PEST-DOMAIN-ENRICHED TYROSINE PHOSPHATASE AND GLUCOCORTICOIDS AS REGULATORS OF MAST CELL SIGNALLING

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ZUSAMMENFASSUNG

Die PEST-domänenreiche Tyrosinphosphatase PEP ist eine cytoplasmatische Tyrosin-Phosphatase, die die Funktion von Immunzellen einschließlich der von Mastzellen reguliert. Mastzellen sind wichtige Effektorzellen, die die allergische Reaktion als Antwort auf ein Antigen auslösen. Die antigenvermittelte Aktivierung von Mastzellen führt zu der Freisetzung von gespeicherten Entzündungsmediatoren, die als Degranulation bezeichnet wird. Außerdem initiiert die Mastzellaktivierung die de novo Synthese und Freisetzung von Lipid-Derivaten und die Synthese und Freisetzung von Zytokinen/Chemokinen. Frühere Studien zeigen, dass PEP eine positive regulatorische Rolle in der Mastzelldegranulation einnimmt, da die Degranulation von aus Knochenmark gewonnenen Mastzellen (BMMCs) von PEP-/- Mäusen, verglichen mit PEP+/+ BMMCs, geschwächt ist. Es war allerdings bislang unbekannt ob PEP auch noch weitere Mastzellfunktionen wie die Lipidmediator-Synthese und Genexpression von Zytokinen/Chemokinen beeinflusst. Weiterhin wird PEP durch Dexamethason, einem steroidalen entzündungshemmenden Glucocorticoid, das zur Behandlung von Allergien eingesetzt wird, hochreguliert. Die Rolle von PEP bei der antiallergischen Wirkung von Glucocorticoiden (GC) war jedoch noch nicht bekannt.

Unter Verwendung von BMMCs von PEP+/+ und PEP-/- Mäusen in RNA-seq Experimenten wurde gezeigt, dass aktivierte PEP-/- BMMCs eine herabgesetzte Zytokin/Chemokin-Genexpression (TNFα, IL13, CSF2) aufweisen im Vergleich zu PEP+/+ BMMCs. Dies bedeutet, dass PEP wichtig für die Zytokin/Chemokin-Synthese in Mastzellen ist. Des Weiteren war die GC-vermittelte Hemmung der Expression von COX-2, einem

proinflammatorischen Gen, welches die Lipidmediator-Synthese fördert, in PEP-/- BMMCs beeinträchtigt. Dies bedeutet, dass PEP für die GC-induzierte negative Regulation der Lipidmediator-Synthese nötig ist. Ähnliche Effekte wie sie in PEP-/- BMMCs beobachtet wurden, konnten in PEP+/+ BMMCs nach Behandlung mit L75NO4, einem chemischen Inhibitor, der die PEP-Aktivität unterdrückt, beobachtet werden. Dies zeigt, dass PEP direkt an der Regulation der Zytokin/Chemokin- und COX-2 Expression beteiligt ist. Zusammengefasst: PEP ist ein positiver Regulator der Zytokin/Chemokin- und COX-2 Expression und ist ebenso für die negative Regulation der COX-2 Expression durch GC nötig.

ABSTRACT

PEST-domain-enriched tyrosine phosphatase (PEP) is a cytoplasmic protein tyrosine phosphatase that regulates immune cell functions, including mast cell functions. Mast cells are the key effector cells that initiate allergic reactions in response to antigen. Antigen-induced activation of mast cells causes the release of preformed inflammatory mediators in a process termed degranulation. There is also the *de novo* synthesis and release of lipid derived mediators, and the synthesis and release of cytokines/chemokines. In previous studies, PEP was shown to be a positive regulator of mast cell degranulation, as PEP-/- bone marrow derived mast cells (BMMCs) showed reduced mast cell degranulation when compared to PEP+/+ BMMCs. However, it is not known whether PEP affects other mast cell functions such as lipid mediator synthesis and cytokine/chemokine gene expression. Also, PEP is upregulated in BMMCs by dexamethasone, a steroidal antiinflammatory glucocorticoid used in the management of allergy, but the role of PEP in the antiallergic action of glucocorticoids (GCs) is not established. Using BMMCs from PEP+/+ and PEP-/- mice in RNA-seq experiments, it was shown that activated PEP-/- BMMCs have reduced cytokine/chemokine gene (TNFα, IL13, CSF2) expression compared to the PEP+/+ BMMCs, meaning that PEP is important for cytokine/chemokine synthesis in mast cells. It was also found that the ability of GCs to negatively regulate the expression of PTGS2/COX-2, a proinflammatory gene that promotes lipid mediator synthesis was inhibited in PEP-/- BMMCs, showing that PEP is needed for glucocorticoid-induced negative regulation of lipid mediator synthesis. Treatment of PEP+/+ BMMCs with L75NO4, a chemical inhibitor of

PEP activity mirrored the effects observed in PEP-/- BMMCs, which means that PEP is directly involved in the regulation of cytokine/chemokine and PTGS2/COX-2 expression. In summary, PEP is a positive regulator of cytokine/chemokine gene expression and is required for negative regulation of PTGS2/COX-2 expression by GCs.

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ABBREVIATIONS

ACK	Ammonium-Chloride-Potassium
AUC	Area under the Curve
bp	Base pair
BMMC	Bone marrow derived mast cell
BMMCs	Bone marrow derived mast cells
cDNA	Complementary DNA
CO_2	Carbon dioxide
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
CSF2/GM-CSF	Colony stimulating factor 2/Granulocyte-macrophage colony stimulating factor
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
FBS	Fetal bovine serum
FceRI	High affinity IgE receptor
GC	Glucocorticoid
GCs	Glucocorticoids
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GREs	Glucocorticoid response elements
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
h	Hour
HEPES	N-2hydroxyethilpiperasine-n′-2-ethansulfonic acid
HRP	Horseradish peroxidase
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
IL13	Interleukin 13
IMDM	Iscove's Modified Dulbecco's Medium

ITAM	Immunoreceptor tyrosine-based activation motif
MAPK	Mitogen-activated protein kinase
MAPKs	Mitogen-activated protein kinases
ml	Millilitre
min	Minutes
mM	Millimolar
mRNA	Messenger RNA
ng	Nanogramme
nGRE	Negative glucocorticoid response element
nGREs	Negative glucocorticoid response elements
O_2	Oxygen
OD	Optical Density
O/N	Over night
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEP	PEST-Domain-Enriched Tyrosine Phosphatase
pM	Picomolar
PTGDS	Prostaglandin D2 synthase
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
+RT	With reverse transcriptase
-RT	Without reverse transcriptase
s	Seconds
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
TAE	Tris-acetate-EDTA
TBST	Tris- buffered saline with Tween-20
TE	Tris/EDTA
TEMED	Tetramethylethylenediamine
Th1	T helper 1

Th2	T helper 2
Tris	Tris-(hydroxymethyl)-aminomethane
TNFα	Tumour necrosis factor alpha
U	Unit
µg	Microgramme
µl	Microlitre
µM	Micromolar
V	Volt
WT	Wild-type
w/v	Weight in volume

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Mast cells are immune cells discovered more than a century ago by the researcher physician Paul Erlich as cells containing cytoplasmic granules that were stained with metachromatic dyes (Gurish and Boyce 2006; Beaven 2009; Shea-Donohue *et al.* 2010; Ghably *et al.* 2015). These cells release a number of substances/mediators in response to extracellular stimuli (Metcalfe *et al.* 2009; Urb and Sheppard 2012; Sibilano *et al.* 2014). Release of mediators protects the body against harm by invading pathogens and other substances (Theoharides *et al.* 2007; DeBruin *et al.* 2015), but the mediators may also exert harmful effects on the body and cause disease (Rao and Brown 2008; Shea-Donohue *et al.* 2010; DeBruin *et al.* 2015). One of the most serious mast cell related diseases is anaphylaxis, which is a severe and life-threatening form of allergy (Theoharides *et al.* 2007; Metcalfe *et al.* 2009; Kalesnikoff and Galli 2010).

Currently, the most potent class of drugs for anaphylaxis are the steroidal antiinflammatory glucocorticoids (GCs) (Lieberman *et al.* ; Simons *et al.* 2011), but their use is fraught with serious side effects including type 2 diabetes, growth retardation, hypertension, osteoporosis and impaired wound healing (Schacke *et al.* 2002; Huscher *et al.* 2009). To deveop GCs with fewer side effects there is the need to understand the mechanisms of GC action. One of the mechanisms by which GCs are known to exhibit antiinflammatory effects is the upregulation of the activity of phosphatases with antiinflammatory functions such as dual specific phosphatase 1 (Dusp 1) (Kassel *et al.* 2001; Lasa *et al.* 2002). Another phosphatase, pest-domain

enriched tyrosine phosphatase (PEP), the subject of this investigation, was more recently found to be upregulated by GCs in mast cells (Maier *et al.* 2007). It is possible that PEP may be a target for GC action, hence its role in mast cell function and glucocorticoid action merits further investigations.

1.2 MAST CELL BIOLOGY

Mast cells (MCs) are immune cells that develop from bone marrow progenitor cells which express both CD34 (cluster of differentiation 34) and the stem cell factor (SCF) receptor c-KIT (CD117) (Rottem *et al.* 1994; Gilfillan *et al.* 2011). These progenitor cells are released into circulation, from where they migrate into peripheral tissues, where they mature in response to local factors such as cytokines found in tissues (Metcalfe *et al.* 1997; Okayama and Kawakami 2006; Dahlin and Hallgren 2015).

The matured mast cells found in peripheral tissues exhibit considerable degree of heterogeneity based on their granule content and tissue of localization. These tissue resident mast cells can therefore be classified into two major phenotypes: connective tissue mast cells (CTMC) and mucosal mast cells (MMC) (Miller and Pemberton 2002; Gurish and Boyce 2006; Jensen *et al.* 2006). CTMC reside mainly in the skin, peritoneum and intestinal tract sub-mucosa and characteristically express mast cell proteases (mMCP) mMCP4, mMCP5, and mMCP6, while MMC located mainly in the lung and gastrointestinal tract epithelia express mMCP1 and mMCP2 (Miller and Pemberton 2002; Gurish and Boyce 2006; Jensen *et al.* 2006).

The growth and survival of mast cells is mainly dependent on SCF (stem cell factor/c-Kit ligand) (Iemura *et al.* 1994; Orinska *et al.* 2010), but other growth factors such as the cytokine interleukin-3 (IL-3) are also important for mast cell differentiation and survival (Razin *et al.* 1984; Westerberg *et al.* 2015).

Currently *in vitro* mouse models are the main stay for mast cell research (Reber and Frossard 2014). Mouse mast cells generated for *in vitro* studies are mostly derived from bone marrow stem cells cultivated in growth medium containing SCF and IL-3. When matured, the bone marrow derived mast cells (BMMCs) release inflammatory mediators upon activation with various stimuli (Radinger *et al.* 2015).

1.3 MAST CELL ACTIVATION

Mast cells are activated by extracellular stimuli via cell surface receptors (Reber and Frossard 2014). Key mast cell receptors whose activation contribute to health and disease include the high-affinity immunoglobulin E (IgE) receptor (FcεRI) (binds antigens) , the c-Kit receptor (binds stem cell factor) and the toll-like receptors (TLRs) (binds bacterial and viral products) (Supajatura *et al.* 2001; Reber *et al.* 2006; Galli and Tsai 2012). They also express receptors that respond to venom peptides (Akahoshi *et al.* 2011) and receptors that are activated by complement proteins (Schafer *et al.* 2013). However, the FcεRI which binds antigen to mediate allergic response, is the most well studied signalling pathway in mast cells (Reber *et al.* 2014).

1.3.1 FcεRI SIGNALLING

FcεRI is a multichain immune recognition receptor (MMIR) that comprises of an α subunit, membrane spanning β subunit, and two disulfide-bonded γ subunits (αβγ2). The α subunit binds IgE, while the β and γ subunits are involved in signal transduction (Metcalfe *et al.* 1997; Garman *et al.* 1998). Prio to FceRI activation, the receptor forms an IgE-FcεRI complex with circulating IgE. Crosslinking of multiple IgE-FcεRI complex occurs after an antigen/allergen binds to the IgE of the complex, leading to mast cell

activation (Blank 2011; Sibilano *et al.* 2014). Mechanistically, receptor cross-linking induces phosphorylation of receptor activation units called immunoreceptor tyrosine-based activation motifs (ITAM) on the β and γ subunits, by tyrosine kinases (notably LYN, FYN, SYK) (Gilfillan and Tkaczyk 2006; Rivera and Gilfillan 2006). This leads to the activation of several adaptor molecules that form molecular scaffolds which organize the downstream signalling events into larger signalling complexes (Gilfillan and Tkaczyk 2006; Rivera and Gilfillan 2006) (Figure 1.1). These downstream signalling complexes are broadly classified into 'principal/primary' and 'complementary/amplification' cascades (Gilfillan and Tkaczyk 2006).

1.3.1.1 The 'Principal/Primary' Cascade

Following cross-linking of FcεRI, the FcεRI proximal kinase LYN becomes auto phosphorylated (Figure 1.1). LYN in turn phosphorylates the ITAMs on the β and γ subunits of the receptor. The phosphorylated ITAMs then recruit and activate SYK (spleen tyrosine kinase) (Kihara and Siraganian 1994). SYK activates key transmembrane adaptor molecules LAT 1/2 (linker for activation of T cells 1/2), leading to the activation of other adaptor molecules that form part of the LAT molecular scaffold such as GRB2 (growth-factor receptor-bound protein 2), GADS (GRB2-related adaptor protein), SLP76 (SH2-domain-containing leukocyte protein of 76 kDa), including the exchange factors VAV and SOS (son of sevenless homologue) (Turner and Kinet 1999; Rivera and Gilfillan 2006). There is also activation of the kinase BTK (Bruton`s tyrosine kinase) whose interaction with the LAT molecular scaffold and the signalling enzyme PLCγ, results in the activation of PLCγ. Activated PLCγ hydrolyses plasma membrane associated lipid, PIP2 [phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2)] to IP3 (inositol -1, 4, 5 –trisphosphate) and DAG

(diacylglycerol). IP3 acts on its receptors on the ER (endoplasmic reticulum) to activate Ca^{2+} release from intracellular stores in the ER. This leads to the activation of the calcium sensor STIM1 (stromal interaction molecule 1) on the ER membrane. STIM1 interacts with plasma membrane Ca^{2+} channel proteins to trigger Ca^{2+} influx through the plasma membrane into the cytoplasm (Gilfillan and Tkaczyk 2006; Kraft and Kinet 2007). On the other hand, DAG activates the signalling enzyme PKC (protein kinase C). Ca^{2+} and PKC induce mast cell degranulation (Gilfillan and Tkaczyk 2006; Kraft and Kinet 2007; Metcalfe *et al.* 2009). Elevation of cytosolic Ca^{2+} levels contributes to the activation of transcription factors such as NFAT (Nuclear factor of activated T-cells). Alternatively, VAV and SOS activate the GTPase RAS leading to the activation of the mitogen activated protein kinase (MAPK) pathway through successive downstream phosphorylation of MAPKKK (MAPKK kinases), MAPKK (MAPK kinases) and the MAPKs (MAP Kinses) ERK1/2 (extracellular signal–regulated kinase 1/2), p38 and JNK (Jun amino-terminal kinase) (Gilfillan and Tkaczyk 2006; Kraft and Kinet 2007). The MAPKs activate transcription factors belonging to the AP1 (Activator protein 1), NFκB (Nuclear factor κB) and NFAT families, to regulate gene expression (Gilfillan and Tkaczyk 2006; Kraft and Kinet 2007). Changes in signalling events in the 'principal cascade' induces the most profound effects on mast cell effector functions such as degranulation, lipid mediator synthesis and release and cytokine/chemokine production (Gilfillan and Tkaczyk 2006; Kraft and Kinet 2007).

1.3.1.2 The 'Complementary/Amplification' Cascade

The complementary pathway involves the regulation of mast cell signalling via the interaction of the kinases SYK and FYN with adaptor molecule

complex that involves GAB2 (growth-factor receptor-bound protein 2 (GRB2)-associated binding protein 2) and the kinase PI3K (phosphatidylinositol 3-kinase), organized by the transmembrane adaptor molecule LAT2 (Figure 1.1). Activated PI3K phosphorylates plasma membrane associated phosphoinositide PIP2 to PIP3 (phosphatidylinositol 3,4,5-triphosphate) resulting in increased cytosolic Ca^{2+} mobilisation through BTK-dependent phosphorylation of PLCγ . Alternatively, PI3K can also increase Ca^{2+} mobilisation via recruitment of PDK1 (phosphoinositide dependent protein kinase 1), which activates PLD (phospholipase D). PLD activates SPHK (sphingosine kinase) to induce the break down of sphingosine to form S1P (sphingosine 1-phosphate), which triggers Ca^{2+} entry through the plasma membrane (Gilfillan and Tkaczyk 2006; Kraft and Kinet 2007). Also, PI3K activation leads to PDK1 activation of the protein kinase AKT. AKT participates in the regulation of various transcription factors (Gilfillan and Tkaczyk 2006; Kraft and Kinet 2007).

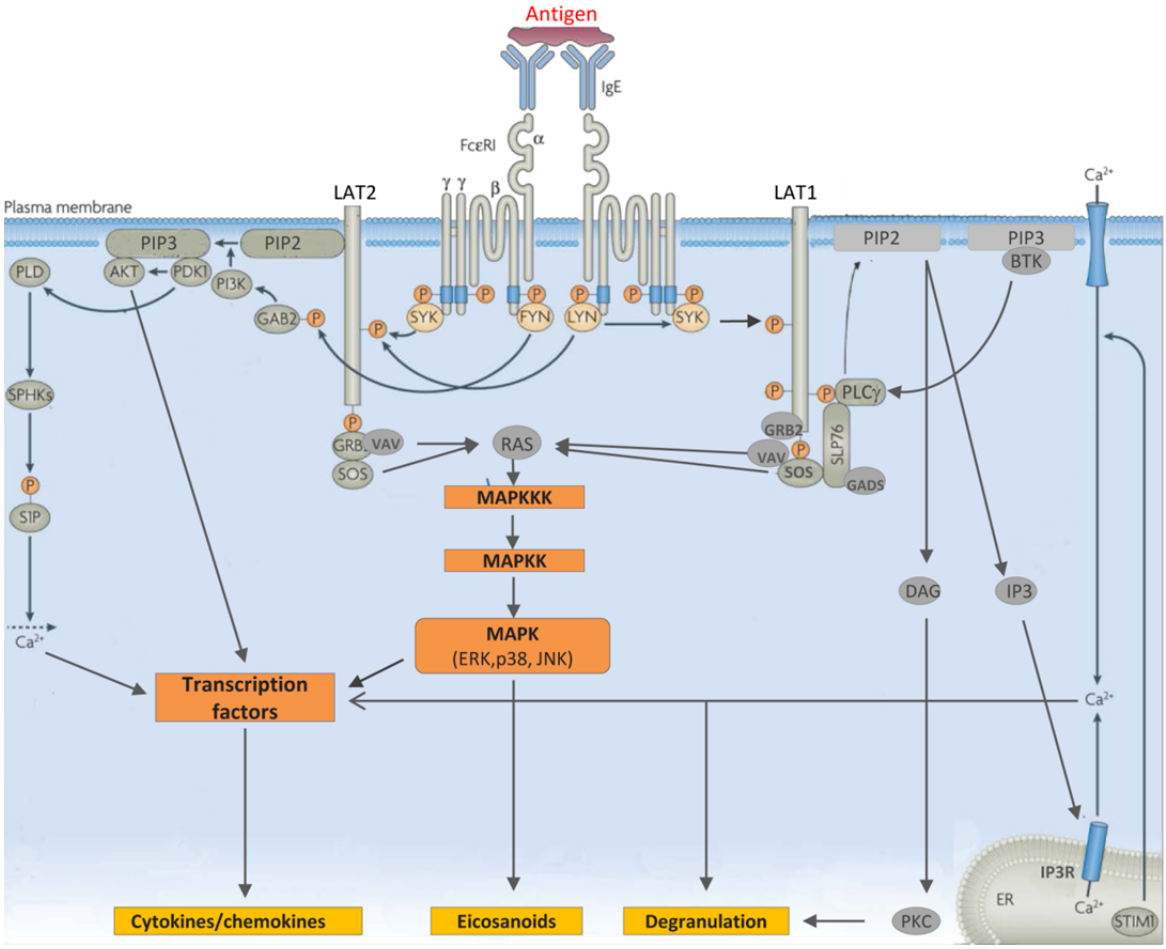


Figure 1.1 Model of FcεRI signalling. In the primary/principal cascade, activated LYN phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) in the β- and γ-chains of the high-affinity Fc receptor for IgE (FcεRI), which results in association of SYK with the γ-chains and subsequent SYK activation. SYK phosphorylates LAT1/2 (linker for activation of T cells 1/2), which recruits GADS (growth-factor-receptor-bound protein 2 (GRB2)-related adaptor protein), SLP76 (SH2-domain-containing leukocyte protein of 76 kDa), VAV and PLCγ (phospholipase Cγ) to form a LAT scaffold complex. PLCγ is activated through the LAT complex via BTK (Bruton's tyrosine kinase) mediated phosphorylation of PLCγ. Activated PLCγ produces IP3 (inositol-1,4,5-trisphosphate) and DAG (diacylglycerol) from PIP_2 [phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2)]. IP3 activates its receptor (IP3R) on the ER (endoplasmic reticulum) to induce Ca^{2+} (calcium) release from the ER stores. ER Ca^{2+} stores depletion leads to STIM1 (stromal interaction molecule 1)-guided opening of plasma membrane Ca^{2+} channels, which causes the influx of extracellular Ca^{2+}. DAG activates PKC (protein kinase C) which collectively with Ca^{2+} mobilization activate degranulation. In the complementary/amplification cascade, FYN and SYK activation induce a signalling complex with GAB2 (growth-factor receptor-bound protein 2 (GRB2)-associated binding protein 2) and the kinase PI3K (phosphatidylinositol 3-kinase), organized by the transmembrane adaptor molecule LAT2. PI3K activation leads to Ca^{2+} mobilisation via BTK-dependent phosphorylation of PLCγ and membrane recruitment of PDK1 (3-phosphoinositide-dependent protein kinase 1) to activate PLD (phospholipase D) which activates SPHK (sphingosine kinase) to induce the break down of sphingosine to form S1P (sphingosine 1-phosphate) leading to Ca^{2+} entry through the plasma membrane. PI3K activation also leads to PDK1 mediated activation of the protein kinase AKT. GRB2 and SOS (son-of-sevenless homologue) are associated with both the primary and complementary cascades and lead to RAS activation, which then leads to the activation of MAPK (mitogen-activated protein kinase) cascade that contributes to cytokine/chemokine and eicosanoid production. Modified from Kraft and Kinet 2007.

1.4 MAST CELL MEDIATORS

Mast cells release a repertoire of mediators when activated (Radinger *et al.* 2015). The release of mediators is generally a consequence of FcεRI activation by antigen/allergen, although other stimuli such as viral and bacteria particles can also contribute to mast cell mediator release (Gilfillan and Tkaczyk 2006).

