University of Strathclyde

Strathclyde Institute of Pharmacy and Biomedical Sciences

The role of individual Protein Kinase C isoforms in mast cell function and their targeting by the immunomodulatory helminth product, ES-62

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A thesis presented in the fulfilment of the requirements for the degree of Doctor of Philosophy

2013

Declaration

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Acknowledgments

Firstly I would like to extend my thanks and gratitude to my supervisor, Professor William Harnett and my second supervisor, Professor Margaret Harnett for their invaluable help, guidance and support over the past 4 years - without them I would not be where I am today.

I also thank SULSA and Strathclyde Institute for Pharmacy and Biomedical Sciences for proving the funding and opportunity to undertake this project.

I would also like to extend my thanks to members of both Harnett laboratory groups (Strathclyde and Glasgow University) for their scientific advice, knowledge and many insightful discussions and suggestions. In particular, I would like to mention members and friends past and present from my research group; Justyna Rzepecka, Lamyaa Al-Riyami, Ivonne Siebeke, Michelle Coates and Felicity Lumb. These colleagues were my primary resource for answering scientific questions (basic or otherwise), discussing ideas and techniques or just generally supplying me with chocolate and conversation when I was having a challenging day. I would also like to thank my fellow PhD students and friends; Jenny Crowe, Rhona Galloway, Nicola McGinely, Debbie Allan and Stuart Woods, and additionally to members of other research groups on the fifth floor. The key to success and sanity throughout your PhD is having friends and colleagues you can turn to for support and advice. I would like to think we have all at some point been there for each other throughout our journeys in the lab. The second most important thing in surviving a PhD is cake - it helps that we have a lot of bakers on our floor!

I also thank my old friends outside of the science world (too many to mention here but you know who you are) for providing the support, love and friendship that I needed during my time here. A special thanks to my childhood friend Steph, she is and always will be my soul mate.

In particular I would like to thank my incredible family. I am lucky to have the most supportive parents in the world, Andrew and Debbie Bell (best not use her birth name she doesn't like that), who have sacrificed so much for my brother and I in order to give us a better life. One of my favourite quotes is from 'The Wedding' by Nicholas Sparks:

"What it's like to be a parent: It's one of the hardest things you'll ever do but in exchange it teaches you the meaning of unconditional love."

My parents are the definition of this. I know I would not have made it this far without them and for that I am eternally thankful. They provided me with constant love and encouragement (and financial aid) and were always there for me when I needed them – even if that was just to be my taxi driver when I needed a night off and a cocktail or two! My beloved brother Ryan, sister in-law Katie and my gorgeous nephew Aaryn – I know thanks to you that I can always rely on my family when things are tough and I am thankful for all the support and understanding especially over the past few months. You mean the world to me. I would also like to add special thanks to the new members of my family – Anne (mum number 2), Daryl and Tracy.

Last, but certainly not least, I would like to thank my best friend, soul-mate and future husband Ross. He appreciates my quirkiness, my sense of humour and has learned to adapt quickly to my new personality traits that have popped up along the way throughout the writing process and final stages! Thank you for helping me to remain calm when it feels my world is collapsing around me - you always find me when I am lost. You have helped me find strength and confidence I never knew I had and provided me with your own when I had none. You have given me faith and reassurance and taught me how to dig that little bit deeper to come out the other side, "Small sacrifices result in big gains". Nothing that's worthwhile is ever easy. I thank you for everything you do for me, for us and for our future together. No matter what happens to us in the future, every day we spend together is the greatest day of my life. I love you and I will always be yours, forever.

Abstract

ES-62, a glycoprotein secreted by the filarial nematode *Acanthocheilonema viteae*, is immunomodulatory via subversion of signal transduction pathways operating in various immune system cells. With respect to human bone-marrow derived mast cells (BMMC), ES-62 has previously been shown to inhibit FcεRI-mediated degranulation by forming a complex with TLR4 to sequester PKC-α away from the plasma membrane, resulting in its degradation. An intriguing additional finding was that ES-62 reduced levels of other PKC isoforms, namely PKC-β, -δ, -ι and –ζ. This project is concerned with establishing if PKC isoforms targeted by ES-62 are critical for BMMC functional responses and if so, whether the absence of any such isoform impacts on ES-62-mediated inhibition of mast cell responses. To establish the role, which each PKC isoform plays in mast cell function, PKC isoform knockout (KO) mice were employed. Simultaneously other types of immune system cell, namely macrophages and dendritic cells were explored, to establish whether any effects observed are mast cell-specific. The data obtained with mast cells indicate that control of pro-inflammatory cytokine production is possibly mediated by a partnership between one conventional and one novel isoform with PKC-α and more importantly, PKC-θ, acting as positive regulators of IL-6 and TNF-α production while on the other hand PKC-β and -ε act as negative regulators of IL-6 production. Although the loss of any one PKC isoform had no clear detrimental effects on ES-62 activity, the absence of PKC-θ may possibly dampen the nematode product's modulatory ability. The utilization of PKC-α, -β and -θ is specific for BMMC functional responses in comparison to macrophages whereby only PKC-ε was

revealed as a positive regulator of IL-6. Interestingly however, PKC-α also appears to be an ES-62 target in macrophages. With respect to dendritic cells, in contrast to antigen-stimulated BMMC, PKC-α negatively regulates LPS-induced IL-6 and TNF-α.

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List of Abbreviations

µg	micro gram
µl	micro litre
µM	micro molar
˚C	degree centigrade
AAM	alternatively activated macrophages
Ab	antibody
AD	atopic dermatitis
Ag	antigen
APC	antigen presenting cells
Av-17	*A. viteae*-derived cystatin
BAL	broncho-alveolar lavage fluid
BCR	B-cell receptor
BMDC	bone marrow-derived dendritic cell
BMM	bone marrow-derived macrophage
BMMC	bone marrow-derived mast cell
BR	blocking reagent

BSA	bovine serum albumin
Ca	calcium
$CaCl_2$	calcium chloride
CCR	C-C chemokine receptor
CD	cluster of differentiation
CTLA-4	cytotoxic T lymphocyte antigen-4
DAG	diacylglycerol
DC	dendritic cell
dH_2O	distilled water
DMAE	dimethylethanolamine
DNP	dinitrophenol
EAE	experimental autoimmune encephalitis
EGF	epidermal growth factor
FcεRI	Fc receptor for IgE
FITC	fluorescein isothiocyanate
Foxp3	forkhead box P3
FSC	forward scatter

g	gravity
GDP	guanine diphosphate
GTP	guanine triphosphate
GM-CSF	granulocyte-macrophage colony stimulating factor
gm	gram
H_20_2	hydrogen peroxide
H_2S0_4	sulphuric acid
HCL	hydrochloric acid
HEL	hen egg lysosome
hr/s	hour/s
HRP	horseradish peroxidase
HSA	human serum albumin
IBD	inflammatory bowel disease
IFN-γ	interferon gamma
IgE	immunoglobulin E
IL	interleukin

iNOS	inducible nitroc oxide synthase
IP_3	inositol 1, 4, 5-triphosphate
ITAM	immunoreceptor tyrosine based activation motif
IU	international units
JNK	c-Jun N-terminal kinase
KCL	potassium chloride
KO	knockout
LPS	lipopolysaccharide
LSC	laser scanning cytometry
LTB4	leukotriene B4
LTC4	leukotriene C4
M	molar
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MBP	major basic protein
MCP	monocyte chemotactic protein
MEM	minimum essential medium

Mf	microfilaria
MFI	mean fluorescence intensity
mg	milligram
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
min	minutes/s
MIP	macrophage inflammatory protein
ml	millilitre
mM	millimolar
MyD88	myeloid differentiaton primary response gene 88
N	normal
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NF-κB	nuclear factor kappa-light chain
ng	nano grams

NGF	nerve growth factor
NK cells	natural killer cells
nm	nano metre
nM	nano molar
NOD	non-obese diabetic
NO	nitric oxide
OVA	ovalbumin
OvAg	antigen of *O. vovulus*
PAF	platelet activating factor
PAS	protein of *A. suum*
PBMC	peripheral blood mononuclear cells
PC	phosphorylcholine
PEC	peritoneal cells
PE	phycoerythrin
pg	pico gram
PGD2	prostaglandin D2
PGDF	platelet derived growth factor

PI	phosphatidylinositol
PIP_2	phosphatidylinositol -4, 5 biphosphate
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PLP	proteolipid protein
PMA	phorbol myristate acetate
PS	phosphatidylserine
ps	pseudosubstrate
PtdCho	phosphatidylcholine
RBL	rat basophilic cell line
RPMI	Roswell Park Memorial Institute medium
SAv	streptavidin
SCF	stem cell factor
SCID	severe combined immunodeficiency disease
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

SHIP	Src homology region 2 domain-containing phosphatase
siRNA	short interfering RNA
SPHK	sphingosine kinase
SSC	side scatter
T1D	type 1 diabetes
TCR	T cell receptor
Tg	transgenic
TGF-β	transforming growth factor beta
Th	T helper
T1R	Toll/Interleukin-1 receptor
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor-α
Treg	T regulatory cell
v/v	volume/volume
v/w	volume/weight
VEGF	vascular endothelial growth factor
w/w	weight /weight

XL	cross-linking
ZAP-70	zeta chain associated protein kinase

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Chapter 1

Introduction

1.1 The Hygiene Hypothesis – the dirt of it all

The 'Hygiene Hypothesis' suggests that the increase in the incidence of allergic and autoimmune diseases such as asthma, hay fever, atopic dermatitis, inflammatory bowel disease (IBD) and type 1 diabetes (T1D) is strongly correlated with a decline in infections during childhood. Strachan was the first to propose this novel link between allergy and infectious agents when he observed an inverse relationship between family size and the development of allergic disease. He observed that hay fever (an allergic disease) was far less common in children from larger families, where presumably these children were more frequently exposed to infectious agents through unhygienic contact with their siblings, than in children from smaller families (Strachan, 1989). Strachan's explanation for the rise in allergic diseases was not only reliant on the idea that fewer siblings resulted in reduced exposure to microbes but it was also attributed to smaller families having improved sanitation, better hygiene and a higher standard of living (Strachan, 1989). Thus, the "Hygiene hypothesis" was born. Initially, the immunological basis of this hypothesis was built on the idea that there was an imbalance between T helper 1 (Th1) and T helper 2 (Th2) responses. Allergic diseases develop when the body responds inappropriately to an otherwise harmless antigen and this inappropriate immune response is driven primarily by Th2 responses. Thus, the balancing of Th1/Th2 immune responses was reliant on the ability of infections such as bacteria and viruses to induce a Th1-mediated immune response which would in turn counteract the over production of antigen-specific Th2 cells in order to maintain a well-regulated immune response (Yazdanbakhsh, et al. 2002; Matricardi & Bonini, 2000; Romagnani,1992). However, this proposition did not take into account two other key observations. Firstly, the incidence of several

Th1-mediated autoimmune diseases such as T1D was increasing in parallel to the rise of allergic diseases, which are associated with an overactive Th2 response. Secondly, allergic diseases are far less predominant in areas such as the tropics where there is an increased infection rate with helminths with such populations displaying strong Th2 responses similar to those witnessed in atopic individuals (Wilson & Maizels, 2004). Therefore, the Th1/Th2 balancing theory where the increase in allergic diseases is attributed to a shift towards pro-allergic Th2 responses through lack of negative regulation by Th1 responses induced by infections is rather controversial and has been shown to be contradictory as both bacterial (Th1 associated) and parasitic (Th2 associated) infections have been found to reduce atopic disease (Wilson & Maizels, 2004; Herz, et al. 2000). A prime example of an infectious agent being able to down-regulate allergic manifestations has been witnessed in a study in Japanese children where positive tuberculin responders were shown to have higher levels of the Th1 cytokine Interferon-γ (IFN- γ) and reduced Th2 cytokines such as IL-4 and IL-13 in addition to lower symptoms of allergic disease (Shirakawa, et al. 1997). On the other hand, children with atopic asthma present larger quantities of IFN- γ in their airways (Brown, et al. 2003). This unusual abundance of a Th1 cytokine in a predominantly Th2-based disease has also been witnessed in atopic dermatitis (Klunker, et al. 2003). These data suggest that this Th1-associated cytokine may act in some cases in conjunction with Th2 responses to maintain exacerbated inflammatory responses in allergic disease rather than help to restrain the overactive Th2 response. Correspondingly, there is evidence that Th1-inducing infections amplify the severity or even directly cause allergy and auto-immune diseases (Kamradt, et al. 2005). For instance, toxins derived from

Staphylococcus aureus known as superantigens (SAs) have been shown to promote disease progression in atopic dermatitis most likely through SA-induced T cell activation (Bunikowski, et al. 2000; Skov, et al. 2000). However, despite such evidence, there are studies which show infections such as *Mycobacterium tuberculosis* can actually prevent the development of atopic diseases (Shirakawa, et al. 1997; von Mutius, et al. 2000; Herz, et al. 2000). Similar protective roles have been observed for parasitic infections in allergic responses. An example of this relationship came from a study by Van de Biggelaar where Gabonese children chronically infected with schistosomes had lower prevalence of skin reactivity to house dust mite than those free of the parasite (Van de Biggelaar, et al. 2000). It seems clear from such examples that the immunological basis of the hygiene hypothesis goes far beyond this Th1/ Th2 balancing act. Instead, it seems likely that the increase in allergic and inflammatory disorders is due to an imbalance between effector cells, whether they are Th1 or Th2 biased, and regulatory cells. Thus, suppression of such aberrant immune responses witnessed in atopic and inflammatory disease is reliant on the induction of a regulatory network that is able to control effector cells through such mechanisms as regulatory cells e.g. T regulatory cells (T Regs) and anti-inflammatory/regulatory cytokines like IL-10 and TGF-β (Yazdanbakhsh, et al. 2002).

More recently, the original formulation of the hygiene hypothesis has been updated to explain the epidemic of inflammatory diseases in evolutionistic terms. Such an explanation suggests that it is the microorganisms that have long been associated with humans when the immune system was evolving (i.e. hunter-gatherer times) and not those more recently evolved or associated with common childhood infections

(e.g. colds, measles, etc.) that are responsible for modulating the development of a properly functioning immune system, however our modernised lifestyle deprives such contact with these organisms (Okada, et al. 2010; Rook, 2008). In conjunction with this, Graham Rook has thus proposed his 'Old friends' hypothesis (Rook, 2008; 2010), which may be considered a strong contender for the observations underlying the explanation of the hygiene hypothesis. This theory suggests that environmental organisms which co-evolved with humans, like helminths and saprophytes such as mycobacteria, stimulate background immunoregulation within the host. These organisms are found in contaminated food, water and soil consequently resulting in them being in constant interaction with humans. This continuous encounter with such groups of organisms drove a need for them to be tolerated by the immune system as a means of protecting the host from damage (Rook, 2008 and 2010). However, the changes of lifestyle in industrialized countries have led to the depletion of such organisms from our environment and perhaps led to immunoregulatory defects, which then cause the associated increase in inflammatory diseases.

Leading on from this interesting adaptation of the hygiene hypothesis, there has been particular focus in recent years on helminthic parasites and their potential ability to diminish allergy prevalence. It has been recognized that there is an inverse worldwide distribution between helminths and allergic diseases: in developing countries, where allergy prevalence is considered low, there is a high infection rate with helminths, whereas allergy and also autoimmune diseases are a growing problem in the developed world (Cooper, 2009; Ruyssers, et al. 2008). This view is supported by many studies including one showing that active infection with common childhood geohelminths such as *Ascaris lumbricoides* or *Ancylostoma duodenale* was

protective against the development of allergen skin test reactivity (Cooper et al, 2002). A similar negative correlation between allergen skin test reactivity and geohelminth infection was found in Vietnamese children who were infected with hookworms (Flohr, et al. 2006). Likewise, schistosomiasis has been shown to have protective properties against atopy in African children (van den Biggelaar, et al. 2000). In parallel to these data, eradication of helminth infection with anti-helminthic treatment has been shown to intensify allergic disease (Lynch, et al. 1993; Flohr, et al. 2006). However, it is surprising that this protective role against hyper-inflammation connected with atopic and also autoimmune diseases is not always consistent. For example, there was no clinical improvement of asthma following deworming treatment of a small cohort of adults and children in a study in Venezuela (Lynch, et al. 1997). On the other hand, it cannot be ignored that worms have provided great benefits for patients in clinical trials with the pig parasite *Trichuris suis* significantly improving the severity of the inflammatory bowel disease ulcerative colitis (Summers, et al. 2005a). Similar success with this helminth was witnessed in patients suffering from Crohn's disease (Summers, et al. 2005b). Table 1.1 Summarises observations in humans with respect to allergic and inflammatory responses in the context of helminth infection.

With helminth infections stereotypically inducing strong Th2 responses (Tawil, et al. 2004), it would be natural to assume that such infection would directly promote atopy perhaps through the increased production of IL-4 stimulating Th2 cells, which in turn can directly or indirectly cause the physiological manifestations of allergic disease. Nevertheless, despite helminth infections and allergic diseases sharing a common immunological phenotype, the clinical outcome is clearly not the same (Fig

1.1). Th2 responses initiate and dominate during allergic diseases leading to pathology, most likely due to inadequate immunoregulatory mechanisms. However the Th2 response is often associated with protection for the host against helminth infections although this aberrant immune response is in turn dampened to prevent pathology (Carvalho et al, 2006), emphasising that helminths are not associated with allergy. Thus, although both helminths and allergy stimulate an almost identical immune response, there are important differences with the most paramount being that helminths (in contrast to allergic responses) also induce a strong anti-inflammatory regulatory response (Maizels et al, 2004), which could be responsible for down-modulating allergic responses.

Allergic inflammation is greatly reduced in the presence of chronic infection with helminths; the parasites are able to modulate an excessive inflammatory response via production of regulatory T cells (Tregs) and anti-inflammatory cytokines IL-10 and TGF-β (Flohr, et al. 2008; Cooper, 2002). More specifically, IL-10 appears to be a key cytokine involved in inhibiting the inflammatory Th2 response associated with atopy as asthma-suffering patients infected with *Schistosoma mansoni* show elevated levels of IL-10 in response to stimulation with the *D. pteronyssinus* antigen 1 when compared to helminth-free patients, with anti-helminthic treatment down-modulating this allergen-specific IL-10 production (Araujo, et al. 2004). Taken together, these results suggest that it is perhaps the control of inflammatory responses through IL-10 and/or TGF-β secreted by Tregs that is more beneficial in down-modulating allergic diseases. This hypothesis is currently the most favoured mechanism by which helminths reduce allergic responses (Maizels, 2005). Conversely, recent data using the filarial nematode derived glycoprotein, ES-62, have reconsidered the idea that

equilibrium of immune responses can be established by counter-regulating the hyperactive Th2 responses associated with allergic phenotype with a Th1-associated response (Rzepecka, et al. 2013). This modulation of OVA-induced airway inflammation was not attributed to the induction of a regulatory network involving increased induction of Treg cells and anti-inflammatory cytokines but rather identified a Th1-like phenotype identifiable by increased IFN-γ production and expression of the Th1-associated transcription factor, Tbet. Thus ES-62 was able to protect against Th2/Th17-associated pathology and airway inflammation by re-establishing a balance between the Th1 and Th2 responses (Rzepecka, et al. 2013).

Table1.1 Correlation between helminth infection and allergic and inflammatory responses.

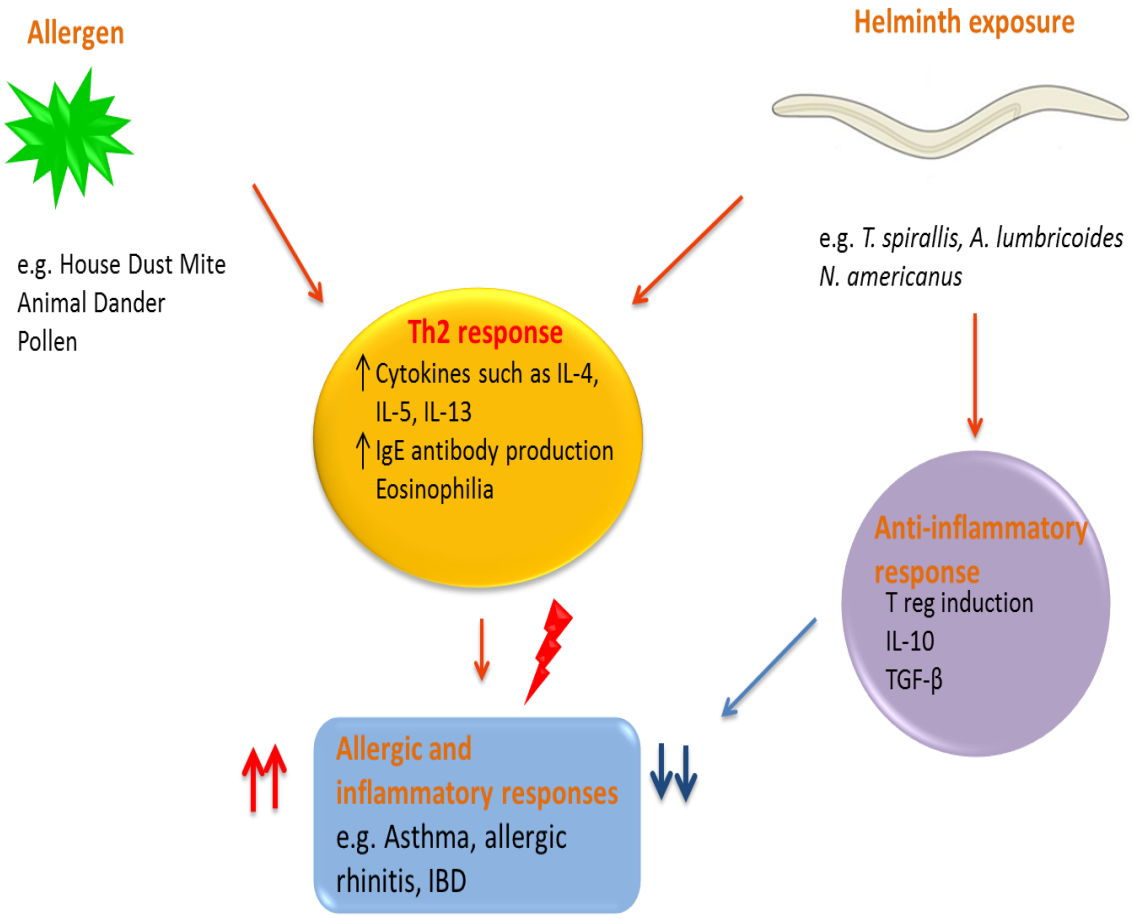
Protective responses induced by helminth infection	**Observation**
Ascaris lumbricoides, Necator americanus **(Selassie, et al. 2000)**	Inverse correlation between incidence of asthma and infection
A. lumbricoides, T. trichiura **(van de Biggelaer, et al. 2004)**	Increased skin reactivity to house dust mites with anti-helminthic treatment
Trichuris suis **(Summers, et al. 2005)**	Protection from IBD
Intestinal parasites such as Hookworm **(Scrivener, et al. 2001)**	Reduced wheezing

Inducing or exacerbating responses by helminth infection	**Observation**
Anisakis simplex **(Audicana & Kennedy, 2008)**	Severe allergic reaction following ingestion of fish contaminated with *A. simplex*
Ascaris spp. **(Dold, et al. 1998)**	Enhances allergic responses. e.g. higher anti-*Ascaris* IgE levels increased asthma

(Adapted from Harnett & Harnett. 2008 and Erb, 2009)

Figure 1.1. Helminths can enhance and reduce allergic responses

Possible mechanism of how helminth infection can both enhance and reduce allergic and inflammatory responses. Allergens induce the production of a potent Th2 response which triggers and exacerbates allergic and inflammatory responses. Helminths are also known to stimulate a strong Th2 response within a host. However, helminth infections tend to be long-lived with evidence suggesting that they are sustained through a parasite-induced immunomodulatory network resulting in an anti-inflammatory response through such actions as inducing the activation of regulatory T cells (Tregs) and the production of anti-inflammatory cytokines. This, in turn, may have a down-regulatory effect on allergic inflammation


Allergen
e.g. House Dust Mite
Animal Dander
Pollen
Helminth exposure
e.g. T. spirallis, A. lumbricoides
N. americanus
Th2 response
↑ Cytokines such as IL-4, IL-5, IL-13
↑ IgE antibody production
Eosinophilia
Anti-inflammatory response
T reg induction
IL-10
TGF-β
Allergic and inflammatory responses
e.g. Asthma, allergic rhinitis, IBD

1.2 Helminths and the Immune Response

Helminth infection is extremely common affecting more than a billion people worldwide (WHO fact sheet No 366, June 2012; Hotez, et al. 2008). Adult worms can live in humans in either the tissues or the gastrointestinal tract. There are many different types of helminths but the most common infecting humans are the soil-transmitted gastrointestinal nematodes such as *A. lumbricoides* (roundworm), *Trichuris trichiura* (whipworm) and the hookworms *Necator americanus* and *A. duodenale*. This is closely followed by infection with schistosomes which burden over 200 million individuals living in tropical and subtropical regions (Hotez et al, 2008). Due to many nematode infections being endemic in one area, it is not uncommon for individuals to harbour more than one of these types of parasite (Brooker, et al. 2006). Parasitic worms display a broad range of characteristics ranging from their complex life cycles, which may involve many hosts, and their mode of transmission such as vector-borne, faecal-oral or transdermal route to the different organs affected by each species (Jackson, et al. 2008). They produce chronic, debilitating infections within humans that are usually non-fatal but have a considerable degree of morbidity. At the present time, there are no vaccines available and little progress has been made towards new effective immunotherapies. Therefore, understanding how helminths interact with the host immune system is imperative to allowing us to make advances towards a new anti-helminthic treatment.

Generally, infection with helminths is characterized by a strong T helper (Th) 2 response. This classical Th2 immune response is broadly characterized by high production of the immunoglobulin IgE, activation of mast cells, basophils and

eosinophils and the production of Th2 associated cytokines such as IL-4, IL-5 and IL-13 (Gause, et al. 2003; Pearce, et al. 2004). The Th2-type environment created by helminths is predominantly linked to protection whereas the induction of a Th1 response is often associated with chronic infection (Else & Finkelman, 1998). However, since each parasite may occupy a specific niche within the host, it is not surprising that each nematode species triggers a selective immune response that may be to some extent be different from that induced by others. Therefore, the development of an appropriate immune response is essential to the outcome of the infection.

In particular, gastrointestinal nematodes are strong inducers of this stereotypical host-protective Th2 immune response (Gause et al, 2003; Finkelman et al, 1997). The cytokine IL-4 directs differentiation of naïve helper T cells (Th0) towards Th2 cells therefore playing a key role in responses against parasitic worm infections. It is known that there is a common constituent of the IL-4 receptor (IL-4R) known as the IL-4 receptor α-chain (IL-4R α) which not only binds IL-4 but can also bind its closely related Th2 cytokine, IL-13 (Callard, et al. 1996). Signal transducer and activator of transcription 6 (STAT6) has been shown to have a central role in IL-4R signalling (Takeda, et al. 1996) and in turn, this signalling pathway has been shown to be fundamental for both IL-13 and IL-4-induced Th2 cell differentiation (Kaplan, et al. 1996). In addition, STAT6 has been publicised to have fundamental effects on Th2 linked functional responses to gut nematodes (Takeda, et al. 1996). Thus, studies have shown that IL-4 is largely responsible for mediating host protection against intestinal nematode infection. Some of the clearest evidence for this has come from *Trichuris muris* and *Heligomosomoides polygyrus* models. For instance, Else and co-

workers (Else, et al. 1994) showed in the mouse-model of *T. muris* that abrogation of IL-4 activity in normally resistant mice with a monoclonal antibody against the IL-4 receptor, which blocks both the IL-13 and IL-4 signalling, abolishes host-mediated protection. The resulting effect was the establishment of a chronic infection, which was characterized by up-regulation of parameters associated with a Th1 response, namely IFN-γ and IgG2a production, along with dampened Th2-related responses such as IgE and IgG1 production in addition to mastocytosis (Else et al, 1994). Similarly, ablation of IFN-γ production promoted larvae expulsion before they could develop into fecund adults. This result correlated with reduced Th1-indicative antibody responses, namely parasite-specific IgG2a (Else et al, 1994). This evidence proposes that with regards to *T. muris*, the outcome of infection is largely reliant on a balance between an important Th2-associated cytokine IL-4, and IFN-γ. Thus, a Th2-biased profile is correlated with protection and a Th1-type response results in the development of chronic infection (Else et al, 1994). In correlation to this, the effects of IL-4 on resistance to host infection have also been demonstrated in *H. polygyrus* in a similar manner to the *T. muris* model. Again, ablation of both IL-4 and the IL-4 receptor eradicated host-protective mechanisms (Urban et al, 1991). Data from IL-4 knockout mice also support the role of this Th-2 indicative parameter in resistance to infection with *H. polygyrus* (Finkelman, et al. 1997). Similar experiments have deemed IL-4 important for the outcome of infection with *Trichinella spiralis* where once more this cytokine fuels protection for the host (Finkelman, et al. 1997). Another observation, which has been shared particularly between *T. muris* and *H. polygyrus* infection is that administration of a prolonged IL-4 complex (IL-4C) facilitated worm expulsion where normally there would be development of chronic

infection (Urban, et al. 1995 and Else, et al. 1994). Interestingly, immunodeficient SCID mice, which lack both B and T cells, were also able to terminate *H. polygyrus* infection following IL-4 treatment indicating that the eviction of worms from the host's intestines is perhaps mediated through non-immune cells (Urban, et al. 1995). On the other hand, *T. muris* infected nude mice administered IL-4 complexes are still unsuccessful at expelling worms (Else, et al. 1994) suggesting the involvement of another cytokine. Thus, although it is obvious from such data that IL-4 is almost imperative for protective immune responses against gut nematode parasites, more evidence is emerging for the role of its closely related counterpart IL-13 in resistance to infections. So far, the function of IL-13 in nematode infection is not entirely resolved, as it appears important in some nematode infections whereas in others its role is far less clear (Else & Finkelman, 1998). For example, IL-13, equivalent to IL-4, has now been shown to be an important cytokine for protection against *T. muris* infection. This is illustrated by IL-13 deficient mice being unable to mount a protective response against *T. muris* infection whereas wild-type controls were able to evict the parasite (Bancroft, et al. 1998). Remarkably, these findings coincide with Else and colleagues previous sightings with nude mice which suggested that IL-4 alone could not confer protection (Else, et al. 1994). Therefore, in *T. muris* infection, both cytokines display a significant role in resistance. Moreover, in the mouse-adapted parasite *N. brasiliensis*, the role of IL-4 in host-protective immunity appears quite complex while IL-13 has been highlighted to have a more dominant role. Firstly, Urban and associates showed that administration of IL-4 to SCID mice facilitated eviction of the parasite from the gut (Urban, et al. 1995). However, further observations in IL-4 knockout mice indicated that surprisingly IL-4 was not

paramount for effective worm expulsion (Urban, et al. 1998). On the other hand, IL-4Rα and STAT6-deficient mice were also largely unsuccessful in expelling *N. brasiliensis* from the host (Urban, et al. 1998). At this point in time, only IL-4 and IL-13 are known to activate STAT6 via IL-4Rα. Therefore, these observations pointed towards IL-13, rather than IL-4, as the key cytokine involved in resisting infection. Indeed, treating *N. brasiliensis*-infected Balb/c mice with a soluble IL-13Rα_2-Fc fusion protein, which obliterates IL-13, radically diminished worm expulsion and egg production (Urban, et al. 1998). Intriguingly, IL-4 knockout mice treated with this neutralizing IL-13 antagonist displayed even higher numbers of adult parasites and greater egg production. Taken together, these observations indicated that IL-4 and IL-13 promote host immunity to *N. brasiliensis* through an IL-4Rα activated, STAT6-dependent mechanism with IL-13 being the more important cytokine (Urban, et al. 1998). Most recently, it has been shown that this protective effect is mediated through effects on non-bone marrow-derived cells such as those found in the gut epithelium and it may include intestinal smooth muscle contractility and an increase in intestinal mucus secretion. These studies showed that *N. brasiliensis* eviction from the gut was promoted in mice which expressed IL-4Rα but most importantly, in mice where the IL-4Rα was expressed on non-bone marrow-derived cells (Urban, et al. 2001). Collectively, these data illustrate that there are opposing amounts of dependence upon the Th2 cytokine IL-4 and that in some nematodes, IL-13 can compensate for IL-4 in immunity to gut helminths. Overall, the fact that these gastrointestinal nematodes have varying needs on both cytokines suggests that these two cytokines have the ability to functionally overlap due to the shared signalling through IL-4Rα.

It is not surprising that mast cells have been implicated in the control or expulsion of nematode parasites due to the classical Th2-associated parameters induced by helminths such as mastocytosis, eosinophilia and increased IgE production. Th2 cytokines such as IL-4, IL-13, IL-3 and IL-9 can influence mast cells, epithelial cells and goblet cells to stimulate physiological processes in the gut such as mucus production, smooth muscle cell contraction, gut permeability and fluid secretion resulting in the expulsion of the parasite from the gut (Maizels & Holland, 1998; Urban, et al. 1998; Vallance & Collins, 1998). However, there are different methods of expulsion to which certain species are susceptible. For example, mast cells are in fact not essential for resistance to *N. brasiliensis* infection. This is shown by *c-kit* mutant W/W^v mice, which are severely deficient in mast cells, retaining their ability to expel the parasite (Crowle & Reed, 1981). Instead, expulsion is mediated through IL-4 and IL-13 stimulated goblet cell hyperplasia which in turn increases mucus production trapping the worms in the gut lumen (Maizels & Holland, 1998). Other mechanisms such as amplified net fluid secretion as well as a decrease in size of the intestinal villi help to effectively eliminate the parasite from the gut lumen (Maizels & Holland, 1998). Similarly, experiments which block IL-3 therefore partially reducing mastocytosis in the gut, along with antibody-induced abolishment of the *c-kit* receptor, which completely diminishes mast cells, showcase that mast cells do not underpin resistance to *T. muris* infection (Else & Finkelman, 1998; Betts & Else, 1999). On the other hand, a prominent role is shown for mast cells in other nematode infections. In particular, strong evidence exists for mast cell-mediated resistance to *T. spiralis*. Constitutive overexpression of IL-9 in transgenic mice enhances Th2-associated responses and enables rapid eviction of *T. spiralis* from the host

(Faulkner, et al. 1997). In parallel, abolishment of the mast cell development-dependent receptor *c-kit* with a monoclonal antibody results in hindered mucosal mastocytosis as well as worm expulsion (Faulkner, et al. 1997). In addition, hosts-protective expulsion mechanisms for the *Strongyloides* species have an absolute requirement for mast cells (Maizels & Holland, 1998). This has been exemplified in the gut parasite *Strongyloides venezuelensis* where eviction was severely postponed in IL-3-deficient mice (Lantz, et al. 1998).

The cytokine IL-5 is crucial for eosinophilia displayed during helminth infections. Despite this, these immune cells appear to have no role in host protection from nematode parasites. Consequently, antibody-mediated neutralisation of IL-5, which reduces eosinophils, has no effect on the levels of gut nematodes such as *T. muris* (Betts & Else, 1999), *T.spiralis* (Herndon, et al. 1992) or *N. brasiliensis* (Coffman, et al. 1989). This non-protective role of eosinophils has also been witnessed in schistosomes (Abbas, et al. 1996). On the contrary, eosinophils have been shown to have protective effects in some tissue-dwelling helminths such as *Brugia pahangi* (Ramalingam, et al. 2005) and *Fasciola hepatica* (Serradell, et al. 2007).

Moreover, filarial nematodes constitute most of the major tissue-dwelling helminths that ultimately cause the largest burden of disease in humans (McSorley & Maizels, 2012). Such filarial nematodes include those that reside in the lymphatic system, such as *Wuchereria bancrofti* and *Brugia malayi*, as well as those that live in subcutaneous tissues, for instance *Onchocerca volvulus* (Cross, 1996; McSorley & Maizels, 2012). The wide spectrum of clinical disease presented in response to filarial nematode infection, which can range from “endemic normal” status (i.e.

uninfected individuals) to patients presenting as asymptomatic but having large quantities of microfilariae (Mf) circulating in their blood (microfilaremic) to patients with extreme pathology and chronic infection resulting in debilitating diseases such as elephantiasis (Brattig, et al. 2002; Ottesen, 1984), has influenced the differential parasite-specific immune phenotype described for such filarial nematode parasites (King, 2001; Brattig, et al. 2002). Thus, the role of the stereotypical protective Th2 response in the outcome of disease has been controversial when studying immunity to filariasis. In human studies, it has been shown that microfilaremic adult patients have suppressed IFN-γ production and decreased parasite-specific T cell proliferation responses, indicating bias of Th2 immune responses through suppression of Th1-associated responses (Piessens, et al. 1980; Dimock, et al. 1996; King, et al. 1993). Additionally, these immune-tolerant persons show increased levels of the regulatory cytokine IL-10 (Mahanty, et al. 1996) and suppressed production of IL-5, a Th2-associated cytokine (Sartono, et al. 1997). On the other hand, amicrofilaremic patients tend to mount strong Th1-like responses to parasite antigen (King, et al. 1993). However, although host protection from *Brugia spp*. has been shown to be critically dependent on T cells (Vincent, et al. 1980), it is unclear which subset, whether Th1 or Th2, is more important for protection for the host. Studies in mice infected with *B. malayi* have shown that in fact different stages of infection are differentially regulated. For example, strong Th2 responses, as characterised by high levels of IL-4 secretion and elevated IgE production in addition to increased IgG1 antibodies, are associated with adult female worms whereas Mf alone have been shown to provoke a Th1 response as evident by high amounts of IFN-γ production (Lawrence, et al. 1994). Despite such high levels of the Th1-type cytokine, adult

female worms continuously secreting Mf were still powerful inducers of a potent Th2 response (Lawrence, et al. 1994). Thus, it was concluded from this study that the overactive Th2 response elicited by the adult female worms can subsequently modulate the initial Mf-induced IFN- γ production (Lawrence, et al. 1994). Further experiments with this parasite to determine the role of Th2 responses in filarial nematode parasites have been conducted using IL-4-deficient mice infected with *B. malayi* (Lawrence, et al. 1995). These experiments demonstrated that as predicted, the immune response to Mf was unaltered from a Th1-biased immune response in the IL-4 knockout mice (Lawrence, et al. 1995). However, in contrast, the absence of this cytokine had a detrimental effect on the Th2 response that would normally be generated by adult stages of this parasite ultimately resulting in the induction of a Th1-like response as indicative of increased IFN-γ production in conjunction with a decrease in IgG1 antibody production (Lawrence, et al. 1995). Interestingly, despite such switching from the Th2 cell phenotype, these alterations did not impede parasite survival for Mf, infective larvae and even adult stages of infection, suggesting that host immunity to *B. malayi* is not exclusively reliant on Th2 responses that are induced by IL-4 (Lawrence, et al. 1995). Furthermore, it is thought that the T helper 1 cell hyporesponsiveness witnessed in microfilaraemic individuals is due, in part; to the regulatory cytokines IL-10 and/or TGF-β as neutralizing these cytokines reverses the hyporesponsive state of T cells that is usually witnessed in microfilaraemic patients (King, et al. 1993; Mahanty, et al. 1996). The expression of such regulatory cytokines has been attributed to the expansion of regulatory T cells as depletion of such cells reversed parasite-antigen induced T cell hyporesposiveness associated with Mf individuals (Wammes, et al. 2012). Additionally, the removal of regulatory

T cells enhanced Th2 associated responses (Wammes, et al. 2012). Together, these data are consistent with studies carried out by Babu and co-workers (Babu, et al. 2006) showing that regulatory networks involving TGF-β and regulatory T cells were important for suppression of both T helper subsets. Interestingly, the natural parasite of rats, *Litomosoides sigmodontis,* which is the only filarial nematode know to be able to undergo its full life cycle in immunocompentent laboratory mice (Hoffmann, et al. 2000), has also been employed to demonstrate that Th1 and Th2 responses are equally important for immunity to this parasite. This has been shown nicely through the use of knockout mice, where mice deficient in IL-4 and IL-5 (representative of Th2 responses) succumb more easily to infection with this parasite (Specht, et al. 2004; Saeftel, et al. 2003) and mice deficient in both the Th1-associated cytokine IFN-γ and IL-5 show even greater susceptibility to infection (Saeftel, et al. 2003).

Expulsion of the parasite (whether through production of a Th2 or Th1/Th2 response) may confer protection for the host but this abruptly ends the life cycle for helminths. Therefore, it is not surprising that some nematodes may be able to modify this protective immune response so that they can induce a more balanced environment in order to survive.

1.3 Immunomodulation by helminths

One well known feature of helminths is their longevity. Adult intestinal worms can survive for up to 4 years where more notably; other nematode species such as the filarial nematode *W. bancrofti* can co-exist with a host in excess of a decade (Brooker et al, 2006; Subramanian, et al. 2004). Therefore, it is acceptable to assume that the long-term survival of helminths is down to their ability to somehow evade and suppress the immune system to prevent their ejection and allow them to live in harmony with the host. Indeed, only a minority of individuals infected with parasitic worms display clinical disease while most remain asymptomatic (Klion et al, 1991). An appropriate immune response must be elicited by the host to control the parasite while limiting any tissue damage towards itself. In parallel, in order for a parasite to be successful it must be able to preserve itself within the host long enough to complete its lifecycle, achieving this establishment most likely through blunting an immune attack from the host. As alluded to earlier, each helminth parasite has evolved to elicit distinct mechanisms ultimately ending in the same result: 1. stimulation of a Th2 response and 2. suppression of an excessive inflammatory immune response (Maizels et al, 2004).

1.3.1 The broad outcome of the specific immune response to helminths

The exposure to helminth parasites results in a spectrum of disease with 3 potential outcomes, each one associated with specific immune responses. This is exemplified in infections with filarial nematodes and schistosomes where chronic disease is usually presented in individuals with a lower worm burden. These individuals

display a dominant Th1 response in correlation with increased levels of B-cell-induced IgE antibody production and low levels of IgG4. This Th1-biased response is assumed to be accompanied by a weak regulatory response therefore leading to chronic inflammation. On the other end of the scale, chronically infected individuals with a persistent parasite burden do not solely display a type 2 response. Instead, it seems there is a strong Th2 response supplemented by an immunoregulatory T cell (Treg) response that restrains anti-parasite inflammatory and effector cells. This 'modified type 2' outcome is characterized by dampened IL-5 and IL-13 cytokine secretion complemented by heightened production of anti-inflammatory cytokines IL-10 and TGF-β as well as altered antibody profiles with low IgE and high IgG4 production (Maizels et al, 2004; Maizels and Yazdanbakhsh, 2003). The third outcome would be the most desired for the host, where the Th1 and Th2 responses reach equilibrium due to their control by T reg activity subsequently resulting in parasite-free individuals (Maizels and Yazdanbakhsh, 2003). There is thus gathering evidence that the immune response in helminth infections is 'modified'. For example, there is differential expression of IgE and IgG4 antibodies in chronic and asymptomatic human filarial infected patients. In human filariasis presenting as elephantiasis, there is significantly high IgE production: in comparison, asymptomatic microfilaraemic patients produce abundant levels of specific IgG4 with relatively low IgE levels (Maizels, et al. 1995). Additionally, evidence of this 'modified Th2 response' can be witnessed in *H. polygyrus* where a potent Th2 response is still unable to aid worm expulsion suggesting the involvement of another type of immune response (Maizels & Yazdanbakhsh, 2003; Wilson, et al. 2005).

1.3.2 Control of pathology by a regulatory network

The specific pathways by which helminths down regulate parasite-specific immune responses in chronically infected individuals is not entirely known. As eluded to previously, the generation of a Th2 response is essential to the outcome of the parasite infection. However, this response must simultaneously be controlled. This suppression of the immune system by helminths has over the years been shown to involve a regulatory network comprising of Treg and B cells as well as altered innate immune cells such as macrophages and dendritic cells, all of which contribute to an anti-inflammatory state characterised by elevated levels of IL-10 and TGF-β (Maizels, 2005; Maizels & Yazdanbakhsh, 2008). Certainly, the anti-inflammatory-associated cytokines IL-10 and/or TGF-β have been shown to be crucial mediators for inducing T cell hyporesponsiveness and enhancing parasite survival within a host (Maizels & Yazdanbakhsh, 2003; Wammes, et al. 2012; Mahanty, et al. 1996). Indeed, *T. muris* infected mice deficient in IL-10 succumbed easily to infection and failed to control pathological reactions resulting in high fatality levels (Schopf, et al. 2002). This level of mortality in IL-10 deficient mice has also been observed in *S. mansoni* infections (Wynn, et al. 1998). However, the predominant source of such cytokines in immune regulation by helminths has been under scrutiny as both Th2 cells and T reg cell populations as well as antigen-presenting cells have all been implicated as cellular sources for IL-10 (Maizels & Yazdanbakhsh, 2003; Smits, et al. 2010; Mitre, et al. 2008; Mahanty, et al. 1996). Thus, particular focus has been on Treg activity due to strong IL-10 production elicited in response to parasite antigens *in vitro* as well as antibodies specific for IL-10 (or TGF-β) being able to restore antigen-specific proliferation *in vivo* (Maizels & Yazdanbakhsh, 2003; Maizels,

2005; Mahanty, et al. 1996; King, et al. 1993). Thus, regulation of the Th2 arm of immunity is thought to be provided largely by T reg cells, whose presence can influence effector T cell populations such as Th1, Th2 or Th17 cells leading to a general T cell hyporesponsiveness (Maizels & Yazdanbakhsh, 2003; Harnett & Harnett, 2008; Maizels, et al. 2009; Babu, et al. 1996). Through use of a *H. polygyrus* model, it was shown that Tregs were able to prevent maximal Th2 responses during infection (Wilson, et al. 2005). However, despite elevated levels of both anti-inflammatory cytokines IL-10 and TGF-β, suppression of infection levels was shown to be IL-10-independent as depletion of Tregs using an antibody against the IL-2Rα chain (CD25) consequently ablated suppression of allergic responses associated with parasitic infection whereas antibodies obstructing the IL-10R had no effect in this environment (Wilson, et al. 2005). Additionally, it has been found that FOXP3 expression (a T reg cell marker) among CD4+ T cells increases soon after infection and that the parasite itself is able to induce CD4+ T cell differentiation into Treg cells through a TGF-β-dependent pathway induced by the release of secretory products (Wilson, et al. 2005; Grainger, et al. 2010). In particular, the significance of the induction of Treg cells for parasite survival has been emphasised in *L. sigmodontis*. Studies in a mouse model of this parasite have shown the expansion of T reg cells during infection (Taylor, et al. 2005) and that depletion of these cells by an antibody against its cell surface marker resulted in both clearance of the parasite and reduced fecundity (Taylor, et al. 2005; Taylor, et al. 2009). Treg cell populations have equally been demonstrated to be a critical factor in establishing chronic infections in humans due to their ability to drive suppression of the host immune responses (Maizels and Yazdanbakhsh, 2003). Indeed, an individual suffering from

onchoceriasis showed increased levels of IL-10 and TGF-β (but not IL-2) secreted from a prepared antigen-parasite-specific T reg clone (Doetze, et al. 2000). Moreover, differential levels of circulating Tregs were witnessed in patients from Gabon who were infected or uninfected with schistosomes where elevated levels of this cell type were observed in schistosome-infected patients (Maizels, et al. 2009). A similar result with this parasite was witnessed in patients from both Kenya and Gabon where infected individuals showed higher expression levels of $CD4^{+}$ $CD25^{hi}$ and $CD4^{+}$ $CD25^{hi}$ FoxP3 T cells (Watanabe, et al. 2007). Taken together, these data emphasise the importance of this cell type for nematode-induced modulation.

1.3.3 Non Treg mechanisms of immunmodulation

More recently, a new form of macrophages known as the alternatively activated macrophages (AAM) has also been implicated in the immune response to helminths and the appearance of this cell type is thought to be triggered by Th2 responses (Kreider, et al. 2007). It has previously been demonstrated that macrophages have the ability to switch phenotype during helminth infection. Thus, as infection with the tapeworm *Taenia crassiceps* progressed in mice, macrophages switched to a phenotype that produced higher levels of IL-6 and PGE2 with supressed IL-12 and nitric oxide production – features associated with AAMs (Rodríguez-Sosa, et al. 2002). Additionally, these cells were weak inducers of $CD4^{+}$ T cell proliferation but encouraged IL-4 cytokine production from $CD4^{+}$ T cell over IFN-γ (Rodríguez-Sosa, et al. 2002). When these APC from chronically infected mice were co-cultured with the same CD4+ T cells, blocking of IL-6 with monoclonal antibodies inhibited the

production of IL-4 (Rodríguez-Sosa, et al. 2002). These data suggest a potential role for an alternative phenotype of macrophages which can induce Th2 cell differentiation in parasitic infections (Rodríguez-Sosa, et al. 2002). Moreover, murine models of nematode infections express a phenotype similar to AAMs known as nematode-elicited macrophages (NEM). These cells have increase the expression of arginase-1, which through a higher affinity for arginine outcompetes the substrate for nitric-oxide production, in conjunction with the expression of proteins YM1 and FIZZ1 (Maizels and Yazdanbakhsh, 2003). Indeed, it has been illustrated that AAMs are able to suppress the immune response to schistosomes and help dampen pathology (Maizels, et al. 2009; Pesce, et al. 2009). This protective effect of AAM was attributed to arginase-1 as deficiency in this enzyme resulted in egg-induced inflammation and a failure to control Th2 responses ultimately contributing to fibrosis and thus a fatal outcome in the mouse (Pesce, et al. 2009). Briefly, another APC known to be modified by helminths and their products are dendritic cells (DCs). DCs are important APC that interact with Pathogen-Associated Molecular Patterns (PAMPs) via Pattern Recognition Receptors (PRRs) such as Toll-Like Receptors (TLR) leading to the activation of naïve T cells and generation of a specific immune response phenotype such as Th1 or Th2. Helminths are capable of interfering with this interaction to influence cytokine secretion by DCs and polarise the immune response towards a Th2/anti-inflammatory response through T cell-priming (Sher et al, 2003). Previous experiments have publicized that DCs exposed to microfilariae of the filarial nematode *B. malayi* showed specific down-regulation of TLR expression, in particular diminishing the responsiveness of TLR-3 and more specifically TLR-4 (Semnani et al, 2008), ultimately resulting in a weaker production of pro-

inflammatory cytokines. Altogether, these data show that helminths are highly capable of interfering with innate cells of the immune system and can manipulate the immune response to their advantage via such mechanisms as modifying DC function and in turn, T cell function.

1.3.4 The "Excretory-secretory" road to parasite survival

The versatile mechanisms by which helminths effectively modulate host immunity have been extensively reviewed (van Riet et al, 2007; Hewitson et al 2009) with particular focus on "excretory –secretory" products released by helminth parasites. The active secretion or excretions of products enables parasitic nematodes to manipulate the host's immune system to their advantage by targeting a range of fundamental immunological networks such as signal transduction pathways, cytokine production and key receptors. For example, cystatins (cysteine protease inhibitors), more specifically those from filarial nematodes, interfere with antigen presentation by monocytes resulting in inhibition of T cell proliferation and responses (Hartmann and Lucius, 2003). These properties along with cystatins inducing large scale IL-10 production (Hartmann and Lucius, 2003) contribute to the induction of an anti-inflammatory environment thus fuelling the success of the parasite's survival. Interestingly, the products of helminths involved in interfering and modulating the immune response appear to occur from the very first stages of infection. Schistosomes can regulate the host's immune response from the earliest infection stage (cercariae) via ES products such as glycocalyx materials and by their inducing immunoregulatory mediators such as the anti-inflammatory cytokine IL-10 (Jenkins

et al, 2005) enabling them to modulate the immune response to persist within the host almost instantaneously. This anti-inflammatory environment is beneficial for both the host and the parasite as this limits a potentially dangerous and uncontrolled inflammatory response allowing optimum reproduction for the host and in turn, the parasite.

1.4 ES-62 – a phosphorylcholine-containing glycoprotein

1.4.1 ES-62 - a filarial nematode-derived immunomodulator

Filarial nematodes and their products are of particular interest due to their intimate contact with the host's immune system, as a consequence of their residing in predominant immunologically rich sites such as tissues and the lymphatic system (Enk, 2006; Ottssen, 1980). As discussed previously, it is widely hypothesized that helminths modulate the host's immune response to be beneficial for both themselves and the host resulting in protection from eradication for the helminths and, in parallel, protecting the host from an excessive inflammatory response that may ultimately lead to tissue damage. One potent immunomodulatory product is ES-62, discovered in the Harnett lab in 1989 as a protein with a molecular mass of ~ 62kDa as determined by SDS-PAGE and secreted by the rodent filarial nematode *Acanthocheilonema viteae* (Harnett, et al. 1989). Additionally, radio-isotope labelling showed that this 62 kDa molecule was the predominant protein in adult-stage *A.viteae* secretions (Harnett, et al. 1989), contributing to greater than 90% of the total protein secreted (Harnett, et al. 1989) and thereby emphasizing the importance of ES-62 during infection of a host.

1.4.2 Structural aspects

Following its discovery, the parasite-derived molecule was shown to be a glycoprotein as it could be biosynthetically radiolabelled with [^{3}H] – glucosamine (Harnett et al, 1989*).* An unusual characteristic of many filarial nematode proteins is that they contain phosphorylcholine (PC), an immunomodulator appearing to play an important role in parasite survival (Grabitzki and Lochnit, 2009). Harnett and colleagues observed that antibodies directed against human filarial parasites could recognize ES-62 and this cross-reactivity was largely due to the presence of antibodies against PC (Harnett et al, 1989). This suggested that the glycoprotein could possibly have a post-translational modification by PC. Further investigation showed that PC is directly attached on the glycoprotein via a carbohydrate, in particular an N-type glycan. This conclusion was based on the observation that when ES-62 biosynthetically radio-labelled with [^{3}H]-glucosamine was exposed to the enzyme N-glycosidase F (an enzyme which is known to cleave N-glycans from proteins) all radioactivity was cleaved from the parasite molecule (Harnett, et al. 1993a). This potent loss of radioactivity through use of a sugar-cleaving enzyme was not mirrored when the enzyme O-glycanase was used (an enzyme which cleaves O-linked glycans from proteins) indicating that ES-62 may lack O-linked glycans (Harnett, et al. 1993a). In parallel, the loss with N-glycanase F was identically witnessed when [^{3}H]-glucosamine was substituted with [^{3}H]-choline (to label PC) thereby indicating that PC is attached to ES-62 via an N-type glycan (Harnett, et al. 1993a). Moreover, further evidence of this specific mode of attachment of PC to the protein backbone of ES-62 was shown when this modification was blocked when cultures of *A. viteae* were incubated with the antibiotic tunicamycin, an inhibitor of

N-type glycan precursors (Houston & Harnett, 1996). Having confirmed that PC is covalently attached to the backbone of ES-62 via an N-type glycan, the next step was to identify the intracellular site of PC transfer. Pulse-chase time-course experiments involving *A. viteae* pulsed with [^{35}S]-methionine or [^{3}H]-choline followed by immunoprecipitation and SDS-PAGE analysis showed that PC addition to ES-62 is fairly quick, occurring within 40-60 minutes after synthesis of the protein thus suggesting that the attachment of PC is almost certainly taking place in the endoplasmic reticulum (ER) or the golgi (Houston et al, 1997). To obtain more detail on the site of PC addition, adult-stage *A. viteae* were cultured in the presence of Brefeldin A, a compound that prevents transfer of proteins from the ER to the golgi (Houston, et al. 1997). ES-62 was detected in worm extracts from these cultures but PC was absent thus Brefeldin A blocked PC transfer to ES-62 thereby favouring the golgi as the site of PC addition to the glycoprotein (Houston, et al. 1997). To further support that the attachment of PC is a post-ER event, inhibitors of N-linked oligosaccharide processing were employed with each reagent targeting specific enzymes involved in glycan processing (Houston, et al. 1997). Thus, deoxymannojirimycin (dNM) which blocks mannosidase I activity and swainsonine which blocks mannosidase II activity were employed. From this experiment, it was observed that PC addition was reliant on mannosidase I activity in the *cis* golgi but not mannosidase II activity in the medial golgi as *A.viteae* worms exposed to dNM were found to lack PC on ES-62 whereas transfer of PC to ES-62 was unaffected in swainsonine cultured worms (Houston, et al. 1997). This finding in conjunction with the theory that PC addition may be dependent on *N*-acetylglucosamine (GlcNAc) transferase I activity, suggest that the golgi, in particular the medial golgi, is the most

likely location for PC transfer to ES-62 (Houston, et al. 1997). These conclusions were confirmed by Fast Atom Bombardment Mass Spectrometry (FAB-MS) – a powerful tool used to provide more information on the structure of glycopolymers. FAB-MS showed ES-62 contained N-type glycans with 3 distinct structures. The 3 major classes of N-glycan structures identified were: a high mannose type structure; a glycan which has been fully trimmed to the trimannosyl core and fucosylated; and a final structure comprised of the trimannosyl core with or without core fucosylation, carrying between one and four additional GlcNac residues (Haslam, et al. 1997). Of the three N-glycan structures revealed in ES-62 only the latter structure contained measurable amounts of PC (Haslam, et al. 1997). Two types of Onchocerca species, *O. volvulus* and *O. gibsoni* have PC-containing glycans of almost identical structure to that of ES-62 (Haslam, et al. 1999) therefore the PC-glycan structure appears to be conserved within filarial nematode species.

The specific role and mechanisms of action of PC-containing carbohydrates in nematode infections awaits full elucidation. Although the general consensus is that PC-containing glycoproteins such as ES-62 serve to modulate the host's immune system by provoking various effects on immune cells (Harnett & Harnett, 2001), this PC modification has also been found to play an important role in the reproduction and development of free-living species (Grabitzki and Lochnit, 2009). This clearly shows that PC- containing carbohydrates do not necessarily serve to have an immunomodulatory role in all nematodes. However, with regards to ES-62, it has been shown that the PC moiety is important for its powerful immunomodulatory properties as PC alone or PC conjugated to bovine serum antigen (BSA) can reproduce many of the properties executed by ES-62 (Harnett & Harnett, 1993).

1.4.3 Immunomodulatory properties

ES-62 exerts its immunomodulatory properties by interacting with various cells of the immune system (Figure 1.2). It modulates the activity of several immune cells such as B cells, macrophages and dendritic cells (DC) towards an anti-inflammatory phenotype (Goodridge, et al. 2005a and 2005b). More recently, this nematode-derived product has also been shown to modulate mast cell functional responses (Melendez, et al. 2007; Ball, et al. 2013). Such ES-62 activity has been shown to be highly dependent on TLR4. Thus, ES-62 was shown to modulate macrophage and DC function in a TLR-4-dependent manner through use of TLR-4 knockout (KO) mice (Goodridge, et al. 2005b). It was previously shown that ES-62 was able to transiently induce low cytokine production from both macrophages and DCs when these cells were incubated with ES-62 alone (Goodridge, et al. 2001; Goodridge, et al. 2004). However, this low level cytokine induction was inhibited in TLR4 and also myeloid differentiation primary response protein 88 (MyD88) KO mice (Goodridge et al, 2005b). Likewise, ES-62 was able to down-regulate cytokine production by TLR ligands, bacterial lipopeptide (BLP) and cytosine-phosphorous-guanine (CpG) oligonucleotides, which are recognized by TLR-2 and TLR-9 respectively, and this was equally dependent on TLR-4. On the other hand, ES-62-mediated effects were independent of TLR-2 and TLR-6 (Goodridge et al, 2005b). Moreover, modulation of the surface expression of MHCII and co-stimulatory molecules CD40, CD80 and CD86 on DCs was supressed in TLR-4 KO mice, with the effects on co-stimulatory molecules being only partially dependent on MyD88. An interesting feature of ES-62, TLR-4-dependent signalling was that macrophages and DCs cultured from C3H/HeJ mice, which have a defective form of TLR-4 due to a point mutation in the

TIR domain, remain responsive to ES-62 as indicated by the ability of this molecule to still modulate both cytokine production and co-stimulatory molecule expression. This result suggests that although ES-62 requires TLR-4 to mediate its effects, TLR-4 does not have to be fully functional (Goodridge et al, 2005b).

1.4.3.1 Antibody Responses

Balb/c mice administered weekly subcutaneous injections of ES-62 in the absence of adjuvant mount an antibody response. The PC-containing glycoprotein was able to selectively modulate this antibody response through evidence of a strong induction of the Th2-associated antibody subclass IgG1 but virtually no Th1-associated isotype IgG2a (Harnett, et al. 1999) therefore implicating ES-62 as a potent immunomodulator with the ability to induce a polarised Th2 immune response. Furthermore, it appears that IL-4 is crucial for this Th2 bias response as IL-4 knockout mice fail to produce IgG1 in response to ES-62 (Harnett, et al. 1999). The effect of ES-62 on the phenotype of the immune response and thus generation of such an antibody profile was attributed to the PC moieties present on this nematode product. This was demonstrated firstly when mice injected with ES-62 lacking PC resulted in the induction of a substantial IgG2a response – an opposing feature to 'normal' ES-62 (Houston, et al. 2000). Additionally, PC-free ES-62 had no effect on IgG1 thereby resulting in a mixed IgG1/IgG2a response. Secondly, suppression of the normal IgG2a response to BSA was witnessed when mice were injected with PC conjugated to BSA (PC-BSA) (Houston, et al. 2000). Taken together, these experiments concluded that PC is responsible for the down-regulation of the Th1

antibody response to ES-62. Furthermore, it is well known that the anti-inflammatory cytokine IL-10 can down-regulate the pro-inflammatory cytokine IFN-γ required for antibody class switching to IgG2a. Injection of ES-62 into IL-10 knockout mice resulted in the generation of an IgG2a response to the parasite molecule indicating a role for this cytokine in the IgG subclass phenotype that is mounted in response to ES-62 (Houston et al, 2000). These data suggest that the skewing of antibody responses and thus the immune response appears to be mediated through inhibition of the Th1 response by the production of IL-10, which in turn is induced by the presence of the PC moiety of ES-62.

1.4.3.2 B cells

When resting spleen-derived murine B cells were incubated with high concentrations (ranging from 25-50 μg/ml) of ES-62, DNA synthesis showed that this caused polyclonal stimulation suggesting that ES-62 was acting as a weak mitogen (Harnett & Harnett, 1993). However, this effect was not mimicked when ES-62 was present in concentrations imitating that of PC-containing molecules that would normally be found in the bloodstream of infected individuals (0.2-2 μg/ml). Instead, ES-62 was able to prevent proliferation of B cells associated with ligation of the antigen receptor (BCR) by up to 60% (Harnett & Harnett, 1993). Through the use of osmotic pumps subcutaneously implanted in mice, the effects of ES-62 *in vivo* were shown to be almost identical with ES-62 having a direct effect on B cell proliferation when conventional splenic (B2) B cells were activated *ex vivo* via ligation of the BCR (Wilson et al, 2003a). On the other hand, unlike conventional B-lymphocytes,

peritoneal B1 cells appear to be activated by ES-62. When these cells were pre-exposed to the parasite-derived glycoprotein released from osmotic pumps *in vivo*, an increase in proliferation was witnessed *ex vivo*, whether these cells were stimulated or not (Wilson et al, 2003b). Furthermore, ES-62 appeared to induce the production of the anti-inflammatory cytokine IL-10 from these B1 cells and this effect was enhanced further when the cells were activated following ligation of the antigen receptor (Wilson et al, 2003b). Probably the best understood mechanism of immunomodulation executed by ES-62 is the disruption of B-cell activation resulting in the suppression of B-cell proliferation following ligation of the BCR. The BCR comprises of an antigen-binding surface Immunoglobulin (sIg) and its accessory immuno-receptor tyrosine-based activatory motif (ITAM)-containing signal-transducing molecules Igα and Igβ. BCR ligation triggers protein tyrosine kinase (PTK) activity subsequently resulting in tyrosine phosphorylation of the ITAMs allowing the BCR to activate a number of signalling pathways including phospholipase C (PLC)-γ, the phosphoinositide-3-kinase (PI-3-K) and the RasErk mitogen activated protein kinase (MAP kinase) signalling pathways – all of which are implicated in BCR-induced B cell activation and proliferation (Dal Porto, et al. 2004). It was shown that ES-62 disrupts the transduction of cellular activation and proliferation by selectively targeting these key signalling events following BCR ligation. For example, ES-62 does not target PLC-γ, which generates the second messengers inositol trisphosphate and diacylglycerol that mobilize intracellular calcium and activate protein kinase C isoforms, but rather appears to down regulate PKC expression and activity (Harnett and Harnett, 1993; Deehan, et al. 1997). ES-62 selectively modulates the expression of such PKC isoforms as shown by the

decreased expression of PKCs -α, -β, -ζ, -ι/λ, and δ but increases γ and ε expression (Deehan, et al. 1997). In particular, PKC isoforms α and β were most strongly targeted by ES-62 and it is thought that ES-62-induced down-regulation of such PKC isoforms is mediated by proteolytic degradation as evident from inhibitor studies (Deehan, et al. 1997). In addition, pre-exposure to ES-62 renders B cells anergic to stimulation via the BCR by uncoupling it from key proliferation pathways such as PI-3K and Ras/MAPK cascades (Deehan, et al. 1998). ES-62 does this not by targeting PTK activation, but rather by inducing the recruitment of two major negative regulators, tyrosine phosphatase SHP-1 and the MAP kinase phosphatase Pac-1, that specifically control BCR coupling to the RasErk MAP kinase cascade (Deehan et al, 2001). Overall, the data clearly show mechanisms by which ES-62 profoundly desensitizes B cells thereby impairing the host's immune response during filarial infection.

