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Molecular mechanisms of regulation of FcεRI signaling in mast cells

Molekulární mechanismy regulace FcεRI signalizace žírných buněk

Ph.D. Dissertation in Biochemistry

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Prague, 2016

Declaration:

This thesis was prepared at the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Laboratory of Signal Transduction. Experimental data were compiled into seven articles (six original, one review with original data).

I hereby declare, that presented thesis has been composed entirely by myself and all used sources are quoted. Neither presented thesis nor any part of it has been submitted to obtain any academic degree.

Prague, April 2016

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Monika Bambousková, M.Sc.

Declaration of co-authors:

This thesis contains seven original papers published in peer-reviewed journals. I, as a corresponding author of all these papers, hereby declare, that Monika Bambousková, a first author or co-author of the publications, substantially contributed to all of them. The studies were performed in our research team and she actively participated in all stages of the work from study design, performing experiments to writing manuscript. Her contribution in the experimental part of the first article "**Down-regulation of protein-tyrosine phosphatases activates an immune receptor in the absence of its translocation into lipid rafts**" published in Journal of Biological Chemistry, 2010, consisted of performing all studies of membrane proteins topography. In the second article "**What precedes the initial tyrosine phosphorylation of the high affinity IgE receptor in antigen-activated mast cells?**", published in FEBS Letters, 2010, she contributed to reviewing the literature and was responsible for all original data on topography of membrane proteins presented in this article. In the third article "**Cross-talk between tetraspanin CD9 and transmembrane adaptor protein non-T-cell activation linker (NTAL) in mast cell activation and chemotaxis**" published in Journal of Biological Chemistry, 2013, she was responsible for all data on topography of membrane proteins and effect of CD9 aggregation on cell spreading. In the fourth article "**Transmembrane adaptor protein PAG/CBP is involved in both positive and negative regulation of mast cell signaling**" Molecular and Cellular Biology, 2014, she prepared lentiviral constructs for overexpression of PAG1 in rescue experiments, and analyzed cell spreading and mast cell content *in vivo.* In the fifth article "**Negative regulatory roles of ORMDL3 in the FcεRI-triggered expression of proinflammatory mediators and chemotactic response in murine mast cells**" published in Cellular and Molecular Life Sciences, 2016, she contributed by analysis of cell spreading and NF-κB nuclear translocation in cells with decreased or increased expression of ORMDL3. In the sixth article "**Ethanol inhibits high-affinity immunoglobulin E (FcεRI) signaling in mast cells by suppressing the function of FcεRI-cholesterol signalosome**" published in PLoS One, 2016, she was responsible for characterization of activated and non-activated cells pretreated with ethanol and/or methyl-β-cyclodextrin; she examined surface LAMP1 expression and multivalent antigen-induced IgE-FcεRI complexes internalization. Experience she obtained during preparing the six articles that are part of this thesis and two methodological articles that are not included in the thesis, she used in the seventh publication "**New regulatory roles of galectin-3 in the high-affinity IgE receptor signaling**",

published in Molecular and Cellular Biology, 2016, in which she is the first author. In this publication she contributed to development and optimization of high-throughput protocol suitable for RNAi screening in mast cells, optimization of lentivirus shRNA delivery for RNAi screening in mast cells, selection and screen of the genes, and collection and analysis of all data. Based on the results she obtained she next examined in detail a new negative regulator of mast cell signaling, galectin-3 (Gal3); she determined the role of Gal3 in FcεRI-mediated degranulation by enzymatic assay and flow cytometry in cells with decreased or increased expression of Gal3 and investigated the role of Gal3 in calcium signaling, F-actin dynamics, phosphorylation of selected signal-transduction proteins, IgE internalization, ubiquitination of FcεRI and function of Gal3 in adhesion and motility.

Prague, April 2016

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RNDr. Petr Dráber, DrSc.

This thesis describes the work carried out at the Laboratory of Signal Transduction, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, during my Ph.D. study. All obtained experimental data during my Ph.D. studies were summarized in ten publications that were published in peer-reviewed journals. Seven of them are included in this thesis.

I would like to thank to my supervisor Petr Dráber for inspirational and motivating guidance and exceptional help during my studies. My many thanks belong to all my colleagues for their support and great work environment, particularly to Ivana Hálová, Viktor Bugajev, Lubica Dráberová, Iva Polakovičová, Lucie Potůčková, Pavol Utěkal, Magda Tůmová, Tomáš Paulenda, Hana Mrázová, Oleksii Redchenko, Filip Franko and Romana Budovičová. I am also very grateful to our collaborators from Center of Computational and Integrative Biology, Masachusets General Hospital and Harvard Medical School in Boston, MA, Ramnik J. Xavier, Agnès Gardet, Aivi Doan and Gautam Goel. Last but not least my thanks belong to my parents and family for their kind and endless support during my studies.

Monika Bambousková

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ABSTRACT

Mast cells are critical component of the immune system. In pathological situations, they are activated and are responsible for allergic reaction. Therefore, detail understanding of mast cell activation at molecular level is important for design of new therapies of allergic diseases. Principal transmembrane receptor of mast cells is the high-affinity Fc receptor for IgE (FcεRI). FcεRI anchors IgE on mast cell surface and upon cross-linking with multivalent antigen it becomes phosphorylated at its intracellular immunoreceptor tyrosine-based activation motifs (ITAMs). This triggers signaling cascade leading to cell degranulation and cytokine production. The antigen-mediated signaling through the FcεRI is critically dependent on interplay with intracellular protein-tyrosine kinases that phosphorylate the ITAM motifs and many other components of the signaling pathway. This study was focused on better understanding of signaling events leading to mast cell activation; emphasis was put on early activation events. First, we examined the role of protein-tyrosine phosphatases (PTP) in FcεRI phosphorylation. We found that upon antigen triggering of FcεRI, PTPs undergo inhibition by oxidation of their active site located tyrosine. Studies of plasma membrane topography of inactivated PTPs showed their proximity to FcεRI receptors and actin cytoskeleton. These and other data allowed us to postulate a new model of FcεRI signal initiation. We also investigated the role of selected proteins (tetraspanin CD9, transmembrane adaptor protein PAG, serine palmitoyl transferase regulator ORMDL3) or selected chemicals as ethanol and methyl-β-cyclodextrin on FcεRI signalosome properties in the course of mast cell activation.

Major aim of this work was to identify new regulators of FcεRI signaling. For this purpose we performed high-throughput screen using RNA interference technology and identified 15 regulators of mast cell activation. We chose galectin-3 (Gal3) for detail functional analysis of its action in FcεRI signaling pathway. Mast cells with reduced Gal3 expression showed increased antigen-mediated degranulation, calcium response and phosphorylation of several signal-transduction proteins. Although phosphorylation of the FcεRI was not affected, cells with Gal3 knockdown showed impaired IgE internalization that was accompanied by decreased receptor ubiquitination. Thus, we identified Gal3 as a critical regulator controlling FcεRI plasma membrane stability and trafficking.

An important role of mast cells in health and disease also depends on their migration. To this end we examined signaling pathways controlling cell movement. We found that Gal3 positively regulates fibronectin-mediated mast cell adhesion and motility but negatively affects antigen-mediated cell chemotaxis. Our data also showed that antigen-mediated mast cell chemotaxis was regulated by CD9, PAG and ORMDL3 proteins.

SOUHRN

Žírné buňky tvoří důležitou součást imunitního systému. Při patologických situacích jsou aktivovány a jsou zodpovědné za alergické reakce. Detailní poznání molekulárních mechanismů vedoucích k jejich aktivaci je proto důležité zejména pro vývoj nových postupů v léčbě alergických reakcí. Nejdůležitějším transmembránovým receptorem žírných buněk je vysokoafinitní Fc receptor pro IgE (FcεRI). FcεRI váže IgE na plasmatické membráně. Po prokřížení polyvalentním antigenem, imunoreceptorové tyrosinové aktivační motivy (ITAM) v intracelularní části receptrou jsou fosforylovány a spouští se signální dráha vedoucí k buněčné aktivaci. Signalizace přes FcεRI po vazbě antigenu je kriticky závislá na interakci s intracelularními protein-tyrosinovými kinasami, které zprostředkovávají fosforylaci ITAM motivů, a zároveň na mnoha dalších komponentách signalizační dráhy. Tato práce byla soustředěna na studium signalizačních procesů vedoucích k aktivaci žírných buněk na několika různých úrovních s důrazem na rané fáze signalizace přes FcεRI. Nejprve jsme se zaměřili na roli protein-tyrosinových fosfatas (PTP) při inicializaci fosforylace FcεRI. Podařilo se nám ukázat, že aktivace FcεRI antigenem vede k oxidaci tyrosinu v aktivním místě PTP a tudíž k jejich inhibici. Topografie inaktivních PTP na plasmatické membráně ukázala jejich bezprostřední blízkost k FcεRI a aktinovému cytoskeletu. Získaná data nám umožnila formulovat nový model inicializace signalizace přes FcεRI. Rovněž jsme se věnovali studiu dalších vybraných proteinů (tetraspaninu CD9, transmembránového adaptorového proteinu PAG a regulátoru serinových palmitoyl transferas ORMDL3) nebo působení vybraných chemikálií jako etanol a metyl-β-cyklodextrin na funkci FcεRI signalosomu během aktivace žírných buněk.

Hlavní náplní této práce bylo nalézt nové regulátory FcεRI signalizace. Za tímto účelem byl proveden “screen” s využitím RNA interference, kde jsme identifikovali 15 regulátorů aktivace žírných buněk. Pro detailní funkční analýzu byl vybrán galectin-3 (Gal3). Žírné buňky se sníženou expresí Gal3 vykazovaly zvýšenou degranulaci, vápníkovou odpověď a fosforylaci některých signálních protein po aktivaci antigenem. Přestože Gal3 neovlivňoval fosforylaci FcεRI jako takového, buňky se sníženou expresí vykazovaly defektní internalizaci IgE, doprovázenou sníženou ubikvitinylací receptoru. Gal3 byl tedy identifikován jako stěžejní regulátor řídící stabilitu aktivovaného FcεRI komplexu na plasmatické membráně a následného intracelulárního transportu.

Fyziologická funkce žírných buněk záleží také na jejich migraci. Dílčím cílem této práce bylo přispet k poznání signálních drah řídících pohyb žírných buněk. Ukázali jsme, že Gal3 pozitivně reguluje adhezi a motilitu na fibronektinu, zároveň však negativně ovlivňuje chemotaxi za antigenem. Získaná data rovněž napomohla objasnit funkci dalších proteinů (CD9, PAG a ORMDL3) v migraci žírných buněk.

Contents

1 ABBREVIATIONS

Ab	antibody
AC	adenylate cyclase
AF	alexa fluor
Ag	antigen
BMMC	bone marrow-derived mast cell
BMMCL	bone marrow-derived mast cell line
BSA	bovine serum albumin
BTK	Bruton's tyrosine kinase
cAMP	cyclic adenosine monophosphate
CBL	Casitas B-cell lymphoma
CBP	CSK-binding protein
CCL	C-C motif ligand
CD	cluster of differentiation
CSK	C-SRC tyrosine kinase
ECM	extracellular matrix
EE1	early endosome antigen 1
EP	E prostanoid
ERK	extracellular signal-regulated kinase
ERM	ezrin, radixin, moesin protein family
F-actin	filamentous actin
FcεRI	high-affinity Fc receptor for IgE
FcγR	Fc receptor for IgG
Gal3	galectin-3
GDP	guanosine diphosphate
GEF	guanosine exchange factor
G_i	inhibitory Gα subunit
GPCR	G protein coupled receptors
GRB2	growth factor receptor-bound protein 2
GTP	guanosine triphosphate
HSC	hematopoietic stem cell
HRP	horse-radish peroxidase
HTS	high-throughput screening

IL	interleukin
Ig	immunoglobulin
ITAM	immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
JNK	JUN amino-terminal kinase
KD	knockdown
KO	knockout
LAMP1	lysosomal-associated membrane protein 1
LAT	linker of activated T cells
LCK	leukocyte C-terminal SRC kinase
MAPK	mitogen-activated protein kinase
MβCD	methyl-β-cyclodextrin
MCP	mast cell progenitor
MMP	matrix metalloproteinase
MOI	multiplicity of infection
mTORC2	mTOR complex 2
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
ORMDL	ORM-like
oxPTP	oxidized protein-tyrosine phosphatase
PAG	phosphoprotein associated with glycosphingolipid-enriched microdomains
PAR2	protease-activate receptor 2
PCA	passive cutaneous anaphylaxis
PCR	polymerase chain reaction
PH	pleckstrin homology
PI3K	phosphatidylinositol-3 kinase
PKA	protein kinase A
PKC	protein kinase C
PLCγ	phospholipase Cγ
PTEN	phosphatase and tensin homolog
PTK	protein-tyrosine kinase
PTP	protein-tyrosine phosphatase
Pv	pervanadate
qPCR	quantitative PCR

RBL	rat basophilic leukemia
RNAi	RNA interference
ROCK	Rho-associated, coiled-coil-containing protein kinase
ROS	reactive oxygen species
RT-PCR	revers transcription PCR
SCF	stem cell factor
SDS	sodium dodecylsulfate
SFK	SRC family kinase
SH	SRC-homology
SHC	SRC homology 2 (SH2)-domain-containing transforming protein C
SHIP	SH2 domain-containing inositol 5-phosphatase 1
SHP	SH2 domain containing phosphatase
shRNA	short hairpin RNA
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
STAT	signal transducer and activator of transcription
SYK	spleen tyrosine kinase
TNF	tumor necrosis factor
TRAP	transmembrane adaptor protein
WT	wild type

2 INTRODUCTION

2.1 Mast cells

Mast cells are bone marrow-derived tissue-homing leukocytes that have been mainly studied as central players in allergic diseases. However recently, the field of mast cell physiology has expanded well beyond the boundaries of atopic disorders. Mast cells revealed to be versatile cells involved in a complex assortment of immunological and non-immunological functions that exert both direct and indirect effects on other cells and their functions in a variety of biological settings [1-3] . The principal mast cell immunoreceptor is high-affinity Fc receptor for immunoglobulin (Ig) E (FcεRI) and its engagement results in cell activation. Disruption of the fine-tuned processes of mast cell activation can lead to the onset of mast cell-driven diseases. Therefore detail comprehension of biochemical events connected to development and function of mast cells is needed for better design of a mast cell-targeted therapeutic strategies and for understanding of immune system homeostasis in general.