The mediators released by activated mast cells belong to a wide range of chemical and biological classes, but they can be classified as (1) preformed/granule mediators which are stored in cytoplasmic granules, the contents of which are released by the process of exocytosis; (2) lipid-derived mediators such as the eicosanoids (prostaglandins, prostacyclin, leukotrienes, thromboxanes) which are synthesized *de novo* and (3) cytokines/chemokines and growth factors generated from induction of gene expression (Da Silva *et al.* 2006; Kalesnikoff and Galli 2010; Reber and Frossard 2014).

1.4.1 PREFORMED/GRANULE MEDIATORS

Mast cell preformed mediators are released within seconds to minutes after cell surface receptor activation (Metcalfe *et al.* 2009; Urb and Sheppard 2012). These mediators include proteases (tryptase, chymase) and biogenic amines (histamine and serotonin), lysosomal enzymes (β-hexosaminidase), and some cytokines such as tmour necrosis factor alpha (TNFα)(Pejler *et al.* 2007; Kunder *et al.* 2009; Wernersson and Pejler 2014). They are stored in granular structures in the cytoplasm and released in a process termed degranulation (Nishida *et al.* 2005; Yamasaki and Saito 2005). These mediators generally induce vasodilation leading to hypotension, and hypothermia that is associated with severe allergic reactions (Kemp and Lockey 2002) such as anaphylaxis.

1.4.2 LIPID-DERIVED MEDIATORS

In addition to preformed mediators, mast cell activation induces *de novo* synthesis and release of lipid-derived mediators (Firestein *et al.* 2015; Radinger *et al.* 2015). The key mast cell lipid derived mediators, PGD_2 (prostaglandin D2), leukotrienes LTC_4 (leukotrienes C4) and LTB_4 are products derived from arachidonic acid (AA) metabolism (Firestein *et al.* 2015; Radinger *et al.* 2015). Upon activation of mast cells, arachidonic acid (AA) is released from membrane phospholipids by the catalytic activities of PLA_2 (phospholipase A2)(Clark *et al.* 1991; Boyce 2007). The AA released is metabolised by COX-1 and COX-2 (cyclooxygenases 1 and 2) to form an unstable intermediate, PGG_2 (prostaglandin G2), which is rapidly converted to PGH_2 (prostaglandin H2) (Figure 1.2). PGH_2 is converted to PGD_2 by the terminal isomerase H-PGDS (haematopoietic prostaglandin D2 synthase), (Figure 1.2). PGD_2 induces Th2 lymphocytes, eosinophils and neutrophil recruitment and contributes to bronchoconstriction and vascular leak associated with allergic conditions such as anaphylaxis (da Silva *et al.* 2014; Firestein *et al.* 2015)

COX-1/2 are the rate liming enzymes for PGD_2 synthesis. Whereas COX-1 is constituvely expressed to maintain normal physiological levels of eicosanoids, COX-2 expression is induced in response to inflammatory stimuli. COX-2 is thus responsible for pathological regulation of PGD_2 synthesis. Consequently, inhibition of COX-2 activity is a key mechanism by which the most commonly used antiinflammatory drugs, the non-steroidal antiinflammatory drugs (NSAIDs) such as aspirin and steroidal antiinflammatory glucocorticoids such as dexamethasone exhibit their therapeutic effects (Goulding 2004; Hayashi *et al.* 2004; Fendrick and Greenberg 2009; Candido *et al.* 2017).

Correspondingly, leukotriene production in mast cells requires the initial conversion of AA to LTA_4 by 5-lipoxygenase (5-LO). LTA_4 is then converted to LTB_4 by LTA_4 hydrolase (LTA4H) or to LTC_4 by LTC4 synthase (LTC4S) (Figure 1.2). These leukotrienes trigger recruitment of eosinophils, neutrophils and T lymphocytes. They also induce increased vascular permeability and bronchoconstriction that occur in allergic diseases (Kim *et al.* 2006; Barrett *et al.* 2011; da Silva *et al.* 2014). Together, the lipid mediators contribute to acute inflammatory resonse associated with mast cell activation (Firestein *et al.* 2015).

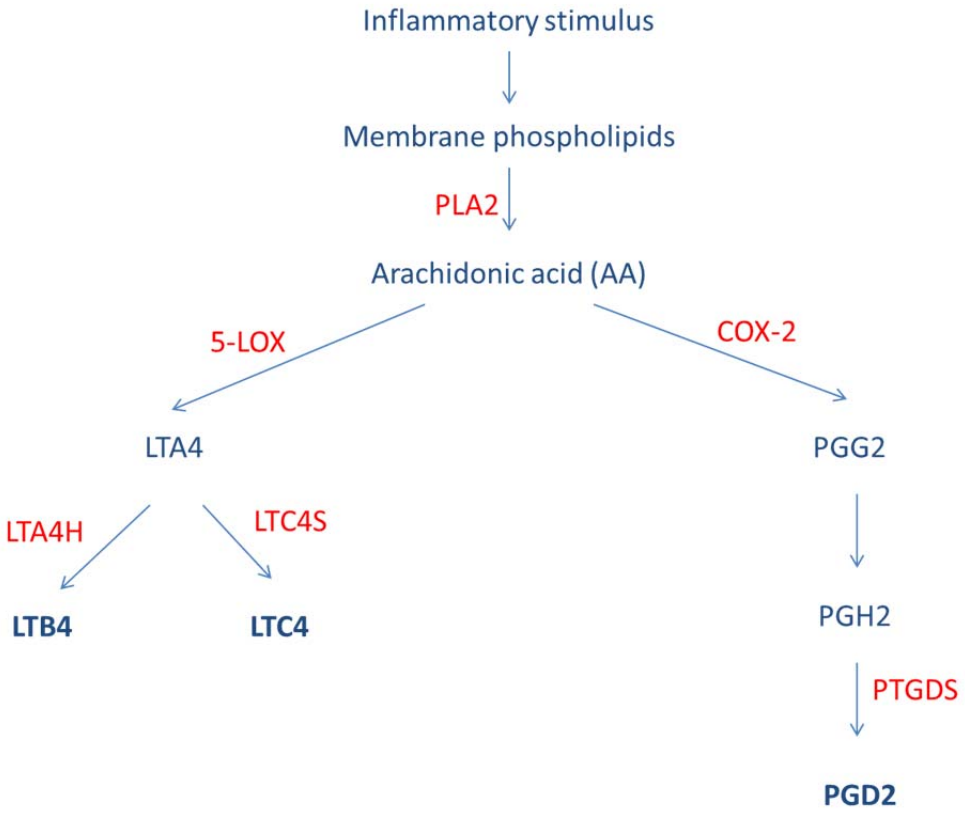


Figure 1.2 COX-2 (Cyclooxygenase-2) pathway for prostaglandin D2 and leukotriene synthesis: COX-1 and COX-2 (cyclooxygenases 1 and 2) catalyses AA (arachidonic acid) released from membrane phospholipids by PLA2 (phospholipase A2) activity to an unstable intermediate, PGG_2 (prostaglandin G2), which is rapidly converted to PGH_2 (prostaglandin H2). PGH_2 is converted to PGD_2 by the terminal isomerase H-PGDS (hematopoietic prostaglandin D2 synthase). 5-LO (5-lipoxygenase) converts AA to LTA_4 (leuckotriene A4) which is then converted to LTB_4 by LTA4H (LTA_4 hydrolase) or to LTC_4 by LTC4S (LTC_4 synthase) Evans et al. (1985).

1.4.3 Cytokines/Chemokines

Mast cell activation induces the synthesis and release mediators that derive from induction of gene expression. Pathologically important classes of such mediators are proinflammatory cytokines/chemokines such as TNFα, IL-1, and IL-6; IL-4, IL-5, IL-10, IL-13; CCL2 and CSF2 (Metcalfe *et al.* 1997; Gilfillan and Tkaczyk 2006; Kraft and Kinet 2007). Some of these cytokines/chemokines notably TNFα, may be prestored and released rapidly after mast cell activation (Firestein *et al.* 2015). These cytokines/chemokines activate other immune cells such as neutrophils, T cells and B cells to release their own mediators, which results in the amplification and perpetuation of the inflammatory response (Kalesnikoff and Galli 2010; Reber and Frossard 2014).

1.5 PROTEIN TYROSINE PHOSPHATASES

The status of mast cell activation at any point in time is a balance between activation and inhibitory pathways (Gilfillan *et al.* 2011; Sibilano *et al.* 2014). The reason being that, inappropriate activation of mast cells can lead to life threatening disease conditions such as anaphylaxis (Peavy and Metcalfe 2008; Gilfillan *et al.* 2011).

Normaly, mast cell FcεRI activation is promoted by protein tyrosine kinases (PTKs) (Gilfillan and Tkaczyk 2006; Rivera and Gilfillan 2006), while inhibition is mediated by various phosphatases including protein tyrosine phosphatases (PTPs) (Kalesnikoff and Galli 2010). PEST-domain-enriched tyrosine phosphatase (PEP), is one of such PTPs, more recently suggested to be involved in the regulation of mast cell functions (Obiri *et al.* 2012).

1.5.1 PEP

PEST-domain-enriched tyrosine phosphatase (PEP in mouse) known as Lyp in humans, is a cytoplasmic PTP. Structually, it is an 802 amino residue protein

with a 200 amino acid C-terminus domain containing four proline rich sequences for protein-protein interactions (Matthews *et al.* 1992; Cloutier and Veillette 1996) and a PEST (proline-glutamic acid-serine-threonine) sequence that may signal the protein for degradation (Matthews *et al.* 1992). The C-terminus shares about 60% homology, while the N-terminus shares 90% homology with Lyp (Cohen *et al.* 1999). The N-terminus contains the phosphatase domain of about 300 amino acid residues (Cohen *et al.* 1999).

PEP is involved in various chronic diseases (Bottini *et al.* 2004; Bottini and Peterson 2014). Disease associated studies showed that alterations in the Lyp gene is a risk factor for human diseases including inflammatory conditions such as rheumatoid arthritis (Vang *et al.* 2008), showing that PEP is involved in immune regulation. In their studies to understand the role of PEP in immune cell functions, Hasegawa and colleagues used murine gene knock-out models to show that PEP deficient mice are viable and fertile. They established that although young PEP -/- mice (4-6 weeks) did not show morphological differences compared to WT, older mice (above 6 months) had larger lymph nodes and spleen (Hasegawa *et al.* 2004). These older mice had higher effector/memory T cell populations and higher number of germinal centres in the spleen and Peyer's patches (Hasegawa *et al.* 2004). Furthermore, T cells from PEP-/- mice exhibited increased proliferation, increased cytokine/chemokine production, and elevated intracellular Ca^{2+} (Hasegawa *et al.* 2004). Also, studies using Jurkat T-cells in which PEP was overexpressed in a luciferase reporter system, showed that induced expression of PEP reduced the activation of proinflammatory transcription factors (NFAT and AP1). It was therefore concluded that PEP is a negative regulator of T cell effector functions (Gjorloff-Wingren *et al.* 1999). Subsequently, it was shown that PEP interacts synergistically with Csk (C-terminal Src kinase) to down regulate the

phosphorylation of the Src kinases Lck (Lyn in mast cells) and Zap-70 (Syk in mast cells) (Cloutier and Veillette 1999). This inhibition of Lck and Zap-70 by PEP were found to be associated with its phosphatase activity in a substrate trapping experiment using catalytically inactive mutants of PEP (Cloutier and Veillette 1999; Gjorloff-Wingren *et al.* 1999), suggesting that PEP phosphatase activity negatively regulates T cell functions. PEP was shown to be upregulated by glucocorticoids (GCs) in mast cells (Maier *et al.* 2007). Initial investigations to understand the role of PEP in mast cells did show that PEP promotes anaphylaxis in mice, and that PEP is needed for GC-mediated inhibition of anaphylaxis (Obiri *et al.* 2012). However, the mechanisms by which PEP regulates mast cell functions and its contribution to the antiinflammatory/antiallergic action of GCs is currently not clear and needs further investigation.

1.6 GLUCOCORTICOIDS

Glucocorticoids (GCs) are a class of steroid hormones produced in the adrenal cortex from cholesterol, by the hypothalamic–pituitary–adrenal (HPA) axis in response to psychogenic, physical and immune challenges (Cain and Cidlowski 2015; Ramamoorthy and Cidlowski 2016; Cain and Cidlowski 2017). The name glucocorticoids derive from the ability of these adrenal steroids to promote the conversion of proteins and lipids into glucose during stress-induced activation of the HPA axis (Smith and Vale 2006; Gross and Cidlowski 2008). They were discovered to exert beneficial effects in inflammatory conditions such as rheumatoid arthritis, for which Kendall, Reichstein and Hench received the Nobel Prize in Physiology and Medicine in 1950 (Burns ; Benedek 2011). Subsequently, various synthetic GCs have been used in the treatment of autoimmune diseases, inflammatory disorders and

cancer, making GCs one of the most widely prescribed classes of drugs (Barnes 2006; Gross and Cidlowski 2008; Cain and Cidlowski 2017). However, their involvement in glucose, fat and protein metabolism causes serious side effects such as type 2 diabetes, growth retardation, impaired wound healing, osteoporosis and hypertension (Barnes 2006; Cain and Cidlowski 2015, 2017). Additionally, a small number of patients do not respond at all or develop reduced response to GCs with chronic use (Ito *et al.* 2006; Caratti *et al.* 2015). Consequently, the need to develop potent GCs with less side effects has generated continuous research into the mechanism of GC action, but for several decades the molecular mechanism of GC action is not fully understood, for a number of reasons. For example, the mechanisms of glucocorticoid action are manifold, as they regulate up to 20% of the transcriptome according to some studies (Galon *et al.* 2002; Cain and Cidlowski 2017). Also, glucocorticoid receptors are expressed by nearly all nucleated cells with some cell-type specific differences in GC functions (Coutinho and Chapman 2011). Thus, many aspects of GC actions remain uncovered. To develop potent GCs with minimal side effects, it is important to have full understanding of the mechanism of GC action.

1.7 MECHANISM OF GLUCOCORTICOID ACTION

GCs diffuse across lipid membranes into cellular compartments such as the cytoplasm where they interact with the glucocorticoid receptor (GR), which belongs to the superfamily of nuclear receptors of transcription factors (Hollenberg *et al.* 1985). GCs induces changes in cellular functions that is broadly classified as either genomic or non-genomic (Gross and Cidlowski 2008; Stahn and Buttgereit 2008; Mitre-Aguilar *et al.* 2015). The genomic mechanisms involve the transport of a cytoplasmic glucococrticoid receptor

(GR) into the nucleus upon ligand (GC) binding, resulting in the activation or repression of gene transcription (Cato and Wade 1996; Cain and Cidlowski 2017). On the other hand, the non-genomic mechanisms involves rapid changes that occur through physiochemical interactions of GC or ligand - activated GR with cellular components independent of GR nuclear transport (Gross and Cidlowski 2008; Stahn and Buttgereit 2008; Buttgereit *et al.* 2011; Cain and Cidlowski 2017). Although both genomic and non-genomic mechanisms modulate cell functions, studies to understand the antiinflammatory action of GCs have focused mainly on the GR mediated genomic effects, since they are the main mode by which GCs exhibit their antiinflammatory effects (Rhen and Cidlowski 2005; Barnes 2006; Desmet and De Bosscher 2017). In the nucleus, glucocorticoid activated GR, represses or activates target gene expression.

1.7.1 REPRESSION OF GENE EXPRESSION

Glucocorticoids repress gene expression via multiple mechanisms, generally termed transrepression. Once inside the nucleus, the GR as a homodimer can bind directly to DNA sequencies called negative glucocorticoid response elements (nGRE), to inhibit target gene expression (Moutsatsou *et al.* 2012; Cain and Cidlowski 2017). This binding of GR as a homodimer to the nGREs denies assess to target gene promoter for other factors involved in transcription, leading to the repression of gene transcription (Subramaniam *et al.* 1998; Zhou and Cidlowski 2005; Zen *et al.* 2011). Example of transcriptional repression through nGRE occurs when the GR binds to nGREs of osteocalcin, a gene involved in bone formation. This prevents binding of transcription factors belonging to the basal transcriptional machinery (BTM), whose binding site overlap with the nGRE of osteocalcin (Morrison and Eisman 1993; Meyer

et al. 1997). This inhibition of osteocalcin gene expression leads to impairement of bone formation as observed in osteoporosis, which is a major adverse effect of GC use (Moutsatsou *et al.* 2012). Likewise, the GC-activated GR in the nucleus also interacts with nGREs to exhibit antiinflammatory effects as observed for TSLP (Thymic stromal Lymphopoietin), a cytokine known to induce atopic dermatitis (Surjit *et al.* 2011).

Alternatively, the GR as a monomer can interfere with the function of DNA bound transcription factors (TFs) via protein-protein interactions termed "tethering", to repress gene expression (Cato and Wade 1996; Ratman *et al.* 2013). This tethering of GR to other TFs alters the assembly of coactivator complexes that are required for gene expression, leading to the repression of target gene expression (Glass and Saijo, 2010; Luecke and Yamamoto, 2005; Ogawa et al., 2005; Rogatsky et al., 2002; Schule et al., 1990). By tethering to immune-regulating transcription factors such as AP-1 (activator protein 1), NF-κB (nuclear factor 'kappa B), NF-AT (nuclear factor of activated T cells), CREB (cyclic AMP-responsive element-binding protein), STAT3 (Signal transducer and activator of transcription 3) STAT6, IRF3 (Interferon Regulatory Factor 3), GATA- 3 (GATA binding protein 3) and t-Bet (T-Box transcription Factor TBX21), GR represses the expression of a number of cytokines/chemokines (TNFα, GM-CSF, IL-1b, IL-2, IL-3, IL-6, IL-8, IL-11, IL13) (Beck et al., 2009; De Bosscher et al., 2003; Langlais et al., 2012; Liberman et al., 2007, 2009a, 2009b; Reily et al., 2006) and COX-2 in immune cells (Revollo and Cidlowski 2009; Cain and Cidlowski 2017).

1.7.2 ACTIVATION OF GENE TRANSCRIPTION

The genomic action of the GR also involves the activation of gene expression via mechanisms collectively termed transactivation (Smoak and Cidlowski

2004). Classically, the GR binds as a homodimer to DNA sequences called positive glucocorticoid response elements (+GREs) to activate the expression of target genes (Beato 1989; Beato *et al.* 1996). This process involves the recruitment of several coactivators with histone acetylase (HAT) activity, such as CBP/p300(CREB-binding protein/p300), and SRC-1 (Smoak and Cidlowski 2004; Tronche *et al.* 2004; Revollo and Cidlowski 2009; King *et al.* 2012), and also non-HAT containing cofactors including SWI/SNF and pCIF to the GR-DNA complex (De Bosscher et al., 2003). Typical examples of genes controlled via this mechanism, include genes involved in glucose and fat metabolism, such as glucose-6-phosphatase and PEPCK (phosphoenolpyruvate carboxykinase), whose expression contributes to the impaired glucocse and fat metablosim associated with GC action (van Raalte *et al.* 2009; Ratman *et al.* 2013). Similarly, genes with antiinflammatory effects such as lipocortin, mitogen-activated protein kinase phosphatase-1/dual specificity phosphatase 1 (MKP-1/Dusp1) (Lu and Cidlowski 2006; Bhattacharyya *et al.* 2007; Moutsatsou *et al.* 2012) and GILZ (glucocorticoid-induced lucine zipper) (Clark, 2007; Newton and Holden, 2007).

Also, the GR may induce target gene expression via interaction with DNA-bound TFs through protein-protein interaction without binding to DNA (Beato 1989; Beato *et al.* 1996; Moutsatsou *et al.* 2012). A typical example for such mechanism is the transactivation of the antiinflammatory cytokine IL-10, which occurs through protein-protein interaction between GR and the transcription factor STAT3 (Unterberger *et al.* 2008).

1.8 AIM

PEP is a protein tyrosine phosphatase that regulates immune cell function. PEP gene knock-out studies have shown that PEP promotes mast cell mediated anaphylaxis. In mast cells, PEP expression is upregulated by the antiinflammatory glucocorticoid (GC) dexamethasone. However, the role of PEP in mast cell function and its contribution to GC action is not clear. Therefore, this project aims to study the role of PEP in antigen-mediated mast cell response, and to investigate whether the antiinflammatory/antiallergic action of GCs in mast cells is mediated by PEP.