1.4.3.3 Antigen Presenting Cells

Macrophages and DCs are important APC involved in directing the host immune response to infection. In particular, these cells prime and activate $CD4^+$ T lymphocytes and through the secretion of cytokines or interactions with co-stimulatory molecules, can direct T lymphocyte differentiation and thus the phenotype of the subsequent immune response (Kou & Babansee, 2010). Stimulation and subsequent activation of macrophages induces the release of several immune effector molecules such as the Th1-inducing cytokine IL-12 and pro-inflammatory cytokines IL-6 and TNF-α (Kou & Babansee, 2010). The release of

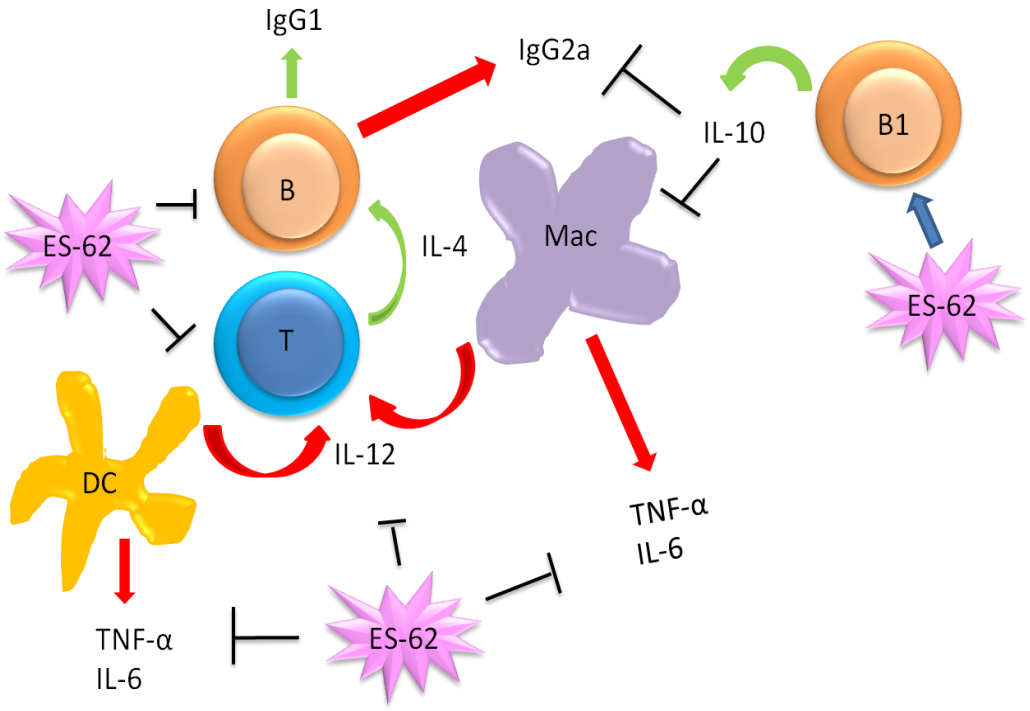
such potent cytokines was shown to be modified when murine macrophages were exposed to ES-62 *in vitro* and subsequently activated by LPS (Goodridge et al, 2001). Intriguingly, as alluded to earlier, initially this parasite-derived product stimulates the production of low levels of IL-12, IL-6 and TNF-α. However, incubation with ES-62 before subsequent stimulation prevents full macrophage activation (Goodridge et al, 2001). These data were further emphasised when the parasite-derived molecule was shown to dampen the production of LPS-induced production of pro-inflammatory and Th1-inducing cytokines when macrophages were exposed to ES-62 *in vivo* via release from osmotic pumps (Goodridge, et al. 2001 & 2004). Taken together, these results suggest that ES-62 is a powerful immunomodulator that acts upon macrophages rendering them hyporesponsive in response to stimuli and in doing so skews the immune response (Goodridge, et al. 2001 & 2004). Interestingly, ES-62 has no effect on nitric oxide release from macrophages suggesting that the helminth molecule does not interfere with all macrophage responses but rather selectively targets the production of Th1/pro-inflammatory cytokine secretion from these cells (Goodridge, et al. 2001 & 2004). It appears that ES-62 impairs the response of macrophages through modulating key signalling events involved in macrophage cytokine production. For example, macrophages treated with ES-62 show suppressed activation of p38 and JNK MAP kinases involved in the production of cytokines such as IL-12 (Goodridge et al, 2005b; Goodridge, et al. 2003). Furthermore, ES-62-induced IL-12 suppression is most likely, in part, facilitated through calcium-dependent ERK MAP kinase-mediated reduction of the P40 subunit (bioactive IL-12 p70 is comprised of P40 and P35 subunits) as ES-62-mediated inhibition of P40 mRNA levels can be rescued by

the ERK MAP kinase (MEK-1) inhibitor PD 98059 (Goodridge, et al. 2003). Also, as mentioned, (i) ES-62 was shown to suppress p38 activation, which is required for the activation of both bioactive IL-12 subunits (p40 and p35) in an ERK-independent manner (Goodridge et al, 2003) and (ii) ES-62 can inhibit JNK MAP kinases and both MAP kinases are required for the production of the pro-inflammatory cytokines IL-6 and TNF-α (Goodridge et al, 2005c). Consistent with these data, electrophoretic mobility shift assay (EMSA) analysis indicated that ES-62 regulates gene induction of IL-12 by modulating the binding of NF-κB to the IL-12 promoter (Goodridge, et al. 2005c). Overall, the data indicate mechanisms by which ES-62 can polarize the immune response towards an anti-inflammatory phenotype through inhibiting the production of pro-inflammatory cytokines by macrophages.

Consistent with this, ES-62 has thus been shown to promote priming of the immune response towards a Th2 phenotype largely by modulating the maturation and function of antigen-presenting cells such as macrophages and DCs (Goodridge, et al. 2005b). With respect to DCs, *in vitro* studies have shown that ES-62 can induce an immature DC phenotype that promotes a Th2 response over a Th1 response (Whelan, et al. 2000). Thus, DCs pre-exposed to ES-62 have the ability to induce Th2 responses to unrelated antigens through increased IL-4 production and inhibition of IFN-γ production (Whelan, et al. 2000). This clearly shows that the PC-containing glycoprotein can differentiate DCs towards a Th2 phenotype. As shown in detail previously, the effects of ES-62 on both macrophage and DC function is highly dependent on TLR-4 (Goodridge, et al. 2005a).

Fig 1.2. Immunomodulatory effects of ES-62.

ES-62 modulates various cells of the immune system towards a Th2/anti-inflammatory phenotype. ES-62 can induce the production of IL-10 from B1 lymphocytes. ES-62 can effect dendritic cell maturation by differentiating DCs towards a Th2 phenotype. ES-62 suppresses the production of the pro-inflammatory cytokines TNF-α and IL-6 and the Th1-inducing cytokine IL-12 by macrophages and DCs. (Adapted from Goodridge, et al. 2005a)


IgG1
IgG2a
IL-10
B1
B
ES-62
IL-4
Mac
ES-62
T
DC
IL-12
TNF-α
IL-6
TNF-α
IL-6
ES-62

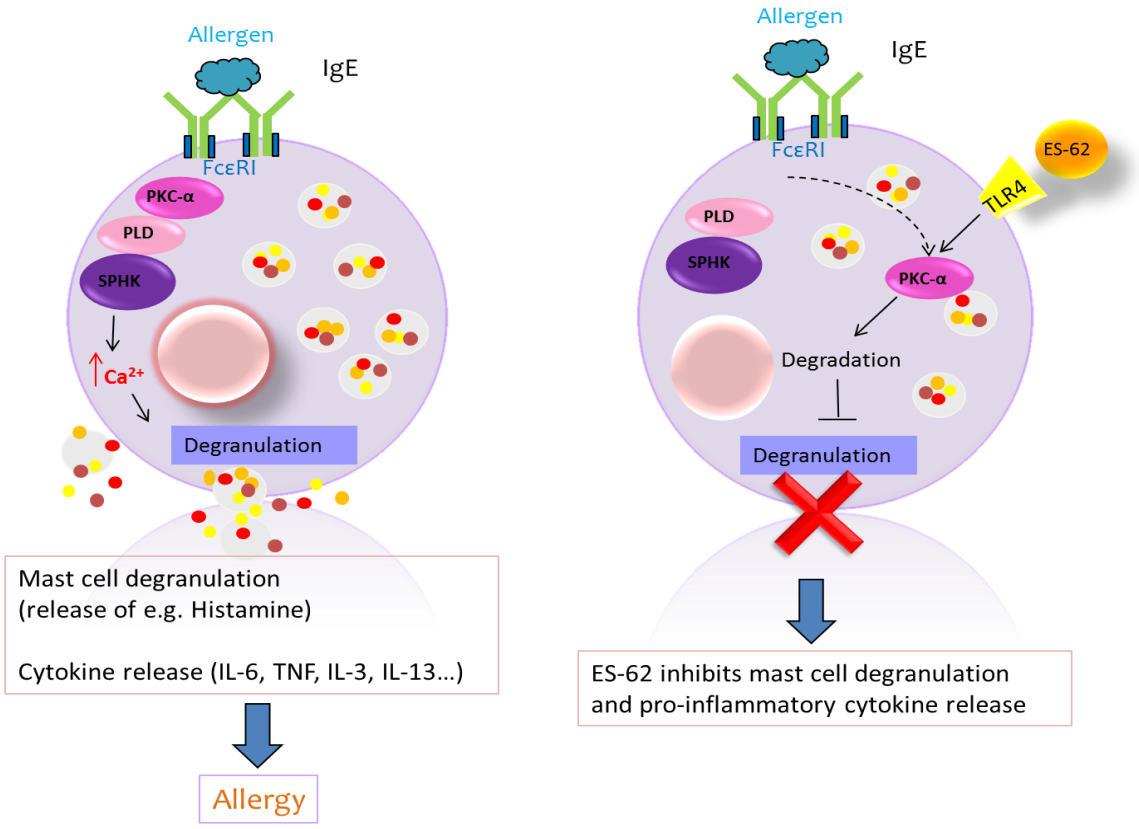
1.4.3.4 Mast cells

Mast cells are bone marrow-derived cells which are activated by aggregation of the high-affinity IgE receptor, FcεRI, by antigen and IgE antibodies subsequently resulting in degranulation and the release of inflammatory mediators (Galli, et al. 2008). The symptoms associated with allergy are largely due to the degranulation of mast cells and subsequent release of various biologically active products (Peavy & Metcalfe, 2008). Considering ES-62 interacts with a range of immune system cells including DCs, macrophages and B cells (Goodridge, et al. 2005b), it was hypothesised that this parasite-derived immunomodulator would interact with this other important immune effector cell – the mast cell. Thus, ES-62 was shown to inhibit FcεRI-mediated mast cell degranulation and the release of allergy-associated mediators such as pro-inflammatory cytokines (IL-6 and TNF-α), leukotrienes and prostaglandins (Melendez, et al. 2007). The mechanism of ES-62-induced inhibition of mast cell functional responses was not due to effects on FcεRI-expression or the early tyrosine phosphorylation events following cross-linking; rather the helminth molecule targeted the initial peak of calcium mobilization triggered by ligation of the FcεRI receptor (Melendez, et al. 2007). ES-62 was thus shown to target key mast cell signalling events such as PLD-coupled, sphingosine-kinase (SPHK) mediated calcium mobilization (initial peak in calcium) and NF-κB activation (Melendez, et al. 2007) in order to mediate its effects on mast cells. In monocytes, FcγRI coupling to PLD is mediated through association with PKC-α (Melendez et al, 2001) and ES-62 was shown to modulate the expression of total PKC and specific PKC isoforms with PKC-α being the isoform most strongly down-regulated (followed by PKC-β) (Melendez, et al. 2007). Following this, the use of antisense RNA to lower PKC-α

expression demonstrated that clearly PKC-α plays an important role in mast cell degranulation. ES-62 was then shown to mediate its effects on mast cells by forming a complex with TLR4 to sequester PKC-α away from the plasma membrane resulting in its degradation in a perinuclear site thus reducing its availability and inhibiting mast cell degranulation (Melendez, et al. 2007). Inhibitor studies showed that PKC-α is degraded in a caveolae/lipid raft-dependent, proteasome-independent pathway of degradation as the inhibitor nystatin, which prevents caveolae/lipid-raft-mediated pathways, subsequently blocks ES-62-mediated PKC-α down regulation (Melendez, et al. 2007). Lastly, an intriguing find was that ES-62 was shown to inhibit other PKC isoforms other than PKC-α. Thus, PKCs -α, -β, -δ, -ζ, and -ι are down regulated by ES-62 while PKC isoforms -γ, -θ and -ε are unchanged (Melendez, et al. 2007). Interestingly, these data are consistent with previous work in B cells where ES-62 was shown to affect key signalling events through such mechanisms as selectively modulating essentially the same PKC isoforms resulting in the suppression of proliferation following BCR cross-linking (Harnett & Harnett, 1993). Most recently, similar results have been witnessed with ES-62 in murine mast cells (Ball, et al. 2013 and Section 3.2). ES-62 has been shown to render these cells hyporesponsive to stimuli (LPS and FcεRI) by inhibiting mast cell degranulation and both pro-inflammatory (IL-6 and TNF-α) and Th2-related (IL-13) cytokine production (Ball, et al. 2013 and Section 3.2). Additionally, it seems ES-62 also targets key signals such as calcium mobilization and PKC-α expression in order to desensitize murine mast cells (Ball, et al. 2013). Interestingly, ES-62 was revealed to target additional signals in murine BMMC, namely PKC-δ and MyD88 (Ball, et al. 2013). The effects of ES-62 on mast cell activation are summarised in Figure 1.3.

Figure 1.3 The effects of ES-62 on mast cell functional responses

Normally, allergen-induced FcεRI cross-linking leads to the activation of a signalling pathway involving the sequential activation of PLD and SPHK. This results in a transient early influx of calcium from intracellular stores and subsequent mast cell degranulation and production of cytokines. ES-62 inhibits this FcεRI-mediated mast cell activation by forming a complex with TLR-4 to sequester PKC-α away from FcεRI and target it for degradation. This results in reduced availability of PKC-α and thus blocks mast cell activities.


Allergen
IgE
FcεRI
PKC-α
PLD
SPHK
Ca2+
Degranulation
Mast cell degranulation
(release of e.g. Histamine)
Cytokine release (IL-6, TNF, IL-3, IL-13…)
Allergy
Allergen
IgE
FcεRI
ES-62
TLR4
PLD
SPHK
PKC-α
Degradation
Degranulation
ES-62 inhibits mast cell degranulation
and pro-inflammatory cytokine release

1.5 Mast cells and mast cell signalling

1.5.1 Mast cells

Mast cells are derivatives of multi-potent hematopoietic progenitors in bone marrow (Kitamura, et al. 1977; Kirshenbaum, et al. 1991; Chen, et al. 2005; Kitamura, et al. 1985). Evidence suggests that these bone-marrow derived progenitors do not mature before they leave the bone marrow but rather circulate in the blood before arriving in mucosal and/or connective tissues to become fully matured (Kitamura, et al. 1985; Kirshenbaum, et al. 1991; Rodewald, et al. 1996; Gurish & Boyce, 2006). Indeed, when $CD34^+$ bone marrow-derived progenitor cells were cultured in the presence of IL-3, these cells gave rise to mast cells that expressed FcεRI and homogenously electron dense granules suggesting a phenotype closely related to immature tissue mast cells (Kirshenbaum, et al. 1991). However, when these precursor cells were co-cultured with 3T3 fibroblasts (known to encourage mast cell differentiation) in the presence of IL-3, they gave rise to a mast cell with diverse granular structures in their cytoplasm - a characteristic more resembling that of mature connective tissue mast cells (Kirshenbaum, et al. 1991). Taken together, these data strongly encouraged the idea that mast cells arise from CD34+ progenitor cells in the bone marrow *in vivo* before maturing in anatomical sites under the influence of other cells such as connective tissue cells (Kirshenbaum, et al. 1991; Kitamura, et al. 1985). Moreover, Nakano and colleagues showed through adoptive transfer studies that cultured mast cells with immature characteristics, as determined by their alcian blue staining, could develop into mast cells of a mucosal or connective tissue phenotype depending on the bodily location these cells were transferred into (Nakano, et al. 1985). Thus,

cultured mast cells adoptively transferred into the skin expressed characteristics of connective-tissue-type mast cells (CTMC) and those in the gastric mucosa showed a phenotype resembling mucosal-type mast cells (MMC), as indicated by their histochemical features (Nakano, et al. 1985). Therefore, it is well recognized that mast cell precursors differentiate and often reside in tissues where these cells, in mature form, share a common morphology with distinctive cytoplasmic granules (Kitamura, et al. 1977; Kirshenbaum, et al. 1991; Kitamura, et al. 1985; Galli, et al. 2011). Although they share a common lineage, it is no surprise that mast cells constitute a heterogeneous cell population that is largely determined by their location and factors present within the local microenvironment such as cytokines, hormones and growth factors (Galli, et al. 2011). Two major murine mast cell subtypes have been identified in rodents based on their localization, staining properties, and granule content: MMC and CTMC (Galli, et al. 1984). These mast cell populations differ greatly in their proteoglycan and protease granule content with MMC having no heparin in addition to little histamine content but CTMC have opposing features with these cells being rich in both heparin and histamine (Gaill, et al. 1984; Galli, et al. 2011). Interestingly, there has been evidence of a further subclass of murine mast cells (BMMC) which are derived from bone marrow hematopoietic stem cells exposed in culture to IL-3. These cells are thought to be the equivalent to MMC but have been shown to be in fact more immature and less granular in nature (Schrader, et al. 1981; Razin, et al. 1981; Galli, et al. 1982) with such immature cultured cells appearing less abundant in histamine levels (Galli, et al. 1982; Schrader, et al. 1981) and expressing lower levels of the high-affinity IgE receptor in comparison to mature mast cells (Galli, et al. 1982). However, the fact that such BMMC have been shown

to be able to differentiate into mature mast cells when adoptively transferred into mast cell deficient-mice (Nakano, et al. 1985; Moon, et al. 2010) highlights that these cells perhaps require signals present in the microenvironment in order to reach a full maturation status. By contrast, there has been evidence that the IL-3 dependent BMMC can change phenotype by altering such components as the culture supplements. For example, co-culture of BMMC with fibroblasts encouraged the generation of a mast cell with more CTMC characteristics (Levi-Schaffer, et al. 1987). Additionally, BMMCs that were grown in the presence of IL-4 and stem cell factor (SCF) (without IL-3) encouraged the generation of CTMC (Karimi, et al. 1999). Thus, these data indicate that bone-marrow precursor cells have the capacity to develop in culture into both mature and "immature" mast cells with factors such as cytokines influencing the changes in their phenotype (Moon, et al. 2010). Such mast cell heterogeneity is also evident in human mast cells where these cells are classified based on their granule protease expression into two major subgroups. The so called T-cell mast cells which only contains trypase (MC_T) and the TC mast cells which are positive for both chymase and trypase (MC_{TC}) (Irani, et al. 1986; Irani, et al. 1989). However, now a third and rare group of human mast cells has been identified where these cells are chymase-only mast cells (Craig & Schwartz, 1989). Human MC_T cells are often found within the mucosa of the gastrointestinal and respiratory tracts and thus share properties common to murine mucosal-type mast cells whereas MC_{TC} appear localized to connective tissues such as the dermis and submucosa of the gastrointestinal tract therefore these cells closely resemble connective tissue-type mast cells (Irani, et al. 1986; Irani, et al. 1989). The most important growth factor for the proliferation and differentiation of mast cells is SCF, also known as the ligand for

c-kit (CD117) (Lorentz & Bischoff, 2001; Lemura, et al. 1994; Mitsui, et al. 1993; Rottem, et al. 1993). However, although this growth factor is essential for human mast cell development (Lorentz & Bischoff, 2001; Mitsui, et al. 1993; Nakahata, et al. 1995), *in vitro* studies have identified other key mast cell growth factors in murine mast cells such as IL-3, IL-4 and IL-10, all of which seem to synergise with SCF to enhance mast cell proliferation and survival (Tsuji, et al. 1991; Nakahata, et al. 1995; Dvorak, et al. 1994; Karimi, et al. 1999). However, IL-3 appears to be an essential component in mice for mast cells development (Lantz & Huff, 1995; Rottem, et al. 1993). More recently, IL-4 has been implicated as an important factor in human mast cell growth and functional responses (Bischoff, et al. 1999). Thus, Bischoff and colleagues laboratory group discovered that IL-4 alone was not sufficient enough to encourage human mast cell survival but in combination with SCF, this cytokine appeared to dramatically up-regulate mast cell proliferation (2-5 fold increase) in comparison to SCF alone (Bischoff, et al. 1999). Interestingly, the secretion of mast cell mediators such as histamine and leukotrienes was additionally enhanced in human mast cells cultured with both SCF and IL-4 (Bischoff, et al. 1999).

Mast cells are widely distributed cells found in tissues throughout the body but they often reside in sites such as epithelial surfaces, where the inner meets the outer milieu (Abraham & St. John, 2010). Such locations are often entry points for many invading pathogens and direct contact points to the environment where antigens and/or other environmental agents can be encountered therefore allowing for their quick recognition by mast cells. Mast cells are involved in many biological responses, playing important roles in both innate and adaptive immune responses to

defend against parasites and bacteria as well as contributing to inflammatory responses (Galli et al, 2008; Metcalfe & Mekori, 1997). As established earlier, mast cells have been shown to be important in the Th2 responses elicited against parasitic worms. Specifically, they have been shown to be indispensable for the expulsion of *T. spiralis* as mast cell-deficient mice suffer from prolonged infections in comparison to their wild-type counterparts or indeed mast cell-deficient mice reconstituted with mast cells (Oku, et al. 1984). In conjunction with this, recent studies have highlighted the importance of mast cells in the defence against harmful pathogens such as bacteria through utilizing a similar approach with the use of mutant mice lacking mast cells. For example, mast cell-deficient mice (W/W^v) showed substantially higher mortality rates than wild-type mice in a model of acute septic peritonitis and this outcome was reversed when W/W^v mice were adoptively transferred with cultured mast cells (Echtenacher, et al. 1996). Additionally, the same mutant mice were less successful than wild-type or W/W^v mice reconstituted with mast cells at clearance of experimentally induced lung enterobacterial infection, with the majority of W/W^v mice ultimately succumbing to death (Malaviya, et al. 1996). Such impaired survival and killing of the infection was attributed to the absence of mast cell-derived TNF-α which would normally be released from mast cells and in turn induce the recruitment of neutrophils into the infected site in the lungs (Malaviya, et al. 1996). Thus, mast cells and their products have been shown to play an important role in host defence against pathogens (Gaill, et al. 2008). More recently, mast cells have been shown to express various TLRs on their cell surface (Matshushima, et al. 2004) which allows them to identify and respond to potentially harmful substances such as bacteria. Such TLRs include TLR-2, -3, -4, -7 and -9 (McCrudy, et al. 2001;

Varadaradjalou, et al. 2003; Matshushima, et al. 2004). Interestingly, through comparing mRNA levels of different TLRs, Matshushima and co-workers showed that different mast cell subpopulations, namely BMMC and murine fetal skin-derived cultured mast cells (FSMC) (a mast cell with features similar to CTMC), express differential TLR profiles (Matshushima, et al. 2004). Furthermore, it has been demonstrated that mast cells can even respond in a different manner to distinct bacterial components. For example, when mast cells derived from human cord blood were stimulated with peptidoglycan or LPS, a TLR-2 or TLR-4 agonist respectively, this induced a Th2-type cytokine response to both stimuli (Varadaradjalou, et al. 2003) but only peptidoglycan stimulation promoted histamine release (Varadaradjalou, et al. 2003). However, as well as expressing TLRs, mast cells also express high-affinity receptors for antibodies, specifically FcεRI, the high affinity receptor for IgE which plays a central role in inducing and maintaining allergic responses and may also protect from parasitic infections (Metcalfe & Mekori, 1997). Following the antigen-dependent aggregation of FcεRI, mast cell degranulation occurs releasing an array of mediators. These mast cell-derived mediators can be grouped into three main classes: (a) preformed mediators stored in cytoplasmic granules such as histamine that are released via exocytosis during degranulation (b) *de novo* synthesis of proinflammatory lipid mediators, namely prostaglandins and leukotrienes, and (c) large amounts of various chemokines, cytokines and growth factors which are synthesised and secreted following mast cell activation (Galli et al, 2008; Metcalfe & Mekori, 1997). These categories are not always mutually exclusive since at least one mast cell-derived mediator, TNF-α, can be both pre-formed in granules and newly synthesised upon mast cell activation (Gordon & Galli, 1991).

Additionally, such responses can be divided into an early and late phase reaction. The early phase involves the fusion of cytoplasmic granules with the plasma membrane and rapid synthesis of eicosanoid mediators from arachidonic acid stores resulting in the release of mediators such as histamine, proteoglycans (e.g. heparin and chondroitin sulfates), proteases (e.g. chymase and tryptase), leukotriene C_4 (LTC_4), prostaglandin D_2 and TNF within minutes into the local environment. The later phase involves newly synthesized mediators such as cytokines and leukotrienes which exhibit a more delayed response (hours) (Gordon & Galli, 1991; Razin, et al. 1983; Charlesworth, et al. 1989; Metcalfe & Mekori, 1997; Stone, et al. 2010). The late phase response is directed by continued mediator release from the activated mast cells and the recruitment and activation of other immune system cells such as neutrophils, eosinophils and basophils (Charlesworth, et al. 1989; Malaviya, et al. 1996; Wershil, et al. 1991; Stone, et al. 2010). The recruitment of additional immune effector cells is usually induced by products released from the early phase such as, for example, the immediate release of mast cell-derived TNF-α directing the influx of neutrophils in a model of IgE-induced cutaneous late phase reaction in mice (Wershil, et al. 1991). The mediators released from activated mast cells following allergen-specific IgE binding and cross-linking of FcεRI result in the pathological consequences of mast cells and are responsible for the symptoms of immediate type hypersensitivity reactions such as allergic rhinitis, allergic, asthma and atopic dermatitis (Peavy and Metcalfe, 2008; Moon, et al. 2010). For example, the use of mast cell deficient mice (W/W^v) and their mast cell "knock in" equivalent has shown that mast cells play a fundamental role in asthmatic responses (Williams & Galli, 2000; Kobayashi, et al. 2000). Upon challenge with antigen in the absence of an

adjuvant, mast cell-deficient mice were shown to have significantly lower lung inflammation and airway hyperactivity compared to wild-type and mast cell-reconstituted mice suggesting a possible critical role for mast cells in asthma (Williams & Galli, 2000). Therefore, in order to understand the mechanisms of allergy and find a potential new therapeutic drug we must be able to understand the intracellular signalling events that lead to such a potent inflammatory response.

1.5.2 Mast cell FcεRI-dependent signalling

Once antigen is bound to IgE, the FcεRI receptor aggregates and the signalling cascade begins (summarised in Figure 1.4). The FcεRI expressed on mast cells (and basophils) is a complex of four subunits comprised of a high-affinity IgE-binding α subunit, a signal-amplifying β subunit that spans the membrane four times and a disulfide-linked homodimeric γ subunit (tetramer – $\alpha\beta\gamma_2$) (Blank, et al. 1989; Blank & Rivera, 2004). Both the β and γ subunits of the FcεRI complex contain specific sequences known as ITAM (immunoreceptor tyrosine-based activation) motifs in their cytoplasmic tails, which following aggregation of the receptor by bound IgE, results in the tyrosine phosphorylation of the ITAMs to initiate the signalling cascade (Reth, 1989; Paolini, et al. 1991; Jouvin, et al. 1994; Gilfillan & Tkaczyk, 2006). Following phosphorylation of the tyrosine residues within the ITAMS by specific tyrosine kinases, the phosphorylated ITAM motifs of the β and γ subunit can bind with high affinity to additional cytoplasmic signalling molecules with src homology 2 (SH2) domains such as the protein tyrosine kinase Syk (Paolini, et al. 1991; Eiseman & Bolen, 1992a; Eiseman & Bolen, 1992b; Jouvin, et al. 1994; Gilfillan &

Tkaczyk, 2006). Thus, one of the earliest responses to receptor activation is tyrosine phosphorylation of the FcεRI β and γ subunits in correlation with association of the tyrosine kinase Syk with the γ chain ITAMs resulting in its activation (Paolini, et al. 1991; Jouvin, et al. 1994; Blank and Rivera, 2004; Gilfillan & Tkaczyk, 2006). It is thought that the γ chains serve to initiate the down-stream signalling cascade whereas the β subunit functions to amplify the signals transduced by the γ chains (Taylor, et al. 1995; Jouvin, et al. 1994; Eiseman & Bolen, 1992b). Phosphorylation of the tyrosine residues of both subunits is thought to occur through the Src family protein tyrosine kinase (PTK) Lyn, which is associated with the β subunit and is activated following FcεRI cross-linking. Thus, Lyn phosphorylates the ITAMS of the β and γ subunits which in turn amplifies its association with the β ITAM and consequently encourages the coupling of Syk with the γ subunits where Syk can then additionally be phosphorylated and activated by Lyn (Eiseman & Bolen, 1992a; Eiseman & Bolen, 1992b; Jouvin, et al. 1994; Gilfillan & Tkaczyk, 2006). The binding of Syk in this manner permits trans/auto phosphorylation of its catalytic domain thereby together with phosphorylation by Lyn, resulting in an increase in catalytic activity of the receptor-bound Syk (Pribluda, et al. 1994). Following its phosphorylation by Lyn, activated Syk can then directly or indirectly phosphorylate downstream signalling molecules essential for mast cell activation such as phospholipase C-γ_1 (PLC-γ_1) (Li, et al. 1992; Pribluda, et al. 1994; Rivera & Brugge, 1995) and linker for activation of T cells (LAT) (Saitoh, et al. 2000). An RBL cell line expressing a chimeric molecule containing the extracellular domain of CD16 and the transmembrane domain of CD7 linked to the complete coding region of the Syk tyrosine kinase was used to demonstrate that Syk has an obligatory role in mast cell

activation as cross-linking of the chimera with anti CD16 antibodies was sufficient for Syk activation and subsequent mast cell responses (Rivera & Brugge, 1995). Consistent with this, studies using Syk deficient-mast cells have shown that these cells fail to induce any downstream signalling pathways in response to antigen stimulation (Costello, et al. 1996). Therefore, these data indicate that the tyrosine kinase Syk plays a crucial role in the initiation of FcεRI-mediated signalling (Rivera & Brugge, 1995; Costello, et al. 1996). Mast cells also express adapter proteins whose primary function is to coordinate the signalling events and interaction of signalling proteins following FcεRI aggregation. Following its phosphorylation, Syk then activates the important adapter molecule, LAT. Saitoh and co-workers were the first laboratory group to show using LAT-deficient mice that LAT functions downstream of the activated PTKs Lyn and Syk (Saitoh, et al. 2000). This group additionally revealed that these mice have impaired degranulation and cytokine responses in response to FcεRI aggregation and that Syk (and/or Lyn)-mediated phosphorylation of LAT was critical for downstream signalling events following FcεRI aggregation (Saitoh, et al. 2000). Thus, upon phosphorylation, the transmembrane LAT acts as a scaffold for several additional signalling molecules containing SH2 domains. From T cell and mast cell studies, it is known that such recruited LAT-binding proteins include the signalling adapter molecules GRB2 (growth-factor-receptor-bound protein2) and GADS (GRB2-related adaptor protein) which are bound to SOS and SLP76 (SH2-domain-containing leukocyte protein of 76 kDa), respectively. Additional molecules include the adapter molecule SHC (SH2-domain-containing transforming protein C) and Vav1 in addition to the signalling enzymes PLCγ_1 and PLCγ_2. Therefore, altogether LAT creates a multi-molecular

signalling complex which allows for diverse signalling pathways leading to differential functional responses such as degranulation, cytokine production, eicosanoid generation and mast cell growth, differentiation and survival (Zhang, et al. 1998; Saitoh, et al. 2000; Zhang, et al. 2000; Paz, et al. 2001; Siraganian, 2003; Gilfillan & Tkaczyk, 2006). Interestingly, although LAT-deficient mice have much weaker responses to receptor aggregation as shown by their reduction in FcεRI-mediated degranulation and cytokine production in comparison to wild-type controls, FcεRI-mediated signalling is still partially intact (Saitoh, et al. 2000). It was observed that BMMCs cultured from LAT-deficient mice showed residual receptor-mediated degranulation, calcium mobilization and cytokine responses (Saitoh, et al. 2000) suggesting the involvement of another adapter molecule that is distinct from LAT. This additional adapter molecule was later identified to be structurally similar to LAT and was thus named LAT2 (formally known as NTAL (non-T cell activation linker) or LAB (linker for activated B cells)) (Brdika, et al. 2002; Janssen, et al. 2003; Gilfillan & Tkaczyk, 2006). This LAT-related adapter protein is found in cells such as B lymphocytes, monocytes and NK cells but not in T lymphocytes (Brdika, et al. 2002; Janssen, et al. 2003). LAT2 has been demonstrated to be equally rapidly tyrosine phosphorylated by the PTKs Lyn and Syk following receptor-induced mast cell activation suggesting that this adapter molecule is involved in FcεRI-mediated mast cell responses (Brdika, et al. 2002). The phosphorylation of LAT2 following antigen stimulation has been observed in both mouse and human studies (Brdika, et al. 2002; Tkaczyk et al, 2004). In co-ordinance with LAT, the LAT2 adapter molecule was also found to associate with Grb2 and SOS1 (Brdika, et al. 2002) but it did not associate with PLC-γ or SLP-76 (Brdika, et al. 2002), a feature which may be

unique to LAT (Zhang, et al. 2000). Additionally, knockout studies have shown that LAT2 may also function as a negative regulator of mast cell functional responses (Volna, et al. 2004). BMMCs derived from LAT2 knockout mice showed enhanced signalling capacities in response to antigen with resultant higher degranulation responses and calcium responses in addition to greater PLC-γ and PI3K activity in comparison to wild-type counterparts (Volna, et al. 2004). This inhibitory role of LAT2 has been proposed to be due to competition between LAT and LAT2 in lipid rafts, as these two adapter molecules may compete for the same pool of signalling molecules within such vicinity (Volna, et al. 2004). However, double knockout studies of LAT and LAT2 showed that the latter adapter molecule simultaneously has a positive role in mast cell functional responses as in the absence of LAT, LAT2 can compensate for some of its functions (Volna, et al. 2004). Although the role of LAT2 is less clear, LAT clearly primarily regulates the signalling pathway leading to PLC-γ activation as observed by LAT-deficient BMMC displaying a reduced calcium response (Saitoh, et al. 2000) most likely through failing to activate PLC-γ (Saitoh et al, 2000). Thus, PLC-γ is the main signalling enzyme that is regulated by PTK phosphorylated LAT (Saitoh, et al. 2000). PLC-γ catalyses the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP_2) in the plasma membrane to generate inositol triphosphate (IP_3) and diaglycerol (DAG) with the latter products inducing calcium mobilization from internal stores and activation of PKC, respectively (Ozawa, et al. 1993a; Ozawa, et al. 1993b; Siraganian, 2003). These signals lead to mast cell degranulation, eicosanoid generation and the synthesis and release of cytokines (Ozawa, et al. 1993a; Ozawa, et al. 1993b; Beaven et al, 1984a; Melendez, et al. 2007). IP_3 rapidly induces calcium mobilization from intracellular stores upon

binding to its receptor in the ER. Although this signal is only transient, the depletion of calcium from intracellular stores induces calcium entry from outside the cells (extracellular calcium) therefore inducing a sustained influx of calcium through store-operated calcium release-activated calcium (CRAC) channels in the plasma membrane (Hoth & Penner, 1992; Kalesnikoff & Galli, 2008). Both isoforms of PLC-γ, PLC-γ_1 and PLC-γ_2, have been identified in RBL cells (Ozawa, et al.1993a) and murine BMMCs (Saitoh, et al. 2000) but so far only PLCγ_1 has been identified in human mast cells (Suzuki, et al. 1997). Nevertheless, PLC-γ-mediated increase in intracellular calcium and PKC activation are critical steps in the signalling pathway to fully activate mast cells (Chang, et al. 1993; Ozawa, et al. 1993a; Ozawa, et al. 1993b; Kimata, et al. 1999).

Moreover, it is been recognized that following aggregation of the FcεRI receptor, an additional Src PTK, Fyn, is activated. As mentioned previously, low levels of PLC-γ and calcium responses were witnessed in LAT-deficient BMMCs (Saitoh, et al. 2000) suggesting the possibility of an alternative signalling pathway. Such an alternative pathway has been shown to involve Fyn kinase and Gab2 (GRB2-associated binding protein 2) (Parravicini, et al. 2002). Parravicini and colleagues showed that in addition to Lyn, Fyn kinase was involved in mast cell degranulation and cytokine production following FcεRI receptor activation (Parravicini, et al. 2002). This latter pathway involving Fyn was shown to be linked to the activation of phosphatidylinositol 3-kinase (PI3K) through a LAT-independent mechanism that did not activate PLC-γ but rather led to the tyrosine phosphorylation of the cytosolic adaptor molecule Gab2. This adaptor molecule was shown to be paramount for PI3K activation in antigen-stimulated mast cells (Gu, et al. 2001). Consistent with this,

Tkaczyk et al showed that degranulation was regulated by two distinct pathways in human mast cells. One pathway was PLCγ1-dependent but PI3K-independent and was thought to be critical to the initial influx of calcium mobilization whereas another latent pathway which was PI3K-dependent was hypothesised to be responsible for maintaining the initial PLC-γ-dependent signals thus contributing to the more sustained calcium response (Tkaczyk, et al. 2003). Following the phosphorylation of Gab2 by Fyn (and/or Syk), Gab2 then binds the SH2 domain-containing p85 subunit of PI3K recruiting it to the plasma membrane where it catalyzes the conversion of PIP_2 to PIP_3 (Rameh, et al. 1995; Scharenberg et al, 1998; Parravicini, et al. 2002; Gu, et al. 2001; Siraganian, 2003). PI3K-induced phosphorylation of membrane bound phosphoinositides acts as a docking site for Plekstrin-homology (PH) domain-containing proteins (Scharenberg et al, 1998). Such PH-containing proteins recruited by PIP_3 include the Tec family kinase Bruton's tyrosine kinase (Btk), Vav, the serine kinase Akt, PLD and both PLCγ isoforms (Sharenberg, et al. 1998; Franke, et al. 1995; Rameh, et al. 1995; Kalesnikoff & Galli, 2008). Overall, it appears that FcεRI requires two Src PTKs, Lyn and Fyn, in order to exhibit a full mast cell degranulation response (Gilfillan & Rivera, 2009). Although both may be required for mast cell activation, they play different roles to one another with Lyn not only being an important positive regulator of mast cell responses, but can also act as a negative regulator through controlling Fyn kinase activity (Odom, et al. 2004). Fyn positively regulates the Gab2 pathway and mast cell responses (Gu, et al. 2001; Gilfillan & Rivera, 2009) but unlike Lyn, cannot phosphorylate the β and γ ITAMs of FcεRI (Gomez et al, 2005).

Moreover, one member of the Tec family kinases in particular, Btk, has additionally been shown to phosphorylate and activate PLC-γ. It has been suggested that PI3K-dependent calcium mobilization and subsequent mast cell degranulation is controlled in part by PI3K recruiting Lyn-activated Btk and PLC-γ to the plasma membrane where PLC-γ can be phosphorylated and activated by Btk (Hata, et al. 1998; Gillfillan & Rivera, 2009). However, as mentioned previously, the initial calcium influx mediated by PLC-γ has been shown to be PI3K-independent (Tkaczyk, et al. 2003). Thus, an alternative pathway independent of PLC-γ and IP_3 involving PLD-coupled, SPHK-mediated calcium influx has now been proposed (Choi, et al. 1996; Melendez, et al. 2007; Kalesnikoff & Galli, 2008). It was first shown in RBL cells that activation of mast cells through FcεRI aggregation resulted in the rapid activation of SPHK and an increase in sphingosine 1 -phopshate (S1P) (Choi, et al. 1996). The resultant S1P-induced calcium response from these cells was shown to originate from intracellular stores (Choi, et al. 1996). It has subsequently been observed that following antigen-induced receptor aggregation, SPHK activation is downstream of PTK and thus is activated by Lyn and Fyn resulting in the phosphorylation of sphingosine to form S1P. However, similar studies with Lyn and Fyn-deficient mice showed that although Lyn and Fyn are required for the immediate activation of SPHK, only Fyn is vital for late SPHK activation (Choi, et al. 1996; Baumruker & Prieschl, 2000; Urtz, et al. 2004; Olivera, et al. 2006). S1P has additionally been shown to have both an intracellular role where S1P acts as a "second messenger" to regulate the initial calcium influx and degranulation in an IP_3, PLCγ-independent pathway in addition to an extracellular role whereby following FcεRI-induced SPHK-mediated S1P secretion from the cell, S1P binds to its surface

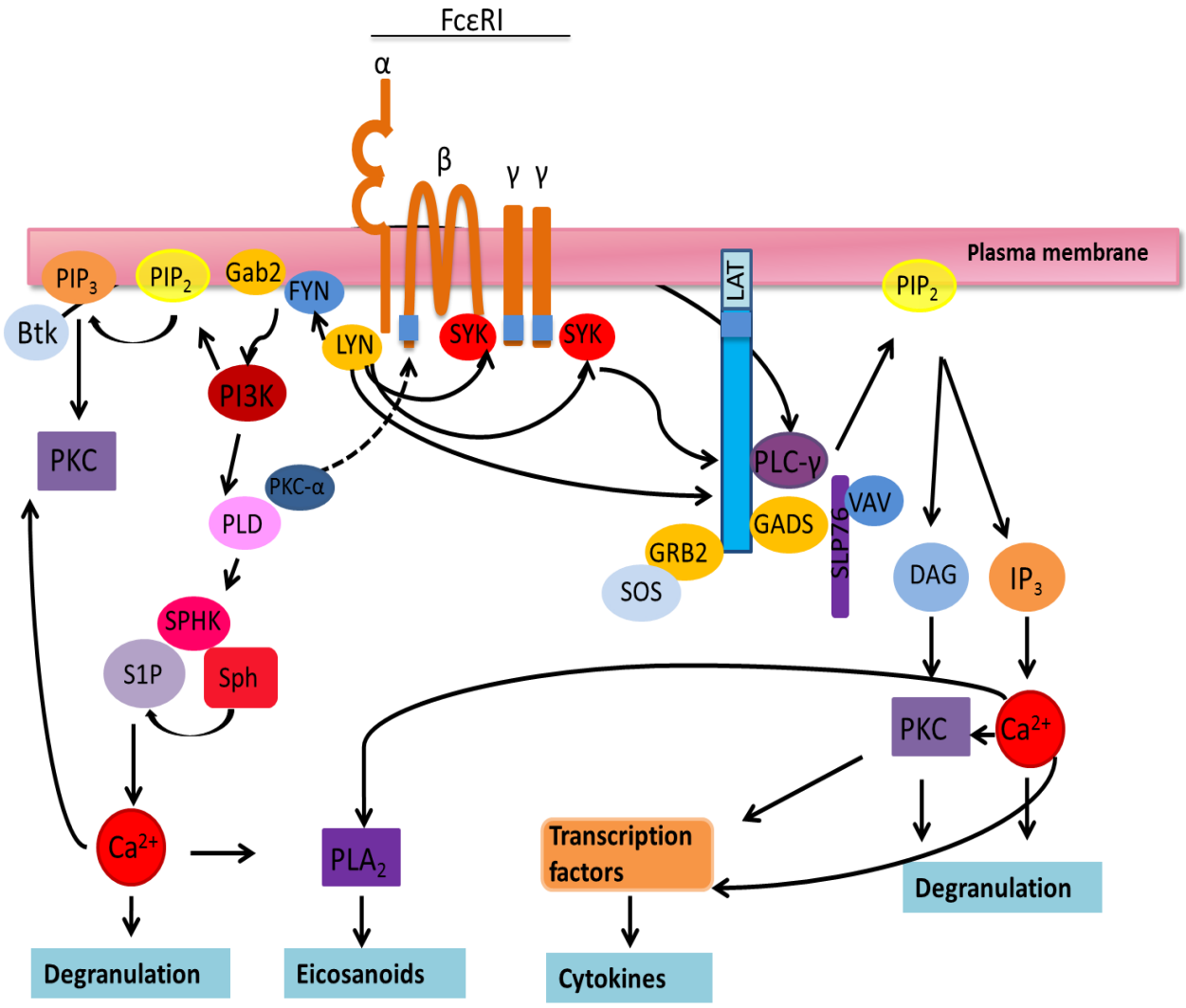
receptors S1P1 and S1P2 (which are present on both human and rodent mast cells) to amplify mast cell functional responses such as degranulation (Choi, et al. 1996; Prieschl, et al. 1999; Jolly, et al. 2004; Kalesnikoff & Galli, 2008). Furthermore, SPHK activity has been shown to be regulated by PLD. PLD is activated in mast cells following IgE-mediated receptor aggregation where it subsequently hydrolyses phosphatidylcholine (PC) to yield phosphatidic acid (PA) and choline. Thus, PLD-derived PA has been shown to have many intracellular signalling functions including the activation of SPHK (Lin, et al. 1991; Delon, et al. 2004; Peng & Beaven, 2005; Olivera & Rivera, 2005; Olivera & Rivera, 2011). However, although it has been shown that PLD may contribute to SPHK activation in mast cells, the specific mechanisms of this signalling pathway are not entirely known (Olivera & Rivera, 2011). On the other hand, PLD has been shown to be undoubtedly important for mast cell degranulation. In mammals, PLD exists as two isoforms: PLD1 and PLD2 (Colley, et al. 1997; Hammond, et al. 1995). However, studies on the cellular location of both isoforms have had various and often contrasting results. Overall, PLD1 appears to be internally localized to intracellular vesicular structures such as peri-nuclear endosomes and in the Golgi where it can associate with secretory granules in mast cells (Strand, et al. 1999; Freyberg, et al. 2001; Choi, et al. 2002) whereas PLD2 is often most associated at the plasma membrane (Slaaby, et al. 1998; Choi, et al. 2002). These membrane-associated isoforms play an important role in mast cell degranulation as catalytically inactive forms of PLD1 and PLD2 show suppressed migration of secretory granules and impaired mast cell exocytosis (Choi et al, 2002). Taken together, these results suggest that each isoform may regulate distinct phases of degranulation but that both are required for optimum mast cell

activity. Additionally, previous studies in murine mast cells observed that knocking down PLD through siRNA resulted in the inhibition of PLD activation and its product DAG, as well as the abrogation of PKC translocation and mast cell degranulation (Peng & Beaven, 2005). Therefore, PLD-derived DAG can regulate enzymes such as PKC (Peng & Beaven, 2005). Additionally, PLD itself can be stimulated by PKC (Chahdi, et al. 2002). Previous studies in monocytes showed that PKC-α was essential for FcγRI coupling to PLD (Melendez, et al. 2001). More recently, in human mast cell studies, it has been proposed that the sequential activation of FcεRI-coupled PLD and SPHK results in an initial peak in calcium and subsequent mast cell activation (Melendez, et al. 2007). Through the use of a filarial nematode molecule, ES-62, it was shown that PKC-α is responsible for FcεRI-coupling to PLD and thus the initial peak in cytosolic calcium ultimately resulting in mast cell degranulation, cytokine and eicosanoid production and NF-κB activation (Melendez, et al. 2007).

Overall, mast cell activation through FcεRI-mediated signalling pathways results in the release of mediators that are associated with allergic responses (summarised in Figure 1.4). Such pathways are complex and controlled by a series of phosphorylation and dephosphorylation events specifically by protein kinases and protein phosphatases which positively or negatively control the signals ultimately leading to full activation of such pathways (Kalesnikoff & Galli, 2008; Gilfillan & Rivera, 2009).

Figure 1.4 Mast cell signalling pathways following activation via FcεRI

Cross-linking of FcεRI results in the release of allergy-associated mediators such as cytokines and eicosanoids. Such antigen-mediated mast cell activation is regulated by a complex series of intracellular signalling cascades. The initial interaction of antigen with the α subunit of FcεRI results in the activation of the receptor-associated protein tyrosine kinase (PTK) LYN which subsequently phosphorylates the ITAM motifs of the β and γ subunits. Phosphorylation of the ITAM motifs recruits the cytosolic PTK SYK which binds to the γ subunits via its SH2 domian resulting in its activation. Activated SYK can then directly or indirectly phosphorylate and activate other proteins. The adaptor molecule LAT is phosphorylated in a LYN and SYK-dependent manner resulting in the recruitment of cytosolic adaptor molecules such as GRB2, GADS, and SLP76 in addition to VAV and SOS and the signalling enzyme PLC-γ. Membrane-associated PLC-γ hydrolyses PIP_2 forming the second messengers DAG and IP_3 leading to release of calcium from intraceullar stores and activation of PKC. Receptor aggregation also results in the activation of another PTK FYN, which phosphorylates the cytosolic adaptor molecule Gab2 leading to the activation of the PI3K pathway. PI3K catalysez the conversation of PIP_2 to form PIP_3 at the plasma membrane. PIP_3 is thought to recruit the Tec family kinase Bruton's tyrosine kinase (Btk) which can phosphorylated and activate PLC-γ resulting in an increase in calcium mobilization. An alternative pathway of calcium mobilization involving PLD and SPHK has been shown to be essential for mast cell degranulation. PKC-α couples FcεRI to PLD and its subsequent signalling pathway leading to calcium mobilization. It is thought that PI3K can also activate PLD and increase intracellular calcium.


FcεRI
α
β
γ γ
Plasma membrane
PIP3
PIP2
Gab2
FYN
Btk
LYN
SYK
SYK
LAT
PIP2
PI3K
PKC
PKC-α
PLD
PLC-γ
VAV
GADS
GRB2
SOS
SLP76
DAG
IP3
SPHK
S1P
Sph
PKC
Ca2+
Ca2+
PLA2
Transcription factors
Degranulation
Degranulation
Eicosanoids
Cytokines

1.6 Protein Kinase C

1.6.1 Protein Kinase C – discovery of a family affair

Protein Kinase C (PKC) is not just one kinase but a family of serine/threonine protein kinases that play a critical role in signal transduction pathways within the cell. They constitute a large group of lipid-dependent kinases that can control the function of other proteins by phosphorylating serine and threonine residues on these proteins (Nishizuka, 1995). PKC can stimulate or inhibit the activity of a variety of enzymes through phosphorylation (Farooqui et al, 1988) suggesting that PKC may have an important role in regulation of many major cellular processes. Thus, PKCs are considered mediators of external agonists which stimulate the production of many lipid second messengers. PKC was discovered by Nishizuka and co-workers as a kinase partially purified from bovine cerebellum. This kinase was found to be able to phosphorylate histone and protamine and was converted from a proenzyme to an active kinase by limited proteolysis in a calcium-dependent manner. Therefore, this was the first time PKC was recognised as a calcium activated enzyme (Inoue, et al. 1977). This discovery was later realised to be of great importance when it was observed that this enzyme was also a major target for DAG (Kishimoto, et al. 1980). Currently, up to 10 PKC isoforms have been identified in mammals and such isoforms have been shown to have extensive tissue distribution and cellular localization suggesting that each isoform may be independently regulated and may possess distinct substrate specificities (Toker, 1998). The first PKC isoforms identified were PKCs- α, - β (I and II – this isoform exists as a splice variant) and γ from brain cDNA libraries (Parker, et al. 1986; Coussens, et al. 1986). Further

analysis of this apparently PKC-rich tissue identified PKCs – ζ, - δ and ε through using a mixture of α, β and γ cDNAs as probes under low-stringency conditions (Ono, et al. 1987). Further low stringency screens of other tissues have now revealed the identification of more PKC isoforms namely PKCs- η, - θ, and ι/ λ (ι is the human homologue and λ is the mouse homologue) (Osada, et al. 1990 & 1992; Selbie, et al. 1993). Based on structure and lipid requirements for activation, PKC family members are divided into three main subsets: (a) Ca2+/DAG-regulated conventional isoforms (cPKCs: α, βI, βII and γ) (b) Ca2+ independent, DAG-dependent novel isoforms (nPKCs: μ, δ, ε, η, and θ) (c) the atypical isoforms which are both Ca2+ and DAG independent (aPKC: ζ and ι/λ) (Ono, et al. 1987; Shearman, et al., 1988; Baier et al, 1993; Osada, et al. 1990 & 1992; Selbie, et al. 1993; Toker, 1998). However, although each subset shows different lipid requirements for activation, all PKC isoforms have an absolute requirement for a co-factor such as a phospholipid, usually phosphatidylserine (PS), in order to be activated (Takai et al, 1979a; Takai et al, 1979b; Nakamura and Nishizuka, 1994). In early studies, PKC was shown to be activated by 'membrane factors' in a calcium-dependent manner (Takai et al, 1979a). Such membrane-associated factors were later exposed to be a phospholipid cofactor such as PS and most importantly, a novel second messenger DAG (Takai et al, 1979b; Kishimoto, et al. 1980). It was revealed that DAG could be generated from PI hydrolysis which may be stimulated by an extracellular agonist (Kishimoto, et al. 1980). Taken together, these data showed that DAG was not merely a by-product of PI turnover but in fact a potent second messenger that utilized PKC as a cellular effector molecule. Thus, PS and DAG were shown to be essential cofactors for PKC regulation and biological functions (Takai et al, 1979b;

Kishimoto, et al. 1980). Following this, it was discovered that PKC was a major target of tumour promoting phorbol esters (Castagna, et al. 1982). For example, phorbol 12-myristate 13-acetate (PMA or occasionally referred to as TPA) was shown to be a potent activator of PKC activity when present with the phospholipid PS (Castagna et al, 1982). This tumour-promoting phorbol ester was shown to contain a DAG-like structure and therefore could activate PKC directly. PMA greatly increases the affinity of PKC for both calcium and phospholipid (Castagna et al, 1982) suggesting a cooperative effect between calcium, DAG and PS for full PKC activation. This discovery showed that such phorbol esters could be used to influence PKC's activation status to uncover substrates and cellular processes regulated by this potent enzyme.

1.6.2 Activation and regulation of PKC

1.6.2.1 Structure, location and phosphorylation

Generally, all PKC family members share a similar primary structure that can be divided into two main regions (Figure 1.5). They all have a highly conserved carboxyl terminal kinase domain that contains a C3 ATP-binding domain and a C4 kinase domain. The C3 and C4 segments are linked by a flexible hinge region to an N-terminal regulatory domain containing C1 and C2 domains which have different lipid and calcium-binding properties therefore giving each subgroup a unique regulatory property (Lee & Bell, 1986; Ono, et al. 1989b; Becker & Hannum, 2005; Toker, 1998). Additionally, the regulatory region also contains an auto-inhibitory pseudosubstrate (ps) sequence. PKC activity is regulated by three highly conserved

serine/threonine residues in the kinase domain and without their subsequent phosphorylation, PKC has little or no activity. The pseudosubstrate binding cavity lacks these critical serine/threonine residues and is instead replaced by an amino acid that cannot be phosphorylated; specifically it is substituted by alanine (House & Kemp, 1987). This sequence, through binding to the substrate-binding site, therefore keeps the PKC in an inactive state in the absence of a suitable allosteric activator (House & Kemp, 1987; Orr, et al. 1992; Kemp, et al. 1994; Orr & Newton, 1994). Moreover, recent data accumulated from conventional isoform studies has shed light on the mechanism by which PKC is activated and thus regulated by lipids at the plasma membrane (Orr, et al. 1992). It is thought that when PKC is bound by PS and DAG in a stereospecific manner, that this interaction results in a conformational change to the enzyme resulting in exposure of the pseudosubstrate region that would usually block the active site of the kinase therefore allowing substrates to bind to this catalytic site (Orr, et al. 1992). Hence, DAG and PS binding to the regulatory domain relieve the auto-inhibition allowing for activation of PKC and phosphorylation of substrates (Orr, et al. 1992). In the presence of calcium, conventional PKCs have an increased affinity for PS as well as a high specificity for PS in the presence of DAG. Thus, enzymatic activity of conventional PKCs has been shown to be highly reliant on the coordinated effect of DAG and PS (Lee & Bell, 1986 and 1989; Newton & Karenen, 1994). Although Orr and co-workers had concluded from their conventional PKC studies that binding of PKC to DAG and PS-containing membranes resulted in the exposure of the pseudosubstrate region which blocks catalytic activity (Orr, et al 1992) the exact mechanism of this synergistic effect is not fully understood. Further studies have thus proposed two models that could

explain such high specificity for PS in the presence of DAG allowing for the conformational change required to expose the pseudosubstrate sequence. One model suggests that through PKC binding PS, a DAG binding site is exposed while another explanation is that binding of DAG exposes a PS-specific binding site (Newton & Karenen, 1994). Through comparing the interaction of a conventional isoform, PKC-α, with monolipid bilayers it has been confirmed that conventional PKCs require calcium for membrane binding as the C2 domain serves as a membrane docking unit. Calcium can then induce a conformational change which allows for a specific binding site for PS to be exposed (Medkova & Cho, 1998a; 1998b). Additionally, PS was then shown to trigger the penetration of PKC into the membrane and by doing so, exposes PKC more efficiently to DAG thus enhancing membrane binding and subsequent PKC activation (Medkova & Cho, 1998a; 1998b). However, using the same approach with a novel PKC isoform, PKC-ε, these experiments showed remarkable differences between membrane-binding and activation requirements other than the obvious differences of calcium dependencies between each subgroup (Medkova & Cho, 1998b). Due to its failure to bind calcium, PKC-ε showed much less specificity for PS therefore the PS-induced penetration of the membrane and subsequent high affinity interaction between PKC and DAG was reduced in comparison to that of conventional PKC membrane-binding (Medkova & Cho, 1998b). Such data suggested that novel isoforms may require another physiological activator (Medkova & Cho, 1998b). Consistent with this, it has been shown that molecules other than PS can induce such conformational change required for membrane binding and DAG targeting in conventional PKCs (Orr & Newton, 1994). Nonetheless, the overall mechanism by which such molecules induce activation of

PKC is through removal of the pseudosubstrate from the active site within the regulatory domain of PKC (Orr & Newton, 1994).

As mentioned previously, Nishisuka and co-workers revealed that phorbol esters were able to activate PKC directly (Castagna et al, 1982). Further studies by other laboratory groups showed that DAG and phorbol esters could compete for a common binding site on PKC (Sharkey, et al. 1984; Hannum & Bell, 1986). This shared site was later defined to be the C1 domain. This domain consists of two closely related tandem zinc finger subdomains (C1a and C1b) that are rich in cysteine and co-ordinate two Zn^{2+} atoms in addition to binding DAG (and specific phorbol ester) (Ono, et al. 1989b; Burns & Bell, 1991; Quest, et al. 1992). This C1 domain is only present in the conventional and novel PKC isoforms. This structure was later confirmed by isolating the crystal structure of the second zinc finger motif of the C1 domain from PKC-δ, when bound or unbound to PMA (Zhang, et al. 1995). This study also revealed that the binding of phorbol ester to the C1 domain did not encourage a conformational change but rather helped to present a hydrophobic surface of the C1 domain to allow its insertion into the membrane (Zhang, et al. 1995). This is consistent with other studies in conventional isoforms where PS binding to its specific domain on PKC resulted in the partial penetration of PKC into the membrane where it could more effectively interact with DAG for activation (Newton & Karenen, 1994; Medkova & Cho, 1998a and 1998b). Most recently, the C1 domains have been redefined where one repeat is classed as C1 and two repeats would be classed as C1A-C1B (Hurley, et al. 1997). In contrast to the conventional and novel isoforms, the atypical isoforms contain a much smaller C1 domain that is 'modified' with only one cysteine-rich domain that is not capable of binding DAG or

PMA (Hurley, et al. 1997). The function of this single zinc finger motif in atypical PKC is currently unknown and it is unsure how atypical PKCs are regulated. However, it has been shown that the atypical isoform PKC-ζ can be activated by ceramide alternatively to DAG-mediated activation (Lozano, et al. 1994). However, it is unknown how or to which domain ceramide binds (Lozano, et al. 1994; Hurley, et al. 1997). In correlation to this, PKC-ζ has also been shown to be activated by both PA and PIP3 (Nakanishi & Exton, 1992; Nakanishi, et al. 1993). Together, these data show that atypical PKCs are still targets of second messengers but they do not specifically interact with DAG. In addition, atypical isoforms ζ and ι/λ have been implicated in protein-protein interactions that may be important for their regulation rather than regulation by DAG or calcium (Diaz-Meco, et al. 1996a; 1996b). It is clear however, that atypical PKCs require PS for activation (Nakanishi & Exton, 1992). Moreover, as discussed earlier, calcium-binding of phospholipids is crucial for kinase activity in conventional PKCs (Lee & Bell, 1989; Newton & Karenen, 1994; Medkova & Cho, 1998b). Therefore, it was assumed that the calcium dependence of conventional PKCs is due to the presence of a C2 domain within the regulatory domain which acts as a calcium sensor (Ono, et al. 1989b; Medkova & Cho, 1998a). The arrangement of the C2 domain in conventional isoforms is such that it follows the C1 domain immediately at the C-terminal end (Ono, et al. 1989b; Mellor & Parker, 1998). This C2 domain is either modified or absent in novel and atypical PKCs, respectively (Ohno, et al. 1988; Ochao, et al. 2001; Sossin, et al. 1993). Novel PKC isoforms are not regulated by calcium therefore it was assumed these isoforms lacked a C2 domain. However, sequence analysis proved that this subgroup had a C2-like sequence patterns that precede the C1 domain (Sossin, et al.

1993). This novel C2 domain lacks key residues that co-ordinate calcium therefore making them insensitive to calcium (Sossin, et al. 1993).

In brief, the phosphorylation and translocation of PKC is essential to its activation (Figure 1.6). The observation that phorbol esters could mimic DAG by penetrating the plasma membrane and binding to PKC (Castagna et al, 1982; Zhang, et al. 1995) showed that through altering the cellular localisation of PKC, this protein kinase could function as an effector for an activator such as DAG or PMA. Thus, the translocation of PKC from the cytosol fraction to the membrane fraction is often representative of its activation and is now widely accepted as the experimental hallmark of PKC activation (Liu et al, 2001; Chang et al, 1997; Ozawa et al, 1993a and 1993b). Additionally, phosphorylation of PKC is critical for its activation. The subsequent activation by second messengers is firstly highly dependent on PKC phosphorylation by both PI-dependent kinase 1 (PDK-1) which phosphorylates residues within the activation loop (Le Good, et al. 1998; Dutil, et al. 1998) and by autophosphorylation at conserved residues in the carboxy terminus (Keranen, et al. 1995). Experiments carried out by Nakhost and colleagues have determined that indeed PKC's enzymatic activity is highly dependent on phosphorylation while its cellular location is critically dependent on sub-cellular binding partners (Nakhost, et al. 1999).

Figure 1.5. Schematic diagram of the structural characteristics associated with the classification of various PKC family members.