2.1.1 Origin and development

Still, only little is known about mast cell functional profile in a phylogenetic perspective. So far it seems that mast cell immune response has evolved to protect body not only against pathogens as parasites but also against toxins and as general inducer of inflammation. Potential phylogenic ancestors of vertebrate mast cells might have appeared in invertebrates, since some invertebrate granulocytes display features related to vertebrate mast cells [4]. Modern mast cells maintain the potential to phagocytose and kill pathogens, and can be activated by bacterial and viral antigens (Ags) through highly conserved pattern recognition molecules and complement receptors, which appeared during invertebrate evolution. Most important feature of modern vertebrate mast cells is content of large amounts of secretory granules filled with wide spectra of biological mediators. Storage of histamine in mast cell granules and its use as inflammatory mediator was probably established in primitive reptiles [5]. When the recombination activating gene 1-mediated adaptive immune system evolved in early vertebrates, mast cell progenitors were integrated into complex networks of adaptive immune responses [1,6,7]. Mast cells then began to cooperate with the new protagonists of the immune system: lymphocytes and dendritic cells.

They became capable of influencing dendritic cells by orchestrating their migration, maturation and function, and of interacting with T cells and B cells by synthesizing cytokines that control lymphocyte differentiation pathways [8]. Development of the FcεRI is a recent acquisition related to the appearance of IgE antibodies in mammals and contributed to defense against parasites, in the context of acquired immune responses. Expression of FcεRI [as well as several Fc receptors for IgG (FcγRs)] provided mast cells with a flexible instrument for fine-tuning in multiple inflammatory settings. Finally, mast cell persistence throughout the evolution of vertebrates indicates a strong selective pressure favoring their survival and indicate that these cells have beneficial health protecting roles.

Mast cells develop from cluster of differentiation (CD)34+/CD117+ pluripotent progenitor in bone marrow (Figure 1). In both, mouse and human, mast cell committed progenitors are released into the bloodstream and subsequently migrate into the peripheral tissues where they terminally differentiate under the influence of local chemical environment [9,10]. Murine bone marrow-deriver mast cells (BMMCs) are dependent on interleukin (IL)-3 and stem cell factor (SCF), thus during *in vitro* mast cell derivation from bone marrow

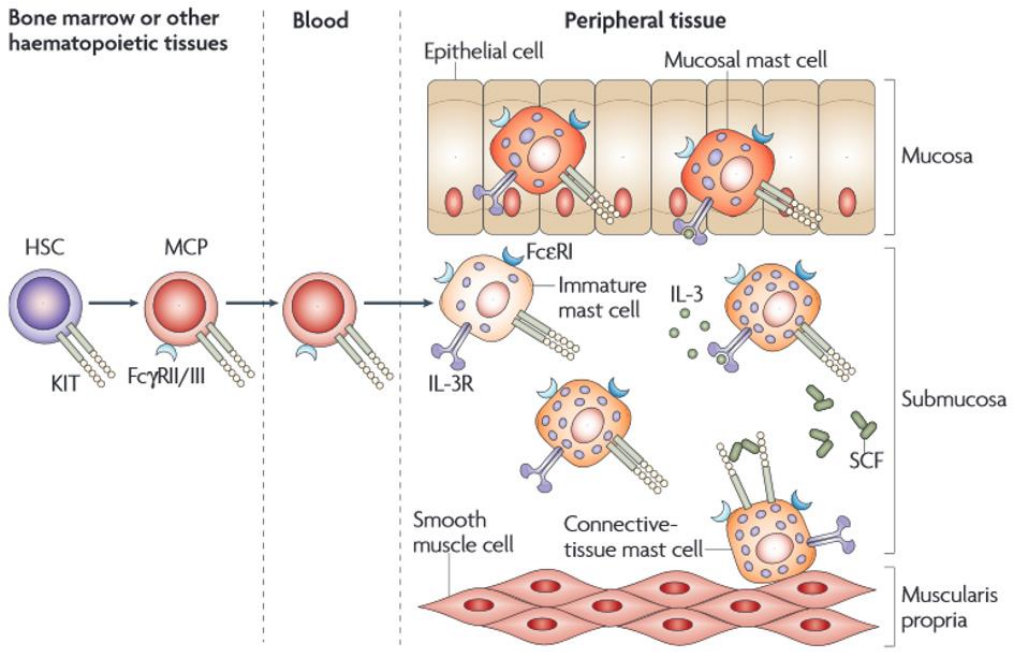

Figure 1. Development and tissue homing of mast cells. Mast cell progenitors (MCPs) develop from hematopoietic stem cells (HSCs) in bone marrow. MCPs migrate into blood and subsequently into homing tissues. There, the development to mature mast cells is finished under the influence of local chemical environment, as presence of IL-3 or SCF. At least two types of mature mast cells have been identified; mucosal mast cells and connective tissue mast cells. Adapted from [11].

these cytokines need to be supplemented. Migration and basal homing of mast cell progenitors is controlled in tissue specific manner. Mast cells are mainly localized in surroundings of blood vessels, in lung and intestines, and tissues close to barriers with external environment where they can be among the first cells that encounter pathogens. Mast cell recruitment is upregulated during inflammation and local accumulation of mast cells has been documented as a hallmark of some diseases [12,13].

2.1.2 Mast cell functions

Mast cells are armed with specialized granules containing an array of biological mediators as histamine, serotonin and proteases. These granulates can be released upon cell activation in process called degranulation [14]. Prototypic trigger of degranulation is recognition of Ag by IgE anchored to FcεRI [15]. Major function of mast cell granule components is summarized in Figure 2. Upon activation, mast cells are capable not only to release pre-stored mediators but also start *de novo* synthesis and production of cytokines [e.g. IL-6, IL-13, tumor necrosis factor (TNF)-α], chemokines [e.g. C-C motif ligand (CCL)3, CCL4], proteoglycans (e.g. heparin) and products of arachidonic acid metabolism (prostaglandins and leukotriens) [16].

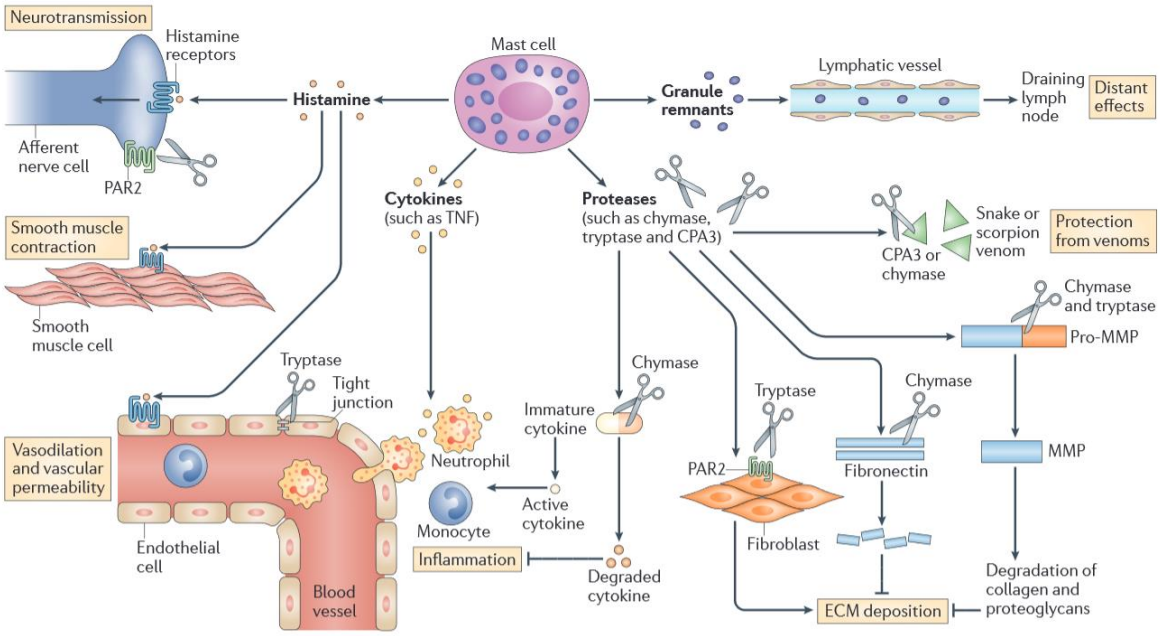

Figure 2. Function of biological mediators stored in mast cell granules. Degranulation of mast cells leads to release of pre-stored granules containing various pro-inflammatory mediators (histamine, cytokines and proteases). Histamine effects are mediated by binding to histamine receptors expressed e.g. on nerve cells,

stimulation of smooth muscle contraction and the induction of vascular permeability and vasodilation. The release of preformed cytokines, such tumor necrosis factor (TNF) contributes substantially to the pro-inflammatory response but there is also a marked contribution of cytokines and lipid mediators that are synthesized *de novo* upon mast cell activation (see main text). Mast cell proteases contribute to the inflammatory response, for example, through the activation of protease-activate receptor 2 (PAR2) or by inducing cytokine maturation through proteolysis. On the other hand mast cell proteases can have also anti-inflammatory activities, mainly through the degradation of pro-inflammatory cytokines and chemokines. Proteases can also affect shape of extracellular matrix (ECM). For example, mast cell proteases can be pro-fibrotic and promote ECM deposition by activating PAR2 on fibroblasts (thereby inducing collagen synthesis). Mast cell proteases can also promote ECM disassembly, either directly through the degradation of ECM components, such as fibronectin, or indirectly by proteolytic activation of matrix metalloproteinase pro-enzymes (pro-MMPs) that subsequently degrade ECM components, such as collagen and proteoglycans. Moreover released granules can be transported through the lymphatic system to the draining lymph nodes, thereby acting as carriers for bioactive compounds (such as TNF). Adapted from [16].

2.1.3 Phenotypic plasticity

Important aspects of mast cell phenotype can vary according to animal species, anatomical location, individual genetics and systemic or local changes in chemical environment [17,18]. At least three mast cell subtypes have been identified in human according to their content of mast cell-specific proteases: mast cells that contain tryptase only, mast cells that contain tryptase, chymase, carboxypeptidase, and cathepsin G, and mast cells that contain only chymase [19]. In rodents, two mast cell populations can be differentiated using morphological, biochemical, and physiological criteria: the connective tissue mast cells and the mucosal mast cells [20]. These mast cell subtypes are present at specific anatomical sites, may respond to different inducers and express fairly distinct functional profiles. Environmental and genetic factors finely control or “tune” many key characteristics of mast cell populations, such as their proliferation, survival and ability to store and/or produce various secreted products and the magnitude and nature of the secretory responses to specific stimuli of activation generated during innate or acquired immune responses [17]. Nevertheless, how the phenotype and function of differentiated mast cells intrinsically variate, either on the level of single cell or the population and what are the implications of such plasticity in health and disease still need to be addressed.

A chief role of mast cells in innate immunity is to enhance the local recruitment of neutrophils, a function that can either enhance host resistance or contribute to pathology

[21]. Although there are many examples of how changes in mast cell phenotype might influence the function of the cells of innate immunity, variation in the ability of mast cells to synthesize and store different proteases or proteoglycans is of particular research interest. Stored serine proteases and proteoglycans represent a substantial fraction of the mass of a mast cell, and individual mast cell subpopulations can store different mixtures of such proteases and proteoglycans. In mice, there is evidence that the ability of mast cells to release large amount of proteases (and perhaps heparin) permits these cells to enhance host resistance to the venoms of poisonous reptiles and arthropods and also to limit the toxicity of certain endogenous peptides, when exposure to such substances induces mast-cell degranulation [22,23].

2.1.4 Mast cells in health and disease

Mast cells are best known for their role in type-I hypersensitivity reactions [11,24]. Recent studies have highlighted their important functions in the innate immunity contributing to protection against parasitic and bacterial infection, angiogenesis, and autoimmunity [3,25,26]. The capacity of mast cells to promptly interact with the microenvironment and respond through the release of an array of biologically active mediators is a delicate balance where the inadequate regulation of mast cell functions can result in devastating effects to the organism. Under pathological conditions, mast cells are involved in immediate hypersensitivity, chronic allergic reactions, asthma, and other inflammatory diseases [13,26,27].

Recently, it has been shown that IgE and mast cells serve not only as the effectors of food anaphylaxis but importantly also as early inducers of Th2 cell responses and suppressors of regulatory T cell responses to food allergens and therefore they might provide an accessible therapeutic target by which to dampen such responses [28].

Single-nucleotide polymorphism association studies showed that chromosome 17q12-q21 region is connected to the risk of asthma and several other inflammatory diseases [29]. This locus contains gene encoding ORM-like (ORMDL)3. Although mast cells are known to be involved in asthmatic diseases, the role of ORMDL3 in mast cells is not known.

In vivo studies facilitating systemic studies of mast cell functions mainly use immediate dermal response called passive cutaneous anaphylaxis (PCA) in mice. In these

studies IgE-Ag interaction is characterized by increased permeability of vessels within the skin. To visualize and quantify the increased permeability characteristic of PCA, the intravenous injection of Evans blue, a dye that binds to and is transported with plasma albumin, is utilized. PCA can be used as a tool to study sensitivity to allergens and compound efficacy in ameliorating these responses [30].