Chapter 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS

General Laboratory Chemicals	Source
5× Green GoTaq reaction buffer	Promega, Mannheim
β-Mercaptoethanol	Carl Roth GmbH & Co., Karlsruhe
Acrylamide/Bis-Acrylamide	Carl Roth GmbH & Co., Karlsruhe
Ammonium persulfate (APS)	Carl Roth GmbH & Co., Karlsruhe
Ammonium Chloride (NH_4Cl)	Carl Roth GmbH & Co., Karlsruhe
Bromophenol blue	BioRad., Heidelberg
Bovine Serum Albumin (Powder)	GE Healthcare Life Science USA
Calcium chloride (CaCl2)	Carl Roth GmbH & Co., Karlsruhe
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH & Co., Karlsruhe
Dithiothreitol (DTT)	Carl Roth GmbH & Co., Karlsruhe
ECL (Western Blotting Substrate)	BioRad, Heidelberg
Ethanol	Carl Roth GmbH & Co., Karlsruhe
Ethidium bromide	Carl Roth GmbH & Co., Karlsruhe
Glucose	Carl Roth GmbH & Co., Karlsruhe
Glycerol	Carl Roth GmbH & Co., Karlsruhe
Glycine	Carl Roth GmbH & Co., Karlsruhe
GoTaq polymerase	Promega, Mannheim
HEPES	Carl Roth GmbH & Co., Karlsruhe
Isopropanol	Carl Roth GmbH & Co., Karlsruhe
Magnesium chloride	Carl Roth GmbH & Co., Karlsruhe
Magnesium sulphate	Carl Roth GmbH & Co., Karlsruhe
Methanol	Carl Roth GmbH & Co., Karlsruhe
Milk powder (dry)	Saliter, Obergünzburg
Moloney Murine Leukemia Virus Reverse Transcriptase, RNase (H-) Point Mutant (M-MLV RT [H–])	Promega , Mannheim
M-MLV RT 5× buffer	Promega, Mannheim
PageRuler™ Prestained Protein Ladder	Thermo Fischer Scientific, USA
PeqGOLD Universal Agarose	PeqLab, Erlangen
Potassium chloride (KCl)	Carl Roth GmbH & Co., Karlsruhe
Potassium hydrogen carbonate ($KHCO_3$)	Carl Roth GmbH & Co., Karlsruhe

General Laboratory Chemicals (Continuation)	Source
Potassium phosphate dibasic (K_2HPO_4)	Carl Roth GmbH & Co., Karlsruhe
Protassium phosphate monobasic (KH_2PO_4)	Carl Roth GmbH & Co., Karlsruhe
Proteinase K	Carl Roth GmbH & Co., Karlsruhe
Sodium acetate	Carl Roth GmbH & Co., Karlsruhe
Sodium chloride	Carl Roth GmbH & Co., Karlsruhe
Sodium dodecylsulphate (SDS)	Carl Roth GmbH & Co., Karlsruhe
Sodium hydroxide	Carl Roth GmbH & Co., Karlsruhe
Tris-base	Carl Roth GmbH & Co., Karlsruhe
Tris hydrochloride (Tris-HCl)	Carl Roth GmbH & Co., Karlsruhe
Tetramethylethylenediamine(TEMED)	Carl Roth GmbH & Co., Karlsruhe
Tween-20	Carl Roth GmbH & Co., Karlsruhe

2.1.2 PEP INHIBITOR

Chemical	Source	Use
L75N04	Kind gift from Dr. Zhong-Yin Zhang, Indiana, USA, (He *et al* 2013)	5 μM

2.1.3 CELL CULTURE MATERIALS

Chemicals	Source
1× Dulbeccos's Phosphate Buffered Saline (PBS)	GIBCO™, Thermo Fisher Scientific, USA
70 μm falcon cell strainer	Corning®, Kaiserslautern
β- mercaptoethanol (Cell culture grade)	Sigma-Aldrich Chemie, Steinheim
Cell culture Dishes, flasks and multiwell plates	Greiner Bio-one GmbH, Frickenhausen
Dexamethasone	Sigma-Aldrich Chemie, Steinheim
DNP-BSA (Albumin from Bovine Serum (BSA), 2,4-Dinitrophenylated)	Molecular Probes, Thermo Fisher Scientific, USA
FBS (South American Origin)	GIBCO®, Thermo Fisher Scientific, USA
Iscove's Modified Dulbecco's Medium (IMDM)	GIBCO®, Thermo Fisher Scientific, USA
L-glutamine	GIBCO®, Thermo Fisher Scientific, USA
Murine monoclonal anti-ditrophenyl IgE (clone SPE-7)	Sigma-Aldrich Chemie, Steinheim
Murine stem cell factor (c-Kit ligand)	Kind gift from Stassen, M (Stassen *et al.* 2006)
Recombinant Murine Interleukin 3 (IL-3)	PreproTech, Rocky Hill, NJ
Sodium Pyruvate	GIBCO®, Thermo Fisher Scientific, USA
Penicillin/Streptomycin 10000 U/ml	GIBCO®, Thermo Fisher Scientific, USA

2.1.4 ANTIBODIES

Primary Antibodies	Source	Use
Anti-PEP	Kind gift from A. Chan (Hasegawa *et al* 2004).	1 : 2000 in 5% milk (in 1× TBST), 4°C O/N
P38α (C-20) sc-535 rabbit polyclonal IgG, 200 μg/ml	Santa Cruz, Heidelberg	1 : 1000 in 5% milk (in 1× TBST), 4°C O/N

Secondary Antibodies	Source	Use
Goat anti-mouse IgG HRP conjugate	Advansta, USA	1 : 10000 in 5% milk (in 1× TBST), 45 min at RT
Goat anti-rabbit IgG HRP conjugate	Advansta, USA	1 : 10000 in 5% milk (in 1× TBST), 45 min at RT

2.1.5 OLIGONUCLEOTIDES

PEP genelocus-specific primers	Sequence
KH-1	5'-AATGCCCGCTTGATGAGCAGAGGTCTG-3'
KH-2	5'-GGCTTCTTTCAGTAGTTGCTGCAGAAT-3'
KH-4	5'-TGCTAAAGCGCATGCTCCAGACTGC-3'

Primers for qRT-PCR	Sequence
CSF2	Forward 5'-TCGTCTCTAACGAGTTCTCCTT-3' Reverse 5'-CGTAGACCCTGCTCGAATATCT-3'
GAPDH	Forward 5'-CTGAGGACCAGGTTGTCTCC-3' Reverse 5'-TGTGAGGGAGATGCTCAGTG-3'
IL13	Forward 5'-TGGCTCTTGCTTGCCTTGGT-3' Reverse 5'-TTTTGGTATCGGGGAGGCTGG-3'
PTGDS/L-PGDS	Forward 5'-GCTCCTTCTGCCCAGTTTTCCT-3' Reverse 5'-GGAGGACCAAACCCATCCAC-3'
PTGS2/COX-2	Forward 5'-TGAGCAACTATTCCAAACCAGC-3' Reverse 5'-GCACGTAGTCTTCGATCACTATC-3'
Ribosomal protein, large, P0 (Rplp0)	Forward 5'-GGACCCGAGAAGACCTCCTT-3' Reverse 5'-GCACATCACTCAGAATTTCA-3'
TNFα	Forward 5'-GATCGGTCCCCAAAGAAGGGATG-3' Reverse 5'-TGATCTGAGTGTGAGGGTCTCG-3'

2.1.6 CELL TYPES AND CULTURE MEDIUM

Cell type	Description	Medium
BMMCs	Bone marrow derived mast cells (primary murine mast cells)	IMDM + 10% FBS, 2 mM L-glutamine, 1 mM Pyruvate, 1% Penicillin/Streptomycin, 100 ng/ml SCF, 10 ng/ml IL3, 50 μM β-mercaptoethanol

2.1.7 ANIMALS

The PEP+/+ and PEP-/- C57BL/6 mice used in this work were generated by successive crossing of PEP+/- C57BL/6 germline mice (obtained from Genentech Inc., South San Franciso) with wild-type C57BL/6 mice, over 10 generations, in the Animal Facility of ITG, KIT.

The C57BL/6 PEP+/- germline was generated by the group of Andrew Chan, by using homologous recombination to disrupt the endogenous PEP gene in embryonic stem cells (ES), to generate PEP+/- ES. The PEP+/- ES were injected into C57BL/6 blastocysts to yield chimeric mice that were crossed with wild-type C57BL/6 mice to generate the PEP+/- germline mice (Hasegawa *et al.* 2004).

To generate PEP+/+ and PEP-/- BALB/c mice, BALB/c mice were crossed with PEP-/- C57BL/6 mice for 10 generations.

All mice were bred in the Animal Facility of ITG (safety level S1, in specific pathogen free conditions and in a 12 hour Light-Dark cycle, with food and water available *ad libitum*. The breeding of mice and animal experiments were carried out in accordance with the German legislation guidelines for care and use of laboratory animals.

2.2 METHODS

2.2.1 NUCLEIC ACID METHODS

2.2.1.1 Genotyping of Mice

a) Isolation of Genomic DNA from Mouse Tail Biopsies

To determine the genotype of mice, mouse tail biopsies were lysed in 200 µl of tail lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg/ml Proteinase K) in 1.5 ml eppendorf tubes, with shaking at 55˚C on a thermal block (Eppendorf Thermomixer 5436, Eppendorf-Netheler-Hinz–GmbH, Hamburg) for 3 h. After lysis, the lysate was centrifuged for 5 min at 14000 rpm (Eppendorf Centrifuge 5417R, Eppendorf AG, Hamburg), to pellet hair and other tissue debris. The supernatant containing the DNA was transferred into 1.5 ml eppendorf tube containing 200 µl of isopropanol to precipitate the DNA. The mixture was centrifuged for 5 min at 14000 rpm and the supernatant discarded, leaving a pellet of DNA. The eppendorf tube was turned upside down on a Kleenex™ paper to dry the pellet. The pellet was further dried for 5 min on thermal block, at 37˚C. The DNA pellet was dissolved in 100 µl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA pH 8) with shaking on the thermal block for 30 min. The DNA was used immediately or stored at -20˚C for future use.

b) PCR with PEP Genelocus-Specific Primers

DNA (section 2.2.1.1a), 4 µl each, was pipetted into two separate PCR tubes. Then 16 µl of a master mix A, containing the PEP genelocus-specific primers KH-1 (forward) and KH-2 (reverse) (4 µl of 5× Go Taq Reaction Buffer, 10 mM dNTPs, 10 pM KH-1, 10 pM KH-2, 25 µl GoTaq Polymerase (5 U/µl), 9.25 µl

dH_20) was added to one of the DNA templates, while 16 µl of a master mix B containing KH1 (forward) and KH4 (reverse) (4 µl of 5× Go Taq Reaction Buffer, 10 mM dNTPs, 10 pM KH-1, 10 pM KH-4, 25 µl GoTaq Polymerase (5 U/µl), 9.25 µl dH_20) was added to the other DNA template.

The PCR was carried out in a thermocycler (GeneAmp™ PCR System 2700, Applied Biosystems), using the following PCR protocol:

PCR Protocol

Step	Temperature	Time	Number of cycles
1	95	3 min	
2	95	1 min	27×
3	55	1 min	27×
4	72	1 min	27×
5	4°C	Hold	

The oligonucleotides KH1 (forward) and KH2 (reverse) anneal within the genomic sequence of PEP gene, outside the left and right border, respectively, of a neo cassette (origionally inserted in the exon 1 to disrupt the PEP gene), while KH4 anneals within the neo cassette sequence (Hasegawa *et al.* 2004). Thus, master mix A (KH1 + KH2) will produce amplified product size of about 550 bp, if the template DNA contained wild-type allele, and master mix B (KH1 + KH4) will produce a product size of about 450 bp, if the template DNA contained mutated allele. The amplication products were subjected to agarose gel electrophoresis for detection.

c) Agarose Gel Electrophoresis

Separation of DNA fragments was carried out using 2% w/v agarose gel

The gel was prepared by heating agarose powder in 1× TAE buffer (40 mM Tris-base pH 7.2, 20 mM sodium acetate, 1 mM EDTA) to boiling. The boiled agarose solution was cooled to 60˚C and ethidium bromide (intercalating

agent added as a fluorescent tag) was added to a final concentration of 0.3 μg/ml. The gel solution was then poured into a horizontal electrophoresis chamber (Owl Seperations Systems) and a comb inserted to make wells whiles the gel polymerises.

The polymerised gel was overlaid with 1× TAE buffer, and PCR samples loaded into wells. The electrophoresis was carried out at 120V for 45 min. DNA bands were thereafter visualised under UV light using a transilluminator fitted with Eagle Eye photo camera system (Peqlab Biotechnologie GmbH, Erlangen).

2.2.1.2 RNA Analysis

a) Phenol:Chlorophorm Extraction of RNA from Bone Marrow Derived Mast Cells (BMMCs)

BMMCs suspension of 2-3 x 10^6 cells/ml in 1.5 ml eppendorf tube was centrifuged (Eppendorf Centrifuge 5417R, Eppendorf AG, Hamburg) at 2000 rpm for 5 min at 4˚C to pellet the cells. The medium was discarded and the cell pellet lysed in 1 ml of PeqGOLD RNAPure™ reagent for 5 min at room temperature (RT). To extract RNA, 200 μl of chloroform was added, vortexed for 15 s and incubated on ice for 5 min. The mixture was then centrifuged at 14000 rpm for 10 min at 4˚C, to separate RNA, DNA and proteins into phases. After centrifugation, 400 μl of the upper RNA containing aqueous phase was transferred into eppendorf tube containing 5 μg glycogen (in 5 μl, as a carrier molecule to enhance RNA precipitation). The resulting mixture was vortexed and incubated at RT for 5 min. Thereafter, 400 μl of isopropanol was added, vortexed, and incubated on ice for 15 min to precipitate RNA. The mixture was centrifuged at 14000 rpm for 10 min at 4˚C to pellet the RNA. After

centrifugation, the supernatant was discarded. Residual isopropanol around the RNA pellet was washed off, twice, with 1 ml 75% ethanol (cold), by vortexing for 5 s and then centrifugation at 14000 rpm for 10 min at 4˚C. After the last wash, eppendorf tube was turned upside down on a Kleenex™ paper under fume chamber for 10 min to dry the pellet. The dried pellet was dissolved in 20 µl sterile RNAse-free water by incubating on heat block (Eppendorf Thermomixer 5436, Eppendorf-Netheler-Hinz– GmbH, Hamburg) at 37˚C for 10 min, and then at 56˚C for 5 min, to denature RNAses. RNA concentration and purity was measured using Nanodrop™ ND-1000 spectrophotometer (PeqLab Biotechnologie GmbH, Germany). The samples were used immediately or stored at -80 for future use.

b) Quantification of RNA Concentration

The concentration of RNA was determined by measuring the optical density (OD) of 2 µl of RNA sample at 230, 260 and 280 nm using NanoDrop™ ND-1000 spectrophotometer (PeqLab Biotechnologie GmbH, Germany). A ratio of OD260/OD280 within 1.8-2.0 indicates a sample of acceptable purity, relatively free of proteins, while OD260/OD230 1.6 and above shows a preparation relatively free of organic chemicals and solvents.

c) cDNA Synthesis

To synthesise cDNA from RNA, first, 1 ug of RNA was incubated with 1 unit of DNAse1, in a total volume of 10 µl in PCR tube, at 37˚C for 30 min, in a thermocycler (GeneAmp™ PCR System 2700, Applied Biosystem), to digest genomic DNA contaminants. After incubation with DNase1, 1 µl of RQ1 DNase Stop Solution™ (Promega, Mannheim) was added and incubated at 65˚C for 10 min in the thermocycler, to inactivate the DNase1. Thereafter, 6 µl and 3 µl portions of the mixture were transferred into seperate PCR tubes,

labeled +RT and –RT respectively. A volume of 6 µl random primer master mix (1 µl random primer [200 ng/µl], 5 µl of sterile RNAse-free water) was pipetted into the +RT, while 3.5 µl was added to the –RT tube. The samples were then incubated at 70˚C for 5 min in the thermocycler for random primers to anneal to RNA. Next, 8 µl of a master mix, containing Reverse Transcriptase (M-MLV RT [H–]) (4 µl M-MLV 5× buffer, 1 µl M-MLV RT [H–](200 U/µl), 2 µl of 10 mM dNTPs, 1 µl sterile RNAse-free water) was added to the +RT PCR tube, while 4 µl of a master mix with no M-MLV RT [H–] (2 µl M-MLV 5× buffer, 1 µl of 10 mM dNTPs, 1 µl sterile RNAse-free water) was added to the –RT PCR tube. The tubes were incubated in parallel in the thermocycler using the following cDNA synthesis protocol:

cDNA Synthesis Protocol

Step	Temperature	Time
1	25°C	10 min
2	42°C	60 min
3	70°C	10 min
4	4°C	Hold

After cDNA synthesis, the content of +RT and –RT PCR were diluted with 80 µl and 40 µl sterile RNAse-free water, respectively. Samples were used immediately or stored in -20 for future use.

The +RT tube contains reverse transcriptase for normal cDNA synthesis, whereas the –RT, having no reverse transcriptase, serves as quality control PCR (QC-PCR), to detect genomic DNA contaminats as decribed below.

d) Quality Control PCR (QC-PCR)

To check for genomic DNA contaminants during RNA extraction, 4 µl of the +RT and -RT PCR products (Section 2.2.1.2, **c**) were taken into separate tubes and 16 µl QC-PCR master mix containing GAPDH gene primers (4 µl GoTag Polymerase 5x buffer, 0.5 µl of 100 nM dNTPs, 1 µl forward primer, 1 µl reverse primer, 0.25 µl of GoTag Polymerase, 9.25 µl water) added to each tube. The samples were incubated in a themocycler (GeneAmp™ PCR System 2700, Applied Biosystem) with the QC-PCR protocol below.

QC-PCR Protocol

Step	Temperature	Time	Number of cycles
1	95°C	2 min	
2	95°C	2 min	30 ×
3	55°C	40 s	30 ×
4	72°C	45 s	30 ×
5	4°C	Hold	

The resultant PCR product were run on agarose gel and visualised (section 2.2.1.1, **c)**. The presence of DNA fragements in the –RT PCR product indicates genomic DNA contamination.

e) Quantitative Real-Time PCR (qRT-PCR)

To carry out gene expression analysis using qRT-PCR, 4 µl of cDNA (+RT PCR product, obtained from Section 2.2.1.2 **c)** was pipetted into qRT-PCR plates (Steinbrenner Laborsysteme, Wiessenbach). Next, 16 µl of qRT-PCR master mix containing gene specific primers (10 µl SYBR Green qPCR-Mix (Promega), 1 µl forward primer, 1 µl reverse primer, 4 µl RNase-free water) was added to the cDNA. The qRT-PCR was run in StepOnePlus (Life Technologie, Carlsbad, California), with an initial denaturation stage carried out at 95°C for 15 min. This was followed by 40 cycles of a two-step stage of 95˚C for 15 s, and 60˚C

for 30 s, for hybridisation and amplification, and finally, a melting curve with a starting temperature of 60˚C, with incremental increase of 0.5˚C to 95˚C, that was maintained for 15 s. The relative mRNA expression of a target gene was calculated as $2^{-(C_{T\ (target\ gene)} - C_{T\ (reference\ gene)})}$.

2.2.2 PROTEIN ANALYSIS

2.2.2.1 Preparation of Whole Cell Lysate

BMMCs (2-3 × 10^6 cells/ml in medium) were centriguged at 2000 rpm (Eppendorf Centrifuge 5417R, Eppendorf AG, Hamburg) for 5 min to pellet the cells. The medium was discarded, and the cell pellet was washed with 1 ml of cold 1× PBS, by centrifugation at 2000 rpm for 5 min. After washing, cells were suspended in 200 µl of 2× SDS sample buffer (160 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 12.5% mM EDTA pH 8.0, 2% β-mercaptoethanol, 0.02% bromophenol blue) and incubated at 95˚C in a heating block (Eppendorf Thermomixer 5436, Eppendorf-Netheler-Hinz– GmbH, Hamburg) for 5 min. After heating, the lysate was centrifuged at 10000 rpm for 5 min and sonicated at an amplitude of 50, 5 pulses (Branson Sonifier Cell disrupter B 15 [G.Heinemann Ultraschall- und Labortechnik, Schwäbisch Gmünd]). The sonified sample was then centrifuged at 10000 rpm at 4 ˚C for 5 min. Volumes of 20 µl supernatant were loaded in SDS-Polyacrylamide gel for electrophoretic separation.

2.2.2.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To analyse proteins by SDS-PAGE, a 10% separating gel was used. In this method, 25 µl of separating gel solution (9.9 ml dH_2O, 8.3 ml 30% Arylamide, 6.3 ml of 1.5 M Tris-HCl pH 8.8, 250 µl of 10% SDS, 250 µl APS, 10 µl TEMED) was poured between two fixed glass plates separated by spacers in a gel casting apparatus (Cast-It M caster apparatus, PeqLab Biotechnologie GmbH, Erlangen). The top of the gel solution was immediately covered with 100% ethanol, and allowed to polymerise at room temperature (RT) for 40 min, to form the separating gel. After polymerisation, the ethanol was washed away with distilled water and the top of the gel overlaid with 8 ml starking gel solution (5.5 ml dH_2O, 1.3 ml of 30% acrylamide, 1 ml of 1.5 M Tris-HCl pH 6.8, 80 µl of 10% SDS, 80 µl APS, 8 µl TEMED). A comb was placed immediately into the starking gel solution to make wells of appropriate depth. The starking gel was allowed to polymerise for 20 min. The comb was removed after polymerisation and the gel placed in a vertical running chamber (PerfectBlue Dual Gel System Twin M [Peqlab, Biotechnologie GmbH, Erlargen]) filled with 1× Laemmli running buffer (25 mM Tris-base, 192 mM glycine, 0.1% (w/v) SDS). The surface of the gel was well covered with the 1x Laemmli running buffer. The first well was loaded with 5 µl protein molecular weight marker (PageRuler™ Prestained Protein Ladder, 10-180 kDa, Thermo Fischer Scientific Inc., Rockford, USA). Thereafter, 20 µl of protein sample prepared in 2x SDS sample buffer (section 2.2.2.1) was loaded into wells.

The protein separation was first run at 90V, for proteins to run equally through the starking gel, and then increased to 160V, for proteins to separate in the separating gel.

2.2.2.3 Transfer of Proteins onto Membranes

The proteins in SDS-polyacrylamide gels were transferred onto PVDF (polyvinylidene fluoride) membranes (Merck Millipore, Darmstadt, Germany) in an electrophoretic wet-transfer chamber (BioRad, Heidelberg).

To do this, the membrane was activated by soaking it in methanol for 10 s, and placed above the gel, which was already laid on three layers of wet filter paper, in a cassette. The membrane was thereafter covered with another three layers of filter paper. The cassette was placed in a transfer chamber filled with Western blot transfer buffer (25 mM Tris-base, 192 mM glycine, 10% methanol). The transfer was carried out at 35V at 4˚C overnight.

2.2.2.4 Immunoblotting and Visualisation of Proteins

For immunoblotting, membranes were cut into pieces according to molecular weight of interest. The membranes were then blocked in 5% milk dissovlved in 1x TBST (Tris buffered saline with Tween-20) (20 mM Tris-base, 150 mM NaCl, 0.05% Tween-20, pH adjusted to 7.6) for 1h. Thereafter, the membranes were incubated overnight with primary antibody at 4˚C. After incubation, the membranes were washed three times at 10 min intervals with 10 ml 1x TBST, with gentle shaking on a shaker (Heidolph Instuments, Polymax 1040) - to wash off unbound primary antibody. Membranes were then incubated with secondary antibodies for 30 min at room temperature (RT), after which unbound secondary antibody was washed off with 10 ml 1x TBST.

To visualise the proteins, the surface of the membrane was covered with enhanced chemiluminiscence (ECL) Western blotting substrate (Biorad, Heidelberg) and signals detected with ChemDoc Touch imager (BioRad, Heidelberg) according to manufacturer instructions.

2.2.3 CELL CULTURE METHODS

2.2.3.1 Isolation and Culture of Bone Marrow Derived Mast Cells (BMMCs)

Male C57BL/6 mice aged 8-10 weeks were sacrificed by cervical dislocation, washed in 70% ethanol and femurs and tibia removed into Iscove's Modified Dulbecco's Medium (IMDM Gibco®). The bones (femurs and tibia) were first washed in 70% ethanol and then in 1x PBS under safety cabinet in S2 cell culture laboratory. Thereafter, tips of bones were cut and the bone marrow flashed into 50 ml falcon tubes with 20 ml of IMDM per animal, using a 20 ml syring and 24-gaige needle.

The cell suspension was centrifuged at 1300 rpm for 10 min (Eppendorf Centrifuge 5804, Eppendorf AG, Harmburg) to pellet the cells. The supernatant was discarded and the cell pellet was washed with 5 ml IMDM by centrifugation at 1300 rpm for 10 min. The washed pellet was then resuspended in 400 µl ACK lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 1 mM EDTA, pH adjusted to 7.3) for 2 min to lyse red blood cells (RBCs). After lysis, 8 ml IMDM was added to inactivate the ACk lysis buffer. The resulting cell suspension was then passed through a cell strainer (Corning®, Kaiserslauten) to remove debris. The seived cell suspension was centrifuged at 1300 rpm for 10 min to pellet the cells. The cell pellet obtained was suspended in 20 ml BMMCs medium (IMDM supplemented with 10% FBS, 2 mML-glutamine, I mM pyruvate, 100 ng stem cell factor/kit ligand, 5 ng/ml IL3, 50 µM β-mecaptoethanol, 1% penicillin/streptomycin [10000 U/ml]) in CELLSTAR® T-75 flask (Greiner Bio-One GmbH, Frickenhausen). Cells were cultured at 37˚C, 5% CO_2 and 95% humidity in an incubator (C 200, Labotech, Göttingen, Germany). BMMC medium was changed every three days. To do this, cell suspension was centrifuged at 1300 rpm for 7 min, to pellet the BMMCs, since BMMCs are suspension cells. Cells were then counted with an Improved

Neubauer haemocytometer under light microscope, and re-cultured in BMMC medium at a maximum concentration of 2×10^6 cells/ ml. Cells were used at 4-7 weeks old.