The kinase domain or catalytic domain of PKC is highly conserved throughout each subfamily. PKC isoforms are subdivided based on their differing regulatory domains. The conventional PKC isoforms are composed of auto-inhibitory pseudosubstrate (ps) domain, two tandem C1 domains (C1A and C1B) that bind DAG and the calcium-binding C2 domain. The novel PKC isoforms can be regulated by DAG due to the presence of the C1 domain similar to the conventional PKCs but in contrast, this subgroup lacks a calcium-binding motif and instead has an extended N terminal domain for receiving regulatory signals. Lastly, the atypical PKCs lack both a calcium and DAG-binding motif and are thought instead to be regulated by protein to protein interactions.

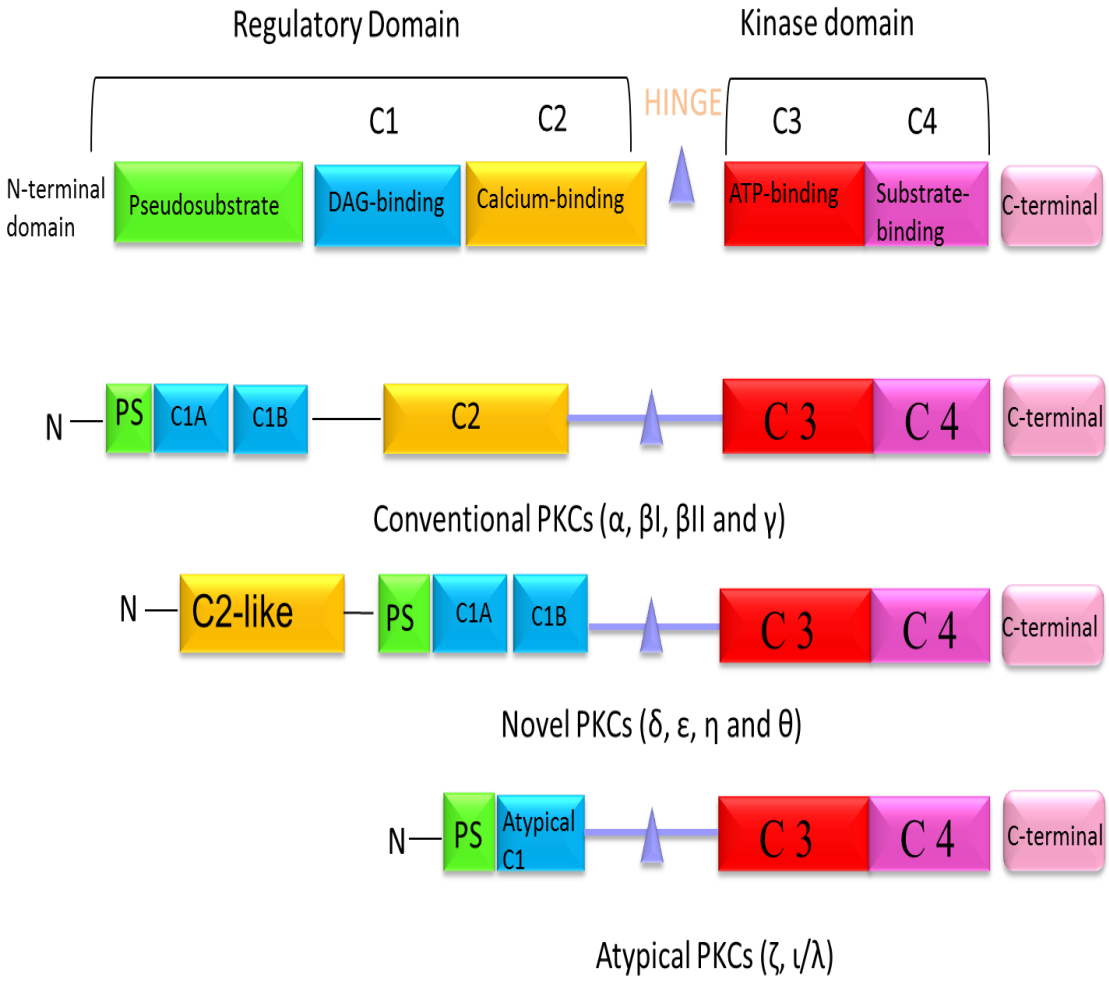
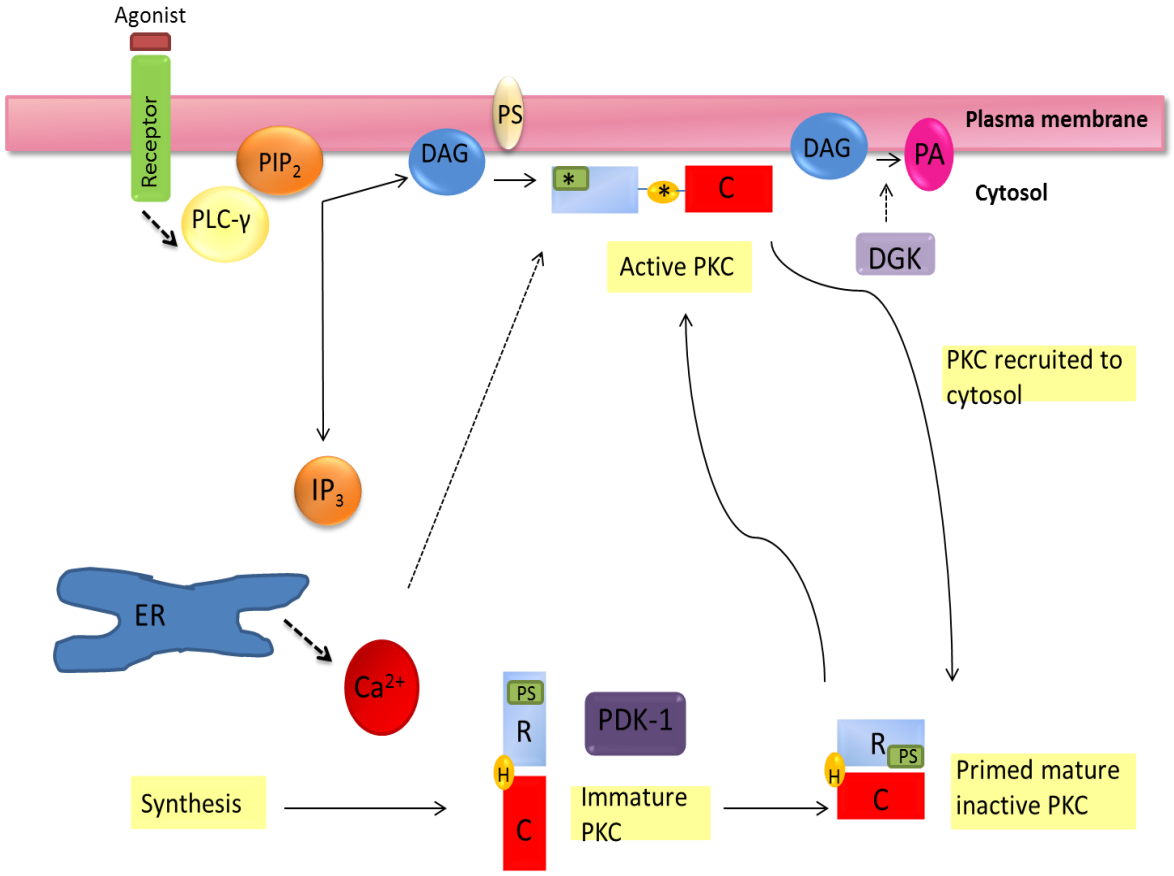

Regulatory Domain
Kinase domain
HINGE
C1
C2
C3
C4
N-terminal domain
Pseudosubstrate
DAG-binding
Calcium-binding
ATP-binding
Substrate-binding
C-terminal
N
PS
C1A
C1B
C2
C 3
C 4
C-terminal
Conventional PKCs (α, βI, βII and γ)
N
C2-like
PS
C1A
C1B
C 3
C 4
C-terminal
Novel PKCs (δ, ε, η and θ)
N
PS
Atypical C1
C 3
C 4
C-terminal
Atypical PKCs (ζ, ι/λ)

Figure 1.6 The classical model of the localization and activation of conventional and novel PKC isoforms.

Following synthesis, PKCs are phosphorylated at key residues including the Activation Loop, which is mediated by PDK-1, and the turn motif and hydrophobic motif. This allows PKC to conform to a closed but enzymatically competent state. It is thought that such phosphorylations occur at the plasma membrane. The ps occupies the substrate-binding site keeping the enzyme inactive and localised to the cytosol where it can readily be activated by the second messenger DAG, or in the case of conventional isoforms, DAG and calcium (Ca^{2+}) (released from IP_3). The binding of an extracellular agonist to the tyrosine kinase-coupled receptor leads to the PLC-mediated hydrolysis of PIP_2 to DAG. Following this, DAG binds to the C1 domain on PKC. Additionally, DAG can then be targeted by DGK to produce PA thus terminating DAG signalling. The binding of second messengers such as DAG and PS is required to release the ps motif from the active site thus activating PKC and allowing access for signalling effectors.

(Adapted from Freeley, et al. 2001; Newton, 1995).



R = Regulatory domain C= conserved Kinase Domain
H= Hine region PS = Pseudosubstrate
* = proteolytically liable exposed hinge or pseudosubstrate

1.6.2.2 Lipid activation

The structural features present within each isoform allow for each PKC family member to be regulated through multiple lipid mediators. So far, the mechanisms for activation and regulation of conventional PKCs appear to be the best understood. Classical PKC activation results from an extracellular agonist binding to tyrosine kinase receptors or G-protein coupled receptors leading to the activation of PLC (Rhee & Choi, 1992). Upon activation, cytosolic PLC is transported to the plasma membrane where it subsequently induces the hydrolysis of membrane inositol phospholipids specifically PIP_2 leading to the generation of membrane bound DAG and soluble IP_3. The latter is responsible for stimulating the release of calcium from intracellular stores allowing for the recruitment of inactive, cytosolic PKC to the plasma membrane. Membrane bound DAG and the acidic phospholipid PS in the presence of Ca2+ can then activate PKC at the plasma membrane (Rhee & Choi, 1992; Ozawa, et al. 1993a and 1993b; Newton & Karenen, 1994; Becker and Hannun, 2005). However, PKC regulation is much more diverse than the classical model of activation. There are many lipid mediators that can stimulate PKC activation and these relationships are highly specific (Bell & Burns, 1991). These lipid mediators activated by membrane phospholipids include IP_3, PIP_3, PA, ceramide, Sphingosine-1-P and free fatty acids (Saito et al, 2002). In particular, the PI3K signalling pathway can also lead to the potent activation of PKCs. The lipid mediator products PI-3-P, PI-3, 4-P_2 (PIP_2) and PI-3, 4, 5-P_3 (PIP_3) from PI3K can activate protein kinases such as protein kinase-1 (PDK-1) and several PKCs including the atypical PKC-ζ and novel PKCs (Saito et al, 2002; Toker, 1998; Nakanishi & Exton, 1992; Nakanishi, et al. 1993). This is further supported by

evidence that PKC-ζ has been shown to specifically require PIP_3 for activation (Nakanishi, et al. 1993), again emphasizing the regulation of PKCs in PI3K signalling pathways (Toker, 1998). Such various lipid requirements for activation of PKCs give an insight into the vast diversity of PKC regulation.

1.6.2.3 PKC and PLD

PKC activation is also regulated through phospholipase D (PLD) (Becker & Hannum, 2005). As mentioned previously, PLD in mammals exists as two isoforms: PLD1 and PLD2 and is generally considered to be located at the membrane (Jenkins & Frohman, 2005; Colley, et al. 1997; Hammond, et al. 1995). PLD is best known to generate PA and subsequently free choline through the hydrolysis of membrane bound PC (Exton, 2002; Jenkins & Frohman, 2005). Additionally, PLD-derived PA can then be converted to DAG by the actions of the enzymes known as PA phosphohydrolases (PAPs) (Sciorra & Morris, 1999; Brindley & Waggoner, 1996). Thus, PLD-specific PA is a significant source of DAG. DAG can then subsequently be converted back to PA by phosphorylation by DAG Kinase (Luo, et al. 2003). Through a transphosphatidylation reaction, PLD can utilize primary alcohols such as 1-butanol to generate phosphatidylbutanol resulting in the inhibition of the formation of the PLD product PA (Ella et al, 1997). This unique reaction can be used as an assay to assess PLD activity. Antigen-stimulated PLD activity can be blocked by both the primary alcohol 1-butanol and through transfection with siRNA for PLD1 and PLD2, through preventing DAG-dependent PKC translocation suggesting that inhibition of PLD and its product PA impairs PKC activation and in turn mast cell

degranulation (Peng & Beaven, 2005). Therefore, PLD plays an essential role in PKC activation and consequent mast cell activation (Peng & Beaven, 2005). This is consistent with other studies using RBL cells where an essential role for both PLD isoforms has been established in mast cell degranulation (Choi, et al. 2002) and thus presumably upstream PKC activation. Most PKC isoforms have been shown to be activated by DAG and translocate from the cytosol to the plasma membrane upon activation (Shearman, et al., 1988; Ozawa, et al. 1993a; Kimata, et al. 1999; Lessman, et al. 2006; Becker & Hannum, 2005). Feng and co-workers showed that upon activation, PKC is initially recruited to the plasma membrane through its C1 and C2 domains before it rapidly returns to the cytosol (Feng, et al. 1998). This brief association of PKC with the plasma membrane has been suggested to be linked to PIP_2-derived DAG and is subsequently followed by trafficking of PKC back to the cytosol which was shown to correlate with autophosphorylation of residues in the carboxy terminus of the enzyme, namely serine 660 (Feng, et al. 1998; Feng, et al. 2000; Becker & Hannum, 2005). Consistent with this, findings in alpha-thrombin-stimulated IIC9 fibroblasts showed that PIP_2-derived DAG was able to activate PKC-α (Leach, et al. 1991). On the other hand, the second phase of DAG generation, which is derived from PLD, has been shown to chronically activate PKC thereby sustaining the presence of this enzyme at the plasma membrane (Ha & Exton, 1993). However, as well as PLD signalling influencing PKC activation, PLD itself has been shown to be a major cellular target of PKC. Although various studies have established that PKC and PLD clearly associate upon activation (Chen & Exton, 2004; Lee, et al. 1997; Min & Exton, 1998) the mechanisms by which PKC regulates PLD still awaits full elucidation and is currently under on-going investigation. In

vitro studies have shown that PLD1 is phosphorylated at specific residues and subsequently activated by PKC-α at specific compartments within the plasma membrane (Kim, et al. 2000). Additionally, activation of PLD1 by the conventional isoforms PKC-α and β has been shown to be ATP-independent and to occur by a non-phosphorylating mechanism (Hammond, et al. 1997; Min, et al. 1998). When ATP was incorporated into the reaction, this resulted in phosphorylation of PLD and subsequent inhibition of the enzyme (Hammond, et al. 1997; Min, et al. 1998). Therefore, taken together, these studies show that there is evidence for a phosphorylation and non-phosphorylation role by PKC in the activation of PLD. It has further been proposed that PLD activation by PKC occurs via direct interaction rather than phosphorylation (Exton, 2002).

1.8 Aims

The aim of this project is to confirm whether PKC isoforms that are targeted by ES-62 are critical for mast cell function and if so, to establish whether absence of the PKC impacts on ES-62 activity. It is intended to use siRNA to knock down these PKC isoforms in mast cells and also to employ mast cells from PKC isoform knockout mice. Monitoring of the effects of PKC isoform loss will be undertaken via measuring cytokine production following FcεRI-mediated or LPS-induced mast cell activation. Some experiments will also be undertaken on LPS-treated macrophages and dendritic cells to determine whether any effects observed are cell-specific. If any PKC isoform is confirmed to be essential for mast cell responses to activation then they can be defined as potential drug targets for the treatment of allergic diseases.

Chapter 2

Materials and Methods

2.1 Reagents

All chemicals were purchased from Sigma-Aldrich, Dorset, U.K and alcohols and acids from Fischer Scientific U.K., Ltd unless otherwise stated. Substrate 4-nitrophenyl-N-acetyl-b-D-glucosaminidase (100MG), Monoclonal anti-DNP IgE antibody produced in mouse, dinitrophenyl albumin (DNP-HSA), Trypan blue, sterile bottled PBS, Lipopolysaccride (LPS) derived from *Salmonella Minnesota*, Sodium pyruvate (100mM) and Hepes (25mM) were purchased from Sigma-Aldrich, Dorset, U.K.

L-glutamine (200mM) and Fetal Calf Serum (FCS) were from Lonza biological plc. Penicillin/Streptomycin was purchased from PAA Laboratories. 2-mercaptoethanol (50 mM), DMEM and RPMI-1640 without L-glutamine were purchased from Gibco (life technologies) Paisley, U.K. Tissue culture suspension culture flasks 75 and 175 cm_2 were purchased from Griener Bio One.

SCF-secreting cell line KLS-C was a kind gift from Dr Xiaoping Zhong, Duke University Medical Centre and produced by the Harnett laboratories at the Universities of Glasgow and Strathclyde. IL-3 secreting cell line TOP3 was a kind gift from Dr Massimo Gadina, NIH and was produced by the Harnett laboratories at the Universities of Glasgow and Strathclyde.

Highly purified, endotoxin free ES-62 was produced from spent culture medium of adult *Acanthochielonema viteae* in the laboratory of Prof. W. Harnett, University of Strathclyde, U.K.

2.2 Tissue culture

2.2.1 Mice

Both male and female BALB/C mice aged between 6-8 weeks old that had been bred and maintained at the University of Strathclyde animal unit were used to generate bone-marrow derived mast cells (BMMC) for certain aspects of this study. Additionally for knockout studies, PKC-α, - β, -θ and –ε knockout mice in conjunction with their age and sex matched wild-type controls were used to generate BMMC, bone marrow-derived macrophages (BMM) and/or bone marrow-derived dendritic cells (BMDC). Each set of PKC knockout mice and their wild-type counterpart were bred and maintained at the specific laboratory group's animal unit before the bones were harvested and shipped to the University of Strathclyde where they were used to generate either BMMC, BMM and/or BMDC.

I would like to thank Michael Leitges of the Biotechnology Centre of Oslo, University of Oslo, for providing bones from PKC-β knockout (KO) and wild-type (WT) control mice (129/Sv/129/Ola background); Alistair Poole, Chris Williams and Graham Britton of the University of Bristol for providing bones from PKC-α (C57Bl/6-Sv129j background) and PKC-θ (B10.PL background) KO and WT control mice and Peter Parker and Tanya Pike from the London Research Institute, Cancer Research UK for providing bones from PKC-ε KO (C57Bl/6Jax) and WT control mice.

All animals used were pathogen- free and all procedures were conducted in accordance with Home Office, U.K. animal guidelines and with the approval of the local ethical committees.

2.2.2 Preparation of progenitor cells from bone marrow

Intact femur and tibia bones were dissected from BALB/c mice (or shipped from collaborator on ice within 24 hours) and soaked in 70% ethanol to remove fibroblasts and connective tissue before the ends of each femur and tibia were snipped and single cell suspensions made from the bone marrow by flushing cold RPMI complete (RPMI 1640 with 10% HI FCS, 2 mM L-gluatmine, 100 U/mL Penicillin and 100 ug/ml streptomycin) through one end of the bone with a 23 gauge sterile needle. The resulting cell suspension was then mixed gently using a 22 gauge sterile needle attached to a syringe to remove any clumps of cells before passing the cell suspension through a sterile BD sieve. The mixture was then centrifuged at 400 g for 5 minutes before the supernatant was discarded and the remaining cell pellet was re-suspended in fresh culture medium and counted using a haemocytometer.

2.2.3 Bone marrow-derived mast cells

BMMC were derived by culture of bone marrow progenitors at 0.5 x 10^6/ mL in RPMI with 10% HI FCS, 2 mM L-glutamine, 100 U/mL Penicillin, 100 µg/ml Streptomycin, 1 mM sodium pyruvate, 10 mM HEPES, and 50 µM β-mercaptoethanol supplemented with conditioned growth medium from KLS-C (1%; SCF) and TOP3 (3%; IL-3) cell lines. Cells were incubated at 37ºC/ 5% CO_2 for 3-6 weeks in total. BMMC were counted and provided with fresh medium and cytokines and transferred to a fresh flask twice a week. After 3-4 weeks maturation, the cells were tested for their expression of the cell surface markers C-kit, FcεRI to ascertain their identity as mature mast cells.

2.2.4 Bone marrow-derived macrophages

To prepare BMM, bone marrow progenitors were cultured for 7 days in 90 mm sterile petri dishes (Sterilin) at 37ºC/5% CO_2 at a cell density of 0.2 x 10^6/ml in Dulbecco's modified Eagle's medium (DMEM) with 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 20% HI FCS supplemented with 20% L929 cell culture supernatant (contains CSF-1). Fresh medium was added on day 4. The cells were harvested at day 7 by gentle scraping and tested for their identity as macrophages by staining for the cell surface markers CD11b and F4/80.

2.2.5 Bone marrow-derived dendritic cells

To prepare BMDC, bone marrow cells were cultured for 6-9 days in 90 mm sterile petri dishes at 37ºC/5% CO_2 at a cell density of 0.2 x 10^6/ml in complete RPMI-1640 supplemented with 10% granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting X63 myeloma cell line-conditioned medium (X63) and 50 µM 2-mercaptoethanol. Fresh medium was provided every 3 days by removing 5 ml of old media from the bottom of the dish and replacing it with fresh media supplemented with 10% X63 and 50 µM 2-mercaptoethanol. After 6-9 days, the cells were harvested by gentle scraping and tested for their surface expression of CD11C and major histocompatibility complex (MHC) class II to identify them as dendritic cells.

2.3 Fluorescence activated cell sorting (FACS) analysis

2.3.1 Staining for surface markers

The presence of cell surface expression of the following markers was confirmed for each cell type by FACS analysis: c-kit and FcεRI on BMMC; CD11b and F4/80 on macrophages and CD11C and MHC II on dendritic cells. Cells were counted (0.1 million cells per sample), washed 1X with cold PBS and re-suspended in 50 µl of FACS buffer (2% BSA (W/V) in 1X PBS) per sample in 5ml polystyrene tubes (Falcon, BD). The aliquots of cells were then incubated with 50 µl of fluorescently-tagged antibodies to confirm the presence of the cell surface markers for 30minutes in the dark on ice. Details of the antibodies used including their dilution, specificity and isotype controls are listed in **Table 2.1**. After labelling, cells were washed a final time with 3 ml FACS buffer at 400 g for 5 minutes before being re-suspended in 250 µl of FACS buffer for flow analysis using a Becton Dickinson BD FACS CANTO™ flow cytometer. Analysis was performed on a minimum of 10, 000 events. Flowjo software (Tree Star Inc., OR, USA, version 7.6.3) was used for final analysis of the flow cytometry data.

2.3.2 Measurement of expression of TLR-4

FACS analysis to determine the presence of TLR-4 on BMMC was carried out in parallel with staining for the cell surface markers of each cell type. The presence of TLR-4 is important for ES-62's effectiveness. As described in 2.3.2, 0.1 million cells in 50 µl of FACS Buffer were incubated with 50 µl of PE-conjugated to anti-TLR-4 or anti-TLR-4/MD-2 complex antibody to detect TLR-4 under the same experimental conditions and treatments. The cells were then analysed for their presence of TLR-4

in conjunction with staining for the other cell surface markers specific for each cell type using a FACS CANTO flow cytometer and Flowjo software. Antibody concentrations and specificities are listed in Table 2.3.

2.4 Stimulation assays

2.4.1 Mast cell stimulation

Unless otherwise stated, mast cells were plated at 1 x 10^6cells/ml on a 12 well sterile tissue culture plate. Cells were then sensitized with anti-DNP IgE (0.5 µg/ml) in the presence or absence of ES-62 (2 µg/ml) or ES-62 alone. Control wells were incubated with medium alone (RPMI complete BMMC medium supplemented with growth factor media 3% TOP3 and 1% KLSC). Cells were incubated for 24 hours at 37ºC/5% CO_2. Following this, samples were collected into 1.5ml sterile eppindorf tubes and centrifuged at 500 g for 5 minutes. The supernatant was then removed and the cells washed twice with sterile PBS (by centrifuging at 500 g for 5 minutes). Cells were then re-suspended in 1 ml of BMMC complete medium supplemented with growth factor medium 3% TOP3 and 1% KLSC.

500 µl of cells were then transferred to the appropriate well in a 24 well plate forming a Basal (unstimulated) and a stimulated well for each experimental condition. Cells were then stimulated by addition of medium alone, 0.5 µg/ml of DNP-HAS to cross-link FcεRI or 1µg/ml of LPS (*Salmonella Minnesota*). Cells were incubated for 24 hours at 37ºC, 5% CO_2. Reactions were terminated after the desired culture period by centrifugation at 500 g for 5 minutes and supernatants

aspirated for determination of cytokine release. The resultant supernatant was stored at –20^{o}C until required.

2.4.2 Macrophage stimulation

Macrophages were scraped in the presence of cold RPMI complete and counted before being plated in triplicate in 96-well plates (100 µL of 2 x 10^6 cells added in triplicate). The cells were rested overnight prior to a pre-incubation step with either ES-62 (2 µg/ml) or medium alone (RPMI complete) for 18 hours at 37ºC, 5% CO_2. Cells were the stimulated with 0.1 µg/ mL LPS (*Salmonella minnesota*) or medium and incubated for a further 24 hours at 37ºC, 5% $CO_{2.}$ Culture supernatants were collected and assayed for cytokines by enzyme-linked immunoabsorbent assays (ELISA) or transferred to fresh plates, covered and stored at –20^{o}C until required.

2.4.3 Dendritic cell stimulation

Dendritic cells were scraped in the presence of cold PBS and counted before 100 µL of 2 x 10^6 cells were plated in triplicate in 96-well ultra-low adherence plates (Costar 3471). The cells were rested overnight prior to a pre-incubation step with either ES-62 (2 µg/ml) or medium alone (RPMI complete) for 18 hours at 37ºC, 5% $CO_{2.}$ Cells were the stimulated with 0.01 µg/ mL LPS (*S. minnesota*) or medium and incubated for a further 24 hours at 37ºC, 5% $CO_{2.}$ Culture supernatants were collected and centrifuged at 400 g for 5 minutes before assayed for cytokines by ELISA or stored at –20^{o}C until required.

2.5 Cytokine analysis

2.5.1 Materials and methods

Cytokine analysis was carried out using ELISA kits from BD Biosciences for IL-6, IL-12, IL-10 and TNF-α, eBioscience for IL-4, and R&D systems for IL-5, IL-1β and IL-13. The concentration of each cytokine in the supernatants obtained from BMMC, macrophages and dendritic cells was determined by cytokine detection kits according to the manufacturer's protocol (as described below in the methods section). The cytokines were detected by biotinylated monoclonal antibodies, Streptavidin horseradish peroxidase (SAv-HRP) and TMB substrate. High-binding 96 well ELISA plates from Greiner bio-one were used for each cytokine ELISA unless otherwise stated.

Wash buffer was prepared using PBS tablets dissolved in distilled water to make up 5 litres of this solution. 0.05% (v/v) Tween 20 was added to complete the washing buffer solution. The coating buffer for TNF-α and IL-6 analysis was 0.1M Sodium carbonate buffer, pH 9.5; for IL-12 and IL-10 analysis was 0.2M Sodium phosphate, pH 9.5; and for IL-13, IL-5, and IL-1β analysis was undertaken with PBS without carrier protein (pH 7.2-7.4). For IL-4 analysis, the coating buffer was ELISA coating buffer powder reconstituted to 1L with distilled water as according to instructions on the manufacturer's protocol.

The assay diluents for IL-6, TNF-α, IL-10, IL-12 and IL-4 were prepared by adding 10% fetal calf serum (FCS) to 1X PBS. Reagent diluents for IL-13 and IL-5 were prepared by adding 1% BSA to 1X PBS and reagent diluent for IL-1β was made by adding 0.1% BSA and 0.05% Tween-20 to Tris-buffered saline. For the Tris-buffered

saline, 4.383g of NaCl (Final conc: 150mM) and 1.2114g of Trizma base (Final conc: 20nM) were dissolved in distilled water to make up 500ml of this solution. Block buffer for IL-1β was made by adding 1% BSA to 1X PBS.

TNF-α, IL-6, IL-12, IL-10 and IL-4 capture antibodies were used at a concentration of 1:250 dilutions in their coating buffer. IL-13, IL-1β and IL-5 capture antibodies were used at a concentration of 1:180 dilutions in PBS without carrier protein. The detection antibodies for IL-13, IL-1β and IL-5 were used at a concentration of 1:180 in Reagent diluent.

Streptavidin (SAv-HRP) was used at a working concentration of 1:200 dilutions for cytokines IL-5, IL-13 and IL-1β as according to the manufacturer's protocol. For IL-6, IL-12, IL-10 and TNF-α SAv-HRP was used at a concentration of 1:250 dilution in assay diluent according to the manufacturer's instructions. For IL-4, Avidin-HRP was used at a concentration of 1:250 dilution.

Substrate solution for the reaction for all cytokines was KPL Sureblue, TMB Microwell Peroxidase Substrate. The reaction was terminated by stop solution, 2N H_2SO_4. The final reactions were measured at 405 nM in an ELISA plate reader.

2.5.2 ELISA procedure

For every incubation step, ELISA plates were covered with cling film and following each washing step, ELISA plates were dried by blotting on blotting paper.

Capture antibody was prepared using the appropriate coating buffer before adding 50 µl to a high-binding 96 well plate and incubating overnight at 4^{o}C for IL-6, IL-12, IL-10, TNF-α and IL-4 or room temperature for IL-13, IL-5 and IL-1β. Plates were then washed following an 18 hour incubation 3 times with wash buffer before blocking with 200 µl of Assay Diluent (IL-4, IL-12, IL-10, IL-6, and TNF-α) or Reagent Diluent (IL-13, IL-5) or block buffer (IL-1β) and incubating at room temperature for one hour. Plates were then washed as before with wash buffer. For all cytokines, 50 µl of sample was added in triplicate to each well in the prepared ELISA plate. For IL-6, IL-12, IL-10 and TNF-α, standards were used at a starting concentration of 1000 pg/ml followed by serial doubling dilutions in assay diluent to generate a standard curve. For IL-1β, IL-5 and IL-13, standards were used at a starting concentration of 1000 pg/ml, 2000 pg/ml and 4000 pg/ml respectively, followed by serial doubling dilutions in their respective Reagent Diluent. For IL-4, a top standard of 400 pg/ml was used followed by serial doubling dilutions in assay diluent. Each ELISA plate was incubated for 2 hours at room temperature before being washed 5 times with wash buffer and dried using blotting paper.

In the case of IL-6, IL-10 and IL-12 50 µl of detection antibody and SAv-HRP was added together as instructed by the manufacturer's protocol and incubated at room temperature for one hour. Elisa plates were then washed 7 times in wash buffer (with 30 second to 1 minute soak). 50 µl of substrate solution was then added and the plates incubated for 30 minutes at room temperature in the dark. 25 µl of Stop solution was then added and the reactions measured at 450nm by an absorbance plate reader. For TNF and IL-4, 50 µl of diluted detection antibody was added to the plates and incubated for one hour at room temperature. For IL-13, IL-5 and IL-1β, 50 µl of

detection antibody was added to the plates and incubated at room temperature for 2 hours. The plates were then washed 5 times with wash buffer. 50 µl of SAv-HRP was then added to the plates for 30 minutes for TNF and 20 minutes for IL-5, IL-13 and IL-1β. 50 µl of Avidin-HRP was added for 30 minutes at room temperature for IL-4. Elisa plates were then washed 7 times (30 second to 1 minute soaks) before 50 µl of substrate solution was added to each well and incubated for 20-30 minutes at room temperature in the dark. 25 µl of stop solution was then added to each well and the reactions measured at 450nm by an absorbance plate reader. The limit detection for all cytokines was no higher than 15pg/ml. The cytokine concentration was determined using the absorbance values obtained for the standards.

2.6 BMMC degranulation assay

The level of degranulation for BMMC was determined using a modified colorimetric assay to assess the release of β-hexosaminidase , a marker of mast cell degranulation. 1 x 10^6 BMMC were incubated in a 96-well ELISA plate at 37ºC overnight (18 hours) in the presence or absence of mouse anti-DNP IgE (0.5 µg/ml) diluted in Tyrode's salt solution supplemented with 1% FCS. Following this step, cells were washed twice by centrifuging at 400G for 5 minutes in PBS (1X) before re-suspended in 200 µl of Tyrode's buffer supplemented with 1% FCS. BMMC were then stimulated with DNP-HSA (1 µg/mL) to induce FcεRI cross-linking or medium alone for one hour at 37ºC. Reactions were terminated by centrifugation (400 g; 5 minutes) and 50 µL aliquots of supernatants assayed for release of β-hexosaminidase and normalised to total cellular β-hexosaminidase following cell lysis by the addition

of 1% Triton X-100 and by incubation with substrate (1mM *p*-nitrophenyl *N*-acetyl-β-$_D$-glucosaminide) in 200 µl 0.05M Citrate Buffer, pH 4.5. After one hour incubation in the dark at 37ºC, the reaction was quenched by mixing 62.5µl of each sample with 125µl of Sodium bicarbonate (0.1M) before measuring the optical density (OD) of the enzyme concentration at 405nm.

The % of β-hexosaminidase release was calculated by the formula:

% = OD of the supernatant / OD of supernatant + OD of total cellular release) X 100

2.7 Transfection of BMMC with siRNA using HiPerfect Transfection Reagent

Following optimization of siRNA conditions such as BMMC cell number, siRNA concentration, HiPerfect Transfection Reagent volume and incubation times of siRNA/HiPerfect complexes the protocol described below was followed for each experiment.

BMMC were counted, washed with 1 X PBS by centrifugation (500 g 5 minutes) and re-suspended in 200 µl of BMMC medium containing serum and antibiotics without growth factors in a 24-well sterile tissue culture plate. 5 nM siRNA was reconstituted in 500 µL of distilled water to obtain a 10 µM working solution. The 10 µM siRNA solution (QIAGEN, UK) was then diluted in 200 µl of culture medium without serum and antibiotics to give siRNA stock solutions of e.g. 600 nM, 1.2 µM and 3 µM. At the end of the experiment this will give final siRNA concentrations of e.g. 100 nM,

200 nM and 500 nM. A siRNA + HiPerfect master-mix was created by adding 12 µl of HiPerfect Transfection Reagent (QIAGEN, UK) to the siRNA stock concentrations. The master-mix was vortexed and incubated at room temperature for 10 minutes to allow complexes to form. Following this, 212 µl of the master-mix was added drop wise onto the cells in the appropriate wells. The plate was then gently swirled upon each drop to ensure uniform distribution. 212 µl of RPMI was added to cells alone as a control and 200 µl of RPMI + 12 µl of HiPerfect were added to the HiPerfect control well. The cells with Transfection complexes were then incubated under normal growth conditions (37°C, 5% CO_2) for 6 hours. Following this, 800 µl of culture medium containing serum and antibiotics supplemented with growth factor medium (3% TOP3 and 1% KLSC) was added to the cells to give a final volume of 1.2 ml and final siRNA concentrations of e.g. 100, 200 and 500 nM siRNA. Cells were then incubated under normal growth conditions for 48 hours. Following the 48 hour incubation, the cells are washed 1 X with PBS by centrifugation (400 g, 5 minutes). The supernatant was discarded and the cell pellet lysed to make total protein extracts before each sample was monitored for gene silencing using SDS-PAGE electrophoresis and Western blot analysis.

2.8 Preparation of total cell protein extracts

Mast cells, macrophages and dendritic cells were all washed in cold 1 X PBS by centrifugation (400 G; 5 minutes) to terminate any reactions and/or to remove serum before cell lysis. Untreated or stimulated cells (as described previously in section 2.4 and 2.7) were lysed by the addition of 20-40 µL (Cell number dependent) of

modified Radio-Immunoprecipitation Assay (RIPA) buffer (RIPA Buffer from SIGMA, USA). Modified RIPA buffer consisted of 100X HALT™ Protease and Phosphatase Inhibitor cocktails (Thermo Scientific, IL, USA) diluted 1 in 100 in plain RIPA buffer. After re-suspending the cell pellet in modified RIPA buffer, cells were incubated on ice for 15 minutes to allow solubilization before centrifugation at 5000 g for 10 minutes. The supernatants containing the whole cell lysates were transferred to fresh tubes and stored at -80˚C for long term storage or -20˚C for short term storage before being analysed by SDS-PAGE gel electrophoresis and Western Blot analysis.

2.9 Western blotting

2.9.1 Materials and methods

All western blot equipment was supplied by Invitrogen unless stated otherwise.

Modified RIPA buffer was prepared by adding 485ul of RIPA buffer solution (SIGMA, USA), 5 µl of 100 X HALT™ Protease inhibiter cocktail, 5 µl of EDTA and 5 µl of HALT™ Phosphate inhibitor cocktail to make a 500 µl solution. This was prepared fresh and stored on ice.

BCA protein assay reagent was prepared by diluting BCA Reagent B in BCA Reagent A in a 1 in 50 dilution, as instructed by the manufacturer's protocol (Thermo Scientific Pierce). BCA protein assay Albumin ampule standards were diluted as instructed by the manufacturer's protocol in water or plain RIPA Buffer and stored at -20℃.

NuPAGE® MOPS SDS Running buffer (20X) was diluted 1 in 20 to make a 500 mL solution. Transfer buffer (1X) was prepared by diluting NuPAGE® Transfer buffer 1 in 20 and supplementing with 10% Methanol to make a 200 mL solution for one blot or 20% methanol for two blots.

2.9.2 Procedure

2.9.2.1 SDS-PAGE gel electrophoresis

All buffers were made up with distilled water and sample lysates diluted with either distilled water or with plain RIPA where appropriate unless otherwise stated.

Equal protein loadings of cell lysates or control extracts (30-40 µg per lane for BMMC unless stated otherwise) determined by BCA protein assay (bicinchoninic acid, Thermo Scientific Pierce) were resolved using XCell *surelock* Mini-cell kit with NuPAGE® Novex® high-performance precast 4-12% Tris-Bis gels and NuPAGE® buffers and reagents. The appropriate volume of 4X NuPAGE® LDS sample buffer and 10X NuPAGE® Reducing agent were added prior to heating samples to 70 ℃ for ten minutes and samples were resolved using NuPAGE® Bis-Tris gels (4-12%) with NuPAGE® MOPS running buffer at 200V for 50 minutes following the manufacturer's instructions. Proteins were then transferred onto a Nitrocellulose membrane (Amersham) using NuPAGE® transfer buffer supplemented with 10% methanol at 30V for 1 hour. The manufacturer's instructions and protocol were followed for setting up the transfer apparatus and transfer stack.

2.9.2.2 Western Blot analysis

All buffers and samples were made up with distilled water and all antibodies were diluted in TBS-Tween with 5% non-fat milk (Marvel) or 5% BSA (antibody dependent). Each washing step consisted of 3 x 5 minute washes with TBS-Tween (0.5M NaCl and 20 mM Tris pH 7.5 with 0.1% Tween-20).

Following transfer, nitrocellulose membranes were stained with Ponceau Red to validate successful transfer of proteins. Membranes were then washed and non-specific binding sites blocked for one hour in TBS-Tween containing 5% non-fat milk protein. Membranes were then incubated with the appropriate primary detection antibody overnight at 4℃ on a shaker. Following primary antibody incubation, nitrocellulose membranes were washed as before and incubated in the appropriate HRP-conjugated secondary antibody (diluted up to 1: 2500 in wash buffer containing 5% non-fat milk) for 1-2 hours at room temperature. Nitrocellulose membranes were then washed as before and protein bands visualised using the NOVEX® ECL HRP Chemiluminescent detection system (Invitrogen, USA). For this, nitrocellulose membranes were incubated in a mixture of equal volumes of ECL HRP Substrate Reagent A and B for 1 minute before exposing membranes to UltraCruz™ Autoradiography film (Santa Cruz biotechnology Inc, USA). The nitrocellulose films were often stripped either at room temperature for 1 hour in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris pH 7.5). Nitrocellulose membranes were washed thoroughly in TBS-Tween before re-starting the Western

blotting protocol. All antibodies and the dilutions utilised in this experimental procedure are listed in **Table 2.2**.

2.10 Densitometry and statistical analysis

The data presented are representative of the stated number of individual experiments involving the indicated statistical analysis using Graphpad Prism software. The statistical analysis for cytokine production was performed in Graphpad PRISM using either a one way ANOVA or a two way ANOVA where * $P<0.05$ ** $P<0.005$ *** $P<0.001$ **** $P<0.0001$. Densitometry was performed using the program ImageJ (NIH).

Table 2.1 Antibodies utilized in FACS analysis

Marker	Dilution	Conjugate	Clone (ID)	Manufacturer	Isotype
FcεRI	1/250	FITC	MAR-1 (11-5898-85)	eBioscience	Armenian hamster IgG
CD117 (C-kit)	1/250	APC	2B8 (17-1171-81)	eBioscience	Rat IgG2bκ
TLR-4	1/100	PE	76B357.1 (ab45104)	Abcam	Mouse IgG2b monoclonal
F4/80	1/50		BM8 (11-4801-81)	eBioscience	Rat IgG2a
CD11b	1/50	Alexa Fluor	M1/70 (557686)	BD Biosciences	Rat IgG2bκ
CD11C	1/50	FITC	HL3 (557400)	BD Biosciences	Hamster IgG1 λ1
MHC II	1/50	APC	M5/114.15.2 (17-5321-81)	eBiosciences	Rat IgG2bκ

CD284/MD-2 (TLR-4/ MD-2 complex)	1/50	PE	MTS510	BD Biosciences	Rat IgG2aκ

Antibodies used to stain for surface markers and TLR-4 on mast cells, macrophages and dendritic cells. All antibodies recognise/react with mouse species.

Table 2.2 Antibodies utilized in western blotting studies.

Specificity	**Host**	**Clone (ID)**	**Dilution**	**Manufacturer**
PKC-α	Rabbit	Ab32376 (Y124)	1/6000	Abcam
PKC-β	Rabbit	NBP1-58914	1/4000	Novus Biologicals
PKC-ε	Rabbit	22B10	1/1000	Cell signalling Technology
PKC-θ	Rabbit	2059S	1/1000	Cell signalling Technology
β-actin	Mouse	C4 (SC-47778)	1/1000	Santa Cruz Biotechnologies
Secondary antibodies	**Host**		**Dilution**	**Manufacturer**
Anti-rabbit HRP	Goat	7074	1/2000	Cell signalling Technology
Anti-mouse HRP	Horse	7076	1/2000	Cell signalling Technology

Chapter 3

Results

3.1 Mast cells

Mast cells originate from hematopoietic stem cells (HSC) (Kitamura, et al. 1977). These bone marrow-derived mast cell progenitors (Franco, et al. 2010; Chen, et al. 2005) do not mature before departing the bone marrow but instead migrate through the blood to vascularized tissues or serosal cavities where they undergo local differentiation and maturation (Galli, et al. 2005; Galli, et al. 2011; Moon, et al. 2010). The completion of development of bone marrow-derived mast cell precursors in tissues (Rodewald, et al. 1996) along with their diverse distribution gives rise to the heterogeneity of this cell population (Galli & Tsai, 2012). Generation of phenotypically distinct populations of mast cells is thought to be greatly influenced by site-specific microenvironment conditions such as the presence of cytokines (IL-4, IL-5 and IFN-γ), hormones and reactive radical species (Moon, et al. 2010; Galli, et al. 2011; Galli, et al. 2005; Prussin and Metcalfe, 2003). However, mast cell activation and functional responses are largely determined by the stimuli they receive (Moon, et al. 2010). Mast cells can participate in a wide variety of physiological and pathological processes through their release of a vast array of biologically active products (Moon, et al. 2010; Kalesnikoff, et al. 2008; Galli, et al. 2011; Galli, et al. 2008). For example, classical activation through IgE-dependent cross-linking of the high-affinity IgE receptor, FcεRI, subsequently results in rapid (minutes) mast cell degranulation leading to the exocytosis of pre-formed mediators such as cytokines, histamine, heparin, prostaglandins and leukotrienes into the extra-cellular environment along with the de novo synthesis of cytokines which exhibit a more delayed (hours) response (Metcalfe, et al. 1997; Kalesnikoff, et al. 2008; Prussin and Metcalfe, 2003). Furthermore, other factors such as cytokines, the bacterial cell wall

component LPS, parasite-derived molecules and allergic stimuli can directly activate mast cells independently of FcεRI (Metcalfe, et al. 1997; Mekori and Metcalfe, 2000). This suggests that mast cells not only play a vital role in the inflammatory response, including inducing allergic reactions, but that they are also required for resolution of bacterial and parasitic infections (Abraham and St.John, 2010; Peavy and Metcalfe, 2008; Galli, et al. 2008).

Mature mast cells have been shown to be long-lived effector cells that can maintain their ability to proliferate in response to appropriate signals (Abraham, et al. 1997; Abraham and St. John 2010). Due to the naturally limited number of mast cells in tissue, isolation of in vivo mature mast cells is highly challenging (Moon, et al. 2010; Arock, et al. 2008). For this reason, it has been necessary to develop means of culturing both human and mouse mast cells in vitro. For example, less than 5% of the cell population from a peritoneal wash is represented by mast cells (Malbec, et al. 2007) but these cells can be expanded in vitro with SCF to create a large population of peritoneal-derived mast cells (PDMCs). These PDMCs are serosal-type mast cells which retain many features attributed to mature mast cells (Malbec, et al. 2007; Galli, et al. 2011). On the other hand, BMMC arise from bone-marrow HSC which are cultured in the presence of both IL-3 and SCF (Tsuji, et al. 1991; Metcalfe, et al. 1995; Dvorak, et al. 1994). These mast cells are often used to mimic MMC (Gurish and Boyce, 2006) however; they are now thought to possess a phenotype similar to immature mast cells (Malbec, et al. 2007; Moon, et al. 2010).

3.1.1. Cell surface expression of C-kit and FcεRI – are the mast cells truly mast cells?

Mast cells are regularly identified by flow cytometry for the cell surface mast cell markers SCF ligand, c-kit, and the high affinity IgE receptor, FcεRI (Chen, et al. 2005; Dvorak, et al. 1994). Mucosal-type BMMC cultured from mice were chosen as the basis for experiments as their primary mast cell characteristics would perhaps mimic human mast cells more closely than a mast cell line (Moon et al, 2010). Furthermore, unlike BMMC, some mast cell lines lack FcεRI rendering them unsuitable for addressing many physiological questions (Moon, et al. 2010).

BMMC were obtained from the femurs of 5-8 week old Balb/c mice. The mast cell progenitors were expanded in vitro in RPMI-1640 complete medium supplemented with 3% Top3 and 1% KLSC conditioned medium-derived growth factors representing IL-3 and SCF respectively to produce mature mast cells over a 4 week period. Flow cytometry analysis shows that mast cells are clearly identifiable on the basis of their forward and side scatter properties and their strong c-kit and FcεRI expression (gating relative to isotype control). On average, mast cell cultures were 89% double positive for c-kit and FcεRI. The BMMC culture example shown was 83.9% double positive for c-kit and FcεRI (Fig. 3.1.1).

3.1.2 Analysis of TLR-4 expression on BMMC

It is well documented that ES-62 activity is critically dependent on TLR-4 as this filarial nematode molecule exerts its effects via subversion of TLR-4 signalling (Goodridge, et al. 2005 a; Goodridge, et al. 2005 b). Therefore, it was important to determine if at week 4 (when the cells generally reach maturity) the BMMCs expressed TLR-4. Thus, in addition to c-kit and FcεRI, the cells were stained for the presence of this Pathogen Recognition Receptor (PRR). Previous studies have shown that BMMC express many TLRs including TLR-4 (McCurdy, et al. 2001) and therefore as predicted, the BMMCs produced for experiments expressed TLR-4 on their surface (Figure. 3.1.2). For the BMMC culture example shown, 83.7% of cells expressed TLR-4 on their surface. The mean fluorescent intensity (MFI) for TLR-4 expression for isotype control and BMMC was 82.5 and 1887, respectively.

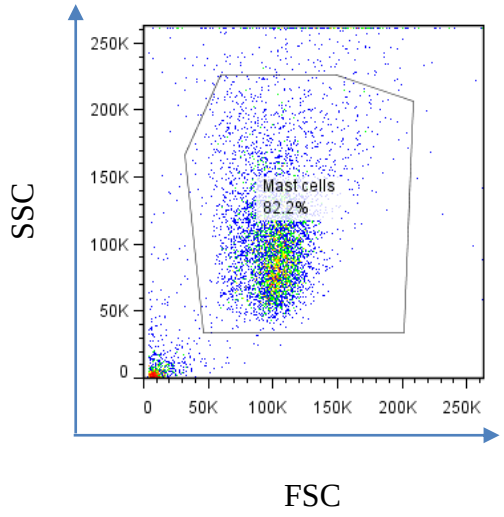
Figure 3.1.1. Flow cytometric analysis of BMMC for expression of mast cell surface markers c-kit and FcεRI

A) Dot plot analysis of forward scatter (FSC) versus side scatter (SSC) allowing gating of live cells on the basis of their size and granularity, respectively.

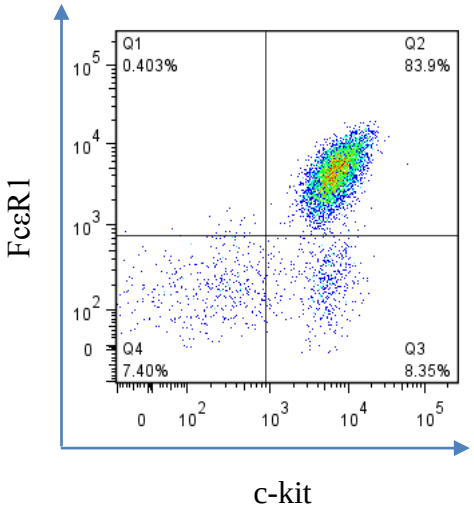
B) Mast cells gated as described in panel A) were examined for their expression of c-kit and FcεRI relevant to appropriate isotype controls (panel C). Expression of c-kit (x-axis) was determined by staining with an APC-conjugated anti-mouse CD117 (c-kit) antibody gated relative to its isotype control (APC-conjugated rat anti-mouse IgG2b; panel C). Expression of FcεRI (y-axis) was determined by staining with FITC-conjugated anti-mouse FITC FcεRI antibody gated relative to its isotype control (FITC-conjugated Armenian Hamster IgG; panel C).

C) Dot plot showing setting of gates for c-kit + and/or FcεRI + cells on the basis of staining of live mast cells (as gated in panel A) with relevant isotype controls.

A.


SSC
FSC
250K
200K
150K
100K
50K
0
0
50K
100K
150K
200K
250K
Mast cells
82.2%

B.


FcεR1
c-kit
Q1
0.403%
Q2
83.9%
Q4
7.40%
Q3
8.35%

C.

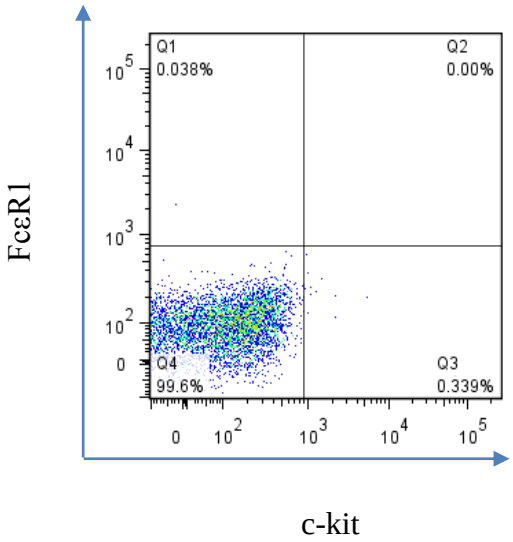
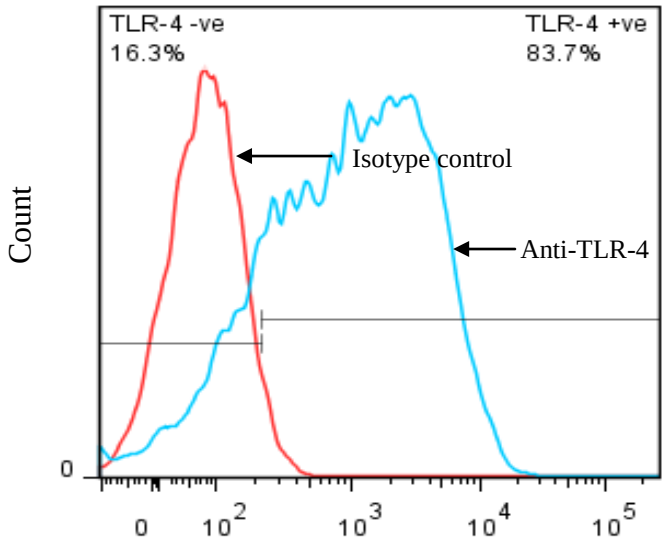

FcεR1
c-kit
Q1
0.038%
Q2
0.00%
Q4
99.6%
Q3
0.339%

Figure 3.1.2 Flow cytometric analysis of TLR-4 expression on BMMC

c-kit + FcεRI+ BMMC gated as described in Figure 3.1.1 were analysed for their expression of TLR-4 using a PE-conjugated mouse monoclonal antibody to TLR-4 relative to an isotype control PE-conjugated mouse IgG2b antibody. Data are presented as a histogram plot of fluorescence intensity of TLR-4 expression (x-axis) versus cell number (count; y-axis). 83.7% of BMMC express TLR-4.


TLR-4 -ve
16.3%
TLR-4 +ve
83.7%
Isotype control
Anti-TLR-4
Count
0
0
10^2
10^3
10^4
10^5

TLR-4

3.2 Selection of an assay for measuring BMMC activation

The key aims of this project were to investigate the role played by individual PKC isoforms in mast cell activation and whether ES-62's mechanism of action is dependent on targeting them. Previously, the effects of ES-62 on human mast cell degranulation had been shown through the use of a degranulation assay involving the cross-linking of anti-DNP IgE antibodies and subsequent FcεRI-induced release of β-hexosaminidase, a mast cell degranulation marker (Melendez, et al. 2007). The release of this granule-stored enzyme was measured on addition of the substrate p-nitrophenyl N-acetyl β-D-glucosaminide. However, studies have reported that different mast cell subsets such as serosal and mucosal-type mast cells, show differential functional responses and these responses can be influenced by both the microenvironment and the stimuli received (Galli, et al. 2011). For instance, PDMC contain more heparin-containing granules and therefore can elicit a stronger degranulation response than BMMC, which primarily produce chemokines and cytokines (Galli, et al. 2011). Recent work from this research group has also produced evidence that the murine mast cell populations PDMC, mucosal-type BMMC and connective tissue-type mast cells (CTMC) exhibit differential responses when stimulated with either antigen (FcεRI cross-linking) or directly with LPS (Ball, et al. 2013). Indeed we found that mature serosal-type mast cells (PDMC) retain the ability to degranulate in response to FcεRI cross-linking but not LPS whereas by contrast, BMMC were weak degranulators but produced cytokines in response to both stimuli (Ball, et al. 2013). PDMC were also weak producers of cytokines in response to both stimuli (Ball, et al. 2013). Therefore, in light of these recent data, it was not surprising that testing of degranulation by BMMC produced inconsistent

results (results not shown) and often displayed low levels of β-hexosaminidase release (Figure 3.2). It would be expected that FcεRI-triggered β-hexosaminidase release would be around 30-40% in BMMC (Razin, et al. 1983) without pre-treatment with ES-62. However, degranulation levels of around 20% were found in the experiments undertaken with BMMC (Figure 3.2). Thus, based on the finding that mucosal-type BMMC seem to successfully secrete cytokines in response to both FcεRI and LPS/TLR-4 signalling (Ball, et al. 2013), it was decided to employ an assay to detect cytokine levels as a measure of BMMC activation.

The remarkable spectrum of cytokines released from mast cells contributes largely to their likely involvement in both pro-inflammatory and regulatory immune functions (Galli, 1993; Mekori and Metcalfe, 2000). These cytokines include IL-1, IL-3, IL-4, IL-5, IL-6, IL-13 and TNF-α (Gordon, et al. 1990; Burd, et al. 1989; Burd, et al. 1995; Gordon and Galli, 1990; Lorentz, et al. 2000). Not only have these mast cell-derived cytokines been shown to be generated via FcεRI-bound IgE-cross-linking (Burd, et al. 1989; Gordon, et al. 1990), but they are now being further dissected to show their effect in both pathological conditions and on other cells of the innate and adaptive immune response (Galli, 1993). For example, both IL-4 and IL-13 have been shown to be secreted by IgE-activated mast cells (Burd, et al. 1995; Bradding, et al. 1992) with the release of such cytokines being reported to stimulate IgE production (Burd, et al. 1995; Coffman, et al. 1986; Pene, et al. 1988; Minty, et al. 1993). Additionally, the mast cell generated cytokines IL-3 and IL-9 (Burd, et al. 1989; Wodnar-filipowicz, et al. 1989; Godfraind, et al. 1998) have been shown to be important mast cell developmental/growth factors (Godfraind, et al. 1998; Stassen, et al. 2001) with IL-3 often being the critical factor for successfully culturing mast cells

in vitro (Tsuji, et al. 1991; Dvorak, et al. 1994; Lantz, et al. 1998). IL-5, which has been shown only to be secreted by mast cells following receptor-induced activation (Lorentz, et al. 2000), has been associated with eosinophil proliferation and activation (Takafuji, et al. 1991). A study by Lorentz et al also showed that a feature of inflammatory conditions such as inflammatory bowel disease was the production of IL-5 from mast cells (Lorentz, et al. 1999). Moreover, both IL-6 and TNF-α produced by mast cells have also been implicated as major factors contributing to the pathology associated with inflammatory responses (Mekori and Metcalfe, 2000). An important study by Gordon and Galli (Gordon and Galli, 1990) offered new insights into mast cells as a source of TNF-α. Thus, this group showed that both murine peritoneal mast cells and BMMC constitutively expressed TNF-α bioactivity and that activation of these cells by antigen-induced IgE receptor aggregation resulted in the further release of TNF-α in conjunction with increased levels of both TNF-α mRNA and bioactivity present within the cells (Gordon and Galli, 1990). These data identified mast cells as not only a cell type that is capable of secreting TNF-α upon stimulation (IgE or other), but as an important source of preformed stored TNF-α which may be released essentially instantaneously (within 60 minutes) without the need for synthesis (Gordon and Galli, 1990). Further analysis showed that IgE-stimulated mast cells (peritoneal and cultured) gave rise to immediate secretion of TNF-α, which was already preformed within the cell in addition to a sustained release of newly synthesized TNF-α (Gordon and Galli, 1991). Together, these unique findings propose that the rapid and sustained release of this mast cell-derived cytokine from both preformed and inducible stores could contribute to biological responses dependent on TNF-α such as inflammation, allergic responses, asthma and

even host defence (Gordon and Galli, 1990; Gordon and Galli, 1991; Wershil, et al. 1991; Bradding, et al. 1994). More specifically, mast cell-derived TNF-α has been shown to be a major contributor to the pathogenesis of mast cell/IgE-dependent allergic inflammation and airway hyperactivity in C57BL/6J mice (Nakae, et al. 2007). Another important cytokine that was demonstrated to be derived from mast cells following IgE and antigen stimulation is IL-6 (Burd, et al. 1989). This cytokine has been implicated in the pathology associated with allergic rhinitis (Bradding, et al. 1993). An approach using immunohistochemistry implicated IL-6 in the pathology associated with allergic rhinitis as this cytokine was localized to mast cells in the nasal mucosa of rhinitic patients (Bradding, et al. 1993). Similar to TNF-α, data obtained have supported a pivotal role for IL-6 production by mast cells in the inflammatory characteristics of asthma (Bradding, et al. 1994). Furthermore, ES-62 inhibited the receptor- induced production of the pro-inflammatory cytokines IL-6 and TNF-α (as well as IL-3) in human mast cells (Melendez, et al. 2007). In lieu of these studies, it was decided to investigate the effect of both the PKC isoform knockout and if applicable, ES-62, on TNF-α and IL-6 production by BMMC. Thus, as a first step, IL-6 and TNF-α cytokine production was measured following IgE-induced FcεRI cross-linking in BMMC and both were found detectable in the culture medium (data not shown). Following this, the effect of ES-62 on BMMC activation was investigated in a similar manner.

3.2.1 Effect of ES-62 on cytokine production from BMMC

Similarly to its effect on mast cell degranulation, ES-62 was shown to inhibit the production of IL-6, TNF-α and IL-3 in human mast cells, but had no effect on certain TH2 type cytokines such as IL-13 (Melendez, et al. 2007). However, the effect of ES-62 on primary mast cells cultured from mice was unknown. ELISAs were therefore used to measure the production of the pro-inflammatory cytokines IL-6 and TNF-α along with IL-13, IL-5, IL-4 and IL-1β and a comparison made of control and ES-62 pre-treated cells. From repeated experiments, it was clear that ES-62 could consistently reduce the production of pro-inflammatory cytokines TNF-α and IL-6 (Figure 3.2.1 A and B). Perhaps surprisingly given the previous data showing no effect on IL-13 in human mast cells (Melendez, et al. 2007) ES-62 was also found to suppress the production of this Th2-associated cytokine in mucosal-type BMMC (Figure 3.2.1 C). The effect of ES-62 on other Th2 cell cytokines produced by BMMCs namely IL-5, IL-4 and in addition, IL-1β could not be determined as no cytokine was detected in each case (data not shown). A similar result was also observed in the human mast cell data obtained with ES-62 treatment with respect to IL-4 (Melendez, et al. 2007).

Figure 3.2 Measurement of the degranulation marker, β-hexosaminidase, following BMMC activation

BMMC were sensitized with 1 µg/ml of anti-DNP IgE before cross-linking with 1µg/ml DNP-BSA then incubated for 1 hour at 37˚C. Degranulation was measured by granule stored enzyme release of β-hexosaminidase, a marker of mast cell degranulation. Culture supernatants containing enzyme were incubated with 200 µl of 1 mM p-nitrophenyl N acetyl β-D-glucosaminide (substrate) for 1 hour at 37˚C before optical density was measured at 405nm. The cells were then lysed with 1% Triton X- 100 to release the remaining β-hexosaminidase in the cells and the % degranulation calculated. The data represent the average of triplicate readings in a single experiment. IgE XL = sample that was sensitized with anti-DNP IgE then cross-linked with antigen (DNP-BSA). N = 1.

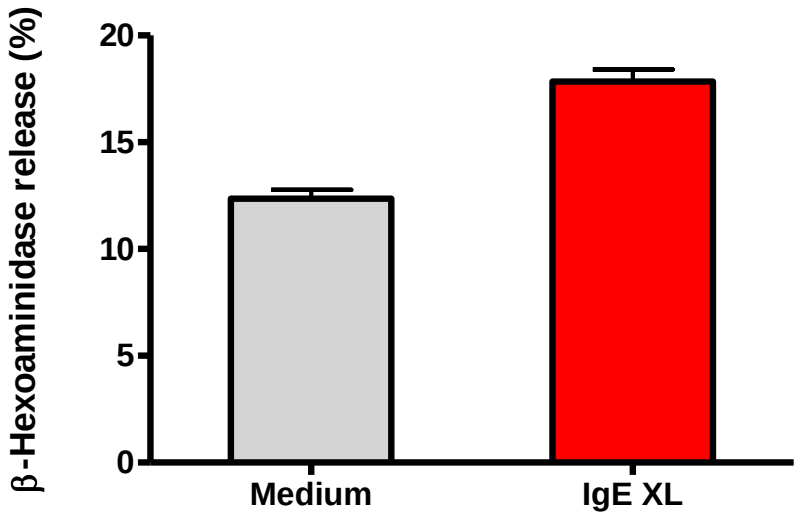

β-Hexoaminidase release (%)
20
15
10
5
0
Medium
IgE XL

Figure 3.2.1 The effects of ES-62, on cytokine production by BMMC in response to FcεRI cross-linking.