2.2 The FcεRI signaling

2.2.1 IgE

IgE, as well as IgG, have been found exclusively in mammals. IgE has unique structure - although it shares basic molecular architecture as antibodies (Abs) of other classes, the heavy ε-chain contains one more domain than the heavy γ-chain of IgG. It was shown by crystal structure studies that native IgE structure adopts bend and compact conformation (Figure 3) [31].

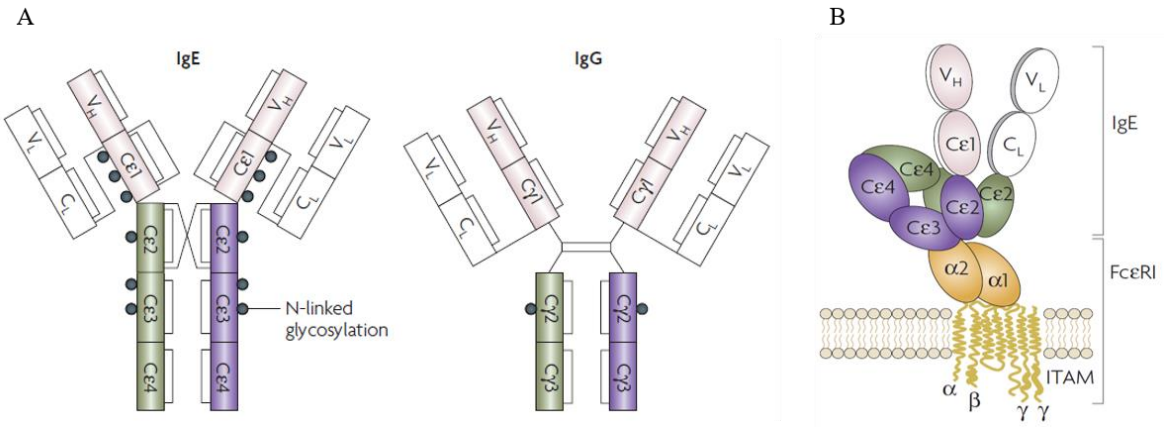

Figure 3. Structure of IgE. (A) Comparison of schematic domain structure of IgE and IgG; intra- and inter-domain disulphide bridges and the sites of N-linked glycosylation are shown. Variable (V) and constant (C) domains of heavy chain (filled) and light chain (white) are denoted. **(B)** Model of IgE structure bound to FcεRI; ITAMs in intracellular part of FcεRI are also shown. Adapted from [32].

Some recent studies discovered that Ag is not needed for certain IgEs to stimulate mast cell signaling. Such IgEs are termed "cytokinergic" and induce different kinetics of mast cell activation [33]. Some studies pointed out the contribution of reactive oxygen species (ROS) in downstream signaling upon treatment with cytokinergic IgE. Conformational isomerism of highly cytokinergic IgEs facilitates binding to structurally unrelated Ags, but if this is the basis of its activity on mast cell activation is not clear.

Similarly these effects could be driven by self-association of FcεRI-bound IgE. The true nature of action of cytokinergic IgEs on mast cell activation remains to be elucidated. Among FcεRI there are also other IgE binding molecules as galectin-3 (Gal3) or CD23. However, these molecules are not likely to mediate cytokinergic activity of IgEs [32].

New data showed that there is absolute requirement for IgE glycosylation in allergic reactions. The obligatory glycan is mapped to a single N-linked oligomannose structure in the constant domain 3 (Cε3) of IgE, at asparagine-394 (N394) in human IgE and N384 in mouse. Genetic disruption of the site or enzymatic removal of the oligomannose glycan altered IgE secondary structure and abrogated IgE binding to FcεRI, rendering IgE incapable of eliciting mast cell degranulation. It has been shown that Gal3 binds different IgEs with different affinity depending on the type of IgE glycosylation. To what extend this can affect allergic reaction is not known [34].

FcεRI receptors on dendritic cells and macrophages lack β-subunit and have been shown to contribute mainly to IgE clearance from the blood [35]. That is accomplished by fast FcεRI internalization and degradation in lysosomes. Interestingly, recently it has been shown that in human cells, IgE mediates internalization of tumor Ags and thus allows cross-presentation of tumor cell-derived peptides to cytotoxic T-cells [36]. These data shed more light on long time-known correlation in epidemiologic studies between increased levels of IgE and resistance to certain types of cancer. This research field now calls "allergooncology" [37].

Allergen-mediated reaction is one of the fastest immune responses. The success of the IgE-specific antibody, omalizumab (Xolair; Novartis Pharmaceuticals Ltd), in the treatment of asthma and other allergic diseases demonstrates the power of targeting IgE in the therapy [38].

2.2.2 FcεRI structure

FcεRI on mast cells and basophils belongs to family of multichain immune recognition receptors (MIRRs). It consists of an α-subunit, a β-subunit, and two disulfide-bridged γ-subunits (Figure 4, page 20) [39]. The α-subunit comprises of a short 17 amino acid cytoplasmic tail and its extracellular part binds to the constant Cε3 region of IgE by its Ig-like domain. The β-subunit belongs to the tetraspanin family and together with the γ-subunits it contains an immunoreceptor tyrosine-based activation motifs (ITAMs) in

cytoplasmic part [40]. FcεRI crosslinking by multivalent Ag causes tyrosine phosphorylation of ITAMs and triggers signaling events by binding to cytoplasmic proteins that contain phosphotyrosine-binding SRC homology (SH)2-domains [41]. FcεRI on murine mast cells always contains β-chain and main function of the β-chain is signal amplification [40]. The exclusive expression of the β-chain-containing FcεRI in murine cells is because all three chains (β and γ dimer) must be present to allow receptor transport to the cell surface. In contrast in human cells, expression of the β-chain is expendable for surface expression of FcεRI and can be expressed with or without β-chain [42]. Unlike the form of human FcεRI, which only comprises α and γ, β-chain-containing FcεRI shows enhanced FcεRI surface expression and stability as well as augmented stimulatory functions.

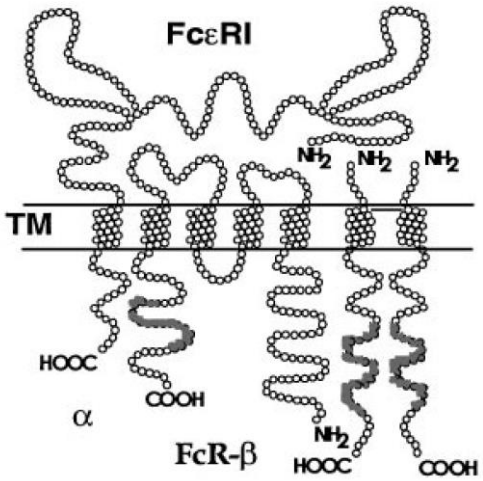

Figure 4. Structure of FcεRI. FcεRI is a transmembrane (TM) tetrameric complex formed by an IgE-binding α-subunit, a signal-amplifying β-subunit with ITAM in the cytoplasmic part (filled), and a homodimer of disulfide-linked γ-subunits with ITAMs. Adapted from [39].

2.2.3 FcεRI signaling pathways

When IgE-FcεRI complexes are crosslinked by multivalent Ag, receptor subunits β and γ are phosphorylated in their ITAMs by SRC family kinases (SFKs), presumably LYN kinase. It has been shown that the full activation of FcεRI mediates its translocation into specialized regions of the plasma membrane enriched in cholesterol and glycosphingolipid, contemporary called lipid rafts [43]. Upon FcεRI triggering membrane microdomains coalesce into larger and more stable structures where engaged receptors are concentrated [17], and can more easily interact with signaling molecules, such as active LYN [44]. Recent

advances in high-resolution microscopy technique and nanofabrication brought important new insights into the mechanisms of spatiotemporal relationship between LYN and FcεRI during activation [45]. How exactly is the phosphorylation initiated is however still not completely understood.

As shown in Figure 5, phosphorylated ITAMs serves as a docking sites for the tandem pair of SH2 domains of the cytoplasmic spleen tyrosine kinase (SYK) that is then activated by tyrosine phosphorylation [46]. When SYK is inhibited by specific inhibitors the FcεRI-mediated signaling is abrogated, therefore SYK has crucial function in activation of mast cells. Activated SYK mediates interaction with its many targets including the linker for activated T cells (LAT) that, once phosphorylated, recruits other SH2-containing adaptors such as SH-2 domain containing leukocyte protein of 76 kDa (SLP-76) and growth factor receptor-bound protein 2 (GRB2), and importantly also enzyme phospholipase Cγ (PLCγ).

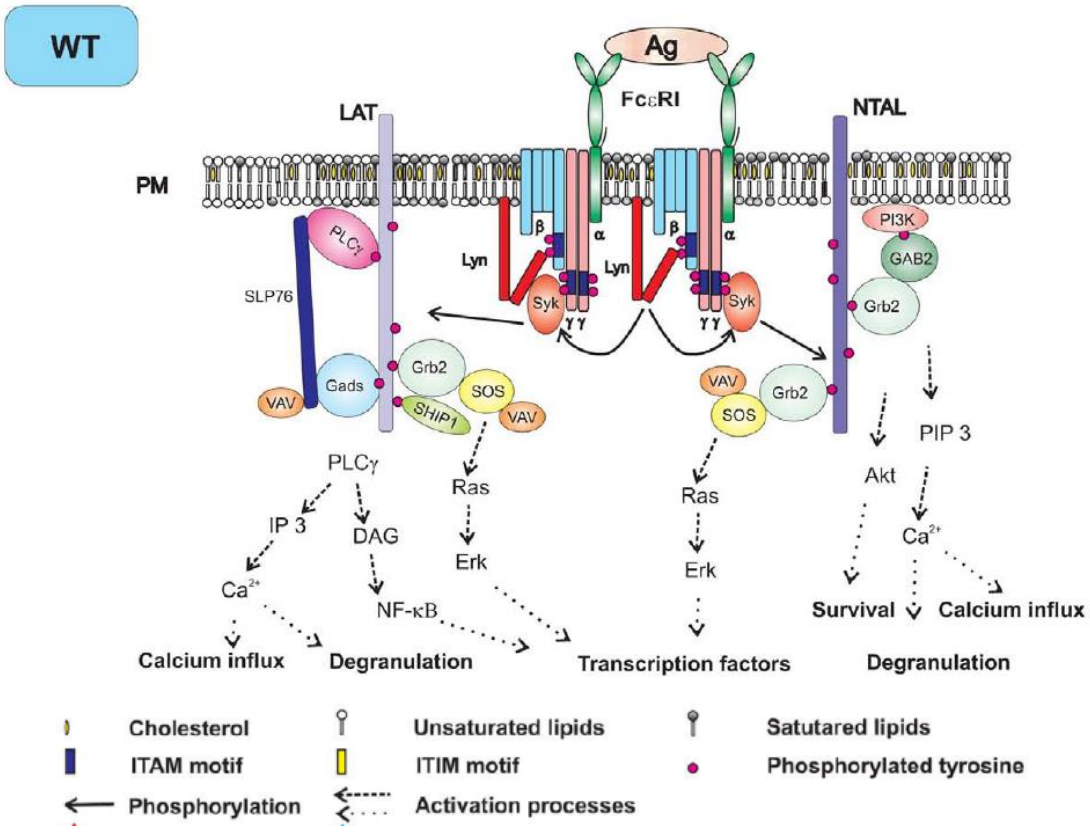

Figure 5. FcεRI signaling in wild type (WT) mast cells. When FcεRI is aggregated with multivalent Ag whole complex translocated to cholesterol-enriched plasma membrane (PM) microdomains β and γ subunits are phosphorylated by LYN kinase and subsequently SYK is recruited and activated by phosphorylation. SYK then mediates phosphorylation of transmembrane adaptor proteins LAT and NTAL. Phosphorylated tyrosines of LAT and NTAL further serve as docking sites for signaling proteins. Adapted from [47].

PLCγ undergoes activating tyrosine phosphorylation when associated with the membrane and then hydrolyzes the membrane phosphatidylinositol-4,5-bisphosphate to form the soluble inositol-1,4,5-trisphosphate and the membrane bound diacylglycerol, which are responsible for intracellular calcium mobilization and protein kinase C (PKC) activation, respectively. SLP-76 and GRB2 recruit guanosine exchange factors (GEFs) as SOS and VAV promoting the activation of the small GTPases, Ras, Rac, and Rho, that then regulate complex networks of signaling pathways leading to cell degranulation.

Another SFK, FYN kinase, can also cooperate with the LYN and SYK pathway in the propagation of FcεRI-mediated signaling. After FcεRI triggering, FYN is capable to facilitate membrane recruitment of the p85 regulatory subunit of phosphatidylinositol-3 kinase (PI3K). Once activated, PI3K catalyzes the formation of phosphatidylinositol-3,4,5-triphosphate that provides docking site for pleckstrin homology (PH) domain-containing proteins such as Bruton's tyrosine kinase (BTK) and PLCγ.

2.2.4 Negative feedback regulatory mechanism in FcεRI pathway

Mast cell degranulation in response to increasing Ag concentrations, follows a bell-shaped dose–response curve, characterized by weak responses at both low (sub-optimal) and high Ag (supra-optimal) concentrations [48]. Reduction of mast cell response after stimulation with supra-optimal Ag concentrations is the result of changes in the interplay of various signal transduction enzymes compared to lower Ag concentrations. Actin polymerization and associated signaling events are effectively supported by both supra-optimal as well as optimal Ag concentrations. Ag-induced actin polymerization is likely to be part of the inhibitory mechanisms at supra-optimal Ag concentrations [49].

SH2 domain-containing inositol 5-phosphatase 1 (SHIP1) has been considered to be the major "gatekeeper" of mast cell activation via catalyzing dephosphorylation of membrane phosphatidylinositol-3,4,5-triphosphate. Mast cells lacking SHIP1 show markedly increased degranulation and cytokine production [50]. Tyrosine sites of β and γ subunits of the FcεRI are dephosphorylated by protein-tyrosine phosphatases (PTPs) as SH2 domain containing phosphatase (SHP)-1 and SHP-2 and thus these enzymes regulate activation of SYK and the extent of mast cell response [46].