2.2.3.2 Activation of BMMCs

To activate BMMCs with antigen, cell suspensions of 2-3 $x10^6$ cells/ml seeded overnight in 24 well cell culture plates (Greiner Bio-One GmbH, Frickenhausen) were sensitised by incubation with 0.05 µg/ml anti-DNP IgE (anti-Dinitrophenol IgE) for 2 h in incubator (C 200, Labotech, Göttingen, Germany). Thereafter, DNP-BSA (dinitrophenyl-bovine serum albumin) was added to activate cells, and the cells placed back into the incubator for 1 h. To stop the DNP action, cells were picked from the incubator and placed on ice. For treatment with dexamethasone (Dex) or the PEP inhibitor L75NO4, seeded cells were pretreated for selected time intervals with DEX or L75NO4 or both Dex and L75NO4, before sensitisation and activation.

2.2.4 CALCIUM MEASUREMENTS

Intracellular free Ca^{2+} concentration $[Ca^{2+}]i$ was measured in 5-7 weeks old BMMCs in 2 mM calcium containing physiological solution (135 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 12 mM glucose, 10 mM HEPES), or 0.1 mM calcium containing physiological solution (135 mM NaCl, 6 mM KCl, 0.1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 12 mM glucose, 10 mM HEPES), or norminally calcium free solution (135 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 12 mM glucose, 10 mM HEPES) where necessary. For measurements of the antigen-induced $[Ca^{2+}]i$ elevation, the cells were sensitised overnight with 300 ng/ml of anti–DNP IgE (anti-dinitrophenol IgE) in BMMCs medium and cultured at 37°C/95%O_2/5%CO_2 in cell culture incubator (Solis-Lopez *et al.* 2017). After

sensitisation, cells were loaded with the calcium dye Fura-2 AM (Invitrogen, Katalog-Nr F-1221), by suspending cells in 2 µM Fura-2 AM (dissolved in 2 mM calcium containing physiological solution) at room temperature (RT) for 30 min. The cell suspension was transferred onto poly-L-lysine-coated coverslips and cells allowed to adhere to coverslips during Fura-2 AM loading time. Excess dye and unattached cells were washed twice with physiological solution. For activation of BMMCs with DNP-HSA (dinitrophenyl-human serum albumin), the DNP-HSA was dissolved in the appropriate physiological solution and released onto the attached cells on the glass slides when necessary. To measure $[Ca^{2+}]i$ levels, the cells were alternatively illuminated with excitation light of 340 nm and 380 nm using a monochromator-based light source, Polychrom V (TILL Photonics, Germany). The fluorescence intensity was measured at 510 nm using Rolera EM-C2 EMCCD Camera (QImaging, Canada) and quantified using Axiovision (Zeiss, Germany) 4.82 software. Signal was acquired every 5 s. The $[Ca^{2+}]i$ level was estimated based on 340/380 ratio. For each experiment, cytosolic $[Ca^{2+}]i$ in non-activated cells was measured for about 100 s to determine basal level $[Ca^{2+}]i$, after which cells were stimulated with physiological solution containing DNP-HSA 100 ng/ml, to measure antigen-induced $[Ca^{2+}]i$ elevation. To measure the calcium releasing response from the pharmacological agent, thapsigargin, 2 µM thapsigargin was made in physiological solution and used in place of DNP-HSA to activate the cells. For dexamethasone treatment, cells were concurrently treated with IgE and dexamethasone in BMMC medium, and then placed back into the incubator for 24 h, before activation with DNP-HSA.

2.2.5 RNA-SEQUENCING (RNA-SEQ) AND ANALYSIS

Total RNA was extracted from 3×10^6 cells for PEP+/+ and PEP-/- BMMCs using innuPREP RNA Mini Kit (Analytik Jena AG, Jena, Germany). RNA samples were first tested on RNA nanochips (Bioanalyzer 2100, Agilent) to ensure there was no sign of degradation. A total of 1 µg RNA was used to prepare mRNA sequencing libraries for each sample using the TruSeq stranded mRNA kit v2 (Illumina), according to the vendor protocol. Next, 10 pM of multiplexed libraries were used to generate clusters in 2 lanes of a high-throughput flowcell. HiSeq 1500 was used to obtain paired-end reads of 50 bp using the SBS v3 kit (Illumina). The cluster dectection and the base calling were done with the software RTA (v1.13) and demultiplexing with the software CASAVA 1.8.1 (Illumina). The sequencing resulted in 576 million reads with a mean quality Phred score of 35.2. The quality of the sequencing data were first assessed with FASTX toolkit (v0.0.13 [http://hannonlab.cshl.edu/fastx_toolkit/]) and no pre-processing was needed. The alignment of the reads was done with Tophat2 v2.0.11 (Kim *et al.* 2013) against the mouse reference genome (GRCm38 v75). The raw counts per gene were calculated using HTSeq v0.5.3 (Anders *et al.* 2014). The normalization of the counts, the differential expression analysis and the downstream analysis was done using the R software packages (including DESeq2 (Love *et al.* 2014), and also other publicly available packages such as 'Database for Annotation, Visualization and Integrated Discovery' (DAVID Bioinformatics Resources 6.8, NIAID/NIH) (Huang *et al.* 2007a; Huang *et al.* 2007b) and Functional Enrichment Analysis Tool (FunRich) (Pathan *et al.* 2015).

2.2.6 IN VIVO METHODS

2.2.6.1 Passive Systemic Anaphylaxis in BALB/c Mice

Female 7 months old BALB/c mice (20-25 g) were sensitised by intra-peritoneal injection (IP) of anti-DNP IgE (1 mg/kg) for 24 h. Anaphylaxis was induced by intravenous injection (IV) of 200 µl DNP-HSA (dinitrophenyl-human serum albumin [1 mg/ml in PBS]) into the dorsal caudal vein (vena coccygealis dorsalis). Thereafter, abdominal body temperature was measured with a vertinary fever thermometer (Micro-life VT 1831 Vet-Temp, Tiershop, Trier, Germany) every 5 min for 60 min.

Chapter 3

RESULTS

3.1 DELETION OF PEP PROTECTS BALB/C MICE AGAINST ANAPHYLAXIS

Anaphylaxis, is a severe and life threathening form of allergy and a major disease condition that is strongly associated with mast cell action (Metcalfe *et al.* 2009). A previous study showed that PEP deficient C57BL/6 mice are less susceptible to passive systemic anaphylaxis (PSA) (Obiri et al. 2012), a mast cell dependent response (Doyle *et al.* 2013). This was rather unexpected as loss of a phosphatase like PEP is expected to cause increased phosphorylation of protein tyrosine kinases (PTKs), which will result in an increased mast cell activation and a corresponding increase in PSA in the PEP-/- mice. However, since some mast cell mediated response can show marked differences between mice of different strains (Finkelman *et al.* 2005; Noguchi *et al.* 2005), this project investigated whether the role of PEP in PSA is dependent on the genetic background of mice.

First, PEP-/- C57BL/6 mice were crossed with BALB/c mice for 10 generations to generate a colony of PEP+/+ and PEP-/- in a pure BALB/c genetic background. PSA was induced by sensitising mice for 24 h with dinitrophenol (DNP)-specific IgE injected intra-peritoneally, after which anaphylactic reaction was triggered by intravenous injection of DNP. Anaphylaxis causes a fall in body temperature that was measured at 5 min intervals over a period of 60 min.

The graph of the anaphylactic response depicted in Figure 3.1A, showed that DNP triggered a sharp fall in body temperature in both PEP+/+ and PEP-/- mice with a peak reduction at about 20 min of DNP treatment, followed by a partial recovery from about 40 min post DNP treatment. Quantification of the

diferences in the anaphylactic response between PEP+/+ and PEP-/- mice determined by the area under the curve (AUC) (Figure 3.1B) showed that the anaphylactic response was about 1.5 -2 fold lower in the PEP-/- BALB/c mice compared to the PEP+/+ mice. The finding that PEP-/- BALB/c mice are less susceptible to anaphylaxis is in agreement with previous report by Obiri and colleagues that PEP-/- C57BL/6 mice are less susceptible to anaphylaxis (Obiri *et al.* 2012). Thus the contribution of PEP to anaphylaxis is not restricted to C57BL/6 mice, and PEP appears to be an essential regulator of anaphylaxis. However, when the present study was compared to the work of Obiri and colleagues, the C57BL/6 mice were more sensitive to anaphylaxis than the BALB/c mice. Thus, C57BL/6 mice were used in all the subsequent experiments carried-out in this project.

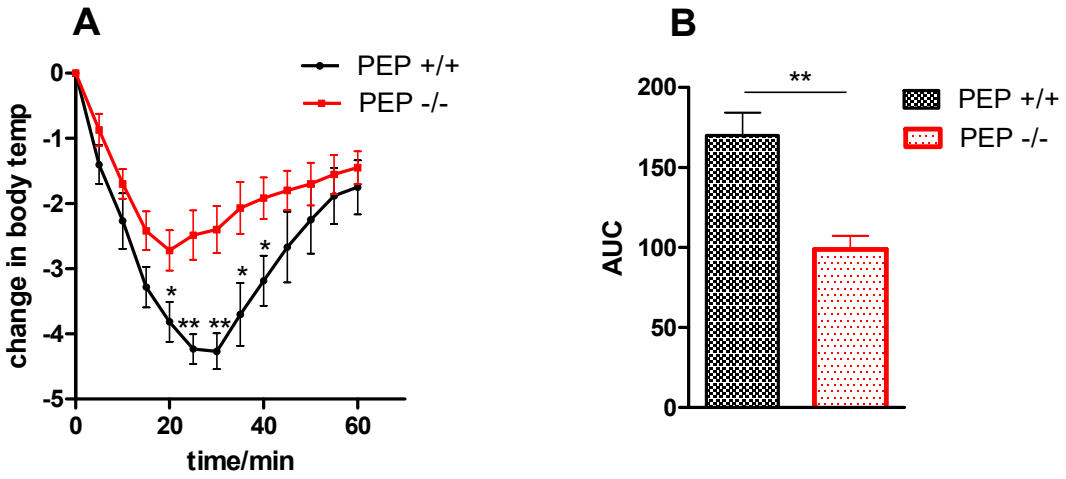


Figure 3.1 Deletion of PEP protects BALB/c mice against anaphylaxis: Seven months old female mice (20–25g) were sensitised by intraperitoneal injection with dinitrophenol (DNP) specific IgE (1 mg/kg) for 24h. Anaphylaxis was then induced with intravenous injection of 200 µl of DNP [1 mg/ml in phosphate-buffered saline (PBS)]. Body temperature was measured every 5 min over a period of 60 min. **A**, shows the curve of temperature changes over time and **B**, the total anaphylactic response calculated in arbitrary units as Area under the curve (AUC) with temperature zero (0) as reference. * $p< 0.05$, ** $p< 0.01$, for unpaired two-tailed t-test. (n=6).

3.2 PEP DEFICIENT BMMCS SHOW REDUCED INTRACELLULAR CALCIUM MOBILISATION IN RESPONSE TO ANTIGEN

Binding of DNP to the high affinity IgE receptor (FcεRI) on mast cells triggers signalling pathways that function synergistically to stimulate mast cell effector functions such as degranulation, which is key to anaphylaxis (Metcalfe et al. 2009). A hallmark of FcεRI-mediated degranulation is intracellular Ca^{2+} $[Ca^{2+}]i$ mobilisation (Baba *et al.* 2008; Harvima *et al.* 2014).

This current work used live cell Ca^{2+} imaging in BMMCs - generated from bone marrow progenitor cells from femurs of 8-10 weeks old PEP+/+ and PEP-/- mice, cultured in IL-3 rich medium in the presence of c-KIT ligand (stem cell factor) for 4-7 weeks (Jensen *et al.* 2006; Stassen *et al.* 2006) - to investigate the role of PEP in $[Ca^{2+}]i$ mobilisation in mast cells.

BMMCs were sensitised overnight with DNP-specific IgE and thereafter loaded with Ca^{2+} sensitive dye in medium (2 mM Ca^2 physiological medium) containing the ratiometric dye, Fura-2 AM. Basal level $[Ca^{2+}]i$ concentration was first measured for 100 s, after which cells were activated by treatment with antigen (DNP) (Figure 3.2A). Although the two cell types did not show any difference in basal level $[Ca^{2+}]i$ mobilisation, activation of the cells with DNP resulted in a transient rise in $[Ca^{2+}]i$, followed by a plateau phase charactarised by a gradual decrease in $[Ca^{2+}]i$ levels. The PEP-/- cells exhibited significant drop (34%, $p \leq 0.001$) in $[Ca^{2+}]i$ mobilisation as shown by the time curve (Figure 3.2A) and the quantification of total $[Ca^{2+}]i$ from the area under the time curve (AUC) (Figure 3.2B). This shows that PEP promotes $[Ca^{2+}]i$ in mast cells.

The role of PEP in $[Ca^{2+}]i$ regulation was therefore further investigated.

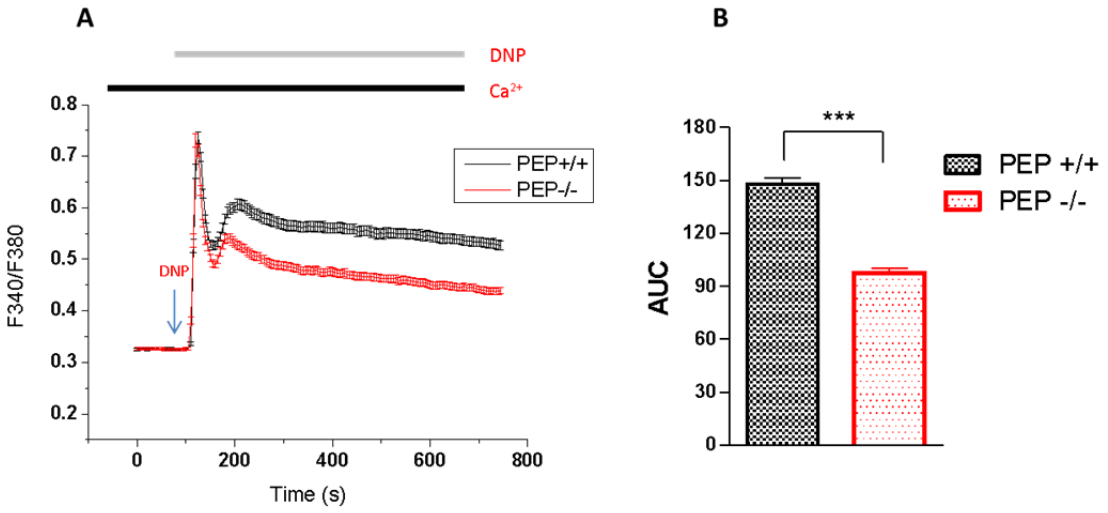


Figure 3.2 PEP deficient BMMCs have reduced Ca^{2+} mobilisation: (**A**) PEP+/+ and PEP-/- BMMCs were sensitized overnight with dinitrophenol human serum albumin (DNP-HSA)-specific IgE (300 ng/ml). Basal level [Ca^{2+}]i concentration was measured before activation with DNP-HSA (100 ng/ml) in optimal (2mM) Ca^{2+} physiological medium. [Ca^{2+}]i concentration was measured ratiometrically (340/380) at 20 s cycles using Fura-2 AM fluorescence dye. (**B**) shows total cytosolic Ca^{2+} levels determined by the area under the curve (AUC) for A. F340/380 is the ratio of Ca^{2+} dye fluorescence intensity at 340 and 380 nm light excitation. The result is presented as mean ± standard error of mean. *** $p< 0.001$, for unpaired two-tailed t test (n=3).

3.3 PEP MODULATES FCεRI SIGNALLING TO REGULATE CALCIUM MOBILISATION IN BMMCs

The current study and a previous report (Obiri *et al.* 2012) do show that activated PEP-/- BMMCs have reduced [Ca^{2+}]i levels, but, as to whether this impairment of [Ca^{2+}]i levels occurs via reduced antigen receptor (FcεRI) signalling or via FcεRI-independent effects, has not been clearly demonstrated.

To determine the mode of [Ca^{2+}]i impairment in the PEP-/- BMMCs, thapsigargin (TG), a pharmacological agent that raises [Ca^{2+}]i levels independent of FcεRI (Huber *et al.* 2000; Bird *et al.* 2008) was employed.

The mechanism of Ca^{2+} mobilisation in mast cells depends on Ca^{2+} release from intracellular stores mainly from the endoplasmic reticulum (ER) and Ca^{2+}

influx into the cell through Ca^{2+} channels in the plasma membrane (Parekh and Penner 1997; Parekh and Putney 2005; Bird *et al.* 2008). Thapsigargin triggers increased levels of $[Ca^{2+}]i$ by intracellular store release, via its inhibition of the ER Ca^{2+} regulating pump, the ER Ca^{2+}-ATPase. The ER Ca^{2+}-ATPase maintains Ca^{2+} equilibrium by pumping Ca^{2+} that is normaly leaked from the ER into the cytosol, back into the ER (Clapham 2007; Bird *et al.* 2008). Inhibition of this pump by thapsigargin causes ER Ca^{2+} extrusion into the cytosol leading to increased $[Ca^{2+}]i$ levels (Bird *et al.* 2008).

To check the effect of PEP deletion on $[Ca^{2+}]i$ mobilisation, basal level $[Ca^{2+}]i$ was first measured in 2 mM Ca^{2+} physiological solution for 100 s, after which $[Ca^{2+}]i$ levels were measured in the presence of thapsigargin (2 μM) under 3 different conditions: **a** = norminally free Ca^{2+} physiological solution, **b** = 0.1 mM Ca^{2+} physiological solution, and **c** = 2 mM Ca^{2+} physiological solution) (Figure 3.3). Ca^{2+} mobilisation in '**a**' depicts Ca^{2+} released from the ER, '**b**' shows Ca^{2+} from ER and entry through highly sensitive membrane channels, and '**c**' for maximal Ca^{2+} mobilisation from the ER, highly sensitive channels and less sensitive membrane channels.

In all three cases, there was no difference in the increase in $[Ca^{2+}]i$ in both PEP+/+ and PEP-/- BMMCs indicating no change in FcεRI independent mechanisms of Ca^{2+} regulation. Hence, the reduced $[Ca^{2+}]i$ mobilisation in DNP treated PEP-/- cells is likely due to modulation at the level of FcεRI-mediated signalling.

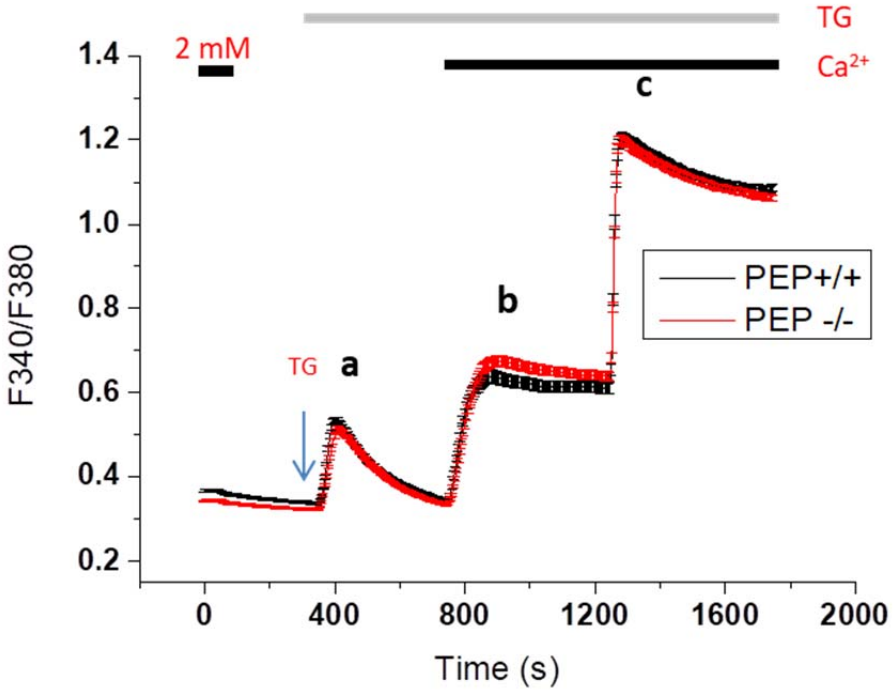


Figure 3.3 PEP gene deletion does not affect thapsigargin (TG)-induced Ca^{2+} mobilisation: a in norminally free Ca^{2+}, **b** in submaximal extracellular Ca^{2+} (0.1mM) and **c** in maximal level of extracellular Ca^{2+} (2 mM). F340/380 is the ratio of Ca^{2+} dye fluorescence intensity at 340 and 380 nm light excitation. The result is presented as the mean at various time points. Thapsigagin was used at 2 μM.

3.4 GENOMEWIDE EFFECTS OF PEP DEFICIENCY IN BMMCs

To identify mast cell effector functions that may be affected by PEP gene deletion, a transcriptomic approach (RNA-sequencing) was adopted.

The main goals in this analysis were:

A. to identify differences in basal (resting) level gene expression between PEP+/+ and PEP-/- BMMCs and
B. to identify differences in antigen-induced gene expression between PEP+/+ and PEP-/- BMMCs that may contribute to changes in mast cell effector functions.

PEP+/+ and PEP-/- BMMCs were sensitised with dinitrophenol (DNP)-specific IgE (500 ng/ml) for 2 h after which cells were either left unactivated

(sensitized) or activated with DNP (200 ng/ml) for 1 h. RNA was extracted from two replicates each, for four experimental conditions:

1) IgE sensitised PEP+/+ (basal),
2) IgE sensitised PEP-/- (basal),
3) DNP treated PEP+/+ (activated) and
4) DNP treated PEP-/- cells (activated)

The gene expression pattern of all the replicates as displayed in a cluster heatmap of the normalised expression (variance transformed) shows genes with low expression (blue) and high (red) under different conditions of treatment (Figure 3.4A).

3.4.1 PEP DEFICIENCY DOES NOT AFFECT BASAL LEVEL GENE EXPRESSION

To identify differences in the pattern of gene expression under basal conditions in the two cell types, PEP+/+ IgE (Figure 3.4A, columns 1 and 2) was compared with that of the PEP-/- IgE (Figure 3.4A, columns 3 and 4). The fold change values (Log2FoldChange) and the level of significance (adjusted p-value ≤ 0.05, Benjamini-Hochberg) are displayed in a volcano plot to highlight genes that are significantly downregulated (blue) or upregulated (red) in the PEP-/- compared to the PEP+/+ cells (Figure 3.4B). This analysis identified PTPN22 (PEP) as the only gene significantly downregulated in the PEP-/- cells compared to PEP+/+ (Figure 3.4B), which means that deletion of PEP does not affect basal level gene expression in BMMCs.

3.4.2 PEP DEFICIENCY ALTERED ANTIGEN-INDUCED GENE EXPRESSION

To determine the effect of PEP gene deletion on the antigen response, a three step analysis was done:

(1) to identify genes that were at least 2 fold misregulated (downregulated or upregulated) in the PEP+/+ in response to antigen (DNP) comparing PEP+/+ IgE (basal, columns 1 and 2) with PEP+/+ DNP (activated, columns 5 and 6).