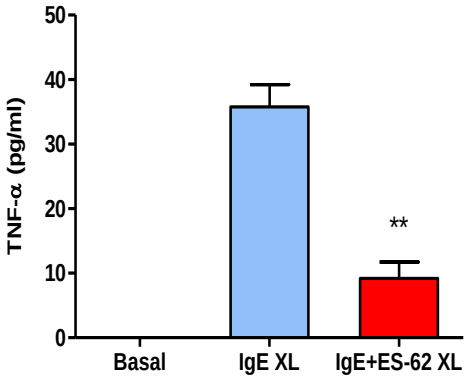
BMMC were sensitized with 500 ng/ml anti-DNP IgE antibodies (IgE) for 24 hours then stimulated with 500 ng/ml DNP-BSA to induce cross-linking (XL) for 24 hours at 37˚C in both ES-62 (2 µg/ml) pre-treated and non-treated cells. Medium alone was taken as the control (basal). Release of IL-6, TNF-α and IL-13 was measured by ELISA. Results are shown as means ± S.D, n=3. The data presented are from a single experiment incorporating triplicate values and are representative of at least 3 independent experiments. The statistical analysis performed is unpaired Students t test where * $P<0.05$ ** $P<0.005$ *** $P<0.001$

a) TNF-α cytokine release measured by ELISA

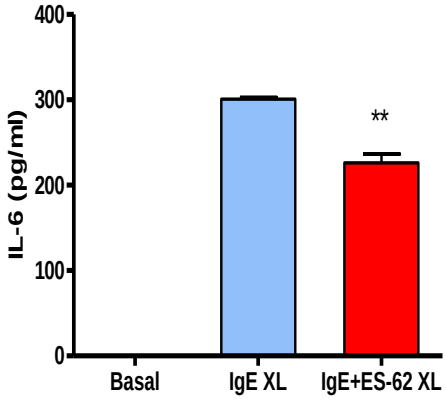
b) IL-6 cytokine release measured by ELISA

c) IL-13 cytokine release measured by ELISA

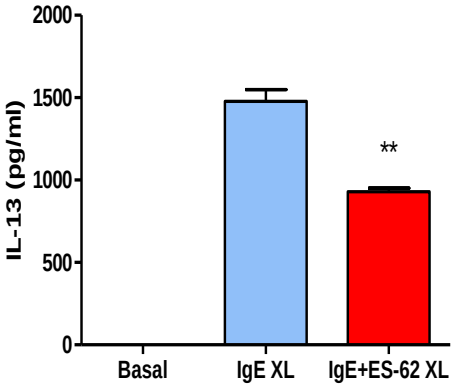
A.


TNF-α (pg/ml)
50
40
30
20
10
0
**
Basal
IgE XL
IgE+ES-62 XL

B.


IL-6 (pg/ml)
400
300
200
100
0
**
Basal
IgE XL
IgE+ES-62 XL

C.


IL-13 (pg/ml)
2000
1500
1000
500
0
**
Basal
IgE XL
IgE+ES-62 XL

3.3 PKC isoforms expression in BMMC and their targeting by ES-62

There are currently 10 PKC isoforms that have been identified in mammalian cells. These mammalian isoforms are sub-divided into 3 PKC subfamilies based on their activation requirements and regulatory domain components. The three subfamilies are: Conventional or classical PKCs (cPKC) which comprises alpha, beta I and the splice variant beta II along with gamma; Novel PKCs (nPKC) include delta, epsilon, eta and theta; and atypical PKCs (aPKC) are represented by zeta and lambda (also known as iota) (Toker, 1998). Both conventional and novel PKCs are thought to be translocated from the cytosol to the plasma membrane where they can be activated by DAG. Translocation can also be mimicked by phorbol esters such as PMA (Mellor and Parker, 1998; Tan and Parker, 2003). Mast cell exocytosis of pre-formed inflammatory mediators is highly dependent on the activation of phospholipase D (PLD) and PKC in addition to calcium mobilization (Ozawa, et al. 1993a; Chahdi, et al. 2002; Kimata, et al. 1999). In particular, PKC translocation and phosphorylation have been demonstrated to be critical for mast cell exocytosis (Kimata, et al. 1999). PLD catalyzes the hydrolysis of phosphatidylcholine (PC) to form phosphatidic acid (PA). This reaction and also the conversion of PA to biologically active molecules such as lysophosphatidic acid and DAG are thought to be vital to various biological and signalling events (Jenkins and Frohman, 2005). In mast cells, it was found that upon addition of the primary alcohol, 1-butanol, the production of PA could be redirected towards phosphatidyl alcohol (the transphosphatidylation reaction) resulting in inhibition of the translocation of DAG-dependent PKC isoforms to the plasma membrane and in turn mast cell degranulation (Peng and Beaven, 2005). These data suggest an important role for the

production of PA by PLD in the activation of PKCs and thus mast cell exocytosis (Peng and Beaven, 2005). All of the classical PKC isoforms have been shown to play an important role in different mast cell responses (Li, et al. 2005; Abdel-Raheem, et al. 2005; Nechushtan, et al. 2000; Ozawa, et al. 1993a). In particular, genetic knockout studies have emphasised the importance of PKC-β in mast cell degranulation (Nechushtan, et al. 2000). In conjunction with this, data obtained when employing ES-62 highlighted that PKC-α is an important regulator of IgE-receptor induced mast cell responses (Melendez, et al. 2007). So far, only two novel PKC isoforms have been identified as essential components of the mast cell signalling pathway. One of the first positive roles assigned to PKC-δ (and incidentally for the classical PKC, PKC-β) in antigen-stimulated mast cell exocytosis was witnessed using PKC reconstitution studies in permeabilized RBL cells (Ozawa, et al. 1993 a). Another novel isoform shown to be clearly associated with FcεRI-induced mast cell activation is PKC-θ (Liu, et al. 2001). Interestingly, out of all 10 PKC isoforms, only one has so far been revealed to have some form of redundancy in mast cells: PKC-ε knockout studies demonstrated that although PKC-ε becomes activated following antigen stimulation, as suggested by its translocation to the plasma membrane, the absence of this isoform does not interfere with important mast cell responses such as IL-6 cytokine secretion and FcεRI triggered exocytosis (Lessman, et al. 2006). Taken together, these data suggest that although there has been significant unveiling of the roles of certain PKC isoforms in mast cell responses, in particular the conventional isoforms α and β, the role of other isoforms awaits full elucidation.

3.3.1 PKC-alpha

It was shown in human mast cells, that the modulation of expression of PKC-alpha (PKC-α) by ES-62 was essential for inhibition of mast cell responses (Melendez et al, 2007). Thus, it was investigated whether ES-62 similarly targets this isoform in murine BMMC. Firstly, it was necessary to confirm that this PKC isoform was expressed in the BMMC under study. Therefore, BMMC whole cell lysates were probed for PKC-α expression using Western blotting. Medium alone was used as a negative control, and BMMC were tested under 3 different conditions: BMMC unstimulated (BMMC), BMMC sensitized with IgE (IgE) and BMMC sensitised with IgE and cross-linked with DNP-BSA (IgE XL). From these data, it was consistently concluded that PKC-α was expressed in these mast cells under all resting and activated conditions tested as a band corresponding to a polypeptide of the correct molecular weight - ~75kD, was observed (Figure 3.3.1 A). A band of ~50kD (and a faint slightly smaller one) were detected in the IgE-sensitised and cross-linked samples: the identity of the corresponding polypeptide was not investigated but it may be a PKC-α fragment or perhaps IgE heavy chain being detected in a non-specific manner. Equal loading is shown by measuring expression of β-actin (Figure 3.3.1 A). Quantitative analysis revealed no changes in expression levels of PKC-α following IgE sensitisation or cross-linking (data not shown).

Following the confirmation of the presence of PKC-α in mouse BMMC, a time-course experiment was undertaken to examine if ES-62 modulates PKC-α expression in the cells and to determine at which time point ES-62 is most effective. Western blotting and densitometric analysis revealed an initial reduction in PKC-α levels

starting at 6 hours which remained at this until 18 hours before being reduced to almost zero by 24 hours (Figure 3.3.1 B). This result suggests that as with human mast cells, ES-62 targets this signalling molecule in mouse BMMC (Figure 3.3.1 B).

3.3.2 PKC-beta

When extracts of human BMMC were pre-treated overnight with ES-62 and then analysed for the expression of various PKC isoforms by western blotting, it was revealed that ES-62 reduced PKC-β levels (Melendez, et al. 2007). It was thus investigated as to whether this conventional isoform was present in mouse BMMC. When BMMC extracts consisting of two high protein quantities (40 and 80 µg) were probed for PKC-β, in a preliminary experiment, it was found that there was no expression of this isoform in either sample (data not shown). Therefore, a PKC-β overexpression lysate positive control (and an associated empty vector negative control) was purchased for future experiments. Following optimization experiments relating to both controls (data not shown); BMMC extracts were tested for PKC-β expression under 3 conditions: BMMC unstimulated (BMMC), BMMC sensitized with IgE (IgE) and BMMC sensitised with IgE and cross-linked with DNP-BSA (IgE XL). An extract from spleen was also included as a possible positive control for this experiment. The results show that the PKC-β antibody is working effectively as a strong band was visible on the blot where PKC-β would be expected to be (~ 90 kDa) in both the positive control and spleen extracts. PKC-β was not visible in the negative control as expected. Considering both controls worked efficiently and PKC-β expression could be detected in the spleen extract but none of the BMMC extracts,

it appears that PKC-β must either not be present in BMMC or that this isoform is expressed at very low amounts that cannot be detected by the western blotting indicator system in use. However, the strong literature evidence suggesting the presence of PKC-β in the RBL-2H3 mast cell line known as RBL cells (Chang, et al. 1997; Ozawa, et al. 1993 a), human mast cells (Melendez, et al. 2007) and indeed BMMC (Li, et al. 2005) led us to continue to further investigate this isoform in BMMC (Section 3.4.2).

3.3.3 PKC-theta

Although PKC-theta was not recorded as an ES-62 target in human BMMC (Melendez, et al. 2007) it is not known if this is true for all human and mouse mast cell phenotypes. Therefore, similar to PKC-α and β, it was investigated as to whether ES-62 targeted PKC-θ in mouse BMMC. Again, the first aim was to investigate the expression of this PKC isoform in murine BMMC by western blotting. A Cell Signalling- recommended positive control was employed for the PKC-θ western blots. This was a total cell extract made from Jurkat cells, which are T lymphocytes that highly express PKC-θ (Baier, et al. 1993). The antibody was able to detect PKC-θ in the control Jurkat cell lysates as predicted (Figure 3.3.3) but there was no detection of PKC-θ expression in the BMMC lysates (Figure 3.3.3). Hence, it proved impossible to show whether this was a target of ES-62 in mouse mucosal-type mast cells by a Western Blot approach. However, it has been shown by other research groups that PKC-θ can be detected in both RBL cells and mouse BMMC (Lui et al, 2001). These researchers also showed in RBL cells that PKC-θ is translocated to the

plasma membrane upon FcεRI activation and consequently plays a role in degranulation, IL-3 gene transcription and ERK activation (Lui, et al. 2001). However, Li and colleagues investigated the PKC isoforms present in BMMC and found PKC-θ to be present at somewhat low levels, especially in comparison to control T cell extract (Li, et al. 2005). Taken together, we can propose that although PKC-θ may be expressed in BMMC and perhaps functionally involved in mast cell signalling, the mouse BMMC lysate employed in the present study may possess too low an amount to be detected by Western blotting. However, the potential importance of this isoform in mast cell- receptor induced signalling as described in current literature (Lui, et al. 2001; Li, et al. 2005) cannot be ignored and therefore this isoform has also been selected to be investigated further using mast cells grown from PKC knockout mice (Section 3.4.3).

3.3.4 PKC-epsilon

Similar to PKC-θ in human mast cells, PKC-ε was expressed in this cell type but expression levels were unaffected by ES-62 (Melendez, et al. 2007). However, as before, it is not known whether this will hold true for all known human and murine mast cell phenotypes. Therefore, using the same approach with the previous PKC isoforms tested, the first step was to investigate the expression levels, if any, of PKC-ε in mouse BMMC. In a preliminary experiment testing two different protein quantities of BMMC extracts (40 and 80 µg) it was found that PKC-epsilon was present but in very low amounts. Therefore, considering that as expected the expression of PKC-ε was more prominent in the higher protein sample on the blot

(data not shown), it was decided to further test these cells using the higher protein quantity and the incorporation of negative and positive controls. Thus, BMMC extracts were probed for PKC-ε where the negative control was medium alone (to measure non-specific binding of the primary antibody and/or second antibody-conjugate to the blot) and positive controls were Jurkat T cells (Cell signalling control as used with PKC-θ experiments), which have previously been shown to express PKC-ε (Tsutsumi, et al. 1993) and an extract from spleen. From these data, it was confirmed that PKC-ε is expressed in murine BMMC (Figure 3.3.4 A). Equal loading is shown by measuring the expression levels of β-actin in these samples (Figure 3.3.4 A).

This confirmation of PKC-ε expression in mouse BMMC led us to consider if this isoform could be targeted by ES-62 in these cells. Thus, considering ES-62 targeted PKC-α expression most strongly at the 24 hour time-point, it was decided to investigate if PKC-ε was similarly targeted by ES-62 at this time-point. Unfortunately, on this occasion the experiments that were carried out could not identify PKC-ε in BMMC samples treated with medium alone or ES-62. As witnessed in the preliminary experiments to identify this isoform in BMMC, higher protein loads (80 µg or more) would need to be used as this isoform is present in very low quantities. Even in an experiment loading 80 µg of protein, PKC-ε could not be detected (Figure 3.3.4 B). It can only be presumed that this is due to primary mast cell culture variations and thus possibly even higher protein amounts would need to be loaded to identify this isoform from these samples in future experiments.

Figure 3.3.1 Investigation of BMMC for their expression of PKC-alpha (PKC-α) and the targeting of this isoform by ES-62

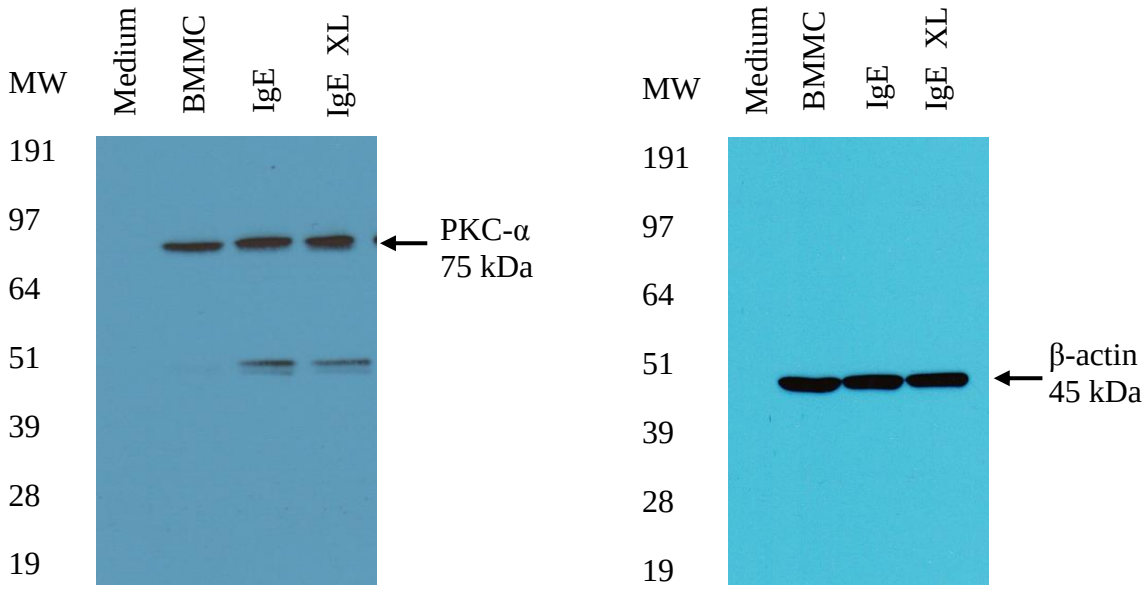
A.

Total protein extracts from BMMC were analysed by Western Blotting for their expression of PKC-α (mw 75 kDa). The samples were also probed for β-actin (45 kDa) as a loading control. Medium alone was used as a control and BMMC sample lysates were BMMC unstimulated (BMMC), BMMC sensitised with IgE for 24 hours (IgE) and BMMC sensitized with IgE and cross-linked with DNP-BSA for 24 hours (IgE XL).

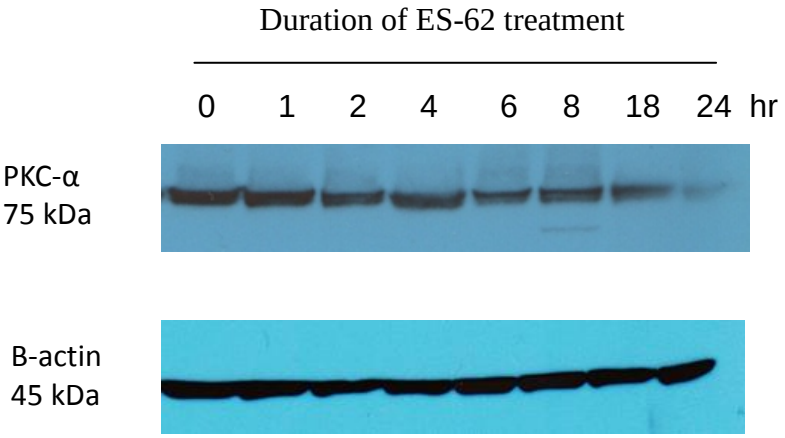
B.

BMMC were sensitised overnight with IgE (500ng/ml) in the presence or absence of ES-62 (2 µg/ml) at the times indicated and PKC-α expression (mw 75 kDa) analysed by Western blotting. The samples were also probed for β-actin (mw 45 kDa) as a loading control. The blot is presented alongside the densitomeric analysis of the protein, which is normalized against the relevent β-actin loading control then adjusted and expressed as a fold change to relative control (0 h time point, normalised to 1).

A.


MW
Medium
BMMC
IgE
IgE XL
191
97
64
51
39
28
19
PKC-α
75 kDa
MW
Medium
BMMC
IgE
IgE XL
191
97
64
51
39
28
19
β-actin
45 kDa

B.


Duration of ES-62 treatment
0 1 2 4 6 8 18 24 hr
PKC-α
75 kDa
B-actin
45 kDa

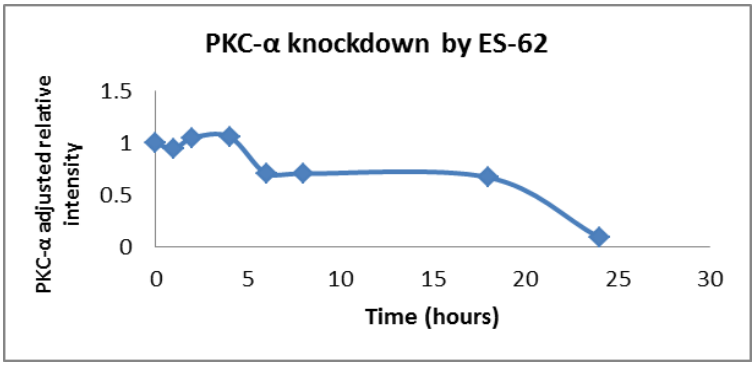
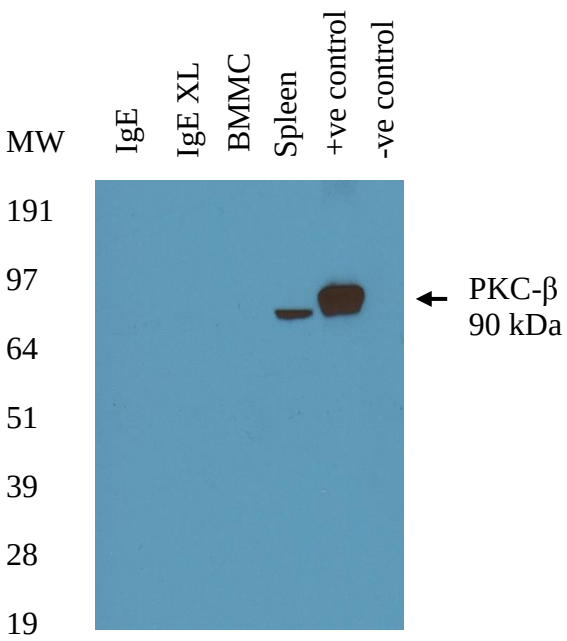

PKC-α knockdown by ES-62
PKC-α adjusted relative intensity
1.5
1
0.5
0
0
5
10
15
20
25
30
Time (hours)

Figure 3.3.2 Investigation of BMMC for their expression of PKC-beta (PKC-β)

Total protein extracts from BMMC were analysed by Western Blotting for their expression of PKC-β (mw 90 kDa). The samples were also probed for β-actin (45 kDa) as a loading control. Spleen extract, PKC-β overexpression lysate positive control (+ve) and empty vector negative control (-ve) were used as controls. BMMC sample lysates were BMMC unstimulated (BMMC), BMMC sensitised with IgE for 24 hours (IgE) and BMMC sensitized with IgE and cross-linked with anti-IgE DNP for 24 hours (IgE XL).


MW
IgE
IgE XL
BMMC
Spleen
+ve control
-ve control
191
97
64
51
39
28
19
PKC-β
90 kDa

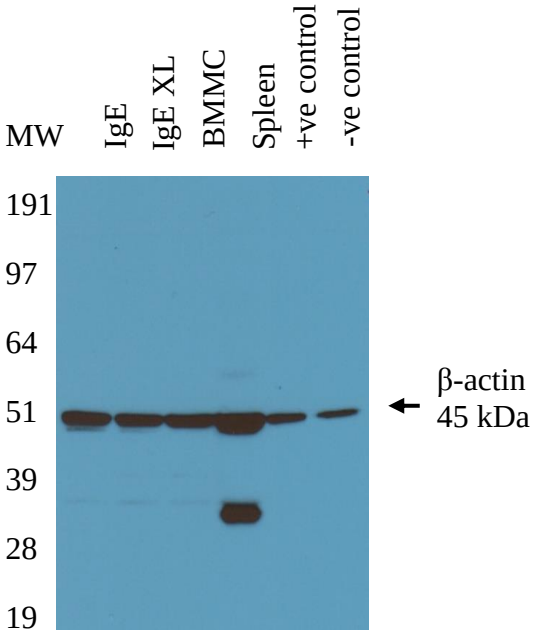
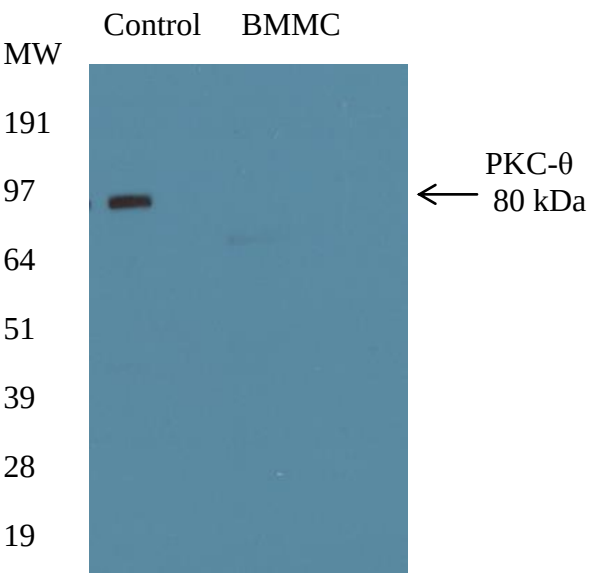

MW
IgE
IgE XL
BMMC
Spleen
+ve control
-ve control
191
97
64
51
39
28
19
β-actin
45 kDa

Figure 3.3.3 Investigation of BMMC for their expression of PKC-theta (PKC-θ)

Total cell protein extracts from BMMC and control cells (Jurkat T cells - Cell signalling) were analysed by Western blotting for PKC-θ expression (mw 80 kDa). The samples were also probed for β-actin (45 kDa) as a loading control.


Control
BMMC
MW
191
97
64
51
39
28
19
PKC-θ
80 kDa

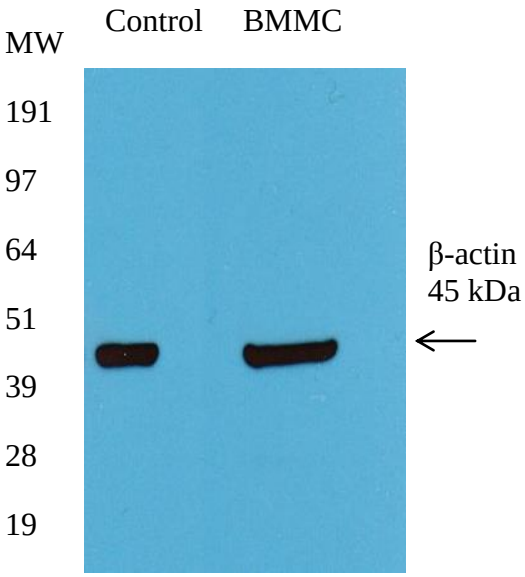
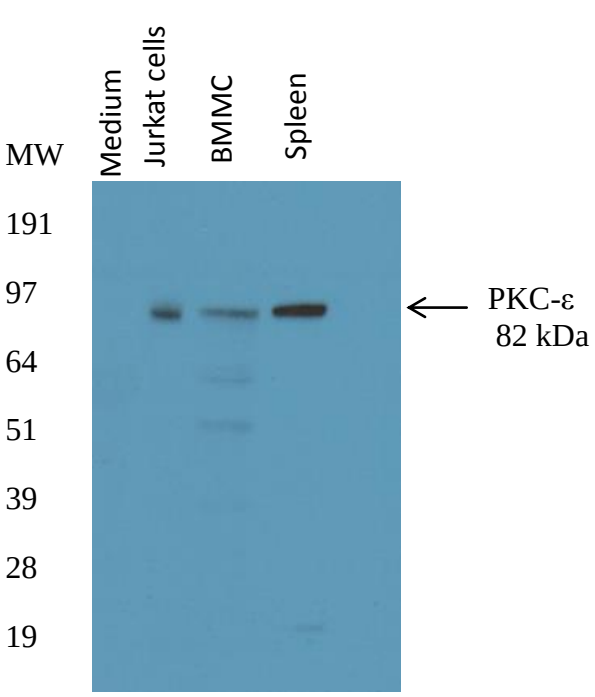

Control
BMMC
MW
191
97
64
51
39
28
19
β-actin
45 kDa

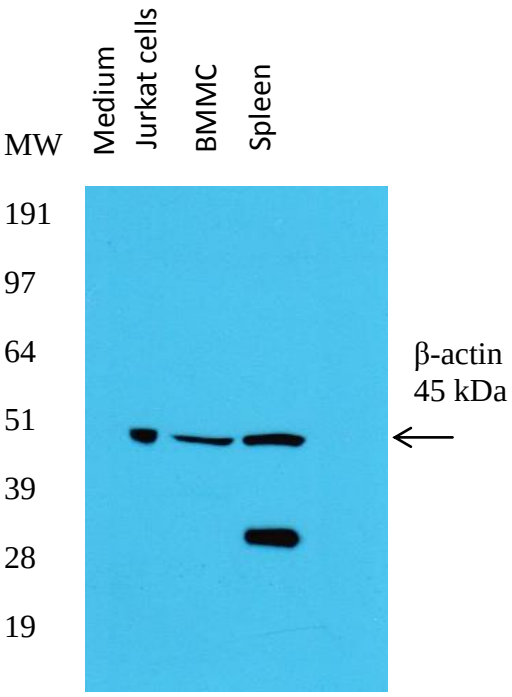
Figure 3.3.4 Investigation of BMMC for their expression of PKC-epsilon (PKC-ε)

A. Total cell protein extracts from BMMC were probed for the expression of PKC-ε (mw 82 kDa). Medium alone (negative control), spleen and Jurkat T cells (positive control) were used as controls. The samples were also probed for β-actin (45 kDa) as a loading control.

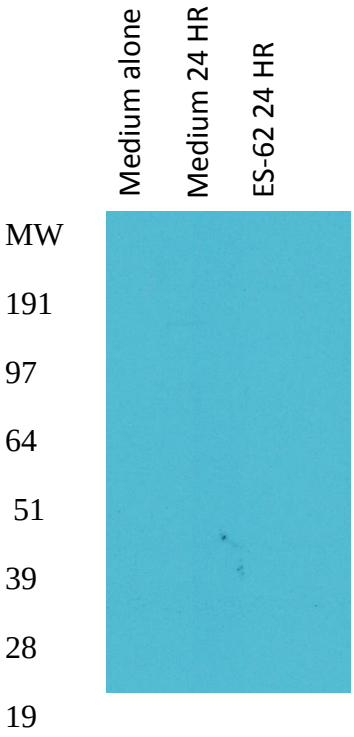
B. BMMC were sensitised overnight with IgE (500ng/ml) in the presence or absence of ES-62 (2ug/ml) for 24 hours and PKC-ε expression (mw 82 kDa) analysed by Western blotting. The samples were also probed for β-actin (mw 45 kDa) as a loading control. Medium alone was used as a negative control.

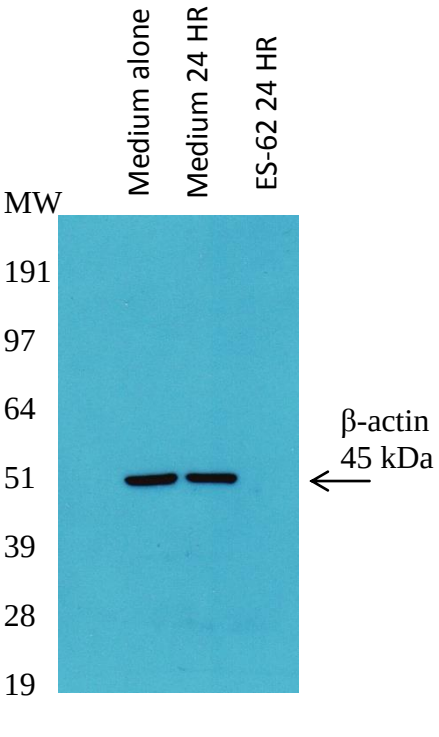
A.


MW
Medium
Jurkat cells
BMMC
Spleen
191
97
64
51
39
28
19
PKC-ε
82 kDa


MW
Medium
Jurkat cells
BMMC
Spleen
191
97
64
51
39
28
19
β-actin
45 kDa

B.


Medium alone
Medium 24 HR
ES-62 24 HR
MW
191
97
64
51
39
28
19


Medium alone
Medium 24 HR
ES-62 24 HR
MW
191
97
64
51
39
28
19
β-actin
45 kDa

3.4 Short interfering RNA (siRNA)

Fire, Mello and colleagues (Fire and Mello, 1998) discovered over a decade ago that double stranded RNA (dsRNA) was the trigger for post-transcriptional gene silencing in nematodes. They found dsRNA to be more effective in silencing gene expression than sense or anti-sense strands and that just a few molecules of dsRNA were enough to almost obliterate the expression of a gene homologous to that dsRNA (Fire and Mello, 1998). They termed this phenomenon RNA interference (RNAi). When dsRNA enters the cell, an RNase-III-like enzyme called Dicer cleaves the dsRNA into short fragments of double stranded small interfering RNAs (siRNA) which are around 21 nucleotides (nt) in length (Bernstein et al, 2001; McManus and Sharp, 2002) (Figure 3.4). SiRNAs typically have a very specific structure consisting of two 21-nt single-stranded RNAs that form a 19-20 base-pair (bp) duplex bearing 2-nucleotide 3' overhangs at each end (Elbashir et al, 2001). The subsequent cleaved siRNA consists of two strands: a guide strand (or anti-sense strand) and a passenger strand (sense strand). The passenger strand will be degraded while the guide strand of the siRNA is incorporated into a protein complex known as RNAi induced silencing complex (RISC) which helps guide the siRNA to the target mRNA with complimentary sequences for its degradation (Shan, 2010; Hammond et al, 2000) (Figure 3.4). It has now been shown that synthetic siRNAs can be transfected into mammalian cells to induce powerful gene silencing (Elbashir et al, 2001). Studies have shown that this potent and specific method of gene silencing is an effective technique to induce gene knockdown in mast cells (Zhang et al, 2010; Peng and Beaven, 2005; Heionen et al, 2002). In particular, this gene-silencing technique was effective for studying mast cell signal transduction pathways following FcεRI cross-

linking (Zhang et al, 2010). Therefore, siRNA was chosen as a method for knocking down PKC isoforms in mouse BMMC. Previously, Melendez and colleagues used an anti-sense oligonucleotide approach to knockdown PKC-α expression in human mast cells (Melendez et al, 2007). Although the end result of both siRNA and anti-sense oligonucleotides is degradation of the target mRNA, there are reports which support siRNA as a more efficient tool for gene silencing. For example, a study comparing a 22 nt long siRNA and an antisense oligonucleotide highlighted that the siRNA had a more potent effect in cell culture experiments (Bertrand et al, 2002). Additionally, in comparison to the oligonucleotides, siRNA duplexes seem to be more stable and are able to resist biodegradation in serum allowing them to have a longer effect (Bertrand et al, 2002). Another interesting study carried out by Miyagishi and co-workers (Miyagishi et al, 2003) similarly concluded that siRNA was more effective at inducing gene silencing than an antisense directed against the same target gene. Therefore, taking such observations into consideration, siRNA was selected for gene silencing experiments.

3.4.1 SiRNA optimization for knocking down PKC isoforms in BMMC

As mentioned previously, synthetic siRNAs with around 21 bp can mimic the natural products of dicer in mammalian cells (Elbashir et al, 2001). These siRNAs can usually be manufactured in many ways but often it is simpler to purchase siRNA through manufacturers who contract the production of synthetic siRNAs such as Dharmacon RNAi technologies or QIAGEN. Additionally, synthetic siRNA needs to be inserted into a cell via the help of an agent such as a transfection solution

(Bertrand et al, 2002; Khvorova et al, 2003). QIAGEN products were chosen as QIAGEN's HiPerfect transfection reagent is seen by many as an established and effective transfection reagent and has often been used as a standard to which all new transfection reagent therapies are compared (Fischer et al, 2010). It has been shown that the HiPerfect transfection reagent can successfully introduce suitable levels of siRNA into the cell thereby inducing highly potent gene knockdown (Fischer et al, 2010; Pallet et al, 2008). SiRNA optimization would require balancing maximal gene silencing with minimal off target effects and toxicity. The RNAi human/mouse starter kit from QIAGEN contains an Allstars HS Cell death control siRNA, which is a mixture of siRNA targeted against genes that are critical for cell survival and therefore can induce high amounts of cell death, which can be monitored by light microscopy. This was the chosen starting point for optimizing the PKC-targeting siRNA. Optimal transfection conditions would be based on the use of 2 different criteria: 1. Maximal knockdown as assessed by the amount of cell death and 2. Minimal toxicity as assessed by the amount of cell death induced by the HiPerfect transfection reagent alone compared to mast cells without HiPerfect transfection reagent. Cell viability would be measured by Trypan Blue staining.

3.4.1.1 Optimizing gene silencing with Cell Death siRNA

There were 3 main parameters that had to be optimized for effective transfection and gene silencing. These were: siRNA concentration, ratio of HiPerfect transfection reagent to siRNA and cell density. Another important factor that had to be considered was the time point at which knock down might occur. This can be

anywhere between 24-96 hours for mammalian cells (Chang et al, 2012). Qiagen's optimization recommendations from their RNAi Human/mouse start kit booklet and online suspension cell protocol were followed during optimization.

Introduction of synthetic siRNA into cells can often result in off-target effects (Jackson and Linsley, 2010) where unintended targets are down-regulated leading to complicated and misleading results. Jackson and colleagues were the first to recognise that siRNA can have off-target effects (Jackson et al, 2003). Using genome-wide microarray profiling, these researchers showed that there were slight changes in the expression of many genes following transfection of an individual siRNA. There were minimal shared complementary levels between the siRNA and off-target genes, with the off-target profile being unique to each siRNA (Jackson et al, 2003). These off-targets effects are usually due to such reasons as the dsRNA lengths present within the siRNA activating the interferon response (Bridge et al, 2003; Sledz et al, 2003), which is a non-specific immune response directed towards foreign genetic material (Reynolds et al, 2006), or certain sequence motifs within an siRNA activating an inflammatory response through TLRs (Judge et al, 2005; Hornung et al, 2005). It has been suggested that by using the lowest concentration of siRNA, it is possible to minimize off-target effects (as well as costs) (Cullen, 2006) and indeed it has been demonstrated that siRNA concentrations below 100 nM are less likely to induce off-target effects within cells but retain high levels of specific silencing activity (Cullen, 2006; Elbashir et al, 2001; Reynolds et al, 2006). Therefore, 3 low siRNA concentrations using the Cell Death control; 10, 50 and 100 nM with 6 µl of HiPerfect transfection reagent and a cell density of 0.2 x106 cells per sample were first tested in BMMC. The cells were counted by light microscopy

after being incubated for 72 hours with the siRNA complexes. Figure 3.4.1.1 A shows that there is obvious cell death at every siRNA concentration with the lower siRNA concentrations (10 and 50 nM) showing 50 and 55% cell death, respectively. Also, the HiPerfect control showed no cell death suggesting that this concentration of transfection reagent is not toxic to the cells. These preliminary optimization data suggested that 6 µl of HiPerfect transfection reagent should be considered for further testing.

Taking into account that siRNAs are usually considered "functional" when the level of gene knockdown is greater than 70% (Khvorova et al, 2003) it was decided to repeat this experiment using increased siRNA concentrations and 2 different volumes of HiPerfect transfection reagent to see if this would produce higher levels of gene knockdown. This experiment investigated siRNA concentrations of 100, 200 and 500 nM with both 6 µl and 9 µl of HiPerfect transfection reagent at cell density of 0.2 $x10^6$ cells per sample. The cells were counted by light microscopy after 0, 24, and 96 hours. Figure 3.4.1.1 B and C show that 96 hours is too long an incubation time with the siRNA complexes as there were very few cells detectable. It is uncertain whether this is due to the Cell Death siRNA itself inducing high levels of cell death or if the cells' health deteriorated due to toxicity from the long incubations with HiPerfect and siRNA complexes. Another reason perhaps, is that the cells died due to lack of sufficient nutrients. Comparing both transfection volumes, it can be seen that 9 µl of HiPerfect produces higher knockdown (Figure 3.4.1.1 C). However, the HiPerfect control showed cell death between 50-60% therefore making it unclear whether the higher volume of HiPerfect was increasing gene knockdown or induced cellular toxicity and thereby causing cell death. Other laboratories using HiPerfect

transfection reagent and siRNA complexes from QIAGEN incubated cells with the siRNA Transfection complexes for 48 hours (Pallet et al, 2008) suggesting that I should perhaps consider reducing the incubation times from 96 hours. Additionally from these experiments, it can be concluded that examination of higher siRNA concentrations (100, 200 and 500 nM) should be undertaken but that the HiPerfect transfection reagent volume should be restricted to 6 µl to minimize cellular toxicity and ensure optimum transfection efficiency with the siRNA. It was also concluded that 0.2 $x10^6$ cells should continue to be employed but perhaps consideration should also be given to testing increased cell density. Therefore, the next experiment tested siRNA concentrations of 100, 200 and 500 nM with 6 µl of HiPerfect at 0.2 and 0.5 $x10^6$ cells per sample. Figure 3.4.1.1 D shows there is greater knockdown of the cell survival with the Cell Death siRNA at 200 nM and 500 nM concentrations compared to 100 nM. There also seems to be greater knockdown at 72 hour compared to 24 hour incubation time suggesting that a longer incubation time than 24 hours is important for transfection of the siRNA. Unfortunately, the results with cell density of 0.5 $x10^6$ cells were inconclusive as the cell numbers increased over both 24 and 72 hour in each sample suggesting unsuccessful transfection (Figure 3.4.1.1 E).

In summary, I tested various siRNA concentrations (10, 50, 100, 200 and 500 nM) with variable amount of HiPerfect Transfection Reagent (6 µl and 9 µl) and seeded different cell numbers (0.1, 0.2, and 0.5 $x10^6$) over different time points of siRNA incubation (24, 72, 96 hour). The suggestion from these experiments with the Cell Death control siRNA is to continue to test the higher siRNA concentrations (100, 200 and 500 nM) in conjunction with transfection with 6 µl of HiPerfect Transfection Reagent for a time-point after 24 hours and before 96 hours such as 48

or 72 hours. Hence I used this information to proceed to attempt optimization using one of the PKC isoforms as this would resemble more closely the exact parameters needed for optimum gene silencing in terms of expression.

3.4.1.2 Optimizing gene silencing using PKC-α siRNA

Previous data obtained using the Cell Death control provided a good starting point for optimizing the siRNA for the PKC isoforms. For the first experiment it was decided to test 0.4 $x10^6$ cells with the same increasing concentrations of PKC-α siRNA (100 nM, 200 nM and 500 nM) transfected with 6 µl of HiPerfect transfection reagent for 48 hours. Other laboratory groups had reported successful transfection with this time point using both the HiPerfect transfection reagent and siRNA from Qiagen (Pallet et al, 2008) and therefore it was decided to reduce the incubation time from 72 to 48 hours to see if this would generate better results. Analysis of PKC-α knockdown was then assessed by Western Blotting with β-actin used as a loading control in all siRNA experiments. Unfortunately, the blot showed that there was little or no visible PKC-α knockdown at any siRNA concentration compared to the HiPerfect transfection control (Figure 3.4.1.2 A). Image J quantitative analysis also confirmed this conclusion of essentially no knockdown, with only 200 nM showing a minimal effect (Figure 3.4.1.2 A).

However, on repeating the experiment, a low level of knockdown was observed with each siRNA concentration (Figure 3.4.1.2 B) and again Image J was employed to quantitatively analyse the Western Blots. On this occasion, ERK was used as a loading control. Regrettably, there was insufficient sample to load the HiPerfect

control and therefore siRNA was compared to the 48 hour BMMC control. As the graph shows (Figure. 3.4.1.2 B), the greatest decrease was observed at 200 nM siRNA compared to 500 and 100 nM respectively. These data suggest that the optimum siRNA concentration based on the work undertaken so far is 200 nM. It was decided to undertake a further repeat experiment and this incorporated a non-silencing Negative control siRNA from QIAGEN to test for any non-specific effects as these should be minimal to ensure reliable RNAi (Jackson and Linsley, 2010). However, it was unclear if there was any significant knockdown of PKC-α compared to 48 hour HiPerfect control in this experiment (Figure 3.4.1.2 C) with the use of Image J and quantitative analysis only showing a small effect at 200 nM siRNA (Figure 3.4.1.2 C). However, it was encouraging to see from these data that there was no knockdown witnessed at any Negative control siRNA concentrations suggesting that there are no off-target effects using the high concentrations of siRNA. This experiment was repeated on a further occasion but almost identical results were obtained with no knockdown at any siRNA concentration and with negative controls (data not shown).

Following these experiments, as an additional approach, it was decided to investigate the functional responses of the BMMC following a 48 hour transfection with siRNA. As before, the experiment incorporated a 48 hour BMMC control, 48 hour HiPerfect Transfection Reagent control along with siRNA concentrations 100, 200 and 500 nM and matching siRNA negative controls. After the 48 hour transfection, one set of cells was lysed to make total cell extracts to be analysed by Western Blotting and one set of cells was then sensitized with IgE (500 ng/ml) for 24 hours followed by cross-linking with DNP (500 ng/ml) for 24 hours before supernatants were collected and

analysed by ELISA for IL-6 cytokine production (Figure 3.4.1.3 A i and ii). From the Western Blot and relative quantitative analysis, it appears there is again no knockdown of PKC-α in either 200 or 500 nM siRNA (Figure 3.4.1.3 A i). Interestingly, however, the cytokine analysis shows that there is no IL-6 production in cells treated with 200 nM of PKC-α-targeting siRNA and 51.5% less IL-6 production in 500 nM siRNA-treated cells compared to the HiPerfect transfection control (Figure 3.4.1.3 A ii). There was no knockdown witnessed in the negative control 200nM (+2%) and minimal knockdown in the 500 nM negative control (14%) again suggesting 200 nM of siRNA is optimal for knockdown without causing off-target or toxic effects. This experiment was repeated to find again no knockdown of PKC-α as analysed by Western blotting (Figure 3.4.1.3 B i) but this time the IL-6 cytokine data also showed no knockdown at either siRNA concentrations (Figure 3.4.1.3 B ii). A third attempt at this experiment also displayed no knockdown according to Western Blot analysis of PKC-α expression but again a reduction in IL-6 cytokine production in both 200 nM and 500 nM siRNA concentrations (data not shown). However, this time, the cytokine analysis also revealed reduced levels of IL-6 in both negative controls, albeit to a lesser extent than the siRNA. This result suggests that there may have been off-target effects from the siRNA in this particular experiment.

Overall, in spite of promising indicators, it was felt that the approach was generating data that were too inconsistent to be useful and hence as an alternative approach, mast cells (BMMC) from PKC isoform knockout mice were investigated.

Figure 3.4 Short interfering RNAs (siRNAs) – post-transcriptional gene silencing

Long double stranded RNA (dsRNA) is cleaved at specific sites into siRNAs by an enzyme called Dicer. siRNAs usually consist of two 21-nucleotide (nt) single stranded RNAs that form a 19 base pair (bp) duplex with 2-nt 3' overhangs. Each siRNA consist of two strands: the sense or passenger strand (orange) and the anti-sense or guide strand (blue). The sense strand is degraded allowing the anti-sense strand to be incorporated into RISC (RNAi-induced silencing complex). The anti-sense strand is then used by RISC to identify the corresponding mRNA (red) for cleavage and degradation thereby ultimately resulting in loss of protein expression.

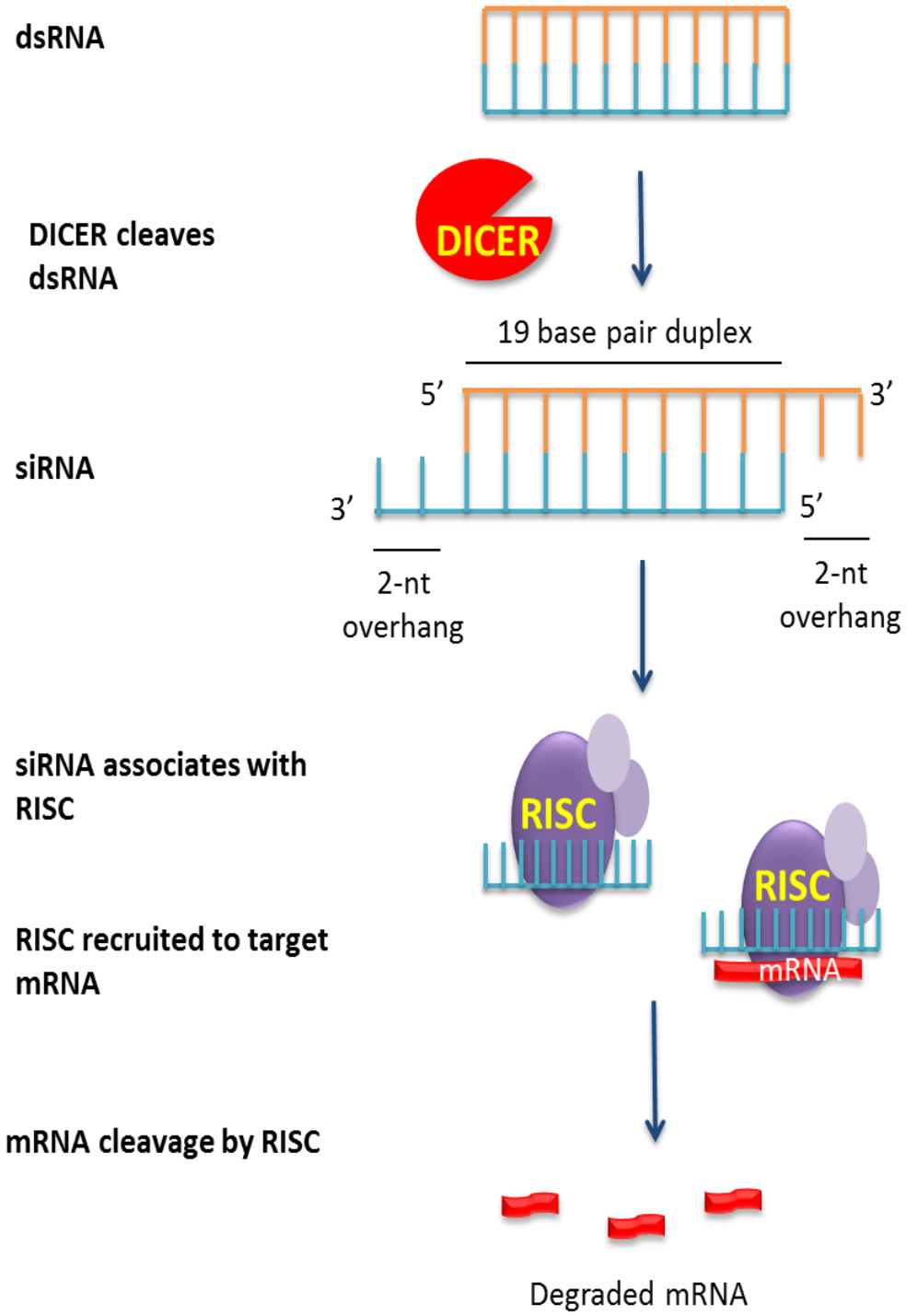

dsRNA
DICER cleaves dsRNA
DICER
19 base pair duplex
siRNA
5'
3'
3'
5'
2-nt overhang
2-nt overhang
siRNA associates with RISC
RISC
RISC recruited to target mRNA
RISC
mRNA
mRNA cleavage by RISC
Degraded mRNA

Figure 3.4.1.1 Optimization of siRNA using Cell Death control

This figure compares knockdown induced by various Cell Death siRNA concentrations using different HiPerfect transfection reagent (HiP) amounts. The cell numbers in each sample were calculated by light microscopy at various time points and compared to the starting cell count, represented as 0 hour.

A. 0.2 x 10^6 BMMC were incubated with 10, 50 and 100 nM of Cell Death siRNA and transfected with 6 µl of HiP for 72 hours. HiP control and cell control were incubated with 6 µl of HiP and RPMI or RPMI alone, respectively.

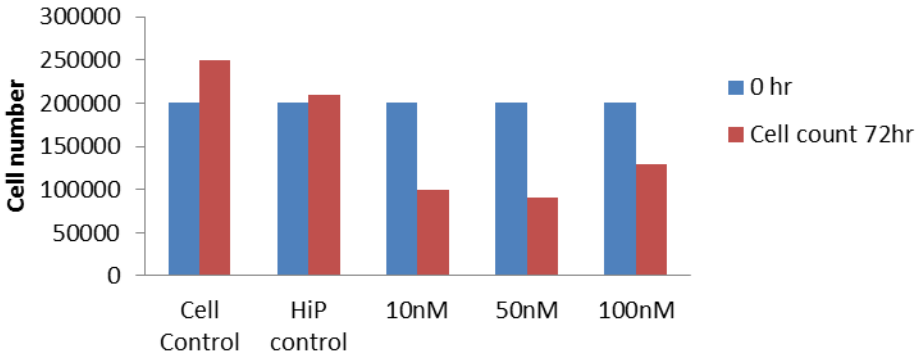
B. 0.2 x 10^6 BMMC were incubated with 100, 200 or 500 nM Cell Death siRNA + 6 µl HiPerfect for 24 or 96 hours. HiP control and cell control were incubated with 6 µl of HiP and RPMI or RPMI alone, respectively.

C. 0.2 x 10^6 BMMC were incubated with 100, 200 or 500 nM Cell Death siRNA + 9 µl HiP for 96 hours. HiP control and cell control were incubated with 9 µl of HiP and RPMI or RPMI alone, respectively.

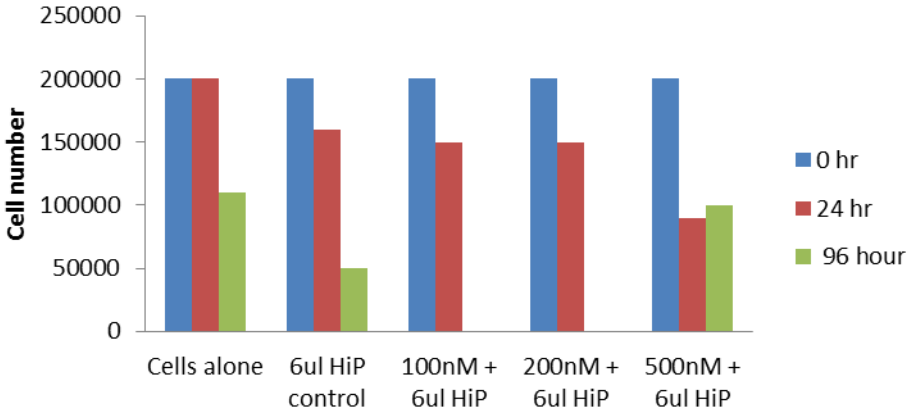
D. 0.2 x 10^6 BMMC were incubated with 100, 200 or 500 nM Cell Death siRNA and transfected with 6 µl of HiP for 0, 24 and 72 hours. HiP control and cell control were incubated with 6 µl of HiP and RPMI or RPMI alone, respectively.

E. 0.5 x 10^6 BMMC were incubated with 100, 200 or 500 nM Cell Death siRNA and transfected with 6 µl of HiP for 0, 24 and 72 hours. HiP and cell control were incubated with 9 µl of HiP and RPMI or RPMI alone, respectively

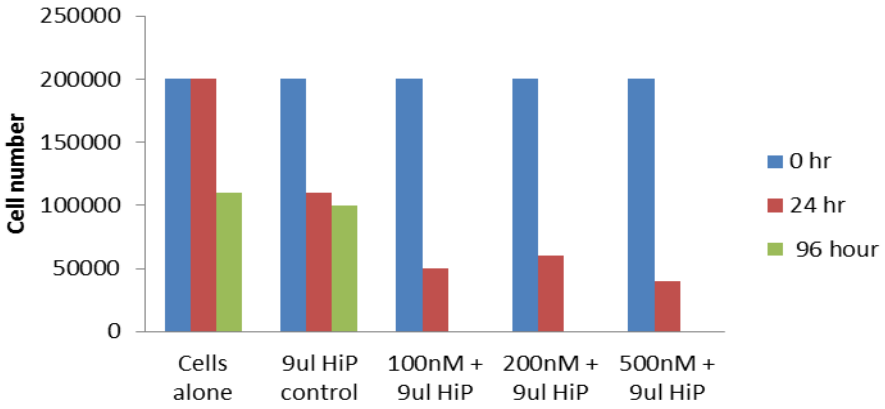
A.


300000
250000
200000
150000
100000
50000
0
Cell number
Cell Control
HiP control
10nM
50nM
100nM
0 hr
Cell count 72hr

B.

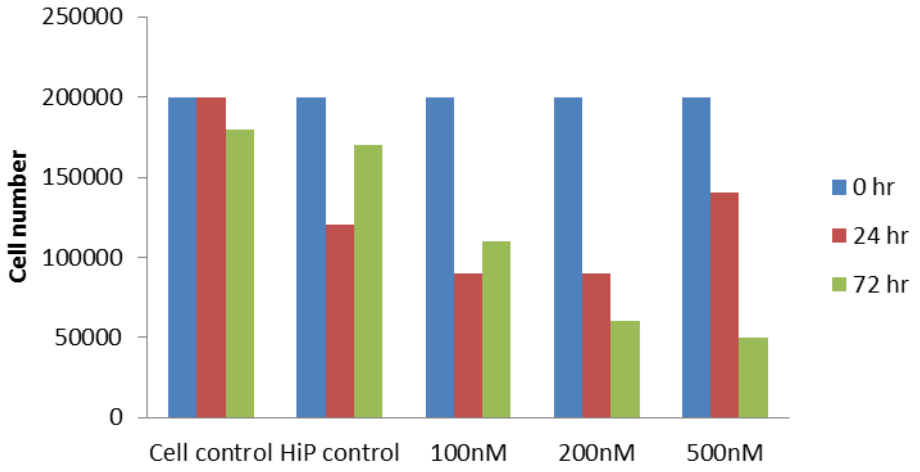

250000
200000
150000
100000
50000
0
Cell number
Cells alone
6ul HiP control
100nM + 6ul HiP
200nM + 6ul HiP
500nM + 6ul HiP
0 hr
24 hr
96 hour

C


250000
200000
150000
100000
50000
0
Cell number
Cells alone
9ul HiP control
100nM + 9ul HiP
200nM + 9ul HiP
500nM + 9ul HiP
0 hr
24 hr
96 hour

.

D.


250000
200000
150000
100000
50000
0
Cell number
Cell control
HiP control
100nM
200nM
500nM
0 hr
24 hr
72 hr

E.

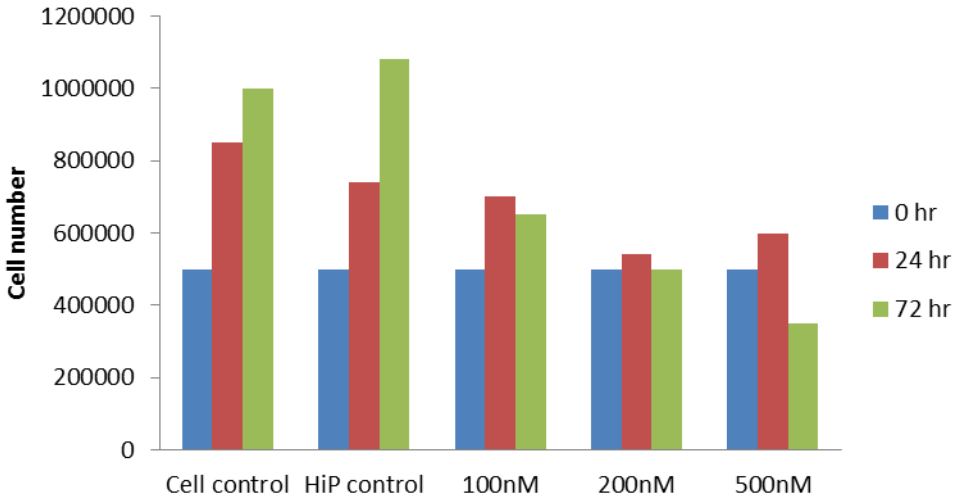

1200000
1000000
800000
600000
400000
200000
0
Cell number
Cell control
HiP control
100nM
200nM
500nM
0 hr
24 hr
72 hr

Figure 3.4.1.2 Optimization of PKC-α siRNA for BMMC

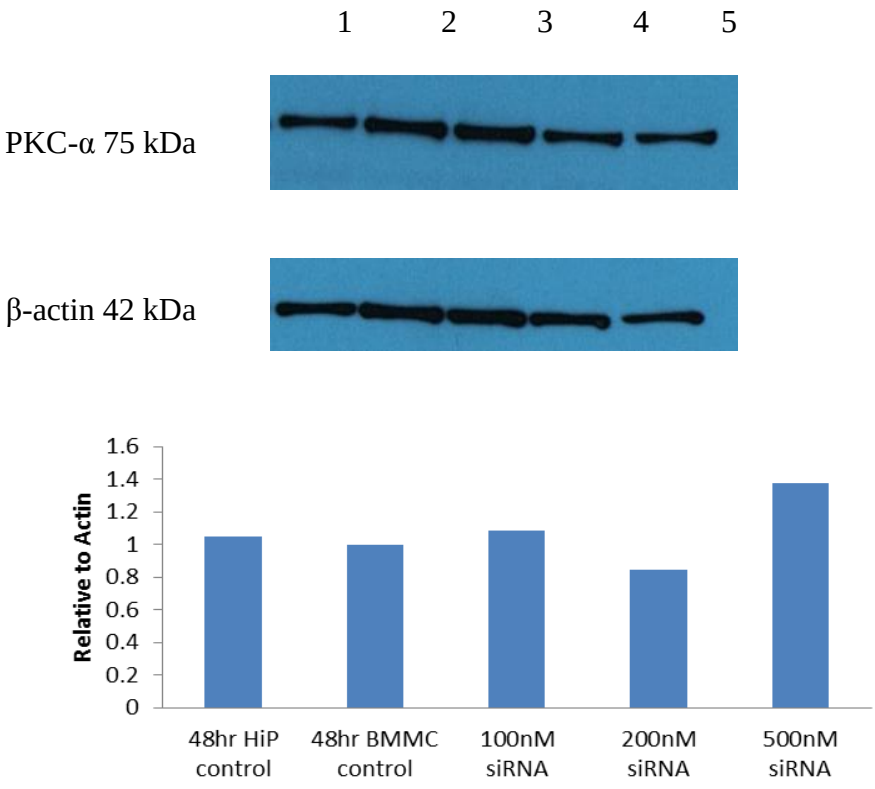
Western blot analysis of PKC-α gene knockdown in BMMC after incubation with various concentrations of PKC-α siRNA transfected with 6 µl of HiPerfect Transfection Reagent (HiP).

A. Total protein extracts from 48 hour control BMMCs (Lane 1) HiP (Lane 2) and siRNA concentrations 100, 200 and 500 nM (Lane 3, 4 and 5) were purified from samples and analysed by western blotting for PKC-α expression. Blots were probed with β-actin for equal loading. Densitometric analysis of PKC-α normalised against the relevant β-actin loading control was undertaken and values expressed as fold change relative to control cells (HiP control normalised to 1)

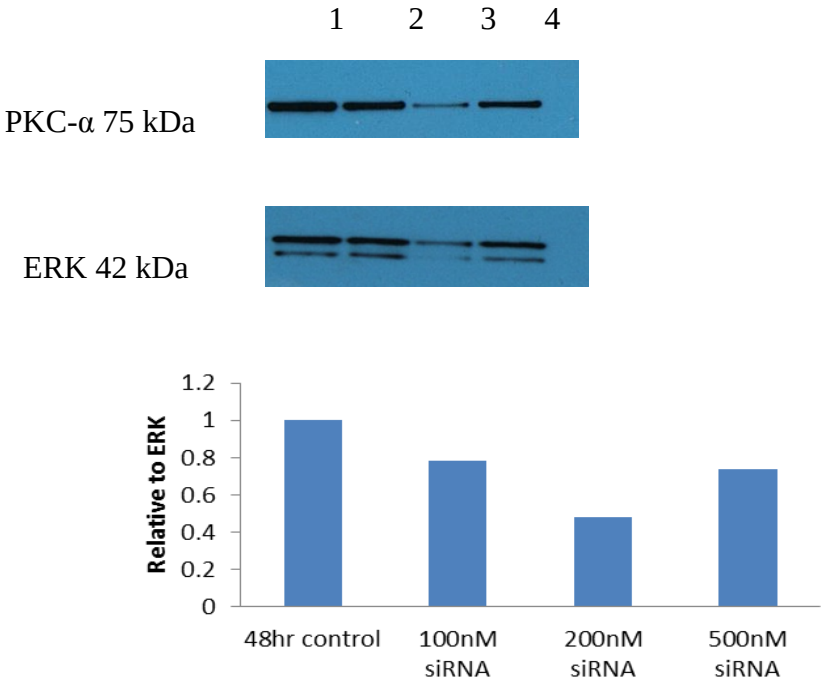
B. Total protein extracts from 48 hour control BMMCs (Lane 1) and siRNA concentrations 100, 200 and 500 nM (Lane 2, 3 and 4) were purified from samples and analysed by western blotting for PKC-α expression. Densitometric analysis of PKC-α normalised against the relevant ERK loading control was undertaken and values expressed as fold change relative to control cells (HiP control normalised to 1)

C. Total protein extracts from 48 hour control BMMC (lane 1), HiPerfect control (Lane 2) and siRNA concentrations 100, 200 and 500 nM (Lane 3, 4 and 5) and negative control siRNA concentrations 100, 200 and 500 nM (Lane 6, 7 and 8) were purified from samples and analysed by western blotting for PKC-α expression. Blots were probed with anti-whole ERK to confirm equal loading. Densitometric analysis of PKC-α as described in Panel B.

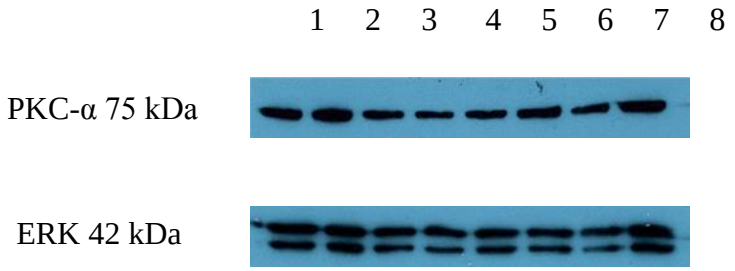
A.


