endocytosis as previously reported for squamous cancer cell carcinoma (Mburu *et al*, 2011). However, it should be pointed out that the conclusion was solely based on the slight, albeit significant, reduction, but not complete abrogation of fluorescently-labelled HBD-3 by microtubule polymerisation inhibitor, nocodazole. In fact, the same concentration of nocodazole (10 μg/mL) only results in a modest inhibition of HBD-3-induced PI uptake (data not showed). Therefore, additional internalisation mechanism(s) would be involved. In fact, in addition to endocytosis-mediated translocation, certain CHDPs and cell-penetrating peptides (CPPs) are able to directly penetrate cellular membrane or translocate through the formation of a transitory structure, such as inverted micelles (Richard *et al*, 2003; Plenat *et al*, 2004; Lau *et al*, 2005; Herce & Garcia, 2007; Mookherjee *et al*, 2009). As suggested by CLSM imaging, it is likely that the cationic HBD-3 initially electrostatically interacts with, then transiently disrupts negatively-charged plasma membrane of cancer cells via local accumulation on outer plasma membrane prior to translocate into the cytoplasm. This may resemble the internalisation mechanism of the trans-activating transcriptional activation (Tat) of HIV-1 virus, the first and one of the best-characterised CPP (Herce & Garcia, 2007). Tat protein possesses a small cationic motif (YGRKKRRQRRR), that facilitates the spontaneous translocation by inserting the charged residues into the plasma membrane to form a transient pore and subsequent diffusion through the pore surface (Herce & Garcia, 2007). It remains to be determined if the early accumulation of HBD-3 leads to similar transient pore during or, perhaps, simply incurs a local charge-based disturbance of cancer cell membrane by promoting the concentration-dependent self-oligomerisation of HBD-3 to allow its subsequent penetration.

The finding that HBD-3 targets membrane lipids and permeabilises cells, as also described previously for selected plant defensins (Baxter *et al*, 2015), suggests an evolutionarily conserved mechanism among membrane-targeting innate immune molecules that possess potential 'phospholipid recognition patterns'. Indeed, the β2-β3 loop motif of HBD-3, NaD1 and TPP3 are reasonably conserved. Based on previous studies (Poon *et al*, 2014; Baxter *et al*, 2015), one might suggest that the positively-charged residues of HBD-3 would not only provide electrostatic attraction but also essentially involve an extensive H-bonding network with the anionic heads of phospholipids to stabilise defensin:lipid interactions. Like NaD1 and TPP3, among

residues within the β2-β3 loop of HBD-3 (S34, T35, R36, R38 and K39), the last basic residue (i.e. K39) is particularly important, as its mutation (to alanine) leads to abolished PI(4,5)P_2 interaction, and ultimately membrane permeabilisation and anticancer activity. It is also suggested that the hydrogen bond donor residues preceding the β2-β3 loop (i.e. K32 in HBD-3, H33 in NaD1) should be included as a possible extension of the 'phospholipid recognition motif'. This is based on the structural conformation observation that H33 in NaD1 participates in hydrogen bonding at the defensin:lipid interface (Poon *et al*, 2014) and mutation thereof results in reduced PI(4,5)P_2 binding as well as impaired defensin activity, together with the finding that HBD-3(K32A) has reduced lipid binding and cell permeabilisation activity. The fact that HBD-3(K32A) and HBD-3(K39A) display impaired PI(4,5)P_2 binding and reduced cytotoxicity but unaffected cancer cell entry further emphasises that PI(4,5)P_2 binding would be the deciding step to mediate HBD-3-induced membrane permeabilisation and cell lysis. Furthermore, unaffected physiological antifungal activity by K32 and K39 mutation, compared to wild-type HBD-3, may not only suggest the structural integrity but also infer additional mechanism(s), possibly PI(4,5)P_2-independent, for antifungal effect of HBD-3 as previously implicated for NaD1 (Bleackley *et al*, 2016). Other residues within the β2-β3 cationic loop do not appear to be crucial for the cancer cell cytotoxicity and cytolysis of HBD-3. However, further studies may be needed to examine if they, particularly the positively-charged residues, play any role in HBD-3 translocation during early local accumulation, as reported previously for the cationic motif of the HIV-1 Tat protein (Herce & Garcia, 2007).

Apart from the β2-β3 loop on HBD-3, NaD1 and TPP3, several other cationic motifs have been implied in phospholipid binding and functional importance. For example, the closely resembling β2-β3 loop (RGFRRR) loop on the plant defensin MtDef4 from *Medicago truncatula* is essential for fungal cell entry, mediated via PA binding (Sagaram *et al*, 2013). The PI(3)P-binding RxLR motif carried by fungal pathogen effector molecules such as Avr is also important for plant cell internalisation (Kale *et al*, 2010; Yaeno *et al*, 2011). The cysteine-flanked KNKEKK segment of serum protein β_2-glycoprotein 1 crucially binds to cardiolipin, enabling its role in anti-cardiolipin-mediated thrombosis, which is completely abolished by triple mutation KNGEGG (Borchman *et al*, 1995; Sheng *et al*, 1996). Likewise, deletion of the highly-conserved polybasic consensus Kx(R/K)xxKQKxK(R/K/Q)(R/K) from the membrane-targeting human Cd42 GTPase-

activating protein results in impaired PI(3,4,5)P_3 interaction and consequently, abolishes Cd42 activity *in vivo* (Karimzadeh *et al*, 2012). Furthermore, although most PIP recognition and binding domains share little sequence similarities, the small cysteine-rich Zn^{2+}-binding 'Fab1, YOTB, Vac1, EEA1' (FYVE) domains exhibit the most obvious (R/K)(R/K)HHCR pattern within their binding pocket for PI(3)P (Kutateladze & Overduin, 2001). Together with our findings, it is suggested that an understanding of these patterns/codes may enable prediction of phospholipid binding partners and functional importance, and the design of phospholipid-targeted therapeutics.

3.5 Conclusion

PI(4,5)P_2-mediated membrane permeabilisation of cancer cells by HBD-3 has been demonstrated via a conserved cationic loop motif. The interaction between HBD-3 and PI(4,5)P_2 suggests a mechanistic conservation among defensins of different species, Indeed, the targeting of membrane lipids by defensins may well be a universal function for this family of innate defense peptides in their activity against various pathogens and could also explain the long-standing question as to why many CHDPS have anti-cancer cell activity. It remains of significant interest to determine whether other defensins, particularly the many human defensins with unknown function, also use this mechanism of action in host defense against pathogens and altered-self such as cancer cells.

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Chapter 4

Cytokine induction, but not chemotactic recruitment, of antigen presenting cells by HBD-3 is $PI(4,5)P_2$-dependent

4.1 Introduction

Host defense peptides (HDPs) are made by most living organisms as a pivotal component of innate immunity, providing the first line of defense against invading microbial pathogens. Despite limited sequence homology, the majority of HDPs are commonly small in size (typically 1–10 kDa), positively-charged, amphipathic, and share a similar disulfide bond-stabilised α-helix and/or β-sheet rich structure (Zasloff, 2002; Pazgier *et al*, 2006). HDPs can be constitutively or inducibly expressed by infectious or inflammatory stimuli, including proinflammatory cytokines, pathogen-associated molecular patterns (Hancock, 2001; Cunliffe & Mahida, 2004). Originally, HDP studies have primarily focused on their antimicrobial activity (Zasloff, 2002; Hancock *et al*, 2016). For certain HDPs, however, their direct microbicidal function may be compromised under physiological and pathological conditions, due to low local peptide concentration, suboptimal ion (*e.g.* Na^+, Ca^{2+}, Mg^{2+}) concentration, polyanions (*e.g.* mucins and glycosaminoglycans) and degradation by proteases (Bals *et al*, 1998; Bowdish *et al*, 2005; Vylkova *et al*, 2007; Nelson *et al*, 2009). Nevertheless, HDPs can also be potent immune effectors, pro- and/or anti-inflammatory, molecules via multifunctional and multimodal orchestration of immune responses to enhance pathogen killing and clearance as well as maintain immune homeostasis (Zasloff, 2002; Harvey *et al*, 2013; Wang *et al*, 2015). Broadly, their immunomodulatory activities involve, but are not limited to, leukocyte chemotaxis, induction of cytokine release, angiogenesis and phagocytosis, modulation of leukocyte development and survival, as well as bacterial lipopolysaccharide (LPS) neutralization (Gudmundsson & Agerberth, 1999; Koczulla *et al*, 2003; Rosenfeld *et al*, 2006; Rohrl *et al*, 2010; Nagaoka *et al*, 2012; Petrov *et al*, 2013; Wan *et al*, 2014). Through chemotactic recruitment of antigen presenting cells and T lymphocytes, induction of immature dendritic cell (iDC) maturation and execution of adjuvant-like functions, they also provide a link between innate and adaptive immunity (Yang *et al*, 1999; Funderburg *et al*, 2007; Cao *et al*, 2011).

Human β-defensin 3 (HBD-3) is one of the most prominent and best-characterised β-defensins which, together with α- and θ-defensins, constitutes the mammalian defensin subfamily. HBD-3 is present at low concentrations in normal physiological settings, measured at 142.9±10.6 pg/mL in serum (Ishimoto *et al*, 2006), 50–935 ng/mL in saliva (Ghosh *et al*, 2007) and 7–700 ng/mL in nasal secretion (Hui *et al*, 2011). However, upon stimulation by infection or proinflammatory cytokines, HBD-3 is upregulated (by

10-1,000 fold, depending on stimuli and site of induction) and secreted by keratinocytes, epithelial cells, monocytes and neutrophils (Garcia *et al*, 2001; Harder *et al*, 2001; Harder *et al*, 2004; Sorensen *et al*, 2006; Yin *et al*, 2010; Zanger *et al*, 2010; Otri *et al*, 2012). HBD-3 is arguably the most potent antimicrobial of the β-defensins, with average minimal inhibitory concentration of approximately 10 μg/mL, against Gram-negative and Gram-positive bacteria, fungi and virus (Garcia *et al*, 2001; Harder *et al*, 2001; Schibli *et al*, 2002; Quinones-Mateu *et al*, 2003; Feng *et al*, 2005; Leikina *et al*, 2005; Sun *et al*, 2005; Pazgier *et al*, 2006; Chen *et al*, 2007; Fazakerley *et al*, 2010; Schroeder *et al*, 2011).

Pro- and anti-inflammatory immunomodulation by HBD-3 have also been reported (Li *et al*, 2009; Semple *et al*, 2010; Semple *et al*, 2011; Semple & Dorin, 2012; Harvey *et al*, 2013; Meisch *et al*, 2013; Lee *et al*, 2015). The biphasic regulation by HBD3, which may depend on concentration tested, exposure timing and pathogenic antigens, is nevertheless believed to ensure pathogen defense and a controlled inflammatory response (Semple & Dorin, 2012; Harvey *et al*, 2013). Anti-inflammatory HBD-3 attenuates LPS-activated Toll-like receptor 4 (TLR4) signalling and resultant proinflammatory gene expression in macrophages, though the inhibitory mechanism remains unexplained (Semple *et al*, 2010; Semple *et al*, 2011). Proinflammatory responses by myeloid dendritic cells upon bacterial hemagglutinin B (HagB) stimulation are also suppressed through direct HBD-3:HagB interaction (Pingel *et al*, 2008). As a proinflammatory mediator, HBD-3 chemotactically recruits leukocytes and triggers cytokine expression and release. HBD-3 was formerly demonstrated to chemoattract monocytes, mast cells, enterocytes, G protein-coupled C-C chemokine receptor type 6 (CCR6) -overexpressing cells and, limitedly, GM-CSF-stimulated monocyte-derived macrophages (Wu *et al*, 2003; Chen *et al*, 2007; Soruri *et al*, 2007; Jin *et al*, 2010; Rohrl *et al*, 2010; Sheng *et al*, 2014). Based on studies done on other β-defensins, HBD-3 is also predicted to recruit iDCs, memory T lymphocytes and neutrophils (Yang *et al*, 1999; Niyonsaba *et al*, 2004; Rohrl *et al*, 2010). These studies also suggested CCR2 and CCR6 as chemotactic receptors for the chemotactic function of HBD-3 (Yang et al, 1999; Chen et al, 2007; Soruri et al, 2007; Rohrl et al, 2010; Sheng et al, 2014). On the other hand, HBD-3 induces proinflammatory cytokine release in monocytes, macrophages and keratinocytes as well as promotes iDC and monocyte maturation by activating NF-κB through CCR2/6-phospholipase C (PLC)-dependent and/or TLR-1/2-dependent pathways (Funderburg *et al*, 2007; Niyonsaba *et al*, 2007; Jin

et al, 2010; Funderburg *et al*, 2011). However, it was later demonstrated that HBD-3 internalisation and subsequent phosphoinositide-3 kinase (PI3K)-protein B (Akt)-dependent NF-κB activation were not inhibited by a G-protein inhibitor pertussis toxin, a MyD88 inhibitor or TLR-1 or TRIF inhibitor, suggesting a G-coupled protein- and TLR-independent HBD-3 induced NF-κB signalling mechanism (Mburu *et al*, 2011). Furthermore, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was named as an intracellular receptor for internalised LL-37, a prominent HDP of cathlecidin family, to stimulate MAPK p38 pathway of interleukin (ILs) release (Mookherjee *et al*, 2009). Thus, an intracellular target may exist for HBD-3-induced PI3K-Akt-NF-κB of cytokine release. In addition, HBD-3 can also induce IL-1β and co-stimulatory marker expression via activation of ATP-gated $P2X_7$ channel (Lioi *et al*, 2015; Wanke *et al*, 2016).

In the previous chapter, HBD-3 was showed to bind to PI(4,5)P_2 and other PIPs of the inner membrane leaflet once entering mammalian cells (sections 2.3.7 and 3.3). Coincidently, PI(4,5)P_2 and its major signalling pathways (PI3K-Akt and PLC-PKC) were reported to mediate NF-κB activation and cytokine induction (Kane *et al*, 1999; Chen & Lin, 2001; Moscat *et al*, 2003; Min *et al*, 2005; Rajaram *et al*, 2006). Moreover, PI(4,5)P_2/PI(3,4,5)P_3 binding and/or metabolising proteins such as cofilin, Cdc42 and phosphoinositide kinases and phosphatases are particularly crucial for cell migration (Van Keymeulen et al, 2006; Roussos et al, 2011; Fets et al, 2014; Wu et al, 2014; Elong Edimo et al, 2016). Therefore, the importance of PI(4,5)P_2 interaction may mediate the proinflammatory functions of HBD-3.

This chapter describe efforts to further define the molecular mechanism(s) of HBD-3-stimulated inflammation. HBD-3 was shown to recruit monocytes, but not macrophages, iDC and memory T lymphocytes, as previously implicated. Induction of different proinflammatory cytokines, such as tumour necrosis factor (TNF) and interleukin 6 (IL-6), by HBD-3 was observed by monocytes and macrophages, consistent with previous findings, but also modestly by iDC and minimally by T lymphocytes. However, for the first time, PI(4,5)P_2 interaction was found to be essential for the PI3P/Akt-dependent cytokine induction, but not the chemotactic recruitment, by a HDP. These data suggest that HBD-3 binds to PI(4,5)P_2 and acts upstream of PI3K to promote PI3K activation, then Akt phosphorylation and NF-κB activation, ultimately leading to TNF and IL-6 induction. This study (i) clarifies an unexplained relevance of HBD-3 internalisation by

leukocytes as described in many previous studies, (ii) provides insights to the currently unresolved mechanism of the multifaceted proinflammatory immunomodulation by HBD-3, and (iii) emphasises the essential role of HDPs in pathogen defense and regulating immune responses.

4.2 Materials and methods

4.2.1 Preparation of endotoxin-free defensins, chemical reagents and plasticwares

HBD-3, HBD-3(K32A) and HBD-3(K39A), recombinantly expressed and purified as per sections 2.2.8 and 3.2.1, were eluted through an Endotrap Red column (Hyglos GmbH, Germany) with endotoxin-free PBS, as per manufacturer's instructions, into 200 µL fractions. BCA protein assay was then performed to determine protein concentration in endotoxin-removed fractions. Earliest fractions without detected protein were retained as a negative control for later assays (Life Technologies).

Escherichia coli LPS (Sigma-Aldrich, MO, USA) and synthetic lipids (PA, PI(4)P, PI(3,4)P_2, PI(4,5)P_2 and PI(3,4,5)P_3; Avanti Polar Lipids, AL, USA) were dissolved in endotoxin-free water (MO BIO Laboratories, CA, USA). Ionomycin, phorbol 12-myristate 13-acetate (PMA), wortmannin and staurosporine were reconstituted in filter-sterilised dimethyl sulfoxide (all from Sigma-Aldrich). Endotoxin-free neomycin was dissolved in appropriate assay buffers immediately before use.

All cell cultures and assays were performed under sterile and endotoxin-free environment with endotoxin-free certified plasticwares, including microtubes (Axygen, CA, USA) and aerosol barrier tips (Interpath Services, Australia).

