PEST-domain enriched tyrosine phosphatase as a positive regulator of mast cell signaling

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I hereby declare that this dissertation is my own independent work. I have only used the given sources and materials and I have cited other's work appropriately

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Karlsruhe, 04/03/2013

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Abstract

PEST-domain enriched tyrosine phosphatase (PEP) is one of three intracellular phosphatases that belong to the PEST (proline, glutamic acid, serine, threonine-rich) group of the non-receptor protein tyrosine phosphatases (PTPs). PEP is a potent negative regulator of T-cell receptor signaling, which acts by suppressing the activity of Src-like family of receptor tyrosine kinases. PEP is also expressed in mast cell and has been identified as positive regulator of antigen-mediated anaphylaxis, but its mode of action is not known. To assess the action of PEP in mast cells, bone marrow derived mast cells (BMMC) from mice with genetic ablation of this gene was used. In co-immunoprecipitation experiments using PEP+/+ and PEP-/- BMMC, PEP was shown to interact with the early signaling components of mast cells such as Csk and Lyn. Sucrose density gradient centrifugation studies showed that in the absence of IgE receptor crosslinking, PEP is present in soluble fractions but migrates to lipid rafts along with Csk upon IgE crosslinking in PEP+/+ BMMC. In PEP-/- BMMC, Csk is already present in the lipid rafts in the absence of antigen stimulation but further accumulates in this fraction following IgE receptor crosslinking. In the lipid rafts, PEP was found to interact with Cbp/PAG and thereby leads to dephosphorylation of Cbp/PAG and reduced recruitment of Csk into the lipid rafts. As Csk negatively regulates the activity of Lyn, the reduced recruitment of Csk to the lipid rafts positively regulates the activation of Lyn. BMMC from PEP-/- mice have reduced Lyn activity and downstream signaling cascades including a reduced NFAT activity and degranulation upon IgE receptor crosslinking. Overexpression of PEP in the PEP-/- BMMC rescued NFAT activity and restored degranulation. Furthermore treatment of PEP+/+ BMMC with L75N04, a compound with a bicyclic benzofuran salicylic acid core mimicked all the signaling defect seen in PEP-/-

BMMC including a reduced NFAT activity and degranulation. Gene expression profile showed that cytokine genes that are regulated by Ca^{2+}/NFAT pathway are down-regulated by the absence of PEP or by the chemical inhibition of PEP activity by compound L75N04. The negative regulation of degranulation by the absence of PEP or by the compound L75N04 leads to inhibition of passive systemic anaphylaxis in mice. These findings together provide mechanistic explanations for the action of PEP as an important positive regulator of anaphylaxis in mice

Zusammenfassung

Die PEST-domänenreiche Tyrosinphosphatase PEP ist eine von drei intrazellulären Phosphatasen der Gruppe der prolin-, glutamin-, serin- und threoninreichen Proteintyrosinphosphatasen (PTP). PEP unterdrückt die Aktivität von Mitgliedern der Familie Src-ähnlichener Rezeptortyrosinkinasen und hemmt auf diese Weise den T-Zellrezeptorsginalweg. PEP ist auch in Mastzellen exprimiert, in denen es die antigenvermittelte anaphylaktische Reaktion verstärkt. Die molekulare Wirkweise von PEP in Mastzellen war bislang nicht bekannt. Um die Rolle von PEP in Matzellen zu untersuchen, wurden Mastzellen aus dem Knochnmark (*bone marrow derived mast cells* -BMMC) von Mäusen mit einer genetischen Ablation von PEP herangezogen. In Koimmunopräzipitationsexperimenten unter Verwendung von BMMC aus wildtype (PEP+/+) und(PEP-/-) Mäusen konnte gezeigt werden, dass PEP mit frühen Komponenten der Mastzellsignalkaskade wie Csk und Lyn interagiert. In Analysen von Zellfraktionierungen nach Zentrifugation über einen Sukrosedichtegradienten konnte nachgewiesen werden, dass PEP in ruhenden Wildtyp-BMMCs in den löslichen Fraktionen lokalisiert ist, während es nach IgE-Rezeptor-Quervernetzung gemeinsam mit Csk in spezialisierte Membranbereiche, den sogenannten‘Lipid Rafts transloziert. In PEP -/- BMMC befindet sich Csk bereits vor der Aktivierung in der Lipid Raft-Fraktion und wird nach IgE-Rezeptor-Quervernetzung weiter angereichert. Innerhalb der Lipid Raft-Fraktion konnte die Interaktion von PEP mit Cbp/PAG gezeigt werden, diese führt zur Dephosphorylierung von Csk und in Folge zur verminderten Rekrutierung des Proteins in die Lipid Rafts. Da Csk die Aktivität von Lyn hemmt, verstärkt die reduzierte Rekrutierung von Csk in die Lipid Rafts auch die Aktivierung von Lyn. BMMC von PEP-/- Mäusen zeigen reduzierte Lyn-Aktivität und damit auch eine verminderte Aktivierung von nachgeordneten Ereignissen wie der NFAT Aktivität

und verminderter Degranulierung nach IgE-Rezeptor-Quervernetzung. In *rescue*-Experimenten konnte die NFAT-Aktivität in PEP-/- BMMC und die Degranulierung durch Überexpression von PEP wiederhergestellt werden. Ähnliche Effekte wie sie in PEP-/- BMMC in Bezug auf die NFAT-Aktivität und Degranulierung beobachtet wurden, konnten in PEP+/+ BMMC nach Behandlung mit L75N04, einem Wirkstoff mit einem bizyklischen Benzfuronsalicylsäurekern, beobachtet werden. Genexpressionsprofile zeigten, dass die Abwesenheit von PEP in BMMC wie auch die Behandlung von Wildtyp BMMCs mit L75N04 die Expression von durch den $Ca^{2+/}$NFAT-Signalweg regulierten Zytokinen reduziert. Die Hemmung der Degranulierung in Folge von PEP-Elimination oder L75N04-Behandlung resultierte konsequenterweise in der Inhibierung der passiven systemischen Anaphylaxis in Mäusen. Diese Befunde liefern einen wichtigen und grundlegenden Erklärungsansatz für die Wirkweise von PEP als Schüsselprotein bei der Regulation der Anaphylaxis in Mäusen.

Abbreviations

bp base pair

CCL-2 chemokine (C-C motif) ligand 2

CCL-12 chemokine (C-C motif) ligand 12

CO_2 carbon-di-oxide

DNA deoxy-ribonucleic acid

dNTP deoxynucleoside triphosphate

EDTA Ethlenediamine-N,N-tetraacetic acid

FcεRI high affinity IgE receptor

GAPDH glyceraldehyde 3-phosphate dehydrogenase

GM1 (monosialotetrahexosylganglioside)

h hour

HEPES N-2hydroxyethilpiperasine-n'-2-ethansulfonic acid

HRP horseradish peroxidase

HRH4 histamine H4 receptor

HSP60 heat shock protein 60

IBD inflammatory bowel disease

IBS irritable bowel syndrome

IgE immunoglobulin E

IFN interferon

IL interleukin

ITAM immunoreceptor tyrosine-based activation motif

LPS lipopolysaccharide

MAPK mitogen-activated protein kinase

M molar

min minute

ml milliliter

μM micromolar

mRNA messenger RNA

PBS phosphate buffered saline

PCR polymerase chain reaction

RT room temperature

S.E.M standard error of mean

SCF stem cell factor

TNF-α tumor necrosis factor α

Th T helper

TLR Toll-like receptor

v/v volume /volume

w/v weight/volume

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1.0 Introduction

Several studies suggest that the prevalence of allergic diseases has increased significantly over the past 20-30 years, a trend that shows no sign of abating. It is estimated that more than 80 million people in Europe suffer from some kind of allergic diseases such as allergic rhinitis, allergic conjunctivitis, food allergy, atopic dermatitis, and asthma and that by 2015 half of all Europeans will be suffering from an allergy. The rise in prevalence also has socio-economic impact, as 70 % of patients state their social and physical activity is limited and it also adds burden to health care cost as it is estimated that asthma alone costs Europe €27 billion per year, with expenditure expected to grow to €50 billion within a decade (www.efanet.org/allergy) (European Academy of Allergology and Clinical Immunology (EAACI) Position paper, 2006).

The spreading epidemic of allergy and asthma has heightened the interest in mast cells, which remain at the core of our understanding of allergic responses as they contribute in the elicitation and subsequent release of potent inflammatory mediators that lead to allergic reaction. As a result they are one of the major targets of investigations and therapeutic interventions in allergy (Nauta et al., 2008).

1.1Mast cell biology

Origin and Development

Mast cells were first described by Paul Ehrlich in the late 19th century based on the metachromatic staining properties of large cytoplasmic granules (Ehrlich, 1878). The “well-fed appearance” led him to designate these cells as mast cells. Today we know that the granule content does not originate from an uptake but from the

synthesis and storage of high amounts of proteoglycans, proteases and other mediators. Mast cells are key components of innate and adaptive immune system. The essential role they play in the immune system is reflected by the fact that no human individual has been identified until today who lacks mast cells (Stevens and Adachi, 2007). Mast cells are involved in the inflammatory response through the combined activities of a variety of cell surface receptors, the important one being the high affinity IgE receptor, FcεRI (Gilfillan and Tkaczyk, 2006). The activation of these receptors on mast cells allows them to be involved in the immune responses to bacterial, viral and parasitic infections (Dawicki and Marshall, 2007).

Mast cells originate from multipotent hematopoietic stem cell precursors in the bone marrow. These multipotent progenitors (MPPs) get differentiated to committed mast cell progenitors (MCPs) in the bone marrow and are then released into the bloodstream and circulate until they extravasate into the connective tissue and serosal/mucosal cavities throughout the body (Okayama and Kawakami, 2006). Upon arrival at peripheral tissues, the committed mast cell progenitors develop into mature mucosal or connective tissue mast cells in response to factors released by the local tissue (Okayama and Kawakami, 2006). The development of MCPs to mature mast cells is strongly dependent on the microenvironment and, thus, on the production of growth factors at the site of differentiation. The cardinal factor for mast cell growth, differentiation, proliferation and survival is the stem cell factor (SCF) that binds to the receptor tyrosine kinase c-Kit and is expressed by stromal cells as a soluble or a membrane bound molecule in various tissues. Mast cells deprived of SCF undergo apoptosis (Galli et al., 1994; Longley et al., 1997; Okayama and Kawakami, 2006). Several other cytokines also participate in the regulation of mast cell proliferation and development under in vitro conditions (Okayama and Kawakami, 2006). The first cytokine identified to drive in vitro

mast cell growth and development is Interleukin-3 (IL-3). This cytokine is able to independently promote the differentiation of murine hematopoietic stem cells into mucosal type BMMCs and is sufficient to support the growth and survival of the resulting culture (Ihle et al., 1983; Razin et al., 1984). In contrast to SCF, IL-3 is dispensable for in vivo mast cell development and proliferation, as IL-3 knockout mice exhibit no reduction in the number of mast cells in their tissues (Lantz et al., 1998). Furthermore, human mast cells do not express the IL-3 receptor and thus do not respond to this cytokine (Valent et al., 1990).

Two types of mast cells (mucosal and connective tissue type) can be differentiated by their tissue of residence and by their differential histological staining properties and the proteases they contain within their secretory granules. Mucosal mast cells are present in mucosal epithelial tissues of the intestine and lungs, while connective tissue mast cells are present in the peritoneal cavity and skin (Hallgren and Gurish, 2007). Mucosal mast cells do not stain with the histological stain safranin and predominantly express mouse mast cell protease (mMCP-1) and (mMCP-2). Connective tissue mast cells stain positively with safranin as a result of heparin being contained within their granules and they express mMCP-3, mMCP-4, mMCP-5 and mMCP-6 (Beil et al., 2000; Gurish et al., 1992).

1.2 Mast cell activation

Stimulation of the mast cell activation, initiated either by interaction of the antigen specific antibodies or the antigen with the corresponding mast cell receptors, is referred to as immunologic activation. Alternatively, the stimulation induced by substances such as neuropeptides or cytokines is termed as non-immunological activation. Both immunological and non-immunological stimulation can elicit distinct, although sometimes overlapping patterns of mediator secretion and degranulation as seen in Fig 1.0

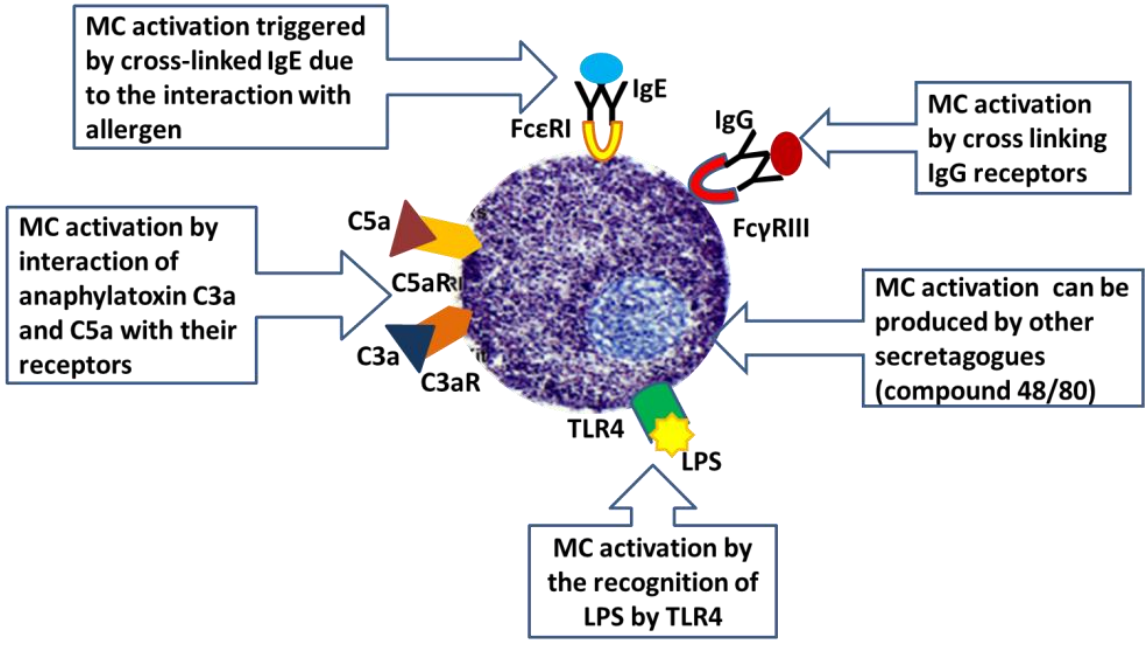


Fig 1.0 Different ways of mast cell activation – A scheme illustrating multiple receptors that are displayed on the mast cells surface. Mast cells express tetrameric high affinity receptor for IgE (FcεRI), interferon gamma (IFN-γ) primed mast cells express FcγRIII, who's cross-linking can induce the release of mediators. These cells also express Toll-like receptor 2 (TLR2), TLR3, TLR4, TLR5, TLR6, TLR7, and TLR9 as well as complement component receptors like C3aR, C5aR.

The classical way of activating mast cells, especially in allergic reactions, is through their high affinity IgE receptors, FcεRI. Apart from activation via FcεRI, they also release mediators following aggregation of low affinity IgG (FcγRIII) receptors (Katz et al., 1992). Mast cells also express multiple receptors for complement components (anaphylatoxins) C3a, C4a and C5a. The complement system is involved in a number of processes contributing to host defense and/or inflammation (Mousli et al., 1994). Mast cells can be activated by different bacterial components through their interactions with toll-like receptors (TLRs) expressed on the mast cell surface (Medzhitov et al., 1997).The mast cell-activating neuropeptides include substance P, calcitonin gene regulated peptide,

vasoactive intestinal peptide and neurotensin (Church et al., 1991). Mast cells can also be activated by different types of cytokines and chemokines, such as macrophage inflammatory protein 1α (MIP-1α) and monocyte chemoattractant-1 (Alam et al., 1994). Other compounds that can directly activate mast cells include calcium ionophores, compound 48/80 (a histamine-releasing agent, its mechanism of action is commonly attributed to a direct, receptor-bypassing property to activate the G(i/o) class of G proteins), synthetic adrenocorticotropic hormone, adenosine and endothelin (Metcalfe et al., 1997).

1.3 FcεRI-mediated mast cell activation

FcεRI-The high affinity receptor for IgE

The classical route of mast cell activation is through the adaptive immune response via antibodies that bind to receptors on the mast cell surface. This is how mast cells act both in the immune defense towards parasites and in mediating hypersensitivity reactions such as allergies and asthma.

Antigen-dependent mast cell activation is regulated by a complex series of intracellular signaling processes which is initiated following FcεRI aggregation. FcεRI is a tetrameric receptor that comprises, α-chain (IgE, binding subunit), as well as a tetra membrane spanning β-chain and a homo-dimeric di-sulphide-linked γ-chain (Fig 1.1) (Kinet, 1999).

The FcεRIα chain is an N-glycosylated type I integral membrane protein which is heavily glycosylated and contains the binding site for the constant region (Fc) of IgE. The assembly, folding and core glycosylation of the immature FcεRIα variant takes place in the endoplasmic reticulum (ER). The homodimeric γ-chain subunit masks the ER-retention signals and thereby enables the export of the FcεRI complex from the ER to the cell surface (Fiebiger et al., 2005; Letourneur et al.,

1995; Ra et al., 1989; Shimizu et al., 1988). When the β chain and γ chain are absent, the immature FcεRIα variant is trapped in the ER by a retention signal.

The FcεRIβ chain amplifies FcεRI signal transduction. It contains an immuno receptor tyrosine-based activation motif (ITAM). β chain does not induce signal transduction by itself rather it amplifies signal-transduction induced by enhancing the phosphorylation of the ITAMs of the γ-chains (Donnadieu et al., 2000; Kurosaki et al., 1992).

The γ-chain dimer is a disulfide-linked homodimer and is shared by various Fc receptors. Each of the FcεRIγ chains consists of a short extracellular and trans-membrane region and a cytoplasmic domain which carries an ITAM. These ITAMs are essential for FcεRI-mediated signal transduction involved in mast cell activation and survival (Ravetch and Kinet, 1991; Sakurai et al., 2004).

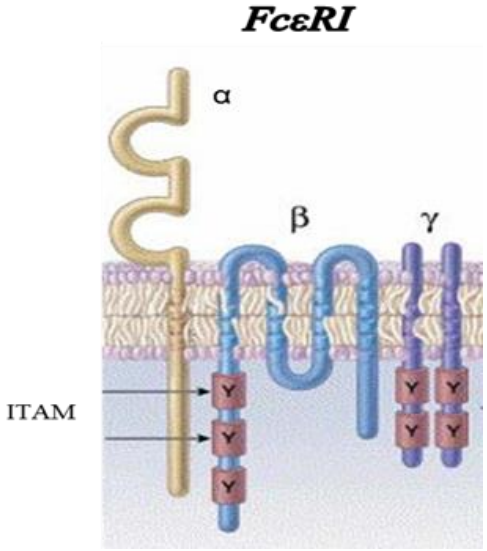


Fig 1.1 Domains of monomeric FcεRI- The high affinity IgE receptor consists of α, β-subunit and a γ dimer. FcεRI activation is initiated by receptor aggregation, which occurs as a result of several IgE molecules binding to a single multivalent antigen molecule. Adapted from (Rivera and Gilfillan, 2006)

The presence of IgE antibodies induces enhanced surface expression levels of FcεRI in vitro and in vivo. IgE-mediated up-regulation of FcεRI is a complex process involving stabilization of FcεRI on the cell surface by IgE-binding and usage of a receptor pool derived from recycled and newly synthesized FcεRI combined with a continued basal level of protein synthesis (Borkowski et al., 2001; Novak et al., 2001; Yamaguchi et al., 1997)

IgE mediated FcεRI signaling

To initiate mast cells activation through FcεRI, IgE antibodies that are bound to FcεRI are cross-linked by the cognate multivalent antigen (allergen). Cross-linkage of IgE by the interaction of allergen with the FcεRI receptors on the surface of mast cells brings the receptors into juxtaposition and initiates mast cell activation. After aggregation, FcεRI coalesces with specialized microdomains of the plasma membrane known as lipid rafts which are glycolipid-enriched membrane domains (Brown and London, 1998; Field et al., 1999), allowing phosphorylation of tyrosine residues within the ITAM sequences YXXL/I (a tyrosine separated from a leucine or isoleucine by any two other amino acids, giving the signature motif YxxL/I) present in the receptor subunits (Pribluda et al., 1994) by the Src kinase Lyn which is potentially activated within these microdomains. When phosphorylated, the β- and γ-chain ITAMs provide high affinity docking sites for the SH2 domains of Lyn and Syk (Benhamou et al., 1993; Chen et al., 1996; Jouvin et al., 1994).

The tethering of Syk to the phosphorylated ITAMs of the γ-chain leads to phosphorylation of Syk at its catalytic domain thereby increasing its catalytic activity which is essential for IgE mediated mast cell signaling (Benhamou et al., 1993; Benhamou and Siraganian, 1992; Costello et al., 1996). For coordination of

the downstream signaling subsequent phosphorylation of adaptor molecule LAT (linker for activation of T-cells) is important (Saitoh et al., 2000).

LAT resides mainly in lipid rafts owing to its juxtamembrane palmitoylation site (Zhang et al., 1998; Zhu et al., 2005). LAT phosphorylation leads to recruitment and binding of several adaptor molecules such as GRB2 (growth factor-receptor-bound protein 2), GADS (GRB2-related adaptor protein), SHC (SH2-domaincontaining transforming protein C), and SLP76 (SH2-domain-containing leukocyte protein of 65k Da), the guanine-nucleotide exchange factors SOS (son of seven less homologue) and VAV and the signaling enzymes phospholipaseCγ (PLCγ). PLCγ hydrolyses the membrane phospholipid (phosphatidylinositol-5-bisphosphate) to form InsP3 (inositol 3, 4, 5-triphosphate) and DAG (diacylglycerol) (Gilfillan and Tkaczyk, 2006) as shown in Fig 1.2.

A complementary activation pathway involves the Fyn-GAB2-PI3K axis. Activation of Fyn results in phosphorylation of GAB2. This leads to the binding of PI3K (phosphatidylinositol 3-OH kinase) and subsequently activation of Btk (Bruton's tyrosine kinase). Rac as well as Btk and Syk are able to activate phospholipaseCγ (PLCγ) leading to formation of inositol triphosphate (IP3) and diacylglycerol (DAG) (Parravicini et al., 2002).

Formation of IP3 leads to calcium depletion of the endoplasmic reticulum (ER) which originates from activation of IP3-receptors. IP3-receptors are transmitter-regulated cation channels of the ER and activation results in calcium release from the ER and an increase of cytosolic calcium. The resulting depletion of the intracellular stores induces calcium entry through the plasma membrane via CRAC (calcium release activated calcium) channels by activation of Orai1 or CRAM1- a plasma membrane protein and the pore forming subunit of (CRAC) channel. This

calcium depletion from ER also leads to translocation of the stromal interaction molecule 1 (Stim1) under the plasma membrane. Stim1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store (ER) to the plasma membrane. After translocation, Stim1 interacts with Orai1, resulting in activation of the CRAC channel and calcium-influx. This increase in intracellular calcium induces degranulation of mast cells (Chang et al., 2008; Metcalfe et al., 2009; Mignen et al., 2007).

In contrast, SLP-76, SHC, GRB2, VAV and SOS are involved in the activation of small GTPases such as Ras. These GTPases further activate members of the mitogen-activated protein kinase (MAPK) family such as ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase), and p38 resulting in synthesis of eicosanoids, chemokines and cytokines (Siraganian, 2003).

Thus FcεRI-mediated mast cell activation results in a complex signaling cascade which induces mast cell degranulation and de novo synthesis of pro-inflammatory mediators involved in allergic reactions.

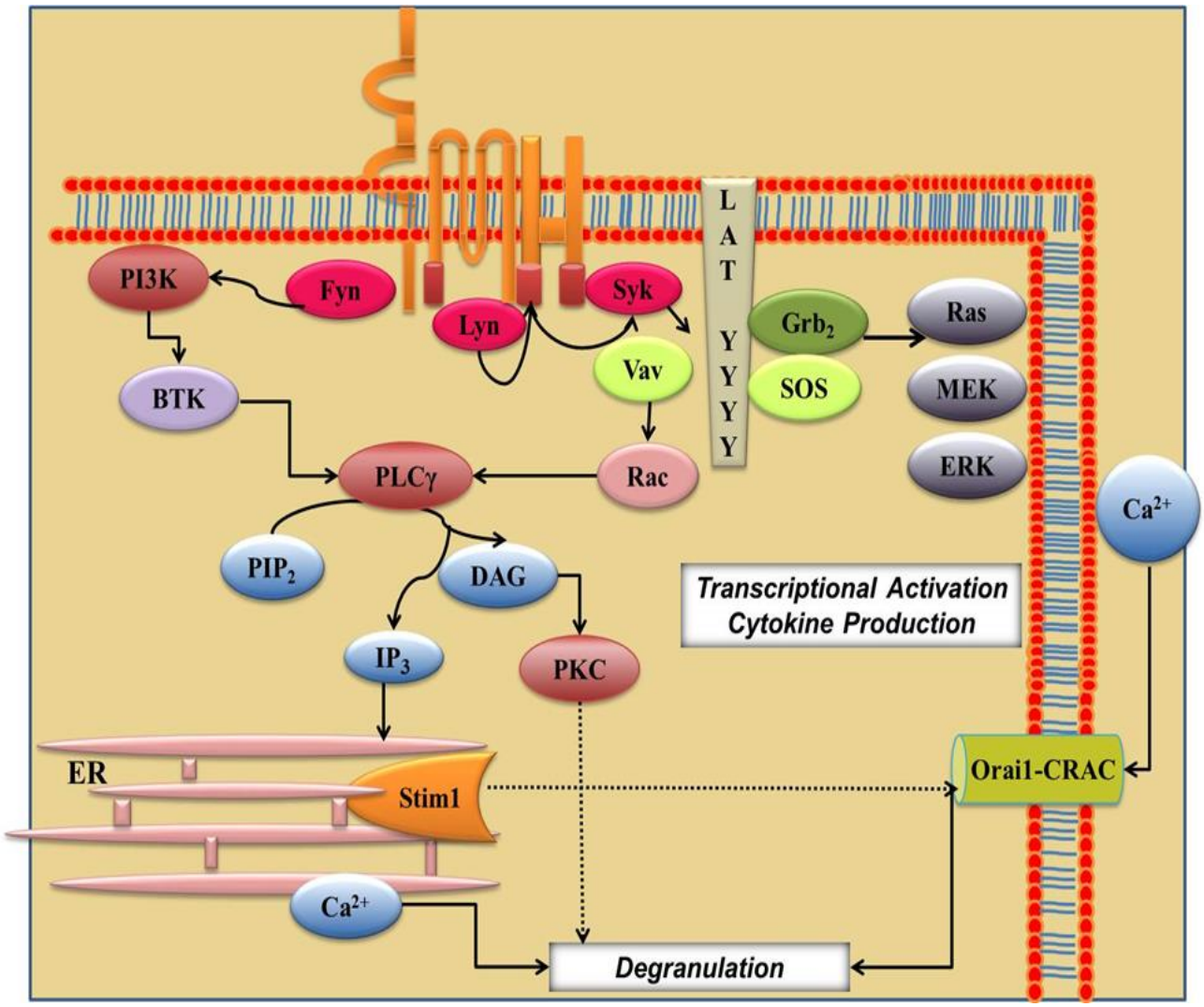


Fig 1.2 The signaling cascade in activated mast cells - Aggregation of FcεRI leads to a Lyn and Syk-dependent phosphorylation of LAT resulting in recruitment and binding of the adaptor molecules GRB2, GADS, SHC, and SLP76, the guanine-nucleotide exchange factors SOS and VAV and the signaling enzymes PLCγ. In parallel, PI3K is activated by Fyn which leads to Btk-PLCγ axis. PLCγ is involved in induction of calcium flux and cellular degranulation whereas the guanine-nucleotide exchange factors promote the induction of the MAPK pathway leading to synthesis of cytokines, chemokines and eicosanoids. Adapted from (Gilfillan and Tkaczyk, 2006)

Kit Receptor Activation Potentiates FcεRI Signaling

Since mast cells respond to multiple signals simultaneously in vivo, it was thought that cross-talk must exist among the key signaling pathways. SCF/c-Kit (Stem cell factor) receptor activation has been shown to cross-talk with the FcεRI signaling cascade, acting to enhance the FcεRI mediated release of histamine and LTC4 (Bischoff and Dahinden, 1992) through synergistic activation of PLCγ, the MAPKs, and Akt (Hundley et al., 2004)

SCF-receptor is a member of the type III receptor tyrosine kinase family encoded by the proto-oncogene, c-kit. It bears five immunoglobulin-like extracellular domains and no ITAM. Unlike FcεRI, SCF-receptor is a single-chain receptor that has inherent protein-tyrosine-kinase activity (Fig 1.3). Binding of SCF results in receptor dimerization and auto phosphorylation (Galli et al., 1993; Zhang et al., 2000).

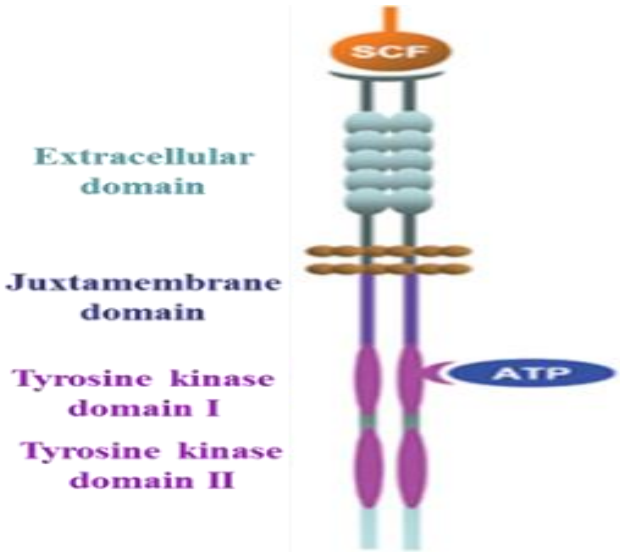


Fig 1.3 Schematic drawing of a SCF-c-KIT receptor complex- The structure of c-Kit receptor is characterized by an extracellular ligand-binding region containing five immunoglobulin-like repeats, a trans-membrane sequence, an auto-inhibitory juxtamembrane domain and two intracellular tyrosine kinase domains: an ATP-binding pocket and a kinase activation loop. Adapted from (Blechman et al., 1995; Chan et al., 2003; Roskoski, 2005)

Tyrosyl-phosphorylated c-Kit recruits signaling molecules containing the Src homology 2 (SH2) domains, such as phosphatidylinositol (PI)-3 kinase, phospholipase Cγ1, Grb2, and the Src kinase, to c-Kit and initiates cytoplasmic signaling. Actin reorganization and the release of Ca^{2+} are triggered, and kinases such as the mitogen-activated protein kinase (MAPK) kinase MEK and its substrates ERK1 and ERK2, as well as survival factors such as Akt/PKB, are activated (Fig 1.4). Thus, a complex set of signaling pathways is activated in mast cells through c-kit stimulation, and not all factors involved in these pathways have been elucidated (Galli et al., 1993; Galli et al., 1994).

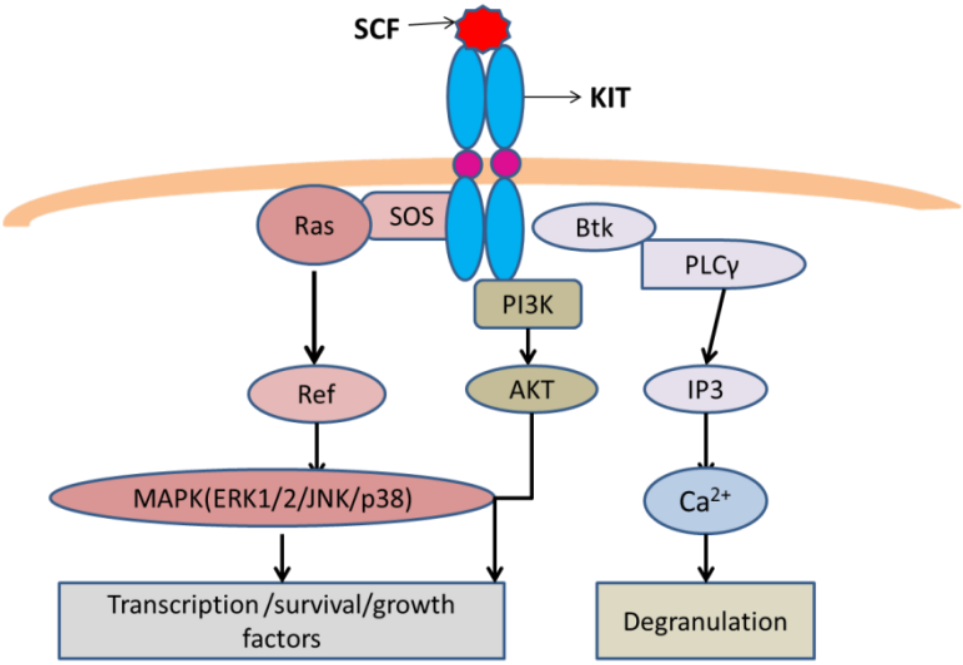


Fig 1.4 SCF-c-Kit receptor mediated mast cell signaling- Stem-cell factor (SCF)-mediated dimerization of KIT induces auto phosphorylation at multiple tyrosine residues in the cytoplasmic tail, resulting in the recruitment of various molecules, including the following: cytosolic adaptor molecules, such as SOS; SRC-family kinases; and signaling enzymes such as phospholipaseCγ (PLCγ) and phosphatidylinositol 3-kinase (PI3K). Subsequent activation of these signaling enzymes, as well as the RAS–RAF–mitogen-activated protein kinase

(MAPK) pathway leads to mast-cell growth, differentiation, survival, and chemotaxis and cytokine production. Adapted from (Gilfillan and Tkaczyk, 2006)

Signaling through c-kit is essential for mast cell development, as well as for other processes in hematopoiesis, erythropoiesis, melanogenesis, and gametogenesis. Mice completely deficient in c-kit or its ligand stem cell factor die during embryogenesis or neonatally of severe anemia. The addition of SCF to cultured mast cells stimulates proliferation, maturation, secretion, cytoskeletal actin rearrangements, membrane ruffling, migration, and cell survival (Galli et al., 1993; Taylor et al., 1995).