Other mechanism of receptor signaling downregulation is receptor endocytosis. Following crosslinking, FcεRI is rapidly internalized by either clathrin-dependent or

clathrin-independent pathways [51]. Interplay between these endocytic pathways is extensively studied and it is clear that there is strong relationship between receptor internalization and signal transduction [52]. Mast cell plasma membrane has multiple specialized domains for endocytosis. Communication between these domains might represent sorting stations that direct cargo to specific endocytic pathways [51]. Endocytic rate and route depend on the complex factors as ligand dose and aggregation state [53]. In some MIRR systems, endocytosis of ligated receptors results in downregulation and signal attenuation [54]. Noteworthy, mutations in receptor tyrosine kinase KIT, found in some mast cell cancers, leads to defective endocytosis and trafficking of the receptor which results in aberrant signaling [55].

Systematic investigation of intracellular trafficking of ligated FcεRI revealed membrane fusion between receptor-containing vesicles and endocytic compartment organelles. Each step is regulated by specific Rab proteins and phosphoinositides generated by the action of PI3K. After internalization from plasma membrane, FcεRI first enter the early endosome antigen 1 (EE1)-positive early endosomes and then traffics through the Rab5-positive late endosomes. Degradation of internalized FcεRI occurs in lysosomal-associated membrane protein 1 (LAMP1)-positive lysosomes. Interestingly kinetics of FcεRI internalization is not affected by SYK, but SYK can modify the rate of FcεRI accumulation and degradation in LAMP1-positive lysosomes [56].

Amplification of FcεRI signaling is also dependent on specific ubiquitin modifications of the activated receptor and cooperating kinases. These modifications are at least from part mediated by Casitas B-cell lymphoma (CBL) family proteins [57].

2.3 Other mast cell receptors

Mast cells express wide variety of surface receptors that either tune FcεRI-mediated activation or act individually to mediate mast cell responses. Some of the other mast cell receptors are e.g. complement receptors, toll-like receptors [58], receptors for neuropeptides and opioids [59], platelet-activating factor receptor, leukocyte Ig-like receptor 4 [60], CD200 receptor 1, CD300a [60], FcγRs [61]. In following text, some of the receptors most relevant to this work are described.

KIT receptor

KIT (CD117), receptor for SCF, is type III transmembrane receptor protein-tyrosine kinase and is expressed by all mast cell types. SCF was described as a potent effector of mast cell activation. SCF is a hematopoietic growth factor that promotes survival, proliferation, and differentiation of hematopoietic cells. Its extracellular part comprises of five Ig-like domains; first three domains bind SCF, the fourth is important for receptor dimerization and the fifth has unknown function. KIT has two catalytic domains separated by a kinase insert and inhibitory cytoplasmic juxtamembrane domain in the intracellular part. Binding of SCF to KIT leads to dimerization of the receptor and autophosphorylation of tyrosine residues (Tyr568 and Tyr570). Then, other residues are phosphorylated and serve as docking sites for SH2 domain-containing molecules [46]. Phosphorylated tyrosine residues of KIT recruit LYN and FYN kinases that leads to activation of PI3K-AKT and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways (Figure 6).

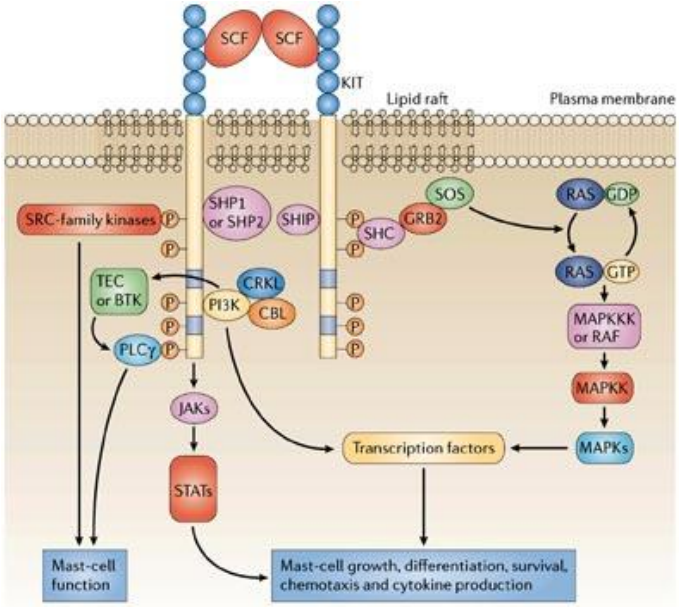

Figure 6. KIT receptor signaling in mast cells. SCF-mediated dimerization of KIT induces autophosphorylation at multiple tyrosine residues in the cytoplasmic tail, resulting in the recruitment of various molecules, including cytosolic adaptor molecules, such as SRC homology 2 (SH2)-domain-containing transforming protein C (SHC) and GRB2; SFKs; and signaling enzymes PLCγ and PI3K. Subsequent activation of these signaling enzymes, as well as the JAK/STAT pathway and the RAS/RAF/mitogen-activated protein kinase (MAPK) pathway leads to mast cell growth, differentiation, survival, chemotaxis and cytokine production. After FcεRI triggering the cascade that leads to activation of the MAPKs extracellular-signal-regulated kinase (ERK)1 and ERK2 is known to be regulated by RAF, the pathways by which KIT regulates

the MAPK kinases (MAPKKs) and the MAPKK kinases (MAPKKKs) that mediate p38 and JUN amino-terminal kinase (JNK) activation in mast cells are less well-defined. Adapted from [62].

Kit gene is frequently used for deletion of mast cells in various mouse models for *in vivo* studies. Despite being indispensable in studies of mast cell physiological functions, KIT expression is not entirely exclusive for mast cells and therefore these models have certain limitations [63].

G protein coupled receptors (GPCR)

GPCRs are 7-*trans*-membrane receptors, which are coupled to heterotrimeric GTP-binding proteins (G proteins). G proteins exist as a complex of Gα and Gβγ subunits. After ligand binding, Gα exchanges guanosine diphosphate (GTP) for guanosine triphosphate (GDP) and detaches from the Gβγ dimer and each activated component interacts with different effectors to initiate various cellular responses [64]. Different Gα subunits can either activate or inhibit (G_i) adenylate cyclase (AC) and therefore promote or decrease production of cyclic adenosine monophosphate (cAMP). Mast cells express various GPCR as sphingosin-1-phosphate receptor [65], adenosine receptors or receptors for complement components [66]. Prostaglandin E_2 (PGE_2) is a potent mast cell chemoattractant and acts through E prostanoid (EP) receptors. PGE_2 acts on four distinct GPCRs - EP1, EP2, EP3, and EP4 which are differ in their Gα subunits and associated signaling pathways and can trigger diverse cellular responses. As EP1–4 may mediate opposing responses, the overall cellular response elicited by PGE_2 depends on the expression pattern of these receptors. Activation of GPCR on mast cells results in cAMP/protein kinase A (PKA)-dependent interference with FcεRI-induced signals and calcium responses (Figure 7, page 26). It was shown that inhibitory effect of PGE_2 on mast cell degranulation is mediated by EP2 [67]. On the other hand, PGE_2 stimulation via EP3 receptors potentiates mast cell Ag-mediated responses and acts as a potent mast cell chemoattractant. Thus, net effect of PGE_2 on mast cell physiology depends strictly on the presence of particular receptors. BMMCs cultivated *in vitro* in presence of SCF and IL-3 express mainly EP3 [68,69].

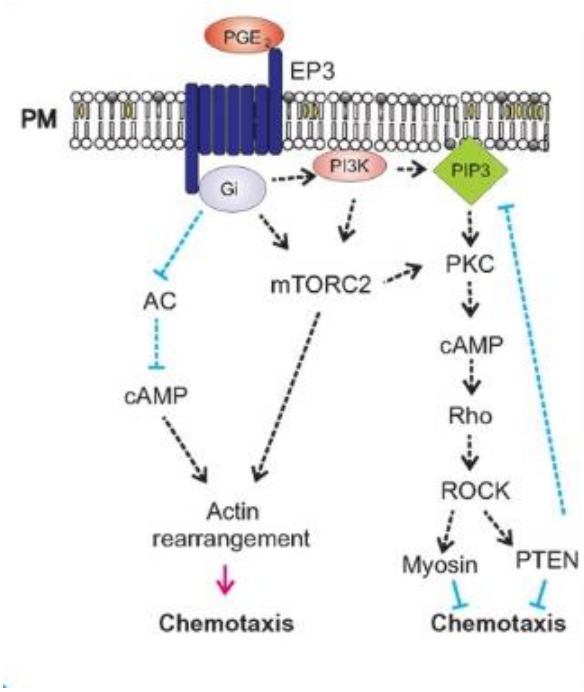

Figure 7. PGE_2 signaling leading to mast cells chemotaxis. In mast cells PGE_2 signals via G_i-coupled EP3 receptor. After activation the α-subunit of G-protein inhibits adenylate cyclase (AC) and results in a decrease of cAMP which stimulates cell migration. This stimulation also results in activation of mTORC2, an important regulator of actin rearrangements and positive regulator of chemotaxis. mTOR complex 2 (mTORC2) can influence PKC-dependent activation of small GTPase Rho and subsequent activation of phosphatase and tensin homolog (PTEN). This can act as a negative feedback for production of PIP3 by PI3K that is also activated by PGE_2 in mast cells. Adapted from [70].

2.4 Transmembrane adaptor proteins (TRAPs)

TRAPs serve as plasma membrane docking sites for cytoplasmic signaling molecules, or as anchors of cytoskeletal components to the plasma membrane. TRAPs are characterized by a short extracellular domain, a single transmembrane domain, and a cytoplasmic tail, which has no intrinsic enzymatic activity but possesses various tyrosine-containing motifs and domains. Extracellular domains are composed of only few amino acids and are therefore unlikely to function as receptors for extracellular ligands. The properties of the transmembrane domains and the presence or absence of juxtamembrane palmitoylation motif, CXXC, determine the solubility of TRAPs in non-ionic detergents, distribution in the plasma membrane, and some other functional properties [71]. In mast cells, individual tyrosine residues in the cytoplasmic tail of the TRAPs are initially phosphorylated by protein-tyrosine kinases (PTKs) of the SRC family and/or SYK kinase and contribute to the function of the TRAPs as molecular scaffolds recruiting downstream effector proteins. Five

TRAPs have been identified in mast cells: LAT, non-T cell activation linker (NTAL), linker for activation of X cells (LAX, X indicates “to be defined”), phosphoprotein associated with glycosphingolipid-enriched membrane microdomains (GEMs) (PAG), and GRB2-binding adaptor protein, transmembrane (GAPT) [47].

LAT and NTAL have high sequence similarity and are only co-expressed in mast cells. That provides unique opportunity to study detail function of these proteins, since mast cells from LAT knockout (KO), NTAL KO or double KO can be obtained. One of the known differences between LAT and NTAL signaling is that NTAL does not poses PLCγ-binding site (Figure 8) [72].

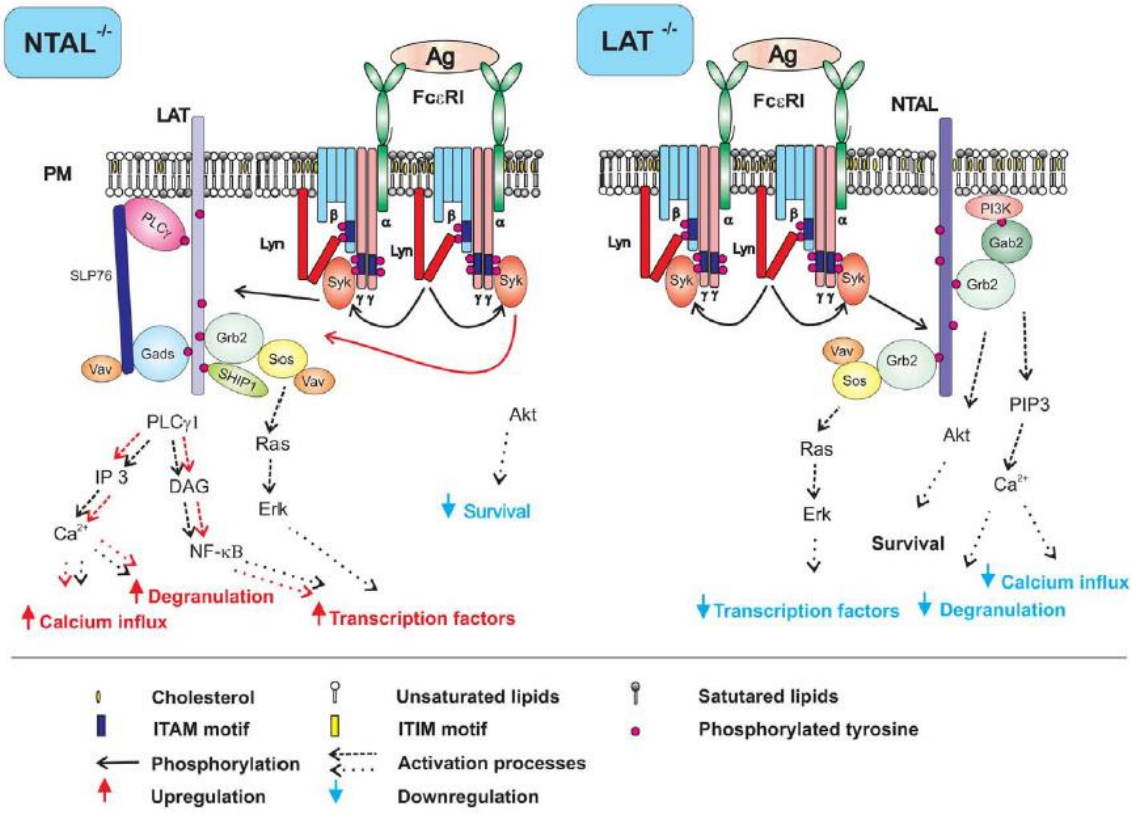

Figure 8. LAT and NTAL TRAPs in mast cell signaling. Phosphorylated NTAL binds GRB2 and some other signaling proteins, except for PLCγ1. However, even in the absence of binding to PLCγ1, NTAL can modulate calcium signaling, degranulation, and cell survival through in direct cross-talk with PI3K activation pathway. In activated NTAL KO (NTAL -/-) cells, an important SYK substrate (NTAL) is missing and LAT is therefore more phosphorylated. This leads to enhanced binding and activity of PLCγ1 and all subsequent PLCγ-dependent events. However, NTAL-dependent activity of AKT is impaired and this could lead to decreased cell survival. Inactivated LAT KO (LAT −/−) cells, decreased calcium influx, degranulation, and production of transcription factors are due to the absence of PLCγ1 anchored to LAT. The remaining weak calcium response, degranulation and production of transcription factors is based on NTAL-mediated activation events. Adapted from [47].