4.2.2 Isolation of PBMC-derived memory T lymphocyte

Every 1×10^7 PBMCs, isolated from fresh buffy coat (as per section 2.2.2), were resuspended in 40 µL of ice-cold isolation buffer (endotoxin-free PBS, 0.5% BSA and 2mM EDTA) prior to addition of 10 µL of human memory $CD4^+$ T cell biotin-antibody cocktail (Miltenyi Biotech, Germany). After 10 min incubation with gentle rolling at 4°C, 30 µL of buffer and 20 µL of anti-biotin microbeads (Miltenyi Biotech) were added, then the mixture was incubated for another 15 min at 4°C. Excess beads were washed with 2 mL buffer at 300 *g* for 10 min. Once resuspended in 500 µL buffer, T lymphocytes were

isolated by magnetic depletion using a MACS LS column with a MACS separator (Miltenyi Biotech).

4.2.3 Isolation of PBMC-derived monocyte

For every 1×10^7 PBMCs, 80 μL of ice-cold isolation buffer (endotoxin-free PBS, 0.5% BSA and 2mM EDTA) and 20 μL of CD14 microbeads (Miltenyi Biotech) were added, and incubated for 15 min at 4°C with gentle rolling. After washing, $CD14^+$ monocytes were obtained by positive magnetic isolation using a MACS LS column with a MACS separator (Miltenyi Biotech).

4.2.4 Cultures of monocyte-derived immature dendritic cell and macrophages

PBMCs were resuspended in pre-warmed complete RPMI-1640 to 2.5×10^7 cells/mL. The cell suspension (5 mL) was transferred into T25 culture flasks (Greiner Bio-One) and monocytes allowed to adhere 5% CO_2 incubator at 37°C for 2 h. After three gentle washes with endotoxin-free PBS, adhered cells were cultured with 5 mL of complete RPMI-1640 containing 10% FCS, 1000 U/mL human GM-CSF (Miltenyi Biotech), and 400 U/mL human IL-4 (Miltenyi Biotech) for immature dendritic cell (iDC), or 5 mL of complete RPMI-1640 containing 5% FCS and 500 U/mL human GM-CSF or 500 U/mL human M-CSF (Miltenyi Biotech) for macrophages. An equal amount of appropriate complete medium was added on the third day of culturing. On the seventh day, iDC were ready to use as a suspension, while the macrophages were detached from culturing flasks using non-enzymatic dissociation solution (Sigma-Aldrich) at 37°C.

4.2.5 Cell surface marker determination

Cells at 1×10^6 cell/mL in PBS containing 0.5% BSA and 2mM EDTA were separately stained with 1:10 dilution of fluorescently-labelled antibodies to cell surface markers (T lymphocyte: CD4 and CD45RO; monocyte: CD14, HLA-DR; iDC: CD14, HLA-DR, CD80 and CD83; macrophages: CD14, HLA-DR, CD163) or appropriate clone-specific IgG controls (**Table 4.1**; Miltenyi Biotech) for 10 min 4°C prior to flow cytometry analysis. For CD68, macrophages were fixed with 2% (v/w) paraformaldehyde (10 min), lysed with 0.5% (w/v) saponin (5 min), and blocked with 3% (w/v) BSA (15 min) prior to CD68 staining and flow cytometry analysis.

Table 4.1 Antibodies used in leukocyte marker determination

All antibodies were purchased from Miltenyi Biotech with specified fluorophores, isotypes, clones and manufacturer catalogue numbers. Isotype controls, which are specific for antigens that are not normally expressed on human cells or cell lines, were used as negative control to distinguish specific from non-specific binding of cell marker-specific antibodies.

	Antigen	Fluorophore	Isotype	Clone	Catalogue #
	CD4	FITC	Mouse IgG2a	M-T466	130-092-358
	CD45RO	PE		UCHL1	130-095-457
	CD14	FITC		TÜK4	130-080-701
	HLA-DR	PE		AC122	130-095-298
	CD80	PE	Mouse IgG1	REA661	130-097-202
	CD83	APC		HB15	130-094-186
	CD163	FITC		REA812	130-097-626
	CD68	APC	Mouse IgG2b	Y1/82A	130-096-431
Isotype control	KLH	FITC	Mouse IgG1	IS5-21F5	130-098-847
		PE			130-098-845
		APC			130-098-846
	Hapten NP	FITC	Mouse IgG2a	S43.10	130-098-877
		PE			130-098-849
	KLH	APC	Mouse IgG2b	IS6-11E5.11	130-098-890

4.2.6 Transwell chemotaxis assay

Transwell chemotaxis assays were performed using 6.5 mm 24-well Transwell® with 5.0 μm (for T lymphocyte and monocyte) or 8 μm (for iDC and macrophage) polycarbonate membrane inserts (Corning Life Sciences). Cells (1×10^5) were seeded in chemotaxis buffer (serum-free RPMI containing 0.5% BSA) in the transwell insert. Chemotaxis buffer containing HBD-3 or mutants were added into the lower compartment, and cells were allowed to migrate for 4 h at 37°C. Migrated cells were harvested and counted using BD FACSCanto II Flow Cytometer and BD FACSDiva Software v6.1.1 (BD Biosciences), gated with forward scatter and side scatter parameters of appropriate freshly-prepared cells. An acquisition time of 2 min was consistently applied across all samples. Chemotaxis index was calculated as the fold difference of the number of migrated cells in treated samples to that of untreated control, to account for free movement by gravity.

In certain experiments, monocytes were pre-treated with 5 mM neomycin or 200 nM wortmannin for 2 h prior to seeding. For lipid inhibition, HBD-3 (20 μg/mL) was pre-incubated with different lipids at a 1:2 ratio for 30 min before adding to the lower chamber. Due to the unavailability of 26-well transwell plates at the time, using HTS

Transwell®-96 system with 5 μm polycarbonate membrane inserts (Corning Life Sciences) was instead used in the lipid inhibition experiments, with 5×10^4 cells seeded in the transwell inserts.

4.2.7 Human proinflammatory cytokine release assay

Human cytokine release assays were conducted using the cytometric bead array (CBA) kit (BD Biosciences) to detect proinflammatory cytokines IL-12p70, TNF, IL-10, IL-6, IL-1β and IL-8. Freshly-prepared leukocytes were seeded into 96-well flat bottom tissue culture plates (Greiner Bio-One) at 2.5×10^5 cells/mL in complete RPMI-1640 with 5% FCS. Defensins, together with ionomycin (1 μg/mL) and PMA (20 ng/mL) or LPS (50 ng/mL) were added to the cells and incubated for 18 h at 37°C. After centrifugation at 500 *g* for 5 min at room temperature, culture supernatants were carefully collected and subjected to CBA as per manufacturer's instructions. Briefly, bead cocktail was prepared by mixing 2 μL each aforementioned cytokine's capture bead and 12 μL PE detection reagent for each sample. To a 96-well V-bottom culture plate (Greiner Bio-One), 24 μL of bead cocktail was incubated with equal volume of cytokine standards (a series of 1:2 dilutions from provided stocks in assay diluent buffer) or culture supernatants in the dark for 3 h at room temperature. When washed twice at 200 *g* for 5 min and resuspended in 80 μL wash buffer, samples were analysed using BD FACSCanto II Flow Cytometer and BD FACSDiva Software v6.1.1, acquiring at least 300 events per capture bead (i.e. minimum of 1,800 total events). Instrument set-up was performed as per manufacturer's protocol prior to flow cytometry. Data analysis was achieved using FCAPArray v.3 (BD Biosciences).

4.2.8 Akt activation detection

Freshly-isolated monocytes at 1.25×10^6 cells/mL were seeded overnight in serum-free RPMI at 37°C, prior to addition of 10 μg/mL HBD-3 or mutants thereof for 30 min. For certain experiments, serum-starved monocytes were pre-treated with 5 mM neomycin or 200 nM wortmannin for 2 h before adding HBD-3. After centrifugation at 500 *g* for 5 min at room temperature, culture supernatant was carefully removed, cells were removed by scraping and lysed at 4°C using ice-cold lysis buffer (20 mM HEPES pH 7.4, 1% IGEPAL® CA-630, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 50 mM NaF, protease inhibitor cocktail tablet, phosphatase inhibitor cocktail tablet; Sigma-Aldrich) with constant vigorous agitation for 45 min. Cell lysates were collected and subjected to BCA protein assay to determine protein concentration. Subsequently, 25 μg of total

protein per sample was subjected to reducing and denaturing SDS-PAGE (as per section 2.2.9) prior to Western transfer (as per section 2.2.10) onto methanol-activated polyvinylidene fluoride membrane (Bio-Rad). Immunoblotting was instead conducted using 5% BSA in PBS containing 0.1% (v/v) Tween®-20, rabbit-anti-phospho-Akt(S473) (1:2000; Cell Signalling Technology, MA, USA) and HRP-conjugated donkey-anti-rabbit IgG (1:10,000; Cell Signalling Technology). After chemiluminescence detection, the membrane was stripped with 70 mM Tris-HCl (pH 6.5) containing 1% (w/v) sodium dodecyl sulfate and 100 mM 2-mercaptoethanol at 45°C for 45 min, and re-probed with rabbit-anti-Akt (pan) (1:1000; Cell Signalling Technology) and then HRP-conjugated donkey-anti-rabbit IgG (1:10,000). Chemiluminescence signal intensity for protein bands was quantitated by densitometry analysis using ImageJ.

4.3 Results

4.3.1 Purity of isolated and cultured leukocytes

To ensure the identity of isolated and cultured leukocytes used in this study, flow cytometry-based immunodetection of cellular markers were performed using fluorescently-labelled marker-specific antibodies. Memory T lymphocyte preparations, purified by magnetic depletion of freshly isolated PBMCs, resulted in ~90% enrichment of characteristic $CD4^+$ and $CD45RO^+$ cells (**Figure 4.1A**), whereas monocyte isolation using positive selection yielded ~95% $CD14^+$ and HLA-DR^+ cells (**Figure 4.2B**), compared to PBMCs. Monocyte-derived iDCs, as expected, showed minimal CD14 but enhanced HLA-DR expression, as well as displayed low levels of maturation markers CD80 and CD83 (**Figure 4.1C**). Monocyte-derived macrophages, on the other hand, possessed characteristic $CD14^+$, HLA-DR^+, macrophage specific markers $CD68^+$ and $CD163^+$ for GM-CSF-stimulated or $CD163^{low}$ for M-SCF-stimulated macrophages (**Figure 4.1D).**

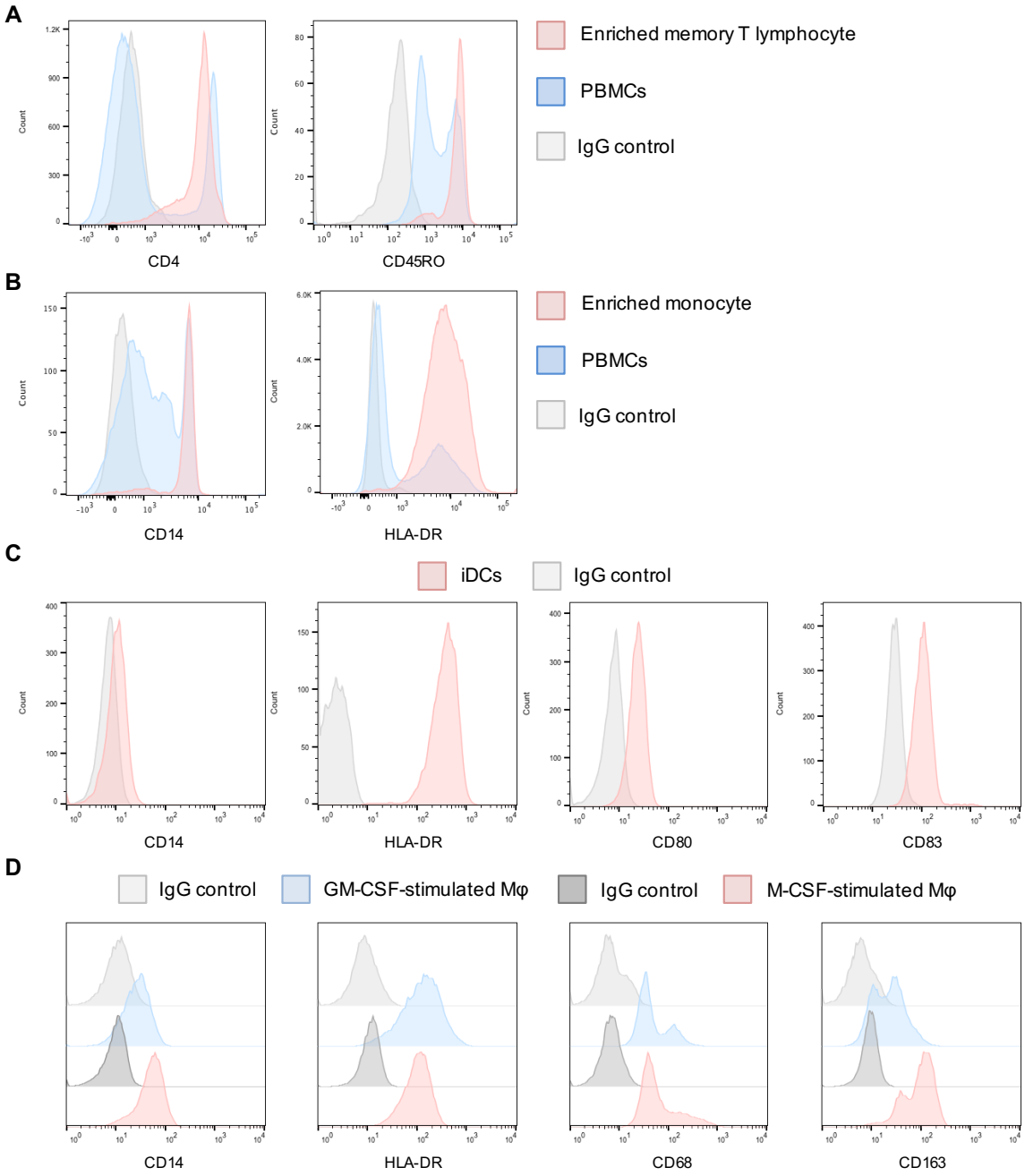

Figure 4.1 Marker confirmation for isolated leukocytes

(A) Memory T lymphocytes, purified by magnetically depletion of PBMCs. **(B)** Monocytes, magnetically isolated from PBMCs using positive selection with CD14 beads. **(C)** iDCs, differentiated from adhered monocytes with 1,000 U/mL GM-CSF and 500 U/mL IL-4 for 7 days. **(D)** Macrophages, by stimulating adhered monocytes with either 500 U/mL GM-CSF or 500 U/mL M-CSF for 7 days. Cells were harvested and stained with indicated fluorescently-labelled antibodies or appropriate IgG controls prior to flow cytometry analysis.

4.3.2 Reduced *in vitro* chemotactic recruitment of monocytes by HBD-3 mutants

Previous studies, though conflicting, demonstrated the ability of HBD-3 to induce chemotactic migration through CCR2 and CCR6 in monocytes, mast cells, enterocytes and, limitedly, GM-CSF-stimulated monocyte-derived macrophages, (Wu *et al*, 2003; Chen *et al*, 2007; Soruri *et al*, 2007; Jin *et al*, 2010; Rohrl *et al*, 2010; Sheng *et al*, 2014). HBD-3 was also implicated to recruit iDCs, memory T lymphocytes and neutrophils (Yang *et al*, 1999; Niyonsaba *et al*, 2004; Rohrl *et al*, 2010). In previous chapters, HBD-3 was demonstrated to bind to PI(4,5)P_2 and, possibly, PI(3,4,5)P_3 (section 2.3.7 and section 3.3) that also play a key role in cell migration (Van Keymeulen et al, 2006; Roussos et al, 2011; Fets et al, 2014; Wu et al, 2014; Elong Edimo et al, 2016). Hence, to verify the chemotactic activity of HBD-3 and to investigate its modulatory role on PI(4,5)P_2, and, potentially, PI(3,4,5)P_3 by interaction with these lipids, in vitro Transwell® chemotaxis assays were performed on memory T lymphocytes, monocytes, monocyte-derived iDCs, GM-CSF- and M-CSF-stimulated macrophages. Of all the tested leukocytes, only monocytes substantially migrated towards HBD-3 at subcytotoxic concentration (**Figure 4.2A**), as shown by the five-times higher than basal migration. The HBD-3 mutants with impaired PIP-binding, HBD-3(K32A) and HBD-3(K39A), all showed around a 50% reduction in migration compared to that of wild-type HBD-3. Interestingly, HBD-3(K39A), did not abolish the chemotactic activity of HBD-3 as expected for its loss of PIP binding. In contrast, the other leukocytes (**Figure 4.2B-E**) showed little or no migration in response to HBD-3.