1 2 3 4 5
PKC-α 75 kDa
β-actin 42 kDa
Relative to Actin
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
48hr HiP control
48hr BMMC control
100nM siRNA
200nM siRNA
500nM siRNA

B.


1 2 3 4
PKC-α 75 kDa
ERK 42 kDa
Relative to ERK
1.2
1
0.8
0.6
0.4
0.2
0
48hr control
100nM siRNA
200nM siRNA
500nM siRNA

C.


1 2 3 4 5 6 7 8
PKC-α 75 kDa
ERK 42 kDa

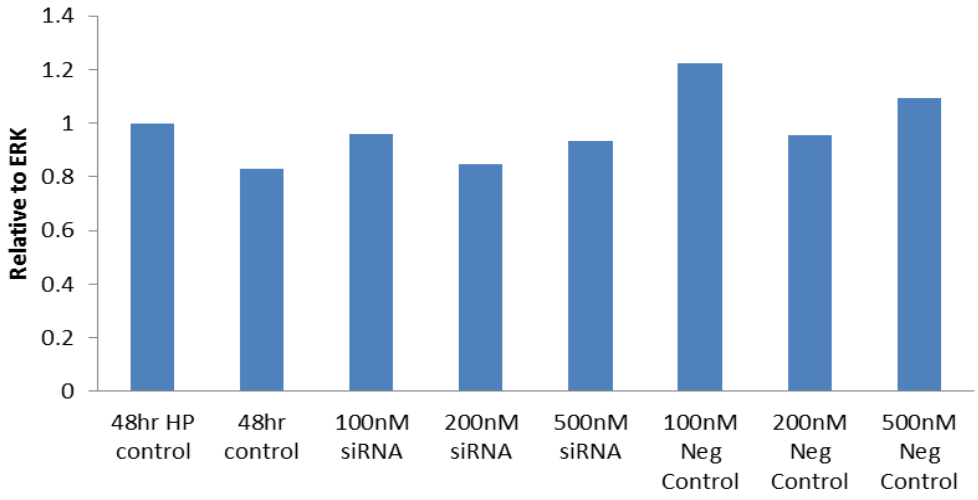

Relative to ERK
1.4
1.2
1
0.8
0.6
0.4
0.2
0
48hr HP control
48hr control
100nM siRNA
200nM siRNA
500nM siRNA
100nM Neg Control
200nM Neg Control
500nM Neg Control

Figure 3.4.1.3 Analysis of BMMC activation following treatment with PKC-α siRNA

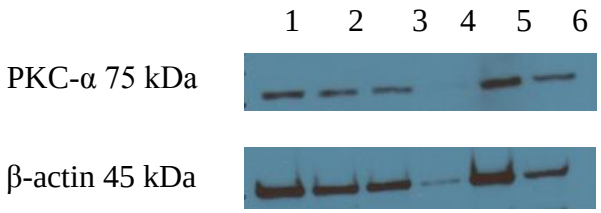
A. Total protein extracts from control BMMC (Lane 1), HiP control (Lane 2) and siRNA concentrations 200 nM and 500 nM cells (lane 3 and 4) and siRNA negative controls 200 nM and 500 nM (Lane 5 and 6) were purified and analysed by western blotting for PKC-α expression. Blots were also probed for β-actin to confirm equal loading.

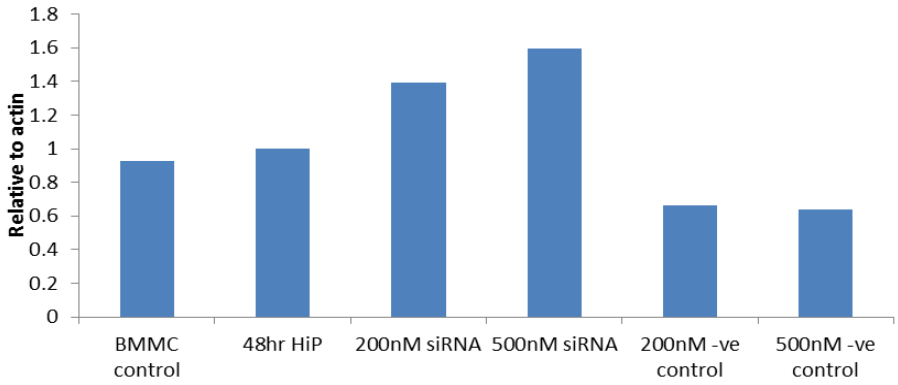
   ii) Following 48hour incubation with siRNA complexes, BMMC were sensitized with 500 ng/ml anti-DNP IgE antibodies (IgE) for 24 hours then stimulated with 500 ng/ml DNP to induce cross-linking (XL) for 24 hours at 37˚C. Cytokine release of IL-6 was measured by ELISA. Results are shown as means ± S.D.

B. i) and ii) All procedures carried out in an identical manner to panel A.

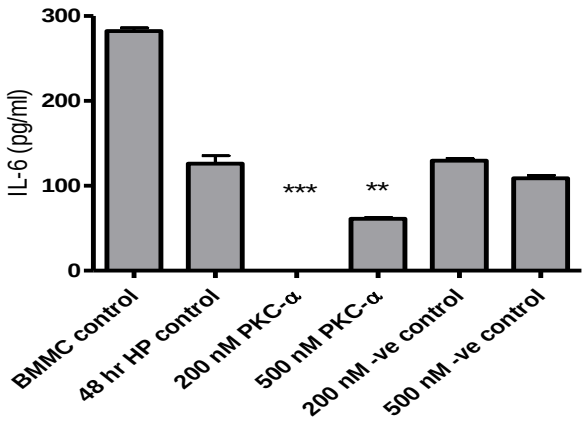
A.

i)


1 2 3 4 5 6
PKC-α 75 kDa
β-actin 45 kDa

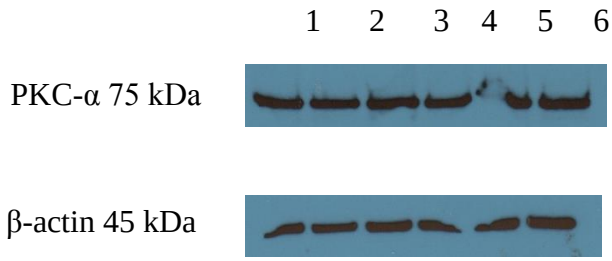

Relative to actin
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
BMMC control
48hr HiP
200nM siRNA
500nM siRNA
200nM -ve control
500nM -ve control

ii)

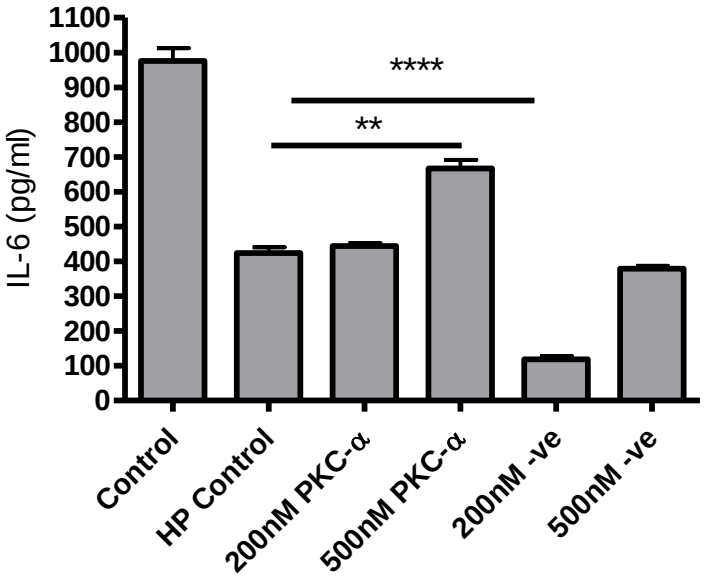

IL-6 (pg/ml)
300
200
100
0
***
**
BMMC control
48 hr HP control
200 nM PKC-α
500 nM PKC-α
200 nM -ve control
500 nM -ve control

B.

i)


1 2 3 4 5 6
PKC-α 75 kDa
β-actin 45 kDa

ii)


IL-6 (pg/ml)
1100
1000
900
800
700
600
500
400
300
200
100
0
****
**
Control
HP Control
200nM PKC-α
500nM PKC-α
200nM -ve
500nM -ve

3.5 PKC knockout studies

As alluded to previously, mast cells are a potential source of multiple cytokines and upon antigen-triggered receptor aggregation these cells synthesise (or indeed have pre-formed) cytokines to be released into the extracellular environment (Mekori & Metcalfe, 2000; Gordon & Galli, 1990). Even though there is a variety of cytokines released from activated mast cells, all of which have been shown to be important in both pathological and protective immune responses (Galli, 1993; Takafuji, et al. 1991; Lorentz, et al. 2000; Mekori & Metcalfe, 2000), extensive cytokine studies have focused primarily on the release of IL-6 and TNF-α following mast cell activation (Ball, et al. 2013; Gagari, et al. 1997; Gordon & Galli, 1990). Indeed, ES-62 has been demonstrated to inhibit the release of both of these cytokines following cross-linking of the high-affinity IgE receptor, FcεRI (Melendez, et al. 2007).

Thus, to establish the role each PKC isoform plays in mast cell function, mast cells grown from PKC knockout (KO) mice were investigated and compared in relation to their wild-type (WT) controls. Similar to previous experiments employing ES-62, the measurement of IL-6 and TNF-α cytokine levels following activation induced by cross-linking of IgE bound to FcεRI in addition to activation with other stimuli, in particular LPS, was used as a method to detect the effects of the absence of a particular PKC isoform on mast cell function. Simultaneously, other immune system cells such as bone-marrow derived macrophages (BMM) and/or dendritic cells (BMDC's) were explored to look for any mast cell-specific effects. Additionally, where a different effect in the KO relative to WT was emerging, both were treated

with ES-62 to investigate whether its mechanism of action is dependent on targeting the particular PKC isoform.

3.5.1 PKC-alpha

PKC-α is probably the best characterized of all PKC isoforms in terms of its role (s) in mast cell responses. PKC-α is often highlighted as one of the PKCs showing the strongest activation following FcεRI cross-linking (Li, et al. 2005; Ozawa, et al. 1993a) suggesting its importance in the subsequent mast cell signalling cascade. Certainly, through employing ES-62, our laboratory group found that PKC-α in particular was a vital isoform for normal mast cell degranulation as anti-sense knockdown inhibits degranulation and the sequestering of this isoform by ES-62-TLR-4 complexes led to the disruption of FcεRI-coupled PLD-SPHK-dependent pathways of calcium mobilization and NF-κB activation (Melendez, et al. 2007). Additionally, inhibitor and activator studies in RBL cells showed that following the direct activation of PKC by the calcium ionophore, Ionomycin, a conventional PKC isoform was most likely involved in the release of TNF (Abdel-Raheem, et al. 2005). Further studies using real-time imaging and western blot analysis revealed that upon addition of the potent histamine antagonist Azelastine, which acts following TNF production at the final processing stage, ionomycin-induced PKC-α translocation to the plasma membrane was blocked (Abdel-Raheem, et al. 2005). Azelastine had previously been shown to inhibit TNF release when used at a low concentration and was particularly effective when cells were stimulated by ionomycin (Hide, et al. 1997). These data collectively implicate the conventional PKC isoform, PKC-α, in

the releasing step of TNF from mast cells (Abdel-Raheem, et al. 2005). Moreover, PKC-α has also been implicated as an important isoform for secretion of other mast cell-derived cytokines following antigen-induced FcεRI aggregation (Li, et al. 2005). Thus, inhibitor and overexpression studies revealed that this conventional isoform was involved in the MAPK signalling pathway, specifically, PKC-α was shown to activate MEKK2 which is involved in the production of IL-6, IL-13 and TNF-α (Li, et al. 2005). Taken together, these studies all implicate PKC-α as an important regulator of FcεRI-stimulated mast cell function, specifically degranulation. However, the effect of genetically knocking out this isoform has been little studied and hence this was the chosen starting point for an alternative approach to studying PKC-α in mast cells.

3.5.1.1 Mast cells

In order to determine if the absence of PKC-α had any effects on the development and maturity of BMMC, the cells were firstly analysed by flow cytometry for their expression of the cell surface markers c-kit and FcεRI. This was followed by a comparison of PKC-α KO and WT BMMC for IL-6 and TNF-α cytokine release following either cross-linking (XL) of bound anti-DNP IgE (IgE) with antigen or stimulation with LPS over several individual experiments. If there was a difference in the response of WT and KO cells emerging as the experiments progressed then BMMC were also analysed following pre-treatment with ES-62 for 24 hours to determine any effect the PKC isoform may have on ES-62 activity.

3.5.1.1.1 Cell surface expression of mast cell markers

PKC-α WT and KO cells were stained for the mast cell surface markers c-kit and FcεRI and then analysed by flow cytometry to look for any differential expression of these surface markers in addition to confirming their identity as mature mast cells. The data suggest that both cultures contain cells visibly recognizable as mast cells based on their forward and side scatter properties and their strong c-kit and FcεRI expression (gating relative to isotype control). The BMMC culture example shown for WT and KO are 86.3% and 92.8% positive respectively (Figure 3.5.1.1.1). These data indicate that the absence of PKC-α does not affect the phenotype of mast cells as both cultures shared near identical expression of both cell surface markers. However, it was interesting to note that for both WT and KO, there appears to be two other small populations of cells that are less mature and for one of these populations, the WT had more cells being c-kit positive but FcεRI negative (represented by around 11.4%) compared to the KO (2.9%). From this, it may suggest that the PKC-α KO may mature slightly quicker than the WT BMMC.

3.5.1.1.2 IL-6 release

The results obtained from 3 separate experiments carried out in an identical manner indicated that PKC-α is important for mast cell functional responses as IL-6 cytokine production was significantly reduced following IgE cross-linking (XL) in 2 out of 3 experiments (Figure 3.5.1.1.2 A), with a small non-significant decrease being observed in the third. One of the experiments showed an atypical significant difference between WT and KO basal levels (data not shown), and although this did

not impact on the significant difference observed between the WT and KO XL samples, it was decided to exclude this from calculations of the percentage change in cytokine production. Thus, from the remaining two experiments, KO cells produced 18% less IL-6 than WT following cross-linking. This result is consistent with previous data obtained using ES-62, which showed that PKC-α is important for fully functional human mast cell responses in response to antigen (Melendez, et al. 2007). However, the data obtained also suggest that although PKC-α is undoubtedly a key component, it is not essential for mast cell IL-6 production as KO BMMC were still able to secrete the cytokine to a lesser extent following antigen stimulation (Figure 3.5.1.1.2 A). An opposing trend was witnessed with LPS-stimulated mast cells where IL-6 production was significantly increased in KO compared to WT in 3 individual experiments (Figure 3.5.1.1.2 B). Overall, KO cells produced 59% ± 8% (Mean ± SEM) more IL-6 following LPS stimulation. Due to the significant differences beginning to emerge in such experiments between the WT and KO, it was decided to further test these cells in the presence or absence of ES-62 to look for any effects knocking out PKC-α might have on ES-62 activity in mast cells. The data obtained when utilizing ES-62 showed that the molecule was able to significantly reduce FcεRI-induced IL-6 cytokine production in both WT and KO cells (Figure 3.5.1.1.3 A), indicating no loss of ES-62 activity in the KO. With respect to LPS-treated cells, ES-62 was unable to significantly inhibit production of IL-6 in the KO cells in two separate experiments but as this was also true of the WT cells in one of the experiments (results not shown) it was not possible to conclude whether ES-62 had a different effect on the KO versus the WT with LPS-stimulated cells.

3.5.1.1.3 TNF-α release

The analysis of PKC-α WT and KO BMMC for TNF-α release was undertaken following either XL of bound anti-DNP IgE with antigen or LPS stimulation. In the XL experiment, secretion of this cytokine was significantly reduced in PKC-α KO mast cells in 3 independent experiments (Figure 3.5.1.1.4). On average, TNF-α production was decreased by 45% ± 10% (Mean ± SEM) in BMMC lacking PKC-α. These data suggest PKC-α plays a role in TNF-α cytokine production by mouse mast cells, yet, KO BMMC were still able to mount some degree of a response to FcεRI cross-linking (Figure 3.5.1.1.4). LPS-induced TNF-α production was decreased in KO BMMC in two experiments, but only in one experiment did the result reach statistical significance (data not shown). A third experiment showed a non-significant increase in cytokine production (data not shown).

The emerging differential behaviour of the WT and KO cells in response to XL prompted investigation of these BMMC following pre-treatment with ES-62 in one of the experiments. ES-62-exposed cells produced less TNF-α in WT mast cells (Figure 3.5.1.1.5) but this did not reach statistical significance as shown previously (Section 3.2.1 and Ball, et al. 2013). Similarly, an inhibition was observed with the KO cells but with this experiment reaching statistical significance (Figure 3.5.1.1.5). Overall, this suggests that ES-62 is active in inhibiting the TNF-α response in the absence of PKC-α.

Fig 3.5.1.1.1 Flow cytometric analysis of WT and PKC-α KO BMMC for expression of mast cell surface markers c-kit and FcεRI

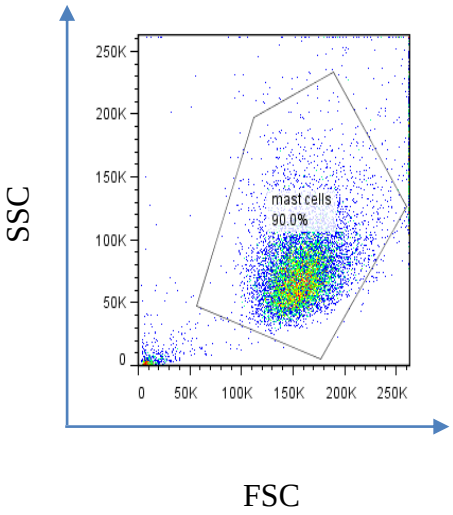
A. Dot plot analysis of FSC versus SSC allowing gating of live cells on the basis of their size and granularity, respectively.

B. Dot plot showing setting of gates for c-kit + and/or FcεRI + cells on the basis of staining of live mast cells (as gated in panel A) with relevant isotype controls.

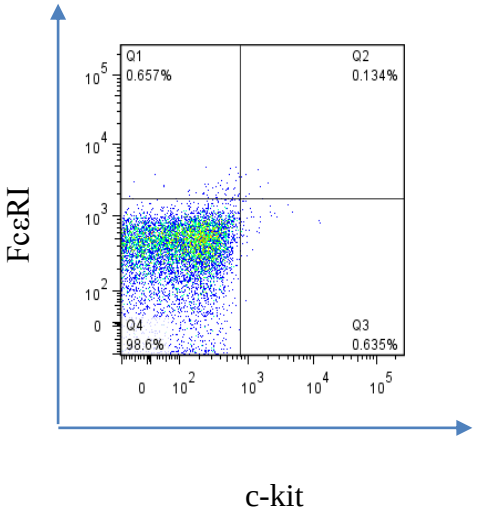
C. WT mast cells gated as described in panel A) were examined for their expression of c-kit and FcεRI relevant to appropriate isotype controls (panel B). Expression of C-kit (x-axis) was determined by staining with an APC-conjugated anti-mouse CD117 (c-kit) antibody gated relative to its isotype control (APC-conjugated rat anti-mouse IgG2b; panel B). Expression of FcεRI (y-axis) was determined by staining with FITC-conjugated anti-mouse FITC FcεRI antibody gated relative to its isotype control (FITC-conjugated Armenian Hamster IgG; panel b).

D. KO mast cells gated as described in panel a) were examined for their expression of c-kit and FcεRI relevant to appropriate isotype controls (panel b) as outlined in panel C.

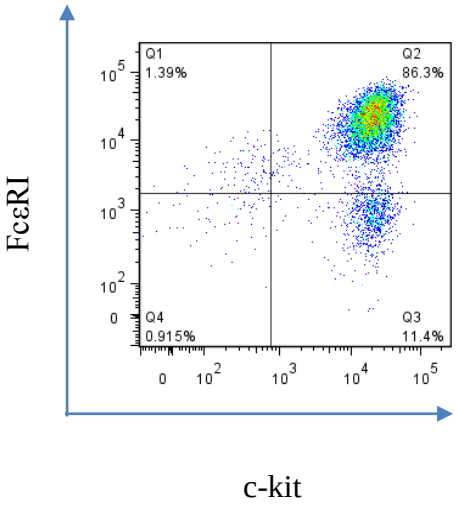
A.


SSC
FSC
mast cells
90.0%
250K
200K
150K
100K
50K
0
0
50K
100K
150K
200K
250K

B.


FcεRI
c-kit
Q1
0.657%
Q2
0.134%
Q4
98.6%
Q3
0.635%

C.


FcεRI
c-kit
Q1
1.39%
Q2
86.3%
Q4
0.915%
Q3
11.4%

D.

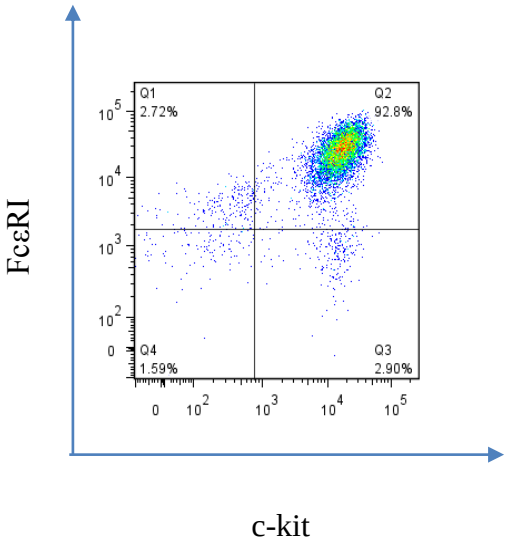
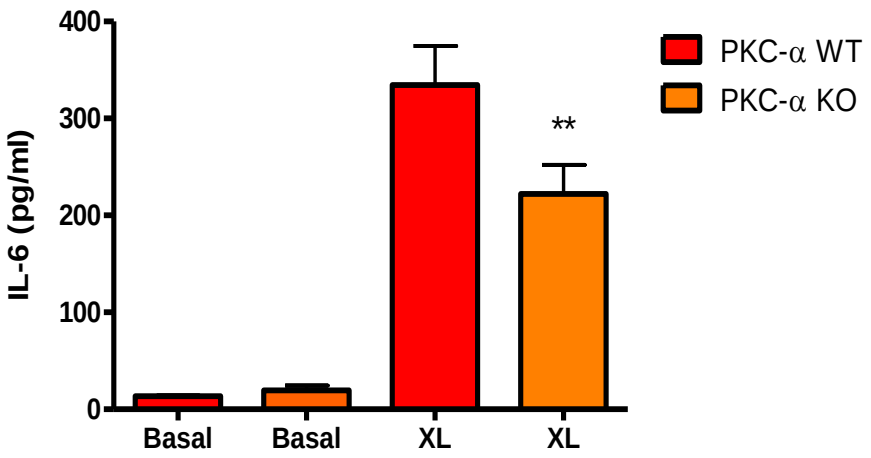

FcεRI
c-kit
Q1
2.72%
Q2
92.8%
Q4
1.59%
Q3
2.90%

Figure 3.5.1.1.2. The effect of PKC-α KO on IL-6 cytokine production in BMMC

A. PKC-α KO and WT BMMC were sensitized with 500 ng/ml anti-DNP IgE antibodies (IgE) for 24 hours and then stimulated with 500 ng/ml DNP–BSA to induce cross-linking (XL) for 24 hours at 37˚C. Release of IL-6 was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 2 independent experiments. A third experiment showed a non-significant decrease in IL-6 production by the KO. The statistical analysis performed is one way ANOVA where ** $P<0.05$ for WT versus KO XL.

B. PKC-α WT and KO BMMC were sensitized with LPS (1μg/ml) for 24 hours at 37˚C. Release of IL-6 was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 3 independent experiments. The statistical analysis performed is one way ANOVA where **** $P<0.0001$ for WT versus KO LPS.

A.


400
300
200
100
0
IL-6 (pg/ml)
**
Basal
Basal
XL
XL
PKC-α WT
PKC-α KO

B.

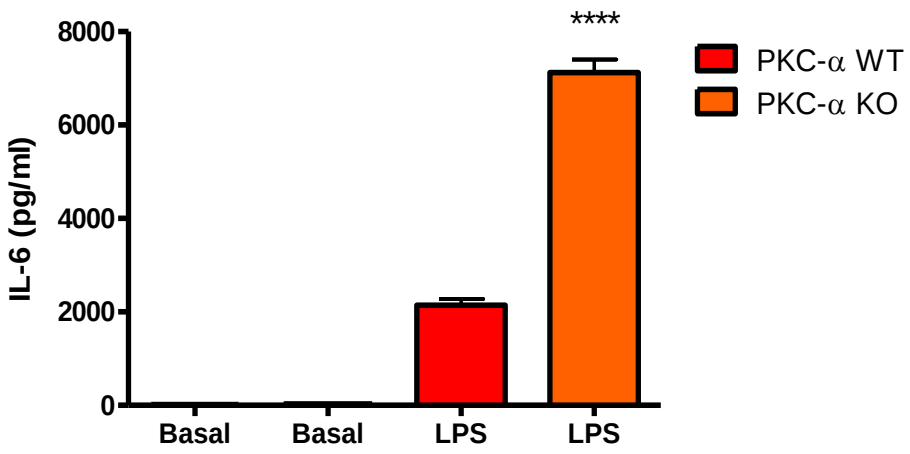
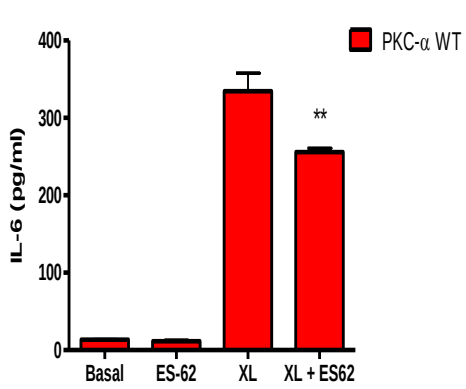

8000
6000
4000
2000
0
IL-6 (pg/ml)
****
Basal
Basal
LPS
LPS
PKC-α WT
PKC-α KO

Figure 3.5.1.1.3 The effect of the absence of PKC-α on ES-62 activity on IL-6 secretion in BMMC

PKC-α WT and KO BMMC were sensitized with 500 ng/ml anti-DNP IgE (IgE) for 24 hours and then stimulated with 500 ng/ml DNP-BSA to induce cross-linking (XL) for 24 hours at 37˚C in the presence or absence of ES-62. Results are shown as means ± S.D. Release of IL-6 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values. The statistical analysis performed is one way ANOVA where ** P <0.005 for WT XL versus WT XL+ES-62 and P<0.005 ** for KO XL versus KO XL+ES-62


PKC-α WT
IL-6 (pg/ml)
400
300
200
100
0
**
Basal
ES-62
XL
XL + ES62

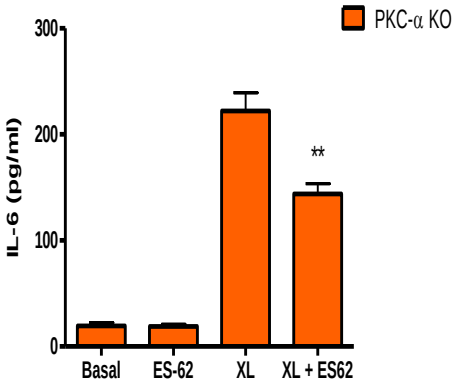

PKC-α KO
IL-6 (pg/ml)
300
200
100
0
**
Basal
ES-62
XL
XL + ES62

Figure 3.5.1.1.4 The effect of the absence of PKC-α on TNF-α cytokine production in BMMC

PKC-α KO and WT BMMC were sensitized with 500 ng/ml anti-DNP IgE antibodies (IgE) for 24 hours and then stimulated with 500 ng/ml DNP-BSA to induce cross-linking (XL) for 24 hours at 37˚C. Release of TNF-α was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 3 independent experiments. The statistical analysis performed is one way ANOVA where ** $P<0.005$ for WT XL versus KO XL.

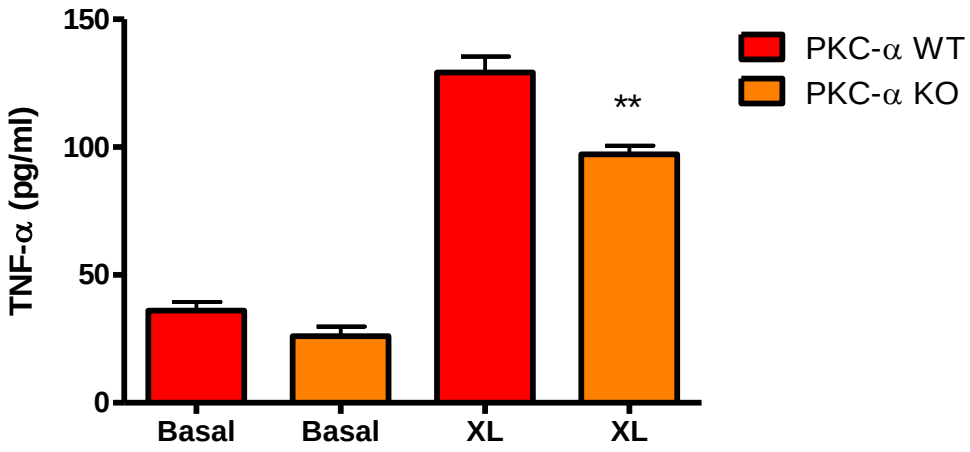
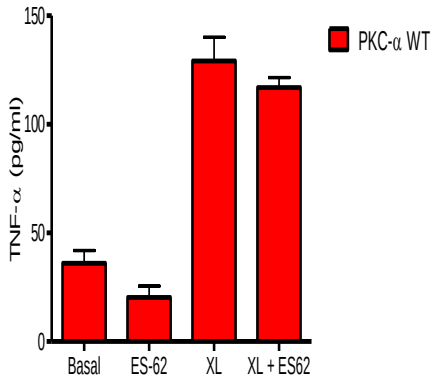
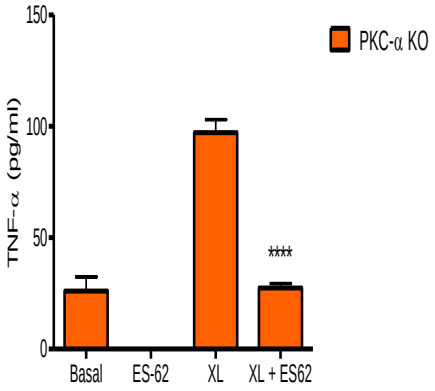

150
100
50
0
TNF-α (pg/ml)
Basal
Basal
XL
XL
**
PKC-α WT
PKC-α KO

Figure 3.5.1.1.5 The effect of the absence of PKC-α on ES-62 activity on TNF-α secretion in BMMC

PKC-α WT and KO BMMC were sensitized with 500 ng/ml anti-DNP IgE (IgE) for 24 hours and then stimulated with 500 ng/ml DNP to induce cross-linking (XL) for 24 hours at 37˚C in the presence or absence of ES-62. Results are shown as means ± S.D. Release of TNF-α was measured by ELISA. The data presented are from a single experiment incorporating triplicate values. The statistical analysis performed is one way ANOVA where **** P<0.0001 for KO XL versus KO XL+ES-62.


150
100
50
0
TNF-α (pg/ml)
PKC-α WT
Basal
ES-62
XL
XL + ES62


150
100
50
0
TNF-α (pg/ml)
PKC-α KO
****
Basal
ES-62
XL
XL + ES62

3.5.1.2 Macrophages

The analysis of PKC-α KO and WT BMM for IL-6, TNF-α, IL-12 and IL-10 cytokine release was undertaken following stimulation with LPS for 24 hours. If a difference was observed between WT and KO, BMM were also analysed following pre-treatment with ES-62 for 18 hours. These particular pro-inflammatory and anti-inflammatory cytokines chosen for analysis was due to the Harnett lab having previously established the effects of ES-62 on them in macrophages (Goodridge, et al. 2001). The data obtained from these experiments could be used as a comparison to the mast cell data to look for any mast cell-specific effects.

3.5.1.2.1 IL-6 release

Analysis of PKC-α KO and WT macrophages following LPS stimulation for 24 hours revealed that the most consistent result - observed in 3 out of 5 experiments - was no significant difference in IL-6 production between WT and KO functional responses (Figure 3.5.1.2.1). With respect to the other two experiments, one showed a significant increase and one a significant decrease (results not shown). Due to the most consistent result being no change between WT and KO macrophages, it was decided not to investigate ES-62 activity in the WT and KO cells.

3.5.1.2.2 TNF-α release

Upon LPS stimulation of macrophages for 24 hours, it was discovered that TNF-α production was significantly up-regulated (Figure 3.5.1.2.2 A) in 3 out of 5 independent experiments in PKC-α KO macrophages compared to WT control cells. The average increase in cytokine production from the 3 experiments was 27 %± 4% (Mean ± SEM). No significant change was observed in the other two experiments (results not shown). The significant difference between the WT and KO cell response to LPS emerging in the majority of the experiments led to later experiments including cells being exposed to ES-62 to look for any effect the KO may have on the ability of this molecule to skew the cytokine response. Analysis of WT macrophages following stimulation with LPS revealed that ES-62 caused a decrease in TNF-α in three experiments although in only one of these did this reach statistical significance (Figure 3.5.1.2.2 B; results now shown). In this last experiment, ES-62 also decreased cytokine levels in the KO cells although this did not reach statistical significance (Figure 3.5.1.2.2 B). Furthermore, the two other experiments showed a non-significant increase in KO cells (results not shown). Overall therefore, although the effects of ES-62 were marginal, there was perhaps a suggestion that the helminth product might possess greater inhibitory activity in the WT macrophages.

3.5.1.2.3 IL-12 release

Similar to the data measuring IL-6 in macrophages, it was found that there was no difference in IL-12 cytokine release following 24 hour LPS stimulation between PKC-α KO and WT macrophages and this was the case in 4 individual experiments

(Figure 3.5.1.2.3). Again, the lack of difference between WT and KO samples meant that no further investigation incorporating ES-62 exposure was undertaken.

3.5.1.2.4 IL-10 release

Following LPS stimulation for 24 hours, the data acquired showed that IL-10 cytokine production was significantly increased in PKC-α KO macrophages in comparison to WT controls in 4 separate experiments under the same conditions (Figure 3.5.1.2.4 A). Overall, PKC-α KO macrophages showed a 40% ± 5% (Mean ± SEM) increase in IL-10 secretion in the 4 experiments. Additionally, the effect of ES-62 on production of this cytokine in these cells was investigated. It was found that pre-treatment of these cells with ES-62 had no significant effect on LPS-induced IL-10 production in both KO and WT macrophages in all experiments performed (Figure 3.5.1.2.4 B). The result with the WT macrophages correlates with previous data where pre-treatment with ES-62 in vivo prompted no elevated levels of IL-10 cytokine production by macrophages when tested ex vivo (Goodridge et al, 2001).

Figure 3.5.1.2.1 The effect of PKC-α KO on IL-6 cytokine production in BMM

PKC-α KO and WT BMM were sensitized with 100 ng/ml LPS for 24 hours at 37˚C. Release of IL-6 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 3 independent experiments. Two other experiments showed a significant increase and significant decrease in IL-6 production, respectively. The statistical analysis performed is one way ANOVA.

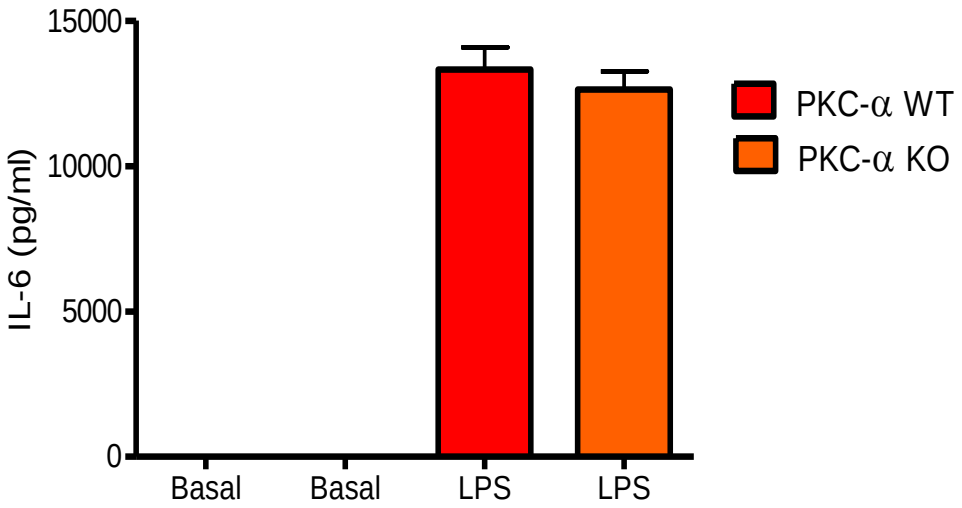
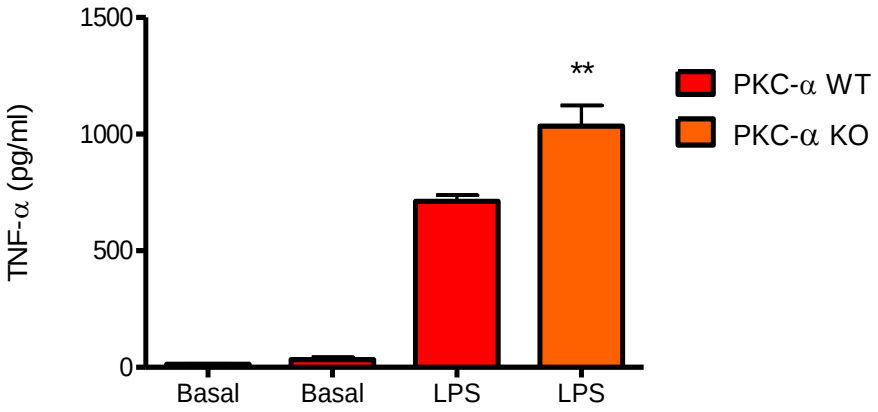

15000
10000
5000
0
IL-6 (pg/ml)
Basal
Basal
LPS
LPS
PKC-α WT
PKC-α KO

Figure 3.5.1.2.2 The effect of PKC-α KO and ES-62 on TNF-α cytokine production in BMM

A. PKC-α WT and KO BMM were sensitized with 100 ng/ml LPS for 24 hours at 37˚C. Release of TNF-α was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and is representative of 3 independent experiments. Two other experiments showed no significant difference between WT and KO cells. The statistical analysis performed one way Anova where ** $P<0.005$ for LPS WT versus KO.

B. PKC-α WT and KO BMM were sensitized with 100 ng/ml LPS for 24 hours following pre-treatment of cells in the presence or absence of ES-62 for 18 hours. Release of TNF-α was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of a single experiment. Two other experiments showed a non-significant decrease in the WT and a small non-significant increase in the KO following ES-62 treatment. The statistical analysis performed is one way ANOVA where *** $P < 0.001$ for WT LPS versus LPS + ES-62.

A.


1500
1000
500
0
TNF-α (pg/ml)
**
PKC-α WT
PKC-α KO
Basal
Basal
LPS
LPS

B.

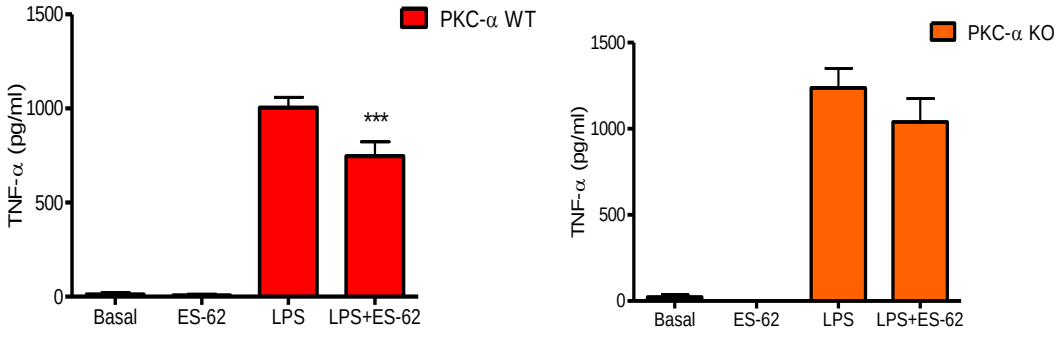

1500
1000
500
0
TNF-α (pg/ml)
PKC-α WT
***
Basal
ES-62
LPS
LPS+ES-62
PKC-α KO
Basal
ES-62
LPS
LPS+ES-62

Figure 3.5.1.2.3 The effect of PKC-α KO on IL-12 cytokine production in BMM

PKC-α KO and WT BMM were sensitized with 100 ng/ml LPS for 24 hours. Release of TNF-α was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 4 independent experiments. The statistical analysis performed is one way ANOVA.

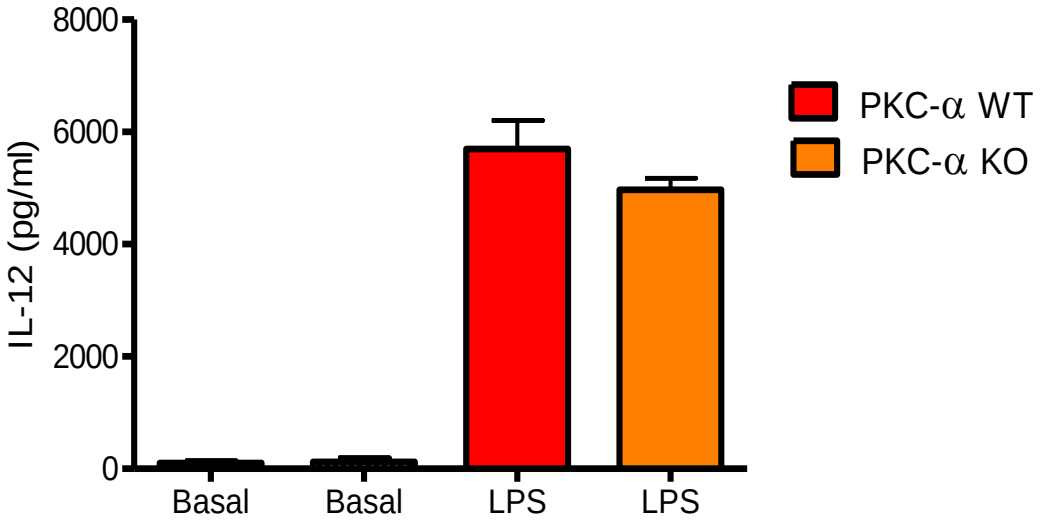
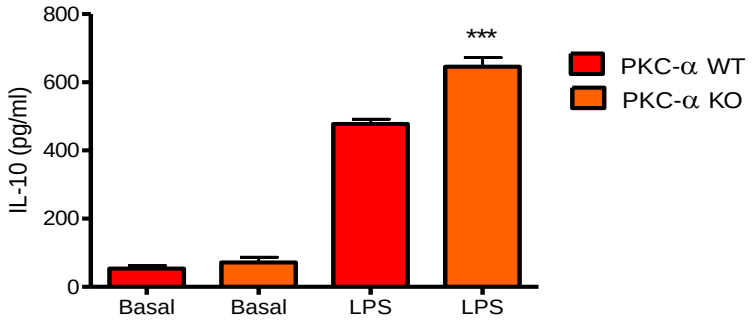

8000
6000
4000
2000
0
IL-12 (pg/ml)
Basal
Basal
LPS
LPS
PKC-α WT
PKC-α KO

Figure 3.5.1.2.4 The effect of PKC-α KO and ES-62 on IL-10 cytokine production in BMM

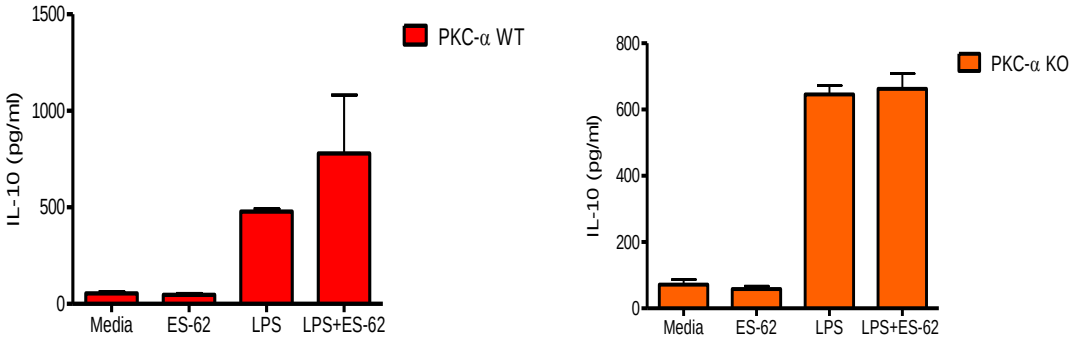
A. PKC-α WT and KO BMM were sensitized with 100 ng/ml LPS for 24 hours. Release of IL-10 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 4 independent experiments. The statistical analysis performed is one way ANOVA where *** $P<0.001$ for LPS WT versus KO.

B. PKC-α WT and KO BMM were sensitized with 100 ng/ml LPS for 24 hours following pre-treatment of cells in the presence or absence of ES-62 for 18 hours. Release of IL-10 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 4 independent experiments. The statistical analysis performed is one way ANOVA.

A.


800
600
400
200
0
IL-10 (pg/ml)
***
PKC-α WT
PKC-α KO
Basal
Basal
LPS
LPS

B.


1500
1000
500
0
IL-10 (pg/ml)
PKC-α WT
Media
ES-62
LPS
LPS+ES-62
800
600
400
200
0
IL-10 (pg/ml)
PKC-α KO
Media
ES-62
LPS
LPS+ES-62

3.5.1.3 Dendritic cells (BMDC)

The same approach with macrophages was used for DCs for PKC-α WT and KO cells. Again, IL-6, TNF-α, IL-12 and IL-10 cytokine release measurement was undertaken following stimulation with LPS for 24 hours. These particular cytokines were chosen, as the effect of ES-62 on their secretion by DCs was known (Goodridge, et al. 2004).

3.5.1.3.1 IL-6

Upon stimulation with LPS (10 ng/ml) for 24 hours, it was witnessed that there was no consistent effect of PKC-α KO on IL-6 cytokine production over 4 individual experiments (data not shown). This therefore resulted in no further testing incorporating the use of ES-62.

3.5.1.3.2 TNF-α

The analysis of TNF-α secretion was also investigated following 24-hour incubation with LPS (10 ng/ml) on 4 separate experiments. The data obtained revealed an increase in TNF-α secretion in PKC-α KO DCs in comparison to WT in all 4 experiments, with 3 out of 4 experiments showing a statistically significant difference (Figure 3.5.1.3.1 A). On average, the experiments revealed a 46% ± 8% (Mean ± SEM) increase in TNF-α production in KO DCs. The emerging data obtained prompted the inclusion of testing of these cells in one experiment with ES-

62. This experiment revealed that WT DCs showed a significant reduction in TNF-α secretion by ES-62 of 54% (Figure 3.5.1.3.1 B). The data obtained from KO DCs similarly showed a significant reduction when incubated with the nematode product (Figure 3.4.1.3.2 B). Thus, it appears that ES-62 is active in DCs lacking PKC-α.

3.5.1.3.3 IL-12

Analysis of IL-12 cytokine production following 24 hour incubation with LPS (10 ng/ml) revealed that there was no difference between PKC-α WT and KO DCs in 4 separate experiments (Figure 3.5.1.3.2). This lack of a differential response between the WT and KO again negated the need for testing with ES-62.

3.5.1.3.4 IL-10

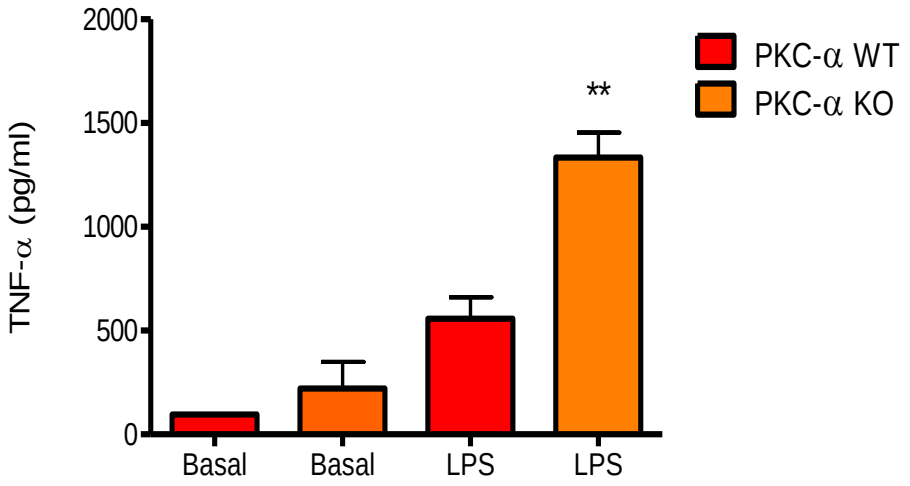
Following LPS stimulation for 24 hours, it was found that PKC-α KO DCs had significantly less IL-10 production compared to WT controls in 3 out of 4 experiments performed (Figure 3.5.1.3.3). This result led to testing of the effects of ES-62 on IL-10 cytokine production in both WT and KO cells in order to look for any differences in the molecule's activity. However, it was found that the effect of ES-62 on the WT cells was surprisingly inconsistent (results not shown) and hence no conclusions could be drawn.

Figure 3.5.1.3.1 The effect of PKC-α KO on TNF- α cytokine secretion from DCs

A. PKC-α WT and KO DCs were sensitized with 10 ng/ml LPS for 24 hours at 37˚C. Release of TNF-α was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 3 independent experiments. A fourth experiment showed a non-significant increase in the KO. The statistical analysis performed is one way ANOVA where ** $P<0.005$ for LPS WT versus KO

B. PKC-α WT and KO DCs were sensitized with 10 ng/ml LPS for 24 hours at 37˚C in the presence or absence of ES-62. Release of TNF-α was measured by ELISA. The data presented are from a single experiment incorporating triplicate values. The statistical analysis performed is one way ANOVA where *** $P<0.001$ for WT LPS versus WT LPS+ES-62 and *** $P<0.001$ for KO LPS versus KO LPS+ES-62.

A.


2000
1500
1000
500
0
TNF-α (pg/ml)
**
PKC-α WT
PKC-α KO
Basal
Basal
LPS
LPS

B.

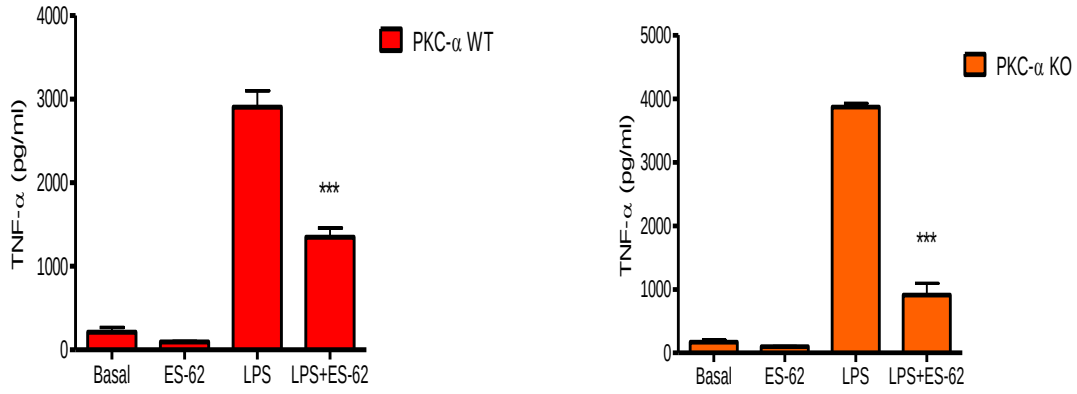

4000
3000
2000
1000
0
TNF-α (pg/ml)
***
PKC-α WT
Basal
ES-62
LPS
LPS+ES-62
5000
4000
3000
2000
1000
0
TNF-α (pg/ml)
***
PKC-α KO
Basal
ES-62
LPS
LPS+ES-62

Figure 3.5.1.3.2 The effect of PKC-α KO on IL-12 cytokine secretion from DCs

PKC-α WT and KO DCs were sensitized with 10 ng/ml LPS for 24 hours at 37˚C. Release of IL-12 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 4 independent experiments. The statistical analysis performed is one way ANOVA

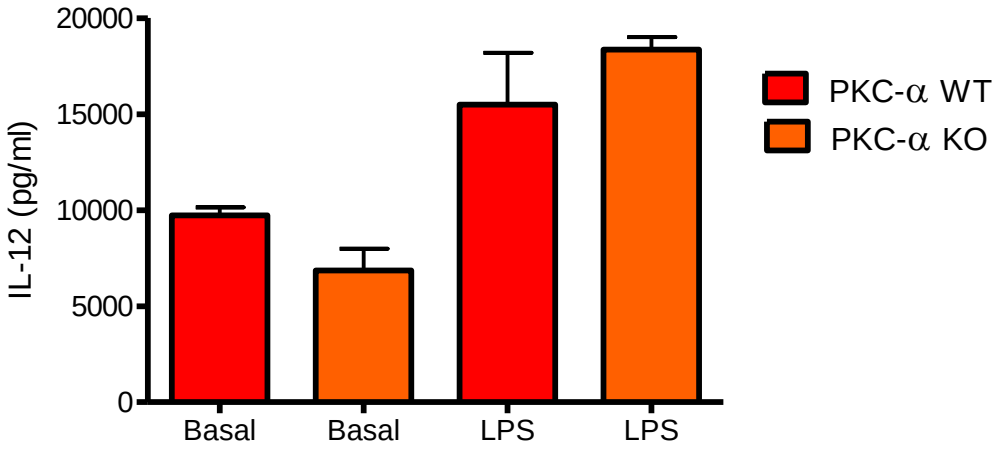
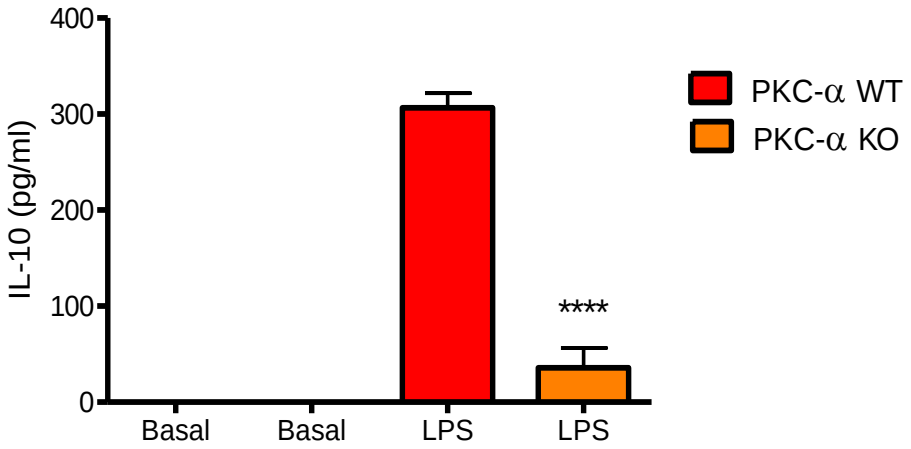

20000
15000
10000
5000
0
IL-12 (pg/ml)
Basal
Basal
LPS
LPS
PKC-α WT
PKC-α KO

Figure 3.5.1.3.3 The effect of PKC-α KO and ES-62 on IL-10 cytokine secretion from DCs

PKC-α WT and KO DCs were sensitized with 10 ng/ml LPS for 24 hours at 37˚C. Release of IL-10 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 3 independent experiments. A fourth experiment showed no significant difference between WT and KO. The statistical analysis performed is one way ANOVA where **** P< 0.0001 for LPS WT versus KO.


400
300
200
100
0
IL-10 (pg/ml)
Basal
Basal
LPS
LPS
****
PKC-α WT
PKC-α KO

3.5.2 PKC-beta

PKC-β strongly parallels PKC-α as a key component of the FcεRI signalling cascade due to its potent translocation to the plasma membrane and strong activation following IgE receptor aggregation (Ozawa, et al. 2005; Li, et al. 2005). A significant role for PKC-β in this mast cell secretory process was discovered by Ozawa and colleagues using RBL cells (Ozawa, et al. 1993a). By reconstituting the PKC-depleted permeabilized RBL cells with PKC-β (or PKC-δ) it was shown that this isoform could potently induce antigen-stimulated mast cell degranulation (Ozawa, et al. 1993a). In parallel, this group further disclosed the importance of PKC-β by highlighting that the activation of this isoform was a positive regulatory signal for antigen-induced FcεRI mast cell exocytosis based on estimated concentrations obtained from comparing total protein extracts from RBL cells with known concentrations of purified recombinant PKC isoforms (Ozawa, et al. 1993b). This finding that PKC-β was a positive regulator of the secretory response in mast cells was supported by studies carried out by Chang et al where the overexpression of the conventional isoform PKC-β positively influenced exocytosis in RBL cells (Chang, et al. 1997). Moreover, a study using PKC-β knockout mice indicated that these mice were far less effective inducers of secretion following antigen stimulation than wild-type cells, with the knockout response inhibited by greater than 50% (Nechushtan, et al. 2000). In addition, a significant decrease in IL-6 cytokine production following IgE-induced FcεRI aggregation was noted in PKC-β knockout cells in comparison to wild-type, suggesting a role for this isoform in IL-6 cytokine production from mast cells (Nechushtan, et al. 2000). It has previously been reported that ES-62 targeted PKC-α for degradation, thereby resulting in inhibition of

degranulation in human mast cells (Melendez, et al. 2007). Interestingly, ES-62 also targeted isoforms other than PKC-α and one of the isoforms most strongly affected after PKC-α was PKC-β (Melendez, et al. 2007). Therefore, this targeting of PKC-β by ES-62 (Melendez, et al. 2007) and the collective data from PKC-β mast cell studies (Nechushtan, et al. 2000; Ozawa, et al. 1993a and 1993 b; Li, et al. 2005) led to PKC-β being chosen as the next isoform to study using BMMC from PKC-β knockout mice.

3.5.2.1 Mast cells

Similar to the PKC-α experiment, as a first step PKC-β WT and KO cells were analysed by flow cytometry for the expression of the mast cell markers FcεRI and c-kit. Following this, PKC-β WT and KO BMMC were sensitized with anti-DNP IgE (500 ng/ml) and stimulated with antigen to induce FcεRI cross-linking (XL) or stimulated with LPS (1μg/ml) for 24 hours before measurement of IL-6 and TNF-α levels was completed. If a differential response was witnessed between WT and KO BMMC, then the cytokine responses of both cells were also analysed following pre-treatment with ES-62 for 24 hours.

3.5.2.1.1 Cell surface expression of mast cell markers

Both WT and KO cells were analysed by flow cytometry for their expression of the cell surface markers, FcεRI and c-kit to not only confirm their identity as mast cells, but also to look for any phenotypic differences between the two cell types. The

results show that mast cells are clearly identifiable on the basis of their forward and side scatter properties and their strong c-kit and FcεRI expression (gating relative to isotype control). On average, BMMC WT cultures were 87.5% and KO BMMC were 85% double positive for c-kit and FcεRI. These data suggest that both WT and KO generated cells were truly mast cells and shared almost identical phenotypic characteristics. The BMMC culture examples shown for WT and KO are 90.6% and 87.6% respectively (Figure 3.5.2.1.1). It was interesting to note that again there was evidence of two other smaller populations with a more immature phenotype in both WT and KO cultures but the WT had less cells being C-kit positive and FcεRI negative (represented by around 4.9%) compared to the KO (8.2%). Thus, it seems with this isoform that the WT BMMC may mature slightly quicker than KO BMMC.

3.5.2.1.2 IL-6

The results obtained revealed that there was very little (3 experiments) or no (one experiment) cytokine production following FcεRI cross-linking in both WT and KO cells over 4 individual experiments. However, despite cytokines levels being below or close to the detection limit, in the 3 experiments where cytokine could be detected there was an increase in IL-6 secretion in KO mast cells in comparison to WT cells; with two experiments showing a statistically significant result (Figure 3.5.2.1.2 A). Overall, the KO mast cells showed an increase in cytokine production by 52% ± 21% (Mean ± SEM). Nechushtan and co-workers found no IL-6 production following FcεRI aggregation in PKC-β deficicent BMMC in their preliminary experiments (Nechushtan, et al. 2006) suggesting this may be a common feature of the KO.

However, this does not explain why such low levels were similarly witnessed in WT BMMC in my experiments. The lack of IL-6 production in the KO witnessed by Nechushtan and colleugues was overcome when the sensitisation step with IgE was increased (Nechushtan, et al. 2006) therefore perhaps such disparity in results could be attributed to experimental differences. Furthermore, it was found that following LPS stimulation, PKC-β KO BMMC showed significantly greater IL-6 production compared to WT in 4 individual experiments (Figure 3.5.2.1.2. B). Overall, KO cells exhibited a 58% ± 4% (Mean ± SEM) increase in IL-6 secretion compared to WT cells. The response of these cells to ES-62 was also assessed and the data obtained showed that following LPS stimulation, ES-62 down-regulated IL-6 production in WT BMMC in 4 experiments (Figure 3.5.2.1.3) where this reached significance on two occasions. However, ES-62's activity was inconsistent in the KO cells with experiments showing significant and non-significant decreases but also significant and non-significant increases in IL-6 secretion for the experiments undertaken (data not shown). Overall therefore, it is not possible to reach any conclusion regarding the nature of ES-62's activity in the KO cells.

3.5.2.1.3 TNF-α

TNF-α cytokine production by PKC-β WT and KO BMMC was undertaken following either cross-linking (XL) of bound anti-DNP IgE with antigen or LPS stimulation for 24 hours. Comparable to IL-6 secretion, the results observed showed that both WT and KO cells failed to produce TNF-α in response to FcεRI XL in 4 separate experiments (data not shown). Again, it was interesting to note, that similar

to IL-6 secretion, LPS-induced TNF-α production was also dramatically increased in KO BMMC in all 4 experiments completed (Figure 3.5.2.1.4 A). The average increase in KO cells was 62% ± 5% (Mean ± SEM). The WT and KO cells were also investigated following exposure to ES-62 (2 μg/ml) for 24 hours. The data obtained showed that the helminth product decreased cytokine production for PKC-β WT across 4 experiments, where this reached significance on one occasion (Figure 3.5.2.1.4 B). However, the results with the PKC- β KO were less consistent. Thus, ES-62 significantly decreased TNF-α production on two occasions (data not shown), but in the other two experiments a significant increase was observed (Figure 3.5.2.1.4 B). Overall, these data may suggest that the absence of PKC-β does not impact on ES-62's ability to inhibit cytokine responses in mast cells. However, the inconsistency witnessed with the KO makes it difficult to be certain about this.

Fig 3.5.2.1.1 Flow cytometric analysis of PKC-β BMMC for expression of mast cell surface markers c-kit and FcεRI

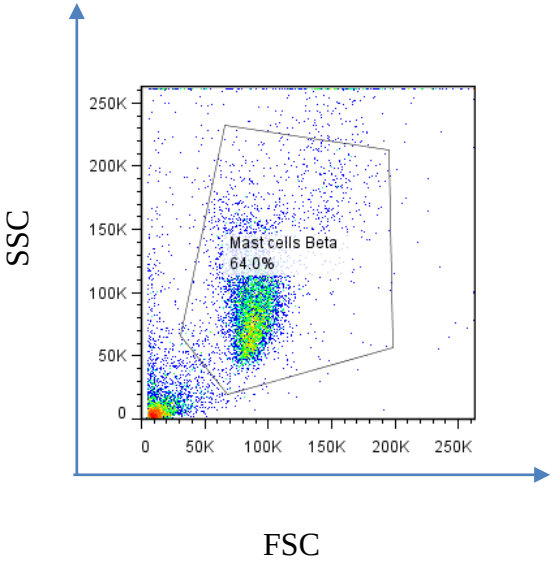
A. Dot plot analysis of FSC versus SSC allowing gating of live cells on the basis of their size and granularity, respectively.

B. Dot plot showing setting of gates for c-kit + and/or FcεRI + cells on the basis of staining of live mast cells (as gated in panel A) with relevant isotype controls.