(2) to identify genes that were at least 2 fold misregulated (downregulated or upregulated in the PEP-/- in response to antigen (DNP) by comparing PEP-/- IgE (basal, columns 3 and 4) against PEP-/- DNP (activated, columns 7 and 8) and

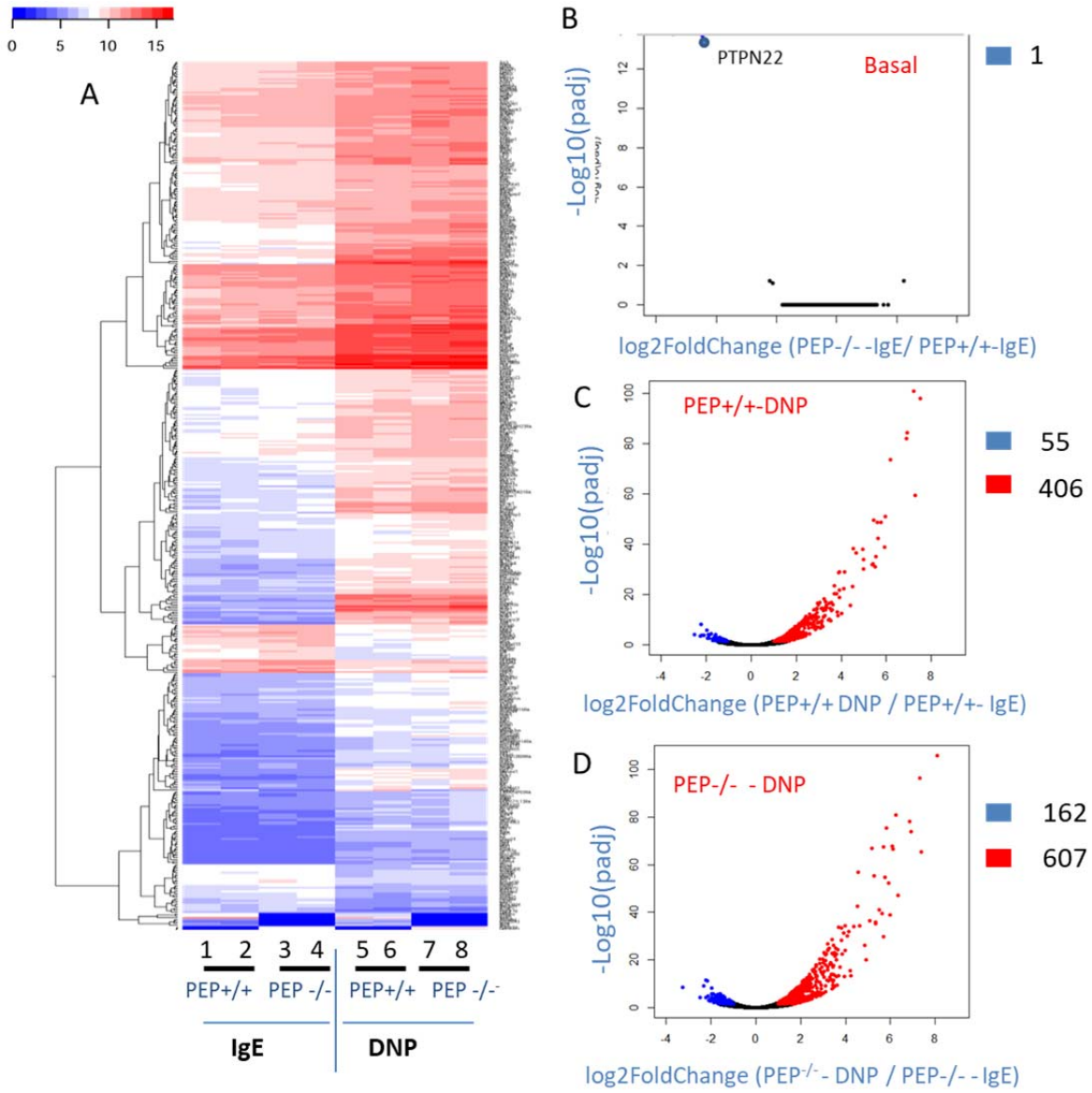
(3) to identify key differences between activated PEP+/+ and PEP-/- cells by comparing the output data from analysis (1) and (2).

The results showed that in both activated PEP+/+ (Figure 3.4C) and PEP-/- BMMCs (Figure 3.4D) many more genes were upregulated (red) than were downregulated (blue) in response to antigen. In activated PEP+/+ BMMCs, 461 genes were misregulated of which 55 (11.9%) were down regulated and 406 (88.1%) were upregulated. Activated PEP-/- BMMCs on the other hand showed 769 genes were misregulated in response to antigen, 162 (21.1%) being down regulated and 607 (78.9%) being upregulated. What is striking is that there were almost twice as many genes misregulated (down or upregulated) in response to antigen in the PEP-/- as compared to the PEP+/+ BMMCs, indicating that PEP deficiency induces remarkable changes in antigen-regulated gene expression, showing that PEP is an essential modulator of FcεRI receptor signalling.

Analysis to identify differences in the gene expression pattern between activated PEP+/+ and PEP-/- BMMCs as illustrated by Venn diagram (Figure 3.4E) showed that almost all the genes that were misregulated in response to

antigen in the PEP+/+ BMMCs were also misregulated in the PEP-/- cells except for 9 genes that were uniquely misregulated in the PEP+/+ cells. About, 452 genes were common to both PEP+/+ and PEP-/- cells, but as many as 317 genes (41.2%) of the total 769 genes misregulated by antigen in PEP-/- BMMCs were unique to PEP-/- BMMCs, which shows that deletion of PEP induces unique changes in antigen-induced gene regulation.

The biological significance of these changes was explored in subsequent analysis described below.



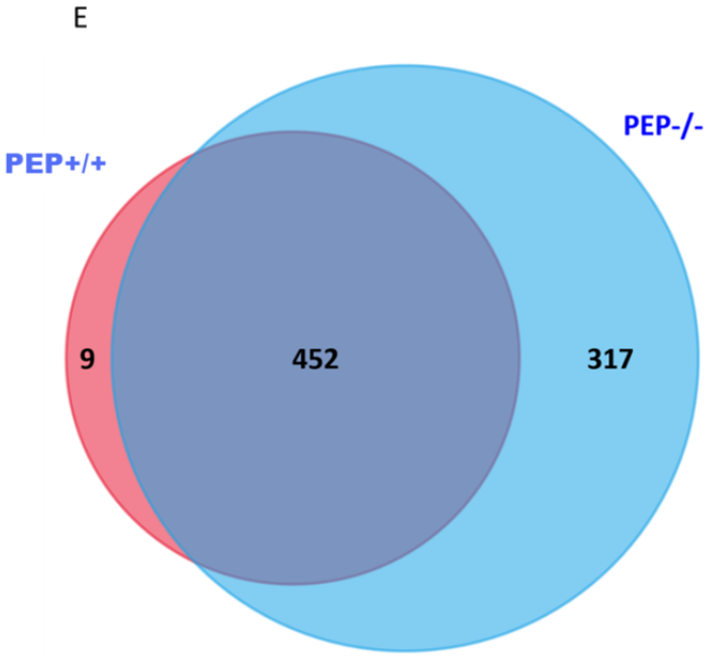


Figure 3.4 Deletion of PEP induces changes in gene expression profile of activated BMMCs: A is a colour-coded heatmap of the normalised expression level of genes in samples representing two replicates each for four different experimental conditions. Genes were hierachically clustered using Euclidean distances in the R software packages. The colour gradient shows blue for low and red for high expression of genes. **B** is a volcano plot showing the distribution of genes that are significantly (adjusted *p* value ≤ 0.05) expressed at least 2 fold lower (blue) or higher (red) in the PEP-/- IgE (basal) when compared to PEP+/+ IgE (basal), **C** shows genes downregulated (blue) or upregulated (red) in the PEP+/+ in response to antigen by comparing PEP+/+ DNP (activated) with PEP+/+ IgE (basal) and **D** genes downregulated (blue) and upregulated (red) in the PEP-/- in response to antigen by comparing the PEP-/- DNP (activated) with PEP-/- IgE (basal). **E** is a scaled Venn diagram showing the similarities and differencies in the antigen-induced gene profile between PEP+/+ and PEP-/- BMMCs.

3.4.3 PEP-/- BMMCs SHOWED REDUCED CYTOKINE/CHEMOKINE GENE EXPRESSION AND MISREGULATED MAPK SIGNALLING IN RESPONSE TO ANTIGEN

To explore the biological significance of the differences between activated PEP+/+ and PEP-/- BMMCs, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was carried-out using the publicly available online program 'Database for Annotation, Visualization and Integrated Discovery' [DAVID Bioinformatics Resources 6.8, NIAID/NIH] (Huang *et al.* 2007a; Huang *et al.* 2007b). A cut-off criterion of adjusted *p*-value ≤ 0.1 (Benjamini-

Hochberg) was used for selection of KEGG pathways of biological significance.

From the results, no KEGG pathway was found for the 9 genes that were uniquely misregulated in PEP+/+ in response to antigen, but some of the 317 genes that were unique to the PEP-/- were associated with KEGG pathways although based on the cut-off criterion none of the pathways was significantly enriched to be considered as biologicaly important for this analysis (Table 3.1). Nevertheless, the assoiated pathways involve immune cell functions such leukocyte transendothelial migration, T cell receptor signaling pathway and natural killer cell mediated cytotoxicity and also pathways such as MAPK signalling which forms an integral part of pathways that mediate immune cell functions (Table 3.1).

For the 452 genes commonly regulated in both cell types, the question was asked whether any of these genes show differences in their pattern of expression between the PEP+/+ BMMCs and PEP-/- cells. Hence their foldchange in response to antigen in the PEP-/- cells was compared with that of the PEP+/+ cells. Genes that were found to be expressed at least 1.2 fold lower or higher in the PEP-/- were filtered out.

In all, 138 genes (30.5%) out of the 452 were identified to have higher expression in the PEP-/-, while 32 (7.1%) showed lower expression when compared to PEP+/+ BMMCs. KEGG pathway analysis using the 32 genes that showed low expression in PEP-/- BMMCs identified 11 KEGG pathways that were significantly enriched with genes (Table 3.2). FcεRI signaling, the main pathway activated by antigen in mast cells was one of the pathways significantly affected. Associated with the FcεRI pathway are cytokines/chemokines [TNFα, IL13 and CSF2 (GM-CSF)], which are downstream gene products of FcεRI activation (Figure 3.5).

Table 3.1 KEGG pathways associated with the 317 genes uniquely misregulated in PEP-/- cells in response to antigen. (Benjamini-Hochberg Adjusted *p*-value ≤ 0.1, considered as significant).

Download File

Term	RT	Genes	Count	%	P-Value	Benjamini
Transcriptional misregulation in cancer	RT		9	2.9	4.7E-3	5.9E-1
MAPK signaling pathway	RT		11	3.6	6.6E-3	4.6E-1
Leukocyte transendothelial migration	RT		7	2.3	1.2E-2	5.4E-1
Osteoclast differentiation	RT		7	2.3	1.5E-2	5.0E-1
T cell receptor signaling pathway	RT		6	2.0	2.4E-2	6.0E-1
Hepatitis B	RT		7	2.3	2.8E-2	5.9E-1
AMPK signaling pathway	RT		6	2.0	5.0E-2	7.5E-1
Natural killer cell mediated cytotoxicity	RT		5	1.6	8.4E-2	8.8E-1
p53 signaling pathway	RT		4	1.3	8.9E-2	8.6E-1
Hippo signaling pathway	RT		6	2.0	9.1E-2	8.3E-1
cAMP signaling pathway	RT		7	2.3	9.1E-2	8.1E-1
Pathways in cancer	RT		11	3.6	9.7E-2	8.0E-1

Table 3.2 KEGG pathways associated with the 32 commonly regulated antigen responsive genes identified to have lower expression in PEP-/- DNP treated BMMCs compared to PEP+/+ DNP treated cells. (Benjamini-Hochberg Adjusted *p*-value ≤ 0.1, considered as significant).

Download File

Term	RT	Genes	Count	%	P-Value	Benjamini
Cytokine-cytokine receptor interaction	RT		8	25.8	1.5E-7	1.0E-5
Rheumatoid arthritis	RT		6	19.4	3.3E-7	1.1E-5
TNF signaling pathway	RT		6	19.4	1.4E-6	3.0E-5
Chemokine signaling pathway	RT		6	19.4	2.4E-5	4.0E-4
Jak-STAT signaling pathway	RT		4	12.9	2.5E-3	3.2E-2
Herpes simplex infection	RT		4	12.9	6.9E-3	7.2E-2
Fc epsilon RI signaling pathway	RT		3	9.7	7.5E-3	6.7E-2
Salmonella infection	RT		3	9.7	9.7E-3	7.6E-2
Hematopoietic cell lineage	RT		3	9.7	1.1E-2	7.8E-2
Toll-like receptor signaling pathway	RT		3	9.7	1.6E-2	9.9E-2
Chagas disease (American trypanosomiasis)	RT		3	9.7	1.7E-2	9.4E-2

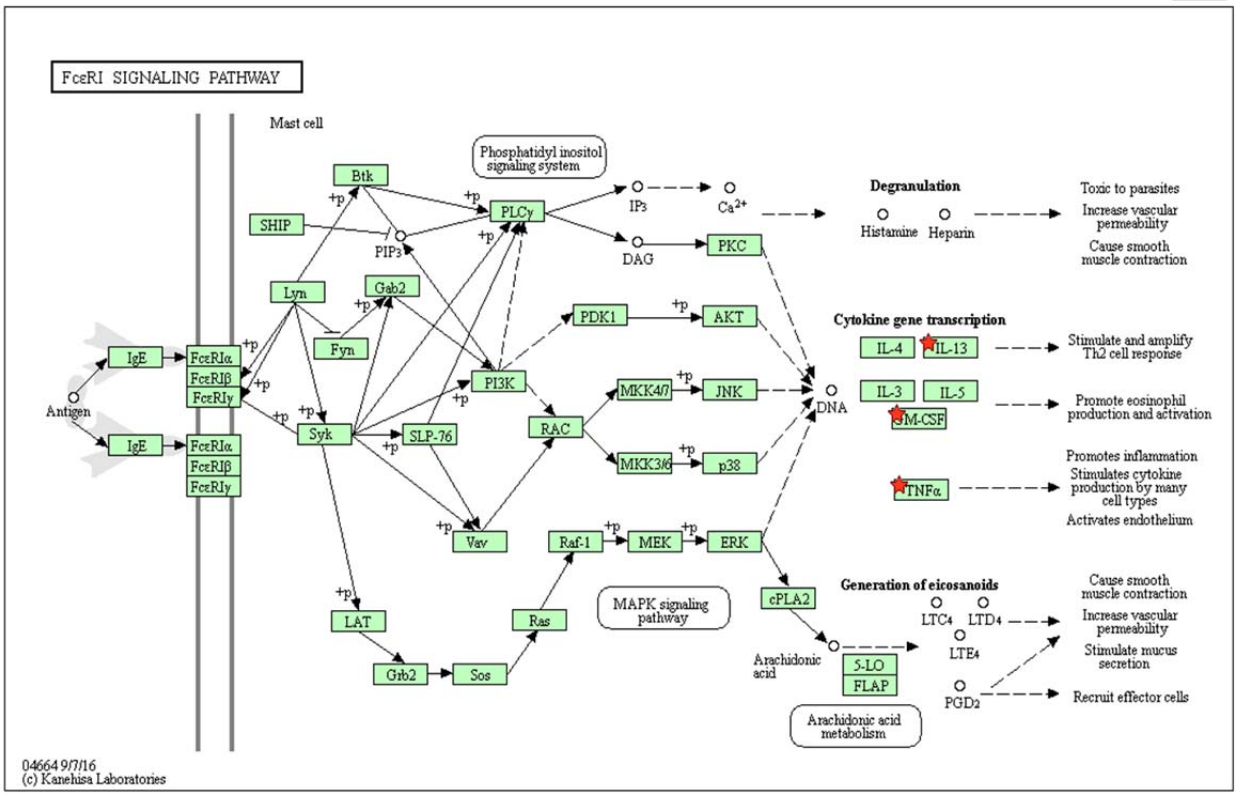


Figure 3.5 Deletion of PEP induces changes in the gene expression profile of activated BMMCs: KEGG pathway analysis using the 32 commonly regulated antigen responsive genes that showed lower expression in PEP-/- BMMCs identified cytokine/chemokine genes involved in FcεRI signalling.

KEGG analysis of the 138 (30.5%) commonly regulated genes that show higher expression in PEP-/- BMMCs identified MAPK signalling, which is part of FcεRI signalling, as the only significantly enriched pathway of importance (Table 3.3). The MARK pathway was associated with 11 genes. Seven (7) (DUSP2, FOS, GADD45B, JUND, MAP3K8, NR4A1, PDGFA) that could promote MAPK signalling and thus enhance inflammation, and four (4) others (DUSP1, DUSP4, DUSP5, DUSP8) which are all dual-specificity phosphatases (DUSP/MKP) that negatively regulate MAPK signalling (Figure 3.6). Therefore in the absence of PEP there is compensatory upregulation of genes that can activate MAPK signalling and genes that inhibit MAPK signalling.

Table 3.3 KEGG pathways associated with 138 commonly regulated antigen responsive genes identified to have higher expression in PEP-/- BMMCs compared to PEP+/+ cells. (Benjamini-Hochberg Adjusted *p*-value ≤ 0.1 considered as significant).

Term	RT	Genes	Count	%	P-Value	Benjamini
MAPK signaling pathway	RT		11	8.3	2.9E-5	3.9E-3
Osteoclast differentiation	RT		6	4.5	3.3E-3	2.0E-1
Leishmaniasis	RT		4	3.0	1.5E-2	4.9E-1
Pertussis	RT		4	3.0	2.1E-2	5.2E-1
FoxO signaling pathway	RT		5	3.8	2.2E-2	4.6E-1
Transcriptional misregulation in cancer	RT		5	3.8	4.4E-2	6.4E-1
Cytokine-cytokine receptor interaction	RT		6	4.5	4.6E-2	6.0E-1
Toll-like receptor signaling pathway	RT		4	3.0	4.7E-2	5.6E-1
Influenza A	RT		5	3.8	4.8E-2	5.3E-1
Chagas disease (American trypanosomiasis)	RT		4	3.0	5.0E-2	5.0E-1

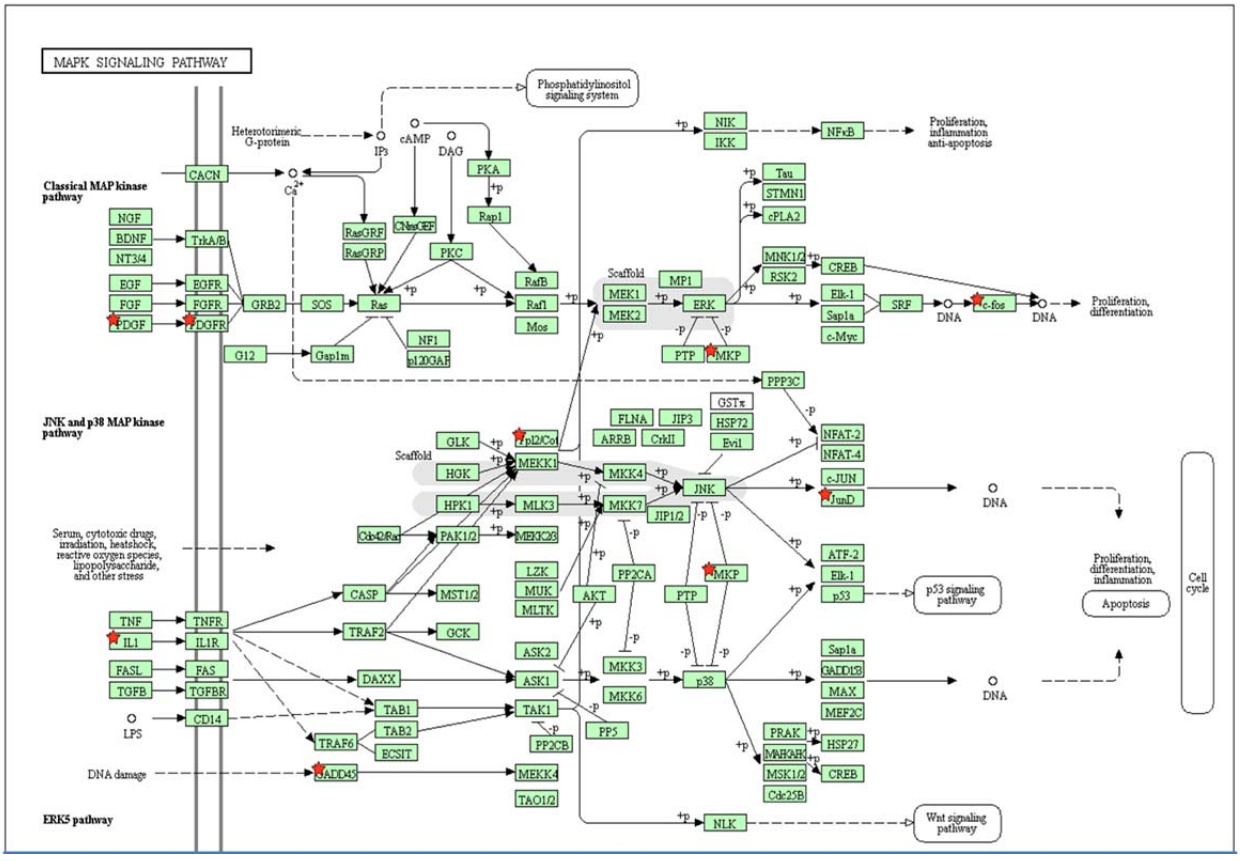


Figure 3.6 MAPK pathway is misregulated in PEP-/- cells in response to antigen in BMMCs: KEGG pathway analysis using the 138 commonly regulated antigen responsive genes that show higher expression in PEP-/- cells, identified 7 genes (DUSP2, FOS, GADD45B, JUND, MAP3K8, NR4A1, PDGFA) that can upregulate MAPK signalling and 4 genes (DUSP1, DUSP4, DUSP5, DUSP8) that can downregulate MAPK signalling.

As secretion of cytokines/chemokines is a key mast cell function, the cytokine/chemokine genes [TNFα, IL13 and CSF2 (GM-CSF)] which showed reduced expression in PEP-/- cells were subsequently validated using gene specific primers in quantitative real-time PCR (qRT-PCR) analysis.

3.4.4 VALIDATION OF CYTOKINE/CHEMOKINE GENES THAT SHOW REDUCED EXPRESSION TO ANTIGEN IN PEP-/- BMMCs

Quantitative real-time PCR (qRT-PCR) was used to validate the expression of cytokine/chemokine genes [TNFα, IL13 and CSF2 (GM-CSF)] that were selectively affected by PEP gene deletion.

BMMCs from PEP+/+ and PEP-/- mice were sensitized with DNP specific IgE (500 ng/ml) for 2 h and thereafter activated with DNP (200 ng/ml) for 1 h. RNA isolated from the cells were then reverse transcribed to cDNA and subjected to qRT-PCR analysis with gene specific primers.

The results showed no significant change in the basal level gene expression. However, antigen (DNP) treatment increased the expression of cytokine/chemokine genes in both cell types, with PEP-/- cells, showing lower expression in response to antigen compared to the PEP+/+ BMMCs in all the genes analysed. This shows that PEP is needed for maximal cytokine/chemokine production in mast cells (Figure 3.7).