FcεRI-LAT-PLCγ signaling axis is major controller of Ag-mediated activation. Therefore, mast cells lacking LAT display decreased activatory characteristics. Conversely, mast cell lacking NTAL showed upregulated degranulation and calcium signaling [47,73]. Interestingly, despite high sequence similarity of these two TRAPs, they localize to different membrane microdomains, that indicates their non-redundant function. Also, NTAL was recently shown to regulate mast cell spreading through activation of Rho-GTPases [74].

PAG, also termed C-SCR tyrosine kinase (CSK)-binding protein (CBP), was first described in 2000 in two studies analyzing a GEMs-associated tyrosine-phosphorylated protein of 80 kDa in cell line Raji [75] and CSK co-precipitating protein of 80–90 kDa from neonatal rat brain [76]. Like other TRAPs, PAG has a short extracellular domain (16 amino acids), a transmembrane domain followed by palmitoylation site, and a cytoplasmic tail containing 10 tyrosines. Furthermore, it has two proline-rich sequences which serve as binding sites for SH3 domains. PAG is a substrate for leukocyte C-terminal SRC kinase (LCK) and FYN but not for Zap-70 or SYK. PAG phosphorylated on tyrosine 317 (human) or tyrosine 314 (mouse) binds CSK via its SH2 domain. Membrane anchored CSK then phosphorylates C-terminal tyrosines of SFKs in the vicinity of PAG, and thus inactivates them. In T cells, activation through the TCR leads to a rapid PAG dephosphorylation, which results in the release of CSK from the membrane and relieve of CSK-mediated inhibition of SRC kinases.

2.5 Role of cytoskeleton in mast cell activation

Actin cytoskeleton has been implicated at early FcεRI-mediated signaling events as well as at later steps leading to degranulation and/or cell migration [77]. In initial biochemical studies aggregation of the FcεRI caused rapid decrease in the amount of filamentous (F)-actin followed by its subsequent increase exceeding the levels typical for non-activated cells [78,79]. Other studies with permeabilized mast cells where F-actin was detected with fluorescently labeled phalloidin confirmed transient changes in F-actin content [80]. Interestingly, IgE binding itself has been shown to increase the content of cellular F-actin [81,82]. Redistribution of actin filaments in activated mast cells is an essential step leading to morphological changes such as formation of membrane ridges, microvilli and cell

spreading. F-actin outlines the cell surface lamellae, which indicates that F-actin complexes are mostly associated with the plasma membrane [74,79].

Most of the experiments aiming at better understanding of the role of F-actin in mast cell signaling utilized drugs which interfere with F-actin formation, namely latrunculin B and cytochalasins. Pretreatment of mast cells with these drugs enhanced Ag-induced tyrosine phosphorylation of the FcεRI β and γ subunits. Latrunculin B alone also caused a small increase in tyrosine phosphorylation of FcεRI subunits. This suggests that microfilaments also regulate the level of receptor tyrosine phosphorylation in resting cells [80]. Actin filaments are involved in the regulation of the kinetics of Ag-mediated tyrosine phosphorylation. Some data indicate that actin filaments are important in limiting the lifetime of FcεRI-LYN interactions [83,84]. However, treatment of mast cells with cytochalasin D or latrunculin A prior to FcεRI aggregation failed to cause any increase in the amount of LYN kinase associated with FcεRI [85].

Detailed studies investigating real-time kinetics of actin and FcεRI in mast cells revealed receptor movement within micron-sized membrane domains defined by actin bundles; this confinement was dynamic over length-scales of microns and time-scales of seconds. The diffusion of FcεRI complexes decreases within seconds after addition of the receptor crosslinking agent [86].

2.6 Mast cell chemotaxis

Chemoattractant-directed migration of mature mast cells or their progenitors might be one of the key mechanisms responsible for local accumulation of these cells under physiological or pathological conditions [70]. Chemotactic mechanisms can be therefore potentially targeted by therapeutic interventions.

The key signal for mast cell homing and recruitment into peripheral tissues is provided by interaction of the SCF with its receptor, the KIT [87]. In rodents another critical factor, IL-3, which binds to its surface receptor is involved [88]. In peripheral tissues mast cell progenitors mature and terminally differentiate under the influence of the local environment. Migration of mast cell progenitors to the site of their residency is directed by various chemokines which bind to chemokine receptors [89]. The expression pattern of these molecules is different in progenitors and mature mast cells and differences are also observed between particular mast cell subtypes [90]. Several other chemotactic ligands affecting mast

cell migration have been identified; these include sphingosine-1-phosphate [65], arachidonic acid metabolites as leukotriene B4 [91], and prostaglandin E_2 (PGE_2) [68,92], as well as Ag, recognized by IgE anchored to FcεRI (Figure 9) [93,94].

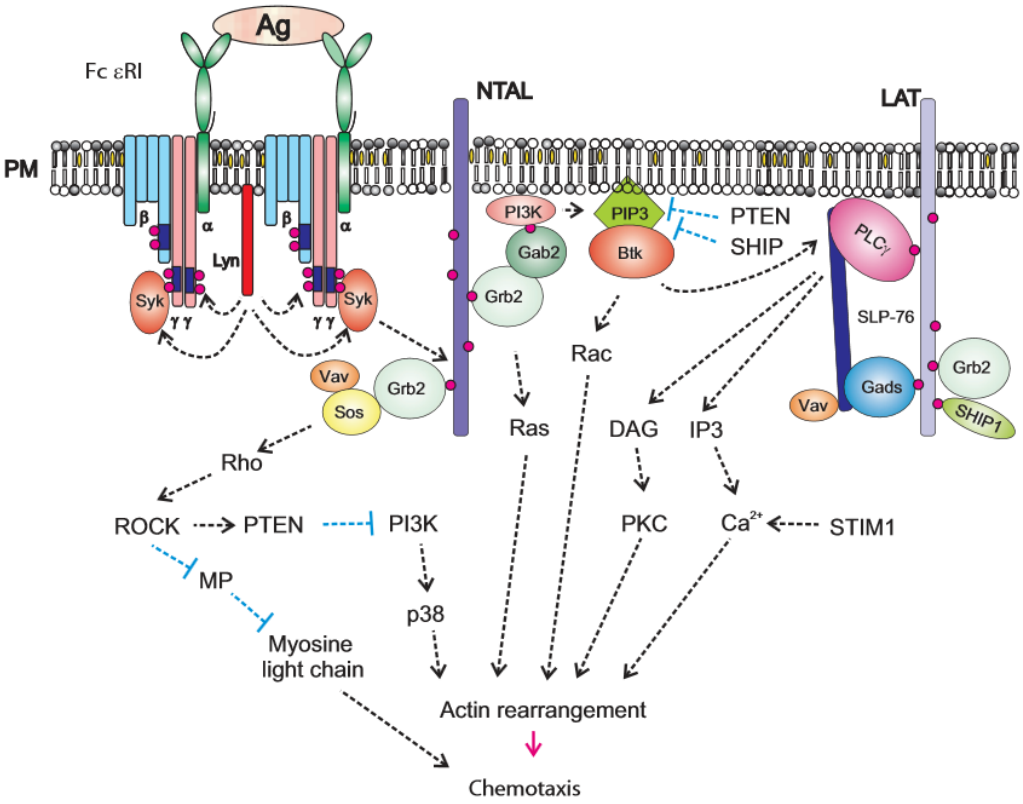

Figure 9. FcεRI-mediated mast cell chemotaxis. Downstream signaling pathways leading to mast cell migration include activated PI3K, activation of PLCγ, resulting in calcium release and actin rearrangement. Another PI3K-dependent pathway contributes to activation of p38 and consequently to enhanced chemotactic response. GRB2 orchestrates activation of Ras by recruiting small GTPases Ras and Rho family GEFs, SOS, VAV, and other signaling molecules, resulting in actin rearrangement and chemotaxis. NTAL could play a negative regulatory role in chemotaxis through activation of Rho/Rho-associated, coiled-coil-containing protein kinase (ROCK) pathway that is responsible for controlling the rear edge of the migrating cell. ROCK could also activate the PTEN phosphatase which inhibits activity of PI3K and in this way decreases the PIP3 levels. Adapted from [95].

In target tissues mast cells participate actively in immune responses against various pathogens by releasing a broad spectrum of mediators, either stored in secretory granules or rapidly synthesized after cell triggering. Mediators produced by mast cells include leukotrienes, prostaglandins, cytokines, and chemokines, which in turn can recruit other immune cells to sites of inflammation [96,97]. Importantly, mature mast cells in sensitized individuals exposed to allergens produce mediators that attract mast cell-committed progenitors to the place of pathogen entry [91]. In healthy tissues the number of mast cells

is stable and can be regulated by proliferation, migration, and mast cell survival. On the other hand, inflamed tissues show an increased number of mast cells, and their enhanced accumulation is characteristic for a number of pathological states [12,98].

3 AIMS

The detail molecular mechanisms leading to mast cell activation are still incompletely unerstood. Major aim of this work was to study signaling pathways induced by FcεRI aggregation with main focus on signaling events associated with plasma membrane. Among these challenges, a specific effort was made to identify novel regulators of FcεRI signaling using the RNA interference (RNAi)-based high throughput screening (HTS) techniques and to elucidate the role of identified regulator Gal3 in context of mast cell activation.
This study included the following specific aims:

1. **To elucidate the role of PTPs in initiation of FcεRI signaling**
    a. Map the topography of redox-regulated PTPs involved in initiation of FcεRI signaling.
    b. Analyze the spatiotemporal relationship of redox-regulated PTPs and FcεRI signalosomes.
    c. Analyze spacial relationship of oxidized PTPs (oxPTPs) and actin cytoskeleton.
    d. Evaluate current models of FcεRI signal initiation with respect to obtained data.

2. **To determine the mechanism of CD9-mediated mast cell activation**
    a. Analyze spationtemporal relationship of CD9 and components of FcεRI signalosomes.
    b. Analyse the role of CD9 in mast cell spreading on fibronectin.

3. **To evaluate the role of PAG adaptor protein in regulation of FcεRI signaling**
    a. Prepare lentiviral constructs encoding Myc-tagged PAG.
    b. Determine plasma membrane topography of PAG and its relationship to FcεRI signalosomes.
    c. Analyze spreading on fibronectin in PAG KO cells.
    d. Analyze mast cell content *in vivo* in PAG KO mice.

4. **Characterize the function of ORMDL3 in mast cells**

a. Analyze the spreading on fibronectecnin of the BMMC with ORMDL3 knockdown (KD) or ORMDL3 overexpressors
b. Set and perform image analysis of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) translocation to nucleus in BMMC with ORMDL3 KD or ORMDL3 overexpressors.

5. Analyze the effect of ethanol on mast cell activation

a. Identify the effect of ethanol treatment on expression of mast cell receptors FcεRI and KIT by flow cytometry.
b. Analyze the degranulation of BMMCs treated by ethanol by measurement of surface LAMP1.
c. Analyze the effect of ethanol on FcεRI internalization

6. To find new regulators of FcεRI signaling using RNAi screening

a. Develop and optimize high-throughput assays suitable for RNAi screening in mast cells.
b. Optimize lentivirus short hairpin RNA (shRNA) delivery for RNAi screening in mast cells.
c. Select and screen the genes with incompletely understood functions in mast cell activation, collect and analyze the data and identify new regulators of FcεRI signaling.
d. Study molecular mechanism of action of newly identified regulator Gal3.
    i. Analyze the role of Gal3 in degranulation by enzymatic assay and by flow cytometry in mast cells with Gal3 KD.
    ii. Prepare the contsructs for Gal3 overexpression and perform flow cytometry analysis with Gal3 overexpressing mast cells.
    iii. Determine Gal3 role in calcium signaling.
    iv. Analyze F-actin levels in BMMCswith Gal3 KD.
    v. Determine Gal3 role in early activation events by phosphoprotein analysis (microscopy and western blot).
    vi. Set the flow cytometry assay for analysis of IgE internalization by acid strip that would facilitatate the studies of Gal3 function in FcεRI internalization.

vii. Set the microscopy assay for analysis of IgE internalization with subsequent image analysis quantification facilitating the stuies of Gal3 role in FcεRI internalization.

viii. Analyze Gal3 function in FcεRI phosphorylation and ubiquitination.

ix. Asses the Gal3 function in mast cell adhesion and motility on fibronectin.

4 SUMMARY OF METHODS

All methods used to solve the particular aims of this study are described in more details in the result sections in the relevant publications.