1.4 Mast cell functions

Mast cells exert their biological functions exclusively by humoral immune mechanism. They exhibit an array of immune-response receptors, adhesion and other surface molecules and also release huge amount of diverse biologically active mediators which can be divided into two categories (I) preformed mediators such as histamine, proteoglycans and neural proteases and certain cytokines like TNF-α, (II) mediators that are newly synthesized following activation like chemokines, lipid mediators, growth and angiogenic factors which can lead to various pro-inflammatory, anti-inflammatory and/or immuno regulatory effects (Galli et al., 2005; Grimbaldeston et al., 2006; Maurer et al., 2003). These biological characteristics and their strategic location at sites that interface with external environment, closely associated with nerves and blood vessels (Dawicki and Marshall, 2007) explains how mast cells can be involved in so many different physiological and pathological conditions as seen in Fig 1.5.

Innate-immune response to Pathogens

Mast cells are equipped with a multitude of receptors serving directly or indirectly as sensors of pathogen invasion. Many of these belong to the large family of pattern recognition receptors (PRR) that bind directly to pathogen associated molecular patterns (PAMPs) which are highly conserved sequences (Yarovinsky et al., 2006) present in exogenously derived molecules which originate from either bacteria or viruses (Anderson, 2000).

Toll-like receptors (TLR) are an important group of the PRR family. Specifically, it has been shown that BMMCs express TLR2, TLR4, TLR6 and TLR8. Through TLR-mediated recognition of exogenous particles, mast cells are involved in the immune response to bacterial, viral and parasitic infections and function to regulate the local tissue immune response to infection by releasing a combination of pre-formed and newly synthesized inflammatory mediators (Dawicki and Marshall, 2007). TLR4 expressed on the surface of mast cells allows mast cell activation in response to stimulation with the major components of bacterial cells walls- the gram negative bacterial component lipopolysaccharide (LPS), and the gram positive derived peptidoglycan (Anderson, 2000). Mast cell activation in reaction to viral products can be mediated by TLR3, TLR7 and TLR8, and serves to initiate the secretion of IL-1β, IL-6, macrophage inflammatory protein (MIP-1α) and (MIP-1β) (King et al., 2000).

Mast cells also play key protective roles in the immune response elicited against parasitic infection, including nematodes, and gastrointestinal helminth. This response is dependent on secreted mast cell proteases, including MCP-1(Lawrence et al., 2004) and MCP-9 (McDermott et al., 2003) for nematodes and gastrointestinal helminth. In addition, mast cells also have a protective role in

sepsis (Mallen-St Clair et al., 2004) and in response to exposure to bee and snake venoms (Rivera, 2006).

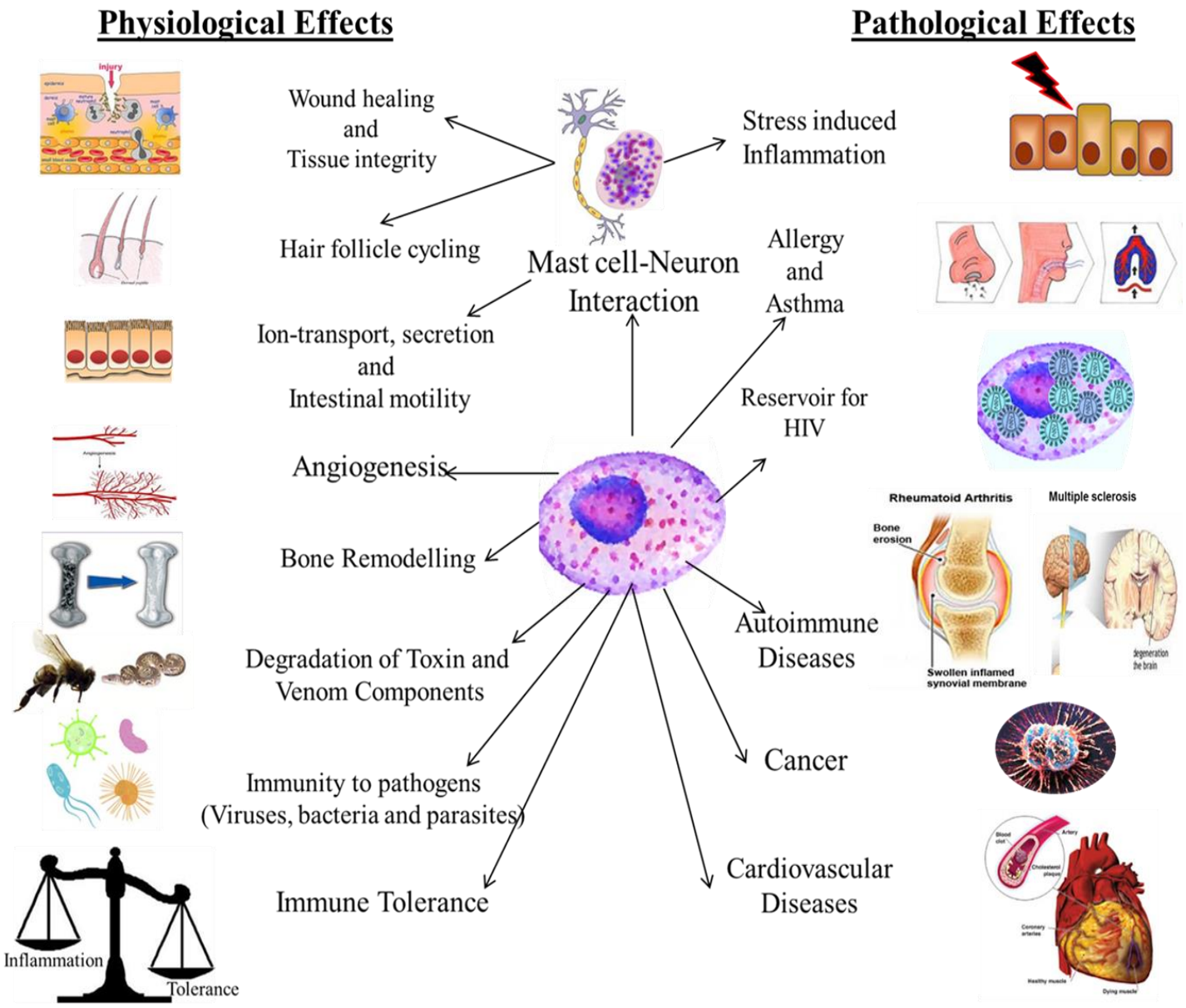


Fig 1.5 Mast cell activation leads to physiological and pathological effects- A hypothetical model for the function of mast cells as pro inflammatory and anti-inflammatory cells. Mast cells are important components of the immune system, essential to fight viruses, bacteria, parasites and other invaders at portals of entry of our bodies as well as for angiogenesis, and immune tolerance. In allergic reactions mast cells can produce havoc with the release of their granules which cause problems ranging from swelling, itching, all the way to lethal anaphylactic

shock. They are also involved in auto-immune inflammatory diseases where they seem to have a detrimental role to play.

Physiological processes

Mast cells play a positive role in non-inflammatory processes such as immune tolerance, angiogenesis and tissue remodeling during wound healing (Henz, 2008). Angiogenic role for mast cells is suspected because of their proximity to blood and lymphatic vessels and their tendency to accumulate in polyps, tumors, and other tissues associated with angiogenesis, and their production of VEGF (vascular endothelial growth factor), TGF-β, TNF-α, histamine and heparin all of which have demonstrated angiogenic activity (Crivellato et al., 2004). Mast cells control all phases of healing from the initial inflammatory response to re-epithelialization and tissue remodeling (Noli and Miolo, 2001).

Mast cell dysregulation and diseases -Pathological effects

Mast cell activation also has detrimental effects which extend to various autoimmune diseases, allergy and asthma.

In allergic hypersensitivity reactions, dysregulation of the levels of T helper (Th) 2 cells produced in response to allergen stimulation results in the hyper secretion of IgE by B cells. This elevated level of circulating IgE causes an increased rate of mast cell activation upon subsequent exposure to the original antigen, which results in an exaggerated mast cell IgE/FcεRI mediated inflammatory response (Montero Vega, 2006). Severe type I hypersensitivity allergic reaction is the anaphylactic shock caused due to increased release of large quantities of immunological mediators leading to systemic vasodilation (associated with a sudden drop in blood pressure and hypothermia in mice) and edema of bronchial mucosa resulting in bronchoconstriction which ultimately lead to death.

Mast cells release PGD2 (Prostaglandin D2), LTC4 (Leukotriene C4) and histamine; these mediators produce many of the symptoms seen in asthma, including mucous secretion, edema of mucosal tissues, and bronchoconstriction. Furthermore, asthmatics show indications of chronic activation of these pathways as well as abnormal accumulation of mast cells in airway smooth muscle tissue (Bradding et al., 2006).

Mast cells have been shown to play a role in the pathogenesis of Rheumatoid arthritis (RA) and Multiple sclerosis (MS). They accumulate in arthritic synovial fluid (Nigrovic and Lee, 2007) while in case of MS they accumulate in the lesions of brain and the cerebral spinal fluid of patients show high levels of histamine and tryptase. Mast cell degranulate in response to myelin basic protein leading to increased permeability of the blood brain barrier that occurs in early stages of MS (Johnson et al., 1988; Zhuang et al., 1996).

Mast cells are also involved in the development of atherosclerosis, a cardiovascular disorder considered to be a chronic inflammatory disease. Since heart is one of the organs rich in mast cells, the mediators released by mast cells after activation are believed to be involved in in the development of atherosclerosis and vascular aneurysm formation (abnormal widening of artery). Moreover it is seen that mast cells reside in the perivascular tissue of healthy arteries and during the progression of atherosclerosis, the cells accumulate in the adventitia and the shoulder region of the atherosclerotic plaque(plaques formed within the arteries) (Libby, 2002).

Increased populations of mast cells are found in tumors such as mammary adenocarcinomas, basal cell carcinomas, melanomas, and neurofibromatosis. Mast cells can promote mammary tumor development by: (1) disturbing the normal stroma-epithelium communication, has been shown in matrix degradation at sites

of tumor invasion in rat mammary adenocarcinoma, (2) facilitating tumor angiogenesis, and (3) releasing growth factors such as stem cell factor (SCF) and nerve growth factor (NGF) that permit the formation of new blood vessels and metastases (Theoharides and Conti, 2004).

Thus these cells have a key role in both induction and elicitation of several chronic inflammatory and autoimmune diseases.

Manifestation of these mast cell driven response are mainly the consequence of the release of pro-inflammatory mediators following antigen induced aggregation of IgE-bound high affinity receptors (FcεRI) and subsequent phosphorylation of Src family kinases (SFKs). Regulated tyrosine phosphorylation of these SFKs is required for appropriate cellular response, and this is achieved by protein tyrosine phosphatases (PTPs). The equilibrium between these SFKs and PTPs define the signal threshold for a given response and is critical for normal immune cell development and function. Any dysregulation in this equilibrium can have pathological consequences resulting in immunodeficiency, autoimmunity and malignancy as stated above in the section on mast cell function.

1.5 Protein Tyrosine Phosphatase (PTPs) in mast cell signaling

Protein tyrosine phosphatases play important role in many pathological situations and also in the regulation of immune cell functions. PTPases family constitutes a large, structurally diverse family of receptor-like and cytoplasmic enzymes which is expressed in all eukaryotes. There are ~100 PTPase genes encoded within the human genome. Every PTPase is composed of at least one conserved domain characterized by a unique PTPase signature motif-C $(X)_5$ R containing the cysteine and arginine residues known to be essential for catalytic activity. Within the PTP (catalytic) domain the cysteine residue functions as a nucleophile and is essential

for catalysis. PTPs fall into three classes: (a) receptor PTP (b) non-receptor (non-trans membrane) PTP (c) dual specificity PTP (DUSPs) as seen in Fig 1.6

Receptor PTPs are predominantly found in the plasma membrane, whereas non-receptor PTPs is localized to a variety of intracellular compartments, including the cytosol, plasma membrane and endoplasmic reticulum (Andersen et al., 2001; Zhang, 1998).

Alterations in the expression or activity of these PTPs have known to cause pathophysiological consequences leading to many human diseases including cancer, diabetes/obesity, and autoimmune disorders

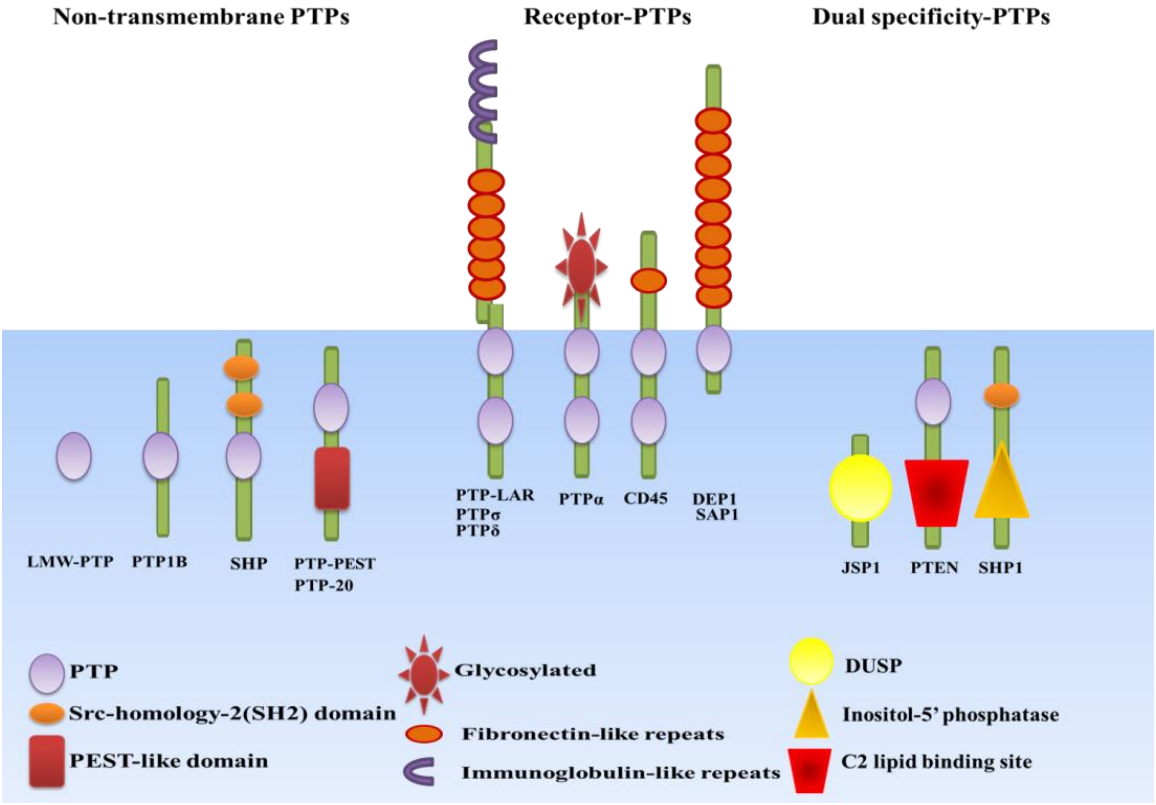


Fig 1.6 Schematic representation of PTP family members- The PTP family includes receptor PTPs (RPTPs), dual-specificity PTPs (DUSPs) and non-receptor/soluble PTPs (NR-PTPs). All the receptor tyrosine phosphatases contain the tyrosine phosphatase motifs often in tandem and are distinguished by differences in their

extracellular domains. The cytosolic/non-receptor tyrosine phosphatases are further divided by the presence of various homology domains such as SH2 or PEST. The PTP catalytic domain is highly conserved and contains a single cysteine that is used in a cysteinyl-phosphate enzyme intermediate during dephosphorylation.DEP1 (high cell-density enhanced PTP-1); JSP1 (Jun amino-terminal kinase (JNK) stimulatory phosphatase); LAR (leukocyte common antigen related); PTP-PEST (PTP-proline, glutamate, serine and threonine-rich domain); SAP1 (stomach cancer-associated PTP); SHP (SH2-domain-containing PTP); PTEN (phosphatase and tensin homologue). Adapted from(Andersen et al., 2001).

Genetic studies on human autoimmune disorders have shown strong association of LYP (Lymphoid tyrosine phosphatase –soluble phosphatase) with a broad spectrum of autoimmune diseases. LYP and its murine orthologs PEP (PEST-domain enriched tyrosine phosphatase) is exclusively expressed in immune cells (Cohen et al., 1999). LYP/PEP functions as a negative regulator in T-cells, a missense C1858T single nucleotide polymorphism in the gene (PTPN22) encoding LYP results in gain-of-function rendering increased inhibition of T-cell signaling leading to autoimmune disorder (Vang et al., 2005). Hence much of the attention has been focused on the function of LYP/PEP and the pathway(s) it regulates which would help in providing new approach to treat autoimmunity.

PEST–domain enriched tyrosine phosphatase- (PEP)

This PTP is characterized by a so-called PEST (proline-glutamic acid-serine-threonine) sequence. The human ortholog lymphoid tyrosine phosphatase (LYP- encoded by PTPN22 gene) and mouse orthologs PEST-domain enriched tyrosine phosphatase (PEP- encoded by PTPN8 gene) share 89% homology between their PTP domains and 61% identity in their non-catalytic portion. LYP/PEP is characterized by 300 amino acid N-Terminal (PTP domain), and C-terminal domain that includes 4 putative poly-proline motifs (P1-P4) (Fig 1.7) (Cote et al., 2002).

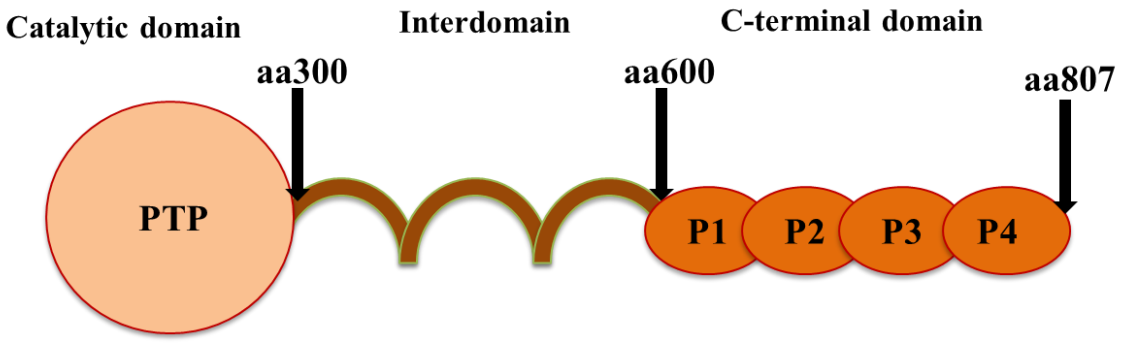


Fig 1.7 Structure of PEP/LYP- PEP/LYP is characterized by an N-terminal phosphatase domain made of 300 amino acid residues and C-terminal domain with 200 amino acid residues, which include four putative poly-proline motifs (P1-P4) which are sites of interaction with SH3 domain containing proteins.

In murine T cells, PEP is a potent negative regulator of T-cell receptor signaling, which acts by suppressing the activity of Src-like family of receptor tyrosine kinases, PEP knockout mice show positive selection in their thymi resulting in clonal expansion of memory and effector T cell pools (Hasegawa et al., 2004).Substrate-trapping experiments in these cells have identified interaction of PEP with Csk (C-terminal Src kinase) which is mediated by the proline-rich motif (P1) in PEP with the SH3 (Src-homology3) domain of Csk (Cloutier and Veillette, 1996). This complex between PEP and Csk has been shown to exert a synergistic inhibition on TCR signaling by acting on the PTKs like Lck and ZAP-70 (Cloutier and Veillette, 1999).

PEP/LYP is a leading example of a genetic variant that confers risk of developing diverse autoimmune diseases, including type-1 diabetes, rheumatoid arthritis, autoimmune thyroid disease and systemic lupus (Hermiston et al., 2009). A single-nucleotide polymorphism that changes arginine 620 to tryptophan (R620W) in this gene product is a common risk factor for Graves' disease, rheumatoid arthritis and systemic lupus erythematous. In T cells the autoimmune-associated LYP-R620W

polymorphism interferes with the formation of the complex between LYP and Csk as a result this is a gain-of-function mutation in which there is more efficient negative regulation of TCR signaling (Fiorillo et al., 2010; Vang et al., 2005; Vang et al., 2012; Veillette et al., 2009).

Association of PTPN22 with autoimmunity has potentially important therapeutic implications, since it has been proposed that inhibition of LYP/PEP activity, which would presumably enhance TCR signaling, could help prevent or treat such diseases(Yu et al., 2007)

PEP has recently been shown, to be expressed in mast cells (Maier et al., 2007) however its function in these cells is still unknown. As mast cell activation is known to have implications in autoimmune disorders, presence of this phosphatase in mast cells suggests that PEP might have an important role in regulating the mast cell activation.

1.6 Aim

Mast cell activation involves IgE mediated FcεRI crosslinking leading to phosphorylation of many PTKs (protein tyrosine kinases) which then initiates downstream signaling cascade culminating in the release of inflammatory mediators. The activity of these PTKs are regulated by the PTPs (protein tyrosine phosphatases), any dysregulation in the function of these PTPs have known to cause autoimmune disorders. The leading example is the LYP/PEP which has strong linkage to autoimmunity. In T-cells, PEP functions as a negative regulator of signaling. PEP has recently been shown, to be expressed in mast cells (Maier et al., 2007) and studies on PEP deletion showed no effect on mast cell numbers (Obiri et al., 2011, 2012) but PEP-/- mice are less susceptible to passive systemic anaphylaxis (Obiri et al., 2011, 2012)

The aim of this study is to identify the molecular mechanisms for the observed differences in the antigen mediated anaphylaxis between the wild-type and PEP knock-out mice .Study the major IgE-FcεRI receptor signaling pathway that lead to mast cell degranulation and eventually causing anaphylaxis in mice.

2.0 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals	Source
Acrylamide/Bis-Acrylamide	Carl Roth Gmbh & Co., Karlsruhe
Agarose	Peqlab ., Erlangen
Ammonium persulfate (APS)	Carl Roth Gmbh & Co., Karlsruhe
Ammonium Chloride	Carl Roth Gmbh & Co., Karlsruhe
β-Mercaptoethanol	Carl Roth Gmbh & Co., Karlsruhe
Bromophenol	Carl Roth Gmbh & Co., Karlsruhe
Bovine Serum Albumin	Sigma-Aldrich Chemical., Taufkirchen
Chloroform	Merck KGaA., Darmstadt
Dimethyl sulfoxide (DMSO)	Carl Roth Gmbh & Co., Karlsruhe
Dithiothreitol (DTT)	Carl Roth Gmbh & Co., Karlsruhe
ECL-Western Blotting Solution	GE HealthCare., Freiburg
Enolase-rabbit muscle	Sigma-Aldrich Chemical., Taufkirchen
Ethanol	Carl Roth Gmbh & Co., Karlsruhe
Ethidium bromide	Sigma-Aldrich Chemical., Taufkirchen
Formaldehyde	Merck KGaA., Darmstadt
Glycerol	Merck KGaA., Darmstadt
Glycine	Carl Roth Gmbh & Co., Karlsruhe
Glucose	Carl Roth Gmbh & Co., Karlsruhe
Glycogen	Peqlab ., Erlangen
HEPES	Carl Roth Gmbh & Co., Karlsruhe
Isopropanol	Merck KGaA., Darmstadt
Magnesium chloride	Carl Roth Gmbh & Co., Karlsruhe
Magnesium sulphate	Carl Roth Gmbh & Co., Karlsruhe

Methanol	Carl Roth Gmbh & Co., Karlsruhe
N,N,N',N',-Tetremethylethylendiamine(TEMED)	Sigma-Aldrich Chemical., Taufkirchen
PeqGOLD	Peqlab ., Erlangen
Sodium acetate	Merck KGaA., Darmstadt
Sodium chloride	Carl Roth Gmbh & Co., Karlsruhe
Sodium dodecylsulfate	Carl Roth Gmbh & Co., Karlsruhe
Sodium hydroxide	Carl Roth Gmbh & Co., Karlsruhe
Tris-base	Carl Roth Gmbh & Co., Karlsruhe
Tris hydrochloride	Carl Roth Gmbh & Co., Karlsruhe
Triton-X-100	Bio-Rad., Munich
Trypan blue	Sigma-Aldrich Chemical., Taufkirchen
Tween-20	Carl Roth Gmbh & Co., Karlsruhe

2.1.2 Cell culture chemicals and cytokines

Chemicals	Source
Dinitrophenyl-Human Serum Albumin(DNP-HSA)	Sigma-Aldrich Chemical.,Taufkirchen
Foetal Bovine Serum(FBS), South American origin	Biowhittakker.,Belgium
L-Glutamine	Gibco., Karlsruhe
Iscove's Modified Dulbecco's Medium (IMDM)	Gibco., Karlsruhe
Murine anti-DNP IgE (Clone SPE-7)	Sigma-Aldrich Chemical.,Taufkirchen
Murine Interleukin-3 (IL-3) recombinant	Biomol Gmbh., Germany
Murine SCF-recombinant	kind gift from Dr.Michael Stassen (Stassen et al ,2006)
Phosphate Buffered Saline(PBS)	Gibco., Karlsruhe
Sodium pyruvate	Gibco., Karlsruhe

2.1.3 Antibodies

Antibody	Source
Primary Antibody	
anti-β Actin	SantaCruz., Heidelberg
anti-AKT	Cell Signalling ., Frankfurt
anti-Cbp /PAG	SantaCruz., Heidelberg
anti-Csk	Millipore., Schwalbach
anti-Fyn	SantaCruz., Heidelberg
anti-Gsk-3β	Cell Signalling ., Frankfurt
anti-JNK	Cell Signalling ., Frankfurt
anti-LAT	SantaCruz., Heidelberg
anti-Lyn	Cell Signalling ., Frankfurt
anti-NFATc1	SantaCruz., Heidelberg
anti-PTPN22 (PEP)	SantaCruz., Heidelberg
anti-PlSrc	SantaCruz., Heidelberg
anti-αTubulin	SantaCruz., Heidelberg
Primary phosphospecific Antibody	
anti-phospho Btk	SantaCruz., Heidelberg
anti-phospho Gsk-3β	Cell Signalling., Frankfurt
anti-phospho JNK	Cell Signalling., Frankfurt
anti-phospho LAT(Y-132)	Abcam., London
anti-phospho LAT(Y-191)	Cell Signalling., Frankfurt
anti-phospho Lyn (Y-505)	Cell Signalling., Frankfurt
anti-phospho Lyn (Y-527)	Cell Signalling., Frankfurt
anti-phospho Vav	SantaCruz., Heidelberg
anti-phospho PLCγ-1	Cell Signalling., Frankfurt
anti-phosphotyrosine (Clone-4G10)	Millipore., Schwalbach
Secondary Antibody	
anti-Goat-HRP	DAKO Diagnostika Gmbh., Hamburg
anti-Rabbit-HRP	DAKO Diagnostika Gmbh., Hamburg

anti-Mouse-HRP	DAKO Diagnostika Gmbh., Hamburg

2.1.4 Oligonucleotides

Primers for genotyping knockout mice	
KH-1	5'-AATGCCCGCTTGATGAGCAGAGGTCTG-3'
KH-2	5'-GGCTTCTTTCAGTAGTTGCTGCAGAAT-3'
KH-4 neo-Cassette	5'-TGCTAAAGCGCATGCTCCAGACTGC-3'
Primers for Real-Time PCR analysis	
36B4(Arbp)	For 5'-GGACCCGAGAAGACCTCCTT-3' Rev 5'-GCACATCACTCAGAATTTCAATGG-3'
IL-1β	For 5'-AAGGAGAACCAAGCAACGACAAAA-3' Rev 5'-TGGGGAACTCTGCAGACTCAAACT-3'
IL-2	For 5'-GAGACTTGTGCTCCTTGTCA-3' Rev 5'-TCAATTCTGTGGCCTGCTTG-3'
IL-4	For 5'-TCGGCATTTTGAACGAGGTC-3' Rev 5'-GAAAAGCCCGAAAGAGTCTC-3'
IL-6	For 5'-GAAATGATGGATGCTTCCAAACTG-3' Rev 5'-GGATATATTTTCTGACCACAGTGATGAGG-3'
IL-10	For 5'-CTTGCACTACCAAAGCCACA-3' Rev 5'-AAGTGTGGCCAGCCTTAGAA-3'
IL-13	For 5'-CCTGGCTCTTGCTTGCCTT-3' Rev 5'-GGTCTTGTGTGATGTTGCTCA-3'
CCL-2 (MCP-1)	For 5'-ATCCCAATGAGTAGGCTGGAGAGC-3' Rev 5'-CAGAAGTGCTTGAGGTGGTTGTG-3'
CCL-12	For 5'-CCTGTGGCCTTG GGCCTCAA-3' Rev 5'-GAGGTGCTGATGTACCAGTTGG-3'
MIP-1α	For 5'-ACCTGCTCAACATCATGAAGG-3' Rev 5'-AGATGGAGCTATGCAGGTGG-3'
TNF-α	For 5'-TACACTTCGGGGTGATCGGTCC-3' Rev 5'-CAGCCTTGTCCCTTGAAGAGAACC-3'
NFATc1	For 5'-CAAAGGAGAGGTCGGACTCG-3' Rev 5'-ACTGTAGTGTTCTTCCTCGGC-3'
PLSCR1	For 5'-GGATCCATGGACAAACAAAA-3' Rev 5'- AAGCTTTTTCATTTTAACATC-3'

PPP3cb	For 5'-TCTGTTCTCAGGGAGGAGAGT-3' Rev 5'-CCAGCCAACACTCCACTAGG-3'
Orai-1	For 5'-CCTGGCGCAAGCTCTACTTA-3' Rev 5'-CATCGCTACCATGGCGAAGC-3'
IP3R	For 5'-TGCCTCCACAATTCTACGACTGA-3' Rev 5'-TCCCACAGTTGCCCACAAAG-3'
STIM1	For 5'-TTGGGCCTCCTCTCTTGACT-3' Rev 5'-TCCTGCTTGGCAAGGTTGAT-3'

2.1.5 Plasmid construct

Plasmid	Description
pUbi-Renilla Luc	Ubi-Renilla-luciferase construct was generated by replacing the growth hormone cDNA in pUbiGH (Schorpp et al. 1996) by Renilla reniformis luciferase cDNA (Promega., Mannheim)
p3xNFAT Luc	Generously provided by Laurie Glimcher, Boston. USA.
p6xNF-kB-tk-Luciferase (3Enh-tk-Luc)	Construct described in (Israel et al., 1992)
pIL-6-Luciferase (–158/111 IL-6- Luc)	Construct described in (Stein and Yang, 1995)
p5xTRE-TATA-Luc (AP-1-Luc)	Construct described in (Jonat et al., 1990)
p3×HA SR α JNK1, p3×HA SR α JNK1-APF, p3×HA SR α JNK2, p3×HA SR α JNK2-APF.	Constructs kindly provided by Dr. Michael Karin, University of California, San Diego, USA (Kallunki et al., 1994).

2.1.6 Inhibitors and other reagents

Inhibitor	Source
C28- Bis (2-cyanoethyl) phenyl phosphine-Au(I)-Cl	Generously provided by Dr. Amy Barrios, Utah, USA.
L75N04- Benzofuran salicylate moiety	Generously provided by Dr. Zhong-Yin Zhang, Indiana, USA.
Reagents	**Source**
Taq Polymerase and Reverse transcriptase	Promega., Mannheim
Protein and DNA ladder and dNTPs	Peqlab., Erlangen

2.1.7 Mouse strain

PEP knockout mice in which PEP was inactivated by homologous recombination was generated in the Lab of Andrew Chan (Hasegawa et al., 2004) and supplied by Genetech Inc., San Francisco. Briefly, PEP+/- embryonic stem cells were generated by homologous recombination, and then injected into C57BL/6 blastocytes to yield chimeric mice that were crossed with pure C57BL/6 strain mice to generate PEP+/- germline mice. Successive crossing of mice yielding the PEP knock-out mice referred as PEP-/- in this thesis were done at ITG Animal Facility. All mice were in the animal laboratory of the ITG (Security Level S1, GenTG) under SPF (specific pathogen free) conditions. The rearing, growing and killing the mice and the animal studies were in compliance with the Animal Welfare Act.

2.2 Methods

2.2.1 Nucleic Acid Methods

2.2.1.1 Genotyping of PEP knock-out mice

Isolation of Genomic DNA

For determining the genotype of the progeny of the PEP-knock-out mouse line-tail biopsies from newborn or adult mice were incubated in 200µl of lysis buffer (100mM Tris pH 8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl, proteinase K 100ug/ml) at 55°C for 2hour. The lysates were subsequently centrifuged for 5 min at 14,000 rpm (Eppendorf centrifuge 5417R) and the supernatant containing the genomic DNA was transferred into fresh 1.5ml eppendorf tube containing 200µl cold isopropanol for DNA precipitation. The DNA was centrifuged for 5 min at 14,000 rpm and dried in a heating block at 37°C and then resuspended in 100µl TE buffer (10mM Tris pH 7.5, 0.1mM EDTA pH 8.0) by and then stored at -20°C.

Genotyping by PCR with specific gene locus primers

Genomic DNA isolated from tail biopsies were used as a template in the PCR reaction, in which sequence specific oligonucleotides (Primers see 2.1.4) were used. The oligonucleotides KH1 (forward) and KH2 (reverse) were used, so that they anneal within the genomic sequence of PTPN22 gene, outside the left and right border, respectively, of the neo-cassette that was originally inserted in the exon-1 of the PTPN22 gene to disrupt it (Hasegawa et al., 2004). KH4 primer instead anneals within the neo-cassette sequence. The PCR reaction was carried out in a reaction volume of 20µl in the thermal cycler machine (GeneAmp^{TM} PCR System 2700, Applied Biosystem). The composition of the PCR master mix and the steps of the PCR programme are listed below.