4.3.3 Inhibition of HBD-3 binding to PI(4,5)P_2 or PI(3,4,5)P_3 does not affect chemotaxis

As PI(4,5)P_2 and PI(3,4,5)P_3 modulation is often indispensable to regulate cell migration (Van Keymeulen *et al*, 2006; Roussos *et al*, 2011; Fets *et al*, 2014; Wu *et al*, 2014; Elong Edimo *et al*, 2016), several inhibitors of HBD-3 binding to PI(4,5)P_2 or PI(3,4,5)P_3 were used to examine if HBD-3 could modulate these lipids to orchestrate chemotactic recruitment of monocyte. The PI(4,5)P_2-binding aminoglycoside antibiotic neomycin, although successfully inhibits anticancer cell permeabilisation (see section 3.3.2), did not alter the chemotactic activity of HBD-3 (Figure 4.3A). Similarly, wortmannin, a well-characterised inhibitor of PI(3,4,5)P_3-metabolising PI3K, also did not alter HBD-3-induced chemotaxis (**Figure 4.3A**). Furthermore, a panel of synthetic phospholipids, including PA, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2 and PI(3,4,5)P_3, did not result in any

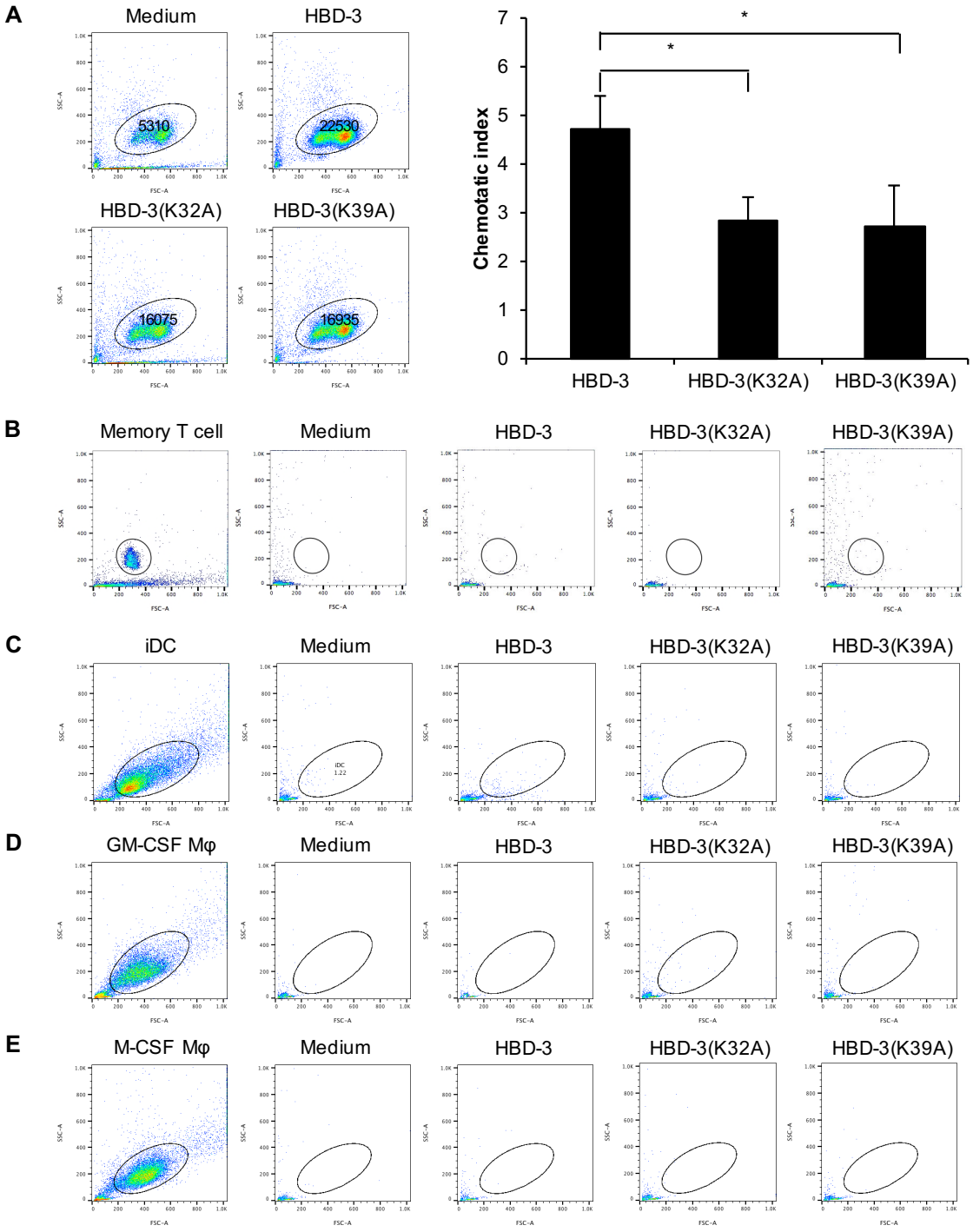

Figure 4.2 Chemotactic effect of HBD-3 and mutants on (A) Memory T lymphocytes, (B) monocytes, (C) iDCs, (D) GMCSF-polarised macrophages, and (E) M-CSF-polarised macrophages

Cells were seeded at 1×10^5 per well at upper chamber of chemotaxis plate, while HBD-3 and mutants were added to lower chamber at 20 µg/mL in RPMI 1640 supplemented with 0.5% BSA. After 4 h incubation at 37°C, 5% CO_2, migrated cells were harvested and counted by flow cytometry. Dot plots, showing counting of migrated cells, are representatives of three independent experiments. Chemotaxis indexes are mean±S.E.M of seven independent experiments, each with triplicates. NS, not significant; *, $p<0.05$; unpaired t-test.

apparent difference in chemotactic migration of monocyte toward HBD-3 (**Figure 4.3B**). These data suggest that binding of HBD-3 to phosphoinositides, particular PI(4,5)P_2 and PI(3,4,5)P_3, does not regulate chemotaxis-associated with PI(4,5)P_2 and/or PI(3,4,5)P_3 modulation, nor is required for HBD-3-induced monocyte migration.

4.3.4 Induction of proinflammatory cytokines in antigen presenting cells, but not memory T lymphocytes by HBD-3

HBD-3 was reported as both proinflammatory and anti-inflammatory, depending on its concentration tested, exposure timing and pathogenic antigens (Li *et al*, 2009; Semple *et al*, 2010; Semple *et al*, 2011; Semple & Dorin, 2012; Harvey *et al*, 2013; Meisch *et al*, 2013; Lee *et al*, 2015). As a proinflammatory mediator, HBD-3 induces cytokine release in monocytes (Funderburg *et al*, 2011), macrophages (Jin *et al*, 2010) and keratinocytes (Niyonsaba *et al*, 2007), by activating NF-κB through G-protein (*e.g.* CCR2)-PLC-dependent and/or TLR-1/2-dependent pathways. However, HBD-3 internalisation and subsequent NF-κB activation, which are not inhibited by a G-protein inhibitor pertussis toxin, a MyD88 inhibitor or TLR-1 or TRIF inhibitor, have challenged the involvement of G-coupled protein receptors and TLRs (Mburu *et al*, 2011). In this thesis, HBD-3 was shown to enter mammalian cells and binds to PI(4,5)P_2 of inner membrane leaflet (section 3.3). Interestingly, PI(4,5)P_2 and its major signalling pathways (PI3K-Akt and PLC-PKC) have been reported to mediate NF-κB activation and cytokine induction (Kane *et al*, 1999; Chen & Lin, 2001; Moscat *et al*, 2003; Min *et al*, 2005; Rajaram *et al*, 2006). It was therefore of interest to study the cytokine induction activity of HBD-3 on different leukocytes as well as to investigate the role of HBD-3:PI(4,5)P_2 interaction, using CBA analysis.

HBD-3 does not induce any substantial cytokine release from memory T lymphocytes, as opposed to the ionomycin and PMA control, which trigger tumour necrosis factor (TNF), interleukins IL-6 and IL-8 induction (**Figure 4.4**). In contrast, for monocytes, HBD-3, promotes high levels of cytokine release, including TNF, IL-6, IL-8 and to a lesser extent, IL-1β, in a dose-dependent manner (**Figure 4.5**). Interestingly, HBD-3 induces only IL-8 release in iDC (**Figure 4.6**) while triggers high level of TNF, IL-6 and IL-8, much lower concentration of IL-10 and minimal level of IL-1β in GM-CSF-activated macrophage (**Figure 4.7**) also in concentration-dependent fashion. LPS was used as a positive control for these types of leukocytes. To control for possible endotoxin contamination, the

no-protein eluted fractions from endotoxin removal showed only background levels of cytokine release (data not showed) that were similar to the medium controls.

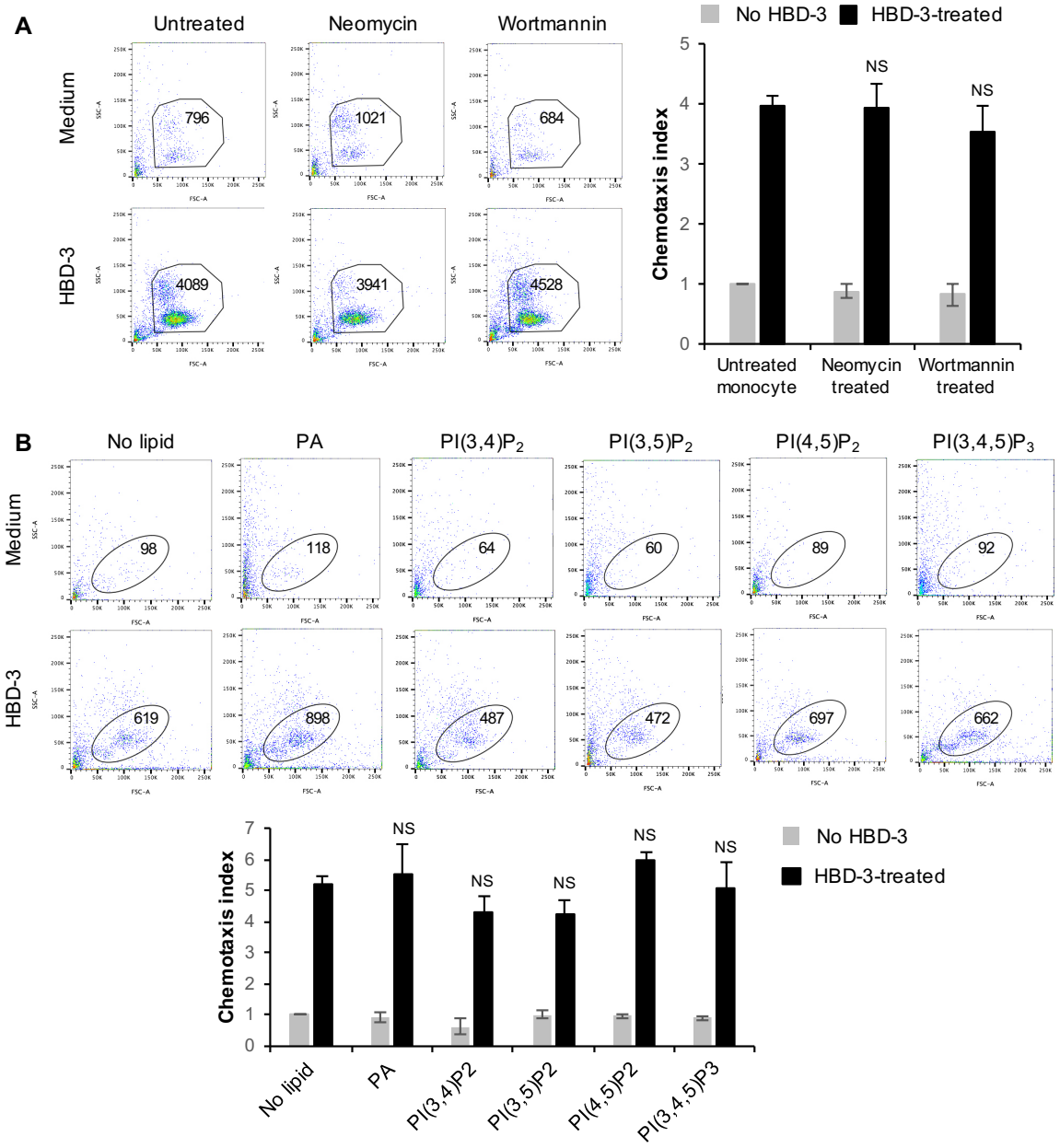

Figure 4.3 Inhibition of HBD2 binding to PI(4,5)P_2 or PI(3,4,5)P_3 does not affect chemotaxis.
(A) PI(4,5)P_2-binding neomycin (5 mM) and PI3K inhibitor wortmannin (200 nM)-treated monocytes, with 20 µg/mL HBD-3. **(B)** HBD-3 (20 µg/mL) was pre-incubated with different lipids at a 1:2 molar ratio. Migrated cells were harvested and counted by flow cytometry. Dot plots, showing numbers of migrated cells, are representatives of two or three independent experiments. Chemotaxis indexes are mean±S.E.M of more than two independent experiments, each with triplicates. P-values were calculated against HBD-3 only treated samples. NS, not significant, unpaired t-test.

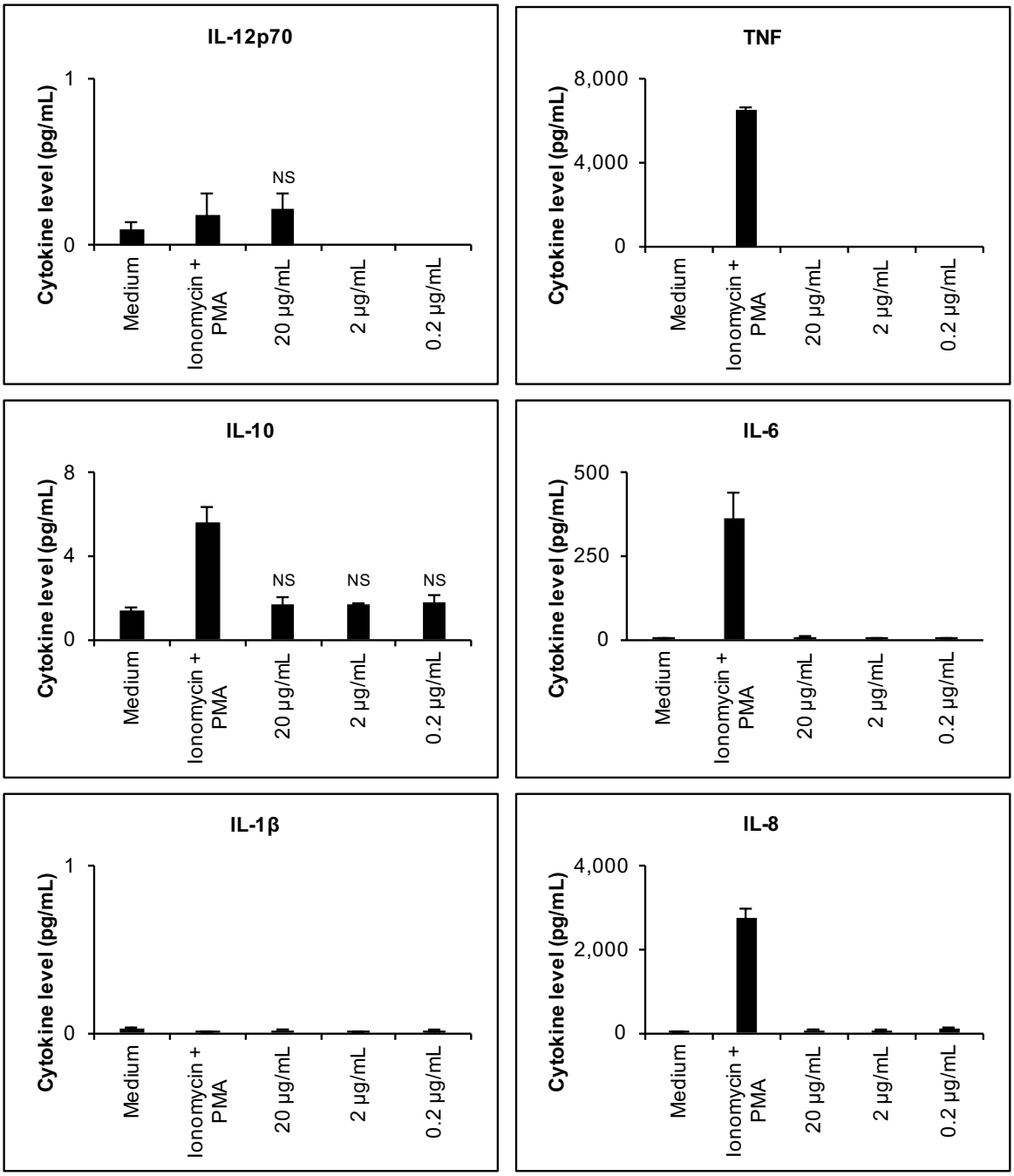

Figure 4.4 Inflammatory cytokine induction profile of HBD-3-treated memory T lymphocyte

Cells were seeded in appropriate culture media and stimulated with ionomycin (1 µg/mL) and PMA (20 ng/mL) or various concentration of HBD-3 for 18 h. Culture supernatant was collected and subjected to cytokine detection using cytometric bead array. Data represent mean ± SEM of three independent experiments. *P*-values were calculated against medium control; NS, not significant.