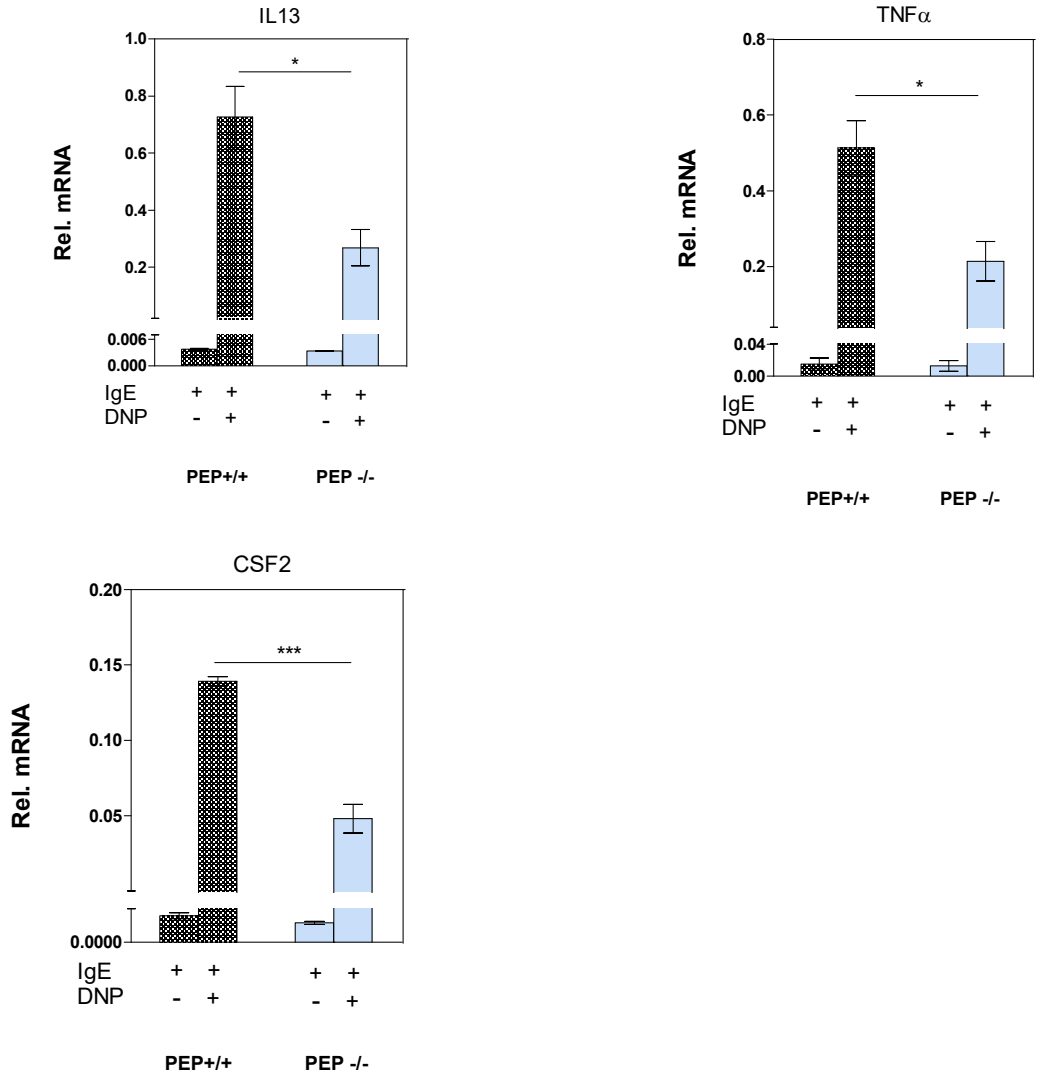


Figure 3.7 PEP-/- BMMCs have reduced cytokine/chemokine gene expression in response to antigen (DNP). Gene expression pattern of cytokine/chemokine genes identified to be differentially misregulated in response to DNP upon PEP gene deletion were analyzed by qRT-PCR. BMMCs were sensitized with anti–dinitrophenyl bovine serum albumin (DNP-BSA) IgE (500 ng/ml) for 2 h. After sensitization, cells were activated with DNP-BSA (200 ng/ml) for 1 h. RNA isolated from the cells were then reverse transcribed to cDNA and subjected to qRT-PCR analysis with gene specific primers. Results are presented as the mean ± standard error of mean. * $p < 0.05$, *** $p < 0.001$ for unpaired two-tailed t test (n=3).

3.4.5 INHIBITION OF PEP ACTIVITY CONTRIBUTES TO REDUCED CYTOKINE/CHEMOKINE GENE EXPRESSION IN PEP-/- BMMCS

To determine whether the reduced cytokine/chemokine gene expression in PEP-/- BMMCs is due to loss of PEP activity or some secondary effects, qRT-PCR experiments were performed with a potent and selective inhibitor of PEP activity. The inhibitor, L75NO4, was shown by He and colleagues to interact

with the catalytic pocket of PEP to inhibit PEP activity, leading to reduced anaphylaxis and mast cell degranulation in PEP-/- mice and BMMCs respectively (He *et al.* 2013).

BMMCs were treated with L75NO4 (5 μM) for 6 h after which cells were sensitized with IgE (500 ng/ml) for 2 h and subsequently activated with the antigen DNP (200 ng/ml) for 1 h. RNA extracted from the cells were reverse transcribed to cDNA for qRT-PCR quantification using TNFα as representative cytokine/chemokine gene.

The results showed that inhibition of PEP catalytic activity in PEP+/+ BMMCs with L75NO4 caused reduced TNFα gene expression in response to antigen (Figure 3.8A), just as was observed for the PEP-/- cells with no inhibitor (Figure 3.8B). Treatment of PEP-/- BMMCs with L75NO4 did not affect basal nor the antigen response (Figure 3.8C) when compared to PEP-/- BMMCs with no inhibitor (Figure 3.8B), showing that the L75NO4 effect on PEP is specific, and that PEP is directly involved in the regulation of antigen-induced cytokine/chemokine gene expression.

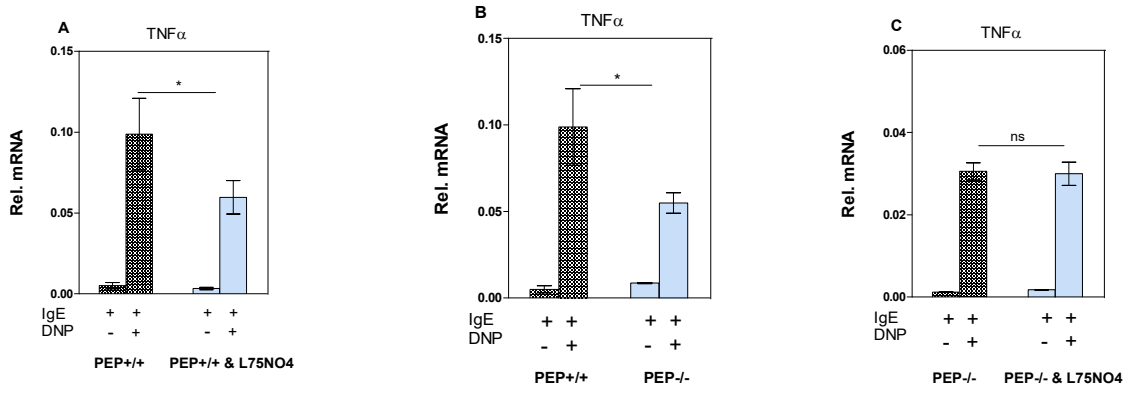


Figure 3.8 Inhibition of PEP activity contributes to the reduced cytokine/chemokine gene expression: BMMCs were first treated with PEP inhibitor L75NO4 (5 μM) for 6 h and thereafter sensitised with IgE (500 ng/ml) for 2 h and subsequently activated with the antigen DNP (200 ng/ml) for 1 h. RNA extracted from the cells was reverse transcribed into cDNA for qRT-PCR quantification of TNFα. Results are presented as the mean ± standard error of mean. * $p < 0.05$, ns (not significant), for unpaired two-tailed t test. (n=3).

3.5 PEP EXPRESSION IS UPREGULATED BY GLUCOCORTICOIDS

Glucocorticoids (GCs) inhibit mast cell functions and upregulate PEP expression in bone marrow derived mast cells (BMMCs) (Maier *et al.* 2007; Obiri *et al.* 2012). To investigate the possibility that GC-mediated regulation of mast cell functions involve PEP, there is the need to establish effective time points for GC-induced PEP expression. This experiment was carried out by treating cells with Dex (10^{-7} M) at given time points (10 min – 24 h). PEP expression was then assayed with whole cell lysate in a Western Blotting analysis. The results showed that GC induces sustained PEP expression from 4-24 h (Figure 3.9).

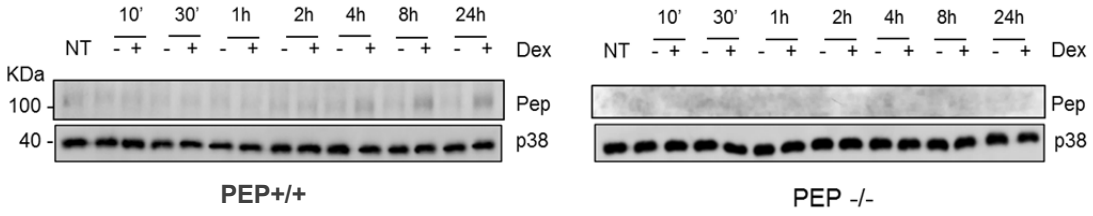


Figure 3.9 Dexamethasone (Dex) induces PEP expression in BMMCs: PEP+/+ and PEP-/- BMMCs were treated with Dex (10^{-7} M) (+) or vehicle control (-) at indicated time points (10 min – 24 h). Whole cell lysates were subjected to Western blot analysis using specific antibodies for PEP and p38 MAPK as control. NT is naïve non-treated control. Data is a representation of 2 independent experiments.

Having shown that GCs are able to induce PEP expression in BMMCs, the role of PEP in GC action in mast cells was further investigated.

3.6 PEP DEFICIENCY DOES NOT AFFECT GLUCOCORTICOID-INDUCED DOWNREGULATION OF CALCIUM MOBILISATION

Glucocorticoids (GCs) exhibit antiallergic/antiinflammatory effects by downregulating mast cell effector functions (Daeron *et al.* 1982; Oppong *et al.* 2013). Inhibition of signalling pathways that mediate Ca^{2+} mobilisation is one mechanism by which GCs downregulate mast cell functions (Obiri *et al.* 2012). As preceeding investigations in this work showed that PEP is involved in Ca^{2+} mobilisation, the question was asked whether PEP is also involved in GC-induced downregulation of Ca^{2+} mobilisation.

To answer this question Ca^{2+} mobilisation in GC treated PEP-/- BMMCs was compared to GC treated PEP+/+ cells. BMMCs bathed in Ca^{2+} (2 mM) containing physiological solution were simultaneously sensitised with DNP specific IgE (300 ng/ml) and treated with either vehicle control (Eth) or dexamethasone (Dex) for 24 h. Thereafter, cells were loaded with the Ca^{2+} sensing dye Fura-2 for 30 min. Thereafter, basal level $[Ca^{2+}]i$ was measured in resting mast cells for 100 s. The results showed that basal level intracellular calcium $[Ca^{2+}]i$ was not significantly different between the experimental groups (Figure 3.10A). Treatment of BMMCs with the antigen DNP triggered rapid increase in $[Ca^{2+}]i$ levels in all the experimental groups (Figure 3.10A) with the PEP-/- vehicle control (PEP-/- Eth) showing lower $[Ca^{2+}]i$ levels when compared to the PEP+/+ vehicle control (PEP+/+ Eth). On the hand, $[Ca^{2+}]i$ levels were downregulated in the BMMCs treated with DEX with no difference between PEP-/- cells treated with Dex when compared to the PEP+/+ treated with Dex (Figure 3.10A).

Quantification of the total $[Ca^{2+}]i$ mobilisation from area under the time curve (AUC) (Figure 3.10B) confirmed that antigen (DNP) treated PEP-/- BMMCs have reduced $[Ca^{2+}]i$ mobilisation in response to antigen when compared to PEP+/+ cells (Figure 3.10B). Nevertheless, deletion of PEP did not affect the

ability of GC to downregulate [Ca^{2+}]i mobilisation (Figure 3.10B), meaning that negative regulation of [Ca^{2+}]i mobilisation by GCs does not involve PEP.

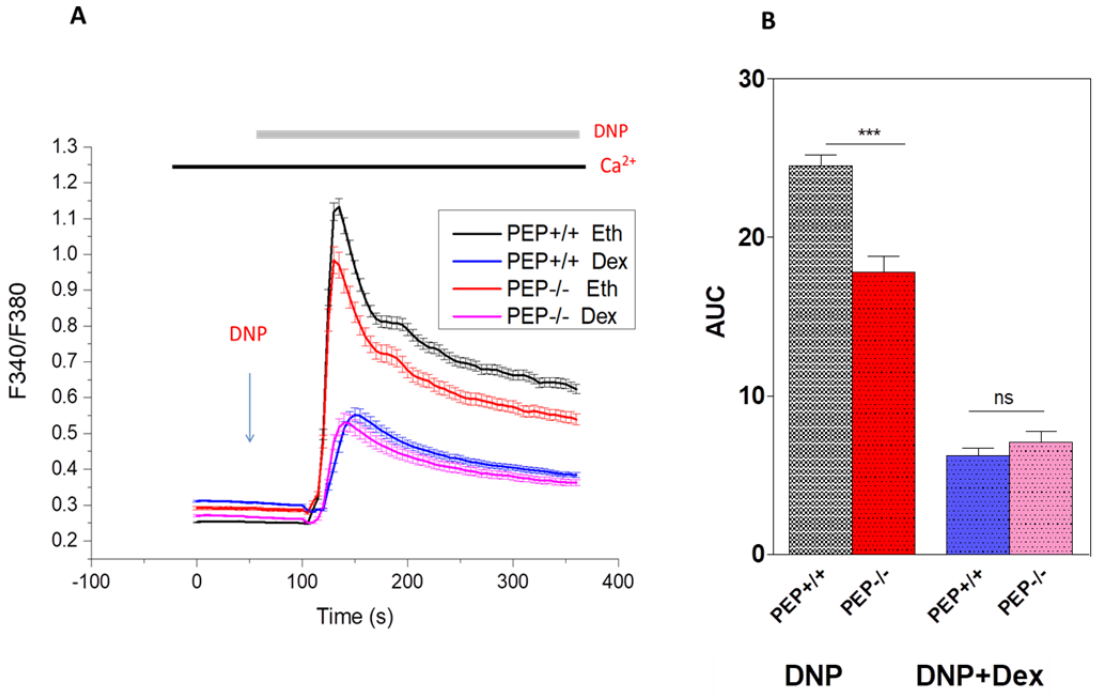


Figure 3.10 PEP deficiency does not impair GC-induced negative regulation of Ca^{2+} mobilisation in BMMCs. PEP+/+ and PEP-/- BMMCs were simultaneously sensitised with DNP-HSA specific IgE (300 ng/ml) and pretreated with either dexamethasone (Dex) (100 nM) or vehicle control (ethanol, Eth) for 24 h before activation with DNP-HSA (100 ng/ml). The cytosolic [Ca^{2+}]i concentration was estimated ratiometrically at two wavelenghs (340/380 nm) using Fura-2 fluorescence dye. **A** is the time curve for [Ca^{2+}]i mobilisation and **B** shows total [Ca^{2+}]i levels determined by the area under the time curve in A (AUC). Data represent the mean ± standard error of mean. *** $p < 0.001$ for unpaired two-tailed t test ns (not significant) (n= 3).

As GCs also downregulate cytokine/chemokine gene expression, the involvement of PEP in this process was analysed.

3.7 DELETION OF PEP DOES NOT AFFECT GLUCOCORTICOID-INDUCED NEGATIVE REGULATION OF CYTOKINE/CHEMOKINE GENE EXPRESSION

PEP+/+ and PEP-/- BMMCs were pretreated with Dex (10^{-7} M) for 5 h and sensitized with DNP specific IgE (500 ng/ml) for 2 h. After sensitization, cells were activated with DNP (200 ng/ml) for 1 h. RNA isolated from the cells were then reverse transcribed to cDNA for qRT-PCR analysis with gene specific primers for the three cytokines/chemokine genes (IL-13, TNFα, CSF2) that were identified to be regulated by PEP. The results showed that antigen induces increased expression of these genes and that their expression is down regulated by GC to the same level in both the PEP+/+ and PEP-/- BMMCs (Figure 3.11), meaning that GC-induced downregulation of these cytokines/chemokines does not involve PEP.

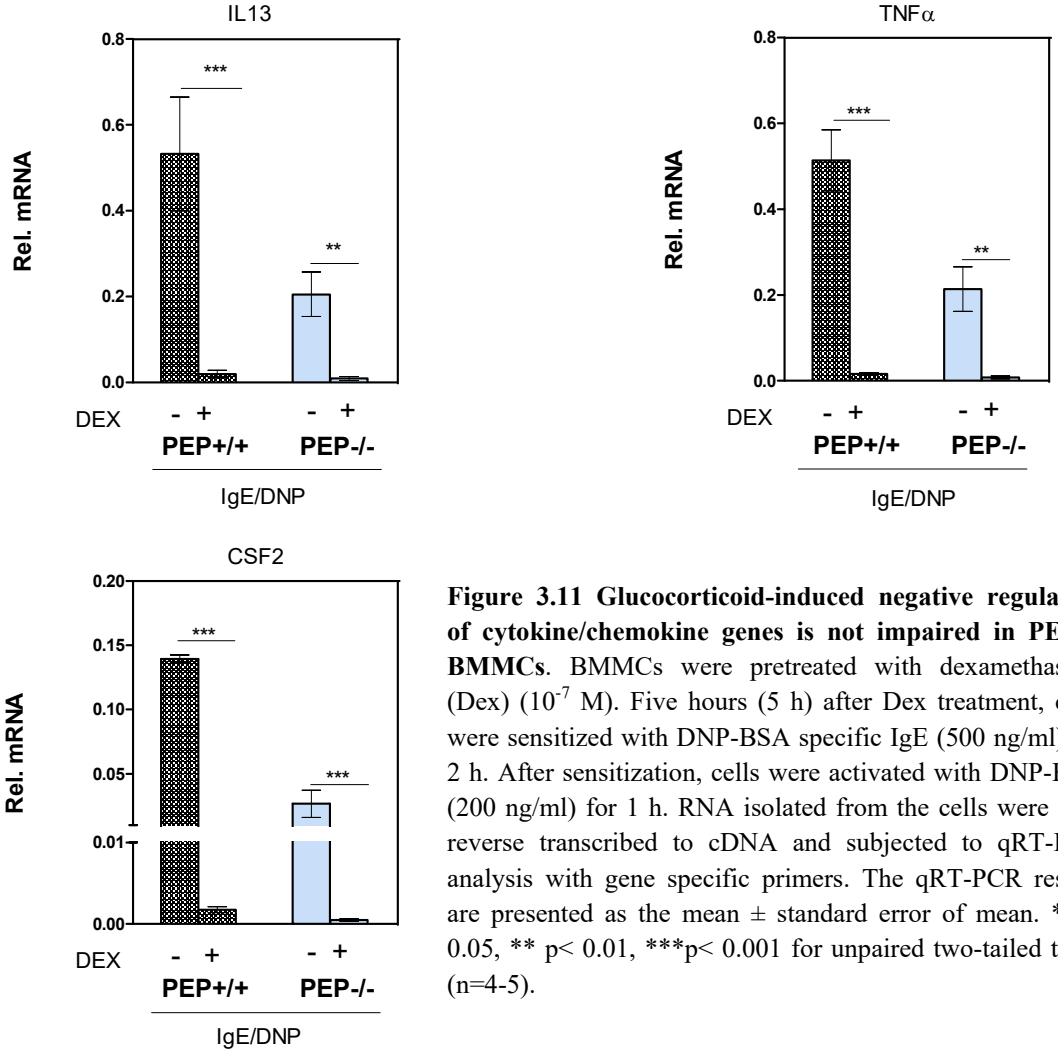


Figure 3.11 Glucocorticoid-induced negative regulation of cytokine/chemokine genes is not impaired in PEP-/- BMMCs. BMMCs were pretreated with dexamethasone (Dex) (10^{-7} M). Five hours (5 h) after Dex treatment, cells were sensitized with DNP-BSA specific IgE (500 ng/ml) for 2 h. After sensitization, cells were activated with DNP-BSA (200 ng/ml) for 1 h. RNA isolated from the cells were then reverse transcribed to cDNA and subjected to qRT-PCR analysis with gene specific primers. The qRT-PCR results are presented as the mean ± standard error of mean. * $p< 0.05$, ** $p< 0.01$, ***$p< 0.001$ for unpaired two-tailed t test (n=4-5).

As GC action on immune cell functions can vary with the dose of GC (Chen *et al.* 2013), the effect of PEP on GC action was analysed in a dose-response experiment, with varying concentrations of Dex (10^{-11}-10^{-6} M), using qRT-PCR analysis. Gene expression of TNFα, one of the cytokines/chemokines regulated by both GC and PEP in response to antigen, was used as a read-out. Treatment of PEP+/+ and PEP-/- BMMCs with antigen and GC was carried out as described ealier for section 3.7.

The results showed that antigen-induced cytokine/chemokine gene expression is lower in the PEP-/- BMMCs compared to the PEP+/+ cells, and that GCs dose-dependently inhibited the antigen response in both cell types

(Figure 3.12). EC_{50} values (effective concentration that produces 50% of maximum response) for GC treatment extrapolated from the dose response curves were statistically similar for both cell-types (PEP+/+ = 1.89E-10 M, PEP-/- = 2.27E-10 M), meaning that PEP may not play a role in GC-induced negative regulation of this cytokine/chemokine expression, in agreement with the previous results in Figure 3.11.

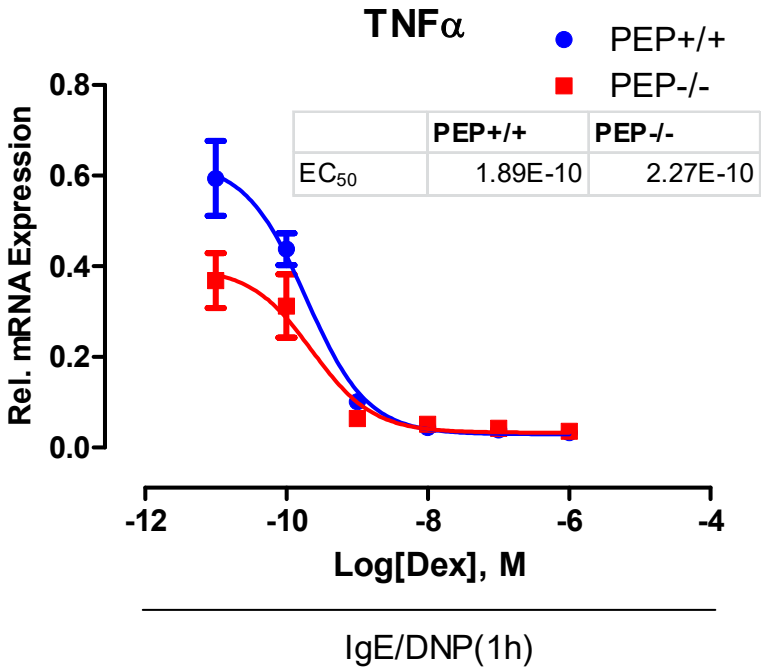


Figure 3.12 Deletion of PEP does not affect glucocorticoid-induced regulation of cytokine/chemokine gene expression. PEP+/+ and PEP-/- BMMCs were pre-treated with dexamethasone (Dex) (10^{-11} – 10^{-6} M). Five hours (5 h) after Dex treatment, cells were sensitized with DNP-BSA specific IgE (500 ng/ml) for 2 h. After sensitization, cells were activated with DNP-BSA (200 ng/ml) for 1 h. RNA isolated from the cells were then reverse transcribed to cDNA and subjected to qRT-PCR analysis with gene specific primers for TNFα. The points on the curve are presented as the mean ± standard error of mean for unpaired two-tailed t test (n=3).