Composition of PCR Master Mix

Reagents	per Reaction
DNA Template	4µl
5x Go Taq Reaction Buffer	4µl
dNTPs (10Mm)	0.5µl
Forward Primer-KH1 (10pmol)	1µl
Reverse Primer- KH2/KH4 (10pmol)	1µl
Taq Polymerase (1.25U)	0.25µl
H_2O	9.25µl

PCR-Programme

Steps	Temperature[°C]	Time
Pre-heating	95	3 min
Denaturing DNA	95	1 min
Annealing- hybridization with primer	55	1 min
Elongation	72	1min
Amplification	Steps 2-4 repeated through 27 cycles	
Hold	4	-

Two separate PCR reactions were set for each template DNA. The amplification products were subsequently separated on a 2% TAE Agarose gel. In the first PCR reaction, KH1 (forward) and KH2 (reverse) primer were used, wherein the amplified product of size 350bp would be obtained only if the template DNA contained a wild-type allele. In the second PCR reaction, KH1 (forward) and KH4 (reverse) primer were used; in this case the product of size about 450 bp would be obtained only if the template DNA contained a mutated allele.

DNA separation by Agarose-gel-Electrophoresis

Separation of DNA fragments was done on a 2% w/v Agarose gel. For this purpose, 2% Agarose was dissolved in 1x TAE buffer (40mM Tris pH 7.2, 2mM sodium acetate, 1mM EDTA pH8) by heating. After cooling of the Agarose solution to about 60°C, Ethidium bromide to a final concentration of 0.3µg/ml was added and the Agarose was poured into a horizontal electrophoresis chamber. The polymerized gel was overlaid with 1x TAE buffer, and samples were loaded into the wells. The separation was carried out at 100 V. Using a trans-illuminator (IL-350, Bachofer, Reutlingen), the DNA bands under UV light (256 nm) were visualized and photographed using Eagle Eye ^{TM} photo camera system.

2.2.1.2 RNA Analysis

Isolation of total RNA from BMMC

2-3 x 10^6 /ml suspension of BMMC were transferred into 1.5 ml eppendorf tube and centrifuged (Eppendorf centrifuge 5415D) for 5 min at 2000 rpm after which the supernatant was removed. To lyse the cells, 1 ml of PeqGOLD RNAPure isolation reagent was added to the cell pellet and after 5 min of incubation at RT, RNA was extracted by adding 200µl of chloroform and vortexed for 15 sec followed by incubation on ice for 5 min and then centrifuged at 14,000 rpm for 10 min at 4°C. 400 µl of the upper phase was taken into a fresh eppendorf tube and 400µl of chloroform was added and vortexed followed by incubation on ice for 10 min and further centrifuged at 14,000 rpm for 10 min at 4°C. To precipitate the RNA, 400µ of the upper phase was taken into fresh eppendorf tube containing 5µl glycogen, mixed and incubated for 5 min at RT. 400µl of isopropanol was added, mixed and incubated for 15 min on ice and then centrifuged at 14,000 rpm for 15 min at 4°C. Supernatant was discarded and the RNA pellet was washed by adding 1 ml of 75% ethanol, then vortex and centrifuged at 14,000 rpm at 4°C, this step was repeated twice. The pellet was dried by keeping at RT for 5 min. To elute the RNA, 20µl sterile RNAse-free water was added to the pellet and vortexed and incubated at 37°C for 10 min and then in heating block at 56°C for 3min. 1µl of the RNA extract was used to measure the RNA concentration. The RNA was then frozen in liquid N_2 and stored at -80°C.

Quantification of RNA

To quantify the amount of RNA, the optical density (OD) at 260, 280 and 230 nm was measured with NanoDrop™ and the software ND-1000(version 3.1.2). An OD_{260}=1 corresponds to 40µg/ml RNA. A ratio of OD_{260} /OD_{280} ranging from 1.8-

2.0 indicates a nucleic acid preparation relatively free from protein contamination.AratioOD_{260} /OD_{230} above 1.6 indicates a preparation free of organic chemicals and solvents.

cDNA-synthesis and RT-PCR

To create first strand of cDNA, 1ug of total RNA was transcribed into cDNA by means of Reverse Transcriptase M-MLV1 (Moloney Murine Leukemia Virus-Reverse Transcriptase Promega., Mannheim). The cDNA preparation involves two steps, in the first step RNA first is incubated with 100pmol random hexameric primer for 5 min at 70°C, and then cooled on ice. In the second step Reverse transcriptase mix is added and the whole mixture was incubated in the thermo cycler machine. The composition of reverse transcriptase mix and the PCR programme used are listed below.

Composition of Reverse Transcriptase- Mix

Reagents	Per Reaction (1µg RNA)
5x M-MLV1-RT buffer	4µl
dNTP (10mM)	2µl.
M-MLV1-RT (200U/µl)	1µl

PCR programme for cDNA synthesis

Steps	Temperature[°C]	Time
1	25	10 min
2	42.	60 min
3	70	10 min

After completion of the cDNA synthesis, the cDNA was diluted with DEPC water at 1:10 and stored at -20°C.A quality control PCR was run with GAPDH primers

using the cDNA as a template and the amplification product was separated by Agarose gel electrophoresis.

Quantitative Real-Time PCR (qRT-PCR)

For the analysis of cDNA, qRT-PCR e was performed using the device ABI StepOnePlus RT-PCR System (Applied Bio systems, Darmstadt, Germany). In an ABgene® PCR plate 5µl cDNA template was added with 18µl of SYBR Master Mix (6µl sterile water, 1µl of gene-specific primer pairs (each 10 pmol) and 10µl SYBR ® Green Mix buffer, Qiagen) end volume 20µl.The steps of PCR programme are listed below.

PCR Programme

Steps	Temperature[°C]	Time
Incubation	50	2 min
Denaturation	95	15 min
Denaturation	95	15 sec
Annealing and Elongation	60	30 sec
Amplification	Steps 3-4 repeated	
Denaturation	95	15 sec
Melting curve	60	1 min

2.2.2 Protein Analysis

2.2.2.1 Preparation of Total cell Lysates

For the extraction of whole cell lysates for the immunoblot analysis NP40 lysis buffer was used. Approximately 2-5×10^6 BMMC was harvested and then transferred into an Eppendorf tube and centrifuged for 5 min at room temperature at 2000 rpm. The medium was discarded and the cells were washed once with 1 ml PBS and centrifuged again. The cell pellet was resuspended in 55µl NP40 lysis and incubated for 20 min on ice. Thereafter, equal volume of 2x SDS sample buffer was mixed (Vortex) and immediately incubated for at least 5 min at 95 ° C in heating block. The lysate was briefly centrifuged at 12000 rpm, and then sonified with 6-8 pulses Ultrasonic disintegrator (level 2-3; Branson Sonifier, G. Heinemann, Schwäbisch Gmünd).

Nonidet P-40 (NP-40) Lysis Buffer	
Nonidet P-40	0.1%
Boric Acid	200mM
NaCl	160mM
EDTA	5mM
PMSF	1mM
Protease Inhibitor Cocktail	1%
2X SDS-Sample Buffer	
SDS	4%
Tris-HCl pH 6.8	160mM
Glycerol	20%
Bromophenol blue	0.02%
B-Mercaptoethanol	4%

2.2.2.2 Separation of Proteins by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For the analysis of proteins by SDS-PAGE 12% separating gel was used. The Penguin Doppelgel System P9DS apparatus (Peqlab, Erlangen, Germany) was used to cast the polyacrylamide gel. About 20 ml of the resolving gel was poured and overlaid with 70% Rotisol. After polymerization the Rotisol was washed off with distill water and the separating gel was covered with 10 ml stacking gel and the comb of the appropriate number of wells were inserted before polymerization. The gel was then fixed in the running chamber and 1X Lammeli buffer was used for run. Around 10-40µl of the sample was loaded per well alongside with protein marker. Gel electrophoresis was carried out initially at 90V in the stacking gel and 120-140V in the separating gel.

SDS-PAGE	**12 % Resolving Gel**	**5 % Stacking Gel**
Acrylamide	12%	5%
Tris-HCl pH 8.8	375mM	---------
Tris-HCl pH 6.8	----------	125
SDS	0.1%	0.1%
APS	0.1%	0.1%
TEMED	0.05%	0.1%

1X Lammeli Buffer	
Tris	25mM
Glycine	200mM
SDS	0.1%

2.2.2.3 Western Blot Analysis

Proteins in SDS-polyacrylamide gels were subjected by electro-transfer using a Wet transfer chamber (Trans-Blot, Bio-Rad laboratories GmbH, Munich) onto a PVDF (polyvinylidene fluoride) membrane (Immobilon, Millipore) presoaked in methanol at 300-350 mA or 35V for minimum 8 h at 4°C in 1X Western Transfer Buffer.

1X Transfer Buffer	
Tris	25mM
Glycine	200mM
Methanol	10% (v/v)

Immunoblotting was performed according to individual instructions for each antibody. In order to reduce unspecific binding of the antibodies to the membrane, the blot was incubated in blocking solution 1X TBST supplemented with either 3-5% (w/v) non-fat dried milk or 5% BSA according to the different antibodies that were going to be used, at RT for 1 h with shaking.

1X TBST	
Tris-HCl pH7.4	10mM
NaCl	150mM
Tween	0.05%

For detection of proteins of interest the membrane was incubated in blocking solution containing the appropriate primary antibody (optimal working dilution was determined empirically) at 4°C overnight under constant rotation. After 3 washes of 10 min each in TBST buffer, the membrane was incubated for 1 hour in

the blocking solution containing a (1:2000) dilution of HRP (Horseradish peroxidase) conjugated secondary antibody. Once the membrane has been washed 3 times 10 min each, detection of specific protein was achieved by enhanced chemiluminescence. This light-releasing reaction is based on the oxidation of a substrate, which is catalyzed by the horseradish peroxidase. For this purpose, the membrane was incubated for 1 min with ECL Western blotting reagent (Amersham, Braunschweig) as directed by manufacture. The signal was developed using X-ray film (Hyper film ECL, or MP, Amersham, Braunschweig).

Western blots were scanned from film with an Epson Expression 1600 digital scanner (Epson, Long Beach, CA), and density was determined using Image J Software; intensities of multiple samples on a single blot were normalized to a single sample in each blot.

2.2.2.4 Stripping of Western blot membranes

To allow more than single use of Western blot membranes, the membranes were incubated in pre-warmed "stripping" solution for 30 min at 55-60°C with shaking. For the removal of β-Mercaptoethanol and SDS the membrane was washed 3 times in the 1xPBS for 15 min each.

Stripping Solution	
Tris-HCl pH 6.8	62.5mM
SDS	2%
β-Mercaptoethanol	0.75%

2.2.2.5 Co-immunoprecipitation

For immunoprecipitation, $10x10^6$BMMC are activated with IgE (1ug/ml) for 2 h and then stimulated with DNP-HSA (200ng/ml) for 30 min. The cells were then harvested, centrifuged for 5 min at 4°C at 1500 rev/min and washed twice with ice-cold PBS + 1mM PMSF + 1% PIC. Subsequently, the cells were suspended in 0.5 ml lysis Buffer (0.1% Nonidet P-40, 200mM boric acid, 160mM NaCl, 5mM EDTA, and 1mM PMSF, 1mM sodium-orthovanadate, 1% PIC) and kept on ice for 20 min. The lysates were centrifuged for 20 min at 4°C 12,000 rpm / min and the supernatant was transferred to a new eppendorf tube. In parallel, 50µl of 50% protein G-Sepharose beads were incubated with 5µg of protein-specific antibodies or with 5ug IgG control antibody for 2 h at 4°C. After incubation, the beads were centrifuged and the unbound antibody is removed and 100µl of cell lysate is added on to the beads and the control input is stored at -20°C. The beads alongwith the lysate is incubated overnight at 4°C (rotating); the beads are then centrifuged for 1min at 1400 rpm at 4°C. Supernatant is carefully removed and the beads are washed 5 times with ice-cold 1 ml lysis buffer. The bound protein is eluted by adding 2% SDS sample buffer and incubating at 95°C for 10 min alongwith the input control. The amount of protein immunoprecipitated was analyzed by performing immunoblot as described in Western Blot Analysis. .

The amount of tyrosine phosphorylated proteins was corrected for the amount of proteins immunoprecipitated as determined by densitometry of immunoblots after stripping the membranes, followed by development with corresponding antibodies.

2.2.2.6 In-Vitro Kinase Assay

Lyn was immunoprecipitated by incubating lysates from 8-10×10^6 BMMC (stimulated and non-stimulated) with 5µg anti-Lyn mouse antibody as described in Co-immunoprecipitation. Immunoprecipitates were then washed twice with lysis buffer without detergent and then once with kinase assay buffer (20mM Tris, pH 7.6, 10mM MgCl2, and 1mM Na3VO4). After washing, Lyn immunoprecipitates were subjected to in vitro kinase assays by adding 200µl of kinase assay buffer containing 1mM ATP and 30µl of prepared substrate Enolase (acid-denatured) as an exogenous substrate. Samples were then incubated at 37 °C for 2 h. Reactions were quenched by the addition of 50µl of 5× non-reducing SDS sample buffer followed immediately by boiling and then subjected to immunoblot analysis. Substrate was prepared by incubating 50µl of 1mg/ml Enolase with 50µl of 50mM sodium acetate at 30°C for 10 min, followed by neutralization with 60µl 1 M Tris (pH 8.8) and 840µl of kinase buffer. Kinase activity was determined as the intensity of phosphorylated Enolase following an in vitro kinase assay divided by the amount of Lyn in the same lane

2.2.3 Cell Culture Methods

2.2.3.1 Cell Culture

All cell culture work was done in a laboratory at a security level Safety cabinet (Class II, Thermo Electron, and Dreieich). BMMC were incubated at 37°C, 5-6% CO_2 and 95% humidity in an incubator (Forma Scientific Labor Tec GmbH, Gottingen). After harvesting the cells were washed either in Iscove's Modified Dulbecco's Medium (IMDM) or sterile magnesium and calcium-free PBS (137mM NaCl, 2.7mM KCl, 6.5mM Na_2HPO_4, and 1.5mM KH_2PO_4) was used.

2.2.3.2 Generation of bone marrow-derived mouse mast cells (BMMC)

For the generation of bone marrow-derived mouse mast cells (BMMC) PEP mice (Section 2.1.7), aged 8-10s week killed by cervical dislocation. Tail biopsies were taken for genotyping (Section 2.2.1.1). The mice were disinfected by immersing in 75% Rotisol. The skeletal muscle was removed from the bones of the posterior limbs and the intact femurs and tibias were isolated and kept in cold IMDM at 4°C. In cell culture laboratory bones were washed once with 75% Rotisol, and three times with PBS. After cutting the Epiphyses of the bone under sterile conditions, the bone marrow was repeatedly flushed through by means of cold IMDM using a 10 ml syringe (0.45 x 25 mm needle) until the Medullary cavity was reasonably free from blood. The cell suspension was collected in a 50 ml falcon tube and was centrifuged for 10 min at 1300 rpm at RT and the supernatant was discarded. The cell pellet was resuspended in 5 ml IMDM and centrifuged again for 10 min at 1300 rpm at RT. To lyse the erythrocytes, the cell pellet was resuspended for 2 min in 2 ml (for 3-5 mice) ACK lysis buffer at RT after which the reaction was terminated by addition of 8 ml of cold IMDM.

ACK Lysis Buffer (sterile filtered)	**pH 7.3**
NH_4Cl	150mM
$KHCO_3$	1mM
EDTA	1mM

For the removal of bone fragments, the Cell suspension was applied on a 70µm nylon Falcon cell strainer (BD Bioscience) and centrifuged again. The cells were then resuspended in 1 ml of the culture medium. After determining the number of cells, the cells were seeded at a density of $2x10^6$ cells / ml of growth medium in 24-well plates (Greiner) at 1 ml / well and cultured for 4 to 5 weeks. Thereby every

2-3 days, half of the medium was carefully removed, and the non-adherent cells with the help of a 1-ml Pipette were transferred after being washed several times in a new 24-well plate and fresh medium supplied. The adherent cells were discarded.

BMMC Culture/Growth Medium	**500 ml**
Iscove's Modified Dulbecco's Medium IMDM	
Fetal Bovine Serum (heat inactivated at 56°C)	10%
L-Glutamine	2mM
Pyruvate	1mM
IL-3	5ng/ml
Stem Cell Factor (SCF)	100ng/ml

2.2.3.3 Stimulation of BMMC

For Phosphorylation and Co-Immunoprecipitation studies BMMC at a cell density of 2-5×10^6 cells/ml were cultured in Growth medium, 2 h prior to harvesting the cells, anti-DNP IgE was added in a final concentration of 1µg / ml. Finally, the cells were transferred into a 1-ml Eppendorf tube, and centrifuged for 5 min at 1500 rpm (Eppendorf bench centrifuge). The medium was discarded and the cells were washed once with 37 ° C warm PBS and again centrifuged and resuspended in 50µl of growth medium and 50µl of DNP HAS was added to a final concentration of 200ng/ml and incubated at 37 ° C heating block at indicated time points.

For stimulation of BMMC by stem cell factor (SCF), the cells were initially cultured in IMDM growth medium and then starved by culturing in growth medium without IL-3 and SCF. Subsequently, the cells were stimulated with SCF at various concentrations ranging from 50-400 ng/ml and for indicated time points.

In some experiments, BMMC were incubated with PEP inhibitors L75N04 (5μM and 20μM) and C28 (5μM) for 1 h. All the inhibitors were dissolved in dimethyl sulfoxide (DMSO), aliquoted and stored at -20 ° C.

Other Experimental Methods

2.2.4 Sucrose Gradient Lipid Raft Fractionation

8-10×10^6 cell/ml BMMC after stimulation were rested on ice for 1min, and then lysed in 1 ml of lysis buffer containing 10mM Tris-HCl (pH 8.0), 50mM NaCl, 10mM EDTA, 1mM Na3VO4, 10mM glycerophosphate, 1mMPMSF and 1% (v/v) Brij-58. After 15 min, the lysate was homogenized by passing 10 times through a 27-gauge needle and adjusted to 40% (w/v) sucrose by adding an equal amount of 80% sucrose. A gradient was formed by successive addition of 0.2 ml of 80% sucrose stock to the bottom of a polyallomer tube (13 by 51 mm), followed by 0.5 ml of 60% sucrose, 1.5 ml of 40% sucrose (containing the cell lysate), 0.8 ml of 35% sucrose, and 0.5-ml aliquots of 30, 20, and 15% sucrose. Sucrose solutions were prepared by mixing the appropriate amount of the gradient buffer (25mM Tris-HCl [pH 7.5], 12mM NaCl, and 2mM EDTA) and 80% sucrose. Tubes were centrifuged at 210,000 g for 12 h at 4°C using a SW55Ti rotor (Beckman Instruments).After centrifugation, 10 fractions of 500μl were collected from top of the tube and then analyzed by immunoblotting ; lipid rafts were found in the upper 4 fractions

2.2.5 Degranulation Assay

5×10^6 cells/ml BMMC were harvested washed and re-suspended in 100 μl of Tyrodes buffer (135mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 5mM Glucose, 20mM HEPES, pH 7.4) at 37°C. Cells were then subjected to centrifugation at 2000 rpm at 4°C for 5min. The supernatant was aspirated and the

cell pellet was carefully resuspended in 200 µl Tyrodes Buffer + 0.5% TritonX-100 and then incubated on ice for 15 min. For complete lysis the cells were then centrifuged for 5min at 13,000 rpm. 50 µl of each sample was transferred to a 96-well plate (Falcon; BD Bioscience), and 50 µl of 1mM pNAG(p-nitrophenyl N-acetyl-β-d-glucosaminidine) is added to each well and incubated for 90 min at 37°C. Finally 150µl of Glycine (0.2M, pH10.7) is added and the color change is measured spectrophotometrically by measuring OD in an Ultra micro- plate reader ELx808IU (Bio-Tec-Instruments, Inc.) at a wavelength of 405 nm. Percent degranulation was calculated as follows (released activity/total activity) ×100.

2.2.6 Adhesion Assay

BMMC were cultured overnight in IL-3 and Kit-ligand (SCF-stem cell factor) free medium. Cells were washed with HEPES buffer + 0.04% bovine serum albumin (BSA) and 3-5×10^6 BMMC were seeded per well in a 96-well tissue culture plate (Falcon; BD Bioscience) precoated with 5µg/ml fibronectin and blocked with 4% BSA. The cells were then either left unstimulated or stimulated with 50 and 100ng of SCF for 30 min. Non-adherent cells were removed by carefully washing the wells once. Wells used for total cells were not washed. Cells were lysed with 0.1 % TritonX-100, and 100µl of the Lysate was then transferred to a 96-well plate for β-hexosaminidase determination as described in Degranulation assay. The percent adherent cells were calculated as [(absorbance of sample – background) / (absorbance of total cell lysates – background)] × 100%.

2.2.7 Transient Transfection of BMMC

The transfection of BMMC with plasmid DNA was carried out by Electroporation using the Nucleofector II (Amaxa), using the Program T-023. For this BMMC were first washed twice with IMDM 5 min and centrifuged at 1500 rpm. Approximately 4-6×10^6 cells in 100 µl were resuspended in 37°C-warm IMDM

resuspended. Around 10µg of Plasmid DNA was filled in this suspension and it was filled by another 100µl IMDM. The 200µl cell suspension was transferred into the cuvette (Amaxa) and treated in the Nucleofector electroporation. Thereafter immediately 800µl of pre-warmed growth medium was added to the cuvette and the cells were pipetted out into a 6-well plate filled with 2 ml of pre-warmed growth medium and incubated for 24 h and then stimulated accordingly.

2.2.8 Luciferase Reporter Gene Analysis

8×10^6 BMMC cells were transfected with 4.3µg of reporter luciferase plasmid and 0.8µg of pUbi-Renilla Luc vectors by electroporation using the Nucleofector II (Amaxa) Program T-023. The cells were then plated in 2 ml culture medium for 24 h to recover from the electroporation. The cells were then left unsensitized or sensitized with anti-DNP IgE for 18 h. They were then left untreated or activated with DNP-HSA for 8 h. Thereafter they were lysed in passive lysis buffer (Promega Mannheim, Germany) and the lysates were centrifuged at 10,000 rpm for 10 min at 40 C. Firefly and Renilla luciferase activity were then measured using a Perkin Elmer luminescence counter using 20µl lysate with 70µl reaction buffer (25mM Glycylglycine, 15mM $MgSO_4$, 4mM EGTA, pH 7.8 with 1mM DTT and 2mM ATP) 30µl D-(-)-luciferin solution (0.28 mg / ml 0.5 M Tris, pH 7.5) were mixed and were measured immediately. For determination of the Renilla luciferase activity were 20µl lysate with 100 coelenterazine µl buffer (0.2 M KH_2PO_4, 0.2 M K_2HPO_4, pH 7.6) and 0.2µM coelenterazine. The luciferin and coelenterazine oxidation released luminescence was measured (relative Light units, RUL), the Quotient from RUL [luciferin] / [coelenterazine] was formed.

2.2.9 Cytosol/Nuclear extraction Assay

Approximately $2\text{-}5\times10^6$ BMMC was harvested and then transferred into an Eppendorf tube and centrifuged for 5 min at 4°C at 2000 rpm. The pellet was

washed twice with ice-cold PBS. The pellet was resuspended in BUFFER A (10mM Tris-HCl pH 8.0, 1mM EDTA, 100mM NaCl, 1% Protease inhibitor cocktail) and kept on ice for 5 min. 2µl of $MgCl_2$ (2mM final concentration) is added to the suspension to keep nuclei intact and then add 10µl of 10% NP-40and vortex for 10 sec and then centrifuge for 3 min at 4000rpm at 4°C. Collect the supernatant which is the NP-40 soluble cytoplasmic fraction, the pellet is resuspended into BUFFER B (10mM Tris-HCl pH 8.0, 1mM EDTA, 1% Protease inhibitor cocktail) and then add salt to extract nuclei (1/10 volume of 2.5 M ammonium sulfate) mix thoroughly and incubate on ice for 45 min and then centrifuge for 30 min at 40,000 rpm at 4°C in Ultracentrifuge and collect the supernatant which contains the nuclear fraction.

2.2.10 Preparation of RNA for Microarray

Total RNA was prepared from 10 X 10^6 cells from resting and activated WT and PEP-/- BMMC using TRIzol reagent (Invitrogen Life Technlogies) according to manufacturer's instructions. Further purification of RNA was done using the RNeasy kit (Qiagen). This RNA sample was then sent to Dr. Ludger Klein-Hitpaß (University Essen) for micro-array analysis. In his Lab following procedures were followed. Approximately 200 ng of total RNA was converted to cDNA using the Superscript II RNA reverse transcriptase (Invitrogen Life Technologies). Double-strand cDNA was produced with an oligo (DT) primer containing the T7 RNA polymerase site at the 5'end. The cDNA was labeled and biotinylated nucleotides with the Affymetrix labeling kit as per the manufacturer's instructions. The cDNA was hybridized for 16 hr to the Affymteric mouse genome chip (430 A) containing 14,000 genes. Chips were washed, stained with Streptavidin – PE and read using the GC Scanner 300 with G7 update according to the manufacturer's instructions.

2.2.11 Passive Systemic Anaphylaxis

8-9 month old female mice (20-25g) were sensitized by intra-peritoneal (i.p) injection of anti-DNP IgE (1mg/kg) for 24 h. C28 (5μM) or L75-NO4 (20μM) or vehicle sterile Magnesium and Calcium –free PBS (137Mm NaCl, 2.7mM KCl, 6.5mM Na_2HPO_4, 1.5mM KH_2PO_4) were administered by IP injection 1 h to the end of sensitization period. Anaphylaxis is then initiated by intravenous (i.v) injection of 200μl dinitrophenyl human serum albumin DNP-HSA (1 mg/ml PBS). Body temperature was measured with a Micro-life VT 1831 Vet-Temp (Tier-shop, Trier, Germany) every 5 min over a period of 60 min.

2.2.12 Statistics

Data are represented as Mean values ± Standard Error of the Mean ($n \geq 3$, n is a positive integer). Group comparisons were done using the Mann-Whitney U-test. Where more than two groups were involved, the results were analyzed for differences using the One-Way Analysis of Variance (ANOVA) and the significant difference between groups were examined by the Newman-Keul's range test at 5% level of significance. All graphs were plotted using the GraphPad Prism software version 5.0 (GraphPad, San Diego, CA).

3.0 Results

3.1 Interaction of PEP with Src-like kinases

PEP is a cytosolic tyrosine phosphatase expressed primarily in hematopoietic tissues like thymus, fetal thymus, spleen and lymphoid tissue, including T and B cells as well as myeloid cell lines (Cohen et al., 1999). Overexpression studies in T cell lines have demonstrated a negative regulatory role for PEP/LYP in T cell receptor signaling and identified its substrate as the activating loop tyrosine of Lck (Cloutier and Veillette, 1999; Gjorloff-Wingren et al., 1999). Substrate trapping experiments in T-cells have shown components of TCR signal transduction machinery such as ZAP-70, CD3ζ, CD3ε, and Vav to be the additional targets of LYP apart from Lck (Wu et al., 2006). PEP has recently been shown to be expressed in mast cells (Maier et al., 2007) and to positively regulate antigen-mediated anaphylaxis (Obiri et al., 2011), but its mode of action is still not clear.

In order to understand the mechanism of action of PEP in mast cell signaling, its interaction partners needs to be determined. Femurs were isolated from 8-10 week old PEP wild type (PEP+/+) and PEP knock-out (PEP-/-) mice and the bone marrow was flushed into the culture medium containing IL-3 and SCF. Bone marrow derived cells (BMMC) were cultured for 4-5 weeks in order to generate homogenous mast cell cultures. These cells were sensitized with antigen-specific immunoglobulin E (IgE anti-dinitrophenyl) for 2 hours and the cells were then stimulated with specific antigen, dinitrophenyl-human serum albumin (DNP-HSA) for 30 min. Cell lysates were prepared and then subjected to immunoprecipitation and then immunoblotted with specific antibodies.

Co-immunoprecipitation studies revealed that PEP in BMMC interacts with src-like kinases (Lyn, LAT and PLC-γ) either in the presence of IgE alone or IgE/DNP

but not in the absence of antigen (Fig 3.1A, compare lanes 5, 6 with 4). There was no interaction in PEP -/- BMMC (Fig 3.1A, lanes 13-15). Hence PEP interacts with kinases like Lyn, LAT, PLC-γ only in the presence of antigen.

Unlike these kinases, another src-like kinase Csk, was constitutively associated with PEP in PEP+/+ BMMC even in the absence of IgE (Fig 3.1A, compare lanes 5, 6 with 4) while no interaction was detected in PEP-/- BMMC (Fig 3.1A, lanes 13-15).

To confirm these findings, Lyn and Csk were immunoprecipitated and interaction of PEP with these kinases was analyzed in a Western blot in PEP+/+ and PEP-/- BMMC (Figs 3.1B and 3.1C). PEP was indeed found to be associated with Lyn only in the presence of antigen (Fig 3.1B, lanes 3-4) while PEP was again constitutively associated with Csk (Fig. 3.1C, lanes 3-4). In these studies no interaction was seen in the absence of PEP in PEP-/- BMMC (Figs 3.1B and 3.1C).

In T cells, PEP and Csk interaction cooperatively inhibit TCR signaling by dephosphorylation of positive regulatory residue Tyr^{394} in Lck by PEP and phosphorylation of negative regulatory residue Tyr^{505} by Csk. In order to study the effects of PEP-Csk interaction on mast cell signaling, association of Lyn (like Lck in T-cells, Lyn initiates the IgE mediated signaling in mast cells) with Csk in PEP+/+ and PEP-/-BMMC was examined. Also to determine whether PEP interacts with Csk and Lyn independently or whether the three proteins can be in a complex, the interaction of Csk and Lyn was analysed.

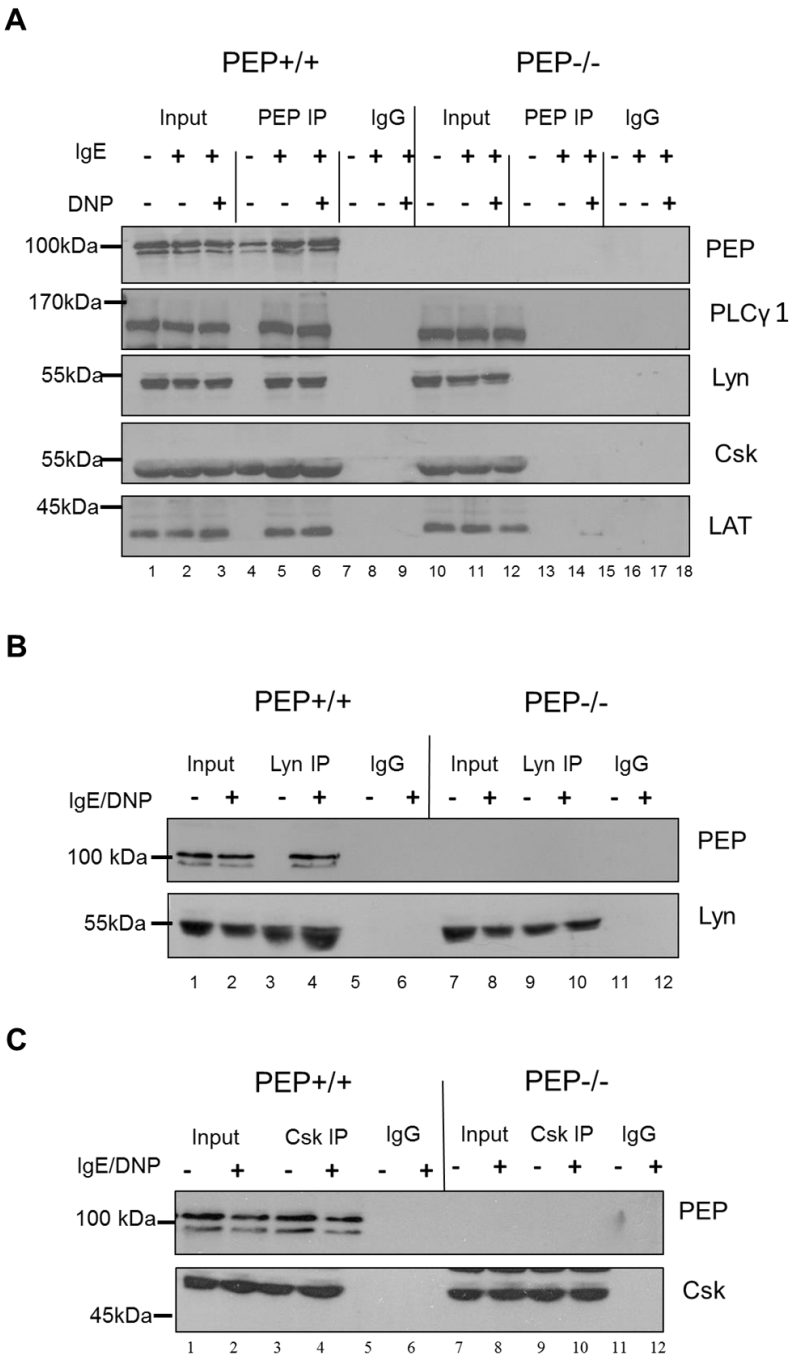


Fig 3.1 Association of PEP with src-like kinases - **(A)** 8-10 x 10^6 PEP+/+ and PEP-/- BMMC were treated with anti-DNP IgE (1µg/ml) for 2 h and activated with DNP-HSA (200 ng/ml) for 30 min. Cell lysates were immuno-precipitated using Protein A sepharose incubated with anti-PEP antibody or IgG control and subjected to immunoblot analysis with the indicated antibodies PEP, PLCγ1, Lyn, Csk and LAT. **(B)** and **(C)** PEP+/+ and PEP-/- BMMC were treated with anti-DNP IgE (1µg/ml) for 2 h and activated with DNP-HSA (200 ng/ml) for 30 min. Cell

lysates were immuno-precipitated with anti-Lyn (B) or with anti-Csk (C) and subjected to immunoblot analysis with anti-Lyn and anti-CSK antibodies. The results are representative of five independent experiments.