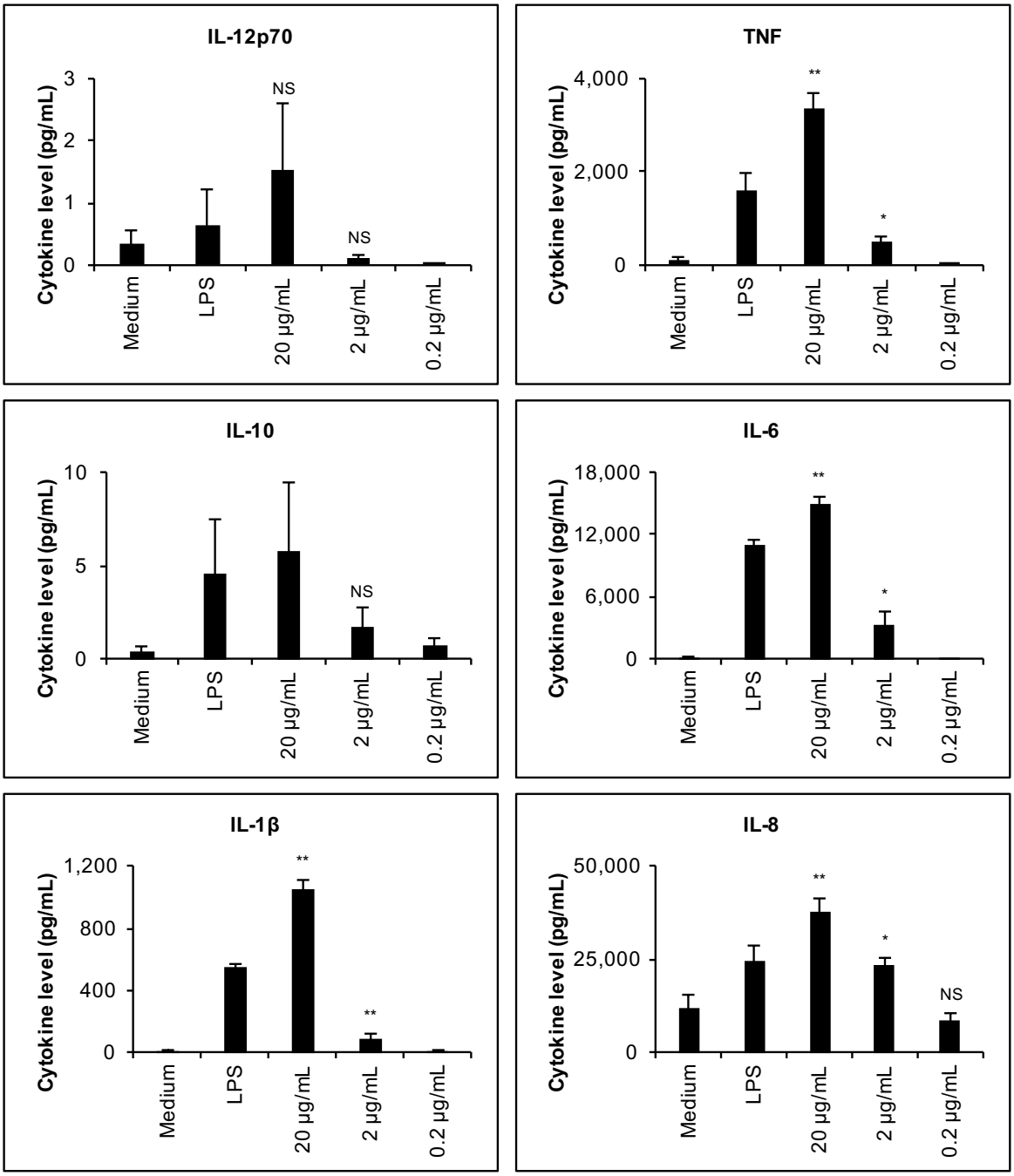

Figure 4.5 Inflammatory cytokine induction profile of HBD-3-treated monocyte

Cells were seeded in appropriate culture media and stimulated with LPS (50 ng/mL) or various concentration of HBD-3 for 18 h. Culture supernatant was collected and subjected to cytokine detection using cytometric bead array. Data represent mean ± SEM of three independent experiments. P-values were calculated against medium control; NS, not significant; *, $p<0.05$; **, $p<0.01$, unpaired t-test.

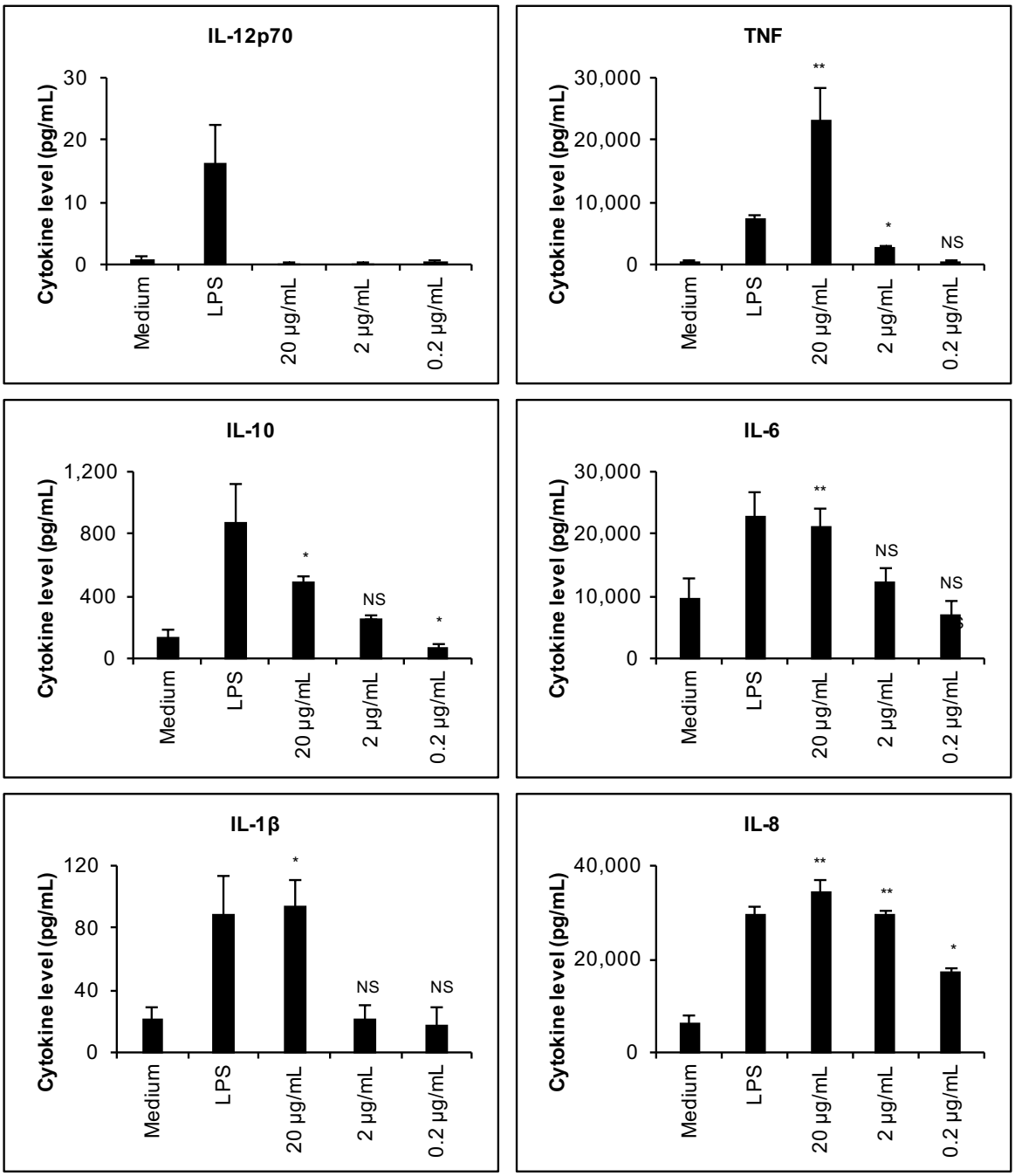

Figure 4.6 Inflammatory cytokine induction profile of HBD-3-treated iDC

Cells were seeded in appropriate culture media and stimulated with LPS (50 ng/mL) or various concentration of HBD-3 for 18 h. Culture supernatant was collected and subjected to cytokine detection using cytometric bead array. Data represent mean ± SEM of three independent experiments. P-values were calculated against medium control; NS, not significant; *, $p<0.05$; unpaired t-test.

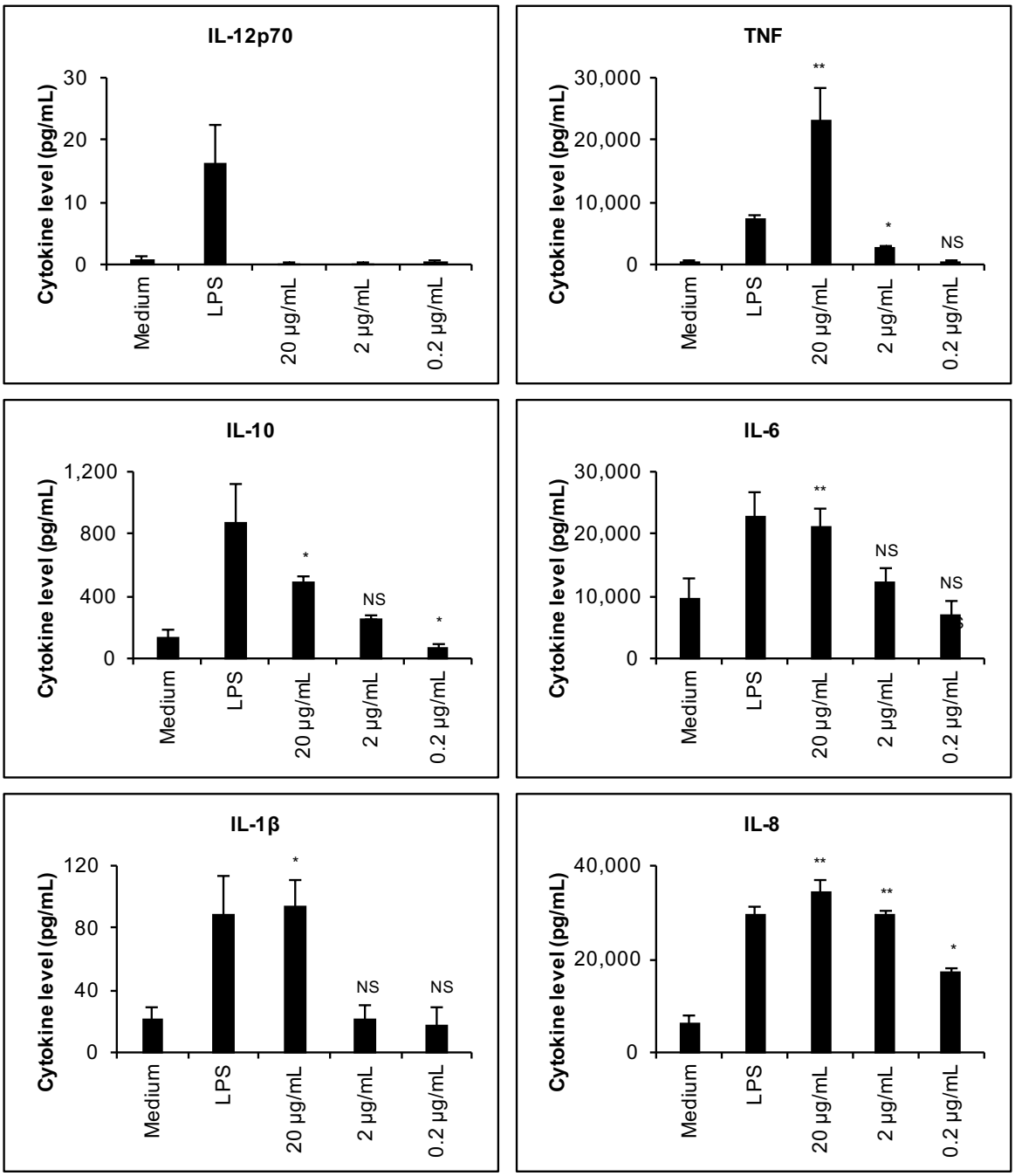

Figure 4.7 Inflammatory cytokine induction profile of HBD-3-treated GM-CSF-polarised macrophage

Cells were seeded in appropriate culture media and stimulated with LPS (50 ng/mL) or various concentration of HBD-3 for 18 h. Culture supernatant was collected and subjected to cytokine detection using cytometric bead array. Data represent mean ± SEM of three independent experiments. P-values were calculated against medium control; NS, not significant; *, $p<0.05$; **, $p<0.01$, unpaired t-test.

4.3.5 Impaired cytokine release by HBD-3 mutants

To investigate the potential relationship between cytokine induction and PI(4,5)P_2 binding by HBD-3, PI(4,5)P_2 binding mutants HBD-3(K32A) and HBD-3(K39A) were tested for their ability to mediate cytokine release. Both HBD-3 mutants at 20 μg/mL resulted in reduced levels of TNF and IL-6 (monocyte and macrophage), as well as to a lesser extent, IL-1β (monocyte) and IL-10 (macrophage) when compared to native HBD-3, whereas no significant difference was observed at lower concentrations (**Figure 4.8 and 4.9**). Generally, the level of diminished cytokine release corresponded with the order of impaired PI(4,5)P_2 binding. However, it should be noted that both mutants still retained the capacity to induce considerable levels of cytokines. In contrast, there was no apparent change in IL-8 induction at any tested concentrations in monocytes and iDCs, or at the higher concentration in macrophages. These data imply a certain correlation between PI(4,5)P_2 binding and HBD-3-induced cytokine release, particularly for TNF and IL-6.

4.3.6 Blocking of HBD-3-induced cytokine release by PI(4,5)P_2 binding inhibition

To further investigate the role of PI(4,5)P_2 binding in HBD-3-stimulated TNF and IL-6 induction in monocytes and macrophages, synthetic phospholipids as well as neomycin were tested in cytokine release assays. The proinflammatory activity of HBD-3 is significantly inhibited by PIPs, but not by PA (**Figure 4.10**). Particularly, PI(4,5)P_2 and PI(3,4,5)P_3 resulted in more than a 50% decrease in released cytokine levels. The inhibitory effect was more dramatic in monocytes than macrophages. On the other hand, none of the tested lipids showed any suppression of TNF and IL-6 release by LPS. Furthermore, in a concentration-dependent manner, neomycin-treated monocytes and macrophages displayed a similar reduced response to HBD-3 stimulation (**Figure 4.11**). The cytokine induction by HBD-3 is almost abolished at 5 mM neomycin, while that of LPS remains unaffected. Therefore, it is likely that HBD-3, but not LPS, essentially requires PI(4,5)P_2 to promote TNF and IL-6 induction.