Mast cell cultures. BMMCs were obtained from femurs and tibias of 6-8 weeks old mice. Cells were cultured in media containing murine recombinant SCF and IL-3 for 8 weeks and then analyzed for surface expression of FcεRI and KIT. The purity of mast cell cultures was usually more than 95%. BMMC-derived mast cell line (BMMCL) was obtained from Dr. M. Hibbs from Ludwig Institute for Cancer Research, Melbourne, Australia and cultured in media supplemented with murine recombinant IL-3.

Transient transfection. BMMCs were transfected using Amaxa nucleofector. Functional assays were than performed usually 48h post-transfection.

Lentiviral transduction. Lentiviral particles containing shRNA sequences or sequences of proteins of interest were packaged using HEK293T cells. Briefly, HEK293T cells were transfected with ViraPower Lentiviral Packaging Mix (Invitrogen) and lentiviral plasmid of interest using polyethylenimine. Viral particles were collected in cell supernatants 48h and 72h post-transfection. BMMCs or BMMCL were then transduced with lentiviral particles in presence of protamine at concentration 10μg/ml for 72h. After that, cells were transferred into fresh media containing puromycin for selection of positively transduced cells. Functional assays were usually performed at least 2 weeks after the start of selection.

Mast cell activation. For Ag-mediated mast cell activation cells were sensitized with IgE overnight in absence of SCF and IL-3. Then, unbound IgE was washed away and cells were activated with Ag and subjected to downstream applications.

β-glucuronidase assay (degranulation). Mast cell degranulation was measured by enzymatic assay for detection of β-glucuronidase released from cell granules into media

during activation. Briefly, cell-free supernatants form activated or control cells were mixed with 4-methylumberylliferyl-β-D-glucuronide substrate and incubated at 37ºC for 1h. Total β-glucuronidase content in samples was determined in Triton-X100 cell lysates. Conversion of substrate into fluorescent product was measured in plate reader at 355 nm excitation and 460 nm emission wavelengths. Degranulation was expressed as percent of β-glucuronidase released into the supernatants from total β-glucuronidase content. Cell degranulation can be already well detected after 5 min of challenge with Ag, in most of the experiments in this study the cells were activated for 30 min.

Calcium measurement. IgE-sensitized cells were loaded with Fura-2AM for 30min in presence of probenecid. After incubation excess of Fura-2AM was washed out and cells were activated in fluorimeter equipped with automated injector. Fluorescent ratio 340/360 nm was monitored at least for 5 min.

Western blot. Whole cell lysates were prepared by sonication of the cells in Laemmli system containing 2-merkaptoethanol and denaturation at 95ºC for 5 min [99]. Proteins in cell lysates were resolved at 10 – 12% sodium dodecylsulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. For most of used Abs, membranes were blocked in buffer containing 2% bovine serum albumin BSA and then incubated with primary Ab in buffer containing 1.5% BSA for 1h or overnight incubation. Primary antibody was detected with horse radish peroxidase (HRP)-conjugated secondary antibody and signal was developed with chemiluminescent reagent. Chemiluminiscence was measured by Luminescent Image Analyzer LAS 3000 (Fuji Photo Film) and quantified with Aida software (Raytest GmbH).

Immunoprecipitation. FcεRI complexes were immunoprecipitated via bound IgE. Protein A-coated beads were incubated with rabbit anti-mouse IgE serum for 2h at 4ºC. Cells were lysed in lysis buffer containing 0.2% Triton-X100 and IgE-FcεRI complexes were immunoprecipitated by incubation with Ab-coated beads for 2h at 4ºC. Beads with captured complexes were then washed and IgE-FcεRI complexes were released by denaturation in non-reducing sample buffer and finally incubated at 95ºC for 5 min.

Confocal microscopy. Samples for confocal microscopy were prepared using multiwell microscopy slides. Cells were attached to glass surface for 30 min at 37°C, then activated or not and fixed in 3% paraformaldehyde. Cells were then permeabilized in 0.1% Triton-X100 (or lysophosphatidylcholine for F-Actin staining) and blocked with 1% BSA. Samples were incubated with various primary Abs in 1% BSA of with alexa fluor (AF)-conjugated phalloidin for detection of F-actin. Primary Abs were detected by appropriate secondary Abs conjugated to various fluorophores. Samples were mounted in glycerol-based mounting media containing Hoechst 33258 stain. Confocal laser scanning microscope (Leica TCS SP5) equipped with an ×63 (numerical aperture, 1.4) oil immersion objective system was used for image acquisition.

Electron microscopy. Rat basophilic leukemia (RBL) cells were grown on glass coverslips, alternatively non-adherent BMMCs were attached to fibronectin-coated coverslips. In some experiments extracellular Ags were labeled with primary Abs followed with gold-conjugated secondary Abs. Nickel electron microscopy grids were glow discharged in argon atmosphere and coated with poly-L-lysine. Cells attached to coverslips were pressed towards the coated grid and ripped. Plasma membrane sheets were rinsed, fixed and subjected to labeling with specific primary Abs and gold-conjugated secondary Abs. Whole samples were then fixed in glutaraldehyde and stained with OsO_4 in cacodylate buffer, followed by tannic acid and finally with uranyl acetate. Images were acquired using FEI Morgagni at 56000x magnification.

Image analysis. Most of the image analysis was performed using FIJI or pipelines built in CellProfiler software (Broad Institute, Boston, MA) that facilitates analysis of large datasets from HTS experiments.

Flow cytometry. For analysis of surface Ags, cells were kept on ice and labeled with appropriate fluorophore-conjugated Abs. For analysis of intracellular Ags, cells were fixed in 2% paraformaldehyde and permeabilized in 0.1% Triton-X100. After blocking with 1% BSA, intracellular Ags were detected with specific Abs followed by fluorophore-labeled secondary Abs. In experiments measuring IgE internalization, cells were activated and split

into two parts. One halve was subjected to acid strip to remove surface-accessible IgE. All samples were then fixed, permeabilized and stained with AF-conjugated mouse anti-Ig Ab. Extent of IgE internalization was expressed as percent of IgE detected in acid stripped samples from total content in non-acid stripped samples. Samples were acquired using LSRII flow cytometer (BD Biosciences) equipped with HTS loading system and data were analyzed in FlowJO sowtware (TreeStar, Ashland, OR).

Motility. BMMCs were attached to fibronectin-coated glass bottom 96-well microscopy plate. Cells were activated with PGE_2 and monitored for 1h using Scan^R System (Olympus). Images were taken in 1 min intervals. Analysis of cell movement using automated tracking was performed in Metamorph software (Molecular Devices).

Cell spreading. BMMCs were attached to fibronectin-coated glass bottom 96-well microscopy plate. Cells were then activated for 30 min with various activators and activation was stopped by fixation in 3% paraformaldehyde. Cells were then permeabilized in lysophosphatidylcholine and stained with AF-conjugated phalloidin to detect F-actin. Samples were kept in PBS supplemented with Hoechst 33258 stain to visualize nuclei. Samples were acquired using Scan^R system (Olympus).

Cell adhesion. BMMCs were loaded with calcein dye and let to attach to fibronectin coated wells in fluorometric 96-well plate. Cells were activated with Ag for 30 min and fluorescent signal was measured. Cells were then subjected to 3 vigorous washes with PBS and fluorescent signal was measured again. Cell adhesion was expressed as percent of fluorescent signal detected after washing from total signal before the wash.

Cloning. Several mammalian expression vectors were prepared during completion of this study. cDNA of protein of interest was obtained from vendor, kindly provided by other researchers or amplified from BMMC cDNA. cDNA was then inserted into pCMV-based vectors or pCDH-based lentiviral vectors containing various protein tags using standard cloning techniques. All constructs were verified by sequencing.

RNA isolation, reverse transcription polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR). RNA from BMMCs or BMMCLs was isolated using an RNeasy mini kit (Qiagen) when single samples were isolated. For RNA isolation from 96-well plates, Macherey Nagel kit for 96-well extraction was used according to the manufacturer's instructions. RNA was reverse transcribed using M-MVL reverse transcriptase (Invitrogen). qPCR reactions were performed in 384-well plates in a LightCycler 480 (Roche Diagnostics). Genes for glyceraldehyde-3-phosphate dehydrogenase, actin, and ubiquitin were used as reference genes, and expression levels of all mRNAs were normalized to the geometric mean of the expression of the reference genes.

5 RESULTS

5.1 LIST OF PUBLICATIONS

Publication included as a part of this doctoral thesis

1. Heneberg P., Dráberová L., **Bambousková M.**, Pompach P., Dráber P.: Down regulation of protein-tyrosine phosphatases activated an immune receptor in absence of its translocation into lipid rafts. *J. Biol. Chem.* **285**(17):12787-802, 2010.

2. Bugajev V., **Bambousková M.**, Dráberová L., Dráber P.: What precedes the initial tyrosine phosphorylation of the high affinity IgE receptor in antigen-activated mast cells? *FEBS Lett.* **84**(24):4949-55, 2010.

3. Hálová I., Dráberová L., **Bambousková M.**, Machyna M., Stegurová L., Smrž D., Dráber P.: Cross-talk between tetraspanin CD9 and transmembrane adaptor protein non-T-cell activation linker (NTAL) in mast cell activation and chemotaxis. *J. Biol. Chem.* **288**(14):9801-14, 2013.

4. Dráberová L., Bugajev V., Potůčková L., Hálová I., **Bambousková M.**, Polakovičová I., Xavier R. J., Seed B., Dráber P.: Transmembrane adaptor protein PAG/CBP is involved in both positive and negative regulation of mast cell signaling. *Mol. Cell. Biol.* **34**(23):4285-300, 2014.

5. Bugajev V., Hálová I., Dráberová L., **Bambousková M.**, Potůčková L., Dráberová H., Paulenda T., Junyent S., Dráber P.: Negative regulatory roles of ORMDL3 in FcεRI-triggered expression of proinflammatory mediators and chemotactic response in murine mast cells. *Cell. Mol. Life Sci.* **73**(6):1265-85, 2016.

6. Dráberová L., Paulenda T., Hálová I., Potůčková L., Bugajev V., **Bambousková M.**, Tůmová M., Dráber P.: Ethanol inhibits high-affinity immunoglobulin E (FcεRI) signaling in mast cells by suppressing the function of FcεRI-cholesterol signalosomes. *PLoS One*. **10**(12):e0144596, 2015.

7. **Bambousková M.**, Polakovičová I., Hálová I., Goel G., Dráberová L., Bugajev V., Doan A., Utěkal P., Gardet A., Xavier R. J., Dráber P.: New regulatory roles of galectin-3 in high-affinity IgE receptor signaling. *Mol. Cell. Biol*. **36**(9):1366-82, 2016.

Publications not included as a part of this doctoral thesis

1. **Bambousková M.**, Hájková Z., Dráber P., Dráber P.: Microscopy assays for evaluation of mast cell migration and chemotaxis. *Basophils and Mast cells: Methods in molecular biology* (Gibbs, B. F., and Falcone, F. eds.), Springer New York, New York, NY. pp *161-176*, 2014.

2. Potůčková L., Franko F., **Bambousková M.**, Dráber P.: Rapid and sensitive detection of cytokines using functionalized gold nanoparticle-based immune-PCR, comparison with immuno-PCR and ELISA. *J. Immunol. Methods.* **371**(1-2):38-47, 2011.

3. Klein O., Nyekel F. N., Stefanache T., Torres R., Salomonsson M., Hallgren J., Rådinger M., **Bambousková M.**, Campbell M., Cohen-Mor S., Dema B., Rose C. G., Abrink M., Charles N., Ainooson G., Paivandy A., Pavlova V. G., Serrano-Candelas E., Yu Y., Hellman L., Jensen B. M., Van Anrooij B., Grootens J., Gura H. K., Stylianou M., Tobio A., Blank U., Öhrvik H., Maurer M,.: Identification of biological and pharmaceutical mast cell and basophil related targets. *Scand. J. Immunol*. Article in press, 2016.

5.2 DOWN REGULATION OF PROTEIN-TYROSINE PHOSPHATASES ACTIVATES AN IMMUNE RECEPTOR IN ABSENCE OF ITS TRANSLOCATION INTO LIPID RAFTS

J Biol Chem. 285(17):12787-802, 2010.

In this work we studied the FcεRI signaling initiation upon inhibition of PTPs by pervanadate (Pv). We found that FcεRI subunits get phosphorylated even in the absence of extensive aggregation and translocation to the lipid rafts, suggesting balance in PTP-PTK activities is a crucial mechanism controlling FcεRI triggering. Using monoclonal Ab specific for oxidized state of PTPs we confirmed that PTPs undergo redox regulation after mast cell activation and identified several redox-regulated PTPs in activated mast cells. We also identified contribution of actin cytoskeleton as a potential regulator of balance in the activities of PTPs and PTKs in mast cells.

5.3 WHAT PRECEDES THE INITIAL TYROSINE PHOSPHORYLATION OF THE HIGH AFFINITY IGE RECEPTOR IN ANTIGEN-ACTIVATED MAST CELLS?

FEBS Lett. 84(24):4949-55, 2010.

In this review we evaluated current models of FcεRI signaling initiation.

5.4 CROSS-TALK BETWEEN TETRASPANIN CD9 AND TRANSMEMBRANE ADAPTOR PROTEIN NON-T-CELL ACTIVATION LINKER (NTAL) IN MAST CELL ACTIVATION AND CHEMOTAXIS

J Biol Chem. 288(14):9801-14, 2013.

In attempts to identify new plasma membrane molecules affecting mast cell activation we generated new Ab recognizing mast cell surface Ag identified as CD9. We found that pretreatment of mast cells with CD9-specific Ab triggered activation events as phosphorylation of some key signaling proteins, calcium flux and dephosphorylation of ezrin, radexin, moesin (ERM) family proteins that is important step facilitating mast cell chemotaxis. After CD9 aggregation, NTAL but not LAT showed increased phosphorylation of tyrosine residues. Ultrasctructural analysis of CD9 topography in plasma membrane showed that upon activation, CD9 colocalized with NTAL but not LAT. The combined data helped to dentify different roles of LAT and NTAL in mast cell activation and chemotaxis.