Co-immunoprecipitation with Csk, showed that in PEP+/+ BMMC, Lyn was associated with Csk only in the presence of antigen (Fig 3.1.1A lanes 3-4). In contrast, in PEP-/- BMMC, Lyn was found in association with Csk in the absence of antigen and this interaction was further enhanced, following IgE crosslinking (Fig 3.1.1A, compare lanes 9 and 10). These observations suggest that in mast cells, under resting conditions, the association of PEP with Csk is unable to interact with Lyn. Unlike other Src family kinases, Csk is devoid of the amino-terminal myristylation signal for localization at the plasma membrane. Thus it is cytoplasmic and interacts with PEP at this cellular localization. To form a complex with Lyn, it needs to be recruited to the plasma membrane (Honda et al., 1997; Yasuda et al., 2002)

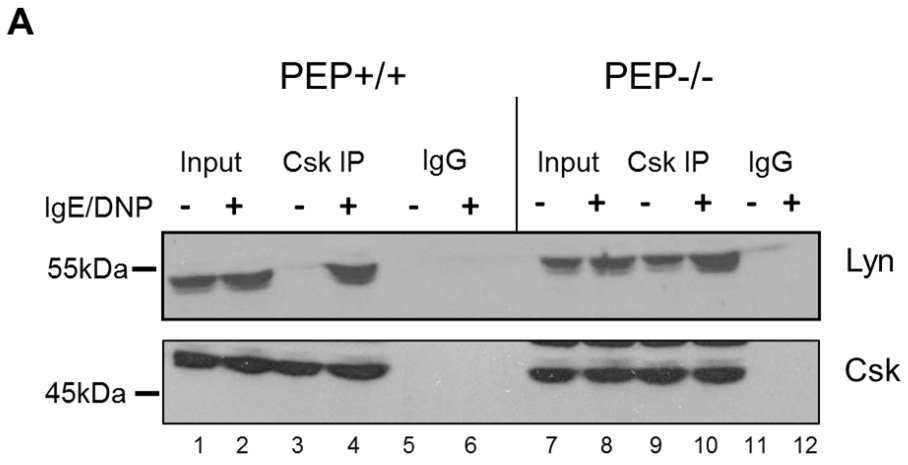


Fig 3.1.1 Interaction of Csk with Lyn - (A) 8-10 x 10^6 PEP+/+ and PEP-/- BMMC were treated with anti-DNP IgE (1µg/ml) for 2 h and activated with DNP-HSA (200 ng/ml) for 30 min. Cell lysates were immuno-precipitated using Protein A sepharose incubated with anti-Csk antibody or IgG control and subjected to immunoblot analysis with anti-Lyn and anti-CSK antibodies. The results are representative of three independent experiments.

3.2 PEP deficiency induces Csk recruitment to the lipid rafts

The plasma membrane contains areas enriched in cholesterol and sphingolipids present in all eukaryotic cells that are referred to as lipid rafts (Draber and Draberova, 2002). Lipid rafts are important sites for protein tyrosine kinase-mediated protein-protein interaction that are involved in the initiation of receptor signaling pathways. These membrane rafts are also involved in mast cells signaling and are enriched with signaling molecules involved in the FcεRI mediated pathway (Silveira et al., 2011). Tyrosine phosphorylation of the high affinity immunoglobulin IgE receptor (FcεRI) by the Src family kinase, Lyn is the first known biochemical step that occurs during activation of mast cells after cross-linking of FcεRI by antigen. This coupling of Lyn and FcεRI is predominantly found in lipid rafts (Kovarova et al., 2001; Radeva and Sharom, 2004; Sheets et al., 1999).

To determine whether the complex of PEP and Csk is recruited to the lipid raft when mast cells are activated, cell lysates from PEP+/+ and PEP-/- BMMC before and after receptor activation were analyzed. The lysates were solubilized in detergent and then subjected to sucrose density gradient centrifugation that separates detergent resistant (rafts/nonsoluble) membrane from the light (soluble) fractions. These fractions were then subjected to western blot analysis and then immunoblotted with specific antibodies and the densitometric quantification of signal were carried out (Fig 3.2)

Under resting conditions, in PEP+/+ BMMC, Csk and PEP are present in the soluble fractions (Fig 3.2A, lanes 5-10) while Lyn and Cbp/PAG (a trans-membrane protein that is known to be present in lipid rafts (Ohtake et al., 2002b)

are present in the raft fractions (Fig 3.2A, lanes 1-4). After IgE/DNP crosslinking, both Csk and PEP are recruited to the lipid rafts (Fig 3.2A, right panel, lanes 1-4). Quantification of the signals in the Western blot experiments show that while no detectable PEP and Csk were present in the lipid rafts at the resting stage, around 20-40% of these proteins were found in the raft following IgE receptor crosslinking.

In case of PEP-/- BMMC under unstimulated conditions around 20-30 % of Csk is already present in the raft fractions (Figs 3.2C, lanes 1-4 and 3.2D) and the rest in soluble fraction (Fig 3.2C, lanes 5-10). In presence of antigen, the proportion of Csk in the lipid raft is increased to more than 40 % (Figs 3.2C, right panel, lanes 1-4 and 3.2D).

These results show that while Lyn is always present in the lipid rafts, differences exist in the level of Csk protein in the lipid rafts of PEP+/+ and PEP-/- BMMCs. In PEP+/+ BMMC, Csk is recruited to the lipid rafts only after activation of the cells while in PEP-/- BMMC, Csk is already present in the lipid rafts but its level is increased upon antigen stimulation. This is in accordance with the immunoprecipitation results where Csk interacts with Lyn only in the presence of antigen stimulation in PEP+/+ BMMC whereas there is interaction of Csk with Lyn under unstimulated conditions which further enhances after IgE/DNP crosslinking in the PEP-/- BMMC.

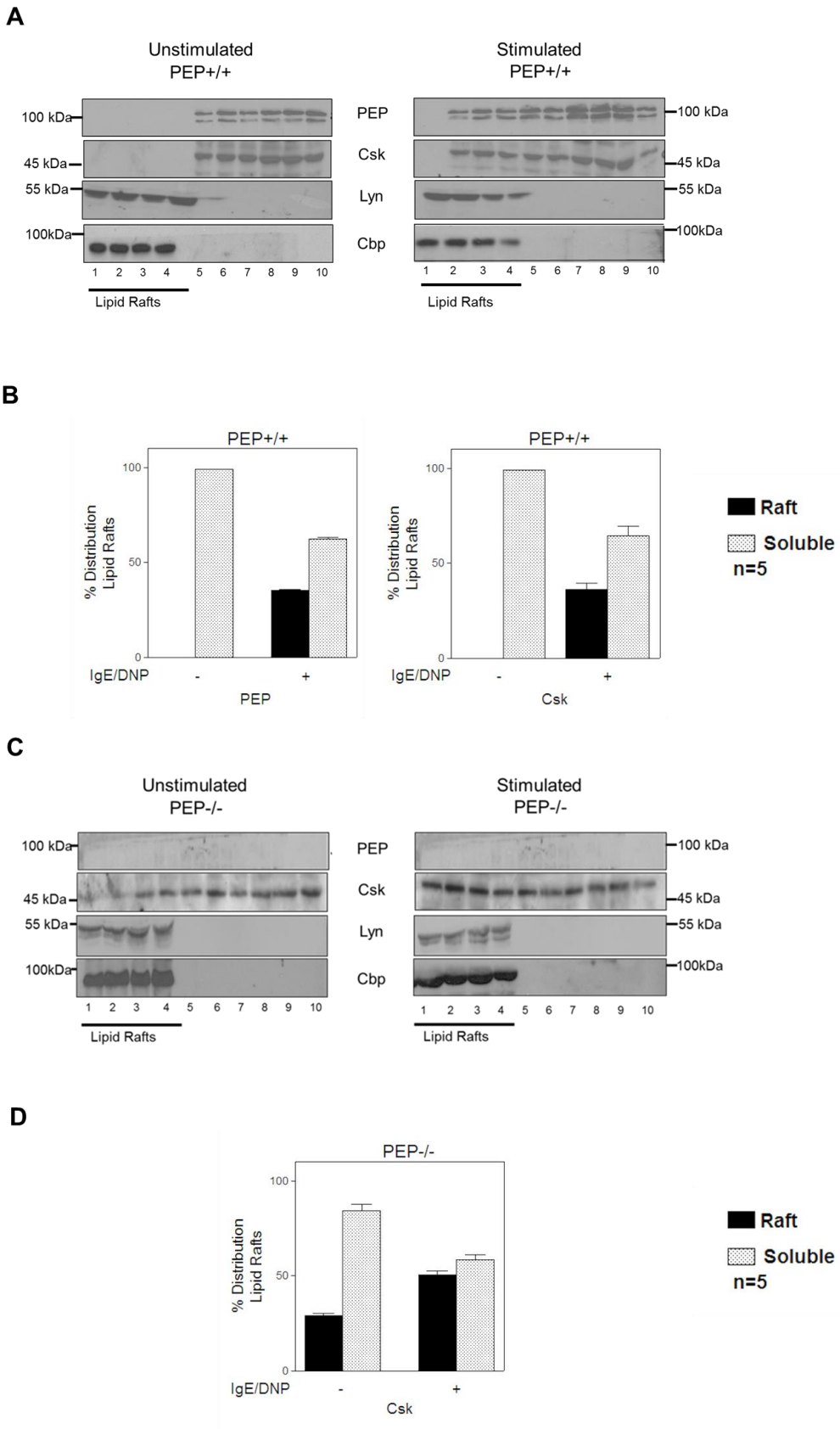

A
Unstimulated
PEP+/+
Stimulated
PEP+/+
100 kDa
45 kDa
55 kDa
100kDa
PEP
Csk
Lyn
Cbp
1 2 3 4 5 6 7 8 9 10
Lipid Rafts
B
PEP+/+
PEP+/+
% Distribution
Lipid Rafts
IgE/DNP
PEP
Csk
Raft
Soluble
n=5
C
Unstimulated
PEP-/-
Stimulated
PEP-/-
D
PEP-/-

Fig 3.2 Absence of PEP leads to increased recruitment of Csk into the Lipid Rafts - **(A)** and **(C)** Cell lysates (unstimulated and stimulated) from wt and PEP knockout BMMC were subjected to sucrose gradient centrifugation and lipid rafts fractions were isolated. The PEP, CSK and Lyn content of raft or non-rafts fractions isolated was visualized by immunoblotting with respective antibodies. An anti-Cbp/PAG antibody serves as a marker of Lipid rafts. Fractions 1-4 represent detergent insoluble while 5-10 represent detergent soluble membrane. **(B)** and **(D)** The graph represents the densitometric quantification of amount of Csk and PEP present in rafts and soluble fractions from 5 independent experiments. (Mean±S.E.M, n=5)

3.3 Impact of PEP deficiency on Cbp/PAG phosphorylation

The ability of Csk to relocate from cytosol to plasma membrane and its involvement in inhibiting Src-PTKs (protein tyrosine kinases) is due to the presence of a plasma-membrane associated, Csk binding molecule, Cbp/PAG that mediates recruitment of Csk to Src-PTK. Cbp is exclusively localized in the GM1 ganglioside (monosialotetrahexosylganglioside) -enriched detergent insoluble membrane (DIM) domain which is important in receptor mediated signaling (Takeuchi et al., 2000). When phosphorylated at Tyr^{314} Cbp/PAG associates with Csk and brings it into proximity with the membrane associated src like kinases (Ohtake et al., 2002b).

As interaction of Csk with Cbp requires tyrosine phosphorylation of Cbp/PAG (Horejsi, 2004), hence Cbp/PAG immunoprecipitates from PEP+/+ and PEP-/- BMMC were probed with phosphotyrosine antibody to determine the phosphorylation status of Cbp/PAG. In the PEP+/+ BMMC, Cbp/PAG is non-phosphorylated but phosphorylation begins 5 min after IgE crosslinking, reaching a peak at 15 min and decreasing slowly at 30 min (Fig 3.3A, lanes 1-6). In contrast, in PEP-/- BMMC, Cbp/PAG is constitutively phosphorylated and IgE crosslinking further increases its level of phosphorylation (Fig 3.3A, lanes 7-12).

In co-immunoprecipitation, Cbp/PAG immunoprecipitates from PEP+/+BMMC showed interaction with PEP and Csk only in presence of antigen (Fig 3.3B, compare lane 4 with 3), while in PEP-/- BMMC Csk interacts with Cbp/PAG even in the absence of antigen and this interaction is enhanced following IgE crosslinking (Fig 3.3B, compare lane 10 with 9).

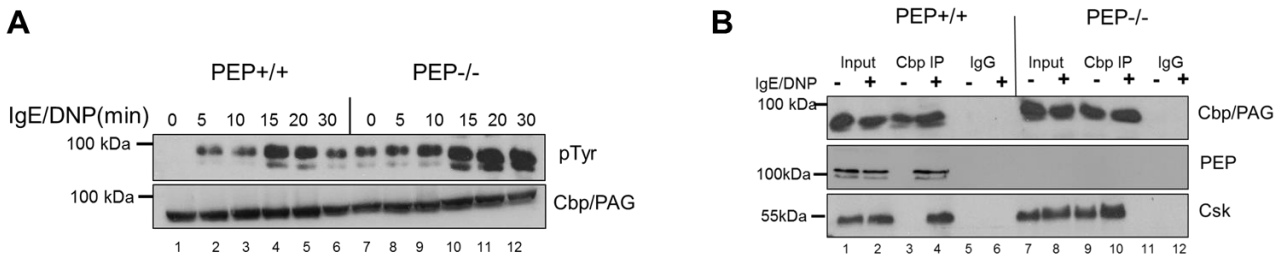


Fig 3.3 Absence of PEP leads to hyper-phosphorylation of Cbp/PAG - (A) 5-6 x 10^6 PEP+/+and PEP-/- BMMC were treated with anti-DNP IgE (1µg/ml) for 2 h and activated with DNP-HSA (200 ng/ml) for indicated time points. Cell lysates were immuno-precipitated with anti-Cbp/PAG antibody and subjected to immunoblot analysis with anti-phosphotyrosine antibody (top panel) and then the filter was stripped and reprobed with anti-Cbp/PAG antibody (bottom panel). **(B)** Cell lysates from PEP+/+ and PEP-/- BMMC were treated as described above and activated with DNP(200ng/ml) for 30 min and immunoprecipitated with anti-Cbp/PAG antibody and then immunoblotted with anti-Cbp/PAG antibody, anti-PEP antibody and anti-Csk antibody respectively. The results are representative of three independent experiments.

These results suggest that Cbp/PAG only in its phosphorylated state can be co-immunoprecipitated with Csk as seen in PEP-/- BMMC but not in the non-phosphorylated state as seen in PEP+/+BMMC.

3.4 Impact on Lyn activity of hyper-phosphorylation of Cbp/PAG and increased recruitment of Csk to lipid rafts in PEP-/-BMMC

Cross-linking of IgE-FcεRI complexes on mast cells by multivalent antigen initiates a series of signaling events that culminate in the exocytosis of mediators of the allergic response. The earliest biochemical event following receptor aggregation is the activation of Lyn and subsequent phosphorylation of the ITAM sequences on FcεRI β and γ subunits (Kinet, 1999; Parravicini et al., 2002).

Lyn like other Src family kinase is regulated by protein interactions through its SH2/SH3domain as well as via phosphorylation events. It possesses a negative-regulatory phosphotyrosine Y-508 located near its carboxyl terminus. This phosphotyrosine is able to interact with Lyn's N-terminal SH2 domain occluding the catalytic site of this kinase from accessing external substrates. Csk negatively regulates Lyn activity by phosphorylating the negative regulatory tyrosine residue at its COOH terminus. This event is facilitated by Cbp/PAG which not only relocates Csk to the membrane but also elevates the affinity of Csk for Lyn (Takeuchi et al., 2000). Activation of Lyn involves dephosphorylation of the C-terminal tyrosine Y-508 to release the auto-inhibitory configuration of the kinase domain. Lyn can then trans-auto-phosphorylate Y-397 within its activation loop to generate a highly active enzyme (Xu et al., 1999). Hence the activation /inactivation regulatory cycle of Lyn is controlled by two proteins; Csk and Cbp/PAG.

Since increased phosphorylation of Cbp/PAG and enhanced recruitment of Csk to the lipid rafts which was observed in the PEP-/- BMMC are implicated in the negative regulation of Lyn, the activity of this kinase was determined in PEP-/-BMMC and compared to the control PEP+/+ BMMC. An in-vitro kinase assay for

determining Lyn activity was performed, also the phosphorylation status of both the positive (Y-397) and negative (Y-508) regulatory sites were determined

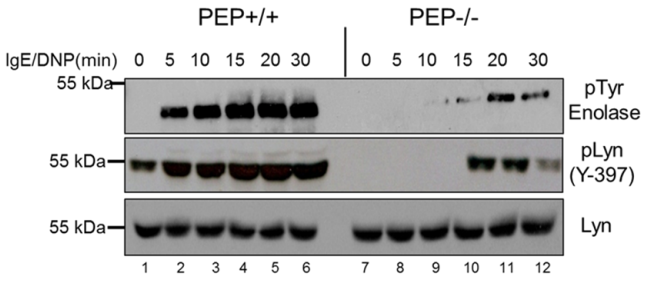
Lyn immunoprecipitates from PEP+/+ and PEP-/- BMMC were subjected to in-vitro kinase assay wherein they were incubated with 1mM ATP and 30µl of 1mg/ml of prepared substrate (acid-denatured enolase) for 2 h at 37°C and then subjected to immunoblot analysis and then probed with anti-phosphotyrosine antibody to detect the phosphorylation of Enolase. Phosphorylation status of Lyn at sites Y-508 and Y-397 was determined by using the same Lyn immunoprecipitates and probing them with specific phospho-Lyn antibodies.

Activity of Lyn in PEP-/- BMMC was markedly reduced compared to PEP+/+ BMMC after receptor crosslinking, as judged by the PTK activity towards exogenous substrate enolase in the in-vitro kinase assay (Fig 3.4A, compare lanes 7-12 to lanes 1-6). In addition, there was a reduced phosphorylation of Lyn at Tyr^{397} in PEP-/- BMMC compared to PEP+/+ BMMC following IgE crosslinking (Fig 3.4A compare lanes 7-12 with 1-6). Quantification of the kinase assay and the Tyr^{397} activity also represents the significant reduction seen in PEP-/- BMMC (Fig 3.4 B).

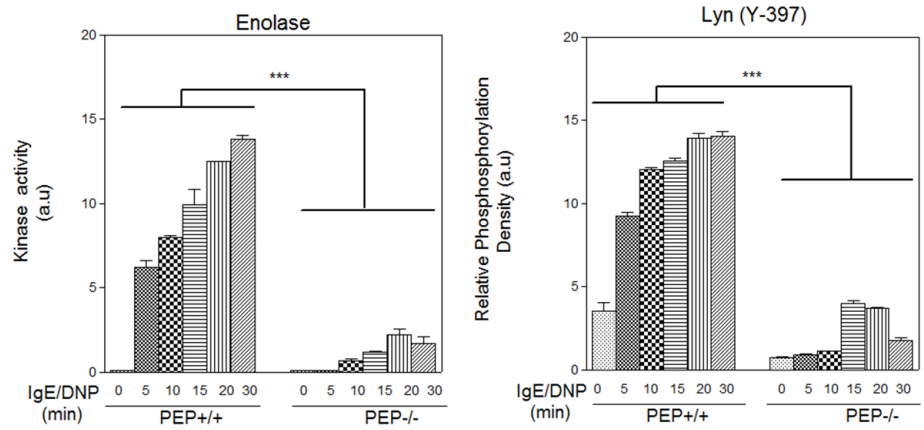
In contrast, the phosphorylation status of Lyn at its negative regulatory site and its quantification showed an increase phosphorylation at Y-508 in PEP-/- BMMC compared to PEP+/+ BMMC (Figs 3.4C and 3.4D). In both the phosphorylation blot and the quantification, a higher level of Y-508 phosphorylation was seen in the PEP-/- compared to the PEP+/+ BMMC in the absence of IgE crosslinking. This is due to the presence of Csk at the lipid raft in the PEP-/- BMMC even in the resting/ unstimulated cells (Figs 3.2C and 3.2D).

Thus the observed increase in Cbp/PAG phosphorylation and thereby recruitment of Csk to the lipid rafts in the absence of PEP leads to inhibition of Lyn kinase activity by Csk which implicates a positive regulatory role of PEP in this signaling event.

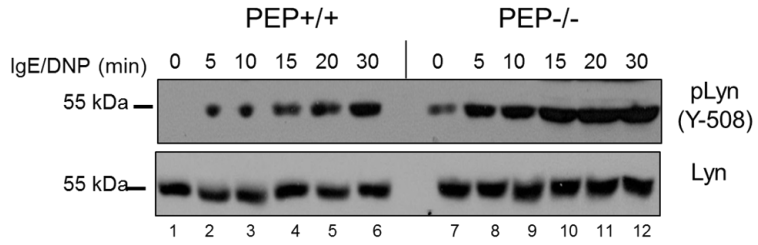
A


PEP+/+
PEP-/-
IgE/DNP(min) 0 5 10 15 20 30 0 5 10 15 20 30
55 kDa
pTyr Enolase
55 kDa
pLyn (Y-397)
55 kDa
Lyn
1 2 3 4 5 6 7 8 9 10 11 12

B


Enolase
Kinase activity (a.u)
***
IgE/DNP (min) 0 5 10 15 20 30 0 5 10 15 20 30
PEP+/+
PEP-/-
Lyn (Y-397)
Relative Phosphosphorylation Density (a.u)
***
IgE/DNP (min) 0 5 10 15 20 30 0 5 10 15 20 30
PEP+/+
PEP-/-

C


PEP+/+
PEP-/-
IgE/DNP (min) 0 5 10 15 20 30 0 5 10 15 20 30
55 kDa
pLyn (Y-508)
55 kDa
Lyn
1 2 3 4 5 6 7 8 9 10 11 12

D

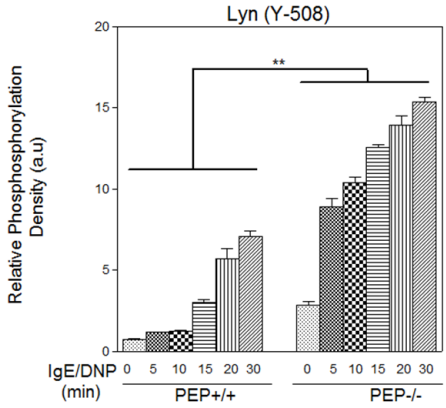

Lyn (Y-508)
Relative Phosphosphorylation Density (a.u)
**
IgE/DNP (min) 0 5 10 15 20 30 0 5 10 15 20 30
PEP+/+
PEP-/-

Fig 3.4 PEP deficiency inhibits enzymatic activity of Lyn - **(A)** 8-10 x 10^6 PEP+/+ and PEP-/- BMMC are treated with anti-DNP IgE (1µg/ml) for 2 h and activated with DNP-HSA (200 ng/ml) for indicated time points. Cell lysates were immuno-precipitated with anti-Lyn antibody and its enzymatic activity was determined by an in-vitro kinase assay using enolase as substrate, followed by immunoblotting with phospho-Lyn (Y-397) antibody and then the filter was stripped and reprobed with anti-Lyn antibody. **(B)** The bar charts are the densitometric quantification of the signals of the kinase activity and phosphorylation signals. They represent mean ±S.E.M of 3 independent experiments ***, p≤0.001) as well as densitometry analysis of phospho-Lyn immunoblot normalized to the amount of protein immunoprecipitated (n=3, ***, ≤p=0.005). **(C)** Cell lysate from above immunoprecipitation were probed for phosphorylation at Y-508 using a phospho-Lyn (Y-508) antibody and then reprobed with anti-Lyn antibody. **(D)** The results of three independent experiments as in C and presented in a bar chart as mean ± S.E.M (n=3**, ≤p=0.02).

3.5 Differential regulation of LAT phosphorylation in the absence of PEP

Signaling by Lyn leads to phosphorylation of phospholipid scramblase 1 (PLSCR1) and LAT which in turn leads to calcium release from intracellular stores that eventually induce calcium influx (Amir-Moazami et al., 2008). As the Lyn kinase activity is reduced in the absence of PEP, it is expected that the downstream signaling pathways will also be down-regulated. To determine this, tyrosine phosphorylation of PLSCR1 and LAT were compared in PEP+/+ and PEP-/- BMMC following IgE crosslinking.

Phosphorylation of PLSCR1 was determined by immunoprecipitation of PLSCR1 followed by immunoblotting with phospho-tyrosine antibody and then reprobed with anti-PLSCR1antibody. Both basal and antigen-mediated transient increase in phosphorylation of PLSCR1 seen in PEP+/+ BMMC was significantly reduced in PEP-/- BMMC (Fig 3.5A, compare lanes 1-4 and 7-10). Quantification of the signals in 3 different experiments confirmed this reduction in phosphorylation (Fig 3.5B).

Phosphorylation of LAT at Tyr ^{136} and Tyr ^{195} is essential for the binding and activation of PLCγ-1 and Grb2 respectively (Takeuchi et al., 2000). To investigate which of these two signaling pathway is affected by PEP knock-out, LAT tyrosine phosphorylation at the two sites was analyzed in LAT immunoprecipitates from PEP+/+ and PEP-/- BMMC. Phosphorylation of LAT at Tyr^{136} needed for PLCγ-1signaling (Saitoh et al., 2000) was reduced in PEP-/- BMMC when compared to PEP+/+BMMC (Figs 3.5C and 3.5D) though phosphorylation slightly increases at 20 min in PEP-/- BMMC but it not that significant as seen in PEP+/+ BMM, while phosphorylation of LAT at Tyr^{195} needed for Grb2-ERK activation (Saitoh et al., 2003) remained unaltered in the PEP+/+ and PEP-/- BMMC (Figs 3.5E and 3.5F). The results of the different effects of the absence of PEP in these signaling pathways were confirmed by quantification of the signals (Figs 3.5D and 3.5F)

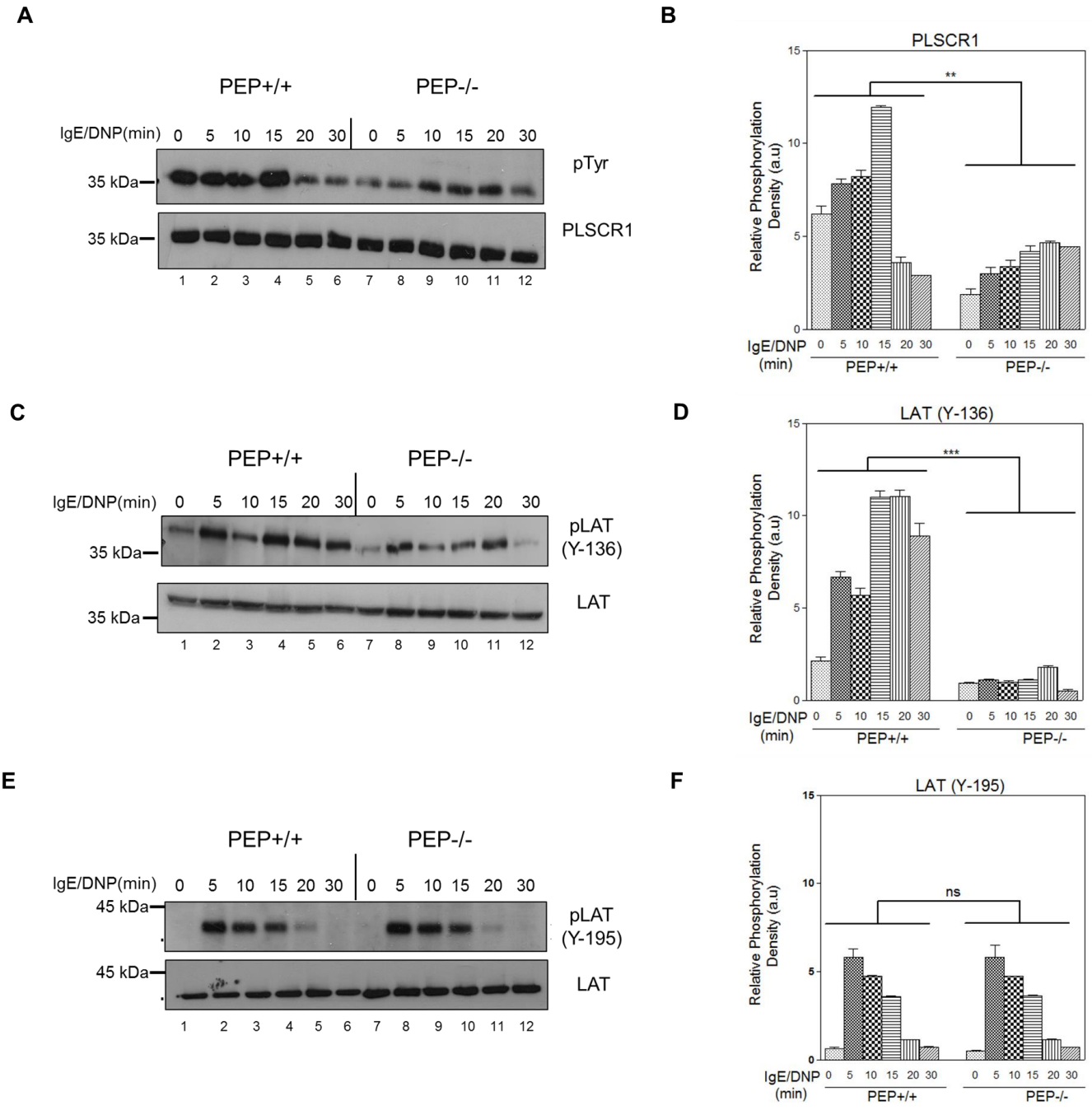


Fig 3.5 Reduced tyrosine phosphorylation of PLSCR1 and LAT in the absence of PEP - (A) 5-6×10^6 PEP+/+ and PEP-/- BMMCs cells were sensitized with anti-DNP IgE (1µg/ml) for 2 h and activated with DNP-HSA (200 ng/ml) for the indicated time points and then immunoprecipitated with anti-PLSRC1 antibody and immunoblotted with anti-phosphotyrosine antibody. After stripping, the filter was reprobed with anti-PLSRC1 antibody. **(B)** Quantification of PLSCR1 phosphorylation was performed by densitometry analysis after

normalizing with the amount of protein immunoprecipitated and data represented as mean ± S.E.M (n=3, **,p≤0.05) **(C)** and **(E)** For LAT phosphorylation, the lysates from PEP+/+ and PEP-/- BMMC were immunoprecipitated with anti-Lyn Ab and then subjected to western blot analysis and the membranes were immunoblotted with phospho-specific Abs that recognize the indicated phosphorylated tyrosine residues in LAT and anti-LAT for loading control. **(D)** and **(F)** Densitometric quantification was used to determine the relative intensity level of the phosphorylation signals and represented as mean ± S.E.M (n=3,***p≤0.001)

3.6 Effect of PEP deficiency on Ca^{2+}, calcineurin/NFAT signaling pathway

Aggregation of FcεRI receptors during mast cell activation coupled with calcium mobilization activates the calcium/calcineurin/NFAT pathway leading to production of inflammatory cytokines and lipid mediators (Monticelli et al., 2004; Yang et al., 2009) .

Receptor ligation leads to the activation of phospholipase Cγ1 (PLCγ1), which hydrolyses phosphatidylinositol-4, 5-bisphosphate to produce inositol-1, 4, 5-trisphosphate (InsP3) and diacylglycerol. InsP3 binds to IP3 receptors (IP3R) in the endoplasmic reticulum and induces release of calcium from intracellular stores which triggers the opening and promotes Ca^{2+} entry through calcium-release-activated calcium (CRAC) channels in the plasma membrane such that the increased levels of intracellular calcium are maintained (Putney and Bird, 1993). Calcium binds to calmodulin, which in turn activates the calmodulin dependent serine phosphatase calcineurin. This enzyme dephosphorylates NFAT (nuclear factor of activated T cells), a transcriptional activator located in the cytosol of unstimulated cells in its phosphorylated form but translocate to the nucleus when dephosphorylated by calcineurin to induce the expression of NFAT-regulated genes (Fig 3.6.1)

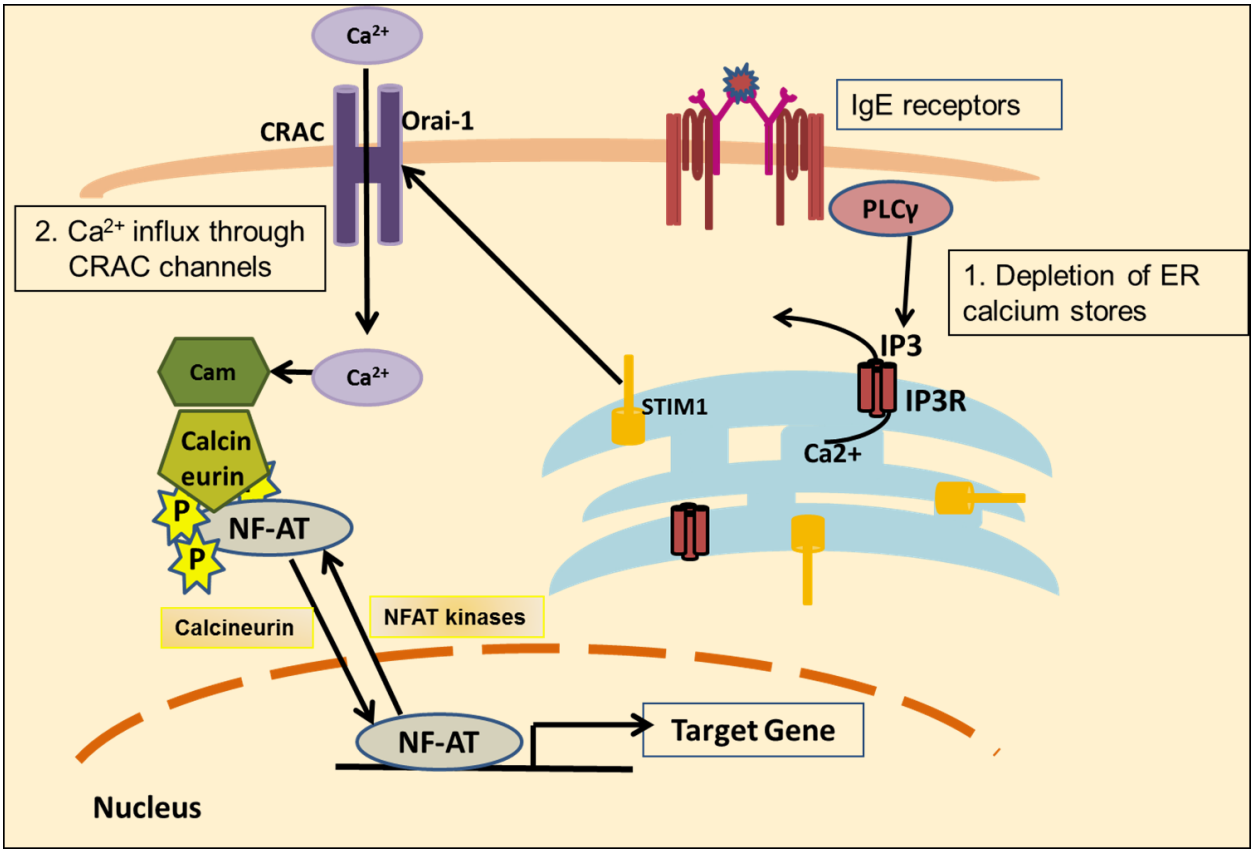


Fig 3.6.1 The Ca^{2+}/ calcineurin/NFAT signaling pathway - NFAT is activated by cell-surface receptors coupled to Ca^{2+} mobilization. $[Ca^{2+}]i$ increases result in activation of many calmodulin (CaM)-dependent enzymes, including the phosphatase calcineurin . Calcineurin dephosphorylates multiple phosphoserine on NFAT, leading to its nuclear translocation and activation. Adapted from (Dolmetsch et al., 2001)

PEP -/- BMMC has been shown to have reduced phosphorylation of PLCγ1 and calcium mobilization when compared to PEP+/+BMMC (Obiri et al., 2011), which can be explained by the reduced Lyn mediated activation of LAT at Tyr^{136} ,the binding site of PLCγ1 seen in PEP-/- BMMC (Figs 3.5).