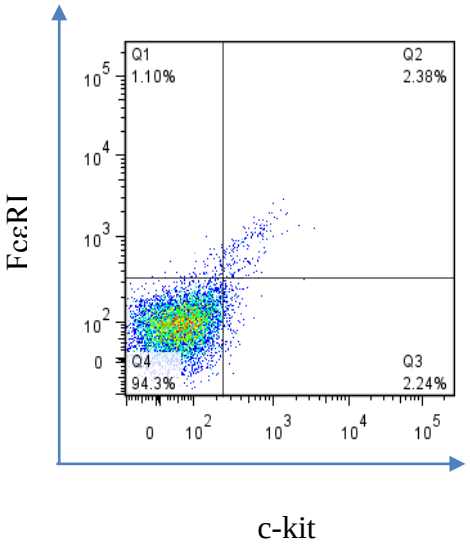
C. WT mast cells gated as described in panel A) were examined for their expression of c-kit and FcεRI relevant to appropriate isotype controls (panel B). Expression of C-kit (x-axis) was determined by staining with an APC-conjugated anti-mouse CD117 (c-kit) antibody gated relative to its isotype control (APC-conjugated rat anti-mouse IgG2b; panel B). Expression of FcεRI (y-axis) was determined by staining with FITC-conjugated anti-mouse FITC FcεRI antibody gated relative to its isotype control (FITC-conjugated Armenian Hamster IgG; panel B).

D. KO mast cells gated as described in panel A) were examined for their expression of c-kit and FcεRI relevant to appropriate isotype controls (panel B). Expression of C-kit (x-axis) was determined by staining with an APC-conjugated anti-mouse CD117 (c-kit) antibody gated relative to its isotype control (APC-conjugated rat anti-mouse IgG2b; panel B. Expression of FcεRI (y-axis) was determined by staining with FITC-conjugated anti-mouse FITC FcεRI antibody gated relative to its isotype control (FITC-conjugated Armenian Hamster IgG; panel B).

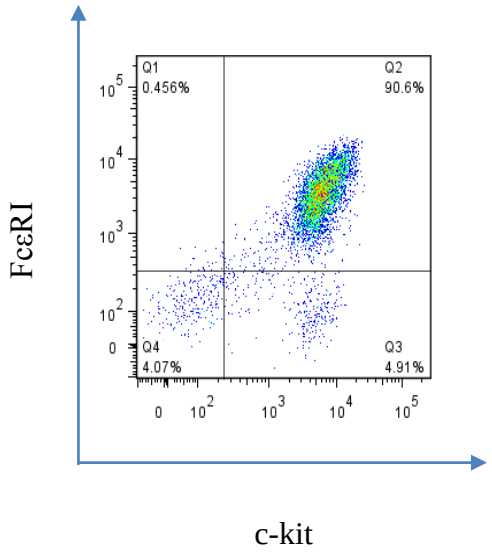
A.


SSC
250K
200K
150K
100K
50K
0
Mast cells Beta
64.0%
0
50K
100K
150K
200K
250K
FSC

B.


FcεRI
Q1
1.10%
Q2
2.38%
Q4
94.3%
Q3
2.24%
c-kit

C.


FcεRI
Q1
0.456%
Q2
90.6%
Q4
4.07%
Q3
4.91%
c-kit

D.

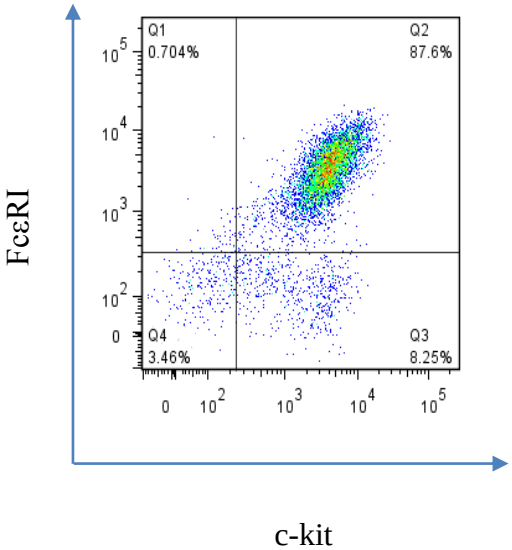
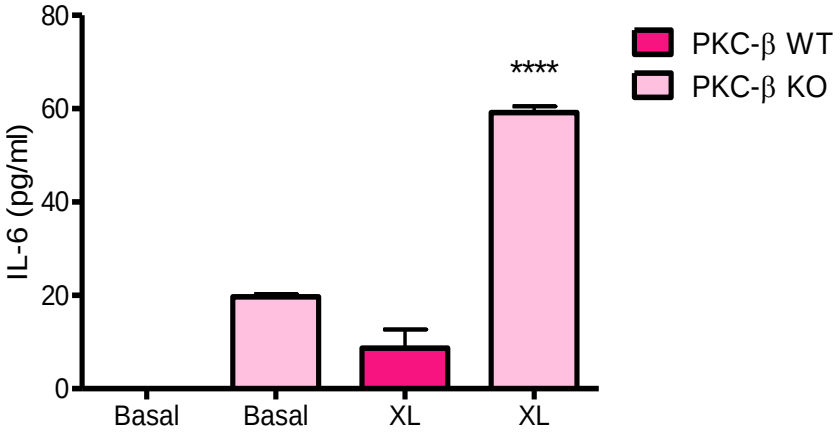

FcεRI
Q1
0.704%
Q2
87.6%
Q4
3.46%
Q3
8.25%
c-kit

Figure 3.5.2.1.2 The effect of PKC-β KO on IL-6 secretion in BMMC

A. PKC-β WT and KO BMMC were sensitised with 500 ng/ml anti-DNP IgE antibodies (IgE) and then stimulated with 500 ng/ml DNP-BSA to induce cross-linking (XL) for 24 hours at 37˚C. Release of IL-6 was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 2 independent experiments. One other experiment showed a non-significant increase while in a fourth experiment; no cytokine production could be detected. The statistical analysis performed is one way ANOVA where **** P< 0.0001 for WT XL versus KO XL.

B. PKC-β WT and KO BMMC were exposed to LPS for 24 hours at 37˚C. Release of IL-6 was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 4 independent experiments. The statistical analysis performed is one way ANOVA where **** P<0.0001 for LPS WT versus KO.

A.


80
60
40
20
0
IL-6 (pg/ml)
****
PKC-β WT
PKC-β KO
Basal
Basal
XL
XL

B.

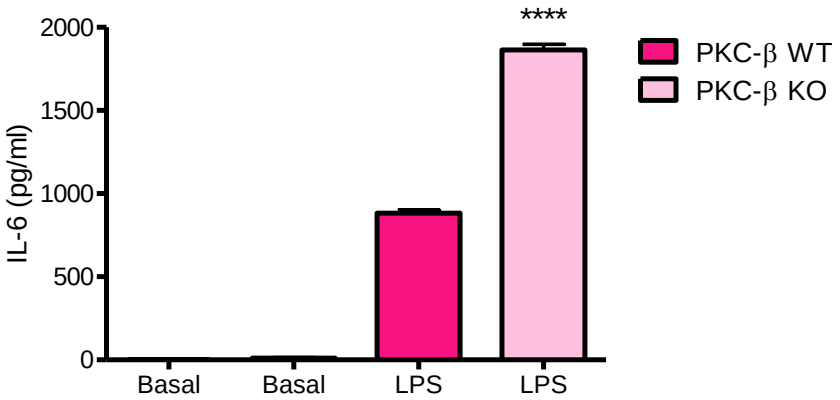

2000
1500
1000
500
0
IL-6 (pg/ml)
****
PKC-β WT
PKC-β KO
Basal
Basal
LPS
LPS

Figure 3.5.2.1.3 The effect of the absence of PKC-β on ES-62 activity on IL-6 secretion in BMMC

PKC-β WT and KO BMMC were exposed to LPS for 24 hours at 37˚C in the presence or absence of ES-62. Release of IL-6 was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 2 independent experiments. Two other experiments showed a non-significant decrease in WT BMMC with a significant and non-significant decrease in KO BMMC, respectively. The statistical analysis performed is one way ANOVA where **** $P<0.0001$ for LPS WT versus KO.

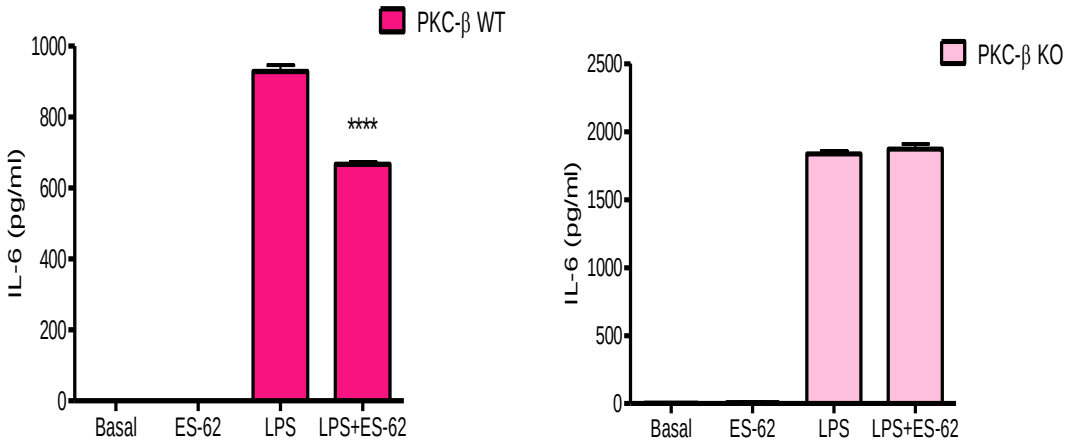
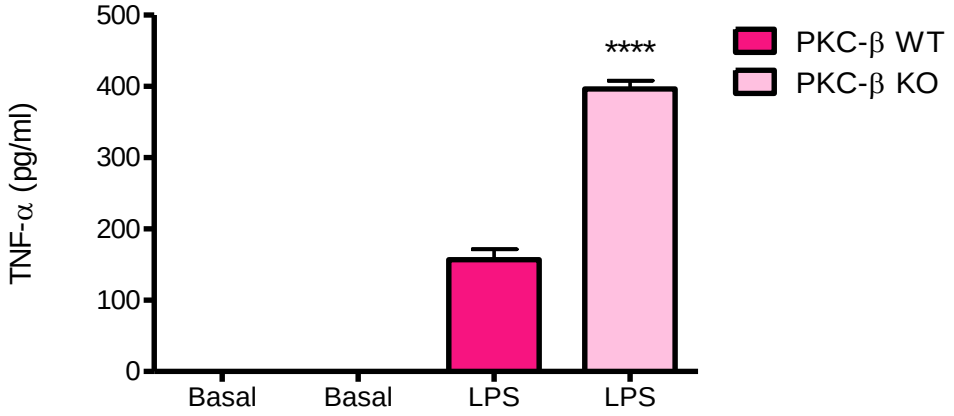

PKC-β WT
IL-6 (pg/ml)
1000
800
600
400
200
0
****
Basal
ES-62
LPS
LPS+ES-62
PKC-β KO
IL-6 (pg/ml)
2500
2000
1500
1000
500
0
Basal
ES-62
LPS
LPS+ES-62

Figure 3.5.2.1.4 The effect of PKC-β KO and ES-62 on TNF-α secretion in BMMC

A. PKC-β WT and KO BMMC were exposed to LPS for 24 hours at 37˚C. Release of TNF-α was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 4 independent experiments. The statistical analysis performed is one way ANOVA where **** P<0.0001 for LPS WT versus KO.

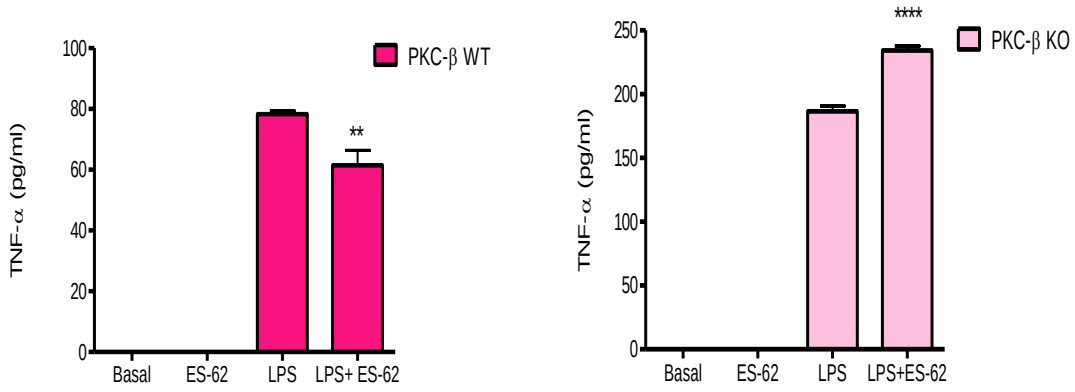
B. PKC-β WT and KO BMMC were exposed to LPS for 24 hours at 37˚C in the presence or absence of ES-62. Release of TNF-α was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of a single experiment. In three other experiments, ES-62 decreased cytokine production in WT BMMC in all experiments undertaken whereas in KO BMMC, a significant increase was observed in one experiment and a significant decrease observed in the two other experiments. The statistical analysis performed is one way ANOVA where ** P < 0.001 for WT LPS verus LPS+ES-62 and **** P<0.0001 for KO LPS versus LPS+ES-62.

A.


TNF-α (pg/ml)
500
400
300
200
100
0
****
PKC-β WT
PKC-β KO
Basal
Basal
LPS
LPS

.

B.


TNF-α (pg/ml)
100
80
60
40
20
0
PKC-β WT
**
Basal
ES-62
LPS
LPS+ ES-62
TNF-α (pg/ml)
250
200
150
100
50
0
****
PKC-β KO
Basal
ES-62
LPS
LPS+ES-62

3.5.2.2 Macrophages

Similar to PKC-α macrophage experiments, the analysis of PKC-β WT and KO BMM for IL-6, TNF-α, IL-12 and IL-10 cytokine release was undertaken following stimulation with LPS. If a differential response was emerging, analysis following pre-treatment with ES-62 for 18 hours was also undertaken.

3.5.2.2.1 IL-6 release

Following stimulation with LPS for 24 hours, the data obtained showed that there was no significant difference between WT and KO cell cytokine response in 3 (Figure 3.5.2.2.1) out of 6 individual experiments performed. A significant decrease was observed in two other experiments and a significant increase in one other. This lack of consistency between the two cell types advocated not investigating them following exposure to ES-62 for this particular cytokine.

3.5.2.2.2 TNF-α release

Upon LPS stimulation, it was discovered that TNF-α production was up-regulated in PKC-β KO macrophages compared to WT control cells in 5 out of 6 experiments, with this result being statistically significant in 2 experiments out of 6 (Figure 3.5.2.2.2 A). The sixth experiment showed a non-significant decrease in TNF-α production in KO cells. Overall in the first 5 experiments, KO cells showed a 24% ± 7% (Mean ± SEM) increase in cytokine production in comparison to WT cells. This

tendency to witness up-regulation of cytokine production in the KO led to the effect of pre-treatment with ES-62 being investigated in 3 of these experiments. The samples were incubated with ES-62 for 18 hours before stimulation, as before, followed by LPS for 24 hours. These data showed a non-significant decrease in cytokine production in WT macrophages in the 3 experiments undertaken and in addition, a decrease was observed in KO cells in 2 of these experiments, where this reached significance on one occasion (Figure 3.5.2.2.2 B) The third experiment showed a significant increase in cytokine secretion (data not shown). Although results are struggling to reach statistical significance and the KO data are less consistent than the WT data, overall these data may suggest that ES-62 can decrease TNF-α production even in the absence of PKC-β.

3.5.2.2.3 IL-12 release

The analysis of IL-12 cytokine secretion following LPS stimulation revealed that there was no significant difference between WT and KO cell cytokine response in 5 separate experiments (Figure 3.5.2.2.3). This similarity between the two cell types supported not investigating effects of ES-62 on secretion of this cytokine.

3.5.2.2.4 IL-10 release

It can be reported that in 5 experiments, no significant differences in IL-10 secretion between WT and KO cells was observed (Figure 3.5.2.2.4). This variability resulted in no investigation of ES-62's activity for this cytokine.

Figure 3.5.2.2.1 The effect of PKC-β KO on IL-6 secretion from BMM

PKC-β WT and KO macrophages were exposed to LPS for 24 hours at 37˚C. Release of IL-6 was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 3 experiments. A significant decrease was observed in two other experiments and a significant increase was observed in a sixth experiment. The statistical analysis performed is one way ANOVA.

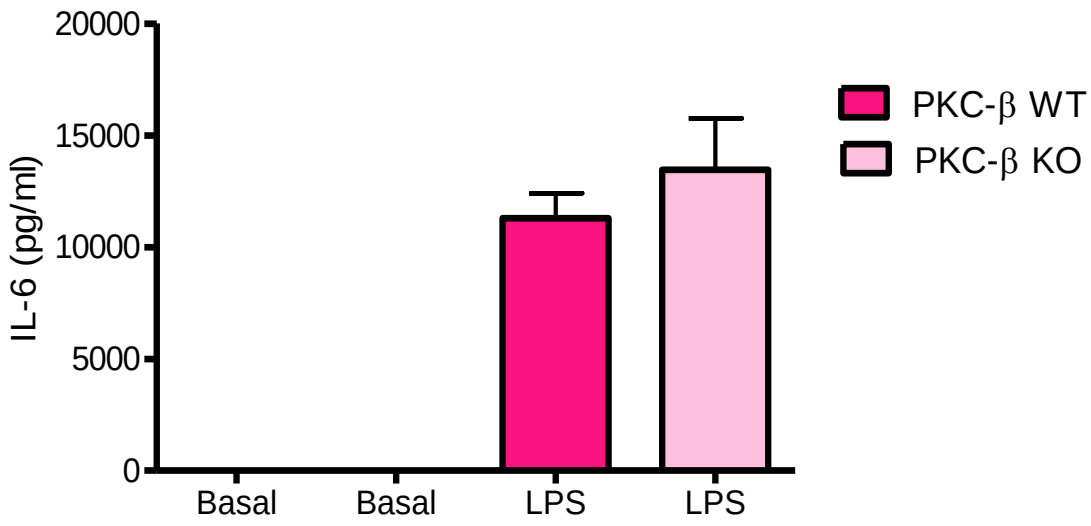
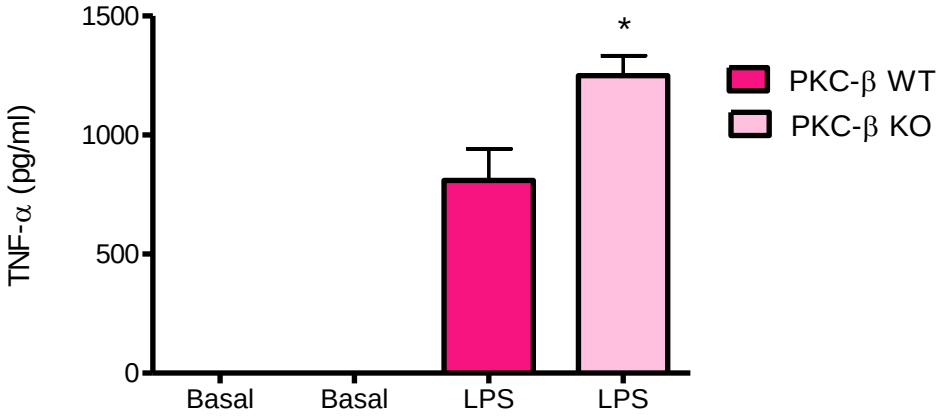

20000
15000
10000
5000
0
IL-6 (pg/ml)
Basal
Basal
LPS
LPS
PKC-β WT
PKC-β KO

Figure 3.5.2.2.2 The effect of PKC-β KO and ES-62 on TNF-α secretion in BMM

A. PKC-β WT and KO BMM were sensitized with LPS for 24 hours at 37˚C. Release of TNF-α was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 2 out of 6 independent experiments. 3 other experiments showed a non-significant increase with a sixth experiment showing a non-significant decrease. The statistical analysis performed is one way ANOVA where * $P<0.05$ for LPS WT versus KO.

B. PKC-β WT and KO BMM were sensitized with LPS for 24 hours at 37˚C in the presence or absence of ES-62. Release of TNF-α was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of a single experiment. A non-significant decrease in cytokine production was observed in WT and KO BMM in one other experiment whereas a thiurd experiment showed a non-significant decrease in the WT and a significant increase in the KO. The statistical analysis performed is one way ANOVA where * $P<0.05$ for LPS WT versus KO.

A.


1500
1000
500
0
TNF-α (pg/ml)
*
PKC-β WT
PKC-β KO
Basal
Basal
LPS
LPS

B.

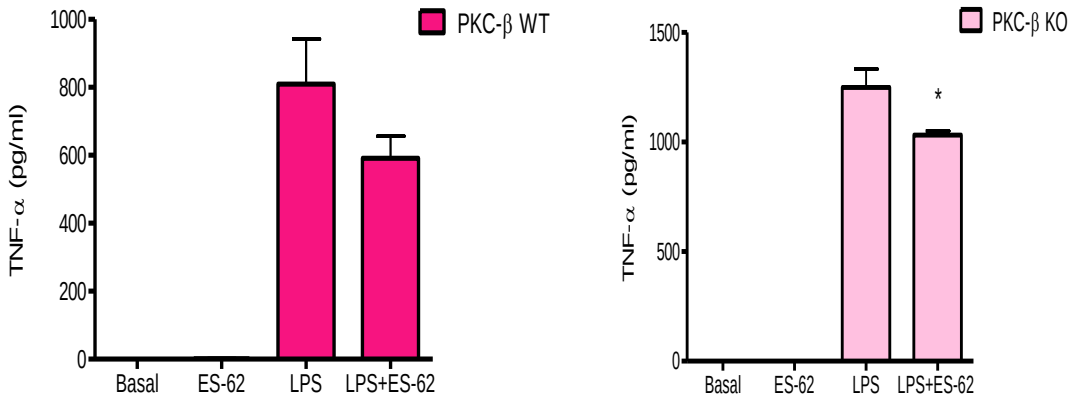

1000
800
600
400
200
0
TNF-α (pg/ml)
PKC-β WT
Basal
ES-62
LPS
LPS+ES-62
1500
1000
500
0
TNF-α (pg/ml)
PKC-β KO
*
Basal
ES-62
LPS
LPS+ES-62

Figure 3.5.2.2.3 The effect of PKC-β KO on IL-12 production in BMM

PKC-β WT and KO BMM were sensitized with LPS for 24 hours at 37˚C. Release of IL-12 was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 5 independent experiments. The statistical analysis performed is one way ANOVA.

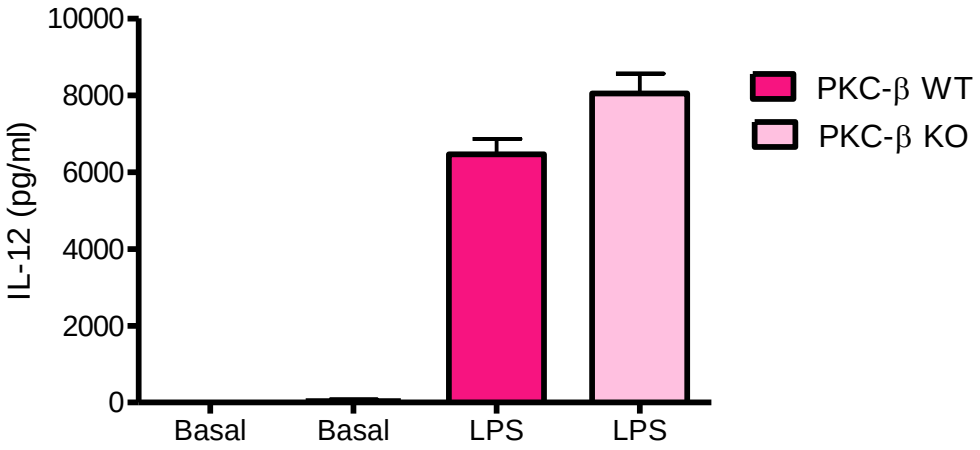
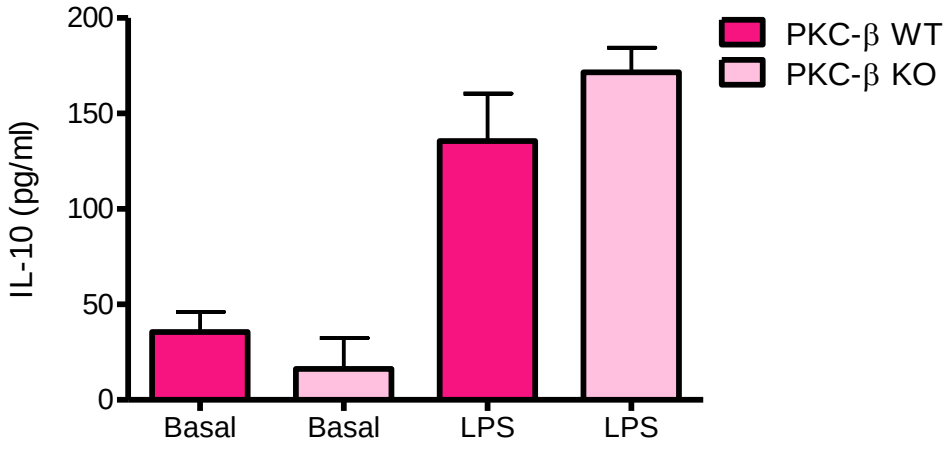

10000
8000
6000
4000
2000
0
IL-12 (pg/ml)
Basal
Basal
LPS
LPS
PKC-β WT
PKC-β KO

Figure 3.5.2.2.4 The effect of PKC-β KO on IL-10 production in BMM

PKC-β WT and KO BMM were sensitized with LPS for 24 hours at 37˚C. Release of IL-10 was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 5 independent experiments. The statistical analysis performed is one way ANOVA.


200
150
100
50
0
IL-10 (pg/ml)
Basal
Basal
LPS
LPS
PKC-β WT
PKC-β KO

3.5.3 PKC-theta

The expression pattern of PKC-θ has been shown to be more tissue-restricted than other isoforms being predominantly abundant in skeletal muscle, T lymphocytes, endothelial cells and megakaryoblasts (Baier, et al. 1993; Osada, et al. 1992; Chang, et al. 1993). On the other hand, more recently this calcium-independent isoform has also been shown to be expressed in additional cell types, in particular, the mast cell line RBL-2H3 and BMMC (Liu, et al. 2001; Li, et al. 2005). Additionally, the novel calcium-independent isoforms δ and ε were of the first members implicated in mast cell antigen-induced exocytosis with PKC-δ considered one of the more potent signal transducers for exocytosis (Ozawa, et al. 1993a). However, as well as revealing the expression of PKC-θ in mast cells, Liu and colleagues were able to show that PKC-θ is involved in mast cell receptor-induced exocytosis as implicated by translocation of this isoform from the cytosol to the plasma membrane upon receptor aggregation (Liu, et al. 2001). Furthermore, PKC-θ was also associated with ERK activation and IL-3 gene transcription in response to FcεRI cross-linking (Liu, et al. 2001). Excluding this study, very little progress has been made in dissecting the role of PKC-θ in mast cells. However, more advances in understanding have been made with this isoform in T cell receptor (TCR) signalling. For example, PKC-θ has been shown to be central for TCR signalling in mature T lymphocytes (Sun, et al. 2000). In vitro studies using PKC-θ deficient mice highlighted a deficiency in the proliferation of peripheral T cells in response to TCR stimulation. This knockout effect was associated with decreased IL-2 secretion and IL-2 receptor expression in addition to defects in activator-protein 1 (AP-1) and nuclear factor–κB transcription factor (NF-κB) activation following TCR ligation (Sun, et al. 2000). Although the

signalling pathway in T cells is not fully delineated, the similarities that exist between TCR signalling and FcεRI signalling in mast cells (Valge, et al,.1988; White, et al. 1985; White & Metzger, 1988) can perhaps give indication to the potential significance of PKC-θ in this context in mast cells. Finally, in spite of PKC-θ being an isoform that was not subject to degradation by ES-62 in human mast cells (Melendez, et al. 2007), it cannot be assumed this is the case for all species and mast cell phenotypes. It is clear from previous studies in RBL cells that PKC-θ is pivotal to FcεRI receptor signalling and certain mast cell functional responses (Liu, et al. 2001). Thus, it was decided that PKC-θ would be a good novel isoform candidate to study with respect to the effect of its absence on BMMC functional responses as determined using PKC-θ deficient mice.

3.5.3.1 Mast cells

As before, any phenotypic differences between the two cell types were analysed by flow cytometry then PKC-θ WT and KO BMMC were analysed for the secretion of IL-6 and TNF-α cytokine following either cross-linking (XL) or activation with LPS after 24 hours. If applicable, BMMC were also analysed following pre-treatment with ES-62 for 24 hours.

3.5.3.1.1 Cell surface expression of mast cell markers

PKC-θ WT and KO cells were analysed for their expression of the cell surface markers, FcεRI and c-kit using flow cytometry. This would enable the confirmation

of their identity as mature mast cells as well as identifying any phenotypic differences relating to these markers that may exist between the two cell types. The results show that mast cells are clearly identifiable on the basis of their forward and side scatter properties and their strong c-kit and FcεRI expression (gating relative to isotype control). On average, BMMC WT cultures were 94.8% and KO BMMC 96.8% double positive for c-kit and FcεRI. These results indicate that both WT and KO cells were truly mast cells of mature phenotype and that both cell types shared almost identical characteristics suggesting that PKC-θ does not affect the development of mast cells. The BMMC culture examples shown for WT and KO are 96.6% and 98% respectively (Figure 3.5.3.1.1). It is noteworthy that a smaller more immature population (c-kit+ FcεRI-) could be witnessed for both WT and KO cells but the percentile variance, 4% and 2.4% respectively, was minimal between these two cell types for this immature population suggesting that both cell types matured at a roughly similar rate.

3.5.3.1.2 IL-6

The analysis of PKC-θ WT and KO BMMC for IL-6 cytokine release was undertaken following either FcεRI cross-linking or exposure to LPS for 24 hours. With respect to the former, the results obtained from 3 individual experiments indicated that PKC-θ potentially plays a major role in mast cell functional responses as IL-6 cytokine production was significantly reduced following cross-linking (XL) in all experiments (Figure 3.5.3.1.2 A). For one experiment, a significant difference between basal WT and KO cells was observed where basal levels were significantly

reduced in the KO (data not shown) but this did not impact on the overall result. Nevertheless, these data were excluded when calculating the average cytokine secretion, which was reduced by 83% in KO cells. The PKC-θ KO data suggest that as with PKC-α, this isoform may be a key component but it is not necessarily essential for IL-6 secretion as KO BMMC were still able to secrete the cytokine (albeit at a greatly reduced amount) following antigen stimulation (Figure 3.5.3.1.2 A). However, interestingly, the loss of this isoform appears more significant than the loss of others such as PKC-α. PKC-θ has already been presented as a functionally important component of mast cell signalling responses such as cytokine production due to the effects witnessed for PKC-θ constructs in RBL cells where cells expressing constitutively active PKC-θ displayed enhanced IL-3 mRNA production by around 30% (Liu et al, 2001). This finding from Liu et al in correlation with the IL-6 cytokine data from the PKC-θ KO BMMC suggests a potential role for PKC-θ in facilitating cytokine production in response to antigen. Furthermore, a similar trend was witnessed with LPS-stimulated KO mast cells where IL-6 production was significantly reduced in KO compared to WT in 3 individual experiments performed (Figure 3.5.3.1.2 B). On average, IL-6 secretion was reduced by 70% ± 9% (Mean ± SEM) in KO BMMC in comparison to WT BMMC.

In addition, the effect of ES-62 on IL-6 production in both WT and KO cells was explored. As expected, ES-62 was able to significantly reduce FcεRI-induced IL-6 cytokine secretion in 3 experiments in WT cells (Figure 3.5.3.1.3 A). ES-62 showed a largely similar effect in KO cells, reducing IL-6 production in two experiments, where one experiment showed a statistically significant result (Figure 3.5.3.1.3 A). The third experiment showed no XL response in the KO cells, therefore ES-62

activity could not be analysed for this sample. Additionally, in the one experiment undertaken, ES-62 was able to significantly reduce IL-6 secretion following LPS stimulation by approximately 29% in WT cells with IL-6 production up-regulated in KO cells, although the latter did not reach significance (Figure 3.5.3.1.3 B). Thus, ES-62 appears to be able to act as an immunomodulator in both the presence and absence of this isoform although the fact that statistical significance was not always reached in the KO following antigen stimulation in addition to the nematode product being unable to rescue LPS-induced IL-6 productrion in the KO BMMC might suggest some loss of effect of the helminth product. Therefore, ES-62 may utilize PKC-θ for its activity in mast cells but it is possibly not essential.

3.5.3.1.3 TNF-α

Following FcεRI cross-linking, it was observed that TNF-α cytokine production was greatly reduced in PKC-θ KO cells in comparison to WT control in 3 individual experiments (Figure 3.5.3.1.4 A). On average, cytokine secretion was reduced by 89% ±7% (Mean ± SEM) in KO cells in comparison to WT. These data suggest that as with IL-6 cytokine production, PKC-θ may play an important role in TNF-α production but this isoform may not be absolutely required as production of this cytokine was not completely inhibited in KO cells (Figure 3.5.3.1.4 A). However, in parallel to IL-6, it appears the absence of this isoform has more detrimental effects on pro-inflammatory cytokine release from BMMC in comparison to PKC-α. Similarly, this trend was identified in PKC-θ KO cells stimulated with LPS where the cells showed significantly less TNF-α production in comparison to WT control

cells in 2 out of 3 separate experiments (Figure 3.5.3.1.4 B). Overall, the 2 experiments showed an average reduction in TNF-α production by 70% in KO BMMC when compared to WT BMMC. However the opposite trend was observed in a third experiment and interestingly, in RBL cells unlike IL-3 production, Liu and co-workers found there was no PKC-θ construct-induced enhancement of TNF-α gene transcription (Liu, et al. 2001). In spite of the inconsistency of some of the data, it was next investigated whether absence of this isoform could potentially affect ES-62 activity. The nematode product significantly reduced TNF-α secretion in 2 experiments employing XL BMMC (Figure 3.5.3.1.5 A). Additionally, ES-62 significantly reduced cytokine production in the KO cells in 2 experiments (Figure 3.5.3.1.5 A). Furthermore, with LPS-stimulated cells, ES-62 inhibited cytokine production on two occasions in WT cells and KO cells, where this reached significance on one occasion for both cell types (Figure 3.5.3.1.5 B). Overall, the data would suggest that absence of this PKC isoform is not essential for ES-62 activity.

Fig 3.5.3.1.1 Flow cytometric analysis of WT and PKC-θ KO BMMC for expression of mast cell surface markers C-kit and FcεRI

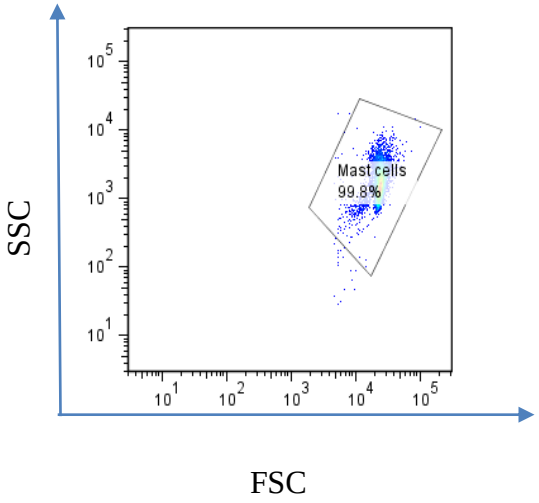
A. Dot plot analysis of FSC versus SSC allowing gating of live cells on the basis of their size and granularity, respectively.

B. Dot plot showing setting of gates for c-kit + and/or FcεRI + cells on the basis of staining of live mast cells (as gated in panel A) with relevant isotype controls.

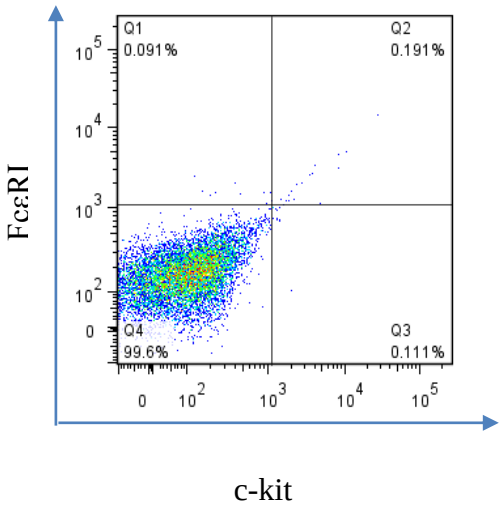
C. WT mast cells gated as described in panel A) were examined for their expression of c-kit and FcεRI relevant to appropriate isotype controls (panel B). Expression of C-kit (x-axis) was determined by staining with an APC-conjugated anti-mouse CD117 (c-kit) antibody gated relative to its isotype control (APC-conjugated rat anti-mouse IgG2b; panel B). Expression of FcεRI (y-axis) was determined by staining with FITC-conjugated anti-mouse FITC FcεRI antibody gated relative to its isotype control (FITC-conjugated Armenian Hamster IgG; panel B).

D. KO mast cells gated as described in panel A) were examined for their expression of c-kit and FcεRI relevant to appropriate isotype controls (panel B). Expression of C-kit (x-axis) was determined by staining with an APC-conjugated anti-mouse CD117 (c-kit) antibody gated relative to its isotype control (APC-conjugated rat anti-mouse IgG2b; panel B. Expression of FcεRI (y-axis) was determined by staining with FITC-conjugated anti-mouse FITC FcεRI antibody gated relative to its isotype control (FITC-conjugated Armenian Hamster IgG; panel B).

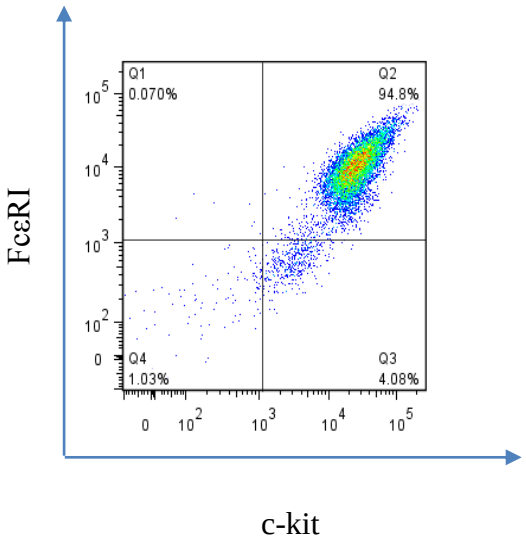
A.


SSC
FSC
Mast cells
99.8%

B.


FcεRI
c-kit
Q1
0.091%
Q2
0.191%
Q4
99.6%
Q3
0.111%

C.


FcεRI
c-kit
Q1
0.070%
Q2
94.8%
Q4
1.03%
Q3
4.08%

D.

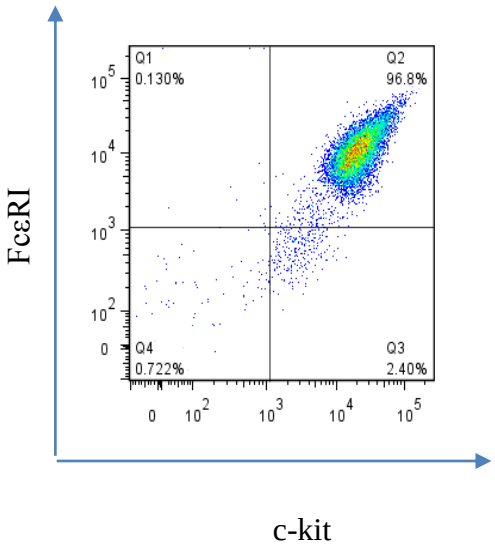
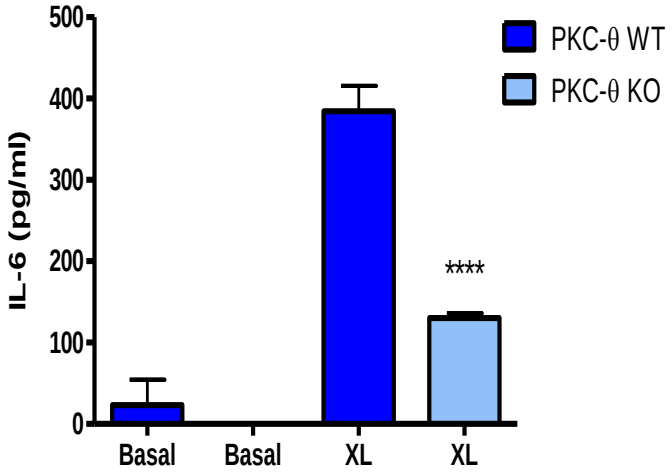

FcεRI
c-kit
Q1
0.130%
Q2
96.8%
Q4
0.722%
Q3
2.40%

Figure 3.5.3.1.2 The effect of PKC-θ KO on IL-6 cytokine production in BMMC

A. PKC-θ WT and KO BMMC were sensitized with 500 ng/ml anti-DNP IgE for 24 hours then stimulated with 500 ng/ml DNP-BSA to induce cross-linking (XL) for 24 hours at 37˚C. IL-6 release was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 3 independent experiments. Statistical analysis was by one way ANOVA where **** P<0.0001 for XL WT versus KO.

B. PKC-θ WT and KO BMMC were sensitized with LPS (1 μg/ml) for 24 hours at 37˚C. IL-6 release was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 3 independent experiments. Statistical analysis was by one way ANOVA where **** P<0.0001 for LPS WT versus KO.

A.


500
400
300
200
100
0
IL-6 (pg/ml)
PKC-θ WT
PKC-θ KO
****
Basal
Basal
XL
XL

B.

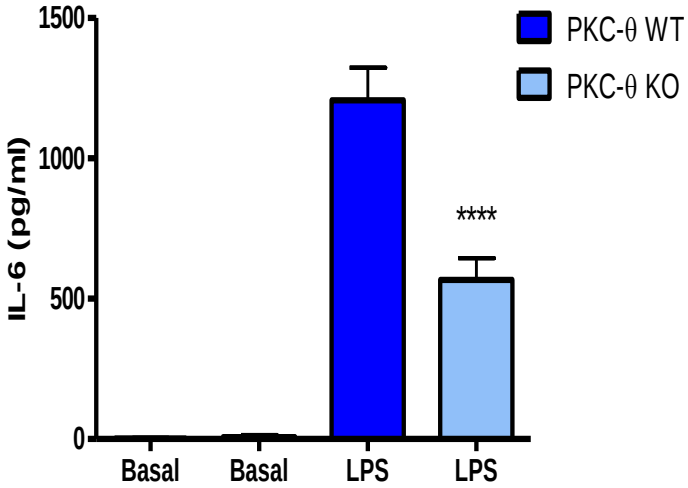
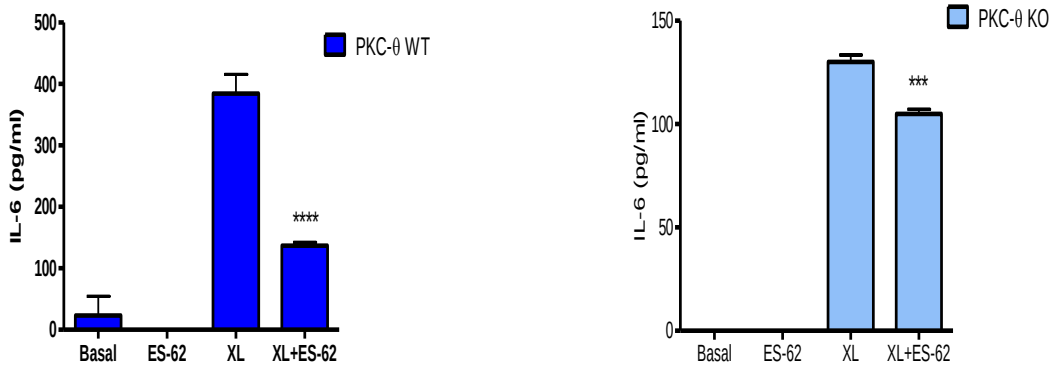

1500
1000
500
0
IL-6 (pg/ml)
PKC-θ WT
PKC-θ KO
****
Basal
Basal
LPS
LPS

Figure 3.5.3.1.3 The effect of the absence of PKC-θ on ES-62 activity on IL-6 secretion in BMMC

A. Cross-linking (XL) of PKC-θ WT and KO BMMC in the presence or absence of ES-62 (2 μg/ml). IL-6 secretion was measured by ELISA. Results are shown as means ± S.D. Data presented are from a single experiment incorporating triplicate values. A second experiment showed a significant decrease by ES-62 in the WT and a non-significant decrease witnessed in the KO. A third experiment showed a significant decrease by ES-62 in the WT but no XL was observed in the KO. Statistical analysis was by one way ANOVA where **** $P<0.0001$ for WT LPS versus WT LPS +ES-62 and *** $P<0.001$ for KO LPS versus KO LPS+ES-62.

B. PKC-θ WT and KO BMMC were sensitized with LPS for 24 hours at 37˚C in the presence or absence of ES-62. IL-6 release was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of a single experiment. Statistical analysis was by one way ANOVA where **** $P<0.0001$ for LPS WT versus KO.

A.


PKC-θ WT
IL-6 (pg/ml)
500
400
300
200
100
0
****
Basal
ES-62
XL
XL+ES-62
PKC-θ KO
150
100
50
0
***
Basal
ES-62
XL
XL+ES-62

B.

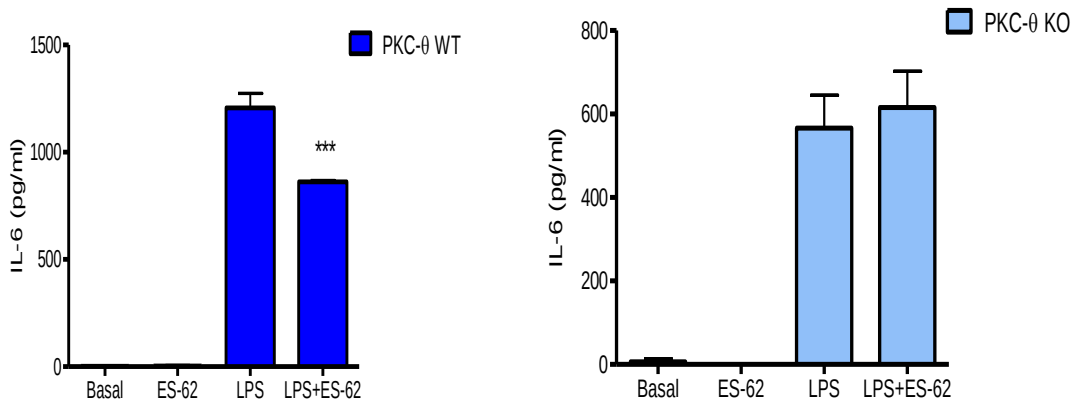
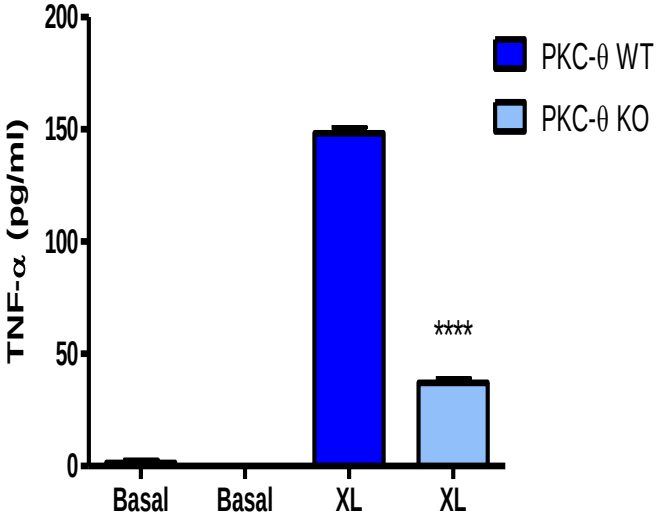

PKC-θ WT
IL-6 (pg/ml)
1500
1000
500
0
***
Basal
ES-62
LPS
LPS+ES-62
PKC-θ KO
800
600
400
200
0
Basal
ES-62
LPS
LPS+ES-62

Figure 3.5.3.1.4 The effect of PKC-θ KO on TNF-α cytokine production in BMMC

A. PKC-θ WT and KO BMMC were sensitized with 500 ng/ml anti-DNP IgE for 24 hours then stimulated with 500ng/ml DNP-BSA to induce cross-linking (XL) for 24 hours at 37˚C. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 3 independent experiments. TNF-α release was measured by ELISA. Statistical analysis was by one way ANOVA where **** P<0.0001 for XL WT versus KO.

B. PKC-θ WT and KO BMMC were sensitized with LPS (1 μg/ml) for 24 hours at 37˚C. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 2 independent experiments. A significant increase was observed in the third experiment. TNF-α release was measured by ELISA. Statistical analysis was by one way ANOVA where **** P<0.0001 for LPS WT versus KO.

A


200
150
100
50
0
TNF-α (pg/ml)
PKC-θ WT
PKC-θ KO
****
Basal
Basal
XL
XL

B.

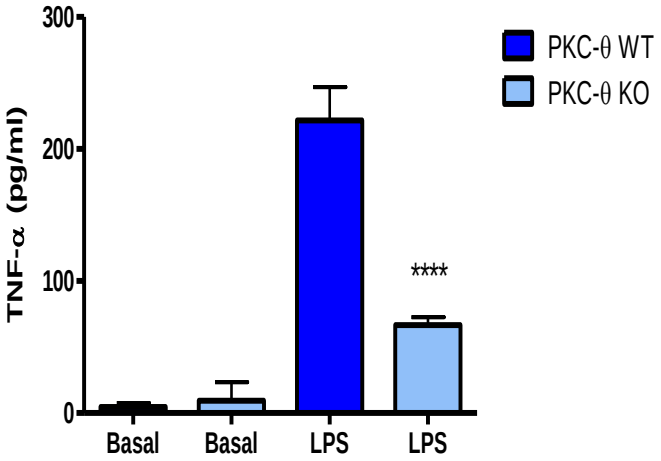
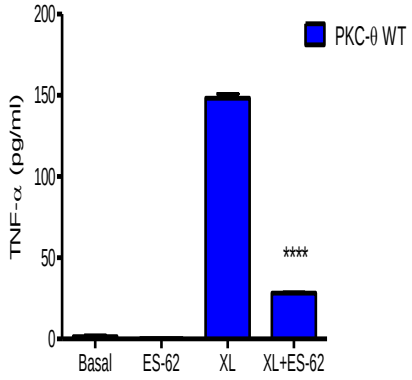

300
200
100
0
TNF-α (pg/ml)
PKC-θ WT
PKC-θ KO
****
Basal
Basal
LPS
LPS

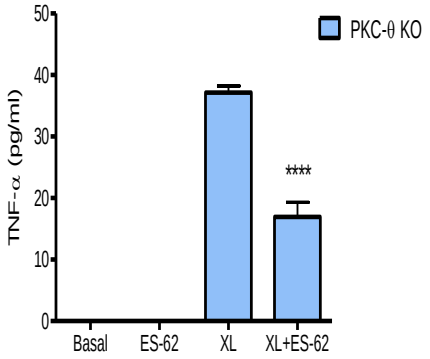
Figure 3.5.3.1.5 The effect of PKC-θ KO on ES-62 activity on TNF-α cytokine production in BMMC

A. PKC-θ WT and KO BMMC were sensitized with IgE and stimulated with 500 ng/ml DNP-BSA to induce cross-linking (XL) for 24 hours at 37˚C in the presence or absence of ES-62. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of two experiments. TNF-α release was measured by ELISA. Statistical analysis was by one way ANOVA where **** P<0.0001 for WT XL versus XL+ES-62 and KO XL versus XL+ES-62.

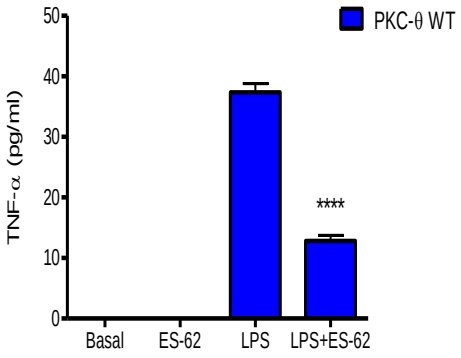
B. PKC-θ WT and KO BMMC were sensitized with LPS for 24 hours at 37˚C in the presence or absence of ES-62. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of a single experiment. In another experiment, a decrease was observed in both the WT and KO where this reached significance in KO BMMC. TNF-α release was measured by ELISA. Statistical analysis was by one way ANOVA where ** P<0.001 for WT LPS versus LPS+ES-62.

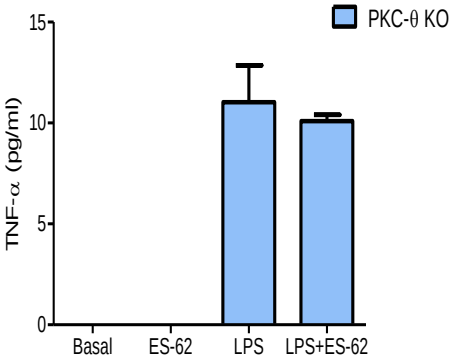
A.


PKC-θ WT
TNF-α (pg/ml)
200
150
100
50
0
****
Basal
ES-62
XL
XL+ES-62


PKC-θ KO
TNF-α (pg/ml)
50
40
30
20
10
0
****
Basal
ES-62
XL
XL+ES-62

B.


PKC-θ WT
TNF-α (pg/ml)
50
40
30
20
10
0
****
Basal
ES-62
LPS
LPS+ES-62


PKC-θ KO
TNF-α (pg/ml)
15
10
5
0
Basal
ES-62
LPS
LPS+ES-62

3.5.3.2 Macrophages

As for all other isoforms, analysis of PKC-θ WT and KO BMM for IL-6, TNF-α, IL-12 and IL-10 cytokine secretion was investigated following LPS stimulation for 24 hours. Where there were differential responses emerging, macrophages were also analysed in the presence or absence of pre-exposure to ES-62 for 18 hours.

3.5.3.2.1 IL-6

Upon stimulation with LPS (100 ng/ml) for 24 hours, it was witnessed that although there was no entirely consistent effect of PKC-θ knockout in comparison to the WT control, there was no differential IL-6 cytokine production in 3 out of 5 individual experiments (Figure 3.5.3.2.1). This result dictated that experiments employing ES-62 were subsequently not undertaken.

3.5.3.2.2 TNF-α

Analysis of TNF-α secretion following LPS stimulation for 24 hours showed that there was a trend of a reduction in production of this cytokine by KO cells in 4 out of 5 experiments performed, however, only 2 of these experiments exhibited a statistically significant decrease in TNF-α (an example of the latter is shown in Figure 3.5.3.2.2). From the 4 experiments, KO cells showed a decrease of 30% ± 12% (Mean ± SEM). A fifth experiment showed a non-significant increase in

production in KO cells. These inconsistencies were the reason investigations using ES-62 were not undertaken.

3.5.3.2.3 IL-12

The data obtained showed that there were no significant differences in IL-12 cytokine secretion between PKC-θ KO and WT macrophages in 3 (Figure 3.5.3.2.3) out of 5 individual experiments. A significant increase in one and a significant decrease in the other were witnessed for the two other experiments. Again, the lack of differential response between WT and KO cells in the majority of experiments dictated that no testing with ES-62 was undertaken.

3.5.2.2.4 IL-10

Following 24-hour LPS stimulation, IL-10 data obtained from PKC-θ WT and KO macrophages were rather inconsistent. There was both a significant increase (2 out of 5 experiments) and decrease (2 out of 5 experiments) witnessed in PKC-θ KO cells in comparison to WT IL-10 secretion (data not shown.) These data supported no subsequent investigations employing ES-62.

Figure 3.5.3.2.1 The effect of PKC- θ IL-6 cytokine production in BMM

PKC-θ WT and KO BMM were exposed to 100 ng/mL LPS for 24 hours at 37˚C. Release of IL-6 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 3 independent experiments. In two further experiments, one showed a significant increase and one a significant decrease in IL-6 production in KO cells. The statistical analysis performed one way ANOVA.

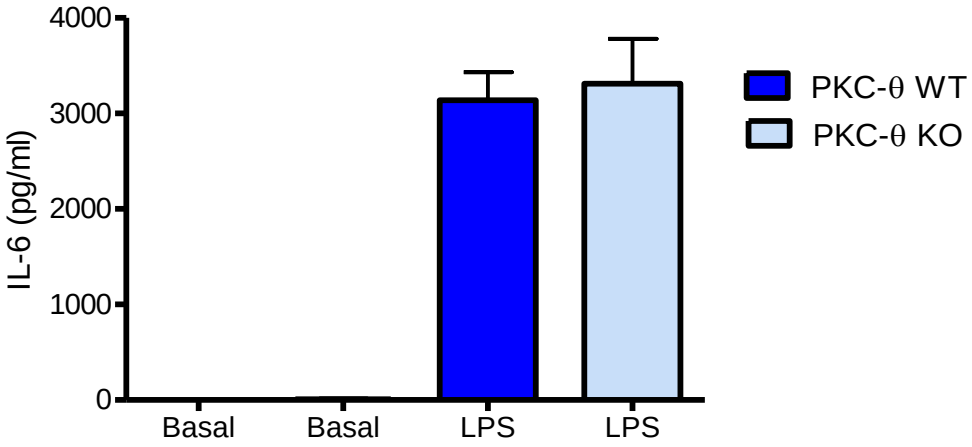

4000
3000
2000
1000
0
IL-6 (pg/ml)
Basal
Basal
LPS
LPS
PKC-θ WT
PKC-θ KO

Figure 3.5.3.2.2 The effect of PKC- θ TNF-α cytokine production in BMM

PKC-θ WT and KO BMM were sensitized with 100 ng/ml LPS for 24 hours at 37˚C. Release of TNF-α was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 2 independent experiments. Two other experiments showed a non-significant decrease and a fifth experiment showed a non-significant increase. The statistical analysis performed is one way ANOVA **** $P<0.0001$ for LPS WT versus KO.

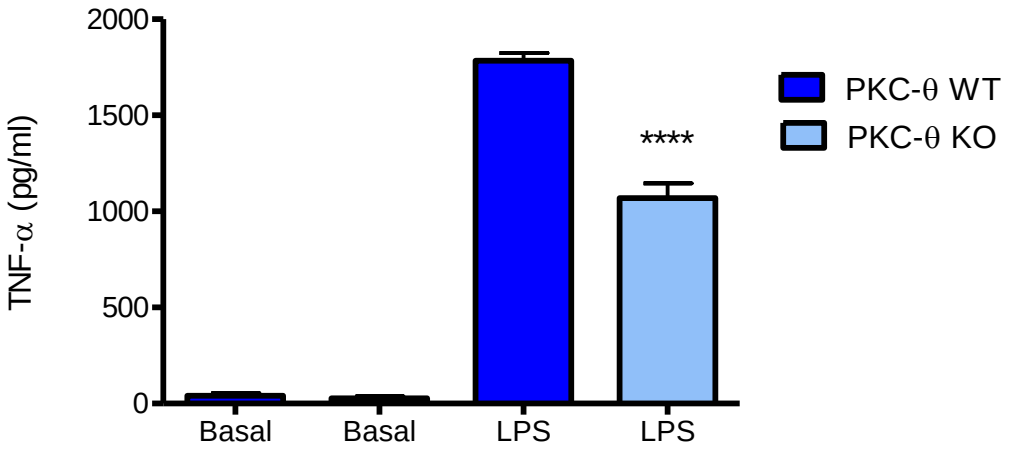
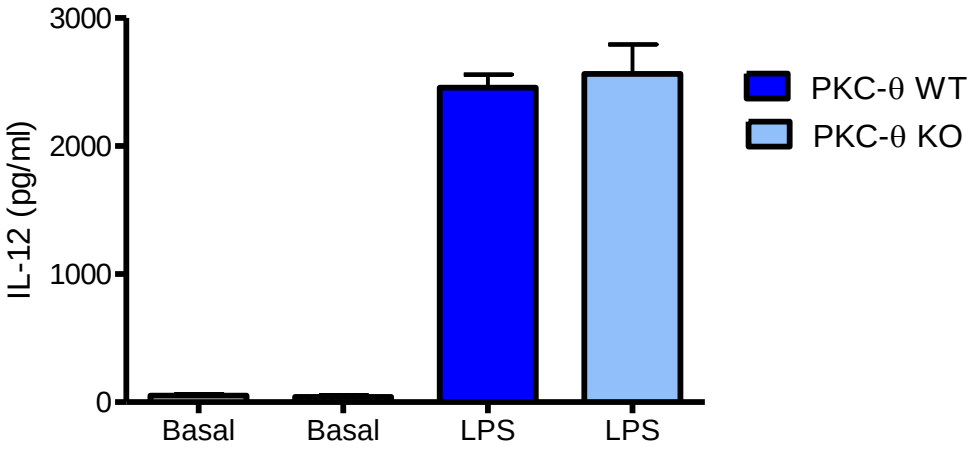

2000
1500
1000
500
0
TNF-α (pg/ml)
****
Basal
Basal
LPS
LPS
PKC-θ WT
PKC-θ KO

Figure 3.5.3.2.2 The effect of PKC-θ IL-12 cytokine production in BMM

PKC-θ WT and KO BMM were sensitized with 100 ng/ml LPS for 24 hours at 37˚C. Release of IL-12 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 3 independent experiments. A fourth and fifth experiment showed a statistically significant increase and statistically significant decrease, respectively in IL-12 production in the KO cells. The statistical analysis performed is one way ANOVA.


3000
2000
1000
0
IL-12 (pg/ml)
Basal
Basal
LPS
LPS
PKC-θ WT
PKC-θ KO

3.5.3.3 Dendritic cells

PKC-θ WT and KO DCs were analysed as described previously over 4 individual experiments. If significant differences were seen to be emerging between WT and KO, DC's were also analysed following pre-treatment with ES-62 for 18 hours.

3.5.3.3.1 IL-6

Upon stimulation with LPS (100 ng/ml) for 24 hours, the data highlighted that there was a reduction in IL-6 secretion in 3 out of 4 experiments by an average of 30% ± 6% (Mean ± SEM) in KO DCs, where this reached significance on two occasions (Figure 3.5.3.3.1 A). The fourth experiment showed a non-significant increase by 3% in KO cells. ES-62 was also tested in this system and it was shown that the nematode product decreased IL-6 secretion in 3 experiments in WT DCs, where this reached significance on one occasion (Figure 3.5.3.3.1 B). ES-62's activity was largely reproducible in the KO where it decreased production of this cytokine on two occasions with a statistically significant decrease witnessed on one occasion (Figure 3.5.3.3.1 B). However, the third experiment showed a non-significant increase in the KO by ES-62 (results not shown). Nevertheless, these data again are broadly suggestive of ES-62's inhibitory activity being retained in the KO.

3.5.3.3.2 TNF-α

TNF-α cytokine release was also investigated following 24-hour incubation with LPS (100 ng/ml) over 4 separate experiments. These data showed a significant reduction in TNF-α secretion in PKC-θ KO DCs in comparison to WT controls in 2 out of 4 experiments (Figure 3.5.3.3.2 A). When these cells were pre-treated with ES-62, it was discovered that it was capable of reducing TNF-α in wild-type DCs in three experiments where this reached significance on two occasions (Figure 3.5.3.3.2 B). Similarly, there was a consistent decrease in KO cells in 3 experiments, where this reached statistical significance on one occasion (Figure 3.5.3.3.2 B).

3.5.3.3.3 IL-12

Following LPS stimulation for 24 hours, analysis of IL-12 cytokine secretion revealed that there was a reduction in IL-12 production by KO DCs in 3 out of 4 experiments performed, although this was only significant in one experiment (Figure 3.5.3.3.3 A). Overall, there was a decrease by 22% ± 8% (Mean ± SEM) in KO cells in comparison to WT controls. These data, although generally statistically non-significant, showed evidence of an inhibitory trend and therefore encouraged additional investigations with ES-62 as the work progressed. When employing the nematode product, it was discovered that this molecule reduced cytokine secretion in PKC-θ WT DCs in 2 experiments, where this was significant on one occasion Figure 3.5.3.3.3 B). However, in KO DCs, ES-62 could only significantly reduce IL-12 production in 1 experiment with a non-significant increase observed in the other (Figure 3.5.3.3.3 B). These data may again suggest that ES-62 is able to reduce IL-12

secretion in both the WT and KO but with the data being rather inconsistent, this is not certain.