4.3.7 Inhibition of HBD-3-stimulated cytokine release in iDC by wortmannin, but not staurosporin

HBD-3-induced cytokine induction in monocyte and macrophage is NF-κB-dependent (Niyonsaba *et al*, 2007; Jin *et al*, 2010; Funderburg *et al*, 2011), which could be activated via PI(4,5)P_2-mediated PI3K/Akt or PLC/PKC signalling cascade (Kane *et al*, 1999; Moscat *et al*, 2003; Min *et al*, 2005; Rajaram *et al*, 2006). To delineate the potential

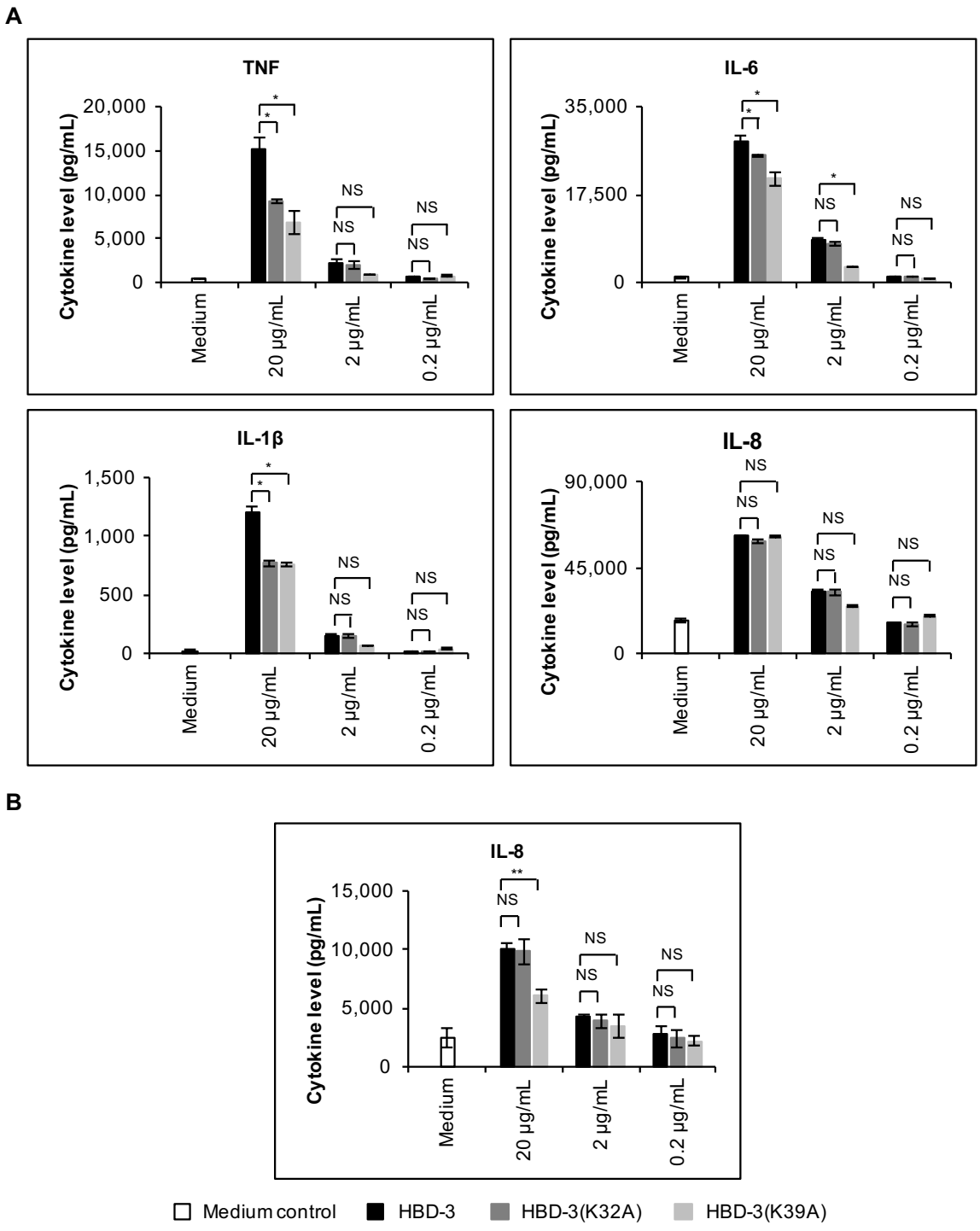

Figure 4.8 Impaired HBD-3-induced cytokine release by PI(4,5)P_2 binding mutants in (A) Monocytes and (B) iDCs

Cells were seeded in appropriate culture media with various concentration of HBD-3, HBD-3(K32A) or HBD-3(K39A) for 18 h. Culture supernatant was collected and subjected to cytokine detection using cytometric bead array. Data represent mean ± SEM of three independent experiments. NS, not significant; *, $p<0.05$; **, $p<0.01$; unpaired t-test.

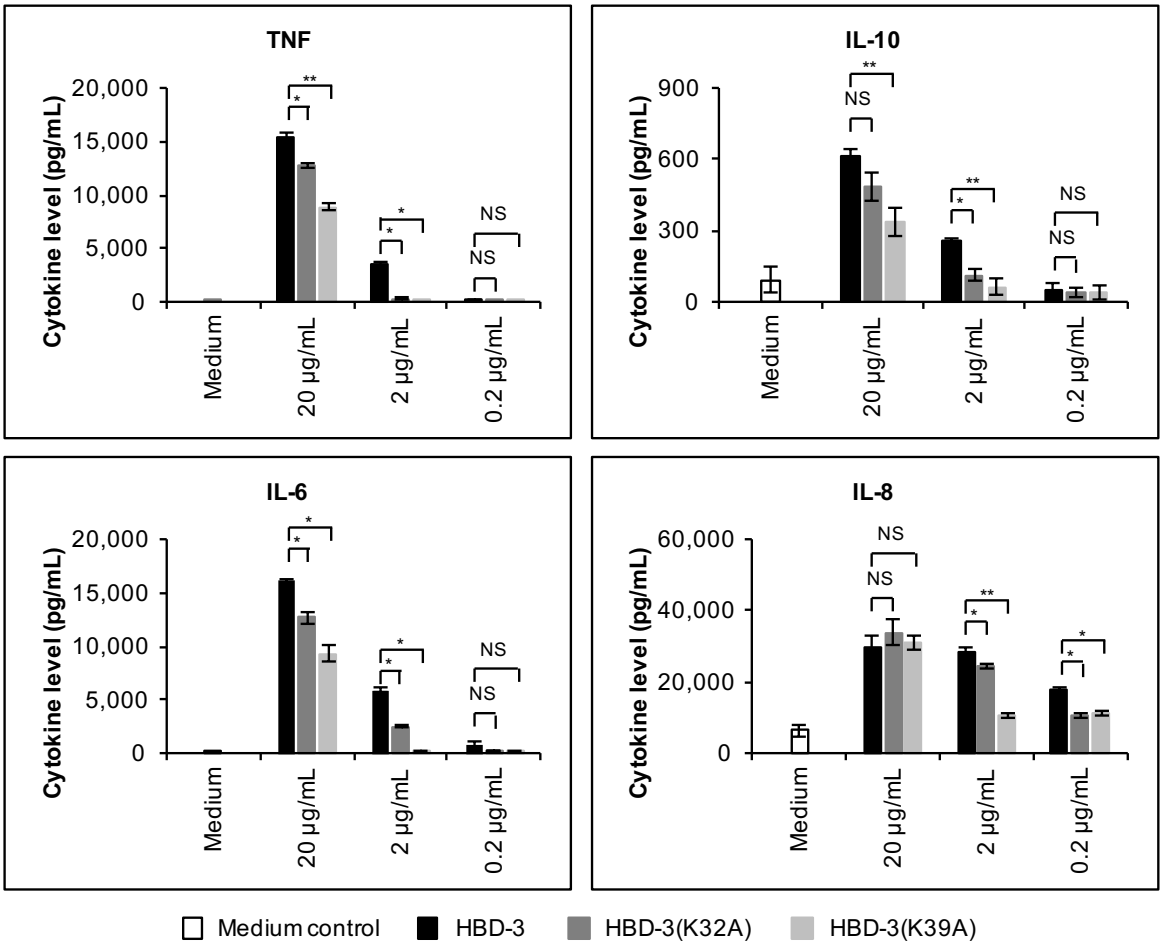

Figure 4.9 Impaired HBD-3-induced cytokine release by PI(4,5)P_2 binding mutants in GM-CSF-polarised macrophage

Cells were seeded in appropriate culture media with various concentration of HBD-3, HBD-3(K32A) or HBD-3(K39A) for 18 h. Culture supernatant was collected and subjected to cytokine detection using cytometric bead array. Data represent mean ± SEM of three independent experiments. NS, not significant; *, $p<0.05$; **, $p<0.01$; unpaired t-test.

relevance of these pathways in HBD-3-stimulated TNF and IL-6 release in the abovementioned antigen presenting cells, signalling inhibitors wortmannin (PI3K inhibitor) and staurosporine (PKC inhibitor) were used in cytokine release assays. Wortmannin substantially suppressed HBD-3-induced, but, negligibly, LPS-stimulated proinflammatory signalling, as demonstrated by a concentration-dependent decrease in levels of TNF and IL-6 from monocytes (**Figure 4.8A**). Similar inhibitory effects where not observed for GM-CSF-activated macrophages, however, wortmannin unexpectedly slightly elevated TNF and IL-6 level by both LPS and HBD-3 (**Figure 4.8B**). Staurosporine does not appear to cause any significant inhibition of TNF and IL-6

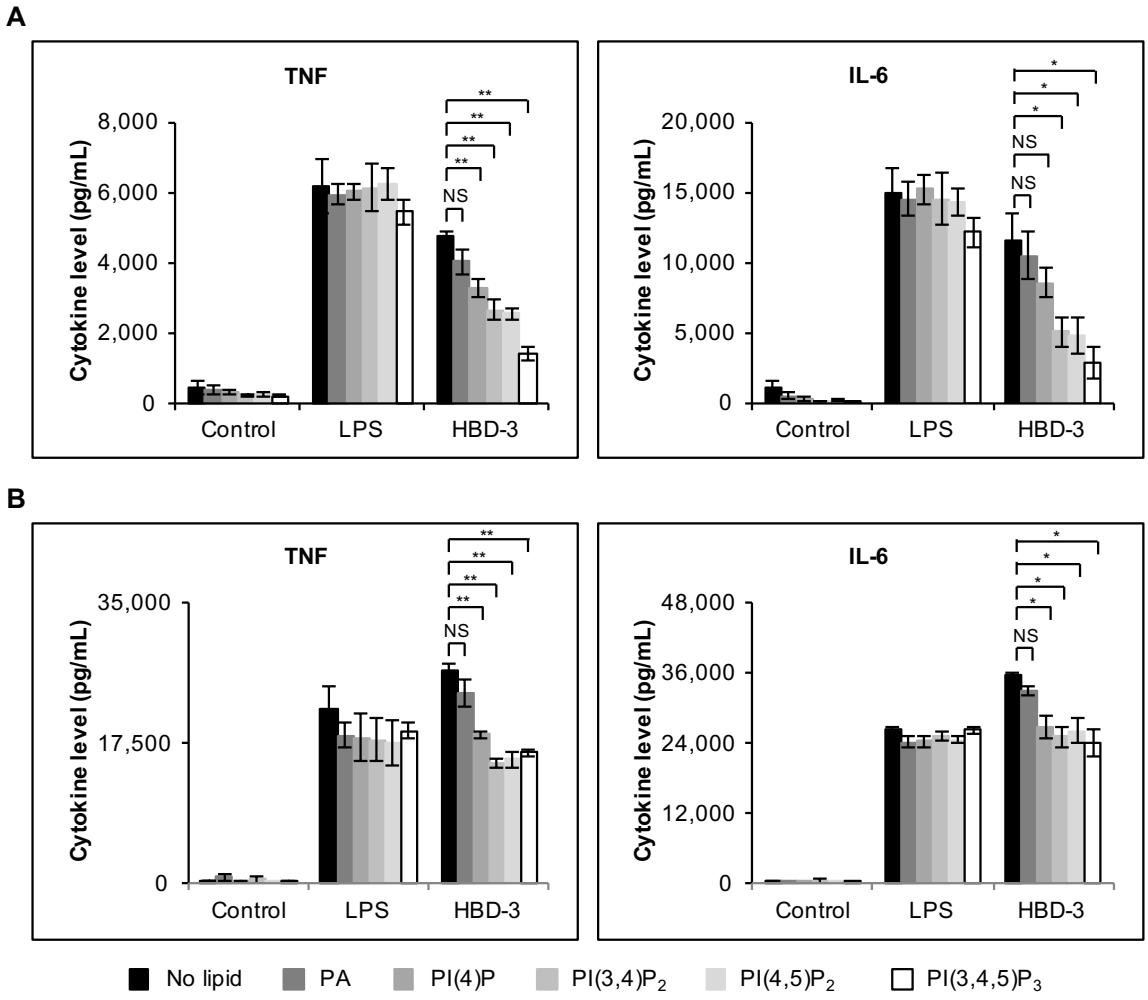

Figure 4.10 Impaired HBD-3-induced cytokine release by phosphoinositides

(A) Monocytes. **(B)** GM-CSF-stimulated macrophages. HBD-3 (10 μg/mL) was pre-incubated with different phospholipids (1:2 molar ratio) prior to stimulate cells for 18 h. Culture supernatant was collected and subjected to cytokine detection using cytometric bead array. Data represent mean ± SEM of three independent experiments. NS, not significant; *, $p<0.05$; **, $p<0.01$; unpaired t-test.

induction in monocyte and macrophage by HBD-3 or LPS (**Figure 4.9**). These data suggest that, unlike LPS, HBD-3 may induce release of TNF and IL-6 from monocytes through the PI3K/Akt signalling pathway, indicating that different cascades are being activated for TNF and IL-6 induction in macrophages.

4.3.8 Impaired Akt activation by HBD-3 mutants

To confirm the effect of HBD-3 mutants and neomycin on HBD-3-induced activation of PI3K/Akt signalling, immunoblotting to detect Akt phosphorylation was performed on stimulated monocyte lysates. While native HBD-3 increased levels of phospho-Akt(S473), suggesting activation of PI3K/Akt signalling, K32A and K39A resulted in

approximately half and complete reduction of Akt phosphorylation, respectively (**Figure 4.14**). Neomycin also abolished HBD-3-induced Akt phosphorylation, and as expected, so did wortmannin. Total Akt was also included as a loading control, confirming the observed differences are attributed to treatments. Therefore, HBD-3 is likely to bind to and modulate PI(4,5)P_2 on the monocyte membrane to activate PI3K/Akt pathway and, ultimately, lead to induction of TNF and IL-6.

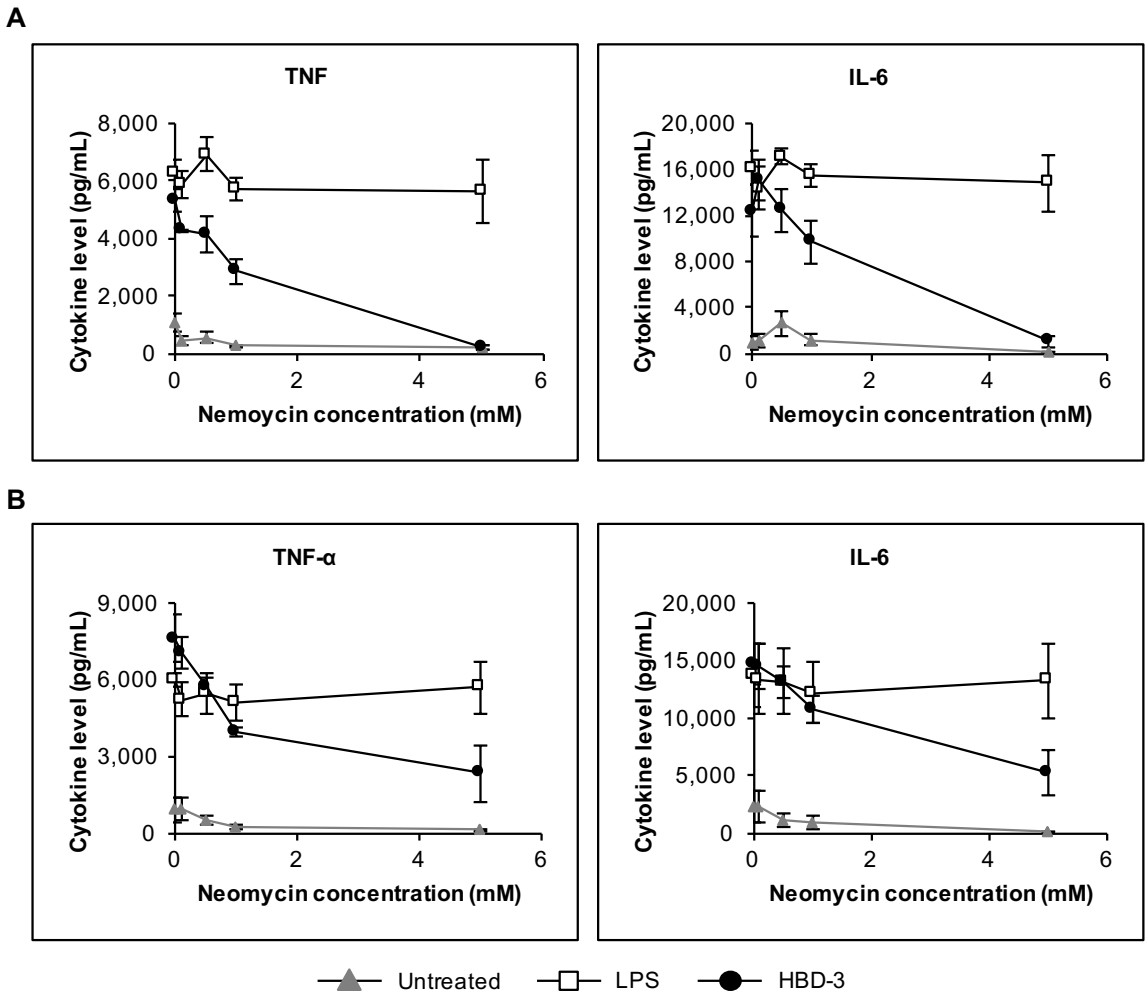

Figure 4.11 Impaired HBD-3-induced cytokine release by neomycin

(A) Monocytes. **(B)** GM-CSF-stimulated macrophages. Cells were seeded in appropriate culture media, pre-treated with various concentration of neomycin prior to stimulation with HBD-3 (10 μg/mL) for 18 h. Culture supernatant was collected and subjected to cytokine detection using cytometric bead array. Data represent mean ± SEM of three independent experiments.