To determine the effects of reduced activation of Lyn/LAT/PLCγ-Ca^{2+} signaling pathway due to absence of PEP on the activity of the NFAT transcription factor, PEP wild-type and knockout BMMC were transfected with a plasmid containing multimerizsed NFAT binding sites driving the expression of a luciferase gene. These cells were sensitized with anti-DNP IgE and activated with DNP-HSA for 8 h. A significant decrease in NFAT luciferase activity was seen in both in basal as well as after DNP-HSA stimulation in PEP deficient BMMC compared with that in wild-type BMMC (Fig 3.6.2A).

Transfection of an IL-6 promoter-luciferase construct, known to be dependent on calcium, in BMMC (Tanaka et al., 2005), also produced similar results in that both basal as well as antigen induced luciferase activity was significantly less in PEP-/- BMMC compared to PEP+/+ BMMC (Fig 3.6.2B). In contrast, the activity of other transcription factors such as NF-κB and AP-1 that are also activated by IgE-receptor aggregation but are not dependent on PLCγ/Ca^{2+} pathway did not show difference in the PEP+/+ and PEP-/- BMMC (Fig 3.6.2C and 3.6.2D). These results demonstrate that absence of PEP leads to reduced NFAT activity.

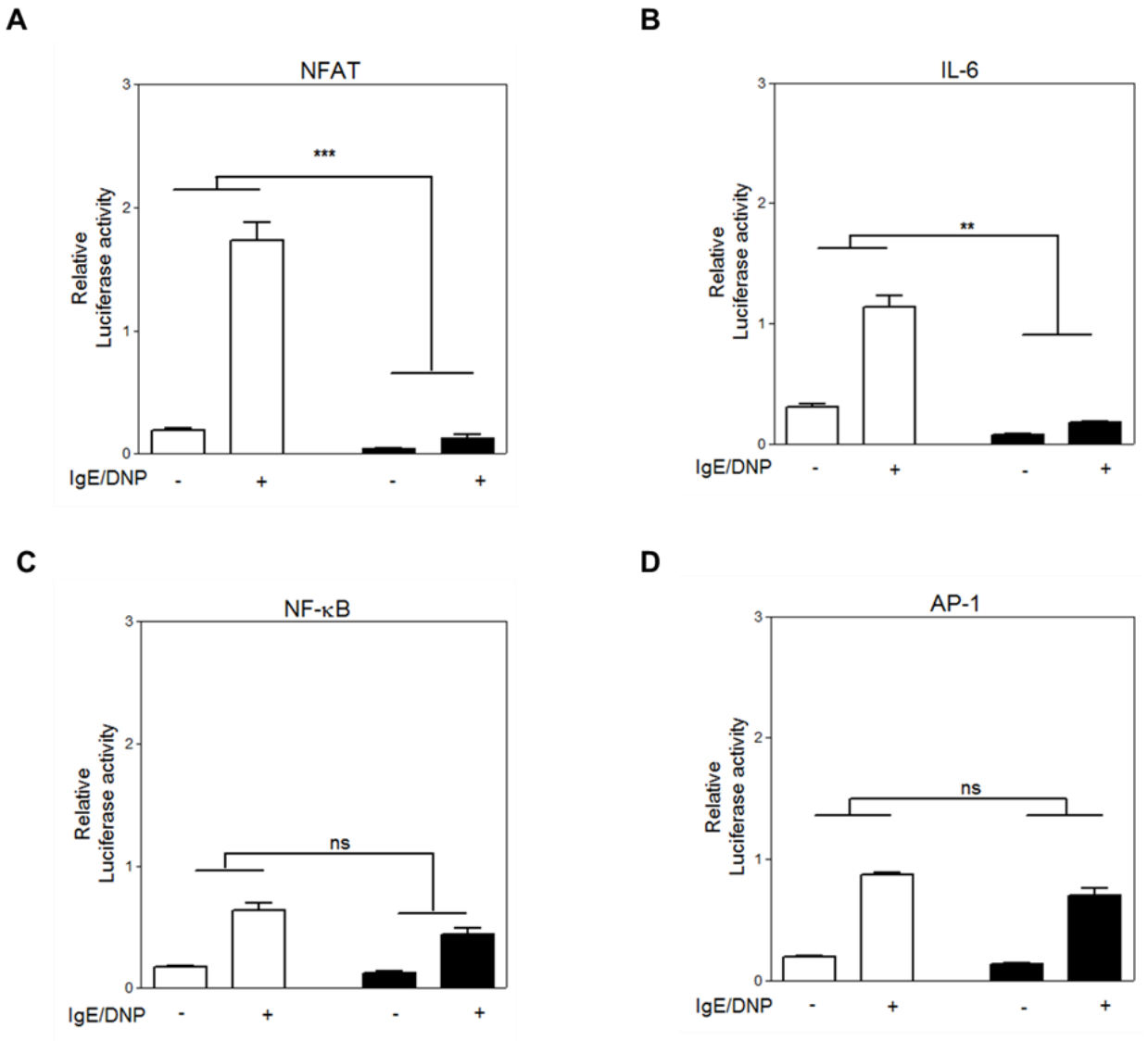


Fig 3.6.2 Reduced NFAT activation in the absence of PEP - 6 - 8 x10^6 PEP+/+ and PEP-/- BMMC were transfected with 0.6µg of Renilla luciferase expression vector and 4.3µg of the reporter construct p3xNFAT-/IL-6-/NF-κB-/AP-1-luciferase. After 24 h, the cells were sensitized with anti-DNP IgE for 18 h. Cells were then stimulated with DNP-HSA (200ng/ml) for 8 h and firefly luciferase activity was measured and normalized to Renilla luciferase activity **(A)** NFAT activity (B) IL6 promoter activity **(C)** NF-κB activity and **(D)** AP-1 activity. Results are shown as bar charts and presented as mean ± SEM (n=3), ***p=0.0003, **p≤0.005 .PEP+/+ (open bars); PEP-/- (closed bars)

To confirm a direct role of PEP on the activity of NFAT, PEP was reintroduced into PEP deficient BMMC to see whether the decreased activity of NFAT in the knockout cells could be rescued. As a control PEP was also introduced into PEP+/+ BMMC. Transfection of a PEP expression vector into PEP-/- BMMC significantly increased both the basal and antigen induced NFAT activation. NFAT activity in DNP stimulated PEP-/- BMMC expressing PEP was equivalent to NFAT activity in PEP+/+ BMMC transfected with empty vector (Fig 3.6.3A). Overexpression of PEP in PEP+/+ BMMC show a further increase in NFAT activity (Fig 3.6.3A).

The expression of PEP in transfected PEP knock-out and control wild-type BMMC was assessed by performing Western blot analysis (Fig 3.6.3B). These experiments clearly demonstrate that over expression of PEP, not only increase NFAT expression in wild-type BMMC but can also rescue the reduced activity of this transcription factor in PEP knock-out BMMC.

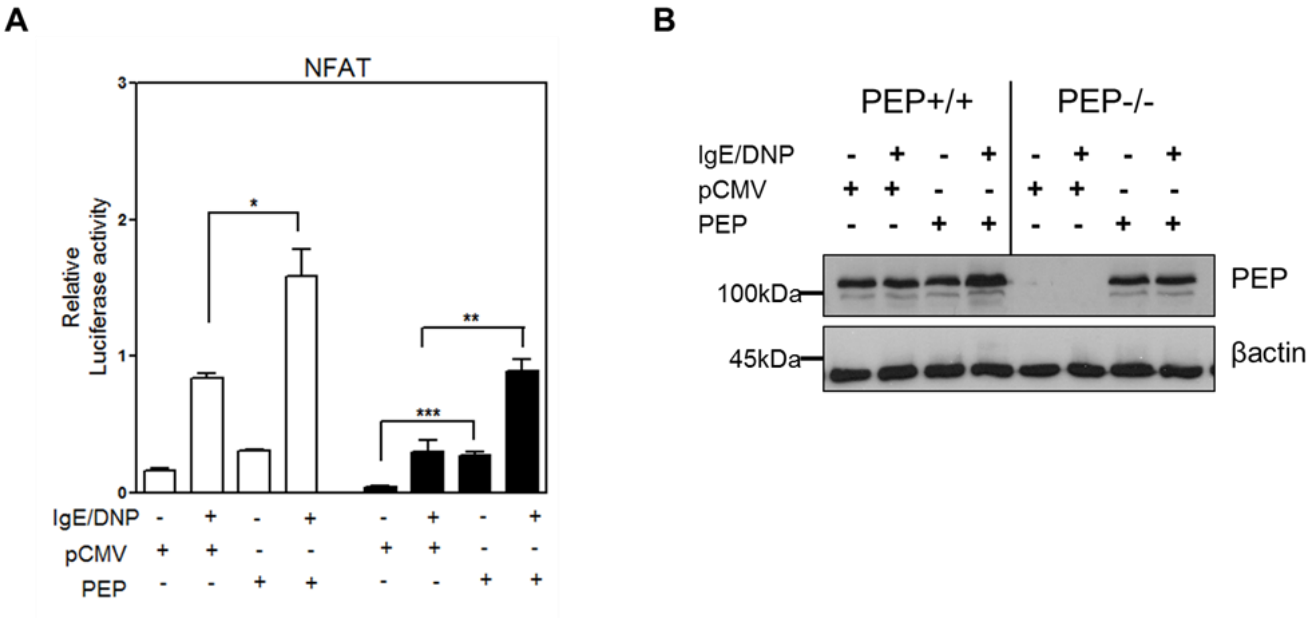


Fig 3.6.3 Reconstitution of PEP expression in PEP-/- BMMC - **(A)** 8-10 × 10^6 PEP+/+ and PEP -/- BMMCs were transfected with 0.6μg Renilla Luciferase expression vector and 4.3μg NFAT-luciferase reporter construct and additionally 4.3μg PEP-pCMV vector or an empty expression vector was transfected. After 24 h, the cells were sensitized with anti-DNP-IgE for 18 h. Cells were stimulated with DNP-HSA (200ng/ml) for 8 h and firefly luciferase activity was measured and normalized to Renilla luciferase activity. Results are presented as mean ± SEM (*p≤0.01, **p=0.007, ***p≤0.0001, n=5) .PEP+/+ (open bars); PEP-/- (closed bars). **(B)** PEP+/+ and PEP-/- BMMCs were treated as shown in the figure and cell lysates were immunoblot using an anti-PEP specific antibody, and βactin as a loading control.

To further demonstrate that it is not only the level of PEP but its activity that controls the action of NF-AT, two known inhibitors of PEP activity C28 and L75N04 were used in the NFAT activity assay.

C28 is a gold (I) phosphine complex (Bis (2-cyanoethyl) phenylphosphine-Au(I)-Cl) that selectively inhibits protein tyrosine phosphatases (PTP) through direct interaction with the catalytic cysteine residues (Karver et al., 2010). L75N04 (He et al., 2013) is benzofuran salicylic acid-based that interacts with the phosphate-binding pocket (catalytic pocket) of Lyp through its benzofuran salicylic acid moiety whereas the distal naphthalene ring interacts with a nearby peripheral site.

Treatment of PEP+/+ BMMC with 5μM C28 reduced the antigen induced activation of NFAT as well as IL-6 response but not to the level of expression in PEP-/- BMMC. Moreover C28 had no effect on the basal activity in PEP+/+BMMC. C28 treatment did not alter the activation level of NFAT and IL-6 in PEP-/- BMMC (Figs 3.6.4A and 3.6.4B). Higher concentration of C28 was not used to further down modulate NFAT/IL-6 activity because they were found to be toxic.

Treatment of the PEP+/+ BMMC with L75N04 showed a dose dependent down-regulation of both the basal and the antigen-mediated increase in NFAT/IL-6

luciferase activity. Though 5μM L75N04 was superior to the same concentration of C28, 20μM of the compound was found to be highly efficacious in inhibiting the PEP activity with no changes observed in PEP-/-BMMC (Figs 3.6.4A and 3.6.4B). Neither C28 nor L75N04 altered the NF-κB and AP-1 luciferase activity determined in both wild-type and PEP deficient BMMC (Figs 3.6.4C and 3.6.4D).

Since no significant effect of both the inhibitors C28 and L75N04 was observed on the residual NFAT/IL-6 activity in the PEP-/- BMMC, this suggests that both compounds function by inactivating the activity of PEP. Additionally, a control immunoblot showed that both inhibitors did not alter the level of expression of PEP (Fig 3.6.4E) indicating that they indeed function through inhibition of activity of the PEP.

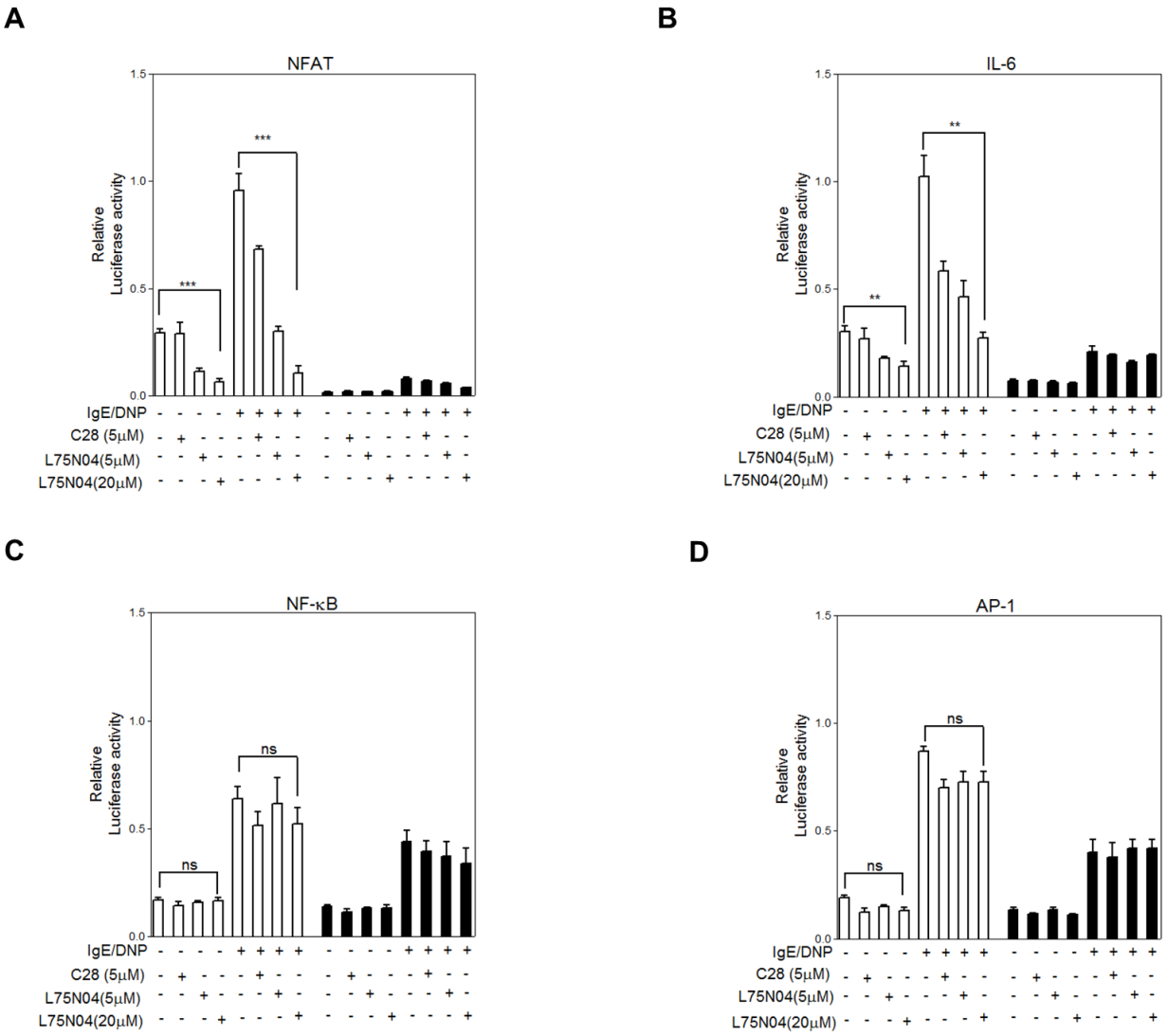

A
NFAT
B
IL-6
C
NF-κB
D
AP-1
Relative Luciferase activity
IgE/DNP
C28 (5μM)
L75N04(5μM)
L75N04(20μM)
***
**
ns
E
IgE/DNP
C28
L75N04
100kDa
55kDa
35kDa
PEP
Lyn
βactin

Fig 3.6.4 NFAT and IL-6 activation in the presence of Lyp/PEP inhibitors - $8x10^6$ PEP+/+ and PEP-/- BMMC were transfected with 4.3µg 3xNFAT- /IL-6 /NF-κB / AP-1 luciferase plasmid and 0.8µg of Renilla luciferase expression. 24 h after transfection, the cells were sensitized with anti-DNP IgE for 18 h. Cells were treated with C-28 (5µM) or L75N04 (5 µM, 20µM) for 1 h to the end of IgE incubation. Cells were then activated with DNP-HAS (200 ng/ml) for 8 h and firefly luciferase activity was measured and normalized to Renilla luciferase activity. **(A)** NF-AT activity **(B)** IL6 promoter activity **(C)** NF-κB activity **(D**) AP-1 activity. Results in bar charts are presented as the mean ± SEM (***p≤0.0001, **p≤0.005, n=3). **(E)** PEP+/+ BMMC were treated as described above and cell lysates were immunoblotted using an anti-PEP antibody, anti-Lyn antibody and an anti- βactin antibody for loading control. Presented is a representative result of three independent experiments PEP+/+ (open bars) and PEP-/- (closed bars).

3.7 Nuclear accumulation of NFATc1 opposed by the increased activation of JNK signaling pathway in PEP deficient BMMC

NFAT family members comprising of four proteins (NFATc1-c4) are expressed in mast cells where they regulate the transcription of cytokines (Hock and Brown, 2003; Hutchinson and McCloskey, 1995).

Calcineurin mediated dephosphorylation and nuclear translocation of NFAT as shown in Fig 3.6.1 are counteracted by NFAT kinases such as JNK1, which act to phosphorylate NFAT proteins, thereby directly antagonizing their nuclear translocation by phosphorylating Serine-Proline repeats present in the motif (Beals et al., 1997a). JNK1 modulates NFATc1 by phosphorylating (Ser^{117} and Ser^{172}) and thus inhibiting calcineurin to bind the calcineurin targeting domain of NFATc1 which is required for the regulation of both nuclear accumulation and transcriptional activity (Chow et al., 2000).

We have previously reported (Obiri et al., 2011) and has again been shown in this thesis (Fig 3.7.1 A, compare lanes 7-12 with lanes 1-6), that PEP deficient BMMC show sustained phosphorylation of JNK1/2 . To determine the consequences of this sustained JNK phosphorylation in PEP-/- BMMC the phosphorylation status of

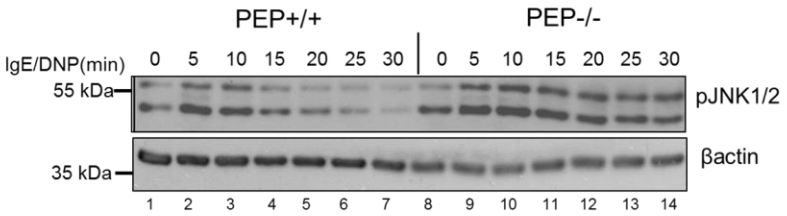
NFATc1 at Ser ^{117} and Ser ^{172} was studied by immunoprecipitating the cell lysates from PEP+/+ and PEP-/- BMMC with NFATc1 and probing them with specific phospho Ser^{117}- and phosphoSer^{172}-NFATc1 antibodies. PEP-/- BMMC showed a significant and sustained increase in phosphorylation at these sites when compared to PEP+/+ BMMC (Figs 3.7.1B compare lanes 7-12 with 1-6).

To investigate whether the sustained JNK1 signaling leads to down-regulation of the nuclear localization of NFATc1 in PEP deficient BMMC compared to wild-type BMMC, cell fractionation studies were carried out to detect the presence of NFATc1 in the nucleus of these cells. Cytosol and nuclear extracts were isolated from PEP-/- and PEP+/+ BMMC which were sensitized by IgE and stimulated with DNP for 30 min and the presence of NFATc1 in the different cellular localizations was detected by Western blotting using βactin as a marker for the cytoplasm and histone H2A, a marker for the nucleus. NFATc1 was detected both in nuclear and cytoplasmic fraction of PEP+/+ BMMC with antigen-receptor crosslinking leading to an enrichment of the signal in the nuclear fraction and a slight decrease in the cytoplasmic fraction. In comparison hardly any NFATc1 is detectable in nuclear extracts of PEP-/- BMMC not even after IgE receptor crosslinking (Fig 3.7.1C). These observations correlate with the enhanced phosphorylation of JNK1/JNK2.

To confirm that JNK signaling does contribute to the impaired NFAT activity observed in the PEP-/- BMMC, wild-type and kinase-deficient JNK1 and JNK2 mutants (Kallunki et al., 1994) were co-transfected in wild-type and PEP knockout BMMC along with the NFAT luciferase plasmid and the activity of NF-AT was determined. While the wild-type JNK constructs down-regulated basal and significantly the antigen induced NFAT luciferase activity in PEP+/+ BMMC, the dominant negative constructs enhanced the NFAT activity in both types of BMMC

(Fig 3.7.1D). Intriguingly NFAT activity that is already low in PEP-/- BMMC could be further reduced by the wild-type JNK1 and JNK2 while the dominant negative kinase deficient JNKs enhanced NFAT activity albeit not to the same level as in the PEP+/+ BMMC (Fig 3.7.1D). Thus sustained JNK activity in PEP deficient BMMC may play a role in inhibiting NFAT transcriptional activity in these cells.

A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PEP-/-
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55 kDa
pJNK1/2
35 kDa
βactin
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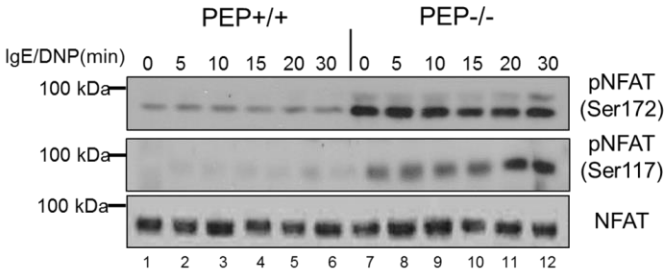
B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PEP-/-
IgE/DNP(min) 0 5 10 15 20 30 0 5 10 15 20 30
100 kDa
pNFAT (Ser172)
100 kDa
pNFAT (Ser117)
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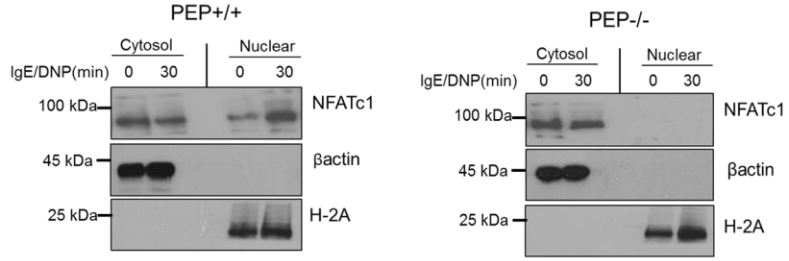
NFAT
1 2 3 4 5 6 7 8 9 10 11 12

C


PEP+/+
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Nuclear
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NFATc1
45 kDa
βactin
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H-2A
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45 kDa
βactin
25 kDa
H-2A

D

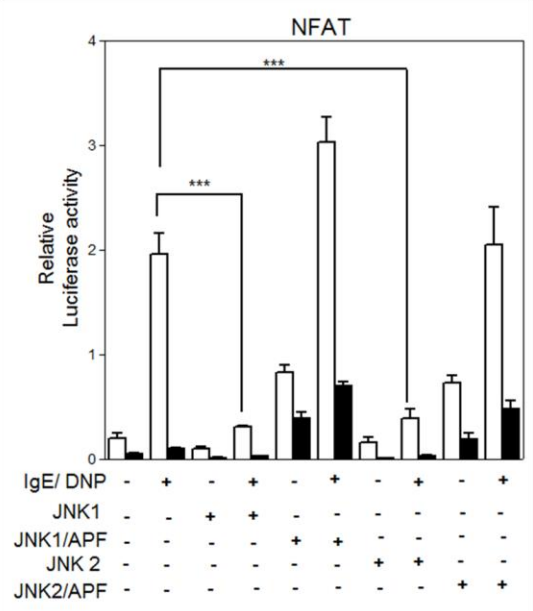

NFAT
***
***
Relative Luciferase activity
0
1
2
3
4
IgE/ DNP - + - + - + - + - +
JNK1 - - + + - - - - - -
JNK1/APF - - - - + + - - - -
JNK 2 - - - - - - + + - -
JNK2/APF - - - - - - - - + +

Fig 3.7.1 Phosphorylation by JNK inhibits NFAT activation in PEP deficient BMMC - (A) $3x10^6$ BMMC from PEP+/+ and PEP-/- mice were sensitized for 2 h with 1µg/ml anti-DNP IgE and then stimulated with 200 ng/ml DNP-HSA for the times indicated. Cell lysates were immunoblotted using the indicated specific phospho-JNK1/2 antibodies and β-actin as loading control. The results are representative of three separate experiments. **(B)** Cell lysates from PEP+/+ and PEP-/- BMMC were stimulated for stipulated time period and then immunoprecipitated with anti-NFATc1 antibody and eventually probed for phospho-Ser^{117}, phospho-Ser^{172} specific antibody and then stripped and reprobed with anti-NFATc1 antibody. **(C)** Nuclear extracts were prepared from 5-10×10^6 PEP+/+ and PEP-/- BMMC after they were left unsensitized or sensitized with 1µg/ml of anti-DNP IgE and then stimulated with 200 ng/ml DNP-HAS for 30 min. The precipitated protein was immunoblotted with anti-NFATc1 antibody. βactin and Histone2A were used as markers for cytosol and nuclei. Presented is a representative result of three independent experiments. **(D)** 6-8 $x10^6$ PEP+/+ and PEP-/- BMMC were transfected with 0.6µg of Renilla luciferase expression vector and 4.3µg 3×NFAT promoter luciferase reporter construct along with 2µg of 3×HA-SRα JNK1 / 3×HA-SRα JNK1-APF / 3×HA-SRα JNK2 / 3×HA-SRα JNK2-APF. After 24 h, the cells were sensitized with anti-DNP-IgE for 18 h. Cells were stimulated with DNP-HSA (200 ng/ml) for 8 h. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. Results are presented as the mean ± SEM ((***$p\leq0.001$, n=3). PEP+/+ (open bars); PEP-/-(closed bar)

To verify the observed effects on JNK signaling which affects the nuclear accumulation of NFATc1 is indeed due to the absence of PEP and that it positively modulates this signaling pathway, Lyp/PEP inhibitor L75N04 was used in the experiments.

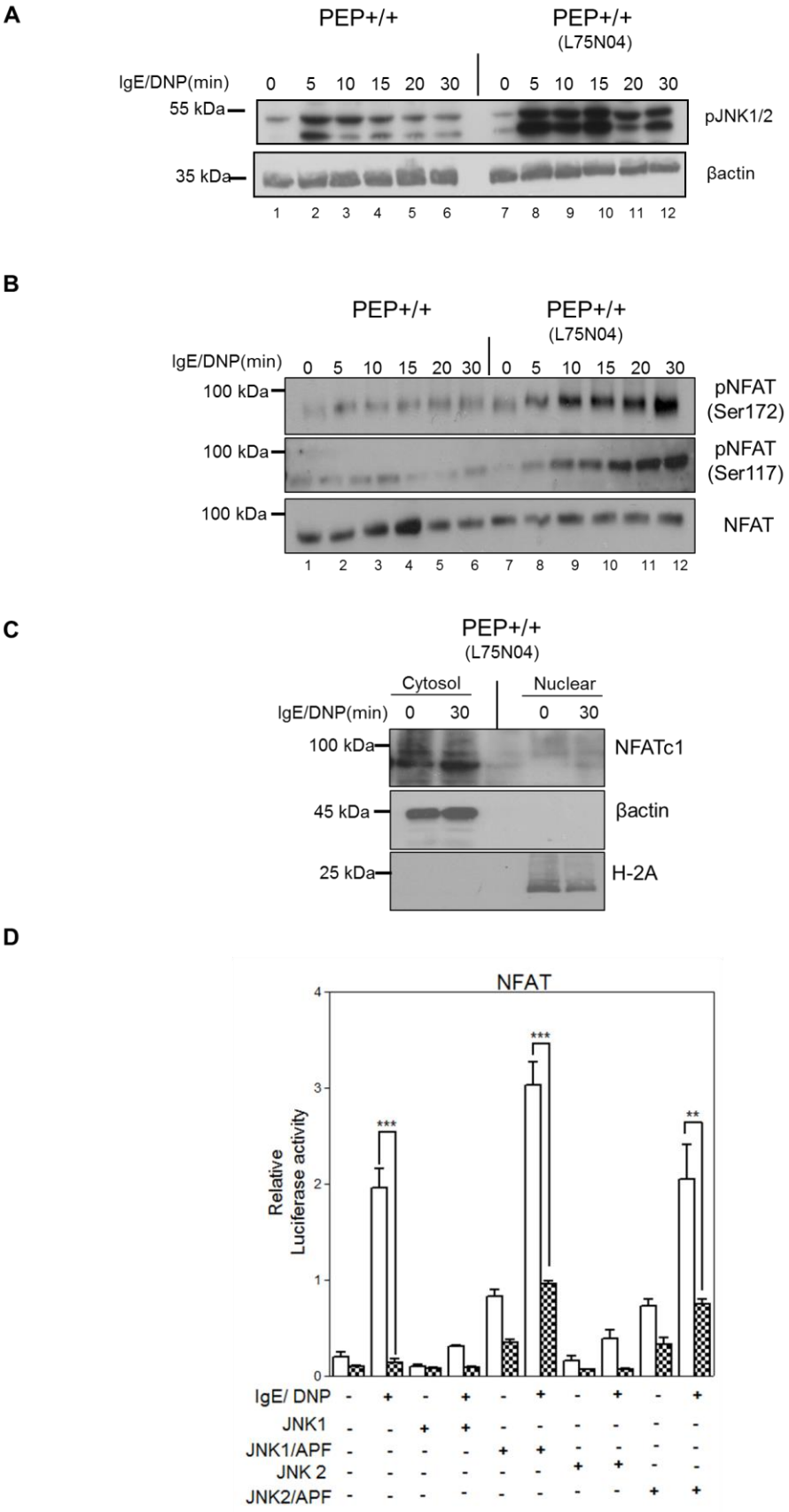
Cell lysates were prepared from PEP+/+ BMMC after having been treated with 20µM L75N04 and analyzed for JNK1/2 phosphorylation as well as for phosphorylation of NFATc1 at Ser^{117} and Ser^{172}.

Similar to PEP-/- BMMC, wild-type BMMC treated with L75N04 showed enhanced and sustained phosphorylation of JNK1/2 as well as of NFATc1 at the above mentioned phosphoserine residues (Figs 3.7.2A and 3.7.2B, compare lanes 1-6 with 7-12).

Also cell fractionation studies in presence of L75N04 revealed decreased or negligible amount of NFATc1 in the nuclear extracts of PEP+/+ BMMC treated with 20μM L75N04 (Fig 3.7.2C).

These results demonstrate that this inhibitor mimics the PEP deficiency in the activation of JNK kinase and thereby resulting in decreased accumulation of NFATc1 in the nucleus of these cells. Additionally transfection experiments carried out with wild type JNK1/2 and dominant negative JNK-APF1/2 kinase deficient mutants in the presence of inhibitor in the wild type showed similar results as in the case of the PEP-/- BMMC. They showed reduced NFAT luciferase activity which can be more or less comparable to PEP deficient BMMC (Fig 3.7.2D).