3.5.3.3.2 IL-10

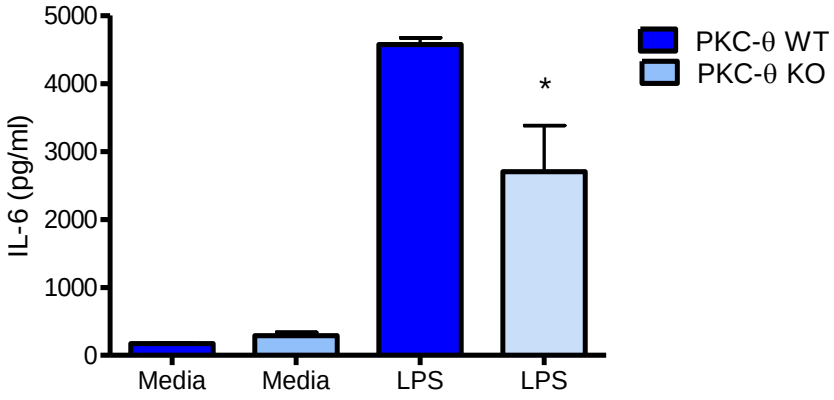
Analysis of IL-10 production following LPS stimulation showed a significant increase in cytokine production in KO cells in 3 out of 4 experiments (Figure 3.5.3.3.4 A). Thus, as these data were emerging, it was decided to undertake additional investigations with ES-62. When employing the helminth product, it was discovered that there was a non-significant increase in IL-10 production in WT cells in 3 experiments (Figure 3.5.3.3.4 B). Somewhat conversely, in KO cells ES-62 showed a non-significant decrease in cytokine secretion in 3 experiments performed, (Figure 3.5.3.3.4 B). Although statistical significance was not reached, the consistency of these data may suggest that ES-62 acts in a different manner in the presence of PKC-θ whereby it promotes something of an anti-inflammatory environment through elevating IL-10 secretion from DCs. This is consistent with previous data employing ES-62 released from osmotic pumps in mice where DCs stimulated with LPS ex vivo showed elevated levels of IL-10 production following exposure to the nematode product (Goodridge, et al. 2004). However, data obtained from the KO experiments may show that this feature is lost in the absence of this isoform, again suggesting that ES-62 may utilize PKC-θ for its activity in DCs.

Figure 3.5.3.3.1 The effect of PKC-θ KO on IL-6 cytokine secretion from DCs

A. PKC-θ WT and KO DCs were sensitized with 10 ng/ml LPS for 24 hours at 37˚C. Release of IL-6 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 2 experiments. A non-significant decrease was witnessed in a third experiment and a non-significant increase was observed in a fourth experiment. The statistical analysis performed is one way ANOVA where * $P<0.05$ for LPS WT versus KO

B. PKC-θ WT and KO DCs were sensitized with 10 ng/ml LPS for 24 hours at 37˚C in the presence or absence of ES-62. Release of IL-6 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values. A second experiment showed a significant decrease in the WT by ES-62 with a non-significant decrease in IL-6 production being witnessed in the KO DCs. A third experiment showed again a significant decrease in IL-6 in WT DCs but this time a non-significant increase was induced by ES-62 in the KO. The statistical analysis performed is one way ANOVA where **** $P<0.0001$ for WT LPS versus WT LPS+ES-62 and *** $P< 0.001$ for KO LPS versus KO LPS+ES-62.

A.


5000
4000
3000
2000
1000
0
IL-6 (pg/ml)
Media
Media
LPS
LPS
*
PKC-θ WT
PKC-θ KO

B.

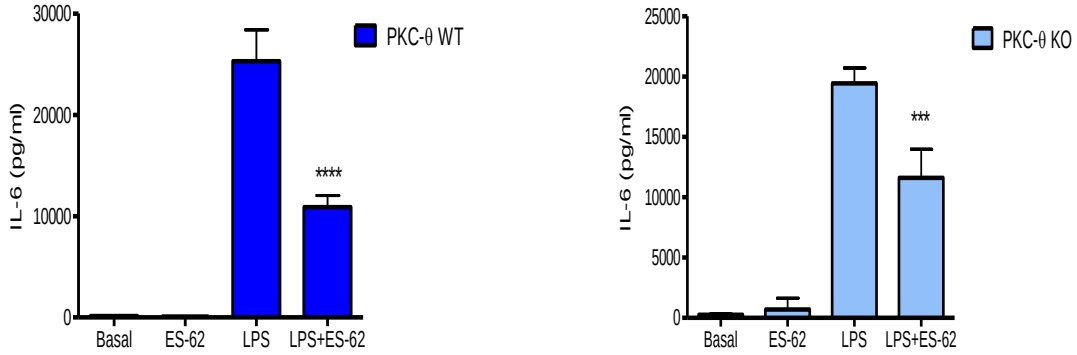
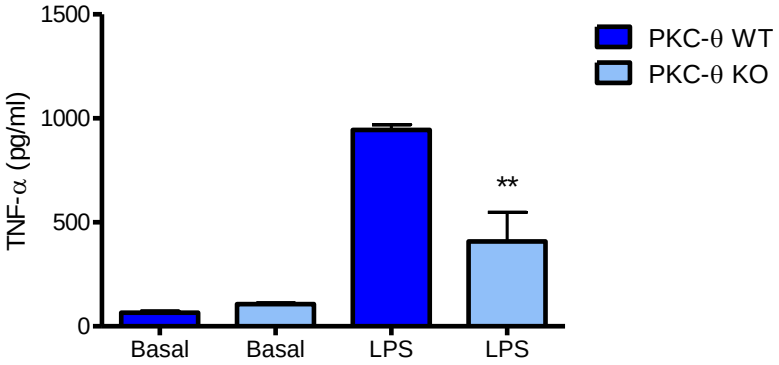

30000
20000
10000
0
IL-6 (pg/ml)
Basal
ES-62
LPS
LPS+ES-62
****
PKC-θ WT
25000
20000
15000
10000
5000
0
IL-6 (pg/ml)
Basal
ES-62
LPS
LPS+ES-62
***
PKC-θ KO

Figure 3.5.3.3.2 The effect of PKC-θ KO and ES-62 on TNF-α cytokine secretion from DCs

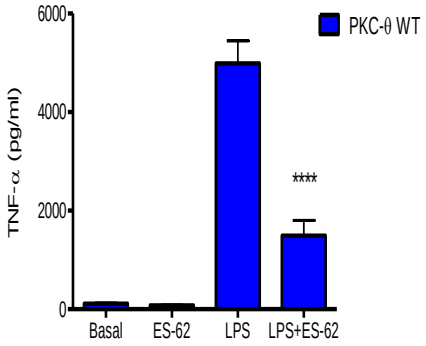
A. PKC-θ WT and KO DCs were stimulated with LPS (10 ng/ml) for 24 hours at 37˚C. TNF-α release was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of 2 independent experiments. No statistically significant difference was observed between WT and KO observed in two other experiments. Statistical analysis is by one way ANOVA where ** $P<0.005$ for LPs WT versus KO.

B. PKC-θ WT and KO DCs were incubated in the presence or absence of ES-62 for 18 h and then stimulated with LPS (10 ng/ml) for 24 hours at 37˚C. TNF-α release was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and is representative of one experiment. A second experiment showed a significant decrease by ES-62 in the WT with a non-significant decrease witnessed in the KO. A third experiment showed a non-significant decrease in both WT and KO IL-6 production by ES-62. Statistical analysis is by one way ANOVA where **** $P<0.0001$ for WT LPS versus WT LPS+ES-62 and * $P<0.05$ for KO LPS versus KO LPS+ES-62.

A.


1500
1000
500
0
TNF-α (pg/ml)
Basal
Basal
LPS
LPS
**
PKC-θ WT
PKC-θ KO

B.


6000
4000
2000
0
TNF-α (pg/ml)
Basal
ES-62
LPS
LPS+ES-62
***
PKC-θ WT

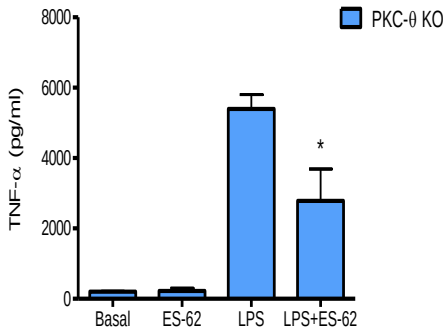
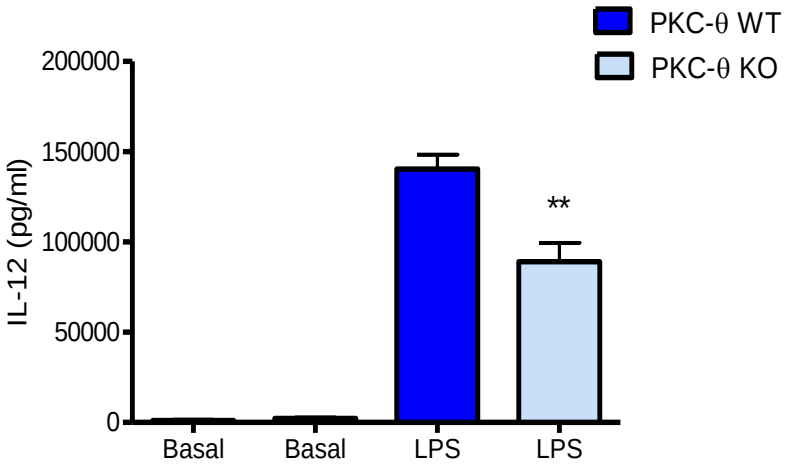

8000
6000
4000
2000
0
TNF-α (pg/ml)
Basal
ES-62
LPS
LPS+ES-62
*
PKC-θ KO

Figure 3.5.3.3.3 The effect of PKC-θ KO and ES-62 on IL-12 cytokine secretion from DCs

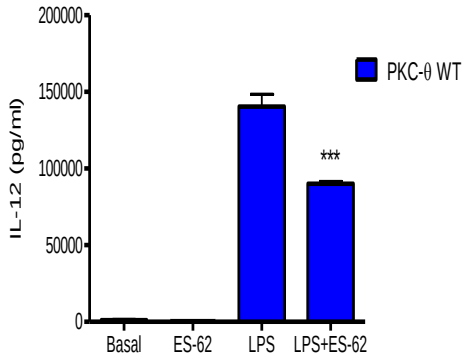
A. PKC-θ WT and KO DCs were stimulated with LPS (10 ng/ml) for 24 hours at 37˚C. IL-12 release was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and are representative of a single experiment. A non-significant decrease was observed in the KO in two other experiments while a non-significant increase was observed in a fourth experiment. Statistical analysis is by one way ANOVA where ** $P<0.001$ for LPS WT versus KO.

B. PKC-θ WT and KO DCs were incubated in the presence or absence of ES-62 for 18 h and then stimulated with LPS for 24 hours at 37˚C. IL-12 release was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and is representative of one experiment. A second experiment showed a non-significant decrease by ES-62 in the WT with a non-significant increase witnessed in the KO. Statistical analysis is by one way ANOVA where *** $P<0.0001$ for WT LPS versus LPS+ES-62.

A.


PKC-θ WT
PKC-θ KO
IL-12 (pg/ml)
200000
150000
100000
50000
0
**
Basal
Basal
LPS
LPS

B.


PKC-θ WT
IL-12 (pg/ml)
200000
150000
100000
50000
0
***
Basal
ES-62
LPS
LPS+ES-62

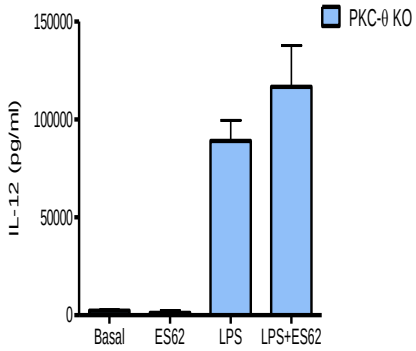
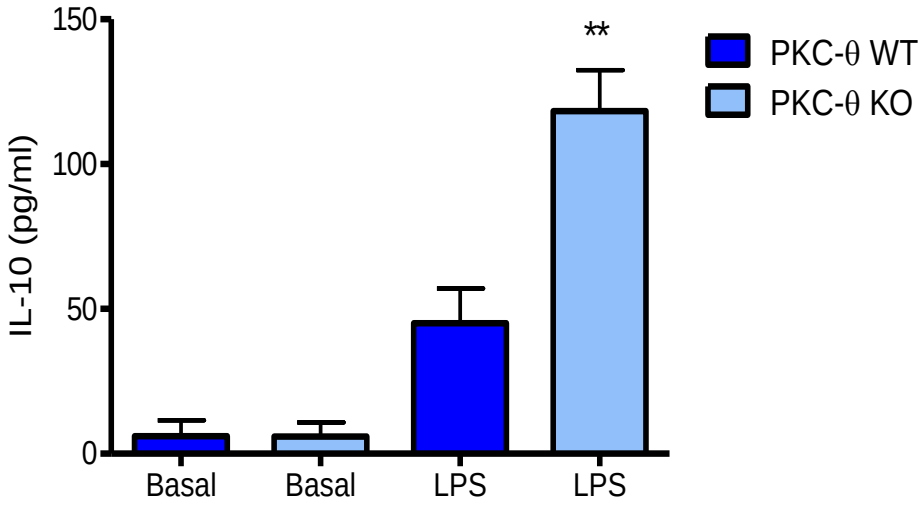

PKC-θ KO
IL-12 (pg/ml)
150000
100000
50000
0
Basal
ES62
LPS
LPS+ES62

Figure 3.5.3.3.4 The effect of PKC-θ KO and ES-62 on IL-10 production from DCs

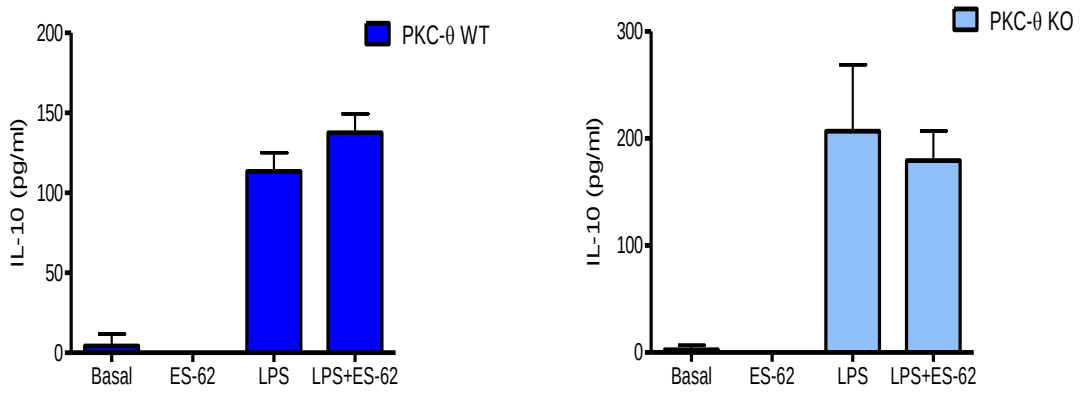
A. PKC-θWT and KO DCs were sensitized with 10 ng/ml LPS for 24 hours at 37˚C. Release of IL-10 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 3 experiments. One other experiment showed no significant difference between WT and KO. The statistical analysis performed is one way ANOVA where ** $P<0.005$ for LPS WT versus KO.

B. PKC-θ WT and KO DCs were incubated in the presence or absence of ES-62 for 18 h and then stimulated with LPS for 24 hours at 37˚C. IL-10 release was measured by ELISA. Results are shown as means ± S.D. The data presented are from a single experiment incorporating triplicate values and is representative of three experiments. Statistical analysis is by one way ANOVA.

A.


150
100
50
0
IL-10 (pg/ml)
**
PKC-θ WT
PKC-θ KO
Basal
Basal
LPS
LPS

B.


200
150
100
50
0
IL-10 (pg/ml)
PKC-θ WT
Basal
ES-62
LPS
LPS+ES-62
300
200
100
0
IL-10 (pg/ml)
PKC-θ KO
Basal
ES-62
LPS
LPS+ES-62

3.5.4 PKC-epsilon

PKC-ε has been shown to be expressed in both human and murine primary mast cells and the mast cell line RBL-2H3 (Ozawa, et al. 1993a and 1993b; Melendez, et al. 2007; Li, et al. 2005; Lessman, et al. 2006). Upon challenge with antigen, this isoform, like many others, has been revealed to be present at the plasma membrane following its translocation from the cytosol suggesting its activation and a possible role of this individual isoform as a transducer of signals for antigen-stimulated mast cell functional responses (Chang, et al. 1997; Ozawa, et al. 1993a and 1993b; Li, et al. 2005; Lessman, et al. 2006). Indeed PKC-ε was shown to be one of the isoforms most strongly associated with the plasma membrane following antigen stimulation with a >50% increase observed (Ozawa, et al. 1993a). Moreover, in parallel with the calcium-dependent conventional isoforms α, β and γ, the novel calcium-independent isoforms δ and ε were implicated in mediating antigen-induced mast cell degranulation in RBL cells (Ozawa, et al. 1993a). Further studies using reconstitution of individual PKC isoforms in washed permeabilized RBL cells have suggested a distinct role for PKC- α and ε in regulating a PKC feedback mechanism of PLC (Ozawa, et al. 1993b). When PKC-α and -ε were re-introduced into the permeabilized cells, these isoforms inhibited antigen-induced hydrolysis of inositol phospholipids by decreasing tyrosine phosphorylation of PLC-γ (Ozawa, et al. 1999b). Moreover, over-expression of PKC-ε (by almost 100 fold) revealed a role for this isoform in the suppression of phospholipase A2 (PLA2) (Chang, et al. 1997). Cytosolic PLA2 (cPLA2) is activated by both calcium mobilization and MAP kinase (Lin, et al. 1993). Thus, this result coordinates well with Ozawa and colleagues' (Ozawa, et al. 1993b) findings suggesting that this isoform can be a negative

regulator of signalling events such as calcium mobilization and/or MAP kinase activation. However, the specific role of this isoform in mast cell signalling appears to be controversial. A study carried out by Lessmann et al showed that as others have found (Chang, et al. 1997; Ozawa, et al. 1993a) PKC-ε is indeed translocated to the plasma membrane in BMMCs (Lessman, et al. 2006). However, further analysis showed that this isoform may be redundant in mast cell effector functions as mast cells derived from PKC-ε deficient mice displayed no changes to key events such as exocytosis and pro-inflammatory cytokine production (IL-6 and TNF-α), in addition to arachidonic acid release (Lessman, et al. 2006). These discrepancies clearly demonstrate the need for verification of the differential functions of individual PKC isoforms such as PKC-ε in mast cells. Therefore, as an alternative approach to permeabilized cells and overexpression studies, a similar approach to that adopted by Lessman et al will be used whereby bone-marrow derived mast cells from PKC-ε wild-type and knockout mice will be examined to help assess if this isoform exerts a critical function in mast cell responses. Mast cell-mediated responses that will be assessed are cytokine production following antigen stimulation and the well-known mast cell stimulus LPS.

3.5.4.1 Mast cells

Again, the first stage in identifying any key differences between the PKC-ε WT and KO cells was to analyse the cells by flow cytometry for the expression of the mast cell markers FcεRI and c-kit. Following this, analysis of PKC-ε WT and KO BMMC for IL-6 and TNF-α cytokine release was carried out following either cross-linking

(XL) of bound anti-DNP IgE (IgE) with antigen or stimulation with LPS after 24 hours over 4 individual experiments. If differential responses were witnessed in the majority of experiments, then BMMC were also analysed following pre-treatment with ES-62 for 24 hours to look for any effect the knockout may have on ES-62's activity in mast cells.

3.5.4.1.1 Cell surface expression of mast cell markers

Both cell types were analysed by flow cytometry for their expression of the cell surface markers, FcεRI and c-kit to confirm their identity as mast cells. The results show that mast cells are clearly distinguishable on the basis of their forward and side scatter properties and their strong c-kit and FcεRI expression (gating relative to isotype control). The BMMC culture example shown for WT and KO are 87% and 95.5% positive respectively (Figure 3.5.4.1.1). These data suggest that both WT and KO cells are truly mast cells and indicate that they exhibit almost equal expression of both cell surface markers. This result is comparable with other data obtained with PKC-ε knockout mice where no differential responses were witnessed with expression of surface markers c-kit and FcεRI as well as toluidine blue staining or the proliferative response of these cells to IL-3 (Lessman, et al. 2006). However, a smaller immature population of mast cells can be witnessed in both WT and KO BMMC, with this population showing more cells in the WT culture (11%) in comparison to the KO culture (3.3%). These data would suggest that KO BMMC might achieve their maturation status quicker than their WT equivalents.

3.5.4.1.2 IL-6

IL-6 cytokine analysis following XL of IgE receptors on mast cells revealed that PKC-ε KO cells showed an increase in IL-6 production relative to WT in 3 separate experiments, where 2 of these experiments showed a statistically significant change (Figure 3.5.4.1.2 A). Overall, the KO cells showed a 58% ± 7% (Mean ± SEM) increase in IL-6 production for all 3 experiments undertaken. This was a surprising result as previous literature using PKC-ε knockout studies has suggested PKC-ε deficiency has no effect on IL-6 cytokine production following antigen stimulation (Lessmann, et al. 2006). With regards to LPS-stimulated mast cells, the results revealed something of an opposing trend to antigen-induced activation where 2 out of 3 experiments showed a significant down-regulation in secretion of IL-6; however, 1 experiment showed a significant increase in KO cells (Figure 3.5.4.1.2 B) The emerging difference witnessed between WT and KO cytokine production following FcεRI-mediated cross-linking led to investigating the effect of ES-62 on these cell types. From these experiments, it was shown that ES-62 reduced IL-6 secretion in WT cells in 3 separate experiments, where two of these results reached statistical significance (Figure 3.5.4.1.3). This result obtained in WT cells was almost identical to that found in the KO where an inhibition in cytokine production was witnessed in 3 experiments and again two of these experiments showed a statistically significant change (Figure 3.5.4.1.3). These data generally suggest that absence of this isoform does not appear to impact on ES-62's action. This outcome is perhaps not unexpected as ES-62 did not reduce the expression of PKC-ε in human mast cells (Melendez, et al. 2007) and therefore it is possible that it may also not target this isoform in BMMC.

3.5.4.1.3 TNF-α

Following FcεRI cross-linking for 24 hours, there was no TNF-α cytokine production detected in either WT or KO cells in 3 experiments (data not shown). Unfortunately, this meant it was not possible to assess any differences that may or may not exist between the two cell types with respect to production of this cytokine. This also meant that the effect this isoform has on TNF-α following pre-treatment with ES-62 could not be investigated. Additionally, LPS-stimulated TNF-α production was measured and it was found that the data obtained were highly inconsistent (data not shown). Thus, one experiment showed no LPS-induced TNF-α production in WT cells, below detection levels in another experiment for both cell types, as well as a significant decrease in the KO response witnessed in a third experiment (data not shown). These results made it impossible to undertake testing with ES-62. Lessmann and co-workers reported a lack of differential response following antigen trigger between WT and PKC-ε KO BMMCs, therefore we can assume that these cells are capable of producing TNF-α in response to stimuli (Lessmann, et al. 2006). This suggests that perhaps the production of this cytokine was too low to detect in both cell types in the current study.

Fig 3.5.4.1.1 Flow cytometric analysis of WT and PKC-ε KO BMMC for expression of mast cell surface markers C-kit and FcεRI

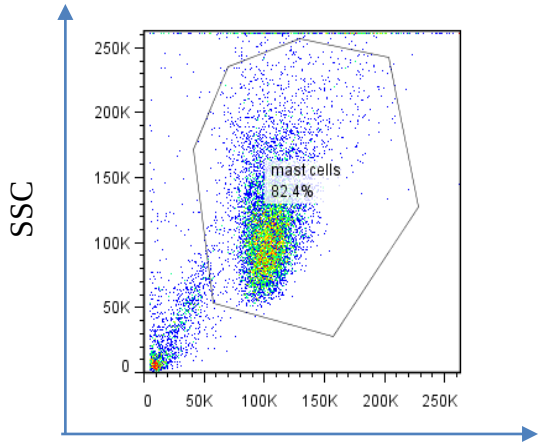
A. Dot plot analysis of FSC versus SSC allowing gating of live cells on the basis of their size and granularity, respectively.

B. Dot plot showing setting of gates for c-kit + and/or FcεRI + cells on the basis of staining of live mast cells (as gated in panel A) with relevant isotype controls.

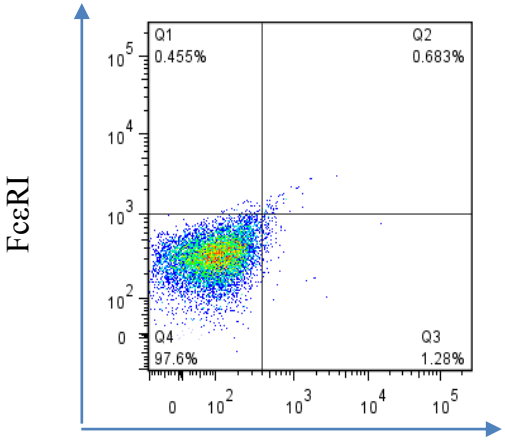
C. WT mast cells gated as described in panel A were examined for their expression of c-kit and FcεRI relevant to appropriate isotype controls (panel B). Expression of C-kit (x-axis) was determined by staining with an APC-conjugated anti-mouse CD117 (c-kit) antibody gated relative to its isotype control (APC-conjugated rat anti-mouse IgG2b; panel B). Expression of FcεRI (y-axis) was determined by staining with FITC-conjugated anti-mouse FITC FcεRI antibody gated relative to its isotype control (FITC-conjugated Armenian Hamster IgG; panel B).

D. KO mast cells gated as described in panel A were examined for their expression of c-kit and FcεRI relevant to appropriate isotype controls (panel B). Expression of C-kit (x-axis) was determined by staining with an APC-conjugated anti-mouse CD117 (c-kit) antibody gated relative to its isotype control (APC-conjugated rat anti-mouse IgG2b; panel b. Expression of FcεRI (y-axis) was determined by staining with FITC-conjugated anti-mouse FITC FcεRI antibody gated relative to its isotype control (FITC-conjugated Armenian Hamster IgG; panel B).

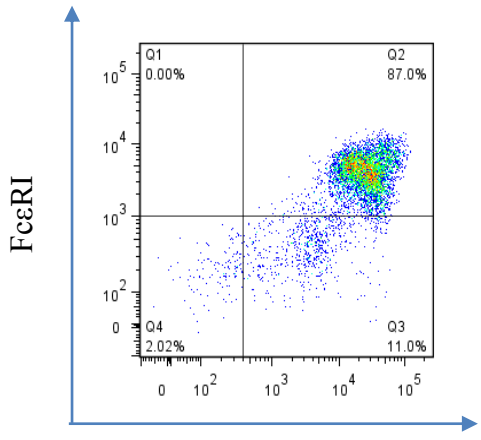
A.


SSC
FSC
250K
200K
150K
100K
50K
0
0
50K
100K
150K
200K
250K
mast cells
82.4%

B.


FcεRI
c-kit
Q1
0.455%
Q2
0.683%
Q4
97.6%
Q3
1.28%

C.


FcεRI
c-kit
Q1
0.00%
Q2
87.0%
Q4
2.02%
Q3
11.0%

D.

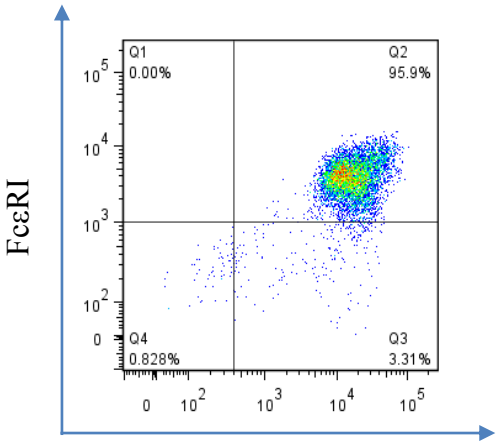
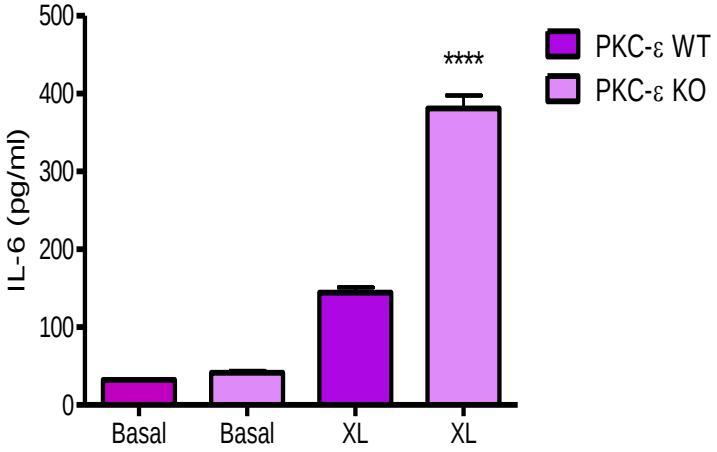

FcεRI
c-kit
Q1
0.00%
Q2
95.9%
Q4
0.828%
Q3
3.31%

Figure 3.5.4.1.2 The effect of PKC-ε KO on IL-6 secretion in BMMC

A. PKC-ε WT and KO BMMC were sensitized with 500ng/ml anti-DNP IgE for 24 hours then stimulated with 500 ng/ml DNP-BSA to induce cross-linking (XL) for 24 hours at 37˚C. Results are shown as means ± S.D. IL-6 release was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 2 independent experiments. A non-significant increase was observed in a third experiment. Statistical significance was analysed by one way ANOVA where **** $P<0.0001$ for both XL WT versus KO and LPS WT versus KO.

B. PKC-ε WT and KO BMMC were sensitized with LPS for 24 hours at 37˚C. Results are shown as means ± S.D. IL-6 release was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 2 independent experiments. A significant increase was observed in a third experiment. Statistical significance was analysed by one way ANOVA where **** $P<0.0001$ for LPS WT versus KO.

A.


500
400
300
200
100
0
IL-6 (pg/ml)
****
PKC-ε WT
PKC-ε KO
Basal
Basal
XL
XL

B.

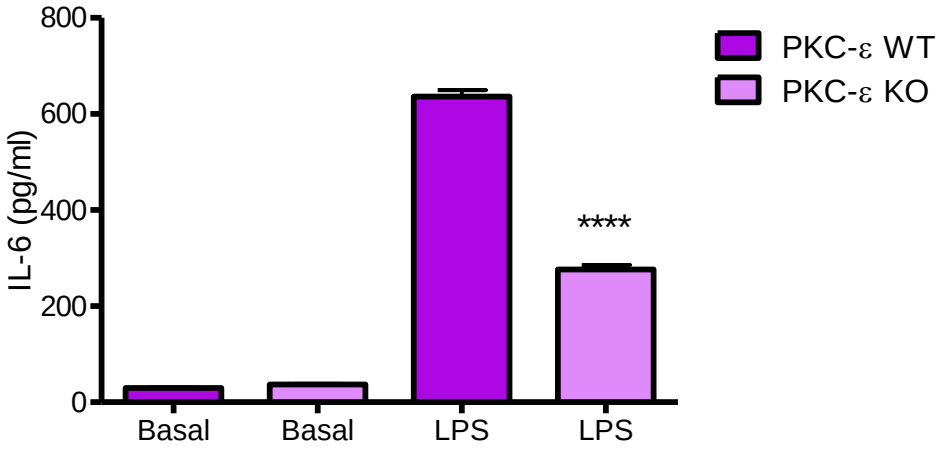
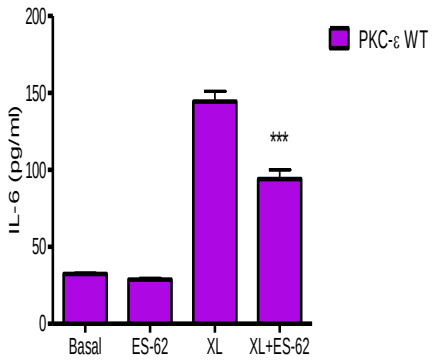
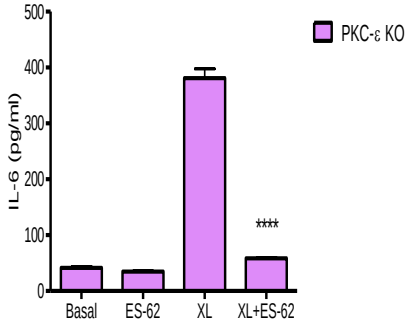

800
600
400
200
0
IL-6 (pg/ml)
****
PKC-ε WT
PKC-ε KO
Basal
Basal
LPS
LPS

Figure 3.5.4.1.3 The effect of the absence of PKC-ε on ES-62 activity on IL-6 secretion in BMMC

Cross-linking (XL) of PKC-ε WT and KO BMMC in the presence or absence of ES-62 (2 μg/ml). Results are shown as means ± S.D. IL-6 release was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 2 independent experiments. A non-significant reduction was observed in a third experiment between XL and XL+ES-62 for both WT and KO BMMC. Statistical significance was analysed by one way ANOVA where *** $P<0.001$ for WT XL versus WT XL+ES-62 and **** $P< 0.0001$ for KO XL versus KO XL+ES-62.


PKC-ε WT
IL-6 (pg/ml)
200
150
100
50
0
***
Basal
ES-62
XL
XL+ES-62


PKC-ε KO
IL-6 (pg/ml)
500
400
300
200
100
0
****
Basal
ES-62
XL
XL+ES-62

3.5.4.2 Macrophages

As previously explained, cytokine analysis of PKC-ε WT and KO BMM was investigated following LPS stimulation. Again, where there were differential responses witnessed between WT and KO cells, further investigations with ES-62 were carried out.

3.5.4.2.1 IL-6

Upon stimulating macrophages with LPS for 24 hours, there was an increase in IL-6 production witnessed in KO cells in 4 experiments, where this result was significant for two experiments (Figure 3.5.4.2.1 A). Overall, there was a 33% ± 5% (Mean ± SEM) increase in KO macrophages. Due to the emerging evidence of a possible difference between WT and KO cells, it was decided in one experiment to further investigate these cell types when employing ES-62. From this experiment, it was observed that ES-62 significantly decreased IL-6 production (Figure 3.5.4.2.1 B). However, the nematode product could not replicate this feature in the KO (Figure 3.5.4.2.1 B). Thus, it may be possible that ES-62 requires PKC-epsilon in order to modulate IL-6 production in macrophages. However, this experiment needs to be repeated.

3.5.4.2.2 TNF-α

The data obtained from WT and KO cells following LPS stimulation showed that there were no significant differential responses observed between WT and KO cells in 4 separate experiments (Figure 3.5.4.2.2). This result meant that no investigation with ES-62 should be undertaken.

3.5.4.2.3 IL-12

Following stimulation with LPS, it was observed that there was a significant increase in IL-12 secretion in PKC-ε KO macrophages in comparison to WT in all 4 experiments (Figure 3.5.4.2.3 A). Overall, these data showed 82% ± 16% (Mean ± SEM) increase in IL-12 production in KO cells across all 4 experiments. Thus, it was decided from these emerging data to further investigate these cells with respect to ES-62. From these subsequent experiments, it was discovered that ES-62 decreased IL-12 secretion in both WT and KO cells in 2 separate experiments undertaken, with results reaching statistical significance in one experiment for both cell types (Figure 3.5.4.2.3 B).

3.5.4.2.4 IL-10

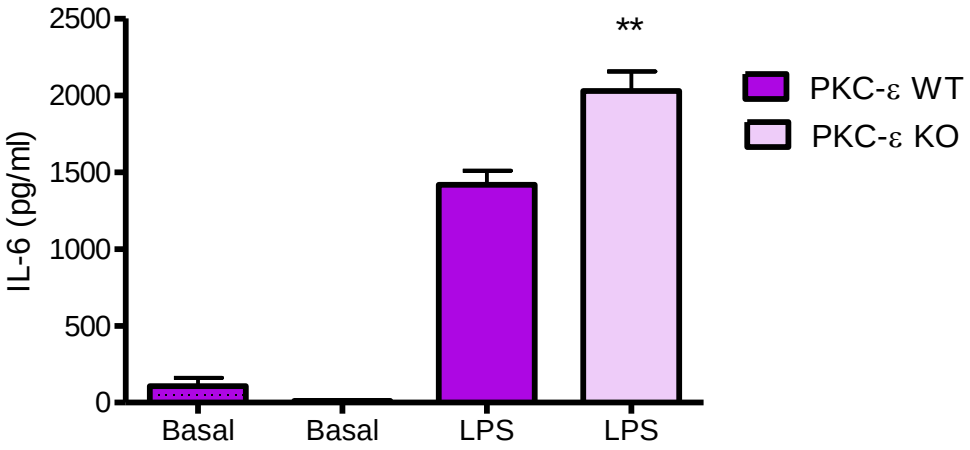
Data obtained from LPS-induced IL-10 cytokine production experiments showed there were no significant differences in cytokine secretion between WT and KO cells in 4 individual experiments undertaken (Figure 3.5.4.2.4). The lack of variance between the two cell types justified no testing with ES-62 under these conditions.

Figure 3.5.4.2.1 The effect of PKC-ε KO and ES-62 on IL-6 secretion in macrophages

A. PKC-ε WT and KO BMM were sensitized with 100 ng/ml LPS for 24 hours at 37˚C. Release of IL-6 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 2 experiments; a non-significant increase was witnessed in 2 other experiments. The statistical analysis performed is one way ANOVA where ** $P<0.005$ for LPS WT versus KO.

B. PKC-ε WT and KO BMM were sensitized with 100 ng/ml LPS for 24 hours at 37˚C in the presence or absence of ES-62 (2 μg/mL). Release of IL-6 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values. The statistical analysis performed is one way ANOVA where ** $P<0.005$ for WT LPS versus LPS + ES-62.

A.


2500
2000
1500
1000
500
0
IL-6 (pg/ml)
**
PKC-ε WT
PKC-ε KO
Basal
Basal
LPS
LPS

B.

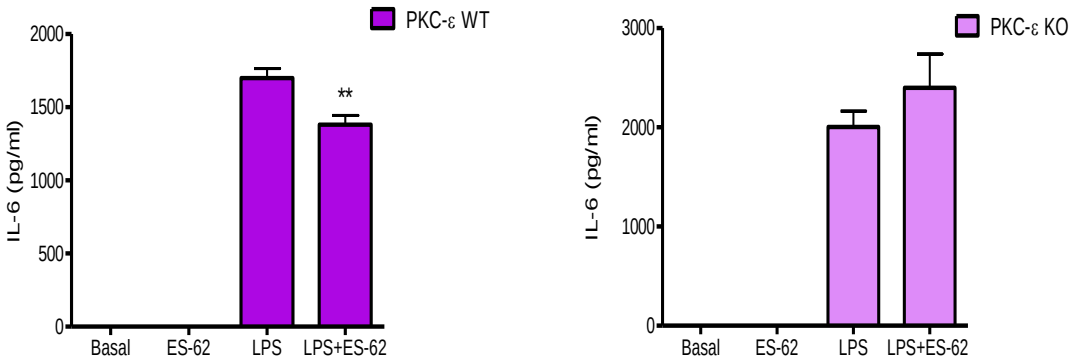

PKC-ε WT
2000
1500
1000
500
0
IL-6 (pg/ml)
**
Basal
ES-62
LPS
LPS+ES-62
PKC-ε KO
3000
2000
1000
0
IL-6 (pg/ml)
Basal
ES-62
LPS
LPS+ES-62

Figure 3.5.4.2.2 The effect of PKC-ε KO on TNF-α secretion in macrophages

PKC-ε WT and KO BMM were sensitized with 100 ng/ml LPS for 24 hours at 37˚C. Release of TNF-α was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 4 experiments. The statistical analysis performed is one way ANOVA.

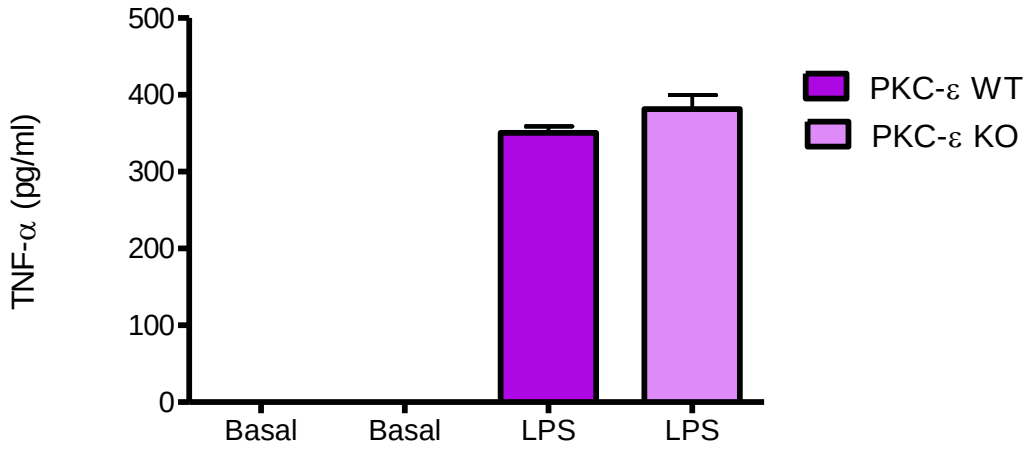
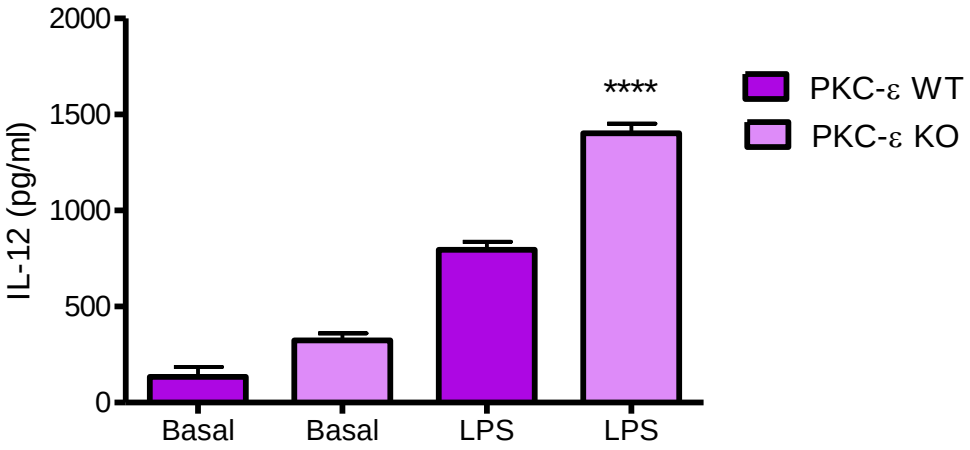

500
400
300
200
100
0
TNF-α (pg/ml)
Basal
Basal
LPS
LPS
PKC-ε WT
PKC-ε KO

Figure 3.5.4.2.3 The effect of PKC-ε KO on IL-12 secretion in macrophages

A. PKC-ε WT and KO BMM were sensitized with 100 ng/ml LPS for 24 hours at 37˚C. Release of IL-12 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 4 experiments. The statistical analysis performed is one way ANOVA where **** P<0.0001 for LPs WT versus KO.

B. PKC-ε WT and KO BMM were sensitized with 100 ng/ml LPS for 24 hours at 37˚C. Release of IL-12 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values. One other experiment showed a non-significant decrease by ES-62 in both WT and KO cells. The statistical analysis performed is one way ANOVA where **** P<0.0001 for both WT LPS versus WT LPS+ES-62 and KO LPS versus KO LPS+ES-62.

A.


2000
1500
1000
500
0
IL-12 (pg/ml)
****
PKC-ε WT
PKC-ε KO
Basal
Basal
LPS
LPS

B.

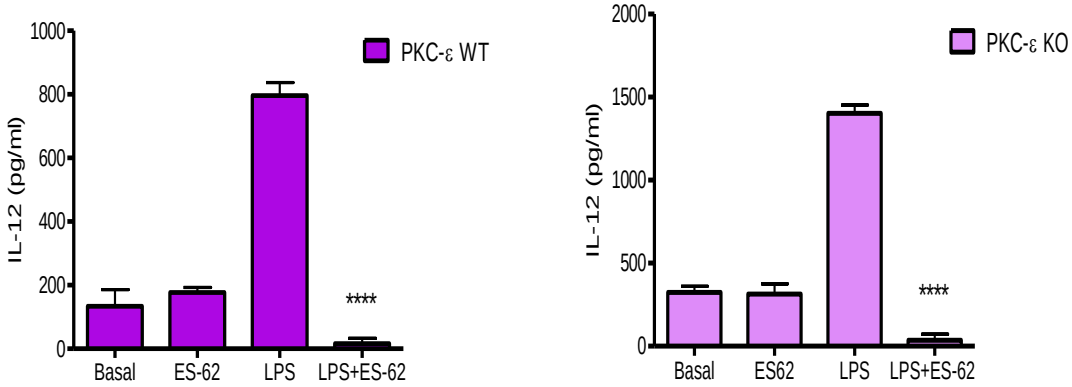
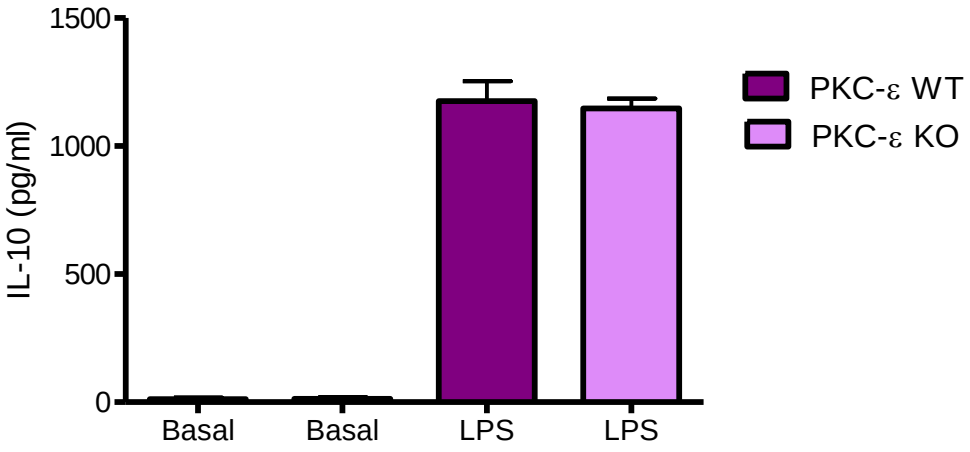

1000
800
600
400
200
0
IL-12 (pg/ml)
PKC-ε WT
****
Basal
ES-62
LPS
LPS+ES-62
2000
1500
1000
500
0
IL-12 (pg/ml)
PKC-ε KO
****
Basal
ES62
LPS
LPS+ES-62

Figure 3.5.4.2.4 The effect of PKC-ε KO on IL-10 secretion in macrophages

PKC-ε WT and KO BMM were sensitized with 100 ng/ml LPS for 24 hours at 37˚C. Release of IL-10 was measured by ELISA. The data presented are from a single experiment incorporating triplicate values and are representative of 4 experiments. The statistical analysis performed is one way ANOVA.


1500
1000
500
0
IL-10 (pg/ml)
Basal
Basal
LPS
LPS
PKC-ε WT
PKC-ε KO

Chapter 4

Discussion

4.1 Introduction

The Hygiene hypothesis states that the increased prevalence of allergic and autoimmune diseases in westernized countries has arisen due to lifestyle changes in these areas with such factors as improvement of hygiene and sanitation as well as decreased exposure to infections being largely responsible for the increasing incidence of diseases including asthma, hay fever and diabetes (Strachan, 1989; Wilson & Maizels, 2004; Cooper, 2009; Okada, et al. 2010). Geographically, it has been recognised that in areas such as the tropics where health standards and hygiene are relatively low, residents of such populations are commonly infected with parasitic helminths. Strikingly, allergy prevalence remains low in these less affluent areas where such common infections have not been eradicated (Wilson & Maizels, 2004; Cooper, 2009; Okada, et al. 2010). Indeed, various studies have supported the role of worms and/or their products in protection against both allergic and autoimmune responses. For example, Cooper and workers observed that an active infection with geohelminths, in particular *A. lumbricoides* or *Ancylostoma duodenale*, had a protective role against allergen skin test reactivity (Cooper, 2002). In addition, Gabonese school children burdened with chronic schistosomiasis showed suppressed allergen skin test reactivity to house dust mite in comparison to children who were uninfected with this helminth parasite, thus supporting a role for parasite-induced suppression of atopy (Van de Biggelaar, et al. 2000). The hypothesis that nematode-induced modulation of host responses (to fuel their own survival) simultaneously results in the suppression of other aberrant immune responses such as autoimmune reactions is supported by clinical trials of IBD where patients showed improvement on the severity of their symptoms following treatment with eggs of the

intestinal worm *Trichuris suis* (Summers, et al. 2005a and 2005b). Unique molecules produced by such parasitic helminths have been used to explain their ability to modulate host immune responses and offer such anti-inflammatory activity. The following are examples of such molecules. A high molecular weight component produced by adult worms of the pig parasitic nematode *A. suum* was shown to modulate the humoral immune response to an unrelated antigen (ovalbumin: OVA) (Soares, et al. 1992). Later, this suppressive effect on antibody responses was shown to be attributed to a single protein of around 200 kDa named PAS-1 (Oshiro, et al. 2004). Through the use of knockout mice, this protein has subsequently been shown to reduce OVA-induced lung allergic inflammation in a mouse model of asthma in an IFN-γ- and IL-10-dependent manner (Araújo, et al. 2008). Additionally, a 17kDa cystatin (AV-17) derived from the filarial nematode *A. viteae* has similarly been shown to possess immunomodulatory properties. In particular, it has the ability to down-regulate T cell responses to both specific and non-specific stimuli and modulate the production of cytokines, above all elevating IL-10 production (Hartmann, et al. 1997). Furthermore, this filarial protein was shown to protect mice against OVA-induced airway allergic hypersensitivity with correlating reduction in total and OVA-specific IgE levels as well as suppression of pro-inflammatory cellular infiltration in the lungs, particularly by eosinophils (Schnoeller, et al. 2008). This cystatin-induced immunomodulation is most likely mediated via an IL-10-dependent manner as blockade of IL-10 by antibodies against the IL-10 receptor reversed such protective effects (Schnoeller, et al. 2008). Additionally, through depleting regulatory T cells by anti-CD25 antibodies and macrophages by clondronate liposomes, this group observed that the main source of cystatin-induced

IL-10 production was macrophages suggesting a pivotal role for this cell type as an effector of cystatin-mediated anti-inflammatory and anti-allergic responses (Schnoeller, et al. 2008). Another filarial nematode-derived molecule showing therapeutic potential is ES-62, a PC-containing glycoprotein that is the subject of my project. ES-62 has been shown to possess both anti-autoimmune and anti-allergic properties. For example, it was observed in DBA/1 mice that this nematode product had powerful anti-inflammatory activity in the Th1/Th17-mediated mouse collagen-induced arthritis (CIA) model of Rheumatoid Arthritis (RA) (McInnes, et al. 2003). Specifically, prophylactic administration of ES-62 showed that this molecule targeted the production of pro-inflammatory and Th1-associated cytokines IL-6, TNF-α and IFN-γ in addition to suppressing collagen-specific IgG2a antibodies (McInnes, et al. 2003). Perhaps more importantly, when ES-62 was given after disease onset (i.e. therapeutically) collagen-induced CIA-associated inflammation and disease progression was significantly reduced where again this nematode product was able to modulate collagen-specific Th1 cytokine responses (McInnes, et al. 2003). More recently, the protective effects of ES-62 in the mouse CIA model were reassessed with respect to IL-17 responses (Pineda, et al. 2012), as Th17 production rather than solely Th1 responses have been increasingly implicated in the pathology of this disease (Koenders, et al. 2005). From these studies, ES-62 was shown to protect against inflammation associated with CIA pathogenesis through supressing IL-17 responses (Pineda, et al. 2012). It was observed that ES-62 specifically targeted two key IL-17-producing populations within the LN of CIA, $CD4^+$ T cells (Th17) and γδ T cells, in order to reduce collagen-induced IL-17 production (Pineda, et al. 2012). Additionally, ES-62 modulated Th17 responses through directly acting

on Th17 cells and by preventing DC-induced Th17 differentiation (Pineda, et al. 2012). Moreover, ES-62 has also been shown to be a powerful modulator of mast cell-dependent allergic responses (Melendez, et al. 2007). In one model, oxazolone was used to initiate an immediate-hypersensitivity reaction in the skin with the consequent inflammatory response, as made evident by ear swelling, being subsequently reduced by ES-62 (Melendez, et al. 2007). This anti-inflammatory activity correlated with ES-62-induced suppression of mast cell responses (Melendez, et al. 2007). In addition, the nematode product was able to reduce inflammation exhibited in the lungs of mice with OVA-induced airway hypersensitivity whereby ES-62's anti-inflammatory activity correlated with inhibition of eosinophilia in the lungs, suppression of the Th2-associated cytokine IL-4 (required for the inflammation exhibited in the lungs), inhibition of mucosal hyperplasia and suppression of bronchial inflammation (Melendez, et al. 2007). Collectively, these experiments show the potential for ES-62 to target mast cells and reduce inflammatory and allergic responses. ES-62's mode of action was attributed to the molecule being able to directly prevent receptor-induced mast cell degranulation and the subsequent release of inflammatory mediators such as leukotrienes, pro-inflammatory cytokines and prostaglandins (Melendez, et al. 2007). It was shown that ES-62 inhibited the PLD-coupled, SPHK-mediated immediate influx of calcium mobilization and subsequent NF-κB activation by forming a complex with TLR-4 to sequester PKC-α, which normally couples FcεRI to the PLD-SPHK pathway, away from the plasma membrane. This results in its caveolae/lipid raft–mediated, proteasome-independent degradation thereby reducing its availability and subsequently abrogating mast cell responses (Melendez, et al. 2007).

Interestingly, although ES-62 most strongly targeted PKC-α in human mast cells, other PKC isoforms were also targeted by this modulatory product (Melendez, et al. 2007). Specifically, following incubation with ES-62 for 24 hours, this molecule degraded PKC-α, -β, -δ, -ζ, and ι, with PKC-β being the isoform most strongly targeted after PKC-α (Melendez, et al. 2007). However, other PKC isoforms such as PKC-γ, -θ and ε were unaffected by ES-62 (Melendez, et al. 2007). As a means of reducing PKC-α levels in human mast cells to allow investigation of the role this isoform played in mast cell responses, anti-sense oligonucleotides were employed *in vitro* (Melendez, et al. 2007). My project was to investigate the roles of PKC isoforms targeted by ES-62, including PKC-α, in promoting mast cell functional responses. Additionally, it was to be investigated whether the absence of any of these isoforms impacted on ES-62's activity in mast cells.

4.2 The measurement of mast cell responses: differences between human and murine BMMC

The experiments conducted on human mast cells by Melendez and co-workers involved measuring β-hexosaminidase release as an indicator of mast cell degranulation following ligation of the high affinity IgE receptor, FcεRI (Melendez, et al. 2007). When using the same experimental approach with BMMC, I found that these cells did not degranulate to levels replicative of human mast cells (Melendez, et al. 2007). Mast cells constitute a heterogeneous cell population and have previously been shown to display differential functional responses. This disparity seems largely due to the differences witnessed in granule content between distinct mast cell

phenotypes with for example, BMMC containing granules in their cytoplasm that appear less mature in comparison to PDMC, CTMC or human mast cells when examined by electron microscopy (Galli, et al. 1982; Nakano, et al. 1985; Galli, et al. 2011). In relation to this, work from my research group has confirmed the differential responses of murine mast cell subsets to various stimuli (Ball, et al. 2013) with PDMCs shown to be successful and potent degranulators in response to antigen-induced receptor cross-linking, but not LPS, whereas BMMC showed very little or no degranulation following activation by all stimuli tested (Ball, et al. 2013). However, this latter cell type was able to routinely secrete IL-6, IL-13 and TNF-α in response to both FcεRI and LPS stimulation, a feature that was absent from PDMCs (Ball, et al. 2013). Therefore, in light of this information, it was perhaps unsurprising that when testing β-hexosaminidase release following FcεRI cross-linking; there was very little activation above basal levels in BMMCs tested in my experiments. Thus, measurement of mast cell responses may need to cater for not only the species i.e. human or mouse, but also for the individual mast cell subset (e.g. CTMC, PDMC or BMMC) under study as clearly mast cells possess differential phenotypical and functional plasticity (Ball, et al. 2013; Galli, et al. 2011; Melendez, et al. 2007). Therefore, with this in mind, experiments with BMMC focused on measuring mast cell pro-inflammatory cytokines rather than degranulation.

4.3 The effect of ES-62 on cytokine release from activated BMMC

As mentioned previously, as a consequence of ES-62 targeting key signalling events such as the PLD-coupled SPHK-mediated calcium mobilization pathway and NF-κB

activation, this nematode molecule was able to inhibit the release of pro-inflammatory cytokines following mast cell activation (Melendez, et al. 2007). Specifically, ES-62 inhibited IL-3, IL-6 and TNF-α secretion following antigen-mediated ligation of IgE bound to FcεRI but had no effect on Th2-associated cytokines (Melendez, et al. 2007). It was hypothesized that ES-62 would similarly target essentially the same cytokines in mouse BMMC but considering ES-62's activity had not yet been explored in this cell type, it was important to establish how it would behave in this system before further investigating the effect, if any, knocking out a particular PKC isoform might have on ES-62's 'normal' activity. ES-62 was shown in my experiments to modulate mast cell responses similarly in BMMC to human mast cells (Melendez, et al. 2007; Figure 3.2.1). Thus, ES-62 was shown to significantly down-regulate the pro-inflammatory cytokines IL-6 and TNF-α following antigen-mediated receptor aggregation. Interestingly, in this system, ES-62 could also target the Th2-associated cytokine IL-13, a result not previously witnessed in human mast cells (Melendez, et al. 2007; Figure 3.2.1). This somewhat unexpected result was not only witnessed in my own experiments, but was confirmed by other members of the research group in both BMMC and another subgroup of mast cells, CTMC (Ball, et al. 2013). This interesting result prompted further investigation of other pro-inflammatory and Th2 cytokines, namely IL-4, IL-5 and IL-1β. However, no other cytokines tested could be detected in this system. This result is in partial agreement with the observations of Melendez and co-workers as IL-4 was also found to be undetectable in human mast cells (Melendez, et al. 2007). The variances in cytokine production witnessed between mouse BMMC and human mast cells are most likely down to species-specific differences and it was interesting

to discover that ES-62 could additionally target the production of IL-13 in mouse BMMC. This molecule is a major Th2 cytokine, which has been shown to be involved in allergic responses (Kasaian & Miller, 2008; Foster et al, 2002) and in particular, IL-13 has been shown to contribute greatly to asthma pathology (Wills-Karp, 2004). These findings propose that IL-13 is a potentially new therapeutic target for the treatment of respiratory diseases such as asthma and other allergic diseases. Overall, my data suggest that ES-62 possesses powerful activity in murine BMMC and subsequently renders such mast cells hyporesponsive to activation via FcεRI. Thus, through suppressing both pro-inflammatory and certain Th2-type cytokine production from mast cells, ES-62 offers a novel therapeutic approach in the treatment of chronic allergic and inflammatory diseases.

4.4 PKC isoform expression in BMMC and the subsequent targeting of such isoforms by the helminth product, ES-62

4.4.1 PKC-α

In the study conducted by Melendez and colleagues, it was shown that human mast cells expressed 8 different PKC isoforms. These isoforms included the conventional isoforms PKC-α, -β and -γ, the novel isoforms including PKC-θ, -ε and -δ as well as the atypical isoforms PKC-ι and -ζ (Melendez, et al. 2007). Comparing a 24-hour incubation period in the presence or absence of ES-62, it was clear that many of these isoforms were targets of the nematode product. It was discovered in BMMC that this murine subset of mast cells also strongly expressed PKC-α (Figure). This result is consistent with findings from members of my own laboratory group (Ball, et

al. 2013) and from other research groups (e.g., Li, et al. 2005). The identification of this isoform in mouse BMMC prompted further investigations with ES-62. Undertaking a time-course experiment and subsequent analysis by Western blotting and densitometric analysis, revealed that ES-62 degraded PKC-α and this was visible following a 6-hour incubation period with expression almost entirely lost by 24 hours. Again, this result resembles that witnessed in human mast cells (Melendez, et al. 2007) and by other members of my research group (Ball, et al. 2013). Therefore, it seems plausible that ES-62 is acting somewhat consistently in both human (Melendez, et al. 2007) and murine mast cells (this thesis; Ball, et al. 2013) and thus, it can perhaps at this stage be assumed that ES-62 is able to suppress the production of both pro-inflammatory and Th2-associated cytokines in BMMCs by targeting PKC-α to disrupt key mast cell signalling pathways that would normally lead to the full activation of these cells as PKC-α has already been implicated as an important isoform for the production of IL-6, IL-13 and TNF-α following antigen stimulation in mast cells (Li, et al. 2005).

4.4.2 PKC-β

Isoforms expressed in mast cells may differ depending on the cell phenotype. This has already been witnessed with the mast cell line RBL differing in isoform expression profile from BMMC (Ozawa, et al. 1993a; Li, et al. 2005). Despite loading a range of amounts of protein and employing appropriate control samples, PKC-β could not be detected in BMMC. Observations from the western blotting studies suggest that the antibody in use was working effectively as a band of the

expected size for PKC-β (approximately 90 kDa) could be witnessed in the positive control (a PKC-β overexpression lysate) and also in spleen cell extracts. This isoform has previously been identified in primary cells of both murine and human origin (Chang, et al. 1997; Ozawa, et al. 1993 a; Melendez, et al. 2007; Li, et al. 2005). Furthermore, PKC-β has already been publicized as one of the isoforms expressed in BMMC in both resting state and following activation by receptor aggregation with the latter determined by use of phospho-specific antibodies to detect the active phosphorylated form of the isoform (Li, et al. 2005). Nonetheless, as this isoform could not be identified in the BMMC under study it suggests that either this isoform is not present or that it is expressed in such low amounts that it cannot be detected by the western blot indicator system in use.

4.4.3 PKC-θ

It is uncertain if ES-62 would target the same isoforms in human and murine mast cells and thus it was decided to investigate if this isoform could be targeted in BMMC by the nematode product. Unfortunately, it was impossible to detect expression of this isoform in the BMMC under investigation. Similar to PKC-β immunoblotting studies, a positive control (Jurkat T cells known to highly express PKC-θ) intended for use with the PKC-θ antibody was utilized to rule out any antibody-related issues as the reasons for lack of detection in our system. Interestingly, another research group who set out to identify PKC isoforms present in BMMC reported that PKC-θ expression was extremely low, especially in comparison to an extract from T cells (Li, et al. 2005) suggesting that this isoform could be

present in BMMC but may be difficult to detect by the western blotting system in use. Indeed, other members from the Harnett laboratory group at Glasgow University were also repeatedly unsuccessful at detecting PKC-θ in BMMC (Dimity Ball, verbal communication). However, a further research group showed that PKC-θ could be detected in both RBL cells and mouse BMMC (Lui, et al. 2001). Overall, these findings indicate that identification of this isoform is possible in murine BMMC but perhaps it was present in too low an amount to be successfully identified by the indicator system in use in the present study.

4.4.4 PKC-ε

PKC-ε could be detected in BMMC lysates, although its expression was relatively low, especially in comparison to Jurkat T cell and spleen extracts (positive controls). This result correlates with other findings in BMMC where PKC-ε was identified as one of the isoforms present in this cell type (Li, et al. 2005; Lessmann, et al. 2006). Due to the confirmation of this isoform being present in BMMC and thus the ability to detect its expression in our system, it was decided to investigate whether ES-62 would target this isoform in BMMC. Unfortunately, with the BMMC extracts that were used, I failed to detect this isoform in both control and ES-62 treated samples. It could be assumed that as alluded too previously, this isoform is expressed in very low amounts and in this particular experiment, too low to detect.