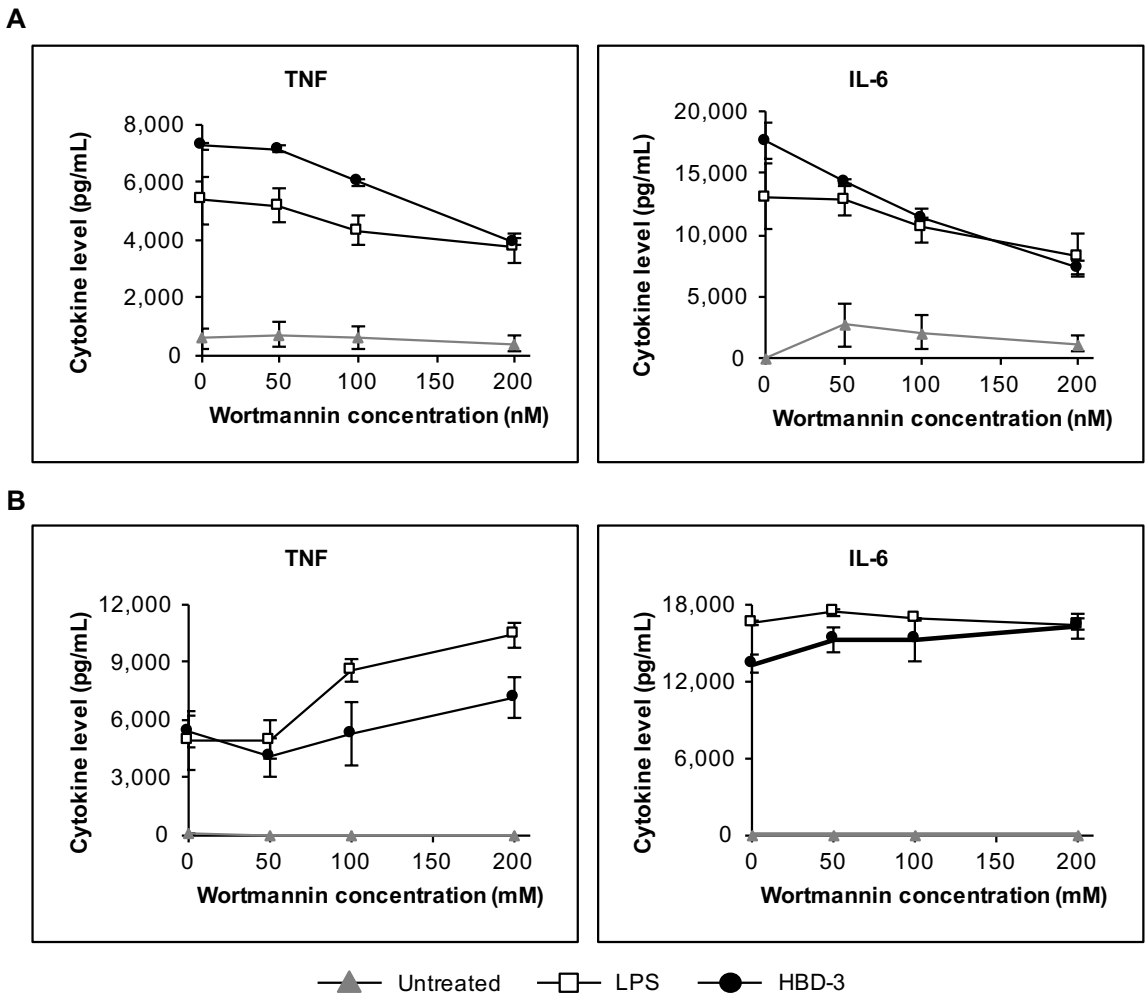

Figure 4.12 Opposite effects of wortmannin on HBD-3-induced cytokine release

(A) Monocytes. **(B)** GM-CSF-stimulated macrophages. Cells were seeded in appropriate culture media, pre-treated with various concentration of wortmannin prior to stimulation with HBD-3 (10 μg/mL) for 18 h. Culture supernatant was collected and subjected to cytokine detection using cytometric bead array. Data represent mean ± SEM of three independent experiments.

4.4 Discussion

HBD-3, a well characterised cationic HDP, can bilaterally modulate immune responses to either trigger or resolve inflammation. As a proinflammatory effector, HBD-3 displays leukocyte chemotaxis and cytokine induction which, however, have only been reported for a limited number of cell types (Yang *et al*, 1999; Chen *et al*, 2007; Soruri *et al*, 2007; Jin *et al*, 2010; Rohrl *et al*, 2010; Sheng *et al*, 2014) (Wu *et al*, 2003; Funderburg *et al*, 2007; Niyonsaba *et al*, 2007; Jin *et al*, 2010; Funderburg *et al*, 2011). A number of signalling pathways have been proposed to mediate these HBD-3-mediated inflammatory responses, including CCR2/6-PLC-NF-κB, TLR1/2-MyD88-IRAK-1-NF-κB and surface

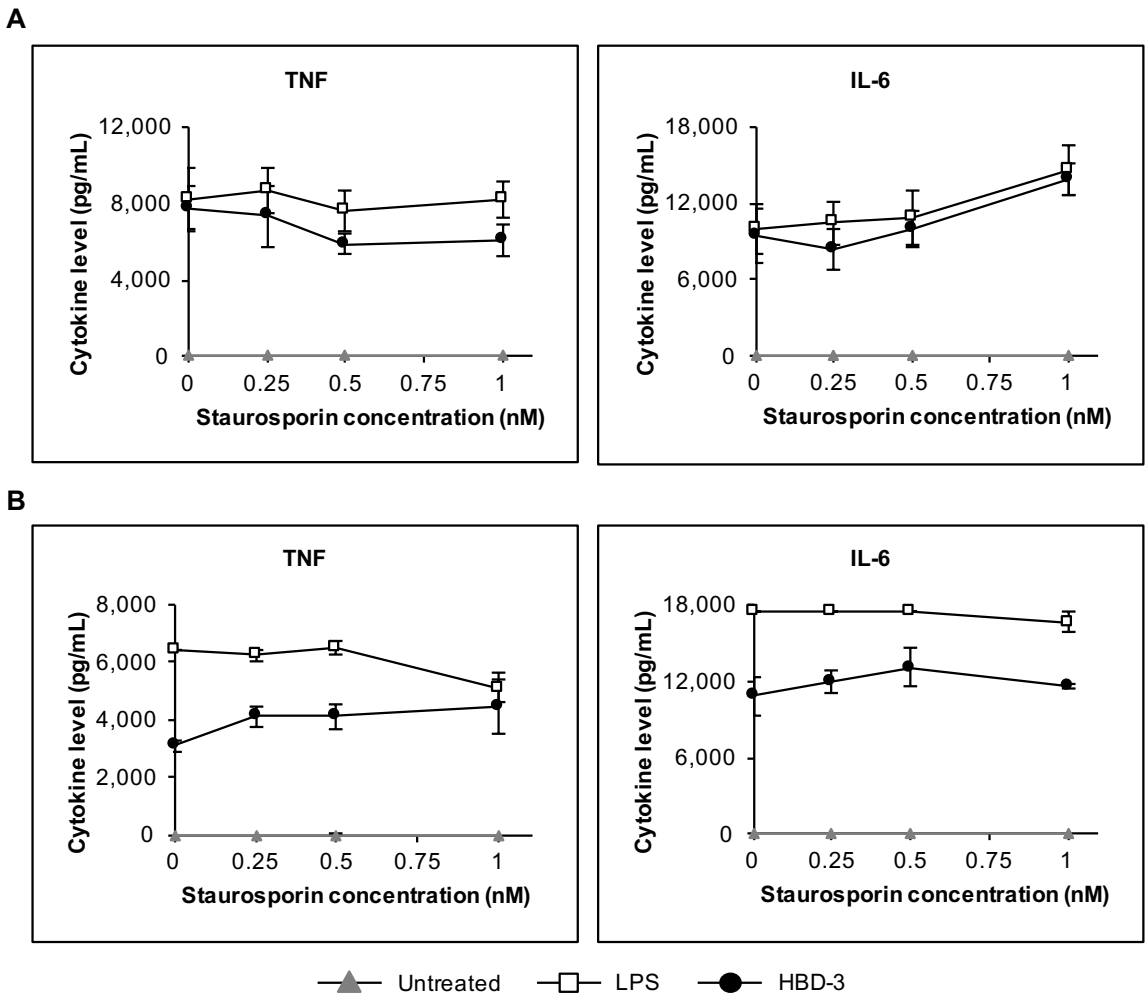

Figure 4.13 No inhibition of staurosporin on HBD-3-induced cytokine release

(A) Monocytes. **(B)** GM-CSF-stimulated macrophages. Cells were seeded in appropriate culture media, pre-treated with various concentration of staurosporin prior to stimulation with HBD-3 (10 µg/mL) for 18 h. Culture supernatant was collected and subjected to cytokine detection using cytometric bead array. Data represent mean ± SEM of three independent experiments.

receptor-independent PI3K-Akt-NF-κB pathways (Funderburg *et al*, 2007; Niyonsaba *et al*, 2007; Jin *et al*, 2010; Funderburg *et al*, 2011; Mburu *et al*, 2011). Further investigation is therefore needed to define the mechanism of action of these immunomodulatory functions of HBD-3 and, more broadly, HDPs.

This chapter expands the investigation of HBD-3-induced chemotactic migration and cytokine induction in different leukocytes. Among all the tested cell types, only monocytes chemotactically responded to HBD-3 at sub-cytotoxic concentrations, which is consistent with that reported by Wu *et al* (2003) and Rhorl *et al* (2010). In contrast,

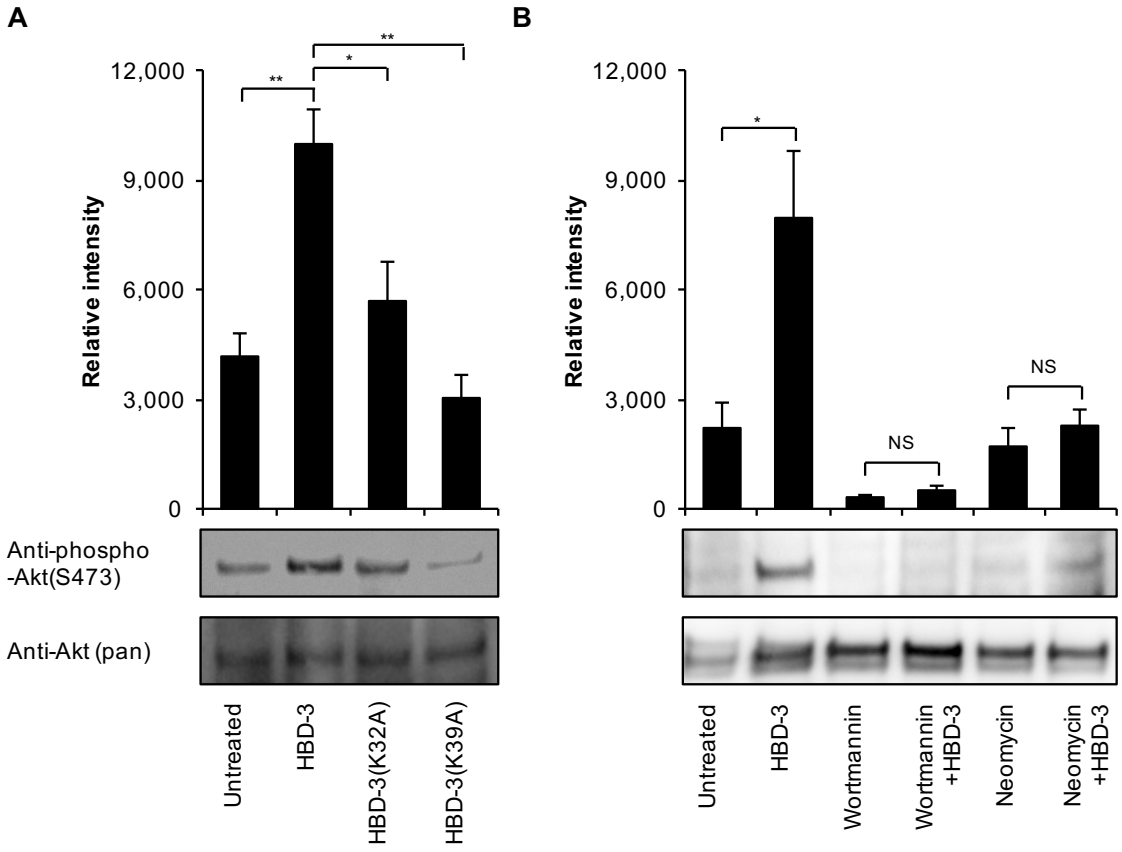

Figure 4.14 Inhibition of HBD-3-induced Akt activation in monocytes by (A) PI(4,5)P_2-binding mutants and (B) wortmannin and neomycin

Serum-starved monocytes were stimulated with HBD-3 and mutants (10 μg/mL) for 30 minutes. For wortmannin (200 nM) and neomycin (5 mM) treatment, monocytes were incubated with the inhibitors for 3 h prior to addition of HBD-3. Cells were then harvested, lysed and subjected to SDS-PAGE and immunoblotting using rabbit-anti-phoso-Akt(S473) (1:2000) or rabbit-anti-Akt (pan) (1:1000) and HRP-conjugated donkey-anti-rabit antibodies (1:10,000). Data represent mean ± SEM of three independent experiments. NS, not significant; *, $p<0.05$; **, $p<0.01$; unpaired t-test.

T lymphocytes, iDCs and GM-CSF/M-CSF-stimulated macrophages showed very little migration toward a HBD-3 gradient. Interestingly, a previous report had suggested that HBD-3-induced chemotaxis for GM-CSF-stimulated macrophages (Soruri *et al*, 2007). However, it was only slight, albeit significant, increase as compared to basal migration (~8×10^3 cells at maximal chemotactic effect vs ~5×10^3 cells) (Soruri *et al*, 2007), suggesting that HBD-3 is not a strong chemoattractant for macrophages. Similarly, it was also speculated that HBD-3, based on observations for HBD-1 and HBD-2, would also recruit iDCs, memory T lymphocytes and neutrophils (Yang *et al*, 1999; Niyonsaba *et al*, 2004; Rohrl *et al*, 2010). However, this study suggests otherwise. Despite similar tertiary structure, β-defensins show little sequence identities, and not always share similar

functions or targets. For example, while HBD-1 and -2 promotes iDC and memory T lymphocyte migration with comparable efficacy and potency, only HBD-2, but not HBD-1, was shown to be a chemotactic agent for TNF-α-treated neutrophils (Yang *et al*, 1999; Niyonsaba *et al*, 2004). Therefore, it may be considered not surprising that HBD-3 shows a different leukocyte chemotaxis profile to the other defensins.

HBD-3 at concentrations > 100 ng/mL, substantially induces cytokine release from antigen presenting cells, namely monocyte (TNF, IL-6, IL-8 and IL-1β), monocyte-derived iDC (IL-8) and GM-CSF-activated macrophage (TNF, IL-6 and IL-8, IL-10 and IL-1β), but not from memory T lymphocytes. Endotoxin contamination was excluded, as control no-protein eluted fractions from endotoxin removal, compared to medium only controls, showed similar negligible levels of cytokine release. Previously, HBD-3 was consistently demonstrated to stimulate the expression of proinflammatory cytokines IL-6, IL-8 and IL-1β, but not IL-10, in human monocytes (Funderburg *et al*, 2011). Similarly, Jin *et al* (2010), although protein levels were not quantitated, showed an increased expression of TNF-α, IL-1β, IL-6, IL-8 and CCL18 gene transcripts in HBD-3-treated human macrophages. Herein, HBD-3 was shown to induce higher levels of cytokine stimulation in macrophages than monocytes, which inversely correlated with chemotaxis. It is tempting to speculate that the compensatory nature of HBD-3 chemoattraction and cytokine induction in monocytes and macrophages may ensure a controlled inflammation in response to microbial pathogen. In addition, HBD-3-induced inflammatory response by monocyte-derived iDC correlates with previously-reported iDC maturation (Funderburg *et al*, 2007; Judge *et al*, 2015). However, despite previous implications based on HBD-1 and HBD-2 (Yang *et al*, 1999), this study shows that HBD-3 is unable to promote inflammatory responses in memory T lymphocytes. This finding reinforces the notion of differential immunomodulatory effects of human β-defensins, probably to specify inflammatory responses against particular stimuli. Taken together, these data further emphasise that the HBD-3 proinflammatory modulation facilitates innate immune responses, primarily via the antigen presenting cells, in particular, monocytes and macrophages.

Proinflammatory effects of HBD-3, like other HBDs, have long been demonstrated to be mediated via promiscuous interactions with surface receptors. Initially, as implicated by HBD-1 and HBD-2, CCR6 was proposed as a receptor for HBD-3 (Yang *et al*, 1999).