Taken together these findings suggest that L75N04 is potent in inhibiting the activity of PEP and thereby inducing effects similar to PEP -/- BMMC on JNK phosphorylation and NFAT activity.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55 kDa
pJNK1/2
35 kDa
βactin
1 2 3 4 5 6 7 8 9 10 11 12
B
PEP+/+
PEP+/+
(L75N04)
IgE/DNP(min)
0 5 10 15 20 30 0 5 10 15 20 30
100 kDa
pNFAT
(Ser172)
100 kDa
pNFAT
(Ser117)
100 kDa
NFAT
1 2 3 4 5 6 7 8 9 10 11 12
C
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(L75N04)
Cytosol
Nuclear
IgE/DNP(min)
0 30 0 30
100 kDa
NFATc1
45 kDa
βactin
25 kDa
H-2A
D
NFAT
Relative
Luciferase activity
0 1 2 3 4
***
***
**
IgE/ DNP - + - + - + - + - +
JNK1 - - + + - - - - - -
JNK1/APF - - - - + + - - - -
JNK 2 - - - - - - + + - -
JNK2/APF - - - - - - - - + +

Fig 3.7.2 Lyp/PEP inhibitor L75N04 inhibits NFATc1 activation in wild-type BMMC- (A) $3x10^6$ BMMC from PEP+/+ were sensitized for 2 h with 1μg/ml anti-DNP IgE and left either treated or untreated with 20μM L75-NO4 for 1 h to the end of IgE incubation and then stimulated with 200 ng/ml DNP-HSA for the times indicated. Cell lysates were immunoblotted using the indicated specific phospho-JNK1/2 antibodies and β-actin as loading control. The results are representative of three separate experiments. **(B)** Cell lysates from PEP+/+ and PEP+/+ BMMC treated with 20μM L75-NO4 for 1 h were stimulated for stipulated time period and then immunoprecipitated with anti-NFATc1 antibody and eventually probed for phospho-Ser^{117}, phospho-Ser^{172} specific antibody and anti-NFATc1 antibody as a control. **(C)** Nuclear extracts were prepared from 5-10×10^6 PEP+/+ and PEP+/+ BMMC treated with L75N04 for 1 h and then stimulated with 200 ng/ml DNP-HAS for 30 min. The precipitated protein was immunoblotted with anti-NFATc1 antibody. βactin and Histone2A were used as markers for cytosol and nuclei. Presented is a representative result of three independent experiments. **(D)** 6-8 $x10^6$ PEP+/+ treated with 20μM L75-NO4 for 1 h were transfected with 0.6μg of Renilla luciferase expression vector and 4.3μg 3×NFAT promoter luciferase reporter construct along with 2μg of 3×HA-SRα JNK1 / 3×HA-SRα JNK1-APF / 3×HA-SRα JNK2 / 3×HA-SRα JNK2-APF. After 24 h, the cells were sensitized with anti-DNP-IgE for 18 h. Cells were stimulated with DNP-HSA (200 ng/ml) for 8 h. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. Results are presented in the graph as the mean ± SEM (***p≤0.0001, **p≤0.005, n=3) PEP+/+ (open bars); PEP+/+ (20μM L75N04) (checkered bars)

3.8 Inhibition of IgE-induced proximal signaling events by L75-NO4 in wild-type BMMC

Attenuation of NFAT activation by L75N04 in wild-type BMMC, suggests that this inhibitor can be used to unravel the events in the early signal pathway of mast cells to answer the question as to how the activity of PEP affects FcεRI signaling and not its presence or interaction with other proteins. Data already established in this work (Section 3.2, 3.3 and 3.4) suggest a positive role of PEP in Lyn-mediated signaling by dephosphorylating Cbp/PAG protein and thereby decreasing the recruitment of negative regulator Csk to the lipid rafts to Lyn.

To identify PEP as a potential Cbp/PAG phosphatase, Cbp/PAG phosphorylation was monitored in PEP+/+ BMMC in presence and absence of L75N04 by immunoprecipitating the cell lysates with anti-Cbp/PAG antibody and probing them with anti-phosphotyrosine antibody. Hyper-phosphorylation of Cbp/PAG was observed in presence of the inhibitor in the wild-type BMMC as previously shown (Section 3.3) in PEP-/- BMMC (Fig 3.8.1A, compare lanes 7-12 with lanes 1-6).

Similar to PEP-/-BMMC, L75N04 treated wild-type BMMC showed phosphorylation of Cbp/PAG in the absence of antigen (Fig 3.8.1A, compare lane 1 with 7), this suggest that L75N04 might be inhibiting the binding of PEP with Csk and Cbp/PAG.

To investigate whether PEP binds to Cbp/PAG and as phosphorylation of Cbp/PAG leads to its interaction with Csk (Ohtake et al., 2002b; Takeuchi et al., 2000), co-immunoprecipitation assay was performed to determine association of Csk with Cbp/PAG from the L75N04 treated PEP+/+BMMC. Lysates from PEP+/+ BMMC treated with or without L75N04 (20μM) were immunoprecipitated with either Cbp/PAG antibody or PEP antibody and then subjected to immunoblot analysis and probed with PEP, Csk and Cbp/PAG antibody.

PEP immunoprecipitates from wild-type BMMC treated with L75N04 showed no interaction with Cbp/PAG and Csk in presence of antigen, also the constitutive interaction of PEP with Csk is lost in presence of this inhibitor (Fig 3.8.1B, compare lanes 9-10 with lanes 3-4) indicating that an active PEP is required to bind to Csk and Cbp/PAG.

Cbp/PAG immunoprecipitates in the absence of inhibitor there was interaction with PEP and Csk following antigen crosslinking in PEP+/+ BMMC, in the presence of L75N04 there was no interaction with PEP as shown above (Fig 3.8.1C compare lane 10 with lane 4). There was however an increased interaction of Cbp/PAG with Csk even in the absence of antigen crosslinking in the treated BMMC (Fig 3.8.1C compare lanes 9-10 with lanes 3-4). This increased interaction of Csk with Cbp/PAG in presence of inhibitor suggest that the recruitment of Csk is influenced by the presence of inhibitor , to investigate whether inhibition of PEP activity does

have a role in this process a sucrose gradient centrifugation assay was carried out in PEP+/+BMMC treated without or with the Lyp/PEP inhibitor L75N04.

In PEP+/+BMMC about 40% Csk remained in the lipid rafts with Cbp/PAG after IgE crosslinking (Figs 3.8.1D left panel and 3.8.1E) while in L75N04 treated PEP+/+ BMMC, around 60-70% increased recruitment of Csk into the lipid rafts was observed (Figs 3.8.1D right panel and 3.8.1E). Under the same conditions although 40% PEP was in the lipid rafts in IgE/DNP activated cells (Figs 3.8.1D left panel and 3.8.1E), in the presence of the inhibitor, hardly any PEP is seen in the rafts fraction. The majority of PEP seems to remain in the soluble fraction (Figs 3.8.1D right panel and 3.8.1E).

This enhanced recruitment of Csk to the lipid rafts in presence of inhibitor suggest a possible higher negative regulation of Lyn activity as seen in PEP-/- BMMC which might explain the potency of L75N04 in attenuating the NFAT activation.

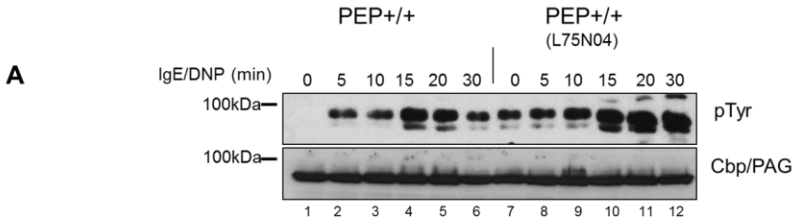
Experiments presented in this thesis on PEP deficient BMMC have demonstrated that an enhanced recruitment of Csk into the lipid rafts results in inhibition of Lyn activity through an increased phosphorylation at its negative regulatory site. A similar effect is expected if the activity of PEP is required for this effect. In-vitro kinase assay as well as phosphorylation on both positive (Y-397) and negative (Y-508) regulatory site of Lyn were determined in the absence and presence of L75N04 in wild-type BMMC after IgE receptor crosslinking.

Lyn activation in PEP+/+BMMC treated with L75N04 was determined by the PTK (Protein Tyrosine Kinase) activity towards the exogenous substrate enolase in the in-vitro kinase assay. Lyn immunoprecipitates from PEP+/+BMMC treated without or with L75N04 were incubated with 1mM ATP and 30µl of prepared substrate (acid-denatured enolase) for 2 h at 37°C and then subjected to

immunoblot analysis and then probed with anti-phosphotyrosine antibody to detect the phosphorylation of Enolase. Phosphorylation status of Lyn at sites Y-508 and Y-397 was determined by using same Lyn immunoprecipitates and probing them with specific phospho-Lyn antibodies.

Wild-type BMMC show reduced Lyn-PTK activity (reduced phosphorylation of enolase) in presence of inhibitor, indicating that the chemical inhibition of PTPase (Protein Tyrosine Phosphatase) activity of PEP causes suppression of Lyn activity (Fig 3.8.2A compare lanes 7-12 with lanes 1-6). Moreover Lyn immunoprecipitates from PEP+/+ BMMC treated with L75N04 show reduced phosphorylation at Tyr^{397}compared to non-treated PEP+/+ BMMC (Fig 3.8.2A compare lanes 7-12 with lanes 1-6). Quantification of the kinase assay and the Tyr^{397} activity also represents the significant reduction seen in L75N04 treated PEP+/+BMMC (Fig 3.8.2B). The phosphorylation status of Lyn at its negative regulatory site and its quantification showed an increase phosphorylation at Tyr^{508} in L75N04 treated PEP+/+BMMC compared to PEP+/+ BMMC.

These findings suggest that the inhibition of phosphatase activity of PEP does not only affect the NFAT activation pathway but also targets the proximal mast cell activation steps that involve Src-like kinases.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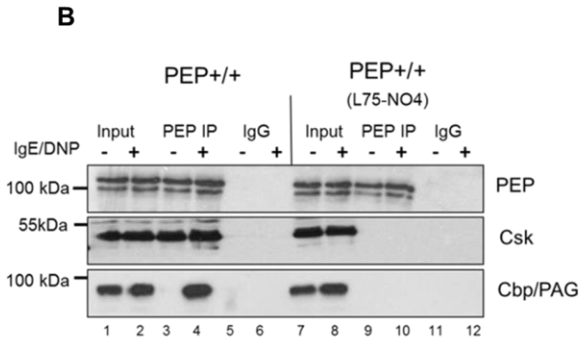
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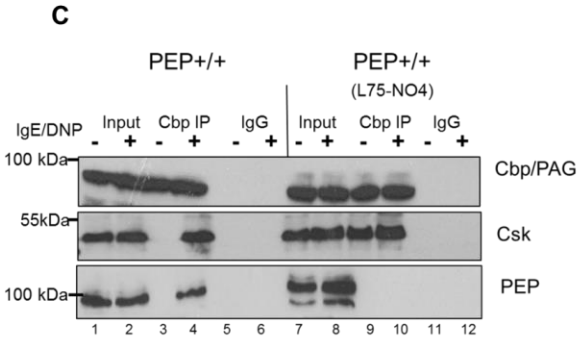
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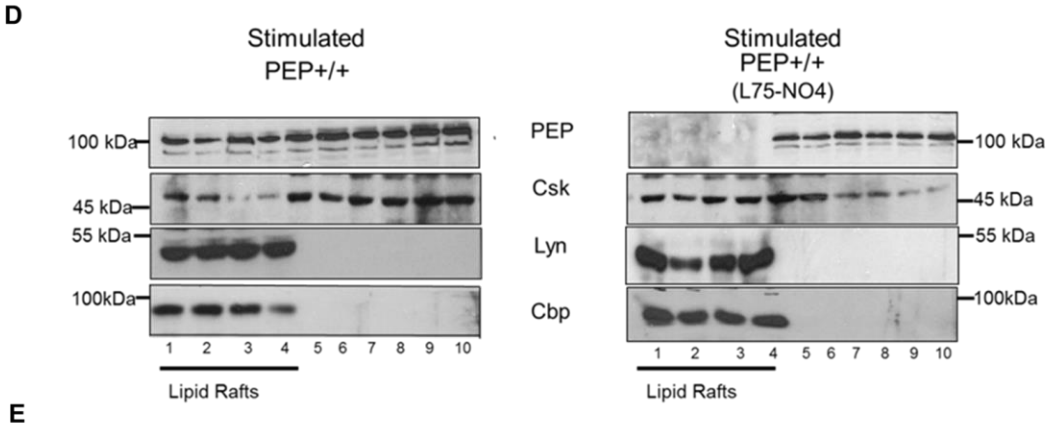
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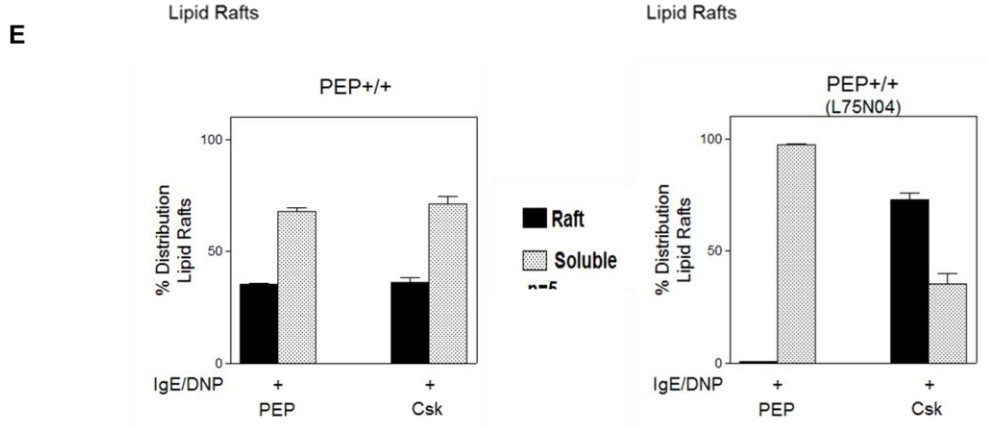
Lipid Rafts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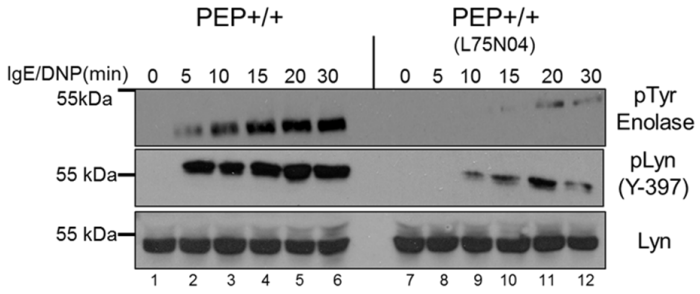
% Distribution
Lipid Rafts
100
50
0
IgE/DNP + +
PEP Csk
Raft
Soluble
PEP+/+
(L75N04)
% Distribution
Lipid Rafts
100
50
0
IgE/DNP + +
PEP Csk

Fig 3.8.1 L75-NO4 enhances recruitment of Csk to the lipid rafts - **(A)** Cell lysates from PEP+/+ were sensitized with anti-DNP IgE (1µg/ml) for 2h and then left untreated or treated with L75-NO4 (20µM) for 1h to the end of IgE incubation, then stimulated with DNP-HAS(200ng/ml) for 30 min. Lysates were immunoprecipitated with anti-Cbp/PAG antibody and immunoblotted to probe for phosphotyrosine, the filter was stripped and reprobed with anti-Cbp/PAG antibody **(B)** and **(C)** PEP+/+BMMCs cells were treated with anti-DNP IgE (1µg/ml) for 2 h and activated with DNP-HSA (200 ng/ml) in the presence and absence of inhibitor .Cell lysates were immuno-precipitated with anti-PEP or anti-Cbp/PAG antibody and subjected to immunoblot analysis with anti-PEP, anti-Csk and anti-Cbp/PAG antibodies. **(D)** Cell lysates (non-stimulated and stimulated) from wt BMMCs in presence and absence of inhibitor were subjected to sucrose gradient centrifugation and lipid rafts fractions were isolated. PEP and Csk content of raft or non- rafts fractions isolated was visualized by immunoblotting with respective antibodies. An anti-Cbp/PAG antibody serves as a marker of Lipid rafts. **(E)** The graph represents the densitometric quantification of amount of Csk and PEP present in rafts and non-rafts (soluble) fractions from 3 independent experiments. Presented is a representative result of three independent experiments.

A


PEP+/+
PEP+/+
(L75N04)
IgE/DNP(min) 0 5 10 15 20 30 0 5 10 15 20 30
55kDa
pTyr
Enolase
55 kDa
pLyn
(Y-397)
55 kDa
Lyn
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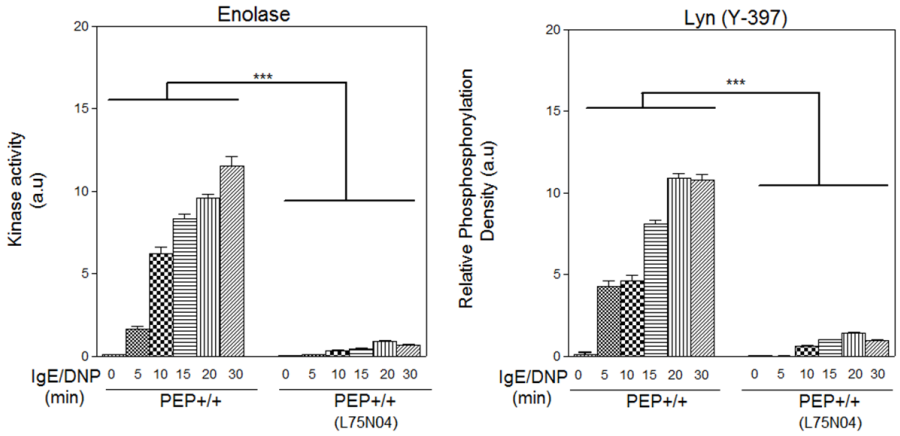
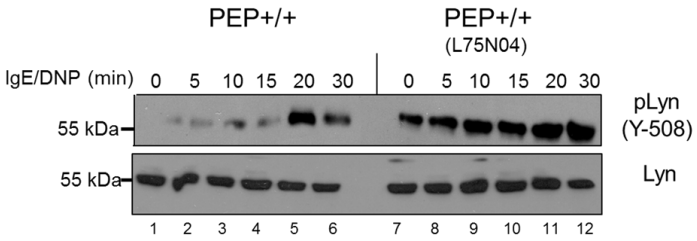
B


Enolase
Kinase activity (a.u)
***
IgE/DNP (min) 0 5 10 15 20 30 0 5 10 15 20 30
PEP+/+
PEP+/+ (L75N04)
Lyn (Y-397)
Relative Phosphosphorylation Density (a.u)
***
IgE/DNP (min) 0 5 10 15 20 30 0 5 10 15 20 30
PEP+/+
PEP+/+ (L75N04)

C


PEP+/+
PEP+/+
(L75N04)
IgE/DNP (min) 0 5 10 15 20 30 0 5 10 15 20 30
55 kDa
pLyn
(Y-508)
55 kDa
Lyn
1 2 3 4 5 6 7 8 9 10 11 12

D

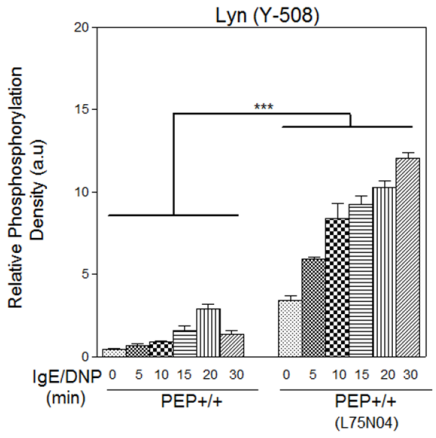

Lyn (Y-508)
Relative Phosphosphorylation Density (a.u)
***
IgE/DNP (min) 0 5 10 15 20 30 0 5 10 15 20 30
PEP+/+
PEP+/+ (L75N04)

Fig 3.8.2 Lyn activation is impaired in presence of L75-NO4 in wild type BMMC- **(A)** 8-10 x 10^6 PEP+/+ were treated with anti-DNP IgE (1µg/ml) for 2 h and left untreated or treated with L75-NO4 (20µM) for 1 h to end of IgE incubation and then activated with DNP-HSA (200 ng/ml) for indicated time. .Cell lysates were immuno-precipitated with anti-Lyn antibody and its enzymatic activity was determined by in-vitro kinase assay using Enolase as substrate, as well as immunoblotted with phospho-Lyn(Y-397)Ab **(B)** The graph represents the densitometric quantification of kinase activity determined in in vitro kinase assay.(mean of 3 independent experiments, ***p≤0.005) as well as densitometry analysis of phosphor-Lyn immunoblot normalized to the amount of protein immunoprecipitated (n=3, ***p≤0.001). **(C)** Cell lysate from above immunoprecipitation were probed for phospho-Lyn(Y-508) Ab that recognizes the indicated phosphorylated tyrosine residues in Lyn. The results of three independent experiments as in C, were quantified by densitometry analysis (n=3, ***p≤0.0002).

3.9 Effect of PEP deficiency on mast cell degranulation

Activated mast cells promote allergic inflammation following the release of biochemical mediators (Bischoff, 2007). IgE mediated signaling through the LAT-PLCγ-Ca2+ pathway does not only end up in activation of the activity of NF-AT but also leads to degranulation. Degranulation occurs through the process of exocytosis whereby cargo proteins and other molecules are loaded into vesicles and shuttled to different subcellular locations for fusion events. It results in the release of preformed mediators like β-hexosaminidase, serotonin, neural proteases and, histamine from the mast cell granules which causes an increase in vascular permeability, bronchoconstriction and inflammatory reaction in the mucosa.

To investigate the impact of PEP gene deletion as well as inhibition of its enzymatic activity by L75N04 on the capacity of mast cells to degranulate, amount of β-hexosaminidase released was measured.

BMMC from PEP+/+ and PEP-/- mice were sensitized with IgE and left non-treated or treated with L75N04 (20µM) and then stimulated with DNP. Cells were suspended in Tyrode's buffer +0.1% TritonX-100 for complete lysis and then centrifuged. Supernatants were then subjected to spectrophotometric quantification

of the released hexosaminidase using fluorescent N-acetyl-β-D-glucosaminidase as a substrate, as a measure for degranulation.

The results show significant difference in the percentage of hexosaminidase released between the wild-type and PEP deficient BMMC. PEP-/- BMMC showed ~ 2 2-fold reduced reduction in degranulation compared to PEP+/+ BMMC at 30 min DNP stimulation (Fig 3.9.A). Overall PEP-/- BMMC showed lower capacity to degranulate.

As seen in the regulation of NF-AT activity, 20μM of L7N04 was able to reduce degranulation by ~ 1.7 fold at 30 min DNP stimulation in PEP+/+BMMC. Even at other time points, the amount of β-hexosaminidase released was significantly reduced. Actually L75N04 treated PEP+/+ BMMC mimicked the effects of PEP-/- BMMC, as the amount of β-hexosaminidase released was similar in these BMMC.

In contrast, L75N04 had no effect on the residual degranulation in PEP-/- BMMC. There was no difference in the amount of β-hexosaminidase released between the treated and untreated PEP-/- BMMC.

To further verify a direct role of PEP in the degranulation process, BMMC from wild-type as well as from PEP deficient mice were transfected with pCMV-based vectors that express PEP to find out whether the defect in the PEP-/- BMMC could be rescued. Cells were sensitized with anti-DNP IgE and then stimulated with DNP-HSA. Cells were suspended in Tyrode's buffer +0.1% TritonX-100 for complete lysis and then centrifuged. Supernatants were then subjected to spectrophotometric quantification of the released hexosaminidase using fluorescent N-acetyl-β-D-glucosaminidase as a substrate, as a measure for degranulation

Transfection of the PEP expression vector into the PEP+/+ BMMC increased both the basal and antigen-induced degranulation (Fig 3.9B). More importantly over

expression of PEP in the PEP-/- BMMC significantly increased both the basal (~ 5 fold) and the antigen induced (~ 2.5 fold) degranulation. Thus reconstitution of PEP-/- BMMC with PEP restored degranulation to levels similar to that seen in the empty vector transfected wild-type BMMC (Fig 3.9B).

Control Western blot experiments with an anti-PEP antibody and a β-action antibody for loading control, show that the transfected PEP is indeed expressed in PEP-/- BMMC. This was more difficult to observe in the PEP+/+ BMMC that endogenously express PEP (Fig 3.9C). Taken together these studies confirm that PEP is required for the degranulation of mast cells.

Intriguingly, both PEP+/+ and PEP-/- showed some level of basal degranulation (Figs 3.9A and 3.9B), which suggest that some other pathway is activated in these BMMC apart from IgE-FcεRI signaling pathway which also leads to degranulation. As these BMMC are cultured in medium supplemented with SCF, Kit signaling pathway seems to be responsible for this process, since it is known that SCF not only enhances antigen mediated release of mediators but can itself stimulate degranulation, synthesis of inflammatory lipids when added to KL-deprived mast cells (Gibbs et al., 1997; Taylor et al., 1995). Hence effect of PEP deficiency on Kit signaling pathway was investigated by carrying out degranulation studies, wherein the amount of β-hexosaminidase released was measured.

For this assay PEP-/- and PEP+/+ BMMC were deprived of SCF overnight and then stimulated with SCF at various concentrations for 30 min and then suspended in Tyrode's buffer +0.1%TritonX-100 and then subjected to spectrophotometric quantification of the released hexosaminidase using fluorescent N-acetyl-β-D-glucosaminidase as a substrate, as a measure for degranulation.

PEP+/+ BMMC showed enhanced degranulation ~ 8 fold from the basal level at 400ng-SCF (Fig 3.9D), while PEP-/- showed only ~2.5 fold increased degranulation from basal level at 400ng (Fig 3.9D). The results reflect that indeed the ability of PEP-/-BMMC to degranulate when stimulated with SCF is compromised compared to PEP+/+BMMC as there is no significant difference in their basal level. Taking 400ng SCF as optimal concentration, the kinetics of degranulation was analyzed in PEP-/- and PEP+/+ BMMC. In PEP+/+ BMMC, degranulation increases and reaches a peak at 20 min (~ 8 fold) and then it levels off at 30 min (Fig 3.9E), while in PEP-/- BMMC, release of hexosaminidase reaches a peak at 15min (~ 2 fold) and then rapidly decreases (Fig 3.9E).

To confirm that the effects observed are indeed due to the absence of PEP, the degranulation assay were carried on PEP+/+BMMC treated with L75N0. IL-3 and SCF starved PEP+/+BMMC were pretreated with L75N04 (20μM) and then stimulated with 400ng SCF for indicated time points. The cells were then subjected to degranulation assay as described above.

Wild-type BMMC treated with L75N04 showed similar degranulation kinetics as seen in PEP-/- BMMC. PEP inhibitor treated BMMC had significantly reduced amount of β-hexosaminidase released compared to non-treated PEP+/+BMMC. Inhibitor treated BMMC showed increase in degranulation at 15 min (~ 3 fold) and then subsided down, mimicking the effects seen in PEP-/-BMMC (Fig 3.9.F).

Taken together, these results reflect that indeed phosphatase activity of PEP is required in both IgE-FcεRI mediated as well as in SCF-Kit activated mast cell degranulation.

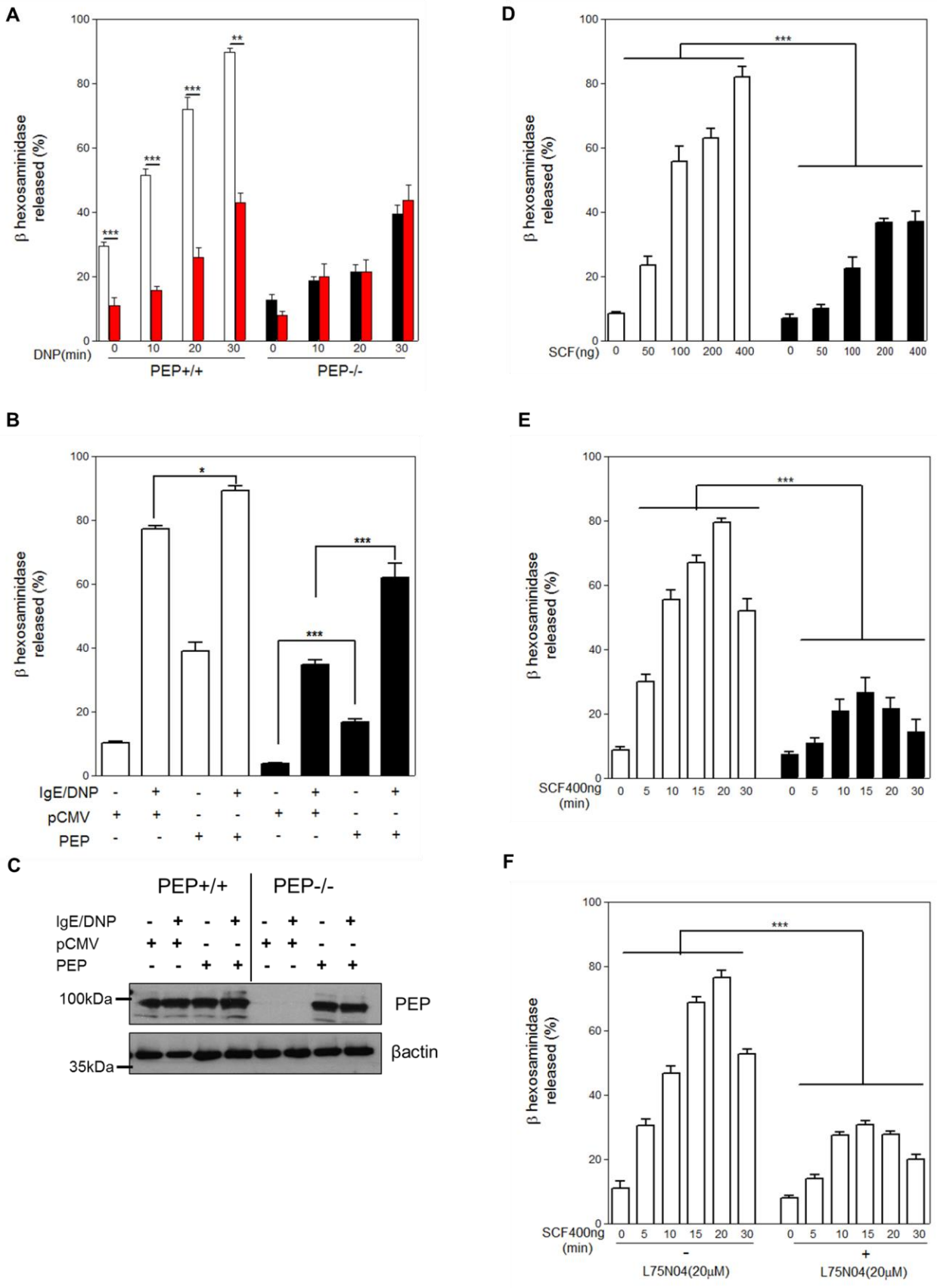

A
β hexosaminidase released (%)
DNP(min)
PEP+/+
PEP-/-
B
IgE/DNP
pCMV
PEP
C
PEP+/+
PEP-/-
IgE/DNP
pCMV
PEP
100kDa
PEP
35kDa
βactin
D
β hexosaminidase released (%)
SCF(ng)
E
β hexosaminidase released (%)
SCF400ng (min)
F
β hexosaminidase released (%)
SCF400ng (min)
L75N04(20μM)
L75N04(20μM)

Fig 3.9 β-Hexosaminidase release in PEP knock-out and L75N04 treated BMMC - (A) 5×10^6 cells /ml BMMC from PEP +/+ and PEP-/- mice were sensitized with anti-DNP-IgE(1μg/ml) for 16 h and treated with L75N04 (20μM) for 1 h at the end of IgE incubation. These cells were then activated with DNP-HSA (200 ng/ml) for various time points. BMMC were pelleted and the amounts of β-hexosaminidase in supernatant and in the solubilized pellet were measured using p-nitrophenyl N-acetyl-β-D-glucosaminidine (p-NAG) as substrate. Percent degranulation was calculated as follows: (released activity/total activity) ×100. The results are represented as mean of ± SEM of 3 independent experiments of % β hexosaminidase release for PEP+/+ (open bars), PEP-/- (closed bars) and L75N04 20μM (red bars) treated cells (**p≤0.05, ***p≤0.0005, n=3). **(B)** 8-10 × 10^6 PEP+/+ and PEP -/- BMMCs were transfected with 4.3μg of PEP-pCMV vector or an empty expression vector. After 24 h, the cells were sensitized with anti-DNP-IgE for 2h. Cells were stimulated with DNP-HSA (200ng/ml) for 30 min and β-hexosaminidase release was measured as described in (A). Results are presented as the mean ± SEM (*p≤0.01, ***p≤0.0001, n=5).PEP+/+ (open bars); PEP-/-(closed bars). **(C)** PEP+/+ and PEP-/- BMMCs were treated as shown in the figure and cell lysates were immunoblot using an anti-PEP specific antibody, and actin as the loading control**.** Presented is a representative result of three independent experiments**. (D)** 8×10^6 cells /ml BMMC from PEP +/+ and PEP-/- mice were cultures overnight in IL-3 and SCF depleted medium overnight and then activated with SCF at different concentration for 30 min. BMMC were pelleted and the amounts of β-hexosaminidase in supernatant and in the solubilized pellet were measured using p-nitrophenyl N-acetyl-β-D-glucosaminidine (p-NAG) as substrate. Percent degranulation was calculated as follows: (released activity/total activity) ×100. The results are represented as mean of ± SEM of 3 independent experiments (***p≤0.005) of % β hexosaminidase release for PEP+/+ (open bars) and PEP-/- BMMC (closed bars). **(E)** 8-10 × 10^6 PEP+/+ and PEP -/- BMMCs were cultured as described in (A) and then stimulated with SCF (400ng/ml) at different time point as indicated in the figure and β-hexosaminidase release was measured as described in (A). Results are presented as the mean ± SEM (***p≤0.0001, n=3) PEP+/+ (open bars); PEP-/- BMMC (closed bars). **(F)** 8×10^6 cells /ml BMMC from PEP +/+ mice were cultured overnight in IL-3 and SCF depleted medium overnight and left either untreated or treated with (20μM) L75N04 for 1 h and then activated with SCF (400ng/ml) at different time point. BMMC were pelleted and the amounts of β-hexosaminidase in supernatant and in the solubilized pellet were measured using p-nitrophenyl N-acetyl-β-D-glucosaminidine (p-NAG) as substrate. Percent degranulation was calculated as follows: (released activity/total activity) ×100. The results are represented as mean of ± SEM of 3 independent experiments (***p≤0.001) of % β hexosaminidase release

3.10 IgE mediated gene expression profiles in wild-type and PEP deficient BMMC

IgE-FcεRI crosslinking activates mast cell signaling pathways and ultimately leads to release of calcium and pro-inflammatory mediators (degranulation). PEP-/- BMMC have reduced activation of Lyn/LAT signaling and reduced release of β-hexosaminidase. To determine whether PEP deficiency has other possible defects, RNA profiling was carried out using Affymetrix gene chip microarray analysis. Two conditions were used: (1) control/non-stimulated and (2) IgE/DNP stimulated for 30 min in wild-type and PEP deficient BMMC.