5.5 TRANSMEMBRANE ADAPTOR PROTEIN PAG/CBP IS INVOLVED IN BOTH POSITIVE AND NEGATIVE REGULATION OF MAST CELL SIGNALING

Mol Cell Biol. 34(23):4285-300, 2014.

Previous studies in the laboratory investigated the role of TRAPs LAT and NTAL in mast cell signaling. Here we focused on deciphering the role of other TRAP, PAG, that has shown variable functions in immunoreceptor signaling across the literature. We used BMMCs derived from PAG KO mice and prepared also BMMCs with PAG KD. BMMCs with decreased expression of PAG showed impaired Ag-mediated activation but increased activation when stimulated via KIT receptor. Observed increased activity of SFKs in resting PAG deficient cells indicate that PAG regulates the basal activity of SFKs and increased SFK activity in PAG KO cells is involved in negative regulatory loop controlling FcεRI signaling.

5.6 NEGATIVE REGULATORY ROLES OF ORMDL3 IN THE FcεRI-TRIGGERED EXPRESSION OF PROINFLAMMATORY MEDIATORS AND CHEMOTACTIC RESPONSE IN MURINE MAST CELLS

Cell Mol Life Sci. 73(6):1265-85, 2016.

In this study we provide evidence that downregulation of ORMDL3 expression in mast cells enhances AKT and NF-κB-directed signaling pathways and chemotaxis and contributes to the development of mast cell-mediated local inflammation *in vivo*.

5.7 ETHANOL INHIBITS HIGH-AFFINITY IMMUNOGLOBULIN E RECEPTOR (FcεRI) SIGNALING IN MAST CELLS BY SUPPRESSING THE FUNCTION OF FcεRI-CHOLESTEROL SIGNALOSOMES

PLoS One. 10(12):e0144596, 2015.

In this study we show that short-term exposure of BMMCs to nontoxic concentrations of ethanol inhibits FcεRI-mediated degranulation, calcium response, and production of several cytokines (TNF-α, IL-6, and IL-13) in a dose-dependent manner. We found that ethanol interferes with the function of FcεRI-cholesterol signalosomes and support the lipid-centric theory of ethanol action in this system at the early stages of cell activation.

5.8 NEW REGULATORY ROLES OF GALECTIN-3 IN HIGH-AFFINITY IgE RECEPTOR SIGNALING

Mol Cell Biol. 36(9):1366-82, 2016

In this study we utilized RNAi-based HTS to identify new regulators of mast cell Ag-mediated activation via FcεRI. We selected 144 genes for screen and identified 15 potential regulators of mast cell degranulation. We chose Gal3 for detail functional analysis. Gal3 negatively regulated mast cell degranulation and cytokine expression. FcεRI signaling in cells with decreased expression of Gal3 was upregulated on the level of SYK kinase, suggesting involvement of Gal3 in early signaling upon FcεRI triggering. We found that Gal3 did not affect phosphorylation of FcεRI receptor subunits but regulated receptor ubiquitination and internalization. Next, Gal3 positively regulated adhesion and motility of mast cells on fibronectin, that is likely to be caused by Gal3-controled surface β1-integrin expression in non-activated and also in Ag-activated mast cells. By contrast Gal3 negatively regulated mast cell chemotaxis towards Ag that is at least by part caused by negative regulatory function of Gal3 in chemokine production.

6 GENERAL DISCUSSION

One of the fundamental questions in research of allergic diseases is how exactly the IgE-mediated responses develop. Most recent studies have demonstrated that mast cells are not only effectors but also initiators of establishing the allergic sensitivity [28]. It has been shown that inhibition of SYK kinase prevents allergic sensitization and in mice with established allergy SYK blockade facilitates desensitization and induction of regulatory T cells. Development of mast cell targeted therapy is considered to be important goal in allergy treatment. The lack of the mast cell specific therapeutic targets calls for a better understanding of the processes leading to mast cell activation on molecular level to bring a new and more efficient therapeutic strategies. On the other hand, mast cells are useful model system for studying of MIRR signaling *in vitro* due to their fast response to defined stimuli that can be relatively easily and quickly measured in simple assays. Therefore the novel findings obtained on FcεRI-mediated activation can, to certain extent, also be applied to general knowledge about signaling through MIRR receptors.

Regulation of early signaling events

As for other MIRRs, FcεRI-mediated signaling is controlled by complex negative signaling loops. Most of the downstream signaling events are propagated as phosphorylation of signaling molecules. Rapid increase in global protein-tyrosine phosphorylation is observed upon FcεRI triggering [100]. These events, reflecting activities of PTKs, are counteracted by activity of PTPs [46]. The most recognized “gatekeeper” of mast cell activation is phosphatase SHIP which has been shown to be responsible for downregulation of IgE-mediated mast cell response [48]. The results presented in this dissertation show, that negative regulation by PTPs is not only crucial for downregulation of signaling events but also for the initiation of the activation. First detectable biochemical step after triggering of FcεRI is phosphorylation of its ITAMs [41]. Studies on growth factor receptors showed, that upon ligand binding, receptors dimerize and transphosphorylate each other at the intracellular tyrosine residues [101]. However phosphorylation of FcεRI and other MIRRs is more complex process depending on cross-talk of plasma membrane-associated kinases and adaptor proteins and it is still not completely clear how aggregation of FcεRIs activates these enzymes. The transphosphorylation model of FcεRI signaling was supported mainly by studies documenting association of the receptor with LYN kinase in non-activated cells

[102]. On the other hand, lipid raft model was mainly supported by observations that LYN is localized in the specialized membrane microdomains, which are devoid of FcεRIs [103]. Further studies of FcεRI activation led to a model in which IgE-FcεRI clustering upon Ag-triggering leads to complex interplay of plasma membrane-bound proteins, lipid components and actin cytoskeleton [104]. The role of PTPs has been largely underestimated in the previous models of FcεRI signaling. Previous studies showed that treatment of cells with Pv, an inhibitor of PTPs, triggers tyrosine protein phosphorylation in mast cells and that Pv is capable to trigger cell degranulation [105]. In our studies presented in publication in J. Biol. Chem. 2010, we showed that Pv triggers phosphorylation of FcεRI ITAM motives in absence of extensive FcεRI aggregation and its translocation into lipid rafts. We also examined which particular PTPs are involved in the process of FcεRI activation and on their changes in the course of FcεRI activation and on their changes in the course of FcεRI triggering. PTPs undergoing redox changes identified in this work were also connected to FcεRI proximal signaling in several other studies [106,107]. Although SHP-1 and SHP-2 are well-characterized mast cell molecules, the regulation of their activity by redox mechanism in FcεRI signaling has not been rigorously examined before. Here we showed that SHP-1, SHP-2 and He-PTP were not only irreversible oxidized by strong action of Pv but also reversibly oxidized after physiological stimulation with Ag. However, changes in PTPs activity are dependent not only on their oxidation state but are also subjected to other ways of regulation, as we showed in the case of SHP-2. The fact that RNAi screen of PTP library in mast cells [108] and also our RNAi screen (published in Mol. Cell. Biol. 2016; that targeted some protein tyrosine phosphatases) did not reveal significant effect of these phosphatases on mast cell degranulation suggests that these PTPs may have redundant functions in mast cell activation or reflects the limitation of RNAi screening techniques.

Our experiments also helped to find novel aspects of PTP regulation in mast cells, mainly – the interplay with actin cytoskeleton. It has been shown that small fraction of FcεRI is immobile in resting cells with marked increase in the immobile fraction upon FcεRI crosslinking [45,109]. These so called protein islands in plasma membrane are restrained by actin cytoskeleton and in this way cytoskeleton significantly contributes to forming of FcεRI signalosomes [86]. The hypotheses presented in this work assumes that PTPs are regulated by actin cytoskeleton that spatially sequesters them from FcεRI at initial phases of FcεRI activation. Finally, the results obtained in our studies allowed us to postulate new model of FcεRI signaling that takes into account local changes in equilibrium of activity of PTK and

PTPs together with actin cytoskeleton which was discussed in detail in a review published in FEBS Lett. 2011. Interestingly, the mechanistic model of early FcεRI-mediated signaling events from Barua and Goldstein supports out hypothesis that FcεRI aggregation and its translocation to membrane microdomains results in protection of FcεRI and LAT from activity of PTPs [110].

Initial phosphorylation of β and γ subunits of FcεRI depends on the spatial organization of FcεRI and LYN kinase in the plasma membrane [111]. We showed, that pretreatment of mast cells with ethanol impaired the Ag-induced phosphorylation of the FcεRI subunits, presumably through interference with formation of FcεRI signalosomes in which cholesterol could play a key role. It has been shown in other cell types, that interplay between membrane cholesterol and ethanol contributes to alterations of the membrane fluidity, viscosity, and redistribution of surface molecules, which affects adhesion, rolling, and tethering behavior of the immune cells [112]. Our conclusions that ethanol caused impaired formation of FcεRI signalosomes was supported by the findings that ethanol potentiated effect of MβCD, a drug disrupting plasma membrane cholesterol, on IgE internalization, which critically depends on function of plasma membrane-associated assemblies [113].

PAG is one of the molecules that acts in negative regulatory feedback on the activity of SFKs [75]. In our work, mast cells derived from PAG-deficient mice exhibited increased activity of SFK after FcεRI triggering but decreased degranulation and some other activation events. This was unexpected finding since the studies in some other cell types showed direct correlation between SFK activity and cell activation [114]. Thus, our data show that PTKs do not possess only activatory effect by mediating phosphorylation of their substrates but also inhibit signaling e.g. by phosphorylation of the inhibitory tyrosine motifs or involvement of inhibitory ITAMs [115]. To analyze the plasma membrane topography of PAG relative to FcεRI we transiently transfected BMMCs with Myc-tagged PAG due to the lack of suitable specific Abs for PAG detection and detected fused proteins with anti-Myc Ab. However we were not able to detect any changes in PAG localization upon cell activation that might have been consequence of strong overexpression of Myc-PAG induced in this system (data not shown).

In contrast to the studies, mast cells with decreased or increased expression of ORMDL3 did not show any changes in Ag-mediated calcium response or degranulation. However since reduced expression of ORMDL3 significantly increased production of

proinflammatory mediators, we focused on AKT/NF-κB signaling axis, which is known to be involved in transcriptional regulation of cytokines and chemokines [116]. It is known that ORMDL proteins in mammalian cells are regulators of *de novo* ceramide synthesis pathways [117]. Another known function of ORMDL proteins is inhibition of serine palmitoyl transferases [118]. However detail molecular mechanism how ORMDL3 affects the AKT/NF-κB signaling pathway is unknown and is now is now under investigation in our laboratory.

Key finding of this study was discovery of Gal3 as a negative regulator of FcεRI signaling. Most profound differences in Ag-induced mast cells degranulation between BMMCs with Gal3 KD and control cells were observed when the cells were activated with sub-optimal concentrations of Ag. Thus, Gal3 seems to be one of the molecules that set the threshold for FcεRI activation. In agreement with published findings [119], we observed, that Gal3 expression and secretion sharply increase upon cell stimulation comparing to the basal state (data not shown). Therefore, controlling of Gal3 expression in mast cells could serve as powerful negative regulatory feedback engaged to control mast cell responsiveness.

The data showing that Gal3 control FcεRI internalization and subsequent subcellular localization of IgE-FcεRI complexes indicate that FcεRI trafficking is tightly associated with downstream signaling. The data obtained in this study are supported by findings on TCR signaling, where cells lacking Gal3 showed upregulated global protein-tyrosine phosphorylation and abolished TCR internalization [120]. Studies on EGFR signaling showed that ligated EGFR discriminated between low-strength and high-strength stimulation by recruiting different endocytic machineries and thus, the way of receptor endocytosis modulates the final signaling outcome. It seems that depending on the strength of stimulation some plasma membrane receptors can be internalized by clathrin mediated pathway or clathrin independent pathway [53]. Interestingly, recent data showed that Gal3 drives local membrane curvatures needed for biogenesis of membrane invaginations. Clathrin-independent route of endocytosis is known to be responsible for internalization of aggregated sphingolipids [121]. Extent of aggregation of cell surface molecules seems to be the key criteria regulating their internalization and this mechanism also applies on Ag-aggregated FcεRI [122]. The combined data suggest that Gal3 is the molecule that controls the clathrin-independent endocytosis of FcεRI; when Gal3 is silenced by RNAi the cells could prefer clathrin-mediated endocytosis to internalize FcεRI that in turn leads to different signaling output. Gal3 has been connected to aberrant trafficking of various receptors in

several cancer cell types that caused drug resistance [123]. The exact molecular mechanism how Gal3 acts on receptor endocytosis in cancer cells is now extensively studied [124]. The importance of the route of endocytosis for overall signaling outcome has been demonstrated in mast cells expressing oncogenic KIT receptor, where mutation in the receptor leads to different receptor trafficking and is responsible for aberrant signaling [55]. How exactly Gal3 acts on FcεRI internalization remains to be elucidated.

Signaling in mast cell chemotaxis

In this study we also examined signaling events leading to mast cell chemotaxis. In agreement with the observed changes in FcεRI- and SCF-mediated cell activation PAG selectively regulated cell migration. However no differences in cell spreading on fibronectin were found in PAG KO when compared to WT cells after activation with Ag or SCF (data not shown) suggesting that PAG is not involved in signaling to actin cytoskeleton. Unique mechanism of FcεRI regulation was revealed in the study focused on CD9. In this case CD9 itself had no effect on cell activation, as shown by RNAi experiments. However, when CD9 was aggregated it co-localized with FcεRI and markedly affected migration towards Ag. CD9 aggregating Ab was capable to selectively induce phosphorylation of NTAL but not LAT. Since mast cells express both LAT and NTAL, otherwise relatively cell specific TRAPs, CD9 aggregation provided unique insight into the function of these molecules in signaling and their interplay and cross-talk with other signaling pathways [47]. Recently it was shown that LAT and NTAL localize to different membrane microdomains and also that some signaling molecules as Tespa1 selectively interacts with NTAL [125]. The data presented in this work further support unique and non-redundant function of NTAL in mast cell activation and chemotaxis.