3.8 RNA-SEQ EXPERIMENT TO INVESTIGATE THE ROLE OF PEP IN GLUCOCORTICOID ACTION

To investigate further the role of PEP in GC action, a global approach using RNA-seq experiments were employed.

The treatment of cells with GC for RNA-seq analysis was carried out in parallel with the RNA-seq experiment for antigen treatment and the analysis carried out in a three-step approach:

(1) to identify genes regulated by GC in antigen treated PEP+/+ cells.

(2) to identify genes regulated by GC in antigen treated PEP-/- cells.

(3) to compare the genes identified in (1) and (2), to show genes whose regulation by GC is altered due to PEP gene deletion.

3.8.1 DELETION OF PEP ALTERS GLUCOCORTICOID RESPONSE TO ANTIGEN IN BMMCS

RNA-seq gene expression data from PEP+/+ and PEP-/- BMMCs activated with DNP were compared with activated cells treated with Dex (10^{-7} M).

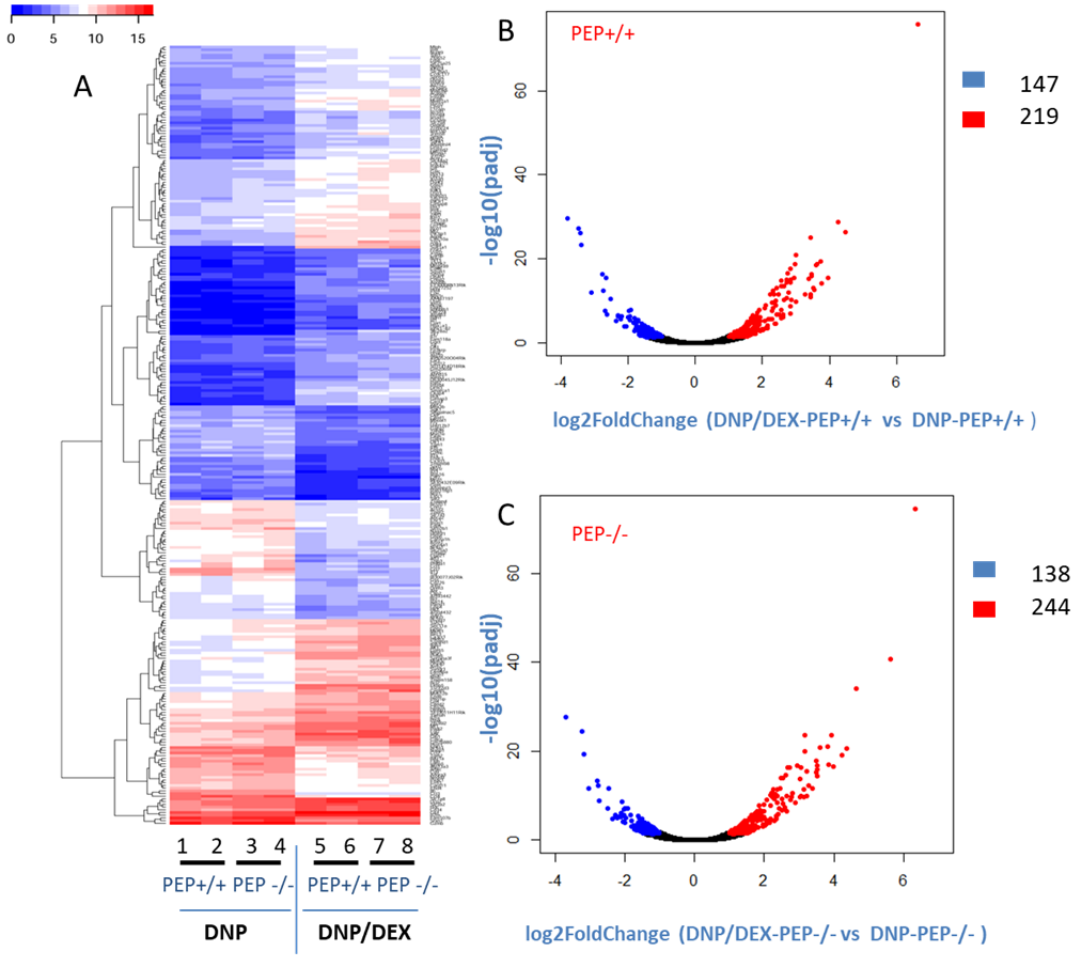
The gene expression pattern of all the replicates as displayed in a cluster heatmap of the normalised expression (variance transformed) shows genes with low (blue) and high (red) expression under different conditions of treatment (Figure 3.13A).

PEP+/+ BMMCs treated with the antigen DNP (PEP+/+ DNP, columns 1and 2) was compared with PEP+/+ cells treated with antigen and Dex (PEP+/+ DNP/DEX, columns 5 and 6) to identify antigen regulated genes that are expressed at least 2 fold lower (blue) or higher (red) in response to GC in PEP+/+ cells (Figure 3.13B). Similarly, DNP treated PEP-/- BMMCs (PEP-/- DNP, culumns 3 and 4) was compared with PEP-/- cells treated with DNP and GC (DEX) (PEP-/- DNP/DEX, columns 7 and 8) to identify antigen-regulated genes that respond to GC treatment in PEP-/- BMMCs (Figure 3.13C).

About 366 antigen regulated genes in PEP+/+ BMMCs were misregulated by Dex, of which 147 (40.2%) were downregulated (blue) while 219 (59.8%) were

upregulated (red) (Figure 3.13B). In PEP-/- BMMCs, 382 genes were misregulated in response to Dex, 138 (36.1%) being downregulated (blue) while 244 (63.9%) were upregulated (red) (Figure 3.13C).

To identify differences in Dex-induced regulation of gene expression between the two cell types, the gene expression pattern between Dex treated activated PEP+/+ BMMCs was compared to activated PEP-/- cells treated with Dex. The results displayed as Venn diagram (Figure 3.13D) showed that 82 genes were uniquely regulated by Dex in the PEP+/+ BMMCs while 98 were exclusively regulated by Dex in the PEP-/- cells. On the other hand, 284 genes were commonly regulated by Dex in both cell types. Pathway analysis was subsequently carried out with these genes to determine the functional significance of these differences.



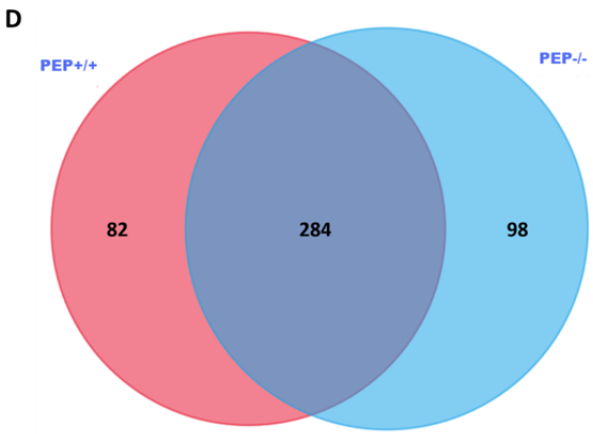


Figure 3.13 Deletion of PEP induces changes in the GC response to antigen: A is a colour-coded heatmap of the normalised expression level of genes in eight (8) samples representing two (2) replicates for each of four (4) different experimental conditions. Genes were hierachically clustered using Euclidean distances in the R software packages. The colour gradient shows blue for low expression and red for high expression. **B** is a volcano plot showing the distribution of genes that are significantly (adjusted *p* value ≤0.05) expressed at least 2 fold lower (blue) or higher (red) in the PEP+/+ DNP/DEX when compared to PEP+/+ DNP. **C** shows genes downregulated (blue) or upregulated (red) in the PEP-/- DNP/DEX when compared to PEP-/- DNP. **D** is a scaled Venn diagram showing the similarities and differencies in GC regulated genes between DEX treated PEP+/+ and DEX treated PEP-/- BMMCs by comparing gene expression pattern in B and C.

3.8.2 DELETION OF PEP ALTERS GLUCOCORTICOID-INDUCED REGULATION OF PTGS2/COX-2 AND PTGDS GENE EXPRESSION

For the 82 genes uniquely regulated by Dex in PEP+/+ BMMCs, 46 (56.1%) were negatively regulated while 36 (43.9%) were upregulated. No KEGG pathway was found to be significantly (adjusted *p*-value ≤ 0.1, Benjamini-Hochberg) enriched with these genes, but the analysis identified amongst these 82 genes, prostaglandin-endoperoxide synthase 2/cyclooxygenase 2 (PTGS2/COX-2), a key enzyme, involved in the production of lipid/eicosanoid mediator synthesis and which is a target of GC action. The expression of COX-2 was upregulated in both PEP+/+ and PEP-/- BMMCs by the antigen DNP, but its negative regulation in the PEP-/- by Dex was abrogated, showing that PEP is needed for the negative regulation of COX-2 by GCs.

Furthermore, of the 98 genes that were exclusively regulated by Dex in PEP-/- cells, 37genes (37.8%) and 61 genes (62.2%) were downregulated and upregulated respectively by GC with no associated KEGG pathway. None of the genes in this group was identified to be of unique importance to mast cell effector functions and GC action, although they are involved in cell signalling.

For the 284 commonly regulated genes, a cut off criterion of 2 fold change identified 3 genes (OLFR224, PTGDS, SLC28a2) whose expression is upregulated in Dex treated PEP-/- cells when compared to Dex treated PEP+/+ cells. Although these genes were also not enriched in any KEGG pathway, one of the genes in this group, PTGDS (lipocalin-type prostaglandin D2 synthase), is an isomerase that promotes lipid mediator synthesis by converting COX-2 derived PGH_2 (prostaglandin H2) to PGD_2 (prostaglandin D2) (Figure 1.2). As the RNA-seq results show that PEP is needed for GC-

induced regulation of PTGS2/COX-2 and PTGDS, the expression of these genes were validated in a qRT-PCR analysis.

3.8.3 QUANTITATIVE REAL-TIME PCR ANALYSIS CONFIRMED IMPAIRMENT OF GLUCOCORTICOID-INDUCED REGULATION OF PTGS2/COX-2 AND PTGDS EXPRESSION BY PEP GENE DELETION

To validate the effect of PEP gene deletion on GC-induced regulation of PTGS2/COX-2 and PTGDS gene expression, BMMCs were treated with Dex and the antigen DNP as described for the RNA-seq analysis in section 3.8. The results showed that antigen induces increased expression of PTGS2/COX-2 in both PEP+/+ and PEP-/- cells (Figure 3.14A), with the PEP-/- BMMCs showing decresed PTGS2/COX-2 expression in response to antigen (Figure 3.14A), meaning that PEP is needed for antigen-induced PTGS2/COX-2 expression. On the other hand, whereas Dex significantly reduces the antigen response in PEP+/+ BMMCs, the Dex response in the PEP-/- cells was lost (Figure 3.14A), suggesting that PEP is needed for GC-mediated negative regulation of antigen-induced COX-2 gene expression.

Furthermore, both PEP+/+ and PEP-/- BMMCs showed low expression of PTGDS in activated cells. However, Dex treatment upregulated the expression of PTGDS gene in both PEP+/+ and PEP-/- BMMCs, but the upregulation of PTGDS expression was enhanced by PEP gene deletion, as Dex treated PEP-/- BMMCs showed significantly higher PTGDS levles when compared to Dex treated PEP+/+ cells (Figure 3.14B). The implication of PTGDS upregulation in PEP-/-BMMCs cannot be easily deduced from the current analysis, because this isoform of prostaglandin D2 synthase is not known to be active in immune cells such as mast cells (Urade and Hayaishi 2000; Firestein *et al.* 2015). But, altogether, the results show that PEP is needed for GC-induced negative regulation of lipid mediator synthesis in mast cells.

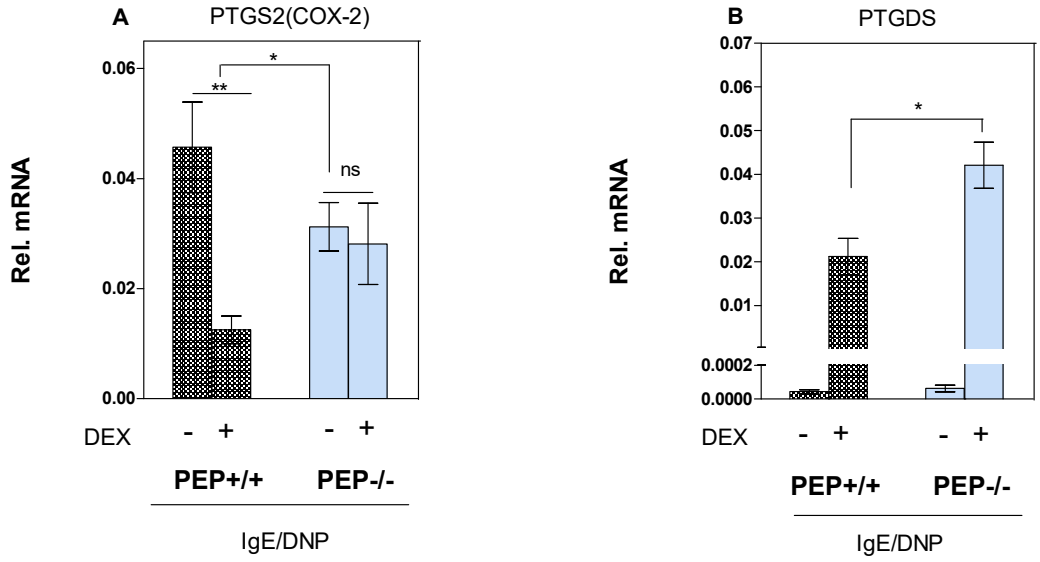


Figure 3.14 GC-induced regulation of PTGS2/ COX-2 and PTGDS is impaired in PEP-/- BMMCs. BMMCs were pretreated with dexamethasone (Dex) (10^{-7} M). Five hours (5 h) after GC treatment, cells were sensitized with DNP-BSA specific IgE (500 ng/ml) for 2 h. After sensitization, cells were activated with DNP-BSA (200 ng/ml) for 1 h. RNA isolated from the cells were then reverse transcribed to cDNA and subjected to qRT-PCR analysis with gene specific primers. The qRT-PCR results are presented as the mean ± standard error of mean. * $p< 0.05$, ** $p< 0.01$ for unpaired two tail t test (n=5).

3.8.4 INHIBITION OF PEP ACTIVITY CONTRIBUTES TO IMPAIRED GLUCOCORTICOID RESPONSE

To determine whether the impairment of GC-induced regulation of lipid mediator synthesis involves direct PEP activity, GC-induced negative regulation of COX-2 expression was analysed in BMMCs treated with the PEP inhibitor L75NO4.

In this analysis, PEP+/+ and PEP-/- BMMCs were pretreated with the L75NO4 for 1 h, after which cells were either treated with ethanol (vehicle control) or Dex (10^{-7} M) for 5 h. Thereafter, cells were sensitized with IgE (500 ng/ml) for 2 h and activated with the antigen DNP (200 ng/ml) for 1 h. RNA extracted from these cells were reverse transcribed to cDNA for qRT-PCR quantification using gene specific primers for COX-2.

The results showed that inhibition of PEP activity wth L75NO4 in PEP+/+ BMMCs caused reduced DNP-induced COX-2 gene expression (Figure 3.15A), just as was observed for the PEP-/- cells with no inhibitor (Figure 3.15B). Also, treatment of PEP-/- BMMCs with L75NO4 did not affect the antigen response when compared with PEP-/- BMMCs with no inhibitor (Figure 3.15C), showing that L75NO4 is specific.

The results also showed that inhibition of PEP activity with L75NO4 in PEP+/+ BMMCs abolished the Dex-induced negative regulation of COX-2 gene expression (Figure 3.15D), similar to what was observed for the PEP-/- cells treated with Dex (Figure 3.15E). Incubation of Dex treated PEP-/- BMMCs with L75NO4 did not cause any significant change in the Dex response when compared with Dex treated PEP-/- BMMCs with no inhibitor (Figure 3.15F), which further confirms that L75NO4 is specfic.

All together, the above observations showed that PEP is important for GC-induced negative regulation of lipid mediator synthesis in mast cells.

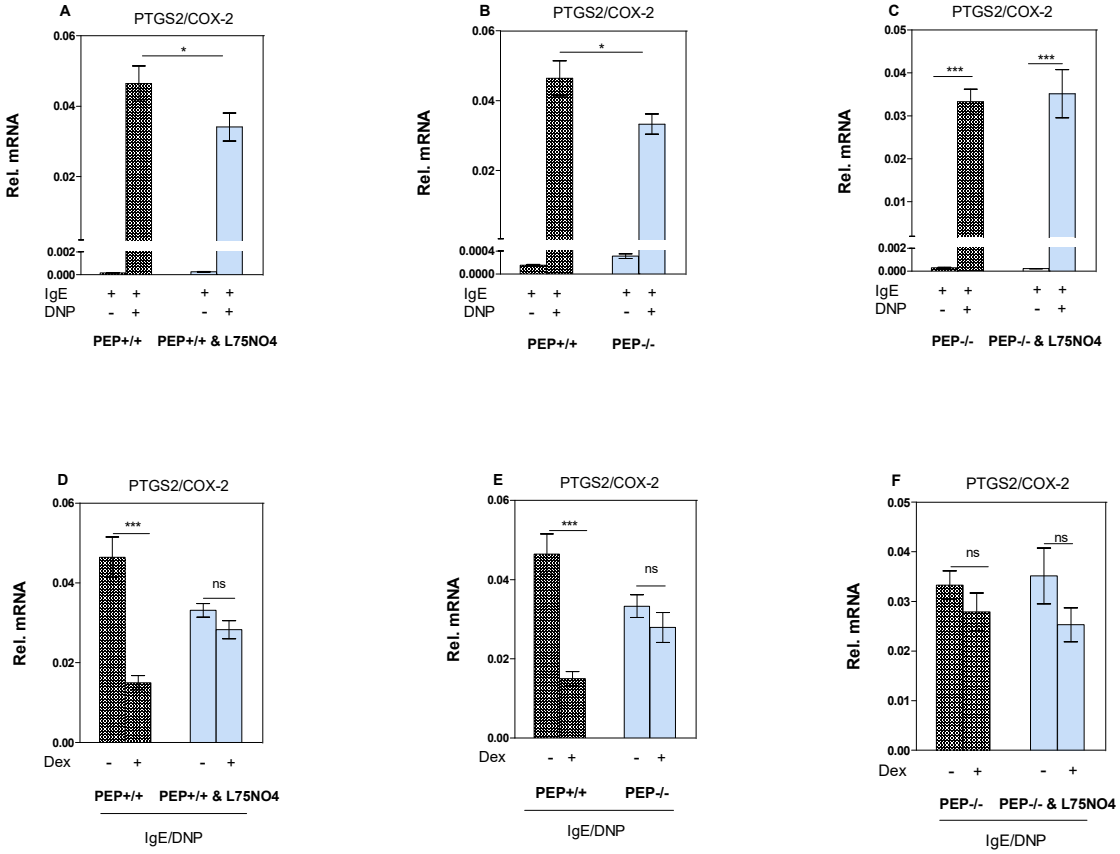


Figure 3.15 Inhibition of PEP phosphatase activity contributes to impaired GC-induced PTGS2/ COX-2 regulation in BMMCs. BMMCs were first treated with the inhibitor L75NO4 (5 uM) for 1 h. After 1 hour cells were either treated with ethanol control or dexamethasone (Dex) (10^{-7} M) for 5 h. After 5 h of Dex treatment, cells were sensitized with IgE (500 ng/ml) for 2 h and then activated with the antigen DNP (200 ng/ml) for 1 h. RNA extracted from the cells were reverse transcribed to cDNA for qRT-PCR quantification. The results are presented as the mean ± standard error of mean.* $p< 0.05$, *** $p< 0.001$, ns (not significant) (n=4).

Chapter 4

DISCUSSION

4.1 GENERAL DISCUSSION

PEP is a protein tyrosine phosphatase exclusively expressed in immune cells (Bottini and Peterson 2014), including mast cells (Maier *et al.* 2007). Disease associated studies showed that single nucleotide polymorphism (SNP) in the human orthologue of PEP (LYP) is a risk fatcor for chronic inflammatory conditions, notably rheumatoid arthritis (Vang *et al.* 2008; Bottini and Peterson 2014; Stanford and Bottini 2014), for which glucocorticoids (GCs) such as dexamethasone are employed as part of a repertoire of therapeutic agents (Kirwan *et al.* 2007). Interestingly, PEP was found to be up regulated by dexamethasone in mast cells (Maier *et al.* 2007), but the role of PEP in mast cell functions and and GC action is not fully understood.

Using PEP+/+ and PEP-/- mice and bone marrow derived mast cells (BMMCs) generated from these mice, the current work showed that PEP promotes antigen-mediated mast cell inflammatory/allergic response, and yet PEP is needed for the antiinflammatory/antiallergic action of GC in mast cells (Figure 4.1).

The finding in the current work that PEP-/- mast cells have reduced response to antigen was rather surprising, because deletion of a phosphatase like PEP is expected to increase the activity of the kinases it regulates and as such enhance mast cell signalling and mast cell action (Kalesnikoff and Galli 2010). This is what was observed in human and murine T cells, where overexpression experiments and studies in PEP -/- T cells showed that PEP is a negative regulator of T cell antigen receptor (TCR) signaling (Hasegawa *et al.* 2004; Brownlie *et al.* 2012; Bottini and Peterson 2014). Nevetheless, a search through

the literature suggests that PEP exhibits cell-type specific responses in murine systems. For instance, in B cells, the work of Hasegawa *et al.* (2004), Zikherman *et al.* (2009) and Dai *et al.* (2013), showed that BCR (B-cell antigen receptor)-induced calcium mobilization, ERK phosphorylation, total tyrosine phosphorylation, and proliferation of B cells was not different between PEP−/− and PEP+/+ cells B cells. This is different from observations in T cells, where deletion of PEP increased the phosphorylation of protein tyrosine kinases (PTKs) that activate T cell antigen receptor signalling, leading to enhanced proliferation and expansion of effector/memory T cells, as well as elevated T cell–dependent Ig (immunoglobulin) production *in vivo* and *in vitro* (Hasegawa *et al.* 2004; Brownlie *et al.* 2012; Bottini and Peterson 2014), meaning that PEP may not be needed for BCR signalling, but is important for TCR signalling.

Interestingly, some other reports suggests that PEP promotes myeloid cell (macrophage, dendritic cells, neutrophils) functions. In mouse marcrophages and dendritic cells, Wang and colleagues showed that PEP promotes type 1 IFN (interferon) production after TLR (Toll-like receptor) ligation (Wang *et al.* 2013). In addition, a very recent work by Vermeren and colleagues showed that PEP is needed for Fcγ-mediated neutrophil activation, as neutrophil effector functions, including production of reactive oxygen species and degranulation induced by immobilized immune complexes, were all reduced in PEP -/- neutrophils when compared to wild-type neutrophils (Vermeren *et al.* 2016). Since mast cells are also myeloid cells, it is not suprising that PEP promotes antigen receptor mediated responses in mast cells, as represented in the current work.