Total RNA was extracted from 10 X 10^6 cells from resting and activated WT and PEP-/- BMMC using TRIzol reagent. Further purification of RNA was done using the RNeasy kit (Qiagen). These RNA samples were then sent to Dr. Ludger Klein-Hitpaß Lab (University Essen) where they performed micro-array analysis.

Briefly, from 200ng of RNA, cDNA synthesis was performed followed by in vitro transcription of biotinylated complementary RNA. The labeled complementary RNA were hybridized to mouse gene array containing 14,000 probe sets (GeneChip Mouse Genome Array 430 2.0 Array) and analyzed with GeneChip Scanner. Affymetrix Statistical Algorithms (MAS5 algorithms) was used in the expression analysis of GeneChip probe arrays for background correction, normalization and calculation of gene expression values. This microarray analysis had no replicates as a result all the expression changes seen in this array were validated with realtime PCR.

Total of 12,390 genes were reliable measured out of which 5046 genes were differentially regulated by IgE in wild type and PEP deficient BMMC. The results are expressed as the ratio between IgE stimulated over control; IgE/Ctrl >/< 1.5

fold, with red indicating an increase in signal and yellow indicating a lower signal. Heat maps were generated using Multiexperiment Viewer Software (Fig 3.10A). Importantly the figure illustrates that absence of PEP leads to positive and negative regulation of gene sets

Out of 5406 genes showing differential regulation by IgE in wild type and PEP-/- BMMC, genes with a fold change cut of 2 for up-regulated genes and down-regulated genes were selected and a Venn diagram was generated. The Venn diagram illustrates that a large number of genes was up-regulated by IgE in presence of PEP while a comparatively reduced number was obtained in PEP-/- BMMC. In case of the repressed genes, only 2-fold difference was seen in the genes that are down-regulated by IgE in wild-type and PEP deficient BMMC (Fig 3.10B).

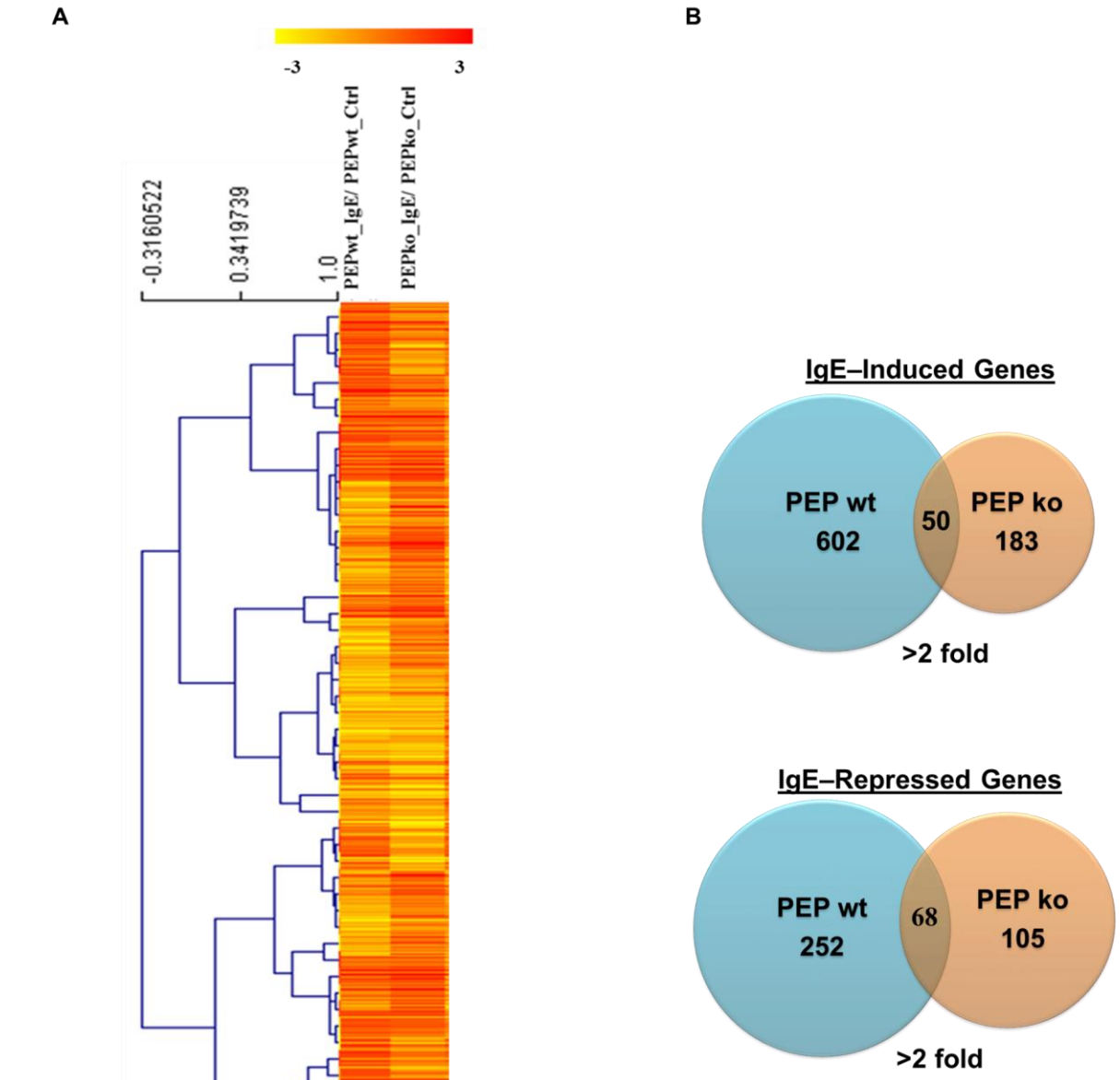
To assess the biological significance of this differential regulation of genes by IgE in PEP+/+ and PEP-/- BMMC, Gene Set Enrichment Analysis (GSEA) was used. GSEA makes use of the Molecular Signature Database (MSigDB) which contains more than 3000 gene sets for use with GSEA ordered into 5 gene set categories (c1-c5). Out of these categories c2 Biocarta gene set category is presented here which shows those biological processes that are up-regulated by IgE in wild-type and PEP knock-out mast cells with FWER p-value < 0.25 or FDR q-value < 0.01. The top 10 processes that showed lowest FDR (false discovery rate) q-value were selected (Fig 3.10C).

In wild-type BMMC, pathways involved in inflammatory processes were up-regulated while in PEP deficient BMMC pathways involved in microtubule formation were enhanced.

The differentially regulated genes showed some interesting functional clustering. For example the gene pool that showed down-regulation in the absence of PEP are genes that belong to calcium signaling pathways that include many cytokines involved in inflammatory processes. Heat map with selected genes that encode proteins involved in calcium signaling and downstream cytokine pathways was generated. The results are expressed as the ratio between IgE stimulated over control; IgE/Ctrl >/< 2 fold, with red indicating an increase in signal and yellow indicating a lower signal (3.10D).

Heat map illustrated indicates that indeed absence of PEP down-regulates genes, whose expression is dependent on calcium for example IL-6, NFATc1, STIM 1 and many inflammatory cytokines which are dependent on NFAT transcription factor (IL-4, IL-13 and IL-2).

Since there were no replicates these data were considered as preliminary and to validate these findings real time PCR was performed.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0.3419739
1.0
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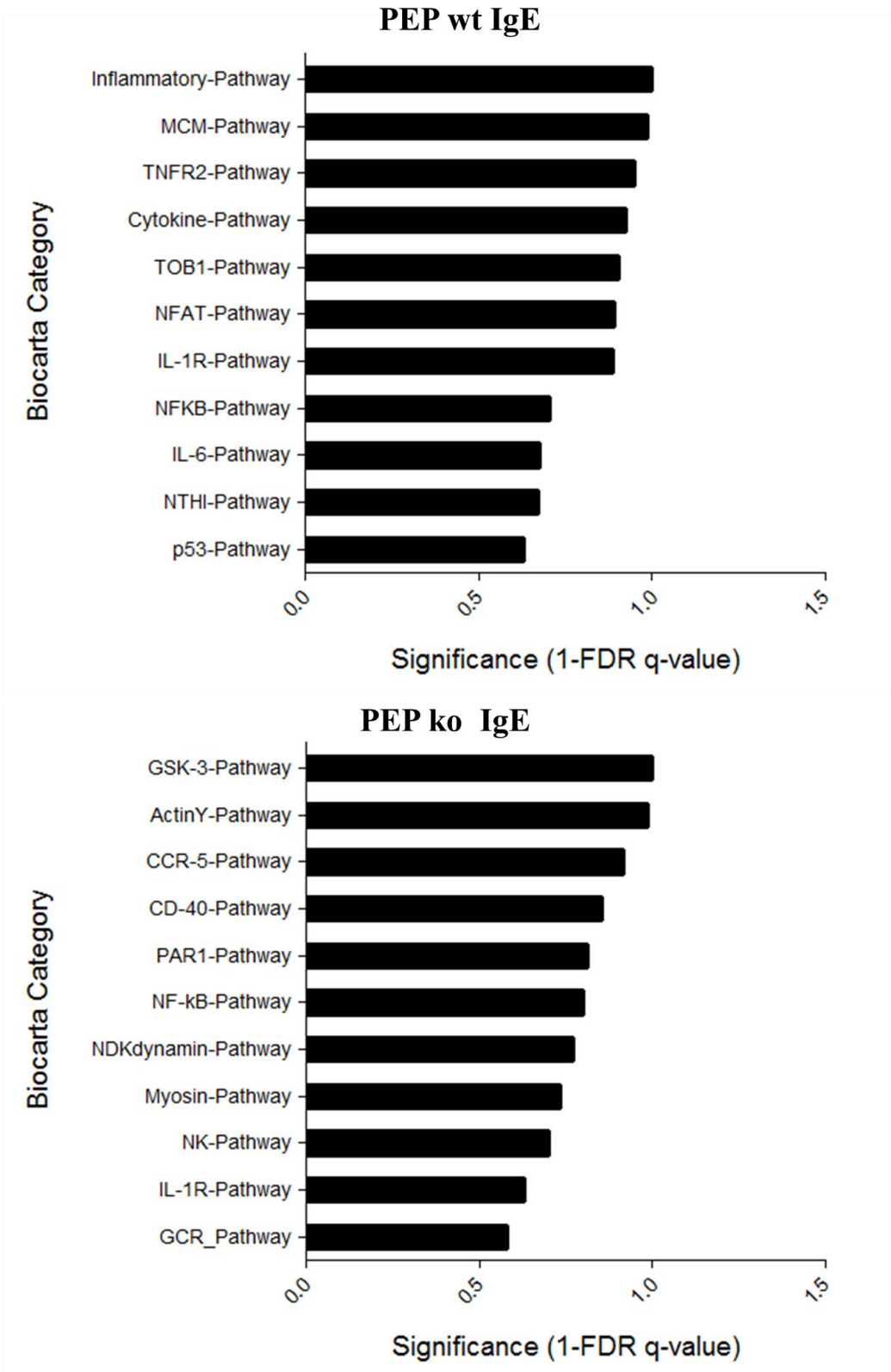
PEPko_IgE/ PEPko_Ctrl
5046 Genes
B
IgE–Induced Genes
PEP wt
602
50
PEP ko
183
>2 fold
IgE–Repressed Genes
PEP wt
252
68
PEP ko
105
>2 fold

C


PEP wt IgE
Inflammatory-Pathway
MCM-Pathway
TNFR2-Pathway
Cytokine-Pathway
TOB1-Pathway
NFAT-Pathway
IL-1R-Pathway
NFKB-Pathway
IL-6-Pathway
NTHI-Pathway
p53-Pathway
Biocarta Category
0.0
0.5
1.0
1.5
Significance (1-FDR q-value)
PEP ko IgE
GSK-3-Pathway
ActinY-Pathway
CCR-5-Pathway
CD-40-Pathway
PAR1-Pathway
NF-kB-Pathway
NDKdynamin-Pathway
Myosin-Pathway
NK-Pathway
IL-1R-Pathway
GCR_Pathway
Biocarta Category
0.0
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Significance (1-FDR q-value)

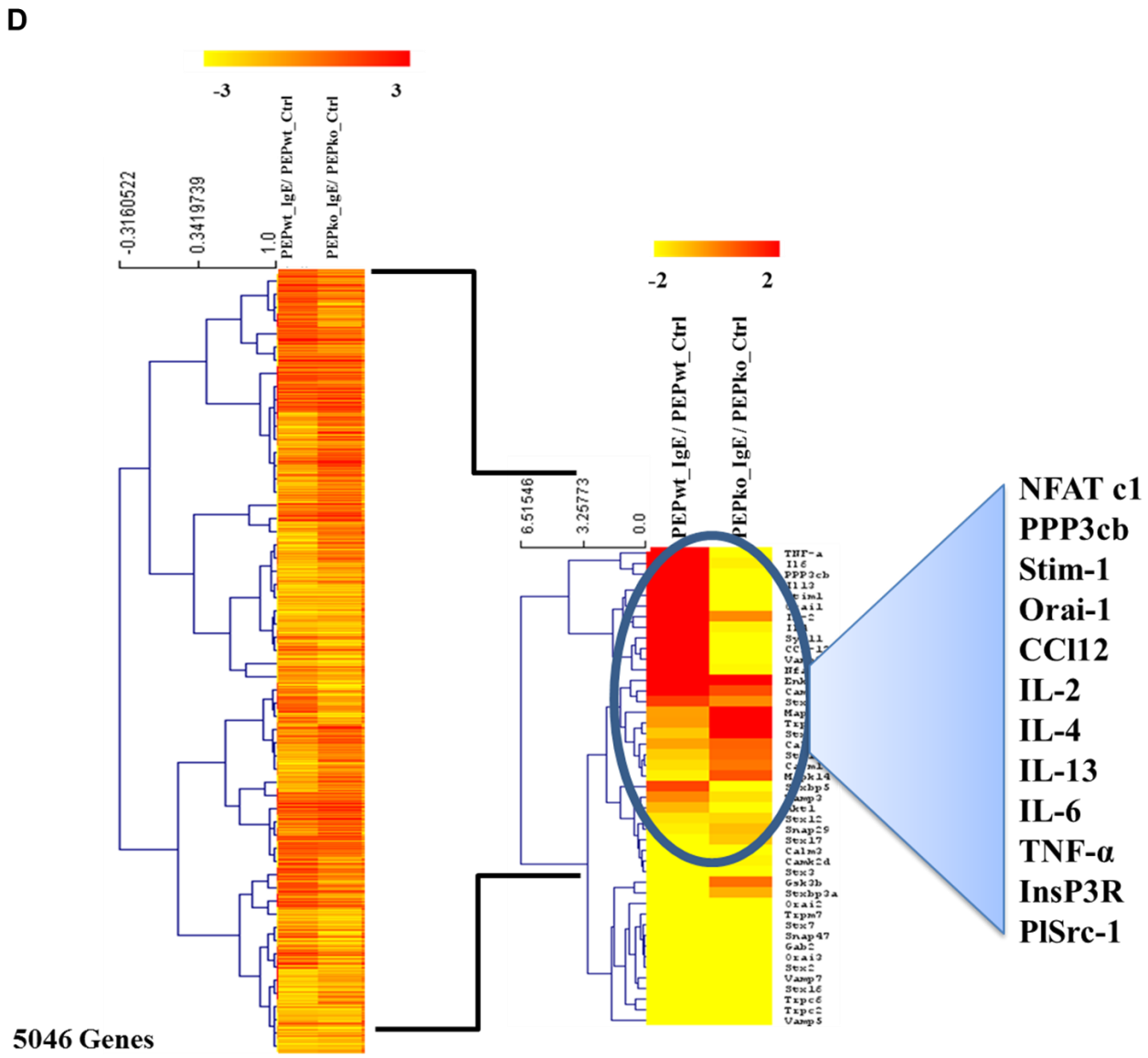


Fig 3.10 Comparison of IgE mediated gene expression profiles in wild-type and PEP deficient BMMC - **(A)** PEP+/+ and PEP-/- BMMC were left unstimulated or stimulated with IgE/DNP for 30 min. Gene expression analysis was performed using Affymetrix mouse genome chip. Control (unstimulated cells) were used as baseline and results are expressed as ratio between IgE stimulated over control; IgE/Ctrl >/< 1.5-fold. Gene expression pattern was generated using Multi experiment viewer. (B) Venn diagrams were generated comparing genes with a fold change cutoff of 2 for induced and repressed genes using Multi experiment viewer. **(C)** Gene set enrichment analysis was performed to obtain the biological functions up-regulated in wild-type and PEP deficient BMMC after IgE/DNP stimulation. Top 10 processes with lowest FDR q-value (false discovery rate) are plotted. **(D)** Heat maps showing relative expression level of genes that encode proteins that are bound by calcium

binding or protein with inflammatory functions. Control (unstimulated cells) were used as baseline and results are expressed as ratio between IgE stimulated over control; IgE/Ctrl >/< 2 -fold. Gene expression pattern was generated using Multi experiment viewer.

3.11 Validation of microarray data- PEP regulates the expression of cytokine /chemokine genes and also genes that are involved in calcium/NFAT signaling pathway

To validate some of the potential targets identified in the heat map that show differential regulation in wild-type and PEP deficient BMMC in the microarray analysis, quantitative real time PCR was carried out.

RNA was extracted from PEP+/+ and PEP-/- BMMC which were sensitized with IgE/DNP and either left untreated or treated with 20μM L75N04 to generate "chemical knockdown" of PEP and the same treatment was used on PEP-/- BMMC as control. Quantitatively, the relative expression of these genes were measured in three independent experiments using primers for the respective genes and normalized against a transcript of the ribosomal subunit 36B4 gene that served as a control.

The first gene set to be analysed included genes that are implicated in the regulation of calcium /NFAT signaling pathway such as PLSCR1 (phospholipid scramblase-, IP3R (inositol-3-phosphate receptor) - these genes are involved in Lyn mediated calcium release from ER stores, STIM1 and Orai1 - controls the opening of CRAC channel for calcium influx, NFATc1 and PPP3CB - encodes NFAT transcription factor and its phosphatase calcineurin respectively.

Expression of these genes in general was reduced in PEP deficient BMMC as well as wild-type BMMC treated with 20μM L75N04 Lyp/PEP inhibitor. The genes that

were drastically affected in PEP -/-BMMC were the NFATc1, STIM1 and Orai-1 these genes showed no up-regulation even after stimulation.

L75N04 treated BMMC showed reduced expression of all the genes, it also reduced the basal expression of PLSCR1, STIM1 and PPP3CB genes. PEP inhibitor not only showed effects on PEP+/+BMMC but it was able to reduce expression of genes such as PLSCR1 and PPP3CB in PEP-/- BMMC (Fig 3.11A).

The other gene set that was analysed by realtime PCR included cytokines/ chemokines that were up-regulated by IgE/DNP stimulation as seen in microarray analysis and were either dependent on calcium/NFAT pathway or not dependent on this pathway.

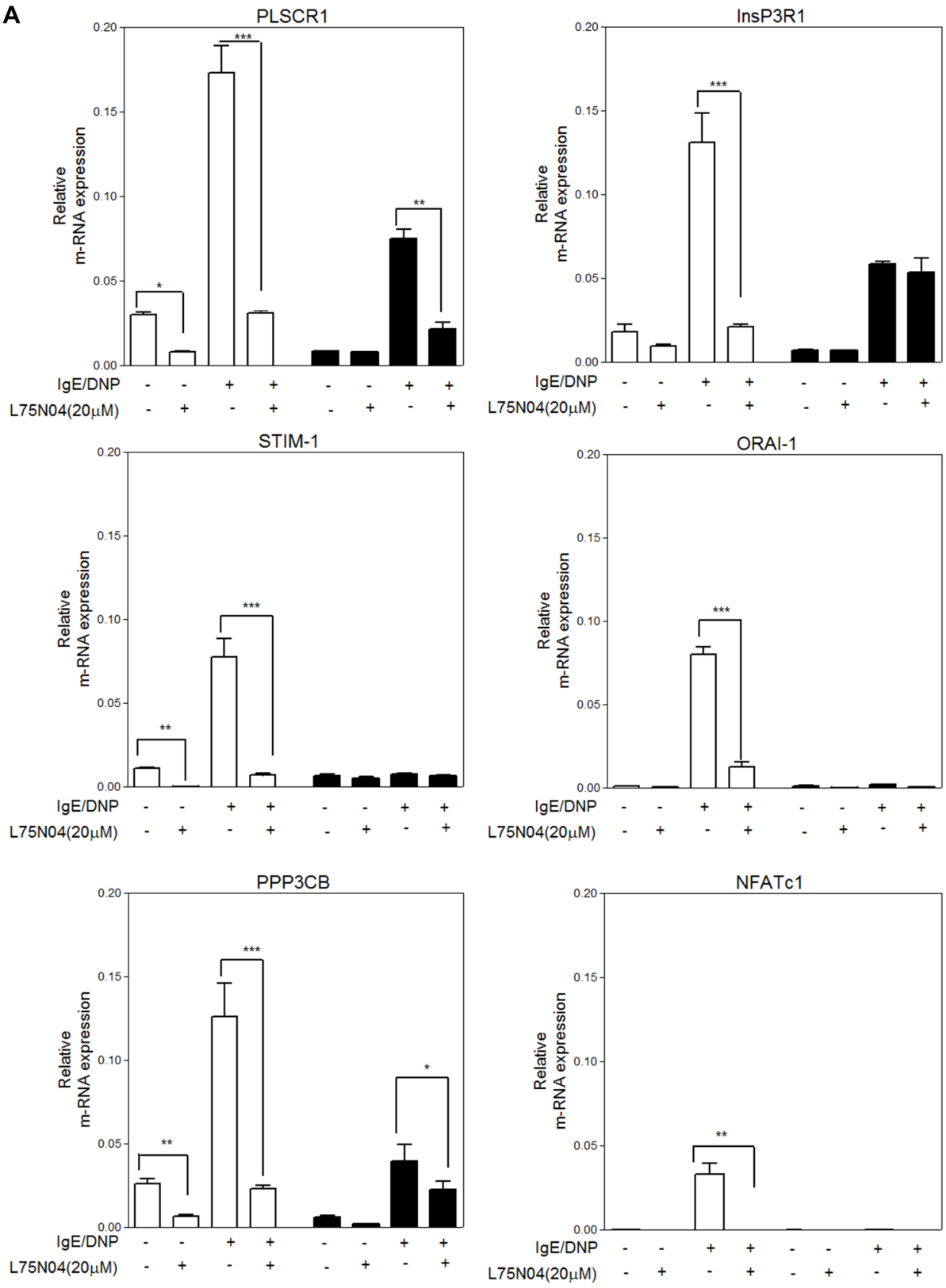
Cytokines /chemokines dependent on calcium /NFAT signaling pathway such as IL-2, IL-4, IL-6, IL-13, TNF-α and CCL-12 showed increase in their expression level in wild-type BMMC after IgE/DNP stimulation while in PEP-/- BMMC , the expression level of these genes both basal and antigen induced, were lower when compared to wild-type BMMC (Fig 3.11B).

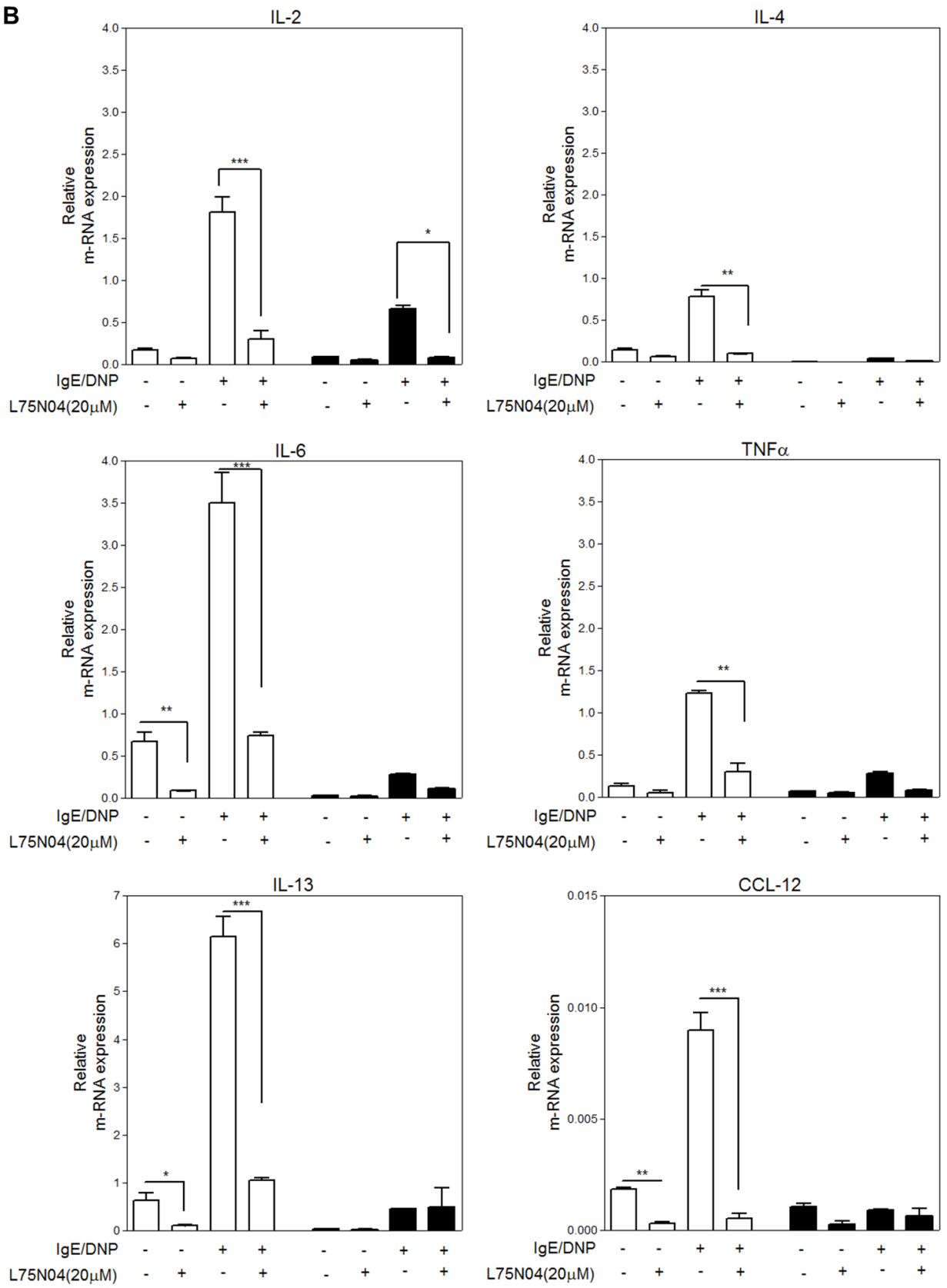
L75N04 treated BMMC showed significant decrease in their expression level, genes such as CCL-12, IL-6 and IL-13 showed reduce expression even at the basal level in the presence of inhibitor in PEP+/+BMMC. In PEP-/- BMMC only IL-2 expression level was reduced in the presence of inhibitor (Fig 3.11B).

m-RNA expression level of cytokines/ chemokines that were not regulated by calcium/NFAT signaling pathway such as IL-1β, IL-10, CCL-2 and MIP-1α were enhanced after IgE/DNP stimulation in both PEP+/+ and PEP-/- BMMC. There was no significant difference in the expression level of these genes in the both the mast cells (Fig. 3.11C)

Moreover L75N04 did not have any effect on the expression level of these genes in both wild-type and PEP knock-out BMMC (Fig 3.11C).

These results, which reflected the data obtained from Lyn/LAT/NFAT activation studies, confirmed that indeed PEP is essential for calcium/NFAT signaling pathway and for the expression of genes that are dependent on this pathway.


A
PLSCR1
InsP3R1
STIM-1
ORAI-1
PPP3CB
NFATc1
Relative m-RNA expression
0.00
0.05
0.10
0.15
0.20
IgE/DNP
L75N04(20μM)
*
**
***


B
IL-2
IL-4
IL-6
TNFα
IL-13
CCL-12
Relative m-RNA expression
IgE/DNP
L75N04(20μM)

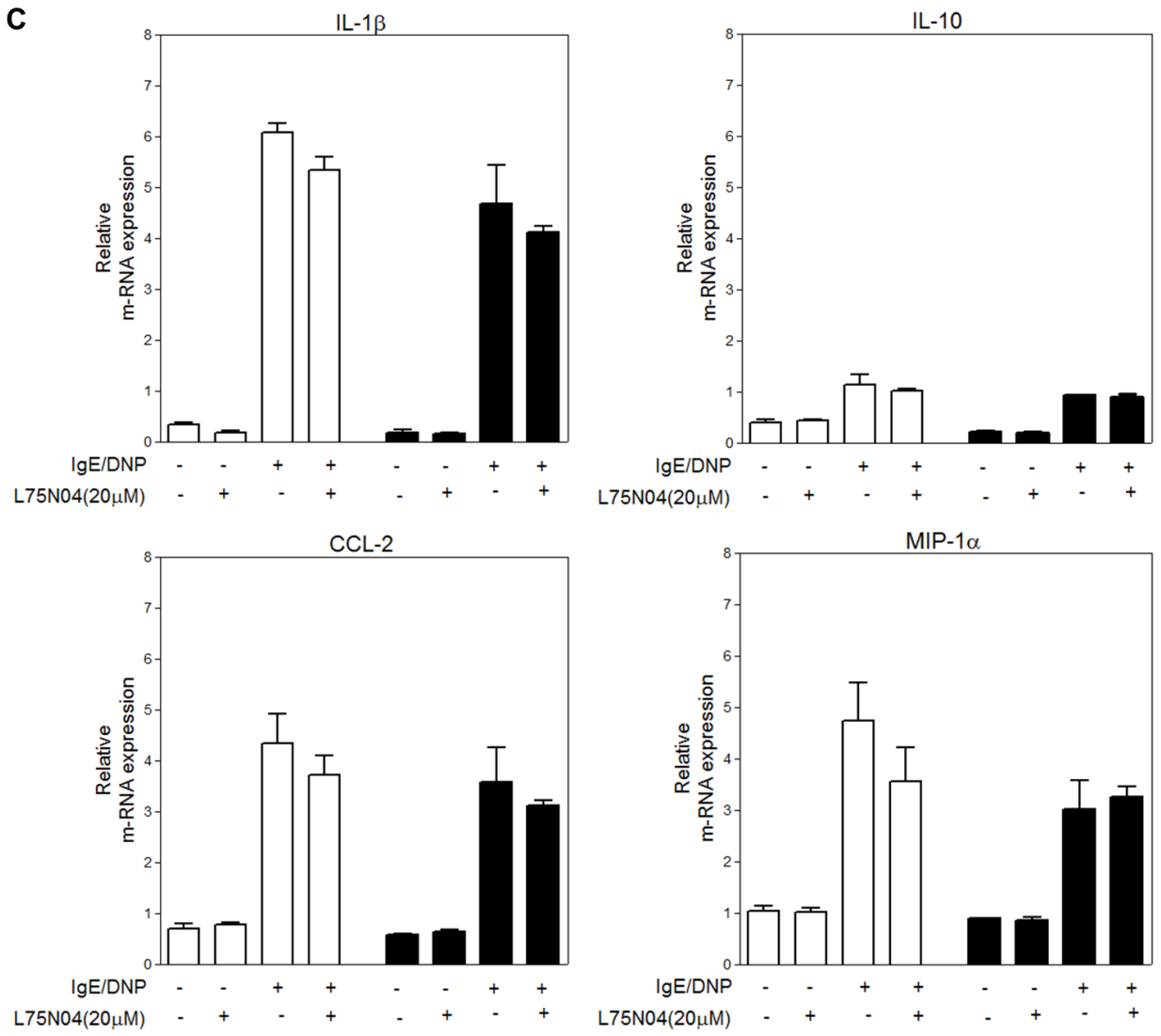


Fig 3.11 Cytokine/Chemokine gene expression - BMMC ($2x10^6$) were sensitized with 1μg/ml anti-DNP IgE for 2 h and left unstimulated or stimulated with 200 ng/ml DNP-HSA for 30 min in the absence or presence of inhibitors L75N04 (20μM). RNA was isolated from these cells, reverse transcribed into cDNA and subjected to real-time quantitative PCR. The expression of the cytokine/chemokine genes was measured using primers for the respective genes **(A)** PLSCR1, IP3R, STIM1, Orai1, NFATc1, PP3CB **(B)** IL-2, IL-4, IL-6,IL-13,TNF-α, CCL-12 **(C)** IL-1β, IL-10, CCL-2, MIP-1α, and normalized against the level of expression of the ribosomal subunit 36B4 gene. (*p≤0.02, **p≤0.005, ***p≤0.0001, n=3) PEP+/+ BMMC (open bars) and PEP-/- BMMC (closed bars)

3.12 L75N04 suppresses Passive Systemic Anaphylaxis in wild-type mice

Aggregation of FcεRI by binding of FcεRI-bound IgE to multivalent allergen activates downstream events that lead to degranulation and synthesis of cytokines and chemokines, which in turn induces the pathophysiologic consequences of anaphylaxis (Galli et al., 2008). Release of vasoactive amines (such as histamine-a potent vasodilator), causes fall in body temperature, which can be monitored as an index of anaphylaxis after antigen stimulation.

It has previously been reported by our laboratory that PEP-/- mice are less susceptible to passive systemic anaphylaxis compared to PEP+/+ mice which was in agreement with degranulation results obtained with PEP+/+ and PEP-/- BMMC (Obiri et al., 2011). As the Lyp/PEP inhibitor L75N04 is more potent in inhibiting IgE receptor signaling compared to C28, its efficacy in inhibiting passive systemic anaphylaxis was investigated.