Indeed, the HBD-3-induced chemotactic migration of CCR6-overexpressing HEK293 cell was later demonstrated (Wu *et al*, 2003). Moreover, HBDs and CCL20, a natural ligand of CCR6, share analogous tertiary CCR6 binding sites despite no sequence homology (Yang *et al*, 1999). HBDs and CCL20 similarly display abundant cationicity, disulfide framework and comparable cysteine-stablised tertiary structures with an α-helix and a triple-stranded β-sheet (Hoover *et al*, 2002). Moreover, the Asp-Cys-Cys-Leu motif, one possible determinant of chemokine-receptor interactions, can be equivalently observed as a conserved Asp…X pair of similar distance (with X denoting hydrophobic residues) in both HBD-1 (as Asp^1…Tyr^3) and HBD-2 (as Asp^4…Val^6) (Hoover *et al*, 2002). Lower CCR6 binding affinity of HBDs may be attributable to narrower potential recognition grooves observed in their structures, as compared to CCL20 (Yang *et al*, 1999; Hoover *et al*, 2002). Nevertheless, the fact that HBD-3 limitedly influences cytokine induction in $CCR6^+$ memory T lymphocyte and iDC, as presented in this chapter, may question the exclusive involvement of CCR6. Intriguingly, HBD-3 (and HBD-2) was also reported as not being able to chemoattract CCR6-overexpressing RBL-2H3 and 300.19 cells and failed to desensitise CCL20-induced migration or induce calcium fluxes in these CCR6 transfectants (Soruri *et al*, 2007). CCR2 is alternatively proposed for HBD-3 immunomodulation, as implied by the evident chemoattraction of $CCR2^+$ monocytes by HBD-3, along with abrogated HBD-3-induced migration of monocytic cell lines upon upon specific blockade of CCR2 (Jin *et al*, 2010), However, the drastically weak chemotactic effect on both GM-CSF-polarised and M-CSF-polarised macrophages, which differentially display expression level of CCR2 (Sierra-Filardi *et al*, 2014), may imply that HBD-3 chemotaxis seems more complicated than just CCR2 and/or CCR6-dependent induction.

Based on the observation that PI(4,5)P_2/PI(3,4,5)P_3 binding and/or metabolising proteins are particularly crucial for cell migration (Van Keymeulen et al, 2006; Roussos et al, 2011; Fets et al, 2014; Wu et al, 2014; Elong Edimo et al, 2016) and that internalised HBD-3 binds to intracellular PI(4,5)P_2 (as showed in chapters 2 and 3), it was attempted to investigate the role of PI(4,5)P_2 modulation in HBD-3-induced leukocyte migration. However, no connection could be established as the basis of (i) monocyte chemotaxis toward HBD-3(K32A) or HBD-3(K39A) did not correlate with the extent of PI(4,5)P_2-binding impairment, (ii) PI(4,5)P_2-binding neomycin did not inhibit monocyte chemoattraction by HBD-3, and (iii) inhibition of PI(3,4,5)P_3-generating PI3K, a key

enzyme in PI(4,5)P_2/PI(3,4,5)P_3-dependent cell migration (Cain & Ridley, 2009; Artemenko *et al*, 2014), resulted in no change in monocyte migration toward a HBD-3 gradient. Canonically, leukocyte chemotaxis is executed through an extensive and tightly regulated signalling cascade that sequentially involves G protein-coupled receptor binding, Ras GTPase activation, PI(4,5)P_2/PI3K/PI(3,4,5)P_3-induced cell polarisation, kinase (TorC2 and Akt) activation and cytoskeleton remodelling (Artemenko *et al*, 2014). However, it has also recently been shown that the PI(4,5)P_2/PI3K/PI(3,4,5)P_3 axis is not necessarily required for accurate leukocyte chemotaxis (Hoeller & Kay, 2007; Smith *et al*, 2007; Volpe *et al*, 2010). Alternative pathways of cell polarisation can be employed, such as alternative kinase activation, PI(3,4)P_2 gradient or PI(3,4,5)P_3-hydrolysing PIP-dependent-5-phosphotase SHIP2-induced polarisation (Kamimura *et al*, 2008; Elong Edimo *et al*, 2016; Li *et al*, 2016). Therefore, it is understandable that neither neomycin nor wortmannin causes significant inhibition on HBD-3-induced monocyte migration. In other words, the chemotactic effect of HBD-3 might be independent PI(4,5)P_2 and PI3K/PI(3,4,5)P_3. Curiously, it remains uncertain as to why HBD-3 mutants diminish monocyte migration; however, K32 and K39 in HBD-3 may play certain roles in migratory interaction that are distinct from PI(4,5)P_2 binding. Taken together, the chemotactic effect of HBD-3 may involve an elaborate signalling cascade, which appears more convoluted than just CCR2 or CCR6 and PI(4,5)P_2/PI3K/PI(3,4,5)P_3 signalling and, probably, entails a non-canonical establishment of cell polarisation prior to migration.

Conversely, PI(4,5)P_2 binding of HBD-3 appears to mediate TNF and IL-6 induction in monocytes and macrophages via PI3K and Akt activation. HBD-3(K32A) and HBD-3(K39A) mutants show a dramatic reduction in TNF and IL-6 release that correlates well with a loss of PI(4,5)P_2 binding, however they have no effect on IL-8 induction. However, the considerable levels of cytokine inducing activity retained by these mutants, particularly at high concentrations, could be associated with the long experimental timeframe (18 h), which may allow an accumulation of PI(4,5)P_2 interaction and signalling. This is consistent with the retained, albeit dramatically diminished, tumour cell cytotoxicity after 48 h incubation, as detailed in the previous chapter. Decreased pro-inflammatory cytokine induction of the HBD-3(K32A) and HBD-3(K39A) also correlates well with reduced levels (50% and 25% respectively) of Akt(S473) phosphorylation (**Figure 4.14A**), a prerequisite of fully-activated Akt for downstream signalling (Vadlakonda *et al*, 2013). Previously, it was demonstrated that cytokine induction by

HBD-3 is NF-κB dependent, and that HBD-3 internalises and activates PI3K/Akt signalling, ultimately leading to parallel activation of transcriptional factors NF-κB and AP-1, independently of G-protein coupled receptors, TLRs and TLR adaptor MyD88 (Mburu *et al*, 2011; Mburu *et al*, 2012; Ferris *et al*, 2013). It is also worth noting that HBD-3 internalisation into leukocytes has been consistently reported, although its relevance remains unanswered (Mburu *et al*, 2011; Semple *et al*, 2011; Mburu *et al*, 2012). In fact, the PI3K/Akt pathway is crucial for HBD-3-induced TNF and IL-6 stimulation, as wortmannin, a well-characterised PI3K inhibitor, causes inhibition of Akt phosphorylation (**Figure 4.14B**) and cytokine release (**Figure 4.12**), noticeably in monocytes, which is however not observed for LPS. Given the importance of the receptor-independent PI3K/Akt pathway, internalised HBD-3 may need to execute its signalling via modulation of intracellular target(s) upstream of PI3K, which is likely to be PI(4,5)P_2, another key effector of PI3K/Akt cascade. Remarkably, substantial inhibition of HBD-3-induced TNF and IL-6 induction by synthetic PIPs, but not PA (**Figure 4.10)** or neomycin in concentration-dependent fashion (**Figure 4.11**) suggests that HBD-3 essentially requires PI(4,5)P_2 to promote TNF and IL-6 induction. Furthermore, neomycin also inhibits Akt phosphorylation by HBD-3 (**Figure 4.14B**), and hence emphasising the importance of PI(4,5)P_2-mediated Akt activation of HBD-3. As reported in Chapters 2 and 3, HBD-3 internalisation is not inhibited by either K32 and K39 mutations or neomycin. Although PI(4,5)P_2 can contribute to NF-κB activation via either PI3K/Akt or PLC/PKC pathways, the latter was ruled out by the use of staurosporine, a potent and specific PKC inhibitor at concentrations below 1 nM (Ward & O'Brian, 1992) as the PKC inhibition does not alter IL-6 and TNF induction by HBD-3 (**Figure 4.13**). Taken together, HBD-3 may induce IL-6 and TNF via PI(4,5)P_2 modulation, independent of known surface receptors, leading to PI3K/Akt transduction and downstream activation of transcriptional factors NF-κB and AP-1.

It remains to be determined how the interaction of HBD-3 with PI(4,5)P_2 leads to PI3K activation. HBD-3 is not known to contain any apparent kinase-binding motif, thus unlikely to involve direct recruitment of PI3K upon PI(4,5)P_2 binding. Furthermore, although intracellular GAPDH was demonstrated as a mononuclear cell intracellular effector for p38 mitogen-activated protein kinase (MAPK p38)-dependent proinflammatory modulation of human cathelicidin LL-37 (Mookherjee *et al*, 2009), no available literature suggests an association between HBD-3, PI(4,5)P_2 binding, PI3K

activation and GAPDH. However, one plausible explanation could be deduced from a similar finding on receptor-independent iDC activation by particulate substances, such as monosodium ureate (Ng *et al*, 2008). Interactions of external particulate substances with membrane lipid might cause membrane rearrangement, leading to aggregation of lipid rafts and subsequent clustering of raft-associated intracellular receptors (*e.g.* immunoreceptor tyrosine receptor-based activation motif, ITAMs) to recruit effector kinases (*e.g.* spleen tyrosine kinase, Syk), leading to PI3K activation and downstream proinflammatory responses, including cytokine induction and iDC activation (Ng *et al*, 2008). Although they have not been formally linked, lipid raft and/or membrane re-organisation by HBDs and PI(4,5)P_2 sequestration/modulation have been reported (Epand *et al*, 2005; Khelashvili *et al*, 2008; Lupyan *et al*, 2010; Morgera *et al*, 2011). Hence, HBD-3 may act through similar pathway(s) associated with PI(4,5)P_2 modulated lipid raft rearrangement-induced clustering of effector kinases to activate PI3K signalling. Intriguingly, this proposal is consistent with the previous suggestion for HBD-2-induced iDC maturation which is not activated by an agonist-receptor-type interaction, but non-canonically membrane internalisation and alteration of membrane composition (Morgera *et al*, 2011). It may also explain why HBD-3 shows a different extent and components of cytokine induction in antigen presenting cells (monocyte, macrophage and iDC) whose display a differential capacity for defensin binding and internalisation (Morgera *et al*, 2011).

Nevertheless, one should be cautious to exclude the involvement of surface receptors, such as the previously reported TLR1/2 (Funderburg *et al*, 2007; Funderburg *et al*, 2011), for HBD-3-induced cytokine release. While neomycin comparably abolishes both Akt phosphorylation and cytokine induction, wortmannin only reduces 50% cytokine level despite the abrogation of Akt activation. HBD-3 may therefore act on multiple signalling pathway. In fact, PI(4,5)P_2 is arguably required for the plasma membrane localisation of the sorting adaptor (TIRAP), and subsequent recruitment of the signalling adaptor MyD88. Hence, the additional participation of MyD88-mediated TLR1/2 signalling may confer the tolerance of cytokine release inhibition by wortmannin, but not by neomycin. LPS, on the other hand, acts primarily via TLR-4 which further transduces through MyD88 or TRIF (which does not require PI(4,5)P_2) to activate NF-κB (Lu *et al*, 2008), implying that the role of PI(4,5)P_2 does not appear critical and, hence, are not affected by wortmannin, neomycin and staurosporine. PI(4,5)P_2 binding of HBD-3 might also explain

IL-1β induction and co-stimulatory marker expression via ATP-gated channel P2X7 (Lioi *et al*, 2015; Wanke *et al*, 2016), which is directly regulated by PI(4,5)P_2 (Zhao *et al*, 2007; Bernier *et al*, 2008).

4.5 Conclusion

The findings in this chapter advance knowledge of HBD-3-stimulated chemotaxis and cytokine induction in different leukocytes. It has been demonstrated that HBD-3 is able to recruit monocytes, and to a lesser extent, macrophages, iDCs and memory T lymphocytes. HBD-3 induction of different cytokines is also showed for monocytes and macrophages, modestly by iDCs and negligibly by T lymphocytes. For the first time, PI(4,5)P_2 interaction is found to be essential for the PI3P/Akt-dependent TNF and IL-6 induction, but not the chemotactic recruitment, by a HDP. It is suggested that internalised HBD-3 binds to PI(4,5)P_2 to promote PI3K activation, possibly via membrane reorganisation and lipid raft-associated effectors, then Akt phosphorylation and NF-κB activation, ultimately leading to cytokine induction. This study also clarifies an unexplained relevance of HBD-3 internalisation into leukocytes in many previous studies. More importantly, it provides insights to currently-unresolved mechanism of the multifaceted proinflammatory immunomodulation of HBD-3, and signifies the elaborate and multifaceted signalling in pathogen defense and alerting immune responses by HDPs.

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Chapter 5

Concluding remarks

5.1 Introduction

Cancer and pathogenic infections are leading causes of disease-related mortality and morbidity in Australia and worldwide, highlighting an urgent need for new anticancer and anti-infective approaches. PIPs are of low abundance but functionally crucial membrane lipids that are commonly subverted by cancer cells and pathogens, and therefore may offer therapeutic opportunities. However, the direct targeting of PIPs or their metabolising enzymes has not been adequately addressed, with most attention instead being invested on PI3Ks. A number of PI3K inhibitors have entered clinical trials, and show promise as anticancer therapeutics. However, the use of these PI3K inhibitors have been challenged by a number of concerning issues, including off-target specificity and detrimental effects on immunity (Rafii *et al*, 2015). Intriguingly, recent reports on cancer cytotoxicity of the antifungal ornamental tobacco defensin NaD1 (Poon *et al*, 2014) and tomato defensin TPP3 (Baxter *et al*, 2015b) via PI(4,5)P_2-mediated membrane permeabilisation have demonstrated the potential anticancer applications of PIP-sequestering host defense peptides (HDPs). PI(4,5)P_2 interaction of these solanaceous plant defensins was mapped to conserved cysteine-flanked, β-strand-spanning, cationic clusters (^{35}S**K**IL**RR**^{40} in NaD1 and ^{37}S**K**LQ**RK**^{42} in TPP3), which resemble the **K/R**-X(3,7)-**K**-X-**K/R**-**K/R** motif found in nuclear PI(4,5)P_2-binding proteins (Lewis *et al*, 2011). Furthermore, blocking the interaction of the matrix protein Gag with PI(4,5)P_2 with the use of monoclonal anti-PI(4,5)P_2 antibodies, has been shown to neutralise HIV-1 by preventing virion release from host cells (Matyas et al, 2010; Jobe et al, 2012). Although additional studies are required, PI(4,5)P_2-sequestering aminoglycosides, neomycin and kanamycin, also inhibited early stage of PI(4,5)P_2-dependent entry of human simplex virus (Langeland *et al*, 1987). Taken together, these data suggest that the direct targeting of PI(4,5)P_2 may represent a new therapeutic approach for treating cancer and infectious diseases. The potential strategies may include the use of PI(4,5)P_2-binding HDPs or aminoglycoside-based drugs. Moreover, as learned from therapeutic blockade of PI3Ks, understanding the immunological effects of PI(4,5)P_2 targeting will also be helpful for future development of this therapeutic approach.

5.2 Key findings and future perspectives

5.2.1 PI(4,5)P_2-binding host defense peptides as potential therapeutics as suggested by HBD-3-induced cancer cell permeabilisation

HBD-3 was identified as a potential PI(4,5)P_2 binding HDP as it contained a cysteine-flanked and highly positively-charged β2-β3 loop (^{34}ST**R**G**RK**^{39}) that is remarkably analogous to those of the solanaceous plant defensins. Chapters 2 and 3 present multiple lines of evidence that suggest that the conserved K39 of the cationic motif as well as the preceding K32 are responsible for its PI(4,5)P_2 binding and relatively selective cytotoxicity against cancer cells at low micromolar concentrations. The underlying mode of action featuring bleb-associated PI(4,5)P_2-dependent membrane permeabilisation, as also described for NaD1 (Poon *et al*, 2014) and TPP3 (Baxter *et al*, 2015b), further accentuates a interspecies conserved mechanism among membrane-targeting innate immune molecules that possess potential 'phospholipid recognition patterns' (Baxter *et al*, 2015a). The findings in this thesis suggest that the hydrogen bond donor residues preceding the β2-β3 loop (i.e. K32 in HBD-3, H33 in NaD1 and H35 in TPP3) should be included as a possible extension of the 'phospholipid recognition pattern'. Hence, the cationic motifs should now be regarded as **K**CST**RG**RK, **H**CS**K**IL**RR** and **H**CS**K**LQ**RK** for HBD-3, NaD1 and TPP3, respectively. Together with the previously-reported pattern of nuclear PI(4,5)P_2-binding proteins (Lewis *et al*, 2011), a potential novel PI(4,5)P_2 binding motif comprising **Bd**-X(n)-**R/K**-X(1,2)-**R/K**-**R/K** (where 'Bd' denotes hydrogen bonding donors such as arginine, lysine and histidine, and 'n' denotes more than two amino acids present) is herein proposed. This study also supports the proof of concept that PI(4,5)P_2-binding HDPs, especially ones with the cationic motif, also display anticancer activity, and hence could be exploitable for combined anticancer and anti-infective therapies.