Gal3 has been reported to regulate cell chemotaxis [126]. In this work, Gal3 facilitated mast cell adhesion and motility on fibronectin but had inhibitory effect on fibronectin-independent migration. Role of Gal3 in cell chemotaxis has been mainly attributed to binding to surface glycans. Importantly, the work of Lakshminarayan at al. shows that galectins regulate clathrin-independent endocytosis of some molecules, including β1-integrin, via interaction with sphingolipids and glycosylated proteins [121]. However our attempts to block Gal3 binding to glycosylated proteins with lactose failed to decrease mast cell adhesion and therefore Gal3 is likely to affect mast cell adhesion and motility by different mechanism, possibly by regulating β1-integrin surface expression.

Finally, our interest in studying signaling pathways regulating mast cell chemotaxis prompted us to develop new techniques for real-time monitoring of cell movement suitable for analysis of non-adherent mast cells. During work on this PhD thesis I successfully set two simple techniques that facilitate real-time recording of mast cell motility and directional chemotaxis. These are now routinely used in various projects and one of them was utilized in article published in *Mol. Cell. Biol. 2016*.

RNAi screening in mast cells and Gal3

One of the major goals of this work was to find new regulators of FcεRI signaling. To this end we utilized RNAi technology. Since mast cells, as other cells of hematopoietic origin are hard to transfect by standard transfection techniques, we optimized shRNA lentiviral transduction suitable for HTS format experiments. In initial phases of the project we optimized several assays as potential functional readouts for HTS experiments. Special emphasis was put on single cell assays such as analysis of cell spreading or phosphorylation of ERK by HTS microscopy. Lentiviral transduction in HTS format provided satisfactory results when BMMCL was used. However, BMMCL shows aberrant cell spreading and increased basal phosphorylation of ERK (data not shown) and therefore we focused on the assays for measuring of calcium signaling and degranulation. In screening experiments we used degranulation and calcium assays in parallel. To minimize potential off-target effects we introduced several quality control criteria to filter the data from degranulation and calcium assays that were crucial for successful separation of positive and negative controls. However calcium assay showed significant level of noise (data not shown), therefore it was not used for identification of potential new mast cell regulators.

Gal3 has been already studied in BMMCs with conclusions that it positively regulates mast cell degranulation [127]. The data obtained by our RNAi screen indicated the opposite effect of Gal3 on mast cell degranulation. The downregulation of MIRR signaling by Gal3 has been shown in several studies on TCR [120,128]. Therefore, we decided to study Gal3 functions in the context of FcεRI signaling in more detail. It was particularly interesting to determine if FcεRI can be regulated by similar Gal3-mediated mechanism as TCR in T-cells, where upon stimulation, Gal3 facilitates internalization of the TCR. By analysis of FcεRI internalization we were able to see significant effect of Gal3 on promoting the FcεRI endocytosis. Moreover, our findings confirm the results of previous studies that gene ablation strategies could lead to different phenotypes, depending on the methodology used.

For example, previous studies from different laboratories showed discrepancies on NTAL functions in mast cells and in our laboratory comparison of BMMCs with NTAL KD and KO also showed different phenotypes [129]. To avoid potential difficulties in experiments with reduced expression of the studied proteins, the experiments focused on PAG were performed in cells with PAG KO and also PAG KD. Gal3 has been shown to affect differentiation and developmental programs in various systems [130-132]. BMMCs derived from Gal3 KO mice exhibited decreased Ag-mediated degranulation and phosphorylation of JUN amino-terminal kinase (JNK)1 but no changes in cytoplasmic calcium levels and phosphorylation of signaling proteins as SYK, LAT and PLCγ [127]. In contrast our data obtained in BMMCs with Gal3 KD showed increased degranulation, increased calcium signaling, phosphorylation of SYK, PLCγ and JNK. Therefore, beside the different methodologies used for BMMC derivation in study of Liu at al. and in our laboratory, the impact of Gal3 deficiency can potentially also reflect developmental compensations leading to the observed differences in phenotypes. Generally, the reproducibility of results obtained in mast cells and also other hematopoietic cells is complicated by inherited phenotype plasticity of these cells that is further affected by genetic background of mouse strains, culture conditions, protocol for differentiation of the cells and source of the cells. The basis of the observed differences between the Gal3 KO and KD phenotype were, however, beyond the scope of this study and remain to be elucidated.

In RBL cells, Gal3 was identified as IgE binding protein. Murine Gal3 is a S-type lectin of 250 amino acids. Its sequence consists from N-terminal domain formed by highly repetitive proline rich sequences and C-terminal carbohydrate recognition domain [133]. It was shown that Gal3 binds only to some IgE glycoforms produced in myeloma cell lines, suggesting possible function of Gal3 in IgE-connected diseases [134]. Further results indicated that binding of extracellular Gal3 to IgE-FcεRI complexes could activate mast cells [135]; however, this was shown only in RBL cells and we were not capable to reproduce this observation in BMMCs. Our results with exposure of IgE-sensitized BMMCs to recombinant Gal3 suggested that negative regulatory function of Gal3 on FcεRI-mediated activation is mediated by intracellular, rather than extracellular, action of Gal3.

Gal3 has important functions in the immune system. For instance, it is known to be a chemoattractant for monocytes and macrophages [136] and its expression in neutrophils plays important role in adhesion and extravasation [137]. Gal3 also modulates growth and apoptosis of T cells [120,138], as well as survival of memory B cells [139]. The role of

endogenous galectin-3 in the modulation of tumor-specific immunity in the host and the mechanisms involved therein need to be further investigated. Despite the fact that Gal3 is one of the known IgE-binding proteins the evidence pointing out its role in allergies is still not completely understood [134,140]. Studies on Gal3 are complicated by the fact that it is synthetized in wide spectra of cell types, and at least by some of them can be experoted to extracellular space. Furthermore, Gal3 can be localized either in the nucleus and/or cytoplasm and in these different sites could have different roles. This complexity could affect communication between immune cells or their communication with other cell types. Specific inhibitors of Gal3 binding have shown therapeutic potential in some diseases [61,141]. However, drugs targeting Gal3 could directly or indirectly affect immune system homeostasis. The results of this study indicate that Gal3 is a negative regulator of FcεRI signaling and therefore could be considered as a potential target of drugs for treatment of mast cell-mediated diseases.

7 CONCLUSIONS

1. Our data showed that PTPs play important role during FcεRI initiation. Based on obtained results we formulated model of FcεRI signaling initiation that proposes equilibrium in activity of PTPs and PTKs in resting state and shift towards the activity of PTKs upon crosslinking of FcεRI with Ag.
    a. Topography of oxPTPs was determined and analyzed before and after FcεRI aggregation and also after Pv treatment. Increased number of oxPTPs was detected upon FcεRI aggregation suggesting that PTPs vulnerable to oxidation might be pre-associated with plasma membrane.
    b. Double labeling studies of oxPTPs and FcεRI showed proximity of these molecules in plasma membrane indicating that PTPs undergoing redox regulation are part of FcεRI signalosomes.
    c. Most of the oxPTPs were found associated with cytoskeletal structures that were identified as actin-containing structures. Therefore, actin cytoskeleton might play important role in FcεRI signaling initiation by sequestering PTPs in plasma membrane.
    d. The proposed model of PTK-PTP equilibrium was summarized and discussed with respect to other models in review article published in *FEBS Lett. 2010*.

2. Results obtained in studies of CD9 helped us to better understand signaling pathways leading to mast cell activation and chemotaxis. We showed, that CD9 can regulate mast cell activation and chemotaxis by interacting with FcεRI and FcεRI signalosomes. Moreover, study on CD9 further supported existence of non-overlapping functions of adaptor proteins LAT and NTAL.
    a. Upon aggregation with CD9-specific Ab, CD9 in plasma membrane co-localized with FcεRI, NTAL but not with LAT. These findings contributed to understanding of some non-redundant functions of LAT and NTAL in FcεRI signaling pathway.
    b. Aggregation of CD9 did not affect mast cells adhesion to fibronectin.

3. We showed, that depending on which signaling pathways are engaged, PAG can act as positive or negative regulator of mast cell signaling. Surprisingly, increased activity of

SFKs in PAG KO cells upon triggering with Ag correlated with decreased cell activation. These findings contributed to understanding of negative regulatory loops controlling mast cell activation.

   a. pCDH-based PAG-containing lentiviral construct was used for rescue studies.
   b. pCMV-based vector containing Myc-tagged PAG was prepared and plasma membrane localization of overexpressed PAG was confirmed by confocal microscopy. However, when plasma membrane topography of PAG was analyzed by electron microscopy, no changes were found upon activation with Ag.
   c. No differences in spreading of PAG KO and WT cells were found suggesting no direct involvement of PAG signaling in regulation of cytoskeleton.
   d. Content of peritoneal mast cells in PAG KO was similar to content in WT mice suggesting that PAG deficiency does not affect mast cell development *in vivo*.

4. BMMCs with decreased or increased expression of ORMDL3 showed no changes in degranulation of early activation events. However, ORMDL3 had significant effect on Ag-mediated production of cytokines and chemokines by regulating AKT/NF-κB signaling pathway. Also, ORMDL3 negatively affected mast cell chemotaxis towards Ag.
   a. BMMC with ORMDL3 KD showed decreased spreading on fibronectin. On the other hand, cells with overexpression of ORMDL3 did not show significant changes in spreading.
   b. The image analysis pipeline suitable for analysis of translocation of proteins between cytoplasm and nucleus was established and successfully used for analysis of localization of p65, a member of NF-κB signaling pathway. The localization of p65 upon Ag-activation BMMCs with ORMDL3 KD and control cells was compared and more p65 translocation to nucleus was detected in cells with KD. The obtained data indicate that ORMDL3 is negative regulator of NF-κB signaling in mast cells.

5. We found that inhibitory effect of ethanol on various mast cell activation events is induced by inhibition of function of cholesterol-dependent FcεRI signalosomes.

a. Ethanol or methyl-β-cyclodextrin (MβCD) had no effect on expression of FcεRI and KIT on mast cells.
b. In agreement with defects in degranulation observed in BMMCs pretreated with ethanol, ethanol pretreatment also abolished LAMP1 surface expression. This effect was further enhanced by simultaneous pretreatment of the cells with MβCD suggesting that ethanol affects cholesterol-dependent FcεRI signalosomes.
c. Ethanol significantly inhibited IgE internalization upon triggering with Ag when used in combination with MβCD.

6. We established RNAi HTS protocol for screening in mast cells. By screening 144 genes, 11 positive and 4 negative regulators of mast cell activation were identified. Combined data indicated, that Gal3 is a negative regulator of mast cell degranulation and acts in early activation events by controlling FcεRI internalization and trafficking. We showed that Gal3 also facilitates mast cell chemotaxis on fibronectin but negatively regulates fibronectin-independent chemotaxis towards Ag or PGE_2.
    a. We chose β-glucuronidase enzymatic assay for analysis of mast cell degranulation for its robustness and suitability for HTS measurements in BMMCL.
    b. The highest transduction efficiency together with good viability were achieved when cells were infected with virus at multiplicity of infection (MOI) of 15 in presence of polybrene, 30 min spin and overnight incubation with virus.
    c. 144 genes were selected based on their abundant expression in mast cells. Data were collected from two independent screen runs and statistical analysis of obtained data identified 12 positive and 4 negative regulators of mast cell activation.
    d. We chose Gal3 for further detail functional analysis of its action in FcεRI signaling for most consistent effect of Gal3-targeted shRNAs in the screen.
        i. Gal3 KD confirmed decreased degranulation in BMMC by enzymatic assay and also by single cell analysis of surface LAMP1 by flow cytometry.
        ii. Construct containing Gal3 fused to EGFP was prepared and transfected into BMMCs. Cells overexpressing Gal3 showed decreased

degranulation, supporting the negative regulatory role of Gal3 in mast cell activation.

iii. BMMCs with Gal3 KD showed increased mobilization of cytoplasmic calcium after Ag activation but no changes after thapsigargin. These results suggest that Gal3 affects FcεRI pathway upstream of calcium signaling.

iv. Microscopy analysis of F-actin content revealed increased depolymerization upon Ag treatment which is in agreement with increased cell activation of BMMCs with Gal3 KD.

v. Global phosphotyrosine analysis was determined by confocal microscopy showing increased phosphorylation in BMMCs with Gal3 KD. Next, phosphorylation of individual signaling proteins was analyzed by western blotting; BMMCs with Gal3 KD showed increased phosphorylation of SYK, PLCγ, AKT, JNK but not ERK. These results suggest that Gal3 affects early activation events dependent on SYK activation.

vi. Gal3 stabilized IgE on the mast cell surface upon Ag activation as shown by impaired IgE internalization in BMMCs with Gal3 KD.

vii. IgE in BMMCs with Gal3 KD did not form the intracellular patches observed in control cells, suggesting that Gal3 is important for normal IgE internalization and degradation in activated mast cells.

viii. Gal3 was shown to positively affect FcεRI ubiquitination that might lead to observed changes in FcεRI internalization. Interestingly, Gal3 did not affect phosphorylation of FcεRI β and γ subunits, which suggest that their ubiquitination does not depend on extent of their phosphorylation.

ix. BMMCs with Gal3 KD showed decreased ability to adhere and move on fibronectin, suggesting that beyond negative regulatory role of Gal3 in FcεRI mediated activation, Gal3 can positively regulate fibronectin-mediated ability to move in mast cells.

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