Wild-type and PEP deficient mice were sensitized with IgE, treated with vehicle alone (0.1% DMSO in PBS) or 5μM C28, or 20μM L75N04 and following intravenous injection of DNP, changes in body temperature were measured every 5 min as a measure of anaphylaxis.

The vehicle treated wild type mice were more susceptible to anaphylaxis than PEP deficient mice as previously reported (Obiri et al., 2011). C28 treated wild type mice rescued the anaphylactic response but no further than the level induced in PEP deficient mice (Figs 3.12A and 3.12 B, blue line).

PEP+/+ mice treated with the inhibitor L75N04 showed a better rescue of anaphylaxis than C28, although its effect in the PEP-/- mice did not significantly differ from that of C28 (Figs 3.12A and 3.12B red line). These results confirm that

compound L75N04 is a more potent inhibitor of anaphylaxis than C28 and that its effect is mediated in part through the inhibition of the action of PEP.

Together, the results show that L75N04 is capable of down-regulating calcium mediated transcription and degranulation in mast cells and inhibiting passive systemic anaphylaxis in mice.

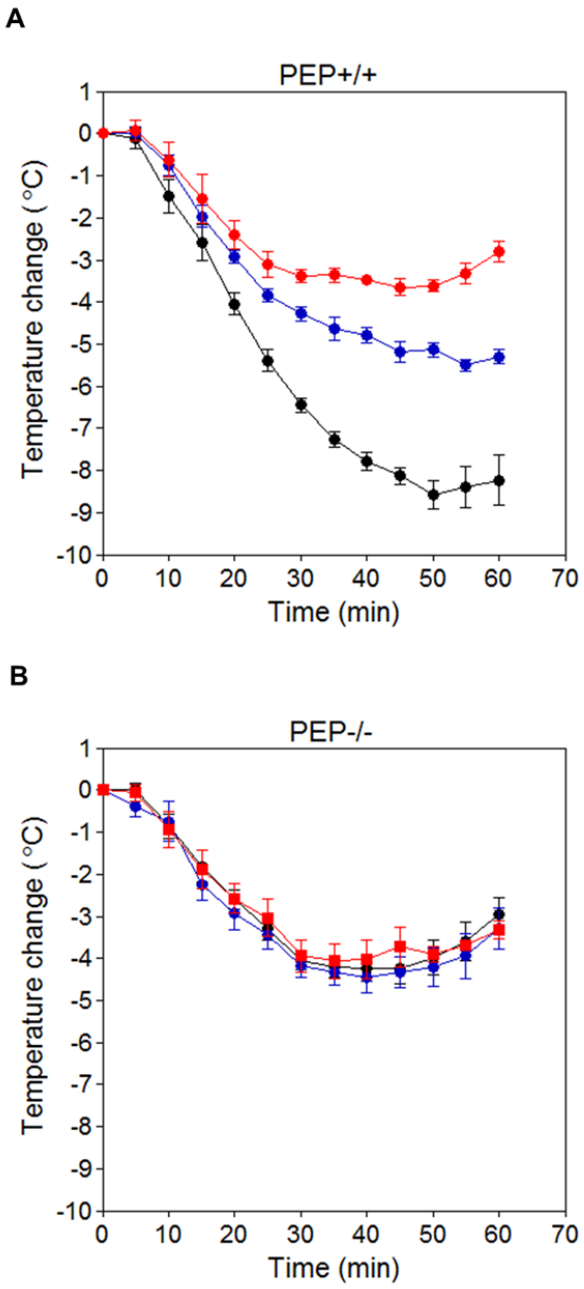


Fig 3.12 Systemic anaphylaxis impaired in the presence of inhibitors - PEP+/+ and PEP-/- mice were sensitized for 24 h with 1 mg/kg IgE and subsequently injected intra-peritoneal (IP) with either vehicle alone (0.01% DMSO-PBS) or C28 (5μM) or L75N04 (20μM) for 1 h to the end of the IgE incubation time. Anaphylaxis was induced with intra-venous injection of 200 μl DNP-HSA and the change in body temperatures measured every 5 min for 1h. Change in body temperature for **(A)** PEP+/+ and **(B)** PEP-/- mice are shown in black (PBS), blue (C28) and red (L75N04) respectively. Results are presented as the mean ± SEM (n=5)

4.0 Discussions

The manifestation of mast cell driven allergic reactions are considered to be mainly a consequence of the release of pro-inflammatory mediators following antigen-induced aggregation of IgE-bound high affinity receptors for IgE (FcεRI) expressed on the mast cell surface. The initial receptor-signaling involves phosphorylation of Src family tyrosine kinase Lyn /Syk. The subsequent Syk and/or Lyn-mediated tyrosine phosphorylation of the trans-membrane adaptor molecule LAT (linker for activation of T cells) is crucial for the downstream signaling required for the release of pro-inflammatory mediator (Gilfillan and Tkaczyk, 2006).

The status of mast cell activation at any point in time is a balance between the signaling pathways discussed above and those that down-regulate these processes.

For maintaining the homeostasis and the normal immune response, a dynamic equilibrium between protein tyrosine kinases (PTKs) and protein tyrosine phosphatase (PTPs) is required. Deregulations in this equilibrium and alterations in the expression or activity of PTPs can have pathophysiological consequences resulting in autoimmune diseases, cancer and diabetes

LYP and its murine ortholog PEP is exclusively expressed in immune cells and is reported to be a negative regulator of T-cell receptor signaling wherein it suppresses the activity of Src-family kinases (Hasegawa et al., 2004). It has previously been reported that PEP is expressed in mast cells (Maier et al., 2007) and that bone marrow derived mast cells from PEP-/- mice show reduced calcium mobilization and PEP positively regulates IgE-mediated anaphylaxis in mice (Obiri et al., 2011). However the molecular mechanism underlying this effect of PEP is

still unknown. In this study the mechanisms involved in the positive regulation of IgE-mediated signaling by PEP in mast cells has been investigated.

The present study provides an insight into the role of PEP in the regulation of FcεRI signaling, degranulation and cytokine production in BMMC. The results demonstrate that PEP primarily acts at a receptor-proximal level by regulating the recruitment of Csk-negative regulator of Src-family kinases like Lyn, to the lipid rafts by acting as a Cbp/PAG phosphatase. This regulation has proven to be essential for Lyn activation and downstream signaling events, since genetic ablation of PEP or the use of a pharmacological inhibitor of PEP showed inhibition of the protein tyrosine kinase activity (PTK activity) of Lyn resulting in the inhibition of phosphorylation of signaling molecules essential for Ca^{2+} influx (such as LAT). Additionally, degranulation mediated through both IgE-FcεRI and SCF-Kit activation is down-regulated alongwith cytokine gene expression in mast cells which are deficient in PEP and those which are treated with the inhibitor. These findings reveal that PEP plays a major role in mast cell signaling.

4.1 PEP functions as a Cbp/PAG phosphatase

Several substrates of PEP have been described in T-cells using substrate-trapping experiments and GST pull down experiments (Wu et al., 2006). Similar studies have not been described for PEP in mast cells. In the work presented here, co-immuprecipitation studies were carried out to identify putative targets of PEP in mast cells. The studies were concentrated on Src-like protein kinase and they revealed that PEP interacts with proximal signaling molecules involved in IgE-mediated mast cell activation like Lyn and LAT only after antigen crosslinking of FcεRI receptor while it is associated with Csk before and after IgE receptor

engagement. PEP deficient BMMC showed association of Csk with Lyn even under resting conditions which was not seen in wild-type BMMC.

It has previously been reported that tyrosine phosphorylation of Src family kinase Lyn present in membrane domains (lipid rafts) initiates a signaling cascade leading to mast cell activation. Csk, a negative regulator of Lyn, is recruited to these membrane domains via a Cbp/PAG protein. This protein when phosphorylated on a specific tyrosine residue, binds the SH2 domain of Csk and thereby elevates the affinity of Csk for Lyn and phosphorylates the negative regulatory phosphotyrosine located near the C-terminus of Lyn (Takeuchi et al., 2000). Lyn activation requires dephosphorylation of the C-terminal tyrosine Tyr^{508} which releases the auto-inhibitory configuration of the kinase domain. Lyn then trans-auto-phosphorylates Tyr^{397} within its activation loop to generate a highly active enzyme. Csk and Cbp/PAG protein regulates this cycle of activation /inactivation (Xu et al., 1999). In this work using PEP deficient BMMC and the Lyp/PEP inhibitor L75N04, PEP was shown to be a phosphatase that overcomes the negative regulation of Lyn brought about by Csk, by dephosphorylating Cbp/PAG.

This is based on the results of lipid rafts fractionation studies wherein Csk was found to be present at higher proportion in raft/detergent insoluble fractions of PEP-/- BMMC as well in PEP+/+ BMMC treated with 20μM L75N04 inhibitor. Increase in Csk protein level in the rafts fraction was due to the hyper-phosphorylation of Cbp/PAG protein observed in PEP deficient BMMC and wild-type treated with PEP inhibitor. In-vitro kinase assay results showed reduced Lyn PTK activity in these BMMC. This reduced PTK activity is due to an increase in the phosphorylation of Lyn at Tyr^{508} in PEP-/- BMMC and L75N04 treated Pep+/+ BMMC which is known to promote an intra-molecular rearrangement whereby the SH2 domain of Lyn binds to the phosphotyrosine and occludes the activation

domain of Lyn thereby generating an inactive kinase confirmation (Ingley, 2012). These results show that PEP regulates Cbp/PAG recruitment of Csk in IgE mediated pathway.

After aggregation of FcεRI receptor, Lyn-mediated signaling pathway leads to phosphorylation of numerous substrates including PLSCR1, phospholipid-scramblase and adaptor molecule LAT(Amir-Moazami et al., 2008).

PLSCR1 is known to be constitutively associated with Lyn in RBL-2H3 mast cells. PLSCR1 knockdown studies in RBL-2H3 shows impaired degranulation and suggested a role of PLSCR1 as an amplifier of LAT/PLCγ1/calcium axis (Amir-Moazami et al., 2008).

The work presented here shows a lower tyrosine phosphorylation of PLSCR1 in PEP deficient BMMC when compared to wild-type BMMC. As PLSCR1 is a downstream target of Lyn its reduced phosphorylation is likely a reflection of the reduced PTK activity of Lyn.

Lyn dependent LAT phosphorylation is essential for calcium release from intracellular stores that eventually induces calcium influx. This is in agreement with the finding that LAT deficiency in mice leads to decreased mast cell degranulation and cytokine release (Saitoh et al., 2000). Mutation studies in BMMC have revealed the importance of phosphorylation of four distal tyrosine residues for LAT-dependent downstream signaling. One of these residues, Tyr^{136} has been shown to be important for PLCγ-binding (Saitoh et al., 2003).

Consistent with this finding, tyrosine phosphorylation of LAT in this study in PEP deficient BMMC showed a lower phosphorylation at Tyr^{136} than wild-type. The finding explains the previously reported reduced phosphorylation of PLCγ1 and decreased calcium mobilization in PEP deficient BMMC (Obiri et al., 2011).

The other LAT tyrosine residue investigated in this study was Tyr^{195}. This site along with Tyr^{175} has been reported by Saitoh et al., 2003 to be involved in Grb-2 binding and thereby activation of the Ras-Erk signaling pathway. Results from the study reported here revealed no change in the phosphorylation of the Tyr^{195} residue in PEP-/- and PEP+/+ BMMC which is in agreement with the previous finding where no change in Erk phosphorylation was detected between wild-type and PEP deficient BMMC (Obiri et al., 2011).

These findings reveal that, signaling events downstream of LAT is dependent on which tyrosine residues are being phosphorylated. PEP deficiency seems to affect the Lyn-mediated phosphorylation of PLCγ1 binding site in LAT, resulting in significantly reduced calcium mobilization. Hence PEP modulates the LAT/PLCγ1/Ca^{2+} axis by positively regulating Lyn activation.

4.2 PEP deficiency affects the activation of NFAT transcription factor

NFAT is a transcriptional activator that has previously been shown to translocate to the nucleus upon allergen stimulation of IgE-primed cells (Pandey et al., 2004). The NFAT transcription factor is made up of a family of five that comprises the four genuine members NFATc1, NFATc2, NFATc3, and NFATc4, as well as a distantly related NFAT5 (Ton EBP). With the exception of NFAT5, which is ubiquitously expressed and activated in response to osmotic stress, nuclear translocation and activation of NFAT proteins is induced by the Ca^{2+}-calmodulin dependent phosphatase calcineurin (Macian, 2005). All NFAT proteins have a highly conserved DNA-binding domain that is structurally related to the DNA-

binding domain of the REL-family transcription factors. This REL-homology region (RHR) is the unifying characteristic of NFAT proteins and confers a common DNA-binding specificity (Rao et al., 1997). Expression of multiple members of the NFAT family such as NFATc1, NFATc2 and NFATc3 in mast cells has already been reported wherein they are known to regulate the cytokine gene expression (Hock and Brown, 2003; Hutchinson and McCloskey, 1995).

The distinguishing feature of NFAT is its regulation by Ca^{2+} and the $Ca^{2+/}$calmodulin-dependent serine phosphatase calcineurin. IgE-FcεRI receptor ligation leads to the activation of phospholipase Cγ1 (PLCγ1), which results in the release of inositol-1, 4, 5-trisphosphate (InsP3) and a transient release of calcium from intracellular stores through the binding of InsP3 to its receptors (IP3 receptor). This initial release of Ca^{2+} is not sufficient to activate NFAT target genes, rather an influx of Ca^{2+} through specialized calcium-release-activated calcium (CRAC) channels in the plasma membrane is required (Putney and Bird, 1993) such that increased levels of intracellular calcium are maintained. Calcium binds calmodulin, which in turn activates the calmodulin dependent phosphatase calcineurin. NFAT proteins are phosphorylated and reside in the cytoplasm in resting cells; upon stimulation, they are dephosphorylated by calcineurin, translocate to the nucleus, and become transcriptionally active, thus providing a direct link between intracellular Ca^{2+} signaling and NFAT-mediated gene transcription.

In this study, experimental results from NFAT activation studies using luciferase assay revealed that PEP deficient BMMC and the L75N04 treated BMMC have significantly reduced activation of NFAT transcription factor. Reconstitution experiment confirmed the direct role of PEP in the activation of NFAT

transcription factor as the activation level in DNP stimulated PEP-/- BMMC transfected with PEP expression vector was restored to the level of empty-vector transfected, DNP stimulated wild-type BMMC. No impairment in the activity NF-κB or AP-1 transcription factors was observed in PEP-/- compared to PEP+/+ BMMC. Wild-type, PEP knockout and L75N04 treated BMMC showed similar activation of these transcription factors. Among these transcription factors only NFAT transcription factor activation, is known to be totally dependent on optimal calcium release, hence it is logical to predict that all gene expression dependent on calcium will be impaired in the PEP-/- BMMC. IL-6 expression is reduced following IgE crosslinking in PEP-/- BMMC compared to PEP+/+ BMMC which could be attributed to the reduced level of calcium release as the expression as well as release of this interleukin is dependent on calcium (Klein et al., 2006; Tanaka et al., 2005).

Studies in T-cells have shown that NFAT activation requires dephosphorylation of the serine residues in a conserved ~300-amino acid region located N-terminal to the DNA-binding domain termed the NFAT regulatory domain. This domain is heavily phosphorylated in resting cells, with the phosphorylated residues (serines) distributed in the SP (serine-proline rich) repeats and in the SRR (serine rich regions) repeats (Beals et al., 1997a; Okamura et al., 2000). Phosphoserines within these regions appear to mask the nuclear localization sequence (NLS) of NFAT. Dephosphorylation by calcineurin exposes the NLS and leads to rapid nuclear import (Beals et al., 1997a).

Interaction of calcineurin with NFAT is shown to be mediated by a targeting domain **PxIxIT** motif (Proline-x-Isoleucine-x-Isoleucine-Threonine) that is present in the NH2-terminal region of the NFAT transcription factor (Aramburu et al.,

1998; Chow et al., 1999). Removal of five phosphates from a conserved serine-rich sequence located immediately adjacent to the **PxIxIT** calcineurin-binding motif exposes the NLS in the regulatory domain and renders additional eight phosphoserine residues in the regulatory domain significantly more accessible to calcineurin (Okamura et al., 2000). Complete dephosphorylation of all 13 residues further results in masking of a nuclear export signal (NES) and promotes the full translocation of NFATc1 to the nucleus. Extensive dephosphorylation is necessary to fully activate the DNA-binding and transcriptional functions of NFAT.

The calcineurin mediated dephosphorylation and nuclear translocation of NFAT are counteracted by NFAT kinases, several kinases have been reported to phosphorylate NFAT proteins and control their nuclear shuttling. These include glycogen-synthase kinase 3 (GSK3), casein kinase1 (CK1) and JUN-N-terminal kinase (JNK). These kinases are distinguished as maintenance kinases which act in the cytosol to keep NFAT proteins in a fully phosphorylated state and prevent their translocation into the nucleus in resting cells. They also function as export kinases to rephosphorylate NFAT in the nucleus and promote its nuclear export, thereby stopping NFAT-mediated transcription after mast cell stimulation is withdrawn and calcineurin activity declines.

CK1 docks at a conserved motif that is near the N terminus of NFAT proteins, and it functions both as a maintenance and an export kinase by dephosphorylating serines present in the SRR motif (Okamura et al., 2004; Zhu et al., 1998). GSK3 functions as an export kinase by phosphorylating the conserved SPxx repeat motifs present in the regulatory domain of NFATc1 (Beals et al., 1997b). Thus these two kinases phosphorylate exactly those residues which are dephosphorylated by calcineurin.

JNK1 functions as maintenance kinase by phosphorylating specific serine residues (Ser^{117} and Ser^{172}) in the PxIxIT calcineurin targeting motif of NFATc1, thus inhibiting the NFATc1-calcineurin interaction required for the regulation of both nuclear accumulation and transcription activity (Chow et al., 2000). In line with these published reports, the massive down-regulation of NFAT activity in PEP-/- BMMC could be a result of one of the NFAT kinase.

In this work, the observed significant reduction in NFAT activation in PEP deficient BMMC and L75N04 treated wild-type BMMC may not only be due to a decrease in calcium release but also due to increased activation of NFAT kinases. Indeed an enhanced and sustained phosphorylation of JNK in PEP knock out and inhibitor treated BMMC was observed. This increased activation of JNK results in a reduced NFAT activity is shown in transfection experiments in which the dominant negative kinase deficient mutants are able to rescue the effect and induce NFAT activation. The increase JNK phosphorylation correlated with an increase in phosphorylation of NFATc1 at Ser^{117} and Ser^{172}, two of the phosphorylation sites known to inhibit nuclear translocation of NFATc1. Additionally nuclear fractionation studies carried on the wild-type and PEP deficient and L75N04 treated BMMC shows that nuclear accumulation is inhibited in the absence of PEP.

These findings show that two different processes (a) a down-regulation of the PLCγ1/calcium dependent pathway and (b) an increase in MAPK-JNK activation pathway regulate the NFAT transcription factor activation in BMMC from PEP deficient mice.

4.3 Cytokine and Chemokine gene expression is differentially regulated by PEP

Affymetrix gene chip analysis was used to determine the genome-wide effect of the absence of PEP on RNA transcript. Comparison of gene expression profile induced by IgE/DNP stimulation in the presence and absence of PEP showed a strong influence of PEP on IgE induced genes.

Gene set enrichment analysis revealed that major class of genes with potential function in inflammatory response, calcium-NFAT signaling as well as cytokine release were up-regulated in wild-type BMMC whereas in PEP knock out BMMC, gene pool with functions in microtubule formation, actin polymerization were up-regulated when stimulated with IgE/DNP. This effect might be due to the reduced calcium/NFAT activation which is required for the inflammatory response.

The microarray analysis also show that the genes involved in calcium/NFAT signaling pathway as well as certain cytokine/chemokines genes were down-regulated in PEP-/- BMMC. To confirm this realtime PCR was conducted, studies from this experiment confirmed that indeed the expression of genes coding for NFATc1 and PPP3cb (calcineurin) are significantly reduced in PEP knockout as well as in L75-N04 treated BMMC which provides mechanistic explanation for decreased activation of NFAT. Also expression of IP3R gene (inositol-3-phosphate receptor) is significantly reduced in absence of PEP due to the reduced expression of PLSCR1 gene (phospholipid scramblase) which is known to activate the transcription of the IP3 receptor 1 gene which regulates the intracellular release of endoplasmic reticulum calcium stores in response to the second messenger inositol-3-phosphate and thus play central role in calcium release(Ben-Efraim et

al., 2004; Zhou et al., 2005). Expression of genes that encode protein involved in opening of CRAC channels like STIM1 and Orai1 (Fahrner et al., 2009) are also down-regulated in PEP deficient and L75N04 treated BMMC. These findings explain the reduced calcium mobilization in these BMMC.

It is well established that NFAT and calcium regulates the expression of many cytokines and chemokines. NFAT is known to regulate cytokine TNFα, IL-13 (Monticelli et al., 2004; Yang et al., 2009) and the promoter activity of cytokines IL-2 and IL-4 is regulated by NFAT while IL-6 is dependent on calcium. Microarray analysis as well as RT-PCR results reveal that the expression of these cytokines are drastically reduced in the absence of PEP and suggest that decrease in NFAT and calcium levels may contribute to a mechanism for decreased cytokine.

Intriguingly, cytokines IL-1β, IL-10 and chemokines like MCP-1 did not show any difference in their expression pattern between the wild-type and PEP deficient or L75N04 treated BMMC. The plausible explanation for this observation is the fact that cytokines are regulated through different pathway and not through PLCγ/calcium/NFAT pathway (Akdis et al., 2011).

IL-1β expression is regulated by NF-κB (Hiscott et al., 1993). This can be explained by the fact that the activation of this transcription factor remains unaffected by the absence of PEP. IL-10 and MCP-1(CCL-2) are regulated by the JAK-STAT pathway (Akdis et al., 2011) and NF-κB-ERK pathway (Thompson and Van Eldik, 2009).

Experimental studies carried out in this work shows that absence of PEP does not have any effect on the MAPK kinase-ERK and p38 pathway (Obiri et al., 2011), moreover phosphorylation studies of LAT reveal that phosphorylation at Tyr^{195} remains unaffected which is needed for Grb2 binding and thereby ERK activation. This might be an explanation for the lack of effect of PEP ablation on MCP-1. Additionally mutation studies in LAT have shown that MCP-1 expression is less affected by Y136F mutation indicating this site is not essential for MCP-1 activation (Saitoh et al., 2003).

Hence, absence of PEP affects only those cytokines and chemokines which are directly or indirectly dependent on calcium/NFAT signaling pathway.

4.4 PEP deficiency impairs mast cell degranulation and passive systemic anaphylaxis in mice.

The key contribution of mast cells to anaphylactic reaction has been widely demonstrated. For example; anti-IgE antibody induced mast cell degranulation is involved in anaphylactic reactions that result in cardiopulmonary changes and mortality in mice (Martin et al., 1989). Functional elimination of certain effector molecules like LAT (Saitoh et al., 2000), Btk (Hata et al., 1998) and Vav (Manetz et al., 2001) causes diminished responses in mast cell mediated anaphylaxis. However, only limited number of phosphatases in FcεRI signaling has been shown to be involved in the anaphylactic response. For example, knock out of DUSP2 (dual specificity phosphatase 2) (Jeffrey et al., 2006) shows reduced susceptibility to anaphylaxis. In this study PEP has been demonstrated as important factor in antigen mediated degranulation and passive systemic anaphylaxis. The reduced

anaphylactic and degranulation response is likely a direct consequence of impaired FcεRI signaling observed in PEP deficient BMMC.

In order to investigate the impact of PEP gene deletion as well as its inhibition of enzymatic activity on the capacity of mast cells to degranulate and thereby trigger anaphylaxis, was assessed in vitro by measuring the amount of β-hexosaminidase released.

Degranulation was impaired in PEP knock out and L75N04 treated BMMC, and this impairment was restored by reconstituting PEP in the PEP deficient BMMC. IgE-mediated signaling pathway in PEP deficient and L75N04 treated BMMC show impairment at receptor proximal event where increased phosphorylation of Cbp/PAG and thereby increased recruitment of Csk is observed. This possibly contributes to the reduced mast cell degranulation response as Cbp/PAG overexpression studies in RBL-2H3 cells have shown reduced degranulation and calcium mobilization in these cells (Ohtake et al., 2002a). Additionally functional mutation studies on LAT have shown that mice expressing Y136F mutation (the site which is essential for PLCγ binding) exhibit reduced serum histamine level, demonstrating the critical function of LAT Y-136 residue in vivo (Saitoh et al., 2003). The functional experiments carried out in this study further support the importance of this tyrosine residue LAT, since reduced phosphorylation of Y136 in PEP deficient BMMC is correlated with reduced degranulation. Moreover the reconstitution experiments restored the degranulation level in these BMMC which shows the importance of PEP in the degranulation process.

SCF(stem cell factor), although primarily required for the growth, differentiation and survival of mast cells, also potentiates the secretory responses elicited via the

FcεRI receptor (Bischoff and Dahinden, 1992). SCF not only enhances antigen mediated release of mediators but can itself stimulate degranulation, synthesis of inflammatory lipids when added to KL-deprived mast cells (Gibbs et al., 1997; Taylor et al., 1995).

As synergistic interaction exists between SCF-Kit and IgE-FcεRI in the release of inflammatory mediators due to the overlapping of signaling pathways, effect of PEP deficiency on this signaling pathway was investigated by performing degranulation assay on BMMC stimulated with SCF.

In the work presented here, BMMC also responded to SCF stimulation, the wild-type BMMC show 80% degranulation when stimulated with 400ng/ml SCF, while only 25 % degranulation is seen in PEP knock-out BMMC, to investigate whether this difference is due to the absence of PEP, wild-type BMMC were treated with L75N04 inhibitor and as expected, L75N04 treated BMMC showed lower degranulation (30%) in comparison to wild-type BMMC. This decrease in degranulation in the absence of PEP suggests that PEP has a role to play in downstream signaling events that lead to degranulation in mast cells.

Anaphylaxis is controlled by rapid lipid mediator release, degranulation and by pro-inflammatory cytokine synthesis in mast cells (Peavy and Metcalfe, 2008).
Decrease in degranulation as well as in the release of pro-inflammatory cytokines explains the observed impairment of systemic anaphylactic response analyzed by the fall in body temperature after antigen stimulation in PEP deficient mice and L75N04 and C28 treated mice. L75N04 compound turns out to be a potent inhibitor than C28 in inhibiting anaphylaxis.
Taken together,these results show that L75N04 is capable of down-regulating calcium mediated transcription and degranulation in mast cells and inhibiting

anaphylaxis in mice. The results reported here are quite encouraging for further studies to be performed with higher concentrations of L75N04.

The current treatment for anaphylactic shock is norepinephrine followed immediately by glucocorticoids and H1-antihistamine, despite a lack of evidence to support these treatments (Choo et al., 2010). Our present finding on L75N04 provide great hopes for a compound with tremendous potential for the rapid treatment of anaphylactic shock that is supported by above experimental evidence.

Ultimately, one of the major goals of animal model based cell signaling research is to provide insight into how biological system in human works, so that novel therapeutics may be designed to manipulate signaling pathways to treat human diseases. Thus, it is important to verify that the results obtained in the murine mast cell system are transferable to human mast cells. LAD2 human mast cell leukemia cell line could be a useful tool in future experiments to verify the results that were obtained in the BMMC model as well as to test the potency of L75N04 on these cell lines.

These investigations will allow for the observations produced by this study to be included in the considerations behind designing strategies for the manipulation of FcεRI pathway during the process of novel drug design in the creation of new therapeutic immunomodulators

4.5 Summary

The present study provides an insight into the molecular mechanism involved in the positive regulation of antigen mediated systemic anaphylaxis by PEP in mice.The data presented here identifies PEP to function at receptor-proximal level in the IgE-FcεRI signaling pathway.

In wildtype BMMC under resting conditions PEP is constitutively associated with Csk (negative regulator of Src-like kinases) in the cytoplasm as shown in Fig 4.A. After antigen stimulation, it gets recruited alongwith Csk to the lipid rafts,where it functions as Cbp/PAG phosphatase and thereby inhibits further recruitment of Csk. This inhibition leads to positive regulation of tyrosine kinase activity of Lyn,which leads to phosphorylation of downstream signaling molecules like LAT and PLCγ1 that results in calcium mobilization. This increase in calcium influx results in the activation of NFAT-transcription factor which ultimately leads to degranulation and cyotkine release which culminates in anaphylactic response (Fig 4A).

On the other hand, in PEP knockout (Fig 4B) and in L75N04 treated BMMC (Fig 4C), Csk is constitutively present in the lipid rafts since Cbp/PAG in these BMMC are phosphorylated under resting conditions. Antigen stimulation leads to higher recruitment of Csk to the lipid rafts causing greater negative regulation of Lyn which leads to reduced phosphorylation of downstream signaling molecules and thereby calcium mobilization and the downstream events are drastically affected which is reflected in the reduced susceptibility of PEP knock-out and L75N04 treated mice to systemic anaphylaxis.

Thus from, these studies it emerges that PEP is indeed a positive regulator of IgE-mediated mast cell signaling and seems to be a potential therapeutic target for autoimmune disorders with L75N04 compound being a starting point for the development of clinically useful PTP inhibitors

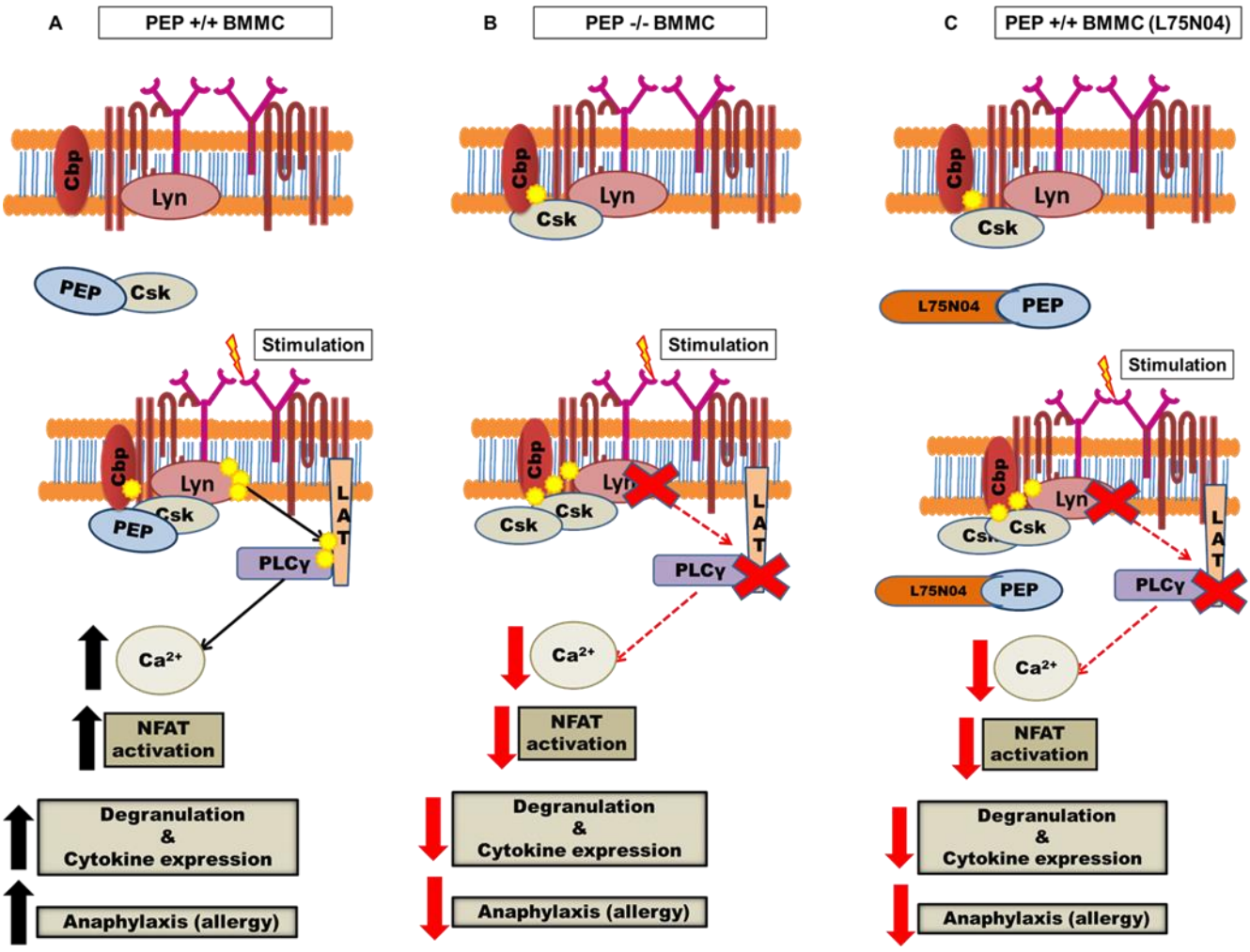


Fig 4 IgE-mediated FcεRI signaling pathway is positively regulated by PEP (A) In wild-type BMMC, PEP-Csk complex is recruited to lipid rafts after stimulation wherein PEP functions as Cbp/PAG phosphatase and Csk as negative regulator of Lyn. Dephosphorylation of Cbp/PAG by PEP inhibits further recruitment of Csk as a result Lyn gets activated and phosphorylates downstream signaling molecule LAT which results in the binding and activation of PLCγ1 which further leads to increase in calcium mobilization, NFAT activation, degranulation as a result, wild-type mice are susceptible to anaphylaxis. On the other side in **(B)** PEP knock-out BMMC and in **(C)** L75N04 treated wild-type BMMC, Cbp/PAG is already phosphorylated and recruitment of Csk to the lipid rafts is enhanced and this leads to increased negative regulation of Lyn which results in the inactivation of Lyn and thereby downstream signaling molecules are affected leading to reduced calcium mobilization is causing inactivation of NFAT and thereby downstream events like degranulation and cytokine production are affected, as a result PEP knock-out mice are less susceptible to anaphylaxis. PEP mediated repression of recruitment of Csk may account for the described activation of the mast cell signaling

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