Indeed, the targeting of membrane lipids, particularly PI(4,5)P_2, by HDPs may be a universal mechanism in their antimicrobial and anticancer properties. As described in Chapter 2, a large number of human defense proteins were identified using computational searches for cationic motifs and PI(4,5)P_2 bead pull-down approaches. Notable candidates included a number of other β-defensins, liver-expressed antimicrobial peptide 2 (LEAP2), human θ-defensin 2 (retrocycin-2 or HTD-2), dermcidin, platelet basic protein, azurocidin, bactericidal/permeability increasing protein, human neutrophil defensin 1 (HNP-1), gasdermin A and S100-A7. While these proteins are well-known to display

broad-spectrum antimicrobial and immunomodulatory functions, their anticancer properties and PIP or P(4,5)P_2 binding, remains largely understudied. Nevertheless, based on the available literature, it is reasonable to propose that the interaction of these proteins with PI(4,5)P_2 is linked to anticancer/cell killing activities, particularly for gasdermin A (Ding *et al*, 2016) and HNP-1 (Lichtenstein *et al*, 1988; Hill *et al*, 1991; Lichtenstein, 1991; Wimley *et al*, 1994) as elaborated in Chapter 2. Therefore, these search results provide sound rationales for future studies to investigate the role of PI(4,5)P_2, not only in cancer cell killing, but also in their physiological functions. The additional investigation of these protein–PI(4,5)P_2 interactions may also identify novel PI(4,5)P_2-binding determinant(s) because none of the proteins identified by bead pull-down experiments appear to possess the cationic motifs proposed in this thesis. The diversity of candidates with provisional PI(4,5)P_2 binding provide a promising platform for therapeutic development.

Defining the molecular and structural basis of the HBD-3–PI(4,5)P_2 interaction may be useful for future engineering of potent anticancer PI(4,5)P_2-binding peptides. It will be interesting to determine why HBD-3 does not appear to oligomerise upon PI(4,5)P_2 binding, in contrast to the solanaceous plant defensins. Instead, HBD-3 appears to self-oligomerise upon increased local concentration (Chapter 3). The inability of HBD-3 to exhibit PI(4,5)P_2-dependent oligomerisation may correlate with the less effective membrane permeabilisation activity, and thus cancer cell killing compared to NaD1 and TPP3. Therefore, understanding the structural and molecular differences between these defensin–lipid interactions will contribute to PI(4,5)P_2-targeting therapeutic development.

Several lysine/arginine-rich clusters of proteins have been shown to confer the ability to penetrate the plasma membrane and gain cell entry to target cytoplasm. For instance, endocytosis-independent cell-penetrating peptides, such as the trans-activating transcriptional activation (Tat) of HIV-1, exploit these basic clusters to induce transient charged-based membrane destabilisation, to facilitate membrane insertion or to form a transient pore for diffusion through the plasma membrane (Herce & Garcia, 2007). Cell-penetrating peptides may represent a promising initiative for drug delivery by countering the issue of drug bioavailability (Munyendo *et al*, 2012). Intriguingly, HBD-3 internalisation into cancer cells, which resembles the cell penetration by Tat, is likely to be mediated through an endocytosis-independent mechanism and possibly involves self-oligomerisation upon local accumulation on the outer plasma membrane (Chapter 3).

Defining the mechanism of direct cell-penetrating activities would not only help answer the fundamental question of how HBD-3 and the plant defensins enter cancer cells, but also provide further information for the therapeutic usability of proteins that contain the $PI(4,5)P_2$-binding cationic motifs.

Another important aspect that remains to be examined is the inhibition of pathogen infection by existing and provisional $PI(4,5)P_2$-binding molecules. In Chapter 1, the importance of $PI(4,5)P_2$ and other PIPs in pathogen invasion, growth, replication and survival were extensively reviewed. Some of these pathogens, such as HIV-1, could be an excellent model for testing viral inhibition through $PI(4,5)P_2$ blocking. Such findings could broaden the therapeutic potential of $PI(4,5)P_2$ targeting for both cancer and infection, conveniently 'killing two birds with one stone'.

5.2.2 Receptor-independent cytokine induction by host defense peptides

As demonstrated in Chapter 4, HBD-3 can stimulate TNF and IL-6 release by direct interaction with $PI(4,5)P_2$ when administered at subacute doses to monocytes and macrophages. Importantly, this is the first HDP demonstrated to stimulate cytokine release independently of conventional cell surface receptors. It is proposed that HBD-3 is internalised and binds to $PI(4,5)P_2$ to promote PI3K activation, possibly via membrane reorganisation and lipid raft-associated effectors, that triggers Akt phosphorylation and NF-κB activation, ultimately leading to cytokine induction. Indeed, the induction of raft-like $PI(4,5)P_2$ clustering has been reported on multiple occasions, notably by Ebola virus protein VP40 to promote viral assembly process (Gc *et al*, 2016; Johnson *et al*, 2016). Furthermore, monosodium urate stimulates inflammatory responses via membrane rearrangement-associated enrichment of intracellular receptors, leading to the activation of effector kinases and eventually PI3Ks (Ng *et al*, 2008).

To further test this proposed model for HBD-3, biophysical studies need to be performed using membrane models and microscopy studies with fluorescent-labels, to assess the degree of $PI(4,5)P_2$ clustering as well as track the localisation of PI3Ks. Additional studies to assess the inhibition of raft formation on TNF and IL-6 induction should also be conducted, for example by using cholesterol synthesis inhibitors, as lipid rafts are accompanied with cholesterol. The intermediate effector molecule(s) that connect the $PI(4,5)P_2$ clustering and PI3K recruitment prior to complete Akt activation, also need to be defined. Together, these findings may provide an explanation for long-standing

observations of HBD-3 internalisation into leukocytes, as well as the absence or only partial inhibition of HBD-3-induced proinflammatory responses upon surface receptor blocking, as reported in many previous studies (Mburu *et al*, 2011; Semple *et al*, 2011; Mburu *et al*, 2012; Ferris *et al*, 2013). Moreover, as canonical receptor-independent proinflammatory modulation has also reported for human cathelicidin LL-37, it is intriguing to determine whether other HDPs, particularly those identified in Chapter 2 with putative PI(4,5)P_2 binding, also use this signalling mechanism.

5.2.3 Neamine core, a minimal structural requirement for PI(4,5)P_2 binding, as a promising prototype for chemical drug design

Neomycin and kanamycin have a strikingly similar neamine core and the 6'-amino group of ring I. In chapter 2, it was shown that neomycin and kanamycin bound to PI(4,5)P_2 and compete with other PI(4,5)P_2-binding molecules (such as NaD1) while other tested aminoglycosides with several differences in their neamine cores, did not. There was a strong correlation between PI(4,5)P_2 binding and anticancer activity, although further studies are required to fully define the PI(4,5)P_2-dependent mechanisms driving cancer cell killing. Indeed, determining the binding affinities and thermodynamic properties of interactions between PI(4,5)P_2 and aminoglycosides would provide important information for further development of PI(4,5)P_2-targeting aminoglycoside-based drugs. For example, thermodynamic and kinetic parameters of PI(4,5)P_2–aminoglycoside interactions can be acquired by thermal shift assays (microscale thermophoresis would be preferred over isothermal titration calorimetry for detection of binding between small molecules) and surface-based methods (dual-polarisation interferometry and quartz crystal microbalance) using PI(4,5)P_2-containing liposomes. Defining the basis of PI(4,5)P_2 selectivity would also be helpful for specific targeting, by comparing interaction between neomycin/kanamycin with PI(4,5)P_2 and other inositol phospholipids. Cell biological assays, including confocal laser scanning microscopy with fluorescent aminoglycosides and genetic disruption or inhibition of PI(4,5)P_2 (*e.g.* siRNA targeting PI(4,5)P_2-generating enzyme PIP5Ks) would also provide additional insights of PI(4,5)P_2 and aminoglycoside interactions in the anticancer activity of PI(4,5)P_2-targeting molecules.

Kanamycin, compared to neomycin, displays more pronounced selectivity against cancer cells (10-fold difference, see Chapter 2), which may be explained by molecular interactions mediated through additional undefined glycosyl substituent(s) outside the neamine core. Therefore, despite their present low potency, aminoglycosides reveal a

structural basis for further therapeutic development. This could involve manipulation of the 5- or 6-substituted residues in ring II whilst retaining the neamine core and its 6-amino group in ring I, in order to generate higher $PI(4,5)P_2$ binding affinity with greater potency and cancer cell specificity. Intriguingly, the neamine core is readily chemically modifiable and thus favourable for drug design (Chou *et al,* 2004; Konno *et al*, 2004). Indeed, it has been shown that neomycin derivatives via conjugation with arginine hexamers or with organometallic complexes can ameliorate its inhibitory effects, observable at low micromolar levels, against HIV entry by blocking receptor binding (Litovchick et al, 2001) and cancer viability via an undetermined mechanism (Grau-Campistany et al, 2013). Therefore, it should be possible to modify kanamycin and/or neomycin around their neamine core to increase $PI(4,5)P_2$ binding affinity, hence improve their $PI(4,5)P_2$-dependent effects.

One approach could involve designing new $PI(4,5)P_2$-binding aminoglycosides based on this information using fragment-based screening. Transitional metal-containing complexes could be promising conjugates, as their ability to form coordinate bonding with phosphate groups may enhance $PI(4,5)P_2$ binding. Furthermore, aminoglycosides can be versatilely conjugated with oligomers or small peptides. It may be possible to increase the $PI(4,5)P_2$-binding affinity of neomycin or kanamycin by conjugating them with known $PI(4,5)P_2$-binding motifs, such as the β2-β3 loop motif of NaD1 and other plant defensins.

Testing the ability of aminoglycoside-based molecules to inhibit $PI(4,5)P_2$-dependent events during viral infection is recommended. To study the link between viral inhibition and $PI(4,5)P_2$ sequestration, aminoglycosides should be tested on HIV-1 as a model, since $PI(4,5)P_2$ is well-characterised to be crucial for HIV assembly post-replication (Ono *et al*, 2004; Chan *et al*, 2008) These studies would involve establishing the infection of monocyte-derived macrophages or cultured T cells by particular HIV-1 strains prior to addition of neomycin, kanamycin, and any novel aminoglycoside derivatives generated as above. Time-course detection of intracellular and extracellular viral p24 capsid protein, could then be used as an indication of viral release. Other assembly factors, such as membrane localisation of Gag, could be examined as further evidence of defects in HIV egress. Electron microscopy will also be useful to visualise differences between infected cells upon aminoglycoside treatment.

5.2.4 Potential roles of PI(4,5)P_2 in other biological processes

The PI(4,5)P_2 bead pull-down experiments (described in Chapter 2) identified three key proteins of blood coagulation, namely fibrinogen, fibronectin and thrombospondin-1 from LPS-stimulated PBMC culture. Fibrin (derived from fibrinogen cleavage), fibronectin, and thrombospondin-1 co-polymerise to provide a physical framework for clot formation and to mediate platelet aggregation (Chow *et al*, 1983; Bale *et al*, 1985; Delvaeye & Conway, 2009). The association of PI(4,5)P_2 with polymerising fibrin, suggested over two decades ago (Vickers *et al*, 1987), has been neglected. The detection of these matrix proteins may have revived the implication of PI(4,5)P_2 in the progression of clot formation, particularly as a defense mechanism to entrap invading microorganisms. PI(4,5)P_2 might directly bind and induce the co-polymerisation of clot matrix proteins, as shown for PI(4,5)P_2-induced oligomerisation and/or fibril formation of the fibroblast growth factor 2 (Steringer *et al*, 2012), MLKL (Dondelinger *et al*, 2014), solanaceous plant defensins (Poon *et al*, 2014; Baxter *et al*, 2015b) and amyloid β (Knight & Miranker, 2004). Biophysical studies, electron microscopy and X-ray crystallography approaches could be employed to determine and visualise the effect of PI(4,5)P_2 on fibrin polymerisation. *In vitro* and *in vivo* detection of fibrin polymers or blood coagulation, with and without PI(4,5)P_2 sequestering agents, in response to pathogen stimuli would provide insight into the importance of PI(4,5)P_2 in blood coagulation. These future studies will rationalise the potential use of PI(4,5)P_2-binding therapeutics as anticoagulating agents in blood clotting-related diseases.

5.3 Concluding remarks

The work presented in this thesis describes the first demonstration of PIP-targeting, particularly PI(4,5)P_2, by a human HDP (HBD-3) to selectively induce cancer cell membrane permeabilisation at low micromolar concentrations. The interaction of HBD-3 with PI(4,5)P_2 was also shown to initiate a proinflammatory PI3K-Akt-NF-κB signalling cascade in antigen presenting cells (**Figure 5.1**). These findings contribute to our understanding of multi-armed defense mechanisms used in host innate immunity in response to pathogens and altered self, such as cancer cells. They also represent a proof of concept for PIPs as multi-faceted therapeutic targets, providing a starting platform for development of PIP-targeting therapeutics based on aminoglycoside antibiotics as well as an arsenal of potential candidate peptides identified herein.

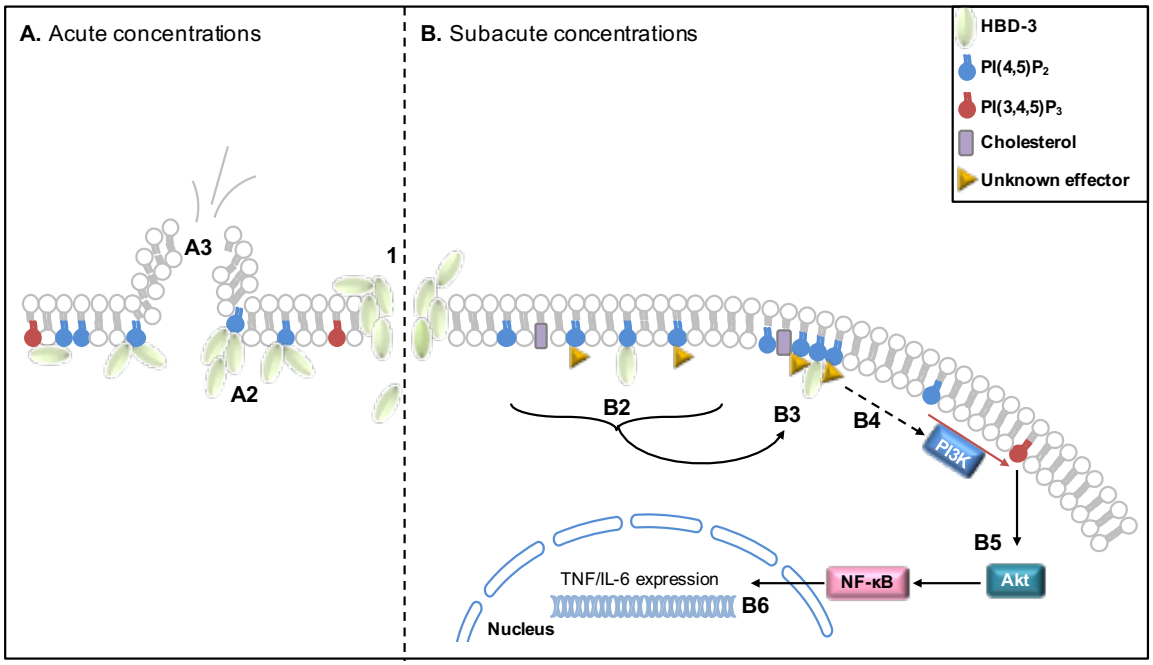

Figure 5.1 Proposed modes of action for HBD-3 at (A) acute and (B) subacute concentrations

HBD-3 electrostatically interacts and accumulates on outer plasma membrane. The increase in local HBD-3 concentration may promote its self-oligomerisation, forming a transient pore allowing its penetration across the plasma membrane **(1)**. Once internalised cancer cells at acute concentration, (**A2**) HBD-3 binds onto $PI(4,5)P_2$ of the inner membrane leaflet and (**A3**) rapidly causes membrane blebbing and permeabilisation, ultimately inducing cell lysis. Alternatively, upon its entry into antigen presenting cells, at subacute concentration, (**B2**) $PI(4,5)P_2$ binding by HBD-3 may lead to membrane rearrangement and $PI(4,5)P_2$ clustering. (**B3**) This may cause activation of unknown raft-associated effector(s) upstream of PI3K, (**B4**) eventually resulting in PI3K activation. (**B5**) Activated PI3K induces the PI3K-Akt signalling cascade, leading to activation and (**B6**) nuclear translocation of NF-κB to induce TNF and IL-6 expression.

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