4.2 PEP PROMOTES ANTIGEN-INDUCED ANAPHYLAXIS

The current work showed that PEP promotes anaphylaxis, as PEP-/- BALB/c mice had reduced anaphylaxis compared to PEP+/+ mice.

This was unexpected for PEP, since the deletion of PEP is expected to increase the activity of protein tyrosine kinases that promote mast cell functions and anaphylaxis. One explanation that was thought of, for the current observation, was that, PEP exhibits strain specific responses. The reason being that, different strains of mice can show differences in immune response, due to their different genetic background (Noguchi *et al.* 2005; Finkelman 2007; Rivera *et al.* 2008). This is common in mast cell related models. For instance, the work of Yamashita and colleagues showed that 129/Sv mice were more susceptible to anaphylaxis than C57BL/6 mice (Yamashita *et al.* 2007). Furthermore, they showed that the protein tyrosine kinase Lyn is a negative regulator of allergic responses contrary to expectation, as mast cells from Lyn deficient 129/Sv mice exhibited enhanced degranulation. In contrast, the work of Kawakami and colleagues showed that mast cells from Lyn deficient (Lyn-/-) mice on a mixed C57BL/6 and 129/Sv genetic background exhibited normal responses (Kawakami *et al.* 2000) meaning that some mast cell responses may be strain dependent. However, the current observation is in agreement with the findings of Obiri *et al.* (2012), who also showed that PEP is a positive regulator of PSA in another mouse train, C57BL/6. This suggests that the role of PEP in anaphylaxis is likely independent of the genetic background of mice, although its role in immune cell response, differs in different cell types.

4.3 PEP MODULATES FcεRI SIGNALLING TO REGULATE MAST CELL FUNCTIONS

In this work, the cell type specific effect of PEP was analysed by studying its role in intracellular calcium ($[Ca^{2+}]i$) mobilisation in bone marrow derived mast cells (BMMCs).

Using thapsigargin – a chemical agent that induceds FcεRI-independent $[Ca^{2+}]i$ mobilisation – the present study showed that PEP promotes FcεRI-mediated $[Ca^{2+}]i$ mobilisation.

Intracellular calcium ($[Ca^{2+}]i$) mobilisation is the central most important signalling event that controls exocytotic release of mast cell preformed mediators (degranulation), which triggers anaphylaxis *in vivo* (Ma and Beaven 2009; Holowka *et al.* 2012). Due to the vital role of $[Ca^{2+}]i$ in antigen receptor signalling, analysis of $[Ca^{2+}]i$ mobilisation is key in the study of molecular components that regulate antigen receptor signalling (Feske 2007; Vig and Kinet 2009).

Most of the work to understand how PEP regulates antigen-induced Ca^{2+} mobilisation in immune cells concentrated on lymphocytes (T cells and B cells) (Bottini and Peterson 2014). In murine T cells, Hasegawa and colleagues found that antigen-induced $[Ca^{2+}]i$ mobilisation in PEP-/- cells was enhanced, compared to control PEP+/+ cells (Hasegawa *et al.* 2004), but antigen-induced Ca^{2+} mobilisation in B cells did not show any difference between the two cell types (Hasegawa *et al.* 2004; Zikherman *et al.* 2009; Dai *et al.* 2013). This shows that PEP exhibits cell-type specific response in $[Ca^{2+}]i$ mobilisation in immune cells. It is therefore not surprising that in this analysis PEP-/- BMMCs showed reduced $[Ca^{2+}]i$ mobilisation contrary to what was observed in T and B cells. Meaning that whereas PEP is a negative regulator of T cell antigen receptor functions, with no effect on B cell antigen receptor signalling (Hasegawa *et al.*

2004; Zikherman *et al.* 2009; Dai *et al.* 2013), PEP is a positive regulator of antigen-mediated mast cell functions.

Mechanistically, the antigen receptor signalling that mediates $[Ca^{2+}]i$ mobilisation is enhanced by the phosphorylation activities of receptor proximal kinases, notably Lck (lymphocyte-specific protein tyrosine kinase) (Lyn in mast cells) and Zap-70 (Zeta-chain-associated protein kinase 70) (Syk in mast cells) (Gjorloff-Wingren *et al.* 1999; Hasegawa *et al.* 2004). PEP gene knock-out studies showed that PEP negatively regulates T cell $[Ca^{2+}]i$ mobilisation by antagonising these kinases.

Although very little is known about the phosphorylation status of these kinases in PEP-/- mast cells, a preceding study by Obiri and colleagues showed that PEP-/- BMMCs have reduced phosphorylation of PLCγ (phospholipase C-gamma) (Obiri *et al.* 2012), an enzyme which indirectly depends on Lck and Zap-70 phosphorylation activities for its activation. In T cells, PEP was shown to regulate $[Ca^{2+}]i$ mobilisation by modulating the phosphorylation of Lck and Zap-70 phosphorylation (Hasegawa *et al.* 2004; Brownlie *et al.* 2012; Bottini and Peterson 2014). It is therefore possible that PEP may regulate $[Ca^{2+}]$ mobilisation in activated mast cells by modulating the activities of Lck (Lyn in mast cells) and Zap-70 (Syk in mast cells).

4.4 PEP PROMOTES CYTOKINE/CHEMOKINE GENE EXPRESSION IN MAST CELLS

In the present study, gene expression analysis (RNA-seq and qRT-PCR) showed that deletion of PEP reduces antigen-induced cytokine/chemokine gene expression, suggesting that PEP promotes cytokine/chemokine gene expression in activated mast cells.

The cytokine/chemokine genes whose expression was impaired were TNFα (Tumour necrosis factor alpha), IL13 (Interleukin 13) and CSF2/GM-CSF (Colony stimulating factor 2/Granulocyte-macrophage colony-stimulating factor). TNFα promotes neutrophil recruitment (Nakae *et al.* 2007b) and dendritic cell migration (Suto *et al.* 2006). It also promotes bacterial clearance in sepsis (Malaviya *et al.* 1996; Piliponsky *et al.* 2010) and induces airway hyperresponsiveness (AHR), lung inflammation, and Th2 (T helper 2) cytokine production in asthma (Nakae *et al.* 2007a). IL13 is well known to be a potent inducer of allergic asthma (Grunig *et al.* 1998; Zhu *et al.* 1999; Wynn 2003; Akdis *et al.* 2011), whereas CSF2 is an important growth factor of immune cells (Wodnar-Filipowicz *et al.* 1989). These genes are key regulators of mast cell allergic response (Kalesnikoff and Galli 2010; Reber and Frossard 2014), showing that PEP plays an important role in mast cell-mediated allergic inflammation.

The expression of these cytokine/chemokine genes is regulated by major transcription factors (TFs) that belong to the NFκB (Nuclear factor κB), AP1 (Activator protein 1) and NFAT (Nuclear factor of activated T cells) families (Marquardt and Walker 2000; Klein *et al.* 2006).

In the current project, the expression of two genes, FOS and JUND which are subunits of the AP-1 family of TFs (Wisdom 1999; Mechta-Grigoriou *et al.* 2001; Hess *et al.* 2004) were found to be higher in activated PEP-/- BMMCs.

Varoius subunits of AP-1 form dimeric TFs that differ in activity, in the regulation of target gene expression (Wisdom 1999; Mechta-Grigoriou *et al.* 2001; Hess *et al.* 2004). In the context of cytokine gene expression, JUND dimers are known to be less potent transcriptional activators, and hence can repress cytokine/chemokine gene expression (Meixner *et al.* 2004). It is therefore possible that increased JUND levels in activated PEP-/- BMMCs resulted in increased JUND dimer formation, which resulted in the repression of antigen-induced cytokine/chemokine gene expression.

Additionally, genes belonging to the family of dual-specificity phosphatases (DUSPs) were also found to be upregulated in PEP-/- BMMCs. A number of these DUSPs (DUSP1, DUSP4, DUSP5, DUSP8) negatively regulate MAPKs such as ERK1/2, p38 MAPK and JNK (Keyse 2000; Clark and Lasa 2003; Dickinson and Keyse 2006), which regulate the activation of NFκB, AP1 and NFAT transcription factors (Karin *et al.* 1997; Schulze-Osthoff *et al.* 1997; Wu *et al.* 2003). Hence, increased DUSPs activity can lead to reduced transcription factor activation and contribute to the reduced cytokine/chemokine gene expression in activated PEP-/- BMMCs.

Moreover, cytokine gene expression is also regulated by $[Ca^{2+}]i$. A number of TFs that regulate cytokine/chemokine gene expression are regulated by signalling pathways that depend directly or indirectly on $[Ca^{2+}]i$ mobilisation (Hogan *et al.* 2003; Feske 2007; Baba *et al.* 2008). In this work, PEP-/- BMMCs showed reduced $[Ca^{2+}]i$ mobilisation, which can lead to reduced activation of TFs such as NFATc (Hogan *et al.* 2003; Feske 2007) and contribute to the reduced cytokine/chemokine gene expression in PEP-/- BMMCs.

4.5 PEP PROMOTES ANTIGEN-INDUCED PTGS2/COX-2 GENE EXPRESSION IN MAST CELLS

Another finding from the current work is that PEP promotes antigen-induced PTGS2/COX-2 gene expression in mast cells, as PEP-/- BMMCs showed reduced PTGS2/COX-2 expression compared to PEP+/+ cells.

Prostaglandin synthases (PTGS) also known as cyclooxygenases (COX) convert arachidonic acid (AA) released from membrane phospholipids by phospholipases to prostaglandin H2 (PGH_2), which is then converted to various prostanoids (prostaglandins, prostacyclin and thromboxanes) (Firestein *et al.* 2015; Rumzhum and Ammit 2016).

There are two major isoforms of PTGS/COX expressed in most cells, PTGS1/COX-1 and PTGS2/COX-2 (DeWitt 1991; Firestein *et al.* 2015). Of the two isoforms, COX-1 is constitutively expressed at high levels in some cells (e.g. endothelium, renal tubules, platelets), to maintain basal level prostanoid, for normal physiological regulation. On the other hand, COX-2, which is induced by a wide range of stimuli in most cells, is the key enzyme for prostanoid synthesis in response to immune cell activation (Firestein *et al.* 2015; Rumzhum and Ammit 2016), including mast cell activation (Reddy *et al.* 2000; Boyce 2007). As the downstream products of COX-2 (prostaglandins, prostacyclin and thromboxanes) promote inflammation (Firestein *et al.* 2015), inhibition of COX-2 activity is a key mechanism of the commonly used antiinflammatory drugs (non-steroidal antiinflammatory drugs (NSAIDs) such as aspirin, and steroidal antiinflammatory glucocorticoids, such as dexamethasone) (Goulding 2004; Hayashi *et al.* 2004; Fendrick and Greenberg 2009; Candido *et al.* 2017).

In mast cells, the COX-2 gene promoter was shown to contain transcriptional regulatory elements for AP-1, C/EBPβ (CAAT Enhancer Binding Protein Beta),

CRE (cyclic AMP response elements) and NF-IL6 (nuclear factor for interleukin 6 expression) (Reddy *et al.* 2000). Moreover MAPK signalling was found to be important for transcriptional regulation of these transcriptional regulatory elements (Reddy *et al.* 2000). In the current study, RNA-seq analysis showed that MAPK pathway was the most significantly misregulated pathway in PEP-/- BMMCs. It is thefore possible that changes in MAPK signalling in PEP-/- BMMCs contributed to the reduced COX-2 gene expression in activated PEP-/- BMMCs.

4.6 PEP IS NEEDED FOR GLUCOCORTICOID-INDUCED NEGATIVE REGULATION OF PTGS2/COX-2 GENE EXPRESSION IN MAST CELLS

The results from this work showed that PEP is needed for glucocorticoid (GC)-induced negative regulation of COX-2 expression in mast cells.

COX-2 is a proinflammatory enzyme whose expression is greatly elevated in response to inflammatory stimuli (Mitchell *et al.* 1995; Herschman 1996; Kang *et al.* 2007; Rumzhum and Ammit 2016). It is the rate-limiting enzyme in the *de novo* synthesis of the prostanoid family of eicosanoids (prostaglandins, prostacyclin and thromboxanes) (Kang *et al.* 2007; Rumzhum and Ammit 2016). Prostanoids such as PGD_2 (prostaglandin D2), LTB_4 (leukotriene B4) and LTC4 (leukotriene C4), which are released by activated mast cells, contribute to increased bronchoconstriction, vascular permeability, recruitment of leukocyte, mucus production, and nerve cell activation associated with allergic conditions such as asthma and anaphylaxis (Galli *et al.* 2005; Weller *et al.* 2007). Consequently, inhibition of COX-2 gene expression is a major mechanism by which GCs exert their therapeutic effects (Goulding 2004; Hayashi *et al.* 2004). The current finding that the ability of

GC to inhibit COX-2 gene expression is lost in PEP-/- BMMCs, shows that PEP is needed for antiinflammatory response by GCs.

In the current work, the gene expression profile from RNA-Seq analysis did show that apart from COX-2, deletion of PEP induced changes in the GC response to many other genes. Although these other genes did not mapped unto any signalling pathway of interest, changes in their expression does show that PEP induces widespread perturbations in GC-induced gene regulation. These as-yet-unknown perturbations could have contributed to the impaired GC response in the regulation of COX-2 gene expression in activated PEP-/- BMMCs.

4.7 PEP MODULATES GLUCOCORTICOID-INDUCED REGULATION OF PTGDS/L-PGDS GENE EXPRESSION IN MAST CELLS

In addition to impaired GC mediated regulation of COX-2 gene expression, the present work also showed that the ability of GCs to regulate another gene involved in the arachidonic acid pathway, PTGDS (prostaglandin D2 synthase) also called L-PGDS (lipocalin-type prostaglandin D2 synthase) was impaired. In this case, GCs induced PTGDS gene expression in mast cells, an effect that was enhanced upon PEP gene deletion.

There are two isoforms of prostaglandin D2 synthases, PTGDS and another synthase, H-PGDS (hematopoietic prostaglandin D2 synthase). They are the terminal enzymes that specifically catalyse the synthesis of PGD_2 (prostaglandin D2) from arachidonic acid metabolites (Helliwell *et al.* 2004; Boyce 2007; Firestein *et al.* 2015).

H-PGDS is responsible for the formation of PGD_2 in immune cells such as mast cells (Helliwell *et al.* 2004; Boyce 2007; Firestein *et al.* 2015), while PTGDS, whose regulation by GC was impaired, as represented in the current results, functions in brain tissues, such as neurons (Urade *et al.* 1985; Urade and Hayaishi 1999).

Studies with neuronal cells have shown that the PTGDS gene promoter has response elements for thyroid hormone (TRE) (García-Fernández *et al.* 1998), estrogen (ERE) and glucocorticoids (GRE) (Garcia-Fernandez *et al.* 2000). It is therefore not surprising that PTGDS expression was upregulated by GCs in mast cells. However, since PTGDS has no known role in the synthesis of prostaglandins in immune cells such as mast cells, the implication of the enhanced GC-induced PTGDS expression in PEP-/- BMMCs cannot be easily deduced.

4.8 PEP MODULATES MAST CELL FUNCTION AND GLUCOCORTICOID ACTION VIA ITS PHOSPHATASE ACTIVITY

Gene knockout models as used in this work are useful tools for studying gene function (Alberts *et al.* 2008). However, complete gene knock-out suffers from the failure to delineate gene specific changes from secondary effects caused by the knock-out (Sudhof 2004; Knight and Shokat 2007). Strategies that helps to delineate primary effects from secondary responses include, the use of chemical inhibitors of the protein product of the targeted gene (Sudhof 2004; Knight and Shokat 2007).

Using PEP activity inhibitor (L75NO4), the present work showed that PEP modulates FcεRI-mediated mast cell functions and GC action via its phosphatase activities, just as was observed for the PEP gene knock-out studies in this work.

A number of groups have shown that PEP can be targeted with chemical/pharmacological inhibitors *in vivo* (Obiri *et al.* 2012; He *et al.* 2013) and *in vitro* (Yu *et al.* 2007; Stanford *et al.* 2011; He *et al.* 2013). Of much interest to the current work, are the findings of Obiri *et al* (2012) and He *et al* (2013). Using a gold (I) phosphine complex (named C28), Obiri and colleagues showed that PEP regulates anaphylaxis, and that PEP is needed for GC action *in vivo* (Obiri *et al.* 2012). On the other, He and colleagues used the present inhibitor (L75NO4) - a benzofuran salicylic acid-based compound, which is chemically more specific inhibitor of PEP activity than C28, to show that PEP is a positive regulator of anaphylaxis (He *et al.* 2013). The findings from these two groups duely confirm the current observation that PEP modulates mast cell functions and the GC response in mast cells via its phosphatase activities.

CONCLUSION

The present work revealed that the role of PEP in anaphylaxis is not strain specific, showing that PEP is an essential modulator of anaphylaxis. Furthermore, it was found that PEP is needed for FcεRI (antigen receptor)-mediated $[Ca^{2+}]i$ mobilisation, cytokine/chemokine and COX-2 gene expression, meaning that PEP is a positive regulator of mast cell effector functions. Nevertheless, it was also found that PEP is needed for GC-induced negative regulation of COX-2 gene expression. These effects were mediated via PEP activity, showing that PEP is directly involved in the regulation of mast cell functions and GC action.

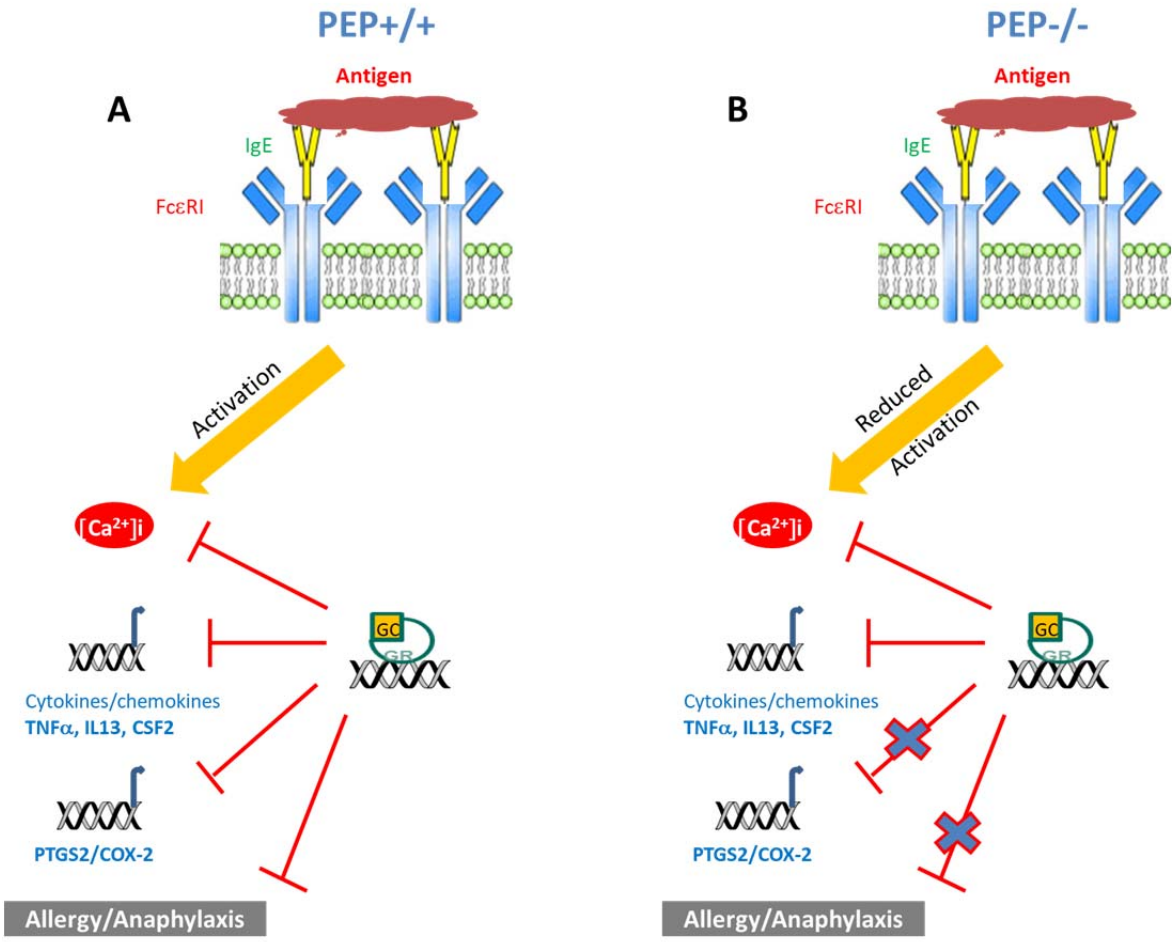


Figure 4.1 PEP is needed for mast cell function and glucocorticoid action. **(A)** In PEP+/+ BMMCs, crosslinking of IgE-bound FcεRI receptors by antigen activates proinflammatory signalling events that results in elevation of intracellular calcium [Ca^{2+}]i, increased cytokine/chemokine gene expression and increased expression of COX-2, all of which contribute to allergy/anaphylaxis. **(B)** In PEP-/- cells, there is reduced activation of these proinflammatory signalling events and loss of GC-induced negative regulation of COX-2 gene expression (current work), which may contribute to impaired GC action in allergy/anaphylaxis as reported by Obiri *et al.* (2012).

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During PhD

- Klein O. *et al* (**2016**) "Identification of Biological and Pharmaceutical Mast Cell- and Basophil-Related Targets." Scand J Immunol 83 (6): 465-472.

Oral presentations and Posters (International conferences/meetings)

- Elena Shuangyu Ma, **George Ainooson**, Rhushikesh A. Kulkarni, Ryan M. Mathews, Andrew C.B. Cato, Amy M. Barrios. "Novel Activity-Based Probes as Tools for Understanding the Mast Cell Signaling Pathway". 2016 FASEB Protein Phosphatase Meeting, Steamboat Springs, Colorado, United States of America, July 17-22, **2016**. (**poster**)
- **George Ainooson**. 'Understanding glucocorticoid action in mast cells: Key to better anti-allergy therapy'. European Cooperation in Science & Technology/Working Group 3 Training School on 'Identification of biological and pharmaceutical mast cell and basophil related targets': February 23-26, **2015** Uppsala, Sweden. (**Oral**)
- **Ainooson G**, Tsvilovskyy V, Geminn J, Stassen M, Freichel M , Cato A C B "Deletion of PEST-domain enriched tyrosine phosphatase (PEP) gene impairs glucocorticoid response in mast cells". 7th EMBRN International Mast cell and Basophil Meeting, Faculté de Médecine de Marseille, Marseille, France, October 21-23, **2015**. (**poster**)
- Volodymyr Tsvilovskyy, **George Ainooson**, Julia Geminn, Michael Stassen, Emmanuel Oppong, Marc Freichel, Andrew C. B. Cato. "PEST-domain enriched tyrosine phosphatase and glucocorticoids in the regulation of calcium homeostasis in murine mast cells". International Mast Cell and Basophil Meeting, Munich, Germany , December 11-12, **2014**. (**poster**)

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