4.5 Establishing a knockdown method by siRNA

Melendez and co-workers were able to successfully knockdown PKC-α expression in human mast cells by around 80% through the use of anti-sense oligonucleotides (Melendez, et al. 2007). Although this method proved to be an effective tool to study the function of this isoform in human mast cells, comparative studies between antisense oligonucleotides and siRNA have suggested that the latter is more effective in cell culture (Bertrand, et al. 2002). Furthermore, this knockdown technique has been shown to be successful in many mast cell studies (Zhang et al, 2010; Peng and Beaven, 2005; Heionen et al, 2002). Therefore, as opposed to the studies carried out by Melendez *et al* (Melendez, et al. 2007); PKC isoforms were chosen to be silenced by siRNA.

4.5.1 Optimization of Cell Death parameters

Due to this being the first attempt at establishing a knockdown method by siRNA in BMMC, it was decided to utilize a Cell Death control siRNA from QIAGEN in order to understand how the siRNAs may function in this cell type and thus form a basis for conclusions from the parameters investigated such as siRNA concentration, cell density and time-point, to then further optimize the protocol when using the siRNAs intended to silence the PKC isoforms. From the Cell Death optimization experiments, it was observed that although the volume of HiPerfect Transfection Reagent being used to facilitate insertion of the siRNA into the cell was being employed at a sufficient concentration and more importantly was not damaging to the cells (due to no cell death being witnessed in the HiPerfect transfection reagent

control with no siRNA), it was clear that when the low siRNA concentrations (below 100 nM) were increased, greater knockdown levels (following a 72 hour incubation) were achieved with expression levels reduced by 70 and 80% for 200 nM and 500 nM, respectively. This could only be witnessed when using a cell density of 0.2 x 10^6 BMMC as the higher cell density experiments were inconclusive. Overall, these experiments established that for this cell type, unfavourable side effects such as toxicity and reduced silencing efficiency could be avoided if higher siRNA concentrations were employed in conjunction with the lower concentration (6 µL) of HiPerfect Transfection Reagent, another important parameter for optimal siRNA uptake into the cells. Therefore, the siRNA concentrations 100, 200 and 500 nM were selected for further testing with the specific PKC isoform siRNAs. Additionally, greater silencing activity could be detected at the 72 hour time-point over 24 hours and therefore it was concluded that a time-point greater than 24 hours was important for witnessing knockdown.

4.5.2 Optimization of PKC isoform siRNA based on Cell Death experimental observations

For optimization experiments involving the PKC isoforms, it was decided to use PKC-α as it is known from previous experiments with human mast cells that this PKC isoform was knocked down by approximately 80% using antisense oligonucleotides with subsequent inhibition of mast cell functional responses (Melendez et al, 2007). Thus, PKC-α gene silencing was intended to be similar if not higher in BMMC using the PKC-α siRNA. Unfortunately, repeated attempts to

knockdown the PKC-α gene produced highly inconsistent results. Despite such apparent failed delivery of siRNA into these mammalian cells to induce silencing, it was decided to subsequently test the functional responses of the mast cells under study through measurement of the mast cell-associated pro-inflammatory cytokine IL-6. Overall, the data from these experiments were inconclusive and a little confusing because on some occasions there was reduced IL-6 production in the apparent absence of PKC-α knockdown as determined by Western blotting. SiRNA treatment has been shown to remain effective for a time span between 3-7 days (Elbashir et al, 2002). Therefore, it is possible that our time point (48 hours) was too early to witness an effect of the siRNA at the PKC protein level but this was then later observed in the cytokine analysis. Furthermore, proteins with a longer half-life will only showcase their silencing phenotype after the protein level is reduced below a certain threshold (Bartlett and Davis, 2007). For example, if a protein has a half-life of 48 hours, it may not be possible to witness the knockdown of that gene until future time points than the frequently used 48-72 hours. Indeed, PKC-α has previously been shown to have a long half-life (6-24 Hr) (Borner et al, 1988) and therefore it is possible that the silencing phenotype would not be observed until later time points than were employed.

4.5.3 General conclusions from siRNA experiments

Despite considerable effort and trouble-shooting, it was impossible to reproducibly knockdown the PKC-α using siRNA. In order for a siRNA experiment to be successful, there must be efficient transport of the siRNA into the cell to allow the

siRNA to be incorporated into RISC (Cullen, 2006). Although RNAi can be induced in mammalian cells using exogenous siRNA, this requires specific conditions and delivery methods unique for each cell type (Caplen et al, 2001; Elshabir et al, 2001). It has been noted by others that siRNA can give a different effect, whether it be specific knockdown or off-target effects, depending on the cell type being studied (Reynolds et al, 2006). Some cells are easily transfectable and can uptake siRNA even at low concentrations whereas others are more difficult to transfect and may only take up minimal amounts even at higher siRNA concentrations. Thus, the transfectability of a cell is the restrictive factor for successful siRNA-mediated knockdown. For example, adherent human cell lines such as HeLa cells are often the mammalian cell of choice when completing RNAi experiments due to their highly reliable and extremely transfectable nature (Echeverri & Perrimon, 2006; McManus & Sharp, 2002). Additionally, other cell lines such as human embryonic kidney derived 293 cells (HEK293) are also more readily transfectable and therefore they are also regularly used for knockdown experiments (Pear et al, 1993; Donzé and Picard, 2002). On the other hand, primary suspension cells are challenging to transfect (Zuhorn et al, 2007; McManus and Sharp, 2002). Since the mast cells used for my siRNA experiments were both primary and suspension cells, it is possible that there was unsuccessful delivery of the siRNA into the cells on many occasions and this may be the explanation for the lack of knockdown witnessed in the majority of the experiments.

4.6 PKC isoform knockout mice studies

4.6.1 PKC-alpha

4.6.1.1 Mast cells

4.6.1.1.1 The effect of the absence of PKC-α on mast cell growth and differentiation

BMMCs from PKC-α KO and WT mice were grown and differentiated in the presence of SCF and IL-3 and then analysed at week 4 by Flow cytometry for the mast cell surface markers c-kit and FcεRI. Both PKC-α positive and negative BMMCs showed almost equal expression of both cell surface markers indicating that the absence of this isoform does not affect mast cell phenotype. However, it was observed that the cell types had slight differences in their maturation status. Thus, PKC-α-deficient BMMC may mature at a slightly faster rate than their WT counterparts due to the presence of two other smaller populations that seemed less mature in nature with respect to their expression of FcεRI, with such populations being larger in the WT. Regardless of KO BMMC possessing more cells of a mature phenotype, this did not positively influence cytokine production exhibited by these cells in response to antigen stimulation. So far, no other studies have indicated an essential role for PKC-α in mast cell proliferation, growth or development and therefore this result was not unexpected.

4.6.1.1.2 The effect of the absence of PKC-α on mast cell functional responses

In studies conducted by Melendez and researchers (Melendez, et al. 2007) the importance of the conventional isoform PKC-α in mast cell responses was unveiled through means of reducing the expression level of this isoform by anti-sense oligonucleotides. Through knocking down PKC-α, coupling of FcεRI, to PLD and the consequent initial peak of calcium mobilization was blocked, ultimately resulting in the inhibition of mast cell degranulation (Melendez, et al. 2007). Through the use of PKC-α WT and KO mice, my studies have additionally demonstrated important roles for this isoform in murine mast cells in response to both FcεRI and LPS/TLR-4 signalling. With respect to antigen stimulation, it was discovered that IL-6 cytokine production was reduced in PKC-α knockout BMMC. Additionally, it was observed that the secretion of another mast cell associated-cytokine, TNF-α was significantly inhibited in cells lacking this isoform following cross-linking. Interestingly, the absence of PKC-α had a greater effect on TNF-α than on IL-6 production from BMMCs with KO cells showing a reduction in TNF-α and IL-6 cytokine production by approximately 45% and 18%, respectively. However, although it was clear that the absence of this isoform impacted on the production of both pro-inflammatory cytokines, the response to antigen-triggered receptor aggregation was clearly not abrogated. Thus, these data suggest that PKC-α is a molecule that is certainly involved in the release of such mast cell mediators, but that it is not the sole molecule required to regulate their production in BMMC. Other research groups have shown that PKC-α was involved in the regulation of mast cell-associated cytokine production (Li, et al. 2005). Through conventional isoform overexpression studies, MEKK2 activity was shown to be clearly augmented by all isoforms tested (PKC-α, -βI and -βII), with the transfection of PKC-α inducing the greatest enhancement of

MEKK2 activity (Li, et al. 2005). In addition, MEKK2 knockout mice showed reduced cytokine secretion of IL-6, IL-13 and TNF-α (by around 50-60%) with the overexpression of PKC isoforms α, βI and βII restoring cytokine levels in MEKK2-absent cells and greatly enhancing cytokine production in wild-type cells following antigen stimulation (Li, et al. 2005). Together, these data suggest that PKC-α is a key component involved in the regulation of MEKK2-dependent and independent pathways for mast cell cytokine production (Li, et al. 2005). Overall, my data are consistent with the current literature that shows PKC-α as being a positive regulator of antigen-dependent pro-inflammatory cytokine responses.

On the other hand, a different effect was observed with the absence of PKC-α for IL-6 and TNF-α secretion in response to LPS stimulation. Thus, KO BMMC displayed significantly enhanced IL-6 production by up to almost 60%, whereas TNF-α responses were reduced in two of the experiments. However, a third experiment with the KO displayed an increase in TNF-α production therefore this inconsistency makes the effect of PKC-α on TNF-α secretion uncertain. Nonetheless, it is clearly evident that PKC-α is acting as a negative regulator for IL-6 cytokine production in response to LPS receptor signalling. Previous studies in BMMC deficient in PKC-α or -β or indeed both isoforms have suggested that such conventional isoforms are not essential for LPS signalling (Zorn, et al. 2009). Thus, the work in my study may represent the first time PKC-α has been shown to play a role in LPS-induced mast cell responses.

4.6.1.1.3 The effect of the absence of PKC-α on ES-62's activity in BMMC

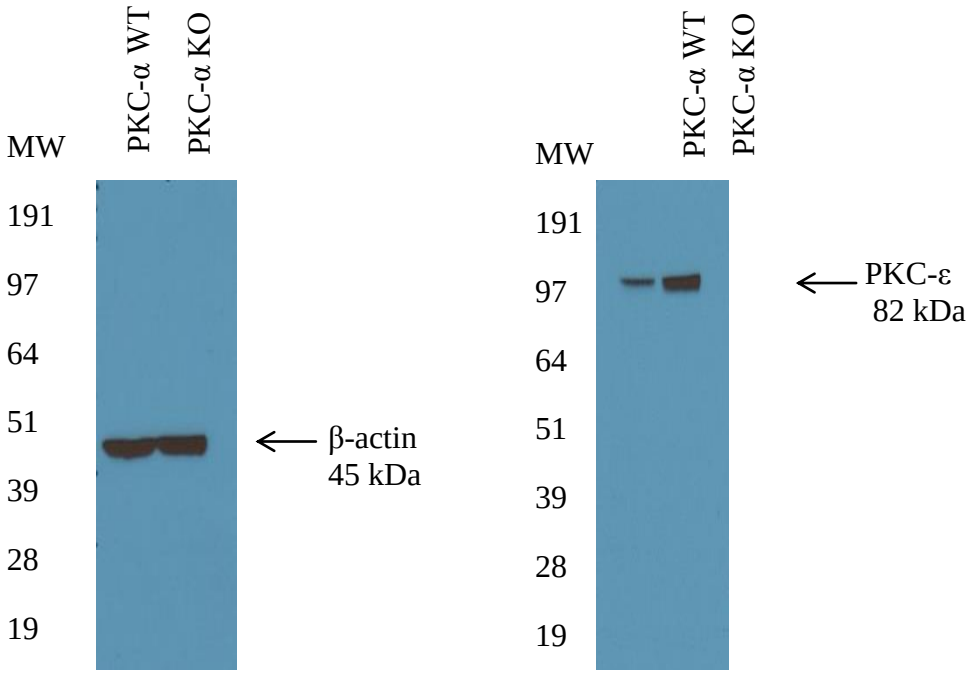
The differential responses that were emerging from studies between the PKC-α KO and WT cells led me to investigate whether ES-62 had altered modulatory ability in the absence of this isoform. A surprising finding from this work was that on some occasions ES-62 was unable to down-regulate cytokine responses in the WT. The reason for this is unknown but one possibility may relate to differences in mouse strain. Previous studies employing ES-62 in mast cells, macrophages and dendritic cells were largely undertaken from cells derived from BALB/c mice (Goodridge, et al. 2001 and 2004; Melendez, et al. 2007; Ball, et al. 2013) whereas all the PKC knockout mice are from different genetic backgrounds such as C57/Bl6. In any case, the purpose of the experiments employing ES-62 was to determine whether KOs mirrored WTs in showing reduced cytokine responses to ES-62, and hence any experiment, which did not show an effect of ES-62 on WT samples was excluded from the study.

On comparing the effect of ES-62 on mast cell cytokine production in response to antigen stimulation, it was found that it could significantly reduce the secretion of IL-6 in KO in addition to WT cells. Similarly, the nematode product could inhibit TNF-α secretion in both cell types, with this result reaching statistical significance in KO BMMC. Together, these data suggest that the absence of this isoform did not alter ES-62's modulatory ability with respect to production of either cytokine. This result was perhaps rather surprising considering ES-62 has been shown to target PKC-α in both human mast cells (Melendez, et al. 2007) and BMMC (this thesis and Ball, et al. 2013) in order to render these cells hyporesponsive to stimuli. However, it

is possible that the nematode product is targeting other molecules involved in generating the cytokine responses and this could include other PKC isoforms. The large number of PKC isoforms that have been identified in mast cells (Ozawa, et al. 1993a; Li, et al. 2005; Melendez, et al. 2007) would suggest that it may be possible for some isoforms to regulate or compensate for one another thus knocking out one isoform may impact on the levels of another. Interestingly, preliminary data from further western blot studies showed that PKC-α KO BMMC have elevated expression of PKC-ε in comparison to their wild-type counterpart (see Figure 4.1). Therefore, based on the idea that PKC-ε is a negative regulator of mast cell IL-6 and TNF-α cytokine responses (results discussed later) it could be hypothesised that the overexpression of PKC-ε witnessed in the absence of PKC-α is causing enhanced negative regulation of such mast cell responses ultimately leading to an overall reduction in cytokine production that is not attributed to ES-62 targeting PKC-α. This theory may seem plausible as PKC-ε is not an ES-62 target in human mast cells (Melendez, et al. 2007) and therefore, assuming similarly that ES-62 does not target PKC-ε for degradation in BMMC, the increased expression of PKC-ε in the PKC-α KO would not be modified by the nematode product, thus allowing for the enhanced negative regulation induced by PKC-ε to proceed. Hence, this could explain why it appears ES-62 remains active in the KO. It could also be the case that other isoforms that are required for mast cell cytokine responses are additionally up-regulated in the absence of PKC-α and ES-62 is targeting them. However, further experiments would have to take place to understand exactly how ES-62 is still able to modulate mast cell-associated effector functions in the PKC-α KO BMMC when the helminth molecule clearly targets this isoform for degradation.

With regards to LPS-stimulation, it was impossible to conclude whether ES-62's behaviour was altered or not in the absence of PKC-α. With respect to TNF-α secretion, ES-62 was not investigated due to the inconsistencies witnessed between the WT and KO data. Additionally, although ES-62 exposure could not significantly decrease IL-6 production in the KO on two occasions, this was also observed for the WT in one of the studies. Thus, it is not known if ES-62 can still remain active in the KO in response to LPS, although this does seem possible for FcεRI signalling.

Figure 4.1. Absence of PKC-α causes up-regulation of another PKC isoform, PKC-ε, in BMMC.



4.6.1.2 Macrophages

Due to the secondary role played by macrophages and DCs in the project and also limited time and availability of cells, less phenotypic analysis was undertaken. However, for the experiments that were undertaken no significant differences were found between WT and KO cells. For example, PKC-ε WT and KO macrophages were 85.5% and 87.4% positive respectively and PKC-θ WT and KO macrophages 74% and 72% positive respectively for the macrophage cell surface markers F4/80 and CD11b. Furthermore, PKC-α WT and KO DCs were both 70% positive and PKC-θ WT and KO DCs 75.6% and 74% positive respectively for the DC cell surface markers CD11c and MHCII.

4.6.1.2.1 The effect of PKC-α KO and ES-62 on macrophage cytokine responses

It was decided to simultaneously investigate the effects of the absence of PKC-α on other cells of the immune system in order to look for mast cell-specific effects. There was generally no difference witnessed between WT and KO macrophages for the production of the pro-inflammatory cytokine IL-6 and the Th1-promoting cytokine IL-12 suggesting that PKC-α plays no role in the production of these cytokines from macrophages. This result is perhaps not unexpected as currently there is no evidence that this conventional isoform plays a role in production of either cytokine. On the other hand, although not always reproducible, TNF-α secretion was increased in the majority of experiments in KO cells suggesting that this isoform is potentially important for negatively regulating the production of this particular pro-inflammatory cytokine. However, current literature suggests that two conventional

isoforms expressed in macrophages, PKC-α and -β, are important for TNF-α production. Foey and Brennan (Foey & Brennan, 2004) observed in human peripheral blood monocyte-derived macrophages that when they employed selective inhibitors of the conventional PKC isoforms α and β, TNF-α secretion was down-regulated in response to both classical LPS signalling and PMA/ionomycin suggesting that these isoforms control the production of this pro-inflammatory cytokine. At this stage, very little is known about the roles of different PKC isoforms in macrophage responses to LPS thus it can only be assumed that such differences witnessed between the two studies could be due to variations between human and mouse species. More studies will have to be undertaken in order to assess the role PKC-α plays in TNF-α production in macrophages. Even so, the difference emerging from such experiments between PKC-α WT and KO macrophages encouraged exposing macrophages to ES-62 in order to compare the modulatory ability of this nematode product in the absence of this isoform in macrophages. As expected, ES-62 was able to consistently down-regulate TNF-α production in WT macrophages across 3 experiments performed. However, in KO cells, ES-62 caused a small decrease in one experiment with a small increase observed in the others. It could thus be concluded from such data that ES-62 is more potent in the WT than in KO macrophages suggesting that as like human and murine mast cells (Melendez, et al. 2007; Ball, et al. 2013) this nematode product might target this isoform in order to render macrophages hyporesponsive to subsequent stimulation.

Furthermore, there was a striking difference observed in IL-10 production where the absence of PKC-α resulted in a significant increase in this cytokine in response to LPS, proposing that PKC-α may negatively regulate IL-10 production in response to

inflammatory stimuli. This would allow for an appropriate level of IL-10 to control any aberrant pro-inflammatory cytokine responses while in turn still allowing a certain threshold to be reached with regards to an inflammatory response in order to control infections. Such findings are opposed to studies conducted by Foey and Brennan (Foey & Brennan, 2004) who discovered that inhibition of the conventional PKC isoforms α and β had no effect on IL-10 production and instead found this cytokine to be regulated by an atypical isoform PKC-ζ. Again, such experiments may not be entirely comparable with the mouse studies described here. Both WT and KO cells showed that ES-62 had no significant effect on IL-10 production. Previous experiments in mice exposed to ES-62 revealed that the nematode product had no effect on IL-10 production from activated macrophages (Goodridge, et al. 2001). Therefore, the results obtained from WT macrophages correlate with previous experiments carried out by Goodridge and researchers. Together, these data suggest that ES-62's lack of activity was unaffected by the absence of PKC-α with respect to IL-10 secretion from macrophages. Overall, it could be concluded that in macrophages, PKC-α controls both a pro- and anti-inflammatory cytokine in order to help provide an effective and appropriate immune response towards invading pathogens. So far, these findings contradict other studies that show that TNF-α and IL-10 are differentially regulated in macrophages by a conventional and atypical isoform respectively (Foey & Brennan, 2004).

4.6.1.3 Dendritic cells

4.6.1.3.1 The effect of PKC-α KO and ES-62 on dendritic cell cytokine responses

Dendritic cells also exhibited similar results to macrophages where both IL-6 and IL-12 production was not modulated by the absence of PKC-α for this cell type. However, differential responses between WT and KO cells could be witnessed with regards to TNF-α and IL-10 cytokine production. KO dendritic cells displayed enhanced production of the pro-inflammatory cytokine TNF-α, a result that mimics data found in macrophages. Such differential responses between WT and KO dendritic cells in response to LPS led to pre-treatment of cells with ES-62. Such an experiment showed that as previously witnessed in dendritic cells (Goodridge, et al. 2004), ES-62 could significantly inhibit TNF-α cytokine secretion in response to LPS stimulation in WT cells; however, this modulatory response was also witnessed in KO cells suggesting that the absence of PKC-α does not impact on ES-62's activity for this cytokine. An opposing trend was observed with respect to IL-10 production in DCs where this cytokine was significantly decreased in KO cells in the majority of experiments undertaken. This result is in contrast to that witnessed in macrophages: thus, PKC-α appears to be acting as a positive regulator in dendritic cells for IL-10 production and a negative regulator of this cytokine in macrophages. ES-62's activity was rather inconsistent in WT DCs therefore no conclusions could be drawn for this cytokine and isoform KO.

4.6.2 PKC-beta

4.6.2.1 Mast cells

4.6.2.1.1 The effect of the absence of PKC-β on mast cell development and differentiation

A similar approach to understand the effect losing PKC-α may have on both maturation and development of mast cells was also used where expression of c-kit and FcεRI cell surface markers showed that the absence of PKC-β had no influence on mast cell phenotype. As before, a smaller population lacked expression of FcεRI with KO BMMC containing more of these cells than the comparable WT population. This suggests that the absence of PKC-β may affect the rate of maturation for mast cells, although this affect appears to be minimal. Nonetheless, PKC-β KO cells showed no alterations in phenotype. This result compares to other studies of BMMC from PKC-β KO mice where the deficiency of this isoform had no effect on the rate of cell proliferation or apoptosis in response to the presence or absence of IL-3 (Nechushtan, et al. 2006), a cytokine important for mast cell differentiation and growth in murine mast cells (Lantz & Huff, 1995; Rottem, et al. 1993). Additionally, it was found that BMMCs lacking PKC-β could retain their ability to differentiate into heparin-containing mast cells as evident by their staining properties (Nechushtan, et al. 2006). Thus, my study is in agreement with others that PKC-β is not essential for mast cell growth and development.

4.6.2.1.2 The effect of the absence of PKC-β on mast cell functional responses

Following PKC-α, the isoform that showed the most degradation induced by ES-62 was one more conventional isoform, PKC-β (Melendez, et al. 2007). This finding, in correlation with the discovery that ES-62 targets PKC-α for degradation to subsequently inhibit mast cell degranulation (Melendez, et al. 2007), would suggest that this isoform is also involved in mast cell functional responses and may like PKC-α, be a target of ES-62 in BMMC. Unfortunately, production of the pro-inflammatory cytokines IL-6 and TNF-α following antigen-mediated cross-linking was either completely absent or very low in both WT and KO BMMC. Thus, no TNF-α secretion could be detected in both phenotypes for all 4 experiments carried out while for IL-6, this cytokine was completely absent for one experiment and very close to or at times below the assay sensitivity level (15 pg/mL) for the 3 other experiments performed. Preliminary studies carried out by Nechushtan and co-workers found that BMMC lacking PKC-β showed no cytokine release following antigen-dependent activation (Nechushtan, et al. 2006). Although this might give insight as to why IL-6 and TNF-α was predominantly absent or extremely low in my experiments for PKC-β KO BMMC, it does not explain why such similar behaviour was witnessed in the WT BMMC. Nechushtan and researchers attributed such lack of responses in the KO to their original short incubation period with IgE (2 hours) and therefore increased this sensitization step to 4 days before subsequent cross-linking and measurement of cytokine mRNA levels (Nechushtan, et al. 2006). It has been reported that IgE can up-regulate the surface expression of FcεRI (Yamaguchi, et al. 1997; Shaikh, et al. 1997) with the expression levels of this IgE receptor allowing for differential strengths in the responses exhibited by mast cells (Yamaguchi, et al.

1997; Yamaguchi, et al. 1999; Kitaura, et al. 2004). In my own experiments, BMMC were sensitized for 24 hours prior to cross-linking. Therefore, the differential cytokine responses witnessed between my studies and those from Nechushtan and colleagues (Nechushtan, et al. 2006) could be attributed to weaker up-regulation of FcεRI expression and subsequently less potent effector responses mediated by antigen stimulation for both cell types under study. Nonetheless, some conclusion could be drawn from the IL-6 cytokine production even at such low levels. It was observed that PKC-β deficient BMMC produced more IL-6 following antigen-mediated activation than WT BMMC where this reached significance in the majority of experiments undertaken. Such data would allow us to suggest that PKC-β is involved in the regulation of this pro-inflammatory cytokine in mast cells. This result is in disagreement to what has been shown in other studies using PKC-β mice where the absence of this isoform resulted in a decrease of antigen-mediated IL-6 cytokine production (as determined by comparison of IL-6 mRNA levels) in addition to inhibition of mast cell degranulation in BMMC (Nechushtan, et al. 2006). Other studies in RBL cells have additionally shown a role for PKC-β in IL-6 secretion through the use of overexpressing PKC isoforms (Chang, et al. 1997). Thus, my data reports for the first time a role for PKC-β in the negative regulation of IL-6 production from BMMC.

Secreted cytokine levels were greater in both WT and KO BMMC in response to LPS. A similar trend to FcεRI-signalling was observed where production of IL-6 and this time TNF-α was significantly up-regulated in KO BMMC in all experiments undertaken in response to LPS/TLR-4 signalling. Together, the data indicate that PKC-β is an important isoform involved in the modulation of pro-inflammatory

cytokine production exhibited from mast cells following LPS (and possibly FcεRI) activation. The extent of up-regulation witnessed for IL-6 (Mean 52%) and TNF-α (Mean 62%) suggests that this isoform is not redundant in this cell type and that PKC-β may play a significant role in the control of the effector responses elicited during pathogenic infection (protective) and allergic disorders (inflammatory).

4.6.2.1.3 The effect of the absence of PKC-β on ES-62's activity in BMMC

When employing ES-62, it was observed that IL-6 production could be inhibited in WT BMMC on all occasions by the nematode product, where this effect was significant in half of the experiments undertaken. Unfortunately, data obtained from the KO BMMCs were less consistent where the nematode product equally induced (significant and non-significant) up-regulation and down-regulation of this cytokine. This result makes it rather difficult to conclude any differences or lack thereof in PKC-β KO BMMC. However, considering ES-62 could reduce IL-6 production in the KO 50% of the time, an effect cannot be ruled out and if present, it can be presumed that the loss of this isoform has no "total" effect on ES-62's modulatory ability with respect to this cytokine. An almost identical relationship was observed in both WT and KO cells with regards to TNF-α where ES-62 decreased TNF-α secretion in the WT in all experiments undertaken (reaching significance on one occasion) with again equal observations of ES-62-induced significant up-regulation and inhibition of this cytokine in KO BMMC. Although the inconsistencies witnessed in the KO makes the relationship between ES-62 and PKC-β uncertain, overall it is surprising that on occasion the lack of PKC-β does not have any

consequences on ES-62's activity even though this isoform was strongly targeted in human mast cells (Melendez, et al. 2007). PKC-β could not be detected in the western blotting studies carried out however and therefore we cannot be certain that degradation is taking place in BMMC. Also, as witnessed in the PKC-α KO BMMC, it could be possible that the expression of other isoforms is modulated in PKC-β KO BMMC and ES-62 may or may not target these modulated PKC isoforms in BMMC in order to still exert its modulatory activity in the KO.

4.6.2.2 Macrophages

4.6.2.2.1 The effect of PKC-β KO and ES-62 on macrophage cytokine responses

Interestingly, there appeared to be no differences between WT and KO macrophages in response to LPS for both IL-6 and IL-12 production, a result that I had previously witnessed for PKC-α. This may suggest that neither conventional isoform plays a role in the production of the pro-inflammatory or Th1-inducing cytokine and indeed there is little evidence in the literature to show otherwise. In correspondence with the PKC-α macrophage data, TNF-α production was up-regulated in almost all experiment in PKC-β KO macrophages. This result indicates that so far, at least two conventional isoforms, PKC-α and β appear to have important roles in negatively regulating this pro-inflammatory cytokine following LPS-induced macrophage activation. Such differences that were evident between WT and KO macrophages prompted pre-exposure to ES-62 with such experiments revealing that although not significant, this molecule could inhibit TNF-α production in the WT over 3 experiments and this effect was also observed in the KO in 2 experiments, with ES-

62-induced inhibition reaching significance on one occasion in the KO. Although there was a general lack of statistical significance reached in the WT unlike previously observed for macrophages (Goodridge, et al. 2001), it could be said that ES-62 is still working in a similar manner in both the WT and KO suggesting the nematode product does not target this isoform in order to modulate cytokine responses in macrophages. Interestingly, there was no differential response between WT and KO macrophages for IL-10 production for this isoform. PKC-ζ has been previously shown to regulate production of this cytokine in macrophages by other researchers (Foey & Brennan, 2004) thus it may not be surprising that IL-10 in unaltered by the conventional isoform.

4.6.3 PKC-theta

4.6.3.1 Mast cells

4.6.3.1.1 The effect of the absence of PKC-θ on mast cell development and differentiation

As with all other PKC isoform knockout cultures, PKC-θ-positive and -negative BMMCs showed almost identical expression of c-kit and FcεRI indicating that the absence of this isoform does not affect mast cell phenotype. Additionally, there also appeared to be one other smaller population of cells however the percentile variance was minimal suggesting that both WT and KO probably matured at a similar rate. So far, no other studies have indicated an essential role for this isoform in mast cell growth or development.

4.6.3.1.1 The effect of the absence of PKC-θ on mast cell functional responses

In spite of inhibiting degranulation and cytokine responses, ES-62 did not target PKC-θ in human mast cells (Melendez, et al. 2007) suggesting that this isoform may not be essential for mast cell functional responses. However, studies in RBL cells have shown that PKC-θ is critically involved in antigen-mediated degranulation and IL-3 gene transcription (Lui, et al. 2001). Therefore, it was decided to investigate this isoform with respect to mast cell cytokine responses in BMMC. It was found that the loss of PKC-θ had a major impact on IL-6 and TNF-α cytokine production. Thus, it was observed that PKC-θ-deficient mast cells had significantly reduced IL-6 and TNF-α production with a loss of 80%-90% in response to antigen stimulation. These data have now further unveiled the involvement of this isoform in supporting important mast cell responses, in particular, the secretion of pro-inflammatory cytokines. However, studies conducted in RBL cells suggested that PKC-θ played no role in TNF-α secretion as constitutively active PKC-θ had only minor effects on TNF-α mRNA levels (Liu, et al. 2001). As a result, my studies indicate for the first time an important role for PKC-θ in the production of this pro-inflammatory cytokine following antigen-stimulation.

The observation that both PKC-α and –θ KO BMMC retained the ability to secrete small levels of IL-6 and TNF-α in response to antigen proposes a role for both a conventional and novel isoform in the regulation of pro-inflammatory cytokine secretion in BMMCs. It was intriguing that the loss of PKC-θ was more detrimental to mast cell effector function than the loss of PKC-α suggesting a more potent and

critical role for the former isoform with respect to such cytokines. Further preliminary studies to understand the differential involvement of PKCs in the production of the same cytokine revealed that PKC-θ KO BMMC displayed reduced PKC-α expression in comparison to WT BMMC (Figure 4.2). Since PKC-α itself is clearly functionally involved in both IL-6 and TNF-α production in response to FcεRI receptor signalling, this would perhaps help explain why PKC-θ KO BMMC suffer a much greater loss of function in response to antigen-stimulation.

Furthermore, IL-6 and TNF-α production was significantly decreased in PKC-θ KO BMMC in response to LPS, albeit less consistent for TNF-α (2 out of 3 experiments) proposing the functional involvement of PKC-θ in the production of pro-inflammatory cytokines from both FcεRI-dependent and independent signals. With respect to IL-6 secretion, this cytokine was up-regulated in PKC-α KO BMMC whilst the opposite trend was observed for PKC-θ KO BMMC. The loss of PKC-α would be expected to increase cytokine production and therefore it seems PKC-θ has the most powerful influence in mast cell effector function. Such findings encourage the idea that PKC isoform utilization can be reliant on the stimuli received with distinct isoforms having the ability to be involved in the production of multiple cytokines and thus effector functions.

4.6.3.1.2 The effect of the absence of PKC-θ on ES-62's activity in BMMC

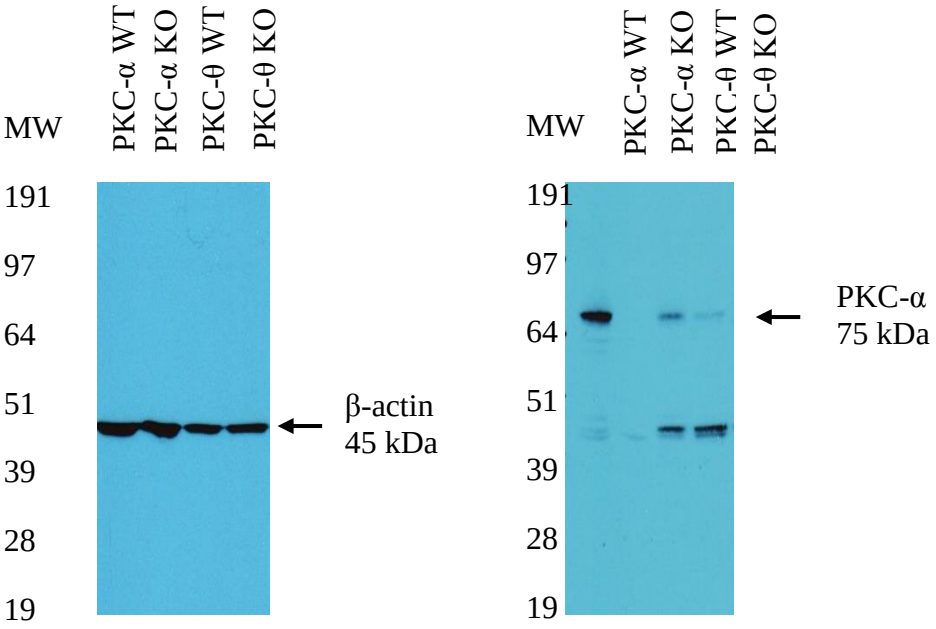
ES-62 was able to consistently significantly inhibit IL-6 production in WT BMMC. Similarly, the nematode product could also decrease IL-6 production in the KO, although this only reached significance on one occasion out of two (No XL

witnessed in the third). Additionally, a similar trend was observed in response to LPS stimulation where ES-62 significantly inhibited this cytokine in a consistent manner. However, in this case even though the molecule could decrease the response in the KO, it also failed to reach statistical significance in all experiments performed. Such results would suggest that ES-62 can still inhibit IL-6 secretion even in the absence of PKC-θ. However, it appears that ES-62 may be more potent in the WT than in the KO based on two findings. Firstly, as just indicated, ES-62-induced down-regulation did not always reach significance in the KO. Secondly, ES-62 showed greater reduction in the WT in comparison to the KO, with an average of approximately 60% inhibition in WT (for 3 experiments) and around 14% in the KO (2 experiments; the third had to be excluded due to a lack of cytokine response in the control). Thus, perhaps ES-62's activity on IL-6 production has some level of dependence on this isoform in BMMC.

With regards to FcεRI-mediated TNF-α production, ES-62 significantly inhibited this cytokine in both WT and KO cells. Additionally, identical inhibitory responses induced by ES-62 were exhibited in both WT and KO cells in response to LPS, where one experiment reached statistical significance for both cell types. Overall, these data would perhaps suggest that as witnessed in human mast cells (Melendez, et al. 2007), PKC-θ is not essential for ES-62 activity in BMMC. The slight variance witnessed between ES-62's potency in WT and KO BMMC for IL-6 production could perhaps be attributed to there being less cytokine production in the KO to begin with. Thus, any modulation by ES-62 in the KO would be minimal (and perhaps therefore not reaching significance) in comparison to WT responses. Unfortunately, due to the expression levels of PKC-θ presumably being so minimal

in BMMC, this isoform could not be detected by Western blotting and therefore the effect of ES-62 on its expression levels could not be investigated. Even so, it could be predicted based on my experiments and what is known about ES-62 PKC isoform targeting from human mast cell studies (Melendez, et al. 2007) that this nematode product would not modulate PKC-θ expression in BMMC. However, this does not take precedence over the importance of this isoform for mast cell functional responses.

Figure. 4.2 The absence of PKC-θ has a knock-on effect on PKC-α expression levels in BMMC



4.6.3.2 Macrophages

4.6.3.2.1 The effect of PKC-θ KO and ES-62 on macrophage cytokine responses

Considering the lack of differential responses that were witnessed from previous PKC isoform KO experiments, it was perhaps not surprising that the absence of PKC-θ had minimal effect across all the cytokines tested. For the majority of experiments carried out, no differential responses could be observed between WT and KO macrophages in response to LPS for production of IL-6 and IL-12 in addition to IL-10. However, experiments measuring the latter cytokine proved extremely inconsistent and therefore no conclusions should perhaps be drawn. On the other hand, TNF-α secretion appeared to be reduced in PKC-θ deficient macrophages in the majority of experiments undertaken suggesting a role for this isoform in LPS-induced TNF-α cytokine production, although this only reached statistical significance on two occasions. Such general indifferences resulted in no investigations employing ES-62. Of note, to date this novel isoform has not been shown to be expressed in macrophages (Webb, et al. 2000) and therefore this could explain the reasons why I witnessed the loss of PKC-θ having no impact on cytokine production for any of the pro-inflammatory (except for possibly TNF-α), anti-inflammatory or Th1-inducing cytokines tested.

4.6.3.3 Dendritic cells

4.6.3.3.1 The effect of PKC-θ KO and ES-62 on dendritic cell cytokine responses

The data obtained from DCs showed that this isoform had some function in the production of the majority of the cytokines tested, a feature that was not witnessed in macrophages. This highlights that PKC isoform expression can vary between cell types and that different members of PKC can have varying roles within a particular cell. It was observed in DCs that IL-6, IL-12 and TNF-α were reduced to some extent following LPS stimulation in PKC-θ KO DCs suggesting this isoform positively regulates pro-inflammatory and Th1-inducing cytokines in DCs. For example, IL-6 was down-regulated by around 30% where this reached statistical significance on two out of 3 occasions. Additionally, TNF-α secretion was significantly inhibited in 2 out of 3 experiments and IL-12 production was reduced in KO DCs with statistical significance reached in one out of 3 occasions. Although these data were not always statistically significant, there was a consistent trend for each cytokine suggestive of PKC-θ being functionally involved in the production of each cytokine in response to pro-inflammatory stimuli. However, with DCs still being able to mount some form of a cytokine response it would suggest the involvement of other isoforms in the regulation of such cytokines. It could be hypothesised that IL-6 and IL-12 are controlled by signals exclusively involving novel isoforms (including PKC-θ) or by a spectrum of other isoforms from different subgroups (excluding PKC-α; this thesis). More experiments would have to be performed to understand the roles of individual isoforms in DC functional responses. With regards to IL-10 production, this cytokine was significantly up-regulated in response to LPS stimulation in 3 out of 4 experiments in KO DCs proposing that PKC-θ negatively regulates this cytokine. Interestingly, from studies with PKC-α, this conventional isoform was thought to be a positive regulator of IL-10 production. Such data propose that IL-10 production

may be controlled by a conventional and novel isoform in order to provide a suitable level of IL-10 towards a particular inflammatory stimulus.

Furthermore, the trend witnessed between WT and KO DCs for IL-6 and IL-12 production led to pre-exposing such cells to ES-62. Although the data were rather inconsistent in the KO and often did not reach statistical significance in the WT as previously shown in DCs pre-incubated with ES-62 (Goodridge, et al. 2004), it was found that the nematode product was able to inhibit IL-6 production in WT cells and in KO cells where statistical significance was reached in one experiment for both. However, KO cells also exhibited a small increase in one experiment. Thus, overall, it could be said that ES-62 can maintain its activity even in the absence of this isoform in DCs although this conclusion is hampered by the inconsistencies witnessed in the KO and general lack of statistical significance in the WT. A more convincing result was obtained with regards to IL-12 production where ES-62 significantly decreased production of this cytokine in both WT and KO cells. Overall, these data suggest that ES-62 does not target PKC-θ in order to exhibit its modulatory activity on IL-6 and IL-12 production in DCs, though such variations witnessed must make this uncertain. With respect to IL-0 production, ES-62 increased IL-10 production consistently, albeit it in a non-significant manner. Even so, this result correlates with previous findings in DCs where pre-exposure to ES-62 caused enhanced IL-10 production *in vitro* and this was replicated *in vivo* through mice exposed to ES-62 released from osmotic pumps (Goodridge, et al. 2004). However, ES-62 caused a non-significant decrease in IL-10 production in KO DCs for all experiments undertaken. These data possibly show that the presence or absence of PKC-θ is a key factor in determining the immune response promoted by

the nematode product. Despite lacking statistical significance, the consistency of the data advocates that ES-62 may utilize PKC-θ to modulate IL-10 production in DCs in order to dampen down a pro-inflammatory response elicited by the host.

4.6.4 PKC-epsilon

4.6.4.1 Mast Cells

4.6.4.1.1 The effect of the absence of PKC-ε on mast cell development and differentiation

A similar pattern was emerging for PKC-ε WT and KO BMMC as compared to other isoforms tested with respect to the maturation and development of mast cells. Based on their expression profiles for c-kit and FcεRI, it appeared BMMC were unaffected by the absence of PKC-ε. Comparable results were obtained for WT and KO cells with both cell types sharing very similar expression of the cell surface markers, although there were marginally more cells expressing both markers in KO BMMC. Again, a smaller population of cells were witnessed for both WT and KO mast cells with such cells lacking expression of the FcεRI where WT BMMC had more cells in this immature population than the comparable KO suggesting that KO BMMC may mature faster than WT BMMC. This is also evident by the KO cell population containing more cells expressing both c-kit and FcεRI. It is possible that with KO BMMC expressing slightly more of the surface markers these cells could exhibit a different level of activation in comparison to WT BMMC that cannot be attributed to a consequence of the isoform KO. However, the difference in cytokine

responses witnessed between WT and KO (> 50% increase in KO) are so great that it can be assumed that this is not the case. Regardless, both WT and PKC-ε KO cells were identifiable as mast cells with almost similar surface marker expression levels indicating no severe alterations in phenotype for the KO BMMC. This result compares to other studies of BMMC from PKC-ε deficient mice where the absence of this isoform had no effect on the proliferative response of BMMC to IL-3 or granule content or to the expression levels of the cell surface markers c-kit and FcεRI (Lessmann, et al. 2006). Therefore the data are in agreement with others that PKC-ε is not essential for mast cell growth and development.

4.6.4.1.2 The effect of the absence of PKC-ε on mast cell functional responses

In human mast cells, much like for the other novel isoform PKC-θ, ES-62 did not degrade PKC-ε (Melendez, et al. 2007). However, as mentioned previously, it is not known if this would hold true for all species and mast cell phenotypes. Given this isoform is expressed across various human and murine mast cell phenotypes (Ozawa, et al. 1993a and 1993b; Chang, et al. 1997; Li, et al. 2005; Melendez, et al. 2007) and its likely involvement in mast cell functional responses in RBL cells due to its translocation to the plasma membrane following antigen stimulation (Ozawa, et al. 1993a and 1993b ; Chang, et al. 1997), it could be hypothesised that PKC-ε is one of the isoforms that are critical for FcεRI-receptor signalling and mast cell effector function. However, so far its role in mast cells has been rather controversial with some studies using PKC-ε deficient mice implicating a redundant role for the isoform (Lessmann, et al. 2006). My studies are in correspondence with the former findings that are suggestive of PKC-ε being somewhat functionally involved in mast cell

effector function following antigen stimulation. For example, it was observed that IL-6 production was up-regulated in KO BMMC following IgE receptor-induced activation, where results reached statistical significance in 2 out of 3 experiments. This result is in direct contrast to studies carried out by Lessmann and co-workers in BMMC where this group found the loss of PKC-ε had no impact on IL-6 or TNF-α secretion (Lessmann, et al. 2006). Unfortunately, in my experiments no TNF-α could be detected in the medium following antigen-mediated cross-linking, meaning no comparable results were obtained. As mentioned, Lessmann *et al* had shown that both PKC-ε positive and deficient BMMC exhibited similar levels of TNF-α secretion in response to receptor aggregation (Lessmann, et al. 2006) and thus it seems such cells are capable of producing measureable levels of this cytokine. This would suggest that perhaps TNF-α expression was too low to detect in the system used in the present work, with variances between studies possibly attributed to differences in experimental procedures. Nonetheless, with regards to IL-6 production, it is clear that PKC-ε may function as a negative regulator of receptor-induced signalling events leading to the production of this cytokine. Interestingly, two other groups have supported a role for PKC-ε as a negative regulator of signalling events such as calcium mobilization and/or MAP kinase activation (Chang, et al. 1997; Ozawa, et al. 1993 b). Specifically, Ozawa and colleagues had noticed that upon removal of PKC isoforms from RBL cells, antigen-mediated exocytosis was completely abrogated while on the other hand, inositol phospholipid hydrolysis was enhanced suggesting the possibility that some members of PKC may act as negative regulators of PLC activity (Ozawa, et al. 1993a). Further studies showed that upon reconstituting the permeabilized RBL cells with PKC-α and PKC-

ε, antigen-induced inositol phospholipid hydrolysis was inhibited (Ozawa, et al. 1993b). Thus, PLC activation was suppressed by around 40-50% by the two isoforms suggesting that such isoforms play a key role in its regulation following antigen-stimulation (Ozawa, et al. 1993b). Since PLC can indirectly stimulate calcium mobilization and activate PKCs through hydrolysing membrane bound PIP_2 to form IP_3 and DAG (Siraganian, 2003), it would appear that the conventional and novel isoforms regulate mast cell effector functions through mediating feedback inhibition of PLC (Ozawa, et al. 1993b). Together, such studies undertaken in RBL cells (Chang, et al. 1997; Ozawa, et al. 1993a and 1993b) in conjunction with my finding that IL-6 production is enhanced in the absence of PKC-ε, would propose that this isoform can potentially negatively regulate different stimulatory signals in order to control mast cell functional responses in response to receptor aggregation such as calcium mobilization and cytokine production. On the other hand, data obtained from WT and KO BMMC following LPS stimulation, were far less consistent where IL-6 production was significantly decreased in 2 experiments while a significant increase was witnessed in another. Although it could be said that KO BMMC exhibited an opposing trend when stimulated with LPS as opposed to FcεRI signalling whereby PKC-ε may be a positive regulator of IL-6 in LPS/TLR-4 signalling, the lack of consistency makes this uncertain. Again, although TNF-α secretion could be detected in some experiments, the data obtained were largely inconsistent and hence no conclusions could be drawn.

4.6.4.1.3 The effect of the absence of PKC-ε on ES-62's activity in BMMC

PKC-ε WT and KO BMMC were pre-exposed to ES-62 subsequent to antigen stimulation to observe ES-62's activity in the absence of PKC-ε. From such experiments, it was shown that ES-62 exhibited identical activity in the WT and KO BMMC. Specifically, it was observed that the nematode product decreased IL-6 production in all experiments where this reached statistical significance on two occasions for both PKC-ε positive and deficient mast cells. Additionally, ES-62 significantly inhibited IL-6 secretion in WT BMMC following stimulation with LPS and although not reaching statistical significance, IL-6 production was reduced in KO BMMC. Interestingly, across the 3 experiments, ES-62 reduced IL-6 production by an average of 50% in WT cells and 65% in KO cells suggesting that ES-62 may actually be more effective in the absence of this isoform. Nonetheless, together these data suggest that ES-62's modulatory activity is unaffected (or possibly enhanced) by the absence of PKC-ε. Thus, we could presume that as found in human mast cells (Melendez, et al. 2007) ES-62 does not target PKC-ε in BMMC. However, this has not yet been confirmed by western blotting analysis and therefore further experiments will have to be performed to address this theory.

4.6.4.2 Macrophages

4.6.4.2.1 The effect of PKC-ε KO and ES-62 on macrophage cytokine responses

Previously all other isoforms tested, namely two conventional isoforms (PKC-α and β) and one novel isoform (PKC-θ) have shown no difference in IL-6 and IL-12 production between WT and KO cells. Although not always statistically significant, there was an obvious trend of enhanced IL-6 secretion in PKC-ε KO macrophages in

addition to IL-12 being significantly up-regulated in response to LPS stimulation in KO macrophages by on average more than 80%. Together, these data propose that a novel isoform, PKC-ε, is functionally involved in negatively regulating a pro-inflammatory cytokine and a Th1-inducing cytokine in macrophages. Clearly, other isoforms that have not been tested in these studies are involved in positively regulating this cytokine. Interestingly, at the other end of the scale, PKC-ε consistently showed no differential responses between WT and KO BMMC in response to LPS stimulation for TNF-α. This result was the first time one of the PKC isoforms tested did not show any involvement in the production of this pro-inflammatory cytokine. Moreover, PKC-ε was additionally shown to play no role in IL-10 production as there was consistently no difference witnessed between WT and KO macrophages in response to LPS. So far, only PKC-α has shown any consistent involvement in IL-10 production from macrophages in this project where this isoform was shown to possibly negatively regulate this cytokine. Such data would propose the involvement of other isoforms not tested in this project for IL-10 production. As mentioned previously, IL-10 production has been shown to be reliant on the atypical isoform PKC-ζ in macrophages (Foey & Brennan, 2004). Therefore, it is perhaps not unexpected that the majority of isoforms tested played no role in IL-10 regulation.

Furthermore, the variance in IL-6 and IL-12 production prompted exposure of WT and KO macrophages to ES-62. Such experiments revealed that concurrent with previous knowledge (Goodridge, et al. 2001), ES-62 significantly inhibited IL-6 production in the WT cells. However, this inhibitory feature was lost in the KO as ES-62 failed to rescue macrophage LPS-induced IL-6 production suggesting that this

nematode molecule may target PKC-ε to modulate IL-6 in macrophages. However, considering such data were only witnessed in a single experiment, more experiments will have to be carried out to confirm this. On the other hand, it appears that ES-62 does not target this isoform for modulating IL-12 production. The nematode product was able to reduce WT and KO macrophage LPS-induced IL-12 production on two occasions where this reached statistical significance for both in one experiment. Thus, although not always reaching statistical significance, it is clear ES-62 had almost identical behaviour in both cell types. Previous studies in B cells showed that ES-62 modulated the expression of several PKC isoforms subsequently leading to suppression of B cell proliferation following BCR cross-linking (Deehan, et al. 1997). In particular the nematode product was found to increase the expression of PKC-ε (Deehan, et al. 1997). In mast cells however, this isoform was unaffected by ES-62 (Melendez, et al. 2007). These data clearly show that the nematode product can differentially regulate PKC isoforms in many cell types with some isoforms being targeted by ES-62 in a manner specific to each cell type in order to ultimately modulate effector function. Thus, it may be possible that ES-62 could also target PKC-ε in macrophages to exert at least some of its effects. However, as yet, it is not known if or how this molecule modulates this isoform in macrophages – it could be degraded like other isoforms such as PKC-α in mast cells (Melendez, et al. 2007) or up-regulated as witnessed for PKC-ε and PKC-γ in B cells (Deehan, et al. 1997). Further experiments would have to be carried out to understand exactly how ES-62 modulates PKC-ε activity in macrophages.

4.7 Conclusions

4.7.1 Mast cells

4.7.1.1 The importance of individual PKC isoforms for mast cell function

It is clear from many studies that several PKC isoforms are expressed in mast cells (Li, et al. 2005; Ozawa, et al. 1993a, Liu, et al. 2001; Melendez, et al. 2007), implicating their involvement in mast cell signalling pathways and effector functions. Although several of these studies have shown essential requirement for PKC activation and indeed specific isoforms in FcεRI-mediated mast cell effector functions such as degranulation and cytokine production (Li, et al. 2005, Nechushtan, et al. 2001; Melendez, et al. 2007), the involvement of several isoforms has similarly been shown to be controversial. For example, PKC-δ has been proposed to positively regulate degranulation in response to antigen in RBL cells (Ozawa, et al. 1993a and 1993b) whereas in BMMC, the use of PKC-δ deficient mice showed a role for this isoform as a negative regulator of receptor-mediated degranulation (Leitges, et al. 2002). Such controversy highlights the requirement to further dissect the relevant roles of PKC isoforms in mast cell functions. Using PKC-deficient BMMC, I found that two conventional (PKC-α and β) and two novel isoforms (PKC-ε and θ) played a role in positively or negatively regulating mast cell functions in response to both FcεRI and/or LPS-mediated stimulation. Interestingly, one conventional and one novel isoform were revealed to possibly work in partnership as positive regulators (PKC-α and θ) or negative regulators (PKC-β and ε) of mast cell cytokine production following antigen stimulation. Thus, both pro-inflammatory cytokines IL-6 and TNF-α were reduced in PKC-α and PKC-θ KO BMMC in response to antigen

stimulation, with the loss of PKC-θ revealed to have a more potent effect on mast cell cytokine production than the absence of PKC-α. Even though the absence of one of these isoforms proved to render mast cells less responsive to FcεRI signalling, minimal cytokine responses to antigen could still be observed for both KO's. Nonetheless, PKC-θ is clearly a critical isoform for fully functional mast cell responses as loss of this isoform resulted in 80-90% reduction of pro-inflammatory cytokines. Additionally, I discovered that PKC isoforms may have the ability to regulate one another with the absence of one isoform impacting on the levels of another. Preliminary data revealed that PKC-α levels were reduced in the absence of PKC-θ. This result may bring some insight as to the reasons why there was a greater loss of function witnessed in PKC-θ KO BMMC. Furthermore, the up-regulation of IL-6 witnessed in response to antigen stimulation for PKC-β and PKC-ε KO BMMC propose these two isoforms as negative regulators of this pro-inflammatory cytokine in mast cells. Again, there was around 50-60% enhanced production of IL-6 in response to antigen for both PKC-β and PKC-ε KO suggesting these two isoforms are equally involved in different stimulatory signals that control this cytokine and cannot simply be replaced by another isoform in their absence. It was interesting to note that the effect of the absence of a particular isoform was replicated in responses to LPS for the majority of isoforms tested where PKC knockout BMMC displayed identical reductions or increases in cytokine production in response to both stimuli. PKC-α was the only isoform that exhibited differential responses depending on the stimuli received with LPS-induced PKC-α deficient BMMC exerting an increase in IL-6 cytokine production.

Together these data suggest that mast cell responses are controlled by multiple PKC isoforms, with the utilization of each isoform depending on the stimulus received. Additionally, some isoforms appear to be affected by the presence or absence of others. This could mean that simply knocking out one isoform may not be enough to render mast cells completely unresponsive to subsequent stimuli. However, it cannot be ignored that specific mast cell functions could be down-regulated by inhibiting or knocking out specific PKC isoforms which in turn could allow for an alternative approach to controlling aberrant inflammatory immune responses such as those seen in allergic disorders. Nonetheless, further dissection of the relevant roles of each PKC isoforms is required with the consideration of how the absence of each individual isoforms impacts on all the others and how overall this may affect mast cell responses. The ability of KO BMMC to adapt to the loss of one isoform by either increasing (as witnessed for PKC-ε up-regulated in PKC-α KO BMMC) or decreasing (as witnessed in PKC-θ KO having less PKC-α expression) the expression of others might help explain the discrepancies that have been observed between PKC knockout studies and studies where PKC overexpression or knockdown studies are used.

4.7.1.2 The importance of PKC isoforms for ES-62 function

It has been shown that ES-62 targets PKC-α for degradation in human mast cells (Melendez, et al. 2007) and subtypes of murine mast cells such as BMMC (Ball, et al. 2013; this thesis) and PDMC (Ball, et al. 2013) with such degradation resulting in inhibition of mast cell functional responses (Melendez, et al. 2007; Ball, et al. 2013).

In addition, in human mast cells ES-62 was shown to target many other isoforms for degradation (Melendez, et al. 207). Thus, it was rather surprising that generally in my experiments, ES-62's action did not seem to be altered in the absence of PKC isosforms. However, ES-62 did not target PKC-θ or PKC-ε in human mast cells (Melendez, et al. 2007) and therefore this result may not be unexpected for these isoforms. Nevertheless, PKC-α and PKC-β were the two isoforms most strongly modulated by the nematode product (Melendez, et al. 2007) and therefore some modification to ES-62's action in the absence of such isoforms might be expected. However, ES-62 was able to inhibit both IL-6 and TNF-α production in PKC-α deficient BMMC and, although less consistent, in PKC-β KO BMMC. Such data would imply that ES-62 does not target these isoforms in BMMC. However, although I could not identify PKC-β in mast cells it was obvious from the western blotting studies that ES-62 degraded PKC-α in BMMC. It could be hypothesised that ES-62 does in fact target these isoforms but that in BMMC, the nematode product additionally targets other signals in order to inhibit mast cell function and that any effect of loss of PKC-α is somewhat compensated for. Our research group has already revealed that in BMMC, ES-62 targets MyD88 and another PKC isoform, PKC-δ (Ball, et al. 2013) in order to help supress the high levels of cytokines exhibited by BMMC (Ball, et al. 2013). Additionally, in the present study, it appears ES-62 may target another novel PKC isoform, PKC-θ, in order to exhibit its inhibitory function in BMMC. As mentioned previously, the lack of differential responses exhibited by ES-62 in WT and KO BMMC for IL-6 and TNF-α production could be attributed to the up-regulation of PKC-ε (negative regulator) in the PKC-α KO and not by the nematode product itself. Therefore perhaps until it can be shown

firstly what other isoforms are targeted by ES-62 in normal BMMC in addition to how the loss of each isoform affects the levels of all the other isoforms present, we cannot be certain of ES-62's activity in PKC KO BMMC.

4.7.2 The importance of individual PKC isoforms in other immune system cells

4.7.2 Macrophages

4.7.2.1 The importance of individual PKC isoforms for macrophage function

IL-6 and IL-12 production was demonstrated to be modified by only one isoform – PKC-ε. Secretion of this cytokine was up-regulated in PKC-ε KO macrophages suggesting that this novel isoform negatively regulates both a pro-inflammatory and Th1-inducing cytokine important for macrophage effector function. IL-12 production was enhanced by greater than 80% illustrating the potency of this isoform in controlling the production of this cytokine. This was not unexpected as so far little evidence has accumulated for conventional isoforms being functionally involved in the production of either cytokine. On the other hand, it was shown that TNF-α production was controlled by at least three isoforms. The two conventional isoforms PKC-α and PKC-β were revealed as negative regulators whereas PKC-θ was revealed as a positive regulator of this cytokine. This may suggest that there can be cross-talk between two subgroups of PKC with one being able to regulate another in order to control a specific signalling pathway. Indeed, it has been shown that upon activation, the conventional isoform PKC-α can inhibit the functional responses of the atypical PKC-ζ (Condrelli, et al. 2001). So far, TNF-α was the only cytokine that

the novel isoform PKC-θ was shown to play a functional role in regulating in macrophages. Furthermore, only one isoform was additionally shown to be utilized in the control of IL-10 production. Thus, PKC-α KO macrophages displayed enhanced IL-10 production in response to LPS stimulation. As mentioned, so far PKC-ζ has been revealed to be the isoform responsible for IL-10 secretion in macrophages (Foey & Brennan, 2004) therefore together these data suggest that IL-10 production may be negatively regulated by a conventional isoform and positively regulated by an atypical isoform since neither of the novel isoforms tested appeared to be involved. Although my studies may have given some insight, little is known about the roles of the various PKC isoforms in macrophage function.

4.7.2.2 The importance of PKC isoforms for ES-62 function in macrophages

ES-62 may have stronger modulatory ability in PKC-α WT macrophages as the nematode product could consistently inhibit TNF-α production yet the results from KO macrophages were suggestive of ES-62 being less potent. This may suggest that as with mast cells (Melendez, et al. 2007) and B cells (Deehan, et al. 1997) ES-62 may target this isoform in order to modulate macrophage functional responses. Conversely, PKC-β deficient macrophages were as responsive to ES-62-induced inhibition as WT macrophages suggesting that ES-62 does not target this isoform for its activity in macrophages. Moreover, for IL-10 production, the absence of this isoform had no impact on ES-62 activity. This result is perhaps not surprising as ES-62 itself is unable to enhance IL-10 production in macrophages (Goodridge, et al. 2001). ES-62 was however shown to possibly target PKC-ε in macrophages to

modulate the secretion of the pro-inflammatory cytokine IL-6 as ES-62-induced inhibition was lost in the KO. This did not appear to be the case for IL-12 production, however.

4.7.3 Dendritic cells

4.7.3.1 The importance of individual PKC isoforms for dendritic cell function

In DCs, PKC-α was revealed to potentially regulate both a pro and anti-inflammatory cytokine. TNF-α production was enhanced in the absence of this isoform suggesting its role as a negative regulator for this cytokine. On the other hand, the loss of PKC-α resulted in the down-regulation of IL-10 production suggesting this isoform may positively regulate this cytokine in DCs. Together, these data may propose that through PKC-α, DC effector function may be controlled to prevent development of excessive inflammatory responses. Furthermore, both pro-inflammatory cytokines IL-6 and TNF-α in addition to the Th1-promoting cytokine IL-12 were all found to be positively regulated in part by the novel isoform PKC-θ. Additionally, IL-10 production appeared to be negatively controlled by this isoform. Together, these data suggest this isoform is critically involved in DC functional responses and is not redundant in this system. The idea that a pro-inflammatory cytokine, TNF-α, and the anti-inflammatory cytokine IL-10 are controlled by one conventional and one novel isoform may suggest that the differential utilization of an isoform can drive the immune response to be of either a pro- or anti-inflammatory nature in dendritic cells.

4.7.3.2 The importance of PKC isoforms for ES-62 function in dendritic cells

Little conclusion could be drawn from investigations employing ES-62 due to the nematode product behaving rather oddly in some experiments. However, from what could be established, the absence of PKC-α had no effect on ES-62-mediated TNF-α inhibition suggesting this isoform is not an ES-62 target in DCs. Similarly, ES-62 does not appear to target PKC-θ in order to decrease IL-6 and IL-12 production, which might be considered surprising since these two cytokines are clearly positively regulated by this isoform. However, it was shown that the nematode molecule may target PKC-θ to modulate IL-10 production from DCs as differential responses were witnessed in WT and KO DC's when pre-exposed to ES-62 where IL-10 production was elevated in the WT and reduced in the KO.

4.8 Summary

The following points can be concluded from the study:

- At least two novel and two conventional isoforms cooperate to control mast cell pro-inflammatory cytokine production following antigen stimulation. PKC-θ, and perhaps to a lesser extent PKC-α, are clearly important positive regulators of IL-6 and TNF-α production with PKC-β and PKC-ε implicated as negative regulators of IL-6 production.

- In response to LPS/TLR-4 signalling, once more PKC-θ is critically involved in the production of both pro-inflammatory cytokines with PKC-β negatively regulating such cytokines. Interestingly, PKC-α has dual roles in response to LPS with this isoform shown to be a negative regulator of IL-6 production and a positive regulator of TNF-α production.

- The absence of any PKC isoform tested appeared to have no "total" effect on ES-62 activity. However, the loss of PKC-θ may suppress ES-62's immunomodulatory ability to a degree.

- PKC-α and PKC-θ acting as positive regulators of IL-6 and TNF-α production and PKC-β acting as a negative regulator of IL-6 production following antigen-mediated activation is a unique feature to mast cells in comparison to macrophages. One isoform, PKC-ε was shown to positively regulate IL-6 in both cell types. Similar to previous findings in human mast

cell studies, PKC-α may be a target of ES-62 in macrophages as this molecule seems more potent in the presence of this isoform when measuring TNF-□. On the other hand, opposing to the mast cell data, ES-62 lost all modulatory activity in the absence of PKC-ε suggesting ES-62 targets this isoform to exert its function in macrophages

- Interestingly, PKC-α was the only isoform to show differential responses between mast cells and DCs with this isoform positively regulating antigen-mediated IL-6 and TNF-α cytokine production in the former and negatively regulating these pro-inflammatory cytokines following activation by LPS in the latter.

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